Host-pathogen interactions in the innate immune response of the nematode *Caenorhabditis elegans*

by

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ABSTRACT

The nematode *Caenorhabditis elegans* has been a powerful experimental organism for almost half a century. Over the past ten years, researchers have begun to exploit the power of *C. elegans* to investigate the biology of a number of human pathogens. This work continues to uncover mechanisms of host immunity and pathogen virulence that are either analogous to those involved during pathogenesis in alternative animal hosts or mechanisms which are, thus far, unique to the worm. In this thesis, we present data that describes an immunological balance in *C. elegans*, whereby heightened tolerance to one pathogen, the enteric bacteria *Salmonella* Typhimurium, comes at the cost of increased susceptibility to another, the fatal fungal human pathogen *Cryptococcus neoformans*. We find that this susceptibility trade-off is mediated by the reciprocal activity of two immune genes: the lysozyme *lys-7* and the tyrosine kinase *abl-1*. We suggest that ABL-1 controls two different DAF-16-dependent pathways to regulate this balance. Both pathways are necessary for wild type resistance to *C. neoformans*, whilst the activity of only one pathway is a requirement for the tolerance phenotype to *S*. Typhimurium. We infer from sequence data that LYS-7 has an atypical mode of action in *C. elegans*, which we hypothesise to be detrimental to the worm during *S*. Typhimurium pathogenesis and thus a contributing factor to the tolerance phenotype. Furthermore, we find that this tolerance has a Salmonella-dependency which we propose to be under the control of the alternative sigma factor, RpoS. Taken together, we describe an immunological balance in *C. elegans* for the first time, one that is mediated by both host and pathogen factors. We therefore suggest that the innate immune response of *C. elegans* has a higher level of immune complexity than previously believed, and that such trade-offs are evolutionarily ancient mechanisms.

For Jane and James

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TABLE OF CONTENTS

LIST OF FIGURES

LIST OF TABLES

LIST OF ABBREVIATIONS

abi	ABL-interacting
AMP	Antimicrobial peptide
ASABF	*Ascaris suum* antibacterial factor
ATP	Adenosine triphosphate
Bus	Bacterially unswollen
cDNA	complementary DNA
CAMP	Cationic antimicrobial peptide
CGC	*Caenorhabditis* genetics center
CNC	*Caenorhabditis* bacteriocin
Ct	Threshold cycle
CTLD	C-type lectin domain
Dar	Deformed anal region
DNA	Deoxyribonucleic acid
dsRNA	double-stranded RNA
EPEC	Enteropathogenic *E. coli*
ERK	Extracellular-signal-regulated kinase
esp	Enhanced susceptibility
FOXO	Forkhead box
GFP	Green-fluorescing protein
HRP	Horseradish peroxidise
IGF	Insulin-like growth factor
IHF	Integration host factor
Int	Integrase
JNK	c-Jun N-terminal kinase
L1 - 4	1^{st}– 4^{th} Larval stage
LB	Luria Bertani medium
LD_{50}	Median lethal dose
LDS	Lithium dodecyl sulfate
LPS	Lipopolysaccharide
lys	Lysozyme
MAPK	Mitogen activated protein kinase
MAPKK	MAP kinase kinase

MAPKKK	MAP kinase kinase kinase
MFα	Mating factor α
mRNA	messenger RNA
MRSA	Methicillin-resistant *Staphylococcus aureus*
NGM	Nematode growth medium
NLP	Neuropeptide-like proteins
ORF	Open reading frame
PAMPs	Pathogen-associated molecular patterns
PBS	Phosphate-buffered saline
PCD	Programmed cell death
PCR	Polymerase chain reaction
PI3K	Phosphoinositide 3-kinase
PRR	Pathogen recognition receptor
qRT-PCR	Quantitative real time PCR
RNA	Ribonucleic acid
RNAi	Ribonucleic acid interference
RNAP	RNA polymerase
SCV	Salmonella-containing vesicle
SDS-PAGE	Sodium dodecyl sulfate polyacrylamide gel electrophoresis
SPI	Salmonella pathogenicity island
T3SS	Type-III secretion system
TCT	Tracheal cytotoxin
TD_{50}	Median survival
TEM	Transmission electron micrograph
TGF-β	Transforming growth factor beta
TIR	Toll/interleukin-1 resistance
TLR	Toll-like receptor
UPR	Unfolded protein response

THESIS OVERVIEW

The nematode *Caenorhabditis elegans* has been utilised as a model organism for over 40 years. These animals offer many experimental advantages that make their application to the field of infectious disease particularly useful: animals can be infected simply by substituting their normal bacterial feeding strain with the pathogen of choice and researchers are able to make use of the wide-reaching investigative tools available for this organism.

In this thesis, I present data that begin to dissect a balance of immunity that we describe for the first time in *C. elegans*. Chapter one introduces our growing understanding of *C. elegans* as a model host in infectious disease since its first description as a model for bacterial infection in 1999 (Tan et al., 1999, Mahajan-Miklos et al., 1999). Next, chapter two describes all the methodology utilised in this work, before I move on to detail my own findings in the results chapters (three to six).

In chapter three, we describe a balance in immunity of a genetic knockout strain that lacks an important defensive lysozyme, LYS-7. We find that these animals are hypersensitive to infection with the fungal pathogen *Cryptococcus neoformans*, but tolerant to the gastrointestinal pathogen, *Salmonella* Typhimurium. The chapter further examines the role of a compensatory mechanism in the nematode that may confer the observed tolerance to this pathogen.

In chapter four, we explore the potential regulation of this balance by the three best characterised immune defence pathways in *C. elegans*; the TGF-β- and IGF-mediated signalling pathways and the p38 MAPK cascade. Conversely, in chapter five, we approach the tolerance phenotype to *S.* Typhimurium from the perspective of the pathogen and examine the bacterial strain dependency of the

phenotype. We return to the *C. elegans* immune response in chapter six, by specifically investigating the LYS-7 protein itself and the potential molecular function of this enzyme.

Finally, in my discussion chapter, I attempt to draw the four results chapters together with reference to the concept of balanced immunity and how it may be regulated and determined by both the host and pathogen. These findings are discussed in relation to published work and with respect to the future experiments needed to continue this project.

1.0 INTRODUCTION

1.1 Host-pathogen interactions

Host-microbe interactions can be studied on many levels, given that not all interactions lead to disease, and those that do have a complex progression that leads to this state. The study of these interactions is becoming increasingly important as a result of recurrent outbreaks of hospital-acquired 'super-bugs', other therapy-resistant infections and the emergence of new pathogens. There are a number of ways of investigating host-pathogen interactions. The various molecular approaches include mutagenesis, cell-based assays with reporters, tissue-based methods, and adhesion-based assays. These methods are complemented by animal studies which examine the infection at the whole organism level. Amidst ever-growing concerns for the welfare of animals in scientific research, there is a heightened need to find ethical organisms in which to study such interactions. Therefore, the discovery that a number of simple and genetically tractable model organisms: *Arabidopsis thaliana* (Dodds and Rathjen, 2010), *Drosophila melanogaster* (Ferrandon et al., 2007), *Caenorhabditis elegans* (Millet and Ewbank, 2004) and Zebrafish (*Danio rerio*) (Trede et al., 2004), are susceptible to a number of human pathogens has been a remarkable advance in this field. This work continues to reveal common mechanisms of immunity across animals and plants including the identification of universal defence genes and the pathways which control their expression in response to infection (Rahme et al., 1995). Thus the use of these models to understand host-pathogen interactions gives insights into the components from both the host and the microbe that are required for a successful interaction. These studies are critical, as understanding the mechanisms involved in immunity may subsequently lead to therapeutic advances for higher animals (Breger et al., 2007, Haine et al., 2008).

1.2 *Caenorhabditis elegans*: a model organism

C. elegans is a free-living nematode that is found in soil and in compost heaps. The population is dominated by self-fertilising hermaphrodites (XX) with a rare occurrence of males (X0) who have a distinct morphology (Figure 1). The animals were first adopted as a laboratory model by Sydney Brenner over 40 years ago (Brenner, 1974). At this time the nematodes were used to study development and behaviour, work which resulted in the Nobel Prize in Physiology or Medicine being awarded to Brenner and his colleagues in 2002 (Frängsmyr, 2003). In the intervening period, *C. elegans* has been used as a model in which to study a wide-range of biological phenomena.

The transparent animals are cultured on a modified agar substrate, termed nematode growth medium (NGM), seeded with an *Escherichia coli* uracil auxotroph, OP50, as a food source (Brenner, 1974). The incubation temperature of this culture significantly alters the progress of the animals through four larval stages to the 1mm-long adult (Figure 2) and as such animal development can be regulated; a feature most advantageous to the laboratory setting. The animals have a generation time of four days and a lifespan of approximately three weeks at 20°C. Interestingly, the animal also has an alternative third larval arrest stage, called dauer. Animals enter this state upon over-crowding, starvation or upon the production of a dauer hormone (Golden and Riddle, 1982). As dauer larvae, nematodes are hyper-resistant to stress and they do not age (Kenyon et al., 1993, Hu, 2007). Given these advantageous characteristics, there has been a large investment in understanding the mechanisms that lead to dauer larvae.

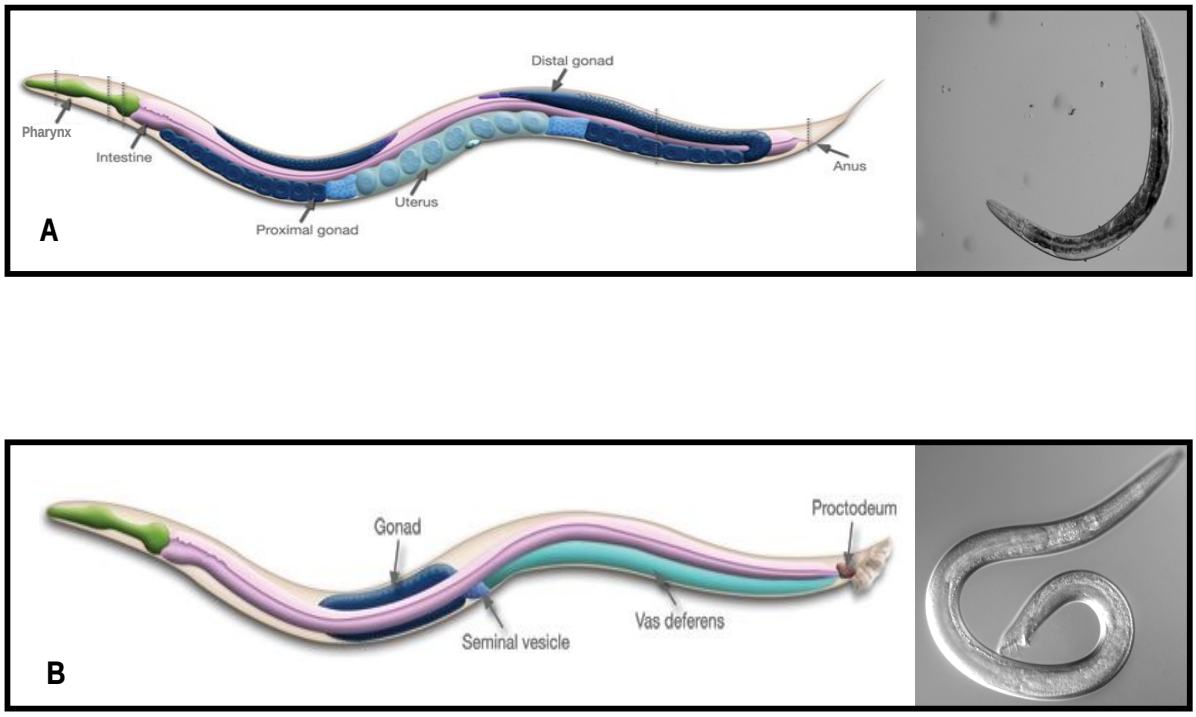

Figure 1: The male and hermaphrodite worm

Schematic and microscope images of hermaphrodite (A) and male (B) animals. Characteristic features include the eggs and vulva in the hermaphrodite and the fan shaped tail of the male.

Images sourced from WormAtlas 1.0

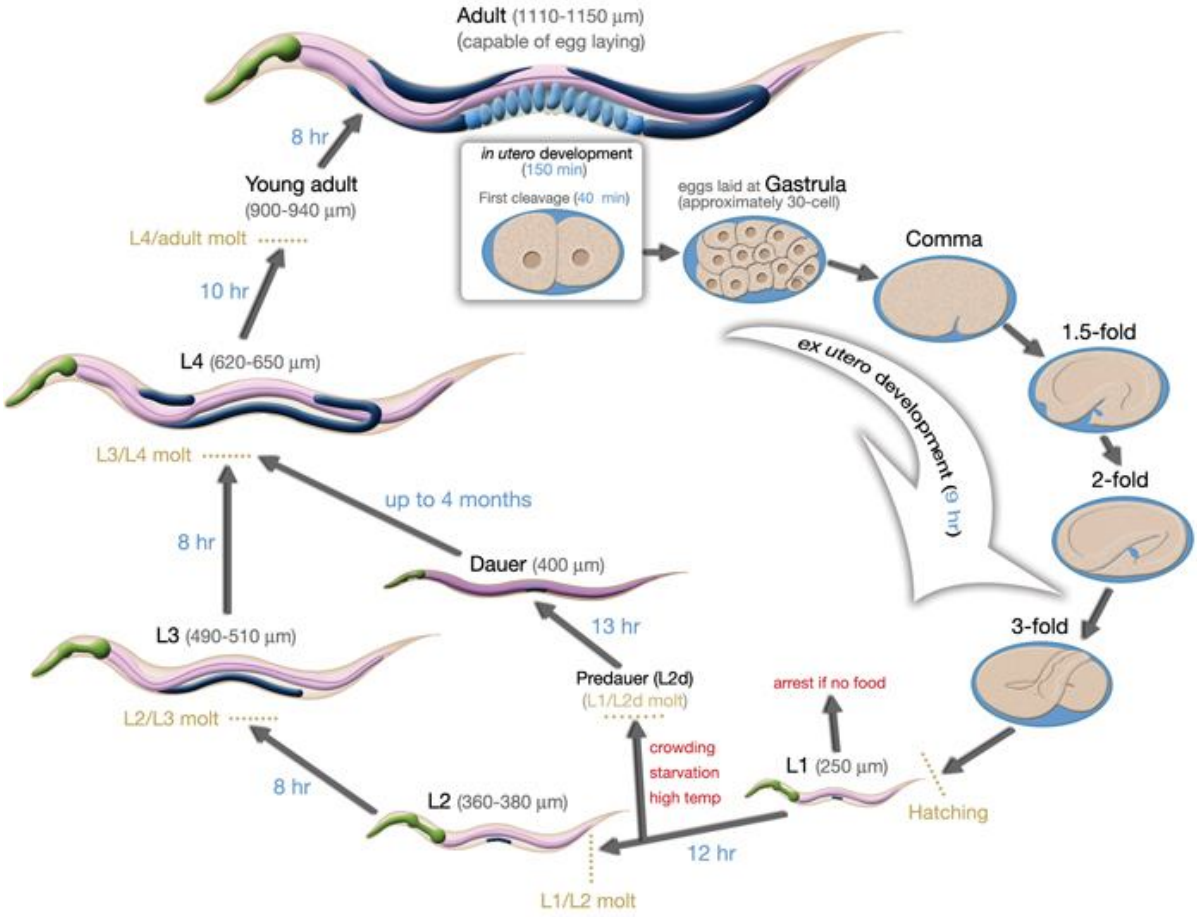

Figure 2: The *C. elegans* lifecycle

The lifecycle of *C. elegans* consists of four larval stages to the fertile adult. This development can be bypassed by an alternative larval stage, called dauer, in unfavourable conditions. These animals later rejoin the cycle when conditions improve.

Image sourced from WormAtlas 1.0

Over the past 40 years, *C. elegans* has been extensively studied: there are vast amounts of genotypic and phenotypic data available as the organism is genetically tractable. The entire nervous system of the animal has been reconstructed (White et al., 1986) and the essentially invariant lineage of each somatic cell is known (Sulston et al., 1983, Kenyon, 1988). Furthermore, *C. elegans* was the organism in which both the process of programmed cell death (Horvitz, 2003) and RNA interference (Fire et al., 1998) were characterised. The genome sequence of the animal was initially published ten years ago identifying approximately 19,000 genes and significantly increasing the volume of information biologists have at their fingertips about the animal (*C. elegans* Sequencing Consortium, 1998).

Like many model organisms, laboratory strains of *C. elegans* have been subjected to a series of genetic bottlenecks with continual adaptation to amenable culture conditions. Potentially the application of wild *C. elegans* isolates to laboratory research may provide one solution to this problem (Barriere and Felix, 2005); in fact, we know surprisingly little about the natural behaviour of *C. elegans*. The advantages that *C. elegans* brings to the laboratory have furthered the development of other, closely-related, *Caenorhabditid* species as model organisms. One other hermaphroditic species is *C. briggsae*, which, despite having an almost indistinct morphology from *C. elegans* (Nigon and Dougherty, 1949), is believed to have diverged around 100 million years ago (Coglan and Wolfe, 2002). The genome of *C. briggsae* was published in 2003, providing a platform for comparative genomics with *C. elegans* (Stein et al., 2003). Despite extensive similarity between the two genomes, around 800 genes were found to be unique to *C. briggsae*, possibly being pseudogenes or genes under very rapid evolution. Moreover, this new sequence provided evidence for over 1,000 new genes in *C. elegans*, centred upon similarities between the species (Stein et al., 2003). Within the genus, the other best-described species are gonochoristic, and include the species *remanei* and *brenneri* (the

latter named in honour of Sydney Brenner). Together, this offers a rich platform for biological study from an evolutionary perspective when utilising the *Caenorhabditis* nematodes.

Model organisms are employed in biological research because understanding biological processes and mechanisms at the simple level of these animals can have significant consequences for the understanding of homologous mechanisms at a more complex level. *C. elegans* shares a number of biological features and pathways with higher vertebrates. Rigorous studies on *C. elegans* can therefore provide important insights into homologous processes in these higher organisms; identifying lead genes and pathways which help to focus the field of investigation.

1.3 *C. elegans* and Immunity

Any organism must be able to defend itself against pathogenic attack to ensure its subsequent survival and propagation. Despite an intense research focus on adaptive immunity, the vast majority of organisms (including *C. elegans*) rely exclusively on the innate immune system for disease resistance, a relatively non-specific response. Even in organisms with adaptive immunity, it is often the innate immune system that triggers the onset of an adaptive immune response.

For a number of years, adaptive immunity has been thought to be restricted to vertebrates, but increasing evidence is suggesting that there is more of a cross-over than previously believed. For instance, the processing of mRNA has been found to confer adaptive features onto invertebrate innate immunity in *Drosophila melanogaster* and *Anopheles gambiae.* In the former case, the immunoglobulin-like Down syndrome cell adhesion molecule is alternatively spliced to create around 18,000 isoforms which have functional diversity in the innate response (Watson et al., 2005); in the

mosquito, alternative splicing is used to produce hypervariable antibody-like molecules to bring a new level of complexity to its immune response (Garver et al., 2008).

Although, as mentioned, the innate immune system is a non-specific response, in some higher organisms there is a remarkable complexity, mediated by the action of phagocytes and the complement system. In addition, it had been assumed that evolutionarily conserved secreted antimicrobial peptides, such as lysozyme, act universally on the front line of innate defence in all organisms upon pathogen exposure. However, a recent study in beetles (*Tenebrio molitor*), has shown that antimicrobial peptides which act against *S. aureus* infection are not induced until the majority of the infection has been cleared from the haemolymph. This suggests that the peptides do not act as general "natural antibiotics", and are instead specifically induced to contain and clear any bacteria that are intrinsically resistant to the constitutive immune defence, and thus act as a more specialised "last line of defence" (Haine et al., 2008).

C. elegans provides a number of benefits as a model host for studying innate immunity. Providing that the pathogen of choice is a suitable nutritional source for the animals, it can simply be substituted in place of the normal feeding bacteria OP50, thus the primary site of the infection is the intestine (Figure 3), although there are some exceptions. Phenotypes such as animal survival, motility, pharyngeal pumping rate, pathogen burden and so forth, can subsequently be easily and non-invasively examined. Work by a number of groups has revealed a complex innate immune approach in *C. elegans* comprising avoidance behaviours and physical barriers. However, systemic immunity is believed to rely purely upon the secretion and action of antimicrobial molecules, including lectins, lysozymes and antibacterial factors (Schulenburg et al., 2004).

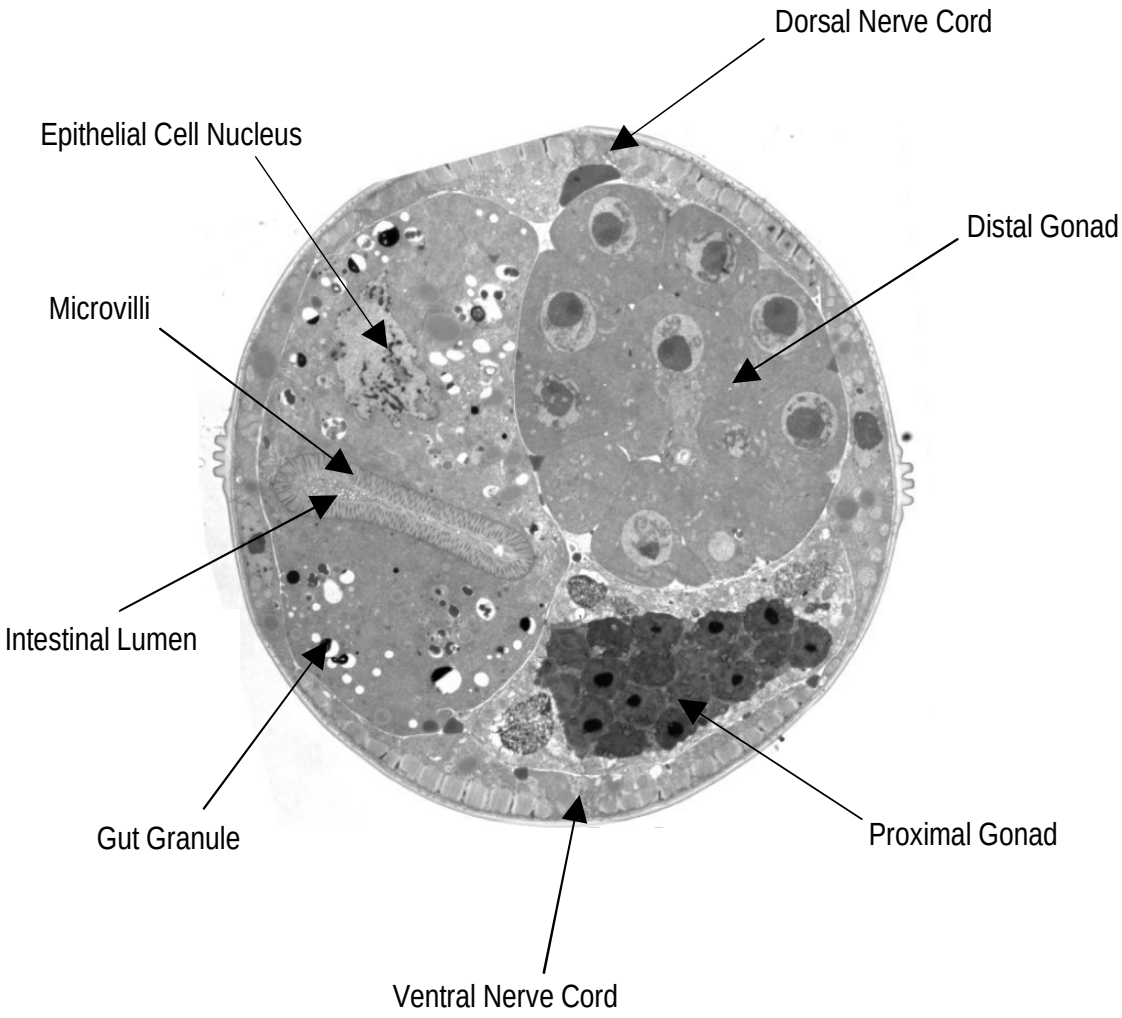

Figure 3: The internal structure of *C. elegans*

This TEM cross-section of the worm highlights the positions of both gonads and the intestine in the body cavity. The intestine is comprised of 20 large epithelial cells that associate as pairs to form a tube around the lumen that runs in parallel to the gonad along the body. At the apical side of each epithelial cell is the microvilli brush border, anchored to the terminal web. This is also where a series of adherens junctions run between cells to provide a tight seal against the contents of the lumen.

Image sourced from WormAtlas 1.0 WormViewer

1.3.1 C-type Lectins

A number of proteins with Calcium-dependent carbohydrate binding C-type lectin domains (CTLD) have been identified in *C. elegans* that are related to, although different from, those found in mammals (Drickamer and Dodd, 1999, Zelensky and Gready, 2005). Certainly in vertebrates CTLD have a key role in the humoral response; the recognition and binding of pathogen-associated molecular patterns (PAMPs) is believed to be one of the most crucial ways of identifying non-self from self and eliciting an immune response (Zelensky and Gready, 2005). However, their function in the worm is less clear. The family comprises 278 genes, the seventh most abundant gene family in this species (*C. elegans* Sequencing Consortium, 1998), that appear to elicit pathogen-specific responses; the proteins have been shown to be induced and secreted in response to infection (Mallo et al., 2002, O'Rourke et al., 2006, Wong et al., 2007, Schulenburg et al., 2008). Thus the proteins are ostensibly involved in the *C. elegans* immune response and may be the source of pathogen-specificity, perhaps by acting as PAMP receptors (Nicholas and Hodgkin, 2004, Schulenburg et al., 2008). In opposition to this hypothesis however, 85% of the proteins are predicted to be non-carbohydrate binding (Zelensky and Gready, 2005) indicating that the animal may simply, yet specifically, respond to the individual stresses caused by the pathogen; a hypothesis that has also been proposed by the Ewbank laboratory (Ewbank, 2006). Together, CTLD proteins have an, as yet, unknown antimicrobial function in the worm.

1.3.2 Lysozyme

Lysozyme is a ubiquitous defence enzyme found across many forms of life. In *C. elegans*, lysozymes are one family of antimicrobial enzymes that are believed to be actively lytic against the invading pathogen; the *lys* gene family appear to be homologous to lysozymes in the protozoan *Entamoeba histolytica*, and are thought to eradicate Gram-positive bacteria by working synergistically with amoebapore-like peptides also found in *C. elegans* (Leippe, 1999, Nickel et al., 1998). Using reporter-

gene constructs, lysozyme proteins in *C. elegans* were found to be localised to the intestine, consistent with the site of bacterial infection (Mallo et al., 2002). Yet the defensive function of these enzymes is thought to be secondary, having evolved from the essential role of these enzymes in the digestion of the animals' bacterial food source (Bachali et al., 2002). Unlike other organisms which have only one or two lysozyme genes, *C. elegans* has a family of ten differentially regulated lysozyme genes of the protist-type, which fall into two clades (Figure 4). Those of clade one are thought to have the primary immune function; these include the lysozymes 1, 2, 3, 7, and 8, which have all been implicated in the immune response of the animal to a range of pathogens (Mallo et al., 2002, O'Rourke et al., 2006, Evans et al., 2008b, Murphy et al., 2003, Troemel et al., 2006, Shapira et al., 2006, Huffman et al., 2004, Wong et al., 2007, Alper et al., 2007). Additionally, the animal has five invertebrate-type lysozyme genes, which together with clade two, are suggested to be involved in digestion. This large gene repertoire is thought to have arisen by both ancient and more recent gene duplications, permitting the unusual functional diversity believed to be present in the animal (Schulenburg and Boehnisch, 2008).

1.3.3 Antibacterial factors

Another antimicrobial gene family homologous to the antibacterial factor found in the human pathogenic nematode *Ascaris suum* (*Ascaris suum* antibacterial factor, ABASF) has been identified in *C. elegans*. The family appears to consist of six genes (WormBase), though only two, *abf-1* and *abf-2,* have been described in detail and of which only the antimicrobial activity of *abf-2* has been confirmed *in vitro* (Kato et al., 2002). In *C. elegans*, *abf-2* expression was found to be localised to the pharynx indicating that the peptide might be secreted into the intestinal lumen of the worm where it may defend

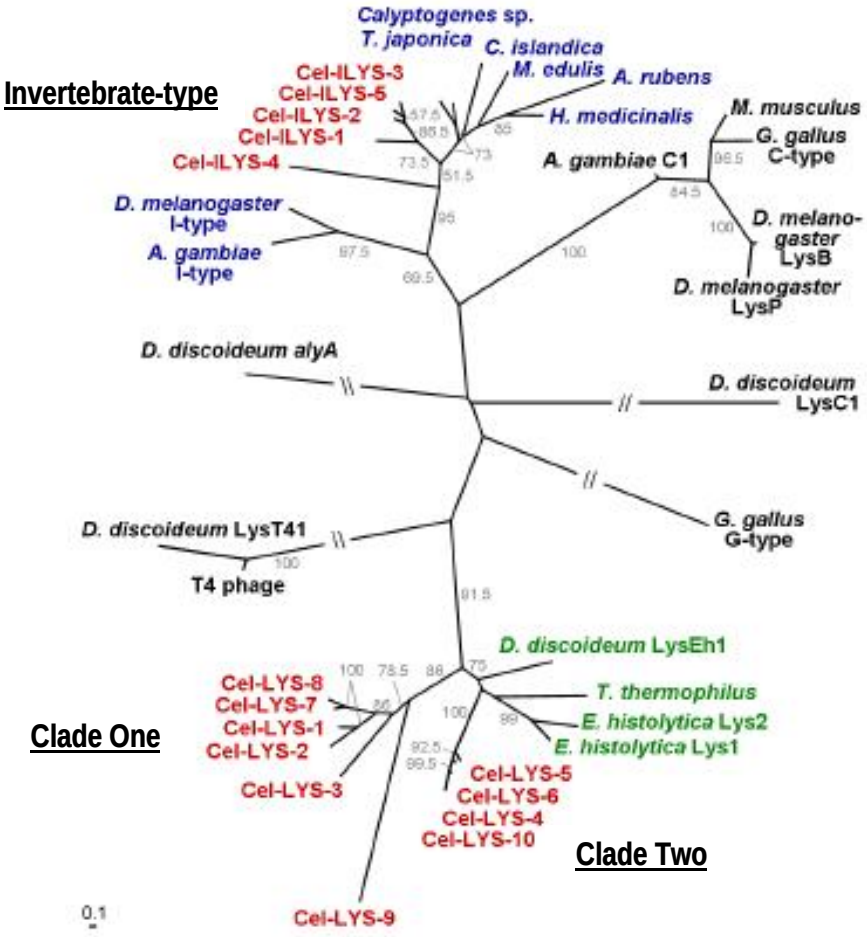

Figure 4: Lysozyme Evolution

Phylogenetic relationships between the *C. elegans* lysozymes and its close relatives; the tree was constructed using amino acid sequences and maximum likelihood, it is unrooted. The *C. elegans* family is grouped into 2 clades, with a third distinctly related group being the invertebrate-type lysozymes.

Image sourced from (Schulenburg and Boehnisch, 2008)

against pathogenic attack: the recombinant protein has potent activity against Gram-positive bacteria and to some extent both Gram-negative bacteria and yeasts are susceptible to its action.

Sequence analysis revealed that the peptides were encoded together on an operon and were believed to have diverged from a common ancestor (Kato et al., 2002). This organisation would allow coordinated expression upon infection, although the genes were not always secreted synchronously (Kato et al., 2002). The antimicrobial function of the five annotated ASABF genes in *A. suum* were investigated further; these nematodes being large enough to easily inject with non-pathogenic bacteria and to subsequently retrieve the pseudocoelomic fluid for analysis. All the ABASF-type peptides tested were induced upon the injection of non-pathogenic OP50 into the intestine, but not after the injection of sterile saline into the gut. This indicated that there must be some form of recognition system in *A. suum*, which enables the nematode to mount a specific immune response to pathogen, as opposed to a more general induction of stress response genes (Pillai et al., 2003). There is potential for the same to be true in *C. elegans*.

As a natural host, only three pathogens of *C. elegans* have been described: the Gram-negative bacteria *Microbacterium nematophilum,* the fungal infection *Drechmeria coniospora* and more recently, the microsporidian parasite *Nematocida parisii*.

1.3.4 *Microbacterium nematophilum*

M. nematophilum was discovered through chance contaminations of *C. elegans* laboratory cultures, as infected animals displayed an unusual and visible tail swelling, or deformed anal region (Dar), previously believed to be a spontaneous, and seemingly heritable, morphological mutation that arose during a routine genetic cross. However, later analysis of these animals, and others, demonstrated

that the Dar phenotype was the result of a novel pathogen of *C. elegans* (Hodgkin et al., 2000). These bacteria establish a specific rectal infection owing to their strong extracellular adherence to the cuticle that, in turn, causes a localised swelling response in the host (Hodgkin et al., 2000, Nicholas and Hodgkin, 2004). Additional work showed that this Dar phenotype was a consequence of the limited activation of the ERK MAPK cascade in the region, perhaps as a defence mechanism raised against the infection (Nicholas and Hodgkin, 2004), although this remains unclear (Hodgkin et al., 2000, Gravato-Nobre et al., 2005). Although not lethal, the animals develop slowly when feeding on pure *M. nematophilum* lawns and show signs of constipation (Hodgkin et al., 2000). The isolation of a series of mutant animals resistant to the infection, exhibiting a bacterially unswollen (Bus) phenotype, demonstrated that a number of *C. elegans* genes are responsible for the Dar response. These mutations have implications for both the host (capability to elicit a swelling response) and the bacteria (ability to adhere and colonise) (Gravato-Nobre et al., 2005) and many of these genes are clustered on the *C. elegans* genome (O'Rourke et al., 2006). Some of the Bus mutants were not colonised by the pathogen, indicating that mutations in the formation of the cuticle may have prevented the establishment of an infection (Hodgkin et al., 2000, Gravato-Nobre et al., 2005). *bus-2* mutant nematodes, for instance, secrete far fewer core-1 *O*-glycans, required for a pathogenic interaction with the bacteria, into the exterior coat of the cuticle and the structure is compensated for with the secretion of alternative carbohydrates (Palaima et al., 2010). The hypothesis is that these carbohydrates mask any other core-1 *O*-glycans present in the cuticle coat and lead to the resistance phenotype (Palaima et al., 2010).

A further study examined the genes induced by the animal in response to a six hour infection with *M. nematophilum*. 89 genes were demonstrated to be differentially expressed, of which 68 were up-regulated and 21 were down-regulated (O'Rourke et al., 2006). The genes induced upon pathogen

infection included a number of proteins with C-type lectin domains, lysozymes and other putative pathogen receptor molecules. Notably, *lys-7* was one of the key genes that was up-regulated in response to infection, and these data were supported by the observation that *lys-7* mutant nematodes were severely constipated and exhibited a strong Dar phenotype when infected with the bacteria. In the same vein, knockdown of selected CTLD genes with RNAi changed the response of the animals to the infection; again these worms were more susceptible to the infection. The induced genes were located in domain-specific clusters on chromosomes IV and V, potentially enabling the synchronised expression of these genes upon infection (O'Rourke et al., 2006).

1.3.5 *Drechmeria coniospora*

D. coniospora, on the other hand, is a nematode parasite that adheres to the mouth and vulva of animals and penetrates throughout the entire worm by means of proteinaceous conidia (Jansson, 1994). This colonisation of *C. elegans* triggers an immune response in the host, predominantly through the induction of neuropeptide-like proteins (NLPs) (Couillault et al., 2004). Some of the 32 NLP genes, identified via their homology to other invertebrate neuropeptides, are thought to act as non-classical neurotransmitters, whilst others have gained alternative functions. Most genes are expressed in the neurons of the worm, but a large number are also expressed in the intestine, perhaps due to a proposed role for them in the animals' behavioural muscle contractions (defecation) (Nathoo et al., 2001). In the immune response, a cluster of these genes, the *nlp-29* cluster, is induced by the activity of the p38 MAPK cascade upon *D. coniospora* infection (Couillault et al., 2004, Pujol et al., 2008a). These proteins not only cluster together on Chromosome V, but also form an independent structural group of *C. elegans* NLPs (Nathoo et al., 2001, Pujol et al., 2008b). The proteins localise to the epidermis of the animal (Pujol et al., 2008a) where they can respond to fungal-induced or

mechanically-induced epidermal wounding; the regulation of these peptides in response to both *D. coniospora* infection and cuticle wounding is discussed later.

Another group of antimicrobial molecules activated in response to infection with *D. coniospora* are the Caenacin (for *Caenorhabditis* bacteriocin, or CNC) proteins (Couillault et al., 2004, Zugasti and Ewbank, 2009). Despite being structurally related to the NLP immune genes, and also being situated in a cluster on Chromosome V, the CNC proteins are a discrete group of antimicrobials. The artificial over-expression of these peptides renders the animal resistant to fungal infection and their induction in response to *D. coniospora* infection is dose-dependent upon TGF-β signalling, by means of the *C. elegans* homologue *dbl-1* (Zugasti and Ewbank, 2009). The specific role of both the NLP and CNC peptides in response to the fungal infection remains elusive.

1.3.6 *Nematocida parisii*

In the case of *Nematocida parisii*, this intracellular parasite is a member of the microsporidia phylum, although it is both a novel genus and species. The pathogen was discovered when a newly isolated wild *C. elegans* strain from a compost heap in Franconville, France was found to be infected with an unknown pathogen that could be transferred horizontally through an animal culture, but not vertically. The microbe was unable to be cultured *in vitro*, thus animal survival was assessed using 'infectious extracts' from an infected plate, and animals at all developmental stages were found to be susceptible to the pathogen. The parasite progressed through its infection cycle, from meront to spore, the latter being the infectious stage though the former appears to do the most damage to the host. The spores cause specific damage to the terminal web of the intestinal epithelium structure, targeting intermediate filaments in particular, but not altering the microvilli structure. Later, the infection overwhelmed the intestine of the animal, and the spores associated together in extracellular vesicles. The host response

to this infection appears to be unique; the infection did not induce fundamental response genes known to be crucial to other pathogenic infections, nor did the abolition of vital components of the immune signalling (p38 MAPK and IGF) pathways have any effect on animal survival. Further, other wild isolates of *C. elegans* from France, Portugal and India, were found to harbour different strains of the parasitic infection, indicating that it may be a relatively common natural pathogen of the worm (Troemel et al., 2008) .

Lessons learned from these studies with natural pathogens of *C. elegans* are particularly informative, as they provide evidence of the 'true' immune response in these animals. The results can then be applied to those collected from pathogenesis studies using human pathogens, to validate these data sets.

1.4 *C. elegans* as a model host

1.4.1 *Pseudomonas aeruginosa*

The Gram-negative bacteria *P. aeruginosa* is ubiquitous to the environment and a common opportunistic pathogen of both animals and plants. In humans, it is of particular danger to immuno-compromised individuals, being strongly associated with hospital acquired infections, cystic fibrosis and burn injuries (Kerr and Snelling, 2009). *C. elegans* was successfully infected and killed with a clinical isolate of the pathogen, strain PA14, known to be virulent in mice and plants. In the worm, its mechanism of pathogenesis was demonstrated to be medium-dependent; when grown on a minimal medium PA14 caused an infection-like process in the intestine of the animal killing over the course of several days, termed "slow" killing. However, PA14 was also found to kill *C. elegans* in a matter of hours, named "fast" killing, when grown on a rich medium (Mahajan-Miklos et al., 1999, Tan et al., 1999).

Slow killing is completely dependent upon the accumulation and active replication of bacteria in the animals' gut, yet animals can recover from the infection if removed from the pathogen source following a brief exposure and providing a 'threshold' has not been breached. Once the pathogenic exposure has reached this threshold, the infection becomes persistent and the animals are unable to recover (Tan et al., 1999). This is in stark contrast to the lethal toxin-based fast killing exhibited on rich media, believed to be mediated by phenazines, pigment compounds secreted by Pseudomonads (Mahajan-Miklos et al., 1999). The work also showed that PA14 mutants with attenuated virulence in mice and plants were also attenuated in *C. elegans*, suggesting that *P. aeruginosa* used common mechanisms to elicit its pathogenic effect across divergent host groups (Mahajan-Miklos et al., 1999, Tan et al., 1999). This, coupled with the rapid identification of multiple factors required for a successful interaction between the host and pathogen, by means of screens for essential genes on both sides of the interaction, implied that *C. elegans* had the potential to be a powerful model for elucidating universal mechanisms of pathogenesis (Tan et al., 1999).

