AN INVESTIGATION INTO THE INTERDOMAIN REGION OF *Caenorhabditis elegans* FATTY ACID SYNTHASE AND ITS IMPLICATIONS AS A DRUG TARGET

Submitted by

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Abstract

Anthelmintics are rapidly losing effectiveness in treating gastrointestinal nematodes (GINs) in small ruminants worldwide, and novel treatments are critically needed. Concurrently, fatty acid synthase (FASN) has emerged as a promising target for human cancer and obesity treatments. To this end, the interdomain (ID) segment of *Caenorhabditis elegans* FASN was investigated with a future view to creating a small peptide to bind to the ID and inhibit the formation of the functional FASN dimer for the treatment of veterinary gastrointestinal nematode infestations. Comparative bioinformatic analysis showed that the *fasn* gene is poorly conserved between *C. elegans* and higher animals, and well conserved amongst members of the *Caenorhabditis* genus. This also showed that the ID is far less evolutionarily constrained than the catalytic domains of FASN in different animals. The ID was amplified using genomic DNA (gDNA) from whole *C. elegans* lysate using different primers to allow ligation into different vectors for mammalian and *C. elegans* expression as tagged fusion proteins. A subcloning strategy was used: first the ID inserts were ligated into generic cloning vectors, and after bacterial transformation they were excised and ligated into expression vectors. Mammalian expression vectors were made and *C. elegans* expression vectors were attempted. FLAG- and GluGlu-tagged ID were found to be expressed in human embryonic kidney (HEK-

293T) cells. This work has demonstrated that the ID region is capable of being expressed on its own, rather than as part of the full enzyme, in cell culture. This is the first time that a tagged domain of *C. elegans* FASN has been expressed in a mammalian cell line, with important implications for future drug design.

Statement of Authorship

Except where reference is made in the text of the thesis, this thesis contains no 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 acknowledgement 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.

(signed)

Christopher Sgro Date:

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Abbreviations

ACP	Fatty acid synthase acyl carrier protein
AT/MT	Fatty acid synthase acyl/malonyl transacyclase
cDNA	Complementary DNA
CoIP	Coimmunoprecipitation
DH	Fatty acid synthase β-hydroxyacyl dehydratase
DMEM	Dulbecco's Modified Eagle Medium
E_2/ER	Oestradiol/oestrogen receptor signalling combination
ER	Fatty acid synthase enoyl reductase
FA	Fatty acid
fasn	Fatty acid synthase gene
FASN	Fatty acid synthase protein
fasn-1	Fatty acid synthase gene of *C. elegans*
FASN1	Fatty acid synthase protein of *C. elegans*

FCS	Fetal calf serum
GABA	γ-aminobutyric acid
gDNA	Genomic DNA extracted from cell lysate
GIN	Gastrointestinal nematode
HEK-293T	Human embryonic kidney cell line
HRP	Horseradish peroxidase
ID	The interdomain of fatty acid synthase
KR	Fatty acid synthase β-hydroxyacyl reductase
KS	Fatty acid synthase β-ketoacyl synthase
MDR	Multiple drug resistance
ML	Macrocyclic lactone anthelmintics
PBS	Phosphate-buffered saline
PVDF	Polyvinylidene fluoride
RNAi	RNA interference
SFM	Serum-free medium
TE	Fatty acid synthase thioesterase

Chapter 1. Introduction

1.1 Fatty acid synthase

1.1.1 Type I & II fatty acid synthase

In all living cells, fatty acid synthase (FASN) is the sole enzyme responsible for the *de novo* synthesis of long-chain fatty acids, which are vital biomolecules (Brignole *et al.* 2009). In animal and yeast cells, two types of FASN exist. Type I FASN catalyses fatty acid formation in the cytosol, and type II FASN does the same in mitochondria (Hiltunen *et al.* 2010). Animal type I FASN is one of the earliest multifunctional proteins to evolve in which one polypeptide houses all of the enzyme activities necessary to make a product (Chirala & Wakil 2004). As the focus of this investigation is to determine the feasibility of animal type I FASN interdomain (ID) as a drug target, type II FASN is mentioned only briefly.

Smith *et al.* (2003) state that in the early 1970s, it was found that yeast and animal FASN were composed of large, yet different multifunctional proteins, unlike the discrete, monofunctional enzymes of *E. coli* FASN discovered in the late 1960s. At this point, the name “type I” was given to animal and yeast FASN, to distinguish their large, multifunctional FASN proteins from the free-standing, individual peptides of

"type II" FASN in bacteria, plants, mitochondria and chloroplasts (Chirala *et al.* 2001, Asturias *et al.* 2005, Brignole *et al.* 2009).

Inactivation of any member of the mitochondrial FASN pathway in *Saccharomyces cerevisiae* leads to a phenotype of respiratory deficiency, indicating that type II FASN is essential for mitochondrial function (Hiltunen *et al.* 2010).

Furthermore, most type II FASN enzymes have homologs in their prokaryotic counterparts. These homologs have even been shown to rescue the respiratory deficient phenotype when targeted to the mitochondria of yeast null mutants (Hiltunen *et al.* 2010).

In contrast to the consequences of type II FASN inactivation, Chirala *et al.* (2003) found that type I FASN is critical for embryonic development. In their experiments, *Mus musculus fasn-1*$^{+/-}$ heterozygotes were interbred and shown to be haploid insufficient. It was found that 100% of the *fasn-1*$^{-/-}$ mutants and around 70% of the *fasn-1*$^{+/-}$ mutants died *in utero*. This occurred even when the heterozygotes were fed a diet high in saturated fatty acids, showing that dietary fats alone cannot supply the fatty acids needed for embryonic development. Chirala & Wakil (2004) later relate another group's experimental inhibition of *Mus musculus* type I FASN with c75. While the mice lost weight and ate less, the FAs in their diet compensated for their lack of FASN activity. More recently, Greer *et al.* (2008) found that exposing *C. elegans* to *fasn-1* RNAi resulted in growth arrest in the larval stage. All these data suggest that animal type I FASN inactivation will lead to the weakening of adult organisms and the death of those in the embryonic stages.

1.1.2 Structure of type I FASN

Type I FASN encompasses both fungal and animal FASN systems, which are organised differently despite both comprising multifunctional proteins (Smith 2006). Both are described here, after which only animal type I FASN is mentioned.

Animal type I FASN is a 540kDa homodimeric enzyme of two 270kDa polypeptides that house all seven partial activities necessary for the biosynthesis of the saturated fatty acid palmitate from malonyl-CoA at two reaction centres (Smith *et al.* 2003, Brignole *et al.* 2009, Maier *et al.* 2006). Approximately one quarter of each monomer is taken up by the interdomain (ID); a non-catalytic region strongly implicated in dimer formation (Chirala & Wakil 2004). The ID is between 600 (Asturias *et al.* 2005) and 650 (Maier *et al.* 2006) amino acids in length, the exact number depends on the species. Much of the work carried out has been on human FASN, and Jayakumar *et al.* (1997) gives the mass of the human native dimer as 544kDa, and the monomer as 272kDa.

The enzymatic activities of animal type I FASN have the following order: β-ketoacyl synthase (KS), acyl/malonyl transacyclase (AT/MT), β-hydroxyacyl dehydratase (DH), enoyl reductase (ER), β-hydroxyacyl reductase (KR), acyl carrier protein (ACP) and thioesterase (TE) (Chirala & Wakil, 2004, Chirala et al. 2001). This was established in the late 1970s (Smith *et al.* 2003). The three domains of Animal type I FASN were determined through proteolysis; Domain I contains KS, AT/MT and DH, Domain II has ER, KR and ACP, leaving Domain III with TE (Chirala & Wakil, 2004, Chirala et al.

2001). A non-catalytic region of approximately 600 residues separates domains I and II in animal FASN: this region is called the core (Asturias *et al.* 2005, Smith *et al.* 2003, Lupu & Menendez 2006) or interdomain (ID) (Chirala et al. 2001). The ID length is larger in mammals, at around 650 residues, although its location among the catalytic domains within FASN remains the same (Ming *et al.* 2002, Maier *et al.* 2006). Fig. 1.1 shows the domain map.

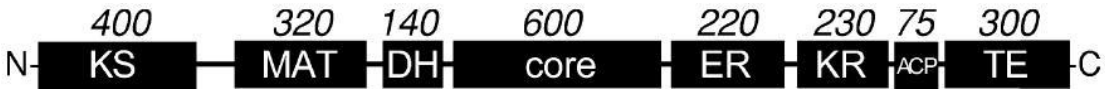

Figure 1.1 Domain map of animal type I FASN. Numbers indicate average numbers of residues in each catalytic domain. MT/AT is labelled as 'MAT', the interdomain region is labelled as 'core'. Adapted from Smith *et al.* (2003).

In every animal, the entire FASN protein is coded for by one gene, and while the sequence differs between animals, all animals' FASN genes encode the same seven activities and the ID in the same order (Chirala et al. 2001).

In the type I FASN system, all the functions are performed by the same multifunctional protein unit (Maier *et al.* 2006). However fungi and animals have evolved two different type I FASN complexes. Fungal type I FASN is a 2.6MDa $\alpha_6\beta_6$ dodecamer of two different proteins, while animal type I FASN is a 540kDa homodimer (Maier *et al.* 2006, Smith 2006). These relationships are summarised in Fig. 1.2.

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Figure 1.2 Organisation of fungal and porcine FASN. From 5 Å resolution structures. This shows the catalytic centres (reaction chambers) and the protein activities involved. Fungal FASN is arranged $\alpha_6\beta_6$ while animal is α_2. Adapted from Smith (2006).

The side-by-side antiparallel arrangement of the animal type I FASN dimer allows two sites of fatty acid synthesis per dimer (Jayakumar *et al.* 1997). This ID-mediated dimerisation allows the FASN dimer's two active sites to work simultaneously and independently of each other (Chirala & Wakil 2004), and have been speculated to function asynchronously (Smith 2006).

If animal type I FASN dimerisation is inhibited, only the KS partial activity is lost, which prevents FASN from making palmitate (Ming *et al.* 2002). Even though the remaining six enzymatic functions of FASN work equally as well as a monomer, all seven activities are required for the catalytic cycle of fatty acid elongation (Maier *et al.* 2006).

1.1.3 Function of type I FASN

As shown in Fig. 1.3, all seven enzymatic activities are needed to synthesise FAs in cells, and different kingdoms of life fill this need in different ways. Animals and fungi have assembled their enzymes into giant multifunctional complexes (type I FASN), while bacteria, plants, chloroplasts and mitochondria have left them as individual proteins (type II FASN) (Smith 2006).

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Figure 1.3 Fatty acid (FA) biosynthesis reaction cycle. Substrate enters the cycle bound to the ACP and exits after seven rounds, each adding two carbon atoms to the elongating FA. Adapted from Brignole *et al.* (2009).

The main product of FASN is palmitate, a long chain fatty acid (16:0) that is built up via cyclic elongation, two carbons at a time by FASN from acetyl-CoA, malonyl-CoA and NADPH (Ming *et al.* 2002, Maier *et al.* 2006). The other product is myristate (14:0) which is built up in the same way (Chirala & Wakil 2004). FASN allows excess food to be converted into lipids as an energy store, generates lipids for membranes and milk lipids during lactation (Chirala & Wakil 2004). FASN is also essential for embryogenesis and homeostasis, which has made it an attractive target in humans for obesity and cancer chemotherapy (Smith 2006).

Malonyl-CoA is a signalling molecule for appetite control and fuel sensing, and FASN inhibition increases the amount of malonyl Co-A with promising anti-obesity effects (Asturias *et al.* 2005). In addition, downregulation of FASN with inhibitors or RNA interference (RNAi) stimulates apoptosis in malignancies (Asturias *et al.* 2005, Chirala & Wakil 2004). The implications of these findings are discussed in section 1.4.

1.1.4 The type I FASN interdomain

An outstanding feature of the research literature dealing with FASN is how little the ID is mentioned. Since the ID is not a catalytic domain, most researchers have paid scant attention to this region, and little is known about its structure.

In 1991, Witkowski *et al.* noted that the 'central region' of animal FASN is poorly conserved, implying the area is under less selection pressure than the catalytic regions. In 1997, Jayakumar *et al.* noted the presence of an interdomain peptide of over 600 residues in the middle of the human FASN protein. They went on to say that its role is unclear, although it was thought that the ID helps in the association and maintenance of the functional dimer. In 2001, Wakil and his team showed that the ID in human FASN is essential for it to function, despite having no catalytic function itself. This finding supports their hypothesis that the ID is structurally necessary in FASN dimerisation, which is required for fatty acid synthesis.

It is relevant to this investigation that *C. elegans* introns are fewer and shorter than those of most other eukaryotes (Blumenthal & Spieth 1996, Bürglin 1998). This finding indicates that the *C. elegans* ID is likely to not contain an intron, so that gDNA can be used in the cloning process, which would eliminate the step of making complementary DNA (cDNA).

Although new drug targets to fight nematode parasites of animals are desperately needed due to the resistance to conventional drugs that is currently evolving, the interdomain of *C. elegans* has not yet been considered as a drug target. This is most likely due to FASN only recently being recognised as a potential drug target for inhibition and the ID being often overlooked in research in favour of the catalytic domains.

1.1.5 Previous FASN cloning work

Many groups have cloned FASN or its sub-domains in the past. Jayakumar *et al.* (1997) showed that expressing recombinant fusion-tagged halves of human FASN in *E. coli* still results in a functional FASN dimer. They demonstrated this by separately cloning domain I on one plasmid and domains II & III on a second plasmid. The expressed human FASN domains were then affinity purified and shown to synthesise long chain fatty acids *in vivo*.

Kridel *et al.* (2004) successfully amplified the FASN TE domain and expressed it in *E. coli*, which shows that a single catalytic domain of FASN can be expressed properly in a prokaryotic expression system.

Roy *et al.* (2005) cloned the bovine fatty acid synthase gene from a bovine bacterial artificial chromosome library. Jayakumar *et al.* (1995) cloned human FAS from a human brain cDNA library. Chirala *et al.* (2001) expressed different combinations of the domains in human FASN and found that palmitate was not produced in absence of the ID.

1.2 Parasitic nematodes and evolution of antinematodal resistance

Members of the phylum Nematoda are enormously diverse in both number and habitat. It is estimated that there are 1-10 million nematode species, but that only around 25,000 of them have been studied (Sommer & Dieterich 2009). Their ubiquity cannot be better described than by Cobb's (1914) celebrated quote "If all the matter in the universe were swept away, except for nematodes, our world would still be dimly recognisable..." (cited in Viney 2009).

Gastrointestinal nematode parasites of ruminant agricultural animals are a global problem (Waller 1997). They cause significant suffering and poor growth (Martin 1997) and have a large economic impact worldwide (Gasser *et al.* 2008). The reasons for parasitic nematode-based financial losses include increased mortality and reduced yield, the cost of purchasing and applying anthelmintic drugs, combined with rising parasite resistance to these drugs (Gasser *et al.* 2008).

Resistance of parasitic nematodes to anthelmintic drugs is an especially serious problem in small ruminants such as goats and sheep, where intensively managed flocks rely heavily on anthelmintics for gastrointestinal nematode (GIN) control (Praslička *et al.* 1994).

Unlike cattle, sheep do not develop sufficient immunoprotection against nematode parasites (Sager *et al.* 2009), making their infection even more costly. Today's widespread sheep GIN resistance is due to uncontrolled long-term use of what were once very efficacious anthelmintics (Sager *et al.* 2009). With resistance to all commercial broad-spectrum anthelmintics becoming a widespread occurrence (Praslička *et al.* 1994), we are now seeing the first sheep farms being shut down (in the UK) due to anthelmintic failure (Sager *et al.* 2009).

There seems to be agreement amongst parasitologists around the world that whilst anthelmintic resistance is a severe agricultural problem, there appear to be no practical alternatives to the current anthelmintic choices. Wolstenholme *et al.* (2004) emphasise that resistant GINs now seriously endanger the profitability of Australia's sheep industry, as well as animal welfare and agricultural income worldwide, but that there are no realistic alternatives to nematode control, other than to understand how resistance occurs and try to stifle its spread through different nematodes in different animals.

1.2.1 Gastrointestinal parasitic nematodes of importance

Livestock around the world is raised for profit, and since parasitism decreases yield, animal wellbeing and survival rates, farmers seek to manage their livestock so that clinical parasitism is not evident (Corwin 1997). Furthermore, most anthelmintics are used in large farms where animals are raised intensively; the situation in which parasitism causes the most serious losses (Demeler *et al.* 2009), and because large farms can afford to use these drugs.

Therefore, intensively grazing livestock increases anthelmintic resistance. Whilst GIN parasitism is the main disease of small ruminants (Burke *et al.* 2009a), completely eliminating all GIN infections is unnecessary both economically and for herd health. This is because subclinical infections have little impact on profits, and they also allow the host animals to acquire immunity to the parasites (Corwin 1997).

Livestock parasites are responsible for over AU$1 billion in lost revenue for the Australian sheep and cattle industries alone (Gasser *et al.* 2008). When the cost of anti-parasitic chemicals is added, the number jumps to the tens of billions of dollars (Gasser *et al.* 2008). GINs also caused losses of US$350 million for ruminants in the USA in 1995 (Chassaing *et al.* 2008). Table 1.1 shows some typical costs of parasitism in the Australian livestock industry.

