Molecular Mechanisms of *Caenorhabditis elegans – Bacillus* Interactions

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List of Publications

Teile der vorliegenden Arbeit wurden bereits veröffentlicht oder wurden zur Veröffentlichung eingereicht:

1. Robbie Rae[*], **Igor Iatsenko**[*], Hanh Witte, Ralf J. Sommer
A subset of naturally isolated *Bacillus* strains show extreme virulence to the free-living nematodes *Caenorhabditis elegans* and *Pristionchus pacificus*.
Environmental Microbiology 2010, 12:3007-3021. doi: 10.1111/j.1462-2920.2010.02278.x.
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2. Amit Sinha, Robbie Rae, **Igor Iatsenko**, Ralf J. Sommer
A system wide analysis of the evolution of innate immunity in the nematode model species *Caenorhabditis elegans* and *Pristionchus pacificus*.
PLoS One 2012, 7(9): e44255. doi:10.1371/journal.pone.0044255.

3. **Igor Iatsenko**, Amit Sinha, Christian Rödelsperger, Ralf J. Sommer
New Role for DCR-1/Dicer in *Caenorhabditis elegans* Innate Immunity against the Highly Virulent Bacterium *Bacillus thuringiensis* DB27.
Infection and Immunity 2013, 81(10), 3942 – 3957. doi:10.1128/IAI.00700-13.

4. **Igor Iatsenko**, Craig Corton, Derek J. Pickard, Gordon Dougan, Ralf J. Sommer
Draft genome sequence of highly nematicidal *Bacillus thuringiensis* DB27.
Genome Announcements 2014, 2(1):e00101-14. doi:10.1128/genomeA.00101-14.

5. **Igor Iatsenko**, Iuliia Boichenko and Ralf J. Sommer
***Bacillus thuringiensis* DB27 produces two novel protoxins, Cry21Fa1 and Cry21Ha1, which act synergistically against nematodes.**
Applied and Environmental Microbiology, in press

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SUMMARY

Pathogens represent strong evolutionary forces driving the complexity of the host defense system. The nematode *Caenorhabditis elegans* has been widely used as a genetically amenable invertebrate for studying host-pathogen interactions. While the *C. elegans* model provided invaluable insights into innate defense pathways against infections, it remains to be discovered what the role of these pathways is in other nematodes and how they shape the evolution of bacterial pathogenicity. The nematode *Pristionchus pacificus* has been extensively used for comparative studies with *C. elegans*, linking developmental biology, ecology and population genetics. In this context, drastic ecological and morphological differences between two nematodes served as a starting point for studying bacterial interactions and immune response of the two nematodes in a comparative manner. Aiming to find suitable pathogens for these comparative studies, I isolated and tested 768 natural *Bacillus* strains for the pathogenicity to nematodes. This resulted in the isolation of the fastest known *C. elegans* killer *B. thuringiensis* DB27, which *P. pacificus* is completely resistant to. Using system wide analysis, we showed that *C. elegans* and *P. pacificus* respond to *B. thuringiensis* DB27 or any other given pathogen in strikingly different ways, regulating a very different set of effector genes. Using the *C. elegans* - *B. thuringiensis* DB27 model, I (i) elucidated *C. elegans* defense mechanisms against the pathogen, revealing a novel role for Dicer in antibacterial immunity; (ii) with the help of whole genome sequencing, discovered that two novel Cry21 protoxins produced by *B. thuringiensis* DB27 act synergistically as the main nematicidal virulence factors; (iii) discovered that *C. elegans* commensal bacterium *B. subtilis* protects the worm from infection via bacteriocin-mediated pathogen inhibition. Taken together, these results not only tackle both sides of *C. elegans* - *B. thuringiensis* DB27 host-pathogen interactions, but also reveal previously unrecognized mechanism of nematode protection by commensal-mediated inhibition of the pathogen.

