UNIVERSITY OF SOUTHAMPTON

FACULTY OF NATURAL AND ENVIRONMENTAL SCIENCES

Centre for Biological Sciences

Probing the bacterial pathogenesis of ESKAPE species utilising *C. elegans* as a model system

by

Euan Scott

Thesis for the degree of Doctor of Philosophy

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ABSTRACT

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PROBING THE BACTERIAL PATHOGENESIS OF ESKAPE SPECIES UTILSING *C.ELEGANS* AS A MODEL SYSTEM

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The nematode *C. elegans* is widely used as a model organism throughout biology, including as a host for bacterial infections. *C. elegans* can be exposed to a range of bacterial strains through presentation of bacteria as potential food sources in the form of a lawn on agar plates and can subsequently be observed. This thesis specifically examined strains from three Gram-negative species of the ESKAPE group, a group of troublesome antibiotic resistant and pathogenic bacterial pathogens. A range of strains from the Gram-negative ESKAPE species *Klebsiella pneumoniae*, *Acinetobacter baumannii* and *Pseudomonas aeruginosa* were examined in *C. elegans,* and compared against *E. coli* OP50, the standard laboratory food source of the animal as a control. This allows similarities and differences between different bacterial strains to be identified as to how they affect *C. elegans* biology. *C. elegans* populations on lawns of OP50 show a progressive increase in leaving the bacterial lawn after extended exposure. This enhanced food-leaving was determined to be driven by *C. elegans* larvae that are produced by adult animals on the bacterial lawn. This was dependent on a homologue of the mammalian hormone oxytocin and indicates that this behaviour is related to signalling that controls parental behaviours in mammals. When *C. elegans* populations were examined on lawns of ESKAPE bacteria, some bacterial strains significantly reduced *C. elegans* lifespan, indicating pathogenicity, with different degrees of virulence being observed. In addition, individual pathogenic bacterial strains were found to generate a food aversion response in *C. elegans*, a previously reported indicator of bacterial pathogenicity. Further investigation revealed that neither colonisation nor the subsequent clearance of bacteria from the *C. elegans* intestine underpins the differential pathogenicity of these bacterial strains. Further analysis showed that neuropeptides are key modulators of the process of colonisation and act in both pathogenic and benign bacteria. Analysis of the food aversion provoked by pathogenic bacteria revealed that signalling by biogenic amines acts to modulate this behaviour. Specifically, serotonin acts to promote the food aversion response and octopamine acts to supress food aversion to specific pathogenic strains. From undertaking all these investigations, it can be seen that individual bacterial strains can exert diverse biological effects on *C. elegans* as a model host, and the comparative approach taken in this thesis allows insight to be made about individual and diverse bacterial strains. The result in this thesis provide further avenues for investigation as to how bacterial pathogenesis is mediated in *C. elegans* as a model host organism, specifically in terms of virulence factors present in different bacterial strains and further neural controls of the *C. elegans* biological response to bacteria.

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Academic Thesis: Declaration Of Authorship

I, Euan Scott, declare that this thesis and the work presented in it are my own and has been generated by me as the result of my own original research.

'Probing bacterial pathogenesis of ESKAPE species utilising *C. elegans* as a model system'

I confirm that:

1. This work was done wholly or mainly while in candidature for a research degree at this University;
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3. Where I have consulted the published work of others, this is always clearly attributed;
4. Where I have quoted from the work of others, the source is always given. With the exception of such quotations, this thesis is entirely my own work;
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6. Where the thesis is based on work done by myself jointly with others, I have made clear exactly what was done by others and what I have contributed myself;
7. Parts of this work have been published as: Scott E, Hudson A, Feist E, Calahorro F, Dillon J, de Freitas R, Wand M, Schoofs L, O'Connor V, Holden-Dye L (2017): An oxytocin-dependent social interaction between larvae and adult *C. elegans*. Sci Rep. 2017 Aug 31;7(1):10122. doi: 10.1038/s41598-017-09350-7.

Signed: Euan Scott

Date:

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Chapter 1: General Introduction

1.1 Introduction to Caenorhabditis elegans

Nematode worms are observed in almost every environmental niche on the planet. Nematodes account for four out of every five animals in the world, with 25,000 currently described species (Zhang, 2013). Many members of the nematode phylum are parasites, with different species being parasitic towards animals and plants and responsible for huge economic and medical costs (Blaxter, 2011). However, many species of nematodes do not require a host organism to survive and are free-living. Since first being utilised in the 1960s, the free-living nematode species *Caenorhabditis elegans* has been as a model organism in virtually all realms of biology (Brenner, 1974; Corsi *et al.*, 2015). The natural niche of *C. elegans* is in fermenting environments in temperature climates across the world. These environments are extremely rich in bacteria, which are the food source of *C. elegans (*Felix and Braendle, 2010). *C. elegans* exists primarily as a self-reproducing hermaphrodite (Figure 1.1), although males are present at a low frequency and can be produced in the lab (Brenner, 1974).

The hermaphroditic nature of *C. elegans* allows genetically identical populations to be produced, and large numbers of worms can be obtained easily as the *C. elegans* life cycle goes from embryo, through four larval stages, to fertile adult in 4 days at the standard laboratory temperature of 20°C (Figure 1.2). This lifecycle occurs quicker at 25°C and slower and 15°C (Lee and Kenyon, 2009). *C. elegans* also has a very well described developmental biology, with the development of each of the 959 cells that are in the *C. elegans* hermaphrodite adult body from the embryo having been described (Sulston and Horvitz, 1977). The adult body also has a very simple anatomy and is translucent, allowing for easy visualisation of individual organs and tissues. *C. elegans* genetics are also well developed, with both a sequenced genome, and large mutant library. The database WormBase contains very well annotated descriptions of gene sequences and mutant phenotypes (www.wormbase.org). The well described genetics of *C. elegans* allows the roles of individual of genes in controlling various aspects of *C. elegans* biology to be identified (Genome sequence of the nematode *C. elegans*: a platform for investigating biology, 1998).

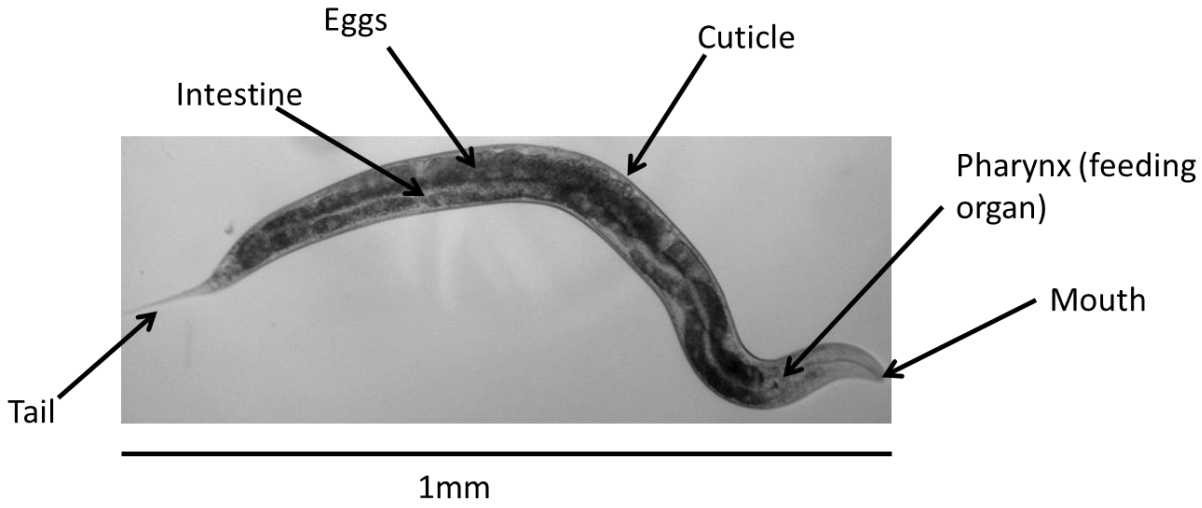


Figure 1.1: A *C. elegans* adult hermaphrodite. Image taken at 100x magnification

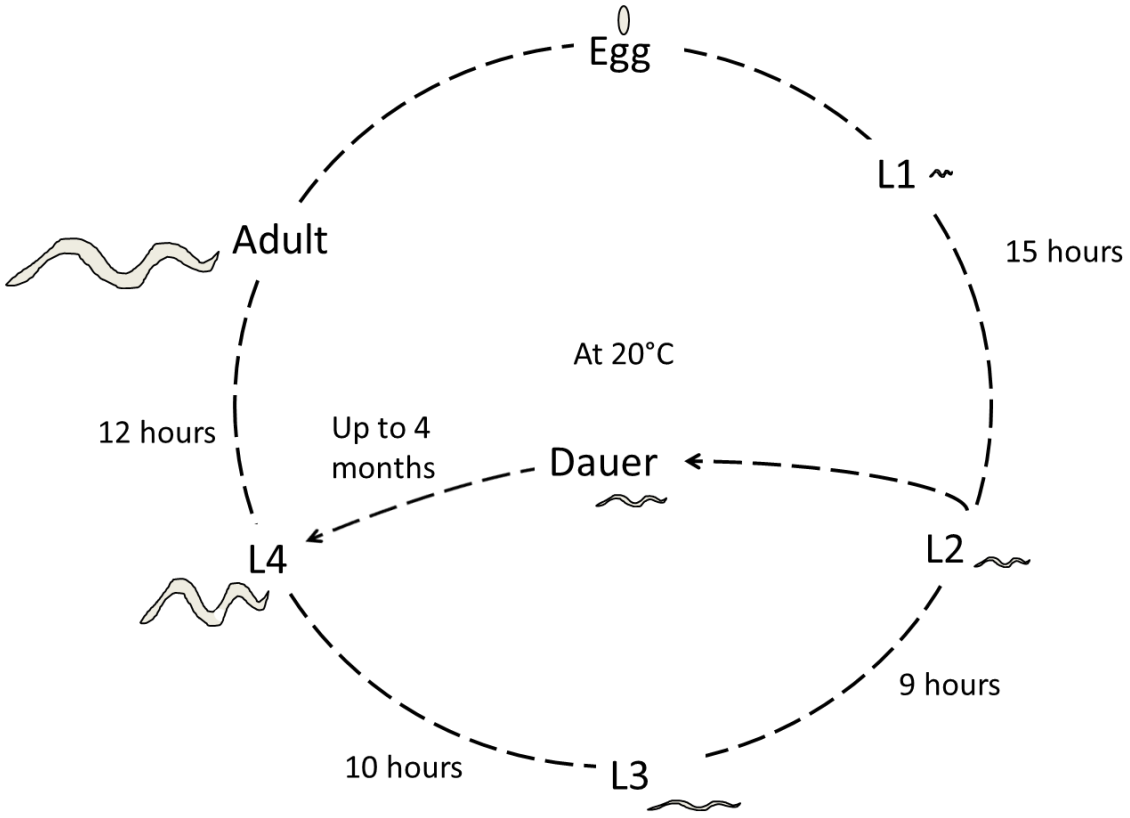


Figure 1.2: The *C. elegans* life cycle at 20°C (L1-4 indicates larval stage 1-4) *C. elegans* larvae form dauer stage upon overpopulation, high temperatures and starvation.

1.1.1 *C. elegans* as a bacterial infection model

Since its initial establishment as a model organism *C. elegans* has been utilised in many fields of biology, including research into ageing, metabolism, neuroscience and developmental biology (Mullaney and Ashrafi, 2009; Hobert, 2010; Kenyon, 2010; Chen *et al.*, 2015). Since 1999, *C. elegans* has been established as a model for a new aspect of biology, bacterial infections (Tan *et al.*, 1999a). This was initially established in studies with the bacteria *Pseudomonas aeruginosa*, a well-defined opportunistic pathogen of humans, and a species which is also present in the natural environments of *C. elegans* (Tan *et al.*, 1999a; Mesaros *et al.*, 2007). In the initial studies animals exposed to the clinical *P. aeruginosa* isolate PA14 died at a faster rate than that which was seen when animals were cultured on the standard laboratory food source of *E. coli* OP50 (Tan *et al.*, 1999a). *C. elegans* has since been used as a model host for human pathogens such as *Salmonella enterica*, *Staphylococcus aureus* and *epidermidis, Serratia marcescens Klebsiella pneumoniae* and *Yersinia pestis*(Aballay *et al.*, 2000; Garsin *et al.*, 2001; Pujol, 2001; Darby *et al.*, 2002; Begun *et al.*, 2007; Kamaladevi and Balamurugan, 2015). In addition to human pathogenic bacteria, *C. elegans* has also been used as a model host for invertebrate and plant pathogens, in addition to pathogenic fungi and viruses (Sifri *et al.*, 2005; Breger *et al.*, 2007; Félix *et al.*, 2011; Marsh and May, 2012; Schulenburg and Félix, 2017).

1.2 Introduction to bacterial infection models

1.2.1 The threat of antibiotic resistant bacteria

In the current clinical environment modelling bacterial infections is essential. Despite the development of antibacterial compounds allowing the successful treatment of bacterial infections, the incredible adaptability of bacteria and the misuse of antibiotics has led to the emergence of antibiotic resistance being a major healthcare threat in the current climate (Spellberg *et al.*, 2008; Chang *et al.*, 2015). The emergence of pathogenic bacteria which are resistant to antibiotics has resulted in bacterial infections being increasingly fatal (Spellberg *et al.*, 2008). The risk of antibiotic resistant bacteria is particularly stark in the case of hospital acquired or nosocomial infections. In healthcare establishments, patients with

weakened immune systems as a result of pre-existing medical conditions can develop potentially lethal infections caused by bacteria which can readily exist on surfaces in environments (Santajit and Indrawattana, 2016). The lack of new antibacterial compounds being developed also means that procedures that are currently benign such as caesarean sections and joint replacements will soon become very risky due to the risk of infections developing (O'Neill, 2016). The risk of antimicrobial resistance is so severe that by the year 2050 bacterial infections are predicted to become responsible for 10 million deaths a year(O'Neill, 2016).

1.2.2 Modelling bacterial pathogenesis using animal models

As a result of the threat posed by antimicrobial resistant, it is essential that further research is undertaken to investigate how bacteria cause infectious disease, and subsequently identify potential routes of treatment for the infections these bacteria cause. Modelling bacterial infections allows identification of the mechanisms of pathogenesis, drug resistance mechanisms and any host responses, in addition to development and identification of novel treatments. This can take a number of approaches, with perhaps the most popular utilising whole animals as surrogates for humans. This allows investigation of how the biology of both the host organism and the pathogen responds following infection. In order to properly study bacterial pathogenesis, it is important that different aspects of these infections are modelled in order to gain a complete understanding about a particular condition. For example it is important in some cases to directly model the human physiology, as many infections occur specifically on certain tissues, such as the respiratory system or the blood stream (Jiminez *et al.* 2015). In addition, it can sometimes be important that a model can be easily manipulated. This allows a broad insight to be gained into the interaction between the host and pathogen. In other cases, especially when different bacterial strains or a range of new treatments is being tested, rapid generation of results and taking a high throughput approach is beneficial.

1.2.2.1 Vertebrates as bacterial infection models

Due to their similar physiology mammalian models tend to be used to model bacterial infections. Mice *(Mus musculus)* is the organism which is most commonly used in

investigations into bacterial pathogenesis (Buer and Balling, 2003; Cossart and Sansonetti, 2004; Mathur *et al.*, 2012; Kidwai *et al.*, 2013). However, mice are not the only vertebrates used in bacterial infection studies. Rats, zebrafish, pigs, and non-human primates have all been used in bacterial infection studies (Jiminez *et al.*, 2015). All of these organisms possess certain traits that are advantages when it comes to modelling bacterial infections, as well as associated disadvantages. For example zebrafish are cheaper than other vertebrate models and are easier to maintain than other organisms, but have different nutritional requirements to mammals (Neely *et al.*, 2002; Jiminez *et al.*, 2015). Alternatively, pigs and primates have much more similar to physiology to humans, but also require a lot of personnel training to handle and can generate overwhelming amounts of tissue for analysis (Jiminez *et al.*, 2015).

1.2.2.2 Invertebrates as alternative infection models

Despite the obvious benefits of using other vertebrates to model human bacterial infections, recent efforts have acted to expand the range of animal infection models utilised in research. This has been driven in part by the increasing awareness of animal welfare issues relating to scientific research as embedded within the 3Rs principle. This principle was established in 1959 by Russel and Burch with the 3Rs representing Replacement, Reduction and Refinement related to the procedures using animals in research (Flecknell, 2002; Graham and Prescott, 2015). Along with changing scientific practice to make it more ethical, the 3Rs principle also acts to address widespread concerns about the numbers of animals as specified in the Animals (Scientific Procedures) Act from 1986 that are used in scientific procedures (Graham and Prescott, 2015).

As a result of the 3Rs principle being enacted in the case of studies investigating bacterial pathogenesis, the model organisms used to investigate bacterial infections in humans have changed. This includes using cell cultures in order to mimic mammalian organs (Xu *et al.*, 2013; Harrison and Diggle, 2016). Increasingly, invertebrates are used for modelling bacterial infections. The use of invertebrates in scientific research is not affected by any ethical legislation governed by the 3Rs (Graham and Prescott, 2015). In addition to these ethical benefits over vertebrate models, invertebrates have a number of other attributes

which makes them attractive biological models. Invertebrates are cheaper to obtain and experiment on than their vertebrate counterparts (Cook and McArthur, 2013). As an example of the costs benefits in using invertebrate models, a strain of *C. elegans* can be obtained for $7 from the *Caenorhabditis* genetics centre (https://cbs.umn.edu/cgc/home) and can be propagated indefinitely. Alternatively, mice have to be ordered individually and subsequent propagated in regulated facilities. This results in a much greater cost than is needed to acquire invertebrates for analysis. In addition, the rapid life cycles and short generation times of invertebrates relative to that of vertebrate organisms allows rapid generation of results and large numbers of subjects to be used, enabling high throughput analysis to be undertaken.

The most common invertebrate models used in bacterial infection studies are *C. elegans*, the fruit fly *Drosophila melanogaster* and the larvae of *Galleria mellonella,* the greater wax moth (D'Argenio *et al.*, 2001; Sifri *et al.*, 2005; Mylonakis *et al.*, 2007; Nehme *et al.*, 2007; Cook and McArthur, 2013). Both *D. melanogaster and G. mellonella* have been used as model hosts for a wide range of pathogens, including bacteria. *D. melanogaster* has been analysed in studies with *P. aeruginosa* and *Serratia marcescens,* and has been utilised to identify both bacterial determinants of infection and key immune pathways(D'Argenio *et al.*, 2001; Nehme *et al.*, 2007; Daisley *et al.*, 2017). *G. mellonella* has been used to investigate the pathogenesis of *Streptococcus pneumoniae, Acinetobacter baumannii, Klebsiella pneumoniae,* numerous *Bacillus* species and *P. aeruginosa* and has enabled effective testing of novel antimicrobial compounds and has highlighted key virulence differences between different bacterial strains (Evans and Rozen, 2012; Wand *et al.*, 2012; Loh *et al.*, 2013; Wand *et al.*, 2013; Benthall *et al.*, 2015).

1.3 Advantages and limitations of *C. elegans* as a bacterial infection model

As summarised in 1.1.1, *C. elegans* has been used for a modelling a variety of different infections. In addition to the advantages of using invertebrate models as described in 1.2.2.2, *C. elegans* possesses individual advantages which make it an attractive organism for modelling bacterial infections. Both the advantages and limitations of *C. elegans* as a bacterial infection model are outlined below.

1.3.1 Advantages of *C. elegans* as a bacterial infection model

1.3.1.1 Experimental exposure of *C. elegans* to bacteria

In its environment *C. elegans* encounters a diverse range of bacteria as prospective food sources, but this will include potential pathogens (Felix and Braendle, 2010; Samuel *et al.*, 2016).Thanks to the natural environment of the organism, *C. elegans* can be easily exposed to different bacteria in the laboratory. Laboratory cultured *C. elegans* are maintained by being plated on Petri dishes filled with NGM (Nematode growth media) agar seeded with lawns of bacteria (Stiernagle, 2006). Traditionally the *E. coli* strain OP50, a derivative of the *E. coli* B strains, is used as the laboratory food source of *C. elegans*. This strain is a uracil auxotroph which allows control of the growth of bacterial lawns, enabling easy visualisation of the nematodes (Brenner, 1974; Brooks *et al.*, 2009; Clark and Hodgkin, 2014). Replacing lawns of OP50 with lawns of potentially pathogenic bacteria allows a simple comparison between the laboratory standard food source and the bacteria in question to be made.

The standard method used for *C. elegans* an experimental bacterial infection involves exposing *C. elegans* matured on *E. coli* OP50 to lawns of other bacteria. The worms are then monitored as they consume the bacteria. To investigate the relative virulence of different bacterial strains measurement of killing time of entire populations of *C. elegans* are made. This method of 'inoculating' *C. elegans* via oral consumption of bacteria provides a contrast to methods of inoculation in other infection models including the other invertebrate alternative infection models *D. melanogaster* and *G. mellonella*, which are injected with the bacteria in question (Lau *et al.*, 2003; Wand *et al.*, 2011).

1.3.1.2 High throughput exposure and genetic tractability

The life cycle of *C. elegans* allows large numbers of synchronised populations to be propagated at one time. The well-defined life cycle of *C. elegans* (Figure 1.2) allows individual larval stages to be identified. Worms can be individually picked the day before the assay when they are at the L4 larval stage, which are recognisable through the presence of a white spot indicating the developing vulva (Figure 1.3). These larvae will develop into reproducing adults the next day. The fact that these synchronised populations can be used removes the

possibility of any gene expression changes due to the different ages of the individual animals. The fast life cycle and high fecundity of *C. elegans* also allows large numbers of individual or populations of animals to be exposed to different bacteria at once. This is useful when it comes to screening a large number of different bacterial strains. In addition, *C. elegans* has a well-developed library of mutant strains that are easily obtainable. This means that *C. elegans* mutants deficient in certain genes can be screened against bacterial pathogens. This leads to the identification of genes involved in the response to infection (Ewbank, 2006). The clonal nature of *C. elegans*, where a single adult hermaphrodite can give rise to hundreds of genetically identical offspring, also allows for consistency of results. Investigating bacterial pathogenesis in different populations of animals with identical genetic backgrounds allows clear conclusions to be made without taking into account the genetic variability of the host organism. In addition, the N2 strain, initially isolated from a compost heap in Bristol, is universally used as a wild type strains in *C. elegans* laboratories across the world, allowing comparison between results obtained in different institutions to be made easily.

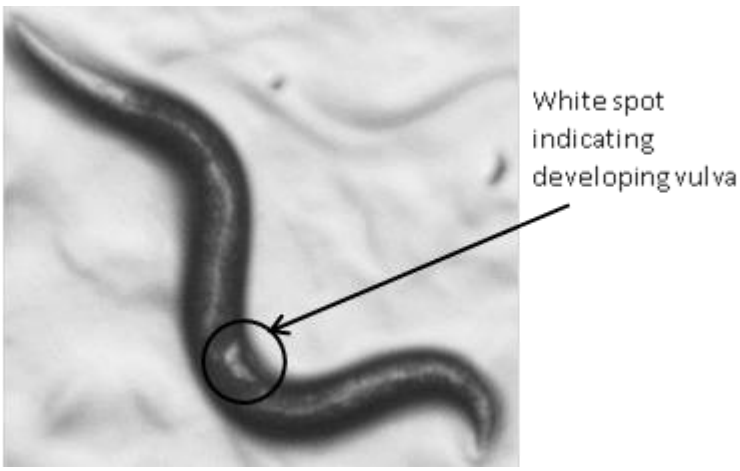


Figure 1.3: A *C. elegans* at larval stage L4. The presence of the developing vulva is used to identify L4 larvae which can be picked to form a population of developmentally synchronised adult animals the next day. Image taken at 60x magnification

1.3.1.3 *C. elegans* morphology

The simple morphology and stereotypical organization of tissues in *C. elegans* can be examined in infection studies (Figure 1.1). These tissues include the cuticle, intestine, gonad and feeding organ (the pharynx). This allows investigation of bacterial infections at distinct host microenvironments. This includes infections based on intestinal colonisation, cuticle adhesion and intracellular invasion (Jansson, 1994; Aballay and Ausubel, 2001; Darby *et al.*, 2002; Sifri *et al.*, 2003; Sifri *et al.*, 2005; Troemel *et al.*, 2008). The presence of bacteria in these different tissues provides routes to more discretely mimic key determinants of infection of vertebrate hosts. In contrast, other infection models like cell cultures and *G. mellonella* lack well defined organs, and thus are not as effective in modelling complex tissue-specific bacterial infections (Cook and McArthur, 2013; Harrison and Diggle, 2016).

The transparent nature of the *C. elegans* body also allows easy visualisation of these tissues. This transparent nature can be exploited when it comes to utilising fluorescent reporters. GFP-labelled bacteria can be used to examine their presence inside *C. elegans* during the infection process (Tan *et al.*, 1999a; Darby *et al.*, 2002).

In addition, genes of *C. elegans* can be engineered to act as fluorescent reporters of gene function as part of the response to infection (Julien-Gau *et al.*, 2014). Being transparent enables these particular reporters to be easily examined non-invasively in distinct morphological compartments under Ultraviolet light.

1.3.1.4 Innate immunity

The well-defined genetics of *C. elegans* have helped detail the multi-faceted innate immune system of *C. elegans* (Ewbank, 2006; Engelmann and Pujol, 2010). This biology reinforces the value of C*. elegans* to understand the host processes that may modulate infection or provide barriers to infection (Labrousse *et al.*, 2000; Ewbank, 2006; Kawli and Tan, 2008; Engelmann and Pujol, 2010). The immune signalling that occurs in *C. elegans* upon bacterial infection is discussed in 1.6.

1.3.1.5 Identification of virulence factors and therapeutic compounds

Mutant bacterial strains can be screened for virulence using *C. elegans* as the host using the high throughput approach described above (1.3.1.1). Identification of bacterial mutants which have attenuated pathogenicity allows the discovery and subsequent investigation of bacterial virulence factors. This has been performed in a range of bacterial species and has identified novel virulence factors as well as those conserved between infection of both vertebrate and invertebrate hosts (Tan *et al.*, 1999b; Kurz *et al.*, 2003; Tenor *et al.*, 2004)(1.5.2). This screening approach has also been used to identify novel antimicrobial compounds for treating of both bacterial and fungal infections (Desalermos *et al.*, 2011; Kong *et al.*, 2016).

1.3.2 Limitations of *C. elegans* as a bacterial infection model

1.3.2.1 Morphology

C. elegans does have some limitations in comparison to other infection models. Firstly, it lacks the precise organisation of tissue and organs that may have important underpinnings to infection of a human host. This is especially true when detailing infection and pathogenicity of nosocomial pathogens, which establish infection in specific tissues of immunocompromised patients. *P. aeruginosa,* is a particular threat in patients with cystic fibrosis or those with pre-existing wounds(Lyczak *et al.*, 2000). *C. elegans* does not have a respiratory system which is required to directly model such infections. Alternatively, mice and other vertebrate models have the lungs, bloodstream and skin necessary for modelling tissue-specific bacterial infections, allowing more accurate modelling of these kinds of infections.

1.3.2.2 Immune responses

Although it possesses a well described innate immune (1.3.1.4), *C. elegans* does not have an adaptive immune system. This is a key determinant of infection modulation in human hosts (Govind, 2008; Engelmann and Pujol, 2010). This means that *C. elegans* cannot inform the on the full range of immune interactions that occurs upon infection of a mammalian host.

However as indicated it has useful insights in wider concepts and specific details of how innate immunity contributes to the response to bacterial infection

1.3.2.3 Incubation temperature

Another limitation of using *C. elegans* as a model for bacterial infection is the incubation temperature. Bacterial cultures are traditionally grown at 37°C, the temperature in which human internal bacterial infections will occur (Bergman and Casadevall, 2010). *C.elegans* is not a thermoregulatory organism and are routinely incubated at 20°C (Stiernagle, 2006). At higher temperatures *C. elegans* undergoes a heat shock response and the lifespan of the organism is progressively reduced (Lee and Kenyon, 2009; Zhang *et al.*, 2015a). It has been demonstrated how temperature regulates production of virulence factors in bacteria, with expression of genes being upregulated at 37°C (Grosso-Becerra *et al.*, 2014; Thomas and Wigneshweraraj, 2014; Guijarro *et al.*, 2015). This temperature incubation limitation means that any temperature sensitive virulence factors secreted by the bacteria will not be produced at the lower temperatures used for *C. elegans* pathogenesis experiments.

1.3.2.4 Precise inoculation of pathogens

C. elegans infection studies involve exposing the worms to potential pathogens as a prospective food source in the form of a bacterial lawn. In contrast, the other invertebrate models *G. mellonella* and *D. melanogaster,* along with mice are often injected with pathogens, allowing the direct inoculation of bacteria entered into the host system to be determined and adjusted(Buer and Balling, 2003; Mylonakis *et al.*, 2007; Wand *et al.*, 2011; Mathur *et al.*, 2012). Alternatively, *D. melanogaster* can have pathogens laced in its food, providing multiple routes of inoculation for this model host (D'Argenio *et al.*, 2001; Mylonakis *et al.*, 2007).

The relative advantages and limitations of *C. elegans* as a bacterial infection model allows a comparison of different relevant aspects of model hosts used for bacterial infection studies (see Table 1.1).

Experimental feature	*M. musculus*	*C. elegans*	*D. melanogaster*	*G. mellonella*
Complex morphology	++	+	+	-
Sequenced genome	++	++	++	-
Mutant libraries	++	++	++	-
Ease of propagation	-	++	+	+
High throughput screening	-	++	-	++
Adaptive immune system	++	-	-	-
37°C incubation	++	-	-	++
Precise inocula	++	-	+	++

Table 1.1: The advantages and limitations of different bacterial infection models. More + indicates a bigger strength in a particular attritbue for an individual model .Advantages of *C. elegans*, as well as the other established invertebrate models *D. melanogaster* and *G. mellonella* are outlined, in comparison to the most commonly used model of mice (*Mus musculus*).Adapted from (Mylonakis *et al.*, 2007).

1.4 Natural interactions between *C. elegans* and bacteria

In this thesis in the interactions between *C. elegans* and bacteria in the context of pathogens will be investigated. However, in order to fully appreciate the interactions between *C. elegans* and bacteria in an infection context, it is important that the other biological interactions between the two organisms are understood.

1.4.1 Bacteria as a *C. elegans* food source

The most biologically relevant interaction between *C. elegans* and bacteria comes from the fact that bacteria are the diet of *C. elegans*. *C. elegans* foraging and feeding behaviour have evolved to identify and exploit food sources for maximum biological benefit (Avery, 1993; Avery and Shtonda, 2003; Avery and You, 2012). The interaction between *C. elegans* and bacterial food can be broken down into two principle components (Avery and You, 2012). The first of these is the actual feeding behaviour whereby *C. elegans* ingests bacteria and thus absorbs nutrients. The second of these is how *C. elegans* locates potential food sources and subsequently discriminates between different food sources.

1.4.1.1 *C. elegans* feeding

C. elegans ingest bacteria via the pharynx. The pharynx is a muscular pump situated between the mouth and intestine of the organism (Avery and You, 2012)(Figure 1.4). The pharynx and associated nervous system and support cells consists of 60 cells. Together these cells form an organ that allow the pharynx to function as a highly coordinated muscular pump underpinning *C. elegans* filter feeding (Avery and You, 2012) . The pharynx itself is made up of three parts: the corpus, isthmus and terminal bulb (Figure 1.4). Pharyngeal pumping consists of muscular contractions which draws in bacteria from the environment suspended in liquid from the outside environment. Contractions are followed by relaxations, which close the pharyngeal lumen, expelling liquid but keeping the bacteria trapped in the pharynx (Avery, 1993; Fang-Yen *et al.*, 2009). The ingested bacteria are carried towards the terminal bulb of the pharynx by a series of contractions of the pharyngeal muscles known as isthmus peristalsis (Fang-Yen *et al.*, 2009). Upon arriving in the terminal bulb, the bacteria are broken cells are disrupted by the plates of the grinder rotating. This allows nutrients that the bacteria provide to be absorbed by *C. elegans* (Fang-Yen *et al.*, 2009).

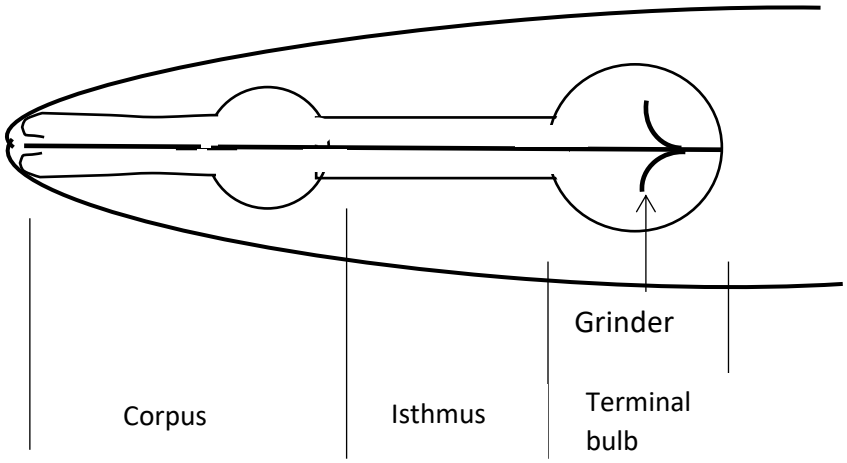


Figure 1.4 : Diagram of the *C. elegans* pharynx, located in the head of the organism adapted from Avery and Shtonda (2003)(see Figure 1.1 for location of the pharynyx in the organism).

1.4.1.2 *C. elegans* food seeking and discrimination

1.4.1.2.1 *C. elegans* foraging

It is essential for *C. elegans* to consume bacteria to thrive. Detection of bacteria and coordinating the response of *C. elegans* is thus important in order to ensure that C. elegans is able to feed. *C. elegans* is able to detect bacteria is through coordination of different sensory pathways. It has been determined how olfactory (sensing of volatile chemicals), gustatory (sensing of water-soluble chemicals) and mechanosensory (sensing of physical stimuli) pathways are all involved in detecting bacterial food sources (Sawin *et al.*, 2000; Bargmann, 2006; Cohen *et al.*, 2009; Kiyama *et al.*, 2012; Song *et al.*, 2013; Stein and Murphy, 2014).

When *C. elegans* are not on a bacterial lawn, animals exhibit a searching strategy in order to locate a food source (Hills *et al.*, 2004; Gray *et al.*, 2005). When *C. elegans* locates a bacterial lawn and feed on it, the animals switch between two distinct behavioural states. Predominantly (about 80% of the time) 'dwelling' behaviour is seen, which involves staying in a restricted area and slow movement speeds (Bargmann, 2006). Occasionally, animals switch to the 'roaming' state, where rapid locomotion and exploration of the bacterial lawn occurs (Bargmann, 2006).

1.4.1.2.2 *C. elegans* food discrimination

Analysis of *C. elegans* exposed to a wide range of bacteria from natural nematode populations has revealed that certain bacteria are beneficial and others are detrimental towards *C. elegans* (Samuel *et al.*, 2016). Bacteria that were classed as 'beneficial' supported *C. elegans* growth better, whereas 'detrimental' bacteria negatively affected *C. elegans* growth and led to upregulation of stress and immune reporter genes (Samuel *et al.*, 2016). It has been determined that isolates from the *Gluconobacter* and *Enterobacter* genera tended to be beneficial, whereas those of the *Xanthomonas, Chryseobacterium, Stenotrophomonas* and *Aeromonas* genera tended to be detrimental (Samuel *et al.*, 2016; Schulenburg and Félix, 2017). In the laboratory environment certain bacterial strains have also been demonstrated to support *C. elegans* growth better than other strains, with

smaller easily digestible bacteria supporting *C. elegans* growth better than bacterial strains larger with larger cell sizes (Avery and Shtonda, 2003). Analysis of the uptake of carboxylate beads by *C. elegans* has demonstrated that the animals more readily take up particles with a diameter between 0.5 and 1μm, further demonstrating there is a preferential for bacteria of a certain size as a *C. elegans* food source (Kiyama *et al.*, 2012).

C. elegans have also been shown to demonstrate a preference for bacteria which better support growth over strains which do not support growth as well (Shtonda and Avery, 2006; Abada *et al.*, 2009). Indeed, indications for this preference can be seen when given a choice between different food sources, and measuring the relative time spent on bacterial lawns. When exposed to lawns of lower quality food, *C. elegans* spend less time on lawns of these bacteria than on lawns of higher quality bacteria (Shtonda and Avery, 2006).

The results from these studies all highlight the ability of *C. elegans* to be able to respond and react to different bacteria. This demonstrates plasticity due to the integration of information obtained through interactions with different bacterial food sources.

1.4.2 Bacteria as *C. elegans* commensals

In addition to being a food source in the natural environment, bacteria have also recently been demonstrated to be a commensal of *C. elegans*. In the laboratory environment, *C. elegans* are sterile and any bacterial and fungal contaminants are removed to avoid the effect of any infection that might have occurred (Stiernagle, 2006). However, the range of bacteria that *C. elegans* encounters in the natural environment, and as a result is exposed to, is very broad (Felix and Braendle, 2010; Felix and Duveau, 2012; Samuel *et al.*, 2016). *C. elegans* are now known to form a microbiome from bacteria which animals encounter in the environment, which then go on to colonise the intestine of the organism, after avoiding mechanical disruption by the grinder (Figure 1.4) (Cabreiro and Gems, 2013). This implies that bacteria are not all broken down by the pharynx and can continue to remain viable in the post pharyngeal intestine. The ability of *C. elegans* to form a microbiome from bacteria that animals have been exposed to has been demonstrated two separate studies. In one study, *C. elegans* were sampled from the natural environment, and the bacteria found to be

associated with the animals was analysed (Dirksen *et al.*, 2016). The second of these studies took germ free *C. elegans* and exposed to collected soil samples, allowing them to assemble their own microbiome (Berg *et al.*, 2016). Both studies found that *C. elegans* develops a complex microbiome. In addition this microbiome remains remarkably similar across individual populations that have been exposed to different substrates (Berg *et al.*, 2016; Dirksen *et al.*, 2016). Indeed, the final composition of the C*. elegans* microbiome is actually quite different from the bacterial signature found in natural bacterial substrates, indicating either a selective ability of bacteria to colonise the C. elegans intestine or regulation by the host organism itself (Schulenburg and Félix, 2017; Zhang *et al.*, 2017). Overall, these microbiome studies revealed that certain families of bacteria have a particular propensity to be enriched in the *C. elegans* microbiome, including *Enterobacteriaceae*, *Pseudomonaceae* and *Xanthomonadaceae* (Schulenburg and Félix, 2017; Zhang *et al.*, 2017).

Up to a 1000 different species making up to 10^{14} cells, have been described in the human microbiome (Zhang *et al.*, 2015c). These bacteria in the intestine contribute to the immune system and regulate mineral absorption and vitamin production (Zhang *et al.*, 2015c). The benefits of colonising bacteria have also been described for the nematode. Certain bacterial commensals have protective effects against pathogen infection (Schulenburg and Félix, 2017). Both *Bacillus megaterium* and *Pseudomonas mendocina* protect against infection with *Pseudomonas aeruginosa* and *Enterobacter faecalis* protects against *Staphylococcus aureus* infection (Montalvo-Katz *et al.*, 2013; King *et al.*, 2016). As part of one of the microbiome studies, it was also demonstrated that *C. elegans* that had been colonised with certain *Pseudomonas* isolates were also protected against infection with the fungal pathogen *Drechmeria coniospora* (Dirksen et al., 2016). This, combined with the fact that bacteria represent food and therefore nutrient sources, demonstrates that the biology of *C. elegans* and environmental bacteria are intrinsically linked, and that bacteria can act to modulate other aspects of *C. elegans* biology.

1.5 Bacteria as *C. elegans* pathogens

The interactions of the worm with bacteria in the environment will also include a range of potentially pathogenic bacteria. Likely natural pathogens of *C. elegans* include *Pseudomonas*

aeruginosa, *Bacillus thuringiensis* and *Serratia marcescens*(Schulenburg and Félix, 2017). The fact that *C. elegans* has developed host pathogen interactions toward classes of bacteria that impact on human health reinforces the potential for its use in analysing the bacterial pathogenesis of clinically relevant bacteria.

A range of pathogens including Gram-negative and Gram-positive bacteria (Darby, 2005; Sifri *et al.*, 2005) have been studied using *C. elegans* as a host. In addition, pathogenic fungi and viruses have also been investigated using *C. elegans* as a host (Mylonakis *et al.*, 2007; Félix *et al.*, 2011).This approach includes examination of a range of human pathogens, in addition to invertebrate and plant pathogens (Tan *et al.*, 1999a; Hodgkin *et al.*, 2000; Bai *et al.*, 2014) As this thesis is focused on examining bacteria known to cause problematic infections in human patients, the subsequent sections will focus on bacteria which are human pathogens. In terms of how *C. elegans* is infections by bacteria occurs, a range of different routes has been determined. This is both in terms the types of pathogenesis that occurs, as well as the different virulence factors used to mediate the infection.

1.5.1 Mechanisms of bacterial pathogenesis in *C. elegans*

Bacterial infections in humans can take a number of forms, with individual species of bacteria having the potential to cause a number of different infections. *P. aeruginosa*, one of the bacterial species that will be examined in thesis (1.10.4) can cause respiratory, skin and urinary tract infections amongst others (Mesaros *et al.*, 2007). Whilst, as discussed in 1.3.2.1, the anatomy of *C. elegans* does not reflect the complex morphology of mammalian systems, different mechanisms of pathogenesis in some cases involving tissues can be observed in this very simple animal model. In initial bacterial pathogenesis studies of *C. elegans*, it was discovered that *P. aeruginosa* can kill *C. elegans* in two different ways (Darby *et al.*, 1999; Mahajan-Miklos *et al.*, 1999; Tan *et al.*, 1999a). These different types of pathogenesis were coined ‘slow’ and ‘fast’ killing and have since been observed with other bacterial pathogens. Slow killing was initially discovered by incubating worms on standard C*. elegans* media (NGM) with lawns of bacteria. During this incubation the killing effect observed as a reduced lifespan is observed on the bacteria over a number of days (Tan *et*

al., 1999a; Sifri *et al.*, 2003). In contrast, fast killing takes place with incubation on richer media and acts to kill following exposure over 48 to 60 hours (Mahajan-Miklos *et al.*, 1999; Tan *et al.*, 1999a).

1.5.1.1 C. elegans infection by intestinal colonisation

One of the mechanisms by which pathogenic bacteria are thought to kill *C. elegans* is through colonisation of the intestine or slow killing. This particular infection occurs upon feeding on a bacterial food source for an extended period of time. Any bacteria that avoid being broken down by the pharynx will be able to pass through the gut of *C. elegans*, and will be able to colonise the intestine. The tight regulation of intestinal pH, avoiding damage of the cells by excessively alkali or acidic conditions, indicates that this organ is a tightly controlled environment (Chauhan *et al.*, 2013). Studies with bacteria-sized particles and GFP labelled bacteria have demonstrated the ability of the *C. elegans* intestine to be colonised with bacteria (Tan *et al.*, 1999a; Kiyama *et al.*, 2012) (Figure 1.5). Studies have demonstrated that after feeding on lawns of pathogenic bacteria for an extended period of time, large numbers of bacteria accumulate in the *C. elegans* intestinal tract. This often results in colonisation induced distention of the intestine (Tan *et al.*, 1999a; Sifri *et al.*, 2003). After colonisation and distension of the intestine, the infection caused by the pathogenic bacteria subsequently leads to damage of host tissues and *C. elegans* death(Irazoqui *et al.*, 2010a).

Both Gram-negative and Gram-positive bacterial species have been described to be pathogens of *C. elegans* through this mechanism (Darby, 2005; Sifri *et al.*, 2005). The Gram-negative bacterial species in which this has been observed includes *Pseudomonas aeruginosa*, *Salmonella enterica*, *Serratia marcescens*, and *Burkholderia pseudomallei* and *cepacia* and *E. coli* (Tan *et al.*, 1999a; Aballay *et al.*, 2000; O'Quinn *et al.*, 2001; Kothe *et al.*, 2003; Kurz *et al.*, 2003; Sifri *et al.*, 2005; Fuursted *et al.*, 2012; Youn *et al.*, 2013; Kamaladevi and Balamurugan, 2015; Kuo *et al.*, 2016). The Gram-positive bacterial pathogens demonstrated to accumulate in the *C. elegans* intestine and cause mortality include *Enterococcus faecalis*, *Staphylococcus aureus* and *Staphylococcus epidermis* (Garsin *et al.*, 2001; Sifri *et al.*, 2003; Sifri *et al.*, 2005; Begun *et al.*, 2007). Symptoms that *C. elegans* display after these infections include reduced motility, a decrease in pharyngeal pumping, a distended pharynx and internal hatching of eggs (Tan *et al.*, 1999a; Sifri *et al.*, 2003;

Kamaladevi and Balamurugan, 2015). Studies with sterile *C. elegans* mutants have revealed that this latter symptom of larvae hatching inside the parent are not the key determinants of killing *C. elegans* (Tan *et al.*, 1999a; Sifri *et al.*, 2003). It has been found that live bacteria are required to cause these particular kinds of infections, and that bacteria that have been killed through heat treatment do not act to kill *C. elegans*, indicating proliferation of live bacteria is central to driving the negative impact of this infection (Tan *et al.*, 1999a; Aballay *et al.*, 2000).

However, differences between the pathogenesis of bacterial species in *C. elegans* have been described (Sifri *et al.*, 2003). For example, it has been demonstrated that *S. enterica* has an ability to elicit death in *C. elegans* germ cells, a phenomenon not reported upon infection with other species (Aballay and Ausubel, 2001). Big differences in the symptoms seen in *C. elegans* upon infection with *Pseudomonas aeruginosa* and *Staphylococcus aureus* have been described (Irazoqui *et al.*, 2010a). Specifically whilst infection with both species led to intestinal distention, *P. aeruginosa* exposure lead to accumulation of extracellular material, intracellular invasion and abnormal autophagy, whereas *S. aureus* infection led to *C. elegans* exhibiting anal deformation and lysis of intestinal cells (Irazoqui *et al.*, 2010a). This demonstrates how even under the umbrella of slow killing infections defined early on in the examination of *C. elegans* bacterial pathogenesis, individual bacterial species have different aspects to their particular mechanisms of pathogenesis (Tan *et al.*, 1999a; Irazoqui *et al.*, 2010a).

1.5.1.1.1 Differential persistence of bacterial pathogens in the *C. elegans* intestine

An important aspect of the pathogenesis of bacteria which is based on intestinal colonisation is the relative ability of the different bacteria to proliferate and maintain a presence in the *C. elegans* intestinal lumen. This can be determined by exposing *C. elegans* to a lawn of pathogenic bacteria and subsequently being removed from the pathogen and presented with a benign bacterial food source. The subsequent levels of bacteria inside the animal can then be analysed (Kawli and Tan, 2008). It has been discovered how *E. faecalis, S. marcescens* and *S. enterica* all elicit these persistent infections (Aballay *et al.*, 2000; Labrousse *et al.*, 2000; Garsin *et al.*, 2001; Kurz *et al.*, 2003). For example, *C. elegans* infected with *S. enterica* subsequently exposed to *E. coli* OP50 show a similar lifespan to

animals infected with the pathogen alone, demonstrating the ability of the *S. enterica* bacteria to persist within the nematode intestine (Aballay *et al.*, 2000; Sifri *et al.*, 2005). Similar effects have also been shown when *C. elegans* fed on a mixture of a small amount of pathogenic bacteria with a large amount of non-pathogenic bacteria, the nematodes still became infected and died as a result of exposure to the pathogen (Aballay *et al.*, 2000; Garsin *et al.*, 2001). In contrast to these more persistent bacteria, it has been determined that *S. aureus* is removed from the *C. elegans* digestive tract very quickly after animals are transferred from *S. aureus* lawns to non-pathogenic bacteria (Sifri *et al.*, 2003). These differences in pathogenesis as well as the relative persistence of these bacteria that have been found to accumulate in the *C. elegans* intestine and cause infections gives insight into how bacterial pathogenesis can be altered in *C. elegans.*

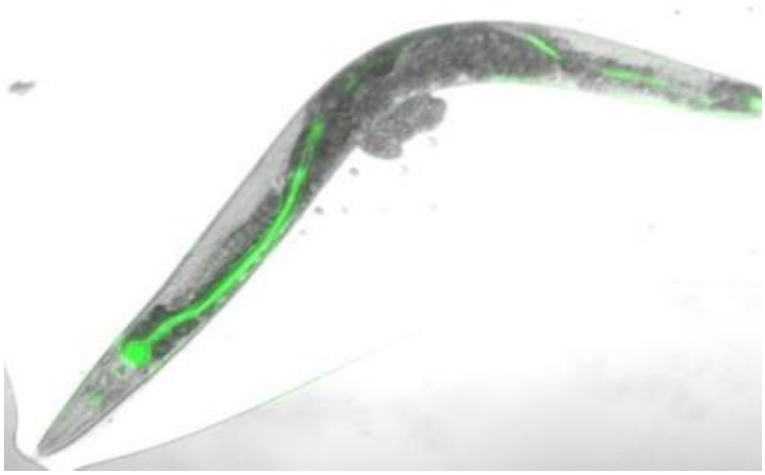


Figure 1.5: The *C. elegans* intestine. Feeding *C. elegans* carboxylate beads with a diameter of 0.5µm (Kiyama *et al.*, 2012) allows visualisation of the *C. elegans* intestine. This represents the environment which can be colonised by bacteria post ingestion, allowing subsequent establishment of an infection. Image taken at 100x magnification

1.5.1.2 Formation of biofilms in *C. elegans* tissues upon bacterial infection

As well as colonisation of the *C. elegans* intestine with planktonic cells upon infection with bacterial pathogens, an additional form of colonisation is also observed in *C. elegans* pathogenesis. This is seen in the form of biofilm formation on various tissues. Bacterial biofilms are aggregates of bacteria with the cells being embedded in a extracellular matrix produced by the bacteria which can form upon attachment to a surface (Stoodley *et al.*, 2002; Flemming *et al.*, 2016) . Bacteria behave differently in biofilms in comparison to existing in the planktonic state. Indeed infections caused by biofilms are harder to treat (Flemming *et al.*, 2016). A number of different species have been shown to form biofilms in *C. elegans* tissues. *Staphylococcus epidermidis* forms biofilms in the *C. elegans* intestine, with the virulence of this particular organism being dependent on key genetic determinant of biofilm formation (Begun *et al.*, 2007). In addition, the plague causing bacteria *Yersina pestis* and the related species *Yersina pseudotuberculosis* form biofilms over the *C. elegans* pharyngeal opening inhibiting feeding (Darby *et al.*, 2002). In addition, whilst not a human pathogen, the bacteria *Microbacterium nematophilum*, a natural pathogen of nematodes, adheres to the rectal and post anal cuticles of *C. elegans*, and leads to swelling in this region, similar to the Dar (deformed anal region) phenotype, a well characterised *C. elegans* phenotype(Hodgkin *et al.*, 2000).

1.5.1.3 Toxin Mediated killing

Another kind of bacterial pathogenesis which has been examined in *C. elegans* is toxin mediated killing, which is what underpins fast killing described in the initial *C. elegans* bacterial pathogenesis studies with *P. aeruginosa* PA14 (Tan *et al.*, 1999a). The production of toxins by the bacteria acts to kill *C. elegans* in this particular form of pathogenesis. Toxin mediated killing is observed upon incubating bacteria and *C. elegans* on a richer medium, which promotes production of bacterial toxins. *P. aeruginosa*, *B. cepacia*, *B. pseudomallei* and *E. coli* have all been identified to produce these 'fast killing' toxins (Table 1.2) (Tan *et al.*, 1999a; O'Quinn *et al.*, 2001; Kothe *et al.*, 2003; Anyanful *et al.*, 2005).

Additional environmental modifications other than incubation on richer media also impact pathogenicity through toxin production. *Enterococcus faecium*, which has been demonstrated not to cause intestinal infections in *C. elegans*, is pathogenic when grown anaerobically. The pathogenesis of this particular species has been demonstrated to be mediated by hydrogen peroxide, which has also been demonstrated to contribute to killing of *C. elegans* by *Streptococcus* species (Garsin *et al.*, 2001; Jansen *et al.*, 2002; Bolm *et al.*, 2004; Moy *et al.*, 2004). It has also been demonstrated that *P. aeruginosa* cells do not need to be alive for toxin mediated killing, as *C. elegans* exposed to heat killed bacteria on rich medium still die (Tan *et al.*, 1999a). This indicates that toxins which act to kill *C. elegans*, including the identified examples of phenazines and hydrogen cyanide, are heat stable (Mahajan-Miklos *et al.*, 1999; Gallagher and Manoil, 2001).

Class of organism	Species	Method(s) of pathogenesis	References
Gram-positive	*Enterococcus faecalis*	Intestinal infection	(Garsin *et al.*, 2001)
	Enterococcus faecium	Toxin mediated	(Moy *et al.*, 2004)
	Staphylococcus aureus	Intestinal infection	(Garsin *et al.*, 2001)
	Staphylococcus epidermidis	Biofilm formation (intestine)	(Begun *et al.*, 2007)
	Streptococcus species	Toxin mediated	(Jansen *et al.*, 2002; Bolm *et al.*, 2004)
Gram-negative	*Burkholderia cepacia*	Intestinal infection, Toxin mediated	(Kothe *et al.*, 2003)
	Burkholderia pseudomallei	Intestinal infection, Toxin mediated	(O'Quinn *et al.*, 2001)
	Escherichia coli	Intestinal infection	(Youn *et al.*, 2013)
	Klebsiella pneumoniae	Intestinal infection	(Kamaladevi and Balamurugan, 2015)
	Pseudomonas aeruginosa	Intestinal infection, Toxin mediated	(Tan et al., 1999a)
	Salmonella enterica	Intestinal infection	(Aballay *et al.*, 2000)
	Serratia marcescens	Intestinal infection	(Kurz *et al.*, 2003)
	Yersina pestis	Biofilm formation (cuticle)	(Darby *et al.*, 2002)
	Yersina pseudotuberculosis	Biofilm formation (cuticle)	(Darby *et al.*, 2002)

Table 1.2: Human bacterial pathogens examined in the *C. elegans* infection model. Distinct modes of pathogenesis as defined in 1.5.1 are depicted. Table adapted from Sifri et al. (2005; Ewbank, 2006) and Darby et al. (2005).

1.5.2 Bacterial virulence factors in *C. elegans* pathogenesis

As well of the modes of action that govern how bacteria cause infections, it is also important to identify the virulence factors, individual genes or groups of genes which act to enhance the ability of bacteria to causes disease by eliciting or mediating the damage caused to host cells. Understanding virulence factors allows deeper understanding about how bacterial infections are controlled by the bacteria themselves. In addition, recent efforts have focused on exploiting virulence factors as new antimicrobial targets (Zhu *et al.*, 2015a). *C. elegans*

infection studies have been important in determining virulence factors necessary to attack the host (Sifri *et al.*, 2005). A range of different bacterial virulence factors have been identified using the bacterial infection model. These have been grouped into three main categories (Sifri *et al.*, 2005). These three categories are genes that regulate virulence by affecting expression of other genes, structures associate with bacterial cell walls and excreted products.

1.5.2.1 Virulence regulators

The first of the groups of virulence factors identified in *C. elegans* are virulence regulators that modulate the virulence of the bacteria by changing gene expression. Specific types of these virulence regulators include quorum sensing regulators, two component regulators and sigma factors.

1.5.2.1.1 Quorum sensing regulators

Quorum sensing is communication between individual bacterial cells, and allows production of different genes based on environmental factors (Rutherford and Bassler, 2012). This relies on the production, detection and response to molecules called autoinducers which act as extracellular signalling molecules (Rutherford and Bassler, 2012). When there is a high level of bacterial population, autoinducers accumulate in the bacteria's environment. Autoinducers are subsequently detected by the bacteria and act to regulate gene expression (Rutherford and Bassler, 2012). Quorum sensing acts to control a wide variety of processes in bacteria such as biofilm formation, sporulation and production of virulence factors (Rutherford and Bassler, 2012). Quorum sensing has been demonstrated to contribute to enhanced virulence in mammalian bacterial pathogens (Garsin *et al.*, 2001; Rutherford and Bassler, 2012). It has been demonstrated how molecules involved in quorum sensing systems contribute to bacterial virulence in *C. elegans* in the Gram-negative *P. aeruginosa, B. cepacia* and *S. marcescens* and the Gram -positive *S. aureus* and *E. faecalis* (Tan *et al.*, 1999b; Garsin *et al.*, 2001; Sifri *et al.*, 2002; Kothe *et al.*, 2003; Sifri *et al.*, 2003; Coulthurst *et al.*, 2004; Papaioannou *et al.*, 2009; Feinbaum *et al.*, 2012). Bacterial mutants deficient in genes utilised in quorum sensing have reduced levels of virulence. This includes genes encoding for autoinducers which act as the signalling molecules in the quorum sensing

process (Kothe *et al.*, 2003; Coulthurst *et al.*, 2004). Furthermore, mutants deficient in the transcriptional regulators which coordinate the response to the signals are also attenuated for virulence(Tan *et al.*, 1999b; Garsin *et al.*, 2001; Sifri *et al.*, 2002; Sifri *et al.*, 2003; Feinbaum *et al.*, 2012). This demonstrates that, as in mammalian hosts, quorum sensing is important in regulating the virulence of bacterial pathogens in a *C. elegans* model.

1.5.2.1.2 Two-component regulators

Two-component regulators are bacterial systems where extracellular signals are converted into cellular responses based changing environmental conditions (Mitrophanov and Groisman, 2008). These systems utilise sensor kinases which respond to environmental changes by modifying the phosphorylation state of regulatory proteins (Mitrophanov and Groisman, 2008). Two-component regulators, like quorum sensing systems, are used by bacteria to regulate a variety of cellular functions like sporulation and antibiotic resistance, in addition to contributing to virulence (Bae *et al.*, 2004; Mitrophanov and Groisman, 2008). Constituents of two-component regulatory systems have been demonstrated to contribute to the virulence of *P. aeruginosa, S. enterica, S. marcescens* and *S. aureus* in a *C. elegans* host, with mutants in the regulatory proteins in exhibiting slower killing of the animals (Tan *et al.*, 1999a; Tan *et al.*, 1999b; Aballay *et al.*, 2000; Gallagher and Manoil, 2001; Kurz *et al.*, 2003; Bae *et al.*, 2004; Coulthurst *et al.*, 2004; Tenor *et al.*, 2004; Feinbaum *et al.*, 2012) (Table 1.3).

1.5.2.1.3 Sigma factors

Sigma factors are utilised by a wide range of microorganisms, and consist of subunits of RNA polymerase which allow regulation of a vast number of genes in response to changing conditions (Kazmierczak *et al.*, 2005). The functions of sigma factors include altering gene expression in response to stressful environments, enabling bacteria to be more tolerant towards insults like hydrogen peroxide and acid treatment (Labrousse *et al.*, 2000; Sifri *et al.*, 2003; Kazmierczak *et al.*, 2005). This is relevant for bacterial pathogens as it enables bacteria to be resistant against any immune responses mounted upon a host organism. In addition to these functions relevant to stress resistance, sigma factors also act to coordinate the production of virulence factors important in bacterial pathogenesis (Kazmierczak *et al.*,

2005). Sigma factors have been demonstrated to contribute to virulence of pathogens in *C. elegans* in infections by *P. aeruginosa, S. enterica* and *S. aureus* (Labrousse *et al.*, 2000; Hendrickson *et al.*, 2001; Sifri *et al.*, 2003).

All of the types of molecules described above involve altering genetic regulation upon changing environmental conditions, with regulation of virulence in mammalian making up part of the functions of these regulators (Kazmierczak *et al.*, 2005; Mitrophanov and Groisman, 2008; Rutherford and Bassler, 2012). The fact that these virulence regulators have been shown to play a role in mediating bacterial pathogenesis in *C. elegans*, demonstrates that bacterial gene regulation is altered upon infection of a range of host organisms.

1.5.2.2 Bacterial cell wall associated structures

Structures that make up part of, or are associated with, the bacterial cell wall have been demonstrated to play an important role in modulating virulence of bacteria in *C. elegans*. *S. aureus* mutants deficient in aspects of the synthesis of the polysaccharides which make up the capsule present on the surface of bacterial cell walls, exhibited reduced virulence towards *C. elegans* (Bae *et al.*, 2004). In addition, the ability of both *S. epidermis* and *Y. pseudotuberculosis* to form biofilms from planktonic cells is a determinant of pathogenesis of these species on *C. elegans* (Darby *et al.*, 2002; Begun *et al.*, 2007). Biofilms are both adapted to exist on surfaces and also makes bacteria more resistant to antibiotic treatment and other environmental stresses (Flemming *et al.*, 2016). The synthesis of lipopolysaccharide (LPS) has also been shown to be a factor involved in *C. elegans* infection by bacteria. LPS is a major component of cell walls in Gram-negative bacteria which are important for pathogen recognition by host organisms, as well as in other non-pathogenic bacterial function like surface adhesion. It has been demonstrated that strains of *S. enterica, P. aeruginosa, K. pneumoniae* and *E. coli* deficient in aspects of LPS synthesis have reduced levels of killing exhibited towards *C. elegans* (Tenor *et al.*, 2004; Lee *et al.*, 2006; Kamaladevi and Balamurugan, 2016b; Kuo *et al.*, 2016)(Table 1.3).

1.5.2.3 Excreted factors

The last class of virulence factors identified to be involved in *C. elegans* infection studies are factors which are excreted by bacterial cells used to mediate infection, including those acting to damage tissues of *C. elegans* during the infection process. Some of these molecules are toxins or enzymes secreted by the bacteria, known as exotoxins or exoenzymes respectively. These secreted effectors are found to be important virulence factors in *S. aureus, E. faecalis*, *P. aeruginosa* and *S. marcescens* infection of *C. elegans* (Tan *et al.*, 1999b; Garsin *et al.*, 2001; Sifri *et al.*, 2002; Kurz *et al.*, 2003; Sifri *et al.*, 2003; McEwan *et al.*, 2012; Zhu *et al.*, 2015a) (Table 1.3). In addition, components of conserved secretion systems, which are important mechanisms of delivering effector molecules into host cells, have been found to be a mediator of infection in *C. elegans* pathogens, these include type III secretion systems in *S. enterica* and type IV pilus proteins in *P. aeruginosa*(Tenor et al., 2004; Feinbaum et al., 2012)(Table 1.3).

Class of Virulence factors	Organism	References
Virulence regulators		
Quorum sensing regulators	*P. aeruginosa*	(Tan *et al.*, 1999b; Papaioannou *et al.*, 2009; Feinbaum *et al.*, 2012)
	B. cepacia	(Kothe *et al.*, 2003)
	S. aureus	(Garsin *et al.*, 2001; Sifri *et al.*, 2003)
	S. marcescens	(Coulthurst *et al.*, 2004)
	E. faecalis	(Sifri *et al.*, 2002)
Two-component regulators	*P. aeruginosa*	(Tan *et al.*, 1999a; Tan *et al.*, 1999b; Gallagher and Manoil, 2001; Feinbaum *et al.*, 2012)
	S. enterica	(Aballay *et al.*, 2000; Tenor *et al.*, 2004)
	S. marcescens	(Kurz *et al.*, 2003; Coulthurst *et al.*, 2004)
	S. aureus	(Bae *et al.*, 2004)
Sigma factors	*P. aeruginosa*	(Hendrickson *et al.*, 2001)
	S. enterica	(Labrousse *et al.*, 2000)
	S. aureus	(Sifri *et al.*, 2003)
Cell wall associated structures		
Cell capsule	*S. aureus*	(Bae *et al.*, 2004)
LPS biosynthesis	*S. enterica*	(Tenor *et al.*, 2004)
	P. aeruginosa	(Lee *et al.*, 2006)
	K. pneumoniae	(Kamaladevi and Balamurugan, 2016b)
	E. coli	(Kuo *et al.*, 2016)
Biofilm formation factors	*Y. pseudotuberculosis*	(Darby *et al.*, 2002)
	S. epidermidis	(Begun *et al.*, 2007)
Secreted products		
Exotoxins and exoenzymes	*S. aureus*	(Garsin *et al.*, 2001; Sifri *et al.*, 2003)
	E. faecalis	(Sifri *et al.*, 2002)
	P. aeruginosa	(Tan *et al.*, 1999b; McEwan *et al.*, 2012; Zhu *et al.*, 2015a)
	S. marcescens	(Kurz *et al.*, 2003)
Type III secretion systems	*S. enterica*	(Tenor *et al.*, 2004)
Type IV pilus protein	*P. aeruginosa*	(Feinbaum *et al.*, 2012)

Table 1.3: Virulence factors identified in the *C. elegans* infection model. Adapted from Sifri et al. (2005).

1.5.2.4 Identification of novel and conserved bacterial virulence factors in *C. elegans*

Some virulence factors identified using *C. elegans* as a model have also been identified as bacterial virulence factors in other model organisms. For example both *gacA*, a two component regulator of *P. aeruginosa,* and alpha-haemolysin, a toxin which causes cell death in *S. aureus* infections, have both been identified to act as virulence factors in a nematode host, in addition to other models (Tan *et al.*, 1999b; Sifri *et al.*, 2003; Feinbaum *et al.*, 2012). The identification of conserved virulence factors being involved in the infection of both mammalian and nematode hosts demonstrates that similar patterns of pathogenesis are elicited upon the infection of diverse host organisms

As well as identifying conserved virulence factors, analysis of transposon mutants of bacteria has allowed novel virulence factors to be identified (Gallagher and Manoil, 2001; Kurz *et al.*, 2003; Tenor *et al.*, 2004; Feinbaum *et al.*, 2012). For example, the study by Feinbaum et al. (2012) identified 41 genes of *P. aeruginosa* as virulence factors in *C. elegans*. 20 of these genes had not been identified as C. elegans virulence factors previously. Identification of these novel virulence factors provides a platform for deeper investigation of bacterial pathogenesis in the *C. elegans* host. In addition, the novel virulence factors determined from this simple animal host can also be investigated in mammalian models in order to gain deeper understanding of how infection is mediated in more clinically relevant hosts.

1.6 The *C. elegans* innate immune response to bacterial infection

As mentioned in 1.3, whilst *C. elegans* does not have an adaptive immune response but it does have a well-defined innate immune system (Ewbank, 2006; Engelmann and Pujol, 2010). The *C. elegans* immune response to infection has been investigated in great detail (Ewbank, 2006; Engelmann and Pujol, 2010; Irazoqui *et al.*, 2010b; Marsh and May, 2012; Cohen and Troemel, 2015). This approach has been particularly useful in revealing the central role of the innate immune system in modulating the response to bacterial infection.

There are three branches of the *C. elegans* immune response to bacterial infection (Engelmann and Pujol, 2010). The first of these are physical barriers, which prevent any bacteria from entering the internal tissues of the animals. These include the cuticle of the animal and the pharynx. Bacteria that go on to establish an intestinal infection, as well as any commensal bacteria must bypass the grinder of the pharynx (Figure 1.4). It has been demonstrated *C. elegans* mutants with defective pharyngeal grinders are more susceptible to infection by bacterial pathogens (Labrousse *et al.*, 2000) highlighting the critical role of this barriers to infection. In addition, a functional cuticle has also been found to be important in the protection against infection by both fungi and bacteria which can adhere to the cuticle (Taffoni and Pujol, 2015).

The second line of protection is a behavioural response observed towards lawns of pathogenic bacteria. *C. elegans* has been demonstrated to learn that pathogenic bacteria are harmful after an extended exposure. This leads to increased avoidance of lawns which are made up of pathogenic bacteria (Pujol, 2001; Zhang *et al.*, 2005; Pradel *et al.*, 2007; Gaglia *et al.*, 2012). This particular response is a main focus of the experiments in this thesis, and will be broken down in 1.7.

The third branch of the *C. elegans* immune response is a variety of signalling pathways which respond upon an infection and initiates molecular cascades which act to protect *C. elegans* against the insult of a pathogen and as a result acts to promote survival of the insult. Studies examining the changes in gene expression upon infection have revealed that the expression of up to 3000 *C. elegans* genes can be stimulated after bacterial infection (Engelmann *et al.*, 2011). The genes which are involved in this response have subsequently been organised into molecular pathways that act to coordinate an immune response. These pathways are detailed in the subsequent sections.

1.6.1 Signalling pathways in the *C. elegans* immune response

1.6.1.1 P38 MAPK pathway

There are number of parallel pathways implicated in *C. elegans* immune signalling, which presumably act to allow redundancy in the response (Irazoqui *et al.*, 2010b). The first of these is the p38 mitogen activated protein kinases (MAPKs). This is among the most conserved pathways in the immune response across phyla (Zarubin and Han, 2005; Engelmann and Pujol, 2010). This particular pathway was first identified as being part of the *C. elegans* response to infection in studies with *P. aeruginosa* (Kim *et al.*, 2002). Subsequently, this pathway has been shown to be active during the response to *S. aureus, S. epidermidis, S. enterica,* and *K. pneumoniae* infections (Aballay *et al.*, 2003; Sifri *et al.*, 2003; Begun *et al.*, 2007; Kamaladevi and Balamurugan, 2015). The components of the *C. elegans* p38 MAPK pathway are NSY-1, SEK-1 and PMK-1 (Figure 1.6). *C. elegans* which are deficient in components of this pathway have been demonstrated to be more susceptible to infection the bacterial pathogens described above (Kim *et al.*, 2002; Aballay *et al.*, 2003; Sifri *et al.*, 2003; Begun *et al.*, 2007; Kamaladevi and Balamurugan, 2015). In addition to the p38 MAPK pathway, *C. elegans* also possesses an extracellular signal-regulated kinase (ERK) pathway and a c-Jun terminal kinase (JNK) pathway. These are involved in response to other stresses, including pathogen infection (Kim *et al.*, 2004; Nicholas and Hodgkin, 2004; Engelmann and Pujol, 2010) . Interactions occur between these different pathways, with the MAPK of the JNK pathway being needed for full PMK-1 activation, revealing cross talk between the different MAPK pathways of *C. elegans (*Kim *et al.*, 2004; Engelmann and Pujol, 2010*).*

1.6.1.2 Toll-like receptors

It has also been found that components of other conserved immune pathways play a role in the p38 MAPK pathway in *C. elegans*. Toll-like receptors (TLRs) have been demonstrated to be important in pathogen detection by *Drosophila* and vertebrates (Ferrandon *et al.*, 2007). The only *C. elegans* homologue, TOL-1, whilst implicated in resistance to *S. enterica* infection, is not involved in the immune response to *S. aureus* or *P. aeruginosa* infection(Pujol, 2001; Tenor and Aballay, 2008; Irazoqui *et al.*, 2010b). This indicates that

this conserved transmembrane protein is not a central component of the *C. elegans* immune response like it is in mammalian and *D. melanogaster* models (Irazoqui *et al.*, 2010b).*C. elegans* also possesses another component of the mammalian TLR pathway, a protein with a TIR (Toll and Interleukin-1 resistance receptor) domain called TIR-1. In mammalian systems, the TLR pathway acts via adaptor proteins with TIR domains which act as signal transducers (Ewbank, 2006). *tir-1* mutant *C. elegans* have been found to be hyper susceptible to infection by *P. aeruginosa* (Liberati *et al.*, 2004). In addition, TIR-1 has been demonstrated to act upstream of NSY-1 in the p38 MAPK kinase pathway (Figure 1.6). The differential immune response observed in *tol-1* and *tir-1* deficient *C. elegans* suggests differential evolution of immune pathways utilising similar components in different model systems (Irazoqui *et al.*, 2010b).

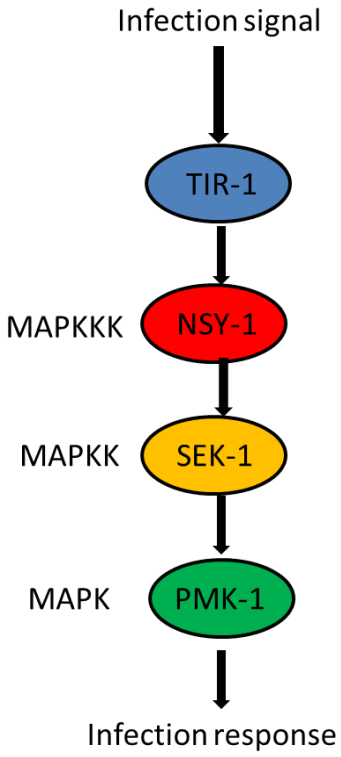


Figure 1.6: The p38 MAPK pathway of *C. elegans.* This pathway has been implicated in the innate immune response to bacterial infection. The individual components of this pathway are the MAP kinase kinase kinase: NSY-1; the MAP kinase kinase: SEK-1; and the MAP kinase PMK-1. In addition the homologue of the mammalian TIR domain containing protein TIR-1 acts upstream of these components in this innate immune signalling pathway. Figure adapted from Ewbank (2006).

