Perennial Ryegrass Microbiome Characterisation and Application

Submitted by

Tongda Li
Bachelor of Engineering (Bioengineering)
Master of Food Science

A thesis submitted in total fulfilment
of the requirement for the degree of

Doctor of Philosophy (Science)

School of Applied Systems Biology
College of Science, Health and Engineering

La Trobe University
Victoria 3086
Australia

February 2021

TABLE OF CONTENTS

LIST OF TABLES

SUMMARY

The health and productivity of agricultural plants is intimately linked to the interactions between plants and their microbiomes. Perennial ryegrass (*Lolium perenne*) is one of the most important pasture grasses, underpinning the performance of the Australian dairy industry. The bacterial microbiome of perennial ryegrass has been profiled recently using 16S ribosomal RNA sequencing, revealing a complex community containing around 500 genera as well as hundreds of isolated strains. Six strains belonging to three of the dominant bacterial genera in the community (*Xanthomonas*, *Paenibacillus* and *Erwinia*) were selected to be characterised in this study as they are commonly associated with agricultural plants. The aim of this study was to develop and conduct assays to characterise the plant growth-promoting (PGP) bioactivities of the selected strains, to examine their interactions with plants and to develop genomic and transcriptomic evidence to understand their functions. Assays developed by this study included *in vitro* (culture-based) and *in planta* (wheat-based) assays to assess the PGP bioactivities (e.g. bioprotection), *in planta* (barley-based) assays to assess the plant-bacteria interactions, as well as *in silico* assays to identify key genes associated with PGP bioactivities. Results demonstrated that all six strains possessed high bioprotection and biofertiliser activity. The three *Xanthomonas* strains represent a novel species based on genomic comparisons with known species. This is the first *Xanthomonas* sp. that has demonstrated strong bioprotection activities against key fungal phytopathogens and is non-pathogenic. The two *Paenibacillus* strains represent a novel species that is closely related to *Paenibacillus polymyxa*. This is believed to be the first report of this novel beneficial bacterium in Australia. The *Paenibacillus* sp. strains possess PGP genes associated with plant nutrient uptake and metabolism (e.g. nitrogen fixation), auxin production and transportation, and secondary metabolite gene clusters associated with bioprotection and other novel functions. Transcriptomic analyses showed strain differentiation, with one strain being more active in expressing key functional genes under stress conditions. The two novel *Paenibacillus* sp. strains and one novel *Erwinia gerundensis* strain were inoculated into barley seedlings to examine the early stage plant-bacteria interaction using dual RNA-seq analyses. Transcriptome profiles suggested that all three strains improved stress response, nutrient uptake and metabolism, and signal transduction of plants, with varied species- and strain-specific responses. Overall, this study further demonstrated the value of PGP bacteria associated with plant microbiome in agriculture. A foundation on the functions of members

of the *L. perenne* microbiome has been generated, identifying strains that have the potential to improve the yield and palatability of pasture grasses and advance the global competitiveness of the Australian dairy industry.

STATEMENT OF AUTHORSHIP

This thesis consists primarily of work by the author that has been published or planned to be submitted for publication as described in the text. Except where reference is made in the text of the thesis, this thesis contains no other material published elsewhere or extracted in whole or in part from a thesis submitted for the award of any other degree or diploma. No other person's work has been used without due acknowledgment in the main text of the thesis. This thesis has not been submitted for the award of any degree or diploma in any other tertiary institution.

Tongda Li

Date: 26/02/2021

SCHOLARSHIP ACKNOWLEDGMENT

Tongda Li was supported by the La Trobe University Full-Fee Research Scholarship, the La Trobe University Postgraduate Research Scholarship and the DairyBio Scholarship to conduct this work.

ACKNOWLEDGMENTS

This PhD study and thesis would not be possible without the guidance from my supervisors, help from my friends, and support from my families.

Firstly, I would sincerely like to thank my principle supervisor Dr. Tim Sawbridge, for offering me such a great research project, for always providing me support, advices and suggestions, and for keeping me on the right path while letting me steer my own work and realise my potential. I would also like to express my sincere gratitude to my co-supervisor Dr. Ross Mann, for your tireless support and motivation to both my laboratory work and data analysis, and for never turning me away whenever I need help. I would never forget the discussions we had when I was drafting my first paper. I could not have asked for a more dedicated supervisor. I would also like to thank my co-supervisor Dr. Simone Rochfort for providing me with feedback and suggestions throughout my PhD study.

I am very honoured to be a member of the great Plant Microbiome team. I would like to thank Dr. Jatinder Kaur for her kind help in answering my questions, and for her valuable input about the technical issues in the laboratory. I am also thankful to Dr. Piyumi Ekanayake, Dr. Dilani De Silva and Desmond Auer for your kind suggestions and support when I was working in the laboratory and drafting my thesis. A special thank you to Ian Tannenbaum, Ankush Chandel and Holly Hone for your help and support during our journey as students, and for making the past four years special to me.

I would also like to thank Dr. Jacqueline Edwards, Dr. Rachel Mann and other members of MSPD for your support throughout my PhD study. To everyone else who has assisted me throughout my thesis, thank you.

Finally, I would like to thank my friends and families, especially to my wonderful wife Dr. Yuhong Liu, for your unconditional love, support and encouragement.

THESIS PREFACE

This thesis is composed of five chapters, with the original experimental content presented in the form of one peer-reviewed and published journal article and two manuscripts that are in preparation for submission in scholarly journals as journal article format. Chapter 1 provides a general overview of the literature in this area of research. The manuscript that has been published is presented in chapter 2, and the manuscripts that are complete and awaiting submission for peer-review are presented in chapter 3 and chapter 4. Each of these chapters contains its own detailed introduction, methodology, results and discussion sections. Each of these chapters also has a preface containing a summary of the work, the publication details of that manuscript, the contribution of co-authors and a statement from a co-author confirming the authorship contribution of the PhD candidate. Supplementary materials for the manuscript featured in chapter 2 are available at the relevant journal websites. Supplementary materials for the manuscripts in chapter 3 and chapter 4 are made available as appendix 2 and appendix 3. Chapter 5 provides a general discussion that integrates the major themes from each of these manuscripts, as well as providing suggestions for future research directions. Because each experimental chapter corresponds to an independently-published manuscript or manuscripts in preparation for submission, some redundancy of content has arisen between the introduction and materials and methods sections of the respective manuscripts. In addition, the individual experimental chapters employ the respective distinct referencing and citation styles of the corresponding journals. In contrast, a single referencing and citation style has been used for chapters 1 and 5, and the bibliography is provided at the end of each chapter.

LIST OF ABBREVIATIONS

ABS	Australian Bureau of Statistics
ACC	1-aminocyclopropane-1-carboxylic acid
ADP	Adenosine 5′-diphosphate
ANI	Average nucleotide identity
ANOVA	Analysis of variance
BLAST	Basic local alignment search tool
BNF	Biological nitrogen fixation
bp	Base pair
cDNA	Complementary DNA
CDS	Coding sequence
CFU	Colony forming unit
COGs	Clusters of orthologous groups
DGE	Differential gene expression
DNA	Deoxyribonucleic acid
dsDNA	Double stranded DNA
GO	Gene ontology
GTP	Guanosine-5'-triphosphate
GWAS	Genome wide association study
HAC	High accuracy
IAA	Indole-3-acetic acid
ISR	Induced systemic resistance
K	Thousand
Kbp	Kilo base pair
KEGG	Kyoto Encyclopedia of Genes and Genomes
M	Million
Mbp	Mega base pair
MALDI	Matrix-assisted laser desorption ionisation
ML	Maximum-likelihood
MS	Mass spectrometry
mRNA	Messenger RNA
N	Nitrogen

NCBI	National Center for Biotechnology Information
NGS	Next-generation sequencing
Nrps	Nonribosomal peptide synthase
OD	Optical density
ONT	Oxford Nanopore Technologies
OTU	Operational taxonomic unit
P	Phosphorus
PBS	Phosphate buffered saline
PCA	Principle components analysis
PCR	Polymerase chain reaction
PE	Paired-end
PGP	Plant growth-promoting
PKS	Polyketide synthase
PSB	Phosphate solubilising bacteria
R	Resistance
RNA	Ribonucleic acid
RNA-seq	RNA sequencing
ROS	Reactive oxygen species
rRNA	Ribosomal RNA
TOF	Time-of-flight
T1SS	Type I secretion system
T2SS	Type II secretion system
T3Es	Type III effectors
T3SS	Type III secretion system
T3PKS	Type III PKS
T6SS	Type VI secretion system
TALEs	Transcription activator-like effectors
UDP	Uridine diphosphate

Chapter 1

Literature review

1.1 Australian dairy industry and pasture grasses

Dairy products, including milk, cheese, yogurt and butter, are consumed worldwide, representing an important source of nutrition for our daily life. As such the dairy industry plays a vital role in the global agriculture sector. Dairy Australia (2015) described the global dairy industry as a dynamic industry which has had an average production growth of 2.2% per annum since 2000. The major driving force behind this growth is the increasing demand for dairy products across the globe, especially in Asia. For example, the Greater China region imported dairy products worth 1,060 million dollars from Australia in 2018–2019 (Dairy Australia, 2019), compared to only 120 million dollars five years ago (Dairy Australia, 2014). A similar import increase was also observed in South East Asia. The Food and Agriculture Organization of the United Nations claimed that there was a significant increase in per capita consumption of dairy products in highly populated countries like China, and concluded that the increasing demand for dairy products was largely due to population growth in developing countries, particularly in Asia (Food and Agriculture Organization, 2010). Such increasing demand has created new opportunities to the global dairy industry, particularly Australia, whose geographic position is close to the emerging Asian markets.

According to Dairy Australia (2019), the Australian dairy industry, which has an estimated farmgate value of 4.4 billion dollars in 2019 and has had a compound annual growth rate of 3.8% since 2010, is Australia's fourth largest rural industry. This growth enabled Australia to become the world's fourth largest exporter of dairy products. Around 30–40% of Australian total dairy production in 2014–2019 was exported, which accounted for 6% share of world dairy trade. Although the whole industry is widely spread across the country, the majority of production is concentrated in the south-east of Australia, with Victoria being the largest producing state.

Although the dairy industry is a complex system which is composed of farms, manufacturing, logistics, supply chain and export, it could also be simply summarised in Australia as the conversion of pasture grasses to milk, which is then converted to other dairy products. As pasture grasses are where the whole production system begins, it can be concluded that, to a large extent, the performance of the Australian dairy industry is underpinned by the performance of pasture grasses.

According to the Australian Bureau of Statistics (ABS) rural census in 2018 (Table 1), around 91.4% of land mainly used for agricultural production in Australia are used for grazing. Amongst all states, Victoria and Tasmania hold very high proportions (75.1% and 67.3%) of grazing land with improved pastures (Table 1). Given the fact that Victoria has 67.4% of the total registered dairy farms in Australia, produces about 63.4% of Australia's milk and accounts for the production of most of Australia's grass-seed crop (Reed, 2014; Dairy Australia, 2019), it is clear that pasture grasses play a vital role in the state's and country's agriculture and economy.

Table 1. Area of improved pastures for grazing in Australia, 2016–2017 (Australian Bureau of Statistics, 2018)

State	Land mainly used for agricultural production (ha)	Improved pastures (ha)	Other grazing land (ha)	Improved pastures (% of total grazing land)
NSW and ACT	51,657,430	8,670,978	33,902,913	20.4
Vic.	9,960,567	4,184,281	1,387,327	75.1
Qld	132,771,840	15,135,244	114,237,256	11.7
SA	47,554,773	2,922,707	39,594,244	6.9
WA	79,609,836	3,855,273	65,944,880	5.5
Tas.	1,245,862	695,407	337,648	67.3
NT	49,920,384	102,991	49,791,971	0.2
Total	372,720,692	35,566,881	305,196,239	10.4

NSW: New South Wales; ACT: Australian Capital Territory; Vic.: Victoria; Qld: Queensland; SA: South Australia; WA: Western Australia; Tas.: Tasmania; NT: Northern Territory.

1.2 Improving the performance of pasture grasses

A series of approaches has been developed to improve the performance of pasture grasses, including both yield and palatability. As one of the earliest approaches, the sowing of seeds of introduced pasture species to replace the underperforming native species has been applied throughout the world. This approach can be dated back to the 16th century when a few farms started sowing pasture seeds in northern Europe (Fussell, 1964). In Australia, although native

grasslands are still a significant component of pasture resources, the importance of introduced pastures has long been recognised since they are generally more digestible and productive when compared with native pastures (Menz, 1984). For example, Groves et al. (2003) compared the growth of seven pasture species, including three Australian native perennial species (*Themeda triandra*, *Poa labillardieri* and *Danthonia carphoides*), two introduced annual species (*Vulpia bromoides* and *Hordeum leporinum*) and two introduced perennial species (*Lolium perenne* and *Dactylis glomerata*), in a short-term glasshouse experiment. Results showed that the native species were less competitive than the introduced species when grown in mixtures, and produced up to 40% less total biomass than the introduced species. Besides, the Australian native grass species are known to have poor seed quality, e.g. low germination rates (Cole and Johnston, 2006). Therefore, to improve the overall performance, the introduced pasture grass species are sown with native species (collectively called 'improved pastures') and they are usually a mixture of grasses and legumes, including annual grasses and legumes (e.g. Italian and hybrid ryegrass, tall wheatgrass, subterranean clover and lucerne) as well as perennial grasses (e.g. perennial ryegrass, *Phalaris*, cocksfoot and tall fescue). Whilst the initial practice was sowing a mixture of common species that could be found on natural grasslands, tailoring the use of particular species according to appropriate land aspects became more common to further improve the productivity (Reed, 2014). For example, in Victoria the dominant improved pastures are the perennial white clover (*Trifolium repens* L.) or the annual subterranean clover (*T. subterraneum* L.) sown with the perennial ryegrass (*L. perenne* L.), which is a key pasture species for the temperate zone due to its ability to respond to varying stresses (Reed, 2014). Such combinations are well received by farmers due to their high nutritional value and simple management (Ludemann et al., 2015).

Besides the underwhelming performance of native pasture species, strong challenges to improving the overall performance of pasture grasses are posed by biotic stresses, e.g. pests and pathogens, and abiotic stresses, e.g. nutrient deficiency. Breeding new cultivars that are resistant to those stresses is an important approach to improve the performance of pasture grasses (Williams et al., 2007), however only limited progress has been achieved thus far. For example, while traditional breeding techniques can be used to select stress-resilient cultivars, the persistency of preferred traits (e.g. yield advantage) might decrease after years of sowing (Parsons et al., 2011). Moreover, since perennial ryegrass is self-incompatible, the seeds are closely related but genetically different individuals (Pembleton et al., 2015), making the

breeding process laborious and costly (Lee et al., 2012a). To overcome the shortcomings of traditional breeding, precision breeding using genome editing or transgenic technologies has been used (Smith et al., 2007; Kim and Kim, 2019). For example, transgenic white clover containing the coat protein gene of *Alfalfa mosaic virus* exhibited resistance to the virus in field conditions (Panter et al., 2012). Transgenic manipulation of 6-glucose fructosyltransferase and sucrose:sucrose 1-fructosyl-transferase in perennial ryegrass led to enhanced accumulation of fructan in leaf blades which are the major parts of plants consumed by grazing ruminants (Panter et al., 2017). There are a few transgenic pasture forage products that are commercially available in the US (Hubbard and Hassanein, 2013). However, this approach is still time-consuming and costly due to the regulations applied to transgenic research and commercialisation (Eriksson, 2019; Giraldo et al., 2019).

Another approach to increase the performance of pasture grasses is to improve the availability of nutrients, such as nitrogen (N) and phosphorus (P), in soil. Both nitrogen and phosphorus are essential elements for the growth and development of plants. They have become key agricultural inputs due to their limited availabilities in most agricultural soils (Dawson and Hilton, 2011). To meet the requirement of such nutrient inputs, the application of a variety of chemical fertilisers has been a common and popular method for decades. In Australia, based on ABS rural census (2018), a total of 4,873,099 tonnes of chemical fertiliser have been applied to 50,733,745 ha of agricultural land in 2016–2017. In Victoria, the numbers were 986,467 tonnes and 8,592,171 ha, which equals to 86.3% of land mainly used for agricultural production. The two most popular fertilisers across the country measured by the amount applied were urea and ammonium phosphate. However, several disadvantages of applying chemical fertilisers have been reported. Since most of chemical fertilisers are either directly derived from fossil fuel or are energy-demanding in the manufacturing process, the cost of applying such fertilisers increases as the cost of fossil fuel increases (Reeve et al., 2010). Moreover, although fertilisers are used as nutritional supplements to plants, not all of them can be utilised by plants. Gourley et al. (2012) reported that the median value of usage efficiency of nitrogen and phosphorus on Australian dairy farms were only 26% and 35%, respectively, with the median value of usage efficiency of other nutrients including potassium and sulfur being around 20%. Generally, it was reported that plants only absorbed 30–50% of the applied nitrogen fertiliser, while the rest being unavailable to plants due to leaching, run-off, volatilisation or being adsorbed as soil organic-nitrogen (Hodge et al., 2000; Mulvaney et al., 2009). Such leaching is not only just a waste of

energy and nutrients. In fact, excess application of chemical fertiliser to boost agricultural production can severely impact arable soils and decrease the soil fertility (Kozdrój et al., 2004), and public concerns have been raised by the side effects of chemical fertiliser on the environment, e.g. eutrophication caused by leaching (Chislock et al., 2013).

Compared to the artificially applied chemical fertilisers, we can take the advantage of bacteria, a group of naturally present organisms, to improve the performance of agricultural plants including pasture grasses. Bacteria are widely spread in soil, with a typical concentration of 10^9–10^{10} microorganisms of more than a million bacterial species per gram dry weight (Gans et al., 2005). Enormous interactions exist between these bacteria and plants. Whilst some of those interactions are undesirable since they lead to plant diseases (Mansfield et al., 2012) such as speck of tomato and bleeding canker of horse-chestnut caused by *Pseudomonas syringae*, and bacterial wilt of forage grasses caused by *Xanthomonas translucens* (Shenge et al., 2007; Green et al., 2010; Wichmann et al., 2013), some bacteria are able to build beneficial interactions with plants therefore improve the performance of plants, including promoting plant growth, speeding up seed germination and improving seedling emergence (Lugtenberg et al., 2002). Such bacteria are known as plant growth-promoting (PGP) bacteria. PGP bacteria can promote plant growth directly by acting as biofertilisers, alleviating abiotic stresses like nutrient deficiency. As biofertilisers, PGP bacteria can convert important nutrient elements from plant-unavailable to plant-available forms via biological processes (Egamberdiyeva, 2007). For example, after forming nodules on the roots of leguminous plants, *Rhizobium* spp. and *Bradyrhizobium* spp. can convert atmospheric nitrogen into ammonia, which is then used by plants as nitrogen sources (Franche et al., 2009). In addition, the mineral uptake by plants can be enhanced by phosphate- and potassium-solubilising bacteria as they can release and convert plant-unavailable phosphorus and potassium from silicate in soil via enzymatic reactions (Sharma et al., 2013; Ahmad et al., 2016). PGP bacteria can also directly promote plant growth by producing phytohormones, e.g. indole-3-acetic acid produced by *Azotobacter* spp. (Patten et al., 2013), and by releasing volatiles, such as 2,3-butanediol and acetoin produced by *Bacillus amyloliquefaciens* (Ryu et al., 2003). In addition to direct promotion, PGP bacteria can also promote plant growth indirectly by reducing the abiotic and biotic stresses, such as protecting plants from soil pollutants and soil-borne plant diseases (Kuiper et al., 2001; Compant et al., 2005a). These growth-promotion effects of PGP bacteria are reviewed more intensively in the relevant sections of this chapter.

There are several advantages to applying PGP bacteria when compared to applying chemicals for agricultural purposes, including safety, quality, efficiency and convenience. From a safety perspective, properly developed PGP bacteria products are believed to be safe to other organisms, and their negative impact on environment is minimal. When using PGP bacteria as bioprotection agents, resistance is harder to be developed by the target organisms when compared to chemical agents due to their complex modes of action (Wu et al., 2005; Grimmer et al., 2015). As for quality, the accumulation of toxic substances or PGP bacteria themselves will not occur in the food chain (Shen, 1997). Applying PGP bacteria is also more efficient than agricultural chemicals due to their complex modes of action. Lastly, due to the self-replication feature, repeated applications of PGP bacteria are minimal compared to conventional chemicals. Given the advantages described above, several PGP bacterial strains are now commercially available. For example, the nitrogen-fixing bacterium *R. leguminosarum* bv. *trifolii* strain WSM1325 is commercially produced and successively utilised in Australia to inoculate a wide range of clovers (Reeve et al., 2010). Consequently, there has been an increasing number of studies of isolating, identifying and characterising more PGP bacterial strains from microorganisms associated with diverse environments, including different soil types and geographical locations, as well as a variety of plant species (Egamberdiyeva, 2007; Ahmad et al., 2008; de Santi Ferrara et al., 2012; Banik et al., 2019; Cherchali et al., 2019).

1.3 Plant microbiome

The term microbiome was first defined in 1988 and has been refined by various studies with different focuses including ecology, host-dependency and genomics (Berg et al., 2020). The currently most cited definition described the microbiome as “the ecological community of commensal, symbiotic and pathogenic microorganisms” that can be found in and on multicellular organisms (Lederberg and McCray, 2001). Based on this definition, a microbiome contains all microorganisms associated with a multicellular organism, including bacteria, archaea, fungi, algae and protozoa. The focus of this study will be bacteria, and hereafter, the term microbiome in this study refers to bacteria only, unless otherwise specified.

Both animals and plants host a diverse range of microorganisms such as bacteria. In animals, the gut is heavily colonised by microorganisms. It has been estimated that the number of

bacterial cells in a human body is at least 10 times more than the number of human cells, and most of those bacteria are living inside the gut (Savage, 1977). Qin et al. (2010) identified over 1,000 prevalent bacterial species by studying the gut microbiome using faecal samples of 124 European individuals. Another recently published research on the pig gut microbiome revealed 719 metagenomic species (Xiao et al., 2016). Similar studies have also been carried out on plants. Hawkes et al. (2007) identified over 1,200 distinguishable bacterial taxa from 14 plant species. Even in the Antarctic which is subject to harsh environments, Teixeira et al. (2010) managed to discover up to 732 operational taxonomic units (OTU) in microorganisms isolated from two vascular plants. Since these microorganisms are ecologically and biologically associated with plants, they can be collectively termed 'the plant microbiome'.

Although nearly all plant tissues are associated with a microbial community, or microbiome (Turner et al., 2013), the abundance and diversity of microorganisms on different part of plant tissues vary. Based on the relative location, plant tissues can be categorised as three parts, i.e. the below-ground tissues, the above-ground tissues and the internal tissues. Each part is surrounded by different environments, leading to differences in their microbiomes.

The below-ground plant tissues are surrounded by the rhizosphere. Defined by Hiltner in 1904, rhizosphere refers to the narrow region of soil that is directly influenced by plant roots (Hartmann et al., 2008). Bulk soil is a heterogeneous environment that contains highly diverse microbial communities, having spatial variability and temporal variability associated with factors like soil pH, nutrient availability and climate (Fierer, 2017). The rhizosphere microbiome is derived from the bulk soil microbiome largely via the migration of microorganisms (Compant et al., 2019). Generally, the rhizosphere microbiome contains 10^7–10^9 colony forming units (CFU) of culturable microbes per gram of soil, and the total number is very likely to be even higher due to the contribution of unculturable microbes (Benizri et al., 2001). Moreover, the rhizosphere also contains an incredibly diverse population of microbes. As reviewed by Mendes et al. (2013), OTU numbers ranging from a couple of hundreds to more than 50,000 have been reported by studying the rhizosphere microbiome using different techniques. Compared to the bulk soil, it has been discovered that the rhizosphere is much richer in bacterial abundance (10 to 1,000 times higher) (Lugtenberg and Kamilova, 2009; Donn et al., 2015). Such a difference is largely due to plant roots, which affect rhizosphere via rhizodeposition of exudates, mucilage and sloughed cells (Paterson et al., 2007). Although the composition of root exudates is dependent on the species, cultivars

and development stages of plant (Cavaglieri et al., 2009; Micallef et al., 2009; Chaparro et al., 2013), in general it contains sugar, organic acids, fatty acids, amino acids, vitamins, phenolics, plant growth regulators, hormones and antimicrobial compounds (Uren, 2007; Saad et al., 2020). It was reported that 5–21% of the carbon fixed by plants is secreted mainly as root exudates (Marschner, 2012). These metabolites, which are rich in nutrients that microorganisms can utilise, make root exudates an important determinant of the microbial structure of rhizosphere (Bais et al., 2006; Shi et al., 2011; Mendes et al., 2013). Moreover, additional biodegradable substances are released into rhizosphere by the release of mucilage and the sloughing of cells, providing extra carbon nutrients to the rhizosphere microbiome (Dennis et al., 2010). All these nutrients clearly contribute to the boost in density of and variety in microbiomes in the rhizosphere.

The distribution of microorganisms on the rhizoplane differs from that in the rhizosphere. Microorganisms only adhere to a small part of the rhizoplane, with major sites being points where lateral roots grow and junctions between root epidermal cells (Bais et al., 2006; Lugtenberg and Kamilova, 2009). Considering the fact that nutrients are relatively rich on the rhizoplane, the rhizoplane colonisation appeared to be affected by microbial chemotaxis toward root exudates and growth rate in root exudates (Lugtenberg et al., 2001; De Weert et al., 2002). Since rhizoplane colonisation is a very competitive process for microorganisms and many genes are involved, further study is required for us to understand it completely (De Weert et al., 2006).

The above-ground plant tissues are surrounded by a completely different environment when compared to the below-ground tissues. Unlike the rhizosphere, the phyllosphere, which is the aerial surface of plants, is an environment which is relatively poor in nutrients and has dynamic and inconsistent changes of temperature, humidity and radiation (Vorholt, 2012). These abiotic stresses, together with biotic factors of plants like leaf structure, directly affect the phyllosphere microbiome (Turner et al., 2013). By profiling the16S ribosomal RNA (rRNA) gene sequences, it has been found that the phyllosphere microbiome is dominated by *Alphaproteobacteria*, *Gammaproteobacteria*, *Bacteroidetes* and *Actinobacteria*, and is low in diversity when compared to the rhizosphere microbiome (Vorholt, 2012; Bodenhausen et al., 2013). Wallace et al. (2018) qualitatively analysed the leaf microbiome of around 300 different maize lines growing in New York and identified the dominating class being *Alphaproteobacteria*, to which 74% of 16S rRNA reads were mapped. Interestingly, unlike

the relationship between the rhizosphere microbiome and the soil microbiome, the phyllosphere microbiome shares little similarities with the air microbiome, to which the leaf surface is exposed (Vokou et al., 2012).

Some bacteria are even capable of entering the internal plant tissues (i.e. the endosphere) as endophytes and then establishing mutualistic associations with plants (Azevedo et al., 2000). Bacterial endophytes have been found in a wide range of plants, including rice (Edwards et al., 2015), maize (Johnston-Monje et al., 2014), wheat (Durán et al., 2014), soybean (Zhang et al., 2011), common bean (Mora et al., 2014), potato (Manter et al., 2010), banana (Andrade et al., 2014) and sugarcane (Magnani et al., 2010). It is most likely that all plant species have their own endophytic microbiome, although whether they can be fully-characterised or not depends on the ease of isolating, culturing and identifying the microorganisms. Similar to the phyllosphere microbiome, endophytic microorganisms of class *Alphaproteobacteria*, *Betaproteobacteria* and *Gammaproteobacteria*, and phylum *Firmicutes*, *Bacteroidetes* and *Actinobacteria* have been isolated (Rosenblueth and Martínez-Romero, 2006). Reinhold-Hurek and Hurek (1998) stated that the bacteria isolated from surface-sterilised plant tissues are not "genuine" endophytes unless they can also be visualised inside plant tissues using microscopic techniques, as bacteria can be protected from chemicals used for surface-sterilisation by niches on the surface of plant tissues.

Bacteria enter plants via naturally occurring cracks like lateral root junctions, root emergence sites and root tips, as well as cracks created by detrimental microorganisms (Reinhold-Hurek and Hurek, 1998; Compant et al., 2005b). They can also enter plants through aerial parts like flowers (Compant et al., 2011). It is notable that this is not a completely passive process. Some bacteria are able to use specific mechanisms, such as expressing plant cell wall degrading enzymes, to penetrate plant cells actively (Hardoim et al., 2008; Monteiro et al., 2012). Once entered the plant, bacteria are most likely to live in the intercellular apoplast and to spread systemically to other parts of the plant via xylem vessels, with an abundance ranging from 10^3 to 10^8 CFU per gram fresh weight (Compant et al., 2010; Bulgarelli et al., 2013a; Turner et al., 2013). However, nodule-forming bacteria can release microbial signals after penetrating root tissues to stimulate the development of a plant organ called nodules (Garg, 2009). These nodules, which typically contain up to 10^{11} CFU per gram fresh weight, are required for the symbiotic lifestyle of nodule-forming bacteria, and are essential in nitrogen fixation (Müller et al., 2001; Compant et al., 2010).

The endosphere microbiome is a subset of the rhizosphere microbiome, and usually has a lower density and diversity than the rhizosphere microbiome (Hallmann and Berg, 2006). Lundberg et al. (2012) stated that 256 of 778 measurable OTUs, which were identified across the soil and the rhizosphere and the endosphere of *Arabidopsis thaliana*, were enriched in the endosphere, whereas 32 of 778 OTUs representing *Acidobacteria*, *Gemmatimonadetes* and *Verrucomicrobia* were depleted in the endosphere. Since not all microbes can enter plant tissues, and those who can enter might alter their metabolism to adapt to the internal environment, the endosphere microbiome has distinctly different characteristics when compared to the rhizosphere microbiome (de Santi Ferrara et al., 2012; Sessitsch et al., 2012).

Besides the microbiomes described above, the seed microbiome has attracted attention since it may be associated with seed germination, root proliferation and plant development (Felestrino et al., 2017; Berg and Raaijmakers, 2018). The seed microbiome mainly contains *Proteobacteria*, *Actinobacteria*, *Firmicutes* and *Bacteroidetes* (Liu et al., 2012; Rodríguez et al., 2018; Tannenbaum et al., 2020). The seed microbiome is inherited from parental plants, proving vertical transmissions of microorganisms (Eyre et al., 2019; Tannenbaum et al., 2020). It has been reported that the seed microbiome is used by plants to transfer a core microbiome containing plant beneficial endophytes (Johnston-Monje et al., 2016; Adam et al., 2018; Bergna et al., 2018; Wassermann et al., 2019). Therefore, Shade et al. (2017) proposed that the seed microbiome represents significant components of the microbiome of parental plants and is crucial for establishment of daughter plants. Pérez-Jaramillo et al. (2016) suggested that the seed microbiome of wild relatives of domesticated plants should be explored to potentially rediscover beneficial microorganisms such as PGP bacteria, which may have been lost through domestication.

1.4 The plant growth promoting functions of plant microbiome

The relationship between the microbiome and its host is not only the close physical distance, but also the complex biological interactions. For example, the gut microbiome has been shown to be able to regulate the expression of human genes responsible for several intestinal functions (Hooper et al., 2001). Another study showed that the human gut microbiome is associated with type 2 diabetes (Qin et al., 2012). Similarly, the plant microbiome plays a remarkable role in determining the health and productivity of their host (Berendsen et al., 2012). In fact, given the deep involvement of microorganisms in major functions of plant

growth and development, Vandenkoornhuyse et al. (2015) proposed that rather than being considered as standalone individuals, plants and their microbiomes should be considered collectively as a holobiont. Such concept required us to have a comprehensive understanding of the biological and ecological role that microbiomes play in the survival and growth of plants, especially the PGP functions of the plant microbiome, which have attracted substantial attentions (Bulgarelli et al., 2013b).

1.4.1 Biological nitrogen fixation

In order to synthesise nucleic acids, proteins and other biological molecules, nitrogen is required by all organisms (Franche et al., 2009). For example, nitrogen is an essential component of chlorophyll, which is the most important pigment required for photosynthesis (Wagner, 2012). However, nitrogen is often the limiting nutrient for plant growth and development (White et al., 2015). Although by volume about 80% of the atmosphere is nitrogen, it exists as dinitrogen gas which is unable to be utilised by plants. In fact, plants take up nitrogen in inorganic forms like nitrates and ammonium as well as organic forms like amino acids from the soil via their roots (Tegeder and Masclaux-Daubresse, 2018). Such bioavailable forms of nitrogen are from fertilisers, organic matter decomposition and conversion of atmospheric nitrogen (Vance, 2001). The contradiction between the increasing demand for higher yield from agricultural plants and the limited bioavailability of nitrogen has led to massive productions of nitrogen fertilisers, whose inefficient use has caused environmental contamination and compromised agricultural sustainability (Herridge et al., 2008). Therefore, agricultural scientists have targeted alternative nitrogen sources, such as the biological nitrogen fixation, to reduce the use of chemical fertilisers.

Biological nitrogen fixation (BNF) is a process which converts the atmospheric dinitrogen gas to ammonia, making this nutrient bioavailable to plants. BNF accounts for around half of annual nitrogen inputs into the biosphere, making it an important nitrogen source for agricultural systems (Kennedy and Islam, 2001). Unkovich (2012) reported that in the Australian dairy system up to 300 kg of nitrogen input per hectare per year are contributed by BNF. Only some prokaryotes are able to perform BNF, with known bacterial strains from *Rhizobium* spp. and *Bradyrhizobium* spp. that are commonly associated with leguminous plants, and *Azospirillum* spp., *Azoarcus* spp., *Herbaspirillum* spp. and *Paenibacillus* spp. that

are commonly associated with non-leguminous plants (Dos Santos et al., 2012; Santi et al., 2013; Baldani et al., 2014; Grady et al., 2016).

The mechanism of BNF is very complex and not yet fully understood (Franche et al., 2009). In general, the reduction of molecular N_2 to NH_3 can be expressed as the following equation:

$$N_2 + 16MgATP + 8e^- + 8H^+ \rightarrow 2NH_3 + H_2 + 16MgADP + 16P_i$$

This reduction reaction is catalysed by nitrogenase which is an oxygen-labile enzyme complex. It is highly conserved in free-living and symbiotic nitrogen-fixing bacteria, or diazotrophs (Santi et al., 2013). The most common form of nitrogenase is referred as the Mo-nitrogenase. It is composed of two multi-subunit metalloproteins, i.e. Component 1 and Component 2. Component 1 is a molybdenum iron protein containing two non-identical subunits α and β (the D and K component proteins), while Component 2 is an iron protein formed by two identical subunits (the H protein) (Newton, 2006; Hartmann and Barnum, 2010). Besides dinitrogen, the Mo-nitrogenase can also reduce several substrates, such as acetylene (reduce acetylene to ethylene) (MacKellar et al., 2016). Other forms of nitrogenase, which contain only iron or contain vanadium in the Component 1 protein, also exist in some diazotrophs, such as *Azotobacter* spp. (Rubio and Ludden, 2005). Despite the differences in the metal content, these forms of nitrogenase are highly related in terms of structure, mechanism and phylogenetics (Dos Santos et al., 2012). It has been discovered that the activity of nitrogenase can be reversibly inhibited by hydrogen gas and carbon monoxide (Hwang et al., 1973). Moreover, the exposure of nitrogenase to oxygen can lead to an irreversible inhibition of activity due to the oxidative damages of metal cofactors (Robson and Postgate, 1980; Imlay, 2006). Consequently, multiple strategies have been developed by nitrogen-fixing organisms to mitigate the effect of oxygen on nitrogenase, as described by Gallon (1981).

Interestingly, a fourth form of nitrogenase, the superoxide-dependent nitrogenase produced by *Streptomyces thermoautotrophicus* UBT1, was reported two decades ago (Ribbe et al., 1997). It then became the only known nitrogenase which doesn't have the patterns described above: 1) the activity was not inhibited by hydrogen gas, carbon monoxide and oxygen; 2) it couldn't reduce acetylene to ethylene; and 3) it had a different subunit structure. However, a recent study discovered that this strain could not incorporate heavy-isotope-labelled $^{15}N_2$ into

its biomass, suggesting no nitrogen was fixed. Moreover, although this strain could grow on NH_4^+ -free media solidified with 0.5–1.0% gellan gum, no growth was observed when gellan gum was replaced by agar. Further analysis showed the gellan gum used in the assay contained 0.095% nitrogen content (MacKellar et al., 2016). Hence, the authors concluded that this strain should be reclassified as non-diazotrophic, even if it could utilise nitrogen at an extremely low concentration.

The genetic basis of BNF has also been explored. Arnold et al. (1988) first reported a 24,206 base pair (bp) DNA fragment containing the nitrogen-fixation genes in a *Klebsiella oxytoca* strain M5a1. A total of 19 *nif* genes (*nifJ*, *nifH*, *nifD*, *nifK*, *nifY*, *nifE*, *nifN*, *nifX*, *nifU*, *nifS*, *nifV*, *nifW*, *nifZ*, *nifM*, *nifF*, *nifL*, *nifA*, *nifB* and *nifQ*) were identified, and their functions have been characterised since then. Specifically, *nifD*, *nifK* and *nifH* encode the D, K and H proteins of the Mo-nitrogenase, whereas *nifY*, *nifE*, *nifN*, *nifX*, *nifU*, *nifS*, *nifV*, *nifB* and *nifQ* are responsible for the synthesis of a prosthetic group, FeMoCo, of the Mo-nitrogenase (Rubio and Ludden, 2008; Franche et al., 2009). Moreover, *nifW*, *nifZ* and *nifM* are involved in the maturation and processing of enzyme components, and *nifF*, *nifJ*, *nifL* and *nifA* regulate the expression of the *nif* cluster (Dixon and Kahn, 2004; Rubio and Ludden, 2005). Other forms of nitrogenase, where molybdenum is substituted by vanadium and iron, contain a subunit G encoded by *vnfG* and *anfG*, respectively (Dos Santos et al., 2012). Although apart from that subunit, the *vnfHDK* and *anfHDK* are homologous to *nifHDK*, these nitrogenases have lower activities and are more sensitive to oxygen when compared with Mo-nitrogenase (MacKellar et al., 2016). Since the analogous subunits from different forms of the nitrogenase share high levels of protein sequence identity, some phylogenetic studies used *nifH* and/or *nifD* (as well as *vnf/anfH* and *vnf/anfD*) to assess the biodiversity of nitrogen fixation (Zehr et al., 2003; Turk et al., 2011; Turk-Kubo et al., 2014).

Numerous efforts have been put to design primers to amplify the *nifH* gene with polymerase chain reaction (PCR) in samples collected from marine and terrestrial sites (Man-Aharonovich et al., 2007; Rösch and Bothe, 2009), making *nifH* the most sequenced of all genes that are involved in BNF. It has also become the most widely used marker gene to study the ecology and evolution of nitrogen-fixing organisms (Raymond et al., 2004). Five primary phylogenetic clusters of genes homologous to the *nifH* gene have been revealed based on gene sequence identity (Table 2) (Raymond et al., 2004; Nomata et al., 2006; Staples et al., 2007; Gaby and Buckley, 2014). Similar to the 16S rRNA sequence database, a

database containing 32,954 *nifH* gene sequences and homologs has been released (Gaby and Buckley, 2014). By analysing such data, researchers have found dramatic differences in the diversity of nitrogen-fixing organisms across different habitats (Zehr et al., 2003; Gaby and Buckley, 2011). Moreover, the diversity and abundance of the *nifH* gene have been found to be related to the rate of BNF (Hsu and Buckley, 2009; Reed et al., 2010). Such diversities of the *nifH* gene, as well as nitrogen-fixing organisms, strongly suggested that more comprehensive studies are required to have a thorough understanding of BNF.

Table 2. Primary phylogenetic clusters of genes homologous to the *nifH* gene

Cluster I	*nifH* genes primarily from aerobic and facultative anaerobic bacteria
Cluster II	alternative nitrogenase genes (*vnfH* and *anfH*)
Cluster III	*nifH* genes from obligate anaerobic bacteria and methanogenic *Archaea*
Cluster IV	genes whose sequence are paralogs of *nifH* but not involved in BNF
Cluster V	

Summarised from Raymond et al. (2004), Nomata et al. (2006), Staples et al. (2007) and Gaby and Buckley (2014)

Given the importance of nitrogen-fixing bacteria, detection and isolation methods based on the genomic and/or phenotypic evidence have been developed. Amplifying fragments of the *nifH* gene using PCR with universal or cluster-specific primers has been widely used to detect the presence of diazotrophic bacteria (Gaby and Buckley, 2012). To gain a deeper insight into the genomic evidence, the genomes of candidate bacteria can be sequenced, and at least six genes encoding the nitrogenase proteins, i.e. *nifB/H/D/K/E/N*, should be identified from the sequenced genomes to categorise the candidate strains as diazotrophs (Dos Santos et al., 2012). The phenotype-based methods use selective media that are completely free of nitrogen. As reviewed by Baldani et al. (2014), diazotrophs form a “growth belt” underneath the surface of the nitrogen-free media where the activity of nitrogenase is not affected by oxygen. Several nitrogen-free media have been developed to detect and isolate diazotrophs from a diverse environment. However, the major limitation of this method is the presence of nitrogen scavengers that can grow with diazotrophs by utilising the fixed nitrogen or the ultra-low nitrogen content present in the media (such as the *S. thermoautotrophicus* UBT1 strain described above). Therefore, nitrogen-free media should be used in combination with other phenotype-based selection approaches, such as antibiotic resistance or heat resistance, to isolate pure colonies of the target diazotrophs. Once isolated, the candidate strains should be assessed for the genomic evidence described above, and/or for further phenotypic

evidence, such as confirming the bioactivity of the nitrogenase using acetylene reduction assays or $^{15}N_2$ assimilation assays (Saiz et al., 2019).

1.4.2 Phosphate solubilisation

Phosphorous is one of the major essential macronutrients for various attributes of plant growth and development, including root development, stalk and stem strength, crop quality, seed formation and diseases resistance (Mohammadi, 2012). Typically, phosphorous is present at levels of 400–1,200 mg per kg of soil as organic and inorganic phosphates (Hayat et al., 2010). Similar to nitrogen, such a pool of phosphorous nutrients can only be utilised by plants in soluble forms. However, a large proportion of that pool is insoluble and less than 5% of the phosphorous in soil is bioavailable (Bulgarelli et al., 2013b). And unlike nitrogen, no atmospheric phosphorous source is available for biological fixation. Therefore, phosphate fertilisers have been widely used in agricultural production (Bakhshandeh et al., 2017). However, it was reported that only 10–25% of applied phosphate fertilisers can be used by plants, while the rest were immobilised through precipitation reactions. Once precipitated, the soluble inorganic phosphates in fertilisers became chemically insoluble therefore biologically unavailable to plants.

Phosphorous could also be immobilised through the formation of organic matter. Richardson (1994) estimated that the organic matter accounted for 20–80% of total soil phosphorous content. The major form of organic phosphorous in soil is inositol phosphate, which is synthesised by microorganisms and plants (Rodríguez and Fraga, 1999). Other forms of soil organic phosphorous compounds include phosphoesters, such as phospholipids and nucleic acids, and xenobiotic phosphonates, which are widely used pesticides and antibiotics. Most of these organic phosphorous compounds have a high molecular weight thus can only be assimilated by plant cells after being converted or degraded to soluble ionic phosphates or low molecular weight substances.

Clearly, a bridge is needed to fill the gap between soil phosphorous and plants. On one hand, the presence of inorganic and organic phosphates makes most agricultural soil large reservoirs of phosphorous. On the other hand, the low levels of bioavailability of soil phosphorous makes the nutrient a limiting factor for plant growth and development. To

become biologically available to plants, the insoluble soil phosphorous needs to be solubilised first.

Luckily, a solution that has the potential to fill this gap has been discovered. There are naturally occurring rhizospheric phosphate-solubilising microorganisms in soil. The evidence of such microorganisms can be dated back to 1903 (Khan et al., 2007). It is estimated that up to 40% of culturable soil microorganisms have the capacity to solubilise phosphate (Bulgarelli et al., 2013b). Compared to fungi, bacteria are far more effective in phosphate solubilisation. It was reported that fungi only account for 0.1–0.5% of the total phosphate solubilisation potential (Chen et al., 2006). Some well-known phosphate-solubilising bacteria (PSB) of genera *Bacillus, Pseudomonas* and *Rhizobium* have been identified from plant microbiomes (Hayat et al., 2010; Bulgarelli et al., 2013b; Otieno et al., 2015). The effects of PSB have been tested in the field. Sundara et al. (2002) reported a study where the application of the PSB strain *B. megatherium* var. *phosphaticum* at 10 kg per hectare on sugarcane farms led to a total yield increase of 12.6% when compared with the control. Moreover, due to PSB, the required dosage of phosphorous fertiliser was reduced by 25%, and 50% of the costly fertiliser (super phosphate) could be replaced by cheap fertilisers (rock phosphate). Similar increases in plant yield and phosphorous uptake have also been observed after applying PSB on maize (Henri et al., 2008). It was reported that applying PSB in conjunction with other PGP bacteria could reduce the usage of phosphorous fertilisers by 50% without any significant reduction in yield (Yazdani et al., 2009). After conducting economic analysis, the value to cost ratio of applying PGP bacteria including PSB was about 2.5 times higher than that of applying chemical fertilisers alone (Jilani et al., 2007). Such results clearly showed that applying PSB is a very promising way to improve plant growth and prevent the ecological damage of excess chemical fertilisers.

The mechanisms used by PSB to solubilise inorganic and organic phosphates have been studied for decades and now are well documented. The ability of solubilising inorganic phosphates (such as tricalcium phosphate, dicalcium phosphate and rock phosphate) has been found in many PSB strains of genera *Pseudomonas, Bacillus, Rhizobium, Agrobacterium, Achromobacter, Burkholderia* and *Erwinia* (Goldstein, 1986; Rodríguez and Fraga, 1999; Rodríguez et al., 2006). Such solubilisation is achieved through the production of organic acids. Gluconic acid is the main organic acid that is related to inorganic phosphates solubilisation by *Pseudomonas* spp., *E. herbicola* and *Burkholderia cepacia* (Rodríguez and

Fraga, 1999). Direct oxidation of glucose to gluconic acid carried out by the glucose dehydrogenase and pyrroloquinoline quinone has been proposed by Goldstein (1994) as the major mechanisms for Gram-negative bacteria to solubilise inorganic phosphates. Several genes and plasmids that are involved in this process have also been identified (Babu-Khan et al., 1995; Kim et al., 1998; Krishnaraj and Goldstein, 2001; Buch et al., 2010). Other organic acids including 2-ketogluconic acid, lactic acid, acetic acid, glycolic acid, oxalic acid, malonic acid and citric acid have also been identified in PSB, and some *Bacillus* spp. strains could use mixtures of them to solubilise mineral phosphates (Chen et al., 2006; Hayat et al., 2010). In addition to acid production, it has been reported that a genetically modified *Escherichia coli* strain with a clone of DNA segment containing the phosphate-solubilisation genes from *Pantoea agglomerans* showed the bioactivity without changing the pH of the medium when compared to the control *E. coli* strain, indicating the absence of excreted acids (Kim et al., 1997). This study signified the complexity of mechanisms used by PSB, and more research is required to get an in-depth understanding.

Enzymes play a central role in solubilising organic phosphates by PSB. The nonspecific phosphatases and phytases are two major enzymes due to the high abundance of their substrates in soil (Rodríguez et al., 2006). The nonspecific phosphatases release phosphorous from soil organic matters via dephosphorylation of the phospho-ester or phospho-anhydride bonds (Thaller et al., 1995). The phytases can release phosphorous from phytate, which is the major form of phosphorous storage in plants (Richardson and Hadobas, 1997). Other enzymes that have been reported to be involved in organic phosphates solubilisation include phosphonatases, C-P lyases, phosphonoacetate hydrolase and D-α-glycerophosphatase (Ohtake et al., 1996; McGrath et al., 1998; Skraly and Cameron, 1998; Lugtenberg and Kamilova, 2009).

Both phenotype- and genotype-based methods have been developed to detect PSB strains. The presence of PSB strains can be easily determined in the lab phenotypically using *in vitro* assays. They form halo zones around the colonies after being cultured onto solid media containing insoluble forms of phosphorus, such as tricalcium phosphate in the Pikovskaya Agar (Doilom et al., 2020; Mohd Din et al., 2020). The genomic evidence of PSB strains can be obtained by assessing the genes involved in phosphate solubilisation, such as glucose dehydrogenase (the *gcd* gene), gluconate dehydrogenase (the *gad* gene) and phytases, using

techniques including PCR , whole genome sequencing and gene expression analyses (de Werra et al., 2009; Rasul et al., 2019; Brito et al., 2020; Silva et al., 2021).

1.4.3 Phytohormone-mediated growth promotion

Phytohormones are organic molecules that regulate the physiological, morphological and biochemical statues of plants (Amara et al., 2015). Phytohormones are categorised in five major classes, i.e. auxin, ethylene, cytokinin, gibberellin and abscisic acid, with other minor classes like jasmonate and salicylic acid (Santner and Estelle, 2009; Maheshwari et al., 2015). Although they are generally present at extremely low concentrations, phytohormones play a critical role in regulating plant metabolism, including cell division and elongation, root initiation and development, leaf expansion, flowering, seed germination and stress response (Hayat et al., 2010; Spaepen, 2015; Egamberdieva et al., 2017). Many PGP bacteria isolated from soil microbiomes or plant microbiomes have been reported to be associated with phytohormone-mediated growth promotion. For example, *Azospirillum* spp. strains are capable of producing all five major classes of phytohormones mentioned above (Cassán et al., 2014). Spaepen et al. (2008) inoculated wheat with the wild-type *A. brasilense* Sp245 strain which led to 14% increase in dry weight when compared to plants inoculated with the mutant strain that was defective in phytohormone production. Tomato seeds inoculated with *Sphingomonas* sp. LK11 which produces auxin and gibberellin were 14.5% higher in chlorophyll content and 37.1% higher in dry weight when compared to the uninoculated control (Khan et al., 2014). Besides direct growth promotion, Kudoyarova et al. (2014) demonstrated that the amino acid deposition of wheat roots can be enhanced by the cytokinin-producing strain *B. subtilis* IB-22, which may lead to improvements of root colonisation by PGP bacteria in soil. It has also been reported that the interactions between leguminous plants and *Rhizobium* spp. are regulated by phytohormones (Ferguson and Mathesius, 2014). Given these beneficial outcomes, the phytohormone-mediated growth promotion of PGP bacteria has been associated with sustainable agriculture and has become a research hotspot (Maheshwari et al., 2015).

Phytohormone-mediated growth promotion is conducted by PGP bacteria mainly via phytohormone biosynthesis whose mechanisms have been extensively studied, especially auxin. It has been discovered that tryptophan plays a central role in the bacterial biosynthesis of indole-3-acetic acid (IAA), which is the most important naturally-occurring auxin (Patten

et al., 2013; Spaepen, 2015). As reviewed by Patten et al. (2013) and Duca et al. (2014), PGP bacteria convert tryptophan to IAA via three major pathways: the indole pyruvate (IPyA) pathway, the indole acetamide (IAM) pathway and the indole acetaldoxime (IAOx)/indole acetonitrile (IAN) pathway. In the IPyA pathway, aminotransferases convert tryptophan to indole-3-pyruvate, which is then converted to indole-3-acetaldehyde by indolepyruvate decarboxylase and finally to IAA by NAD-dependent aldehyde dehydrogenase. In the IAM pathway, tryptone 2-monooxygenases convert tryptophan to amide indole-3-acetamide, which is subsequently converted to IAA by indole-3-acetamide hydrolase. In the IAOx/IAN pathway, tryptophan is converted first to indole acetaldoxime and then to indole acetonitrile, which is consequently converted to IAA by nitrilases. However, compared to the IPyA and IAM pathways, the bacterial enzymes involved in the IAOx/IAN pathway have not been well characterised (Duca et al., 2014). These biosynthesis pathways have been discovered from a diverse range of PGP bacteria, including *Azospirillum* spp. (Cassán and Diaz-Zorita, 2016), *Arthrobacter* spp. (Li et al., 2018), *Streptomyces* spp. (Lin and Xu, 2013) and *Bacillus* spp. (Shao et al., 2015). It is notable that a bacterial strain can have more than one of the IAA biosynthesis pathways described above. For example, an *A. brasilense* strain which was defective in producing indolepyruvate decarboxylase was still able to produce about 10% of IAA when compared to the wildtype strain (Spaepen et al., 2007). Furthermore, IAA not only plays an important role in plants, but also acts as signal molecules in bacteria and contributes to bacterial resistance to stress conditions, suggesting that producing IAA via multiple pathways can be an evolutionary advantage for bacteria associated with plants (Duca et al., 2014). As for other classes of phytohormone, although the biosynthesis pathways used by PGP bacteria have been proposed based on the biosynthesis pathways used by plants and fungi, the genetic evidence still requires further examination and assessment (Maheshwari et al., 2015; Spaepen, 2015).

Phytohormone-mediated growth promotion is also conducted by PGP bacteria via repressing ethylene biosynthesis in plants. Ethylene is a gaseous phytohormone which has positive effects on almost all stages of plant development when at low concentrations (Maheshwari et al., 2015). However, the biosynthesis of ethylene by plants is stimulated by stress conditions, leading to inhibitory effects on plant development such as root growth (Gamalero and Glick, 2012). The biosynthesis of ethylene in plants consists of two reaction steps, with 1-aminocyclopropane-1-1carboxylic acid (ACC) being the intermediate products (Houben and Van de Poel, 2019). PGP bacteria lower the ethylene levels by acting as an alternative sink

for ACC using ACC deaminases which convert ACC to ammonia (Glick, 2014). This enzyme has been discovered from many PGP bacterial strains isolated from soil and plant microbiomes (Bal et al., 2013; Gupta and Pandey, 2019). Ali et al. (2014) inoculated tomato plants growing under salt stress with ACC deaminase-producing *P. fluorescens* strains and observed significant increases in biomass, chlorophyll content and number of flowers and buds. Besides, PGP bacteria also lower the ethylene levels by repressing the expression of plant ACC oxidase which converts ACC to ethylene (Houben and Van de Poel, 2019). A transcriptome analysis conducted by Camilios-Neto et al. (2014) showed that inoculating an *A. brasilense* strain into wheat was able to repress the expression of ACC oxidase by 3.1-fold while increasing the root mass by up to 30%, when compared to the uninoculated control. Overall, by lowering the accumulation of excessive ethylene in plants using the two mechanisms, PGP bacteria can enhance stress response and promote growth of plants.

Similar to biological nitrogen fixation and phosphate solubilisation, *in vitro* assays have been developed to determine the phytohormone-mediated growth promotion activities of PGP bacteria. Usually, candidate strains are cultured in liquid media, from which supernatant is extracted and analysed using colorimetric assays or high-performance liquid chromatography for the production of phytohormones (Patten and Glick, 2002; Chaiharn and Lumyong, 2011). The genomic evidence can be obtained by sequencing the genome and/or transcriptome of candidate strains, and screening for the genes encoding the enzymes described above, such as the *ipdC* gene for indolepyruvate decarboxylase and the *iaaM* gene for tryptone 2-monooxygenases.

1.4.4 Bioprotection

It was estimated that 10–16% of global agricultural harvest, which equals to around 220 billion US dollars, is lost every year because of plant diseases caused by fungi, bacteria and viruses (Strange and Scott, 2005; Oerke, 2006). In Australia, such phytopathogens are responsible for an estimated average loss of 252 million dollars per year in the barley industry and 913 million dollars per year in the wheat industry during the period of 1998–2009 (Murray and Brennan, 2009a; b). A survey conducted in 2005–2006 season showed that the crown and root disease caused by fungi and oomycetes destroyed up to 50% of strawberry plants on the worst-infected farms in Western Australia, which accounted for up to 72% of Australia's strawberry exports (Phillips and Golza, 2008). As for the diseases of pasture

grasses in Australia, although they were categorised as insignificant and not worth controlling before 1970 (Sloane and King, 1988), huge attention has been paid to them since the introduction of foreign pasture species and emerging evidences of diseases. Early studies pointed out that the diseases related to perennial ryegrass included bacterial wilt, crown rust, stem rust and blind seed disease, with related pathogens including *Xanthomonas translucens* pv. *graminis*, *Puccinia* spp., *Drechslera* spp., *Helminthosporium* spp. and *Fusarium culmorum* (Schmidt, 1988; Clarke and Eagling, 1994). Another recent review showed that 21 viruses, which could infect 36 pasture or forage grass species and 59 wild grass species, have been identified in Australia (Jones, 2013). Several approaches have been used to control phytopathogens and diseases. Agrochemicals are trusted by most farmers as a relatively reliable control method. However, the increased and prolonged usage of agrochemicals could lead to the development of resistance in pathogens and other off-target environmental impacts, thus is negatively perceived by consumers (Gerhardson, 2002; Compant et al., 2005a; Grimmer et al., 2015). Hence, similar to nitrogen and phosphorous fertilisers, studies have been conducted to substitute the artificial chemicals with naturally occurring bioprotectant microorganisms isolated from plant and soil microbiomes. Compared with agrochemicals, using bioprotectant microorganisms is environmentally friendly and is subject to less regulatory scrutiny (Bach et al., 2016), representing a promising way to minimize the effects of phytopathogens on agricultural crops and pasture grasses.

Bioprotection, or biological control, refers to the use of living organisms to supress deleterious or pathogenic organisms (Bulgarelli et al., 2013a). Many PGP bacterial strains, especially those belong to genera *Bacillus* and *Pseudomonas*, have been proved to have bioprotection activities against phytopathogens and have been commercialised for years (Berg, 2009). It has long been discovered that mixing a small amount of suppressive soil, which contains bioprotectant bacteria that could protect plant from diseases, with a large amount of conducive soil, which contains pathogens that cause diseases, would make the latter soil suppressive (Schroth and Hancock, 1982; Lugtenberg and Kamilova, 2009). Whilst the bioprotection activity against phytopathogens is a complex process that involves the PGP bacterial strains, the pathogen strains, the plant host, the soil as well as the corresponding microflora (other soil bacteria) and macroflora (such as protozoa and nematodes), some of the major mechanisms used by PGP bacteria to achieve bioprotection have been revealed (Compant et al., 2005a; Haas and Défago, 2005; Berg et al., 2014).

PGP bacteria protect plants directly via microbial antagonism. Such antagonism is conducted by competing for nutrients and spaces for colonisation on the root surface (Kamilova et al., 2005). Moreover, PGP bacteria also synthesise allelochemicals, such as secondary metabolites like antibiotics and siderophores, to supress pathogens (Beneduzi et al., 2012). Antibiotics are a diverse group of low molecular weight organic compounds that have detrimental effects on the metabolic activities of other organisms (Duffy et al., 2003). Producing antibiotics is the most common weapon used by PGP bacteria to fight against phytopathogens (Glick et al., 2007). Antibiotics that have been found to be related to the bioprotection activities of PGP bacteria included phenazines (e.g. phenazine-1-carboxylic acid and phenazine-1carboxamide), phloroglucinols, pyoluteorin, pyrrolnitrin, cyclic lipopeptides, hydrogen cyanide, zwittermycin A, kanosamine, 2-hexyl-5-propyl resorcinol, gluconic acid and lipopeptide biosurfactants (Milner et al., 1996; Nielsen and Sørensen, 2003; Emmert et al., 2004; Haas and Défago, 2005; Cazorla et al., 2006; Kaur et al., 2006; Mavrodi et al., 2006; Ongena et al., 2007; Choi et al., 2009). The modes of action of such antibiotics include inhibiting the cell wall synthesis, altering the structure of membranes and interfering in the formation of the small subunit of the ribosome (Maksimov et al., 2011). Siderophores are small peptides that can solubilise and extract iron from most mineral or organic complexes due to the presence of side chains and functional groups that have a high affinity when coordinating ferric ions (Crosa and Walsh, 2002; Wandersman and Delepelaire, 2004). Iron can be chelated by siderophores from surrounding environments and then moved back to the cell surface of the microorganisms (Andrews et al., 2003). It has been discovered that some plants can enhance their iron uptake by recognising and utilising bacterial ferric-siderophore complex (Vansuyt et al., 2007; Dimkpa et al., 2009). Given the fact that bacterial siderophores have a higher affinity to iron when compared to fungal siderophores (Compant et al., 2005a), PGP bacteria produce siderophores under iron-limiting environments to competitively acquire iron and consequently deprive fungal pathogens of this essential element. A good example of such PGP bacterial strains are fluorescent pseudomonads, whose bioprotection activities on fungal pathogens like *F. oxysporum* via producing siderophores have been reported during the past decades (Kloepper et al., 1980; Dwivedi and Johri, 2003). Other allelochemicals used by PGP bacteria to attack phytopathogens included bacteriocins and lytic enzymes (Singh et al., 1999; Abriouel et al., 2010). In addition to microbial antagonism, PGP bacteria can also protect plants indirectly through induced systemic resistance (ISR) in the plant. The interactions between plants and PGP bacteria can induce resistance against phytopathogens via the production of signal molecules (van Loon, 2007;

Pascale et al., 2020). Many ISR-inducing PGP bacterial strains of genera *Pseudomonas* and *Bacillus* have been well characterised and reviewed (Kloepper et al., 2004; van Loon, 2007; Beneduzi et al., 2012).

Phenotypic assays, both *in vitro* and *in planta*, have been designed to detect bioprotectant PGP bacteria from plant and soil microbiomes. In *in vitro* assays, candidate bacterial strains are co-cultured with phytopathogens and growth reduction or elimination of the pathogens are recorded (Diaz Herrera et al., 2016). In *in planta* assays, plants are inoculated with both candidate bacterial strains and phytopathogens and disease symptoms are assessed (Li et al., 2020; Pellegrini et al., 2020) Assessing the genomic evidence of bioprotection requires sequencing and annotating the genome of candidate strains to identify the biosynthetic genes of secondary metabolites associated with their bioactivity, and characterizing the bioactive compounds using metabolomics techniques (Zachow et al., 2015; Vater et al., 2018).

1.5 Comprehensive examination of interactions between host plant and plant microbiome

It is known that complex interactions involving genetic and metabolic regulations exist between host plant and its microbiome (Carvalho et al., 2016). Although the studies described above provided detailed characterisations of specific bioactivities and aspects of the interaction (such as BNF) of PGP bacteria, most of them failed to provide a comprehensive profile of the interactions between the host plant and bacteria, e.g. regulated expression of genes involved in the plant-bacteria interaction. Such detailed information can be obtained by conducting transcriptome- and proteome-based assays, and can enhance our understandings of the plant-bacteria interaction (Schenk et al., 2012). For example, Drogue et al. (2014) inoculated two rice cultivars with two PGP bacterial strains, *A. lipoferum* 4B and *Azospirillum* sp. B510, and profiled the root transcriptome after seven days. They discovered 34 genes that were regulated by both strains in both cultivars, suggesting the presence of representative gene markers associated with rice-*Azospirillum* interactions. Moreover, compared to the seed-associated strain *A. lipoferum* 4B, the endophytic strain *Azospirillum* sp. B510 repressed the expression of multiple plant stress and defence genes. Similar repressions of plant defence genes were also observed from *A. thaliana* inoculated with the PGP bacterium *B. subtilis* FB17 when compared to the uninoculated control, suggesting the potential associations between repressed plant defence genes and stable root colonisation of PGP bacteria (Lakshmanan et al., 2013). Banaei-Asl et al. (2015) conducted proteome

analyses of root of canola inoculated with *P. fluorescens* and identified 55 proteins that may be associated with the increases of dry weight under salt stress in inoculated plants.

Comprehensive profiles of plant-bacteria interactions can be conducted by examining both the plant and bacteria simultaneously. Lery et al. (2011) conducted a proteome study of interactions between two sugarcane cultivars and an endophytic PGP bacterium *Gluconacetobacter diazotrophicus*. A total of 78 differentially expressed plant proteins and nine differentially expressed bacterial proteins were identified. Moreover, it was discovered that the cultivars of sugarcane were associated with the varying responses to the inoculation of *G. diazotrophicus* as well as the BNF activity of *G. diazotrophicus*. As for transcriptome-based studies, Camilios-Neto et al. (2014) conducted the first dual RNA-seq profiling of plants and PGP bacteria using wheat roots inoculated with an *A. brasilense* FP2 strain. A total of 23,215 and 702 expressed wheat and bacterial genes were identified, respectively. The differentially expressed wheat genes caused by the bacterial strain were associated with plant defence, biosynthesis of phytohormones and nutrient transport. The differentially expressed bacterial genes caused by wheat roots were associated with chemotaxis, biofilm formation and BNF. The revealed transcriptome profiles also provided possible explanations to the overall PGP bioactivities exhibited by the bacterial strain, such as the increased root length. The second dual RNA-seq study of plants and PGP bacteria was conducted recently by Liu et al. (2020). Inoculating tobacco with *P. polymyxa* YC0136 induced the expression of phytohormone-related genes in both the plant and the bacterium. Moreover, the bacterium also induced the expression of stress resistance and systemic resistance genes in plant. These studies have demonstrated that transcriptome- or proteome-based assays should be conducted alongside with assays that focus on specific traits to obtain a comprehensive understanding of interactions between plant and PGP bacteria.

1.6 Perennial ryegrass microbiome

Studies of the microbiome of pasture grasses can be dated back to decades ago, with a focus on the symbiotic *Epichloë* fungal endophytes (Bacon et al., 1977). *Epichloë* spp. endophytes form a symbiotic relationship with pasture grasses of *Poaceae* species, such as perennial ryegrass (*L. perenne*) and tall fescue (*L. arundinaceum* or *Festuca arundinacea*) and produce a wide range of bioactive alkaloids. Whilst some of the produced alkaloids like lolitrem B are toxic to mammals and cause ryegrass staggers in grazing animals (Philippe, 2015; 2016), it

has been discovered that other alkaloids like peramine are associated with improved resistance of plants to biotic stresses, such as invertebrate pests, and abiotic stresses, such as drought (Bush et al., 1997; Tanaka et al., 2012; Ma et al., 2015). Moreover, these *Epichloë* fungal endophytes from the parent plants can be vertically transmitted to progeny seeds, providing persistent beneficial activities. Such unique characteristics have made pasture grass seeds with introduced *Epichloë* spp. endophytes commercially available to farmers (Johnson et al., 2013), and have oriented studies that discovered novel strains of *Epichloë* endophytes and enhanced the beneficial associations of known strains (Kaur et al., 2015; Hettiarachchige et al., 2019).

Unlike the fungal microbiome, the bacterial microbiome of pasture grasses has only been characterised recently. Chen et al. (2016) characterised the root-associated bacterial microbiome of perennial ryegrass in a rhizobox experiment using two soil types (red soil vs. fluvo-aquic soil) under two CO_2 conditions (ambient vs. elevated). The results of this study demonstrated a spatial gradient from the bulk soil to the plant endosphere associated with the bacterial microbiome. For instance, while little structural differences were observed between the bacterial microbiome of bulk soil and outer rhizosphere, the diversity of the bacterial microbiome decreased along the spatial gradient, with the endosphere microbiome having the lowest phylogenetic diversity and observed species richness regardless of the soil type or the CO_2 condition. Furthermore, an enrichment of *Methylobacterium* spp., *Rhizobium* spp., *Pseudomonas* spp., *Stenotrophomonas* spp. and *Enterobacter* spp. that may be associated with PGP bioactivities was identified along the spatial gradient. Overall, the study stated that soil types are a key determinant of the structure of bacterial microbiome when compared to CO_2 conditions, and supported the two-step selection model utilised by plants to recruit microorganisms from soil (bulk soil – rhizoplane – endosphere).

Tannenbaum et al. (2020) recently characterised the bacterial microbiome associated with perennial ryegrass for a complete life cycle, namely the original seeds (generation 1), the subsequent mature plants and the seeds produced by those mature plants (generation 2). Two commercial seed batches were used, including one batch containing a fungal endophyte *Epichloë festucae* subsp. *lolii* and another batch being free of fungal endophytes. Results showed that the bacterial microbiome of generation 1 seeds was dominated by *Gammaproteobacteria*. However, mature plants had a much more diverse and complex bacterial microbiome when compared to that of generation 1 seeds, having 18 to 37 classes in

total and unique classes of bacteria associated with different growth conditions, i.e. sand and soil. Moreover, most of the detected OTUs in the microbiome of mature plants were absent from the microbiome of generation 1 seeds, suggesting the recruitment of bacteria from growth media. Such results clearly demonstrated the effects of growth conditions on the microbiome composition. Interestingly, despite the differences in the microbiome of mature plants, the microbiome of generation 2 seeds resembled the microbiome of generation 1 seeds with the addition of class *Bacilli*. Such similarities suggested that plants have strict selection criteria to control the composition of the microbiome of seeds during seed generation. This study also identified that the microbiome of shoots was in general a subset of the microbiome of roots, and the fungal endophyte had a weak effect on the microbiome of mature plants, which is similar to the study conducted by Nissinen et al. (2019). Overall, this study revealed a core microbiome of perennial ryegrass that persisted through two plant generations, which is subject to future characterisation for potential PGP bioactivities.

1.7 Bacterial strains characterised in this study

Given hundreds of bacterial strains have been isolated from the perennial ryegrass microbiome (Tannenbaum et al., 2020), the next key question awaiting to be addressed is their functions. Preliminary identification using matrix-assisted laser desorption ionization time-of-flight (MALDI-TOF) mass spectrometry suggested that they represented complex phylogenetic diversities (Li et al., 2021). Six strains were selected to be characterised in this PhD study since they are commonly associated with agricultural plants and crops, including three strains representing a novel species of *Xanthomonas* spp., two strains representing a novel species of *Paenibacillus* spp. that is genetically closely related to *Paenibacillus polymyxa*, as well as one novel strain of *Erwinia gerundensis*. This section will briefly introduce these species.

1.7.1 *Xanthomonas* spp.

Xanthomonas spp. are rod-shaped, Gram-negative bacteria belonging to the class *Gammaproteobacteria*. They produce a pigment called xanthomonadin which leads to their characteristic yellow colonies (Poplawsky et al., 2000). The genus *Xanthomonas* consists of at least 27 known species, and is commonly associated with plants (Ryan et al., 2011). *Xanthomonas* spp. have been well characterised as phytopathogens. They can infect more

than 400 plant species, including many important agricultural crops and pasture grasses, causing diseases like bacterial wilts, blights, cankers and spots (Dunger et al., 2016). Moreover, diseases caused by *Xanthomonas* spp. are known to be highly host- and tissue-specific, suggesting the adaptation to different plants by *Xanthomonas* spp. (Ryan et al., 2011; Hersemann et al., 2017). Interestingly, while many strains of *Xanthomonas* spp. have been described as pathogenic, there has been an increasing number of non-pathogenic strains being isolated from plants. For example, Garita-Cambronero et al. (2017) reported a non-pathogenic strain of *X. arboricola* which causes bacterial spot on stone fruits. Other non-pathogenic strains have also been reported from novel *Xanthomonas* species isolated from watercress and walnut (Vicente et al., 2017; Martins et al., 2020). Li et al. (2020) reported three strains of a novel *Xanthomonas* species isolated from perennial ryegrass that are not only non-pathogenic due to the lack of essential pathogenicity factors, but also beneficial because of their bioprotection activities against phytopathogens. Since *Xanthomonas* spp. have been discovered from the microbiome of many plant species (Bouffaud et al., 2014; Bulgarelli et al., 2015; Tannenbaum et al., 2020), the presence of non-pathogenic strains and beneficial strains suggested that future studies are required to expand our understanding of the ecological role of *Xanthomonas* spp. in plant microbiome.

1.7.2 *Paenibacillus polymyxa*

Paenibacillus spp. are Gram-positive, sporulating bacteria that are commonly associated with soil and plants. As the type species of the genus *Paenibacillus*, *P. polymyxa* strains have been isolated from the rhizosphere or plants in Europe, Asia and North America (Trüper, 2005; Grady et al., 2016). Unlike *Xanthomonas* spp., *P. polymyxa* strains have long been described as PGP bacteria which colonise the root of plants and form beneficial interactions with plants, with bioactivities mainly associated with BNF and bioprotection (Timmusk et al., 2005; Xie et al., 2016; Hao and Chen, 2017; Wang et al., 2020). It has been discovered that *P. polymyxa* strains produce a diverse range of secondary metabolites, including the bioprotectant peptides like polymyxin and fusaricidin (Choi et al., 2009; Lee et al., 2012b) as well as novel secondary metabolites whose functions remain to be characterised (Wang et al., 2020). Moreover, some *P. polymyxa* strains also have genes associated with phosphate solubilisation and IAA production (Eastman et al., 2014). Some *P. polymyxa* strains have been well-characterised. For instance, the PGP activities of *P. polymyxa* P2b-2R have been validated on canola, maize, tomato, cedar and pine (Anand and Chanway, 2013; Anand et al., 2013; Padda

et al., 2016b; a; Puri et al., 2016). There has been ten published genome and proteome studies of *P. polymyxa* E681, with focuses on its bioactive secondary metabolites and production of IAA. The results of these studies suggested that *P. polymyxa* strains have a wide spectrum of PGP bioactivities, representing ideal candidates to be developed as biofertilisers and bioprotectants. As of the time of writing, there are 56 published genomes of *P. polymyxa* strains on NCBI, none of which was isolated from Australia. The chapters 3 and 4 of this thesis described the isolation of two novel *Paenibacillus* sp. strains that were genetically closely related to *P. polymyxa* in experiments to isolate nitrogen-fixing bacteria from perennial ryegrass seeds, and their further characterisation. This work may promote the local application of this novel PGP bacterial species.

1.7.3 *Erwinia gerundensis*

Erwinia spp. are rod-shaped, Gram-negative and non-spore-forming bacteria that are commonly associated with plants. Similar to *Xanthomonas* spp., while a large number of *Erwinia* species are phytopathogens (e.g. *E. amylovora* and *E. persicina*), some *Erwinia* spp. have been characterised as non-pathogenic plant epiphytes (Geider et al., 2006; Smits et al., 2011; Zhang and Nan, 2014). *E. gerundensis* is a newly identified *Erwinia* species reported by Rezzonico et al. (2016). The strain was initially isolated together with more than 200 isolates from plants in Spain and was found to have no bioactivity in the bioprotection assay. Preliminary phylogenetic analyses showed that the strain represented a separated clade when compared with known *Pantoea* spp. and *Erwinia* spp., suggesting a possible new species. The identification of a novel species was later confirmed by genomic evidence. Further analyses conducted by this study suggested that additional strains of *E. gerundensis* have been isolated from *Pyrus* sp., *Malus* sp., and *Triticum* sp. in Australia. Moreover, novel strains of *E. gerundensis* have been isolated from the recently profiled perennial ryegrass microbiome (Tannenbaum et al., 2020). Preliminary results of assays showed that one of the novel strains has bioprotection activities against phytopathogens and is able to promote root growth of barley under glasshouse conditions (Appendix 1, section 1.3). The identification of these novel strains demonstrated the genetic and ecological diversity of *E. gerundensis*. Further studies are required to assess the possible PGP activities associated with this species.

1.8 Research plan

To date, in depth studies have been conducted to characterise individual bacterial and fungal strains and their interactions with plants, with focuses on phytopathogens and symbiotic diazotrophic bacteria such as *Rhizobium* spp. (Mendes et al., 2013). However, such knowledge is still limited for other members of the plant microbiome. Understanding the functions of these microorganisms is critical for improving plant health and growth. The microbiome of perennial ryegrass (*L. perenne* L. cv. Alto) has been recently profiled, revealing a complex bacterial community containing around 500 genera and unique community structures being associated with seeds as well as mature plants grown in different media (Tannenbaum et al., 2020). This study also isolated 284 bacterial strains from seeds, and more bacterial strains have subsequently been isolated from mature plants. These bacterial strains represented an excellent source from which potential PGP bacteria could be identified and characterised. Six strains representing three species that are commonly associated with agricultural plants and crops were selected to be characterised in this PhD study. The fundamental aim is to develop and conduct various assays to evaluate the PGP bioactivities of these strains as well as examine their interactions with plants, and to develop the genomic and transcriptomic evidence to support the bioactivity and interaction. The outcome of this study will lay a solid foundation to enable further characterisation of other bacterial members of the perennial ryegrass microbiome, provide invaluable insights to the molecular basis of PGP bioactivities and eventually enhance the beneficial bioactivities and interactions to improve the performance of perennial ryegrass as well as other agricultural crops and pasture grasses.

This project will initially develop *in vitro* and *in planta* assays to assess the bioprotection activities of bacterial strains against a wide range of phytopathogens of agricultural crops and pasture grasses. *In vitro* assays will be conducted first to select highly bioactive strains, which are then tested using *in planta* assays. *In vitro* assays using nitrogen-free media and PCR assays using published primers will be used to detect nitrogen-fixing bacteria. To examine the early stage plant-bacteria interactions, dual RNA-seq assays will be designed using bacterial strains and barley seedlings co-incubated in different media. *In silico* assays will be developed and conducted to provide the genomic and transcriptomic evidence to support the bioactivities identified in the *in vitro* and *in planta* assays. The genomes of all strains used in this study will be sequenced, assembled and annotated. Comparative genomics

analyses will be performed to taxonomically identify the strains. Genes associated with PGP bioactivities and the biosynthesis of secondary metabolites will be identified. Transcriptomic analyses will be used to examine the activity of the *nif* operon associated with biological nitrogen fixation and the core biosynthetic genes of secondary metabolite associated with bioprotection. The transcriptomic response of barley seedlings and bacterial strains during their early stage interactions will also be examined. Using perennial ryegrass seedlings in the assay requires a large number of biological replicates due to their heterozygosity. As barley cultivars are largely homozygous, a barley-based assay reduces host genotype variability and can validate the design of the assay which can be then adapted for perennial ryegrass in future.

1.9 Project aims

The microbiome of perennial ryegrass seeds and mature plants has been profiled, from which over 300 strains have been isolated. Six bacterial strains, including three novel *Xanthomonas* sp. strains, two novel *Paenibacillus* sp. strains and one novel *E. gerundensis* strain, were characterised using a series of *in vitro*, *in planta* and *in silico* assays, addressing the following project aims:

To design *in vitro* and *in planta* assays to evaluate the bioprotection activities of the three novel *Xanthomonas* sp. strains, and characterise the three strains using genomic techniques, including genome sequencing, assembly, annotation and comparison, to demonstrate the absence of genes associated with pathogenicity and to reveal genes and secondary metabolites may be associated with their bioprotection activities (Chapter 2).

To detect and isolate nitrogen-fixing bacterial strain(s) associated with perennial ryegrass seeds and evaluate their bioprotection activities using *in vitro* assays. To characterise the isolated strain(s) using genomic and transcriptomic techniques to identify genes associated PGP bioactivities, such as BNF, and bioprotection activities, such as the production of bioactive secondary metabolites (Chapter 3).

To design *in planta* assays as well as dual RNA-seq analyses to reveal the molecular basis of the early stage plant-bacteria interactions under different conditions using barley seedlings and three bacterial strains isolated from perennial ryegrass seeds, which will enhance our

knowledge of the potential mechanisms utilised by PGP bacterial strains of the perennial ryegrass microbiome (Chapter 4).

1.10 References

Abriouel, H., Franz, C.M., Omar, N.B., and Gálvez, A. (2010). Diversity and applications of *Bacillus* bacteriocins. *FEMS Microbiology Reviews* 35**,** 201-232.

Adam, E., Bernhart, M., Müller, H., Winkler, J., and Berg, G. (2018). The *Cucurbita pepo* seed microbiome: genotype-specific composition and implications for breeding. *Plant and Soil* 422**,** 35-49.

Ahmad, F., Ahmad, I., and Khan, M.S. (2008). Screening of free-living rhizospheric bacteria for their multiple plant growth promoting activities. *Microbiological Research* 163**,** 173-181.

Ahmad, M., Nadeem, S.M., Naveed, M., and Zahir, Z.A. (2016). "Potassium-solubilizing bacteria and their application in agriculture," in *Potassium Solubilizing Microorganisms for Sustainable Agriculture,* eds. V.S. Meena, B.R. Maurya, J.P. Verma & R.S. Meena. (New Delhi: Springer India), 293-313.

Ali, S., Charles, T.C., and Glick, B.R. (2014). Amelioration of high salinity stress damage by plant growth-promoting bacterial endophytes that contain ACC deaminase. *Plant Physiology and Biochemistry* 80**,** 160-167.

Amara, U., Khalid, R., and Hayat, R. (2015). "Soil bacteria and phytohormones for sustainable crop production," in *Bacterial Metabolites in Sustainable Agroecosystem,* ed. D.K. Maheshwari. (Cham: Springer International Publishing), 87-103.

Anand, R., and Chanway, C. (2013). N2-fixation and growth promotion in cedar colonized by an endophytic strain of *Paenibacillus polymyxa*. *Biology and Fertility of Soils* 49**,** 235-239.

Anand, R., Grayston, S., and Chanway, C. (2013). N2-fixation and seedling growth promotion of lodgepole pine by endophytic *Paenibacillus polymyxa*. *Microbial Ecology* 66**,** 369-374.

Andrade, L.F., Larisse, G., De Souza, D., Nietsche, S., Xavier, A.A., Costa, M.R., Maria, A., Cardoso, S., Cristian, M., and Pereira, T. (2014). Analysis of the abilities of endophytic bacteria associated with banana tree roots to promote plant growth. *Journal of Microbiology* 52**,** 27.

Andrews, S.C., Robinson, A.K., and Rodríguez-Quiñones, F. (2003). Bacterial iron homeostasis. *FEMS Microbiology Reviews* 27**,** 215-237.

Arnold, W., Rump, A., Klipp, W., Priefer, U.B., and Pühler, A. (1988). Nucleotide sequence of a 24,206-base-pair DNA fragment carrying the entire nitrogen fixation gene cluster of *Klebsiella pneumoniae*. *Journal of Molecular Biology* 203**,** 715-738.

Australian Bureau of Statistics (2018). "Land management and farming in Australia, 2016-2017". 26/06/2018 ed.: Australian Bureau of Statistics).

Azevedo, J.L., Maccheroni Jr, W., Pereira, J.O., and De Araújo, W.L. (2000). Endophytic microorganisms: a review on insect control and recent advances on tropical plants. *Electronic Journal of Biotechnology* 3**,** 15-16.

Babu-Khan, S., Yeo, T.C., Martin, W.L., Duron, M.R., Rogers, R.D., and Goldstein, A.H. (1995). Cloning of a mineral phosphate-solubilizing gene from *Pseudomonas cepacia*. *Applied and Environmental Microbiology* 61**,** 972-978.

Bach, E., Seger, G.D.D.S., Fernandes, G.D.C., Lisboa, B.B., and Passaglia, L.M.P. (2016). Evaluation of biological control and rhizosphere competence of plant growth promoting bacteria. *Applied Soil Ecology* 99**,** 141-149.

Bacon, C.W., Porter, J.K., Robbins, J.D., and Luttrell, E.S. (1977). *Epichloë* typhina from toxic tall fescue grasses. *Applied and Environmental Microbiology* 34**,** 576-581.

Bais, H.P., Weir, T.L., Perry, L.G., Gilroy, S., and Vivanco, J.M. (2006). The role of root exudates in rhizosphere interactions with plants and other organisms. *Annual Review of Plant Biology* 57**,** 233-266.

Bakhshandeh, E., Pirdashti, H., and Lendeh, K.S. (2017). Phosphate and potassium-solubilizing bacteria effect on the growth of rice. *Ecological Engineering* 103**,** 164-169.

Bal, H.B., Das, S., Dangar, T.K., and Adhya, T.K. (2013). ACC deaminase and IAA producing growth promoting bacteria from the rhizosphere soil of tropical rice plants. *Journal of Basic Microbiology* 53**,** 972-984.

Baldani, J.I., Reis, V.M., Videira, S.S., Boddey, L.H., and Baldani, V.L.D. (2014). The art of isolating nitrogen-fixing bacteria from non-leguminous plants using N-free semi-solid media: a practical guide for microbiologists. *Plant and Soil* 384**,** 413-431.

Banaei-Asl, F., Bandehagh, A., Uliaei, E.D., Farajzadeh, D., Sakata, K., Mustafa, G., and Komatsu, S. (2015). Proteomic analysis of canola root inoculated with bacteria under salt stress. *Journal of Proteomics* 124**,** 88-111.

Banik, A., Dash, G.K., Swain, P., Kumar, U., Mukhopadhyay, S.K., and Dangar, T.K. (2019). Application of rice (*Oryza sativa* L.) root endophytic diazotrophic *Azotobacter* sp. strain Avi2 (MCC 3432) can increase rice yield under green house and field condition. *Microbiological Research* 219**,** 56-65.

Beneduzi, A., Ambrosini, A., and Passaglia, L.M. (2012). Plant growth-promoting rhizobacteria (PGPR): their potential as antagonists and biocontrol agents. *Genetics and Molecular Biology* 35**,** 1044-1051.

Benizri, E., Baudoin, E., and Guckert, A. (2001). Root colonization by inoculated plant growth-promoting rhizobacteria. *Biocontrol Science and Technology* 11**,** 557-574.

Berendsen, R.L., Pieterse, C.M., and Bakker, P.A. (2012). The rhizosphere microbiome and plant health. *Trends in Plant Science* 17**,** 478-486.

Berg, G. (2009). Plant–microbe interactions promoting plant growth and health: perspectives for controlled use of microorganisms in agriculture. *Applied Microbiology and Biotechnology* 84**,** 11-18.

Berg, G., Grube, M., Schloter, M., and Smalla, K. (2014). Unraveling the plant microbiome: looking back and future perspectives. *Frontiers in Microbiology* 5**,** 148.

Berg, G., and Raaijmakers, J.M. (2018). Saving seed microbiomes. *The ISME Journal* 12**,** 1167-1170.

Berg, G., Rybakova, D., Fischer, D., Cernava, T., Vergès, M.-C.C., Charles, T., Chen, X., Cocolin, L., Eversole, K., Corral, G.H., Kazou, M., Kinkel, L., Lange, L., Lima, N., Loy, A., Macklin, J.A., Maguin, E., Mauchline, T., Mcclure, R., Mitter, B., Ryan, M., Sarand, I., Smidt, H., Schelkle, B., Roume, H., Kiran, G.S., Selvin, J., Souza, R.S.C.D., Van Overbeek, L., Singh, B.K., Wagner, M., Walsh, A., Sessitsch, A., and Schloter, M. (2020). Microbiome definition re-visited: old concepts and new challenges. *Microbiome* 8**,** 103.

Bergna, A., Cernava, T., Rändler, M., Grosch, R., Zachow, C., and Berg, G. (2018). Tomato seeds preferably transmit plant beneficial endophytes. *Phytobiomes Journal* 2**,** 183-193.

Bodenhausen, N., Horton, M.W., and Bergelson, J. (2013). Bacterial communities associated with the leaves and the roots of *Arabidopsis thaliana*. *PLOS One* 8**,** e56329.

Bouffaud, M.L., Poirier, M.A., Muller, D., and Moenne-Loccoz, Y. (2014). Root microbiome relates to plant host evolution in maize and other Poaceae. *Environmental Microbiology* 16**,** 2804-2814.

Brito, L.F., López, M.G., Straube, L., Passaglia, L.M.P., and Wendisch, V.F. (2020). Inorganic phosphate solubilization by rhizosphere bacterium *Paenibacillus sonchi*: gene expression and physiological functions. *Frontiers in Microbiology* 11.

Buch, A., Archana, G., and Naresh Kumar, G. (2010). Heterologous expression of phosphoenolpyruvate carboxylase enhances the phosphate solubilizing ability of fluorescent pseudomonads by altering the glucose catabolism to improve biomass yield. *Bioresource Technology* 101**,** 679-687.

Bulgarelli, D., Garrido-Oter, R., Munch, P.C., Weiman, A., Droge, J., Pan, Y., Mchardy, A.C., and Schulze-Lefert, P. (2015). Structure and function of the bacterial root microbiota in wild and domesticated barley. *Cell Host Microbe* 17**,** 392-403.

Bulgarelli, D., Rott, M., Schlaeppi, K., Van Themaat, E.V.L., Ahmadinejad, N., Assenza, F., Rauf, P., Huettel, B., Reinhardt, R., and Schmelzer, E. (2013a). Revealing structure and assembly cues for *Arabidopsis* root-inhabiting bacterial microbiota. *Nature* 501**,** S25.

Bulgarelli, D., Schlaeppi, K., Spaepen, S., Van Themaat, E.V.L., and Schulze-Lefert, P. (2013b). Structure and functions of the bacterial microbiota of plants. *Annual Review of Plant Biology* 64**,** 807-838.

Bush, L.P., Wilkinson, H.H., and Schardl, C.L. (1997). Bioprotective alkaloids of grass-fungal endophyte symbioses. *Plant Physiology* 114**,** 1-7.

Camilios-Neto, D., Bonato, P., Wassem, R., Tadra-Sfeir, M.Z., Brusamarello-Santos, L.C.C., Valdameri, G., Donatti, L., Faoro, H., Weiss, V.A., Chubatsu, L.S., Pedrosa, F.O., and Souza, E.M. (2014). Dual RNA-seq transcriptional analysis of wheat roots colonized by *Azospirillum brasilense* reveals up-regulation of nutrient acquisition and cell cycle genes. *BMC Genomics* 15**,** 378.

Carvalho, T., Ballesteros, H., Thiebaut, F., Ferreira, P., and Hemerly, A. (2016). Nice to meet you: genetic, epigenetic and metabolic controls of plant perception of beneficial associative and endophytic diazotrophic bacteria in non-leguminous plants. *Plant Molecular Biology* 90**,** 561-574.

Cassán, F., and Diaz-Zorita, M. (2016). *Azospirillum* sp. in current agriculture: from the laboratory to the field. *Soil Biology and Biochemistry* 103**,** 117-130.

Cassán, F., Vanderleyden, J., and Spaepen, S. (2014). Physiological and agronomical aspects of phytohormone production by model plant-growth-promoting rhizobacteria (PGPR) belonging to the genus *Azospirillum*. *Journal of Plant Growth Regulation* 33**,** 440-459.

Cavaglieri, L., Orlando, J., and Etcheverry, M. (2009). Rhizosphere microbial community structure at different maize plant growth stages and root locations. *Microbiological Research* 164**,** 391-399.

Cazorla, F.M., Duckett, S.B., Bergström, E.T., Noreen, S., Odijk, R., Lugtenberg, B.J., Thomas-Oates, J.E., and Bloemberg, G.V. (2006). Biocontrol of avocado dematophora root rot by antagonistic *Pseudomonas fluorescens* PCL1606 correlates with the production of 2-hexyl 5-propyl resorcinol. *Molecular Plant-Microbe Interactions* 19**,** 418-428.

Chaiharn, M., and Lumyong, S. (2011). Screening and optimization of indole-3-acetic acid production and phosphate solubilization from rhizobacteria aimed at improving plant growth. *Current Microbiology* 62**,** 173-181.

Chaparro, J.M., Badri, D.V., Bakker, M.G., Sugiyama, A., Manter, D.K., and Vivanco, J.M. (2013). Root exudation of phytochemicals in *Arabidopsis* follows specific patterns

that are developmentally programmed and correlate with soil microbial functions. *PLOS One* 8**,** e55731.

Chen, L., Brookes, P.C., Xu, J., Zhang, J., Zhang, C., Zhou, X., and Luo, Y. (2016). Structural and functional differentiation of the root-associated bacterial microbiomes of perennial ryegrass. *Soil Biology and Biochemistry* 98**,** 1-10.

Chen, Y., Rekha, P., Arun, A., Shen, F., Lai, W.-A., and Young, C. (2006). Phosphate solubilizing bacteria from subtropical soil and their tricalcium phosphate solubilizing abilities. *Applied Soil Ecology* 34**,** 33-41.

Cherchali, A., Boukhelata, N., Kaci, Y., Abrous-Belbachir, O., and Djebbar, R. (2019). Isolation and identification of a phosphate-solubilizing *Paenibacillus polymyxa* strain GOL 0202 from durum wheat (*Triticum durum* Desf.) rhizosphere and its effect on some seedlings morphophysiological parameters. *Biocatalysis and Agricultural Biotechnology* 19**,** 101087.

Chislock, M.F., Doster, E., Zitomer, R.A., and Wilson, A. (2013). Eutrophication: causes, consequences, and controls in aquatic ecosystems. *Nature Education Knowledge* 4**,** 10.

Choi, S.K., Park, S.Y., Kim, R., Kim, S.B., Lee, C.H., Kim, J.F., and Park, S.H. (2009). Identification of a polymyxin synthetase gene cluster of *Paenibacillus polymyxa* and heterologous expression of the gene in *Bacillus subtilis*. *Journal of Bacteriology* 191**,** 3350-3358.

Clarke, R.G., and Eagling, D.R. (1994). Effects of pathogens on perennial pasture grasses. *New Zealand Journal of Agricultural Research* 37**,** 319-327.

Cole, I.A., and Johnston, W.H. (2006). Seed production of Australian native grass cultivars: an overview of current information and future research needs. *Australian Journal of Experimental Agriculture* 46**,** 361-373.

Compant, S., Clément, C., and Sessitsch, A. (2010). Plant growth-promoting bacteria in the rhizo- and endosphere of plants: their role, colonization, mechanisms involved and prospects for utilization. *Soil Biology and Biochemistry* 42**,** 669-678.

Compant, S., Duffy, B., Nowak, J., Clément, C., and Barka, E.A. (2005a). Use of plant growth-promoting bacteria for biocontrol of plant diseases: principles, mechanisms of action, and future prospects. *Applied and Environmental Microbiology* 71**,** 4951-4959.

Compant, S., Mitter, B., Colli-Mull, J.G., Gangl, H., and Sessitsch, A. (2011). Endophytes of grapevine flowers, berries, and seeds: identification of cultivable bacteria, comparison with other plant parts, and visualization of niches of colonization. *Microbial Ecology* 62**,** 188-197.

Compant, S., Reiter, B., Sessitsch, A., Nowak, J., Clément, C., and Ait Barka, E. (2005b). Endophytic colonization of *Vitis vinifera* L. by plant growth-promoting bacterium *Burkholderia* sp. strain PsJN. *Applied and Environmental Microbiology* 71**,** 1685-1693.

Compant, S., Samad, A., Faist, H., and Sessitsch, A. (2019). A review on the plant microbiome: ecology, functions, and emerging trends in microbial application. *Journal of Advanced Research* 19**,** 29-37.

Crosa, J.H., and Walsh, C.T. (2002). Genetics and assembly line enzymology of siderophore biosynthesis in bacteria. *Microbiology and Molecular Biology Reviews* 66**,** 223-249.

Dairy Australia (2014). *The Australian dairy industry in focus 2014*. Available: https://www.dairyaustralia.com.au/-/media/dairyaustralia/documents/about-dairy-australia/key-publications/2014-in-focus.pdf?la=en&hash=79F1C521F91DA07C21795F81D01EF587D00DCE8F [Accessed 21/09/2020]. ISSN 2202-7467.

Dairy Australia (2015). *Investment and the Australian dairy industry*. Available: http://www.dairyaustralia.com.au/~/media/Documents/Industry%20overview/About%20the%20industry/Investment%20guide/0538%20Investment%20and%20Australian%20Dairy%20Industry-20151218.pdf [Accessed 02/03/2017].

Dairy Australia (2019). *The Australian dairy industry in focus 2019*. Available: https://www.dairyaustralia.com.au/industry/farm-facts/in-focus [Accessed 22/01/2020]. ISSN 1839-0781.

Dawson, C.J., and Hilton, J. (2011). Fertiliser availability in a resource-limited world: production and recycling of nitrogen and phosphorus. *Food Policy* 36**,** S14-S22.

De Santi Ferrara, F.I., Oliveira, Z.M., Gonzales, H.H.S., Floh, E.I.S., and Barbosa, H.R. (2012). Endophytic and rhizospheric enterobacteria isolated from sugar cane have different potentials for producing plant growth-promoting substances. *Plant and Soil* 353**,** 409-417.

De Weert, S., Dekkers, L.C., Bitter, W., Tuinman, S., Wijfjes, A.H., Van Boxtel, R., and Lugtenberg, B.J. (2006). The two-component colR/S system of *Pseudomonas fluorescens* WCS365 plays a role in rhizosphere competence through maintaining the structure and function of the outer membrane. *FEMS Microbiology Ecology* 58**,** 205-213.

De Weert, S., Vermeiren, H., Mulders, I.H., Kuiper, I., Hendrickx, N., Bloemberg, G.V., Vanderleyden, J., De Mot, R., and Lugtenberg, B.J. (2002). Flagella-driven chemotaxis towards exudate components is an important trait for tomato root colonization by *Pseudomonas fluorescens*. *Molecular Plant-Microbe Interactions* 15**,** 1173-1180.

De Werra, P., Pechy-Tarr, M., Keel, C., and Maurhofer, M. (2009). Role of gluconic acid production in the regulation of biocontrol traits of *Pseudomonas fluorescens* CHA0. *Applied and Environmental Microbiology* 75**,** 4162-4174.

Dennis, P.G., Miller, A.J., and Hirsch, P.R. (2010). Are root exudates more important than other sources of rhizodeposits in structuring rhizosphere bacterial communities? *FEMS Microbiology Ecology* 72**,** 313-327.

Diaz Herrera, S., Grossi, C., Zawoznik, M., and Groppa, M.D. (2016). Wheat seeds harbour bacterial endophytes with potential as plant growth promoters and biocontrol agents of *Fusarium graminearum*. *Microbiological Research* 186-187**,** 37-43.

Dimkpa, C., Merten, D., Svatoš, A., Büchel, G., and Kothe, E. (2009). Siderophores mediate reduced and increased uptake of cadmium by *Streptomyces tendae* F4 and sunflower (*Helianthus annuus*), respectively. *Journal of Applied Microbiology* 107**,** 1687-1696.

Dixon, R., and Kahn, D. (2004). Genetic regulation of biological nitrogen fixation. *Nature Reviews Microbiology* 2**,** 621.

Doilom, M., Guo, J.-W., Phookamsak, R., Mortimer, P.E., Karunarathna, S.C., Dong, W., Liao, C.-F., Yan, K., Pem, D., Suwannarach, N., Promputtha, I., Lumyong, S., and Xu, J.-C. (2020). Screening of phosphate-solubilizing fungi from air and soil in Yunnan, China: four novel species in *Aspergillus*, *Gongronella*, *Penicillium*, and *Talaromyces*. *Frontiers in Microbiology* 11**,** 2443.

Donn, S., Kirkegaard, J.A., Perera, G., Richardson, A.E., and Watt, M. (2015). Evolution of bacterial communities in the wheat crop rhizosphere. *Environmental Microbiology* 17**,** 610-621.

Dos Santos, P.C., Fang, Z., Mason, S.W., Setubal, J.C., and Dixon, R. (2012). Distribution of nitrogen fixation and nitrogenase-like sequences amongst microbial genomes. *BMC Genomics* 13**,** 162.

Drogue, B., Sanguin, H., Chamam, A., Mozar, M., Llauro, C., Panaud, O., Prigent-Combaret, C., Picault, N., and Wisniewski-Dyé, F. (2014). Plant root transcriptome profiling

reveals a strain-dependent response during *Azospirillum*-rice cooperation. *Frontiers in Plant Science* 5**,** 607.
Duca, D., Lorv, J., Patten, C.L., Rose, D., and Glick, B.R. (2014). Indole-3-acetic acid in plant–microbe interactions. *Antonie van Leeuwenhoek* 106**,** 85-125.
Duffy, B., Schouten, A., and Raaijmakers, J.M. (2003). Pathogen self-defense: mechanisms to counteract microbial antagonism. *Annual Review of Phytopathology* 41**,** 501-538.
Dunger, G., Llontop, E., Guzzo, C.R., and Farah, C.S. (2016). The *Xanthomonas* type IV pilus. *Current Opinion in Microbiology* 30**,** 88-97.
Durán, P., Acuña, J.J., Jorquera, M.A., Azcón, R., Paredes, C., Rengel, Z., and De La Luz Mora, M. (2014). Endophytic bacteria from selenium-supplemented wheat plants could be useful for plant-growth promotion, biofortification and *Gaeumannomyces graminis* biocontrol in wheat production. *Biology and Fertility of Soils* 50**,** 983-990.
Dwivedi, D., and Johri, B. (2003). Antifungals from fluorescent pseudomonads: biosynthesis and regulation. *Current Science***,** 1693-1703.
Eastman, A.W., Heinrichs, D.E., and Yuan, Z.-C. (2014). Comparative and genetic analysis of the four sequenced *Paenibacillus polymyxa* genomes reveals a diverse metabolism and conservation of genes relevant to plant-growth promotion and competitiveness. *BMC Genomics* 15**,** 851.
Edwards, J., Johnson, C., Santos-Medellín, C., Lurie, E., Podishetty, N.K., Bhatnagar, S., Eisen, J.A., and Sundaresan, V. (2015). Structure, variation, and assembly of the root-associated microbiomes of rice. *Proceedings of the National Academy of Sciences* 112**,** E911-E920.
Egamberdieva, D., Wirth, S.J., Alqarawi, A.A., Abd_Allah, E.F., and Hashem, A. (2017). Phytohormones and beneficial microbes: essential components for plants to balance stress and fitness. *Frontiers in Microbiology* 8**,** 2104.
Egamberdiyeva, D. (2007). The effect of plant growth promoting bacteria on growth and nutrient uptake of maize in two different soils. *Applied Soil Ecology* 36**,** 184-189.
Emmert, E.A., Klimowicz, A.K., Thomas, M.G., and Handelsman, J. (2004). Genetics of zwittermicin A production by *Bacillus cereus*. *Applied and Environmental Microbiology* 70**,** 104-113.
Eriksson, D. (2019). The evolving EU regulatory framework for precision breeding. *Theoretical and Applied Genetics* 132**,** 569-573.
Eyre, A.W., Wang, M., Oh, Y., and Dean, R.A. (2019). Identification and characterization of the core rice seed microbiome. *Phytobiomes Journal* 3**,** 148-157.
Felestrino, É.B., Santiago, I.F., Freitas, L.D.S., Rosa, L.H., Ribeiro, S.P., and Moreira, L.M. (2017). Plant growth promoting bacteria associated with *Langsdorffia hypogaea*-rhizosphere-host biological interface: a neglected model of bacterial prospection. *Frontiers in Microbiology* 8**,** 172.
Ferguson, B.J., and Mathesius, U. (2014). Phytohormone regulation of legume-rhizobia interactions. *Journal of Chemical Ecology* 40**,** 770-790.
Fierer, N. (2017). Embracing the unknown: disentangling the complexities of the soil microbiome. *Nature Reviews Microbiology* 15**,** 579-590.
Food and Agriculture Organization (2010). "Global dairy sector: status and trends," in *Status and prospects for smallholder milk production a global perspective,* eds. T. Hemme & J. Otte.).
Franche, C., Lindström, K., and Elmerich, C. (2009). Nitrogen-fixing bacteria associated with leguminous and non-leguminous plants. *Plant and Soil* 321**,** 35-59.
Fussell, G.E. (1964). The grasses and grassland cultivation of britain. *Grass and Forage Science* 19**,** 49-54.

Gaby, J.C., and Buckley, D.H. (2011). A global census of nitrogenase diversity. *Environmental Microbiology* 13**,** 1790-1799.
Gaby, J.C., and Buckley, D.H. (2012). A comprehensive evaluation of PCR primers to amplify the *nifH* gene of nitrogenase. *PLOS One* 7**,** e42149.
Gaby, J.C., and Buckley, D.H. (2014). A comprehensive aligned *nifH* gene database: a multipurpose tool for studies of nitrogen-fixing bacteria. *Database* 2014**,** bau001.
Gallon, J. (1981). The oxygen sensitivity of nitrogenase: a problem for biochemists and micro-organisms. *Trends in Biochemical Sciences* 6**,** 19-23.
Gamalero, E., and Glick, B.R. (2012). "Ethylene and abiotic stress tolerance in plants," in *Environmental adaptations and stress tolerance of plants in the era of climate change*, eds. P. Ahmad & M.N.V. Prasad. (New York, NY: Springer New York), 395-412.
Gans, J., Wolinsky, M., and Dunbar, J. (2005). Computational improvements reveal great bacterial diversity and high metal toxicity in soil. *Science* 309**,** 1387-1390.
Garg, N. (2009). "Symbiotic nitrogen fixation in legume nodules process and signaling: a review," in *Sustainable Agriculture*. Springer), 519-531.
Garita-Cambronero, J., Palacio-Bielsa, A., Lopez, M.M., and Cubero, J. (2017). Pan-genomic analysis permits differentiation of virulent and non-virulent strains of *Xanthomonas arboricola* that cohabit *Prunus* spp. and elucidate bacterial virulence factors. *Frontiers in Microbiology* 8**,** 573.
Geider, K., Auling, G., Du, Z., Jakovljevic, V., Jock, S., and Völksch, B. (2006). *Erwinia tasmaniensis* sp. nov., a non-phytopathogenic bacterium from apple and pear trees. *International Journal of Systematic and Evolutionary Microbiology* 56**,** 2937-2943.
Gerhardson, B. (2002). Biological substitutes for pesticides. *Trends in Biotechnology* 20**,** 338-343.
Giraldo, P.A., Shinozuka, H., Spangenberg, G.C., Cogan, N.O.I., and Smith, K.F. (2019). Safety assessment of genetically modified feed: is there any difference from food? *Frontiers in Plant Science* 10**,** 1592.
Glick, B.R. (2014). Bacteria with ACC deaminase can promote plant growth and help to feed the world. *Microbiological Research* 169**,** 30-39.
Glick, B.R., Cheng, Z., Czarny, J., and Duan, J. (2007). Promotion of plant growth by ACC deaminase-producing soil bacteria. *European Journal of Plant Pathology* 119**,** 329-339.
Goldstein, A.H. (1986). Bacterial solubilization of mineral phosphates: historical perspective and future prospects. *American Journal of Alternative Agriculture* 1**,** 51-57.
Goldstein, A.H. (1994). "Involvement of the quinoprotein glucose dehydrogenase in the solubilization of exogenous phosphates by gram-negative bacteria," in *Phosphate in microorganisms: cellular and molecular biology*, eds. A. Torriani-Gorini, E. Yagil & S. Silver. (Washington, DC: ASM Press), 197-203.
Gourley, C.J.P., Dougherty, W.J., Weaver, D.M., Aarons, S.R., Awty, I.M., Gibson, D.M., Hannah, M.C., Smith, A.P., and Peverill, K.I. (2012). Farm-scale nitrogen, phosphorus, potassium and sulfur balances and use efficiencies on Australian dairy farms. *Animal Production Science* 52**,** 929-944.
Grady, E.N., Macdonald, J., Liu, L., Richman, A., and Yuan, Z.-C. (2016). Current knowledge and perspectives of *Paenibacillus*: a review. *Microbial Cell Factories* 15**,** 203.
Green, S., Studholme, D.J., Laue, B.E., Dorati, F., Lovell, H., Arnold, D., Cottrell, J.E., Bridgett, S., Blaxter, M., and Huitema, E. (2010). Comparative genome analysis provides insights into the evolution and adaptation of *Pseudomonas syringae* pv. *aesculi* on *Aesculus hippocastanum*. *PLOS One* 5**,** e10224.

Grimmer, M.K., Van Den Bosch, F., Powers, S.J., and Paveley, N.D. (2015). Fungicide resistance risk assessment based on traits associated with the rate of pathogen evolution. *Pest Management Science* 71**,** 207-215.

Groves, R.H., Austin, M.P., and Kaye, P.E. (2003). Competition between Australian native and introduced grasses along a nutrient gradient. *Austral Ecology* 28**,** 491-498.

Gupta, S., and Pandey, S. (2019). ACC deaminase producing bacteria with multifarious plant growth promoting traits alleviates salinity stress in french bean (*Phaseolus vulgaris*) plants. *Frontiers in Microbiology* 10**,** 1506.

Haas, D., and Défago, G. (2005). Biological control of soil-borne pathogens by fluorescent pseudomonads. *Nature Reviews Microbiology* 3**,** 307-319.

Hallmann, J., and Berg, G. (2006). Spectrum and population dynamics of bacterial root endophytes. *Microbial Root Endophytes***,** 15-31.

Hao, T., and Chen, S. (2017). Colonization of wheat, maize and cucumber by *Paenibacillus polymyxa* WLY78. *PLOS One* 12**,** e0169980.

Hardoim, P.R., Van Overbeek, L.S., and Van Elsas, J.D. (2008). Properties of bacterial endophytes and their proposed role in plant growth. *Trends in Microbiology* 16**,** 463-471.

Hartmann, A., Rothballer, M., and Schmid, M. (2008). Lorenz Hiltner, a pioneer in rhizosphere microbial ecology and soil bacteriology research. *Plant and Soil* 312**,** 7-14.

Hartmann, L.S., and Barnum, S.R. (2010). Inferring the evolutionary history of Mo-dependent nitrogen fixation from phylogenetic studies of *nifK* and *nifDK*. *Journal of Molecular Evolution* 71**,** 70-85.

Hawkes, C.V., Deangelis, K.M., and Firestone, M.K. (2007). "Root interactions with soil microbial communities and processes," in *The rhizosphere, an ecological perspective*. Elsevier, New York), 1-3.

Hayat, R., Ali, S., Amara, U., Khalid, R., and Ahmed, I. (2010). Soil beneficial bacteria and their role in plant growth promotion: a review. *Annals of Microbiology* 60**,** 579-598.

Henri, F., Laurette, N.N., Annette, D., John, Q., Wolfgang, M., Franccedil, E., and Dieudonne, N. (2008). Solubilization of inorganic phosphates and plant growth promotion by strains of *Pseudomonas fluorescens* isolated from acidic soils of Cameroon. *African Journal of Microbiology Research* 2**,** 171-178.

Herridge, D.F., Peoples, M.B., and Boddey, R.M. (2008). Global inputs of biological nitrogen fixation in agricultural systems. *Plant and Soil* 311**,** 1-18.

Hersemann, L., Wibberg, D., Blom, J., Goesmann, A., Widmer, F., Vorhölter, F.J., and Kölliker, R. (2017). Comparative genomics of host adaptive traits in *Xanthomonas translucens* pv. *graminis*. *BMC Genomics* 18**,** 35.

Hettiarachchige, I.K., Elkins, A.C., Reddy, P., Mann, R.C., Guthridge, K.M., Sawbridge, T.I., Forster, J.W., and Spangenberg, G.C. (2019). Genetic modification of asexual *Epichloë* endophytes with the *perA* gene for peramine biosynthesis. *Molecular Genetics and Genomics* 294**,** 315-328.

Hodge, A., Robinson, D., and Fitter, A. (2000). Are microorganisms more effective than plants at competing for nitrogen? *Trends in Plant Science* 5**,** 304-308.

Hooper, L.V., Wong, M.H., Thelin, A., Hansson, L., Falk, P.G., and Gordon, J.I. (2001). Molecular analysis of commensal host-microbial relationships in the intestine. *Science* 291**,** 881-884.

Houben, M., and Van De Poel, B. (2019). 1-aminocyclopropane-1-carboxylic acid oxidase (ACO): the enzyme that makes the plant hormone ethylene. *Frontiers in Plant Science* 10**,** 695.

Hsu, S.-F., and Buckley, D.H. (2009). Evidence for the functional significance of diazotroph community structure in soil. *The ISME Journal* 3**,** 124-136.

Hubbard, K., and Hassanein, N. (2013). Confronting coexistence in the United States: organic agriculture, genetic engineering, and the case of Roundup Ready® alfalfa. *Agriculture and Human Values* 30**,** 325-335.

Hwang, J., Chen, C., and Burris, R. (1973). Inhibition of nitrogenase-catalyzed reductions. *Biochimica et Biophysica Acta (BBA)-Bioenergetics* 292**,** 256-270.

Imlay, J.A. (2006). Iron‐sulphur clusters and the problem with oxygen. *Molecular Microbiology* 59**,** 1073-1082.

Jilani, G., Akram, A., Ali, R.M., Hafeez, F.Y., Shamsi, I.H., Chaudhry, A.N., and Chaudhry, A.G. (2007). Enhancing crop growth, nutrients availability, economics and beneficial rhizosphere microflora through organic and biofertilizers. *Annals of Microbiology* 57**,** 177-184.

Johnson, L.J., De Bonth, A.C.M., Briggs, L.R., Caradus, J.R., Finch, S.C., Fleetwood, D.J., Fletcher, L.R., Hume, D.E., Johnson, R.D., Popay, A.J., Tapper, B.A., Simpson, W.R., Voisey, C.R., and Card, S.D. (2013). The exploitation of epichloae endophytes for agricultural benefit. *Fungal Diversity* 60**,** 171-188.

Johnston-Monje, D., Lundberg, D.S., Lazarovits, G., Reis, V.M., and Raizada, M.N. (2016). Bacterial populations in juvenile maize rhizospheres originate from both seed and soil. *Plant and Soil* 405**,** 337-355.

