

**The genetic architecture of immune defence in the
nematode *Caenorhabditis elegans***

Dissertation

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1. Thesis content

I The mysterious role of the *C. elegans* nuclear hormone receptor NPR-1 in pathogen defence

R. Nakad, B. Snoek, S. Ellendt, F. Schneider, T. Mohr, L. Rösingh, A. Masche, K. Dierking, JE. Kammenga, H. Schulenburg

II Trans-generational immune priming of pathogen avoidance behaviour in the nematode *C. elegans*

R. Nakad, F. Schneider, H. Schulenburg

III Activation of the *Caenorhabditis elegans* FOXO family transcription factor DAF-16 by pathogenic *Bacillus thuringiensis*

J. Wang, R. Nakad, H. Schulenburg

2. Authors contribution

R. Nakad: development of original ideas, establishment of the study, the general data interpretation (**I and II**), optimization of experimental protocols and development of new phenotypic measurements (**I and II**), performance of experiments (**I, II, III**), supervision of experiments (**I and II**), data analysis (**I and II**), and preparation of chapters for publications (**I and II**)

H Schulenburg: original ideas, supervision of experiments, project discussion, data analysis and interpretation, and preparation of chapters for publication (**all chapters**).

B. Snoek: contribution to development of original ideas, data analysis and interpretation (QTL and epistatic analysis), project discussion (**I**)

JE. Kammnaga: contribution to development of original ideas, data analysis and interpretation (QTL and epistatic analysis), project discussion (**I**)

S. Ellendt: performance of experiments (survival assays), data analysis and development of the experimental set-up, project discussion (**I**)

F. Schneider: performance of experiments (leaving assays), development of the experimental set-up, project discussion (**I and II**)

K. Dierking: performance of experiments (leaving and survival assays), project discussion (**I**)

T. Mohr: performance of experiments (leaving and survival assays), project discussion (**I**)

L. Rösingh: performance of experiments (leaving assays), project discussion (**I**)

A. Masche: performance of experiments (survival assays), project discussion (**I**)

J. Wang: performance of experiments (**III**)

3. Summary

To enhance fitness, any living organism should evaluate how much to invest in defence against pathogen threats. One likely advantageous strategy is pathogen avoidance behaviour. This defence response should be highly cost-effective, because it minimizes exposure to infection (and thus tissue damage), it minimizes spread of harmful pathogens, and it also minimizes self-damage caused by immunopathology upon activation of physiological immunity.

Pathogen avoidance behaviour, as any other defence mechanism, is subject to strong selective pressure that results in specific variation in the underlying genes. Characterizing the genetics of these variations should provide information as to the type of genes and associated specific gene functions that are targeted by parasite-mediated selection.

One of the tools employed to locate and identify the involved genes is Quantitative Trait Locus (QTL) analysis.

For this PhD project, QTL analysis was used to characterize the genetic architecture of natural variation in *C. elegans* avoidance behaviour against the Gram-positive bacterium *Bacillus thuringiensis*. The analysis identified several genomic regions associated with variation in avoidance of pathogenic and/or non-pathogenic bacteria. Furthermore, our analysis showed significant epistatic interactions between some of the identified loci, suggesting a role of epistasis in determining behavioural defences.

The main effect QTL for avoidance behaviour towards all tested bacteria encompasses the neuropeptide receptor gene *npr-1*, which has previously been shown to mediate behavioural defence against *Pseudomonas aeruginosa*. Interestingly, our results show that

npr-1 is not the only gene mediating avoidance behaviour toward *B. thuringiensis*, and that different alleles of this gene have opposite effects on resistance (physiological and behavioural) in *C. elegans*. Moreover, our functional genetic analysis revealed that *npr-1* influences survival and avoidance behaviour toward *B. thuringiensis* in the opposite direction than toward *P. aeruginosa*. Our findings thus suggest that *npr-1* plays a central role in fine-tuning nematode behavioural defences to alternative stimuli.

In addition, we evaluated whether *C. elegans* shows transgenerational immune priming of avoidance behaviour against *B. thuringiensis*. Interestingly, our results show that behavioural defences can be inherited from parents to offspring, with this response being dependent on the *C. elegans* isolate. This suggests that *C. elegans* can respond to pathogen challenge not only by individual activation of protective defences (e.g. avoidance behaviour) but also by transfer of such responses to the next generation.

In parallel, we examined the role of one of the highly conserved signaling pathways, the insulin-like signaling cascade, in *C. elegans*' defence. We specifically tested activation of the Foxo transcription factor DAF-16 by pathogenic strains of *B. thuringiensis*. Our results indicate that DAF-16 is directly activated by pathogens and that its activity is mainly determined by pathogenicity level and bacterial strain.

Taken together, our observations from the different studies emphasize that *C. elegans* possesses a repertoire of alternative defence mechanisms that appear to be coordinated by a few central regulators (e.g., the neuropeptide receptor NPR-1 or the FOXO transcription factor DAF-16), that include physiological immune responses, avoidance behaviours and also transgenerational effects, and that also allows the nematode to differentiate between different pathogen taxa and additionally different strains of the same pathogen species.

4. Zusammenfassung

Um eine hohe Fitness zu erzielen, müssen Organismen entscheiden, wieviel sie in die Abwehr gegen Pathogene investieren wollen. Eine potentiell vorteilhafte Abwehrstrategie ist das Pathogen-Vermeidungsverhalten. Dieser Abwehrmechanismus sollte sehr kosteneffizient sein, da er die Exposition zu Pathogenen (und somit Gewebeschäden), die Ausbreitung schädlicher Agentien und den Schaden durch Immunopathologie minimiert.

Wie jeder andere Verteidigungsmechanismus ist das Pathogen-Vermeidungsverhalten einem hohen Selektionsdruck ausgesetzt, was zu Variation in den verantwortlichen Genen führt. Eine Charakterisierung dieser genetischen Variations sollte Informationen liefern zu der Art Genen und den assoziierten spezifischen Genfunktionen, die Pathogen-spezifischer Selektion unterliegen. Eines der Werkzeuge, um die zu Grunde liegenden Abwehrgene zu identifizieren, ist die "Quantitative Trait Locus (QTL)" Analyse.

Für diese Doktorarbeit wurde die QTL Analyse eingesetzt, um die genetische Architektur des *C. elegans* Vermeidungsverhalten gegenüber dem Gram-positiven Bakterium *Bacillus thuringiensis* zu charakterisieren. Mit Hilfe dieses Ansatzes konnten mehrere chromosomale Regionen identifiziert werden, die eine Veränderung im Verhalten der Würmer gegenüber pathogenen und/oder nicht-pathogenen Bakterien beeinflussen. Unsere Analyse weist daneben auf signifikante epistatische Interaktionen zwischen verschiedenen Regionen hin, die somit eine wichtige Rolle bei der Ausprägung von Vermeidungsverhalten spielen sollten.

Der QTL mit dem größten Effekt auf das Pathogen-Vermeidungsverhalten umfasst das Neuropeptid-Rezeptor Gen *npr-1*, welches zuvor bereits mit dem Vermeidungsverhalten

von *C. elegans* gegenüber *Pseudomonas aeruginosa* in Verbindung gebracht wurde. Eine genauere funktional genetische Untersuchung dieses Gens belegt, dass *npr-1* nicht das einzige Gen in *C. elegans* ist, welches Vermeidungsverhalten gegenüber *B. thuringiensis* vermittelt und dass verschiedene Allele dieses Gens gegensätzliche Effekte auf Resistenz (physiologische wie verhaltensmäßige) besitzen. Die funktionelle Analyse zeigt daneben, dass *npr-1* konträre Effekte im Überleben und Vermeiden von zwei verschiedenen Pathogenen verursacht, in diesem Fall *B. thuringiensis* im Vergleich zu *P. aeruginosa*. Diese Ergebnisse deuten darauf hin, dass *npr-1* eine zentrale Rolle in der Feinabstimmung von Verhaltens- Immunantworten in *C. elegans* spielt.

Zusätzlich haben wir getestet, ob *C. elegans* Trans-generationen Effekte im Vermeidungsverhalten gegenüber *B. thuringiensis* aufweist. Interessanterweise haben unsere Ergebnisse gezeigt, dass eine verhaltensmäßige Immunantwort, abhängig vom *C. elegans* Stamm, vererbt werden kann. Der Fadenwurm scheint somit nicht nur individuell auf Pathogene zu reagieren, sondern die relevanten Mechanismen auch an die Nachfolgeneration weitergeben zu können.

Parallel haben wir die Rolle eines der hoch konservierten Signalwege des *C. elegans* Immunsystems, der Insulin-ähnlichen Signalkaskade, untersucht. Konkret haben wir dabei die Aktivierung vom Foxo-Transkriptionsfaktor DAF-16 nach Pathogenexposition charakterisiert. Unsere Ergebnisse deuten darauf hin, dass DAF-16 direkt durch Pathogene aktiviert wird und dass der Grad der Aktivierung hauptsächlich durch die Pathogenität des Bakterienstammes bestimmt wird.

Abschließend lässt sich aus unseren Beobachtungen in den verschiedenen Studien schließen, dass *C. elegans* ein Repertoire alternativer Abwehrmechanismen besitzt, die anscheinend durch wenige zentrale Regulatoren koordiniert werden (z. B. dem

Neuropeptid-Rezeptor NPR-1 oder dem Foxo Transkriptionsfaktor DAF-16), die physiologische Immunantworten, Vermeidungsverhalten und auch Transgenerationen-Effekte umfassen und die es dem Fadenwurm ermöglichen, zwischen verschiedenen Pathogen-Taxa wie auch verschiedenen Stämmen einer bestimmten Pathogenart zu differenzieren.

5. Introduction

5.1 *Caenorhabditis elegans* as a model system for a broad variety for biological studies

Caenorhabditis elegans was introduced as a model by Sydney Brenner in 1965 to study development and neurobiology (Brenner, 1974). Nowadays, this nematode is used in a broader variety of biological studies including basic and medical biology. Some examples of these biological processes include studies about apoptosis, cell signaling, cell cycle, cell polarity, gene regulation, metabolism, ageing and sex determination (Kaletta and Hengartner, 2006; Riddle et al., 1997).

C. elegans has numerous features of a powerful model organism for such a huge variety of biological studies. It is easy to culture and maintain in the lab. Worms are raised on petri dishes with Nematode Growth medium (NGM) agar seeded with the Gram-negative bacterium *Escherichia coli* strain OP50 as a food source. It has a short generation time of 3 days at 20°C and a high proliferation rate. One self-fertilizing hermaphrodite can produce 300 progenies, whereas mating between one male and one hermaphrodite can lead to the production of more than 1000 progenies. This means that several million animals can be produced in one day. *C. elegans* has a small size of 1mm length allowing large cultures of worms or high throughput assays to be carried out with either small petri dishes or microtitre plates. Moreover, it has a transparent colour which makes visualization of *in vivo* fluorescence markers, infection and disease progress easy to track in real time in worms. *C. elegans* survives freezing in glycerol solution at -80°C. This allows long term storage of worms especially for later analysis of mutants or use of materials generated from

previous experimental work. From a genetic point of view, *C. elegans* has a completely sequenced genome which was published in 1998 (Consortium, 1998). It was the first animal to have its genome completely sequenced which facilitates the identification of genetic determinants underlying any phenotype of interest in this nematode.

C. elegans is easily used for genetic manipulation including forward and reverse genetic screens. The discovery of RNA interference (RNAi) in *C. elegans* in 1998 by Fire *et al.* further facilitated *in vivo* functional genetic analysis in this nematode (Fire *et al.*, 1998). RNAi can be performed either by injecting worms with double-stranded RNA (dsRNA) (Fire *et al.*, 1998), by soaking worms in a solution of dsRNA (Tabara *et al.*, 1998), or by feeding worms on *E. coli* expressing dsRNA of the target gene (Timmons and Fire, 1998). The available feeding RNAi library can target up to 86% of predicted genes in the *C. elegans*' genome (Fraser *et al.*, 2000; Kamath and Ahringer, 2003). In parallel to the RNAi library, numerous mutants of *C. elegans* generated by mutagenesis using ethyl-methyl sulfate (EMS) as a mutagen are available at the *Caenorhabditis* Genetic Center (CGC), the most extensive public resource for *C. elegans* strains (<http://www.cbs.umn.edu/cgc>). Furthermore, transgenic worms can be easily generated by microinjection of DNA. In addition to all of these listed features, information about *C. elegans*' biology, genomics and proteomics are publically available at the central online platform called "wormBase" which was founded in 2000 (Chen *et al.*, 2005; Stein *et al.*, 2001).

5.2 *Caenorhabditis elegans* as a model system for studying host-parasite interactions and innate immunity

There are several methods applied in *C. elegans* for the study of *C. elegans*-pathogen interactions. The impact pathogens have on *C. elegans* as a host organism can be revealed

by scoring different phenotypic and/or genetic readouts. Phenotypic readouts include scoring: i) worms' survival (Tan et al., 1999 (a)); ii) morphological changes such as “Dar” phenotype (Hodgkin et al., 2000) or lumen distension after accumulation and/or persistence of bacterial cells in the gut (Aballay et al., 2000); and iii) behavioural responses (Hasshoff et al., 2007; Pradel et al., 2007; Zhang et al., 2005). Whereas genetic readouts include: i) microarrays and quantitative reverse transcription polymerase chain reaction (qRT-PCR) (Mallo et al., 2002; Troemel et al., 2006); and ii) RNA-sequencing (Engelmann et al., 2011).

C. elegans was described for the first time as a model susceptible to bacterial infection in 1999 by the Ausubel group after observing that worms could be infected with *Pseudomonas aeruginosa* (strain PA14), a Gram-negative, human opportunistic pathogen, also known to infect mice, insects, plants and humans (Mahajan-Miklos et al., 1999; Tan et al., 1999 (a)). These studies showed that several virulence factors or genes in *P. aeruginosa*, required for pathogenicity in mice and plants, were required for killing *C. elegans* as well. This finding indicated that using *C. elegans* as a model host to study killing by the clinical isolate of *Pseudomonas aeruginosa* should allow us the identification of novel virulence factors required for mammalian pathogenesis. Before the findings of Tan *et al.* and Miklos *et al.* (Mahajan-Miklos et al., 1999; Tan et al., 1999 (a)), it was still unclear whether *C. elegans* had an immune system for defence responses against potential pathogens or whether pathogenic microbes were completely deactivated in the worm's grinder during oral uptake. This elegant finding stimulated subsequent studies on *C. elegans*-pathogen interactions. For more than ten years now, this nematode has proven to be a powerful model for investigating diverse bacterial and fungal infections (reviewed in (Simonsen et al., 2012)).

C. elegans was thought for a long time to be a soil dwelling nematode till recent findings showed that it colonizes microbe rich habitats such as rotting fruits and decaying plant materials where it feeds on bacteria and yeasts (Felix and Braendle, 2010). These microbes can be either a food source or harmful pathogens. Since it was shown that exposure to pathogenic microorganisms activates defence mechanisms in *C. elegans*, this nematode has become a suitable model for investigating microbial pathogenesis.

For more than a decade now, detailed knowledge has accumulated on defence mechanisms and their genetic basis in *C. elegans* through experimental infection of worms with a diverse range of pathogenic microbes, including human pathogenic bacteria.

C. elegans relies solely on its innate immune system for defence against pathogenic microbes. It does not possess an adaptive immune system, or cells such as macrophages, neutrophils, or even cells equivalent to phagocytes which are found in other invertebrates (e.g. *Drosophila*). Protective responses in *C. elegans* thus rely on physical barriers, behavioural avoidance and non-cellular physiological immune responses.

Physical barriers

Physical barriers in *C. elegans* include the cuticle and the grinder. The cuticle consists of collagen and chitin. It protects *C. elegans* from the outside due to its multi-layered structure. Worms are then exposed to the environment only at three different body openings, the mouth, the anus and the vulva. Most bacteria access the gut of worms through the mouth. The grinder causes bacterial disruption after oral uptake of bacterial cells which should not then reach the intestine intact.

Physical barriers in *C. elegans* are thought to minimize invasion rates of parasites (Schulenburg et al., 2009).

Behavioural defences

Pathogen avoidance behaviour is an economic defence strategy compared with physiological immunity. It reduces contact with parasites, thus the inflicted tissue damage (Schulenburg and Ewbank, 2007); the costly energy required for the activation of physiological immune responses (Schulenburg and Ewbank, 2007); and the self-damage caused upon activation of physiological immunity, such as autoimmunity or immunopathology (Schmid-Hempel, 2003). Moreover, behavioural defences reduce the risk of contaminants' spread from infected to non infected individuals influencing then parasite transmission rate and host survival.

Although behavioural defences economize animal's resources, they still come with their own cost. Avoiding pathogen rich compounds either results in reduced energy intake due to decreased feeding or requires energy for moving away and searching for pathogen free food patches. Such a cost is reduced when animals can chose between pathogen rich or pathogen free areas within the same location.

C. elegans has been recently intensively used to decipher the genetics underlying behavioural defences. This allowed the generation of a comprehensive data set showing that pathogen avoidance responses are of major importance in the nematode's defence system.

C. elegans shows different behavioural defence responses: i) physical pathogen avoidance behaviour mediated by either G-protein signaling in chemosensory neurons, the insulin-like signaling pathway, or HECT domain-containing E3 ubiquitin ligase (Chang et al., 2012; Hasshoff et al., 2007; Pradel et al., 2007); ii) learning of pathogen avoidance behaviour based on serotonin signaling (Zhang et al., 2005); and iii) reduced oral uptake of pathogenic microbes mediated by the insulin-like signaling pathway (Hasshoff et al.,

2007). Such aversive responses were exhibited toward several pathogenic microbes including Gram-negative and Gram-positive bacteria such as *Serratia marcesens* (Pradel et al., 2007; Pujol et al., 2001; Zhang et al., 2005), *Pseudomonas aeruginosa* (Beale et al., 2006; Chang et al., 2012; Laws et al., 2006; Yook and Hodgkin, 2007; Zhang et al., 2005), *Burkholderia pseudomallei* (Smith et al., 2002), *Yersinia pestis* (Smith et al., 2002), *Bacillus thuringiensis* (Hasshoff et al., 2007; Schulenburg and Ewbank, 2004; Wang et al., 2012), *Photobacterium luminescens* (Sicard et al., 2007) and *Micobacterium nematophilum* (Yook and Hodgkin, 2007).

C. elegans can specifically detect natural products of pathogenic bacteria such as surfactants secreted by *Serratia marcesens* and acylated homoserine lactone autoinducers expressed by *Pseudomonas aeruginosa* (discussed in (Schulenburg and Ewbank, 2007)).

The response toward *S. marcesens* seemed to be specific as *C. elegans* reacts differently to different types of Serrawettin surfactant molecules produced by this bacterium. Worms are attracted by Serrawetin W1 and W3, whereas they are repulsed by Serrawetin W2 (Pradel et al., 2007). This specificity in response might be mediated by the genetically diverse repertoire of G-protein coupled chemoreceptors (GPCRs). *C. elegans* possesses about 1000 genes encoding orphan worm specific receptors. Most of these receptors are thought to be chemoreceptors (Bargmann, 1998). These GPCRs supplemented with sensory pathways are very likely to be involved in bacteria recognition knowing that *C. elegans* senses and reacts toward natural products of bacterial metabolism. Other promising candidate receptors for mediating specific pathogen recognition in *C. elegans* are proteins with leucine-rich repeat domains, C-type lectin domains or F-box domains (discussed in (Schulenburg and Ewbank, 2007)).

Although *C. elegans* can discriminate between different bacterial species (Shtonda and Avery, 2006), it is still unclear whether this discrimination can be entirely coupled with bacterial pathogenesis, especially that avoidance may be due to low food quality of the bacterium, as it is the case with *Bacillus megaterium* (Shtonda and Avery, 2006). To date, there is still no clear evidence showing that *C. elegans* exhibits aversive responses due to a direct recognition of pathogenic bacteria, or to tissue damage caused by infection or to the combination of both. However, Pradel *et al.* showed that a mutant strain of *Serratia* (*swrA*) deficient for the production of Serrawettin W2 had a reduced ability to elicit avoidance behaviour in *C. elegans* compared with another strain of *Serratia* able to produce Serrawettin W2; whereby both strains had equal pathogenic effect on *C. elegans* (Pradel *et al.*, 2007). This means that *C. elegans* might have the ability to recognize pathogens by detecting specific pathogen-associated molecules. Still, this finding does not exclude the hypothesis that cellular damage and stress provoked by bacterial infection might be coupled with avoidance responses in *C. elegans*. Lots of work still awaits us before we enhance our understanding on the mechanisms underlying pathogen recognition and the effect of infection on aversive behaviour in *C. elegans*.

Moreover, it is still unknown whether the different molecular mechanisms underlying behavioural defence interact with each other or whether they act independently from each other. Previous findings indicated that there is a link between chemosensation and insulin-like signaling pathway, both involved in aversive responses in *C. elegans*. Liang *et al.* showed that a stress dependant activation of the transcription factor of the insulin-like pathway DAF-16 was affected by serotonin release in the nervous system. Daf-16 activation was enhanced upon serotonin release in NSM neurons and suppressed upon serotonin release in ADF neurons (Liang *et al.*, 2006) which by itself was influenced by DAF-16 activity (Estevez *et al.*, 2006). Moreover, the sensory Gi-like protein ODR-3

expressed in AWB neurons was shown to extend life span via the transcription factor DAF-16 (Lans and Jansen, 2007).

Based on the findings that aversive learning is regulated by serotonin signaling in ADF neurons (Zhang et al., 2005), that physical avoidance is controlled by DAF-16 activation (Hasshoff et al., 2007) and Gi-like protein ODR-3 in AWB neurons (Pradel et al., 2007), how does the interaction between chemosensory neurons and insulin-like signaling pathway affect behavioural defences in *C. elegans*? Is there any genetic redundancy between the different mechanisms? And is the interaction between these mechanisms affected differently according to the pathogen taxa *C. elegans* is exposed to?

To date, we still lack full knowledge about the relationship between the different described molecular mechanisms underlying behavioural defences in *C. elegans* and about their functions in the whole organism. It is still even unclear whether the different types of behavioural defences are independent one from one another. For instance, do worms choose to reduce oral uptake as a behavioural defence strategy in an environment where physical avoidance is not possible?

Physiological innate immune responses

Numerous studies have characterized the genetics of physiological immune responses in *C. elegans*. These studies showed the involvement of several signaling pathways in the nematode's defence system including for example: i) p38 MAPK involved in the host response towards several pathogens including *P. aeruginosa* (Kim et al., 2002) and *B. thuringiensis* (Bischof et al., 2008; Huffman et al., 2004); ii) the JNK MAPK involved in resistance towards for example *B. thuringiensis* (Kao et al., 2011); iii) the ERK pathway involved in the response of worms to at least *M. nematophilum* (Nicholas and Hodgkin, 2004), iv) the TGF β involved in resistance of worms to infection by for example *P.*

aeruginosa and *S. marcesens* (Mallo et al., 2002; Tan, 2001), v) the insulin-like signaling pathway mediating resistance and pathogen avoidance behaviour to bacterial pathogens such as *P. aeruginosa*, *B. thuringiensis*, *E. faecalis* and *S. aureus* (Evans et al., 2008 (a); Evans et al., 2008 (b); Garsin et al., 2003; Wang et al., 2012); vi) the TLR-pathway signalling which contributes to defence only against infection by the bacterium *Salmonella enterica* in the pharynx but not against other pathogens such as *S. aureus*, *P. aeruginosa* and the fungus *Drechmeria coniospora* (reviewed in (Irazoqui et al., 2010)).

In addition to all these signaling pathways, *C. elegans* relies as well on the activity of antimicrobial peptides for defence against pathogens including NLP or CNC peptides (Couillault et al., 2004), caenopores such as SPP-5, SPP-3 and SPP-12 (Hoeckendorf and Leippe, 2012; Hoeckendorf et al., 2012 ; Roeder et al., 2010) and lysozymes such as LYS-2, LYS-5 and LYS-7 (Boehnisch et al., 2011).

Other immune effectors of the defence system in *C. elegans* include proteins with C-type lectin domain, CUB domains and DUF (proteins with domains of unknown functions) (reviewed in (Simonsen et al., 2012)).

Several pathways and effector molecules are conserved from *C. elegans* to human. Therefore, studying innate immune mechanisms in *C. elegans* may on the one hand enhance our understanding of the evolution and function of human innate immunity; and on the another hand provide new insight into conserved virulence strategies.

One of the highly conserved pathways in *C. elegans* is the insulin-like signaling pathway (focus of chapter 3). This pathway controls the worm's metabolism, reproduction, development, lifespan and resistance to a variety of environmental stresses including resistance toward pathogens (discussed in (Irazoqui et al., 2010)). The insulin receptor DAF-2 negatively regulates the transcription factor DAF-16 through phosphorylation of a

kinase cascade that includes the phosphoinositide 3-kinase AGE-1 and the four serine threonine kinases AKT-1, AKT-2, PDK-1 and SGK-1. The downregulation of DAF-2 induces the nuclear translocation and regulatory activity of DAF-16 (Antebi, 2007; Landis and Murphy, 2010) (Figure 1).

Mutations in *daf-2* or *age-1* induce the activation of *daf-16*, leading then to a prolonged lifespan, delayed ageing and increased resistance toward pathogenic bacteria (Garsin et al., 2003). This enhanced resistance was observed toward different pathogens such as *S. epidermis*, *Staphylococcus aureus* (Amrit et al., 2010; Garsin et al., 2003), *Enterococcus faecalis* (Chávez et al., 2007; Garsin et al., 2003), *Pseudomonas aeruginosa* (Amrit et al., 2010; Evans et al., 2008 (a); Evans et al., 2008 (b); Garsin et al., 2003; Kawli and Tan, 2008), *Salmonella enterica* (Jia et al., 2009), *Yersinia pestis* (Singh and Aballay, 2006), *Cryptococcus neoformans* (Amrit et al., 2010; van den Berg et al., 2006), enteropathogenic *Escherichia coli* (Anyanful et al., 2009), *Bacillus subtilis* (Garsin et al., 2003) and *Bacillus thuringiensis* (Hasshoff et al., 2007).

Insulin-like signalling pathway seemed to control defence responses through DAF-16 in *C. elegans* in three different ways: i) directly activating antimicrobial genes such as *spp-1* and *lys-7* (Evans et al., 2008 (a)); ii) protecting against immune pathological effect, such as cellular damage caused upon infection, by mediating oxidative stress responses (Chávez et al., 2007; Mohri-Shiomi and Garsin, 2008); iii) enhancing behavioural defence responses toward pathogen rich locations that includes physical avoidance and reduced feeding (Hasshoff et al., 2007).

Despite all these findings, it was still unclear whether the nuclear translocation and regulatory activity of DAF-16 were directly induced by pathogens. Most previous cases suggested that DAF-16 involvement in pathogen defence seem to be as part of a general

stress response that is not directly activated by pathogens but by other cues (Troemel et al., 2006). Examples of these factors modulating insulin-like signaling pathway activity are heat stress and starvation.

Only recently, two different studies showed that DAF-16 activity may be directly regulated by pathogens. An increase in DAF-16::GFP nuclear localization was observed after exposure to enteropathogenic *E. coli* for up to 3 hours (Anyanful et al., 2009) or to pathogenic *Pseudomonas fluorescens* for 12 hours (Neidig et al., 2011).

In our study (chapter 3 of the thesis), we investigated whether DAF-16 nuclear translocation can directly be induced by pathogenic *Bacillus thuringiensis*. We therefore looked at the nuclear translocation of DAF-16::GFP, relating this read out to the level of BT pathogenicity by looking at worms' survival, and BT infectivity by measuring infection load in worms' gut. Our results showed that the two distinct strains of pathogenic *B. thuringiensis* we used had different levels of pathogenicity and infectivity. Pathogenicity correlated positively with the extent of nuclear translocation and activation of DAF-16. Our study shows in agreement with Anyanful and Neidig's findings that insulin-like signaling pathway responds directly to pathogens. This finding in addition to the previous establishment of the role of this cascade in both physiological and behavioural immune responses (Hasshoff et al., 2007) highlights the strong involvement of this pathway in defence responses toward distinct pathogens.

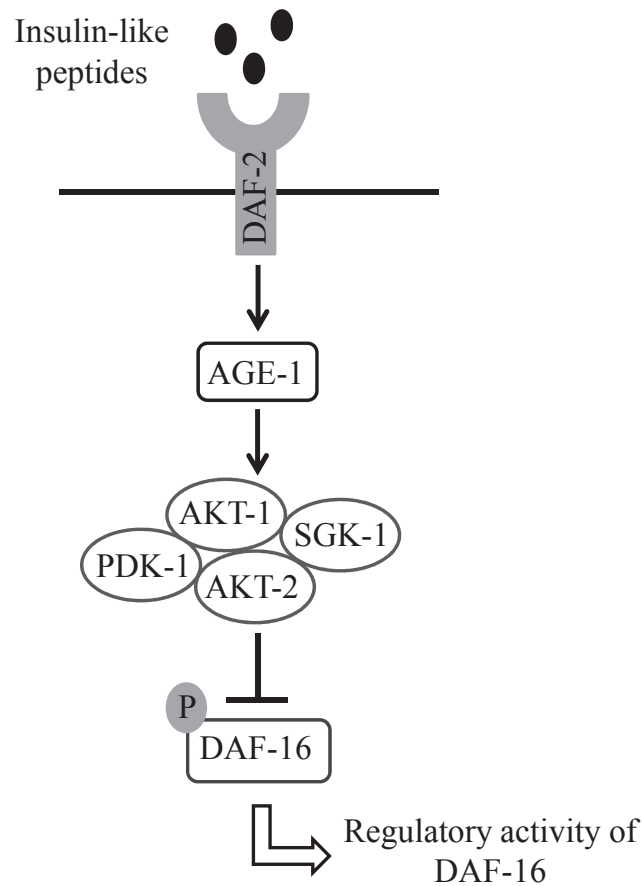


Figure 1: Schematic of the insulin-like signaling pathway in *C. elegans*. The nuclear translocation, thus the regulatory activity of DAF-16 is inhibited by DAF-2.

5.3 *Bacillus thuringiensis* as a natural pathogen of *C. elegans*

Bacillus thuringiensis is a Gram-positive soil dwelling bacterium. It is widely used as an insecticide bacterium (Wei et al., 2003). It multiplies by division of vegetative cells which sporulate under adverse conditions. Spores may germinate after favorable conditions prevail again. During sporulation, *B. thuringiensis* produces one or more crystal proteins (Borgonie et al., 1995), called δ -endotoxins; some of which are pathogenic against nematodes, including *C. elegans* (Wei et al., 2003).

The nematocidal activity of *B. thuringiensis* depends mainly on the presence of toxins associated to spores which are the infectious stage of this bacterium. Infection occurs upon

oral uptake of spore-toxin mixtures. Spores associated to toxins usually accumulate in the gut of *C. elegans*. This is followed by toxins solubilization, proteolitical activation and binding to specific receptors on the intestinal membrane where they form pores. Pore formation leads to cell lysis. Ingested spores can germinate into vegetative cells which can replicate inside infected worms (Griffitts and Aroian, 2005) (Figure 2).