This work was replicated for a second *P. aeruginosa* clinical strain, PAO1, one which is known to be less pathogenic than PA14, and a third mechanism of killing was described; that of a rapid and lethal paralysis mediated by an unidentified diffusible toxin (Darby et al., 1999). By screening mutant libraries of both the pathogen and the host, the lethal pathogenesis was shown to be dependent upon regulation of the toxin by two quorum-sensing genes, LasR and RhlR, in the bacterium, combined with the expression of the egg-laying protein EGL-9 in the host. At this time, the toxin was unknown as bacterial mutants for a number of known secreted proteins, including exotoxin A, were unchanged in their pathogenesis of *C. elegans* (Darby et al., 1999). It has since been described that PAO1 fast-killing is mediated by cyanide poisoning (Gallagher and Manoil, 2001). On the part of the host, further molecular characterisation of *egl-9* suggested that the gene was involved in the contraction of both

pharyngeal and body muscles. As loss-of-function mutants for *egl-9* became resistant to the infection, the authors propose that the bacterial toxin activated host muscle paralysis through the activity of EGL-9 (Darby et al., 1999). These animals are also resistant to killing by cyanide in the absence of pathogen, suggesting that wild type animals' susceptibility to PAO1 may involve the suppression of a hypoxia response by EGL-9, or conversely the activation of a stress-response pathway by EGL-9 that induces death (Gallagher and Manoil, 2001).

PAO1 is also able to induce the rapid onset of "red death", the presence of a red coloured material in the *C. elegans* pharynx and intestine, in nematodes exposed to physiological stress (such as starvation or heat-shock) and subsequently infected with PAO1 grown on low phosphate media (Zaborin et al., 2009). Transcriptomic analysis of PAO1 grown on high or low phosphate media identified 323 genes that were up-regulated in the response to low phosphate, and which may be causative of the "red death" phenotype. Three regulatory systems were found to be essential in the induction of "red death" in both worms and mice: the bacteria activate their phosphate uptake system through PhoB, induce a number of quorum sensing-associated genes, including phenazines, and activate pyoverdin biosynthesis in order to acquire iron and initiate phosphate signalling (Zaborin et al., 2009).

A comparative genomic and transcriptomic analysis for both PA14 and PAO1 confirmed that the two strains, despite being remarkably similar, had distinct mechanisms for virulence in *C. elegans* (Lee et al., 2006).

An alternative approach highlighted an evasive mechanism by *P. aeruginosa* to aid *C. elegans* pathogenesis (Kawli and Tan, 2008, Evans et al., 2008b). Long-lived *daf-2* (an insulin-like receptor; Figure 5) mutant animals are resistant to a number of bacterial infections, including *P. aeruginosa*

(Garsin et al., 2003). However it appears that *P. aeruginosa* can suppress the *C. elegans* immune response by activating DAF-2-mediated signalling, inhibiting the downstream DAF-16-directed activation of immunity genes by sequestering this forkhead transcription factor in the cytoplasm of epithelial cells, thus preventing nuclear transcription (Kawli and Tan, 2008). The intestinal expression of DAF-16 was shown to be essential for *C. elegans* resistance to the pathogen (Evans et al., 2008b). The host genes specifically down-regulated by *P. aeruginosa* include the lysozyme *lys-7*, the saposin-like protein *spp-1*, and, perhaps surprisingly, an antifungal protein *thn-1*. This suppression pattern was not mirrored upon exposure to other bacteria, indicating that it is an immune adaptation specific to *P. aeruginosa*, and it was shown to be dependent upon the quorum sensing regulators LasR and RhlR once more, and a two-component response regulator, GacA (Evans et al., 2008b). Given this adaptation, there is some potential that *P. aeruginosa* may be a natural pathogen of *C. elegans*.

From the host perspective, a screen of *C. elegans* immunity genes, which also have possible candidates for mammalian and fly immunity orthologues, identified 59 genes required for strong expression of *clec-85* (Alper et al., 2008), which is strongly expressed by *C. elegans* during the pathogenesis of *P. aeruginosa* (Alper et al., 2007), and nematode survival during the infection. In addition, these genes have some potential to be developed as novel targets for pharmaceutical development as these immuno-modulated 'control' genes identified in *C. elegans* were also found to have a role in regulating cytokine production during the mammalian defence response against *E. coli* in murine macrophages (Alper et al., 2008).

More recently, the pathology of the PA14-mediated slow killing of *C. elegans* has been described through intestinal cross-sections imaged by transmission electron microscopy (Irazoqui et al., 2010a). The initial stages of the infection appeared to have involved intestinal distension but little bacterial

colonisation, although those bacteria that had begun to accumulate appeared to be surrounded by outer membrane vesicle-like structures. As the infection progressed to 24 hours, large numbers of bacteria formed sizeable clumps in the distended intestine, surrounded by an unknown extracellular matrix structure, and associated with microvilli. By 48 hours, some of the bacteria appeared to have invaded the epithelium and spread to other tissues, the intestine was grossly distended, the microvilli had shortened and a large number of autophagosomes were present. These pathologies were not observed in a *P. aeruginosa gacA* mutant, which is attenuated for *C. elegans* killing (Irazoqui et al., 2010a).

1.4.2 *Salmonella enterica*

The genus Salmonella encompasses a number of species, further subdivided into serovars, of Gram-negative bacteria capable of causing enteric disease in animals and humans. The acute systemic illness typhoid fever, characterised by malaise, high fever and non-bloody diarrhoea, is caused by *Salmonella enterica* serovar Typhi, and is transmitted by contaminated water sources, foodstuffs and poor hygiene (Tam et al., 2008). The World Health Organisation has recently reported the global burden of the disease to be 21.5 million illnesses with over 200,000 cases proving to be fatal during the year 2000 alone (Crump et al., 2004). The pathogen is, however, restricted purely to humans, thus presenting the problem of studying the host-microbe interaction and subsequent infection on a whole-organism scale in an ethical manner.

Another serovar, *Salmonella* Typhimurium, causes a gastric infection in humans that can become severe and occasionally systemic in the immunocompromised. As a principal cause of gastroenteritis world-wide (Haraga et al., 2008), there is good reason to study the infection in its own right. Additionally, the bacterium causes a systemic disease analogous to 'human' typhoid fever in

susceptible mice with a polymorphism at the Slc11a1 (Nramp1) locus, an innate immunity gene responsible for bacterial resistance and macrophage activation. Thus the bacterium *S*. Typhimurium is exploited together with the murine host, and alongside tissue-culture systems, as a model for investigating the more critical human pathogen, *S*. Typhi (Tam et al., 2008).

The progression of *S*. Typhimurium infection in the murine model is now well understood. Briefly, the bacteria are consumed orally and immediately begin to evade innate host defences, such as the low pH of the stomach (Haraga et al., 2008). The site of colonisation and infection for Salmonella is the small intestine; here the bacteria preferentially utilise the protective role of specialised epithelial microfold (M) cells which engulf and transport the bacterial cells directly to lymphatic Peyer's patches within the mucosa (Haraga et al., 2008, Tam et al., 2008). The bacteria are also able to cross the epithelial barrier and disrupt tight-junctions by inducing membrane ruffles and their subsequent endocytosis into non-phagocytic epithelial cells (Tam et al., 2008). This substantial change in the ordered structure of the intestinal brush border is a contributing factor towards diarrhoea production (Haraga et al., 2008, Viswanathan et al., 2009). Following invasion, typhoidal serovars exploit macrophages to assist their systemic spread through the blood (Tam et al., 2008, Mastroeni et al., 2009). Non-typhoidal strains remain localised to the intestine and the subsequent host inflammatory response leads to a restricted enteric disease, which, in healthy individuals, is generally cleared within a few days (Haraga et al., 2008). Salmonellae make use of a number of virulence factors to achieve their translocation across the epithelium indicating how crucial this step is in the progression of the mammalian infection.

One such major regulator of *S*. Typhimurium virulence is the PhoP/PhoQ trans-membrane two-component system which controls the expression of many genes involved in invasion, survival and

lipopolysaccharide (LPS) composition amongst others (Groisman, 2001, Prost and Miller, 2008). Upon encountering low cation concentrations after internalisation into the host, it is understood that the membrane-bound sensor kinase, PhoQ, undergoes a conformational change which phosphorylates PhoP, its response module in the cytosol, enabling PhoP to modulate the transcription of downstream genes; hundreds of virulence genes are regulated in this manner (Groisman, 2001, Prost and Miller, 2008). Moreover, it has been shown that sub-lethal concentrations of host-produced antimicrobial peptides (AMPs) are able to compete with the cation binding sites, act as PhoQ ligands and subsequently initiate PhoP-directed cellular responses resulting in the activation of a number of virulence mechanisms (Bader et al., 2003, Kindrachuk et al., 2007, Prost and Miller, 2008, Groisman and Mouslim, 2006).

One of the genes regulated by the PhoP/PhoQ system is HilA (Groisman, 2001), a Salmonella pathogenicity island one (SPI-1) transcriptional regulator that, with others, controls the expression of the SPI-1 Type III secretion system (T3SS) (Cornelis, 2006). This system encodes a needle-like mechanism used to inject effector proteins directly into the host cell in order to drive the manipulation of their host cell targets; for instance, the induction of ruffling through the manipulation of the host actin cytoskeleton which facilitates the uptake of Salmonellae by non-phagocytic cells (Cornelis, 2006, McGhie et al., 2009). The *S*. Typhimurium genome encodes around fourteen of these pathogenicity islands, although the two best-described systems are SPI-1 and SPI-2, both of which encode independent T3SSs (McGhie et al., 2009). The SPI-1 system is involved in the invasion of bacteria into the host cells, whereas the SPI-2-encoded T3SS is required for survival and proliferation within macrophages and is induced independently of PhoP/PhoQ once the Salmonellae are internalised (Beuzon et al., 2001, Waterman and Holden, 2003).

Another such virulence factor, the stationary-phase sigma factor RpoS (previously known as KatF), is induced to control the expression of a number of genes upon starvation and other stresses. This sigma factor is found in a number of other bacteria, including *Escherichia coli* where the regulon has been heavily studied (Dong et al., 2009, Patten et al., 2004, Tian et al., 2008, Hengge-Aronis, 2002). In Salmonella, this alternative sigma factor appears to be dispensable in *S*. Typhi where mutants for *rpoS* appear frequently in clinical isolates; 15 out of the 41 strains tested. However, the same is not true for *S*. Typhimurium, suggesting that these mutants were selected against and thus indicating that RpoS is essential for the pathogenesis of this serovar (Robbe-Saule et al., 2003).

Indeed, RpoS controls the expression of a number of virulence genes in *S*. Typhimurium including the plasmid genes *spvRABCD*, which are important for the systemic spread of the bacterial infection (Fang et al., 1992, Norel et al., 1992); the role of *rpoS* in *S*. Typhimurium infection appears to be in the establishment of a systemic infection and not in the production of a local enteric illness (Fang et al., 1992, Nickerson and Curtiss, 1997). In mice, an *rpoS* mutant strain requires a 1000-fold greater oral lethal dose than wild type *S*. Typhimurium (Fang et al., 1992). Further, a common laboratory reference strain for *S*. Typhimurium, LT2, is avirulent in mice; an avirulence entirely attributable to an alternative start codon (UUG) of the *rpoS* gene that significantly reduces the quantity of RpoS protein produced (Swords et al., 1997a, WilmesRiesenberg et al., 1997).

1.4.2.1 Salmonella infection in *C. elegans*

Following the discovery that the human pathogen *P. aeruginosa* kills *C. elegans*, it was postulated that *S*. Typhimurium infections may also be modelled in the worm. Two back-to-back publications demonstrated that this specialised bacterium was capable of persistently infecting *C. elegans* with a colonisation process that required only a small bacterial load to become established. The infection of

the animals was found to be limited to the gut; the bacteria were not internalised into the epithelium. This colonisation caused the intestine to become distended before being subsequently destroyed by live bacteria that had escaped the action of the grinder. This persistent infection exhibited by *S*. Typhimurium is unlike the other model infections of *C. elegans* tested to date, and indicates that the bacteria may form a strong interaction with the host reminiscent of a 'true' infection of the worm (Aballay et al., 2000, Labrousse et al., 2000).

The observation that some animals displayed a strong endomitotic oocyte phenotype, where the parent animal dies containing fertilised eggs that never develop (Aballay et al., 2000), indicated that germ line development may somehow be involved with *S*. Typhimurium infection. The protective function of germ line cell death mediated by the action of *ced-3* and *ced-4* was later described (Aballay and Ausubel, 2001), though these data have not been supported by our laboratory or others (Jia et al., 2009). This immune role of a programmed cell death (PCD) response may activate an unknown defence pathway given that it seems unlikely that protection from a strictly intestinal infection would be dependent upon gonadal cell death. The role of the PCD response was upheld in a further study examining the replication of vaccinia virus in *C. elegans*: animals lacking components of the cell death pathway had significantly higher viral loads; however, this did not translate to survival rates (Liu et al., 2006).

As with *C. elegans* susceptibility to *P. aeruginosa* (Kim et al., 2002), animals with mutations in the p38 MAPK cascade are hypersensitive to *S*. Typhimurium infection. It was subsequently shown by epistasis analysis that this pathway activates the *Salmonella*-elicited PCD response (Aballay et al., 2003). Interestingly, the attenuated *S*. Typhimurium PhoP/PhoQ mutants did not instigate germ line PCD or a persistent infection (Aballay and Ausubel, 2001, Aballay et al., 2003, Alegado and Tan,

2008), implying that the PhoP/PhoQ regulon manages essential virulence factors for nematode pathogenesis. Indeed, its expression (along with other virulence factors such as SPI-1 and SPI-2), are induced by the bacteria upon colonisation of *C. elegans* (Alegado and Tan, 2008). One of the crucial factors for Salmonella pathogenesis could be lipopolysaccharide (LPS), whose synthesis is under the control of PhoP/PhoQ. In support of this hypothesis, bacterial mutants with a modified LPS structure were not able to induce an immune response or establish a persistent infection in the host. This suggests that a component of the LPS may be the pathogen-associated recognition factor for *S*. Typhimurium infection in *C. elegans*, as it is in mammals (Aballay et al., 2003).

A screen of 960 *S*. Typhimurium insertion mutants identified 15 strains with attenuated killing in *C. elegans*, which, in each case, were attributable to a single Tn*phoA* insertion. Seven of these mutations, in turn, corresponded to a number of novel virulence genes, including *spi4-F*, *cstA* and *leuO*. Most of these strains were also attenuated in a tissue culture cell-based mammalian infection model, as they were significantly reduced in their ability to both induce polymorphonuclear leukocyte migration and invade epithelial cells in comparison to the wild type strain SL1344; characteristics of *S*. Typhimurium infection (Tenor et al., 2004). A further four of these *S*. Typhimurium mutants encoded components of the SPI-1 T3SS: *hilA* and *hilD*, both of which regulate the expression of the T3SS, *invH* which is required for its assembly, and *sptP* which is one of the translocated effector proteins of the system ((McGhie et al., 2009, Cornelis, 2006, Cain et al., 2008). Together, this suggests that the T3SS has a significant role in *C. elegans* killing. In support of this, *S*. Typhimurium strains lacking either *hildD* or the entire SPI-1 gene cluster are significantly attenuated in *C. elegans* killing. Conversely, mutants that over-express *hilA* kill *C. elegans* faster than the wild type control (Tenor et al., 2004). SptP is responsible for inhibiting the p38 MAPK response in mammalian cells (McGhie et al., 2009); is this feature conserved across phyla? Ectopic expression of SptP in the intestinal epithelium of *C.*

elegans significantly reduced PMK-1 activation by western analysis and these animals subsequently became more sensitive to *S*. Typhimurium-mediated killing. The contact between the host and bacteria could be mediated by the T3SS and its subsequent translocation of SptP into epithelial cells; indeed it may be this interaction that causes the persistent lethal infection of *C. elegans* by *S*. Typhimurium (Tenor et al., 2004).

The persistence of the infection may also be a result of *S*. Typhimurium resistance to host antimicrobial compounds. Upon infection with *S*. Typhimurium, *C. elegans* responds with the defensive expression of two innate immunity peptides *abf-2* (Kato et al., 2002) and *spp-1* (Alper et al., 2007) to limit the colonisation of the bacteria (Alegado and Tan, 2008). Additionally, persistent-defective bacterial mutants (for SPI-2, *phoP* and Δ*pSLT*) could be rescued by the suppression of *spp-1* by RNAi in the host, implying that the resistance of Salmonella to *spp-1* is a feature of colonisation and persistence in the worm (Alegado and Tan, 2008).

Genes involved in the unfolded protein response (UPR) are also required for *S*. Typhimurium infection where they are regulated by the apoptotic regulator CED-1. *C. elegans* mutants for *ced-1* are hypersensitive to Salmonella and *Candida*-mediated killing (Haskins et al., 2008, Means et al., 2009) and microarray analysis of these mutants identified a cluster of *pqn/apu* UPR genes that were significantly down-regulated (Haskins et al., 2008). Survival analyses of knock-down mutants for a number of the genes in this cluster via RNAi additionally showed that these animals were hypersensitive to *S*. Typhimurium infection, suggesting a role in *C. elegans* immunity to these bacteria. Additionally, there is greater pharyngeal destruction and invasion by *S*. Typhimurium in *ced-1* mutant animals, thus it appears that the UPR defence is a response to *S*. Typhimurium-induced damage in the animal, and the UPR pathway acts to preserve the integrity of organ structures (Haskins et al., 2008).

C. elegans mutants for the autophagy genes *bec-1* and *lgg-1* are also hypersensitive to infection with *S.* Typhimurium (Jia et al., 2009). Autophagy, the process of recycling cellular components via lysosomal degradation, is regulated by the DAF-2 pathway in *C. elegans* and is implicated in the clearance of intracellular bacteria in the mammalian innate immune response. The basis of this hypersensitivity was not mediated by the link between autophagy and apoptosis, but instead the autophagy pathway appeared to limit bacterial replication in the intestinal epithelium (Jia et al., 2009). On day two of a *S.* Typhimurium infection, a small number of intracellular bacteria were seen in wild type worms, predominantly as debris in autophagosomes, although some were intact, whereas *bec-1* RNAi animals were overwhelmed by the infection and had a number of Salmonella-containing vacuoles (SCVs) and intact bacteria in the epithelial layer. By day four of the infection, the epithelial cells were almost entirely destroyed in the *bec-1* knock-down animals and there was intracellular replication of the bacteria; this was not true in the wild type animals suggesting that autophagy protects the structure of the intestine from *S.* Typhimurium (Jia et al., 2009).

Taken together, there are limitations of the *C. elegans* infection model with respect to mammalian pathogenesis due to the predominantly extracellular nature of the infection. However, the fact that Salmonella is one of the few human pathogens to initiate a persistent infection in the worm is encouraging and, once understood, may be particularly informative.

1.4.3 *Serratia marcescens*

It was a study with the Gram-negative bacterium *S. marcescens* which first suggested that *C. elegans* has an inducible immune response (Mallo et al., 2002). *S. marcescens* is an environmental bacterium, which causes disease in a number of organisms; plants, invertebrates and vertebrate hosts. In humans it is an opportunistic pathogen associated with hospital-acquired infections and, as many

strains are intrinsically antibiotic resistant, the pathogen represents an ongoing public health challenge (Kurz et al., 2003).

In *C. elegans*, *S. marcescens* establishes a persistent intestinal infection, arising from an avoidance of the pharyngeal grinder. The bacteria progressively destroy the animal tissues, resulting in a grossly distended intestine, before the worm dies with a maximum survival of just 6 days (Mallo et al., 2002, Kurz et al., 2003). Notably, a small number of bacteria become intracellular, although this is a very rare event (Kurz et al., 2003). The *C. elegans* response to the bacteria involves the up-regulation of a number of putative pathogen-response proteins, including lysozymes, NLPs and lectins, a subset of which appeared to be largely under the control of the DBL-1/TGF-β pathway (Mochii et al., 1999). *dbl-1* knockout animals, which are unable to signal through the TGF-β cascade, are hypersensitive to infection suggesting a central role of this pathway in the host response to Serratia (Mallo et al., 2002). A comparative approach found that the expression profiles of genes induced following *S. marcescens* infection were distinct from those induced upon infection with the natural nematode pathogen *M. nematophilum*, although the gene families induced were similar, suggesting that *C. elegans* can mount responses tailored to the specific infection and must therefore have a pathogen recognition system of some sort (Mallo et al., 2002, O'Rourke et al., 2006). Indeed, the over-expression of one principal response lysozyme, *lys-1*, was enough to increase the animals' resistance to infection (Mallo et al., 2002).

This work was followed by the exploitation of *C. elegans* as a high-throughput screen for *S. marcescens* transposon mutants with attenuated virulence (Kurz et al., 2003). Some of the mutants identified in the screen were also attenuated in the alternative infection models *D. melanogaster* and an *in vitro* cell-based assay. Characterisation of these mutants revealed novel mechanisms required

for full virulence of the pathogen; these included genes involved in iron uptake, LPS synthesis (the bacteria lacked the *O*-antigen) and haemolysin production. Additionally, this screen was indicative of *P. aeruginosa* pathogenesis, as orthologues of two genes identified in the *S. marcescens* mutants (one uncharacterised, the other involved in sequestering iron) were also attenuated for virulence in *C. elegans* when disrupted in *P. aeruginosa* (Kurz et al., 2003).

To evaluate the interaction between *C. elegans* and *S. marcescens* from an evolutionary perspective, the relationship between natural worm and pathogen isolates was examined (Schulenburg and Ewbank, 2004). The interaction varied with respect to the pathogen strain and the *C. elegans* micro-population in question. Although animals from the same population responded in a similar way to each pathogen strain, the four populations assayed showed clear differences in resistance and susceptibility phenotypes to the various *S. marcescens* strains (there was no general trend), thus supporting a genetic basis for host resistance, pathogen virulence and the interaction itself. It was postulated that these observed differences are likely to represent the co-evolution of the bacteria and host as both struggle to get ahead in the evolutionary "arms race" (Schulenburg and Ewbank, 2004). This work showed the potential that *C. elegans* has for a model to study the adaptations the host and pathogen make as they co-evolve together, although whether *C. elegans* and *S. marcescens* interact in their natural environments remains to be seen.

1.4.4 *Escherichia coli*

In the laboratory, nematodes are cultured using *E. coli* OP50 as the sole food source. This is a non-pathogenic food source, and animals live for 2-3 weeks in these conditions (Brenner, 1974). However, there is evidence that even OP50 is pathogenic to both aging animals in standard growth conditions and to younger animals when grown on rich media (Couillault and Ewbank, 2002). This is a feature

held in common with the infection of higher vertebrates with these Gram-negative bacteria: generally, *E. coli* is found in the intestine of humans and other mammals as part of the commensal gastro-intestinal flora, where it is harmless and can even offer protection to the host against other virulent pathogens (Crossman et al., 2010). Even virulent enteric isolates rarely cause anything more than an acute bout of food poisoning in the healthy host; however, these isolates are opportunistic pathogens which can cause severe disease in immuno-compromised individuals (Crossman et al., 2010).

C. elegans has been applied as a pathogenesis model for understanding the molecular basis of Enteropathogenic *E. coli* (EPEC) infection. Killing of *C. elegans* by EPEC correlated with the accumulation of bacteria in the animal gut over a few days, rather than by a toxin-mediated mechanism (Mellies et al., 2006). This colonisation is dependent upon the global regulator, Ler, and although nematodes infected with a *ler* mutant strain did not die any slower than other EPEC isolates, far fewer bacteria were found in the animals' intestine suggesting that only a few number of bacteria are required for a successful colonisation and that this interaction with the host is lethal. The *C. elegans*-EPEC model has its limitations, however, as other virulence factors required in mammalian systems, such as the T3SS, are not essential for the *C. elegans* model, hence the modes of EPEC infection of invertebrate and vertebrate hosts appear to be far removed (Mellies et al., 2006).

The EPEC infection process described above is one of "slow" killing, analogous to the mechanism of "slow" killing by *P. aeruginosa*. Similarly, EPEC also exhibits a "fast" killing process, mediated by secreted toxins when grown on a rich medium supplemented with tryptophan. *C. elegans* killing was modulated by p38 MAPK and insulin signalling; *daf-2* and *age-1* mutations (insulin signalling) rendered the animals resistant to the infection, whereas *mek-1* and *sek-1* mutations (p38 MAPK signalling) led to hypersensitivity (Anyanful et al., 2005).

This group went on to show that a 30 minute exposure to fast-killing EPEC, followed by a recovery period in normal culture conditions, led to a 4 fold increase in animal survival upon a subsequent 3 hour "fast kill" exposure, a phenomenon they describe as 'conditioning'. A three hour recovery on OP50 was optimal; any more or less time reduced the subsequent animal survival rate during the three hour secondary infection. It was further shown that a primary exposure to avirulent EPEC strains and to other bacterial species could still provide protection against a secondary wild type EPEC infection. The conditioning response was dependent upon both insulin and p38 MAPK signalling, and mediated by the DAF-16 regulated genes the saposin *spp-1* and the aquaporin *aqp-1*, which suppress the conditioning response when mutated (Anyanful et al., 2009).

1.4.5 *Staphylococcus aureus*

S. aureus is a common Gram-positive bacterium that causes a range of minor infections, which occasionally become serious, in many animals. It is a major pathogen of livestock, and in humans a leading cause of hospital-acquired infections, for both of which it employs an array of virulence factors. In humans, the bacteria are commensals in around 30% of the population. There are 10 dominant lineages of *S. aureus*, although no one lineage in associated with infectious disease, indicating that the host background is extremely important for the progression of the disease. Its relevance becomes more and more critical given the establishment of extensively antibiotic resistant strains, notably the emergence of Methicillin-resistant *Staphylococcus aureus* (MRSA) during the 1990s, reviewed in (Lindsay, 2010).

In *C. elegans*, intact bacteria accumulate in the gut of the animal and it is this colonisation that eventually overwhelms the host, leading to death (Garsin et al., 2001, Sifri et al., 2003). Nematodes can be rescued from the lethality of the infection by transfer to a non-pathogenic food source, enabling

the clearing of the bacteria from the animals' intestines. This recovery is dependent upon a critical exposure threshold, the length of which is strain-dependent, not having been reached. This correlates with the observation that infected animals have normal feeding behaviour and locomotion until this threshold is achieved and the infection becomes lethal (Sifri et al., 2003).

The infection of *C. elegans* by *S. aureus* appeared to result from an active process rather than from the formation of an evasive biofilm, which is the principal component of *S. epidermidis*-mediated killing in *C. elegans* (Begun et al., 2007). Indeed, there was a rapid initial colonisation of the intestine by *S. aureus*, followed by additional bacterial accumulation at the rectum and an apparent adhesion of the intestinal bacteria to the apical surface of the epithelium. This colonisation was coupled with gross intestinal distension and a Dar (deformed anal region) phenotype, dependent upon both β-catenin and ERK MAPK signalling (Irazoqui et al., 2010a). As the infection progressed to 12 hours, the architecture of the epithelium was disrupted: the microvilli had shortened and the epithelial cells themselves had lost volume and had started to 'bleb' into the lumen. Later, the architecture was completely destroyed by the bacteria and later still, the entire animal had been degraded leaving just a ghost of the cuticle. Interestingly, this systematic destruction was not dependent upon the *S. aureus* cytolysins, as bacterial strains lacking these virulence factors still caused the same cytopathology as wild type (Irazoqui et al., 2010a).

Isolates of *S. aureus* mutated for crucial virulence factors, such as the global virulence regulators *agr* and *sarA*, in mammalian hosts are attenuated in *C. elegans* killing, indicating that these genes are also required for full pathogenesis in the worm model. From the host perspective, nematodes which are unable to signal through the p38 MAPK cascade (*sek-1* and *nsy-1* mutants) are hypersensitive to infection with *S. aureus*, indicating that the p38 MAPK pathway is largely responsible for eliciting a host

immune response to infection (Sifri et al., 2003). Another signalling cascade, analogous to the vertebrate β-catenin (*bar-1*) and homeobox (*egl-5*) transcription factors, also up-regulates *C. elegans* immune factors in response to *S. aureus* infection, particularly *clec-60* and *lys-5* (Irazoqui et al., 2008). Mutant animals for *bar-1* and *egl-5* are more susceptible to *S. aureus* than wild type; the bacteria actively divide in the intestine of these animals and they mount a repressed defence response that is not capable of lysing the bacterial cells: together this leads to increased damage of the intestinal epithelium. This phenotype is more severe than the hypersensitivity of p38 MAPK mutants, suggesting that the independent *bar-1/egl-5* pathway is the predominant pathway for initiating the immune response to *S. aureus* infection. The importance of this pathway in *S. aureus* pathogenesis is mirrored in higher vertebrates, where it activates NFκB-mediated gene expression (Irazoqui et al., 2008), again highlighting the potential *C. elegans* has as a model for pathogenesis.

Microarrays for wild type animals' gene expression at eight hours of *S. aureus* infection showed a further 382 genes whose expression was modulated upon infection (Irazoqui et al., 2010a). Of these, a subset of the genes up-regulated more than 4-fold above expression levels in non-pathogenic conditions were putative antimicrobial and detoxification peptides. These genes appeared to be induced in a series of induction waves in at least two different groups (Irazoqui et al., 2010a) and could be indicative of the severity of *S. aureus* infection, facilitating further pathogenesis screens with the *C. elegans* model.

1.4.6 *Cryptococcus neoformans*

C. neoformans is an encapuslated yeast, ubiquitous in the environment. As a pathogen, it causes disease in a number of animals. In humans it is primarily a pathogen of the immuno-compromised, notably co-infecting AIDS patients. After the primary infection, the fungus can disseminate to the

central nervous system and cause severe meningitis; a key component of this spread is the ability of the yeast to survive and reproduce in macrophages. However, *Cryptococcus* is beginning to emerge as a pathogen of immuno-competent individuals since a hypervirulent *C. gattii* variant is responsible for a continuing outbreak of cryptococcosis on Vancouver Island, Canada, and thus has significant clinical relevance (Ma and May, 2009).

Killing of *C. elegans* by *C. neoformans* is rapid, with considerable killing from just day 2 of infection (Mylonakis et al., 2002, van den Berg et al., 2006). The yeast cells accumulate in the intestine of the host causing gross distension, but the infection is not persistent as animals can be rescued from the pathogen by transfer to normal culture conditions. The mechanism of pathogenesis is not clear, but a number of genes and features required for mammalian pathogenesis are also essential for the worm model (Mylonakis et al., 2002, Tang et al., 2005). A surprising exception was the finding that acapsular yeast, although attenuated, did not impede nematode killing, but that instead this mutation inhibited the accumulation of intestinal yeast cells, implying that neither the capsule nor the fungal accumulation are essential for the lethality (Mylonakis et al., 2002). This, coupled with the discovery that heat-killed yeast also killed *C. elegans*, suggested that the pathogenesis of the worm may be mediated by a toxic interaction between the host and pathogen. The lethality generated by *C. neoformans* was not thought to be a by-product of the inability of the animals to utilise yeast as a food source: other *Cryptococcus* species such as *C. laurentii* and *C. kuetzingii* did not impair *C. elegans* survival, and animals were readily able to propagate on these lawns when they were the sole food source (Mylonakis et al., 2002).

C. elegans appears to be a particularly effective model host for *C. neoformans* pathogenesis. Significantly, infected animals are cultured at 25°C, which is the ideal temperature for cryptococcal growth. As the yeast infects mammalian hosts at 37°C and is carried in avian hosts at 42°C, modelling

the infection at 25°C could be informative of novel and 'authentic' pathogenic behaviour of the yeast itself. Further, as mentioned above, there is much cross-over between the virulence factors required for mammalian and *C. elegans* pathogenesis. Remarkably, the production of mating factor α (MFα) pheromone, which is essential for full virulence and is thus activated in mammals, is also activated in *C. elegans* (Mylonakis et al., 2002). In the mammalian host, macrophages have an essential role in eliminating the infection. Two host scavenger receptors, SCARF1 and CD36 are required to recognise β-glucans on the invading yeast and elicit a host defence by activating macrophages. In *C. elegans*, the orthologues of these receptors, CED-1 and C03F11.3, are crucial to activating a defence response following recognition of the pathogen (Means et al., 2009). This highly specific conservation between two seemingly divergent host groups underscores the significance of innate immunity in response to fungal pathogens.

1.5 Signalling in the *C. elegans* immune response

1.5.1 MAP-kinase pathways

The Mitogen-Activated Protein Kinase (MAPK) pathways utilise a sequential cascade to amplify and transmit a signal elicited upon an external stimulus, with a biological response. In *C. elegans*, there are three main cascades: the ERK MAPK, JNK MAPK and p38 MAPK pathways, which each respond to different stimuli and trigger distinct responses, such as proliferation, differentiation, broad stress responses and apoptosis (Seger and Krebs, 1995). In contrast, the pathways have some basal activity, which in the case of the p38 MAPK pathway is dependent upon long-chain fatty acids (Nandakumar and Tan, 2008).

A forward screen of 1,400 mutant worms for an enhanced susceptibility phenotype (*esp*) to *P. aeruginosa*, strain PA14, identified two mutations as being crucial for the host response to the

infection. The *esp* genes were identified by single nucleotide mapping and subsequent gene sequencing and were found to be a MAPKK homologue, *sek-1* and a MAPKKK homologue, *nsy-1*. The immune function of the p38 MAPK cascade was confirmed by RNAi knockdown of *pmk-1*, a p38 orthologue in *C. elegans*, as this too resulted in the *esp* phenotype (Kim et al., 2002).

Subsequent work from this group examined the potential role of c-Jun N-terminal kinase (JNK) signalling in the immune response, as it had been implicated in the animals' response to heavy metal stress (Mizuno et al., 2004). By utilising knockout animals for components of the JNK pathway in *C. elegans* to identify positive regulators of the pathway, combined with a reverse genetic screen to identify negative regulators, the group described interactions between the JNK and p38 MAPK cascades at two levels. Firstly, that the activation of p38 (PMK-1) required the synergistic activity of the upstream MAPKK, SEK-1, and the corresponding component of the JNK pathway, MEK-1, to achieve maximal bacterial resistance, though SEK-1 is sufficient to activate PMK-1 alone. Secondly, that the MAP Phosphatase VHP-1 negatively regulates both cascades at the level of the MAPK, either by inhibiting PMK-1 or KGB-1 (Kim et al., 2004). This seemingly substantial and significant cross-talk between pathways suggests that complex immune and stress responses are regulated by the integration of diverse signals in *C. elegans* (Figure 5).

In the example of the soil-dwelling bacterium *Bacillus thuringiensis*, a pathogen that *C. elegans* is likely to encounter in its natural environment, two key genes are induced during the host response to the Bacillus toxin Cry5B; *ttm-1* and *ttm-2*, both of which are under the regulation of the p38 MAPK pathway (Huffman et al., 2004). Although their function is essential for resistance to the toxin (RNAi knockdown of these genes results in animals unchanged from wild type in standard culture conditions, but renders the animals hypersensitive to *E. coli* expressing the toxin) their method for host protection remains

unknown (Huffman et al., 2004). In support of cross-talk between the MAPK pathways, there also appears to be a role for the JNK cascade in regulating immunity to *B*. *thuringiensis*, but this is unclear and may only arise from the mis-regulation of a coordinated stress response by both the p38 and JNK pathways, as animals lacking components of the JNK pathway were hypersensitive to both Cry5B and $CdCl_2$ which had been used as a stress control (Huffman et al., 2004).

Next, the induction of *nlp-1* in the epidermis by *D. coniospora* is dependent upon the p38 MAPK cascade. An upstream kinase regulator, NIPI-3, was identified through a forward screen in this work; NIPI-3 acts specifically to induce *nlp-1* expression in response to fungal infection, rather than its induction in response to injury (Pujol et al., 2008a). From the same screen, the group characterised another mutant that failed to induce *nlp-1* upon *Drechmeria* infection. Epistasis analysis placed this gene, *tpa-1* a homologue of protein kinase Cδ, downstream of *nipi-3*, but upstream of the MAPKK *sek-1* (Figure 5) to regulate the antifungal and wounding response of *C. elegans* (Ziegler et al., 2009).

Additionally, expression of *nlp-29* is dependent upon NIPI-3, and an even greater level of specificity to *D. coniospora* infection occurs downstream of *nipi-3* in the form of the *C. elegans* Protein Kinase C homologues, *tpa-1* and *pkc-3*; activation of the pathway through TPA-1 or PKC-3 is essential for an antifungal response by the host. The role of PKC in this system is dependent upon G-protein signalling, principally via the Gα subunit, *gpa-12*, which itself acts upstream of two phospholipase C genes, *plc-3* and *egl-8* (Ziegler et al., 2009). Thus the activation of *C. elegans* innate immunity in response to *D. coniospora* infection is remarkably complex and continues to reflect the mechanisms of immunity in vertebrate systems.

Furthermore, TPA-1 was found to regulate a diacylglycerol (DAG)-controlled immune response by influencing its downstream component Protein kinase D (DFK-2; Figure 5). This immune regulation was both p38-dependent and –independent (Ren et al., 2009). However, as with many mutant phenotypes, questions remain as to the specificity of the pathogen sensitivity phenotype of *dfk-2* mutant animals, since the loss of Protein kinase D is likely to lead to universal disruption of immune regulation.

The p38 MAPK signal is transduced by a single downstream transcription factor; ATF-7, a member of the activating basic-region leucine zipper transcription factors. It appears as though ATF-7 typically acts to repress PMK-1-controlled genes, but switches to an activating form upon phosphorylation by PMK-1 (Shivers et al., 2010).

The third of the MAPK cascades, the Extracellular-signal-regulated kinase (ERK) pathway, has been described in the host response to infection with *M. nematophilum* and *S. aureus* where it was shown that the limited activation of the ERK MAPK response causes the Dar phenotype (Hodgkin et al., 2000, Irazoqui et al., 2010a). It is yet unclear whether this phenotype is a defence mechanism of the host or a virulence mechanism by the bacteria (Nicholas and Hodgkin, 2004).

The conservation of the MAPK cascades in *C. elegans* has demonstrated that the pathways are an ancient mechanism for defence against pathogenic attack (Kim et al., 2002). The discovery that *C. elegans* does not have an NFκB orthologue, although it does employ a ubiquitin/proteasome pathway (Alper et al., 2008), implies that the p38 cascade preceded the NFκB response during evolution (Wang et al., 2006).