Johnston-Monje, D., Mousa, W.K., Lazarovits, G., and Raizada, M.N. (2014). Impact of swapping soils on the endophytic bacterial communities of pre-domesticated, ancient and modern maize. *BMC Plant Biology* 14**,** 233.

Jones, R.a.C. (2013). Virus diseases of pasture grasses in Australia: incidences, losses, epidemiology, and management. *Crop and Pasture Science* 64**,** 216-233.

Kamilova, F., Validov, S., Azarova, T., Mulders, I., and Lugtenberg, B. (2005). Enrichment for enhanced competitive plant root tip colonizers selects for a new class of biocontrol bacteria. *Environmental Microbiology* 7**,** 1809-1817.

Kaur, J., Ekanayake, P.N., Tian, P., Van Zijll De Jong, E., Dobrowolski, M.P., Rochfort, S.J., Mann, R.C., Smith, K.F., Forster, J.W., Guthridge, K.M., and Spangenberg, G.C. (2015). Discovery and characterisation of novel asexual *Epichloë* endophytes from perennial ryegrass (*Lolium perenne* L.). *Crop and Pasture Science* 66**,** 1058-1070.

Kaur, R., Macleod, J., Foley, W., and Nayudu, M. (2006). Gluconic acid: an antifungal agent produced by *Pseudomonas* species in biological control of take-all. *Phytochemistry* 67**,** 595-604.

Kennedy, I., and Islam, N. (2001). The current and potential contribution of asymbiotic nitrogen fixation to nitrogen requirements on farms: a review. *Australian Journal of Experimental Agriculture* 41**,** 447-457.

Khan, A.L., Waqas, M., Kang, S.-M., Al-Harrasi, A., Hussain, J., Al-Rawahi, A., Al-Khiziri, S., Ullah, I., Ali, L., Jung, H.-Y., and Lee, I.-J. (2014). Bacterial endophyte *Sphingomonas* sp. LK11 produces gibberellins and IAA and promotes tomato plant growth. *Journal of Microbiology* 52**,** 689-695.

Khan, M.S., Zaidi, A., and Wani, P.A. (2007). Role of phosphate-solubilizing microorganisms in sustainable agriculture—a review. *Agronomy for Sustainable Development* 27**,** 29-43.

Kim, J.-I., and Kim, J.-Y. (2019). New era of precision plant breeding using genome editing. *Plant Biotechnology Reports* 13**,** 419-421.

Kim, K., Mcdonald, G., and Jordan, D. (1997). Solubilization of hydroxyapatite by *Enterobacter agglomerans* and cloned *Escherichia coli* in culture medium. *Biology and Fertility of Soils* 24**,** 347-352.

Kim, K.Y., Jordan, D., and Krishnan, H.B. (1998). Expression of genes from *Rahnella aquatilis* that are necessary for mineral phosphate solubilization in *Escherichia coli*. *FEMS Microbiology Letters* 159**,** 121-127.

Kloepper, J.W., Leong, J., Teintze, M., and Schroth, M.N. (1980). *Pseudomonas* siderophores: a mechanism explaining disease-suppressive soils. *Current Microbiology* 4**,** 317-320.

Kloepper, J.W., Ryu, C.-M., and Zhang, S. (2004). Induced systemic resistance and promotion of plant growth by *Bacillus* spp. *Phytopathology* 94**,** 1259-1266.

Kozdrój, J., Trevors, J., and Van Elsas, J. (2004). Influence of introduced potential biocontrol agents on maize seedling growth and bacterial community structure in the rhizosphere. *Soil Biology and Biochemistry* 36**,** 1775-1784.

Krishnaraj, P., and Goldstein, A. (2001). Cloning of a *Serratia marcescens* DNA fragment that induces quinoprotein glucose dehydrogenase‐mediated gluconic acid production in *Escherichia coli* in the presence of stationary phase *Serratia marcescens*. *FEMS Microbiology Letters* 205**,** 215-220.

Kudoyarova, G.R., Melentiev, A.I., Martynenko, E.V., Timergalina, L.N., Arkhipova, T.N., Shendel, G.V., Kuz'mina, L.Y., Dodd, I.C., and Veselov, S.Y. (2014). Cytokinin producing bacteria stimulate amino acid deposition by wheat roots. *Plant Physiology and Biochemistry* 83**,** 285-291.

Kuiper, I., Bloemberg, G.V., and Lugtenberg, B.J. (2001). Selection of a plant-bacterium pair as a novel tool for rhizostimulation of polycyclic aromatic hydrocarbon-degrading bacteria. *Molecular Plant-Microbe Interactions* 14**,** 1197-1205.

Lakshmanan, V., Castaneda, R., Rudrappa, T., and Bais, H.P. (2013). Root transcriptome analysis of *Arabidopsis thaliana* exposed to beneficial *Bacillus subtilis* FB17 rhizobacteria revealed genes for bacterial recruitment and plant defense independent of malate efflux. *Planta* 238**,** 657-668.

Lederberg, J., and Mccray, A.T. (2001). Ome SweetOmics--A genealogical treasury of words. *The Scientist* 15**,** 8-8.

Lee, J.M., Matthew, C., Thom, E.R., and Chapman, D.F. (2012a). Perennial ryegrass breeding in New Zealand: a dairy industry perspective. *Crop and Pasture Science* 63**,** 107-127.

Lee, S.H., Cho, Y.E., Park, S.-H., Balaraju, K., Park, J.W., Lee, S.W., and Park, K. (2012b). An antibiotic fusaricidin: a cyclic depsipeptide from *Paenibacillus polymyxa* E681 induces systemic resistance against Phytophthora blight of red-pepper. *Phytoparasitica* 41**,** 49-58.

Lery, L.M.S., Hemerly, A.S., Nogueira, E.M., Von Krüger, W.M.A., and Bisch, P.M. (2011). Quantitative proteomic analysis of the interaction between the endophytic plant-growth-promoting bacterium *Gluconacetobacter diazotrophicus* and sugarcane. *Molecular Plant-Microbe Interactions* 24**,** 562-576.

Li, M., Guo, R., Yu, F., Chen, X., Zhao, H., Li, H., and Wu, J. (2018). Indole-3-acetic acid biosynthesis pathways in the plant-beneficial bacterium *Arthrobacter pascens* ZZ21. *International Journal of Molecular Sciences* 19**,** 443.

Li, T., Mann, R., Sawbridge, T., Kaur, J., Auer, D., and Spangenberg, G. (2020). Novel *Xanthomonas* species from the perennial ryegrass seed microbiome – Assessing the bioprotection activity of non-pathogenic relatives of pathogens. *Frontiers in Microbiology* 11**,** 1991.

Li, T., Tannenbaum, I., Chandel, A., Kaur, J., Vincent, D., Hone, H., Sawbridge, T., Mann, R., and Spangenberg, G. (2021). *Plant microbiome and methods for profiling plant microbiome*. PCT/AU2020/050733. 28/01/2021.

Lin, L., and Xu, X. (2013). Indole-3-acetic acid production by endophytic *Streptomyces* sp. En-1 isolated from medicinal plants. *Current Microbiology* 67**,** 209-217.

Liu, H., Wang, J., Sun, H., Han, X., Peng, Y., Liu, J., Liu, K., Ding, Y., Wang, C., and Du, B. (2020). Transcriptome profiles reveal the growth-promoting mechanisms of *Paenibacillus polymyxa* YC0136 on tobacco (*Nicotiana tabacum* L.). *Frontiers in Microbiology* 11**,** 2384.

Liu, Y., Zuo, S., Xu, L., Zou, Y., and Song, W. (2012). Study on diversity of endophytic bacterial communities in seeds of hybrid maize and their parental lines. *Archives of Microbiology* 194**,** 1001-1012.

Ludemann, C., Jacobs, J., and Smith, K. (2015). The economic significance of maintaining pasture production at its peak value. *Crop and Pasture Science* 66**,** 205-213.

Lugtenberg, B., and Kamilova, F. (2009). Plant-growth-promoting rhizobacteria. *Annual Review of Microbiology* 63**,** 541-556.

Lugtenberg, B.J., Chin-a-Woeng, T.F., and Bloemberg, G.V. (2002). Microbe–plant interactions: principles and mechanisms. *Antonie van Leeuwenhoek* 81**,** 373-383.

Lugtenberg, B.J., Dekkers, L., and Bloemberg, G.V. (2001). Molecular determinants of rhizosphere colonization by *Pseudomonas*. *Annual Review of Phytopathology* 39**,** 461-490.

Lundberg, D.S., Lebeis, S.L., Paredes, S.H., Yourstone, S., Gehring, J., Malfatti, S., Tremblay, J., Engelbrektson, A., Kunin, V., and Del Rio, T.G. (2012). Defining the core *Arabidopsis thaliana* root microbiome. *Nature* 488**,** 86.

Ma, M., Christensen, M.J., and Nan, Z. (2015). Effects of the endophyte *Epichloë festucae* var. *lolii* of perennial ryegrass (*Lolium perenne*) on indicators of oxidative stress from pathogenic fungi during seed germination and seedling growth. *European Journal of Plant Pathology* 141**,** 571-583.

Mackellar, D., Lieber, L., Norman, J.S., Bolger, A., Tobin, C., Murray, J.W., Oksaksin, M., Chang, R.L., Ford, T.J., Nguyen, P.Q., Woodward, J., Permingeat, H.R., Joshi, N.S., Silver, P.A., Usadel, B., Rutherford, A.W., Friesen, M.L., and Prell, J. (2016). *Streptomyces thermoautotrophicus* does not fix nitrogen. *Scientific Reports* 6**,** 20086.

Magnani, G., Didonet, C., Cruz, L., Picheth, C., Pedrosa, F., and Souza, E. (2010). Diversity of endophytic bacteria in Brazilian sugarcane. *Genetics and Molecular Research* 9**,** 250-258.

Maheshwari, D.K., Dheeman, S., and Agarwal, M. (2015). "Phytohormone-producing PGPR for sustainable agriculture," in *Bacterial Metabolites in Sustainable Agroecosystem,* ed. D.K. Maheshwari. (Cham: Springer International Publishing), 159-182.

Maksimov, I., Abizgil'dina, R., and Pusenkova, L. (2011). Plant growth promoting rhizobacteria as alternative to chemical crop protectors from pathogens. *Applied Biochemistry and Microbiology* 47**,** 333-345.

Man‐Aharonovich, D., Kress, N., Zeev, E.B., Berman‐Frank, I., and Béjà, O. (2007). Molecular ecology of *nifH* genes and transcripts in the eastern Mediterranean Sea. *Environmental Microbiology* 9**,** 2354-2363.

Mansfield, J., Genin, S., Magori, S., Citovsky, V., Sriariyanum, M., Ronald, P., Dow, M., Verdier, V., Beer, S.V., and Machado, M.A. (2012). Top 10 plant pathogenic bacteria in molecular plant pathology. *Molecular Plant Pathology* 13**,** 614-629.

Manter, D.K., Delgado, J.A., Holm, D.G., and Stong, R.A. (2010). Pyrosequencing reveals a highly diverse and cultivar-specific bacterial endophyte community in potato roots. *Microbial Ecology* 60**,** 157-166.

Marschner, P. (2012). "Rhizosphere biology," in *Marschner's mineral nutrition of higher plants,* ed. P. Marschner. 3 ed: Academic Press, 369-388.

Martins, L., Fernandes, C., Blom, J., Dia, N.C., Pothier, J.F., and Tavares, F. (2020). *Xanthomonas euroxanthea* sp. nov., a new xanthomonad species including pathogenic and non-pathogenic strains of walnut. *International Journal of Systematic and Evolutionary Microbiology* 70**,** 6024–6031.

Mavrodi, D.V., Blankenfeldt, W., and Thomashow, L.S. (2006). Phenazine compounds in fluorescent *Pseudomonas* spp. biosynthesis and regulation. *Annual Review of Phytopathology* 44**,** 417-445.

Mcgrath, J.W., Hammerschmidt, F., and Quinn, J.P. (1998). Biodegradation of phosphonomycin by *Rhizobium huakuii* PMY1. *Applied and Environmental Microbiology* 64**,** 356-358.

Mendes, R., Garbeva, P., and Raaijmakers, J.M. (2013). The rhizosphere microbiome: significance of plant beneficial, plant pathogenic, and human pathogenic microorganisms. *FEMS Microbiology Reviews* 37**,** 634-663.

Menz, K. (1984). "Australia's pasture resource base: history and current issues", in: *28th annual conference of the Australian Agricultural Economics Society'.* Australian Agricultural Economics Society.

Micallef, S.A., Shiaris, M.P., and Colón-Carmona, A. (2009). Influence of *Arabidopsis thaliana* accessions on rhizobacterial communities and natural variation in root exudates. *Journal of Experimental Botany* 60**,** 1729-1742.

Milner, J.L., Silo-Suh, L., Lee, J.C., He, H., Clardy, J., and Handelsman, J. (1996). Production of kanosamine by *Bacillus cereus* UW85. *Applied and Environmental Microbiology* 62**,** 3061-3065.

Mohammadi, K. (2012). Phosphorus solubilizing bacteria: occurrence, mechanisms and their role in crop production. *Resources and Environment* 2**,** 80-85.

Mohd Din, A.R.J., Rosli, M.A., Mohamad Azam, Z., Othman, N.Z., and Sarmidi, M.R. (2020). *Paenibacillus polymyxa* role involved in phosphate solubilization and growth promotion of *Zea mays* under abiotic stress condition. *Proceedings of the National Academy of Sciences, India Section B: Biological Sciences* 90**,** 63-71.

Monteiro, R.A., Balsanelli, E., Wassem, R., Marin, A.M., Brusamarello-Santos, L.C., Schmidt, M.A., Tadra-Sfeir, M.Z., Pankievicz, V.C., Cruz, L.M., and Chubatsu, L.S. (2012). *Herbaspirillum*-plant interactions: microscopical, histological and molecular aspects. *Plant and Soil* 356**,** 175-196.

Mora, Y., Díaz, R., Vargas-Lagunas, C., Peralta, H., Guerrero, G., Aguilar, A., Encarnación, S., Girard, L., and Mora, J. (2014). Nitrogen-fixing rhizobial strains isolated from common bean seeds: phylogeny, physiology, and genome analysis. *Applied and Environmental Microbiology* 80**,** 5644-5654.

Müller, J., Wiemken, A., and Boller, T. (2001). Redifferentiation of bacteria isolated from *Lotus japonicus* root nodules colonized by *Rhizobium* sp. NGR234. *Journal of Experimental Botany* 52**,** 2181-2186.

Mulvaney, R., Khan, S., and Ellsworth, T. (2009). Synthetic nitrogen fertilizers deplete soil nitrogen: a global dilemma for sustainable cereal production. *Journal of Environmental Quality* 38**,** 2295-2314.

Murray, G.M., and Brennan, J.P. (2009a). Estimating disease losses to the Australian barley industry. *Australasian Plant Pathology* 39**,** 85-96.

Murray, G.M., and Brennan, J.P. (2009b). Estimating disease losses to the Australian wheat industry. *Australasian Plant Pathology* 38**,** 558-570.

Newton, W.E. (2006). "Physiology, biochemistry and molecular biology of nitrogen fixation," in *Biology of the nitrogen cycle,* eds. H. Bothe, S. Ferguson & W. Newton. (Amsterdam: Elsevier), 109-130.

Nielsen, T.H., and Sørensen, J. (2003). Production of cyclic lipopeptides by *Pseudomonas fluorescens* strains in bulk soil and in the sugar beet rhizosphere. *Applied and Environmental Microbiology* 69**,** 861-868.

Nissinen, R., Helander, M., Kumar, M., and Saikkonen, K. (2019). Heritable *Epichloë* symbiosis shapes fungal but not bacterial communities of plant leaves. *Scientific Reports* 9**,** 5253.

Nomata, J., Mizoguchi, T., Tamiaki, H., and Fujita, Y. (2006). A second nitrogenase-like enzyme for bacteriochlorophyll biosynthesis: reconstitution of chlorophyllide a reductase with purified X-protein (BchX) and YZ-protein (BchY-BchZ) from *Rhodobacter capsulatus*. *Journal of Biological Chemistry* 281**,** 15021-15028.

Oerke, E.C. (2006). Crop losses to pests. *Journal of Agricultural Science* 144**,** 31-43.

Ohtake, H., Wu, H., Imazu, K., Anbe, Y., Kato, J., and Kuroda, A. (1996). Bacterial phosphonate degradation, phosphite oxidation and polyphosphate accumulation. *Resources, Conservation and Recycling* 18**,** 125-134.

Ongena, M., Jourdan, E., Adam, A., Paquot, M., Brans, A., Joris, B., Arpigny, J.L., and Thonart, P. (2007). Surfactin and fengycin lipopeptides of *Bacillus subtilis* as elicitors of induced systemic resistance in plants. *Environmental Microbiology* 9**,** 1084-1090.

Otieno, N., Lally, R., Kiwanuka, S., Lloyd, A., Ryan, D., Germaine, K., and Dowling, D. (2015). Plant growth promotion induced by phosphate solubilizing endophytic *Pseudomonas* isolates. *Frontiers in Microbiology* 6**,** 745.

Padda, K.P., Puri, A., and Chanway, C.P. (2016a). Effect of GFP tagging of *Paenibacillus polymyxa* P2b-2R on its ability to promote growth of canola and tomato seedlings. *Biology and Fertility of Soils* 52**,** 377-387.

Padda, K.P., Puri, A., and Chanway, C.P. (2016b). Plant growth promotion and nitrogen fixation in canola (*Brassica napus*) by an endophytic strain of *Paenibacillus polymyxa* and its GFP-tagged derivative in a long-term study. *Botany* 94**,** 1209-1217.

Panter, S., Chu, P.G., Ludlow, E., Garrett, R., Kalla, R., Jahufer, M.Z.Z., De Lucas Arbiza, A., Rochfort, S., Mouradov, A., Smith, K.F., and Spangenberg, G. (2012). Molecular breeding of transgenic white clover (*Trifolium repens* L.) with field resistance to *Alfalfa mosaic virus* through the expression of its coat protein gene. *Transgenic Research* 21**,** 619-632.

Panter, S., Mouradov, A., Badenhorst, P., Martelotto, L., Griffith, M., Smith, K.F., and Spangenberg, G. (2017). Re-programming photosynthetic cells of perennial ryegrass (*Lolium perenne* L.) for fructan biosynthesis through transgenic expression of fructan biosynthetic genes under the control of photosynthetic promoters. *Agronomy* 7**,** 36.

Parsons, A.J., Edwards, G.R., Newton, P.C.D., Chapman, D.F., Caradus, J.R., Rasmussen, S., and Rowarth, J.S. (2011). Past lessons and future prospects: plant breeding for yield and persistence in cool-temperate pastures. *Grass and Forage Science* 66**,** 153-172.

Pascale, A., Proietti, S., Pantelides, I.S., and Stringlis, I.A. (2020). Modulation of the root microbiome by plant molecules: the basis for targeted disease suppression and plant growth promotion. *Frontiers in Plant Science* 10**,** 1741.

Paterson, E., Gebbing, T., Abel, C., Sim, A., and Telfer, G. (2007). Rhizodeposition shapes rhizosphere microbial community structure in organic soil. *New Phytologist* 173**,** 600-610.

Patten, C.L., Blakney, A.J.C., and Coulson, T.J.D. (2013). Activity, distribution and function of indole-3-acetic acid biosynthetic pathways in bacteria. *Critical Reviews in Microbiology* 39**,** 395-415.

Patten, C.L., and Glick, B.R. (2002). Role of *Pseudomonas putida* indoleacetic acid in development of the host plant root system. *Applied and Environmental Microbiology* 68**,** 3795-3801.

Pellegrini, M., Ercole, C., Di Zio, C., Matteucci, F., Pace, L., and Del Gallo, M. (2020). *In vitro* and *in planta* antagonistic effects of plant growth-promoting rhizobacteria consortium against soilborne plant pathogens of *Solanum tuberosum* and *Solanum lycopersicum*. *FEMS Microbiology Letters* 367**,** fnaa099.

Pembleton, L., Shinozuka, H., Wang, J., Spangenberg, G., Forster, J., and Cogan, N. (2015). Design of an F1 hybrid breeding strategy for ryegrasses based on selection of self-incompatibility locus-specific alleles. *Frontiers in Plant Science* 6**,** 764.

Pérez-Jaramillo, J.E., Mendes, R., and Raaijmakers, J.M. (2016). Impact of plant domestication on rhizosphere microbiome assembly and functions. *Plant Molecular Biology* 90**,** 635-644.

Philippe, G. (2015). Ergot alkaloids produced by endophytic fungi of the genus *Epichloë. Toxins* 7**,** 773-790.

Philippe, G. (2016). Lolitrem b and indole diterpene alkaloids produced by endophytic fungi of the genus *Epichloë* and their toxic effects in livestock. *Toxins* 8**,** 47.

Phillips, D., and Golza, H. (2008). *Strawberry root and crown rot disease survey 2005 and 2006 seasons.* Perth, Western Australia: Department of Agriculture and Food. [Accessed 21/09/2020]

Poplawsky, A.R., Urban, S.C., and Chun, W. (2000). Biological role of xanthomonadin pigments in *Xanthomonas campestris* pv. *campestris*. *Applied and Environmental Microbiology* 66**,** 5123.

Puri, A., Padda, K.P., and Chanway, C.P. (2016). Seedling growth promotion and nitrogen fixation by a bacterial endophyte *Paenibacillus polymyxa* P2b-2R and its GFP derivative in corn in a long-term trial. *Symbiosis* 69**,** 123-129.

Qin, J., Li, R., Raes, J., Arumugam, M., Burgdorf, K.S., Manichanh, C., Nielsen, T., Pons, N., Levenez, F., Yamada, T., Mende, D.R., Li, J., Xu, J., Li, S., Li, D., Cao, J., Wang, B., Liang, H., Zheng, H., Xie, Y., Tap, J., Lepage, P., Bertalan, M., Batto, J.-M., Hansen, T., Le Paslier, D., Linneberg, A., Nielsen, H.B., Pelletier, E., Renault, P., Sicheritz-Ponten, T., Turner, K., Zhu, H., Yu, C., Li, S., Jian, M., Zhou, Y., Li, Y., Zhang, X., Li, S., Qin, N., Yang, H., Wang, J., Brunak, S., Doré, J., Guarner, F., Kristiansen, K., Pedersen, O., Parkhill, J., Weissenbach, J., Meta, H.I.T.C., Bork, P., Ehrlich, S.D., and Wang, J. (2010). A human gut microbial gene catalog established by metagenomic sequencing. *Nature* 464**,** 59-65.

Qin, J., Li, Y., Cai, Z., Li, S., Zhu, J., Zhang, F., Liang, S., Zhang, W., Guan, Y., Shen, D., Peng, Y., Zhang, D., Jie, Z., Wu, W., Qin, Y., Xue, W., Li, J., Han, L., Lu, D., Wu, P., Dai, Y., Sun, X., Li, Z., Tang, A., Zhong, S., Li, X., Chen, W., Xu, R., Wang, M., Feng, Q., Gong, M., Yu, J., Zhang, Y., Zhang, M., Hansen, T., Sanchez, G., Raes, J., Falony, G., Okuda, S., Almeida, M., Lechatelier, E., Renault, P., Pons, N., Batto, J.-M., Zhang, Z., Chen, H., Yang, R., Zheng, W., Li, S., Yang, H., Wang, J., Ehrlich, S.D., Nielsen, R., Pedersen, O., Kristiansen, K., and Wang, J. (2012). A metagenome-wide association study of gut microbiota in type 2 diabetes. *Nature* 490**,** 55-60.

Rasul, M., Yasmin, S., Suleman, M., Zaheer, A., Reitz, T., Tarkka, M.T., Islam, E., and Mirza, M.S. (2019). Glucose dehydrogenase gene containing phosphobacteria for biofortification of Phosphorus with growth promotion of rice. *Microbiological Research* 223-225**,** 1-12.

Raymond, J., Siefert, J.L., Staples, C.R., and Blankenship, R.E. (2004). The natural history of nitrogen fixation. *Molecular Biology and Evolution* 21**,** 541-554.

Reed, K. (2014). Perennial pasture grasses—an historical review of their introduction, use and development for southern Australia. *Crop and Pasture Science* 65**,** 691-712.

Reed, S.C., Townsend, A.R., Cleveland, C.C., and Nemergut, D.R. (2010). Microbial community shifts influence patterns in tropical forest nitrogen fixation. *Oecologia* 164**,** 521-531.

Reeve, W., O'hara, G., Chain, P., Ardley, J., Brau, L., Nandesena, K., Tiwari, R., Copeland, A., Nolan, M., Han, C., Brettin, T., Land, M., Ovchinikova, G., Ivanova, N., Mavromatis, K., Markowitz, V., Kyrpides, N., Melino, V., Denton, M., Yates, R., and Howieson, J. (2010). Complete genome sequence of *Rhizobium leguminosarum* bv. *trifolii* strain WSM1325, an effective microsymbiont of annual Mediterranean clovers. *Standards in Genomic Sciences* 2**,** 347-356.

Reinhold-Hurek, B., and Hurek, T. (1998). Interactions of gramineous plants with *Azoarcus* spp. and other diazotrophs: identification, localization, and perspectives to study their function. *Critical Reviews in Plant Sciences* 17**,** 29-54.

Rezzonico, F., Smits, T.H.M., Born, Y., Blom, J., Frey, J.E., Goesmann, A., Cleenwerck, I., De Vos, P., Bonaterra, A., Duffy, B., and Montesinos, E. (2016). *Erwinia gerundensis* sp. nov., a cosmopolitan epiphyte originally isolated from pome fruit trees. *International Journal of Systematic and Evolutionary Microbiology* 66**,** 1583-1592.

Ribbe, M., Gadkari, D., and Meyer, O. (1997). N2 fixation by *Streptomyces thermoautotrophicus* involves a molybdenum-dinitrogenase and a manganese-superoxide oxidoreductase that couple N2 reduction to the oxidation of superoxide produced from O2 by a molybdenum-CO dehydrogenase. *Journal of Biological Chemistry* 272**,** 26627-26633.

Richardson, A. (1994). "Soil microorganisms and phosphorus availability," in *Soil biota: management in sustainable farming systems*, ed. B.M.D. C.E. Pankhurst, V.V.S.R. Gupta, P.R. Grace. (Victoria, Australia: CSIRO), 50-62.

Richardson, A., and Hadobas, P. (1997). Soil isolates of *Pseudomonas* spp. that utilize inositol phosphates. *Canadian Journal of Microbiology* 43**,** 509-516.

Robson, R.L., and Postgate, J.R. (1980). Oxygen and hydrogen in biological nitrogen fixation. *Annual Review in Microbiology* 34**,** 183-207.

Rodríguez, C.E., Mitter, B., Barret, M., Sessitsch, A., and Compant, S. (2018). Commentary: seed bacterial inhabitants and their routes of colonization. *Plant and Soil* 422**,** 129-134.

RodríGuez, H., and Fraga, R. (1999). Phosphate solubilizing bacteria and their role in plant growth promotion. *Biotechnology Advances* 17**,** 319-339.

Rodríguez, H., Fraga, R., Gonzalez, T., and Bashan, Y. (2006). Genetics of phosphate solubilization and its potential applications for improving plant growth-promoting bacteria. *Plant and Soil* 287**,** 15-21.

Rösch, C., and Bothe, H. (2009). Diversity of total, nitrogen‐fixing and denitrifying bacteria in an acid forest soil. *European Journal of Soil Science* 60**,** 883-894.

Rosenblueth, M., and Martínez-Romero, E. (2006). Bacterial endophytes and their interactions with hosts. *Molecular Plant-Microbe Interactions* 19**,** 827-837.

Rubio, L.M., and Ludden, P.W. (2005). Maturation of nitrogenase: a biochemical puzzle. *Journal of Bacteriology* 187**,** 405-414.

Rubio, L.M., and Ludden, P.W. (2008). Biosynthesis of the iron-molybdenum cofactor of nitrogenase. *Annual Review of Microbiology* 62**,** 93-111.

Ryan, R.P., Vorhölter, F.-J., Potnis, N., Jones, J.B., Van Sluys, M.-A., Bogdanove, A.J., and Dow, J.M. (2011). Pathogenomics of *Xanthomonas*: understanding bacterium–plant interactions. *Nature Reviews Microbiology* 9**,** 344-355.

Ryu, C.-M., Farag, M.A., Hu, C.-H., Reddy, M.S., Wei, H.-X., Paré, P.W., and Kloepper, J.W. (2003). Bacterial volatiles promote growth in *Arabidopsis*. *Proceedings of the National Academy of Sciences* 100**,** 4927-4932.

Saad, M.M., Eida, A.A., and Hirt, H. (2020). Tailoring plant-associated microbial inoculants in agriculture: a roadmap for successful application. *Journal of Experimental Botany* 71**,** 3878-3901.

Saiz, E., Sgouridis, F., Drijfhout, F.P., and Ullah, S. (2019). Biological nitrogen fixation in peatlands: comparison between acetylene reduction assay and $^{15}N_2$ assimilation methods. *Soil Biology and Biochemistry* 131**,** 157-165.

Santi, C., Bogusz, D., and Franche, C. (2013). Biological nitrogen fixation in non-legume plants. *Annals of Botany* 111**,** 743-767.

Santner, A., and Estelle, M. (2009). Recent advances and emerging trends in plant hormone signalling. *Nature* 459**,** 1071-1078.

Savage, D.C. (1977). Microbial ecology of the gastrointestinal tract. *Annual Review in Microbiology* 31**,** 107-133.

Schenk, P.M., Carvalhais, L.C., and Kazan, K. (2012). Unraveling plant–microbe interactions: can multi-species transcriptomics help? *Trends in Biotechnology* 30**,** 177-184.

Schmidt, D. (1988). Prevention of bacterial wilt of grasses by phylloplane bacteria. *Journal of Phytopathology* 122**,** 253-260.

Schroth, M.N., and Hancock, J.G. (1982). Disease-suppressive soil and root-colonizing bacteria. *Science* 216**,** 1376-1381.

Sessitsch, A., Hardoim, P., Döring, J., Weilharter, A., Krause, A., Woyke, T., Mitter, B., Hauberg-Lotte, L., Friedrich, F., and Rahalkar, M. (2012). Functional characteristics of an endophyte community colonizing rice roots as revealed by metagenomic analysis. *Molecular Plant-Microbe Interactions* 25**,** 28-36.

Shade, A., Jacques, M.-A., and Barret, M. (2017). Ecological patterns of seed microbiome diversity, transmission, and assembly. *Current Opinion in Microbiology* 37**,** 15-22.

Shao, J., Li, S., Zhang, N., Cui, X., Zhou, X., Zhang, G., Shen, Q., and Zhang, R. (2015). Analysis and cloning of the synthetic pathway of the phytohormone indole-3-acetic acid in the plant-beneficial *Bacillus amyloliquefaciens* SQR9. *Microbial Cell Factories* 14**,** 130.

Sharma, S.B., Sayyed, R.Z., Trivedi, M.H., and Gobi, T.A. (2013). Phosphate solubilizing microbes: sustainable approach for managing phosphorus deficiency in agricultural soils. *SpringerPlus* 2**,** 587.

Shen, D. (1997). Microbial diversity and application of microbial products for agricultural purposes in China. *Agriculture, Ecosystems & Environment* 62**,** 237-245.

Shenge, K., Mabagala, R., Mortensen, C.N., Stephan, D., and Wydra, K. (2007). First report of bacterial speck of tomato caused by *Pseudomonas syringae* pv. *tomato* in Tanzania. *Plant Disease* 91**,** 462-462.

Shi, S., Richardson, A.E., O'callaghan, M., Deangelis, K.M., Jones, E.E., Stewart, A., Firestone, M.K., and Condron, L.M. (2011). Effects of selected root exudate components on soil bacterial communities. *FEMS Microbiology Ecology* 77**,** 600-610.

Silva, U.C., Cuadros-Orellana, S., Silva, D.R.C., Freitas-Júnior, L.F., Fernandes, A.C., Leite, L.R., Oliveira, C.A., and Dos Santos, V.L. (2021). Genomic and phenotypic insights into the potential of rock phosphate solubilizing bacteria to promote millet growth *in vivo*. *Frontiers in Microbiology* 11.

Singh, P.P., Shin, Y.C., Park, C.S., and Chung, Y.R. (1999). Biological control of *Fusarium* wilt of cucumber by chitinolytic bacteria. *Phytopathology* 89**,** 92-99.

Skraly, F.A., and Cameron, D.C. (1998). Purification and characterization of a *Bacillus licheniformis* phosphatase specific ford-α-glycerophosphate. *Archives of Biochemistry and Biophysics* 349**,** 27-35.

Sloane, C., and King, P. (1988). *The economic impact of pasture weeds, pests and diseases on the australian wool industry*. Melbourne, Australia: Australian Wool Corporation. [Accessed 01/02/2018].

Smith, K.F., Forster, J.W., and Spangenberg, G.C. (2007). Converting genomic discoveries into genetic solutions for dairy pastures - An overview. *Australian Journal of Experimental Agriculture* 47**,** 1032-1038.

Smits, T.H.M., Rezzonico, F., and Duffy, B. (2011). Evolutionary insights from *Erwinia amylovora* genomics. *Journal of Biotechnology* 155**,** 34-39.

Spaepen, S. (2015). "Plant hormones produced by microbes," in *Principles of plant-microbe interactions: microbes for sustainable agriculture*, ed. B. Lugtenberg. (Cham: Springer International Publishing), 247-256.

Spaepen, S., Dobbelaere, S., Croonenborghs, A., and Vanderleyden, J. (2008). Effects of *Azospirillum brasilense* indole-3-acetic acid production on inoculated wheat plants. *Plant and Soil* 312**,** 15-23.

Spaepen, S., Versées, W., Gocke, D., Pohl, M., Steyaert, J., and Vanderleyden, J. (2007). Characterization of phenylpyruvate decarboxylase involved in auxin production of *Azospirillum brasilense*. *Journal of Bacteriology* 189**,** 7626-7633.

Staples, C.R., Lahiri, S., Raymond, J., Von Herbulis, L., Mukhophadhyay, B., and Blankenship, R.E. (2007). Expression and association of group IV nitrogenase NifD and NifH homologs in the non-nitrogen-fixing archaeon *Methanocaldococcus jannaschii*. *Journal of Bacteriology* 189**,** 7392-7398.

Strange, R.N., and Scott, P.R. (2005). Plant disease: a threat to global food security. *Annual Review of Phytopathology* 43**,** 83-116.

Sundara, B., Natarajan, V., and Hari, K. (2002). Influence of phosphorus solubilizing bacteria on the changes in soil available phosphorus and sugarcane and sugar yields. *Field Crops Research* 77**,** 43-49.

Tanaka, A., Takemoto, D., Chujo, T., and Scott, B. (2012). Fungal endophytes of grasses. *Current Opinion in Plant Biology* 15**,** 462-468.

Tannenbaum, I., Kaur, J., Mann, R., Sawbridge, T., Rodoni, B., and Spangenberg, G. (2020). Profiling the *Lolium perenne* microbiome: from seed to seed. *Phytobiomes Journal* 4**,** 281-289.

Tegeder, M., and Masclaux-Daubresse, C. (2018). Source and sink mechanisms of nitrogen transport and use. *New Phytologist* 217**,** 35-53.

Teixeira, L.C., Peixoto, R.S., Cury, J.C., Sul, W.J., Pellizari, V.H., Tiedje, J., and Rosado, A.S. (2010). Bacterial diversity in rhizosphere soil from Antarctic vascular plants of Admiralty Bay, maritime Antarctica. *The ISME Journal* 4**,** 989.

Thaller, M.C., Berlutti, F., Schippa, S., Iori, P., Passariello, C., and Rossolini, G.M. (1995). Heterogeneous patterns of acid phosphatases containing low-molecular-mass polypeptides in members of the family *Enterobacteriaceae*. *International Journal of Systematic and Evolutionary Microbiology* 45**,** 255-261.

Timmusk, S., Grantcharova, N., and Wagner, E.G.H. (2005). *Paenibacillus polymyxa* Invades Plant Roots and Forms Biofilms. *Applied and Environmental Microbiology* 71**,** 7292-7300.

Trüper, H. (2005). The type species of the genus *Paenibacillus* Ash et al. 1994 is *Paenibacillus polymyxa*. Opinion 77. Judicial Commission of the International Committee on Systematics of Prokaryotes. *International Journal of Systematic and Evolutionary Microbiology* 55**,** 17.

Turk-Kubo, K.A., Karamchandani, M., Capone, D.G., and Zehr, J.P. (2014). The paradox of marine heterotrophic nitrogen fixation: abundances of heterotrophic diazotrophs do

not account for nitrogen fixation rates in the Eastern Tropical South Pacific. *Environmental Microbiology* 16**,** 3095-3114.

Turk, K.A., Rees, A.P., Zehr, J.P., Pereira, N., Swift, P., Shelley, R., Lohan, M., Woodward, E.M.S., and Gilbert, J. (2011). Nitrogen fixation and nitrogenase (*nifH*) expression in tropical waters of the eastern North Atlantic. *The ISME Journal* 5**,** 1201.

Turner, T.R., James, E.K., and Poole, P.S. (2013). The plant microbiome. *Genome Biology* 14**,** 209.

Unkovich, M. (2012). Nitrogen fixation in Australian dairy systems: review and prospect. *Crop and Pasture Science* 63**,** 787-804.

Uren, N.C. (2007). Types, amounts, and possible functions of compounds released into the rhizosphere by soil-grown plants. *The rhizosphere. Biochemistry and organic substances at the soil-plant interface. Marcel Dekker, New York***,** 1-21.

Van Loon, L.C. (2007). Plant responses to plant growth-promoting rhizobacteria. *European Journal of Plant Pathology* 119**,** 243-254.

Vance, C.P. (2001). Symbiotic nitrogen fixation and phosphorus acquisition. Plant nutrition in a world of declining renewable resources. *Plant Physiology* 127**,** 390-397.

Vandenkoornhuyse, P., Quaiser, A., Duhamel, M., Le Van, A., and Dufresne, A. (2015). The importance of the microbiome of the plant holobiont. *New Phytologist* 206**,** 1196-1206.

Vansuyt, G., Robin, A., Briat, J.-F., Curie, C., and Lemanceau, P. (2007). Iron acquisition from Fe-pyoverdine by *Arabidopsis thaliana*. *Molecular Plant-Microbe Interactions* 20**,** 441-447.

Vater, J., Herfort, S., Doellinger, J., Weydmann, M., Borriss, R., and Lasch, P. (2018). Genome mining of the lipopeptide biosynthesis of *Paenibacillus polymyxa* E681 in combination with mass spectrometry: discovery of the lipoheptapeptide paenilipoheptin. *Chembiochem* 19**,** 744-753.

Vicente, J.G., Rothwell, S., Holub, E.B., and Studholme, D.J. (2017). Pathogenic, phenotypic and molecular characterisation of *Xanthomonas nasturtii* sp. nov. and *Xanthomonas floridensis* sp. nov., new species of *Xanthomonas* associated with watercress production in Florida. *International Journal of Systematic and Evolutionary Microbiology* 67**,** 3645-3654.

Vokou, D., Vareli, K., Zarali, E., Karamanoli, K., Constantinidou, H.-I.A., Monokrousos, N., Halley, J.M., and Sainis, I. (2012). Exploring biodiversity in the bacterial community of the Mediterranean phyllosphere and its relationship with airborne bacteria. *Microbial Ecology* 64**,** 714-724.

Vorholt, J.A. (2012). Microbial life in the phyllosphere. *Nature Reviews Microbiology* 10**,** 828.

Wagner, S. (2012). Biological nitrogen fixation. *Nature Education Knowledge* 3**,** 15.

Wallace, J.G., Kremling, K.A., Kovar, L.L., and Buckler, E.S. (2018). Quantitative genetics of the maize leaf microbiome. *Phytobiomes Journal* 2**,** 208-224.

Wandersman, C., and Delepelaire, P. (2004). Bacterial iron sources: from siderophores to hemophores. *Annual Review of Microbiology* 58**,** 611-647.

Wang, B., Cheng, H., Qian, W., Zhao, W., Liang, C., Liu, C., Cui, G., Liu, H., and Zhang, L. (2020). Comparative genome analysis and mining of secondary metabolites of *Paenibacillus polymyxa*. *Genes & Genetic Systems* 95**,** 141-150.

Wassermann, B., Cernava, T., Müller, H., Berg, C., and Berg, G. (2019). Seeds of native alpine plants host unique microbial communities embedded in cross-kingdom networks. *Microbiome* 7**,** 108.

White, J.F., Chen, Q., Torres, M.S., Mattera, R., Irizarry, I., Tadych, M., and Bergen, M. (2015). Collaboration between grass seedlings and rhizobacteria to scavenge organic nitrogen in soils. *AoB Plants* 7**,** plu093.

Wichmann, F., Vorhölter, F.J., Hersemann, L., Widmer, F., Blom, J., Niehaus, K., Reinhard, S., Conradin, C., and Kölliker, R. (2013). The noncanonical type III secretion system of *Xanthomonas translucens* pv. *graminis* is essential for forage grass infection. *Mol Plant Pathol* 14**,** 576-588.

Williams, W.M., Easton, H.S., and Jones, C.S. (2007). Future options and targets for pasture plant breeding in New Zealand. *New Zealand Journal of Agricultural Research* 50**,** 223-248.

Wu, S., Cao, Z., Li, Z., Cheung, K., and Wong, M. (2005). Effects of biofertilizer containing N-fixer, P and K solubilizers and AM fungi on maize growth: a greenhouse trial. *Geoderma* 125**,** 155-166.

Xiao, L., Estellé, J., Kiilerich, P., Ramayo-Caldas, Y., Xia, Z., Feng, Q., Liang, S., Pedersen, A.Ø., Kjeldsen, N.J., and Liu, C. (2016). A reference gene catalogue of the pig gut microbiome. *Nature Microbiology* 1**,** 16161.

Xie, J., Shi, H., Du, Z., Wang, T., Liu, X., and Chen, S. (2016). Comparative genomic and functional analysis reveal conservation of plant growth promoting traits in *Paenibacillus polymyxa* and its closely related species. *Scientific Reports* 6**,** 21329.

Yazdani, M., Bahmanyar, M.A., Pirdashti, H., and Esmaili, M.A. (2009). Effect of phosphate solubilization microorganisms (PSM) and plant growth promoting rhizobacteria (PGPR) on yield and yield components of corn (*Zea mays* L.). *Proceedings of the World Academy of Science, Engineering and Technology* 37**,** 90-92.

Zachow, C., Jahanshah, G., De Bruijn, I., Song, C., Ianni, F., Pataj, Z., Gerhardt, H., Pianet, I., Lammerhofer, M., Berg, G., Gross, H., and Raaijmakers, J.M. (2015). The novel lipopeptide poaeamide of the endophyte *Pseudomonas poae* RE*1-1-14 is involved in pathogen suppression and root colonization. *Molecular Plant-Microbe Interactions* 28**,** 800-810.

Zehr, J.P., Jenkins, B.D., Short, S.M., and Steward, G.F. (2003). Nitrogenase gene diversity and microbial community structure: a cross-system comparison. *Environmental Microbiology* 5**,** 539-554.

Zhang, Y.Z., Wang, E.T., Li, M., Li, Q.Q., Zhang, Y.M., Zhao, S.J., Jia, X.L., Zhang, L.H., Chen, W.F., and Chen, W.X. (2011). Effects of rhizobial inoculation, cropping systems and growth stages on endophytic bacterial community of soybean roots. *Plant and Soil* 347**,** 147.

Zhang, Z., and Nan, Z. (2014). *Erwinia persicina*, a possible new necrosis and wilt threat to forage or grain legumes production. *European Journal of Plant Pathology* 139**,** 349-358.

Chapter 2

Novel *Xanthomonas* species from the perennial ryegrass seed microbiome – Assessing the bioprotection activity of non-pathogenic relatives of pathogens

2.1 Chapter preface

This chapter details the characterisation of three *Xanthomonas* sp. strains (GW, SS and SI) isolated from the perennial ryegrass microbiome, representing the first published paper of this thesis. The three strains exhibited strong bioprotection activities against key fungal pathogens of grasses in *in vitro* assays, and the best performing strain (GW) provided prolonged protection (up to 39 days) in *in planta* assays. Complete circular genomes were generated for all three strains, and comparative genomics analyses of closely related strains showed they represented a novel species of the genus *Xanthomonas*. Further genomic analyses identified gene clusters associated with the production of bioprotectant secondary metabolites (e.g. siderophore and nonribosomal peptide synthetase) as well as genes associated with an endophytic lifestyle (e.g. Type VI secretion system). Moreover, no genes associated with the pathogenicity of *Xanthomonas* spp. (e.g. Type III secretion system and effectors) were identified. Overall, this novel species represented by the three strains is the first *Xanthomonas* sp. that is bioprotectant and non-pathogenic. The standardised methods developed in this chapter, such as the *in vitro* and *in silico* assays, have also been used in the characterisation of other bacterial strains isolated from perennial ryegrass, including *Paenibacillus* spp. (Chapter 3).

This chapter is presented in its final published format. The supplementary materials featured in this chapter are available at the journal's website. This chapter also contributed to a patent for the three strains (Appendix 1, section 1.2).

2.2 Publication details

Title: Novel *Xanthomonas* species from the perennial ryegrass seed microbiome – Assessing the bioprotection activity of non-pathogenic relatives of pathogens

Journal details: Frontiers in Microbiology, 26 August 2020, doi: 10.3389/fmicb.2020.01991

Stage of publication: Published

Authors: Tongda Li, Ross Mann, Timothy Sawbridge, Jatinder Kaur, Desmond Auer, German Spangenberg

2.3 Statement of contribution of joint authorship

TL designed and conducted the *in vitro* bioprotection assays and the *in silico* assays to analyse genomic data. TL also conducted genomic sequencing (long reads), and assisted JK to conduct genomic sequencing (short reads). DA designed and conducted the *in planta* bioprotection assays. TL prepared the manuscript. TS conceptualised the study. TL, TS, and RM designed the experiment. RM, TS, DA, and GS reviewed and edited the manuscript. TS and RM supervised the study. GS contributed to the funding acquisition.

2.4 Statement from the co-author confirming the authorship contribution of the PhD candidate

"As co-author of the manuscript 'Li, T., Mann, R., Sawbridge, T., Kaur, J., Auer, D., and Spangenberg, G. (2020). Novel *Xanthomonas* species from the perennial ryegrass seed microbiome – Assessing the bioprotection activity of non-pathogenic relatives of pathogens. Frontiers in Microbiology 11, 1991.', I confirm that Tongda Li made the following contributions,

- Designed and conducted the *in vitro* bioprotection assays
- Conducted genomic sequencing (long reads)
- Assisted Jatinder Kaur to conduct genomic sequencing (short reads)
- Designed and conducted the *in silico* assays to analyse genomic data
- Generated all figures and tables
- Prepared the manuscript, critical appraisal of content and response to reviewers"

Principal Research Scientist Timothy Sawbridge

Date: 09/02/2021

frontiers in Microbiology

ORIGINAL RESEARCH
published: 26 August 2020
doi: 10.3389/fmicb.2020.01991

Novel *Xanthomonas* Species From the Perennial Ryegrass Seed Microbiome – Assessing the Bioprotection Activity of Non-pathogenic Relatives of Pathogens

Tongda Li[1,2,3], Ross Mann[1,2], Timothy Sawbridge[1,2,3], Jatinder Kaur[1,2], Desmond Auer[1] and German Spangenberg[1,2,3]*

[1] Agriculture Victoria, AgriBio, Centre for AgriBioscience, Bundoora, VIC, Australia, [2] DairyBio, Bundoora, VIC, Australia, [3] School of Applied Systems Biology, La Trobe University, Bundoora, VIC, Australia

OPEN ACCESS

Edited by:
Christina Cowger, Plant Science Research Unit (USDA-ARS), United States

Reviewed by:
Jeffrey Jones, University of Florida, United States
Xianjun Yuan, Nanjing Agricultural University, China
Alison Jean Popay, AgResearch Ltd., New Zealand
Ralf Koebnik, Institut de Recherche pour le Développement (IRD), France

***Correspondence:**
Tongda Li
tongda.li@agriculture.vic.gov.au

Specialty section:
This article was submitted to Microbe and Virus Interactions with Plants, a section of the journal Frontiers in Microbiology

Received: 18 April 2020
Accepted: 28 July 2020
Published: 26 August 2020

Citation:
Li T, Mann R, Sawbridge T, Kaur J, Auer D and Spangenberg G (2020) Novel Xanthomonas Species From the Perennial Ryegrass Seed Microbiome – Assessing the Bioprotection Activity of Non-pathogenic Relatives of Pathogens. Front. Microbiol. 11:1991. doi: 10.3389/fmicb.2020.01991

The productivity of the Australian dairy industry is underpinned by pasture grasses, and importantly perennial ryegrass. The performance of these pasture grasses is supported by the fungal endophyte *Epichloë* spp. that has bioprotection activities, however, the broader microbiome is not well characterized. In this study, we characterized a novel bioprotectant *Xanthomonas* species isolated from perennial ryegrass (*Lolium perenne* L. cv. Alto). *In vitro* and *in planta* bioassays against key fungal pathogens of grasses (*Sclerotium rolfsii*, *Drechslera brizae* and *Microdochium nivale*) indicated strong bioprotection activities. A complete circular chromosome of ∼5.2 Mb was generated for three strains of the novel *Xanthomonas* sp. Based on the 16S ribosomal RNA gene, the strains were closely related to the plant pathogen *Xanthomonas translucens*, however, comparative genomics of 22 closely related xanthomonad strains indicated that these strains were a novel species. The comparative genomics analysis also identified two unique gene clusters associated with the production of bioprotectant secondary metabolites including one associated with a novel nonribosomal peptide synthetase and another with a siderophore. The analysis also identified genes associated with an endophytic lifestyle (e.g., Type VI secretion system), while no genes associated with pathogenicity were identified (e.g., Type III secretion system and effectors). Overall, these results indicate that these strains represent a novel, bioactive, non-pathogenic species of the genus *Xanthomonas*. Strain GW was the designated type strain of this novel *Xanthomonas* sp.

Keywords: bioprotection, *Xanthomonas*, perennial ryegrass, microbiome, non-pathogenic, secondary metabolite

INTRODUCTION

In Australia, the dairy industry has a farmgate value of $4.4 billion (2018 – 2019) and is ranked fourth for global market share (Dairy Australia, 2019). Despite its complexity in operation, the dairy industry can be summarized as the conversion of pastures grasses to milk and other dairy products. As such, the Australian dairy industry is underpinned by

the performance of pasture grasses, and importantly perennial ryegrass.

The productivity of pasture grasses can be severely affected by plant pathogens. The major bacterial grass pathogen globally is *Xanthomonas translucens* pv. *graminis*, which causes bacterial wilt of pasture grasses (Egli and Schmidt, 1982), however, this pathovar is not present in Australia. According to the Victorian Plant Pathogen Herbarium (VPRI, Bundoora, Victoria, Australia), fungal grass pathogens are more common in Australia, including *Pyrenophora* spp., *Sclerotium* spp., *Phoma* spp., *Bipolaris* spp. and *Microdochium nivale*. The successful management of these pathogens is important for improving pasture productivity.

Biological controls (or biopesticides) are one management strategy that uses living organisms (e.g., microorganisms) to suppress deleterious or pathogenic organisms (Bulgarelli et al., 2013a). These bioprotection agents represent around 6.8% of the global pesticides market (2016) and are predicted to be worth $79.3 billion by 2022 (Chen, 2018). This growing area has seen more than 1320 bioprotection products registered in the US Environmental Protection Agency in 2014 (Mehrotra et al., 2017). For example, the fungal endophyte *Epichloë* spp. is a biological control that protects pasture grasses from herbivore via the production of bioactive compounds (Kauppinen et al., 2016). In addition, many bacteria have bioprotection activities, including *Bacillus* spp. and *Pseudomonas* spp. (Berg, 2009).

The plant microbiome provides an excellent reservoir where potential microbial bioprotection agents could be discovered. The diverse range of microorganisms associated with plant (plant microbiome) play a remarkable role in determining the health and productivity of the host (Berendsen et al., 2012). Therefore, substantial attention has been put on studying the bioprotection activities of these microorganisms (Bulgarelli et al., 2013b).

Next-generation sequencing technologies have led to fundamental changes to bacterial genomics by lowering cost and increasing throughput (Metzker, 2010). Recent advances in long read sequencing platforms like Oxford Nanopore Technologies (ONT) have made generating complete circular genomes for bacteria much easier (Koren et al., 2017). The availability of complete genome sequences underpins both the taxonomic identification and characterisation of novel microbial bioprotection agents, including the putative mode of action (i.e., identification of secondary metabolite gene clusters) and non-pathogenicity (i.e., absence of pathogenicity factors).

To gain insight into the broader microbiome of pasture grasses, we have profiled the microbiome of perennial ryegrass (*Lolium perenne* L. cv. Alto) and isolated bacterial strains (Tannenbaum et al., 2020), which were assessed for their beneficial activities (e.g., bioprotection). Three closely related strains (strain GW, seed-associated; strain SS and SI, mature-plant associated) exhibited excellent bioprotection activities against phytopathogens (*in vitro* and *in planta*). Complete genome assemblies were generated for these bacteria, and genome analysis showed that they represent a novel species of the genus *Xanthomonas*. Further bioinformatics analysis was conducted to determine the production of secondary metabolites that are putatively associated with bioprotection activities and to examine the presence/absence of pathogenicity related genes.

MATERIALS AND METHODS

Bacterial Strain Isolation

Bacterial strains were isolated from perennial ryegrass (*Lolium perenne* L. cv. Alto, Barenbrug Agriseeds NZ). To isolate seed-associated bacteria, surface-sterilized seeds (3% NaOCl for 3 min, followed by 3 × sterile dH_2O washes) were germinated under sterile conditions (on moistened sterile filter paper in sealed Petri dish). Germinated seedlings (5–7 days old) were harvested and sectioned into aerial and root tissue. Tissues were suspended in sterile Phosphate Buffered Saline (PBS), and ground using a Qiagen TissueLyser II (2 × 1 min at 30 Hz). Plant macerates were serial diluted (1:10, 100 μL in 900 μL), and plated onto Reasoners 2 Agar (R2A, Oxoid or Amyl Media, Australia) to isolate pure separated colonies. To isolate mature plant-associated bacteria, plants were grown in pots in a glasshouse for at least 60 days with standard potting mix and harvested for leaf and root tissues. Root tissues were washed in PBS to remove soil particles and then sonicated for 10 min to remove soil particulates and the rhizosphere. Tissue maceration, serial dilutions and bacterial isolations were prepared as above. All isolated bacterial strains were taxonomically classified using matrix assisted laser desorption ionization time-of-flight mass spectrometry (Bruker ultrafleXtreme MALDI-TOF/TOF MS and Biotyper System) (Tannenbaum et al., 2020), and stored in nutrient broth with 15% glycerol (v/v) at −80°C.

Bioprotection Assay (*in vitro*)

An assay was designed to assess the *in vitro* bioprotection activity of bacterial strains against fungal phytopathogens of *Poaceae* species. The bacterial strains assessed included three xanthomonads (GW, SS, SI) and one *Paenibacillus* sp. (BU). Six fungal phytopathogens of *Poaceae* species (**Supplementary Table S1**) were obtained from the Victorian Plant Pathogen Herbarium (VPRI, Bundoora, VIC, Australia). Each bacterial strain was cultured in Nutrient Broth (BD Bioscience) overnight (OD = 1.0) and drop-inoculated (20 μL) onto four equidistant points on a Nutrient Agar (BD Bioscience) plate, which was then incubated overnight at 28°C. Then, a 6 × 6 mm plug of the phytopathogen (actively growing hyphae) was placed at the center of the plate and incubated at 28°C in dark. The incubation time varied to accommodate the differences in growth rate of the fungal pathogens (**Table 1**). The diameter of the fungal colony on the plate was measured twice. One reading was taken from the straight line that was defined by two inoculation points and the center of the plate, and the other reading was taken after rotating the plate for 45 degrees. The average of the two readings was used for statistical analysis. For each treatment, three plates were prepared as biological replicates. For the blank control, sterile Nutrient Broth was used to replace the bacteria. Statistical analysis (One-way ANOVA and Tukey Test) was conducted using OriginPro 2018 (Version b9.5.1.195) for any significant difference ($P < 0.05$) between treatments.

TABLE 1 | The average colony diameter (±standard error) of fungal pathogens when exposed to the three xanthomonads in a bioprotection assay (*in vitro*).

Pathogen ID	$T_{incubation}$/day	GW/cm	BU/cm	Blank/cm	SS/cm	SI/cm	Blank/cm
P. sorghina	9	2.83 ± 0.12^{a}	3.90 ± 0.06^{b}	4.43 ± 0.07^{b}	N/A	N/A	N/A
D. brizae	8	3.13 ± 0.07^{a}	3.67 ± 0.03^{b}	3.90 ± 0.06^{b}	2.63 ± 0.30^{a}	2.33 ± 0.42^{a}	4.50 ± 0.21^{b}
S. rolfsii	5	2.13 ± 0.14^{a}	6.10 ± 0.10^{b}	8.47 ± 0.03^{c}	2.13 ± 0.27^{a}	1.87 ± 0.14^{a}	8.46 ± 0.03^{b}
B. gossypina	7	2.27 ± 0.24^{a}	3.07 ± 0.07^{a}	5.00 ± 0.12^{b}	6.08 ± 0.22^{a}	5.95 ± 0.05^{a}	7.05 ± 0.41^{a}
F. verticillioides	10	4.67 ± 0.07^{a}	6.47 ± 0.09^{b}	6.90 ± 0.25^{b}	5.03 ± 1.09^{a}	6.43 ± 0.72^{a}	7.97 ± 0.03^{a}
M. nivale	6	2.37 ± 0.18^{a}	6.70 ± 0.12^{b}	7.37 ± 0.07^{b}	7.83 ± 0.12^{a}	6.90 ± 1.05^{a}	7.97 ± 0.03^{a}

^{a,b,c}*: Different letters are statistically significantly ($P < 0.05$) different. Strain GW/SS/SI: Xanthomonas sp. Strain BU: Paenibacillus sp.*

Bioprotection Assay (*in planta*)

An assay was designed to assess the *in planta* bioprotection activity of the bacterial strains against the fungal phytopathogen *Bipolaris sorokiniana* (VPRI 42684). The xanthomonad strain GW was used in this assay. Wheat seeds were surface-sterilized as per section 2.1. The bacterial strain was cultured in Nutrient Broth (BD Bioscience) for 6 h (OD = 0.5). Sterile seeds were imbibed in the bacterial culture for 18 h, removed from the culture, dried under sterile conditions and then germinated in dark at room temperature (23°C) for 4 days for root and shoot development. Germinated seedlings were transferred into pots with standard potting mix (4 seeds per pot, 4 pots per treatment) in a glasshouse (**Supplementary Table S2**) for 39 days. A 7 cm segment of the lowest leaf that was green and fully extended from each plant was excised and placed on 0.5% water agar. A sterile sharp needle was used to create a wound at the center of each leaf, to which 1 μL of *B. sorokiniana* spore suspension (8.5×10^3 spores/mL) was added. Plates were then sealed and left at room temperature (23°C) for 3 days. To assess the bioprotection activity, the size (measured in mm^2) of the lesion, chlorotic zones and fungal hyphal growth was recorded. For the blank control, sterile Nutrient Broth was used. Statistical analysis (One-way ANOVA and Tukey Test) was conducted using OriginPro 2018 (Version b9.5.1.195) for any significant difference ($P < 0.05$) between treatments.

Genome Sequencing

DNA was extracted from bacterial pellets of GW, SS and SI (overnight cultures) using a Wizard® Genomic DNA Purification Kit (A1120, Promega, Madison, WI, United States), and assessed for quality (average molecular weight ≥ 30 Kb) on an Agilent 2200 TapeStation (Agilent Technologies, Santa Clara, CA, United States).

Genomic sequencing libraries (short reads) were prepared from the DNA using the Illumina Nextera XT DNA library preparation kit (Cat# FC-131-1096) and sequenced on an Illumina HiSeq 3000 platform. Genomic sequence data (raw reads) were assessed for quality and filtered to remove any adapter and index sequence, and low-quality bases using fastp (Chen et al., 2018) with the following parameters: *-w 8 -3 -5*.

Genomic sequencing libraries (long reads) were prepared from the DNA using the Oxford Nanopore Technologies (ONT) transposases-based library preparation kit with minor modifications (SQK-RAD004, ONT, Oxford, United Kingdom) and sequenced on a MinION Mk1B platform (MIN-101B) with R9.4 flow cells (FLO-MIN106). Genomic sequence data (raw read signals) were basecalled using ONT's Albacore software (Version 2.3.4), and assessed for quality using NanoPlot (De Coster et al., 2018). Basecalled data was filtered to remove adapter sequences using Porechop (Version 0.2.3[1]), while reads shorter than 300 bp and the worst 5% of reads (based on quality) were discarded using Filtlong (Version 0.2.0[2]).

Genome Assembly, Classification and Alignment

The whole genome of GW, SS, and SI were assembled with filtered long and short reads using Unicycler (Wick et al., 2017). Long reads were used for primary assembly and to resolve repeat regions in the genome, whereas short reads were used to correct small base-level errors. Assembly graphs were visualized using Bandage (Wick et al., 2015). Assembled genomes were taxonomically classified by Kraken2 (Wood and Salzberg, 2014) using a custom database containing all completed bacterial reference genomes in NCBI (20/03/2020). Genomes of GW, SS, and SI were aligned using LASTZ (Version 1.04.00[3]), and visualized using AliTV (Ankenbrand et al., 2017).

Genome Annotation and Characterisation

The assembled genome of GW, SS and SI were annotated using Prokka (Seemann, 2014) with a custom *Xanthomonas* protein database (based on Kraken2 classification) to predict genes and corresponding functions. A further functional characterisation of annotated genomes was conducted using KEGG BlastKOALA (Kanehisa et al., 2016). Identification of secondary metabolite gene clusters from annotated genomes was conducted using antiSMASH (Weber et al., 2015) with the following options: *–clusterblast –asf –knownclusterblast –subclusterblast –smcogs –full-hmmer*. An evaluation of the presence of pathogenicity-related genes from the annotated genomes of all three strains (GW, SS, and SI) was conducted using BLAST (Camacho et al., 2009) (blastp and tblastn, *e*-value > $1e^{-10}$). Initially, pathogenicity-related genes previously reported in *Xanthomonas* spp. were targeted (133 genes), including secretion systems (Type I/II/III/VI), pili (Type IV), flagella, pathogenicity regulatory factors, xanthan biosynthesis and lipopolysaccharide biosynthesis. A further comparison of 36 genes involved

[1]https://github.com/rrwick/Porechop

[2]https://github.com/rrwick/Filtlong

[3]http://www.bx.psu.edu/~rsharris/lastz/

in Type III secretion systems (T3SS) from six pathogenic strains, including *X. translucens* pv. *translucens* DSM18974, *X. translucens* pv. *undulosa* Xtu4699, *X. translucens* pv. *cerealis* CFBP2541, *X. translucens* DAR61454, *X. translucens* pv. *graminis* Xtg29 and *X. translucens* pv. *graminis* ICMP6431, and the three strains was conducted, including structural and regulatory genes, as well as conserved and variable Type III effectors (T3Es). Transcription activator-like effectors (TALEs) were predicted from the three strains (GW, SS and SI) and three pathogenic strains using annoTALE (Grau et al., 2016). Since TALE genes usually have multiple near-perfect repeats in the sequence and multiple copies of sequences in the genome (White et al., 2009), short reads often struggle to properly assemble the TALEs regions (Peng et al., 2016). Therefore, only pathogenic strains whose genome was completely assembled, i.e., *X. translucens* pv. *translucens* DSM18974, *X. translucens* pv. *undulosa* Xtu4699 and *X. translucens* pv. *cerealis* CFBP2541, were used in the prediction of TALE genes. The genome of strain GW and *X. translucens* pv. *undulosa* Xtu4699 were aligned using BLAST (Camacho et al., 2009). The alignment as well as the T3SS, T3Es and TALE genes that were detected on the genome of *X. translucens* pv. *undulosa* Xtu4699 were visualized using BRIG (Alikhan et al., 2011).

Phylogeny and Comparative Genomics

Eighteen Group 1 *Xanthomonas* spp. genomes and one *X. campestris* genome (Group 2 *Xanthomonas*) that were publicly available on NCBI (**Supplementary Table S3**) were downloaded and used for phylogenetic analysis (Young et al., 2008). These genomes were annotated *de novo* using the method above. Genes that were shared by all strains were identified using Roary and aligned (codon aware) using PRANK (Löytynoja, 2014). A maximum-likelihood phylogenetic tree was inferred using FastTree (Price et al., 2010) with Jukes-Cantor Joins distances, the Generalized Time-Reversible substitution model and the CAT approximation model. Local branch support values were calculated using 1000 resamples with the Shimodaira–Hasegawa test.

RESULTS

Bioprotection Assay (*in vitro*)

Xanthomonas sp. strain GW significantly ($P < 0.05$) reduced the average colony diameter of all six fungal pathogens compared to the blank control, and four pathogens compared to *Paenibacillus* sp. strain BU (**Table 1**). Strain GW reduced the growth of *S. rolfsii, M. nivae, D. brizae, P. sorghina, F. verticillioides* and *B. gossypina* by 74.9, 67.8, 54.6, 36.1, 32.3, and 19.7%, respectively, compared to the blank control. Strain SI reduced the growth of *S. rolfsii* and *D. brizae* by 77.9 and 48.2%, respectively, and strain SS reduced the growth of *S. rolfsii* and *D. brizae* by 74.8 and 41.6%, respectively, when compared to the blank control. When comparing across the three xanthomonads, only strain GW significantly inhibited the growth of all pathogens, indicating its broad-spectrum bioprotection activity (**Supplementary Figures S1, S2**).

Bioprotection Assay (*in planta*)

Xanthomonas sp. strain GW significantly ($P < 0.05$) reduced the average size of lesion and fungal hyphal growth compared to the blank control (**Figure 1** and **Table 2**). The lesion size was reduced by 96.7%, and the area of fungal hyphal growth was reduced by 94.7%.

Genome Sequencing, Assembly and Annotation

A total of 9,674,929,775 bp short reads and 761,078,031 bp long reads were generated (**Supplementary Table S4**). Complete

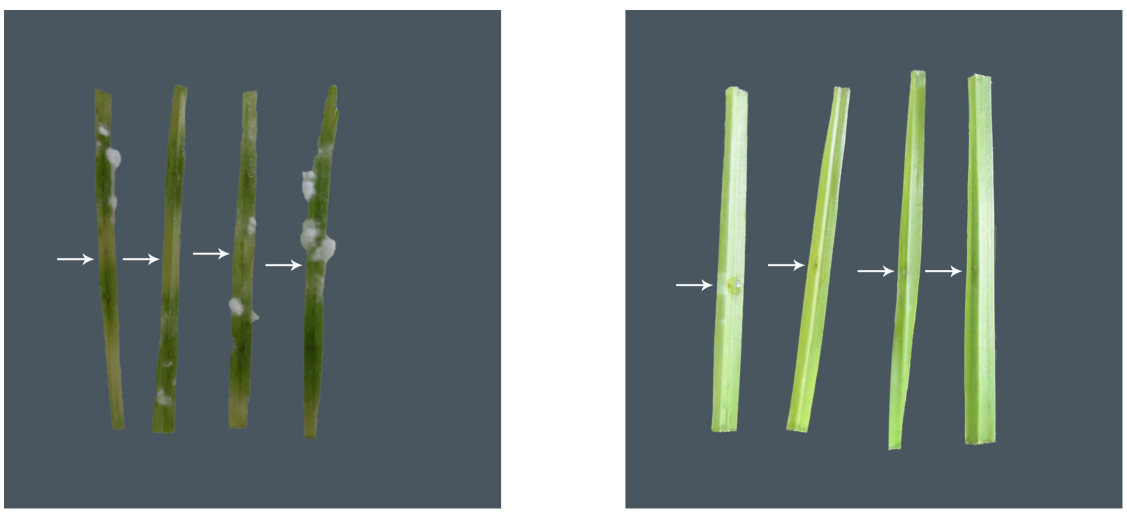


FIGURE 1 | Representative images of the *in planta* bioprotection assay for the blank control group (left) and the treatment group (inoculated with strain GW, right), with white arrows representing the point of inoculation of the pathogen *B. sorokiniana* (VPRI 42684) in wheat. Extensive leaf discoloration and white fungal hyphal growth are seen away from the point of inoculation in the blank control leaves, but not in the GW inoculated leaves.

TABLE 2 | The average size of area showing disease symptoms (±standard error) of *B. sorokiniana* when exposed to strain GW in a bioprotection assay (*in planta* in wheat).

Strain ID	Lesion/mm^2	Chlorosis/mm^2	Fungal hyphal growth/mm^2
GW	1.33 ± 0.25^a	34.44 ± 10.72^a	2.00 ± 1.37^a
Blank	42.75 ± 10.26^b	68.88 ± 22.50^a	37.63 ± 20.45^b

^{a,b}*: Different letters are statistically significantly ($P < 0.05$) different.*

circular genome sequences were produced for all three strains. The genome size for strain GW, SS and SI were 5,233,349 bp (4358 CDSs), 5,185,085 bp (4227 CDSs) and 5,246,417 bp (4290 CDSs), respectively, (**Table 3**). The percent GC content ranged from 68.37% to 68.55%. There were no plasmids present in any strain.

Phylogeny and Comparative Genomics

The three *Xanthomonas* strains (GW, SS, and SI) were phylogenetically related to *Xanthomonas translucens* (strain XT2, Genbank Accession: NR_036968.1) with a sequence coverage of 100% and homology of 99.53 – 99.73% based on the 16S ribosomal RNA gene. The genomes of the three xanthomonads were also classified as *X. translucens* pv. *cerealis* (NCBI:txid 152263) by Kraken2, suggesting their close relationship with *X. translucens*.

A comparative genomics analysis indicated that the three *Xanthomonas* strains (GW, SS, SI) belonged to the Group 1 *Xanthomonas* based on a sequence homology comparison of 68 genes shared by all 22 strains (**Figure 2**). The topology of the tree was consistent with Young et al. (2008), with unique clades/branches apparent for *X. albilineans*, *X. sacchari*, *X. theicola*, *X. hyacinthi* and *X. translucens*, with the three *Xanthomonas* strains (GW, SS, SI) between *X. hyacinthi* and *X. translucens*. The tree showed the three *Xanthomonas* strains (GW, SS, SI) formed a unique clade adjacent to *X. translucens* pathovars and were separated with a strong local support value (100%). The *X. translucens* clade were divided into a subclade consisting of *X. translucens* pv. *translucens* DSM18974, *X. translucens* pv. *undulosa* Xtu4699, *X. translucens* pv. *undulosa* ICMP11055 and *X. translucens* DAR61454 (**Figure 2**, yellow, barley and wheat pathogens) and a subclade consisting of *X. translucens* pv. *arrhenatheri* LMG727, *X. translucens* pv. *poae* LMG728, *X. translucens* pv. *phlei* LMG730 and all *X. translucens* pv. *graminis* strains (**Figure 2**, blue, pasture grass pathogens).

Average nucleotide identity (ANI) was calculated to further elucidate the relationship between the three *Xanthomonas* strains (GW, SS and SI) and *X. translucens* pathovars (**Supplementary Table S5**). The results showed 97.20 – 97.39% similarities between the three xanthomonads, and 92.97 – 94.07% similarities between the three xanthomonads and *X. translucens* pathovars.

Pathogenicity-Related Gene Analysis

The genomes of the three *Xanthomonas* strains (GW, SS and SI) were found to have a reduced complement of pathogenicity-related genes. The assessment of 133 pathogenicity-related genes identified that the three *Xanthomonas* strains (GW, SS and SI) was devoid of the T3SS that is critical for pathogenicity of most *Xanthomonas* species (White et al., 2009; Wichmann et al., 2013) (**Supplementary Table S6**). A comprehensive assessment of the T3SS structural and regulatory genes and T3Es across the three *Xanthomonas* strains (GW, SS and SI) and six pathogenic *X. translucens* strains identified that the three strains had 0 of 37 T3SS genes and T3Es (**Table 4**; **Figure 3**). This included an absence of the *hrc* genes, which encode the injectisome (Wagner et al., 2018), and the *hrp* genes, which are essential to suppress host plant defense responses for *Xanthomonas* species (Kay and Bonas, 2009). The *hrpF* gene, which encodes a translocon protein complex that is required to deliver T3Es (Chatterjee et al., 2013), was missing in all nine strains, which was supported by previous research (Pesce et al., 2017). However, the *hpaT* gene, which was described to encode an undescribed translocon protein complex of *X. translucens* strains (Pesce et al., 2017), was detected in all pathogenic strains but not in the three *Xanthomonas* strains (GW, SS and SI).

Similar to the T3SS and T3Es genes, no TALE genes could be identified in the genome of the three *Xanthomonas* strains (GW, SS and SI). Eight TALE genes were predicted for strain *X. translucens* pv. *undulosa* Xtu4699 (**Figure 3**) and *X. translucens* pv. *translucens* DSM18974, and two TALE genes were predicted for strain *X. translucens* pv. *cerealis* CFBP2541.

Secondary Metabolite

The *in vitro* and *in planta* bioprotection activity of the three *Xanthomonas* strains (GW, SS and SI) indicated that they could produce biocidal secondary metabolites. Furthermore, it has been demonstrated that both live culture and cell-free extracts of strain GW have biocidal activity against fungal phytopathogens (unpublished data). Secondary metabolite gene analysis identified three clusters (Clusters 1 – 3), with strain GW having all three clusters, and strain SI and SS having two of the three clusters. These clusters contain all the genes (core/additional biosynthetic genes, regulatory genes, transport-related genes and other genes) required for complete function (**Figures 4A–C**).

Cluster 1 contained a nonribosomal peptide synthetase (Nrps), and the entire cluster was unique to strain GW. Cluster 1 was located between bases 1,997,794 and 2,067,075 in

TABLE 3 | General genomic characteristics of the three *Xanthomonas* strains.

Strain ID	Genome size (bp)	GC content (%)	No. of tRNA	No. of tmRNA	No. of rRNA	No. of gene	No. of CDS
GW	5,233,349	68.37	60	1	6	4425	4358
SS	5,185,085	68.55	57	1	6	4291	4227
SI	5,246,417	68.44	63	1	6	4360	4290

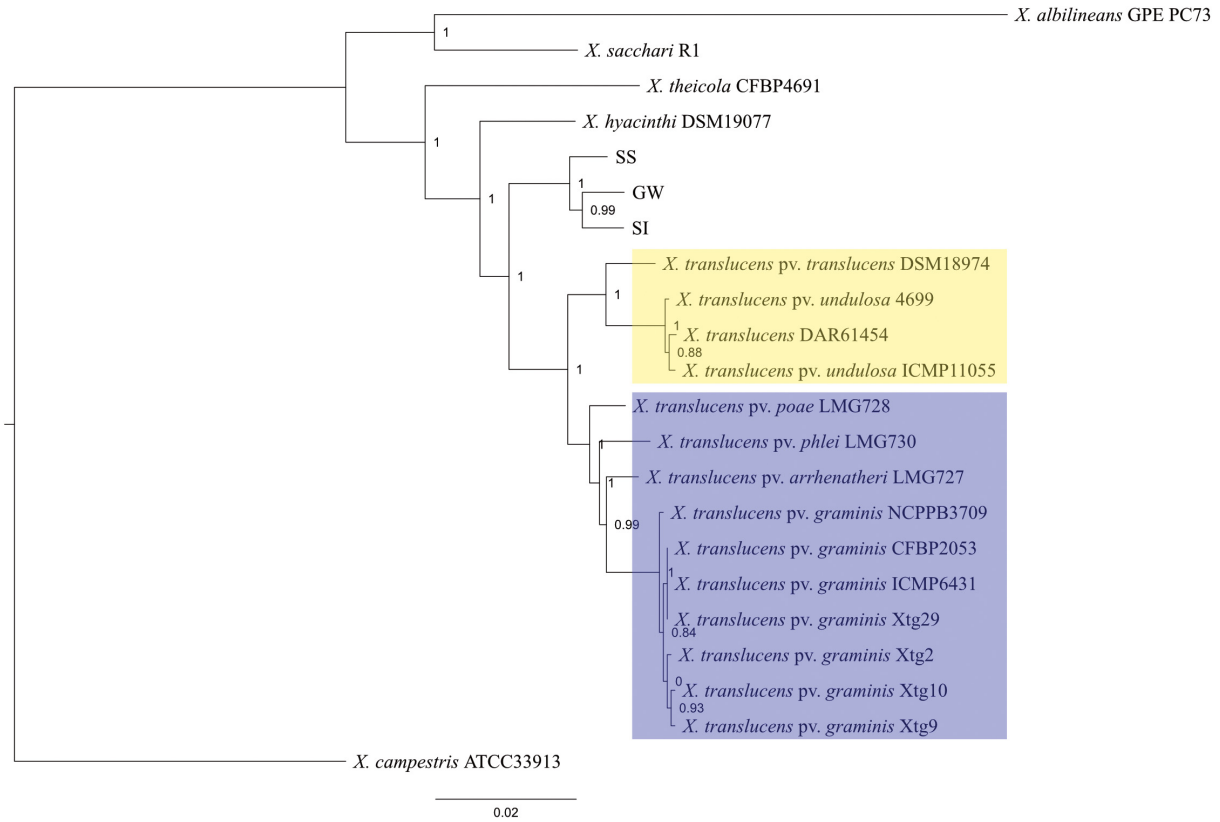


FIGURE 2 | Phylogeny of Group 1 *Xanthomonas* species and strain GW, SS and SI. This maximum-likelihood tree was inferred based on 68 genes conserved among 22 genomes. Values shown next to branches were the local support values calculated using 1000 resamples with the Shimodaira–Hasegawa test. Strain GW, SS and SI formed a clade that was well separated from *X. translucens* pathovars that are pathogenic on crop species (yellow) and grass species (blue).

the genome of strain GW, while this region was absent from strain SS and SI (**Figure 4D**). Cluster 1 appears novel based on sequence homology searching against the antiSMASH gene clusters database. Cluster 2 contained a siderophore synthetase and the entire cluster was present in all three strains. Cluster 2 was located between bases 1,300,000 and 1,380,000 in the genomes of strain GW, SS and SI (**Figure 4D**). Cluster 2 has sequence homology to the xanthoferrin biosynthesis gene cluster. Cluster 3 contained an aryl polyene synthase and the entire cluster was present in all three strains, however, slight variations in the cluster structure were observed (**Figure 4C**). Cluster 3 was located between bases 4,860,000 and 4,980,000 in the genomes of strain GW, SS and SI (**Figure 4D**). Cluster 3 has sequence homology to the xanthomonadin biosynthesis gene cluster.

DISCUSSION

Plant microbiomes are a repository from which plant beneficial bacteria can be isolated and identified. In this study, we compared three related *Xanthomonas* strains from the *L. perenne* microbiome. These had differing *in vitro* bioprotection activities, and the strain with the strongest activities against a wide range of phytopathogens (GW) became the focus of this study. Based on the complete genome assembly, strain GW possesses a novel Nrps cluster compared to the other two strains. All three strains lack many of the genes that are essential for pathogenicity in pathogenic *Xanthomonas* strains. Such characteristics made strain GW a promising candidate to be developed as a bioprotection agent for crops and grasses.

Identification of a Novel Xanthomonas Species

Taxonomic identification of bacterial species often uses 16S ribosomal RNA, whole genome sequence homology and ANI, with each technique providing varying degrees of taxonomic resolution. Taxonomic assignment based on the 16S ribosomal RNA gene provides genus level resolution, whereas whole genome techniques provide species or sub-species resolution. In this study, the initial classification based on 16S ribosomal RNA and whole genome sequence against the NCBI RefSeq database suggested that strain GW, SS and SI were most likely representatives of the plant pathogenic *X. translucens*. However, comparative genome analysis demonstrated the three strains formed a cluster that was separated from other *X. translucens* pathovars. Most importantly, the ANI between these three strains and *X. translucens* pathovars was lower than the species boundary, which is 95 – 96% ANI

TABLE 4 | T3SS and T3Es genes in the genome of the three *Xanthomonas* strains (GW, SS and SI) and other *X. translucens* strains.

	Gene	GW SS SI	DSM 18974 Barley US	Xtu4699 Wheat US	CFBP2541 Bromegrass US	DAR61454 Wheat AU	Xtg29 Forage grass CH	ICMP6431 Perennial ryegrass NZ
T3SS gene components	*hrcC*	–	+	+	+	+	+	+
	hrcJ	–	+	+	+	+	+	+
	hrcN	–	+	+	+	+	+	+
	hrcQ	–	+	+	+	+	+	+
	hrcR	–	+	+	+	+	+	+
	hrcS	–	+	+	+	+	+	+
	hrcT	–	+	+	+	+	+	+
	hrcU	–	+	+	+	+	+	+
	hrcV	–	+	+	+	+	+	+
	hrpB1	–	+	+	+	+	+	+
	hrpB2	–	+	+	+	+	+	+
	hrpE	–	+	+	+	+	+	+
	hrpF	–	–	–	–	–	–	–
	hpaT	–	+	+	+	+	+	+
Conserved T3Es	*XopB*	–	+	+	+	+	+	+
	XopC2	–	+	+	+	+	+	+
	XopF	–	+	+	+	+	+	+
	XopG	–	+	+	+	+	+	+
	XopK	–	+	+	+	+	+	+
	XopN	–	+	+	+	+	+	+
	XopQ	–	+	+	+	+	+	+
	XopV	–	+	+	+	+	+	+
	XopX	–	+	+	+	+	+	+
	XopY	–	+	+	+	+	+	–
	XopZ	–	+	+	+	+	+	+
	XopAA	–	+	+	+	+	–	–
	XopAD	–	+	+	+	+	+	+
	XopAM	–	+	+	+	+	+	+
Variable T3Es	*AvrBs1*	–	–	–	+	–	–	–
	AvrBs2	–	+	+	+	+	+	+
	XopE1	–	–	+	+	+	+	+
	XopL	–	+	+	+	+	+	–
	XopP	–	+	+	+	+	+	+
	XopR	–	+	+	+	+	+	+
	XopAF	–	+	+	+	+	+	+
	XopAH	–	+	+	–	+	–	–

Gray (+)/white (–): presence/absence of gene in genome. Blue: strains that have a complete genome sequence available, Yellow: strains that have no complete genome sequence available.

(Richter and Rosselló-Móra, 2009; Chun et al., 2018). Therefore, the three xanthomonads used in this study represent a novel species of the genus *Xanthomonas*. This clearly demonstrated the limitations of 16S ribosomal RNA-based classification (Klenk and Goker, 2010). Due to the technical limitations of the short-read sequencing platforms, most microbiome studies only used a variable region of the 16S ribosomal RNA (Pollock et al., 2018). It was likely that such novel, bioactive *Xanthomonas* species were present in the samples but were overlooked since they were classified as the known pathogenic *Xanthomonas* species. Moreover, this study also emphasized the importance of available whole genome sequences. The hybrid assembly approach used here combined the advantages of both short reads and long reads to produce high quality genome sequences for all three strains, which underpinned the downstream analysis including taxonomic identification and functional characterisation of the genomic resources.

Absence of Pathogenicity-Related Genes in the Three Xanthomonads

An analysis of pathogenicity-related genes clarified that the three xanthomonads were highly likely non-pathogenic. The T3SS and T3SS-related effector proteins (T3Es and TALEs) were completely absent from the genome of the three xanthomonads. The T3SS is a needle and syringe-like system that delivers (i) T3Es that suppress plant innate immunity and modulate plant cellular pathways to enhance bacterial infection (Büttner, 2016), and (ii) TALEs that induce host susceptibility genes to enhance

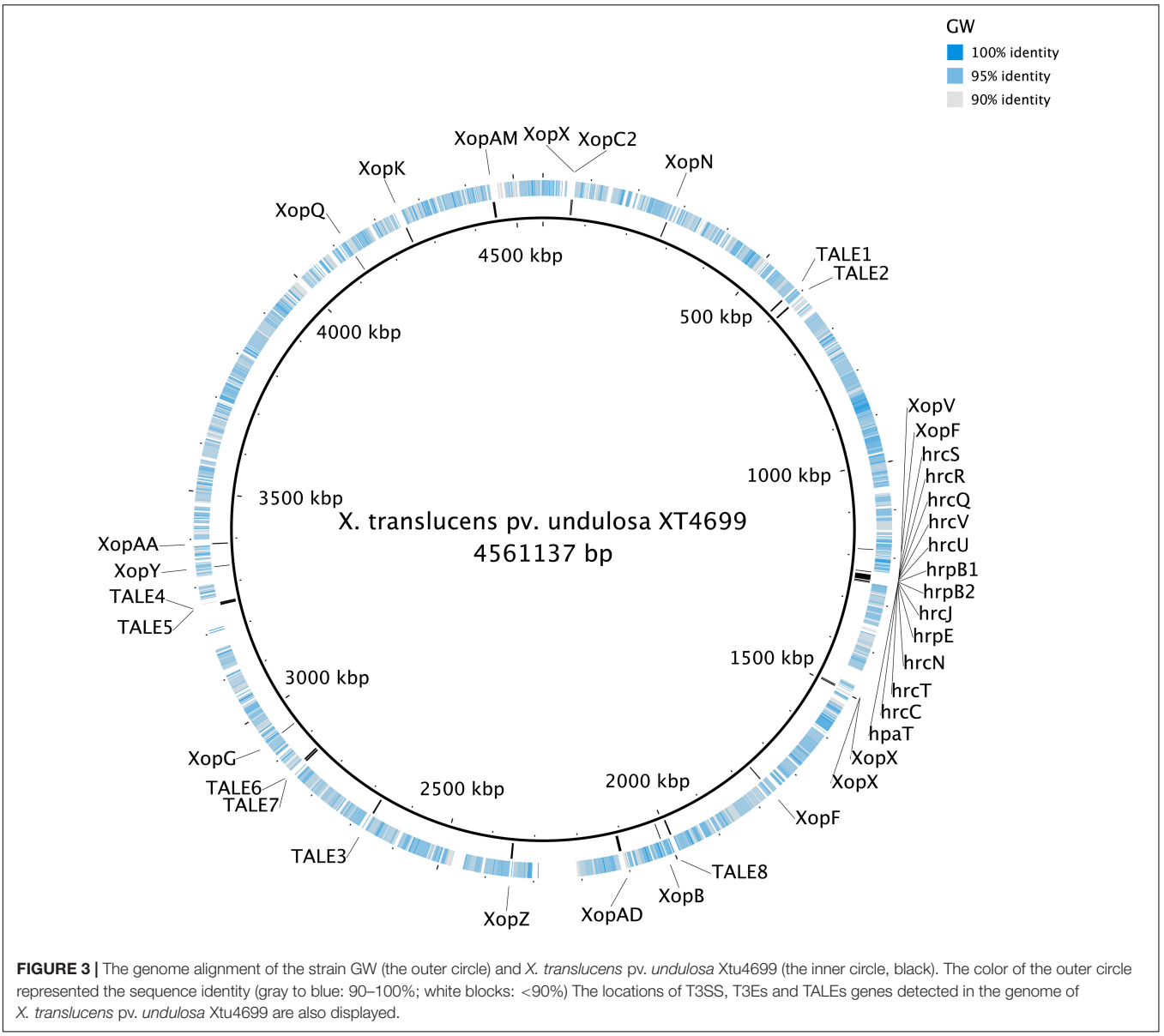


FIGURE 3 | The genome alignment of the strain GW (the outer circle) and *X. translucens* pv. *undulosa* Xtu4699 (the inner circle, black). The color of the outer circle represented the sequence identity (gray to blue: 90–100%; white blocks: <90%) The locations of T3SS, T3Es and TALEs genes detected in the genome of *X. translucens* pv. *undulosa* Xtu4699 are also displayed.

virulence (Cernadas et al., 2014; Hu et al., 2014), both of which are important for pathogenicity in *Xanthomonas* species (Green and Mecsas, 2016). For instance, deletion mutations of the T3SS structural genes *hrpE* or *hrcR* showed significantly reduced symptoms of *X. translucens* pv. *graminis* Xtg29 when compared with the wildtype strain (Wichmann et al., 2013). Furthermore, complete loss of symptoms was observed for a *X. translucens* pv. *undulosa* Xtu4699 strain with an insertion mutation in the T3SS structure gene *hrcC* (Peng et al., 2016). Complete absence of the T3SS and T3Es has been reported in other *Xanthomonas* species, such as *X. arboricola* strains (Group 2 *Xanthomonas*) which were referred to as non-pathogenic (Garita-Cambronero et al., 2017).

It must be stated that some xanthomonads that lacked the Hrp T3SS were found to be associated with diseased plants including *X. cannabis* NCPPB3735 and *X. cannabis* NCPPB2877 strains (Group 2 *Xanthomonas*) that could cause symptoms on hemp, barley and tobacco (Jacobs et al., 2015). While they lacked the Hrp T3SS, they had *HrpG* and *HrpX*, which are two key Hrp pathogenicity regulator genes (Büttner and Bonas, 2010), that were absent from the genome of the three xanthomonads. Moreover, there is another pathogenic strain of the same species, *X. cannabis* pv. *phaseoli* (Nyagatare strain), that has been reported to have both regulator genes, the full Hrp T3SS and T3Es (Aritua et al., 2015). In Group 1 *Xanthomonas*, *X. albilineans* GPE PC73, which is a xylem-limited pathogen, also lacked the Hrp T3SS (Pieretti et al., 2009). However, this strain had a *Salmonella* pathogenicity island-1 (SPI-1) containing an alternate T3SS and a gene cluster that encodes the phytotoxic albicidin, neither of which was detected in the three xanthomonads used in this study. *X. sacchari*, which is also a Group 1 *Xanthomonas*, lacked the Hrp T3SS and the SPI-1 T3SS (Studholme et al., 2011). However, the strain was isolated from an insect from a diseased banana plant

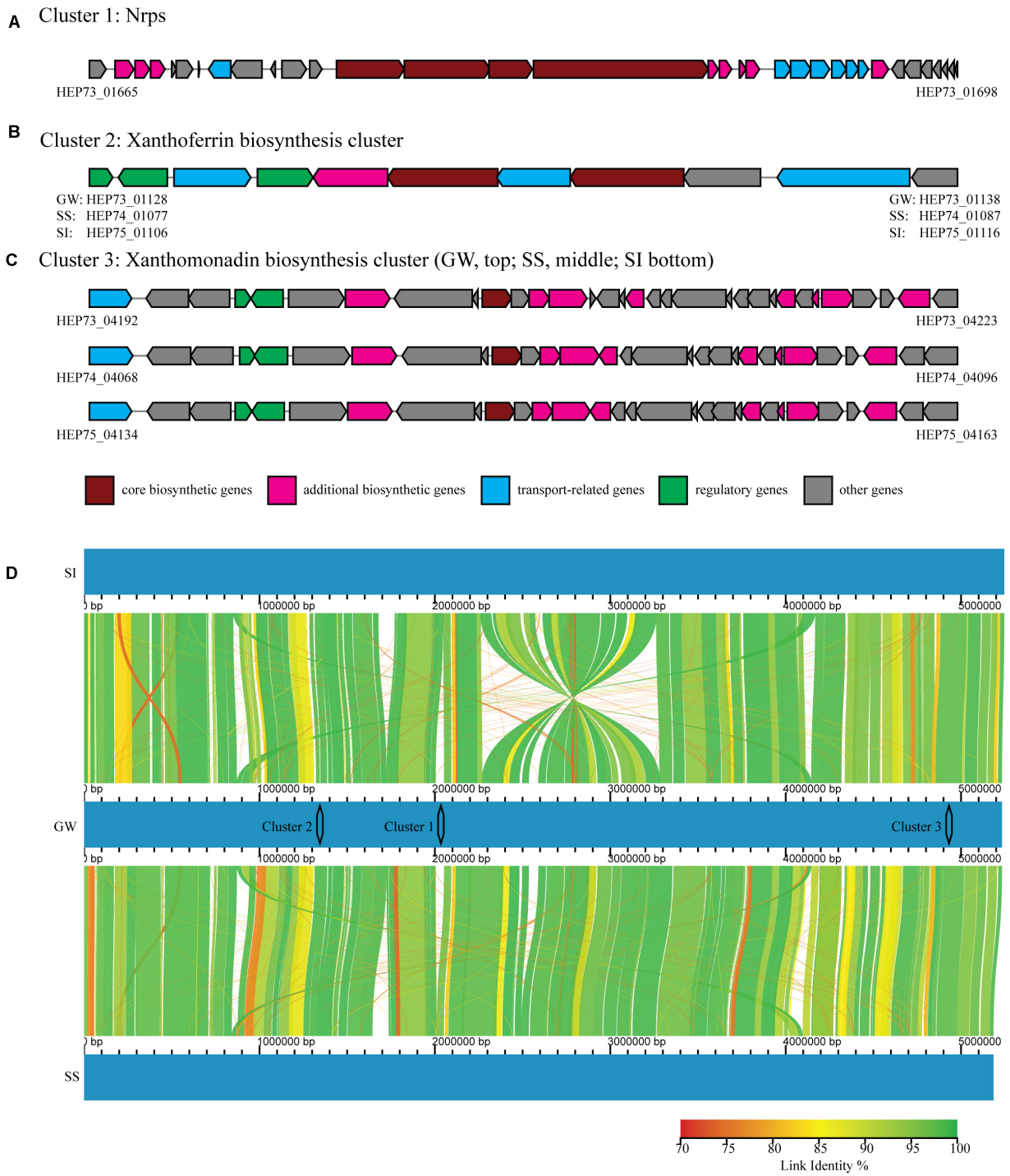


FIGURE 4 | Three secondary metabolite gene clusters **(A–C)** identified by antiSMASH, including core biosynthetic genes (maroon), additional biosynthetic genes (pink), regulatory genes (green), transport-related genes (blue) and other genes (gray). The NCBI identifiers were shown for genes located at both ends of each cluster. **(D)** Whole genome comparison of strain GW (middle), SI (top) and SS (bottom), with color graduation representing nucleotide percentage similarity between genomes (from 70 to 100%, red to green). The locations of the three clusters were also represented.

and there was no evidence of plant pathogenicity, which could be explained by the missing T3SS. T3SS has been proven to be essential for pathogenicity for *X. translucens* (Wichmann et al., 2013), which has the closest phylogenetic relationship amoug all known *Xanthonomas* species to the three xanthomonads in this study. Therefore, without any known type of T3SS, T3Es and

TALEs the three xanthomonads are highly likely non-pathogenic, and no symptoms have been seen in inoculated wheat, barley and ryegrass plants. Furthermore, given the fact that these genes are widely distributed across the whole chromosome (**Figure 3**), they are highly unlikely to acquire all the genes necessary to become pathogenic through horizontal gene transfer.

The three xanthomonads contained gene clusters (T1SS, T2SS, T6SS, type 4 pilus, flagella) linked to pathogenicity of *X. translucens* pathovars (**Supplementary Table S6**), however, these clusters have also been reported to possess functions associated with an endophytic lifestyle. For example, the T1SS was associated with biofilm formation (Tseng et al., 2009), the T2SS was used to secrete enzymes that facilitate environmental adaptation (Green and Mecsas, 2016), and the T6SS was involved in communication between bacteria or bacteria and the symbiotic host plant (Boyer et al., 2009). The three xanthomonads also had a type IV pilus cluster and a flagellar gene cluster that are associated with adherence and motility (Dunger et al., 2016; Hersemann et al., 2017). The presence of these gene clusters is supportive of the endophytic lifestyle proposed for the three xanthomonads.

Bioprotection Activity and Putative Mode of Action

Biological controls agents (e.g., bioprotectant bacteria) have been widely adopted globally for managing plant diseases as they are an effective and environmentally sustainable alternative to agrochemicals (Glare et al., 2012). For instance, biological control agents offer unique, complex modes of action, whereas agrichemicals have specific mode of action that can more easily lead to the development of resistance (Grimmer et al., 2015). Furthermore, there is less regulatory burden associated with biological control agents, in contrast to some agrichemicals that are under increased regulatory scrutiny as they have increasing environmental and public health concerns (Bach et al., 2016; Droby et al., 2016). Many *Bacillus*- and *Pseudomonas*- based biological control products have been commercialized globally for controlling bacterial and fungal phytopathogens (e.g., *Bacillus subtilis* for controlling *Fusarium* spp., and *Pseudomonas fluorescens* for controlling *Erwinia amylovora*) (Berg, 2009). These types of bacteria protect plants from phytopathogens directly via microbial antagonism, either endophytically (within the plant) or on the rhizosphere and phyllosphere (on the plant surface) (Eljounaidi et al., 2016; O'brien, 2017). Such antagonism can be carried out by competing for nutrients and spaces for colonization on the plant surface (Kamilova et al., 2005), and/or synthesizing allelochemicals such as antibiotics and siderophores to suppress pathogens (Beneduzi et al., 2012).

Bioprotection activity against fungi has not been reported to be associated with xanthomonads. *Xanthomonas* spp. are commonly associated with plants as either endophytes (Bouffaud et al., 2014; Bulgarelli et al., 2015; Zarraonaindia et al., 2015; Mitter et al., 2017) or as phytopathogens (An et al., 2019). In this study, we isolated three xanthomonads that had bioprotection activity, providing inhibitory activity against fungal pathogens from a broad taxonomic range (2 Phyla, 5 Families) in *in vitro* and *in planta* assays. The activity observed in the assays was a reduction in growth of the pathogen, and while no complete control was observed the xanthomonad strains restricted growth up to 77.9% of some pathogens. Strain GW also provided prolonged protection (up to 39 days) against the pathogen in the *in planta* assay. This suggests two methods of plant protection including (1) localized microbial colonization of a plant tissue from which antibiotic compounds are produced that are translocated systemically throughout the plant, or (2) systemic microbial colonization of the plant from which the bacteria either competes for nutrients or produces antibiotic compounds. Method 1 is utilized by *Epichloë* spp. endophytes to protect *Poaceae* species against pests and pathogens (Johnson et al., 2013), whereas method 2 is utilized by *Erwinia* and *Pantoea* species (Born et al., 2016; Smits et al., 2019). Given the *in vitro* bioprotection assay indicated production of a bioactive suppressant, we propose that the *in planta* activity is analogous. Further experiments including *in planta* assays and glasshouse and field assays have been planned to explore the potential bioactivity of strain GW.

Xanthomonas spp. have been shown to produce an array of bioactive secondary metabolites including the siderophore xanthoferrin, the pigment xanthomonadin, and the polysaccharide xanthan gum (Poplawsky et al., 2000; He et al., 2011; Palaniraj and Jayaraman, 2011; Huang et al., 2015; Pandey et al., 2017; Madden et al., 2019). A genomics-based assessment identified two secondary metabolite gene clusters that could be linked to the bioactivity of strain GW, SS and SI. A xanthoferrin siderophore synthesis cluster was detected in all three strains. First described in *X. campestris* pv. *campestris*, xanthoferrin is a vibrioferrin-type siderophore which facilitate iron uptake of bacteria by binding ferric iron from the environment (Andrews et al., 2003). Bacterial siderophores have higher affinity to iron compared to fungal siderophores (Compant et al., 2005), and therefore they can act as bioprotection agents under iron-limiting environments by depriving fungi of this essential element. This has been observed in fluorescent pseudomonads against the fungal pathogen *Fusarium oxysporum* (Kloepper et al., 1980; Dwivedi and Johri, 2003). Therefore, xanthoferrin could be responsible for the *in vitro* bioprotection activity that was observed from the three xanthomonads. Given that siderophores are predominantly produced locally (Saha et al., 2016), the mode of action of such bioprotection activity could be explained by method 2 described above. Moreover, strain GW showed a stronger and broader spectrum bioprotection activity against phytopathogens compared to strain SS and SI. Given that a novel Nrps cluster that was unique to strain GW but was missing from strain SS and SI, we proposed a hypothesis that the product of this novel Nrps cluster was responsible for the broad-spectrum of bioprotection activity of strain GW. Further research is needed to prove this hypothesis, including creating mutants of the Nrps cluster and evaluate the bioprotection activity (*in vitro*), along with identifying, purifying and characterizing the active compound(s). The mode of action of the bioprotection activity that provide by this Nrps could be either method described above.

DATA AVAILABILITY STATEMENT

Annotated genome sequences of all strains were deposited in the NCBI GenBank with the accession numbers: CP051189 for GW, CP051190 for SS, and CP051261 for SI.

AUTHOR CONTRIBUTIONS

TS conceptualized the study. TL prepared the manuscript. TL, TS, and RM designed the experiment. TL, JK, and DA contributed to the laboratory work. RM, TS, DA, and GS reviewed and edited the manuscript. TS and RM supervised the study. GS contributed to the funding acquisition. All authors have read and agreed to the submitted version of the manuscript.

FUNDING

This research was supported by the Agriculture Victoria, Dairy Australia, and Gardiner Foundation.

ACKNOWLEDGMENTS

TL received La Trobe University Full-Fee Research Scholarship, La Trobe University Postgraduate Research Scholarship and DairyBio Scholarship. The authors wish to thank Dr. Jacqueline Edwards for access to the Victorian Plant Pathogen Herbarium.

SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fmicb.2020.01991/full#supplementary-material

FIGURE S1 | Representative images of the *in vitro* bioprotection assay when challenging strain GW and BU with *Microdochium nivale*.

FIGURE S2 | Representative images of the *in vitro* bioprotection assay when challenging strain SS and SI with *Microdochium nivale*.

TABLE S1 | Pathogens used in the *in vitro* bioprotection assay.

TABLE S2 | Programmed conditions of the glasshouse used in this study.

TABLE S3 | *Xanthomonas* spp. genomes used in phylogeny and comparative genomics.

TABLE S4 | Summary of reads available for genome assembly.

TABLE S5 | The average nucleotide identity (ANI) between *Xanthomonas* spp. genomes used in comparative genomics.

TABLE S6 | Pathogenicity-related gene clusters identified in *Xanthomonas* spp.

REFERENCES

Alikhan, N.-F., Petty, N. K., Ben Zakour, N. L., and Beatson, S. A. (2011). BLAST ring image generator (BRIG): simple prokaryote genome comparisons. *BMC Genomics* 12:402. doi: 10.1186/1471-2164-12-402

An, S. Q., Potnis, N., Dow, M., Vorhölter, F. J., He, Y. Q., Becker, A., et al. (2019). Mechanistic insights into host adaptation, virulence and epidemiology of the phytopathogen *Xanthomonas*. *FEMS Microbiol. Rev.* 44, 1–32.

Andrews, S. C., Robinson, A. K., and Rodríguez-Quiñones, F. (2003). Bacterial iron homeostasis. *FEMS Microbiol. Rev.* 27, 215–237. doi: 10.1016/s0168-6445(03)00055-x

Ankenbrand, M. J., Hohlfeld, S., Hackl, T., and Förster, F. (2017). AliTV—interactive visualization of whole genome comparisons. *PeerJ Comput. Sci.* 3:e116. doi: 10.7717/peerj-cs.116

Aritua, V., Musoni, A., Kabeja, A., Butare, L., Mukamuhirwa, F., Gahakwa, D., et al. (2015). The draft genome sequence of *Xanthomonas* species strain Nyagatare, isolated from diseased bean in Rwanda. *FEMS Microbiol. Rev.* 362, 1–4.

Bach, E., Seger, G. D. D. S., Fernandes, G. D. C., Lisboa, B. B., and Passaglia, L. M. P. (2016). Evaluation of biological control and rhizosphere competence of plant growth promoting bacteria. *Appl. Soil Ecol.* 99, 141–149. doi: 10.1016/j.apsoil.2015.11.002

Beneduzi, A., Ambrosini, A., and Passaglia, L. M. (2012). Plant growth-promoting rhizobacteria (PGPR): their potential as antagonists and biocontrol agents. *Genet. Mol. Biol.* 35, 1044–1051. doi: 10.1590/s1415-47572012000600020

Berendsen, R. L., Pieterse, C. M., and Bakker, P. A. (2012). The rhizosphere microbiome and plant health. *Trends Plant Sci.* 17, 478–486. doi: 10.1016/j.tplants.2012.04.001

Berg, G. (2009). Plant-microbe interactions promoting plant growth and health: perspectives for controlled use of microorganisms in agriculture. *Appl. Microbiol. Biotechnol.* 84, 11–18. doi: 10.1007/s00253-009-2092-7

Born, Y., Remus-Emsermann, M. N., Bieri, M., Kamber, T., Piel, J., and Pelludat, C. (2016). Fe2+ chelator proferrorosamine A: a gene cluster of *Erwinia rhapontici* P45 involved in its synthesis and its impact on growth of *Erwinia amylovora* CFBP1430. *Microbiology* 162, 236–245. doi: 10.1099/mic.0.000231

Bouffaud, M. L., Poirier, M. A., Muller, D., and Moenne-Loccoz, Y. (2014). Root microbiome relates to plant host evolution in maize and other Poaceae. *Environ. Microbiol.* 16, 2804–2814. doi: 10.1111/1462-2920.12442

Boyer, F., Fichant, G., Berthod, J., Vandenbrouck, Y., and Attree, I. (2009). Dissecting the bacterial type VI secretion system by a genome wide in silico analysis: what can be learned from available microbial genomic resources? *BMC Genomics* 10:104. doi: 10.1186/1471-2164-12-104

Bulgarelli, D., Garrido-Oter, R., Munch, P. C., Weiman, A., Droge, J., Pan, Y., et al. (2015). Structure and function of the bacterial root microbiota in wild and domesticated barley. *Cell Host Microb.* 17, 392–403. doi: 10.1016/j.chom.2015.01.011

Bulgarelli, D., Rott, M., Schlaeppi, K., Van Themaat, E. V. L., Ahmadinejad, N., Assenza, F., et al. (2013a). Revealing structure and assembly cues for *Arabidopsis* root-inhabiting bacterial microbiota. *Nature* 501:S25.

Bulgarelli, D., Schlaeppi, K., Spaepen, S., Van Themaat, E. V. L., and Schulze-Lefert, P. (2013b). Structure and functions of the bacterial microbiota of plants. *Annu. Rev. Plant Biol.* 64, 807–838. doi: 10.1146/annurev-arplant-050312-120106

Büttner, D. (2016). Behind the lines-actions of bacterial type III effector proteins in plant cells. *FEMS Microbiol. Rev.* 40, 894–937. doi: 10.1093/femsre/fuw026

Büttner, D., and Bonas, U. (2010). Regulation and secretion of *Xanthomonas* virulence factors. *FEMS Microbiol. Rev.* 34, 107–133.

Camacho, C., Coulouris, G., Avagyan, V., Ma, N., Papadopoulos, J., Bealer, K., et al. (2009). BLAST+: architecture and applications. *BMC Bioinform.* 10:421. doi: 10.1186/1471-2164-12-421

Cernadas, R. A., Doyle, E. L., Nino-Liu, D. O., Wilkins, K. E., Bancroft, T., Wang, L., et al. (2014). Code-assisted discovery of TAL effector targets in bacterial leaf streak of rice reveals contrast with bacterial blight and a novel susceptibility gene. *PLoS Pathog.* 10:e1003972. doi: 10.1371/journal.ppat.1004126

Chatterjee, S., Chaudhury, S., Mcshan, A. C., Kaur, K., and De Guzman, R. N. (2013). Structure and biophysics of type III secretion in bacteria. *Biochemistry* 52, 2508–2517. doi: 10.1021/bi400160a

Chen, J. (2018). *Biopesticides: GLOBAL MARKETs to 2022. BCC Research*. Available: https://www.bccresearch.com/market-research/chemicals/biopesticides-global-markets-report.html (accessed September 9, 2019).

Chen, S., Zhou, Y., Chen, Y., and Gu, J. (2018). fastp: an ultra-fast all-in-one FASTQ preprocessor. *Bioinformatics* 34, i884–i890. doi: 10.1093/bioinformatics/bty560

Chun, J., Oren, A., Ventosa, A., Christensen, H., Arahal, D. R., Da Costa, M. S., et al. (2018). Proposed minimal standards for the use of genome data for the taxonomy of prokaryotes. *Int. J. Syst. Evol. Microbiol.* 68, 461–466. doi: 10.1099/ijsem.0.002516

Compant, S., Duffy, B., Nowak, J., Clément, C., and Barka, E. A. (2005). Use of plant growth-promoting bacteria for biocontrol of plant diseases: principles, mechanisms of action, and future prospects. *Appl. Environ. Microbiol.* 71, 4951–4959. doi: 10.1128/aem.71.9.4951-4959.2005

Dairy Australia, (2019). *The Australian Dairy Industry In Focus 2019*. Available: https://www.dairyaustralia.com.au/industry/farm-facts/in-focus (accessed January 22, 2020).

De Coster, W., D'hert, S., Schultz, D. T., Cruts, M., and Van Broeckhoven, C. (2018). NanoPack: visualizing and processing long read sequencing data. *Bioinformatics* 34, 2666–2669. doi: 10.1093/bioinformatics/bty149

Droby, S., Wisniewski, M., Teixidó, N., Spadaro, D., and Jijakli, M. H. (2016). The science, development, and commercialization of postharvest biocontrol products. *Postharvest Biol. Technol.* 122, 22–29. doi: 10.1016/j.postharvbio.2016.04.006

Dunger, G., Llontop, E., Guzzo, C. R., and Farah, C. S. (2016). The *Xanthomonas* type IV pilus. *Curr. Opin. Microbiol.* 30, 88–97. doi: 10.1016/j.mib.2016.01.007

Dwivedi, D., and Johri, B. (2003). Antifungals from fluorescent pseudomonads: biosynthesis and regulation. *Curr. Sci.* 85, 1693–1703.

Egli, T., and Schmidt, D. (1982). Pathogenic variation among the causal agents of bacterial wilt of forage grasses. *J. Phytopathol.* 104, 138–150. doi: 10.1111/j.1439-0434.1982.tb00520.x

Eljounaidi, K., Lee, S. K., and Bae, H. (2016). Bacterial endophytes as potential biocontrol agents of vascular wilt diseases - review and future prospects. *Biol. Control* 103, 62–68. doi: 10.1016/j.biocontrol.2016.07.013

Garita-Cambronero, J., Palacio-Bielsa, A., Lopez, M. M., and Cubero, J. (2017). Pan-genomic analysis permits differentiation of virulent and non-virulent strains of *Xanthomonas arboricola* that cohabit *Prunus* spp. and elucidate bacterial virulence factors. *Front. Microbiol.* 8:573. doi: 10.3389/fmicb.2017.00573

Glare, T., Caradus, J., Gelernter, W., Jackson, T., Keyhani, N., Kohl, J., et al. (2012). Have biopesticides come of age? *Trends Biotechnol.* 30, 250–258. doi: 10.1016/j.tibtech.2012.01.003

Grau, J., Reschke, M., Erkes, A., Streubel, J., Morgan, R. D., Wilson, G. G., et al. (2016). AnnoTALE: bioinformatics tools for identification, annotation, and nomenclature of TALEs from *Xanthomonas* genomic sequences. *Sci. Rep.* 6:21077.

Green, E. R., and Mecsas, J. (2016). Bacterial secretion systems: an overview. *Microbiol. Spectr.* 4:10.1128/microbiolsec.VMBF-0012-2015. doi: 10.1128/microbiolspec.VMBF-0012-2015

Grimmer, M. K., Van Den Bosch, F., Powers, S. J., and Paveley, N. D. (2015). Fungicide resistance risk assessment based on traits associated with the rate of pathogen evolution. *Pest Manag. Sci.* 71, 207–215. doi: 10.1002/ps.3781

He, Y.-W., Wu, J. E., Zhou, L., Yang, F., He, Y.-Q., Jiang, B.-L., et al. (2011). *Xanthomonas campestris* diffusible factor is 3-hydroxybenzoic acid and is associated with xanthomonadin biosynthesis, cell viability, antioxidant activity, and systemic invasion. *Mol. Plant Microb. Interact.* 24, 948–957. doi: 10.1094/mpmi-02-11-0031

Hersemann, L., Wibberg, D., Blom, J., Goesmann, A., Widmer, F., Vorhölter, F. J., et al. (2017). Comparative genomics of host adaptive traits in *Xanthomonas translucens* pv. *graminis*. *BMC Genomics* 18:35. doi: 10.1186/1471-2164-12-35

Hu, Y., Zhang, J., Jia, H., Sosso, D., Li, T., Frommer, W. B., et al. (2014). Lateral organ boundaries 1 is a disease susceptibility gene for citrus bacterial canker disease. *Proc. Natl. Acad. Sci. U.S.A.* 111, E521–E529.