Both, *B. thuringiensis* and *C. elegans* are present in terrestrial environments. *B. thuringiensis* strains have not been yet isolated from natural isolates of worms, but they could be isolated together with *C. elegans* from a compost heap in France (Personal communication, Marie-Anne Félix, Paris).

Under laboratory conditions, *B. thuringiensis* and *C. elegans* can specifically interact with each other. Previous findings showed that different isolates of *C. elegans* showed significant differences in survival rate, infection load, physical evasion and pumping rate when infected with pathogenic *B. thuringiensis*. These phenotypic differences between the isolates were observed irrespectively of environmental conditions (Schulenburg and Muller, 2004).

From the worm perspective, several distinct mechanisms have been identified to be involved in defence against pathogenic *B. thuringiensis*. Studies focused on defence mechanisms in *C. elegans* against either one particular toxin, mainly Cry5B, or the entire infectious stages of *B. thuringiensis*, which include a mixture of different toxins associated to spores.

The response against Cry5B depends on intestinal glycosphingolipid receptors, cellular GDP-fucose and plasma membrane repair mechanism mediated by RAB-5 and RAB-11 trafficking pathways (Barrows et al., 2007; Griffitts and Aroian, 2005; Griffitts et al., 2003; Ideo et al., 2009; Los et al., 2011). Immunity against Cry5B also involves hypoxia

responses (Bellier et al., 2009), two mitogen-activated protein kinase (MAPK) pathways, p38 and JNK-like MAPK, and the transcription factor activating protein 1 (AP-1) (Huffman et al., 2004; Kao et al., 2011). Moreover, recent findings showed that behavioural avoidance (e.g. inhibition of feeding) and physiological immune responses against Cry5B require the neuronal protein Go α subunit *goa-1* and that maintenance of these responses depend on calcium activator for protein secretion (CAPS) homolog *unc-31* (Los et al., 2013).

Studies on defence against the entire infectious stages of *B. thuringiensis* showed the involvement of the insulin-like signaling pathway (Hasshoff et al., 2007; Wang et al., 2012) and at least three lysozymes LYS-2, LYS-5 and LYS-7 (Boehnisch et al., 2011).

None of the previous studies looked at the molecular genetics underlying the observed natural variation in defence mechanisms between different isolates of *C. elegans*. Moreover, we still lack knowledge about how specific the response is toward different strains of *B. thuringiensis* and whether worms react to certain produced toxins or to *Bacillus* cells.

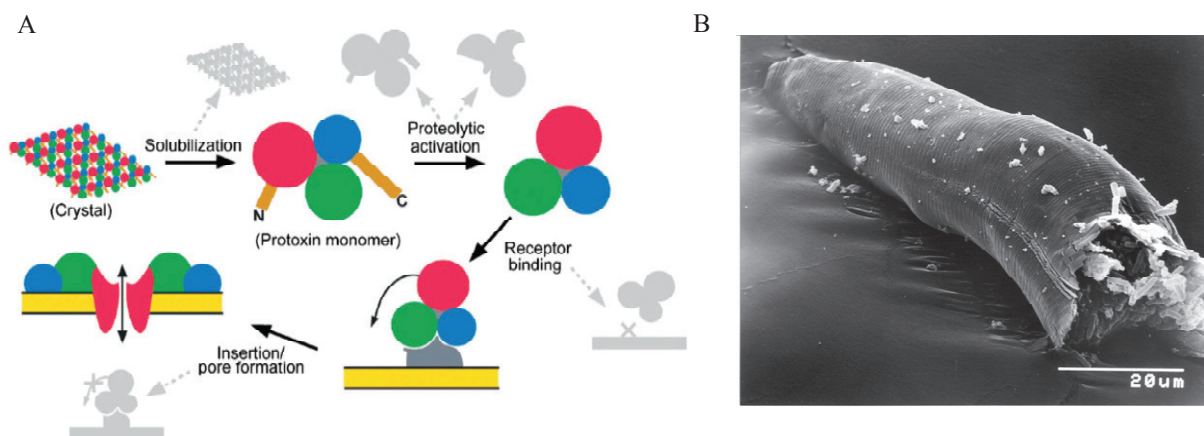


Figure 2: **A.** BT infection process inside the gut of *C. elegans* according to Griffiths & Aroian 2005 Bioessays 27.614-24. **B.** Photo of an infected worm with BT taken by Joe Lange (electron microscopy). The worm is heavily filled with BT particles.

5.4 *Pseudomonas aeruginosa* as a medically relevant pathogen model of *C. elegans*

Pseudomonas aeruginosa is a Gram-negative human opportunistic pathogen. It grows in soil, marshes, coastal marine habitat, and on plants as well as on animal tissues (Hardalo, 1997).

P. aeruginosa strain PA14 was shown to kill *C. elegans* through two distinct mechanisms, called fast and slow killing. These mechanisms depend on the composition of the agar on which PA14 is grown (Tan et al., 1999 (b)). Slow killing is induced when PA14 is grown on minimal medium and worm death is observed over the course of several days. It is characterized by an infection-like process associated with the accumulation of bacteria inside the worm's intestine. In contrast, fast killing is induced when PA14 is grown on high osmolarity medium leading to nematode death within several hours (Tan et al., 1999 (b)). Fast killing is not associated with the accumulation of bacteria in the gut of *C. elegans* but due to the production of one or more toxins such as phenazines (Mahajan-Miklos et al., 1999).

A different strain of *P. aeruginosa*, PA01, was also shown to rapidly kill and paralyse *C. elegans*. PA01 killing ability is due to the production of a typical secondary pseudomonad metabolite such as hydrogen cyanide (Darby et al., 1999; Gallagher and Manoil, 2001).

The p38 MAPK pathway is a key determinant of *C. elegans*' defence against *P. aeruginosa*. Strains with mutations in distinct components of this cascade, including SEK-1 (a MAPK kinase), NSY-1 (a MAPK kinase kinase) and PMK-1, had a shortened lifespan on *P. aeruginosa* compared with non-pathogenic *E. coli* (Kim et al., 2002; Kim et al., 2004; Mizuno et al., 2004). The insulin-like signaling pathway was also shown to

contribute to defence against *P. aeruginosa*. In particular, *daf-2* mutants were resistant to killing by *P. aeruginosa* (Garsin et al., 2003).

Despite the extensive studies focusing on the role of these two conserved pathways in *C. elegans*' immunity, little information is known about the interconnectivity between DAF-16 and PMK-1 p38 MAPK cascades.

Microarray analysis in *C. elegans* showed that there was essentially no overlap between genes positively regulated by PMK-1 and DAF-16 upon infection with *P. aeruginosa*. This suggests that the p38 MAPK and the insulin-like signaling pathways function in parallel to promote innate immunity in *C. elegans* (Troemel et al., 2006).

Additionally, several genes were induced upon infection by *P. aeruginosa* independently from PMK-1 and DAF-16 suggesting that there might be other immune pathways regulating genes induced by *Pseudomonas* infection (Troemel et al., 2006). Further research is required to identify such additional pathway(s) and to understand how do the p38 MAPK and insulin-like signaling pathways control immunity? Although, the study by Troemel *et al.* (Troemel et al., 2006) suggested that these two pathways function in parallel, we still cannot exclude the possibility that the two cascades interact with each other by controlling similar pathogen response genes to promote innate immune responses in *C. elegans*, especially that Troemel *et al.* (Troemel et al., 2006) tested this hypothesis using only *P. aeruginosa* without testing other pathogens as well.

5.5 Immune priming

One of the most remarkable properties of host defence is the ability to learn from a previous challenge and increase the efficiency of a second immune response to a

subsequent challenge (Schmid-Hempel, 2011). In the field of immunology, this ability is called immune memory.

Immune memory does not depend on the persistence of a primary response between successive antigenic challenges but it depends on the preexistence of a clonally expanded population of antigen-specific lymphocytes (Janeway CAJ, 2001; Schmid-Hempel, 2011). Such enhanced protection can be individual (occurring within the lifetime of one individual), or trans-generational (transmitted from parents to offspring), as it is the case in mammals when antibodies produced after an immune challenge of the mother are transferred before birth through the placenta and after birth via breast milk (Hanson, 1999; Schmid-Hempel, 2011).

Individual immune memory can be either specific or cross-reactive. Specific memory is given when the secondary response is elicited by the same or very similar antigens to the primary challenge. It is cross-reactive when the secondary response is activated by antigens different from the primary challenge.

Immune memory was first recorded by the ancient Greeks and has been routinely exploited in vaccination programs for over 200 years (Janeway CAJ, 2001).

Though invertebrates lack the cellular components of the adaptive immune memory, such as lymphocytes, specific immune responses and immune memory-like phenomena were also observed in these organisms. Such memory-like phenomena of the innate immune system are usually called immune priming.

Immune priming (individual and/or trans-generational) were previously demonstrated in various invertebrate taxa including cockroaches (Faulhaber and Karp, 1992; Rheins et al., 1980), shrimps (Huang and Song, 1999; Swain and Nayak, 2009; Witteveldt et al., 2006), copepods (Kurtz and Franz, 2003), *Drosophila melanogaster* (Pham et al., 2007), *Daphnia*

(Little et al., 2003; Stjernman and Little, 2011), the mealworm beetle *Tenebrio molitor* (Moret, 2006; Moret and Siva-Jothy, 2003), the social insect *Bombus terrestris* (Sadd et al., 2005; Sadd and Schmid-Hempel, 2006) and the beetle *Tribolium castaneum* (Roth et al., 2009 (b); Roth et al., 2009 (a)). In most of these studies, the molecular mechanisms underlying immune priming were not characterized. The priming effect was only confirmed at the phenotypic level, mostly through measuring survival as a proxy for resistance to infection by pathogens. To our knowledge, only few studies showed that priming effect involved either an elevated activity of antimicrobial molecules in *T. molitor* (Moret, 2006; Moret and Siva-Jothy, 2003), or phagocytes in addition to the Toll pathway in *D. melanogaster* (Pham et al., 2007).

Recent research conducted on priming in *C. elegans* yielded new insight into the underlying molecular mechanisms.

Most of these studies focused on the epigenetic transfer of information from parents to offspring and considered a number of different life-history traits.

Trans-generational epigenetic inheritance in *C. elegans* can occur in various ways. One example is the inheritance of histone methylation pattern which was shown for the methylation site H3K4 for histone 3 lysine 4. In *C. elegans*, the histone H3 lysine 4 trimethylation (H3K4me3) complex regulates life span. This complex is composed of the following chromatin modifiers ASH-2, WDR-5 and the histone methyltransferase SET-2 (reviewed in (Greer and Shi, 2012)). Deficiencies in any of these components in the parental generation led to an extension of lifespan in their descendants. This epigenetic inheritance of longevity was maintained for three generations (Greer et al., 2011). This result contrasted with the previous belief that histone methylation marks are erased between generations by epigenetic programming (Hajkova et al., 2008). The molecular

mechanism underlying such example of trans-generational epigenetic memory remains unclear (Greer and Shi, 2012).

Several other studies showed that trans-generational epigenetic inheritance in *C. elegans* can be mediated through transmission of RNAi effects across generations (Alcazar et al., 2008; Fire et al., 1998; Grishok et al., 2000; Rechavi et al., 2011; Vastenhouw et al., 2006). Most of these studies reported transmission of gene-silencing effect purely at a molecular level without assessing the biological relevance, the physiological context and the effect of such epigenetic memory on life-history traits in *C. elegans*. In addition to the study by Greer *et al.* on epigenetic inheritance of longevity (Greer et al., 2011), only one other study connected the presence of epigenetic memory with a phenotypic effect. In particular, Rechavi *et al.* demonstrated trans-generational inheritance of an antiviral protection through transmission of viral silencing agents (ViRNAs) (Rechavi et al., 2011). To our knowledge, this study identified for the first time the molecular mechanism responsible for trans-generational immune priming in an invertebrate species.

For *C. elegans*, several additional studies examined immune priming at the individual level and not across generations. Examples for these studies include: i) an associative learning of avoidance towards the pathogen *Pseudomonas aeruginosa* (PA14) mediated by an increase of serotonin production in chemosensory neurons through which worm's pre-challenge modified their olfactory preferences (Zhang et al., 2005); and ii) an increased survival against enteropathogenic *E. coli* (EPEC) in pre-exposed worms relative to naive worms (Anyanful et al., 2009). In the latter case, conditioning depended on the insulin/IGFR-1 pathway and the p38 MAPK pathway, and was mediated by dopaminergic neurons (Anyanful et al., 2009).

5.6 Natural variation in *C. elegans*' defence and analysis of quantitative trait loci

Parasite-mediated selection is a driving force in the evolution of animal's immune system. Hosts usually respond to such a selective pressure by showing intra-specific variation in their immune defence including physical barriers, physiological immune responses and behavioural defences (e.g. pathogen avoidance behaviour). Therefore, deciphering the genetics of such resulting natural variation would enhance our knowledge on the evolution of immune defence.

Mutagenesis is the classical approach for identifying genes responsible for a wild-type expression of a trait, especially in the case of monogenic traits. However, when it comes to complex trait analysis, mutagenesis has shown some limitations. The dissection of complex traits has proved to be very challenging because it is very difficult to follow all genomic regions and epistasis interactions that are responsible for variation in a phenotype of interest.

A key development in the field of complex trait analysis was the establishment of Quantitative Trait Locus (QTL) mapping.

QTL mapping has several advantages over mutagenesis as a strategy to characterize genetic variation underlying complex phenotypes. It covers a large spectrum of mutations including point mutations, deletions, insertions, duplications and genome rearrangements in comparison with mutagenesis.

A QTL is a region of any genome that is responsible for variation in the quantitative trait of interest and which can be used to study the genetic architecture of traits (Doerge, 2002; Snoek et al., 2013). QTL mapping is a statistical method providing information about the

location of genes underlying quantitative phenotypes. It includes different types of analysis: single marker mapping, interval mapping, composite interval mapping and multiple trait mapping. These statistical analyses test for the presence of associations between phenotype and genotype in order to dissect regions in the genome affecting variation in complex traits (reviewed in (Doerge, 2002)).

QTL methodology requires:

- Two or more parental strains that differ genetically with regard to the trait of interest
- Genetic markers (e.g. SNPs) that differ between these parental lines

The analysis is based on crossing the inbred parental lines resulting in heterozygous (F_1) individuals. F_1 are crossed among themselves to produce Recombinant Inbred lines (RILs) containing different fractions of the genome of each parental line (Figure 3A). The RILs are then genotyped using molecular markers (most commonly SNPs) before being screened for the phenotype of interest and mapped for candidate QTLs (Figure 4). The recombined regions in the RILs include large genomic fragments (Li et al., 2006), which should help to identify epistatic interactions or redundancy effects.

In parallel to the use of RILs, the generation of introgression lines (ILs), which can be called as well Introgression lines (ILs), increases usually the resolution of mapping. The genome of each IL is mainly composed of one of the parental genomes (called recipient genome) with an introgression of one homozygote segment (called donor genome) coming from the other genetically distinct parent. Consequently, the phenotypic differences observed between recipient lines and ILs can be attributed to the introgressed locus (Doroszuk et al., 2009) (Figure 3B).

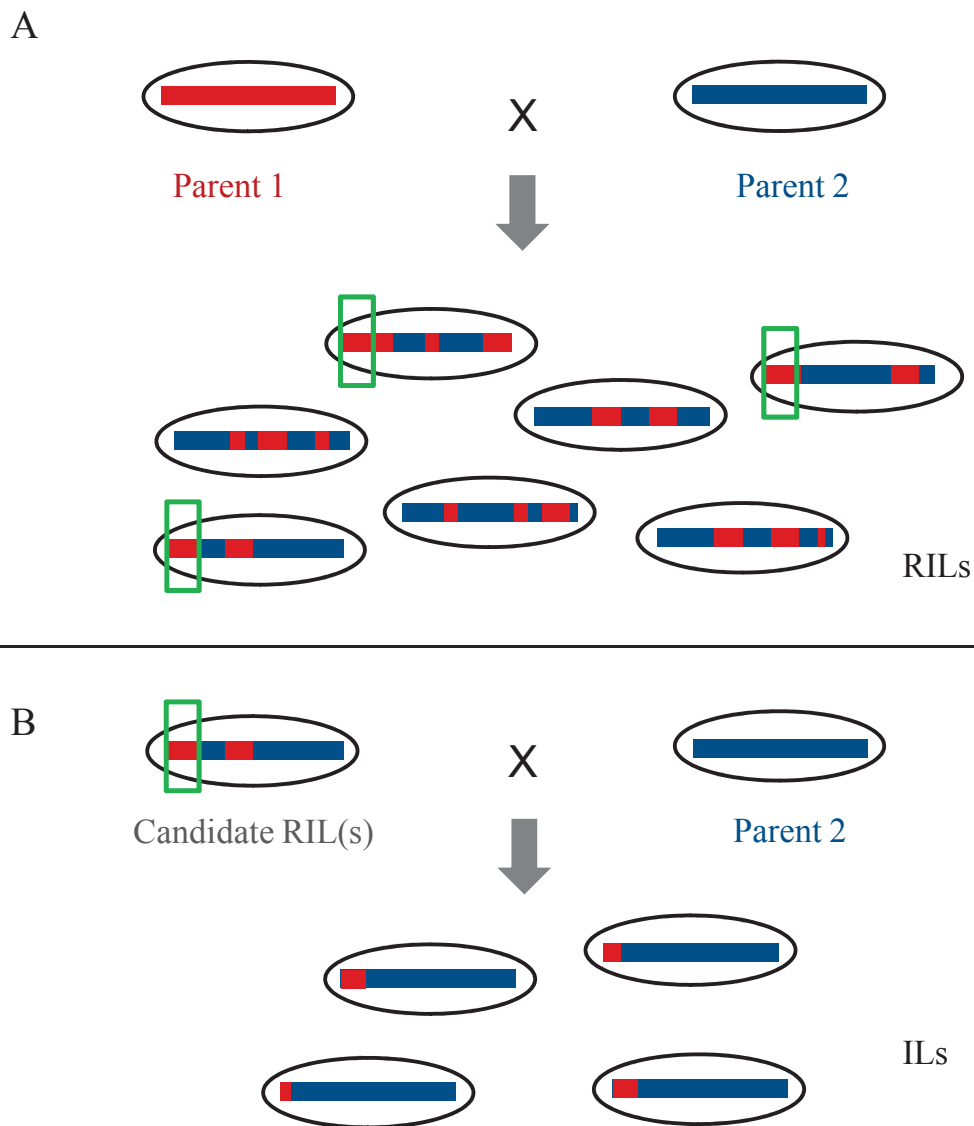


Figure 3: Quantitative trait locus (QTL) mapping requirements. **A.** Recombinant Inbred Lines (RILs) generated from backcrosses between two parental strains (red and blue lines) that differ genetically for the trait of interest. The RILs with a green rectangle marking a red region expressed the phenotype of interest. **B.** Introgression Lines (ILs) generated from backcrossing RILs carrying the candidate region (green rectangle) with one of the parental strain.

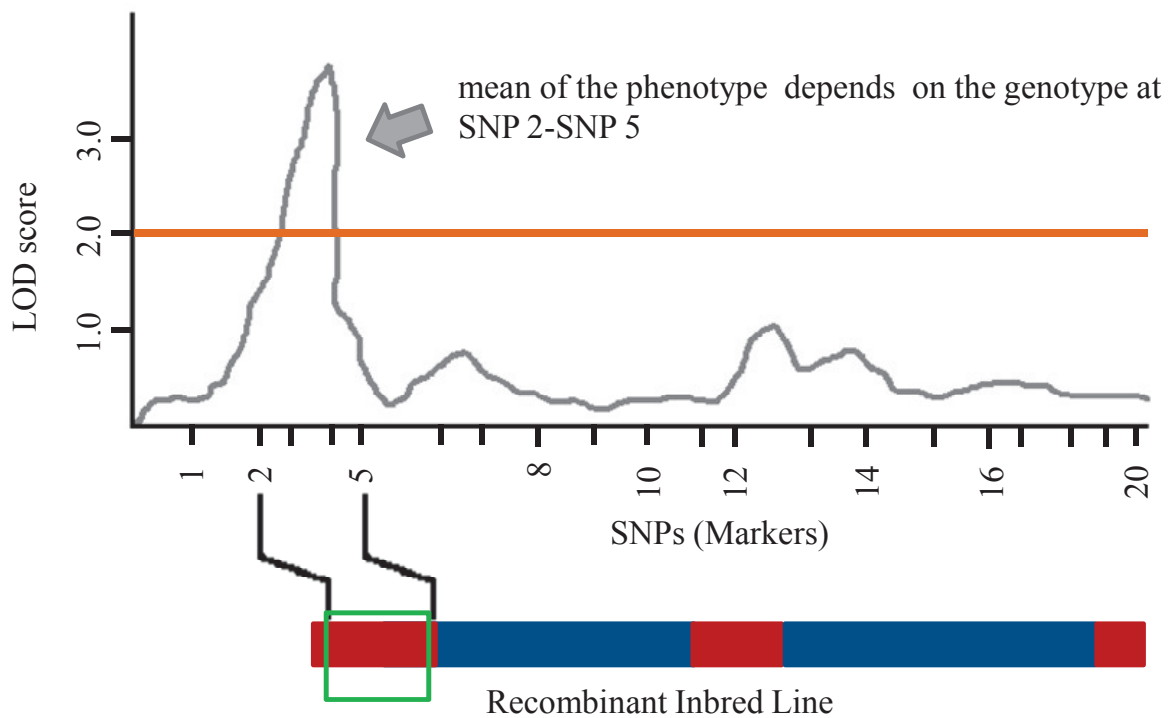


Figure 4: An example of QTL mapping graph. The results of such analysis are presented as a plot of LOD scores profile (Y axis) against the distribution of SNP markers across the genome (X axis). LOD gives the probability that a marker at a given position in the genome is associated with the phenotype of interest. The horizontal orange line marks the significance threshold. Likelihood ratios above this line are significant. Thus this figure shows one QTL between SNP 2 and SNP 5.

Several invertebrate model species have been shown to be suitable for such a type of natural variation analysis, especially in immunological traits. These species include *D. melanogaster* (Lazzaro et al., 2006; Sackton et al., 2010), the flour beetle *Tribolium castaneum* (Wegner et al., 2009), the bumble bee *B. terrestris* (Wilfert et al., 2007) and the mosquito *A. Gambiae* (Riehle et al., 2006).

The nematode *Caenorhabditis elegans* has proven to be an ideal model for characterizing the genetic basis of quantitative traits. Its easy manipulation in the lab, completely sequenced genome and short generation time (allowing a fast construction of a library of RILs and ILs) makes it a suitable model for QTLs analysis. Several studies demonstrated that *C. elegans* shows comprehensive natural variation in several phenotypic traits

including for example: life-history characteristics (Ayyadevara et al., 2003; Gutteling et al., 2007; Gutteling et al., 2006; Knight et al., 2001), behavioural responses (Bendesky et al., 2012; de Bono and Bargmann, 1998; McGrath et al., 2009), copulatory plug polymorphism (Palopoli et al., 2008), and resistance to RNAi (Pollard and Rockman, 2013).

Most importantly, *C. elegans* shows natural variation in pathogen defence. This natural variation between distinct isolates of *C. elegans* was shown for several defence mechanisms including: i) physiological resistance on pathogenic bacteria such as *S. marcescens* (Schulenburg and Ewbank, 2004), *B. thuringiensis* (Schulenburg and Muller, 2004) and *Photorhabdus luminescens* (Sicard et al., 2007); ii) behavioural defences including physical pathogen avoidance behaviour on *B. thuringiensis* (Schulenburg and Muller, 2004) and *P. aeruginosa* (Chang et al., 2012); and reduced feeding on *B. thuringiensis* (Schulenburg and Muller, 2004) and *Photorhabdus luminescens* (Sicard et al., 2007).

To our knowledge, none of these studies characterized the genetic architecture of the observed natural variation in physiological or behavioural immune responses. So far only one study characterized natural variation in *C. elegans*' resistance against the pathogenic bacterium *P. aeruginosa* (PA14). This study was based on QTL analysis of RILs generated from crosses between the two divergent *C. elegans*' isolates N2 and CB4856. N2 showed a higher resistance on PA14 compared to CB4856 (Reddy et al., 2009). The observed difference in survival was indirectly related to differences in aerotaxis behaviour between the two isolates. The conclusion was that low oxygen concentrations on a PA14 lawn compared with a free PA14 space led to a higher leaving behaviour in N2 compared with CB4856, as the latter isolate shows preferences for low O₂ concentrations. As a consequence, CB4856 was more exposed to PA14 compared with N2, which made it

highly susceptible (Reddy et al., 2009). To confirm this conclusion, Reddy *et al.* showed that under experimental conditions which suppressed physical avoidance behaviour, the difference in survival between N2 and CB4856 was no longer observed (Reddy et al., 2009). The QTLs analysis identified a polymorphism in the gene *npr-1* as the main locus underlying the observed difference in resistance.

Similar results were obtained in another study based on a reverse genetic screen, even though the conclusions drawn varied (Styer et al., 2008). In particular, Styer *et al.* (Styer et al., 2008) used their data to propose that lawn avoidance mediated by *npr-1* polymorphism does not account for all of the observed difference in resistance between N2 and CB4856. The residual difference in resistance should then be caused by an *npr-1*-dependent neuronal regulation of physiological innate immunity that acts in addition to and independently from the behavioural responses (Figure 5). Support for this conclusion was provided through the results of gene expression microarray analysis comparing the loss of function mutant *npr-1(ad609)* with the wild-type N2. These results highlighted a role for the NPR-1 neural circuit in modulating the activity of PMK-1, which was lower in animals with a *npr-1* loss of function mutation than N2 wild-type (Aballay, 2009; Styer et al., 2008).

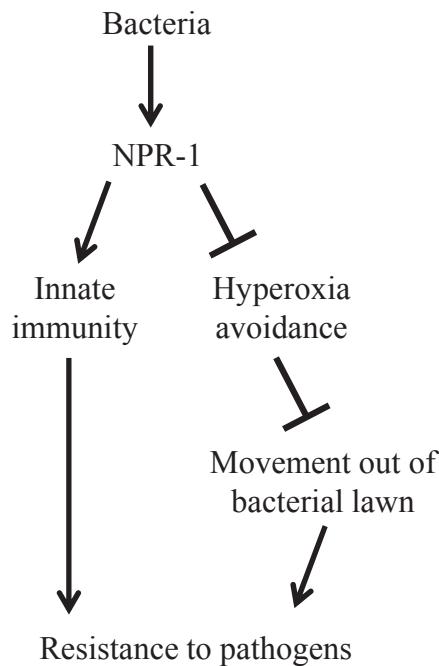


Figure 5: Role of NPR-1 in mediating resistance to pathogens in *C. elegans*. According to Reddy *et al.*, Science, 2009, NPR-1 mediates resistance only through its suppressive effect on hyperoxia avoidance (right panel of the model). In contrast, Styer *et al.*, Science, 2008, suggest that NPR-1 influences both hyperoxia avoidance and innate immunity (left and right panel of the model).

5.7 NPR-1 in *C. elegans*

In *C. elegans*, NPR-1 is a homologue of the mammalian neuropeptide Y receptor and can occur in two different isoforms. These isoforms result from a single amino acid change at position 215 where the amino acid is valine in isoform 215V and phenylalaline in isoform 215F (de Bono and Bargmann, 1998). N2 has the isoform 215V, whereas CB4856 has the isoform 215F. These isoforms differ in their activity level with *npr-1* 215V having a higher activity than *npr-1* 215F (de Bono and Bargmann, 1998).

The *npr-1* polymorphism accounts for variation in foraging behaviour in response to oxygen concentrations. The strains carrying the allele 215V (e.g. N2) disperse evenly across a bacterial lawn showing then a solitary behaviour, whereas strains carrying the

allele 215F (e.g. CB4856) aggregate in clumps and remain at the edge of a bacterial lawn exhibiting a “social” behaviour (de Bono and Bargmann, 1998; Rogers et al., 2006) (Figure 6). Thus *npr-1* seems to be a major determinant of foraging behaviour in *C. elegans*, such as “clumping” and “bordering” expressed in “social strains”. *npr-1* mediates aerotaxis behaviour in response to metabolically active bacteria (de Bono and Bargmann, 1998; Gray et al., 2004). Such behaviours might affect physical exposure of worms toward bacteria including pathogens. For example, social strains might stay more in physical contact with pathogenic bacteria than solitary strains due to their preference for low O₂ concentrations on bacterial lawns. Therefore, this gene might play as well a major role in pathogen avoidance behaviour against a diverse range of pathogen species.

NPR-1 is mainly expressed in the nervous system in approximately 20 neuron types (Coates and de Bono, 2002) and is activated by at least two ligands. Previous findings demonstrated that NPR-1 215F and NPR-1 215V receptors are differentially activated by FMRFamide-related neuropeptides (FaRPs) encoded by the *flp-18* and *flp-21* genes. For example, overexpression of *flp-21* transforms social worms into solitary worms (Rogers et al., 2003).

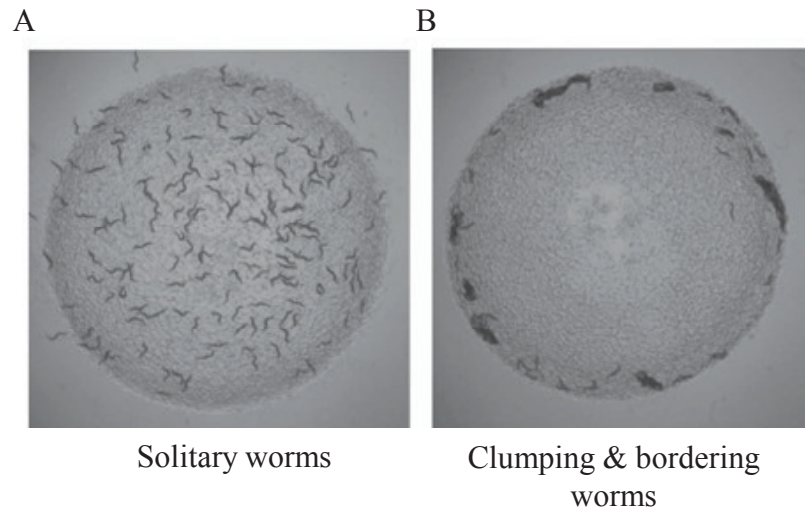


Figure 6: **A.** Solitary behaviour in 150 N2 worms. **B.** Aggregation behaviour in 150 worms carrying *npr-1* loss of function mutation. Photos taken by Macosko *et al.*, Nature, 2009.

5.8 Objectives of the thesis

Objectives of chapter 1

Most studies in the immunological field focused on understanding how host organisms respond to infection by microbes through activation of physiological immune responses, neglecting one important defence manifestation, behavioural responses.

Behavioural defences such as pathogen avoidance responses are seen across animal taxa and are probably of major importance (Schulenburg and Ewbank, 2007). They reduce host contact with pathogenic microbes and the necessity of upregulating costly physiological immune responses. Their genetic basis and the underlying molecular mechanisms remains poorly understood.