1.6.1.3 DAF-2 Pathway

Another molecular pathway that functions in the *C. elegans* immune response to bacterial infection is the Insulin-like signalling pathway. This utilises the Insulin like receptor DAF-2 and the nuclear transcription factor DAF-16. DAF-2 can be activated by a number of peptides which are homologues of the human insulin peptide (1.8.3.2) (Pierce *et al.*, 2001). Activation of DAF-2 by these ligands acts via phosphorylation of proteins in a signalling cascade resulting in the deactivation of DAF-16 by retaining it in the cytoplasm (Figure 1.7A). When DAF-2 is not activated, DAF-16 is localised in the nucleus and regulates gene expression (Figure 1.7B) (Ewbank, 2006). This pathway has been well described with respect to how it regulates *C. elegans* lifespan. *daf-2* mutants exhibit a longer lifespan than wild type animals, due to the constitutive activation of DAF-16 (Murphy *et al.*, 2003). In addition *daf-2* mutant animals are resistant to *P. aeruginosa* and *E. faecalis* infection (Garsin *et al.*, 2003). This is likely due to the inactivation of DAF-2 leading to enhanced transcription of a number of effectors involved in coordinating an immune response (Irazoqui *et al.*, 2010b). The role that this pathway plays in the *C. elegans* immune response is also demonstrated by the fact that *P. aeruginosa* stimulates activation of DAF-2 by provoking enhanced production of the insulin like peptide INS-7. This supresses the subsequent signalling responses coordinated by DAF-16 (Evans *et al.*, 2008). However, analysis of both *pmk-1* (1.6.1.1) and *daf-2* mutant animals has revealed that they likely function in parallel as part of an immune response to bacterial infection, with analysis of DAF-16 targets revealing this pathway is responds to general stresses rather than specific immune stresses (Murphy *et al.*, 2003; Troemel *et al.*, 2006).

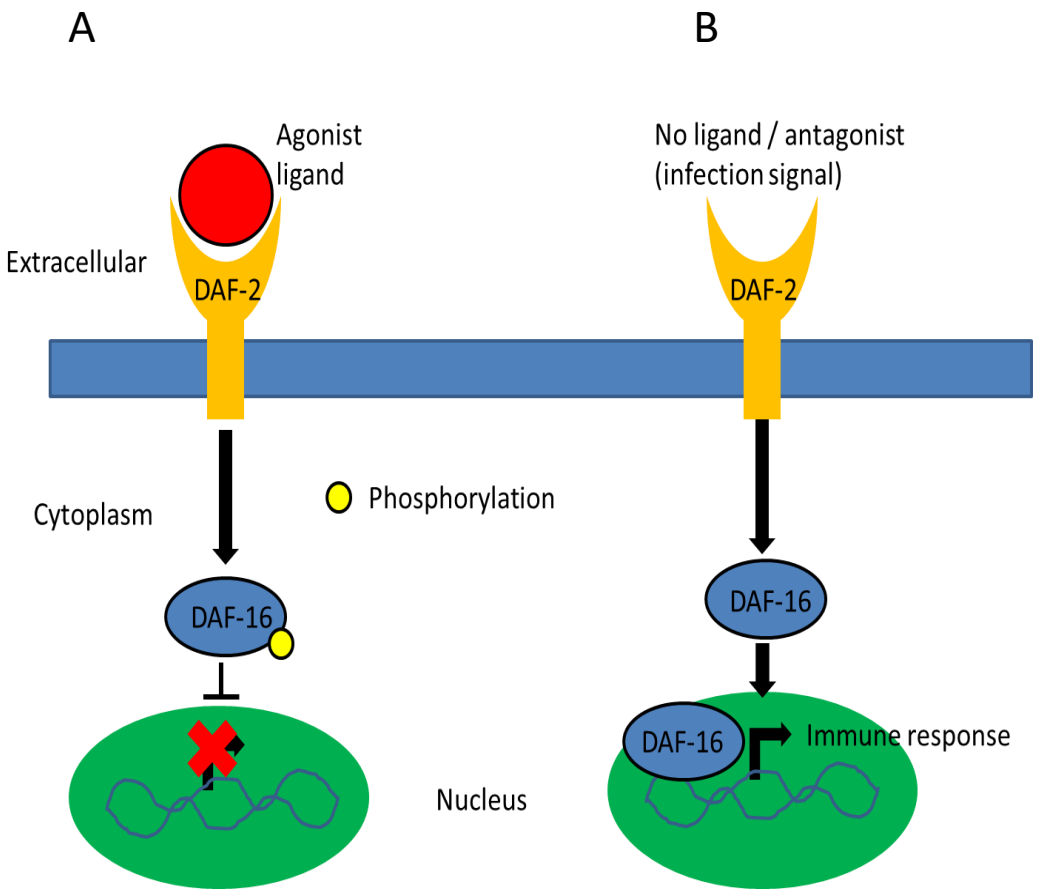


Figure 1.7: The DAF-2/DAF-16 signalling pathway in *C. elegans.* Binding of an insulin-like ligand (A) leads to activation of DAF-2, which through a signalling cascade phosphorylates the transcription factor DAF-16, meaning it cannot translocate to the nucleus and regulate gene expression. No binding of a ligand or an antagonist (B) means that DAF-16 is not phosphorylated, meaning that it can translocate to the nucleus and regulate expression of genes. In the case of a bacterial infection, this leads to upregulation of immune response effector genes. Figure adapted from Ewbank (2006).

1.6.1.4 The TGF-β/ DBL-1 pathway

Another signalling pathway in which *C. elegans* mutant strains are hyper susceptible to infection is the DBL-1 pathway. The DBL-1 gene encodes for a homologue of a Transforming growth factor β (TGF-β) ligand. The TGF-β family contains a number of molecules required for intercellular signalling which help control cell growth, differentiation and death throughout biology (Zhang and Zhang, 2012). In *C. elegans*, the DBL-1 ligand binds to a receptor made up of the SMA-6 and DAF-4 proteins and subsequently phosphorylates a molecular complex made up of the proteins SMA-2, SMA-3 and SMA-4, allowing translocation of this complex to the nucleus to control gene expression (Figure 1.8) (Ewbank,

2006). This particular pathway has been found to enhance resistance to *S. enterica, S. marcescens* and *P. aeruginosa* infection, with *dbl-1* mutants being hyper susceptible to bacterial infection with these species (Mallo *et al.*, 2002; Ewbank, 2006; Tenor and Aballay, 2008; Portal-Celhay *et al.*, 2012).

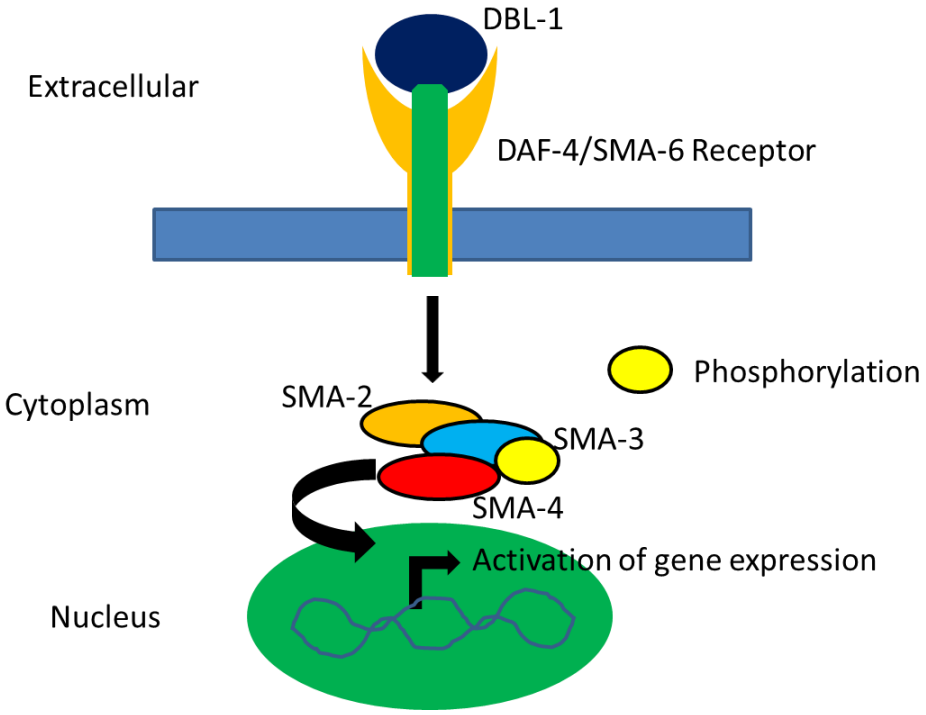


Figure 1.8: The DBL-1 pathway of *C. elegans*. Binding of DBL-1 to the DAF-4/SMA-6 receptor leads to phosphorylation of the complex containing, SMA-2, SMA-3 and SMA-4 which leads to translation to the nucleus and activation of gene expression. In the case of bacterial infection this leads to expression of immune effector genes. Figure adapted from Ewbank (2006) and Irazoqui et al. (2010b).

1.6.1.5 The WNT signalling pathway

β-catenin, a component of the *C. elegans* WNT signalling pathway, and a protein necessary for developmental processes, has a role in the immune signalling seen upon *S. aureus* infection (Irazoqui *et al.*, 2008). Detailed analysis of this particular interaction revealed the WNT pathway acts alongside the p38 MAPK pathway (1.6.1.1). Consequently, analysis of bacterial infection with two different species revealed that the WNT pathway is more strongly implicated in the *S. aureus* immune response , with the p38 pathway implicated more in the response to *P. aeruginosa* infection (Irazoqui *et al.*, 2008). This indicates that

infection with different bacterial pathogens stimulates different immune pathways in *C. elegans*, demonstrating that *C. elegans* can deal with bacterial infection in different ways depending on the nature of the infection (Irazoqui *et al.,* 2008).

1.6.1.6 The FSHR-1 pathway

Another pathway found to function in parallel to both the PMK-1 and DAF-2 pathways is one controlled by the G protein coupled receptor FSHR-1 a homologue of mammalian follicle stimulating hormone receptor. This particular receptor acts in the intestine to enhance resistance to infections by *P. aeruginosa*, *S. aureus* and *E. faecalis* (Powell *et al.*, 2009; Miller *et al.*, 2015).

The discovery of these different immune pathways that function in the *C. elegans* immune response suggest that there is a large amount of parallel signalling that is stimulated upon bacterial infection of *C. elegans* (Irazoqui *et al.*, 2010b). This demonstrates that that even a very simple animal like *C. elegans* can execute immune signalling through a number of pathways upon infection by a bacterial pathogen.

1.6.2 Transcription factors in the immune response

As all of the pathways discussed above act to regulate gene expression in response to bacterial infection, the primary target of these pathways is transcription factors. Activation of transcription factors by these signalling pathways alters gene expression of effectors molecules and other downstream components (1.6.3). Roles for transcription factors in regulating the longevity of *C. elegans*, including upon response to infection has been identified through studies with DAF-16 (1.6.1.3). The main transcription factor which functions to govern the response to infection in mammals is NF-kB. However, this particular protein is not present in *C. elegans* and it has been demonstrated that a number of transcription factors are involved in the worm immune response (Engelmann and Pujol, 2010). In addition to DAF-16, one of these transcription factors are the intestinal GATA transcription factor ELT-2, which is an important regulator of intestinal development but

also helps to protect *C. elegans* from bacterial infection (Shapira *et al.*, 2006; Block *et al.*, 2015). Another example is the bZIP transcription factor ZIP-2, which is triggered by the *P. aeruginosa* virulence factors Exotoxin A, and regulates the expression of a wide variety of response genes (Estes *et al.*, 2010; McEwan *et al.*, 2012).

1.6.3 Downstream components of the immune response

As well as the different signalling pathways and transcription factors which are stimulated upon activation of the *C. elegans* immune response upon bacterial infection, there has also been examination of the downstream components of the pathway, which are the actual components involved in protecting the organism against the insult presented by bacterial infection and act to attack the bacterial cells. These downstream components have a number of functions and are described below.

1.6.3.1 Antimicrobial peptides

Many molecules whose activity is upregulated in the response to bacterial infection are peptides which act to attack bacterial cells in an attempt to remove the infection (Engelmann and Pujol, 2010). Groups of these antimicrobial peptide which demonstrate antibacterial activity include caenopores, lysozymes, lectins and ABF peptides (Table 1.4) (Couillault *et al.*, 2004; Alegado and Tan, 2008; Ewbank and Zugasti, 2011).

Group	Number of peptides	Mechanism of action	Bacteria against which action is seen	References
Caenopores	33	Permeabilization of membrane	*S. enterica, P. aeruginosa*	(Alegado and Tan, 2008; Evans *et al.*, 2008; Roeder *et al.*, 2010)
Lysoszymes	15	Cell wall lysis	*S. marcescens, M. nematophilum*	(Mallo *et al.*, 2002; O'Rourke *et al.*, 2006)
Lectins	250+	Prevents binding of toxins	*M. nematophilum, P. aeruginosa, S. marcescens*	(Mallo *et al.*, 2002; O'Rourke *et al.*, 2006; Troemel *et al.*, 2006)
ABF peptides	6	Membrane disruption	*S. aureus, S. enterica*	(Alper *et al.*, 2007; Alegado and Tan, 2008)

Table 1.4: The classes of antimicrobial peptides of *C. elegans* involved in the immune response to bacterial infection. The number of peptides known to be present in each class, as well as the mechanism of action and the species in which activity is seen is indicated.

1.6.3.2 Apoptosis and the unfolded protein response (UPR)

It has been determined that *C. elegans* initiates apoptosis upon infection with *S. enterica* mediated by induction of the unfolded protein response (UPR) (Aballay and Ausubel, 2001). This response has also been demonstrated to form part of the *C. elegans* defence to the invertebrate pathogen *Bacillus thuringeinsis*, specifically in response to the pore forming toxin Cry5B(Bischof *et al.*, 2008). Distinct UPRs have been observed to occur in both the endoplasmic reticulum and mitochondria in response to *P. aeruginosa* infection (Richardson *et al.*, 2010; Pellegrino *et al.*, 2014). This particular protective response has been speculated to be mounted to counteract any stress placed on the endoplasmic reticulum which occurs through production of antimicrobial peptides, which could elicit further damage to *C. elegans* tissues(Richardson *et al.*, 2010; Pellegrino *et al.*, 2014).

1.6.3.3 Autophagy

C. elegans has been demonstrated to exhibit autophagy as a protective response upon bacterial infection. Following S*. enterica* infection, *C. elegans* mutants deficient in autophagy are more susceptible to infection (Jia *et al.*, 2009) . This increased susceptibility is due to the role of autophagy in the removal of damaged cellular components following infection (Jia *et al.*, 2009; Cohen and Troemel, 2015). Protective autophagy in *C. elegans* has also been observed in response to both *S. aureus* and *P. aeruginosa* infection (Visvikis *et al.*, 2014; Zou *et al.*, 2014). It has also been demonstrated that *C. elegans* undergo mitophagy, where mitochondria are removed through autophagy, in response to the iron siderophore pyoverdin produced during *P. aeruginosa* infection (Kirienko *et al.*, 2015).

1.6.3.4 Reactive Oxygen Species.

Reactive oxygen species (ROS) have also been demonstrated to exhibit bactericidal activity against infection of *C. elegans,* as part of the downstream component the DAF-2 signalling pathway (1.6.1.3) (Chavez *et al.*, 2007; Chavez *et al.*, 2009). ROS production by phagocytes is an important aspect of pathogen defence in the mammalian immune system (Chavez *et*

al., 2009). Infection of *C. elegans* by *E. faecalis* with has been shown to activate the BLI-3 protein *(Chavez et al., 2007)*. BLI-3 is an oxidase which has both peroxidase and NADPH oxidase domains (Edens *et al.*, 2001). Activation of BLI-3 leads to the production of ROS which acts in the intestine to protect against the *E. faecalis* infection (Chavez *et al.*, 2007; Chavez *et al.*, 2009). To prevent damage of *C. elegans* tissues, this response is coupled with the production of the superoxide dismutase SOD-3 and the catalase CTL-2, which act to detoxify the ROS generated (Chavez *et al.*, 2007).

1.7 The *C. elegans* behavioural response to bacterial pathogens

1.7.1 *C. elegans* aversive behaviours

As mentioned in 1.6, another component of the *C. elegans* immune response in addition to signalling pathways and physical barriers has been described. This is the behavioural avoidance of pathogenic bacteria. In order to avoid damage to the organism, *C. elegans* has evolved to determine that certain stimuli are harmful and can thus learn to avoid these stimuli upon future exposure (1.6). It was demonstrated in 1993 that *C. elegans* responds to a range of volatile chemicals in different ways, with some being attractants and others being repellents, driving *C. elegans* away (Bargmann *et al.*, 1993). Since this initial study, it has been found that *C. elegans* acts to avoid a wide range of different stimuli. *C. elegans* avoid undesirable level of metabolic gases, with high concentration of carbon dioxide, as well as both high (>12%) and low (<5%) concentrations of oxygen eliciting avoidance in *C. elegans* (Gray *et al.*, 2004; Hallem and Sternberg, 2008). *C. elegans* also actively avoid environments with a pH of above 10.5, as well as toxic metals like cadmium and copper (Sambongi *et al.*, 1999; Sassa *et al.*, 2013).

1.7.2 *C. elegans* food aversion to lawns of pathogenic bacteria

In addition to avoidance of these harmful environmental stimuli mentioned above, *C. elegans* also demonstrates aversion towards pathogenic bacteria. This particular behavioural phenomenon has been observed in two distinct kinds of assays. The first approach involves placing populations of *C. elegans* on a single lawn of bacteria and measuring the relative proportion of the animals on the bacterial lawn (Figure 1.9A). Upon

exposure to the S*. marcescens* strains Db10 and Db11, and P*. aeruginosa* PA14, these *C. elegans* populations increasingly avoid the bacterial lawns, with phenomenon usually being observed after *C. elegans* have been on the bacterial lawn for between 12 and 24 hours (Pujol, 2001; Pradel *et al.*, 2007; Reddy *et al.*, 2009; Chang *et al.*, 2011; Gaglia *et al.*, 2012)(Figure 1.9A).

The alternative approach is by examining populations of *C. elegans* which have been preconditioned to lawns of bacteria, allowing potential aversive learning to occur. Aversion can subsequently be examined in a chemotaxis assay where animals are given a choice between two bacterial lawns on opposite sides of an NGM plate. This usually involves *E. coli* OP50 and the potentially aversive bacteria that is being investigated. Following presentation of the animals to the bacterial lawns the proportion of animals migrating to each lawn is measured over a 1 hour period. When this approach was undertaken with a lawns of pathogenic *P. aeruginosa* and *S. marcescens*, *C. elegans* which have been previously exposed to this pathogen express a conditioned aversion to bacteria are harmful and thus avoid the lawn (Tan *et al.*, 1999a; Zhang *et al.*, 2005) (Figure 1.9B). These 'trained' animals were raised with exposure to lawn of pathogenic bacteria along with a supplemental lawn of OP50 as a food source (Zhang *et al.*, 2005). However, these aversive responses are not observed in *C. elegans* populations which have not previously experienced PA14 ('naive' populations) (Zhang *et al.*, 2005). In these populations the lawns of pathogens are initially attractive, and a four hour exposure to pathogenic bacteria is necessary to elicit negative chemotaxis in the subsequent assay (Zhang *et al.*, 2005)(Figure 1.9C).

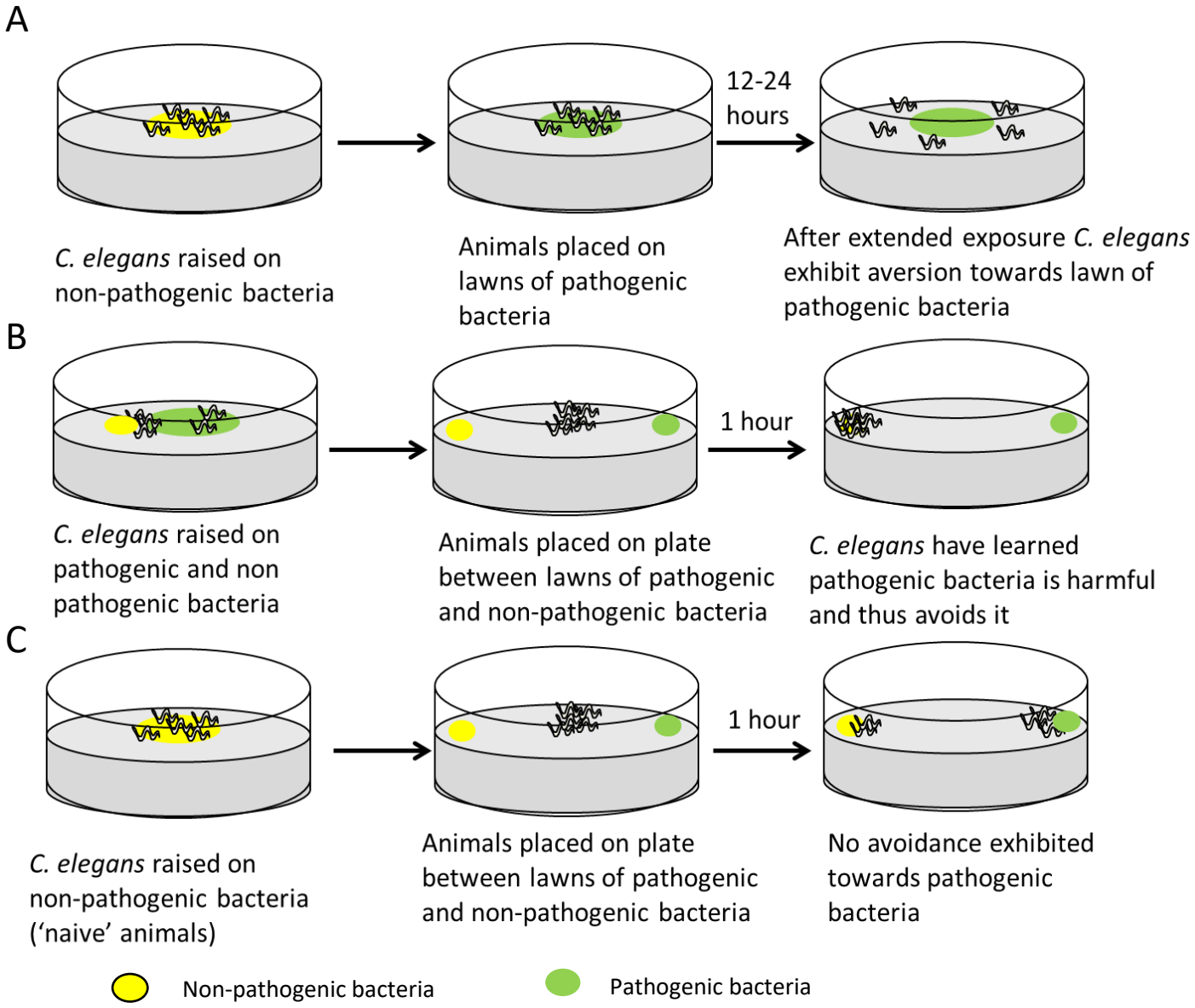


Figure 1.9: *C. elegans* avoidance of lawns of pathogenic bacteria. After being transferred to single lawns of pathogenic bacteria after being raised on non-pathogenic bacteria, an extended period of exposure to the pathogen leads to animals increasingly avoiding the bacterial lawn (A). This effect is also observed when *C. elegans* that have been raised on pathogenic bacteria and are then given a choice between pathogenic and non-pathogenic bacteria for 1 hour (B). This period of exposure is necessary to elicit a negative chemotaxis away from the lawn of pathogenic bacteria. Performing this same kind of assay on *C. elegans* that have not previously experienced the pathogenic bacteria ('naive' animals) do not elicit this avoidance and initially find the lawn of pathogenic bacteria attractive (C). Adapted from Meisel and Kim (2014).

In both of the approaches mentioned above, *C. elegans* populations which are exposed to lawns of pathogenic bacteria increasingly exist off the bacterial lawn after being exposed to the pathogenic bacteria for an extended period of time. This response is presumably elicited upon ingesting bacteria, with damage occurring as a result of bacterial infection as

summarised in 1.5, and is a form of aversive learning, through modifying of olfactory preferences of the animals through chemosensation of the bacteria (Zhang *et al.*, 2005; Meisel and Kim, 2014). This aversion that *C. elegans* exhibits towards bacteria has been demonstrated to be part of the survival strategy of *C. elegans* to cope with a pathogenic insult, as mutant strains which are deficient in this aversive learning are also more susceptible towards infection by these bacteria (Reddy *et al.*, 2009; Shivers *et al.*, 2009; Chang *et al.*, 2011; Meisel *et al.*, 2014). In addition to pathogenic bacteria, *C. elegans* exposed to otherwise benign *E. coli* which are expressing dsRNA which acts to silence various important cellular processes such as mitochondrial function or larval development, avoid these lawns of bacteria over a similar time course as they would a pathogen (Melo and Ruvkun, 2012). In this study, the RNAi that was occurring was representative of cellular damage that can occur upon a bacterial infection (Melo and Ruvkun, 2012; Meisel and Kim, 2014). The fact that *C. elegans* has been demonstrated to exhibit this food aversion response to lawns of harmful bacteria, indicates that this form of behaviour is a response to sickness as a result of a bacterial infection. In addition, this aversion of bacterial lawns will also be a survival response, driving animals away from harmful food sources in favour of benign or beneficial food sources (Zhang *et al.*, 2005).

1.7.2.1 Microbial cues stimulating *C. elegans* food aversion

The food aversion behavioural effect elicited in *C. elegans* which is induced by bacteria described in this section involves the *C. elegans* nervous system sensing stimuli and executing pathways. This particular aversive behaviour can be stimulated not just by lawns of harmful bacteria as described above. Aversive *C. elegans* behaviour has been observed when purified bacterial toxicants, such as zeocin, antimycin A and violacein were used to lace benign *E. coli* lawns has (Melo and Ruvkun, 2012; Ballestriero *et al.*, 2016). It has also been observed that bacteria produce specific chemicals which act to repel *C. elegans* away from bacterial lawns. This includes homoserine lactones, molecules involved in bacterial signalling, and phenazines which act to repel *C. elegans* away from *P. aeruginosa* and the serrawettin W2 induces *C. elegans* avoidance of lawns of *S. marcescens* (Beale *et al.*, 2006; Pradel *et al.*, 2007; Meisel *et al.*, 2014). This food aversion behaviour of *C. elegans* in response to pathogenic bacteria is similar to an aversion effect seen in *Drosophila* upon

exposure to pathogens, with certain microbial compounds, like geosmin acting to promote this, suggesting such aversive behaviours may be a conserved among invertebrates (Stensmyr *et al.*, 2012).

1.8 The *C. elegans* nervous system and its role in controlling aversion towards pathogenic bacteria

In addition to identifying different bacterial products or bacterial metabolites which drive food aversion, the neurons and pathways involved to promote the food aversion response to pathogenic bacteria in *C. elegans* have been investigated (Meisel and Kim, 2014). The food aversion of pathogenic bacterial lawns by *C. elegans* will involve detection of any stimuli, followed by the processing and coordination of a response by various aspects of the *C. elegans* nervous system. Understanding this particular behaviour, which may be a form of sickness behaviour, is important as it reveals more insight into the response to bacterial infection by the host organism. The *C. elegans* nervous system has been studied in great detail. The nervous system is the largest individual tissue in *C. elegans*, with 302 of the 959 cells in the adult hermaphrodite body being neurons (Hobert, 2010). The neurons of *C. elegans* form two distinct systems, with 282 neurons in the hermaphrodite making up the somatic nervous and the remaining 20 making up the pharyngeal nervous system (White *et al.*, 1986). The majority of *C. elegans* neurons are located in the head of the organism, arranged in sensory organs called sensilla, with the largest collection being found in the nerve ring, located just anterior to the terminal bulb of the pharynx (White *et al.*, 1986). In addition, neurons are also located in the ventral nerve cord which spans the length of the organism, the tail and the pharynx. As a further example of the advantages of using *C. elegans* as a biological model, individual neurons can be ablated to specifically examine the roles that they each provide to the overall biology of the animal. As mentioned above, the final behavioural output of avoiding a lawn of pathogenic bacteria requires detection of sensory inputs, signalling via neurotransmitters and a behavioural output in the form of *C. elegans* leaving the food lawn. Studies have been undertaken to identify components of neural circuits which act to control this, particularly the sensory neurons involved and the transmitters involved (Meisel and Kim, 2014).

1.8.1 The *C. elegans* chemosensory network

Any chemical cues which act to drive *C. elegans* aversion away from bacterial lawns will be detected by the chemosensory neurons of *C. elegans* .The chemosensory network of *C. elegans* is highly developed, and allows detection of a vast array of both volatile (olfactory) and water soluble (gustatory) chemical cues, including those that come from food, other organisms or hazardous chemicals (Bargmann, 2006). The chemicals which are detected by the chemosensory network can modulate *C. elegans* chemotaxis, motility and entry and exit into dauer stage(Bargmann, 2006). There are 32 chemosensory neurons in *C. elegans*, with 22 being paired neurons of the amphids in the head, four paired neurons of the phasmids in the tail and 6 neurons of the inner labia (all IL2 neurons). All of these chemosensory neurons are exposed to the environment through opening made up by glial cells (Figure 1.10)(Bargmann, 2006).

The neurons in the amphids are particularly key components of the *C. elegans* chemosensory system (Bargmann, 2006). The amphids are pair of sensilla located in the head of the animal (Mori and Ohshima, 1997). Each amphid harbours 12 neurons, all of which function in chemosensation with the exception of the thermosensory AFD neuron (Figure 1.10, Table 1.5). Of the eleven types of amphid chemosensory neurons, each has the ability to detect multiple chemicals that *C. elegans* may encounter in the environment and each chemical is in turn detected by multiple neurons (Bergarnasco and Bazzicalupo, 2006) (Table 1.5).

As well as being involved in chemosensory processes, the neurons of the amphids in particular have been found to be involved in nociception. Nociception is defined as the neural processes encoding and processing noxious stimuli, basically the ability to recognise potentially harmful components in the environment (Loeser and Treede, 2008). This allows the *C. elegans* to avoid these potential harmful stimuli and thus survive. *C. elegans* nociception has been described as a reversal response in response to an aversive cue, which could be a toxic chemical (Tobin and Bargmann, 2004; Hapiak *et al.*, 2013). Examples of these aversive cues include being touched, heavy metals, acidic pH, and noxious temperatures (Tobin and Bargmann, 2004). It could be suggested that the signals such as

those summarised in 1.7 from pathogenic bacteria and the food avoidance by *C. elegans* that it leads to could be an example of a nociceptive cue.

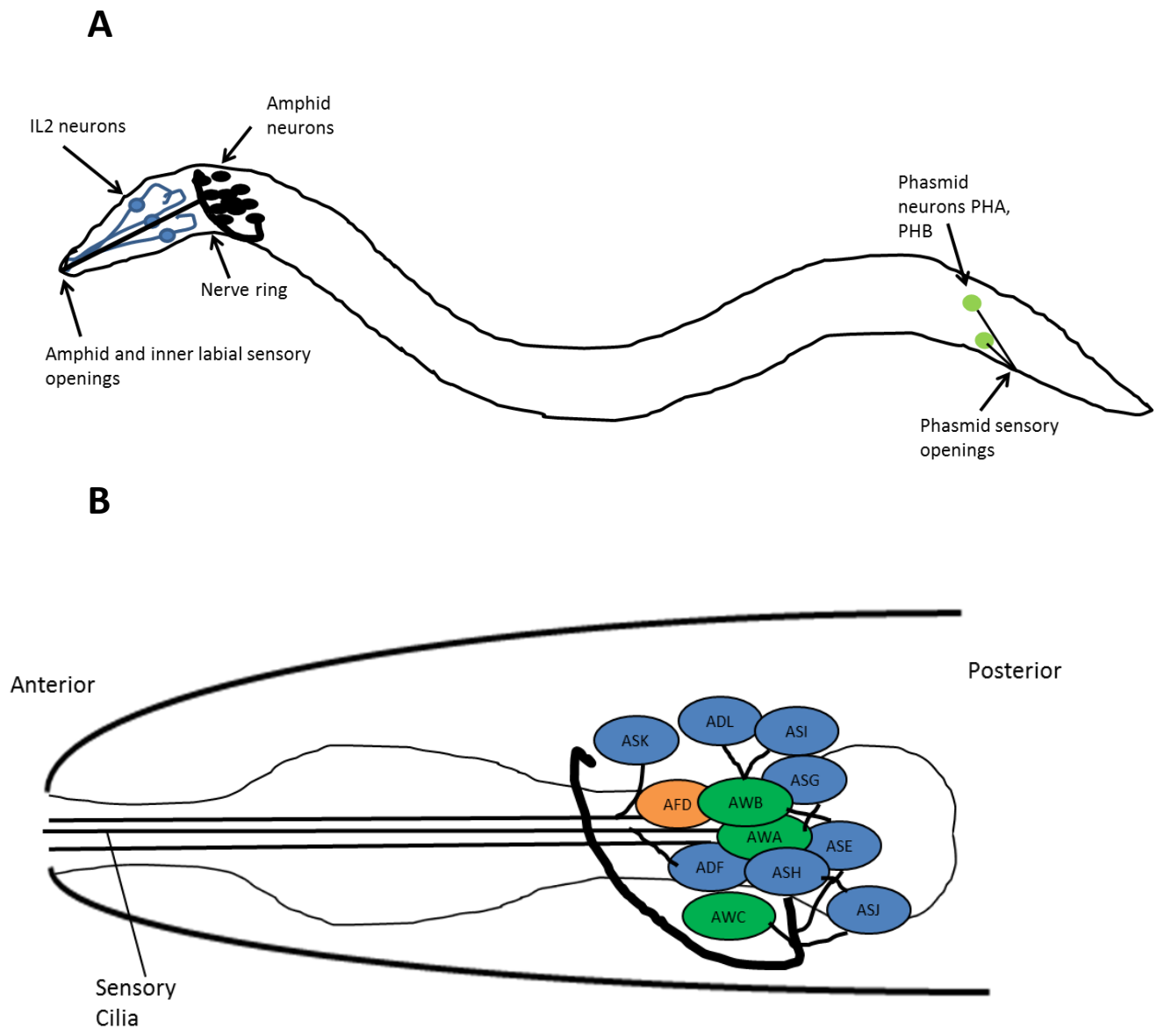


Figure 1.10: The chemosensory neurons of *C. elegans.* Diagram showing the location of the *C. elegans* sensory neurons (A). Chemosensory neurons are located in the amphids, the inner labia and the phasmids. This diagram represents one side of the animal, with the same neurons being present on the other side of the organism. Diagram adapted from Bargmann (2006). The amphid sensory neurons of *C. elegans* (B), there are 12 types of amphid sensory neurons, 11 of which are chemosensory with the other (AFD) being a thermosensory neuron (orange). Out of the 11 chemosensory neurons, 3 respond to volatile/olfactory cues (green) and 8 respond to water soluble/ gustatory cues (blue). Each of the neurons is found in each of the two amphids of the organisms, with the final nomenclature of each neuron having L or R indicating left or right. adapted from Ortiz et al. (2006).

Neuron	Roles	
ADF	Chemotaxis to water-soluble attractants Dauer entry or exit	Respond to Gustatory cues (taste) (cilia exposed to outside)
ASE	Chemotaxis to water-soluble attractants- main sensor, two neurons have distinct function R= chloride/potassium L= sodium Interaction between food detection and egg laying Minor role in avoidance	
ASG	Chemotaxis to water-soluble attractants Dauer entry or exit	
ASI	Chemotaxis to water-soluble attractants Dauer entry or exit Navigation of movement	
ASJ	Chemotaxis to water-soluble attractants Dauer formation and recovery	
ASH	Main nociceptors, mediate avoidance Nose touch, acid, high osmotic strength, metals, octanol etc.	
ASK	Chemotaxis to water soluble attractants Cadmium and copper avoidance	
ADL	Minor role in osmosensation and avoidance from octanol, copper and cadmium	
AWA	Chemotaxis to volatile attractants	Respond to Olfactory cues (smell) (cilia embedded in sheath)
AWC	Chemotaxis to volatile attractants	
AWB	Avoidance of volatile repellents	
AFD	Thermotaxis	Thermosensor (cilia embedded in sheath)

Table 1.5: The amphid sensory neurons of *C. elegans* and their functions. Adapted from Bargmann (2006)

1.8.2 The *C. elegans* neurons involved in controlling food aversion

Utilising the knowledge summarised above about the *C. elegans* chemosensory system (1.8.1), it has been determined specifically which neurons are involved in coordinating the aversion displayed towards pathogenic bacteria. These studies have implicated the AWB and AWC neurons in the amphids, which are involved in avoidance and attraction to volatile chemicals respectively(Bargmann, 2006). The circuits utilising these individual neurons become modified following learning that *P. aeruginosa* PA14 is pathogenic (Ha *et al.*, 2010). In addition, the AWB neurons have been implicated in avoidance of the serrawettin W2 which is an important component of driving *C. elegans* away from lawns of pathogenic *S. marcescens* (Pradel et al., 2007). As well as these two types of neuron, the ADF amphid sensory neurons have also been identified in modulating *C. elegans* food aversion. A circuit utilising ADF neurons is involved in the aversive learning response towards *P. aeruginosa* (Zhang *et al.*, 2005; Ha *et al.*, 2010).This particular functional pathway has also been implicated in the aversive response elicited towards non-pathogenic food sources expressing plasmids which drive RNAi of essential cellular processes in *C. elegans* (Melo and Ruvkun, 2012). The final group of chemosensory neurons implicated in pathogen avoidance are the ASI neurons, along with their previously implicated roles in food sensing (Chen *et al.*, 2013).

In addition to the sensory neurons involved in detecting cues which drive an aversive response, interneurons and motor neurons acting to integrate and executing the aversive behavioural output have also been identified. Ha *et al*. (2010) identified neural components which act downstream of the AWB/AWC and ADF cells. Specifically, the AIY, AIB and AIZ interneurons, and the RIM and SMD motor neurons act downstream of signalling from AWC and AWB neurons. These neurons are all located in the nerve ring of *C. elegans* and act to enhance the aversive response (Figure 1.11A) (Ha *et al.*, 2010). The SMD motor neuron also functions downstream of signalling from the ADF neurons alongside the RIA interneuron, also located in the nerve ring (Figure 1.11) (Ha *et al.*, 2010).

As well as neurons located in the nerve ring, in order to coordinate the movement of *C. elegans* away from the bacterial lawns, the motor neurons which act to coordinate the locomotion of the organism are involved, which are also involved in the food discrimination behaviour described in 1.4.1.2. The 75 neurons which act to drive the undulatory movement of *C. elegans* are distributed along the ventral nerve cord of the animal (White *et al.*, 1986; Zhen and Samuel, 2015).These neurons act to stimulate the dorsal and ventral muscles and subsequently coordinate both backward and forward *C. elegans* movements(de Bono and Maricq, 2005; Zhen and Samuel, 2015). In the case of avoiding harmful bacterial lawns, this locomotory system of *C. elegans* will also be involved to drive the animals away from the lawn of bacteria.

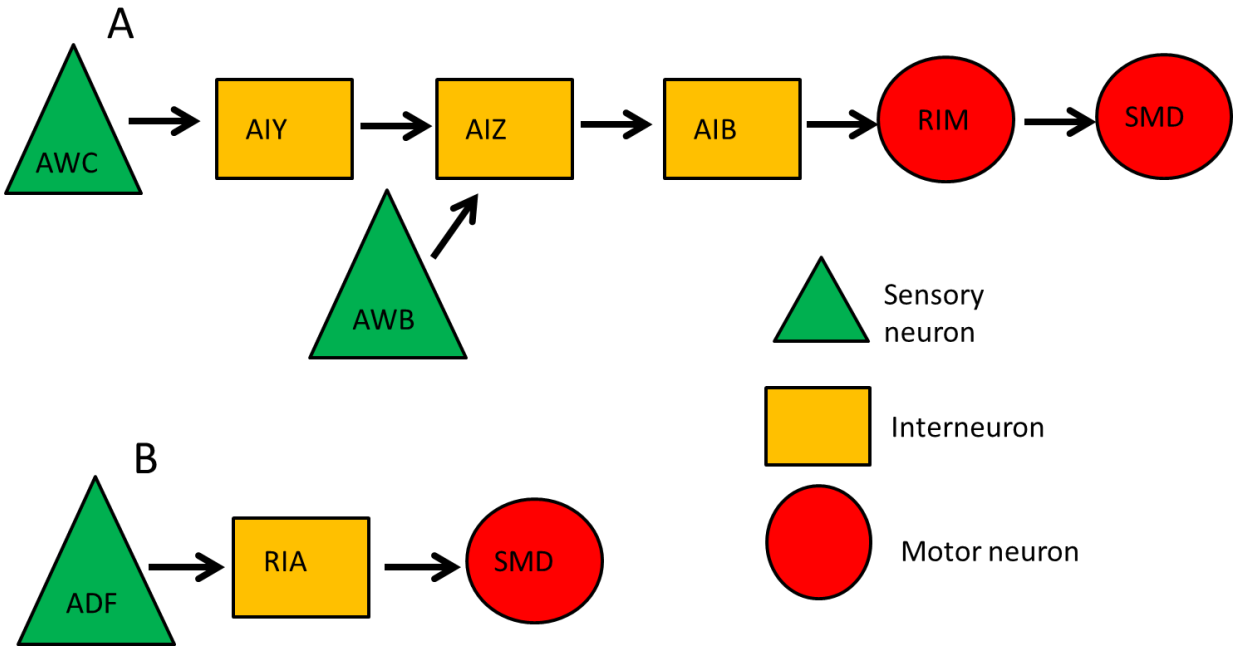


Figure 1.11: Stylised representation of neural circuits involved in controlling *C. elegans* aversion towards pathogens. This is adapted from the circuits identified in modulating aversion towards *P. aeruginosa* in Ha *et al.* (2010). Specifically, the circuits described utilise the AWC and AWB sensory neurons (A) and the ADF sensory neuron (B). Arrows indicate the transmission of signalling from one neuron to another in the *C. elegans* aversive response.

1.8.3 Neurotransmitters involved in modulating *C. elegans* food aversion in response to pathogens

Examples of the neurotransmitters which act as modulators between the neurons involved in the aversive responses to pathogenic bacteria in *C. elegans* (1.8.2) have also been identified. Identification of these neurotransmitters allows further detailing about the circuits which controls the aversion of lawns of pathogenic bacteria.

1.8.3.1 Serotonin signalling

The biogenic amines of *C. elegans* (tyramine, octopamine and dopamine), are all important neurotransmitters in the biology of the animal, and are involved in coordinating the response of the animal upon changing environments (Chase and Koelle, 2007). Two of these molecules, serotonin and dopamine, are also key neurotransmitters in the mammalian brain, and have been implicated in clinical conditions such as depression, schizophrenia and Tourette's (Sanyal *et al.*, 2004; Cools *et al.*, 2008). Analysing the specific roles of the biogenic amines of *C. elegans* has revealed that serotonin acts to control a number of *C. elegans* behaviours by modulating locomotion behaviour (Chase and Koelle, 2007). These functions include modulating the *C. elegans* response to bacterial food sources, by promoting dwelling behaviour and enhancing feeding rate by increasing pharyngeal pumping (Avery and Horvitz, 1990; Sawin *et al.*, 2000; Flavell *et al.*, 2013; Song *et al.*, 2013). In addition to these functions regulating interactions with non-pathogenic food, serotonin has also been implicated in the avoidance of lawns of aversive bacteria (Zhang *et al.*, 2005).. *tph-1* mutant *C. elegans*, deficient in the biosynthetic enzyme tryptophan hydroxylase needed to synthesise serotonin from the precursor molecule tryptophan, do not exhibit a negative chemotaxis towards *P. aeruginosa* lawns, with serotonin signalling originating from the ADF neurons (Zhang *et al.*, 2005). In addition to being shown to promote this particular aversive behaviour, this same kind of signalling has also been shown to drive the aversion elicited towards lawns of non-pathogenic *E. coli* laced with toxic RNAi plasmids (Melo and Ruvkun, 2012).

1.8.3.2 Neuropeptide signalling

Neuropeptides are an important class of signalling molecules throughout biology, including in *C. elegans*. There are over 250 individual neuropeptides produced by *C. elegans*, originating from 115 genes (Li and Kim, 2008; Holden-Dye and Walker, 2013). These can be grouped into three broad families, the FMRF amide like peptides (the FLP family), Insulin-like peptides (the INS family), which act as ligands in the DAF-2 pathway described earlier (1.6.1.3) and the neuropeptide like peptides (the NLP family) (Nathoo *et al.*, 2001; Pierce *et al.*, 2001; Kim and Li, 2004) (Table 1.6). Whilst FLP and INS peptides are defined by their similarity to well defined peptides, NLP peptides are defined by their lack of similarity of either of these. 90 individual G-protein couple receptors have been identified for their roles in transmitting neuropeptide signals. Neuropeptides are synthesised in a range of sensory, inter and motor neurons in *C. elegans* (Holden-Dye and Walker, 2013). However, there are a number of NLP neuropeptides which are not expressed in the nervous system. This includes some expressed in the intestine, where they can function as antimicrobial peptides to combat infections (Holden-Dye and Walker, 2013).

Family	Number of genes	Number of peptides
FLP	32	80
INS	40	42
NLP	47	125

Table 1.6: The categories of neuropeptides present in *C. elegans*

As in other organisms, neuropeptides are processed in *C. elegans* by a proprotein convertase. *C. elegans* has a proprotein convertase analogue encoded by the *egl-3* gene. Mutants of this gene have been analysed for implicating neuropeptides in *C. elegans* behaviours (Kass *et al.*, 2001). Analysis of neuropeptide mutants in *C. elegans*, has revealed a number of roles in which neuropeptides function in the biology of the animal. These include roles important for overall biological functioning of *C. elegans*, including coordinating egg-laying and locomotion, feeding behaviour and metabolism, especially with respect to lipids (Kass *et al.*, 2001; Ashrafi *et al.*, 2003; Papaioannou *et al.*, 2005). In

addition, neuropeptides have a role in coordinating the sexual behaviours of male *C. elegans* (Liu *et al.*, 2007). As well as being involved in these general biological roles of *C. elegans*, neuropeptides are also involved in some chemosensory responses of the organism. These particular roles include chemotaxis towards salt, aversive behaviours shown against the repellent octanol and the response to hypoxia (Tomioka *et al.*, 2006; Harris *et al.*, 2010; Pocock and Hobert, 2010). As well as these particular aversive responses, neuropeptides have also been implicated in the avoidance of pathogenic bacteria. Two insulin like peptides, INS-6 and INS-7, act antagonistically to regulate the aversive response to *P. aeruginosa,* signalling through the aforementioned ADF pathway (1.8.2) (Chen et al., 2013; Meisel and Kim, 2014). Specifically, signalling through INS-6 acts to promote aversion, whereas INS-7 acts to suppress aversion through DAF-2 antagonism.

In addition, the TGF-β ligand DBL-1(1.6.1.4) has also been implicated in avoidance of *P. aeruginosa* lawns by *C. elegans*. In addition to DBL-1, the TGF- β protein DAF-7, an important regulator of key developmental processes in *C. elegans*, has been demonstrated to detect secondary metabolites produced by bacterial lawns through the ASJ amphid sensory neurons of *C. elegans* to promote avoidance of harmful bacterial lawns (Meisel et al., 2014). In this particular case, it was demonstrated that recognition of bacterial lawns alters *C. elegans daf-7* expression which leads to promotion of aversive behaviour towards pathogenic *P. aeruginosa* lawns (Meisel et al., 2014).

As well as neuropeptides themselves being implicated in the avoidance of pathogenic bacterial lawns, neuropeptide receptors have also been demonstrated to mediate this particular behaviour. In particular, the neuropeptide and G-protein coupled receptor NPR-1, an important regulator of *C. elegans* social behaviours (ref) has been demonstrated to contribute to the resistance of *C. elegans* towards pathogenic *P. aeruginosa*, through mediating aversion towards lawns of this bacteria (Reddy et al., 2009). Specifically, increased activity of the *npr-1* gene, has been found to be coupled with both decreased time on pathogenic bacterial lawn through aerotaxis and increses resistance to the pathogenic bacteria (Reddy et al., 2009). Intriguingly, it has also been demonstrated that mucoid bacteria are able to suppress the pathogen avoidance behaviours that are driven by

NPR-1, demonstrating that bacteria can act to intefer with this particular neuronal signalling which promotes *C. elegans* avoidance of harmful bacterial lawns (Reddy et al., 2011).

1.8.4 Commonalities between the *C. elegans* pathogen aversion and the innate immune responses

Through identifying the neural circuits which are involved in controlling the *C. elegans* food aversion response seen upon exposure to lawns of pathogenic bacteria, it has been observed that there are certain shared characteristics between this particular kind of behavioural signalling and that seen in the innate immune response elicited upon pathogen infection (1.6). This includes the main immune signalling pathway of *C. elegans*, the p38 MAPK signalling pathway (1.6.1.1), which is needed for serotonin signalling in promoting aversion towards *P. aeruginosa* (Shivers *et al.*, 2009). Another MAPK pathway, the Jnk like pathway described in 1.6.1.1 is involved in controlling aversion behaviour towards RNAi-stimulating non-pathogenic bacteria through the *C. elegans* intestine and hypodermis (Melo and Ruvkun, 2012). In the same study, this circuit was also discovered to require serotonin signalling (Melo and Ruvkun, 2012). Functioning in a similar serotoninergic circuit is DBL-1, the TGF-β ligand (1.6.1.4), which has been demonstrated to be a component involved in the aversive learning towards *P. aeruginosa* PA14 lawns (Zhang and Zhang, 2012). In addition, the insulin like peptide INS-7, involved in negatively regulating the food aversion response (1.8.3.2), is stimulated by *P. aeruginosa* to suppress the immune response upon infection (1.6.1.3)(Evans *et al.*, 2008; Chen *et al.*, 2013). In addition, the toll-like like receptor TOL-1, whilst not demonstrated to be a major component of the *C. elegans* immune response to bacterial infection (1.6.1.2), is involved in the aversive response towards *S. marcescens* lawns (Pujol, 2001; Irazoqui *et al.*, 2010b; Meisel and Kim, 2014).

The work summarised in this section demonstrates how the *C. elegans* nervous system acts to drive *C. elegans* food aversion in response to pathogenic bacteria. It has been determined how there is not necessarily a single circuit functioning alone to control this response, similar to the range of signalling pathways functioning in parallel in the immune response as described in 1.6. Both different transmitters and different neurons have been implicated in modulating this aversive behaviour (1.8.2, 1.8.3). It has been found how signalling originating from chemosensory neurons located in the amphids acts to promote food

aversion, responding to chemicals sensed in the external environment (Zhang *et al.*, 2005; Pradel *et al.*, 2007; Ha *et al.*, 2010). However, signals emanating from the gut have also been shown to promote *C. elegans* food aversion (Melo and Ruvkun, 2012). These signals include the Jnk-like MAPK pathway mentioned in 1.6.1.1, which subsequently effects the expression of other immune genes expressed in the intestine. These intestinal signals are presumably responding to the presence of pathogenic bacteria in the intestine following consumption, and will act via any neurons which have projections into the intestine. The different tissues involved in stimulating *C. elegans* food aversion indicates that this behaviour can be stimulated by multiple modalities (Melo and Ruvkun, 2012). These signals are then integrated by neurons, presumably by those in the nerve ring based on obtained evidence, but possibly from the other ganglia in the *C. elegans* nervous system (Ha *et al.*, 2010). This integration of stimuli then results in a behavioural output transmitted through motor neurons, subsequently acting to drive *C. elegans* away from a pathogenic bacterial lawn (Figure 1.12). This behavioural response could be defined as both a survival strategy to avoid further consumption of harmful bacteria and sickness behaviour in response to infection.

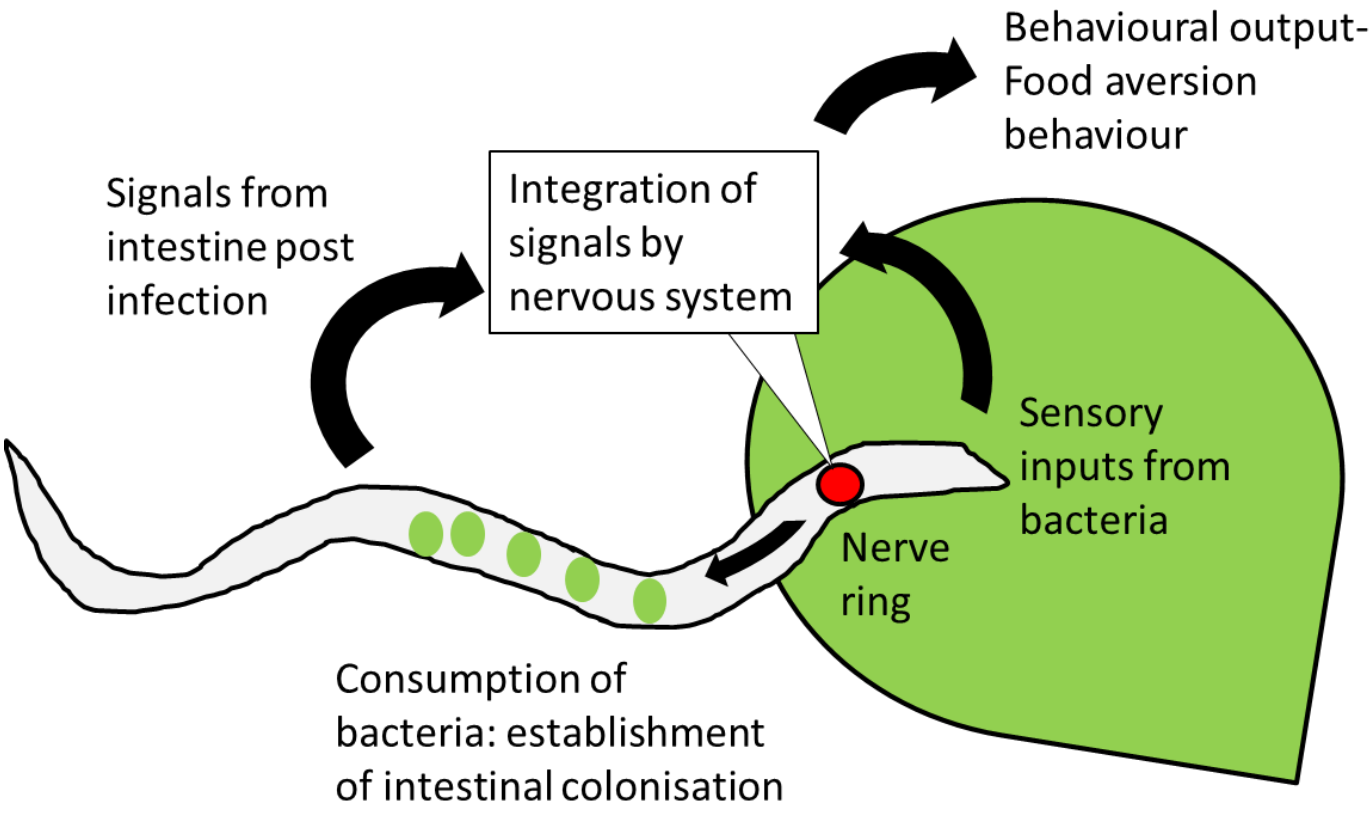


Figure 1.12 : Model of C*. elegans* food aversion behaviour. Exposure of *C. elegans* to pathogen bacteria leads to detection of chemical cues by the animal, along with signalling resulting from feeding and subsequent colonisation of the intestine by the bacteria. Integration of these signals by the nervous system of *C. elegans* results in determination that this particular bacterial lawn is harmful. The nervous system subsequently acts to coordinate the behavioural output of food aversion by driving *C. elegans* away from the bacterial lawn. Adapted from Melo and Ruvkun (2012).

1.8.5 The Gut-brain axis in mammals

The research summarised above has highlighted how a central mode of bacterial pathogenesis in *C. elegans* involves interacting with the *C. elegans* intestine (1.5.1.1). It has also been established that bacteria can influence *C. elegans* behaviour and biology, in its roles as a food source (1.4.1), a commensal (1.4.2) and a pathogen (1.5, 1.6, 1.7). As well as influencing invertebrate behaviour, bacteria have been demonstrated to influence behaviour of vertebrates as part of the gut-brain axis. The health effects of the human microbiota have been well described, and as summarised in 1.4.2, commensal bacteria have also been shown to have a positive impact in *C. elegans* (Zhang *et al.*, 2015c). This parallels

research into the mammalian microbiome which has revealed it can influence behaviour (Mayer *et al.*, 2014). It has been demonstrated from analysis of rodent models that animals which lack a microbiota (germ free animals) exhibit anxiety and depression like behaviours, and an altered microbiota has been implicated in conditions with psychological components such as autism spectrum disorder and irritable bowel syndrome (Mayer *et al.*, 2014; Mayer *et al.*, 2015).

There is also evidence that bidirectional signalling occurs between the microbiota in the gastrointestinal tract and the central nervous system of mammalian host organisms. Signalling from the host nervous system and the enteric nervous system can affect the environment of the gastrointestinal tract (Mayer *et al.*, 2015). This includes modulating the microbiota by changing the environment in which the bacteria exist, for example by changing the makeup of the mucus layer on the surface of the tract (Rhee *et al.*, 2009; Mayer *et al.*, 2015). In addition, it has been found that various signalling molecules such as cytokines and catecholamines produced by neurons, immune cells and enterochromaffin cells, coordinated by the central nervous system, acts to influence the intestinal environment and as a result the microbiota.

On other hand, it has also been determined that signalling molecules produced by bacteria can communicate with systems of the host organism (Rhee *et al.*, 2009; Mayer *et al.*, 2015). A number of bacterial products have been shown to communicate with host cells in the gut. These molecules include those involved in quorum sensing, metabolites like short chain fatty acids and bile acid metabolites, and a range of neuroactive substances like the neurotransmitter GABA, serotonin and catecholamines, as well as cytokines (Mayer *et al.*, 2015). These signals emanate from the bacteria in the intestine, and can act via neurocrine and endocrine pathways to targets well beyond the intestine, including receptors in the brain (Mayer *et al.*, 2015). The existence of the gut brain axis demonstrates that bacteria can act to influence neural function in higher organisms, and thus the behavioural reactions triggered in *C. elegans* upon exposure to pathogenic bacteria is an example of bacteria influencing behaviour in a simple vertebrate model host.

1.9 Ascaroside signalling in *C. elegans*

In addition to *C. elegans* aversive responses being stimulated by aversive substances and lawns of bacteria, *C. elegans* can also act to drive its own aversive behaviour. *C. elegans* has evolved to produce a number of molecules which act as cues to drive inter-organismal signalling to alter the behaviour of the animal, including in response to aversive environments. These particular molecules in *C. elegans* are called ascarosides. Ascarosides are multifunctional small molecules produced by *C. elegans* and are based on the sugar ascarylose with an additional head group, a fatty acid side chain and terminus group acting as modifications (Figure 1.13) (Hollister *et al.*, 2013; Butcher, 2017). Modification of the core structures of ascarosides comes from cycles of peroxisomal β- oxidation of the fatty acid side chains. This is done through four classes of enzymes: three different acyl-CoA oxidases (ACOX-1,2 and 3), an enoyl-CoA hydratase (MAOC-1), a(3R)-hydroxyacyl-CoA dehydrogenase (DHS-28) and a 3-ketoacyl-CoA thiolase (DAF-22)(Zhang *et al.*, 2015b).

The ascarosides were first discovered as the chemical cue which drives the formation of dauers from *C. elegans* larvae (Jeong *et al.*, 2005). This cue, the dauer pheromone, has been demonstrated to be a mixture of different molecules, with four different ascarosides shown to be present, with this make up varying depending on different environmental conditions (Jeong *et al.*, 2005; Ludewig and Schroeder, 2013) . Dauer formation in *C. elegans* is triggered by a high population density and starvation (Golden and Riddle, 1982). After formation, *C. elegans* dauers have a thickened cuticle and a closed mouth cavity, which helps to protect against a number of environmental insults, in addition to profound shifts in metabolism (Ludewig and Schroeder, 2013; Butcher, 2017).

The identification of individual ascarosides and the necessary biosynthetic pathways allows roles for individual molecules to be determined. These include attraction of male *C. elegans* by ascarosides produced by both L4 and adult hermaphrodites in order elicit mating (Simon and Sternberg, 2002; Srinivasan *et al.*, 2008). There are also examples of ascarosides that both hermaphrodite and male *C. elegans* produce which can attract hermaphrodites (Izrayelit *et al.*, 2012; Srinivasan *et al.*, 2012). Ascarosides have also been shown to modify how *C. elegans* responds to chemicals, specifically the organic compound benzaldehyde

(Yamada *et al.*, 2010). In this study, *C. elegans* exposed to more ascarosides exhibited higher levels of olfactory plasticity (Yamada *et al.*, 2010).

In addition, as mentioned at the beginning of this section, ascarosides have been shown to trigger dispersal of *C. elegans*, which is a similar behavioural output to that elicited upon exposure to harmful environments (1.7.1). This particular behavioural phenomenon is observed upon exposure to both ascaroside produced by dauer *C. elegans* as well as well-fed L1 larvae (Kaplan *et al.*, 2012; Artyukhin *et al.*, 2013b). It has also been identified that these kind of dispersal driving ascarosides also exist in other nematode species, thus suggesting there is conserved role for these kind of molecules (Kaplan *et al.*, 2012).

It has also been identified that individual ascaroside molecules selectively drive different *C. elegans* behaviours. Slight chemical modifications can lead to big changes in functional sequences (Hollister *et al.*, 2013). For example, the ascaroside ascr#3, which has saturated fatty acid side chains, attracts males upon release. In contrast, it acts as a repellent to hermaphrodite animals. The addition of one double bond into the fatty acid side chain of the molecule leads to this molecule also attracting *C. elegans* hermaphrodites (Izrayelit *et al.*, 2012). It has also been determined that different concentrations of the same molecules can act to antagonistically affect *C. elegans* behaviour. A low concentration of a blend of the ascarosides icas#3 and ascr#3, acts as a *C. elegans* attractant, whereas a high concentration of the same molecules acts as a repellent (Srinivasan *et al.*, 2012). It was also determined that these two molecules were signalling antagonistically against each other, with icas#3 being an attractant and ascr#3 acting as a repellent when *C. elegans* are exposed to them individually (Srinivasan *et al.*, 2012).

It has also been determined as *C. elegans* progress through the four larval stages and adulthood in the *C. elegans* life cycle, different blends of ascarosides are produced, and this blend can also be influenced by diet (Kaplan *et al.*, 2011). This includes specific ascarosides being identified as being produced by L1 larvae, with a particular chemical, osas#9 acting as a dispersal signal in adult animals (Artyukhin *et al.*, 2013b). In addition, it has recently been demonstrated that different natural isolates of *C. elegans* have different sensitivities to an ascaroside which acts to suppress *C. elegans* exploration of a bacterial lawn (Greene *et al.*, 2016).

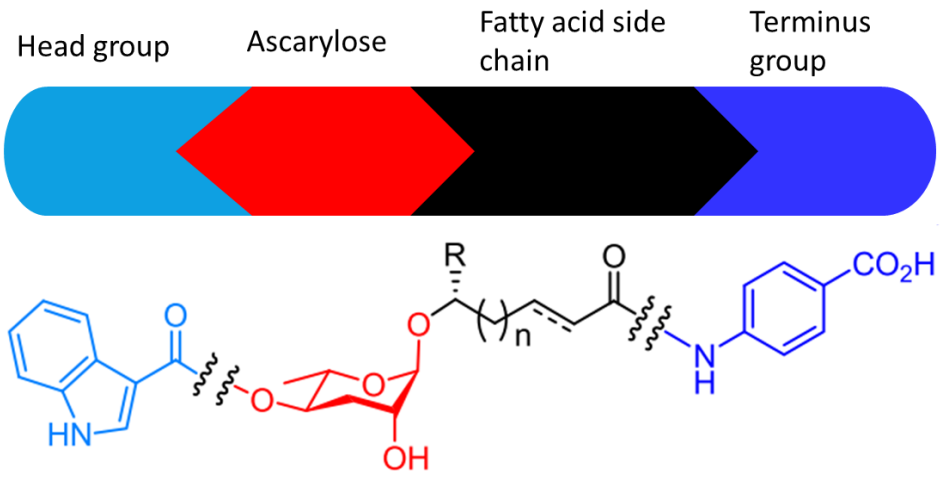


Figure 1.13: The structure of ascarosides in *C. elegans*. *C. elegans* are based on the sugar ascarylose acting as a backbone for the molecule. Modifications of ascarylose, leading to ascarosides having different structures come from the additions of a head group, a fatty acid side chain and a terminus group. Figure adapted from Hollister et al. (2013).

1.10 The bacterial species analysed in this thesis

1.10.1 Introduction to the ESKAPE pathogens

In section 1.2.1 the healthcare issues surrounding antimicrobial resistance were summarised. In the current climate, a particular group of bacteria has emerged that are of the most concern in terms of hospital acquired infections in both the developed and developing world. These are known as the ESKAPE pathogens, and are common causes of a range serious nosocomial infections amongst vulnerable individuals in healthcare environments (Santajit and Indrawattana, 2016). This group consists of the species *Enterococcus faecium, Staphylococcus aureus, Klebsiella pneumoniae, Acinetobacter baumannii, Pseudomonas aeruginosa* and members of the *Enterobacter* genus, and a referred to as ESKAPE pathogens because of their ability to 'escape' from antibacterial treatments (Rice, 2008; Boucher *et al.*, 2009; Bassetti *et al.*, 2011). In addition to the initial six species of bacteria, both non-typhoidal *Salmonella* species and non-enteric *E. coli* are also emerging as responsible for a range of multi-drug resistant infections, and are sometimes included in the group (Hassani, 2014). As a result of their drug resistant properties, and their clinical relevance, ESKAPE bacteria are currently the main focus for

investigation into antimicrobial resistance (Rice, 2008; Hassani, 2014). In addition, the members of the ESKAPE group represent a range of pathogenesis, transmission and resistance types. The range of biology exhibited by these different species means that understanding aspects of how these particular bacteria cause infections and can be managed can be applied to other bacterial species (Rice, 2008).

To demonstrate the ability of *C. elegans* to be used as an effective model for problematic bacterial infections in the current clinical climate, the ESKAPE pathogens *E. faecium*, *S. aureus, K. pneumoniae, A. baumannii* and *P. aeruginosa* have all been analysed for some aspects of pathogenesis in the nematode model (Tan *et al.*, 1999a; Garsin *et al.*, 2001; Moy *et al.*, 2004; Peleg *et al.*, 2008b; Fuursted *et al.*, 2012).

Among the ESKAPE species, *K. pneumoniae, A. baumannii, P. aeruginosa* and *Enterobacter* are all Gram-negative organisms. Gram-negative bacterial pathogens are of particular concern when it comes to antimicrobial resistance because current antimicrobial development is mostly developing compounds effective for treating Gram-positive bacteria, meaning that the lack of compounds is even starker for Gram-negative species. The work in this thesis has focused on examining three of these Gram-negative ESKAPE species utilising *C. elegans* as the host: *K. pneumoniae, A. baumannii and P. aeruginosa.*

1.10.2 *Klebsiella pneumoniae*

K. pneumoniae is speculated to be found in environmental niches like surface water, sewage and soil. In addition, *K. pneumoniae* can be found at the mucosal surfaces of mammalian hosts, and has long been considered an opportunistic pathogen (Podschun and Ullmann, 1998). *K. pneumoniae* is responsible for a range of clinical condition including urinary tract and soft tissue infections, bacteraemia ,pneumonia and pyogenic liver abcesses (Hennequin and Robin, 2015).

K. pneumoniae is a particularly important pathogen in the current clinical climate. The organism is naturally resistant to penicillins , meaning a well-established class of antibiotics is already ineffective in treating infections caused by *K. pneumoniae* before any additional resistant is obtained through mutation or horizontal gene transfer (Hennequin and Robin,

2015). In addition, clinical isolates of *K. pneumoniae* are resistant to carbapenems and cephalosporins, two important classes of antibiotics (Broberg *et al.*, 2014). This issue highlights the need for new antimicrobial development against this particular bacteria (Pitout *et al.*, 2015).

The investigation of *K. pneumoniae* pathogenesis using *C. elegans* is in its infancy relative to other bacterial species such as *P. aeruginosa, S. aureus* and *S. enterica*. The earliest study analysing *K. pneumoniae* in *C. elegans* was published in 2012, where numerous clinical strains of the species were analysed for pathogenicity in *C. elegans* alongside a murine sepsis model (Fuursted *et al.*, 2012). In addition, three more recent studies have been undertaken where *K. pneumoniae* is analysed in the *C. elegans* model, specifically with respect to the innate immune response generated upon infection (Kamaladevi and Balamurugan, 2015, 2016a, 2016b).

1.10.3 *Acinetobacter baumannii*

Acinetobacter baumannii is involved in various clinical conditions including pneumonia, bacteraemia, bacterial meningitis and urinary tract infections (Bergogne-Berezin and Towner, 1996; Perez *et al.*, 2007). Patients who have undergone surgery or have pre-existing conditions are more susceptible to being infected with *A. baumannii* (Maragakis and Perl, 2008). *A. baumannii* tends to thrive in hospital environments, thanks to the ability of the organism to be resistant to desiccation (Jawad *et al.*, 1998). This allows persistence of the bacteria on surfaces, thus providing a route for infection (Santajit and Indrawattana, 2016). The species also exists in the human skin, having a long survival time on human hands, but does not appear to be an organism typically found in the natural environment (Bergogne-Berezin and Towner, 1996; Peleg *et al.*, 2008a; Santajit and Indrawattana, 2016).

A. baumannii is currently of particular clinical concern as strains are being identified which are resistant to all available antibiotics, including colistin and imipenem, both important drugs in the current system (Vila and Pachon, 2011; Santajit and Indrawattana, 2016; Kroger

et al., 2017). This high level of antibiotic resistance makes infections and ensuing issues caused by such isolates even more difficult to treat.

Out of the three species analysed in this thesis, *A. baumannii* has been analysed the least in the *C. elegans* model, with no studies really focusing on the pathogenesis of the bacteria in the nematode. However, *A. baumannii* has been analysed in *C. elegans* for protective properties in a coinfection model with the pathogenic fungus *C. albicans*, and also for its ability to affect *C. elegans* reproduction (Peleg *et al.*, 2008b; Beceiro *et al.*, 2014). In addition, *C. elegans* has also been used as a screening for platform for antimicrobials for treatment of *A. baumannii* infected animals (Jayamani *et al.*, 2015; Girardello *et al.*, 2017).

1.10.4 *Pseudomonas aeruginosa*

P. aeruginosa is a major cause of hospital acquired infections in immunocompromised individuals in particular those with cystic fibrosis. In addition to respiratory tract infections *P. aeruginosa*, also infects skin, soft tissue, urinary tract and bone and joint infections (Mesaros *et al.*, 2007). *P. aeruginosa* can exist in a range of environmental niches, including plant surfaces, soil and aquatic habitats (Kerr and Snelling, 2009). *P. aeruginosa* can also exist in hospital environments due to its ability to form biofilms on a range of surfaces including those that are water related (Kerr and Snelling, 2009). As a result of its adaptability, *P. aeruginosa* is increasingly acquires a range of antibiotic resistances and its infections are increasingly difficult to treat (Hassani, 2014).

P. aeruginosa is a particular problem with respect to antibiotic resistance because the outer membrane is up to 100 times less permeable than *E. coli* (Breidenstein et al., 2011). As a result of this a large number of potential antimicrobials are excluded from entering the cell (Hancock, 1998; Breidenstein *et al.*, 2011). In addition to this *P. aeruginosa* has an ability to very quickly acquire further antimicrobial resistance. This likely arises as a result of the large size and variability of the *P. aeruginosa* genome, allowing rapid acquisition of genetic material from other species or strains (Mesaros *et al.*, 2007).

