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CAENORHABDITIS ELEGANS AS A MODEL TO ELUCIDATE HOST-PATHOGEN INTERACTIONS FOR HUMAN BACTERIAL PATHOGENS

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to my dearest family and love

“The Universe is one great kindergarten for man.

Everything that exists has brought with it its own peculiar lesson.”

Orison Swett Marden

ABSTRACT

The soil nematode *Caenorhabditis elegans* is a popular host utilized to model bacterial virulence and microbial pathogenesis *in vivo*. This thesis explores the use of *C. elegans* for the study of host-pathogen interactions for two Gram-negative bacteria, *Burkholderia thailandensis* and *Salmonella enterica.*

We conducted a RNA interference screen to identify host genes capable of modulating the infection outcomes of *C. elegans* infected with *B. thailandensis*. We discovered that during infection, the cell junction protein LIN-7 appeared to modulate the evolutionarily conserved DAF-2 insulin/IGF-1 signalling pathway, culminating on both the FOXO transcription factor DAF-16 and the heat-shock factor 1. Moreover, LIN-7 regulated nematode survival during infection with other Gram-negative bacteria. Tissue-specific experiments also revealed that this interaction between LIN-7 and the DAF-2 signalling pathway operated mainly in nematode tissues outside the intestine (Paper I).

Through a forward genetics screen using ultraviolet light, we identified *pt1* as a novel allele of the *unc-7* innexin gene. We found that the *pt1* mutant exhibited enhanced survival only when infected with *Burkholderia* spp. We further defined a specific subclass of *unc-7* interacting genes, *unc-9* and *goa-1*, in a unique pathway which probably involves calcium ion fluxes (Paper II).

Next we characterized a new aspect of *S. enterica* virulence. We observed that *S. enterica* provoked oxidative stress in the hypodermal tissues of infected *C. elegans* even though there was no apparent invasion beyond the intestinal epithelium. Via chemical and mutational interference, we found this phenomenon to be deleterious to the host. Genetic inactivation of the bacterial thioredoxin 1 strongly abrogated pathogenicity of *S. enterica* as well as the emergence of oxidative stress, thereby suggesting a novel role for this virulence factor (Paper III).

Finally, we investigated the combinatorial effects of the proton pump inhibitor omeprazole and the salicylidene acylhydrazide INP0010 during *S. enterica* infection. We observed disparate effects when they were used in combination and applied to different infection models including the epithelial and macrophage-like cell lines and *C. elegans*. The nematode can thus provide a platform for testing virulence inhibitors, allowing the elucidation of their mechanisms in the context of a whole organism (Paper IV).

LIST OF PUBLICATIONS

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IV. Speranta Puiac, XIAOHUI SEM, Aurel Negrea and Mikael Rhen. **Small-molecular virulence inhibitors show divergent and immunomodulatory effects in infection models of *Salmonella enterica* serovar Typhimurium.** *International Journal of Antimicrobial Agents* 2011 Nov; 38(5):409-16.

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LIST OF ABBREVIATIONS

B. thailandensis	*Burkholderia thailandensis*
B. pseudomallei	*Burkholderia pseudomallei*
C. elegans	*Caenorhabditis elegans*
cfu	Colony-forming unit
dsRNA	Double-stranded RNA
E. faecalis	*Enterococcus faecalis*
GFP	Green fluorescent protein
L4	Larva-4, fourth larva stage
LPS	Lipopolysaccharide
MAPK	Mitogen-activated protein kinase
NF-κB	Nuclear factor kappa light chain enhancer of activated B cells
NHP	Non-human primate
NO	Nitric oxide
P. aeruginosa	*Pseudomonas aeruginosa*
RFP	Red fluorescent protein
RNAi	RNA interference
ROS	Reactive oxygen species
RTK	Receptor tyrosine kinase
S. enterica	*Salmonella enterica*
S. Dublin	*Salmonella enterica* serovar Dublin
S. Typhi	*Salmonella enterica* serovar Typhi
S. Typhimurium	*Salmonella enterica* serovar Typhimurium
S. marcescens	*Serratia marcescens*
SCV	*Salmonella*-containing vacuole
SPI	*Salmonella* pathogenicity island
T3SS	Type three secretion system

1 INTRODUCTION

1.1 HOST-PATHOGEN INTERACTIONS

Despite remarkable progression in medical research and higher standards of sanitation into the 21st century, infectious diseases caused by bacteria, viruses, fungi and parasites continue to be one of the top major causes of death, disability, and social and economic disruption for millions of people each year [1]. In addition to a permanent background of existing pathogens with evolving abilities for transmission, pathogenesis and drug resistance, newly-emerging and re-emerging pathogens also greatly contribute to the global burden of infection. Strengthening basic and applied research to understand how pathogens interact with their hosts at the molecular and cellular level would enable us to place pathogenesis in different perspectives and thereafter develop more efficient diagnostics, vaccines and therapies. The interaction between the host and the pathogen is a continuous battle: on one end is the pathogen with expression of virulence factors to elicit direct tissue damage, evoke immunopathology or evade host responses; on the other is the host with defence pathways and effector molecules to restrict or eradicate the pathogen, or tolerate the damage caused by the pathogen or the induced immune response. Determining these mechanisms and the outcome of their interactions are crucial to understanding disease pathogenesis.

1.2 *IN VIVO* INFECTION MODELS

The use of animal models complements existing molecular and cellular approaches to study host-pathogen interactions and provide further insights into the various host and pathogenic components required for a successful infection at the whole organism level. In contrast to studies carried out on cultured cell monolayers, those using whole organism models have allowed us to understand the importance of spatial complexity and inter-tissue communication and signalling during infection.

In vivo animal models are continuously being developed to understand disease pathogenesis and can also function as a system to assess the efficacy of antimicrobial compounds and virulence inhibitors. We should be cautious and aware that no single animal model can answer all scientific questions – selection of a suitable host model

can be based on the virulence trait or host infection response to be studied and the amount of available resources, physical space and time commitment. Alternatively, multiple host models can also be utilized in a study to complement each finding and observation [2].

Some pathogens such as the human immunodeficiency virus, hepatitis C and human malaria parasites are strictly host-specific and often cannot be tested on lower mammals. To some degree, these pathogens can infect **non-human primates (NHPs)** such as chimpanzees, macaques and baboons because of their genetic similarities with humans. However this approach poses serious ethical problems because of the increased likelihood that during scientific experiments, NHPs may experience pain and suffering in ways similar to humans [3].

The **murine model** has been by far a popular mammalian host system but the lengthy reproduction time and the difficulties and financial expenses associated with obtaining and maintaining sufficient numbers of animals for experiments are discouraging. Moreover, approaches using mice to screen and discover new antimicrobial compounds and virulence inhibitors or conduct large mutagenesis screens for host infection factors, although not impossible, remain very laborious and extremely costly [4].

Hence, amidst persistent ethical considerations regarding the welfare of mammals used for experimentation and the need to have more tractable and cost-effective model systems to study host-pathogen interactions, non-mammalian models have gained significant attention over the past decade.

1.2.1 Non-mammalian models

1.2.1.1 Vertebrates

The **zebrafish*, Danio rerio***, been used to model infection for a number of human pathogenic bacteria, *Listeria monocytogenes* [5] and *Streptococcus pyogenes* [6], the zoonotic *Mycobacterium marinum* [7] and the human opportunistic fungal pathogen *Candida albicans* [8,9]. The small size and transparency of zebrafish larvae provide the unique possibility to image infection dynamics at the cellular level for both the host and

the pathogen. While the larvae only possess innate immunity, adult fish also has an additional adaptive arm [10,11]. Currently many more tools are still being developed for the use of zebrafish as an infection model, but transgenic larvae with fluorescing innate immune cells can already be used to identify specific cell types involved in infection. Modified anti-sense oligonucleotides or morpholinos are also used to knock down various host defence components [8,12].

1.2.1.2 Invertebrates

The short life cycle and simple anatomy of invertebrates make them useful and convenient models for the study of host-pathogen interactions. Invertebrates are also often cost-effective and generation of large numbers of individuals is relatively easy. Even though invertebrate models only possess innate immunity and lack higher adaptive immune systems of vertebrates, they are still highly attractive in the field as it is believed that the central concept of innate immunity is present throughout the animal kingdom, and that most importantly, successful strategies against pathogens have been conserved throughout evolution [2,13].

The **common fruit fly *Drosophila melanogaster*** was first reported in 1972 to have an antibacterial defence system [14] but only emerged as a model organism to study host-pathogen interactions a couple of decades later [15,16,17]. The innate immune system in the fruit fly involves both cellular (specialized cells dedicated to encapsulating, engulfing and eliminating pathogens [18]) and humoral components (secretion of antimicrobial peptides into the hemolymph [19,20]). More importantly, the fruit fly was used to study how innate immunity against pathogenic microorganisms can be induced and sensed by the Toll receptor, the invertebrate counterpart to mammalian Toll-like receptor 4, and strongly demonstrates how successful defence strategies have been conserved from insects to mammals [19,20].

More recently, the **larvae of *Galleria mellonella* (Greater Wax Moth)** have been utilized as infection models because similar to the fruit fly, the wax moth larvae also possess specialized innate immune cells. The greatest advantage is that the wax moth larvae can be infected at the physiological temperature for human infections and are cheap and easy to maintain and do not require feeding [21,22].

The **soil nematode *Caenorhabditis elegans*** has been used extensively for the past decade for the study of host defence strategies and bacterial virulence mechanisms. This thesis describes the use of *C. elegans* for these studies and details of this system will be further elaborated in Section 1.3.

1.3 *C. ELEGANS* AS AN INFECTION MODEL

Sydney Brenner's 1974 seminal paper introduced *C. elegans* as a model organism to study a variety of biological questions regarding cell division and differentiation, muscular assembly and function and the nervous system [23]. This came about from a deliberate search for a multicellular organism that could be analyzed with the ease and resolution similar to that of unicellular organisms. Biological events can also be dissected by means of genetic, cellular and molecular approaches at almost single-cell resolution in an intact whole organism.

For the past decade, researchers have begun to exploit the soil nematode *C. elegans* as a host organism to model virulence and microbial pathogenesis *in vivo* [24,25,26,27]. Nearly 50 different microorganisms, including bacteria, fungi, viruses and microsporidia, have been shown to be pathogenic to *C. elegans.* This thesis explores the use of *C. elegans* for the study of human bacterial pathogens and the sections which follow will largely focus on discussing this model in the context of bacterial pathogenesis.

Although the last ancestor between humans and *C. elegans* appeared more than five hundred million years ago, we share common susceptibilities to many human bacterial pathogens [28]. *C. elegans*, being a bacterivore, has also interacted and co-evolved with these microorganisms over a similar amount of time and virulence-related traits of bacterial pathogens may have evolved from both defensive and offensive mechanisms to avoid predation by *C. elegans* and other larger animals [13,29].

Likewise, for *C. elegans* to survive the detrimental effects of ingesting harmful bacteria, host defence mechanisms must have been developed and executed. The most successful defence strategies are highly likely to be re-used and conserved across animal species [28]. In higher organisms the host defence mechanisms become more sophisticated with the recruitment of additional components to cope with more complex

network of cellular tissues and processes. In an essence, this hints that the basic mechanisms by which bacterial pathogens establish infections in *C. elegans* and higher organisms may be very similar.