ZUSAMMENFASSUNG

Pathogene sind starke evolutionäre Kräfte, die die Komplexität des Abwehrsystems des Wirts vorantreiben. Der Nematode *Caenorhabditis elegans* wurde oft als genetisch zugänglicher Invertebrat verwendet, um Wirt-Pathogen-Wechselwirkungen zu untersuchen. Während das *C. elegans* Modell äußerst wertvolle Einblicke in die Signalwege der angeborenen Abwehr von Infektionen ermöglicht hat, bleibt die Frage nach der Rolle dieser Signalwege in anderen Nematoden und ihrem Einfluss auf die Evolution der bakteriellen Pathogenität noch unerforscht. Der Nematode *Pristionchus pacificus* wurde vielfach für vergleichende Studien mit *C. elegans* verwendet, um Entwicklungsbiologie, Ökologie und Populationsgenetik miteinander zu verknüpfen. In diesem Zusammenhang dienten gravierende ökologische und morphologische Unterschiede zwischen diesen beiden Nematoden als Ansatzpunkt, um bakterielle Wechselwirkungen und Immunreaktionen der beiden Arten zu vergleichen. Um geeignete Pathogene für diese vergleichenden Untersuchungen zu finden, isolierte ich 768 natürliche *Bacillus* Stämme und testete diese auf Pathogenität bezüglich Nematoden. Dies führte zur Entdeckung des Stammes *B. thuringiensis* DB27, welcher *C. elegans* schneller tötet als alle bisher bekannten *Bacillus* Stämme. *P. pacificus* ist jedoch vollkommen resistent gegen *B. thuringiensis* DB27. Mithilfe einer systemweiten Analyse zeigten wir, dass *C. elegans* und *P. pacificus* auf *B. thuringiensis* DB27 sowie auf jedes andere beliebige Pathogen auf extrem unterschiedliche Art und Weise reagieren, indem sie eine deutlich andere Gruppe von Effektorgenen regulieren. Unter Verwendung des *C. elegans* - *B. thuringiensis* DB27 Modells konnte ich (i) die *C. elegans* Abwehrmechanismen gegen das Pathogen aufklären, was zur Entdeckung einer neuen Rolle von Dicer in der antibakteriellen Immunität führte; (ii) mithilfe der Genomsequenzierung zwei neue Cry21-Protoxine entdecken, welche *B. thuringiensis* DB27 produziert und welche zusammen als Hauptvirulenzfaktoren gegen Nematoden wirken; (iii) feststellen, dass das mit *C. elegans* kommensale Bakterium *B. subtilis* den Fadenwurm durch bacteriocin-vermittelte Pathogeninhibition vor einer Infektion schützt. Insgesamt nehmen diese Ergebnisse nicht nur beide Seiten der *C. elegans* - *B. thuringiensis* DB27 Wirt-Pathogen-Wechselwirkungen in Angriff, sondern zeigen auch einen bisher unbekannten Mechanismus des Nematodenschutzes durch die kommensal vermittelte Inhibition des Pathogens.

INTRODUCTION

1.1 Evolutionary perspective in innate immunity and virulence

Pathogens represent powerful selective agents, responsible for the generation of a number of evolutionary patterns, ranging from the maintenance of genetic variation to the shifts in the genetic composition of both host and pathogen populations (Schulte et al., 2010, Woolhouse et al., 2002). Infection by pathogens is one of the major threats to living organisms. Therefore, the evolution of a defense system, which allows recognition and elimination of pathogens, is a strong adaptive trait. Vertebrate defense systems reach the highest complexity and are represented by the adaptive (acquired) response and the innate immune response (Medzhitov and Janeway, 1998). Although the adaptive system is slow, it is highly specific, long lasting and is able to generate immune memory. Excluding vertebrates, other animals rely exclusively on innate immunity, which represents an immediate response to infection. Interestingly, an extensive homology between vertebrates and invertebrates has been found for the innate defense system, suggesting that many features of the innate system are of common origin and evolved early in animal history and remained conserved across major taxa (Hemmrich et al., 2007; Salzet, 2001). Consequently, invertebrate models may be used to study innate immunity functions in higher vertebrates (Irazoqui et al., 2010). Importantly, some of the invertebrate model systems (*Caenorhabditis elegans*, *Drosophila melanogaster*) allow faster genetic analysis compared to typical vertebrate models (mice, zebra fish) and permit the study of innate immunity without confounding forces of acquired immunity (Schulenburg et al., 2004; Irazoqui et al., 2010). Being less complex and having lower redundancy in gene regulation, invertebrate models allow to infer the evolutionary history of immune components and permit identification of conserved, variable and novel elements of innate immune system (Schulenburg et al., 2004).