Huang, C.-L., Pu, P.-H., Huang, H.-J., Sung, H.-M., Liaw, H.-J., Chen, Y.-M., et al. (2015). Ecological genomics in *Xanthomonas*: the nature of genetic adaptation with homologous recombination and host shifts. *BMC Genom.* 16:188. doi: 10.1186/1471-2164-12-188

Jacobs, J. M., Pesce, C., Lefeuvre, P., and Koebnik, R. (2015). Comparative genomics of a cannabis pathogen reveals insight into the evolution of pathogenicity in *Xanthomonas*. *Front. Plant Sci.* 6:431. doi: 10.3389/fmicb.2017.00431

Johnson, L. J., De Bonth, A. C. M., Briggs, L. R., Caradus, J. R., Finch, S. C., Fleetwood, D. J., et al. (2013). The exploitation of epichloae endophytes for agricultural benefit. *Fungal Divers.* 60, 171–188. doi: 10.1007/s13225-013-0239-4

Kamilova, F., Validov, S., Azarova, T., Mulders, I., and Lugtenberg, B. (2005). Enrichment for enhanced competitive plant root tip colonizers selects for a new class of biocontrol bacteria. *Environ. Microbiol.* 7, 1809–1817. doi: 10.1111/j.1462-2920.2005.00889.x

Kanehisa, M., Sato, Y., and Morishima, K. (2016). BlastKOALA and GhostKOALA: KEGG tools for functional characterization of genome and metagenome sequences. *J. Mol. Biol.* 428, 726–731. doi: 10.1016/j.jmb.2015.11.006

Kauppinen, M., Saikkonen, K., Helander, M., Pirttila, A. M., and Wali, P. R. (2016). *Epichloë* grass endophytes in sustainable agriculture. *Nat. Plants* 2:15224.

Kay, S., and Bonas, U. (2009). How *Xanthomonas* type III effectors manipulate the host plant. *Curr. Opin. Microbiol.* 12, 37–43. doi: 10.1016/j.mib.2008.12.006

Klenk, H. P., and Goker, M. (2010). En route to a genome-based classification of Archaea and Bacteria? *Syst. Appl. Microbiol.* 33, 175–182. doi: 10.1016/j.syapm.2010.03.003

Kloepper, J. W., Leong, J., Teintze, M., and Schroth, M. N. (1980). *Pseudomonas* siderophores: a mechanism explaining disease-suppressive soils. *Curr. Microbiol.* 4, 317–320. doi: 10.1007/bf02602840

Koren, S., Walenz, B. P., Berlin, K., Miller, J. R., Bergman, N. H., and Phillippy, A. M. (2017). Canu: scalable and accurate long-read assembly via adaptive k-mer weighting and repeat separation. *Genome Res.* 27, 722–736. doi: 10.1101/gr.215087.116

Löytynoja, A. (2014). "Phylogeny-aware alignment with PRANK," in *Multiple Sequence Alignment Methods*, ed. D. J. Russell, (Totowa, NJ: Humana Press), 155–170. doi: 10.1007/978-1-62703-646-7_10

Madden, K. S., Jokhoo, H., Conradi, F., Knowles, J., Mullineaux, C., Whiting, A. J. O., et al. (2019). Using nature's polyenes as templates: studies of synthetic xanthomonadin analogues and realising their potential as antioxidants. *Organic Biomol. Chem.* 17, 3752–3759. doi: 10.1039/c9ob00275h

Mehrotra, S., Kumar, S., Zahid, M., and Garg, M. (2017). "Biopesticides," in *Principles and Applications of Environmental Biotechnology for a Sustainable Future*, ed. R. L. Singh, (Berlin: Springer), 273–292.

Metzker, M. L. (2010). Sequencing technologies—the next generation. *Nat. Rev. Genet.* 11:31.

Mitter, E. K., De Freitas, J. R., and Germida, J. J. (2017). Bacterial root microbiome of plants growing in oil sands reclamation covers. *Front. Microbiol.* 8:849. doi: 10.3389/fmicb.2017.00849

O'brien, P. A. (2017). Biological control of plant diseases. *Austral. Plant Pathol.* 46, 293–304.

Palaniraj, A., and Jayaraman, V. (2011). Production, recovery and applications of xanthan gum by *Xanthomonas campestris*. *J. Food Eng.* 106, 1–12. doi: 10.1016/j.jfoodeng.2011.03.035

Pandey, S. S., Patnana, P. K., Rai, R., and Chatterjee, S. (2017). Xanthoferrin, the alpha-hydroxycarboxylate-type siderophore of *Xanthomonas campestris* pv. campestris, is required for optimum virulence and growth inside cabbage. *Mol. Plant Pathol.* 18, 949–962. doi: 10.1111/mpp.12451

Peng, Z., Hu, Y., Xie, J., Potnis, N., Akhunova, A., Jones, J., et al. (2016). Long read and single molecule DNA sequencing simplifies genome assembly and TAL effector gene analysis of *Xanthomonas translucens*. *BMC Genom.* 17:21. doi: 10.1186/1471-2164-12-21

Pesce, C., Jacobs, J. M., Berthelot, E., Perret, M., Vancheva, T., Bragard, C., et al. (2017). Comparative genomics identifies a novel conserved protein, HpaT, in proteobacterial Type III secretion systems that do not possess the putative translocon protein HrpF. *Front. Microbiol.* 8:1177. doi: 10.3389/fmicb.2017.01177

Pieretti, I., Royer, M., Barbe, V., Carrere, S., Koebnik, R., Cociancich, S., et al. (2009). The complete genome sequence of *Xanthomonas albilineans* provides new insights into the reductive genome evolution of the xylem-limited *Xanthomonadaceae*. *BMC Genomics* 10:616. doi: 10.1186/1471-2164-12-616

Pollock, J., Glendinning, L., Wisedchanwet, T., and Watson, M. (2018). The madness of microbiome: attempting to find consensus "best practice" for 16S microbiome studies. *Appl. Environ. Microbiol.* 84:e02627-17.

Poplawsky, A. R., Urban, S. C., and Chun, W. (2000). Biological role of xanthomonadin pigments in *Xanthomonas campestris* pv. *campestris*. *Appl. Environ. Microbiol.* 66:5123. doi: 10.1128/aem.66.12.5123-5127.2000

Price, M. N., Dehal, P. S., and Arkin, A. P. (2010). FastTree 2–approximately maximum-likelihood trees for large alignments. *PLoS One* 5:e9490. doi: 10.1371/journal.ppat.009490

Richter, M., and Rosselló-Móra, R. (2009). Shifting the genomic gold standard for the prokaryotic species definition. *Proc. Natl. Acad. Sci. U.S.A.* 106, 19126–19131. doi: 10.1073/pnas.0906412106

Saha, M., Sarkar, S., Sarkar, B., Sharma, B. K., Bhattacharjee, S., and Tribedi, P. (2016). Microbial siderophores and their potential applications: a review. *Environ. Sci. Pollut. Res.* 23, 3984–3999. doi: 10.1007/s11356-015-4294-0

Seemann, T. (2014). Prokka: rapid prokaryotic genome annotation. *Bioinformatics* 30, 2068–2069. doi: 10.1093/bioinformatics/btu153

Smits, T. H. M., Duffy, B., Blom, J., Ishimaru, C. A., and Stockwell, V. O. (2019). Pantocin A, a peptide-derived antibiotic involved in biological control by plant-associated *Pantoea* species. *Archiv. Microbiol.* 201, 713–722. doi: 10.1007/s00203-019-01647-7

Studholme, D. J., Wasukira, A., Paszkiewicz, K., Aritua, V., Thwaites, R., Smith, J., et al. (2011). Draft Genome Sequences of *Xanthomonas sacchari* and two banana-associated *Xanthomonads* reveal insights into the *Xanthomonas* Group 1 Clade. *Genes* 2, 1050–1065. doi: 10.3390/genes2041050

Tannenbaum, I., Kaur, J., Mann, R., Sawbridge, T., Rodoni, B., and Spangenberg, H. (2020). Profiling the *Lolium perenne* microbiome: from seed to seed. *Phytobiome J.* 4, 281–289. doi: 10.1094/PBIOMES-03-20-0026-R

Tseng, T. T., Tyler, B. M., and Setubal, J. C. (2009). Protein secretion systems in bacterial-host associations, and their description in the gene ontology. *BMC Microbiol.* 9(Suppl. 1):S2. doi: 10.1186/1471-2180-9-S1-S2

Wagner, S., Grin, I., Malmsheimer, S., Singh, N., Torres-Vargas, C. E., and Westerhausen, S. (2018). Bacterial type III secretion systems: a complex device for the delivery of bacterial effector proteins into eukaryotic host cells. *FEMS Microbiol. Lett.* 365:fny201.

Weber, T., Blin, K., Duddela, S., Krug, D., Kim, H. U., and Bruccoleri, R. (2015). antiSMASH 3.0-a comprehensive resource for the genome mining of biosynthetic gene clusters. *Nucleic Acids Res.* 43, W237–W243.

White, F. F., Potnis, N., Jones, J. B., and Koebnik, R. (2009). The type III effectors of *Xanthomonas*. *Mol. Plant Pathol.* 10, 749–766.

Wichmann, F., Vorhölter, F. J., Hersemann, L., Widmer, F., Blom, J., Niehaus, K., et al. (2013). The noncanonical type III secretion system of *Xanthomonas translucens* pv. graminis is essential for forage grass infection. *Mol. Plant Pathol.* 14, 576–588. doi: 10.1111/mpp.12030

Wick, R. R., Judd, L. M., Gorrie, C. L., and Holt, K. E. (2017). Unicycler: resolving bacterial genome assemblies from short and long sequencing reads. *PLoS Computat. Biol.* 13:e1005595. doi: 10.1371/journal.ppat.1005595

Wick, R. R., Schultz, M. B., Zobel, J., and Holt, K. E. (2015). Bandage: interactive visualization of de novo genome assemblies. *Bioinformatics* 31, 3350–3352. doi: 10.1093/bioinformatics/btv383

Wood, D. E., and Salzberg, S. L. (2014). Kraken: ultrafast metagenomic sequence classification using exact alignments. *Genome Biol.* 15:R46.

Young, J. M., Park, D. C., Shearman, H. M., and Fargier, E. (2008). A multilocus sequence analysis of the genus *Xanthomonas*. *Syst. Appl. Microbiol.* 31, 366–377. doi: 10.1016/j.syapm.2008.06.004

Zarraonaindia, I., Owens, S. M., Weisenhorn, P., West, K., Hampton-Marcell, J., Lax, S., et al. (2015). The soil microbiome influences grapevine-associated microbiota. *mBio* 6:e02527-14.

Conflict of Interest: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Chapter 3

Transcriptomics differentiate two novel bioactive strains of *Paenibacillus* sp. isolated from the perennial ryegrass seed microbiome

3.1 Chapter preface

This chapter details the detection, isolation and characterisation of two *Paenibacillus* sp. strains. Two strains (S02 and S25) were isolated from perennial ryegrass seeds using selective media based on the genomic evidence obtained from PCR and genome sequencing. Complete circular genomes were generated for both strains, and comparative genomics analyses showed they represented a novel species that is closely related to *Paenibacillus polymyxa*, a well-known PGP bacterial species. This is believed to be the first report of this novel beneficial bacterium in Australia. Genomic analyses showed they possess PGP genes associated with plant nutrient uptake and metabolism (e.g. biological nitrogen fixation), as well as auxin production and transportation. Secondary metabolite gene clusters associated with bioprotection and other novel functions were also identified from both strains, whose bioprotection activities were confirmed in the *in vitro* assays. Transcriptomic analyses showed strain differentiation, with strain S02 being more active in expressing the *nif* operon in nitrogen-free medium, and the secondary metabolite gene clusters when being exposed the fungal phytopathogen *Fusarium verticillioides*. Such bioactivities made strain S02 an ideal candidate to be further developed as biofertiliser/bioprotectant for sustainable agriculture. The standardised methods of transcriptome sequencing and analysis developed in this chapter have also been used in the examination of early stage plant-bacteria interactions (Chapter 4).

This chapter is presented in submission-ready format for the journal Scientific Reports. The supplementary materials are presented in Appendix 2, and will be available at the journal's website once this chapter is published. This chapter also contributed to a patent for the two strains that will be publicly available in 2022.

3.2 Publication details

Title: Transcriptomics differentiate two novel bioactive strains of *Paenibacillus* sp. isolated from the perennial ryegrass seed microbiome

Journal details: Scientific Reports

Stage of publication: Submitted

Authors: Tongda Li, Ross Mann, Jatinder Kaur, German Spangenberg, Timothy Sawbridge

3.3 Statement of contribution of joint authorship

TL designed and conducted the detection and isolation of bacterial strains from seeds. TL conducted the *in vitro* bioprotection assays and the *in silico* assays to analyse genomic and transcriptomic data. TL also conducted genomic sequencing (long reads) and transcriptome sequencing, as well as assisted JK to conduct genomic sequencing (short reads). TL prepared the manuscript. TS conceptualised the study. TL, TS, and RM designed the experiment. RM, TS, and GS reviewed and edited the manuscript. TS and RM supervised the study. GS contributed to the funding acquisition.

3.4 Statement from the co-author confirming the authorship contribution of the PhD candidate

"As co-author of the manuscript 'Li, T., Mann, R., Kaur, J., Spangenberg, G. and Sawbridge, T. (2021). Transcriptomics differentiate two novel bioactive strains of *Paenibacillus* sp. isolated from the perennial ryegrass seed microbiome', I confirm that Tongda Li made the following contributions,

- Designed and conducted the detection and isolation of bacterial strains from seeds
- Conducted the *in vitro* bioprotection assays
- Conducted genomic sequencing (long reads) and transcriptome sequencing
- Assisted Jatinder Kaur to conduct genomic sequencing (short reads)
- Designed and conducted the *in silico* assays to analyse genomic and transcriptomic data
- Generated all figures and tables
- Prepared the manuscript, critical appraisal of content"

Principal Research Scientist Timothy Sawbridge

Date: 09/02/2021

Transcriptomics differentiate two novel bioactive strains of *Paenibacillus* sp. isolated from the perennial ryegrass seed microbiome

Tongda Li[*,1,2,3], Ross Mann[1,2], Jatinder Kaur[1,2], German Spangenberg[1,2,3], Timothy Sawbridge[1,2,3]

[1]Agriculture Victoria, AgriBio, Centre for AgriBioscience, Bundoora, Victoria, Australia
[2]DairyBio, Bundoora, Victoria, Australia
[3]School of Applied Systems Biology, La Trobe University, Bundoora, Victoria, Australia

*Correspondence:
Tongda Li
(+61)-3-9032 7088
tongda.li@agriculture.vic.gov.au

Abstract

Paenibacillus species are Gram-positive bacteria that have been isolated from a diverse array of plant species and soils, with some species exhibiting plant growth-promoting (PGP) activities. Here we report two strains (S02 and S25) of a novel *Paenibacillus* sp. that were isolated from perennial ryegrass (*Lolium perenne*) seeds. Comparative genomics analyses showed this novel species was closely related to *P. polymyxa*. Genomic analyses revealed that strains S02 and S25 possess PGP genes associated with biological nitrogen fixation, phosphate solubilisation and assimilation, as well as auxin production and transportation. Moreover, secondary metabolite gene cluster analyses identified 13 clusters that are shared by both strains and three clusters unique to S25. *In vitro* assays demonstrated strong bioprotection activity against phytopathogens (*Colletotrichum graminicola* and *Fusarium verticillioides*), particularly for strain S02. A transcriptomics analysis evaluating nitrogen fixation activity showed both strains carry an expressed *nif* operon, but strain S02 was more active than strain S25 in nitrogen-free media. Another transcriptomics analysis evaluating the interaction of strains with *F. verticillioides* showed strain S02 had increased expression of core genes of secondary metabolite clusters (fusaricidin, paenilan, tridecaptin and polymyxin) when *F. verticillioides* was present and absent, compared to S25. Such bioactivities make strain S02 a promising candidate to be developed as a combined biofertiliser/bioprotectant.

Keywords: bioprotection, biofertilisation, *Paenibacillus*, perennial ryegrass, microbiome, RNA-seq

1. Introduction

Microorganisms associated with plants (collectively the plant microbiome) are one of the key factors that determine plant health and productivity[1]. Plant growth-promoting (PGP) bacteria competitively colonise plant tissues and build beneficial interactions with plant hosts by acting as biofertilisers or bioprotectants[2], such as commercial products based on *Pseudomonas* spp. and *Rhizobium* spp. that have been utilised globally of the past 30-40 years[3]. Compared with other bacteria, the endospore-forming *Bacillus* spp. and *Paenibacillus* spp. could survive longer in soils with varying conditions such as pH, temperature and salinity, representing promising candidates for agricultural usage as biologicals[4].

Paenibacillus spp. are Gram-positive, facultative anaerobic bacteria that are commonly found in soil from diverse geographic environments[4,5]. As the type species of the genus *Paenibacillus*[6], *Paenibacillus polymyxa* inhabits the rhizosphere or root tissues of a wide range of agricultural crops like wheat and barley[7,8], agricultural pastures like perennial ryegrass[9] and forest trees like pine and cedar[10]. *P. polymyxa* has been reported to promote the growth of many plants, with significant improvements in nutrient uptake and increases in total biomass and/or seeding height[11-15]. Genomic analyses of *P. polymyxa* strains have revealed that they are capable of acting as both biofertilisers and bioprotectants[5,16-19]. As biofertilisers, *P. polymyxa* possesses genes involved in biological nitrogen (N) fixation, phosphate solubilisation and assimilation, iron assimilation and auxin production. As bioprotectants, *P. polymyxa* carries gene clusters that synthesise bioactive compounds including polymyxin and fusaricidin, as well as novel clusters of unknown functions. Furthermore, *P. polymyxa* also plays an active role in the biotechnology sector due to its ability to produce cell wall degrading enzymes and exopolysaccharides[20]. Such diverse properties have made *P. polymyxa* a prominent commercially useful PGP bacterium for sustainable agriculture[21,22].

The microbiome of perennial ryegrass (*Lolium perenne* L. cv. Alto) has been profiled recently by our laboratory, which suggested the presence of *Paenibacillus* spp. within the community[23]. This study aimed to confirm the presence of *Paenibacillus* spp. through genomic sequencing of the seed-associated bacterial community, and to isolate strains using selective media (antibiotics and nitrogen-free media). The biological nitrogen fixation ability of the community was assessed with *nifH* PCR. The genome of the isolated strains was

assembled, which was in turn used to confirm the taxonomy and confirm the presence of PGP genes and secondary metabolite gene clusters. Further assays were conducted to determine the *in vitro* bioprotection activities against phytopathogens and to analyse the changes in transcriptome profiles associated with biological nitrogen fixation and early stage bacteria-pathogen interactions of the isolated strains.

2. **Results**

2.1. Seed-associated N-fixing bacterial strain detection and isolation

The presence of bacteria containing the *nifH* gene was confirmed from the seed of perennial ryegrass, as amplicons of the expected size (~ 400 bp) were produced by the *nifH* gene PCR. Amplicons were generated using DNA extracted from a suspension of ground perennial ryegrass seeds that was serially diluted (10^{0}–10^{-3}), including from two of eight replicates of the 10^{-2} dilution (Supplementary Figure S1), while no amplicon was produced from all eight replicates of the 10^{-3} dilution. Amplicons were sequenced and identified as partial sequences of the *nifH* gene of *P. polymyxa* CR1 (Accession ID: CP006941.2, 1,087,670–1,088,026 bp; coverage = 97%, identity = 99%) using BLASTn search against the nt database.

Long read sequence data was also generated from the DNA of the seed suspension, and then classified by Kraken2[24]. The reads had sequence homology to multiple bacterial species including *Bacillus* spp. (high read abundance), *Pseudomonas* spp., *Massilia* spp. and *Paenibacillus* spp. (low read abundance). Despite the low *Paenibacillus* spp. read abundance, a single 110 Kb read containing the entire *P. polymyxa nif* operon (nine genes) was identified (Supplementary Figure S2), which confirmed the presence of *P. polymyxa* in the two dilutions.

The confirmation of a *P. polymyxa*-like bacterial strain in the seed suspension provided guidance regarding its isolation and purification. *P. polymyxa* produces the antibiotic polymyxin, which is biocidal against Gram-negative bacteria[25]. Supplementing media with polymyxin B aided in the isolation of the low abundant *Paenibacillus* spp. strains from the dominant bacteria. Two bacterial strains (S02 and S25) were isolated using Burk's N-free medium supplemented with polymyxin B. Both strains are rod-shaped and Gram-positive under microscopic examination and form heaped, small- to medium-sized colonies on agar

plates. Strain S02 produces white and mucoid colonies, whilst strain S25 produces translucent colonies. Both strains were stored in 15% glycerol at −80 ℃.

2.2. Genome sequencing, assembly and annotation

A total of 2,536,823,196 bp short reads and 13,203,686,400 bp long reads were generated for *Paenibacillus* sp. strains S02 and S25 (Supplementary Table S1). Complete circular genome sequences were produced for both strains. The genome size of *Paenibacillus* sp. S02 was 6,060,529 bp (5,310 CDSs), with a G+C content of 45.60%, while the genome size of *Paenibacillus* sp. S25 was 5,958,851 bp (5,177 CDSs), with a G+C content of 45.72% (Supplementary Table S2). There were no plasmids present in either strain.

2.3. Phylogeny and comparative genomics

The results of the *nifH* gene PCR and the mixed culture read analysis described in section 2.1 suggested that the isolated strains were closely related to the species *P. polymyxa*. The 16S ribosomal RNA genes showed both strains were phylogenetically related to *P. polymyxa* DSM36 (GenBank Accession: NR_117732.2) with a sequence homology of 99.45% and coverage of 100%. The close relationship between the two strains and *P. polymyxa* was further supported by genome-based identifications where both strains were classified by Kraken2 as *P. polymyxa* E681 (NCBI:txid 349520).

The average nucleotide identity (ANI) was compared between the genomes of the two isolated strains (S02 and S25) and 44 known *P. polymyxa* strains (Supplementary Table S3). The ANI dendrogram-heatmap revealed three major (Clades 1–3) and two minor clades (Figure 1). The ANI between strains within the same clade was at least 95%. Among the three major clades, strains from Clade 2 and 3 had ANI < 95%, while Clade 1 was further separated from the other two clades (ANI < 91%). *Paenibacillus* sp. strains S02 and S25 were in Clade 1 and had an ANI of 97.78% to one another. Strain S02 was most similar to *P. polymyxa* TD94 (ANI = 98.11%) which was isolated from *Scutellaria* spp. rhizosphere[26], while strain S25 was most similar to *P. polymyxa* YC0136 (ANI = 99.29%) which was isolated from tobacco rhizosphere[19]. Both strains were clustered with 16 known *P. polymyxa* strains isolated from various geographic regions including Asia, North America and Europe. The majority of the Clade 1 strains (14) were isolated from plant rhizosphere or soil, with the

exceptions being *P. polymyxa* CCI-25 isolated from vermicompost[18], and *P. polymyxa* J isolated from the phloem of a chilli plant. Clades 2 and 3 contained 18 and seven *P. polymyxa* strains respectively, and these strains were mainly isolated from plant rhizosphere or soil in Asia, Europe and North America. The type strain of *P. polymyxa* (ATCC 842) was place in Clade 2. *P. polymyxa* ZF197 and ND24 formed a minor clade that had an ANI of 92.45–92.61% and 92.93–93.09% when compared to Clade 2 and 3, respectively. There was also another minor clade containing a single strain *P. polymyxa* NCTC4744 isolated from the UK, which had low ANI values (< 89%) when compared to other strains.

A pan genome Roary[27] analysis was conducted comparing the sequence similarity of genes shared by *Paenibacillus* sp. strains S02 and S25, along with 13 other *P. polymyxa* strains with complete genomes available on NCBI. The analysis identified 2,059 shared genes by all 15 strains. A maximum-likelihood phylogenetic tree was inferred based on the sequence homology of the shared genes (Figure 2). The topology of the tree consisted of three major clades and was consistent with the ANI analysis (Figure 1, Clade 1–3). All clades were separated with a strong local support value (100%). Clade 1 consisted of eight strains, including the two *Paenibacillus* sp. strains (S02 and S25), and were separated from Clades 2 and 3 at the root node. Clades 2 and 3 formed adjoining clades on the same primary root node, and each had three strains. Strain ZF197 also clustered with Clade 2 and 3 but formed its own branch. Clade 1 consisted of strains from across a broad geographic range, including Asia (China, South Korea), the Pacific (Australia), North America (Canada) and Europe (Belgium), whereas Clades 2 and 3 were largely from Asia (China), except strain Sb3-1 in Clade 3 that was isolated from Egypt. All strains were either associated with plants or soil.

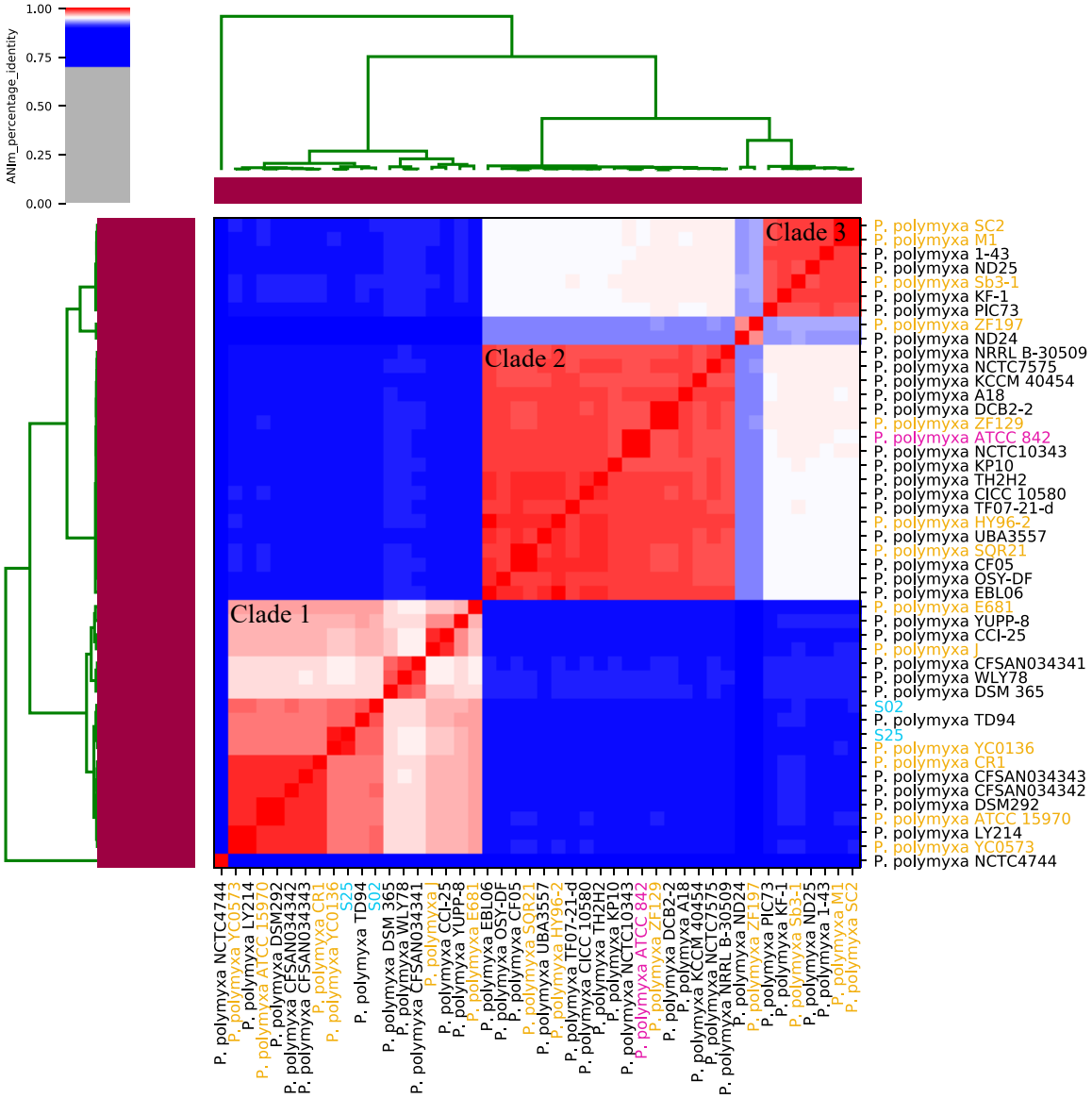


Figure 1. Phylogeny of *Paenibacillus* species based on a heatmap with row and column dendrograms from the average nucleotide identity (ANI) of genomes of *Paenibacillus* sp. strains S02 and S25 and 44 *P. polymyxa* strains from NCBI. Clustering across the dendrograms were based on overall genomic sequence similarity, forming three major clades and two minor clades (intra-cluster ANI > 95%). Clade 1 contained *Paenibacillus* sp. strains S02 and S25 as well as 16 known *P. polymyxa* strains. Clade 2 and 3 contained 18 and seven known *P. polymyxa* strains, respectively. *P. polymyxa* ZF197 and ND24 formed a minor clade, and *P. polymyxa* NCTC4744 formed another minor clade.
Blue label: *Paenibacillus* sp. strains isolated in this study
Yellow label: *P. polymyxa* strains with complete circular genome sequences
Purple label: The type strain of *P. polymyxa*

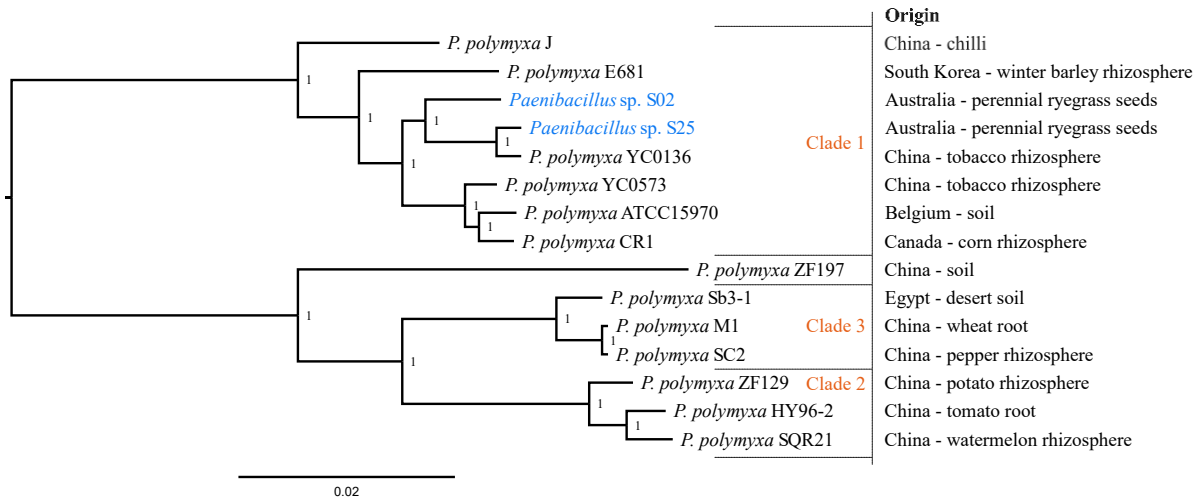


Figure 2. Phylogeny of *Paenibacillus* species based on a pan genome Roary analysis of strains S02 and S25 and13 *P. polymyxa* strains with complete circular genome sequences. The maximum-likelihood tree was inferred based on 2,059 genes conserved among 15 genomes. Values shown next to branches were the local support values calculated using 1,000 resamples with the Shimodaira-Hasegawa test. *Paenibacillus* sp. strains S02 and S25 clustered with other six *P. polymyxa* strains in Clade 1. Clades 2 and 3 separated from Clade 1 at the root node, and consisted of three *P. polymyxa* strains each.

2.4. Plant growth-promoting genes

The genomes of both *Paenibacillus* sp. strains (S02 and S25) were assessed for the presence of 30 plant growth-promoting (PGP) genes and were found to possess a comprehensive set of genes (Supplementary Table S4). A 10.54 Kb region containing a *nif* operon of nine genes (*nifB/H/D/K/E/N/X*, *hesA/moeB* and *nifV*) was identified, including all the genes necessary for encoding the Mo-nitrogenase (molybdenum-dependent nitrogenase) that catalyses biological nitrogen fixation[28]. In addition, 16 genes associated with phosphate solubilisation and assimilation were identified, including the glucose-1-dehydrogenase (*gcd*) gene for inorganic phosphate solubilisation[29], the *phn* cluster of nine genes for organic phosphate (phosphonates) solubilisation[2] and the phosphate-specific transport system of six genes for phosphate assimilation[30]. However, the gluconic acid dehydrogenase (*gad*) gene for inorganic phosphate solubilisation[29] was not found in either of the two strains. Additionally, genes involved in indole-3-acetic acid (IAA) production and transportation were identified, including the *ipdC* gene that encodes a key enzyme in the IAA biosynthetic pathway[31], as well as three auxin efflux carrier genes. Sequence comparison of the PGP genes between the two strains (S02 and S25) showed sequence similarity of 95.39–99.54%, while the two strains

showed sequence similarity of 94.69–99.78% when compared to *P. polymyxa* strain CR1 (Supplementary Table S5).

2.5. Secondary metabolite genes

Genes associated with secondary metabolite production were identified using antiSMASH[32]. The analyses identified 16 clusters (designated C1–C16) consisting of 13 clusters that were shared by both strains and three clusters that only strain S25 possessed (Supplementary Table S6). All clusters contained all the genes (core/additional biosynthetic genes, regulatory genes, transport-related genes and other genes) required for complete function.

Secondary metabolite gene clusters that were shared by both strains consisted of four that were identical to known clusters, including three nonribosomal peptide synthetase (Nrps) clusters (C1, fusaricidin B; C10, tridecaptin; C15, polymyxin) and one lanthipeptide cluster (C7, paenilan). The products of all four clusters have been reported to have antimicrobial bioactivities[25,33-35]. A further four clusters had partial sequence homology to known clusters included a lassopeptide cluster (C5), a Nrps cluster (C6), a Nrps/transAT-polyketide synthase (PKS) cluster (C11) and a Nrps/Type III (T3) PKS/transAT-PKS cluster (C14), which had homology to paeninodin, marthiapeptide A, paenilipoheptin and aurantinin B/C/D, respectively. Among these four clusters, cluster C11 had the highest similarity (S02, 73%; S25, 76%) to a known cluster of *P. polymyxa* E681 that produces paenilipoheptin[36]. There were also five clusters that appear novel based on sequence homology, including a siderophore cluster (C2), a bacteriocin cluster (C3), a Nrps/transAT-PKS cluster (C4), a Nrps-like cluster (C9) and a phosphonate cluster (C16).

Paenibacillus sp. S25 had three unique secondary metabolite gene clusters that were missing in the genome of *Paenibacillus* sp. S02, including a lanthipeptide cluster (C8) and two novel Nrps clusters (C12, C13). While the two Nrps clusters appear novel based on sequence homology, the lanthipeptide cluster had a similarity of 71% to a known paenicidin B cluster, which was a novel lantibiotic peptide active against Gram-positive bacteria produced by *Paenibacillus terrae*[35].

Secondary metabolite gene cluster analyses were also conducted using the 13 *P. polymyxa* strains with complete circular genome sequences (Figure 1, yellow labels), and their presence

was compared to *Paenibacillus* sp. strains S02 and S25. The total number of secondary metabolite gene clusters of each strain varied between 11 to 16 (Table 1). Clade-specific clusters were identified, including one associated with a novel siderophore and another associated with a novel phosphonate being exclusive to Clade 1 strains, and a nostamide A (betalactone) cluster being exclusive to Clade 2 strains. Moreover, the paenilipoheptin cluster was only identified in some Clade 1 and 3 strains but no Clade 2 strain. More than half of the identified clusters were Nrps or PKS, including a tridecaptin cluster that was shared by all strains regardless of the phylogenetic clades. The fusaricidin B cluster was identified in all strains except *P. polymyxa* CR1, and the polymyxin cluster was identified in all strains except *P. polymyxa* strains YC0573, ATCC 15970 and CR1. Nrps/PKS clusters producing novel products were also identified in all strains. Lanthipeptide clusters producing both novel and known products were also widely distributed in all strains. Specifically, the paenilan cluster was identified in all strains except *P. polymyxa* strains J and ATCC 15970 (Clade 1), however the paenibacillin cluster and the paenicidin A/B cluster were less common. The paenibacillin cluster was only identified in *P. polymyxa* ZF129 (Clade 2), and the paenicidin A/B cluster was only identified in all Clade 2 strains and one Clade 1 strain (*P. polymyxa* J). Other widely distributed secondary metabolite gene clusters included one novel lassopeptide cluster and one novel bacteriocin cluster.

Table 1. Secondary metabolite gene clusters identified in *Paenibacillus* sp. strains S02 and S25 and 13 *P. polymyxa* strains with complete circular genome sequences

	Nrps - fusaricindin B	Nrps - tridecaptin	Nrps - polymyxin	Nrps/PKS - paenilipoheptin	lanthipeptide - paenilan	lanthipeptide - paenibacillin	lanthipeptide - paenicidin A/B	lassopeptide - novel	siderophore - novel	bacteriocin - novel	phosphonate - novel	betalactone - nostamide A	Total - known products	Total - novel products	Total	
***P. polymyxa* J**	1	1	1	0	0	0	1	1	1	1	1	0	0	8	5	13	Clade 1
***P. polymyxa* E681**	1	1	1	1	1	0	0	0	1	0	1	2	0	9	2	11	
***Paenibacillus* sp. S02**	1	1	1	1	1	0	0	0	1	1	1	1	0	9	4	13	
***Paenibacillus* sp. S25**	1	1	1	1	1	0	0	1	1	1	1	1	0	10	6	16	
***P. polymyxa* YC0136**	1	1	1	0	1	0	0	0	1	1	1	1	0	8	6	14	
***P. polymyxa* YC0573**	1	1	0	1	1	0	0	0	1	1	1	1	0	8	5	13	
***P. polymyxa* ATCC 15970**	1	1	0	0	0	0	0	1	1	1	1	1	0	7	5	12	
***P. polymyxa* CR1**	0	1	0	0	1	0	0	0	1	1	1	1	0	6	6	12	
***P. polymyxa* Sb3-1**	1	1	1	0	1	0	0	2	1	0	1	0	0	8	8	16	Clade 3
***P. polymyxa* M1**	1	1	1	1	1	0	0	1	1	0	1	0	0	8	4	12	
***P. polymyxa* SC2**	1	1	1	1	1	0	0	1	1	0	1	0	0	8	4	12	
***P. polymyxa* ZF129**	1	1	1	0	1	1	1	0	1	0	1	0	1	9	5	14	Clade 2
***P. polymyxa* HY96-2**	1	1	1	0	1	0	1	1	1	0	1	0	1	9	7	16	
***P. polymyxa* SQR21**	1	1	1	0	1	0	1	1	1	0	1	0	1	9	7	16	

The type and product of each secondary metabolite gene cluster were shown in the first row. Numbers are the total count of each secondary metabolite gene cluster. The length of the orange bar represents the total count of each secondary metabolite gene cluster.
Novel: Similarity ≤ 70% when compared to the most similar known cluster in the antiSMASH database
Nrps: Nonribosomal peptide synthetase
PKS: Polyketide synthase
Clade 1/2/3: Clades identified in phylogeny and comparative genomics study (section 2.3)

2.6. Bioprotection assay (*in vitro*)

A dual culture *in vitro* assay was established to compare the biocidal activity of *Paenibacillus* sp. strains S02 and S25 against three fungal pathogens, *Colletotrichum graminicola*, *Fusarium verticillioides* and *Microdochium nivale*. *Paenibacillus* sp. S02 significantly ($P < 0.05$) reduced the average colony diameter of the fungal pathogens *C. graminicola* and *F. verticillioides* compared to the blank control and *Paenibacillus* sp. S25 (Supplementary Table S7, Supplementary Figure S3). It reduced the growth of *C. graminicola* and *F. verticillioides* by up to 74.9% and 56.9%, respectively. *Paenibacillus* sp. S25 significantly ($P < 0.05$) reduced the growth of *F. verticillioides* by 9.6% compared to the blank control, however no similar activity was observed for *C. graminicola*. Neither of the two strains could significantly reduce the average colony diameter of *M. nivale*.

2.7. Transcriptome sequencing and analysis – N-fixation activity assay

A transcriptome sequencing experiment was designed to confirm the expression of the *nif* operon of *Paenibacillus* sp. strains S02 and S25 when the nitrogen source (NH_4Cl) was removed from the media. A 150 bp PE library prepared from cDNA from strains used in the N-fixation activity assay generated an average of 13.8 million clean reads per sample. Differential gene expression (DGE) analysis successfully identified genes that were differentially expressed under different conditions in the assay. A total of 5,059 and 4,745 genes passed the abundance filter for strain S02 and S25, respectively, and were used in the subsequent DGE analysis. Among those genes, 2,467 and 2,479 genes were differentially expressed when nitrogen was removed from the media for strain S02 and S25, respectively. Biological replicates of the nitrogen treatment (+/−) formed distinctive clusters along the PC1 axis for both strains, suggesting the presence/absence of nitrogen impacted the transcriptome profiles of both strains (Figure 3). Specifically, when nitrogen was present in the media, the *nif* operon comprising nine genes was expressed by both strains, and there was no significant difference in expression levels of any *nif* gene between the two strains. However, the expression levels of the *nif* operon varied when nitrogen was removed from the media (Table 2). Gene expression levels when nitrogen was removed were represented as fold-changes in relation to the expression levels when nitrogen was present. The expressions of all nine genes of the *nif* operon of *Paenibacillus* sp. S02 were upregulated by 8.62–22.50 folds. For *Paenibacillus* sp. S25, the *nifB/H/D/K/E* genes were differentially expressed with 1.76- to 3.90-fold increase. The remaining four genes of the *nif* operon also had fold-changes in expression level, however they failed to be considered as differentially expressed (*q-value* < 0.05 and absolute fold-change ≥ 1.5). Such results confirmed that both strains likely carry a biologically functional *nif* operon that enables biological nitrogen fixation.

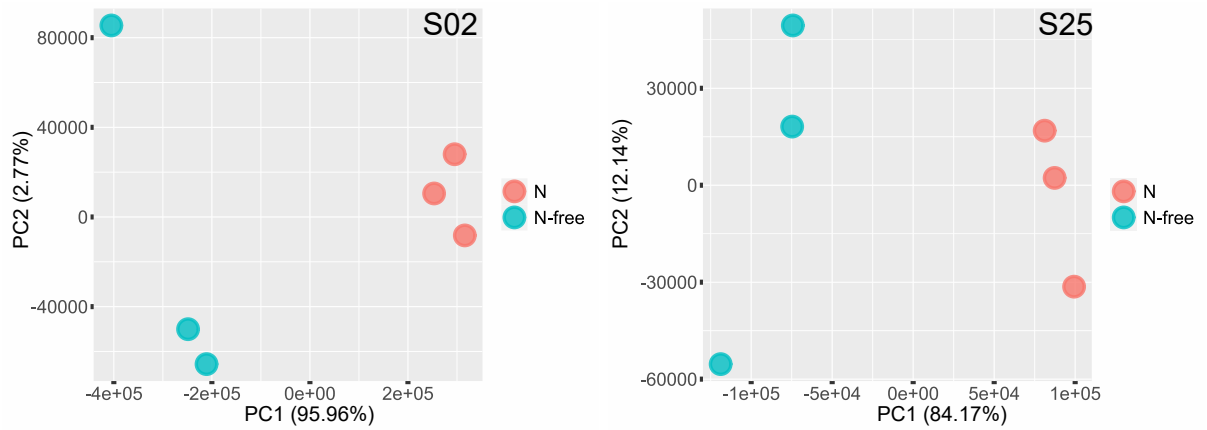


Figure 3. PCA plots of transcriptome profiles of *Paenibacillus* sp. strains S02 (left) and S25 (right) when grown in media with nitrogen (N) and without nitrogen (N-free) in the N-fixation activity assay. Percentage variance explained by each axis are given in brackets. Distinctive clusters indicate that the presence/absence of a nitrogen source (NH_4Cl) in the media changed the transcriptome profiles of both strains.

Table 2. Changes in expression levels of the *nif* operon of *Paenibacillus* sp. strains S02 and S25 when NH_4Cl was removed from the media.

***nif* genes**	**S02** fold-change	**S25** fold-change
nifB	22.50*	2.46*
nifH	20.21*	3.90*
nifD	15.80*	2.06*
nifK	17.51*	2.01*
nifE	15.86*	1.76*
nifN	18.16*	1.59
nifX	8.62*	1.19
hesA/moeB	15.13*	1.56
nifV	11.01*	-1.46

*: Genes that were differentially expressed (*q-value* < 0.05 and absolute fold-change ≥ 1.5) when nitrogen was removed from the media.

2.8. Transcriptome sequencing and analysis – Bacteria-pathogen interactions assay

A transcriptome sequencing experiment was designed to explore the early stage interactions between *Paenibacillus* sp. strains S02 and S25 and the fungal pathogen *F. verticillioides*. A 150 bp PE library prepared from cDNA from strains used in the bacteria-pathogen interactions assay generated an average of 31.1 million clean reads per sample. DGE analysis successfully identified genes that were differentially expressed under different conditions in the assay. A total of 5,201 and 4,817 genes passed the abundance filter for strain S02 and S25, respectively, and were used in the subsequent DGE analysis. Among those genes, only 61 genes were differentially expressed by strain S02 when *F. verticillioides* was present. In contrast, 2,706 genes were differentially expressed by strain S25 when *F. verticillioides* was present. Moreover, distinctive clustering of the three biological replicates based on

transcriptome profiles was identified for strain S25 along the PC1 axis, but the clustering was not as evident for strain S02, particularly when the pathogen was present (Figure 4). The Clusters of Orthologous Groups (COGs) of proteins[37] encoded by the genes mentioned above rendered an overview of functions of those genes. For *Paenibacillus* sp. S02, 29 genes were associated with cellular processes and signalling, and 14 genes were associated with metabolism. There were also 18 genes with unknown functions. For *Paenibacillus* sp. S25, 368 genes were associated with cellular processes and signalling, and 889 genes were associated with metabolism. In addition, there were 416 genes associated with information storage and processing, 43 genes associated with multiple function groups and 990 genes with unknown functions. Given the complexity of transcriptome profiles, this study focused on the expression levels of the core biosynthetic genes of secondary metabolite gene clusters identified in section 2.5 to demonstrate the early stage bacteria-pathogen interactions.

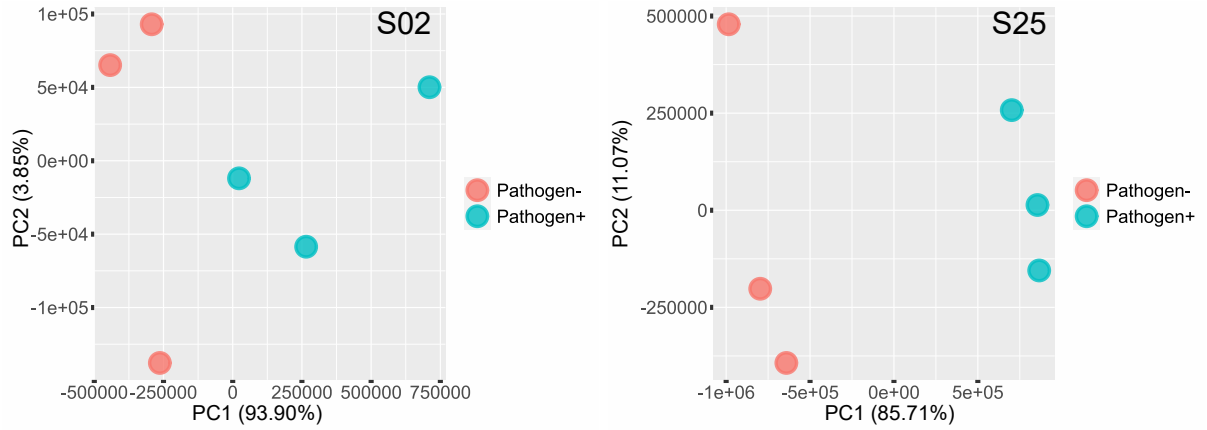


Figure 4. PCA plots of transcriptome profiles of *Paenibacillus* sp. strains S02 (left) and S25 (right) when grown in media with *F. verticillioides* (Pathogen+) and without *F. verticillioides* (Pathogen−) in the bacteria-pathogen interactions assay. Percentage variance explained by each axis are given in brackets. Distinctive clusters indicate that the presence/absence of *F. verticillioides* in the media changed the transcriptome profiles of strain S25 (along the PC1 axis).

When *F. verticillioides* was absent, *Paenibacillus* sp. S02 showed increased expressions of the core biosynthetic genes of secondary metabolite gene clusters compared to *Paenibacillus* sp. S25. Gene expression levels of those genes of strain S02 were represented as fold-changes in relation to the expression levels of the corresponding homolog of each gene of strain S25 (Table 3). Among the 44 core biosynthetic genes of secondary metabolite gene clusters shared by the two strains, the majority (41) were expressed in increased levels ranging from 1.92- to 486.58-fold increase, including the four clusters that produce known antimicrobial compounds (C1, C7, C10 and C15). Only one cluster had a decreased expression, with C9

exhibiting a 7.42-fold decrease in the expression level. Two core biosynthetic genes of cluster C4 were not differentially expressed when comparing the two strains.

Table 3. Fold-changes in expression levels of the core biosynthetic genes of secondary metabolite gene clusters of strain S02 compared to strain S25 when *F. verticillioides* was absent

ID	Type	Most similar known cluster (similarity)	*Paenibacillus* sp. S02	
			Gene ID	Fold-change
C1	Nrps	**fusaricidin B (100%)**	KAI36_00078	486.58*
			KAI36_00083	20.36*
C2	siderophore	–	KAI36_00955	11.71*
			KAI36_00956	13.93*
			KAI36_00959	4.64*
C3	bacteriocin	–	KAI36_01103	46.95*
C4	Nrps transAT-PKS	–	KAI36_01166	18.88*
			KAI36_01170	2.43*
			KAI36_01172	1.93*
			KAI36_01173	3.31*
			KAI36_01175	2.35*
			KAI36_01176	2.30*
			KAI36_01178	2.27*
			KAI36_01179	1.92*
			KAI36_01180	-1.25
			KAI36_01181	1.27
C5	lassopeptide	paeninodin (40%)	KAI36_01236	134.38*
			KAI36_01240	37.82*
C6	Nrps	marthiapeptide A (33%)	KAI36_01339	239.45*
			KAI36_01340	159.93*
			KAI36_01341	119.29*
C7	lanthipeptide	**paenilan (100%)**	KAI36_01558	60.94*
			KAI36_01560	271.12*
			KAI36_01562	69.08*
C9	Nrps-like	–	KAI36_01944	-7.42*
C10	Nrps	**tridecaptin (100%)**	KAI36_02333	7.74*
			KAI36_02334	32.36*
C11	Nrps transAT-PKS	paenilipoheptin (S02, 73%; S25, 76%)	KAI36_02506	17.20*
			KAI36_02507	29.10*
			KAI36_02508	23.54*
			KAI36_02509	22.65*
			KAI36_02510	10.63*
C14	Nrps T3PKS transAT-PKS	aurantinin B/C/D (35%)	KAI36_03362	118.38*
			KAI36_03363	46.83*
			KAI36_03365	125.65*
			KAI36_03366	98.69*
			KAI36_03367	31.92*
			KAI36_03368	77.19*
			KAI36_03371	134.46*
			KAI36_03372	113.90*
C15	Nrps	**polymyxin (100%)**	KAI36_04684	9.61*
			KAI36_04687	8.61*
			KAI36_04688	9.86*
C16	phosphonate	–	KAI36_05277	15.10*

Clusters in bold: Known antimicrobial compounds

*: Genes that were differentially expressed (*q-value* < 0.05 and absolute fold-change ≥ 1.5) when comparing the two strains.

The presence of *F. verticillioides* had different effects in expressions of the core biosynthetic genes of secondary metabolite gene clusters on the two strains. Gene expression levels when *F. verticillioides* was present were represented as fold-changes in relation to the expression levels when *F. verticillioides* was absent (Table 4). For *Paenibacillus* sp. S02, only three of 44 core biosynthetic genes of secondary metabolites were differentially expressed, while for *Paenibacillus* sp. S25, 32 of 51 genes were differentially expressed. Despite clusters C1, C7, C10 and C15, which produce known antimicrobial compounds, being shared by both strains, the expression levels of core biosynthetic genes of secondary metabolite varied. For instance, the four genes of the polymyxin cluster (C15) were not differentially expressed by strain S02 but were differently expressed with 1.45- to 2.61-fold decrease by strain S25. Strain S25 had a gene of the fusaricidin B cluster (C1) that was differentially expressed with a 5.21-fold decrease, and two genes of the tridecaptin cluster (C10) that were differentially expressed (one with a 1.92-fold decrease and the other with a 1.79-fold increase), whereas these gene clusters were not affected in strain S02. The three core biosynthetic genes of the paenilan cluster (C7) were not differentially expressed in either strain. As for the four clusters that had the best matches in the antiSMASH gene cluster database (C5, C6, C11 and C14), although the core genes had differing levels of fold-change, the trend of changes (i.e. upregulated or downregulated) were consistent between strains. Similar consistencies were also observed from the core biosynthetic genes of three shared novel clusters (C4, C9 and C16). Furthermore, the core gene of the bacteriocin cluster (C3) was not differentially expressed in either strain. However, two genes of the siderophore cluster (C2) of strain S25 were differentially expressed with a 2.06- and 2.60-fold increase, respectively, unlike strain S02. Moreover, amongst the three novel clusters (C8, C12 and C13) that were unique to strain S25, two (C12 and C13) had differentially expressed core genes.

Table 4. Fold-changes in expression levels of the core biosynthetic genes of secondary metabolite gene clusters identified in *Paenibacillus* sp. strains S02 and S25 when *F. verticillioides* was present

ID	Type	Most similar known cluster (similarity)	***Paenibacillus* sp. S02**		***Paenibacillus* sp. S25**	
			Gene ID	Fold-change	Gene ID	Fold-change
C1	Nrps	**fusaricidin B (100%)**	KAI36_00078	-1.28	KAI37_00078	-1.50
			KAI36_00083	-1.36	KAI37_00083	-5.21*
C2	siderophore	–	KAI36_00955	-1.23	KAI37_00927	2.06*
			KAI36_00956	-1.15	KAI37_00928	2.60*
			KAI36_00959	-1.12	KAI37_00931	1.04
C3	bacteriocin	–	KAI36_01103	1.10	KAI37_01049	-1.19
C4	Nrps transAT-PKS	–	KAI36_01166	-1.25	KAI37_01130	-1.17
			KAI36_01170	-1.89*	KAI37_01134	-1.08
			KAI36_01172	-1.54	KAI37_01136	-1.43
			KAI36_01173	-1.65*	KAI37_01137	-1.49
			KAI36_01175	-1.64	KAI37_01139	-1.52
			KAI36_01176	-1.48	KAI37_01140	-1.56
			KAI36_01178	-1.41	KAI37_01142	-1.51*
			KAI36_01179	-1.40	KAI37_01143	-1.55
			KAI36_01180	-1.43	KAI37_01144	-2.34*
			KAI36_01181	-1.33	KAI37_01145	-1.90*
C5	lassopeptide	paeninodin (40%)	KAI36_01236	-1.20	KAI37_01200	-3.63*
			KAI36_01240	-1.30	KAI37_01204	-2.07
C6	Nrps	marthiapeptide A (33%)	KAI36_01339	1.19	KAI37_01293	4.34*
			KAI36_01340	1.15	KAI37_01294	2.43*
			KAI36_01341	1.12	KAI37_01295	2.02*
C7	lanthipeptide	**paenilan (100%)**	KAI36_01558	-1.04	KAI37_01518	1.32
			KAI36_01560	-1.00	KAI37_01520	1.10
			KAI36_01562	1.02	KAI37_01522	1.18
C8	lanthipeptide	**paenicidin B (71%)**			KAI37_01661	-1.73
					KAI37_01663	-1.19
C9	Nrps-like	–	KAI36_01944	1.81*	KAI37_01854	2.07*
C10	Nrps	**tridecaptin (100%)**	KAI36_02333	1.02	KAI37_02322	-1.92*
			KAI36_02334	1.00	KAI37_02323	1.79*
C11	Nrps transAT-PKS	paenilipoheptin (S02, 73%; S25, 76%)	KAI36_02506	1.14	KAI37_02476	15.43*
			KAI36_02507	1.16	KAI37_02477	23.42*
			KAI36_02508	1.16	KAI37_02478	11.77*
			KAI36_02509	1.21	KAI37_02479	7.92*
			KAI36_02510	1.11	KAI37_02480	3.23*
C12	Nrps	–			KAI37_02516	-1.91*
C13	Nrps betalactone	–			KAI37_02623	1.50*
					KAI37_02624	2.02*
					KAI37_02633	2.10*
C14	Nrps T3PKS transAT-PKS	aurantinin B/C/D (35%)	KAI36_03362	-1.29	KAI37_03372	1.71
			KAI36_03363	-1.35	KAI37_03373	-1.78
			KAI36_03365	-1.32	KAI37_03375	-1.66*
			KAI36_03366	-1.34	KAI37_03376	-1.82*
			KAI36_03367	-1.26	KAI37_03377	-2.00*
			KAI36_03368	-1.05	KAI37_03378	-1.78*
			KAI36_03371	-1.05	KAI37_03381	N/A
			KAI36_03372	-1.03	KAI37_03382	-4.68*
C15	Nrps	**polymyxin (100%)**	KAI36_04684	-1.02	KAI37_04566	-2.61*
					KAI37_04567	-2.33*
			KAI36_04687	1.10	KAI37_04570	-1.45*
			KAI36_04688	1.12	KAI37_04571	-1.92*
C16	phosphonate	–	KAI36_05277	-1.38	KAI37_05154	-1.77*

Clusters in bold: Known antimicrobial compounds

N/A: Genes that didn't pass the abundance filter described in section 4.8

*: Genes that were differentially expressed (*q-value* < 0.05 and absolute fold-change ≥ 1.5) when *F. verticillioides* was present.

Fungal transcripts of *F. verticillioides* were also assessed as a part of the bacteria-pathogen interactions assay. Transcript quantification using the transcriptome sequences of *F. verticillioides* 7600 as the reference reflected the differences in bioprotection activities between the two strains (Supplementary Table S8). The percentage of mapped reads in the treated samples for *Paenibacillus* sp. S02, which showed stronger bioprotection activities in the *in vitro* assay (section 2.6), was much lower than that for *Paenibacillus* sp. S25. Furthermore, amongst the treated samples for *Paenibacillus* sp. S02, the percentage of mapped reads of the replicate 1 (S02_treated_1) was even comparable to the untreated samples.

3. Discussion

3.1. Isolation and identification of novel *Paenibacillus* sp. strains

Two N-fixing bacterial strains were isolated in this study using a combined approach that took advantage of both microbiological techniques (N-free medium) and genomic resources (sequencing). Various N-free media have been widely used to isolate N-fixing microorganisms from natural environments on the basis that nitrogen is required by all organisms to survive[28,38]. However, being able to grow in N-free media does not necessarily guarantee the ability to fix atmospheric nitrogen by a microorganism. For instance, *Streptomyces thermoautotrophicus* UBT1 was initially reported as a N-fixing bacterium[39], however further analysis revealed that the solidifying agent used to prepare the N-free medium contained 0.095% nitrogen content[40]. Hence, instead of being a N-fixing bacterium, this strain was a N-scavenger that could utilise nitrogen present in ultra-low concentrations. Furthermore, even if the selective medium is N-free, those scavengers could still grow in combination with other N-fixing microorganisms present, hence hindering the isolation process, which was observed in this study. The N-free medium and the *nifH* gene PCR used in this study confirmed the presence of N-fixing bacteria in the culture. Moreover, the sequencing results provided possible identities of the N-fixing bacteria, which greatly assisted the isolations of the two strains (S02 and S25). The long-read sequencing data of dilutions confirmed the presence of *Paenibacillus* spp. and also revealed the possible identity of other bacterial species in dilutions, leading to the addition of polymyxin B, which removed all N-scavengers in the culture.

Taxonomic identification of bacterial species based on the 16S ribosomal RNA and whole genome sequence homology suggested that the two strains used in this study (S02 and S25) were closely related to *P. polymyxa*. Furthermore, the ANI analysis showed that the two strains were not genetically identical to any of the known *P. polymyxa* strains, and hence represent two novel strains of *Paenibacillus* spp. There are currently (27/07/2020) genome sequences of 56 *P. polymyxa* strains publicly available on NCBI, none of which was originally isolated from Australia. Therefore, this is the first study that reported Australian strains of this species. *P. polymyxa* has been extensively studied, and some strains have been developed as commercially available biopesticides or biofertilisers[5]. The two strains (S20 and S05) reported by this study will be of great benefit to the local application of this bacterial species as they represent indigenous *Paenibacillus* sp. strains.

Interestingly, the phylogeny and comparative genomics analyses of this study suggested possible future taxonomic subdivision of the species *P. polymyxa*. The dendrograms based on ANI of *P. polymyxa* genomes and the ML tree based on the sequence homology of conserved genes of *P. polymyxa* genomes shared the same topology consisting of three major clades. Moreover, the comparative genomics analysis showed that the ANI values between strains from different clades were lower than 95%, which is the proposed prokaryotic species boundary[41,42]. While both Clade 1 and Clade 2 contained 18 *P. polymyxa* strains, the type strain of *P. polymyxa*, which is ATCC 842, was in Clade 2. There was no apparent patterns of geographic locations or environment origins of strains associated with each clade (Supplementary Table S3). Such results demonstrated that current *P. polymyxa* strains might need to be reclassified into three species based on ANI, including the "original" *P. polymyxa* species (Clade 2). The two novel *Paenibacillus* sp. strains (S02 and S25) are representatives of Clade 1 and represent a new *Paenibacillus* species. Similarly, the nine strains identified in Clade 3 also represent a new *Paenibacillus* species. Strains from the three clades were genetically closely related, and shared some PGP genes (e.g. IAA production)[16] and secondary metabolite gene clusters (e.g. fusaricidin and tridecaptin, Table 1). However, the differences between strains from the three clades were demonstrated by the absence of some PGP genes from strains of a clade (e.g. the *nif* operon, Clade 3)[16] and the presence of unique secondary metabolite gene clusters in strains of a clade (e.g. betalactone, Clade 2). Such phylogenomic reclassifications of *P. polymyxa* have been proposed in a previous study[43]. One of the reasons that caused this taxonomic ambiguity was the molecular markers used for taxonomic assignment. Prokaryotic taxonomy identification has been relying on the 16S

ribosomal RNA over the last four decades, however the current standard is now shifting to genome-scale data[44]. For example, *P. polymyxa* E681 (Clade 1), which was isolated in 1990s and was the most studied *P. polymyxa* strain, was identified as *P. polymyxa* solely based on the 16S ribosomal RNA gene sequences[43]. However, the ANI analysis using its genome published in Kim, et al.[8] only showed a similarity of 89.98% compared to the type strain *P. polymyxa* ATCC 842, whose genome was published in 2011[45]. Molecular phylogeny based on the 16S ribosomal RNA gene sequences led to the discovery of the novel genus *Paenibacillus*[46], which now comprised more than 200 species and this number is still growing[4]. With more and more *Paenibacillus* spp. genomes becoming available, molecular taxonomy based on genome-scale data including ANI will clarify the complexity of *Paenibacillus* species.

3.2. PGP genes and secondary metabolite gene clusters in the two *Paenibacillus* sp. strains

P. polymyxa has long been described as a PGP bacterium. The mode of action of plant growth promotion utilised by *P. polymyxa* has been proposed by Jeong, et al.[43], including (1) direct promotion by producing phytohormones and enhancing nutrient uptake by plants, and (2) indirect promotion by providing bioprotection against phytopathogens. Method 1 is implemented via the expression of PGP genes. In this study, *Paenibacillus* sp. strains S02 and S25 were found to possess multiple PGP genes associated with biological nitrogen fixation, inorganic and organic phosphate solubilisation, phosphate assimilation and IAA production. Comparative genomics studies conducted by Eastman, et al.[16] and Xie, et al.[5] showed that these genes are highly conserved in *P. polymyxa* strains. For example, the *nif* operon carried by *P. polymyxa* strains contained nine genes and had a high sequence homology (> 80%)[4]. Interestingly, it has been reported that the ancestral *Paenibacillus* species could not fix atmospheric nitrogen, and the *nif* operon was acquired by N-fixing strains from possibly *Frankia* spp. via horizontal gene transfer[26]. Hence. these PGP genes were highly likely acquired by *Paenibacillus* spp. during the evolution to adapt to their plant-associated lifestyle[17].

In this study, the two *Paenibacillus* sp. strains (S02 and S25) exhibited strong inhibitory activities against several fungal phytopathogens in *in vitro* assays. Such bioprotection (method 2) activities of *P. polymyxa* have been extensively studied. It has been discovered that *P. polymyxa* strains colonise plant roots and form biofilms, hence preventing the

colonisation of phytopathogens[22]. Moreover, *P. polymyxa* strains also produce a wide variety of secondary metabolites associated with bioprotection. The two strains used in this study were found to carry 13 and 16 secondary metabolite gene clusters synthesising both antimicrobial ribosomal peptides, e.g. paenilan and paenicidin (lanthipeptide), and nonribosomal peptides including fusaricidin B, polymyxin and tridecaptin. Comparative analyses conducted in this study revealed that these clusters are conserved in *P. polymyxa* strains regardless of the phylogenetic clades. Furthermore, the compounds synthesised by more than half of the secondary metabolite gene clusters were novel, and they were commonly detected in many *P. polymyxa* strains[5,16,17]. These novel clusters represented a repository where more potential bioactive antimicrobial compounds could be discovered. Future research is required to identify and characterise these novel compounds.

Furthermore, some *P. polymyxa* strains have been reported to produce volatile compounds that promote plant growth by utilising both methods described above. For example, the volatile compounds emitted by *P. polymyxa* E681 increased the total leaf surface area of *Arabidopsis* seedlings and induced plant resistance to *Pseudomonas syringae*[47]. Thirteen volatile compounds produced by *P. polymyxa* WR-2 not only inhibited the growth of *F. oxysporum*, but also prevented the germination of *F. oxysporum* spores[48]. Hence, further studies are needed to identify and characterise bacterial volatile compounds produced by the novel *Paenibacillus* sp. strains S02 and S25.

3.3. Transcriptome sequencing confirmed the activities of the *nif* operon and provided insights into the early stage bacteria-pathogen interactions

Transcriptome sequencing revealed that the presence/absence of nitrogen greatly affected the transcriptome profiles of the two *Paenibacillus* sp. strains (S02 and S25). The expression levels of all nine genes of the *nif* operon were regulated, and mostly increased, for both strains when nitrogen was removed from the medium. However, the level of changes of those genes caused by removing nitrogen varied between the two strains. There were much higher increases in expression levels of the *nif* genes in strain S02 when compared with strain S25. Given that the two strains shared similar expression levels of the *nif* operon before the removal of nitrogen, it could be concluded that strain S02 is more active in biological nitrogen fixation. Such increased bioactivity could be explained by the difference in growth kinetics. It has been found that strain S02 grows faster than strain S25 in both Burk’s medium

and Nutrient Broth (based on OD_{600} readings after 24 h). It could be postulated that *Paenibacillus* sp. S02 requires more nitrogen to meet the demand of cell multiplication, which leads to increased bioactivities of the *nif* operon when nitrogen is removed from the growth medium. To validate this increased activity, further experiments using the acetylene reduction assay or similar methods[49] are required to quantify the N-fixation rate of both strains.

The two *Paenibacillus* sp. strains (S02 and S25) were bioactive against *F. verticillioides* (growth reduction) in the *in vitro* assays, with strain S02 being significantly more bioactive than S25. Genomic analyses demonstrated that the two strains were capable of producing at least two antifungal secondary metabolite compounds, i.e. fusaricidin[50] and bacterial siderophore[51], as well as a wide range of novel secondary metabolite compounds. We proposed a hypothesis that these compounds were associated with the bioprotection activities against *F. verticillioides* exhibited by both strains. To support results from the bioassay and genomic analysis, a transcriptome sequencing experiment was designed to explore the early stage interactions between the two strains and the pathogen. The stronger bioprotection activities of strain S02 were reflected by the percentages of mapped *F. verticillioides* transcripts in the treated samples. One of the biological replicates of strain S02 even produced a mapping rate that was comparable to the untreated samples. Moreover, the treated samples of strain S02 were plated on Potato Dextrose Agar plates, and no visual evidence of fungal growth was observed for that biological replicate (Supplementary Figure S4). Such results suggested that, for this specific biological replicate, the growth of *F. verticillioides* was highly likely completely inhibited. Furthermore, the results of DGE analysis showed that the core biosynthetic genes of secondary metabolite gene clusters were actively expressed by strain S02 comparing to strain S25 before the introduction of *F. verticillioides*, including clusters producing two known antifungal compounds (C1: fusaricidin B; C2: bacterial siderophore) as well as clusters producing novel compounds (e.g. C6 and C14), which may have also contributed to the bioactivity. Hence, we proposed a hypothesis that the stronger bioprotection activities provided by strain S02 was related to the higher concentrations of antifungal bioactive compounds that were produced even before the introduction of *F. verticillioides.* In addition, changes in transcriptome profiles suggested that strain S02 was more resilient to the introduction of *F. verticillioides* when compared with strain S25. Such highly active and stable transcriptome profiles would make strain S02 a promising candidate to be developed as a bioprotection agent. Future experiments, including *in vitro* and *in planta*

assays and field assays, are needed to further validate the potential of this strain. The bioactive compounds produced by the two strains should also be identified, purified and characterised.

It is notable that 32 of 51 core biosynthetic genes of secondary metabolite clusters of strain S25 were differentially expressed when *F. verticillioides* was present, with 17 being downregulated including those from clusters producing known antifungal compounds. Given this strain was bioactive against *F. verticillioides* in the *in vitro* assays, a possible explanation was the incubation time. Whilst the two strains and *F. verticillioides* were co-incubated for five days in the *in vitro* bioassays, they were only co-incubated for six hours before RNA was extracted in the transcriptome sequencing experiment. The short incubation time may not be enough for strain S25 to produce significant amounts of antifungal compounds. It has been reported that the expressions of secondary metabolites of *Bacillus* spp. were enhanced when fungal pathogens (including some *Fusarium* spp.) were present[52], however such information is still missing for *P. polymyxa* and *F. verticillioides*. It is possible that the decreased expressions of those genes observed in this study was related to the bacteria-pathogen interactions. Future studies should incorporate a longer incubation time or a series of time points, to further elucidate the changes in transcriptome profiles of the two strains after the introduction of *F. verticillioides.*

The two *Paenibacillus* sp. strains reported by this study were genetically highly similar (ANI = 97.78%) despite the apparent differences in bioactivities and transcriptome profiles. Interestingly, our laboratory has previously isolated and characterised three novel *Xanthomonas* sp. strains from the perennial ryegrass microbiome which were also genetically similar but phenotypically different[53]. Hence, we proposed a hypothesis that plant hosts recruit and take advantages of multiple genetically similar strains of the same species with a diverse range of bioactivities. Future studies, especially *in planta* assays, are required to further characterise those strains to gain a deeper understanding of the perennial ryegrass microbiome.

4. Materials and Methods

4.1. Seed-associated N-fixing bacterial strain detection and isolation

A PCR assay was designed to detect the presence of seed-associated N-fixing bacteria by amplifying the *nifH* gene of nitrogenase. Approximately 1,000 perennial ryegrass seeds (*L. perenne*, cv. Alto, with standard endophytes, Barenbrug Agriseeds NZ) were washed using sterile water and then ground and soaked in 30 mL of Burk's N-free medium ($MgSO_4$, 0.2 g/L; K_2HPO_4, 0.8 g/L; KH_2PO_4, 0.2 g/L; $CaSO_4$, 0.13 g/L; $FeCl_3$, 0.00145 g/L; Na_2MoO_4, 0.000253 g/L; sucrose, 20 g/L). The suspension was incubated for two days at 26 °C and 200 rpm, and then serial diluted using sterile Burk's N-free medium (1:10, 100 µL in 900 µL, eight replicates per dilution). Genomic DNA was extracted from the 10^{-2} and 10^{-3} dilutions using a Wizard® Genomic DNA Purification Kit (A1120, Promega, Madison, WI, USA), and assessed for quality on a NanoDrop 2000 (Thermo Scientific, Waltham, MA, USA). PCR conditions were as per Gaby and Buckley [54]. In brief, OneTaq@ Hot Start 2× Master Mix (M0484, Promega, Madison, WI, USA) was used with a universal *nifH* gene PCR primer pair (IGK3: 5'- GCIWTHTAYGGIAARGGIGGIATHGGIAA-3'; DVV: 5'-ATIGCRAAICCICCRCAIACIACRTC-3'; final concentration = 0.4 µM)[54] and 50 ng of extracted DNA. For the no template control, nuclease-free water was used. For the positive control, the genomic DNA of *Rhizobium leguminosarum* bv. *trifolii* WSM1325[55] was used. PCR products (~ 400 bp) were visualised on an Agilent 2200 TapeStation (Agilent Technologies, Santa Clara, CA, USA), sequenced by Macrogen and then analysed using BLAST[56].

Genomic DNA of dilutions that produced PCR amplicons were sequenced using long read sequencing technology. A library was prepared using the Oxford Nanopore Technologies (ONT) ligase-based library preparation kit (SQK-LSK109, ONT, Oxford, UK) and sequenced on a MinION Mk1B platform (MIN-101B) with R10 flowcells (FLO-MIN110). Genomic sequence data (raw read signals) were basecalled using ONT's Guppy software (Version 3.4.3, HAC basecalling model), and assessed for quality using NanoPlot[57]. Basecalled data was filtered to remove adapter sequences using Porechop (Version 0.2.3, https://github.com/rrwick/Porechop), while reads shorter than 300 bp and the worst 5% of reads (based on quality) were discarded using Filtlong (Version 0.2.0, https://github.com/rrwick/Filtlong). Sequencing reads were taxonomically classified by

Kraken2[24] using a custom database containing all completed bacterial reference genomes in NCBI (20/03/2020) and were analysed using BLAST[56]. In addition, 50 µL of those dilutions were inoculated into vials containing 5 mL of Burk's N-free medium supplemented with 1.6 g/L agar and incubated for up to five days at 26 ℃. Cultures were checked daily for a band of microbial growth below the surface of medium, which indicated the presence of N-fixing bacteria[38]. Microbes were streaked onto Burk's N-free medium supplemented with 15 g/L agar and Burk's N-free medium supplemented with 15 g/L agar and 100 IU/mL polymyxin B (P4932-1MU, Sigma-Aldrich, St. Louis, MO, USA) and incubated for up to five days at 26 ℃ to isolate pure colonies.

4.2. Genome sequencing

DNA was extracted from bacterial pellets (overnight cultures) using a Wizard® Genomic DNA Purification Kit (A1120, Promega, Madison, WI, USA), and assessed for quality (average molecular weight ≥ 30 Kb) on an Agilent 2200 TapeStation (Agilent Technologies, Santa Clara, CA, USA). Genomic sequencing libraries (short reads) were prepared from the DNA using the PerkinElmer NEXTFLEX® Rapid XP DNA-Seq Kit (Cat# NOVA-5149-03) and sequenced on an Illumina NovaSeq 6000 platform. Genomic sequence data (raw reads) were assessed for quality and filtered to remove any adapter and index sequence, and low-quality bases using fastp[58] with the following parameters: *-w 8 -3 -5 --detect_adapter_for_pe*. In addition, genomic sequencing libraries (long reads) were prepared from the DNA as per section 4.1.

4.3. Genome assembly and classification

The whole genomes of bacterial strains were assembled with filtered long and short reads using Unicycler[59]. Long reads were used for primary assembly and to resolve repeat regions in the genome, whereas short reads were used to correct small base-level errors. Assembly graphs were visualised using Bandage[60]. Assembled genomes were taxonomically classified by Kraken2[24] as per section 4.1.

4.4. Genome annotation and characterisation

The assembled genomes of bacterial strains were annotated using Prokka[61] with a custom *Paenibacillus* protein database (based on Kraken2 classification) to predict genes and corresponding functions. Identification of secondary metabolite gene clusters from annotated genomes was conducted using antiSMASH[32] with the following options: *--clusterblast --asf --knownclusterblast --subclusterblast --smcogs --full-hmmer*. The presence of PGP genes in the annotated genomes was conducted using BLAST[56] (blastn and tblastn, e value > $1e^{-10}$). PGP genes previously reported in *P. polymyxa* strains[5,16] were targeted (30 genes), including biological nitrogen fixation (nine genes), phosphate solubilisation and assimilation (17 genes) and indole-3-acetic acid production and auxin transportation (four genes). The PGP gene identification compared the sequence homology of genes from strains S02 and S25 with a closely related *P. polymyxa* strain CR1.

4.5. Phylogeny and comparative genomics

A comparative genomic analysis was performed by calculating the average nucleotide identity (ANI) of the genomes of isolated strains to 44 *P. polymyxa* genomes that were publicly available on NCBI (Supplementary Table S3) using a python package pyani (Version 0.2.8, https://widdowquinn.github.io/pyani/). Moreover, a pan-genome analysis was conducted using Roary[27] to compare the isolated strains to 13 *P. polymyxa* strains with complete circular genome sequences (Figure 1, yellow labels) and to identify shared genes. A maximum-likelihood (ML) phylogenetic tree was inferred using FastTree[62] with Jukes-Cantor Joins distances, the Generalized Time-Reversible substitution model and the CAT approximation model. Local branch support values were calculated using 1,000 resamples with the Shimodaira-Hasegawa test.

4.6. Bioprotection assay (*in vitro*)

An assay was conducted to assess the *in vitro* bioprotection activity of isolated strains against fungal pathogens of *Poaceae* species. Three fungal pathogens of *Poaceae* species (Supplementary Table S9) were obtained from the National Collection of Fungi (VPRI, Bundoora, Victoria, Australia). The setup of the *in vitro* assay was described in detail in Li, et al.[53]. Briefly, bacterial strains, which were drop-inoculated onto four equidistant points on a

Nutrient Agar plate, and pathogens, which were placed at the centre of the plate as a plug containing actively growing hyphae, were co-incubated at 28 ℃ in the dark for five days. The diameter of the fungal colony was measured twice, and the average of the two readings was used for statistical analysis. Three plates were prepared for each treatment as biological replicates. Sterile medium was used as the blank control. Statistical analysis (One-way ANOVA and Tukey Test) was conducted using OriginPro 2020 (Version SR1 9.7.0.188) to detect the presence of any significant difference ($P < 0.05$) between treatments.

4.7. Transcriptome sequencing

Transcriptome sequencing experiments were designed to confirm the expression of the *nif* operon and to explore the early stage of bacteria-pathogen interactions. For the N-fixation activity assay, bacterial strains were cultured in Burk's N-free medium overnight (OD_{600} = 1.0). Cultures were diluted using Burk's N-free medium to OD_{600} = 0.7 and further cultured for six hours to produce actively growing cells for extracting high quality RNA. Burk's N-free medium supplemented with 10 g/L NH_4Cl was used for culturing the bacterial strains as the control. For the bacteria-pathogen interactions assay, bacterial strains and the phytopathogen VPRI42586a *Fusarium verticillioides* were cultured in Nutrient Broth overnight (OD_{600} = 1.0). Bacterial cultures were diluted using Nutrient Broth to OD_{600} = 0.7. 20 mL of such culture was mixed with 200 µL of the pathogen culture and was further incubated for six hours. For the control, the pathogen culture was replaced by sterile Nutrient Broth. Three biological replicates were prepared for each treatment.

Total RNA was extracted form cell pellets using a TRIzol™ Plus RNA Purification Kit (12183555, Thermo Fisher Scientific). On-column treatments were conduct using a PureLink™ DNase Kit (12185010, Thermo Fisher Scientific) to ensure the complete removal of genomic DNA that would affect the downstream analyses, and ribosomal RNA was depleted using a NEBNext® rRNA Depletion Kit (E7860L, NEB, Ipswich, MA, USA). Directional RNA-seq libraries were prepared using a NEBNext Ultra™ II Directional RNA Library Prep Kit (E7765) and sequenced on an Illumina NovaSeq 6000 platform. RNA-seq data (raw reads) were assessed for quality and filtered as per described in section 4.2.

4.8. Transcriptome analysis

Salmon[63] was used to quantify transcripts using the clean RNA-seq reads with the following parameters: *-l A --validateMappings --numBootstraps 1000 --seqBias*. The references used for transcript quantification were the gene sequences generated by Prokka (section 4.4) or the gene sequences of *F. verticillioides* 7600 downloaded from NCBI GenBank (Accession ID: SAMN02953630). A total of 1,000 rounds of bootstraps were performed during transcript quantification to minimise the impact of technical variations. Differential gene expression (DGE) analysis was conducted using a R package sleuth[64]. Likelihood ratio tests were conducted to detect the presence of any significant difference (*q-value* < 0.05) in transcript abundances between treatments, and Wald tests were conducted to determine an approximation of the fold-change in transcript abundances between treatments. Transcripts that were of ultra-low abundance (defined by having less than 20 mapped reads or were only present in less than three samples) were removed prior DGE analysis. The differentially expressed genes were defined to be significant at *q-value* < 0.05 and absolute fold-change ≥ 1.5.

Data availability statement

Annotated genome sequences of strains S02 and S25 were deposited in the NCBI GenBank with the accession number PRJNA720481.

References

1 Berendsen, R. L., Pieterse, C. M. & Bakker, P. A. The rhizosphere microbiome and plant health. *Trends Plant Sci.* **17**, 478-486 (2012).
2 Lugtenberg, B. & Kamilova, F. Plant-growth-promoting rhizobacteria. *Annu. Rev. Microbiol.* **63**, 541-556 (2009).
3 Berg, G. Plant–microbe interactions promoting plant growth and health: perspectives for controlled use of microorganisms in agriculture. *Appl. Microbiol. Biotechnol.* **84**, 11-18 (2009).
4 Grady, E. N., MacDonald, J., Liu, L., Richman, A. & Yuan, Z.-C. Current knowledge and perspectives of *Paenibacillus*: a review. *Microb. Cell. Fact.* **15**, 203, doi:10.1186/s12934-016-0603-7 (2016).
5 Xie, J. *et al.* Comparative genomic and functional analysis reveal conservation of plant growth promoting traits in *Paenibacillus polymyxa* and its closely related species. *Sci. Rep.* **6**, 21329, doi:10.1038/srep21329 (2016).

6 Trüper, H. The type species of the genus *Paenibacillus* Ash et al. 1994 is *Paenibacillus polymyxa*. Opinion 77. Judicial Commission of the International Committee on Systematics of Prokaryotes. *Int. J. Syst. Evol. Microbiol.* **55**, 17 (2005).
7 Cherchali, A., Boukhelata, N., Kaci, Y., Abrous-Belbachir, O. & Djebbar, R. Isolation and identification of a phosphate-solubilizing *Paenibacillus polymyxa* strain GOL 0202 from durum wheat (*Triticum durum* Desf.) rhizosphere and its effect on some seedlings morphophysiological parameters. *Biocatal. Agric. Biotechnol.* **19**, 101087, doi:10.1016/j.bcab.2019.101087 (2019).
8 Kim, J. F. *et al.* Genome sequence of the polymyxin-producing plant-probiotic rhizobacterium *Paenibacillus polymyxa* E681. *J. Bacteriol.* **192**, 6103, doi:10.1128/JB.00983-10 (2010).
9 Holl, F. B., Chanway, C. P., Turkington, R. & Radley, R. A. Response of crested wheatgrass (*Agropyron cristatum* L.), perennial ryegrass (*Lolium perenne*) and white clover (*Trifolium repens* L.) to inoculation with *Bacillus polymyxa*. *Soil Biol. Biochem.* **20**, 19-24, doi:10.1016/0038-0717(88)90121-6 (1988).
10 Bal, A., Anand, R., Berge, O. & Chanway, C. P. Isolation and identification of diazotrophic bacteria from internal tissues of *Pinus contorta* and *Thuja plicata*. *Can. J. For. Res.* **42**, 807-813, doi:10.1139/x2012-023 (2012).
11 Zhou, C. *et al. Paenibacillus polymyxa* BFKC01 enhances plant iron absorption via improved root systems and activated iron acquisition mechanisms. *Plant Physiol. Biochem.* **105**, 162-173, doi:10.1016/j.plaphy.2016.04.025 (2016).
12 Puri, A., Padda, K. P. & Chanway, C. P. Evidence of nitrogen fixation and growth promotion in canola (*Brassica napus* L.) by an endophytic diazotroph *Paenibacillus polymyxa* P2b-2R. *Biol. Fertility Soils* **52**, 119-125, doi:10.1007/s00374-015-1051-y (2015).
13 Puri, A., Padda, K. P. & Chanway, C. P. Seedling growth promotion and nitrogen fixation by a bacterial endophyte *Paenibacillus polymyxa* P2b-2R and its GFP derivative in corn in a long-term trial. *Symbiosis* **69**, 123-129, doi:10.1007/s13199-016-0385-z (2016).
14 Anand, R., Grayston, S. & Chanway, C. N2-fixation and seedling growth promotion of lodgepole pine by endophytic *Paenibacillus polymyxa*. *Microb. Ecol.* **66**, 369-374, doi:10.1007/s00248-013-0196-1 (2013).
15 Xu, S. & Kim, B.-S. Evaluation of *Paenibacillus polymyxa* strain SC09-21 for biocontrol of Phytophthora blight and growth stimulation in pepper plants. *Trop. Plant. Pathol.* **41**, 162-168, doi:10.1007/s40858-016-0077-5 (2016).
16 Eastman, A. W., Heinrichs, D. E. & Yuan, Z.-C. Comparative and genetic analysis of the four sequenced *Paenibacillus polymyxa* genomes reveals a diverse metabolism and conservation of genes relevant to plant-growth promotion and competitiveness. *BMC Genomics* **15**, 851, doi:10.1186/1471-2164-15-851 (2014).
17 Wang, B. *et al.* Comparative genome analysis and mining of secondary metabolites of *Paenibacillus polymyxa*. *Genes Genet. Syst.* **95**, 141-150, doi:10.1266/ggs.19-00053 (2020).
18 Aleti, G. *et al.* The draft genome sequence of *Paenibacillus polymyxa* strain CCI-25 encompasses high potential for secondary metabolite production. *Genome Announc.* **4**, e00366-00316, doi:10.1128/genomeA.00366-16 (2016).
19 Liu, H. *et al.* Complete genome sequence of *Paenibacillus polymyxa* YC0136, a plant growth–promoting rhizobacterium isolated from tobacco rhizosphere. *Genome Announc.* **5**, e01635-01616, doi:10.1128/genomeA.01635-16 (2017).

20 Lal, S. & Tabacchioni, S. Ecology and biotechnological potential of *Paenibacillus polymyxa*: a minireview. *Indian J. Microbiol.* **49**, 2-10, doi:10.1007/s12088-009-0008-y (2009).
21 Padda, K. P., Puri, A. & Chanway, C. P. in *Agriculturally Important Microbes for Sustainable Agriculture: Volume 2: Applications in Crop Production and Protection* (eds Vijay Singh Meena, Pankaj Kumar Mishra, Jaideep Kumar Bisht, & Arunava Pattanayak) 165-191 (Springer Singapore, 2017).
22 Timmusk, S., Grantcharova, N. & Wagner, E. G. H. *Paenibacillus polymyxa* invades plant roots and forms biofilms. *Appl. Environ. Microbiol.* **71**, 7292-7300, doi:10.1128/aem.71.11.7292-7300.2005 (2005).
23 Tannenbaum, I. *et al.* Profiling the *Lolium perenne* microbiome: From seed to seed. *Phytobiomes J.* **4**, 281-289, doi:10.1094/pbiomes-03-20-0026-r (2020).
24 Wood, D. E. & Salzberg, S. L. Kraken: ultrafast metagenomic sequence classification using exact alignments. *Genome Biol.* **15**, R46, doi:10.1186/gb-2014-15-3-r46 (2014).
25 Choi, S. K. *et al.* Identification of a polymyxin synthetase gene cluster of *Paenibacillus polymyxa* and heterologous expression of the gene in *Bacillus subtilis*. *J. Bacteriol.* **191**, 3350-3358, doi:10.1128/jb.01728-08 (2009).
26 Xie, J.-B. *et al.* Comparative genomic analysis of N2-fixing and non-N2-fixing *Paenibacillus* spp.: Organization, evolution and expression of the nitrogen fixation genes. *PLOS Genet.* **10**, e1004231, doi:10.1371/journal.pgen.1004231 (2014).
27 Page, A. J. *et al.* Roary: rapid large-scale prokaryote pan genome analysis. *Bioinformatics* **31**, 3691-3693, doi:10.1093/bioinformatics/btv421 (2015).
28 Franche, C., Lindström, K. & Elmerich, C. Nitrogen-fixing bacteria associated with leguminous and non-leguminous plants. *Plant Soil* **321**, 35-59 (2009).
29 de Werra, P., Pechy-Tarr, M., Keel, C. & Maurhofer, M. Role of gluconic acid production in the regulation of biocontrol traits of *Pseudomonas fluorescens* CHA0. *Appl. Environ. Microbiol.* **75**, 4162-4174, doi:10.1128/AEM.00295-09 (2009).
30 Yuan, Z.-C., Zaheer, R. & Finan, T. M. Regulation and properties of PstSCAB, a high-affinity, high-velocity phosphate transport system of *Sinorhizobium meliloti*. *J. Bacteriol.* **188**, 1089-1102, doi:10.1128/jb.188.3.1089-1102.2006 (2006).
31 Spaepen, S., Vanderleyden, J. & Remans, R. Indole-3-acetic acid in microbial and microorganism-plant signaling. *FEMS Microbiol. Rev.* **31**, 425-448, doi:10.1111/j.1574-6976.2007.00072.x (2007).
32 Blin, K. *et al.* antiSMASH 5.0: updates to the secondary metabolite genome mining pipeline. *Nucleic Acids Res.* **47**, W81-W87, doi:10.1093/nar/gkz310 (2019).
33 Li, J. & Jensen, S. E. Nonribosomal biosynthesis of fusaricidins by *Paenibacillus polymyxa* PKB1 involves direct activation of a D-amino acid. *Chem. Biol.* **15**, 118-127, doi:10.1016/j.chembiol.2007.12.014 (2008).
34 Park, J. E., Kim, H. R., Park, S. Y., Choi, S. K. & Park, S. H. Identification of the biosynthesis gene cluster for the novel lantibiotic paenilan from *Paenibacillus polymyxa* E681 and characterization of its product. *J. Appl. Microbiol.* **123**, 1133-1147, doi:10.1111/jam.13580 (2017).
35 Lohans, C. T. *et al.* Biochemical, structural, and genetic characterization of tridecaptin A_1, an antagonist of *Campylobacter jejuni*. *ChemBioChem* **15**, 243-249, doi:10.1002/cbic.201300595 (2014).
36 Vater, J. *et al.* Genome mining of the lipopeptide biosynthesis of *Paenibacillus polymyxa* E681 in combination with mass spectrometry: Discovery of the lipoheptapeptide paenilipoheptin. *ChemBioChem* **19**, 744-753, doi:10.1002/cbic.201700615 (2018).

37 Galperin, M. Y., Makarova, K. S., Wolf, Y. I. & Koonin, E. V. Expanded microbial genome coverage and improved protein family annotation in the COG database. *Nucleic Acids Res.* **43**, D261-269, doi:10.1093/nar/gku1223 (2015).
38 Baldani, J. I., Reis, V. M., Videira, S. S., Boddey, L. H. & Baldani, V. L. D. The art of isolating nitrogen-fixing bacteria from non-leguminous plants using N-free semi-solid media: a practical guide for microbiologists. *Plant Soil* **384**, 413-431, doi:10.1007/s11104-014-2186-6 (2014).
39 Ribbe, M., Gadkari, D. & Meyer, O. N_2 fixation by *Streptomyces thermoautotrophicus* involves a molybdenum-dinitrogenase and a manganese-superoxide oxidoreductase that couple N_2 reduction to the oxidation of superoxide produced from O2 by a molybdenum-CO dehydrogenase. *J. Biol. Chem.* **272**, 26627-26633 (1997).
40 MacKellar, D. *et al. Streptomyces thermoautotrophicus* does not fix nitrogen. *Sci. Rep.* **6**, 20086, doi:10.1038/srep20086 (2016).
41 Richter, M. & Rosselló-Móra, R. Shifting the genomic gold standard for the prokaryotic species definition. *Proc. Natl. Acad. Sci.* **106**, 19126-19131 (2009).
42 Chun, J. *et al.* Proposed minimal standards for the use of genome data for the taxonomy of prokaryotes. *Int. J. Syst. Evol. Microbiol.* **68**, 461-466, doi:10.1099/ijsem.0.002516 (2018).
43 Jeong, H., Choi, S.-K., Ryu, C.-M. & Park, S.-H. Chronicle of a soil bacterium: *Paenibacillus polymyxa* E681 as a tiny guardian of plant and human health. *Front. Microbiol.* **10**, 467, doi:10.3389/fmicb.2019.00467 (2019).
44 Klenk, H. P. & Goker, M. En route to a genome-based classification of *Archaea* and *Bacteria*? *Syst. Appl. Microbiol.* **33**, 175-182, doi:10.1016/j.syapm.2010.03.003 (2010).
45 Jeong, H. *et al.* Draft genome sequence of the *Paenibacillus polymyxa* type strain (ATCC 842), a plant growth-promoting bacterium. *J. Bacteriol.* **193**, 5026, doi:10.1128/JB.05447-11 (2011).
46 Ash, C., Priest, F. G. & Collins, M. D. Molecular identification of rRNA group 3 bacilli (Ash, Farrow, Wallbanks and Collins) using a PCR probe test. *Antonie Van Leeuwenhoek* **64**, 253-260 (1993).
47 Lee, B. *et al.* Induced resistance by a long-chain bacterial volatile: Elicitation of plant systemic defense by a C_{13} volatile produced by *Paenibacillus polymyxa*. *PLOS One* **7**, e48744, doi:10.1371/journal.pone.0048744 (2012).
48 Raza, W., Yuan, J., Ling, N., Huang, Q. & Shen, Q. Production of volatile organic compounds by an antagonistic strain *Paenibacillus polymyxa* WR-2 in the presence of root exudates and organic fertilizer and their antifungal activity against *Fusarium oxysporum* f. sp. *niveum*. *Biol. Control* **80**, 89-95, doi:10.1016/j.biocontrol.2014.09.004 (2015).
49 Das, S. & De, T. K. Microbial assay of N_2 fixation rate, a simple alternate for acetylene reduction assay. *MethodsX* **5**, 909-914, doi:10.1016/j.mex.2017.11.010 (2018).
50 Lee, S. H. *et al.* An antibiotic fusaricidin: a cyclic depsipeptide from *Paenibacillus polymyxa* E681 induces systemic resistance against Phytophthora blight of red-pepper. *Phytoparasitica* **41**, 49-58, doi:10.1007/s12600-012-0263-z (2012).
51 Compant, S., Duffy, B., Nowak, J., Clément, C. & Barka, E. A. Use of plant growth-promoting bacteria for biocontrol of plant diseases: Principles, mechanisms of action, and future prospects. *Appl. Environ. Microbiol.* **71**, 4951-4959 (2005).

52 Andrić, S., Meyer, T. & Ongena, M. *Bacillus* responses to plant-associated fungal and bacterial communities. *Front. Microbiol.* **11**, 1350, doi:10.3389/fmicb.2020.01350 (2020).
53 Li, T. *et al.* Novel *Xanthomonas* species from the perennial ryegrass seed microbiome – assessing the bioprotection activity of non-pathogenic relatives of pathogens. *Front. Microbiol.* **11**, 1991, doi:10.3389/fmicb.2020.01991 (2020).
54 Gaby, J. C. & Buckley, D. H. A comprehensive evaluation of PCR primers to amplify the *nifH* gene of nitrogenase. *PLOS One* **7**, e42149 (2012).
55 Reeve, W. *et al.* Complete genome sequence of *Rhizobium leguminosarum* bv. *trifolii* strain WSM1325, an effective microsymbiont of annual Mediterranean clovers. *Stand. Genomic Sci.* **2**, 347-356, doi:10.4056/sigs.852027 (2010).
56 Camacho, C. *et al.* BLAST+: architecture and applications. *BMC Bioinformatics* **10**, 421, doi:10.1186/1471-2105-10-421 (2009).
57 De Coster, W., D'Hert, S., Schultz, D. T., Cruts, M. & Van Broeckhoven, C. NanoPack: visualizing and processing long read sequencing data. *Bioinformatics*, 2666–2669, doi:10.1093/bioinformatics/bty149 (2018).
58 Chen, S., Zhou, Y., Chen, Y. & Gu, J. fastp: an ultra-fast all-in-one FASTQ preprocessor. *Bioinformatics* **34**, i884-i890, doi:10.1093/bioinformatics/bty560 (2018).
59 Wick, R. R., Judd, L. M., Gorrie, C. L. & Holt, K. E. Unicycler: Resolving bacterial genome assemblies from short and long sequencing reads. *PLOS Comp. Biol.* **13**, e1005595, doi:10.1371/journal.pcbi.1005595 (2017).
60 Wick, R. R., Schultz, M. B., Zobel, J. & Holt, K. E. Bandage: interactive visualization of de novo genome assemblies. *Bioinformatics* **31**, 3350-3352, doi:10.1093/bioinformatics/btv383 (2015).
61 Seemann, T. Prokka: rapid prokaryotic genome annotation. *Bioinformatics* **30**, 2068-2069, doi:10.1093/bioinformatics/btu153 (2014).
62 Price, M. N., Dehal, P. S. & Arkin, A. P. FastTree 2--approximately maximum-likelihood trees for large alignments. *PLOS One* **5**, e9490, doi:10.1371/journal.pone.0009490 (2010).
63 Patro, R., Duggal, G., Love, M. I., Irizarry, R. A. & Kingsford, C. Salmon provides fast and bias-aware quantification of transcript expression. *Nat. Methods* **14**, 417-419, doi:10.1038/nmeth.4197 (2017).
64 Pimentel, H., Bray, N. L., Puente, S., Melsted, P. & Pachter, L. Differential analysis of RNA-seq incorporating quantification uncertainty. *Nat. Methods* **14**, 687-690, doi:10.1038/nmeth.4324 (2017).

Acknowledgements

Tongda Li received the La Trobe University Full-Fee Research Scholarship, the La Trobe University Postgraduate Research Scholarship and the DairyBio Scholarship. The authors wish to thank Dr. Jacqueline Edwards for access to the Victorian Plant Pathogen Herbarium and Desmond Auer for editing the manuscript.

Author contributions

TS conceptualised the study. TL, TS, and RM designed the experiment. TL and JK contributed to the laboratory work. TL prepared the manuscript. RM, TS, and GS reviewed and edited the manuscript. TS and RM supervised the study. GS contributed to the funding acquisition. All authors have read and agreed to the submitted version of the manuscript.

Additional information - Competing interests statement

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Chapter 4

Transcriptome analyses of barley roots inoculated with novel *Paenibacillus* sp. and *Erwinia gerundensis* strains reveal beneficial early stage plant-bacteria interactions

4.1 Chapter preface

Understanding the interactions between agricultural plants and PGP bacteria is essential to enhance the beneficial interactions and eventually improve the performance of plant. This chapter details the examination of early stage plant-bacteria interactions using barley seedlings and three PGP bacterial strains isolated from the perennial ryegrass microbiome, including two novel *Paenibacillus* sp. strains (S02 and S25) and one novel *Erwinia gerundensis* strain (AR). Differentially expressed bacterial genes and barley transcripts were identified from dual RNA-seq analyses. Overall, transcriptome profiles suggested all three strains improved stress response, nutrient uptake and metabolism, as well as signal transduction of plants, with varied species- and strain-specific responses. This study is one of the few studies that examined the interactions between plants and PGP bacteria using dual RNA-seq analysis. It provided the molecular basis of PGP activities of the three novel bacterial strains in barley, leading to further characterisations of the three strains and future development of the three strains as biofertilisers.

This chapter is presented in submission-ready format for the journal Frontiers in Microbiology. The supplementary materials are presented in Appendix 3, and will be available at the journal's website once this chapter is published. This chapter also contributed to two patents for the three strains, including one for strain AR (Appendix 1, section 1.3) and another for strains S02 and S25 (publicly available in 2022).

4.2 Publication details

Title: Transcriptome analyses of barley roots inoculated with novel *Paenibacillus* sp. and *Erwinia gerundensis* strains reveal beneficial early stage plant-bacteria interactions

Journal details: Frontiers in Microbiology

Stage of publication: Pre-submission

Authors: Tongda Li, Ross Mann, Jatinder Kaur, German Spangenberg, Timothy Sawbridge

4.3 Statement of contribution of joint authorship

TL designed and conducted the assay to examine the early stage plant-bacteria interaction. TL also conducted transcriptome sequencing and the *in silico* assays to analyse transcriptomic data. JK assisted TL to conduct transcriptome sequencing. TL prepared the manuscript. TS conceptualised the study. TL, TS, and RM designed the experiment. RM, TS, and GS reviewed and edited the manuscript. TS and RM supervised the study. GS contributed to the funding acquisition.

4.4 Statement from the co-author confirming the authorship contribution of the PhD candidate

"As co-author of the manuscript 'Li, T., Mann, R., Kaur, J., Spangenberg, G. and Sawbridge, T. (2021). Transcriptome analyses of barley roots inoculated with novel *Paenibacillus* sp. and *Erwinia gerundensis* strains reveal beneficial early stage plant-bacteria interactions', I confirm that Tongda Li made the following contributions,

- Designed and conducted the assay to examine the early stage plant-bacteria interactions
- Conducted transcriptome sequencing
- Designed and conducted the *in silico* assays to analyse transcriptomic data
- Generated all figures and tables
- Prepared the manuscript, critical appraisal of content"

Principal Research Scientist Timothy Sawbridge

Date: 09/02/2021

Transcriptome analyses of barley roots inoculated with novel *Paenibacillus* sp. and *Erwinia gerundensis* strains reveal beneficial early stage plant-bacteria interactions

Tongda Li[*,1,2,3], Ross Mann[1,2], Jatinder Kaur[1,2], German Spangenberg[1,2,3], Timothy Sawbridge[1,2,3]

[1]Agriculture Victoria, AgriBio, Centre for AgriBioscience, Bundoora, Victoria, Australia
[2]DairyBio, Bundoora, Victoria, Australia
[3]School of Applied Systems Biology, La Trobe University, Bundoora, Victoria, Australia

*Correspondence:
Tongda Li
(+61)-3-9032 7088
tongda.li@agriculture.vic.gov.au

Abstract

Plant growth-promoting bacteria can improve host plant traits including nutrient uptake and metabolism and tolerance to biotic and abiotic stresses. Understanding the molecular basis of plant-bacteria interactions using dual RNA-seq analyses provides key knowledge of both hosts and bacteria simultaneously, leading to future enhancements of beneficial interactions. In this study, dual RNA-seq analyses were performed to provide insights into the early stage interactions between barley seedlings and three novel bacterial strains (two *Paenibacillus* sp. strains and one *Erwinia gerundensis* strain) isolated from the perennial ryegrass seed microbiome. Differentially expressed bacterial and barley genes/transcripts involved in plant-bacteria interactions were identified, with varying species- and strain-specific responses associated with processes including (1) initial contact between the bacteria and plant, (2) bacterial plant growth-promoting genes, plant nutrient uptake and metabolism, and (3) bacterial secondary metabolites. Overall, transcriptome profiles suggested that all three strains improved stress response, signal transduction and nutrient uptake and metabolism of barley seedlings. Transcriptome profiles also suggested potential improvements in seedling root growth via repressing ethylene biosynthesis in roots. Bacterial secondary metabolite gene clusters producing compounds that are potentially associated with interactions with the barley endophytic microbiome and associated with stress tolerance of plants under nutrient limiting conditions were also identified. The results of this study provided the molecular basis of plant growth-promoting activities of three novel bacterial strains in barley, laid a solid foundation to the future development of these three bacterial strains as biofertilisers, and identified key differences between bacterial strains of the same species in their responses to plants.

Keywords: *Paenibacillus*, *Erwinia gerundensis*, barley, growth-promotion, interaction, RNA-seq

1. Introduction

Plants and bacteria can establish mutualistic beneficial interactions or undesirable pathogenic interactions (Soto et al., 2011), leading to great impacts on the performance of agriculturally important crops and pastures. Plant growth-promoting (PGP) bacteria possess genes conferring beneficial traits to their host plants, and can act as biofertilisers and bioprotectants, leading to significant increases in yield and improved tolerance to both biotic and abiotic stresses in plants (Compant et al., 2010; Carvalho et al., 2016). *Paenibacillus polymyxa* strains have long been described as PGP bacteria that can improve the nutrient uptake and metabolism of plants via biological nitrogen fixation and phytohormone production, and protect plants form phytopathogens via synthesising bioactive secondary metabolites (Son et al., 2009; Xie et al., 2016; Wang et al., 2020). *Erwinia gerundensis* is a newly identified species that was originally isolated from pome fruit trees and is associated with multiple plant hosts across different continents (Rezzonico et al., 2016). Understanding the interactions between plants and bacteria, especially PGP bacteria, has the potential to improve the overall performance of agricultural plants.

The beneficial interactions between legumes and rhizobia have been extensively studied, revealing the molecular basis and regulatory pathways of each stage of their interaction (Udvardi and Poole, 2013; Liu et al., 2018). However, such knowledge remains to be discovered for many other PGP bacteria and non-leguminous plants. An ideal method to study the molecular basis of plant-bacteria interactions is dual RNA-seq analysis, which can provide transcriptome profiles of both host plants and bacteria simultaneously (Wolf et al., 2018). While dual RNA-seq analysis has been widely used to reveal the interactions between plants and phytopathogens (Westermann et al., 2012; Hayden et al., 2014; Liao et al., 2019), its application in studying the interactions between plants and PGP bacteria is limited. Camilios-Neto et al. (2014) reported the first case of using dual RNA-seq analyses to demonstrate the interactions between wheat and a PGP bacterium *Azospirillum brasilense*, revealing improvements in plant nutrient acquisition and metabolism. Recent work by Liu et al. (2020) demonstrated that interactions between *P. polymyxa* YC0136 and tobacco plants enhanced phytohormone transduction and systemic resistance against pathogens in the plant as well as stimulated auxin biosynthesis in the bacterial strain. Such promising results suggest that dual RNA-seq analyses should be used to deepen our understandings of interactions

between more plant species, especially agricultural crops, and other novel PGP bacteria including *Paenibacillus* spp. and *E. gerundensis*.

In this study we utilised two novel *Paenibacillus* sp. strains (S02 and S25, unpublished data) and one novel *E. gerundensis* strain (AR) isolated from the perennial ryegrass (*Lolium perenne* L. cv. Alto) microbiome (Tannenbaum et al., 2020). Preliminary characterisation showed that the two *Paenibacillus* sp. strains were genetically closely related to *P. polymyxa* and had strong bioprotection and biological nitrogen fixation activities *in vitro*, and the *E. gerundensis* strain was able to grow in low nitrogen conditions *in vitro* and enhance plant root development (Appendix 1, section 1.3), making them ideal candidates for further characterisation. An early stage plant-bacteria interaction assay was conducted using barley seedlings and the three strains. Barley seedlings and bacterial strains were co-incubated for six hours and harvested for RNA extraction. Dual RNA-seq analyses were then performed to identify differentially expressed genes/transcripts associated with the early stage plant-bacteria interaction and to provide insights into the molecular basis of the interaction, with focuses on (1) initial contact between the bacteria and plant, (2) bacterial plant growth-promoting genes, plant nutrient uptake and metabolism, and (3) bacterial secondary metabolites.

2. Materials and Methods

2.1. Assay design

An assay was designed to examine the transcriptional response in early stage plant-bacteria interactions. Barley (*Hordeum vulgare*, cv. Hindmarsh) seeds and three bacterial strains isolated from the perennial ryegrass (*L. perenne* L. cv. Alto) microbiome (Tannenbaum et al., 2020) were used in this study, including two novel *Paenibacillus* sp. strains (S02 and S25) and one novel *E. gerundensis* strain (AR). Two media were utilised as the substrates for the assay, with either a standard medium (Nutrient Broth) or a nitrogen-free medium (Burk's).

Barley seeds were surface-sterilised (80% ethanol for three minutes, followed by 3 × sterile dH_2O washes, each one minute) and germinated under sterile conditions (on moistened sterile filter paper in sealed Petri dish). Bacterial strains were cultured in Nutrient Broth (NB, BD Bioscience) overnight (OD_{600} = 1.0) and cultures were diluted using fresh NB to OD_{600} = 0.7

(final volume = 50 mL). Seedlings (five days old) had their roots submerged in the bacterial culture and were incubated at 26 ℃ for six hours with shaking (100 rpm). Moreover, preliminary characterisations showed that *Paenibacillus* sp. strain S02 is able to actively fix atmospheric nitrogen when growing in Burk's N-free medium ($MgSO_4$, 0.2 g/L; K_2HPO_4, 0.8 g/L; KH_2PO_4, 0.2 g/L; $CaSO_4$, 0.13 g/L; $FeCl_3$, 0.00145 g/L; Na_2MoO_4, 0.000253 g/L; sucrose, 20 g/L) (Baldani et al., 2014). Hence another assay was prepared for strain S02 as described above using Burk's N-free medium to replace NB. For the blank control (seedlings), seedlings had their roots submerged in sterile media (either NB or Burk's N-free medium). For the blank control (bacteria), bacteria were cultured without the presence of a seedling. Three samples were prepared as biological replicates for each treatment and control. Plant root tissues were separated from the bacterial culture after six hours of co-incubation. Bacterial cultures were centrifuged to collect pellets. Plant roots and bacterial pellets were used for RNA extraction.

2.2. Transcriptome sequencing

Total RNA was extracted using a TRIzol™ Plus RNA Purification Kit (12183555, Thermo Fisher Scientific). On-column treatments were conducted using a PureLink™ DNase Kit (12185010, Thermo Fisher Scientific) to ensure the complete removal of genomic DNA. RNA samples were assessed for quality (RIN, the RNA integrity number ≥ 7) on an Agilent 2200 TapeStation (Agilent Technologies, Santa Clara, CA, USA). For bacterial RNA samples, ribosomal RNA (rRNA) was depleted using a NEBNext® rRNA Depletion Kit (E7860L, NEB, Ipswich, MA, USA). For plant RNA samples, messenger RNA (mRNA) was enriched using a NEBNext® Poly(A) mRNA Magnetic Isolation Module (E7490L). Directional RNA-seq libraries were prepared using a NEBNext® Ultra™ II Directional RNA Library Prep Kit (E7765) and sequenced on an Illumina NovaSeq 6000 platform. RNA-seq data (raw reads) were assessed for quality and filtered to remove any adapter and index sequence using fastp (Chen et al., 2018) with the following parameters: *-w 8 -3 -5 --detect_adapter_for_pe*.

2.3. Transcriptome analyses

Salmon (Patro et al., 2017) was used to quantify transcripts using the filtered RNA-seq reads with the following parameters: *-l A --validateMappings --numBootstraps 1000 --seqBias*.

Complete circular genome sequences were generated and annotated for all three bacterial strains using the methods described in Li et al. (2020) (Table 1), which were then used as references for transcript quantification. For the plant samples, a high quality barley reference transcript dataset (BaRTv1.0) containing 60,444 genes with 177,240 transcripts published by Rapazote-Flores et al. (2019) was used as the reference. A total of 1,000 rounds of bootstraps were performed during transcript quantification to minimise the impact of technical variations. Differential gene expression (DGE) analyses were conducted using a R package sleuth (Pimentel et al., 2017). Likelihood ratio tests were conducted to detect the presence of any significant difference (*q-value* < 0.05) in abundances of each transcript between treatments, and Wald tests were conducted to determine an approximation of the fold-change in abundances of each transcript between treatments. Transcripts that were of ultra-low abundance (defined by having less than 20 mapped reads or were only present in less than three samples) were removed prior DGE analyses. The differentially expressed transcripts were defined to be significant at *q-value* < 0.05 and absolute fold-change ≥ 1.5. Gene Ontology (GO) enrichment analyses were conducted using g:Profiler (Raudvere et al., 2019). Venn diagrams were generated using BioVenn (Hulsen et al., 2008).