Table 1.1 Costs of parasitism to the Australian livestock industries. Values are millions of Australian dollars in 1994. Note that nematode parasitism of sheep is responsible for the highest cost out of all forms of parasitism listed. Adapted from McLeod (1995).

	Cattle Ticks	Sheep Worms	Sheep Lice	Sheep Blow fly
Control				
Chemical	7	55	31	11
Labour	20	26	44	115
Other	14	0	39	4
Production loss				
Meat loss	63	19	0	0
Wool loss	0	81	55	14
Reduced fertility	0	0	0	5
Mortality	28	41	0	12
Total cost	132	222	169	161

Resistant GINs are a major problem in small ruminants (sheep, goats), but resistance is not seen as often in cattle GINs, with possible reasons including the lower frequencies of treatment cattle receive, and the different nature of their faecal pats that could leave different numbers of resistant infective larvae on the pasture (Williams 1997). However, that does not prevent gastrointestinal nematodes from affecting cattle production. The annual losses they inflict on the dairy cattle industry in the Netherlands is estimated at US$120 million (Corwin 1997). Infestations in growing lambs and kids are considered the direst of all, since they severely impact growth and productivity, complicated by the smaller immune responses of younger animals (Burke & Miller 2006).

Humbert *et al.* (2001) put the three main gastrointestinal nematodes of small ruminants as being *Teladorsagia circumcincta, Trichostrongylus colubriformis* and *Haemonchus contortus*. Suter *et al.* (2005), with an Australian emphasis, has the same opinion, but uses *Trichostrongylus* spp. instead of *T. colubriformis*.

Besier (2006) agrees with Humbert *et al.*, adding that these three nematode species are also the ones most resistant to anthelmintic chemotherapy around the world, beginning with resistance to broad-spectrum anthelmintics in the 1960s.

The main parasites affecting sheep production around the world are gastrointestinal nematodes, with *Teladorsagia circumcinta* as the single main gastrointestinal sheep parasite in temperate climates causing considerable mortality and loss of production (Coltman et al. 2001). The main gastrointestinal nematodes affecting cattle are *Ostertagia ostertagi*, as well as *Cooperia* spp. and *Nematodirus* spp. (Corwin 1997).

The low-pathogenicity *Cooperia* produce more eggs than *Ostertagia*, but they usually occur together as a mixed infection, which potentiates the clinical effects of both GINs (Demeler *et al.* 2009). Williams (1997) reminds us that, although GINs of small ruminants are more commonly found to harbour resistance, cattle have sporadically been host to resistant *Ostertagia* spp. and *Cooperia* spp. in Australia and New Zealand. Demeler *et al.* (2009) similarly report that anthelmintic resistance is seen frequently in small ruminants and very infrequently in cattle, although an increasing number of reports of resistance in cattle GINs have come mostly from New Zealand and South America, with isolated cases in the UK. They attributed this to the different management systems used in cattle production, including less frequent anthelmintic treatment and different grazing schemes (Demeler *et al.* 2009).

H. contortus, a GIN found in warm climates, is the single most important sheep pathogen (Burke *et al.* 2009b). It accounts for the overwhelming majority of sheep and goat faecal egg output in the south of the USA (Burke *et al.* 2009b), where anthelmintic resistance is also prevalent (Burke & Miller 2006). In addition, multiple drug resistant (MDR) *H. contortus* exists in much of South America, South Africa, Malaysia, the south-eastern USA, Australia and New Zealand, and seriously threatens their small ruminant industries (Kaplan 2004).

1.2.2 The status of broad-spectrum antinematodal chemotherapy

Parasitic disease in man has been recognised for thousands of years, but use of chemotherapy (drugs of known composition) to treat parasitised livestock is a relatively recent occurrence (Bennet-Jenkins & Bryant 1996). Many early treatments were herbal, such as the oil of the herb *Chenopodium* that was given as an anthelmintic for centuries, and in 1908 it was found that the active constituent was ascaridol (Bennet-Jenkins & Bryant 1996). These herbal dewormers are making a commercial comeback today; however their effectiveness in livestock GIN control is mostly unexamined (Burke *et al.* 2009a). From the 1920s to the 1970s, halogenated hydrocarbons were used in a string of continually more efficacious anthelmintics, until their underlying host toxicity was revealed (Bennet-Jenkins & Bryant 1996). The modern broad-spectrum anthelmintics were developed by pharmaceutical companies that can afford the screening programs and testing systems that modern drug development involves (Bennet-Jenkins & Bryant 1996, Williams 1997).

Table 1.2 The three families of broad-spectrum anthelmintics. Adapted from Gilleard 2006.

Class	Examples	Major mode of action
Benzimidazoles	Albendazole, Fenbendazole, Thiabendazole, Oxfendazole, Febental, Netobimin	Microtubule disruption
Imidazothiazoles Tetrahydropyrimidines	Levamisole, Tetramisole Morantel, Pyrantel	Nicotinic acetylcholine receptor agonists
Macrocyclic lactones (a) Avermectins (b) Milbemycins	(a) Ivermectin, Doramectin, Eprinomectin, Abamectin (b) Milbemycin, Moxidectin	Glutamate-gated chloride channel agonists

As shown in Table 1.2, Gilleard (2006) gives the three classes of broad-spectrum anthelmintics as being benzimidazoles, imidazothiazoles/tetrahydropyrimidines and macrocyclic lactones. They disrupt microtubules, nicotinic acetylcholine receptors (nAChR) and glutamate-gated Cl^- channels respectively. Benzimidazoles have been used since the 1960s and resistance to them is widespread: a mutated β-tubulin nucleotide changes one residue and renders benzimidazoles ineffective (Humbert *et al.* 2001). Wolstenholme *et al.* (2004) report that these three broad-spectrum anthelmintic groups routinely encounter resistant GINs. Farmers around the world rely on these increasingly ineffective drugs to help ensure the health and productivity of their animals.

1.2.2.1 Benzimidazole use and resistance

The introduction of thiabendizole in 1961 heralded the beginning of modern anthelmintic chemotherapy, but after a few years resistance developed, first in *Haemonchus contortus* and then in the other two major small ruminant GINs, *Teladorsagia circumcincta* and *Trichostrongylus colubriformis* (Kaplan 2004).

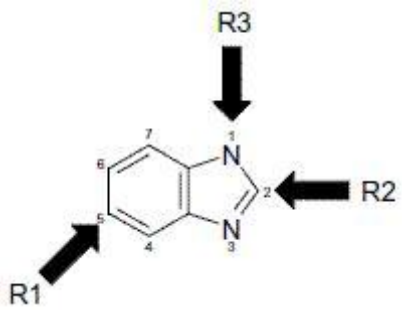


Figure 1.4 The benzimidazole nucleus. All benzimidazole anthelmintics have different substitutions of different groups at any of substitution sites R1, R2 or R3. Adapted from Velík *et al.* 2004.

The benzimidazoles were discovered by chance. During research on vitamin B_{12}, the benzimidazole nucleus (Fig. 1.4) was found to be a stable platform on which drugs could be developed (Bennet-Jenkins & Bryant 1996). The benzimidazole anthelmintics were developed throughout the 1970s, with mebendazole the first to be used extensively (Horton 2003). Benzimidazoles were the first of the modern broad-spectrum anthelmintics which combined efficacious antinematodal activity with low host toxicity (Kaplan 2004).

Benzimidazoles as a class encompass many derivatives that bind tubulin, an evolutionarily conserved and vital part of the cytoskeleton and mitotic spindle (Wang 1984).

Benzimidazoles are selectively toxic towards parasitic nematodes, selectively binding and depolymerising their tubulins with up to 400 times greater affinity than bovine brain tubulin (Wang 1984, Wolstenholme *et al.* 2004).

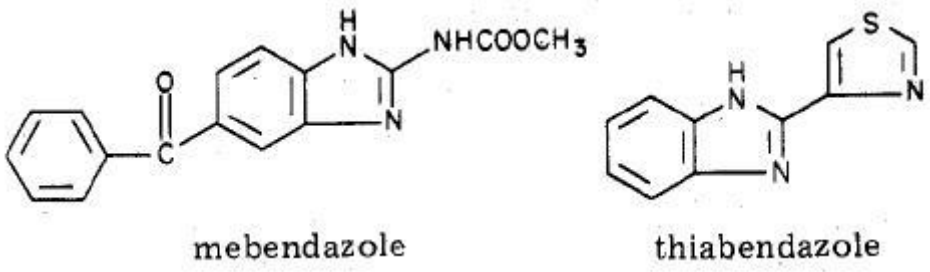


Figure 1.5 Structures of two benzimidazole derivatives. Adapted from Wang (1984).

Thiabendazole (Fig. 1.5) is also a fungicide (through binding fungal tubulin) and resistant *Aspergillus nidulans* mutants were found in the gene coding for β-tubulin, which was reversible by a mutation in the gene for α-tubulin, which shows that thiabendazole binds to both α- and β-tubulin (Wang 1984).

Benzimidazoles were rapidly accepted throughout the livestock industry after their inception (Kaplan 2004). In Europe, where anthelmintics have always been heavily used, livestock producers have moved away from benzimidazoles and towards macrocyclic lactone (ML) anthelmintics in recent years (Demeler *et al.* 2009).

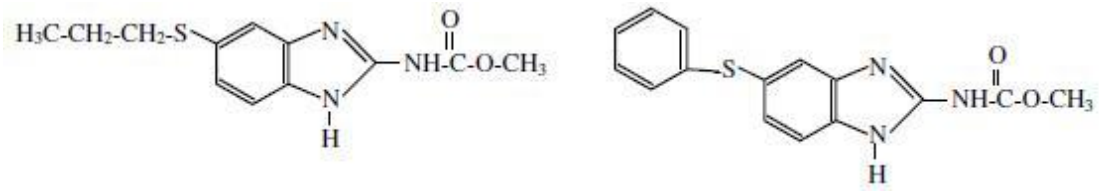


Figure 1.6 Structures of albendazole (left) and fenbendazole. Adapted from Capece *et al.* (2009).

Benzimidazoles are poorly absorbed by the host and have generally inactive metabolites (Horton 2003). The development of albendazole (Fig. 1.6) was a triumph because its sulfoxide metabolite is also an anthelmintic agent in itself (Horton 2003).

With respect to the development of resistance to benzimidazoles, there is evidence to suggest that the cell membrane efflux protein, p-glycoprotein, may play a part in resistant trichostrongyles (Wolstenholme *et al.* 2004, Gilleard 2006). P-glycoprotein confers resistance by reducing the amount of benzimidazole-class drugs in the target cells (Blackhall *et al.* 2008). This active drug efflux also occurs in human cells with the human p-glycoprotein, ABCB1, causing multiple drug resistant cancer (Blackhall *et al.* 2008). Furthermore, β-tubulin mutations correlate with benzimidazole resistance in *H. contortus*, *C. elegans* and even fungi (Wolstenholme *et al.* 2004, Blackhall *et al.* 2008, Humbert *et al.* 2001).

The overwhelming majority of sheep farms surveyed in Uruguay, Paraguay and Brazil detected resistance to benzimidazoles in all three of the most important sheep GINs (Nari *et al.* 1996, Maciel *et al.* 1996, Echevarria *et al.* 1996).

1.2.2.2 Tetrahydropyrimidine use and resistance

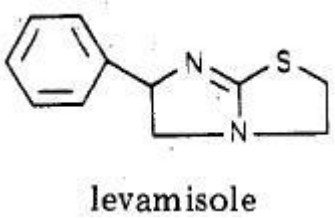
The tetrahydropyrimidine group includes pyrantel (which was first marketed in 1966) and oxantel, which are nicotinic acetylcholine receptor agonists with exceedingly low systemic absorption by the host (Kopp *et al.* 2008, Wang 1984), making them only useful at eliminating parasites residing in the gut. Tetrahydropyrimidines depolarise parasites' neurons 100x greater than acetylcholine, causing sustained muscular contraction, leading to death by spastic paralysis (Kopp *et al.* 2008, Wang 1984). This anthelmintic group is commonly used to treat domestic animals as well as livestock, and resistance has been reported in both groups of animals (Kopp *et al.* 2008).

Tetrahydropyrimidine treatment may select for resistant strains that express a different type of, or fewer nicotinic acetylcholine receptors, and resistance has been reported in pigs, horses, and in sheep trichostrongyloids (Kopp *et al.* 2008).

1.2.2.3 Thiazole use and resistance

This group includes levamisole (Fig. 1.7) and butamisole, which are also nicotinic acetylcholine receptor agonists, similarly effective in depolarising nematode neuromuscular junctions and causing spastic paralysis (Wang 1984). They entered the small ruminant anthelmintic market in 1965 (Harder 2002). Resistance to levamisole is common in New Zealand sheep farms (Waghorn *et al.* 2006).

The overwhelming majority of sheep farms surveyed in Uruguay, Paraguay and Brazil detected levamisole resistance in all three of the most important sheep GINs (Nari *et al.* 1996, Maciel *et al.* 1996, Echevarria *et al.* 1996).



levamisole

Fig 1.7 Structure of levamisole. Adapted from Wang (1984).

1.2.2.4 Macrocyclic lactone use and resistance

Macrocyclic lactones (MLs) include ivermectin, doramectin and moxidectin (Wolstenholme *et al.* 2004) which bind glutamate-gated chloride channels in nematodes, leading to spastic paralysis as studied in *C. elegans* (Blackhall *et al.* 2008). Ivermectin was released in 1980, with moxidectin and doramectin in 1992 and 1993 (Harder 2002). Allele frequency changes corresponding to ML resistance have been observed in the glutamate-gated chloride channel α-subunit gene of *H. contortus* and *Cooperia oncophora*, as well as in the *H. contortus* genes coding for p-glycoprotein and the γ-aminobutyric acid (GABA) receptor (Blackhall *et al.* 2008). P-glycoprotein is the transmembrane drug efflux pump that is also partially responsible for GIN benzimidazole resistance Blackhall *et al.* 2008, Wolstenholme *et al.* 2004.

Ivermectin is a lipophilic anthelmintic widely used to control parasites of cattle and small ruminants due to its retention in the host plasma, however it remains active in the dung which can kill non-target organisms such as soil nematodes (Iglesias *et al.* 2006). Suter *et al.* (2005) report that of the three main small ruminant GINs, *H. contortus* and *Teladorsagia circumcincta* are becoming resistant to ivermectin in Australia, although this is not yet reported for *Trichostrongylus* spp. in Australia. Maciel *et al.* (1996) found the majority of surveyed Paraguayan sheep farms had the three most important GINs resistant to ML drugs.

In many European countries, benzimidazoles have been replaced by ML anthelmintics, even though sub-therapeutic ML treatment can lead to resistance developing in sheep (Demeler *et al.* 2009). However, all ML drugs are highly efficacious towards GINs of ruminants (Harder 2002), although ML resistance is now common on sheep and cattle farms in New Zealand (Leathwick *et al.* 2006).

In 2006, Iglesias *et al.* published data showing that ivermectin is present in cattle faeces which reduces the invertebrate colonisation of faecal pats in both numbers and diversity, thus hindering the natural breakdown process.

1.2.3 Antinematodal resistance is widespread

Resistance to antinematodal (anthelmintic) drugs is an evolutionary process where surviving parasites contribute their genes to the next generation (Blackhall *et al.* 2008). Common practices that can lead to resistance include over- and under-dosing anthelmintics, and lack of *refugia* - which refers to the part of the parasite population not exposed to the anthelmintic - and therefore not undergoing selection for resistance (Suter *et al.* 2005). Some researchers contend that ensuring some worms are in *refugia* is essential to slow the spread of resistance (Pomroy 2006).

Table 1.3 Global anthelmintic resistance and its mechanisms. Sheep, goats, cattle and horses are included. Adapted from Kaplan (2004), Wolstenhome *et al.* (2004) & Kopp *et al.* (2008).

Drug class	Hosts with high resistance[a,b]	Hosts with emerging resistance[c]	Areas where drug is still highly effective[d]	Mechanism of resistance
Benzimidazoles	Sheep, goats, horses	Cattle	None	β-tubulin mutations
<u>Imidothiazoles & tetrahydropyrimidines</u>				
Levamisole	Sheep, goats	Cattle	None	nAChR changes (e.g. decreased expression)
Pyrantel	Horses (USA only)	Horses	Unknown outside USA	
<u>MLs</u>				
Ivermectin	Sheep, goats, cattle	Cattle	Horses: worldwide	P-glycoprotein overexpression Mutations in Glu-gated Cl channel genes and/or GABA-R genes
			Sheep, goats: Europe & Canada	
Moxidectin	goats	Sheep, goats, cattle	Horses: worldwide Sheep: most regions	

Note a= in ruminants, this specifically means resistance in trichostrongylid nematodes.
Note b= high resistance means that, for at least one GIN species, the drug in question would not be used without first testing for efficacy.
Note c= emerging resistance means that, resistance is known but is not prevalent enough to be a severe problem.
Note d= this column records the major livestock-producing areas where the drug remains very effective in sheep, goats and horses only.

As indicated in Table 1.3, it is impossible to overstate the importance of the spread of anthelmintic resistance. Anthelmintic chemotherapy is costly and all major parasites in all major hosts have varying degrees of resistance worldwide (Coltman *et al.* 2001). Furthermore, there is no way around this problem. At the moment, in conventional intensive livestock grazing, anthelmintic drugs are the only way to control parasitic nematode infestations (Wolstenholme *et al.* 2004).