1.3.1 Features of the *C. elegans* system

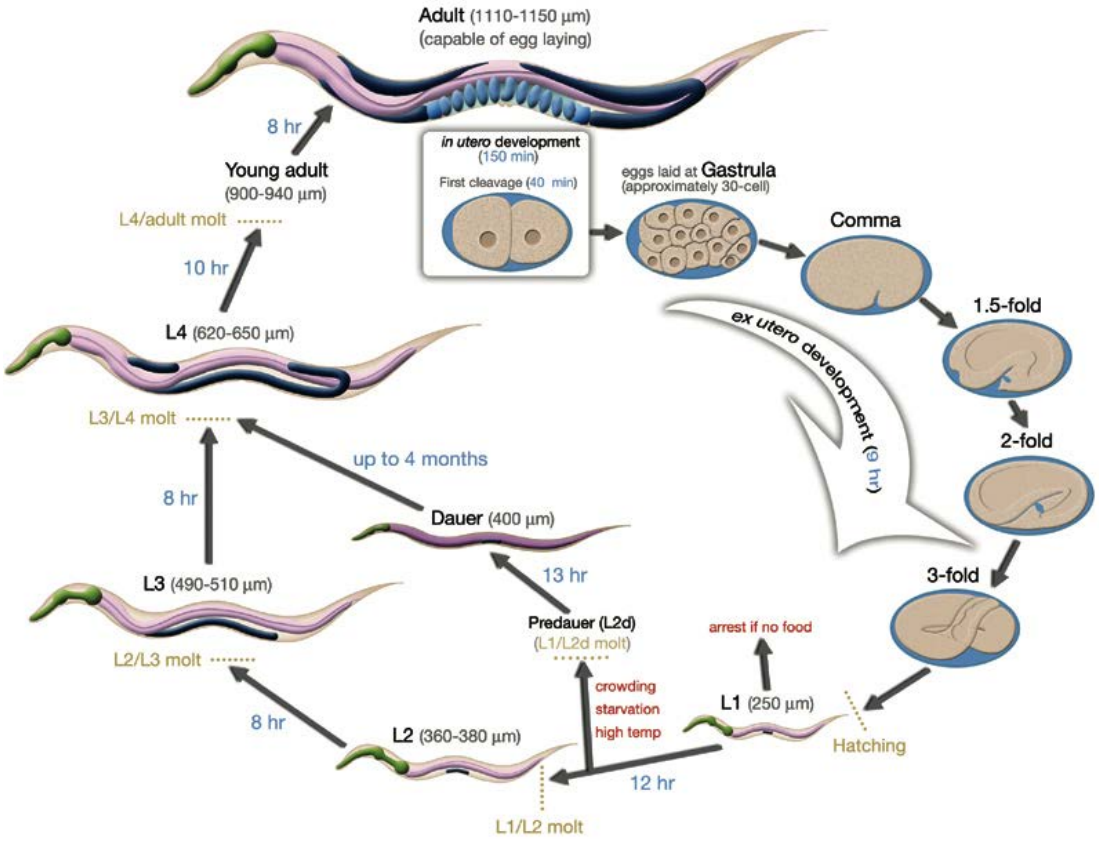

Figure 1. Life cycle of C. elegans [30]

The *C. elegans* life cycle is simple and consists of 4 larval stages till the fertile adult nematode (Figure 1). Its short generation time of less than 3 days has greatly facilitated genetic analysis and generation of genetic crosses. *C. elegans* hermaphrodites reproduce by self-fertilization, allowing the production of nearly 300 genetically identical progeny and favouring new mutations to become homozygous automatically. The cost-effective soil nematode can be simply propagated and maintained on agar plates with non-pathogenic *Escherichia coli* as food source [23]. In addition, the ease and robustness to freeze *C. elegans* and recover them thereafter allow strains to be maintained indefinitely [31].

C. elegans is also the first multicellular organism to have its whole genome sequenced [32], thereby allowing extensive forward and reverse genetics screens to be developed. The entire cell lineage, from egg to adult, is also visible under the light microscope and has been defined precisely [33,34,35,36], meaning that any process involving the behaviours of individual cells in a multicellular context can be analyzed at the singular cell level.

C. elegans is one of the more genetically tractable model organisms which would allow us to rapidly understand the impact of host genes on the outcome of an infection – transgenic strains can be easily generated by microinjection or bombardment with DNA [37,38]; gene expression can also be conveniently knocked down by feeding nematodes with gene-specific double-stranded RNA (dsRNA) expressed by non-pathogenic *E. coli* [39]. Further development of this RNA interference (RNAi) technology in the nematode have now allowed tissue-specific gene knockdowns, making it easier to determine the spatial requirement for a specific gene and to study genes that have pleitropic effects in different tissues [40].

Homologous features between the innate immune systems of *C. elegans* and mammals make the nematode an attractive host model. These similarities would be further discussed in Section 1.3.4.3. *C. elegans* only has an innate immune system and this allows the unprecedented elucidation of innate immune functions without confounding influences from the adaptive arm present in higher organisms [28].

The relatively inexpensive and small-sized nematode system is amenable to the development and execution of automated high-throughput whole-animal assays and screens and thus enables compound screening from large chemical banks and libraries even with minute quantities of valuable leads [41].

1.3.2 Readouts for host-pathogen interactions

Using *C. elegans* as a host to model bacterial pathogenesis can generate a rich repertoire of data in various forms [28,42] and enable better delineation of the infection. Selected examples are described below:

The effectiveness of host defence mechanisms and pathogen virulence can be monitored by quantifying the survival of nematodes over time under controlled conditions, observing morphological and behavioural changes of the infected organisms and measuring the persistence of live pathogens in the nematode intestine.

The transparency of *C. elegans* also allows for direct visualization of bacterial pathogens tagged with fluorescent proteins, and in combination with the many established transgenic nematode strains with specific fluorescent proteins as cell or tissue-specific markers, enables the direct observation of host-pathogen interactions at the cellular level (Figure 2).

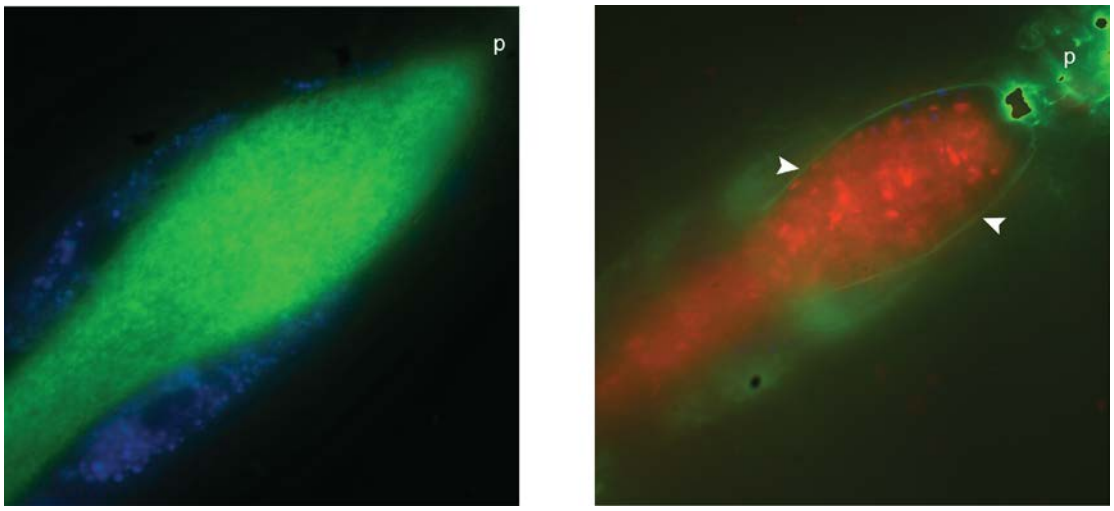

Figure 2. Examples of in vivo visualization of infection. (Left) Wild-type N2 nematodes were fed S. Typhimurium 14028::GFP, intestinal autofluorescence was shown in blue; (Right) SU159 nematodes expressing AJM-1::GFP were infected with S. Typhimurium 14028::RFP. Images are adapted from Paper III [43].

Host gene expression changes upon infection can be monitored using microarrays, quantitative polymerase chain reaction and specific reporter constructs in transgenic nematode strains which are easy to generate. Conversely, infecting nematodes with transgenic bacterial strains carrying reporter constructs can yield *in vivo* images of microbial gene expression in the infected host.

1.3.3 Limitations

Here, several limitations of using *C. elegans* as a model organism to study host-pathogen interactions should be considered.

As *C. elegans* can be infected by simply replacing the normal *E. coli* food source with the specific bacterial pathogen [42], the precise inoculum or multiplicity of infection for each nematode at the start of the infection experiment cannot be precisely determined.

The maximum growth temperature of *C. elegans* is 25^{o}C. Hence there is a concern that certain virulence factors from human bacterial pathogens usually expressed at the physiological temperature of 37^{o}C may not be optimally expressed in the nematode. Recently, an alternative nematode model, *Panagrellus redivivus,* has been proposed for human bacterial pathogens – *P. redivivus* is viable for several days at 37^{o}C but does not reproduce at this temperature [44]. A need for continuous efforts to develop this model and hopefully recapitulate the established approaches for *C. elegans* is necessary.

It is important to note that *C. elegans* appears to lack several characteristics of mammalian innate immunity:

Due to the observations that phagocytic cells called coelomocytes of a larger nematode, *Ascaris summ,* are capable of phagocytosing invading organisms [45], it had been suggested that the six coelomocytes of the adult *C. elegans* hermaphrodite could also serve as scavenging immune cells akin to mammalian neutrophils, monocytes and macrophages [46]. However this has been proven not to be the case as *C. elegans* coelomocytes are not motile nor have been observed to phagocytose any pathogen [47].

The Toll/NF-κB (nuclear factor-kappa B) signalling pathway has been shown to play a very critical role in innate immune responses of vertebrates and even the fruit fly [20,48]. However *C. elegans* appears to lack many components of this pathway in its genome [49]. For the few homologues present, they appear to be involved in independent host defence mechanisms divergent from the well-studied Toll/NFκB signalling [49,50].

In spite of these limitations, *C. elegans* remains as a very attractive model for studying host-pathogen interactions because previous studies are strongly suggestive of the existence of a very competent and efficient network of host defence mechanisms in the nematode even when lacking many classical features of mammalian innate immunity. Further understanding and delineation using *C. elegans* as a host model may reveal previously unrecognized aspects of host defences and add on to the rich knowledge pool for host-pathogen interactions.

1.3.4 Host defence strategies

1.3.4.1 Avoidance

A host susceptible to infections can protect itself by avoidance, hence reducing the risk of pathogen exposure. *C. elegans* possesses a complex chemosensory system to sense and respond to a repertoire of chemicals including microbial products. This olfactory chemotaxis is mediated by G protein-coupled receptors and could result in either an attraction or an aversive behavior - some Gram-negative bacteria produce acylated homoserine lactone autoinducers to attract *C. elegans* [51] while *Serratia marcescens* produces serrawettin which *C. elegans* avoids [52]. In the case of acylated homoserine lactone autoinducers, both pathogenic and non-pathogenic Gram-negative strains have been reported to produce such molecules and the nematode can further associate the specific autoinducer with the experience it has (deleterious or not after ingestion) and learn to avoid the specific pathogen in the future [53]. The *C. elegans* single Toll receptor gene *tol-1* was initially reported to aid recognition and avoidance of *S. marcescens* [49]. *tol-1* loss-of-function mutants were defective in distinguishing wild-type *S. marcescens* and mutant *S. marcescens* incapable of producing serrawettin but could not differentiate non-pathogenic *E. coli* spiked with or without serrawettin, suggesting that the nematode Toll receptor can probably function to integrate both attractive and repulsive stimuli from *S. marcescens* [52].