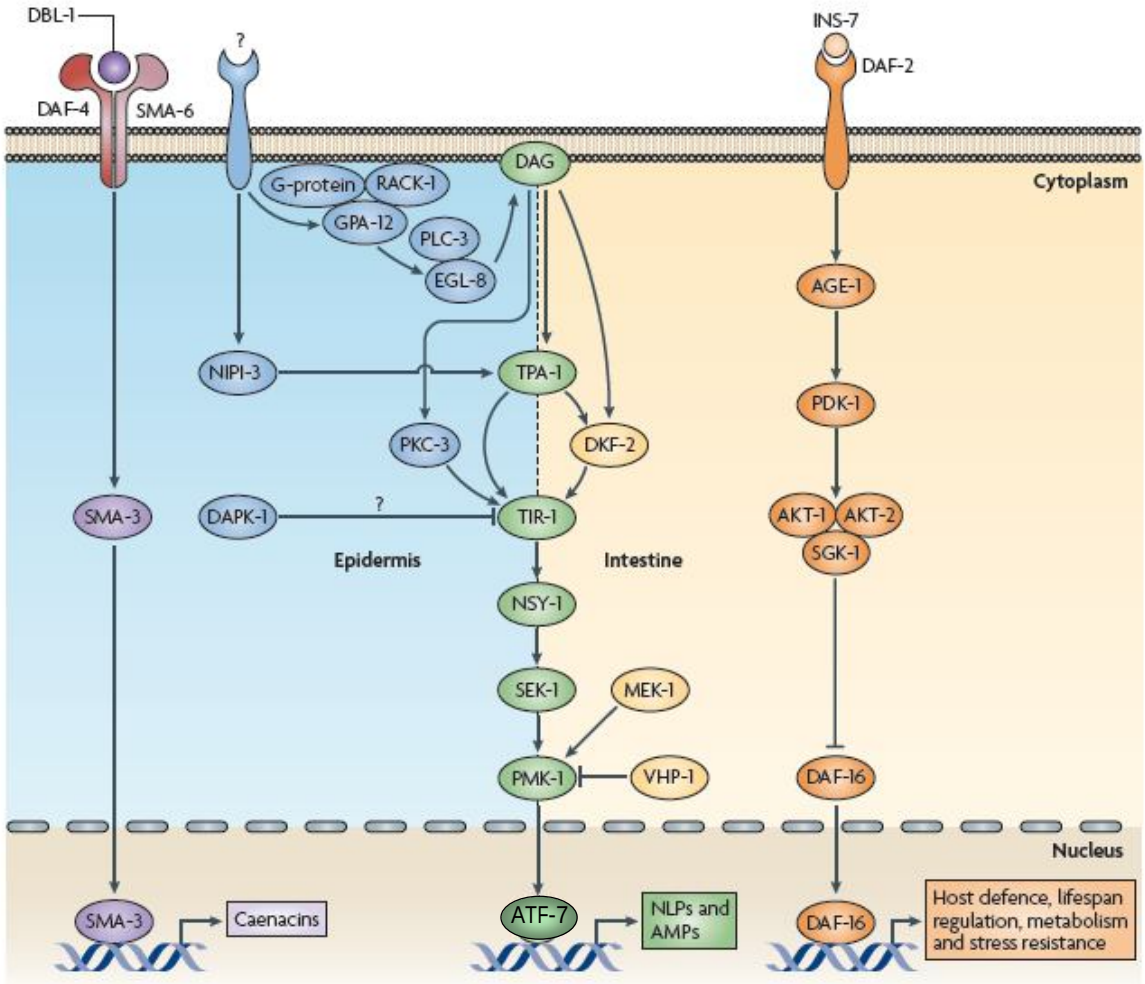

Figure 5: Signalling pathways activated during the innate immune response of *C. elegans*

Four main signalling pathways operate in parallel in the immune response of *C. elegans* to a range of pathogens. From left to right: the TGF-β/DBL-1 pathway (purple), the Toll-like/TIR-1 response which operates in hand with the p38 MAPK cascade (blue and green), and the IGF/DAF-2 pathway (orange).

Image modified from (Irazoqui et al., 2010b)

1.5.2 Toll-like Pathway

The identification of a major immunity signalling receptor, Toll, was heralded in 1995 (Lemaitre et al., 1995), 10 years after the receptor was first described in *Drosophila* as being essential for dorsal-ventral polarity during development (Anderson et al., 1985). This further work with Toll mutant flies identified a role for the receptor in innate immunity; the animals were found to be hypersusceptible to fungal infection due to the down-regulation of the antifungal peptide drosomycin by the absence of the Spätzle-Toll-Cactus pathway, presumably caused by the subsequent mis-regulation of the downstream transcription factor, Dorsal (Lemaitre et al., 1995, Lemaitre et al., 1996).

Later, mammalian Toll-like receptors (TLRs) were described with orthologous roles in the vertebrate immune response. Interestingly, the TLRs of mammals are more canonical Pathogen Recognition Receptors (PRRs) than their *Drosophila* counterparts, responding directly to microbial signatures, rather than the host-produced cytokine spätzle. These receptor-complexes are now known to signal through the Toll/IL-1 resistance (TIR) domains of their associated cytoplasimc adapter proteins, and the signal is transduced either through the p38 MAPK pathway, or the NFκB pathway (Lemaitre, 2004). As *C. elegans* has no NFκB protein (Wang et al., 2006, Medzhitov, 2001), the Toll-pathway must therefore signal through the p38 MAPK pathway if it is orthologous to the *Drosophila*/mammalian Toll pathway. The only two homologues for the Toll-signalling pathway in *C. elegans* are *tol-1*, a TLR gene essential for the early development of the worm, and *tir-1*, a homologue of the vertebrate TIR-protein SARM, which is the adapter protein for TLR4 (WormBase).

Down-regulation of TIR-1 by RNAi was found to reduce expression of the aforementioned infection-inducible antimicrobial NLPs, NLP-31 and NLP-29, whereas there was no modulation of *nlp-31* expression by TOL-1, indicating that TIR-1 acts independently of TOL-1 to activate antifungal

responses in *C. elegans* (Couillault et al., 2004). The work further showed that TOL-1 and TIR-1 do not even physically interact. Inactivation of *tir-1* alone also significantly reduced wild type animals' survival with both *D. coniospora* and *S. marcescens*. A validated yeast-two-hybrid screen produced two proteins that could physically interact with *tir-1*, and may therefore transduce the immune response signal, although these proteins, RAB-1 (a small GTPase) and R53.4 (a mitochondrial ATP-subunit f-synthase), had, and still have, no other described function in innate immunity in *C. elegans* (Couillault et al., 2004)(WormBase).

A publication from the Ausubel group just one month later placed *tir-1* as an upstream activator of the p38 MAPK pathway, as the activated form of *pmk-1* was significantly decreased in animals lacking *tir-1* (Liberati et al., 2004). In this study, the TIR protein is operating in a fashion more comparable to Toll signalling in *Drosophila* and mammals. A further link between the Toll pathway and p38 MAPK cascade has been described through the collective activation of the *C. elegans* ABC transporter, *pgp-5*, which is induced in, and required for, the animals' immune (notably to *P. aeruginosa*) and stress responses (especially to heavy metals). Animals combining mutations for *pgp-5* and components of the p38 MAPK pathway or *tir-1* were hypersensitive to PA14 infection, confirming *tir-1* as an upstream component of the p38 MAPK cascade (Figure 5; (Kurz et al., 2007). This hypersensitivity was not found when *pgp-5* mutation was combined with others corresponding to key components of the JNK MAPK and other main immunity pathways (Kurz et al., 2007) implying that *C. elegans* can generate specific and highly regulated responses.

In all three examples, the association between the Toll receptor and TIR adapter are uncoupled in the *C. elegans* immune response, suggesting that Toll-mediated immunity arose after the split from pseudocoelomates and acoelomates.

Concurrently, the concept arose that animals were able to distinguish between different bacterial and fungal food sources, actively seeking non-pathogenic, high quality food, and avoiding pathogenic and other poorer food sources, all mediated under neurological control. Animals lacking *tol-1* were unable to avoid *S. marcescens*, but there appeared to be no other role of the Toll receptor in *C. elegans* immunity (Pujol et al., 2001). One further publication examined the function of the TOL-1 protein in *S. enterica* pathogenesis, and found a direct role for TOL-1 in the innate immune response of *C. elegans* to Gram-negative pathogens. In this study, TOL-1 prevented the pharyngeal invasion of *S. enterica* and was required to induce two antimicrobial peptides, *abf-2* and *hsp-16*, which are both strongly expressed in the pharynx (Tenor and Aballay, 2008).

Alternative work supported the 'active food choice' hypothesis. It was shown that the hunt for 'good' food is enhanced if the animals have prior exposure to 'good' food (Shtonda and Avery, 2006), a study which corroborated the learning behaviour that the Bargmann group had observed the year before (Zhang et al., 2005). In this work, the group showed that the olfactory behaviour of *C. elegans* changed after exposure to pathogenic bacteria; an observed increase in serotonin was thought to be a negative stimulus associated with the odour of pathogenic food sources (Zhang et al., 2005). The TIR-1 protein was found to mediate this olfactory choice by regulating the asymmetry of the two AWC neurons, located on the left and right side of the head. Briefly, TIR-1 patterns NSY-1 (the *C. elegans* p38 MAPKKK) at the post-synaptic regions, suggesting that the olfactory choice behaviour is instigated at the AWC synapse (Chuang and Bargmann, 2005). Further work confirmed that *tol-1* does mediate avoidance behaviour against *S. marcescens* in *C. elegans* (Pradel et al., 2007). Here, animal genetics was combined with bacterial knockouts to identify components both in the host and pathogen that contribute to this behaviour. The detection and avoidance of *S. marcescens* required the AWB chemosensory neurons and TOL-1, which operate in complementary pathways, in the host and the

production of cyclic lipodepsipentapeptide, serrawettin W2 in the bacteria. Surprisingly, animals lacking *tol-1* are still able to learn aversive olfactory behaviour against other bacteria however, so there must be a comprehensive mechanism or mechanisms that feed into an animal's choice of food (Pradel et al., 2007).

1.5.3 TGF-β-like pathway

It is well known that the neuronally-expressed gene *dbl-1* (previously named *cet-1*) encodes one of four ligands in the *C. elegans* Transforming Growth Factor (TGF)-β-like signalling pathway, that binds and activates the Sma-6/DAF-4 receptor complex. Together, this pathway controls body size and the position of the male *vas deferens* in a dose-dependent manner, mediated downstream by a number of Smad proteins (Suzuki et al., 1999, Morita et al., 1999). However, there is also evidence of the role of *dbl-1*-regulated signalling in the *C. elegans* immune response.

The pathway appears to control a number of immune response factors, including lectins and lysozymes both constitutively (Mochii et al., 1999) and upon *S. marcescens* infection (Mallo et al., 2002). In support of this, there is expression of both DBL-1 and SMA-6 in the intestine, where together they appear to directly regulate the expression of these antimicrobial genes (Mochii et al., 1999), although the mechanism of this DBL-1-mediated activation remains unknown. In support of this, knock-down of *dbl-1* results in a heightened susceptibility of these mutant animals to *S. marcescens* and *P. aeruginosa* infection (Mallo et al., 2002, Ewbank, 2006).

Upon *D. coniospora* infection, paracrine signalling through neuronally expressed *dbl-1* activates the *cnc* cluster in the epidermis in a manner which provides pathogen resistance in the cuticle, analogous to cytokine-based signalling in higher vertebrates (Zugasti and Ewbank, 2009). Although the SMA-6

receptor was required for the up-regulation of *cnc-2* in response to the fungal infection, the other downstream Smad genes were not required, suggesting that *dbl-1* utilises an alternative signalling transduction cascade in this instance (Figure 5). The strength of *cnc-2* induction was also directly dependent upon the expression levels of *dbl-1* (Zugasti and Ewbank, 2009). The neuroimmune regulation theory in *C. elegans* continues to gather substance (Kawli and Tan, 2008, Styer et al., 2008, Zugasti and Ewbank, 2009, Reddy et al., 2009) and further study into such phenomena may help unearth the identity of the pathogen receptors in the animals, which remain elusive to date.

1.5.4 Insulin-like pathway

The endocrine signalling pathway is remarkably conserved across phyla and is one of the best characterised pathways in *C. elegans* (Mukhopadhyay et al., 2006). Here, DAF-2 is the single receptor of this Insulin Growth Factor I (IGF)-like cascade and its activity is to shorten life span by sequestering the forkhead transcription factor, DAF-16, in the cytoplasm and thus DAF-2 negatively regulates the activation of downstream effectors (Figure 5). DAF-16 directs the transcription of a number of target genes when IGF activity is inhibited through limited signalling, dietary restriction, increased JNK signalling (which can directly phosphorylate DAF-16) (Oh et al., 2005) and mutations in the DAF-2 receptor or in other upstream kinases, such as *age-1* (a PI3-kinase) and *akt-1/2* (Lee et al., 2001, Lin et al., 2001, Baumeister et al., 2006). The dephosphorylation of DAF-16 enables it to translocate into the nucleus and regulate an array of genes that include those involved in development, metabolism, longevity, stress and immune responses (Jensen et al., 2006, Murphy et al., 2003, McElwee et al., 2003, Yu et al., 2008a, Lee et al., 2003, Bolm et al., 2004).

Interestingly, long-lived *daf-2* mutant animals were found to be resistant to a number of bacterial pathogens (Garsin et al., 2003), providing the first evidence that ageing and immunity may be coupled

in *C. elegans*. Further work has corroborated this hypothesis, including the identification of a number of DAF-16 regulated genes as antimicrobials (McElwee et al., 2003, Yu et al., 2008a, Murphy et al., 2003). On the other hand, a microarray study investigating the transcriptional outputs of the parallel DAF-2 and p38 MAPK pathways, with respect to immunity, found that PMK-1 and not DAF-16, directly regulated a plethora of antimicrobial genes, suggesting that the p38 MAPK pathway has a critical role in immunity whereas the role of DAF-2 signalling is simply a consequence of the more general stress response elicited through this pathway (Troemel et al., 2006). Indeed, *daf-16* mutant animals have comparable survival rates to wild type animals on a number of pathogens (Garsin et al., 2003, van den Berg et al., 2006), suggesting that insulin signalling is not a vital component of the immune response of wild type animals in the laboratory setting. Instead, the group propose that DAF-16 is activated through a non-canonical pathway that they call the "defence response pathway" induced in animals destined to become sterile, which may be a link between immunity and reproduction (Miyata et al., 2008).

However, it is believed that continuous over-expression of *daf-16* has deleterious effects on pathogen susceptibility (Singh and Aballay, 2009). *daf-2(e1370);daf-16::gfp* animals which have continuous nuclear accumulation of DAF-16 become more sensitive to *P. aeruginosa* infection than the resistant *daf-2(e1370)* or *daf-16::gfp* animals alone, though this is not suppressed to wild type levels. It was found that the nuclear export of DAF-16 is required to achieve the optimal amount of the active protein in the nucleus; a translocation dependent upon the heat-shock family transcription factor HSF-1 (Singh and Aballay, 2009), a protein that had already been linked to the DAF-2 pathway in the immune response (Singh and Aballay, 2006).

Work from our laboratory examined the survival of male worms to infection with *C. neoformans* (van den Berg et al., 2006). It was already known that male animals have an extended lifespan, partially attributable to the costs of reproduction but largely dependent upon the depression of insulin-like signalling in the animal (Gems and Riddle, 2000). The work showed that phenotypically (interestingly not genotypically) male animals have extended survival upon exposure to *C. neoformans*. This resistance is also principally, but not entirely, dependent upon the activity of DAF-16, indicating that longevity and immunity may have co-evolved through the DAF-2 pathway (van den Berg et al., 2006). Our laboratory went on to show that the constitutively higher DAF-16 expression levels in the gonochoristic *Caenorhabditis* nematodes of *remanei* and *brenneri*, resulted in both a longer lifespan and a stronger immune response than found in their hermaphroditic counterparts (Amrit et al., 2010).

Additionally, it is well-established that germline ablation has a resounding effect on lifespan extension in DAF-16 expressing worms (Lin et al., 2001, Smelick and Ahmed, 2005, Arantes-Oliveira et al., 2002, Hsin and Kenyon, 1999, Berman and Kenyon, 2006). The germline also appears to negatively modulate DAF-16 activation and thus confers sensitivity to *P. aeruginosa*, such that sterile mutants with a proliferating germline are resistant to the infection (Evans et al., 2008a). In these cases, the DAF-2 pathway influences both aging and immunity through similar means.

On the other hand, DAF-2-mediated responses to aging and immunity can be uncoupled. For instance, mutants for components of the pathway downstream of *daf-2* and *age-1,* such as *pdk-1* and *sgk-1*, uphold their extended lifespan, but are not resistant to bacterial pathogens in the same way as mutant animals lacking their upstream kinases. Similarly, *akt-1* and *-2* mutant animals show enhanced survival to bacterial infections, but only a slightly longer lifespan than wild type (Evans et al., 2008a). The resistant mutants from this pathway are better able to clear the infection, have fewer bacteria

colonising the intestine and respond harder to the infection with a much greater induction of antimicrobial genes than wild type worms and *sgk-1* mutants. Together this suggests that DAF-2 signalling in the immune response utilises different components than those exploited during lifespan regulation (Evans et al., 2008a) in order to tailor appropriate biological responses.

DAF-2 is also suggested to negatively regulate the role of autophagy in pathogen resistance, especially in response to *S*. Typhimurium. *daf-2(e1370)* animals show enhanced lysosomal degradation of bacteria and have fewer bacteria in the intestine. Further, the bacterial resistance of DAF-2 mutants or DAF-16 over-expressors is suppressed to wild type survival with *bec-1* and *lgg-1* RNAi, genes that are involved in the autophagy response, implying that autophagy, through the DAF-2 pathway, has a significant role in *C. elegans* pathogenesis by *S*. Typhimurium (Jia et al., 2009).

1.6 Conclusion

C. elegans has been shown to be susceptible to a wide range of bacterial and fungal pathogens and these virulent pathogens cause a number of symptoms and utilise a number of mechanisms for killing in the worm model. One of the most advantageous functions of the *C. elegans* system is its suitability for high-throughput screening for both mutated pathogens, in order to identify attenuated and hyper-virulent strains, and as a first stage for testing novel pharmaceutical compounds.

However, the application of the *C. elegans* model towards human disease has significant limitations that must be recognised. Firstly, although there is some conservation of the mechanisms of pathogenesis between *C. elegans* and higher vertebrates (Alper et al., 2008), there are huge differences. A critical mechanism of virulence in mammalian hosts is for these pathogens to become internalised and then spread throughout the host; whereas in *C. elegans* the infections, save for only a

couple of examples to date, do not become intracellular. Further, in mammalian systems, pathogenic infection does not occur solely at one site, as is the case with *C. elegans* pathogenesis; infections are generally tissue-specific before spreading systemically, and thus cause wide-reaching symptoms. Therefore, it is still unclear as to which pathogens actually elicit a *bona fide* infection in *C. elegans*, rather than simply causing lethality via accumulation in the intestine.

Secondly, many of the human pathogens that are studied in *C. elegans* have adapted to 37°C. Exposure to this temperature for extended periods of time is lethal to *C. elegans*, thus assays are carried out at 25°C. We must therefore recognise that a number of pathogen virulence factors may not be expressed as they are induced only at 37°C. In the same vein, the "novel" virulence mechanisms of pathogens identified through the *C. elegans* model may simply be the adaptation of the pathogen to this new temperature, which could explain why some of the hits are not conserved in tissue culture systems or in the mouse model.

Thirdly, there are considerable differences between the immunity profiles of *C. elegans* and of higher vertebrates. *C. elegans* has a remarkably simple innate immune system and is thus dependent upon the secretion of antimicrobial peptides to counter pathogen attack alone. Higher vertebrates, on the other hand, have both a more complex innate system and an additional, highly specialised, adaptive system, which together permits great versatility in the immune response. Although there is some conservation in the pathways that control the immune response in both animals; since the vertebrate response has likely evolved from a common predecessor to the primitive *C. elegans* response, there are still large inconsistencies. One example is the Toll-like receptor pathway which represents a significant arm of innate immunity that was first identified in *Drosophila* (Medzhitov, 2001). In the

vertebrate immune response it has a fundamental role and yet its function in *C. elegans* is limited (Pujol et al., 2001, Aballay et al., 2003, Liberati et al., 2004, Tenor and Aballay, 2008).

Perhaps the solution is for the exploitation of the *C. elegans* model in pathogenesis to not have a human disease focus. Rather than looking at animal survival and the process that achieves the end-point of mortality in these studies, it may be more informative to utilise the wealth of genetic resources in this animal and closely examine the earlier processes of infection in order to understand further the molecular interaction between the pathogen and the host. Subsequently, instead of relating results to human disease, it may be more fruitful if they were examined in the light of the evolution of the immune system throughout the animal and plant kingdoms.

1.7 Scope of this thesis

The nematode *Caenorhabditis elegans* is now firmly established as a powerful model system for the study of host-pathogen interactions (Sifri et al., 2005, Schulenburg et al., 2004, Ewbank, 2002). The animal shares many of the mechanisms of innate immunity with higher vertebrates, including several pathways that control the expression of universal defence factors in response to pathogenic infection (Ewbank, 2006). There are key differences, however, including the lack of a cell-mediated immune system or a complement-like response in *C. elegans*. Instead, the animal relies upon secreted antimicrobial molecules for a systemic immune response to pathogenic challenge (Schulenburg et al., 2004).

One such factor, lysozyme, is an evolutionarily ancient defence enzyme that is believed to be actively lytic against a range of microbes (Bachali et al., 2002). Whereas many organisms have only one or two lysozyme genes, *C. elegans* has a family of at least ten differentially regulated genes (Figure 4) that are predicted to show significant functional diversity (Thomas, 2006, Schulenburg and Boehnisch, 2008). Within the *C. elegans* lysozyme family, *lys-7* has been the most extensively studied. Expression of this molecule is strongly induced upon exposure of the animal to the pathogenic bacteria *Serratia marcescens* (Mallo et al., 2002), *Microbacterium nematophilum* (O'Rourke et al., 2006) and *Salmonella* Typhimurium (Evans et al., 2008b). Furthermore, *lys-7* knockout animals show enhanced susceptibility to *M. nematophilum,* indicating that *lys-7* has a protective function against this pathogen (O'Rourke et al., 2006).

We, and others, have previously shown that the fatal fungal human pathogen *Cryptococcus neoformans* kills *C. elegans* (Mylonakis et al., 2002, van den Berg et al., 2006). A preliminary gene expression study of two *C. elegans* strains, *daf-2(e1370)* and *fem-1(hc17)*IV, that are intrinsically

resistant to killing by the fungus revealed that *lys-7* expression was strongly and constitutively induced in these animals (R. C. May, unpublished data). We therefore hypothesised that LYS-7 may play an important role in mediating resistance towards *C. neoformans.*

This thesis examines:

I. The role of LYS-7 in the innate immune response of *C. elegans* to a range of human pathogens, with particular emphasis on both *C. neoformans* and the enteric bacteria *Salmonella* Typhimurium

II. The upstream signalling pathways that control the expression of LYS-7 during the immune response, including signalling mediated by the tyrosine kinase ABL-1 and the TGF-β and IGF pathways

III. The molecular determinants of immune interactions between *S. Typhimurium* and *C. elegans*

2.0 MATERIALS AND METHODS

2.1 Maintenance of *C. elegans*

2.1.1 *C. elegans* strains used

The strains used for this work were N2 (*C. elegans* wild type; Bristol isolate), RB1285 *lys-7(ok1834)*, RB1286 *lys-7(ok1385)*, XR1 *abl-1(ok171)*, RB1252 *rga-6(ok1316)*, BX107 *fat-5(tm420)*, CF1038 *daf-16(mu86)*, RB2071 *ced-3(ok2734)*, NU3 *dbl-1(nk3)*, KU4 *sek-1(km4)* and RB829 *abi-1(ok640)* which were kindly provided by the *C. elegans* Genetics Center and the *C. elegans* Knockout Consortium. The strains *clec-60(tm2319)* and *abi-1(tm494)* were kindly provided by Shohei Mitani's laboratory in Tokyo. Two further strains, *abl-1(n1691)* and *abl-1(n1963)* were provided by the Horvitz laboratory, in Boston, MA. Additionally, 14 double mutant animals were generated during the study by crossing (Hope, 1999): FB0016 and FB0017 both *lys-7(ok1384)abl-1(ok171)*, FB0018 *lys-7(ok1384)rga-6(ok1316)*, FB0019 and FB0020 both *lys-7(ok1384)fat-5(tm420)*, FB0021 and FB0022 both *lys-7(ok1384)daf-16(mu86)*, FB0023 and FB0024 both *abl-1(ok171)daf-16(mu86)*, FB0025 and FB0026 both *abl-1(ok171)ced-3(ok2734),* FB0027 *abl-1(ok171)sek-1(km4)*, FB0028 *lys-7(ok1384)sek-1(km4)* and FB0029 *abl-1(ok171)dbl-1(nk3)*.

2.1.2 Culturing and maintenance of animal stocks

Nematodes were cultured using standard methods as described previously (Brenner, 1974, Hope, 1999). Briefly, animals were maintained in 9cm petri dishes on a modified agar substrate, nematode growth medium (NGM; 1.7% agar, 0.25% peptone, 0.3% NaCl, 5µg/ml Cholesterol, with the addition of 1mM $CaCl_2$, 1mM $MgSO_4$ and 25mM KPO_4, pH6, after autoclaving). These plates were seeded with a lawn of *Escherichia coli* OP50, a natural uracil mutant strain and the standard food source of the

Caenorhabditis nematodes, and incubated at 15-20°C. Animals were transferred to new plates as food sources became limited.

2.1.3 *C. elegans* bleaching protocol

This protocol was used to generate staged animal cultures and to remove any contamination from *C. elegans* stock plates. Animals were washed off newly starved stock plates (9cm) in 3ml M9 buffer (22mM KH_2PO_4, 42mM Na_2HPO_4, 80mM NaCl, 1mM $MgSO_4$ (Hope, 1999)) and the suspension was transferred into 1.5ml centrifuge tubes and centrifuged at 6,500g for 30s. The supernatant was removed to leave 0.5ml in the tube. 80µl 5M NaOH and 160µl Sodium hypochlorite was added and the tubes were shaken vigorously for 2 min. The tubes were then filled with M9, centrifuged as above and the supernatant removed; this washing step was repeated twice and on the 2nd time 200-400µl M9 buffer was added to the tube. The tubes were rolled overnight to keep the animals oxygenated and transferred onto OP50 plates the following day, where the cultures developed synchronously.

2.1.4 Generation of male animals by heat-shock treatment

5 late larval four (L4) stage hermaphrodites were transferred onto 6cm NGM plates seeded with OP50 and incubated at 30°C for 6h before being allowed to self-fertilise at 20°C. The frequency of spontaneous male generation by loss of the second X chromosome is increased 10-fold under these conditions (Hope, 1999). F1 male progeny were picked from plates 3-4 days after heat-shock.

2.1.5 Generation of double mutant animals by crossing

Five or more L4 male animals (of strain one) were transferred onto a 6cm NGM OP50 plate onto which two hermaphrodite L4 animals (of strain two) had already been transferred. The animals were allowed to mate at 20°C for 48h. Approximately 50% male progeny indicated the mating had been successful

and subsequently four L4 F_1 hermaphrodites from each cross were singled onto small seeded plates and allowed to self. More than thirty F_2 progeny from each of these F_1 worms were typed using the single worm lysis method and deletion PCR reaction, described in 2.4.1-2.4.2, and the aplimers were separated by electrophoresis on a 1% agarose gel. Animals with the genotype of interest were selected, grown up and frozen down.

2.1.6 Freezing of *C. elegans* stocks

Frozen stocks of worm strains were produced by washing off freshly starved L1 animals in 4ml M9 buffer, and aliquoted into 4x1.8ml labelled cryovials. 500µl sterile M9 buffer with 30% glycerol (v/v) was added to this worm suspension. Cryovials were then insulated in a freezing blanket to achieve 8°C cooling per hour and frozen at -80°C. One vial was thawed after 24 hours, put on NGM plates and left to incubate overnight at 20°C. The plate was examined the following day to check the viability of the frozen stock. Once this was ascertained, the remaining tubes were then transferred from the freezing blanket into cryo boxes and stored at -80°C.

2.2 Characterisation of mutant animals

2.2.1 Lifespan analysis

15-30 animals at L4 stage were picked from 9cm animal stock plates onto 6cm NGM plates seeded with 30µl OP50 and incubated at 20ºC. Animals were transferred to new seeded plates every one to two days during the fertile stage. The plates were scored for survival each day using a Leica M7.5 stereomicroscope and death was determined as a failure to respond to a mechanical stimulus. Data are representative of 100-200 animals, exact numbers are presented in the figure legends.

2.2.2 Brood size analysis

L4 hermaphrodites were singled onto 6cm NGM plates seeded with OP50; three plates of each strain. The animals were transferred to new plates one to two times daily during the fertile period. The progeny on the previous plates were allowed to grow up to around the L3 stage when they were counted using a Leica M7.5 stereomicroscope; the total brood size for each hermaphrodite was determined once all plates had been counted.

2.2.3 Statistical analysis

Lifespan replicates were checked for consistency prior to being combined into single survival curves in Microsoft Excel using a macro-based template to calculate Kaplan-Meier survival probabilities every 24 hours. The curves represent data from at least three individual experiments. Differences in survival were tested with a non-parametric log-rank analysis and assessed for significance using Chi squared. P-values below 0.05, after correcting for multiplicity, were taken to be significant. The data in the lifespan curves are presented as the proportion of animals surviving.

2.3 *C. elegans* infection assays

2.3.1 Bacterial and fungal strains used and their respective growth conditions

Strains, sources and growth requirements are shown in Tables 1 and 2.

2.3.2 Bacterial survival method

20µl of an overnight bacterial culture was inoculated onto 6cm NGM plates, supplemented with the appropriate antibiotic if required, and lawns were allowed to grow at room temperature for 12h. 15-30 animals at the L4 stage were picked onto each plate and transferred to newly seeded plates every one-

two days as eggs were laid. Animal survival was examined at 25°C and scored every 24h using a Leica M7.5 stereomicroscope. Death was determined as a failure to respond to a mechanical stimulus.

2.3.3 Fungal survival method

20µl of an overnight fungal culture was inoculated onto 6cm NGM plates supplemented with kanamycin (30 µg/ml) and lawns were allowed to grow at room temperature for 12h. 15-30 L4 animals were picked onto each plate and incubated at 25ºC. Survival was scored every 24h using a Leica M7.5 stereomicroscope. Death was determined as a failure to respond to a mechanical stimulus.

2.3.4 *C. elegans* bacterial CFU analysis

Infection load was assessed through viable counts. The assay was modified from (Kawli and Tan, 2008); L4 animals were infected with *S*. Typhimurium strain L1019 as per survival assays. After a defined period, ten replicates of six animals each were incubated in 200µl M9 buffer containing 25mM levamisole hydrochloride (Sigma) and ampicillin (1mg/ml) for one hour. This was to paralyse the animals, preventing pharyngeal pumping and defecation, and kill any bacteria present on the cuticle of the worm. The ampicillin was subsequently removed by three washes of 200µl M9 with 25mM levamisole hydrochloride. The animals were lysed in this buffer for 10s using the Precellys 24 Lysis and Homogeniser. Lysates were serially diluted in M9 and then plated onto LB plates containing kanamycin (30µg/ml) to select for L1019. Colonies were counted by eye and scaled to the original concentration per nematode.

	Strain	Strain details	Overnight Culture Medium	*C. elegans* media	Source/Reference
Escherichia coli	OP50	Uracil auxotroph	LB (1% tryptone, 1% NaCl, 0.5% yeast extract), shaking at 37°C	NGM	(Brenner, 1974)
Cryptococcus neoformans	H99 (serotype A)	Clinical isolate from Hodgkin's disease patient, USA	YPG (1% yeast extract, 1% peptone, 2% glucose) + kanamycin (30µg/ml), shaking at 25°C.	NGM + Kan (30µg/ml)	Joe Heitman, Duke University (Franzot et al., 1999)
Pseudomonas aeruginosa	PAO1	Standard reference strain, wound isolate	LB, shaking at 37°C	NGM	Piddock Lab, University of Birmingham (Stover et al., 2000)
Staphylococcus aureus	NCTC 8532 (ATCC 12600)	Standard type strain, clinical isolate from pleural fluid	LB, shaking at 37°C	NGM	Piddock Lab, University of Birmingham (Piddock et al., 1999)

Table 1: Bacterial and fungal strains and their respective growth requirements

	Strain	Details	Overnight Growth Conditions	*C. elegans* media	Source/Reference
***Salmonella enterica* serovar Typhimurium**	SL1344	Standard reference strain, calf isolate, 1978	LB, shaking at 37°C	NGM	Piddock Lab, University of Birmingham (Wray and Sojka, 1978)
	LT2 (ATCC 700720)	Attenuated reference strain, clinical isolate 1948	LB, shaking at 37°C	NGM	Piddock Lab, University of Birmingham (Lilleengen, 1948)
	14028s (ATCC 14028)	Standard reference strain, isolated from chicken tissues	LB, shaking at 37°C	NGM	Piddock Lab, University of Birmingham (Bailey et al., 2008)
	L1019	SL1344 + GFP*mut2*	LB + kanamycin (30µg/ml), shaking at 37°C	NGM + Kan (30µg/ml)	Blair and Piddock, Unpublished, University of Birmingham
	JF2691	SL1344 *rpoS* mutant	LB + ampicillin (50µg/ml), shaking at 37°C	NGM + Amp (50µg/ml)	Foster Lab, University of South Alabama (Lee et al., 1995)
	χ4996	LT2 *rpoS* mutant	LB + ampicillin (50µg/ml), shaking at 37°C	NGM + Amp (50µg/ml)	Curtiss Lab, Washington University (WilmesRiesenberg et al., 1997)
	χ8000	LT2 $rpoS^{SL1344}$	LB + tetracycline (12.5µg/ml in EtOH), shaking at 37°C	NGM + Tet (12.5µg/ml)	Curtiss Lab, Washington University (WilmesRiesenberg et al., 1997)

Table 2: *S*. Typhimurium strains and their respective growth conditions

2.3.5 *S.* Typhimurium persistence analysis

L4 animals were infected with *S.* Typhimurium SL1344 as per the survival assays. After a six hour exposure, these animals were washed 3 times in M9 solution and shifted onto NGM plates seeded with 20µl OP50. Animals were transferred to newly seeded plates every one-two days as eggs were laid. Survival was examined at 25°C and scored every 24h using a Leica M7.5 stereomicroscope. Death was determined as a failure to respond to a mechanical stimulus.

2.3.6 *C. elegans* bacterial load microscopy analysis

Animals at the L4 stage were infected with GFP-expressing *S.* Typhimurium strain L1019, as per the survival assays, either as a constant or persistent-shift infection. After a defined period, five replicates of six worms were transferred onto agarose pads on a microscope slide and paralyzed with an M9 solution containing 10mM Sodium azide. These slides were then imaged on a Nikon SMZ1000 stereomicrosope at 4x magnification, with a HR Plan Apo lens. An intensilight C-HGFI (Nikon) with a GFP filter set were used for fluorescence illumination. Digital images were captured with Nikon DS-Fi1 camera and NIS elements (v3.0) software. Images were processed in Adobe Photoshop. Those images presented are representative of the data set.

2.3.7 Statistical analysis

The bacterial CFU analysis was carried out in Microsoft Excel and subsequently tested for significance using the Students' T-test (2 tailed, equal variance). All survival replicates were checked for consistency prior to being combined into single survival curves in Microsoft Excel using a macro-based template to calculate Kaplan-Meier survival probabilities every 24 hours. Each curve represents at least three independent experiments. Differences in survival were tested with a non-parametric log-rank analysis and assessed for significance using Chi squared. P-values below 0.05, after correcting

for multiplicity, were taken to be significant. The data in the survival curves are presented as the proportion of animals surviving.

2.4 Molecular Biology

2.4.1 Single worm lysis

Single worms were lysed in a 5µl reaction comprising 1xPCR buffer (0.1M Tris-Hcl pH 8.3, 0.5M KCl, 15mM MgCl2, 200µg/ml gelatine), 60µg/ml proteinase K and ddH2O, incubated at 60°C for 90 min then 95°C for 15 min. Lysates were stored at 4°C for up to a month, if not used immediately.

2.4.2 Confirmation of deletion alleles by PCR

1µl of single worm lysate was added to 9µl PCR mix (1xDreamTaq buffer, 0.01mM dNTPs, 0.4mM of each primer, ddH2O and 0.5 units DreamTaq DNA polymerase (Fermentas)). PCR was carried out on a SensoQuest Labcycler and the product was amplified through 35 cycles (94°C, 0.2s; 55°C, 0.2s; 72°C, 120s). Nested PCRs used 0.3µl of the first product as a template for the second reaction. The *fat-5* products required alternative parameters for amplification (Wicks et al., 2001) (35 cycles: 95°C, 40s; 58°C, 40s; 72°C, 40s). Primer sequences can be found in Table 3. The products were electrophoresed along a 1% agarose gel to confirm the presence of each genetic knockout.

2.4.3 Deletion mapping by DNA sequencing

PCR reactions were carried out as above. 10ng of this amplified product was mixed with 3.2pmol primer and ddH2O to a final volume of 10µl. This sample was labelled, purified and sequenced (on the capillary sequencer ABI 3730) using the full sequencing service provided by the Genomics Facility in the Biosciences Department at the University of Birmingham. Sequences were analysed by ChromasPro v1.42 and mapped to the *C. elegans* genome using WormBase (www.wormbase.org).

Gene	Name	Primer sequence (5'-3')	Product size (bp)	Reference/Source
lys-7	Forward	TCCATCAAAATTGGCAACAA	WT: 2400 KO: 1600	Kindly provided by Robin May
	Reverse	CGGCGAAATAAATTTTGGAA		
abl-1	External Forward	TTTTGCTTTCAACTCGCCTT	WT: 3300 KO: 800	*C. elegans* Knockout Consortium
	External Reverse	ATATGCCTCCCTCCTTTGCT		
	Internal Forward	GTCTTCTGCTTTCGAATCGG		
	Internal Reverse	TTCATATATCCACCGGCCAT		
fat-5	Forward	TTGCCTCCGGCAAACAGACT	WT: 1100 KO: 300	(Brock et al., 2006)
	Reverse	ATTCTCAGGCTTGAGCTCAG		
clec-60	Forward	TCTGCCATCCAGACTGCAGT	WT: 1400 KO: 900	WormBase
	Reverse	CTTCCTATACCGACCGTCGA		
C01F4.2	External Forward	ACTGATTTTGAGGTGGTGGC	WT: 3200 KO: 2400	WormBase
	External Reverse	TAAAACCGGGAATGGAGTTG		
	Internal Forward	GTCTCGCCACGACGAATTAT		
	Internal Reverse	AAATTTCAGTTCGCATTCCG		
daf-16	Forward	GCGCCTTTGTCTCTCTATCG	F+R WT: 11744 KO: 764 I+R WT: 1343 KO: No product	Primer 3 (v0.4.0)
	Internal – F	AAATGATTCAGGGGGAAAGG		
	Reverse	GACGATCCAGGAATCGAGAG		
ced-3	External Forward	CAAAAGGACGCTCTGCCTAT	WT: 1100 KO: 400	WormBase
	External Reverse	TGTCGTGTCGAGACCAGGTA		
	Internal Forward	AGCAGATCGATTGTTGTTCAAG		
	Internal Reverse	TTGGTCCCAAAAACCAAAAA		
abi-1	Forward	TCTCCAAAAATACCTTTTTCCAA	F+R WT: 1171 KO: 61 I+R WT: 950 KO: No band	Primer 3 (v0.4.0)
	Internal – F	TGGAAGATCATGTCGGTTCA		
	Reverse	CGAACGGCTACGTTAGGAAG		
sek-1	Forward	AACGCAGGTCACTCGTTTCT	F+R WT: 2470 KO: 393 F+I WT: 984 KO: No band	Primer 3 (v0.4.0)
	Internal – R	GTACCTTGGCCATGCTGTTT		
	Reverse	ATTTATTCCGTCCACGTTGC		
dbl-1	The deletion is ~6kb and not sequenced, thus the allele was followed via the recessive *sma* phenotype			

Table 3: Primers for single-worm PCR

2.4.4 RNA isolation and cDNA preparation

Total RNA was extracted from each strain on three independent occasions. Full plates of staged L4 animals were homogenised in 400µl lysis buffer (Qiagen) using the Precellys 24 Lysis and Homogeniser. RNA was isolated from these lysates with the column-based "RNeasy Mini Kit" (Qiagen) according to the manufacturer's instructions save for one change: at the final elution step, the flow-through was pipetted back onto the column membrane instead of using a further 30µl RNAse-free water to achieve a more concentrated sample. Each RNA sample was DNase treated with "DNA-*free*" (Ambion, Inc.) after isolation, in accordance with the manufacturer's instructions, and subsequently quantified using the Nanodrop ND1000 microspectrophotometer (NanoDrop Technologies, Inc.). cDNA was synthesised from all RNA extractions using the SuperScript II method (Invitrogen) with random primers (Promega) in a SensoQuest Labcycler, assuming a 1:1 conversion. The absence of genomic DNA was confirmed by PCR of these cDNA products (34 cycles: 94°C, 25s; 55°C, 30s; 72°C, 60s), the amplimers of which were separated by electrophoresis on a 2.5% agarose gel.

2.4.5 Quantitative real-time PCR

Quantitative real-time PCR (qRT-PCR) was performed on each cDNA sample in triplicate using SYBR Green chemistry in accordance with the manufacturer's instructions. The 2x SensiMix (dU) SYBR Green kit (Quantace) was chosen to provide increased sensitivity in the assay, although reactions were run at a volume of 25µl, not 50µl. In each reaction $MgCl_2$ had a final concentration of 3mM, primers (sequences can be found in Table 4) were used at 20mM and a template concentration of 2.5ng was used. Each plate contained a set of negative controls. Each plate was run using the ABI Prism 7000 sequence detection system with the following thermal cycling conditions: 37°C, 10min; 95°C, 10 min; 95°C, 15s; 55°C, 30s; 72°C, 30s; steps 3-5 were repeated for 40 cycles.