In any environment where antinematodal drugs are present, selection for resistant nematodes is a constant occurrence. In 2004, Kaplan reported that drug resistance exists in all livestock hosts and to all anthelmintic drug classes. He attributed this to the fact that parasitic nematodes have high genetic diversity due to large populations and fast rates of nucleotide sequence evolution. Between the late 1950s, when *H. contortus* was the first nematode to develop resistance, and today, there has been an explosion of anthelmintic resistance, including multi-drug resistance (Kaplan 2004).

Drug resistance in parasitic nematodes has been noted in livestock in Australia, New Zealand, the UK, Canada, the USA (Kaplan 2004), Germany, the Netherlands (Molento *et al.* 2008), France (Hoste *et al.* 2002), Belgium (Praslička *et al.* 1994), Morocco, Algeria (Bentounsi *et al.* 2006), Denmark, Slovakia, Switzerland (Artho *et al.* 2007), Greece and Ireland (Cernanska *et al.* 2008), and Turkey (Köse *et al.* 2007). It is also prevalent in all major sheep GINs in Argentina (Eddi *et al.* 1996), Brazil (Echevarria *et al.* 1996), Uruguay (Nari *et al.* 1996) and Paraguay, where on sheep farms anthelmintics are approaching the end of their usefulness in the face of widespread resistance (Maciel *et al.* 1996).

In sub-Saharan Africa, anthelmintic resistance has been slower to develop due to insufficient use of anthelmintic drugs. Anthelmintic drugs are rarely used because they are not readily available, and there would be little gain in using them due to the practice of communal grazing that ensures rapid re-infection (Sissay *et al.* 2006). However, Sissay *et al.* (2006) sum up reported resistance of sheep and goat parasites in Tanzania, Zimbabwe, Cameroon, Mozambique, Nigeria and Zambia, whilst Keyyu *et al.* (2009) argue that coordinated, community-based GIN control should be implemented to slow the spread of resistance.

MDR to the three major broad-spectrum anthelmintic classes (benzimidazoles, imidothiazole-tetrahydropyrimidine and avermectin-milbemycin) in the three main GINs of small ruminants (*T. circumcincta, T. colubriformus* & *H. contortus*) have been reported since the early 1980s and have now spread throughout the world.

It has long been known that anthelmintic resistance is more prevalent and evolves quicker in the tropics because the parasites develop better and have shorter generation times there, although the drenching frequency of livestock is also a factor in how quickly resistance evolves; the more often they are treated, the more rapidly resistance results (Cernanská *et al.* 2008). In Argentina it was found that resistance was highest on farms that only carried sheep and farms where drenching is frequent (Eddi *et al.* 1996). Table 1.4 shows the time-based relationships involved.

Table 1.4. Broad-spectrum anthelmintics: the time between release and resistance. Adapted from Kaplan (2004).

Drug	Host	Year drug first approved[a]	Year resistance first documented[a]
Benzimidazoles			
Thiabendazole	Sheep	1961	1964
	Horse	1962	1965
Imidothiazoles-tetrahydropyrimidines			
Levamisole	Sheep	1970	1979
Pyrantel	Horse	1974	1996
MLs			
Ivermectin	Sheep	1981	1988
	Horse	1983	2002[b]
Moxidectin	Sheep	1991	1995
	Horse	1995	2003[b]

Note a= in any country
Note b= suspected as of 2004

Techniques to slow the spread of resistance include the rotation of anthelmintics used, weighing animals to ensure that drug doses (mg per kg) are sufficient, and keeping pastures parasite-free by rotation of grazing (Praslička *et al.* 1994).

1.2.4 The need for new anthelmintics

From the 1950s to the 1980s, a new class of effective (and inexpensive) anthelmintics was released into the market every decade, leading to excessive use throughout agriculture in the absence of any alternative anti-nematodal strategies (Kaplan 2004).

It was assumed that ongoing development of anthelmintics would always keep "one step ahead" of the inevitable resistance that followed and thereby maintain animals at maximum health and profitability. Kaplan (2004) argues that this strategy was successful in the short term, but short-sighted in the long-term as the anthelmintics were taken for granted and used excessively. It is significant that not one new class of anthelmintics has entered the market in the last three decades. The MLs (avermectins and milbemycins) of the 1980s were the last groups in what had been until then a continual flow of novel and effective anthelmintics (Kaplan 2004).

As a result, anthelmintic resistance in small ruminant GINs is now at crisis point, showing that exclusive use of anthelmintic drugs as a nematode control strategy is completely unsustainable (Kaplan 2004).

Interestingly, Besier (2006) argues that the reason no novel anthelmintics have been introduced into the commercial livestock industry in the past thirty years is not because no new compounds have been found, but because ruminants are only a small market for anthelmintics and it is not in companies' interests to sell drugs where the returns are low. Among the new anthelmintics that have not been applied to farm animals are the cyclic depsipeptides, paraherquamide compounds, and tribendimidine (Besier 2006, Kaplan 2004). Williams (1997) reports that not only have there not been any new anthelmintics, but also that no recent innovations or new systems for controlling parasitic nematodes have been introduced. However earlier this year, Novartis researchers published a paper showing the efficacy of a member of a new anthelmintic class, the amino-acetonitrile derivatives, in treating sheep GINs (Sager *et al.* 2009).

The paper does not mention a timeline for commercial release, so it may be some years yet. Williams (1997) also notes that current anthelmintic-based strategies are causing additional problems; including environmental toxicity, weakened immunity to GIN infestation, and the substantial costs involved in finding novel anthelmintics which will be inevitably needed as resistance to current chemotherapy spreads.

Oliveira *et al.* (2009) outline the reasons why alternatives to anthelmintics are urgently required. These include the continuing emergence of resistant nematodes, their cost, which can be prohibitively high for farmers in developing nations, and the risk of contamination of the animal products and the environment. Medicinal plants were traditionally used as antiparasitic agents long before the active ingredients were found, and organic farmers need anthelmintic-free methods of parasite control. Consequently, research into traditional treatments has increased, with some agents passing (Oliveira *et al.* 2009) and others failing (Burke *et al.* 2009a, 2009b) preliminary testing. However the fact remains that there are no new anthelmintics currently expected to be released over the next few years (Devaney 2006).

Until new anthelmintics enter the marketplace, and even after they arrive, producers simply have to accept that anthelmintics are finite resources that must be managed well to safeguard their efficacy, and used as one part of a multi-faceted antiparasitism strategy on farms (Kaplan 2004).

1.3 *C. elegans* as a model organism

The investigation reported in this thesis have utilised the model nematode *C. elegans*. This is one of the most powerful model organisms in developmental biology and in recent years has been widely used to study the gene function of parasitic nematodes (Gilleard 2004). *C. elegans* was proposed as a model organism by Sydney Brenner in 1965, and in 1998 became the first animal to have its genome sequenced (Wilson 1999). Although Gilleard (2004) admits that debate exists on the subject of using *C. elegans* in parasitic nematode research, it is argued that *C. elegans* has an important role in parasitic nematode research; just as yeast genetics has a profound role in mammalian cell research.

C. elegans is a free-living, bacteriovorous nematode of the order Rhabditida, and a highly important research model (Bürglin *et al.* 1998) that has well-defined characteristics that make it such a valuable model organism. At 25°C, a zygote can mature and produce its own zygotes in less than 3 days, with embryogenesis taking 14 hours (Bürglin *et al.* 1998). Over their 15-17 day lifespan, the worms are maintained on agar plates with a lawn of *E. coli*, or they can be stored in liquid nitrogen (Bürglin *et al.* 1998). The adults are about 1.2mm long, the hermaphrodites having 959 somatic nuclei while the males have 1031 (the fate of every cell is known), and strains can be maintained as self-fertilising hermaphrodites (XX), with males (XO) only necessary for crosses (Bürglin *et al.* 1998, Fire 1986).

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Figure 1.8 The *C. elegans* life cycle. Adapted from Bürglin *et al.* (1998).

As shown in Fig. 1.8, there is a fork in the road of *C. elegans* larval development. It can move steadily through the larval stages, or it can take the alternative route of becoming a dauer larva. Dauer larvae do not feed, grow or age; the stimuli for entering the dauer pathway are low food supplies and high temperatures (Viney 2009).

It is thought that this pathway is one of the most important steps in the evolution of parasitism because free-living larvae may have formed relationships with animals, becoming dauer larvae while resident, and then evolving to feed off the host (Viney 2009).

Naturally, experimenting on *Caenorhabditis elegans* is not exactly the same as experimenting on the parasitic nematodes of interest. However, at a molecular level, more is known about *C. elegans* than any other multicellular organism (Geary & Thompson 2001). Using *C. elegans* in our studies means that we have access to more genetic information, our results will be more reproducible, and we can draw on more experiments and ideas than experimenting on the actual parasites directly. Moreover, the vast number of protocols for propagating, breeding and manipulating *C. elegans* are clear and well-tested (Hashmi *et al.* 2001). This is why such model organisms are used. In any case, it is reasonable to assume that the relevance of *C. elegans* experiments to our parasitic nematode of interest will correlate accordingly with its evolutionary relatedness to *C. elegans*.

The publication of the gene sequence of *C. elegans* in 1998 was a significant milestone in the history of genetics (Thomas 2008) because of its wide use as a model organism. On the basis of EST data, Geary & Thompson (2001) suggest that ≥30% of the genes of parasitic nematodes have no homologs in *C. elegans*. Alternatively, Hashmi *et al.* (2001) have reported that >40% of parasitic nematode genes do have homologs in *C. elegans*. Because FASN is an essential enzyme, it is extremely likely that the FASN sequence, including the ID, is highly conserved between nematodes.

This was tested bioinformatically during the course of the investigation reported in this thesis.

As of mid-2008, it was estimated that *C. elegans* has around 20,000 genes, whilst estimates in *Drosophila* ranged from 14,000-17,000 genes, with *Homo sapiens* estimated at 20,500, showing very similar protein length distributions between the three organisms (Thomas 2008). In terms of protein coding capacity, *C. elegans* can no longer be seen as 'simplistic' compared to mammals (Thomas 2008).

C. elegans was first used in parasitology in anthelmintic screening, although that process has not been very successful in finding novel anthelmintics (Geary & Thompson 2001). However, studies of anthelmintics such as benzimidazole and its derivatives on *C. elegans* demonstrated that they kill nematodes via interference with β-tubulin polymerisation. Likewise, *C. elegans* showed how macrocyclic lactones open glutamate-gated Cl^- channels, and how imidothiazoles such as levamisole act on nematode acetylcholine receptors (Geary & Thompson 2001).

In summing up reviews of the use of *C. elegans* as a model for parasitic nematodes, Gilleard (2004) agrees that *C. elegans* is a useful and widely-used model, just as yeast is used as a model for eukaryotic biochemistry, and indicates that the reason parasitic nematodes are not used in the lab is due to the practical difficulties of working with them. These difficulties include the dearth of *in vitro* parasitic nematode culturing systems, and a lack of parasite DNA sequences.

Using examples such as understanding parasite regulatory elements, Gilleard (2004) shows how working with *C. elegans* can give results that are relevant to even distantly-related parasitic nematodes, thereby confirming the advantages of working with such a well-defined model organism.

Transgenic *C. elegans* progeny are commonly created through plasmid microinjection of the syncytial cytoplasm in the hermaphrodite gonad (Praitis *et al.* 2001). The syncytium is a large cytoplasm-filled structure containing around 1000 nuclei that form a queue in order of maturity and resides in the gonad's distal arm (Fire 1986). The more mature nuclei around the turn region of the distal arm are incorporated into the oocytes first (Fire 1986). Microinjection (Fig. 1.9) is regarded as a reliable way to express exogenous genes in *C. elegans* and has been used widely over the last 20 years (Praitis *et al.* 2001, Fire 1986). The injected DNA forms semistable multicopy extrachromosomal arrays in the oocytes, which are transmitted to the next generation with a frequency of anywhere between 10-90% (Praitis *et al.* 2001).

Figure 1.9 Microinjection of DNA into *C. elegans* hermaphrodite gonad. Introducing DNA into the syncytium creates F1 worms with heritable extrachromosomal arrays of transgenic DNA. Adapted from Stinchcomb *et al.* 1985.

1.3.1 Using *C. elegans* as an experimental model for parasitic nematodes

As shown in Fig. 1.10, nematodes are an extremely diverse group of organisms (Gasser & Nikolaou 2006).

Figure 1.10 Cladogram of major nematode orders. The relationships were determined through analysis of 18S rRNA genes. Note the proximity of free-living and parasitic orders. Adapted from Bürglin *et al.* (1998).

Sommer and Dieterich (2009) single out parasitic nematodes as the group that will benefit most in research through the use of *C. elegans* in defining nematodal biology and methods. As figure 1.12 shows, *C. elegans* resides in the same clade as the important animal parasitic group, the Strongylida.

In arguing that microtubules and nervous systems make attractive anthelmintic targets in parasitic nematodes, Wang (1984) indicates that free-living and parasitic nematodes share identical neuronal systems and microtubules and are equally susceptible to the thiazole levamisole, piperazine and MLs. For these targets, what will kill *C. elegans* will also kill GINs.

Figure 1.11 The five clades of phylum Nematoda. Note the interspersal of parasitic species alongside free-living species. (AP: animal parasite, BV: bacteriovore, FV: fungivore, OM: omnivore, PP: plant parasite) Adapted from Sommer & Dieterich (2009).

Because animal parasitism is so widespread amongst nematodes, some parasites are going to have greater similarity to *C. elegans* than others. The *Strongylida*, which include the important *Trichostrongyloid* small ruminant parasites, are very similar to the *Rhabditida*, of which *C. elegans* is a member (Geary & Thompson 2001).

Fig. 1.11 shows they are both members of clade V. It is expected that the usefulness of *C. elegans* as a model for a parasite would depend on the proximity of their evolutionary relationship (Geary & Thompson 2001). Furthermore, the distinction must be drawn between lumenal and invasive parasitism (Geary & Thompson 2001). Geary & Thompson (2001) argue that the evolutionary strategy of living in a tube (the GIT in lumenal parasitism) is more similar to living freely than invasive parasitism, which invokes the evolution of tissue penetration, and usually the utilisation of an intermediate host.

Another important feature of phylum Nematoda is the independent evolution of parasitism multiple times in different species (Sommer & Dieterich 2009, Viney 2009), which is also shown in Fig. 1.11 by the distribution of parasites and free-living nematodes within the same clade. Both free-living and parasitic nematodes share a pathway of developmental arrest, (called the dauer pathway in *C. elegans*) which is an important similarity in the life cycles of parasitic nematodes and our model organism (Devaney 2006). Signals that lead to dauer formation such as raised temperature (e.g. inside a host) and a higher pheromone:food ratio are detected by the amphids - sensory organs shared by all nematodes (Devaney 2006). Thus, the larval development of *C.* elegans is arrested in unfavourable conditions (Devaney 2006). This is of interest to us due to the suggestion that there are some inherent qualities of nematodes that facilitate the evolution of parasitism; qualities that all nematodes may share (Viney 2009).

1.4 FASN as a drug target for inhibition

While FASN, especially in humans, has been thought of as a possible drug target for inhibition (Maier *et al.* 2006), the ID itself goes unmentioned. We have seen earlier that inhibition of the ID should destroy a cell's ability to synthesise palmitate, an essential fatty acid. If a small peptide inhibitor could be designed to specifically target the nematodal ID and not allow FASN dimerisation, then that could be delivered to livestock as an anthelmintic drug.

From the outset of this investigation, it was known that there has been very little work done on the ID of FASN, and no proposals to use it as a drug target for a novel anthelmintic or any other treatment. In the literature, the ID has been consistently ignored in favour of the catalytic regions of FASN. Although we have seen that parasitic nematodes are increasingly resistant to antinematodal treatments, and that *C. elegans* is a good model for parasitic nematodes, there has been no work done towards exploiting unorthodox drug targets such as the ID of FASN.

However, FASN is beginning to be exploited as a drug target for inhibition in humans in two significant ways: as a treatment for cancer and also obesity.

1.4.1 Human FASN as a target for anti-cancer chemotherapy

Cancers can begin with cells that aggressively up-regulate the production of pro-survival and/or anti-death proteins, and it has been shown that FASN can be one of these oncogenic proteins if it too is up-regulated (Lupu & Menendez 2006a). In particular, FASN is up-regulated in carcinomas of the breast, prostate and ovaries (Zhang *et al.* 2008).

Under normal conditions, FASN is only highly expressed in the liver and is minimally expressed in other tissues, since these prefer to obtain their FAs from the bloodstream (Lupu & Menendez 2006a). New research has shown that some aggressive carcinomas express constitutively high levels of FASN while ignoring down-regulating signals: this occurs early in cancer development, is more noticeable in advanced tumours, and is an indicator for poor prognosis (Lupu & Menendez 2006a). Currently, inhibition of FASN is being studied in relation to preventing breast and endometrial cancer, and has been shown to improve the effectiveness of the anti-cancer drugs paclitaxel and trastuzumab (Herceptin™) (Zhang *et al.* 2008, Vazquez-Martin *et al.* 2007).