In the first manuscript of this thesis, we focus on behavioural defences in *C. elegans* and address the following hypothesis:

i) whether *C. elegans* show comprehensive natural variation in physical pathogen avoidance behaviour against the Gram-positive bacterium *B. thuringiensis*

We therefore used two genetically divergent isolates of *C. elegans* N2 and CB4856 coming from two different environments. N2 was isolated in Bristol, whereas CB4856 was isolated in Hawaii. Therefore, it is likely that N2 and CB4856 have been exposed to different microbial communities including pathogenic bacteria. Thus, N2 and CB4856 might show natural variation in their defence genes resulting from the different selective pressures exerted on these isolates by naturally co-occurring pathogens such as *B. thuringiensis*.

ii) whether *C. elegans* shows specific behavioural responses toward distinct strains of *B. thuringiensis* (BT)

We therefore evaluated leaving behaviour towards pathogenic and non-pathogenic strains of BT

iii) whether pathogen avoidance behaviour is exhibited after a certain time of worms exposure

We therefore scored leaving behaviour at several time points of exposure.

iv) whether there are genetic basis responsible for natural variation in pathogen avoidance behaviour

We therefore used a quantitative genetic approach based on a large-scale analysis of Quantitative Trait Loci (QTLs) using Recombinant Inbred Lines (RILs) and Introgression Lines (ILs) generated from crosses between N2 and CB4856 (Doroszuk et al., 2009; Li et al., 2006) (Figure 7).

The approach we used allows us to:

- enhance our understanding on the evolution of behavioural defences in *C. elegans* by identifying defence genes targeted by parasite-mediated selection

- identify novel central regulators of behavioural defence responses in *C. elegans* which have not been previously covered using a traditional mutagenesis approach
- identify potential pathogen recognition receptors in *C. elegans* as behavioural responses require pathogen recognition
- identify epistatic interactions between different components of defence responses in order to understand their connectivity and function in the whole organism
- reveal how important are trade-offs among different defence system components

Pathogen recognition and synergistic interaction between different immune components have not been yet properly addressed in previous studies on immune defence in *C. elegans*.

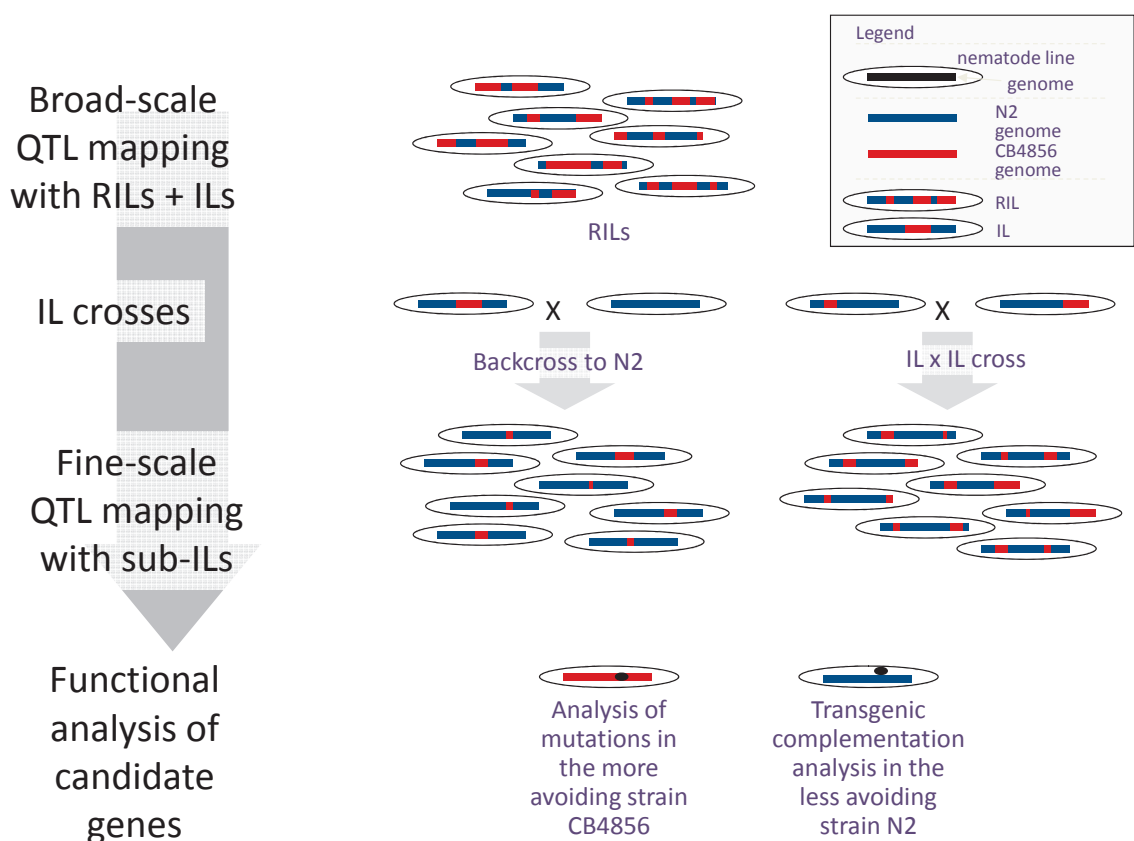


Figure 7: Schematic of the quantitative genetic approach. This methodology was used to characterize natural genetic variation in pathogen avoidance response of *C. elegans*. The figure was prepared by Hinrich Schulenburg.

Objectives of chapter 2

Any host organism can be repeatedly exposed in its natural environment to the same pathogenic microbes which might have negative impact on host's fitness. Therefore there should be selection for mechanisms (e.g. immune priming) that reduce the impact of secondary exposure. The nematode *C. elegans* is one of these host organisms. It proliferates in microbe-rich habitats, meaning that parents and offspring are very likely to develop on the same bacterial patches including pathogenic ones. Hence, trans-generational priming of defence responses in *C. elegans* should reduce impact of repeated infections across generations on worm's fitness; especially those defences which economize animal's resources such as pathogen avoidance behaviour.

In the second manuscript of this thesis, we investigated:

i) whether pathogen avoidance behaviour toward *B. thuringiensis* can be transmitted from parents to offspring

We tested whether offspring coming from parents primed on pathogenic BT would show a higher leaving response against BT compared with offspring of naïve parents.

ii) whether *C. elegans* shows natural variation in trans-generational priming of avoidance behaviour.

We used the two genetically divergent isogenic isolates N2 and CB4856 which differ in their avoidance responses toward BT. We tested whether the *C. elegans* isolate with a low leaving response (e.g. N2, shown in chapter 1) uses trans-generational priming of avoidance behaviour in order to enhance behavioural defences in their offspring against pathogenic BT.

Objectives of chapter 3

The insulin-like signaling pathway (DAF-2/DAF-16) was previously shown to mediate behavioural and physiological defences in *C. elegans* (Chávez et al., 2007; Evans et al.,

2008 (a); Evans et al., 2008 (b); Hasshoff et al., 2007; Mohri-Shiomi and Garsin, 2008). Until recently, it was still unclear whether DAF-16 nuclear translocation (and thus regulatory activity) is directly induced by pathogens, or indirectly through other cues such as heat stress and starvation.

To answer this question, we tested in this study DAF-16 activation by pathogenic *B. thuringiensis* through its experimental overexpression. Such approach provides us a direct observation of DAF-16 localization which was related in our study to the level of pathogenicity and infectivity of BT. We used different strains of pathogenic BT to compare their ability in infecting and killing *C. elegans*. We scored survival and infection load as a proxy for physiological immunity and oral uptake for behavioural avoidance.

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6. Chapter 1:

The mysterious role of the *C. elegans* nuclear hormone receptor NPR-1 in pathogen defence

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6.1 Abstract

The nematode *Caenorhabditis elegans* feeds on microbes in its natural environment. Some of these microbes are pathogenic and thus harmful to *C. elegans*. To minimize resulting fitness reductions, *C. elegans* has evolved various defence mechanisms including behavioural responses (e.g. avoidance behaviour) that reduce contact with the infectious microbes. In this study, we characterized the genetic architecture of natural variation in *C. elegans* avoidance behaviour against the infectious stages of the Gram-positive bacterium *Bacillus thuringiensis*. We performed an analysis of quantitative trait loci (QTLs) using recombinant inbred lines (RILs) and introgression lines (ILs) generated from a cross of two genetically as well as phenotypically distinct natural isolates N2 and CB4856. The analysis identified several QTLs that underlie variation in the behavioural response to pathogenic and/or non-pathogenic bacteria. One of the candidates is the *npr-1* gene. This gene encodes a homolog of the mammalian neuropeptide receptor. *Npr-1* was previously indicated to fully contribute to behavioural defence against the Gram-negative bacterium *Pseudomonas aeruginosa* and food patch-leaving behaviour on *Escherichia coli*. Interestingly, in our study, *npr-1* is not the only gene mediating avoidance behaviour toward *Bacillus thuringiensis*. Moreover, our functional analyses show that *npr-1* alleles appear to influence survival and avoidance behaviour toward *Bacillus thuringiensis* in exactly the opposite way than toward *Pseudomonas aeruginosa*. Our findings highlight the role of *npr-1* in fine-tuning nematode behaviour in an ecological context depending on the microbe to which *C. elegans* is exposed.

6.2 Introduction

Pathogen avoidance behaviour is found in most living organisms as a consequence of past selection by pathogenic antagonists upon host parasite-interaction (e.g. host-parasite coevolution). During such a type of interaction, hosts have evolved behavioural defences including pathogen avoidance behaviour required for reducing contact with pathogens and avoiding contagion (Loehle, 1997). Avoidance behaviour can have various manifestations. Examples include: guarding nest entrance in honeybees (Arechavaleta-Velasco and Hunt, 2003; Moore et al., 1987), avoiding infected corpses in termites (Kramm et al., 1982), waste management in ants (Cremer et al., 2007), avoidance of contaminated flowers by bumblebees (Fouks and Lattorff), avoidance of parasitoid wasp-infested sites by *Drosophila melanogaster* and *D. simulans* (Lefevre et al., 2012), increased fly switching by elephants when flies are present in order to reduce the number of blood-sucking flies (reviewed in (Hart, 2011)), evading, dropping or rejection of disgusting objects in humans (Angyal, 1941; Nordin et al., 2004; Rozin and Fallon, 1987).

Behavioural defences require pathogen recognition and are thought to be advantageous for living organisms as they economize animals' resources by reducing their contact with pathogens (thus the inflicted tissue damage), and by saving the highly costly energy invested upon activation of physiological immune responses (reviewed in (Schulenburg and Ewbank, 2007)) Therefore, behavioural defences should lead to fitness advantages in animals susceptible to encountered pathogens (Kiesecker et al., 1999). In spite of their potential benefits, behavioural defences have been largely neglected in the immunological field until the last few years, as most of the research was focusing on physiological immunity. Only recently, several studies focused on pathogen avoidance behaviour in

order to provide more insight into these defence mechanisms and to decipher the underlying genetics which are still to date not well characterized.

One of the most promising model organisms to reveal the underlying genetics of behavioural defences is the nematode *Caenorhabditis elegans*. In the wild, *C. elegans* colonizes microbe rich habitats as rotting fruits and decaying plant materials where it feeds on bacteria and yeasts (Felix and Braendle, 2010). These microorganisms can be pathogenic to the worms; therefore *C. elegans* evolved an immune defence system consisting of: i) physiological responses involving antimicrobial factors and various signaling processes reviewed in (Bogaerts et al., 2010; Ewbank and Zugasti, 2011; Irazoqui et al., 2010) ii) physical barriers like the cuticle (Cox et al., 1981), iii) behavioural responses reviewed in (Schulenburg and Ewbank, 2007). In its natural environment, we still lack the knowledge about the different responses (e.g. behavioural defences) *C. elegans* may exhibit towards pathogenic microorganisms. In contrast, under laboratory conditions, several studies showed already that *C. elegans* exhibits different types of pathogen avoidance behaviours. These include: i) physical avoidance of different pathogens, including for example the Gram-negative bacterium *Serratia marcescens*, mediated by G-protein coupled receptors expressed in chemosensory neurons (Pradel et al., 2007), and also the Gram-positive bacterium *Bacillus thuringiensis*, in this case influenced by insulin-like signaling (Hasshoff et al., 2007). ii) Associative learning of avoidance towards the Gram-negative bacterium *Pseudomonas aeruginosa* through an increase of serotonin production in chemosensory neurons (Zhang et al., 2005). iii) Reduced oral uptake on pathogenic *B. thuringiensis* (Hasshoff et al., 2007; Wang et al.) and on the pathogens *Pseudomonas aeruginosa*, *Burkholderia pseudomallei* and *Yersinia pestis* (Smith et al., 2002).

Caenorhabditis elegans does not only show avoidance behaviour towards different parasites, it even exhibits specific behavioural responses upon exposure to different types of Serrawettin surfactants produced by *S. marcesens*. The worms are attracted by Serrawetin W1 and W3, whereas they are repelled by Serrawetin W2 (Pradel et al., 2007). Such molecule-specific behavioural responses are consistent with the recent demonstrations of high specificity in the innate immune system of invertebrates (reviewed in (Kurtz, 2004; Schulenburg et al., 2007)), the characteristic which was for a long time only attributed to the adaptive immune system in vertebrates.

Natural variation in pathogen defence between different *C. elegans* strains is consistent with the idea of high immune specificity. In particular, previous studies demonstrated: i) significant variation in host susceptibility of different *C. elegans* isolates towards *Serratia marcesens*, of which the underlying genetics remain unknown (Schulenburg and Ewbank, 2004), ii) significant differences in survival, infection rate, oral uptake and physical pathogen avoidance behaviour between different *C. elegans* isolates upon exposure to *Bacillus thuringiensis*; with unrevealed underlying genetics (Schulenburg and Muller, 2004), iii) significant differences in survival and ingestion rate between two isolates of *C. elegans* exposed to *Photorhabdus luminescens* where the genetics of these natural variation remains unknown (Sicard et al., 2007), iv) significant differences in pathogen avoidance behavior between *C. elegans* strains towards *Pseudomonas aeruginosa* due to two distinct polymorphisms in the gene *HECW-1* (Chang et al., 2012). None of these studies addressed the genetic architecture of these natural variation underlying physiological or behavioural immune responses in *C. elegans*. To our knowledge, only a single study characterized the genetics of natural resistance variation against the pathogen *P. aeruginosa*. This study identified the involvement of the neuropeptide receptor gene *npr-1* to account for resistance through mediation of aerotaxis behaviour (Reddy et al., 2009). This finding

came in consistence with a previous study demonstrating a role of *npr-1* in variation in resistance against *P. aeruginosa* not only through aerotaxis behaviour but also a direct role of this gene in physiological immune responses (Styer et al., 2008). NPR-1 is a homologue of the mammalian neuropeptide Y receptor for which two different isoforms have been characterized in *C. elegans*. These isoforms result from a single amino acid change at position 215 where the amino acid is valine in isoform 215V and phenylalaline in isoform 215F (de Bono and Bargmann, 1998). This natural variation was shown to be responsible for differences in foraging behaviour in response to oxygen concentrations between different isolates of *C. elegans*. The strains carrying the allele 215V disperse evenly across a bacterial lawn showing then a solitary behaviour, whereas strains carrying the allele 215F accumulate in clumps and remain at the edge of a bacterial lawn exhibiting a social behaviour (de Bono and Bargmann, 1998; Rogers et al., 2006). The effect of *npr-1* polymorphism on foraging behaviour and variation in pathogen resistance was characterized using mostly the two *C. elegans* isolates N2 (solitary strain) and CB4856 (social strain).

To identify genetic loci underlying natural variation in pathogen avoidance behaviour, we used in this study a quantitative genetic approach based on a screen of 200 recombinant inbred lines (RILs) and 90 introgression lines (ILs) generated from crosses between isolates N2 from Bristol and CB4856 from Hawaii, which show very large genetic differentiation within the genotype network of *C. elegans* isolates (Doroszuk et al., 2009; Li et al., 2006). We used as a microparasite the Gram-positive bacterium *B. thuringiensis*. This bacterium is soil dwelling and could previously be isolated together with *C. elegans* from a compost heap in France, indicating that *C. elegans* and *B. thuringiensis* coexist in nature (MA Félix and P. Dirksen, personal communication). Some *B. thuringiensis* (BT) strains can produce toxins with a specific pathogenic effect towards nematodes including

C. elegans (Borgonie et al., 1996; Borgonie et al., 1995). BT infection occurs after oral uptake of toxins associated to spores which accumulates in the gut and then binds specifically to receptors on intestinal membranes forming then pores into the intestinal cells and leading to the lysis of the gut thus the death of the worm (Griffitts and Aroian, 2005; Wei et al., 2003). Little information is known about the genetics of defence mechanisms used by *C. elegans* against *B. thuringiensis*. These mechanisms involve: i) the insulin-like signaling pathway for behavioural and physiological defences (Hasshoff et al 2007), ii) two mitogen-activated protein kinase (MAPK) pathways (namely the p-38 and the JNK-like MAPK) in addition to the activating protein 1 (AP-1) transcription factor (Huffman et al., 2004; Kao et al., 2011) and at least three lysozymes, LYS-2 , LYS-5, LYS-7 (Boehnisch et al., 2011) for physiological immune responses. Nevertheless, the genetics of natural variation in defence responses against *B. thuringiensis* is still poorly understood.

Our results from the QTL analysis identified *npr-1* as one of the candidate genes for natural variation in pathogen avoidance behaviour. Therefore, we investigate for the first time in this study specifically the role of *npr-1* in mediating behavioural and physiological immune defences against *B. thuringiensis* with a comparative analysis of this gene's effect as well on defence against the Gram-negative bacterium *Pseudomonas aeruginosa*.

6.3 Results

Two *C. elegans* wild-type strains differ significantly in bacterial lawn leaving behaviour

Variation in behavioural defences was assessed with lawn leaving assays (Figure S1) using two nematocidal *B. thuringiensis* strains B-18247 and B-18679 and two non nematocidal bacteria *B. thuringiensis* strain DSM350 and *Escherichia coli* strain OP50 as controls. Leaving was scored at different time points of worms' exposure.

N2 and CB4856 differed significantly in avoidance of both pathogenic and non-pathogenic *B. thuringiensis* as well as *E. coli* (Figure 1). Lawn leaving behaviour was significantly higher for CB4856 compared to N2 on all tested bacterial strains and for all exposure time periods (Table S1). For both *C. elegans* strains, we observed a significant increase of leaving across time points (Figure 1). Importantly, for N2 avoidance response was stronger towards the two pathogens (B-18247 & B-18679) compared to the two controls (DSM 350 & OP50) especially at the two later time points of exposure (14h & 24h) (Table S2).

The observed pathogen avoidance response was even more pronounced for N2 and CB4856 towards the most pathogenic strain (B-18679) than the less pathogenic strain (B-18247) (Table S2, S3).

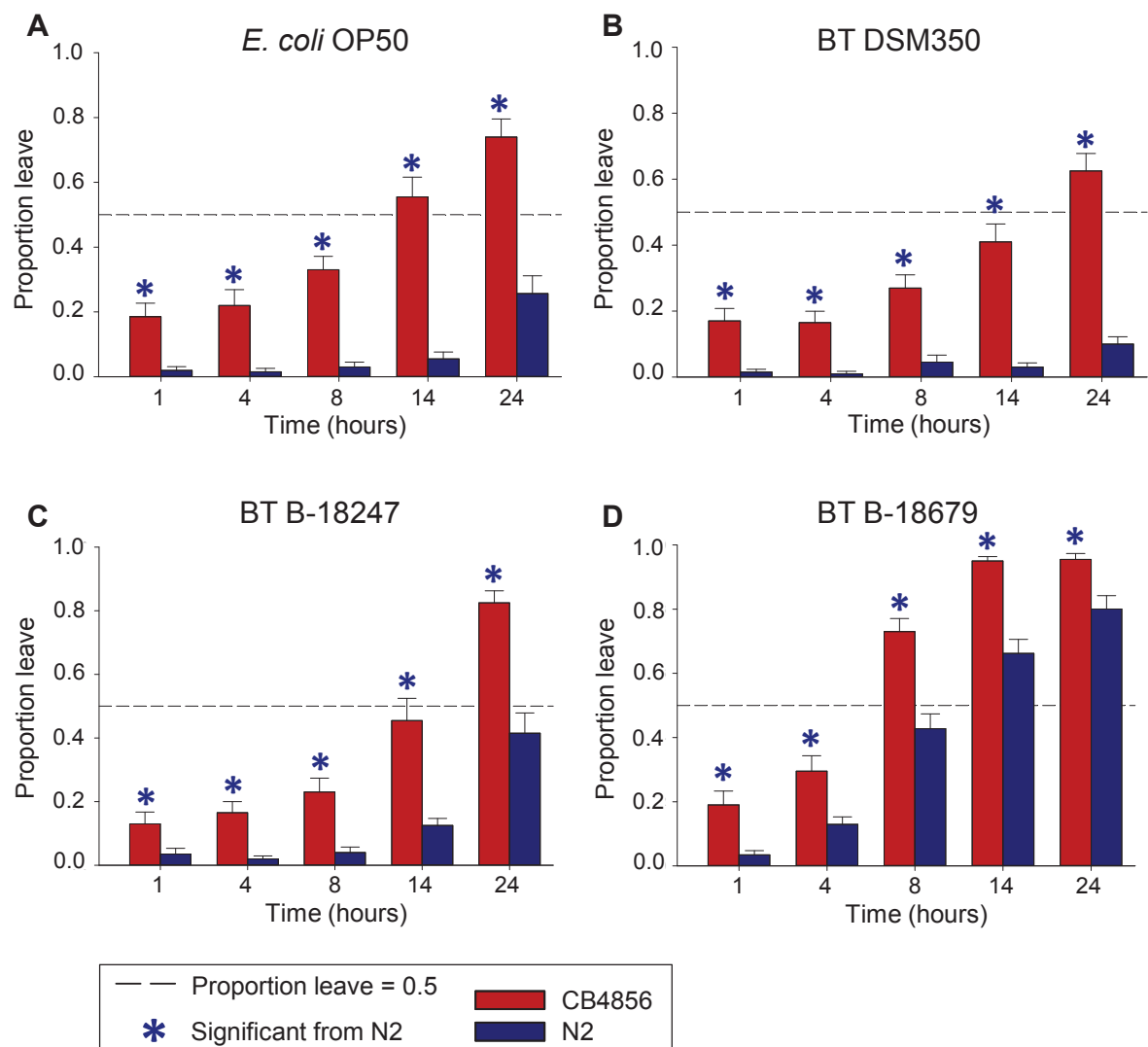


Figure 1. Lawn leaving behaviour was scored for N2 and CB4856 at different time points against **A.** *E. coli* strain OP50 as a control. **B.** Non nematocidal *B. thuringiensis* strain DSM350 as a control. **C.** Nematocidal *B. thuringiensis* strain B-18247. **D.** Highly nematocidal *B. thuringiensis* strain B-18679. The Y axis shows proportion of N2 (blue) or CB4856 (red) nematodes which left the bacterial lawn; the X axis the different exposure time points. The asterisk indicates the treatments for which leaving behaviour of CB4856 is significantly different from N2. The dotted reference line shows a proportion of leaving of 0.5. Error bars represent standard error of the means. Detailed statistical results are shown in Supplementary Table S1.

Multiple quantitative trait loci (QTLs) and their epistatic interactions account for natural variation in avoidance behaviour

The genetic architecture of the observed variation in avoidance behaviour was characterized with the help of a total of 200 recombinant inbred lines (RILs) and 90 introgression lines (ILs) (Doroszuk et al., 2009; Li et al., 2006). The avoidance response was studied towards the two pathogenic *B. thuringiensis* strains (B-18247 & B-18679) and the two controls BT DSM350 and *E. coli* OP50. The lines were screened in parallel using a randomized block design and including their parental strains N2 and CB4856. Avoidance response was scored at two different time points of exposure (14h and 24h) at which the response of N2 and CB4856 was particularly pronounced (see above; Figure 1).

Our QTL analysis uncovered several regions with strong effect on avoidance behaviour: i) one region on chromosome II for which the CB4856 allele(s) affect(s) food patch leaving behaviour only at time point 24h exposure (Figure 2A, B); ii) a different region on chromosome II for which the N2 allele(s) affect(s) specifically avoidance of the more pathogenic strain B-18679 especially at time point 24h exposure (Figure 2D); iii) one region on the left arm of chromosome IV for which the N2 allele(s) affect(s) leaving behaviour towards food and pathogen patches at the two exposure time points (14h & 24h) (Figure 2A, B, C, D); iv) one region on the right arm of chromosome IV for which the CB4856 allele(s) affect(s) specifically pathogen avoidance behaviour at both time points of exposure (14h & 24h) (Figure 2C, D); v) one region on chromosome X with the strongest effect on leaving behaviour towards food and pathogen patches (Figure 2A, B, C, D) for which the CB4856 allele(s) has or have an effect at 14h & 24h of respective exposure but with a significant time effect on pathogen avoidance responses (Figure 2C, D). The very strong effect of the CB4856 region on chromosome X was confirmed by the ILs with the respective introgression (Figure S2C, D).

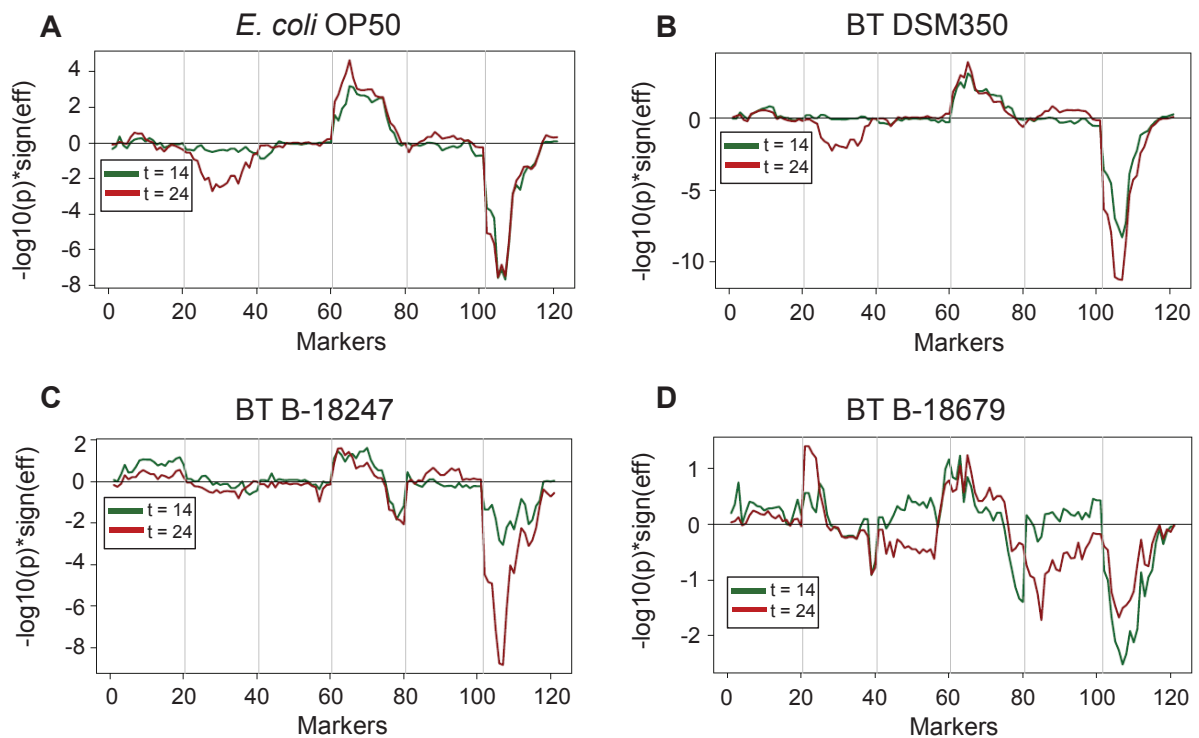


Figure 2. QTL profiles for single marker mapping for avoidance behaviour of A. *E. coli* strain OP50. B. Non-nematocidal *B. thuringiensis* strain DSM350. C. Nematocidal *B. thuringiensis* strain B-18247. D. Highly nematocidal *B. thuringiensis* strain B-18679. The X-axis shows the markers along the five autosomes and the X chromosome. The Y-axis indicates the association between the chromosomal markers and variation in avoidance. A value above 0 indicates an influence of the N2 allele; a value below 0 that of the CB4856 allele. By convention, values above +2 or below -2 are considered to indicate a significant influence. Green and red lines show the results after either 14h or 24h exposure, respectively.

To assess the possible influence of epistatic interactions between QTLs, we used a standard interaction model: $\text{phenotype} \sim \text{time} + \text{marker1} * \text{marker2}$. The analysis revealed several interaction effects for the different bacteria. For *E. coli*, we found at least significant interactions between: i) the beginning of chromosome I and the first half of chromosome X (Figure 3A); and ii) the end of chromosome II and almost the entire IV chromosome (Figure 3A). For BT DSM350, the analysis identified interaction effects between: i) the end of chromosome IV and the first quarter of chromosome X (Figure 3B); and ii) the end of chromosome II and almost the entire IV chromosome (Figure 3B). The latter one seems

to be specific for food patch leaving behaviour as it was identified for both of the non-pathogenic bacteria *E. coli* OP50 and BT DSM350 (Figure 3A, B). For the nematocidal BT B-18247 we found interaction effects at least between: i) the beginnings of chromosome II and V (Figure 3C); and ii) the second quarter of chromosome II and the beginning of chromosome X (Figure 3C). For the highly nematocidal BT B-18679, several interaction effects were identified including among others between: i) the ends of chromosome I and II (Figure 3D); and ii) the second quarter and the middle of chromosome IV (Figure 3D).