This avoidance behaviour exhibited by *C. elegans* is also being exploited by pathogenic bacteria. *Bacillus nematocida* produces food-like volatile organic compounds which attract nematodes even more than its usual food source but once ingested, *B. nematocidal* secretes toxic proteases to kill the nematode [54].

1.3.4.2 Physical mechanisms

The *C. elegans* epidermis (also known as the hypodermis) encapsulates the nematode and secretes an external collagenous cuticle which is relatively impermeable and prevents access to nematode cells and tissues [55]. The cuticle thus acts as the primary barrier against any pathogen it encounters and access beyond the cuticle can only be gained through the mouth, anus, vulva or sensory openings. The natural nematocidal fungal pathogen *Drechmeria coniospora* is able to bypass this primary barrier by attaching to the cuticle and forming penetration tubes which pierce through and transverse the epidermis to colonize the whole nematode [56].

Pathogens entering via the mouth of the nematode next encounter the grinder of the pharynx which consists of contracting muscles and mechanically disrupts microbes as they pass to the intestine. Grinder-defective *C. elegans* mutants have been reported to be more susceptible to *Pseudomonas aeruginosa* [57,58].

1.3.4.3 Innate immune signalling

In *C. elegans,* several innate immune signalling pathways, at least in part resembling that of higher organisms, may function as host defence mechanisms against bacterial pathogens. Three of them which are involved during infection with several pathogens are represented in Figure 3.

One of the two well-studied transforming growth factor beta-like pathways in *C. elegans*, the Sma pathway, is involved in host defences against *Serratia marcescens* and *P. aeruginosa.* Mutants defective in *dbl-1*, the ligand of the Sma pathway, were highly susceptible to both pathogens. Several genes found highly induced after exposure to *S. marcescens* were also under the regulation of the *dbl-1* ligand [59].

An important signalling cascade downstream of Toll-like receptors in insects and mammals involves the p38 mitogen-activated protein kinase (MAPK) module. Even though *C. elegans* lacks Toll receptors, a conserved p38 MAPK cascade, involving NSY-1, SEK-1 and PMK-1, was found to be functional in the nematode for host defences against multiple bacterial pathogens [57]. Activation signals and transcriptional outputs of this conserved p38 MAPK cascade are still not well

characterized but possible mechanisms are being revealed in recent and ongoing studies. During *Enterococcus faecalis* infection, studies suggested that cell-permeable reactive oxygen species (ROS) produced by the infected host may activate the p38

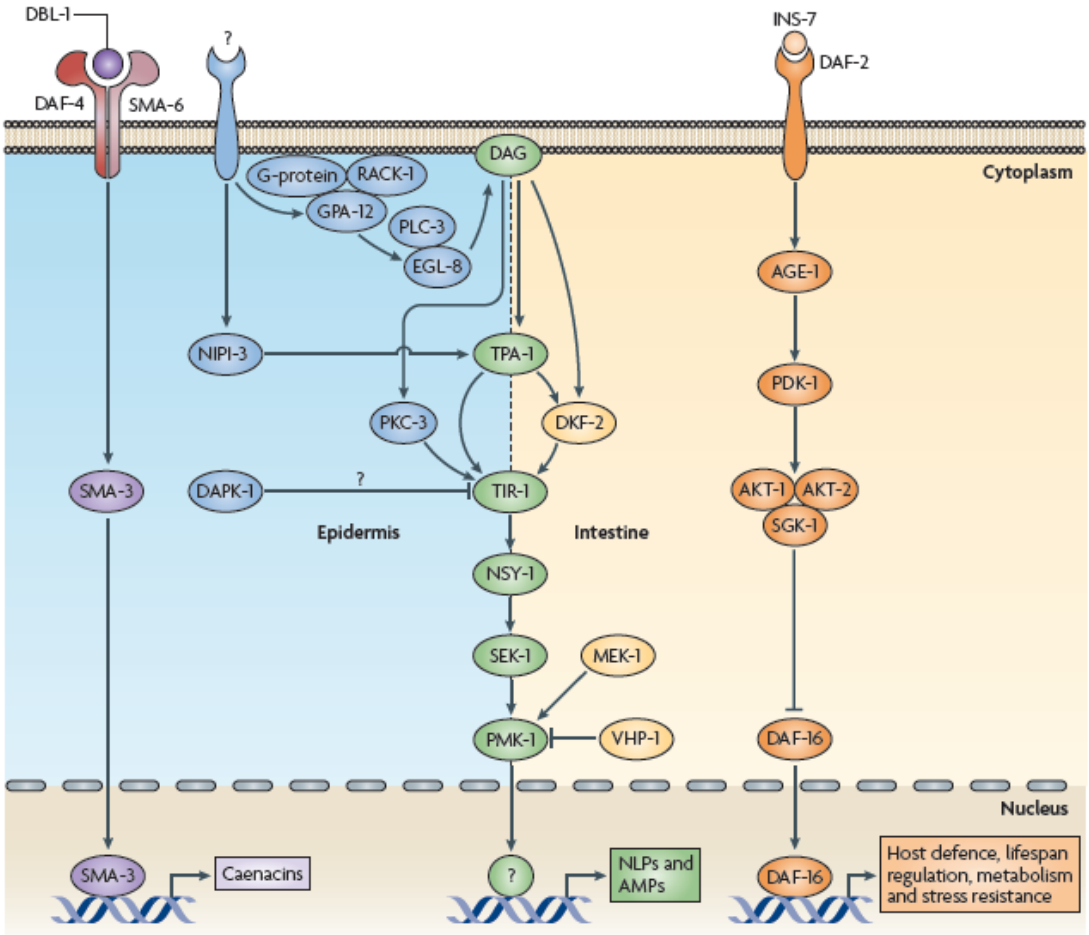

Figure 3. Innate immune signalling pathways in C. elegans [28].

MAPK signalling pathway, resulting in phosphorylation and nuclear localization of transcription factor SKN-1. SKN-1 activates the transcription of genes with detoxification functions to neutralize host ROS produced during *E. faecalis* infection [60]. Another study reported that the pore-forming toxin Cry5B produced by *Bacillus thuringiensis* initiates the unfolded protein response in the endoplasmic reticulum to maintain cellular homeostasis during infection and this process requires the upstream activation of the p38 MAPK cascade [61].

The DAF-2 insulin/IGF-1 signalling pathway depicted on the rightmost of Figure 3 will be further described and discussed in Paper I [62].

Mammalian innate immunity is often thought to be non-specific to different pathogens as it is constitutively present and ready to be mobilized upon infection. *C. elegans* relies solely on its innate immune system to execute host defence mechanisms. Despite being lower in complexity and lacking classical features of mammalian innate immunity, it has been shown in targeted and genome-wide studies that upon infection of *C. elegans* by different pathogens, both pathogen-specific responses and responses shared by several pathogens can be triggered and induced [63]. This indicates that the nematode is highly capable of recognizing and responding to different pathogens despite its simplicity.

1.3.4.4 Infection resistance or tolerance

Host defence strategies are classically understood to detect and eliminate pathogenic microorganisms *per se*. However, it is important to distinguish between two distinct and relatively independent mechanisms, resistance and tolerance, because accurately defining and describing each host-pathogen interaction in these contexts may influence our perception of understanding how both sides interact and further impact on the progression of therapeutic and prophylactic interventions. Resistance strategies reduce pathogen burden during infection by neutralizing, disabling or elimination of invading microbes while tolerance strategies do not necessarily affect pathogen burden but rather limit the effect of direct host damage inflicted by the pathogen or collateral host damage caused by immune responses [64].

The summation of both resistance and tolerance would ultimately define the defensive capacity of the host. Resistance mechanisms have been well described but knowledge about tolerance strategies is still limited in the field. From an evolutionary perspective, resistance strategies are highly likely to place strong selective pressures on the pathogen, driving the latter to evolve and subvert host defences – we have already observed such dynamics with antibiotic-resistant pathogens. Conversely, host tolerance places less selective pressure on pathogens and drugs designed to increase host tolerance would less likely be subverted by the pathogen [65].

A striking example of host tolerance studied in *C. elegans* was demonstrated by Mohri-Shiomi and Garsin where they infected nematode strains carrying fluorescent aggregation-prone polyglutamine proteins [66]. The amount of polyglutamine protein

aggregation correlates with protein quality which is easily perturbed by changes in protein synthesis, trafficking or degradation [67] and accumulating such aggregates is detrimental to the host. During *Entercoccus faecalis* infection, ROS produced by host defence mechanisms cause protein aggregation in the intestine and reduction or prevention of this aggregation via expression of genes modulated by the DAF-2 insulin signalling pathway and heat-shock factor 1 (HSF-1) appears to be advantageous to the infected host [66,68]. HSF-1 is a transcription factor regulating several heat shock proteins which can act as chaperones binding to and possibly clearing unfolded or damaged proteins [69]. The observations made during *E. faecalis* infection could be mediated independently by either DAF-2 signalling or HSF-1, or as a combinatorial effort since DAF-2 signalling can act upon HSF-1 as described in the context of aging by Chiang *et al.* [70].

In Paper I [62], we attempt to discuss whether the observations made pertain to infection resistance or tolerance. Much further work is warranted to have a better insight into this. We are also cautious in Paper II not to use such terminology without sufficient evidence.

1.3.5 Bacterial virulence mechanisms

The *C. elegans* host model has been previously used to identify novel bacterial virulence factors and importantly, bacterial mutants exhibiting reduced virulence in nematodes also displayed attenuated virulence in murine models [25,71,72]. Bacterial pathogenesis in the nematode can occur via several mechanisms and a few selected ones will be briefly discussed in this section.

1.3.5.1 Cuticle infection

Yersinia pestis, the causative agent of bubonic plague, produces a polysaccharide-rich biofilm that attaches to the cuticle of *C. elegans*, especially on the head, and blocks the pharyngeal opening. Feeding is hence inhibited, thereby “starving” and killing the nematodes. This is similar to how *Y. pestis* in nature blocks feeding of its vector, the flea, in order to transmit to mammals [73] .

1.3.5.2 Intestinal infection

A majority of the human bacterial pathogens colonizes and accumulates in the intestinal lumen as individual bacteria, ultimately leading to the death of infected *C. elegans*. Numerous bacterial mutants exhibiting reduced intestinal colonization and thus reduced virulence have been uncovered but the exact role of how these virulence factors mediate intestinal colonization and what modes of pathogenesis they elicit to cause nematode death remains to be largely elucidated [25].

Alegado and Tan found that during infection by *Salmonella enterica* serovar Typhimurium (*S.* Typhimurium), expression of two antimicrobial peptides are induced to limit bacterial proliferation and several virulence factors such as PhoP and the virulence plasmid are responsible to mediate resistance to the antimicrobial peptides, enabling continuous persistence in the intestinal lumen [74]. Still, how this persistence elicits death in the host is not understood.

We attempt to show in Paper III [43] how a virulence-associated factor from *S.* Typhimurium manifests pathogenesis via inducing a host oxidative response that is too overwhelming and detrimental for the infected nematode [43].

Pathogen burden remain relatively high and killing rates remain consistent even in *C elegans* infected with *S.* Typhimurium for a few hours and thereafter transferred to non-pathogenic *E. coli* [75]. But this does not occur during *Staphylococcus aureus* and *Burkholderia pseudomallei* infection [76,77], suggesting that certain bacterial pathogens like *S.* Typhimurium can probably adhere to specific receptors in the intestine in order to persist and colonize to a high degree. It would be interesting to further elucidate such interactions in the nematode system.