An important pathway for cancer growth and survival is the oestradiol/oestrogen receptor (E_2/ER) signal transduction pathway, with nearly all premalignant breast neoplasms expressing increased levels of ER (Lupu & Menendez 2006a). In breast and endometrial cancer cells showing large amounts of this type of signalling, increased lipogenesis due to increased FASN expression was shown to increase the cells' sensitivity to E_2-dependent growth and survival (Lupu & Menendez 2006a). Both RNAi and pharmacological inhibition of FASN were found to promote apoptosis in these cancer cells (Lupu & Menendez 2006a). FASN was effectively blocked by the naturally-occurring mycotoxin cerulenin, and its synthetic analog C75 (Fig. 1.12). Both molecules effectively inhibit FA production by covalently and irreversibly binding and inhibiting the active site cysteine of the FASN KS domain (Lupu & Menendez 2006a, 2006b). C75 has been shown to have significant anti-tumour activity (Lupu & Menendez 2006b).

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Figure 1.12 Structures of estradiol (E_2), cerulenin and C75. E_2 binds to ER which is overexpressed in most breast cancers. The mycotoxin cerulenin and its synthetic analog C75 are two inhibitors of the FASN KS catalytic domain. Adapted from Lupu & Menendez (2006a).

Neither cerulenin nor C75 are possible anthelmintics, because they would inhibit the KS domain of FASN in host and parasite alike. Furthermore, unlike antiobesity therapy, no FASN inhibitors have yet been used in cancer chemotherapy (Zhang *et al.* 2008), as they are still being trialled.

1.4.2 Human FASN as a target for anti-obesity chemotherapy

The over-the-counter weight-loss medication orlistat (tetrahydrolipstatin) (Fig1.13) is an irreversible inhibitor of both pancreatic lipase and the TE domain of FASN (Zhang *et al.* 2008, Lupu & Menendez 2006b). Kridel *et al.* (2004) were surprised to find orlistat was a FASN inhibitor, which also makes orlistat an antineoplastic agent. A member of the reactive β-lactone class of compounds, orlistat covalently binds to the TE domain, inhibiting the release of palmitate from the ACP (Lupu & Menendez 2006b).

Orlistat's FASN blockade has been found to specifically inhibit the growth and survival of cultured cancer cells (Lupu & Menendez 2006b) which has led to the search for novel β-lactone FASN inhibitors based on the structure of orlistat (Richardson *et al.* 2008), due in part to orlistat's poor solubility and oral bioavailability (Lupu & Menendez 2006b).

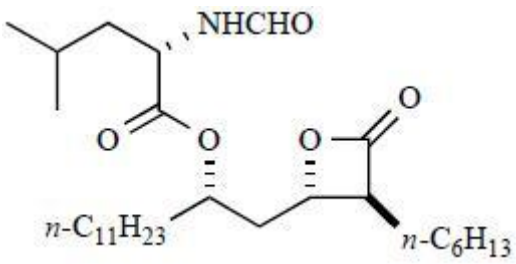

Figure 1.13 Structure of orlistat. Orlistat is marketed as Xenical®. Adapted from Lupu & Menendez 2006b.

1.5 FASN ID as a drug target in GINs

Bennet-Jenkins & Bryant (1996) recognise that, being animals, the nematode parasite and its host share many cellular pathways and processes. However they have markedly different characteristics that a developer of a novel anthelmintic could exploit as a drug target. These include processes related to the rapid growth and cell division that nematodes enjoy, as well as muscle activity, sensory and feeding processes, and energy metabolism and nutrient uptake (Bennet-Jenkins & Bryant 1996).

The FASN ID was chosen to be investigated in this paper due to its recent emergence as a novel drug target (Vazquez-Martin *et al.* 2007, Lupu & Menendez 2006b, Richardson *et al.* 2008) and the fact that FASN inhibition alone does not appear to have deleterious effects on mammalian cells (Lupu & Menendez 2006a, Richardson

et al. 2008). After its release to the public, orlistat was shown to also inhibit FASN (Kridel *et al.* 2004), which demonstrates that FASN inhibition in mammals does not lead to death. Also of significance are the findings of Greer *et al.* (2008) who showed that exposure of *C. elegans* to *fasn-1* RNAi resulted in larval growth arrest. The evidence suggests a drug inhibiting a FASN subunit could be given to livestock to kill off, or at least lessen their intestinal parasite load, while leaving the animal unharmed. For these reasons, including the fact that the FASN ID has never been comprehensively investigated, the ID was chosen for this study.

1.6 The aims of this investigation

In the face of widespread anthelmintic resistance, nematode parasitism of ruminants is currently the most serious disease problem in grazing livestock (Waller 2004). Concurrently, there is a great deal of interest in FASN as a drug target for inhibition in human antineoplastic and anti-obesity therapy (Kridel *et al.* 2004, Lupu & Menendez 2006a, 2006b). Whilst specific drug development is the ultimate goal of this line of research, it is beyond the scope of this investigation. Consequently, as a first step, the question that was addressed during this investigation is whether cloned *C. elegans* ID can be expressed in mammalian and/or *C. elegans* cells such that the monomers come together to form a dimer in the same way that the full-length FASN monomers dimerise in *C. elegans in vivo*. Successful expression of the ID alone would allow future studies to be done, such as phage display, to find an ID-binding protein that could become a drug.

The investigation documented within this thesis details what is possibly the very first in-depth study of the *C. elegans* fatty acid synthase interdomain, in both DNA and protein, both bioinformatically and experimentally. The ID sequence was compared to those of other animals and subsequently cloned and expressed in cultured mammalian cells. Taking into account the growing resistance of parasitic nematodes of livestock to anthelmintic drugs, it is suggested that a protein inhibitor of nematode FASN ID may be feasible in future. This thesis therefore examines the *C. elegans* FASN ID with a view towards its utilisation as a future drug target against parasitic nematodes of livestock.

Chapter 2. Materials and methods

2.1 Materials

2.1.1 Buffers, solutions and media

Table 2.1 details all buffers, solutions and media used throughout this investigation.

PBS, LB broth, LB plate media, glycerol and H_2O were autoclaved for 20 min at 121°C. All H_2O used was MilliQ H_2O unless otherwise specified.

Table 2.1 Composition of all buffers, solutions and media used and their applications to this study

Application	Name	Composition	Notes
C. elegans DNA extraction	Lysis solution	100mM NaCl 100mM Tris-HCl 50mM EDTA (pH 7.5) 1% SDS 1% β-mercaptoethanol 200µg/mL proteinase K	3mL total volume
DNA manipulation	TAE	40mM tris-acetate 1mM EDTA	Agarose gel electrophoresis running buffer
	DNA loading buffer	Bioline 5x DNA loading buffer, blue	Cat. No.: BIO-37045
	GelRed	Biotium GelRed 10,000X in H_2O	Used instead of EtBr for UV DNA visualisation Cat. No.: 41003-0.5mL
	DNA ladder	Invitrogen TrackIt™ 1kb plus	Cat. No.: 10488-085
	Water	Sigma-Aldrich CHROMASOLV® water for HPLC	Pure water used in PCR Cat. No.: 270733-1L

Bacterial	Cracking solution	H_2O 1230µL 4M NaOH 20µL 10% SDS 75µL 0.5M EDTA 15µL Glycerol 150µL 2% Bromophenol blue 10µL	For determination of successful ligations in transformed colonies. Made fresh for each use
	LB broth	10g bacto-tryptone 5g bacto yeast extract 10g NaCl 1mL 1M NaOH H_2O up to 1L	When used to culture successful transformants, 1:1000 carbenicillin was added
	Carbenicillin	Carbenicillin 25µg/mL	Antibiotic used in media for growth of plasmid-transformed cells only
	LB-carbenicillin plates	10g bacto-tryptone 5g bacto yeast extract 10g NaCl 1mL 1M NaOH 15g agar 1mL carbenicillin H_2O up to 1L	For selection of successful transformants
	Glycerol stock	60% Glycerol 375µL Transformed *E. coli* culture 750µL	To store plasmid-transformed bacterial cultures at -80°C
Mammalian cell transfection	SFM (Serum-Free Medium)	DMEM (Dulbecco's Modified Eagle Medium) (low glucose)	For 293T transfection
	DMEM	DMEM (low glucose) 10% FCS (Fetal Calf Serum)	For 293T growth Contains Phenol Red
	PBS (Phosphate-buffered saline)	137 mM NaCl 2.7 mM KCl 10 mM Na2HPO4 2 mM KH2PO4	pH 7.4
	Fugene	Roche Fugene HD	For plasmid transfection of 293T cells Cat. No.: 04709705001

Western Blot	Blotto	5 % skim milk powder in PBS/Tween 20	For blocking PVDF membranes
	ONYX lysis buffer	20 mM Tris-HCl 135 mM NaCl 1.5 mM $MgCl_2$ 1 mM EDTA 1% Triton X-100 10% glycerol 2 mM $NaVO_4$ 50 mM NaF 1 µg/ml pepstatin 1 µg/ml aprotinin 1 µg/ml leupeptin	pH 7.4
	2x SDS loading dye	1M tris-HCl 1.6mL 10% SDS 4mL Glycerol 2mL β-mercaptoethanol 1mL Bromophenol blue 4mg H_2O up to 10mL	
	10x running buffer	tris-glycine 151g glycine 720g SDS 50g H_2O to 5L	10x stock solution of buffer in which protein gels were run
	Transfer buffer	tris-glycine 1.4g glycine 7.2g methanol 200mL H_2O to 1L	Buffer in which gel → PVDF transfer was run

2.1.2 Primers, plasmids and enzymes

Primers (Table 2.2) were made by GeneWorks Pty Ltd; the chosen production parameters were 'PCR/sequencing purity' and the 40nmol scale. Pure CHROMASOLV (Table 2.1) H_2O was added to make 50mM master solutions, from which 10mM working solutions were made as required. Restriction enzymes, other enzymes and plasmids are shown in Tables 2.3, 2.4, and 2.5 respectively.

Table 2.2 Primer sequences used. All sequences are shown 5' → 3'

Name	Sequence	Application
Nesting Fwd	GGA CTC CCT TCT TCA AAC TGC TCT TCT	Nesting PCR
Nesting Rev	TAA CCA AAG CAT AAT AAG CGG TGG TGT A	Nesting PCR
pCAN-EE Fwd	GGA TCC AAC TCG CGT TCG TCA T	Amplify ID compatible with pCAN-EE Encodes BamHI restriction site
pCAN-EE Rev	TCT AGA ACT AGT CGT GGC AAG AGC TTG	Amplify ID compatible with pCAN-EE Encodes XbaI restriction site, stop codon
pEF-FLAG Fwd	GGA TCC ACT CGC GTT CGT CAT	Amplify ID compatible with pEF-FLAG Encodes BamHI restriction site
pEF-FLAG Rev	TCT AGA ACT AGT CGT GGC AAG AGC TTG	Amplify ID compatible with pEF-FLAG Encodes XbaI restriction site, stop codon
pPD 49.78-MCS FLAG Fwd	CAC CGC GGC CGC TAT GGA CTA CAA AGA CGA TGA CGA TAA ACG TCT TCC AAC TCG CGT TCG TCA T	Amplify Encodes FLAG tag, NotI restriction site
pPD 49.78-MCS Glu Glu Fwd	CAC CGC GGC CGC TAT GGA ATA CAT GCC AAT GGA ACG TCT TCC AAC TCG CGT TCG TCA T	Encodes glu-glu tag, NotI restriction site
pPD 49.78-MCS c-myc Fwd	CAC CGC GGC CGC TAT GGC AGA AGA ACA AAA ACT CAT CTC AGA AGA GGA TCT GCT GCG TCT TCC AAC TCG CGT T	Encodes c-myc tag, NotI restriction site, start codon
pPD Rev	GAC GTC ACT CAT GAC AGT GGT GGC AAG AGC TTG	Encodes PstI restriction site, stop codon
M13 Fwd	GTA AAA CGA CGG CCA G	Sequencing primers for *C. elegans* plasmid pPD49.78 provided by Dr. Matt Crook.
M13 Rev	CAG GAA ACA GCT ATG AC	

The nesting PCR primers in Table 2.2 amplify a region 50 base pairs larger in each direction than the ID region.

Table 2.3 Restriction endonucleases and their uses in DNA manipulation. All restriction enzymes were sourced from New England Biolabs.

Name	Uses
BamHI	Preparation of pCAN-EE, pEF-FLAG, ID insert Liberation of compatible ID insert from pGEM-T Easy
NotI	Preparation of pPD49.78-MCS and compatible ID insert Liberation of compatible ID insert from pGEM-T Easy
PstI	Preparation of pPD49.78-MCS and compatible ID insert Liberation of compatible ID insert from pGEM-T Easy
XbaI	Preparation of pCAN-EE, pEF-FLAG, ID insert Liberation of compatible ID insert from pGEM-T Easy

Table 2.4 Enzymes used in DNA manipulation.

Enzyme	Notes
T4 DNA ligase	Invitrogen catalogue number 15224-017
Phusion™ high-fidelity DNA polymerase	Finnzymes catalogue number F530S, 100U
Platinum® *Taq* DNA polymerase	Invitrogen catalogue number 10966-026

Table 2.5 Plasmids used in this investigation

Name	Purpose	Supplied by
pCAN-EE	Mammalian cell expression	Dr. Hamsa Puthalakath
pEF-FLAG	Mammalian cell expression	Dr. Hamsa Puthalakath
pPD 49.78-MCS	*C. elegans* expression	Dr. Matt Crook
pGEM-T Easy	Subcloning vector	Promega (catalogue number A1360)

2.1.3 Cells

E. coli bacterial cells used for plasmid manufacture were α-select chemically competent cells (Bioline) $\geq 10^8$ transformation efficiency, catalogue number BIO-85046.

Cells used for mammalian ID expression were HEK-293T cells, kindly donated by Dr. Hamsa Puthalakath and cultured by Diane Moujalled.

2.1.4 Commercial kits

The two kits used in DNA purification and clean-ups are shown in Table 2.6.

Table 2.6 Commercial kits used in the process of DNA manipulation.

Name	Company	Catalogue No.	Purpose
Qiaprep spin miniprep kit	Qiagen	27106	Purification of plasmid DNA from transformed bacterial cell cultures
Wizard SV gel and PCR clean-up system	Promega	A9281	Purification of ID insert DNA after gel purification for inserting into expression vectors after subcloning

2.1.5 Equipment

For determination of DNA concentration during DNA manipulation experiments, a NanoDrop® ND-1000 spectrophotometer was used. PCRs were performed using a Takara TP-600 thermocycler.

2.1.6 Proteins, antibodies and detection reagents used in Western Blotting

A protein ladder was run on the protein gel alongside transfected and untransfected 293T cell lysates so that the size of any expressed proteins could be estimated. The FLAG-tagged protein BIM-Long was also run on the gel as a positive control for the anti-FLAG Western Blot. The sizes of all proteins are shown in Table 2.7, the antibodies used in the Western Blot are shown in Table 2.8.

Table 2.7 Proteins used in protein gels and Western Blotting. Protein ladder used in western blots, and positive control protein for FLAG-tagged expression. All proteins courtesy of Dr. Hamsa Puthalakath and Diane Moujalled.

Protein	Size	Purpose
Myosin	180 kDa	Protein ladder
Bovine Serum Albumin	62 kDa	Protein ladder
Alcohol Dehydrogenase	38 kDa	Protein ladder
Carbonic Anhydrase	28 kDa	Protein ladder
Lysozyme	14 kDa	Protein ladder
BIM Long (FLAG tagged)	27 kDa	Positive control for anti-FLAG western blot

Table 2.8 Antibodies used to confirm 293T expression of tagged ID. These antibodies were used for Western Blotting.

Antibody	Details
1° anti-FLAG	Anti-FLAG IgG, whole antibody (from mouse) Supplied by Dr. Hamsa Puthalakath
1° anti-Glu-Glu	Anti-Glu-Glu IgG, whole antibody (from mouse) Supplied by Dr. Hamsa Puthalakath
2° anti-mouse	Anti-mouse IgG, horseradish peroxidase-linked whole antibody (from sheep) Supplied by GE Healthcare Catalogue number NXA931-1ML

Protein bands were transferred from the gel to a piece of Millipore Immobilon-P transfer membrane (catalogue number IPVH00010) which was treated with the antibodies listed above. Chemiluminescent detection reagents from GE Healthcare (catalogue numbers RPN2106V1 and RPN2106V2) were then used for visualisation of horseradish peroxidase- (HRP) conjugated IgG. The membrane was then exposed to Amersham Hyperfilm ECL high performance chemiluminescence film (catalogue number 28-9068-38) to visualise the chemiluminescence produced.

2.1.7 Bioinformatics software

For viewing the chromatograms of sequencing results, Invitrogen Vector NTI version 10 for Windows was used.

2.2 Methods

2.2.1 Flowchart of methodology

Fig. 2.1 provides an overall flowchart of methodology used throughout the course of this investigation.