As in the previous sections of this chapter, *P. aeruginosa* has been extensively investigated in the *C. elegans* model. Since being first identified as a *C. elegans* pathogen, as indicated by increased mortality relative to *E. coli* in 1999, *P. aeruginosa*, and the strain PA14 in particular has been used as a 'gold standard' in *C. elegans* pathogenesis studies, and is highly virulent in the nematode model (Tan *et al.*, 1999a). *P. aeruginosa* has subsequently been analysed in all aspects of *C. elegans* infection studies, including identification of virulence factors and immune effectors (1.5, 1.6). In addition to the PA14 strain, the other well-defined *P. aeruginosa* strain PA01, which is less virulent than PA14, has also been analysed in *C. elegans* pathogenesis studies(Gallagher and Manoil, 2001; Lee *et al.*, 2006). Studies examining *P. aeruginosa* pathogenesis in *C. elegans* have also expanded beyond the examination of fast and slow killing described in 1.5.1, with traditional assays being altered to reflect different aspects of the pathogenesis of *P. aeruginosa* in mammalian systems (Utari and Quax, 2013). As alluded to in 1.7, lawns of *P. aeruginosa* PA14 also acts to stimulate food aversion in *C. elegans* populations, both from a chemotaxis assay with a choice between two bacterial lawns and from a single bacterial lawn (Zhang *et al.*, 2005; Gaglia *et al.*, 2012).

1.11 Aims of the project

As summarised above, *C. elegans* has been established in the last 18 years as a viable platform for investigating aspects of bacterial pathogenesis. As part of these studies, it has been determined that upon exposure to lawns of certain pathogenic bacteria, populations demonstrate an aversive response in the form of food aversion (1.7). However, this particular response has only been reported for lawns of *P. aeruginosa* PA14 and two strains

of *S. marcescens* (Pujol, 2001; Zhang *et al.*, 2005; Pradel *et al.*, 2007; Gaglia *et al.*, 2012). As summarised in 1.5 and 1.6, *C. elegans* has been used as a model for other clinically relevant bacterial species, including the ESKAPE species*, K. pneumoniae* and *S. aureus*. Whether lawns of pathogenic strains of these species also stimulate food aversion in *C. elegans* has not been reported. Studies where *C. elegans* are exposed to lawns of bacteria that are otherwise benign which express toxic RNAi plasmids which affect cellular processes of the animals and *E. coli* lawns laced with bacterial toxicants have also led animals exhibiting this same aversive behaviour (Melo and Ruvkun, 2012; Ballestriero *et al.*, 2016). These studies could be suggesting that *C. elegans* food aversion is a general behavioural effect which is observed upon exposure to pathogenic bacterial lawns as a form of sickness behaviour.

There is not a published example of where strains from different bacterial species which exhibited a range of virulence in *C. elegans* have been examined for their ability to generate food aversion response. Performing this kind of study, which takes advantage of the ability of *C. elegans* to be used as a platform for screening bacteria, subsequently allows deeper investigation into the biological mechanisms which drive food aversion. Systematic analysis of this will investigate how the pathogenesis of the bacteria is mediated in order to drive the behavioural responses in the host organism, as well as the neural signalling which occurs in *C. elegans*. As mentioned in 1.8, the well annotated nervous system of *C. elegans* provides multiple avenues for investigation as to how behavioural responses during a bacterial infection are mediated.

The principle aim of this thesis was to investigate a range of clinically relevant bacteria in a *C. elegans* model. Specifically, this was in order to investigate how any food aversion behaviours elicited by exposed populations of *C. elegans* related to the relative pathogenicity of the same bacteria in a *C. elegans* host.

The objectives of this thesis, which are covered in chapters 3 to 6 are:

- Develop experimental paradigms which can be used to measure behavioural responses of *C. elegans* to bacterial lawns, specifically with respect to food aversion.
- Compare the food aversion exhibited by *C. elegans* upon exposure to a range of ESKAPE bacterial strains with their respective pathogenicity.
- Investigate the mechanistic underpinning of bacterial pathogenesis in *C. elegans*.

- Investigate the neuronal controls of *C. elegans* food aversion to pathogenic bacteria

Investigating these aspects of the interactions of *C. elegans* with bacteria will give further insight into interactions between a simple animal host and bacterial pathogens. This will also lead to determination as to whether measuring simple behaviours of *C. elegans* can be predictive of the pathogenicity of clinically relevant bacterial species.

Chapter 2: Materials and Methods

2.1 Basic maintenance methods

2.1.1 Maintenance of *C. elegans* populations

C. elegans strains were stored at -80°C as frozen stocks in media consisting of equal volumes of worm freezing buffer (2.7.3) and M9 Buffer(2.7.4). These were thawed and were cultured on *E. coli* OP50 lawns on 5.5cm petri dishes (Corning) filled with NGM according to standard methods(Stiernagle, 2006). Fungal or bacterial infected nematode populations were decontaminated using bleach solution. Gravid adults were lysed in a 10µl bleaching solution (2.7.5) outside a food lawn on a seeded NGM plate for 5 minutes and dragged out of the solution, allowing the release of bleach resistant eggs from which uncontaminated *C. elegans* were generated. Other *Caenorhabditis* species were maintained in this manner. All individuals to be assayed were picked at L4 larval stage as defined by the presence of a developing vulva, then maintained overnight on *E. coli* OP50 to become L4+1s (young adults), at which point they were ready to assay unless otherwise indicated. All assays began with all *C. elegans* being L4+1s.

2.1.2 Storage of *C. elegans*

C. elegans strains were prepared for freezing by growing a population on NGM plates until the *E. coli* OP50 lawn was just depleted in order for a high number of L1 larvae to be present. Animals were washed off the plate using 2ml M9 Buffer and pipetted into a 15ml falcon tube. 2ml of worm freezing buffer (2.7.3) was added to the tube and mixed well. This mixture was separated into 4x1ml aliquots in cryovial tubes (Corning) placed in a Styrofoam box and left overnight in a -80°C freezer. Following freezing, one of the tubes was defrosted and to room temperature. The contents of the tube were then pipetted onto seeded 5.5cm NGM plates. Successful recovery of *C. elegans* resulted in the remaining tubes being put into permanent storage in the -80°C freezer. Animals defrosted for experimental analysis were also defrosted using this method.

2.1.3 Maintenance and preparation of bacterial cultures

All bacterial cultures were made from glycerol stocks stored at -80°C. Overnight cultures were prepared in LB broth at 37°C overnight from and a single isolated colony picked off from a freshly streaked LB agar plate. Overnight cultures were then diluted 1 in 100 in LB and then grown in a rotary incubator at 37°C until an OD_{600} of 0.8 was reached. These cultures were then stored at 4°C and used for up to 4 weeks. For all strains of *Klebsiella pneumoniae*, *Acinetobacter baumannii* and *Pseudomonas aeruginosa* (2.7.8) this was done under category level 2 containment conditions.

2.1.4 Setup of NGM assay plates

5.5cm petri dishes (Corning) plates had 10ml of NGM media (2.7.1) aliquoted individually using a peristaltic pump (Jencons). Once poured, NGM plates were stored at 20°C for a maximum of two weeks, and were left to dry for at least 2 days before seeding with bacteria. NGM plates used in assays were seeded with 50µl of indicated bacterial cultures (OD_{600} of 0.8) directly onto the centre of the agar. These lawns were allowed to dry and grow overnight in order to form a solid lawn on the agar. All plates were prepared in this way, and inoculated with worms the day after they were first seeded.

2.2 Behavioural assays

2.2.1 Food aversion assay

NGM plates were set up as described above (2.1.4). On the day of the assay seven young L4+1 *C. elegans* were transferred onto the middle of the bacterial lawn and observed at 2,6,24 and 48 hours after initial transfer (Figure 2.1). The food aversion level of the animals was indexed by recording the number of the original seven worms that were off the bacterial lawn as measured by visual representation. This was then plotted as the proportion of *C. elegans* off the food against the total number of adult animals.

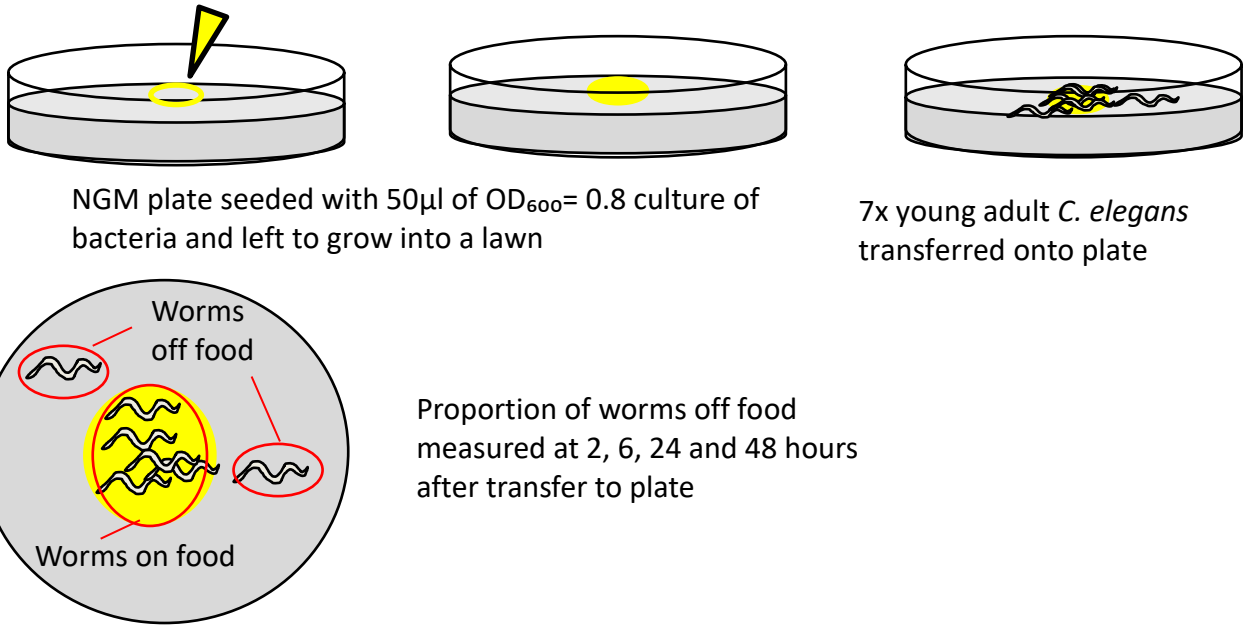


Figure 2.1: Setup of a *C. elegans* food aversion assay

2.2.2 Food-leaving assay

Seven L4+1 *C. elegans* hermaphrodites of indicated strains were picked onto the middle of the bacterial lawn. Once the worms had been placed on the plate, they were observed for 30 minutes, with the number of leaving events counted. A leaving event was recorded when the whole body of an individual *C. elegans* completely exited the food patch, as observed by visual inspection. Following this initial measurement in the first 30 minutes of the assay the number of food-leaving events was also recorded over 30 minutes after 2, 6 and 24 hours (Figure 2.2). When multiple *C. elegans* strains were analysed, mutant and N2 animals were always run in parallel to provide a proper control.

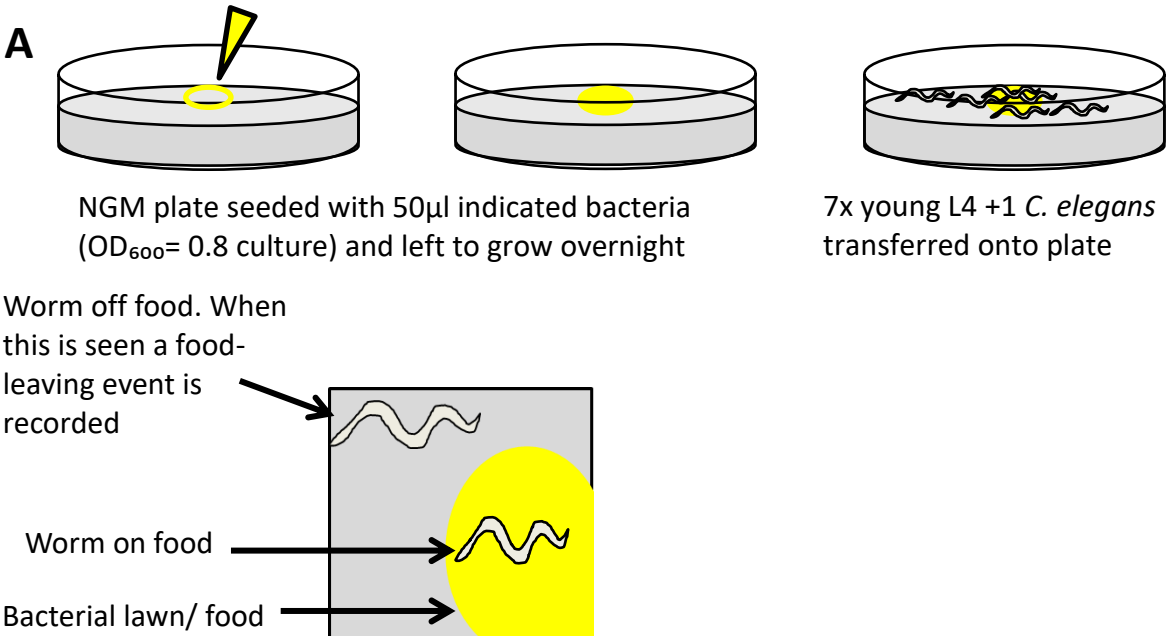


Figure 2.2: Diagrammatic representation of a *C. elegans* food-leaving assay (A). Picture of a bacterial lawn demonstrating individual *C. elegans* on and off food (B).

2.2.3 Loading of OP50 lawns with *C. elegans* larvae

Assay plates were prepared as above (2.1.4). To examine how progeny produced during the 24 hour time course influences food-leaving bacterial lawns were laced with eggs before

adding the adult worms (Figure 2.3). L4+1 *C. elegans* were placed on a bacterial lawn and were left to lay defined numbers of eggs on the food patch before being removed. The eggs were then left overnight to hatch into larvae (Figure 2.3). The number of eggs placed on each plate ranged from 5 up to 140. The top value was equivalent to the number of larvae present on each lawn after it had been populated by seven adult worms for 24 hours. The next day, approximately 18 hours after removing the adults, a food-leaving assay was performed as above, by measuring the food-leaving behaviour of L4 +1 *C. elegans* (2.2.2). When the experiment was performed using C*. briggsae* and *C. remanei* larvae, larvae were loaded in the same manner. When *G. pallida* juveniles were used, hatched larvae collected from cysts were added directly to the food lawns 30 minutes prior to addition of adult *C. elegans*. For some further experiments, in order to remove any traces of adult worms, eggs were directly pipetted onto the lawn. Eggs were prepared from gravid adults by washing them off plates in 1ml M9 into an Eppendorf containing 500ml bleaching solution (2.7.5). The tube was left for 5 min and was then pelleted by centrifuging at 1500 rpm for 2 min. The supernatant was removed and replaced with 100µl M9. 25µl of this solution was pipetted onto the food lawn and eggs were left to hatch resulting in 130-140 L1 larvae the following day. For experiments where adult leaving on lawns laced with L4s was measured, L3s were picked onto the lawn and left for 10 hours to develop into L4s, with L4+1 adults to be assayed being added after 8 hours. Following this 10 hours, food-leaving was measured as described above (2.2.2).

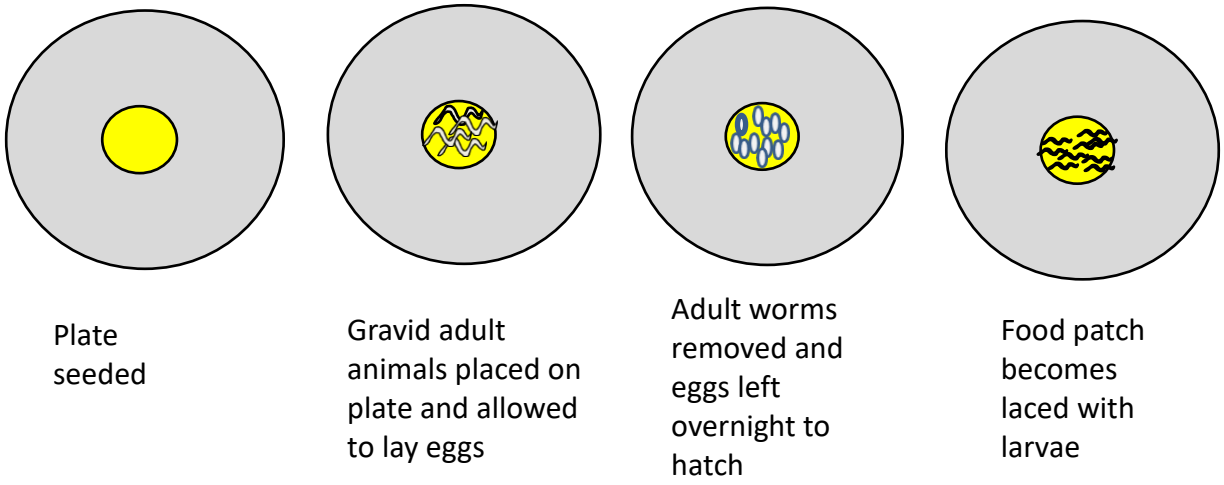


Figure 2.3: Diagrammatic representation of NGM plates used to investigate progeny induced food leaving.

2.2.4 Measurement of *C. elegans* pharyngeal pumping

The Pharyngeal pumping rate of C. elegans on food was analysed whilst the animals where on bacterial lawns and were therefore feeding. Whilst undergoing visualisation at 60x magnification, one contraction and relaxation of the grinder portion of the terminal bulb was considered one pharyngeal pump, with this being measured for one minute (60 seconds) at a time. The pharyngeal pumping rate was measured using a handheld counter.

2.2.5 Progeny production and viability assay

Lawns of bacteria were set up as described above (2.1.4). Individual L4 +1 *C. elegans* were picked onto the lawns of bacteria, and allowed to lay eggs for 24 hours (Figure 2.4). The adult animals were then removed. Visual observation was used to count the number of eggs and larvae on each individual plate. 24 hours later, the numbers of eggs and larvae was also counted. From these sequential observations the percentage of larvae that hatched from eggs after 48 hours was also calculated. This was done by combining the number of larvae and eggs at 24 hours to give a predicted total number of larvae, and comparing the number of larvae at 48 hours as a percentage of this number.

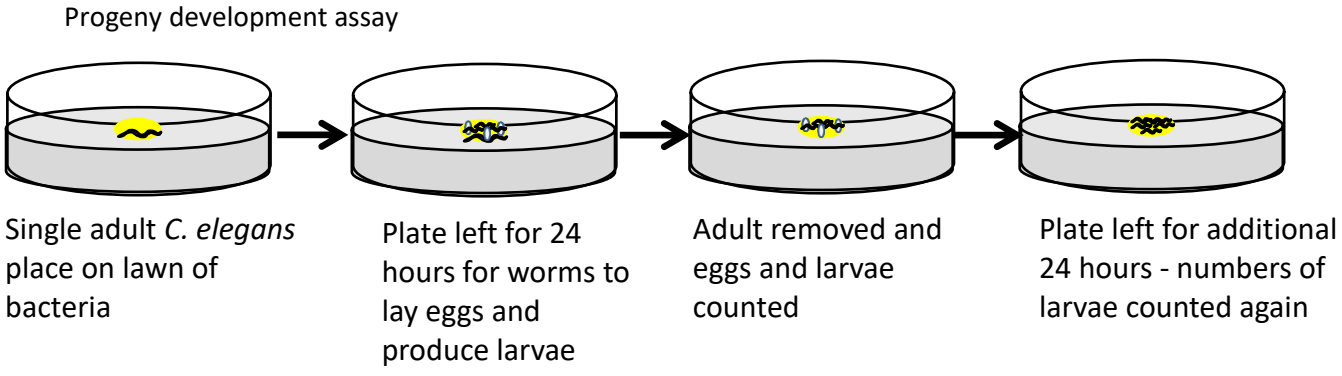


Figure 2.4: *C. elegans* progeny production assay

Lawns of bacteria were set up on NGM plates as described (2.1.4). On the day the NGM plates were seeded with bacteria, L4+1 *C. elegans* were picked onto an OP50- seeded NGM plate and allowed to lay eggs for approximately six hours. The adult worms were removed to allow synchronisation of the subsequent larvae. The eggs were left overnight to hatch and the next day approximately 20 L1 larvae (the exact number being recorded) were

passaged onto the bacterial lawns. The developmental stage of the larvae on each plate was recorded each day, and continued until all the animals reached maturity.

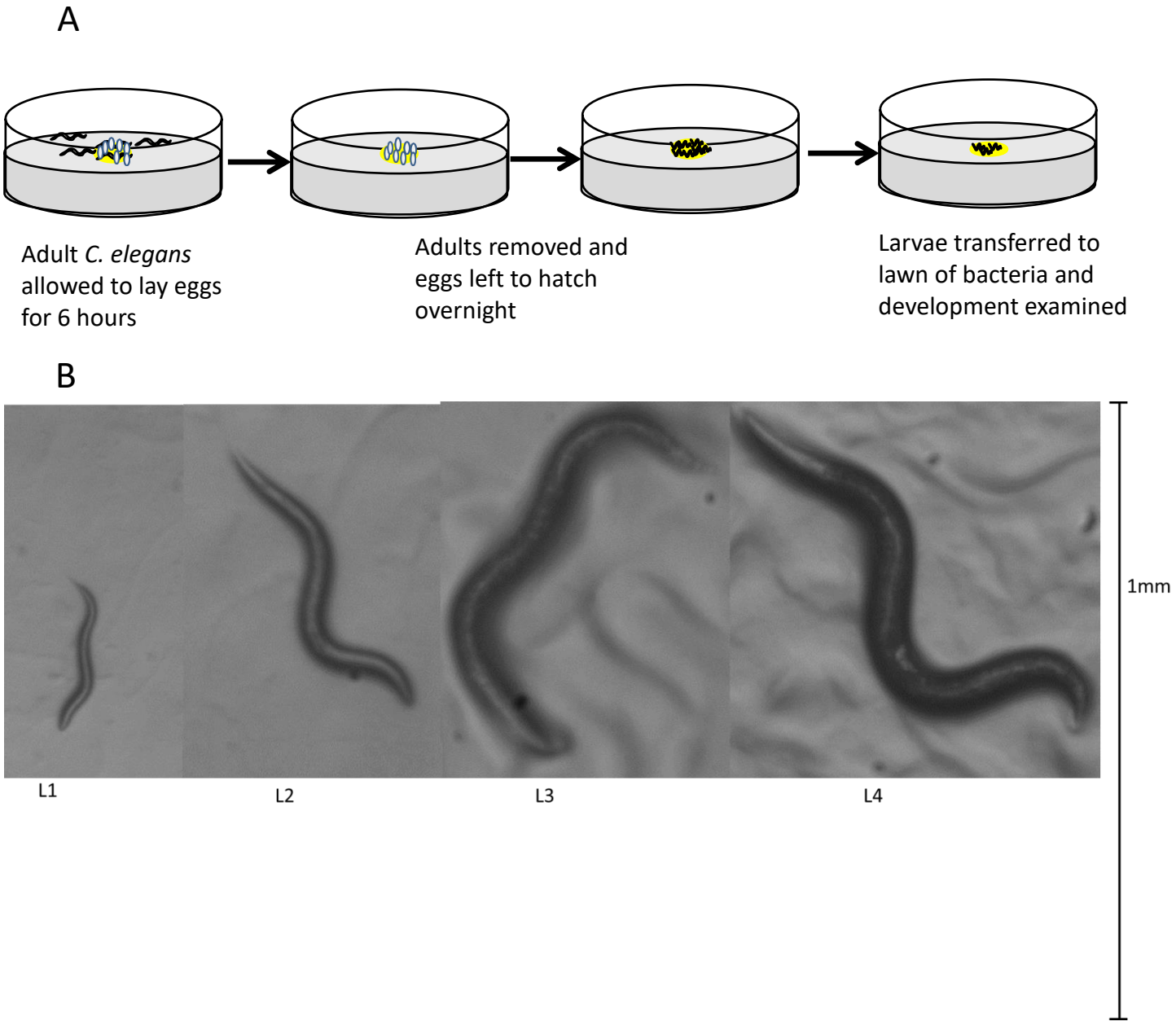


Figure 2.5: *C. elegans* progeny development assay (A). The different developmental stages of *C. elegans* larvae analysed in the progeny development assay (B). Images taken at 60x magnification

2.2.6 Octanol avoidance assay

Avoidance of 30% octanol was performed as previously described (Chao *et al.*, 2004; Wragg *et al.*, 2007; Mills *et al.*, 2012a) (Figure 2.6). Briefly, a 30% solution of octanol was prepared by diluting neat octanol in 100% ethanol. L4+1 *C. elegans* were picked onto an unseeded 5.5cm NGM plate (cleaning plate) for 10 minutes to remove any bacteria from the outside of the animals and then picked onto an additional unseeded plate (testing plate) for another 10 minutes. A Pasteur pipette with a paintbrush hair taped to the end was dipped in the 30% octanol solution and presented to the head of a forward moving animal and the time for the animal to generate a backwards movement was recorded. A cut-off was set at 20 seconds as N2 C. elegans spontaneously reverse every twenty seconds. Any individuals that did not move after this 20 second period were deemed non responders (Chao *et al.*, 2004).

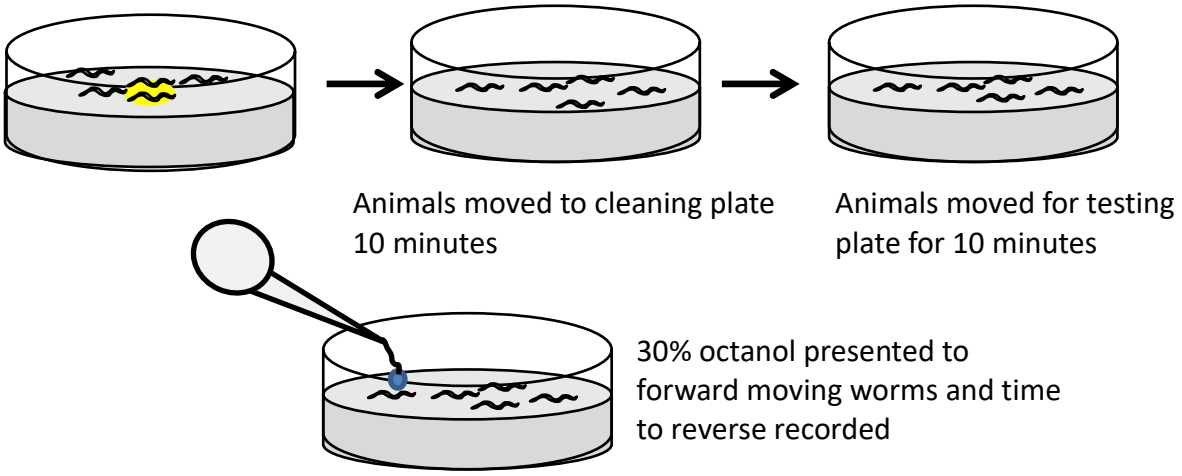


Figure 2.6: Measuring *C. elegans* avoidance towards 30% octanol

2.3 Assays based on continued exposure of *C. elegans* to bacterial lawns

2.3.1 *C. elegans* killing assay

15-20 L4 plus 1 *C. elegans* were transferred onto plates prepared as described above (2.1.4). Worms were observed each day and recorded as dead when they did not respond to being touched with a platinum wire. Animals that had crawled off the side of the plate were censured and removed from the assay. Individual worm populations were transferred to a new NGM plate with a fresh lawn of the same bacteria every other day to ensure the

original population was the only one being observed (Figure 2.7). The number of the original worms surviving against time in terms of the number of days was plotted as a Kaplan Meier plot.

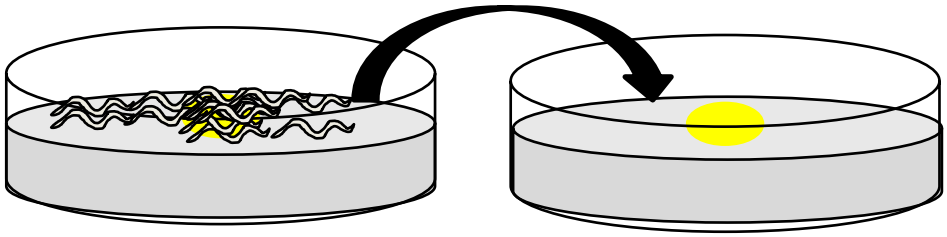


Figure 2.7: Set up of a *C. elegans* killing assay

2.3.2 Determination of bacterial colonisation

The determination of the number of bacterial cells present in the *C. elegans* intestine was based on previous protocols (Begun *et al.*, 2007; Kawli and Tan, 2008; Portal-Celhay *et al.*, 2012). Following an indicated time of exposure to a lawn of bacteria, set up as previously described (2.1.4). 10 worms were picked from these inoculation plates into a 5µl drop of 25mM levamisole in M9 (LM solution) on an unseeded 5cm NGM plate. This treatment paralysed worms and inhibited pharyngeal pumping. These worms were washed three times with 5µl drops of 1mM sodium azide in LM solution. The worms were then washed twice with 5µl drops of M9. Whilst *C. elegans* were being washed the liquid added was allowed to dry before the next solution was added. For homogenisation the washed C*. elegans* were picked into an Eppendorf containing 50µl PBS made from PBS tablets (Thermo Fisher) with 1% Triton X-100 and lysed using a glass pestle. Complete lysis of *C. elegans* was ensured by visual inspection, to allow accurate recording of CFUs per individual animal. The worm lysates were then serially diluted in PBS and were plated on LB agar. Following overnight growth at 37°C the number of colony forming units per animal was calculated (Figure 2.8).

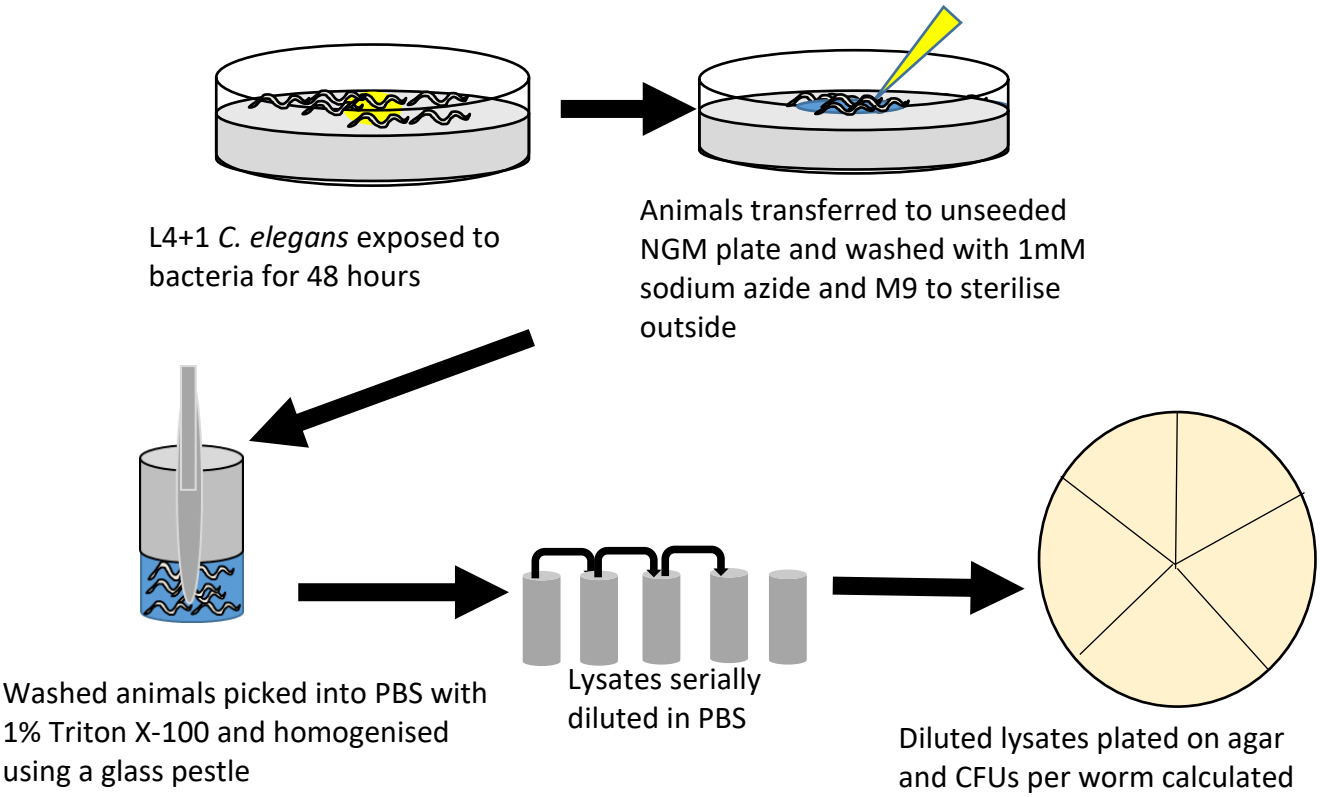


Figure 2.8: Determining bacterial colonisation of *C. elegans*

2.3.3 Measuring bacterial clearance from the *C. elegans* intestine

P. aeruginosa persistence inside *C. elegans* was estimated by measuring the amount cleared from *C. elegans* after transfer to OP50 (Kawli and Tan, 2008) (Figure 2.9). Animals were exposed to lawns of *P. aeruginosa* for 24 hours and then analysed for bacterial colonisation as described above to gain the number of bacteria colonising *C. elegans* at 24 hours (2.3.2). In parallel *C. elegans* exposed to *P. aeruginosa* for 24 hours were transferred to a lawn of OP50 for an additional 24 hours. Animals were analysed for *P. aeruginosa* CFUs as above by being plated on cetrimide agar (Sigma Aldrich), which specifically selects for *P. aeruginosa*. This allowed calculation of number of *P. aeruginosa* CFUs present inside *C. elegans* after a 24 hour incubation followed by a transfer to OP50 and additional 24 hour incubation, deemed the 'transfer' step. In order to calculate the efficacy with which *P. aeruginosa* cells are removed from *C. elegans*, the fold clearance was calculated by dividing the number of *P. aeruginosa* CFUs at 24 hours by the number of *P. aeruginosa* CFUs after the transfer to OP50.

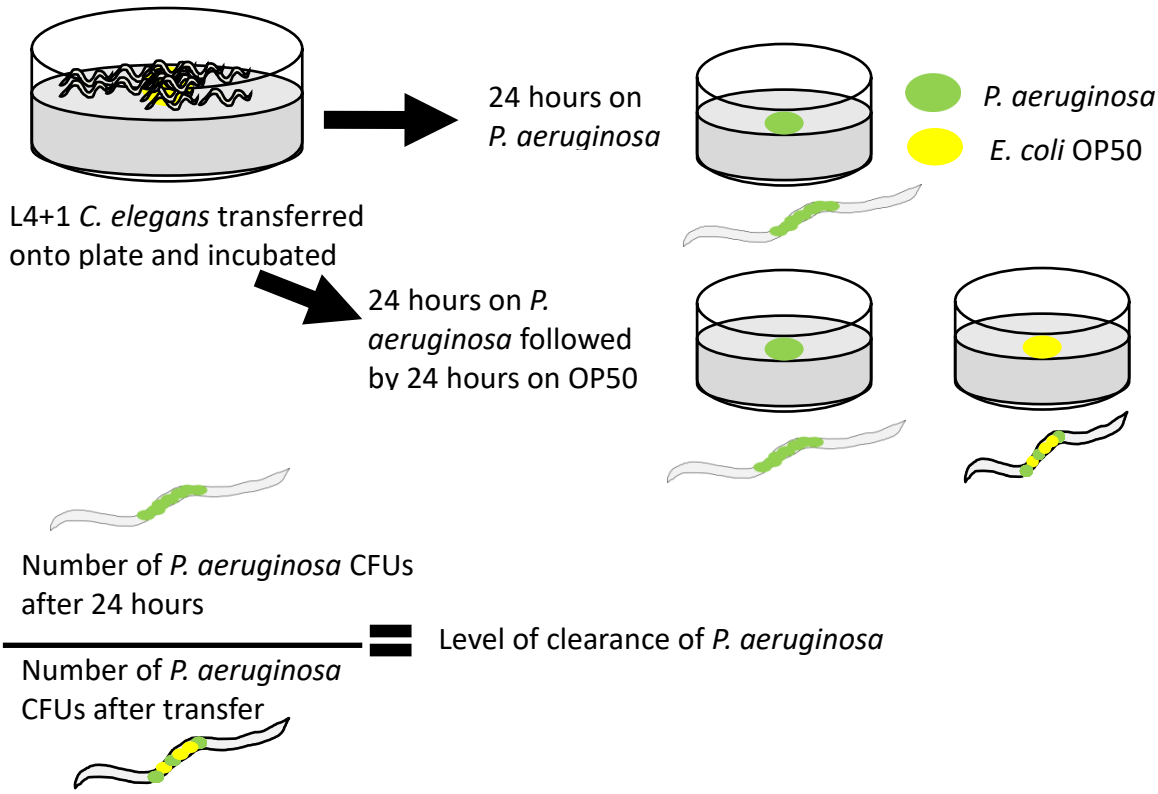


Figure 2.9: Measuring bacterial clearance from *C. elegans*

2.4 Molecular Biology Methods

2.4.1 Fosmid Purification

The fosmid containing the *C. elegans tbh-1* gene, along with the native promoter (clone CBGtg9050G1018D), expressed in *E. coli* EPI300 was obtained from the *C. elegans* TransgeneOme Resource (MPI-CBG) from Source Biosciences. The clones were stored as instructed and the 43kb fosmid was purified following a Qiagen Maxi prep protocol, following induction with Fosmid Autoinduction solution. Following purification, the identity of the fosmid was confirmed by PCR. Primers were designed to span the beginning of the first exon of the tbh-1 gene, in addition to the 5' UTR (2.7.9). The gap between the primers was 821 bp. The results of the PCR reaction (2.7.10,2.7.11) were examined by agarose gel electrophoresis on a 0.7% agarose gel run at 90V. 8µl samples of DNA were run with 2µl of 5x loading buffer to allow visualisation in a UV lightbox.

2.4.2 *C. elegans* microinjection

Injection mixes of 10ng/µl of the *tbh-1* fosmid and 30ng/µl of *pmyo-3:: gfp* were prepared, with the latter acting as an injection control. Both injection mixtures were made up in ultrapure water (Sigma). Microinjection was performed according to standard methods(Kadandale *et al.*, 2009). Injection mixes were centrifuged at 14000 rpm for 25 minutes and the resulting supernatant was used for injection. Injection Needles were made from SM100F10 glass capillaries pulled on a Narishige PC-10 needle puller. These injection pipettes were back filled with 1µl of injection mixed pipetted and allowed to reach the exit point by capillary action. L4+1 *tbh-1(n3247) C. elegans* were immobilised on a 2% agarose pad, being dipped in halocarbon oil beforehand in order to prevent the animals from being desiccated. Animals were injected in the syncytial gonad arm. Following injection worms were lifted off the agarose pad using M9 buffer and picked individually onto OP50 seeded 5.5cm NGM plates. 3-4 days after injection injected worms were analysed for green offspring. Any green worms (F1 generation) present were plated individually and allowed to reproduce. Any lines of the next generation (F2 generation) that produced green offspring were considered to be stable injected lines (Figure 2.11). All worms analysed in subsequent behavioural experiments were picked after being identified as having GFP-expressing body-wall muscle when examined under UV light, consistent with expression of the GFP-tagged *myo-3* gene (Figure 2.10).

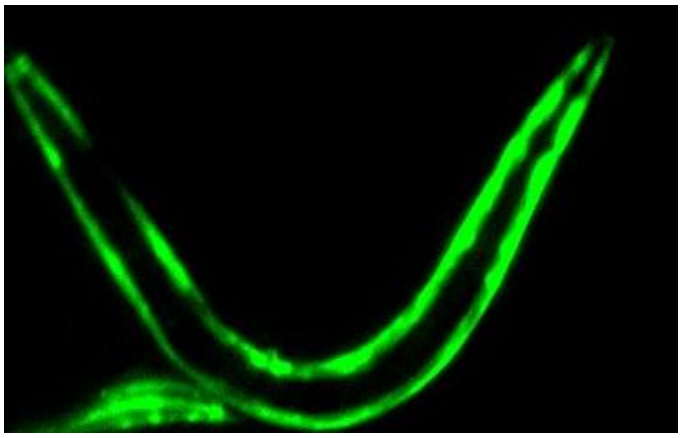


Figure 2.10: *C. elegans* expressing pmyo-3::GFP after injection visualised under blue UV light. The presence of this marker was used as criteria for successful microinjected *C. elegans*. Image taken at 100x magnification.

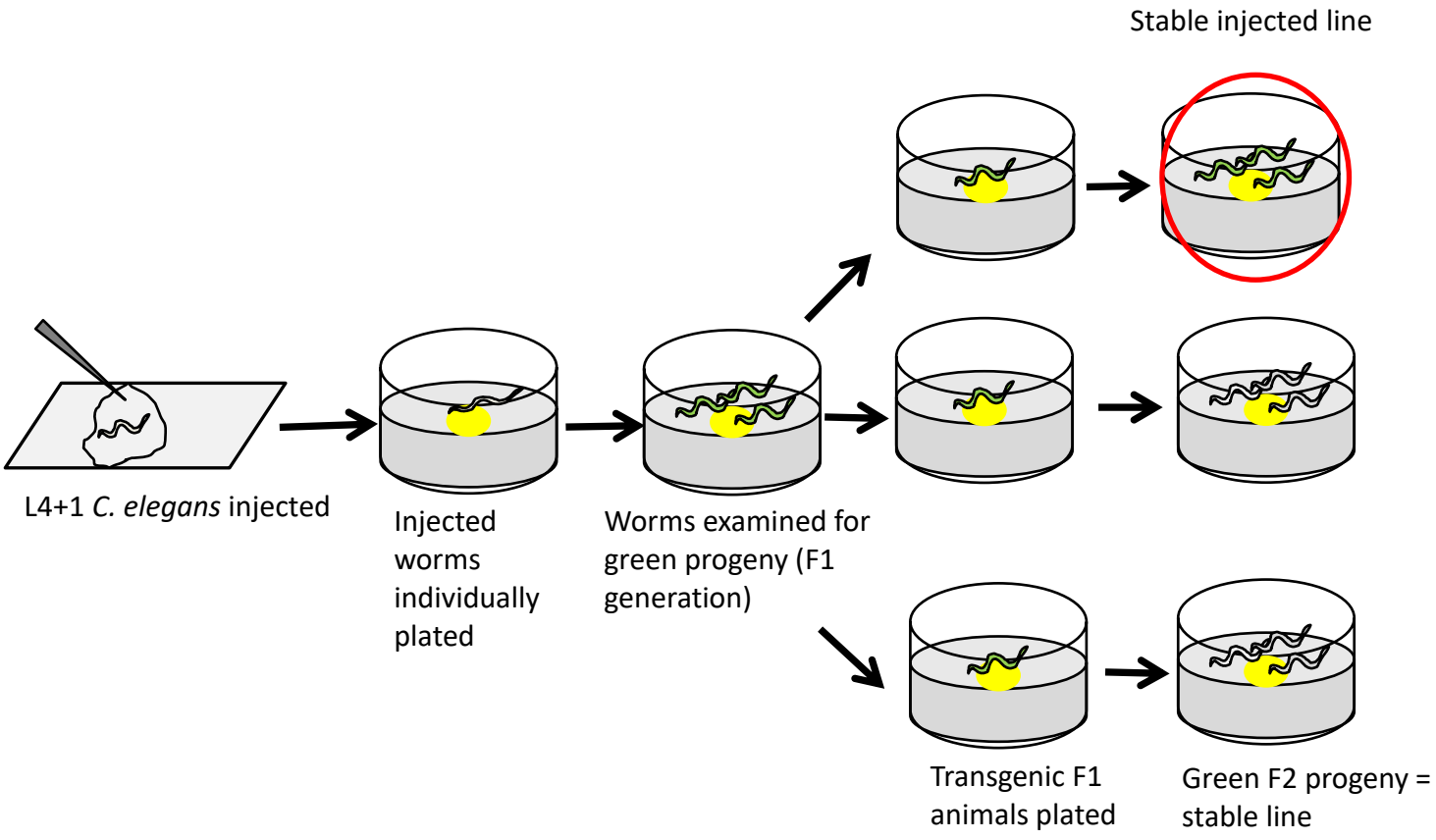


Figure 2.11: *C. elegans* microinjection and selection of transformed lines

2.5 Microscopy

All experiments were performed with under visualisation of a dissecting microscope at 10X magnification

2.6 Data analysis and Statistics

All graphs presented in thesis were plotted using GraphPad Prism. Statistical analysis was also performed in GraphPad Prism. Individual statistical tests are described in figure legends. For all statistical tests in the figures *P<0.05, **P<0.01, ***P<0.001 and ****P<0.0001

2.7 Materials

2.7.1 NGM

For 4 litres of agar:

Agar	80g
Sodium Chloride (NaCl)	12g
Peptone	10g
ddH_2O	3888ml

Following sterilisation the following was added

Sterilised 1M Calcium Chloride ($CaCl_2$)	4ml
Sterilised 1M Magnesium Sulphate ($MgSO_4$)	4ml
Sterilised Potassium Phosphate Buffer	100ml
5mg/ml Cholesterol in Ethanol	4ml

2.7.2 Potassium Phosphate buffer for NGM

For 1 litre:

Dipotassium phosphate (K_2HPO_4)	23g
Monopotassium phosphate (KH_2PO_4)	118.12g
ddH_2O	To 1 litre

Buffer was separated into 100ml aliquots and sterilised by autoclaving.

2.7.3 Worm freezing solution

For 500ml:

Sodium Chloride (NaCl)	2.9g
Monopotassium Phosphate (KH_2PO_4)	3.4g
Glycerol	150ml
1M Sodium Hydroxide (NaOH)	2.8ml
ddH_2O	To 500ml

Buffer was separated into 100ml aliquots and sterilised by autoclaving. 0.03ml of sterile 1M Magnesium Sulphate ($MgSO_4$) was added to each aliquot before use.

2.7.4 M9 Solution

For 1 Litre:

Monopotassium Phosphate (KH_2PO_4)	3g
Sodium phosphate dibasic (Na_2HPO_4)	6g
Sodium Chloride (NaCl)	5g
Sterile 1M Magnesium Sulphate ($MgSO_4$)	1ml
ddH_2O	To 1 Litre

Buffer was separated into 100ml aliquots and sterilised by autoclaving.

2.7.5 Bleaching solution

For 1ml:

Sodium Hypochlorite (NaClO)	200µl
1M Sodium Hydroxide (NaOH)	250µl
ddH_2O	550µl

2.7.6 *C. elegans* strains used

Strain	Genotype	Source
N2	Wild-type	University of Southampton
MT14984	*tph-1 (n4622) II*	*Caenorhabditis* Genetics centre, University of Minnesota
MT9455	*tbh-1 (n3247) X*	*Caenorhabditis* Genetics centre, University of Minnesota
CB1112	*cat-2 (e1112) II*	*Caenorhabditis* Genetics centre, University of Minnesota
MT13113	*tdc-1 (n3419) II*	*Caenorhabditis* Genetics centre, University of Minnesota
XA3441	*egl-3 (ok979)*	*Caenorhabditis* Genetics centre, University of Minnesota
CB4568	*Hawaiian* wild isolate	*Caenorhabditis* Genetics centre, University of Minnesota

DR476	*daf-22(m130)*	*Caenorhabditis* Genetics centre, University of Minnesota
TM2385	*ntc-1(tm2385)*	National BioResource Project
TM2765	*ntr-1 (tm2765)*	National BioResource Project
TM2243	*ntr-2 (tm2243)*	National BioResource Project
LSC42	*ntc-1 (tm2385)*	Schoofs Lab, KU Leuven
LSC48	*ntr-1 (tm2765)*	Schoofs Lab, KU Leuven
LSC455	*LSC42 [Pntc-1::ntc-1:: SL2 gfp 50ng/µl; Pelt-2::gfp]*	Schoofs Lab, KU Leuven
LSC402	*LSC48 [Pntr-1::ntr-1:: SL2 gfp; Pelt-2::gfp]*	Schoofs Lab, KU Leuven
DA509	*unc-31 (e928)*	*Caenorhabditis* Genetics centre, University of Minnesota
tbh-1 control 1.5	*tbh-1 (n3247) pmyo-3::GFP* 30ng/µl	Created in this thesis, Chapter 6
tbh-1 rescue 1.1	*tbh-1 (n3247) pmyo-3::GFP* 30ng/µl *tbh-1* 10ng/µl	Created in this thesis, Chapter 6
tbh-1 rescue 1.3	*tbh-1 (n3247) pmyo-3::GFP* 30ng/µl *tbh-1* 10ng/µl	Created in this thesis, Chapter 6
CX13079	*octr-1 (ok371) X*	*Caenorhabditis* Genetics centre, University of Minnesota
DA1774	*ser-3 (ad1774) I*	*Caenorhabditis* Genetics centre, University of Minnesota
Y54G2A.35	*ser-6 (tm2146)*	Alkema Lab, University of Massachusetts Medical School

2.7.7 Other *Caenorhabditis* strains used

Strain	Genotype	Source
HK104	*C. briggsae* wild isolate from Okayama, Japan	*Caenorhabditis* Genetics centre, University of Minnesota
AF16	*C. briggsae* wild isolate from Ahmedabad, India	*Caenorhabditis* Genetics centre, University of Minnesota
JU724	*C. remanei* wild isolate	*Caenorhabditis* Genetics centre, University of Minnesota

2.7.8 Bacterial strains used

All bacterial strains utilised in this thesis were obtained from Matthew Wand at Public Health England. *E. coli* OP50 was maintained from pre-existing laboratory stock

Species	Strain
E. coli	OP50
Klebsiella pneumoniae	NCTC 9633/ ATCC 13883
	NCTC 13438
	MGH 78578/ ATCC 700721
	NCTC 13439
	NCTC 13368/ ATC 700603
Acinetobacter baumannii	UKA15
	UKA2
	AYE/ ATCC BAA-1710
	ATCC 17978
	W1
Pseudomonas aeruginosa	PA01
	GH56
	GH12
	GH97
	ATCC 15442
	PA14

2.7.9 Primers used for PCR of *tbh-1* fosmid

Name	Sequence (5'→3')
Forward Primer	CTACTGGTAGAGTTGATGGTTGC
Reverse Primer	GTGAATACCCTGTGACGCAGTG

2.7.10 PCR reaction set up

Step	Temperature (°C)	Time (seconds)
Initial denaturation	95	30
Denaturation	95	30
Annealing	55	60
Extension	68	60
Final extension	72	300
Hold	4	Until machine stopped

2.7.11 PCR mixture

Component	Volume
Forward primer (100µM stock)	3µl
Reverse primer (100µM stock)	3µl
Fosmid (1.4µg/µl stock)	1µl
Taq polymerase	0.5µl
2mM dNTPs	5µl
5xGC Buffer	10µl
Nuclease free water	27.5µl
Final mixture	**50µl**

Chapter 3: The food-leaving dynamics of *C. elegans* on *E. coli* OP50 and the role of progeny in controlling adult food-leaving

3.1 Introduction

All bacteria that *C. elegans* encounters in the natural environment are potential food sources, therefore examining the responses between them allows evolutionary conserved controls of feeding behaviour to be studied (Avery and Shtonda, 2003). Investigation of the interactions of *C. elegans* with food sources includes food sources used in the lab. The most common of these is the *E. coli* strain OP50 (Brenner, 1974). As mentioned in 1.3.1.1, OP50 is a uracil auxotroph and as a result forms thin lawns of on NGM plates (Brenner, 1974; Brooks *et al.*, 2009; Clark and Hodgkin, 2014). This enables easy visualisation of individual *C. elegans* when feeding on lawns of OP50. The use of this bacterial strain allows for investigation of how *C. elegans* interacts with a non-pathogenic food source (Shtonda and Avery, 2006; Harvey, 2009). This approach has also been extended to understand how animals respond to an otherwise non-pathogenic food source laced with bacterial toxins and other harmful insults (Melo and Ruvkun, 2012; Ballestriero *et al.*, 2016).

C. elegans do not initially exhibit any aversive behaviour towards lawns of OP50, tending to dwell on the lawns and eat the bacteria. However, over time animals will increasingly leave the food patch more often and stay off the food for longer. This response has been attributed to the animal's interpretation of nutrient depletion and the animals therefore interpreting this food source as less favourable. This drives a foraging strategy to seek other available food sources. This behavioural response or "decision" has previously been discussed as arising from a depleted food source (Harvey, 2009; Bendesky *et al.*, 2011; Milward *et al.*, 2011; Busch and Olofsson, 2012). The levels of metabolically important gases also affects food-leaving with high carbon dioxide and oxygen levels causing worms to increasingly leave a food lawn (Bretscher *et al.*, 2008). This suggests that *C. elegans* can integrate their response based on the benefits of feeding versus the danger of potentially toxic ambient air conditions (Busch and Olofsson, 2012).

Assays have been developed using a small lawn of bacteria on an NGM agar plate, with clear agar around the bacterial lawn in which the *C. elegans* can roam and explore the external environment. These assays have been developed to investigate foraging in *C. elegans* and have been coupled with genetic analyses to provide insight into the molecular substrates

that underpin the worm's decision of whether or not to leave a food patch. Some studies have taken advantage of the observation that different strains of *C. elegans* have distinct foraging behaviours. Specifically, N2 *C. elegans*, the most commonly used wild isolate of *C. elegans*, has a lower tendency to leave a bacterial lawn than another *C. elegans* wild isolate the Hawaiian (CB4856) strain, initially isolated from pineapple fields in Hawaii (Harvey, 2009; Bendesky *et al.*, 2011). The striking differences in the level of food-leaving between these strains are linked to a plethora of genes to controlling this behaviour (Zhu *et al.*, 2015b). Indeed, an enhanced food-leaving represents one of several sub-behaviours associated with the Hawaiian strain in which the neuropeptide Y receptor NPR-1 and the tyramine receptor TYRA-3 are both significant determinants (de Bono and Bargmann, 1998; Gloria-Soria and Azevedo, 2008; Harvey, 2009; Bendesky *et al.*, 2011; Milward *et al.*, 2011)

In addition to being regulated by food density, quality and indicators of pathogenicity, foraging is also modified by factors relating to reproduction and fitness. Thus male *C. elegans* will leave a food patch in order to locate a mate (Lipton *et al.*, 2004) highlighting the neural drive to reproduce can over-ride an otherwise potent nutritional cue to remain on the lawn. It has also been found that both arrested L1 or dauer larvae, which are *C. elegans* life stages generated under starvation conditions, produce signals that trigger adult food-leaving or dispersal, as an example of the ascaroside signalling summarised in Chapter 1 (1.9).

In addition to solely examining how *C. elegans* interacts on lawns of bacteria, the effect of other *Caenorhabditis* species on triggering *C. elegans* behaviour was examined. The genus *Caenorhabditis* is part of a diverse order of terrestrial nematodes called Rhabitida (Blaxter, 2011). This order includes free living species such as *C. elegans*, as well as others which associate with arthropods and parasites (Kiontke and Sudhaus, 2006; Blaxter, 2011). The *Caenorhabditis* genus consists of species which all live in decaying organic material, like the soil and rotting fruit. The particular group in which *C. elegans* exists is known as the *Elegans* group. This group also includes the species *C. briggsae, C. remanei* and *C. brenneri*, which are all equally closely related to *C. elegans* in terms of the genetic identity (Figure 3.1) (Kiontke and Fitch, 2005; Kiontke and Sudhaus, 2006). In this chapter, two other species in the *Elegans* group of the *Caenorhabditis* genus were examined, *C. briggsae* is a

hermaphroditic relation of *C. elegans* that shares habitats with *C. elegans* (Kiontke and Sudhaus, 2006; Felix and Duveau, 2012). *C. remanei*, like both *C. elegans* and *C. briggsae* is found in fermenting environments but unlike the other two species naturally exists as males and females and reproduces sexually (Kiontke and Sudhaus, 2006).

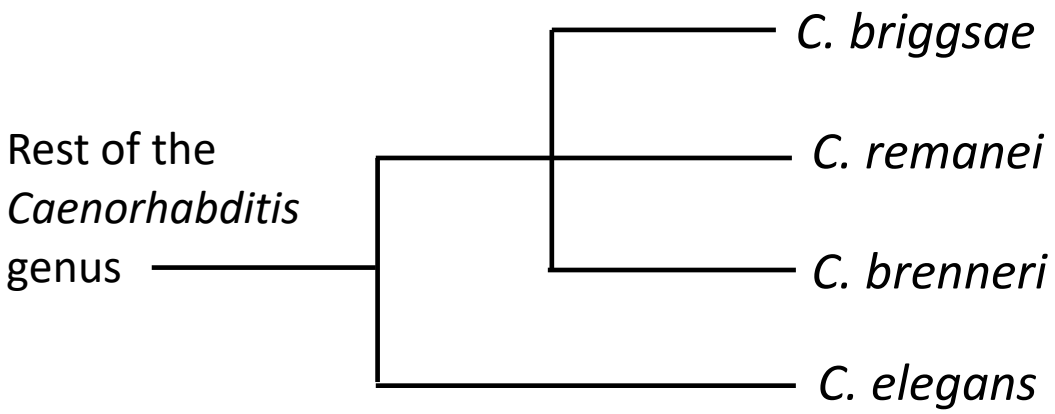


Figure 3.1: The *Elegans* group of the *Caenorhabditis* genus and the different species in the group, including *C. elegans.* Adapted from Kiontke and Fitch (2005).

3.2 Aims and objectives

The principle aim of this chapter was to develop assays which examine *C. elegans* aversive behaviours based on exposing populations of *C. elegans* to lawns of *E. coli* OP50. This would create control conditions to monitor aversive behaviours upon an extended bacterial incubation, which will be undertaken in the later chapters of this thesis. These assays examined both *C. elegans* exploration away from food (as measured by proportion of animals off a bacterial lawn) and food-leaving rate (the number of times *C. elegans* individuals leave a bacterial lawn over a defined time period). This also allowed identification of important components of *C. elegans* behaviours upon exposure to a lawn of inert and nutritious bacteria. Undertaking this approach revealed a novel component which acts to stimulate *C. elegans* food-leaving. *C. elegans* larval progeny act to induce this food-leaving, which resulted in an increase in both food aversion and food-leaving. This behaviour was underpinned by a selective cue to leave a bacterial food lawn from offspring that emerge during an assay.

3.3 Results

3.3.1 *C. elegans* exhibit an enhanced food-leaving rate after extended exposure to lawns of *E. coli* OP50

Previous studies identified that *C. elegans* transiently leaves a defined bacterial lawn with initially a very low rate but this shows a steady increase over time (Milward *et al.*, 2011). To investigate this phenomenon, standard assays were generated utilising a lawn of OP50 grown from a culture with an OD_{600} of 0.8 (2.1.4). These assays included two measures. The first of these was a measure of the level of *C. elegans* exploration away from food. This involved counting the proportion of the adult animals used in the assay that were off the bacterial lawn at the various time points (2.2.1). The second measure was a measure of food-leaving. This involved observing the food-leaving rate of the *C. elegans* adults being assayed (as defined by food-leaving events/worm/minute (2.2.2).

When both of these measures were taken through examining L4+1 wild type *C. elegans* behaviour on an OP50 lawn, both the food aversion levels and the food-leaving rate significantly increased after 24 hours of exposure to the lawn (Figure 3.2 A,B). The fact that both food aversion and food-leaving exhibited this increase demonstrates that these measurements are surrogates of each other, and that measuring food aversion represents the levels of food-leaving. The time dependent food-leaving increase exhibited here demonstrated a similar time-dependent increase in *C. elegans* food-leaving as previously described (Milward *et al.*, 2011).

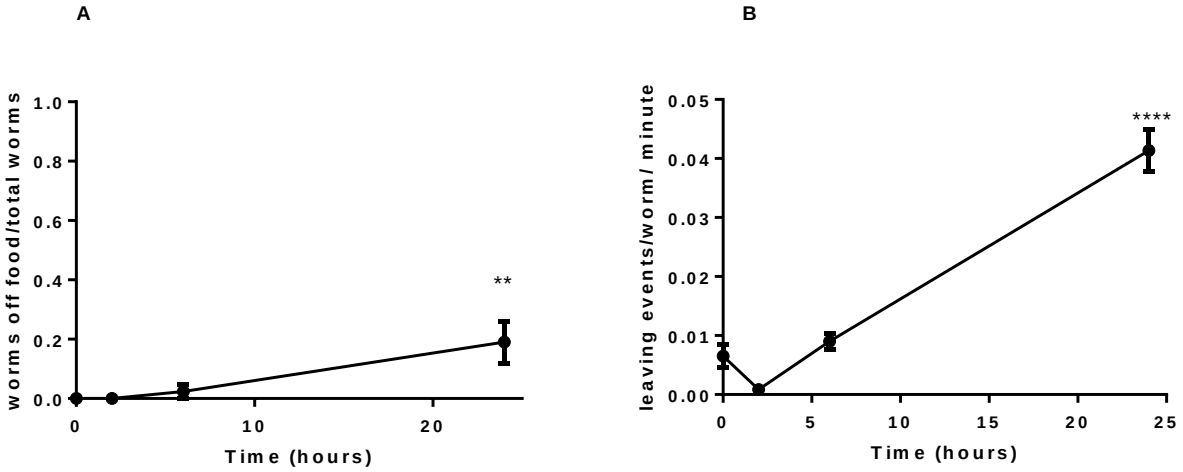


Figure 3.2: Food-leaving behaviour of adult wild-type N2 *C. elegans* on lawns of *E. coli* OP50. Seven one day old adult wild-type (N2) *C. elegans* were placed on a defined bacterial lawn of *E. coli* OP50 and the proportion of worms off the bacterial lawns (A) and number of food leaving events scored for a period of 30 minutes (B) was measured. Both measurements were taken immediately after exposure and 2, 6 and 24 hours after exposure. n=6 lawns. Error bars represent ±SEM. Data analysed by one-way ANOVA with Tukey's multiple comparisons test. *P<0.05, **P<0.01, ***P<0.001 and ****P<0.0001

3.3.2 *C. elegans* enhanced food-leaving is not a result of food depletion

The bacterial lawns after 24 hours are similar in the experimental makeup and nematode exposure to those reported in the literature to have undergone depletion, and as a result driving food-leaving (Figure 3.3A,B) (Milward *et al.*, 2011). To resolve whether there was significant change in the density of the bacterial lawn bacterial growth curves were measured for OP50 lawns that had been cultivated for 24 hours with 7 young adult worms; that is, the conditions under which there was a progressive increase in food-leaving (Figure 3.2). These were compared to lawns incubated for 24 hours without addition of 7 worms. The growth curves for both samples in LB broth were identical (Figure 3.3C) suggesting that the bacterial lawn is not depleted by accumulated feeding of the seven adults, and that the increase in food aversion is not driven by food depletion or starvation of the animals.

This preliminary conclusion was reinforced by measuring the food-leaving rate of *C. elegans* on OP50 lawns. Doing this revealed that there was no difference in the food-leaving events,

despite greater than 10-fold differences in optical density of the bacteria used to make the food patch (Figure 3.3D). Taken together, these data indicate that there is not significant depletion in the food lawn during the period of enhanced food-leaving. The dilution of the bacterial lawns is not a significant driver of enhanced food-leaving observed in adult *C. elegans* over the 24 hour period. It has also been demonstrated that even a very dilute bacterial lawn still contains enough bacteria for a *C. elegans* to feed on (S. Zarroug, personal communication). During the period in which the adult *C. elegans* exhibit this enhanced rate of food-leaving they also sustained an active feeding rate whilst they are on the bacterial lawn as observed by their high frequency of pharyngeal pumping, which was sustained throout 24 hours of exposure on an *E. coli* lawn (Figure 3.4).

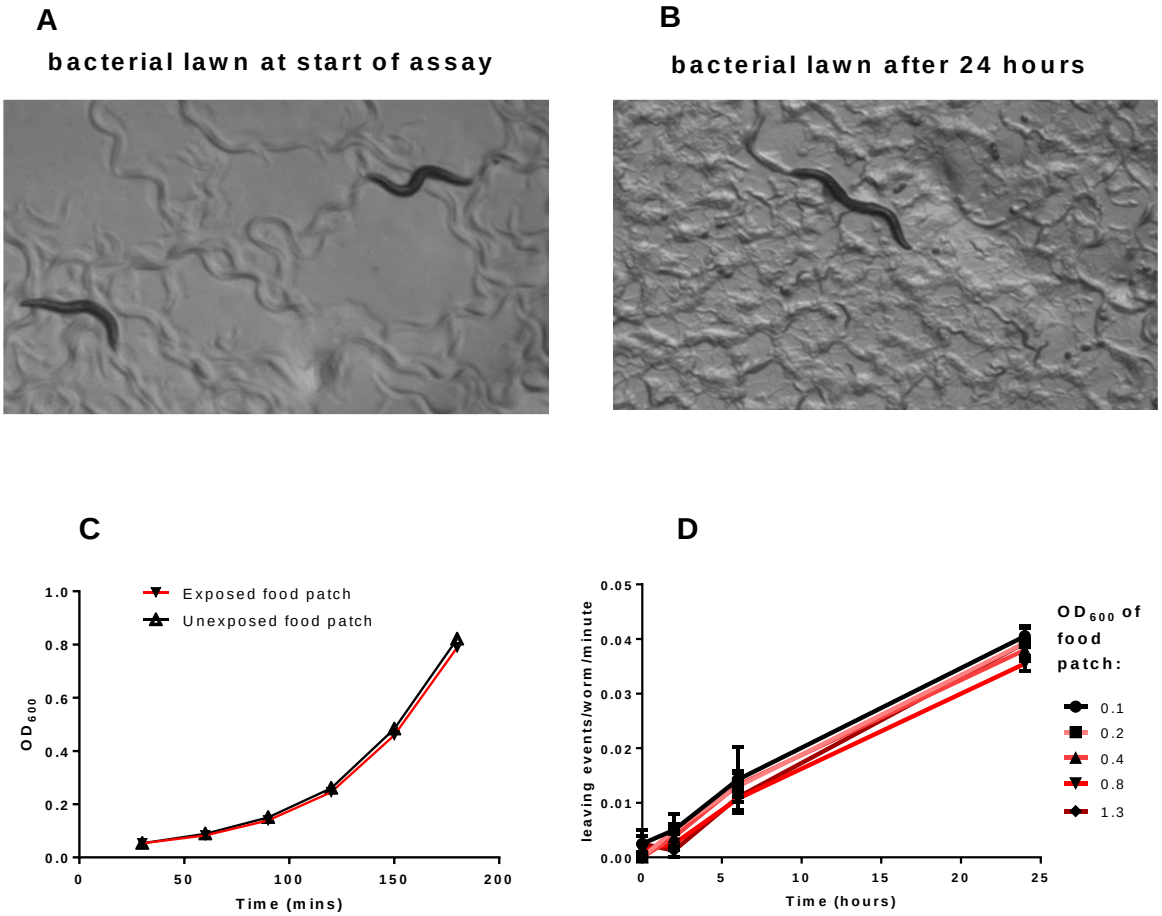


Figure 3.3: *C. elegans* food-leaving is not due to food depletion. Pictures of the food lawn at the beginning (A) and after 24 hours (B) of a food leaving assay. At the end of the food-leaving assay (Figure 3.2) the bacterial lawns were cut out of the agar plates and grown in LB broth at 37°C (C). The growth rate of the bacterial lawns that had been exposed to worms (food patch plus worms) was compared to bacterial lawns recovered from plates cultured in an identical manner except in the absence of worms (food patch minus worms) (D). Data are mean ± SEM; n=4. D. One day old adult *C. elegans* were exposed to bacterial lawns of different optical densities and food-leaving scored as for (A). Data are mean ± SEM for n=4 lawns.

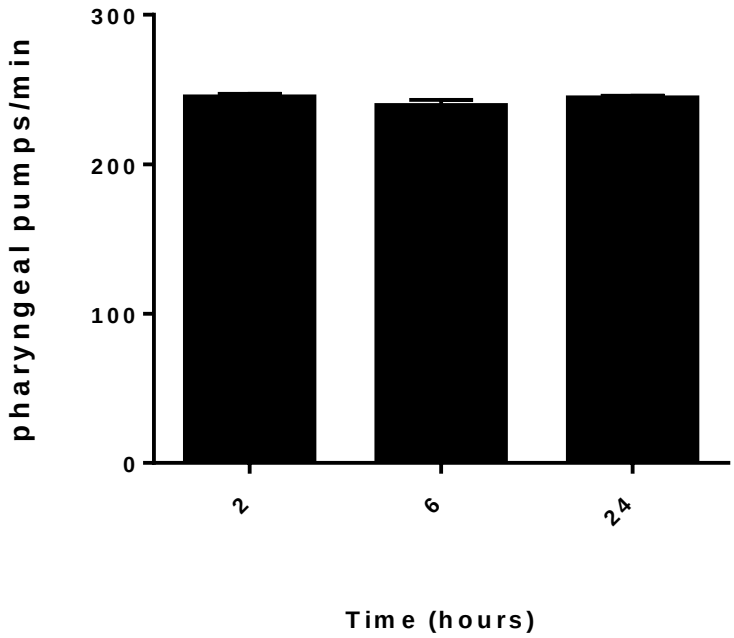


Figure 3.4: The pharyngeal pumping rate of *C. elegans* on lawns of *E. coli* OP50 across a 24 hour time course. Error bars representative of ±SEM. Data analysed by one-way ANOVA with Tukey's multiple comparison test.

3.3.3 *C. elegans* larvae stimulate adult food-leaving

As is shown in the images of the lawns after 24 hours (Figure 3.3B) of an assay the adult *C. elegans*, which are gravid adults, lay eggs which subsequently hatch during the 24 hour time course of the assay. Typically *C. elegans* larvae take 6 to 8 hours to hatch after being laid so L1 larvae begin to appear on the bacterial lawn between the 6 and 24 hour time-point in the food-leaving assay based on the *C. elegans* life cycle (1.1). By 24 hours they start to transition to the L2 stage. Thus, at the 24 hour time-point there was a mixed population containing both the original seven adults, over 200 eggs and around 120 L1 and L2 larvae (Figure 3.5). As there was no evidence to support depletion of the food source as a stimulus for enhanced food-leaving, the progressive increase in progeny of the bacterial lawn might provide a drive to enhance food-leaving.

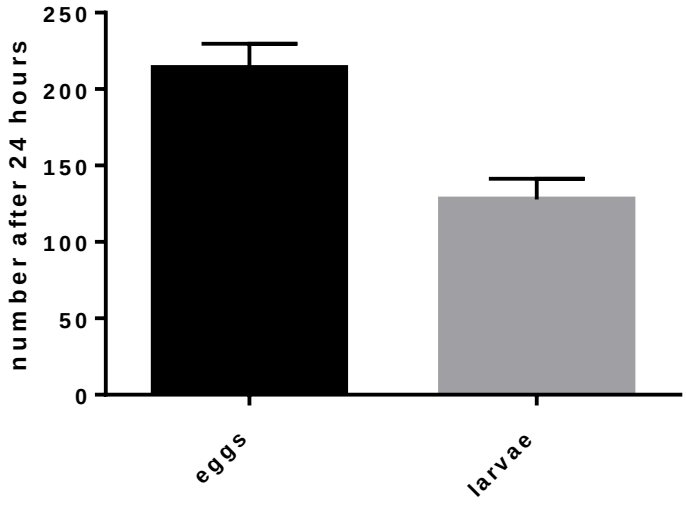


Figure 3.5: The numbers of eggs and larvae produced from seven adult N2 animals after 24 hours on an *E. coli* OP50 lawn. Data representative of n=6 trials. Error bars represent ±SEM

3.3.3.1 Pre-loading bacterial lawns with *C. elegans* larvae stimulates adult food-leaving

To test the effect of larvae on driving *C. elegans* food-leaving, one day old hermaphrodite *C. elegans* on were placed on food patches that had been pre-loaded with increasing numbers of eggs (between 0 and 140) 24 hours before the assay (2.2.3) and which had subsequently developed into larvae. Adult *C. elegans* placed on bacterial lawns that had been populated with 140 larvae showed an immediate high rate of food-leaving, similar to the food-leaving rate of worms placed on bacterial lawns without progeny after 24 hours (Figure 3.6A,Figure 3.2B). This had the appearance of dose-dependency with a threshold of between 20 and 70 larvae driving this increased level of food-leaving (Figure 3.6A). Additionally, the food-leaving of the adult worms placed on lawns pre-loaded with progeny was slightly higher after 24 hours than that seen on plates which were initially not populated with progeny, as in the assays performed in 3.3.1 (Figure 3.6A). At this point in the assay the animals being assayed have populated the plates with their own progeny. In the case of the pre-loaded plates the production of further progeny, increasing the number of larvae on the lawn may be acting to further increase food-leaving (Figure 3.6A). However, the relative small increase between the experimental groups, control and pre-loaded with 140 progeny at the 24 hour time point, where the animals on the lawns laced with larvae have produced their own larvae,

suggests that there may be a plateauing effect with it reaching a near maximal level in the presence of 140 plus progeny. For plates preloaded with progeny the increase in food-leaving was accompanied by an increase in the proportion of adult *C. elegans* off the bacterial food lawn (Figure 3.6B).