Besides facilitating studies on evolution of innate immunity, invertebrate models have also been used to reveal conserved virulence strategies of pathogens (Kurz et al., 2003; Feinbaum et al., 2012). Considering the evolutionary age and ecological dominance of invertebrates, it is very likely that they are not only the reservoir of present human pathogens, but also shaped their evolution (Waterfield et al., 2004). While antagonistic interactions between bacteria and simple eukaryotes have driven the evolution of many virulence factors, multicellular animals evolved and gained a more complex and effective immune system (Hemmrich et al., 2007). This arms race consequently led to the evolution of bacterial virulence strategies that are optimized for survival under more effective immune pressure. Considering striking similarities of vertebrate and invertebrate innate immune systems (Salzet 2001), it is not surprising that their

respective pathogens use conserved virulence factors (Kurz et al., 2003; Feinbaum et al., 2012), since they have to overcome similar defense systems. Besides influencing the evolution of existing mammalian pathogens, invertebrate-associated pathogens might be candidates for emerging human pathogens (Waterfield et al., 2004). This is mainly supported by the extreme similarities between causative agents of vertebrate and invertebrate diseases. For example, the causative agent of antrax, *Bacillus antracis*, is closely related to the insect-associated bacteria *B. thuringiensis* and *B. cereus* (Helgason et al, 2000). Some strains are so similar that only plasmid content and composition of plasmid-encoded virulence genes are distinct (Helgason et al, 2000). Considering numerical abundance of invertebrate-associated bacteria, intensity of horizontal gene transfer between microbes, and that only minor molecular changes are required to markedly change the host range, invertebrate pathogens represent an extensive reservoir of existing and shape the evolution of emerging mammalian pathogens. Thus, the study of invertebrates might provide invaluable insights into the evolution of pathogen virulence and aid our understanding of innate immunity of higher vertebrates.

1.2 Nematodes as model organisms

1.2.1 Advantages of *C. elegans* as a model organism

Nematodes, also called roundworms, are a large animal phylum characterized by species richness, functional diversity, ecological omnipresence and numerical abundance (Lee, 2002). While only a small number of nematodes have been described to date, some of them are extensively used as model organisms in basic research. For instance, the nematode *C. elegans* was introduced in the second half of the twentieth century by Sydney Brenner as a model to study development and neurobiology (Brenner, 2009). Today, *C. elegans* is used as a model system to address fundamental questions in multiple fields of biology, including apoptosis, cell signalling, cell cycle, gene regulation, metabolism, ageing and sex determination (Riddle et al., 1997; Wormbook, The *C. elegans* Research Community, www.wormbook.org). In recent years, this nematode has been applied to the study of microbial pathogenesis and host innate immunity (Irazoqui et al., 2010), and contributed to drug discovery and development (Ewbank and Zugasti, 2011). Together, these studies uncovered a remarkably strong conservation in molecular and cellular pathways between nematodes and mammals. Comparison of the *C. elegans* and human genomes showed that many human disease genes and disease pathways are present in the nematode (The *C. elegans* Sequencing Consortium, 1998).