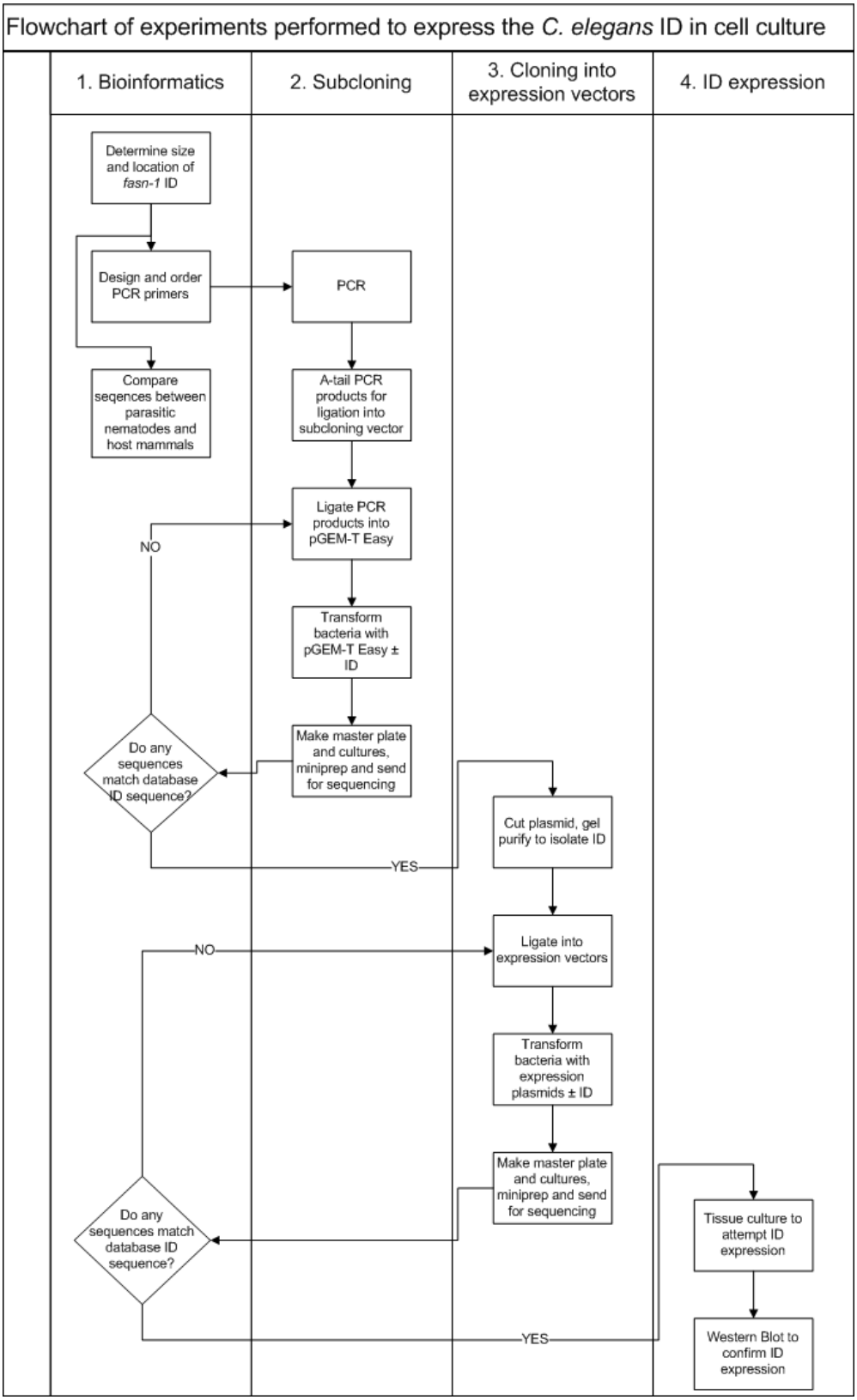

Figure 2.1 Final experimental strategy of this investigation. Using the generic subcloning vector pGEM-T Easy (Promega), a subcloning strategy was pursued because no successful clones were achieved after ligating the ID into expression vectors directly.

2.2.2 Bioinformatics

2.2.2.1 Identification of FASN ID

This was achieved by utilising the annotated sequence repositories at http://www.wormbase.org.

2.2.2.2 Comparative bioinformatics

C. elegans fasn-1 was compared to other *fasn* of animals (including nematodes) using online bioinformatics tools. The *C. elegans* ID was used as a query sequence in the blastn similarity search algorithm (Altschul *et al.* 1997). The Entrez gene database was searched to provide *fasn* sequences from all other animals (Maglott *et al.* 2005). Tools on the ExPASy proteomics server (Gasteiger *et al.* 2003) were used to predict FASN and ID protein characteristics. This included the GOR IV protein secondary structure prediction tool (Garnier *et al.* 1996) as part of the network protein sequence analysis suite (Combet *et al.* 2000).

2.2.3 *C. elegans* DNA manipulation

2.2.3.1 Extracting DNA from Bristol N2 strain *C. elegans*

Genomic DNA was prepared from frozen stocks of *C. elegans* for amplification of the fasn-1 ID. Complimentary DNA (cDNA) was not made because examination of the *fasn-1* gene sequence showed no introns were present in the segment to be amplified.

One pellet each of *C. elegans* (Bristol N2 strain) adult and L1 stage were taken from storage at -80°C and left to thaw on ice. Each pellet was then digested in 3mL of lysis solution (table 2.1). The digestion was performed at 65°C for 2 hours with occasional mixing.

The worm lysate was successively extracted using 3mL phenol, followed by 3mL phenol/chloroform/isoamyl alcohol (25/24/1) and finally 3mL chloroform/isoamyl alcohol. The resultant mixture was centrifuged between each extraction for 20 minutes at 3500 rpm (1100 x *g*) until debris accumulated at the interface.

The aqueous layer was recovered from the final extraction and the DNA contained therein was precipitated through the addition of 2 volumes of cold absolute ethanol and 0.1 volume of 3M sodium acetate. The precipitated DNA was pelleted by centrifugation at 4600 rpm (3400 x *g*) in a Sorvall centrifuge for 30 minutes.

The pellets were washed twice in 70% ethanol and allowed to dry completely each time. The pellets were suspended in 300µL of TE at 65°C. The DNA solutions were finally Nanodropped to ascertain their concentrations. The concentration of the gDNA from the adult *C. elegans* was 970 ng/µL, while the gDNA from the L1 *C. elegans* was 130 ng/µL. This solution was further diluted with another 300µL of TE to give a concentration of 64.8 ng/µL. This gDNA was used as the template DNA in subsequent PCRs.

2.2.3.2 PCR

Table 2.9 shows the components of PCR amplifications that were performed to amplify the *C. elegans* ID, while Table 2.10 shows the thermocycler program used.

PCR products and plasmids were sequenced at the Brisbane node of the Australian Genome Research Facility (AGRF) using an Applied Biosystems 3730*xl* 96-capillary sequencer. This was done to ensure the ID was amplified with no random base pair mutations, and inserted correctly into the vector. Generic subcloning vectors were sequenced after ligation and 'cracking' of colonies.

Table 2.9 PCR protocol. Used for all PCRs, as suggested by the Finnzymes Phusion product insert.

Component	Amount
H_2O[a]	12.5µL
5X Phusion™ HF buffer[b]	4 µL
10mM dNTP mix	0.4 µL
Phusion™ Polymerase[b] (2U/µL)	0.1 µL
10mM forward primer	1 µL
10mM reverse primer	1 µL
DNA[c,d]	1 µL
Total	20µL

Note a= Sigma-Aldrich CHROMASOLV® water (Table 2.1)
Note b= In kit of Finnzymes Phusion™ High-Fidelity DNA Polymerase (Table 2.4)
Note c= In round 1 of nesting PCR, gDNA from lysed L1-stage *C. elegans* was used, diluted to a concentration of 64.8ng/µL
Note d= In round 2 of nesting PCR, 1µL of a 1:10 dilution of the round 1 product was used.

Table 2.10 PCR program used in two rounds of nesting PCR. This program amplified plasmid-specific ID regions over two rounds of nesting PCR. Stages of denaturation, annealing and extension were repeated for 30 cycles.

PCR stage	Temperature (°C)	Duration (min:sec)	Cycles
Initial denaturation	98	0:30	1
Denaturation	98	0:10	30
Annealing	62	0:30	30
Extension	72	1:00	30
Final extension	72	10:00	1

2.2.3.3 A-tailing the ID for subcloning vector ligation

Phusion polymerase, unlike *Taq* polymerase, does not leave 5' A-tailed ends on PCR products. The generic subcloning vector pGEM-T Easy requires these A-tails to successfully ligate the insert. The A-tailing protocol is shown in Table 2.11. A thermocycler was used to heat the mixture to 72°C for 30 minutes.

Table 2.11 A-tailing protocol.

Component	Amount
10x PCR buffer[a]	2µL
50mM $MgCl_2$	0.6µL
100µM dATP	4µL
Taq polymerase[a]	0.1µL
H_2O	3.3µL
PCR product	10µL

Note a= Platinum *Taq* was used, see Table 2.4

2.2.3.4 Ligating ID insert into subcloning and expression vectors

Two different types of ligations were done, both according to the manufacturer's protocol. The first round of ligations aimed at ligating the ID insert into the generic cloning vector pGEM-T Easy. The second round of ligations combined expression vectors with the appropriate insert which had been excised from the generic cloning vector. A description of a typical set of ligation reactions is given in Table 2.13, with conditions used in Table 2.12.

Table 2.12 Ligation reaction conditions using T4 DNA ligase. ID was ligated first into the subcloning vector. Once successful clones were confirmed, the ID was cut from that vector and ligated into the relevant expression vector. For DNA ligase used see Table 2.4.

Reaction condition	For cohesive ends
5x ligase reaction buffer	4µL
Insert:vector ratio	3:1 (range 90ng:30ng to 150ng:50ng)
T4 DNA ligase (1U/µL)	0.1µL
Temperature	24°C
Time	16-24h

Table 2.13 Representative set of DNA ligation reactions. Incorporating a ligase-free negative control (group 1), an insert-free control (group 2) and the insert ligation (group 3).

Group	Component	Amount
1	5x ligase reaction buffer	4 µL
	Vector	1 µL
	H_2O	15 µL
2	5x ligase reaction buffer	4 µL
	T4 DNA ligase	0.1 µL
	Vector	1 µL
	H_2O	14.9 µL
3	5x ligase reaction buffer	4 µL
	T4 DNA ligase	0.1 µL
	Vector	1 µL
	Insert	3 µL
	H_2O	11.9 µL

2.2.3.5 Transformation of bacterial cells

Once the ligations had been performed, 'silver efficiency' α-select chemically competent *E. coli* cells (Bioline) were transformed. These transformations were performed with both the generic cloning vector pGEM-T Easy and later, the expression vectors.

The transformations were performed according to the manufacturer's protocol, with the following changes. Each 50µL aliquot of chemically competent cells from Bioline was split equally between the three transformations: negative control, positive control and experimental transformations (Table 2.13 groups 1, 2 and 3.)

As per the Bioline protocol, the ligation mixtures were added to the thawed cells, and 200µL of each 1mL mixture was plated on LB-carbenicillin medium and incubated for 16 hours. Colonies from reaction conditions 2 and 3 were used to determine colonies transformed by plasmid containing the ID insert by "cracking".

2.2.3.6 Analysis of transformants by "cracking"

Transformed bacterial colony "cracking" was performed as a rapid method to determine if plasmid-transformed bacterial colonies had taken up a plasmid containing the ligated ID insert. Colonies that took up the plasmid were able to grow in the presence of carbenicillin. These colonies were picked onto a master plate and numbered. Control colonies from the insert-free transformation were also picked and plated two or three to a plate to provide a control band for the cracking gel. These master plates were incubated overnight at 37°C. The next day, fresh cracking solution (Table 2.1) was made and a 25µL aliquot was dispensed into PCR tubes for each colony to be tested. Autoclaved toothpicks were used to transfer and mix a small amount of each colony into the PCR tube. The tubes were incubated for 30 minutes at 68°C using a thermocycler and then left to cool. 15µL of each sample was then loaded into a 1% agarose gel containing GelRed for electrophoresis. The gel was photographed under UV light. Colonies that had taken up a plasmid containing an insert could be distinguished by their plasmid bands on the gel, which were around 2kb heavier than in colonies whose plasmids lacked an ID insert. Transformed colonies found to contain the insert were cultured overnight (around 16 hours) at 37°C with shaking in approximately 5mL LB broth containing 1:1000 25µg/mL carbenicillin. The plasmids were then extracted by miniprep.

2.2.3.7 Excising the ID from subcloning plasmids

Insert-bearing plasmids were sent for sequencing at the AGRF, Brisbane. The sequence was viewed using Vector NTI and compared to the *C. elegans* ID sequence. Each nucleotide was checked to ensure no random mutations had occurred during PCR. Only plasmids in which the ID insert was properly ligated were used in this investigation.

The appropriate restriction enzymes were added to the plasmid solution. A digestion was run for 60 minutes according to the manufacturer's protocol in order to free the insert from the plasmid. Following the digest, DNA loading buffer (Table 2.1) was added to the digested DNA solution which was loaded into a 1% agarose gel containing GelRed (Table 2.1). Gel electrophoresis was performed at 80 volts for 45 minutes. The gel was viewed under UV light and the insert bands cut out with a scalpel blade. Finally, the ID band underwent clean-up using the Wizard SV kit (Table 2.6).

2.2.3.8 Inserting the ID into the expression vectors

The processes of ligation, transformation, 'cracking', sequencing and miniprep were repeated with expression vectors. They were then stored at -20°C until used to transform cultured mammalian cells. Glycerol stocks (Table 2.1) of colonies found to have been transformed by plasmids with the correct insert were made and kept at -80°C.

2.2.4 Mammalian cell culture ID expression

With a view to coimmunoprecipitation, mammalian cell expression was performed using the constructs pCAN-EE and pEF-FLAG which express GluGlu- and FLAG-tagged fusion proteins with the expressed insert, which in this case was the *C. elegans* ID.

HEK-293T cells were seeded at $1x10^6$ cells / 10cm plate. Two plates were used for each construct's transfection, with one untransfected control plate. Therefore five plates were used in total per attempt. The plates were incubated for 24 hours at 37°C in 10% CO_2.

The next day, 3µg DNA, 9µL of fugene and 100µL SFM were added per plate. This was accomplished by mixing 400µL SFM and 36µL fugene together first. 109µL of this SFM and fugene mixture was then added to each of the four plasmid solutions, two tubes of each plasmid. The plates were left to incubate at room temperature for 15 minutes.

The 293T cell plate media was then aspirated and replaced with 6mL DMEM. The fugene-DNA mixture was added drop-wise to each of the four plates, which were then incubated along with the control plate for 48 hours at 37°C in 10% CO_2.

After 48 hours, cells were loosened from the plate by gentle scraping. The plates' DMEM liquid containing the loose scraped cells was then transferred to separate 10mL tubes and centrifuged at 1500 rpm (365 x *g*) for 5 minutes at 4°C. The supernatant was removed via suction pump and the 293T cell pellets were resuspended in 1mL PBS. The tubes were again centrifuged for 5 minutes at 1500 rpm (365 x *g*) and 4°C.

The PBS was removed via aspiration, the pellets were spun for 2 minutes at 1500 rpm (365 x *g*) and 4°C, and stored at -80°C.

A Western Blot was then performed to check for tagged ID expression. The two pellets of cells transfected by the same construct were combined into one 1.5mL microfuge tube each and treated with 200µL of onyx P1 PO1 lysis buffer containing protease inhibitor and phosphatase inhibitor, and left on rocking rollers for 45 minutes. The lysed cells were finally centrifuged for 60 seconds at 13,200 rpm (17200 x *g*) and 4°C.

A lysate of untransfected 293T cells was supplied by Diane Moujalled from the Puthalakath laboratory. 5µL of each lysate was mixed and heated to 100°C for 5 minutes along with 5µL of 2x SDS loading dye. The three lysates (one from each of untransfected 293T, FLAG-tagged ID 293T, Glu-Glu-tagged ID 293T cells) were run on an Invitrogen pre-cast tris-glycine 4-20% gel.

The gel apparatus (an Invitrogen Novex mini-cell) was filled with running buffer made from a 10x stock solution. The gel was run for 140 minutes at 100 volts. 10µL of protein ladder was also run.

Following the running of the gel, the protein bands were transferred to a PVDF membrane. The membrane was treated with methanol for 20 seconds and then water. The transfer apparatus contained, from bottom to top, two sponges, two Whatman filters, the protein gel, the PVDF membrane, two more Whatman filters, and four sponges on top. The apparatus was filled with transfer buffer. The transfer set-up was surrounded by water and ice to keep the temperature low.

The protein transfer was carried out at 25 volts for 150 minutes. The PVDF membrane was then removed and left to dry at room temperature.

The next day, the PVDF was blocked with 5mL of blotto (5% skim milk powder in PBS/Tween 20) for 1 hour with rolling. Anti-FLAG IgG, the primary antibody (1° Ab), was added 1:500 in 2.5mL blotto. The PVDF membrane was left for 60 minutes rolling the 1° Ab mixture, followed by three 10 minute rolling washes with PBS/Tween 20. After this, the 2° Ab was added - anti-mouse IgG (from sheep), conjugated with horseradish peroxidase. This was diluted 1:1000 in 5% blotto, in which the membrane was rolled for 45 minutes, again followed by three 10 minute PBS/Tween 20 washes. The membrane was then kept in PBS/Tween 20 until the chemiluminescence test was performed.

1mL of each detection reagent was added to the membrane and incubated at room temperature for 1 minute. The membrane was exposed to the chemiluminescence film for 1 to 2 minutes as required.

Following this, the anti-Glu-Glu Western Blot was performed, without any protein stripping of the membrane. This second Western was carried out as described above, except the anti-Glu-Glu antibody was diluted 1:1000 when applied to the membrane.