To further test whether or not the cue for adult food-leaving is offspring derived, instead of an enduring signal permeating the lawn left by the adults that were used to preload the lawns with eggs prior to their removal, another method was used to load the plates with progeny. For this, isolated *C. elegans* eggs from gravid adults were pipetted onto the bacterial lawn. This removed any potential chemical cue that could be left on the bacterial lawn by adults moving on the lawn whilst eggs are being laid. Adult food-leaving on lawns preloaded with progeny in this manner was identical as that for lawns preloaded by allowing gravid adults to lay eggs before their removal (Figure 3.6C).

These results suggested that a cue from the *C. elegans* larvae, rather than from the adults that supplied the eggs for preloading the plates, drives the enhanced food-leaving response in adults. In order to determine whether early stage larvae specifically enhanced food-leaving of adult *C. elegans*, a similar experiment was undertaken with bacterial lawns were loaded with L4 animals (2.2.3). This was done in two different approaches. The first of these was picking L4s onto the bacterial lawn and leaving for 2 hours before the adult animals were added. The second approach involved picking L3 stage larvae onto the lawn and leaving them for 10 hours to allow them to develop into L4s. When food-leaving of adult *C. elegans* was measured on both of these types of lawns loaded with L4s, there was no significant enhancement of food-leaving (Figure 3.6D).

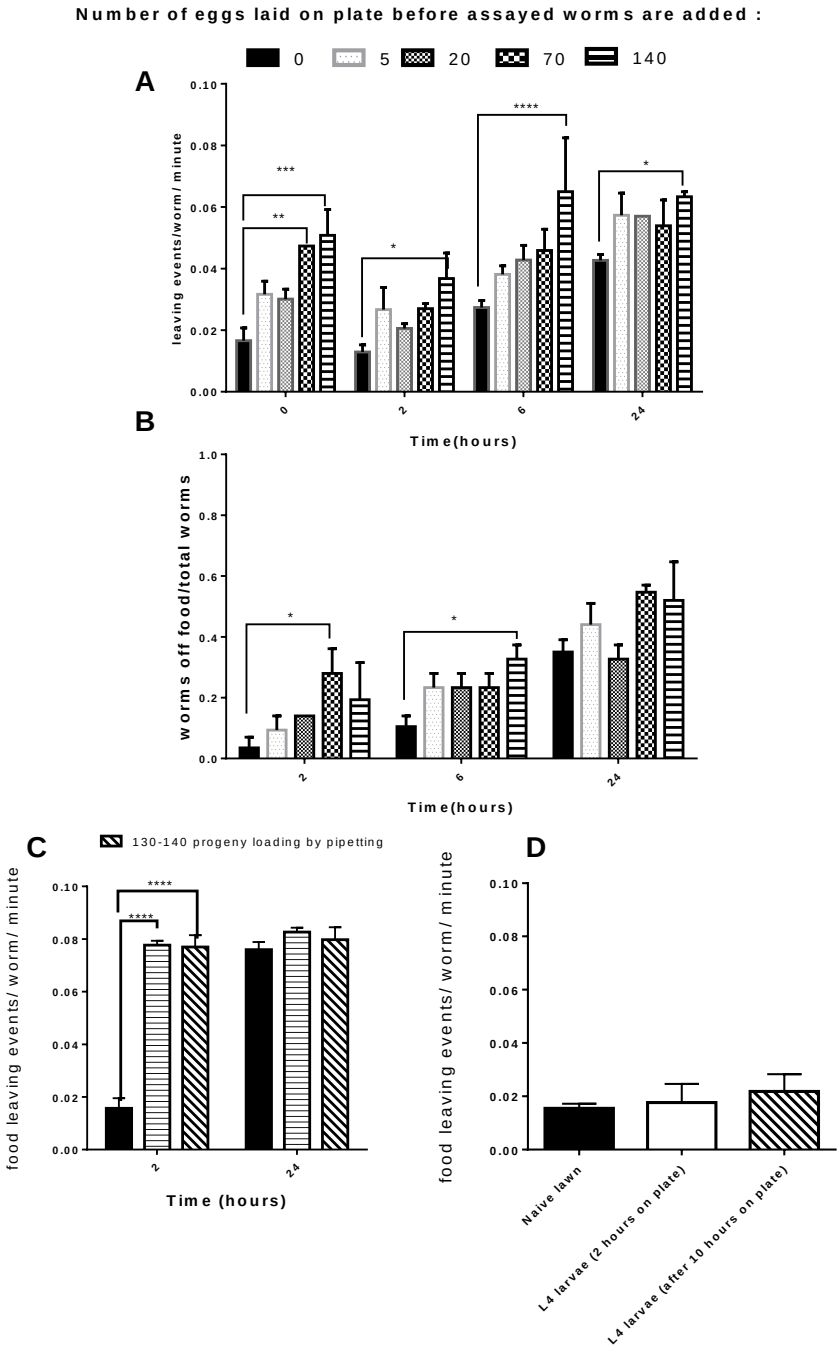


Figure 3.6: ***C. elegans*** **L1 larvae enhance adult food-leaving. Bacterial lawns were loaded with *C. elegans* eggs at increasing density, ranging from 5 to 140, as indicated by allowing gravid adults to lay eggs on the lawn for a period of time following which the adults were removed. The eggs were left overnight to hatch into larvae and the food-leaving assay instigated by placing seven adults on each lawn. Food-leaving (A) and proportion (B) of worms off food were scored as described for Fig. 1A and B. 'n' number for treatment group '0', n= 4, all other treatments n=3. Data analysed by two-way ANOVA with Tukey's multiple comparisons test; *C. elegans* larvae enhance adult food-leaving from lawns that have never been exposed to adults (C). In this experiment, isolated eggs were pipetted onto the lawn and the effect of the resulting larvae on adult food-leaving compared to that on plates prepared by eggs laid from gravid adults as described in A. 'n' number for '0' progeny treatment group and for '140' progeny loaded by the method described in A =3, 'n' for progeny preloaded by pipetting =4. Data analysed two-way ANOVA with Tukey's multiple comparisons test. L4 larvae did not enhance adult food-leaving (D). Bacterial lawns were conditioned with 120 L4s for 2 or 10 hours after which adult food-leaving was scored. Error bars represent ±SEM. 'n' = 3 for each experimental group. Data analysed by one-way ANOVA with Tukey's multiple comparison test. *P<0.05, **P<0.01, ***P<0.001 and ****P<0.0001**

3.3.3.2 Neither larvae nor sterile adults mimic the enhanced food-leaving observed in fertile adult *C. elegans*

The proportions of both adults and larvae on the lawn were also measured after 24 hours on the lawn of *E. coli* OP50. This enabled examination of the location of the larvae produced during a food-leaving assay which were hypothesised to drive food-leaving in adult animals. This showed a significantly higher level of adults off the food lawn than larvae off the food lawn. This supports enhanced food-leaving is specifically executed in adult *C. elegans*. Furthermore, it supports the notion that this adult behaviour is driven by, but not elicited in *C. elegans* larvae (Figure 3.7A). To investigate the possibility that viable early stage larvae, not the eggs produced by adult *C. elegans*, enhanced adult food-leaving young adult worms were sterilised by pre-treating them with the DNA synthesis inhibitor 0.1mg/ml 5-fluoro-2'-deoxyuridine (FUdR)((Mitchell *et al.*, 1979; Aitlhadj and Sturzenbaum, 2010). FUdR treated animals laid less eggs that untreated animals after 24 hours, but the numbers of eggs after 2 and 6 hours, which would otherwise hatch to be L1 larvae after 24 hours, were not affected Figure 3.7B). Any eggs that were laid by FUdR treated animals did not hatch, and the treated animals did not exhibit enhanced food-leaving or proportion of worms off food over time (Figure 3.7C, D). This indicates that it is the larvae produced by the adult animals during the time course of a standard assay that are largely responsible for the enhanced food-leaving effect. Altogether, these results show that well fed *C. elegans* L1 and L2 larvae provide a significant drive to enhance the food-leaving behaviour of adult animals.

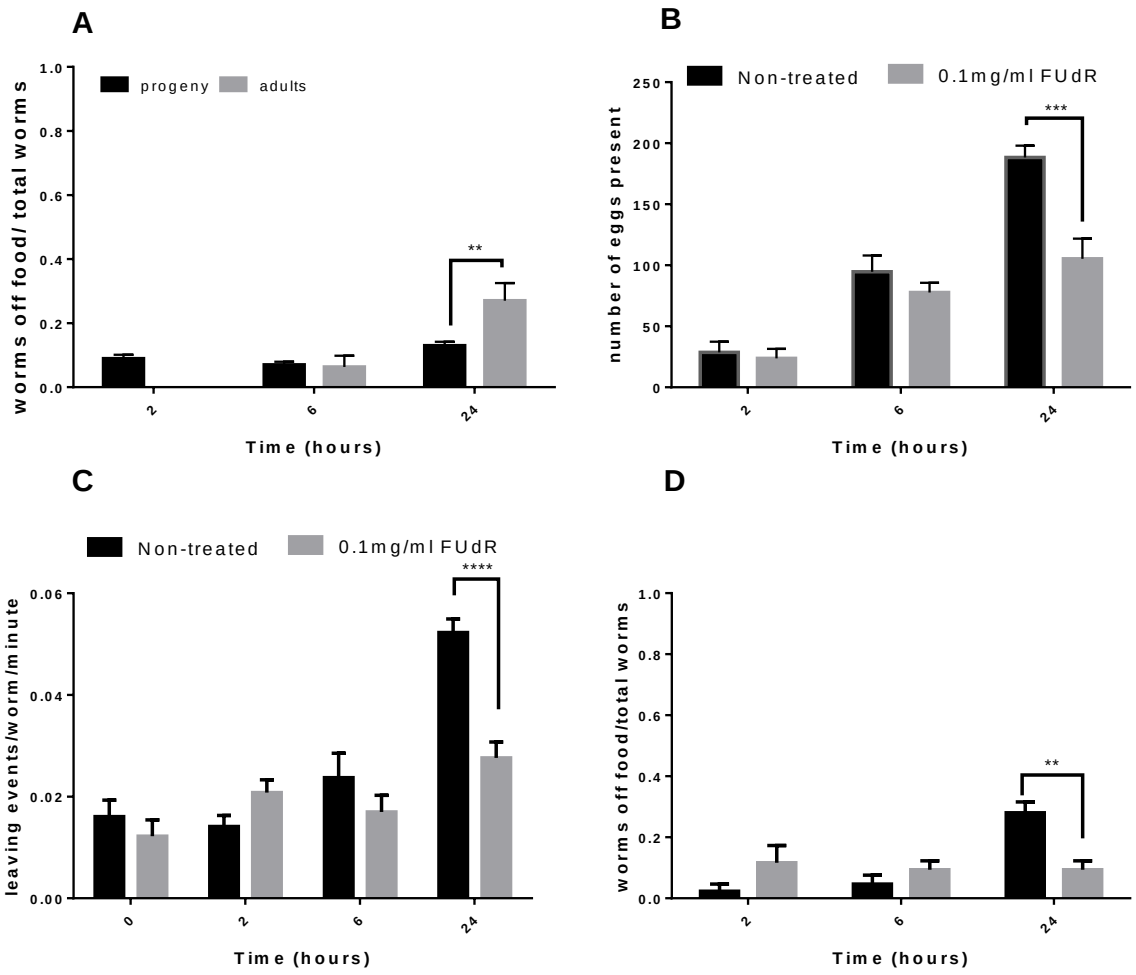


Figure 3.7: The food-leaving response is not seen in larvae or in sterile worms. The proportion of worms off food scored as in Figure 3.2 except that in these assays both adult worms and larvae were scored in parallel (A). n=4 bacterial lawns. Data are mean ±SEM. Data analysed by one-way ANOVA with Bonferroni multiple comparisons. In addition C*. elegans* were pre-treated with 0.1mg/ml FUdR to induce sterilisation. The number of eggs of non-treated and FUdR animal populations were measured (B). Eggs laid by FUdR treated animals did not hatch. Non-treated and FUdR treated animals were subsequently analysed in a food leaving assay as in Figure 3.2. The proportion of worms off bacterial lawns (C) and the number of food-leaving events (D) were measured over 24 hours as in Figure 3.2.Error bars represent ±SEM, n=5 lawns for both treatment groups. Data analysed by two-way ANOVA with Bonferroni multiple comparisons. *P<0.05, **P<0.01, ***P<0.001 and ****P<0.0001

3.3.3.3 Larvae of other nematode species does not trigger *C. elegans* food-leaving

As the data above indicate that adult *C. elegans* will increasingly leave a food source that is populated by predominantly L1 larvae in the absence of any obvious depletion in the quantity of food it was next considered whether changing of the quality of the food might provide an explanation for the behaviour. This change in quality could be due to the chemical makeup of the lawn changing. This could then lead to any chemical signals which

are integrated by the *C. elegans* nervous system being altered, resulting in a change of behaviour. To investigate this, the larvae of two *C. briggsae* wild isolates, AF16 and HK104 and the *C. remanei* wild isolate JU724 were examined. In addition to *Caenorhabditis* larvae, J2 juveniles of *Globodera pallida* were also tested. *G. pallida* is a plant parasitic nematode that infects and proliferates inside potato roots, and unlike the three *Caenorhabditis* species is not a bacteriovore (Perry and Moens, 2013). As before, the presence of N2 larvae increased the food-leaving of N2 adults (Figure 3.8A) as indicated by the increase in leaving rate in the first 30 minutes after the adults were placed on the lawns with the progeny. In contrast only a weak enhancement of food-leaving was observed for *C. briggsae* larvae (Figure 3.8B,C) whilst for *C. remanei* (Figure 3.8D) and *G. pallida* (Figure 3.8E) there was no significant effect. Thus, the ability of larvae to drive the adult food-leaving response in *C. elegans* is specifically triggered by *C. elegans* larvae.

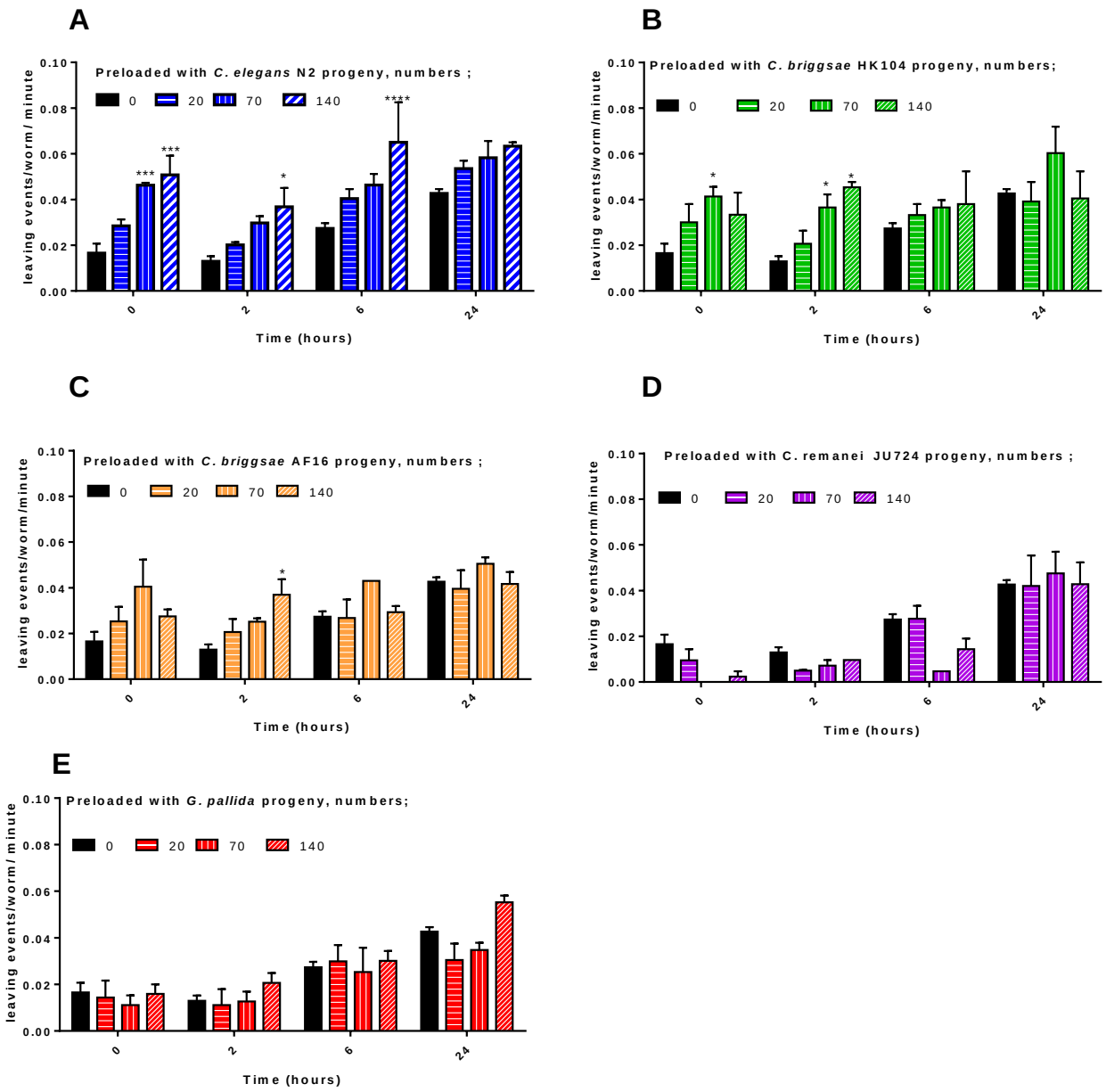


Figure 3.8: Progeny enhanced food-leaving response in adult *C. elegans* is conspecific. Different numbers of larvae, as indicated, from *C. elegans* (A), *C. briggsae* (B,C), *C. remanei* (D) and *G. pallida* (E) were pre-loaded onto bacterial lawns before adult *C. elegans* were added and assayed for food-leaving. Data are mean ± SEM. n=3 lawns for each experimental group. Data analysed by two-way ANOVA with Bonferroni multiple comparisons to animals on 'naive' lawns. *P<0.05, **P<0.01, ***P<0.001 and ****P<0.0001

3.3.4 Genetic determinants controlling *C. elegans* enhanced food-leaving

The preceding sections have demonstrated that *C. elegans* larvae act to promote the food-leaving behaviour of adult animals. In order to unpick the neural circuits that function to elicit this behaviour in adult *C. elegans*, mutants deficient in different aspects of neural signalling where examined in the food-leaving assay established above. In addition, the nature of any cue which is produced by the larvae was also investigated.

3.3.4.1 Progeny induced food-leaving is biogenic amine independent

The four biogenic amines which *C. elegans* synthesises (serotonin, dopamine, tyramine and octopamine) are key neurotransmitters of the organism (Chase and Koelle, 2007). Between them, these molecules are synthesised in a range of *C. elegans* neurons and regulate a range of behaviours based on how the worms interact with changing in environments. These behaviours include how *C. elegans* interacts with its food, and serotonin has also been implicated in avoidance of pathogenic bacteria and dwelling states on food lawns (Zhang *et al.*, 2005; Melo and Ruvkun, 2012; Flavell *et al.*, 2013; Ballestriero *et al.*, 2016). In order to analyse this, *C. elegans* mutant strains that were deficient in the biosynthetic enzymes needed to make serotonin *(tph-1 (n4622)),* tyramine *(tdc-1 (n3419))* and octopamine *(tbh-1 (n3247))* were examined for food-leaving (Figure 3.9) (Alkema *et al.*, 2005; Wragg *et al.*, 2007) (Sze *et al.*, 2000). Mutants for *tdc-1* and *tbh-1* showed the same food-leaving as wild-type adults (Figure 3.10A) therefore tyramine and octopamine are not involved in promoting the enhanced food-leaving response driven by larvae. There was however a reduction in food-leaving in *tph-1(n4622) C. elegans* (Figure 3.10A). To investigate this further, *tph-1* animals were tested on a lawn laced with N2 larvae. In this format *tph-1* mutants *C. elegans* subsequently behaved in the same way as N2 adults (Figure 3.10B). The difference in food-leaving behaviour between *tph-1* adult animals exposed to *tph-1* larvae (Figure 3.10A) and N2 larvae (Figure 3.10B) could be explained by the signal produced by larvae which drives food-leaving in adult animals being modified and therefore not as potent. This reinforces the suggestion that the progeny enhanced food-leaving in adults is not an aversive response to poor quality or noxious food.

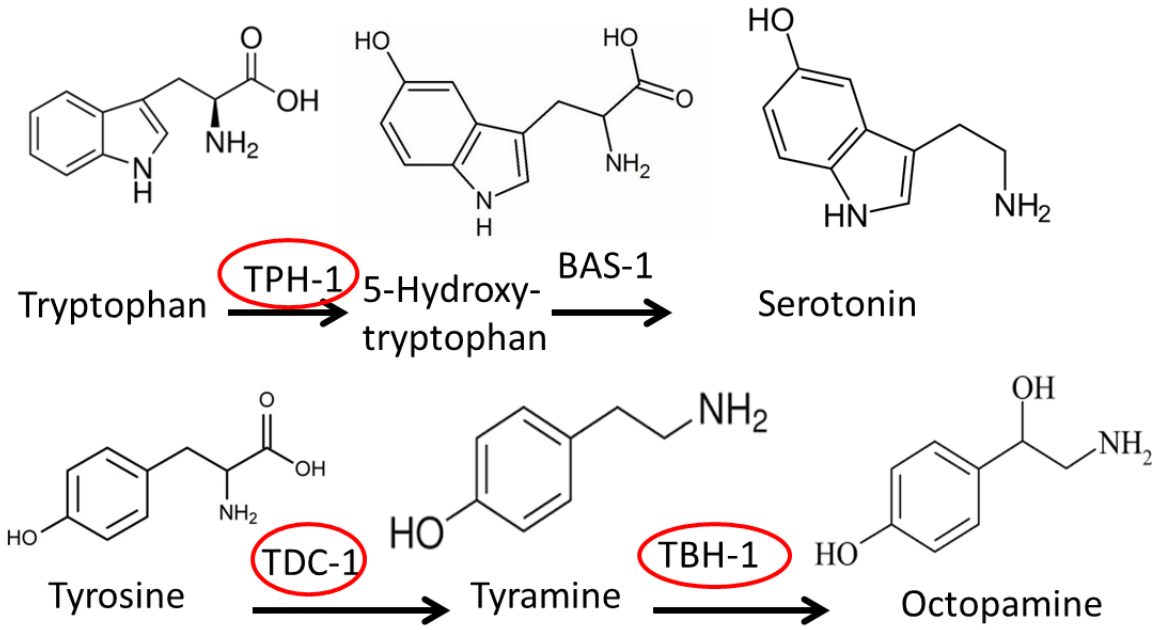


Figure 3.9: The biosynthetic pathways of serotonin, tyramine and octopamine in *C. elegans* with the enzymes TPH-1, TDC-1 and TBH-1 highlighted. *C. elegans* mutants in these enzymes were subsequently measured for their food-leaving

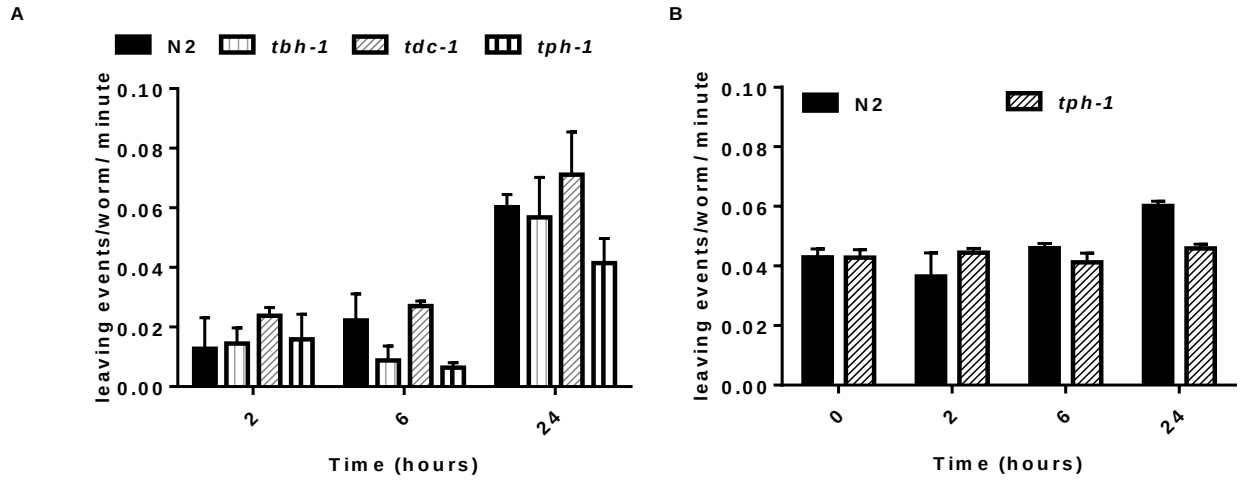


Figure 3.10: The progeny induced food-leaving response in adult *C. elegans* is not dependent on serotonin or octopamine. Food-leaving for N2, *tbh-1 (n3247)*, *tdc-1 (n3419)* or *tph-1(n4622)* adults placed on unlaced OP50 lawns (A). Food-leaving was subsequently analysed for seven wild-type N2 or *tph-1(n4622)* adults placed on bacterial lawns preloaded with 140 N2 larvae (B). N=4 bacterial lawns for each *C. elegans* strains. Error bars represent ±SEM. Data analysed by two-way ANOVA with Bonferroni multiple comparisons.

3.3.4.2 Progeny induced food-leaving is ascaroside dependent

As described in Chapter 1, signals triggered by ascarosides produced by dauer and well-fed larvae can trigger a dispersal response (1.9). In order to investigate the contribution of ascarosides in progeny stimulated food-leaving *daf-22 (m130)* mutant *C. elegans* were analysed for their food-leaving behaviour. The *C. elegans daf-22* gene encodes a homologue of the thiolase domain of the human SCP2 protein, which catalyses the final step in fatty acid oxidation, and is required for the biosynthesis of ascarosides (Butcher *et al.*, 2009) (1.9). *daf-22 (m130)* animals did not exhibit enhanced food-leaving after 24 hours on an OP50 lawn (Figure 3.11A). To test whether the deficit in the progeny stimulated food-leaving behaviour can be ascribed to a loss of signal from *daf-22(m130)* larvae to the adults the food-leaving rate of N2 adults on bacterial lawns that had been preloaded with either N2 or *daf-22(m130)* progeny was tested (Figure 3.11B). Food-leaving was elicited to a significantly lesser extent in response to *daf-22(m130)* larvae, supporting the idea that a *daf-22(m130)* dependent signal from the larvae elicits food-leaving behaviour in adults (Figure 3.11B).

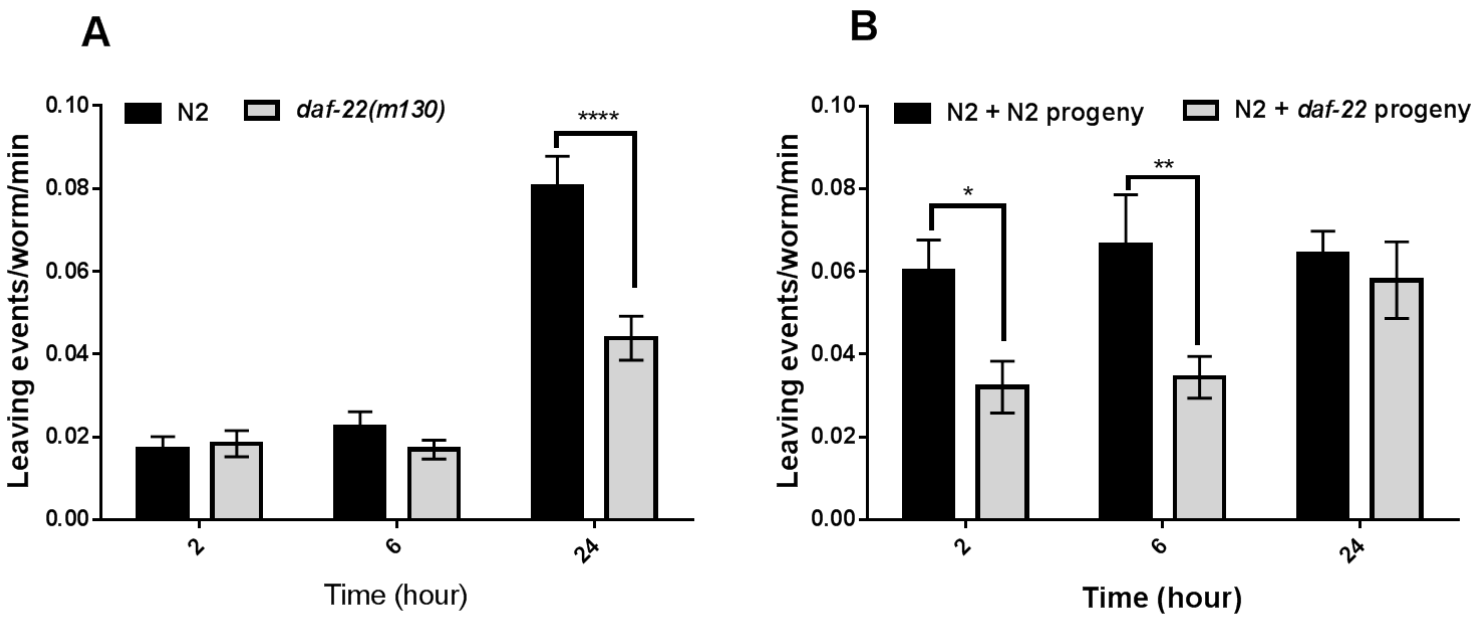


Figure 3.11: Progeny stimulated *C. elegans* food leaving is ascaroside dependent. Food-leaving of wild-type and the ascaroside deficient *daf-22 (m130) C. elegans* on lawns of *E. coli* OP50 n=7 lawns for each *C. elegans* strain (A)Food-leaving for wild-type N2 worms in the presence of either wild-type larvae or *daf-22* larvae (B). N2 adults were placed on bacterial lawns without pre-loaded larvae (n=7) or with 130 N2 larvae (n=5) or 130 *daf-22* larvae (n=4) larvae. Error bars represent ±SEM. Data analysed by two-way ANOVA with Bonferroni multiple comparisons. *P<0.05, **P<0.01, ***P<0.001 and ****P<0.0001

3.3.4.3 Hawaiian strain *C. elegans* are not effected in progeny driven enhanced food-leaving

In addition to the N2 strain, the Hawaiian/CB4856 strain is another wild *C. elegans* isolate. This particular strain was originally isolated from pineapple fields in Hawaii and has been demonstrated to have a high food-leaving rate than N2 animals (Bendesky *et al.*, 2011). This increased tendency to leave a bacterial lawn has provided a route to Quantitative Trait Loci analysis (QTL) to identify genetic determinants of this polygenic behaviour (de Bono and Bargmann, 1998; Gloria-Soria and Azevedo, 2008; Harvey, 2009; Bendesky *et al.*, 2011; Zhu *et al.*, 2015b). The base-line for the food-leaving response in Hawaiian strain animals was elevated compared to N2 across all the time-points (Figure 3.12). Previous analysis of the increased food-leaving of Hawaiian strain animals has suggested that this may at least in part be explained by increased motility of the Hawaiian strain compared to N2 (Bendesky *et al.*, 2011). However, like N2 animals, the food-leaving rate of Hawaiian strain animals was enhanced on lawns laced with N2 larvae (Figure 3.12). This indicates that a distinct circuit is involved in controlling *C. elegans* progeny stimulated rather than the TYRA-3 and NPR-1

circuit previously identified to drive enhanced food-leaving in Hawaiian strain animals (Bendesky *et al.*, 2011).

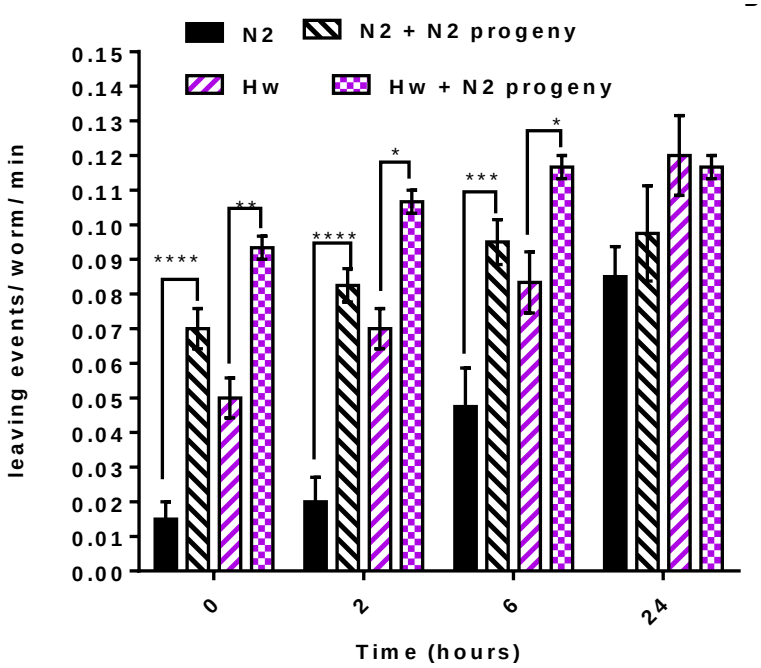


Figure 3.12: N2 and Hawaiian strain (CB4856) *C. elegans* both exhibit progeny stimulated enhanced food leaving. Food-leaving was scored for wild-type N2 adults and Hawaiian strain as described in Figure3. 1A. in the absence and presence of 140 wild-type N2 larvae. n=4 individual experiments for N2 and n=3 for Hawaiian Error bars represent ±SEM. Data analysed by two-way ANOVA with Dunnett's multiple comparison test. *P<0.05, **P<0.01, ***P<0.001 and ****P<0.0001

3.3.4.4 Progeny stimulated food-leaving is neuropeptide dependent

Neuropeptides are key regulators of behaviour in *C. elegans* (1.8.3.2). To investigate the contribution of neuropeptides in controlling progeny stimulated food-leaving *egl-3 (ok979)*, were examined. *egl-3* mutants have a global reduction in neuropeptide content as it is deficient in a proprotein convertase needed for processing of numerous neuropeptides in *C. elegans* (Kass *et al.*, 2001; Husson *et al.*, 2006). The food-leaving behaviour of adult *egl-3 C. elegans*, as with the other mutant strains, was carried out on lawns laced with N2 larvae. This was done in order to avoid any change in the signal produced by larvae which drives food-leaving in adult animals as a result of genetic mutation. Upon performing this experiment it was observed that *egl-3* worms were deficient in food-leaving included upon

being analysed on lawns that had been laced with N2 larvae (Figure 3.13B). This deficiency in progeny induced food-leaving was rescued in mutant animals injected with the native *C. elegans egl-3* gene on via cosmid C26B6 (Figure 3.13B)(Mitchell *et al.*, 2010). This is consistent with a role for neuropeptide signalling in *C. elegans* as a major determinant of the food-leaving response although this could be an indirect consequence of an effect on locomotory behaviour, which is reduced in *egl-3* mutant animals (Mitchell *et al.*, 2010).

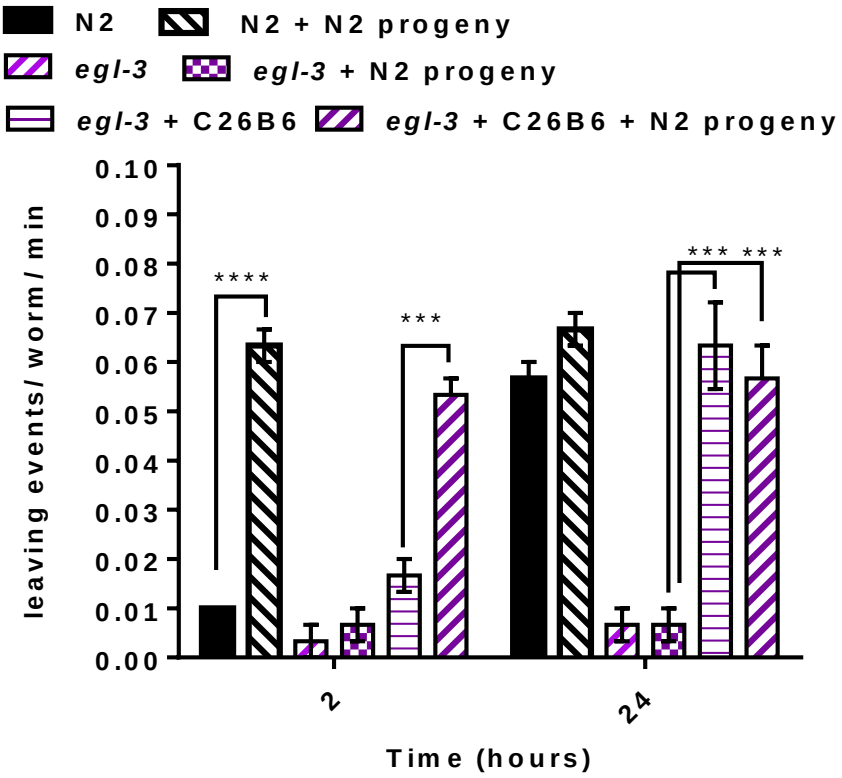


Figure 3.13: Progeny enhanced food-leaving is not observed in the neuropeptide deficient mutant *egl-3*. Food-leaving was compared between wild-type N2, *egl-3* and transgenic *egl-3* mutants expressing the cosmid C26B6 which harbours genomic sequence for *egl-3*. For this assay each strain tested was assayed in the absence or presence of 140 *C. elegans* larvae as indicated. n=3 lawns for each experimental group. Error bars represent ±SEM. Data analysed by two-way ANOVA with Dunnett's multiple comparison test. *P<0.05, **P<0.01, ***P<0.001 and ****P<0.0001

3.3.4.5 Nematocin is involved in controlling progeny driven enhanced food-leaving

The involvement of neuropeptide signalling in a Hawaiian strain independent manner indicates that the well-defined *npr-1*-dependent circuit does not underpin the progeny stimulated food-leaving. The nature of the food-leaving behaviour described in this chapter hinted at a parental response, there was speculation that nematocin, the *C. elegans*

homologue of the mammalian peptide hormone oxytocin, may underpin the progeny enhanced food-leaving response. In mammals oxytocin is an important regulator of social behaviours, including parental bonding(Marlin *et al.*, 2015). Nematocin has been shown to control mate searching and mating behaviours in male *C. elegans,* as well as gustatory learning in the form of salt chemotaxis (Beets *et al.*, 2012; Garrison *et al.*, 2012). Moreover, unlike *egl-3* mutants, no movement deficits have been reported for nematocin signalling mutants (Garrison *et al.*, 2012). To investigate the role that nematocin signalling may play in progeny stimulated food-leaving this *C. elegans* deficient in the nematocin gene *ntc-1*, and its two receptors, *ntr-1* and *ntr-2*, were examined.

Initial analysis of the reproductive ability of these mutants revealed a significant reduction in the number of larvae produced by *ntc-1(tm2385)*, *ntr-1* (*tm2765*) and *ntr-2* (*tm2243*) (Figure 3.14A). As the nematocin mutant strains exhibited this reproductive defect which might confound interpretation of a progeny enhanced food-leaving response these mutants were analysed in lawns pre-loaded with N2 larvae, as before, and then compared the food-leaving of adult wild-type and the nematocin signalling mutants. Undertaking this revealed that all three nematocin mutant strains were deficient in progeny stimulated food-leaving relative to N2 animals (Figure 3.14B).

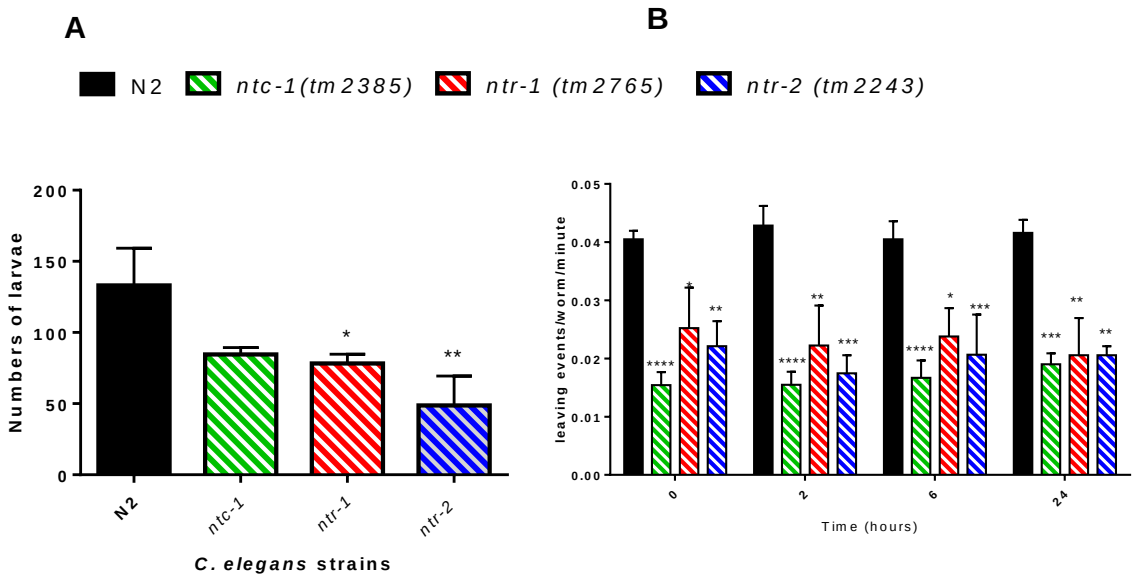


Figure 3.14: *C. elegans* deficient in nematocin have reproductive and progeny stimulated food leaving deficits, The number of larvae produced by the nematocin mutants and N2 *C. elegans* (A) Data analysed by One-way ANOVA with Bonferroni's multiple comparison tests). Food leaving of N2 and nematocin mutants on lawns laced with 140 N2 larvae (B). Data analysed by two-way ANOVA with Dunnett's multiple comparison test. N=3 for all strains in all experiments, Error bars represent ±SEM. *P<0.05, **P<0.01, ***P<0.001 and ****P<0.0001

To confirm this effect the assay was repeated in outcrossed and rescue strains for *ntc-1* and *ntr-1*. Mutants for *ntc-1(tm2385)* and *ntr-1(tm2765)* showed reduced food-leaving both after being on unlaced OP50 lawns for 24 hours and on lawns laced with N2 *C. elegans* larvae in comparison to N2 animals (Figure 3.15). This was rescued by expression of *ntc-1* or *ntr-1*, respectively from their native promoters. Therefore, it can be proposed that signalling through nematocin in adult worms mediates a *daf-22* dependent signal emanating from their larvae and drives the adults to leave the food patch with increasing frequency.

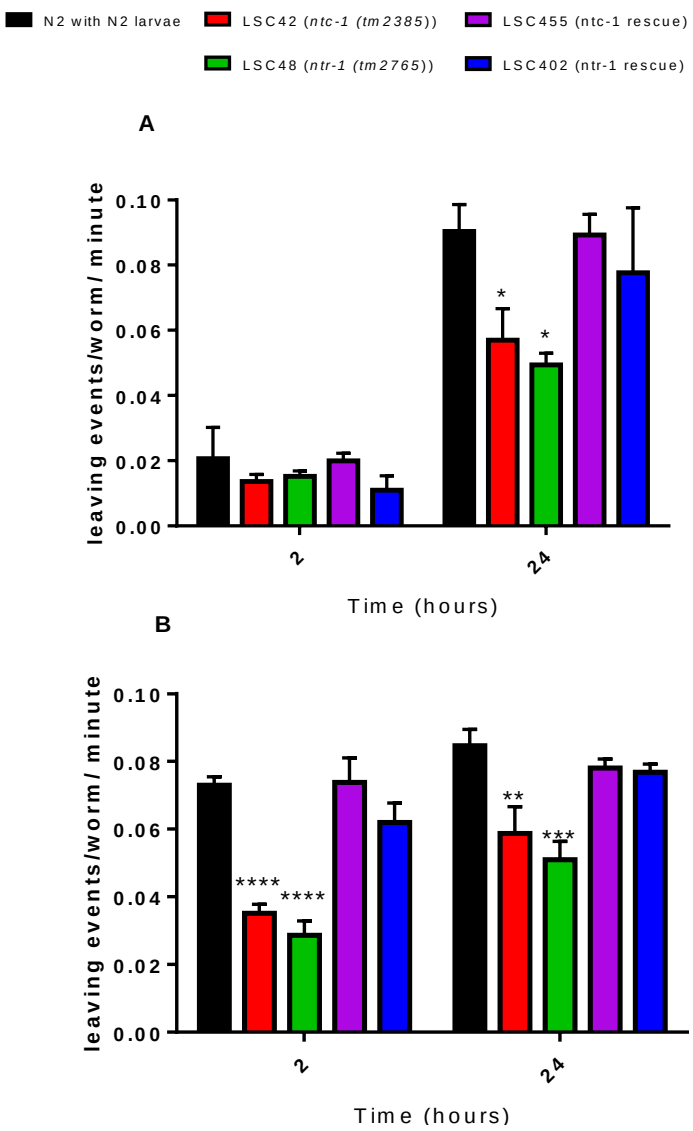


Figure 3.15:Food-leaving for wild-type N2 adults and outcrossed *ntc-1* and *ntr-1* mutants along with the respective rescue lines (A). The comparison between wild-type N2, nematocin mutants and rescue lines was repeated on bacterial lawns pre-loaded with 140 N2 progeny. N2 *ntc-1(tm2385),*(LSC42) *ntr-1(tm2765)*(LSC48) n= 5; *Pntc-1::ntc-1*(LSC455) ; *Pntr-1::ntr-1* (LSC402) (B) N≥4 for all *C. elegans* strains Data are expressed as mean ± SEM. Data analysed by two-way ANOVA with Dunnett's multiple comparisons test. *P<0.05, **P<0.01, ***P<0.001 and ****P<0.0001

3.4 Discussion

In this chapter, an important modulator of adult *C. elegans* food-leaving behaviour has been investigated, in addition to that triggered by pathogens, RNAi and starvation (Zhang *et al.*, 2005; Pradel *et al.*, 2007; Kaplan *et al.*, 2012; Melo and Ruvkun, 2012). Specifically, this modulator is larval progeny that drive a food-leaving response in adult animals. This behaviour is reflected both in snapshot measurements of food aversion and in the more dynamic measurement of food-leaving rate. Specific data here provide a detailed investigation into this behaviour, including the genetic controls coordinating the *C. elegans* response.

3.4.1 *C. elegans* food-leaving increases after continued exposure to an *E. coli* OP50 lawn

The enhanced dispersal of wild type *C. elegans* larvae after extended exposure to a bacterial lawn of benign bacteria has previously been described (Gloria-Soria and Azevedo, 2008; Harvey, 2009; Milward *et al.*, 2011). These experiments originally utilised N2 worms and dense OP50 bacterial lawns to provide conditions that converge to ensure a relatively low rate of initial food-leaving. Indeed the initial rate of leaving from a lawn of 50µl of OP50(OD_{600} = 0.8) was in the region of 0.02 leaving events/worm/minute which is comparable to the leaving rate previously reported for the same number of N2s on a lawn of 10µl of HB101 (OD_{600} =2.0) in the region of 0.01 leaving events/worm/minute(Bendesky *et al.*, 2011). However, this rate of *C. elegans* food-leaving dramatically increases between 6 and 24 hours of exposure to an OP50 lawn (3.3.1).

The adult food-leaving that is driven by the worm's progeny is distinct from a previously described food-leaving behaviour which has been previously been speculated to be driven by both food depletion and previous nutritional deprivation (Milward *et al.*, 2011; Olofsson, 2014). In the assays performed in this chapter adults and the larvae were well fed and the assays were conducted in the presence of abundant food (3.3.2). It is also distinct in terms of the magnitude of effect, which is greater in nutritionally deprived worms. This argues for

discrete modulation of adult foraging decisions by the immediate proximity of their progeny on the food patch.

A number of possible explanations for progeny enhanced adult food-leaving have also been investigated. In particular, it was considered whether or not the negative impact of the increase in population density on either the amount of bacterial food availability or the quality of the same bacteria has a role in controlling this behaviour. The measurements of the growth curves of bacterial lawns conditioned for 24 hours with or without worms did not reveal any indication of significant food depletion during the assay time period, this indicated that the enhanced food-leaving seen upon extended exposure to the bacterial lawn is not due to the lawn becoming depleted (Milward *et al.*, 2011) (3.3.2). In addition to food depletion, ascaroside signalling, high levels of metabolic gases and pathogenic bacteria all act to trigger *C. elegans* dispersal (Zhang *et al.*, 2005; Pradel *et al.*, 2007; Srinivasan *et al.*, 2008; Bretscher *et al.*, 2011).

3.4.2 Adult *C. elegans* food-leaving is stimulated by *C. elegans* larvae

Pre-loading the bacterial lawns with progeny, which are mostly L1 larvae, immediately drives enhanced adult *C. elegans* food-leaving in comparison with that seen after 24 hours on a lawn not laced with bacteria (3.3.3.1,3.3.1). This demonstrates that it is the production and accumulation of larvae on the bacterial lawn, which drives an enhanced food-leaving effect, similar to that seen after 24 hours of a standard food-leaving assay.

Measuring the relative food aversion in adults and larvae after the adults where on a 'naive' lawn for 24 hours demonstrates that this effect is purely exhibited in the adult animals, with any signal being produced by the larvae and detected by the adult and driving a behaviour in the adult animals, but not in the larvae themselves (3.3.3.2). In addition, adults treated with FUdR, which leads to laying of unfertilised eggs, thus removing the contribution of larval progeny on the plate, did not exhibited enhanced food-leaving after extended time on the OP50 lawns (Mitchell *et al.*, 1979). These results further suggested that that the increase in population of larvae drives food-leaving specifically in adults. In addition, this provides

further insight into this specific behaviour by showing that viable larvae are required to drive then enhanced food-leaving, and unfertilised eggs, as produced by worms which have been treated with FUdR, do not drive this.

3.4.3 Progeny induced food-leaving is species specific

Whilst on its own the data discussed above does not negate the possibility that there is an undetectable change in food quantity or quality, such a change is unlikely to provide an explanation for progeny enhanced food-leaving in adult *C. elegans*: If this were the case one might expect to see the same food-leaving response regardless of the species of nematode progeny that were used to pre-condition the bacterial lawn. Analysis of *C. elegans* food-leaving on OP50 lawns that were preloaded with larvae of other nematode species, including the other *Caenorhabditis* species *C. briggsae* and *C. remanei*, and the plant parasitic nematode *Globodera pallida,* revealed that food-leaving was not enhanced by the larvae of these species in comparison to *C. elegans* larvae (3.3.3.3) (Kiontke and Sudhaus, 2006; Felix and Duveau, 2012; Perry and Moens, 2013). This indicates that despite living in similar niches to and being close relations to *C. elegans,* enhanced *C. elegans* food-leaving is species- specific and not driven by larvae of other *Caenorhabditis* species (Felix and Duveau, 2012).

This species-specific nature of the food-leaving behaviour in adult *C. elegans* in, further argues that this is not an indirect consequence of depletion or deterioration of the food lawn. This result also leads to the conclusion that the signal which is produced by the larvae in order to stimulate food-leaving in the adult animals is produced specifically by *C. elegans* larvae, and not from larvae of the related species, indicating that this particular signal is not conserved amongst other *Caenorhabditis* species. Previous studies have determined how even different *C. elegans* natural isolates can produce different mixtures of ascarosides, and that these different *C. elegans* lines also elicit different responses to the individual ascarosides (Diaz *et al.*, 2014; Greene *et al.*, 2016). In addition, the ascaroside production by N2 *C. elegans* is influenced by the dietary status and development stage of the animals (Kaplan *et al.*, 2011). The results obtained with the other nematode wild isolates in 3.3.3.3

together with these earlier studies suggests that whilst conserved roles for dispersal signals have previously been suggested, the signal which drives the enhanced food-leaving stimulated by progeny appears to have a strong genetic component (Kaplan *et al.*, 2012; Artyukhin *et al.*, 2013b).

3.4.4 Genetic analysis of progeny driven *C. elegans* food-leaving

3.4.4.1 Enhanced food-leaving is not driven by biogenic amines

After identifying that it is a signal produced by *C. elegans* larvae which drive food-leaving in the adult animals, it was important to investigate the genetic controls which drive this behaviour. Previous studies have implicated the biogenic amine serotonin, in controlling *C. elegans* aversive behaviours to a range of stimuli, including avoiding pathogenic bacteria and toxic bacterial products (Zhang *et al.*, 2005; Melo and Ruvkun, 2012; Ballestriero *et al.*, 2016). Serotonin has also been implicated to play an important role in promoting *C. elegans* feeding behaviour, specifically acting to keep animals on lawns of nutritious bacteria (Avery and You, 2012; Flavell *et al.*, 2013; Song *et al.*, 2013). In addition, biogenic amine signalling has been implicated in food-leaving behaviour previously, with non-coding polymorphisms in the tyramine G-protein coupled receptor TYRA-3 affecting the activity of the receptor and acting to promoting enhanced food-leaving on *E. coli* HB101 by Hawaiian strain animals (Bendesky *et al.*, 2011). However, examining food aversion on serotonin deficient animals, along with mutants deficient in the other *C. elegans* biogenic amines octopamine and tyramine revealed that biogenic amines are not involved in controlling the enhanced food-leaving response elicited upon progeny production (3.3.4.1). This observation that this behaviour is not modified by serotonin signalling, a known regulator of aversive behaviour, provides further argument that the enhanced response does not arise because the presence of the larvae modifies the bacteria making the lawn aversive or noxious towards the to the adults(Zhang *et al.*, 2005; Melo and Ruvkun, 2012). However, the fact that upon food-leaving of *tph-1 C. elegans* being measured in food-leaving assay on an unlaced lawn, and therefore with *tph-1* larvae being present at the 24 hour time point, these serotonin deficient animals exhibited lower levels of food-leaving than wild type *C. elegans*. This could

possibly be explained by the fact that serotonin has been shown to affect *C. elegans* egg laying, which could therefore lead to serotonin deficient animals producing less progeny. Alternatively, it has previously been shown that biogenic amines make up part of ascarosides, and thus serotonin may be a component of any L1-derived molecular signal which drives food-leaving in adult *C. elegans* (Artyukhin *et al*., 2013). In addition, the fact that signalling by other biogenic amines octopamine and tyramine is not implicated in progeny stimulated food-leaving, indicating a different circuit is involved to that which acts to control enhanced food-leaving in Hawaiian strain animals relative to N2 (Bendesky *et al.*, 2011).

3.4.4.2 The progeny derived cue is an ascaroside signal

Progeny enhanced food-leaving could be interpreted as a parental response in the adults to the increasing population density. Arguably, this would be beneficial to the larvae allowing them to take full advantage of the food source on which they hatched. The data presented here suggests that a signal is transmitted from the larvae to the adults on the bacterial lawn to induce them to leave the food patch. As described in 1.9, the ascarosides are an important class of molecules in *C. elegans*, with individual molecules with even subtle differences in structure controlling a diverse range of *C. elegans* behaviours, with some effects also being found to depend on the gender and developmental stage of the animals (Srinivasan *et al.*, 2008; Kaplan *et al.*, 2011; Artyukhin *et al.*, 2013b; Hollister *et al.*, 2013). There is also evidence for an ascaroside independent signal that promotes survival of L1 larvae subjected to starvation, (Artyukhin *et al.*, 2013a). The fact that *daf-22* mutant *C. elegans,* which are deficient in ascaroside production did not show enhanced food-leaving is consistent with the idea that an ascaroside signal from *C. elegans* larvae enhances food-leaving in adults as part of a parental behavioural response (3.3.4.2) (Butcher *et al.*, 2009). This distinguishes progeny enhanced food-leaving from that observed in nutritionally deprived worms as the latter is not *daf-22* dependent (Olofsson, 2014). In addition, the fact that *daf-22* larvae, which are deficient in producing ascarosides, do not promote enhanced food-leaving in wild type adults, further suggests that it is an ascaroside signal being produced by the larvae which drives the food-leaving response in adult *C. elegans* (3.3.1). The experimental paradigm established here for progeny enhanced food-leaving, where a

bacterial lawn is laced with *C. elegans* larvae, establishes a tractable platform for resolving further chemical cues underpinning conspecific interactions. It is likely that an ascaroside signal is being produced by the *C. elegans* larvae onto the bacterial lawn. This signal is then detected by the nervous system of the adult animals, and leads to the exhibiting of the enhanced food-leaving behaviour.

3.4.4.3 Progeny enhanced food-leaving is neuropeptide dependent and involves nematocin

Intriguingly, the progeny enhanced food-leaving described in this chapter is mediated by a neural circuit that appears independent of a well described circuit previously discovered to regulate foraging decisions (de Bono and Bargmann, 1998; Bendesky *et al.*, 2011). This supposition is based on consideration of the observations made in the Hawaiian strain. Hawaiian strain animals have previously been demonstrated to show a higher rate of food-leaving N2 animals (Bendesky *et al.*, 2011). Analysis of Hawaiian strain animals revealed that *C. elegans* larvae also generated an enhanced food-leaving response in this particular strain. This reveals that the progeny stimulated food-leaving described in this chapter is not driven in by the mutations in the *npr-1* and *tyra-3* genes which act to increase food-leaving in Hawaiian strain *C. elegans* in comparison to N2 animals (3.3.4.3)(Bendesky *et al.*, 2011). These results, combined with those discussed in (3.4.4.1) suggest a distinct neural circuit involved in complex decision making in *C. elegans* adults.

Analysis of food-leaving in animals deficient in neuropeptide maturation (*egl-3(ok979*)) reveals that there is a neuropeptide component involved in coordinating the adult food-leaving response (Kass *et al.*, 2001; Husson *et al.*, 2006). Due to the evidence supporting a role for neuropeptides in controlling the *C. elegans* food-leaving response driven by progeny, the role of the neuropeptide nematocin and its receptors were investigated. Nematocin is the homologue of the mammalian neuropeptide oxytocin. Oxytocin, along with the neuropeptide vasopressin is involved in pair bonding in mammals and helps to control bonding between offspring and parents, as well as other reproductive behaviours (Young and Wang, 2004; Beets *et al.*, 2013; Marlin *et al.*, 2015). Oxytocin homologues are seen throughout biology, and studies with nematocin have revealed the conserved roles of

these similar peptides in controlling reproductive behaviour (Elphick and Rowe, 2009; Garrison *et al.*, 2012; Beets *et al.*, 2013; Lockard *et al.*, 2017).

Here it is described that nematocin signalling is required in the adults for them to engage the progeny induced food-leaving behaviour (3.3.4.5). This signal involves both the neuropeptide itself, and the receptor NTR-1. The additional receptor NTR-2 may also be involved, but the lack of a properly outcrossed mutant means this claim cannot be made definitively. Given that the null nematocin hermaphrodites have previously been analysed to exhibit both normal locomotion speed and chemotaxis it is unlikely that this deficit is due to an indirect effect on a sub-behaviour required for the response (Garrison *et al.*, 2012) (Beets *et al.*, 2012). Rather it suggests that nematocin is required in circuits that integrate a chemical cue from the larvae in the context of the food source to drive dispersal in the adults. The fact that oxytocin signalling is recognised for its intimate role in social interactions in general means that it is possible that nematocin signalling between adults could be involved in population density affects previously reported for food-leaving behaviours (Olofsson, 2014). These results obtained with nematocin deficient animals indicates that distinct circuits act to drive a similar behavioural output (enhanced food-leaving) in *C. elegans*. Polymorphisms in both the *npr-1* and *tyra-3* genes act to enhance food-leaving of *C. elegans* and in this chapter a distinct circuit, mediated by nematocin has been identified which acts to drive food-leaving in response to *C. elegans* larvae (de Bono and Bargmann, 1998; Gloria-Soria and Azevedo, 2008; Bendesky *et al.*, 2011).

Nematocin and its receptors are quite broadly expressed in *C. elegans*, being present in sensory neurons, interneurons and motor neurons in both the head and tail of hermaphrodite and male animals (Beets *et al.*, 2012; Garrison *et al.*, 2012). This places nematocin signals in neural circuits that are involved in detecting and responding to environmental cues. One class of neurons in which the nematocin gene is present is the amphid sensory neuron ADF (Garrison *et al.*, 2012). This particular pair of neurons is involved in the *C. elegans* interaction with food and has also been implicated in avoidance of aversive insults through serotoninergic signalling (1.8.3.1) (Zhang *et al.*, 2005; Melo and Ruvkun, 2012; Song *et al.*, 2013). This indicates that signalling from distinct ligands from the same neurons may act to drive a similar response in terms of an increased tendency to leave

a bacterial lawn. In addition, it will be interesting to understand how the signalling is organised and to what extent it deploys neurohormonal versus local transmission compared to mammalian oxytocin signalling (Grinevich *et al.;* Leng and Ludwig, 2008).

3.4.5 Summary

The data generated in this chapter demonstrates how populations of adult *C. elegans* behave on a replete lawn of *E. coli* OP50. Specifically, well fed early stage larvae generate potent inter-organismal signalling that drives behaviour in the form of increasingly leaving the bacterial lawn in the adult animals. This is in addition to the previously reported signalling that emanates from starved larvae (Artyukhin *et al.*, 2013a). This signal, which may reflect differential ascaroside activity, exhibits a dose-dependent modulation of food-leaving activity. Previous determinants implicated in food-leaving were not attributed to this context(Milward *et al.*, 2011) .This particular observation, that the behaviour is dependent on intact nematocin signalling, points to a novel neural circuit mediating an offspring-dependent social interaction in *C. elegans.*

The role of nematocin signalling controlling the progeny driven food-leaving effect indicates that the adult animals are undergoing a form of parental response upon the production of larvae. Biologically, this behaviour could relate to adult animals reproducing on a lawn of bacteria that could be found in the environment. Undergoing reproduction, and ascaroside signalling from the progeny subsequently increasingly driving adult animals off a bacterial food source, will act to preserve the genetics of the animals, with the larvae being present on a non-harmful food source, and thus avoiding premature death by infection with pathogenic bacteria or starvation. On the other hand the adult animals can then leave the food source which is now populated with larvae, this being made easier by the increased movement speed of adult animals. This will then allow the adult *C. elegans* to explore the surrounding environment, and potentially populate another bacterial food source. This repopulation and foraging strategy also avoids competition between genetically identical populations over limited food resources.

In addition to providing information on a new circuit identifying aspects of *C. elegans* social interactions, this chapter also provides a useful benchmark for how *C. elegans* can behave differently on a lawn of bacteria over time. As lawns of OP50 will be used as a control for all of the strains of potentially pathogenic bacteria in this thesis, understanding how a small population of wild type adult *C. elegans* behave on a bacterial lawn of a controlled density of a relatively well described bacterial food source, which is not pathogenic towards *C. elegans* under standard conditions. This particular behaviour will act as an informative comparison for strains of clinically relevant bacteria in the next results chapters. Defining that the food-leaving behaviour investigated in this chapter is driven by the production of progeny allows a comparison of any *C. elegans* behaviour seen on lawns of clinically relevant pathogenic bacteria in the subsequent chapters. However, this has revealed that larvae that are produced by adult animals consuming a bacterial lawn can affect the behaviour of the adult animals. Specifically, production of larvae leads to the adult animals not only leaving the food lawn more but also showing an enhanced proportion of animals off the bacterial lawn, which is also a behaviour that is exhibited in *C. elegans* upon exposure to pathogenic bacteria or other harmful (Pradel *et al.*, 2007; Gaglia *et al.*, 2012). Thus, it is important to bear this phenomenon in mind when *C. elegans* populations will be exposed to lawns of pathogenic bacteria in the subsequent chapters, as the contribution of progeny may confound any aversive response driven by the bacteria.

Chapter 4: Systematic comparison of strains of ESKAPE bacteria on *C. elegans* biology

4.1 Introduction

Previous studies analysing pathogenic bacterial strains using *C. elegans* as the host have identified that certain bacterial strains produce an aversive effect in *C. elegans* populations exposed towards lawns of these strains, demonstrated by spending increasing time away from the bacterial lawn (Zhang *et al.*, 2005; Pradel *et al.*, 2007; Gaglia *et al.*, 2012a). However, the subsequent investigation of how these particular behaviours are controlled is typically conducted by focusing on individual bacterial strains , particularly *P. aeruginosa* PA14, (Zhang *et al.*, 2005; Zhang and Zhang, 2012). The genetic tractability and high fecundity of *C. elegans* facilitates use of the organism in wider screening approaches to investigate bacterial pathogenesis, with mutants of individual pathogenic bacterial strains used to identify virulence factors (Tan *et al.*, 1999b; Gallagher and Manoil, 2001; Kurz *et al.*, 2003; Tenor *et al.*, 2004; Feinbaum *et al.*, 2012). This approach has been extended and been utilised to screen for antimicrobials (Kong *et al.*, 2014; Rajamuthiah *et al.*, 2014). In addition, *C. elegans* has been exposed to a range of bacteria presented as potential food sources and bacteria isolated from the *C. elegans* natural habitats (Avery and Shtonda, 2003; Samuel *et al.*, 2016). However, there have been relatively few studies where multiple clinical strains from individual, let alone multiple species have been studied for virulence in *C. elegans* (Garsin *et al.*, 2001; Sifri *et al.*, 2003; Irazoqui *et al.*, 2010a; Lavigne *et al.*, 2013). This approach has been undertaken in the other invertebrate infection model *G. mellonella* and allows similarities and differences between panels of strains to be identified in terms of how pathogenic the different strains are and how they affect the host organism (Wand et al., 2011; Wand et al., 2012; Wand et al., 2013; Benthall et al., 2015).

4.2 Aims and objectives

The primary aim of this chapter was to analyse the relative pathogenicity of a range of *K. pneumoniae, A. baumannii* and *P. aeruginosa* strains in the *C. elegans* model. Pathogenicity was assessed by measuring the impact of exposure to the bacterial strains on *C. elegans* lifespan. In addition, the more discrete effect of the bacterial pathogens on behaviour that could be signals of pathogenicity before *C. elegans* dies was also investigated. These

'preclinical measurements' included the effects of the bacterial strains on food aversion, reproduction and feeding during and after a maximum 48 hour exposure to bacteria. All these measures were taken in order to make a comparison with the bacterial virulence and preclinical signs to assess which, if any, of the preclinical measurements were predictors of the relative pathogenicity of investigated strains.

Five strains from each of the Gram negative ESKAPE pathogens *Klebsiella pneumoniae*, *Acinetobacter baumannii* and *Pseudomonas aeruginosa* were examined. The individual strains analysed are included in 2.7.8 and Table 4.1. These strains were selected as they had already been analysed in the alternative infection model *Galleria mellonella*. They represent important clonal types demonstrating distinct antimicrobial resistance phenotypes (Wand *et al.*, 2012; Wand *et al.*, 2013; Benthall *et al.*, 2015). The species were indicated by single letter code and a number of 1-5 being assigned. Throughout the rest of this thesis, the original numbering system will be used (strains will be referred to as A4, P3 etc.). In addition to these strains, the well described *C. elegans* pathogen PA14 was also analysed. PA14 is a clinical *P. aeruginosa* isolate, first being identified as highly pathogenic in *C. elegans* in 1999, having previously been shown to be pathogenic in both murine and *Arabidopsis* models (Rahme *et al.*, 1995; Tan *et al.*, 1999a; Mikkelsen *et al.*, 2011). PA14 has since been widely used as a *C. elegans* pathogen, being used for identification of virulence factors as well as for investigation of the food aversion phenotype it elicits in *C. elegans* when animal populations are exposed to bacterial lawns (Zhang *et al.*, 2005; Feinbaum *et al.*, 2012; Gaglia *et al.*, 2012; McEwan *et al.*, 2012; Melo and Ruvkun, 2012; Zhang and Zhang, 2012; Cohen and Troemel, 2015; Clamens *et al.*, 2017).

Strain number	Species	Identity
K1	*Klebsiella pneumoniae*	NCTC 9633/ ATCC 13883
K2		NCTC 13438
K3		MGH 78578/ ATCC 700721
K4		NCTC 13439
K5		NCTC 13368/ ATC 700603
A1	*Acinetobacter baumannii*	UKA15
A2		UKA2
A3		AYE/ ATCC BAA-1710
A4		ATCC 17978
A5		W1
P1	*Pseudomonas aeruginosa*	PA01
P2		GH56
P3		GH12
P4		GH97
P5		ATCC 15442

Table 4.1: The strains of *K. pneumoniae, A. baumannii and P. aeruginosa* analysed in the *C. elegans* virulence model in this and subsequent chapters.

4.3 Results

4.3.1 Pathogenicity of strains of *K. pneumoniae*, *A. baumannii* and *P. aeruginosa* in *C. elegans* as determined by killing assays

4.3.1.1 Killing assays performed at 20°C

Pathogenic bacteria have been demonstrated to shorten the lifespan of *C. elegans* (Tan *et al.*, 1999a; Aballay *et al.*, 2000; Pujol, 2001). Therefore, to understand which bacterial strains were more pathogenic than others *C. elegans* synchronised populations were exposed to the indicated bacterial strain from L4+1 at 20°C until all the animals were dead (2.3.1,Figure 4.1). These killing experiments are similar to slow killing experiments described in the literature and were designed to mimic the conditions on which a standard lifespan of *C. elegans* would be measured with the animals feeding on OP50 (Tan *et al.*, 1999a; Pradel *et al.*, 2007).

Populations of worms fed on OP50 were completely dead after 16 days. In comparison, every *K. pneumoniae* strain showed a reduced lifespan OP50 (Figure 4.1A), with total mortality being observed between 10 and 14 days. However only exposure to K4 and K5 resulted in a significant decrease in *C. elegans* lifespan relative to OP50.

Exposure to the *A. baumannii* strains resulted in complete *C. elegans* mortality between 11 and 14 days of exposure. However, none of these lifespans were significantly different compared to OP50-fed *C. elegans* (Figure 4.1B). Exposure to *P. aeruginosa* resulted in total mortality between 8 and 14 days. Four strains of *P. aeruginosa* (P1-4) killed *C. elegans* significantly quicker than OP50 did (Figure 4.1C). P5 was the only *P. aeruginosa* strain which did not have a significantly quicker kill time than OP50, with a lifespan of 14 days observed in these exposed animals (Figure 4.1C).