1.3.5.3 Toxin-mediated killing

Bacillus thuringiensis produces spores associated with pore-forming crystal toxins (Cry and Cyt) [78]. One of the nematocidal toxins widely characterized, Cry5B, binds to glycolipids on intestinal epithelial cells [79,80,81]. Subsequently, membrane pores are formed followed by cellular disintegration [82].

When *P. aeruginosa* is cultured on specific media, several strain-dependent toxins capable of killing *C. elegans* are produced. *P. aeruginosa* strain PA14 produces small secondary metabolites of the phenazine pyocyanin class of toxins which generate ROS harmful to the nematodes [26] while *P. aeruginosa* strain PA01 produces volatile hydrogen cyanide to rapidly paralyze and kill nematodes [83,84].

Gram-positive bacteria such as *Streptococci* spp. [85,86,87] and anaerobically-grown *Enterococcus faecium* [88] are nematocidal via the production of hydrogen peroxide (H_2O_2). The ability to produce H_2O_2 and the amount of H_2O_2 produced was tightly correlated to the killing capacity of each specific bacteria strain. H_2O_2 production has been linked to several aspects of pneumococcal diseases including bacterial colonization, direct oxidative damage to brain and epithelial cells and growth inhibition of other competing organisms in the specific niche. Given that there are conserved programmed cell death pathways in both *C. elegans* and mammals, the nematode model could be useful to further elucidate the virulence mechanisms of human pneumococcal strains [85].

1.3.5.4 Suppression of host defence mechanisms

Bacterial pathogens are also capable of evolving mechanisms to evade and overcome host defence mechanisms. This form of active virulence may involve inhibiting host defence signalling pathways or restricting the expression of antimicrobial effectors. The *C. elegans* platform can facilitate such studies as a large repertoire of molecular and cellular approaches have been developed to quantify and measure the relative effectiveness of host infection responses. One example was the finding that *P. aeruginosa* infection results in the suppression of a subset of DAF-16-regulated immune genes in the infected nematode. These observations are tightly linked to the fact that during *P. aeruginosa* infection, there is upregulation of INS-7 an insulin-like peptide agonist for the DAF-2 insulin/IGF-1 signalling pathway. This study also showed that the immune downregulation requires *P. aeruginosa* factors such as the two-component response regulator GacA and the quorum-sensing regulators LasR and RhlR [89].

1.4 BACTERIAL PATHOGENS AS TOOLS TO PROBE THE SYSTEM

1.4.1 Burkholderia pseudomallei and *Burkholderia thailandensis*

The Gram-negative bacillus *Burkholderia pseudomallei* is an environmental saprophyte commonly isolated from wet soils and stagnant waters and is the causative agent of melioidosis in both humans and animals [90,91]. The bacterium can be acquired by inhalation, ingestion or skin penetration of contaminated soil or ground water. Person-to-person transmission is very rare. [90].

Melioidosis is endemic to a number of tropical regions (Figure 4) and disease incidence is particularly high in Southeast Asia, India and northern Australia [92]. The disease is particularly problematic in Thailand where it accounts for up to 20% of community-acquired septicemias and causes a significant number of deaths despite vigorous antibiotic treatments [90]. Melioidosis is dubbed as the "Great Mimicker" as it presents a wide spectrum of clinical outcomes ranging from pneumonia, skin abscesses in internal organs and soft tissues to highly fatal septic shock and is even often

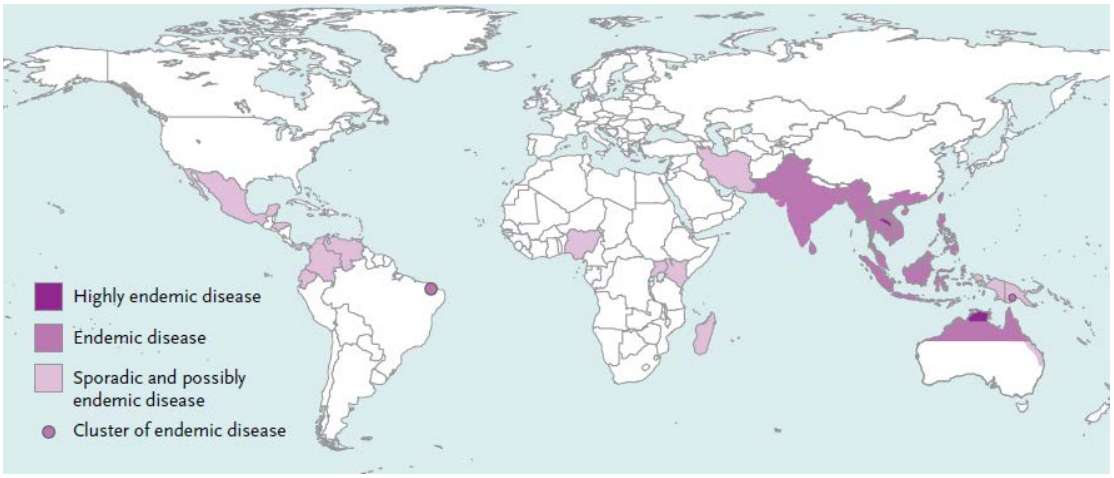

Figure 4. Global distribution of melioidosis [92].

misdiagnosed as tuberculosis and other ailments [90,93]. Asymptomatic infections have also been recorded but many of them progress to acute melioidosis much later on. The disease has been shown to develop in human patients up to 62 years after exposure, indicating a remarkable capability of the bacterium to remain latent in the host [94]. Treatment of the disease with antibiotics is usually long and difficult because *B.*

pseudomallei is intrinsically resistant to a diverse group of antibiotics and cases of relapse are common even after initial successful treatments [95]. Currently, there is still no available vaccine.

B. pseudomallei has also been implicated as a bioterrorism threat and classified by the Centers for Disease Control and Prevention as a category B biological agent [96].. Experimental manipulation of *B. pseudomallei* requires Biosafety Level 3 containment but unfortunately, many endemic regions do not have adequate facilities for research [97]. Due to the importance of melioidosis and the fact that *B. pseudomallei* is not well characterized, there has been an increased interest in the identification and characterization of bacterial virulence determinants and the molecular basis of its interaction with the host.

B. thailandensis [98] is considered to supplement as a tractable model system for *B. pseudomallei* and by contrast, can be easily handled under Biosafety Level 1 conditions as it is considered avirulent and very rarely pathogenic to humans and animals [90,99,100]. It has been isolated from similar environments and regions as *B. pseudomallei* and prior to its classification, was often mistaken for *B. pseudomallei*, owing to similarities between their biochemical, morphological and antigenic profiles.

In spite of a smaller genome size, the two chromosomes of *B. thailandensis* exhibit high degree of synteny with *B. pseudomallei* [101] and appear to share an extensive repertoire of genes involved in core metabolism, accessory pathways, structure-based superfamilies and even bacterial virulence factors including lipopolysaccharide (LPS), adhesion factors, virulence-associated Type II and III secretion systems and complex quorum-sensing systems [101,102,103]. Similar to *B. pseudomallei*, *B. thailandensis* can survive and replicate intracellularly within eukaryotic cell lines and polymerize host cell actin to facilitate intra- and intercellular spread [104,105,106].

We and others have shown that *C. elegans* is susceptible to both *B. thailandensis* and *B. pseudomallei* [62,71,107]. More importantly, during a screen of *B. pseudomallei* mutants created via transposon insertion [108], mutants attenuated in their ability to kill *C. elegans* were also found to exhibit delayed and significantly reduced virulence in BALB/c mice [71], validating the use and relevance of *C. elegans* as an alternative

host for these pathogens. Hence given i) the high degree of genomic similarity between *B. thailandensis* and *B. pseudomallei* and ii) the synonymous infectivity of *C. elegans* by both pathogens, it raises the possibility that *B. thailandensis* can be used as a tool to study selected aspects of *B. pseudomallei* biology in the nematode model.

Clinical and experimental observations suggest that host factors play important roles in determining the outcome of a *B. pseudomallei* infection – a significant proportion of infected individuals exhibit no apparent symptom or mild illness; infections by identical *B. pseudomallei* strains have also been shown to elicit distinct survival outcomes in different strains of mice [109]. Still, not much knowledge has been generated from this perspective.

1.4.2 *Salmonella enterica* serovar Typhimurium

Salmonella enterica serovar Typhimurium (*S.* Typhimurium) belongs to the bacterial genus *Salmonellae*, a group of Gram-negative bacilli capable of causing enteric disease in a wide range of different organisms ranging from humans and mice to nematodes, amoebae and plants [75,110,111,112]. *S. enterica* serovars, such as *S. enterica* serovar Typhi (*S.* Typhi) and *S.* Typhimurium, have the ability to infect mammals and be transmitted by the feco-oral route via contaminated food and water and ultimately result in salmonellosis ranging from mild gastroenteritis to life-threatening systemic infections including typhoid fever [113]. On a global scale, there is an annual estimation of 3 billion human infections and typhoid fever makes up 22 million of these cases, and is responsible for nearly 0.2 million deaths annually [114].

S. Typhi is the aetiologic agent responsible for invasive typhoid fever which is characterized by high fever, great discomfort and abdominal pain [113]. It has no animal reservoir and is transmitted from human to human. The strict adaptation to the human host limits the studying of *S.* Typhi to NHPs such as chimpanzees. *S.* Typhi is not virulent to lower primates and non-primate vertebrates [115]. On the other hand, *S.* Typhimurium infection of mice is commonly used as a model to study the pathogenesis of *S.* Typhi infection in humans as *S.* Typhimurium causes a systemic disease in mice, much resembling human typhoid fever [116,117]. However, *S.* Typhimurium causes only gastroenteritis in humans.

During invasive salmonellosis in humans and mice, *S. enterica* invades the Peyer's patches of the small intestine via M cells [118,119]. M cells are specialized epithelial cells which transcytose *Salmonella* antigens to phagocytic immune cells, initiating inflammation. *S. enterica* also acts as a facultative intracellular pathogen and replicates in macrophages and dendritic cells [120,121,122] for subsequent dissemination to the mesenteric lymph nodes and eventually to the liver and spleen [123]. By persisting in the mesenteric lymph nodes, bone marrow and gall bladder, *S. enterica* infects chronically and may occasionally reseed via the bile ducts or lymph nodes (Figure 5).