Table 1. General genomic characteristics of the three bacterial strains used in the assay

Strain ID	Genome size (bp)	No. of gene
S02 (*Paenibacillus* sp.)	6,060,529	5,436
S25 (*Paenibacillus* sp.)	5,958,851	5,306
AR (*Erwinia gerundensis*)	4,437,426	4,091

3. Results

3.1. Transcriptome sequencing – An overview

A 150 bp PE library prepared from cDNA from samples used in the early stage plant-bacteria interaction assay generated an average of 61.7 million clean reads per bacterial sample and 132.4 million per plant sample (Supplementary Table S1). Transcript quantification showed that 80–90% and 85–90% reads from bacterial and plant samples were mapped to the corresponding transcriptome reference (bacteria: Prokka annotation; plant: BaRTv1.0), respectively. Biological variability was checked by comparing the normalised counts of mapped reads within the biological replicates generated by DGE analyses using Pearson correlation coefficients. All biological replicates had a correlation coefficient of 0.92–0.99,

except the root samples of barley co-incubated with *Paenibacillus* sp. strain S02 in Burk's N-free medium (correlation coefficient: 0.84–0.98), suggesting high data reproducibility of this study and the robust nature of the methodology.

3.2. Transcriptome analyses – An overview

DGE analyses clearly demonstrated changes in transcriptome profiles caused by plant-bacteria interactions. For bacterial samples, the biological replicates of all three strains formed distinctive clusters along the PC1 axis based on the presence/absence of barley seedlings (Figure 1). For plant samples, four distinctive clusters containing the biological replicates of each treatment (the presence/absence of bacteria in different media) were identified (Figure 2). Seedlings co-incubated with strain AR (*E. gerundensis*) were separated from seedlings co-incubated with strain S02 and S25 (*Paenibacillus* sp.) along the PC1 axis. Moreover, seedlings co-incubated with strain S02 in Burk's N-free medium were separated from other seedlings co-incubated with bacterial strains in NB along the PC2 axis. These results suggested that the transcriptome profiles of seedlings were affected by both the bacterial species/strain they were co-incubated with and the medium used in the assay. Moreover, when comparing seedlings co-incubated with bacterial strains in NB, seedlings co-incubated with strain AR or S25 formed distinct clusters that were separated from the control seedlings along all three axes (PC1–PC3, Figure 3). Conversely, seedlings co-incubated with strain S02 formed a cluster with the control seedlings along axes PC1 and PC2, only separating along the PC3 axis (Figure 3, right) which accounted for only 4.29% of the total variances. These results suggested seedlings co-incubated with strain S02 produced transcriptome profiles similar to the control seedlings, unlike strain AR and strain S25 which have triggered more obvious changes in transcriptome profiles of barley seedlings.

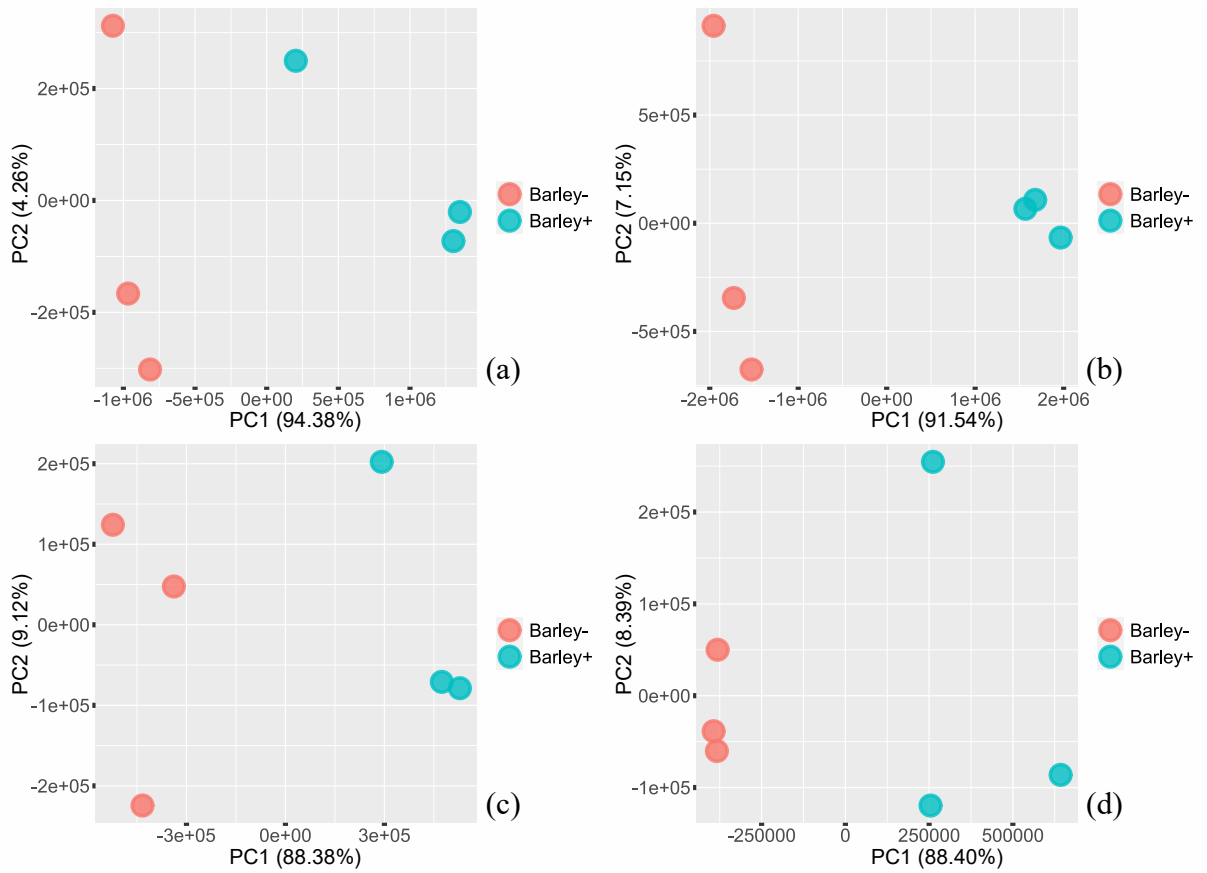


Figure 1. PCA plots representing the clustering of biological replicates based on gene expression levels of (a) strain AR (*E. gerundensis*) in NB, (b) S25 (*Paenibacillus* sp.) in NB, (c) S02 (*Paenibacillus* sp.) in NB, and (d) S02 (*Paenibacillus* sp.) in Burk's N-free medium. Percentage variance explained by each axis are given in brackets. Distinctive clusters that represented the presence (Barley+)/absence (Barley−) formed along the PC1 axis, demonstrating the changes in transcriptome profiles caused by the plant-bacteria interactions.

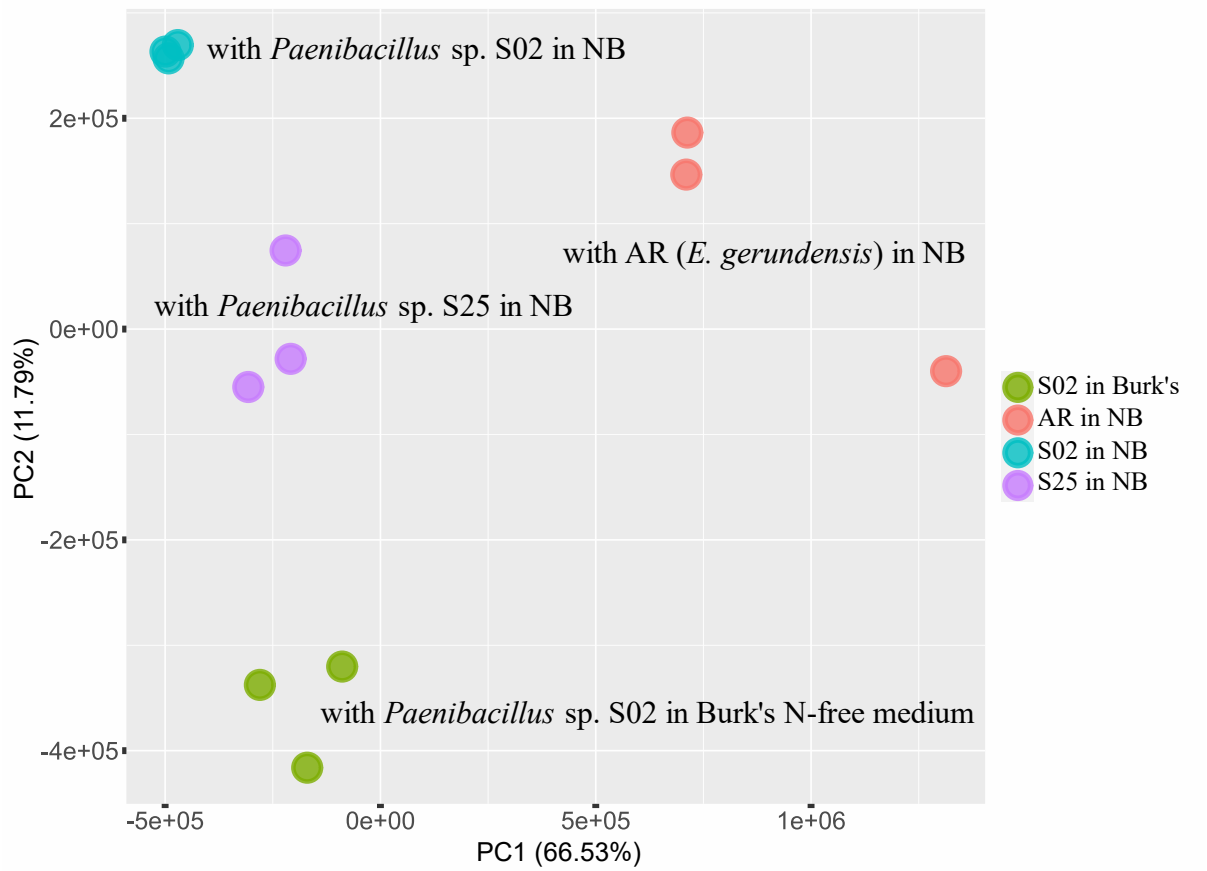


Figure 2. PCA plots representing the clustering of biological replicates based on gene expression levels of barley seedling roots. Percentage variance explained by each axis are given in brackets. Seedlings co-incubated with strain AR (*E. gerundensis*) are separated from the seedlings co-incubated with strain S02 and S25 (*Paenibacillus* sp.) along the PC1 axis, suggesting the effects of two different bacterial species. Seedlings co-incubated with strain S02 in Burk's N-free medium are separated from the seedlings co-incubated with bacterial strains in NB along the PC2 axis, suggesting the effects of two different media. Seedlings co-incubated with strains S02 and S25 in NB are also separated, suggesting the effects of different strains of the same species.

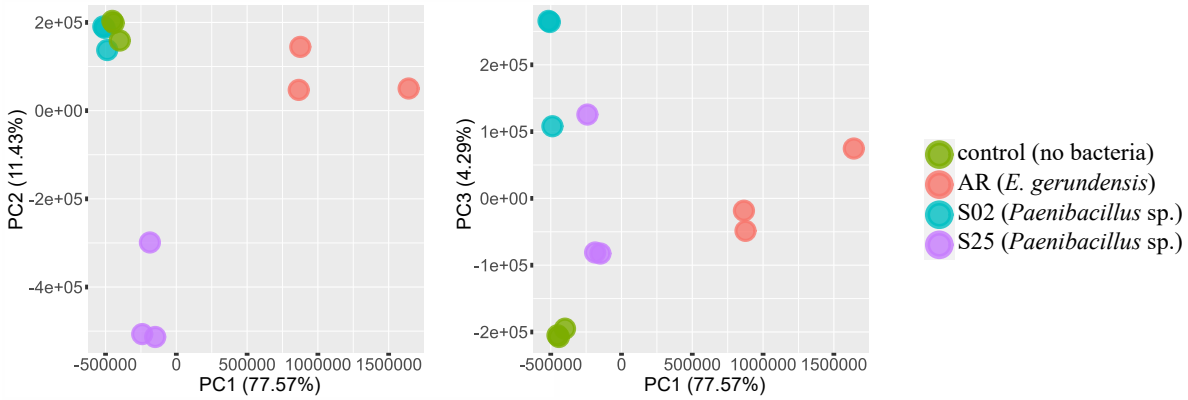


Figure 3. PCA plots representing the clustering of biological replicates based on gene expression levels of barley seedling roots when using NB. Percentage variance explained by each axis are given in brackets. Seedlings co-incubated with strain AR (*E. gerundensis*) or S25 (*Paenibacillus* sp.) are separated from the control seedlings along all three axes (PC1–PC3). However, seedlings co-incubated with strain S02 (*Paenibacillus* sp.) are separated from the control seedlings only along the PC3 axis, which accounts for only 4.29% of the total variances, suggesting strains AR and S25 triggered more obvious changes in transcriptome profiles of barley seedlings when compared with strain S02.

DGE analyses successfully identified genes that were differentially expressed caused by plant-bacteria interactions (Table 2). For bacteria, the DGE analyses compared transcriptome profiles of bacteria when barley seedlings were present and absent. When NB was used, strain AR (*E. gerundensis*) had 4,009 genes that passed the abundance filter, 1,380 of which were differentially expressed when seedlings were present. For *Paenibacillus* sp. strains S25 and S02, 5,013 and 5,266 genes passed the abundance filter, respectively, and 2,945 and 2,890 genes were differentially expressed when seedlings were present, respectively. Moreover, strain S02 cultured in Burk's N-free medium had 5,032 genes that passed the abundance filter and 2,524 genes that were differentially expressed when seedlings were present. Interestingly, strain-specific responses were identified with the two *Paenibacillus* sp. strains in NB (Figure 4) despite the fact that the two strains are genetically highly similar (average nucleotide identity = 97.78%) and share 4,332 conserved genes (unpublished data). Amongst 4,332 conserved genes, there were 997 genes that were only differentially expressed by strain S02 and 1,104 genes that were only differentially expressed by strain S25. There were also 1,317 genes that were differentially expressed by both strains, including 228 genes that were induced in strain S02 but repressed in strain S25 and another 228 genes that were repressed in strain S02 but induced in strain S25. There were also 490 genes that were upregulated in both strains and 371 genes that were downregulated in both strains, and 914 genes that were not differentially expressed by either strain.

For barley, the DGE analyses compared transcriptome profiles of seedlings inoculated with the bacterial strains (absence vs. presence). Barley seedlings co-incubated with strains AR, S25 and S02 in NB had 37,073, 35,365 and 34,798 genes that passed the abundance filter respectively, and 13,948, 13,648 and 9,129 genes that were differentially expressed when bacterial strains were present. When Burk's N-free medium was used, seedlings co-incubated with strain S02 had 31,502 genes that passed the abundance filter and 10,806 genes that were differentially expressed when the strain was present. Overall, 22,015 barley genes were differentially expressed during the plant-bacteria interaction assay using NB, including 3,862 genes that were shared by interactions with all three strains, and 5,117, 4,020 and 2,030 genes that were unique to interactions with strain AR, S25 and S02, respectively (Figure 5). GO enrichment analysis using 3,862 differentially expressed barley genes shared by all three strains identified an overrepresented ($P < 0.05$) GO category associated with sequence-specific DNA binding (GO:0043565), suggesting the transcriptional regulation of plant-bacteria interactions. There were no overrepresented GO categories associated with disease responses and plant defence mechanisms detected using those barley genes. GO enrichment analysis of the 8,067 differentially expressed barley genes that were associated with the two *Paenibacillus* sp. strains (S02 and S25) revealed overrepresented ($P < 0.05$) GO categories associated with nitrogen metabolism, including nitrogen compound transport (GO:0015112) and organonitrogen compound metabolic process (GO:1901564). Moreover, compared with seedlings inoculated with *Paenibacillus* sp. strain S02, seedlings inoculated with *Paenibacillus* sp. strain S25 shared more differentially expressed genes with seedlings inoculated with *E. gerundensis* strain AR. GO enrichment analysis of the 7,611 genes shared by seedlings inoculated with strain S25 and AR revealed overrepresented ($P < 0.05$) GO categories associated with stress responses (GO:0006950, 0006979).

Table 2. Bacterial and plant genes that passed the abundance filter and were differentially expressed identified by DGE analyses

	Sample	Treatment	Medium	No. of genes passed the abundance filter	No. of differentially expressed genes
Bacteria	AR	Barley seedling	NB	4,009	1,380
	S25			5,013	2,945
	S02			5,266	2,890
			Burk's N-free	5,032	2,524
Plant	Barley seedling	AR	NB	37,073	13,948
		S25		35,365	13,648
		S02		34,798	9,129
			Burk's N-free	31,502	10,806

AR: Novel *E. gerundensis* strain
S02/S25: Novel *Paenibacillus* sp. strains

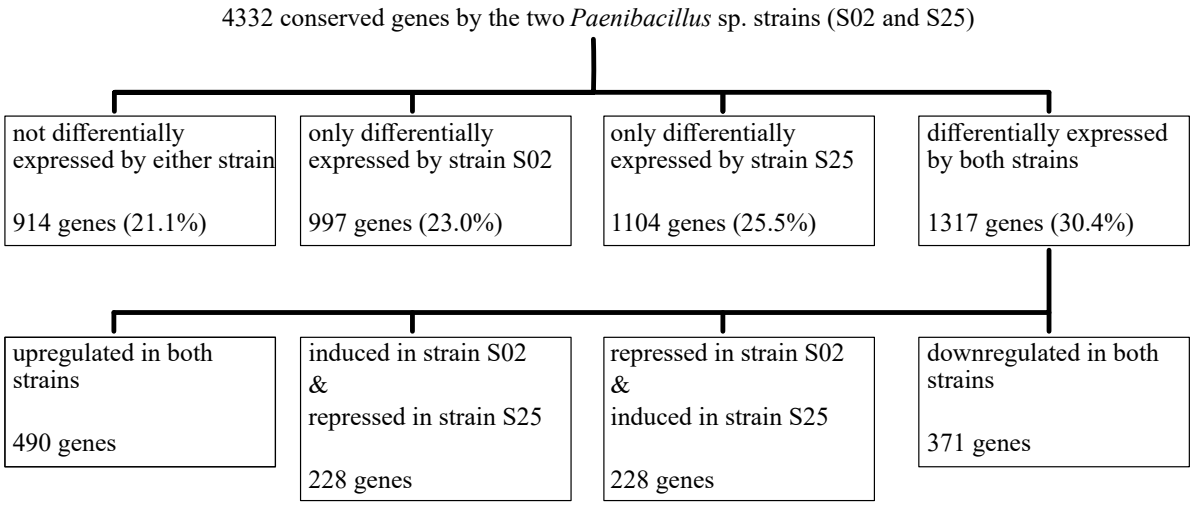


Figure 4. Regulated expressions of 4,332 conserved genes of the two *Paenibacillus* sp. strains (S02 and S25) when co-incubated with barley seedlings in NB. Number of genes and the corresponding percentage of total conserved genes are shown for each category. Despite being genetically closely related (average nucleotide identity = 97.78%), the two strains showed strain-specific responses when interacting with barley seedlings.

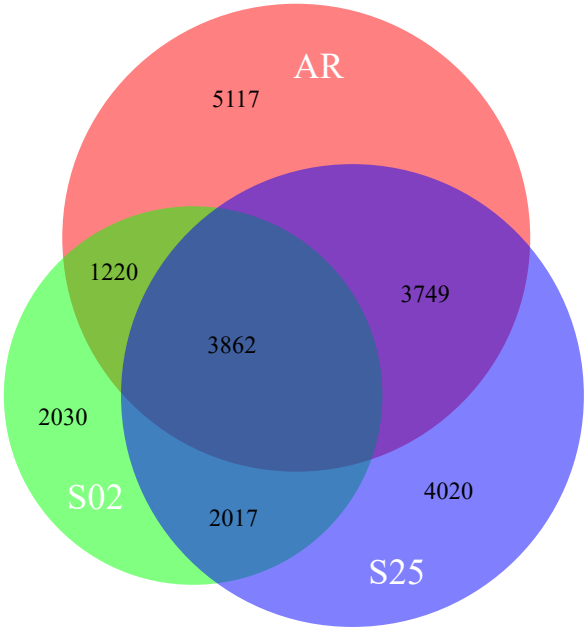


Figure 5. A Venn diagram that shows the amount of barley genes that were differentially expressed in roots during the plant-bacteria interaction assay for all three strains in NB. A total of 22,015 genes were differentially expressed.
AR: Novel *E. gerundensis* strain; S02/S25: Novel *Paenibacillus* sp. strains

3.3. Transcriptome analyses – Functional genes associated with plant-bacteria interaction

The results of DGE analyses clearly demonstrated that the transcriptome profiles of the bacterial strains and roots of barley seedlings were shaped by the interactions between them, causing significant changes in expression levels of some genes. Specific genes that may be involved in plant-bacteria interaction are described and discussed below. Gene expression levels (or transcripts expression levels for plant data) when bacteria/plants were present were represented as approximate fold-changes in relation to the expression levels when bacteria/plants were absent, unless otherwise specified.

Bacterial initial contact with plants

Bacterial genes that are involved in the initial contact with plants (chemotaxis and biofilm formation) were differentially expressed by all three strains (AR, S02 and S25) in NB (Supplementary Table S2). The expressions of methyl-accepting chemotaxis proteins, which are the predominant chemoreceptors that sense the presence of signal molecules and nutrients

produced by plants as root exudates (Salah Ud-Din and Roujeinikova, 2017), were downregulated (up to a 3-fold decrease) in strain AR but were upregulated (up to a 3.9-fold increase) in strain S02 and S25. In addition, functional annotation also identified other chemotaxis proteins that were downregulated in strain AR and S25 but were upregulated in strain S02. The flagellar motor switch proteins, which are utilised by bacteria to move towards favourable environments (Minamino et al., 2019), were highly expressed by strain AR and S25 (up to a 5.01-fold increase) but not by strain S02. Moreover, transporter proteins for sugars, which are the major content of root exudates (Chaparro et al., 2013), were upregulated in all three strains (up to a 2.17-fold increase).

Biofilm formation has been described as an adaptive strategy used by bacteria to enable successful host colonisation (Castiblanco and Sundin, 2016). In this study, the *Paenibacillus* sp. strain S02 even formed visible biofilms on the root surface within three hours of co-incubation with barley seedlings (Figure 6). DGE analyses showed that genes that are involved in biofilm formation, including the biosynthesis of exopolysaccharide, glycogen and cellulose, were highly expressed by strain AR and S25 (up to a 17.61-fold increase) but not by strain S02 (Supplementary Table S3). However, comparisons of the control (i.e. when the barley seedlings were absent) of the two *Paenibacillus* sp. strains (S02 and S25) suggested that those genes were actively expressed by strain S02 with up to a 39.39 -fold increase (Supplementary Table S4).

Figure 6. A representative image showing the visible biofilm formed by the *Paenibacillus* sp. strain S02 on the root surface of barley seedlings after three hours of co-incubation.

Plant growth-promoting genes

The two *Paenibacillus* sp. strains used in this study (S02 and S25) have been characterised by our laboratory, revealing the presence of a comprehensive set of plant growth-promoting genes including biological nitrogen fixation, inorganic and organic phosphate solubilisation and transportation, as well as phytohormone (indole-3-acetic acid) production and transportation. DGE analyses showed that most of these genes were either not differentially expressed or downregulated in expression levels when barley seedlings were present (Supplementary Table S5). However, the expression of one of the auxin efflux carriers genes was upregulated with a 2.34- and 986.13-fold increase for strain S02 and S25, respectively. Furthermore, it is known that biological nitrogen fixation of *P. polymyxa* strains requires low environmental nitrogen content (Wang et al., 2013), therefore a plant-bacteria interaction assay using Burk's N-free medium was conducted for *Paenibacillus* sp. strain S02. It has been shown that strain S02 carries a highly active *nif* operon when growing in Burk's N-free medium. DGE analyses showed that the expression levels of the *nif* operon were upregulated with up to an 11.12-fold increase when barley seedlings were present (Supplementary Table S6). Moreover, the transporting and binding proteins of molybdenum, which is an essential part of the nitrogenase (Hernandez et al., 2009), were also highly expressed (up to a 7.22-fold increase, Supplementary Table S6), suggesting that they were co-induced with the *nif* operon under low N conditions.

Compared to the two *Paenibacillus* sp. strains (S02 and S25), the *E. gerundensis* strain AR carries a reduced set of plant-growth promoting genes (phosphate transporters and auxin efflux carriers). However, similar to the two *Paenibacillus* sp. strains, these genes were either not differentially expressed or downregulated in expression levels when barley seedlings were present (Supplementary Table S7).

Secondary metabolite biosynthesis gene clusters

Previous analyses conducted by our laboratory suggested that the three strains used in this study (AR, S02 and S25) possess multiple secondary metabolite biosynthesis gene clusters, and DGE analyses showed that their expression levels were regulated by plant-bacteria interactions. For the two *Paenibacillus* sp. strains (Supplementary Table S8), the expression levels of the core biosynthetic genes of secondary metabolite gene clusters encoding known

antimicrobial compounds polymyxin (C15) (Choi et al., 2009) and tridecaptin (C10) (Lohans et al., 2014) were upregulated in strain S02 but were downregulated in strain S25. The expression level of the core biosynthetic genes of another antimicrobial compound paenilan (C7) (Park et al., 2017) was not changed in strain S02 but was increased by 2.73-fold in strain S25. Both strains carry an antifungal compound fusaricidin cluster (C1) (Li and Jensen, 2008) whose core biosynthetic gene was downregulated in expression. Strain S25 also carries a unique lanthipeptide cluster with the core biosynthetic genes being downregulated. As for the secondary metabolite gene clusters that encode novel products, whilst the expression levels of the core biosynthetic genes were either not changed or downregulated, the expression levels of the core biosynthetic genes encoding a novel non-ribosomal peptide (C11) were increased by up to 2.53-fold in strain S02 and up to 13.60-fold in strain S25. The expression levels of a siderophore cluster (C2) were also upregulated in both *Paenibacillus* sp. strains.

Interestingly, the core biosynthetic genes of most of the secondary metabolite gene clusters were highly expressed by strain S02 in Burk's N-free medium when barley seedlings were present, including all clusters encoding known antimicrobial compounds with up to a 12.29-fold increase and clusters encoding novel compounds with up to a 269.16-fold increase (Supplementary Table S9).

The *E. gerundensis* strain AR carries a carotenoid biosynthesis cluster that was upregulated when barley seedlings were present (up to a 2.37-fold increase in expression levels). The expression levels of the core biosynthetic genes of the remaining six secondary metabolite gene clusters were either not changed or downregulated (Supplementary Table S10).

Defence and stress response mechanisms utilised by barley seedlings

DGE analyses revealed differentially expressed barley transcripts associated with plant defence and stress response mechanisms. The expressions of defence related proteins, including disease resistance proteins and heat shock proteins (Park and Seo, 2015), were regulated by all three strains, however the *Paenibacillus* sp. strain S02 induced less of those proteins that were differentially expressed when compared to the other *Paenibacillus* sp. strain S25 and the *E. gerundensis* strain AR (Supplementary Table S11). Moreover, transcripts encoding the R-gene-coded resistance protein leucine rich repeat receptor kinase (Kourelis and van der Hoorn, 2018) were only upregulated by strain AR (a 1.75-fold

increase, Supplementary Table S12). Similarly, whilst transcripts encoding inhibitors of bacterial degradative enzymes such as the polygalacturonase (Bashi et al., 2012) and xylanase (Moscetti et al., 2013) were differentially expressed with all three strains, only strain AR upregulated the expression levels (Supplementary Table S12). Furthermore, strain AR also induced the increased expressions of more transcripts encoding endoglucanases, which are released by plants to degrade the cell wall of pathogens (Rose et al., 2002), when compared with strains S02 and S25 (Supplementary Table S13).

The expressions of stress response related proteins were also regulated by all three strains (Supplementary Table S12). The *E. gerundensis* strain AR induced the increased expressions of more transcripts encoding caffeoyl CoA O-methyltransferase, a key enzyme involved in the biosynthesis of lignin that supports the mechanical strength of plant cells (Li et al., 2013), when compared to the two *Paenibacillus* sp. strains (S02 and S25). The same trend was also observed for the stress response proteins glutamate decarboxylase (Mei et al., 2016) and ubiquitin-activating enzyme E1 (Stone, 2014) when using NB as the medium. However, strain S02 induced more differentially expressed transcripts encoding glutamate decarboxylase when using Burk's N-free medium. Furthermore, only strain AR induced differentially expressions of transcripts encoding ascorbate peroxidase, an enzyme which scavenges reactive oxygen species (ROS) released by plants under environmental stress (Pandey et al., 2017).

Differentially expressed barley transcripts associated with signal transduction and ethylene biosynthesis

DGE analyses revealed differentially expressed barley transcripts associated with plant signal transduction (Supplementary Table S14). The expression of transcripts encoding GTP binding proteins and ADP-ribosylation factors, which are involved in plant cellular processes by controlling and relaying signals (Reyes et al., 2011; Ku et al., 2018), were upregulated by all three strains, especially the *E. gerundensis* strain AR.

DGE analyses also revealed differentially expressed barley transcripts associated with ethylene biosynthesis (Supplementary Table S14). To synthesise the phytohormone ethylene, plants first use ACC synthase to convert S-adenosyl-L-methionine into 1-aminocyclopropane-1-carboxylic acid, which is then converted into ethylene by

aminocyclopropanecarboxylate oxidase (Houben and Van de Poel, 2019). The expressions of transcripts encoding aminocyclopropanecarboxylate oxidase were greatly suppressed by all three strains (up to a 314.69-fold decrease).

Differentially expressed barley transcripts associated with nutrient uptake and metabolism

DGE analyses revealed differentially expressed barley transcripts associated with nutrient uptake and metabolism. Expressions of transcripts encoding high affinity transporters associated with nitrate, iron, potassium, sulphate and inorganic phosphate were regulated (Supplementary Table S15). All three strains greatly repressed the expression of transcripts encoding high affinity sulfate transporters (up to a 493.77-fold decrease). A similar repression of expression was also identified from transcripts encoding high affinity nitrate transporters (up to a 478.53-fold decrease), however, strain AR still induced the expression of three transcripts encoding the protein (a 128.40-fold increase). Expressions of transcripts encoding high affinity potassium transporters were induced by both strain AR and S25 (up to a 37.21-fold increase) but were repressed by strain S02 (a 1.72-fold decrease). Moreover, only strains AR and S02 upregulated the expression of transcripts encoding high affinity iron transporters, and only the two *Paenibacillus* sp. strains downregulated the expression of transcripts encoding high affinity inorganic phosphate transporters.

Expressions of transcripts associated with nitrogen transport and metabolism were also regulated (Supplementary Table S16). Compared to the two *Paenibacillus* sp. strains S02 and S25, the *E. gerundensis* strain AR caused the differential expressions of more transcripts encoding ammonium transporters, including one with a 160.67-fold increase and another with a 457.99-fold decrease. Expressions of transcripts encoding glutamine synthetase, which is the principle enzyme involved in nitrogen assimilation (Howitt and Udvardi, 2000), and transcripts encoding aspartate aminotransferase, which plays an important role in nitrogen metabolism (Zhou et al., 2009), were upregulated by all three strains. Interestingly, only strain AR greatly induced the expression of transcripts encoding anthocyanidin -O-glucosyltransferase involved in anthocyanin biosynthesis (up to a 1,063.42-fold increase), which has been reported to be induced by low nitrogen stress (Liang and He, 2018).

Expressions of transcripts associated with carbohydrate metabolism were also regulated (Supplementary Table S16). The *E. gerundensis* strain AR greatly induced the expression of

transcripts encoding UDP-glucose pyrophosphorylase (up to a 753.55-fold increase), which is involved in the biosynthesis of sucrose and the plant cell wall (Kleczkowski et al., 2010). All three strains also induced the expression of transcripts encoding sucrose synthase (up to a 30.36-fold increase), which is the key enzyme of cellulose synthesis (Fujii et al., 2010).

4. Discussion

4.1. Dual RNA-seq analyses of bacterial strains and barley seedling roots

This is the first study that utilised dual RNA-seq analyses to investigate the early stage interactions between barley and *E. gerundensis* and *P. polymyxa* strains. *E. gerundensis* is a newly identified species (Rezzonico et al., 2016) and there has been no research focusing on the transcriptome of the bacterial strain or the inoculated plants as yet. There are a few published studies of the transcriptome or proteome of plants inoculated with *P. polymyxa* strains (Kwon et al., 2016; Zhou et al., 2016; E et al., 2017), but only Liu et al. (2020) reported changes in the bacterial transcriptome associated with plant-bacteria interactions. By sequencing the transcriptome of both the host barley seedlings and the inoculated bacterial strains, this study was able to identify over 20,000 barley genes and over 2,800 bacterial genes that were differentially expressed caused by the plant-bacteria interaction. It provided a comprehensive transcriptome profile of both the bacteria and the plant that could be examined to understand the molecular basis of plant-bacteria interactions, especially between PGP bacteria and agriculturally important crops and pastures. Future dual RNA-seq studies are required to deepen our understanding of such interactions and to potentially contribute to the development of bacterial biofertilisers and improved breeds of plants.

The quantification of expressed genes/transcripts of RNA-seq analysis relies on high quality transcriptome references. While acquiring high quality bacterial transcriptome references has become easier due to recent advances in sequencing technologies (Metzker, 2010; Koren et al., 2017), the availability of high quality plant transcriptome references is still limited (Brown et al., 2017). One of the limitations of the first dual RNA-seq research between plant and PGP bacteria was the lack of a high quality wheat transcriptome reference (Camilios-Neto et al., 2014). In this study, a high quality barley transcriptome reference described by Rapazote-Flores et al. (2019) was used, leading to improved mapping rates in transcripts quantification (~ 90%) when compared to the previous transcriptome reference (high-

confidence gene set only: ~ 55%; high- and low-confidence gene sets combined: ~ 75–80%) described by Mascher et al. (2017). Further research is required to release and improve the reference transcriptomes/genomes of all agriculturally important crops and pastures that underpin RNA-seq analysis studies.

4.2. Initial contact between bacteria and plants – Chemotaxis, biofilm formation and plants' defence and stress response

Plants produce root exudates containing rich nutrients, which have been reported as a key determinant of the composition of the rhizosphere microbiome (Bais et al., 2006; Shi et al., 2011). Bacteria sense the presence of root exudates via chemoreceptors and move towards plants via chemotaxis (Feng et al., 2018; O'Neal et al., 2020), thus creating initial contact between the microbes and plants. In this study, the two *Paenibacillus* sp. strains (S02 and S25) showed higher activities in expressing chemotaxis proteins than the *E. gerundensis* strain (AR) induced by barley seedlings. However, barley seedlings induced active expression of sugar transporters of all three strains and the motor switch proteins of strain AR and S25. While *P. polymyxa* strains are known to colonise the rhizosphere and root tissues (Cherchali et al., 2019), *E. gerundensis* was initially described as a cosmopolitan epiphyte of various plants (Rezzonico et al., 2016). Hence, it is possible that the *E. gerundensis* strain AR is less capable of sensing root exudates when compared to the two *Paenibacillus* sp. strains S02 and S25 but is still able to move towards and utilise root exudates.

Biofilm formation has been described to be important for successful interactions between plants and PGP bacteria (Seneviratne et al., 2011). *P. polymyxa* strains are known to form biofilms on plant roots within two hours under gnotobiotic systems and after seven days under soil systems (Timmusk et al., 2005), but such information is still missing for *E. gerundensis* strains. In this study, the *Paenibacillus* sp. strain S02 actively expressed genes associated with biofilm formation even when barley seedlings were absent and formed visible biofilms on the root surface in just three hours when barley seedling were present. The rapid formation of biofilms could also saturate the capacity of the root surface for future colonisation, which could possibly explain the absence of increased expressions of motor switch proteins induced by barley seedlings. For the other *Paenibacillus* sp. strain (S25) and the *E. gerundensis* strain (AR), although no visible biofilm formation was observed, the induced expression of corresponding genes by barley seedlings suggested that the biofilm

formation was still ongoing when materials were harvested. Further experiments are required to confirm the presence of such biofilms formed by those two strains using microscopic techniques.

Upon successful colonisation on root surface, some bacteria are capable of entering plant internal tissues via naturally occurring cracks like root tips or via active production of plant cell wall degrading enzymes (Hardoim et al., 2008; Monteiro et al., 2012), thus creating stress condition to plants. A previous research has shown that *P. polymyxa* strains invade plant roots (Timmusk et al., 2005). The *E. gerundensis* strain AR was isolated from seedlings grown from surface-sterilised seeds of perennial ryegrass (Tannenbaum et al., 2020), suggesting its ability to enter plant tissues. In this study, strain S02 was more active in biofilm formation and root colonisation but induced the expressions of less resistance proteins and stress proteins in barley seedlings when compared to strains S25 and AR, which was consistent with the GO enrichment analysis. Such results suggested that strain S02 is more adaptive to barley, leading to enhanced interactions with hosts and alleviated stresses in hosts. Conversely, inoculating strain AR induced the expressions of more resistance proteins and stress proteins in barley seedlings when compared to strain S02 and S25. Moreover, it also induced the expressions of inhibitors of plant cell wall degrading enzymes (such as xylanase), as well as the enzyme supporting the strength of plane cell wall (caffeoyl CoA O-methyltransferase). Such results suggested that the *E. gerundensis* strain AR created more intense stress conditions in barley seedlings when compared to the two *Paenibacillus* sp. strains S02 and S25. Furthermore, strain AR also triggered the expressions of plant defence and stress response genes such as endoglucanase (Ferreira et al., 2007) and ascorbate peroxidase (Anjum et al., 2016), suggesting this strain could improve the plant responses to fungal phytopathogens and excessive oxidative stress.

4.3. Interactions between bacteria and plants – Plant growth-promoting genes, plant nutrient uptake and metabolism, signal transduction and ethylene biosynthesis

Bacterial plant growth-promoting genes are a key contributor to the beneficial plant-bacteria interactions. In this study, the expression of biological nitrogen fixation genes (the *nif* operon) of *Paenibacillus* sp. strain S02 was greatly enhanced by barley seedlings under low nitrogen conditions (Burk's N-free medium), making the strain a promising candidate to be developed as a biofertiliser. Interestingly, the presence of barley seedlings did not enhance

the expressions of other plant growth-promoting genes of the three strains. This could be explained by the relatively short period of time of plant-bacteria interactions (six hours). Further research is required to track the expression of those genes in long-term interactions between plants and the three strains.

The beneficial plant-bacteria interactions can greatly improve the nutrient uptake and metabolism of plants (Pii et al., 2015). Expressions of barley transcripts encoding high affinity transporters, which are utilised by plants under low environmental nutrient concentrations (Rodríguez-Navarro and Rubio, 2006; Sun et al., 2014), were downregulated by all three strains for sulphate and nitrate. The *Paenibacillus* sp. strain S02 also repressed the expression of high affinity potassium transporters. Such results may indicate increased concentrations of these nutrients in the medium released by the action of the bacteria. Transcripts of barley genes associated with nitrogen metabolism and carbohydrate metabolism were observed to be increased when the bacterial strains were present, indicating increased core metabolic activity in the plant. Overall, these results indicated the improved nutrient availability and metabolism in barley seedlings when the three strains were present. Counterintuitively, whilst the nutrient availability was improved, expressions of most of the nutrient transporters in barley seedlings were not induced by the three strains. For instance, expressions of most ammonium transporters were repressed by the two diazotrophic *Paenibacillus* sp. strains (S02 and S05). However, such downregulations were reasonable since excess ammonium can be cytotoxic (Howitt and Udvardi, 2000; Courty et al., 2015; Tegeder and Masclaux-Daubresse, 2018). Species-specific responses were also observed in this study. The two *Paenibacillus* sp. strains (S02 and S25) downregulated the expression of barley high affinity inorganic phosphate transporters, indicating the improved bioavailability of phosphate to seedlings. Such improvement could be explained by the plant growth-promoting genes associated with phosphate solubilisation and transportation carried by the two strains. Besides, the *E. gerundensis* strain AR induced the expression of barley anthocyanidin -O-glucosyltransferase. Anthocyanidins are flavonoid pigments that are associated with plants growing under nitrogen deficient environments (Diaz et al., 2006; Soubeyrand et al., 2014; Quan et al., 2016). The induced expressions of the anthocyanidin biosynthesis gene and the GO enrichment analysis of differentially expressed barley genes triggered by the two *Paenibacillus* sp. strains suggested that the *E. gerundensis* strain AR is less capable of improving nitrogen bioavailability and metabolism in barley seedlings when compared to the *Paenibacillus* sp. strains S02 and S25.

In addition to nutrient uptake and metabolism, inoculating the three strains promoted the expressions of plant signal transduction proteins including GTP binding proteins and ADP-ribosylation factors. These proteins have been reported to be related to plants' responses to abiotic and biotic stresses (Joshi et al., 2014; Ku et al., 2018). Thus, the induced expressions of these proteins may increase of resilience of inoculated seedlings to stresses. Furthermore, all three strains repressed the ethylene biosynthesis protein aminocyclopropanecarboxylate oxidase in barley seedlings. Ethylene supresses the root cell elongation and lateral root development (Swarup et al., 2007; Lewis et al., 2011). Camilios-Neto et al. (2014) inoculated wheat with *Azospirillum brasilense* and identified a 3.1-fold decrease in expressions of aminocyclopropanecarboxylate oxidase and up to 30% increases in root mass in three days. Preliminary glasshouse experiment results have shown that the root length of five-day-old barley seedlings inoculated with the *E. gerundensis* strain AR was 21.8% longer when compared to the uninoculated control (Appendix 1, section 1.3). Hence, we proposed that the root growth-promoting activity exhibited by strain AR is analogous, and the two *Paenibacillus* sp. strains (S02 and S25) are also capable of promoting the root growth of barley based on the transcriptome data. Future research (e.g. *in planta* inoculation) is required to validate such root growth-promoting activities of all three strains on more crops such as wheat.

4.4. Bacterial secondary metabolite

Bacteria produce a wide range of secondary metabolites that have important biological and ecological functions (Tyc et al., 2017; Blin et al., 2019). The results of this study demonstrated that the expressions of some bacterial secondary metabolites are potentially associated with plant-bacteria interactions. The *E. gerundensis* strain AR has a carotenoid biosynthesis cluster, and the expressions of the core biosynthetic gene of the cluster was induced by 2.37-fold by barley seedlings. Carotenoids play an important role in both pigmentation and cell signalling (Sui et al., 2013). Bible et al. (2016) reported that a PGP bacterium strain *Pantoea* sp. YR343 became defective in biofilm formation and root colonisation when its carotenoid biosynthesis was impaired. Given the close phylogenetic relationships between *Erwinia* spp. and *Pantoea* spp. (Zhang and Qiu, 2015), we proposed a hypothesis that carotenoid biosynthesis is also necessary for the root colonisation by strain AR. Further targeted experiments, e.g. creating mutants of the carotenoid biosynthesis cluster, is required to corroborate this hypothesis.

P. polymyxa strains are known to produce a diverse range of secondary metabolites, including ones associated with bioprotection and others associated with novel functions (Xie et al., 2016; Wang et al., 2020). The two *Paenibacillus* sp. strains used in this study (S02 and S25) carry multiple secondary metabolite gene clusters encoding known antimicrobial compounds, whose expressions were induced by barley seedlings despite some strain-specific variations. Such increased expression could be associated with the endophytic bacteria of barley seedlings. Although the barley seeds used in this study were surface-sterilised, only epiphytic bacteria would be killed. Barley is known to host a complex endophytic bacterial community (Bulgarelli et al., 2015). Moreover, the presence of unmapped reads in the bacterial transcriptome sequencing samples also suggested the presence of other bacterial strains. Hence, we postulate that the induced expressions of those antimicrobial secondary metabolites of the two *Paenibacillus* sp. strains were caused by the interactions between these inoculated strains and the endophytic bacterial strains in barley seedlings. To validate this hypothesis, sterile seedlings that are free of both epiphytic and endophytic microbes must be used to repeat the experiment.

Interestingly, the expression of secondary metabolites encoding novel compounds were either not changed or downregulated by barley seedlings when NB was used but were highly increased by barley seedlings when Burk's N-free medium was used. Compared with NB, Burk's N-free medium contains less nutrients and represents a stress environment to barley seedlings. It has been previously suggested that the secondary metabolites produced by PGP bacteria can enhance the tolerance to abiotic and biotic stress of plants (Bacon et al., 2015; Mishra et al., 2018). Therefore, we submit that these novel secondary metabolites are associated with the stress tolerance response of plants. It is notable that given the complexity of biosynthesis of secondary metabolites, this study focused on the expression levels of core biosynthetic genes of secondary metabolite gene clusters to represent the expression of those metabolites. Future research is required to prove this hypothesis, including purifying, quantifying and characterising the novel compounds with mass spectrometry, in conjunction with *in planta* glasshouse experiments.

4.5. Specific responses associated with bacterial strains and bacteria/plant species, and their implications for enhanced plant-bacteria interactions

Plants recruit and interact with beneficial bacteria by using complex signalling and comprehensive genetic and metabolic controls (Carvalho et al., 2016). As discussed above, all three strains used in this study were isolated from the same host plant species (*L. perenne*) but the two *Paenibacillus* sp. strains exhibited strain-specific behaviours when interacting with barley and caused varied beneficial responses. There were also clear differences between *E. gerundensis* and these strains. Such strain- and species-specific responses of barley transcriptome included different genes associated with stress responses, nutrient uptake and metabolism and phytohormone biosynthesis. Furthermore, strain- and species-specific responses were also proven since different bacterial strains triggered the expression of different transcripts (isoforms) of genes in barley (e.g. ammonium transporter, Supplementary Table S16). Whilst such varying responses are anticipated in bacterial strains belonging to different species, it is interesting to observe such varying responses from genetically closely related strains (e.g. *Paenibacillus* sp. strain S02 and S25). Since other Australian *E. gerundensis* strains are available (Rezzonico et al., 2016; Tannenbaum et al., 2020), further experiments should be conducted to characterise the interactions between those strains and barley seedlings to identify potential strain-specific responses within this species, comparing responses to our *E. gerundensis* strain AR.

Liu et al. (2020) recently reported the first dual RNA-seq analyses of interactions between *P. polymyxa* YC0136 and tobacco (*Nicotiana tabacum* L.). *P. polymyxa* YC0136 is closely related to the two *Paenibacillus* sp. strains used in this study, having an average nucleotide identity of 97.81% and 99.29% when compared to strain S02 and S25, respectively (unpublished data). The results from that study are largely consistent with those presented here. Differences in the results presented include fewer differentially expressed bacterial genes (187 vs. 1,380 and 2,945 in this study) and upregulated expression of the bacterial *pst* genes associated with phosphate transportation. These differences are likely associated with different methodologies used by the two studies, including differing ages of plants and length of co-incubation time. Another reason for the lower number of differentially expressed bacterial genes may be the host plant used. *P. polymyxa* YC0136 was isolated from the tobacco rhizosphere (Liu et al., 2017), whereas the bacteria described in this study were isolated from perennial ryegrass and tested on a different species, namely barley. Thus *P.*

polymyxa YC0136 may be more adapted to the host tested. It remains unclear that if this strain would interact with other plant species in a similar pattern when compared to its interactions with the original host plant species. Similarly, will the three strains used in study interact with their original host (perennial ryegrass) in a similar pattern when compared to their interactions with barley? Will they exhibit a universal pattern of interactions with plants, or a host-specific pattern? Further research is required to answer these questions by repeating the experiment using other *P. polymyxa* strains on a wider range of host plant species, which was outside the scope of this study.

Whilst barley seedlings used in this study were only inoculated with a single bacterial strain, it has been demonstrated that co-inoculation of multiple bacterial strains in plants may have synergistic effects (Yu et al., 2012; Korir et al., 2017; Razzaghi Komaresofla et al., 2019). The microbiome of perennial ryegrass has been profiled recently by our laboratory, revealing a complex community of microbes (Tannenbaum et al., 2020). Given the fact that strain-specific responses have been observed in this study, a key question that should orient future studies is how these microbes interact with plants collectively as a community. As the three strains used in this study (S02, S25 and AR) have shown that each has their own role in promoting the growth of barley seedlings, we postulate that a consortia of S02, S25 and AR would be of maximum benefit to the growth of barley seedlings. To affirm this, dual RNA-seq analyses would need to be performed with barley seedlings in the presence of all three strains, with associated differential time points to tease out the interactions as time progresses. Such knowledge is required to characterise these plant-associated bacteria, understand various strain-specific and host-specific responses and eventually enhance the beneficial interactions to improve the productivity of plants.

Data availability statement

Annotated genome sequences of strains used in this study were deposited in the NCBI GenBank with the accession number PRJNA720480 (AR) and PRJNA720481 (S02 and S25).

Acknowledgements

Tongda Li received the La Trobe University Full-Fee Research Scholarship, the La Trobe University Postgraduate Research Scholarship and the DairyBio Scholarship. The authors wish to thank Ms. Desmond Auer for editing the manuscript.

Author contributions

TS conceptualised the study. TL, TS, and RM designed the experiment. TL and JK contributed to the laboratory work. TL prepared the manuscript. RM, TS, and GS reviewed and edited the manuscript. TS and RM supervised the study. GS contributed to the funding acquisition. All authors have read and agreed to the submitted version of the manuscript.

Funding

This research was supported by Agriculture Victoria, Dairy Australia and Gardiner Foundation

Competing interests statement

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Supplementary materials

The supplementary materials for this manuscript include:

Supplementary Table S1. Yield (number of clean reads) generated by transcriptome sequencing

Supplementary Table S2. Differentially expressed genes associated with chemotaxis of the three strains when barley seedlings were present

Supplementary Table S3. Differentially expressed genes associated with biofilm formation of the three strains when barley seedlings were present

Supplementary Table S4. Differentially expressed genes associated with biofilm formation of *Paenibacillus* sp. S02 compared to *Paenibacillus* sp. S25 when barley seedlings were absent

Supplementary Table S5. Differentially expressed genes associated with plant growth promotion of strain S02 and S25 when barley seedlings were present

Supplementary Table S6. Differentially expressed genes associated with biological nitrogen fixation of strain S02 when barley seedlings were present (in Burk's N-free medium)

Supplementary Table S7. Differentially expressed genes associated with plant growth promotion of strain AR when barley seedlings were present

Supplementary Table S8. Differentially expressed core biosynthetic genes of secondary metabolite gene clusters of strain S02 and S25 when barley seedlings were present

Supplementary Table S9. Differentially expressed core biosynthetic genes of secondary metabolite gene clusters of strain S02 when barley seedlings were present (in Burk's N-free medium)

Supplementary Table S10. Differentially expressed core biosynthetic genes of secondary metabolite gene clusters of strain AR when barley seedlings were present

Supplementary Table S11. Differentially expressed transcripts encoding disease resistance proteins and heat shock proteins in barley seedlings when the three bacterial strains were present

Supplementary Table S12. Differentially expressed transcripts associated with defence and stress responses in barley seedlings when the three bacterial strains were present

Supplementary Table S13. Differentially expressed transcripts encoding endoglucanase in barley seedlings when the three bacterial strains were present

Supplementary Table S14. Differentially expressed transcripts associated with signal transduction and ethylene biosynthesis in barley seedlings when the three bacterial strains were present

Supplementary Table S15. Differentially expressed transcripts encoding high affinity transporters in barley seedlings when the three bacterial strains were present

Supplementary Table S16. Differentially expressed transcripts associated with nutrient uptake and metabolism in barley seedlings when the three bacterial strains were present

Reference

Anjum, N.A., Sharma, P., Gill, S.S., Hasanuzzaman, M., Khan, E.A., Kachhap, K., Mohamed, A.A., Thangavel, P., Devi, G.D., Vasudhevan, P., Sofo, A., Khan, N.A., Misra, A.N., Lukatkin, A.S., Singh, H.P., Pereira, E., and Tuteja, N. (2016). Catalase and ascorbate peroxidase—representative H2O2-detoxifying heme enzymes in plants. *Environmental Science and Pollution Research* 23**,** 19002-19029.

Bacon, C.W., Palencia, E.R., and Hinton, D.M. (2015). "Abiotic and biotic plant stress-tolerant and beneficial secondary metabolites produced by endophytic *Bacillus* species," in *Plant microbes symbiosis: applied facets*, ed. N.K. Arora. (New Delhi: Springer India), 163-177.

Bais, H.P., Weir, T.L., Perry, L.G., Gilroy, S., and Vivanco, J.M. (2006). The role of root exudates in rhizosphere interactions with plants and other organisms. *Annual Review of Plant Biology* 57**,** 233-266.

Baldani, J.I., Reis, V.M., Videira, S.S., Boddey, L.H., and Baldani, V.L.D. (2014). The art of isolating nitrogen-fixing bacteria from non-leguminous plants using N-free semi-solid media: a practical guide for microbiologists. *Plant and Soil* 384**,** 413-431.

Bashi, Z.D., Rimmer, S.R., Khachatourians, G.G., and Hegedus, D.D. (2012). *Brassica napus* polygalacturonase inhibitor proteins inhibit *Sclerotinia sclerotiorum* polygalacturonase enzymatic and necrotizing activities and delay symptoms in transgenic plants. *Canadian Journal of Microbiology* 59**,** 79-86.

Bible, A.N., Fletcher, S.J., Pelletier, D.A., Schadt, C.W., Jawdy, S.S., Weston, D.J., Engle, N.L., Tschaplinski, T., Masyuko, R., Polisetti, S., Bohn, P.W., Coutinho, T.A., Doktycz, M.J., and Morrell-Falvey, J.L. (2016). A carotenoid-deficient mutant in

Pantoea sp. YR343, a bacteria isolated from the rhizosphere of *Populus deltoides*, is defective in root colonization. *Frontiers in Microbiology* 7**,** 491.

Blin, K., Shaw, S., Steinke, K., Villebro, R., Ziemert, N., Lee, S.Y., Medema, M.H., and Weber, T. (2019). antiSMASH 5.0: updates to the secondary metabolite genome mining pipeline. *Nucleic Acids Research* 47**,** W81-W87.

Brown, J.W.S., Calixto, C.P.G., and Zhang, R. (2017). High-quality reference transcript datasets hold the key to transcript-specific RNA-sequencing analysis in plants. *New Phytologist* 213**,** 525-530.

Bulgarelli, D., Garrido-Oter, R., Munch, P.C., Weiman, A., Droge, J., Pan, Y., Mchardy, A.C., and Schulze-Lefert, P. (2015). Structure and function of the bacterial root microbiota in wild and domesticated barley. *Cell Host Microbe* 17**,** 392-403.

Camilios-Neto, D., Bonato, P., Wassem, R., Tadra-Sfeir, M.Z., Brusamarello-Santos, L.C.C., Valdameri, G., Donatti, L., Faoro, H., Weiss, V.A., Chubatsu, L.S., Pedrosa, F.O., and Souza, E.M. (2014). Dual RNA-seq transcriptional analysis of wheat roots colonized by *Azospirillum brasilense* reveals up-regulation of nutrient acquisition and cell cycle genes. *BMC Genomics* 15**,** 378.

Carvalho, T., Ballesteros, H., Thiebaut, F., Ferreira, P., and Hemerly, A. (2016). Nice to meet you: genetic, epigenetic and metabolic controls of plant perception of beneficial associative and endophytic diazotrophic bacteria in non-leguminous plants. *Plant Molecular Biology* 90**,** 561-574.

Castiblanco, L.F., and Sundin, G.W. (2016). New insights on molecular regulation of biofilm formation in plant-associated bacteria. *Journal of Integrative Plant Biology* 58**,** 362-372.

Chaparro, J.M., Badri, D.V., Bakker, M.G., Sugiyama, A., Manter, D.K., and Vivanco, J.M. (2013). Root exudation of phytochemicals in *Arabidopsis* follows specific patterns that are developmentally programmed and correlate with soil microbial functions. *PLOS One* 8**,** e55731.

Chen, S., Zhou, Y., Chen, Y., and Gu, J. (2018). fastp: an ultra-fast all-in-one FASTQ preprocessor. *Bioinformatics* 34**,** i884-i890.

Cherchali, A., Boukhelata, N., Kaci, Y., Abrous-Belbachir, O., and Djebbar, R. (2019). Isolation and identification of a phosphate-solubilizing *Paenibacillus polymyxa* strain GOL 0202 from durum wheat (*Triticum durum* Desf.) rhizosphere and its effect on some seedlings morphophysiological parameters. *Biocatalysis and Agricultural Biotechnology* 19**,** 101087.

Choi, S.K., Park, S.Y., Kim, R., Kim, S.B., Lee, C.H., Kim, J.F., and Park, S.H. (2009). Identification of a polymyxin synthetase gene cluster of *Paenibacillus polymyxa* and heterologous expression of the gene in *Bacillus subtilis*. *Journal of Bacteriology* 191**,** 3350-3358.

Compant, S., Clément, C., and Sessitsch, A. (2010). Plant growth-promoting bacteria in the rhizo- and endosphere of plants: their role, colonization, mechanisms involved and prospects for utilization. *Soil Biology and Biochemistry* 42**,** 669-678.

Courty, P.E., Smith, P., Koegel, S., Redecker, D., and Wipf, D. (2015). Inorganic nitrogen uptake and transport in beneficial plant root-microbe interactions. *Critical Reviews in Plant Sciences* 34**,** 4-16.

Diaz, C., Saliba-Colombani, V., Loudet, O., Belluomo, P., Moreau, L., Daniel-Vedele, F., Morot-Gaudry, J.-F., and Masclaux-Daubresse, C. (2006). Leaf yellowing and anthocyanin accumulation are two genetically independent strategies in response to nitrogen limitation in *Arabidopsis thaliana*. *Plant and Cell Physiology* 47**,** 74-83.

E, Y., Yuan, J., Yang, F., Wang, L., Ma, J., Li, J., Pu, X., Raza, W., Huang, Q., and Shen, Q. (2017). PGPR strain *Paenibacillus polymyxa* SQR-21 potentially benefits watermelon growth by re-shaping root protein expression. *AMB Express* 7**,** 104.

Feng, H., Zhang, N., Du, W., Zhang, H., Liu, Y., Fu, R., Shao, J., Zhang, G., Shen, Q., and Zhang, R. (2018). Identification of chemotaxis compounds in root exudates and their sensing chemoreceptors in plant-growth-promoting rhizobacteria *Bacillus amyloliquefaciens* SQR9. *Molecular Plant-Microbe Interactions* 31**,** 995-1005.

Ferreira, R.B., Monteiro, S., Freitas, R., Santos, C.N., Chen, Z., Batista, L.M., Duarte, J., Borges, A., and Teixeira, A.R. (2007). The role of plant defence proteins in fungal pathogenesis. *Molecular Plant Pathology* 8**,** 677-700.

Fujii, S., Hayashi, T., and Mizuno, K. (2010). Sucrose synthase is an integral component of the cellulose synthesis machinery. *Plant and Cell Physiology* 51**,** 294-301.

Hardoim, P.R., Van Overbeek, L.S., and Van Elsas, J.D. (2008). Properties of bacterial endophytes and their proposed role in plant growth. *Trends in Microbiology* 16**,** 463-471.

Hayden, K.J., Garbelotto, M., Knaus, B.J., Cronn, R.C., Rai, H., and Wright, J.W. (2014). Dual RNA-seq of the plant pathogen *Phytophthora ramorum* and its tanoak host. *Tree Genetics & Genomes* 10**,** 489-502.

Hernandez, J.A., George, S.J., and Rubio, L.M. (2009). Molybdenum trafficking for nitrogen fixation. *Biochemistry* 48**,** 9711-9721.

Houben, M., and Van De Poel, B. (2019). 1-aminocyclopropane-1-carboxylic acid oxidase (ACO): the enzyme that makes the plant hormone ethylene. *Frontiers in Plant Science* 10**,** 695.

Howitt, S.M., and Udvardi, M.K. (2000). Structure, function and regulation of ammonium transporters in plants. *Biochimica et Biophysica Acta (BBA) - Biomembranes* 1465**,** 152-170.

Hulsen, T., De Vlieg, J., and Alkema, W. (2008). BioVenn – a web application for the comparison and visualization of biological lists using area-proportional Venn diagrams. *BMC Genomics* 9**,** 488.

Joshi, R., Ramanarao, M.V., Lee, S., Kato, N., and Baisakh, N. (2014). Ectopic expression of ADP ribosylation factor 1 (SaARF1) from smooth cordgrass (*Spartina alterniflora* Loisel) confers drought and salt tolerance in transgenic rice and *Arabidopsis*. *Plant Cell, Tissue and Organ Culture (PCTOC)* 117**,** 17-30.

Kleczkowski, L.A., Kunz, S., and Wilczynska, M. (2010). Mechanisms of UDP-glucose synthesis in plants. *Critical Reviews in Plant Sciences* 29**,** 191-203.

Koren, S., Walenz, B.P., Berlin, K., Miller, J.R., Bergman, N.H., and Phillippy, A.M. (2017). Canu: scalable and accurate long-read assembly via adaptive k-mer weighting and repeat separation. *Genome Research* 27**,** 722-736.

Korir, H., Mungai, N.W., Thuita, M., Hamba, Y., and Masso, C. (2017). Co-inoculation effect of rhizobia and plant growth promoting rhizobacteria on common bean growth in a low phosphorus soil. *Frontiers in Plant Science* 8**,** 141.

Kourelis, J., and Van Der Hoorn, R.a.L. (2018). Defended to the nines: 25 years of resistance gene cloning identifies nine mechanisms for r protein function. *The Plant Cell* 30**,** 285.

Ku, Y.-S., Sintaha, M., Cheung, M.-Y., and Lam, H.-M. (2018). Plant hormone signaling crosstalks between biotic and abiotic stress responses. *International Journal of Molecular Sciences* 19**,** 3206.

Kwon, Y.S., Lee, D.Y., Rakwal, R., Baek, S.-B., Lee, J.H., Kwak, Y.-S., Seo, J.-S., Chung, W.S., Bae, D.-W., and Kim, S.G. (2016). Proteomic analyses of the interaction

between the plant-growth promoting rhizobacterium *Paenibacillus polymyxa* E681 and *Arabidopsis thaliana*. *Proteomics* 16**,** 122-135.

Lewis, D.R., Negi, S., Sukumar, P., and Muday, G.K. (2011). Ethylene inhibits lateral root development, increases IAA transport and expression of PIN3 and PIN7 auxin efflux carriers. *Development* 138**,** 3485.

Li, J., and Jensen, S.E. (2008). Nonribosomal biosynthesis of fusaricidins by *Paenibacillus polymyxa* PKB1 involves direct activation of a D-amino acid. *Chemistry & Biology* 15**,** 118-127.

Li, T., Mann, R., Sawbridge, T., Kaur, J., Auer, D., and Spangenberg, G. (2020). Novel *Xanthomonas* species from the perennial ryegrass seed microbiome – Assessing the bioprotection activity of non-pathogenic relatives of pathogens. *Frontiers in Microbiology* 11**,** 1991.

Li, X., Chen, W., Zhao, Y., Xiang, Y., Jiang, H., Zhu, S., and Cheng, B. (2013). Downregulation of caffeoyl-CoA O-methyltransferase (*CCoAOMT*) by RNA interference leads to reduced lignin production in maize straw. *Genetics and Molecular Biology* 36**,** 540-546.

Liang, J., and He, J. (2018). Protective role of anthocyanins in plants under low nitrogen stress. *Biochemical and Biophysical Research Communications* 498**,** 946-953.

Liao, Z.-X., Ni, Z., Wei, X.-L., Chen, L., Li, J.-Y., Yu, Y.-H., Jiang, W., Jiang, B.-L., He, Y.-Q., and Huang, S. (2019). Dual RNA-seq of *Xanthomonas oryzae* pv. *oryzicola* infecting rice reveals novel insights into bacterial-plant interaction. *PLOS One* 14**,** e0215039.

Liu, A., Contador, C.A., Fan, K., and Lam, H.-M. (2018). Interaction and regulation of carbon, nitrogen, and phosphorus metabolisms in root nodules of legumes. *Frontiers in Plant Science* 9**,** 1860.

Liu, H., Liu, K., Li, Y., Wang, C., Hou, Q., Xu, W., Fan, L., Zhao, J., Gou, J., Du, B., and Ding, Y. (2017). Complete genome sequence of *Paenibacillus polymyxa* YC0136, a plant growth–promoting rhizobacterium isolated from tobacco rhizosphere. *Genome Announcements* 5**,** e01635-01616.

Liu, H., Wang, J., Sun, H., Han, X., Peng, Y., Liu, J., Liu, K., Ding, Y., Wang, C., and Du, B. (2020). Transcriptome profiles reveal the growth-promoting mechanisms of *Paenibacillus polymyxa* YC0136 on tobacco (*Nicotiana tabacum* L.). *Frontiers in Microbiology* 11**,** 2384.

Lohans, C.T., Van Belkum, M.J., Cochrane, S.A., Huang, Z., Sit, C.S., Mcmullen, L.M., and Vederas, J.C. (2014). Biochemical, structural, and genetic characterization of tridecaptin A_1, an antagonist of *Campylobacter jejuni*. *Chembiochem* 15**,** 243-249.

Mascher, M., Gundlach, H., Himmelbach, A., Beier, S., Twardziok, S.O., Wicker, T., Radchuk, V., Dockter, C., Hedley, P.E., Russell, J., Bayer, M., Ramsay, L., Liu, H., Haberer, G., Zhang, X.Q., Zhang, Q., Barrero, R.A., Li, L., Taudien, S., Groth, M., Felder, M., Hastie, A., Simkova, H., Stankova, H., Vrana, J., Chan, S., Munoz-Amatriain, M., Ounit, R., Wanamaker, S., Bolser, D., Colmsee, C., Schmutzer, T., Aliyeva-Schnorr, L., Grasso, S., Tanskanen, J., Chailyan, A., Sampath, D., Heavens, D., Clissold, L., Cao, S., Chapman, B., Dai, F., Han, Y., Li, H., Li, X., Lin, C., Mccooke, J.K., Tan, C., Wang, P., Wang, S., Yin, S., Zhou, G., Poland, J.A., Bellgard, M.I., Borisjuk, L., Houben, A., Dolezel, J., Ayling, S., Lonardi, S., Kersey, P., Langridge, P., Muehlbauer, G.J., Clark, M.D., Caccamo, M., Schulman, A.H., Mayer, K.F.X., Platzer, M., Close, T.J., Scholz, U., Hansson, M., Zhang, G., Braumann, I., Spannagl, M., Li, C., Waugh, R., and Stein, N. (2017). A chromosome conformation capture ordered sequence of the barley genome. *Nature* 544**,** 427-433.

Mei, X., Chen, Y., Zhang, L., Fu, X., Wei, Q., Grierson, D., Zhou, Y., Huang, Y., Dong, F., and Yang, Z. (2016). Dual mechanisms regulating *glutamate decarboxylases* and accumulation of gamma-aminobutyric acid in tea (*Camellia sinensis*) leaves exposed to multiple stresses. *Scientific Reports* 6**,** 23685.

Metzker, M.L. (2010). Sequencing technologies—the next generation. *Nature Reviews Genetics* 11**,** 31.

Minamino, T., Kinoshita, M., and Namba, K. (2019). Directional switching mechanism of the bacterial flagellar motor. *Computational and Structural Biotechnology Journal* 17**,** 1075-1081.

Mishra, J., Fatima, T., and Arora, N.K. (2018). "Role of secondary metabolites from plant growth-promoting rhizobacteria in combating salinity stress," in *Plant microbiome: stress response*, eds. D. Egamberdieva & P. Ahmad. (Singapore: Springer Singapore), 127-163.

Monteiro, R.A., Balsanelli, E., Wassem, R., Marin, A.M., Brusamarello-Santos, L.C., Schmidt, M.A., Tadra-Sfeir, M.Z., Pankievicz, V.C., Cruz, L.M., and Chubatsu, L.S. (2012). *Herbaspirillum*-plant interactions: microscopical, histological and molecular aspects. *Plant and Soil* 356**,** 175-196.

Moscetti, I., Tundo, S., Janni, M., Sella, L., Gazzetti, K., Tauzin, A., Giardina, T., Masci, S., Favaron, F., and D'ovidio, R. (2013). Constitutive expression of the xylanase inhibitor TAXI-III delays fusarium head blight symptoms in durum wheat transgenic plants. *Molecular Plant-Microbe Interactions* 26**,** 1464-1472.

O'neal, L., Vo, L., and Alexandre, G. (2020). Specific root exudate compounds sensed by dedicated chemoreceptors shape *Azospirillum brasilense* chemotaxis in the rhizosphere. *Applied and Environmental Microbiology* 86**,** e01026-01020.

Pandey, S., Fartyal, D., Agarwal, A., Shukla, T., James, D., Kaul, T., Negi, Y.K., Arora, S., and Reddy, M.K. (2017). Abiotic stress tolerance in plants: myriad roles of ascorbate peroxidase. *Frontiers in Plant Science* 8**,** 581.

Park, C.-J., and Seo, Y.-S. (2015). Heat shock proteins: a review of the molecular chaperones for plant immunity. *The Plant Pathology Journal* 31**,** 323-333.

Park, J.E., Kim, H.R., Park, S.Y., Choi, S.K., and Park, S.H. (2017). Identification of the biosynthesis gene cluster for the novel lantibiotic paenilan from *Paenibacillus polymyxa* E681 and characterization of its product. *Journal of Applied Microbiology* 123**,** 1133-1147.

Patro, R., Duggal, G., Love, M.I., Irizarry, R.A., and Kingsford, C. (2017). Salmon provides fast and bias-aware quantification of transcript expression. *Nature Methods* 14**,** 417-419.

Pii, Y., Mimmo, T., Tomasi, N., Terzano, R., Cesco, S., and Crecchio, C. (2015). Microbial interactions in the rhizosphere: beneficial influences of plant growth-promoting rhizobacteria on nutrient acquisition process. A review. *Biology and Fertility of Soils* 51**,** 403-415.

Pimentel, H., Bray, N.L., Puente, S., Melsted, P., and Pachter, L. (2017). Differential analysis of RNA-seq incorporating quantification uncertainty. *Nature Methods* 14**,** 687-690.

Quan, X., Zeng, J., Ye, L., Chen, G., Han, Z., Shah, J.M., and Zhang, G. (2016). Transcriptome profiling analysis for two Tibetan wild barley genotypes in responses to low nitrogen. *BMC Plant Biology* 16**,** 30.

Rapazote-Flores, P., Bayer, M., Milne, L., Mayer, C.D., Fuller, J., Guo, W., Hedley, P.E., Morris, J., Halpin, C., Kam, J., Mckim, S.M., Zwirek, M., Casao, M.C., Barakate, A., Schreiber, M., Stephen, G., Zhang, R., Brown, J.W.S., Waugh, R., and Simpson, C.G. (2019). BaRTv1.0: an improved barley reference transcript dataset to determine accurate changes in the barley transcriptome using RNA-seq. *BMC Genomics* 20**,** 968.

Raudvere, U., Kolberg, L., Kuzmin, I., Arak, T., Adler, P., Peterson, H., and Vilo, J. (2019). g:Profiler: a web server for functional enrichment analysis and conversions of gene lists (2019 update). *Nucleic Acids Research* 47**,** W191-W198.

Razzaghi Komaresofla, B., Alikhani, H.A., Etesami, H., and Khoshkholgh-Sima, N.A. (2019). Improved growth and salinity tolerance of the halophyte *Salicornia* sp. by co–inoculation with endophytic and rhizosphere bacteria. *Applied Soil Ecology* 138**,** 160-170.

Reyes, F.C., Buono, R., and Otegui, M.S. (2011). Plant endosomal trafficking pathways. *Current Opinion in Plant Biology* 14**,** 666-673.

Rezzonico, F., Smits, T.H.M., Born, Y., Blom, J., Frey, J.E., Goesmann, A., Cleenwerck, I., De Vos, P., Bonaterra, A., Duffy, B., and Montesinos, E. (2016). *Erwinia gerundensis* sp. nov., a cosmopolitan epiphyte originally isolated from pome fruit trees. *International Journal of Systematic and Evolutionary Microbiology* 66**,** 1583-1592.

Rodríguez-Navarro, A., and Rubio, F. (2006). High-affinity potassium and sodium transport systems in plants. *Journal of Experimental Botany* 57**,** 1149-1160.

Rose, J.K.C., Ham, K.-S., Darvill, A.G., and Albersheim, P. (2002). Molecular cloning and characterization of glucanase inhibitor proteins: coevolution of a counterdefense mechanism by plant pathogens. *The Plant Cell* 14**,** 1329-1345.

Salah Ud-Din, A.I.M., and Roujeinikova, A. (2017). Methyl-accepting chemotaxis proteins: a core sensing element in prokaryotes and archaea. *Cellular and Molecular Life Sciences* 74**,** 3293-3303.

Seneviratne, G., Weerasekara, M.L.M.a.W., Seneviratne, K.a.C.N., Zavahir, J.S., Kecskés, M.L., and Kennedy, I.R. (2011). "Importance of biofilm formation in plant growth promoting rhizobacterial action," in *Plant growth and health promoting bacteria,* ed. D.K. Maheshwari. (Berlin, Heidelberg: Springer Berlin Heidelberg), 81-95.

Shi, S., Richardson, A.E., O'callaghan, M., Deangelis, K.M., Jones, E.E., Stewart, A., Firestone, M.K., and Condron, L.M. (2011). Effects of selected root exudate components on soil bacterial communities. *FEMS Microbiology Ecology* 77**,** 600-610.

Son, S.H., Khan, Z., Kim, S.G., and Kim, Y.H. (2009). Plant growth-promoting rhizobacteria, *Paenibacillus polymyxa* and *Paenibacillus lentimorbus* suppress disease complex caused by root-knot nematode and fusarium wilt fungus. *Journal of Applied Microbiology* 107**,** 524-532.

Soto, M.J., Nogales, J., Pérez-Mendoza, D., Gallegos, M.-T., Olivares, J., and Sanjuán, J. (2011). Pathogenic and mutualistic plant-bacteria interactions: ever increasing similarities. *Central European Journal of Biology* 6**,** 911-917.

Soubeyrand, E., Basteau, C., Hilbert, G., Van Leeuwen, C., Delrot, S., and Gomès, E. (2014). Nitrogen supply affects anthocyanin biosynthetic and regulatory genes in grapevine cv. Cabernet-Sauvignon berries. *Phytochemistry* 103**,** 38-49.

Stone, S.L. (2014). The role of ubiquitin and the 26S proteasome in plant abiotic stress signaling. *Frontiers in Plant Science* 5**,** 135.

Sui, X., Kiser, P.D., Lintig, J.V., and Palczewski, K. (2013). Structural basis of carotenoid cleavage: from bacteria to mammals. *Archives of Biochemistry and Biophysics* 539**,** 203-213.

Sun, J., Bankston, J.R., Payandeh, J., Hinds, T.R., Zagotta, W.N., and Zheng, N. (2014). Crystal structure of the plant dual-affinity nitrate transporter NRT1.1. *Nature* 507**,** 73-77.

Swarup, R., Perry, P., Hagenbeek, D., Van Der Straeten, D., Beemster, G.T.S., Sandberg, G., Bhalerao, R., Ljung, K., and Bennett, M.J. (2007). Ethylene upregulates auxin biosynthesis in *Arabidopsis* seedlings to enhance inhibition of root cell elongation. *The Plant Cell* 19**,** 2186.

Tannenbaum, I., Kaur, J., Mann, R., Sawbridge, T., Rodoni, B., and Spangenberg, G. (2020). Profiling the *Lolium perenne* microbiome: from seed to seed. *Phytobiomes Journal* 4**,** 281-289.

Tegeder, M., and Masclaux-Daubresse, C. (2018). Source and sink mechanisms of nitrogen transport and use. *New Phytologist* 217**,** 35-53.

Timmusk, S., Grantcharova, N., and Wagner, E.G.H. (2005). *Paenibacillus polymyxa* invades plant roots and forms biofilms. *Applied and Environmental Microbiology* 71**,** 7292-7300.

Tyc, O., Song, C., Dickschat, J.S., Vos, M., and Garbeva, P. (2017). The ecological role of volatile and soluble secondary metabolites produced by soil bacteria. *Trends in Microbiology* 25**,** 280-292.

Udvardi, M., and Poole, P.S. (2013). Transport and metabolism in legume-rhizobia symbioses. *Annual Review of Plant Biology* 64**,** 781-805.

Wang, B., Cheng, H., Qian, W., Zhao, W., Liang, C., Liu, C., Cui, G., Liu, H., and Zhang, L. (2020). Comparative genome analysis and mining of secondary metabolites of *Paenibacillus polymyxa*. *Genes & Genetic Systems* 95**,** 141-150.

Wang, L., Zhang, L., Liu, Z., Zhao, D., Liu, X., Zhang, B., Xie, J., Hong, Y., Li, P., Chen, S., Dixon, R., and Li, J. (2013). A minimal nitrogen fixation gene cluster from *Paenibacillus* sp. WLY78 enables expression of active nitrogenase in *Escherichia coli*. *PLOS Genetics* 9**,** e1003865.

Westermann, A.J., Gorski, S.A., and Vogel, J. (2012). Dual RNA-seq of pathogen and host. *Nature Reviews Microbiology* 10**,** 618-630.

Wolf, T., Kämmer, P., Brunke, S., and Linde, J. (2018). Two's company: studying interspecies relationships with dual RNA-seq. *Current Opinion in Microbiology* 42**,** 7-12.

Xie, J., Shi, H., Du, Z., Wang, T., Liu, X., and Chen, S. (2016). Comparative genomic and functional analysis reveal conservation of plant growth promoting traits in *Paenibacillus polymyxa* and its closely related species. *Scientific Reports* 6**,** 21329.

Yu, X., Liu, X., Zhu, T.-H., Liu, G.-H., and Mao, C. (2012). Co-inoculation with phosphate-solubilzing and nitrogen-fixing bacteria on solubilization of rock phosphate and their effect on growth promotion and nutrient uptake by walnut. *European Journal of Soil Biology* 50**,** 112-117.

Zhang, Y., and Qiu, S. (2015). Examining phylogenetic relationships of *Erwinia* and *Pantoea* species using whole genome sequence data. *Antonie van Leeuwenhoek* 108**,** 1037-1046.

Zhou, C., Guo, J., Zhu, L., Xiao, X., Xie, Y., Zhu, J., Ma, Z., and Wang, J. (2016). *Paenibacillus polymyxa* BFKC01 enhances plant iron absorption via improved root systems and activated iron acquisition mechanisms. *Plant Physiology and Biochemistry* 105**,** 162-173.

Zhou, Y., Cai, H., Xiao, J., Li, X., Zhang, Q., and Lian, X. (2009). Over-expression of aspartate aminotransferase genes in rice resulted in altered nitrogen metabolism and increased amino acid content in seeds. *Theoretical and Applied Genetics* 118**,** 1381-1390.

Chapter 5

General discussion

5.1 Context of study

The interactions between plants and their microbiomes play a remarkable role in determining the health and productivity of host plants, with individual members of a plant microbiome being able to not only alter the community structure of the microbiome, but also promoting the growth and overall performance of the host plant (Compant et al., 2019; Jiménez et al., 2020). The term ‘plant holobiont’ proposed by Vandenkoornhuyse et al. (2015) further emphasised the irreplaceable role that PGP bacteria play in the survival and fitness of plants. Some PGP bacterial strains have been commercialised for agricultural use for decades, and there has been an increasing number of novel bioactive strains being isolated from plant microbiomes (Berg, 2009). In order to develop new bioprotectants and/or biofertilisers using those novel strains, it is essential to characterise the strains with *in vitro, in planta* and *in silico* assays to gain phenotypic, genomic and transcriptomic evidence that underpin the beneficial bioactivities.

The microbiomes of perennial ryegrass seeds and mature plants have been recently profiled, with 284 bacterial strains being isolated from seeds and more strains being subsequently isolated from mature plants (Tannenbaum et al., 2020). The community structure of the profiled microbiomes suggested the presence of known PGP bacterial species (e.g. *Paenibacillus* spp.) and bacterial species that are commonly associated with plants and potentially have PGP bioactivities (e.g. *Erwinia* spp. and *Xanthomonas* spp.). As such, these Australian strains represented promising candidates to be characterised for possible PGP bioactivities, e.g. bioprotection and biofertilisation. The overall aim of this PhD study was to isolate, identify and characterise these strains for PGP bioactivities based on the phenotypic, genomic and transcriptomic evidence obtained from *in vitro, in planta* and *in silico* assays.

5.2 Summary of key findings

5.2.1 Identification and characterisation of three novel non-pathogenic, bioprotectant *Xanthomonas* sp. strains

This study identified and characterised three novel *Xanthomonas* sp. strains (GW, SS and SI) isolated from perennial ryegrass seeds and mature plants (Chapter 2; Li et al., 2020). The three strains inhibited the growth of phytopathogens of agricultural crops and pasture grasses belonging to five fungal families in *in vitro* assays and one strain (GW) alleviated disease symptoms of *B. sorokiniana* on wheat leaves in *in planta* assays, demonstrating their broad spectrum bioprotection activities. Genome sequencing and comparative genomics analyses showed that the three strains are genetically different to any known species of the genus *Xanthomonas*, representing a novel species. Further genomic analyses showed that the strain (GW) with the highest bioprotection activities has a unique secondary metabolite biosynthesis (Nrps) cluster that encodes a novel product, suggesting the associations between the Nrps cluster and its unique bioprotection activity compared to the other strains. This study also demonstrated the absence of pathogenicity-associated genes (such as T3SS and T3Es) of the genus *Xanthomonas* in the genomes of all three strains. Although non-pathogenic *Xanthomonas* spp. strains have been previously reported (Garita-Cambronero et al., 2017; Martins et al., 2020), this novel *Xanthomonas* species represented by the three strains is the first bioprotectant *Xanthomonas* sp., a key finding of this study. The results of this study provided solid phenotypic and genomic foundations to further develop strains from this novel *Xanthomonas* sp. as bioprotectants.

5.2.2 Isolation, identification and characterisation of two novel *Paenibacillus* sp. strains with PGP bioactivities

This study isolated, identified and characterised two novel *Paenibacillus* sp. strains (S02 and S25) associated with perennial ryegrass seeds (Chapter 3). The microbiome profile (Tannenbaum et al., 2020) and *nifH* gene PCR assay (Chapter 3) suggested the presence of seed-associated diazotrophic bacterial strains, and two strains were subsequently isolated using a combined approach of a selective medium and genomic sequencing of mixed cultures. Comparative genomics analyses showed that the two strains are closely related to *P. polymyxa* and may represent a novel species. Genomic analyses showed that the two strains

have a wide range of PGP genes associated with biological nitrogen fixation, phosphate solubilisation and assimilation, as well as auxin production and transportation. Further analyses revealed that the two strains have at least 13 secondary metabolite biosynthesis clusters associated with both known antimicrobial compounds (e.g. polymyxin and fusaricidin) and novel compounds. The bioprotection activities of both strains were subsequently confirmed in the *in vitro* bioprotection assays. Transcriptome sequencing and analyses showed while both strains have an expressed *nif* operon for biological nitrogen fixation, strain S02 was more active in expressing the operon when compared to strain S25. Moreover, compared to strain S25, strain S02, which showed stronger activities in bioprotection assays, was more active in expressing secondary metabolites biosynthesis genes, and had a more stable transcriptome profile when cultured with the fungal phytopathogen *F. verticillioides*. This is the first study that reported Australian strains of *Paenibacillus* sp. with excellent bioactivities, providing solid foundations to future local applications of these indigenous strains. One highlight of this study was the incorporation of genomic techniques with bacterial isolation. Sequencing the total DNA of mixed bacterial cultures isolated from seeds identified a single 110 Kb read containing the entire *nif* operon (nine genes) with sequence homology similar to a known *P. polymyxa* strain, which led to the decision of supplementing polymyxin in the N-free medium to accelerate the isolation of pure colonies of the target strains. Another highlight of this study was the strain-specific variations identified in bioprotection and transcriptome assays. While the two strains are genetically closely related, obvious variations were observed in both bioprotection assays and transcriptomic responses to the absence of nitrogen and the presence of a fungal phytopathogen. Such intriguing results also led to further characterisations of interactions between the two *Paenibacillus* sp. strains and plants (Chapter 4).

5.2.3 Examination of early stage plant-bacteria interactions

This study designed and conducted dual RNA-seq analyses that examined the transcriptomic responses of both host plant (barley seedlings) and three bacterial strains (S02 and S25: *Paenibacillus* sp.; AR: *E. gerundensis*) during the early stage plant-bacteria interaction (Chapter 4). Overall, changes in transcriptome profiles suggested beneficial plant-bacteria interactions, including the improvement of nutrient uptake and metabolism, stress response as well as signal transduction of barley seedlings induced by the three bacterial strains. All three strains repressed ethylene biosynthesis in roots, potentially leading to increases in root

growth. Further analyses also identified bacterial secondary metabolites that may be associated with interactions with barley endophytic microbiome and associated with stress tolerance of plants in nutrient limiting conditions. The highlight of study was the successful identification of both plant and bacterial genes that are probably associated with beneficial plant-bacteria interactions, leading to further characterisations of the three strains and future development of the three strains as biofertilisers. Another highlight of study was the strain-specific changes in transcriptome profiles of the two genetically closely related *Paenibacillus* sp. strains, which have not been reported in previous studies. These strain-specific responses of plant-bacteria interactions demonstrated the necessity of further experiments to characterise and eventually enhance their beneficial interactions.

5.2.4 Development of *in vitro, in planta* and *in silico* assays enabling the standardised characterisation of bacteria associated with perennial ryegrass

The outcomes of bacterial isolation, identification and characterisation described above are underpinned by a series of *in vitro, in planta* and *in silico* assays designed and conducted in this PhD study. Such assays included the *in vitro* and *in planta* assays that enabled rapid evaluations of bioprotection activities of candidate strains against a wide range of phytopathogens curated by the Victorian Plant Pathogen Herbarium (Chapter 2 and 3), and the *in planta* assay that enabled dual RNA-seq analyses for early stage plant-bacteria interactions (Chapter 4). These assays have been adapted and implemented to characterise more bacteria associated with other agricultural plants and crops, including the novel *Curtobacterium flaccumfaciens* and *Arthrobacter* sp. strains that promote growth of wheat under drought conditions (Hone et al., 2021), further demonstrating the value of assays designed in this PhD study.

It is notable that barley and wheat plants were used in the *in planta* assays of this study despite the fact that those bacterial strains were isolated from perennial ryegrass. Unlike barley and wheat, perennial ryegrass is heterozygous with high levels of genetic diversity across different individuals (Brazauskas et al., 2011; Pembleton et al., 2015). Such genetic diversity is known to affect phenotypes of different individuals (Yu et al., 2013; Kovi et al., 2015), introducing additional variations to the interpretation of assays. Hence, using barley and wheat plants enabled us to design standardised and robust assays to characterise the candidate bacterial strains, which is one of the major outcomes of this research. Moreover,

barley and wheat are important agricultural crops, which *Xanthomonas* spp., *Paenibacillus* spp. and *Erwinia* spp. are commonly associated with. For instance, *Paenibacillus* spp. has been reported as part of the core seed endophytic microbiome of five barley cultivars (Yang et al., 2017). Rahman et al. (2018) reported that a *Xanthomonas* spp. OTU dominated the seed microbiome of three different barley cultivars, and *Paenibacillus* spp. strains were also isolated from non-sterilised seeds, exhibiting PGP bioactivities including phosphate solubilisation, nitrogen fixation, IAA biosynthesis and bioprotection in *in vitro* assays. A recent microbiome study conducted by Yang et al. (2020) reported an enrichment of *Erwinia* spp., *Pantoea* spp. and *Pseudomonas* spp. in the roots of barley plants under drought stress, suggesting the associations between these bacterial genera and plant stress responses. Similarly, Kuźniar et al. (2020) described *Paenibacillus* spp. as a member of obligatory bacteria in the seeds of several cultivars of wheat (*Triticum aestivum* L. and *Triticum spelta* L.). Such studies demonstrated that these bacterial genera are integral parts of microbiomes of barley and wheat, warranting further studies on their biological and ecological functions. The results of *in planta* assays using barley and wheat in this study provided valuable information of bacterial strains of those genera, including their compatibility with these plants and the corresponding PGP bioactivities. Such information is critical for the further development of these bacterial strains as biofertilisers/bioprotectants. The results of this study also validated the design of these assays, which could be adapted for use on more plants including perennial ryegrass, with adjustments to the number of replicates to account for the inherent genetic variability in this plant.

The *in silico* assays underpin the genetic resource discovery of this study, including the identifications of novel bacterial species (Chapter 2 and 3) and the transcriptomic characterisations of the plant-bacteria interaction (Chapter 4). A complete workflow of *in silico* assays for microbial identification and characterisation has been developed as part of this PhD study, including genome sequencing, assembly, annotation, functional gene screening, comparative genomics and transcriptome analysis. Curated databases, such as reference bacterial genomes, were also established in this study. One highlight of the *in silico* assays of this study is the incorporation of the latest sequencing technologies, such as the NovaSeq platform for short-read sequencing and the ONT MinION platform for long-read sequencing. Data generated by these platforms enabled rapid generation of complete, circular bacterial genomes (Lu et al., 2016; Wick et al., 2017), which served the fundamental role in subsequent characterisations of bacterial members of plant microbiome including this study

(Peng et al., 2016; Vater et al., 2018; Li et al., 2020). Overall, such established *in silico* assays can be used in future characterisations of other bacterial strains isolated from plants, providing reliable and comparable results.

5.3 Future directions and recommendations

5.3.1 The beneficial functions of non-pathogenic relatives of phytopathogens

A major finding of this PhD study is the beneficial bioactivities of the non-pathogenic strains that belong to bacterial genera/species that are commonly described as phytopathogens, such as *Xanthomonas* spp. and *Erwinia* spp. The three xanthomonad strains (GW, SS and SI) reported in this study represented a novel species (Chapter 2). The novel *E. gerundensis* strain (AR) showed bioprotection and root growth promotion activities in preliminary characterisation (Appendix 1, section 1.3) as well as demonstrated transcriptome profiles that may be associated with PGP bioactivities (Chapter 4), neither of which has been identified from the type strain (EM595) of this species (Rezzonico et al., 2016). These bacterial genera/species are routinely detected in plant microbiome studies (Bulgarelli et al., 2015; Chen et al., 2016; Wang et al., 2016; Hamonts et al., 2018), which were based on analysing a variable sequence region of the bacterial 16S rRNA gene. Consequently, the potential presence of these beneficial non-pathogenic strains could be overlooked due to the limited resolution of 16S rRNA gene if no bacterial isolation and characterisation was conducted. For example, Rahman et al. (2018) isolated a *X. translucens* strain from barley seeds. However, since the bacterial identification was based on the 16S rRNA gene sequence, it remains unclear if that is a genuine pathogenic *X. translucens* strain or a possible non-pathogenic and beneficial *Xanthomonas* spp. strain like the one described in this study (Chapter 2). Therefore, in future plant microbiome studies, bacterial isolation should be conducted if possible alongside with 16S rRNA-based profiling, e.g. Tannenbaum et al. (2020). The isolated candidate strains can be preliminary identified using robust methods such as matrix-assisted laser desorption ionization time-of-flight (MALDI-TOF) mass spectrometry (Stets et al., 2013; Ziegler et al., 2015), and then further analysed in details using *in vitro*, *in planta* and *in silico* assays to recover more beneficial strains that may promote the growth of plants, e.g. Li et al. (2020). Furthermore, long-read sequencing technologies should be used for metagenomics and transcriptomics at large read depth to identify genetic markers other than 16S rRNA that provide enhanced taxonomic resolution to benefit future plant microbiome

studies. For example, the high-accuracy method recently developed by Karst et al. (2021) was able to sequence the full length of the bacterial and fungal rRNA using unique molecule identifiers with long-read sequencing technologies, which may provide species-level taxonomic identification.

5.3.2 Strain-specific phenotypes/transcriptome of genetically closely related bacterial strains

Varying phenotypic bioactivities and transcriptome profiles have been consistently identified in this study when comparing genetically closely related strains. Such variations include the bioprotection activities of the three *Xanthomonas* sp. strains (Chapter 2) and the two *Paenibacillus* sp. strains (Chapter 3), as well as the transcriptome profiles of the two *Paenibacillus* sp. strains and the corresponding inoculated barley seedlings (Chapter 3 and 4). While possible explanations were proposed for some variations, e.g. the unique Nrps cluster possessed by the strain with the highest bioprotection activities (Chapter 2), the underlying molecular mechanisms of most variations described above remain to be discovered. Similar strain-specificity associated with PGP bacteria have been reported before. For instance, strain-specific transporter protein profiles that were potentially linked to modulation of root architecture and nutrients transportation of *Populus tremuloides* seedlings were identified in three *P. fluorescens* strains (Shinde et al., 2017). Another study conducted by Weston et al. (2012) identified both strain-specific and strain-independent responses induced by two *P. fluorescens* strains in *Arabidopsis thaliana* that were associated with plant functions including defence, hormone metabolism and general fitness. Drogue et al. (2014) reported strain-specific responses induced by two *Azospirillum* spp. strains in rice, with the endophytic strain causing repressed expression of genes associated with plant defence and stress. Such intriguing results warrant further investigation of closely related strains of the *E. gerundensis* strain AR used in this study as well as more strains isolated from perennial ryegrass and other plant species.

Plants are known to have complex genetic as well as metabolic controls and signalling when recruiting and interacting with PGP bacteria (Carvalho et al., 2016). The bacterial strains used in this study showed different bioactivities, leading to different roles that they play in the perennial ryegrass microbiome as well as different contributions that they make in the overall performance of perennial ryegrass. While bacterial strains were characterised as

individuals in this study, future studies are required to characterised them collectively by inoculating plants with multiple strains in several combinations (Molina-Romero et al., 2017; Razzaghi Komaresofla et al., 2019). With the knowledge of strain-specific bioactivities and co-inoculation of multiple strains, we can tailor the composition of biofertilisers/bioprotectants to take the advantage of different PGP bacterial strains based on the environment of application (such as soil type), which is known to affect the effectiveness of PGP bacteria (Schreiter et al., 2014; Wang et al., 2018).

While this study demonstrated varying phenotypic bioactivities and transcriptome profiles of genetically closely related strains on one cultivar of wheat and barley, it is known that similar variations could also be observed between the interactions of different genotypes of a plant species and a bacterial strain. For instance, the effects of plant genotypes on their interactions with plant pathogens have been extensively examined using genome-wide association studies (GWAS). As reviewed by Bartoli and Roux (2017), such studies used a wide range of genetic lines of a plant species to determine their disease resistance under both laboratory and field conditions, which was then correlated with their genotypes using GWAS to identify genetic markers that are associated with disease resistance. The identified genetic markers could be subsequently used for marker-assisted-selection to improve resistance breeding (Wille et al., 2019). Besides pathogens, a few studies also examined the effect of plant genotypes on other members of the plant microbiome. A GWAS study using field experiments conducted by Horton et al. (2014) showed that the composition of the leaf microbiome of *A. thaliana* was affected by the plant loci associated with defence and plant cell wall integrity. Moreover, they also identified plant loci that may affect the species richness of the leaf microbiome. Inspired by this study, Beilsmith et al. (2019) proposed a workflow for comprehensive examinations of effects of plant genotypes on the plant phyllosphere microbiome using GWAS. Moreover, studies have also demonstrated that plant genotypes can also affect the plant-bacteria interactions. Curtin et al. (2017) evaluated ten genes of *Medicago truncatula* previously identified to be associated with the nodulation of rhizobia in legumes based on GAWS and validated the functions of three genes using gene-disruption platforms. The expression of the *acdS* gene that enables deamination of 1-aminocyclopropane-1-carboxylate of a PGP bacterial strain *P. fluorescens* F113 was found to be higher on maize line FV2 (inbred) and PR37Y15 (hybrid) than that on EP1 (inbred) and teosinte, which is close to ancestral maize (Vacheron et al., 2016). The same strain was later inoculated to 192 ancient and modern wheat genotypes, and was found to have improved interactions with the ancient

wheat varieties (Valente et al., 2020). Such improved interactions included enhanced root colonisation and higher expression of the *phl* genes encoding 2,4-Diacetylphloroglucinol that is associated with bioprotection and plant hormone balance (Almario et al., 2017). Moreover, the PGP strain was only able to improve the growth of certain wheat genotypes (Valente et al., 2020). Similarly, a recent study using 288 ecotypes of *A. thaliana* showed that a third of ecotypes did not respond to the inoculation of a PGP bacterial strain *Bacillus pumilus* TUAT-1, leading to no significant changes in root development when compared to the control (Cotta et al., 2020). Such results clearly demonstrated the importance of plant genotypes in plant-bacteria interactions, which should be further examined in future studies. With such knowledge we can identify genetic markers that are associated with plant-bacteria interactions and select for plant genotypes with enhanced interactions. Moreover, the combination of plant genotypes and PGP bacterial strains can be further tailored to improve the performance of plants.

5.3.3 Implications of novel bacterial species and strains on biosecurity

Novel bacterial species and strains have been one of the major outcomes of this PhD study. The three xanthomonads (GW, SS and SI) themselves described in this thesis represent a novel *Xanthomonas* species (Li et al., 2020). While the two *Paenibacillus* sp. strains (S02 and S25) isolated in this study were described to be closely related to *P. polymyxa*, the phylogenetic analyses showed they were clustered with 16 other *Paenibacillus* sp. strains and formed a separated clade when compared with the type strain of this species, suggesting the presence of a novel species and possible future taxonomic subdivision of the species *P. polymyxa* (Chapter 3). Since they represent novel species, new names will be assigned for taxonomic purposes. This poses new challenges to current biosecurity regulations, which are based on the name (taxonomy) of pathogens. The pathogens are isolated, identified, named and characterised, providing necessary information for biosecurity regulations. However, such workflow may overlook the emerging new pathogens that have not been described. Moreover, this workflow may limit the applications of beneficial strains of novel species which belong to genera that also contain known pathogens, such as *Xanthomonas* and *Paenibacillus*. Such limitations of current name-based biosecurity regulations have been identified for fungi (McTaggart et al., 2016). This PhD study demonstrated that these limitations also exist for bacteria. With the rapid advancement of sequencing technologies, biosecurity regulations should shift from the taxonomy of an organism to the key genes that

defined the pathogenicity of an organism. Moreover, this PhD study emphasised the importance of identifying and characterising endemic strains of novel species for testing, which will benefit the local applications of beneficial microorganisms under current biosecurity regulations.

5.3.4 Further development of assays

Apart from the wider implications of this study described above, future studies based on further development of assays should be conducted to enhance our understanding of the outcomes of this PhD study. For example, while the three novel *Xanthomonas* sp. strains (Chapter 2) and the two novel *Paenibacillus* sp. strains (Chapter 3) have shown strong bioprotection activities in *in vitro* and *in planta* assays, more data should be collected to further develop those strains as bioprotectants, including *in planta* assays using more plant species and field experiments. Moreover, all strains used in this study are capable of synthesising secondary metabolites, which are believed to be associated with bioactivities including bioprotection, root colonisation and plant stress alleviation (Chapter 2, 3 and 4). The role of these bacterial secondary metabolites should be examined and confirmed, such as purifying, identifying and characterising the bioactive compounds using metabolomics techniques (Zachow et al., 2015). In addition, the long-read nanopore sequencing technologies (ONT MinION) played a critical role in genomic characterisation of bacterial strains in this study. Recent studies have shown that the nanopore sequencing is capable of identifying full-length isoforms of plant transcripts and preserving valuable information about RNA modifications, neither of which could be done using the short-read RNA-Seq approaches (Zhao et al., 2019; Zhang et al., 2020). Therefore, future studies should look to utilise nanopore sequencing to characterise plant-bacteria interactions, providing novel insights that may explain the strain-specific responses of plant transcriptome profiles. Finally, the *in planta* assays designed in this study should be refined to be applied on more plant species including perennial ryegrass in future, leading to enhanced understanding of PGP bioactivities of the candidate bacterial strains. To overcome the effects of genetic variations of perennial ryegrass plants, clonal replicates propagated vegetatively from a single plant should be used in small scale assays (Yates et al., 2019). For large scale assays, such as field experiments with multiple cultivars of perennial ryegrass, an increased number of replicates should be used for each treatment and control to reduce the effects of genetic variations.

5.4 Concluding remarks

The plant microbiome is an excellent source from which potential PGP bacterial strains can be isolated. These PGP strains represent an emerging method to improve the health and productivity of agricultural crops and pasture grasses, leading to sustainable agriculture. Establishing a thorough understanding of PGP bioactivities of candidate strains underpins their future applications in agriculture. Such thorough understandings were approached in this PhD study by designing and conducting a series of *in vitro, in planta* and *in silico* assays, leading to comprehensive characterisations of six novel PGP bacterial strains isolated from the perennial ryegrass microbiome. The promising traits of theses strains, such as non-pathogenicity, PGP bioactivities and beneficial interactions with plants, laid a solid foundation to their further development as commercial biofertilisers and bioprotectants. The *Xanthomonas* sp. strain GW is a promising candidate to be developed as bioprotectants due to its strong and broad spectrum bioactivity. The two *Paenibacillus* sp. strains S02 and S25 are key candidates to be developed as all-arounder biofertilisers/bioprotectants due to their comprehensive PGP bioactivities. Overall, this PhD study further demonstrated the value of PGP bacteria associated with plant microbiomes in agriculture. The methods and resources that were developed in this PhD study form a robust pipeline for characterising more candidate strains isolated from plants, providing enhanced understandings of beneficial bioactivities. With such knowledge, there will continue to be more novel PGP bacteria isolated, characterised and eventually applied to improve the performance of agricultural crops and pasture grasses.

5.5 References

Almario, J., Bruto, M., Vacheron, J., Prigent-Combaret, C., Moënne-Loccoz, Y., and Muller, D. (2017). Distribution of 2,4-Diacetylphloroglucinol biosynthetic genes among the *Pseudomonas* spp. reveals unexpected polyphyletism. *Frontiers in Microbiology* 8**,** 1218.

Bartoli, C., and Roux, F. (2017). Genome-wide association studies in plant pathosystems: toward an ecological genomics approach. *Frontiers in Plant Science* 8**,** 763.

Beilsmith, K., Thoen, M.P.M., Brachi, B., Gloss, A.D., Khan, M.H., and Bergelson, J. (2019). Genome-wide association studies on the phyllosphere microbiome: embracing complexity in host–microbe interactions. *The Plant Journal* 97**,** 164-181.

Berg, G. (2009). Plant–microbe interactions promoting plant growth and health: perspectives for controlled use of microorganisms in agriculture. *Applied Microbiology and Biotechnology* 84**,** 11-18.

Brazauskas, G., Lenk, I., Pedersen, M.G., Studer, B., and Lübberstedt, T. (2011). Genetic variation, population structure, and linkage disequilibrium in European elite germplasm of perennial ryegrass. *Plant Science* 181**,** 412-420.

Bulgarelli, D., Garrido-Oter, R., Munch, P.C., Weiman, A., Droge, J., Pan, Y., Mchardy, A.C., and Schulze-Lefert, P. (2015). Structure and function of the bacterial root microbiota in wild and domesticated barley. *Cell Host Microbe* 17**,** 392-403.

Carvalho, T., Ballesteros, H., Thiebaut, F., Ferreira, P., and Hemerly, A. (2016). Nice to meet you: genetic, epigenetic and metabolic controls of plant perception of beneficial associative and endophytic diazotrophic bacteria in non-leguminous plants. *Plant Molecular Biology* 90**,** 561-574.

Chen, L., Brookes, P.C., Xu, J., Zhang, J., Zhang, C., Zhou, X., and Luo, Y. (2016). Structural and functional differentiation of the root-associated bacterial microbiomes of perennial ryegrass. *Soil Biology and Biochemistry* 98**,** 1-10.

Compant, S., Samad, A., Faist, H., and Sessitsch, A. (2019). A review on the plant microbiome: ecology, functions, and emerging trends in microbial application. *Journal of Advanced Research* 19**,** 29-37.

Cotta, M.S., Do Amaral, F.P., Cruz, L.M., Wassem, R., De Oliveira Pedrosa, F., Yokoyama, T., and Stacey, G. (2020). Genome-wide association studies reveal important candidate genes for the *Bacillus pumilus* TUAT-1-*Arabidopsis thaliana* interaction. *bioRxiv***,** 2020.2005.2026.117002.

Curtin, S.J., Tiffin, P., Guhlin, J., Trujillo, D.I., Burghardt, L.T., Atkins, P., Baltes, N.J., Denny, R., Voytas, D.F., Stupar, R.M., and Young, N.D. (2017). Validating genome-wide association candidates controlling quantitative variation in nodulation. *Plant Physiology* 173**,** 921-931.

Drogue, B., Sanguin, H., Chamam, A., Mozar, M., Llauro, C., Panaud, O., Prigent-Combaret, C., Picault, N., and Wisniewski-Dyé, F. (2014). Plant root transcriptome profiling reveals a strain-dependent response during *Azospirillum*-rice cooperation. *Frontiers in Plant Science* 5**,** 607.

Garita-Cambronero, J., Palacio-Bielsa, A., Lopez, M.M., and Cubero, J. (2017). Pan-genomic analysis permits differentiation of virulent and non-virulent strains of *Xanthomonas arboricola* that cohabit *Prunus* spp. and elucidate bacterial virulence factors. *Frontiers in Microbiology* 8**,** 573.

Hamonts, K., Trivedi, P., Garg, A., Janitz, C., Grinyer, J., Holford, P., Botha, F.C., Anderson, I.C., and Singh, B.K. (2018). Field study reveals core plant microbiota and relative importance of their drivers. *Environmental Microbiology* 20**,** 124-140.

Hone, H., Mann, R., Yang, G., Kaur, J., Tannenbaum, I., Li, T., Spangenberg, G., and Sawbridge, T. (2021). Profiling, isolation and characterisation of beneficial microbes from the seed microbiomes of drought tolerant wheat. *Scientific Reports* 11.

Horton, M.W., Bodenhausen, N., Beilsmith, K., Meng, D., Muegge, B.D., Subramanian, S., Vetter, M.M., Vilhjálmsson, B.J., Nordborg, M., Gordon, J.I., and Bergelson, J. (2014). Genome-wide association study of *Arabidopsis thaliana* leaf microbial community. *Nature Communications* 5**,** 5320.

Jiménez, J.A., Novinscak, A., and Filion, M. (2020). Inoculation with the plant-growth-promoting rhizobacterium *Pseudomonas fluorescens* LBUM677 impacts the rhizosphere microbiome of three oilseed crops. *Frontiers in Microbiology* 11**,** 2534.

Karst, S.M., Ziels, R.M., Kirkegaard, R.H., Sørensen, E.A., Mcdonald, D., Zhu, Q., Knight, R., and Albertsen, M. (2021). High-accuracy long-read amplicon sequences using unique molecular identifiers with Nanopore or PacBio sequencing. *Nature Methods* 18**,** 165-169.

Kovi, M.R., Fjellheim, S., Sandve, S.R., Larsen, A., Rudi, H., Asp, T., Kent, M.P., and Rognli, O.A. (2015). Population structure, genetic variation, and linkage disequilibrium in perennial ryegrass populations divergently selected for freezing tolerance. *Frontiers in Plant Science* 6**,** 929.

Kuźniar, A., Włodarczyk, K., Grządziel, J., Woźniak, M., Furtak, K., Gałązka, A., Dziadczyk, E., Skórzyńska-Polit, E., and Wolińska, A. (2020). New insight into the composition of wheat seed microbiota. *International Journal of Molecular Sciences* 21**,** 4634.

Li, T., Mann, R., Sawbridge, T., Kaur, J., Auer, D., and Spangenberg, G. (2020). Novel *Xanthomonas* species from the perennial ryegrass seed microbiome – Assessing the bioprotection activity of non-pathogenic relatives of pathogens. *Frontiers in Microbiology* 11**,** 1991.

Lu, H., Giordano, F., and Ning, Z. (2016). Oxford Nanopore MinION sequencing and genome assembly. *Genomics Proteomics Bioinformatics* 14**,** 265-279.

Martins, L., Fernandes, C., Blom, J., Dia, N.C., Pothier, J.F., and Tavares, F. (2020). *Xanthomonas euroxanthea* sp. nov., a new xanthomonad species including pathogenic and non-pathogenic strains of walnut. *International Journal of Systematic and Evolutionary Microbiology* 70**,** 6024–6031.

Mctaggart, A.R., Van Der Nest, M.A., Steenkamp, E.T., Roux, J., Slippers, B., Shuey, L.S., Wingfield, M.J., and Drenth, A. (2016). Fungal genomics challenges the dogma of name-based biosecurity. *PLOS Pathogens* 12**,** e1005475.

Molina-Romero, D., Baez, A., Quintero-Hernández, V., Castañeda-Lucio, M., Fuentes-Ramírez, L.E., Bustillos-Cristales, M.D.R., Rodríguez-Andrade, O., Morales-García, Y.E., Munive, A., and Muñoz-Rojas, J. (2017). Compatible bacterial mixture, tolerant to desiccation, improves maize plant growth. *PLOS One* 12**,** e0187913.

Pembleton, L., Shinozuka, H., Wang, J., Spangenberg, G., Forster, J., and Cogan, N. (2015). Design of an F1 hybrid breeding strategy for ryegrasses based on selection of self-incompatibility locus-specific alleles. *Frontiers in Plant Science* 6**,** 764.

Peng, Z., Hu, Y., Xie, J., Potnis, N., Akhunova, A., Jones, J., Liu, Z., White, F.F., and Liu, S. (2016). Long read and single molecule DNA sequencing simplifies genome assembly and TAL effector gene analysis of *Xanthomonas translucens*. *BMC Genomics* 17**,** 21.

Rahman, M.D.M., Flory, E., Koyro, H.-W., Abideen, Z., Schikora, A., Suarez, C., Schnell, S., and Cardinale, M. (2018). Consistent associations with beneficial bacteria in the seed endosphere of barley (*Hordeum vulgare* L.). *Systematic and Applied Microbiology* 41**,** 386-398.

Razzaghi Komaresofla, B., Alikhani, H.A., Etesami, H., and Khoshkholgh-Sima, N.A. (2019). Improved growth and salinity tolerance of the halophyte *Salicornia* sp. by co–inoculation with endophytic and rhizosphere bacteria. *Applied Soil Ecology* 138**,** 160-170.

Rezzonico, F., Smits, T.H.M., Born, Y., Blom, J., Frey, J.E., Goesmann, A., Cleenwerck, I., De Vos, P., Bonaterra, A., Duffy, B., and Montesinos, E. (2016). *Erwinia gerundensis* sp. nov., a cosmopolitan epiphyte originally isolated from pome fruit trees. *International Journal of Systematic and Evolutionary Microbiology* 66**,** 1583-1592.

Schreiter, S., Ding, G.-C., Grosch, R., Kropf, S., Antweiler, K., and Smalla, K. (2014). Soil type-dependent effects of a potential biocontrol inoculant on indigenous bacterial communities in the rhizosphere of field-grown lettuce. *FEMS Microbiology Ecology* 90**,** 718-730.

Shinde, S., Cumming, J.R., Collart, F.R., Noirot, P.H., and Larsen, P.E. (2017). *Pseudomonas fluorescens* transportome is linked to strain-specific plant growth promotion in aspen seedlings under nutrient stress. *Frontiers in Plant Science* 8**,** 348.

Stets, M.I., Pinto, A.S., Huergo, L.F., De Souza, E.M., Guimarães, V.F., Alves, A.C., Steffens, M.B.R., Monteiro, R.A., Pedrosa, F.D.O., and Cruz, L.M. (2013). Rapid identification of bacterial isolates from wheat roots by high resolution whole cell MALDI-TOF MS analysis. *Journal of Biotechnology* 165**,** 167-174.

Tannenbaum, I., Kaur, J., Mann, R., Sawbridge, T., Rodoni, B., and Spangenberg, G. (2020). Profiling the *Lolium perenne* microbiome: from seed to seed. *Phytobiomes Journal* 4**,** 281-289.

Vacheron, J., Combes-Meynet, E., Walker, V., Gouesnard, B., Muller, D., Moënne-Loccoz, Y., and Prigent-Combaret, C. (2016). Expression on roots and contribution to maize phytostimulation of 1-aminocyclopropane-1-decarboxylate deaminase gene *acdS* in *Pseudomonas fluorescens* F113. *Plant and Soil* 407**,** 187-202.

Valente, J., Gerin, F., Le Gouis, J., Moënne-Loccoz, Y., and Prigent–Combaret, C. (2020). Ancient wheat varieties have a higher ability to interact with plant growth-promoting rhizobacteria. *Plant, Cell & Environment* 43**,** 246-260.

Vandenkoornhuyse, P., Quaiser, A., Duhamel, M., Le Van, A., and Dufresne, A. (2015). The importance of the microbiome of the plant holobiont. *New Phytologist* 206**,** 1196-1206.

Vater, J., Herfort, S., Doellinger, J., Weydmann, M., Borriss, R., and Lasch, P. (2018). Genome mining of the lipopeptide biosynthesis of *Paenibacillus polymyxa* E681 in combination with mass spectrometry: discovery of the lipoheptapeptide paenilipoheptin. *Chembiochem* 19**,** 744-753.