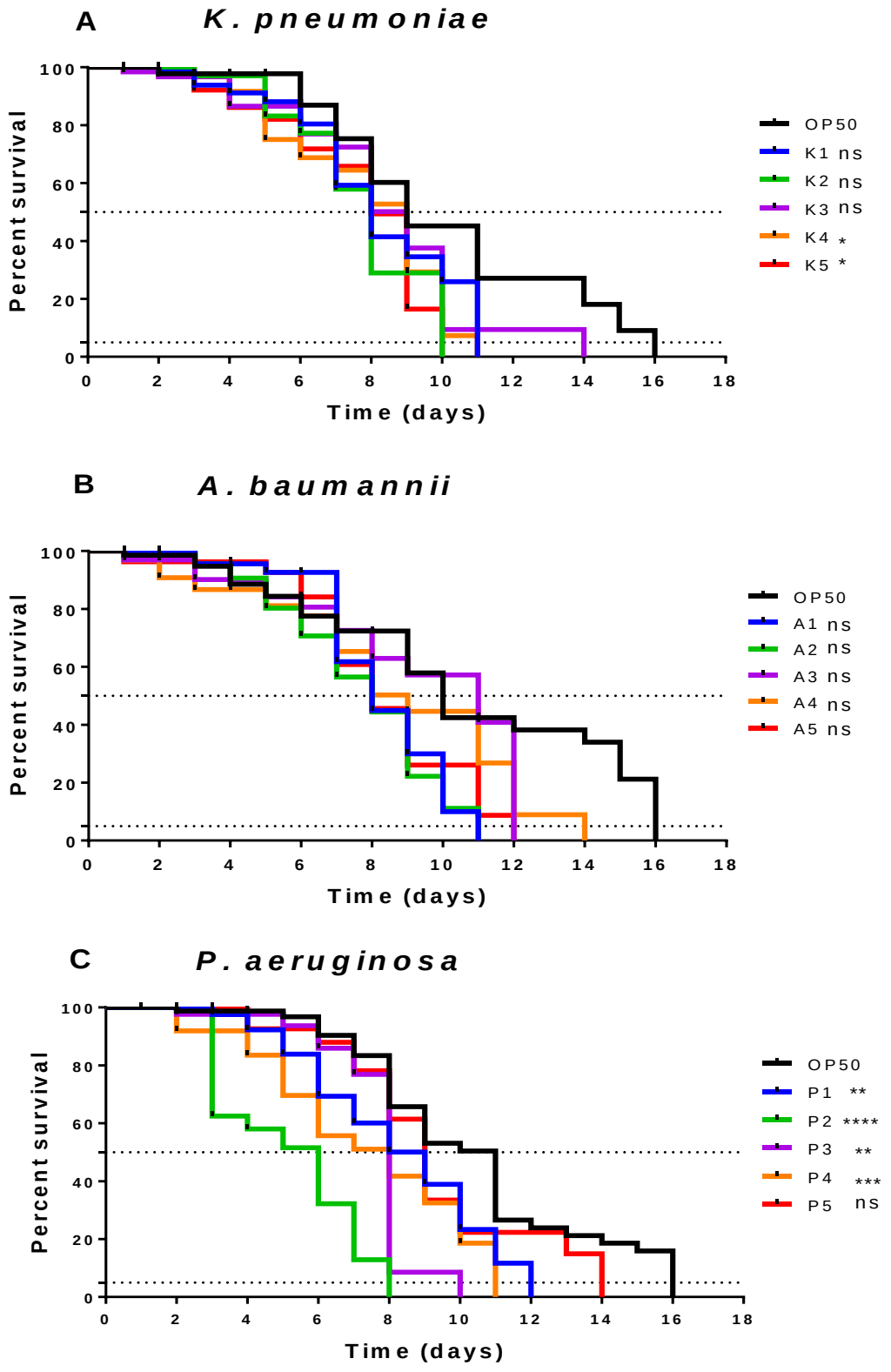


Figure 4.1: *C. elegans* killing at 20°C. Populations of L4+1 *C. elegans* were exposed to lawns of *K. pneumoniae* (A), *A. baumannii* (B) and *P. aeruginosa* (C) and the time for the animals to die was measured relative to *E. coli* OP5O. n≥3 assays with between 15-20 animals in each repeat for all bacterial strains. Analysis by log-rank (Mantel Cox) test in comparison to OP50 exposed animals. Dotted lines show median (50%) lifespan and 5% survival times. *P<0.05, **P<0.01, ***P<0.001 and ****P<0.0001.

4.3.1.2 Killing assays performed at 25°C

The initial analysis of the pathogenicity of the range of bacterial strains analysed for food aversion was based on continued exposure at 20°C, the standard maintenance temperature of *C. elegans*. However, some previous pathogenesis studies on *C. elegans* have used incubation temperatures of 25°C (Fuursted *et al.*, 2012). However incubating adult *C. elegans* at 25°C reduces the lifespan of the animals (Zhang *et al.*, 2015a). Therefore, to compare the data obtained with the strains examined in this thesis, experiments were also performed at 25°C This revealed that the lifespan of *C. elegans* on OP50 was 3 days shorter upon incubation at 25°C in comparison to 20°C, decreasing from 16 to 13 days (Figure 4.2). In terms of how *C. elegans* responded to the range of clinical strains at 25°C, exposure to all 5 *K. pneumoniae* and *P. aeruginosa* strains significantly shortened lifespan in comparison to OP50 (Figure 4.2A, C, Table 4.2) , with all of these strains completely killing the *C. elegans* populations between 7 and 11 days. In comparison, upon exposure to four out of the five *A. baumannii* strains (A1-A4) no significant shortening of lifespan was observed, with the lifespans between 10 and 12 days. A5 was the only *A. baumannii* strain which killed *C. elegans* significantly quicker than OP50, with a lifespan of 10 days observed (Figure 4.2B).

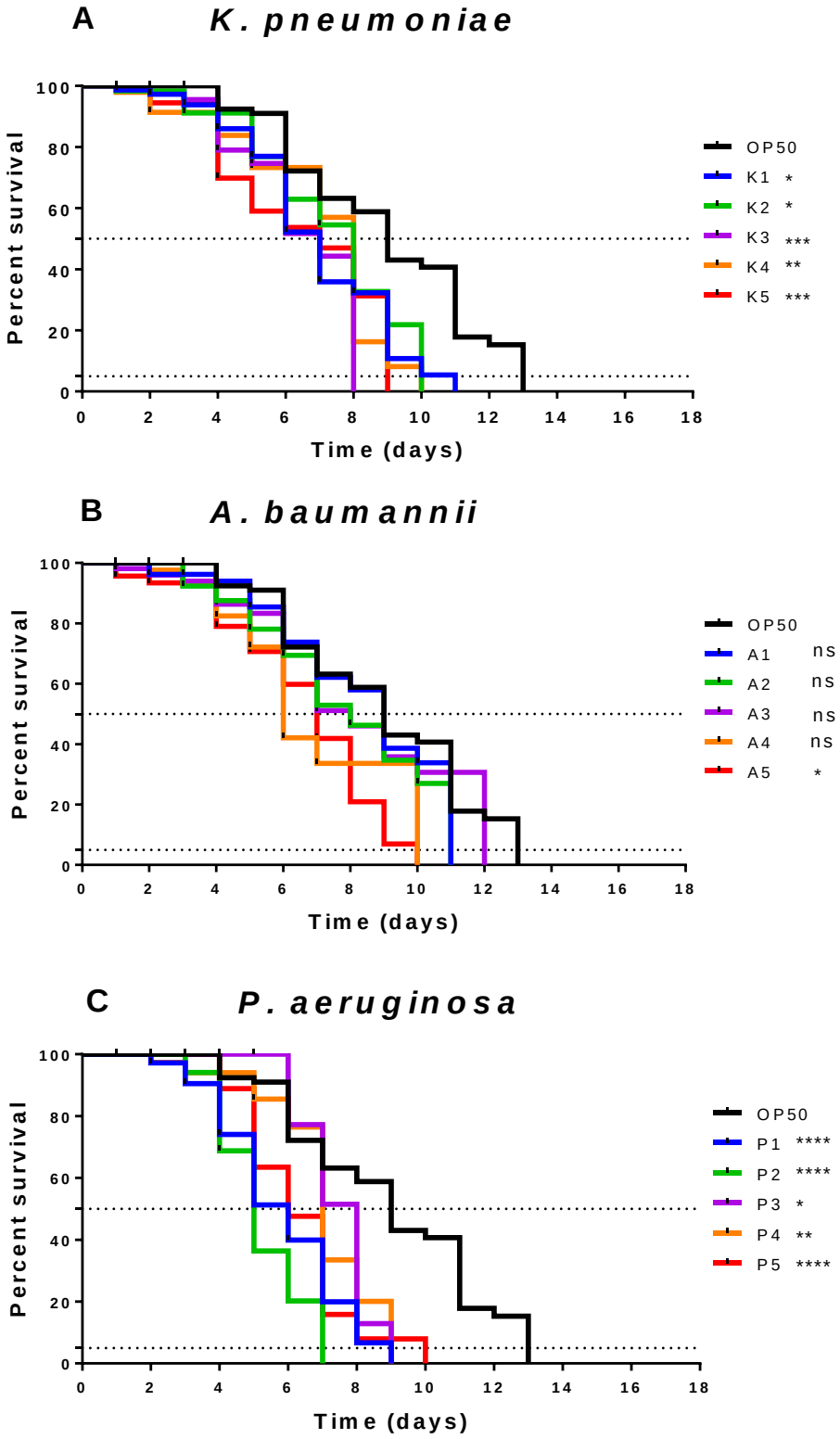


Figure 4.2: ***C. elegans*** **killing at 25°C. Populations of L4+1** ***C. elegans*** **were exposed to lawns of** ***K. pneumoniae*** **(A)** ***A. baumannii*** **(B) and** ***P. aeruginosa*** **(C) in comparison to** ***E. coli*** **OP50 as a control n≥3 assays with 15-20 animals each for all bacterial strains. Analysis by Log-rank (Mantel-Cox) test in comparison to OP50. Dotted lines show Median lifespan (50% survival) and 5% survival times. *P<0.05, **P<0.01, ***P<0.001 and ****P<0.0001.**

In order to further compare the effect of temperature on the lifespan of *C. elegans* in the presence of a range of bacterial strains, the data from the killing assays at the different temperatures was compared (Figure 4.1,Figure 4.2). This comparison from the killing experiments examined both the median and 5% survival times (Table 4.2). This revealed that increasing the incubating temperature from 20°C to 25°C significantly enhanced the virulence of 6 strains. These are *E. coli* OP50, *K. pneumoniae* K1 and K3, *A. baumannii* A5, and *P. aeruginosa* P1 and P5 (Table 4.2). These data demonstrate that not only is *C. elegans* lifespan significantly shortened on the standard laboratory food source *E. coli* OP50 by shifting the incubation temperature from 20 to 25°C, but this effect is also seen in the clinically relevant bacterial species included in this study. However, this effect was not conserved for all strains from the same species. Comparison of the data at 20 and 25°C also reveals that in 8 out of 16 strains the median killing time was reduced following the 5°C increased incubating temperature, and 10 out of 16 strains the 95% killing time was reduced. (Table 4.3). This provides further evidence that temperature increase has a negative effect on *C. elegans* survival.

A *K. pneumoniae*

	20°C						25°C					
Strain	OP50	K1	K2	K3	K4	K5	OP50	K1	K2	K3	K4	K5
Median survival time (days)	9	8	8	9	9	8	9	8	8	7	8	7
5% survival time (days)	16	11	10	14	11	10	13	11	10	8	10	9
Significance of lifespan shortening (compared to OP50 at same temperature)		ns	ns	ns	*	*	n/a	*	*	***	**	***
Signifance of lifespan shortening (compared to same strain at lower temperature)							*	*	ns	**	ns	ns

B *A. baumannii*

	20°C						25°C					
Strain	OP50	A1	A2	A3	A4	A5	OP50	A1	A2	A3	A4	A5
Median survival time (days)	10	8	8	11	9	8	9	9	8	8	6	7
5% survival time (days)	15	11	11	12	14	12	11	11	11	12	10	10
Significance of lifespan shortening (compared to OP50 at same temperature)		ns	ns	ns	ns	ns	n/a	ns	ns	ns	ns	*
Signifance of lifespan shortening (compared to same strain at lower temperature)							*	ns	ns	ns	ns	*

C *P. aeruginosa*

	20°C						25°C					
Strain	OP50	P1	P2	P3	P4	P5	OP50	P1	P2	P3	P4	P5
Median survival (days)	11	9	6	8	8	9	9	6	5	8	7	6
5% survival (days)	16	12	8	10	11	14	13	9	7	9	9	10
Significance of lifespan shortening (compared to OP50 at same temperature)	n/a	*	****	***	***	ns	n/a	****	****	*	**	****
Signifance of lifespan shortening (compared to same strain at lower temperature)							*	***	ns	ns	ns	***

Table 4.2: Data from *C.elegans* killing experiments. Data are collated from Figure 4.1 and Figure 4.2 showing Median and 5% survival, and the effect of modulating temperature on *C. elegans* lifespan for strains of *K. pneumoniae* (A), *A. baumannii* (B) and *P. aeruginosa* (C) on the same bacteria allowing direct comparison between experiments done at different temperatures . All statistical comparison is analysis by Log-rank (Mantel- Cox) test.

A

Median killing time

	Strain				
Species	1	2	3	4	5
K. pneumoniae					
A. baumannii					
P. aeruginosa					
E. coli (OP50)					

B

95% killing time

	Strain				
Species	1	2	3	4	5
K. pneumoniae					
A. baumannii					
P. aeruginosa					
E. coli (OP50)					

Table 4.3: The effect of increasing temperature on *C. elegans* median (A) and 95% killing time (B) upon exposure to bacterial strains. Pink highlighting indicates the relative measure of *C. elegans* incubated on the same bacteria is reduced upon increasing incubation temperature to 25°C, as indicated in Table 4.2 yellow highlighting indicates no change in lifespan. Any reduction is based on the amount of time elapsed. Statistical difference is presented in Table 4.2.

4.3.2 The *C. elegans* food aversion response to a range of *K. pneumoniae*, *A. baumannii* and *P. aeruginosa* strains

C. elegans has been shown to exhibit the phenomenon of food aversion when exposed to pathogenic bacteria (1.7) (Zhang *et al.*, 2005; Pradel *et al.*, 2007; Gaglia *et al.*, 2012; Zhang and Zhang, 2012). *C. elegans* populations were exposed to the range of strains of *Klebsiella pneumoniae, Acinetobacter baumannii* and *Pseudomonas aeruginosa* and the resulting food aversion was measured relative to OP50(Figure 4.3). This utilised the number of bacteria found on and off food at the given time points.

Individual species were grouped and investigated compared to a paired OP50 control. In these controls, *C. elegans* populations exposed OP50 lawns exhibited an increase in food aversion across the 48 hours. As discussed in chapter 3, this increase in the proportion of *C.*

elegans off a bacterial lawn was found to be representative of the enhanced *C. elegans* food-leaving driven by larval progeny.

Exposure of *C. elegans* populations to the *K. pneumoniae* strains resulted in four out of the five strains generating similar levels of food aversion (as measured by the proportion of adult animals off food) as animals exposed to lawns of OP50. This pattern was seen across all the time points at which food aversion was monitored (Figure 4.3A). However, exposure of *C. elegans* to strain K5 (NCTC 13368), showed a significant increase in food aversion at the 48 hour time point.

The *A. baumannii* strains elicited a complex pattern of food aversion. *C. elegans* exhibited lower levels of food aversion after exposure to A4 lawns for 24 hours in comparison to OP50. However, this reduction was not sustained at the 48 hour time point. Food aversion was also blunted in the A3 and A5 strains at the 48 hour time point. In contrast to these strains *C. elegans* exhibited enhanced food aversion to A2, with a significantly enhanced food-leaving compared to OP50 at 24 hours that was maintained at the 48 hour time point (Figure 4.2B).

Exposure of *C. elegans* to the *P. aeruginosa* strains, strains P1, 3 and 5 showed no variation in food aversion relative to OP50 at any of the time points investigated. In contrast, strains P2 and 4 elicited a clear increase in the levels of food aversion (Figure 4.3C). For strain P2, increased food aversion was observed from 6 hours of exposure onwards, with this increasing in significance relative to OP50 as the assay progressed. In the case of P4 food aversion was only significantly after 48 hours, similar to the effect seen in K5 (Figure 3C).

The data described above provide support for the conclusion that strains K5, A2, P2 and P4 generated an aversive response, measured by an increased propensity of *C. elegans* to reside off a bacterial lawn.

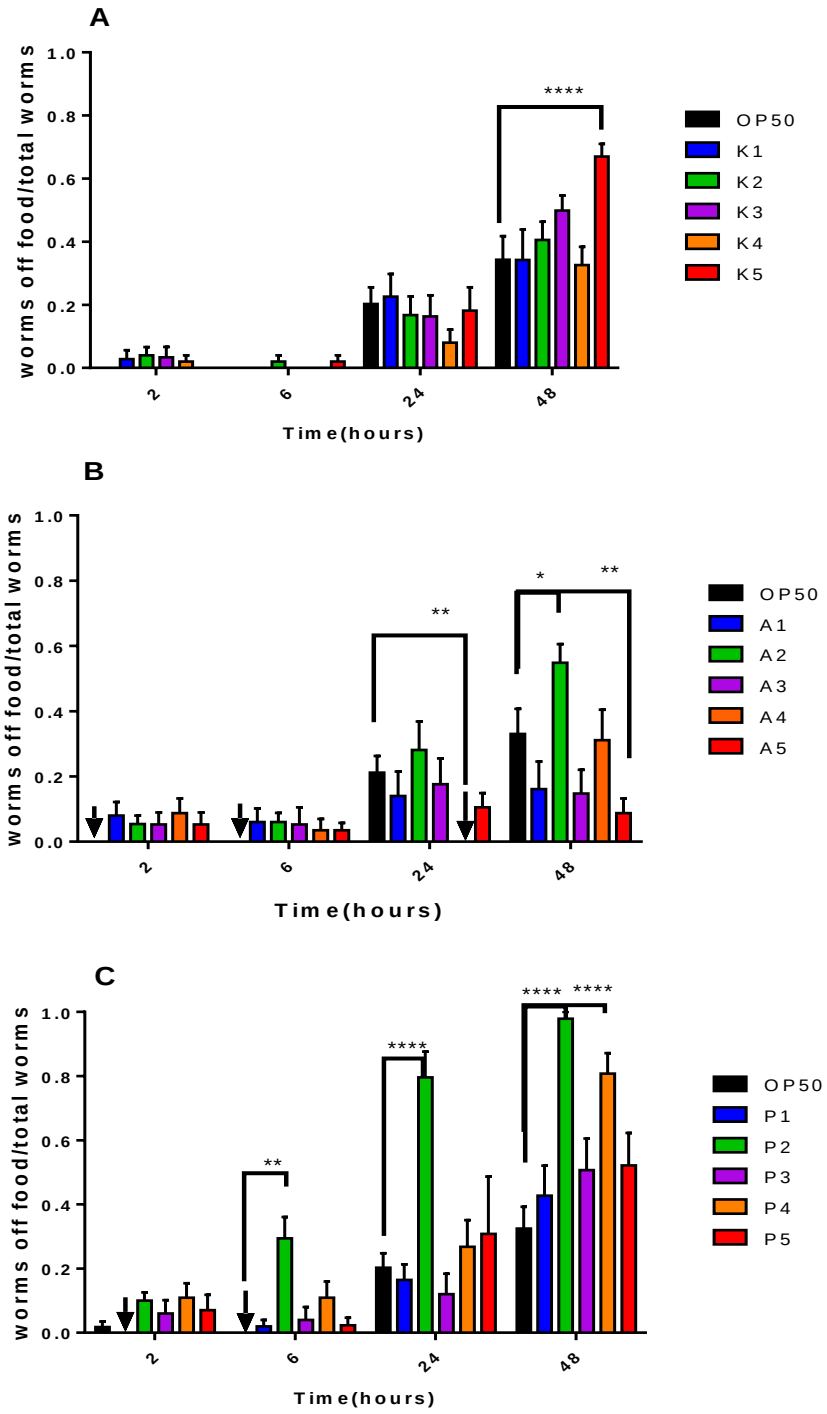


Figure 4.3: *C. elegans* food aversion levels upon exposure to strains of ESKAPE bacterial strains. The proportion of 7 adult *C. elegans* off the bacterial food lawns was measured over 48 hours and compared to animals on lawns of OP50. Food aversion was measured on lawns of 5 strains each of *Klebsiella pneumoniae* (A), *Acinetobacter baumannii* (B) and *Pseudomonas aeruginosa* (C). n=7 for all strains. Data analysed by two-way ANOVA with Dunnett's multiple comparisons to OP50. Error bars represent ±SEM. *P<0.05, **P<0.01, ***P<0.001 and ****P<0.0001.

4.3.3 Examination of the *P. aeruginosa* strain PA14 in behavioural and killing assays

In order to compare the effect of the strains described above on *C. elegans* behaviour and survival a strain previously identified to be a *C. elegans* pathogen, the *P. aeruginosa* strain PA14 was also analysed in both killing and food aversion assays (4.2). To allow a direct comparison to the other results obtained in *C. elegans* behavioural assays both in this chapter and Chapter 3 the further killing assays presented in this thesis were all performed at 20°C. Continued exposure to PA14 at 20°C led to highly significant shortening of the *C. elegans* lifespan in comparison to OP50. In fact, PA14 killed *C. elegans* more rapidly than in any of the other strains tested (Figure 4.4A). Exposure of young adult *C. elegans* to lawns of PA14 over a 48 hours, elicited a significant food aversion response at both 24 and 48 hours(Figure 4.4B) similar to the effect observed upon exposure to strains K5, A2, P2 and P4 reported earlier (4.3.2). These data provide a benchmark to assist in the interpretation of the results presented earlier in this chapter. Whilst the results presented earlier are less profound, the significant enhancement of food aversion and significant decrease in killing time in comparison to OP50 for K5, P2 and P4 (4.3.1,4.3.2), are similar to that exhibited by a well described *C. elegans* pathogen (Figure 4.5).

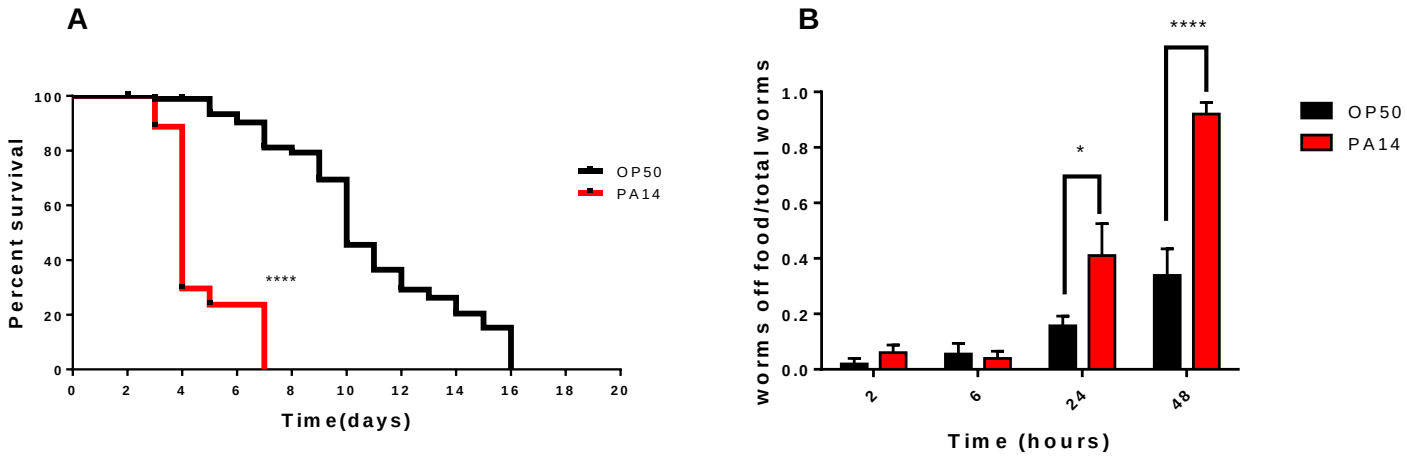


Figure 4.4: *C. elegans* responses towards the pathogenic strain of *Pseudomonas aeruginosa, PA14*. Responses were measured by the lifespan of *C. elegans* constantly exposed to PA14 at 20°C, as in Figure 4.1(A) and by measuring food aversion, by measuring the proportion of adult *C. elegans* off the bacterial lawn, as in Figure 4.3 (B)). N= 3 for (A), n=7 for (B) (A) analysed by Log-rank (Mantel-Cox) test, (B) analysed by two-way ANOVA with Sidak's multiple comparisons. All error bars represent ±SEM. *P<0.05, **P<0.01, ***P<0.001 and ****P<0.0001.

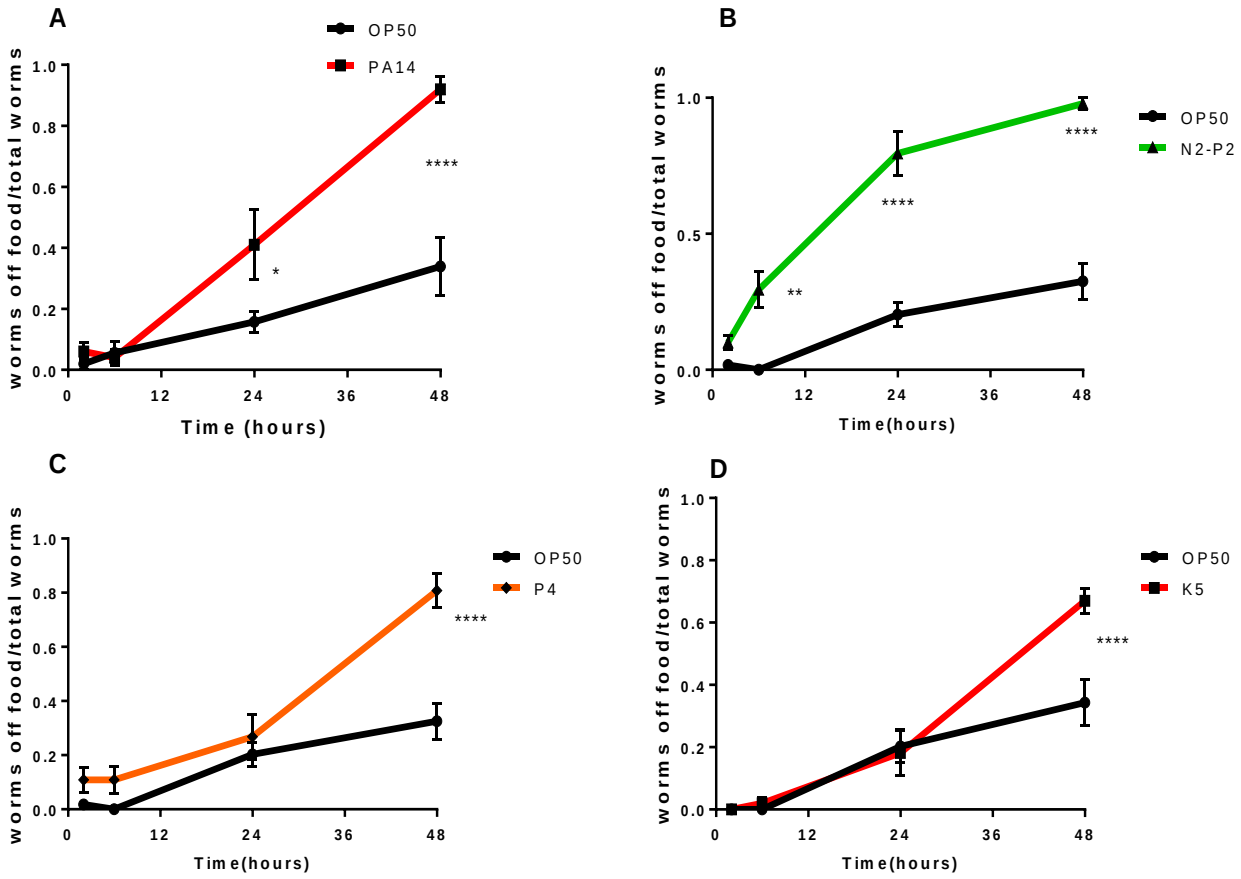


Figure 4.5: *C. elegans* food aversion of aversive pathogenic bacterial strains. Alongside the well described C. *elegans* pathogen *P. aeruginosa* PA14 (Figure 4.4), Prior experiments in this chapter has identified the strains P2, P4 and P5 also being aversive towards *C. elegans*, as well as being pathogenic (Figure 4.3,Figure 4.1). *P<0.05, **P<0.01, ***P<0.001 and ****P<0.0001.

4.3.4 The effect of *K. pneumoniae, A. baumanni*i and *P. aeruginosa* strains on C. elegans reproduction

4.3.4.1 C. elegans egg laying

C. elegans egg laying and hatching, and the ability to develop larval progeny are good measures of *C. elegans* health. To measure this individual L4+1 adult *C. elegans* were transferred onto lawns of the indicated bacteria for 24 hours. The adult animals were then removed and the number of eggs and L1 larvae counted. 24 hours later, the numbers of larvae were counted again (2.2.4). No eggs were found to be present after 48 hours indicating that most of the eggs had hatched. Analysis of the numbers of eggs revealed that

C. elegans egg laying was not significantly altered upon exposure to any of the clinical bacterial strains relative to OP50 (Figure 4.6).

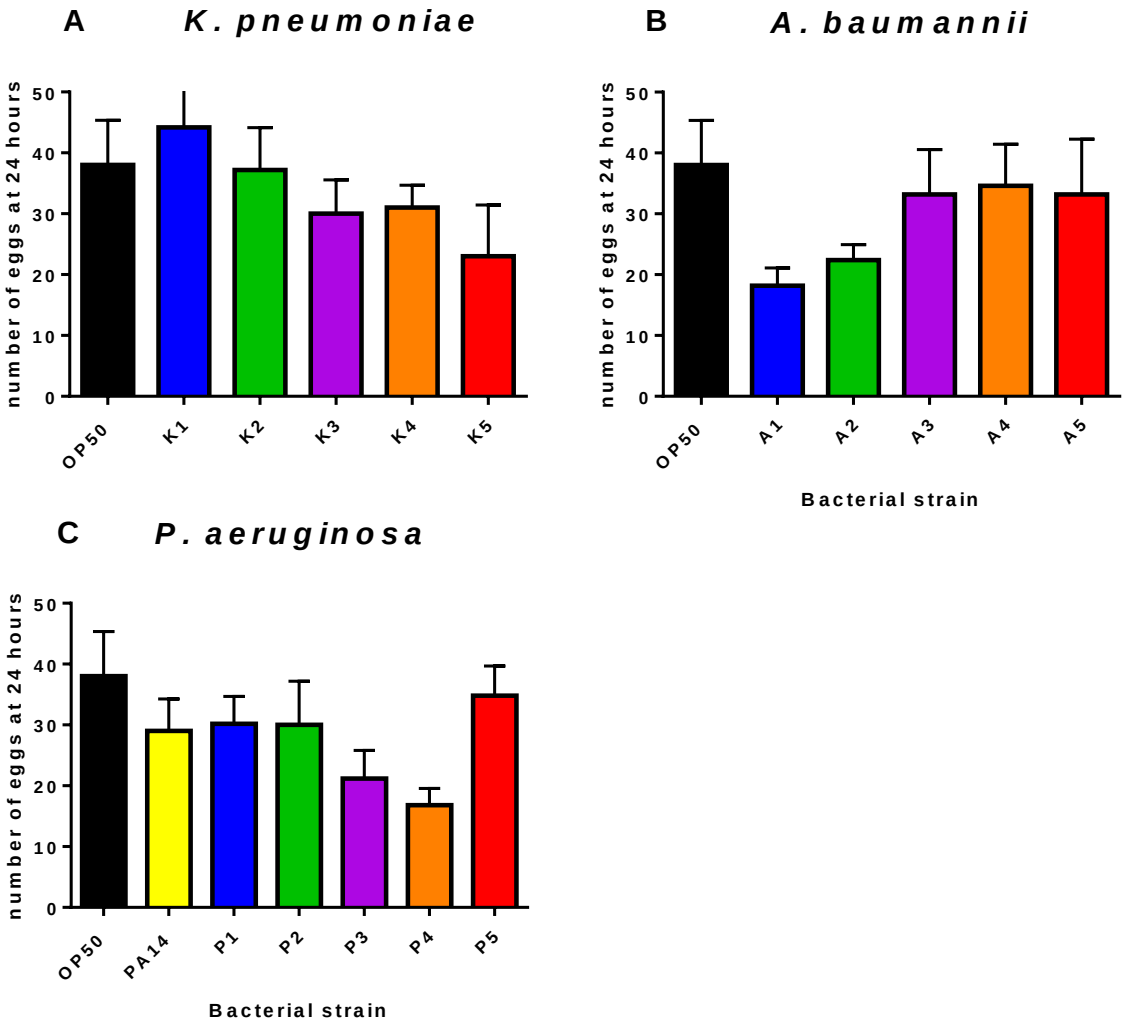


Figure 4.6: Number of eggs produced by individual *C. elegans* on bacterial lawns. Single L4+1 animals were placed on lawns of *K. pneumoniae* (A), *A. baumannii* (B) and *P. aeruginosa* (C) and left for 24 hours after which the numbers of eggs present on the plate was counted and compared to lawns of *E. coli* OP50. N=5 for all strains. Error bars are ±SEM. Data analysed by one-way ANOVA with Dunnett's multiple comparisons to OP50.

4.3.4.2 *C. elegans* larvae production

Exposure to the bacterial strains led to some variation in the numbers of *C. elegans* larvae produced over a 48 hours period (Figure 4.7). However, these variations were not significant. These data together demonstrate that exposure to the range of *K. pneumoniae*, *A.*

baumannii and *P. aeruginosa* strains did not significantly modulate *C. elegans* reproductive capability.

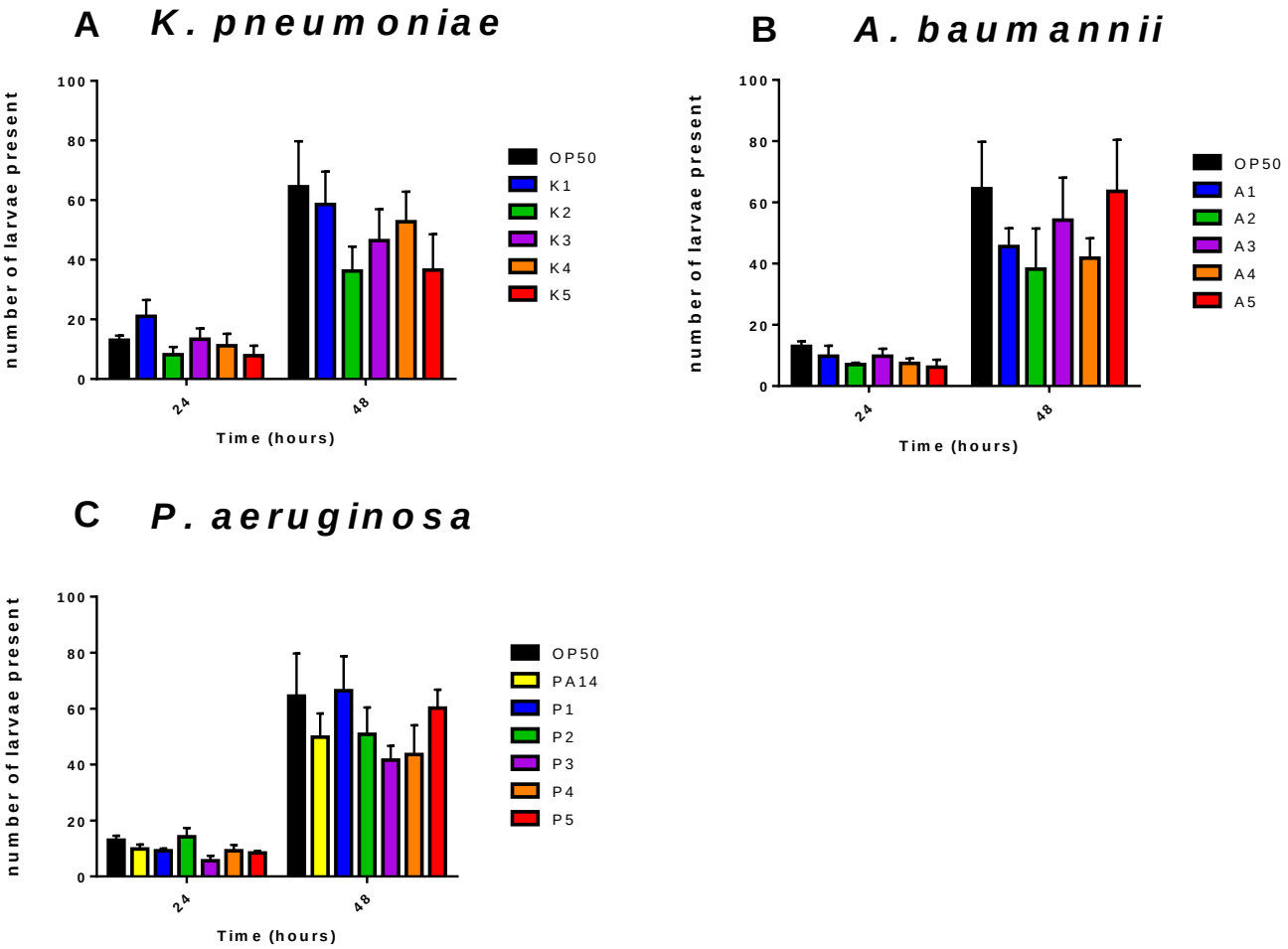


Figure 4.7: Numbers of *C. elegans* larvae produced from single adult animals on lawns of ESKAPE bacteria. Eggs were laid on lawns of *Klebsiella pneumoniae* (A), *Acinetobacter baumannii* (B) and *Pseudomonas aeruginosa* (C). N=5 for all strains. Error bars are ±SEM. Data analysed by two-way ANOVA with Dunnett's multiple comparisons to animals on OP50 lawns.

The assays and measurements described above allowed an estimate of the ability of the various bacterial strains to affect egg hatching. The percentage of the eggs present at 24 hours which hatched was calculated. This showed all of the 17 investigated bacterial strains supported eggs hatching to live larvae at a rate of at least 80% and this was not a significant reduction compared to OP50 (Figure 4.8). This was reinforced at 48 hours in which no eggs were found on the plates. This result showed that exposure to the strains of *K. pneumoniae,*

A. baumannii and *P. aeruginosa* did not affect the viability or overtly impact on viability of eggs laid onto any of the bacterial lawns investigated.

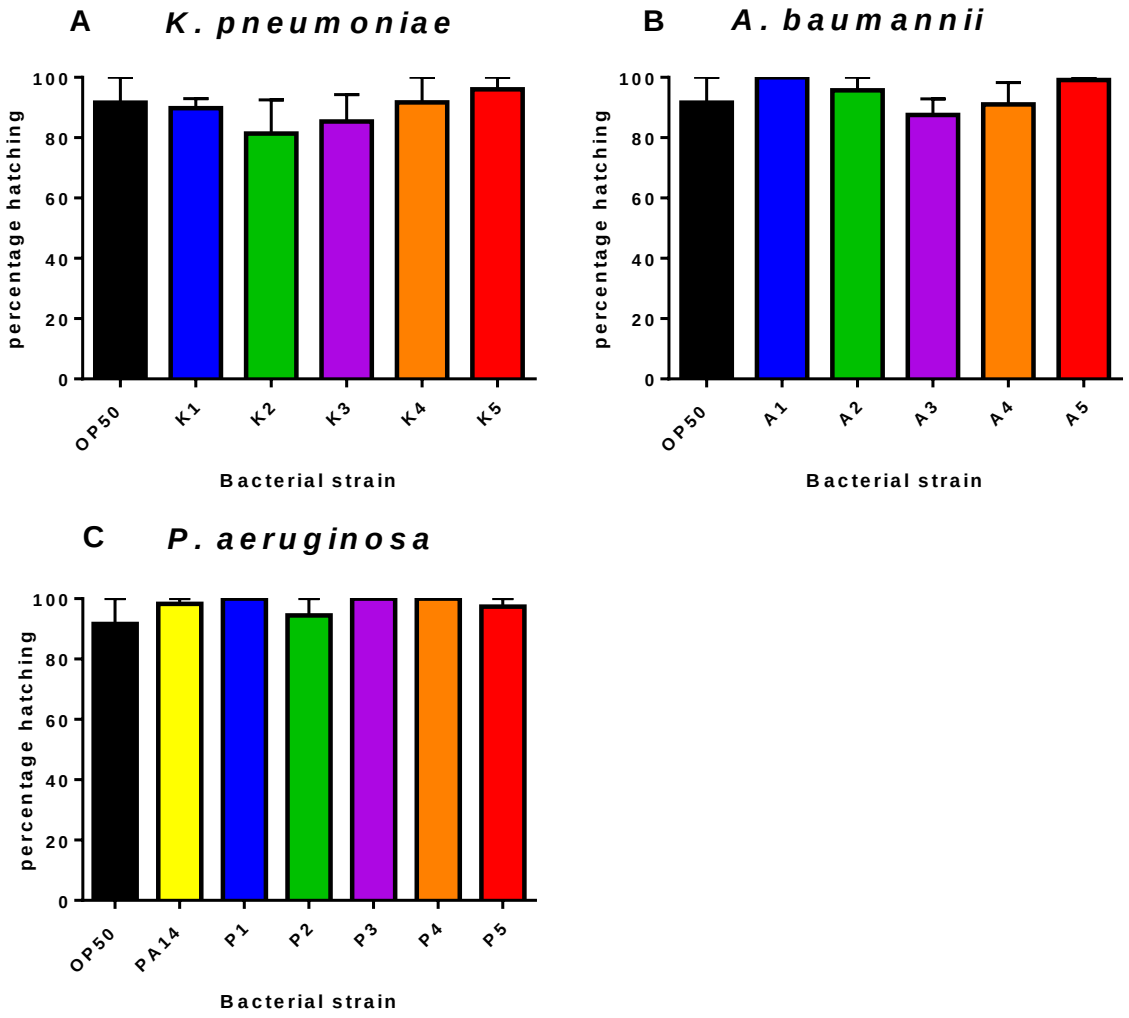


Figure 4.8: Hatching of *C. elegans* eggs laid on lawns of ESKAPE bacteria. Single adult *C. elegans* were exposed to lawns of indicated K. *pneumoniae* (A), *A. baumannii* (B) and *P. aeruginosa* (C). Data presents hatching of eggs produced from single adult *C. elegans* on a bacterial lawn for 24 hours. Data analysed by one-way ANOVA with Dunnett's multiple comparison to animals on OP50. Error bars represent ±SEM.

4.3.5 *C. elegans* progeny development upon exposure to *K. pneumoniae, A. baumannii* and *P. aeruginosa*

Exposure to harmful substances can affect the development and maturation of *C. elegans* larvae although this has not been addressed in context of comparing multiple bacterial strains (Karmacharya *et al.*, 2009). The ability of the various bacterial strains to affect the time-course of *C. elegans* development was examined. To investigate this L1 larvae were transferred onto bacteria and their subsequent development examined (0). One day after

placing L1s on the bacterial lawns there were L2 and L3 stage larvae on the plates. Two days after exposure there were L4 and young adults. The percentage of the population at each larval stage was measured by defining the developmental stage and plotting each as a percentage of the total population. One day of bacterial exposure led to between 80 to 100% of animals being L3s, and two days of exposure resulted in 75 to 100% of animals being adults. Statistical analysis performed on the percentage of L3 animals after 1 day (Figure 4.9A,C), and percentage of adults after 2 days of bacteria exposure (Figure 4.9B,D) between the *K. pneumoniae, A. baumannii* and *P. aeruginosa* strains and *E. coli* OP50 demonstrated that there was no significant variation in the development of *C. elegans* exposed to different strains of bacteria.

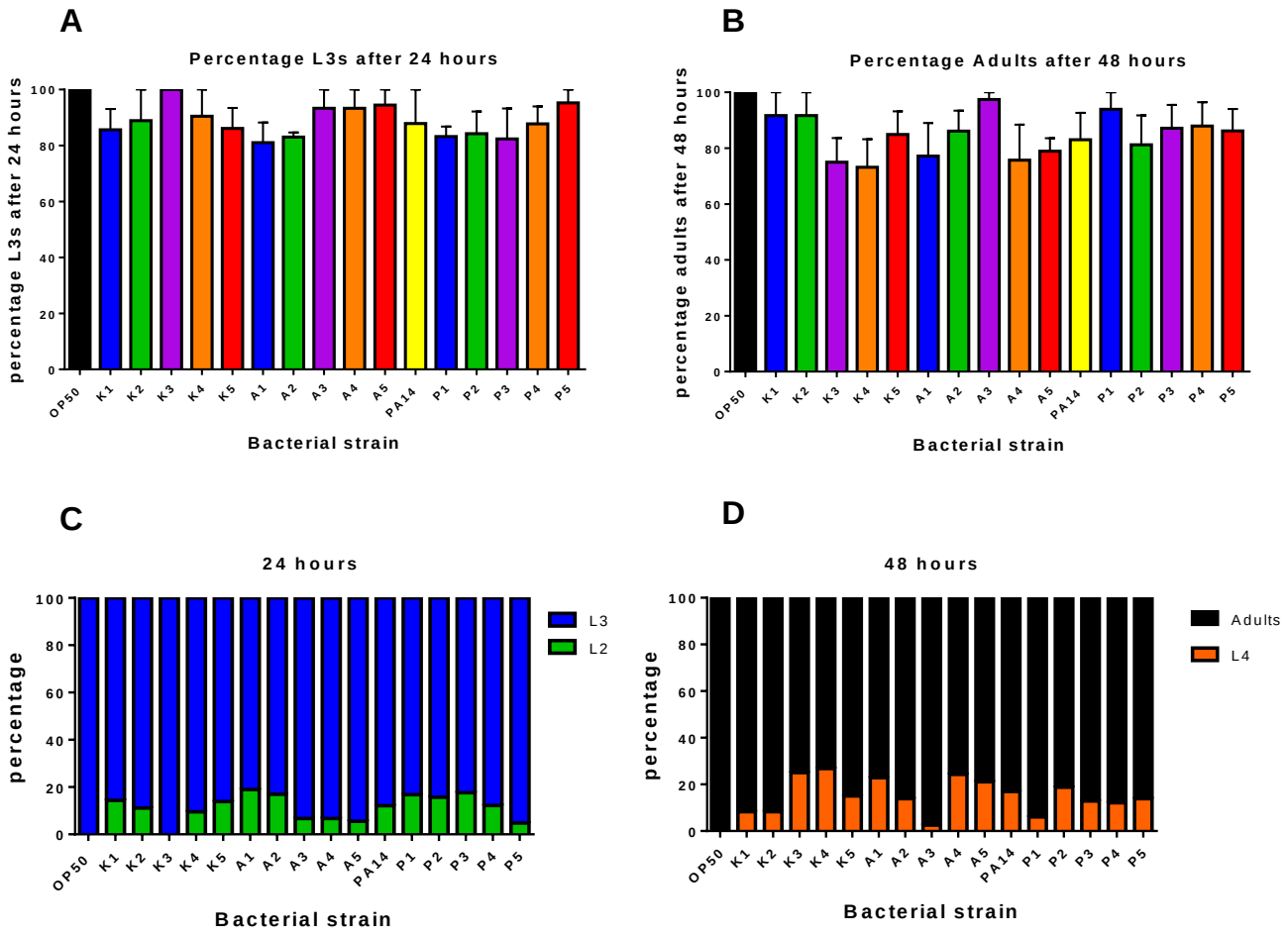


Figure 4.9: Development of *C. elegans* larvae on lawns of ESKAPE bacteria. L1 larvae were placed on lawns of strains of *K. pnuemoniae, A. baumannii* and *P. aeurignosa* and subsequent development was measured relative to *E. coli* OP50. These data are represented as the percentage of L3 larvae stage after 24 hours on the lawn (A) and percentage of adults after 48 hours on the lawn (B), as well as the proportion of L3s and L2 after 24 hours (C) and L4s and adults after 48 hours (D) n=3 for all strains. Error bars represent ± SEM. Data analysed by one-way ANOVA with Dunnett's multiple comparisons to results gained with animals exposed to OP50 lawns.

4.3.6 Analysis of *C. elegans* pharyngeal pumping

Pharyngeal pumping is regulated by the presence or absence of food (1.4.1.1) (Avery and Horvitz, 1990). Ingestion of pathogenic food may lead to a modulation of pharyngeal pumping. The food aversion exhibited by *C. elegans* as described in 4.3.2 may be driven by a reduction in feeding. To test the rate of consumption of the bacteria by *C. elegans*, adult animals were exposed to bacterial lawns for 48 hours after which pharyngeal pumping was measured. This timing recapitulated the experimental conditions used for the food aversion experiments described earlier (4.3.2). Analysis of pharyngeal pumping on bacterial lawns revealed that wild type *C. elegans* exposed to OP50 as well as the strains of the ESKAPE species exhibited pharyngeal pumping of around 240 pumps per minute (Figure 4.10). Only the *P. aeruginosa* strain PA14 exposed animals had reduced pharyngeal pumping with a rate of 223 pumps per minute.

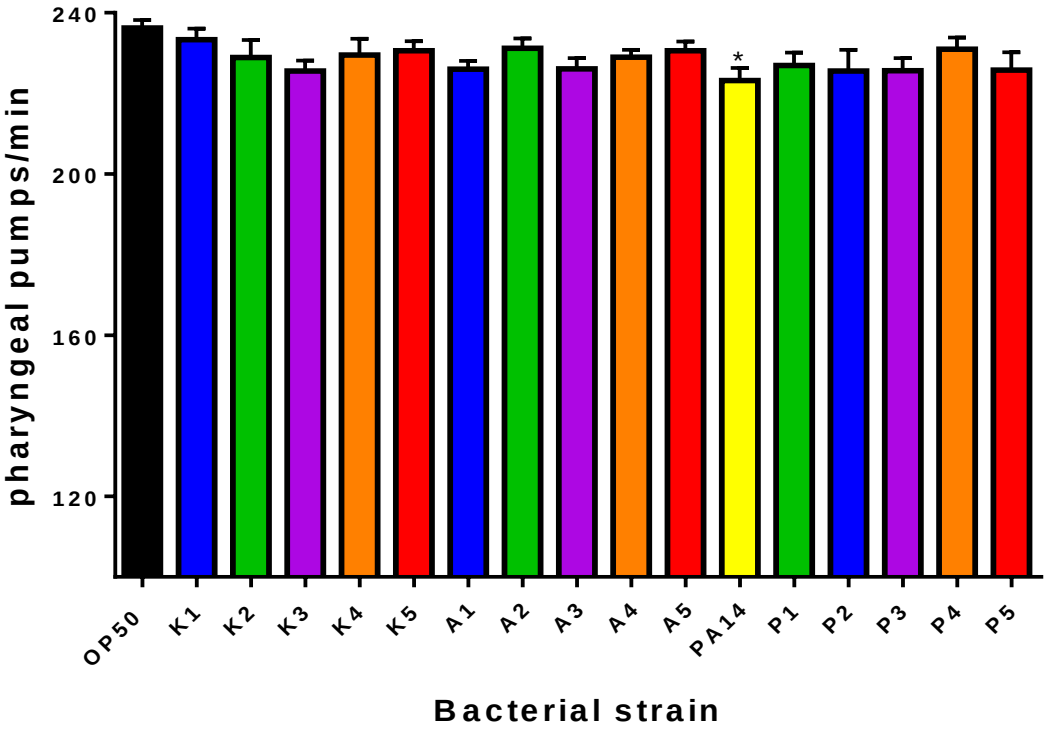


Figure 4.10: Pharyngeal pumping of *C. elegans* after 48 hours on bacterial lawns of *Klebsiella pneumoniae, Acinetobacter baumannii* and *Pseudomonas aeruginosa* in comparison to *E. coli* OP50. n≥10 for all strains. Error bars represent ±SEM. Analysis by one-way ANOVA with multiple comparisons.

4.4 Discussion

In this chapter a comparative approach in which numerous *C. elegans* behaviours were examined in order to inform the potential for bacterial pathogenicity was undertaken. Initially, the virulence of the individual bacterial strains towards *C. elegans* was determined by comparing the lifespans of animals constantly exposed to the respective bacterial lawns (4.3.1). The lifespan of *C. elegans* is used as a measure of the 'health' the individual animals. Both genetic and environmental and factors modulate *C. elegans* lifespan (Labrousse *et al.*, 2000; Begun *et al.*, 2007; Pradel *et al.*, 2007; Zheng *et al.*, 2016). This was followed by an examination of the behavioural responses of populations of adult *C. elegans* towards *Klebsiella pneumoniae*, *Acinetobacter baumannii* and *Pseudomonas aeruginosa* strains, especially with respect to any aversion exhibited by adult animals towards bacterial lawns. This pathogen avoidance or food aversion effect has previously been described in *C. elegans*

and has been demonstrated to be a part of how *C. elegans* responds to pathogen infection as part of the immune response (1.7) (Zhang *et al.*, 2005; Pradel *et al.*, 2007; Engelmann and Pujol, 2010; Gaglia *et al.*, 2012).

Undertaking both these measurements allowed a comparison to be made between the virulence of different bacteria towards *C. elegans* and the behaviour exhibited towards bacterial lawns. This approach with a range of strains also allows a comparison to be made between strains of the same and different bacterial species.

In addition to these two measures, the ability of the bacterial strains to affect *C. elegans* reproduction and feeding rate was measured. It has previously been discussed in the literature how the availability of food and exposure to a number of harmful insults, including pathogenic bacteria, high levels of environmental CO_2 and treatment with experimental drugs can impact on the fecundity of *C. elegans* (Schafer, 2005; Kumar *et al.*, 2010; Fenk and de Bono, 2015; Kamaladevi and Balamurugan, 2015). The ability of *C. elegans* to produce viable larvae has also been shown to be effected with drug treatment, most notably with the DNA synthesis inhibitor FUdR (Mitchell *et al.*, 1979). Therefore, in addition to scoring egg production per worm and the numbers of larvae that hatched, the effect of the bacterial strains on the development of *C. elegans* larvae from the L1 stage to viable adults was also examined. It has previously been reported that *C. elegans* larvae can exhibit developmental defects upon treatment with antipsychotic drugs and that adult *C. elegans*' growth is inhibited by eating less palatable bacteria (Avery and Shtonda, 2003; Donohoe *et al.*, 2006; Karmacharya *et al.*, 2009). Taking these different measurements related to fecundity and feeding may be representative of the levels of pathogenicity exhibited by the different bacterial strains.

4.4.1 Killing of *C. elegans* by strains of *K. pneumoniae, A. baumannii* and *P. aeruginosa*

To directly compare the virulence of the bacteria to an OP50 control it was decided to perform a continued incubation on the same media (NGM) and initially compare two temperatures (20 and 25°C). From the resulting killing assays, a couple of clear conclusions can be made. Firstly, animals constantly exposed to OP50 showed a consistent lifespan of

16 days at 20°C and 13 days at 25°C. These lifespans are shorter than reported in the literature, likely due to the differences in experimental protocols (Zhang *et al.*, 2015a; Zheng *et al.*, 2016; Abergel *et al.*, 2017). In the killing assays in this chapter, the animals being analysed were transferred to a new plate of with a lawn of the same bacteria (2.3.1). The animals used in killing assays were also not treated with FUdR to sterilise the animals which has been done in previous killing experiments with pathogenic bacteria, with FUdR acting to prolong *C. elegans* lifespan (Mitchell *et al.*, 1979; Reddy *et al.*, 2009; Shivers *et al.*, 2009). How often worms are manipulated, specifically with the mechanical stimulation of picking and drug treatment may also affect the lifespan of the animals.

Exposure to certain bacteria strains at both temperatures resulted in significantly shorter *C. elegans* lifespans than *E. coli* OP50 (4.3.1), with exposure to 6 strains at 20°C (2 *K. pneumoniae* and 4 *P. aeruginosa*) and 11 at 25°C (all 5 strains of both *K. pneumoniae* and *P. aeruginosa* and one strain of *A. baumannii*) resulting in a significantly decreased *C. elegans* lifespan than exposure to OP50. Previous work has identified that bacteria which significantly reduce lifespan have been classed as pathogenic (Tan *et al.*, 1999a; Begun *et al.*, 2007; Pradel *et al.*, 2007). The results from the killing assays results in any bacterial strains which significantly reduced *C. elegans* lifespan relative to OP50 being classified as pathogenic. This classification for these strains will subsequently be used for the rest of this thesis.

Results from both sets of killing assays reveal that *P. aeruginosa* tends to be more pathogenic than both *K. pneumoniae* and especially *A. baumannii*. At 20°C all but one of the *P. aeruginosa* strains were pathogenic (based on the definition above) towards *C. elegans* with two *K. pneumoniae* and no *A. baumannii* strains being pathogenic at this temperature. *P. aeruginosa* has previously been found in the natural environments of *C. elegans*, and thus may be better adapted to causing an infection in *C. elegans* than the other Gram-negative species (Schulenburg and Félix, 2017).

Using mutant *C. elegans* deficient in certain aspects of the organism's biology will help to elucidate the *C. elegans* responses to infection, beginning to unpick the differences in the pathogenicity of the bacterial strains screened here. This potentially allows identification as to whether the responses described here upon bacterial infection are driven by conserved or distinct biological pathways. The differences which contribute to the differential virulence

between PA14 and PA01 strains in *C. elegans* have previously been investigated (Lee *et al.*, 2006). In addition, studies have been taken which identify key determinants of virulence in individual bacterial strains, by identifying mutants with reduced levels of virulence using a *C. elegans* model(Tan *et al.*, 1999b; Gallagher and Manoil, 2001; Tenor *et al.*, 2004; Feinbaum *et al.*, 2012). In addition there has been a study where differences between *S. aureus* and *P. aeruginosa* infection in their pathogenesis, virulence control and recognition in *C. elegans* has been investigated (Irazoqui *et al.*, 2010a). Exposing *C. elegans* to a range of strains from three different species bacteria and gaining range of results, as done in this chapter, allows a deeper comparison between strains of the same and other species to be made and gain more insight into how bacterial infection is controlled in the *C. elegans* model.

4.4.2 Temperature modulates the pathogenicity of ESKAPE pathogens in the *C. elegans* infection model

The data from the killing assays reveals that the temperature increase alone has a deleterious effect on *C. elegans* as worms exposed to OP50 have significantly shorter lifespans at 25°C (4.3.1.2). Incubating adult worms at 25°C triggers a thermosensitive pathway which acts to reduce the lifespan of the animals (Zhang *et al.*, 2015a). It is also interesting that out of the 15 bacterial strains analysed in this way 11 of the clinical strains killed *C. elegans* significantly more rapidly than OP50 at 25°C compared to 6 strains at 20°C. In the case of the five strains with significantly shorter lifespans than OP50 at 25°C but not 20°C (K1, K2, K3, A5 and P5) the temperature increase clearly makes these strains more virulent towards *C. elegans*. Additionally, the fact that 6 strains, including OP50, kill *C. elegans* significantly more rapidly at 25°C than at 20°C (Table 4.2), and that median and 95% kill times are altered in 8 and 10 strains respectively (Table 4.3), it is apparent that increasing the temperature increases the ability of the bacterial strains to kill *C. elegans*. It is important to note that all four strains where this faster killing at 25°C was not observed were *A. baumannii* strains. This combined with the fact that no *A. baumannii* strains significantly decrease *C. elegans* lifespan at 20°C and only one (A5) does at 25°C indicates *that K. pneumoniae* and *P. aeruginosa* strains in general are more pathogenic food sources, which gives credence to the fact that *P. aeruginosa* in particular is being increasingly

investigated in the *C. elegans* infection model. Investigating a wider range of strains representative of each species will enable this conclusion of differential pathogenicity between species between species to be made more firmly.

There are a number of possibilities that might explain the greater virulence of the bacteria at 25°C. First it could be that as a result of the metabolic change that occurs in the animals upon the temperature increase, which could be akin to a mild heat shock response, *C. elegans* are less able to deal with a pathogenic insult. In addition to temperature affecting lifespan, when *C. elegans* undergo heat shock, as in all organisms, heat shock proteins (HSPs) expression and activity is upregulated (Prahlad *et al.*, 2008; Rodriguez *et al.*, 2013; Zhang *et al.*, 2015a). However, accumulation of large amounts of HSPs can be detrimental in the interactions between different tissues (Prahlad *et al.*, 2008). Because of this the immune response of *C. elegans* may be down regulated thus making the animals more susceptible to pathogen infection. When dealing with opportunistic pathogens, which *K. pneumoniae, A. baumannii* and *P. aeruginosa* are all in humans, increased host susceptibility will be magnified (Podschun and Ullmann, 1998; Perez *et al.*, 2007; Breidenstein *et al.*, 2011).

Alternatively, the raised incubation temperature may allow the bacteria to produce a greater suite of virulence factors which could help to actively kill *C. elegans.* Previous studies demonstrated that incubating *C. elegans* with PA14 on a higher osmolarity media than NGM, such as PGS (peptone glucose sorbitol) and BHI (Brain Heart Infusion) leads to fast, toxin-mediated killing (1.5.1.3), different from the slow killing which is occurring in this chapter (1.5.1.1). Individual toxins which help to mediate fast killing have been identified (1.5.1.3) (Cezairliyan *et al.*, 2013). Increasing the temperature of the bacterial incubation from 20 to 25°C, therefore getting closer to the internal temperature of a mammalian host of 37°C may lead to the bacteria producing more toxins or virulence factors which help to increase the relative pathogenicity of the bacteria towards *C. elegans.*

It has been demonstrated in this chapter that temperature plays an important role in host pathogen interactions in the *C. elegans* model. However, it was also determined that a range of pathogenicity can be resolved at 20°C. This represents conditions suited to standard *C. elegans* behavioural assays and demonstrates that bacterial pathogenesis of C. elegans can be investigated at 20°C.

4.4.3 *C. elegans* food aversion exhibited towards strains of *K. pneumoniae, A. baumannii and P. aeruginosa*

The 48 hour behavioural experiments were designed to compare how the exposure of a range of clinical bacterial strains to OP50 being impacted worms incubated at 20°C. Previous studies in which *C. elegans* killing was observed in a 48 hour period involved incubating *C. elegans* with the bacteria at an elevated temperature in addition to incubation on a higher osmolarity medium than NGM (Darby *et al.*, 1999; Tan *et al.*, 1999a; Pellegrino *et al.*, 2014). This means that the behavioural assays carried out in this chapter were designed to act as pre-death measurements for *C. elegans* infected with bacteria allowing a comparison with food aversion seen upon *C. elegans* exposure to pathogenic bacteria previously described, in addition to the enhanced proportion of animals off food which reflects progeny enhanced food-leaving as described in chapter 3 (Zhang *et al.*, 2005; Pradel *et al.*, 2007; Gaglia *et al.*, 2012; Melo and Ruvkun, 2012). Food aversion was measured by the proportion of adult *C. elegans* off a bacterial lawn throughout a 48 hour exposure. In terms of the food aversion effect that is seen in the 48 hour exposure to the range of bacterial strains, the observations that immediately become apparent is that there are the four strains which gave significantly higher food aversion levels relative to animals exposed to lawns of OP50. In the case of these OP50-exposed animals, as mentioned in chapter 3, the increasing proportion of worms off food is not a response to an aversive stimulus, but rather a parental response towards the larval progeny. These four strains are the two *P. aeruginosa* strains P2 and P4, the *K. pneumoniae* strain K5 and the *A. baumannii* strain A2. This food aversion effect is strikingly observed after populations of animals after being exposed to lawns of the respective bacteria for 48 hours. As exposure to the P2, P4, K5 and A2 strains have an enhanced level of food aversion relative to animals exposed to OP50, these bacterial strains will subsequently be classed as aversive.

The aversive behaviour seen here is similar to other bacterial aversion behaviours described where after a period of extended exposure to bacteria *C. elegans* populations show increasing levels of aversion towards bacterial lawns of *P. aeruginosa* PA14 and *S. marcescens* Db11 and Db10 relative to *E. coli* OP50 (Zhang *et al.*, 2005; Pradel *et al.*, 2007; Gaglia *et al.*, 2012; Melo and Ruvkun, 2012). In addition to these aversive strains, two *A.*

baumannii strains in which food aversion levels were lower than OP50 were also identified with exposure to the remaining 11 strains resulting in similar levels of food aversion as the OP50 control populations' (4.3.2). The two *A. baumannii* strains in which lower levels of food aversion than seen upon OP50 exposure (A4 and A5) may be preferential food sources towards *C. elegans* in comparison with OP50, or any chemical signals given off by these bacteria may be disguising the postulated ascaroside signal produced by larvae which drives adult food-leaving (Chapter 3).

These results demonstrate the *C. elegans* can exhibit differential food aversion levels to a range of bacterial strains, including strains of the same species. This is particularly evident when examining the *P. aeruginosa* strains, with exposure of *C. elegans* to two strains resulting in significantly higher levels of food aversion relative to OP50-exposed animals. These results also indicate that food aversion in *C. elegans* is elicited in a distinct strain by strain basis. Previous studies have demonstrated how *C. elegans* exhibits differential responses to different bacterial strains. This phenomenon is seen both in terms of feeding preferences and the growth stimulated by bacteria isolated from the natural environment (Shtonda and Avery, 2006; Samuel *et al.*, 2016).

Visual inspection of the lawns showed that there was no noticeable depletion of the bacterial lawns throughout the 48 hour assay, with the lawns of some strains, specifically some *K. pneumoniae* strains, becoming thicker throughout the assay. This indicates that the enhanced food aversion levels seen upon exposure to certain bacterial strains is unlikely to be caused by food depletion, previously described to drive *C. elegans* food aversion (Milward *et al.*, 2011). The response that was observed here is more likely to be driven by an aversive stimulus similar to previously described *C. elegans* to bacterial pathogens or xenobiotics (Zhang *et al.*, 2005; Pradel *et al.*, 2007; Gaglia *et al.*, 2012; Melo and Ruvkun, 2012; Ballestriero *et al.*, 2016).

Noticeably, three of the four strains which resulted in significant food aversion, specifically *K. pneumoniae* K5 and *P. aeruginosa* P2 and P4, all significantly affect the lifespan of *C. elegans* upon exposure. This indicates that *C. elegans* exhibiting a significantly higher level of food aversion upon exposure to a bacterial food source when compared to the standard, benign food source *E. coli* OP50, is an indication that the food source in question is pathogenic. This is similar behaviour to that previously described when populations when *C.*

elegans are exposed to certain strains of pathogenic bacteria (Zhang *et al.*, 2005; Pradel *et al.*, 2007; Gaglia *et al.*, 2012). As the strains K5, P2 and P4 all exhibited significantly increased killing of *C. elegans* and exhibited increased food aversion relative to OP50 (4.3.1, 4.3.2), they are both pathogenic and aversive with regards to a *C. elegans* host. This leads to these strains being classed as aversive pathogens, which they will be referred to as in the rest of this thesis.

As mentioned above, food aversion elicited upon exposure to bacterial strains is very specific. Not all of the bacteria which were identified to be pathogenic also elicit a food aversion response. There are a range of host pathways stimulated upon bacterial infection, and avoidance a lawn of pathogenic bacteria is only one of these (1.5,1.6, 1.7) (Ewbank, 2006). In addition, some of these pathogenic strains may not be producing a chemical cue, which acts to drive the food aversion response through neuronal signalling in the aversive strains. It has been discussed previously how a mutant *S. marcescens* strain which does not make a natural product (the serrawettin W2) does not provoke an aversion response seen upon exposure to the wild type strain, but is still equally pathogenic (Pradel *et al.*, 2007). It is possible that the pathogenic strains in the screen in this chapter which do not elicit avoidance may be deficient in a similar avoidance-stimulating product. In addition, the relative aversion elicited by *C. elegans* upon infection with other pathogenic bacteria such as *S. aureus* and *S. enterica* has not been examined. It is plausible that whilst these bacteria have been demonstrated to kill *C. elegans,* populations of animals do not exhibit and aversive response like that reported upon exposure to PA14 and *S. marcescens* (and in this chapter, *K. pneumoniae*). (Aballay *et al.*, 2000; Garsin *et al.*, 2001; Pujol, 2001; Zhang *et al.*, 2005; Pradel *et al.*, 2007; Gaglia *et al.*, 2012). This acts to further corroborate the idea that the production and detection of certain chemical cues is important for stimulating an aversive response in *C. elegans.* Alternatively, the enhanced aversion that *C. elegans* populations exhibit upon exposure to these specific bacterial strains may be a survival strategy executed by the animals to lessen the pathogenic effect harmful bacterial strains may exert by spending less time on the lawns of the particular bacterial strain, thus preventing further bacterial exposure.

4.4.4 Comparison of food aversion with bacterial pathogenicity

In order to determine whether food aversion could be used as a direct predictor of pathogenicity a comparison was made between the killing time and level of food aversion at 48 hours for each of the bacterial strains investigated Figure 4.11. Interestingly, no clear correlation is seen, with the highest R^2 value (0.38), and the only significant slope value being found for the *P. aeruginosa* strains (P=0.0013)(Figure 4.11C). The linear regression for both the *K. pneumoniae* and the *A. baumannii* strains re resulted in lower R^2 values and nonsignificant slopes (Figure 4.11A,B). This indicates that food aversion does not directly correlate with the pathogenicity of bacteria However, as is the case for K5, P2 and P4, high levels of food aversion can be representative of bacterial pathogenicity, indicating that this behavioural phenomenon can be used to identified a subset of *C. elegans* pathogens.

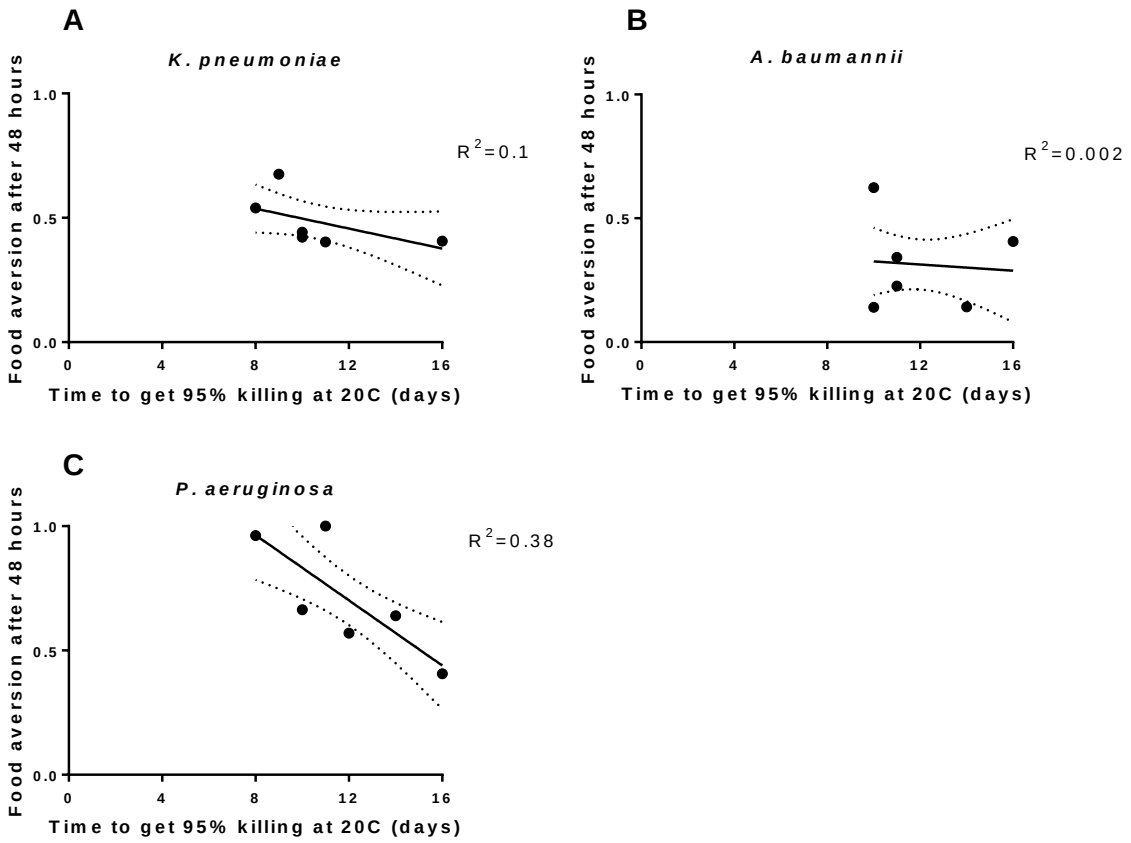


Figure 4.11: Correlation of *C. elegans* 95% killing time against food aversion after 48 hours of bacterial exposure. These data are taken from Figure 4.1, Table 4.2 and Figure 4.3 resulting from exposure of young adult *C. elegans* to strains of *K. pneumoniae* (A), *A. baumannii* (B) and *P. aeruginosa* strains (C), with the control of *E. coli* OP50 also being included in each analysis. Data analysed by linear regression.