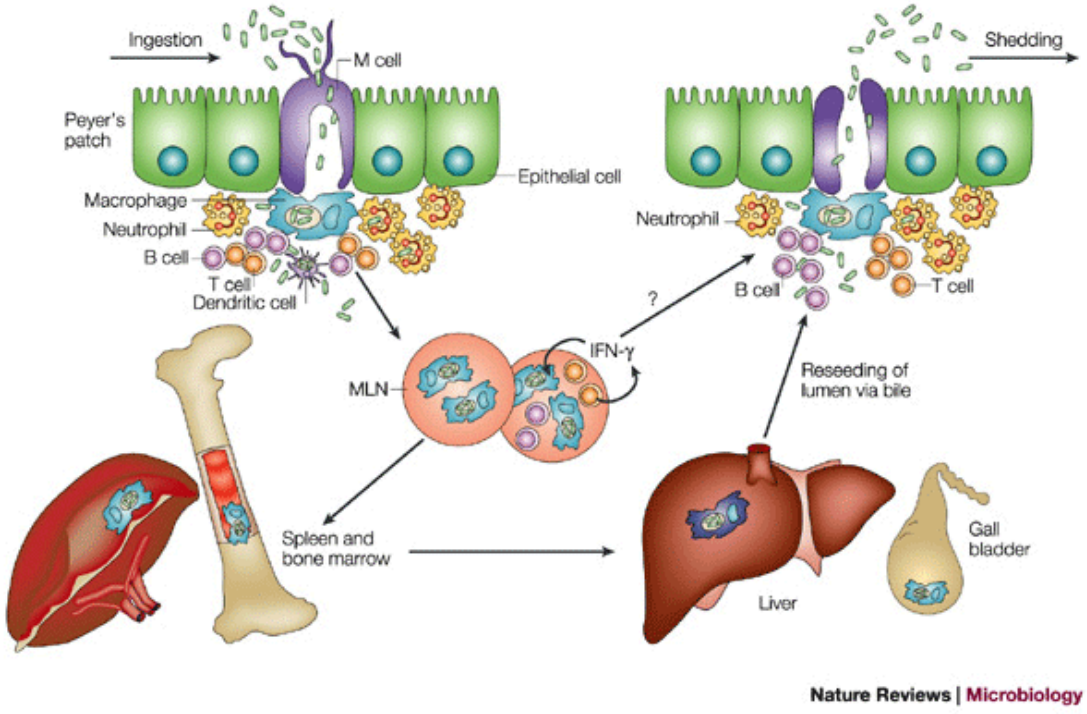

Figure 5. Pathogenesis of S. Typhi in humans or S. Typhimurium in mice [124]

This pathogenesis relies on several sets of virulence genes, many of which are contained on horizontally acquired genetic inserts called *Salmonella* pathogenicity islands or SPIs [125]. SPI1 and SPI2 code for two separate type III secretion systems (T3SS) essential for virulence in mammals. The T3SS on SPI1 is needed for invasion of the intestinal barrier and initiation of enteropathogenesis [126]. 13 effectors secreted via the SPI1 T3SS have been identified – three of them, SipB, SipC and SipD are postulated to form a complex in the host cell membrane to facilitate translocation of the other effectors into the host cell cytoplasm. These effectors rearrange the actin cytoskeleton, causing membrane ruffling and internalization of the bacteria into epithelial cells [127]. Following invasion into host cells, *S. enterica* resides in acidified

membrane-bound *Salmonella*-containing vacuoles (SCV) and effector proteins secreted by the SPI2 T3SS manipulate vesicular trafficking and delay apoptosis, allowing the bacteria to replicate intracellularly in the SCV [128]. A *spv* gene cluster carried on the virulence plasmid of *S. enterica* has also been shown to be essential in the systemic phase of the infection and promotes replication in liver and spleen macrophages [121]. Besides these, *S. enterica* also contains defence mechanisms against antimicrobial host strategies – for example, detoxification of harmful substances such as ROS and reactive nitrogen species, repair of damage caused by these substances and manipulation of host responses to facilitate intracellular survival and replication.

Importantly, *S.* Typhimurium uses many of its virulence factors, originally defined in murine infection models and thought to be specifically targeted towards mammalian hosts, to infect *C. elegans* as well [72,75,111]. Using the nematode model system, it has been observed that *S.* Typhimurium intestinal colonization results in an accelerated level of *ced-3*- and *ced-4*-mediated cell death in the gonad [129]. Consequently, *ced-3* and *ced-4* mutants were killed significantly faster by *S.* Typhimurium. But how germline cell death protects the host from the pathogen is still not clear. Another study shows that autophagy, an evolutionarily conserved lysosomal degradation pathway, plays an important role during *S.* Typhimurium infection – when autophagic genes were knocked down, the few bacteria which can invade the intestinal epithelial cells but are usually efficiently targeted to the lysosomal pathway, could now establish an intracellular replicative niche leading to death [130]. However, the mechanisms by which *S.* Typhimurium elicits death in *C. elegans* have not been fully clarified nor well understood.

2 SCOPE OF THESIS

This thesis aims to characterize the mechanisms by which the model host *C. elegans* protects itself from invading bacterial pathogens and by which the pathogen mounts an *in vivo* infection.

2.1 SPECIFIC AIMS

Paper I:

We identified the specific route used by *B. thailandensis* during infection of *C. elegans* and thereafter, used the RNAi feeding library to conduct a targeted screen of host genes expressed along this route. We verified that this reverse genetic approach was capable of identifying *C. elegans* genes important in modulating host infection outcomes. Using *in silico* analyses, genetic and biochemical methods, we also further delineate how a positive hit from the screen modulates host infection outcomes.

Paper II:

We performed a forward genetic screen to identify host genes relevant to melioidosis and identified a new allele of a known gap junction gene which has previously never been implicated during host-pathogen interactions. We attempt to use our genetic findings and observations to postulate how such junctions may play an important role in influencing disease susceptibility in higher eukaryotes.

Paper III:

S. Typhimurium infection of *C. elegans* does not seem to involve the traditional invasive and intracellular phenotype of the pathogen. We sought to clarify the mechanisms by which *S.* Typhimurium elicits death in the nematode and delineate the pathogenic factors which may be involved.

Paper IV:

In combination with other infection models, we investigated the combinatorial effects of two small-molecular virulence inhibitors. Using *C. elegans* as a whole organism model, we attempt to use some preliminary findings to suggest that the nematode can be useful in elucidating mechanistic activities of such inhibitors.

3 EXPERIMENTAL PROCEDURES

3.1 SURVIVAL ASSAYS

Unless specified otherwise, pathogenic strains or *E. coli* strain OP50 were grown overnight in LB at 37°C and lawns were prepared by spreading overnight culture on modified NGM agar. Stage-synchronized nematodes were added to each lawn and infected as per described [42]. Nematodes were placed down on bare agar before transferring to pathogen-containing lawns to minimize the transfer of *E. coli*. No visible *E. coli* growth on pathogen-containing lawns was observed at locations where nematodes were added nor was there any crowding of nematodes at such locations. To further test for *E. coli* contamination, nematodes were removed 24 h post infection; pathogen-containing lawns were harvested, diluted appropriately in M9 buffer [23] and tested for *E. coli* by plating on neat LB agar and LB agar supplemented with an appropriate antibiotic for the specific pathogen. No contamination on pathogen-containing lawns had been observed. Nematode survival was scored at 24°C and nematodes were considered dead upon failure to respond to gentle touch by a platinum wire. Each survival curve is representative of three independent experiments, each with three plates per strain. Survival curves were analysed using the PRISM (version 5.0) software and Kaplan-Meier survival curves with p values < 0.05 were considered significantly different from the control.

For experiments involving chemicals or antibiotics, modified NGM agar was impregnated with each compound to ensure maximum exposure to the nematodes.

3.1.1 Liquid infection assays

Pathogenic strains or *E. coli* strain OP50 were grown overnight in LB at 37^{o}C. The overnight culture was washed and resuspended in S Basal liquid media [23] and 800 μl of the suspension was aliquoted into each well of a 48-well flat-bottomed plate. When necessary, 190 U of catalase was added into each well and mixed thoroughly. L4-staged N2 nematodes were transferred to each well and incubated at 24°C for 24 h. Subsequently, nematodes were examined with a light microscope and considered dead when neither body twitching nor pharyngeal pumping could be observed. Nematodes

were also transferred to bare agar to verify the phenotype. Results are representative of three independent assays, each with triplicates.

3.2 IMMUNOFLUORESCENCE ASSAYS

Infected nematodes were prepared for immunohistochemical staining using a freeze-crack method [131] and fixed using 50% methanol and 50% acetone. After washing, slides were blocked in 5% bovine serum albumin in antibody buffer, followed by 1 h primary antibody incubations. Secondary antibody incubations were performed at a dilution of 1:500 for 4 h. All incubations were performed at 24°C. Slides were mounted in anti-photobleaching media with 4',6-diamidino-2-phenylindole (DAPI).

For live imaging, fluorescent pathogenic or *E. coli* lawns were prepared by spreading overnight LB culture on modified NGM agar. Nematodes were added to these lawns and infected as described in 3.1. At each time point, live nematodes were harvested and mounted for microscopy in phosphate buffered saline (PBS) with 25 mM sodium azide (NaN_3).

All slides were visualized on a LEICA DMRE microscope and images were analyzed by GNU Image Manipulation Program (version 2.6.3). Images are representative of at least 20 nematodes from 3 independent assays.

3.3 RNA INTERFERENCE ASSAYS

Unless specified otherwise, RNAi assays were carried out at 20°C by feeding nematodes with parental *E. coli* HT115 (DE3) strain or *E. coli* HT115 clones expressing gene-specific dsRNA [39]. Each clone identity was verified by direct sequencing using specific oligonucleotides targeting the L4440 vector [132]. RNAi assays were performed by growing each clone for 8 h in LB supplemented with ampicillin and seeding on isopropyl β-D-1-thiogalactopyranoside (IPTG)-containing modified NGM agar. Nematode embryos, generated by hypochlorite treatment, were propagated on these seeded plates until the L4 stage. Nematodes were subsequently transferred to pathogen-containing lawns (Figure 6).

For experiments involving sterile nematodes, embryos were exposed to *cdc-25.1* dsRNA at late embryogenesis till day 1 adult stage, before transferring them to pathogen-containing lawns. Under such conditions, *cdc-25.1* RNAi resulted in nematodes with an Emb phenotype [133,134].

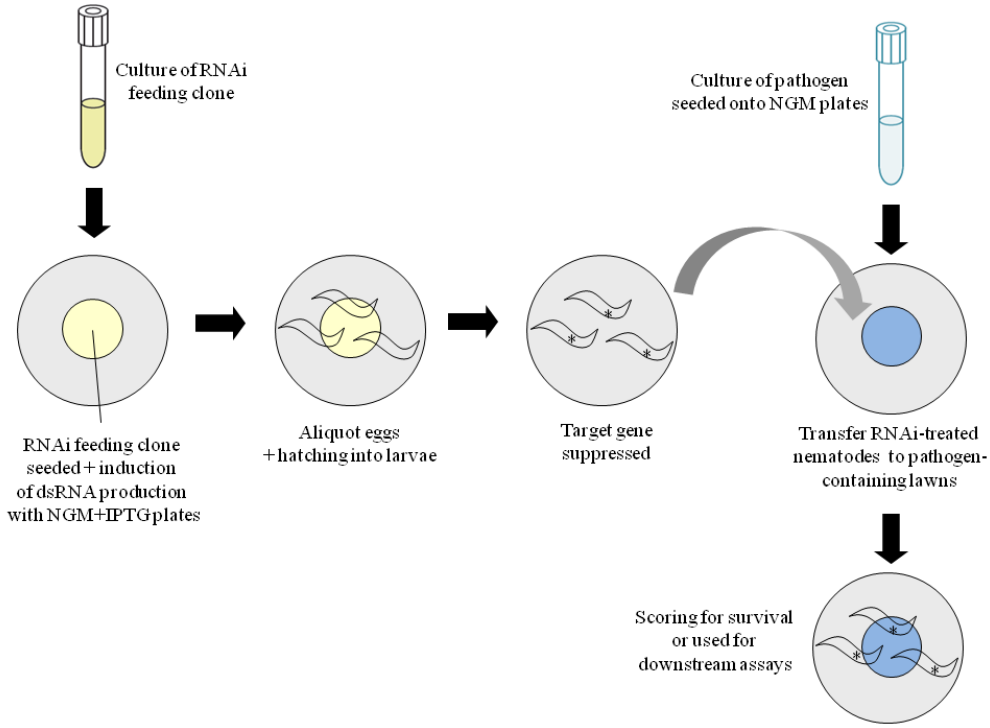

Figure 6. Schematic diagram of RNAi coupled to infection assays.