Wang, J., Li, Q., Xu, S., Zhao, W., Lei, Y., Song, C., and Huang, Z. (2018). Traits-based integration of multi-species inoculants facilitates shifts of indigenous soil bacterial community. *Frontiers in Microbiology* 9**,** 1692.

Wang, W., Zhai, Y., Cao, L., Tan, H., and Zhang, R. (2016). Endophytic bacterial and fungal microbiota in sprouts, roots and stems of rice (*Oryza sativa* L.). *Microbiological Research* 188-189**,** 1-8.

Weston, D.J., Pelletier, D.A., Morrell-Falvey, J.L., Tschaplinski, T.J., Jawdy, S.S., Lu, T.-Y., Allen, S.M., Melton, S.J., Martin, M.Z., Schadt, C.W., Karve, A.A., Chen, J.-G., Yang, X., Doktycz, M.J., and Tuskan, G.A. (2012). *Pseudomonas fluorescens* induces strain-dependent and strain-independent host plant responses in defense networks, primary metabolism, photosynthesis, and fitness. *Molecular Plant-Microbe Interactions* 25**,** 765-778.

Wick, R.R., Judd, L.M., Gorrie, C.L., and Holt, K.E. (2017). Unicycler: resolving bacterial genome assemblies from short and long sequencing reads. *PLOS Computational Biology* 13**,** e1005595.

Wille, L., Messmer, M.M., Studer, B., and Hohmann, P. (2019). Insights to plant–microbe interactions provide opportunities to improve resistance breeding against root diseases in grain legumes. *Plant, Cell & Environment* 42**,** 20-40.

Yang, L., Danzberger, J., Schöler, A., Schröder, P., Schloter, M., and Radl, V. (2017). Dominant groups of potentially active bacteria shared by barley seeds become less abundant in root associated microbiome. *Frontiers in Plant Science* 8**,** 1005.

Yang, L., Schröder, P., Vestergaard, G., Schloter, M., and Radl, V. (2020). Response of barley plants to drought might be associated with the recruiting of soil-borne endophytes. *Microorganisms* 8**,** 1414.

Yates, S., Jaškūnė, K., Liebisch, F., Nagelmüller, S., Kirchgessner, N., Kölliker, R., Walter, A., Brazauskas, G., and Studer, B. (2019). Phenotyping a dynamic trait: leaf growth of perennial ryegrass under water limiting conditions. *Frontiers in Plant Science* 10**,** 344.

Yu, X., Bai, G., Liu, S., Luo, N., Wang, Y., Richmond, D.S., Pijut, P.M., Jackson, S.A., Yu, J., and Jiang, Y. (2013). Association of candidate genes with drought tolerance traits in diverse perennial ryegrass accessions. *Journal of Experimental Botany* 64**,** 1537-1551.

Zachow, C., Jahanshah, G., De Bruijn, I., Song, C., Ianni, F., Pataj, Z., Gerhardt, H., Pianet, I., Lammerhofer, M., Berg, G., Gross, H., and Raaijmakers, J.M. (2015). The novel lipopeptide poaeamide of the endophyte *Pseudomonas poae* RE*1-1-14 is involved in pathogen suppression and root colonization. *Molecular Plant-Microbe Interactions* 28**,** 800-810.

Zhang, S., Li, R., Zhang, L., Chen, S., Xie, M., Yang, L., Xia, Y., Foyer, C.H., Zhao, Z., and Lam, H.-M. (2020). New insights into *Arabidopsis* transcriptome complexity revealed by direct sequencing of native RNAs. *Nucleic Acids Research* 48**,** 7700-7711.

Zhao, L., Zhang, H., Kohnen, M.V., Prasad, K.V.S.K., Gu, L., and Reddy, A.S.N. (2019). Analysis of transcriptome and epitranscriptome in plants using pacbio iso-seq and nanopore-based direct RNA sequencing. *Frontiers in Genetics* 10**,** 253.

Ziegler, D., Pothier, J.F., Ardley, J., Fossou, R.K., Pflüger, V., De Meyer, S., Vogel, G., Tonolla, M., Howieson, J., Reeve, W., and Perret, X. (2015). Ribosomal protein biomarkers provide root nodule bacterial identification by MALDI-TOF MS. *Applied Microbiology and Biotechnology* 99**,** 5547-5562.

Appendix 1

Patents

1.1 Appendix preface

Perennial ryegrass (*Lolium perenne*) is one of the most important pasture grasses in Australia and New Zealand, as it is the primary feedbase for the dairy and livestock industries. The grass forms a symbiotic relationship with an endophytic fungus, *Epichloë festucae* subsp. *lolii*, that provides tolerance against abiotic and biotic stresses, however it is unclear what other beneficial microbes have formed mutualistic relationships with perennial ryegrass. These patents described four novel plant associated bacterial species (*Xanthomonas* sp. – 3 strains; *Erwinia gerundensis* – 1 strain; *Pseudomonas poae* – 1 strain; *Stenotrophomonas rhizophila* – 1 strain) isolated from perennial ryegrass plants that have bioprotectant and/or biofertiliser activities. The three *Xanthomonas* sp. strains (GW, SS and SI) described in patent 1 have been described in this thesis as a published journal article (Chapter 2). The *E. gerundensis* strain (AR), which was used in the dual RNA-seq analyses of early stage plant-bacteria interactions described in chapter 4, was described in patent 2, providing supporting evidences of the beneficial activities of the strain (e.g. *in planta* root growth promotion). Patent 3 and 4 described a *P. poae* strain (EY) and a *S. rhizophila* strain (JB), which were other findings of this PhD study. They were characterised using the *in vitro*, *in planta* and *in silico* assays described in this thesis. The two strains were both bioprotectants (*in vitro*) and biofertilisers (*in vitro* and *in planta*). In addition, they were able to produce various secondary metabolites, including both known antimicrobial compounds and novel compounds. Besides, the patents presented in this appendix also provided additional evidence to support the application of these strains, e.g. the optimal concentration of inoculum for *in planta* inoculations. Overall, these patents further demonstrated the beneficial values of PGP bacteria associated with perennial ryegrass and their potential applications in sustainable agriculture.

This appendix is presented in patent format.

1.2 Patent 1 – *Xanthomonas* sp.

1.2.1 Publication details

Title: Novel bacterial strain (2)

Details: https://patentscope.wipo.int/search/en/detail.jsf?docId=WO2021012001

Stage of publication: Full patent (publicly available)

Authors: Li, Tongda; Tannenbaum, Ian Ross; Kaur, Jatinder; Krill, Christian; Sawbridge, Timothy Ivor; Mann, Ross C.; Spangenberg, German Carlos

1.2.2 Statement of contribution of joint authorship

TL performed all work relating to genomics of the strains, *in vitro* bioprotectant assays, and design of strain specific PCR primers. TL generated all figures associated with the above works (1, 3–12) and drafted the majority of the experimental section of the patent associated with these works. TL conducted all statistical and data analysis of these works. RM, GS, TS, JK and TL all conceptualised the patent and assisted in editing the patent. Experimental and analysis work conducted by TL is highlighted in yellow.

Appendix 1

AUSTRALIA

Patents Act 1990

PROVISIONAL SPECIFICATION

Applicants: Agriculture Victoria Services Pty Ltd
Dairy Australia Limited
Geoffrey Gardiner Dairy Foundation Limited

Title: NOVEL BACTERIAL STRAIN (2)

The invention is described in the following statement:

NOVEL BACTERIAL STRAIN (2)

Field of the Invention

The present invention relates to novel plant microbiome strains, plants infected with such strains and related methods.

Background of the Invention

Microbes represent an invaluable source of novel genes and compounds that have the potential to be utilised in a range of industrial sectors. Scientific literature gives numerous accounts of microbes being the primary source of antibiotics, immune-suppressants, anticancer agents and cholesterol-lowering drugs, in addition to their use in environmental decontamination and in the production of food and cosmetics.

A relatively unexplored group of microbes known as endophytes, which reside e.g. in the tissues of living plants, offer a particularly diverse source of novel compounds and genes that may provide important benefits to society, and in particular, agriculture.

Endophytes may be fungal or bacterial. Endophytes often form mutualistic relationships with their hosts, with the endophyte conferring increased fitness to the host, often through the production of defence compounds. At the same time, the host plant offers the benefits of a protected environment and nutriment to the endophyte.

Important forage grasses perennial ryegrass (*Lolium perenne*) are commonly found in association with fungal and bacterial endophytes. However, there remains a general lack of information and knowledge of the endophytes of these grasses as well as of methods for the identification and characterisation of novel endophytes and their deployment in plant improvement programs.

Knowledge of the endophytes of perennial ryegrass may allow certain beneficial traits to be exploited in enhanced pastures, or lead to other agricultural advances, e.g. to the benefit of sustainable agriculture and the environment.

There exists a need to overcome, or at least alleviate, one or more of the difficulties or deficiencies associated with the prior art.

Summary of the Invention

In one aspect, the present invention provides a substantially purified or isolated endophyte strain isolated from a plant of the Poaceae family, wherein said endophyte is a strain of *Xanthomonas* sp. which provides bioprotection and/or biofertilizer phenotypes to plants into which it is inoculated. In a preferred embodiment, the *Xanthomonas* sp. strain may be a strain selected from the group consisting of GW, SS and SI as described herein and as deposited with The National Measurement Institute on 17th May 2019 with accession numbers V19/009902, V19/009905 and V19/009909, respectively.

As used herein the term “endophyte” is meant a bacterial or fungal strain that is closely associated with a plant. By “associated with” in this context is meant that the bacteria or fungus lives on, in or in close proximity to a plant. For example, it may be endophytic, for example living within the internal tissues of a plant, or epiphytic, for example growing externally on a plant.

As used herein the term “substantially purified” is meant that an endophyte is free of other organisms. The term includes, for example, an endophyte in axenic culture. Preferably, the endophyte is at least approximately 90 % pure, more preferably at least approximately 95 % pure, even more preferably at least approximately 98 % pure, even more preferably at least approximately 99 % pure.

As used herein the term ‘isolated’ means that an endophyte is removed from its original environment (e.g. the natural environment if it is naturally occurring). For example, a naturally occurring endophyte present in a living plant is not isolated, but the same endophyte separated from some or all of the coexisting materials in the natural system, is isolated.

As used herein the term “bioprotection and/or biofertilizer” means that the endophyte possesses genetic and/or metabolic characteristics that result in a beneficial phenotype in a plant harbouring, or otherwise associated with, the endophyte. Such beneficial properties include improved resistance to pests and/or diseases, improved tolerance to water and/or nutrient stress, enhanced biotic stress tolerance, enhanced drought tolerance, enhanced water use efficiency, reduced toxicity and enhanced vigour in the plant with which the endophyte is associated, relative to an organism not harbouring the endophyte or harbouring a control endophyte such as standard toxic (ST) endophyte.

The pests and/or diseases may include, but not limited to, fungal and bacterial pathogens. In a particularly preferred embodiment, the endophyte may result in the production of the bioprotectant compound in the organism with which it is associated.

As used herein, a bioprotectant compound is meant as a compound that provides bioprotection to the plant or aids the defence of the plant with which it is associated against pests and/or diseases, such as fungal and/or bacterial pathogens. A bioprotectant compound may also be known as a 'biocidal compound'. In a particularly preferred embodiment, the endophyte produces a bioprotectant compound and provides bioprotection to the organism against fungal and/or bacterial pathogens. The terms bioprotectant, bioprotective and bioprotection (or any other variations) may be used interchangeably herein.

In a particularly preferred embodiment the bioprotectant compound is selected from the group consisting of siderophore xanthoferrin, and/or xanthomonadin or a derivative, isomer and/or salt thereof.

The endophyte may be suitable as a biofertilizer to improve the availability of nutrients to the plant with which the endophyte is associated, including but not limited to improved tolerance to nutrient stress.

The nutrient stress may be lack of or low amounts of a nutrient such as phosphate and/or nitrogen. The endophyte is capable of growing in conditions such as low nitrogen and/or low phosphate and enable these nutrients to be available to the plant with which the endophyte is associated.

The endophyte may result in the production of organic acids and/or the solubilisation of phosphate in the organism with which it is associated and/or provide a source of phosphate to the plant.

Alternatively, or in addition, the endophyte is capable of nitrogen fixation. Thus, an endophyte is capable of nitrogen fixation, the plant in which the endophyte is associated is capable of growing in low nitrogen conditions and/or provide a source of Nitrogen to the plant.

Alternatively, or in addition, the endophyte is capable of nitrogen fixation. Thus, if an endophyte is capable of nitrogen fixation, the organism in which the endophyte is associated is capable of growing in low nitrogen conditions.

As used herein the term "plant of the Poaceae family" is a grass species, particularly a pasture grass such as ryegrass (*Lolium*) or fescue (*Festuca*), more particularly perennial ryegrass (*Lolium perenne* L.) or tall fescue (*Festuca arundinaceum*, otherwise known as *Lolium arundinaceum*).

In another aspect, the present invention provides a plant or part thereof infected with an endophyte as hereinbefore described. In preferred embodiments, the plant or part thereof infected with the endophyte may produce a bioprotectant compound. Preferably, the bioprotectant compound is selected from siderophore xanthoferrin and xanthomonadin.

Also in preferred embodiments, the plant or part thereof includes an endophyte-free host plant or part thereof stably infected with said endophyte.

The plant inoculated with the endophyte may be a grass or non-grass plant suitable for agriculture, specifically a forage, turf, or bioenergy grass, or a grain crop or industrial crop.

Preferably, the plant is a grass species plant, specifically a forage, turf, bioenergy, grain crop or industrial crop grass.

The forage, turf or bioenergy grass may be those belonging to the *Brachiaria-Urochloa* species complex (panic grasses), including *Brachiaria brizantha, Brachiaria decumbens, Brachiaria humidicola, Brachiaria stolonifera, Brachiaria ruziziensis, B. dictyoneura, Urochloa brizantha, Urochloa decumbens, Urochloa humidicola*, *Urochloa mosambicensis* as well as interspecific and intraspecific hybrids of *Brachiaria-Urochloa* species complex such as interspecific hybrids between *Brachiaria ruziziensis* x *Brachiaria brizantha*, *Brachiaria ruziziensis* x *Brachiaria decumbens*, [*Brachiaria ruziziensis* x *Brachiaria decumbens*] x *Brachiaria brizantha*, [*Brachiaria ruziziensis* x *Brachiaria brizantha*] x *Brachiaria decumbens*.

The forage, turf or bioenergy grass may also be those belonging to the genera *Lolium* and *Festuca*, including *L. perenne* (perennial ryegrass) and *L. arundinaceum* (tall fescue) and *L. multiflorum* (Italian ryegrass).

The grain crop may be a non-grass species, for example, any of soybeans, cotton and grain legumes, such as lentils, field peas, fava beans, lupins and chickpeas, as well as oilseed crops, such as canola.

Thus, the grain crop or industrial crop species may selected from the group consisting of wheat, barley, oats, chickpeas, triticale, fava beans, lupins, field peas, canola, cereal rye, vetch, lentils, millet/panicum, safflower, linseed, sorghum, sunflower, maize, canola, mungbeans, soybeans, and cotton.

The grain crop or industrial crop grass may be those belonging to the genus *Triticum*, including *T. aestivum* (wheat), those belonging to the genus *Hordeum*, including *H. vulgare* (barley), those belonging to the genus *Zea*, including *Z. mays* (maize or corn), those belonging to the genus *Oryza*, including *O. sativa* (rice), those belonging to the genus *Saccharum* including *S. officinarum* (sugarcane), those belonging to the genus *Sorghum* including *S. bicolor* (sorghum), those belonging to the genus *Panicum*, including *P. virgatum* (switchgrass), and those belonging to the genera *Miscanthus*, *Paspalum*, *Pennisetum*, *Poa*, *Eragrostis* and *Agrostis*.

A plant or part thereof may be infected by a method selected from the group consisting of inoculation, breeding, crossing, hybridisation, transduction, transfection, transformation and/or gene targeting and combinations thereof.

Without wishing to be bound by theory, it is believed that the endophyte of the present invention may be transferred through seed from one plant generation to the next. The endophyte may then spread or locate to other tissues as the plant grows, i.e. to roots. Alternatively, or in addition, the endophyte may be recruited to the plant root, e.g. from soil, and spread or locate to other tissues.

Thus, in a further aspect, the present invention provides a plant, plant seed or other plant part derived from a plant or part thereof as hereinbefore described. In preferred embodiments, the plant, plant seed or other plant part may produce a bioprotectant compound. Preferably, the bioprotectant compound is selected from siderophore xanthoferrin and xanthomonadin or derivative, isomer and/or salt thereof.

In another aspect, the present invention provides the use of an endophyte as hereinbefore described to produce a plant or part thereof stably infected with said endophyte. The present invention also provides the use of an endophyte as hereinbefore described to produce a plant or part thereof as hereinbefore described.

In another aspect, the present invention provides a bioprotectant compound produced by an endophyte as hereinbefore described, or a derivative, isomer and/or a salt thereof. Preferably, the bioprotectant compound is selected from siderophore xanthoferrin and xanthomonadin.

The bioprotectant compound may be produced by the endophyte when associated with a plant, e.g. a plant of the Poaceae family as described above. Thus, in another aspect, the present invention provides a method for producing a bioprotectant compound, said method including infecting a plant with an endophyte as hereinbefore described and cultivating the plant under conditions suitable to produce the bioprotectant compound. The endophyte-infected plant or part thereof may be cultivated by known techniques. The person skilled in the art may readily determine appropriate conditions depending on the plant or part thereof to be cultivated. Preferably, the bioprotectant compound is selected from siderophore xanthoferrin and xanthomonadin or derivative, isomer and/or salt thereof.

The bioprotectant compound may also be produced by the endophyte when it is not associated with a plant. Thus, in yet another aspect, the present invention provides a method for producing a bioprotectant compound, said method including culturing an endophyte as hereinbefore described, under conditions suitable to produce the bioprotectant compound. Preferably, the bioprotectant compound is selected from siderophore xanthoferrin and xanthomonadin or derivative, isomer and/or salt thereof.

The conditions suitable to produce the bioprotectant compound may include a culture medium including a source of carbohydrates. The source of carbohydrates may be a starch/sugar-based agar or broth such as potato dextrose agar, potato dextrose broth or half potato dextrose agar or a cereal-based agar or broth such as oatmeal agar or oatmeal broth. Other sources of carbohydrates may include endophyte agar, Murashige and Skoog with 20 % sucrose, half V8 juice/half PDA, water agar and yeast malt extract agar. The endophyte may be cultured under aerobic or anaerobic conditions and may be cultured in a bioreactor. Preferably, the bioprotectant compound is selected from siderophore xanthoferrin and xanthomonadin.

In a preferred embodiment of this aspect of the invention, the method may include the further step of isolating the bioprotectant compound from the plant or culture medium. Preferably, the bioprotectant compound is selected from siderophore xanthoferrin and xanthomonadin or derivative, isomer and/or salt thereof.

The endophyte-infected plant or part thereof may be cultivated by known techniques. The person skilled in the art may readily determine appropriate conditions depending on the plant or part thereof to be cultivated.

In another aspect, the present invention provides a method of increasing phosphate use efficiency or increasing phosphate solubilisation by a plant, said method including infecting a plant with an endophyte as hereinbefore described, and cultivating the plant.

In yet another aspect, the present invention provides a method of reducing phosphate levels in soil, said method including infecting a plant with an endophyte as hereinbefore described, and cultivating the plant in the soil.

In yet a further aspect, the present invention provides a method of increasing nitrogen use efficiency or increasing nitrogen availability to a plant, said method including infecting a plant with an endophyte as hereinbefore described, and cultivating the plant.

In yet another aspect, the present invention provides a method of reducing nitrogen levels in soil, said method including infecting a plant with an endophyte as hereinbefore described, and cultivating the plant in the soil.

The production of a bioprotectant compound has particular utility in agricultural plant species, in particular, forage, turf, or bioenergy grass species, or grain crop species or industrial crop species. These plants may be cultivated across large areas of e.g. soil where the properties and biological processes of the endophyte as hereinbefore described and/or bioprotectant compound produced by

the endophyte may be exploited at scale. Preferably, the bioprotectant compound is selected from siderophore xanthoferrin and xanthomonadin or derivative, isomer and/or salt thereof.

The part thereof of the plant may be, for example, a seed.

In preferred embodiments, the plant is cultivated in the presence of soil phosphate and/or nitrogen, alternatively or in addition to applied phosphate and/or nitrogen. The applied phosphate and/or applied nitrogen may be by way of, for example, fertiliser. Thus, preferably, the plant is cultivated in soil.

In preferred embodiments, the endophyte may be a *Xanthomonas* sp. strain selected from the group consisting of GW, SS and SI as described herein and as deposited with The National Measurement Institute on 17th May 2019 with accession numbers V19/009902, V19/009905 and V19/009909, respectively.

Preferably, the plant is a forage, turf, bioenergy grass species or, grain crop or industrial crop species, as hereinbefore described.

The part thereof of the plant may be, for example, a seed.

In preferred embodiments, the plant is cultivated in the presence of soil phosphate and/or applied phosphate. The applied phosphate may be by way of, for example, fertiliser. Thus, preferably, the plant is cultivated in soil.

Alternatively, or in addition, the plant is cultivated in the presence of soil nitrogen and/or applied nitrogen. The applied nitrogen may be by way of, for example, fertiliser. Thus, preferably, the plant is cultivated in soil.

The present invention will now be more fully described with reference to the accompanying Examples and drawings. It should be understood, however, that the description following is illustrative only and should not be taken in any way as a restriction on the generality of the invention described above.

Brief Description of the Drawings/Figures

Figure 1 - 16S Amplicon sequence of novel bacterial strain GW.

Figure 2 - Phylogenetic analysis of the gyrase B gene from the *Xanthomonas* sp. novel bacterial strains GW, SS and SI, in comparison to 11 related *Xanthomonas* species and the outgroups *Lysobacter enzymogenes* and *Pseudoxanthomonas suwonensis.*

Figure 3 - Phylogeny of *X. translucens* pathovars and *Xanthomonas* sp. novel bacterial strains GW, SS and SI. This maximum-likelihood tree was inferred based on 97 genes conserved among 19 genomes. Values shown next to branches were the local support values calculated using 1000 resamples with the Shimodaira-Hasegawa test.

Figure 4 - Secondary metabolite biosynthesis gene clusters in the *Xanthomonas* sp. novel bacterial strains GW, SS and SI identified using antiSMASH (Weber et al. 2015). The gene clusters have sequence homology and structure to (A) the xanthoferrin gene cluster, (B) the xanthomonadin gene cluster and (C) an unknown gene cluster.

Figure 5 - Whole genome sequence comparison of the *Xanthomonas* sp. novel bacterial strains GW (middle), SI (top) and SS (bottom). The links between genome sequences indicated percentage similarity (from 70 % to 100 %). Genetic variations, including non-identical regions and insertions/deletions/inversions, suggested that the novel bacterial strains GW, SI and SS are genetically different. The star indicates the site of the genome of novel bacterial strain GW where the unique secondary metabolite biosynthesis gene cluster is located.

Figure 6 - Type I and Type III secretion systems of bacteria.

Figure 7 - Gene clusters of bacterial secretion systems in: A. the endophyte *Xanthomonas* sp. novel bacterial strain GW from perennial ryegrass, B. the pathogen *Xanthomonas translucens* pv. *translucens* from barley, and C. the pathogen *Xanthomonas translucens* pv. *undulosa* from wheat. Grey shading indicates presence of gene in gene cluster.

Figure 8 - Genome alignment of *Xanthomonas* sp. novel bacterial strain GW (the outer circle, greys) and *X. t. pv. undulosa* Xtu4699 (the inner circle, black). The colour of the outer circle represented the sequence identity (dark grey: 90 % -100 %; white: <90 %). The locations of T3SS, T3Es and TALEs genes that were detected on the genome of *X. t. pv. undulosa* Xtu4699 are designated.

Figure 9 - Image of 5 day old seedlings inoculated with the *Xanthomonas* sp. novel bacterial strain GW and an untreated control.

Figure 10 - Average shoot length of barley seedlings inoculated with bacterial strains of *Xanthomonas* sp. (strain GW) and non-Xanthomonads (Strain 1, 2, 3), and grown for 5 days. The * indicates significant difference in the mean at $P < 0.05$ between the control and the bacterial strains.

Figure 11 - Average root length of barley seedlings inoculated with bacterial strains of *Xanthomonas* sp. (strain GW) and non-Xanthomonads (Strain 1, 2, 3), and grown for 5 days. The * indicates significant difference in the mean at $P < 0.05$ between the control and the bacterial strains.

Figure 12 - Agarose gel electrophoresis (2 % [w/v]) of PCR amplicons generated using the GW strain-specific primers on *Xanthomonas* sp. strains GW, SS, SS, a negative control (NC) and a 2 kb DNA molecular ladder (M)

Figure 13 - Average root length of barley seedlings inoculated with bacterial strains of *Xanthomonas* sp. (strain GW) and non-Xanthomonads (Strain 1, 2, 3, 4), and grown for 4 days on nitrogen free media. The star indicates significant difference in the mean at $P < 0.05$ between the control and the bacterial strains.

Figure 14 - Average shoot length of barley seedlings inoculated with bacterial strains of *Xanthomonas* sp. (strain GW) and non-Xanthomonads (Strain 1, 2, 3, 4), and grown for 4 days on nitrogen free media. The star indicates significant difference in the mean at $P < 0.05$ between the control and the bacterial strains.

Figure 15 - Average root length of barley seedlings inoculated with bacterial strains of *Xanthomonas* sp. (strain GW) and non-Xanthomonads (Strain 1, 2, 3, 4), and grown for 4 days on media containing insoluble phosphate. The star indicates significant difference in the mean at $P < 0.05$ between the control and the bacterial strains.

Figure 16 - Average shoot length of barley seedlings inoculated with bacterial strains of *Xanthomonas* sp. (strain GW) and non-Xanthomonads (Strain 1, 2, 3, 4), and grown for 4 days on media containing insoluble phosphate. The star indicates significant difference in the mean at $P < 0.05$ between the control and the bacterial strains.

Figure 17 - Average root and shoot length of barley seedlings inoculated with novel *Xanthomonas* sp. bacterial strain GW at different concentrations (10^0, 10^{-1}, 10^{-2}), and grown for 7 days.

Detailed Description of the Embodiments

Discovery and characterisation of plant associated *Xanthomonas* sp. novel bacterial strains providing bioprotection phenotypes to plants.

Three novel plant associated *Xanthomonas* sp. bacterial strains GW, SS and SI were isolated from perennial ryegrass (*Lolium perenne*) plants. They display the ability to inhibit the growth of plant fungal pathogens in plate assays. The genomes of the three novel *Xanthomonas* sp. bacterial strains have been sequenced and are shown to be novel species, related to other Xanthomonad bacteria including *Xanthomonas translucens*. Analysis of the genome sequence has shown that all three *Xanthomonas* bacterial strains do not contain the type III secretion system shown to be essential for pathogenesis in pathogenic strains but do contain a type IV secretion system that has been implicated in an endophytic life cycle. Although the bacterial strains are closely related, they have differing biocidal activities, with one strain antagonistic to more fungi than the other strains. These bacterial strains have been used to inoculate barley (*Hordeum vulgare*) seeds under glasshouse conditions and have been demonstrated not to cause disease in these barley plants. These barley plants are also able to produce seed. Novel bacterial strain GW also enhances root growth in nitrogen limiting conditions and in insoluble phosphate. The optimal concentration of inoculum for novel bacterial strain GW is a dilution of an overnight culture (10^{-1}, 10^{-2}). Overall, novel plant associated *Xanthomonas* sp. bacterial strains GW, SS and SI offer both bioprotectant and biofertilizer activity (GW only).

Example 1 – Isolation of Bacterial Strains

Seed associated bacterial strains

Seeds from perennial ryegrass (*Lolium perenne*) were surface-sterilised by soaking in 80 % ethanol for 3 mins, then washing 5 times in sterile distilled water. The seeds were then plated onto sterile filter paper soaked in sterile water in sterile petri dishes. These plates were stored at room temperature in the dark to allow seedlings to germinate for 1 – 2 weeks. Once the seedlings were of sufficient size, the plants were harvested. In harvesting, the remaining seed coat was discarded, and the aerial tissue and root tissue were harvested. The plant tissues were submerged in sufficient Phosphate Buffered Saline (PBS) to completely cover the plant tissue, and ground using a Qiagen TissueLyser II, for 1 minute at 30 Hertz. A 10 µL aliquot of the macerate was added to 90 µL of PBS. Subsequent 1 in 10 dilutions of the 10^{-1} suspension were used to create additional 10^{-2} to 10^{-4} suspensions. Once the suspensions were well mixed 50 µL aliquots of each suspension were plated onto Reasoners 2 Agar (R2A) for growth of bacteria. Dilutions that provided a good separation of bacterial colonies were subsequently used for isolation of individual bacterial colonies through re-

streaking of single bacterial colonies from the dilution plates onto single R2A plates to establish a pure bacterial colony.

Mature plant associated bacterial strains

Leaf and root tissue were harvested from mature plants grown in the field or grown in pots in a greenhouse. Root tissue was washed in PBS buffer to remove soil particles and sonicated (10 mins) to remove the rhizosphere. The harvested tissues were placed into sufficient PBS to completely cover the tissue and processed as per the previous section to isolate pure bacterial cultures.

Around 300 bacterial strains were obtained from sterile seedlings, and 300 strains from mature plants. The novel bacterial strain GW was collected from seed of perennial ryegrass, while SS and SI were collected from mature plants.

Example 2 – Identification of *Xanthomonas* sp. novel bacterial strain

Amplicon (16S rRNA gene) Sequencing

A phylogenetic analysis of the novel bacterial strain GW was undertaken by sequence homology comparison of the 16S rRNA gene. The novel bacterial strain GW was grown overnight in Reasoners 2 Broth (R2B) media. DNA was extracted from pellets derived from the overnight culture using a DNeasy Blood and Tissue kit (Qiagen) according to manufacturer's instructions. The 16S rRNA gene amplification used the following PCR reagents: 14.8 µL H_2O, 2.5 µL 10× reaction buffer, 0.5 µL 10 mM dNTPs, 2.5 µL each of the 5 µM 27F primer (5'- AGAGTTTGATCMTGGCTCAG -3') and 5 µM reverse primers 1492R (5'- GGTTACCTTGTTACGACTT -3'), 0.2 µL of Immolase enzyme, and template to a final volume of 25 µL. The PCR reaction was then run in an Agilent Surecycler 8800 (Applied Biosystems) with the following program; a denaturation step at 94 °C for 15 min; 35 cycles of 94 °C for 30 sec, 55 °C for 10 sec, 72 °C 1 min; and a final extension step at 72 °C for 10 min.

Shrimp alkaline phosphatase (SAP) exonuclease was used to purify the 16S rRNA gene PCR amplicon. The SAP amplicon purification used the following reagents: 7.375 µL H_2O, 2.5 µL 10× SAP, and 0.125 µL Exonuclease I. The purification reaction was incubated at 37 °C for 1 hr, followed by 15 min at 80 °C to deactivate the exonuclease.

The purified 16S rRNA gene amplicon was sequenced using the BigDye® Terminator v3.1 Cycle Sequencing Kit (Thermofisher) with the following reagents; 10.5 µL H_2O, 3.5 µL 5× Seq buffer, 0.5 µL BigDye®, 2.5 µL of either the 3.2 µM Forward (27F) and 3.2 µM Reverse primers (1492R), and 4.5 µL of PCR amplicon as template, to a final reaction volume of 20 µL. The sequencing PCR reaction was then run in an Agilent Surecycler 8800 (Applied Biosystems) with the following

program; denaturation step at 94 °C for 15 min; followed by 35 cycles of 94 °C for 30 sec, 55 °C for 10 sec, 72 °C 1 min; and one final extension step at 72 °C for 10 min. The 16S rRNA gene amplicon from novel bacterial strain GW was sequenced on an ABI3730XL (Applied Biosystems). A 1269 bp 16S rRNA gene sequence was generated (Figure 1). The sequence was aligned by BLASTn on NCBI against the non-redundant nucleotide database and the 16S ribosomal RNA database.

BLASTn hit against database nr; *Xanthomonas* sp. strain PRd6 16S ribosomal RNA gene, partial sequence

Max Score	Total Score	Query Coverage	E-Value	% Identity	Accession
2289	2289	100 %	0	100.00 %	KY203971.1

BLASTn hit against database 16S ribosomal RNA; *Xanthomonas translucens* strain XT 2 16S ribosomal RNA gene, partial sequence

Max Score	Total Score	Query Coverage	E-Value	% Identity	Accession
2271	2271	100 %	0	99.68 %	NR_036968.1

The preliminary taxonomic identification of the novel bacterial strain GW was a novel *Xanthomonas* sp., closely related to *Xanthomonas transluscens*.

Genomics

The genome of the novel bacterial strain GW was sequenced, along with two additional *Xanthomonas* strains SS and SI. These novel bacterial strains were retrieved from the glycerol collection stored at -80 °C by streaking on R2A plates. Single colonies from these plates were grown overnight in Nutrient Broth and pelleted. These pellets were used for genomic DNA extraction using the bacteria protocol of Wizard® Genomic DNA Purification Kit (A1120, Promega). DNA sequencing libraries were generated for Illumina sequencing using the Illumina Nextera XT DNA library prep protocol. All libraries were sequenced using an Illumina MiSeq platform or HiSeq platform. Raw reads from the sequencer were filtered to remove any adapter and index sequences as well as low quality bases using Trimmomatic (Bolger, Lohse & Usadel 2014) with the following options: ILLUMINACLIP: NexteraPE-PE.fa:2:30:10 LEADING:3 TRAILING:3 SLIDINGWINDOW:4:15 MINLEN:36. To enable full genome assembly, long reads were generated for the three *Xanthomonas* sp. novel bacterial strain by sequencing DNA using Oxford Nanopore Technologies (ONT) MinION platform. The DNA from the Wizard® Genomic DNA Purification Kit was first assessed with the genomic assay on Agilent 2200 TapeStation system (Agilent Technologies, Santa

Clara, CA, USA) for integrity (average molecular weight ≥30 Kb). The sequencing library was prepared using an in-house protocol modified from the official protocols for transposases-based library preparation kits (SQK-RAD004/SQK-RBK004, ONT, Oxford, UK). All libraries were sequenced on a MinION Mk1B platform (MIN-101B) with R9.4 flow cells (FLO-MIN106) and under the control of MinKNOW software. After the sequencing run finished, the fast5 files that contain raw read signals were transferred to a separate, high performance computing Linux server for local basecalling using ONT's Albacore software (Version 2.3.1) with default parameters. For libraries prepared with the barcoding kit (SQK-RBK004), barcode demultiplexing was achieved during basecalling. The sequencing summary file produced by Albacore was processed by the R script minion qc (https://github.com/roblanf/minion_qc) and NanoPlot (De Coster et al. 2018) to assess the quality of each sequencing run, while Porechop (Version 0.2.3, https://github.com/rrwick/Porechop) was used to remove adapter sequences from the reads. Reads which were shorter than 300 bp were removed and the worst 5 % of reads (based on quality) were discarded by using Filtlong (Version 0.2.0, https://github.com/rrwick/Filtlong).

The whole genome sequence of the three *Xanthomonas* sp. novel bacterial strains were assembled using Unicycler (Wick et al. 2017). Unicycler performed hybrid assembly when both Illumina reads and MinION reads were available. MinION reads were mainly used to resolve repeat regions in the genome, whereas Illumina reads were used by Pilon (Walker et al. 2014) to correct small base-level errors. Multiple rounds of Racon (Vaser et al. 2017) polishing were then carried out to generate consensus sequences. Assembly graphs were visualised by using Bandage (Wick et al. 2015).

A complete circular chromosome sequence was produced for the three *Xanthomonas* sp. novel bacterial strains. The genome size for the novel bacterial strains GW, SS and SI were 5,233,349 bp, 5,185,085 bp and 5,246,417 bp respectively (Table 1). The percent GC content ranged from 68.37 % - 68.55 %. These novel bacterial strains were annotated by Prokka (Seemann 2014) with a custom, genus-specific protein database to predict genes and corresponding functions, which were then screened manually to identify specific traits.

The number of genes for the novel bacterial strains GW, SS and SI were 4,425, 4,291 and 4,290 genes respectively (Table 2).

Table 1 – Summary of properties of the final genome sequence assembly

Strain ID	**Genome size (bp)**	**GC content (%)**	**Coverage Illumina reads**	**Coverage ONT MinION**
GW	5,233,349	68.37	105.6x	97x
SS	5,185,085	68.55	1167.9x	23.7x
SI	5,246,417	68.44	584.5x	24.9x

Table 2 – Summary of genome coding regions

Strain ID	Genome size (bp)	No. of tRNA	No. of tmRNA	No. of rRNA	No. of CDS	No. of gene
GW	5,233,349	60	1	6	4358	4425
SS	5,185,085	57	1	6	4227	4291
SI	5,246,417	63	1	6	4290	4360

The gyrase B gene was extracted from the genome sequences of the *Xanthomonas* sp. novel bacterial strains GW, SS and SI, and a multiple sequence alignment was performed with 20 gyrase B genes from *X. translucens* (9 strains), *X. sacchari* (1), *X. albilineans* (2), *X. cassavae* (1), *X. campestris* (2), *X. hortorum* (1), *X. gardeneri*, *X. oryzae*, *X. vasicola* (1), *X. citri* (2), *X. axonopodis* (2) and the outgroups *Lysobacter enzymogenes* and *Pseudoxanthomonas suwonensis*. A neighbour joining tree was generated from this alignment with 100 bootstraps performed (Figure 2). The strains GW, SS and SI formed a distinct clade from *X. translucens* and *X. sacchari* and *X. albilineans* strains, which supports that these three strains are from a novel *Xanthomonas* species.

Fifteen *X. translucens* genome sequences and one *X. campestris* genome sequence that are publicly available on NCBI were acquired and used for pan-genome/comparative genome sequence analysis alongside *Xanthomonas* sp. novel bacterial strains GW, SS and SI. A total of 97 genes that are shared by all 19 strains were identified by running Roary (Page et al. 2015). PRANK (Löytynoja 2014) was then used to perform a codon aware alignment. A maximum-likelihood phylogenetic tree (Figure 3) was inferred using FastTree (Price, Dehal & Arkin 2010) with Jukes-Cantor Joins distances and Generalized Time-Reversible and CAT approximation model. Local support values for branches were calculated using 1000 resamples with the Shimodaira-Hasegawa test. The novel bacterial strains GW, SS and SI clustered tightly together, suggesting a close phylogenetic relationship between these bacterial strains. Moreover, this cluster was separated from other *X. translucens* pathovars. with strong local support value (100 %). This separation supports that these three bacterial strains are from a novel *Xanthomonas* species, but closely related to *X. translucens* pathovars.

Example 3 – Bioprotection activity (*in vitro*) of *Xanthomonas* sp. strains

In vitro bioassays were established to test the bioactivity of the *Xanthomonas* sp. novel bacterial strains GW, SS and SI against five plant pathogenic fungi (Table 3). An unrelated bacterial strain (Strain X) was used as a negative control. The fungal pathogens were all isolated from monocot species, and were obtained from the National Collection of Fungi (Herbarium VPRI) and the AVR collection. Each bacterial strain was cultured in Nutrient Broth (BD Biosciences) overnight at 28 °C in a shaking incubator (200 rpm). Each bacterial strain was drop-inoculated (20 µL) onto four

equidistant points on a Nutrient Agar (BD Biosciences) plate, which was then incubated overnight at 28 °C. A 6 mm × 6 mm agar plug of actively growing mycelia from the pathogen was placed at the centre of the plate. The bioassay was incubated for at least 5 days at 28 °C in the dark, and then the diameter of the fungal colony on the plate was recorded. For each treatment three plates were prepared as biological triplicates. OriginPro 2018 (Version b9.5.1.195) was used to carry out One-way ANOVA and Tukey Test to detect the presence of any significant difference ($P < 0.05$) between treatments.

Table 3 – Pathogens used in the bioprotection bioassay

VPRI Accession No.	**Taxonomic Details**	**Host Taxonomic Details**	**State**	**Collection Date**
12962	*Drechslera brizae* (Y.Nisik.) Subram. & B.L.Jain	*Briza maxima* L.	Vic.	24-Oct-85
32148	*Sclerotium rolfsii* Sacc.	*Poa annua* L.	Vic.	1-Jan-05
42586a	*Fusarium verticillioides* (Sacc.) Nirenberg	*Zea mays* L.	Vic.	27-Feb-15
42563	*Bipolaris gossypina*	*Brachiaria*	Qld	
N/A	*Microdochium nivale*	*Lolium perenne* L.	Vic	

The *Xanthomonas* sp. novel bacterial strain GW inhibited the growth of all five pathogens, indicating it had broad spectrum biocidal activity, unlike novel bacterial strains SS and SI that only inhibited the growth of three and four pathogens respectively (Table 4, grey shading). Novel bacterial strain GW significantly inhibited the growth of *Sclerotium rolfsii* (74.80 %) in comparison to Strain X, *Microdochium nivale* (67.87 %) compared to bacterial strains SS, SI and X, and *Bipolaris gossypina* (54.67 %), compared to bacterial strains SS, SI.

Table 4 – Bioprotection bioassay indicating the percentage inhibition (versus the control) of the *Xanthomonas* sp. novel bacterial strains GW, SS and SI against 5 plant pathogenic fungi, *Bipolaris gossypina, Sclerotinia rolfsii, Drechslera brizae, Fusarium verticillioides and Microdochium nivale*. An unrelated bacterial strain (strain X) was the negative control. The grey shading indicates strains that were significantly different ($P < 0.05$, Tukey Test) from one another in bioassays against a given pathogen.

Pathogen ID	GW	SS	SI	Strain X
Bipolaris gossypina VPRI 42563	**62.38±3.99**	-0.97±3.66	1.24±0.83	**49.10±1.11**
Sclerotium rolfsii VPRI 32148	**74.80±1.72**	**74.80±3.22**	**77.95±1.72**	27.95±1.18
Drechslera brizae VPRI 12962	25.40±1.59	37.30±7.05	44.44±9.94	12.70±0.79
Fusarium verticillioides VPRI 42586	37.21±0.90	32.29±14.70	13.45±9.65	13.00±1.19
Microdochium nivale	**69.13±2.30**	-2.17±1.57	10.00±13.70	12.61±1.51

Example 4 – Genome sequence features supporting the bioprotection niche of the *Xanthomonas* sp. novel bacterial strains

Secondary metabolite biosynthesis gene clusters

The genome sequences of the three *Xanthomonas* sp. novel bacterial strains GW, SS and SI were assessed for the presence of features associated with bioprotection. The annotated genome sequences were analysed by antiSMASH (Weber et al. 2015) to identify secondary metabolite biosynthesis gene clusters that are commonly associated with the production of biocidal compounds that aid in their defence. Annotated genome sequences were passed through antiSMASH with the following options: *--clusterblast --asf --knownclusterblast --subclusterblast --smcogs --full-hmmer*. A total of three secondary metabolite gene clusters were identified in the genome sequences of the three *Xanthomonas* sp. novel bacterial strains (Figure 4). A biosynthetic gene cluster was identified in all three novel bacterial strains that had sequence homology and structure to the xanthoferrin gene cluster that produces the bioprotectant siderophore xanthoferrin (Figure 4A). This gene cluster had the non-ribosomal peptide synthases (NRPS) essential for the biosynthesis of the nonribosomal peptide xanthoferrin and was identical in structure across the strains. A biosynthetic gene cluster was also shared by all three novel bacterial strains that had sequence homology and structure to the xanthomonadin gene cluster that produces the bioprotectant pigment xanthomonadin (Figure 4B). This gene cluster had the polyketide synthase (PKS) essential for the biosynthesis of the polyketide

xanthomonadin, but the cluster had slight variations in structure across three novel bacterial strains. A gene cluster was identified that was unique to novel bacterial strain GW, and had a NRPS essential for biosynthesis a nonribosomal peptide, but showed no sequence homology to other gene clusters in the antiSMASH database (Figure 4C). This gene cluster is of interest as it may be linked to the biosynthesis of a compound that explains the biocidal activity of novel bacterial strain GW.

Genome sequence alignment

The genome sequences of the novel bacterial strains GW, SS and SI were aligned using LASTZ (Version 1.04.00, http://www.bx.psu.edu/~rsharris/lastz/) and visualised using AliTV (Ankenbrand et al. 2017) to validate the absence of the unique secondary metabolite gene cluster from novel bacterial strains SS and SI. A region of the genome of novel bacterial strain GW was identical between bases 1,997,794 and 2,067,075 that contained the unique secondary metabolite gene cluster, but was absent from novel bacterial strains SS and SI (Figure 5, star).

Example 5 – Genome sequence features supporting the endophytic niche of the *Xanthomonas* sp. novel bacterial strains

There have been nine virulence-related gene clusters identified in the *X. translucens* genome that are important for the pathogenicity of this species (Table 5). These include gene clusters that regulate biosynthesis of secretion systems (T1, T2, T3 and T6), pili (Type 4), flagella, xanthan and lipopolysaccharides (Table 5). The presence of these clusters in the genome of the *Xanthomonas* sp. novel bacterial strain GW was assessed through homology searches of gene sequences (BLASTp, KEGG) and gene names in a custom pathogenesis database (Table 5, Figure 6 & 7). The novel bacterial strain GW had genes in seven of the nine virulence-related gene clusters, however it had an incomplete type 1 secretion system (1 of 3 genes necessary for function) and no type III secretion system (0 of 10 genes necessary for function). These two secretion systems are important for the secretion of toxins and cell degrading enzymes into the host (type I), along with effectors (type III) (Figure 6). The type III secretion system is complete in the pathogens *X. translucens pv. translucens* and *X. translucnes pv. undulosa*, whereas the type I secretion system is only in *X. translucens pv. translucens* (Figure 7). The type III secretion system is known to be integral for virulence in *X. translucens*, as demonstrated in *X. translucens pv. undulosa* (Xtu4699) (Peng et al. 2016). The type III secretion system genes are either involved in the structure (*Ysc/Hrc F, O, P, X, C, W, J, R, S, T, U, V, N, Q, L* and *HrpE*) or the transport of effectors (Hrp B1, B2 and HpaT). These genes are normally localised in the genome of pathogenic *Xanthomonas translucens* strains (Figure 8), but are completely absent in *Xanthomonas* sp. novel bacterial strain GW. There was also an absence of all conserved Type III effectors (*XopAA, AD, AM, B, C2, F, G, K, N, O, V, X, Y, Z*), variable Type III effectors (*Xop, AF, AH, E1 L, P, AvrBs1, AvrBs2*) and transcription activator-like effectors (TALEs 1 – 8) in *Xanthomonas* sp. novel bacterial strain GW. In pathogenic *Xanthomonas*

strains these genes supress plant innate immunity and modulate plant cellular pathways to enhance bacterial infection (Buttner, 2016). Given that the *Xanthomonas* sp. novel bacterial strain GW did not have the type III secretion system and effectors it is thought that the strain is not a pathogen, but occupies an endophytic niche within perennial ryegrass. Furthermore, given that the Type III secretion system and effector genes are widely distributed across the whole chromosome of pahogenic *Xanthomonas* strains (Figure 8), it is highly unlikely that *Xanthomonas* sp. novel bacterial strain GW would acquire all the genes necessary to become pathogenic through horizontal gene transfer.

Table 5 – Virulence-related gene clusters identified in *X. translucens* pathovars

Virulence-related gene cluster	**Reference**	**Presence of genes in strain GW**
T1SS	Lee, S-W et al. (2006)	Incomplete
T2SS	Lee, HM et al. (2001)	Yes
T3SS	Wichmann et al. (2013)	No
T6SS	Boyer et al. (2009)	Yes
Type IV pilus	Dunger et al. (2016)	Yes
Flagellum	Darrasse et al. (2013)	Yes
Pathogenicity regulatory factors	Tang et al. (1991)	Yes
Xanthan biosynthesis	Katzen et al. (1996)	Yes
Lipopolysaccharide biosynthesis	Vorhölter, Niehaus and Pühler (2001)	Yes
Conserved T3SS Effectors	Buttner (2016)	No
Variable T3SS Effectors	Buttner (2016)	No
Transcription activator-like Effectors	Cernadas et al., (2014); Hu et al., (2014)	No

Example 6 – *In planta* inoculations supporting the endophytic niche of the *Xanthomonas* sp. novel bacterial strains

To assess direct interactions between the *Xanthomonas* sp. novel bacterial strain GW and plants, an early seedling growth assay was established in barley. A total of 4 bacterial strains (GW – *Xanthomonas* sp.; Isolate 1, Isolate 2, Isolate 3 – non Xanthomonads) were cultured in Lysogeny Broth (LB) overnight at 26 °C. The following day seeds of barley (cultivar Hindmarsh) were surface-sterilised by soaking in 80 % ethanol for 3 mins, then washing 5 times in sterile distilled water. The seeds were then soaked in the overnight cultures for 4 hours at 26 °C in a shaking incubator. For control seedlings, seeds were soaked in LB without bacteria for 4 hours at 26 °C in a shaking incubator. The seeds were planted into a pot trial, with three replicates (pots) per strain/control, with a randomised design. A total of 20 seeds were planted per pot, to a depth of 1 cm. The potting

medium contained a mixture of 25 % potting mix, 37.5 % vermiculite and 37.5 % perlite. The plants were grown for 5 days and then removed from the pots, washed, assessed for health (i.e. no disease symptoms) and photographed. The lengths of the longest root and the longest shoot were measured. Data was statistically analysed using a One-way ANOVA and Tukey test to detect the presence of any significant difference ($P < 0.05$) between treatments using OriginPro 2018 (Version b9.5.1.195).

Seedlings inoculated with the *Xanthomonas* sp. novel bacterial strain GW were healthy with no disease symptoms recorded on leaves or roots (Figure 9). The length of the shoots of seedlings inoculated with the *Xanthomonas* sp. novel bacterial strain GW were equivalent to the control (Figure 10). The length of the roots of seedlings inoculated with the *Xanthomonas* sp. novel bacterial strain GW were significantly shorter than the control (Figure 11).

Example 7 – *In planta* inoculations supporting the bioprotection niche of the *Xanthomonas* sp. novel bacterial strain GW

An *in planta* bioprotection assay was established in wheat to evaluate the activity of *Xanthomonas* sp. novel bacterial strain GW against the fungal phytopathogen *Bipolaris sorokiniana* (VPRI 42684). The bacterial strain was cultured in nutrient broth (BD Bioscience) for 6 hours. Wheat seeds were surface sterilised (3 % NaOCl for 3 mins, 3 x sterile dH2O wash), imbibed in bacterial culture for 18 hours, and then germinated in dark for 4 days for root and shoot development. Germinated seedlings were transferred in pots (4 seeds per pot, 4 pots per treatment) in a glasshouse for 39 days. A 7 cm segment of the lowest leaf that was green and fully extended from each plant was excised and placed on 0.5 % water agar. A sterile sharp needle was used to create a wound at the centre of the leaf, to which 1 µL of *B. sorokiniana* spore suspension was added. Plates were then sealed and left at room temperature for 2 days. To assess the bioprotection activity, the size of lesion, chlorotic zones and fungal hyphal growth were recorded (measured in mm^2). For the control, sterile Nutrient Broth was used. Statistical analysis (One-way ANOVA and Tukey Test) was conducted using OriginPro 2018 (Version b9.5.1.195) to detect the presence of any significant difference ($P < 0.05$) between treatments.

Xanthomonas sp. novel bacterial strain GW significantly ($P < 0.05$) reduced the average size of lesion and fungal hyphal growth compared to the control (Table 6). The lesion size was reduced by 96.7 %, and the area of fungal hyphal growth was reduced by 94.7 %.

Table 6 – Bioprotection assay (*in planta*) results for *Xanthomonas* sp. novel bacterial strain GW (average ± standard error)

Isolate ID	Lesion/mm^2	Chlorosis/mm^2	Fungal hyphal growth / mm^2
GW	1.33±0.25^a	34.44±10.72^a	2.00±1.37^a
Blank	42.75±10.26^b	68.88±22.50^a	37.63±20.45^b

Example 8 – *In planta* inoculations supporting colonisation and localisation of the *Xanthomonas* sp. novel bacterial strain GW in wheat and perennial ryegrass

Strain-specific primers were designed for X*anthomonas* sp. novel bacterial strain GW targeting the 1997794 bp – 2067075 bp region of the genome, which related to a section of the unique non-ribosomal peptide synthase of strain GW (GW-F CCACGCCGAATACAATGCAG; GW-R CATGGATGACTGGCACTGGT; 5'→3'). An *in silico* analysis using Primer-BLAST and a sequence homology comparison to strain SS and SI indicated that the primers were strain-specific.

The strain-specific primer for GW was evaluated on cultures of strains X*anthomonas* sp. novel bacterial strains GW, SS and SI. Initially, bacterial cultures were grown in nutrient broth (BD Bioscience) and grown overnight at 22 °C in the dark in a shaking incubator. The Promega Wizard® genomic DNA purification kit was used with the following modifications: initial centrifugation of 1 mL of overnight culture at 13,000 – 16,000× g for 2 mins was performed twice to pellet bacterial cells; incubations were conducted at -20 ºC for 10 mins to enhance protein precipitation; DNA pellets were rehydrated in 50 mL rehydration solution at 65 °C for 10 mins followed by overnight incubation at 4 °C. Final DNA concentration was measured using a Quantus™ Fluorometer and stored at 4 °C until further processing. The 25 µL reaction mixture contained: 12.5 µL of OneTaq™ Hot Start 2× master mix with standard buffer (New England BioLabs®), 2 µL of each primer (10µM/µL), 8.5 µL of nuclease-free water and 2 µL of template DNA sample. The thermocycling conditions were: initial denaturation at 94 °C for 1 min, followed by 30 cycles of denaturation at 94 °C for 30 sec, annealing at 58 °C for 1 min, elongation at 72 °C for 2 min, and a final extension at 72 °C for 10 min. PCR products were separated at 120 V in a 2 % (w/v) agarose gel containing 0.05 µL mL-1 SYBR safe stain in 1× TAE running buffer and visualized under UV light next to a 2 kb DNA ladder. The strain-specific primer generated an amplicon of the correct size (943 bp) for X*anthomonas* sp. novel bacterial strain GW only (Figure 12).

The strain-specific primer for GW was evaluated on wheat and perennial ryegrass plants inoculated with X*anthomonas* sp. novel bacterial strain GW. Initially, perennial ryegrass and wheat seeds were sterilized in 70 % ethanol for 3 minutes, followed by rinsing with sterilized distilled water (SDW) for three times. The bacterial strain was cultured in nutrient broth (BD Bioscience) overnight, while

seeds were imbibed in nutrient broth overnight in the dark. Seeds and the bacterial culture were combined for 4 hours in dark in a shaking incubator. For the controls, seeds were not inoculated with bacteria. A total of three seeds were sown per pot into potting mix and grown in a glasshouse. For perennial ryegrass, plants were harvested at three time points (12, 22 and 33 days after planting, DAP), while for wheat, plants were harvested at only one time point (7 DAP). For perennial ryegrass inoculated with GW, 20 replicates were maintained for each time point, while for wheat inoculated with GW 10 replicates were maintained. For the uninoculated control treatments (perennial ryegrass and wheat) 5 replicates were maintained for each time point. At harvest, plants were uprooted, washed thoroughly (roots only) and then sectioned into roots, pseudostem and leaves (ryegrass - 12 & 22 DAP; wheat - 7 DAP). However, for perennial ryegrass at 33 DAP, plants were sectioned into roots, pseudo-stem, lower leaves and upper leaves as plants were larger. Each section comprised three pieces (~0.5 cm^2) of plant tissue, which was placed into collection microtubes (2 mL) and stored at -80 °C. The 22 and 33 DAP (perennial ryegrass) samples were freeze-dried for 48 hours, while the 7 (wheat) and 12 DAP (perennial ryegrass) samples were not freeze-dried. The Qiagen® MagAttract® 96 DNA plant core kit (Qiagen®, Hilden, Germany) was utilized to extract plant DNA using the Biomek® FXP lab automation workstation linked to Biomek software version v. 4.1 and Gen 5 (v. 2.08) software (Biotek Instruments, USA) with the following modifications to the manufacturer's instructions: to each well of the 96 well microplate, a 33 µL aliquot of RB buffer and 10 µL of resuspended MegAttract suspension G was added. A touch-down PCR (TD-PCR) was performed to enhance the sensitivity and specificity of primers *in planta*, compared to *in vitro* pure cultures. The PCR reaction mixture was prepared as per *in vitro* cultures. Touch-down PCR amplification was performed in two phases. In phase I, initial denaturation was carried out at 94 °C for 1 min, followed by 10 cycles of denaturation at 94 °C for 30 sec, annealing for at 65 – 55 °C (dropping 1 °C for each cycle) and 72 °C for 2 mins. In phase II, it was 20 cycles of denaturation at 94 °C for 30 sec, annealing at 58 °C for 1 min and extension at 72 °C for 2 min, with a final extension at 72 °C for 10 min. For perennial ryegrass, the presence of the X*anthomonas* sp. novel bacterial strain GW was detected at 12, 22 and 33 DAP, with the highest rates of incidence recorded 22 DAP (20 – 85 %) and the lowest at 7 DAP (0 – 1 %) (Table 7). The most detections were recorded in consistently in roots (2 – 80 %), followed by pseudostem (28 – 85 %; 22 and 33 DAP only) and leaves (0 – 44 %; 22 and 33 DAP only). There were no detections in the control. For wheat, the presence of the X*anthomonas* sp. novel bacterial strain GW was detected at 7 DAP, with the highest rates of incidence recorded in roots (90 %), followed by pseudostem (20 %) and leaves (10 %) (Table 8). Overall, X*anthomonas* sp. novel bacterial strain GW appears to inoculate into both perennial ryegrass and wheat, where it colonises all tissues, but appears to preferentially colonise roots, and persists for at least 33 DAP.

Table 7 – Incidence of GW in perennial ryegrass at three harvest time points. The incidence is indicated as the number of plants showing the presence of GW per total number of replicates inoculated or uninoculated (R - roots; P - pseudostem; L - leaves; LL - lower leaves; UL - upper leaves).

	12 DAP			22 DAP			33 DAP			
	R	**P**	**L**	**R**	**P**	**L**	**R**	**P**	**LL**	**UL**
GW	2/20	0/20	0/20	16/20	17/20	4/20	13/18	5/18	4/18	8/18
Control	0/5	0/5	0/5	0/5	0/5	0/5	0/5	0/5	0/5	0/5

Table 8 – Incidence of GW in wheat at one harvest time point. The incidence is indicated as the number of plants showing the presence of GW per total number of replicates inoculated or uninoculated (R - roots; P - pseudostem; L - leaves).

	7 DAP		
	R	**P**	**L**
GW	9/10	2/10	1/10
Control	0/5	0/5	0/5

Example 9 – *In planta* inoculations supporting the biofertilizer (nitrogen) niche of the *Xanthomonas* sp. novel bacterial strain GW

An *in planta* biofertilizer assay was established in barley to evaluate the ability of *Xanthomonas* sp. novel bacterial strain GW to aid growth under nitrogen limiting conditions. Initially, bacterial strains (5, including GW) were cultured in 20 mL nutrient broth (BD Bioscience) overnight at 26 °C whilst rotating at 200 rpm. The following day cultures were pelleted via centrifugation at 4000 rpm for 5 minutes, washed three times in 10 mL Phosphate Buffered Saline (PBS), resuspended in 20 mL PBS, quantified via spectrophotometry (OD_{600}) and diluted (1:10). Barley seeds were sterilized in 70 % ethanol for 5 minutes, followed by rinsing with sterilized distilled water (SDW) for five times. These sterile seeds were submerged in the dilution for 4 hours in a dark incubator at room temperature whilst rotating at 200 rpm. The seeds were subsequently transferred to moistened sterile filter paper and allowed to germinate for three days. The three-day-old seedlings were individually transferred to 60 mm plates with semi-solid Burks media (HiMedia) (5 g/L Agar). Seedlings were allowed to grow for a further 4 days, before the shoots and roots were measured for each seedling. There was a total of 6 treatments (5 bacterial strains including GW; 1 blank media control) containing 10 seedlings per treatment. Statistical analysis (One-way ANOVA and Tukey Test) was conducted using OriginPro 2018 (Version b9.5.1.195) to detect the presence of any significant difference ($P < 0.05$) between treatments.

The root growth of seedlings inoculated with novel bacterial strain GW and grown under nitrogen limiting conditions was significantly greater than the control ($P < 0.05$), with an average increase of 27.6 % (Figure 13). The shoot growth of seedlings inoculated with novel bacterial strain GW was not significantly greater than the control ($P < 0.05$) (Figure 14). Overall, results indicate that novel bacterial strain GW can aid in the growth of seedlings grown under nitrogen limiting conditions.

Example 10 – *In planta* inoculations supporting the biofertilizer (phosphate solubilisation) niche of the *Xanthomonas* sp. novel bacterial strain GW

An *in planta* biofertilizer assay was established in barley to evaluate the ability of *Xanthomonas* sp. novel bacterial strain GW to aid growth under conditions with insoluble phosphate. Initially, bacterial strains (5, including GW) were cultured in 30 mL R2B overnight at 26 °C whilst rotating at 200 rpm. The following day the barley seeds were sterilized in 70 % ethanol for 5 minutes, followed by rinsing with SDW for five times. These sterile seeds were submerged in the overnight cultures for 4 hours in a dark incubator at room temperature whilst rotating at 200 rpm. The seeds were subsequently transferred to moistened sterile filter paper to be allowed to germinate for three days. These three-day-old seedlings were individually transferred to 60 mm plates with semi-solid Pikovskaya's media which contains yeast extract (0.5 g/L), D-glucose (5.0 g/L), calcium phosphate (5.0 g/L), ammonium sulphate (0.5 g/L), potassium chloride (0.2 g/L), magnesium sulphate (0.1 g/L), manganese sulphate (0.1 mg/L), ferrous sulphate (0.1 mg/L) and agar (5.0 g/L). These seedlings were allowed to grow for another 4 days, before the shoots and roots were measured for each seedling. There was a total of 6 treatments (5 bacterial strains including GW; 1 blank media control) containing 10 seedlings per treatment. Statistical analysis (One-way ANOVA and Tukey Test) was conducted using OriginPro 2018 (Version b9.5.1.195) to detect the presence of any significant difference ($P < 0.05$) between treatments.

The root growth of seedlings inoculated with novel bacterial strain GW and grown under conditions with insoluble phosphate was significantly greater than the control ($P < 0.05$), with an average increase of 36.5 % (Figure 15). The shoot growth of seedlings inoculated with novel bacterial strain GW was not significantly greater than the control ($P < 0.05$) (Figure 16). Overall, results indicate that novel bacterial strain GW can aid in the growth of seedlings grown under conditions with insoluble phosphate.

Example 11 – *In planta* inoculations identifying optimal concentrations of *Xanthomonas* sp. novel bacterial strain GW

An *in planta* biofertilizer assay was established in perennial ryegrass to evaluate the optimal concentration in which *Xanthomonas* sp. novel bacterial strain GW would support seedling growth. Initially, the bacterial strain was cultured overnight in 20 mL nutrient broth (BD Bioscience) at 26 °C

whilst rotating at 200 rpm. The following day the culture was pelleted via centrifugation at 4000 rpm for 5 minutes, washed three times in 10 mL PBS, resuspended in 20 mL PBS, quantified via spectrophotometry (OD_{600}). The culture was diluted (1:10) twice to create three concentrations (10^0, 10^{-1} and 10^{-2}). The perennial ryegrass seeds were sterilized in 70 % ethanol for 5 minutes, followed by rinsing five times with SDW. These sterile seeds were submerged in the dilutions for 4 hours in a dark incubator at room temperature whilst rotating at 200 rpm. After inoculation, 10 seeds were transferred to moistened sterile filter paper for germination from each dilution. After seven days, the roots and shoots were measured.

There was a trend observed whereby root and shoot growth increased as the concentration of novel bacteria GW decreased (Figure 17). The greatest root growth was observed at the 10^{-2} dilution, which was 19.7 % greater than 10^{-1} dilution and 45.2 % greater than the 10^{-0} dilution. The greatest shoot growth was observed at the 10^{-2} dilution, which was 14.5 % greater than 10^{-1} dilution and 45.9 % greater than the 10^{-0} dilution.

It is to be understood that various alterations, modifications and/or additions may be made without departing from the spirit of the present invention as outlined herein.

As used herein, except where the context requires otherwise, the term "comprise" and variations of the term, such as "comprising", "comprises" and "comprised", are not intended to be in any way limiting or to exclude further additives, components, integers or steps.

Reference to any prior art in the specification is not, and should not be taken as, an acknowledgment or any form of suggestion that this prior art forms part of the common general knowledge in Australia or any other jurisdiction or that this prior art could reasonably be expected to be combined by a person skilled in the art.

References

1. Ankenbrand, MJ, Hohlfeld, S, Hackl, T & Förster, F 2017, 'AliTV—interactive visualization of whole genome comparisons', *PeerJ Computer Science*, vol. 3.

2. Bolger, AM, Lohse, M & Usadel, B 2014, 'Trimmomatic: a flexible trimmer for Illumina sequence data', *Bioinformatics*, vol. 30, no. 15, pp. 2114-20.

3. Boyer, F, Fichant, G, Berthod, J, Vandenbrouck, Y & Attree, I 2009, 'Dissecting the bacterial type VI secretion system by a genome wide in silico analysis: what can be learned from available microbial genomic resources?', *BMC genomics*, vol. 10, p. 104.

4. Buttner, D. 2016. Behind the lines-actions of bacterial type III effector proteins in plant cells. FEMS Microbiol Rev 40, 894-937.

5. Cernadas, R.A., Doyle, E.L., Nino-Liu, D.O., Wilkins, K.E., Bancroft, T., Wang, L., Schmidt, C.L., Caldo, R., Yang, B., White, F.F., Nettleton, D., Wise, R.P., and Bogdanove, A.J. 2014. Code-assisted discovery of TAL effector targets in bacterial leaf streak of rice reveals contrast with bacterial blight and a novel susceptibility gene. PLoS Pathog 10, e1003972.

6. Darrasse, A, Carrère, S, Barbe, V, Boureau, T, Arrieta-Ortiz, ML, Bonneau, S, Briand, M, Brin, C, Cociancich, S, Durand, K, Fouteau, S, Gagnevin, L, Guérin, F, Guy, E, Indiana, A, Koebnik, R, Lauber, E, Munoz, A, Noël, LD, Pieretti, I, Poussier, S, Pruvost, O, Robène-Soustrade, I, Rott, P, Royer, M, Serres-Giardi, L, Szurek, B, van Sluys, M-A, Verdier, V, Vernière, C, Arlat, M, Manceau, C & Jacques, M-AJBG 2013, 'Genome sequence of Xanthomonas fuscans subsp. fuscansstrain 4834-R reveals that flagellar motility is not a general feature of xanthomonads', vol. 14, no. 1, p. 761.

7. De Coster, W, D'Hert, S, Schultz, DT, Cruts, M & Van Broeckhoven, C 2018, 'NanoPack: visualizing and processing long read sequencing data', *Bioinformatics*.

8. Dunger, G, Llontop, E, Guzzo, CR & Farah, CS 2016, 'The Xanthomonas type IV pilus', *Curr Opin Microbiol*, vol. 30, pp. 88-97.

9. Katzen, F, Becker, A, Zorreguieta, A, Pühler, A & Ielpi, LJJob 1996, 'Promoter analysis of the Xanthomonas campestris pv. campestris gum operon directing biosynthesis of the xanthan polysaccharide', vol. 178, no. 14, pp. 4313-8.

10. Hu, Y., Zhang, J., Jia, H., Sosso, D., Li, T., Frommer, W.B., Yang, B., White, F.F., Wang, N., and Jones, J.B. 2014. Lateral organ boundaries 1 is a disease susceptibility gene for citrus bacterial canker disease. Proc Natl Acad Sci U S A 111, E521-529.

11. Lee, HM, Tyan, SW, Leu, WM, Chen, LY, Chen, DC & Hu, NT 2001, 'Involvement of the XpsN protein in formation of the XpsL-xpsM complex in Xanthomonas campestris pv. campestris type II secretion apparatus', *J Bacteriol*, vol. 183, no. 2, pp. 528-35.

12. Lee, S-W, Han, S-W, Bartley, LE & Ronald, PCJPotNAoS 2006, 'Unique characteristics of Xanthomonas oryzae pv. oryzae AvrXa21 and implications for plant innate immunity', vol. 103, no. 49, pp. 18395-400.

13. Löytynoja, A 2014, 'Phylogeny-aware alignment with PRANK', in DJ Russell (ed.), *Multiple Sequence Alignment Methods*, Humana Press, Totowa, NJ, pp. 155-70, DOI 10.1007/978-1-62703-646-7_10, <https://doi.org/10.1007/978-1-62703-646-7_10>.

14. Page, AJ, Cummins, CA, Hunt, M, Wong, VK, Reuter, S, Holden, MT, Fookes, M, Falush, D, Keane, JA & Parkhill, J 2015, 'Roary: rapid large-scale prokaryote pan genome analysis', *Bioinformatics*, vol. 31, no. 22, pp. 3691-3.

15. Peng, Z, Hu, Y, Xie, J, Potnis, N, Akhunova, A, Jones, J, Liu, Z, White, FF & Liu, S 2016, 'Long read and single molecule DNA sequencing simplifies genome assembly and TAL effector gene analysis of Xanthomonas translucens', *BMC genomics*, vol. 17, p. 21.

16. Price, MN, Dehal, PS & Arkin, AP 2010, 'FastTree 2--approximately maximum-likelihood trees for large alignments', *PLoS One*, vol. 5, no. 3, p. e9490.

17. Seemann, T 2014, 'Prokka: rapid prokaryotic genome annotation', *Bioinformatics*, vol. 30, no. 14, pp. 2068-9.

18. Tang, J-L, Liu, Y-N, Barber, CE, Dow, JM, Wootton, JC, Daniels, MJJM & MGG, GG 1991, 'Genetic and molecular analysis of a cluster of rpf genes involved in positive regulation of synthesis of extracellular enzymes and polysaccharide in Xanthomonas campestris pathovar campestris', vol. 226, no. 3, pp. 409-17.

19. Vaser, R, Sovic, I, Nagarajan, N & Sikic, M 2017, 'Fast and accurate de novo genome assembly from long uncorrected reads', *Genome Res*, vol. 27, no. 5, pp. 737-46.

20. Vorhölter, F-J, Niehaus, K & Pühler, A 2001, 'Lipopolysaccharide biosynthesis in Xanthomonas campestris pv. campestris : a cluster of 15 genes is involved in the biosynthesis of the LPS O-antigen and the LPS core', *Molecular Genetics and Genomics*, vol. 266, no. 1, pp. 79-95.

21. Walker, BJ, Abeel, T, Shea, T, Priest, M, Abouelliel, A, Sakthikumar, S, Cuomo, CA, Zeng, Q, Wortman, J, Young, SK & Earl, AM 2014, 'Pilon: an integrated tool for comprehensive microbial variant detection and genome assembly improvement', *PLoS One*, vol. 9, no. 11, p. e112963.

22. Weber, T, Blin, K, Duddela, S, Krug, D, Kim, HU, Bruccoleri, R, Lee, SY, Fischbach, MA, Muller, R, Wohlleben, W, Breitling, R, Takano, E & Medema, MH 2015, 'antiSMASH 3.0-a comprehensive resource for the genome mining of biosynthetic gene clusters', *Nucleic Acids Res*, vol. 43, no. W1, pp. W237-43.

23. Wichmann, F, Vorholter, FJ, Hersemann, L, Widmer, F, Blom, J, Niehaus, K, Reinhard, S, Conradin, C & Kolliker, R 2013, 'The noncanonical type III secretion system of Xanthomonas translucens pv. graminis is essential for forage grass infection', *Mol Plant Pathol*, vol. 14, no. 6, pp. 576-88.

24. Wick, RR, Judd, LM, Gorrie, CL & Holt, KE 2017, 'Unicycler: Resolving bacterial genome assemblies from short and long sequencing reads', *PLOS Computational Biology*, vol. 13, no. 6, p. e1005595.

25. Wick, RR, Schultz, MB, Zobel, J & Holt, KE 2015, 'Bandage: interactive visualization of de novo genome assemblies', *Bioinformatics*, vol. 31, no. 20, pp. 3350-2.

THE CLAIMS DEFINING THE INVENTION ARE AS FOLLOWS:

1. A substantially purified or isolated endophyte strain isolated from a plant of the Poaceae family, wherein said endophyte is a strain of *Xanthomonas* sp. which provides bioprotection and/or biofertilizer phenotypes to plants into which it is inoculated.

2. An endophyte according to claim 1, wherein the bioprotection and/or biofertilizer phenotype includes production of the bioprotectant compound in the plant into which the endophyte is inoculated.

3. An endophyte according to any one of claims 1 to 2, wherein the endophyte is strain is GW as described herein and as deposited with The National Measurement Institute on 17th May 2019 with accession number V19/009902.

4. An endophyte according to any one of claims 1 to 2, wherein the endophyte is strain is SS as described herein and as deposited with The National Measurement Institute on 17th May 2019 with accession number V19/009905.

5. An endophyte according to any one of claims 1 to 2, wherein the endophyte is strain is SI as described herein and as deposited with The National Measurement Institute on 17th May 2019 with accession number V19/009909.

6. An endophyte according to any one of claims 1 to 5, wherein the plant from which the endophyte is isolated is of the Poaceae family is a pasture grass.

7. An endophyte according to claim 6, wherein the pasture grass is from the genus *Lolium* or *Festuca*.

8. An endophyte according to claim 7, wherein the pasture grass is from the species *Lolium perenne* or *Festuca arundinaceum.*

9. An endophyte according to any one of claims 1 to 8, wherein the plant into which the endophyte is inoculated includes an endophyte-free host plant or part thereof stably infected with said endophyte.

10. An endophyte according to any one of claims 1 to 9, wherein the plant into which the endophyte is inoculated is an agricultural plant species selected from one or more of forage grass, turf grass, bioenergy grass, grain crop and industrial crop.

11. An endophyte according claim 10, wherein the plant into which the endophyte is inoculated is a forage, turf or bioenergy grass selected from the group consisting of those belonging to the genera *Lolium* and *Festuca*, including *L. perenne* (perennial ryegrass), *L. arundinaceum* (tall fescue) and *L. multiflorum* (Italian ryegrass), and those belonging to the *Brachiaria-Urochloa* species complex (panic grasses), including *Brachiaria brizantha, Brachiaria decumbens, Brachiaria humidicola, Brachiaria stolonifera, Brachiaria ruziziensis, B. dictyoneura, Urochloa brizantha, Urochloa decumbens, Urochloa humidicola*, *Urochloa mosambicensis* as well as interspecific and intraspecific hybrids of *Brachiaria-Urochloa* species complex such as interspecific hybrids between *Brachiaria ruziziensis* x *Brachiaria brizantha*, *Brachiaria ruziziensis* x *Brachiaria decumbens*, [*Brachiaria ruziziensis* x *Brachiaria decumbens*] x *Brachiaria brizantha*, [*Brachiaria ruziziensis* x *Brachiaria brizantha*] x *Brachiaria decumbens*.

12. An endophyte according claim 10, wherein the plant into which the endophyte is inoculated is a grain crop or industrial crop grass selected from the group consisting of those belonging to the genus *Triticum*, including *T. aestivum* (wheat), those belonging to the genus *Hordeum*, including *H. vulgare* (barley), those belonging to the genus *Zea*, including *Z. mays* (maize or corn), those belonging to the genus *Oryza*, including *O. sativa* (rice), those belonging to the genus *Saccharum* including *S. officinarum* (sugarcane), those belonging to the genus *Sorghum* including *S. bicolor* (sorghum), those belonging to the genus *Panicum*, including *P. virgatum* (switchgrass), those belonging to the genera *Miscanthus*, *Paspalum*, *Pennisetum*, *Poa*, *Eragrostis* and *Agrostis*,

13. An endophyte according to claim 10, wherein the plant into which the endophyte is inoculated is a grain crop or industrial crop selected from the group consisting of wheat, barley, oats, chickpeas, triticale, fava beans, lupins, field peas, canola, cereal rye, vetch, lentils, millet/panicum, safflower, linseed, sorghum, sunflower, maize, canola, mungbeans, soybeans, and cotton.

14. A plant or part thereof infected with one or more endophytes according to any one of claims 1 to 10.

15. A plant, plant seed or other plant part derived from a plant or part thereof according to claim 14 and stably infected with said one or more endophytes.

16. Use of an endophyte according to any one of claims 1 to 13 to produce a plant or part thereof stably infected with said one or more endophytes.

17. A bioprotectant compound produced by an endophyte according to any one of claims 1 to 13.

18. A bioprotectant compound according to claim 17, wherein the compound is selected from siderophore xanthoferrin and xanthomonadin or a derivative, isomer and/or salt thereof.

19. A method for producing a bioprotectant compound, said method including infecting a plant with an endophyte according to any one of claims 1 to 13 and cultivating the plant under conditions suitable to produce the bioprotectant compound.

20. A method according to claim 19, wherein the conditions include a culture medium including a source of carbohydrates.

21. A method according to claim 20, wherein the source of carbohydrates is selected from one or more of the group consisting of a starch/sugar-based agar or broth, a cereal-based agar or broth, endophyte agar, Murashige and Skoog with 20 % sucrose, half V8 juice/half PDA, water agar and yeast malt extract agar.

22. A method according to any one of claims 19 to 21, wherein the method further includes isolating the bioprotectant compound from the plant or culture medium.

23. A method according to claim 22, wherein the bioprotectant compound is selected from siderophore xanthoferrin and xanthomonadin or a derivative, isomer and/or salt thereof.

ABSTRACT

The present invention relates to an endophyte strain isolated from a plant of the Poaceae family, wherein said endophyte is a strain of *Xanthomonas* sp. which provides bioprotection and/or biofertilizer phenotypes to plants into which it is inoculated. The present invention also discloses plants infected with the endophyte and related methods.

Figures

TCTACCTTTTCGTGGGGGATAACGTAGGGAAACTTACGCTAATACCGCATACGACCTTAGGGTGAAAGCGGA
GGACCTTCGGGCTTCGCGCGGATAGATGAGCCGATGTCGGATTAGCTAGTTGGCGGGGTAAAGGCCCACCA
AGGCGACGATCCGTAGCTGGTCTGAGAGGATGATCAGCCACACTGGAACTGAGACACGGTCCAGACTCCTA
CGGGAGGCAGCAGTGGGGAATATTGGACAATGGGCGCAAGCCTGATCCAGCCATGCCGCGTGGGTGAAGA
AGGCCTTCGGGTTGTAAAGCCCTTTTGTTGGGAAAGAAAAGCAGTCGGTTAATACCCGATTGTTCTGACGGT
ACCCAAAGAATAAGCACCGGCTAACTTCGTGCCAGCAGCCGCGGTAATACGAAGGGTGCAAGCGTTACTCG
GAATTACTGGGCGTAAAGCGTGCGTAGGTGGTTGTTTAAGTCCGTTGTGAAAGCCCTGGGCTCAACCTGGG
AATTGCAGTGGATACTGGGCAACTAGAGTGTGGTAGAGGATGGCGGAATTCCCGGTGTAGCAGTGAAATGC
GTAGAGATCGGGAGGAACATCTGTGGCGAAGGCGGCCATCTGGACCAACACTGACACTGAGGCACGAAAG
CGTGGGGAGCAAACAGGATTAGATACCCTGGTAGTCCACGCCCTAAACGATGCGAACTGGATGTTGGGTGC
AACTTGGCACGCAGTATCGAAGCTAACGCGTTAAGTTCGCCGCCTGGGGAGTACGGTCGCAAGACTGAAAC
TCAAAGGAATTGACGGGGGCCCGCACAAGCGGTGGAGTATGTGGTTTAATTCGATGCAACGCGAAGAACCT
TACCTGGTCTTGACATCCACGGAACTTTCCAGAGATGGATTGGTGCCTTCGGGAACCGTGAGACAGGTGCTG
CATGGCTGTCGTCAGCTCGTGTCGTGAGATGTTGGGTTAAGTCCCGCAACGAGCGCAACCCTTGTCCTTAGTT
GCCAGCACGTAATGGTGGGAACTCTAAGGAGACCGCCGGTGACAAACCGGAGGAAGGTGGGGATGACGTC
AAGTCATCATGGCCCTTACGACCAGGGCTACACACGTACTACAATGGTTAGGACAGAGGGCTGCAAACTCGC
GAGAGTAAGCCAATCCCAGAAACCTAATCTCAGTCCGGATTGGAGTCTGCAACTCGACTCCATGAAGTCGGA
ATCGCTAGTAATCGCAGATCAGCATTGCTGCGGTGAATACGTTCCCGGGCCTTGTACA

Figure 1

Appendix 1

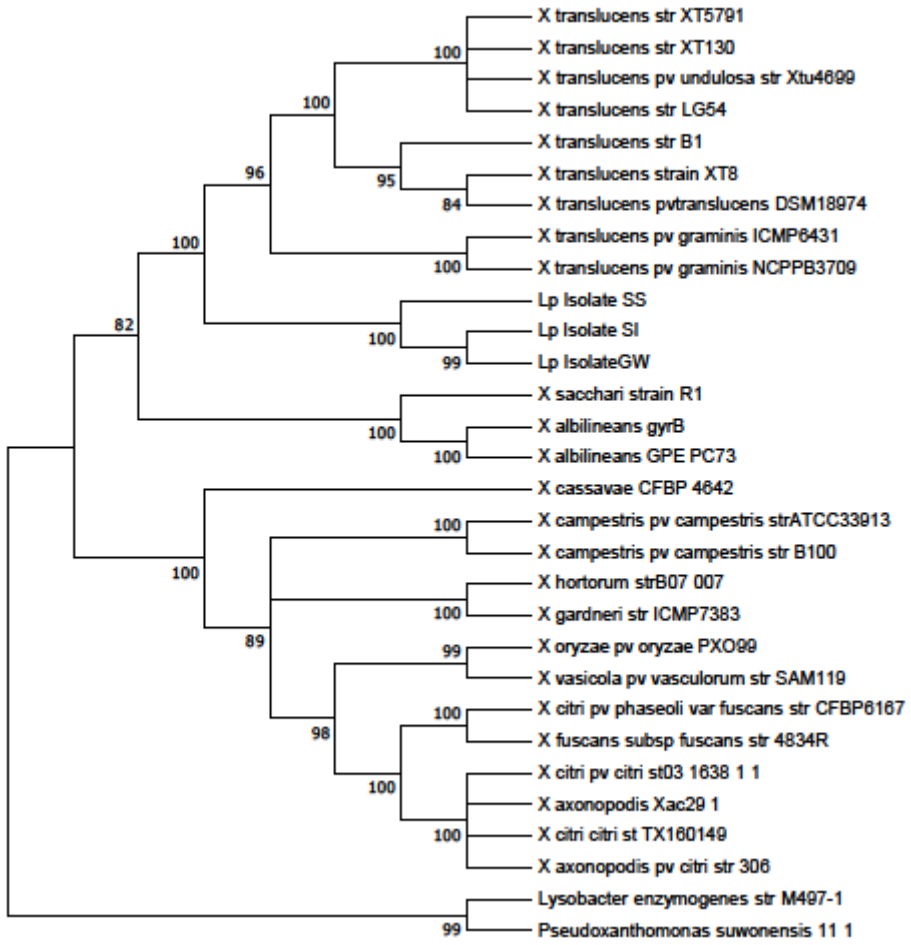


Figure 2

Appendix 1

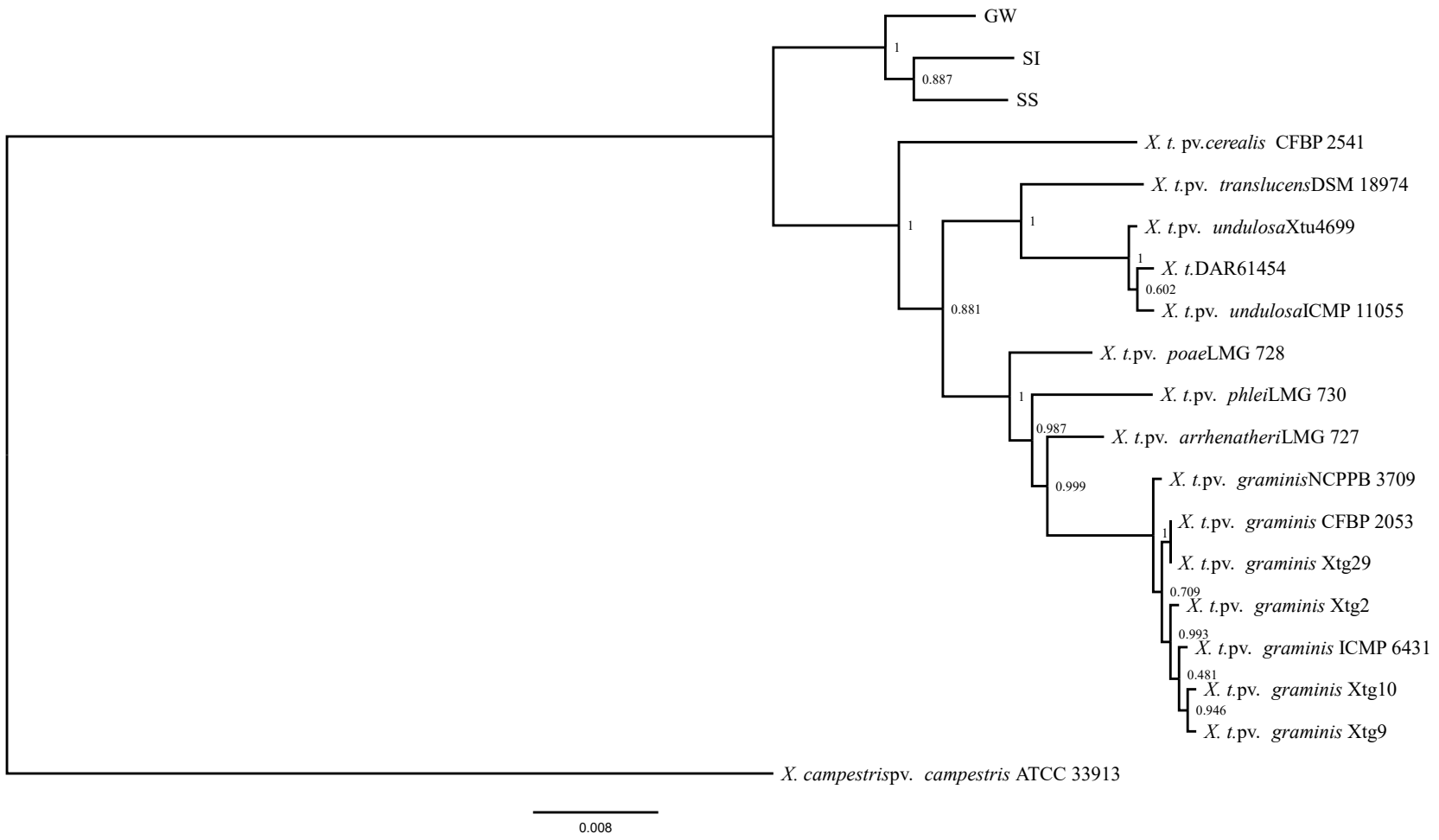


Figure 3

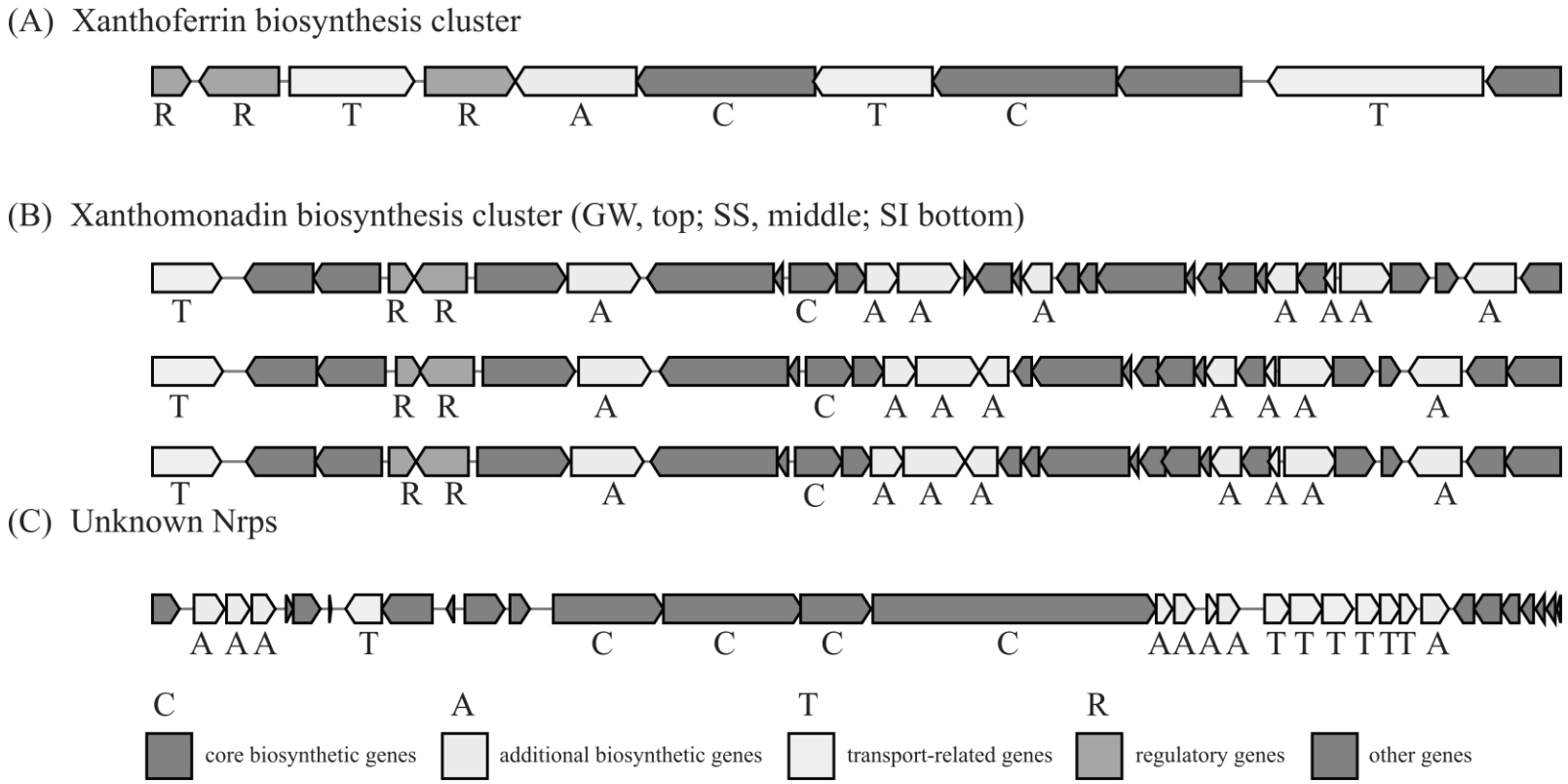

(A) Xanthoferrin biosynthesis cluster
R R T R A C T C T
(B) Xanthomonadin biosynthesis cluster (GW, top; SS, middle; SI bottom)
T R R A C A A A A A A A
T R R A C A A A A A A A
T R R A C A A A A A A A
(C) Unknown Nrps
A A A T C C C C A A A A T T T T T T A
C core biosynthetic genes
A additional biosynthetic genes
T transport-related genes
R regulatory genes
other genes

Figure 4

Appendix 1

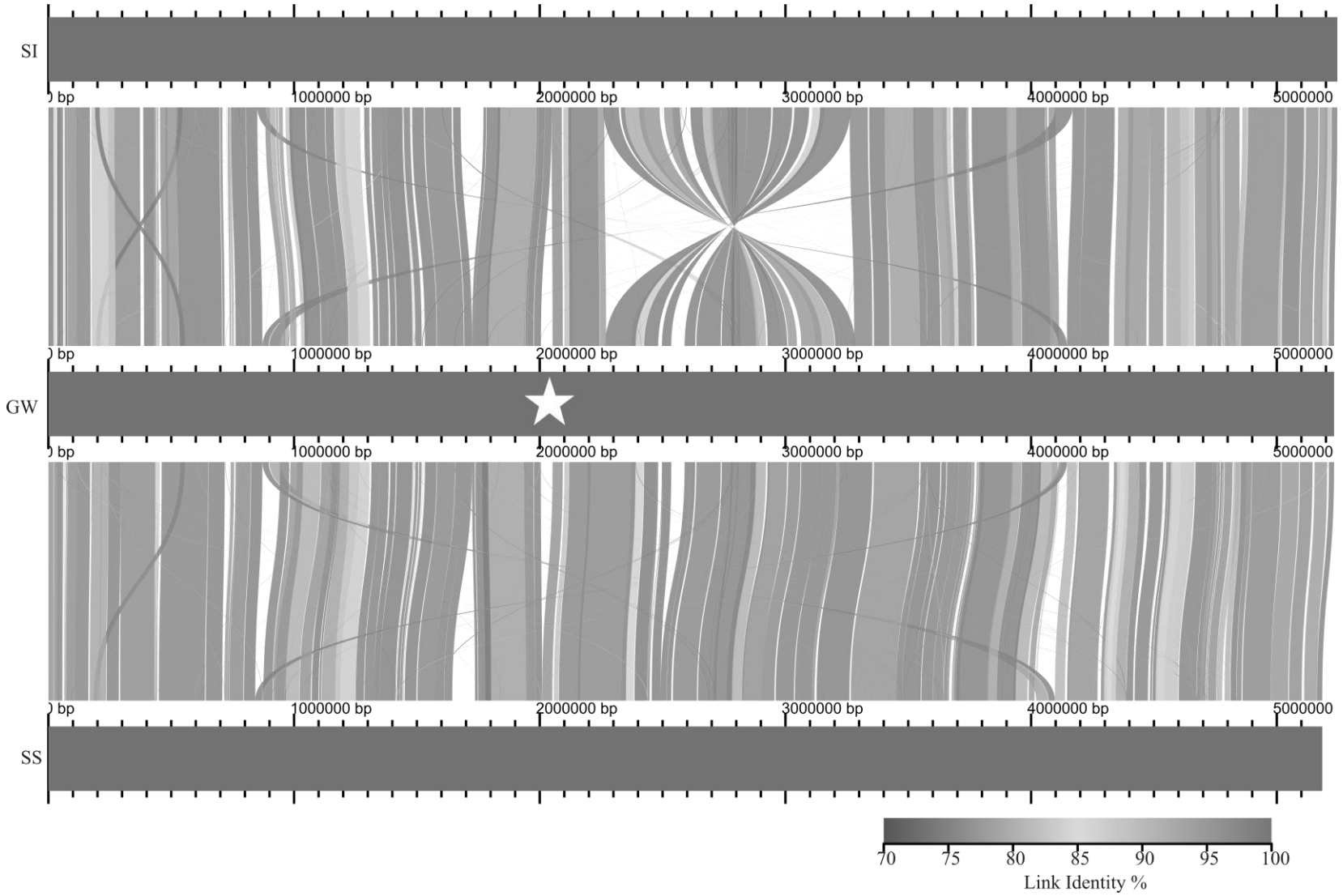


Figure 5

Appendix 1

6/17

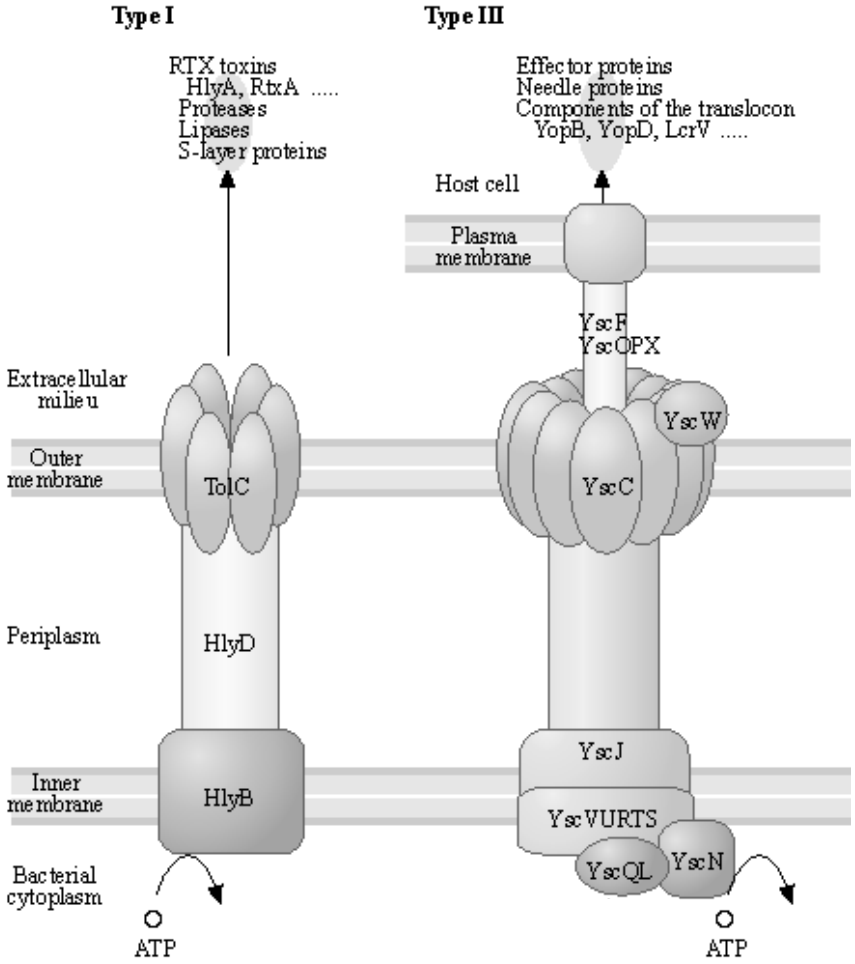
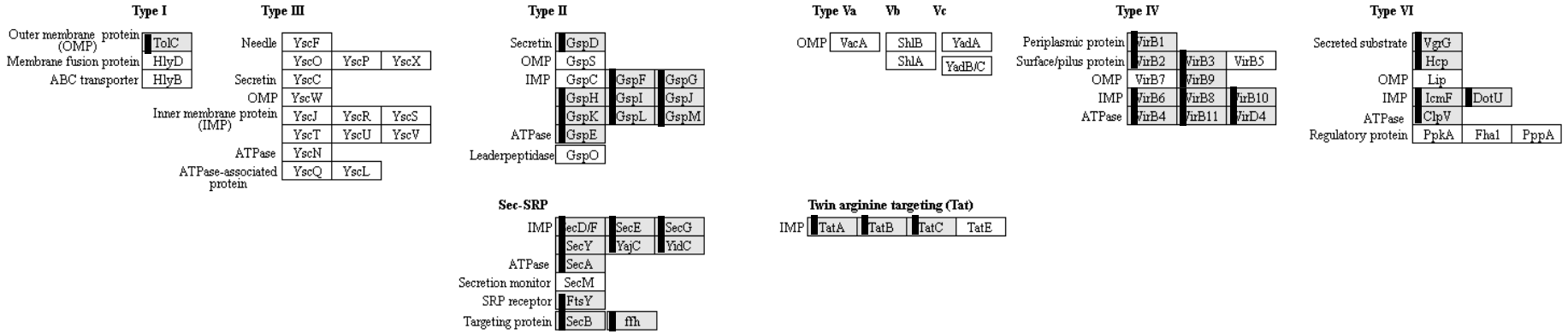
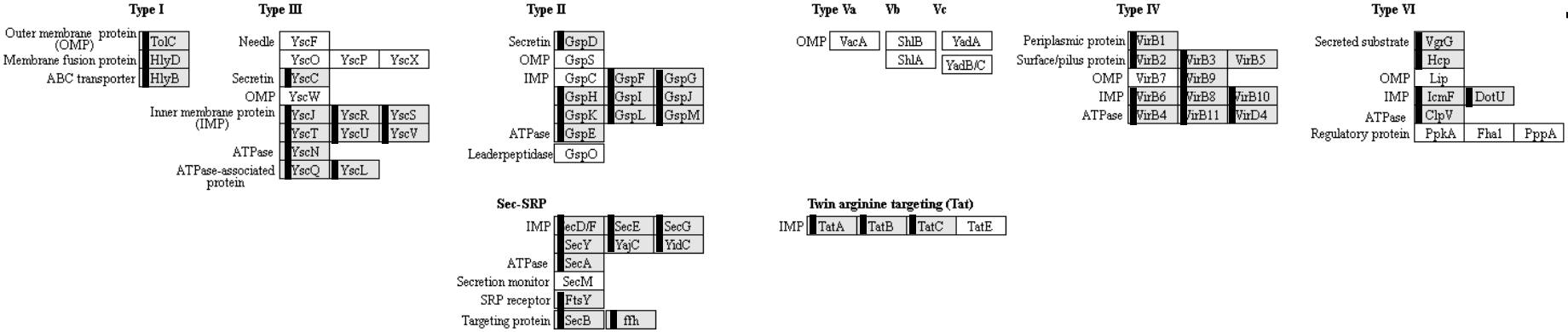


Figure 6

Appendix 1



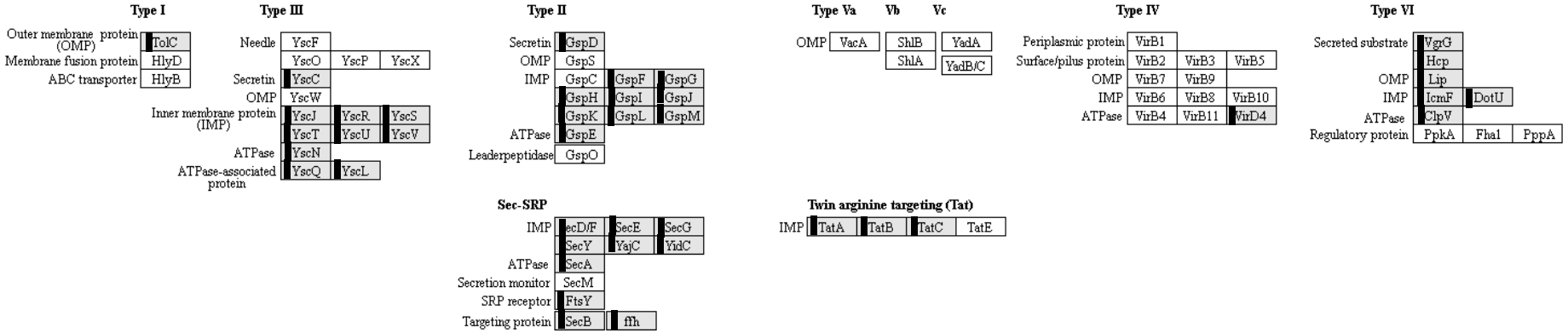
A. Strain GW



B. *X. translucens* pv. *translucens* DSM 18974 (Host: Barley)

Figure 7

Appendix 1



C. *X. translucens* pv. *undulosa* Xtu4699 (Host: Wheat)

Figure 7 (cont.)