The success of *C. elegans* as a model is substantially facilitated by the simplicity of maintenance and availability of diverse functional tools. This nematode is a self-fertilizing hermaphrodite with a rapid generation time. With a length of approximately 1 mm at adult stage, it can produce around 300 genetically identical progeny under laboratory conditions in a 3-day life cycle (Brenner, 1974). This facilitates the rapid expansion of strains and the establishment of large uniform populations. Under lab conditions, *C. elegans* is maintained on agar plates or in liquid media with the auxotrophic *Escherichia coli* mutant strain OP50 (Brenner, 1974), and can live up to 3 weeks at room temperature. The *C. elegans* genome sequence is available (The *C. elegans* Sequencing Consortium, 1998) and many functional genomic approaches have been developed (Lamitina, 2006). Notably, RNA interference (RNAi) can be used systemically by feeding worms with bacteria that express double-stranded RNA (dsRNA) targeting any gene of interest (Timmons and Fire, 1998). Given that RNAi libraries covering almost 90% of the 20,000 *C. elegans* genes are available, numerous genome-wide RNAi screens have been conducted (see Lamitina, 2006 for review). Moreover, transgenic *C. elegans* strains can be easily generated via microinjection of DNA (plasmids and/or PCR products) (Mello et al., 1991), and its transparency facilitates the use of fluorescent reporter genes *in vivo*, as well as permits direct real-time observation of infectious processes (Aballay et al., 2000).

1.2.2 *C. elegans* as a model host

C. elegans is a free-living, bacteriovorus nematode that is found in soil, compost heaps and rotting fruits (Félix and Duveau, 2012). In these environments, nematodes interact with diverse microbes, some of which are pathogenic. And indeed, multiple microbes, including human pathogens, can cause infections in *C. elegans* that result in death (Marsh and May, 2012; Engelmann and Pujol, 2010). This discovery accelerated the application of *C. elegans* as a surrogate host for studying virulence mechanisms of human pathogens. There are number of benefits that facilitated establishment of the worm as a model host for studying innate immunity. *C. elegans* can be infected by simply substituting the normal feeding bacterium *E. coli* OP50 with the pathogen of choice. Given that the nematode body is transparent, it is possible to monitor infections in real time (Abbalay et al., 2000). Multiple readouts can be easily and noninvasively used to follow pathogenesis, including animal survival (Tan et al., 1999), motility, morphological and behavioural changes (Hodgkin et al., 2000; Zhang et al., 2005), pathogen accumulation and persistence in the intestine (Aballay et al., 2000); monitoring changes in gene expression upon infection using microarrays, quantitative reverse transcription PCR (Troemel et al., 2006) and transgenic worms with reporter constructs (Irazoqui et al., 2008). These methods

allow the study of host-pathogen interactions at different levels in the context of the whole organism and on a large scale. Considering growing concerns for the welfare of animals in scientific research, there is an increasing need to find organisms in which to study such interactions ethically. All these *C. elegans* advantages have been used to elucidate genetic components of the host response to infection and to understand the virulence determinants used by pathogens to overcome host defenses.

1.2.2.1 Diverse pathogens of *C. elegans*

In 1999 *C. elegans* was used for the first time as a model for bacterial infection (Tan et al., 1999). Since then, great expansion of this model has been achieved. In the first studies, *C. elegans* has been employed as an alternative model to study virulence factors of human pathogens. In some cases, e.g. *Pseudomonas aeruginosa* and *Serratia marcescens* (Kurz et al., 2003; Feinbaum et al., 2012), this approach succeed in identification of virulence factors relevant to pathogenesis in mammalian models. Following this discovery, a diversity of other human pathogens, including *Salmonella enterica*, *E. coli*, *Enterococus faecalis*, *Staphylococcus aureus* and others (Marsh and May, 2012; Engelmann and Pujol, 2010), has been used to infect *C. elegans* (Fig.1). While bacterial pathogens clearly dominate in *C. elegans* host-pathogen studies, other microbes, including fungi, microsporidia and viruses, have been shown to be pathogenic to the nematode as well.