Data in this chapter has revealed that not all of the bacteria which were identified to be pathogenic also elicit a food aversion response. There are a range of host pathways stimulated upon bacterial infection, and avoidance a lawn of pathogenic bacteria is only one of these (1.5,1.6, 1.7) (Ewbank, 2006). In addition, some of these pathogenic strains may not be producing a chemical cue, which acts to drive the food aversion response through neuronal signalling in the aversive strains. It has been discussed previously how a mutant *S. marcescens* strain which does not make a natural product (the serrawettin W2) does not provoke an aversion response seen upon exposure to the wild type strain, but is still equally pathogenic (Pradel *et al.*, 2007). It is possible that the pathogenic strains in the screen in this chapter which do not elicit avoidance may be deficient in a similar avoidance-stimulating product. In addition, the relative aversion elicited by *C. elegans* upon infection with other pathogenic bacteria such as *S. aureus* and *S. enterica* has not been examined. It is plausible that whilst these bacteria have been demonstrated to kill *C. elegans,* populations of animals do not exhibit and aversive response like that reported upon exposure to PA14 and *S. marcescens* (and in this chapter, *K. pneumoniae*). (Aballay *et al.*, 2000; Garsin *et al.*, 2001; Pujol, 2001; Zhang *et al.*, 2005; Pradel *et al.*, 2007; Gaglia *et al.*, 2012). This acts to further corroborate the idea that the production and detection of certain chemical cues is important for stimulating an aversive response in *C. elegans.*

4.4.5 Comparison of pathogenicity and food aversion results with *P. aeruginosa* PA14

In this chapter three bacterial strains were identified as having both significantly higher food aversion and significantly faster *C. elegans* killing time than *E. coli* OP50. These strains are the *K. pneumoniae* strain K5 and the *P. aeruginosa* strains P2 and P4. Whilst these could be putatively described as aversive pathogenic strains it was important to compare all of these strains to the well-defined *P. aeruginosa* strain PA14. PA14 has been found to be a conserved pathogen for nematodes, mice and plants (Rahme *et al.*, 1995; Tan *et al.*, 1999a). Since being identified as a *C. elegans* pathogen PA14 has been used as a 'gold standard' for studying *C. elegans* pathogenesis. This comes from extensive analaysis of this particular strain, with PA14 having been used to identify virulence factors controlling infection, host factors involved in the immune response and neural controls of the food aversion behaviour

which it elicits in *C. elegans* (Mahajan-Miklos *et al.*, 1999; Tan *et al.*, 1999b; Zhang *et al.*, 2005; Lee *et al.*, 2006; Feinbaum *et al.*, 2012; Gaglia *et al.*, 2012; McEwan *et al.*, 2012; Zhang and Zhang, 2012; Cohen and Troemel, 2015; Kirienko *et al.*, 2015; Zhu *et al.*, 2015a; Clamens *et al.*, 2017).When PA14 was examined in both food aversion and killing assays both a significant increase in food aversion and significantly quicker *C. elegans* killing time were observed mirroring the similar effects in the strains classified as aversive pathogens mentioned above (4.3.3,4.4.3). These results using *P. aeruginosa* PA14 as a potential food source in the assays developed for examining other clinical strains of *P. aeruginosa*, in addition to strains of *K. pneumoniae* and *A. baumannii*, demonstrate that *P. aeruginosa* is both highly pathogenic and aversive towards *C. elegans*, similar to results of previous studies (Zhang *et al.*, 2005; Gaglia *et al.*, 2012). Even though PA14 killed *C. elegans* quicker than the other three strains, the fact that all four of these bacterial strains reduced *C. elegans* lifespan and generated a food aversion response allows the strains K5, P2 and P4 to still be classified as aversive pathogens, as determined in 4.4.3.

These results all demonstrate that a simple food aversion assay can be used to identify aversive pathogens. Snapshots of food aversion based on the proportion of adult *C. elegans* off the food lawn over 48 hours have revealed not only the well described food aversion phenotype of *P. aeruginosa* PA14 but has also identified this in a range of newly identified pathogenic strains, two *P. aeruginosa* strains (P2 and P4) and one *K. pneumoniae* strain (K5). It is also worthy of note that strain P1 is *P. aeruginosa* strain PA01, a widely studied strain that was initially a wound isolate (Table 4.1)(Gallagher and Manoil, 2001). Like PA14, the pathogenesis of PA01 on *C. elegans* has also been investigated (Tan *et al.*, 1999a; Gallagher and Manoil, 2001; Lee *et al.*, 2006; Mikkelsen *et al.*, 2011). As part of these studies, it has been determined that PA14 is more virulent than PA01 in *C. elegans* in addition to other models, with genetic analysis identifying key genetic components controlling this enhanced virulence (Rahme *et al.*, 1995; Lee *et al.*, 2006; Mikkelsen *et al.*, 2011). It is another advantage of the screening approach taken here that this reported difference is mimicked with P1 exposed animals having a longer lifespan than PA14 infected animals.

4.4.6 C*. elegans* compared to *Galleria mellonella* as an infection model

The strains analysed in this chapter have been analysed in a *Galleria mellonella* virulence assays, with the results being summarised in Table 4.4 (1.3, 4.1) (Peleg *et al.*, 2009; Wand *et al.*, 2012; Wand *et al.*, 2013). In these assays *G. mellonella* caterpillars are injected with set inoculations of bacteria, and the subsequent pathogenicity of the different strains is measured by melanisation of the animals. The fact that these strains have been analysed in both *C. elegans* and *G. mellonella* allows a direct comparison between two distinct invertebrate infection models. Performing this analysis revealed that the 15 strains had a range of virulence in the *G. mellonella* model with 3 *K. pneumoniae*, 3 *A. baumannii* and all 5 *P. aeruginosa* strains showing pathogenic effects in the moth larvae. These data have been compared to the *C. elegans* data in this chapter (4.3.1, Table 4.4).

Strain	Virulence score in the *Galleria mellonella* model	Virulence in *C. elegans*
K1	_	+
K2	_	+
K3	++	+
K4	+++	++
K5	+++	++
A1	+/-	+
A2	-	+
A3	++	+
A4	+++	+
A5	+++	+
P1	+++	+++
P2	+++	+++
P3	+++	+++
P4	+++	+++
P5	+++	+

Table 4.4: The relative virulence of K*. pneumoniae, A. baumannii* and *P. aeruginosa* strains in *G. mellonella* and *C. elegans. C. elegans* virulence was determined from Figure 4.1 (+= non-significant reduction of lifespan, ++=significant reduction in lifespan (P≤0.05), +++= significant reduction in lifespan (P≤0.01)).

All the *P. aeruginosa* strains kill the moth larvae to a similarly rapid degree as defined by melanisation (Table 4.4)(Benthall *et al.*, 2015). However in the assays with *C. elegans*, a wider range of virulence as defined by the number of days of bacterial exposure is needed to kill the animals, is observed (4.3.1). This is in part due to the length of the killing assays described here, the lifespans of *C. elegans* exposed to the *P. aeruginosa* strains vary in length from 8 to 14 days at 20°C, and 7 to 10 days at 25°C. The range of results obtained with *C. elegans* are based on a long term exposure, and are thus representative of the chronic infections that *K. pneumoniae*, *A. baumannii* and *P. aeruginosa* cause in the clinic (Podschun and Ullmann, 1998; Perez *et al.*, 2007; Breidenstein *et al.*, 2011). However, bacterial pathogenesis is examined in *G. mellonella* on injection assays which are generally examined after 24 hours (Evans and Rozen, 2012; Loh *et al.*, 2013; Utari and Quax, 2013). These differences between to the models demonstrate the ability of *C. elegans* to act as a

model host for opportunistic bacterial pathogens. These results also demonstrate how *C. elegans* can be used to differentiate levels of virulence between different bacterial strains similar to studies performed on *G. mellonella* (Peleg *et al.*, 2009; Wand *et al.*, 2012; Wand *et al.*, 2013; Benthall *et al.*, 2015). Comparing the results gained with the different models demonstrates that whilst both *G. mellonella* and *C. elegans* can be used to identify bacterial pathogens from a selection of different strains, *G. mellonella* is good for rapid detection of pathogens, whereas *C. elegans* is better for identifying different degrees of virulence between different strains. Comparing the results gained in both invertebrate model hosts reveals that *P. aeruginosa* is the species which is most pathogenic in both models, demonstrating that diverse biological models can be used to gain corroborating results with bacterial strains.

4.4.7 Exposure to pathogenic bacteria does not aFffect *C. elegans* reproduction, larval development or feeding

As all the worms used in the assays to investigate the bacterial strain were L4+1s, the earliest stage at which *C. elegans* can reproduce, exposure to the bacterial food sources would lead to both laying of eggs and the subsequent production of progeny. It has previously been discussed in this thesis the effect that *C. elegans* larvae can have on adult behaviour (3.3.3). Food availability, and exposure to environmental or external toxic insults, including strains of pathogenic bacteria, have been shown to affect *C. elegans* egg laying and subsequent progeny production after (Schafer, 2005; Kumar *et al.*, 2010; Beceiro *et al.*, 2014; Fenk and de Bono, 2015; Kamaladevi and Balamurugan, 2015).

Analysis of *C. elegans* reproductive outputs (egg laying and larvae production development) demonstrated that exposure to none of the collection of *K. pneumoniae*, *A. baumannii* and *P. aeruginosa* strains was sufficient to cause a reduction in the numbers of *C. elegans* eggs or larvae produced by adult animals (4.3.4) when compared to the standard laboratory food source of *E. coli* OP50.

Analysis of the hatching efficiency of the eggs, measured by percentage hatching demonstrated that at least 80% of all eggs laid after 24 hours had hatched into viable larvae 24 hours later. These results indicate that whilst some of these bacterial strains have already been identified as pathogenic towards *C. elegans,* exposure of adult animals to lawns of the

bacteria at standard *C. elegans* maintenance temperature (20°C) is not enough to trigger a reproductive defect (4.3.4). Even though there are some cases where up to a 20% discrepancy between eggs and larvae numbers are observed, the fact that no eggs were observed 48 hours after initial exposure of the adults to the bacterial lawns suggests that these eggs did indeed hatch and either the larvae were nonviable or crawled off the side of the plate, meaning the larvae in this case where not visible to be counted.

C. elegans larvae show developmental defects and even arrest upon experiencing stressful environments or drug treatment (Donohoe *et al.*, 2006; Karmacharya *et al.*, 2009). The analysis of larval development presented in this chapter demonstrates that growing up on lawns of strains of *K. pneumoniae*, *A. baumannii* or *P. aeruginosa* does not affect the development of *C. elegans* larvae relative to OP50.

The data discussed above suggest that whilst members of the panel of Gram negative bacteria screened in *C. elegans* are clearly pathogenic they do not markedly effect reproduction or development of larval *C. elegans*. This demonstrates that these bacterial strains do not show an acute toxicity effect in the *C. elegans* model over a 48 hour period, and thus any differences that may occur upon an extended exposure to do not manifest themselves over a similar time course in which high levels of food aversion are exhibited (4.3.2, 4.4.3). These enhanced food aversion phenotypes, present themselves after 6 hours, are established after 24 hours and are clearly expressed after 48 hours (Figure 4.3). From the measurements taken here it is clear that the food aversion measurements are the earliest indicators of infection with pathogenic bacteria that have been examined in this chapter.

Examining the consumption of the bacteria by *C. elegans* is also not a reliable indicator of bacterial pathogenicity. When young adult *C. elegans* are on a lawn of bacteria they will consistently pump at around 240-250 times per minute(Avery and You, 2012). The rate of pharyngeal pumping has been used to quantify food intake and estimating how much bacteria is being ingested by the animals (Avery, 1993). Analysis of pharyngeal pumping rate reveals that in the case of exposure to all of the bacterial strains but PA14 for 48 hours (4.3.6), *C. elegans* feeding rate on the lawns of bacteria is not significantly modulated. Thus *C. elegans* are still actively consuming these bacteria even though some of these bacteria are pathogenic as determined by the impact on lifespan (4.3.24.4.1). This also further reinforces the point made earlier (4.4.3) that the food lawns are not significantly depleted in

the 48 hours in which the assay runs, to generate an aversive response caused by food depletion (Milward *et al.*, 2011). The fact that PA14 is the only bacterial strain which elicits a significant reduction in pharyngeal pumping relative to OP50 is not surprising as it is the most pathogenic of all the strains of all three species (4.3.3). Pharyngeal pumping has previously been demonstrated to reduce as *C. elegans* infected with pathogenic bacteria progress towards death (Tan *et al.*, 1999a; Garsin *et al.*, 2001). PA14 affecting pharyngeal pumping within a relatively short exposure demonstrates the enhanced pathogenicity of this strain compared to the other strains analysed in this chapter.

However, it is important to take into account the time limitations of the experiments. The food aversion measurements discussed earlier (4.4.3) all develop over the 48 hours for which the assay runs, demonstrating that any pathogenic effects of the bacterial infection obviously take time to develop. This is also demonstrated by the fact that no noticeable killing effect is observed in the killing assays until after 3 days. It is very likely that in the case of the other measures discussed in this section related to both reproduction and progeny, significant alterations may be manifested upon prolonged exposure to the bacterial strains. It has previously been demonstrated how bacterial infection of *C. elegans* with *S. enterica* leads to apoptosis of germ cells, and infection of *C. elegans* in liquid with *K. pneumoniae* reduces the number of eggs produced (Aballay and Ausubel, 2001; Kamaladevi and Balamurugan, 2015). These studies indicate that a continued bacterial infection may result in a striking reproductive phenotype. As *C. elegans* continues to consume bacteria they will become 'sicker' as a result of any toxins being secreted by the bacteria, as well as by any possible bacterial proliferation occurring in the gut. This 'sickness' may lead to any upregulated immune response to infection, which may manifest in more striking differences being observed in the range of *K. pneumoniae*, *A. baumannii* or *P. aeruginosa* strains.

4.4.8 Summary

This chapter has described how a selection of Gram-negative bacteria that are pathogenic towards *C. elegans* as indicated by reduced lifespan also elicit a food aversion behavioural effect based on measuring the proportion of adult animals off a lawn of bacteria, subsequently being classed as aversive pathogens. This is similar to the aversive learning

towards lawns of pathogenic bacteria previously described (1.7). There is one *K. pneumoniae* strain (K5) and two *P. aeruginosa* strains (P2 and P4) which fit this criteria. However, in addition to the results gained with these strains, a wide spectrum of results was seen with respect to both pathogenicity and food aversion. Exposing *C. elegans* to five strains each of three Gram-negative ESKAPE species, *K. pneumoniae*, *A. baumannii* and *P. aeruginosa* demonstrate the range of results that can be obtained from screening multiple bacterial strains in the *C. elegans* model rather than focusing on a single strain of bacteria for more detailed study of the pathogenesis (Tan *et al.*, 1999b; Kurz *et al.*, 2003; Feinbaum *et al.*, 2012) . In this chapter it has been observed that *C. elegans* populations can respond very differently to different bacterial strains including those of the same species. This is similar to other studies where *C. elegans* responses to bacteria from natural environments, and as potential food sources were measured which resulted in a broad range of responses being observed (Avery and Shtonda, 2003; Samuel *et al.*, 2016).Whilst the results in this chapter lead to certain avenues of further investigation it has been demonstrated that a simple food aversion assay utilising *C. elegans* can generate a range of responses which can be representative of the pathogenicity of bacteria

Chapter 5: Mechanistic analysis of *C. elegans* infection with ESKAPE bacteria

5.1 Introduction

In this project one objective was to ascertain what aspects of the strains of ESKAPE bacteria mediate the pathogenesis of *C. elegans*(1.11). Examining the bacteria in the interaction helps to define mechanistic basis of pathogenicity that is expressed by a reduced time to kill *C. elegans*. Specific factors could either be determinants of pathogenicity in individual strains or genetic determinants that are shared across a range of pathogenic bacterial strains. *C. elegans*, along with other model host organisms, has been used to determine a range of virulence factors which help to mediate the pathogenesis of a range of bacterial species (1.5.2). This chapter focuses on how the different bacterial strains utilised in this thesis (2.7.8, 4.2) interact with the *C. elegans* intestine, specifically in terms of colonisation and clearance, to begin to identify potential mediators of virulence of these strains. Utilising a comparative approach, as undertaken in this thesis, allows for differences in these particular interactions to be identified, thus providing potential for differences in pathogenesis to be resolved.

5.1.1 Bacterial Colonisation of *C. elegans*

Both bacterial pathogens as well as commensals colonise host organisms (Mathur et al., 2012; Zhang et al., 2015c; Berg et al., 2016; Dirksen et al., 2016). The ability of bacteria to invade specific tissues of mice has long been established, with individual organs like the spleen and liver analysed for bacterial colonisation (Cossart and Sansonetti, 2004; Mathur et al., 2012). With regards to invertebrate models, previous studies utilising *G. mellonella* have demonstrated that bacterial strains which are more virulent have an increased presence inside the haemocoel of the organism (Wand et al., 2011; Wand et al., 2013).

The ability of different species of bacteria to colonise the *C. elegans* intestine in bacterial infections has been analysed (1.5.1.1). Bacteria which are not broken down by the grinder of the pharynx upon ingestion by *C. elegans* can subsequently proceed to colonise the intestine and exert deleterious effects on the nematode host (Engelmann and Pujol, 2010). In some studies analysing the bacterial pathogenesis of *C. elegans*, it has been found that knocking out certain genes in the bacteria reduces their ability to colonise the *C. elegans* gut. This is associated with a reduction in the virulence of the bacteria (Begun et al., 2007;

Kuo et al., 2016). These studies suggest that the number of bacteria which are found in the *C. elegans* intestine shows strong correlation with bacterial virulence.

However, the recent demonstration of the microbiome of the *C. elegans* intestine has cast doubt on whether the assumption that increased bacterial colonisation directly relates to pathogenicity is true (1.5). These initial microbiome studies demonstrated that C. elegans can be colonised by a broad spectrum of bacteria which are not pathogenic (Berg et al., 2016; Dirksen et al., 2016). In addition, the assembled microbiome, which consists of many different genera of bacteria, provides health benefits to C. elegans (Berg et al., 2016; Dirksen et al., 2016). These benefits include protection against fungal and bacterial pathogens and increases the ability of the animals to reproduce (Dirksen et al., 2016). These benefits are similar to the health benefits that the human microbiome provides to the host organism(Bull and Plummer, 2014). Chapter 4 demonstrated the range of pathogenicity in the different *K. pneumoniae, A. baumannii* and *P. aeruginosa* strains. Examining the ability of the different strains to colonise the *C. elegans* intestine may act to give insight about controls of pathogenicity in *C. elegans*.

5.1.2 Clearance of bacteria from *C. elegans* tissues

The ability of an infected host to remove bacteria that may have colonised tissues is important when dealing with a bacterial infection. Removing bacteria from any colonised tissues allows the host to recover, with antibiotic treatment acting to speed up this process (Moy *et al.,* 2006). As mentioned in 1.5.1.1.1, different bacterial pathogens which colonise the *C. elegans* intestine exhibit different levels of persistence. As described in 1.6, an important component of the *C. elegans* innate immune response is effector molecules such as antimicrobial peptides which act to remove bacteria from the intestine upon infection. In addition, some bacterial *C. elegans* pathogens exhibit different levels of persistence in the intestine (1.5.1.1.1). In this chapter, the ability of *C. elegans* to remove the range of *P. aeruginosa* strains was examined. This allows not only investigation of how the ability of *C. elegans* to remove *P. aeruginosa* corresponds with the relative virulence of the same strains towards the nematode, but gives more insight into the complex intestinal dynamics of *C. elegans*.

5.1.3 Genetic modulation of the *C. elegans* intestinal environment

It has been demonstrated that *C. elegans* mutants with diminished neurosecretory activity, are resistant to infection with *P. aeruginosa* PA14 and also exhibit increased levels clearance of this strain (Kawli and Tan, 2008). These mutants support a role for increased clearance of bacteria is a process associated with resistance to bacterial infection (Kawli and Tan, 2008). Among these mutants were those that are fundamental to neurosecretion, including *unc-31* animals. The *C. elegans unc-31* gene encodes the single *C. elegans* orthologue of the mammalian CAPS protein. CAPS (Ca^2+ dependent activator protein for secretion) are required for dense core vesicle mediated exocytosis (DCVs). DCVs package monoamines and neuropeptides which modulate both pre and post synaptic function by activating G-protein coupled receptors (Speese *et al.*, 2007; Lin *et al.*, 2010). In this chapter *unc-31 (e928)* animals were analysed for their ability to clear a range of *P. aeruginosa* strains in order to compare to the altered immune function of the animals previously described (Kawli and Tan, 2008).

A subset of the cargo of DCVS is neuropeptides. Subsequently the role of neuropeptides in controlling bacterial proliferation was also examined in this chapter. In *C. elegans*, neuropeptides regulate neuronal signalling that underpins locomotion, feeding, reproduction and responses to infection (1.8.3.2). In mammalian systems neuropeptides are central to the gut-brain axis described in 1.8.5 (Holzer and Farzi, 2014). In addition, neuropeptides are also an important part of the mammalian innate immune system and perform key functions in response to infection (Sternberg, 2006). To investigate the role of neuropeptides in controlling bacterial clearance in *C. elegans*, *egl-3 (ok979)* mutants, deficient in neuropeptide maturation, were examined (Kass *et al.*, 2001; Mitchell *et al.*, 2010).

5.2 Aims and objectives

In this chapter an investigation in colonisation of bacteria initially analysed in Chapter 4, by specifically examining bacteria found inside *C. elegans* post infection. Further, the ability of *C. elegans* to subsequently remove bacteria from the intestine after the bacteria has been consumed was also measured. In addition, *C. elegans* mutants deficient in aspects of neuronal signalling where examined for bacterial colonisation and clearance. This allows the molecular controls of bacterial colonisation and clearance to begin to be examined. Undertaking the comparative approach as in Chapter 4, where a range of different bacterial strains can be analysed at once, allows comparisons between different bacterial strains to be made based on their interactions with the *C. elegans* intestine.

5.3 Results

5.3.1 Cell numbers in bacterial lawns used for *C. elegans* assays

Before any measurements of bacteria inside *C. elegans* were measured, the numbers of bacteria in cultures used to make the lawns in all the experiments in Chapter 4 was measured. Viable cell counts of 50µl of bacterial cultures with an OD_{600} of 0.8 were performed on all strains of *K. pneumoniae, A. baumannii* and *P. aeruginosa* analysed in chapter 4. This provided a measure of the potential input of bacteria into *C. elegans* which could then lead to intestinal colonisation. This revealed that all of the bacterial strains had between $2x10^7$ and $6x10^7$ cells in these 50µl cultures (Figure 5.1). In comparison OP50 had ~$4x10^7$ cells in 50µl of liquid culture. There were six bacterial strains out of the 16 in total examined which had significantly lower viable cell counts than OP50. These strains were *K. pneumoniae* strains K1 (Figure 5.1A), the *A. baumannii* strains A1, A3 and A4 (Figure 5.1B) and the *P. aeruginosa* strains P2 (Figure 5.1C). In addition, the *P. aeruginosa* strain P1 had a significantly higher viable cell count than OP50, and this was the only strain to exhibit this.

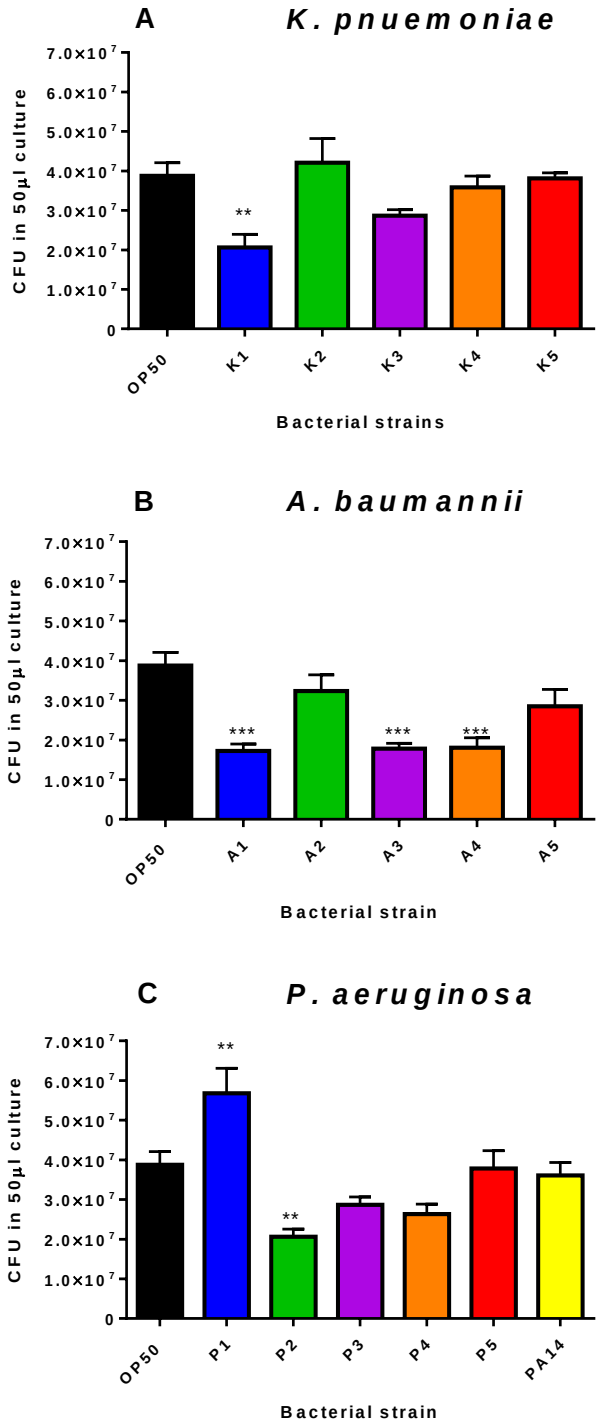


Figure 5.1: Viable cell counts of culture of *K. pneumoniae*, *A. baumannii* and *P. aeruginosa* strains. The number of cells present in 50µl of an OD_{600} = 0.8 culture of strains of *Klebsiella pneumoniae* (A) *Acinetobacter baumannii* (B) and *Pseudomonas aeruginosa* (C). n≥9 for all strains. Analysis by one-way ANOVA with- Dunnett's multiple comparisons to OP50. Error bars represent ±SEM. *P<0.05, **P<0.01, ***P<0.001 and ****P<0.0001.

5.3.2 Colonisation of *C. elegans* by the range of bacterial strains

Previous studies have speculated that bacteria which are pathogenic towards *C. elegans* exhibited increased colonisation (Begun *et al.*, 2007; Portal-Celhay *et al.*, 2012; Kuo *et al.*, 2016). To this end *C. elegans* populations were exposed to lawns of the bacterial strains for 48 hours and were subsequently lysed to estimate the number of bacteria inside the intestine and compared to the levels of OP50 found in control populations, similar to other protocols (2.3.2). This exposure time was chosen so the level of colonisation could be examined when the range of 'preclinical measures' were measured in chapter 4. The colonisation levels of the individual strains of *K. pneumoniae, A. baumannii* and *P. aeruginosa* demonstrated that no individual strain of these bacteria colonised *C. elegans* significantly more than *E. coli* OP50 (Figure 5.2A,B,C). The colonisation levels of the different bacteria varied between OP50 having 10^4 CFUs per animal and A1 and P1 having around $5x10^5$ CFUs per animal. This indicates that a range of different bacterial strains colonise *C. elegans* to similar levels. Grouping all strains from one species together showed that *A. baumannii* and *P. aeruginosa* colonised *C. elegans* intestines more than *K. pneumoniae* (Figure 5.2D).

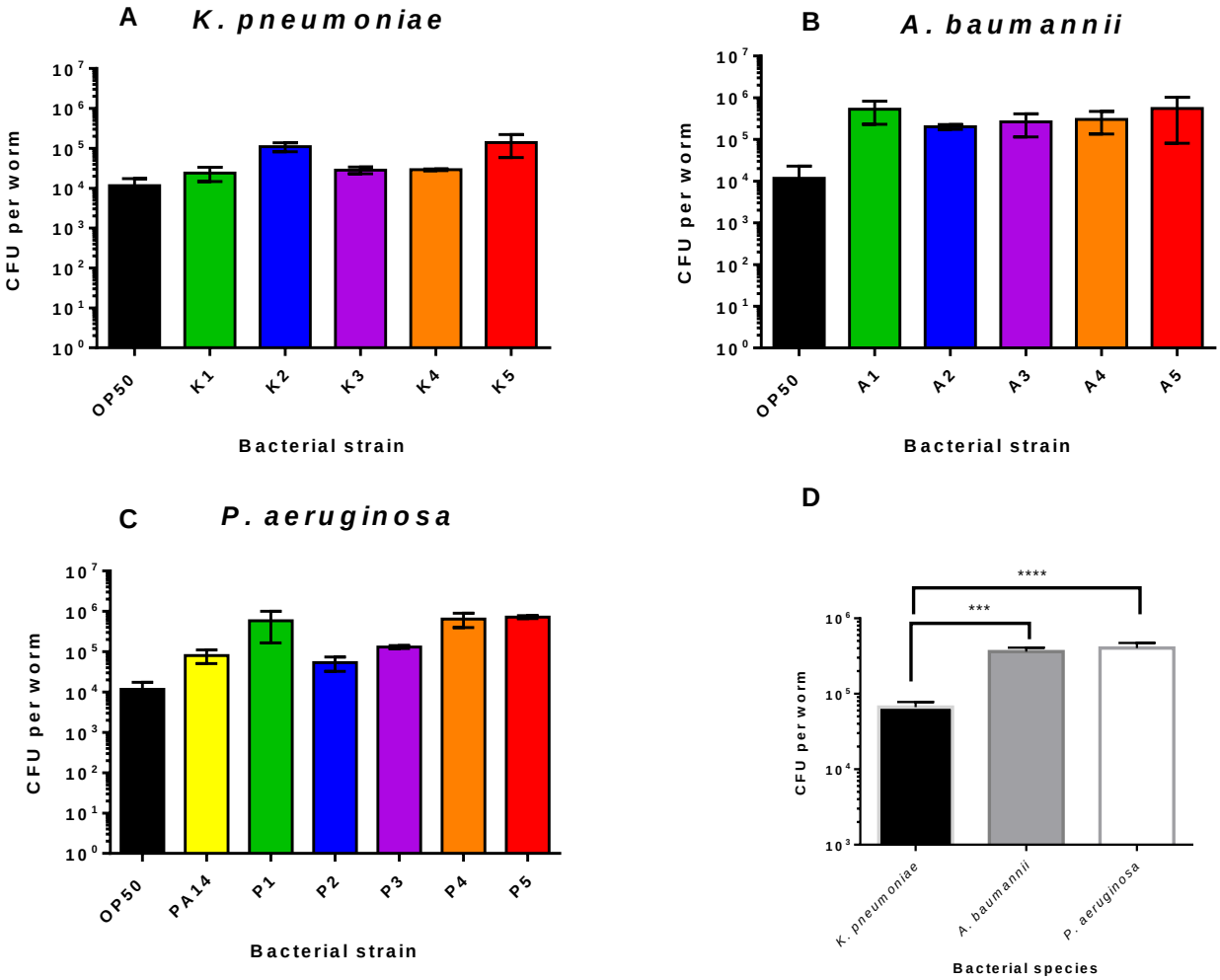


Figure 5.2: Colonisation levels of *C. elegans* after 48 hours of exposure to lawns of Gram-negative ESKAPE bacteria. Numbers of bacteria were isolated from *C. elegans* exposed to strains of *K. pneumoniae* (A), *A. baumannii* (B) and *P. aeruginosa* (C) All strains from the same species were subsequently grouped together and the average colonisation for each species was analysed (D) n≥3 independent experiments for all strains, with between 5 and 10 individual animals lysed per individual repeat. Data analysed by one-way ANOVA with Tukey's multiple comparison tests to animals fed OP50. Error bars represent ±SEM. *P<0.05, **P<0.01, ***P<0.001 and ****P<0.0001.

5.3.3 Colonisation correlation with earlier measures

The colonisation levels of the strains of *K. pneumoniae, A. baumannii* and *P. aeruginosa* in the C*. elegans* intestine were correlated against the measures of pathogenicity and behavioural results described in chapter 4 (4.3.1,4.3.2). A linear regression analysis comparing the CFU per animal against both *C. elegans* food aversion at 48 hours and killing time was carried out (Figure 5.3). This resulted in an R^2 value of 0.030 for colonisation (Figure 5.3A) against food aversion and 0.047 for colonisation against killing time (Figure 5.3B). In

addition, the P value for both linear regression analyses indicated that there was no correlation between bacterial colonisation and food aversion or killing time. These data provide evidence that the assessment of colonisation levels of *C. elegans* by *K. pneumoniae*, *A. baumannii* and *P. aeruginosa* do not underpin pathogenicity of the different bacterial strains (4.3.1).

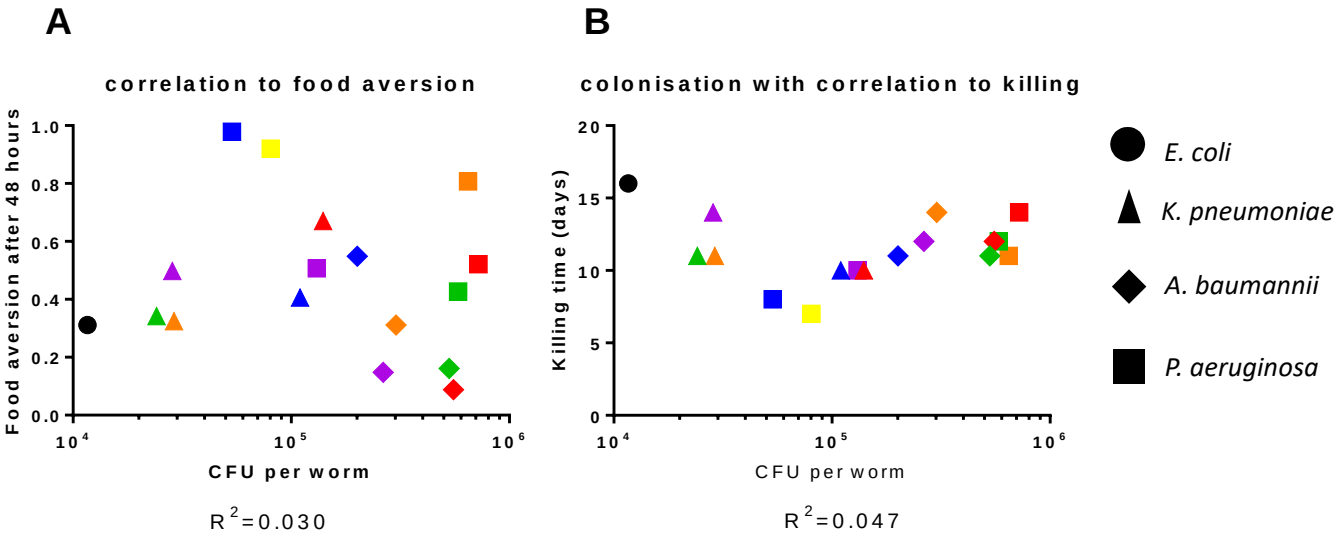
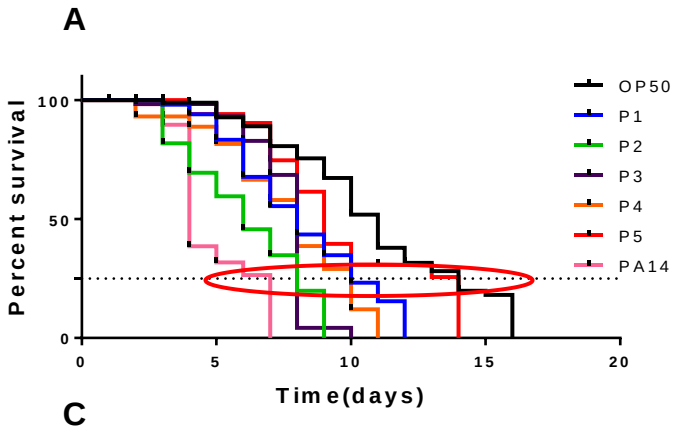


Figure 5.3: Correlation of bacterial colonisation of *C. elegans* with food aversion at 48 hours (A) and with killing time (B). Colour coding is the same as in Figure 5.2. Data analysed by linear regression analysis.

5.3.4 Bacterial colonisation of *C. elegans* near death with *P. aeruginosa*

The next investigation was concerned with examining the colonisation of *C. elegans* with bacteria as they approached death. Progressing through the killing assays presented in chapter 4 may result in a proliferation in the amount of bacteria present in the *C. elegans* intestine, and this could be associated with succumbing to infection. As discussed in chapter 4, the time to kill *C. elegans* varied for the range of bacterial strains. To address whether bacterial colonisation was altered with prolonged bacterial exposure animals were analysed for bacterial colonisation that had been exposed to the six *P. aeruginosa* strains and *E. coli* OP50 until the point of the killing assays representative of 75% death survival curve (4.3.1,Figure 5.4A). The times chosen for colonisation were as follows: 5 days for PA14, 7 days for P2, 8 days for P3, 10 days for P4, 11 days for P1 and 13 days for both OP50

and P5 (Figure 5.4B). There was no increase in the number of bacteria inside *C. elegans* relative to the colonisation levels observed after a 48 hour exposure upon extended incubation (Figure 5.4C).Despite a wide range in the amount of time the animals were exposed, all strains showed colonisation levels of between 10^5 and $7x10^5$ bacteria per worm (Figure 5.4D). These results strongly suggests that the level of bacteria in the *C. elegans* intestine is not a key determinant of bacterial pathogenesis in *C. elegans.*



B

Bacterial strain	Time of exposure to reach 75% death (days)
E. coli OP50	13
P. aeruginosa P1	11
P. aeruginosa P2	7
P. aeruginosa P3	8
P. aeruginosa P4	10
P. aeruginosa P5	13
P. aeruginosa PA14	5

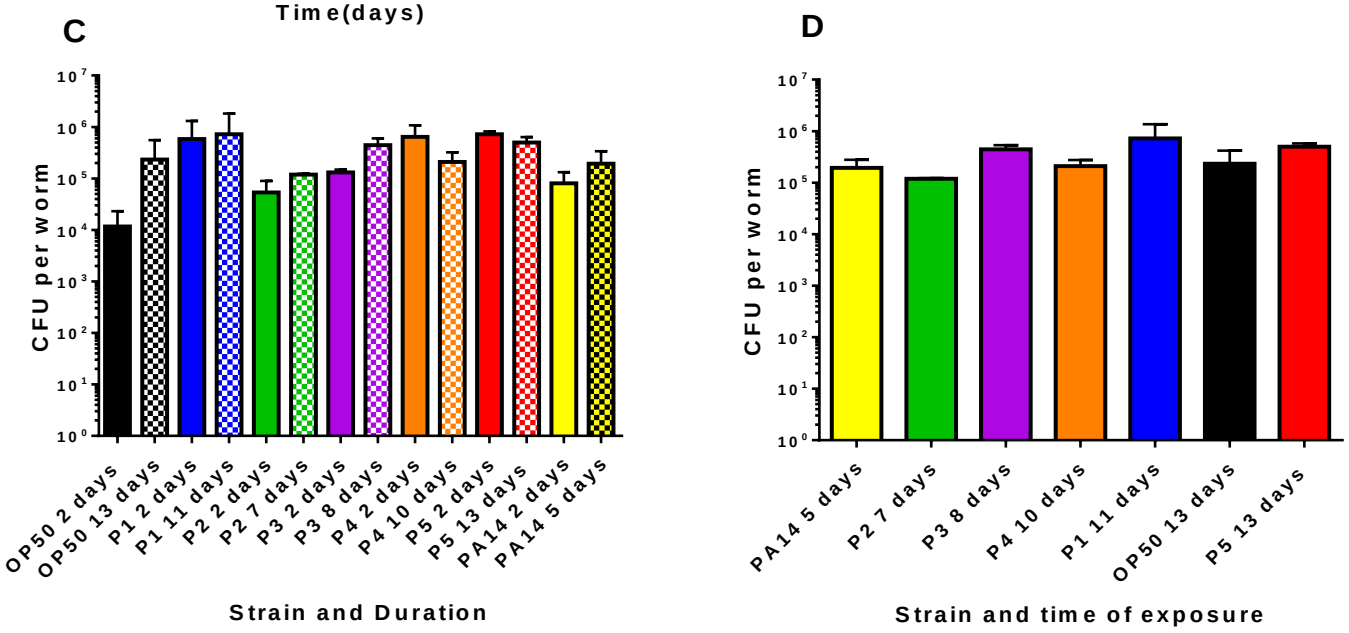


Figure 5.4: *P. aeruginosa* colonisation of *C. elegans* after an extended exposure to bacterial lawns. Lifespan of *C. elegans* exposed to all six *P. aeruginosa* strains along with OP50, adapted from Figure 4.1 (A). This allowed determination of the number of days to which exposure to each strain was required for 75% killing (25% survival) was required (B). *P. aeruginosa* CFUs per animal were compared to the colonisation levels of the respective strains (bar of matching colour) after 48 hours (hatched fill) and after an incubation consistent with a 75% death (filled bar) (C). CFUs per worms of animals that were '75% dead' in sequential order (D). All data representative of n=3 repeats, with between 5 and 10 individual C. elegans being analysed for each individual treatment. Error bars represent ±SEM. Data analysed for C and D by one-way ANOVA with Sidak's multiple comparisons.

5.3.5 Clearance of *P. aeruginosa* from the *C. elegans* intestine

Colonisation of the *C. elegans* intestine reflects ingested bacteria which remain intact and viable by resisting lysis from the pharyngeal grinder. Species that can proliferate in the intestine post colonisation will remain whereas other bacteria will be able to be removed from the intestine by expulsion through defecation or an immune response. The ability of *C. elegans* to clear bacteria from the intestine was measured. In order to examine this the number of CFUs of *P. aeruginosa* were analysed after 24 hours of exposure of *C. elegans* to bacterial lawns (24 hours column Figure 5.5A), after 24 hours on *P.aeruginosa* lawns followed by 24 hours on *E. coli* OP50 (Transfer column Figure 5.5A). Comparison of these raw CFU values with levels determined after 48 hours of bacterial exposure (Figure 5.2/48 hour column Figure 5.5A) showed that there were no significant reductions in *P. aeruginosa* CFU numbers after the transfer to OP50 (Figure 5.5A). The 24 hours and transfer values were subsequently used to calculate a level of bacterial clearance from the *C. elegans* intestine (2.3.3) based on the ratio of the bacterial numbers present before and after the transfer to OP50.Examining clearance of the different *P. aeruginosa* strains revealed a broad range of clearance levels of *P. aeruginosa* strains from the *C. elegans* intestine, but statistical comparison revealed there was no significant differences in these clearance levels (Figure 5.5B).

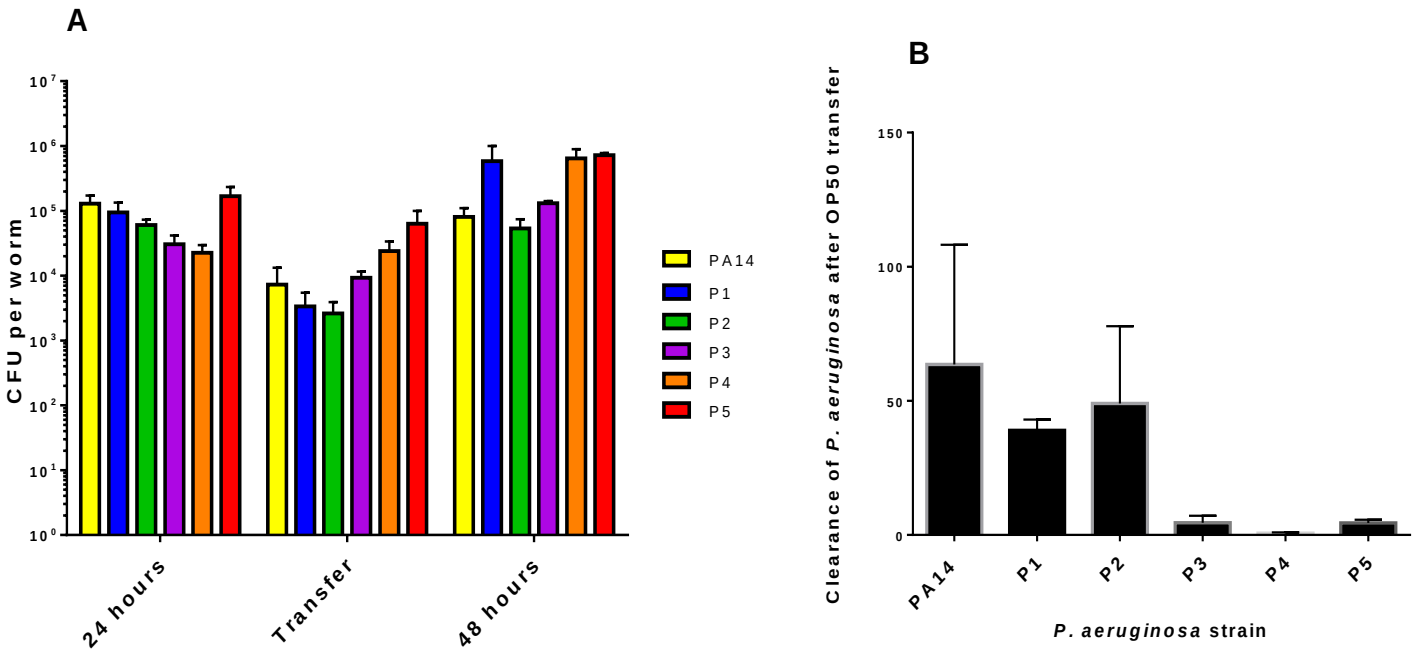


Figure 5.5: The relative clearance of *P. aeruginosa* strains from the *C. elegans* intestine. Colonisation was measured after 24 hours of exposure to lawns of *P. aeruginosa* (24 hour column), 24 hours of exposure to lawns of *P. aeruginosa* followed by 24 hours on lawns of *E. coli* OP50 (Transfer column) and 48 hours on *P. aeruginosa* using data from Figure 5.2 (48 hour column). (A) Following analysis of colonisation, clearance of *P. aeruginosa* from the intestines of *C. elegans* was analysed by calculating the fold reduction in *P. aeruginosa* CFUs present after the transfer step relative to the number present after 24 hours of incubation(B). N=3 experiments with 5-10 *C. elegans* being lysed for each individual repeat, error bars represent ±SEM. Data analysed by two-way ANOVA with Dunnett's multiple comparison test to N2 animals.

5.3.6 *P. aeruginosa* clearance is enhanced in neuropeptide deficient *C. elegans*

C. elegans mutants deficient in aspects of neuronal signalling have been demonstrated to show enhanced clearance of *P. aeruginosa* PA14 (Kawli and Tan, 2008). To examine how conserved this upregulation in clearance was, colonisation and clearance of the different *P. aeruginosa* strains was performed as in 5.3.5 in *unc-31 (e928)* and *egl-3 (ok979)* mutant *C. elegans*. Analysing the *P. aeruginosa* colonisation levels of the different *C. elegans* strains revealed that there was no significant alterations in the numbers of bacteria in the intestines of *unc-31(e928)* or *egl-3 (ok979)* animals relative to N2 after 24 hours of bacterial exposure or after the subsequent transfer to OP50 (Figure 5.6,). Analysis of *P. aeruginosa* CFU numbers after 48 hours in the mutant *C. elegans* strains revealed that both *egl-3* and *unc-31* animals had reduced colonisation levels after incubation with both P4 and P5 strains (Figure 5.6 E,F).

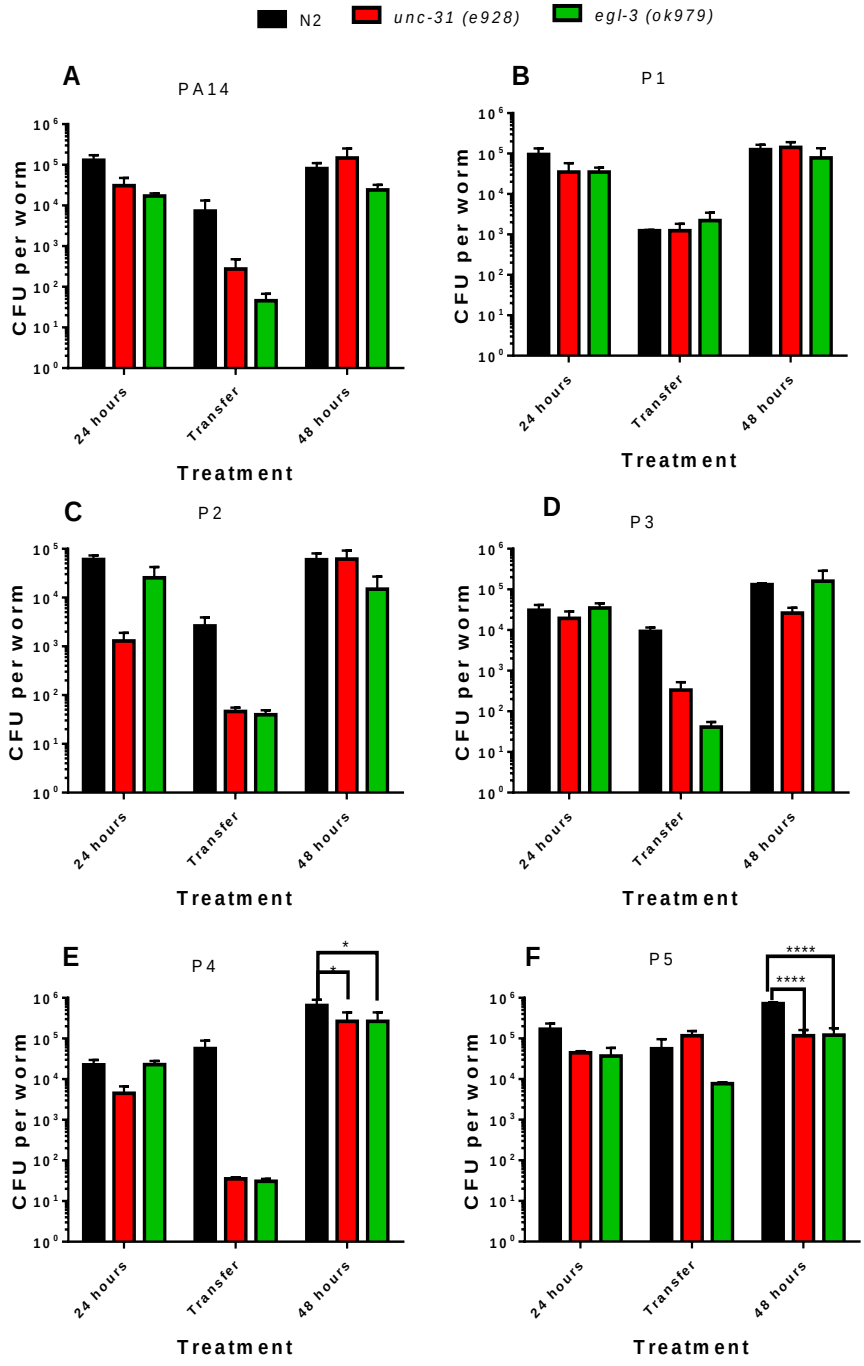


Figure 5.6: Colonisation of *unc-31* and *egl-3 C. elegans* by *P. aeruginosa*. Colonisation was measured for both wild type , *unc-31 (e928)* and *egl-3 (ok979) C. elegans* after 24 hours of exposure to lawns o *P. aeruginosa (*24 hour column*)*, 24 hours of exposure to lawns of *P. aeruginosa* followed by 24 hours on lawns of *E. coli* OP50 (Transfer column) and 48 hours on *P. aeruginosa* (48 hour column*).* Wild type and indicated mutant *C. elegans* were incubated on the indicated strains. N=3 experiments for all strains, with between 5-10 individual animals being lysed in each individual experiment . Error bars represent ±SEM. Data analysed by two-way ANOVA with Dunnett's multiple comparisons to colonisation levels in N2 animals. *P<0.05, **P<0.01, ***P<0.001 and ****P<0.0001.

Subsequently, as in 5.3.5, the relative clearance levels were calculated using the data from Figure 5.6. Examining clearance levels of *unc-31 (e928)* animals revealed that they cleared PA14 more effectively than N2 animals, as previously reported (Figure 5.7A) (Kawli and Tan, 2008). However, the clearance of the other *P. aeruginosa* strains in *unc-31* mutants was not different from N2 animals (Figure 5.7A).

Analysis of bacterial clearance from *egl-3 (ok979)* animals demonstrated that these mutants cleared four *P. aeruginosa* strains more effectively than N2 animals (Figure 5.7B). The only *P. aeruginosa* strains which were not cleared more readily in *egl-3 (ok979) C. elegans* relative to N2 animals were strains P1 and P5 (Figure 5.7). These results suggest that neurosecretory signalling, in particular that neuropeptides, act to suppress clearance of bacteria in the wild type *C. elegans* intestine.

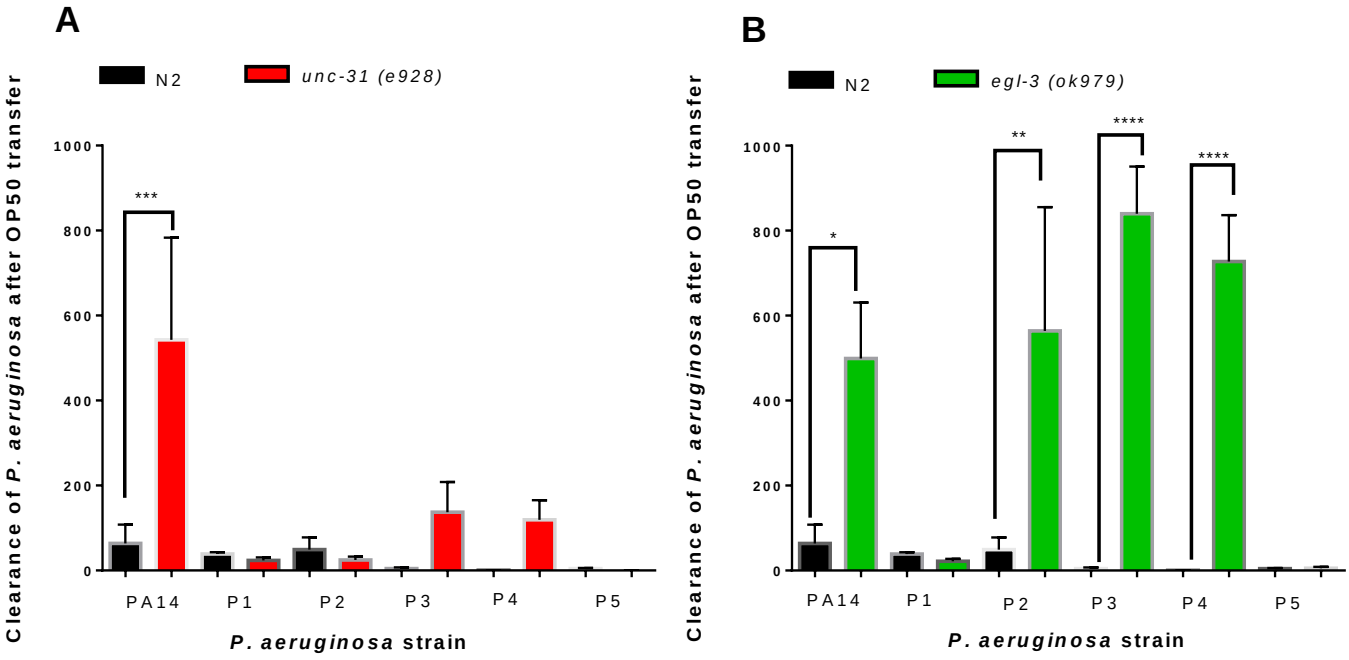


Figure 5.7: Clearance of *P. aeruginosa* by *unc-31* and *egl-3 C. elegans*. Following analysis of colonisation (Figure 5.6), clearance of *P. aeruginosa* from the intestines of N2, *unc-31(e928)*(A) and *egl-3(ok979)* (B) *C. elegans* was analysed by calculating the fold reduction in P. aeruginosa CFUs after 24 hours on lawns of *E. coli* following 24 hours on lawns of *P. aeruginosa* N=3 individual repeats for all strains. Error bars represent ±SEM. Data analysed by one-way ANOVA with Tukey's multiple comparison tests to N2 animals. *P<0.05, **P<0.01, ***P<0.001 and ****P<0.0001.

5.4 Discussion

5.4.1 Viable cell numbers in bacterial cultures

Performing a viable cell count analysis on the liquid cultures used to make the bacterial lawns revealed that there was variation in the number of viable cells in these lawns (5.3.1). This demonstrates that optical density does not equate to the number of cells in the liquid cultures (5.3.1). This variation in cell density is probably due to extracellular material that is produced when the bacteria are growing in liquid culture. It is likely that the bacteria are producing mucus and extracellular polysaccharide which contributes to the optical density of the cultures. This variation in cell numbers could affect bacterial input into the *C. elegans* system through uptake of bacterial cells. It has previously been reported that as an OP50 lawn is depleted *C. elegans* show an increased tendency to leave the lawn possibly in search of a new food source (Milward *et al.*, 2011). However, data from chapter 4 demonstrates that *C. elegans* actively consumes all of these bacteria even after an extended incubation (4.3.6). Also the number of cells in the bacterial lawn is not likely to act as a determinant of the food aversion behaviour observed in *C. elegans*, as previous data in this thesis has shown that *C. elegans* tend to remain on dilute OP50 lawns (3.3.2).

5.4.2 Colonisation of the *C. elegans* intestine

Colonisation and subsequent proliferation of bacteria inside host organisms has previously been described as a key determinant of the mechanism of bacterial pathogenesis (Garsin *et al.*, 2001; Sifri *et al.*, 2003; Begun *et al.*, 2007). Pathogenic bacteria that avoid lysis by the grinder of the pharynx intact can colonise the post pharyngeal digestive tract and establish infection (1.5.1.1). Upon analysing the bacterial colonisation levels of *C. elegans* which have been exposed to bacterial lawns for 48 hours, in order to corroborate with the analysis of food aversion undertaken in chapter 4 (4.3.2), it became clear the different bacterial strains were equivalent in their colonisation (5.3.2). Comparing the colonisation levels with food aversion and killing times showed no significant correlation (5.3.3). This contrasts with claims in the literature that an increased presence of bacteria in the *C. elegans* intestine underpins relative pathogenicity of different bacteria. However, in the context of subsequent studies, the claims in these studies appear to be an over interpretation. Recent

studies have implicated both the presence and benefits of the *C. elegans* microbiome (1.4.2), whereas previous to the microbiome investigations it was presumed that *C. elegans* intestines were generally sterile with any bacteria being present causing an infectious insult. In addition, the studies by both Begun *et al.*(2007) and Kuo *et al*. (2016) implicate genes that are expressed on cell surfaces, intercellular adhesin in *S. epidermidis* and LPS genes in *E. coli* respectively, in modulating both pathogenicity and colonisation levels. These suggest that adhesion of bacteria to the surface of the *C. elegans* intestine is important to establish colonisation in the case of these bacteria. Disrupting this leads to bacteria not attaching to the *C. elegans* intestinal surface as readily and therefore not able to establish an infection as readily. This subsequently leads to a reduction in virulence, indicating that these cell surface genes are important regulators of the pathogenicity of bacteria. However, it has been determined through studies examining virulence factors in *C. elegans* bacterial pathogenesis that there are other classes of genes which act to mediate *C. elegans* infection, for example secreted toxins (1.5.2.3). Presumably the absence of these different categories of these genes would not affect colonisation of the *C. elegans* intestine but would affect virulence. The colonisation results obtained in 5.3.2 add to this hypothesis, with bacterial strains with varying virulence having similar levels of colonisation.

5.4.2.1 Certain species are better equipped to *C. elegans* colonisation

Whilst the previous section discusses how virulence of different bacterial strains is not related in their ability to colonise the *C. elegans* intestine, there is evidence that the ability of different bacteria to colonise *C. elegans* is species rather than strain dependent. The *C. elegans* gut is more permissible to *A. baumannii* or *P. aeruginosa* colonisation than *K. pneumoniae* (5.3.2). This could be related to *A. baumannii* and *P. aeruginosa* more efficiently avoid lysis by the *C. elegans* pharynx. Alternatively, certain species may more readily adhere to the *C. elegans* gut (Kawli and Tan, 2008; Lin *et al.*, 2010). The environmental conditions of the *C. elegans* intestine are tightly controlled through the homeostasis of the animals (Chauhan et al., 2013). As an example of this the *C. elegans* intestine has a well-defined pH range, which may make the microenvironment of the intestine unsuitable for colonisation by certain bacteria (Chauhan *et al.*, 2013). In addition, the nature of some of the strains analysed in this thesis, being clinical isolates, may mean

they are adapted to certain specific niches, which could affect the ability of the bacteria to attach to the *C. elegans* intestinal surface. As mentioned above (5.4.2), it has previously been demonstrated that genes expressed on the surface of bacterial cells are important for colonisation of the *C. elegans* intestine (Begun *et al.,*2007, Kuo *et al.,*2016). Certain strains that lack such genes will not be able to colonise the intestine as readily as other strains which do.

The recent analysis of the *C. elegans* microbiome further suggests selective colonisation from bacteria. This is exemplified by recent observations that animals exposed to bacteria from a diverse range of soils and assemble very similar microbiotas (Berg *et al.*, 2016; Dirksen *et al.*, 2016). These studies revealed that Enterobacteriaceae are particularly suited for proliferation in *C. elegans* (Berg *et al.*, 2016; Dirksen *et al.*, 2016; Samuel *et al.*, 2016). Interestingly, the *Klebsiella* genus belongs in the Enterobacteriaceae family but the lack of any identification of individual species in the *C. elegans* microbiome demonstrates that *Klebsiella* species may be among Enterobacteriaceae which do not colonise the *C. elegans* gut as well (Berg *et al.*, 2016; Dirksen *et al.*, 2016).

5.4.2.2 Bacteria do not proliferate in the *C. elegans* intestine animals approach death

Previous studies have described how numbers of bacteria in the intestine increase as an infection proceeds to death in *C. elegans* (Tan *et al.*, 1999a; Sifri *et al.*, 2003; Portal-Celhay *et al.*, 2012). This bacterial proliferation allows the bacteria to cause more cellular damage to the nematode intestine, and more of the *C. elegans* energy resources will be needed to combat the infection. However, analysis of *P. aeruginosa* CFUs in *C. elegans* approaching death revealed that there was no significant increase in the amount of bacteria present in the intestine compared to the numbers found after a 24 hour exposure (5.3.4). This suggests that the range of virulence seen in the *P. aeruginosa* strains described in the previous chapter is not coupled with a change in bacterial proliferation, based on the numbers of bacteria found in the intestine. This indicates that *C. elegans* which succumb to a bacterial infection are not dying as a result of a high level of *P. aeruginosa* proliferation in the intestine after extended bacterial exposure.

5.4.2.3 Virulence factors are likely to be the key modulators of *C. elegans* bacterial pathogenesis

The results discussed above raise the issue of the determining factors of bacterial pathogenicity. This is likely to be as a result of secretion of toxins or the production of other bacterial virulence factors (1.5.2). When *C. elegans* are fed a 1000:1 ratio of non-pathogenic *E. faecium* to pathogenic *E. faecalis,* this small inoculum of *E. faecalis* is still able to proliferate in the *C. elegans* intestine and acts to kill the animals (Garsin *et al.*, 2001). This is a good indication that the virulence determinants of pathogenic bacteria are more important than numbers of bacteria alone in controlling pathogenesis. In the case of the *P. aeruginosa* infections examined here, it is likely the nature of the bacteria, specifically with relation to the virulence factors that they possess, which acts to drive the different levels of pathogenicity based on killing time in chapter 4. The differences in virulence between PAO1 and PA14, including in *C. elegans* virulence have previously been examined (Lee *et al.*, 2006; Mikkelsen *et al.*, 2011). These studies revealed that PA14 possesses a number of virulence factors, which increase the pathogenicity of PA14 relative to PA01. Data in this chapter demonstrates that PAO1 (strain P1) and PA14 colonise *C. elegans* to a similar degree (5.3.2), demonstrating the important contribution of these virulence factors (Lee *et al.*, 2006; Mikkelsen *et al.*, 2011). In addition, studies have identified other aspects of bacterial infections, which contribute to virulence which are coupled with an intestinal colonisation. These include death of *C. elegans* germ cells upon *S. enterica* infection, anal deformation and lysis of intestinal cells by *S. aureus* infection, and intracellular invasion and abnormal autophagy in *P. aeruginosa* infection (Aballay and Ausubel, 2001; Irazoqui *et al.*, 2010a). These different symptoms might all be occurring in the time course that bacterial colonisation has been measured in this thesis, but measuring the numbers of bacteria present in the intestine alone does not allow measurement of death of germ cells, lysis of intestinal cells or autophagy. Analysis of individual *C. elegans* tissues for these other signs of bacterial infections will allow for examination of whether any of these symptoms are undertaken in the infections examined in this thesis.

5.4.3 Clearance from the *C. elegans* intestine

The results discussed in 5.4.2 demonstrate varying virulence of the *K. pneumoniae, A. baumannii* and *P. aeruginosa* in *C. elegans* cannot be determined by the number of bacteria in the intestinal tract alone. Removal of bacteria either through excretion or antimicrobial treatment is a key component of recovering from infections (Moy *et al.,* 2006). Like mammals, *C. elegans* treated with antibiotics can recover from bacterial infections (Moy *et al.*, 2006). The ability of different bacterial strains to remain in the *C. elegans* intestine by avoiding the innate immune response may be a determinant of pathogenicity. It has been demonstrated how different *C. elegans* bacterial pathogens have different levels of persistence within the intestine (Sifri *et al.*, 2005). Alternatively, immune function may be upregulated in response to pathogenic bacteria. The ability of *C. elegans* to clear pathogenic bacteria from their intestines can be measured by exposure to a pathogen followed by a transfer to a lawn of the non-pathogenic laboratory standard food source *E. coli* OP50 (Kawli and Tan, 2008). This transfer to OP50 allows for clearance of bacterial cells by the immune response of *C. elegans*. Any bacteria that remain after this clearance can then continue to reside as commensals or continue to establish further infection in the case of pathogens. Analysis of bacterial clearance of *P. aeruginosa* strains in N2 wild type *C. elegans* revealed that there was no significant alteration in the level of bacterial clearance of the different *P. aeruginosa* strains (5.3.5). However, the large degree of variability, possibly due to biological variability, which is present in the data presented in Figure 5.5 makes statistical significance difficult to deduce. This leads to the conclusion that the ability of *C. elegans* to clear bacteria from the intestine is not indicative of the relative pathogenicity of the bacteria.

5.4.4 Analysis of intestinal dynamics in *C. elegans* neurosecretory mutants

Previous studies have identified a role for neurosecretory pathways acting to modulated clearance of bacterial pathogens from the *C. elegans* intestine (Kawli and Tan, 2008). In addition, antimicrobial peptides are important components of the *C. elegans* innate immune system (1.6). Signalling from the intestine from neuropeptides, along with serotonin, have also been demonstrated to contribute to the behavioural avoidance of pathogens (Melo and Ruvkun, 2012; Chen *et al.*, 2013). To address the contribution of neurosecretory mutants

were analysed for both colonisation by and clearance of *P. aeruginosa* (5.3.6)(Kass *et al.*, 2001; Speese *et al.*, 2007).

Analysing the bacterial colonisation levels of *unc-31 (e928)* and *egl-3 (ok979) C. elegans* revealed that these mutants were deficient in colonisation of two *P. aeruginosa* strains after 48 hours but no deficiencies in colonisation were observed during the 24 hour or transfer treatments (5.3.6).

Analysis of the bacterial clearance levels of both *unc-31* and *egl-3* mutants revealed enhanced levels of clearance of *P. aeruginosa* (5.3.6). *unc-31* animals exhibited enhanced of PA14 more readily than wild type animals, in agreement with previous studies, whereas *egl-3* animals cleared four strains significantly more than wild type *C. elegans* (Kawli and Tan, 2008). This demonstrates that even though *unc-31* animals removed PA14 more than wild-type animals, this is not sustained across the other pathogenic strains, suggesting that this *unc-31* dependent modulation of bacterial clearance previously demonstrated is not a universal response to pathogen exposure (Kawli and Tan, 2008). This is likely to reflect the relative ability of the different bacteria to combat the *C. elegans* immune response, rather than as a direct indicator of general bacterial pathogenicity. This again points to genetic determinants of pathogenicity being crucial, as mentioned in 5.4.2.3.

Comparison of the clearance levels of *unc-31* and *egl-3* mutant *C. elegans* reveals that neuropeptide signalling has a bigger impact on bacterial clearance than DCV signalling mediated by *unc-3*1, building in previous studies (Kawli and Tan, 2008). All four *P. aeruginosa* strains which were removed more in *egl-3 C. elegans*, were pathogenic, as measured by the killing assays undertaken in Chapter 4 (4.3.1). The two strains in which this enhanced clearance was not seen, P1 and P5, were two strains in which *C. elegans* lifespan was weakly reduced and not significantly reduced relative to OP50 respectively (Chapter 4). As *egl-3* animals are deficient in aspects of neuropeptide maturation this indicates a suppression of bacterial clearance of pathogenic strains in wild type *C. elegans* by neuropeptides (Kass *et al.*, 2001; Husson *et al.*, 2006). In addition, this neuropeptide dependent regulation has a broader effect than DCV mediated exocytosis does (Speese *et al.*, 2007; Kawli and Tan, 2008).

5.4.4.1 Neuropeptides are involved in the suppression of the removal of bacteria

Neuropeptides are critical modulators of neuronal functions in mammals gut hormones (Holzer and Farzi, 2014). Among these functions for neuropeptides is a role as gut hormones (Holzer and Farzi, 2014). The importance of neuropeptide mediators in the gut brain axis is well established and several important neuropeptide classes have *C. elegans* orthologues (Holzer and Farzi, 2014). Neuropeptides of *C. elegans*, particularly members of the *nlp* family have been demonstrated to elicit antibacterial activity (Couillault *et al.*, 2004; Li and Kim, 2008). However; the data obtained in 5.3.6 demonstrate that neuropeptides are involved in supressing the clearance of bacteria from the *C. elegans* intestine. This idea initially seems counterintuitive, due to the antimicrobial activity of certain neuropeptides. However, previous studies have determined that pathogenic bacteria can interact with *C. elegans* neuropeptides during an infection. It has been demonstrated that PA14 is able to supress the *C. elegans* immune response by activating the DAF-2 pathway (1.6.1.3) that increases expression of the insulin like peptide INS-7, which results in suppression of DAF-16 (Evans *et al.*, 2008). This demonstrates that in addition to the antimicrobial function that neuropeptides can elicit, they can also be exploited by pathogenic bacteria upon infection to down regulate the immune response. Also, whilst some neuropeptides demonstrate antibacterial activity (1.6.3.1), neuropeptides only make up a fraction of the effector proteins implicated in the *C. elegans* immune response (1.6.3). Other classes of effector proteins involved in the immune responses includes lysozymes or the caenopores (1.6.3.1) (Roeder *et al.,* 2010). These non-neuropeptide effector molecules will not be dependent on EGL-3 for their maturation (Alegado and Tan, 2008; Evans *et al.*, 2008; Roeder *et al.*, 2010). Such immune response genes have been found to be expressed to higher levels in neurosecretory mutants which display enhanced pathogen resistance and removal (Kawli and Tan, 2008). Expression of these particular kinds of immune effectors could be regulated by neuropeptides. A lack of this regulation could lead to upregulated immune function in the *egl-3* animals, leading to enhanced clearance of pathogenic bacteria. This indicates an up-regulated immune response due to lack of neuropeptide function. This enhanced level of immune activity is undoubtedly an advantage when it comes to dealing with bacterial infections. However, analysis of other neurosecretory mutants with enhanced bacterial removal has suggested that the other phenotypic effects that come with mutations, such as

locomotory and sensory deficits, are not beneficial to the general biology of *C. elegans* and will not confer a selective advantage outside the laboratory environment (Kawli and Tan, 2008; Mitchell *et al.*, 2010). As mentioned in Chapter 1 and investigated in Chapter 4, the recognition and subsequent avoidance of pathogenic bacteria is a key aspect of the *C. elegans* infection response, as it avoids colonisation by pathogenic bacteria in the first instance, thus removing the need for a high level of clearance.

5.4.4.2 Implications for the *C. elegans* microbiome

As suggested above, removal of neuropeptides could lead to an up-regulated *C. elegans* immune response, resulting in higher clearance of bacteria upon a pathogenic insult (5.4.4.1). However, it is important to note that upon exposure to the strains P1 and P5, a weakly pathogenic and non-pathogenic strain respectively (4.3.1), *egl-3* mutant *C. elegans* did not exhibit enhanced clearance towards these bacteria (5.3.6). This demonstrates that *C. elegans* can respond differently upon colonisation with different bacteria. It will be a waste of resources to expel non-pathogenic or beneficial bacteria from the intestine, (1.4.2) (Berg *et al.*, 2016; Dirksen *et al.*, 2016; Samuel *et al.*, 2016). This demonstrates that even in a mutant background in which the immune response is enhanced, such as *egl-3*, *C. elegans* can still alter intestinal function to keep non-harmful bacteria in the digestive tract. In this case, these benign bacteria will help to protect against any future challenges with pathogens (*Dirksen et al., 2016*). These findings from this chapter further builds on the initial studies examining the *C. elegans* microbiome and the health benefits it provides to the host organism.

5.4.4.3 Wider biological significance

In the clearance experiments undertaken with N2, *unc-31* and *egl-3* all demonstrate a range of clearance phenotypes upon incubation with different *P. aeruginosa* strains (5.3.5,5.3.6). When taken together these data demonstrate that a multi-faceted response occurs during bacterial gut colonisation. It is likely that there is dynamic balance occurring in the *C. elegans* intestinal environment between maintenance of a healthy, beneficial microbiota and removal of pathogenic bacteria. In the natural environment of *C. elegans,* animals are constantly moving between different food sources and selection and preference of different

bacteria can result in a biological advantage (Samuel *et al.*, 2016). This balance of the microbiome will be true in higher organisms as well, where retaining a healthy gut microbiome, whilst avoiding infection by pathogenic bacteria, will be essential for the health of the host organism. The evidence obtained here indicates that neuropeptides appear to be important in modulating the balance between maintaining a mutually beneficial, healthy microbiome and removing harmful bacteria.