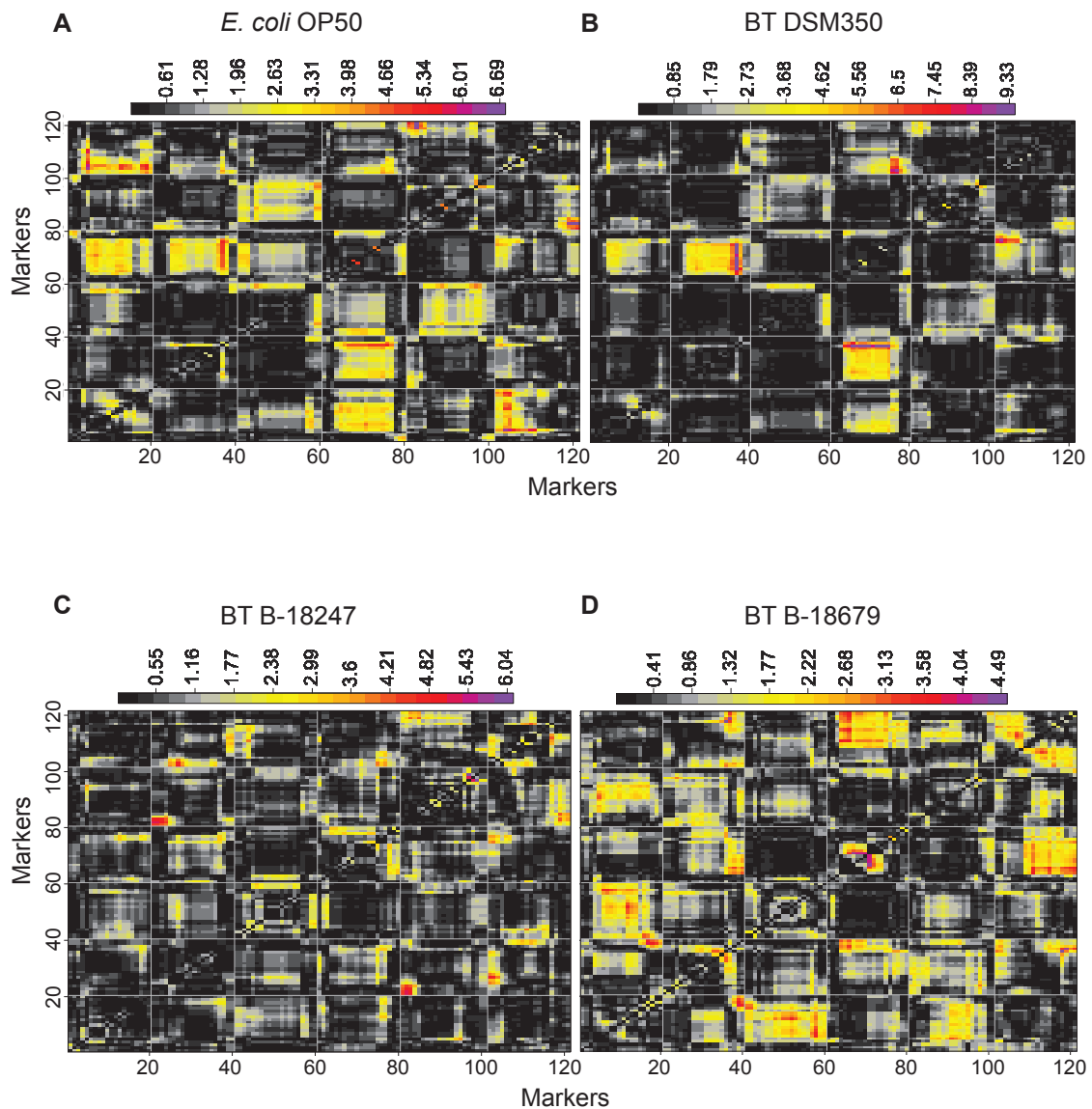


Figure 3. Heat-map of interaction effects for avoidance behaviour of A. *E. coli* strain OP50. B. Non-nematocidal *B. thuringiensis* strain DSM350. C. Nematocidal *B. thuringiensis* strain B-18247. D. Highly nematocidal *B. thuringiensis* strain B-18679. The distribution of markers across the genome is shown on both axis X and Y. Colour legend for significance of the interaction between markers is shown on top of the panels.

Significant influence of the *npr-1* gene from the X chromosome on avoidance behaviour

As the QTL on chromosome X with the strongest effect on leaving behaviour encompasses the gene *npr-1*, the exact role of this gene was tested with the help of two different alleles, *npr-1(ad609)* and *npr-1(ur89)*. The loss of *npr-1* function in these mutants was previously shown to decrease pathogen avoidance behaviour against the Gram-negative bacterium *P. aeruginosa* (Reddy et al., 2009). We furthermore tested the involvement of the gene *tyra-3* on leaving behaviour using the knock-out mutant *tyra-3(ok325)*. This gene was previously demonstrated to influence food patch leaving behaviour (Bendesky et al., 2012). Its possible contribution to pathogen avoidance behaviour has not as yet been assessed.

Interestingly, we found opposite behavioural phenotypes of the two tested *npr-1* alleles (Figure 4). Similarly to CB4856, the loss of function mutant *npr-1(ur89)* showed higher leaving behaviour than N2 on food (*E. coli* & BT DSM350) (Figure 4A, B) at both time points 14h and 24h (Table S4). *npr-1(ad609)* always showed a low food patch leaving behaviour similar to the wild type N2 (Figure 4A, B, Table S5). On the pathogen B-18247, we saw the same pattern of avoidance behaviour as on the controls with a very pronounced response at time point 24h of exposure (Figure 4C). The mutant *npr-1(ur89)* avoided this strain more than N2 at 14h and 24h (Table S4), whereas the mutant *npr-1(ad609)* had always a similar avoidance response to N2 (Table S5). Surprisingly, on the highly pathogenic strain BT B-18679 we didn't see any significant differences in avoidance behaviour between the tested *C. elegans* strains (Figure 4D, Table S4, S5).

The mutant *tyra-3* always showed a similar response than the wild type N2, irrespective of the bacterium and the exposure time (Figure 4, Table S6).

Unexpectedly, at time point 14h of exposure CB4856 did not show a significantly higher leaving behaviour on *B. thuringiensis* (B-18247, B-18679 & DSM350) compared to N2

(Figure 4B, C, D, Table S7) in contrast to our earlier results (Figure 1B, C, D). However, the differences between these two strains were again highly significant after 24h exposure (Figure 4B, 4C, 4D, Table S7).

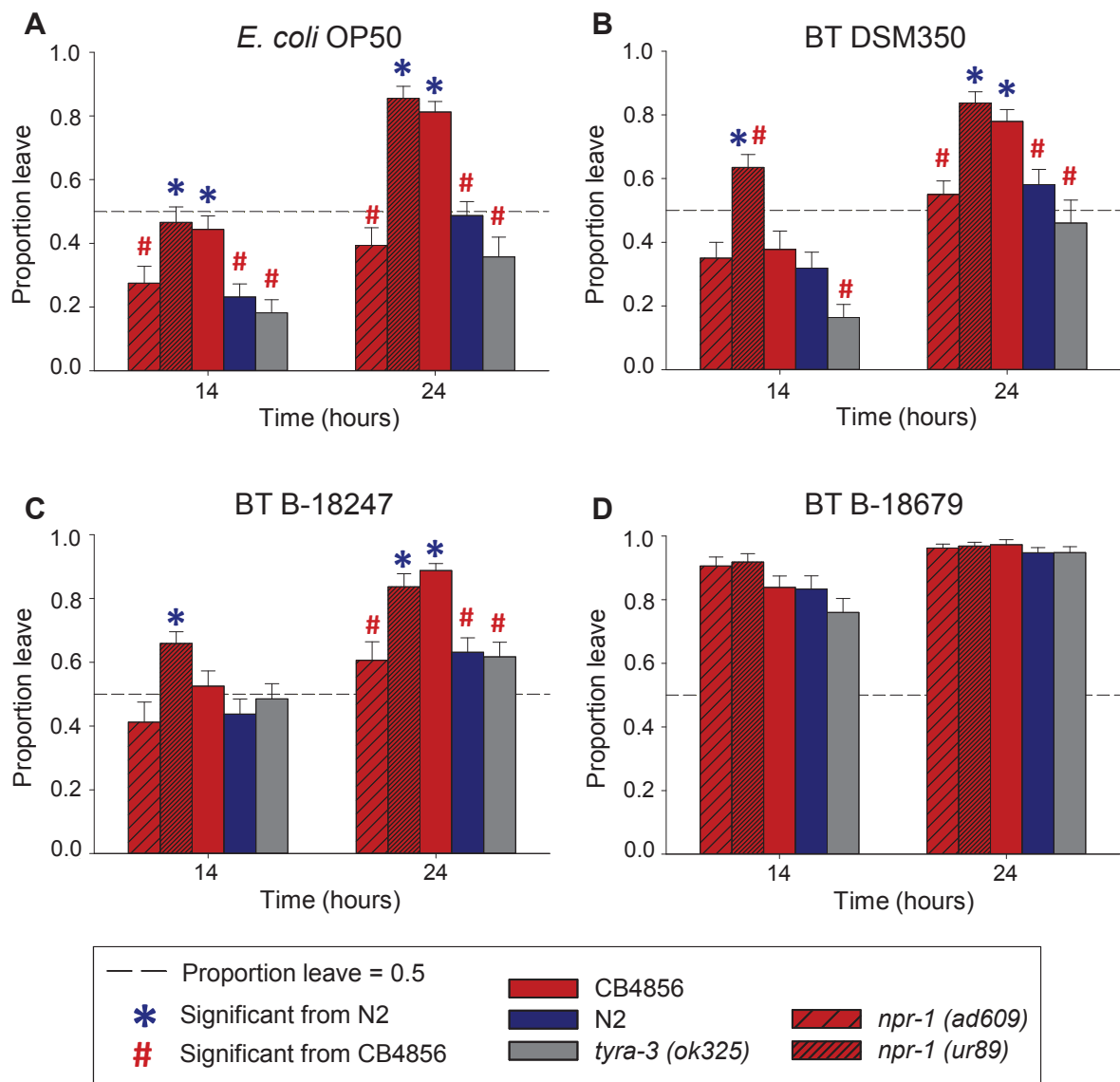


Figure 4. Lawn leaving behaviour was scored for N2, CB4856, two *npr-1* mutants and one *tyra-3* mutant at two different time points against **A.** *E. coli* strain OP50. **B.** Non nematocidal *B. thuringiensis* strain DSM350. **C.** Nematocidal *B. thuringiensis* strain B-18247. **D.** Highly nematocidal *B. thuringiensis* strain B-18679. The Y axis shows the proportion of escaped worms; the X axis shows the two time points, at which leaving was scored. The asterisk (*) is shown for the treatments which differ significantly from N2 whereas the hash sign (#) indicates significant differences from CB4856. The dotted reference line shows a leaving proportion of 0.5. Error bars represent standard error of the means. Detailed statistical results are given in Supplementary Table S4, S5, S6 & S7.

Positive relationship between avoidance behaviour and survival on pathogenic *B. thuringiensis*

We assessed the relationship between avoidance behaviour and survival rate, the latter of which is often used as a proxy for immunity. We performed survival assays on the same bacterial strains we used during our lawn leaving assays. We first focused on N2 and CB4856 and identified significantly higher resistance in CB4856 compared to N2 on both pathogenic strains (Figure 5A, B, Table 1). On the controls BT DSM350 (Figure S3) and *E. coli* OP50 (data not shown), we didn't see any mortality or any differences in survival between N2 and CB4856 (Figure S3, Table 1).

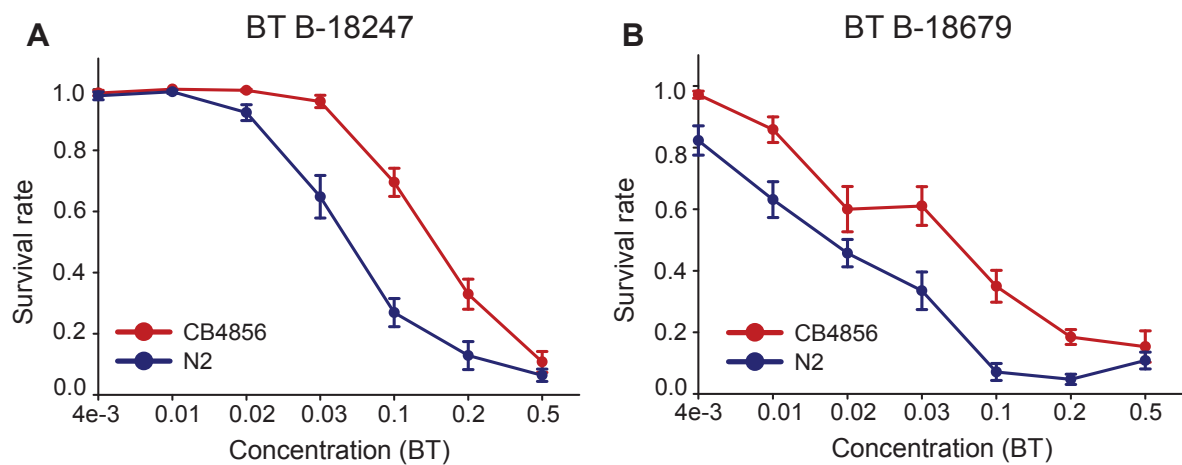


Figure 5. Survival of N2 and CB4856 on A. Nematocidal *B. thuringiensis* strain B-18247. **B.** Highly nematocidal *B. thuringiensis* strain B-18679. Survival on the Y axis was plotted against BT concentration on the X axis. Error bars represent standard error of the means.

Table 1. Statistical analysis of survival rate for the comparison of N2 with CB4856 on three different bacteria¹

Bacterium ¹	Factor ²	Survival	
		χ^2	<i>p</i>
B-18247	<i>C. elegans</i> strain	4.36	0.0367
	BT concentration	49.22	<0.0001
	Interaction (BT conc. x <i>C. elegans</i>)	0.088	0.7657
B-18679	<i>C. elegans</i> strain	4.6	0.0319
	BT concentration	27.58	<0.0001
	Interaction (BT conc. x <i>C. elegans</i>)	0.72	0.3948
DSM350	<i>C. elegans</i> strain	0.77	0.3799
	BT concentration	1.15	0.2818
	Interaction (BT conc. x <i>C. elegans</i>)	1.8	0.1792

¹ Bacteria, for each of which the analysis was done separately.

² Factors for which effect tests were calculated as a part of a general linear model, using ordinal logistic regression analysis. Degrees of freedom (DF) = 1 for all effect tests. Significant probabilities are given in bold. Significance level was adjusted using Bonferroni correction for multiple pairwise comparisons.

We next repeated the survival analysis for the *npr-1* mutants. The survival assays confirmed the higher resistance previously shown by CB4856 compared to N2 on both pathogenic BT strains (Figure 6, Table 2, 3). On B-18247, the mutant *npr-1(ur89)* was significantly more resistant than N2 (Figure 6A, Table 2) and as resistant as CB4856 (Figure 6A, Table S8). On B-18679, this mutant similarly did not differ in survival to CB4856 (Table S9) but whereas it was significantly more resistant than N2 (Figure 6B, Table 3). The mutant *npr-1(ad609)* was as susceptible as N2 on both pathogenic strains B-18247 (Figure 6A, Table 2) and B-18679 (Figure 6B, Table 3). We did not see any mortality on the control bacteria DSM350 (Figure S4) and *E. coli* (data not shown).

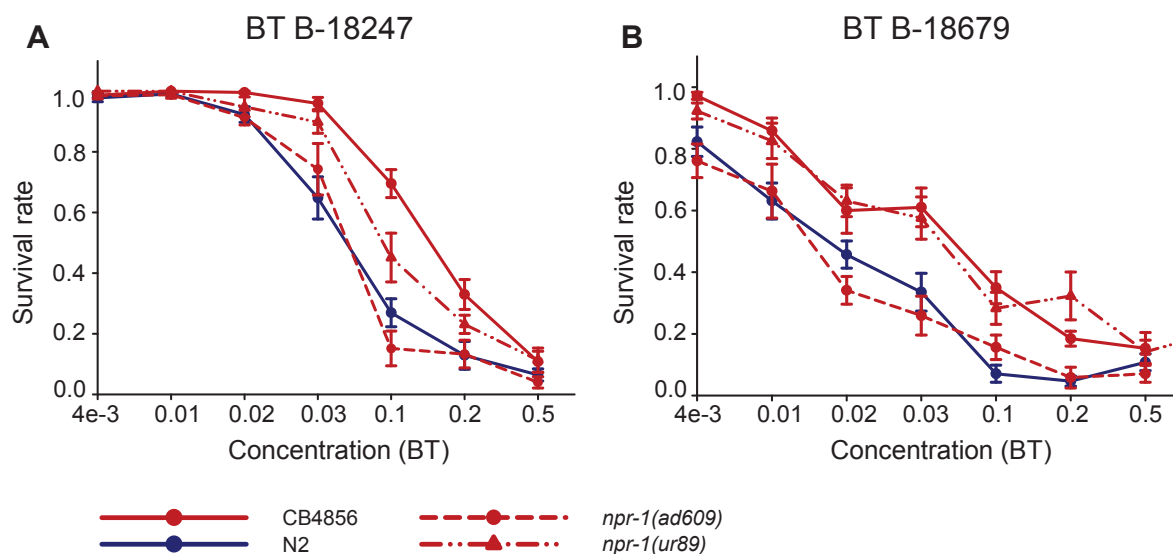


Figure 6. Survival of N2, CB4856, *npr-1(ad609)* and *npr-1(ur89)* on **A.** Nematocidal *B. thuringiensis* strain B-18247. **B.** Highly nematocidal *B. thuringiensis* strain B-18679. Survival on the Y axis was plotted against BT concentration on the X axis. Error bars represent standard error of the means.

Table 2. Statistical analysis of survival rate on BT B-18247 for the comparison of N2 with either CB4856^(a) or *npr-1(ur89)*^(b) or *npr-1(ad609)*^(c)

Bacterium ¹	Factor ²	Survival	
		χ^2	<i>p</i>
B-18247	N2 vs. CB4856 ^(a)	16.8	<0.0001
	BT concentration	114.99	<0.0001
	Interaction (BT conc. x <i>C. elegans</i>)	0.118	0.7307
	N2 vs. <i>npr-1(ur89)</i> ^(b)	6.6	0.0102
	BT concentration	105.66	<0.0001
	Interaction (BT conc. x <i>C. elegans</i>)	0.11	0.7361
	N2 vs. <i>npr-1(ad609)</i> ^(c)	0.3	0.5813
	BT concentration	87.31	<0.0001
	Interaction (BT conc. x <i>C. elegans</i>)	1.48	0.2233

¹ Bacteria, for each of which the analysis was done separately.

² Factors for which effect tests were calculated as a part of a general linear model, using ordinal logistic regression analysis. Degrees of freedom (DF) = 1 for all effect tests. Significant probabilities are given in bold. Significance level was adjusted using Bonferroni correction for multiple pairwise comparisons.

Table 3. Statistical analysis of survival rate on BT B-18679 for the comparison of N2 with either CB4856^(a) or *npr-1(ur89)*^(b) or *npr-1(ad609)*^(c)

Bacterium ¹	Factor ²	Survival	
		χ^2	<i>p</i>
B-18679	N2 vs. CB4856 ^(a)	14.5	0.0001
	BT concentration	59.2	<0.0001
	Interaction (BT conc. x <i>C. elegans</i>)	1.04	0.3066
	N2 vs. <i>npr-1(ur89)</i> ^(b)	14.42	0.0001
	BT concentration	52.72	<0.0001
	Interaction (BT conc. x <i>C. elegans</i>)	0.21	0.641
	N2 vs. <i>npr-1(ad609)</i> ^(c)	1.27	0.2584
	BT concentration	1.54	0.2133
	Interaction (BT conc. x <i>C. elegans</i>)	3.18	0.0744

¹ Bacteria, for each of which the analysis was done separately.

² Factors for which effect tests were calculated as a part of a general linear model, using ordinal logistic regression analysis. Degrees of freedom (DF) = 1 for all effect tests. Significant probabilities are given in bold. Significance level was adjusted using Bonferroni correction for multiple pairwise comparisons.

Opposite avoidance and survival phenotypes are expressed against the Gram-negative pathogen *Pseudomonas aeruginosa*

Previous work reported an opposite effect of the wild-type and also *npr-1* alleles towards *P. aeruginosa* PA14. We re-evaluated the previous analyses under our laboratory conditions. We first used the lawn leaving assay to assess directly the expression of an avoidance response against PA14. We began with a comparison of the *C. elegans* wild-type strains N2 and CB4856 at four different exposure time points (8h, 14h, 24h & 48h). On PA14, N2 showed for all but one time point (exception refers to 8h exposure) a significantly higher avoidance response compared to CB4856 (Figure 7B, Table 4). No significant variation was observed on the *E. coli* control (Figure 7A, Table 4). Interestingly, N2 expressed a significantly higher avoidance response against PA14 than against *E. coli* (significant effect from 14h onwards; Figure 7, Table S10). In contrast,

avoidance behaviour of CB4856 did not vary significantly between bacterial strains (Figure 7, Table S10).

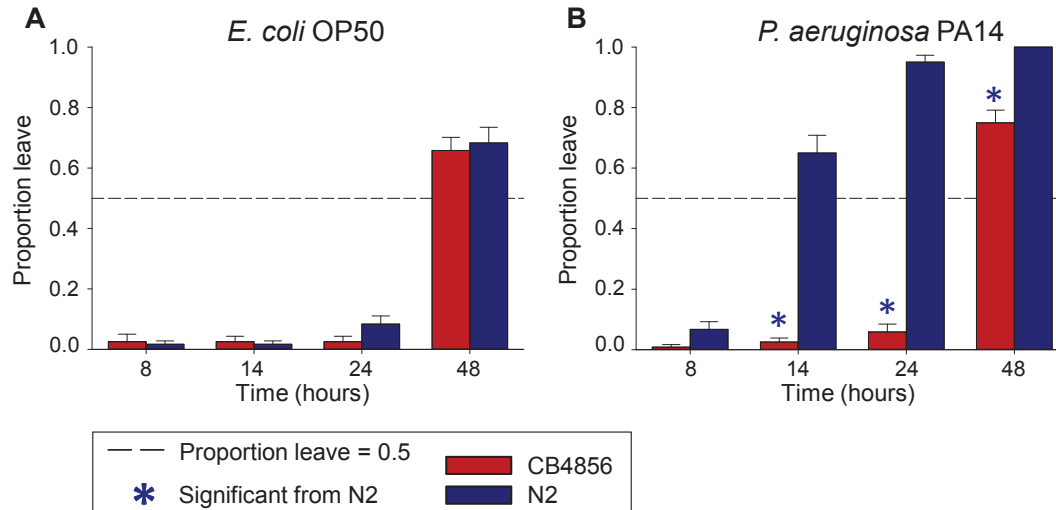


Figure 7. Lawn leaving behaviour was scored for N2 and CB4856 at four different time points against **A.** *E. coli* strain OP50. **B.** *P. aeruginosa* strain PA14. The asterisk (*) points to treatments for which leaving behaviour is significantly different from N2. The dotted reference line indicates the 0.5 avoidance response. Statistical results are shown in Table 4.

Table 4. Comparison between N2 and CB4856 as to their leaving behaviour from two different bacteria² at different time points¹

Time point ¹	Bacteria ²	Proportion leave	p
		χ^2	
8 h	PA14	3,7681	0,0522
	OP50	0,2525	0,6153
14 h	PA14	18,4	<0.0001
	OP50	0,0079	0,929
24 h	PA14	18,5028	<0.0001
	OP50	3,0023	0,0831
48 h	PA14	15,1565	<0.0001
	OP50	0,3139	0,5753

¹ Time point, for each of which the analysis was done separately.

² Bacteria for which effect tests were calculated, using the non parametric test Kruskal Wallis. Degrees of freedom (DF) = 1 for all effect tests. Significant probabilities are given in bold. Significance level was adjusted using Bonferroni correction for multiple pairwise comparisons.

We subsequently tested the influence of *npr-1* and *tyra-3*. On PA14, both *npr-1* mutants and CB4856 expressed significantly lower pathogen avoidance than N2 across all time points (Figure 8B, Table S11, S12 & S13). For the 48h time point the mutant *npr-1(ad609)* even had a lower leaving response than CB4856 (Figure 8B). On OP50, leaving behaviour was similar for all *C. elegans* strains at all time points of exposure except at time point 14h, when the mutant *npr-1(ad609)* showed a more pronounced leaving behaviour than N2 (Figure 8A, Table S12).

The gene *tyra-3* had a similar leaving response to N2 independently from the bacterium or the time point of exposure except at time point 48h where it showed a lower leaving response against PA14 compared to N2 (Figure 8B, Table S14).

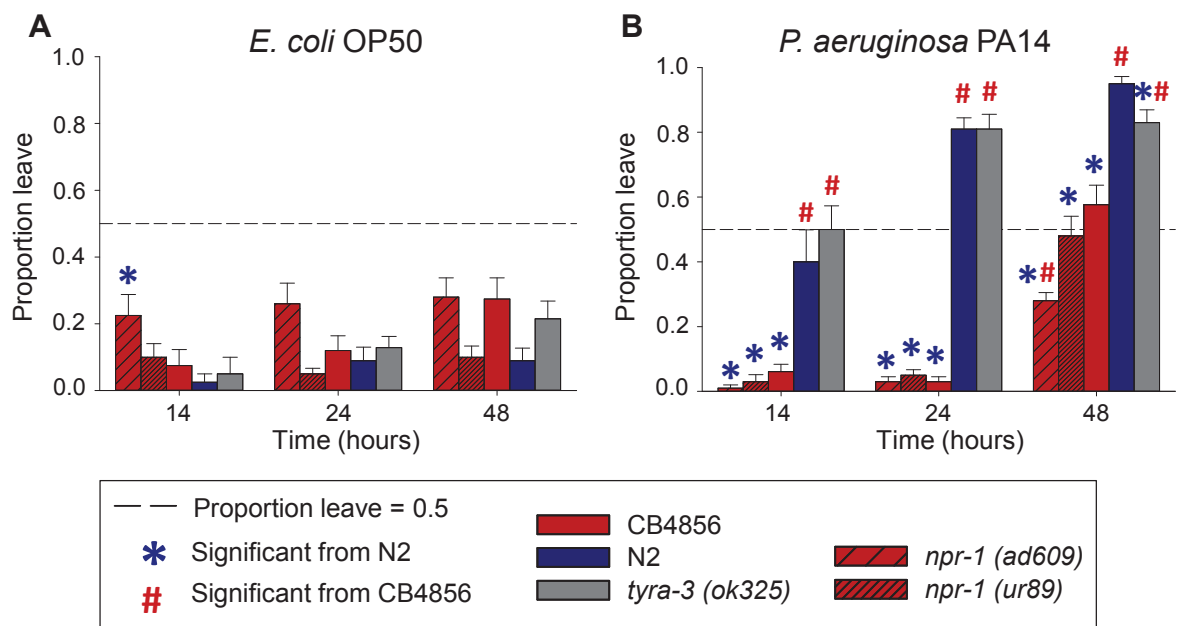


Figure 8: Lawn leaving behaviour was scored for N2, CB4856, two *npr-1* mutants and one *tyra-3* mutant at three different time points against A. *E. coli* strain OP50. B. *P. aeruginosa* strain PA14. The Y axis shows the proportion of escaped worms; the X axis shows the three time points, at which leaving was scored. The asterisk (*) is shown for the treatments which differ significantly from N2 whereas the hash sign (#) indicates significant differences from CB4856. The dotted reference line shows a leaving proportion of 0.5. Error bars represent standard error of the means. Detailed statistical results are given in Supplementary Table S11, S12, S13 & S14.

We second evaluated whether the observed pathogen avoidance against PA14 affects resistance in *C. elegans* by performing survival assays.

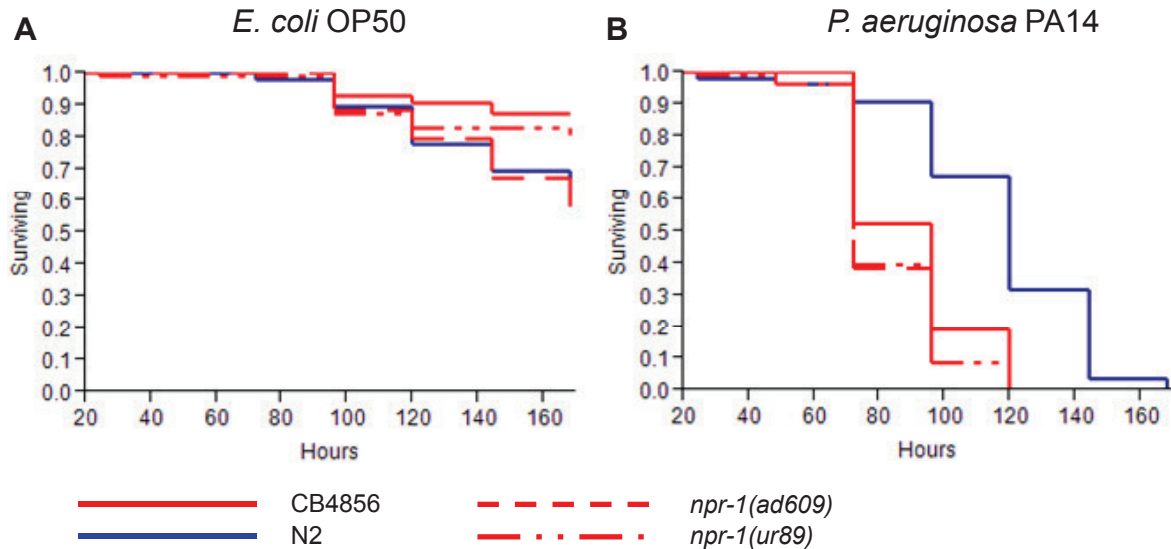


Figure 9. Survival of N2, CB4856, *npr-1(ad609)* and *npr-1(ur89)* on A. *E. coli* strain OP50. B. *P. aeruginosa* strain PA14. Survival shown on the Y axis was plotted against time in hours shown on the X axis. We used Kaplan-Meier method to calculate survival fractions.

On PA14, N2 showed a higher resistance compared to CB4856, *npr-1(ur89)* and *npr-1(ad609)* (Figure 9B, Table 5)

Table 5. Statistical analysis of survival rate on *P. aeruginosa* PA14 for the comparison of N2 with either CB4856 or *npr-1(ur89)* or *npr-1(ad609)*

Groups	Survival	
	χ^2	<i>p</i>
N2 vs. CB4856	58.32	<0.0001
N2 vs. <i>npr-1(ur89)</i>	93.38	<0.0001
N2 vs. <i>npr-1(ad609)</i>	93.01	<0.0001

Probabilities were calculated using Kaplan-Meier method and the Log-rank test for each pairwise comparison (Groups) separately. Significant probabilities are given in bold. Significance level was adjusted using Bonferroni correction for multiple pairwise comparisons.

All *C. elegans* strains showed a significantly higher survival on the control OP50 compared to the pathogen PA14 (Figure 9A, Table S15)

6.4 Discussion

Our study demonstrates that natural variation affects defence mechanisms in *C. elegans* such as avoidance behaviour and physiological resistance. These defence manifestations depend on the combination of pathogen taxa and worm's genotype. The study of the genetics of natural variation should on one hand yield insight into the evolution of defence system and on another hand enable us to reveal in which way these variations affect host resistance

Our experiments on lawn leaving behaviour show variation in such a response between the Bristol (N2) and the Hawaii (CB4856) isolates. CB4856 showed a higher leaving response compared with N2 on pathogenic and non pathogenic *B. thuringiensis* as well as on *E.coli* OP50 (Figure 1, Table S1). This observation is consistent with the results of a previous study where they showed that CB4856 has a higher OP50-patch leaving behaviour compared to N2 (Bendesky et al., 2012).

Interestingly, our results on variation in pathogen avoidance between N2 and CB4856 against *B. thuringiensis* are in contrast with previous studies using *P. aeruginosa* (Chang et al., 2012; Reddy et al., 2009). Moreover, in our case, the observed pathogen avoidance behaviour was more pronounced against stronger pathogens (B-18679) compared with less pathogenic BT strain (B-18247) (Figure 1C, D, Table S2, S3) indicating that *C. elegans* can differentiate between different levels of pathogenicity of the same bacterial species. This pronounced response towards B-18679 can be explained on the one hand by the fact that pathogenicity of *B. thuringiensis* depends mainly on the production of crystal toxins (Griffitts and Aroian, 2005; Wei et al., 2003) which are encoded by cry-toxin genes and differ between the two pathogenic BT strains we used in this study (unpublished results

from the Schulenburg lab). On the other hand, by the QTL we uncovered on chromosome II as associated with variation in avoidance behaviour towards only B-18679. The latter result indicates a certain level of specificity as the extent of avoidance response is dependent on both host and pathogen genotype. Such highly specific interactions were previously demonstrated by *Schulte et al.* and *Masri et al.* after experimental tests of co-evolution between *C. elegans* and *B. thuringiensis* (Masri et al., 2013; Schulte et al., 2010, 2011).