3.3.1 Tissue-specific RNA interference assays

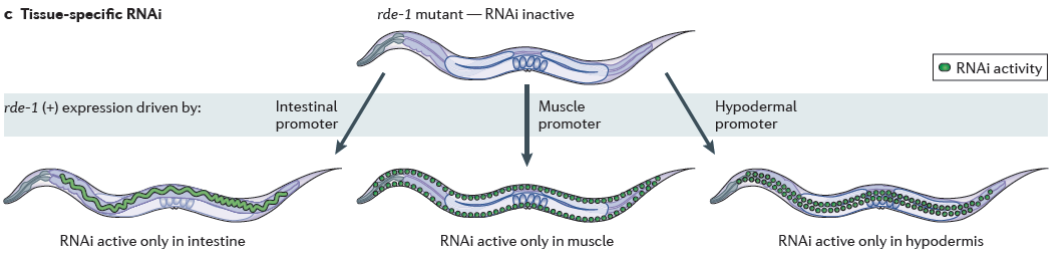

Figure 7. Principles of tissue-specific RNAi [135].

rde-1 encodes a member of the Argonaute protein family, whose expression is necessary to initiate RNAi in a cell-autonomous manner [136]. Using *rde-1 (ne219)* mutants, tissue-specific RNAi was achieved by expressing in these mutants the wild-type *rde-1* transgene under the control of tissue-specific promoters: the intestine-specific promoter *pnhx-2* [137] or the hypodermis-specific promoter *plin-26* [40].

When these nematodes were fed *E. coli* HT115 clones expressing gene-specific dsRNA, they are RNAi-proficient only in the tissue where wild-type *rde-1* was expressed (Figure 7). Tissue specificity of RNAi in these two strains was confirmed by feeding them with *unc-22* dsRNA. As *unc-22* expression is restricted to the muscles [138], neither strain showed the characteristic *unc-22* twitching phenotype as seen in wild-type nematodes. Control RNAi clones relevant to the downstream assay were also included to further confirm the tissue specificity of gene knockdown.

3.4 INTESTINAL BACTERIAL LOADS

Nematodes were infected as described in Section 3.1. At 24 h post infection, infected nematodes were harvested and set down on bare agar before transferring to M9 buffer to minimize the contamination of uningested bacteria. Nematodes were washed thrice with M9 buffer, followed by 1 h incubation in M9 buffer containing trypsin-EDTA to remove bacteria present on the exterior of the nematode. Nematodes were then washed thrice with M9 buffer alone to remove trypsin-EDTA, and subsequently lysed by vortexing with silicon-carbide sharp particles and 0.2% sodium dodecyl sulfate. Lysates were diluted appropriately in M9 buffer and plated on LB agar supplemented with the appropriate antibiotic to select for the specific pathogenic strain. After 1 to 2 days of incubation at 37°C, amount of live bacteria per nematode was determined by colony-forming units (cfu) counts. At least 25 nematodes were harvested per set and experiments were performed in triplicates.

3.5 DETECTION OF REACTIVE OXYGEN SPECIES

2',7'-dichlorodihydrofluorescein diacetate (H_2DCFDA) is used to visualize intracellular ROS in nematodes. 2 mM stock aliquots of H_2DCFDA were prepared in dimethyl sulfoxide and stored in the dark at -80^{o}C. Nematodes were infected as described in Section 3.1. At each time point, infected nematodes were harvested into tubes and washed twice with M9 buffer. Nematodes were subsequently incubated with 25 μM H_2DCFDA in M9 buffer, in the dark for 30 min in a 20^{o}C water bath. After staining, nematodes were washed thrice with M9 buffer and mounted for microscopy in PBS with NaN_3 and visualized as described in Section 3.2.

4 RESULTS AND DISCUSSIONS

4.1 PAPER I

At the beginning, we set out to perform a reverse genetic screen in *C. elegans* to identify novel factors modulating host infection outcomes to *B. thailandensis*. There are 16 256 *E. coli* clones in the Ahringer RNAi feeding library [139] and we chose to adopt a targeted approach and screen for genes expressed in the intestine where colonization of *B. thailandensis* was observed (Figure 1). We discovered that RNAi knockdown of *lin-7*, coding for a cell junction protein, resulted in enhanced *C. elegans* survival after infection with *B. thailandensis* (Figure 2). This study highlighted the advantages of an RNAi screen over classical mutagenesis approaches. We would not have found *lin-7* as a potential candidate if we have chosen the latter approach because the vulvaless *lin-7* hermaphrodite would not have outlived the wild-type nematode during infection due to matricidal hatching [140]. Using RNAi has allowed us to partially reduce *lin-7*'s activity down to 40% (Sem *et al.*, unpublished) before pathogen exposure and without any confounding influence from vulval developmental defects. This extent of gene silencing was sufficient to generate an infection phenotype with *B. thailandensis* (Figure 2).

We verified the positive hit from the RNAi screen with various loss-of-function genetic mutants of *lin-7* and similar infection phenotypes were also observed when testing loss-of-function mutations in *lin-2* and *lin-10*. LIN-7 physically associates with LIN-2 and LIN-10 to form a tripartite complex regulating the function of signalling receptors in both mammals and nematodes [141,142,143,144]. Potential confounding factors including aberrant lifespan and the matricidal nature of *lin-7* hermaphrodites were also investigated and they did not deviate from the initial observations. We also observed that the pathogen burden in *lin-7* mutants did not differ significantly much from that in the wild-type (Figure 2).

Previous studies have shown that the LIN-2/7/10 complex modulates the localization and activity of LET-23 receptor tyrosine kinase (RTK), a regulator of vulval development in *C. elegans* vulval epithelial cells [141,142]. This is mediated by LIN-7's PDZ domain binding to the C-terminus of LET-23. Considering these previous

observations, we carried out *in silico* predictions and highlighted DAF-2, the insulin/IGF-1 RTK as a potential signalling receptor which could be regulated by the LIN-2/7/10 complex (Figure 3). Supporting this, yeast two-hybrid assays confirmed that the LIN-2 PDZ domain indeed physically binds to the DAF-2 C-terminus. Subsequently, we used a combination of genetic mutations, RNAi knockdowns and nuclear localization/egression kinetics to show that during infection, *lin-7* may be acting upstream of *daf-2* in the insulin/IGF-1 signalling pathway and that the infection phenotype exhibited by the *lin-7* mutant is likely to be, at least in part, dependent on *daf-16* and *hsf-1* activity (Figures 4 and 5).

Although our initial RNAi screen focused on genes expressed in the nematode intestine, we found that silencing *lin-7* in the hypodermis, but not the intestine, was protective against infection (Figure 6). When previous studies expressed a LIN-7::GFP full-length translational fusion protein in the *lin-7* mutant, they did not detect any LIN-7 expression outside the intestine [142]. Despite this, they did not exclude the possibility that LIN-7 was also expressed outside the intestine *in vivo* as they were able to rescue the vulvaless phenotype of *lin-7* mutants. This indicated that although LIN-7::GFP expression in these cells was not high enough to give detectable fluorescence, it was sufficient to provide cell-autonomous wild-type LIN-7 and hence LET-23 activity. Similarly, our tissue-specific RNAi experiments implicated the hypodermal expression of *lin-7* as a key factor in influencing infection outcomes. Additionally, this suggests that even though the host-pathogen interface is primarily localized to the intestine, tissues outside the intestine (in this case, the hypodermis) clearly can respond and contribute to the overall infection outcome.

The infection phenotype exhibited by *lin-7* mutants probably encompasses, as least in part, increased levels of HSF-1 activity and decreased levels of DAF-16 activity in the hypodermis: Elevated HSF-1 levels could help counter infection-induced protein aggregation [66,68] and suggest a form of infection tolerance in these tissues; DAF-16 regulates genes with antimicrobial activities such as lysozymes (*lys-7*), catalases (*ctl-1, ctl-2*), saposins (*spp-1, spp-12*) and superoxide dismutases (*sod-3*) [145] but it is also a double-edged sword and needs to be tightly regulated to prevent its activity from reaching an undesired threshold [146], and *lin-7* mutants may have the ability to maintain advantageous levels of DAF-16 in the hypodermal tissues and this confers protection to the whole nematode.

Finally, consistent with the DAF-2 pathway being a defence mechanism against multiple pathogens [147], *lin-7* mutants also exhibited enhanced survival upon infection by *P. aeruginosa* and *S.* Typhimurium.

4.2 PAPER II

We performed a forward genetic screen to identify host genes relevant to melioidosis and identified a mutant, *pt1*, which exhibited enhanced survival when exposed to multiple *Burkholderia* spp., but not to other Gram-negative pathogens such as *P. aeruginosa* and *S.* Typhimurium (Figures 2 and 3). In contrast to the infection phenotype exhibited by the *lin-7* mutant in Paper I [62], the infection phenotype exhibited by *pt1* is pathogen-specific and thus, as discussed in Section 1.3.4.3, provide further evidence that the nematode is highly capable of recognizing and responding to distinct pathogens. We show that *pt1* has a loss-of-function mutation in the *unc-7* innexin, a member of an evolutionarily conserved class of gap junction genes (Figure 4). In addition, our observations suggest that *unc-7* and two of its interacting genes, another innexin *unc-9* and a G protein alpha subunit *goa-1* (Figure 5), may function in an interconnected pathway modulating host infection outcomes to *Burkholderia* spp.

All these three genes are expressed in the neurons [148,149,150]. Interestingly, recent studies in *C. elegans* have started to unravel and delineate the molecular mechanisms by which the nervous system influences host defence mechanisms [151,152]. Given the complexity of the nervous and immune systems of mammals, the precise mechanisms by which the two systems influence each other remain understudied and the nematode thus provides an excellent platform to study these relationships. Our study also hints at a possible neuronal involvement in host defence.

We postulate, in Figure S6, that *B. thailandensis* infection results in the dissemination of a tissue-damage signal in the neurons. When *unc-7* or *unc-9* is mutated, the rate of signal spread is reduced, resulting in enhanced survival. Conversely, *goa-1* mutations seem to accelerate or increase neurotransmission of the signal, leading to accelerated death. This is reminiscence of the study by Kawli and Tan where they showed that during *P. aeruginosa* infection, loss of *goa-1* resulted in increased exocytosis and neurotransmission of the insulin-like neuropeptide, INS-7, from dense core vesicles [152]. INS-7 subsequently

acted, in a non-cell-autonomous manner, to hyperactivate the DAF-2 insulin signalling pathway in the intestine, leading to nuclear egression of DAF-16 which when transcriptionally active, usually protects the nematode against *P. aeruginosa.* As a result, the nematode was hypersensitive to infection. *goa-1* is likely to be involved in regulating neurotransmission of more than one molecule since the proposed *unc-7/unc-9/goa-1* pathway in this study is specific to *Burkholderia* spp. and is not involved in *P. aeruginosa* infection (Figure 3). In addition, *goa-1* has been proposed as a negative regulator of calcium channels [149] while innexins are known to facilitate intercellular calcium ion flux [153,154], suggesting that the dissemination of the proposed tissue-damage signal may be correlated with the establishment of calcium ion gradients.