Gene	Name	Primer sequence (5'-3')	Product size (bp)	Reference/Source
lys-7	Forward	GTCTCCAGAGCCAGACAATCC	143	Kindly provided by Claudia Boehnisch
	Reverse	CCAGTGACTCCACCGCTGTA		
abl-1	Forward	TGCATCAGAGGAGTTTGAGA	178	Primer 3 (v0.4.0)
	Reverse	ATTGTCAGCGTCAGTGCTT		
fat-5	Forward	CGATTTGTACGAGGATCCGGTG	201	(Brock et al., 2006)
	Reverse	CAGTGGGAGACACTGTTGATGC		
clec-60	Forward	TCACAACGGACTCAGTATGG	197	Primer 3 (v0.4.0)
	Reverse	CAGTCGTCAGTGGCTTGATA		
C01F4.2	Forward	AAAGTCAATGGCGTCTTTTC	154	Primer 3 (v0.4.0)
	Reverse	GCATTCTCATCAACATCCAA		
daf-16	Forward	GCGAATCGGTTCCAGCAATTCCA	171	Kindly provided by Francis Amrit (Amrit et al., 2010)
	Reverse	ATCCACGGACACTGTTCAACTCGT		
gpd-3	Forward	TGAAGGGAATTCTCGCTTACACC	154	Kindly provided by Francis Amrit (Amrit et al., 2010)
	Reverse	GAGTATCCGAACTCGTTATCGTAC		

Table 4: Primers for qRT-PCR

2.4.6 Statistical analysis

The qRT-PCR was normalised to *gpd-3* expression using the Comparative Ct method in Microsoft Excel (Bookout and Manglesdorf, 2003, Amrit et al., 2010), and tested for statistical significance using the Students' T-test (2 tailed, equal variance, paired).

2.5 Recombinant lysozyme

2.5.1 Gateway Cloning

The *lys-2*, *lys-5* and *lys-7* genes were cloned from N2 cDNA using sequence-specific Worm ORFeome primers (sequences can be found in Table 5) (Lamesch et al., 2004, Walhout et al., 2000), which are compatible with the Invitrogen Gateway Cloning System (Hartley, 2006, Hartley, 2002, Walhout et al., 2000, Nallamsetty and Waugh, 2007). 5µl of cDNA was added to 45µl PCR mix (1xPhusion HF buffer, 800µM dNTPs, 0.5µM of each primer, ddH2O and 0.5 units Phusion DNA polymerase (Finnzymes)). PCR was carried out on an MJ Research TETRAD2 DNA Engine (Bio-Rad) and the product was amplified through 35 cycles (98°C, 0.1s; 72°C, 30s) following an initial 30s denaturation at 98°C. The PCR amplicons were sequenced at the DNA Core Facility at Massachusetts General Hospital, Boston, MA. Sequences were analysed using ApE v1.17 and WormBase (www.wormbase.org).

Subsequently, the One Tube Format Gateway (Invitrogen) recombination reaction was used. Briefly, 2µl *att*B DNA (PCR product), 1.3µl *att*P DNA (pDONR 201 entry vector (Invitrogen); 150ng/µl), 3µl BP Clonase II enzyme (Invitrogen) and 8.7µl TE Buffer pH 8.0 was gently mixed in a 1.5ml centrifuge tube and incubated overnight at 25°C. 10µl of this reaction was added to 2µl destination vector (pDEST-527 (addgene); 150ng/µl) and 3µl LR Clonase II enzyme (Invitrogen) to add a His-tag to the protein. The mixture was vortexed briefly and incubated at 25°C for 8h. 2µl 20mg/ml Proteinase K solution (Invitrogen) was added to the mixture and the tube incubated at 37°C for 10 min. 1µl of the

Gene	Name	Primer sequence (5'-3')	Product size (bp)	Reference/Source
lys-2	Forward	GGGGACAAGTTTGTACAAAAAAGCAGGCTTGATCAAGCTTCTAGTTTCTTTTAC	840	Worm ORFeome v3.1 (Lamesch et al., 2004)
	Reverse	GGGGACCACTTTGTACAAGAAAGCTGGGTAGTTTCCGACAAATCCTCCGACA		
lys-5	Forward	GGGGACAAGTTTGTACAAAAAAGCAGGCTTGAAGCATTTTTTCATTACAATCCTTC	648	Worm ORFeome v3.1 (Lamesch et al., 2004)
	Reverse	GGGGACCACTTTGTACAAGAAAGCTGGGTATGGAATGTAGTTCATATCAACAG		
lys-7	Forward	GGGGACAAGTTTGTACAAAAAAGCAGGCTTGGCCCACAAATCGATTGTCAT	852	Worm ORFeome v3.1 (Lamesch et al., 2004)
	Reverse	GGGGACCACTTTGTACAAGAAAGCTGGGTAAATTTTCAGACTTCCTTGCACAAA		
pDESTsequenceoligo		GGCGTAGAGGATCGAGATCG	For sequencing	ApE 1.17

Table 5: Primers for lysozyme cloning

Red text represents the *att*B cloning sites for Gateway recombination technology

recombination mix was used to transform 50µl One Shot TOP10 Chemically Competent *E. coli* cells (Invitrogen) according to the manufacturer's instructions. Recombinants were allowed to grow on LB-ampicillin (50µg/ml) plates at 37°C overnight. Colonies were grown overnight in 4ml LB broth, 37°C shaking, and the pDEST-527 vectors containing the lysozyme sequence of interest were extracted using a QIAprep miniprep kit (Qiagen) according to the manufacturer's instructions. The plasmids were sequenced using the pDESTsequenceoligo (sequence in Table 5) at the DNA Core Facility at Massachusetts General Hospital, Boston, MA. Sequences were analysed and mapped using ApE v1.17 and WormBase (www.wormbase.org).

2.5.2 BL21-AI transformation

BL21-AI One Shot Chemically Competent *E. coli* cells (Invitrogen) were transformed, by heat shock, with 5ng recombined pDEST-527 vectors, according to the manufacturer's instructions. Transformants could be stored on LB-ampicillin plates at 4°C for up to one week without significant loss of protein induction.

2.5.3 BL21-AI expression

5ml test inductions were assayed in accordance with the manufacturer's expression guidelines. After a 3h induction at 37°C, the samples were spun down and the cell pellets were resuspended in LDS sample buffer (106mM Tris HCl, 141mM Tris Base, 2% Lithium Dodecyl Sulfate, 10% (w/v) glycerol, 0.51mM EDTA, 0.22mM SERVA Blue G250, 0.175 mM Phenol Red, pH 8.5), 500mM DTT and heated at 70°C for 10 min.

2.5.4 Western blot analysis

Clones were analysed by western blot to determine the best expressers. The NuPAGE Novex Bis-Tris System (Invitrogen) was used in an XCell *SureLock* Mini-Cell (Invitrogen); samples were run on a NuPAGE Novex 12% Bis-Tris Gel 1.0mm 10 well, with NuPAGE MOPS SDS running buffer and NuPAGE Antioxidant (for reducing samples in the cathode chamber). The proteins were resolved down the gel for 50 min at 200V. The proteins were transferred onto 0.22µm nitrocellulose membrane using the XCell II Blot Module Kit (Invitrogen) and NuPAGE transfer buffer, in accordance with the manufacturer's instructions; proteins were allowed to migrate for 1h at 32V. The membrane was blocked in 10% milk powder with PBS-T (1xPBS tablets (Oxoid), ddH_20, Tween 1:1000) for 1h or overnight and then incubated in 1° antibody, mouse-anti-His (Sigma) at 1:2000 in PBS-T for 1h or overnight. The membrane was washed three times in PBS-T for 10 minutes each, and incubated in 2° antibody, rabbit-anti-mouse-HRP (Jackson ImmunoResearch) at 1:5000 in PBS-T, for 45 min. The membrane was washed three times as before and incubated in 10ml Immobilon Western Chemiluminescent HRP substrate (Millipore) for two min. The membrane was drip-dried, exposed to BioMax MR Autoradiography Film (Kodak) and developed using an X-OMAT 2000A automatic processor (Kodak).

2.5.5 LYS-7 predicted structure

The LYS-7 283 amino acid sequence (cosmid C02A12.4.1; WormBase) was submitted to the Phyre (Protein homology/analogy recognition engine) Program v0.2 from Imperial College London (www.sbg.bio.ic.ac.uk/phyre)(Kelley and Sternberg, 2009). This method utilises threading and homology modelling to predict the protein structure. The model with the highest sequence ID is presented in this thesis.

2.5.6 LYS-7 sequence analysis in comparison to other GH25 lysozymes

C. elegans LYS-2, LYS-5 and LYS-7, *S. coelicor* cellosyl, *A. fumigatus* GH25, and *E. histolytica* lysozyme amino acid sequences were taken from the Universal Protein Resource (UNIPROT; www.uniprot.org)(Apweiler et al., 2010) and the subsequent .clustalw file was aligned using T-COFFEE (Tree based consistency objective function for alignment evaluation; www.tcoffee.org) multiple sequence alignment program (Notredame et al., 2000). The sequences were analysed with ESPript 2.2 (Easy sequencing in postscript; www.espript.ibcp.fr/ESPript/ESPript/) in order to identify and annotate secondary structures, similarities and conserved sequences (Gouet et al., 1999). The alignment was further annotated for signal and pro-peptide sequences, and catalytic and functional residues by eye (A. L. Lovering, personal communication).

3.0 BALANCED IMMUNITY IN *C. ELEGANS*

3.1 Introduction

The defensive enzyme LYS-7, a member of the *C. elegans* lysozyme gene family, is believed to be particularly important in the immune response of the nematode to a number of pathogens (Mallo et al., 2002, O'Rourke et al., 2006, Evans et al., 2008b).

Preliminary work from our group examined the host-basis for the heightened resistance of two *C. elegans* strains, *daf-2(e1370)* and *fem-1(hc17)*IV, to killing by the fungal pathogen, *Cryptococcus neoformans*. This work revealed that *lys-7* expression was strongly and constitutively induced in these animals (R. C. May, unpublished data). We therefore hypothesised that LYS-7 may play an important role in mediating resistance of the worm towards *C. neoformans.*

This chapter examines the immunological function of LYS-7 during *C. elegans* infection with a number of human pathogens, including *C. neoformans*. It subsequently describes an immunological balance, with respect to *C. neoformans* and the bacteria *S*. Typhimurium, which is mediated by a compensatory relationship between LYS-7 and the tyrosine kinase, ABL-1. We further consider the physiology of this balance, finding it to be independent of pathogen load, immune priming or infection persistence.

3.2 Results

3.2.1 *lys-7* mutant animals are hyper-susceptible to killing by *Cryptococcus neoformans*

Given the published role of LYS-7 as an important immunity factor (O'Rourke et al., 2006, Evans et al., 2008b, Mallo et al., 2002), coupled with the finding by our group that it is over-expressed in two *C. neoformans*-resistant *C. elegans* strains (R. C. May, unpublished), we tested whether the loss of LYS-7

would render animals hypersensitive to cryptococcal killing. In line with this prediction, a *lys-7* knockout strain (ok1384) showed severely reduced survival following exposure to the fungus (Figure 6). However, this phenotype does not reflect a general loss of fitness in these animals, since neither brood size (Table 6) nor longevity under non-pathogenic conditions (Figure 7) differed from that of wild type animals.

3.2.2 *lys-7* mutant animals are resistant to killing by *Salmonella* Typhimurium

In order to establish whether the loss of LYS-7 influenced immunity to other pathogens, we exposed *lys-7* knockout animals to three organisms previously shown to be pathogenic towards *C. elegans*; the Gram-positive bacterium *Staphylococcus aureus* (Sifri et al., 2003) and two Gram-negative bacteria *Pseudomonas aeruginosa* (Mahajan-Miklos et al., 1999, Tan et al., 1999, Darby et al., 1999) and *Salmonella enterica* serovar Typhimurium (Labrousse et al., 2000, Aballay et al., 2000). The susceptibility of *lys-7* knockout animals to *S. aureus* (Figure 8) and *P. aeruginosa* (Figure 9) was indistinguishable from that of the wild type strain, suggesting that the loss of LYS-7 does not damage the worm immune response to all pathogens. In marked contrast, however, *lys-7* knockout animals are strongly resistant to killing by *S*. Typhimurium (Figure 10). Moreover, the hypersensitivity and resistance survival phenotypes of the *C. elegans* mutants observed on *C. neoformans* and *S*. Typhimurium were upheld with an additional, independent, allele: *lys-7(ok1385)* (Figures 11 and 12). We refer to this phenomenon as “balanced immunity”, to indicate a process whereby increased resistance to one pathogen comes at the cost of increased susceptibility to another.

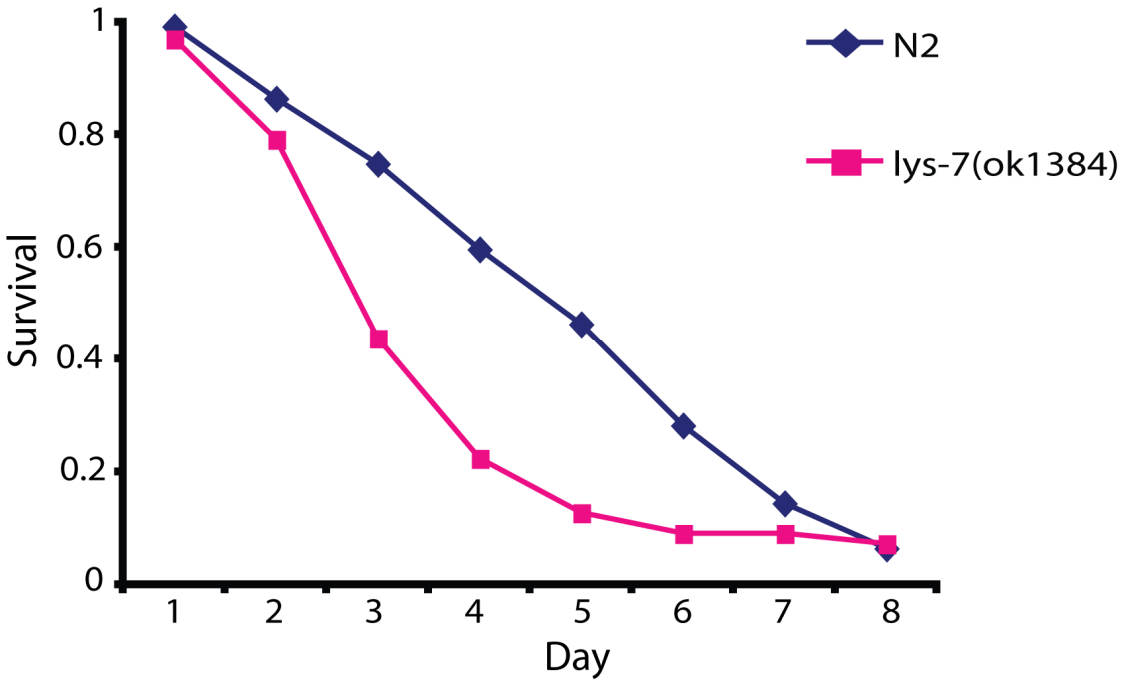

Figure 6: *lys-7(ok1384)* mutant animals are hypersensitive to *C. neoformans* infection

Animals were exposed to *C. neoformans* and monitored daily for survival. The survival of these animals is significantly lower than wild type animals, $p<0.0001$, [*lys-7(ok1384)* n=106; N2 n=151]

	Brood Size
N2	240.3 ± 12.7
lys-7(ok1384)	275.5 ± 6.36

Table 6: *lys-7(ok1384)* brood size in non-infectious conditions

lys-7 knockout animals are unimpaired in their ability to produce young, data represent mean ± S.D.

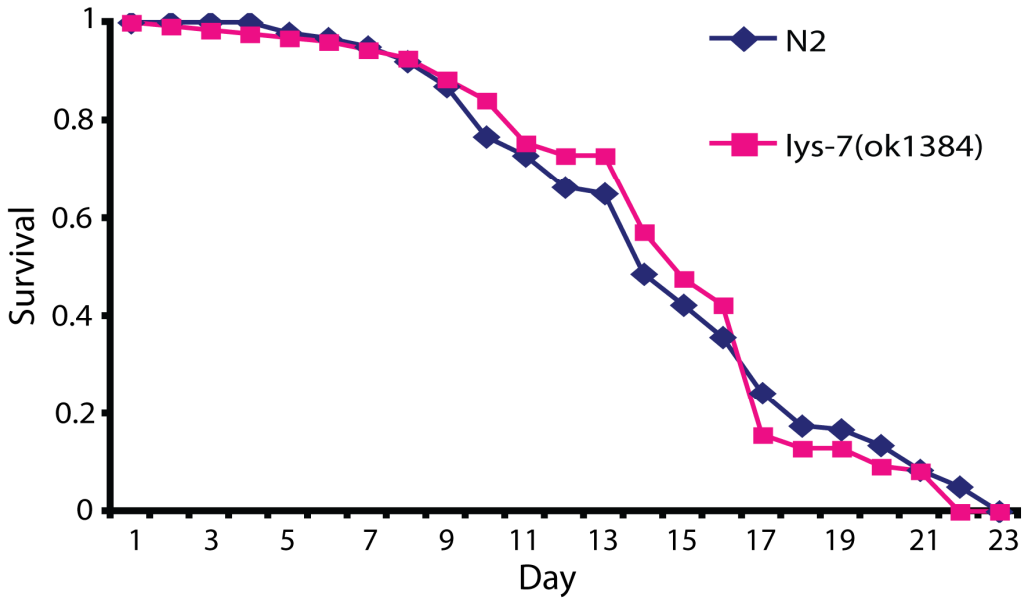

Figure 7: *lys-7(ok1384)* survival in non-infectious conditions

lys-7(ok1384) knockout animals have a normal lifespan in non-infectious conditions, p>0.2 [*lys-7(ok1384)* n=129; N2 n=199]

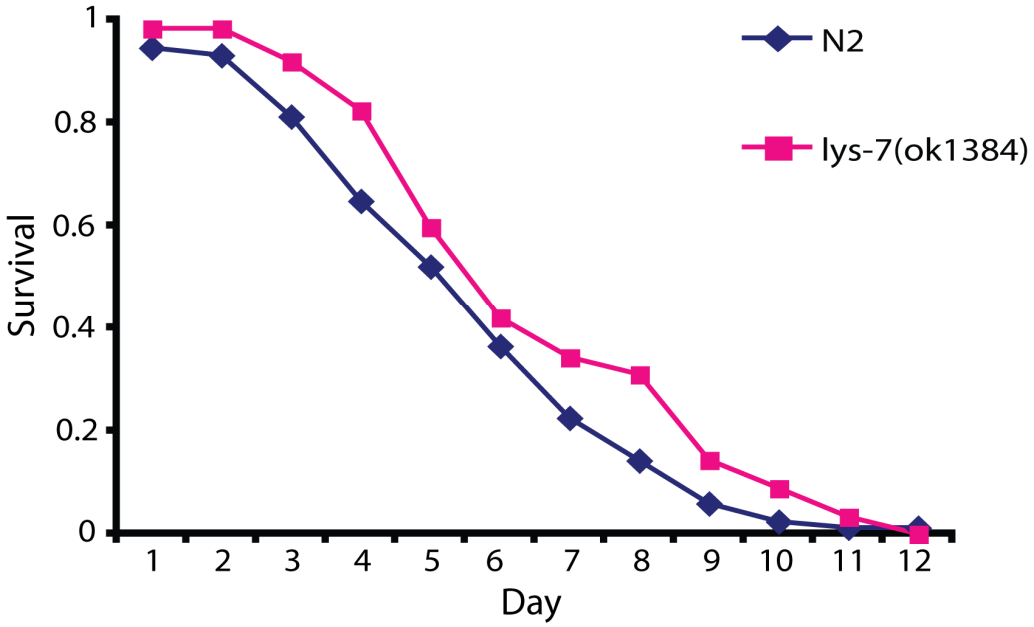

Figure 8: *lys-7(ok1384)* survival on *S. aureus*

Animals were exposed to *S. aureus* and monitored daily for survival. No difference between *lys-7(ok1384)* and wild type survival was detected, p>0.05, [*lys-7(ok1384)* n=131; N2 n=168].

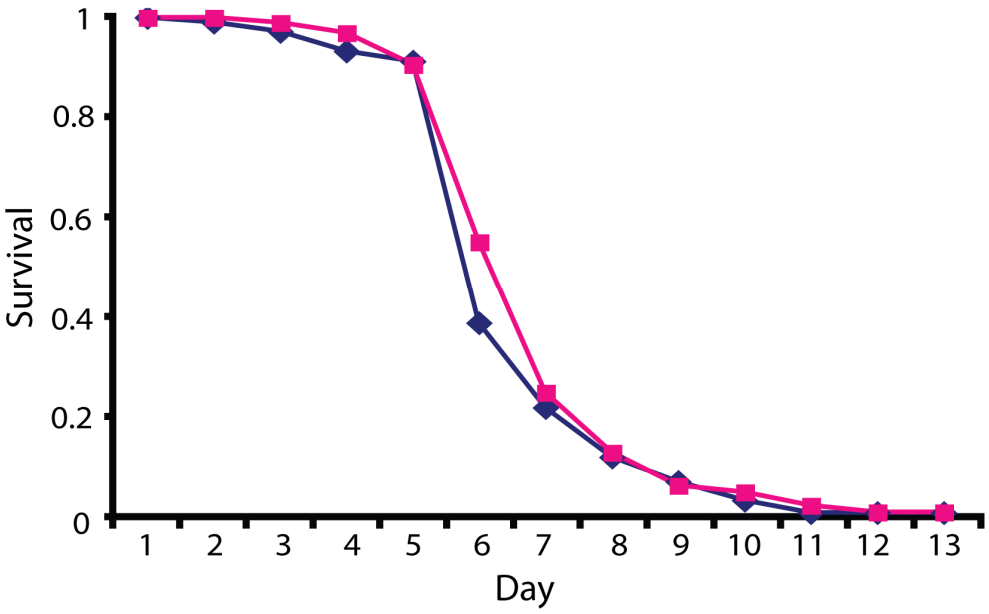

Figure 9: *lys-7(ok1384)* survival on *P. aeruginosa*

Animals were exposed to *P. aeruginosa* and monitored daily for survival. No difference between the two strains was detected, p>0.2, [*lys-7(ok1384)* n=104; N2 n=111].

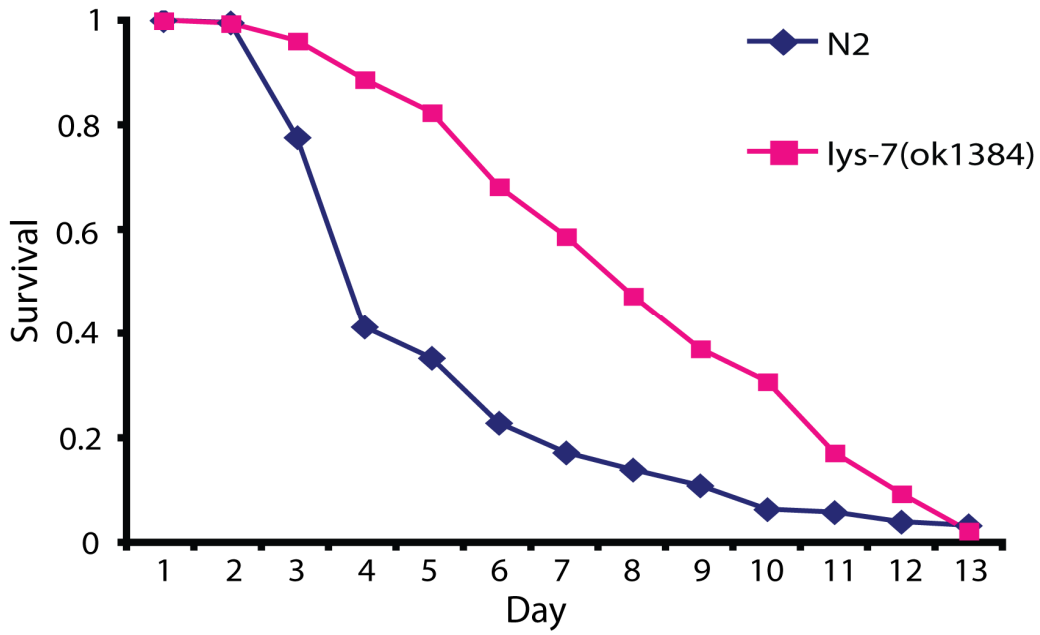

Figure 10: *lys-7(ok1384)* survival on *S.* Typhimurium

Animals were exposed to *S.* Typhimurium SL1344 and monitored daily for survival. The mutant animals exhibited an enhanced survival to *S.* Typhimurium; $p<0.0001$, [*lys-7(ok1384)* n=191; N2 n=242].

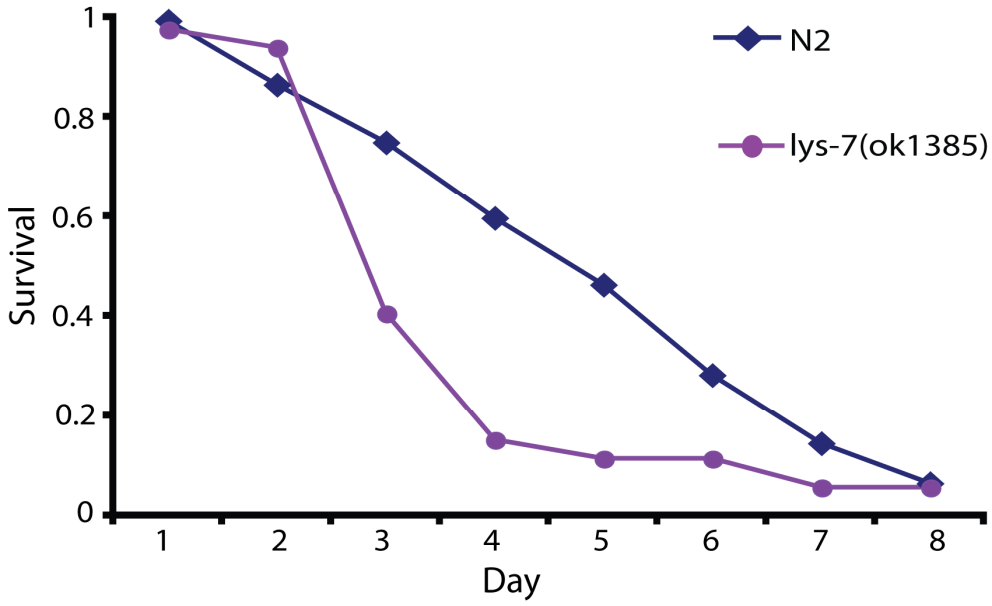

Figure 11: *lys-7(ok1385)* survival on *C. neoformans*

Animals were exposed to *C. neoformans* and monitored daily for survival. This independent *lys-7* knockout strain showed a similar hypersensitivity to the infection, p<0.001 [*lys-7(ok1385)* n=101; N2 as Figure 6].

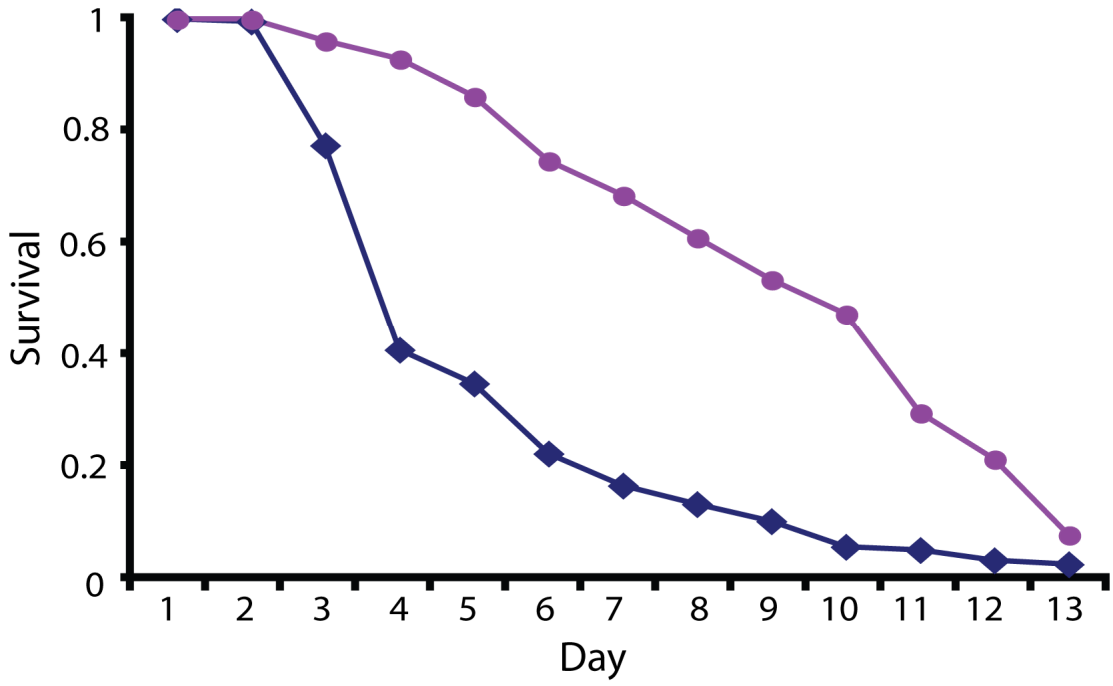

Figure 12: *lys-7(ok1385)* survival on *S.* Typhimurium

Animals were exposed to *S.* Typhimurium SL1344 and monitored daily for survival. These animals were again resistant to the infection, p<0.0001 [*lys-7(ok1385)* n=169; N2 as Figure 10].

3.2.3 Resistance to *S.* Typhimurium is mediated by genetic compensation

In order to identify genes that may compensate for the loss of *lys-7* and thus confer the observed resistance to *S.* Typhimurium, we characterised gene expression changes in the *lys-7* mutant animals with a rudimentary whole-genome microarray analysis (see Appendix 1.0). This analysis identified only four genes that are up-regulated in both *lys-7* knockout strains (ok1384 and ok1385) in comparison to wild type animals. These genes were: *abl-1* (2.65 ± 0.94 fold up-regulation), a tyrosine kinase (Deng et al., 2004) which additionally has a putative immunity role in *C. elegans* (Burton et al., 2006); *fat-5* (1.82 ± 0.15 fold up-regulation), a fatty acid desaturase (Watts and Browse, 2000); *clec-60* (1.59 ± 0.08 fold up-regulation), a C-type lectin (O'Rourke et al., 2006) that is a member of a gene family proposed to be putative immune recognition factors (Schulenburg et al., 2008); and *rga-6* (1.92 ± 0.089 fold up-regulation), a Rho-GTPase activating protein with high orthology to the human Rho-GTPase activating protein ARHGAP6 (Potet et al., 2009).

We obtained knockout strains for each of these genes and tested their susceptibility to killing by *C. neoformans* (Figure 13) and *S.* Typhimurium (Figure 14). Remarkably, animals lacking either *abl-1* or *rga-6* were hypersensitive to *C. neoformans* but resistant to *S.* Typhimurium infection; a survival pattern which phenocopies that of the *lys-7* knockout animals. *fat-5* knockout animals show slight resistance to *S.* Typhimurium but a normal susceptibility to *C. neoformans*, whilst *clec-60* mutant animals are indistinguishable from wild type animals in their survival following exposure to either pathogen.

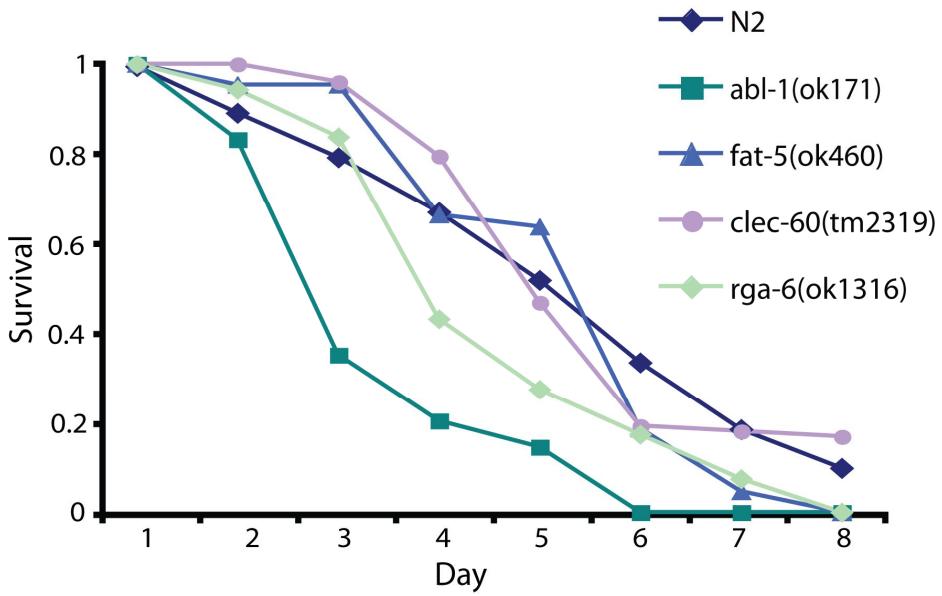

Figure 13: *abl-1* and *rga-6* mutant animals phenocopy *lys-7* susceptibility to *C. neoformans*

Knockout strains for the four genes which were identified by a microarray to be up-regulated in the *lys-7* mutant background were tested for sensitivity to *C. neoformans*. The survival of *abl-1(ok171)* and *rga-6(ok1316)* animals is significantly lower than wild type animals, $p<0.0001$ and $p<0.01$ respectively. The remaining animals are unchanged from wild type survival, $p>0.2$ [N2 n=186; *abl-1(ok171)* n=130; *rga-6(ok1316)* n=192; *fat-5(ok460)* n=100; *clec-60(tm2319)* n=110].

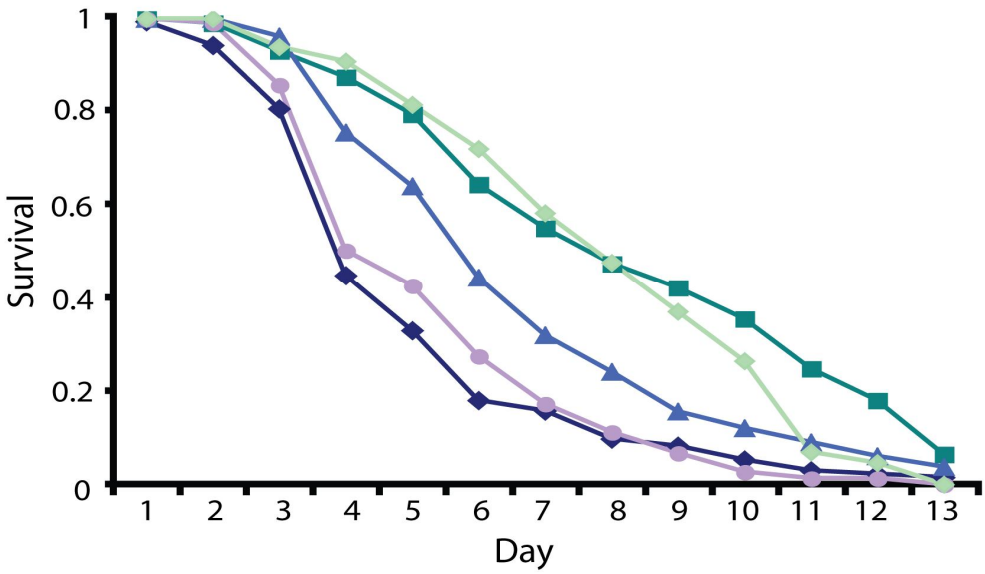

Figure 14: *abl-1*, *rga-6* and *fat-5* mutant animals phenocopy *lys-7* resistance to *S.* Typhimurium

Animals were exposed to *S.* Typhimurium and monitored daily for survival. *abl-1(ok171)*, *rga-6(ok1316)* and *fat-5(tm420)* animals exhibit enhanced resistance to the pathogen, $p<0.0001$ in each case. *clec-60(tm2319)* animals have survival patterns that reflect those of wild type animals, $p>0.2$ [N2 n=170; *abl-1(ok171)* n=222; *rga-6(ok1316)* n=100; *fat-5(ok460)* n=162; *clec-60(tm2319)* n=125].

We next used qRT-PCR to assess whether *lys-7* expression was altered in these knockout animals under normal culture conditions. *lys-7* expression remained unchanged in *fat-5, clec-60* and *rga-6* knockout animals, but, interestingly, was significantly up-regulated in *abl-1* mutants (Figure 15).

To address the compensation in survival between animals lacking *lys-7* and the three candidate genes (*abl-1*, *fat-5* and *rga-6*) identified by resistance analysis, we generated a series of double mutants and tested their susceptibility to infection with *C. neoformans* and *S.* Typhimurium. Whilst *lys-7(ok1384);abl-1(ok171)* double mutants remained sensitive to killing by *C. neoformans* (Figure 16), the resistance to *S*. Typhimurium exhibited by both single mutants was completely abolished (Figure 17). In contrast however, the other double mutants (*rga-6(ok1316);lys-7(ok1384)* and *fat-5(ok460);lys-7(ok1384)*) remained sensitive to killing by *C. neoformans* (Figure 18) and resistant to killing by *S*. Typhimurium (Figure 19) suggesting that the loss of *lys-7* is dominant in these animals, although we do note that the degree of resistance has been slightly reduced in the case of *rga-6*. Taken together, these data indicate that, of the three candidate genes, only *abl-1* shows full phenocopying and reciprocal regulation with *lys-7* in response to these two pathogens.

In support of these data, we tested the survival of two further *abl-1* null alleles, *n1961* and *n1963* (Hurwitz et al., 2009), on *C. neoformans* (Figure 20) and *S*. Typhimurium (Figure 21) and, in both cases, observed a consistent balanced immunity phenotype. Further, there is no loss or gain of fitness in *abl-1(ok171)* animals; their survival upon *S. aureus* infection (Figure 22), lifespan in normal culture conditions (Figure 23) and brood size (Table 7) analyses were all unchanged from those of wild type animals.

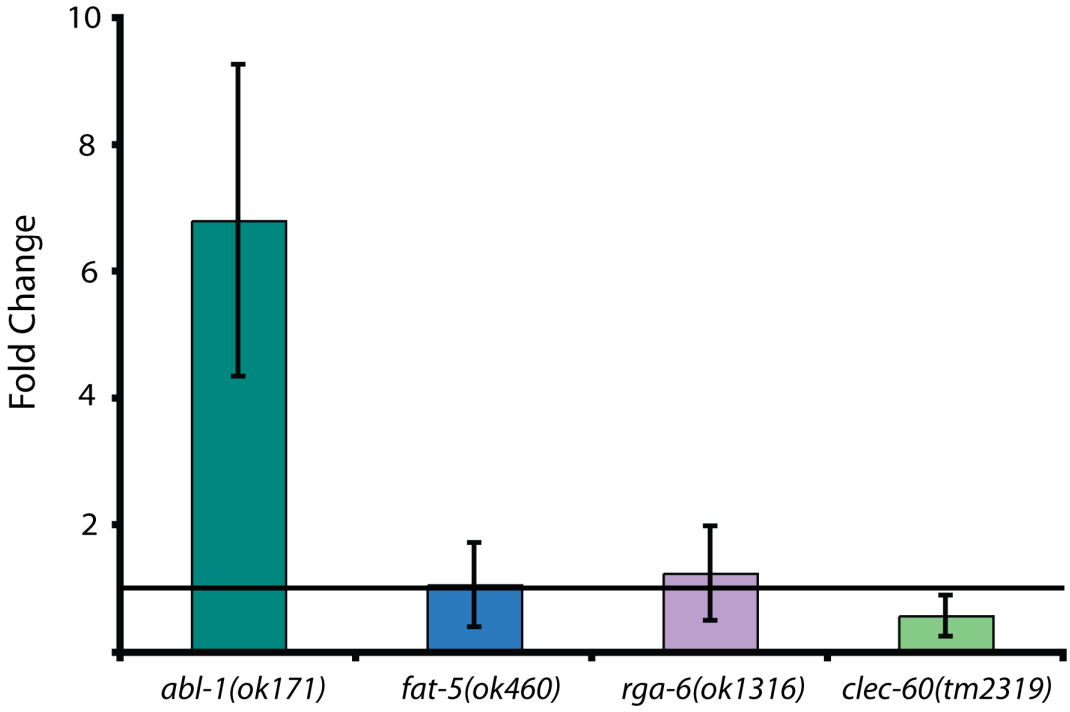

Figure 15: *abl-1* mutant animals up-regulate *lys-7* expression

Changes in *lys-7* expression between the four array genes and N2 were determined by the qRT-PCR analysis of total RNA for each strain. *abl-1(ok171)* animals significantly up-regulate *lys-7* expression with an average induction of 6.8 fold, $p<0.05$, whereas *lys-7* expression is unchanged from wild type in the remaining mutant backgrounds. These data represent the mean of three independent experiments ± S.E.M.