Our QTLs analysis based on a main effects model showed that the observed variation in lawn leaving behaviour is a polygenic trait (Figure 2). The location of the region underlying variation in leaving response depends on the species and strain of the bacterium, on whether it is a pathogen or not, and on the time point of worms' exposure.

Moreover, our interaction analysis indicated several significant epistatic effects between the different uncovered candidate regions, for example between the QTL region on chromosome X and the end-of-chromosome-IV region (Figure 3). This interaction was identified for the response against the two controls (*E. coli* OP50 and BT DSM350) (Figure 3A, B) and the pathogen BT B-18247 (Figure 3C). Our results represent the first evidence for the involvement of epistatic interactions in mediating variation in behavioural avoidance.

Interestingly, the regions we uncovered on chromosome II and X involved in food patch leaving behaviour against *E. coli* OP50 and BT DSM350 (Figure 2A, B) were also identified by *Bendesky et al.* (Bendesky et al., 2012) to be involved in leaving a patch of OP50. *Bendesky et al.* additionally showed that the QTL region on chromosome X contains the G-protein-coupled catecholamine receptor gene *tyra-3*, for which sequence polymorphisms between N2 and CB4856 associate with variation in food-leaving

(Bendesky et al., 2012). Interestingly, our functional analysis of *tyra-3* did not indicate a role in either food-patch leaving or pathogen avoidance behaviour (Figure 4). One possible reason for these differences is that we directly characterized leaving behaviour, whereas Bendesky *et al.* scored activity as an indirect measure of leaving behaviour (Bendesky et al., 2012).

This suggests that the region on chromosome X we uncovered with a strong association with leaving behaviour against food and pathogens must contain different gene(s), yet not identified, responsible for the observed variation in behavioural responses. Taken together, these findings suggest that worm behavioural defence has complex mechanisms.

Our functional genetic analysis focused mainly on the gene *npr-1* as it is present in the region we mapped on chromosome X. The QTLs analysis of the RILs (Figure 2) and the ILs (Figure S2) showed that this region had the strongest association with leaving behaviour among all the other mapped candidate regions. This was expected from previous studies, which identified an effect from *npr-1* on different behavioural phenotypes such as aggregation, aerotaxis, locomotion in the presence of food, and also pathogen avoidance (Chang et al., 2006; de Bono and Bargmann, 1998; Reddy et al., 2011). Intriguingly, pathogen avoidance was previously found to be influenced in opposite form to our analyses with pathogenic *B. thuringiensis*. In particular, the N2 215V *npr-1* allele enhances resistance against pathogenic *P. aeruginosa* PA14 compared to the 215F *npr-1* allele of the wild-type CB4856. The higher resistance in N2 was due to either a pathogen-associated change in aerotaxis behaviour (Reddy et al., 2009) or an increased physiological immune response (Styer et al., 2008). Accordingly, our functional analysis of *npr-1* using *P. aeruginosa* as a pathogen reproduced the results of Reddy *et al.* (Reddy et al., 2009) and Styer *et al.* (Styer et al., 2008). Similarly, we also confirmed that the N2 allele led to higher survival than the CB4856 allele and the two loss-of-function alleles *npr-1(ad609)* and *npr-*

I(ur89) (Figure 9B, Table 5). Importantly, our study is the first to demonstrate directly that the N2 allele additionally causes higher PA14 avoidance in comparison to the other alleles (Figure 8B, Table S11, S12 & S13). These results are consistent with those from Reddy *et al.* (Reddy *et al.*, 2009), who only obtained an indirect measure for the lawn leaving response, and they confirm the finding of PA14 avoidance by Chang *et al.* (Chang *et al.*, 2012). Moreover, our analysis revealed that the *E. coli* control does not induce any variation in leaving behaviour among the considered strains, except between N2 and *npr-1(ad609)* at 14h exposure (Figure 8A, Table S11, S12 & S13). This indicates that the high avoidance of N2 against PA14 is not due to a general aerotaxis response as proposed by Reddy *et al.* (Reddy *et al.*, 2009). Instead, it seems that *C. elegans* does express a specific avoidance response against this pathogenic bacterium.

Taken together, we conclude that the N2 wild-type *npr-1* allele enhances both resistance and behavioural defence against pathogenic *P. aeruginosa*.

Interestingly, on peptone free NGM escape assay plates, N2 and CB4856 showed high variation in their leaving response against OP50 (Figure 1A, Table S1). Whereas on NGM escape plates, this variation in leaving response observed between N2 and CB4856 against OP50 were not present anymore (Figure 7A, Table 4). This can be explained by the fact that the absence or presence of bacteria proliferation on assay plates might affect animals' behavioural responses due to production of some metabolites or change in oxygen concentration. On peptone free NGM plates OP50 cells cannot proliferate as they do on Nematode Growth Medium plates.

In clear contrast to these findings, it is the CB4856 allele that enhances defence against nematocidal *B. thuringiensis*, including both lawn leaving behaviour (Figure 1, Table S1) and also resistance (Figure 5, Table 1). These results are consistent with our own previous

findings, based on a slightly different resistance assay in liquid medium (Schulenburg and Muller, 2004). Our results identified a similar pattern for the highly pathogenic BT strain B-18679 (Figure 5B).

Interestingly, the two loss-of-function *npr-1* alleles varied in their effect on avoidance behaviour and resistance. In particular, food-patch leaving, pathogen avoidance behaviour and resistance in *npr-1(ur89)* were similar to CB4856, whereas in *npr-1(ad609)* they were similar to N2 (Figure 4A, B, C, 6A Table 2, S4, S6). This suggests that different alleles of the same gene can have different effect on the same phenotype. Moreover, the potential positive relationship between variation in avoidance behaviour and resistance across the different *C. elegans* strains shows nicely that the strains showing a strong physical pathogen avoidance behaviour are better at resisting pathogenic bacteria.

Unexpectedly, this potential positive relationship could not be observed in the case of the highly nematocidal strain B-18679. This is due to our results from the lawn leaving assays with B-18679 which did not show any variation in avoidance between all *C. elegans* strains (Figure 4D, Table S4, S5, S6). This result can be explained by the high level of pathogenicity of this strain which led to a strong avoidance response in all *C. elegans* strains. The assay plates were inoculated with BT always coming from the same culture of spores with toxins aliquotted in tubes and frozen at -20°C. The culture used for this assay had higher virulence, inducing high leaving responses in all strains. However, the results of the survival assays with B-18679 showed a differential effect of *npr-1* alleles as on B-18247. *Npr-1(ur89)* had a similar survival to CB4856, whereas *npr-1(ad609)* had a similar survival to N2 (Figure 6B, Table 3).

The observed opposite allelic effect of *npr-1* against *B. thuringiensis* comes in contradiction to Styer *et al.* conclusion stating that *npr-1* is required for immune responses

to pathogens in general (Styer et al., 2008). Apart from resistance against *P.aeruginosa*, *S. enterica* and *E. faecalis*, *npr-1* seems to have a partial effect on resistance against *B. thuringiensis*.

Taken together, these results suggest that the gene *npr-1* either has a minor effect on resistance against *B. thuringiensis* and/or its effect depends on an epistatic interaction with an allele at another locus as shown by our QTLs and epistatic interaction analysis.

6.5 Materials and methods

C. elegans strains

We used in our study the following *C. elegans* strains: i) the Bristol (N2) and the Hawaii (CB4856) isolates as controls to study pathogen avoidance behaviour; ii) 200 Recombinant Inbred Lines (RILs) and 90 Introgression lines (ILs) generated from crosses between N2 and CB4856 in Jan E. Kammenga's lab following the strategy described by Li *et al.* and Doroszuk *et al.* (Doroszuk *et al.*, 2009; Li *et al.*, 2006); iii) two loss of function *npr-1* mutants, *npr-1(ur89)* and *npr-1(ad609)*, and one *tyra-3* knock out mutant *tyra-3(ok325)*, all obtained from the Caenorhabditis Genetics Center (CGC; <http://www.cbs.umn.edu/CGC/>). All worms' strains were maintained at 20°C on Nematode Growth Medium (NGM) plates with the non pathogenic *E. coli* OP50 as an *ad libitum* food according to protocols described by Stiernagle (Stiernagle, 2006).

All mutants were backcrossed at least three times to the wild type strain before the start of the functional analysis. Presence of the target mutation was checked for the two *npr-1* mutants by sequencing the gene *npr-1* at the location of the mutations and for the knock-out mutant *tyra-3* by PCR.

Bacteria strains

During our experiments, worms were exposed to the following bacteria: i) two nematocidal strains of *B. thuringiensis*, NRRL B-18247 and NRRL B-18679 (mentioned as BT B-18247 & BT B-18679), provided by the Agriculture Service Patent Culture Collection (United States Department of Agriculture, Peoria, Illinois, USA); ii) one non nematocidal strain of *B. thuringiensis* named DSM 350 (mentioned as BT DSM350) obtained from the

German Collection of Microorganisms and Cell Cultures (Deutsche Sammlung von Mikroorganismen und Zellkulturen GmbH, DSMZ, Braunschweig, Germany), iii) the pathogen *P. aeruginosa* strain PA14 obtained from Dennis H. Kim, Boston, USA.

The non nematocidal strain DSM 350 and *E. coli* strain OP50 were used as controls. Before the start of this study, the three strains of *B. thuringiensis* (B-18247, B-18679 & DSM 350) were cultured in large quantities as described in (Borgonie et al., 1995). The produced cultures contained mainly spores associated to toxins for the nematocidal strains and only spores for the BT control. These cultures were frozen in aliquots at -20°C as toxicity is not affected by freezing at that temperature (Leyns et al., 1995). Aliquots were thawed directly before each experiment.

Escape assays

The assay was designed for 9 cm petri dishes with either Peptone free NGM for escape assays done with *B. thuringiensis* (Figure S1) as described previously in (Hasshoff et al., 2007) or NGM for escape assays done with *P. aeruginosa*. Peptone free NGM prevents BT growth and germination, this way worms are challenged with spores associated to toxins on the assay plates. The test bacterium (BT or PA14) was mixed with *E. coli* OP50 from an ON culture at 37°C suspended in PBS and adjusted to an OD=5. 30 µl of this mixture were spotted onto the center of the plate and 80 µl of *E. coli* only with OD=5 were additionally placed in a shape of a ring surrounding the central bacterial lawn (Figure S1). This ring of OP50 prevents escaping worms from starving minimizing then their return to the lawn in the center. The BT strains were diluted in 1:250 from a BT stock frozen at -20°C, consisting of a spore-toxin preparation with a concentration of 1.5×10^{10} particles/ml. PA14 was diluted in 4:1 from an ON culture at 37°C. The assay plates were left to dry overnight (9-15h) at 20°C when seeded with BT and at 22.5°C when seeded with PA14

following the protocol of Chang *et al.* (Chang et al., 2012). 10 hermaphroditic fourth instar larvae (L4) were picked onto the test lawn. We transferred only a small number of worms to suppress clumping behaviour which is expressed by some *C. elegans* strains at high densities complicating then worm scoring. The experiments with BT were performed at 20°C and with PA14 at 22.5°C.

The leaving behaviour of the tested *C. elegans* strains and lines was recorded by counting the number of worms remaining on the lawn at different time points of exposure and calculated according to the following formula:

$$\text{Leaving index} = \frac{10 - \text{number of worms on the lawn}}{10}$$

The escape assays which were done with N2 and CB4856 included 12 replicates of each treatment (Figure 1, Figure 7). The assays which screened the RILs (200 lines) and ILs (90 lines) were done using a randomized block design on 17 dates including always the parental strains of these lines, N2 and CB4856, as internal controls. Each *C. elegans* strain-bacteria-time point combination was assayed in three replicates, resulting in a total of 7008 individual data points. The escape assays screening *C. elegans* mutants (for the genes *npr-1* and *tyra-3*) with N2 and CB4856 as controls included 12 replicates of each treatment (Figure 4, Figure 8).

Survival assays

Survival assays with *Bacillus thuringiensis*

The assays were done on 6 cm plates with peptone free NGM to prevent BT germination outside worms. Each plate was inoculated with 75 µl of *B. thuringiensis* mixed with *E. coli* OP50 (OD=5). BT was prepared in a series of seven different dilutions: 1:2, 1:5, 1:10,

1:30, 1:50, 1:100, and 1:250, from a spore-toxin stock culture at a concentration of 1.5×10^{10} particles/ml. The assay plates were left to dry overnight (9-15h) at 20°C. 30 L4 hermaphrodites were picked onto each assay plates which were stored then at 20°C in the dark. After 24 hours, survival was recorded by counting alive, dead and missing worms. Each treatment group was replicated 8 times across 8 runs (one replicate per run).

Survival assays with *Pseudomonas aeruginosa*

The assays were done on 3 cm plates with modified NGM. The plates were inoculated with 5 µl of *P. aeruginosa* (PA14), grown overnight at 37°C grown in LB. As a control assay plates were inoculated with 5 µl of an overnight culture of *E. coli*. The assay plates were incubated for one overnight at 37°C and then another overnight at 25°C. 30 L4 hermaphrodites were picked onto each assay plates which were stored then at 25°C in the dark. Every 24 hours alive and dead worms were counted and survival worms were transferred to new assay plates. The assays were stopped when all worms died on the pathogen *P. aeruginosa*. Each treatment group was replicated 10 times across two runs.

Statistical analysis:

Escape assays: We used the non parametric test Kruskal Wallis to assess differences in escape behaviour between the different *C. elegans* strains. The analysis was done for each time point and bacterium separately. We also used this test to compare the leaving behaviour of each *C. elegans* strain against pathogens compared to control bacteria per time points of exposure. A Bonferroni based adjustment was used to correct for multiple testing.

Survival assays with PA14: We used Kaplan Meier analysis applying the Log Rank test to assess differences in survival between *C. elegans* strains. The analysis was done for PA14 and the control OP50 separately. We even compared the survival of each *C. elegans*

strain on the pathogen (PA14) versus the control bacterium (OP50). A Bonferroni based adjustment was used to correct for multiple testing.

Survival assays with BT: We used GLM ordinal logistic regression analysis to assess differences in survival between the different *C. elegans* strains, using *C. elegans* strains and BT concentration as factors. We even tested the presence of interaction effect between these two factors. The analysis was done for each strain of BT separately. A Bonferroni based adjustment was used to correct for multiple testing.

All escape and survival assays data were analyzed using the program JMP version 9.0 (SAS Institute Inc.), while graphic illustrations were produced with the program SIGMAPLOT version 12.0 (Systat Software Inc.).

Quantitative trait loci analysis: the analysis was performed on the average of three replicates per genotype/line of the calculated proportion “leave” (see assay method) of 200 Recombinant Inbred Lines (RILs) (Elvin et al., 2011; Gutteling et al., 2007; Li et al., 2006; Li et al., 2010; Rodriguez et al., 2012; Snoek et al., 2013; Viñuela et al., 2010, 2012) QTLs were calculated by single marker mapping using a linear model (trait~marker+error) for each marker using a custom written script in the statistical programming language “R” (REF).. Significance levels were estimated from 1000 permutations of the data. The analysis calculated the significance of the linkage between the genetic marker and the trait one by one for each bacterium and exposure time point separately.

We furthermore evaluated epistatic interactions between each two markers across the genome for each bacterium separately using the following model: phenotype~ time + marker1 * marker2.

6.6 Conclusion:

Our study on lawn leaving behaviour demonstrate an opposite effect of the two alleles depending on the pathogen: the CB4856 allele is associated with faster lawn leaving behaviour and higher resistance than the N2 allele on *B. thuringiensis*. Whereas, the CB4856 allele is associated with lower lawn leaving behaviour and lower resistance compared with the N2 allele on *P. aeruginosa*. Moreover, it shows that the observed variation in survival and leaving behaviour on *B. thuringiensis* are not exclusively determined by *npr-1*, as it is the case with *P. aeruginosa*, but by also another/other gene(s) present either in the same QTL region or in another one through an epistatic interaction.

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6.9 Supplementary material

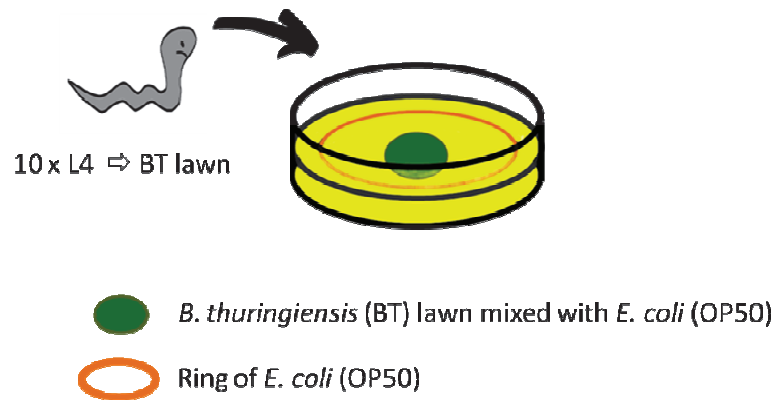


Figure S1. Schematic of the lawn leaving assay. 10 hermaphrodites at the stage L4 were picked to 9 cm peptone free NGM plates containing a lawn of the tested bacteria, each mixed with *E. coli* OP50 and surrounded by a ring of 80 μ l of OP50.

Table S1. Comparison between N2 and CB4856 as to their leaving behaviour from four different bacteria² at different time points¹

Time point ¹	Bacteria ²	Proportion leave χ^2	<i>p</i>
1 h	B-18247	5,4041	0,0201
	B-18679	10,7164	0,0011
	DSM350	13,9306	0,0002
	OP50	12,112	0,0005
4 h	B-18247	12,3981	0,0004
	B-18679	5,9083	0,0151
	DSM350	15,6593	<0.0001
	OP50	15,2311	<0.0001
8 h	B-18247	10,7441	0,001
	B-18679	16,1618	<0.0001
	DSM350	19,6375	<0.0001
	OP50	26,4735	<0.0001
14 h	B-18247	13,4096	0,0003
	B-18679	17,6981	<0.0001
	DSM350	24,9556	<0.0001
	OP50	24,885	<0.0001
24 h	B-18247	17,7271	<0.0001
	B-18679	9,2485	0,0024
	DSM350	26,6702	<0.0001
	OP50	19,416	<0.0001

¹ Time point, for each of which the analysis was done separately.

² Bacteria for which effect tests were calculated, using the non parametric test Kruskal Wallis. Degrees of freedom (DF) = 1 for all effect tests. Significant probabilities are given in bold. Significance level was adjusted using Bonferroni correction for multiple pairwise comparisons.

Table S2. Pairwise comparison² of leaving response in N2 on nematocidal *B. thuringiensis* (B-18247 & B-18679) and non nematocidal BT DSM350 vs. *E.coli* OP50 at different time points¹

Time point ¹	Pairwise comparison ²	Proportion leave χ^2	<i>p</i>
1 h	B-18247 vs. OP50	0,2609	0,6095
	B-18679 vs. OP50	1,2364	0,2662
	DSM350 vs. OP50	0	1
4 h	B-18247 vs. OP50	0,6171	0,4321
	B-18679 vs. OP50	15,792	<0.0001
	DSM350 vs. OP50	0,0027	0,9585
8 h	B-18247 vs. OP50	0,3691	0,5435
	B-18679 vs. OP50	26,0565	<0.0001
	DSM350 vs. OP50	0,1662	0,6835
14 h	B-18247 vs. OP50	7,3681	0,0066
	B-18679 vs. OP50	29,917	<0.0001
	DSM350 vs. OP50	0,9157	0,3386
24 h	B-18247 vs. OP50	3,2961	0,0694
	B-18679 vs. OP50	23,6421	<0.0001
	DSM350 vs. OP50	3,0823	0,0792

¹ Time point, for each of which the analysis was done separately.

² Pairwise comparisons for which effect tests were calculated, using the non parametric test Kruskal Wallis. Degrees of freedom (DF) = 1 for all effect tests. Significant probabilities are given in bold. Significance level was adjusted using Bonferroni correction for multiple pairwise comparisons.

Table S3. Pairwise comparison² of leaving response in CB4856 on nematocidal *B. thuringiensis* (B-18247 & B-18679) and non nematocidal BT DSM350 vs. *E.coli* OP50 at different time points¹

Time point ¹	Pairwise comparison ²	Proportion leave χ^2	<i>p</i>
1 h	B-18247 vs. OP50	0,9461	0,3307
	B-18679 vs. OP50	0,0031	0,9558
	DSM350 vs. OP50	0,0279	0,8673
4 h	B-18247 vs. OP50	0,3699	0,543
	B-18679 vs. OP50	1,1752	0,2783
	DSM350 vs. OP50	0,3535	0,5521
8 h	B-18247 vs. OP50	2,3572	0,1247
	B-18679 vs. OP50	21,9162	<0.0001
	DSM350 vs. OP50	1,0117	0,3145
14 h	B-18247 vs. OP50	1,2751	0,2588
	B-18679 vs. OP50	26,9248	<0.0001
	DSM350 vs. OP50	3,1323	0,0768
24 h	B-18247 vs. OP50	0,8285	0,3627
	B-18679 vs. OP50	10,668	0,0011
	DSM350 vs. OP50	2,4218	0,1197

¹ Time point, for each of which the analysis was done separately.

² Pairwise comparisons for which effect tests were calculated, using the non parametric test Kruskal Wallis. Degrees of freedom (DF) = 1 for all effect tests. Significant probabilities are given in bold. Significance level was adjusted using Bonferroni correction for multiple pairwise comparisons.

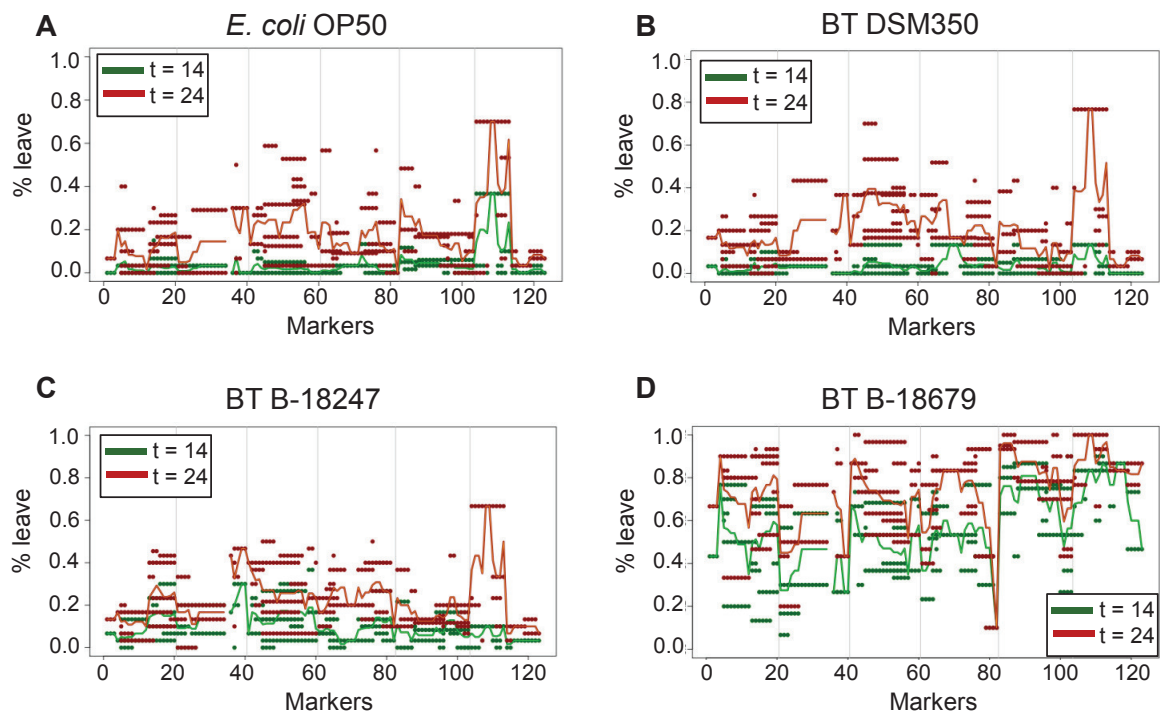


Figure S2. ILs' leaving phenotype plotted against their introgression position shown on the X axis for the tested bacteria **A.** *E. coli* strain OP50. **B.** Non nematocidal *B. thuringiensis* strain DSM350. **C.** Nematocidal *B. thuringiensis* strain B-18247. **D.** Highly nematocidal *B. thuringiensis* strain B-18679. Green and red lines show the results after either 14h or 24h exposure.

Table S4. Comparison between N2 and *npr-1(ur89)* as to their leaving behaviour from four different bacteria² at two different time points¹

Time point	Treatment	Proportion leave χ^2	<i>p</i>
14 h	B-18247	10,0357	0,0015
	B-18679	3,4626	0,0628
	DSM350	14,2642	0,0002
	OP50	10,0906	0,0015
24 h	B-18247	11,1077	0,0009
	B-18679	1,0684	0,3013
	DSM350	12,7068	0,0004
	OP50	18,4095	<0.0001

¹ Time point, for each of which the analysis was done separately.

² Bacteria for which effect tests were calculated, using the non parametric test Kruskal Wallis. Degrees of freedom (DF) = 1 for all effect tests. Significant probabilities are given in bold. Significance level was adjusted using Bonferroni correction for multiple pairwise comparisons.

Table S5. Comparison between N2 and *npr-1(ad609)* as to their leaving behaviour from four different bacteria² at two different time points¹

Time point	Treatment	Proportion leave χ^2	<i>p</i>
14 h	B-18247	0,0033	0,9545
	B-18679	2,6964	0,1006
	DSM350	0,023	0,8796
	OP50	0,8978	0,3434
24 h	B-18247	0,0937	0,7595
	B-18679	0,5396	0,4626
	DSM350	0,4002	0,527
	OP50	1,5278	0,2164

¹ Time point, for each of which the analysis was done separately.

² Bacteria for which effect tests were calculated, using the non parametric test Kruskal Wallis. Degrees of freedom (DF) = 1 for all effect tests. Significant probabilities are given in bold. Significance level was adjusted using Bonferroni correction for multiple pairwise comparisons.

Table S6. Comparison between N2 and *tyra-3* as to their leaving behaviour from four different bacteria² at two different time points¹

Time point	Treatment	Proportion leave χ^2	<i>p</i>
14 h	B-18247		0.5426
	B-18679		0.187
	DSM350		0.0265
	OP50		0.4033
24 h	B-18247		0.8618
	B-18679		0.8477
	DSM350		0.203
	OP50		0.1058

¹ Time point, for each of which the analysis was done separately.

² Bacteria for which effect tests were calculated, using the non parametric test Kruskal Wallis. Degrees of freedom (DF) = 1 for all effect tests. Significant probabilities are given in bold. Significance level was adjusted using Bonferroni correction for multiple pairwise comparisons.

Table S7. Comparison between N2 and CB4856 as to their leaving behaviour from four different bacteria² at two different time points¹

Time point	Treatment	Proportion leave χ^2	p
14 h	B-18247	1,3501	0,2453
	B-18679	0,006	0,9382
	DSM350	0,395	0,531
	OP50	9,3181	0,0023
24 h	B-18247	19,4927	<0.0001
	B-18679	1,7789	0,4823
	DSM350	8,8199	0,003
	OP50	17,9222	<0.0001

¹ Time point, for each of which the analysis was done separately.

² Bacteria for which effect tests were calculated, using the non parametric test Kruskal Wallis. Degrees of freedom (DF) = 1 for all effect tests. Significant probabilities are given in bold. Significance level was adjusted using Bonferroni correction for multiple pairwise comparisons.

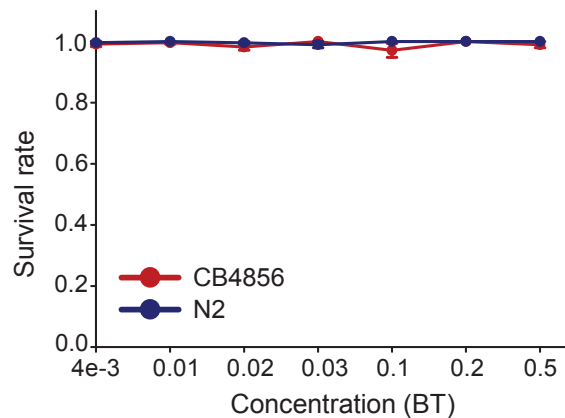


Figure S3. Survival of N2 and CB4856 on the non nematocidal *B. thuringiensis* strain DSM350. Survival shown on the Y axis was plotted against BT concentration shown on the X axis.

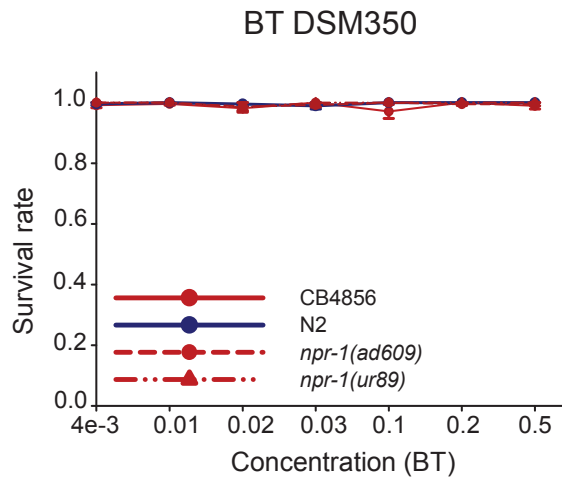


Figure S4. Survival of N2, CB4856, *npr-1(ur89)* and *npr-1(ad609)* on the non nematocidal *B. thuringiensis* strain DSM350. Survival shown on the Y axis was plotted against BT concentration shown on the X axis.

Table S8. Statistical analysis of survival rate on BT B-18247 for the comparison of CB4856 with either *npr-1(ur89)*^(a) or *npr-1(ad609)*^(b)

Bacterium ¹	Factor ²	Survival	
		χ^2	<i>p</i>
B-18247	CB4856 vs. <i>npr-1(ur89)</i> ^(a)	2.81	0.0931
	BT concentration	135.91	<0.0001
	Interaction (BT conc. x <i>C. elegans</i>)	0.005	0.9408
	CB4856 vs. <i>npr-1(ad609)</i> ^(b)	15.33	<0.0001
	BT concentration	105.42	<0.0001
	Interaction (BT conc. x <i>C. elegans</i>)	0.27	0.597

¹ Bacteria, for each of which the analysis was done separately.

² Factors for which effect tests were calculated as a part of a general linear model, using ordinal logistic regression analysis. Degrees of freedom (DF) = 1 for all effect tests. Significant probabilities are given in bold. Significance level was adjusted using Bonferroni correction for multiple pairwise comparisons.

Table S9. Statistical analysis of survival rate on BT B-18679 for the comparison of CB4856 with either *npr-1(ur89)*^(a) or *npr-1(ad609)*^(b)

Bacterium ¹	Factor ²	Survival	
		χ^2	<i>p</i>
B-18679	CB4856 vs. <i>npr-1(ur89)</i> ^(a)	0.013	0.9068
	BT concentration	81.94	<0.0001
	Interaction (BT conc. x <i>C. elegans</i>)	0.25	0.6146
	CB4856 vs. <i>npr-1(ad609)</i> ^(b)	20.17	<0.0001
	BT concentration	64.64	<0.0001
	Interaction (BT conc. x <i>C. elegans</i>)	0.109	0.7404

¹ Bacteria, for each of which the analysis was done separately.