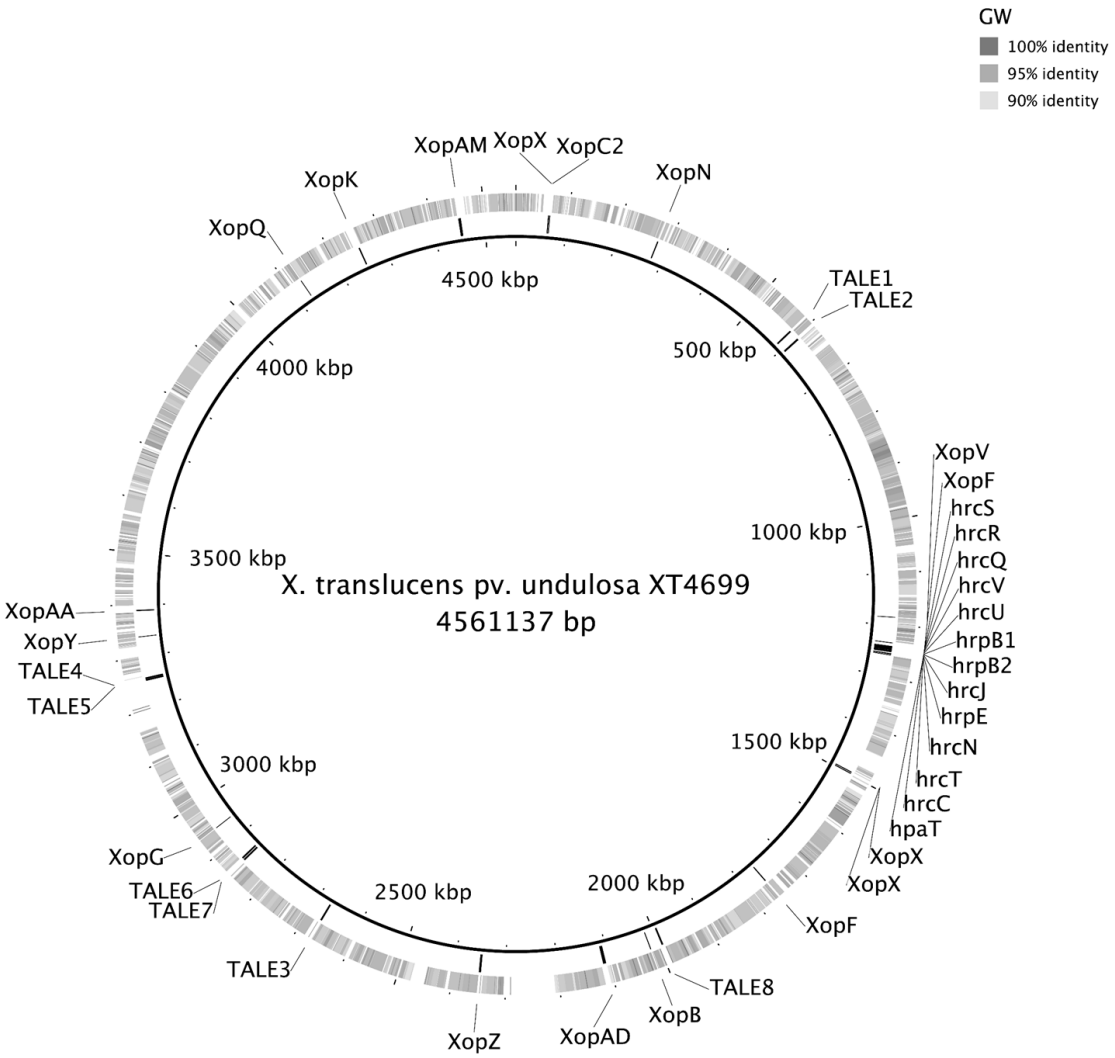

GW
100% identity
95% identity
90% identity
X. translucens pv. undulosa XT4699
4561137 bp
4500 kbp
500 kbp
1000 kbp
1500 kbp
2000 kbp
2500 kbp
3000 kbp
3500 kbp
4000 kbp
XopAM
XopX
XopC2
XopN
XopK
XopQ
TALE1
TALE2
XopV
XopF
hrcS
hrcR
hrcQ
hrcV
hrcU
hrpB1
hrpB2
hrcJ
hrpE
hrcN
hrcT
hrcC
hpaT
XopX
XopX
XopF
TALE8
XopB
XopAD
XopZ
TALE3
XopG
TALE6
TALE7
XopAA
XopY
TALE4
TALE5

Figure 8

9/17

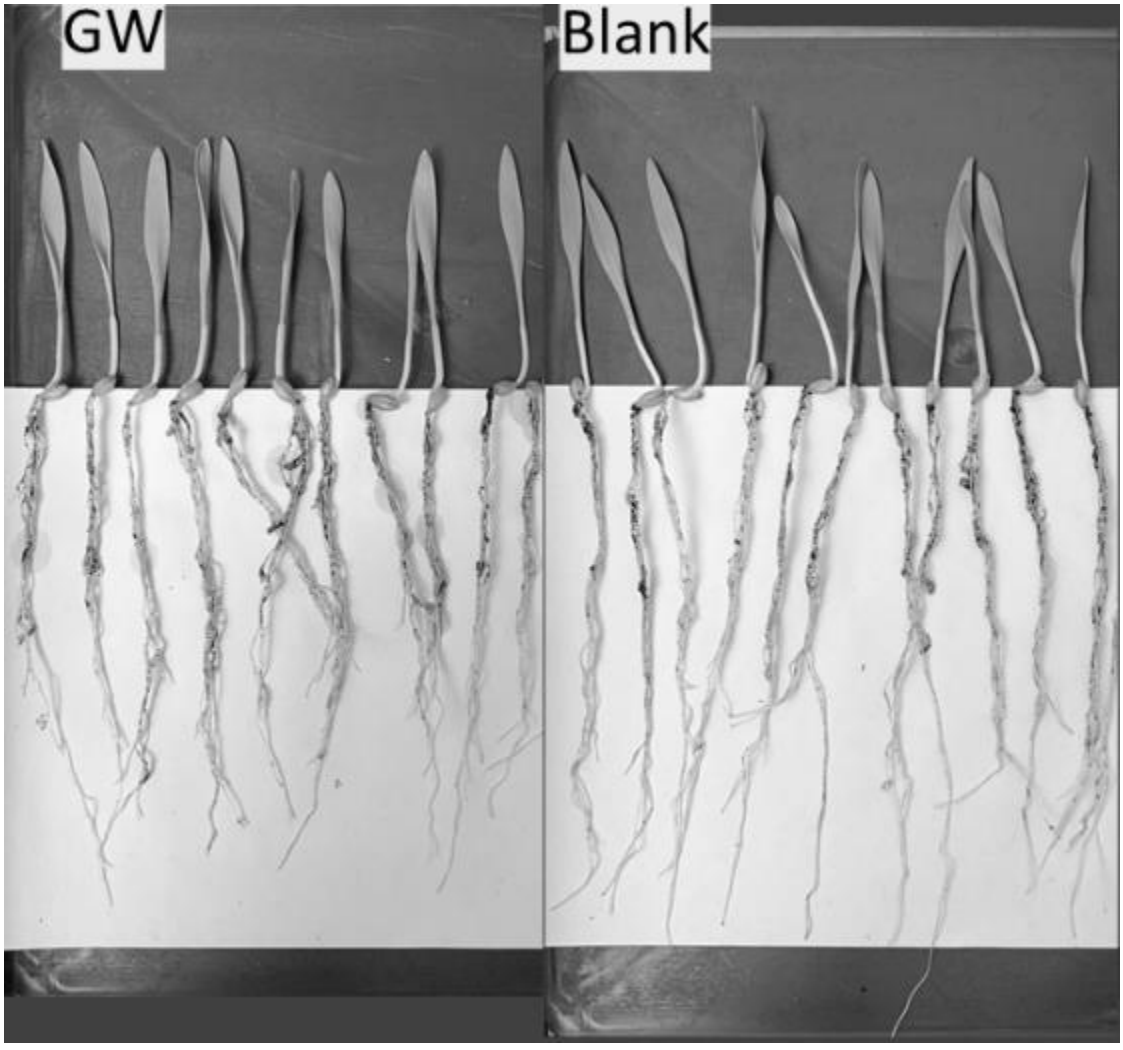


Figure 9

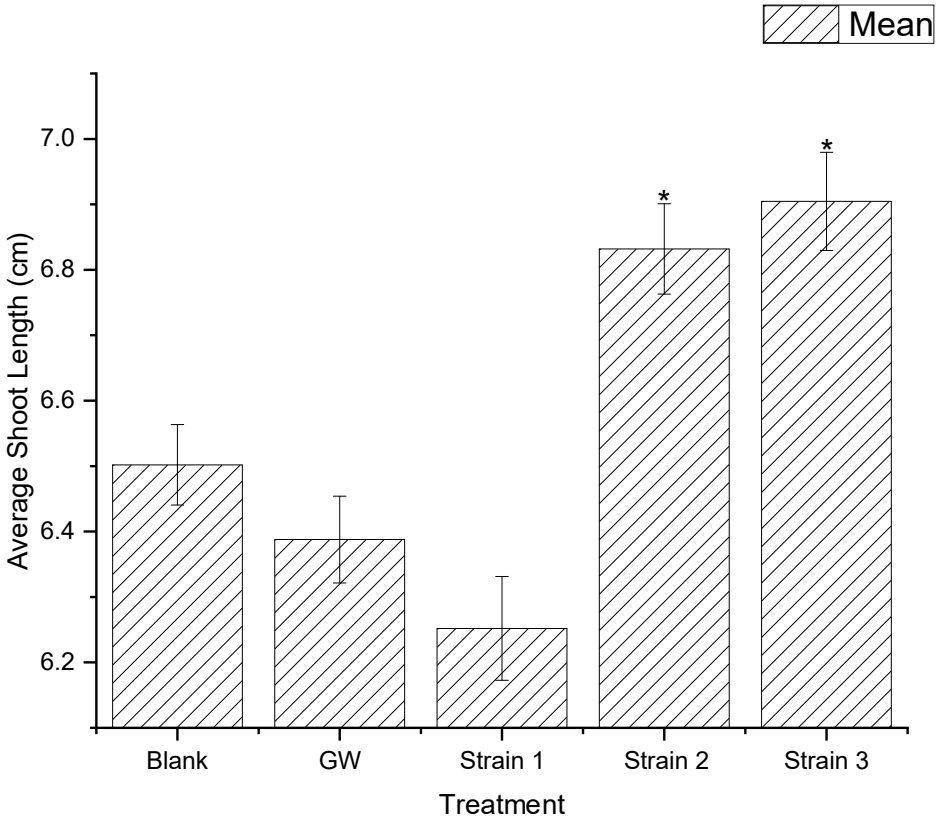

Mean
7.0
6.8
6.6
6.4
6.2
Average Shoot Length (cm)
*
*
Blank
GW
Strain 1
Strain 2
Strain 3
Treatment

Figure 10

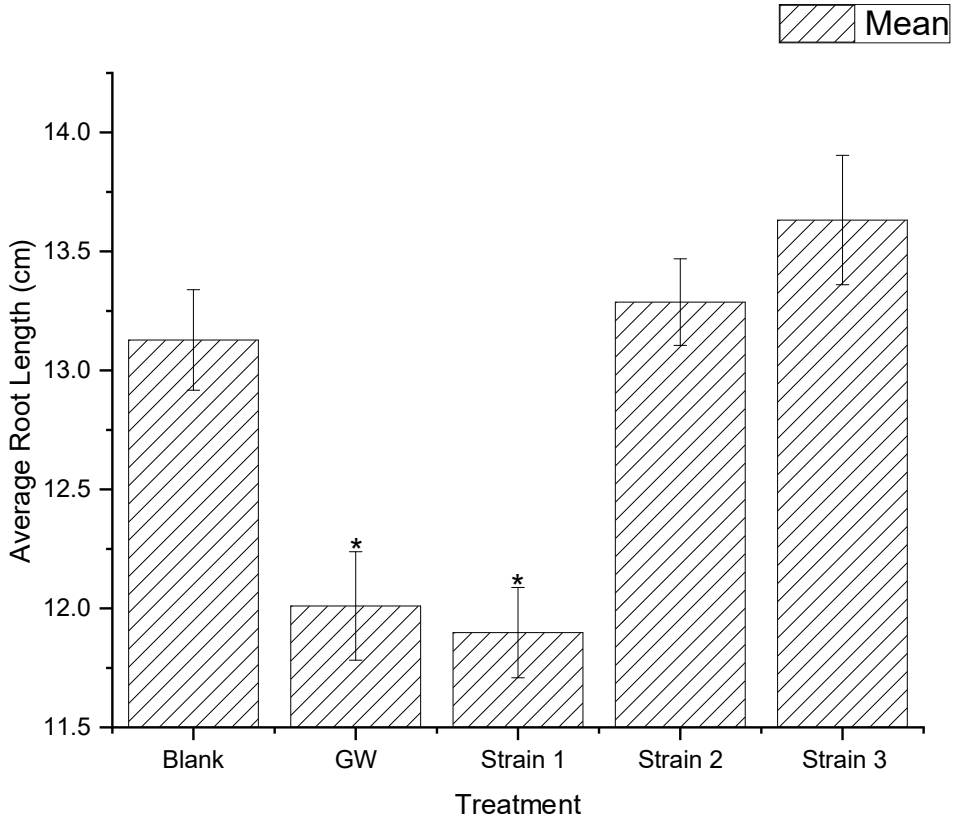

Mean
Average Root Length (cm)
14.0
13.5
13.0
12.5
12.0
11.5
*
*
Blank
GW
Strain 1
Strain 2
Strain 3
Treatment

Figure 11

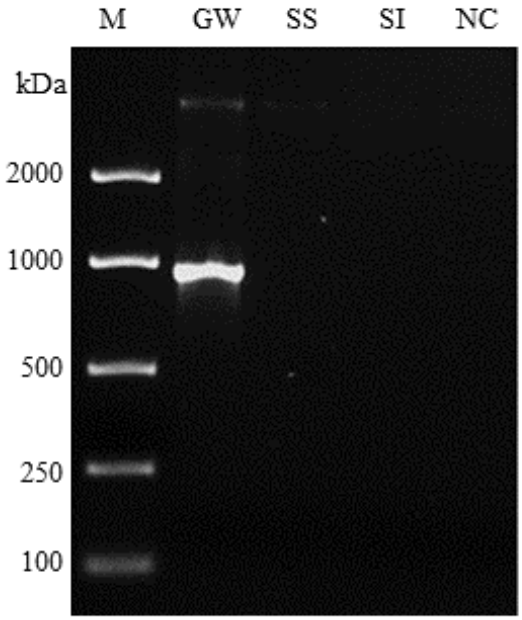


Figure 12

Appendix 1

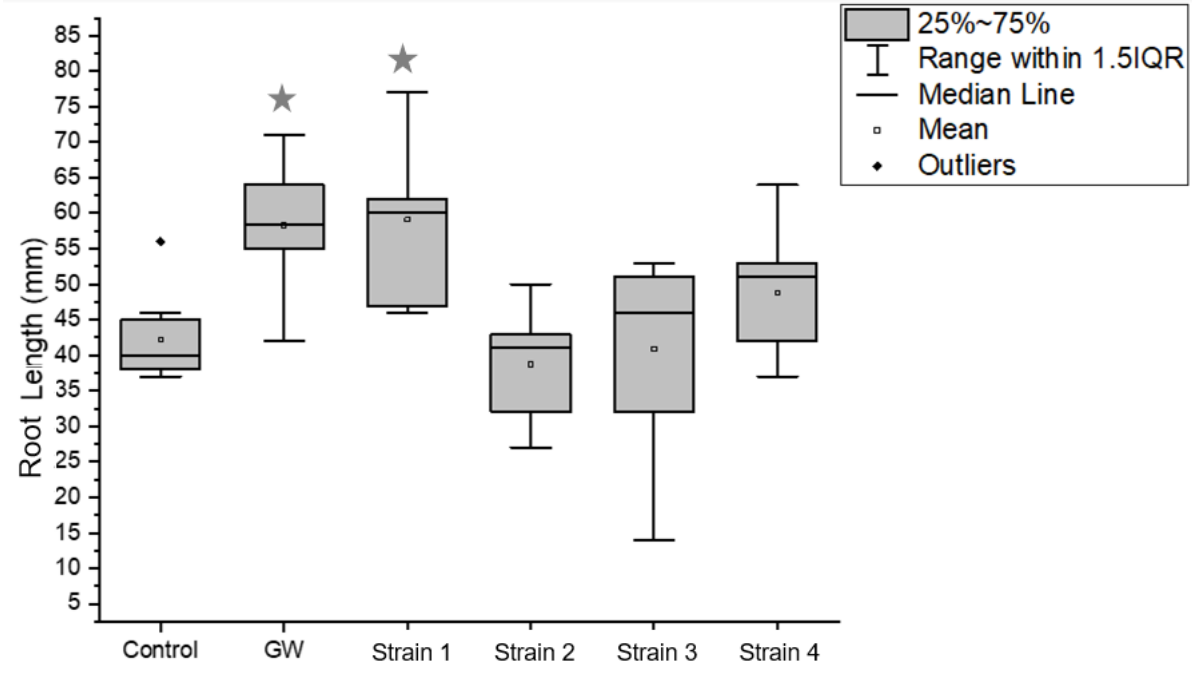


Figure 13

Appendix 1

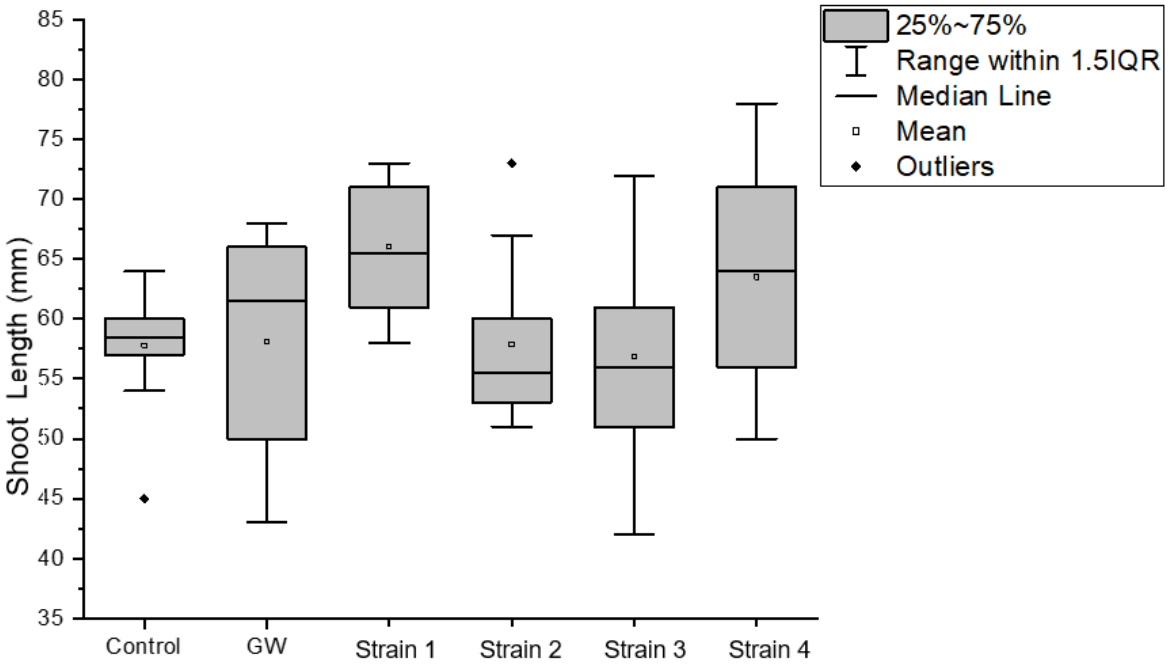


Figure 14

Appendix 1

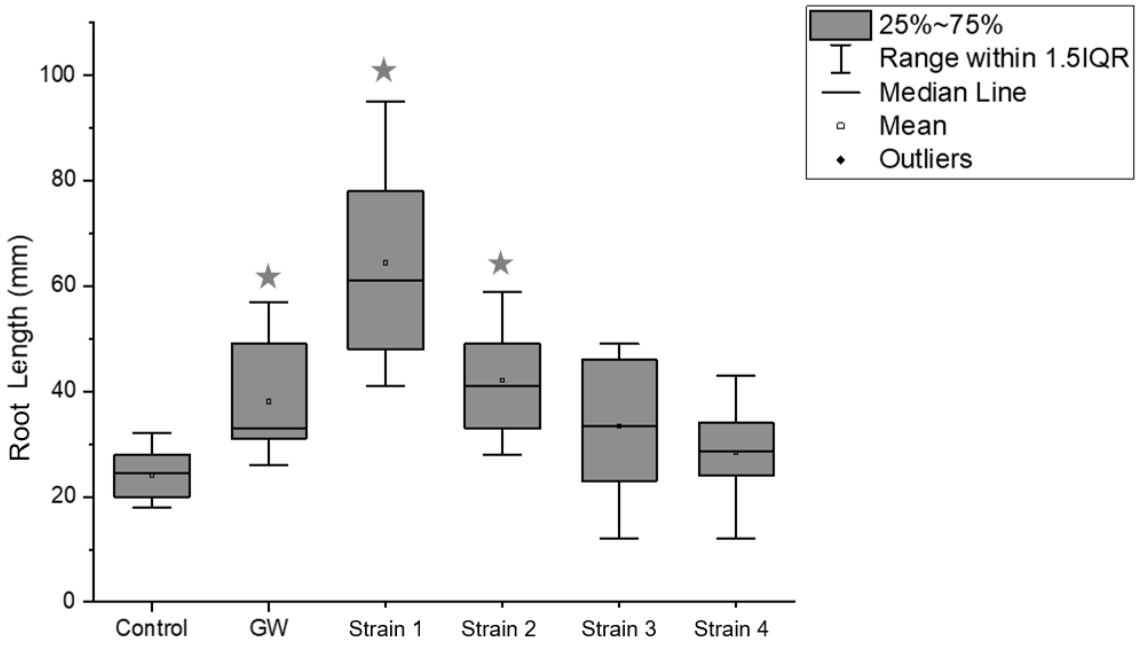


Figure 15

Appendix 1

16/17

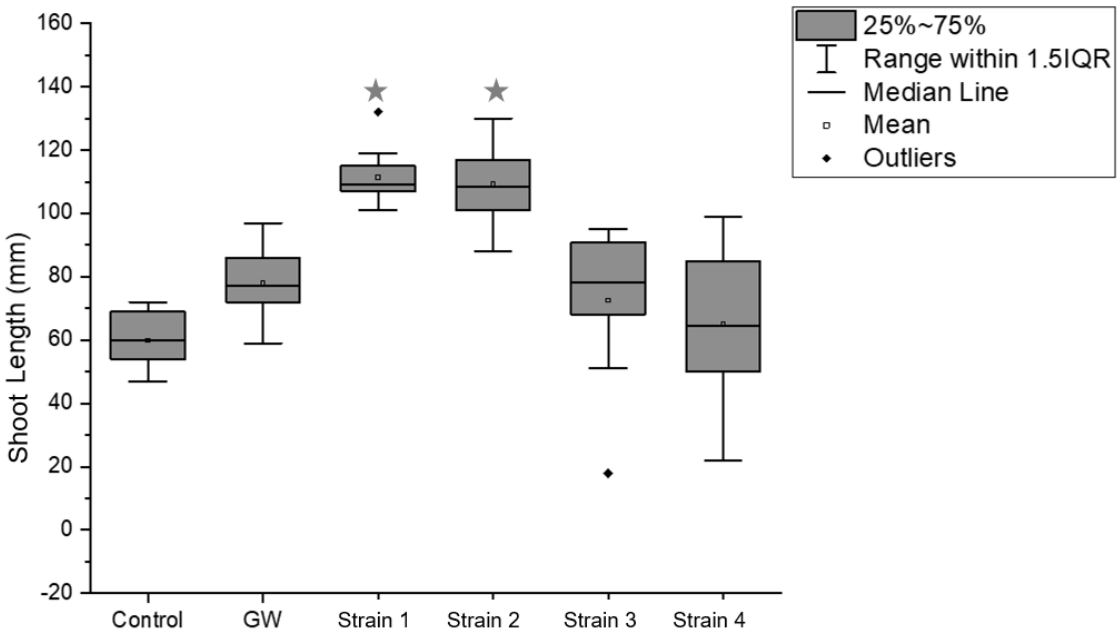


Figure 16

17/17

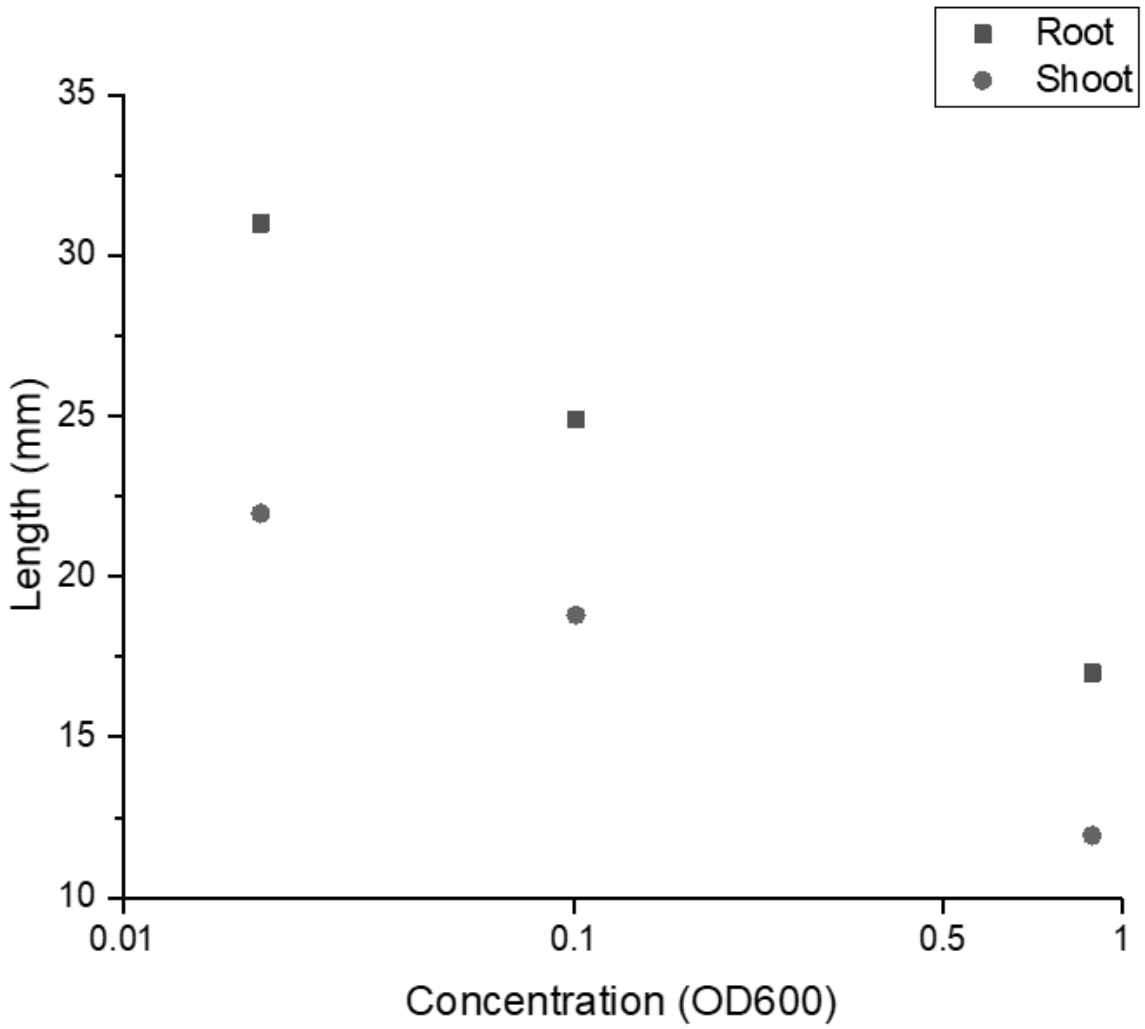


Figure 17

1.3 Patent 2 – *Erwinia gerundensis*

1.3.1 Publication details

Title: Novel bacterial strain (4)

Details: https://patentscope.wipo.int/search/en/detail.jsf?docId=WO2021011998

Stage of publication: Full patent (publicly available)

Authors: Li, Tongda; Tannenbaum, Ian Ross; Kaur, Jatinder; Krill, Christian; Sawbridge, Timothy Ivor; Mann, Ross C.; Spangenberg, German Carlos

1.3.2 Statement of contribution of joint authorship

TL performed all work relating to genomics of the strains, *in vitro* bioprotectant assays, *in vitro* biofertiliser activity assays and *in planta* inoculation assays (Example 5). TL generated all figures associated with the above works (1–6) and drafted the majority of the experimental section of the patent associated with these works. TL conducted all statistical and data analysis of these works. RM, GS, TS, JK and TL all conceptualised the patent and assisted in editing the patent. Experimental and analysis work conducted by TL is highlighted in yellow.

Appendix 1

AUSTRALIA

Patents Act 1990

PROVISIONAL SPECIFICATION

Applicants: Agriculture Victoria Services Pty Ltd
Dairy Australia Limited
Geoffrey Gardiner Dairy Foundation Limited

Title: NOVEL BACTERIAL STRAIN (4)

The invention is described in the following statement:

NOVEL BACTERIAL STRAIN (4)

Field of the Invention

The present invention relates to novel plant microbiome strains, plants infected with such strains and related methods.

Background of the Invention

Microbes represent an invaluable source of novel genes and compounds that have the potential to be utilised in a range of industrial sectors. Scientific literature gives numerous accounts of microbes being the primary source of antibiotics, immune-suppressants, anticancer agents and cholesterol-lowering drugs, in addition to their use in environmental decontamination and in the production of food and cosmetics.

A relatively unexplored group of microbes known as endophytes, which reside e.g. in the tissues of living plants, offer a particularly diverse source of novel compounds and genes that may provide important benefits to society, and in particular, agriculture.

Endophytes may be fungal or bacterial. Endophytes often form mutualistic relationships with their hosts, with the endophyte conferring increased fitness to the host, often through the production of defence compounds. At the same time, the host plant offers the benefits of a protected environment and nutriment to the endophyte.

Important forage grasses perennial ryegrass (*Lolium perenne*) are commonly found in association with fungal and bacterial endophytes. However, there remains a general lack of information and knowledge of the endophytes of these grasses as well as of methods for the identification and characterisation of novel endophytes and their deployment in plant improvement programs.

Knowledge of the endophytes of perennial ryegrass may allow certain beneficial traits to be exploited in enhanced pastures, or lead to other agricultural advances, e.g. to the benefit of sustainable agriculture and the environment.

There exists a need to overcome, or at least alleviate, one or more of the difficulties or deficiencies associated with the prior art.

Summary of the Invention

In one aspect, the present invention provides a substantially purified or isolated endophyte strain isolated from a plant of the Poaceae family, wherein said endophyte is a strain of *Erwinia gerundensis* which provides bioprotection and/or biofertilizer phenotypes to plants into which it is inoculated. In a preferred embodiment, the *Erwinia gerundensis* strain may be strain AR as described herein and as deposited with The National Measurement Institute on 17th May 2019 with accession number V19/009908.

As used herein the term “endophyte” is meant a bacterial or fungal strain that is closely associated with a plant. By “associated with” in this context is meant that the bacteria or fungus lives on, in or in close proximity to a plant. For example, it may be endophytic, for example living within the internal tissues of a plant, or epiphytic, for example growing externally on a plant.

As used herein the term “substantially purified” is meant that an endophyte is free of other organisms. The term includes, for example, an endophyte in axenic culture. Preferably, the endophyte is at least approximately 90 % pure, more preferably at least approximately 95 % pure, even more preferably at least approximately 98 % pure, even more preferably at least approximately 99 % pure.

As used herein the term ‘isolated’ means that an endophyte is removed from its original environment (e.g. the natural environment if it is naturally occurring). For example, a naturally occurring endophyte present in a living plant is not isolated, but the same endophyte separated from some or all of the coexisting materials in the natural system, is isolated.

As used herein the term “bioprotection and/or biofertilizer” means that the endophyte possesses genetic and/or metabolic characteristics that result in a beneficial phenotype in a plant harbouring, or otherwise associated with, the endophyte. Such beneficial properties include improved resistance to pests and/or diseases, improved tolerance to water and/or nutrient stress, enhanced biotic stress tolerance, enhanced drought tolerance, enhanced water use efficiency, reduced toxicity and enhanced vigour in the plant with which the endophyte is associated, relative to an organism not harbouring the endophyte or harbouring a control endophyte such as standard toxic (ST) endophyte.

The pests and/or diseases may include, but not limited to, fungal and/or bacterial pathogens, preferably fungal. In a particularly preferred embodiment, the endophyte may result in the production of the bioprotectant compound in the plant with which it is associated.

As used herein, the term ‘bioprotectant compound’ is meant as a compound that provides or aids bioprotection to the plant with which it is associated against pests and/or diseases, such as bacterial and/or fungal pathogens. A bioprotectant compound may also be known as a ‘biocidal compound’.

In a particularly preferred embodiment, the endophyte produces a bioprotectant compound and provides bioprotection to the plant against bacterial and/or fungal pathogens. The terms bioprotectant, bioprotective and bioprotection (or any other variations) may be used interchangeably herein.

The endophyte may be suitable as a biofertilizer to improve the availability of nutrients to the plant with which the endophyte is associated, including but not limited to improved tolerance to nutrient stress.

The nutrient stress may be lack of or low amounts of a nutrient such as phosphate and/or nitrogen. The endophyte is capable of growing in conditions such as low nitrogen and/or low phosphate and enable these nutrients to be available to the plant with which the endophyte is associated.

The endophyte may result in the production of organic acids and/or the solubilisation of phosphate in the organism with which it is associated and/or provide a source of Phosphate to the plant.

Alternatively, or in addition, the endophyte is capable of nitrogen fixation. Thus, if an endophyte is capable of nitrogen fixation, the organism in which the endophyte is associated is capable of growing in low nitrogen conditions and/or provide a source of Nitrogen to the plant.

In a particularly preferred embodiment, the endophyte provides the ability of the organism to grow in low nitrogen.

Alternatively, or in addition, the endophyte is capable of nitrogen fixation. Thus, if an endophyte is capable of nitrogen fixation, the plant in which the endophyte is associated is capable of growing in low nitrogen conditions.

As used herein the term “plant of the Poaceae family” is a grass species, particularly a pasture grass such as ryegrass (*Lolium*) or fescue (*Festuca*), more particularly perennial ryegrass (*Lolium perenne* L.) or tall fescue (*Festuca arundinaceum*, otherwise known as *Lolium arundinaceum*).

In another aspect, the present invention provides a plant or part thereof infected with an endophyte as hereinbefore described. In preferred embodiments, the plant or part thereof infected with the endophyte may produce a bioprotectant compound. Also in preferred embodiments, the plant or part thereof includes an endophyte-free host plant or part thereof stably infected with said endophyte.

The plant inoculated with the endophyte may be a grass or non-grass plant suitable for agriculture, specifically a forage, turf, or bioenergy grass, or a grain crop or industrial crop.

The forage, turf or bioenergy grass may be those belonging to the *Brachiaria-Urochloa* species complex (panic grasses), including *Brachiaria brizantha, Brachiaria decumbens, Brachiaria humidicola, Brachiaria stolonifera, Brachiaria ruziziensis, B. dictyoneura, Urochloa brizantha, Urochloa decumbens, Urochloa humidicola*, *Urochloa mosambicensis* as well as interspecific and intraspecific hybrids of *Brachiaria-Urochloa* species complex such as interspecific hybrids between *Brachiaria ruziziensis* x *Brachiaria brizantha*, *Brachiaria ruziziensis* x *Brachiaria decumbens*, [*Brachiaria ruziziensis* x *Brachiaria decumbens*] x *Brachiaria brizantha*, [*Brachiaria ruziziensis* x *Brachiaria brizantha*] x *Brachiaria decumbens*.

The forage, turf or bioenergy grass may also be those belonging to the genera *Lolium* and *Festuca*, including *L. perenne* (perennial ryegrass) and *L. arundinaceum* (tall fescue) and *L. multiflorum* (Italian ryegrass).

The grain crop or industrial crop may be a non-grass species, for example, any of soybeans, cotton and grain legumes, such as lentils, field peas, fava beans, lupins and chickpeas, as well as oilseed crops, such as canola.

Thus, the grain crop or industrial crop species may be selected from the group consisting of wheat, barley, oats, chickpeas, triticale, fava beans, lupins, field peas, canola, cereal rye, vetch, lentils, millet/panicum, safflower, linseed, sorghum, sunflower, maize, canola, mungbeans, soybeans, and cotton.

The grain crop or industrial crop grass may be those belonging to the genus *Triticum*, including *T. aestivum* (wheat), those belonging to the genus *Hordeum*, including *H. vulgare* (barley), those belonging to the genus *Zea*, including *Z. mays* (maize or corn), those belonging to the genus *Oryza*, including *O. sativa* (rice), those belonging to the genus *Saccharum* including *S. officinarum* (sugarcane), those belonging to the genus *Sorghum* including *S. bicolor* (sorghum), those belonging to the genus *Panicum*, including *P. virgatum* (switchgrass), and those belonging to the genera *Miscanthus*, *Paspalum*, *Pennisetum*, *Poa*, *Eragrostis* and *Agrostis*.

A plant or part thereof may be infected by a method selected from the group consisting of inoculation, breeding, crossing, hybridisation, transduction, transfection, transformation and/or gene targeting and combinations thereof.

Without wishing to be bound by theory, it is believed that the endophyte of the present invention may be transferred through seed from one plant generation to the next. The endophyte may then spread

or locate to other tissues as the plant grows, i.e. to roots. Alternatively, or in addition, the endophyte may be recruited to the plant root, e.g. from soil, and spread or locate to other tissues.

Thus, in a further aspect, the present invention provides a plant, plant seed or other plant part derived from a plant or part thereof as hereinbefore described. In preferred embodiments, the plant, plant seed or other plant part may produce a bioprotective compound.

In another aspect, the present invention provides the use of an endophyte as hereinbefore described to produce a plant or part thereof stably infected with said endophyte. The present invention also provides the use of an endophyte as hereinbefore described to produce a plant or part thereof as hereinbefore described.

In another aspect, the present invention provides a bioprotective compound produced by an endophyte as hereinbefore described, or a derivative, isomer and/or a salt thereof.

The bioprotective compound may be produced by the endophyte when associated with a plant, e.g. a plant of the Poaceae family as described above. Thus, in another aspect, the present invention provides a method for producing a bioprotective compound, said method including infecting a plant with an endophyte as hereinbefore described and cultivating the plant under conditions suitable to produce the bioprotective compound. The endophyte-infected plant or part thereof may be cultivated by known techniques. The person skilled in the art may readily determine appropriate conditions depending on the plant or part thereof to be cultivated.

The bioprotective compound may also be produced by the endophyte when it is not associated with a plant. Thus, in yet another aspect, the present invention provides a method for producing a bioprotective compound, said method including culturing an endophyte as hereinbefore described, under conditions suitable to produce the bioprotective compound.

The conditions suitable to produce the bioprotective compound may include a culture medium including a source of carbohydrates. The source of carbohydrates may be a starch/sugar-based agar or broth such as potato dextrose agar, potato dextrose broth or half potato dextrose agar or a cereal-based agar or broth such as oatmeal agar or oatmeal broth. Other sources of carbohydrates may include endophyte agar, Murashige and Skoog with 20 % sucrose, half V8 juice/half PDA, water agar and yeast malt extract agar. The endophyte may be cultured under aerobic or anaerobic conditions and may be cultured in a bioreactor.

In a preferred embodiment of this aspect of the invention, the method may include the further step of isolating the bioprotectant compound from the plant or culture medium.

The endophyte of the present invention is capable of nitrogen fixation. Thus, in yet another aspect, the present invention provides a method of growing the plant in low nitrogen containing medium, said method including infecting a plant with an endophyte as hereinbefore described, and cultivating the plant. Preferably, the low nitrogen medium is low nitrogen containing soil.

In yet a further aspect, the present invention provides a method of increasing nitrogen use efficiency or increasing nitrogen availability to a plant, said method including infecting a plant with an endophyte as hereinbefore described, and cultivating the plant.

In yet another aspect, the present invention provides a method of reducing nitrogen levels in soil, said method including infecting a plant with an endophyte as hereinbefore described, and cultivating the plant in the soil.

In yet another aspect, the present invention provides a method of increasing phosphate use efficiency or increasing phosphate solubilisation by a plant, said method including infecting a plant with an endophyte as hereinbefore described, and cultivating the plant.

In yet another aspect, the present invention provides a method of reducing phosphate levels in soil, said method including infecting a plant with an endophyte as hereinbefore described, and cultivating the plant in the soil.

The endophyte-infected plant or part thereof may be cultivated by known techniques. The person skilled in the art may readily determine appropriate conditions depending on the plant or part thereof to be cultivated.

The production of a bioprotectant compound has particular utility in agricultural plant species, in particular, forage, turf, or bioenergy grass species, or grain crop species or industrial crop species. These plants may be cultivated across large areas of e.g. soil where the properties and biological processes of the endophyte as hereinbefore described and/or bioprotectant compound produced by the endophyte may be exploited at scale.

The part thereof of the plant may be, for example, a seed.

In preferred embodiments, the plant is cultivated in the presence of soil phosphate and/or nitrogen, alternatively or in addition to applied phosphate and/or nitrogen. The applied phosphate and/or applied nitrogen may be by way of, for example, fertiliser. Thus, preferably, the plant is cultivated in soil.

In preferred embodiments, the endophyte may be a *Erwinia gerundensis* strain AR as described herein and as deposited with The National Measurement Institute on 17th May 2019 with accession number V19/009908.

Preferably, the plant is a forage, turf, bioenergy grass species or, grain crop or industrial crop species, as hereinbefore described.

The part thereof of the plant may be, for example, a seed.

In preferred embodiments, the plant is cultivated in the presence of soil phosphate and/or applied phosphate. The applied phosphate may be by way of, for example, fertiliser. Thus, preferably, the plant is cultivated in soil.

Alternatively, or in addition, the plant is cultivated in the presence of soil nitrogen and/or applied nitrogen. The applied nitrogen may be by way of, for example, fertiliser. Thus, preferably, the plant is cultivated in soil.

The present invention will now be more fully described with reference to the accompanying Examples and drawings. It should be understood, however, that the description following is illustrative only and should not be taken in any way as a restriction on the generality of the invention described above.

Brief Description of the Drawings/Figures

Figure 1 - 16S Amplicon sequence of novel bacterial strain AR.

Figure 2 - Phylogeny of *Erwinia* spp., *Pantoea* spp. and novel bacterial strain AR. This maximum-likelihood tree was inferred based on 103 genes conserved among 10 genomes. Values shown next to branches were the local support values calculated using 1000 resamples with the Shimodaira-Hasegawa test.

Figure 3 - Bioprotection bioassay indicating the growth of 11 bacterial stains (including *Erwinia gerundensis* novel bacterial strain AR, star) against 6 plant pathogenic fungi, *Fusarium verticillioides* (10 days post inoculation, dpi), *Bipolaris gossypina* (7 dpi), *Sclerotinia rolfsii* (5 dpi), *Drechslera brizae* (8 dpi), *Phoma sorghina* (9 dpi) and *Microdochium nivale* (6 dpi). Bars represent the mean diameter of fungal colonies from three replicate plates of each treatment. Different superscript letters indicate significant differences ($P < 0.05$) between treatments.

Figure 4 - Biofertiliser activity (*in vitro*) of the *Erwinia gerundensis* novel bacterial strain AR on semi-solid NFb medium. Activity recorded as a change in absorbance at 615 nm over 84 hours (12 hour intervals) relative to absorbance at 615 nm at time 0 hours. The *Erwinia gerundensis* novel bacterial strain AR was compared to an *Escherichia coli* negative control strain, and a no growth control (NGC – NFb media only).

Figure 5 - Image of 5 day old seedlings inoculated with the *Erwinia gerundensis* novel bacterial strain AR and an untreated control.

Figure 6 - Average shoot and root length of barley seedlings inoculated with the *Erwinia gerundensis* novel bacterial strain AR and an untreated control (blank), and grown for 5 days. The root length was significantly different ($P < 0.05$) between the two treatments, but not the shoot length.

Figure 7 - Average root length of barley seedlings inoculated with bacterial strains of *Erwinia gerundensis* (strain AR) and non-*Erwinia* strains (Strain 1, 2, 3, 4) and grown for 4 days on media under nitrogen limiting conditions. The star indicates significant difference in the mean at $P < 0.05$ between the control and the bacterial strains.

Figure 8 - Average shoot length of barley seedlings inoculated with bacterial strains of *Erwinia gerundensis* (strain AR) and non-*Erwinia* strains (Strain 1, 2, 3, 4) and grown for 4 days on media under nitrogen limiting conditions. The star indicates significant difference in the mean at $P < 0.05$ between the control and the bacterial strains.

Detailed Description of the Embodiments

Discovery and characterisation of plant associated *Erwinia gerundensis* novel bacterial strains providing bioprotection and biofertilizer phenotypes to plants.

The novel plant associated *Erwinia gerundensis* bacterial strain AR has been isolated from perennial ryegrass (*Lolium perenne*) plants. It displays the ability to inhibit the growth of plant fungal pathogens, grow under low N conditions in plate assays, and have some plant growth promotion abilities. The genome of the *Erwinia gerundensis* novel bacterial strain AR has been sequenced and is shown to be novel, related to the species *Erwinia gerundensis.* This novel bacterial strain has been used to inoculate barley (*Hordeum vulgare*) seeds under glasshouse conditions and has been demonstrated not to cause disease in these barley plants. These barley plants are also able to produce seed. Novel bacterial strain AR also enhances root and shoot growth in nitrogen limiting conditions. Overall, novel plant associated *Erwinia gerundensis* novel bacterial strain AR offer both bioprotectant and biofertilizer activity.

Example 1 – Isolation of Bacterial Strains

Seed associated bacterial strains

Seeds from perennial ryegrass (*Lolium perenne*) were surface-sterilised by soaking in 80 % ethanol for 3 mins, then washing 5 times in sterile distilled water. The seeds were then plated onto sterile filter paper soaked in sterile water in sterile petri dishes. These plates were stored at room temperature in the dark to allow seedlings to germinate for 1 – 2 weeks. Once the seedlings were of sufficient size, the plants were harvested. In harvesting, the remaining seed coat was discarded, and the aerial tissue and root tissue were harvested. The plant tissues were submerged in sufficient Phosphate Buffered Saline (PBS) to completely cover the tissue, and ground using a Qiagen TissueLyser II, for 1 minute at 30 Hertz. A 10 µL aliquot of the macerate was added to 90 µL of PBS. Subsequent 1 in 10 dilutions of the 10^{-1} suspension were used to create additional 10^{-2} to 10^{-4} suspensions. Once the suspensions were well mixed 50 µL aliquots of each suspension were plated onto Reasoners 2 Agar (R2A) for growth of bacteria. Dilutions that provided a good separation of bacterial colonies were subsequently used for isolation of individual bacterial colonies through re-streaking of single bacterial colonies from the dilution plates onto single R2A plates to establish a pure bacterial colony.

Mature plant associated bacterial strains

Leaf and root tissue were harvested from mature plants grown in the field or grown in pots in a greenhouse. Root tissue was washed in PBS buffer to remove soil particles and sonicated (10 mins) to remove the rhizosphere. The harvested tissues were placed into sufficient PBS to completely cover the tissue and processed as per the previous section to isolate pure bacterial cultures.

Around 300 bacterial strains were obtained from sterile seedlings, and 300 strains from mature plants. The novel bacterial strain AR was collected from seed of perennial ryegrass.

Example 2 – Identification of *Erwinia gerundensis* novel bacterial strain

Amplicon (16S rRNA gene) Sequencing

A phylogenetic analysis of the novel bacterial strain AR was undertaken by sequence homology comparison of the 16S rRNA gene. The novel bacterial strain AR was grown overnight in Reasoners 2 Broth (R2B) media. DNA was extracted from pellets derived from the overnight culture using a DNeasy Blood and Tissue kit (Qiagen) according to manufacturer's instructions. The 16S rRNA gene amplification used the following PCR reagents: 14.8 µL H_2O, 2.5 µL 10× reaction buffer, 0.5 µL 10 mM dNTPs, 2.5 µL each of the 5 µM 27F primer (5'- AGAGTTTGATCMTGGCTCAG -3') and 5 µM

reverse primers 1492R (5'- GGTTACCTTGTTACGACTT -3'), 0.2 µL of Immolase enzyme, and template to a final volume of 25 µL. The PCR reaction was then run in an Agilent Surecycler 8800 (Applied Biosystems) with the following program; a denaturation step at 94 °C for 15 min; 35 cycles of 94 °C for 30 sec, 55 °C for 10 sec, 72 °C 1 min; and a final extension step at 72 °C for 10 min.

Shrimp alkaline phosphatase (SAP) exonuclease was used to purify the 16S rRNA gene PCR amplicon. The SAP amplicon purification used the following reagents: 7.375 µL H_2O, 2.5 µL 10× SAP, and 0.125 µL Exonuclease I. The purification reaction was incubated at 37 °C for 1 hr, followed by 15 min at 80 °C to deactivate the exonuclease.

The purified 16S rRNA gene amplicon was sequenced using the BigDye® Terminator v3.1 Cycle Sequencing Kit (Thermofisher) with the following reagents; 10.5 µL H_2O, 3.5 µL 5× Seq buffer, 0.5 µL BigDye®, 2.5 µL of either the 3.2 µM Forward (27F) and 3.2 µM Reverse primers (1492R), and 4.5 µL of PCR amplicon as template, to a final reaction volume of 20 µL. The sequencing PCR reaction was then run in an Agilent Surecycler 8800 (Applied Biosystems) with the following program; denaturation step at 94 °C for 15 min; followed by 35 cycles of 94 °C for 30 sec, 55 °C for 10 sec, 72 °C 1 min; and one final extension step at 72 °C for 10 min. The 16S rRNA gene amplicon from novel bacterial strain AR was sequenced on an ABI3730XL (Applied Biosystems). A 1282 bp 16S rRNA gene sequence was generated (Figure 1). The sequence was aligned by BLASTn on NCBI against the non-redundant nucleotide database and the 16S ribosomal RNA database.

BLASTn hit against database nr; *Erwinia sp.* strain KUDC3014 16S ribosomal RNA gene, partial sequence

Max Score	Total Score	Query Coverage	E-Value	% Identity	Accession
2313	2313	100 %	0	100.00 %	MK070133.1

BLASTn hit against database 16S ribosomal RNA; *Erwinia gerundensis* strain EM595 16S ribosomal RNA gene, partial sequence

Max Score	Total Score	Query Coverage	E-Value	% Identity	Accession
2264	2264	100 %	0	98.91 %	NR_148820.1

The preliminary taxonomic identification of the novel bacterial strain AR was *Erwinia gerundensis.*

Genomics

The genome of novel bacterial strain AR was sequenced. This novel bacterial strain was retrieved from the glycerol collection stored at -80 °C by streaking on R2A plates. Single colonies from these plates were grown overnight in Nutrient Broth and pelleted. These pellets were used for genomic DNA extraction using the bacteria protocol of Wizard® Genomic DNA Purification Kit (A1120, Promega). A DNA sequencing library was generated for Illumina sequencing using the Illumina Nextera XT DNA library prep protocol. The library was sequenced using an Illumina MiSeq platform or HiSeq platform. Raw reads from the sequencer were filtered to remove any adapter and index sequences as well as low quality bases using Trimmomatic (Bolger, Lohse & Usadel 2014) with the following options: ILLUMINACLIP: NexteraPE-PE.fa:2:30:10 LEADING:3 TRAILING:3 SLIDINGWINDOW:4:15 MINLEN:36. To enable full genome assembly, long reads were generated for novel bacterial strain AR only by sequencing DNA using Oxford Nanopore Technologies (ONT) MinION platform. The DNA from the Wizard® Genomic DNA Purification Kit was first assessed with the genomic assay on Agilent 2200 TapeStation system (Agilent Technologies, Santa Clara, CA, USA) for integrity (average molecular weight ≥ 30 Kb). The sequencing library was prepared using an in-house protocol modified from the official protocols for transposases-based library preparation kits (SQK-RAD004/SQK-RBK004, ONT, Oxford, UK). The library was sequenced on a MinION Mk1B platform (MIN-101B) with R9.4 flow cells (FLO-MIN106) and under the control of MinKNOW software. After the sequencing run finished, the fast5 files that contain raw read signals were transferred to a separate, high performance computing Linux server for local basecalling using ONT's Albacore software (Version 2.3.1) with default parameters. The sequencing summary file produced by Albacore was processed by the R script minion qc (https://github.com/roblanf/minion_qc) and NanoPlot (De Coster et al. 2018) to assess the quality of the sequencing run, while Porechop (Version 0.2.3, https://github.com/rrwick/Porechop) was used to remove adapter sequences from the reads. Reads which were shorter than 300 bp were removed and the worst 5 % of reads (based on quality) were discarded by using Filtlong (Version 0.2.0, https://github.com/rrwick/Filtlong).

The whole genome sequence of novel bacterial strain AR was assembled using Unicycler (Wick et al. 2017). Unicycler performed hybrid assembly when both Illumina reads and MinION reads were available. MinION reads were mainly used to resolve repeat regions in the genome, whereas Illumina reads were used by Pilon (Walker et al. 2014) to correct small base-level errors. Multiple rounds of Racon (Vaser et al. 2017) polishing were then carried out to generate consensus sequences. Assembly graphs were visualised by using Bandage (Wick et al. 2015).

A complete circular chromosome sequence and two plasmid sequences were produced for the *Erwinia gerundensis* novel bacterial strain AR. The genome size for the novel bacterial strain AR was 3,748,909 bp (Table 1). The percent GC content ranged from 55 % to 53 % for the genome and

plasmids. The novel bacterial strain AR was annotated by Prokka (Seemann 2014) with a custom, genus-specific protein database to predict genes and corresponding functions, which were then screened manually to identify specific traits. The number of genes for the novel bacterial strain AR was 4,091 (Table 2).

Table 1 – Summary of properties of the final genome sequence assembly

Strain ID	**Genome size (bp)**	**GC content (%)**	**Coverage Illumina reads**	**Coverage ONT MinION**
AR chromosome	3,748,909	55	150×	150×
AR plasmid 1	580,656	55	150x	150x
AR plasmid 2	107,871	53	150x	150x

Table 2 – Summary of genome coding regions

Strain ID	**Genome size (bp)**	**No. of tRNA**	**No. of tmRNA**	**No. of rRNA**	**No. of CDS**	**No. of gene**
AR	4,437,426	78	0	22	3,991	4,091

Nine *Erwinia* and *Pantoea* spp. (*Pantoea* sp PSNIH2*, P. ananatis* L*MG20103, P. vagans* C9-1*, P. agglomerans* C410P1, L15 and TH81*, E. amylovora* CFBP1430*, E. persicina* NBRC102418, *and E. gerundensis* EM595*)* genome sequences that are publicly available on NCBI were acquired and used for pan-genome/comparative genome sequence analysis alongside the novel bacterial strain AR (*E. gerundensis*). A total of 103 genes that are shared by all 10 bacterial strains were identified by running Roary (Page et al. 2015). PRANK (Löytynoja 2014) was then used to perform a codon aware alignment. A maximum-likelihood phylogenetic tree (Figure 2) was inferred using FastTree (Price, Dehal & Arkin 2010) with Jukes-Cantor Joins distances and Generalized Time-Reversible and CAT approximation model. Local support values for branches were calculated using 1000 resamples with the Shimodaira-Hasegawa test. The novel bacterial strain AR clustered tightly with the *Erwinia gerundensis* bacterial strain EM595, suggesting a close phylogenetic relationship between these two bacterial strains. Moreover, this cluster was separated from other *Pantoea and Erwinia* spp. with strong local support value (100 %). This separation supports that bacterial strain AR is novel and from the species *Erwinia gerundensis.*

Example 3 – Bioprotection activity (*in vitro*) of the *Erwinia gerundensis* novel bacterial strain AR

In vitro bioassays were established to test the bioactivity of the *Erwinia gerundensis* novel bacterial strain AR, against six plant pathogenic fungi (Table 3). A plate with only the pathogen was used as a negative control (blank). The fungal pathogens were all isolated from monocot species, and were obtained from the National Collection of Fungi (Herbarium VPRI) and the AVR collection. Each bacterial strain was cultured in Nutrient Broth (BD Biosciences) overnight at 28 °C in a shaking incubator (200 rpm). Each bacterial strain was drop-inoculated (20 µL) onto four equidistant points on a Nutrient Agar (BD Biosciences) plate, which was then incubated overnight at 28 °C. A 6 mm × 6mm agar plug of actively growing mycelia from the pathogen was placed at the centre of the plate. The bioassay was incubated for at least 5 days at 28 °C in the dark, and then the diameter of the fungal colony on the plate was recorded. For each treatment three plates were prepared as biological triplicates. OriginPro 2018 (Version b9.5.1.195) was used to carry out One-way ANOVA and Tukey Test to detect the presence of any significant difference ($P < 0.05$) between treatments.

Table 3 – Pathogens used in the bioprotection bioassay

VPRI Accession No.	**Taxonomic Details**	**Host Taxonomic Details**	**State**	**Collection Date**
12962	*Drechslera brizae* (Y.Nisik.) Subram. & B.L.Jain	*Briza maxima* L.	Vic.	24-Oct-85
32148	*Sclerotium rolfsii* Sacc.	*Poa annua* L.	Vic.	1-Jan-05
10694	*Phoma sorghina* (Sacc.) Boerema, Dorenbosch, van Kesteren	*Cynodon dactylon* Pers.	Vic.	19-Apr-79
42586a	*Fusarium verticillioides* (Sacc.) Nirenberg	*Zea mays* L.	Vic.	27-Feb-15
42563	*Bipolaris gossypina*	*Brachiaria*	Qld	
N/A	*Microdochium nivale*	*Lolium perenne* L.	Vic	

The *Erwinia gerundensis* novel bacterial strain AR inhibited the growth of four of the six fungal pathogens compared to the control (Figure 3). The *Erwinia gerundensis* novel bacterial strain AR was active against *Bipolaris gossypina*, *Sclerotium rolfsii* and *Phoma sorghina*, and *Microdochium nivale*.

Example 4 – Biofertiliser activity (*in vitro*) of the *Erwinia gerundensis* novel bacterial strain AR

Nitrogen (N) is an important nutrient for plant growth and a key component of fertilisers. Plant associated bacteria able to grow under low nitrogen conditions may be useful in plant growth as they can pass this N onto the plant. The ability to grow under low nitrogen conditions was assessed by using the nitrogen-free NFb medium (Dobereiner 1980) and Burks medium (Wilson & Knight 1952). One litre of NFb medium contains 5 g DL-malic acid, 0.5 g dipotassium hydrogen orthophosphate, 0.2 g magnesium sulfate heptahydrate, 0.1 g sodium chloride, 0.02 g calcium chloride dehydrate, 2 mL micronutrients solution [0.4 g/L copper sulfate pentahydrate, 0.12 g/L zinc sulfate heptahydrate, 1.4 g/L boric acid, 1 g/L sodium molybdate dehydrate, 1.5 g/L manganese(II) sulfate monohydrate], 1 mL vitamin solution (0.1 g/L biotin, 0.2 g/L pyridoxol hydrochloride), 4 mL iron(III) EDTA and 2 mL bromothymol blue (0.5 %, dissolved in 0.2 N potassium hydroxide). For solid NFb medium, 15 g/L bacteriological agar was added, otherwise 0.5 g/L was added for semi-solid medium. The pH of medium was adjusted to 6.8. The contents of Burks medium include 10 g/L dextrose, 0.41 g/L potassium dihydrogen phosphate, 0.52 g/L dipotassium hydrogen orthophosphate, 0.05 g/L sodium sulfate, 0.2 g/L calcium chloride, 0.1 g/L magnesium sulfate heptahydrate, 0.005 g/L iron(II) sulfate heptahydrate, 0.0025 g/L sodium molybdate dehydrate and 15 g/L bacteriological agar. The pH of medium was adjusted to 7. To detect the nitrogen fixation ability, bacterial strains, including *E. coli* as a negative control, were inoculated onto solid medium plates. For each inoculation, triplicates were prepared. All NFb medium plates were incubated at 30 °C, whereas Burks medium plates were incubated at 28 °C. After 96 hours, the colour change of NFb medium plates was recorded, with development of blue colour an indication of growth under limiting N. The physical growth of bacteria on Burks medium plates was the indicator for this assay. To evaluate if the nitrogen is the limiting factor in Burks medium, a control group whose Burks medium was supplemented with 10 g/L tryptone or ammonia chloride was added to the bioassay.

In the high throughput automated method to detect nitrogen fixation ability semi-solid media NFb was used. Bacterial strains (including *E. coli* negative control) were inoculated into 20 mL R2B medium (0.5 g/L yeast extract, 0.5 g/L proteose peptone, 0.5 g/L casein hydrolysate, 0.5 g/L glucose, 0.5 g/L starch, 0.3 g/L dipotassium hydrogen orthophosphate, 0.024 g/L magnesium sulphate and 0.3 g/L sodium pyruvate) and incubated at 28 °C and 200 rpm overnight. Th cell pellet was collected by centrifuging at 4000× g for 3 minutes, and then was twice with 1× PBS to remove the nitrogen residue from R2B. Then cell pellet was resuspended in 10 mL semi-solid NFb medium. 1 µL of cell suspension was added to a well containing 199 µL semi-solid NFb medium on a 96-well cell culture plate. For each bacterial strain, the cell suspension was added to six consecutive wells of the same column, representing six biological replicates. After inoculating all bacterial strains, the plate was examined by plate reader immediately by obtaining a reading at 615 nm wavelength. Wells located in rows A and H, and columns 1 and 12 were excluded during the examination due to the edge effect

which may lead to an unreliable reading. The plate was incubated at room temperature for 84 hours, during which it was examined by plate reader every 12 hours. Values were expressed as differences in absorbance at 615 nm relative to the absorbance at 615 nm in the well at time zero. An increase in absorbance represented an increase in growth under low nitrogen conditions.

The *Erwinia gerundensis* novel bacterial strain AR was able to grow under low N, as evident from the colour change in the NFb media, growth on Burks media (without supplementary N source) and elevated absorbance levels at a wavelength of 615 nm in comparison to the *E. coli* negative control and no growth control (NFb media only) (Figure 4).

Example 5 – *In planta* inoculations supporting endophytic niche of the *Erwinia gerundensis* novel bacterial strain AR

To assess direct interactions between the *Erwinia gerundensis* novel bacterial strain AR and plants, an early seedling growth assay was established in barley. The *Erwinia gerundensis* novel bacterial strain AR was cultured in Lysogeny Broth (LB) overnight at 26 °C. The following day seeds of barley (cultivar Hindmarsh) were surface-sterilised by soaking in 80 % ethanol for 3 mins, then washing 5 times in sterile distilled water. The seeds were then soaked in the overnight cultures for 4 hours at 26 °C in a shaking incubator. For control seedlings, seeds were soaked in LB without bacteria for 4 hours at 26 °C in a shaking incubator. The seeds were planted into a pot trial, with three replicates (pots) per strain/control, with a randomised design. A total of 20 seeds were planted per pot, to a depth of 1 cm. The potting medium contained a mixture of 25 % potting mix, 37.5 % vermiculite and 37.5 % perlite. The plants were grown for 5 days and then removed from the pots, washed, assessed for health (i.e. no disease symptoms) and photographed. The lengths of the longest root and the longest shoot were measured. Data was statistically analysed using a t test to detect the presence of any significant difference ($P < 0.05$) between treatments using Excel.

Seedlings inoculated with the *Erwinia gerundensis* novel bacterial strain AR were healthy with no disease symptoms recorded on leaves or roots (Figure 5). The mean length of the shoots inoculated with the *Erwinia gerundensis* novel bacterial strain AR were equivalent to the control (Figure 6) at 53.5 to 54.4 mm. The length of the roots of seedlings inoculated with the *Erwinia gerundensis* novel bacterial strain AR were significantly longer than the control (Figure 6) at 131.2 mm to 107.7 mm (T-test 0.001675013).

Example 6 – *In planta* inoculations supporting the biofertilizer (nitrogen) niche of the *Erwinia gerundensis* novel bacterial strain AR

An *in planta* biofertilizer assay was established in barley to evaluate the ability of *Erwinia gerundensis* novel bacterial strain AR to aid growth under nitrogen limiting conditions. Initially, bacterial strains (5, including AR were cultured in 20 mL nutrient broth (BD Bioscience) overnight at 26 °C whilst rotating at 200 rpm. The following day cultures were pelleted via centrifugation at 4000 rpm for 5 minutes, washed three times in 10 mL Phosphate Buffered Saline (PBS), resuspended in 20 mL PBS, quantified via spectrophotometry (OD_{600}) and diluted (1:10). Barley seeds were sterilized in 70 % ethanol for 5 minutes, followed by rinsing with sterilized distilled water (SDW) for five times. These sterile seeds were submerged in the dilution for 4 hours in a dark incubator at room temperature whilst rotating at 200 rpm. The seeds were subsequently transferred to moistened sterile filter paper and allowed to germinate for three days. The three-day-old seedlings were individually transferred to 60 mm plates with semi-solid Burks media (HiMedia) (5 g/L Agar). Seedlings were allowed to grow for a further 4 days, before the shoots and roots were measured for each seedling. There was a total of 6 treatments (5 bacterial strains including AR; 1 blank media control) containing 10 seedlings per treatment. Statistical analysis (One-way ANOVA and Tukey Test) was conducted using OriginPro 2018 (Version b9.5.1.195) to detect the presence of any significant difference ($P < 0.05$) between treatments.

The root growth of seedlings inoculated with novel bacterial strain AR and grown under nitrogen limiting conditions was not significantly greater than the control ($P < 0.05$), despite increasing root growth by 13.6 % (Figure 7). The shoot growth of seedlings inoculated with novel bacterial strain AR was not significantly greater than the control ($P < 0.05$), despite increasing shoot growth by 9.0 % (Figure 8). Overall, results indicate that novel bacterial strain AR can aid in the growth of seedlings grown under nitrogen limiting conditions.

It is to be understood that various alterations, modifications and/or additions may be made without departing from the spirit of the present invention as outlined herein.

As used herein, except where the context requires otherwise, the term "comprise" and variations of the term, such as "comprising", "comprises" and "comprised", are not intended to be in any way limiting or to exclude further additives, components, integers or steps.

Reference to any prior art in the specification is not, and should not be taken as, an acknowledgment or any form of suggestion that this prior art forms part of the common general knowledge in Australia or any other jurisdiction or that this prior art could reasonably be expected to be combined by a person skilled in the art.

References

1. Bolger, AM, Lohse, M & Usadel, B 2014, 'Trimmomatic: a flexible trimmer for Illumina sequence data', *Bioinformatics*, vol. 30, no. 15, pp. 2114-20.

2. De Coster, W, D'Hert, S, Schultz, DT, Cruts, M & Van Broeckhoven, C 2018, 'NanoPack: visualizing and processing long read sequencing data', *Bioinformatics*.

3. Dobereiner, J 1980, 'Forage grasses and grain crops', in *Methods for evaluating biological nitrogen fixation/edited by FJ Bergersen*, John Wiley & Sons Ltd, pp. 535-55.

4. Löytynoja, A 2014, 'Phylogeny-aware alignment with PRANK', in DJ Russell (ed.), *Multiple Sequence Alignment Methods*, Humana Press, Totowa, NJ, pp. 155-70, DOI 10.1007/978-1-62703-646-7_10, <https://doi.org/10.1007/978-1-62703-646-7_10>.

5. Page, AJ, Cummins, CA, Hunt, M, Wong, VK, Reuter, S, Holden, MT, Fookes, M, Falush, D, Keane, JA & Parkhill, J 2015, 'Roary: rapid large-scale prokaryote pan genome analysis', *Bioinformatics*, vol. 31, no. 22, pp. 3691-3.

6. Price, MN, Dehal, PS & Arkin, AP 2010, 'FastTree 2--approximately maximum-likelihood trees for large alignments', *PLoS One*, vol. 5, no. 3, p. e9490.

7. Seemann, T 2014, 'Prokka: rapid prokaryotic genome annotation', *Bioinformatics*, vol. 30, no. 14, pp. 2068-9.

8. Vaser, R, Sovic, I, Nagarajan, N & Sikic, M 2017, 'Fast and accurate de novo genome assembly from long uncorrected reads', *Genome Res*, vol. 27, no. 5, pp. 737-46.

9. Walker, BJ, Abeel, T, Shea, T, Priest, M, Abouelliel, A, Sakthikumar, S, Cuomo, CA, Zeng, Q, Wortman, J, Young, SK & Earl, AM 2014, 'Pilon: an integrated tool for comprehensive microbial variant detection and genome assembly improvement', *PLoS One*, vol. 9, no. 11, p. e112963.

10. Wick, RR, Judd, LM, Gorrie, CL & Holt, KE 2017, 'Unicycler: Resolving bacterial genome assemblies from short and long sequencing reads', *PLOS Computational Biology*, vol. 13, no. 6, p. e1005595.

11. Wick, RR, Schultz, MB, Zobel, J & Holt, KE 2015, 'Bandage: interactive visualization of de novo genome assemblies', *Bioinformatics*, vol. 31, no. 20, pp. 3350-2.

12. Wilson, PW & Knight, SG 1952, *Experiments in Bacterial Physiology*, Burgess.

THE CLAIMS DEFINING THE INVENTION ARE AS FOLLOWS:

1. A substantially purified or isolated endophyte strain isolated from a plant of the Poaceae family, wherein said endophyte is a strain of *Erwinia gerundensis* which provides bioprotection and/or biofertilizer phenotypes to plants into which it is inoculated.

2. An endophyte according to claim 1, wherein the bioprotection and/or biofertilizer phenotype includes production of the bioprotectant compound in the plant into which the endophyte is inoculated.

3. An endophyte according to claim 1, wherein the bioprotection and/or biofertilizer phenotype includes nitrogen fixation in the plant into which the endophyte is inoculated.

4. An endophyte according to any one of claims 1 to 3, wherein the endophyte is strain AR as described herein and as deposited with The National Measurement Institute on 17th May 2019 with accession number V19/009908.

5. An endophyte according to any one of claims 1 to 3, wherein the plant from which the endophyte is isolated is of the Poaceae family is a pasture grass.

6. An endophyte according to claim 5, wherein the pasture grass is from the genus Lolium or Festuca.

7. An endophyte according to claim 6, wherein the pasture grass is from the species *Lolium perenne* or *Festuca arundinaceum.*

8. An endophyte according to any one of claims 1 to 7, wherein the plant into which the endophyte is inoculated includes an endophyte-free host plant or part thereof stably infected with said endophyte.

9. An endophyte according to any one of claims 1 to 8, wherein the plant into which the endophyte is inoculated is an agricultural plant selected from one or more of forage grass, turf grass, bioenergy grass, grain crop and industrial crop.

10. An endophyte according claim 9, wherein the plant into which the endophyte is inoculated is a forage, turf or bioenergy grass selected from the group consisting of those belonging to the genera *Lolium* and *Festuca*, including *L. perenne* (perennial ryegrass), *L. arundinaceum* (tall fescue) and *L. multiflorum* (Italian ryegrass), and those belonging to the *Brachiaria-Urochloa* species complex (panic grasses), including *Brachiaria brizantha, Brachiaria decumbens, Brachiaria*

humidicola, Brachiaria stolonifera, Brachiaria ruziziensis, B. dictyoneura, Urochloa brizantha, Urochloa decumbens, Urochloa humidicola, *Urochloa mosambicensis* as well as interspecific and intraspecific hybrids of *Brachiaria-Urochloa* species complex such as interspecific hybrids between *Brachiaria ruziziensis* x *Brachiaria brizantha*, *Brachiaria ruziziensis* x *Brachiaria decumbens*, [*Brachiaria ruziziensis* x *Brachiaria decumbens*] x *Brachiaria brizantha*, [*Brachiaria ruziziensis* x *Brachiaria brizantha*] x *Brachiaria decumbens*.

11. An endophyte according claim 9, wherein the plant into which the endophyte is inoculated is a grain crop or industrial crop grass selected from the group consisting of those belonging to the genus *Triticum*, including *T. aestivum* (wheat), those belonging to the genus *Hordeum*, including *H. vulgare* (barley), those belonging to the genus *Zea*, including *Z. mays* (maize or corn), those belonging to the genus *Oryza*, including *O. sativa* (rice), those belonging to the genus *Saccharum* including *S. officinarum* (sugarcane), those belonging to the genus *Sorghum* including *S. bicolor* (sorghum), those belonging to the genus *Panicum*, including *P. virgatum* (switchgrass), those belonging to the genera *Miscanthus*, *Paspalum*, *Pennisetum*, *Poa*, *Eragrostis* and *Agrostis*.

12. An endophyte according to claim 9, wherein the plant into which the endophyte is inoculated is a grain crop or industrial crop selected from the group consisting of wheat, barley, oats, chickpeas, triticale, fava beans, lupins, field peas, canola, cereal rye, vetch, lentils, millet/panicum, safflower, linseed, sorghum, sunflower, maize, canola, mungbeans, soybeans, and cotton.

13. A plant or part thereof infected with one or more endophytes according to any one of claims 1 to 12.

14. A plant, plant seed or other plant part derived from a plant or part thereof according to claim 13 and stably infected with said one or more endophytes.

15. Use of an endophyte according to any one of claims 1 to 12 to produce a plant or part thereof stably infected with said one or more endophytes.

16. A bioprotectant compound produced by an endophyte according to any one of claims 1 to 12, or a derivative, isomer and/or a salt thereof.

17. A method for producing a bioprotectant compound, said method including infecting a plant with an endophyte according to any one of claims 1 to 12 and cultivating the plant under conditions suitable to produce the bioprotectant compound.

18. A method for producing a bioprotectant compound, said method including culturing an endophyte according to any one of claims 1 to 12 under conditions suitable to produce the bioprotectant compound.

19. A method according to claim 18, wherein the conditions include a culture medium including a source of carbohydrates.

20. A method according to claim 19, wherein the source of carbohydrates is selected from one or more of the group consisting of a starch/sugar-based agar or broth, a cereal-based agar or broth, endophyte agar, Murashige and Skoog with 20 % sucrose, half V8 juice/half PDA, water agar and yeast malt extract agar.

21. A method according to any one of claims 17 to 20, wherein the method further includes isolating the bioprotectant compound from the plant or culture medium.

22. A method of growing a plant in a low nitrogen medium, said method including infecting a plant with a bioprotectant compound -producing endophyte according to any one of claims 1 to 12, and cultivating the plant.

23. A method according to claim 22, wherein the low nitrogen medium is low nitrogen soil.

ABSTRACT

The present invention relates to an endophyte strain isolated from a plant of the Poaceae family, wherein said endophyte is a strain of *Erwinia gerundensis* which provides bioprotection and/or biofertilizer phenotypes to plants into which it is inoculated. The present invention also discloses plants infected with the endophyte and related methods.

Figures

AGTAATGTCTGGGGATCTGCCCGATGGAGGGGGATAACCACTGGAAACGGTGGCTAATACCGCATAACGTC
GCAAGACCAAAGTGGGGGACCTTCGGGCCTCACACCATCGGATGAACCCAGATGGGATTAGCTAGTAGGTG
GGGTAACGGCTCACCTAGGCGACGATCCCTAGCTGGTCTGAGAGGATGACCAGCCACACTGGAACTGAGAC
ACGGTCCAGACTCCTACGGGAGGCAGCAGTGGGGAATATTGCACAATGGGCGCAAGCCTGATGCAGCCATG
CCGCGTGTATGAAGAAGGCCTTCGGGTTGTAAAGTACTTTCAGCGGGGAGGAAGGGGATGAGGTTAATAAC
CTCGTTCATTGACGTTACCCGCAGAAGAAGCACCGGCTAACTCCGTGCCAGCAGCCGCGGTAATACGGAGG
GTGCAAGCGTTAATCGGAATTACTGGGCGTAAAGCGCACGCAGGCGGTCTGTTAAGTCAGATGTGAAATCC
CCGGGCTTAACCTGGGAACTGCATTTGAAACTGGCAGGCTTGAGTCTTGTAGAGGGGGGTAGAATTCCAGG
TGTAGCGGTGAAATGCGTAGAGATCTGGAGGAATACCGGTGGCGAAGGCGGCCCCCTGGACAAAGACTGA
CGCTCAGGTGCGAAAGCGTGGGGAGCAAACAGGATTAGATACCCTGGTAGTCCACGCCGTAAACGATGTCG
ACTTGGAGGCTGTGAGCATGACTCGTGGCTTCCGGAGCTAACGCGTTAAGTCGACCGCCTGGGGAGTACGG
CCGCAAGGTTAAAACTCAAATGAATTGACGGGGGCCCGCACAAGCGGTGGAGCATGTGGTTTAATTCGATG
CAACGCGAAGAACCTTACCTGCTCTTGACATCCACGGAATTCGGCAGAGATGCCTTAGTGCCTTCGGGAACC
GTGAGACAGGTGCTGCATGGCTGTCGTCAGCTCGTGTTGTGAAATGTTGGGTTAAGTCCCGCAACGAGCGC
AACCCTTATCCTTTGTTGCCAGCGATTCGGTCGGGAACTCAAAGGAGACTGCCGGTGATAAACCGGAGGAA
GGTGGGGATGACGTCAAGTCATCATGGCCCTTACGAGCAGGGCTACACACGTGCTACAATGGCGCATACAA
AGAGAAGCGACCTCGCGAGAGCAAGCGGACCTCACAAAGTGCGTCGTAGTCCGGATCGGAGTCTGCAACTC
GACTCCGTGAAGTCGGAATCGCTAGTAATCGTGGATCAGAATGCCACGGTGAATACGTTCCCGGGCCTTGTA
CAC

Figure 1

Appendix 1

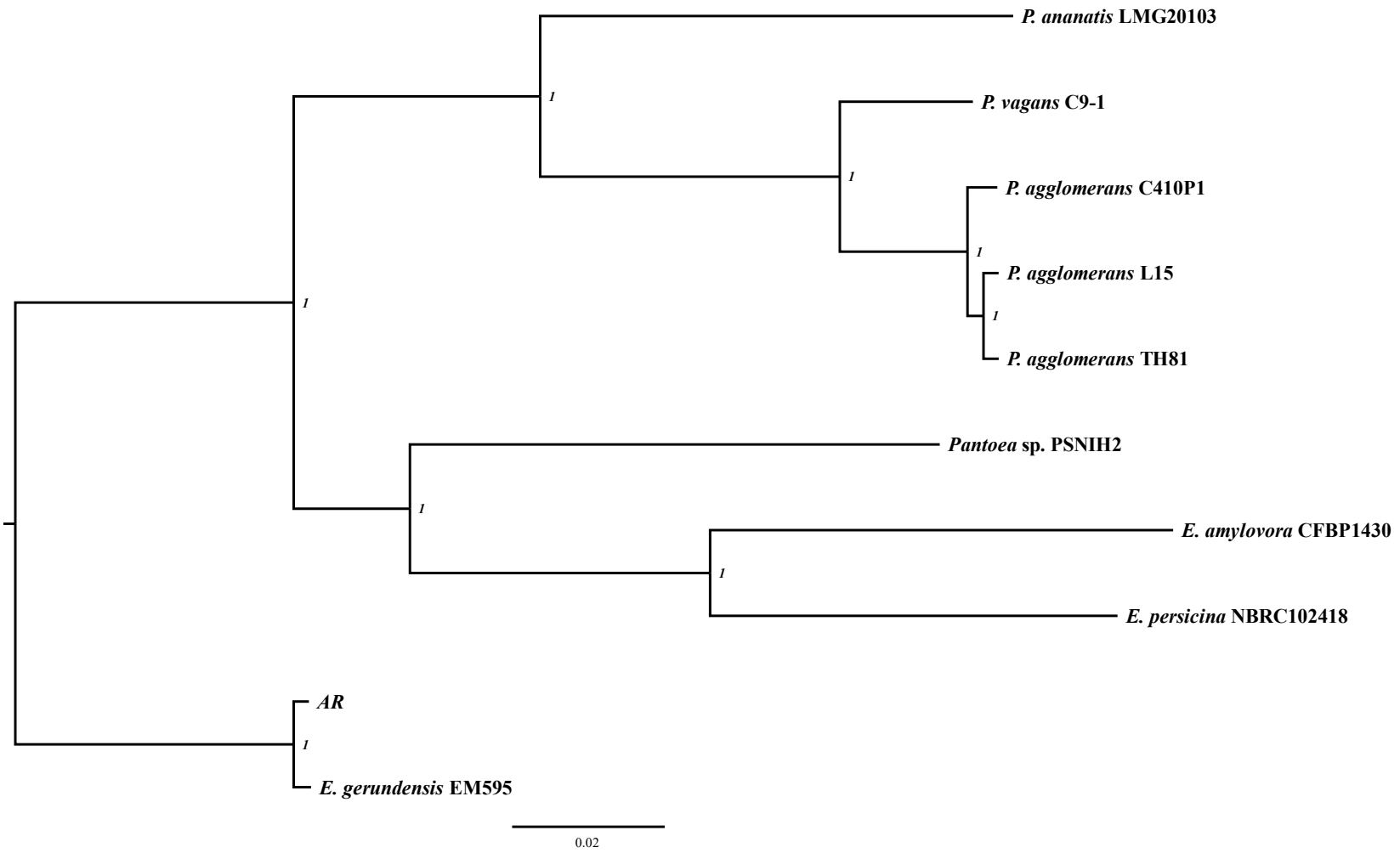


Figure 2

Appendix 1

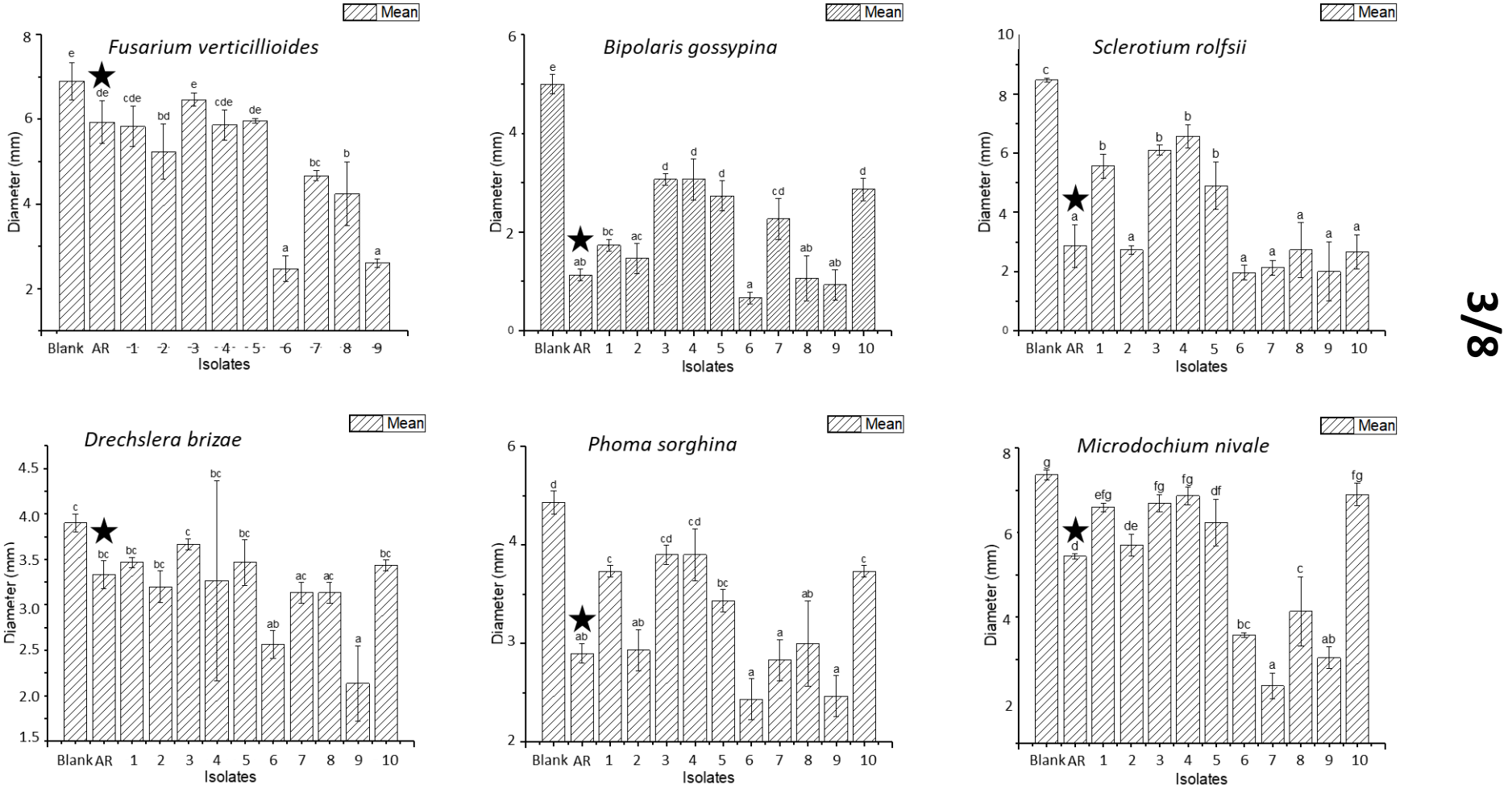


Figure 3

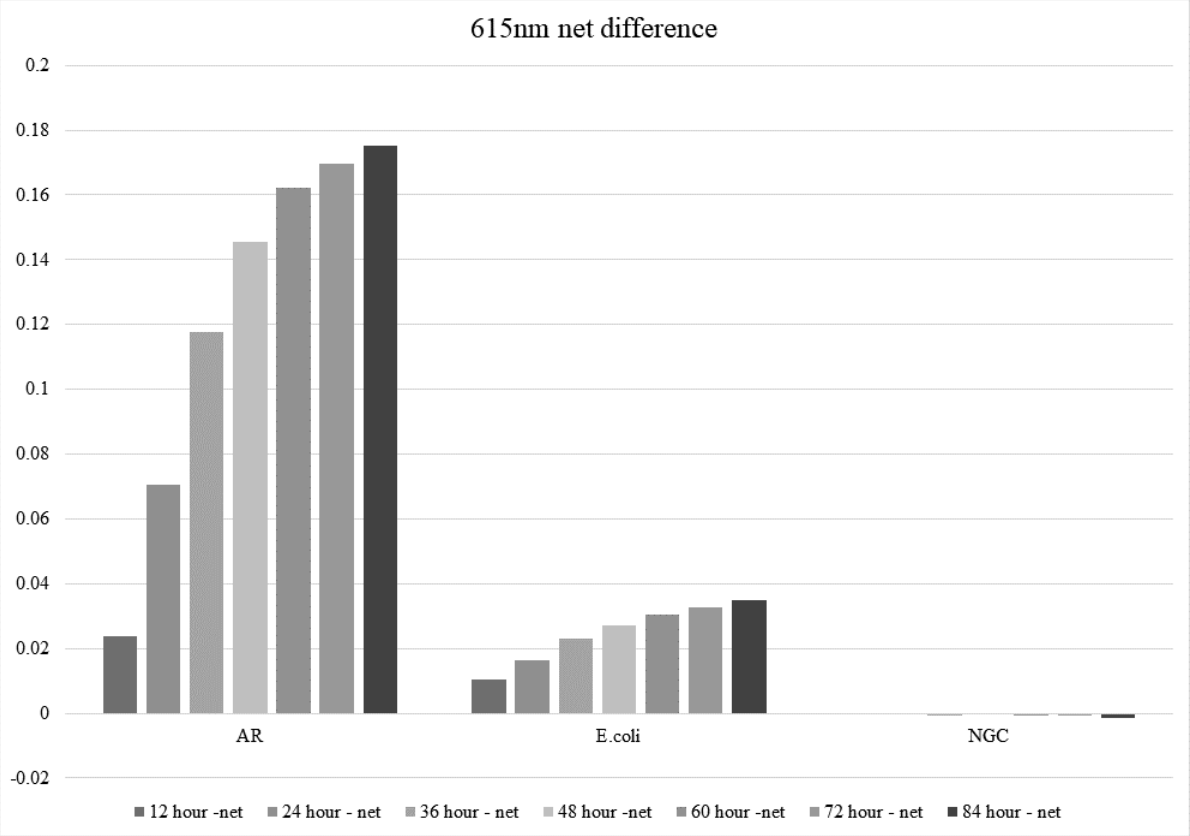

615nm net difference
0.2
0.18
0.16
0.14
0.12
0.1
0.08
0.06
0.04
0.02
0
-0.02
AR
E.coli
NGC
12 hour -net
24 hour - net
36 hour - net
48 hour -net
60 hour -net
72 hour - net
84 hour - net

Figure 4

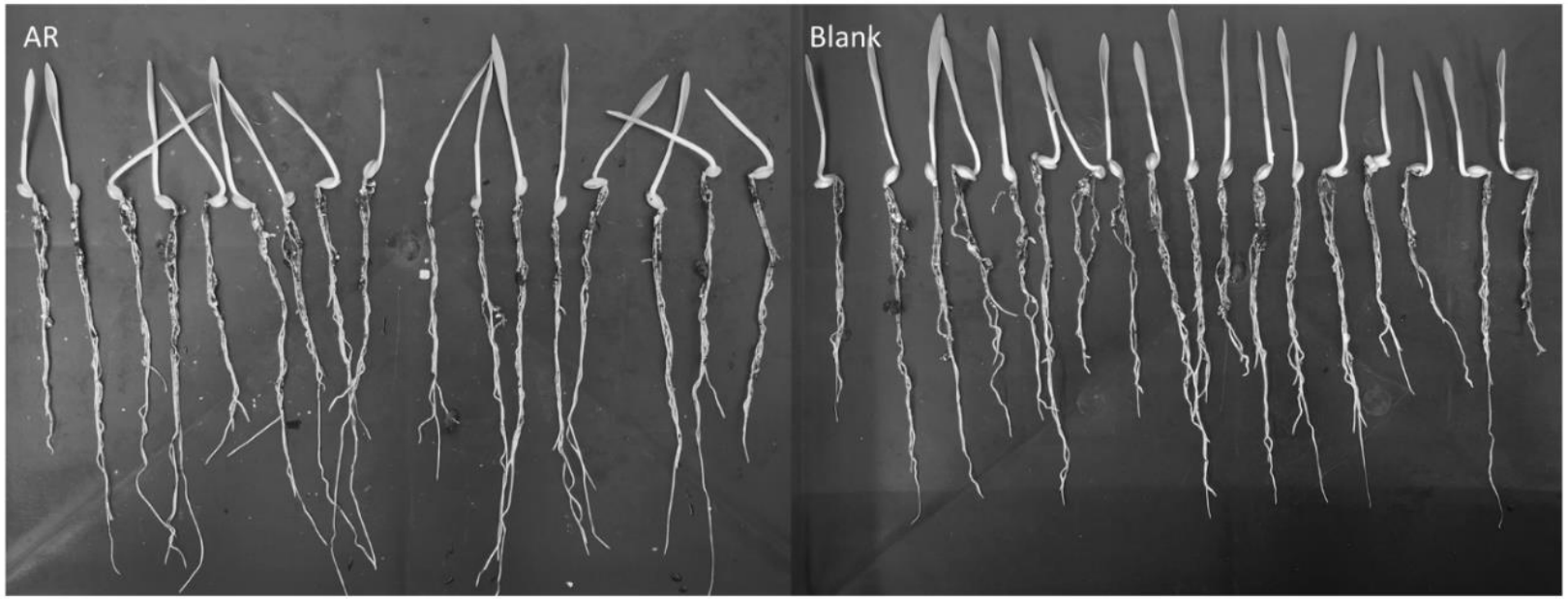


Figure 5

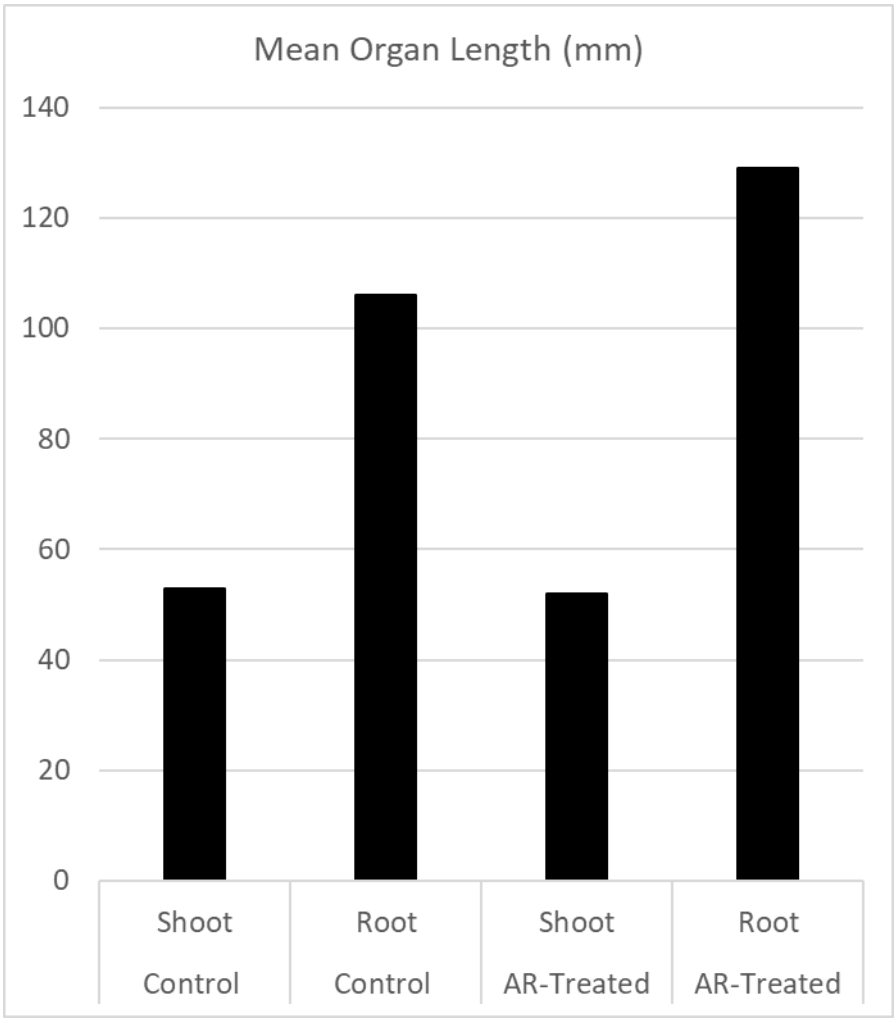

Mean Organ Length (mm)
140
120
100
80
60
40
20
0
Shoot Control
Root Control
Shoot AR-Treated
Root AR-Treated

Figure 6

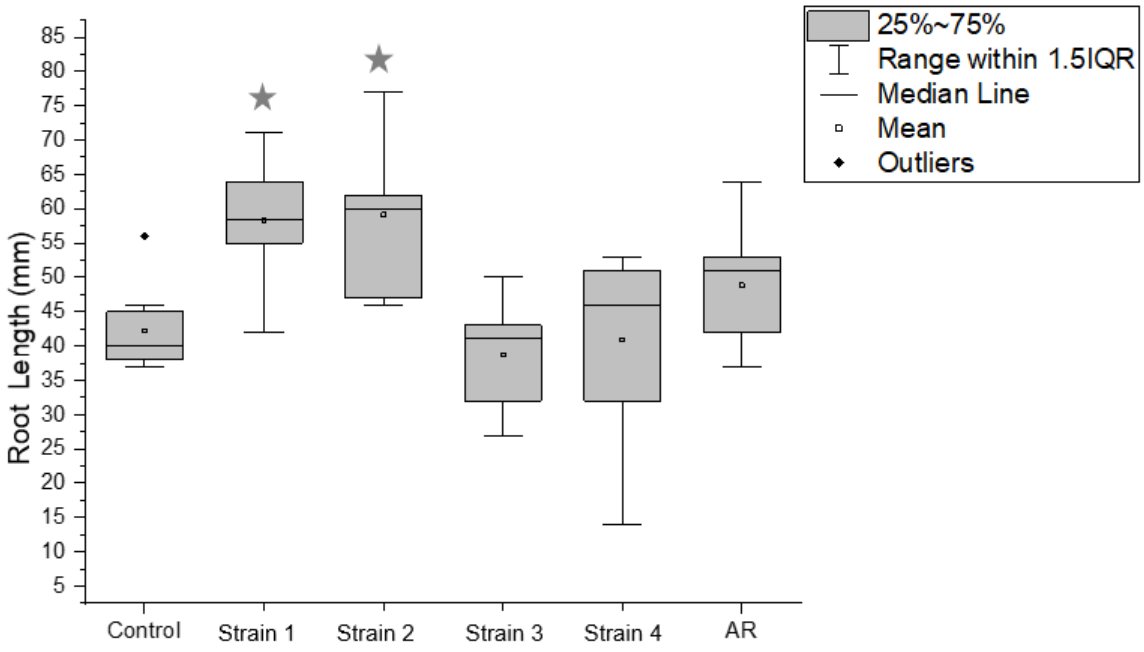

25%~75%
Range within 1.5IQR
Median Line
Mean
Outliers
Root Length (mm)
85
80
75
70
65
60
55
50
45
40
35
30
25
20
15
10
5
Control
Strain 1
Strain 2
Strain 3
Strain 4
AR

Figure 7

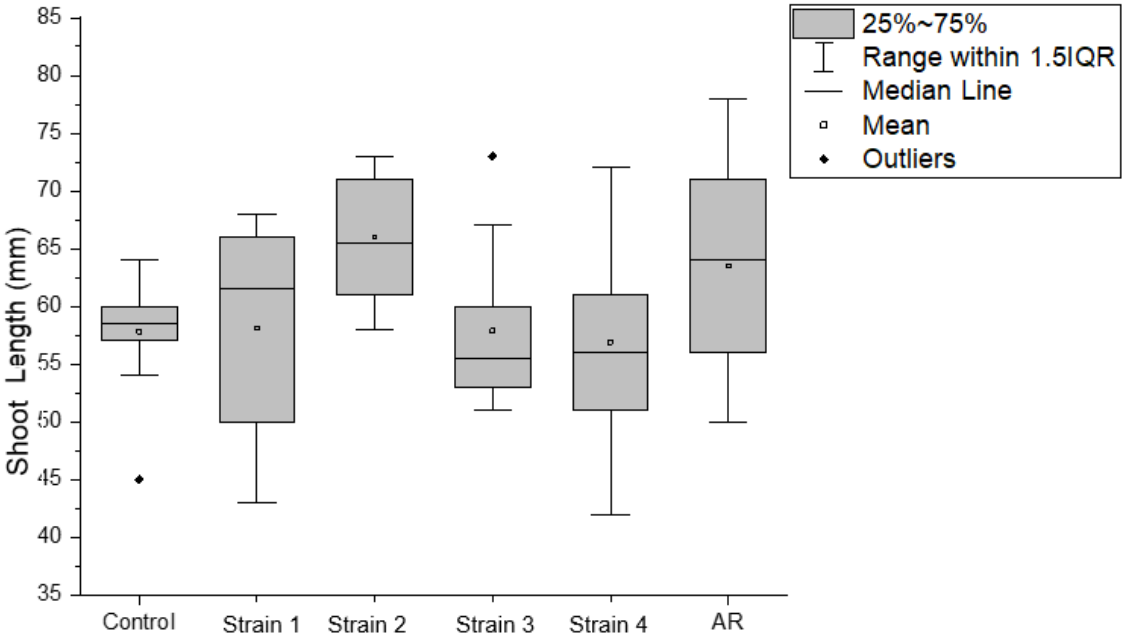

25%~75%
Range within 1.5IQR
Median Line
Mean
Outliers
Shoot Length (mm)
85
80
75
70
65
60
55
50
45
40
35
Control
Strain 1
Strain 2
Strain 3
Strain 4
AR

Figure 8

1.4 Patent 3 – *Pseudomonas poae*

1.4.1 Publication details

Title: Novel bacterial strain (1)

Details: https://patentscope.wipo.int/search/en/detail.jsf?docId=WO2021011999

Stage of publication: Full patent (publicly available)

Authors: Li, Tongda; Tannenbaum, Ian Ross; Kaur, Jatinder; Krill, Christian; Sawbridge, Timothy Ivor; Mann, Ross C.; Spangenberg, German Carlos

1.4.2 Statement of contribution of joint authorship

TL performed all work relating to genomics of the strains, *in vitro* bioprotectant assays, *in vitro* biofertiliser activity assays, design of strain specific PCR primers and *in planta* inoculation assays (Example 8). TL generated all figures associated with the above works (1–10) and drafted the majority of the experimental section of the patent associated with these works. TL conducted all statistical and data analysis of these works. RM, GS, TS, JK and TL all conceptualised the patent and assisted in editing the patent. Experimental and analysis work conducted by TL is highlighted in yellow.

Appendix 1

AUSTRALIA

Patents Act 1990

PROVISIONAL SPECIFICATION

Applicants: Agriculture Victoria Services Pty Ltd
Dairy Australia Limited
Geoffrey Gardiner Dairy Foundation Limited

Title: NOVEL BACTERIAL STRAIN (1)

The invention is described in the following statement:

NOVEL BACTERIAL STRAIN (1)

Field of the Invention

The present invention relates to novel plant microbiome strains, plants infected with such strains and related methods.

Background of the Invention

Microbes represent an invaluable source of novel genes and compounds that have the potential to be utilised in a range of industrial sectors. Scientific literature gives numerous accounts of microbes being the primary source of antibiotics, immune-suppressants, anticancer agents and cholesterol-lowering drugs, in addition to their use in environmental decontamination and in the production of food and cosmetics.

A relatively unexplored group of microbes known as endophytes, which reside e.g. in the tissues of living plants, offer a particularly diverse source of novel compounds and genes that may provide important benefits to society, and in particular, agriculture.

Endophytes may be fungal or bacterial. Endophytes often form mutualistic relationships with their hosts, with the endophyte conferring increased fitness to the host, often through the production of defence compounds. At the same time, the host plant offers the benefits of a protected environment and nutriment to the endophyte.

Important forage grasses perennial ryegrass (*Lolium perenne*) are commonly found in association with fungal and bacterial endophytes. However, there remains a general lack of information and knowledge of the endophytes of these grasses as well as of methods for the identification and characterisation of novel endophytes and their deployment in plant improvement programs.

Knowledge of the endophytes of perennial ryegrass may allow certain beneficial traits to be exploited in enhanced pastures, or lead to other agricultural advances, e.g. to the benefit of sustainable agriculture and the environment.

There exists a need to overcome, or at least alleviate, one or more of the difficulties or deficiencies associated with the prior art.

Summary of the Invention

In one aspect, the present invention provides a substantially purified or isolated endophyte strain isolated from a plant of the Poaceae family, wherein said endophyte is a strain of *Pseudomonas poae* which provides bioprotection and/or biofertilizer phenotypes to plants into which it is inoculated. In a preferred embodiment, the *Pseudomonas poae* strain may be strain EY as described herein and as deposited with The National Measurement Institute on 17th May 2019 with accession number V19/009907.

As used herein the term “endophyte” is meant a bacterial or fungal strain that is closely associated with a plant. By “associated with” in this context is meant that the bacteria or fungus lives on, in or in close proximity to a plant. For example, it may be endophytic, for example living within the internal tissues of a plant, or epiphytic, for example growing externally on a plant.

As used herein the term “substantially purified” is meant that an endophyte is free of other organisms. The term includes, for example, an endophyte in axenic culture. Preferably, the endophyte is at least approximately 90 % pure, more preferably at least approximately 95 % pure, even more preferably at least approximately 98 % pure, even more preferably at least approximately 99 % pure.

As used herein the term ‘isolated’ means that an endophyte is removed from its original environment (e.g. the natural environment if it is naturally occurring). For example, a naturally occurring endophyte present in a living plant is not isolated, but the same endophyte separated from some or all of the coexisting materials in the natural system, is isolated.

As used herein the term “bioprotection and/or biofertilizer” means that the endophyte possesses genetic and/or metabolic characteristics that result in a beneficial phenotype in a plant harbouring, or otherwise associated with, the endophyte. Such beneficial properties include improved resistance to pests and/or diseases, improved tolerance to water and/or nutrient stress, enhanced biotic stress tolerance, enhanced drought tolerance, enhanced water use efficiency, reduced toxicity and enhanced vigour in the plant with which the endophyte is associated, relative to an organism not harbouring the endophyte or harbouring a control endophyte such as standard toxic (ST) endophyte.

The pests and/or diseases may include, but not limited to, bacterial and/or fungal pathogens, preferably fungal. In a particularly preferred embodiment, the endophyte may result in the production of the bioprotectant compound in the plant with which it is associated.

As used herein, the term ‘bioprotectant compound’ is meant as a compound that provides or aids bioprotection to the plant with which it is associated against pests and/or diseases, such as bacterial and/or fungal pathogens. A bioprotectant compound may also be known as a ‘biocidal compound’.

In a particularly preferred embodiment, the endophyte produces a bioprotectant compound and provides bioprotection to the plant against bacterial and/or fungal pathogens. The terms bioprotectant, bioprotective and bioprotection (or any other variations) may be used interchangeably herein.

In a particularly preferred embodiment the bioprotectant compound is poaeamide or poaeamide derivative, isomer and/or salt thereof.

The endophyte may be suitable as a biofertilizer to improve the availability of nutrients to the plant with which the endophyte is associated, including but not limited to improved tolerance to nutrient stress.

The nutrient stress may be lack of or low amounts of a nutrient such as phosphate and/or nitrogen. The endophyte is capable of growing in conditions such as low nitrogen and/or low phosphate and enable these nutrients to be available to the plant with which the endophyte is associated.

In a particularly preferred embodiment, the endophyte may result in the production of organic acids and/or the solubilisation of phosphate in the plant with which it is associated and/or provide a source of phosphate to the plant.

Alternatively, or in addition, the endophyte is capable of nitrogen fixation. Thus, an endophyte is capable of nitrogen fixation, the plant in which the endophyte is associated is capable of growing in low nitrogen conditions and/or provide a source of Nitrogen to the plant.

As used herein the term “plant of the Poaceae family” is a grass species, particularly a pasture grass such as ryegrass (*Lolium*) or fescue (*Festuca*), more particularly perennial ryegrass (*Lolium perenne* L.) or tall fescue (*Festuca arundinaceum*, otherwise known as *Lolium arundinaceum*).

In another aspect, the present invention provides a plant or part thereof infected with an endophyte as hereinbefore described. In preferred embodiments, the plant or part thereof infected with the endophyte may produce a bioprotectant compound, preferably poaeamide or derivative, isomer and/or salt thereof. Also in preferred embodiments, the plant or part thereof includes an endophyte-free host plant or part thereof stably infected with said endophyte.

The plant inoculated with the endophyte may be a grass or non-grass plant suitable for agriculture, specifically a forage, turf, or bioenergy grass, or a grain crop or industrial crop.

The forage, turf or bioenergy grass may be those belonging to the *Brachiaria-Urochloa* species complex (panic grasses), including *Brachiaria brizantha, Brachiaria decumbens, Brachiaria humidicola, Brachiaria stolonifera, Brachiaria ruziziensis, B. dictyoneura, Urochloa brizantha, Urochloa decumbens, Urochloa humidicola*, *Urochloa mosambicensis* as well as interspecific and intraspecific hybrids of *Brachiaria-Urochloa* species complex such as interspecific hybrids between *Brachiaria ruziziensis* x *Brachiaria brizantha*, *Brachiaria ruziziensis* x *Brachiaria decumbens*, [*Brachiaria ruziziensis* x *Brachiaria decumbens*] x *Brachiaria brizantha*, [*Brachiaria ruziziensis* x *Brachiaria brizantha*] x *Brachiaria decumbens*.

The forage, turf or bioenergy grass may also be those belonging to the genera *Lolium* and *Festuca*, including *L. perenne* (perennial ryegrass) and *L. arundinaceum* (tall fescue) and *L. multiflorum* (Italian ryegrass).

The grain crop may be a non-grass species, for example, any of soybeans, cotton and grain legumes, such as lentils, field peas, fava beans, lupins and chickpeas, as well as oilseed crops, such as canola.

Thus, the grain crop or industrial crop species may be selected from the group consisting of wheat, barley, oats, chickpeas, triticale, fava beans, lupins, field peas, canola, cereal rye, vetch, lentils, millet/panicum, safflower, linseed, sorghum, sunflower, maize, canola, mungbeans, soybeans, and cotton.

The grain crop or industrial crop may be a grass belonging to the genus *Triticum*, including *T. aestivum* (wheat), those belonging to the genus *Hordeum*, including *H. vulgare* (barley), those belonging to the genus *Zea*, including *Z. mays* (maize or corn), those belonging to the genus *Oryza*, including *O. sativa* (rice), those belonging to the genus *Saccharum* including *S. officinarum* (sugarcane), those belonging to the genus *Sorghum* including *S. bicolor* (sorghum), those belonging to the genus *Panicum*, including *P. virgatum* (switchgrass), and those belonging to the genera *Miscanthus*, *Paspalum*, *Pennisetum*, *Poa*, *Eragrostis* and *Agrostis*.

A plant or part thereof may be infected by a method selected from the group consisting of inoculation, breeding, crossing, hybridisation, transduction, transfection, transformation and/or gene targeting and combinations thereof.

Without wishing to be bound by theory, it is believed that the endophyte of the present invention may be transferred through seed from one plant generation to the next. The endophyte may then spread

or locate to other tissues as the plant grows, i.e. to roots. Alternatively, or in addition, the endophyte may be recruited to the plant root, e.g. from soil, and spread or locate to other tissues.

Thus, in a further aspect, the present invention provides a plant, plant seed or other plant part derived from a plant or part thereof as hereinbefore described. In preferred embodiments, the plant, plant seed or other plant part may produce a bioprotectant compound, preferably a poaeamide, or derivative, isomer and/or salt thereof.

In another aspect, the present invention provides the use of an endophyte as hereinbefore described to produce a plant or part thereof stably infected with said endophyte. The present invention also provides the use of an endophyte as hereinbefore described to produce a plant or part thereof as hereinbefore described.

In another aspect, the present invention provides a bioprotectant compound, preferably poaeamide, produced by an endophyte as hereinbefore described, or a derivative, isomer and/or a salt thereof.

The bioprotectant compound, preferably poaeamide, may be produced by the endophyte when associated with a plant, e.g. a plant of the Poaceae family as described above. Thus, in another aspect, the present invention provides a method for producing a bioprotectant compound, preferably poaeamide, or a derivative, isomer and/or a salt thereof, said method including infecting a plant with an endophyte as hereinbefore described and cultivating the plant under conditions suitable to produce the bioprotectant compound, preferably poaeamide, or a derivative, isomer and/or a salt thereof. The endophyte-infected plant or part thereof may be cultivated by known techniques. The person skilled in the art may readily determine appropriate conditions depending on the plant or part thereof to be cultivated.

The bioprotectant compound, preferably poaeamide, or a derivative, isomer and/or a salt thereof may also be produced by the endophyte when it is not associated with a plant. Thus, in yet another aspect, the present invention provides a method for producing a bioprotectant compound, preferably poaeamide, or a derivative, isomer and/or a salt thereof, said method including culturing an endophyte as hereinbefore described, under conditions suitable to produce the bioprotectant compound.

The conditions suitable to produce the bioprotectant compound, preferably poaeamide, or a derivative, isomer and/or a salt thereof, may include a culture medium including a source of carbohydrates. The source of carbohydrates may be a starch/sugar-based agar or broth such as potato dextrose agar, potato dextrose broth or half potato dextrose agar or a cereal-based agar or broth such as oatmeal agar or oatmeal broth. Other sources of carbohydrates may include endophyte agar, Murashige and Skoog with 20 % sucrose, half V8 juice/half PDA, water agar and

yeast malt extract agar. The endophyte may be cultured under aerobic or anaerobic conditions and may be cultured in a bioreactor.

In a preferred embodiment of this aspect of the invention, the method may include the further step of isolating the bioprotectant compound, preferably poaeamide, or a derivative, isomer and/or a salt thereof from the plant or culture medium.

The endophyte of the present invention displays the ability to solubilise phosphate.

Thus, in yet another aspect, the present invention provides a method of increasing phosphate use efficiency or increasing phosphate solubilisation by a plant, said method including infecting a plant with an endophyte as hereinbefore described, and cultivating the plant.

In yet another aspect, the present invention provides a method of reducing phosphate levels in soil, said method including infecting a plant with an endophyte as hereinbefore described, and cultivating the plant in the soil.

In yet a further aspect, the present invention provides a method of increasing nitrogen use efficiency or increasing nitrogen availability to a plant, said method including infecting a plant with an endophyte as hereinbefore described, and cultivating the plant.

In yet another aspect, the present invention provides a method of reducing nitrogen levels in soil, said method including infecting a plant with an endophyte as hereinbefore described, and cultivating the plant in the soil.

The endophyte-infected plant or part thereof may be cultivated by known techniques. The person skilled in the art may readily determine appropriate conditions depending on the plant or part thereof to be cultivated.

The production of a bioprotectant compound has particular utility in agricultural plant species, in particular, forage, turf, or bioenergy grass species, or grain crop species or industrial crop species. These plants may be cultivated across large areas of e.g. soil where the properties and biological processes of the endophyte as hereinbefore described and/or bioprotectant compound produced by the endophyte may be exploited at scale.

The part thereof of the plant may be, for example, a seed.

In preferred embodiments, the plant is cultivated in the presence of soil phosphate and/or nitrogen, alternatively or in addition to applied phosphate and/or nitrogen. The applied phosphate and/or

applied nitrogen may be by way of, for example, fertiliser. Thus, preferably, the plant is cultivated in soil.

In preferred embodiments, the endophyte may be a *Pseudomonas poae* strain EY as described herein and as deposited with The National Measurement Institute on 17th May 2019 with accession number V19/009907.

Preferably, the plant is a forage, turf, bioenergy grass species or, grain crop or industrial crop species, as hereinbefore described.

The part thereof of the plant may be, for example, a seed.

In preferred embodiments, the plant is cultivated in the presence of soil phosphate and/or applied phosphate. The applied phosphate may be by way of, for example, fertiliser. Thus, preferably, the plant is cultivated in soil.

Alternatively, or in addition, the plant is cultivated in the presence of soil nitrogen and/or applied nitrogen. The applied nitrogen may be by way of, for example, fertiliser. Thus, preferably, the plant is cultivated in soil.

The present invention will now be more fully described with reference to the accompanying Examples and drawings. It should be understood, however, that the description following is illustrative only and should not be taken in any way as a restriction on the generality of the invention described above.

Brief Description of the Drawings/Figures

Figure 1 - 16S Amplicon sequence of novel bacterial strain EY.

Figure 2 - Phylogeny of *Pseudomonas* spp. and novel bacterial strain EY. This maximum-likelihood tree was inferred based on 21 genes conserved among 19 genomes. Values shown next to branches were the local support values calculated using 1000 resamples with the Shimodaira-Hasegawa test.

Figure 3 - Bioprotection bioassay indicating the growth of 11 strains (including *Pseudomonas poae* novel bacterial strain EY, star) against 6 plant pathogenic fungi, *Fusarium verticillioides* (10 days post inoculation, dpi), *Bipolaris gossypina* (7 dpi), *Sclerotinia rolfsii* (5 dpi), *Drechslera brizae* (8 dpi), *Phoma sorghina* (9 dpi) and *Microdochium nivale* (6 dpi). Bars represent the mean diameter of

fungal colonies from three replicate plates of each treatment. Different superscript letters indicate significant differences ($P < 0.05$) between treatments.

Figure 4 - Secondary metabolite biosynthesis gene clusters in *Pseudomonas poae* novel bacterial strain EY identified using antiSMASH (Weber et al. 2015). The gene clusters have sequence homology and structure to (A) the *poaA* gene cluster and (B) the *poaB* and *poaC* gene cluster. An additional 4 genes are present in the *poaA* gene cluster in strain EY, including an ABC transporter binding protein (i), ABC transporter permease (ii), cyclodehydratase (iii) and an oxidoreductase (iv) that are all involved in microcin biosynthesis.

Figure 5 - Whole genome sequence comparison of *Pseudomonas poae* novel bacterial strain EY (top) and *Pseudomonas poae* bacterial strain RE1-1-14 (bottom). The links between genome sequences indicated percentage similarity (from 70 % to 100 %). Genetic variations, including non-identical regions and insertions/deletions/inversions, suggest that *Pseudomonas poae* bacterial strains EY and RE1-1-14 are genetically different. Stars represent genomic regions unique to *Pseudomonas poae* bacterial strains EY (dark grey stars) or RE1-1-14 (light grey stars).

Figure 6 - Biofertiliser activity (*in vitro*) of the *Pseudomonas poae* novel bacterial strain EY on Pikovskaya's Agar, which determines the ability of bacteria to solubilise inorganic phosphate.

Figure 7 - Image of 5 day old seedlings (11) inoculated with the *Pseudomonas poae* novel bacterial strain EY and an untreated control.

Figure 8 - Average shoot length of barley seedlings inoculated with bacterial strains of *Pseudomonas poae* (novel strain EY) and non-Pseudomonads (Strain 1, 2, 3), and grown for 5 days. The * indicates significant difference in the mean at $P < 0.05$ between the control and the bacterial strains.

Figure 9 - Average root length of barley seedlings inoculated with bacterial strains of *Pseudomonas poae* (novel strain EY) and non-Pseudomonads (Strain 1, 2, 3), and grown for 5 days. The * indicates significant difference in the mean at $P < 0.05$ between the control and the bacterial strains.

Figure 10 - Agarose gel electrophoresis (2 % [w/v]) of PCR amplicons generated using the EY strain-specific primers on *Pseudomonas poae* bacterial strain EY, closely related strains (DP, HC, CT14) a negative control (NC) and a 2 kb DNA molecular ladder (M)

Figure 11 - Average root length of barley seedlings inoculated with bacterial strains of *Pseudomonas poae*. (strain EY) and non-Pseudomonads (Strain 1, 2, 3, 4), and grown for 4 days on nitrogen free

media. The star indicates significant difference in the mean at $P < 0.05$ between the control and the bacterial strains.

Figure 12 - Average shoot length of barley seedlings inoculated with bacterial strains of *Pseudomonas poae*. (strain EY) and non-Pseudomonads (Strain 1, 2, 3, 4), and grown for 4 days on nitrogen free media. The star indicates significant difference in the mean at $P < 0.05$ between the control and the bacterial strains.

Figure 13 - Average root length of barley seedlings inoculated with bacterial strains of *Pseudomonas poae*. (strain EY) and non-Pseudomonads (Strain 1, 2, 3, 4) and grown for 4 days on media containing insoluble phosphate. The star indicates significant difference in the mean at $P < 0.05$ between the control and the bacterial strains.

Figure 14 - Average shoot length of barley seedlings inoculated with bacterial strains of *Pseudomonas poae*. (strain EY) and non-Pseudomonads (Strain 1, 2, 3, 4) and grown for 4 days on media containing insoluble phosphate. The star indicates significant difference in the mean at $P < 0.05$ between the control and the bacterial strains.

Figure 15 - Average root and shoot length of barley seedlings inoculated with novel *Pseudomonas poae* bacterial strain EY at different concentrations (10^0, 10^{-1}, 10^{-2}), and grown for 7 days.

Detailed Description of the Embodiments

Discovery and characterisation of plant associated *Pseudomonas poae* novel bacterial strains providing bioprotection and biofertilizer phenotypes to plants.

The novel plant associated *Pseudomonas poae* bacterial strain EY has been isolated from perennial ryegrass (*Lolium perenne*) plants. It displays the ability to inhibit the growth of plant fungal pathogens and solubilise phosphate in plate assays. The genome of the *Pseudomonas poae* bacterial strain EY has been sequenced and is shown to be novel, related to bioprotectant *Pseudomonas poae* strains and not pathogenic Pseudomonad bacteria. Analysis of the genome sequence has shown that the *Pseudomonas poae* novel bacterial strain EY has gene clusters for the biosynthesis of the bioprotectant compound poaeamide, genes involved in biofertilisation via the production of organic acids and the solubilisation of phosphate, while there is an absence of virulence-related genes (effectors) suggesting the strain has an endophytic life cycle. This novel bacterial strain has been used to inoculate barley (*Hordeum vulgare*) seeds under glasshouse conditions and has been demonstrated not to cause disease in these barley plants. These barley plants are also able to produce seed. Novel bacterial strain EY also enhances root and shoot growth in nitrogen limiting conditions and in insoluble phosphate. The optimal concentration of inoculum for novel bacterial

strain EY is a dilution of an overnight culture (10^{-1}, 10^{-2}). Overall, novel plant associated *Pseudomonas poae* bacterial strain EY offer both bioprotectant and biofertilizer activity.

Example 1 – Isolation of Bacterial Strains

Seed associated bacterial strains

Seeds from perennial ryegrass (*Lolium perenne*) were surface-sterilised by soaking in 80 % ethanol for 3 mins, then washing 5 times in sterile distilled water. The seeds were then plated onto sterile filter paper soaked in sterile water in sterile petri dishes. These plates were stored at room temperature in the dark to allow seedlings to germinate for 1 – 2 weeks. Once the seedlings were of sufficient size, the plants were harvested. In harvesting, the remaining seed coat was discarded, and the aerial tissue and root tissue were harvested. The plant tissues were submerged in sufficient Phosphate Buffered Saline (PBS) to completely cover the tissue, and ground using a Qiagen TissueLyser II, for 1 minute at 30 Hertz. A 10 µL aliquot of the macerate was added to 90 µL of PBS. Subsequent 1 in 10 dilutions of the 10^{-1} suspension were used to create additional 10^{-2} to 10^{-4} suspensions. Once the suspensions were well mixed 50 µL aliquots of each suspension were plated onto Reasoners 2 Agar (R2A) for growth of bacteria. Dilutions that provided a good separation of bacterial colonies were subsequently used for isolation of individual bacterial colonies through re-streaking of single bacterial colonies from the dilution plates onto single R2A plates to establish a pure bacterial colony.

Mature plant associated bacterial strains

Leaf and root tissue were harvested from mature plants grown in the field or grown in pots in a greenhouse. Root tissue was washed in PBS buffer to remove soil particles and sonicated (10 mins) to remove the rhizosphere. The harvested tissues were placed into sufficient PBS to completely cover the tissue and processed as per the previous section to isolate pure bacterial cultures.