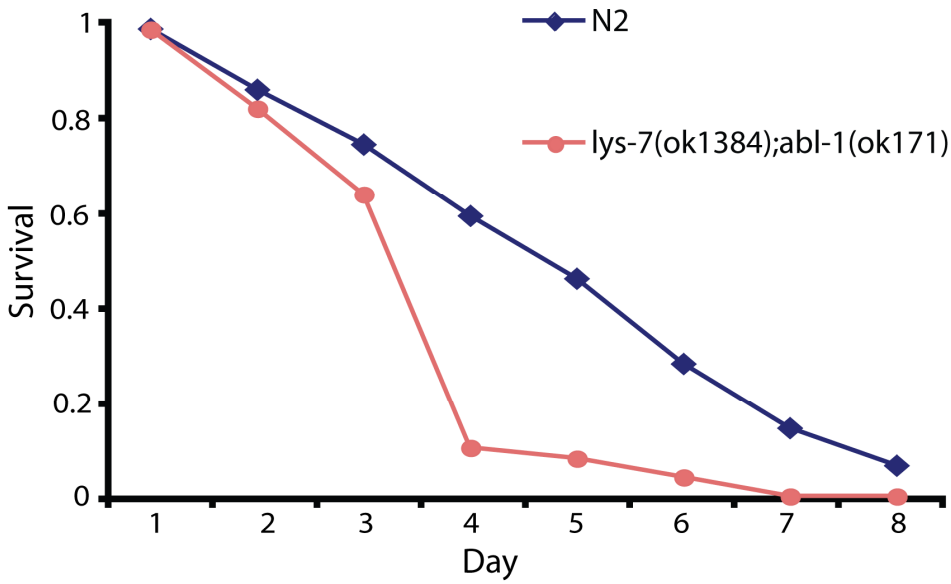

Figure 16: *lys-7;abl-1* double mutants are sensitive to *C. neoformans*

abl-1(ok171)lys-7(ok1384) double mutants were infected with *C. neoformans* at the L4 stage and monitored daily for survival. The double mutant nematodes were hypersensitive to the infection, p<0.0001, similar to their corresponding single mutants [*abl-1(ok171)lys-7(ok1384)* n=133; N2 n=150].

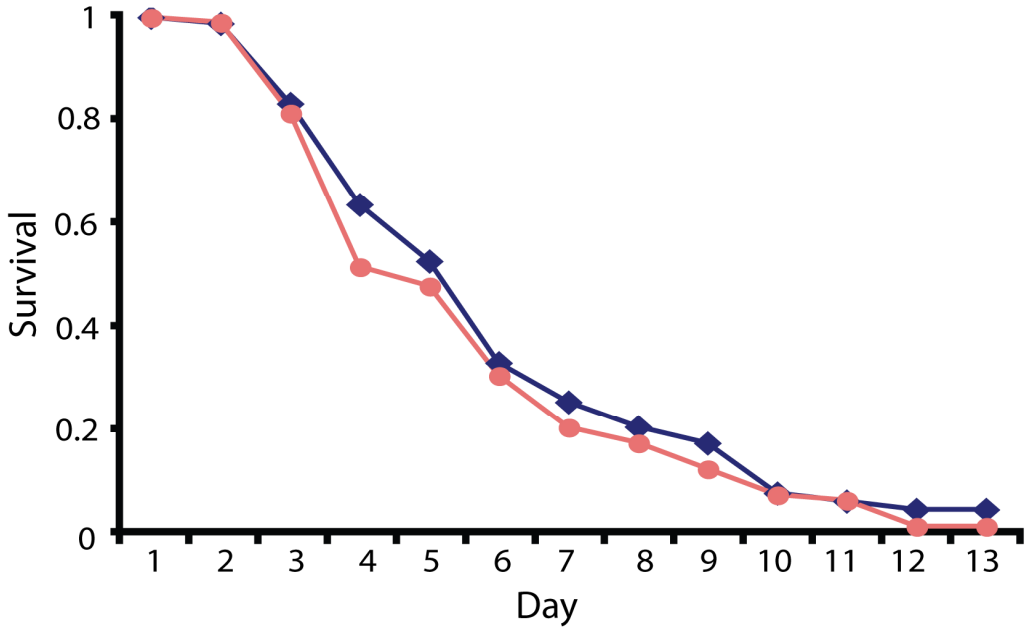

Figure 17: *lys-7;abl-1* double mutants are sensitive to *S.* Typhimurium

abl-1(ok171)lys-7(ok1384) double mutants were infected with *S.* Typhimurium at the L4 stage and monitored daily for survival. The resistance exhibited by the single mutants to the infection has been completely suppressed to wild type survival levels in the double mutants; p>0.2, [*abl-1(ok171)lys-7(ok1384)* n=110; N2 n=94].

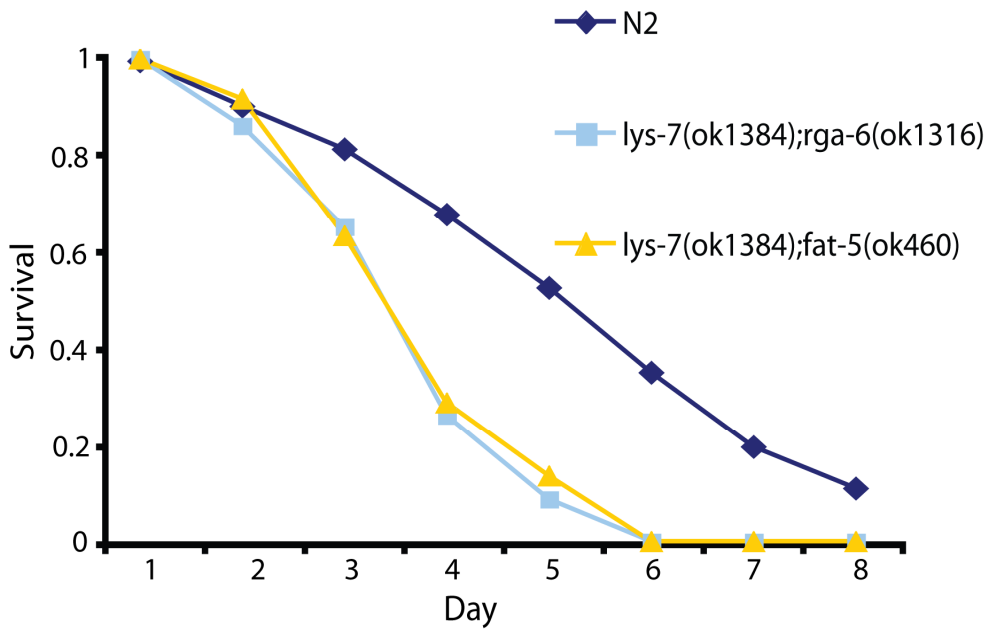

Figure 18: *lys-7;fat-5* and *lys-7;rga-6* double mutants phenocopy *lys-7* on *C. neoformans*

Animals were exposed to *C. neoformans* and monitored daily for survival. Double mutants between *lys-7* and *fat-5* or *lys-7* and *rga-6* were hypersensitive to the infection in comparison to wild type survival, p<0.0001 in both cases, [N2 n=207; *lys-7(ok1384)fat-5(tm420)* n=100; *lys-7(ok1384)rga-6(ok1316)* n=100].

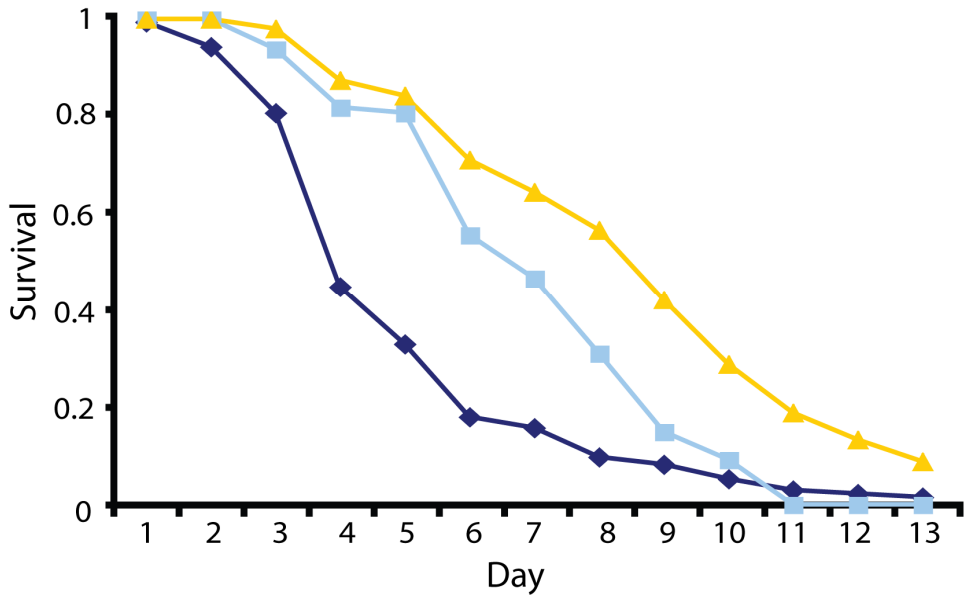

Figure 19: *lys-7;fat-5* and *lys-7;rga-6* double mutants phenocopy *lys-7* on *S.* Typhimurium

Animals were exposed to *S.* Typhimurium and monitored daily for survival. The resistance to *S.* Typhimurium exhibited by the *lys-7* knockout strain, was either unaltered (*fat-5*) or only slightly reduced (*rga-6*) by these secondary mutations [N2 n=170; *lys-7(ok1384)fat-5(tm420)* n=100; *lys-7(ok1384)rga-6(ok1316)* n=100].

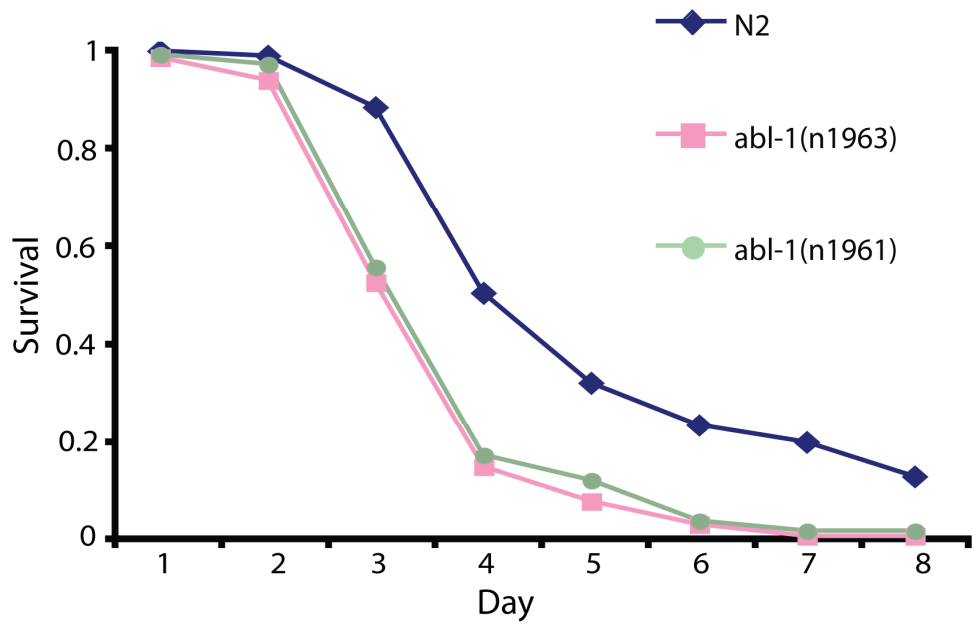

Figure 20: *abl-1(n1961) and abl-1(n1963)* survival on *C. neoformans*

Animals were exposed to *C. neoformans* and monitored daily for survival. These independent *abl-1* alleles showed a similar hypersensitivity to the infection as *abl-1(ok171)* animals, p<0.0001 in both cases, [*abl-1(n1961)* n=150; *abl-1(n1963)* n=152; N2 n=325].

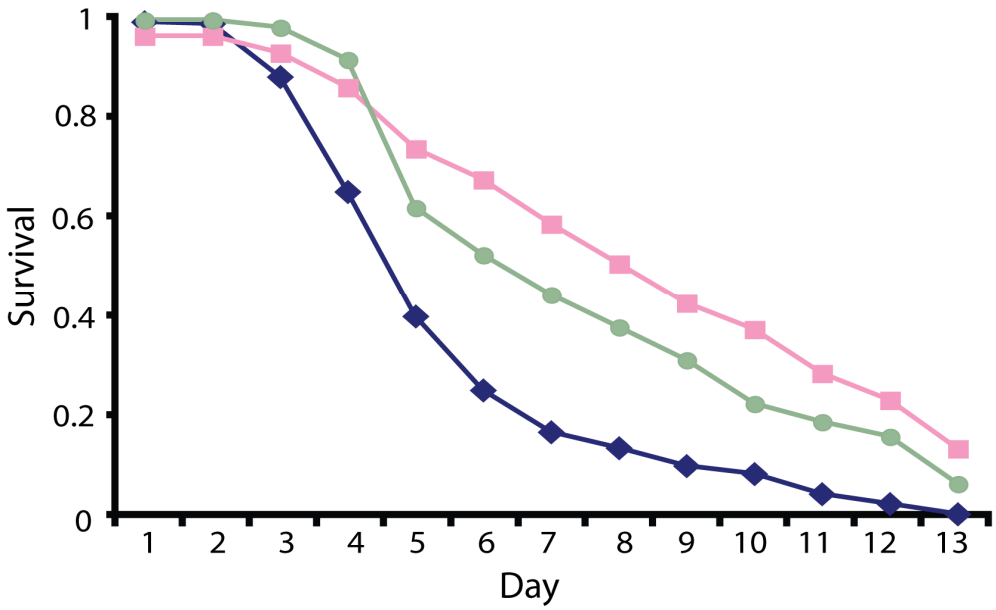

Figure 21: *abl-1(n1961) and abl-1(n1963)* survival on *S.* Typhimurium

Animals were exposed to *S.* Typhimurium SL1344 and monitored daily for survival. These animals were resistant to the infection in a manner similar to the *abl-1(ok171)* animals, p<0.0001 in both cases, [*abl-1(n1961)* n=150; *abl-1(n1963)* n=127; N2 n=278].

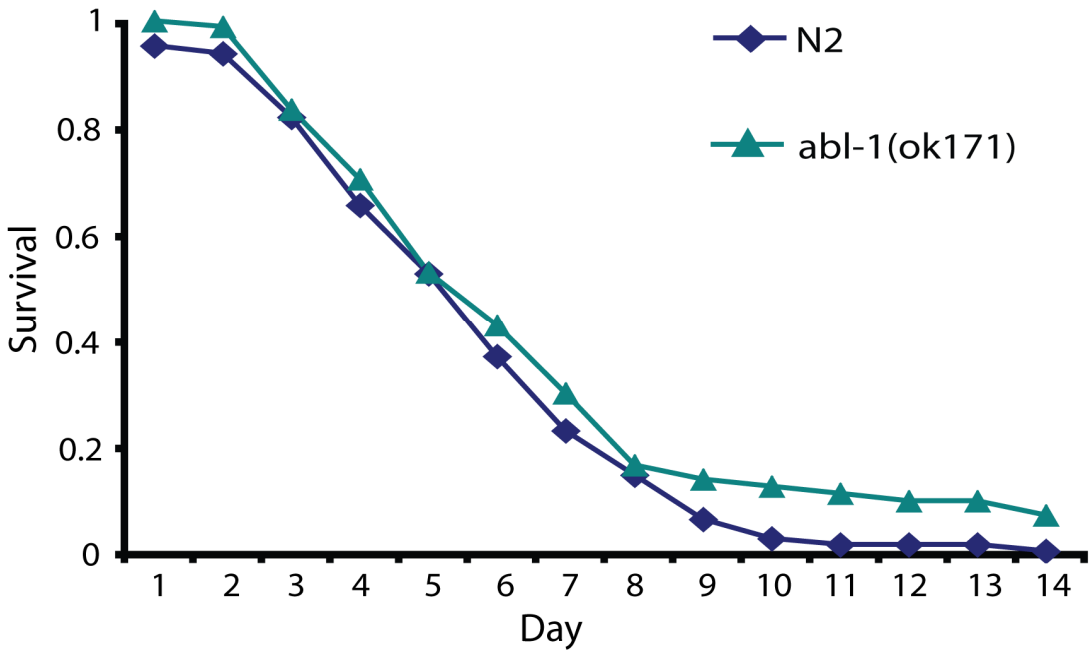

Figure 22: *abl-1(ok171)* survival on *S. aureus*

Animals were exposed to *S. aureus* and monitored daily for survival. No difference between *abl-1(ok171)* and wild type survival was detected, p>0.2, [*abl-1(ok171)* n=120; N2 as Figure 8].

	Brood Size
N2	240.3 ± 12.7
abl-1(ok171)	325.5 ± 23.67

Table 7: *abl-1(ok171)* brood size in non-infectious conditions

abl-1 knockout animals are unimpaired in their ability to produce young, data represent mean ± S.D.

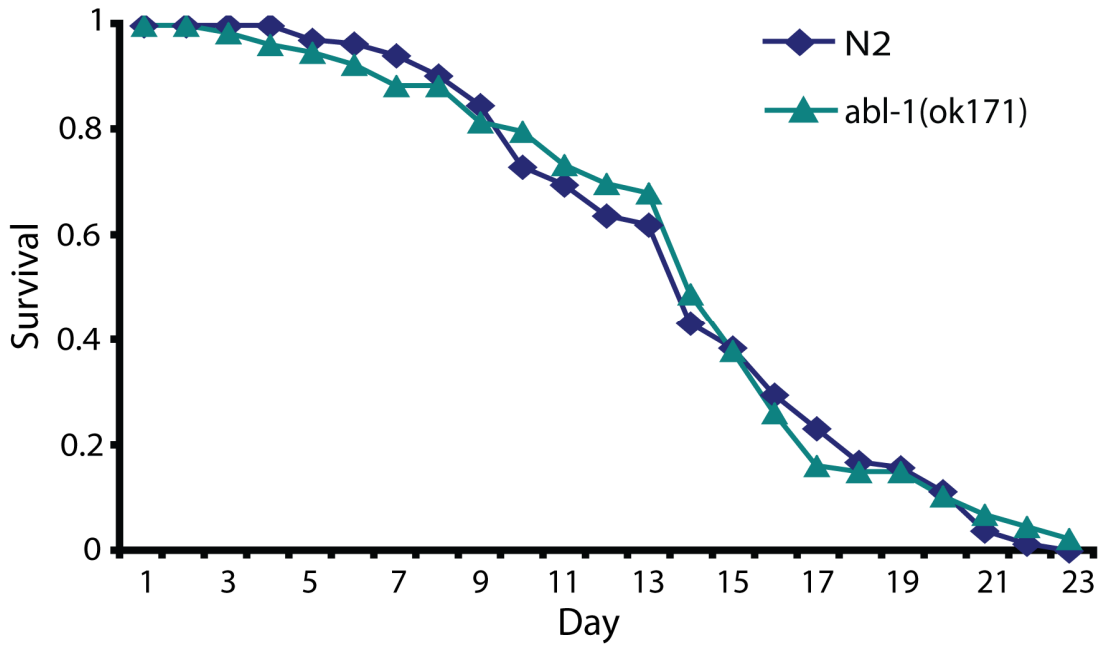

Figure 23: *abl-1(ok171)* survival in non-infectious conditions

abl-1(ok171) knockout animals have a normal lifespan in non-infectious conditions, p>0.2 [*abl-1(ok171)* n=142; N2 n=159].

It should be noted here that we have been unable to validate the up-regulation of *abl-1* in either *lys-7* mutant background by qRT-PCR, where expression levels appear unchanged from wild type (Figure 24). Unfortunately the lack of a functional antibody for *C. elegans* ABL-1 has also prevented us from determining the protein levels of ABL-1 in *lys-7* mutant animals. Thus we presume that the loss of LYS-7 induces ABL-1 activity via a post-transcriptional mechanism, such as the activation of its kinase activity.

3.2.4 The physiology of *S.* Typhimurium infection in the mutant animals is unchanged from wild type

We wondered whether the resistance of *lys-7(ok1384)* and *abl-1(ok171)* single mutant animals to *S.* Typhimurium challenge may result from a lower bacterial load, for instance due to a reduced pharyngeal pumping rate or more effective clearance from the intestinal tract. To test this, we infected wild type, *lys-7* and *abl-1* animals with *S.* Typhimurium L1019 (a *S.* Typhimurium SL1344 strain carrying the pUA66*pacpP* plasmid which encodes GFP*mut2*; a kind gift from Jessica Blair and Laura Piddock, University of Birmingham) and examined animals for infection differences both microscopically (Figure 25) and through viable counts (Figure 26). We found no discernable difference between any of the strains, save for a slight disparity between the knockout strains on day 4 of the infection that had resolved by day 5.

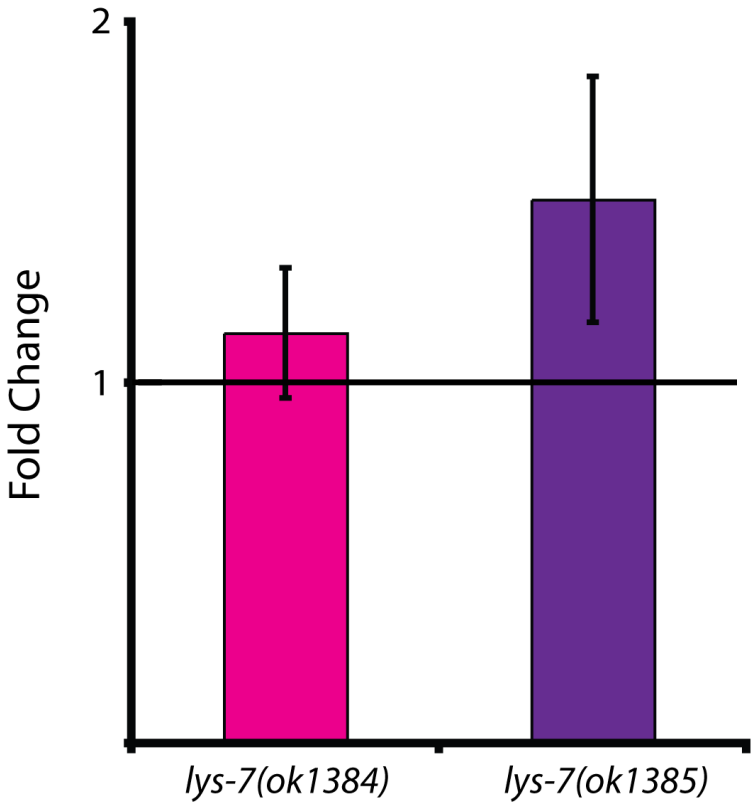

Figure 24: *lys-7* mutant animals do not change *abl-1* expression

abl-1 mRNA expression is unchanged from wild type in both *lys-7* mutant backgrounds, determined by the qRT-PCR analysis of total RNA for each strain. These data represent the mean of three independent experiments ± S.E.M.

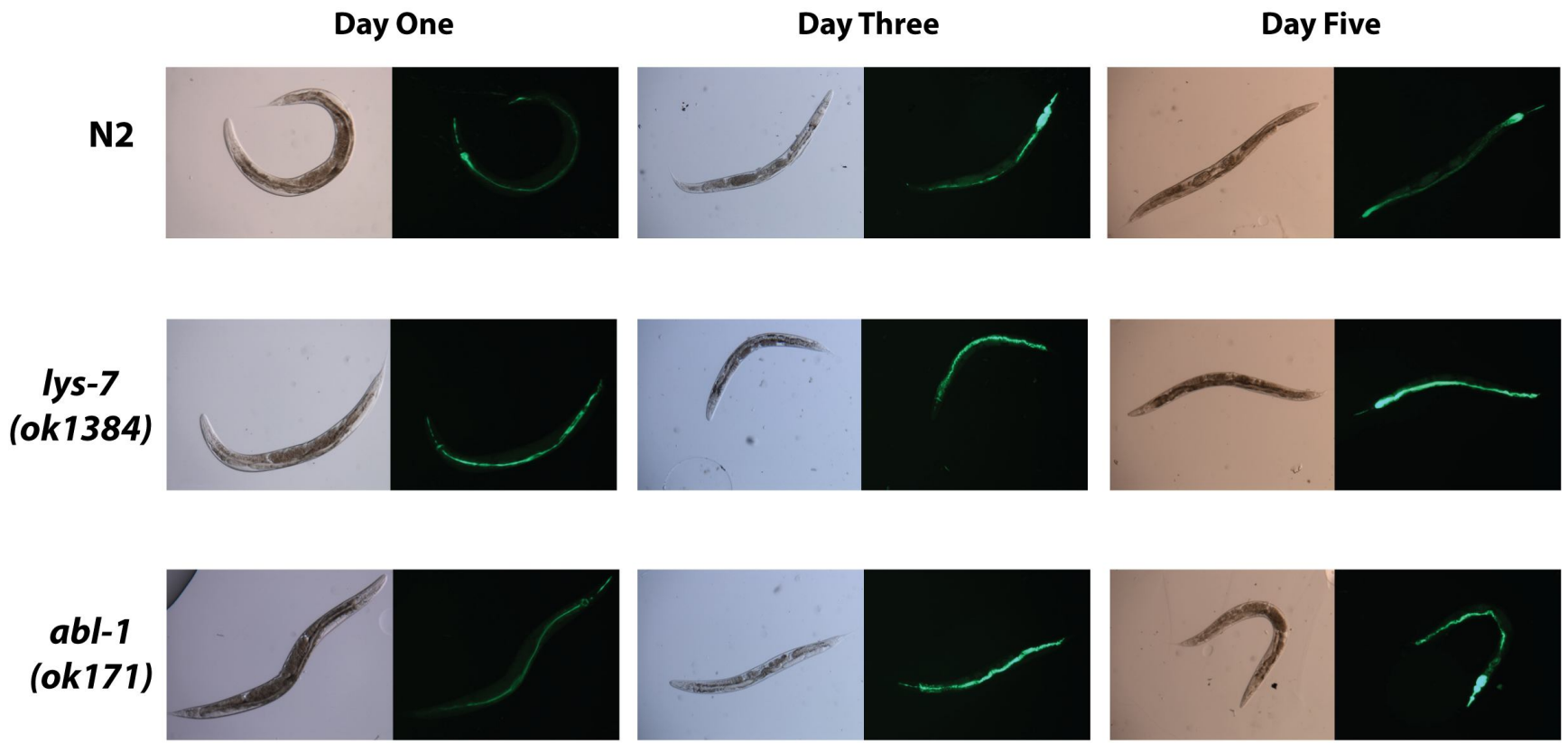

Figure 25: Microscopic analysis of a constant *S.* Typhimurium infection in *C. elegans*

Bacterial load was assessed microscopically using a GFP-expressing *S*. Typhimurium, strain L1019, across a five day infection. No difference in infection load was identified between the strains.

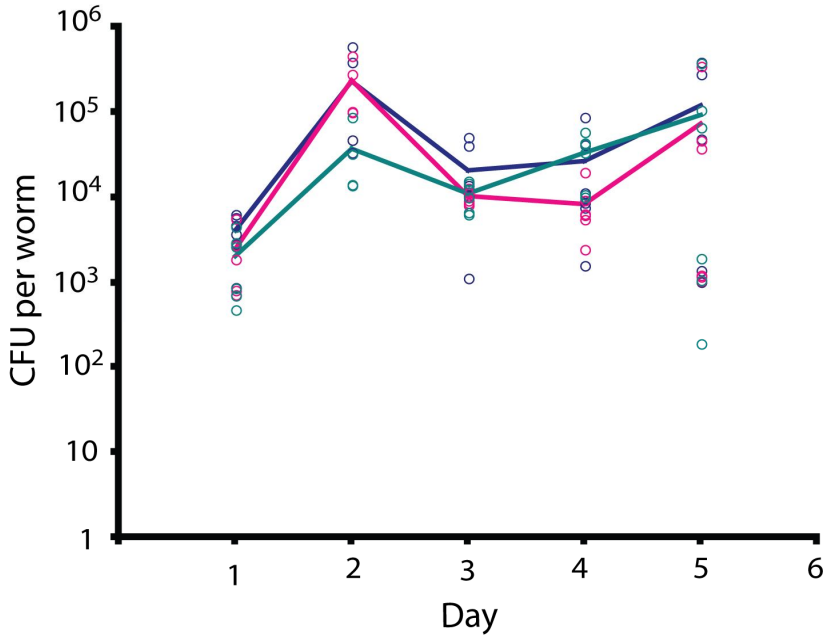

Figure 26: *C. elegans* bacterial load during a constant *S.* Typhimurium infection

Bacterial load was assessed through viable counts per worm. Generally, there was no difference between any of the strains, p>0.2, although there is a slight disparity between *abl-1(ok171)* and *lys-7(ok1384)* on day 4 of the infection, p<0.05 [*abl-1(ok171)* n=60; *lys-7(ok1384)* n=60; N2 n=60].

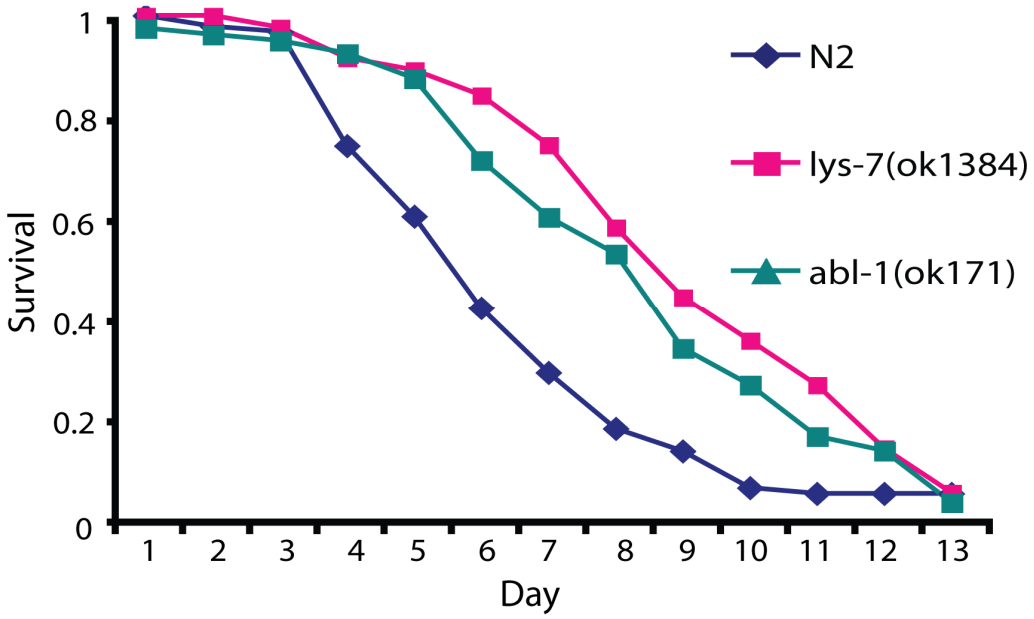

Figure 27: *C. elegans* survival following a six hour *S.* Typhimurium infection

Bacterial persistence following a six hour *S.* Typhimurium infection was examined by daily survival. The *lys-7(ok1384)* and *abl-1(ok171)* single mutants were tolerant to the infection under these conditions in comparison with wild type animals' survival, p<0.01 and p<0.0001 respectively, [*lys-7(ok1384)* n=90; *abl-1(ok171)* n=86; N2 n=96].

We also considered the possibility that the difference in susceptibility may result from *lys-7* and *abl-1* knockout animals being better able to limit *S*. Typhimurium proliferation within the gut. To test this, we restricted the animals' exposure to the pathogen to just six hours and then shifted them to the normal feeding bacteria *E. coli* OP50. This regime has previously been shown to lead to persistent colonisation of *C. elegans* by *S*. Typhimurium (Labrousse et al., 2000). Under these conditions both *lys-7* and *abl-1* animals retain their strong resistance phenotypes (Figure 27), but we observed no consistent difference in *S.* Typhimurium colonisation between the mutants and wild type animals microscopically (Figure 28). Indeed, as we find no difference in *S*. Typhimurium colonisation between the two mutant animal strains and wild type, we conclude that *lys-7* and *abl-1* knockout animals are more "tolerant" (i.e. a gain in survival with no change in pathogen levels, dependent upon damage limitation) to the *S*. Typhimurium infection as opposed to being "resistant" (Raberg et al., 2009).

Since *C. elegans* are fed on a live bacterial foodstuff (*E. coli* OP50) prior to infectious challenge, we considered the possibility that the loss of *lys-7* or *abl-1* may result in an altered response of *C. elegans* to OP50, such that there is a general up-regulation of defensive enzymes in these animals that subsequently enhances tolerance to *S*. Typhimurium. To investigate this further, animals were raised on antibiotic-killed OP50 to the fourth larval stage, and then exposed to *S*. Typhimurium infection and assayed daily for survival. However, under these conditions *lys-7* and *abl-1* mutant animals show the same strong tolerance phenotype to Salmonella infection (Figure 29).

We thus deduce that neither Salmonella load nor Salmonella persistence are altered in *lys-7* or *abl-1* mutant animals, nor can the heightened tolerance of these animals be explained by a pre-existing induction of the animals' immune response in response to *E. coli* OP50.

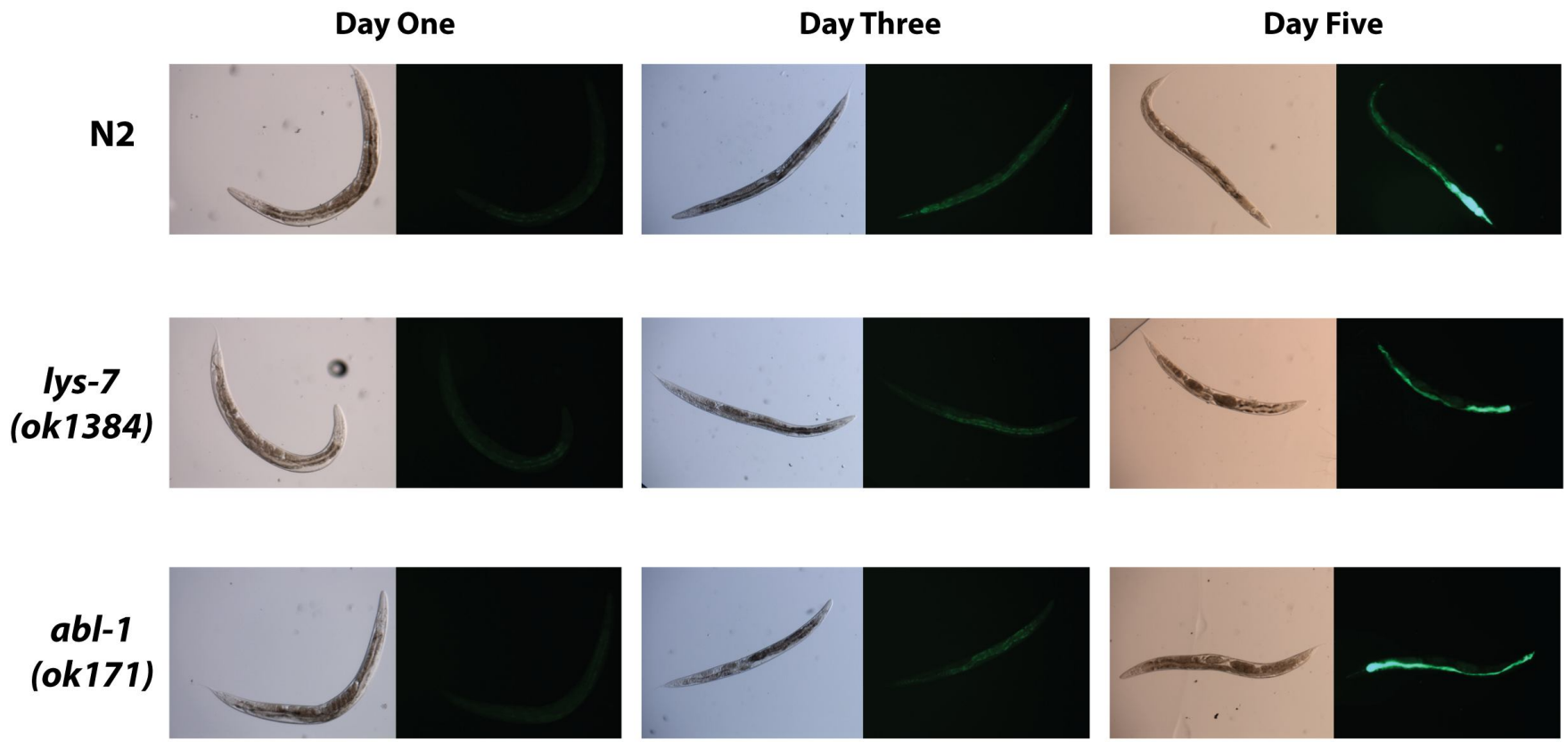

Figure 28: Microscopic analysis of a six hour *S.* Typhimurium infection in *C. elegans*

Bacterial load was assessed microscopically using a GFP-expressing *S*. Typhimurium, strain L1019, across a five day infection. No difference in infection load was identified between the strains.

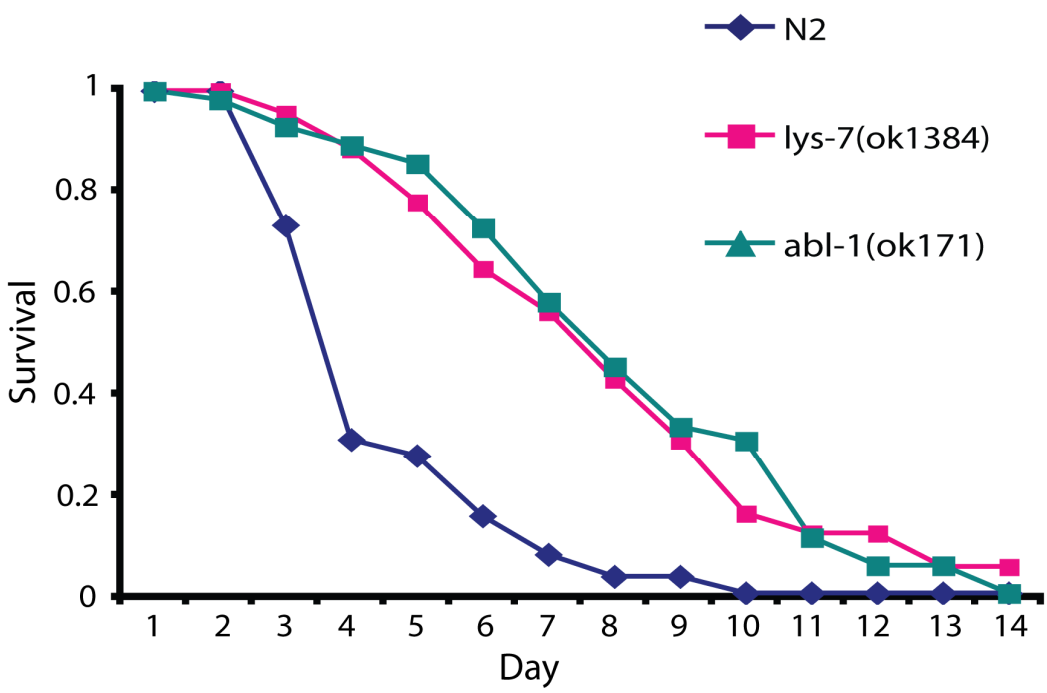

Figure 29: Immune priming is not causative of *C. elegans* tolerance to *S.* Typhimurium

lys-7 or *abl-1* mutant animals reared to L4 stage on kanamycin-killed OP50, show identical patterns of *S.* Typhimurium tolerance, $p<0.0001$ versus N2 in both cases, as those reared on normal (live) OP50 [*lys-7(ok1384)* n=93; *abl-1(ok171)* n=113; N2 n=96].