4.3 PAPER III

The mechanisms by which *S.* Typhimurium elicits death in *C. elegans* have not been fully clarified. Similar to previous studies, we observed that *S.* Typhimurium pathogenesis in the nematode did not involve the classical invasive or intracellular phenotype of the pathogen (Figure 2). Our results show that pathogenicity of *S.* Typhimurium in *C. elegans* involves, at least in part, a specific *S. enterica*-induced emergence of ROS in the nematode hypodermal tissues (Figure 3). The production of ROS appeared initially as independent foci and finally culminated together in the hypodermis, despite the lack of any detectable *S. enterica* at these sites. Exposure of nematodes to non-pathogenic *E. coli* and another nematocidal pathogen, *B. thailandensis*, did not result in such a pathogenesis (Figures 3 and 4).

Production of ROS is one of the most primitive defense mechanisms against many invading microbes [155,156,157]. Even in higher organisms with more specific adaptive immune systems, lack of an efficient phagocytic NADPH oxidase-mediated oxidative burst sensitizes mammals to infections with *Salmonella* spp. [158]. *C. elegans* also has the ability to mount oxidative responses mediated by NADPH oxidases [68]. However, when we added ascorbic acid or catalase in our infection experiments, the emergence of ROS was abrogated and the treatments extended rather than shortened the lifespan of infected nematodes (Figure 5). All these strongly imply that the *S. enterica*-induced ROS mediated the infection pathogenesis. This resembles septic shock in mammals where overwhelming ROS production has been implicated as a crucial pathological effector [159].

We could furthermore implicate the bacterial redox enzyme thioredoxin 1 (TrxA) [160] as being essential in evoking this pathogenic ROS emergence (Figures 6 and 7). As *E. coli* also possesses an identical TrxA, the virulence input by TrxA during *S.* Typhimurium infection relies on some *Salmonella*-specific factors. In mammalian cells, it is well-established that TrxA is co-induced with SPI2 and needed for the proper activity of SPI2 during infection [161]. Essentially, virulence of the *trxA* mutant is attenuated similarly to a SPI2 mutant in the murine infection model. However, in *C. elegans,* the same SPI2 mutant, deficient in a T3SS apparatus protein SsaV, did not exhibit the same infection phenotype as the *trxA* mutant (Figure 6) nor was there any additional effect when both *trxA* and *ssaV* were mutated (Sem *et al.*, unpublished). This suggests that with regards to the pathogenesis in *C. elegans*, TrxA was functionally connected to other *Salmonella*-specific factor other than SPI2.

We are critical to note that the disseminated oxidative stress is not the only mechanism mediating pathogenesis in *S.* enterica-infected nematodes as the *trxA* mutant was still more virulent than non-pathogenic *E. coli,* abrogation of ROS rescued infected nematodes only by a certain extent and a LPS O-antigen-deficient mutant still evoked the ROS response despite being attenuated in virulence. Hence, we have identified a new mechanism by which pathogens, specifically *S. enterica,* mediate lethality in *C. elegans* and our findings also suggest a novel and unique aspect of bacterial TrxA.

4.4 PAPER IV

For the past two decades, *S. enterica* started to show multidrug resistance and now pose a serious public health problem [162], reiterating the need to search for compounds which, in contrast to antibiotics, interfere with the expression or functionality of bacterial virulence factors [163]. Somewhat similar to the discussion in Section 1.3.4.4, such compounds would perhaps place a lesser amount of selective pressure on the pathogen. As such, one of the strategies is to conduct high-throughput screens of chemical compounds. From such screens, it was shown previously that two small-molecular virulence inhibitors, the salicylidene acylhydrazide INP0010 and the proton pump inhibitor omeprazole, reduced SPI2-mediated intracellular replication of *S.* Typhimurium in murine macrophage-like RAW264.7 cells without affecting *in vitro* bacterial growth or the viability of the host cells [161,164]. Here, we observed disparate effects when they were used in combination and applied to different infection

models including epithelial MDCK cells, macrophage-like RAW264.7 cells and *C. elegans*.

When both INP0010 and omeprazole were applied during *S.* Typhimurium infection of macrophage-like cells, an antagonistic rather than synergistic combined effect on bacterial intracellular growth inhibition was observed (Figure 1). Interestingly, INP0010 and omeprazole did not reduce bacterial intracellular replication in epithelial cells, suggesting that these small-molecular virulence inhibitors may also have the ability to modulate host immune responses. Indeed, the antagonistic effect exerted by both compounds was correlated with similar patterns observed in levels of inducible nitric oxide synthase expression and nitric oxide (NO) production from compound-treated and infected macrophage-like cells (Figure 2). Epithelial cells, on the other hand, do not produce detectable amounts of NO.

When analyzing either ROS production in infected macrophage-like cells (Figure 3) or survival (non-paralysis) of infected *C. elegans* (Figure 6), we observed that omeprazole alone exerted a small enhancement of the parameters measured. This time, it acted in synergy with INP0010, which alone already significantly increased the ROS production and nematode survival to a larger extent. On the contrary to mammalian cells, we observed that the compounds reduced induction of ROS in infected nematodes (Sem *et al.*, unpublished), in accordance to Paper III [43] where we showed that oxidative stress is detrimental to *C. elegans* during *S.* Typhimurium infection. Nevertheless, these observations strongly illustrated that when used in combination, INP0010 and omeprazole may converge upon certain oxidative pathways, giving rise to different effects in different host models used. Our results also suggest that these small molecules, initially selected for their abilities to inhibit specific virulence determinants of the pathogen, can also modulate host immune responses to enhance survival of the infected host.

Hence, in addition to being amenable for high-throughput whole-animal assays for chemical compound screening, the *C. elegans* system is also useful to elucidate mechanistic activities of positive hits. In conjunction with Paper III [43], our observations also open up the possibility to use *C. elegans* to screen pharmaceutical regimens to control overwhelming ROS responses during sepsis.

5 EPILOGUE

5.1 "IN THE BEGINNING WAS SYDNEY BRENNER"

C. elegans has a low number of cells – hermaphrodites have 959 somatic cells while males have 1031. In contrast to *Drosophila* and higher animals, nematode cells also do not move much during development. Hence the fate of every cell in the adult nematode from the moment the egg hatched was easily mapped out during early work by John Sulston and H. Robert Horvitz [34,35]. *C. elegans* embryos are also transparent and this has allowed tracking of the fate of all 671 embryonic cells that are born, and the 113 which die during early growth [36]. With the *C. elegans* cell lineage completed, researchers are now able to map a cell's predecessors and successors by tracing the tree.

"In the beginning was the worm" - I quote here the title of the 2009 *Genetics* paper [165] authored by Sydney Brenner, who is the nematode's main man and instrumental in establishing this novel experimental system in the 1970s; **"In the beginning was Sydney Brenner"** - all *C. elegans* research essentially originated from him and many scientists working with the model organism share a close connection, having either worked in his lab or in the lab of someone who previously worked with him and so forth. Using information adapted from the WormBase, I attempt to map out the "cell lineage" related to this thesis (Figure 8).

5.2 *C. ELEGANS* I SVERIGE

Sweden has played a significant role in paying homage to *C. elegans* and honouring the work done in this field. The award-winning scientists are highlighted with asterisks in Figure 8.

In 2002, the Nobel Assembly at **KI** awarded the Nobel Prize in Physiology or Medicine jointly to Sydney Brenner, H. Robert Horvitz and John Sulston for their discoveries on the genetic regulation of organ development and programmed cell death [23,35,166]. They developed *C. elegans* as a novel model organism and identified key genes regulating these processes. Sydney Brenner in his Nobel lecture crowned *C. elegans* as the "4th winner of the Nobel Prize" that year, given that the nematode has been

"Nature's gift to Science". And hence the quote at the beginning of this thesis: *"The Universe is one great kindergarten for man. Everything that exists has brought with it its own peculiar lesson."* – referring not just to *C. elegans* but also to the microbes.

In 2006, **KI** awarded the Nobel Prize in Physiology or Medicine jointly to Andrew Fire and Craig Mello for their discoveries of RNAi in *C. elegans* using dsRNA [167]. Without their discoveries and further developments, one quarter of this thesis would not have been possible.

In 2008, the **Royal Swedish Academy of Sciences** awarded the Nobel Prize in Chemistry jointly to Osamu Shimomura, Martin Chalfie and Roger Tsien for their discoveries and development of the green fluorescent protein. Martin Chalfie worked on GFP in *C. elegans* [168]. These discoveries have greatly accelerated microscopic studies in living organisms.

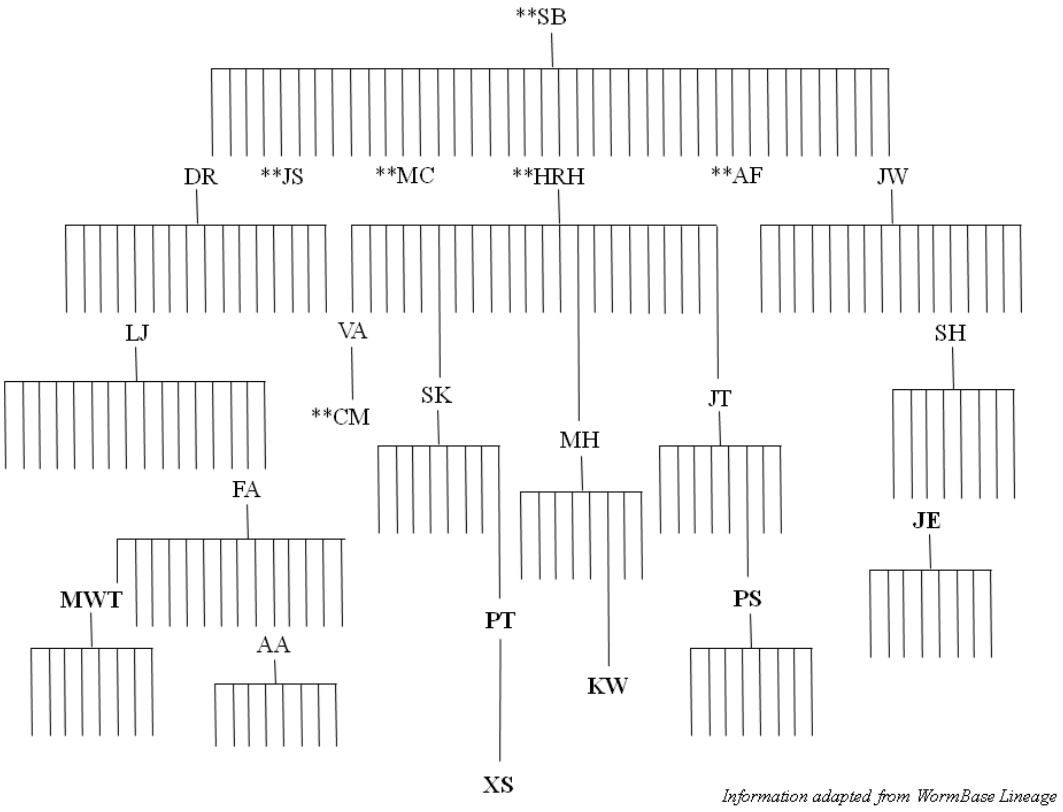

Figure 8. Highlights of the thesis "cell lineage"

5.3 *C. ELEGANS* RNAI CLONE LOCATOR

During my second year, I signed up for a “Practical programming for scientists” course #2215, organised by Sten Linnarsson, MBB. The course aimed to teach the C# programming language in a practical context for solving problems in research. I learnt how powerful and helpful programming can be but was not too ambitious to become an expert after this. Initially I was very excited and satisfied after completing some small application exercises on the .NET platform. But as we advanced further into the course and started working on the assignment, I found it an extremely taunting experience to learn this new language as well as meet the assignment deadline. Learning Swedish, on the other hand, seemed relatively much easier even though I’m still nowhere near proficient; similarly the rich repertoire of European languages embracing my stint at KI appeared less intimidating as well. At the end of the course, we each submitted a functional application relevant to our research or domain of expertise. Here in my thesis, I would like to share this little application which greatly facilitated my experiment planning and execution for the remaining of the PhD journey – certainly living up to the definition of “practical”. I would like to thank the instructor and several programming friends for their technical advice and patient coaching.