5.4.5 Summary

In this chapter it has been found that the number of bacteria found inside *C. elegans* after an initial incubation does not relate to the relative virulence of the bacteria. In addition, the progression towards death of *C. elegans* is not accompanied by bacterial proliferation in the intestine, in contrast with previous claims made in the literature. These results all indicate that it is not the number of bacteria inside the nematode intestine that is important in terms of an infection, but rather the relative virulence of the bacteria that determines infection and subsequent death of *C. elegans* exposed to these bacteria. This opens the door to taking a similar approach that has previously been undertaken in other bacterial strains, where genetic determinants of virulence have been identified (Tan *et al.*, 1999b; Gallagher and Manoil, 2001; Kurz *et al.*, 2003; Tenor *et al.*, 2004; Lee *et al.*, 2006; Feinbaum *et al.*, 2012). Identification of *C. elegans* virulence factors, either those that are novel or those previously identified, are likely to be key determinants of modulating the relative pathogenicity of the collection of bacterial strains first analysed in Chapter 4.

In this chapter, it has also been demonstrated that the clearance of bacteria is differentially regulated in *C. elegans* depending on whether the bacteria is benign or pathogenic. Mutants deficient in aspects of neurohormonal signalling have enhanced bacterial clearance. This suggests that the wild type *C. elegans* gut is permissive to bacterial colonisation and maintenance of a bacterial population. The results with both the *unc-31* and *egl-3* mutants indicate that there is an aspect of neurohormonal regulation in the *C. elegans* intestinal environment which may act to suppress the immune response towards and clearance of bacteria.

Chapter 6: Investigating biogenic amine signalling controlling *C. elegans* food aversion in response to pathogenic bacteria

6.1 Introduction

Selective pathogenic strains of *K. pneumonaie* and *P. aeruginosa* were found in Chapter 4 to generate a food aversion response in *C. elegans* similar to that previously described in the literature (1.7).To investigate neural circuits in *C. elegans* that control this behavioural response (1.8.3.1), *C. elegans* deficient in biogenic amine signalling where examined. biogenic amines are key signalling molecules in *C. elegans*, and serotonin signalling has been demonstrated to control *C. elegans* food aversions towards *P. aeruginosa* PA14.

6.1.1 The Biogenic amines of *C. elegans*

The four signalling biogenic amines of *C. elegans*, serotonin, octopamine, dopamine and tyramine act to modulate *C. elegans* behaviour (Chase and Koelle, 2007). These neurotransmitters control locomotion, reproduction, feeding processes and olfaction in *C. elegans* (Chase and Koelle, 2007). Of the four biogenic amines of *C. elegans*, both serotonin and dopamine have neurotransmitter roles throughout the animal phyla including in the mammalian nervous system. In contrast octopamine and its precursor tyramine are more restricted to invertebrates (Roeder, 2005). They are classified as trace amines in the mammalian nervous system and do not have a major role in neurotransmission (Chase and Koelle, 2007; Khan and Nawaz, 2016). Octopamine and tyramine have been described as the invertebrate equivalents of the adrenergic system (Roeder, 2005). The individual biogenic amines are synthesised in distinct sets of cells in *C. elegans*, some of which are overlapping (Table 6.1)(Chase and Koelle, 2007).

The genetic tractability of *C. elegans* and simple nature of the biology of the organism means mutants can be easily obtained which are deficient in the biosynthetic enzymes needed to make biogenic amines. Examples of these enzymes are *cat-2* (tyrosine hydroxylase)*, tph-1*(tryptophan hydroxylase)*, tbh-1* (tyramine β-hydroxylase) and *tdc-1* (tyrosine decarboxylase), biosynthetic enzymes needed to make dopamine, serotonin, octopamine and tyramine respectively (Figure 6.1). Utilising these mutants means that the worms are deficient in the respective biogenic amines neurotransmitters for which the enzymes encode. This approach can be extended to investigation of the distinct classes of

receptors for each neurotransmitter, of which there are multiple examples for each molecule (Table 6.2). This genetic approach utilising mutant *C. elegans* is often supplemented by ablating individual neurons or other cells where the neurotransmitters are either made or where they act. This approach leads to a deeper understanding of the cellular interactions that allow distinct biogenic amine functions to be expressed (Chase and Koelle, 2007). This also gives a basis for the cellular circuits involved in controlling certain biogenic amine-dependent behaviours to be identified.

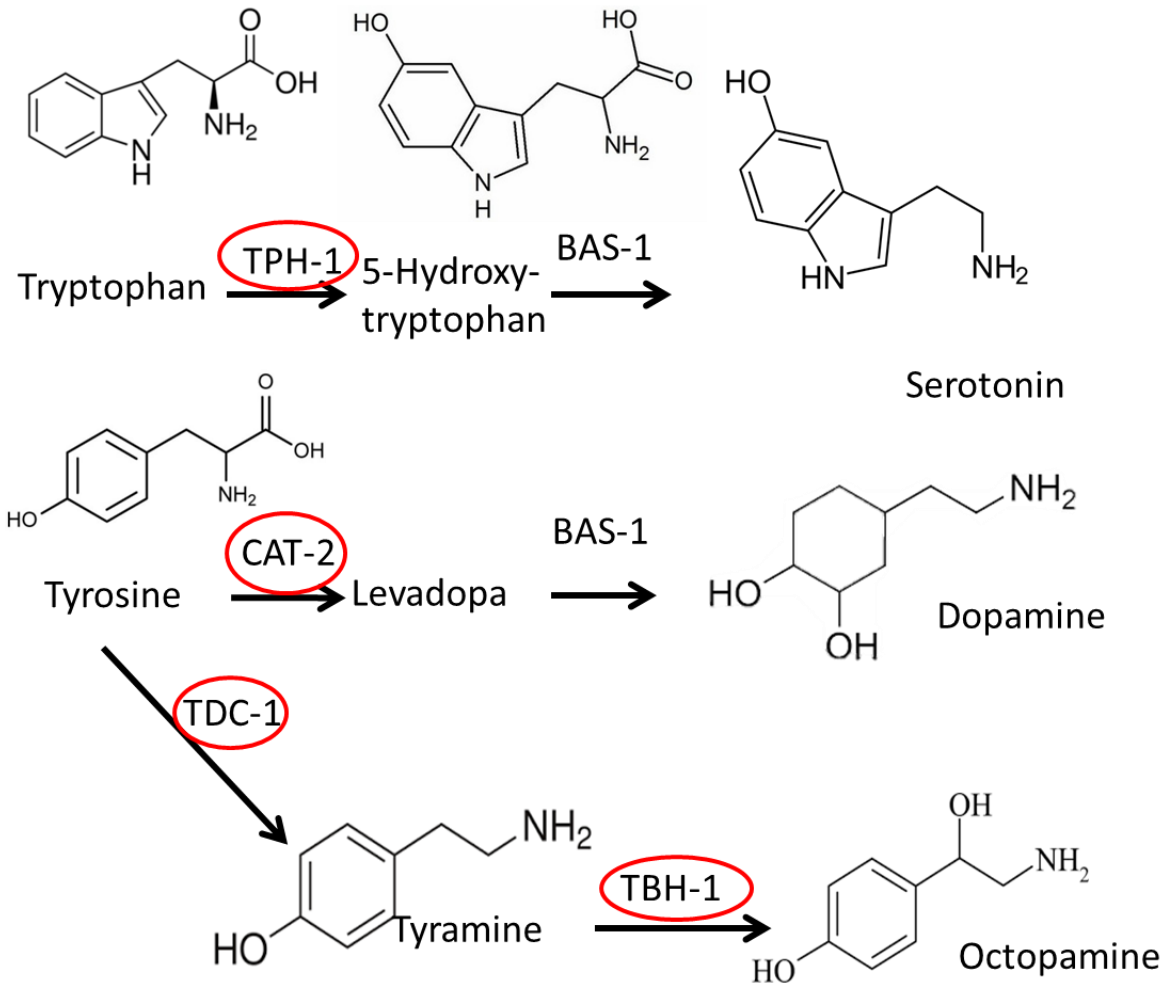


Figure 6.1: Biogenic amine neurotransmitters of *C. elegans* showing key biosynthetic enzymes utilised. The enzymes TPH-1, CAT-2, TDC-1 and TBH-1 are required for the synthesis of serotonin, dopamine, tyramine and octopamine respectively. Mutant *C. elegans* in defective in these enzymes were used in this study to investigate the role of biogenic amines in controlling *C. elegans* food aversion upon exposure to pathogenic bacteria.

Molecule	Neurons/ cells where molecule is synthesised
Dopamine	**ADE x 2 , PDE x 2, CEP x 4**
Serotonin	**NSM x 2, HSN x 2, VC4, VC5, ADF x 2, RIH, AIM x 2**
Octopamine	**RIC x2, *Gonad Sheath***
Tyramine	**RIC x2, *Gonad Sheath*, RIM x 2, *UV1***

Table 6.1: The neurons and other cells which synthesis the biogenic amines of *C. elegans.* Adapted from Chase and Koelle (2007). Figure 6.2 shows the location of these individual cells using the same colour scheme. Cells which are not neurons are italicised

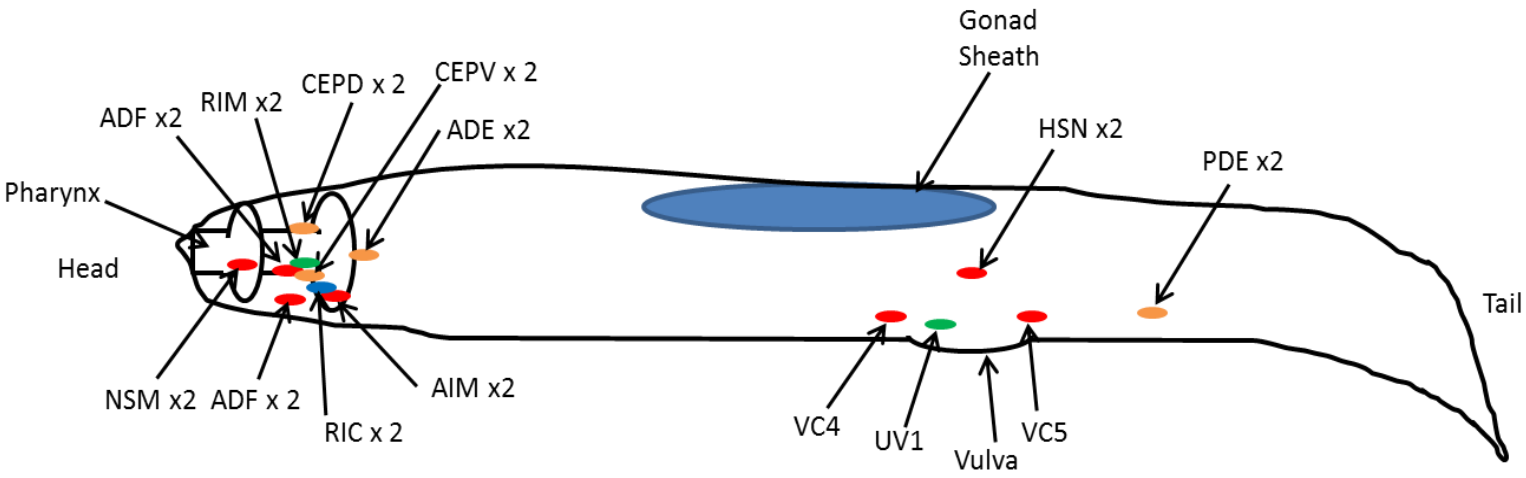


Figure 6.2: Pictorial representation of the cells which synthesise biogenic amines in *C. elegans* using the same colour coding as shown Table 6.1 and their location within *C. elegans*. All cells except the gonad sheath and UV1 are neurons.

	Gene	Receptor Type	Expression pattern	References
Dopamine Receptors	*dop-1*	7TM GPCR	Head neurons, head muscles, excretory gland cells, amphid and labial support cells, cholinergic ventral cord motor neurons, tail neurons	(Chase *et al.*, 2004) ,(Sanyal *et al.*, 2004)
	dop-2	7TM GPCR	Head and tail neurons	(Suo *et al.*, 2003),(Tsalik *et al.*, 2003)
	dop-3	7TM GPCR	ventral cord motor neurons	(Chase *et al.*, 2004)
	dop-4	7TM GPCR	Pharyngeal neurons, vulval, intestine and rectal cells	(Sugiura *et al.*, 2005)
Serotonin Receptors	*ser-1*	7TM GPCR	pharyngeal and vulval muscles, head, tail and ventral cord motor neurons, cells of the posterior intestine	(Tsalik *et al.*, 2003; Dempsey *et al.*, 2005),(Carnell *et al.*, 2005; Carre-Pierrat *et al.*, 2006),
	ser-4	7TM GPCR	pharyngeal, tail and head neurons	(Tsalik *et al.*, 2003),(Carre-Pierrat *et al.*, 2006)
	ser-7	7TM GPCR	Pharyngeal neurons and vulval muscles	(Hobson *et al.*, 2003; Hobson *et al.*, 2006)
	mod-1	ligand gated chloride channel	Head, ventral cord and tail neurons	(Ranganathan *et al.*, 2000)
Octopamine receptors	*octr-1*	7TM GPCR	head and tail neurons	(Wragg *et al.*, 2007)
	ser-3	7TM GPCR	Pharynx, Head and tail neurons, head muscles, phasmid sockets, nerve ring and intestine, spermatheca, eggs, gonad and vulva.	(Mills *et al.*, 2012b)
	ser-6	7TM GPCR	Head neurons, posterior Ventral Cord Neurons and intestine.	(Mills *et al.*, 2012b)
Tyramine receptors	*tyra-3*	7TM GPCR	Head and tail neurons and vulva	(Wragg *et al.*, 2007; Bendesky *et al.*, 2011)
	ser-2	7TM GPCR	Some pharyngeal cells and head muscles.	(Rex and Komuniecki, 2002)
	lgc-55	ligand gated chloride ion channel	Head, tail and body neurons, vulval cells	(Pirri *et al.*, 2009)

Table 6.2: The *C. elegans* biogenic amine receptors. Adapted from (Chase and Koelle, 2007)

The following section will describe the roles that the individual biogenic amines have in have been implicated in the biology of C. *elegans*. In particular roles which have been implicated specifically in regard to interacting with pathogenic bacteria are highlighted.

6.1.1.1 Dopamine

Dopamine is synthesised in mechanosensory neurons of *C. elegans* (Table 6.1,Figure 6.2)(Chase and Koelle, 2007). The essential role of dopamine in mechanosensory modalities is illustrated by disruption in behaviours such as the slowing in response to food and behavioural plasticity in the form of habituation to mechanical stimuli (Sawin *et al.*, 2000; Sanyal *et al.*, 2004; Chase and Koelle, 2007; Kindt *et al.*, 2007). Dopamine has also been found to be involved in the search for new food sources and has also been recently been determined to have a role in controlling body size (Hills *et al.*, 2004; Nagashima *et al.*, 2016) . The fact that dopamine is involved in habituation to mechanical stimuli in the form of plate tapping is an example of dopamine being involved in *C. elegans* plasticity. A further example of this is dopamine deficient animals having reduced adaptation towards odorants (Bettinger and McIntire, 2004).

6.1.1.2 Serotonin

Serotonin is synthesised in seven types of neurons in *C. elegans* including those in the ventral cord and the pharynx (Table 6.1, Figure 6.2)(Chase and Koelle, 2007). Serotonin acts to decrease the locomotion of starved *C. elegans* as they encounter a bacterial lawn (Sawin *et al.*, 2000). Serotonin also acts to keep *C. elegans* on a food lawn by promoting dwelling behaviour (Chase and Koelle, 2007; Flavell *et al.*, 2013). In a related behaviour, serotonin is needed to stimulate *C. elegans* feeding, and this signalling drives enhanced feeding of familiar bacterial food as determined through pharyngeal pumping (Avery and Horvitz, 1990; Song *et al.*, 2013).Serotonin is also involved in the heat shock response of *C. elegans,* (Tatum *et al.*, 2015). Intriguingly, serotonin has also been implicated in aversive learning behaviour of *C. elegans* towards pathogenic bacteria. It has previously been found that serotonin deficient animals have reduced levels of aversive learning towards *P. aeruginosa* PA14, and this effect is mirrored when exposed to otherwise non-pathogenic bacteria which

deactivated essential cellular processes by RNAi (Zhang *et al.*, 2005; Melo and Ruvkun, 2012).

6.1.1.3 Tyramine and Octopamine

Compared to the wide-ranging expression of dopamine and serotonin, octopamine and its precursor tyramine are expressed in just one class of neuron, the pair of the RIC interneurons in the head ring and also in cells of the gonad sheath (Alkema *et al.*, 2005; Chase and Koelle, 2007). In addition to these two cell types, tyramine is also made in the RIM motor neurons, and the UV1 cells, which are neuroendocrine cells which help to form the connection between the uterus and the vulva (Alkema *et al.*, 2005; Chase and Koelle, 2007; Gupta *et al.*, 2012)(Figure 6.2, Table 6.1) Despite being in the same biosynthetic pathway, octopamine and tyramine have distinct roles in controlling *C. elegans* behaviour, through acting through different receptors (Alkema *et al.*, 2005).

6.1.1.3.1 Tyramine

Analysis of *tdc-1* mutant *C. elegans* has revealed that tyramine imparts negative regulation of egg laying and positive modulation of both head oscillations in response to touch and spontaneous reversals in response to nose touch (Rex *et al.*, 2004; Alkema *et al.*, 2005; Chase and Koelle, 2007).

6.1.1.3.2 Octopamine

Octopamine negatively modulates reversal of *C. elegans* away from chemical deterrents, which has been proposed as an analogue for the signalling involved in modulation of chronic pain in mammals (Wragg *et al.*, 2007; Mills *et al.*, 2012a; Mills *et al.*, 2012b). It has also been found that under fasting conditions *C. elegans* octopamine levels are increased. Octopamine has also been implicated in controlling the *C. elegans* oxidative stress response (Suo *et al.*, 2009; Hoshikawa *et al.*, 2017). In addition, studies involving exogenous octopamine have identified physiological antagonism between octopamine and serotonin, showing that *C. elegans* exposed to exogenous octopamine have inhibition of both egg laying and pharyngeal pumping (Horvitz *et al.*, 1982; Alkema *et al.*, 2005). As these behaviours are promoted by serotonin, and compounded by the fact that both molecules have opposite

effects on the firing rate of rate of muscles in the pharynx, this suggests that octopamine and serotonin signalling can directly act to counteract each other (Niacaris and Avery, 2003; Alkema *et al.*, 2005). In addition, both egg laying and pharyngeal pumping are stimulated by the presence of food, suggesting that octopamine may act to oppose the neural signalling which is triggered by food (Chase and Koelle, 2007).

6.1.2 Biogenic amine signalling in the immune response

Biogenic amines have been implicated in signalling that drives innate immune pathways in *C. elegans* (1.6). The octopamine receptor OCTR-1 (Table 6.2) acts to suppress immune responses and causes changes in protein synthesis levels in response to *P. aeruginosa* PA14 infection (Sun *et al.*, 2011; Liu *et al.*, 2016). In addition, it has been found that serotonin signalling is involved in supressing the innate immune response upon infection with the natural *C. elegans* pathogen *M. nematophilum* (Anderson *et al.*, 2013).

6.2 Aims and objectives

From the summary above it is evident that the biogenic amines of *C. elegans* all have important roles in controlling the behaviour of the organism. The expressions of these behaviours often underpin adaptive responses to an environmental challenge. The time course of behavioural paradigms involving biogenic amines implies they mediate their modulatory function on medium and longer term exposure to bacteria (Chapter 4) (Zhang *et al.,* 2005). In the context of this thesis it is noteworthy that they have a potential role in immune responses following exposure to pathogenic bacteria (6.1.2). In addition, the fact that serotonin has previously been implicated in avoidance of pathogenic bacteria in a conditioning paradigm, highlights the possibility that it may also be involved in the food aversion behaviour to pathogenic bacterial strains (Zhang *et al.*, 2005).

The specific aims of this chapter were to investigate the role of biogenic amines as determinants of the response to pathogenic bacteria developed in chapters 4 and 5.

6.3 Results

6.3.1 Exposure of biogenic amine mutants to pathogenic bacteria leads to differential levels of food aversion

In chapter 4 an enhanced level of food aversion upon exposure to a subset of bacterial strains was observed compared to the standard laboratory food source *E. coli* OP50 (Figure 6.3). To investigate how biogenic amines contribute to the food aversion response upon exposure to pathogenic bacteria, *C. elegans* mutants deficient in biosynthetic enzymes needed to synthesise serotonin (*tph-1(n4622)*), octopamine (*tbh-1(n3247)*), dopamine (*cat-2(e1112)*) and tyramine (*tdc-1(n3419)*) were investigated. This was initially focused on the bacterial strains previously identified as strongly aversive. These strains were *P. aeruginosa* PA14, P2 and P4 and the *K. pneumoniae* strain K5 (4.3.2). The rationale was to investigate the bacterial strains that had the most penetrant food aversion phenotypes for wild type animals (4.3.2). Figure 6.3 summarizes the data from the established food aversion assay showing that exposure to strains PA14, P2, P4 and K5 significantly stimulates higher levels of food aversion when compared to OP50.

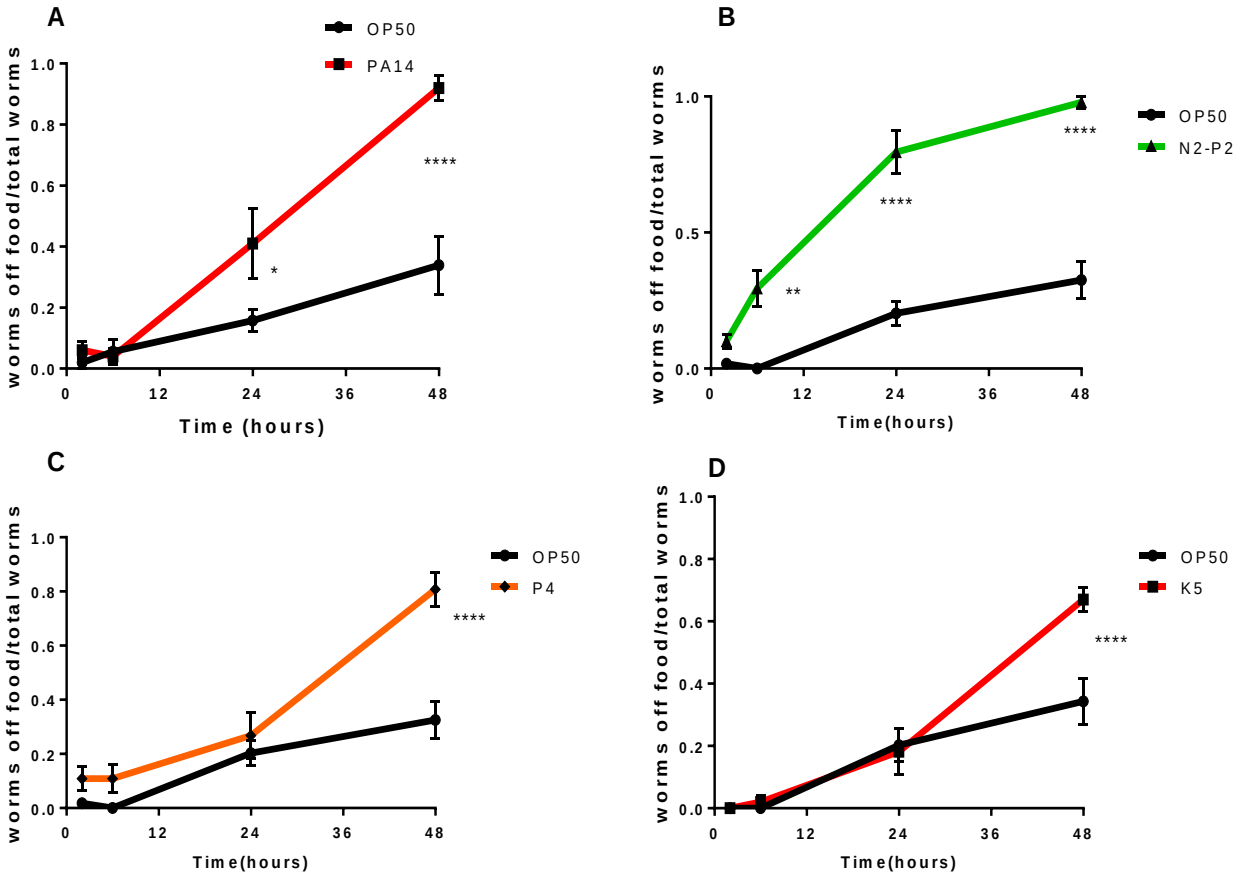


Figure 6.3: *C. elegans* aversive pathogens. Exposure of wild type (N2) adult *C. elegans* to *P. aeruginosa* strains PA14 (A) P2 (GH56) (B) and P4 (GH97) (C) and *K. pneumoniae* K5 (D) led to an enhancement in the level of food aversion compared to OP50. Food aversion was measured as the proportion of adult animals off a bacterial lawn over a 48 hour period compared to behaviour measured against a paired *E. coli* OP50 control. N=7 independent assays for all strains. Error bars represent ±SEM. Data analysed by two-way ANOVA with Dunnett's multiple comparisons to OP50. *P<0.05, **P<0.01, ***P<0.001 and ****P<0.0001. Figure adapted from Figure 4.3 and Figure 4.4.

In order to investigate the role of biogenic amines in controlling food aversion to pathogenic bacteria, populations of biogenic amine deficient mutants were exposed to lawns of the aversive bacteria and the food aversion levels were measured over a 48 hour period as in Figure 6.3. Assays for wild type animals were conducted in parallel. When the biogenic amine mutants were exposed to OP50, there was no significant modulation of food aversion relative to N2 animals (Figure 6.4A). This food aversion effect is representative of the progeny driven food-leaving described in chapter 3 and is not subject to regulation by biogenic amine signalling (3.3.4.1).

In contrast to food aversion on OP50, the response of animals to pathogenic bacteria exhibited varying levels of regulation by biogenic amines. This is revealed as a difference in the proportion of worms off the bacterial lawn over time of exposure for N2 wild type compared to the biogenic amine mutants (Figure 6.4 B-E). *tph-1 (n4622)* animals exhibited reduced levels of food aversion relative to N2 *C. elegans* after 24 hours of exposure to all pathogenic strains of bacteria (Figure 6.4B-E). *tph-1* animals also exhibited significant reduction in food aversion after 48 hours of exposure to K5 (Figure 6.4E). This indicates that serotonin is required for driving *C. elegans* aversive behaviour to pathogenic bacteria and is consistent with earlier reports that serotonin is a key player in responses to pathogenic bacteria(Zhang *et al.*, 2005) .

Exposure of both *tbh-1 (n3247)* and *tdc-1 (n3419)* animals to lawns of PA14 and P2, led to wild type levels of food aversion in both strains (Figure 6.4B, C). However, exposure of *tbh-1 (n3247)* and *tdc-1 (n3419)* animals to lawns of *P. aeruginosa P4* and *K. pneumoniae K5*, resulted in higher levels of food aversion after 24 hours of exposure to lawns of both bacterial strains compared to wild type animals (Figure 6.4D, E). This same phenotype was also observed after 48 hours of exposure on lawns of K5 (Figure 6.4E). These results indicate that octopamine signalling selectively acts to suppress the food aversion response to these bacterial strains. This appears to act in an antagonistic manner to serotonin signalling which acts to promote food aversion to pathogenic bacteria. Dopamine deficient animals *(cat-2(e1112)*, similar to *tph-1 (n4622)* animals also exhibited reduced food aversion in comparison to wild type after 48 hours of exposure to PA14, and 24 hours on K5, indicating that dopamine may play a role in stimulating food aversion behaviour. However, this effect is less consistent than that seen for *tph-1 (n4622*) (Figure 6.4B, E). These data indicate a complex, differential role for biogenic amine signalling in regulating food aversion to distinct strains of pathogenic bacteria.

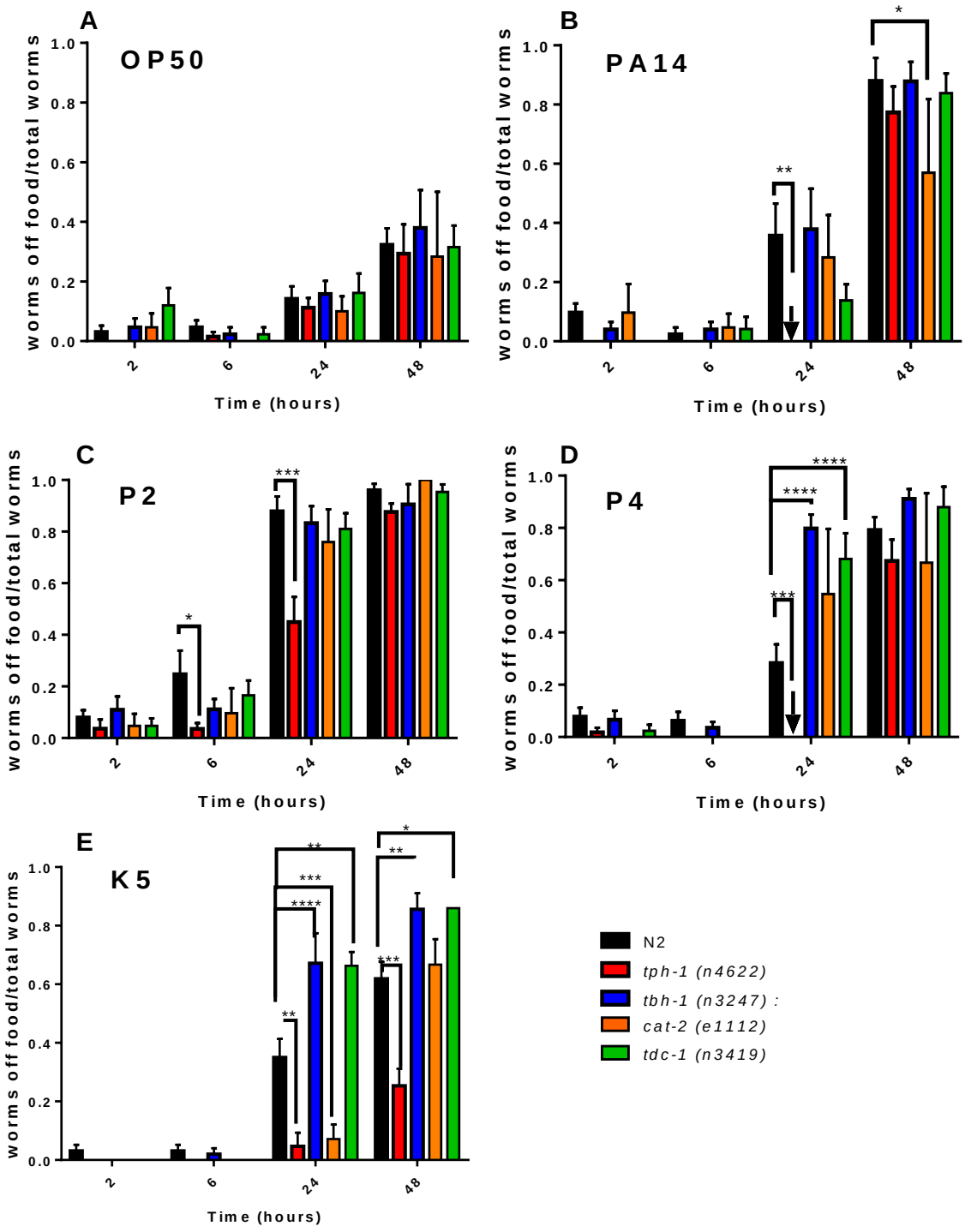


Figure 6.4: Comparison of food aversion between wild type N2 and biogenic amine mutant *C. elegans* in the presence of different bacterial strains. The biogenic amine mutants were *tph-1 (n4622), tbh-1 (n3247), cat-2 (e1112)* and *tdc-1 (n3419*) and the bacterial strains tested were *E. Coli* OP50 as a control (A), *Pseudomonas aeruginosa* PA14 (B), P2 (C) and P4 (D) and *K. pneumoniae* (K5) in comparison to N2 animals. The number of worms off food at indicated time points and mutant behaviour were compared relative to N2. N≥4 independent assays for all experiments. Error bars represent ±SEM. Data analysed by two-way ANOVA with Dunnett's multiple comparison test. *P<0.05, **P<0.01, ***P<0.001 and ****P<0.0001.

6.3.2 The role of biogenic amine signalling on *C. elegans* lifespan in the presence of pathogenic bacteria

The bacterial strains analysed in the previous section have all been shown in Chapter 4 to be pathogenic towards *C. elegans.* As biogenic amine mutants exhibited differential food aversion phenotypes this will influence the proportion of time that they dwell on the bacterial lawn and thus might be predicted to alter the pathogenic impact of the bacteria on worm survival time by changing the duration of exposure to the pathogen (6.3.1). To investigate this, killing assays were performed on *tph-1, tbh-1, tdc-1* and *cat-2* mutants as well as wild-type *C. elegans* (2.3.1). These killing assays were carried out with a constant incubation temperature at 20°C as described in chapter 4. These experiments were conducted as before and the N2 life span is consistent with that reported in chapter 4. *C. elegans* biogenic amine mutants on OP50 lawns had a similar lifespan to N2 animals (Figure 6.5A). Moreover, the lifespan of the biogenic amine mutants on pathogenic bacteria was reduced in a similar manner to N2. This was consistently observed across all four aversive pathogenic strains (Figure 6.5B, C, D, E), with no significant alteration in lifespan being observed. This indicates, somewhat surprisingly, that deficiencies in biogenic amine signalling can alter food aversion to pathogenic bacteria without affecting the virulence of the same strains upon an extended exposure.

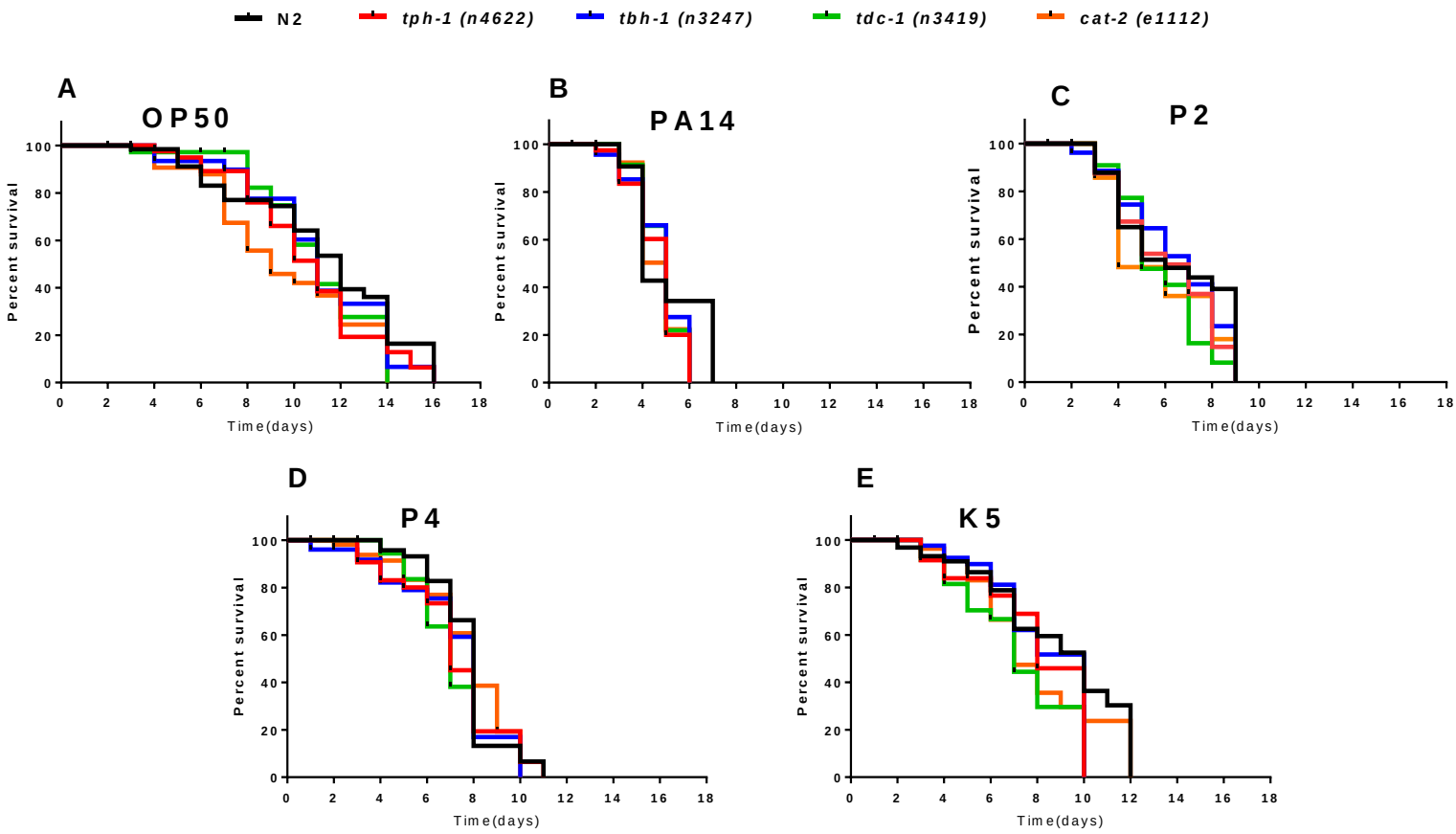


Figure 6.5: The survival of biogenic amine mutants upon exposure to bacterial pathogens. N2*, tph-1 (n4622), tbh-1 (n3247), tdc-1 (n3419)* and *cat-2 (e1112) C. elegans* upon exposure to *E. coli* OP50 (A) and the aversive pathogens *Pseudomonas aeruginosa* PA14 (B), P2 (C) and P4 (D) and *Klebsiella pneumoniae* K5. Data representative of n≥3. Data analysed by Log-rank (Mantel-Cox) test. *P<0.05, **P<0.01, ***P<0.001 and ****P<0.0001.

6.3.3 Colonisation and clearance of *P. aeruginosa* from the *C. elegans* intestine in serotonin and octopamine mutants

As summarised in chapters 1 and 5 of this thesis, previous work has suggested bacterial colonisation of the *C. elegans* intestine is an important determinant of pathogenesis (see chapter 5). The results described earlier provided evidence for a role of neuropeptides in modulating clearance of bacteria from the *C. elegans* intestine, thus providing a potentially important determinant of host pathogen interactions. As the results above demonstrate biogenic amines to be involved in modulating a behavioural response to bacteria, it was investigated if these molecules were also involved in controlling bacterial colonisation or clearance.

In the context of this investigation it is interesting to note the results obtained in chapter 5 which demonstrated that *egl-3* and *unc-31 C. elegans* mutants exhibit increased clearance of pathogenic bacteria from the intestine. The contribution of these particular signalling pathways that is affected in these mutants may be indicative of a systemic neuromodulatory component controlling bacterial proliferation in the *C. elegans* gut. Furthermore, UNC-31 is a key component of biogenic amine signalling. As both *tph-1* and *tbh-1* animals exhibited striking food aversion phenotypes in comparison to wild type *C. elegans*, these strains were analysed in the same colonisation and clearance experiments described in chapter 5.

6.3.3.1 *P. aeruginosa* colonisation

P. aeruginosa colonisation levels of *tph-1 (n4622)* and *tbh-1 (n3247) C. elegans* were analysed after 24 hours on lawns of *P. aeruginosa* (24 hour column), 24 hours on *P. aeruginosa* lawns followed by 24 hours on *E. coli* OP50 (transfer column) and 48 hours on lawns of *P. aeruginosa* (48 hour column) as in chapter 5 (2.3.2, 5.3.2). Analysis of these colonisation levels revealed that generally both serotonin and octopamine deficient animals had the same levels of *P. aeruginosa* in their intestines as N2 animals, including upon exposure to all strains for 24 hours and after the transfer step. Some variation in colonisation levels was seen after 48 hours of exposure to certain *P. aeruginosa* strains. Both *tbh-1(n3247)* and *tph-1(n4622)* mutant *C. elegans* had reduced levels of *P. aeruginosa* CFUs after 48 hours of exposure to both P4 and P5 relative to N2 animals (Figure 6.6E,F), and *tbh-1(n3247)* animals had increased CFUs after 48 hours on PA14 in comparison to N2 animals(Figure 6.6A).

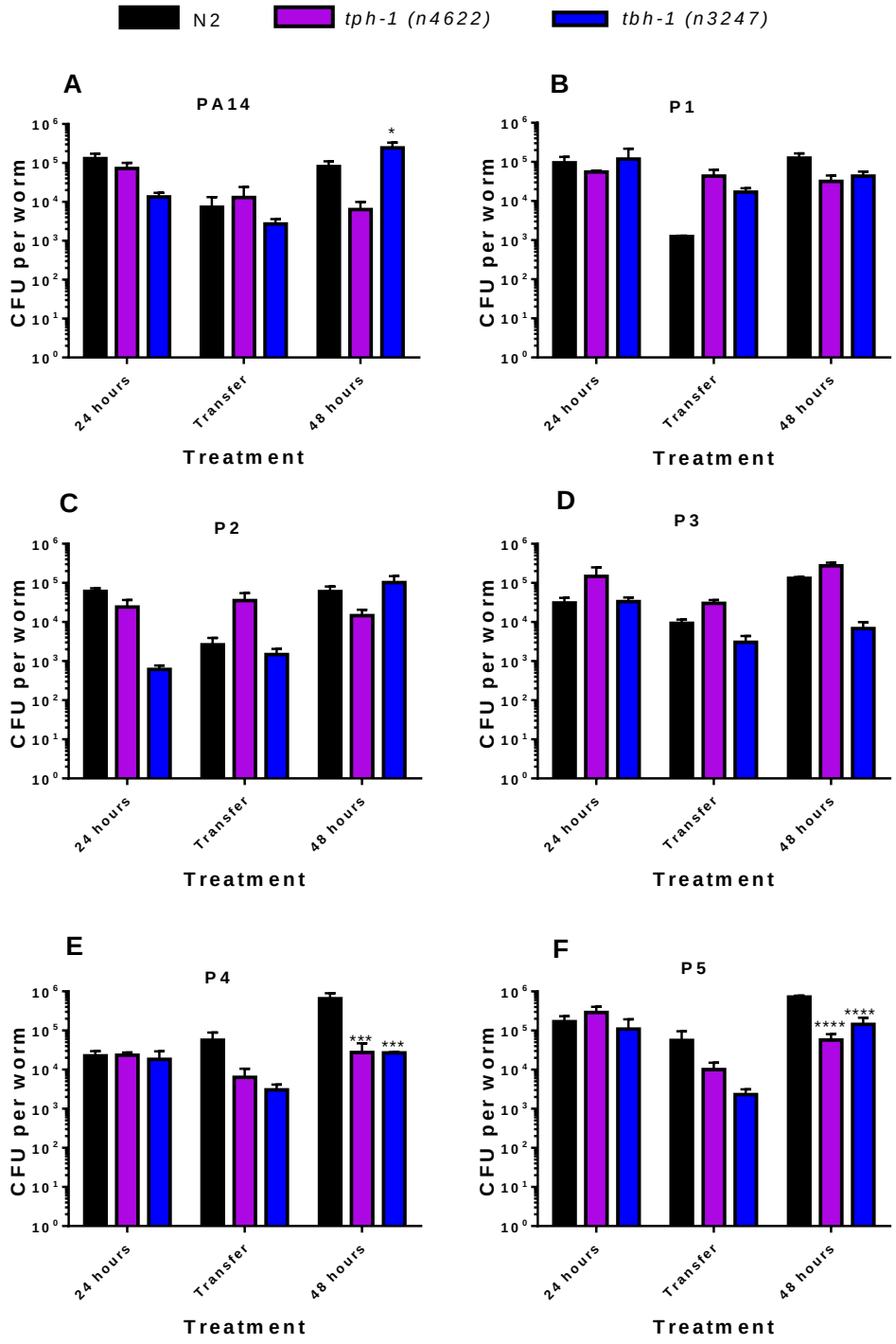


Figure 6.6: The colonisation of *P. aeruginosa* in N2, *tph-1 (n4622)* and *tbh-1 (n3247) C. elegans.* Colonisation was measured after 24 hours of exposure to lawns of *P. aeruginosa (*24 hour column*)*, 24 hours of exposure to lawns of *P. aeruginosa* followed by 24 hours on lawns of *E. coli* OP50 (Transfer column) and 48 hours on *P. aeruginosa* (48 hour column). N=3 experiments with 5-10 *C. elegans* being lysed for each individual experiment, error bars represent ±SEM. Data analysed by two-way ANOVA with Dunnett's multiple comparison test to N2. *P<0.05, **P<0.01, ***P<0.001 and ****P<0.0001.

6.3.3.2 *P. aeruginosa* clearance

Transferring *C. elegans* that have been exposed to pathogenic bacteria to a lawn of non-pathogenic OP50 allows investigation of how well the colonising pathogenic bacteria are removed from the *C. elegans* intestine (2.3.3,5.3.5). Analysis of *the P. aeruginosa* clearance of both *tph-1 (n4622)* and *tbh-1 (n3247)* mutant *C. elegans* was based on the colonisation levels displayed in Figure 6.6. This clearance analysis revealed that there was no significant increase or decrease in the bacterial clearance of *P. aeruginosa* from the intestine relative to wild type animals (Figure 6.7). This demonstrates that neither serotonin nor octopamine are involved in the control of the removal of bacteria following bacterial exposure. These data suggest that the fluctuation in PA14 clearance seen in the *unc-31* mutant animals in chapter 5 is not due to the modulated biogenic amine release seen in this mutant strain, despite biogenic amines being among the cargo released by DCVs which are controlled by UNC-31 (Speese *et al.,* 2007; Lin *et al.,* 2010).

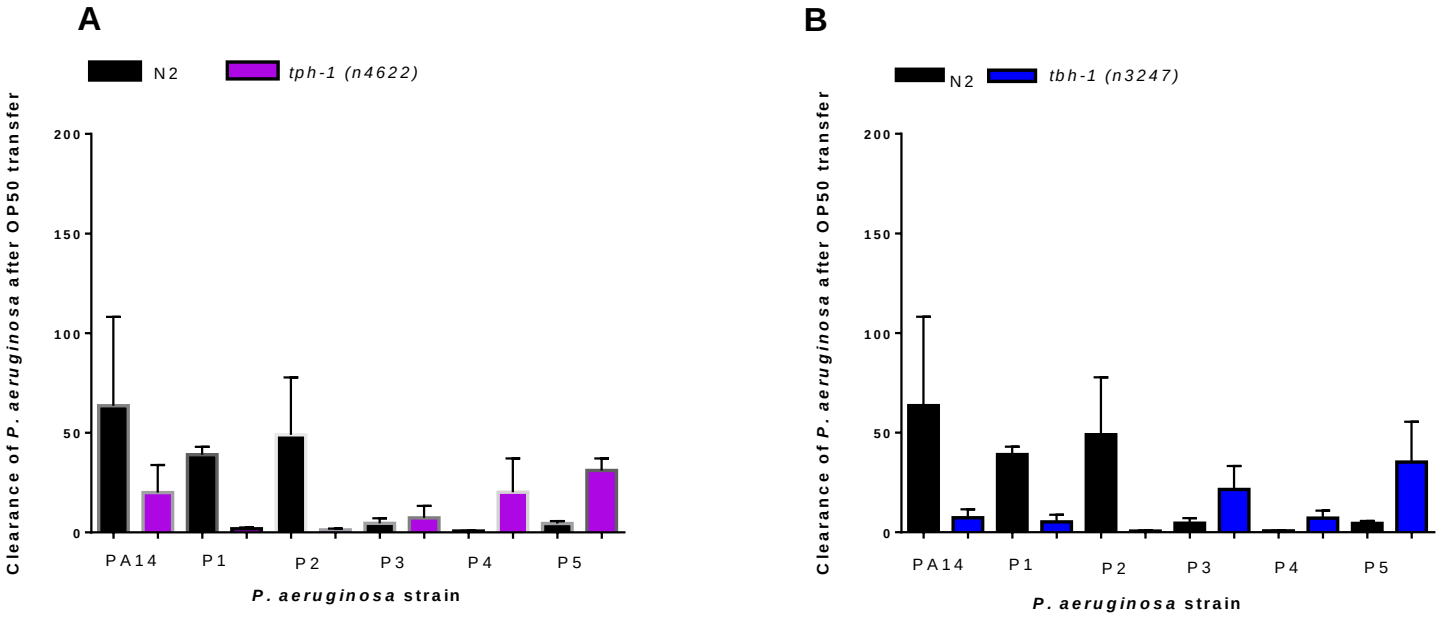


Figure 6.7: Clearance of the *Pseudomonas aeruginosa* strains from N2 and biogenic amine mutants. Clearance of *P. aeruginosa* from the intestines of N2, *tph-1 (n4622*) (A) and *tbh-1(n3247*) (B) animals was analysed by calculating the fold reduction in *P. aeruginosa* CFUs after 24 hours on lawns of *E. coli* following 24 hours on lawns of *P. aeruginosa*. N=3 for each experiment. Error bars represent ±SEM. Data analysed by one-way ANOVA with Tukey's multiple comparison test.

6.3.4 Octopamine modulates *C. elegans* food aversion in other non-aversive bacteria

In the experiments described above *tbh-1 (n3247)* exhibited higher food aversion to two aversive pathogenic bacterial strains (6.3.1). These specific strains were *K. pneumoniae* K5 and *P. aeruginosa* P4. This suggests potential negative regulation of the aversive behaviour by octopamine for these strains of pathogenic bacteria. To investigate whether this octopamine dependence extends to other strains of pathogenic bacteria the behaviour of *tbh-1 (n3247)* animals was compared to wild type animals across the whole panel of the remaining *K. pneumoniae*, *A. baumannii* and *P. aeruginosa* strains.

Analysis of *tbh-1(n3247) C. elegans* upon exposure to these strains over 48 hour in comparison to N2 animals reveals that the majority of bacterial strains lead to no significant difference in food aversion between N2 and *tbh-1 C. elegans* (Table 6.3) (for raw data for all strains see appendix). This is similar to the behaviour observed upon exposure to OP50 and the *P. aeruginosa* strains P2 and PA14 (6.3.1). This provides further evidence that *tbh-1 (n3247) C. elegans* do not universally exhibit higher food aversion than wild type animals. However, an exception to this is the *K. pneumoniae* strain K4 for which *tbh-1 (n3247) C. elegans* exhibited enhanced food aversion in comparison to N2 (Table 6.3). This is similar to the response observed upon exposure to both *K. pneumoniae* strain K5 and *P. aeruginosa* strain P4. This indicates that in addition to these strains identified earlier (6.3.1), there is another *K. pneumoniae* strains which elicits enhanced food aversion in octopamine deficient animals.

	Pathogenicity	N2 food aversion	enhanced *tbh-1* food aversion
K1			
K2			
K3			
K4			
K5			
A1			
A2			
A3			
A4			
A5			
PA14			
P1			
P2			
P3			
P4			
P5			

Table 6.3: Summarising the pathogenicity, aversion and the octopamine regulation of aversion of the strains of *K. pnuemoniae*, *A. baumannii* and *P. aeruginosa.* In the first column (pathogenicity) green indicates that the strain is not pathogenic, based of the lifespan measurements undertaken in chapter 4, whereas red indicates the strain is pathogenic. The second column indicates whether the strains are aversive towards N2 animals, based on the 48 hour food aversion experiments undertaken in chapter 4, yellow indicates not aversive, and orange indicates aversive. The third column (enhanced *tbh-1* food aversion) indicates the upregulation of food aversion relative to wild type animals seen in *tbh-1 (n3247)* mutants, with grey indicating no upregulation and blue indicating an upregulation. The data in the right hand column are based on the data in Figure 8.1 (see Appendix), with an n=3 independent assays and statistical comparison of the data by two-way ANOVA with Sidak's multiple comparison test.

Combining the data from this screen with the earlier analysis demonstrates that exposure to three individual bacterial strains in the collection (Table 4.1) leads to enhanced food aversion in a 48 hour assay in *tbh-1 (n3247)* animals, the *K. pneumoniae* strains K4 and K5, and the *P. aeruginosa* strain P4. All three of these strains have previously been found to be pathogenic and two of them are also aversive (4.3.2, Table 6.3). This indicates that octopamine is involved in suppressing the food aversion levels in *C. elegans* upon exposure to a specific subset of pathogenic bacteria which have previously been analysed in this thesis (4.3).

Repeating food aversion assays with *tbh-1* animals exposed to K4 revealed that *tbh-1 (n3247)* animals exhibit a highly significant increase in food aversion relative to N2 animals after 48 hours of exposure (Figure 6.8B). The time course of enhanced K4 aversion does differ from that seen upon exposure to P4 and K5 (6.3.1). Exposure of *tbh-1* animals to *K. pneumoniae* K5 and *P. aeruginosa* P4 reveals that the biggest differential between wild type and mutant *C. elegans* was observed after 24 hours exposure (Figure 6.8A, C). This indicates that whilst *tbh-1 (n3247)* mutants display enhanced food aversion towards multiple bacterial strains, this behaviour is not always expressed over the same time course.

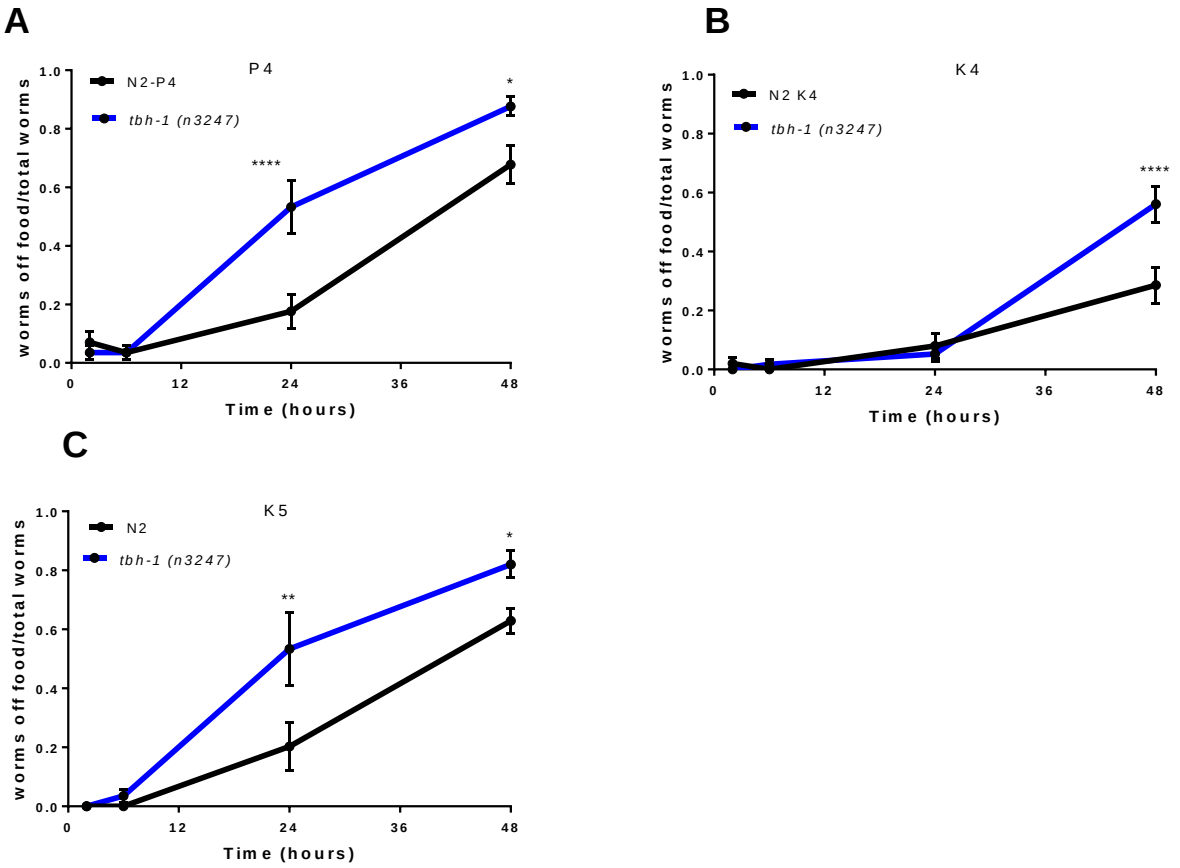


Figure 6.8: Bacterial strains in which *tbh-1 (n3247)* animals display enhanced food aversion. The three bacterial strains previously screened which elicited enhanced food aversion in *tbh-1 (n3247)* *C. elegans* in comparison to N2 wild type animals *P. aeruginosa strain* P4 (A) and the *K. pneumoniae* strains K4 (B) and K5 (C). N=8 for all strains. Error bars represent ±SEM. Data analysed by two-way ANOVA with Sidak's multiple comparison test. *P<0.05, **P<0.01, ***P<0.001 and ****P<0.0001.

6.3.5 Confirmation of the role of octopamine in aversion to a subset of pathogenic bacteria

6.3.5.1 Construction of *tbh-1* rescue lines

To confirm the dependence of the enhanced food aversion behaviour on octopamine as revealed by the behaviour of *tbh-1(n3247)* animals, microinjection was performed to produce transgenic rescue of the *tbh-1* deficiency. The *C. elegans tbh-1* gene is encoded by the sequence H13N06.6 (www.wormbase.org). The clone CBGtg9050G1018D/WRM0617D_D11, which contains H13N06.6, and the predicted the *tbh-1* gene was sourced from the *C. elegans* TransgeneOme Resource of Source Biosciences.

The fosmid containing the *tbh-1* gene was purified to a final concentration of 1.4µg/µl. The presence of the sequence was validated using PCR (2.7.9,2.7.10,2.7.11). As shown in Figure 6.9, primers were designed to span the beginning of the first exon of the *tbh-1* gene, in addition to the 5' UTR. The distance between the primers was 821 bp (Figure 6.9). The resulting products of the PCR reaction were run on an agarose gel, alongside the products of an identical PCR reaction with no Taq polymerase. This revealed a band from the *tbh-1* fosmid being level with the 800bp band on the Bioline HyperLadder 1kb used as a marker, thus indicating successful amplification of the genetic product (Figure 6.10).

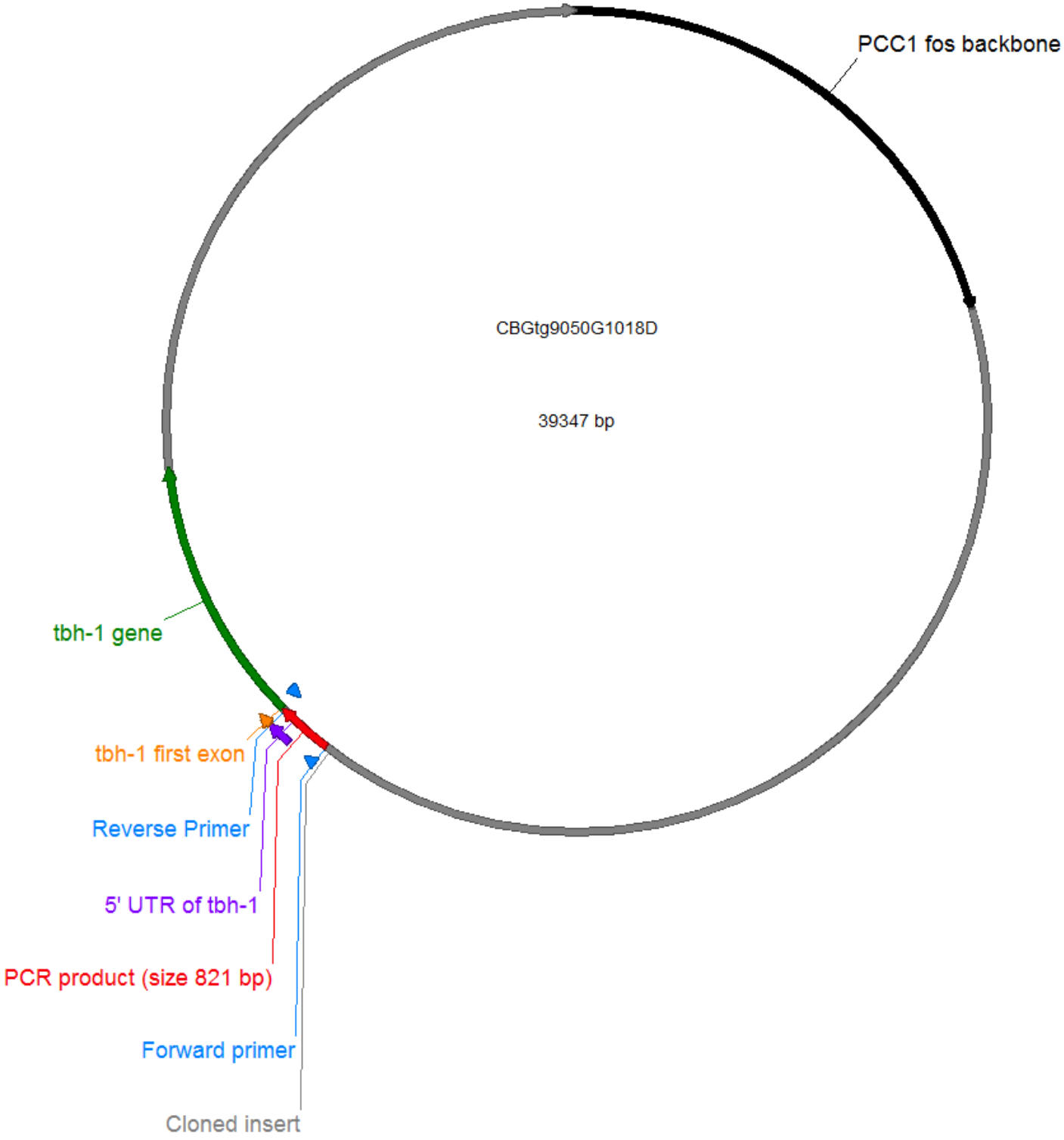


Figure 6.9: A Map of the Fosmid containing the native *C. elegans tbh-1* gene, showing the location of the gene of interest and the location of the region amplified by PCR for product authentication.

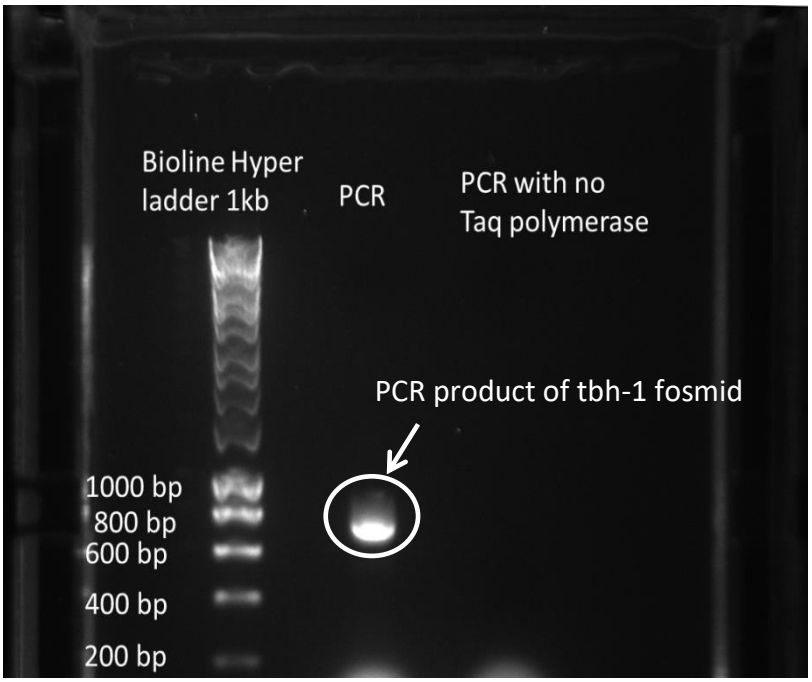


Figure 6.10: Selective amplification of the 5' end of the *C. elegans tbh-1* gene within the *tbh-1* fosmid gene by PCR. The product was run and imaged on an agarose gel.

The PCR authenticated fosmid was injected into *C. elegans* based on a standard injection protocol (2.4.2).The fosmid was injected at a final concentration of 10ng/µl along with 30ng/µl *pmyo-3::GFP* (2.4.2). The presence of *pmyo-3::GFP* in the body wall muscle was used as an indicator of successful transformants (2.4.2,Figure 2.10). Another set of *C. elegans* were also injected with *Pmyo-3::*GFP alone to act as a control. This approach lead to the establishment of one control line (*pmyo-3::GFP* alone) and two transgenic rescue lines (1.1 and 1.3) potentially expressing (*tbh-1* and pmyo-3::GFP) in a *tbh-1 (n3247)* mutant background. Having two potential rescue lines allowed comparison in the subsequent sections of both of these lines for successful rescue of the *tbh-1* gene.

6.3.5.2 Phenotypic analysis of the *tbh-1* rescue lines

6.3.5.2.1 Avoidance of dilute octanol

Following injection, the transgenic lines were behaviourally analysed. Previous work has identified that octopamine deficient *C. elegans* are hypersensitive to an acute aversive response to dilute octanol (Chao *et al.*, 2004; Wragg *et al.*, 2007; Mills *et al.*, 2012b). This is a robust phenotype which was used to investigate the injected transgenic lines for rescue of the *tbh-1* mutant defect. This was done by performing a 'smell on a stick test' in which 30% octanol is positioned close to an advancing worm and time to reverse recorded (2.2.6). When this test was carried out in N2 animals they initiated backward movement after 10 seconds (Figure 6.11). Both *tbh-1* (n3247) and control injected strains initiated backward movement from 30% octanol significantly faster than N2 animals , consistent with previous studies (Wragg *et al.*, 2007)(Figure 6.11). In contrast, the injected lines expressing *tbh-1* from the injected fosmid initiated reversals with the same delay as wild type animals strongly pporting a robust functional rescue from the transgene (Figure 6.11).

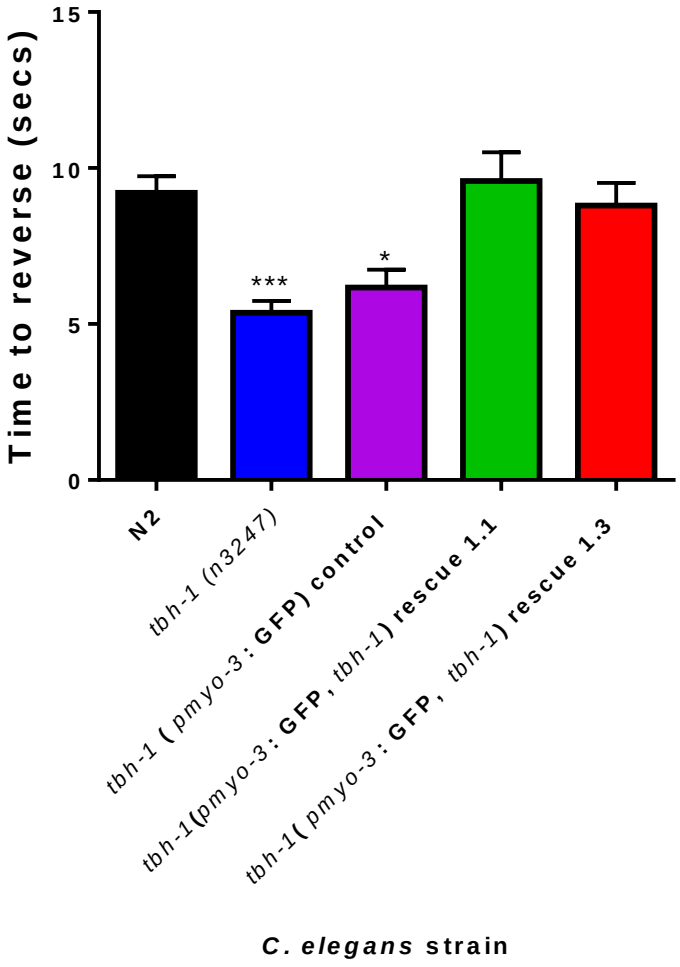


Figure 6.11: Octanol induced reversal time of transgenic *C. elegans*. Reversal from 30% octanol in N2*, tbh-1 (n3247), tph-1 (n4622)* and injected *tbh-1 (n3247) C. elegans*. n≥10 individual animals analysed for all strains. Error bars represent ±SEM. Data analysed by ordinary one-way ANOVA with multiple comparisons to N2 animals. *P<0.05, **P<0.01, ***P<0.001 and ****P<0.0001.

6.3.5.2.2 Aversion of pathogenic bacteria by *tbh-1* rescue lines

Following the characterisation of the injected lines in the octanol reversal experiment (6.3.5.2.1), the lines were analysed in the food aversion assay on the strains in which *tbh-1 (n3247)* animals display enhanced food aversion in comparison to N2, namely *P. aeruginosa* P4, *K. pneumoniae* K4 and *K. pneumoniae* K5 (6.3.4). In addition to these bacterial strains OP50 and *P. aeruginosa* PA14 were used as controls. These food aversion assays were conducted as before (4.3.2,6.3.1). However, the time course of the assay was extended, with measurements being taken 2, 6, 18, 24, 30,42 and 48 hours after initial exposure to the indicated bacteria (Figure 6.12). The assay was expanded in this way because the initial experiments where enhanced food aversion was observed in *tbh-1* animals was observed

after 24 hours of bacterial exposure, with no further measurements being taken until 48 hours. Expanding the food aversion assay allowed better resolution of the *tbh-1* food aversion phenotype. Measuring food aversion at these extra time points revealed that in *tbh-1* mutants enhanced food aversion is elicited between 24 and 42 hours upon exposure to lawns of both P4 and K5 (Figure 6.12 C,E) and from 30 hours onwards upon exposure to K4 (Figure 6.12D).

Analysing the injected lines on lawns of both OP50 and *P. aeruginosa* PA14 resulted in both rescue lines and the control line all having identical food aversion levels to both N2 and *tbh-1 (n3247)* animals. This indicates that injection of these lines did not affect the progeny stimulated food-leaving effect seen on lawns of OP50, nor the food aversion effect seen upon exposure to the pathogen *P. aeruginosa* PA14 (Figure 6.12A,B).

The transgenic control line consistently exhibited the same level of food aversion as the *tbh-1(3247)* animals (Figure 6.12C,D,E). This indicates that injecting *tbh-1 (n3247)* worms with the *pmyo-3::GFP* transgene does not affect the enhanced food aversion seen in *tbh-1* mutant animals upon exposure to select bacterial strains. Importantly this shows that the selection marker of pmyo-3::GFP does not impact on the nature of pathogen induced food leaving as determined by it response to the bacterial strains P4, K4 and K5.

In contrast, the lines expressing wild type *tbh-1* in the *tbh-1* mutant background both exhibited higher levels of food aversion compared to N2 animals on lawns of P4 (Figure 6.12C). However, these levels of food aversion were lower than that seen in both mutant *tbh-1 (n3247)* animals and the control line (Figure 6.12C). In contrast, both rescue lines did not have altered food aversion to *K. pneumoniae* strains (K4 and K5) relative to N2 *C. elegans,* throughout the 48 hour time course (Figure 6.12D,E).