The two Western Blots were repeated with slight modifications. A 293T cell lysate containing FLAG-tagged Bim-Long protein was used as a positive control for the FLAG Western Blot. Following this, the PVDF membrane was kept in the 1:1000 solution of anti-Glu-Glu antibody overnight instead of for 1 hour.

Chapter 3. Results

3.1 Bioinformatics

3.1.1 *C. elegans* FASN ID identification

At the start of the investigation, the exact location of the FASN ID of *C. elegans* was not known. Firstly, outer limits had to be set on the *C. elegans* ID DNA sequence region so that the ID could be cloned. Wormbase.org states that the FASN protein in *C. elegans* is 2613 amino acids in length, encoded by 7842 nucleotides. Including introns, the *fasn-1* gene is 11143 base pairs long.

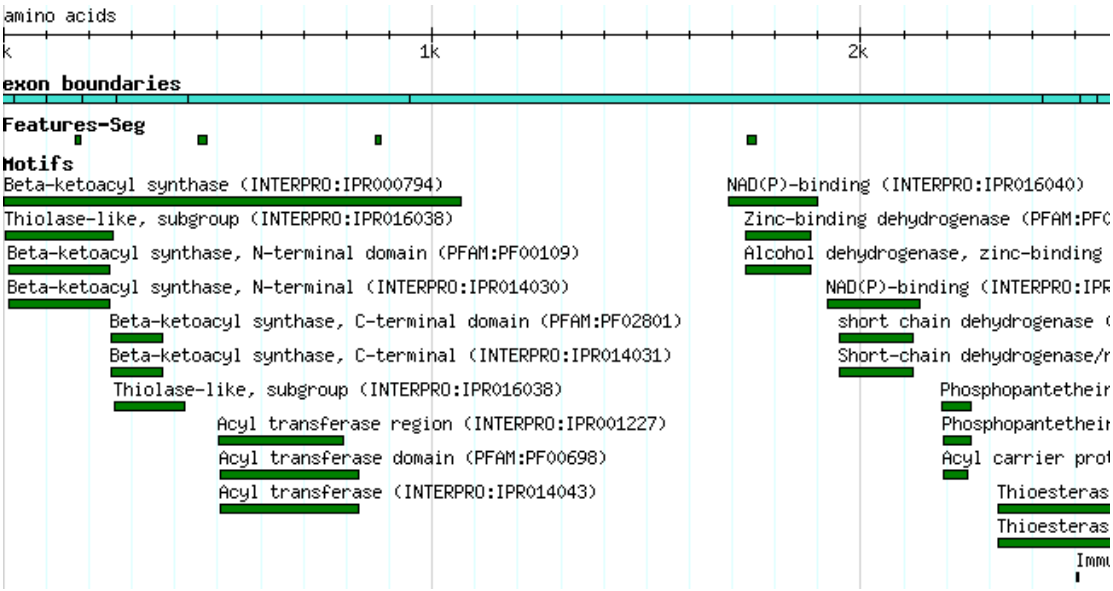

Figure 3.1 *C. elegans* FASN motif map. Adapted from the 'protein report for WP:CE09880' on wormbase.org.

The *C. elegans* FASN motif map generated by wormbase.org (Fig. 3.1) shows a region free of motifs from approximately 1075-1690 amino acids. The details in the motif summary give the exact residues of the interdomain boundaries. The β-ketoacyl synthase motif ends at residue 1066, leaving a 626 residue motif-free region until

the beginning of a NAD(P)-binding motif at residue 1693.

This was defined as the ID. The 626 residues are coded for by 1878 base pairs in *fasn-1*, base pairs 3280-5157. Wormbase shows no introns between base pairs 3280 and 5157. Therefore, making cDNA is not necessary, because the whole ID can be amplified from *C. elegans* gDNA as one continuous piece of exonic DNA.

It must be emphasised that, as there is no clearly defined functional motif in the ID region, the conservative approach was taken and the entire un-annotated region between the two annotated domains was included in this definition of the ID. Since no residues or groups of residues have defined as necessary for ID dimerisation, the choice of boundary for the ID has been governed by the location of known domains surrounding the ID.

The *C. elegans* ID DNA sequence was entered into BLAST to be compared with a non-redundant DNA sequence database (Altschul *et al.* 1997). Results are shown in Table 3.1.

Table 3.1 BLAST results from FASN ID query. Non-redundant database. Only significant hits shown (Altschul *et al.* 1997).

Pair-wise BLAST was then performed with all *fasn* sequences available on Entrez gene database (Maglott *et al.* 2005), results are shown in Table 3.2. A table of overall identity and dot plots to visualise alignments over the whole gene are reproduced here. The *C. elegans* gene *fasn-1* is always on the X-axis, the ID is between 3280-5157 base pairs.

Table 3.2 Pair-wise BLAST output. *fasn-1* query compared to other animals' *fasn*. Blastn algorithm used to search for 'somewhat similar sequences' (Altschul *et al.* 1997).

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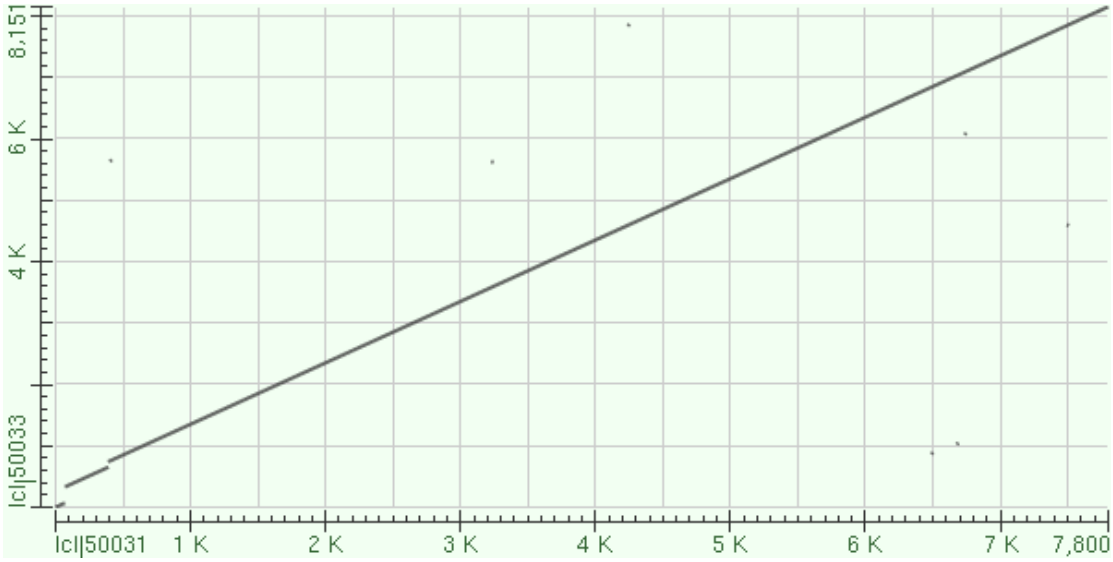

Figure 3.2 Dot matrix plot of alignment between *C. elegans fasn* and *C. briggsae fasn*. An example of a good alignment, *C. elegans* is on the X-axis, gaps in the line equal breaks in the alignment. The ID region 3280-5157 base pairs have high identity between the species, as indicated by the unbroken straight line in that region.

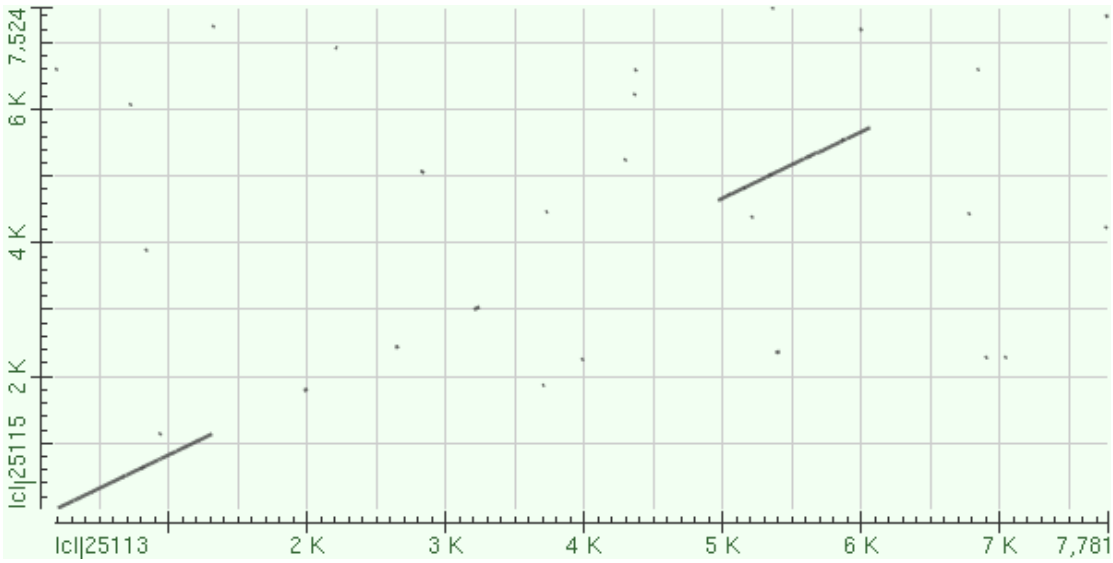

Figure 3.3 Dot matrix plot of alignment between *C. elegans fasn* and *G. gallus fasn.* An example of a poorer alignment, there are only two large stretches of identity between the two species' genes, which include only a small part of the ID at around 5000 base pairs.

The ProtParam tool on the ExPASy proteomics server (Gasteiger *et al.* 2003) was used to compare the translated protein sequences of both the whole *C. elegans* FASN and the FASN ID. Results from this tool are presented in Table 3.3.

Table 3.3 Predicted protein parameters for whole FASN and ID only. Data from ProtParam tool on ExPASy proteomics server (Gasteiger *et al.* 2003).

Parameter	*C. elegans* FASN	*C. elegans* FASN ID
Amino acids	2613	626
Molecular weight	289225.9 Da	70540.5 Da
Theoretical pI	5.71	5.70
Estimated half-life in *E. coli*	>10 hours	2 minutes
Instability index	35.97 (stable)	34.16 (stable)
Grand average of hydropathicity	-0.195	-0.233

In order to determine if the ID fragment could be stably expressed alone, its secondary structure was predicted. The GOR IV prediction algorithm (Garnier *et al.* 1996), part of the network protein sequence analysis (NPS@) suite (Combet *et al.* 2000) was used. These results are shown in Table 3.4.

Table 3.4 Secondary structure predictions for whole FASN and ID only.

Secondary Structure	*C. elegans* FASN	*C. elegans* FASN ID
Random coil	45.77%	47.76%
α-helix	34.71%	35.14%
Extended strand	19.52%	17.09%

Table 3.4 shows that the secondary structure properties of *C. elegans* ID are very similar to those of the whole FASN. We have seen that the whole FASN can be expressed (Chirala *et al.* 2001, Jayakumar *et al.* 1997, Jayakumar *et al.* 1995, Roy *et al.* 2005) and these data show that the ID should also be expressible on its own without any other part of the FASN.

A literature search was carried out to find researchers that had determined the size and position of the FASN ID. It was found that only one published measurement of FASN ID position existed. Chirala *et al.* (2001) write that in human FASN, which is 2504 amino acids long, the ID is between domains I and II and comprises residues 981 to 1630, thus making it 649 amino acids in length. This is also referenced by Ming *et al.* (2002). Roy *et al.* (2005) cloned and characterised bovine FASN, showing it to be 2513 amino acids long, but did not study the ID specifically.

Maier *et al.* (2006) noted that the FASN ID sequence was marked by lower sequence conservation than those of the catalytic domains. Roy *et al.* (2005) agree, stating that the ID is the region of greatest difference between human, bovine and rat sequences.

For a future anthelmintic drug to work through specifically binding the ID of gastrointestinal nematode parasite FASN, the ID sequences between the host and the parasite must be different enough so that the drug specifically binds to the parasite's ID. Furthermore, if the drug is to have broad-spectrum activity, the ID sequences between different GIN species must be similar enough so that they also bind the drug. Table 3.5 lists previously published results of similarity studies regarding animal FASN.

Table 3.5 Results of similarity studies published on metazoan FASN.

FASN sequence comparison	% identity	Reference
H. sapiens v. Rattus norvegicus	79	Jayakumar *et al.* 1995
H. sapiens v. Gallus gallus	63	Jayakumar *et al.* 1995
Bos taurus v. H. sapiens	73	Roy *et al.* 2005
Bos taurus v. Rattus norvegicus	73	Roy *et al.* 2005
Bos taurus v. Gallus gallus	63	Roy *et al.* 2005

3.1.2 *In silico* ID digests

In order to determine which restriction endonuclease sites to incorporate into the plasmid-specific ID PCR primers, and to decide which plasmid site to ligate into, *in silico* digests were performed using NEBcutter as per Vincze *et al.* (2003). Results are shown in Fig. 3.4.

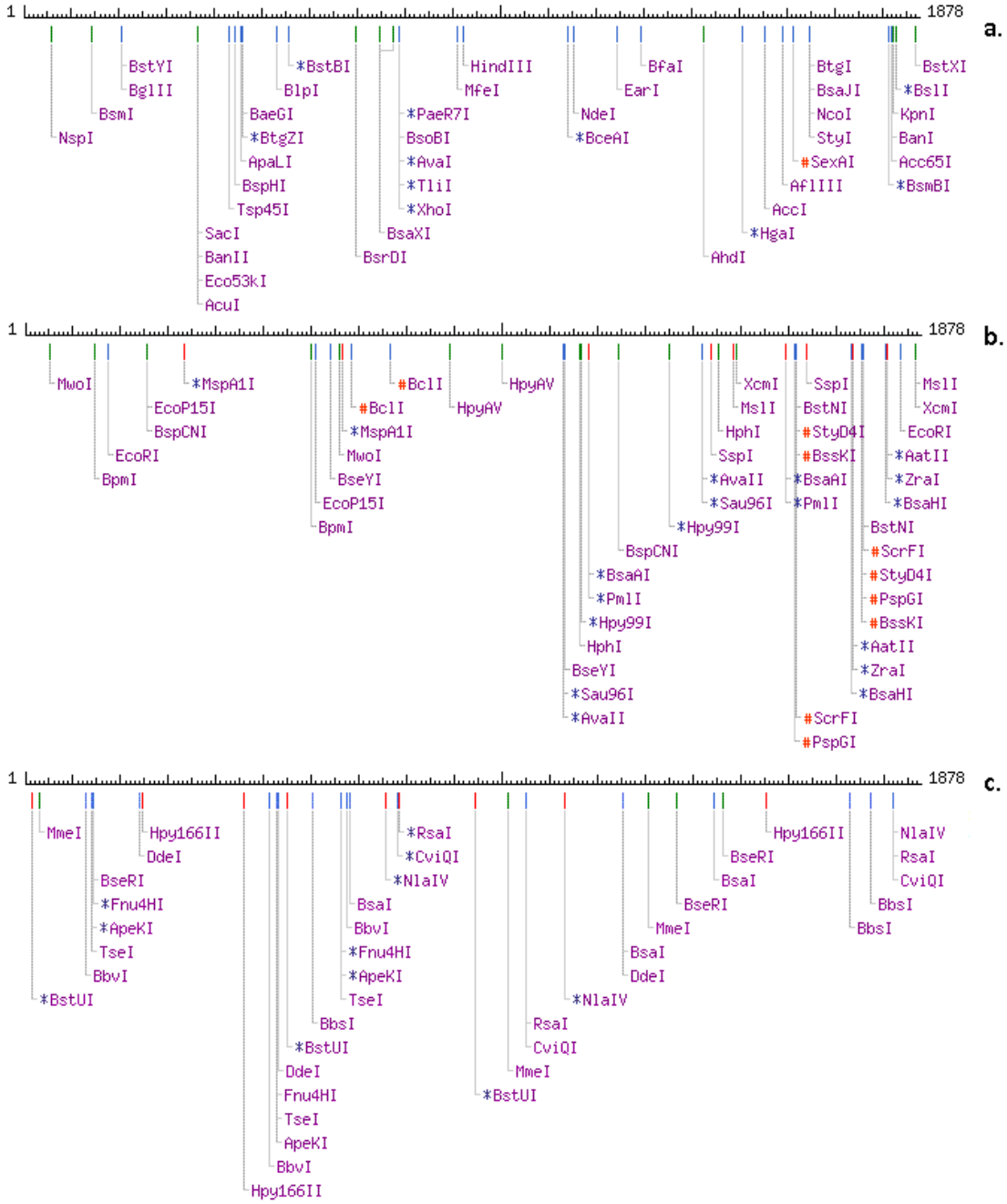

Figure 3.4 *In silico* digests showing predicted cut sites in ID sequence. These enzymes were therefore ruled out as potential enzymes to use for ligation into plasmids. (a) shows enzymes that cut once, (b) shows those that cut twice, and (c) shows those that cut three times in the sequence. Diagrams made by NEBcutter (Vincze *et al.* 2003).

3.2 PCR and cloning of the FASN ID

Figure 3.5 shows in detail the order in which laboratory work was conducted from the C. elegans gDNA being obtained to the experimental verification of the expression of the ID in HEK-293T cells with Western Blotting.