3.3 Conclusion

The lysozyme LYS-7 has been well-described in *C. elegans* as an essential antimicrobial molecule (Mallo et al., 2002, O'Rourke et al., 2006, Evans et al., 2008b, Schulenburg and Boehnisch, 2008) that is assumed to actively degrade invading pathogens (Kerry et al., 2006, Murphy et al., 2003). In addition, *lys-7* expression is known to be up-regulated in long-lived *daf-2* mutant animals (Murphy et al., 2003), which are resistant to a number of pathogens (Garsin et al., 2003).

In this work, we show that LYS-7 is a specialised defence molecule that protects animals against *C. neoformans*-mediated killing, since *lys-7* knockout animals are hyper-susceptible to this organism; a function that is presumably attributable to the secondary chitinase (anti-fungal) activity exhibited by most lysozymes (Jolles, 1996). Remarkably, however, *lys-7* acts simultaneously as a susceptibility factor for *C. elegans* to the Gram-negative bacterium *Salmonella enterica* serovar Typhimurium, as the loss of *lys-7* more than doubles the median survival of Salmonella-challenged animals.

C. elegans raise an immune response that consists entirely of defensive molecules upon pathogenic challenge (Schulenburg et al., 2004). Generally, the secretion of these molecules is maintained in balance, such that the loss of a defensive factor by mutation causes susceptibility to one (or a small number) of pathogens (Mallo et al., 2002, O'Rourke et al., 2006). We now find, however, that the loss of an immune factor can simultaneously trigger greater tolerance to another pathogen through genetic compensation; in this example we show that the susceptibility trade-off requires a balancing mechanism mediated by the activity of LYS-7 and the tyrosine kinase ABL-1.

As a non-receptor tyrosine kinase that is expressed in the tail ganglia of the nematode (WormBase) we hypothesised that ABL-1 would act upstream of LYS-7 in the signalling pathway controlling the

balanced immunity phenotype; a hypothesis that we explore further in chapter four. However, in a manner similar to the *lys-7* mutant animals, we find that *abl-1* mutant worms are hypersusceptible to killing by *C. neoformans*. Since these animals show up-regulation of *lys-7*, a gene that we demonstrate to be important for cryptococcal resistance, we must therefore conclude that ABL-1 regulates immunity at two levels (by the up-regulation of *lys-7* and by a second, *lys-7* independent pathway), both of which are required for wild type resistance to *C. neoformans*.

In the response to *S*. Typhimurium, *lys-7* and *abl-1* mutant animals display an increased tolerance to the pathogen, a phenotype that is suppressed in the double mutant. The physiological rationale for this tolerance in the *lys-7* and *abl-1* single mutants remains unclear, since these animals did not exhibit any differences in bacterial colonisation following either a constant or minimal exposure to the pathogen. Further, the phenotype cannot be attributed to immunological 'priming' due to growth on live *E. coli* OP50. These data therefore suggest that the loss of *lys-7* or *abl-1* enhances tolerance to Salmonella at the level of the host (for instance, in repairing tissue damage or neutralising bacterial toxins), rather than by a bacteriocidal mechanism.

4.0 SIGNALLING CONTROL OF BALANCED IMMUNITY

4.1 Introduction

In the previous chapter we describe an immunological balance in *C. elegans*, whereby increased tolerance to one pathogen comes at the cost of increased sensitivity to another. We found that this susceptibility trade-off was mediated by two genes, *lys-7* and *abl-1*; but what are the underlying signals that control this phenotype?

C. elegans innate immunity is regulated by four major signalling cascades; the p38 MAPK (Kim et al., 2002, Shivers et al., 2010), IGF (Garsin et al., 2003, McElwee et al., 2003, Troemel et al., 2006), TGF-β (Mochii et al., 1999, Mallo et al., 2002, Zugasti and Ewbank, 2009), and Toll-like pathways (Couillault et al., 2004, Pujol et al., 2001, Pradel et al., 2007) (Figure 5). In addition, there is also a published role for a protective PCD response to *S*. Typhimurium (Aballay and Ausubel, 2001, Aballay et al., 2003), which, given the regulatory function of ABL-1 in apoptosis (Deng et al., 2004, Salinas et al., 2006), may be important for balanced immunity.

We have described an increased tolerance of *lys-7* and *abl-1* mutant animals to *S*. Typhimurium, in which the mutants exhibit improved survival to the pathogen without any change in pathogen levels. As Toll-mediated signalling likely renders animals resistant to infections (i.e. a gain in survival with a decrease in pathogen load) mediated through avoidance behaviour (Pujol et al., 2001) and olfactory learning (Pradel et al., 2007) we did not pursue the function of the Toll pathway in this example of immunological balance.

In this chapter we examine the role of three of the main immunity signalling pathways in *C. elegans*, as well as two ABL-network proteins, CED-3 and ABI-1, in the balanced immunity phenotype. We find that the balance is independent of p38 MAPK and apoptotic signalling; instead, it appears to be mediated by the activity of the IGF and TGF-β pathways.

4.2. Results

4.2.1 The balanced immunity phenotype is independent of apoptotic signalling

ABL-1 is known to regulate apoptosis in *C. elegans* (Deng et al., 2004, Salinas et al., 2006). Since germ line apoptosis has previously been reported to protect *C. elegans* against *S.* Typhimurium infection (Aballay and Ausubel, 2001), (although we note that conflicting data have recently been reported by others (Jia et al., 2009)), we postulated that the loss of *abl-1* may lead to an increase in apoptosis that, in turn, protects the animal against killing by *S*. Typhimurium. Instead, we find that *ced-3(ok2734)* mutant animals are marginally tolerant to a *S*. Typhimurium infection. Furthermore, an *abl-1;ced-3* double mutant, in which apoptosis is blocked by the loss of the caspase CED-3, did not differ from the *abl-1* single mutant in sensitivity towards either *C. neoformans* (Figure 30) or *S.* Typhimurium (Figure 31). Thus, the role of *abl-1* in regulating immunity does not appear to involve its function as an apoptotic factor.

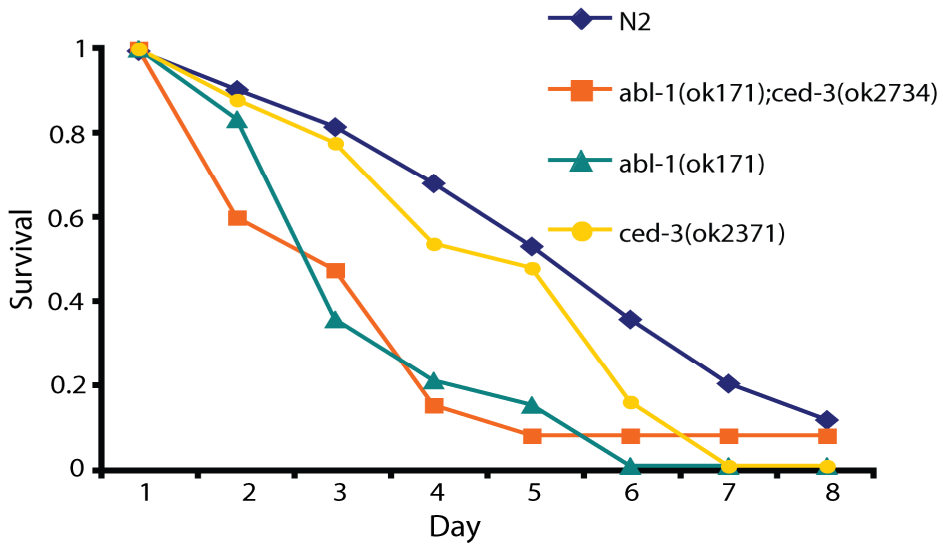

Figure 30: *abl-1(ok171)* hypersensitivity to *C. neoformans* is independent of apoptosis

abl-1(ok171)ced-3(ok2734) double mutants, in which apoptosis is blocked, were infected with *C. neoformans* at the L4 stage and monitored on a daily basis. The survival of these animals did not deviate from that of the *abl-1* single mutant, p>0.2, on this pathogen [*abl-1(ok171)ced-3(ok2734)* n=100; *ced-3(ok2734)* n=100; N2 as Figure 18; *abl-1(ok171)* as Figure 13].

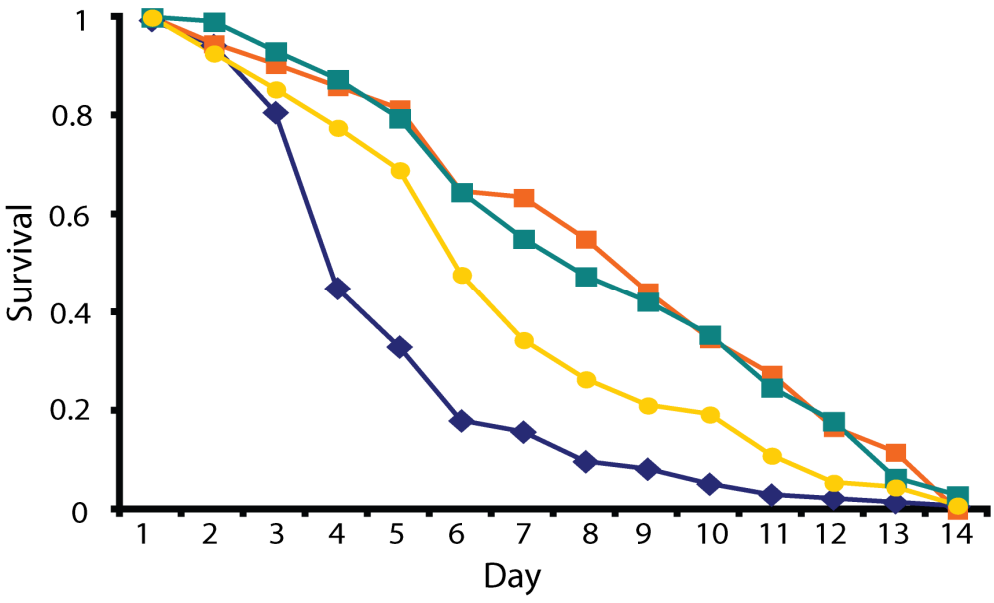

Figure 31: *abl-1(ok171)* tolerance to *S.* Typhimurium is independent of apoptosis

abl-1(ok171)ced-3(ok2734) double mutants, in which apoptosis is blocked, were infected with *S.* Typhimurium and monitored daily for survival. The survival of these animals phenocopied the tolerance of the *abl-1* single mutants, p>0.2 [*abl-1(ok171)ced-3(ok2734)* n=125; *ced-3(ok2734)* n=125; N2 as Figure 19; *abl-1(ok171)* as Figure 14].

4.2.2 The ABL-1 interacting protein (ABI-1) phenocopies the ABL-1 immunity profile

In vertebrates, several ABL-interacting (abi) proteins act in concert with ABL to mediate diverse downstream effects (Dai and Pendergast, 1995, Dai et al., 1998, Tani et al., 2003, Proepper et al., 2007, Stuart et al., 2006). In *C. elegans* there is only a single abi homologue, ABI-1, which has recently been shown to interact with ABL-1 *in vitro* (Hurwitz et al., 2009). We therefore tested a possible function for ABI-1 in *C. elegans* immunity by challenging *abi-1(ok640)* mutant animals with *C. neoformans* and *S*. Typhimurium.

The susceptibility of these nematodes to *C. neoformans* (Figure 32) and resistance to *S*. Typhimurium (Figure 33) is very similar to that of *abl-1* mutant animals, suggesting that ABI-1 and ABL-1 act in the same way to mediate immunological balance in *C. elegans*. Regrettably, we have thus far been unable to generate an *abl-1;abi-1* double mutant for epistasis analysis, suggesting that this combination may be lethal.

4.2.3 Balanced immunity is mediated through the activity of DAF-16 and DBL-1

To further tease apart the mechanism regulating immunological balance in the *lys-7* and *abl-1* knockout animals, we examined the potential role of three of the best characterized immunity pathways in *C. elegans*; the p38 MAPK pathway (Kim et al., 2002, Troemel et al., 2006), the TGF/DBL-1 signalling pathway (Kurz et al., 2003, Sifri et al., 2003, Zugasti and Ewbank, 2009) and the IGF/DAF-2 signalling pathway (Garsin et al., 2003, van den Berg et al., 2006, Evans et al., 2008a). We generated a series of double mutants lacking essential components of each of these pathways in combination with a secondary mutation for either *lys-7* or *abl-1* and then tested the susceptibility of these double mutants to both *C. neoformans* and *S*. Typhimurium.

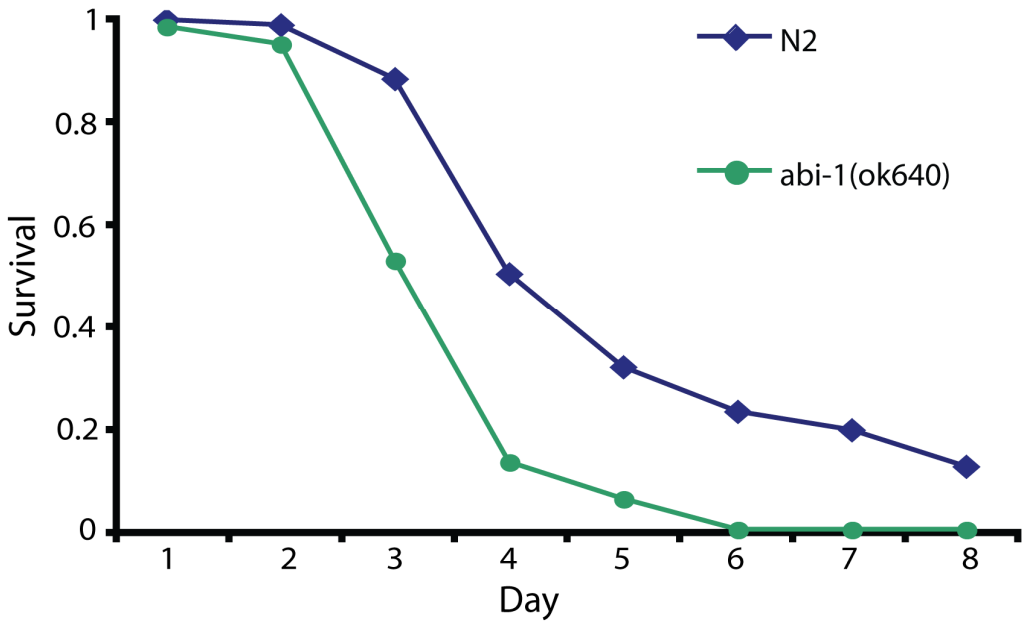

Figure 32: *abi-1(ok460)* animals are hypersensitive to *C. neoformans* infection

Animals lacking the *abl-1* interacting protein, *abi-1(ok460)* were infected with *C. neoformans* and monitored for survival. These animals phenocopied the *abl-1(ok171)* hypersensitivity in comparison with wild type survival, p<0.0001 [*abi-1(ok640)* n=150; N2 as Figure 20].

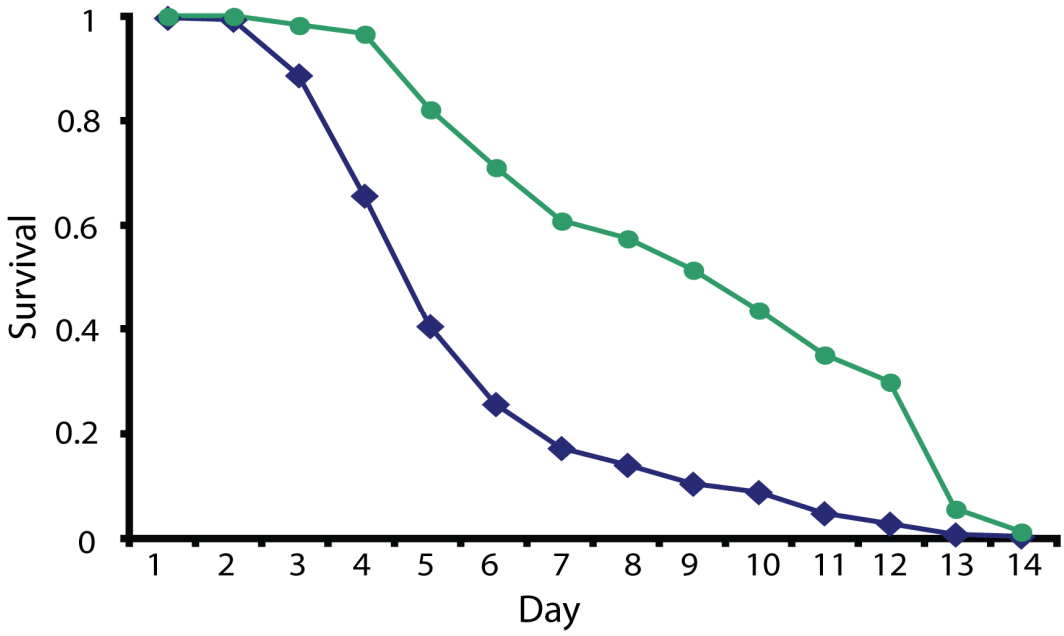

Figure 33: *abi-1(ok460)* animals are tolerant to *S*. Typhimurium infection

abi-1(ok460) animals phenocopy the *abl-1(ok171)* tolerance to *S*. Typhimurium in comparison with wild type animals, p<0.0001 [*abi-1(ok640)* n=127; N2 as Figure 21], indicating that *abi-1* acts in a similar manner to *abl-1* in regulating immunological balance.

Single mutants lacking the *C. elegans* homologue of the p38 MAPKK, *sek-1*, exhibited an enhanced susceptibility phenotype to infection with both *C. neoformans* (Figure 34) and *S*. Typhimurium (Figure 35), consistent with previous reports (Kim et al., 2002, Troemel et al., 2006). However, this sensitivity to *S*. Typhimurium is completely rescued upon the introduction of a secondary mutation for *lys-7*; these double knockouts instead mimic the strong resistance exhibited by the *lys-7* single mutants. Conversely, *lys-7;sek-1* double mutants behave like neither single mutant upon *C. neoformans* infection. Regrettably, *abl-1;sek-1* double knockout animals are very sick even under non-infectious conditions (Figure 36), precluding us from testing these animals upon pathogen exposure. Nevertheless, our data exclude the possibility that the *S*. Typhimurium resistance exhibited by *lys-7* animals is mediated by *sek-1*.

The *C. elegans* TGF-β homologue, *dbl-1*, is hypersensitive to infection with *C. neoformans* (Figure 37), but unchanged from wild type survival when infected with *S*. Typhimurium (Figure 38). As *dbl-1* is closely linked to *lys-7* on chromosome V, we were unable to generate a *dbl-1;lys-7* double knockout mutant. However, we were able to produce *dbl-1;abl-1* double mutant animals. These animals were extremely sensitive to both *C. neoformans* (Figure 37) and *S*. Typhimurium (Figure 38), suggesting a role for the TGF-β pathway in the balance mechanism.

Finally, since the forkhead transcription factor DAF-16 acts downstream of the DAF-2/IGF pathway to regulate the expression of many genes involved in stress, immunity and aging (Mukhopadhyay et al., 2006), we asked whether DAF-16 may play a role in the resistance of *abl-1* and *lys-7* mutant animals towards *S*. Typhimurium. Indeed, double mutants lacking either *abl-1* or *lys-7* together with an additional mutation for *daf-16* remained hypersensitive to infection with *C. neoformans* (Figure 39), but tolerance to *S*. Typhimurium was suppressed to levels similar to *daf-16* single mutants (Figure 40).

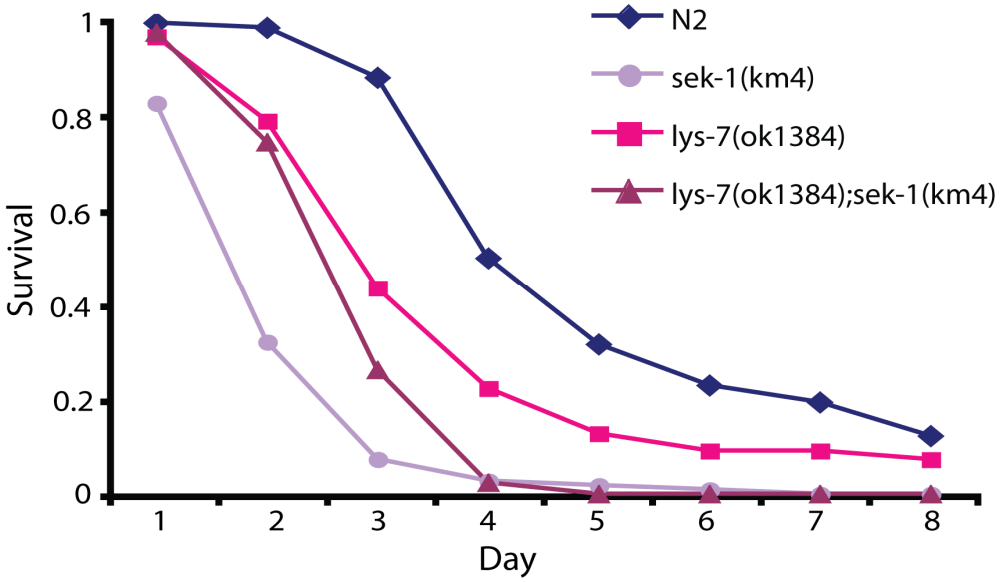

Figure 34: *sek-1(km4)* a p38 MAPK pathway mutant is hypersensitive to *C. neoformans*

lys-7(ok1384)sek-1(km4) double mutant animals behave neither as *lys-7(ok1384)* nor *sek-1(km4)* single mutants, p<0.0001 in both cases, and instead find an intermediate-susceptible phenotype when challenged with cryptococcal infection [*lys-7(ok1384)sek-1(km4)* n=100; *sek-1(km4)* n=100; N2 as Figure 20; *lys-7(ok1384)* as Figure 6 which was a temporally independent experiment].

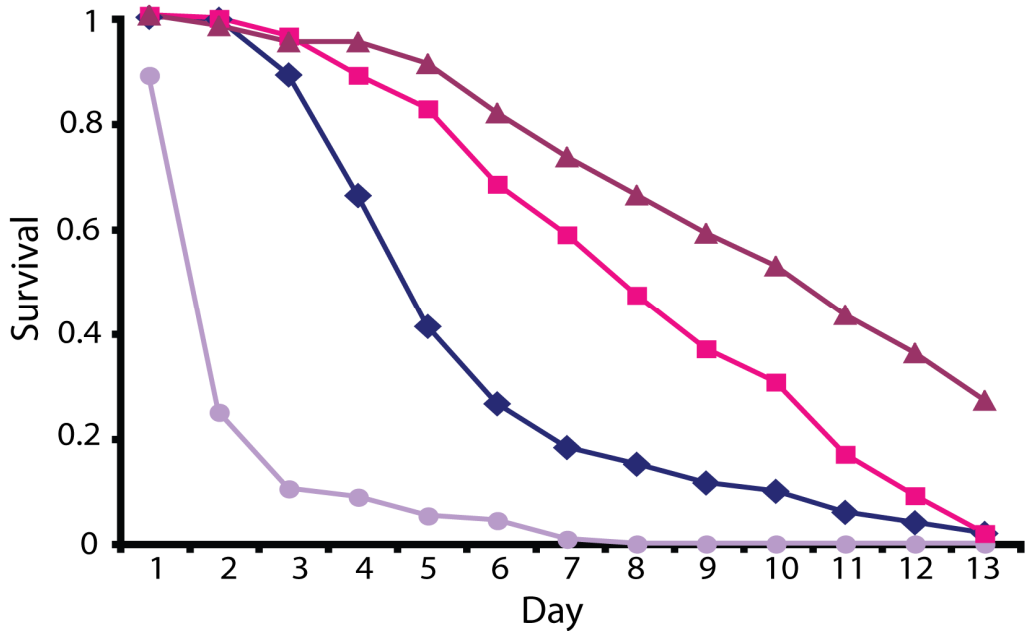

Figure 35: The p38 MAPK pathway has no role in the tolerance phenotype to *S.* Typhimurium

lys-7(ok1384) mutation rescues the hypersensitivity of the *sek-1(km4)* mutants, leading to a resistant phenotype in comparison to the wild type strain, p<0.0001, [*lys-7(ok1384)sek-1(km4)* n=100; *sek-1(km4)* n=150; N2 as Figure 21; *lys-7(ok1384)* as Figure 10, which was a temporally independent experiment], indicating that the p38 MAPK pathway does not mediate the balance mechanism.

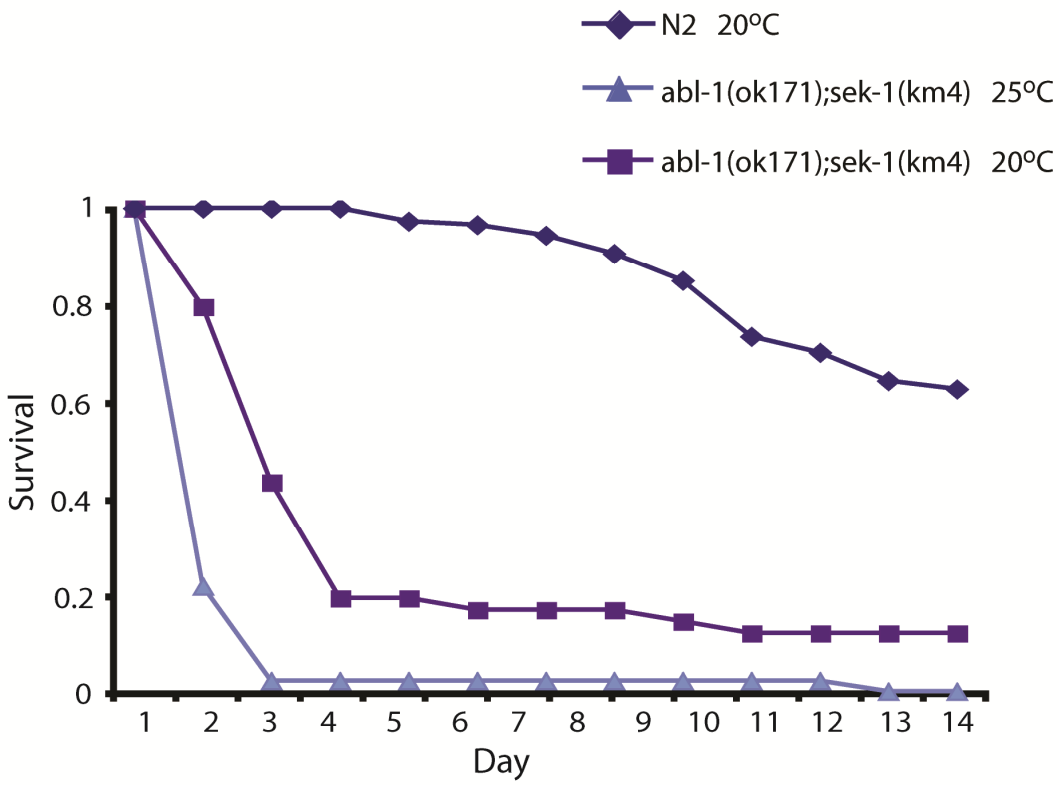

Figure 36: *abl-1;sek-1* nematodes have a greatly reduced lifespan in non-infectious conditions

abl-1(ok171)sek-1(km4) animals are very sick, clear, mutants even in non-infectious conditions. Temperatures of both 20°C and 25°C were examined to rule out any temperature-based effect. Representative data for N2 survival is shown for comparison [*abl-1(ok171)sek-1(km4)* 25°C n=50; *abl-1(ok171)sek-1(km4)* 20°C n=50; N2 as Figure 23].

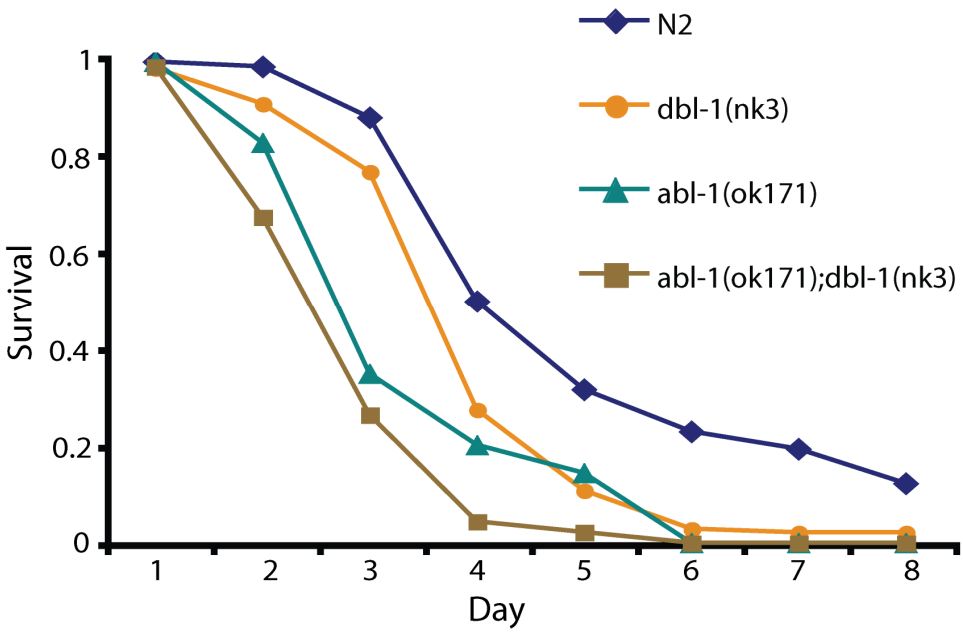

Figure 37: The TGF-β pathway is important in *C. neoformans* pathogenesis

Although both *abl-1(ok171)* and *dbl-1(nk3)* single mutants are hypersensitive to *C. neoformans* infection in comparison with wild type worms, p<0.0001 in both cases, this effect is exacerbated when the mutations are coupled together [*abl-1(ok171)dbl-1(nk3)* n=100; *dbl-1(nk3)* n=100; N2 as Figure 20; *abl-1(ok171)* as Figure 13].

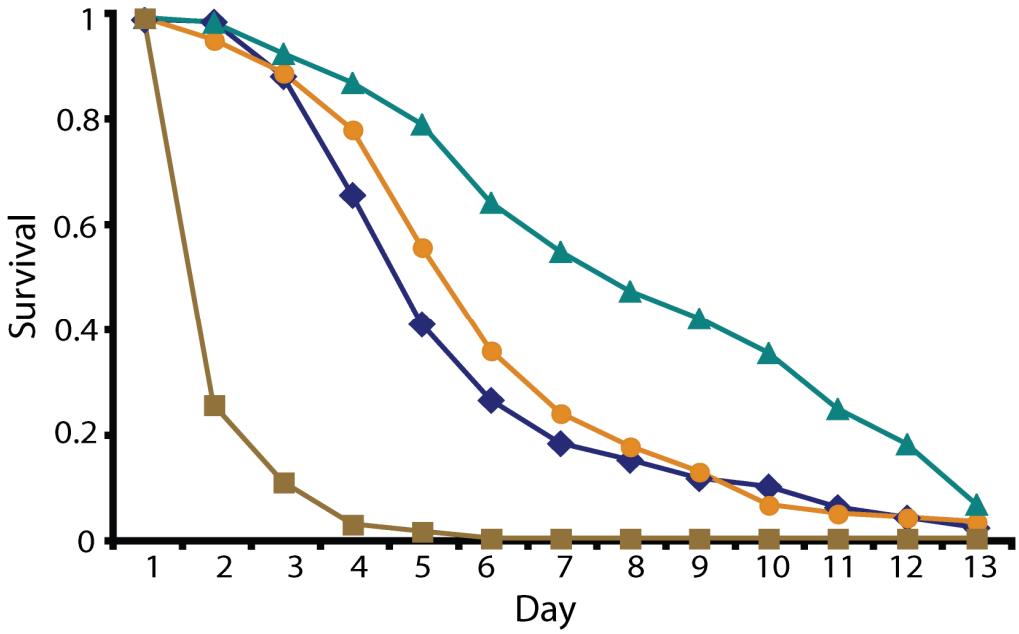

Figure 38: The TGF-β pathway greatly suppresses *S.* Typhimurium tolerance

abl-1(ok171)dbl-1(nk3) double knockout animals are hypersensitive to *S.* Typhimurium infection, p<0.0001, indicating that TGF-β is required for the balance mechanism [*abl-1(ok171)dbl-1(nk3)* n=100; *dbl-1(nk3)* n=150; N2 as Figure 21; *abl-1(ok171)* as Figure 14].

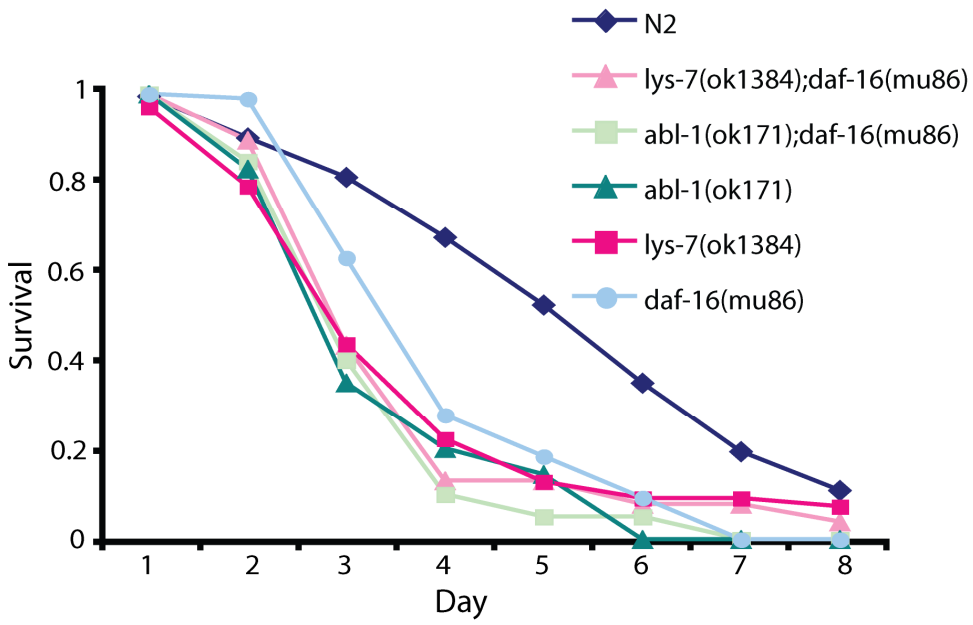

Figure 39: *C. neoformans* hypersensitivity is dependent upon insulin signalling

Animals were exposed to *C. neoformans* and monitored daily for survival. Both *daf-16(mu86)* double mutants remain hypersusceptible to cryptococcal infection and their survival is unchanged from that of the single parent strains; p>0.2, [*abl-1(ok171)daf-16(mu86)* n=100; *lys-7(ok1384)daf-16(mu86)* n=100; *daf-16(mu86)* n=100; N2 as Figure 18; *abl-1(ok171)* as Figure 12; *lys-7(ok1384)* as Figure 6].

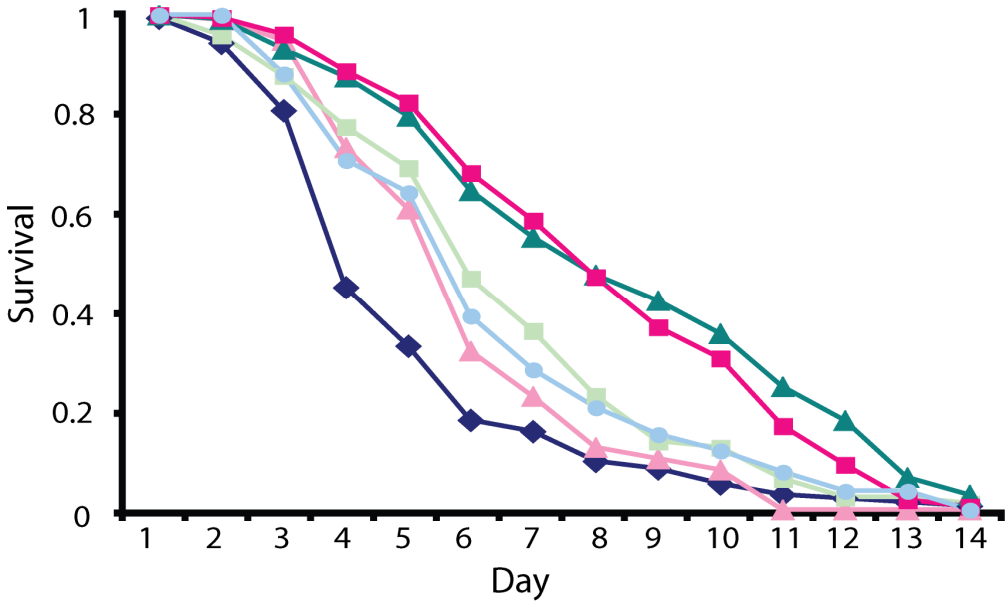

Figure 40: *S.* Typhimurium tolerance is modulated by the forkhead transcription factor *daf-16*

daf-16(mu86) mutation significantly suppresses the tolerance phenotypes of the single mutants when infected with *S.* Typhimurium; p<0.0001 in both cases, indicating that DAF-16 is required for immunological balance [*abl-1(ok171)daf-16(mu86)* n=101; *lys-7(ok1384)daf-16(mu86)* n=100; *daf-16(mu86)* n=100; N2 as Figure 19; *abl-1(ok171)* as Figure 13; *lys-7(ok1384)* as Figure 10].

4.3 Conclusion

With respect to the regulation of this balance, the picture is highly complex. *abl-1* is expressed broadly during embryogenesis but is more limited to the pharynx and tail ganglia post-development (Deng et al., 2004). These data are consistent with a signalling function for *abl-1* in regulating this immunological balance; indeed there is a precedent for neuronal regulation of immunity (Chuang and Bargmann, 2005, Styer et al., 2008, Reddy et al., 2009, Zugasti and Ewbank, 2009). In support of this, we show that the loss of the ABL-1 interacting protein ABI-1 produces a similar phenotype to the loss of ABL-1, suggesting that it too has a role in the regulation of immunity.

These data rule out the involvement of the p38 MAPK pathway, since its loss does not suppress the *abl-1* or *lys-7* tolerance phenotypes. Instead, however, both TGF-β and DAF-16 appear to play important roles in the regulation of immunological balance. As a single mutant, *dbl-1* is unchanged from wild type survival upon infection with *S*. Typhimurium, although these animals are hypersensitive to *C. neoformans* infection. Yet once this knockout is coupled with a further mutation for *abl-1*, the double mutant animals become extremely sensitive to both infections. Moreover, the data here show a strong modulation in the resistance of the *lys-7* and *abl-1* mutants to *S*. Typhimurium infection by DAF-16, since a secondary mutation for *daf-16* suppresses the tolerance phenotype of these single knockout animal strains. Taken together, these data suggest that the loss of *abl-1* or *lys-7* confers resistance towards *S*. Typhimurium through interplay between DAF-16 and TGF-β. Interestingly, *lys-7* itself is a known DAF-16 target (Yu et al., 2008a, McElwee et al., 2003, Murphy et al., 2003), thereby providing a potential feedback mechanism to regulate the activity of the pathway (Figure 49).

5.0 THE ROLE OF RpoS

5.1 Introduction

The *C. elegans*-*S*. Typhimurium interaction is particularly interesting as it is one of the few human pathogens to initiate a persistent infection in the worm (Labrousse et al., 2000, Aballay et al., 2000). Despite primarily being an extracellular interaction, and as such not a *bona fide* Salmonella infection, the model has revealed a number of virulence factors from the bacteria and immunity genes from the worm that are essential for a successful host-pathogen interaction, some of which are also required in mammalian infections (Aballay and Ausubel, 2001, Aballay et al., 2003, Alegado and Tan, 2008, Tenor et al., 2004).

Our observations and data from chapter three have shown that the host expression of the antimicrobial proteins *lys-7* and *abl-1* in wild type *C. elegans* during an *S*. Typhimurium infection is detrimental, since inactivation of either gene more than doubles median animal survival. Similarly, the data in chapter four examines the host mechanisms that underlie this increased tolerance. In this chapter, we turn our attention to the pathogen and examine the Salmonella dependence of the balanced immunity phenotype.