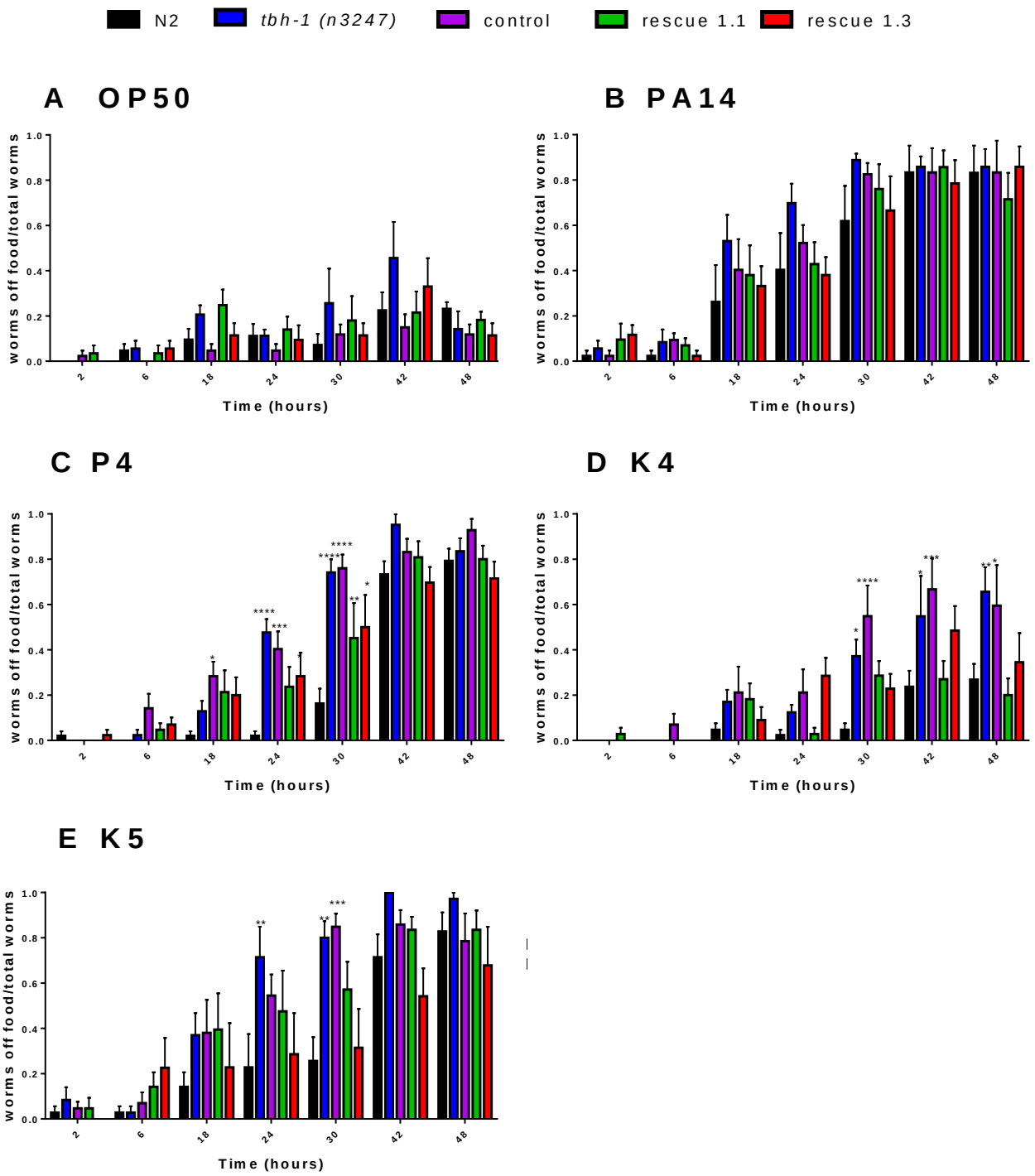


Figure 6.12: Investigating transgenic rescues of the *tbh-1* dependent upregulation of *C. elegans* food aversion. The food aversion exhibited by transgenic *tbh-1 (n3247) C. elegans* in response to *E. coli* OP50 (A) the *Pseudomonas aeruginosa* strain PA14 (B) and P4(C) and the *Klebsiella pneumoniae* strains K4 (D) and K5 (E). P4, K4 and K5 modulated aversion to in *tbh-1 (n3247) C. elegans* compared to N2 animals. n≥5 for all experiments. Error bars represent ±SEM. Data analysed by two-way ANOVA with Dunnett's multiple comparison to N2 animals. *P<0.05, **P<0.01, ***P<0.001 and ****P<0.0001.

These results described above demonstrate that there is a clear rescue of the *tbh-1* enhanced food aversion phenotype after exposure to the *K. pneumoniae* strains K4 and K5 (Figure 6.12D,E). In contrast, for the P4 strain this rescue is less profound (Figure 6.12C). To further investigate this, further statistical analysis was undertaken whereby which the food aversion of the transgenic lines was compared to that elicited by *tbh-1* mutant *C. elegans* (Figure 6.13). This analysis revealed that both rescue lines had significantly lower food aversion than *tbh-1(n3247)* mutants after 30 hours of exposure on *P. aeruginosa* P4, the point at which the differential between wild type and *tbh-1 (n3247) C. elegans* is greatest (Figure 6.13A). This differential in food aversion was also seen with the rescue 1.3 line at 42 hours (Figure 6.13B) another time point at which a differential between wild type and *tbh-1* mutant animals is observed. These data are consistent with a partial rescue of the *tbh-1* dependent enhanced food aversion.

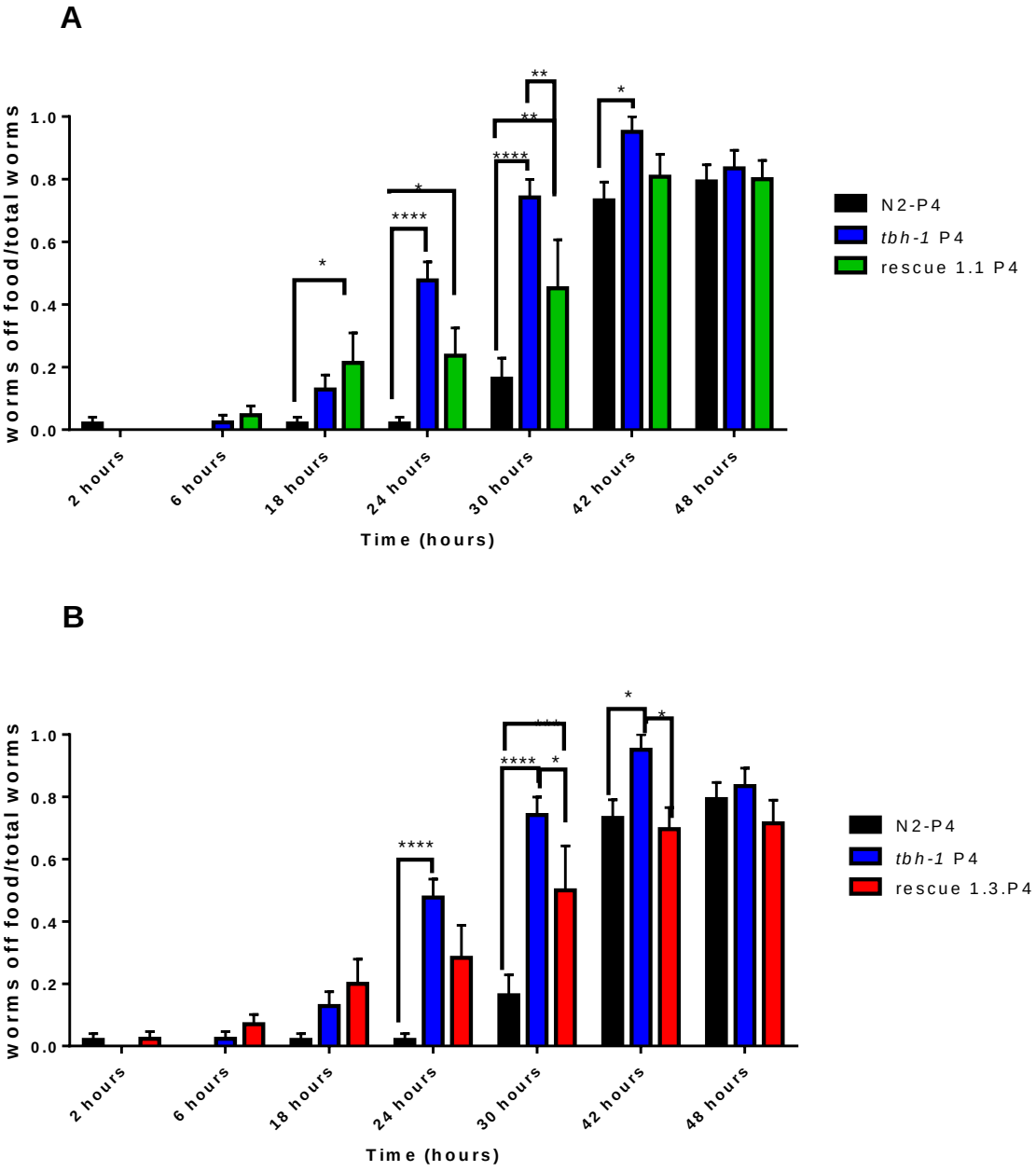


Figure 6.13: Food aversion of injected *tbh-1* lines on *P. aeruginosa* strain P4. Food aversion levels of *tbh-1 (n3247) C. elegans* injected with pmyo-3::GFP alone (A) and pmyo-3::GFP and the native *C. elegans tbh-1* gene (B,C) in comparison to N2 and tbh-1 (n3247) animals upon exposure to the *P. aeruginosa* strain P4. N= 6 for all strains, data analysed by two-way ANOVA with Dunnett's multiple comparisons. Error bars represent ±SEM. *P<0.05, **P<0.01, ***P<0.001 and ****P<0.0001.

6.3.6 Investigating the role of octopamine receptors in supressing food aversion

The data above provide evidence that octopamine signalling is required for suppressing aversion to a subset of pathogenic bacteria in wild type animals As indicated above the receptors for octopamine in *C. elegans* have been identified (Table 6.2)(6.1.1) and are termed OCTR-1, SER-3 and SER-6 (Wragg *et al.*, 2007; Mills *et al.*, 2012b)(Table 6.4). These receptors have been shown to both individually and collectively contribute to a range of *C. elegans* behaviours (Table 6.4). To examine the effects of octopamine receptors in controlling the food aversion *C. elegans* strains with individual mutations in these three octopamine receptors were analysed in the food aversion assay as performed earlier (6.3.1). These experiments where performed alongside N2 and *tbh-1 (n3247)* animals.

	Gene	Receptor Type	Expression pattern	Roles	References
Octopamine receptors	*octr-1*	7TM GPCR	Subset of head and tail neurons,	Modulates the octopamine mediated suppression of 5-HT sensitised aversive responses in ASH.	(Wragg *et al.*, 2007)
	ser-3	7TM GPCR	Pharynx, Head and tail neurons, head muscles, phasmid sockets, nerve ring and intestine, spermatheca, eggs, gonad and vulva.	Acts antagonistically against octr-1 signalling at high exogenous octopamine concentrations	(Wragg *et al.*, 2007)
	ser-6	7TM GPCR	Head neurons posterior VCN and intestine.	Inhibits ASH mediated aversive responses to 100% octanol through peptidergic cascade.	(Mills *et al.*, 2012b)

Table 6.4: The *C. elegans* octopamine receptors, expanded from Table 6.2

The *C. elegans* receptor mutants were exposed to *E. coli* OP50 as a control, along with the three bacterial strains *P. aeruginosa* P4 and *K. pneumoniae* K4 and K5 which showed enhanced food aversion in octopamine deficient animals (6.3.4). Exposure to OP50 revealed that there was no significant modulation in the level of the progeny stimulated food aversion as described in Chapter 3 in any of the octopamine receptor deficient *C. elegans,* further indicating that octopamine signalling is not involved in this phenomenon (Figure 6.14A).

ser-3 (ad1774) C. elegans exhibited significantly enhanced food aversion to *P. aeruginosa* P4 after 24 hours on the bacterial lawn relative to N2, mirroring the enhanced food aversion seen in *tbh-1 (n3247)* (Figure 6.14B). *ser-6 (tm2146)* and to a lesser degree *ser-3 (ad1774) C.*

elegans also showed significant enhancement of food aversion to *K. pneumoniae* K4 lawns after 48 hours of exposure similar to *tbh-1* mutants (Figure 6.14C). These results suggest a role for these receptors being involved in mediating the octopamine dependent suppression of food aversion.

In contrast, exposure of the receptor mutants to lawns of *K. pneumoniae* K5 showed that the absence of any receptor exhibited enhanced food aversion food aversion in comparison to N2 animals (Figure 6.14D). This suggested that in the case of the enhanced food aversion seen in *tbh-1* animals upon exposure to the K5 *K. pneumoniae* strain a combination of octopamine receptors rather than specific receptors is required to modulate this response in wild type *C. elegans.*

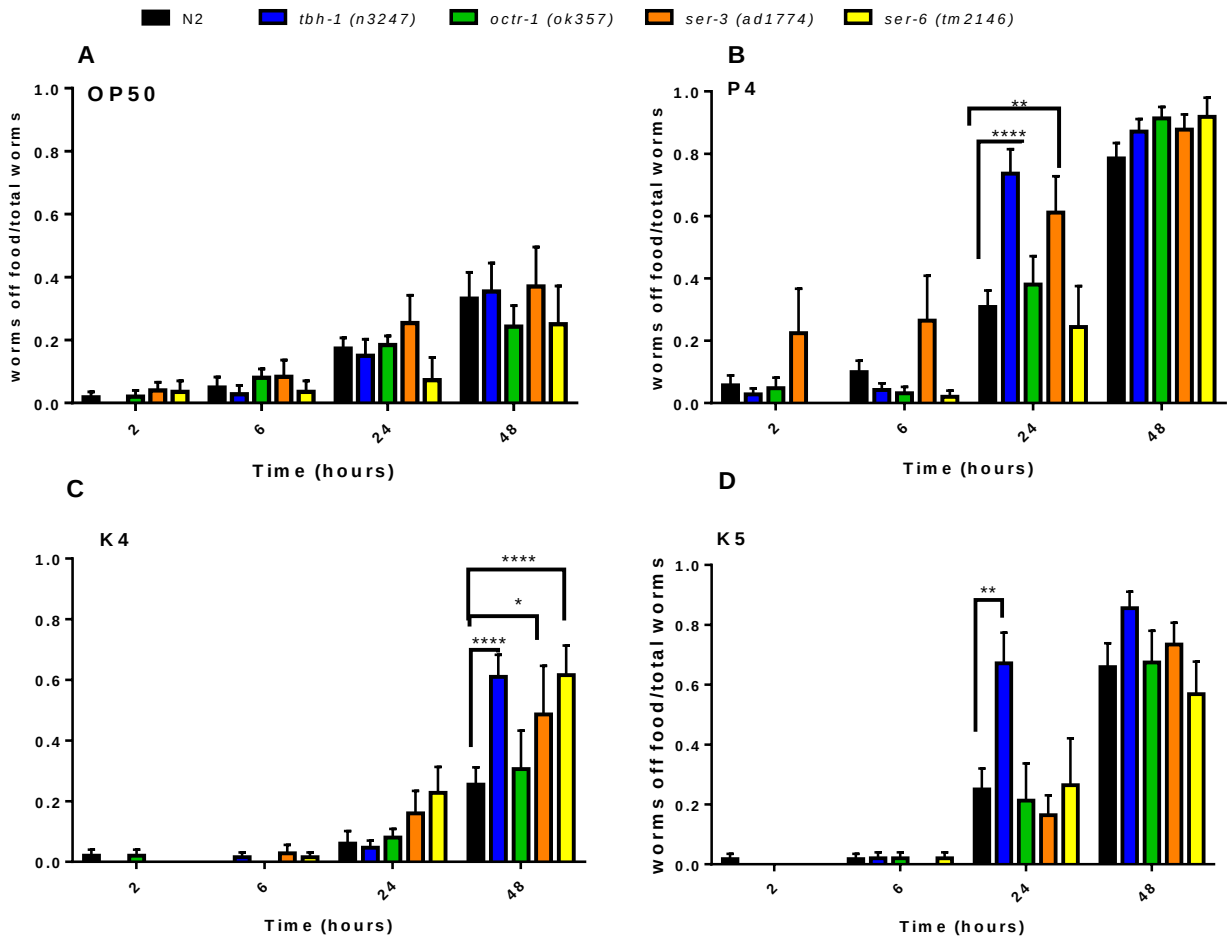


Figure 6.14: The food aversion levels of *C. elegans* octopamine receptor mutants. N2, *tbh-1 (n3247), octr-1 (ok371), ser-3 (ad1774*) and *ser-6 (tm2146)* in response to exposure to lawns of *E. coli* OP50 (A) and the three strains which *tbh-1 (n3247)* show enhanced aversion to: *P. aeruginosa* P4 (B) and *K. pneumoniae* K4 (C) and K5 (D). Data representative n≥3 trials. Error bars represent ±SEM. Data analysed by two-way ANOVA with Dunnett's multiple comparison test to N2. *P<0.05, **P<0.01, ***P<0.001 and ****P<0.0001.

6.4 Discussion

6.4.1 Amine signalling alters food aversion induced by pathogenic bacteria

It has previously been reported how important the four biogenic amines serotonin, octopamine, dopamine and tyramine are in *C. elegans* biology (Chase and Koelle, 2007)(6.1.1). This includes coordinating responses to harmful stimuli, such as heat shock and noxious chemicals (Chao *et al.*, 2004; Wragg *et al.*, 2007; Mills *et al.*, 2012b; Tatum *et al.*, 2015). More relevant to this thesis, serotonin has been shown to promote aversive responses in *C. elegans* (Chao et al., 2004). This includes responses to pathogenic bacteria where *tph-1* animals are deficient in aversive learning towards *P. aeruginosa* PA14 (Zhang *et al.*, 2005; Melo and Ruvkun, 2012).

In this chapter, it has been observed that serotonin deficient *(tph-1 (n4622)) C. elegans* exhibited reduced levels of food aversion upon exposure to bacterial strains identified as being both aversive and pathogenic towards *C. elegans* (6.3.1); the *K. pneumoniae* strain K5 and the *P. aeruginosa* strains P2 and P4, as well as the previously identified aversive pathogenic *P. aeruginosa* PA14. This confirms the idea that serotonin is required for food aversion upon exposure to pathogenic bacteria as has been previously described (Zhang *et al.*, 2005). The assays measuring C*. elegans* food aversion levels demonstrated that the most marked difference in food aversion between wild type and *tph-1 (n4622) C. elegans* was generally seen at 24 hours. This differential is less marked after 48 hours of exposure hours with only the K5 strain leading to a significant variation between N2 and *tph-1 C. elegans*. This indicates that serotonin signalling occurs in promoting food aversion upon exposure to different aversive bacterial strains. This also indicates that there is an additional signal from a different molecule occurring which acts to drive food aversion other than serotonin as *tph-1 (n4622)* exhibit delayed rather than consistently reduced food aversion behaviour.

In contrast to the reduced food aversion seen in *tph-1 (n4622)* animals when *tbh-1 (n3247)* (octopamine deficient) and *tdc-1 (n3419)* (tyramine and octopamine deficient) were screened against pathogenic bacterial strains, enhanced food aversion was observed in the P4 and K5 strains, with the strongest differential being seen after 24 hours of exposure (6.3.1). The enhanced food aversion to P4 and K5 appears to be octopamine dependent as the behaviour of *tdc-1 (n3419)* animals in these assays has an identical phenotype to *tbh-1*

(n3247) animals. This all suggests that octopamine signalling acts in wild type animals to suppress food aversion upon exposure to these bacterial strains.

The opposing phenotypes observed in serotonin deficient *(tph-1)* and octopamine deficient *(tbh-1)* animals suggests physiological between these biogenic amines as previously described in *C. elegans* (Horvitz *et al.*, 1982; Niacaris and Avery, 2003). The results reported here show that amine signalling is involved in controlling food aversion to the aversive pathogenic bacterial strains identified earlier, with serotonin acting to promote food aversion and octopamine acting selectively to reduce it in wild type animals. The fact that enhanced food aversion was only observed in *tbh-1 (n3247)* mutants for two out of the four bacterial strains the *C. elegans* tested indicates that there is complex neuronal signalling that follows exposure to bacteria. This is supported by the observation that no biogenic amine mutant displayed consistently lower or higher levels pathogen induced food aversion in comparison to N2 *C. elegans* (6.3.1) (Figure 6.4).

In addition to the roles implicated for serotonin and octopamine, the results in this chapter provide evidence for dopamine also having a selective role in controlling food aversion, with *cat-2* mutants having reduced food aversion levels upon exposure to selective strains (PA14 and K5).

6.4.2 Amine-deficient *C. elegans* do not have altered immune function in the context of bacterial infection.

As the biogenic amine mutants displayed an array of food aversion phenotypes, it was important to understand whether this change in behavioural output corresponded with the relative virulence of the bacteria. In Chapter 4 it was described how strains of *K. pneumoniae*, *A. baumannii* and *P. aeruginosa* can in a strain selective fashion displayed different levels of pathogenicity and food aversion in *C. elegans.*

It has previously been reported that serotonin is involved in the modulation of immune function upon infection of *C. elegans* with the natural *C. elegans* pathogen *Microbacterium nematophilum,* and *octr-1* deficient animals showed an elevated immune response and

enhanced resistance to PA14 infection(Anderson *et al.*, 2013; Liu *et al.*, 2016). However, when *tph-1, tbh-1, tdc-1* and *cat-2* mutant lifespans were measured on the aversive bacteria these mutants had no altered lifespan compared to wild type *C. elegans* (6.3.2). This indicates that any impact the biogenic amines do exert on *C. elegans* innate immunity is not expressed in the ability of bacterial strains to kill *C. elegans*. Further evidence that amines do not significantly alter the *C. elegans* immune function are provided by examining the colonisation and clearance data obtained after exposure to *P. aeruginosa* strains (6.3.3), which suggests that additional barometers of innate immune output are not modulated by any class of biogenic amine signalling. Whilst modulation in the numbers of bacterial CFUs inside exposed animals was observed in both *tph-1* and *tbh-1* mutants after exposure to three of the *P. aeruginosa* strains there was no significant alteration in the clearance of *P. aeruginosa* in either serotonin or octopamine deficient *C. elegans* in comparison to wild type.

Together these data suggest that biogenic amines have a role in controlling a behavioural response towards bacterial pathogenicity, consistent with the literature (Zhang *et al.,* 2005, Melo and Ruvkun, 2012). However, this effect is not exhibited in terms of pathogen resistance or in the modulation of the intestinal environment after bacterial infection, which previous data in this thesis have implicated a role for other neurosecretory pathways acting in (5.3.6) (Kawli and Tan, 2008).

6.4.3 Octopamine acts to modulate food aversion in *C. elegans* to other bacteria

Two of the three aversive pathogenic bacterial strains identified in Chapter 4, namely K5 and P4, generated enhanced aversion in *tbh-1 (n3247)* (6.3.1,6.4.1). *tbh-1 (n3247)* were also exposed to lawns of the bacterial strains that were characterised in results chapter 2 as non-aversive. This revealed that exposure of octopamine deficient *C. elegans* to the *K. pneumoniae* strain K4, previously identified to be non-aversive yet pathogenic (4.3.2), led to enhanced food (6.3.4). This shows that octopamine is not only suppressing the food aversion response in wild type *C. elegans* elicited by highly aversive strains, but also regulates food-leaving in the context of less aversive bacteria. However, the bacteria strains which selectively elicit this response in the animals are pathogenic towards *C. elegans*, as

determined by reduction of the lifespan of the animals in 4.3.1 (Table 6.3). Interestingly, enhanced food aversion elicited by K4 in *tbh-1 C. elegans* is most noticeable after 48 hours of exposure, whereas for both K5 and P4 the biggest differential between *tbh-1* and N2 *C. elegans* was observed after 24 hours of exposure. This shows that even though a range of pathogenic strains elicit a similar behavioural phenotype the expression this phenotype follows different time courses upon exposure to the different bacterial strains. This indicates that octopamine signalling acts to suppress food aversion in wild type *C. elegans* according to a different time course depending on the individual bacterial strain the animals are being exposed to. This may be representative of the fact that the signal which drives the responses builds up on a different time course upon exposure of *C. elegans* to the different strains. Alternatively, the underlying octopamine signalling that drives the behavioural response in the animals might be acting via a different mechanism.

6.4.4 Fosmid rescue of *C. elegans* tbh-1 enhanced food aversion

As the enhanced food aversion phenotype was seen in octopamine deficient animals but not in tyramine and octopamine double mutants (*tdc-1* mutants), this selective behavioural response appears to be octopamine dependent. Rescuing the native *C. elegans tbh-1* phenotype in *tbh-1 (n3247)* animals enable this octopamine dependency to be validated. Transgenic lines were generated and functional rescue was first checked for using a simple and established *tbh-1* dependent behaviour, namely the octanol avoidance test. This particular experiment, along with a related experiment which examines avoidance from 100% octanol, has demonstrated that octopamine signalling acts antagonistically against serotonin stimulated aversive behaviours, such that *tbh-1* deficient *C. elegans* are more sensitive towards these aversive odours (Wragg *et al.*, 2007; Mills *et al.*, 2012a). These octanol avoidance experiments have been utilised to identify the roles of the individual *C. elegans* octopamine receptors and the physiological antagonism between serotonin and octopamine signalling in *C. elegans.* Specifically, octopamine acts to prevent the reversal response, stimulated by food and serotonin, by signalling through the OCTR-1 receptor on the ASH sensory neurons, signalling from the other octopamine receptor SER-3 directly antagonising this action. In addition, this simple experimental set up has also been utilised

to identify the role of food and neuropeptides in controlling this behaviour (Chao *et al.*, 2004; Wragg *et al.*, 2007; Harris *et al.*, 2010; Mills *et al.*, 2012b). As the food aversion shown upon exposure to lawns of pathogenic bacteria is also an aversive behaviour, this experiment is a good analogue for the octopamine dependent behaviour described in this chapter. The results of this 'smell on a stick' test demonstrated that the mutant animals injected with *pmyo-3::GFP* alone (the control line) initiated backward movement with a shorter latency than wild type N2 animals, the same effect as was seen in the *tbh-1 (n3247)*. Examination of two *tbh-1* rescue lines showed that both of these lines initiated reversal in response to dilute octanol in a similar time to what is seen in wild type animals.

As discussed above, the enhanced food aversion effect observed in the *tbh-1* animals was time dependent, with the biggest differential being observed at a snapshot measurement after 24 hours of exposure on the bacterial lawn (6.3.1). Undertaking analysis of a more detailed time course revealed more detail about the enhanced *tbh-1(n3247)* mutant aversion over a 48 hour exposure to bacterial lawns, by developing a clearer time course through which the enhanced food aversion phenotype is generated. Performing this more detailed analysis revealed that the enhanced food aversion is generated at a faster rate upon exposure to lawns of both P4 and K5, whereas upon exposure to K4 this response is generated more slowly (6.3.5.2.2). This elucidates more detail about the time course discussed in 6.4.3. This further indicates that there are differential forms of neuronal signalling within *C. elegans*, upon exposure to lawns of different bacterial including the strains in which *tbh-1* mutant animals elicit enhanced food aversion. As described in chapter 1, different neurons are involved in coordinating the *C. elegans* response to different chemical signals (1.8.2). This includes the differential response to aversive pathogenic bacteria, where distinct circuits involving AWB and AWC neurons in one circuit and the ADF neurons in another (Ha *et al.*, 2010). It may be that signalling from different neurons which function in circuits involving octopamine as a neurotransmitter act to mediate these food aversion responses.

This more detailed time-course was deployed for the characterisation of the *C. elegans tbh-1* rescue lines. In terms of the lines injected with the *tbh-1* fosmid, statistical comparison to N2 animals alone was sufficient to determine successful rescue of the *tbh-1* enhanced phenotype in the case of the two *K. pneumoniae* strains (K4 and K5) (Figure 6.13). This leads

to the conclusion that for both K*. pneumoniae* strains, the enhanced food aversion exhibited in octopamine deficient *C. elegans* is due to the absence of the *tbh-1* gene. However, the phenotype seen in the rescue lines upon exposure to the *P. aeruginosa* P4 strain is more complex. Significant differences in food aversion are seen between both rescue lines and wild type *C. elegans*. However, comparison to *tbh-1 (3247)* animals revealed that both rescue lines did exhibit significantly lower food aversion than octopamine deficient animals. This indicates at least a partial rescue of the enhanced food aversion phenotype. This at least partial rescue for both of the *tbh-1* injected lines indicates that the enhanced food aversion exhibited by *tbh-1* mutant *C. elegans* is octopamine dependent. The fact that the level of rescue is not consistent across the three strains of bacteria could be due to mosaic expression of the *tbh-1* gene in the transgenic lines. The localisation of the expression of the *tbh-1* gene, and as a result the relative levels of octopamine produced, is impossible to determine through microinjection. This is especially true when dealing with octopamine, being synthesised in only two sets of cells in the entirety of *C. elegans*, the RIC neurons and the cells in the gonad sheath(Chase and Koelle, 2007). Performing cell specific rescue of RIC in *tbh-1* mutants or cell specific ablation in wild type *C. elegans* might act as an additional indicator of the octopamine modulation of enhanced food aversion behaviour, as good supplanting *tbh-1* mutants with exogenous octopamine. Nonetheless there is sufficient evidence here to suggest that the enhanced food aversion seen in *tbh-1 (n3247)* mutants can be rescued by the reintroduction of the native *C. elegans tbh-1* gene by microinjection.

6.4.5 *C. elegans* octoapamine receptors differentially modulate enhanced food aversion

From the results obtained with the *tbh-1* mutant *C. elegans* described earlier in the chapter, it is clear that differential amine signalling is involved in the behavioural response to the aversive pathogenic strains characterised in chapter 4. This is even the case when examining the bacterial strains which generated an enhanced food aversion response in *tbh-1 (n3247)* mutants, with different time courses and different levels of rescue for this phenotype (6.4.3, 6.4.4). The receptors which coordinate octopamine dependent behaviours in *C. elegans* have been identified (6.4.4) (Wragg *et al.*, 2007; Mills *et al.*, 2012b).

In agreement with the results discussed above it appears that there are differential roles for octopamine signalling in controlling enhanced food aversion to exposure to P4, K4 and K5. For P4, there is a clear role for *ser-3* being involved in transmitting octopamine dependent suppression of food aversion, with *ser-3* animals mimicking the enhanced food aversion exhibited by *tbh-1* animals. In addition, K4 food aversion involves both the *ser-3* and *ser-6* receptors. However, there is no detectable role for any individual receptor in modulating aversion upon K5 exposure. This suggests that signalling through multiple or even all three octopamine receptors is required in this case. This indicates that rather than individual sub classes of octopamine receptors are involved in suppression of pathogen-induced food aversion, multiple receptors are needed to transmit the signal in order to generate this response. In this context it is interesting to note that distinct, even antagonising roles have been previously described for discrete octopamine signalling pathways (Mills *et al.*, 2012b). Specifically, OCTR-1 and SER-3 signal antagonistically in coordinating the *C. elegans* response to dilute octanol, whereas SER-6 acts on its own pathway entirely to coordinate the response to 100% octanol. This comparison is underpinned by the different octopamine receptors being expressed in different neurons (Table 6.4) (Mills *et al.*, 2012b). In light of this, and combined with the two sets of results discussed above, the fact that different receptors are implicated in controlling the *C. elegans* aversive responses to three different bacterial strains, two of *K. pneumoniae* and one of *P. aeruginosa* may not be surprising.

6.4.6 Differential octopamine signalling acts to modulate *C. elegans* food aversion

As indicated in (6.1.1.3.2) octopamine has been implicated in controlling a repertoire of behavioural responses including modulating feeding, egg laying, the oxidative stress response and aversion to noxious chemicals (6.1.1.3.2)(Horvitz *et al.*, 1982; Niacaris and Avery, 2003; Alkema *et al.*, 2005; Chase and Koelle, 2007; Wragg *et al.*, 2007; Suo *et al.*, 2009; Mills *et al.*, 2012a; Mills *et al.*, 2012b; Hoshikawa *et al.*, 2017). The different profiles of the enhanced food aversion seen in *tbh-1 (n3247)* for the different strains of bacteria examined in this chapter in terms of the time course expressed, the level of rescue and the receptors involved could reflect this range of signalling. It is also important to note it is not clear exactly which interaction between the host *C. elegans* and the pathogenic bacteria

triggers this food aversion. Olfactory (smell), gustatory (taste) mechanosensory (touch) and the colonisation of the bacteria, or a combination of these may all trigger the food aversion response first investigated in chapter 4, by signalling through a range of octopamine driven pathways. All of the range of bacteria examined could smell, taste and interact with the *C. elegans* intestine very differently, and thus could differentially stimulate a wide range of octopamine signalling pathways. Despite TBH-1 being synthesised in only one pair of neurons (RIC) and the cells of the gonad sheath (Table 6.1,6.1.1.3), the potential reach of octopamine signalling is very broad, with the three octopamine receptors being expressed in a wide range of *C. elegans* tissues (Table 6.2, Table 6.4) suggesting that the three bacterial strains in which an octopamine dependence is observed may be stimulating neurons in distinct circuits resulting in the wide range of responses observed. The data presented here also implicate a role for biogenic amine independent signalling occurring upon exposure to bacteria, due to the different responses observed.

6.4.7 A model of biogenic amine mediated food aversion of pathogenic bacteria

The data described in this chapter have been used to formulate the following model to explain the food aversion behaviour in the context of pathogenic strains of bacteria (Figure 6.15). For two of the pathogenic bacterial strains (P4 and K5) the observations are consistent with a model in which serotonin acts to promote food aversion whereas octopamine acts to suppress food aversion upon exposure to pathogenic food sources. Thus, it would seem that serotonin and octopamine are acting as physiological antagonists to regulate the behavioural response. This is consistent with reports of physiological antagonism between serotonin and octopamine in other behavioural assays with it being postulated that serotonin and octopamine are used to signal the presence and absence of food respectively (Horvitz *et al.*, 1982; Niacaris and Avery, 2003; Alkema *et al.*, 2005; Wragg *et al.*, 2007). Following exposure to the bacteria sensory neurons are stimulated which leads to the release of serotonin and octopamine. This release could depend on a cue driven by consumption of the bacteria, leading to signals being processed in the intestine, or by mechanosensory or chemosensory (olfactory or gustatory) cues. In this model the relative

level of serotonin signalling against octopamine signalling will determine the behavioural fate of the animals, in a see-saw like manner (Figure 6.15). Stronger serotonin signalling, as seen for example in the case of exposure to the *P. aeruginosa* strains P2 and PA14, may lead to a higher level of food aversion. Conversely stronger octopamine signalling, as seen upon exposure to *K. pneumoniae* K5, K4 and *P. aeruginosa* P4, leads to a higher proportion of worms remaining on the food lawn (food attraction). However, when *tbh-1 (n3247)* mutants are exposed to these bacterial strains, the lack of octopamine signalling leads to higher levels of food aversion than observed with wild type animals (Figure 6.15).

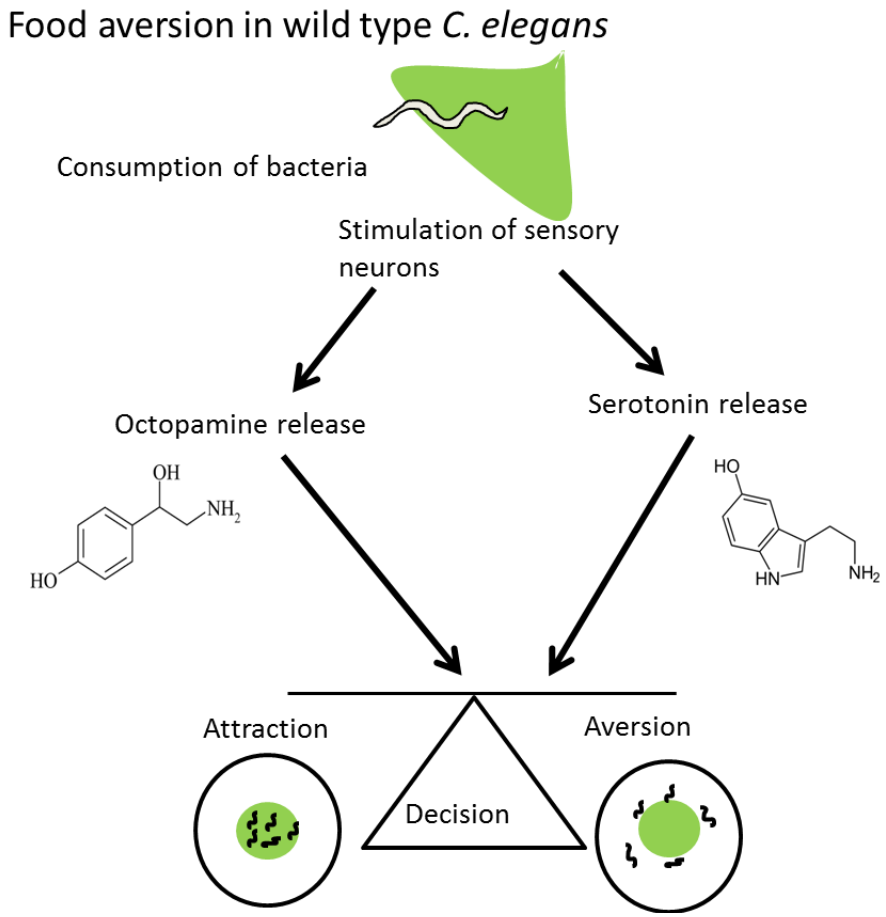


Figure 6.15: A model of serotonin and octopamine signalling controlling the *C. elegans* food aversion response

6.4.8 Summary

In this chapter it has been demonstrated that both serotonin and octopamine play roles in controlling the aversive behaviour towards lawns of pathogenic bacterial strains. Serotonin acts to promote food aversion towards pathogenic bacteria, demonstrating that the serotonin dependent response previously described to drive aversion towards *P. aeruginosa* PA14 is conserved following exposure to a range of pathogenic bacteria (6.3.1) (Zhang *et al.*, 2005; Zhang and Zhang, 2012). A novel role for octopamine has also been described here, specifically that this neurotransmitter selectively acts to supress *C. elegans* food aversion upon exposure to certain strains of pathogenic bacteria. This effect is seen upon exposure to three of the bacterial strains which have been examined in this thesis. These strains are the aversive pathogenic strains *P. aeruginosa* P4 and *K. pneumoniae* K5, and the less aversive but still pathogenic *K. pneumoniae* K4. Not only is this effect only seen during exposure to certain bacterial strains (6.3.4), but this is also a variable response. This particular behaviour differs in terms of the time course elicited (6.4.3), the level to which these phenotypes can be rescued (6.4.4) and also in the receptors involved in coordinating the neural signals (**Error! Reference source not found.**). This all strongly suggests that exposure to these d ifferent bacterial strains stimulate different or overlapping neural responses and circuits in *C. elegans*.

That a differential response is observed in different bacterial strains is not surprising as infection with different bacteria has previously been demonstrated to provoke a range of immune signalling pathways in *C. elegans* (1.6). It should also be noted that when serotonin deficient *C. elegans* are exposed to lawns of bacteria, even though the mutant animals exhibit lower levels of food aversion after 24 hours of exposure, in three out of the four of the aversive pathogenic strains, wild type levels of food aversion are observed after 48 hours. A similar effect is also seen with regards to the octopamine mutants, with the wild type and octopamine deficient animals exhibiting similar food aversion levels after 48 hours of exposure to both the K5 and P4 strains. This suggests that there are other pathways which control *C. elegans* food aversion upon exposure to pathogenic bacteria alongside amine signalling. It has previously been discussed how peptides, along with biogenic amines act to drive food aversion (Chen *et al.*, 2013; Meisel and Kim, 2014). This suggestion of

parallel and compensatory signalling is not surprising. Neurons which have a number of functions could have potential roles in coordinating the food aversion response. Pathways involved in regulating *C. elegans* feeding and immune response could be involved in addition to those related to chemosensation. This signalling could trigger response pathways that may well overlap in their components and biological effects. It was described in chapter 1 how parallel signalling occurring in different pathways occurs in the immune response (1.6) (Irazoqui *et al.*, 2010b). This could also be the case in food aversion. As food aversion behaviour is related to avoiding harmful food sources, this behaviour will be crucial for survival of *C. elegans* in the natural environment. Because of this, the presence of parallel signalling by different pathways, as implied from the discussion of results in this chapter pertaining to biogenic amines, governing this crucial behaviour is not surprising.

Chapter 7: General Discussion

7.1 Principal findings of this thesis

The main aims of this thesis were to develop *C. elegans* as a bacterial infection model utilising a comparative approach. This involved investigating how *C. elegans* responded to lawns of Gram-negative ESKAPE bacteria. In particular the aversive behaviours expressed when *C. elegans* are exposed to pathogenic bacteria was examined. Following on from these measurements, further analysis was undertaken in order to determine the biological controls of both bacterial pathogenicity and bacteria- induced food aversion in *C. elegans*.

The main findings, as described in Chapters 3-6 were:

- *C. elegans* adults elicit an enhanced food-leaving response from an *E. coli* OP50 lawn as a result of a cue emitted from larval progeny.
- Strains of *K. pneumoniae, A. baumannii* and *P. aeruginosa* exhibit a range of virulence in the *C. elegans* infection model.
- The virulence in some strains of bacteria is reflected through food aversion behaviour.
- The *C. elegans* intestine can be colonised be a range of both pathogenic and non-pathogenic bacteria.
- Neuropeptides are involved in modulating the clearance of bacteria from the *C. elegans* intestine.
- The biogenic amines serotonin and octopamine regulate the food aversion response elicited towards bacterial lawns.

7.2 *C. elegans* elicits different biological responses upon extended exposure to different kinds of bacteria

The results gained in chapter 3 show that whilst *E. coli* OP50 is not pathogenic towards *C. elegans* at standard laboratory conditions, as determined by previous studies, extended exposure to lawns of *E. coli* OP50, leads to the animals eliciting an enhanced food-leaving response (3.3) (Garsin *et al.*, 2001). This effect is mirrored by an increased proportion of *C. elegans* off a bacterial lawn. Aversive *C. elegans* behaviours are exhibited in response to a

number of aversive insults, including high levels of metabolic gases, alkaline pH and toxic metals, in addition to dispersal triggered by ascaroside signalling (Sambongi *et al.*, 1999; Gray *et al.*, 2004; Hallem and Sternberg, 2008; Srinivasan *et al.*, 2012; Sassa *et al.*, 2013). As part of the studies examining *C. elegans* as a bacterial infection model, it has been described how *C. elegans* populations learn to avoid lawns of pathogenic *P. aeruginosa* and *S. marcescens*, as well as otherwise benign bacterial lawns that can elicit RNAi in nematodes or bacterial toxicants (Pujol, 2001; Zhang *et al.*, 2005; Pradel *et al.*, 2007; Melo and Ruvkun, 2012).

However, exposing *C. elegans* populations to the aversive pathogens (as initially classified in chapter 4) all generates higher levels of food aversion through aversive learning than that which is seen on lawns of OP50 which is driven by OP50. This indicates that whilst the progeny induced food-leaving on lawns of *E. coli* OP50 could be a contributing factor whilst analysing populations of *C. elegans* adults on bacterial lawns, this does not confound the more profound food aversion effect which is elicited by *C. elegans* on lawns of aversive pathogenic bacteria.

7.3 *C. elegans* exhibits differential biological responses to lawns of ESKAPE bacteria

In this thesis a comparative approach was taken in which several strains of bacteria were examined in *C. elegans* populations. Taking this approach also allowed a range of different *C. elegans* responses to the bacteria to be examined. It has previously been described how bacterial infections can be differentially elicited in different bacterial pathogens, and how *C. elegans* can respond differentially upon infection (1.6). In addition, previous studies have demonstrated that *C. elegans* responds differently to different non-pathogenic bacteria, including those from the natural environment (Shtonda and Avery, 2006; Samuel *et al.*, 2016). In chapter 4 it was shown that strains of *K. pneumoniae, A. baumannii* and *P. aeruginosa* generated different responses in *C. elegans* in relation to how pathogenic the various strains were with respect to lifespan and the food aversion response that is exhibited by *C. elegans* exposed to bacterial lawns for 48 hours. Deeper analysis of the enhanced food aversion phenotype in chapter 6 revealed that this response to being

exposed to bacterial pathogens can be modulated differentially through different aspects of biogenic amine signalling. Analysis of the regulation of bacteria in the intestine in chapter 5 also revealed that the strains of *P. aeruginosa* were removed from the intestine to a differential degree. The differences between the pathogenesis of *C. elegans* by the moderately pathogenic *P. aeruginosa* strain P1 and the highly pathogenic PA14 have previously been investigated, and the results obtained in this thesis demonstrate that differences can be seen when an array of strains are examined (Mikkelsen *et al.*, 2011). For instance, examining six different *P. aeruginosa* strains (including the well described PA14) in chapter 4 revealed a range of *C. elegans* lifespans, with total worm death being observed between 8 and 14 days upon exposure to the different bacterial strains. Furthermore three *P. aeruginosa* strains were subsequently classed as aversive pathogens, and the levels of clearance of the strains of *P. aeruginosa* also varied. All of these investigations have revealed that bacteria can be treated very differently after *C. elegans* is exposed to them. The fact that this variation is also observed between strains of the same species rather than just different species of bacteria, builds on previous studies where different bacterial strains were analysed in the *C. elegans* pathogenesis model (Garsin *et al.*, 2001; Mikkelsen *et al.*, 2011; Lavigne *et al.*, 2013).

7.4 Neuronal signalling identified to coordinate *C. elegans* behaviours identified in this thesis

This thesis has examined mutant *C. elegans* strains to begin to unpick the biological controls of progeny stimulated food leaving, clearance of bacteria and pathogen triggered food aversion. This analysis has identified neuronal signalling utilising both neuropeptides and biogenic amines.

7.4.1 Neuropeptide signalling

As described in 1.8.3.2, neuropeptides have wide ranging functions in *C. elegans* biology, including previously defined roles in the *C. elegans* immune response upon bacterial

infection (Mallo *et al.*, 2002; O'Rourke *et al.*, 2006; Troemel *et al.*, 2006; Alper *et al.*, 2007; Alegado and Tan, 2008; Evans *et al.*, 2008; Li and Kim, 2008; Roeder *et al.*, 2010; Holden-Dye and Walker, 2013). Among these functions that neuropeptides perform in the interactions between *C. elegans* and bacteria include both neuropeptides themselves as well as the neuropeptide receptor NPR-1 acting to promote the aversion response of *C. elegans* towards pathogenic bacteria (Reddy *et al.,* 2009, Chen *et al.,* 2013, Meisel *et al.*, 2014).

In this thesis, neuropeptide signalling has been implicated in two different neuromodulatory contexts *C. elegans*. The first of these is the nematocin signalling acting to enhance *C. elegans* food leaving on a non-pathogenic food bacterial lawn, in response to a cue generated by *C. elegans* larval progeny, as described in Chapter 3. The second of these is neuropeptide signalling acting to reduce clearance of pathogenic bacteria from the *C. elegans* intestine, which as discussed in chapter 5 could be through disruption of neuropeptide signalling by infecting pathogens.

7.4.2 Serotonin and octopamine signalling

In addition to neuropeptide signalling, the results in chapter 6 implicate a role for neuronal signalling utilising both serotonin and octopamine in controlling the food aversion response to aversive bacteria first described in Chapter 4. Again, this signalling is an example of the differential response that occurs in *C. elegans* upon interacting with lawns of distinct bacterial strains as discussed in 7.3. This investigation has also begun to reveal how *C. elegans* aversion of pathogenic bacterial lawns appears to be controlled differently on a strain by strain basis, with different neurotransmitters, receptors and being seen to control behaviours and with different time courses being observed with differential transmitters, receptors and time courses being demonstrated (6.4.8). In addition, this aspect of the investigation provides another example of the antagonistic signalling previously described to occur between serotonin and octopamine (Horvitz *et al.*, 1982; Niacaris and Avery, 2003; Alkema *et al.*, 2005; Wragg *et al.*, 2007). This reinforces the established idea of serotonin signalling in controlling aversion towards pathogenic insults (Zhang *et al.*, 2005; Chase and Koelle, 2007; Melo and Ruvkun, 2012; Zhang and Zhang, 2012; Flavell *et al.*, 2013; Song *et al.*, 2013).

However, whilst biogenic amine signalling acts to control the aversive response to pathogenic bacteria, results gained in chapter 3 indicate that biogenic amines are not involved in promoting enhanced food leaving in response to larval progeny. The differing neurochemical basis of coordinating different *C. elegans* behaviours which both result in an increase proportion of animals off a bacterial food lawn indicates that different circuits are involved in controlling different behaviours in *C. elegans*.

Evidence for this idea of different circuits driving *C. elegans* behaviours also comes from the fact that it has been demonstrated how there are different cues which act to drive the different aversive responses. It has previously been described how different neuronal circuits are involved in controlling the *C. elegans* aversion of PA14 lawns (Ha *et al.,* 2010). The enhanced food leaving behaviour described in chapter 3 has been hypothesised to be driven by an ascaroside signal emanating from *C. elegans* larvae. Alternatively the aversion stimulated by pathogenic bacterial lawns, as discussed in 7.1, is not confounded by progeny driven food leaving and instead is driven by the bacteria through either the of the detection of aversive chemicals through chemosensation or as a sickness behaviour after consumption of the bacteria (Pradel *et al.*, 2007; Melo and Ruvkun, 2012; Meisel *et al.*, 2014) (Figure 1.12). The fact that different cues are utilised to trigger the behavioural response also indicates that different *C. elegans* neurons are involved in sensing and integrating responses. This thesis has demonstrated that different neurotransmitters are subsequently utilised in the neuronal signalling, with neuropeptide signalling activing to mediate progeny driven food-leaving and serotonin and octopamine signalling mediating aversion of pathogenic bacterial lawns.

7.5 Future implications for using *C. elegans* as a model for bacterial pathogenesis

As described in chapter 1, *C. elegans* has been used to investigate several aspects of bacterial pathogenesis of animal host organisms including mechanisms, virulence factors and host immunity (1.5,1.6). Whilst exposure of *C. elegans* to bacterial lawns did lead to differential responses in some of the measurements taken as mentioned in 7.3, it was also observed that some aspects of the *C. elegans* responses to pathogenic and non-pathogenic bacteria are similar. In chapter 4 it was determined that the feeding rate of *C. elegans*, along

with the reproduction ability, was not affected by exposure by pathogenic bacteria. In addition, in chapter 5 it was determined that both pathogenic and non-pathogenic bacteria colonise the *C. elegans* intestine to a similar degree, with the species of the bacteria determining the level of colonisation rather than the relative pathogenicity of the different strains. These findings demonstrate that taking these particular measurements, at least based on the short-term exposure undertaken in the studies here, of how different bacteria affect different aspects of *C. elegans* biology does not reflect the relative pathogenicity of the different strains. Thus, in the case of the NGM-plate based assays with constant incubation at 20°C utilised in this thesis, examining *C. elegans* feeding, reproduction and bacterial colonisation levels is not a reliable measurement of bacterial pathogenesis in this host organism. This brings up one of the limitations of *C. elegans* as an infection model first laid out in Chapter 1 (1.3.2). If *C. elegans* could be incubated at 37°C, mimicking the internal temperature of a mammalian host, like the alternative invertebrate model *G. mellonella* can, this would more closely resemble mammalian infection and perhaps aspects of the bacterial pathogenesis process would be expressed more readily.

Previous studies have suggested that the ability of different bacteria to colonise *C. elegans* upon infection is an important determination of the relative pathogenicity of the bacteria (Begun *et al.*, 2007; Kuo *et al.*, 2016). The results regarding intestinal colonisation in chapter 5 demonstrate that it is not the number of bacteria inside the *C. elegans* intestine that is important when it comes to bacterial infections, but rather the nature of the bacteria that is important in determining the pathogenicity of the bacteria. The recent studies examining the *C. elegans* microbiota have determined how a large variety of different bacteria can colonise the *C. elegans* intestine, and that this colonisation is not only benign but can also provide health benefits (Berg *et al.*, 2016; Dirksen *et al.*, 2016). In addition, analysis of the slow killing of *C. elegans* by bacteria, which has been examined in this thesis, has determined that it is the bacterial cells themselves which are important in eliciting this kind of infection (Tan *et al.*, 1999a; Labrousse *et al.*, 2000). This further indicates, as mentioned in 5.4.5, that further investigating particular aspects of the biology of *C. elegans* bacterial pathogens, such as the virulence factors present, is more important in understanding bacterial pathogenesis of *C. elegans*, than simple measuring the ability of bacteria to colonise the host.

Although this thesis has addressed some of the shortcomings for using a short term bacterial exposure in *C. elegans* to investigate bacterial pathogenesis, the results in this thesis have nonetheless demonstrated further the effectiveness of as a platform for investigating the bacterial pathogenesis of the ESKAPE species. Exposure of populations of a simple animal to lawns of bacteria can provide very useful insights into the pathogenesis of bacterial species.

7.6 Future directions

7.6.1 Identification of genetic differences between bacterial strains

7.6.1.1 Identification of virulence factors

To fully understand the pathogenesis of the different bacteria analysed in this thesis in the *C. elegans* model, it is important that genetic determinants of the pathogenicity of the strains is determined. This allows identification of what causes the variation in pathogenicity, food aversion and clearance of bacteria from the intestine discussed in the previous section. As set out chapter 1, the *C. elegans* has been used to identify a range of bacterial virulence factors. This includes *P. aeruginosa* and *K. pneumoniae* examples of which were examined in this thesis, as well as the other Gram-negative species *S. enterica, S. marcescens* and *B. cepacia* (Tan *et al.*, 1999b; Labrousse *et al.*, 2000; Gallagher and Manoil, 2001; Hendrickson *et al.*, 2001; Kothe *et al.*, 2003; Kurz *et al.*, 2003; Coulthurst *et al.*, 2004; Tenor *et al.*, 2004; Lee *et al.*, 2006; Papaioannou *et al.*, 2009; Feinbaum *et al.*, 2012; McEwan *et al.*, 2012; Zhu *et al.*, 2015a; Kamaladevi and Balamurugan, 2016b). This approach has previously identified genetic differences which effect virulence in *P. aeruginosa* PA14 and PAO1 (Harrison *et al.*, 2010; Mikkelsen *et al.*, 2011). In this thesis it has been determined that differences between the bacteria as determined by genetics, particular with respect to the virulence factors present in the different strains, is likely to be important in modulating the pathogenicity of the different strains (Chapter 5). In this thesis, the advantages of using a comparative approach have been laid out, allowing comparison of different bacterial strains. However, this approach can also be used to screen different mutant bacterial strains, as has been done in previous studies (Tan *et al.*, 1999b; Gallagher and Manoil, 2001; Kurz *et al.*, 2003; Tenor *et al.*, 2004; Feinbaum *et al.*, 2012). Identification

of mutant bacterial strains which have reduced levels of virulence subsequently allows identification of virulence factors which contribute to the pathogenesis of the pathogenic strains identified in this thesis.

Using bioinformatics to identify the presence or absence of virulence factors identified in these earlier studies in the strains analysed in this thesis may help to gain insight to the controls of pathogenicity in the various strains. Examination of the literature reveals a range of virulence factors that have been identified in *P. aeruginosa* previously, in both PA14 and PA01 (Table 7.1)(Tan *et al.*, 1999b; Gallagher and Manoil, 2001; Feinbaum *et al.*, 2012; Kirienko *et al.*, 2015).

Gene	Function of virulence factor	Strain identified in	References
gacA	Two component regulator	PA14	(Tan *et al.*, 1999b; Feinbaum *et al.*, 2012)
lasR	Quorum sensing regulator	PA14	(Tan *et al.*, 1999b) Feinbaum *et al.*, 2012)
rhiR	Quorum sensing regulator	PA14	Feinbaum *et al.*, 2012)
ptsP	Phosphoenolpyruvate-protein phosphotransferase	PA14	Feinbaum *et al.*, 2012)
pepP	Proline aminopeptidase	PA14	Feinbaum *et al.*, 2012)
PA0745	enoyl-CoA hydratase isomerase	PA14	Feinbaum *et al.*, 2012)
clpA	Chaperone and ATPase	PA14	Feinbaum *et al.*, 2012)
pvdF	Pyoverdin biosynthesis	PA14	(Kirienko *et al.*, 2015)
pvdP	Pyoverdin biosynthesis	PA14	(Kirienko *et al.*, 2015)
hcnC	Hydrogen cyanide synthase	PAO1	(Gallagher and Manoil, 2001)
lasB	Zinc metalloprotease elastase	PAO1	(Gallagher and Manoil, 2001)

Table 7.1: Virulence factors previously identified to mediate *P. aeruginosa* pathogenesis in *C. elegans*. Bioinformatics was used to determine the presence/absence of the genes encoding these factors in the *P. aeruginosa* strains utilised in this thesis.

Bioinformatic based analysis in the *P. aeruginosa* strains analysed in this thesis has been initiated. This was done by comparing the sequences of the virulence factors in Table 7.1 compared against the genomes of strains P1-5. This analysis revealed that nearly all of the virulence factors investigated are present in strains P1-5 (Table 7.2). The only case in which virulence factors are not present, is the two pyoverdin biosynthesis strains pvdP and pvdF being absent from the P4 strain. This indicates that the genes encoding virulence factors previously demonstrated to enhance bacterial pathogenesis in *C. elegans* are present in the P. aeruginosa strains analysed in this thesis. However, this analysis does not give any clues about the expression of these genes and thus there is no definitive indication as to how these genes contribute to pathogenesis of *P. aeruginosa* in *C. elegans*. Examining mutants of the bacterial strains deficient in the genes in Table 7.1 and may give greater insight about the roles these genes play in modulating virulence.

	gacA	lasR	rhiR	PtsP	pepP	PA0745	clpA	PvdF	PvdP	hcnC	LasB
P1											
P2											
P3											
P4											
P5											

Table 7.2: The presence/ absence of the genes described in Table 7.1 in P. aeruginosa strains P1-5. Green indicates gene is present, red indicates gene is absent and amber indicates gene is present but the gene length is different from that found in PA01 (strain P1).

7.6.1.2 Other genetic differences between bacterial strains

In addition to identification of genetic differences which lead to differences of pathogenicity in the bacterial strains, the comparison of the genetic makeup of the different bacterial strains analysed in this thesis allows the deeper investigation about what modulates the differential behavioural responses *C. elegans* exhibit towards the bacterial strains as mentioned in 0. It was identified in chapter 4 that not all strains which are pathogenic towards *C. elegans* generate food aversion responses (4.3.2). In addition, in chapter 6 it was found that octopamine acts to supress food aversion in wild type *C. elegans* upon exposure to specific bacterial strains, but not others. This indicates that bacteria may be giving of

different chemical signals that are then used by the animals to mediate the aversive response which is observed upon *C. elegans* learning that lawns of bacteria are harmful(Zhang *et al.*, 2005). It has previously been how the serrawettin W2, a lipopeptide, which is produced by the pathogenic *S. marcescens* strain Db10, acts to promote *C. elegans* aversion. However another *S. marcescens* strain JESM267 which does not produce W2 is still as pathogenic towards *C. elegans* as Db10 but less aversive (Pradel *et al.*, 2007). The fact that in this thesis pathogenic bacterial strains were discovered which did not elicit aversion in wild-type *C. elegans* indicates that the aversive strains may be producing chemicals or metabolites which *C. elegans* subsequently interprets as aversive. Further analysis of the genomes of the bacterial strains analysed in this thesis may reveal that the aversive strains identified in chapter 4 produce natural products which act to stimulate aversion in *C. elegans*, perhaps similar to those previously identified (Pradel *et al.*, 2007; Meisel *et al.*, 2014). In addition, this type of analysis may also reveal more about the nature of the strains which generate enhanced aversion in octopamine deficient animals. These particular strains (K4, K5 and P4) may all generate a particular or related metabolites or products not produced by the other strains which engage the octopaminergic circuit of C. elegans which was identified in chapter 6.

7.6.2 Further investigation of neural circuits identified in this thesis

7.6.2.1 The specific neuropeptides influencing bacterial clearance

In chapter 5 the role of neuropeptides acting in wild type animals to suppress clearance of bacteria from the nematode intestine was discussed. This analysis utilised *egl-3* animals, deficient in aspects of general neuropeptide maturation. This could be developed to investigate *C. elegans* mutant strains deficient in individual neuropeptides (Kass *et al.*, 2001). It has been discussed how a number of neuropeptides, specifically of the NLP family, are not expressed in the nervous system (Holden-Dye and Walker, 2013). 16 genes encoding for NLPs are expressed in the intestine (*nlp-1, 2, 3, 6, 8, 9, 13, 14, 15, 16, 18, 20, 21, 27, 29* and *37*), and any neuropeptides expressed there could interact with bacteria that colonise that tissue (Li and Kim, 2008; Holden-Dye and Walker, 2013). Examining *C. elegans* mutants in these particular genes could reveal about more about the roles of neuropeptides in modulating clearance of bacteria from the intestine. In addition, a hypothesis was also put

forward in Chapter 5 that infection with pathogenic bacteria leads to upregulated neuropeptide activity, which subsequently leads to a suppression of the *C. elegans* immune response. The study by Evans *et al.* (2008), demonstrated a circuit in which infection with *P. aeruginosa* PA14 upregulates production of the insulin like peptide INS-7, which in turn activates DAF-2 and suppresses upregulation of genes controlled by DAF-16, including those involved with encoding for proteins with an antimicrobial function (Figure 7.1). Examining *C. elegans* mutants deficient in aspects of this particular circuit with the different pathogenic bacterial strains would help to identify whether this circuit is involved in the bacterial infections of *C. elegans* examined in this thesis. This could provide further information about the specificity and generality of responses towards bacterial infection in *C. elegans.*

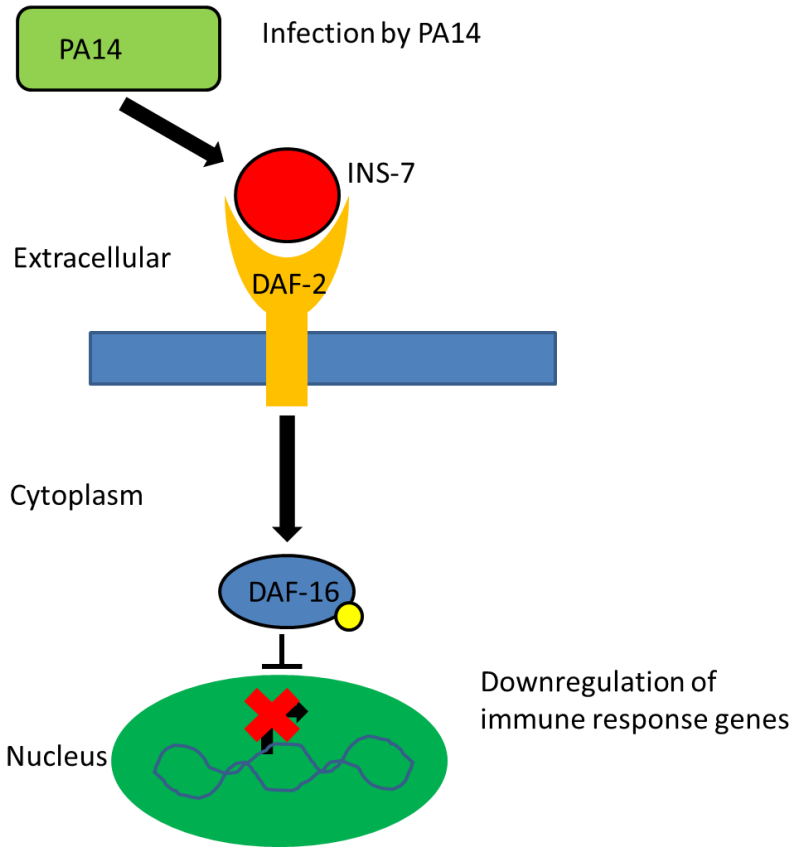


Figure 7.1: The circuit identified by Evans et al. (2008) demonstrating the pathogen stimulated suppression of *C. elegans* immune responses. Infection by *P. aeruginosa* PA14 causes activation of the DAF-2 receptor (section) by the insulin like peptide INS-7 leads to the phosphorylation of the transcription factor DAF-16 and subsequent downregulation of immune response genes, making *C. elegans* more susceptible to infection by the bacteria. This circuit provides an avenue of future investigation of the neuropeptide regulation of clearance of bacteria from the *C. elegans* intestine.

7.6.2.2 The neuronal circuits promoting aversion towards bacterial pathogens

Chapter 4 demonstrated that *C. elegans* is able to exhibit food aversion towards novel strains of pathogenic bacteria in addition to the previously reported examples of *P. aeruginosa* PA14 and *S. marcescens* Db10 and Db11 (Pujol *et al.,* 2001; Zhang *et al.,* 2005; Pradel *et al.,* 2007; Gaglia *et al.,* 2012). In Chapter 6 the neuronal signalling which controls this behavioural phenomenon was investigated and it was found that the two biogenic amines serotonin and octopamine have antagonistic roles in modulating this. However, whilst transmitters acting in this particular behavioural response were identified, no cellular basis for any circuits controlling this was identified.

In previous studies two separate circuits were identified to mediate the food aversion response of *C. elegans* to PA14 (Ha *et al.*, 2010). In order to fully understand the neuronal signalling that occurs to drive *C. elegans* food aversion, the role of individual neurons need to be investigated. This includes neurons which act to sense stimuli, and to coordinate and drive the behavioural response. This includes neurons which function downstream of signalling by serotonin and octopamine identified in chapter 6. As mentioned in chapter 1, successful driving of a food aversion response requires integration of detection of different stimuli (1.8), to learn that lawns of pathogenic bacteria are harmful. Therefore, it is possible that a range of different neurons which are involved in detecting different stimuli, may be involved.

In terms of sensory neurons, which have been most extensively investigated for their roles in controlling *C. elegans* food aversion, the ADF amphid sensory neurons are good candidates for investigation (1.8.1). This pair of neurons has been demonstrated to act in a serotoninergic circuit to promote the aversive learning in chemotaxis assays involving PA14 lawns (Zhang *et al.*, 2005; Ha *et al.*, 2010). Other sensory neurons which may be involved are the AWC and AWB neurons, involved in the attraction to and avoidance of volatile chemicals respectively. These neurons have been implicated in a circuit which becomes modified upon learning that *P. aeruginosa* PA14 is aversive (Ha *et al.*, 2010). In addition, AWB is involved in mediating the avoidance of *S. marcescens* lawns through detection of the serrawettin W2 (Pradel *et al.*, 2007). These previous roles, along with the fact that signalling from these neurons acts antagonistically, with AWC mediated attraction and AWB mediating avoidance, may implicate these neurons in the octopamine and serotonin signalling circuit, which also involves antagonistic signalling as identified in chapter 6. Another amphid sensory neuron

which may be involved is ASH, which is the main nociceptive neuron of *C. elegans*, and controls avoidance from mechanical and chemical stimuli (Bargmann, 2006).

In the octopamine signalling pathway, due to the fact that it is the only class of neuron which expresses *tbh-1*, RIC neurons are very likely to be involved. However, as RIC is an interneuron, identification of the sensory neurons acting upstream of this is also required. However, as discussed in chapter 6, the enhanced food aversion responses observed in octopamine deficient *C. elegans* do not all follow the same time course (6.4.8). This suggests that different neurons may be acting upstream of the RIC neurons to coordinate the suppression of food aversion meditated by octopamine in wild type animals, explaining the differential time course of food aversion observed in *tbh-1 (n3247) C. elegans*.

There is also the additional role that other neurotransmitters may play in mediating the food aversion response. It was mentioned in chapter 6 that there is likely to be parallel signalling acting to coordinate the food aversion response to pathogenic bacteria. Neuropeptide signalling, specifically that by INS-6 and DBL-1, have previously been demonstrated to drive aversion of *C. elegans* to lawns of pathogenic bacteria, in addition to other aversive responses (Zhang and Zhang, 2012; Chen *et al.*, 2013). Investigating mutant *C. elegans* deficient in genes needed to make these peptides, possibly in combination with deficiencies in biogenic amine production, may give further insight into the neurotransmitters involved in coordinating the *C. elegans* aversive response. In addition, results in Chapter 3 have demonstrated how neuropeptides deficient *C. elegans* do have reduced levels of progeny stimulated food-leaving. This suggests that neuropeptides may be important in transmitting and integrating the expression of diverse *C. elegans* behaviours which are involved with driving adult animals off a food lawn. This gives an indication into the scope of neuropeptide signalling in *C. elegans*

If it were the case that signalling from both biogenic amines and neuropeptides were controlling the aversion response it is would not be the only case where these two kinds of molecules have been demonstrated to function in a combined circuit. Hawaiian/C4856 animals have mutations relative to N2 C. elegans in the *tyra-3* and *npr-1* genes. These genes encode for tyramine receptor and a neuropeptide receptor respectively, and these mutations have been demonstrated to contribute to the enhanced food-leaving rate of Hawaiian strain *C. elegans* relative to N2 animals (0). Whilst work undertaken in chapter 3

demonstrated that this particular circuit is not involved in modulating the enhanced food-leaving response to *C. elegans* larvae, a similar circuit in which both neuropeptide and serotonin/ octopamine signalling may be acting to control the food aversion behavioural response to bacterial pathogens examined in this thesis.

In conclusion, this thesis has built on the work of other by further demonstrating that the simple nematode worm *C. elegans* can be used as a platform for investigating species of the ESKAPE group of bacterial pathogens, in addition to other non-pathogenic bacteria. Insights can be gained about the pathogenesis of different bacteria by investigating the behaviour of exposed animals, as well as other aspects of their biology. The results presented and discussed here provide further insight into the pathogenesis of bacteria in a simple animal host and provides opportunities for further investigation of this clinically important phenomenon.

Appendix

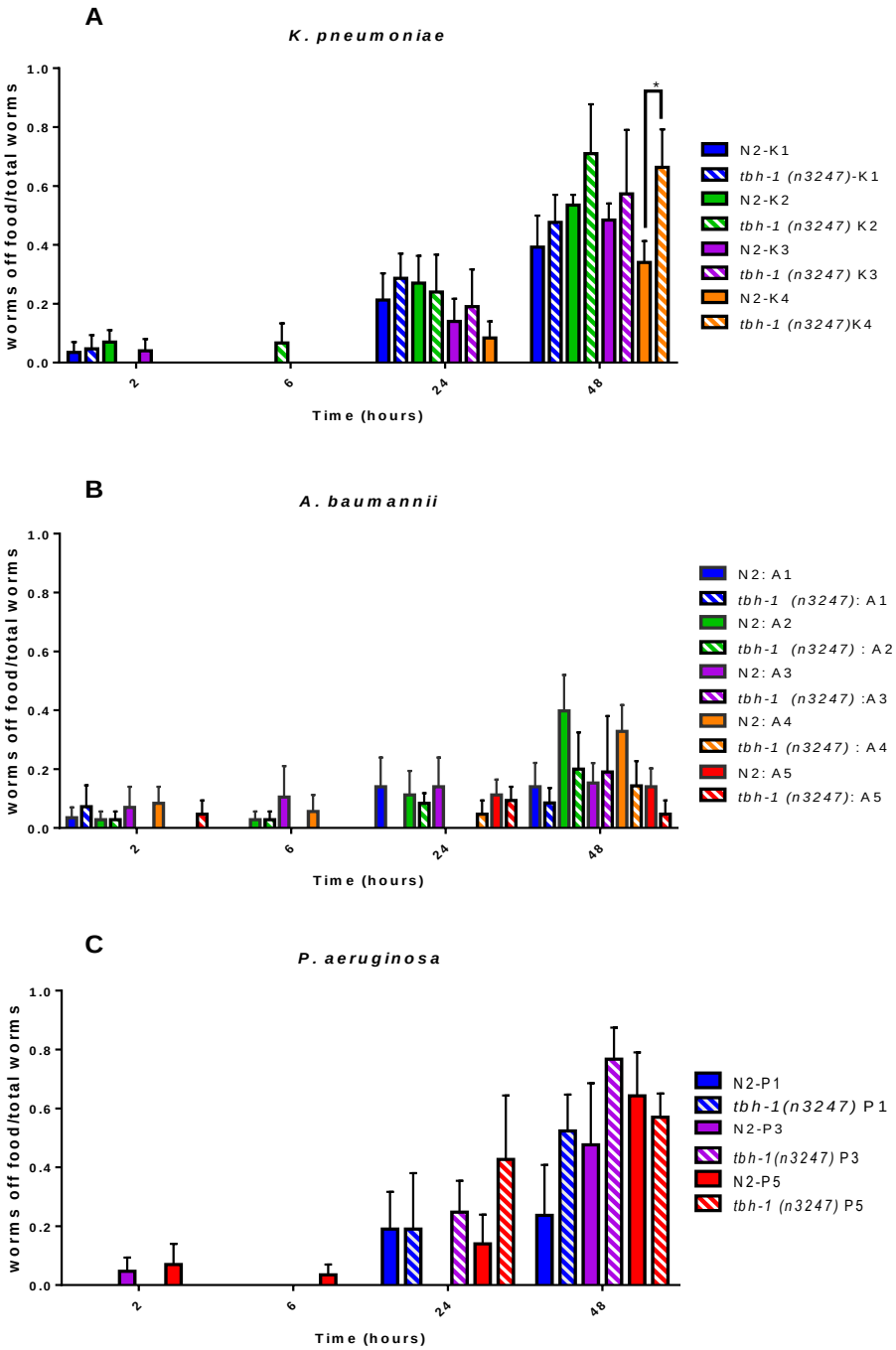


Figure 8.1: Food aversion analysis of non-aversive strains of *K. pneumoniae (A)*, *A. baumannii* (B)and *P. aeruginosa* (C) which were examined in Chapter 4 , in wild type and *tbh-1 (n3247) C. elegans.* Data representative of N≥3 trials. Error bars represent ±SEM. Data analysed by Two-way ANOVA with Sidak's multiple comparisons between wild type and *tbh-1 (n3247) C. elegans* on the same strain of bacteria. *P<0.05, **P<0.01, ***P<0.001 and ****P<0.0001. This data was used to generate the last column of Figure 6.8.

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