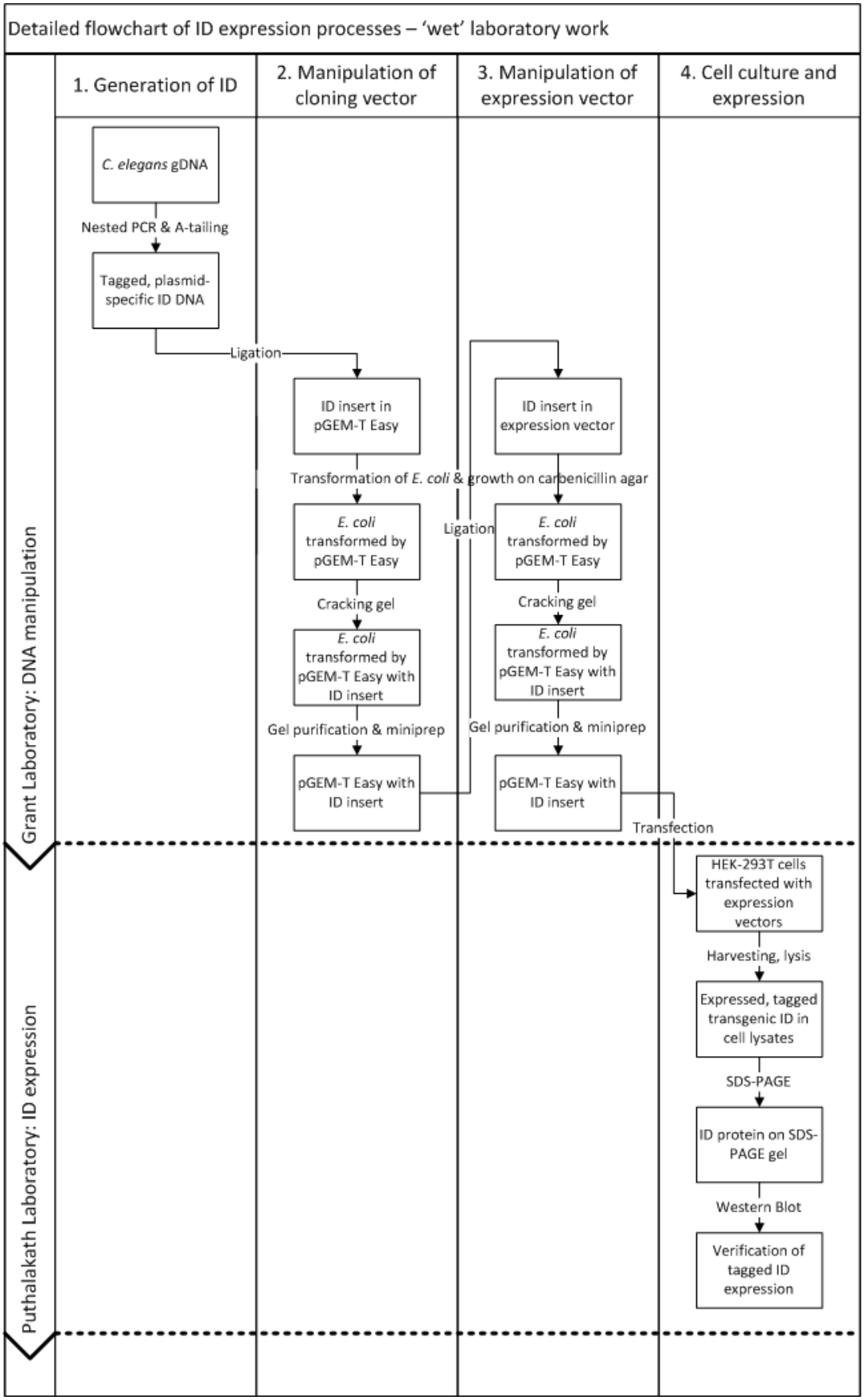

Figure 3.5 Detailed flowchart of laboratory work.

Before experimental work began, it was envisioned that the *C. elegans* ID could be amplified directly with restriction endonuclease-compatible ends and (in the case of plasmids for pPD 49.78-MCS) the DNA encoding the affinity tag (table 2.5). However, most likely due to the presence inadequate complimentary sequence for sufficient annealing, this could not be achieved. Therefore, nesting PCRs were performed, and ID compatible with plasmids pCAN-EE, pEF-FLAG, and pPD 49.78-MCS tagged with both glu-glu and c-myc tags were produced this way. Nesting PCR increases the amount of substrate by amplifying the area of interest. This is visualised by Figure 3.6(B).

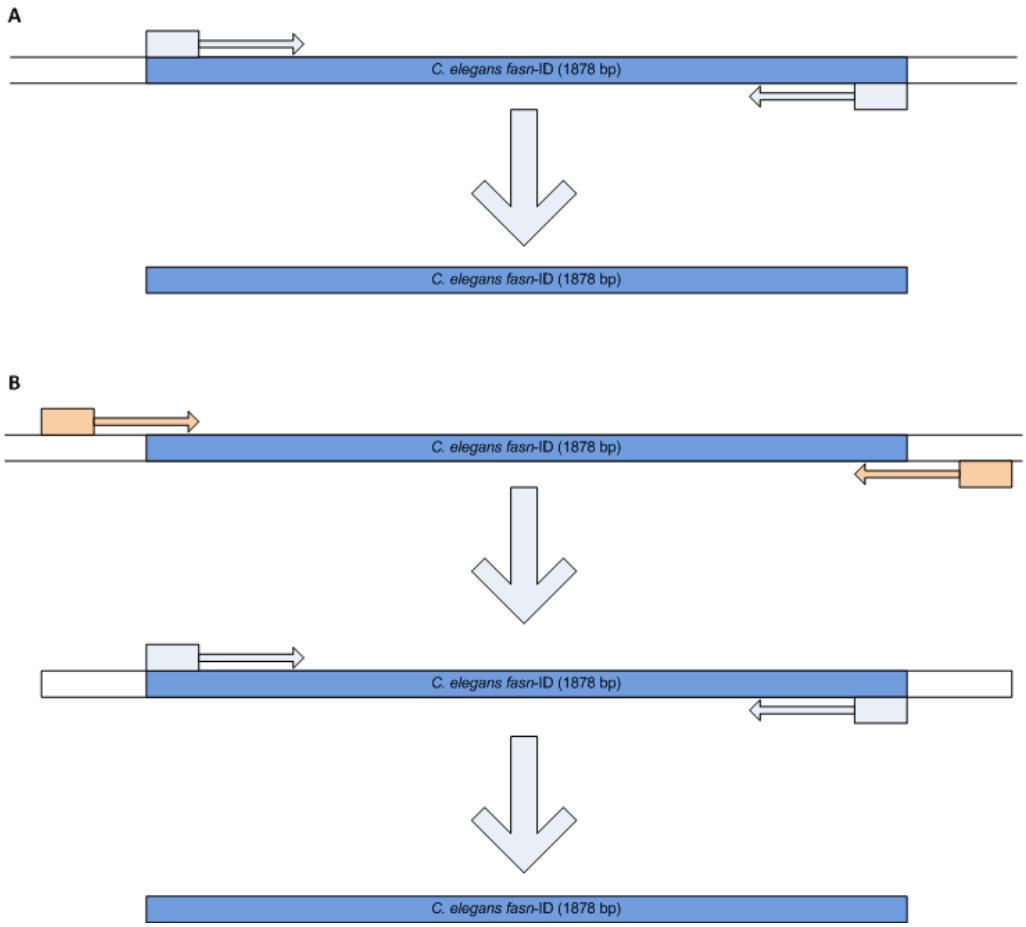

Figure 3.6 (A) A standard PCR and (B) a nesting PCR. Nesting PCRs were used to generate ID DNA after normal PCR failed. Nesting primers (orange) were complementary to a region around 50 base pairs before and after the ID. The resulting PCR product was then used as a template for a normal PCR.

These inserts were ligated into the plasmids for which they were designed. At this point, inserts were ligated directly into the mammalian expression vectors pCAN-EE and pEF-FLAG, as well pPD 49.78-MCS inserts tagged with glu-glu and c-myc. Attempts to transform chemically competent *E. coli* with these plasmids failed. Therefore, the unsuccessful single-step direct cloning strategy was changed into the successful two-step subcloning strategy, utilising the subcloning vector pGEM-T Easy (Figure 3.7).

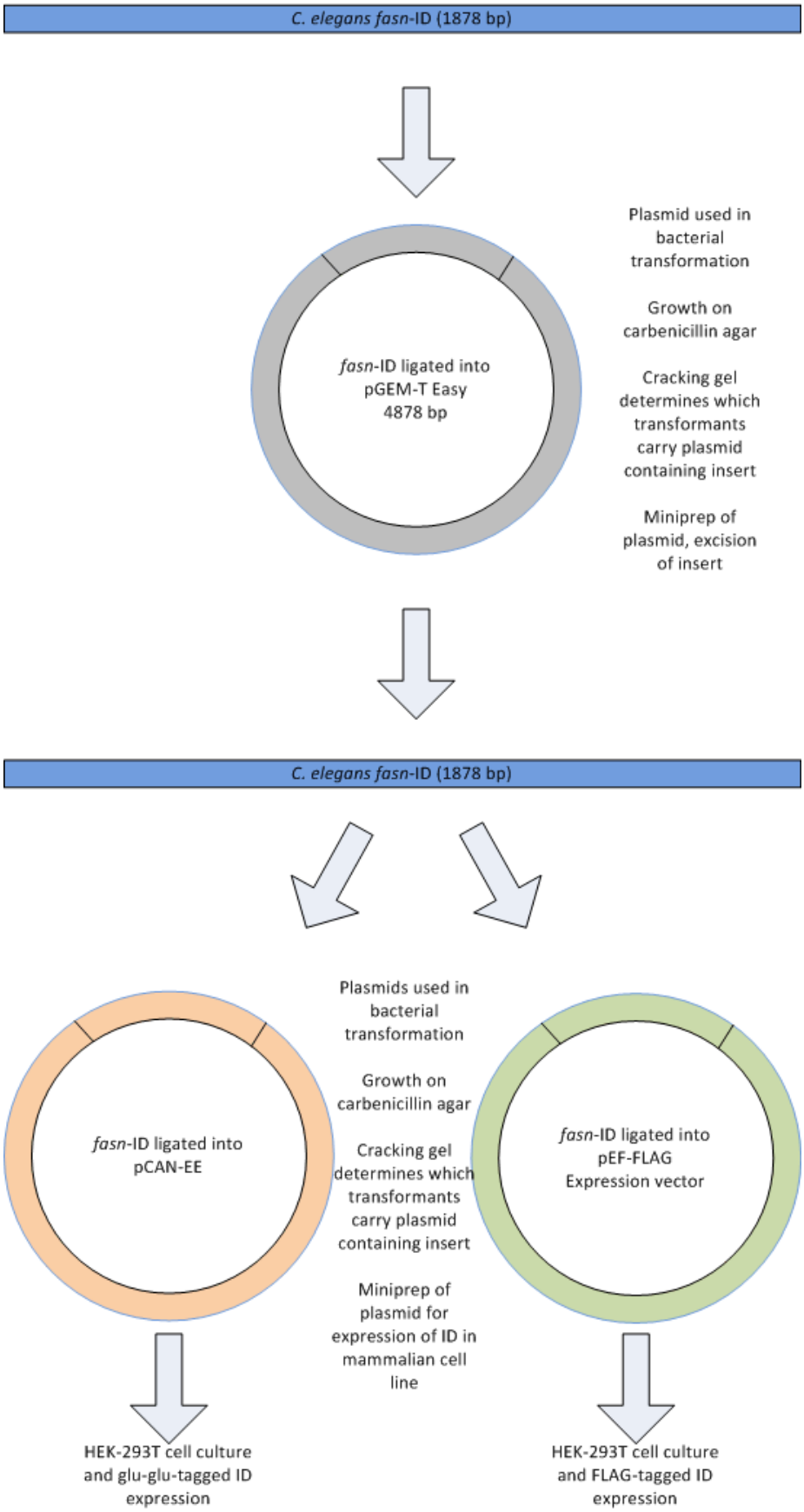

Figure 3.7 Subcloning strategy. Used after expression vector direct ligation failure.

Because of these failed attempts at direct ligation, a subcloning strategy was pursued. The ID was first ligated into the generic cloning vector pGEM-T Easy. After ligation, bacterial cells were transformed with these plasmids. Colonies found to have taken up a plasmid containing the ID insert were cultured and miniprepped. The resulting plasmid solution was digested with the appropriate restriction enzymes to free the insert. The DNA was then gel-purified and the insert was physically excised from the gel (Fig. 3.8).

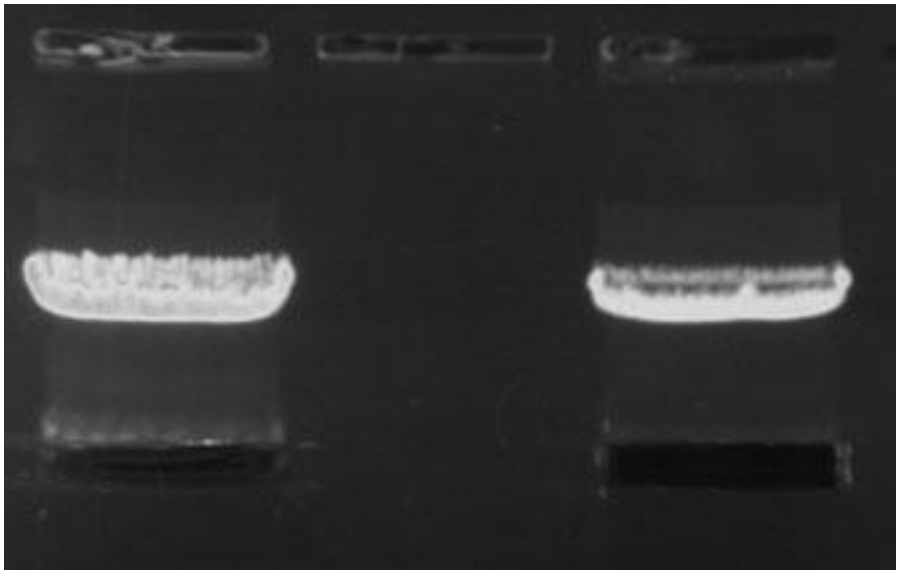

Figure 3.8 Removal of ID bands from pGEM-T Easy following gel purification. Both pCAN-EE compatible (left) and pEF-FLAG compatible ID (right) were excised from the generic cloning plasmid using restriction enzymes BamHI and XbaI.

However, even after the adoption of the subcloning strategy, attempts to transform chemically competent *E. coli* with the *C. elegans* plasmid pPD 49.78-MCS all failed. Over twenty different transformation experiments yielded no transformants in which the insert was faithfully copied.

3.3 Ligation and bacterial transformations

A representative sample of numbers of colonies grown after 16 hours is given in Table 3.6. Table 2.11 lists the compositions of reactions 1, 2 and 3.

Table 3.6 Typical numbers of transformants after overnight incubation.

Ligation reaction added to competent cells	Average numbers of colonies seen
1 (ligase-free negative control)	≤10
2 (insert-free control)	≤20
3 (experimental insert ligation)	≤100

Cracking gels were a rapid and straightforward method of determining which transformed colonies contained plasmids that had taken up the ID insert. Fig. 3.9 is an example of a representative cracking gel. Even with large numbers of transformants, it is easy to see which plasmids have shifted upwards on the gel, which signifies the presence of an insert.

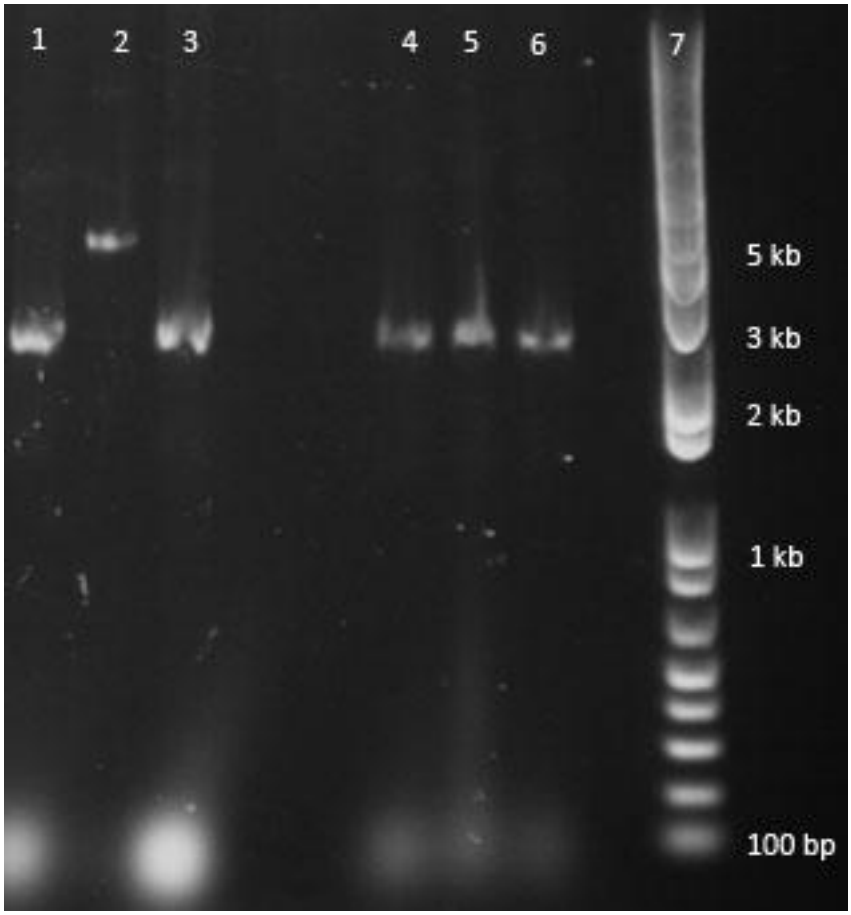

Figure 3.9 Representative cracking gel. The pGEM-T Easy plasmid in lane 2 contains a pCAN-EE-compatible ID insert. Note how it is approximately 2 kb heavier than the plasmids without inserts in lanes 1, 3, 4, 5 and 6. Lane 7 contains DNA ladder (Table 2.1).