² Factors for which effect tests were calculated as a part of a general linear model, using ordinal logistic regression analysis. Degrees of freedom (DF) = 1 for all effect tests. Significant probabilities are given in bold. Significance level was adjusted using Bonferroni correction for multiple pairwise comparisons.

Table S10. Comparison of leaving response in each of N2 and CB4856 on *P. aeruginosa* (PA14) vs *E. coli* (OP50) at different time points

<i>C. elegans</i>	Time point	Proportion leave	<i>p</i>
		χ^2	
N2	8 h	2,3	0,1294
	14 h	18,7649	<0.0001
	24 h	18,3321	<0.0001
	48 h	19,8323	<0.0001
CB4856	8 h	0,0036	0,952
	14 h	0,135	0,7133
	24 h	1,5134	0,2186
	48 h	1,6669	0,1967

The analysis was done separately for each time point and each *C. elegans* strain.

Effect tests were calculated, using the non parametric test Kruskal Wallis. Degrees of freedom (DF) = 1 for all effect tests. Significant probabilities are given in bold. Significance level was adjusted using Bonferroni correction for multiple pairwise comparisons.

Table S11. Comparison between N2 and *npr-1(ur89)* as to their leaving behaviour from two different bacteria² at three different time points¹

Time point ¹	Bacteria ²	Proportion leave χ^2	<i>p</i>
14 h	PA14	10,6641	0,0011
	OP50	1,4657	0,226
24 h	PA14	14,9724	0,0001
	OP50	0,2852	0,5933
48 h	PA14	14,9606	0,0001
	OP50	0,0063	0,9367

¹ Time point, for each of which the analysis was done separately.

² Bacteria for which effect tests were calculated, using the non parametric test Kruskal Wallis. Degrees of freedom (DF) = 1 for all effect tests. Significant probabilities are given in bold. Significance level was adjusted using Bonferroni correction for multiple pairwise comparisons.

Table S12. Comparison between N2 and *npr-1(ad609)* as to their leaving behaviour from two different bacteria² at three different time points¹

Time point ¹	Bacteria ²	Proportion leave χ^2	<i>p</i>
14 h	PA14	12,6951	0,0004
	OP50	7,4102	0,0065
24 h	PA14	15,2122	<0,0001
	OP50	4,9623	0,0259
48 h	PA14	14,9606	0,0001
	OP50	0,7767	0,3781

¹ Time point, for each of which the analysis was done separately.

² Bacteria for which effect tests were calculated, using the non parametric test Kruskal Wallis. Degrees of freedom (DF) = 1 for all effect tests. Significant probabilities are given in bold. Significance level was adjusted using Bonferroni correction for multiple pairwise comparisons.

Table S13. Comparison between N2 and CB4856 as to their leaving behaviour from two different bacteria² at three different time points¹

Time point ¹	Bacteria ²	Proportion leave χ^2	<i>p</i>
14 h	PA14	8,3889	0,0038
	OP50	2,0309	0,1541
24 h	PA14	15,2122	<0,0001
	OP50	2,716	0,0993
48 h	PA14	13,0955	0,0003
	OP50	1,5867	0,2078

¹ Time point, for each of which the analysis was done separately.

² Bacteria for which effect tests were calculated, using the non parametric test Kruskal Wallis. Degrees of freedom (DF) = 1 for all effect tests. Significant probabilities are given in bold. Significance level was adjusted using Bonferroni correction for multiple pairwise comparisons.

Table S14. Comparison between N2 and *tyra-3(ok325)* as to their leaving behaviour from two different bacteria² at three different time points¹

Time point ¹	Treatment ²	Proportion leave χ^2	<i>p</i>
14 h	PA14		0.2490
	OP50		0.3022
24 h	PA14		0.5488
	OP50		0.0873
48 h	PA14		0.0086
	OP50		0.7009

¹ Time point, for each of which the analysis was done separately.

² Bacteria for which effect tests were calculated, using the non parametric test Kruskal Wallis. Degrees of freedom (DF) = 1 for all effect tests. Significant probabilities are given in bold. Significance level was adjusted using Bonferroni correction for multiple pairwise comparisons.

Table S15. Statistical analysis of survival rate of each *C. elegans* strain N2, CB4856, *npr-1(ur89)* and *npr-1(ad609)* on *P. aeruginosa* PA14 compared to the control *E. coli* OP50

<i>C. elegans</i> strain	Survival	
	χ^2	<i>p</i>
N2	114.72	<0.0001
CB4856	134.95	<0.0001
<i>npr-1(ur89)</i>	103.77	<0.0001
<i>npr-1(ad609)</i>	158.09	<0.0001

We used Kaplan-Meier method to calculate survival fractions and compare survival curves of each *C. elegans* strain on pathogenic *P. aeruginosa* vs. the control *E. coli* OP50 applying a Log-rank test.

7. Chapter 2:

Trans-generational immune priming of pathogen avoidance behaviour in the nematode *C. elegans*

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7.1 Abstract

Over the last years, an increasing number of invertebrates have been shown to rely on “memory”-like phenomena in immune defence as a fast and efficient protection against secondary exposures to pathogens. Such immune priming was demonstrated for physiological responses either within the lifetime of an individual or across generations within the same family (i.e., individual or trans-generational immune priming). Here we investigate the trans-generational priming of a behavioural avoidance response using the nematode *Caenorhabditis elegans* and its Gram-positive pathogen *Bacillus thuringiensis* (BT). This nematode is known to behaviourally evade pathogenic microbes to protect itself against damage and infection. Our results now demonstrate that pathogen avoidance can increase significantly among the offspring of parents challenged with pathogenic as well as non-pathogenic BT compared with offspring of naïve parents. The priming effect varies between two genetically distinct, isogenic *C. elegans* isolates, strongly suggesting a genetic contribution to its expression. We conclude that trans-generational immune priming extends to behavioural traits and most likely has a genetic foundation.

7.2 Introduction

Parasites are omnipresent. They infect host organisms, exploit their resources and reduce their fitness. Hosts resist to parasites by activating their defence system through either behavioural avoidance or physiological immune responses.

For our understanding of immunity, it is important to reiterate that a single host organism may not only face a range of different pathogens during its life-time, but that it can repeatedly encounter the same infectious microbe. In vertebrates, it is the activation of “immune memory” which combats repeated infections and ensures lasting protection. Such immune memory is a feature of the adaptive immune system and is based on the persistence of antigen-specific lymphocytes generated by clonal expansion (Janeway CAJ, 2001). Even though invertebrates lack an adaptive immune system, “memory”-like phenomena, usually termed “immune priming”, have been repeatedly observed in these organisms (Schmid-Hempel, 2005). Immune memory in invertebrates seems to have two facets: an enhanced protection in a single individual within its lifetime (individual immune priming), and a transmitted enhanced protection of offspring after a pre-challenge of the parents (trans-generational immune priming).

Individual immune priming has been previously demonstrated in various invertebrate taxa. For example, in cockroaches, immunization with killed *Pseudomonas aeruginosa* induced significant later protection compared with unchallenged ones (Faulhaber and Karp, 1992). Even an immunization with soluble toxins produced highly specific protection during secondary toxin exposure (Rheins et al., 1980). In the mealworm beetle *Tenebrio molitor*, larvae showed an increased resistance against the fungus *Metarhizium anisopliae* after being challenged with lipopolysaccharides (LPS). This enhanced resistance was based on

an elevated antimicrobial activity in the haemolymph (Moret and Siva-Jothy, 2003). In shrimps, survival increased against viral proteins after oral vaccination (Witteveldt et al., 2006). In copepods, a reduction of secondary infection by the tapeworm *Schistocephalus solidus* was observed when individuals were pre-infected with antigenically similar parasites compared to unrelated parasites (Kurtz and Franz, 2003). In the social insect *Bombus terrestris*, a pre-challenge increased protection against infectious bacteria upon secondary exposure (Sadd and Schmid-Hempel, 2006). In *Drosophila melanogaster*, it was shown that priming flies with sublethal doses of *Streptococcus pneumoniae* led to their protection against a secondary infection with lethal doses of *S. pneumoniae*. This priming response required the involvement of the Toll pathway and phagocytes (Pham et al., 2007). In the beetle *Tribolium castaneum*, a previous study showed that individuals survived better a second bacterial infection homologous to the heat killed bacteria used during priming than a heterologous exposure (Roth et al., 2009 (a)). All these findings enhance our knowledge on the ability of the innate immune system in invertebrates in learning from previous challenges in order to mount faster and more efficient secondary immune responses.

Apart from individual priming, several studies focused as well on transgenerational immune priming in invertebrates. One of the first evidences for maternal transmission of immunity in invertebrates was demonstrated in shrimps. The results showed that larvae derived from glucan injected spawners had an increase in survival compared to larvae derived from unprimed spawners. This increase in survival was observed against white spot syndrome associated virus (WSSV) (Huang and Song, 1999; Swain and Nayak, 2009). In *Daphnia magna*, Little *et al.* (Little et al., 2003) demonstrated that *Daphnia* is able to show an inherited strain-specific immune response toward the bacterium *Pasteuria ramosa*. The results of this study indicated that offspring had an enhanced fitness when

they encountered the same bacterium their mother already experienced compared with offspring infected by a different bacterium than their mother was (Little et al., 2003). Another recent study on *Daphnia* showed even an effect of food stress in mothers on resistance responses in offspring. Offspring coming from poorly fed mothers showed a higher ability of preventing parasite establishment relative with offspring of well fed mothers (Stjernman and Little, 2011). Parental diet was shown in another study to have an effect on offspring immunity as well in the moth *Plodia interpunctella*. In this case, the effect of poor parental diet influenced offsprings' immunity in the opposite way. A poor diet of either both parents or one of them led in both cases to a reduction in offspring immunity (Triggs and Knell, 2012). In *Bombus terrestris*, Sadd et al. showed evidence for transgenerational immune priming (Sadd et al., 2005). They had a direct evaluation of transfer of immunity through measuring antibacterial activity which increased in offspring coming from a queen mother who experienced a corresponding bacterial immune challenge prior to colony founding (Sadd et al., 2005). Transgenerational immune priming was also studied in the insect *Tenebrio molitor*. Moret et al. (Moret, 2006) injected two generations of insects with lipopolysaccharides (LPS). They observed that offspring of challenged parents had a higher induction of antimicrobial peptides (AMPs) in the haemolymph (Moret, 2006). This maternal transfer of high levels of AMPs to the eggs was later found to be negatively correlated with the maternal immune response depending on female's body size (Moreau et al., 2012). Further work on maternal transfer of immunity in *Tenebrio* showed that only a limited number of produced eggs were protected against bacterial challenge with a sufficient level of transferred AMPs (Zanchi et al., 2012). In *Tribolium castaneum*, Roth et al showed that a pre-challenge of parents with heat-killed bacteria led to an increase of resistance in their offspring upon exposure to live

bacteria. This study showed first evidence for paternal transmission of immune priming in an invertebrate (Roth et al., 2009 (b)).

Despite all these evidences for priming in invertebrates, we still lack knowledge of the molecular mechanisms underlying these “memory”-like phenomena. It is even thought that such priming could be mainly due to the activation of a primary response which does not disappear entirely (Little and Kraaijeveld, 2004).

We have more insight into these mechanisms in the nematode *Caenorhabditis elegans* through studies which focused on: i) multigenerational epigenetic inheritance, ii) individual and/or transgenerational immune priming upon exposure to pathogens. Several studies addressed epigenetic inheritance in *C. elegans* after. Fire *et al.* (Fire et al., 1998) showed that genetic interference by double stranded RNA (dsRNA) was observed in progeny of worms injected with dsRNA. Following this finding, a subsequent study showed that this inheritance depended on the transmission of an extragenic silencing factor and the implication of the RNAi pathway genes (*rde-1* and *rde-4*) in the formation of this inherited agent (Grishok et al., 2000). Inheritance of transcriptional silencing effect in *C. elegans* was shown to occur indefinitely in the absence of the original trigger (Vastenhouw et al., 2006) and to be promoted and maintained in somatic tissues by nuclear RNAi (Nrde) pathway (Burton et al., 2011). Inheritance of interference in *C. elegans* is constantly multigenerational exceeding in some cases five generations, but can as well be non constant over generational time (e.g. inheritance of silencing of the oocyte maturation factor) (Alcazar et al., 2008). RNAi inheritance was recently found to depend on the agronaute protein HRDE-1 through its regulatory effect on gene silencing events in germ-cell nuclei (Buckley et al., 2012). This nuclear agronaute was also shown to be required for long-term silencing in *C. elegans* triggered by piRNAs and inherited for at least 20 generations (Ashe et al., 2012).

The biological relevance in terms of adaptive benefits of this epigenetic inheritance in *C. elegans* is still not well understood. So far, two studies looked at epigenetic inheritance in *C. elegans* from a physiological context. The first addressed transgenerational inheritance of longevity traits, showing that deficiencies in the chromatin modifiers of the H3K4 trimethylation complex members (ASH-2, WDR-5 and SET-2), only in the parental generation, led to an extended lifespan of descendants up until the third generation (Greer et al., 2011). The second showed a transgenerational inheritance of an antiviral protection through transmission of viral silencing agents (viRNAs) in *C. elegans* (Rechavi et al., 2011).

Olfactory memory is also another inherited acquired trait in *C. elegans*. This inheritance can be stable through many following generations (Remy, 2010).

In parallel to transgenerational priming, *C. elegans* also shows individual priming. Worms exhibit an associative learning of avoidance towards the pathogen *P. aeruginosa* (PA14) through an increase of serotonin production in chemosensory neurons. Worm's pre-challenge modified their olfactory preferences (Zhang et al., 2005). Furthermore, worms pre-exposed to enteropathogenic *E. coli* (EPEC) showed an increased survival against EPEC relative to naive worms (Anyanful et al., 2009).

In this study, we investigate trans-generational priming of pathogen avoidance behaviour in *C. elegans*. Such behaviour is important for *C. elegans* and used as a defence strategy against pathogenic microbes (Hasshoff et al., 2007; Pradel et al., 2007; Wang et al.; Zhang et al., 2005). Behavioural defences in animals are thought to be less costly than the activation of physiological immune responses as avoidance reduces contact with pathogenic microbes. In the wild *C. elegans* feeds on bacteria and yeasts (Felix and Braendle, 2010). Nematode offspring usually develop on the same bacterial patches as

their parents. Therefore, a transmission of behavioural defences from parents to offspring might be beneficial for enhancing offspring survival. We investigated trans-generational priming of avoidance behaviour in the two genetically divergent isogenic strains of *C. elegans*, the Bristol isolate N2 and the Hawaii isolate CB4856. These strains differ in their pathogen avoidance behaviour toward both pathogenic bacteria *Bacillus thuringiensis* as shown in Nakad et al, (manuscript in preparation) (Nakad) and *Pseudomonas aeruginosa* (Chang et al., 2012). For challenging parents and offsprings, we used the pathogenic bacterium *B. thuringiensis* (BT) strain B-18247 and as bacterial control either the non pathogenic BT strain DSM350 or *Escherichia coli* strain OP50. *B. thuringiensis* is a soil dwelling Gram-positive bacterium which co-interact with *C. elegans* in nature as both could be previously isolated together from a compost heap in France (MA Félix, personal communication).

7.3 Materials and methods

(a) Host-pathogen system

As host model two isogenic strains of nematodes were used in this study: the Bristol isolate N2 and the Hawaii isolate CB4856. *C. elegans* was maintained at 20°C on Nematode Growth Medium (NGM) plates with the non pathogenic *E. coli* OP50 as an *ad libitum* food according to protocols described by Stiernagle (Stiernagle, 2006).

As a pathogen model, we used the nematocidal strain of *B. thuringiensis* NRRL B-18247, provided by the Agriculture Service Patent Culture Collection (United States Department of Agriculture, Peoria, Illinois, USA).

As controls we used the non-nematocidal strain of *B. thuringiensis* DSM 350 and the non-pathogenic *E. coli* strain OP50. DSM 350 was obtained from the German Collection of Microorganisms and Cell Cultures (Deutsche Sammlung von Mikroorganismen und Zellkulturen GmbH, DSMZ, Braunschweig, Germany).

The *B. thuringiensis* strains were cultured in large quantities as described in Borgonie *et al.* (Borgonie *et al.*, 1995) before the start of the project. These cultures, containing mainly spores associated with toxins for the nematocidal strains and only spores for the BT control, were kept in aliquots at -20°C according to Leyns *et al.* (Leyns *et al.*, 1995). Directly before each experiment, BT aliquots were thawed whereas *E. coli* OP50 was grown in liquid Luria-Bertani (LB) medium overnight (ON) at 37°C.

(b) Parental exposure

For exposure of the parental generation (P0) to pathogens, we used hermaphrodite worms at the fourth instar larval stage (L4). These worms (P0) were washed off the growth NGM

plates (figure 1A) with a Phosphate-buffered saline (PBS) solution and transferred onto priming plates inoculated with the test bacterium (figure 1B). The test bacterium was always mixed with *E. coli* OP50 (as food) when it was either the pathogenic strain B-18247 or the non-pathogenic strain DSM350 (figure 1B). Mixtures were prepared with 1:400 dilution from a frozen BT stock with a concentration of 1.5×10^{10} particles/ml. Dilutions were made in a suspension of *E. coli* OP50 from an ON culture at 37°C suspended in PBS and adjusted to an OD = of 5. Naïve priming plates were seeded with only *E. coli* OP50 with OD = of 5 (figure 1B).

The priming step was done for 48 hours on peptone free Nematode Growth Medium (PFM) plates at 20°C. Peptone free NGM prevents BT growth and germination, this way worms are challenged on the assay plates with either spores associated to toxins for nematocidal strains or only spores for non-nematocidal strains.

After 48 hours of priming, worms (P0) filled with eggs were washed off the priming plates and bleached with a mixture of 5M NaOH and NaClO (12%). This step is used for decontamination and synchronization of worm cultures. It is only the eggs which survive the bleaching procedure, whereas worms and bacterial cells are killed.

The bleached eggs (F1) were transferred to growth (PFM) plates (figure 1C) and kept at 20°C until the fourth instar larvae stage (L4).

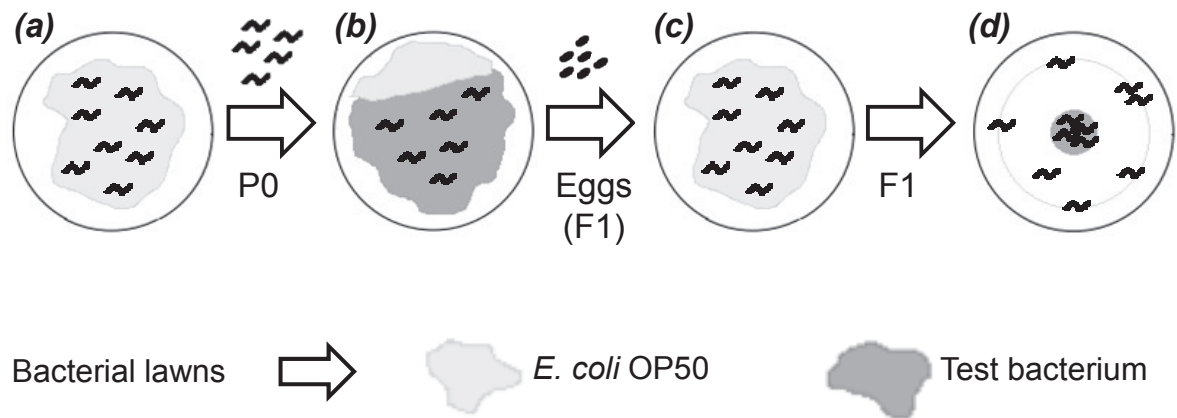


Figure 1. Schematic of the experimental design of trans-generational priming of pathogen avoidance behaviour. (a) NGM plate where parents (P0) were maintained until they reached their fourth larval stage (L4) feeding on *Escherichia coli* strain OP50. (b) Priming plates where P0 were exposed for 48 hours to the test bacterium. (c) PFM plate where offspring were raised to the L4 stage on *E. coli* OP50. (d) PFM escape assay plates on which offspring were tested for their leaving response toward the test bacterium.

(c) Lawn leaving assay

The assay was designed for 9 cm petri dishes of Peptone free NGM (figure 1D), as described previously (Hasshoff et al., 2007). The assay plates were inoculated in the center with the test bacterium (either B-18247, DSM350 or OP50). BT central lawns were mixed with *E. coli* OP50 from an ON culture at 37°C suspended in PBS and adjusted to an OD = of 5. The mixture was prepared by adding BT at a dilution of 1:250. 30 µl of either this mixture (B-18247 + OP50 or DSM350 + OP50) or just OP50 were spotted onto the center of the plate and 80 µl of *E. coli* OP50 with OD=5 were additionally placed at the boundary of the plates (figure 1D). This *E. coli* "ring" prevents escaping worms from starving, thus minimizing their subsequent return to the central test lawn. The assay plates were left to dry overnight (9-15h) at 20°C.

Hermaphroditic L4 (F1) were pipetted onto the test lawn. The number of pipetted worms (F1) was counted at time point 0h, the time point at which the worms touched the central

lawn. Leaving behaviour was scored at three different time points: 1 hour, 14 hours and 24 hours after worms' exposure. The assay was performed at 20°C and included 5 replicates of each treatment.

The leaving behaviour was assessed by counting the number of worms remaining on the lawn at the different exposure times. A leaving index was calculated as:

$$\text{Leaving index} = \frac{\text{starting number of worms} - \text{number of worms on the lawn}}{\text{starting number of worms}}$$

(d) Statistical analysis

We analyzed our data from the leaving assays using a fully factorial ANOVA model. Our analysis showed for each time point that the defined model provided a significantly better fit to the data than a minimal model (ANOVA, $F_{11,116/117} > 13$, $p < 0.0001$).

We first performed the ANOVA effect tests for the factors *C. elegans* strain (CE), Treatment (Treat) and parents' exposure (PE). For the effect tests which showed significant p-values, we then performed pairwise comparisons applying the posthoc test Tukey HSD.

The statistics were done using the program JMP version 9.0 (SAS Institute Inc.), whereas graphs were produced with the program SIGMAPLOT version 12.0 (Systat Software Inc.).

7.4 Results

*Testing whether priming parents on *B. thuringiensis* affects pathogen avoidance behaviour in their offspring*

For all time points of offspring exposure 1h, 14h and 24h, CB4856 had a higher leaving response than N2 on both BT strains; the pathogen B-18247 and the control DSM350 (figure 2, table 1, table 2). Pathogen avoidance behaviour was only observed in N2 and at time points 14h and 24h where leaving was higher toward the B-18247 compared with DSM350 (figure 2, table 3). Leaving response was very high in CB4856 which didn't avoid the pathogenic BT strain more than the control (figure 2, table 3).

Our results from the fully factorial ANOVA analysis show a significant interaction term between the factors *C. elegans* (CE) and treatment (Treat) only at time point 14h (table 1). The significance levels of pairwise comparisons between the different *C. elegans* and bacterial treatment strains were calculated using the post hoc test Tukey HSD (table 4).

Parents' exposure (PE) didn't have any effect on the leaving response of offspring at time point 1h (table 1). This result changed across exposure time showing a trend effect of priming at 14h and a highly significant effect at time point 24h (table 1).

At the latter time point, priming effect was tested performing pairwise comparisons between the three bacterial treatments used to prime parents (B-18247, DSM350 and OP50) and the two *C. elegans* strains using a Tukey HSD test (table 5). The results showed significant priming effect only in N2 and not in CB4856. This effect depended on the bacterial taxa and not the strain, as it was only significant for the comparison B-18247 vs

OP50 and DSM350 *vs* OP50 but not for the comparison between both BT strains, B-18247 *vs* DSM350 (table 5).

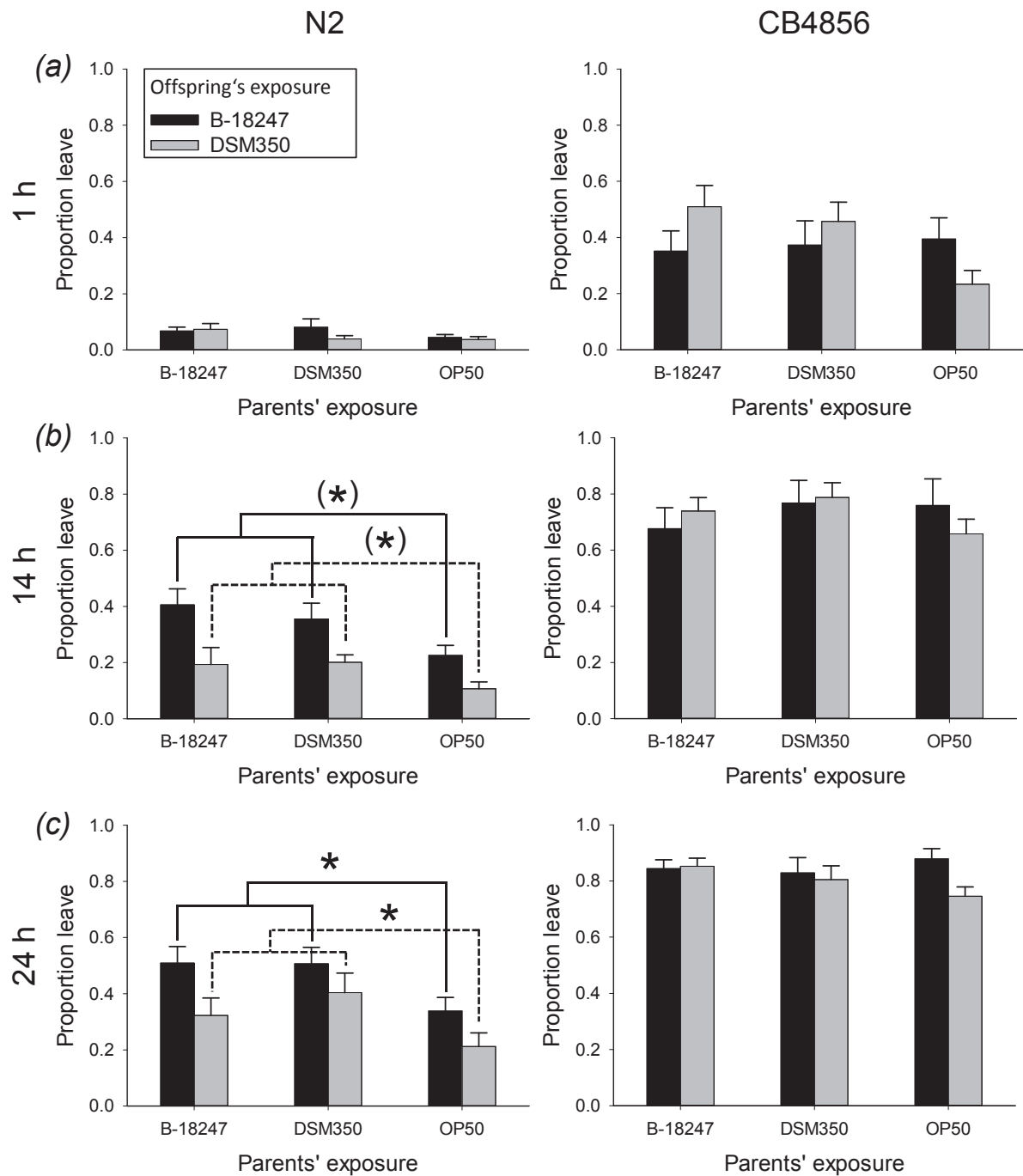


Figure 2. Lawn leaving behaviour scored in offspring at time points (a) 1h, (b) 14h and (c) 24h exposure. Left column shows results for N2, whereas the right column shows those for CB4856. The y axis gives the proportion of worms, which left the lawn. The x axis shows the priming treatment toward which parents were exposed, including either pathogenic B-18247, non-pathogenic DSM350, or *E. coli* OP50. The histograms show the offspring leaving response after exposure to nematocidal B-18247 (in black) or the control DSM350 (in grey). A star indicates a significant difference ($p < 0.05$) and a star in brackets a trend of a difference ($p < 0.1$). Error bars represent standard error of the means.

Table 1. Effect tests from the fully factorial analysis.

Time point	Factor	DF	F ratio	<i>p</i>
1 h	Treatment (Treat)	1	0	0.9242
	<i>C. elegans</i> strain (CE)	1	135.96	<0.0001
	Parents' exposure (PE)	2	2.43	0.0919
	Treat * CE	1	0.73	0.394
	Treat * PE	2	2.8	0.0645
	CE * PE	2	1.02	0.3611
	Treat * CE * PE	2	3.02	0.0524
14 h	Treatment (Treat)	1	6.48	0.012
	<i>C. elegans</i> strain (CE)	1	215.42	<0.0001
	Parents' exposure (PE)	2	2.71	0.0704
	Treat * CE	1	5.58	0.0197
	Treat * PE	2	0.16	0.8519
	CE * PE	2	1.45	0.2381
	Treat * CE * PE	2	1.29	0.2772
24 h	Treatment (Treat)	1	10.65	0.0014
	<i>C. elegans</i> strain (CE)	1	231.34	<0.0001
	Parents' exposure (PE)	2	4.28	0.016
	Treat * CE	1	2.44	0.1202
	Treat * PE	2	0.46	0.6303
	CE * PE	2	3.14	0.0467
	Treat * CE * PE	2	1.1	0.3357

DF = degrees of freedom, SS = sum of squares. Significant *p* values are shown in bold. Results from the fully factorial ANOVA model with interaction fitted with a GLM (general linear model) procedure for time point 1 hour of offsprings' exposure. The table shows the different main effects (Treat, CE and PE) and the interaction effects (Treat * CE, Treat * PE, CE * PE and Treat * CE * PE), both with their significance levels.

Table 2. Pairwise comparisons between N2 and CB4856 as to their leaving behaviour toward each treatment and per time point of exposure.

Time point	Treatment	t	df	<i>p</i>
1 h	B-18247	-6.82	34.03	<0.0001
	DSM350	-7.88	30.37	<0.0001
14 h	B-18247	-7.24	51.53	<0.0001
	DSM350	-14.73	56.8	<0.0001
24 h	B-18247	-9.89	60.46	<0.0001
	DSM350	-11.26	51.89	<0.0001

Significant *p*-values are shown in bold. False discovery rate (FDR) was used to adjust for multiple pairwise comparisons

Table 3 Comparison of leaving behaviour in N2 and CB4856 separately toward pathogenic BT (B-18247) vs non pathogenic BT (DSM350).

<i>C. elegans</i>	Time point	t	df	<i>p</i>
N2	14 h	-4.09	63.11	<0.0001
	24 h	-2.75	64.79	0.0075
CB4856	14 h	-0.04	49.79	0.9638
	24 h	-1.67	58.8	0.0495

Significant p-values are shown in bold. False discovery rate (FDR) was used to adjust for multiple pairwise comparisons. The p-value 0.0495 was not significant anymore after FDR correction.

Table 4. Tukey HSD post hoc test for the interaction effect between the factor treatment (treat) and the factor *C. elegans* strain (CE) at time point 14h of offspring exposure.