Around 300 bacterial strains were obtained from sterile seedlings, and 300 strains from mature plants. The novel bacterial strain EY was collected from seed of perennial ryegrass.

Example 2 – Identification of *Pseudomonas poae* novel bacterial strain

Amplicon (16S rRNA gene) Sequencing

A phylogenetic analysis of the novel bacterial strain EY was undertaken by sequence homology comparison of the 16S rRNA gene. The novel bacterial strain EY was grown overnight in Reasoners 2 Broth (R2B) media. DNA was extracted from pellets derived from the overnight culture using a

DNeasy Blood and Tissue kit (Qiagen) according to manufacturer's instructions. The 16S rRNA gene amplification used the following PCR reagents: 14.8 µL H_2O, 2.5 µL 10× reaction buffer, 0.5 µL 10 mM dNTPs, 2.5 µL each of the 5 µM 27F primer (5'- AGAGTTTGATCMTGGCTCAG -3') and 5 µM reverse primers 1492R (5'- GGTTACCTTGTTACGACTT -3'), 0.2 µL of Immolase enzyme, and template to a final volume of 25 µL. The PCR reaction was then run in an Agilent Surecylcer 8800 (Applied Biosystems) with the following program; a denaturation step at 94 °C for 15 min; 35 cycles of 94 °C for 30 sec, 55 °C for 10 sec, 72 °C 1 min; and a final extension step at 72 °C for 10 min.

Shrimp alkaline phosphatase (SAP) exonuclease was used to purify the 16S rRNA gene PCR amplicon. The SAP amplicon purification used the following reagents: 7.375 µL H_2O, 2.5 µL 10× SAP, and 0.125 µL Exonuclease I. The purification reaction was incubated at 37 °C for 1 hr, followed by 15 min at 80 °C to deactivate the exonuclease.

The purified 16S rRNA gene amplicon was sequenced using the BigDye® Terminator v3.1 Cycle Sequencing Kit (Thermofisher) with the following reagents; 10.5 µL H_2O, 3.5 µL 5× Seq buffer, 0.5 µL BigDye®, 2.5 µL of either the 3.2 µM Forward (27F) and 3.2 µM Reverse primers (1492R), and 4.5 µL of PCR amplicon as template, to a final reaction volume of 20 µL. The sequencing PCR reaction was then run in an Agilent Surecylcer 8800 (Applied Biosystems) with the following program; denaturation step at 94 °C for 15 min; followed by 35 cycles of 94 °C for 30 sec, 55 °C for 10 sec, 72 °C 1 min; and one final extension step at 72 °C for 10 min. The 16S rRNA gene amplicon from novel bacterial strain EY was sequenced on an ABI3730XL (Applied Biosystems). A 1278 bp 16S rRNA gene sequence was generated (Figure 1). The sequence was aligned by BLASTn on NCBI against the non-redundant nucleotide database and the 16S ribosomal RNA database.

BLASTn hit against database nr; *Pseudomonas poae* strain HTM601-1 16S ribosomal RNA gene, partial sequence

Max Score	Total Score	Query Coverage	E-Value	% Identity	Accession
2361	2361	100 %	0	100.00 %	MG835948.1

BLASTn hit against database 16S ribosomal RNA; *Pseudomonas poae* strain P 527/13 16S ribosomal RNA gene, partial sequence

Max Score	Total Score	Query Coverage	E-Value	% Identity	Accession
2355	2355	100 %	0	99.92 %	NR_028986.1

The preliminary taxonomic identification of the novel bacterial strain EY was *Pseudomonas poae*.

Genomics

The genome of novel bacterial strain EY was sequenced. This novel bacterial strain was retrieved from the glycerol collection stored at -80 °C by streaking on R2A plates. Single colonies from these plates were grown overnight in Nutrient Broth and pelleted. These pellets were used for genomic DNA extraction using the bacteria protocol of Wizard® Genomic DNA Purification Kit (A1120, Promega). A DNA sequencing library was generated for Illumina sequencing using the Illumina Nextera XT DNA library prep protocol. The library was sequenced using an Illumina MiSeq platform or HiSeq platform. Raw reads from the sequencer were filtered to remove any adapter and index sequences as well as low quality bases using Trimmomatic (Bolger, Lohse & Usadel 2014) with the following options: ILLUMINACLIP: NexteraPE-PE.fa:2:30:10 LEADING:3 TRAILING:3 SLIDINGWINDOW:4:15 MINLEN:36. To enable full genome assembly, long reads were generated for novel bacterial strain EY only by sequencing DNA using Oxford Nanopore Technologies (ONT) MinION platform. The DNA from the Wizard® Genomic DNA Purification Kit was first assessed with the genomic assay on Agilent 2200 TapeStation system (Agilent Technologies, Santa Clara, CA, USA) for integrity (average molecular weight ≥30 Kb). The sequencing library was prepared using an in-house protocol modified from the official protocols for transposases-based library preparation kits (SQK-RAD004/SQK-RBK004, ONT, Oxford, UK). The library was sequenced on a MinION Mk1B platform (MIN-101B) with R9.4 flow cells (FLO-MIN106) and under the control of MinKNOW software. After the sequencing run finished, the fast5 files that contain raw read signals were transferred to a separate, high performance computing Linux server for local basecalling using ONT's Albacore software (Version 2.3.1) with default parameters. The sequencing summary file produced by Albacore was processed by the R script minion qc (https://github.com/roblanf/minion_qc) and NanoPlot (De Coster et al. 2018) to assess the quality of the sequencing run, while Porechop (Version 0.2.3, https://github.com/rrwick/Porechop) was used to remove adapter sequences from the reads. Reads which were shorter than 300 bp were removed and the worst 5 % of reads (based on quality) were discarded by using Filtlong (Version 0.2.0, https://github.com/rrwick/Filtlong).

The whole genome sequence of novel bacterial strain EY was assembled using Unicycler (Wick et al. 2017). Unicycler performed hybrid assembly when both Illumina reads and MinION reads were available. MinION reads were mainly used to resolve repeat regions in the genome, whereas Illumina reads were used by Pilon (Walker et al. 2014) to correct small base-level errors. Multiple rounds of Racon (Vaser et al. 2017) polishing were then carried out to generate consensus sequences. Assembly graphs were visualised by using Bandage (Wick et al. 2015).

A complete circular chromosome sequence was produced for the novel bacterial strain EY. The genome size for the novel bacterial strain EY was 5,469,454 bp (Table 1). The percent GC content was 60.99 %. The novel bacterial strain EY was annotated by Prokka (Seemann 2014) with a

custom, genus-specific protein database to predict genes and corresponding functions, which were then screened manually to identify specific traits. The number of genes for the novel bacterial strain EY was 4,877 (Table 2).

Table 1 – Summary of properties of the final genome sequence assembly

Strain ID	Genome size (bp)	GC content (%)	Coverage Illumina reads	Coverage ONT MinION
EY	5,469,454	60.99	115×	40×

Table 2 – Summary of genome coding regions

Strain ID	Genome size (bp)	No. of tRNA	No. of tmRNA	No. of rRNA	No. of CDS	No. of gene
EY	5,469,454	69	1	16	4,791	4,877

Eighteen *Pseudomonas* spp. (*P. fluorescens*, *P. chlororaphis*, *P. syringae*, *P. putida*, *P. stutzeri*, *P. aeruginosa*, *P. oryzihabitans*) genome sequences that are publicly available on NCBI were acquired and used for pan-genome/comparative genome sequence analysis alongside the novel bacterial strain EY. A total of 21 genes that are shared by all 19 *Pseudomonas* spp. bacterial strains were identified by running Roary (Page et al. 2015). PRANK (Löytynoja 2014) was then used to perform a codon aware alignment. A maximum-likelihood phylogenetic tree (Figure 4) was inferred using FastTree (Price, Dehal & Arkin 2010) with Jukes-Cantor Joins distances and Generalized Time-Reversible and CAT approximation model. Local support values for branches were calculated using 1000 resamples with the Shimodaira-Hasegawa test. The novel bacterial strain EY clustered tightly with the bioprotectant *Pseudomonas poae* bacterial strain RE1-1-14, suggesting a close phylogenetic relationship between these two bacterial strains. Moreover, this cluster was separated from other *Pseudomonas* spp. with strong local support value (100 %). This separation supports that bacterial strain EY is novel and from the species *Pseudomonas poae*.

The average nucleotide identity (ANI) was calculated for novel bacterial strain EY against *Pseudomonas poae* bacterial strain RE1-1-14. The genome sequences were aligned and compared using minimap2 (Li 2018). The ANI between bacterial strains EY and RE1-1-14 was 99.46 %. Based on a species boundary of 95 – 96 % (Chun et al. 2018; Richter & Rosselló-Móra 2009) bacterial strain EY is a novel strain of the species *Pseudomonas poae* (Müller et al. 2013).

A maximum-likelihood tree was inferred based on 21 genes conserved among 19 genomes (Figure 2).

Example 3 – Bioprotection activity (*in vitro*) of the *Pseudomonas poae* novel bacterial strain EY

In vitro bioassays were established to test the bioactivity of 11 plant associated bacterial strains including *Pseudomonas poae* novel bacterial strain EY, against six plant pathogenic fungi (Table 3). A plate with only the pathogen was used as a negative control (blank). The fungal pathogens were all isolated from monocot species, and were obtained from the National Collection of Fungi (Herbarium VPRI) and the AVR collection. Each bacterial strain was cultured in Nutrient Broth (BD Biosciences) overnight at 28 °C in a shaking incubator (200 rpm). Each bacterial strain was drop-inoculated (20 µL) onto four equidistant points on a Nutrient Agar (BD Biosciences) plate, which was then incubated overnight at 28 °C. A 6 mm × 6 mm agar plug of actively growing mycelia from the pathogen was placed at the centre of the plate. The bioassay was incubated for at least 5 days at 28 °C in the dark, and then the diameter of the fungal colony on the plate was recorded. For each treatment three plates were prepared as biological triplicates. OriginPro 2018 (Version b9.5.1.195) was used to carry out One-way ANOVA and Tukey Test to detect the presence of any significant difference ($P < 0.05$) between treatments.

Table 3 – Pathogens used in the bioprotection bioassay

VPRI Accession No.	**Taxonomic Details**	**Host Taxonomic Details**	**State**	**Collection Date**
12962	*Drechslera brizae* (Y.Nisik.) Subram. & B.L.Jain	*Briza maxima* L.	Vic.	24-Oct-85
32148	*Sclerotium rolfsii* Sacc.	*Poa annua* L.	Vic.	1-Jan-05
10694	*Phoma sorghina* (Sacc.) Boerema, Dorenbosch, van Kesteren	*Cynodon dactylon* Pers.	Vic.	19-Apr-79
42586a	*Fusarium verticillioides* (Sacc.) Nirenberg	*Zea mays* L.	Vic.	27-Feb-15
42563	*Bipolaris gossypina*	*Brachiaria*	Qld	
N/A	*Microdochium nivale*	*Lolium perenne* L.	Vic	

The *Pseudomonas poae* novel bacterial strain EY inhibited the growth of all six fungal pathogens compared to the control and many of the other test bacterial strains, indicating it had broad spectrum biocidal activity (Figure 3). The *Pseudomonas poae* novel bacterial strain EY was the most active bacterial strain against *Fusarium verticillioides*, *Bipolaris gossypina*, *Sclerotium rolfsii* and *Phoma sorghina*, while it was the second most active strain against *Drechslera brizae* and *Microdochium nivale*.

Example 4 – Genome sequence features supporting the bioprotection niche of the *Pseudomonas poae* novel bacterial strain EY

Secondary metabolite biosynthesis gene clusters

The genome sequence of *Pseudomonas poae* novel bacterial strain EY was assessed for the presence of features associated with bioprotection. The annotated genome was analysed by antiSMASH (Weber et al. 2015) to identify secondary metabolite biosynthesis gene clusters that are commonly associated with the production of biocidal compounds that aid in their defence. An annotated genome was passed through antiSMASH with the following options: *--clusterblast --asf --knownclusterblast --subclusterblast --smcogs --full-hmmer*. A total of two secondary metabolite gene clusters were identified in the genome sequence of the *Pseudomonas poae* novel bacterial strain EY. (Figure 4). The two biosynthetic gene clusters (cluster 1 – *poaA*; cluster 2 – *poaB* and *poaC*) had sequence homology (99 %) and structure to the poeamide gene cluster that produces the bioprotectant non-ribosomal peptide poaeamide (Figure 4). This gene cluster had the non-ribosomal peptide synthases (NRPS - *poaA*, *poaB*, *poaC*) essential for the biosynthesis of poaeamide and was similar in structure compared to the reference strain (RE1-1-14). In the *poaA* gene cluster of EY there is the presence of an additional four genes with sequence homology to genes involved in microcin biosynthesis, including an ABC transporter binding protein, ABC transporter permease, cyclodehydratase and an oxidoreductase (Figure 4A). Some of these additional genes are likely to interact with poaeamide to alter the structure and produce a slightly different compound to poaeamide.

Genome sequence alignment

The genome sequences of *Pseudomonas poae* novel bacterial strain EY and the bioprotectant *Pseudomonas poae* strain RE1-1-14 were aligned using LASTZ (Version 1.04.00, http://www.bx.psu.edu/~rsharris/lastz/) and visualised using AliTV (Ankenbrand et al. 2017) to determine the genomic similarity between the two strains. The genome sequences of the two strains were similar, but there were large genomic regions unique to the novel bacterial strain EY (red stars) or the bacterial strain RE1-1-14 (yellow stars) (Figure 5).

Example 5 – Biofertiliser activity (*in vitro*) of the *Pseudomonas poae* novel bacterial strain EY

Phosphate is an essential ion for plant growth. Phosphate is applied to fields to improve plant growth and yield. A large amount of applied phosphate is not accessible to plants. Some bacteria have been shown to have the ability to mobilise some of this inaccessible phosphate. The P-solubilisation ability of bacterial strains was detected by using the Pikovskaya's Agar (Sundar ORacand & Sinha 1963), which contains inorganic phosphate in the form of calcium phosphate (5 g/L). *Pseudomonas poae*

novel bacterial strain EY and *Escherichia coli* (negative control) were inoculated onto Pikovskaya's Agar at three equidistant points on a plate. All plates were then incubated for 72 hours at room temperature, and inspected visually for the formation of a clear zone around the colony. For each strain three plates were prepared as biological triplicates. The *Pseudomonas poae* novel bacterial strain EY was able to solubilise inorganic phosphate, as evidenced by a zone of clearing around the colony (Figure 6).

Example 6 – Genome sequence features supporting the biofertiliser niche of the *Pseudomonas poae* novel bacterial strain EY

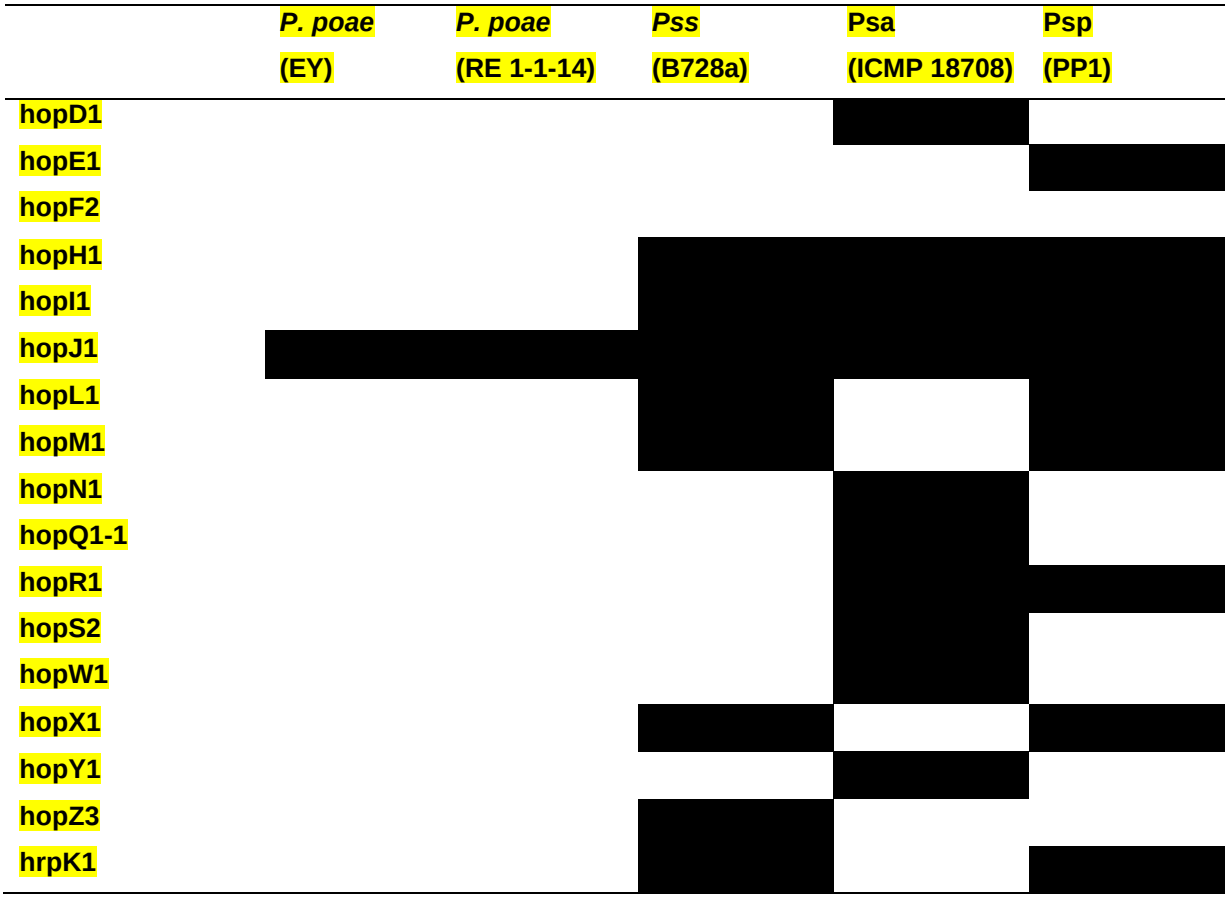
A number of bacterial and fungal species have been reported to solubilise inorganic phosphate. The mechanism of inorganic phosphate solubilization is via the production of mineral dissolving compounds such as organic acids (i.e. oxalic acid, citric acid, lactic acid, gluconic acid), siderophores, protons, hydroxyl ions and CO_2 (Rodríguez & Fraga 1999; Sharma, Kumar & Tripathi 2017). Organic acids together with their carboxyl and hydroxyl ions chelate cations or reduce the pH to release Phosphorous (Tallapragada & Seshachala 2012). A total of 4,877 genes in the annotated genome sequence of *Pseudomonas poae* novel bacterial strain EY were assessed for nomenclature consistent with the production of organic acids and the solubilisation of phosphate. Enzymes involved in organic acid production were identified including glucose dehydrogenases (gluconic acid), gluconate dehydrogenase (2-ketogluconic acid) and lactate dehydrogenase (lactic acid).

Example 7 – Genome sequence features supporting the endophytic niche of the *Pseudomonas poae* novel bacterial strain EY

There have been 57 virulence-related type III effector repertoires (genes) identified in *Pseudomonas syringae* pathovars that are important for the pathogenicity of this species (Lindeberg, Cunnac & Collmer 2012). These effectors are important for invading the host, suppressing the host immune system and altering host physiology for the benefit of the pathogen (Henry et al. 2017). A total of 50 type III effector repertoires were assessed for presence/absence in the genome sequence of novel bacterial strain EY (*Pseudomonas poae*), along with bacterial strains RE1-1-14 (*Pseudomonas poae*), B28a (*Pseudomonas syringae* pv. *syringae*), ICMP18708 (*Pseudomonas syringae* pv. *actinidae*) and PP1 (*Pseudomonas syringae* pv. *pisi*) through sequence homology searches (BLASTp, 80 % similarity, e-value 1^{-10}) (Table 4). The *Pseudomonas poae* novel bacterial strain EY had only one of the 50 type III effector repertoires (*HopJ*). There was an absence of many of the key effectors involved in the pathogenicity of *Pseudomonas syringae*, including *AVRE1* and *HopI* (Wei, Zhang & Collmer 2018).

Table 4 – Fifty type III effector repertoires (genes) identified in *Pseudomonas syringae* pathovars and *Pseudomonas poae* strains (EY and RE1-1-14)

	P. poae **(EY)**	***P. poae*** **(RE 1-1-14)**	***Pss*** **(B728a)**	**Psa (ICMP 18708)**	**Psp (PP1)**
avrB3			■		■
avrB4-1					
avrE1			■	■	■
avrPphB					
avrPto					
avrRpm1			■	■	
avrRps4					■
hopA1				■	
hopAA1			■		■
hopAA1-1				■	
hopAA1-2				■	
hopAB1			■		
hopAC1			■	■	■
hopAE1			■		■
hopAF1			■		■
hopAG::ISPssy				■	
hopAG1			■	■	■
hopAH1			■	■	■
hopAH2			■		■
hopAH2-1				■	
hopAH2-2				■	
hopAI1				■	
hopAJ1					■
hopAJ2			■	■	■
hopAK1			■	■	■
hopAM1-1				■	■
hopAN1			■	■	■
hopAO1					
hopAS1				■	
hopAU1				■	
hopAV1				■	
hopAW1				■	
hopC1					■



Example 8 – *In planta* inoculations supporting endophytic niche of the *Pseudomonas poae* novel bacterial strain EY

To assess direct interactions between the *Pseudomonas poae* novel bacterial strain EY and plants, an early seedling growth assay was established in barley. A total of 4 bacterial strains (EY – *Pseudomonas poae*; Strain 1, Strain 2, Strain 3) were cultured in Lysogeny Broth (LB) overnight at 26 °C. The following day seeds of barley (cultivar Hindmarsh) were surface-sterilised by soaking in 80 % ethanol for 3 mins, then washing 5 times in sterile distilled water. The seeds were then soaked in the overnight cultures for 4 hours at 26 °C in a shaking incubator. For control seedlings, seeds were soaked in LB without bacteria for 4 hours at 26 °C in a shaking incubator. The seeds were planted into a pot trial, with three replicates (pots) per strain/control, with a randomised design. A total of 20 seeds were planted per pot, to a depth of 1 cm. The potting medium contained a mixture of 25 % potting mix, 37.5 % vermiculite and 37.5 % perlite. The plants were grown for 5 days and then removed from the pots, washed, assessed for health (i.e. no disease symptoms) and photographed. The lengths of the longest root and the longest shoot were measured. Data was statistically analysed using a One-way ANOVA and Tukey test to detect the presence of any significant difference ($P < 0.05$) between treatments using OriginPro 2018 (Version b9.5.1.195).

Seedlings inoculated with the *Pseudomonas poae* novel bacterial strain EY were healthy with no disease symptoms recorded on leaves or roots (Figure 7). The length of the shoots inoculated with the *Pseudomonas poae* novel bacterial strain EY were equivalent to the control (Figure 8). The length of the roots of inoculated with the *Pseudomonas poae* novel bacterial strain EY were significantly shorter than the control (Figure 9).

Example 9 – *In planta* inoculations supporting colonisation and localisation of the *Pseudomonas poae* novel bacterial strain EY in wheat and perennial ryegrass

Strain-specific primers were designed for *Pseudomonas poae* novel bacterial strain EY targeting the 3440768-3441879 bp region of the genome, which related to an insertion the *paoA* gene of the poaeamide biosynthetic gene cluster of EY (EY-F TGTTAAACACGCAACTCGCC; EY-R AAAGGTGCACTCACAACCTCTG; 5'→3'). An *in silico* analysis using Primer-BLAST indicated that the primers were strain-specific.

The strain-specific primer for EY was evaluated on cultures of strains *Pseudomonas poae* novel bacterial strain EY, along with closely related strains (DP, HC, CT14). Initially, bacterial cultures were grown in nutrient broth (BD Bioscience) and grown overnight at 22 °C in the dark in a shaking incubator. The Promega Wizard® genomic DNA purification kit was used with the following modifications: initial centrifugation of 1 mL of overnight culture at 13,000 – 16,000× g for 2 mins was performed twice to pellet bacterial cells; incubations were conducted at -20 ºC for 10 mins to enhance protein precipitation; DNA pellets were rehydrated in 50 mL rehydration solution at 65 °C for 10 mins followed by overnight incubation at 4 °C. Final DNA concentration was measured using a Quantus™ Fluorometer and stored at 4 °C until further processing. The 25 µL reaction mixture contained: 12.5 µL of OneTaq™ Hot Start 2× master mix with standard buffer (New England BioLabs®), 2 µL of each primer (10 µM/µL), 8.5 µL of nuclease-free water and 2 µL of template DNA sample. The thermocycling conditions were: initial denaturation at 94 °C for 1 min, followed by 30 cycles of denaturation at 94 °C for 30 sec, annealing at 58 °C for 1 min, elongation at 72 °C for 2 min, and a final extension at 72 °C for 10 min. PCR products were separated at 120 V in a 2 % (w/v) agarose gel containing 0.05 µL mL-1 SYBR safe stain in 1× TAE running buffer and visualized under UV light next to a 2 kb DNA ladder. The strain-specific primer generated an amplicon of the correct size (1112 bp) for *Pseudomonas poae* novel bacterial strain EY and DP (likely duplicate of EY) (Figure 10).

The strain-specific primer for EY was evaluated on wheat plants inoculated with *Pseudomonas poae* novel bacterial strain EY. Initially, wheat seeds were sterilized in 70 % ethanol for 3 minutes, followed by rinsing with sterilized distilled water (SDW) for three times. The bacterial strain was cultured in nutrient broth (BD Bioscience) overnight, while seeds were imbibed in nutrient broth overnight in the dark. Seeds and the bacterial culture were combined for 4 hours in dark in a shaking

incubator. For the controls, seeds were not inoculated with bacteria. A total of three seeds were sown per pot into potting mix and grown in a glasshouse. For wheat, plants were harvested at only one time point (7 days after planting, DAP). For wheat inoculated with EY 10 replicates were maintained. For the uninoculated control treatments (wheat) 5 replicates were maintained for each time point. At harvest, plants were uprooted, washed thoroughly (roots only) and then sectioned into roots, pseudostem and leaves (wheat - 7 DAP). Each section comprised three pieces (~0.5 cm^2) of plant tissue, which was placed into collection microtubes (2 mL) and stored at -80 °C. The Qiagen® MagAttract® 96 DNA plant core kit (Qiagen®, Hilden, Germany) was utilized to extract plant DNA using the Biomek® FXP lab automation workstation linked to Biomek software version v. 4.1 and Gen 5 (v. 2.08) software (Biotek Instruments, USA) with the following modifications to the manufacturer's instructions: to each well of the 96 well microplate, a 33 µL aliquot of RB buffer and 10 µL of resuspended MegAttract suspension G was added. A touch-down PCR (TD-PCR) was performed to enhance the sensitivity and specificity of primers *in planta*, compared to *in vitro* pure cultures. The PCR reaction mixture was prepared as per *in vitro* cultures. Touch-down PCR amplification was performed in two phases. In phase I, initial denaturation was carried out at 94 °C for 1 min, followed by 10 cycles of denaturation at 94 °C for 30 sec, annealing for at 65 - 55 °C (dropping 1 °C for each cycle) and 72 °C for 2 mins. In phase II, it was 20 cycles of denaturation at 94 °C for 30 sec, annealing at 58 °C for 1 min and extension at 72 °C for 2 min, with a final extension at 72 °C for 10 min. For wheat, the presence of the *Pseudomonas poae* novel bacterial strain EY was detected at 7 DAP, with the highest rates of incidence recorded in roots (80 %), followed by pseudostem (30 %), however it was not detected in the leaves (0 %) (Table 9). Overall, *Pseudomonas poae* novel bacterial strain EY appears to inoculate into wheat, where it colonises subterranean and aerial tissue, but appears to preferentially colonise roots.

Table 9 – Incidence of *Pseudomonas poae* novel bacterial strain EY in wheat at one harvest time point. The incidence is indicated as the number of plants showing the presence of EY per total number of replicates inoculated or uninoculated (R - roots; P - pseudostem; L - leaves).

	7 DAP		
	R	**P**	**L**
EY	8/10	3/10	0/10
Control	0/5	0/5	0/5

Example 10 – *In planta* inoculations supporting the biofertilizer (nitrogen) niche of the *Pseudomonas poae* novel bacterial strain EY

An *in planta* biofertilizer assay was established in barley to evaluate the ability of *Pseudomonas poae* novel bacterial strain EY to aid growth under nitrogen limiting conditions. Initially, bacterial strains (5, including EY were cultured in 20 mL nutrient broth (BD Bioscience) overnight at 26 °C

whilst rotating at 200 rpm. The following day cultures were pelleted via centrifugation at 4000 rpm for 5 minutes, washed three times in 10 mL Phosphate Buffered Saline (PBS), resuspended in 20 mL PBS, quantified via spectrophotometry (OD_{600}) and diluted (1:10). Barley seeds were sterilized in 70 % ethanol for 5 minutes, followed by rinsing with sterilized distilled water (SDW) for five times. These sterile seeds were submerged in the dilution for 4 hours in a dark incubator at room temperature whilst rotating at 200 rpm. The seeds were subsequently transferred to moistened sterile filter paper and allowed to germinate for three days. The three-day-old seedlings were individually transferred to 60 mm plates with semi-solid Burks media (HiMedia) (5 g/L Agar). Seedlings were allowed to grow for a further 4 days, before the shoots and roots were measured for each seedling. There was a total of 6 treatments (5 bacterial strains including EY; 1 blank media control) containing 10 seedlings per treatment. Statistical analysis (One-way ANOVA and Tukey Test) was conducted using OriginPro 2018 (Version b9.5.1.195) to detect the presence of any significant difference ($P < 0.05$) between treatments.

The root growth of seedlings inoculated with novel bacterial strain EY and grown under nitrogen limiting conditions was significantly greater than the control ($P < 0.05$), with an average increase of 28.6 % (Figure 11). The shoot growth of seedlings inoculated with novel bacterial strain EY was not significantly greater than the control ($P < 0.05$), despite increasing shoot growth by 12.5 % (Figure 12). Overall, results indicate that novel bacterial strain EY can aid in the growth of seedlings grown under nitrogen limiting conditions.

Example 11 – *In planta* inoculations supporting the biofertilizer (phosphate solubilisation) niche of the *Pseudomonas poae* novel bacterial strain EY

An *in planta* biofertilizer assay was established in barley to evaluate the ability of *Pseudomonas poae* novel bacterial strain EY to aid growth under conditions with insoluble phosphate. Initially, bacterial strains (5, including EY) were cultured in 30 mL R2B overnight at 26 °C whilst rotating at 200 rpm. The following day the barley seeds were sterilized in 70 % ethanol for 5 minutes, followed by rinsing with SDW for five times. These sterile seeds were submerged in the overnight cultures for 4 hours in a dark incubator at room temperature whilst rotating at 200 rpm. The seeds were subsequently transferred to moistened sterile filter paper to be allowed to germinate for three days. These three-day-old seedlings were individually transferred to 60 mm plates with semi-solid Pikovskaya's media which contains yeast extract (0.5 g/L), D-glucose (5.0 g/L), calcium phosphate (5.0 g/L), ammonium sulphate (0.5 g/L), potassium chloride (0.2 g/L), magnesium sulphate (0.1 g/L), manganese sulphate (0.1 mg/L), ferrous sulphate (0.1 mg/L) and agar (5.0 g/L). These seedlings were allowed to grow for another 4 days, before the shoots and roots were measured for each seedling. There was a total of 6 treatments (5 bacterial strains including EY; 1 blank media control) containing 10 seedlings per treatment. Statistical analysis (One-way ANOVA and Tukey Test) was

conducted using OriginPro 2018 (Version b9.5.1.195) to detect the presence of any significant difference ($P < 0.05$) between treatments.

The root growth of seedlings inoculated with novel bacterial strain EY and grown under conditions with insoluble phosphate was significantly greater than the control ($P < 0.05$), with an average increase of 62.5 % (Figure 13). The shoot growth of seedlings inoculated with novel bacterial strain EY was significantly greater than the control ($P < 0.05$), with an average increase of 46.2 % (Figure 14). Overall, results indicate that novel bacterial strain EY can aid in the growth of seedlings grown under conditions with insoluble phosphate.

Example 12 – *In planta* inoculations identifying optimal concentrations of *Pseudomonas poae* novel bacterial strain EY

An *in planta* biofertilizer assay was established in perennial ryegrass to evaluate the optimal concentration in which *Pseudomonas poae* novel bacterial strain EY would support seedling growth. Initially, the bacterial strain was cultured overnight in 20 mL nutrient broth (BD Bioscience) at 26°C whilst rotating at 200 rpm. The following day the culture was pelleted via centrifugation at 4000 rpm for 5 minutes, washed three times in 10 mL PBS, resuspended in 20 mL PBS, quantified via spectrophotometry (OD_{600}). The culture was diluted (1:10) twice to create three concentrations (10^0, 10^{-1} and 10^{-2}). The perennial ryegrass seeds were sterilized in 70 % ethanol for 5 minutes, followed by rinsing five times with SDW. These sterile seeds were submerged in the dilutions for 4 hours in a dark incubator at room temperature whilst rotating at 200 rpm. After inoculation, 10 seeds were transferred to moistened sterile filter paper for germination from each dilution. After seven days, the roots and shoots were measured.

Root growth of seedlings inoculated with novel bacterial strain EY was greatest with the 10^{-1} dilution, which was 4.4 % greater than 10^{-2} dilution and 14.0 % greater than the 10^{-0} dilution (Figure 15). Shoot growth of seedlings inoculated with novel bacterial strain EY was greatest with the 10^{-2} dilution, which was 13.3 % greater than 10^{-1} dilution and 16.7 % greater than the 10^{-0} dilution. Overall, results indicate that novel bacterial strain EY has the greatest effects on root and shoot growth at lower concentrations.

It is to be understood that various alterations, modifications and/or additions may be made without departing from the spirit of the present invention as outlined herein.

As used herein, except where the context requires otherwise, the term "comprise" and variations of the term, such as "comprising", "comprises" and "comprised", are not intended to be in any way limiting or to exclude further additives, components, integers or steps.

Reference to any prior art in the specification is not, and should not be taken as, an acknowledgment or any form of suggestion that this prior art forms part of the common general knowledge in Australia or any other jurisdiction or that this prior art could reasonably be expected to be combined by a person skilled in the art.

5

References

1. Ankenbrand, MJ, Hohlfeld, S, Hackl, T & Förster, F 2017, 'AliTV—interactive visualization of whole genome comparisons', *PeerJ Computer Science*, vol. 3.

2. Bolger, AM, Lohse, M & Usadel, B 2014, 'Trimmomatic: a flexible trimmer for Illumina sequence data', *Bioinformatics*, vol. 30, no. 15, pp. 2114-20.

3. Chun, J, Oren, A, Ventosa, A, Christensen, H, Arahal, DR, da Costa, MS, Rooney, AP, Yi, H, Xu, XW, De Meyer, S & Trujillo, ME 2018, 'Proposed minimal standards for the use of genome data for the taxonomy of prokaryotes', *Int J Syst Evol Microbiol*, vol. 68, no. 1, pp. 461-6.

4. De Coster, W, D'Hert, S, Schultz, DT, Cruts, M & Van Broeckhoven, C 2018, 'NanoPack: visualizing and processing long read sequencing data', *Bioinformatics*.

5. Henry, E, Toruño, TY, Jauneau, A, Deslandes, L & Coaker, G 2017, 'Direct and indirect visualization of bacterial effector delivery into diverse plant cell types during infection', *The Plant Cell*, vol. 29, no. 7, pp. 1555-70.

6. Li, H 2018, 'Minimap2: pairwise alignment for nucleotide sequences', *Bioinformatics*, pp. bty191-bty.

7. Lindeberg, M, Cunnac, S & Collmer, A 2012, 'Pseudomonas syringae type III effector repertoires: last words in endless arguments', *Trends Microbiol*, vol. 20, no. 4, pp. 199-208.

8. Löytynoja, A 2014, 'Phylogeny-aware alignment with PRANK', in DJ Russell (ed.), *Multiple Sequence Alignment Methods*, Humana Press, Totowa, NJ, pp. 155-70, DOI 10.1007/978-1-62703-646-7_10, <https://doi.org/10.1007/978-1-62703-646-7_10>.

9. Müller, H, Zachow, C, Alavi, M, Tilcher, R, Krempl, PM, Thallinger, GG & Berg, G 2013, 'Complete Genome Sequence of the Sugar Beet Endophyte Pseudomonas poae RE*1-1-14, a Disease-Suppressive Bacterium', *Genome Announc*, vol. 1, no. 2, p. e0002013.

10. Page, AJ, Cummins, CA, Hunt, M, Wong, VK, Reuter, S, Holden, MT, Fookes, M, Falush, D, Keane, JA & Parkhill, J 2015, 'Roary: rapid large-scale prokaryote pan genome analysis', *Bioinformatics*, vol. 31, no. 22, pp. 3691-3.

11. Price, MN, Dehal, PS & Arkin, AP 2010, 'FastTree 2--approximately maximum-likelihood trees for large alignments', *PLoS One*, vol. 5, no. 3, p. e9490.

12. Richter, M & Rosselló-Móra, R 2009, 'Shifting the genomic gold standard for the prokaryotic species definition', *Proceedings of the National Academy of Sciences*, vol. 106, no. 45, pp. 19126-31.

13. Rodríguez, H & Fraga, R 1999, 'Phosphate solubilizing bacteria and their role in plant growth promotion', *Biotechnology Advances*, vol. 17, no. 4–5, pp. 319-39.

14. Seemann, T 2014, 'Prokka: rapid prokaryotic genome annotation', *Bioinformatics*, vol. 30, no. 14, pp. 2068-9.

15. Sharma, S, Kumar, V & Tripathi, RB 2017, 'Isolation of phosphate solubilizing microorganism (PSMs) from soil', *Journal of microbiology and Biotechnology Research*, vol. 1, no. 2, pp. 90-5.

16. Sundar ORacand, W & Sinha, M 1963, 'Phosphate dissolving microorganisms in the rhizosphere and soil', *Indiaj Agric Sci*, vol. 33, no. 7, pp. 272-8.

17. Tallapragada, P & Seshachala, U 2012, 'Phosphate-solubilizing microbes and their occurrence in the rhizospheres of Piper betel in Karnataka, India', *Turkish Journal of Biology*, vol. 36, no. 1, pp. 25-35.

18. Vaser, R, Sovic, I, Nagarajan, N & Sikic, M 2017, 'Fast and accurate de novo genome assembly from long uncorrected reads', *Genome Res*, vol. 27, no. 5, pp. 737-46.

19. Walker, BJ, Abeel, T, Shea, T, Priest, M, Abouelliel, A, Sakthikumar, S, Cuomo, CA, Zeng, Q, Wortman, J, Young, SK & Earl, AM 2014, 'Pilon: an integrated tool for comprehensive microbial variant detection and genome assembly improvement', *PLoS One*, vol. 9, no. 11, p. e112963.

20. Weber, T, Blin, K, Duddela, S, Krug, D, Kim, HU, Bruccoleri, R, Lee, SY, Fischbach, MA, Muller, R, Wohlleben, W, Breitling, R, Takano, E & Medema, MH 2015, 'antiSMASH 3.0-a comprehensive resource for the genome mining of biosynthetic gene clusters', *Nucleic Acids Res*, vol. 43, no. W1, pp. W237-43.

21. Wei, H-L, Zhang, W & Collmer, A 2018, 'Modular study of the type III effector repertoire in Pseudomonas syringae pv. tomato DC3000 reveals a matrix of effector interplay in pathogenesis', *Cell reports*, vol. 23, no. 6, pp. 1630-8.

22. Wick, RR, Judd, LM, Gorrie, CL & Holt, KE 2017, 'Unicycler: Resolving bacterial genome assemblies from short and long sequencing reads', *PLOS Computational Biology*, vol. 13, no. 6, p. e1005595.

23. Wick, RR, Schultz, MB, Zobel, J & Holt, KE 2015, 'Bandage: interactive visualization of de novo genome assemblies', *Bioinformatics*, vol. 31, no. 20, pp. 3350-2.

THE CLAIMS DEFINING THE INVENTION ARE AS FOLLOWS:

1. A substantially purified or isolated endophyte strain isolated from a plant of the Poaceae family, wherein said endophyte is a strain of *Pseudomonas poae* which provides bioprotection and/or biofertilizer phenotypes to plants into which it is inoculated.

2. An endophyte according to claim 1, wherein the bioprotection and/or biofertilizer phenotype includes production of a bioprotectant compound in the plant into which the endophyte is inoculated.

3. An endophyte according to claim 2, wherein the bioprotectant compound is poaeamide or a derivative, isomer and/or salt thereof.

4. An endophyte according to any one of claims 1 to 3, wherein the bioprotection and/or biofertilizer phenotype includes production of organic acids and/or the solubilisation of phosphate in the plant into which the endophyte is inoculated.

5. An endophyte according to any one of claims 1 to 4, wherein the endophyte is strain EY as described herein and as deposited with The National Measurement Institute on 17th May 2019 with accession number V19/009907.

6. An endophyte according to any one of claims 1 to 5, wherein the plant from which the endophyte is isolated is of the Poaceae family is a pasture grass.

7. An endophyte according to claim 6, wherein the pasture grass is from the genus Lolium or Festuca.

8. An endophyte according to claim 7, wherein the pasture grass is from the species *Lolium perenne* or *Festuca arundinaceum.*

9. An endophyte according to any one of claims 1 to 8, wherein the plant into which the endophyte is inoculated includes an endophyte-free host plant or part thereof stably infected with said endophyte.

10. An endophyte according to any one of claims 1 to 9, wherein the plant into which the endophyte is inoculated is an agricultural plant species selected from one or more of forage grass, turf grass, bioenergy grass, grain crop and industrial crop species.

11. An endophyte according claim 10, wherein the plant into which the endophyte is inoculated is a forage, turf or bioenergy grass selected from the group consisting of those belonging to the

genera *Lolium* and *Festuca*, including *L. perenne* (perennial ryegrass), *L. arundinaceum* (tall fescue) and *L. multiflorum* (Italian ryegrass), and those belonging to the *Brachiaria-Urochloa* species complex (panic grasses), including *Brachiaria brizantha, Brachiaria decumbens, Brachiaria humidicola, Brachiaria stolonifera, Brachiaria ruziziensis, B. dictyoneura, Urochloa brizantha, Urochloa decumbens, Urochloa humidicola*, *Urochloa mosambicensis* as well as interspecific and intraspecific hybrids of *Brachiaria-Urochloa* species complex such as interspecific hybrids between *Brachiaria ruziziensis* x *Brachiaria brizantha*, *Brachiaria ruziziensis* x *Brachiaria decumbens*, [*Brachiaria ruziziensis* x *Brachiaria decumbens*] x *Brachiaria brizantha*, [*Brachiaria ruziziensis* x *Brachiaria brizantha*] x *Brachiaria decumbens*.

12. An endophyte according claim 10, wherein the plant into which the endophyte is inoculated is a grain crop or industrial crop selected from the group consisting of those belonging to the genus *Triticum*, including *T. aestivum* (wheat), those belonging to the genus *Hordeum*, including *H. vulgare* (barley), those belonging to the genus *Zea*, including *Z. mays* (maize or corn), those belonging to the genus *Oryza*, including *O. sativa* (rice), those belonging to the genus *Saccharum* including *S. officinarum* (sugarcane), those belonging to the genus *Sorghum* including *S. bicolor* (sorghum), those belonging to the genus *Panicum*, including *P. virgatum* (switchgrass), those belonging to the genera *Miscanthus*, *Paspalum*, *Pennisetum*, *Poa*, *Eragrostis* and *Agrostis*,

13. An endophyte according to claim 10, wherein the plant into which the endophyte is inoculated is a grain crop or industrial crop selected from the group consisting of wheat, barley, oats, chickpeas, triticale, fava beans, lupins, field peas, canola, cereal rye, vetch, lentils, millet/panicum, safflower, linseed, sorghum, sunflower, maize, canola, mungbeans, soybeans, and cotton.

14. A plant or part thereof infected with one or more endophytes according to any one of claims 1 to 13.

15. A plant, plant seed or other plant part derived from a plant or part thereof according to claim 14 and stably infected with said one or more endophytes.

16. Use of an endophyte according to any one of claims 1 to 13 to produce a plant or part thereof stably infected with said one or more endophytes.

17. A bioprotectant compound produced by an endophyte according to any one of claims 1 to 13, or a derivative, isomer and/or a salt thereof, preferably the bioprotectant compound is poaeamide.

18. A method for producing a bioprotective compound, or a derivative, isomer and/or a salt thereof, said method including infecting a plant with an endophyte according to any one of claims 1 to 13 and cultivating the plant under conditions suitable to produce the bioprotective compound.

19. A method for producing a bioprotective compound, or a derivative, isomer and/or a salt thereof, said method including culturing an endophyte according to any one of claims 1 to 13 under conditions suitable to produce the bioprotective compound.

20. A method according to claim 19, wherein the conditions include a culture medium including a source of carbohydrates.

21. A method according to claim 20, wherein the source of carbohydrates is selected from one or more of the group consisting of a starch/sugar-based agar or broth, a cereal-based agar or broth, endophyte agar, Murashige and Skoog with 20 % sucrose, half V8 juice/half PDA, water agar and yeast malt extract agar.

22. A method according to any one of claims18 to 21, wherein the method further includes isolating the bioprotective compound from the plant or culture medium.

23. A method of increasing phosphate use efficiency or increasing phosphate solubilisation by a plant, said method including infecting a plant with an endophyte according to any one of claims 1 to 13, and cultivating the plant.

24. A method according to claim 23, wherein the plant is cultivated in the presence of soil phosphate and/or applied phosphate.

25. A method according to claim 24, wherein the applied phosphate includes phosphate applied by fertiliser.

26. A method according to any one of claims 23 to 25, wherein the plant is cultivated in soil.

ABSTRACT

The present invention relates to an endophyte strain isolated from a plant of the Poaceae family, wherein said endophyte is a strain of *Pseudomonas poae* which provides bioprotection and/or biofertilizer phenotypes to plants into which it is inoculated. The present invention also plants infected with the endophyte and related methods.

Figures

TGCCTAGGAATCTGCCTGGTAGTGGGGGATAACGTTCGGAAACGGACGCTAATACCGCATACGTCCTACGG
GAGAAAGCAGGGGACCTTCGGGCCTTGCGCTATCAGATGAGCCTAGGTCGGATTAGCTAGTTGGTGGGGTA
ATGGCTCACCAAGGCGACGATCCGTAACTGGTCTGAGAGGATGATCAGTCACACTGGAACTGAGACACGGT
CCAGACTCCTACGGGAGGCAGCAGTGGGGAATATTGGACAATGGGCGAAAGCCTGATCCAGCCATGCCGCG
TGTGTGAAGAAGGTCTTCGGATTGTAAAGCACTTTAAGTTGGGAGGAAGGGCAGTTACCTAATACGTGATTG
TTTTGACGTTACCGACAGAATAAGC\ACCGGCTAACTCTGTGCCAGCAGCCGCGGTAATACAGAGGGTGCAA
GCGTTAATCGGAATTACTGGGCGTAAAGCGCGCGTAGGTGGTTTGTTAAGTTGGATGTGAAATCCCCGGGC
TCAACCTGGGAACTGCATTCAAAACTGACTGACTAGAGTATGGTAGAGGGTGGTGGAATTTCCTGTGTAGCG
GTGAAATGCGTAGATATAGGAAGGAACACCAGTGGCGAAGGCGACCACCTGGACTAATACTGACACTGAGG
TGCGAAAGCGTGGGGAGCAAACAGGATTAGATACCCTGGTAGTCCACGCCGTAAACGATGTCAACTAGCCG
TTGGAAGCCTTGAGCTTTTAGTGGCGCAGCTAACGCATTAAGTTGACCGCCTGGGGAGTACGGCCGCAAGG
TTAAAACTCAAATGAATTGACGGGGGCCCGCACAAGCGGTGGAGCATGTGGTTTAATTCGAAGCAACGCGA
AGAACCTTACCAGGCCTTGACATCCAATGAACTTTCTAGAGATAGATTGGTGCCTTCGGGAACATTGAGACA
GGTGCTGCATGGCTGTCGTCAGCTCGTGTCGTGAGATGTTGGGTTAAGTCCCGTAACGAGCGCAACCCTTGT
CCTTAGTTACCAGCACGTCATGGTGGGCACTCTAAGGAGACTGCCGGTGACAAACCGGAGGAAGGTGGGGA
TGACGTCAAGTCATCATGGCCCTTACGGCCTGGGCTACACACGTGCTACAATGGTCGGTACAGAGGGTTGCC
AAGCCGCGAGGTGGAGCTAATCCCATAAAACCGATCGTAGTCCGGATCGCAGTCTGCAACTCGACTGCGTG
AAGTCGGAATCGCTAGTAATCGCGAATCAGAATGTCGCGGTGAATACGTTCCCGGGCCTTGTACAC

Figure 1

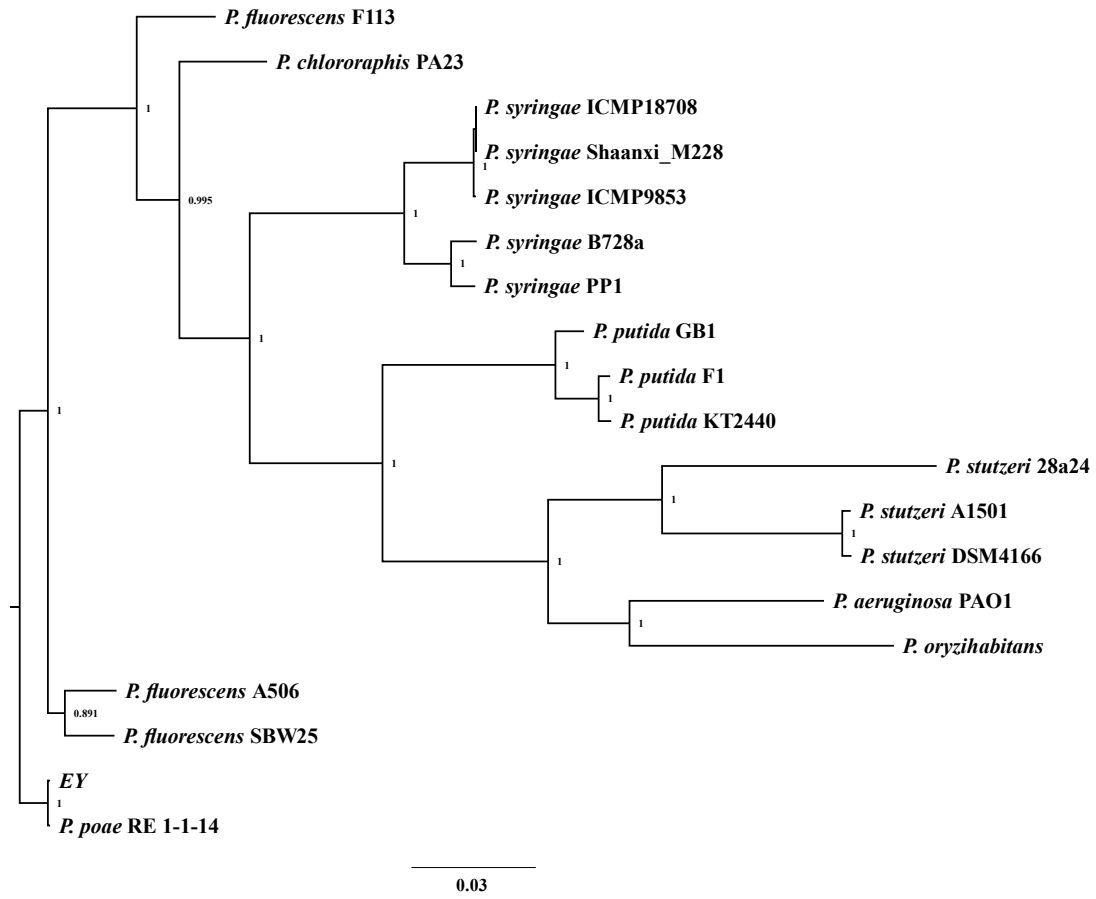

P. fluorescens F113
P. chlororaphis PA23
P. syringae ICMP18708
P. syringae Shaanxi_M228
P. syringae ICMP9853
P. syringae B728a
P. syringae PP1
P. putida GB1
P. putida F1
P. putida KT2440
P. stutzeri 28a24
P. stutzeri A1501
P. stutzeri DSM4166
P. aeruginosa PAO1
P. oryzihabitans
P. fluorescens A506
P. fluorescens SBW25
EY
P. poae RE 1-1-14
0.995
0.891
0.03

Figure 2

Appendix 1

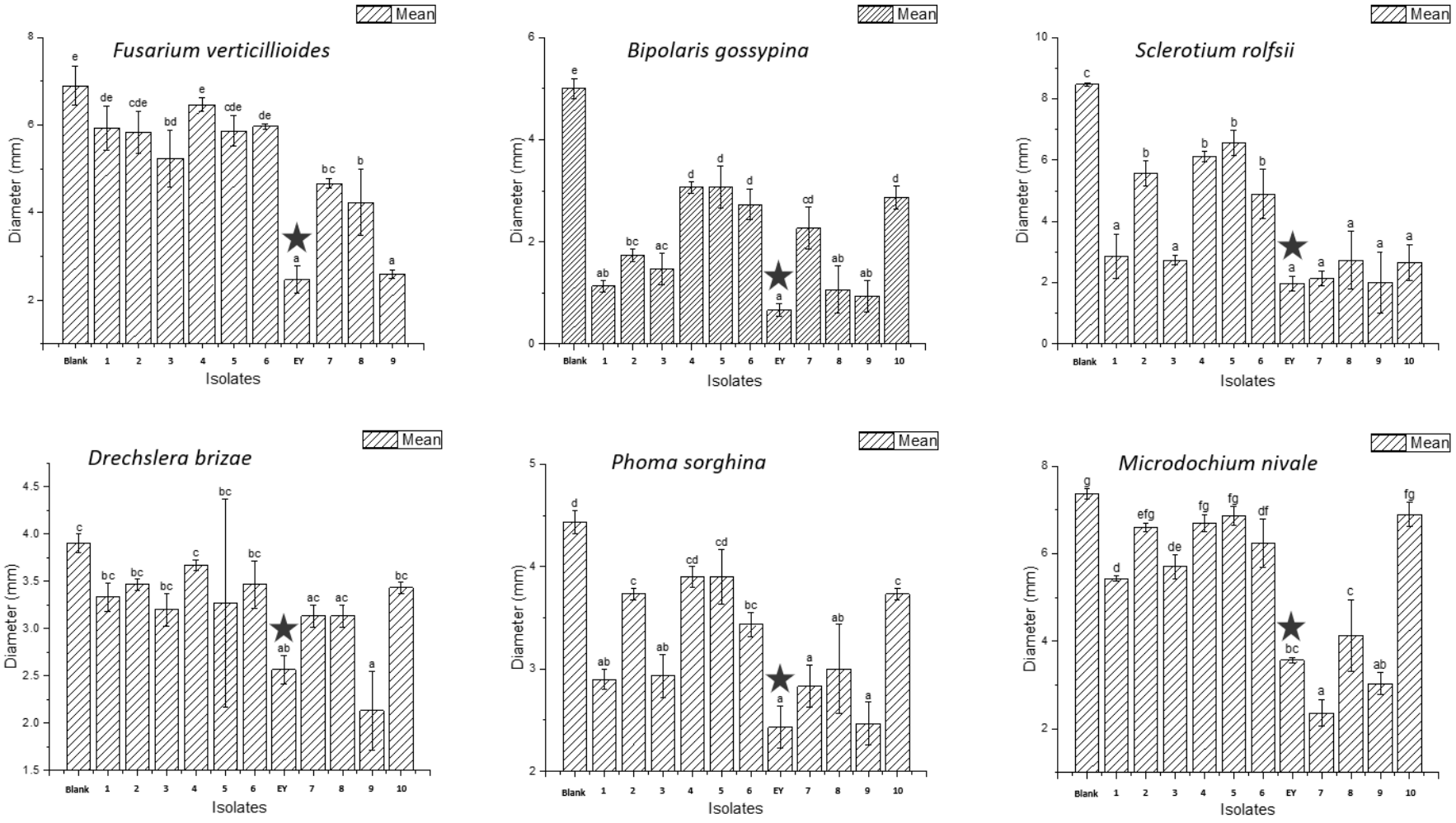
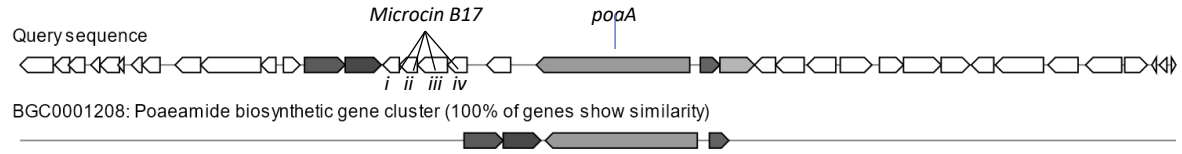


Figure 3

A. *poaA* Gene Cluster



B. *poaB* and *poaC* Gene Cluster

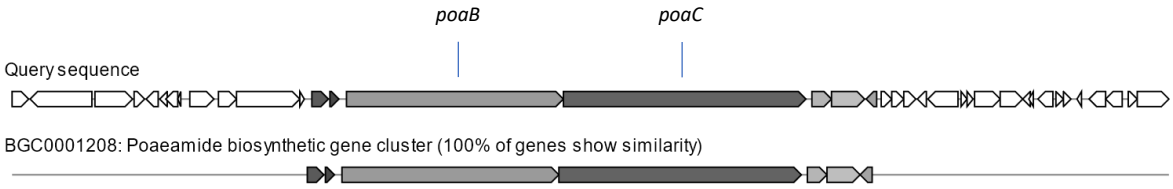


Figure 4

Appendix 1

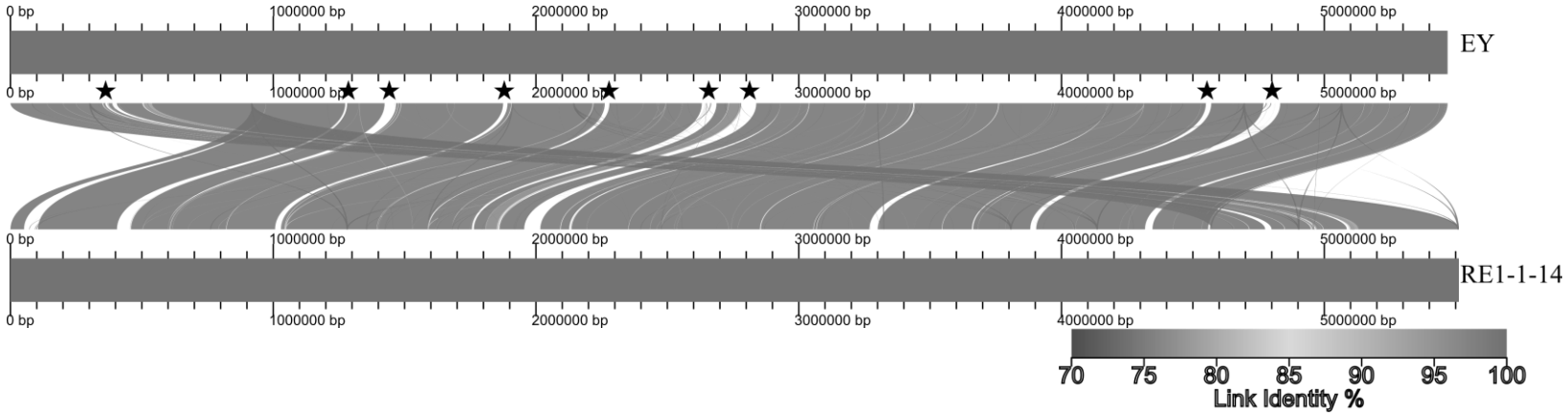


Figure 5

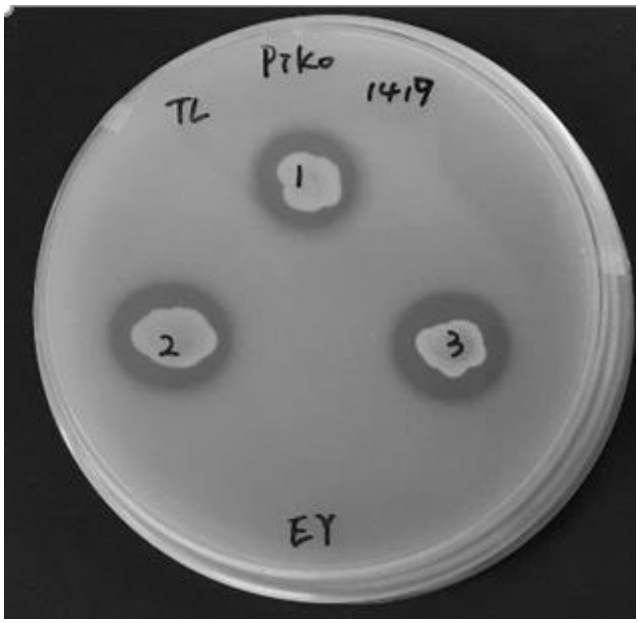


Figure 6

Appendix 1

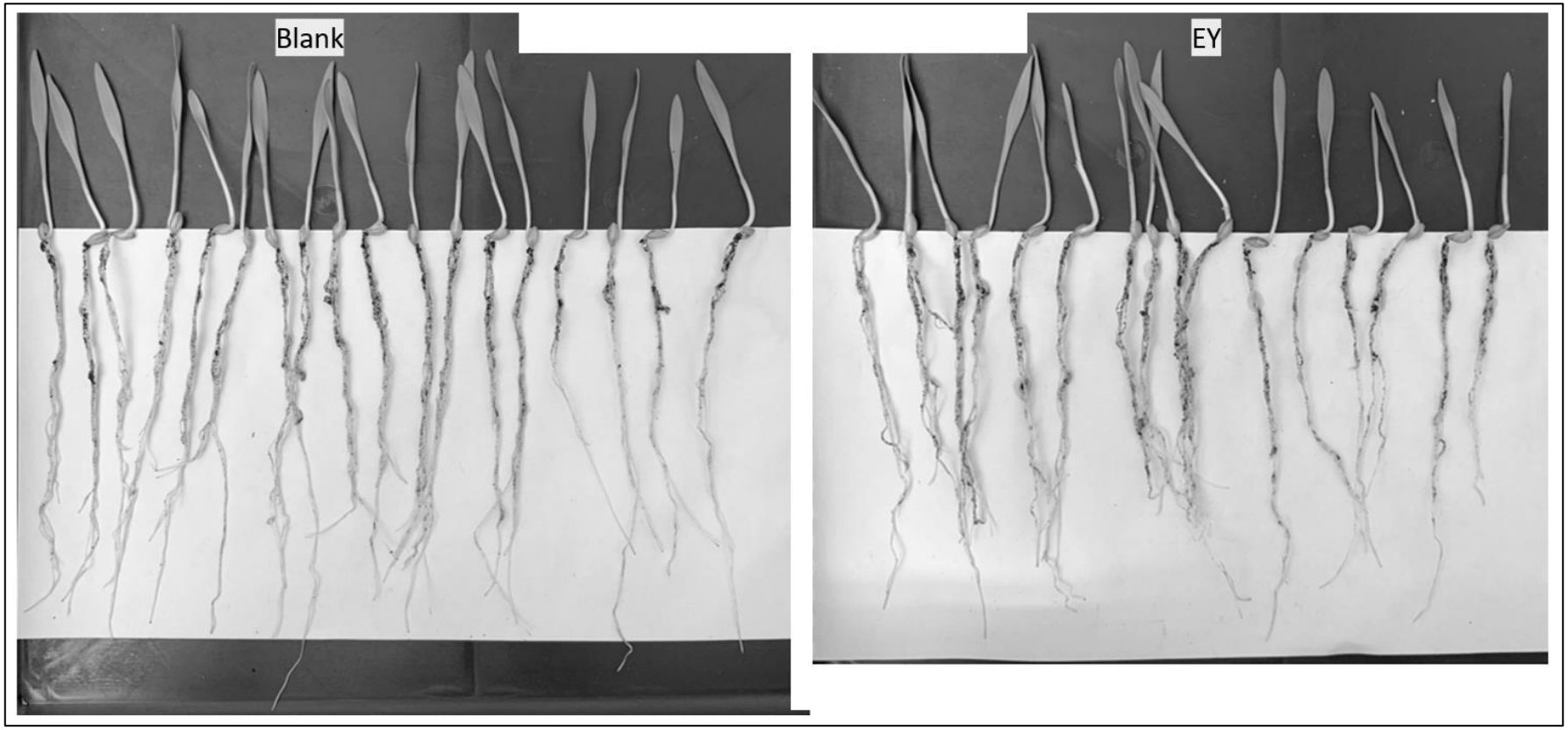


Figure 7

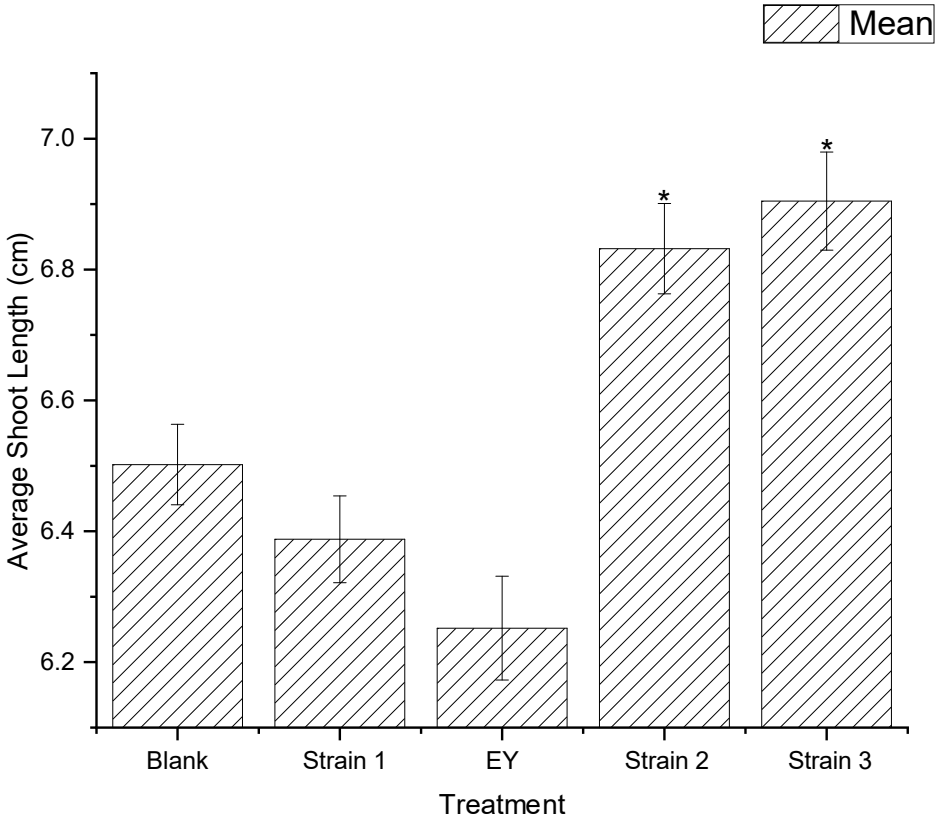

Mean
Average Shoot Length (cm)
7.0
6.8
6.6
6.4
6.2
*
*
Blank
Strain 1
EY
Strain 2
Strain 3
Treatment

Figure 8

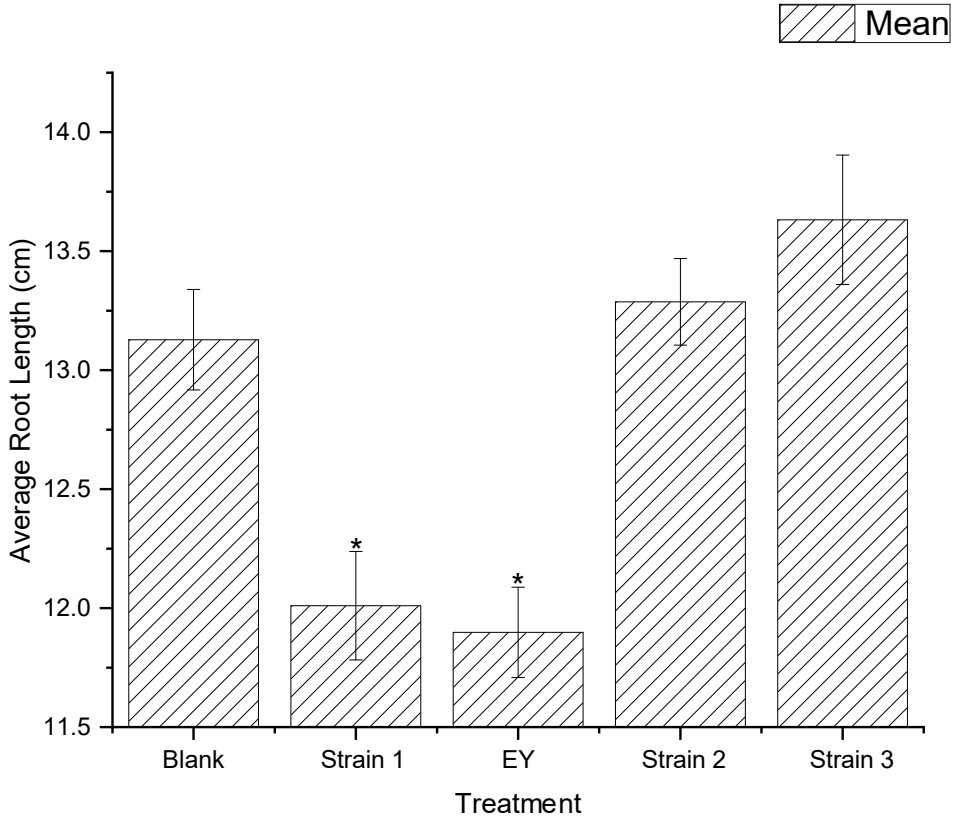

Mean
Average Root Length (cm)
14.0
13.5
13.0
12.5
12.0
11.5
*
*
Blank
Strain 1
EY
Strain 2
Strain 3
Treatment

Figure 9

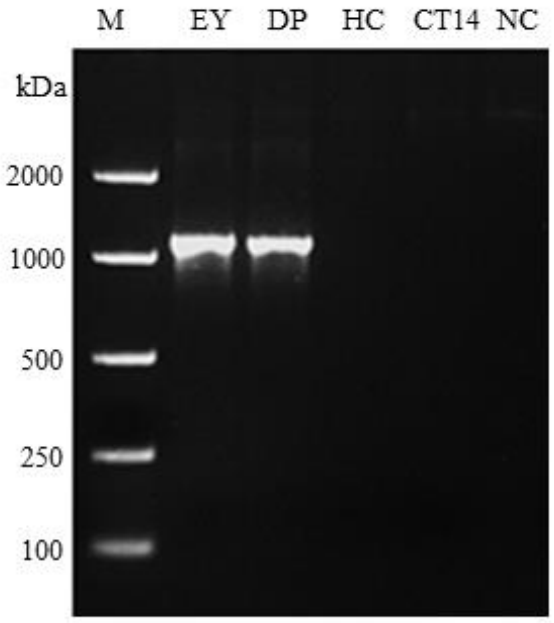


Figure 10

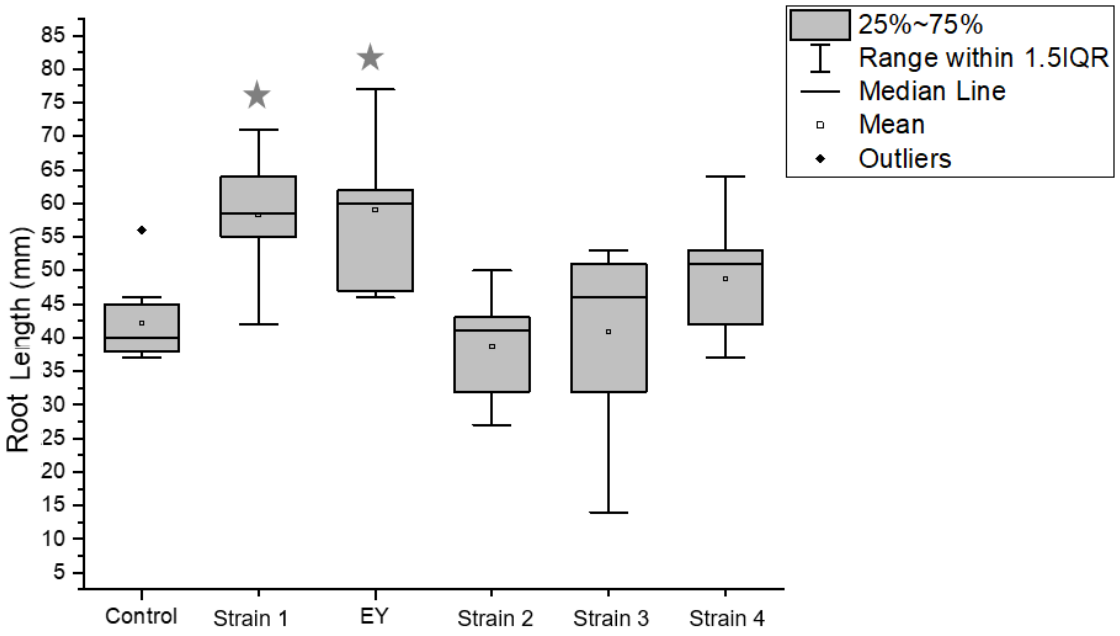

25%~75%
Range within 1.5IQR
Median Line
Mean
Outliers
Root Length (mm)
85
80
75
70
65
60
55
50
45
40
35
30
25
20
15
10
5
Control
Strain 1
EY
Strain 2
Strain 3
Strain 4

Figure 11

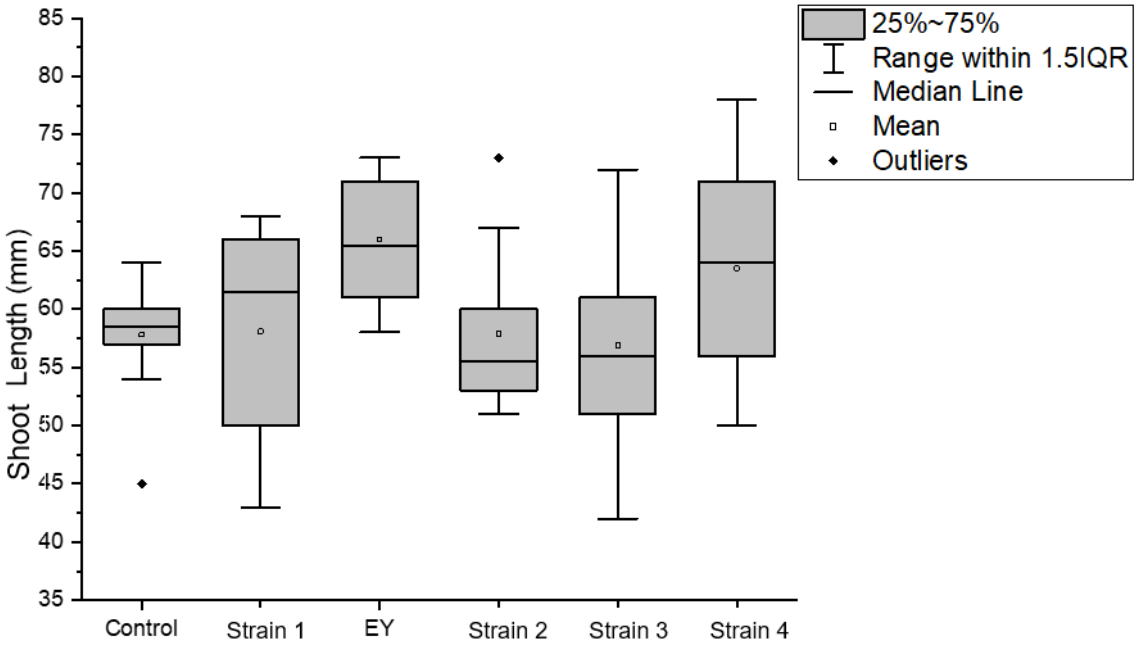

Shoot Length (mm)
85
80
75
70
65
60
55
50
45
40
35
Control
Strain 1
EY
Strain 2
Strain 3
Strain 4
25%~75%
Range within 1.5IQR
Median Line
Mean
Outliers

Figure 12

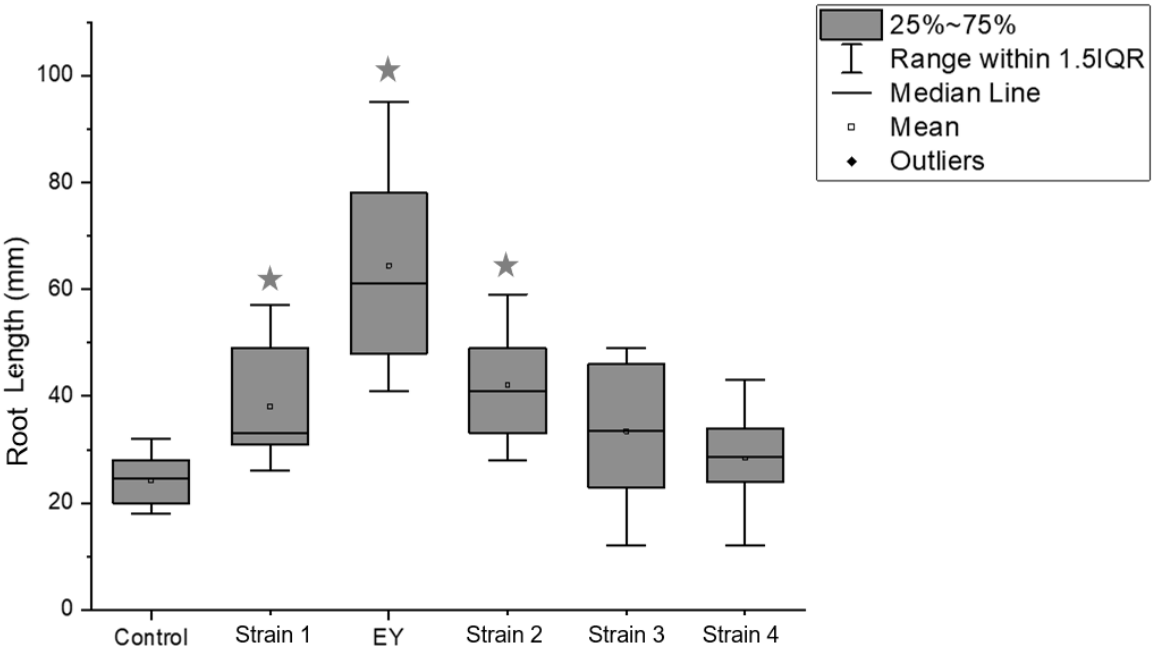

25%~75%
Range within 1.5IQR
Median Line
Mean
Outliers
Root Length (mm)
0
20
40
60
80
100
Control
Strain 1
EY
Strain 2
Strain 3
Strain 4

Figure 13

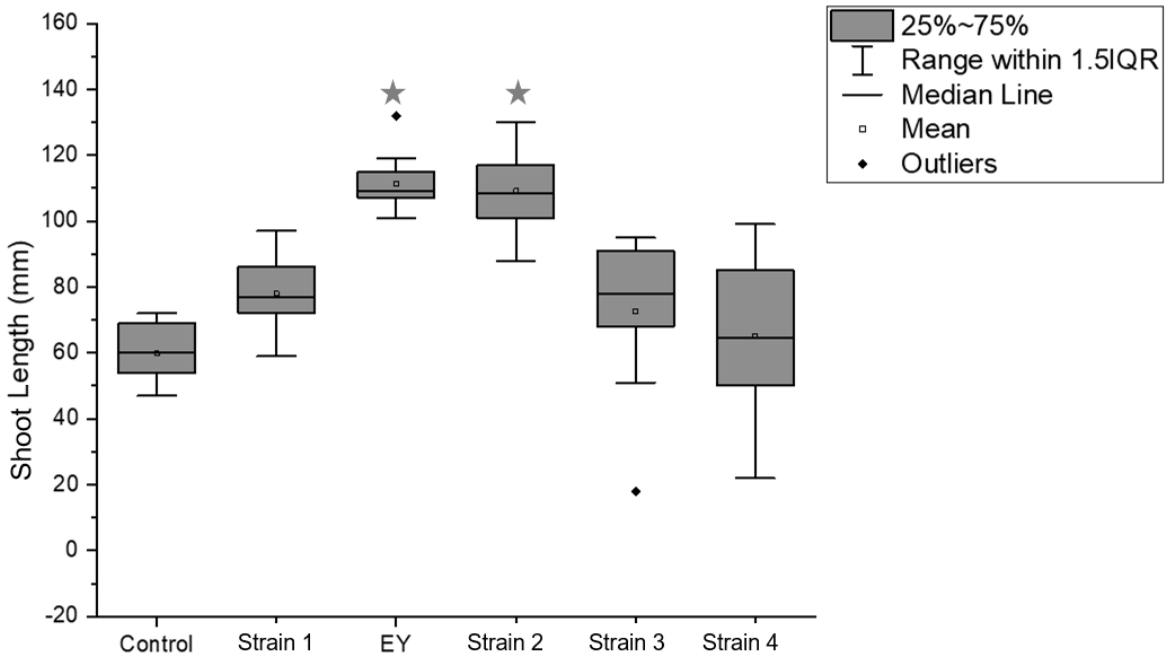

25%~75%
Range within 1.5IQR
Median Line
Mean
Outliers
Shoot Length (mm)
160
140
120
100
80
60
40
20
0
-20
Control
Strain 1
EY
Strain 2
Strain 3
Strain 4

Figure 14

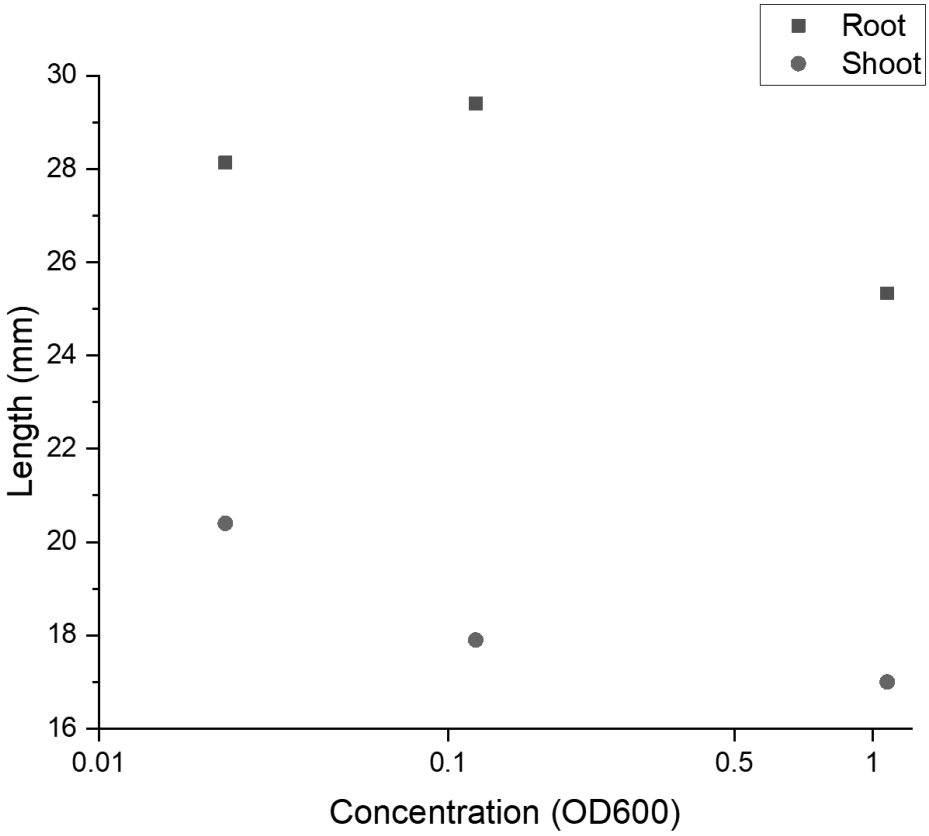

Root
Shoot
Length (mm)
30
28
26
24
22
20
18
16
0.01
0.1
0.5
1
Concentration (OD600)

Figure 15

1.5 Patent 4 – *Stenotrophomonas rhizophila*

1.5.1 Publication details

Title: Novel bacterial strain (3)

Details: https://patentscope.wipo.int/search/en/detail.jsf?docId=WO2021012000

Stage of publication: Full patent (publicly available)

Authors: Li, Tongda; Tannenbaum, Ian Ross; Kaur, Jatinder; Krill, Christian; Sawbridge, Timothy Ivor; Mann, Ross C.; Spangenberg, German Carlos

1.5.2 Statement of contribution of joint authorship

TL performed all work relating to genomics of the strains, *in vitro* bioprotectant assays, *in vitro* biofertiliser activity assays and *in planta* inoculation assays (Example 7). TL generated all figures associated with the above works (1–9) and drafted the majority of the experimental section of the patent associated with these works. TL conducted all statistical and data analysis of these works. RM, GS, TS, JK and TL all conceptualised the patent and assisted in editing the patent. Experimental and analysis work conducted by TL is highlighted in yellow.

Appendix 1

AUSTRALIA

Patents Act 1990

PROVISIONAL SPECIFICATION

Applicants: Agriculture Victoria Services Pty Ltd
Dairy Australia Limited
Geoffrey Gardiner Dairy Foundation Limited

Title: NOVEL BACTERIAL STRAIN (3)

The invention is described in the following statement:

NOVEL BACTERIAL STRAIN (3)

Field of the Invention

The present invention relates to novel plant microbiome strains, plants infected with such strains and related methods.

Background of the Invention

Microbes represent an invaluable source of novel genes and compounds that have the potential to be utilised in a range of industrial sectors. Scientific literature gives numerous accounts of microbes being the primary source of antibiotics, immune-suppressants, anticancer agents and cholesterol-lowering drugs, in addition to their use in environmental decontamination and in the production of food and cosmetics.

A relatively unexplored group of microbes known as endophytes, which reside e.g. in the tissues of living plants, offer a particularly diverse source of novel compounds and genes that may provide important benefits to society, and in particular, agriculture.

Endophytes may be fungal or bacterial. Endophytes often form mutualistic relationships with their hosts, with the endophyte conferring increased fitness to the host, often through the production of defence compounds. At the same time, the host plant offers the benefits of a protected environment and nutriment to the endophyte.

Important forage grasses perennial ryegrass (*Lolium perenne*) are commonly found in association with fungal and bacterial endophytes. However, there remains a general lack of information and knowledge of the endophytes of these grasses as well as of methods for the identification and characterisation of novel endophytes and their deployment in plant improvement programs.

Knowledge of the endophytes of perennial ryegrass may allow certain beneficial traits to be exploited in enhanced pastures, or lead to other agricultural advances, e.g. to the benefit of sustainable agriculture and the environment.

There exists a need to overcome, or at least alleviate, one or more of the difficulties or deficiencies associated with the prior art.

Summary of the Invention

In one aspect, the present invention provides a substantially purified or isolated endophyte strain isolated from a plant of the Poaceae family, wherein said endophyte is a strain of *Stenotrophomonas rhizophila* which provides bioprotection and/or biofertilizer phenotypes to plants into which it is inoculated. In a preferred embodiment, the *Stenotrophomonas rhizophila* strain may be strain JB as described herein and as deposited with The National Measurement Institute on 17th May 2019 with accession number V19/009906.

As used herein the term “endophyte” is meant a bacterial or fungal strain that is closely associated with a plant. By “associated with” in this context is meant that the bacteria or fungus lives on, in or in close proximity to a plant. For example, it may be endophytic, for example living within the internal tissues of a plant, or epiphytic, for example growing externally on a plant.

As used herein the term “substantially purified” is meant that an endophyte is free of other organisms. The term includes, for example, an endophyte in axenic culture. Preferably, the endophyte is at least approximately 90 % pure, more preferably at least approximately 95 % pure, even more preferably at least approximately 98 % pure, even more preferably at least approximately 99 % pure.

As used herein the term ‘isolated’ means that an endophyte is removed from its original environment (e.g. the natural environment if it is naturally occurring). For example, a naturally occurring endophyte present in a living plant is not isolated, but the same endophyte separated from some or all of the coexisting materials in the natural system, is isolated.

As used herein the term “bioprotection and/or biofertilizer” means that the endophyte possesses genetic and/or metabolic characteristics that result in a beneficial phenotype in a plant harbouring, or otherwise associated with, the endophyte. Such beneficial properties include improved resistance to pests and/or diseases, improved tolerance to water and/or nutrient stress, enhanced biotic stress tolerance, enhanced drought tolerance, enhanced water use efficiency, reduced toxicity and enhanced vigour in the plant with which the endophyte is associated, relative to an organism not harbouring the endophyte or harbouring a control endophyte such as standard toxic (ST) endophyte.

The pests and/or diseases may include, but not limited to, fungal and/or bacterial pathogens, preferably fungal. In a particularly preferred embodiment, the endophyte may result in the production of the bioprotectant compound in the plant with which it is associated.

As used herein, the term ‘bioprotectant compound’ is meant as a compound that provides or aids bioprotection to the plant with which it is associated against pests and/or diseases, such as bacterial and/or fungal pathogens. A bioprotectant compound may also be known as a ‘biocidal compound’.

In a particularly preferred embodiment, the endophyte produces a bioprotectant compound and provides bioprotection to the plant against bacterial and/or fungal pathogens. The terms bioprotectant, bioprotective and bioprotection (or any other variations) may be used interchangeably herein.

In a particularly preferred embodiment the bioprotectant compound is spermidine or derivative, isomer and/or salt thereof.

The endophyte may be suitable as a biofertilizer to improve the availability of nutrients to the plant with which the endophyte is associated, including but not limited to improved tolerance to nutrient stress.

The nutrient stress may be lack of or low amounts of a nutrient such as phosphate and/or nitrogen. The endophyte is capable of growing in conditions such as low nitrogen and/or low phosphate and enable these nutrients to be available to the plant with which the endophyte is associated.

The endophyte may result in the production of organic acids and/or the solubilisation of phosphate in the plant with which it is associated and/or provide a source of phosphate to the plant.

Alternatively, or in addition, the endophyte is capable of nitrogen fixation. Thus, if an endophyte is capable of nitrogen fixation, the organism in which the endophyte is associated is capable of growing in low nitrogen conditions and/or provide a source of Nitrogen to the plant.

In a particularly preferred embodiment, the endophyte provides the ability of the organism to grow in low nitrogen.

As used herein the term “plant of the Poaceae family” is a grass species, particularly a pasture grass such as ryegrass (*Lolium*) or fescue (*Festuca*), more particularly perennial ryegrass (*Lolium perenne* L.) or tall fescue (*Festuca arundinaceum*, otherwise known as *Lolium arundinaceum*).

In another aspect, the present invention provides a plant or part thereof infected with an endophyte as hereinbefore described. In preferred embodiments, the plant or part thereof infected with the endophyte may produce a bioprotectant compound, particularly spermidine or derivative, isomer and/or salt thereof. Also in preferred embodiments, the plant or part thereof includes an endophyte-free host plant or part thereof stably infected with said endophyte.

The plant inoculated with the endophyte may be a grass or non-grass plant suitable for agriculture, specifically a forage, turf, or bioenergy grass, or a grain crop or industrial crop.

The forage, turf or bioenergy grass may be those belonging to the *Brachiaria-Urochloa* species complex (panic grasses), including *Brachiaria brizantha, Brachiaria decumbens, Brachiaria humidicola, Brachiaria stolonifera, Brachiaria ruziziensis, B. dictyoneura, Urochloa brizantha, Urochloa decumbens, Urochloa humidicola*, *Urochloa mosambicensis* as well as interspecific and intraspecific hybrids of *Brachiaria-Urochloa* species complex such as interspecific hybrids between *Brachiaria ruziziensis* x *Brachiaria brizantha*, *Brachiaria ruziziensis* x *Brachiaria decumbens*, [*Brachiaria ruziziensis* x *Brachiaria decumbens*] x *Brachiaria brizantha*, [*Brachiaria ruziziensis* x *Brachiaria brizantha*] x *Brachiaria decumbens*.

The forage, turf or bioenergy grass may also be those belonging to the genera *Lolium* and *Festuca*, including *L. perenne* (perennial ryegrass) and *L. arundinaceum* (tall fescue) and *L. multiflorum* (Italian ryegrass).

The grain crop or industrial crop may be a non-grass species, for example, any of soybeans, cotton and grain legumes, such as lentils, field peas, fava beans, lupins and chickpeas, as well as oilseed crops, such as canola.

Thus, the grain crop or industrial crop species may be selected from the group consisting of wheat, barley, oats, chickpeas, triticale, fava beans, lupins, field peas, canola, cereal rye, vetch, lentils, millet/panicum, safflower, linseed, sorghum, sunflower, maize, canola, mungbeans, soybeans, and cotton.

The grain crop or industrial crop grass may be those belonging to the genus *Triticum*, including *T. aestivum* (wheat), those belonging to the genus *Hordeum*, including *H. vulgare* (barley), those belonging to the genus *Zea*, including *Z. mays* (maize or corn), those belonging to the genus *Oryza*, including *O. sativa* (rice), those belonging to the genus *Saccharum* including *S. officinarum* (sugarcane), those belonging to the genus *Sorghum* including *S. bicolor* (sorghum), those belonging to the genus *Panicum*, including *P. virgatum* (switchgrass), and those belonging to the genera *Miscanthus*, *Paspalum*, *Pennisetum*, *Poa*, *Eragrostis* and *Agrostis*.

A plant or part thereof may be infected by a method selected from the group consisting of inoculation, breeding, crossing, hybridisation, transduction, transfection, transformation and/or gene targeting and combinations thereof.

Without wishing to be bound by theory, it is believed that the endophyte of the present invention may be transferred through seed from one plant generation to the next. The endophyte may then spread

or locate to other tissues as the plant grows, i.e. to roots. Alternatively, or in addition, the endophyte may be recruited to the plant root, e.g. from soil, and spread or locate to other tissues.

Thus, in a further aspect, the present invention provides a plant, plant seed or other plant part derived from a plant or part thereof as hereinbefore described. In preferred embodiments, the plant, plant seed or other plant part may produce a bioprotectant compound, particularly spermidine or derivative, isomer and/or salt thereof.

In another aspect, the present invention provides the use of an endophyte as hereinbefore described to produce a plant or part thereof stably infected with said endophyte. The present invention also provides the use of an endophyte as hereinbefore described to produce a plant or part thereof as hereinbefore described.

In another aspect, the present invention provides a bioprotectant compound, produced by an endophyte as hereinbefore described, particularly spermidine or a derivative, isomer and/or a salt thereof.

The bioprotectant compound, may be produced by the endophyte when associated with a plant, e.g. a plant of the Poaceae family as described above. Thus, in another aspect, the present invention provides a method for producing a bioprotectant compound, particularly spermidine, or a derivative, isomer and/or a salt thereof, said method including infecting a plant with an endophyte as hereinbefore described and cultivating the plant under conditions suitable to produce a bioprotectant compound, particularly spermidine. The endophyte-infected plant or part thereof may be cultivated by known techniques. The person skilled in the art may readily determine appropriate conditions depending on the plant or part thereof to be cultivated.

The bioprotectant compound, may also be produced by the endophyte when it is not associated with a plant. Thus, in yet another aspect, the present invention provides a method for producing a bioprotectant compound, particularly spermidine, or a derivative, isomer and/or a salt thereof, said method including culturing an endophyte as hereinbefore described, under conditions suitable to produce the bioprotectant compound, particularly spermidine.

The conditions suitable to produce the bioprotectant compound may include a culture medium including a source of carbohydrates. The source of carbohydrates may be a starch/sugar-based agar or broth such as potato dextrose agar, potato dextrose broth or half potato dextrose agar or a cereal-based agar or broth such as oatmeal agar or oatmeal broth. Other sources of carbohydrates may include endophyte agar, Murashige and Skoog with 20 % sucrose, half V8 juice/half PDA, water agar and yeast malt extract agar. The endophyte may be cultured under aerobic or anaerobic conditions and may be cultured in a bioreactor.

In a preferred embodiment of this aspect of the invention, the method may include the further step of isolating a bioprotectant compound from the plant or culture medium.

The endophyte of the present invention is capable of nitrogen fixation. Thus, in yet another aspect, the present invention provides a method of growing the plant in low nitrogen containing medium, said method including infecting a plant with an endophyte as hereinbefore described, and cultivating the plant. Preferably, the low nitrogen medium is low nitrogen containing soil.

In yet a further aspect, the present invention provides a method of increasing nitrogen use efficiency or increasing nitrogen availability to a plant, said method including infecting a plant with an endophyte as hereinbefore described, and cultivating the plant.

In yet another aspect, the present invention provides a method of reducing nitrogen levels in soil, said method including infecting a plant with an endophyte as hereinbefore described, and cultivating the plant in the soil.

in yet another aspect, the present invention provides a method of increasing phosphate use efficiency or increasing phosphate solubilisation by a plant, said method including infecting a plant with an endophyte as hereinbefore described, and cultivating the plant.

In yet another aspect, the present invention provides a method of reducing phosphate levels in soil, said method including infecting a plant with an endophyte as hereinbefore described, and cultivating the plant in the soil.

The endophyte-infected plant or part thereof may be cultivated by known techniques. The person skilled in the art may readily determine appropriate conditions depending on the plant or part thereof to be cultivated.

The production of a bioprotectant compound has particular utility in agricultural plant species, in particular, forage, turf, or bioenergy grass species, or grain crop species or industrial crop species. These plants may be cultivated across large areas of e.g. soil where the properties and biological processes of the endophyte as hereinbefore described and/or bioprotectant compound produced by the endophyte may be exploited at scale.

The part thereof of the plant may be, for example, a seed.

In preferred embodiments, the plant is cultivated in the presence of soil phosphate and/or nitrogen, alternatively or in addition to applied phosphate and/or nitrogen. The applied phosphate and/or

applied nitrogen may be by way of, for example, fertiliser. Thus, preferably, the plant is cultivated in soil.

In preferred embodiments, the endophyte may be a *Stenotrophomonas rhizophila* strain JB as described herein and as deposited with The National Measurement Institute on 17th May 2019 with accession number V19/009906.

Preferably, the plant is a forage, turf, bioenergy grass species or, grain crop or industrial crop species, as hereinbefore described.

The part thereof of the plant may be, for example, a seed.

In preferred embodiments, the plant is cultivated in the presence of soil phosphate and/or applied phosphate. The applied phosphate may be by way of, for example, fertiliser. Thus, preferably, the plant is cultivated in soil.

Alternatively, or in addition, the plant is cultivated in the presence of soil nitrogen and/or applied nitrogen. The applied nitrogen may be by way of, for example, fertiliser. Thus, preferably, the plant is cultivated in soil.

The present invention will now be more fully described with reference to the accompanying Examples and drawings. It should be understood, however, that the description following is illustrative only and should not be taken in any way as a restriction on the generality of the invention described above.

Brief Description of the Drawings/Figures

Figure 1 - 16S Amplicon sequence of novel bacterial strain JB.

Figure 2 - Phylogeny of *Stenotrophomonas* spp. and the novel bacterial strain JB. This maximum-likelihood tree was inferred based on 196 genes conserved among 10 genomes. Values shown next to branches were the local support values calculated using 1000 resamples with the Shimodaira-Hasegawa test.

Figure 3 - Whole genome sequence comparison of the *Stenotrophomonas rhizophila* novel bacterial strain JB (bottom) and the type *Stenotrophomonas rhizophila* strain DSM14405 (top). The links between genome sequences indicated percentage similarity (from 70 % to 100 %). Genetic variations, including non-identical regions, insertions / deletions / inversions and rearrangements, suggest that the novel bacterial strain JB and the bacterial strain DSM14405 are genetically different.

The stars represent genomic regions unique to the novel bacterial strain JB or the bacterial strain DSM14405. The triangle represents genomic regions with 70 % sequence homology between the novel bacterial strain JB or the bacterial strain DSM14405. The square represents genomic regions that have undergone rearrangement.

Figure 4 - Bioprotection bioassay indicating the growth of 11 strains (including the *S. rhizophila* novel bacterial strain JB, star) against 6 plant pathogenic fungi, *Fusarium verticillioides* (10 days post inoculation, dpi), *Bipolaris gossypina* (7 dpi), *Sclerotinia rolfsii* (5 dpi), *Drechslera brizae* (8 dpi), *Phoma sorghina* (9 dpi) and *Microdochium nivale* (6 dpi). Bars represent the mean diameter of fungal colonies from three replicate plates of each treatment. Different superscript letters indicate significant differences ($P < 0.05$) between treatments.

Figure 5 - Secondary metabolite biosynthesis gene clusters in the *Stenotrophomonas rhizophila* novel bacterial strain JB identified using antiSMASH (Weber et al. 2015). The gene clusters have sequence homology and structure to (A) a bacteriocin-like gene cluster; (B) a lantipepetide-like gene cluster; (C) an unknown NRPS gene cluster; (D) an arylpolyene-like gene cluster. The core biosynthetic genes of each cluster are designated by a black line.

Figure 6 - Biofertiliser activity (*in vitro*) of the *Stenotrophomonas rhizophila* novel bacterial strain JB and other bacterial strains on semi-solid NFb medium, which determines the ability of bacteria to grow under low N. A) Absorbance readings across a wavelength range of 300 – 800 nm (615 nm – optimal wavelength for bioassay) for 8 bacterial strains and a no growth control (NGC – NFb media only). B) Growth of 8 bacterial strains and a NGC in semi-solid NFb media in a 96 well plate, indicating strains capable of growing under low N (dark – strains 2, 3, 4, 5, 6, JB) and those strains that cannot (light – 1, 7, -ve control, NGC).

Figure 7 - Gene clusters of *Stenotrophomonas rhizophila* (strains JB and DSM14405) responsible for the regulation of the important plant polyamine spermidine. The spermidine synthase is designated by a start, while the triangle designates regions that differ between the two strains.

Figure 8 - Image of 5 day old seedlings inoculated with the *Stenotrophomonas rhizophila* novel bacterial strain JB and an untreated control (blank).

Figure 9 - Average shoot and root length of barley seedlings inoculated with the *Stenotrophomonas rhizophila* novel bacterial strain JB and an untreated control (blank), and grown for 5 days. There was no significant difference ($P < 0.05$) between the two treatments.

Figure 10 - Average root length of barley seedlings inoculated with bacterial strains of *Stenotrophomonas rhizophila* (strain JB) and non-*Stenotrophomonas* strains (Strain 1, 2, 3, 4) and

grown for 4 days on media containing insoluble phosphate. The star indicates significant difference in the mean at $P < 0.05$ between the control and the bacterial strains.

Figure 11 - Average shoot length of barley seedlings inoculated with bacterial strains of *Stenotrophomonas rhizophila* (strain JB) and non-*Stenotrophomonas* strains (Strain 1, 2, 3, 4) and grown for 4 days on media containing insoluble phosphate. The star indicates significant difference in the mean at $P < 0.05$ between the control and the bacterial strains.

Detailed Description of the Embodiments

Discovery and characterisation of plant associated *Stenotrophomonas rhizophila* novel bacterial strains providing bioprotection and biofertilizer phenotypes to plants.

The novel plant associated *Stenotrophomonas rhizophila* bacterial strain JB has been isolated from perennial ryegrass (*Lolium perenne*) plants. It displays the ability to inhibit the growth of plant fungal pathogens and an ability to grow in low nitrogen in plate assays. The genome of the *Stenotrophomonas rhizophila* bacterial strain JB has been sequenced and is shown to be novel, related to bioprotectant *Stenotrophomonas rhizophila* strains and not pathogenic *Stenotrophomonas maltophilia* strains. Analysis of the genome sequence has shown that the *Stenotrophomonas rhizophila* novel bacterial strain JB has gene clusters for the biosynthesis of the antibacterial and antifungal bioprotectant compounds and genes involved in plant growth/endophytic niche via the production of spermidine. This novel bacterial strain has been used to inoculate barley (*Hordeum vulgare*) seeds under glasshouse conditions and has been demonstrated not to cause disease in these barley plants. These barley plants are also able to produce seed. Novel bacterial strain JB also enhances root and shoot growth in insoluble phosphate. Overall, novel plant associated *Stenotrophomonas rhizophila* bacterial strain JB offer both bioprotectant and biofertilizer activity.

Example 1 – Isolation of Bacterial Strains

Seed associated bacterial strains

Seeds from perennial ryegrass (*Lolium perenne*) were surface-sterilised by soaking in 80 % ethanol for 3 mins, then washing 5 times in sterile distilled water. The seeds were then plated onto sterile filter paper soaked in sterile water in sterile petri dishes. These plates were stored at room temperature in the dark to allow seedlings to germinate for 1 – 2 weeks. Once the seedlings were of sufficient size, the plants were harvested. In harvesting, the remaining seed coat was discarded, and the aerial tissue and root tissue were harvested. The plant tissues were submerged in sufficient Phosphate Buffered Saline (PBS) to completely cover the tissue, and ground using a Qiagen TissueLyser II, for 1 minute at 30 Hertz. A 10 µL aliquot of the macerate was added to 90 µL of PBS.

Subsequent 1 in 10 dilutions of the 10^{-1} suspension were used to create additional 10^{-2} to 10^{-4} suspensions. Once the suspensions were well mixed 50 µL aliquots of each suspension were plated onto Reasoners 2 Agar (R2A) for growth of bacteria. Dilutions that provided a good separation of bacterial colonies were subsequently used for isolation of individual bacterial colonies through re-streaking of single bacterial colonies from the dilution plates onto single R2A plates to establish a pure bacterial colony.

Mature plant associated bacterial strains

Leaf and root tissue were harvested from mature plants grown in the field or grown in pots in a greenhouse. Root tissue was washed in PBS buffer to remove soil particles and sonicated (10 mins) to remove the rhizosphere. The harvested tissues were placed into sufficient PBS to completely cover the tissue and processed as per the previous section to isolate pure bacterial cultures.

Around 300 bacterial strains were obtained from seeds of perennial ryegrass, and 300 strains from mature perennial ryegrass plants. The novel bacterial strain JB was collected from seed of perennial ryegrass.

Example 2 – Identification of *Stenotrophomonas rhizophila* novel bacterial strain

Amplicon (16S rRNA gene) Sequencing

A phylogenetic analysis of the novel bacterial strain JB was undertaken by sequence homology comparison of the 16S rRNA gene. The novel bacterial strain JB was grown overnight in Reasoners 2 Broth (R2B) media. DNA was extracted from pellets derived from the overnight culture using a DNeasy Blood and Tissue kit (Qiagen) according to manufacturer's instructions. The 16S rRNA gene amplification used the following PCR reagents: 14.8 µL H_2O, 2.5 µL 10× reaction buffer, 0.5 µL 10 mM dNTPs, 2.5 µL each of the 5 µM 27F primer (5'- AGAGTTTGATCMTGGCTCAG -3') and 5 µM reverse primers 1492R (5'- GGTTACCTTGTTACGACTT -3'), 0.2 µL of Immolase enzyme, and template to a final volume of 25 µL. The PCR reaction was then run in an Agilent Surecycler 8800 (Applied Biosystems) with the following program; a denaturation step at 94 °C for 15 min; 35 cycles of 94 °C for 30 sec, 55 °C for 10 sec, 72 °C 1 min; and a final extension step at 72 °C for 10 min.

Shrimp alkaline phosphatase (SAP) exonuclease was used to purify the 16S rRNA gene PCR amplicon. The SAP amplicon purification used the following reagents: 7.375 µL H_2O, 2.5 µL 10× SAP, and 0.125 µL Exonuclease I. The purification reaction was incubated at 37 °C for 1 hr, followed by 15 min at 80 °C to deactivate the exonuclease.

The purified 16S rRNA gene amplicon was sequenced using the BigDye® Terminator v3.1 Cycle Sequencing Kit (Thermofisher) with the following reagents; 10.5 µL H_2O, 3.5 µL 5× Seq buffer, 0.5 µL BigDye®, 2.5 µL of either the 3.2 µM Forward (27F) and 3.2 µM Reverse primers (1492R), and 4.5 µL of PCR amplicon as template, to a final reaction volume of 20 µL. The sequencing PCR reaction was then run in an Agilent Surecycler 8800 (Applied Biosystems) with the following program; denaturation step at 94 °C for 15 min; followed by 35 cycles of 94 °C for 30 sec, 55 °C for 10 sec, 72 °C 1 min; and one final extension step at 72 °C for 10 min. The 16S rRNA gene amplicon from novel bacterial strain JB was sequenced on an ABI3730XL (Applied Biosystems). A 1546 bp 16S rRNA gene sequence was generated (Figure 1). The sequence was aligned by BLASTn on NCBI against the non-redundant nucleotide database and the 16S ribosomal RNA database.

BLASTn hit against database nr; *Stenotrophomonas rhizophila* strain e-p10 16S ribosomal RNA gene, partial sequence

Max Score	Total Score	Query Coverage	E-Value	% Identity	Accession
2850	2850	100 %	0	99.94 %	NR_121739.1

BLASTn hit against database 16S ribosomal RNA; *Stenotrophomonas rhizophila* strain e-p10 16S ribosomal RNA gene, partial sequence

Max Score	Total Score	Query Coverage	E-Value	% Identity	Accession
2850	2850	100 %	0	99.94 %	NR_121739.1

The preliminary taxonomic identification of the novel bacterial strain JB was *Stenotrophomonas rhizophila.*

Genomics

The genome of the *Strenotrophomonas rhizophila* novel bacterial strain JB was sequenced. This novel bacterial strain was retrieved from the glycerol collection stored at -80 °C by streaking on R2A plates. Single colonies from these plates were grown overnight in Nutrient Broth and pelleted. These pellets were used for genomic DNA extraction using the bacteria protocol of Wizard® Genomic DNA Purification Kit (A1120, Promega). A DNA sequencing library was generated for Illumina sequencing using the Illumina Nextera XT DNA library prep protocol. The library was sequenced using an Illumina MiSeq platform. Raw reads from the sequencer were filtered to remove any adapter and index sequences as well as low quality bases using Trimmomatic (Bolger, Lohse & Usadel 2014) with the following options: ILLUMINACLIP: NexteraPE-PE.fa:2:30:10 LEADING:3 TRAILING:3

SLIDINGWINDOW:4:15 MINLEN:36. To enable full genome assembly, long reads were generated for novel bacterial strain JB by sequencing DNA using Oxford Nanopore Technologies (ONT) MinION platform. The DNA from the Wizard® Genomic DNA Purification Kit was first assessed with the genomic assay on Agilent 2200 TapeStation system (Agilent Technologies, Santa Clara, CA, USA) for integrity (average molecular weight ≥30 Kb). The sequencing library was prepared using an in-house protocol modified from the official protocols for transposases-based library preparation kits (SQK-RAD004/SQK-RBK004, ONT, Oxford, UK). The library was sequenced on a MinION Mk1B platform (MIN-101B) with R9.4 flow cells (FLO-MIN106) and under the control of MinKNOW software. After the sequencing run finished, the fast5 files that contain raw read signals were transferred to a separate, high performance computing Linux server for local basecalling using ONT's Albacore software (Version 2.3.1) with default parameters. The sequencing summary file produced by Albacore was processed by the R script minion qc (https://github.com/roblanf/minion_qc) and NanoPlot (De Coster et al. 2018) to assess the quality of the sequencing run, while Porechop (Version 0.2.3, https://github.com/rrwick/Porechop) was used to remove adapter sequences from the reads. Reads which were shorter than 300 bp were removed and the worst 5 % of reads (based on quality) were discarded by using Filtlong (Version 0.2.0, https://github.com/rrwick/Filtlong).

The whole genome sequence of novel bacterial strain JB was assembled using Unicycler (Wick et al. 2017). Unicycler performed hybrid assembly when both Illumina reads and MinION reads were available. MinION reads were mainly used to resolve repeat regions in the genome sequence, whereas Illumina reads were used by Pilon (Walker et al. 2014) to correct small base-level errors. Multiple rounds of Racon (Vaser et al. 2017) polishing were then carried out to generate consensus sequences. Assembly graphs were visualised by using Bandage (Wick et al. 2015).

A complete circular chromosome sequence was produced for the novel bacterial strain JB. The genome size for the novel bacterial strain JB was 4,667,358 bp (Table 1). The percent GC content was 67.27 %. The novel bacterial strain JB was annotated by Prokka (Seemann 2014) with a custom, genus-specific protein database to predict genes and corresponding functions, which were then screened manually to identify specific traits. The number of genes for the novel bacterial strain JB was 4,141 (Table 2).