The problem: A total of 16 256 *E. coli* clones in the Ahringer RNAi feeding library [139] were stored as glycerol stocks in 384-well plates. A robotics platform has systematically arrayed these plates into independent copies of 96-well plates. Each time I need to retrieve a RNAi clone from the library, I had to manually search for its location in the 384-well library and translate it into a 96-well library location using different worksheets in an Excel file. Often this is tedious and highly prone to human error, especially when I may need to retrieve many clones at one time for screening purposes. Thus I would like to write an application which allows me to computationally retrieve these locations in the most accurate and efficient method. These RNAi clones are identified by their GenePairs Name and I would need to translate their corresponding GeneService Location (position in the original 384-well library, Figure 9) to an output which is more useful to me (their new position in the 96-well library).

The components of the GeneService Location (384-well library location) are broken down here, explaining how they are individually translated to the final 96-well library

plate and well location. For the gene *lin-7*, the 384-well library location is II-9I09 and the 96-well location is 0209021-E5.

	A	B	C	D	G
1	Plate	Well	Chrom	GenePairs Name	Geneservice _Location
2	1	A01	I	K10E9.1	I-1A01
3	1	A03	I	F56C11.1	I-1A05
4	1	A04	I	F56C11.2	I-1A07
5	1	A05	I	F56C11.3	I-1A09
6	1	A06	I	F56C11.5	I-1A11
7	1	A07	I	F56C11.6	I-1A13
8	1	A08	I	F53G12.1	I-1A15
9	1	A09	I	F53G12.2	I-1A17
10	1	A10	I	F53G12.3	I-1A19
11	1	A11	I	F53G12.4	I-1A21
12	1	A12	I	F53G12.5	I-1A23
13	1	B01	I	F53G12.6	I-1C01
14	1	B02	I	F53G12.7	I-1C03
15	1	B03	I	F53G12.8	I-1C05
16	1	B04	I	F53G12.9	I-1C07
17	1	B05	I	F53G12.10	I-1C09
18	1	B06	I	F53G12.11	I-1C11
19	1	B07	I	C53D5.a	I-1C13
20	1	B08	I	C53D5.b	I-1C15
21	1	B10	I	C53D5.d	I-1C19
22	1	B11	I	C53D5.e	I-1C21
23	1	B12	I	C53D5.f	I-1C23
24	1	C01	I	C53D5.g	I-1E01
25	1	C03	I	C53D5.i	I-1E05

Figure 9. List of GeneService / 384-well library locations of each E. coli clone

96 Format (Quadrant A) 1

	1	2	3	4	5	6	7	8	9	10	11	12
A	A1	A3	A5	A7	A9	A11	A13	A15	A17	A19	A21	A23
B	C1	C3	C5	C7	C9	C11	C13	C15	C17	C19	C21	C23
C	E1	E3	E5	E7	E9	E11	E13	E15	E17	E19	E21	E23
D	G1	G3	G5	G7	G9	G11	G13	G15	G17	G19	G21	G23
E	I1	I3	I5	I7	I9	I11	I13	I15	I17	I19	I21	I23
F	K1	K3	K5	K7	K9	K11	K13	K15	K17	K19	K21	K23
G	M1	M3	M5	M7	M9	M11	M13	M15	M17	M19	M21	M23
H	O1	O3	O5	O7	O9	O11	O13	O15	O17	O19	O21	O23

96 Format (Quadrant C) 3

	1	2	3	4	5	6	7	8	9	10	11	12
A	B1	B3	B5	B7	B9	B11	B13	B15	B17	B19	B21	B23
B	D1	D3	D5	D7	D9	D11	D13	D15	D17	D19	D21	D23
C	F1	F3	F5	F7	F9	F11	F13	F15	F17	F19	F21	F23
D	H1	H3	H5	H7	H9	H11	H13	H15	H17	H19	H21	H23
E	J1	J3	J5	J7	J9	J11	J13	J15	J17	J19	J21	J23
F	L1	L3	L5	L7	L9	L11	L13	L15	L17	L19	L21	L23
G	N1	N3	N5	N7	N9	N11	N13	N15	N17	N19	N21	N23
H	P1	P3	P5	P7	P9	P11	P13	P15	P17	P19	P21	P23

96 Format (Quadrant B) 2

	1	2	3	4	5	6	7	8	9	10	11	12
A	A2	A4	A6	A8	A10	A12	A14	A16	A18	A20	A22	A24
B	C2	C4	C6	C8	C10	C12	C14	C16	C18	C20	C22	C24
C	E2	E4	E6	E8	E10	E12	E14	E16	E18	E20	E22	E24
D	G2	G4	G6	G8	G10	G12	G14	G16	G18	G20	G22	G24
E	I2	I4	I6	I8	I10	I12	I14	I16	I18	I20	I22	I24
F	K2	K4	K6	K8	K10	K12	K14	K16	K18	K20	K22	K24
G	M2	M4	M6	M8	M10	M12	M14	M16	M18	M20	M22	M24
H	O2	O4	O6	O8	O10	O12	O14	O16	O18	O20	O22	O24

96 Format (Quadrant D) 4

	1	2	3	4	5	6	7	8	9	10	11	12
A	B2	B4	B6	B8	B10	B12	B14	B16	B18	B20	B22	B24
B	D2	D4	D6	D8	D10	D12	D14	D16	D18	D20	D22	D24
C	F2	F4	F6	F8	F10	F12	F14	F16	F18	F20	F22	F24
D	H2	H4	H6	H8	H10	H12	H14	H16	H18	H20	H22	H24
E	J2	J4	J6	J8	J10	J12	J14	J16	J18	J20	J22	J24
F	L2	L4	L6	L8	L10	L12	L14	L16	L18	L20	L22	L24
G	N2	N4	N6	N8	N10	N12	N14	N16	N18	N20	N22	N24
H	P2	P4	P6	P8	P10	P12	P14	P16	P18	P20	P22	P24

Figure 10. Each E. coli clone is systemically arrayed into 96-well plates.

"II" corresponds to the chromosome on which the gene exists. The five autosomes (I, II, III, IV, V) and one X chromosome translate to the first two digits of the 96-well plate identity (01, 02, 03, 04, 05) and (10) respectively. The "9" after the chromosome number and hyphen, indicates the 9th arrayed 384-well plate in this chromosome series and thus gives the next two digits "09". "02" is always, by default, the 5th and 6th digit in the final 96-well location output as the 96-well libraries

were gridded from the "2nd" 384-well library copy stamped by the robotics system. The final digit for the 96-well library identity is deciphered as I09 is the original position in the 384-well library and this is represented in the pink quadrant A or 1 (Figure 10). Finally, I09 corresponds to E05 in the 96-well library quadrant 1.

The application "C. elegans RNAi clone locator": Using C# programming, this tedious process is now automated by various scripts and presented as an executable program shown in Figure 11. Instructions in chronological order are shown in red.

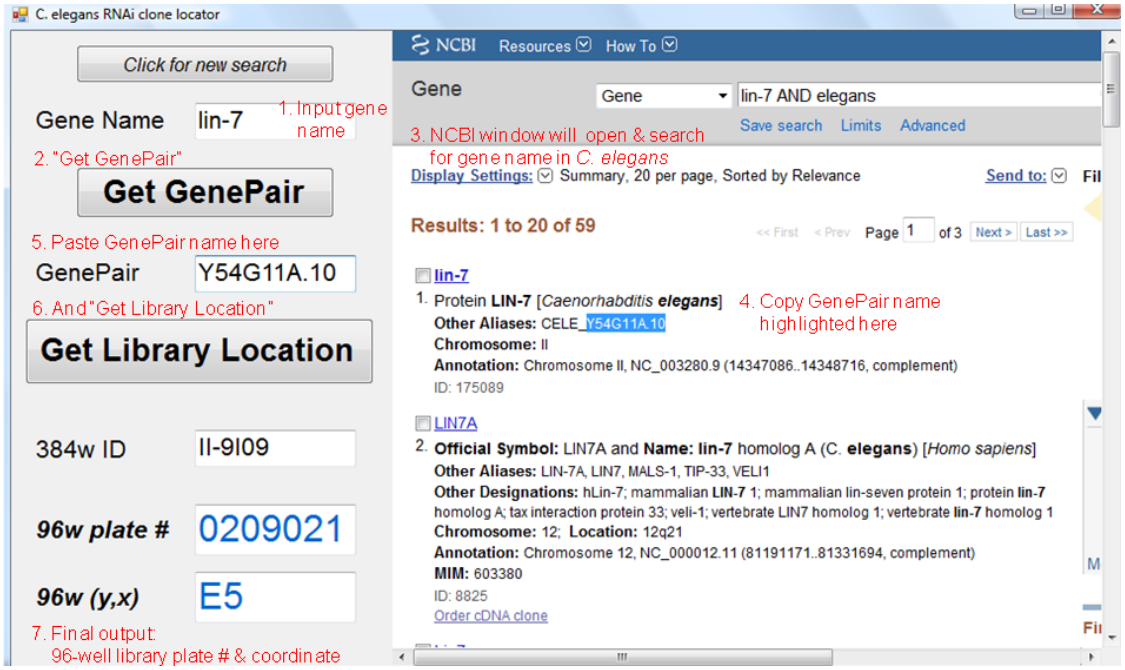

Figure 11. The C. elegans RNAi clone locator.

Bugs to fix when time allows:

- While clearing all text boxes for a new search ("Click for new search"), the web browser should also return to an empty page to reduce confusion.
- If a space was included behind the GenePair input (this occurs frequently while copying and pasting), the program was not able to find this value and would show "Not present in RNAi library!"
- Hope to query a list of gene names and obtain as output a list of 96-well library locations.
- Application depended on internet connection to find the GenePair; hope to integrate with the sequenced genome, enabling offline usage.

6 ACKNOWLEDGEMENTS

This journey could have been the longest learning process in my life so far – learning about science, about research, about the world, about life, about friends, about family and about love. Maybe it's just the age, maybe it's just the era or maybe it's just the PhD. Nevertheless there was not one moment to regret, as they eventually prepared me for the next stage of life. There are a number of people without whom this body of work would not have been possible and I would like to take this opportunity to express my heartfelt gratitude and appreciation.

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Our ex- and current corridor neighbours: **Martin, Big Berit, Small Berit, Frank and Suman**; also **Benedict our favourite mentor, everyone from BHN group especially Ye Ying, Katrin, Jonas and Peter -** for all your warm and comforting "hej hej!" and chitchats in the corridors and all the help and advice rendered during my time at MTC. Special mention to **Jeffni -** for being such a nice girl friend, inviting me to join in your family events…one day our double date will materialize!

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Before I go on to thank the last-but-not-least important person, I must thank IRTG1273, Mikael, Oliver and Prof. Sebastian Suerbaum, for without whom both **Chee Han**'s and my paths would never have crossed anywhere else in the world. Thank YOU for coming into my life and setting things right. For effortlessly and endlessly making sure I sleep properly and wake up promptly every day, regardless of the time difference and geographical location; and for scavenging all my stress and frustrations. The last one year of this journey would not have been any possible without your unconditional love and most importantly, your being more persistent than our favourite *Salmonella*!

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