	DSM, N2	B-18247, N2	DSM, CB4856	B-18247, CB4856
DSM, N2		0.0028	< 0.0001	< 0.0001
B-18247, N2			< 0.0001	< 0.0001
DSM, CB4856				0.993

This interaction effect was significant according to the results from our fully factorial ANOVA analysis (table 1). Significant p values are shown in bold. False discovery rate (FDR) was used to adjust for multiple pairwise comparisons.

Table 5. Tukey HSD post hoc test for the interaction effect between the factor *C. elegans* strain (CE) and the factor parents' exposure (PE). This interaction effect was significant according to the results from our fully factorial ANOVA analysis.

	OP50, N2	B-18247, N2	DSM350, N2	OP50, CB4856	DSM350, CB4856
B-18247, CB4856	< 0.0001	< 0.0001	< 0.0001	0.9825	0.9912
DSM350, CB4856	< 0.0001	< 0.0001	< 0.0001	1	
OP50, CB4856	< 0.0001	< 0.0001	< 0.0001		
DSM350, N2	0.0036				
B-18247, N2	0.0751		0.9401		

This interaction effect was significant according to the results from our fully factorial ANOVA analysis (table 1). Significant p values are shown in bold. False discovery rate (FDR) was used to adjust for multiple pairwise comparisons.

7.5 Discussion

Trans-generational priming is common in invertebrates but has mainly been so far described for physiological immunity, whereas it should also be expressed for behavioural defences. A recent study demonstrated priming of a behavioural immune response used by *Drosophila melanogaster* against endoparasitoid wasps. This study showed a change in oviposition behaviour in flies after they saw wasps. Flies switched egg laying to alcohol-laden food sources that serve as a protection of hatched larvae from infection (Kacsoh et al., 2013).

Trans-generational priming should impact positively on *C. elegans* fitness and thus be favoured by natural selection. This nematode reproduces via either mating or selfing. Compared to outcrossing, clonal or selfing organisms are limited in their ability to produce genetically diverse offspring that have novel abilities to resist pathogens. Moreover, *C. elegans* is a colonizer of microbe-rich habitat where it feeds on bacteria and yeasts (Felix and Braendle, 2010). Secondary encounters affecting *C. elegans*' lifespan are highly likely to occur in its natural environment. As a consequence of these two points, trans-generational priming should be highly beneficial for *C. elegans* in order to enhance offspring survival. Upregulating physiological immune responses and keeping these responses upregulated for an extended period for protection against secondary encounters might be very costly for short-lived animals. Therefore, behavioural defences might be beneficial, as these types of defence are usually fast, reduce contact with harmful microorganisms and decrease inflicted tissue damage. Hence, transmitting such energy saving defences across generations should additionally enhance overall fitness.

Our results show a higher leaving behaviour in CB4856 compared with N2 on both BT strains B-18247 and DSM350 and for all time points of exposure confirming recent findings by (Nakad) (manuscript in preparation). BT treatment didn't have any effect on leaving in CB4856 as this strain had a pronounced equal leaving response on nematocidal and non nematocidal BT in contrary to N2 which left pathogenic BT (B-18247) more than the control (DSM350). This as well correlates with our previous results from a recent study (Nakad) (manuscript in preparation).

Parents' exposure to pathogenic and non-pathogenic BT had a trend effect on offspring leaving response after 14h and a significant effect at the subsequent 24h time point (table 1), indicating a time-dependant decision or response process in *C. elegans*.

Interestingly, only the Bristol isolate of *C. elegans* showed trans-generational priming effect of leaving behaviour. Offspring of parents exposed to either BT B-18247 or BT DSM350 exhibited an increase in pathogen avoidance response compared with offspring of naïve parents (parents only exposed to OP50 during the priming step) (figure 2c, table 5). This parental priming effect was not observed in the Hawaii isolate CB4856.

N2 and CB4856 are genetically divergent isogenic isolates and differ by one single-nucleotide polymorphism (SNP) per 840bp (Koch et al., 2000; Swan et al., 2002). This natural divergence has been shown in different studies to underlie several phenotypic differences between these two isolates. Some examples are related to either differences in certain life history traits (Gutteling et al., 2007) or to differences in behavioural and immunity phenotypes such as foraging behaviour like clumping and bordering (de Bono and Bargmann, 1998), food-patch and pathogen avoidance behaviour (Bendesky et al., 2012; Chang et al., 2012), or pathogen resistance (Reddy et al., 2009). This difference

between these two isogenic and genetically distinct lines strongly indicates that trans-generational immune priming has a genetic basis which is not given in all natural isolates.

Our results show that priming of leaving behaviour was independent from BT pathogenicity. Exposing the parents to either B-18247 or DSM350 increased leaving behaviour in their offspring compared with offspring of parents which encountered only *E. coli* OP50. This might be an indication that *C. elegans* can distinguish between different types of bacteria (e.g., Gram-positive BT versus Gram-negative *E. coli*). The increase in pathogen avoidance response, which did not depend on whether the BT treatment during priming was pathogenic or not, might be partially explained by the spore concentration we used for parents' exposure. The pathogen B-18247 had a concentration which did not induce any mortality or paralysis of worms. Survival was similarly unaffected on pathogenic (B-18247) vs non pathogenic BT (DSM350). This might partially explain why priming effect of leaving behaviour was dependant on the genus and not the pathogenicity of the tested bacteria. BT might have been then recognized as a bad food source which led to this increase in BT avoidance response. Though the concentration of pathogenic spores did not induce mortality, still offspring of naive parents showed a higher leaving response on B-18247 compared with DSM350. This suggests that even at low concentrations, pathogenic BT can still affect worm fitness without causing mortality.

Taken all together, our results suggest that trans-generational priming of pathogen avoidance behaviour results from a combination of at least two factors: BT's pathogenicity and BT's recognition as a bad food source by *C. elegans*.

7.6 Conclusion

Our results demonstrate that pathogen avoidance can increase significantly among the offspring of parents challenged with pathogenic as well as non-pathogenic BT compared with offspring of naïve parents. This priming effect is very likely to have genetic basis as it varied between two genetically divergent *C. elegans* isolates. Transgenerational inheritance of defence responses should be beneficial for offspring's survival and fitness as they are likely to encounter in nature similar bacterial patches as their parents.

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8. Chapter 3:

Activation of the FOXO family transcription factor DAF-16 by pathogenic *Bacillus thuringiensis*

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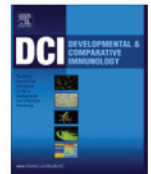
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Activation of the *Caenorhabditis elegans* FOXO family transcription factor DAF-16 by pathogenic *Bacillus thuringiensis*

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ABSTRACT

The FOXO family of transcription factors have recently been implicated in innate immunity, especially in case of DAF-16 from the nematode *Caenorhabditis elegans*. However, previous studies with this nematode proposed that DAF-16 is not directly activated by pathogens. Rather, DAF-16 mediates resistance if activated by some other cue as part of a general stress response. We specifically tested this notion by analysis of DAF-16 nuclear translocation and thus regulatory activity upon exposure to pathogenic *Bacillus thuringiensis*. Our results demonstrate that DAF-16 nuclear translocation is indeed particularly induced in response to bacterial pathogenicity, whereas infection load alone has little effect. Translocation is strongest at an early time point, suggesting a role during the immediate immune response. The increased DAF-16 availability is associated with higher resistance and a reduction in feeding behaviour. Taken together, our data highlight that a FOXO transcription factor directly responds to pathogens and thus contributes to immune defence.

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1. Introduction

The transcription factors of the FOXO family play a central regulatory role in animals, where they influence diverse traits such as metabolism, stress resistance, or longevity (Arden, 2008). In the nematode *Caenorhabditis elegans*, the best studied member of these transcription factors is DAF-16, which is one of the central targets of the insulin-like signalling cascade, characterised by the insulin-like receptor DAF-2. DAF-2 negatively regulates DAF-16 through phosphorylation of the phosphoinositide 3-kinase AGE-1, and subsequent activation of four serine threonine kinases AKT-1, AKT-2, PDK-1, and SGK-1. Thus, DAF-16 nuclear translocation and regulatory activity can be induced by DAF-2 downregulation (reviewed in Antebi, 2007; Baumeister et al., 2006; Landis and Murphy, 2010). DAF-16 activity is further modulated by the MAPK c-Jun N-terminal kinase-1 (JNK-1, Oh et al., 2005; Wolf et al., 2008), the MST1-homologue *ste20*-like protein kinase CST-1 (Lehtinen et al., 2006), or the E3 ubiquitin ligase RLE-1 (Li et al., 2007). Upon nuclear translocation, DAF-16 regulates expression of a large number of genes (Halaschek-Wiener et al., 2005; Lee et al., 2003; McElwee et al., 2004; Murphy et al., 2003; Oh et al., 2006), ultimately influencing diverse phenotypes, ranging from increases in stress resistance (e.g. against heat, oxidative stress, and heavy metals),

longevity, fat storage, formation of the long-lived dauer stage, to the reduction of reproductive and mating rates, body size, overall fitness, and a shift in metabolic processes (e.g., Antebi, 2007; Ashrafi et al., 2003; Barysytė et al., 2001; Baumeister et al., 2006; Brys et al., 2007; Gems et al., 1998; Jenkins et al., 2004; Landis and Murphy, 2010). As such, this transcription factor appears to act as an important life-history switch, which mediates between alternative life-history strategies in response to both environmental and endogenous cues.

One of the previously characterized resistance phenotypes is directed against pathogens. This phenotype was first suggested after identification of the genes, for which expression is enhanced by DAF-16 and which include several putative antimicrobial factors, e.g. the lysozyme genes *lys-7*, *lys-8*, *ilys-3*, or the saposin-like protein gene *spp-1* (Halaschek-Wiener et al., 2005; McElwee et al., 2004; Murphy et al., 2003). Indeed, activation of DAF-16 in DAF-2 loss-of-function mutants or in transgenic strains, which overexpress DAF-16, enhances survival in the presence of different pathogens such as *Staphylococcus aureus*, *Enterococcus faecalis*, *Pseudomonas aeruginosa*, *Salmonella enterica*, *Yersinia pestis*, *Cryptococcus neoformans*, *Bacillus thuringiensis*, and enteropathogenic *Escherichia coli* (Amrit et al., 2010; Anyanful et al., 2005; Begun et al., 2007; Chavez et al., 2007; Evans et al., 2008a,b; Garsin et al., 2003; Hasshoff et al., 2007; Jia et al., 2009; Kawli and Tan, 2008; Kerry et al., 2006; Miyata et al., 2008; Mohri-Shiomi and Garsin, 2008; Singh and Aballay, 2006, 2009; Troemel et al., 2006; van den Berg et al., 2006).

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Interestingly, insulin-like signalling via DAF-16 appears to mediate pathogen defence in three ways. Firstly, an important aspect is the direct activation of antimicrobial genes like the saposin-like protein gene *spp-1* and the lysozyme gene *lys-7* (Evans et al., 2008a). This antimicrobial response is regulated slightly differently to the effect on longevity, whereby the former is more strongly influenced by the serine threonine kinases AKT-1 and AKT-2, whereas the latter depends more strongly on the serine threonine kinases PDK-1 and SGK-1, which all act downstream of DAF-2 and upstream of DAF-16 (Evans et al., 2008a). Upstream of DAF-2, the antimicrobial response is regulated by neuroendocrine signals, which include the insulin ligand INS-7 (Kawli and Tan, 2008) and which are influenced by G protein α q subunit EGL-30 and the phospholipase C β EGL-8 (Kawli et al., 2010). The DAF-16 dependent immune response can be suppressed by pathogenic *P. aeruginosa* through the bacterial two-component response regulator GacA and the quorum-sensing regulators LasR and RhlR (Evans et al., 2008b). Secondly, DAF-16 mediates protection against immuno-pathological effects. *C. elegans* uses reactive oxygen species (ROS) to fight pathogenic *Enterococcus faecalis*. These ROS account for protein and thus cellular damage at the site of infection, which can be mitigated by the activity of DAF-16 (Chavez et al., 2007; Mohri-Shiomi and Garsin, 2008). Thirdly, DAF-16 also contributes to enhanced behavioural avoidance of pathogens, in this case pathogenic *Bacillus thuringiensis* (Hasshoff et al., 2007). The avoidance response was fast and consisted of both physical evasion of pathogen-rich locations and reduced feeding rates. Such behavioural defences represent an alternative and widespread *C. elegans* defence mechanism against pathogens (reviewed in, Schulenburg and Ewbank, 2007).

All of the above examples were inferred from mutant analysis, in which DAF-16 activity is manipulated genetically – either through downregulation of DAF-2 or experimental DAF-16 overexpression. To date, it is unclear whether DAF-16 nuclear translocation (and thus regulatory activity) is directly induced by pathogens. Some DAF-16 targets appear upregulated after exposure to pathogenic *P. aeruginosa* in a DAF-2/DAF-16 dependent manner (e.g. the antimicrobial gene *abf-2*, Evans et al., 2008b). However, in the case of *P. aeruginosa*, exposure to this pathogen failed to result in nuclear localization of DAF-16 (Shapira et al., 2006; Troemel et al., 2006). Instead, *P. aeruginosa* virulence factors appear to interfere with insulin signalling, ultimately causing DAF-16 delocalization from the nucleus and thus increased susceptibility (Evans et al., 2008b; Kawli and Tan, 2008). In consistency with these results, lower nuclear DAF-16 localization was also observed in the presence of pathogenic *C. neoformans* if compared to the *E. coli* control (TeKippe and Aballay, 2010). In these cases, DAF-16 appears to function in pathogen defence as part of a general stress response that is not directly activated by pathogens but instead by other cues (Troemel et al., 2006). To our knowledge, only two studies suggest that DAF-16 is directly activated by pathogens. Here, an increased nuclear localization of DAF-16::GFP was observed after exposure to enteropathogenic *E. coli* for up to 3 h (Anyanful et al., 2009) or to pathogenic *Pseudomonas fluorescens* for 12 h (Neidig et al., 2011).

In this study, we specifically tested DAF-16 activation by pathogenic strains of *B. thuringiensis*. We challenged transgenic *C. elegans* strains, in which DAF-16 is coupled to the green fluorescent protein, thus allowing direct observation of DAF-16 localization. This approach was used to relate DAF-16 nuclear translocation with the level of pathogenicity and infectivity of the bacteria.

2. Materials and methods

DAF-16 nuclear translocation was monitored in two transgenic *C. elegans* strains: TJ356 (*zls356[Pdaf-16::daf-16::gfp;rol-6(su1006)]*)

and GR1352 (*mgDf47;xrls87[Pdaf-16::gfp::daf-16b;rol-6(su1006)]*). Each of these strains harbours a DAF-16::GFP construct integrated into the genome of the standard laboratory strain N2 (Henderson and Johnson, 2001; Lee et al., 2001). The standard *C. elegans* strain N2 was used as a control to study infection load and survival. As a specific control for feeding behaviour, we examined the strain HE1006. This strain carries the same *rol-6(su1006)* allele that is present in the transgenic strains, for which it was used as an easily identifiable co-injection marker, and that also affects movement behaviour. The *C. elegans* strains were obtained from the Caenorhabditis Genetics Center (CGC; <http://www.cbs.umn.edu/CGC/>). Their maintenance in our laboratory followed standard protocols (Stiernagle, 2006). Non-pathogenic *E. coli* OP50 was used as an *ad libitum* worm food during maintenance and all experiments (concentration of 10^{10} bacteria/ml). Worms were challenged with two nematocidal *B. thuringiensis* strains, NRRL B-18247 and NRRL B-18679 (in the following: BT18247 and BT18679), provided by the Agricultural Research Service Patent Culture Collection (United States Department of Agriculture, Peoria, Illinois, USA). The non-nematocidal strain DSM 350, obtained from the German Collection of Microorganisms and Cell Cultures (Deutsche Sammlung von Mikroorganismen und Zellkulturen GmbH, DSMZ, Braunschweig, Germany), was used as a control. Before start of the experiments, large quantities of spore-toxin mixtures (or only spore mixtures for the control) were produced as previously described. These mixtures were frozen in small aliquots at -20°C , at which temperature they can be stored for up to a year without losing pathogenicity (unpublished data, Leyns et al., 1995).

In all experiments, the various treatment combinations were always evaluated in parallel and in fully randomized order. Observer bias was minimized by coding of samples. To characterize disease dynamics, we examined nematode survival rate and infection load in 4 h intervals within a 24 h period. For each time point, independent populations of 20–30 fourth instar larvae (L4) of the strain N2 were exposed to either BT18247 (final concentration of 1.5×10^8 particles/ml) or BT18679 (concentration of 1.5×10^7 particles/ml). After the respective time intervals, the survival rate was measured as the number of living worms divided by the total number of worms on the plate. The infection load was determined for four up to ten surviving worms from each population, using six categories and generally following the previously described approach (Hasshoff et al., 2007): (i) no infection; (ii) no more than 20 spores in the gut; (iii) more than 20 and no more than 100 spores, accumulation in either the anterior or posterior part of the gut, no vegetative cells; (iv) more than 100 spores with massive accumulations in the anterior and/or posterior part of the gut, first intestinal cells destroyed, no vegetative cells; (v) as category iv, but with vegetative cells inside the gut; (vi) as category v but spores and vegetative cells in high concentrations in all parts of the gut and body. For each population, we then inferred an overall infection load index by calculating the average across the measured individuals. One particular run of the experiment always included all of the different time points and treatments. The whole experiment was based on a total of four independent repetitions, performed on different dates. The statistical analysis of the results was based on a general linear model (GLM) based on ordinal logistic regression and the restricted maximum likelihood method (REML), using a factorial design, including the factors *B. thuringiensis* strain, time, and the interaction between the two.

DAF-16 translocation was studied in two independent experiments, one with the transgenic strain TJ356 and the other with GR1352. Note that the second experiment had to be performed with new preparations of the pathogens, resulting in slightly different pathogenicity levels (see Section 3). In each experiment, DAF-16 translocation was inferred at two time points (4 and 12 h) after exposure to the two pathogen strains (BT18247 and

BT18679), each at three concentrations (1.5×10^8 , 0.9×10^8 , and 0.3×10^8 particles/ml). As negative controls, we used exposure to *E. coli* OP50 only or *E. coli* OP50 plus the non-pathogenic *B. thuringiensis* DSM 350 (concentration of 1.5×10^8 particles/ml). As a positive control, the nematodes were exposed for the same time periods to heat shock at 30 °C. For each treatment and time point, separate populations of 20 animals were set up and analysed. A total of four and five repetitions were performed for TJ356 and GR1352, respectively. The repetitions were performed on different dates. DAF-16 translocation was monitored in four up to six surviving animals and, in each case, separately for four body regions, which should be particularly exposed to the pathogens, including: (i) the head (mouth up to the second bulb of the pharynx); (ii) the front gut (the intestinal area that directly follows the pharynx); (iii) the mid gut (the intestinal area which is adjacent to the vulva); and (iv) the anal region of the gut. The level of translocation was determined using six categories: (i) no translocation; (ii) weak nuclear localization in no more than five cells per body region; (iii) weak nuclear localization in more than five cells per region; (iv) as in category iii but with one up to five cells with strong nuclear localization per region; (v) strong nuclear localization in more than five and no more than 10 cells per region; (vi) strong nuclear localization in more than 10 cells per body region. In parallel, we also determined the infection load of the animals, as described above. For each population, we then calculated an overall translocation index for the different body regions as well as the overall body and an overall infection load index, using in all cases the average across the examined individuals per population. We compared each concentration of each pathogenic strain to the non-pathogenic control DSM 350 using GLM statistics, based on ordinal logistic regression and a factorial model, which included pathogen strain as a factor. The different body regions and the two time points were evaluated separately. Because of multiple testing, we applied the false discovery rate (FDR, Benjamini and Hochberg, 1995) to adjust the significance level. Variation in infection load was similarly analysed with a GLM ordinal logistic regression using *B. thuringiensis* strain and concentration nested within strain as factors.

Parallel to each run of the DAF-16 translocation assay, we also always checked pathogenicity and infectivity of the pathogens by analysis of survival and infection load in N2 worms and the respective transgenic strain after 24 h exposure, following exactly the same protocol described above for characterization of disease dynamics. In this case, statistical analysis was based on a GLM ordinal logistic regression model including the factors: nematode strain, bacterial strain, the interaction between nematode and bacterial strain, and bacterial concentration nested within bacterial strain.

In addition to the above experiments, we evaluated a behavioural response to pathogens, namely the enhanced reduction in feeding activity after pathogen exposure, which we previously demonstrated to be influenced by DAF-16 (Hasshoff et al., 2007). We tested whether this enhanced reduction is shown by the transgenic line TJ356, which bears the DAF-16::GFP reporter construct and at the same time should overexpress DAF-16, supposedly leading to a stronger reduction than shown by the wild-type. TJ356 additionally carries the *rol-6(su1006)* allele as an easily scorable co-injection marker. Since this allele affects movement behaviour, we used a mutant strain with the same allele (strain HE1006 with *rol-6(su1006)*) as a control for this assay. Feeding activity was examined as described previously (Hasshoff et al., 2007). Briefly, ten L4 hermaphroditic larvae were transferred to bacterial lawns with *E. coli* OP50 and either of the two pathogenic or the non-pathogenic *B. thuringiensis* strain. Bacterial concentrations were as above, including a high, medium, and low concentration treatment. Worms were allowed to acclimatize to the environment. Feeding

activity was measured after 1 h and 6 h by counting the number of grinder movements within a 20 s period under a dissecting microscope. We only considered nematodes which were inside the bacterial lawn. Feeding activity was scored for five worms per replicate, followed by calculation of the average per replicate for further statistical analysis. A total of six replicates were included per treatment combination. We used GLM ordinal logistic regression analysis to assess differences between the two *C. elegans* strains, using nematode strain as the factor in the model. We also compared a specific concentration of one of the pathogens with exactly the same concentration of the non-pathogenic control, again using GLM and pathogen strain as a factor in the model. The two time points were analysed separately. An FDR-based adjustment of significance levels was applied to account for multiple testing.

All statistical analysis was done with the program JMP version 8.0 (SAS Institute Inc.), while graphic illustrations were produced with SIGMAPLOT version 12.0 (Systat Software Inc.).

3. Results

The experiment on disease dynamics was used to assess whether the two *B. thuringiensis* strains differ in the infection process when pathogenicity levels are similar, i.e. there is no significant difference in host survival rate. In a pilot study, we determined that similar pathogenicity levels are obtained if BT18247 is applied at a 10× higher concentration than BT18679. Using such different concentrations in our experiment, the results indeed confirmed that the factor pathogen strain did not affect host survival rate (Table 1), even though towards the end of the observation period BT18679-mediated host survival rates appear lower than those for BT18247 (Fig. 1). In fact, the model-based analysis showed that the only factor with significant influence is time (Table 1). In contrast, under these conditions infection load in surviving animals is influenced by both the factors time and *B. thuringiensis* strain (Fig. 1, Table 1). Interestingly, the more pathogenic strain BT18679 associated with the significantly smaller infection load in surviving animals. The value close to one indicates that these survivors either lack presence of pathogens or contain only few spores (infection categories 1 and 2; see Section 2). All animals killed by either pathogen strain always and invariably show an infection load index of six (the highest category). Taken together, we conclude that at similar pathogenicity levels the two *B. thuringiensis* strain vary in their infection dynamics.

In the subsequent experiments, we evaluated whether pathogenicity and/or infection dynamics lead to an activation of DAF-16. We separately analysed two transgenic *C. elegans* strains, TJ356 and GR1352, which both carry a *daf-16::gfp* reporter gene construct that allows direct evaluation of the localization of DAF-16 (Fig. 2). These *C. elegans* strains were exposed to two pathogenic *B. thuringiensis* strains.

Table 1

Statistical results of the influence of *B. thuringiensis* strain and time on nematode survival rate and infection load index.

Factor ^a	DF	Survival		Infection	
		χ^2	P	χ^2	P
<i>B. thuringiensis</i> strain	1	1.26	0.2616	61.64	<0.0001
Time	1	46.52	<0.0001	17.49	<0.0001
Interaction (<i>B. thuringiensis</i> × time)	1	1.66	0.1980	<0.01	0.9827

^a Effect tests were calculated as part of a general linear model, using ordinal logistic regression and the restricted likelihood method, and the following factors in the model: *B. thuringiensis* strain, time, and the interaction between the two. Survival and infection load were analysed separately. In both cases, the full model was significantly better than a minimal model ($P < 0.0001$), but not significantly worse than a saturated model ($P > 0.99$). DF, degrees of freedom. Significant probabilities are given in bold.

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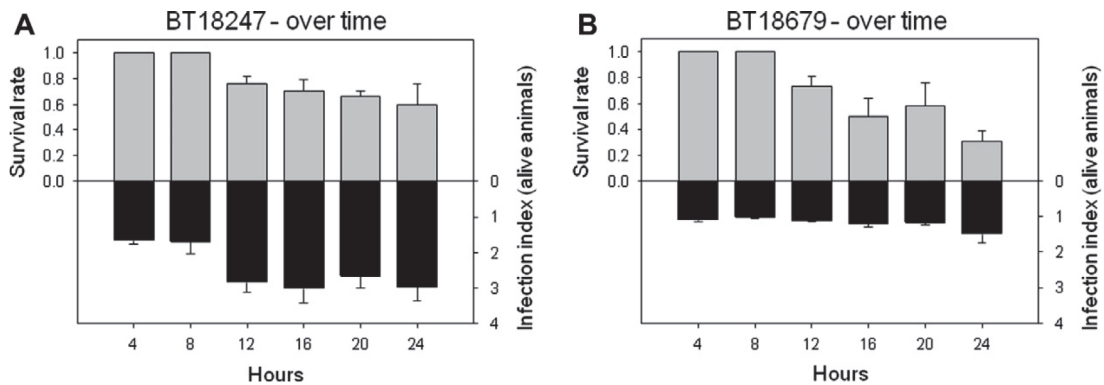


Fig. 1. Survival rate (top part of graphs) and infection load index (bottom part of graphs) in N2 strain nematodes exposed to either (A) *B. thuringiensis* strain BT18247 or (B) strain BT18679. Both traits were measured over time in 4 h intervals. Bars show the average plus standard error. Detailed statistics are given in Table 1.

giansis at identical concentration ranges, including a high, medium and low concentration. In both analyses, we examined survival rate and infection load after 24 h. For this evaluation, we additionally included the *C. elegans* wild-type N2 as a control. In parallel, DAF-16 nuclear translocation and infection load was scored in

the transgenic strains after 4 and 12 h. The two separate analyses generally produced consistent results.

In particular, nematode survival was always significantly lower upon exposure to *B. thuringiensis* BT18679 than BT18247 (Figs. 3 and 4A, B, Table 2). At the same time, the transgenic *C. elegans* lines always survived significantly better than the N2 wild-type (Figs. 3 and 4A, B, Table 2). In the experiment with the transgenic strain GR1352, the interaction term and also the concentration of *B. thuringiensis* had a significant influence on the variation in survival (Table 2). The former may be due to a larger survival difference between the *C. elegans* strains in response to *B. thuringiensis* BT18679 (Fig. 4B), which is not reflected to the same extent in response to BT18247 (Fig. 4A). Nematode infection load showed comparatively little variation at the 24 h time point. In general, *B. thuringiensis* BT18679 associates with lower infection rates in alive animals than BT18247 (Figs. 3 and 4A, B). This effect was significant in the experiment with *C. elegans* strain TJ356 and still showed a trend ($0.1 > P > 0.05$) in the experiment with GR1352 (Table 2, factor *B. thuringiensis* strain). All other factors did not have a significant effect on the observed variation in infection load (Table 2). We conclude that in consistency with our previous data (Schulte et al., 2010) BT18679 is more pathogenic than BT18247. Moreover, we also conclude that the increased availability of DAF-16 in the transgenic lines associates with higher resistance.

During the evaluation of DAF-16 translocation, we could confirm that *B. thuringiensis* BT18679 produces significantly lower infection rates than BT18247 – at both 4 and 12 h time points (Figs. 3 and 4, Table 3). Significant DAF-16 nuclear translocation was observed primarily in the gut, especially the front and medium part of the intestines, in response to medium and high doses of the more pathogenic *B. thuringiensis* strain BT18679 (Figs. 3 and 4C, D). For one of the transgenic *C. elegans* strain, namely GR1352, significant translocation was additionally observed after 4 h but not 12 h exposure to the high dose of *B. thuringiensis* BT18247 (Fig. 4C, D). If DAF-16 nuclear translocation occurred, then it was always more pronounced after 4 h than after 12 h pathogen exposure (Figs. 3 and 4C, D). Note that in all cases, the two negative controls (exposure to non-pathogenic bacteria) always produced the lowest scores and did not differ significantly from each other. Similarly, the positive control (exposure to heat shock) always produced translocation scores of six, strongly suggesting high reliability of the experimental material and procedures used. Consequently, we conclude that DAF-16 nuclear translocation is affected by pathogenic *B. thuringiensis* at the site of contact, namely the gut. Moreover, it is associated with the level of *B. thuringiensis* pathogenicity but not infection load.

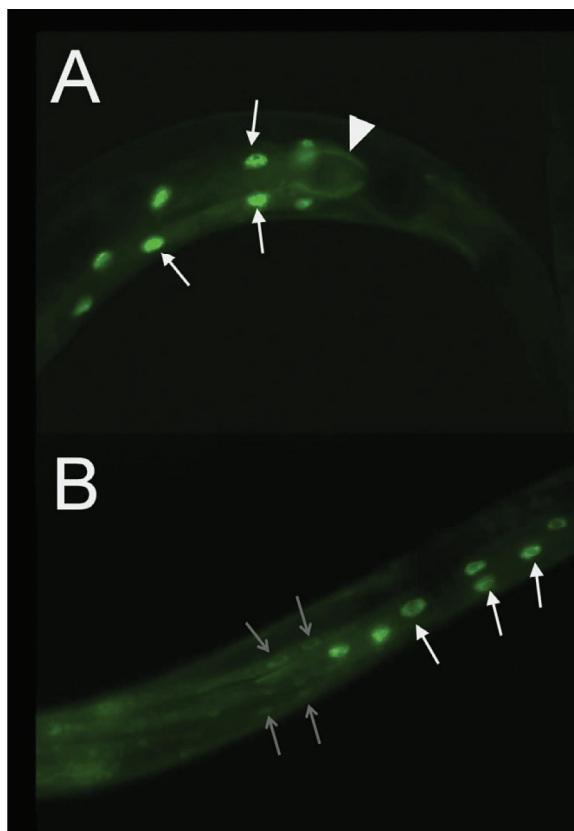


Fig. 2. Fluorescent microscopy photograph of two strain TJ356 nematodes after 4 h exposure to *B. thuringiensis* BT18679, showing: (A) cells with strong nuclear DAF-16 localization (filled arrows); the extended front part of the gut is indicated by the triangle; and (B) some cells with strong (filled arrows) and some with weak nuclear DAF-16 localization (open arrows). In both cases, the front tip of the worm is towards the right.