Table 1 – Summary of properties of the final genome sequence assembly

Strain ID	**Genome size (bp)**	**GC content (%)**	**Coverage Illumina reads**	**Coverage ONT MinION**
JB	4,667,358	67.27	698×	72×

Table 2 – Summary of genome coding regions

Strain ID	Genome size (bp)	No. of tRNA	No. of tmRNA	No. of rRNA	No. of CDS	No. of gene
JB	4,667,358	74	1	10	4,056	4,141

Nine *Stenotrophomonas* spp. (*S. rhizophila, S. maltophilia, S. pavanii*) genome sequences that are publicly available on NCBI were acquired and used for pan-genome/comparative genome sequence analysis alongside the novel bacterial strain JB. A total of 196 genes that are shared by all 10 strains were identified by running Roary (Page et al. 2015). PRANK (Löytynoja 2014) was then used to perform a codon aware alignment. A maximum-likelihood phylogenetic tree (Figure 2) was inferred using FastTree (Price, Dehal & Arkin 2010) with Jukes-Cantor Joins distances and Generalized Time-Reversible and CAT approximation model. Local support values for branches were calculated using 1000 resamples with the Shimodaira-Hasegawa test. The novel bacterial strain JB clustered tightly with the bioprotectant *S. rhizophila* strain DSM14405 (type strain of this species), suggesting a close phylogenetic relationship between these two bacterial strains. Moreover, this cluster was separated from other *Stenotrophomonas* spp. with strong local support value (100 %), including the human pathogen *S. maltophilia*. This separation supports that bacterial strain JB is novel and from the species *S. rhizophila*.

The average nucleotide identity (ANI) was calculated for novel bacterial strain JB against the other nine *Stenotrophomonas* spp. strains (Table 3). The genome sequences of the ten strains were aligned and compared using minimap2 (Li 2018). Based on a species boundary of 95 – 96 % (Chun et al. 2018; Richter & Rosselló-Móra 2009) the bacterial strain JB is from *S. rhizophila*, but is novel and a different strain to the type strain of this species (DSM14405) (Wolf et al. 2002).

Table 3 – Average nucleotide identity (ANI) of ten strains of *Stenotrophomonas* spp. including novel bacterial strain JB and the type *S. rhizophila* strain DSM14405

		S. rhizophila JB	*S. rhizophila* DSM14405	*S. rhizophila* USBA_GBX_843	*S. rhizophila* BIGb0145	*S. rhizophila* Sp952	*S. rhizophila* OG2	*S. rhizophila* QL_P4	*S. pavanii* LMG25348	*S. maltophilia* JV3	*S. maltophilia* R551-3
S. rhizophila	JB		96.44%	86.19%	86.33%	86.12%	86.13%	86.12%	82.73%	82.76%	82.57%
S. rhizophila	DSM14405			86.41%	86.33%	86.25%	86.21%	86.19%	82.77%	82.83%	82.63%
S. rhizophila	USBA_GBX_843				93.41%	85.21%	85.23%	85.21%	82.47%	82.51%	82.34%
S. rhizophila	BIGb0145					85.08%	85.13%	85.07%	82.30%	82.46%	82.18%
S. rhizophila	Sp952						97.78%	97.43%	82.86%	82.90%	82.62%
S. rhizophila	OG2							97.74%	82.83%	82.88%	82.60%
S. rhizophila	QL_P4								82.78%	82.92%	82.61%
S. pavanii	LMG25348									92.35%	90.28%
S. maltophilia	JV3										91.27%
S. maltophilia	R551-3										

Genome sequence alignment

The genome sequences of *Stenotrophomonas rhizophila* strain JB and the type strain DSM14405 were aligned using LASTZ (Version 1.04.00, http://www.bx.psu.edu/~rsharris/lastz/) and visualised using AliTV (Ankenbrand et al. 2017) to determine the genomic similarity between the two strains. The genomes of the two strains were similar, but there were large genomic regions unique to the novel bacterial strain JB or the bacterial strain DSM14405 (Figure 3 - stars). Similarly, there are a large number of genomic regions that have undergone rearrangements (Figure 3 – square) or have low sequence homology (e.g. 70 % homology, Figure 3 – triangle).

Example 3 – Bioprotection activity (*in vitro*) of the *Stenotrophomonas rhizophila* novel bacterial strain JB

In vitro bioassays were established to test the bioactivity of 11 plant associated bacterial strains including *Stenotrophomonas rhizophila* novel bacterial strain JB, against six plant pathogenic fungi (Table 4). A plate with only the pathogen was used as a negative control (blank). The fungal pathogens were all isolated from monocot species, and were obtained from the National Collection of Fungi (Herbarium VPRI) and the AVR collection. Each bacterial strain was cultured in Nutrient Broth (BD Biosciences) overnight at 28 °C in a shaking incubator (200 rpm). Each bacterial strain was drop-inoculated (20 µL) onto four equidistant points on a Nutrient Agar (BD Biosciences) plate, which was then incubated overnight at 28 °C. A 6 mm × 6 mm agar plug of actively growing mycelia from the pathogen was placed at the centre of the plate. The bioassay was incubated for at least 5 days at 28 °C in the dark, and then the diameter of the fungal colony on the plate was recorded. For each treatment three plates were prepared as biological triplicates. OriginPro 2018 (Version b9.5.1.195) was used to carry out One-way ANOVA and Tukey Test to detect the presence of any significant difference ($P < 0.05$) between treatments.

Table 4 – Pathogens used in the bioprotection bioassay

VPRI Accession No.	Taxonomic Details	Host Taxonomic Details	State	Collection Date
12962	*Drechslera brizae* (Y.Nisik.) Subram. & B.L.Jain	*Briza maxima* L.	Vic.	24-Oct-85
32148	*Sclerotium rolfsii* Sacc.	*Poa annua* L.	Vic.	1-Jan-05
10694	*Phoma sorghina* (Sacc.) Boerema, Dorenbosch, van Kesteren	*Cynodon dactylon* Pers.	Vic.	19-Apr-79
42586a	*Fusarium verticillioides* (Sacc.) Nirenberg	*Zea mays* L.	Vic.	27-Feb-15
42563	*Bipolaris gossypina*	*Brachiaria*	Qld	
N/A	*Microdochium nivale*	*Lolium perenne* L.	Vic	

The *Stenotrophomonas rhizophila* novel bacterial strain JB inhibited the growth of all six fungal pathogens compared to the control and many of the other test bacterial strains, indicating it had broad spectrum biocidal activity (Figure 4). The *S. rhizophila* novel bacterial strain JB was the most active bacterial strain against *Drechslera brizae*, while it was the second most active strain against *Fusarium verticillioides, Bipolaris gossypina*, *Sclerotium rolfsii, Phoma sorghina* and *Microdochium nivale*.

Example 4 – Genome sequence features supporting the bioprotection niche of the *Stenotrophomonas rhizophila* novel bacterial strain JB

Secondary metabolite biosynthesis gene clusters

The genome sequence of the *Stenotrophomonas rhizophila* novel bacterial strain JB was assessed for the presence of features associated with bioprotection. The annotated genome was analysed by antiSMASH (Weber et al. 2015) to identify secondary metabolite biosynthesis gene clusters that are commonly associated with the production of biocidal compounds that aid in their defence. An annotated genome was passed through antiSMASH with the following options: *--clusterblast --asf --knownclusterblast --subclusterblast --smcogs --full-hmmer*. A total of four secondary metabolite gene clusters were identified in the genome sequence of the *Stenotrophomonas rhizophila* novel bacterial strain JB. (Figure 5A-D). These included a bacteriocin-like gene cluster (cluster 1), a lantipeptide-like (bacteriocin) gene cluster (cluster 2), an unknown non-ribosomal peptide synthase (NRPS) gene cluster (cluster 3), and an arylpolyene-like gene cluster (cluster 4). Cluster 1 had one core biosynthetic gene and showed 34 % similarity to a cluster in the genome sequence of the type strain of *Stenotrophomonas rhizophila* (DSM14405) (Figure 5A). Cluster 2 had two core biosynthetic genes

and showed 48 % similarity to a cluster in the genome sequence of the type strain of *Stenotrophomonas rhizophila* (DSM14405) (Figure 5B). Cluster 3 had six core biosynthetic genes and showed 100 % similarity to a cluster in the genome sequence of the type strain of *Stenotrophomonas rhizophila* (DSM14405) (Figure 5C). Cluster 4 had eight core biosynthetic genes and showed 61 % similarity to a cluster in the genome sequence of *Stenotrophomonas maltophilia* (EPM1 G2RA73Z01B2RDT) (Figure 5D). The proposed function of clusters 1 and 2 is thought to involve the biosynthesis of bacteriocins, which have antimicrobial activity against similar or closely-related bacterial strains. The proposed function of cluster 3 is unclear. The proposed function of cluster 4 is thought to involve the biosynthesis of an arylpolyene, some of which have antimicrobial activity against fungi.

Example 5 – Biofertiliser activity (*in vitro*) of the *Stenotrophomonas rhizophila* novel bacterial strain JB

Nitrogen (N) is an important nutrient for plant growth and a key component of fertilisers. Plant associated bacteria able to grow under low nitrogen conditions may be useful in plant growth as the bacteria can pass this N onto the plant. This was assessed by using the nitrogen-free NFb medium (Dobereiner 1980). One litre of NFb medium contains 5 g DL-malic acid, 0.5 g dipotassium hydrogen orthophosphate, 0.2 g magnesium sulfate heptahydrate, 0.1 g sodium chloride, 0.02 g calcium chloride dehydrate, 2 mL micronutrients solution [0.4 g/L copper sulfate pentahydrate, 0.12 g/L zinc sulfate heptahydrate, 1.4 g/L boric acid, 1 g/L sodium molybdate dehydrate, 1.5 g/L manganese(II) sulfate monohydrate], 1 mL vitamin solution (0.1 g/L biotin, 0.2 g/L pyridoxol hydrochloride), 4 mL iron(III) EDTA and 2 mL bromothymol blue (0.5 %, dissolved in 0.2 N potassium hydroxide). For solid NFb medium, 15 g/L bacteriological agar was added, otherwise 0.5 g/L was added for semi-solid medium. The pH of medium was adjusted to 6.8. To detect the nitrogen fixation ability, bacterial strains were inoculated onto solid medium plates. For each inoculation, triplicates were prepared. All NFb medium plates were incubated at 30 °C. After 96 hours, the colour change of NFb medium plates was recorded, with development of blue colour an indication of growth under limiting N.

In the high throughput automated method to detect nitrogen fixation ability semi-solid media NFb was used. Bacterial strains were inoculated into 20 mL R2B medium (0.5 g/L yeast extract, 0.5 g/L proteose peptone, 0.5 g/L casein hydrolysate, 0.5 g/L glucose, 0.5 g/L starch, 0.3 g/L dipotassium hydrogen orthophosphate, 0.024 g/L magnesium sulphate and 0.3 g/L sodium pyruvate) and incubated at 28 °C and 200 rpm overnight. The cell pellet was collected by centrifuging at 4000× g for 3 minutes, and then was twice with 1× PBS to remove the nitrogen residue from R2B. Then cell pellet was resuspended in 10 mL semi-solid NFb medium. 1 µL of cell suspension was added to a well containing 199 µL semi-solid NFb medium on a 96-well cell culture plate. For each strain, cell suspension was added to six consecutive wells of the same column, representing six biological replicates. Wells that are located in row A and H, and column 1 and 12 were excluded during the

examination due to the edge effect which may lead to unreliable reading. The plate was incubated at room temperature for 27 hours, after which the plate was examined by the plate reader by conducting a spectrum scan (300 nm – 750 nm wavelength, 10 nm increment). An increase in absorbance represented an increase in growth under low N conditions.

The *Stenotrophomonas rhizophila* novel bacterial strain JB was able to grow under low N, as evident from the colour change in the NFb media and elevated absorbance levels at a wavelength of 615 nm (Figure 6A and 6B).

Example 6 – Genome sequence features supporting the endophytic niche of the *Stenotrophomonas rhizophila* novel bacterial strain JB

Spermidine is an important polyamine involved in seed and embryo development, regulation of plant growth (particularly roots), and tolerance against drought and salinity (Gill & Tuteja 2010; Hummel et al. 2002; Imai et al. 2004). The biosynthesis of spermidine is regulated by spermidine synthases that catalyse the production of spermidine from putrescine and decarboxylated S-adenosylmethionine (dcSAM). Spermidine synthases have been identified in plant associated bacteria including *Stenotrophomonas rhizophila* and have been shown to be critical for the plant growth promotion activity of the bacterium (Alavi et al. 2014; Xie et al. 2014). The genome sequence of the *Stenotrophomonas rhizophila* novel bacterial strain JB was analysed and a spermidine synthase gene was identified (Figure 7). The gene showed 100 % sequence homology to a complementary gene in the genome of the type *Stenotrophomonas rhizophila* strain DSM14405 (Figure 7 – stars), however the surrounding genes showed significant variability including the addition of a hypothetical gene (Figure 7 – triangle).

Example 7 – *In planta* inoculations supporting endophytic niche of the *Stenotrophomonas rhizophila* novel bacterial strain JB

To assess direct interactions between the *Stenotrophomonas rhizophila* novel bacterial strain JB and plants, an early seedling growth assay was established in barley (*Hordeum vulgare)*. The *Stenotrophomonas rhizophila* novel bacterial strain JB was cultured in Lysogeny Broth (LB) overnight at 26 °C. The following day seeds of barley (cultivar Hindmarsh) were surface-sterilised by soaking in 80 % ethanol for 3 mins, then washing 5 times in sterile distilled water. The seeds were then soaked in the overnight cultures for 4 hours at 26 °C in a shaking incubator. For control seedlings, seeds were soaked in LB without bacteria for 4 hours at 26 °C in a shaking incubator. The seeds were planted into a pot trial, with three replicates (pots) per strain/control, with a randomised design. A total of 20 seeds were planted per pot, to a depth of 1 cm. The potting medium contained a mixture of 25 % potting mix, 37.5 % vermiculite and 37.5 % perlite. The plants were grown for 5 days and then removed from the pots, washed, assessed for health (i.e. no disease symptoms) and

photographed. The lengths of the longest root and the longest shoot were measured. Data was statistically analysed using a t test to detect the presence of any significant difference ($P < 0.05$) between treatments using Excel.

Seedlings inoculated with the *Stenotrophomonas rhizophila* novel bacterial strain JB were healthy with no disease symptoms recorded on leaves or roots (Figure 8). The length of the shoots inoculated with the *Stenotrophomonas rhizophila* novel bacterial strain JB were equivalent to the control (Figure 9).

Example 8 – *In planta* inoculations supporting the biofertilizer (phosphate solubilisation) niche of the *Stenotrophomonas rhizophila* novel bacterial strain JB

An *in planta* biofertilizer assay was established in barley to evaluate the ability of *Stenotrophomonas rhizophila* novel bacterial strain JB to aid growth under conditions with insoluble phosphate. Initially, bacterial strains (5, including JB) were cultured in 30 mL R2B overnight at 26 °C whilst rotating at 200 rpm. The following day the barley seeds were sterilized in 70 % ethanol for 5 minutes, followed by rinsing with SDW for five times. These sterile seeds were submerged in the overnight cultures for 4 hours in a dark incubator at room temperature whilst rotating at 200 rpm. The seeds were subsequently transferred to moistened sterile filter paper to be allowed to germinate for three days. These three-day-old seedlings were individually transferred to 60 mm plates with semi-solid Pikovskaya's media which contains yeast extract (0.5 g/L), D-glucose (5.0 g/L), calcium phosphate (5.0 g/L), ammonium sulphate (0.5 g/L), potassium chloride (0.2 g/L), magnesium sulphate (0.1 g/L), manganese sulphate (0.1 mg/L), ferrous sulphate (0.1 mg/L) and agar (5.0 g/L). These seedlings were allowed to grow for another 4 days, before the shoots and roots were measured for each seedling. There was a total of 6 treatments (5 bacterial strains including JB; 1 blank media control) containing 10 seedlings per treatment. Statistical analysis (One-way ANOVA and Tukey Test) was conducted using OriginPro 2018 (Version b9.5.1.195) to detect the presence of any significant difference ($P < 0.05$) between treatments.

The root growth of seedlings inoculated with novel bacterial strain JB and grown under conditions with insoluble phosphate was significantly greater than the control ($P < 0.05$), with an average increase of 42.6 % (Figure 10). The shoot growth of seedlings inoculated with novel bacterial strain JB was significantly greater than the control ($P < 0.05$), with an average increase of 45.2 % (Figure 11). Overall, results indicate that novel bacterial strain JB can aid in the growth of seedlings grown under conditions with insoluble phosphate.

It is to be understood that various alterations, modifications and/or additions may be made without departing from the spirit of the present invention as outlined herein.

As used herein, except where the context requires otherwise, the term "comprise" and variations of the term, such as "comprising", "comprises" and "comprised", are not intended to be in any way limiting or to exclude further additives, components, integers or steps.

Reference to any prior art in the specification is not, and should not be taken as, an acknowledgment or any form of suggestion that this prior art forms part of the common general knowledge in Australia or any other jurisdiction or that this prior art could reasonably be expected to be combined by a person skilled in the art.

References

1. Alavi, P, Starcher, MR, Thallinger, GG, Zachow, C, Müller, H & Berg, G 2014, 'Stenotrophomonas comparative genomics reveals genes and functions that differentiate beneficial and pathogenic bacteria', *BMC genomics*, vol. 15, no. 1, p. 482.

2. Ankenbrand, MJ, Hohlfeld, S, Hackl, T & Förster, F 2017, 'AliTV—interactive visualization of whole genome comparisons', *PeerJ Computer Science*, vol. 3.

3. Bolger, AM, Lohse, M & Usadel, B 2014, 'Trimmomatic: a flexible trimmer for Illumina sequence data', *Bioinformatics*, vol. 30, no. 15, pp. 2114-20.

4. Chun, J, Oren, A, Ventosa, A, Christensen, H, Arahal, DR, da Costa, MS, Rooney, AP, Yi, H, Xu, XW, De Meyer, S & Trujillo, ME 2018, 'Proposed minimal standards for the use of genome data for the taxonomy of prokaryotes', *Int J Syst Evol Microbiol*, vol. 68, no. 1, pp. 461-6.

5. De Coster, W, D'Hert, S, Schultz, DT, Cruts, M & Van Broeckhoven, C 2018, 'NanoPack: visualizing and processing long read sequencing data', *Bioinformatics*.

6. Dobereiner, J 1980, 'Forage grasses and grain crops', in *Methods for evaluating biological nitrogen fixation/edited by FJ Bergersen*, John Wiley & Sons Ltd, pp. 535-55.

7. Gill, SS & Tuteja, N 2010, 'Polyamines and abiotic stress tolerance in plants', *Plant signaling & behavior*, vol. 5, no. 1, pp. 26-33.

8. Hummel, I, Couée, I, El Amrani, A, Martin-Tanguy, J & Hennion, F 2002, 'Involvement of polyamines in root development at low temperature in the subantarctic cruciferous species Pringlea antiscorbutica', *Journal of Experimental Botany*, vol. 53, no. 373, pp. 1463-73.

9. Imai, A, Matsuyama, T, Hanzawa, Y, Akiyama, T, Tamaoki, M, Saji, H, Shirano, Y, Kato, T, Hayashi, H & Shibata, D 2004, 'Spermidine synthase genes are essential for survival of Arabidopsis', *Plant physiology*, vol. 135, no. 3, pp. 1565-73.

10. Li, H 2018, 'Minimap2: pairwise alignment for nucleotide sequences', *Bioinformatics*, pp. bty191-bty.

11. Löytynoja, A 2014, 'Phylogeny-aware alignment with PRANK', in DJ Russell (ed.), *Multiple Sequence Alignment Methods*, Humana Press, Totowa, NJ, pp. 155-70, DOI 10.1007/978-1-62703-646-7_10, <https://doi.org/10.1007/978-1-62703-646-7_10>.

12. Page, AJ, Cummins, CA, Hunt, M, Wong, VK, Reuter, S, Holden, MT, Fookes, M, Falush, D, Keane, JA & Parkhill, J 2015, 'Roary: rapid large-scale prokaryote pan genome analysis', *Bioinformatics*, vol. 31, no. 22, pp. 3691-3.

13. Price, MN, Dehal, PS & Arkin, AP 2010, 'FastTree 2--approximately maximum-likelihood trees for large alignments', *PLoS One*, vol. 5, no. 3, p. e9490.

14. Richter, M & Rosselló-Móra, R 2009, 'Shifting the genomic gold standard for the prokaryotic species definition', *Proceedings of the National Academy of Sciences*, vol. 106, no. 45, pp. 19126-31.

15. Seemann, T 2014, 'Prokka: rapid prokaryotic genome annotation', *Bioinformatics*, vol. 30, no. 14, pp. 2068-9.

16. Vaser, R, Sovic, I, Nagarajan, N & Sikic, M 2017, 'Fast and accurate de novo genome assembly from long uncorrected reads', *Genome Res*, vol. 27, no. 5, pp. 737-46.

17. Walker, BJ, Abeel, T, Shea, T, Priest, M, Abouelliel, A, Sakthikumar, S, Cuomo, CA, Zeng, Q, Wortman, J, Young, SK & Earl, AM 2014, 'Pilon: an integrated tool for comprehensive microbial variant detection and genome assembly improvement', *PLoS One*, vol. 9, no. 11, p. e112963.

18. Weber, T, Blin, K, Duddela, S, Krug, D, Kim, HU, Bruccoleri, R, Lee, SY, Fischbach, MA, Muller, R, Wohlleben, W, Breitling, R, Takano, E & Medema, MH 2015, 'antiSMASH 3.0-a comprehensive resource for the genome mining of biosynthetic gene clusters', *Nucleic Acids Res*, vol. 43, no. W1, pp. W237-43.

19. Wick, RR, Judd, LM, Gorrie, CL & Holt, KE 2017, 'Unicycler: Resolving bacterial genome assemblies from short and long sequencing reads', *PLOS Computational Biology*, vol. 13, no. 6, p. e1005595.

20. Wick, RR, Schultz, MB, Zobel, J & Holt, KE 2015, 'Bandage: interactive visualization of de novo genome assemblies', *Bioinformatics*, vol. 31, no. 20, pp. 3350-2.

21. Wolf, A, Fritze, A, Hagemann, M & Berg, G 2002, 'Stenotrophomonas rhizophila sp. nov., a novel plant-associated bacterium with antifungal properties', *International Journal of Systematic and Evolutionary Microbiology*, vol. 52, no. 6, pp. 1937-44.

22. Xie, S-S, Wu, H-J, Zang, H-Y, Wu, L-M, Zhu, Q-Q & Gao, X-W 2014, 'Plant growth promotion by spermidine-producing Bacillus subtilis OKB105', *Molecular plant-microbe interactions*, vol. 27, no. 7, pp. 655-63.

THE CLAIMS DEFINING THE INVENTION ARE AS FOLLOWS:

1. A substantially purified or isolated endophyte strain isolated from a plant of the Poaceae family, wherein said endophyte is a strain of *Stenotrophomonas rhizophila* which provides bioprotection and/or biofertilizer phenotypes to plants into which it is inoculated.

2. An endophyte according to claim 1, wherein the bioprotection and/or biofertilizer phenotype includes production of the bioprotectant compound in the plant into which the endophyte is inoculated.

3. An endophyte according to claim 2, wherein the bioprotectant compound is spermidine, or a derivative, isomer and/or salt thereof.

4. An endophyte according to claim any one claims 1 to 3, wherein the bioprotection and/or biofertilizer phenotype includes nitrogen fixation in the plant into which the endophyte is inoculated.

5. An endophyte according to any one of claims 1 to 4, wherein the endophyte is strain JB as described herein and as deposited with The National Measurement Institute on 17th May 2019 with accession number V19/009906.

6. An endophyte according to any one of claims 1 to 5, wherein the plant from which the endophyte is isolated is of the Poaceae family is a pasture grass.

7. An endophyte according to claim 6, wherein the pasture grass is from the genus Lolium or Festuca.

8. An endophyte according to claim 7, wherein the pasture grass is from the species *Lolium perenne* or *Festuca arundinaceum.*

9. An endophyte according to any one of claims 1 to 8, wherein the plant into which the endophyte is inoculated includes an endophyte-free host plant or part thereof stably infected with said endophyte.

10. An endophyte according to any one of claims 1 to 9, wherein the plant into which the endophyte is inoculated is an agricultural plant selected from one or more of forage grass, turf grass, bioenergy grass, grain crop and industrial crop.

11. An endophyte according claim 9, wherein the plant into which the endophyte is inoculated is a forage, turf or bioenergy grass selected from the group consisting of those belonging to the

genera *Lolium* and *Festuca*, including *L. perenne* (perennial ryegrass), *L. arundinaceum* (tall fescue) and *L. multiflorum* (Italian ryegrass), and those belonging to the *Brachiaria-Urochloa* species complex (panic grasses), including *Brachiaria brizantha, Brachiaria decumbens, Brachiaria humidicola, Brachiaria stolonifera, Brachiaria ruziziensis, B. dictyoneura, Urochloa brizantha, Urochloa decumbens, Urochloa humidicola*, *Urochloa mosambicensis* as well as interspecific and intraspecific hybrids of *Brachiaria-Urochloa* species complex such as interspecific hybrids between *Brachiaria ruziziensis* x *Brachiaria brizantha*, *Brachiaria ruziziensis* x *Brachiaria decumbens*, [*Brachiaria ruziziensis* x *Brachiaria decumbens*] x *Brachiaria brizantha*, [*Brachiaria ruziziensis* x *Brachiaria brizantha*] x *Brachiaria decumbens*.

12. An endophyte according claim 9, wherein the plant into which the endophyte is inoculated is a grain crop or industrial crop grass selected from the group consisting of those belonging to the genus *Triticum*, including *T. aestivum* (wheat), those belonging to the genus *Hordeum*, including *H. vulgare* (barley), those belonging to the genus *Zea*, including *Z. mays* (maize or corn), those belonging to the genus *Oryza*, including *O. sativa* (rice), those belonging to the genus *Saccharum* including *S. officinarum* (sugarcane), those belonging to the genus *Sorghum* including *S. bicolor* (sorghum), those belonging to the genus *Panicum*, including *P. virgatum* (switchgrass), those belonging to the genera *Miscanthus*, *Paspalum*, *Pennisetum*, *Poa*, *Eragrostis* and *Agrostis*.

13. An endophyte according to claim 10, wherein the plant into which the endophyte is inoculated is a grain crop or industrial crop selected from the group consisting of wheat, barley, oats, chickpeas, triticale, fava beans, lupins, field peas, canola, cereal rye, vetch, lentils, millet/panicum, safflower, linseed, sorghum, sunflower, maize, canola, mungbeans, soybeans, and cotton.

14. A plant or part thereof infected with one or more endophytes according to any one of claims 1 to 13.

15. A plant, plant seed or other plant part derived from a plant or part thereof according to claim 14 and stably infected with said one or more endophytes.

16. Use of an endophyte according to any one of claims 1 to 13 to produce a plant or part thereof stably infected with said one or more endophytes.

17. A bioprotectant compound produced by an endophyte according to any one of claims 1 to 13, or a derivative, isomer and/or a salt thereof, preferably the bioprotectant compound is spermidine or derivative, isomer and/or salt thereof.

18. A method for producing a bioprotectant compound, said method including infecting a plant with an endophyte according to any one of claims 1 to 13 and cultivating the plant under conditions suitable to produce the spermidine.

19. A method for producing a bioprotectant compound, said method including culturing an endophyte according to any one of claims 1 to 13 under conditions suitable to produce the bioprotectant compound.

20. A method according to claim 19, wherein the conditions include a culture medium including a source of carbohydrates.

21. A method according to claim 20, wherein the source of carbohydrates is selected from one or more of the group consisting of a starch/sugar-based agar or broth, a cereal-based agar or broth, endophyte agar, Murashige and Skoog with 20 % sucrose, half V8 juice/half PDA, water agar and yeast malt extract agar.

22. A method according to any one of claims 18 to 21, wherein the method further includes isolating the bioprotectant compound from the plant or culture medium.

23. A method of growing a plant in a low nitrogen medium, said method including infecting a plant with a bioprotectant compound -producing endophyte according to any one of claims 1 to 13, and cultivating the plant.

24. A method according to claim 23, wherein the low nitrogen medium is low nitrogen soil.

ABSTRACT

The present invention relates to an endophyte strain isolated from a plant of the Poaceae family, wherein said endophyte is a strain of *Stenotrophomonas rhizophila* which provides bioprotection and/or biofertilizer phenotypes to plants into which it is inoculated. The present invention also discloses plants infected with the endophyte and related methods.

Figures

AAAGGAGGTGATCCAGCCGCACCTTCCGATACGGCTACCTTGTTACGACTTCACCCCAGTCATCGGCCACACC
GTGGCAAGCGCCCTCCCGAAGGTTAAGCTACCTGCTTCTGGTGCAACAAACTCCCATGGTGTGACGGGCGGT
GTGTACAAGGCCCGGGAACGTATTCACCGCAGCAATGCTGATCTGCGATTACTAGCGATTCCGACTTCATGG
AGTCGAGTTGCAGACTCCAATCCGGACTGAGATAGGGTTTCTGGGATTGGCTTGCCCTCGCGGGTTTGCAGC
CCTCTGTCCCTACCATTGTAGTACGTGTGTAGCCCTGGTCGTAAGGGCCATGATGACTTGACGTCATCCCCAC
CTTCCTCCGGTTTGTCACCGGCGGTCTCCTTAGAGTTCCCACCATTACGTGCTGGCAACTAAGGACAAGGGTT
GCGCTCGTTGCGGGACTTAACCCAACATCTCACGACACGAGCTGACGACAGCCATGCAGCACCTGTGTTCGA
GTTCCCGAAGGCACCAATCCATCTCTGGAAAGTTCTCGACATGTCAAGACCAGGTAAGGTTCTTCGCGTTGCA
TCGAATTAAACCACATACTCCACCGCTTGTGCGGGCCCCCGTCAATTCCTTTGAGTTTCAGTCTTGCGACCGTA
CTCCCCAGGCGGCGAACTTAACGCGTTAGCTTCGATACTGCGTGCCAAATTGCACCCAACATCCAGTTCGCAT
CGTTTAGGGCGTGGACTACCAGGGTATCTAATCCTGTTTGCTCCCCACGCTTTCGTGCCTCAGTGTCAGTGTT
GGTCCAGGTAGCTGCCTTCGCCATGGATGTTCCTCCCGATCTCTACGCATTTCACTGCTACACCGGGAATTCC
ACTACCCTCTACCACACTCTAGTCGTCCAGTATCCACTGCAATTCCCAGGTTGAGCCCAGGGCTTTCACAACA
GACTTAAACAACCACCTACGCACGCTTTACGCCCAGTAATTCCGAGTAACGCTTGCACCCTTCGTATTACCGC
GGCTGCTGGCACGAAGTTAGCCGGTGCTTATTCTTTGGGTACCGTCAGAACAACCGAGTATTAATCGACTGC
TTTTCTTTCCCAACAAAAGGGCTTTACAACCCGAAGGCCTTCTTCACCCACGCGGTATGGCTGGATCAGGCTT
GCGCCCATTGTCCAATATTCCCCACTGCTGCCTCCCGTAGGAGTCTGGACCGTGTCTCAGTTCCAGTGTGGCT
GATCATCCTCTCAGACCAGCTACGGATCGTCGCCTTGGTGGGCCTTTACCCCGCCAACTAGCTAATCCGACAT
CGGCTCATCTATCCGCGCAAGGCCCGAAGGTCCCCTGCTTTCACCCGAAGGTCGTATGCGGTATTAGCGTAA
GTTTCCCTACGTTATCCCCCACGAAAAGGTAGATTCCGATGTATTCCTCACCCGTCCGCCACTCGCCACCCATA
AGAGCAAGCTCTTACTGTGCTGCCGTTCGACTTGCATGTGTTAGGCCTACCGCCAGCGTTCACTCTGAGCCAG
GATCAAACTCTTCACTT

Figure 1

Appendix 1

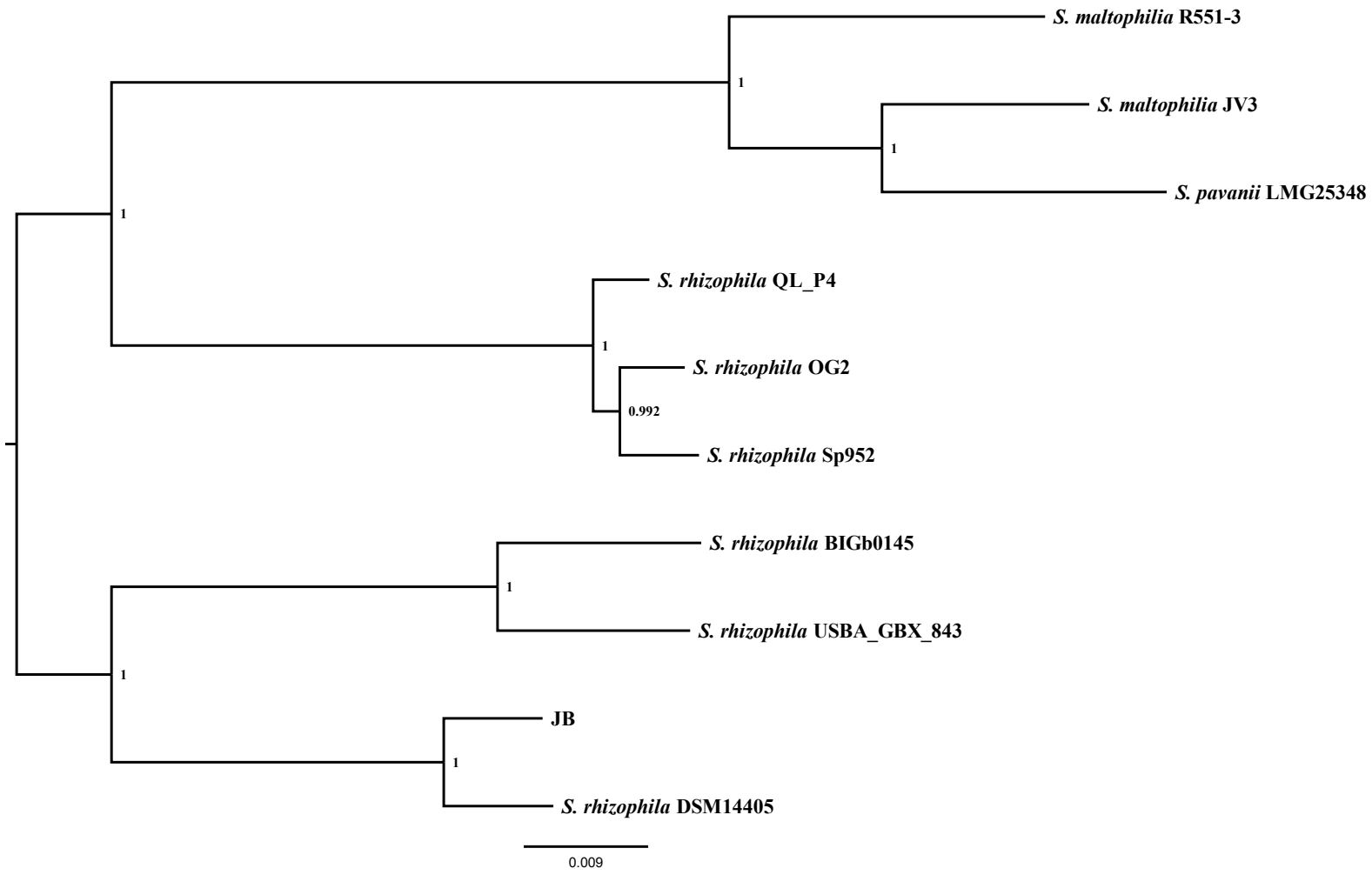


Figure 2

Appendix 1

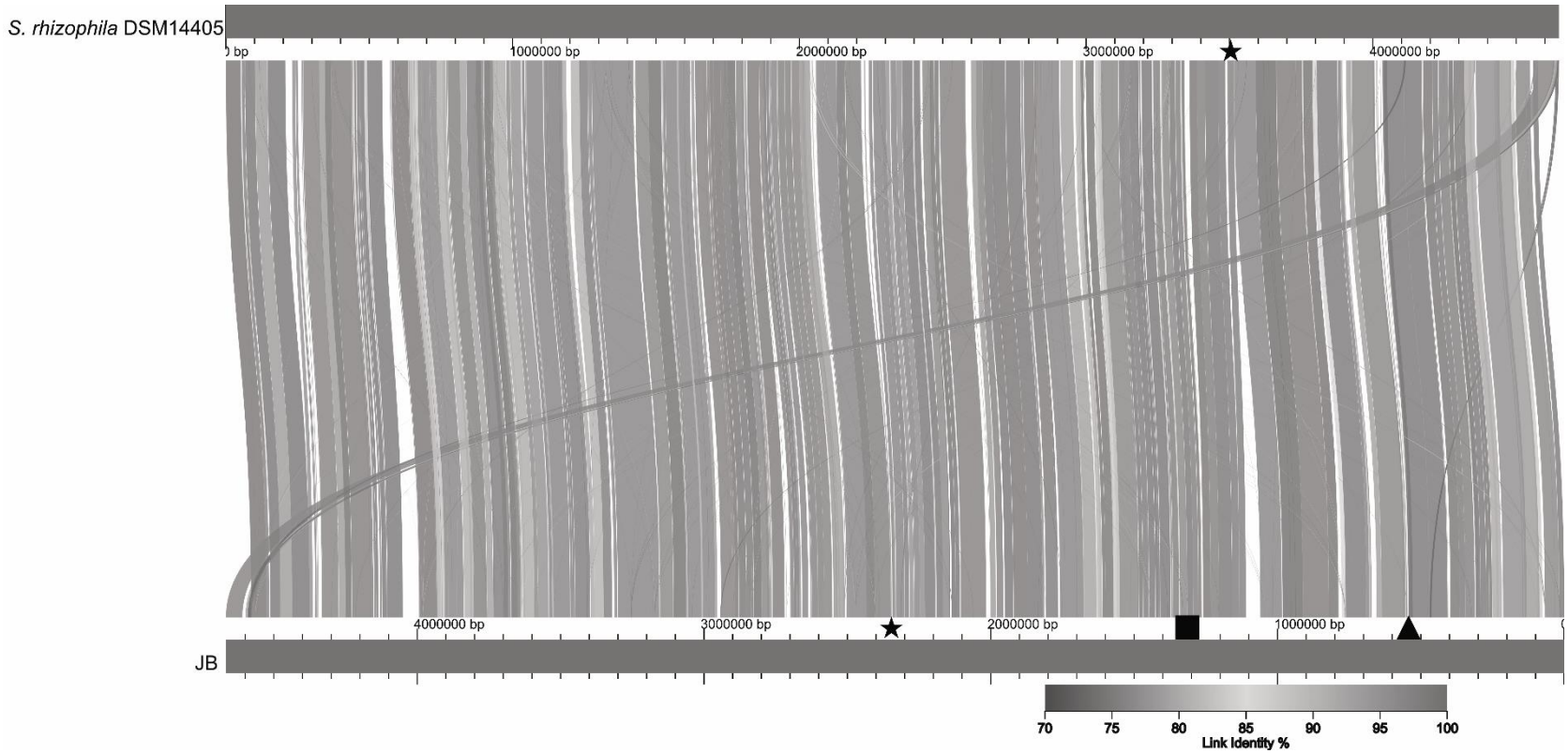


Figure 3

Appendix 1

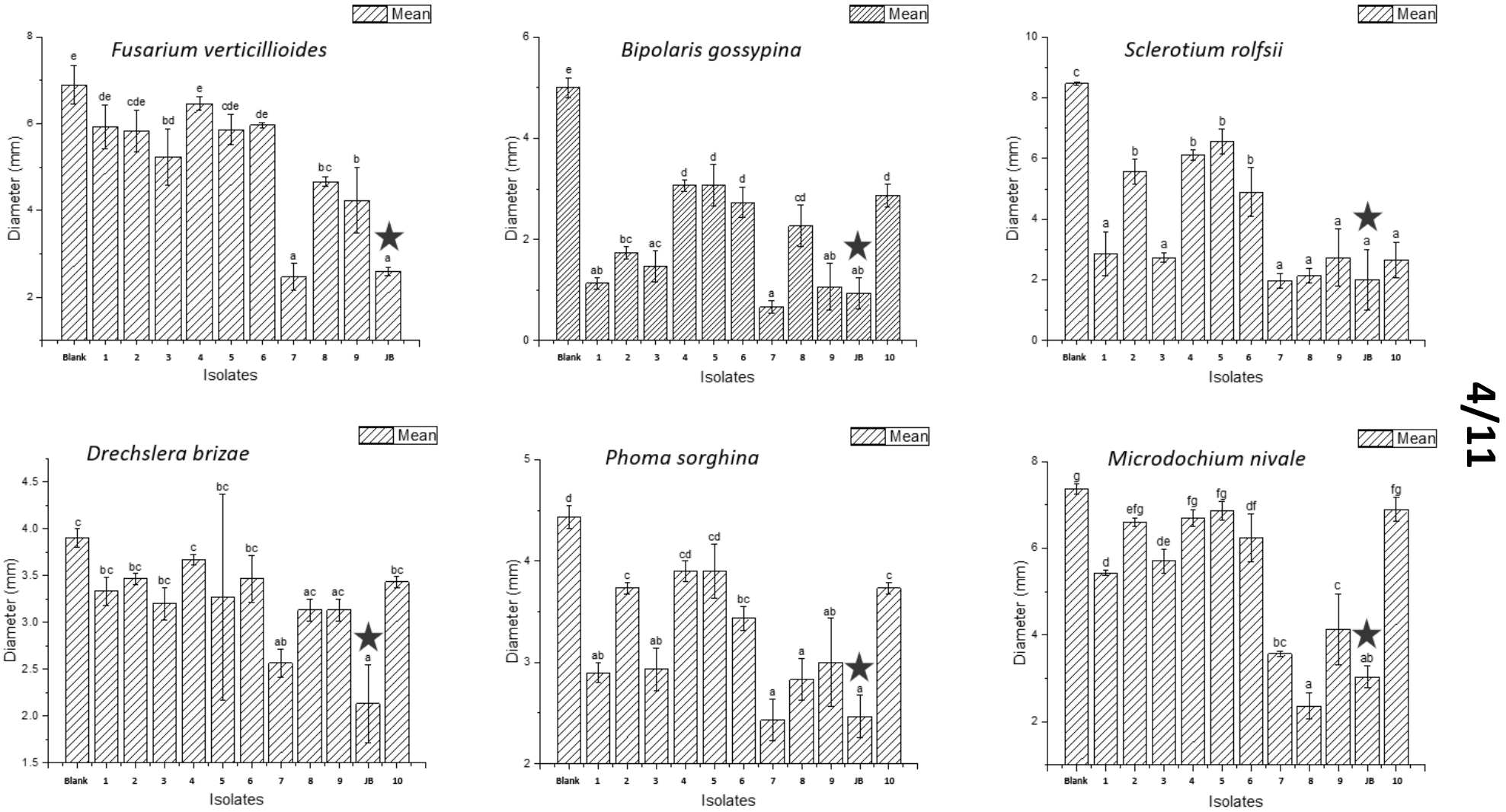
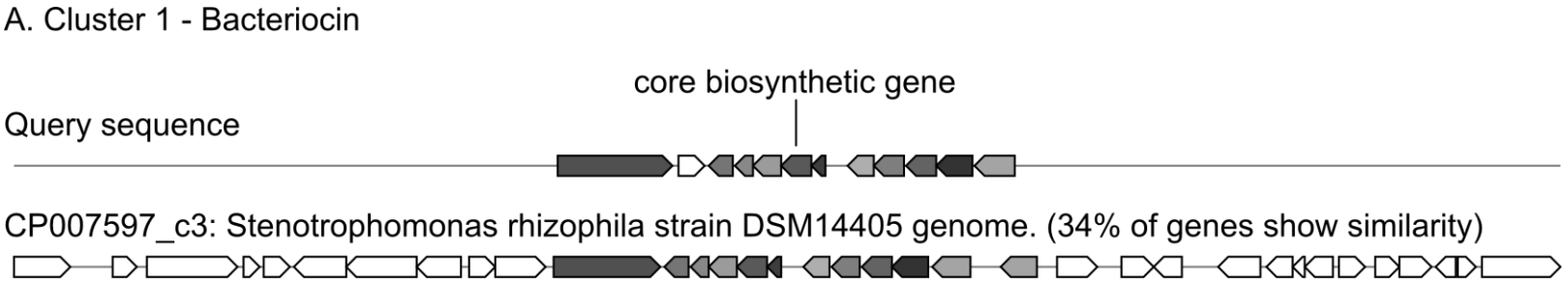


Figure 4


A. Cluster 1 - Bacteriocin
core biosynthetic gene
Query sequence
CP007597_c3: Stenotrophomonas rhizophila strain DSM14405 genome. (34% of genes show similarity)

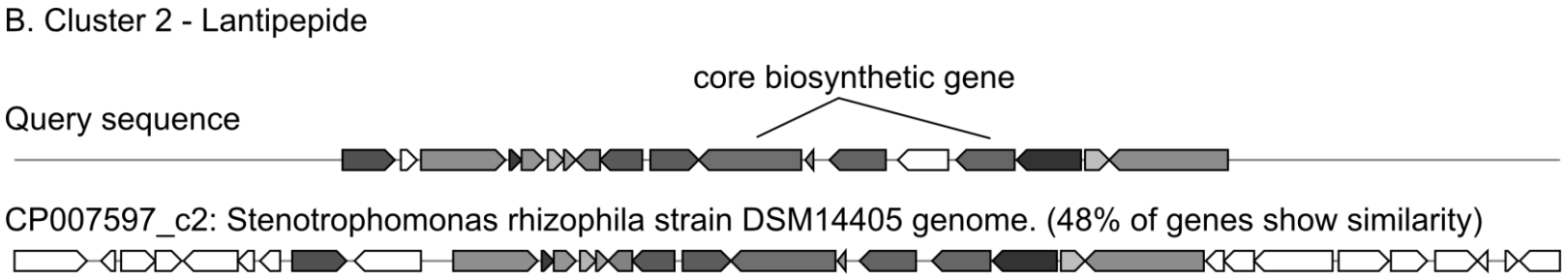
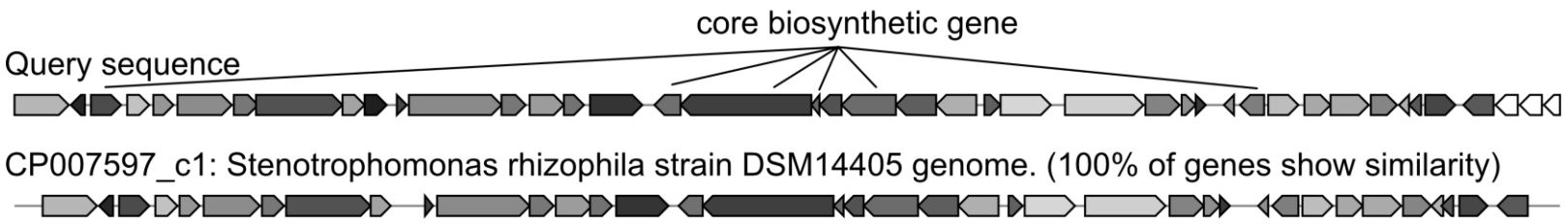

B. Cluster 2 - Lantipepide
core biosynthetic gene
Query sequence
CP007597_c2: Stenotrophomonas rhizophila strain DSM14405 genome. (48% of genes show similarity)

Figure 5

C. Cluster 3 - Non-ribosomal peptide synthase



D. Cluster 4 - Arylpolyene

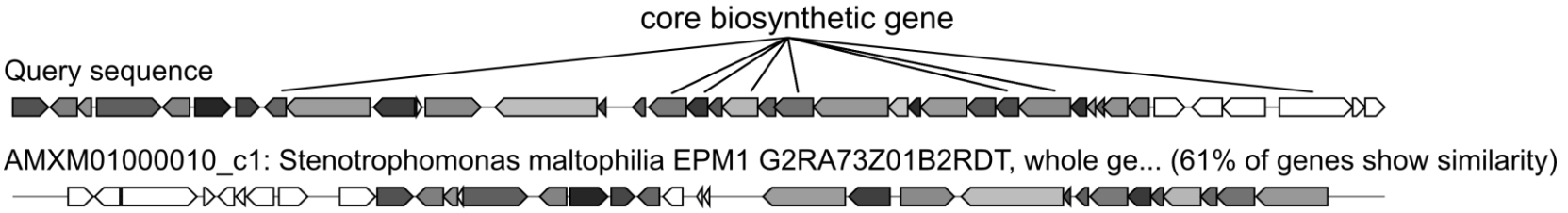


Figure 5 (cont.)

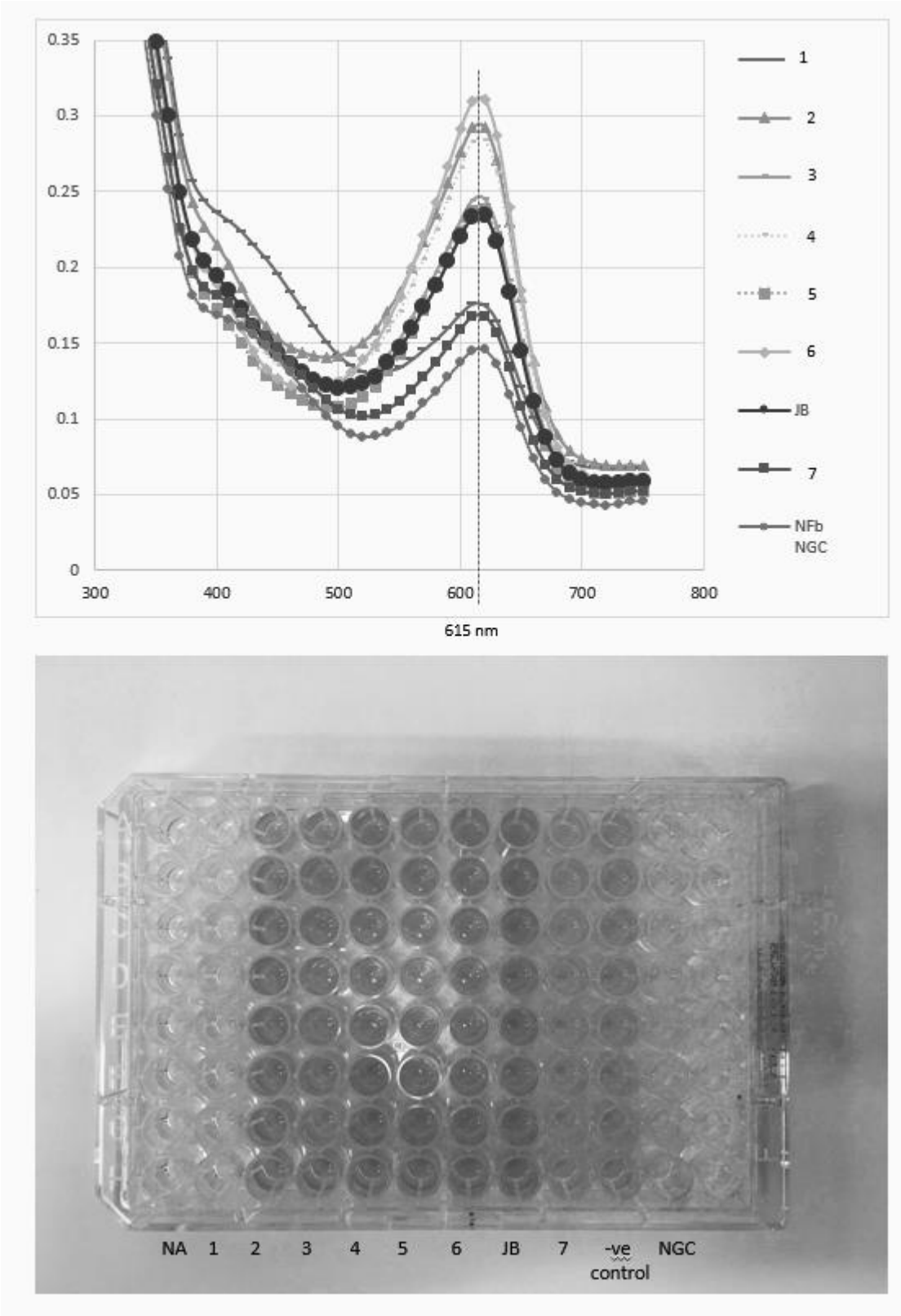

0.35
0.3
0.25
0.2
0.15
0.1
0.05
0
300
400
500
600
700
800
615 nm
1
2
3
4
5
6
JB
7
NFb
NGC
NA 1 2 3 4 5 6 JB 7 -ve control NGC

Figure 6

Appendix 1

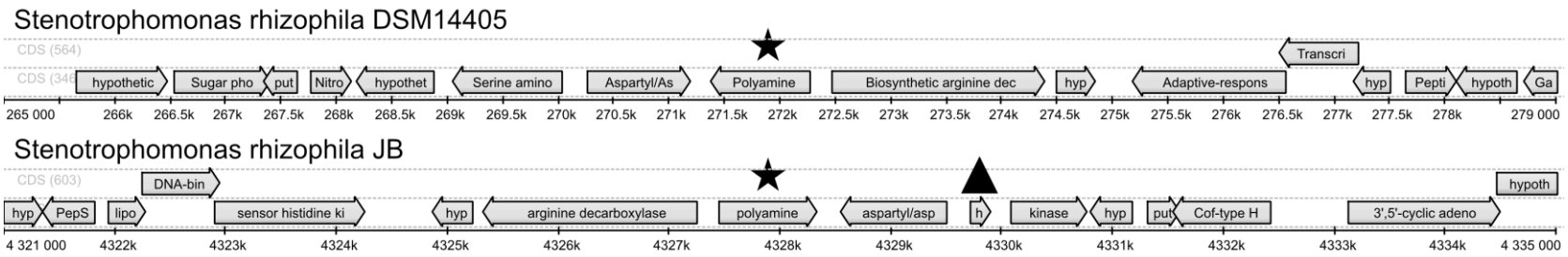


Figure 7

Appendix 1

Figure 8

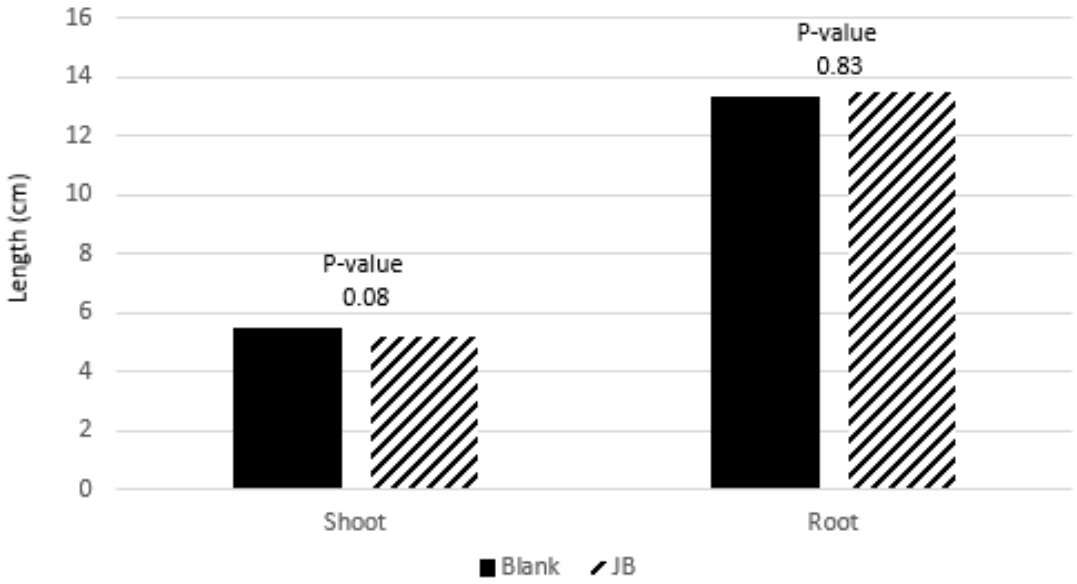

16
14
12
10
8
6
4
2
0
Length (cm)
P-value
0.08
P-value
0.83
Shoot
Root
Blank
JB

Figure 9

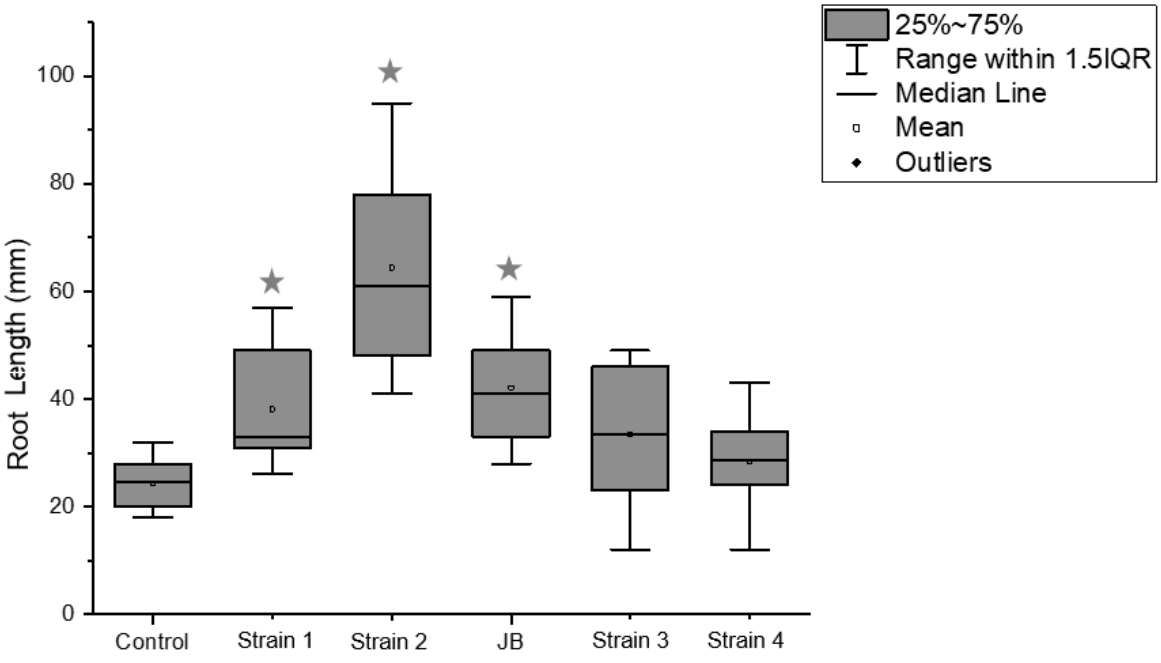

25%~75%
Range within 1.5IQR
Median Line
Mean
Outliers
Root Length (mm)
0
20
40
60
80
100
Control
Strain 1
Strain 2
JB
Strain 3
Strain 4

Figure 10

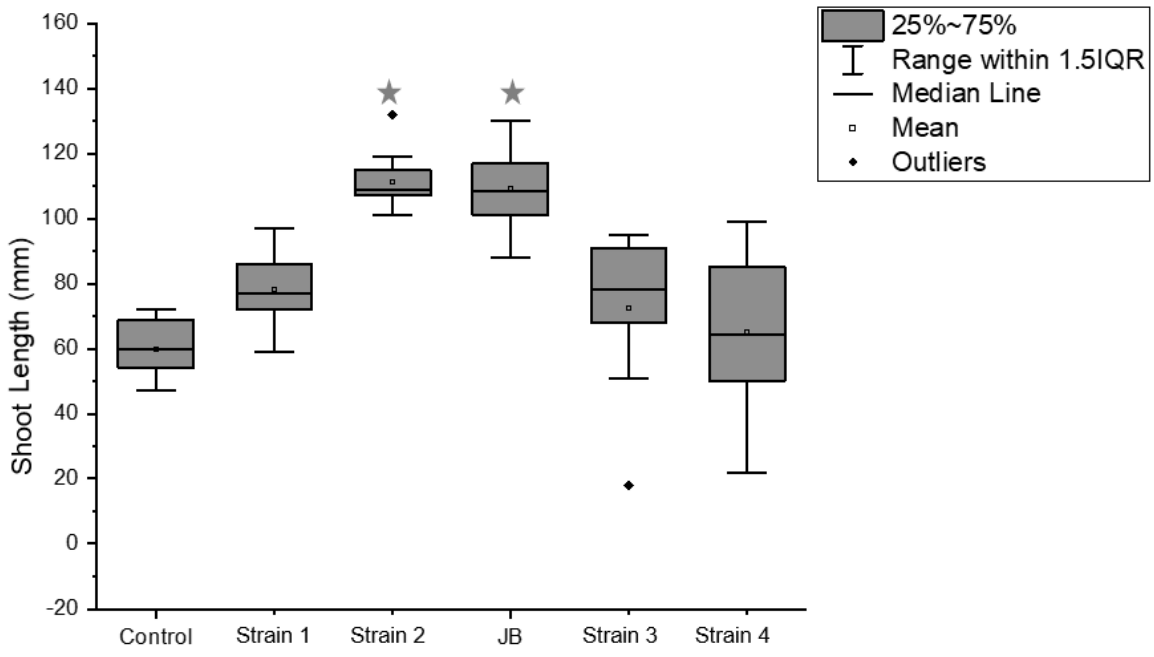

160
140
120
100
80
60
40
20
0
-20
Shoot Length (mm)
Control
Strain 1
Strain 2
JB
Strain 3
Strain 4
25%~75%
Range within 1.5IQR
Median Line
Mean
Outliers

Figure 11

Appendix 2

Supplementary materials relating to chapter 3 manuscript: Transcriptomics differentiate two novel bioactive strains of *Paenibacillus* sp. isolated from the perennial ryegrass seed microbiome

Supplementary Table S1. Summary of reads available for genome assembly

Strain ID	Yield (bp) Illumina	Yield (bp) ONT	Mean length (bp) ONT	Coverage Illumina	Coverage ONT
S02	1,555,571,650	8,073,984,100	1,144	256.7×	1332.2×
S25	981,251,546	5,129,702,300	5,088	164.7×	860.8×

Supplementary Table S2. General genomic characteristics of *Paenibacillus* sp. strains S02 and S25

Strain ID	Genome size (bp)	GC content (%)	No. of tRNA	No. of tmRNA	No. of rRNA	No. of gene	No. of CDS
S02	6,060,529	45.60	92	1	33	5,436	5,310
S25	5,958,851	45.72	92	1	36	5,306	5,177

Supplementary Table S4. Plant growth-promoting genes in the genomes of *Paenibacillus* sp. strains S02 and S25, and *P. polymyxa* strain CR1

Trait	Gene Name	*Paenibacillus* sp. S02	*Paenibacillus* sp. S25	*P. polymyxa* CR1
Nitrogen fixation				
	nifB	KAI36_01039	KAI37_00991	YP_008910495
	nifH	KAI36_01040	KAI37_00992	YP_008910496
	nifD	KAI36_01041	KAI37_00993	YP_008910497
	nifK	KAI36_01042	KAI37_00994	YP_008910498
	nifE	KAI36_01043	KAI37_00995	YP_008910499
	nifN	KAI36_01044	KAI37_00996	YP_008910500
	nifX	KAI36_01045	KAI37_00997	YP_008910501
	hesA/moeB	KAI36_01046	KAI37_00998	YP_008910502
	nifV	KAI36_01047	KAI37_00999	YP_008910503
Phosphate solubilization				
	gcd	KAI36_02793	KAI37_02644	YP_008912273
	gad	–	–	–
Phosphonate cluster (*phn*)				
	phnA	KAI36_05309	KAI37_05177	YP_008914717
	phnB	KAI36_00880	KAI37_00854	YP_008910326
	phnC	KAI36_04607	KAI37_04494	YP_008913947
	phnD	KAI36_04606	KAI37_04493	YP_008913946
	phnE	KAI36_04608	KAI37_04495	YP_008913948
	phnW	KAI36_05275	KAI37_05152	YP_008914692
	phnX	KAI36_00485	KAI37_00496	YP_008909947
	ppd	KAI36_05276	KAI37_05153	YP_008914693
	pepM	KAI36_05277	KAI37_05154	YP_008914694
Phosphate transporter (*pst*)				
	pstS	KAI36_01689	KAI37_01595	YP_008911198
	pstA	KAI36_01691	KAI37_01597	YP_008911200
	pstB	KAI36_01692	KAI37_01598	YP_008911201
	pstC	KAI36_01690	KAI37_01596	YP_008911199
	phoP	KAI36_01703	KAI37_01609	YP_008911212
	phoR	KAI36_01702	KAI37_01608	YP_008911211
Indole-3-acetic acid production				
	ipdC	KAI36_01475	KAI37_01435	YP_008911027
	auxin efflux carriers	KAI36_02845 KAI36_03330 KAI36_05253	KAI37_02683 KAI37_03351 KAI37_05129	YP_008912813 YP_008912323 YP_008911849

Gene identifiers for *Paenibacillus* sp. strains S02 and S25 were from the annotated genomes described in section 2.2. Gene identifiers for *P. polymyxa* CR1 were from Eastman, et al.[16]

Supplementary Table S5. Percentage identity of plant growth-promoting genes in the genome of strains *Paenibacillus* sp. S02/S25 and *P. polymyxa* CR1

Trait	Gene Name	S02 VS S25	S02 VS CR1	S25 VS CR1
Nitrogen fixation				
	nifB	97.13	98.00	96.80
	nifH	95.39	98.15	94.69
	nifD	98.96	98.62	98.27
	nifK	98.24	97.45	97.39
	nifE	98.24	98.60	98.16
	nifN	97.25	97.55	97.63
	nifX	98.46	97.69	97.69
	hesA/moeB	96.47	98.04	96.34
	nifV	98.50	98.59	97.80
Phosphate solubilization				
	gcd	98.11	97.60	98.39
	gad	-	-	-
Phosphonate cluster (*phn*)				
	phnA	98.53	98.53	98.82
	phnB	98.21	98.43	99.78
	phnC	97.66	97.81	98.39
	phnD	98.14	99.07	97.63
	phnE	98.25	97.78	97.43
	phnW	98.74	98.47	98.20
	phnX	98.04	97.92	98.81
	ppd	98.97	98.62	98.79
	pepM	99.33	99.44	99.00
Phosphate transporter (*pst*)				
	pstS	98.92	98.48	99.13
	pstA	96.99	97.55	98.22
	pstB	98.81	98.70	98.22
	pstC	99.14	98.71	98.82
	phoP	98.09	97.81	97.94
	phoR	98.28	97.45	97.89
Indole-3-acetic acid production				
	ipdC	99.54	99.48	99.14
	auxin efflux carriers 1	99.13	98.15	98.37
	auxin efflux carriers 2	97.92	97.60	98.85
	auxin efflux carriers 3	98.58	98.96	99.05

Supplementary Table S6. Secondary metabolite gene clusters identified in *Paenibacillus* sp. strains S02 and S25

ID	Type	Location		Most similar known cluster (similarity)
		S02	S25	
C1	Nrps	62,712-130,949	62,863-131,149	**fusaricidin B (100%)**
C2	siderophore	1,060,830-1,078,231	1,021,525-1,038,926	–
C3	bacteriocin	1,226,685-1,236,921	1,163,825-1,174,061	–
C4	Nrps transAT-PKS	1,276,170-1,374,849	1,234,262-1,333,113	–
C5	lassopeptide	1,410,732-1,434,848	1,369,083-1,393,199	paeninodin (40%)
C6	Nrps	1,496,857-1,557,694	1,452,621-1,513,241	marthiapeptide A (33%)
C7	lanthipeptide	1,752,471-1,779,477	1,717,117-1,742,305	**paenilan (100%)**
C8	lanthipeptide	–	1,865,337-1,891,786	**paenicidin B (71%)**
C9	Nrps-like	2,147,919-2,191,265	2,068,326-2,110,857	–
C10	Nrps	2,564,994-2,657,512	2,538,234-2,631,151	**tridecaptin (100%)**
C11	Nrps transAT-PKS	2,800,573-2,881,430	2,762,852-2,843,624	paenilipoheptin (S02, 73%; S25, 76%)
C12	Nrps	–	2,847,977-2,929,143	–
C13	Nrps betalactone	–	3,004,139-3,056,727	–
C14	Nrps T3PKS transAT-PKS	3,755,116-3,856,856	3,756,437-3,858,120	aurantinin B/C/D (35%)
C15	Nrps	5,189,939-5,270,981	5,092,876-5,173,931	**polymyxin (100%)**
C16	phosphonate	5,879,383-5,920,282	5,775,191-5,816,090	–

Nrps: Nonribosomal peptide synthetase
transAT-PKS: transAT-polyketide synthase
T3PKS: Type III polyketide synthase
Clusters in bold: Known antimicrobial compounds

Supplementary Table S7. The average colony diameter (± standard error) of fungal pathogens when exposed to the two *Paenibacillus* sp. strains (S02 and S25) in a bioprotection assay (*in vitro*)

Pathogen ID	S02/cm	S25/cm	Blank/cm
Colletotrichum graminicola	1.03 ± 0.02^{b}	3.77 ± 0.04^{a}	4.10 ± 0.20^{a}
Fusarium verticillioides	2.93 ± 0.04^{c}	6.15 ± 0.14^{b}	6.80 ± 0.08^{a}
Microdochium nivale	5.12 ± 0.11^{a}	5.18 ± 0.12^{a}	5.27 ± 0.04^{a}

$^{a, b, c}$: Different letters are statistically significantly ($P < 0.05$) different.
S02/S25: *Paenibacillus* sp. strains

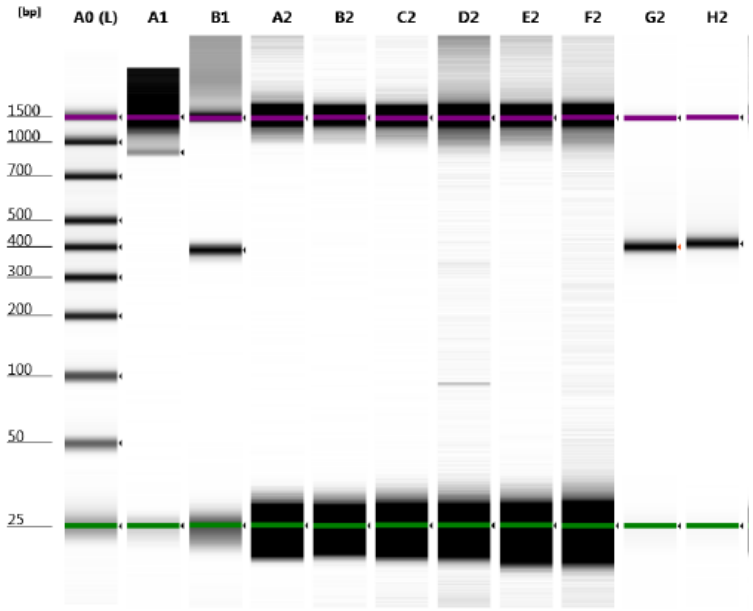
Supplementary Table S8. The percentage of mapped reads when quantifying transcript of each sample using the transcriptome sequences of *F. verticillioides* 7600 as the reference

Sample	No. of mapped reads	Mapping rate
S02_ctrl_1	4	too low
S02_ctrl_2	1	too low
S02_ctrl_3	2	too low
S02_treated_1	3	too low
S02_treated_2	1,492	0.0082%
S02_treated_3	1,366	0.0089%
S25_ctrl_1	1	too low
S02_ctrl_2	0	0%
S02_ctrl_3	0	0%
S25_treated_1	33,944	0.2817%
S25_treated_2	17,987	0.1407%
S25_treated_3	22,684	0.1395%

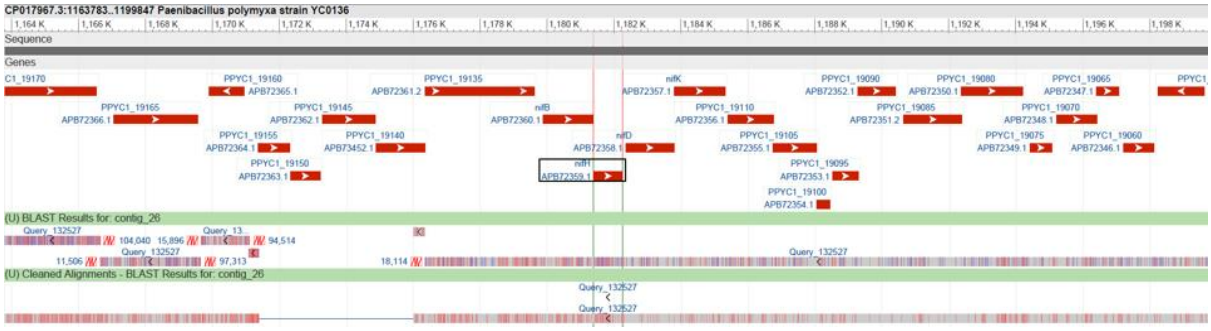
too low: 0–0.0001%; ctrl: Without *F. verticillioides*; treated: With *F. verticillioides*
S02/S25: *Paenibacillus* sp. strains isolated in this study

Supplementary Table S9. Pathogens used in the *in vitro* bioprotection assay.

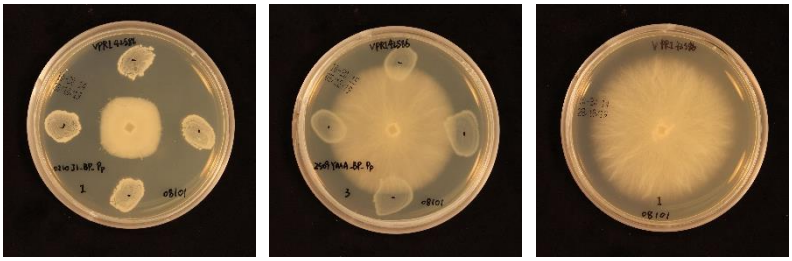
VPRI Acc. No.	Taxonomic Details	Host Taxonomic Details	State	Collection Date
32315	*Colletotrichum graminicola*	*Cynosurus echinatus*.	Vic.	27-Apr-05
42586a	*Fusarium verticillioides*	*Zea mays* L.	Vic	27-Feb-15
43403	*Microdochium nivale*	*Lolium perenne*	Vic.	11-Nov-17



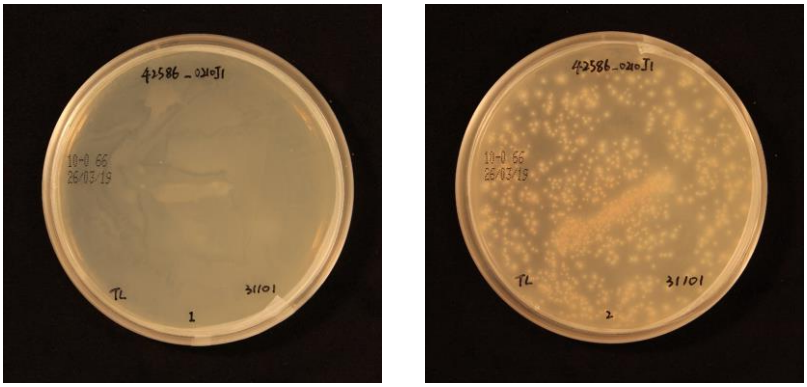
Supplementary Figure S1. Amplicons of the expected size (~ 400 bp) were produced using the DNA extracted from two (G2 and H2) of eight replicates of the 10^{-2} dilution (A2–H2). Results of the 10^{-3} dilutions were not shown since no amplicon was produced.
A0: DNA ladder; A1: No template control; B1: Positive control, *Rhizobium leguminosarum* bv. *trifolii* WSM1325 DNA



Supplementary Figure S2. A single 110 Kb read containing the entire *P. polymyxa nif* operon (nine genes) of *P. polymyxa* YC0136 identified via BLAST analyses. The *nifH* gene was highlighted.



Supplementary Figure S3. Representative images of the in vitro bioprotection assay when challenging strain S02 (left) and S25 (middle) with *Fusarium verticillioides*. The control plate (pathogen-only) was shown on the right.



Supplementary Figure S4. Images of Potato Dextrose Agar plates containing samples of *Paenibacillus* sp. S02 co-incubated *F. verticillioides* (six hours). The first biological replicate (S02_treated_1, left) showed no visual growth of the pathogen, and only had three mapped fungal reads. The second biological replicate (S02_treated_2, right) had 1,492 mapped fungal reads.

Appendix 3

Supplementary materials relating to chapter 4 manuscript: Transcriptome analyses of barley roots inoculated with novel *Paenibacillus* sp. and *Erwinia gerundensis* strains reveal beneficial early stage plant-bacteria interactions

Supplementary Table S1. Yield (number of clean reads) generated by transcriptome sequencing

	Sample ID	Strain ID	Treatment	Medium	No. of clean reads (Million)
Bacteria	AR-C-NB-1	AR *Erwinia gerundensis*	control (no barley seedling)	Nutrient Broth	134.4
	AR-C-NB-2				98.7
	AR-C-NB-3				138.6
	AR-B-NB-1		with barley seedlings		121.4
	AR-B-NB-2				145.9
	AR-B-NB-3				106.0
	P25-C-NB-1	S25 *Paenibacillus* sp.	control (no barley seedling)		31.8
	P25-C-NB-2				29.4
	P25-C-NB-3				29.1
	P25-B-NB-1		with barley seedlings		110.3
	P25-B-NB-2				91.6
	P25-B-NB-3				90.5
	P02-C-NB-1	S02 *Paenibacillus* sp.	control (no barley seedling)		26.7
	P02-C-NB-2				30.8
	P02-C-NB-3				41.7
	P02-B-NB-1		with barley seedlings		29.4
	P02-B-NB-2				44.8
	P02-B-NB-3				62.4
	P02-Bu-1		control (no barley seedling)	Burk's N-free	17.7
	P02-Bu-2				26.0
	P02-Bu-3				16.2
	P02-B-Bu-1		with barley seedlings		4.9
	P02-B-Bu-2				22.2
	P02-B-Bu-3				30.1
Plant	B-C-NB-r-1	N/A	control (no bacteria)	Nutrient Broth	155.6
	B-C-NB-r-2				154.5
	B-C-NB-r-3				137.7
	B-C-Bu-r-1			Burk's N-free	122.8
	B-C-Bu-r-2				129.3
	B-C-Bu-r-3				127.9
	B-AR-NB-r-1	AR *Erwinia gerundensis*	with bacteria	Nutrient Broth	146.8
	B-AR-NB-r-2				148.9
	B-AR-NB-r-3				130.4
	B-P25-NB-r-1	S25 *Paenibacillus* sp.			137.9
	B-P25-NB-r-2				93.53
	B-P25-NB-r-3				176.0
	B-P02-NB-r-1	S02 *Paenibacillus* sp.			147.9
	B-P02-NB-r-2				138.7
	B-P02-NB-r-3				165.3
	B-P02-Bu-r-1			Burk's N-free	97.64
	B-P02-Bu-r-2				92.52
	B-P02-Bu-r-3				79.52

Supplementary Table S2. Differentially expressed genes associated with chemotaxis of the three strains when barley seedlings were present

Strain ID	Gene ID	Annotation	Fold change
AR	AGEIDLEC_00382	methyl-accepting chemotaxis protein	-2.33
	AGEIDLEC_00704		-2.33
	AGEIDLEC_01195		-1.93
	AGEIDLEC_01334		-1.53
	AGEIDLEC_01896		-1.84
	AGEIDLEC_03604		-3.00
	AGEIDLEC_03627		-1.90
	AGEIDLEC_03746		-1.73
	AGEIDLEC_01893	chemotaxis protein	-2.17
	AGEIDLEC_01201	flagellar motor switch protein	1.95
	AGEIDLEC_01208		5.01
	AGEIDLEC_01659	sugar ABC transporter permease	1.57
S02	CGFHABJE_01390	methyl-accepting chemotaxis protein	2.18
	CGFHABJE_04010		2.05
	CGFHABJE_03756		1.77
	CGFHABJE_01945		1.73
	CGFHABJE_02050	chemotaxis protein	1.64
	CGFHABJE_04625		2.09
	CGFHABJE_02039		1.60
	CGFHABJE_00103		1.50
	CGFHABJE_02026	flagellar motor switch protein	-2.03
	CGFHABJE_04651	sugar efflux transporter	1.94
S25	KKIAGPJH_03945	methyl-accepting chemotaxis protein	3.90
	KKIAGPJH_01846		2.96
	KKIAGPJH_01348		2.89
	KKIAGPJH_02413		2.41
	KKIAGPJH_03955		2.25
	KKIAGPJH_03695		2.13
	KKIAGPJH_03201		1.84
	KKIAGPJH_01957	chemotaxis protein	-2.57
	KKIAGPJH_04513		-1.77
	KKIAGPJH_01947		-1.85
	KKIAGPJH_00106		-2.01
	KKIAGPJH_01934	flagellar motor switch protein	1.57
	KKIAGPJH_04538	sugar efflux transporter	2.17

AR: Novel *E. gerundensis* strain
S02/S25: Novel *Paenibacillus* sp. strains

Supplementary Table S3. Differentially expressed genes associated with biofilm formation of the three strains when barley seedlings were present

Strain ID	Gene ID	Annotation	Fold change
AR	AGEIDLEC_00073	cellulose biosynthesis	1.98
	AGEIDLEC_03135	glycogen biosynthesis	1.62
	AGEIDLEC_03136		1.55
	AGEIDLEC_00235	exopolysaccharide biosynthesis protein	2.77
S02	CGFHABJE_02209	glycogen biosynthesis	-2.62
	CGFHABJE_02210		-2.90
	CGFHABJE_02211		-2.40
	CGFHABJE_01529	exopolysaccharide biosynthesis protein	-1.69
	CGFHABJE_02778		2.70
S25	KKIAGPJH_02201	glycogen biosynthesis	17.61
	KKIAGPJH_02202		11.66
	KKIAGPJH_02709		3.37
	KKIAGPJH_01490	exopolysaccharide biosynthesis protein	-2.31

AR: Novel *E. gerundensis* strain
S02/S25: Novel *Paenibacillus* sp. strains

Supplementary Table S4. Differentially expressed genes associated with biofilm formation of *Paenibacillus* sp. S02 compared to *Paenibacillus* sp. S25 when barley seedlings were absent

Strain ID	Gene ID	Annotation	Fold change
S02	CGFHABJE_02210	glycogen biosynthesis	39.39
	CGFHABJE_02211		24.74
	CGFHABJE_02209		33.13
	CGFHABJE_02873		5.97
	CGFHABJE_02871		7.05
	CGFHABJE_02778	exopolysaccharide biosynthesis protein	-3.54

S02: Novel *Paenibacillus* sp. strain

Supplementary Table S5. Differentially expressed genes associated with plant growth promotion of strain S02 and S25 when barley seedlings were present

Trait	Gene Name	S02		S25	
Nitrogen fixation		**Gene ID**	**Fold change**	**Gene ID**	**Fold change**
	nifB	CGFHABJE_01039	NDE	KKIAGPJH_00991	NDE
	nifH	CGFHABJE_01040	-2.84	KKIAGPJH_00992	NDE
	nifD	CGFHABJE_01041	-2.48	KKIAGPJH_00993	NDE
	nifK	CGFHABJE_01042	-3.37	KKIAGPJH_00994	-2.23
	nifE	CGFHABJE_01043	-3.38	KKIAGPJH_00995	-1.87
	nifN	CGFHABJE_01044	-4.83	KKIAGPJH_00996	-2.18
	nifX	CGFHABJE_01045	-4.79	KKIAGPJH_00997	NDE
	hesA/moeB	CGFHABJE_01046	-3.61	KKIAGPJH_00998	NDE
	nifV	CGFHABJE_01047	-2.45	KKIAGPJH_00999	NDE
Phosphate solubilization					
	gcd	CGFHABJE_02793	NDE	KKIAGPJH_02644	NDE
Phosphonate cluster (*phn*)					
	phnA	CGFHABJE_05309	2.27	KKIAGPJH_05177	NDE
	phnB	CGFHABJE_00880	NDE	KKIAGPJH_00854	2.75
	phnC	CGFHABJE_04607	NDE	KKIAGPJH_04494	-2.78
	phnD	CGFHABJE_04606	-1.54	KKIAGPJH_04493	-2.45
	phnE	CGFHABJE_04608	NDE	KKIAGPJH_04495	NDE
	phnW	CGFHABJE_05275	-3.31	KKIAGPJH_05152	NDE
	phnX	CGFHABJE_00485	NDE	KKIAGPJH_00496	NDE
	ppd	CGFHABJE_05276	-2.73	KKIAGPJH_05153	NDE
	pepM	CGFHABJE_05277	-2.00	KKIAGPJH_05154	NDE
Phosphate transporter (*pst*)					
	pstS	CGFHABJE_01689	NDE	KKIAGPJH_01595	-5.91
	pstA	CGFHABJE_01691	-1.79	KKIAGPJH_01597	-2.30
	pstB	CGFHABJE_01692	-1.52	KKIAGPJH_01598	-3.51
	pstC	CGFHABJE_01690	NDE	KKIAGPJH_01596	-2.19
	phoP	CGFHABJE_01703	1.73	KKIAGPJH_01609	NDE
	phoR	CGFHABJE_01702	1.54	KKIAGPJH_01608	2.70
Indole-3-acetic acid production					
	ipdC	CGFHABJE_01475	NDE	KKIAGPJH_01435	NDE
	auxin efflux carriers	CGFHABJE_02845 CGFHABJE_03330 CGFHABJE_05253	NDE NDE 2.34	KKIAGPJH_02683 KKIAGPJH_03351 KKIAGPJH_05129	NDE -2.65 986.13

S02/S25: Novel *Paenibacillus* sp. strains
NDE: Not differentially expressed

Supplementary Table S6. Differentially expressed genes associated with biological nitrogen fixation of strain S02 when barley seedlings were present (in Burk's N-free medium)

Strain ID	Gene ID	Annotation	Fold change
S02	CGFHABJE_01039	*nifB*	7.34
	CGFHABJE_01040	*nifH*	10.50
	CGFHABJE_01041	*nifD*	11.12
	CGFHABJE_01042	*nifK*	8.54
	CGFHABJE_01043	*nifE*	10.86
	CGFHABJE_01044	*nifN*	6.07
	CGFHABJE_01045	*nifX*	NDE
	CGFHABJE_01046	*hesA/moeB*	NDE
	CGFHABJE_01047	*nifV*	4.13
	CGFHABJE_03691	molybdate-binding protein	4.27
	CGFHABJE_03812		7.22

S02: Novel *Paenibacillus* sp. strain
NDE: Not differentially expressed

Supplementary Table S7. Differentially expressed genes associated with plant growth promotion of strain AR when barley seedlings were present

Strain ID	Gene ID	Annotation	Fold change
AR	AGEIDLEC_03437	phosphate transporter (*pstS*)	NDE
	AGEIDLEC_03438	phosphate transporter (*pstC*)	NDE
	AGEIDLEC_03439	phosphate transporter (*pstA*)	-1.61
	AGEIDLEC_03440	phosphate transporter (*pstB*)	-1.73
	AGEIDLEC_03441	phosphate transport system regulator (*phoU*)	NDE
	AGEIDLEC_02162	auxin efflux carriers	-1.54

AR: Novel *E. gerundensis* strain
NDE: Not differentially expressed

Supplementary Table S8. Differentially expressed core biosynthetic genes of secondary metabolite gene clusters of strain S02 and S25 when barley seedlings were present

ID	Type	Most similar known cluster (similarity)	S02		S25	
			Gene ID	Fold change	Gene ID	Fold change
C1	Nrps	fusaricidin B (100%)	CGFHABJE_00078	NDE	KKIAGPJH_00078	NDE
			CGFHABJE_00083	-2.50	KKIAGPJH_00083	-3.44
C2	siderophore	N/A	CGFHABJE_00955	NDE	KKIAGPJH_00927	3.71
			CGFHABJE_00956	NDE	KKIAGPJH_00928	4.23
			CGFHABJE_00959	1.50	KKIAGPJH_00931	NDE
C3	bacteriocin	N/A	CGFHABJE_01103	1.51	KKIAGPJH_01049	4.07
C4	Nrps transAT-PKS	N/A	CGFHABJE_01166	-1.75	KKIAGPJH_01130	-1.63
			CGFHABJE_01170	-1.82	KKIAGPJH_01134	2.35
			CGFHABJE_01172	-2.03	KKIAGPJH_01136	NDE
			CGFHABJE_01173	-1.58	KKIAGPJH_01137	NDE
			CGFHABJE_01175	NDE	KKIAGPJH_01139	NDE
			CGFHABJE_01176	NDE	KKIAGPJH_01140	NDE
			CGFHABJE_01178	-2.72	KKIAGPJH_01142	NDE
			CGFHABJE_01179	-2.04	KKIAGPJH_01143	NDE
			CGFHABJE_01180	-2.52	KKIAGPJH_01144	-2.32
			CGFHABJE_01181	-2.17	KKIAGPJH_01145	-2.27
C5	lassopeptide	paeninodin (40%)	CGFHABJE_01236	-1.58	KKIAGPJH_01200	NDE
			CGFHABJE_01240	-1.69	KKIAGPJH_01204	NDE
C6	Nrps	marthiapeptide A (33%)	CGFHABJE_01339	NDE	KKIAGPJH_01293	6.70
			CGFHABJE_01340	NDE	KKIAGPJH_01294	3.00
			CGFHABJE_01341	-2.23	KKIAGPJH_01295	2.61
C7	lanthipeptide	paenilan (100%)	CGFHABJE_01558	NDE	KKIAGPJH_01518	NDE
			CGFHABJE_01560	NDE	KKIAGPJH_01520	2.73
			CGFHABJE_01562	NDE	KKIAGPJH_01522	NDE
C8	lanthipeptide	paenicidin B (71%)			KKIAGPJH_01661	-2.25
					KKIAGPJH_01663	-1.67
C9	Nrps-like	N/A	CGFHABJE_01944	1.60	KKIAGPJH_01854	-1.52
C10	Nrps	tridecaptin (100%)	CGFHABJE_02333	1.55	KKIAGPJH_02322	-2.89
			CGFHABJE_02334	NDE	KKIAGPJH_02323	NDE
C11	Nrps transAT-PKS	paenilipoheptin (S02, 73%; S25, 76%)	CGFHABJE_02506	2.53	KKIAGPJH_02476	12.81
			CGFHABJE_02507	NDE	KKIAGPJH_02477	13.60
			CGFHABJE_02508	1.90	KKIAGPJH_02478	2.41
			CGFHABJE_02509	2.09	KKIAGPJH_02479	1.59
			CGFHABJE_02510	1.74	KKIAGPJH_02480	NDE
C12	Nrps	N/A			KKIAGPJH_02516	-2.69
C13	Nrps betalactone	N/A			KKIAGPJH_02623	NDE
					KKIAGPJH_02624	1.87
					KKIAGPJH_02633	-1.92
C14	Nrps T3PKS transAT-PKS	aurantinin B/C/D (35%)	CGFHABJE_03362	NDE	KKIAGPJH_03372	NDE
			CGFHABJE_03363	NDE	KKIAGPJH_03373	NDE
			CGFHABJE_03365	NDE	KKIAGPJH_03375	NDE
			CGFHABJE_03366	-2.23	KKIAGPJH_03376	NDE
			CGFHABJE_03367	NDE	KKIAGPJH_03377	-2.62
			CGFHABJE_03368	NDE	KKIAGPJH_03378	-1.90
			CGFHABJE_03371	1.65	KKIAGPJH_03381	NDE
			CGFHABJE_03372	NDE	KKIAGPJH_03382	-2.77
C15	Nrps	polymyxin (100%)	CGFHABJE_04684	1.57	KKIAGPJH_04566	-2.34
					KKIAGPJH_04567	-1.63
			CGFHABJE_04687	1.74	KKIAGPJH_04570	NDE
			CGFHABJE_04688	1.53	KKIAGPJH_04571	NDE
C16	phosphonate	N/A	CGFHABJE_05277	-2.00	KKIAGPJH_05154	NDE

S02/S25: Novel *Paenibacillus* sp. strains

NDE: not differentially expressed; Clusters in blue: known antimicrobial compounds

Supplementary Table S9. Differentially expressed core biosynthetic genes of secondary metabolite gene clusters of strain S02 when barley seedlings were present (in Burk's N-free medium)

ID	Type	Most similar known cluster (similarity)	S02	
			Gene ID	Fold change
C1	Nrps	fusaricidin B (100%)	CGFHABJE_00078	4.94
			CGFHABJE_00083	8.31
C2	siderophore	N/A	CGFHABJE_00955	NDE
			CGFHABJE_00956	NDE
			CGFHABJE_00959	-2.42
C3	bacteriocin	N/A	CGFHABJE_01103	NDE
C4	Nrps transAT-PKS	N/A	CGFHABJE_01166	53.46
			CGFHABJE_01170	236.34
			CGFHABJE_01172	269.16
			CGFHABJE_01173	247.75
			CGFHABJE_01175	244.58
			CGFHABJE_01176	174.31
			CGFHABJE_01178	134.82
			CGFHABJE_01179	79.06
			CGFHABJE_01180	75.55
			CGFHABJE_01181	51.95
C5	lassopeptide	paeninodin (40%)	CGFHABJE_01236	2.96
			CGFHABJE_01240	NDE
C6	Nrps	marthiapeptide A (33%)	CGFHABJE_01339	84.91
			CGFHABJE_01340	60.46
			CGFHABJE_01341	48.96
C7	lanthipeptide	paenilan (100%)	CGFHABJE_01558	7.22
			CGFHABJE_01560	12.29
			CGFHABJE_01562	11.36
C9	Nrps-like	N/A	CGFHABJE_01944	NDE
C10	Nrps	tridecaptin (100%)	CGFHABJE_02333	4.20
			CGFHABJE_02334	5.36
C11	Nrps transAT-PKS	paenilipoheptin (S02, 73%; S25, 76%)	CGFHABJE_02506	NDE
			CGFHABJE_02507	3.21
			CGFHABJE_02508	4.95
			CGFHABJE_02509	7.65
			CGFHABJE_02510	5.29
C14	Nrps T3PKS transAT-PKS	aurantinin B/C/D (35%)	CGFHABJE_03362	4.72
			CGFHABJE_03363	17.63
			CGFHABJE_03365	10.78
			CGFHABJE_03366	8.93
			CGFHABJE_03367	8.27
			CGFHABJE_03368	9.10
			CGFHABJE_03371	4.62
			CGFHABJE_03372	6.49
C15	Nrps	polymyxin (100%)	CGFHABJE_04684	3.80
			CGFHABJE_04687	2.89
			CGFHABJE_04688	2.12
C16	phosphonate	N/A	CGFHABJE_05277	NDE

S02: Novel *Paenibacillus* sp. strain
NDE: Not differentially expressed
Clusters in blue: Known antimicrobial compounds

Supplementary Table S10. Differentially expressed core biosynthetic genes of secondary metabolite gene clusters of strain AR when barley seedlings were present

ID	Type	Most similar known cluster (similarity)	AR	
			Gene ID	Fold change
C1	linear azol(in)e-containing peptides	N/A	AGEIDLEC_01604	NDE
			AGEIDLEC_01622	NDE
C2	arylpolyene hserlactone	aryl polyenes (88%)	AGEIDLEC_01995	NDE
			AGEIDLEC_01998	NDE
			AGEIDLEC_02030	NDE
C3	thiopeptide	N/A	AGEIDLEC_02102	NDE
			AGEIDLEC_02105	-1.85
C4	Nrps	N/A	AGEIDLEC_02933	NDE
			AGEIDLEC_02940	-1.54
C5	terpene	carotenoid (100%)	AGEIDLEC_03208	1.55
			AGEIDLEC_03210	2.37
C6	hserlactone	N/A	AGEIDLEC_03481	NDE
C7	siderophore	N/A	AGEIDLEC_03599	NDE
			AGEIDLEC_03601	NDE

AR: Novel *E. gerundensis* strain
NDE: Not differentially expressed

Supplementary Table S11. Differentially expressed transcripts encoding disease resistance proteins and heat shock proteins in barley seedlings when the three bacterial strains were present

Strain ID	Annotation	No. of upregulated transcripts	No. of downregulated transcripts
AR	Disease resistance proteins	84	96
	Heat shock proteins	31	12
S02	Disease resistance proteins	39	41
	Heat shock proteins	12	9
S25	Disease resistance proteins	64	103
	Heat shock proteins	24	17
S02 (in Burk's N-free medium)	Disease resistance proteins	58	35
	Heat shock proteins	32	6

AR: Novel *E. gerundensis* strain
S02/S25: Novel *Paenibacillus* sp. strains

Supplementary Table S12. Differentially expressed transcripts associated with defence and stress responses in barley seedlings when the three bacterial strains were present

Strain ID	Transcript ID	Annotation	Fold change
AR	BART1_0-p09460.001	leucine-rich receptor kinase	1.75
	BART1_0-p51301.001	polygalacturonase inhibitor	6.36
	BART1_0-p07261.001	xylanase inhibitor	145.89
	BART1_0-p50355.001	caffeoyl CoA O-methyltransferase	8.76
	BART1_0-p52030.004		-12.71
	BART1_0-p52031.001		3.49
	BART1_0-p12652.001	glutamate decarboxylase	48.58
	BART1_0-p12655.001		2.80
	BART1_0-p17187.003		2.43
	BART1_0-p17187.004		1.81
	BART1_0-p30229.001		6.77
	BART1_0-p08022.001	ubiquitin-activating enzyme E1	1.89
	BART1_0-p35458.001		1.65
	BART1_0-p35458.005		2.24
	BART1_0-p35458.009		1.61
	BART1_0-p14610.004	ascorbate peroxidase	-40.11
	BART1_0-p14611.002		43.98
	BART1_0-p14611.004		38.10
S02	BART1_0-p59228.001	leucine-rich receptor kinase	-2.87
	BART1_0-p09955.001	polygalacturonase inhibitor	-13.96
	BART1_0-p50356.001	caffeoyl CoA O-methyltransferase	-33.51
	BART1_0-p30229.001	glutamate decarboxylase	8.31
	BART1_0-p35458.005	ubiquitin-activating enzyme E1	1.55
	BART1_0-p35458.008		-163.22
	BART1_0-p35458.009		1.61
S25	BART1_0-p09955.001	polygalacturonase inhibitor	-6.43
	BART1_0-p15038.001		-3.57
	BART1_0-p37179.001	caffeoyl CoA O-methyltransferase	-2.80
	BART1_0-p52031.001		2.75
	BART1_0-p12655.001	glutamate decarboxylase	-2.31
	BART1_0-p17187.003		2.72
	BART1_0-p17187.005		2.32
	BART1_0-p17187.011		-625.82
	BART1_0-p26684.001		42.12
	BART1_0-p30229.001		-1.85
	BART1_0-p08024.002	ubiquitin-activating enzyme E1	-2.45
	BART1_0-p35458.001		1.75
S02 (in Burk's N-free medium)	BART1_0-p09955.001	polygalacturonase inhibitor	-13.96
	BART1_0-p09460.001	leucine-rich receptor kinase	-1.90
	BART1_0-p59228.001		-6.26
	BART1_0-p37179.001	caffeoyl CoA O-methyltransferase	-2.82
	BART1_0-p52030.001		-3.26
	BART1_0-p52030.003		-4.90
	BART1_0-p52031.001		-4.06
	BART1_0-p12654.004	glutamate decarboxylase	37.48
	BART1_0-p12654.006		93.49
	BART1_0-p12655.001		2.93
	BART1_0-p17187.004		-2.14
	BART1_0-p26684.001		-3.17
	BART1_0-p30229.001		4.56
	BART1_0-p08022.001	ubiquitin-activating enzyme E1	-1.74
	BART1_0-p27782.001		-1.62
	BART1_0-p29625.001		-1.70

AR: Novel *E. gerundensis* strain; S02/S25: Novel *Paenibacillus* sp. strains

Supplementary Table S13. Differentially expressed transcripts encoding endoglucanase in barley seedlings when the three bacterial strains were present

Strain ID	Transcript ID	Fold change
AR	BART1_0-p02416.002	2.74
	BART1_0-p13013.001	1.89
	BART1_0-p13221.001	1.56
	BART1_0-p13221.002	7.07
	BART1_0-p13221.003	2.03
	BART1_0-p13224.001	8.98
	BART1_0-p16546.001	-51.59
	BART1_0-p26867.004	-169.13
	BART1_0-p35786.001	-2.70
	BART1_0-p35791.001	138171.33
	BART1_0-p36297.001	134.32
	BART1_0-p36298.001	180.65
	BART1_0-p36300.001	110.53
	BART1_0-p37935.001	2.97
	BART1_0-p37939.001	25.44
	BART1_0-p38044.002	-137.95
	BART1_0-p38044.019	-174.56
	BART1_0-p42923.003	-3.93
	BART1_0-p42924.003	755.12
	BART1_0-p42924.004	1275.61
	BART1_0-p46758.001	1.88
	BART1_0-p50604.001	-198.34
	BART1_0-p50609.001	16.40
	BART1_0-p51464.001	1.50
	BART1_0-p51464.002	2.21
	BART1_0-p59306.001	35.34
S25	BART1_0-p02416.002	2.43
	BART1_0-p13013.001	1.65
	BART1_0-p13059.001	-2.21
	BART1_0-p13221.002	4.85
	BART1_0-p13224.001	15.77
	BART1_0-p29302.001	1.62
	BART1_0-p37930.001	-109.68
	BART1_0-p38044.001	-1.50
	BART1_0-p43284.001	272.20
	BART1_0-p46514.001	-7.18
	BART1_0-p50604.001	-196.79
	BART1_0-p50607.001	-150.94
	BART1_0-p51464.002	3.10
	BART1_0-p53733.001	-6.40
	BART1_0-p55128.003	-1.60
	BART1_0-p59304.001	-62.31
S02	BART1_0-p05997.001	-3.67
	BART1_0-p19258.001	-2.11
	BART1_0-p38044.001	-1.55
	BART1_0-p50607.001	-12.19
	BART1_0-p53733.001	-2.32
	BART1_0-p55128.003	-1.55
S02 (in Burk's N-free medium)	BART1_0-p02416.002	2.02
	BART1_0-p05997.001	-13.56
	BART1_0-p13059.001	-5.57
	BART1_0-p13221.001	-2.83
	BART1_0-p13221.003	-1.89
	BART1_0-p16188.001	-13.24
	BART1_0-p19258.001	-8.17
	BART1_0-p35786.001	-15.32
	BART1_0-p37930.001	-30.63
	BART1_0-p38044.001	-1.70
	BART1_0-p43284.001	260.24
	BART1_0-p46514.001	-3.19
	BART1_0-p46758.001	-1.60
	BART1_0-p51464.001	-2.16
	BART1_0-p51466.001	-2.49
	BART1_0-p53733.001	-6.94
	BART1_0-p55128.001	2.45

AR: Novel *E. gerundensis* strain
S02/S25: Novel *Paenibacillus* sp. strains

Supplementary Table S14. Differentially expressed transcripts associated with signal transduction and ethylene biosynthesis in barley seedlings when the three bacterial strains were present

Strain ID	Transcript ID	Annotation	Fold change
AR	BART1_0-p22808.001	ADP-ribosylation factor	2.03
	BART1_0-p32313.002		10670.18
	BART1_0-p32313.003		4579.19
	BART1_0-p32313.004		244.17
	BART1_0-p40410.001		1.58
	BART1_0-p59482.001		1.98
	BART1_0-p59482.003		1.89
	BART1_0-p27744.001	GTP-binding proteins	-57.23
	BART1_0-p35339.001		310.46
	BART1_0-p36228.006		-2.52
	BART1_0-p38804.001		1.60
	BART1_0-p38805.001		78.48
	BART1_0-p38805.002		138.92
	BART1_0-p27326.001	aminocyclopropanecarboxylate oxidase	-72.03
	BART1_0-p27327.002		-2.83
S02	BART1_0-p22808.001	ADP-ribosylation factor	1.56
	BART1_0-p22808.009		1.72
	BART1_0-p27743.001	GTP-binding proteins	3.88
	BART1_0-p36228.006		-1.84
	BART1_0-p27326.001	aminocyclopropanecarboxylate oxidase	-3.70
	BART1_0-p27327.001		2.64
S25	BART1_0-p22808.001	ADP-ribosylation factor	1.75
	BART1_0-p42864.001		1.61
	BART1_0-p59482.001		1.63
	BART1_0-p59482.003		1.66
	BART1_0-p59482.006		2.86
	BART1_0-p27743.001	GTP-binding proteins	2.53
	BART1_0-p38805.001		19.51
	BART1_0-p27326.001	aminocyclopropanecarboxylate oxidase	-314.69
	BART1_0-p27327.002		-15.25
S02 (in Burk's N-free medium)	BART1_0-p40410.004	ADP-ribosylation factor	-30.77
	BART1_0-p59482.002		26.67
	BART1_0-p59482.003		1.61
	BART1_0-p36228.001	GTP-binding proteins	-1.79
	BART1_0-p27326.001	aminocyclopropanecarboxylate oxidase	-9.00

AR: Novel *E. gerundensis* strain
S02/S25: Novel *Paenibacillus* sp. strains

Supplementary Table S15. Differentially expressed transcripts encoding high affinity transporters in barley seedlings when the three bacterial strains were present

Strain ID		Transcript ID	Fold change
AR	nitrate	BART1_0-p42069.001	-92.78
		BART1_0-p42070.001	-324.24
		BART1_0-p42072.001	9.36
		BART1_0-p42073.001	-41.16
		BART1_0-p42077.001	128.40
		BART1_0-p42082.001	41.67
		BART1_0-p55377.001	-80.43
		BART1_0-p40717.001	-10.69
		BART1_0-p45131.001	-5.72
	iron	BART1_0-p53866.001	1.83
	potassium	BART1_0-p14481.001	8.05
		BART1_0-p14481.002	8.40
		BART1_0-p14476.001	2.46
		BART1_0-p59178.002	37.21
	sulphate	BART1_0-p30667.002	-93.09
		BART1_0-p30667.004	-12.07
		BART1_0-p30667.005	-18.58
		BART1_0-p30667.006	-493.77
		BART1_0-p30667.007	-37.07
S25	nitrate	BART1_0-p42069.001	-126.57
		BART1_0-p42070.001	-478.53
		BART1_0-p42072.001	-17.42
		BART1_0-p42073.001	-123.42
		BART1_0-p42079.001	-321.70
		BART1_0-p42084.001	-2.22
		BART1_0-p42092.001	-5.27
		BART1_0-p42093.001	-6.37
		BART1_0-p55377.001	-201.97
		BART1_0-p40717.001	-11.20
		BART1_0-p45131.001	-4.93
	potassium	BART1_0-p14481.001	19.52
		BART1_0-p14481.002	19.05
		BART1_0-p14476.001	4.60
		BART1_0-p59179.001	4.76
	sulphate	BART1_0-p30667.002	-9.51
		BART1_0-p30667.005	-5.13
	inorganic phosphate	BART1_0-p31346.001	-514.99
		BART1_0-p31572.001	-33.95
S02	nitrate	BART1_0-p40717.001	-1.69
		BART1_0-p42069.001	-150.75
		BART1_0-p42070.001	-71.16
		BART1_0-p42073.001	-9.55
		BART1_0-p42079.001	2.65
		BART1_0-p42084.001	-7.89
		BART1_0-p42092.001	-31.39
		BART1_0-p42093.001	-11.37
		BART1_0-p45131.001	-2.35
		BART1_0-p40717.001	-1.69
	iron	BART1_0-p53866.001	1.80
	potassium	BART1_0-p59179.001	-1.72
	sulphate	BART1_0-p30667.002	-8.88
		BART1_0-p30667.004	-3.70
		BART1_0-p30667.005	-4.61
		BART1_0-p30670.001	3.29
	inorganic phosphate	BART1_0-p31346.001	-15.73
		BART1_0-p31572.001	-18.34
S02 (in Burk's N-free medium)	nitrate	BART1_0-p42069.001	-193.11
		BART1_0-p42070.001	-97.43
		BART1_0-p42078.001	-2.13
		BART1_0-p42079.001	-909.93
		BART1_0-p42084.001	-650.54
		BART1_0-p42092.001	-983.39
		BART1_0-p42093.001	-143.76
		BART1_0-p45131.001	-2.53
		BART1_0-p45134.001	-3.99
	potassium	BART1_0-p14476.001	-2.48
		BART1_0-p59179.001	4.17
	sulphate	BART1_0-p30667.002	-30.78
		BART1_0-p30667.004	-3.03
		BART1_0-p30668.001	3.26
		BART1_0-p30670.001	-12.50

AR: Novel *E. gerundensis* strain
S02/S25: Novel *Paenibacillus* sp. strains

Supplementary Table S16. Differentially expressed transcripts associated with nutrient uptake and metabolism in barley seedlings when the three bacterial strains were present

Strain ID	Transcript ID	Annotation	Fold change
AR	BART1_0-p04828.001	ammonium transporter	-457.99
	BART1_0-p04829.001		160.67
	BART1_0-p40957.001		-7.40
	BART1_0-p45407.001		-3.08
	BART1_0-p58582.001		-4.47
	BART1_0-p01562.001	anthocyanidin-O-glucosyltransferase	91.46
	BART1_0-p01563.001		2.11
	BART1_0-p40352.001		-2.30
	BART1_0-p50969.002		-6.28
	BART1_0-p38221.001		1.82
	BART1_0-p38224.001		174.97
	BART1_0-p22047.004		1063.42
	BART1_0-p26633.001	glutamine synthetase	4.49
	BART1_0-p26633.002		2.51
	BART1_0-p26633.003		6.65
	BART1_0-p26633.004		3.03
	BART1_0-p46549.002		1.89
	BART1_0-p46549.007		1.65
	BART1_0-p22331.001	aspartate aminotransferase	4.74
	BART1_0-p22331.002		2.16
	BART1_0-p22331.008		5.06
	BART1_0-p22331.011		23.73
	BART1_0-p22331.013		67.08
	BART1_0-p48209.001	sucrose synthase	2.80
	BART1_0-p48951.001		4.61
	BART1_0-p50918.003		2.69
	BART1_0-p50918.004		2.33
	BART1_0-p50919.002		2.20
	BART1_0-p04906.023	UDP-glucose pyrophosphorylase	753.55
	BART1_0-p04906.031		2.71
S02	BART1_0-p04827.001	ammonium transporter	2.30
	BART1_0-p40957.001		-3.21
	BART1_0-p58582.001		-2.59
	BART1_0-p22331.001	aspartate aminotransferase	2.69
	BART1_0-p22331.002		1.73
	BART1_0-p54667.001		1.92
	BART1_0-p58839.006		3.27
	BART1_0-p58839.015		43.44
	BART1_0-p09325.001	sucrose synthase	-3.05
	BART1_0-p50918.003		1.77
	BART1_0-p50918.013		2.35
	BART1_0-p50919.003		-2.15
	BART1_0-p50919.018		1.72

S25	BART1_0-p04828.001	ammonium transporter	-5.66
	BART1_0-p40957.001		-8.34
	BART1_0-p45407.001		-4.09
	BART1_0-p58582.001		-3.34
	BART1_0-p26633.001	glutamine synthetase	5.72
	BART1_0-p26633.002		6.38
	BART1_0-p26633.003		15.22
	BART1_0-p26633.004		6.16
	BART1_0-p46549.001		1.92
	BART1_0-p46549.002		3.87
	BART1_0-p46549.007		2.84
	BART1_0-p46549.008		2.77
	BART1_0-p46549.010		3.18
	BART1_0-p22331.001	aspartate aminotransferase	3.56
	BART1_0-p22331.002		1.57
	BART1_0-p22331.007		4.84
	BART1_0-p22331.008		6.08
	BART1_0-p22331.011		12.17
	BART1_0-p58839.001		-2.42
	BART1_0-p58839.015		57.59
	BART1_0-p48209.001	sucrose synthase	1.88
	BART1_0-p48951.001		4.45
	BART1_0-p48951.009		4.64
	BART1_0-p50918.013		3.53
	BART1_0-p50918.016		30.21
	BART1_0-p50919.002		2.70
S02 (in Burk's N-free medium)	BART1_0-p23459.001	ammonium transporter	-3.47
	BART1_0-p40957.001		-9.80
	BART1_0-p45407.001		-16.10
	BART1_0-p46549.007	glutamine synthetase	2.37
	BART1_0-p22331.001	aspartate aminotransferase	3.14
	BART1_0-p22331.002		1.91
	BART1_0-p22331.011		3.57
	BART1_0-p54667.001		-2.16
	BART1_0-p55159.001		7.04
	BART1_0-p50918.004	sucrose synthase	-2.12
	BART1_0-p50918.013		1.90
	BART1_0-p50919.017		30.36

AR: Novel *E. gerundensis* strain
S02/S25: Novel *Paenibacillus* sp. strains