We first considered *S*. Typhimurium strain dependence for the animals' augmented survival. This analysis identified altered tolerance profiles in the worm model for three common *S*. Typhimurium reference strains, SL1344, 14028s and LT2. Based on these differences, we hypothesised that *C. elegans* sensitivity may, in part, be regulated by the activity of the Salmonella alternative sigma factor RpoS. In the final part of this chapter, we present preliminary data in support of this hypothesis, and propose future experiments to test this in more detail.

5.2 Results

5.2.1 The tolerance phenotype is suppressed upon S. Typhimurium LT2 infection

As described in chapter three, the *lys-7* and *abl-1* mutant animals are tolerant to an infection with the *S*. Typhimurium isolate SL1344. In order to determine the strain dependence of this phenotype, two further isolates were tested, 14028s and LT2. *lys-7(ok1384)* and *abl-1(ok171)* animals show a similar enhancement of survival upon 14028s infection (Figure 41), $p<0.01$ and $p<0.001$, respectively. However, this tolerance is suppressed to wild type survival levels when the animals are infected with the strain LT2 (Figure 42), $p>0.2$. Thus these mutant animals exhibit enhanced tolerance to *S*. Typhimurium in a strain dependent manner.

5.2.2 The sigma factor RpoS influences *C. elegans* survival to *S*. Typhimurium

LT2 is an *rpoS* mutant *S*. Typhimurium strain and is considered avirulent, whereas SL1344 and 14028s are fully virulent in all tested models (WilmesRiesenberg et al., 1997). Briefly, ATCC 700720, Lilleengen type 2 (LT2), is a clinical *S*. Typhimurium isolate originally isolated in the United Kingdom during 1948 from an individual presenting with gastroenteritis (Lilleengen, 1948). It was used in one of the first phage-typing studies in Salmonella (Lilleengen, 1948, Rabsch, 2007) and has since been a significant strain in the development of the cellular and molecular understanding of Salmonellosis (McClelland et al., 2001). Although once a virulent isolate in mice and humans (Lilleengen, 1948, Herzberg et al., 1965, Herzberg et al., 1972, Baumberg and Freeman, 1971), most LT2 descendents have become avirulent in mice, believed to be due to the inability of the bacteria to withstand the acidic environment of the host gastrointestinal tract and, in the case of systemic infection, the macrophage (Fang et al., 1992).

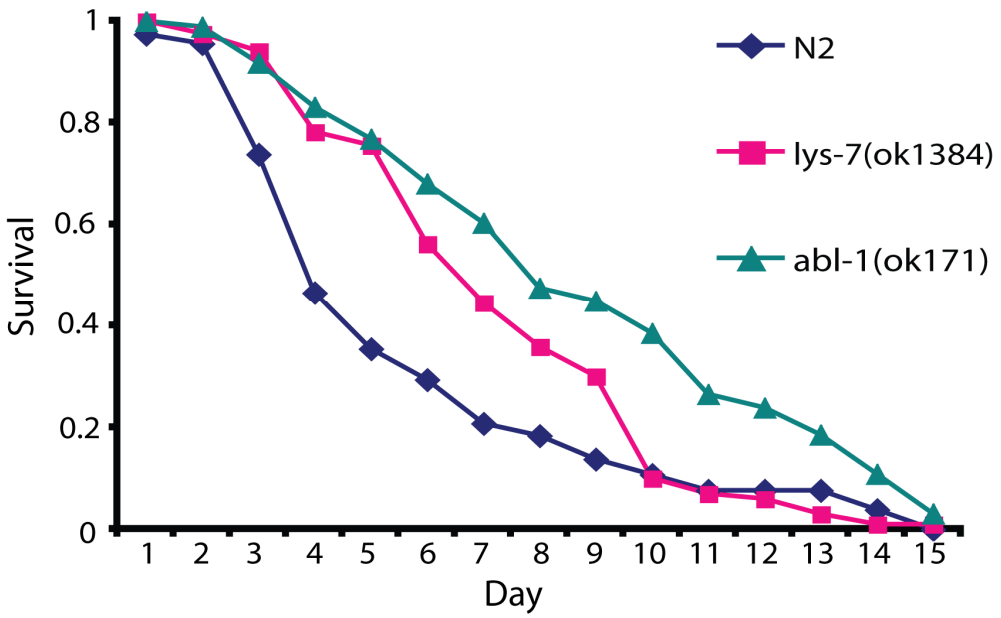

Figure 41: *C. elegans* survival on *S.* Typhimurium 14028s

Animals were exposed to *S.* Typhimurium 14028s and monitored daily for survival. Both strains *lys-7(ok1384)* and *abl-1(ok171)*, were tolerant to the infection, p<0.01 and p<0.001 respectively, in comparison to the wild type strain [N2 n=120; *lys-7(ok1384)* n=123; *abl-1(ok171)* n=90].

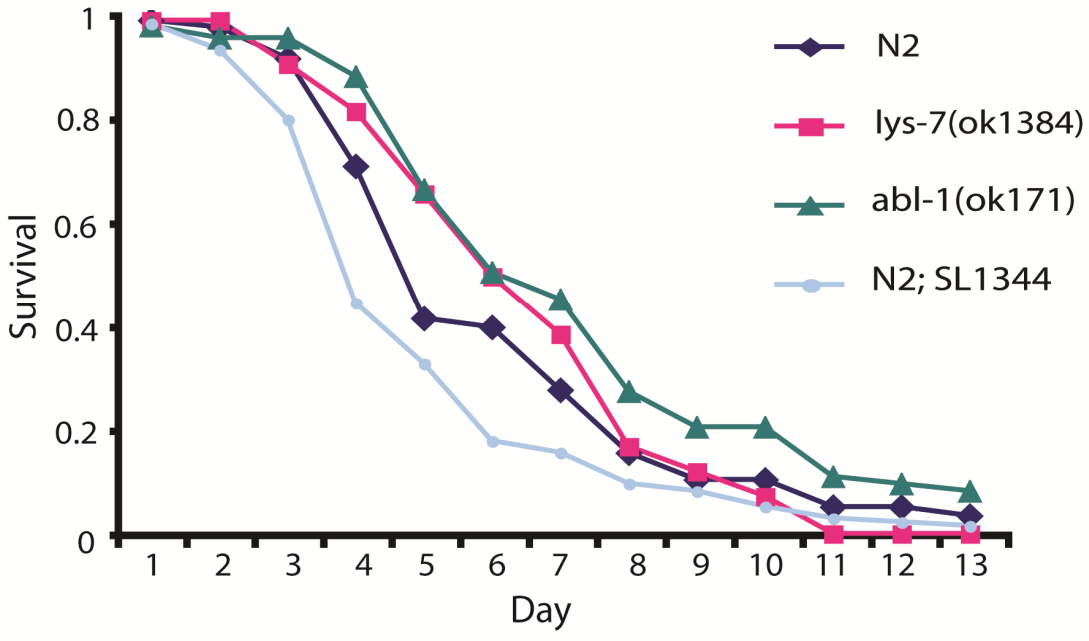

Figure 42: *C. elegans* survival on *S.* Typhimurium LT2

Animals were exposed to *S.* Typhimurium LT2 and monitored daily for survival. No difference between any of the three strains was detected, p>0.2 in each case. Representative data for N2 survival on *S.* Typhimurium SL1344 is shown for comparison [N2 n=80; *lys-7(ok1384)* n=71; *abl-1(ok171)* n=92; N2 SL1344 as Figure 14].

This avirulence is entirely dependent upon the acquisition of a single mutation in mouse virulence gene B (*mviB*) during laboratory passage that has since been described as a point mutation in the start codon of *rpoS*, an alternative sigma factor, that substantially reduces the expression levels of RpoS (Lee et al., 1995, WilmesRiesenberg et al., 1997, Swords et al., 1997a). Interestingly, *C. elegans* survival data from a previous study, which included a superficial examination of the pathogen requirements for the Salmonellae-*C. elegans* interaction, suggested that the RpoS sigma factor was important in *S*. Typhimurium virulence in the worm (Labrousse et al., 2000). We therefore asked whether the RpoS locus was necessary for the augmented survival of the mutant animals.

We hypothesised that the tolerance of the *lys-7* and *abl-1* knockout animals to a virulent *S*. Typhimurium infection would return when these animals were infected with the strain χ8000. This strain is LT2 with a complemented $rpoS^{SL1344}$ allele in cis ($rpoS^{SL1344}$zxx-5181::Tn*10*(dTc)). Indeed, upon complementation of the LT2 *rpoS* mutation, the tolerance phenotype of the *lys-7(ok1834)* and *abl-1(ok171)* animals strongly returns (Figure 43), $p<0.0001$. Thus the restoration of RpoS is enough to restore the increased survival of the *C. elegans* mutants, although it should be noted that the survival patterns of the wild type and mutant animals do not entirely reflect those upon exposure to SL1344.

In the same vein, we anticipated that the tolerance phenotype would be lost upon infection of the animals with an SL1344 *rpoS* insertional inactivated mutant strain. However, this strain JF2691 (*rpoS::Ap*^{r} from P22(SF1005) x SL1344) continues to induce the tolerance phenotype of the mutant animals (Figure 44), $p<0.0001$. These data therefore suggest that either RpoS does not determine the strain dependence of the *C. elegans* infection tolerance, or that an intermediate level of RpoS expression, as found in LT2, is critical to the phenotype.

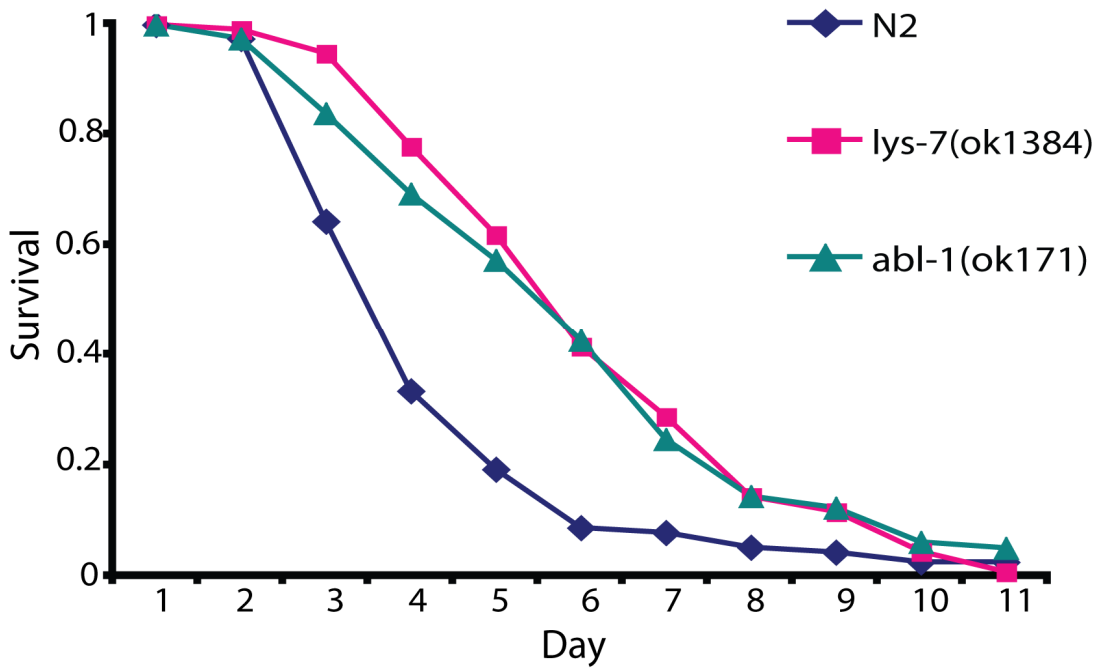

Figure 43: *C. elegans* survival on *S.* Typhimurium χ8000

rpoS^{SL1344} was complemented in χ4996 (LT2, *rpoS::Ap*^{r}), to restore RpoS expression to wild type in LT2. Animals were infected with this strain and monitored daily for survival. Virulence was restored and *lys-7(ok1384)* and *abl-1(ok171)* were once more tolerant to the infection, $p<0.0001$ in both cases [N2 n=120; *lys-7(ok1384)* n=120; *abl-1(ok171)* n=120].

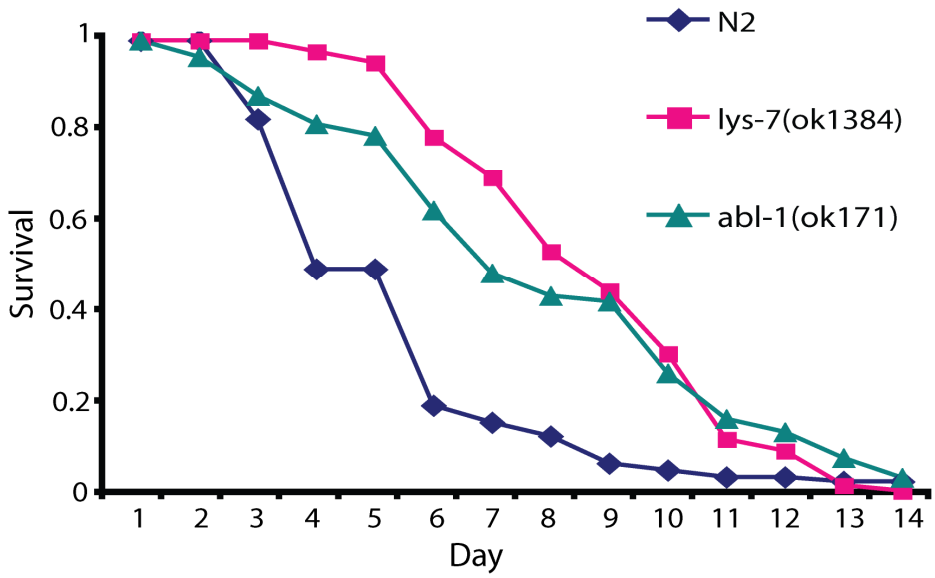

Figure 44: *C. elegans* survival on *S.* Typhimurium JF2691

Animals were exposed to *S*. Typhimurium JF2691, an *rpoS* insertional inactivated mutant in an SL1344 background, and monitored daily for survival. The two mutant *C. elegans* strains were tolerant to the infection, p<0.0001 in both cases [N2 n=142; *lys-7(ok1384)* n=82; *abl-1(ok171)* n=83].

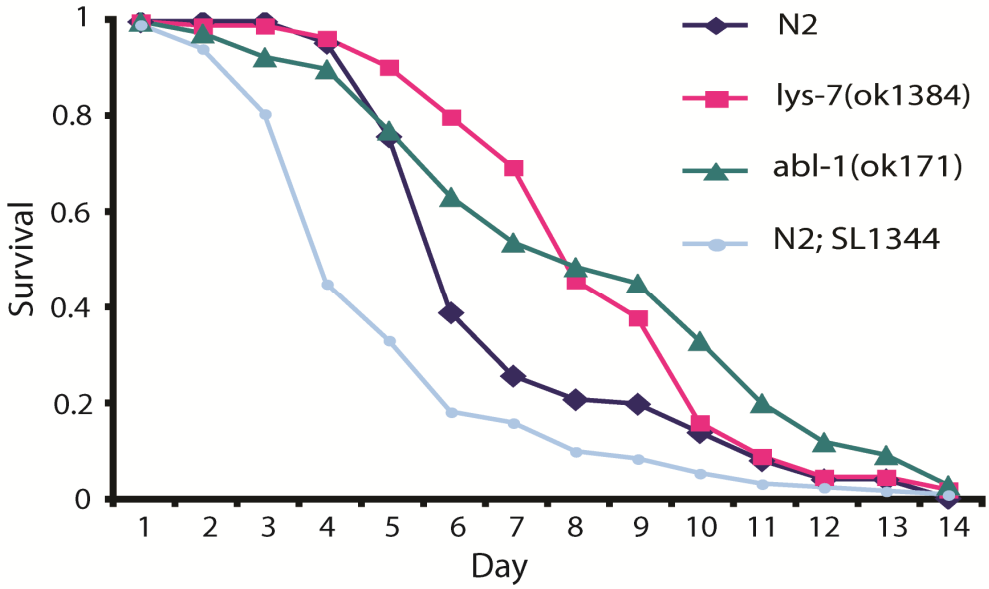

Figure 45: *C. elegans* survival on *S.* Typhimurium χ4996

Animals were infected with χ4996, a strain with an *rpoS* insertional inactivation mutation in an *S*. Typhimurium LT2 background, and monitored daily for survival. Both mutant strains were tolerant to the infection, p<0.05, in comparison to the wild type strain. Representative data for N2 survival on *S*. Typhimurium SL1344 is shown for comparison [N2 n=121; *lys-7(ok1384)* n=121; *abl-1(ok171)* n=122; N2 SL1344 as Figure 14].

To address this, the animals' survival was next assessed on strain χ4996 which has an *rpoS* insertional inactivation mutation in an LT2 background (*rpoS::Ap*^{r} from P22(JF2690) x LT2). In this case, the tolerance phenotype of the mutant strains returns (Figure 45) $p<0.05$, suggesting that the sigma factor RpoS does indeed influence *C. elegans* survival to *S*. Typhimurium. However, the increased survival of the animals to this strain is not as marked as that observed against SL1344; this may be due to the attenuated survival of the wild type animals, N2, which have an TD_{50} of 6 days upon χ4996 infection versus 4 days upon SL1344 infection.

Together, these data suggest that the most plausible hypothesis is that balanced immunity is dependent upon the level of RpoS expression in Salmonella.

5.3 Conclusion

The observation that the augmented survival of the mutant animals is present in SL1344 and 14028s infection but absent upon LT2 infection reveals that the heightened tolerance of these animals to *S*. Typhimurium infection is not dependent upon a strain effect as both SL1344 and 14028s represent the paradigm of Salmonella infection; instead, the suppression of tolerance in LT2 must be specific to this isolate. Based on previous data demonstrating the importance of RpoS to mouse (Swords et al., 1997b, Fang et al., 1992) and *C. elegans* (Labrousse et al., 2000) virulence, and as the level of RpoS is reduced in LT2 (WilmesRiesenberg et al., 1997), we tested the involvement of RpoS in the *C. elegans* balanced immunity phenotype.

RpoS is a principal subunit of RNA polymerase, which is believed to be a 'master regulator' of bacterial responses to a number of stress conditions (Hengge-Aronis, 2002). It is particularly important in the stationary growth phase response, but is also required in the early stages of a systemic *S*.

Typhimurium infection (Nickerson and Curtiss, 1997). In mammalian infections, RpoS mutations limit intracellular proliferation of the bacteria (Cano et al., 2001) and RpoS activity is important for inducing the expression of a number of virulence factors, including the *spv* virulence plasmid (Fang et al., 1992, Norel et al., 1992, Guiney et al., 1995, Gulig et al., 1993, Heiskanen et al., 1994) that is crucial for *S*. Typhimurium virulence (Gulig et al., 1992, Gulig and Doyle, 1993, Williamson et al., 1988b, Williamson et al., 1988a).

Indeed, RpoS does appear to be important in Salmonella tolerance by *C. elegans*. In LT2 isolates, the level of RpoS, and thus murine virulence, is compromised by the inefficiency of its unusual UUG start codon (Lee et al., 1995, Swords et al., 1997a, WilmesRiesenberg et al., 1997). This mutation appears to be enough to suppress the extended survival of the *C. elegans* mutants when infected with this strain, given that the expression of $rpoS^{SL1344}$ in LT2 is enough to recover the tolerance phenotype. In this way, our data from *C. elegans* reflect behavioural observations of *S*. Typhimurium isolates in other models, including mammalian cells and hosts.

Moreover, from our data it is possible to assemble the survival phenotype of the *lys-7(ok1384)* and *abl-1(ok171)* animals into three groups, which are dependent upon the condition of the Salmonella *rpoS* gene:

1. Wild type RpoS activity (strains SL1344, 14028s and χ8000); *lys-7* and *abl-1* mutant animals have increased tolerance to the infection
2. No RpoS activity (strains JF2691 and χ4996); the mutant animals have increased tolerance to the infection
3. A low level of RpoS activity (strain LT2); the mutant animals are sensitive to the infection.

Interestingly, *rpoS* insertional inactivation mutations in both SL1344 and LT2 do not suppress the tolerance of the mutants. It is now well established that a total absence of RpoS expression in *S*. Typhimurium results in avirulence (Fang et al., 1992, Norel et al., 1992). However, RpoS expression that is too high and/or inappropriate can also lead to avirulence (Swords et al., 1997a). Thus it appears to be the intermediate level of RpoS expression that is crucial for the tolerance phenotype in *C. elegans*. Expression levels of the *spvABCD* locus are reduced to an intermediate level (i.e. higher than *rpoS::Ap*^{r}, but lower than wild type) in LT2 (Chen et al., 1995, Obyrne and Dorman, 1994, WilmesRiesenberg et al., 1997). Potentially the intermediate expression of one of these genes mediates *S*. Typhimurium tolerance in *C. elegans*; in *rpoS* insertional inactivation mutant strains it is not expressed, in wild type strains it is expressed too highly; thus the optimal level of expression for *C. elegans* survival is in an LT2 background.

In order to test this hypothesis, it would be interesting to examine the *C. elegans* survival phenotype upon infection with an *rpoS* mutant in SL1344, whereby the single point mutation in the start codon of LT2 (Lee et al., 1995) has been recapitulated in this wild type background by site-directed mutagenesis. An alternative approach would be to make use of an inducible promoter on *rpoS* in order to titrate the level of induction and assess the parameters that are required for balanced immunity. Work is on-going in the laboratory to address these essential experiments.

6.0 RECOMBINANT LYSOZYME

6.1 Introduction

In chapter three we established that the loss of the antimicrobial protein LYS-7 in *C. elegans* renders animals highly vulnerable to a fungal infection from *C. neoformans*, yet this mutation simultaneously confers resistance to another pathogen, the bacterium *S.* Typhimurium. We considered the mechanism of action of LYS-7, where we assume that the enzyme hydrolyses the β-1,4 linkages of N-acetylglucosamine present in both peptidoglycan and chitin (Jolles, 1996), and how this could contribute to the balanced immunity phenotype. However, very little is known about the activity of *C. elegans* antimicrobials secreted during the innate immune response and most descriptions are inferred from sequence data and through orthologous protein comparisons. Thus we sought to express LYS-7 *in vitro*, along with LYS-2 (another clade one lysozyme; see figure 4) and LYS-5 (a clade two lysozyme) as controls.

With the onset of whole genome sequencing during the 1990s, a new era of biology was born; that of functional genomics. The *C. elegans* genome was the first multicellular genome to be sequenced (*C. elegans* Sequencing Consortium, 1998) and it subsequently spawned the *C. elegans* ORFeome project; an attempt to clone and characterise each predicted ORF from the *C. elegans* sequence (Walhout et al., 2000). To facilitate such an enormous task, the team utilised a novel cloning strategy called Gateway recombinational cloning (Walhout et al., 2000, Hartley et al., 2000), now available commercially from Invitrogen (Figure 46). This method enables a gene to be cloned and easily transferred into a number of expression vectors by site-specific recombination based on the integration and excision of the lambda bacteriophage into, and out of, the *E. coli* genome (Hartley et al., 2000).

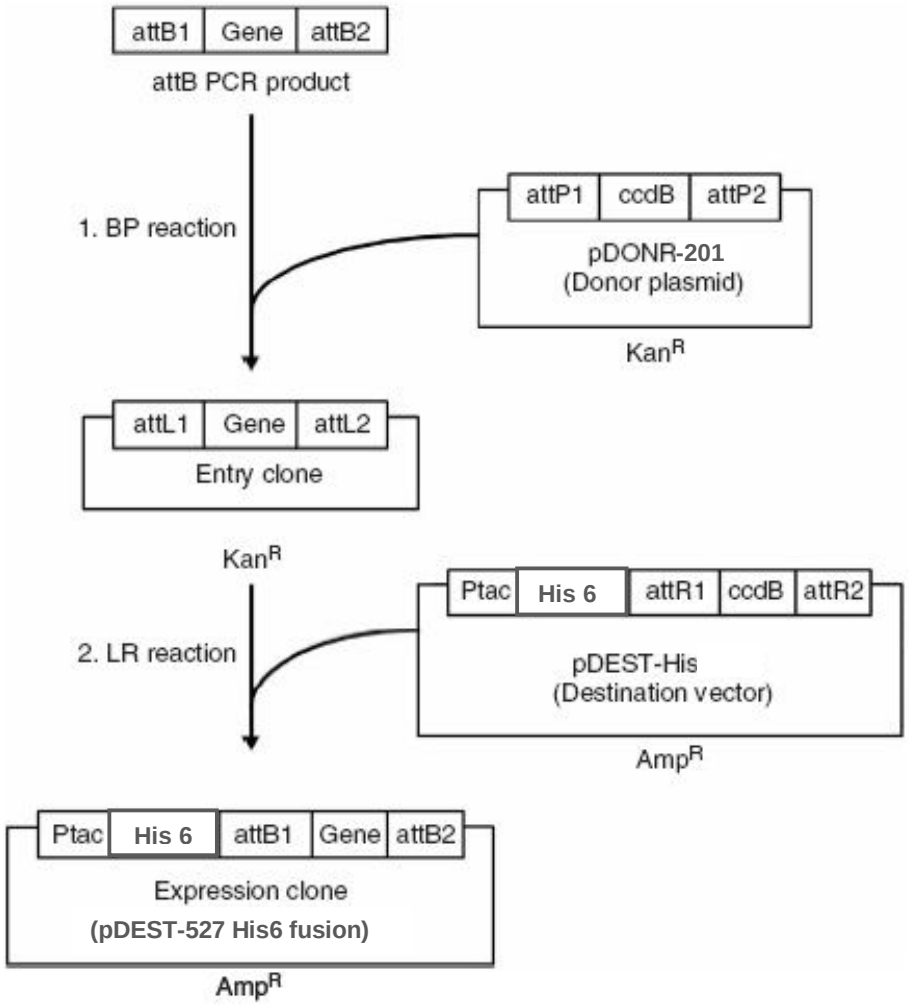

Figure 46: The Invitrogen Gateway Cloning System

In the BP reaction, the PCR product is recombined with the donor vector, pDONR-201, to produce an entry clone. At this point, the entry clone intermediate can be isolated from DH5α cells in order to facilitate the recombination of this clone into a number of different expression vectors. In our protocol, however, the LR reaction was performed immediately after the BP reaction, giving rise to an N-terminal His-tagged ORF in pDEST-527.

Image adapted from (Nallamsetty and Waugh, 2007)

We sought to exploit the Vidal ORFeome and Gateway technology in order to express *C. elegans* recombinant lysozyme, as the method is specific, simple and amenable to multiple expression hosts. It is important to note that the *att*B-tailed primer design of the ORFeome eliminates all sequence motifs between *att* sites, preventing any internal translation initiation and enabling entry vectors to be cloned into both N- and C-terminal fusion expression vectors (Walhout et al., 2000), thus, should expression in *E. coli* fail, the system could be adapted to an alternative host such as baculovirus. However, it should be recorded that the Vidal group have thus far failed to produce a LYS-7 Gateway clone, although LYS-2 and LYS-5 are successfully part of the ORFeome v3.1 (http://worfdb.dfci.harvard.edu/).

6.2 Results

6.2.1 *In vitro* expression of *C. elegans* genes *lys-2*, *lys-5* and *lys-7* in *E. coli*

In order to generate recombinant lysozyme proteins we generated clones for expression in *E. coli*, as it is a fast, relatively inexpensive and scalable system. Firstly, the three lysozyme genes were successfully amplified from *C. elegans* N2 cDNA with the *C. elegans* ORFeome primers (Table 5). These *att*B-flanked PCR products were cloned into the destination vector pDEST-527 (Figure 47) using Gateway technology (Invitrogen) in order to generate His-tagged recombinant protein. From here, the vectors were transformed into a number of *E. coli* expression hosts and were induced for expression at a range of temperatures (16°C, 25°C, 30°C and 37°C) and times (3h, 6h, 8h and overnight; data not shown).

These preliminary investigations revealed the best expression to occur with BL21-AI One Shot Chemically Competent *E. coli* (Invitrogen), a host designed specifically for the expression of toxic genes, such as lysozyme, due to the tight regulation of the T7 RNA polymerase; in BL21-AI cells, the T7 RNAP is under the control of the *araBAD* promoter and is thus inducible by L-arabinose.

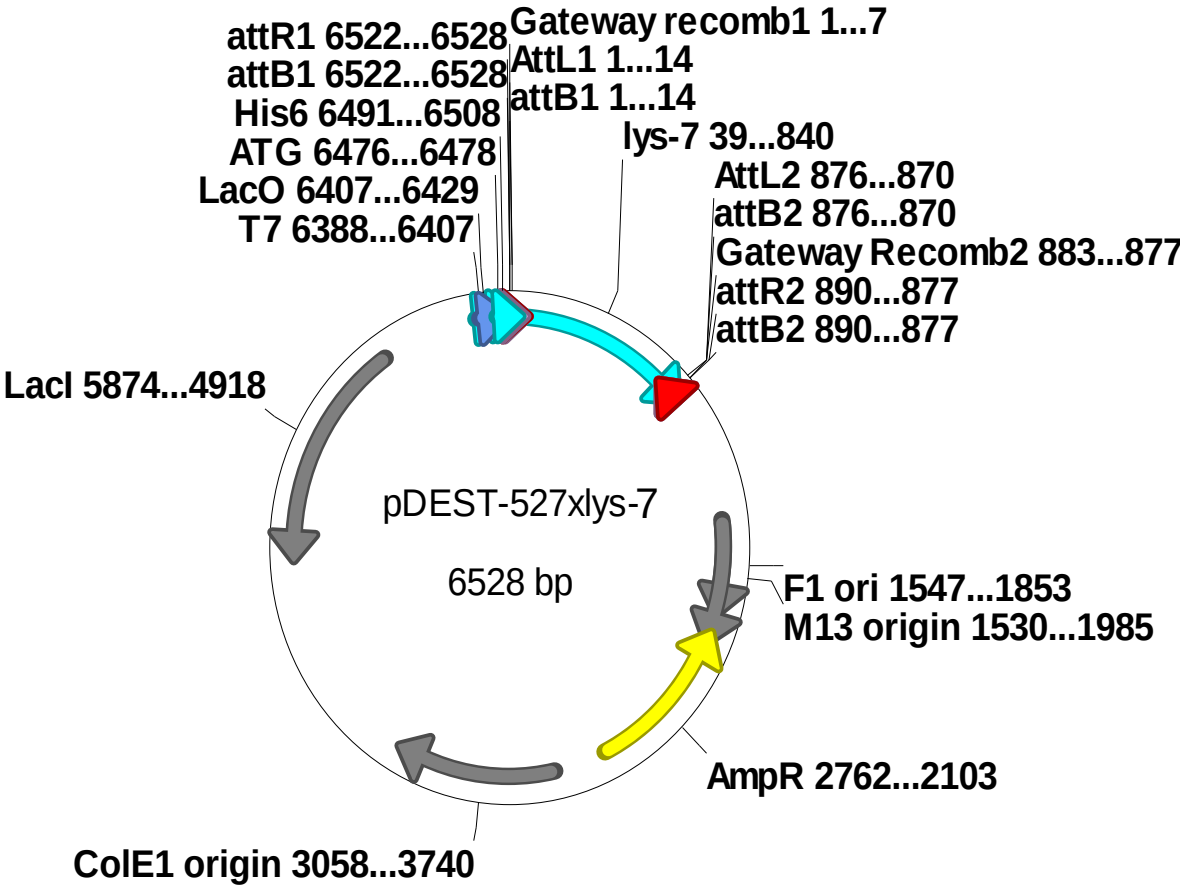

Figure 47: pDEST-527x*lys-7* plasmid map

The *lys-7* ORF was recombined into pDEST-527 using the Gateway site-specific recombination system (Invitrogen).

Plasmid map created in ApE v1.17.
The *lys-7* sequence is taken from WormBase and the pDEST-527 sequence from AddGene Inc.

SDS-PAGE and immunoblotting analysis demonstrated that small-scale pilot inductions of LYS-2, LYS-5 and LYS-7 in BL21-AI cells could produce polyhistidine-tagged proteins for all three cDNAs using this system (Figure 48), offering the potential for large-scale experiments and subsequent protein purification.

6.3 Conclusion

We have been able to express three *C. elegans* lysozymes in *E. coli*, indicating that the Gateway and BL21-AI expression system we describe here is a reliable and successful method. Unfortunately, due to limitations of time, we have been unable to develop this part of the project much further. The next stage will be to induce large-scale preparations to increase protein expression, before target protein isolation and subsequent purification with a nickel-affinity column. Provisional experiments have shown that overnight inductions at 16°C is a regime that produces consistent expression with minimal background, and that the proteins are amenable to isolation by sonication (W. Grey, unpublished).

Once we have achieved pure protein, it would be very interesting to assess the *in vitro* hydrolysis activity of the three proteins against a benchmark of hen egg-white lysozyme (using a standard *Micrococcus lysodeiltikus* lysis assay) (Shugar, 1952). Having assayed the standard activity, we shall look to test the lysozyme and chitinase activity of the enzymes against a range of bacteria and fungi, including *S*. Typhimurium and *C. neoformans*.

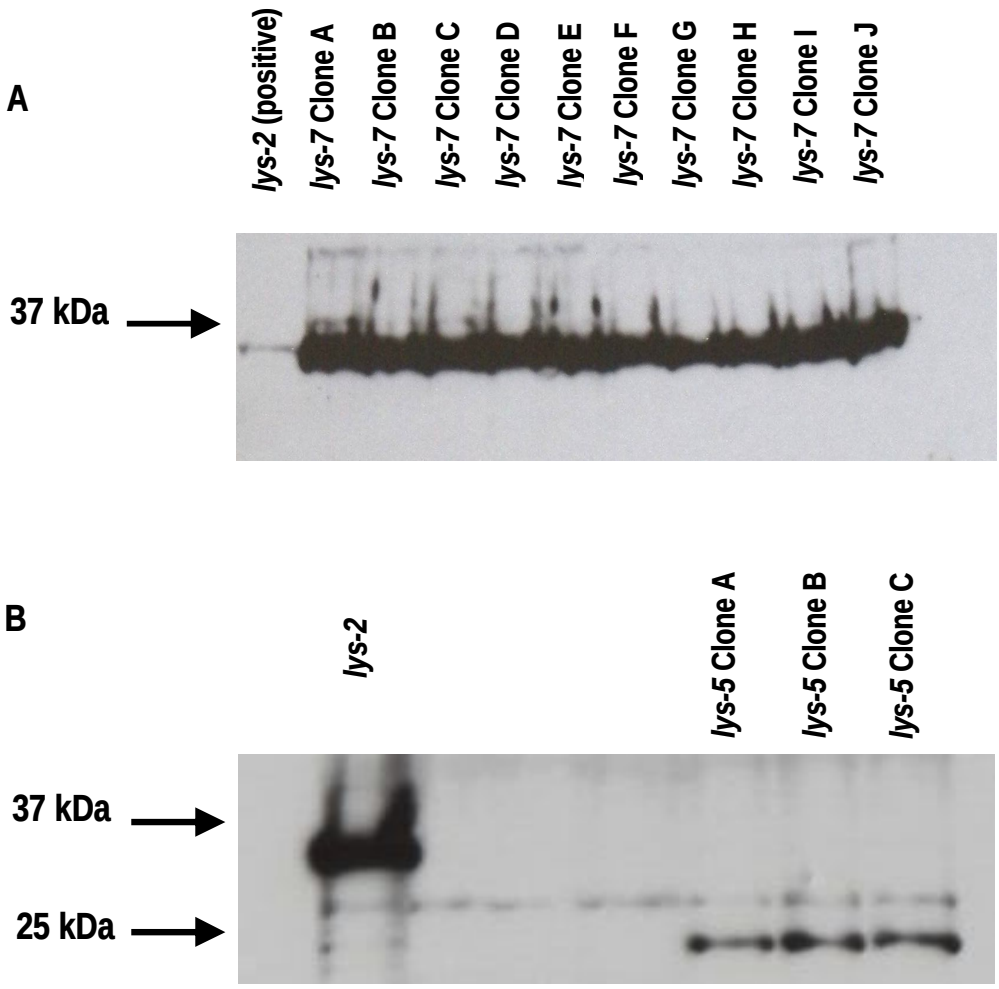

Figure 48: Expression of recombinant lysozymes *lys-2, lys-5* and *lys-7* in BL21-AI cells

C. elegans lysozymes *lys-2*, *lys-5* and *lys-7* were cloned into pDEST-527 vectors using the Gateway (Invitrogen) system and transformed into BL21-AI cells. 5ml log-phase cells were induced for 3 hours at 37°C to test protein induction. Cells were lysed in LDS buffer and clones analysed using SDS-PAGE and immunoblotting with an anti-His antibody. Expression of all three lysozymes was achievable using the BL21-AI system; blot A for *lys-7* and blot B for *lys-2* and *lys-5*.

The sequence alignment between LYS-7 and five other lysozyme-like proteins (Figure 52) shows that LYS-7 and LYS-2 have no DXE motif, whereas the sequences of the other enzymes all do. DXE is most likely one of the essential catalytic components of the enzyme (Korczynska et al., 2010) and the LYS-7 residues at this point, Q150 X151 T152, will not compensate for the nonexistence of DXE (A. L. Lovering, personal communication). What then, is the significance of this absence of the DXE motif in LYS-7? Interestingly, the D59 catalytic residue is conserved in LYS-7; together, these suggest that LYS-7 may be an atypical lysozyme (A. L. Lovering, personal communication). It is essential, therefore, to produce a high-resolution structure of LYS-7, as this is likely to indicate a mode of action for the protein due to the absence of DXE. The predicted structure (Figure 51) identifies a *Chalaropis*-type lysozyme fold, which enables these enzymes to hydrolyse β-1,4 N-, 6 O-diacetylmuramidase such as those residues present in the *S. aureus* cell wall, in addition to their classical action on β-1,4 N-acetylglucosamine linkages (Rau et al., 2001).

However, there are some problems with the *lys-7* construct as it currently stands. Firstly, the bioinformatic software WoLF PSORT (http://psort.nibb.ac.jp/) predicts that LYS-7 is a secreted protein. This makes sense given the predicted antimicrobial function of this protein acting in the intestinal lumen of *C. elegans*. Moreover, the conserved cysteine residues in the sequence alignment imply that disulphide bonds are utilised to stabilise the secreted protein (A. L. Lovering, personal communication) and, additionally, there is the precedent that most lysozymes are secreted (Jolles, 1996, Nickel et al., 1998). Consistent with this hypothesis, the sequence alignment suggests that the first 50 residues of LYS-7 are a putative signal and pro-peptide sequence. Currently, the signal sequence is presumably retained in the recombinant protein, which may disturb the folding of the protein and its subsequent activity; the cloning and crystallisation attempt need only be of the mature, isolated protein. Second, we had omitted the introduction of a cleavage site between the *lys-7* ORF and the *att*B sites,

preventing the cleavage of the His-fusion protein to liberate the native target protein. Consequently work is on-going in the laboratory to re-clone and express the truncated protein, in order to alleviate both of these concerns.

7.0 DISCUSSION

Many studies have made use of the nematode *C. elegans* to study the molecular basis of host-pathogen interactions. Although the nematode immune system is much simpler than that of vertebrates, it is nonetheless clear that many mechanisms and pathways are conserved between phyla.

7.1 Balanced Immunity

In vertebrates the phenomenon of immunological trade-off, whereby resistance to one class of pathogens comes at the cost of increased susceptibility to others, has been well documented. For example, it is well established that the activity of one immune pathway can inhibit that of another, so as to ensure the correct regulation of the system overall (Kim et al., 2007). In humans, immunological balance is typified by the Th1/Th2 response, which influences predisposition to autoimmune disease (Nicholson and Kuchroo, 1996) and sensitivity to particular classes of pathogenic infection (Koguchi and Kawakami, 2002).

Recently, analogous balance phenotypes have been described in the fly model. For instance, a mutation in the melanisation cascade of *Drosophila* can increase both tolerance and resistance to some infections whilst simultaneously decreasing tolerance and resistance to other pathogens (Ayres and Schneider, 2008). Similarly, mutations in the tumour necrosis factor pathway (Schneider et al., 2007, Brandt et al., 2004), the Toll-Dorsal pathway (Gordon et al., 2008) or global changes in a series of immunity genes (Lazzaro et al., 2006), lead to increased susceptibility to some pathogens but improved immunity to others. However, as yet there are few reports of similar balance mechanisms in

other invertebrates, nor has the underlying mechanism for invertebrate immunological balance been fully elucidated.