3.4 Western Blots

The Western Blots were performed to visualise the expression of *C. elegans* ID in cultured HEK-293T cells. The HEK-293T cells were lysed, and the lysate was run on an SDS-PAGE gel. This was performed twice, because the first time Western Blotting was attempted, no specific antibody binding to proteins of interest was observed. A repeat of transfection, growth and harvesting of the cells, and lysis, SDS-PAGE and Western Blotting yielded good results (Fig. 3.10, 3.11). The PVDF membrane was first probed for presence of the FLAG tag (Fig. 3.10), followed by the glu-glu tag.

This can be seen in Fig 3.8 which shows chemiluminescence still given off by antibodies bound to the FLAG tag even though the membrane was being probed for the glu-glu tag at that stage.

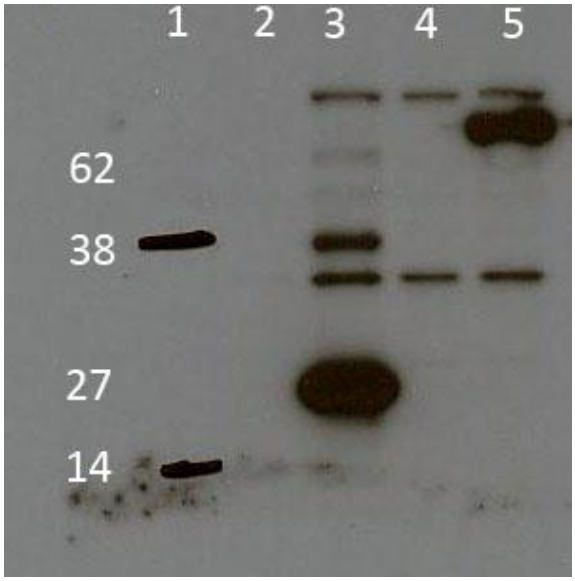

Figure 3.10 Successful Western Blot for FLAG tag. Numbers across top indicate lane numbers. Lane 1: protein ladder. Lane 2: untransfected lysate. Lane 3: FLAG-tagged Bim-Long control lysate. Lane 4: EE-tagged ID lysate. Lane 5: FLAG-tagged ID lysate. Numbers running vertically indicate sizes of protein ladder members in kDa. Exposure time: 1 minute.

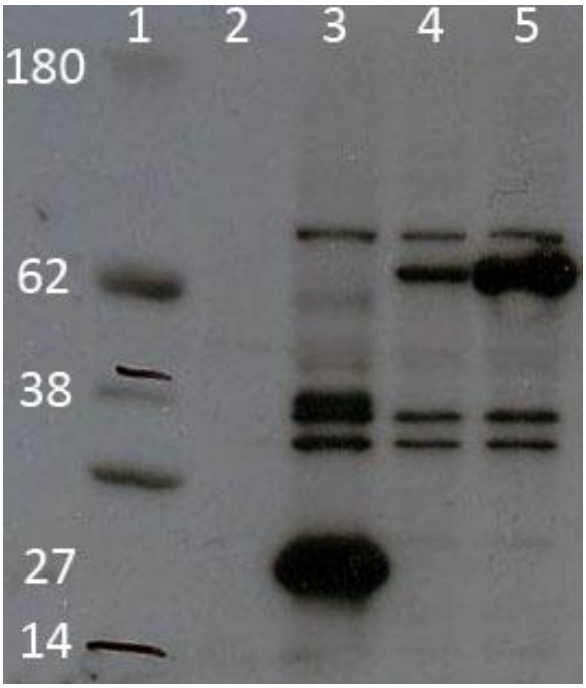

Figure 3.11 Successful Western Blot for EE tag. Exposed after probe for FLAG tag (lane 5). Numbers across top indicate lane numbers. Lane 1: protein ladder. Lane 2: untransfected lysate. Lane 3: FLAG-tagged Bim-Long control lysate. Lane 4: EE-tagged ID lysate. Lane 5: FLAG-tagged ID lysate. Numbers running vertically indicate sizes of protein ladder members in kDa. Exposure time: 2 minutes.

Chapter 4. Discussion

4.1 Bioinformatics

Bioinformatics was a key area of study in this investigation. Bioinformatic databases and techniques yielded the ID position (Fig. 3.1), predicted its secondary structure (Table 3.4) and characteristics (Table 3.3), and allowed sequence similarity searches (Table 3.1, Table 3.2). Without these findings, this investigation would not have progressed. Unfortunately, no genome sequences have been completed for GINs, and their *fasn* genes could not be found.

4.2 DNA manipulation

The process of amplifying plasmid-specific ID inserts involved designing various primers with differing restriction enzyme cut sites, stop and start codons, and tags for inserts to be ligated into pPD49.78-MCS. At the beginning of the project, it was thought that the ID could be amplified from *C. elegans* gDNA and ligated directly into the expression vectors. Two problems, however, arose from this strategy. Firstly, the plasmid-specific primers failed to amplify the ID. Secondly, the amplified ID would not ligate into the expression vectors. These two problems prompted two far-reaching changes to the PCR and cloning strategy.

A nesting PCR strategy was chosen after initial failures in amplifying ID directly from *C. elegans* gDNA. In place of gDNA, a 1:10 dilution of nested PCR product was successfully used as the template DNA from which the ID was amplified.

The nested primers bound to sites approximately 50 base pairs further out from the ID than the previous primers. The nested primers consisted wholly of complementary sequence, which increased their ability to anneal to the gDNA template.

A subcloning strategy was chosen because no ID-containing clones were created after numerous attempts at transforming competent bacterial cells with amplified ID. When the expression vectors were sequenced, they were found to contain either no insert, or an insert of a different sequence to the ID. An extra step was added, in which the ID was ligated into the generic cloning vector pGEM-T Easy. This vector was found to transform the competent bacterial cells much more efficiently than the expression vectors did. Once the presence of the ID in pGEM-T Easy was confirmed via sequencing, the transformed colony was miniprepped to isolate the plasmid. The ID insert was excised, and was able to be ligated into the expression vector at much higher efficiencies than ligating amplified ID.

With this strategy, the mammalian expression vectors pCAN-EE and pEF-FLAG were produced with the ID insert, and successfully transfected HEK-293T cells, which expressed the ID. However, even with these two changes to procedure, no plasmids to express the ID in *C. elegans* had been made by the time this investigation had to come to an end.

4.3 Mammalian ID expression

HEK-293T cells successfully expressed the transfected ID. This was shown in the Western Blots, which shows the presence of tagged protein at around 70kDa, approximately the size of the ID protein expected (Table 3.3). However, the HEK-293T transfections were repeated after the first set of Western Blots failed to show the presence of any tagged protein at the size expected. It is unknown why the first group of transfections failed while the second group succeeded, as the same plasmid source and transfection conditions were used in both cases.

The HEK-293T cells used to express the ID protein are a very popular and commonly used cell line. They were developed from human embryonic kidney cells transformed through the addition of DNA from human adenovirus type 5; these cells are called HEK 293 cells (Graham *et al.* 1977). Simian virus 40 tumour antigen (T antigen) DNA was further added to these HEK 293 cells to make HEK-293T cells which feature improved foreign DNA replication and expression (Stillman & Gluzman 1985).

Techniques used to transfect mammalian cells include electroporation, viral based methods, and chemical based methods. To transfect HEK-293T cells, FuGENE® HD (Roche) was chosen as chemical techniques are technically simple and do not need expensive specialist equipment (Yalvac *et al.* 2009).

The successful expression of tagged *C. elegans* FASN ID is the most important finding arising from this investigation. It shows that the region selected is capable of being expressed on its own, not as part of the full enzyme, and in a mammalian cell line.

Although human FASN and various fragments of it have been expressed previously in *E. coli* (Jayakumar *et al.* 1997, Wakil et al., 2001, Kridel *et al.* 2004), so far as the author is aware, this is the first time that a tagged domain of *C. elegans* FASN has been expressed in a mammalian expression system. This shows that codon bias does not prevent expression of the *C. elegans* protein in HEK-293T cells.

4.4 Conclusions and future directions

4.4.1 Conclusions

This work has shown that the ID in *C. elegans* can be estimated, amplified, cloned, and be confirmed to express in a mammalian cell line via Western Blot. This is significant because, as far as the author is aware, it marks the first comprehensive study of the animal FASN ID that begins with locating the sequence and ends with ID sequences expressed on their own without any other FASN domain.

Since the genomes of important parasitic nematodes have not yet been sequenced, there is no way to tell how similar their ID sequences are, or if a drug designed to bind to *C. elegans* ID will bind to GIN ID. However, with new genome sequences constantly becoming available, the most important nematode parasites should be sequenced within the next few years.

As stated at the beginning of this thesis, this project has been a first step towards the possible development of a completely novel veterinary anthelmintic. This investigation has elucidated the probable sequence of the *fasn-1* ID in *C. elegans.* Moreover, as far as the author is aware, this study demonstrates the first amplification and expression (in any expression system) of the FASN ID without any other catalytic domains.

4.4.2 Future directions

Time constraints left some work incomplete, and it is these experiments that should come first in any further investigations building on the work documented in this thesis. These include coimmunoprecipitation to determine whether the expressed ID is capable of dimerising on its own. Also discussed is *in vivo C. elegans* expression of ID, for which the plasmids could not be created in the course of this investigation. Finally, some aspects of possible drug creation are discussed.

4.4.2.1 Coimmunoprecipitation

Coimmunoprecipitation (coIP) is a critical experiment that is the next logical step in the investigation of the *C. elegans* FASN ID. Its purpose would be to show whether or not the ID expressed is capable of dimerisation. It is vital for the continuation of this line of research that ID expressed can dimerise. If it can, then it can be assumed that its structure remains the same whether it is expressed as part of FASN or, as done in this thesis, even though it is expressed alone and in different cells. This is important because peptides will be tested for binding affinity to the expressed ID. Currently, the dimerisation of the ID monomers expressed in 293T cells is untested.

Because the transfected 293T lysates have been retained, a coIP experiment would be simple. Phizicky & Fields (1995) describe briefly that, after cell lysates are generated, antibodies are added, antigens are precipitated and washed, and bound proteins are eluted and analysed. In our case, this analysis would entail another Western Blot.

Protein-protein interactions are at the core of most cellular processes (Phizicky & Fields 1995), and the understanding of them is central to understanding the role of the ID in animal FA biosynthesis. Phizicky & Fields (1995) call coIP a 'classical' method in detecting protein-protein interactions, and one that has been popularly used in research since its inception. It has advantages over other methods of inferring protein-protein interactions; because a lysate is used, the proteins of interest are likely to be folded correctly with correct post-translational modifications. However, one disadvantage is that coIP is not as sensitive as other methods such as affinity chromatography, because of lower antigen concentrations in the cell lysate used in coIP (Phizicky & Fields 1995).

A coIP procedure using the tagged ID expressed in this investigation is summarised here. FLAG- and glu-glu-tagged ID are expressed and mixed together. Antibodies against one of the tags (e.g. FLAG) and added to the ID mix. Protein G-sepharose is added, rendering the antigen-antibody complex insoluble and able to be pelleted by centrifugation. A Western Blot is then performed using the precipitated protein, and the presence of the other tag is detected by HRP-conjugated antibodies (in this case, anti-glu-glu antibodies).

The principle here is that if ID protein is precipitated using one antibody, and detected on a Western using the other antibody, then the ID has dimerised. The precipitated ID dimer in this case is essentially a heterodimer of FLAG- and glu-glu-tagged ID monomers. In this manner, the dimerisation of expressed ID can be demonstrated. The ID must pass this test in order to move on to finding binding proteins for therapeutic use, because binding implies that the ID is expressed principally in its native state and will behave the same way *in vitro* as *in vivo*.

4.4.2.2 *In vivo* ID overexpression in *C. elegans*

At the same time as plasmids were being made to express *C. elegans* FASN ID in 293T cells, attempts were made to utilise the *C. elegans* expression plasmid pPD49.78-MCS. Unfortunately, the plasmids were never successfully produced. The relevant ID inserts were amplified from gDNA, and they were successfully ligated into the generic subcloning vector, but no bacterial colonies were found to have been transformed by pPD49.78-MCS that contained the insert. It would be relevant to the project if the plasmids could be produced so ID could be expressed in *C. elegans*. It is hoped that this will be done by future researchers of this line of study.

It has been hypothesised that the heat shock-induced overexpression of the *C. elegans* ID after pPD49.78-MCS-ID microinjection would result in death through the binding of the overexpressed ID to the ID regions of endogenous FAS, thus preventing dimerisation of the functional enzyme (Grant, 2009).

However, no tagged ID could be successfully ligated into the pPD vectors before the end of the project, although FLAG and c-myc tagged ID for ligation into pPD were successfully ligated into pGEM-T Easy.

The pPD vectors containing the tagged ID sequence were to have been microinjected into Bristol N2 strain *C. elegans* to create stable transgenic lines as previously described (Fire, 1986). This work should be undertaken in future.

4.4.2.3 Creation of a drug

As previously discussed, the end goal for this line of research, of which this thesis is merely a first step, is to create a marketable veterinary anthelmintic that binds to the GIN FASN ID and inhibits dimerisation. This inhibitor will likely be a small peptide. To find a small peptide inhibitor, known ligands can be tested, and phage display can be employed to search for binding peptides.

The use of rationally-designed small peptides as drugs for protein-protein binding inhibition can face challenges such as low stability and short plasma half-lives, but also have some advantages over non-peptide drugs such as high selectivity and low toxicity (Ricklin & Lambris 2008).

Protein-protein interactions make difficult targets for small-molecule inhibition, and there are few reports of small molecular drugs disrupting protein-protein interactions (Cochran 2000). However, researchers have been able to inhibit protein dimerisation by using peptides derived from the dimerisation domain (Cochran 2000).

One example is the homodimeric HIV protease being prevented from dimerising when peptides derived from the dimerisation interface are bound (Cochran 2000). Consequently, candidate peptides to inhibit FASN dimerisation in nematode parasites of ruminants could possibly be found in the ID. The ID consists of at most 626 amino acids; a conservative estimate and a number large enough to warrant further investigation. The other source of dimerisation inhibitors are random peptide libraries of unknown binding affinity to the ID. At least 10^8 random peptides are panned against a protein to check for binding using phage display. A binding peptide may be found to be similar to a known ligand, or it may be a completely novel peptide whose binding could not have been predicted (Cochran 2000). Once binding peptides are found, further optimisation can allow them exquisite binding specificity (Cochran 2000), which is very desirable in a drug.

4.4.2.4 Phage display – the search for binding peptides

Smith & Petrenko (1997) describe the principle of phage display (Fig. 4.1) as a process of *in vitro* evolution, where a ligand's 'fitness' depends on its ability to bind to a target protein. With non-binding peptides being discarded, progress towards finding strongly-binding peptides is 'automatic' (Smith & Petrenko 1997). While many alternative peptide display platforms have been developed, the original phage display system (Fig. 4.1) remains the most commonly used (Sidhu & Koide 2007).

Peptide libraries of enormous size must be used to find binding ligands. When Li *et al.* (2002) used phage display to find inhibitors of the malarial parasite *P. falciparum* surface protein AMA1 (involved in erythrocyte invasion), a phage library displaying $>10^8$ random 15-mer peptides was panned and yielded three binding peptides.

Figure 4.1 Phage display is evolution in a closed system. A pool of filamentous bacteriophage is panned against a protein of interest. The selection pressure is binding to the immobilised antigen. Adapted from Sidhu & Koide (2007).

Foreign peptides are inserted into the genes coding for coat proteins in a filamentous phage strain such as M13 (Smith & Petrenko 1997). The encoded polypeptide sequences are then expressed on the outside of the phage protein coats, giving clear genotype-phenotype linkage (Sidhu & Koide 2007).

Ricklin & Lambris (2008) set out a straightforward pathway for the rational design of small peptide inhibitors using the phage display technique. They describe the discovery of compstatin, a small cyclic peptide found via phage display to bind and inhibit a critical protein in the complement activation cascade.

This involved the panning of $2x10^8$ random clones in a 27-mer peptide library for binding affinity to a complement-activating protein. When a peptide was found, they found the smallest peptide fragment of the 27-mer that resulted in no loss in binding affinity. After that, the peptide was gradually improved by residue substitution.

The peptide was then optimised for activity, specificity, stability and ease of expression in *E. coli* by at least 6 different competing research groups. Phase I clinical trials for compstatin are now in progress. Their review article gives us a basic program of drug development that can be followed in any future searches for ID-binding anthelmintics.

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