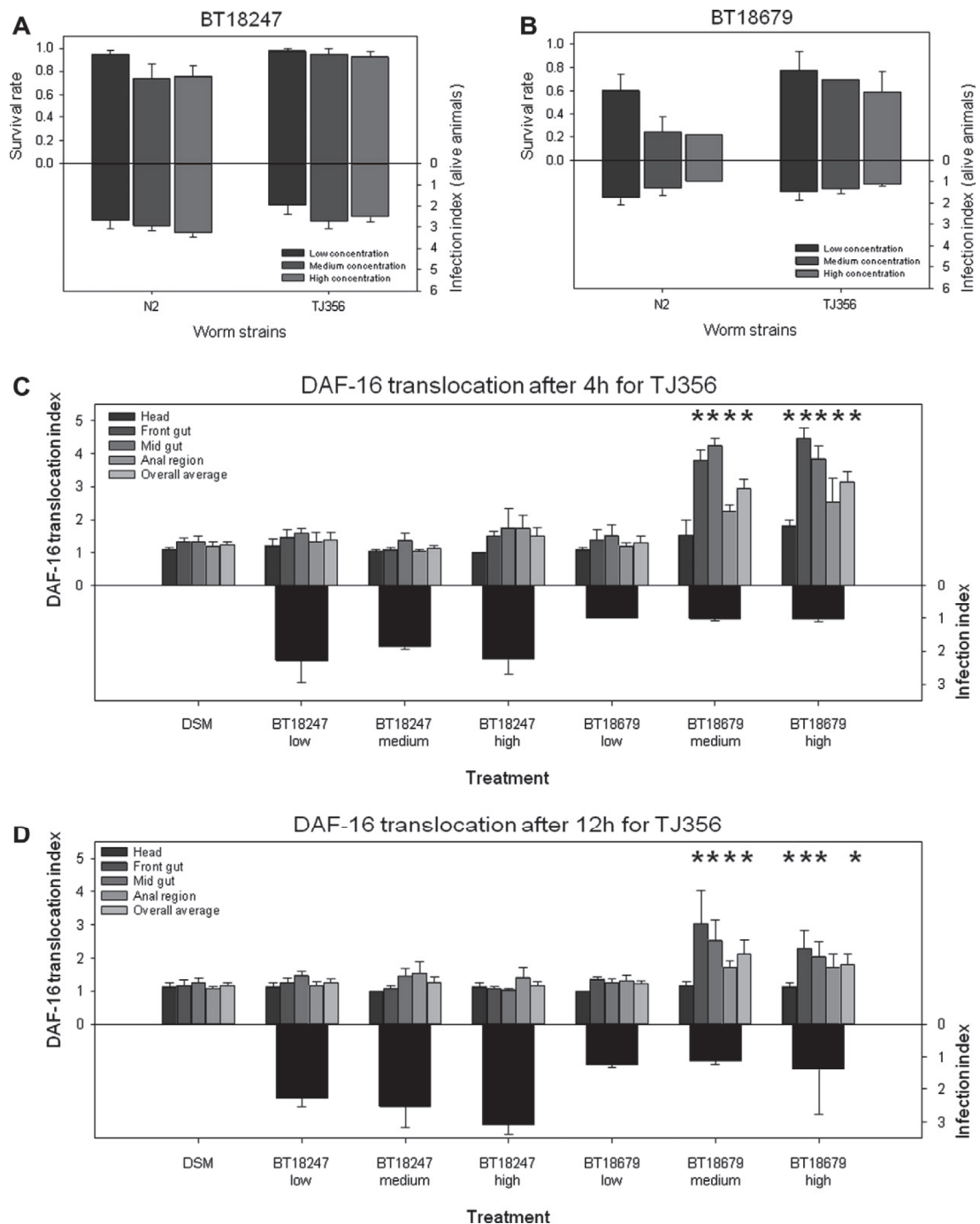


Fig. 3. Survival rate, infection load, and DAF-16 nuclear translocation for the *C. elegans* strain TJ356. In parallel to the DAF-16 translocation assay, survival rate (top part of graphs A and B) and infection load index (bottom part of graphs A and B) were measured for separate populations of *C. elegans* strains N2 and TJ356 after 24 h exposure to different concentrations (different shades of gray) of the *B. thuringiensis* strains BT18247 (A) and BT18679 (B). The DAF-16 translocation index was measured after (C) 4 h and (D) 12 h exposure to different concentrations of BT18247 and BT18679 (X-axis of graphs) for different parts of the gut (different shades of gray). The infection load index (bottom part of graphs) was determined for the same animals. The non-pathogenic *B. thuringiensis* strain DSM-350 (abbreviated DSM) was used as a control. In all cases, bars show the average plus standard error. Asterisks indicate significant differences in DAF-16 translocation after exposure to the respective pathogen versus the non-pathogenic control (DSM). Statistical significance was adjusted with the help of the false discovery rate.

We additionally evaluated whether the elevated availability of DAF-16 in the transgenic lines also affects other DAF-16 mediated responses to pathogens like the inhibition of feeding behaviour.

The activity of feeding was significantly reduced after exposure to pathogens if compared with the non-pathogenic control of the same dose (Fig. 5A, B; GLM, *P* values significant after FDR adjust-

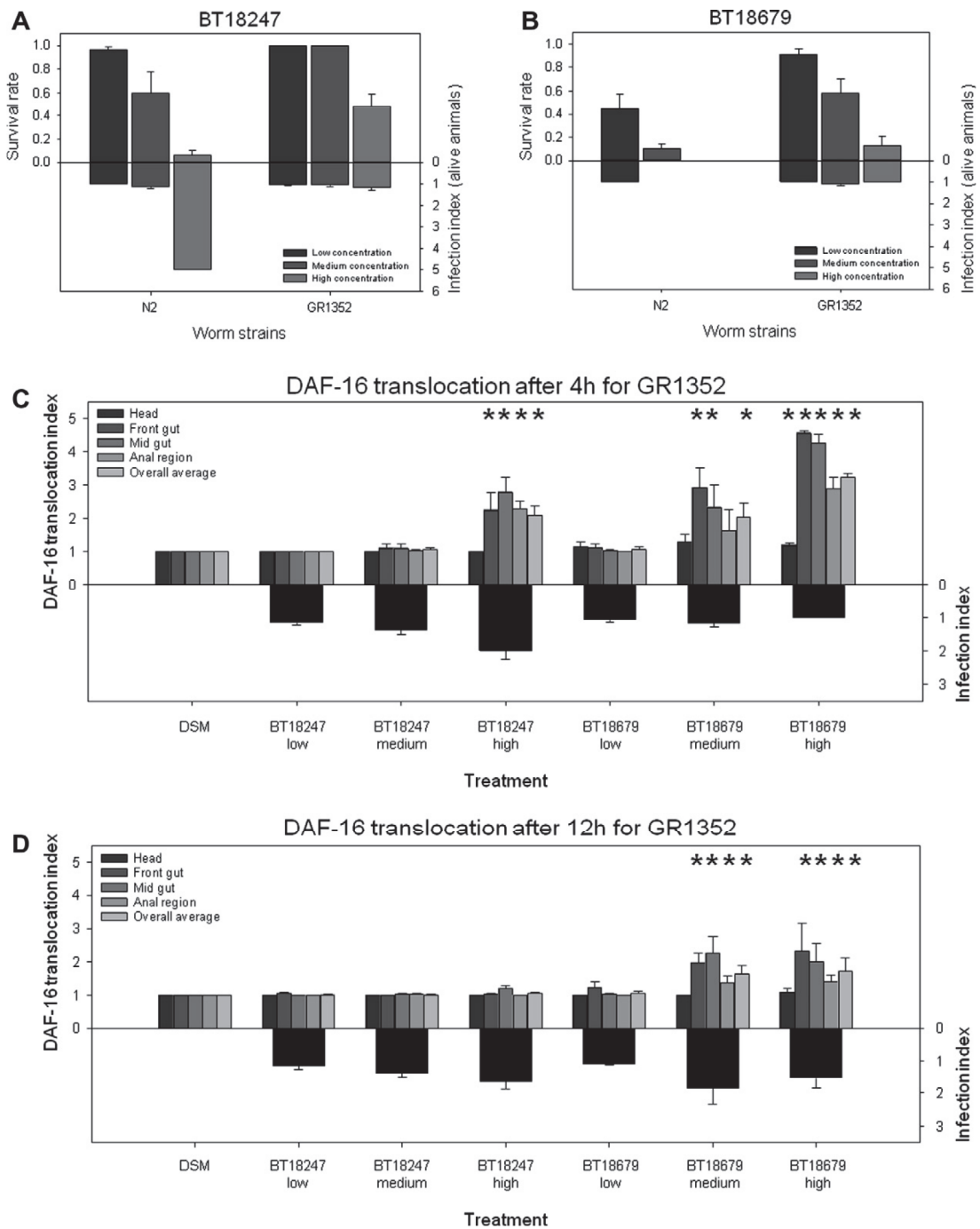


Fig. 4. Survival rate, infection load, and DAF-16 nuclear translocation for the *C. elegans* strain GR1352. In parallel to the DAF-16 translocation assay, survival rate (top part of graphs A and B) and infection load index (bottom part of graphs) were measured for separate populations of *C. elegans* strains N2 and GR1352 after 24 h exposure to different concentrations (different shades of gray) of the *B. thuringiensis* strains BT18247 (A) and BT18679 (B). The DAF-16 translocation index was measured after (C) 4 h and (D) 12 h exposure to different concentrations of BT18247 and BT18679 (X-axis of graphs) for different parts of the gut (different shades of gray). The infection load index (bottom part of graphs) was determined for the same animals. The non-pathogenic *B. thuringiensis* strain DSM-350 (abbreviated DSM) was used as a control. For further details, see legend of Fig. 3.

ments). The only exceptions were observed for the control *C. elegans* strain HE1006 in the comparisons between low doses of BT18247 and DSM after 1 h exposure (Fig. 5A), between all doses of BT18247 and DSM after 6 h (Fig. 5B), and between the low doses

of BT18679 and DSM after 6 h (Fig. 5B). Importantly, we observed a significantly stronger reduction in feeding activity for the transgenic *C. elegans* strain TJ356 if compared with HE1006. These cases referred to high dose of BT18247 after 1 h exposure and low dose

Table 2Statistical analysis of nematode survival rate and infection load for the comparison of the *C. elegans* wild-type N2 and either strain TJ356 (top part) or GR1352 (bottom part).

Factor ^a	DF	Survival		Infection	
		χ^2	<i>P</i>	χ^2	<i>P</i>
<i>N2 vs. TJ356</i>					
<i>B. thuringiensis</i> strain	1	9.48	0.0021	23.97	<0.0001
<i>C. elegans</i> strain	1	13.79	0.0002	0.83	0.3614
Interaction (<i>B. thuringiensis</i> × <i>C. elegans</i>)	1	1.29	0.2565	1.35	0.2445
<i>B. thuringiensis</i> concentration	2	4.78	0.0918	3.59	0.1665
<i>N2 vs. GR1352</i>					
<i>B. thuringiensis</i> strain	1	24.19	<0.0001	3.53	0.0604
<i>C. elegans</i> strain	1	20.76	<0.0001	0.66	0.4171
Interaction (<i>B. thuringiensis</i> × <i>C. elegans</i>)	1	5.21	0.0224	3.63	0.0566
<i>B. thuringiensis</i> concentration	2	36.88	<0.0001	4.56	0.1021

^a Measurements were made in parallel to the DAF-16 translocation assay. Effect tests were calculated as part of a general linear model, using logistic regression and the restricted likelihood method, and the following factors in the model: *B. thuringiensis* strain, *C. elegans* strain, the interaction between the two, and *B. thuringiensis* concentration nested within *B. thuringiensis* strain. Survival and infection load were analysed separately. For TJ356 and GR1352 survival rate, the full models were significantly better than the corresponding minimal models ($P < 0.003$), but not significantly worse than the saturated models ($P > 0.99$). For infection load in GR1352, the defined model did not provide a better fit than the minimal model ($P = 0.1188$), but also not a worse fit in comparison to the saturated model ($P = 0.4299$), indicating lack of significant variation among treatment groups. DF, degrees of freedom. Significant probabilities are given in bold.

Table 3Statistical analysis of infection load measured in parallel to DAF-16 translocation for the comparison of the *C. elegans* wild-type N2 and either strain TJ356 (top part) or GR1352 (bottom part).

Factor ^a	DF	Infection χ^2	4 h <i>P</i>	Infection χ^2	12 h <i>P</i>
<i>N2 vs. TJ356</i>					
<i>B. thuringiensis</i> strain	1	18.76	<0.0001	12.99	0.0003
<i>B. thuringiensis</i> concentration	4	2.03	0.7296	4.06	0.3986
<i>N2 vs. GR1352</i>					
<i>B. thuringiensis</i> strain	1	15.85	<0.0001	0.06	0.8108
<i>B. thuringiensis</i> concentration	2	9.57	0.0084	4.06	0.1309

^a Effect tests were calculated as part of a general linear model, using logistic regression and the restricted likelihood method, and the following factors in the model: *B. thuringiensis* strain, and *B. thuringiensis* concentration nested within *B. thuringiensis* strain. The two time points (4 and 12 h) were analysed separately. In all cases, the full model was significantly better than a minimal model ($P < 0.008$), but not significantly worse than a saturated model ($P > 0.93$). DF, degrees of freedom. Significant probabilities are given in bold.

of BT18679 after 6 h exposure (asterisks in Fig. 5A, B). There was still a trend of a difference for the medium dose of BT18679 after 1 h and all doses of BT18247 after 6 h (asterisks in brackets in Fig. 5A, B). For the non-pathogenic *B. thuringiensis* strain DSM, the *C. elegans* strain TJ356 similarly produced a trend of reduced feeding activity after 1 h exposure to the high dose, but none of the other non-pathogenic treatments (Fig. 5A and B). These results are generally consistent with previous data showing that DAF-16 has a visible although statistically weak effect on the pathogen-induced reduction in feeding behaviour (Hasshoff et al., 2007).

4. Discussion

Our analysis demonstrates that the infectious stages of a pathogen can manipulate nuclear translocation and thus most likely the regulatory activity of the DAF-16 transcription factor. Thus, the DAF-16 defence functions are likely to be directly induced by pathogen challenge. Consistent with this conclusion, we found that the high pathogen-mediated nuclear translocation of DAF-16 in the

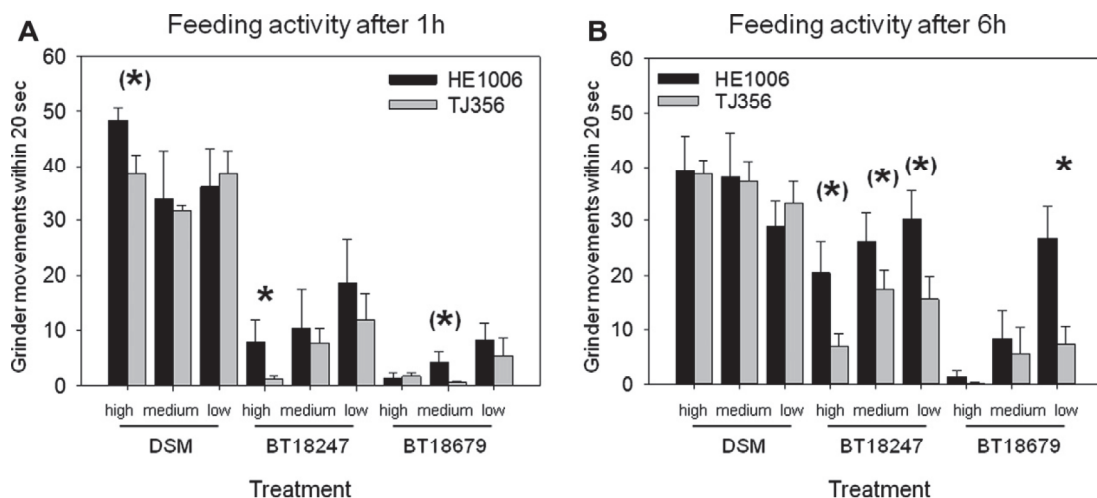


Fig. 5. Variation in feeding activity of the *C. elegans* strains TJ356 and HE1006 after exposure to *B. thuringiensis* for (A) 1 h or (B) 6 h. The nematode strain TJ356 contains additional copies of DAF-16, whereas HE1006 served as the control. Both strains were exposed to either pathogenic BT18247, BT18679, or the non-pathogenic DSM at three different doses (high, medium, low). Feeding activity was determined by counting grinder movements within a 20 s period. The bars give the average plus standard error. Asterisks point to significant and asterisks in brackets point to a trend of a difference between the *C. elegans* strains TJ356 and HE1006 for a particular *B. thuringiensis* strain at a given concentration. In all cases, significance and trend levels were adjusted using the false discovery rate.

transgenic strains is associated with comparatively high resistance and the reduced feeding activity response. Our results are also consistent with two previous studies, which demonstrated nuclear DAF-16 translocation after 3 h exposure to enteropathogenic *E. coli* (Anyanful et al., 2009) or 12 h exposure to a pathogenic strain of *P. fluorescens* (Neidig et al., 2011). It thus contradicts the previous notion that the transcription factor only contributes to immunity as part of a stress response, which itself is activated by something else than pathogens (Shapira et al., 2006; Troemel et al., 2006). However, this conclusion was drawn from analysis of the interaction between *C. elegans* and pathogenic *P. aeruginosa*, but not in consideration of other pathogen taxa.

The exact mechanism of pathogen-mediated activation of DAF-16 is currently unknown. First of all, we still lack an understanding of the mechanism of pathogen recognition, which could be direct by detection of pathogen-associated molecular patterns (PAMPs) or indirect through perception of pathogen-induced stress or damage (discussed in Schulenburg and Ewbank, 2007). In the case of *B. thuringiensis*, it is particularly interesting to note that the main virulence factors appear to be bacterial endotoxins (de Maagd et al., 2001; Griffiths and Aroian, 2005), which induce cellular stress and damage, thus potentially providing a simple mechanism for pathogen detection, irrespective of the availability of specific PAMP recognition receptors. This idea is supported by our observation that DAF-16 translocation is strongest at the general site of *B. thuringiensis* infection, namely the gut, where induced damage should be most pronounced. In contrast, similarly strong DAF-16 translocation is absent in the head region, where we may expect to find the PAMP recognition receptors as part of the chemosensory organs, which are known to mediate responses to other bacteria, including food bacteria (Shtonda and Avery, 2006) and also pathogenic *Serratia marcescens* (Pradel et al., 2007). Upon pathogen recognition, the signal could subsequently be transduced by downregulation of the insulin-like pathway or other modulators of DAF-16 nuclear translocation such as JNK-1 or RLE-1 (Li et al., 2007; Oh et al., 2005; Wolf et al., 2008). Detailed future dissection of these signalling processes should provide important insights into immune system activation in this nematode.

Nuclear DAF-16 localization is reduced after 12 h compared to 4 h exposure to both pathogen strains used. This pattern may indicate pathogen-mediated suppression of DAF-16 regulatory activity over time, in analogy with the manipulative effects of pathogenic *P. aeruginosa* (Evans et al., 2008b) or *C. neoformans* (TeKippe and Aballay, 2010). Such manipulative effects have as yet not been reported for *B. thuringiensis*. At the same time, it cannot be excluded that DAF-16 delocalization may be caused by an intrinsic negative feedback loop. In both cases, the pattern is consistent with our previous observation that DAF-16-dependent pathogen-specific behavioural defences are more pronounced directly after pathogen exposure rather than at later time points (Hasshoff et al., 2007).

Our results also highlight that different isolates of the same pathogen species can cause distinct infection patterns and survival rates in this nematode host. Interestingly, infectivity in surviving animals does not correlate with DAF-16 translocation or the overall survival rate. Whereas the strain BT18679 is highly pathogenic (i.e., high induced host mortality), the hosts surviving its presence show low or absent infection and at the same time a comparatively high level of DAF-16 translocation. This pattern may have two non-exclusive explanations: (i) Infection by this pathogen is only possible after host death, which is induced by the highly effective and/or more abundant BT18679 toxins. Surviving hosts have not been killed yet and therefore lack infection. (ii) This pathogen's highly pathogenic toxins cause immediate activation of immune responses, yielding surviving hosts with cleared infections. Our current data favours the first hypothesis, since the second should have produced a somewhat higher infection load shortly after exposure

and before the immune system could be activated. Moreover, the second hypothesis should also be associated with a generally higher survival rate, especially at later time points when the immune system has been upregulated, which is clearly not the case.

In contrast, the strain BT18247 generates a clearly different pattern. Although it generally shows lower pathogenicity, increased host death over time (or across increasing pathogen concentrations) correlates positively with infection load as well as DAF-16 translocation. Thus, pathogenicity and induced host responses directly depend on the infection process. Since the two *B. thuringiensis* strains are known to bear different toxin genes (own unpublished data, Payne, 1992; Payne et al., 1993; Schnepf et al., 2001), these differences could depend on toxin composition. A non-exclusive alternative explanation may be variation in toxin concentration.

Taken together, our observations indicate that DAF-16 activity is mainly determined by pathogenicity. Since the studied *B. thuringiensis* strains differ in this trait, distinct host immune responses emerge, thereby producing the pattern of high immune specificity (cf. Schulenburg et al., 2007). Such immune specificity could be at the basis of highly specific host genotype \times pathogen genotype interactions, as recently demonstrated for experimentally co-evolved populations of *C. elegans* and *B. thuringiensis* (Schulte et al., 2010, 2011). Thus, our results support the previous notion that invertebrates possess the capacity to produce immune specificity that differentiates between different natural isolates of the same pathogen species (reviewed in Kurtz, 2004; Schulenburg et al., 2007), even though they lack the adaptive immune system commonly assumed necessary for such specific responses. Consequently, our model may also be of value for further elucidation of the genetics of immune specificity patterns in invertebrates.

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10. Curriculum Vitae

Personal information

Name	Rania Nakad
Date of birth	07.02.1984, Lebanon
Nationalities	Lebanese

Education

October 2008 – August 2013

PhD at Christian-Albrechts University, Department of Evolutionary Ecology and Genetics, Kiel, Germany

Research field: Genetics of behavioural and physiological defences in *Caenorhabditis elegans*

September 2007 – July 2008

Master thesis at Saint Joseph University, Faculty of medicine, Medical Genetics Unit, Beirut, Lebanon

Research field: Genetics of neurological disorders, particularly “cerebellar ataxia” in human

October 2005 – July 2008

Masters in molecular biology at Saint Joseph University, Faculty of Sciences, Beirut Lebanon

Masters in animal physiology at the Lebanese University, Faculty of Sciences, Beirut, Lebanon

October 2002 – June 2005

Bachelor in biology at the Lebanese University, Faculty of sciences, Beirut, Lebanon

11. List of Publications

Nakad R, Snoek B L, Ellendt S, Schneider F, Mohr T G, Rösingh L, Masche A C, Dierking K, Kammenga J E, Schulenburg H, (manuscript in preparation), The mysterious role of the *C. elegans* nuclear hormone receptor NPR-1 in pathogen defence. Chapter 1 of the thesis.

Nakad R, Schneider F, Schulenburg H, (manuscript in preparation), Transgenerational priming of pathogen avoidance behaviour in *Caenorhabditis elegans*. Chapter 2 of the thesis.

Wang J, **Nakad R**, Schulenburg H, (2011), Activation of the *Caenorhabditis elegans* FOXO family transcription factor DAF-16 by pathogenic *Bacillus thuringiensis*. *Dev. Comp. Immunol.* Chapter 3 of the thesis.

Sheppard A E, **Nakad R**, Saebelfeld M, Masche A C, Dierking K, Schulenburg H (manuscript in preparation), Plasmid instability in the Gram-positive bacterium *Bacillus thuringiensis*.

12. Appendix

Chemicals

Name	Supplier
Agar-Agar	Roth
Agarose	Biolab
Bacteriological peptone	Fluka
Calcium chloride ($\text{CaCl}_2 \times 2\text{H}_2\text{O}$)	Roth
Cholesterol	Roth
Di-potassium hydrogen phosphate (K_2HPO_4)	Roth
Di-sodium hydrogen phosphate dihydrate ($\text{Na}_2\text{HPO}_4 \times 2\text{H}_2\text{O}$)	Roth
Ethanol	
Glucose monohydrate	Merck
Glycerol	Roth
Iron II sulphate 7-hydrate ($\text{FeSO}_4 \times 7\text{H}_2\text{O}$)	Roth
LB-agar	Roth
LB-medium	Roth
Magnesium sulfate heptahydrate ($\text{MgSO}_4 \times 7\text{H}_2\text{O}$)	Roth
Manganese (II) – sulfate monohydrate ($\text{MnSO}_4 \times \text{H}_2\text{O}$)	Roth
Potassium chloride (KCl)	Roth
Potassium dihydrogen phosphate (KH_2PO_4)	Roth
Peptone ex meat	Roth
Sodium chloride (NaCl)	Roth
Sodium di-hydrogen phosphate monohydrate ($(\text{NaH}_2\text{PO}_4 \times \text{H}_2\text{O})$)	Roth
Sodium hydroxide (NaOH)	Roth
Zinc sulphate 7-hydrate ($\text{ZnSO}_4 \times 7\text{H}_2\text{O}$)	Roth

Media

NGM (Nematode Growth Medium)

- 1) Add to Duran bottle (1 l):
 - 34.0 g Agar
 - 2.5 g Peptone
 - 3.0 g NaCl
- 2) Fill up with 1 l deionised H₂O
- 3) Add: 1 ml Cholesterol/Ethanol (5 g Cholesterol in 1 l 95% Ethanol)
- 4) Invert bottle a couple of times
- 5) Autoclave the mixture (20 min at 121°C)
- 6) Let it cool down to approximately 50°C
- 7) Add:
 - 1 ml 1M MgSO₄
 - 25 ml 1M KPO₄
- 8) Mix by inverting the bottle
- 9) Add: 1 ml 1M CaCl₂
- 10) Mix again
- 11) Pour NGM plates

PFM (Peptone-Free nematode growth Medium)

PFM is prepared in exactly the same way as NGM without peptone!

LB-medium

LB-broth [SIGMA L3022]	20 g
Deionised H ₂ O	1 l

stir and heat up to dissolve, aliquot: 5 x 200 ml (250 ml Duran bottles) and autoclave 15/20 min 121°C

LA-medium

LB Agar [SIGMA L2897]	35 g
Deionised H ₂ O	1 l

stir and heat to boiling to dissolve all ingredients, aliquot: 5 x 200 ml (250 ml Duran bottles) and autoclave 15 min 121°C

Bacillus thuringiensis culturing medium and protocol

Media and buffers preparation

BT medium

Bacto Peptone (Sigma) 7.5 g
Glucose 1.0 g
KH₂PO₄ 3.4 g
K₂HPO₄ 4.35 g
add deionised H₂O to 1l, adjust pH to 7.2 and autoclave

Salts Solution (100 ml)

MgSO₄·7H₂O 2.46 g
MnSO₄·H₂O 0.04 g
ZnSO₄·7H₂O 0.28 g
FeSO₄·7H₂O 0.40 g
add deionised H₂O to 100 ml and filtersterilise

1M CaCl₂

See below, section buffers and solutions

PBS- buffer

See below, section buffers and solutions

All solutions (incl. PBS) should be freshly prepared (not older than 2 weeks)

Culturing BT

- Incubate cultures at 28 °C (max. 30°C) on rotary shaker at 150 rpm for 7 days
- Use 2000ml volumes
- Use Erlenmeyer flasks with chicanes

- At the time of inoculation, add to each 1000 ml BT-medium
 - 5 ml salts solution
 - 1250 µl 1M CaCl₂
 - 1 ml of an overnight culture of BT in LB medium at 28 °C
- On day 4, add to each 1000 ml BT-medium
 - 5 ml salt-solution
 - 1250 µl 1M CaCl₂
- After 7 days
 - Centrifuge in 50 ml Falcontubes for 10 min - 3000 rpm
 - Discard supernatant and resuspend the pellet in each Falcon tube in 0,5 ml **PBS**
 - Collect BT suspension in sterile glas and mix well
- Aliquot 1.0 or 0.5 or 0.1 ml volumes in eppendorf tubes and store at -20°C

Buffers, solutions and media

1M CaCl₂

73.5 g CaCl₂ x 2H₂O (MW 147.0)
in 500 ml deionised H₂O

filtersterilise, aliquot in 50 ml vials and store at 4°C

Cholesterol in EtOH

1 g Cholesterol (Merck 1.03672)
in 200 ml 95% EtOH

store at room temperature (RT)

1M KPO₄

108.3 g KH₂PO₄ (MW 136.09)
35.6 g K₂HPO₄ (MW 174.18)
in 1 l deionised H₂O

adjust pH to 6.0, autoclave, store at RT

M9 buffer

3 g KH₂PO₄ (MW 136.09)
6 g Na₂HPO₄ 2H₂O (MW 177.99)
5 g NaCl (MW 58.44)
1 ml 1M MgSO₄

add deionised H₂O to a final volume of 1 l, autoclave and store at RT

1M MgSO₄

123.24 g MgSO₄ x 7H₂O (MW 246.48)
in 500 ml deionised H₂O

filtersterilise, aliquot in 50 ml vials and store at RT

5 M NaOH

100 ml	200 ml		
20 g	40 g	NaOH	(MW 40.0)

in autoclaved container (do NOT autoclave) and store at RT

PBS- buffer

500 ml	1 l	component	
4.385 g	8.77 g	NaCl	(MW 58.44)
1.12 g	2.24 g	KCl	(MW 74.56)
0.34 g	0.69 g	NaH ₂ PO ₄ x H ₂ O	(MW 137.99)
0.44 g	0.89 g	Na ₂ HPO ₄ x 2 H ₂ O	(MW 177.99)

adjust to pH 7.0 and autoclave and store at RT

S-buffer

5.85 g	NaCl	(MW 58.44)
1.123 g	K ₂ HPO ₄	(MW 174.18)
5.926 g	KH ₂ PO ₄	(MW 136.09)
in 1 l deionised H ₂ O		

autoclave and store at RT

S-buffer + glycerol

(*C. elegans* – freezing)

100 ml	200 ml	500ml	
70 ml	140 ml	350 ml	S-buffer
30 ml	60 ml	150 ml	glycerol [end concentration: 30% (v/v)]

autoclave or filtersterilise and store at RT

Molecular reagents

Reaction buffer: 5x green Go Taq (Promega)

5x colour less Go Taq (Promega)

dNTP (100mM) (Invitrogen)

dream Taq DNA polymerase (Thermo Scientific)

Devices

Name	Company
Clean banch 200	Kojair Biowizard SL-
M80 dissecting scope	Leica
KL200 dissecting scope	Leica
Dialux 20 microscope	Laitz
Centrifuge 5415R	Eppendorf
Centrifuge 5810R	Eppendorf
Spectrophotometer Jenway 6300	Eydam
pH-meter HI221	Hanna Instruments
Thermomixer	Eppendorf
PCR thermocycler	Biolab
Incubator 37°C	Binder
Incubator (15-25°C)	Lovibond
Incubator (80°C)	Memmert
Shaking incubator	Sartorius
Thermomixer	Eppendorf

13. Declaration

I, Rania Nakad, declare that:

- apart from my supervisor's guidance and supervision the content and design of the thesis is all my own work;
- my PhD project was supported by colleagues; their contribution is specified in detail in the section "Authors contribution";
- the whole thesis have not yet been submitted neither partially nor wholly as part of a doctoral degree to another examining body. Chapter I and II have not yet been published nor submitted for publication. Chapter III is already published in the journal "Developmental & Comparative Immunology", volume 37, Issue 1, May 2012
- the thesis has been prepared subject to the Rules of Good Scientific Practice of the German Research Foundation.

Rania Nakad