In chapter three, we present data which demonstrate that innate immunity in *C. elegans* also involves immunological balance. This response is mediated by two genes, the lysozyme *lys-7* and the tyrosine kinase *abl-1,* which appear to be reciprocally regulated. We have shown that single knockouts of these genes initiate a specific susceptibility trade-off by rendering animals hypersensitive to infection with *C. neoformans*, but tolerant to *S*. Typhimurium. In addition, we show in chapter five that the tolerance towards *S*. Typhimurium depends on the function of the bacterial sigma factor RpoS. LYS-7 has been described as an essential antimicrobial molecule in the *C. elegans* immune response (Mallo et al., 2002, O'Rourke et al., 2006, Evans et al., 2008b, Schulenburg and Boehnisch, 2008) that is assumed to actively degrade invading pathogens (Kerry et al., 2006, Murphy et al., 2003). Previous work in our group had identified LYS-7 as an important anti-cryptococcal immune factor (R. C. May, unpublished), but its dual role as a susceptibility factor in *S*. Typhimurium pathogenesis was surprising. In contrast, the role of the *abl-1* in *C. elegans* immunity is still unclear.

In vertebrates, ABL1 is a recognized oncogene (Sirvent et al., 2008), but its only known involvement in infection is as a target for manipulation by invading pathogens (Backert et al., 2008). Its *C. elegans* homologue, *abl-1*, has a multifaceted role in germline apoptosis: ABL-1 specifically inhibits germline apoptosis in animals following ionizing radiation, but does not prevent apoptosis in response to other mutagens (Deng et al., 2004). Conversely, ABL-1 also acts as a pro-apoptotic factor upon the subjection of nematodes to abiotic stress (Salinas et al., 2006). Additionally, ABL-1 inhibits the engulfment of apoptotic cells by acting independently of the two described cellular engulfment pathways (Hurwitz et al., 2009). In the immune response, *abl-1* expression is down-regulated in the

animals' response to infection with the Gram-negative pathogen *Serratia marcescens* (Wong et al., 2007). Furthermore, *abl-1* is central to a successful infection of the animal with *S. flexneri* (Burton et al., 2006), although the mechanism that the authors propose for this involvement is unclear, given that these bacteria are not intracellular pathogens in the nematode. We recognise that our finding that *abl-1* mutants are resistant to killing by *S*. Typhimurium contrasts with this previous publication (Burton et al., 2006). Furthermore, there are significant discrepancies between the lifespans of the *C. elegans* strains reported in this work, and those determined by our laboratory and others (Mylonakis et al., 2002, Styer et al., 2005, Diard et al., 2007). There are at least two possible reasons for these differences; firstly problems with the strains used in this work, although we have confirmed the identity of our *C. elegans* lines by PCR and utilised a number of distinct bacterial reference strains. Next, we note that the Aballay group both maintain their *C. elegans* strains and run their survival assays on modified NGM; containing 0.35% peptone, instead of 0.25% (Burton et al., 2006). It is known that a richer growth medium elicits a more virulent pathogenesis of the nematode host by the infecting bacteria (Tan et al., 1999), yet as the survival of wild type animals on SL1344 is comparable in both our laboratories (TD_{50} of approximately 4 days) this media modification cannot account for the survival differences we observe. Thus the rationale for these discrepancies remains unclear.

7.2 The regulation of balanced immunity in *C. elegans*

Based on the expression profile of ABL-1 in adult nematodes we suggested in chapter three that the role of ABL-1 in balanced immunity is regulatory, through a signalling response. We find that *abl-1* mutant *C. elegans* strains are hypersusceptible to cryptococcal killing whilst showing up-regulation of *lys-7*, a gene that we demonstrate to be important for cryptococcal resistance. In this manner and given that *lys-7;abl-1* double mutant animals remain overly susceptible to *C. neoformans* whilst losing the tolerance phenotype to *S*. Typhimurium, the data suggest that the function of ABL-1 in the trade-off

is two-fold. Firstly, we find that ABL-1 regulation is mediated through a LYS-7-dependent pathway which must operate in conjunction with an, as yet uncharacterised secondary, LYS-7-independent pathway. We find that both of these pathways are required for wild type resistance to *C. neoformans*, but that the activation of either downstream cascade is required for the tolerance response to Salmonella.

Having described this susceptibility trade-off between *C. neoformans* and *S.* Typhimurium pathogenesis, we then provide data in chapter four that probe the downstream nature of the ABL-1-regulated pathways that mediate the immunological balance response upon the loss of *abl-1* or *lys-7*.

Firstly, our data rule out the involvement of the p38 MAPK pathway, as the loss of *sek-1* does not suppress the *abl-1* or *lys-7* phenotypes. Instead, the data are consistent with a role for the ABL-1-interacting protein, ABI-1, alongside ABL-1 in the regulation of the balance, since knockout animals for *abi-1* phenocopy the balanced immunity response of *lys-7* and *abl-1* knockout animals to *C. neoformans* and *S.* Typhimurium. ABI-1 can act both downstream and upstream of ABL-1 (Hurwitz et al., 2009, Dai and Pendergast, 1995, Yu et al., 2008b, Juang and Hoffmann, 1999, Tani et al., 2003, Lin et al., 2009), although based on our data we feel that the most likely state is that ABI-1 acts upstream of ABL-1 to influence a downstream cascade, and subsequently, LYS-7 activity.

We next examined the role of the two other major immunity pathways, TGF-β/DBL-1 and IGF/DAF-2, in the balance mechanism. We were able to produce *dbl-1;abl-1* double mutant animals which were found to be extremely sensitive to both *C. neoformans* and *S.* Typhimurium infection, suggesting a role for the TGF-β pathway in the balance. Furthermore, in the tolerance response to *S.* Typhimurium, we found both immunomodulatory effects of *lys-7* and *abl-1* to act through the forkhead transcription factor

DAF-16, as a mutation for *lys-7* or *abl-1* coupled with one for *daf-16*, suppresses the tolerance phenotype. The most likely explanation is that the loss of either *abl-1* or *lys-7* must activate DAF-16 in an attempt to compensate for the immunological impairment.

We believe that each of these pathway components is required for wild type sensitivity to *C. neoformans*, but not for wild type sensitivity to *S*. Typhimurium infection; in this latter case the pathways act to initiate the heightened tolerance response. This hypothesis is confirmed by our observations that the *daf-16* and *dbl-1* single mutants, as well as the *lys-7;abl-1* double mutants (where the compensatory mechanism is suppressed), have wild type resistance to *S*. Typhimurium infection, but are sensitive to *C. neoformans*. With a similar assumption, we believe that the *abl-1;dbl-1* hypersensitivity to both pathogens could be due to a number of pleiotropic effects, thus we interpret these results purely as a suppression of the *abl-1* tolerance phenotype to wild type survival levels.

Collectively, our data suggest a signalling cascade mediated by the ABL-1-dependent inhibition of TGF-β/DBL-1 (Naka et al., 2010), which results in the activation of DAF-16 by DBL-1, since TGF-β/DBL-1 is known to activate FOXO/DAF-16 in vertebrates (Lee et al., 2001, Seoane et al., 2004). Together this culminates in the induction of *lys-7* expression. The secondary ABL-1-dependent, LYS-7-independent, uncharacterised pathway must also be dependent upon DAF-16 and its subsequent activation of immune effectors (Figure 49). Interesting, *lys-7* itself is a known DAF-16 target (Yu et al., 2008a, McElwee et al., 2003, Murphy et al., 2003), thereby providing a potential feedback mechanism to regulate the activity of the pathways.

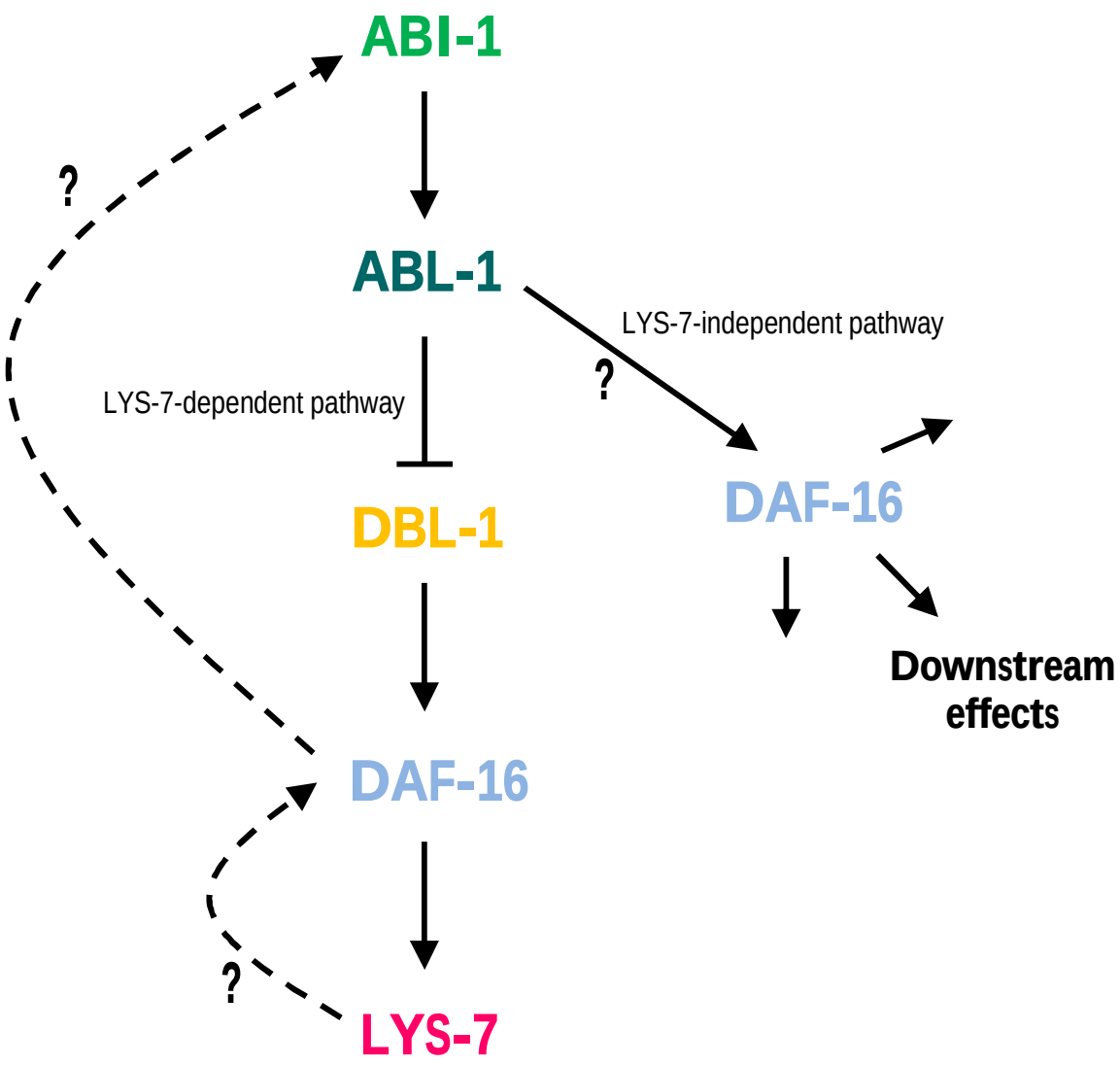

Figure 49: Proposed pathway regulating balanced immunity

We propose that ABL-1 inhibits DBL-1-mediated activation of DAF-16, having inferred this interaction from that of their mammalian homologues (Naka et al., 2010). Upon the loss of *abl-1*, the repression of DBL-1 is removed and the transcription factor drives the activation of *lys-7*, resulting in increased tolerance to *S.* Typhimurium infection. On the other hand, upon the loss of *lys-7*, there is a feedback mechanism that triggers an ABL-1-mediated response, which is also DAF-16-dependent, that ultimately leads to the reciprocal resistance phenotype through the activation of downstream targets. It is necessary for either one of these pathways to be activated through the loss of *lys-7*, *abl-1* or *abi-1* to confer tolerance to Salmonella. Conversely, during *C. neoformans* infection, both response pathways must be intact for wild type resistance to the fungus. The position of *abi-1* in this model remains to be confirmed, both by epistatic analysis and through the qRT-PCR analysis of downstream gene expression.

In this manner, our data suggest the modulation of the *C. elegans* immune response by *lys-7* and may, accordingly, introduce a novel function for this defensive enzyme. Currently, there is only one report of non-enzymatic activity of invertebrate lysozyme (Zavalova et al., 2006), but the immunomodulatory role of cationic antimicrobial compounds (CAMPs) has been previously described in vertebrate systems, although the mechanisms of this effect are not yet understood (Bowdish et al., 2005, Yu et al., 2007). In this context, we note that the considerable lysozyme repertoire in *C. elegans* (Schulenburg and Boehnisch, 2008, Ito et al., 1999) could be partly explained by their divergence into modulators of the immune response. Moreover, the sequence comparisons would support such a hypothesis, given that LYS-7 lacks the catalytic DXE motif and is therefore likely to have atypical lysozyme activity (Figure 52).

Previous work from our group has shown that DAF-16 activity does not modulate animal sensitivity to *S*. Typhimurium; this pathogen shows similar levels of lethality in both the hermaphroditic *Caenorhabditis* species and gonochorisitic species, which have higher levels of DAF-16 (Amrit et al., 2010). Furthermore, DAF-2 mutants also show the same patterns of susceptibility to *S*. Typhimurium as wild type animals (Amrit et al., 2010). Thus it would appear that DAF-16 is involved with regulating the immunological balance *per se*, consistent with the hypothesis that the IGF pathway does not confer pathogen-specific responses in *C. elegans* but is associated with the basal innate immune response as a whole (Troemel et al., 2006). The finding that *daf-2* mutants are not resistant to *S*. Typhimurium infection is surprising in this context, as *daf-2* knockout animals are known to up-regulate *lys-7* expression (Murphy et al., 2003). It would seem, therefore, that the balanced immunity phenotype is not dependent upon the up-regulation of a single component of the pathway, rather that it is reliant upon the trade-off mechanism as a whole. One way to test this would be to examine the survival of a *lys-7* over-expressing *C. elegans* strain, recently made in our laboratory (C. M. L. Boehnisch,

unpublished), to *S*. Typhimurium in order to establish whether the up-regulation of *lys-7* is enough to confer a tolerance phenotype.

Given time, there are a number of potential experiments that would help test the model outlined in Figure 49. For instance, it will be important to quantify the levels of *lys-7* expression in *abi-1* mutant worms by qRT-PCR, as our model currently suggests that ABI-1 regulates immune balance through the LYS-7-dependent pathway. If so, an *abi-1;lys-7* double mutant should phenocopy the survival of *abl-1;lys-7* double mutants. Furthermore, we were unable to test a *dbl-1;lys-7* double mutant due to the close proximity of these two genes on chromosome V, but a double knockdown should be achievable using a single knockout worm coupled with RNAi to suppress the expression of the second gene. If DBL-1 does indeed regulate the DAF-16-mediated activation of LYS-7, *dbl-1;lys-7* double mutants should be tolerant to *S*. Typhimurium through the compensatory activity of the secondary ABL-1-dependent pathway. Lastly, expression levels of *lys-7* should also be measured in the *abl-1;dbl-1* double knockouts to verify that *abl-1* negatively regulates the TGF-β pathway, as Figure 49 suggests.

One further thing to note here is an additional discrepancy between the data presented in this thesis and that published by the Aballay laboratory. We find that *dbl-1(nk3)* knockout animals are not more sensitive to *S*. Typhimurium infection than wild type animals and hence conclude that the TGF-β pathway is not involved in the *C. elegans* immune response to this pathogen in the wild type scenario. Conversely, the Aballay group have published that *dbl-1(nk3)* mutants are hypersensitive to a number of human pathogens, including *S*. Typhimurium (Tenor and Aballay, 2008). Again, having confirmed our animal and bacterial strains we conclude that the animals and bacterial strains must have undergone microevolution in both laboratories and that these changes could account for the survival differences we observe.

7.3 LYS-7-mediated toxicity in *S.* Typhimurium pathogenesis

In the balanced immunity phenotype, we describe a gain in tolerance of *lys-7(ok1384)* and *abl-1(ok171)* animals to *S*. Typhimurium infection, as these strains have a pathogen burden comparable to that of N2 worms. Hence there is no difference in resistance, since bacterial burden is the same, yet N2 animals succumb much faster to the infection. We hypothesise that the ABL-1-dependent compensatory signalling is the means by which the tolerance mechanism is regulated in the host, yet what is the physiological nature of the gain of tolerance in the knockout animals?

We postulated that immunopathology, a major cause of disease that significantly impacts the fitness of both the host and pathogen (Graham et al., 2005), may be the basis of the N2 sensitivity to *S*. Typhimurium infection and that the *lys-7* and *abl-1* mutant animals were better able to limit the damage caused by these bacteria during pathogenesis. We have already established that the role of LYS-7 in *C. neoformans* pathogenesis is protective, whereas in *S*. Typhimurium infection, LYS-7 acts as a susceptibility factor. We therefore hypothesise that there are deleterious consequences of the immune response elicited through the action of LYS-7 in defence against Salmonella infection, which together is actually detrimental to the host.

There are at least two ways in which LYS-7 could generate such a damaging pathology. For instance, LYS-7 may cleave a bacterial product from *S.* Typhimurium that is toxic to *C. elegans* or induces a harmful inflammatory response in a manner reminiscent of lipopolysaccharide (LPS)-mediated toxic shock in humans. Alternatively, the action of LYS-7 may induce a stress response in the bacterium that, in turn, renders the infection more virulent. In either scenario, a host that lacks LYS-7 does not experience this detrimental interaction and thus the animals are tolerant to *S*. Typhimurium-induced killing. We know that LYS-7 is strongly induced upon *S*. Typhimurium infection (Evans et al., 2008b),

but this delayed induction may be too late to elicit a positive immune response against the pathogen; instead, it is possible that the constitutively higher level of LYS-7 present in *abl-1* knockout animals allows them to overcome this negative interaction and limit Salmonella pathogenicity. We know that *abl-1(ok171)* mutant animals do not experience a bactericidal resistance effect from increased LYS-7 expression as its pathogen burden is unchanged from wild type of *lys-7(ok1384)* animals; rather this is a bacteriostatic response (Mine et al., 2004). Together, this adds evidence for an alternative function for LYS-7 in *C. elegans* immunity. In this respect, the dual nature of *lys-7* is analogous to that of the *Drosophila* tumor necrosis factor homolog *eiger*, which modulates the pathogenesis of *S*. Typhimurium and other intracellular pathogens in the fly (Brandt et al., 2004, Schneider et al., 2007).

Additionally, there is a precedent for such antimicrobial activity that is capable of promoting bacterial pathogenicity in the host. For instance, when *S*. Typhimurium is exposed to sub-lethal concentrations of cationic antimicrobial peptides (AMPs) *in vitro* using a mammalian tissue culture system (we suggest this is analogous to LYS-7 expression levels in N2 worms), the bacteria respond by globally changing their protein composition by primarily activating their PhoP/PhoQ two-component virulence regulon, rendering the bacteria more resistant to antimicrobial action through major changes in the bacterial cell surface (Bader et al., 2003). Indeed, these AMPs are able to directly activate the PhoP/PhoQ system and initiate the bacterial stress response (Bader et al., 2003, Kindrachuk et al., 2007). RpoS activity was additionally induced by the antimicrobial peptides, leading to the transcription of its downstream genes, including *katE*, which causes the bacteria to become more resistant to oxidative stress generated as a defence mechanism by the host (Bader et al., 2003). RpoS is itself stabilised by PhoP under low magnesium conditions in order to direct catalase activity (Tu et al., 2006), thus there has been some suggestion that this is also true during the AMP resistance response (Groisman and Mouslim, 2006). Moreover, resistance to antimicrobial peptides has been shown to significantly

contribute towards the persistence of *S*. Typhimurium in the *C. elegans* intestine (Alegado and Tan, 2008). It would be very interesting to investigate whether recombinant LYS-7 is also capable of activating the PhoP/PhoQ regulon to elicit a comparable response *in vitro*.

It is interesting to note that the sigma factor RpoS has a pivotal role in this antimicrobial resistance phenotype as our data further show that the *C. elegans* tolerance phenotype is dependent upon the level of RpoS activity of the *S*. Typhimurium strain in question. As *rpoS* is important for inducing the expression of a number of virulence factors in *S*. Typhimurium-mediated pathogenesis, it is inviting to postulate that RpoS may be responsible for modulating the cell wall structure and hence the expression of our proposed structural motif with which LYS-7 interacts to produce the lethal phenotype. As suggested above, perhaps the cleavage of this product by LYS-7 releases a toxic compound or activates a stress response from the bacteria. Either way, it would appear that the mis-regulation, and thus the intermediate level of this product as found in an LT2 background, is a contributing factor to the tolerance phenotype (Figure 50). However, the RpoS-dependency of the balanced immunity phenotype must still be held in concert with the immunity status of the host. Such that in strains where there is no up-regulation of *lys-7* (for instance in *lys-7;daf-16* double mutants) the animals are still not tolerant to the *S*. Typhimurium infection; in these animals there can be no activation of the secondary LYS-7-independent pathway to compensate for the loss of *lys-7*.

An outstanding question from the data is the nature of the catalytic mechanism of LYS-7. As a lysozyme, albeit an unconventional one, LYS-7 is predicted to have 1,4 β-N-acetylmuraminidase activity (Figure 51). The main target of this hydrolysis action is the peptidoglycan structure of the colonising pathogen, notably of Gram-positive bacteria, although lysozymes are also known to have antimicrobial activity against Gram-negative bacteria (Mine et al., 2004). However, we have already

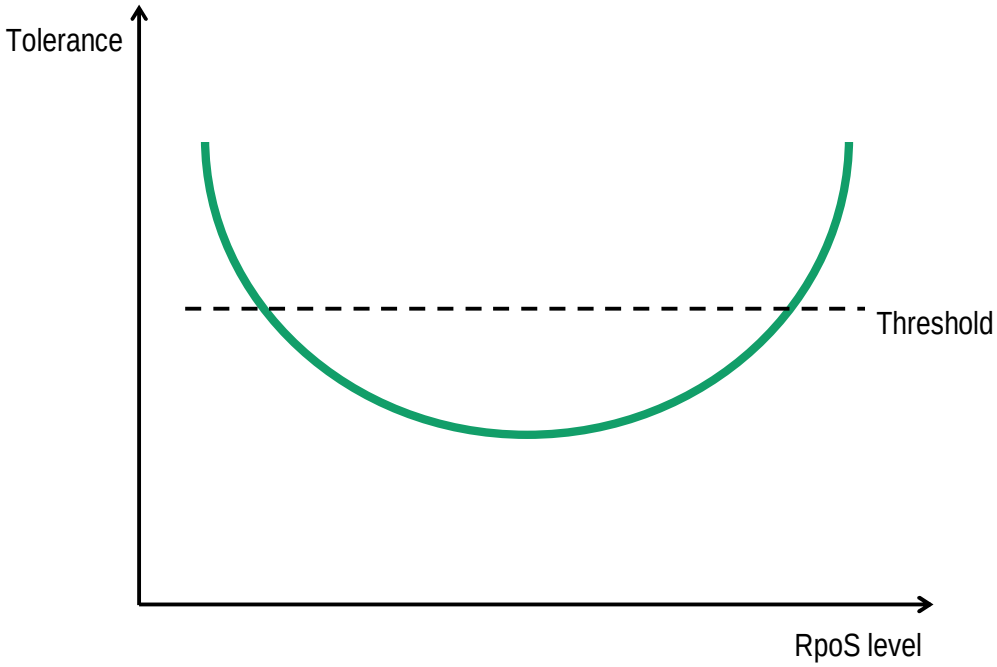

Figure 50: RpoS levels mediate tolerance in *S.* Typhimurium pathogenesis of *C. elegans*

Low and high levels of RpoS such as those found in knockdown or wild type *S.* Typhimurium isolates respectively, facilitate the tolerance phenotype of the *lys-7* and *abl-1* knockout strains. However, in *S.* Typhimurium isolates with intermediate levels of *rpoS* expression, such as LT2, the tolerance phenotype is not induced.

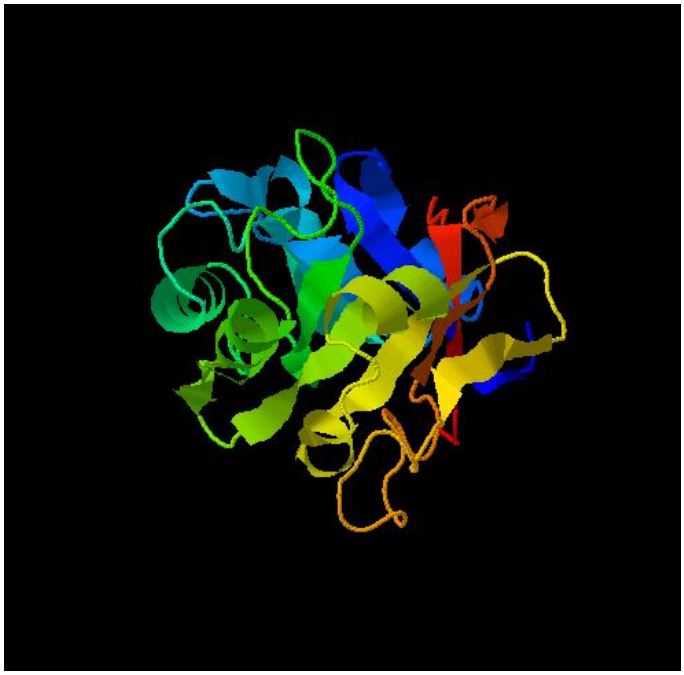

Figure 51: Predicted structure of LYS-7

Threading and homology modelling was used to produce a predicted structure of the LYS-7 protein. This structure has an E number of $6.5e^{-24}$ and a 100% estimated precision. The structure predicts the protein to be a member of the transglyscosidase superfamily with 1,4 β-N-acetylmuraminidase activity. The *Chalaropsis* lysozymes, of which LYS-7 is a member, display a specialist Ch-type fold; a modified α/β-barrel-like fold in which an eight-stranded β-barrel is flanked by just five α-helices.

Structure produced using the Phyre program from Imperial College London, http://www.sbg.bio.ic.ac.uk/phyre/

alluded to a crucial mutation in the principal DXE motif in the LYS-7 sequence (Figure 52) which is likely to substantially alter the catalytic mechanism of LYS-7 (A. L. Lovering, personal communication).

This motif is critical to the two-step hydrolysis reaction of the GH25/*Chalaropsis* family lysozymes of which LYS-7 is a member (Figure 51) (Rau et al., 2001, Nickel et al., 1998, Martinez-Fleites et al., 2009, Korczynska et al., 2010), whereby the first reaction consists of the DXE residue attacking the peptidoglycan sequence to generate a 1,2 cyclic intermediate, followed by the secondary stabilisation of this structure by hydrolysis, which is conducted by the conserved Asp; residue D59 in LYS-7 (Figure 52) (Martinez-Fleites et al., 2009). As LYS-7 does not appear to have a conserved DXE motif, it cannot achieve the initial step in this reaction and must therefore rely on the generation of a cyclic intermediate by an alternative lysozyme.

An alternative hypothesis is that LYS-7 may act on a peptide such as tracheal cytotoxin (TCT), which, interestingly, is naturally cyclic (Luker et al., 1993). TCT is a 1,6 anhydro-cyclic muramic peptide; a native peptidoglycan fragment from Gram-negative bacteria that acts concurrently as a virulence factor of animal hosts, an immune response elicitor and, further, is associated with the pathogenesis of *Bordetella pertussis* (whooping cough) (Luker et al., 1993, Stenbak et al., 2004, Chang et al., 2006, Gentryweeks et al., 1988), amongst other infections. This anhydro form of the monomer is crucial for achieving maximum immune-stimulatory activity (Stenbak et al., 2004, Chang et al., 2006). However, as TCT is naturally cyclic, an enzyme with only one catalytic residue (such as LYS-7) would be able to reduce the 1,6 anhydro monomer. If TCT is the moiety involved in the toxicity response to *C. elegans* during *S*. Typhimurium infection, we postulate that it is the LYS-7-treated, non-cyclic form of the peptide which elicits the toxic response in the host, a hypothesis which is consistent with the finding in

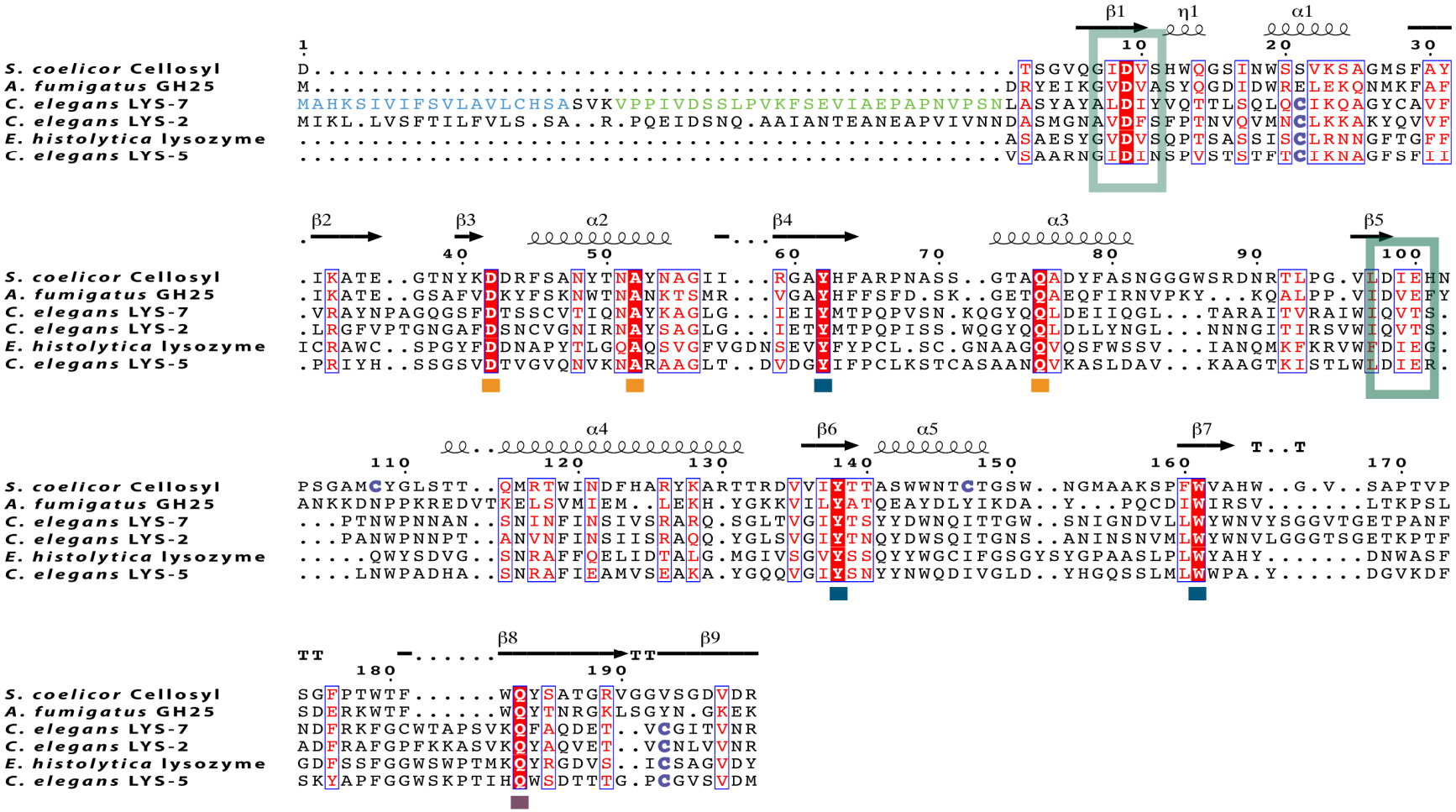

Figure 52: Sequence alignment of LYS-7 with 5 other related lysozymes

A clustalw file was generated using UNIPROT sequences, aligned with T-COFFEE and analysed with ESPript. The signal sequence for LYS-7 is shown in blue, with the pro-peptide in green before the mature enzyme starts at LASY. The catalytic residues are highlighted within green boxes: firstly the conserved Asp at residue 9, and secondly the DXE motif at position 98-100. Surprisingly, this residue is missing in LYS-7 and LYS-2. Other conserved residues are structural (orange blocks), presumed substrate binding (blue blocks) and involved in the orientation of the substrate and catalytic residues (maroon block). Conserved Cys residues for disulphide bonds are highlighted in purple.

B. pertussis that the dehydration of TCT at carbons 1 and 6 is not important in toxicity (Luker et al., 1993).

In combination with the additional experiments I have suggested in chapter six, the *in vitro* testing of recombinant LYS-7 with mutations at conserved residues such as D59 and the crystal structure of the wild type protein, complexed with its natural substrate, will reveal the mechanism of action of LYS-7 and hence help to solve the complex enigma of its function in the balanced immune response, and in *C. elegans* immunity in general.

7.4 Conclusion

In this thesis we describe a complex balance of immunity in *C. elegans,* mediated by a trade-off in the activity of ABL-1 and LYS-7 during the immune response to two human pathogens, *C. neoformans* and *S*. Typhimurium. We find that there is a multifaceted role for ABL-1 in controlling the balance through two DAF-16-dependent pathways. LYS-7, too, appears to directly modulate the host response to these pathogens: firstly, as a factor offering protection to the host in the case of *C. neoformans* infection, whilst concurrently acting as a susceptibility factor during *S*. Typhimurium pathogenesis. In this latter role, LYS-7 most probably acts through the generation of a toxic peptidoglycan intermediate. Furthermore, we find that the tolerance phenotype of the *lys-7* and *abl-1* knockout worms is *S*. Typhimurium-dependent; given that this dependency corresponds to the activity of RpoS in the bacteria. We therefore suggest that the pathogen-basis of the elicitor-based mechanism is under the control of RpoS.

In conclusion we find that, as in vertebrates, a complex, reciprocally-regulated network of genes from both the host and pathogen, are involved in maintaining balanced immunity in *C. elegans*. These data

suggest that immunological balance is an evolutionarily ancient phenomenon; therefore a more detailed investigation of this phenomenon in invertebrates may have significant implications for our understanding of orthologous mechanisms in vertebrates.

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APPENDIX

1.0 Microarray analysis

The following work formed part of my undergraduate dissertation.

Briefly, RNA was isolated (as described) and converted into cDNA probes. These were indirectly labelled using a CyScribe post-labelling kits (Amersham GE Healthcare) according to the manufacturer's instructions. However, there were some slight modifications to the protocol as follows: at each elution step, 65µl elution buffer was used (instead of 60µl), in addition the labelling of amino allyl-modified cDNA with CyDYE reaction was carried out overnight. Long oligomer-based spotted microarrays for the *C. elegans* genome were obtained from the Genome Sequencing Centre. The pre-soak and pre-hybridisation protocol was followed. The probes were mixed; Control N2 (labelled with Cy3) with *lys-7(ok1384)* (labelled with Cy5) and N2 with *lys-7(ok1385)* (labelled with Cy5). 80pmol of each labelled cDNA was required to run the array; this determined the number of µl of each labelled probe used. The hybridisation procedure for Eukaryotic DNA was followed. The following day the slides were washed using the AdvaWash (Implen) system according to the standard operating procedure. The wash buffers used were 1: 2xSSC 0.1% SDS; 2: 0.1xSSC 0.1% SDS; 3: 0.1xSSC. The washed slides were dried by centrifuging for 10 min at 1,500g. The slides were scanned at the Functional Genomics and Proteomics Unit at the University of Birmingham, on the Axon 4000 Scanner according to the manufacturer's instructions. Each microarray was lined up with the *C. elegans* gal. file (from the *C. elegans* genome sequencing centre) and stored. Throughout the protocol, RNA and cDNA quality and yield was confirmed by gel electrophoresis and OD260/280 analysis. Once the microarray results were obtained, the data were normalized using the DNMAD program found on the GEPAS website (http://www.gepas.org) and used for subsequent analysis. Genes of significant

change were determined – defined by an expression change of 1.5 fold. Only those genes consistently up- or down-regulated in both *lys-7* mutants versus N2 were identified. The WormBase website (http://www.wormbase.org/) was used to annotate the genes of interest. Additionally, the Babelomics website tool (http://www.babelomics.org) was used to examine gene ontology.

1.1 Microarray data

1.1.2 4 genes were consistently up-regulated across both arrays:

Sequence ID	Gene name	Average fold induction	Description
M79.1c	*abl-1*	2.65 ± 0.94	Src Homology, regulator of apoptosis
W06D12.3	*fat-5*	1.82 ± 0.15	Fatty acid desaturase, predicted to be mitochondrial
C01F4.1	*rga-6*	1.93 ± 0.08	Rho-GTPase activating protein
ZK666.6	*clec-60*	1.59 ± 0.089	C-type lectin domain, required for resistance to *M. nematophilum*

1.1.3 43 genes were consistently down-regulated across both arrays:

Sequence ID	Gene name	Average fold reduction	(Predicted) Function
Y48E1B.10	*gst-20*	-1.75 ± 0.3	Glutathione S-transferase
F28H7.8		-2.16 ± 0.14	Phosphatidylinositol transfer protein
Y48E1B.8		-2.18 ± 0.62	Not characterised
Y19D10A.9	*clec-209*	-2.38 ± 0.92	C-type lectin domain
ZK1025.6	*nhr-244*	-2.21 ± 0.09	Nuclear hormone receptor family
F57F4.3	*gfi-1*	-5.63 ± 0.5	Contains 21 ET modules
K10C2.3		-2.40 ± 0.72	Aspartyl protease
EGAP7.1	*dpy-3*	-2.88 ± 1.56	Cuticular collagen
Y45G12C.3		-2.05 ± 0.75	Not characterised
R11A5.3		-1.74 ± 0.17	Not characterised
C14F5.2	*zig-3*	-2.37 ± 0.16	Immunoglobulin superfamily
Y116A8C.5		-1.98 ± 0.17	Neprilysin
ZK470.1		-1.93 ± 0.25	Peroxisomal membrane protein
F43D9.1		-3.07 ± 0.82	Predicted membrane protein
C23H5.8a		-1.95 ± 0.41	Not characterised
F41A4.1	*cutl-28*	-3.71 ± 1.02	Not characterised
F59F3.6		-1.93 ± 0.55	K^+ ion transport domain
F53C3.8		-2.12 ± 0.82	Not characterised
D1053.1	*gst-42*	-1.66 ± 0.2	Glutathione S-transferase
Y4C6B.6		-1.80 ± 0.25	Beta-glucocerebrosidase
F52B11.3	*noah-2*	-2.58 ± 0.74	PAN and ZP domain-containing
M03A1.3		-2.67 ± 1.08	~10 predicted transmembrane sequences
K10H10.2		-1.95 ± 0.03	Cystathionine beta-synthase
C44H4.3	*sym-1*	-2.43 ± 0.79	Leucine rich repeats
B0218.8	*clec-52*	-2.07 ± 0.63	C-type lectin domain
F15D4.5		-2.61 ± 0.29	Not characterised
F08F3.9		-1.82 ± 0.01	snRNA activating complex
C32H11.12	*dod-24*	-2.70 ± 0.57	Acts downstream of Daf-16
F22E5.1		-2.36 ± 1.14	Not characterised
C38D9.2		-3.82 ± 0.43	Not characterised
W01F3.3	*mlt-11*	-2.54 ± 0.99	Molting defective, serine protease inhibitor
F41E7.4	*fip-5*	-1.85 ± 0.16	Fungus-induced protein
Y46H3A.2	*hsp-16.41*	-2.01 ± 0.17	16-kD heat shock protein, expressed broadly
D1086.3		-2.69 ± 0.22	Not characterised
F16F9.2	*dpy-6*	-2.00 ± 0.25	Novel, conserved protein, calcium binding
K02E10.4		-1.71 ± 0.16	Not characterised
Y46H3A.3	*hsp-16.2*	-2.09 ± 0.1	Heat shock protein
ZK829.3		-1.87 ± 0.51	Not characterised
F46F2.3		-2.09 ± 0.05	Not characterised
C37E2.2		-1.57 ± 0.01	Beta-2-glycoprotein
C34G6.6a	*noah-1*	-2.25 ± 1.01	PAN and ZP domain-containing
T14B4.6	*dpy-2*	-2.51 ± 1.26	Unusual cuticular collagen
T08G5.10	*mtl-2*	-1.59 ± 0.11	Metallothionein