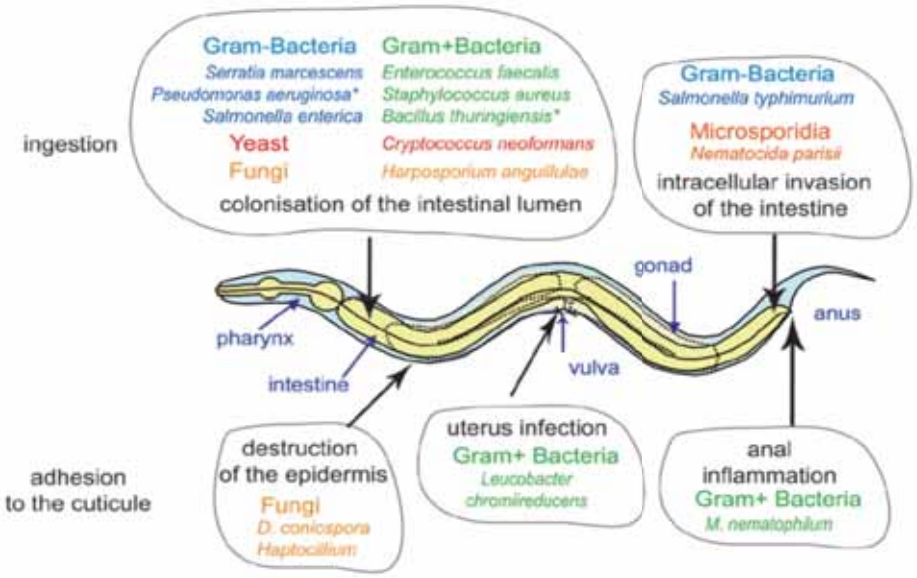

Figure 1. Some pathogens of *C. elegans* and their routes of infection. Reprinted from Engelmann and Pujol 2010, with kind permission from Springer Science and Business Media. Copyright © 2010, Springer Science and Business Media.

For example, the microsporidia *Nematocida parisii* was isolated from *C. elegans* wild-caught strain, and causes lethal intestinal infection in the host (Troemel et al., 2008). *N. parisii* is the first intracellular natural pathogen identified in *C. elegans*. Microsporidia are obligate intracellular pathogens, most closely related to fungi, that can cause lethal infections in humans and animals, but mechanism of infection are poorly investigated. Therefore, *C. elegans* is a promising model to study mechanisms of virulence of these intracellular pathogens.

Efforts to use *C. elegans* to study anti-viral immunity, however, have been limited by the lack of natural viruses able to infect and replicate in *C. elegans*. Nonetheless, artificial methods, like introducing animal virus e. g. Flock House virus genomes into nematodes, have been used and revealed a crucial role for the RNAi machinery in anti-viral response (Lu et al., 2005). The discovery of first *C. elegans* natural viruses (Felix at al., 2011), related to Nodaviridae family, is a first step towards the understanding of crucial aspects of nematode-virus interactions.

Drechmeria coniospora is a natural fungal pathogen that infects *C. elegans*. The spores of the fungus attach to specific parts of the *C. elegans* cuticle and then send out hyphal processes that penetrate the nematode body (Jansson, 1994). This is followed by a rapid proliferation of hyphae that spread through the host, eventually killing it. The *D. coniospora - C. elegans* model has been extensively used to reveal anti-fungal innate immunity pathways (Ziegler et al., 2009).

Diverse pathogens of *C. elegans* employ different routes of invasion (Fig.1). Most bacterial pathogens infect worms via oral up-take and establish infection it the intestine. While most intestinal pathogens remain extracellular, *Salmonella typhimurium* and *N. parisii* establish intracellular infection in intestinal cells (Jia et al., 2009; Troemel et al., 2008). Fungal infection with *D. coniospora* involves adhesion to cuticle and destruction of the epidermis (Jansson, 1994). Other microbes use more sophisticated routes of infection, for example *M. nematophilum* adheres to the anal region of *C. elegans* and causes hindgut swelling (Hodgkin et al., 2000). *Leucobacter chromiireducens* enters the reproductive tract through the external vulval opening and initiates a lethal uterine infection (Muir and Tan, 2008). Two other *Leucobacter* spp, called Verde1 and Verde2, were isolated from the same wild-caught *Caenorhabditis* worms that exhibited a swollen tail (Dar) phenotype (Hodgkin et al., 2013). Worms exposed to Verde1 in liquid culture were trapped by their tails together to form the so-called “worm-stars” and were eventually killed. In contrast, Verde2 caused severe rectal infection to wild-type worms, while mutant worms that are resistant to Verde2 become hypersusceptible to Verde1. Thus, worms cannot be resistant to Verde2 without being susceptible to Verde1. This study showed for the first time natural co-infection of *C. elegans* with two pathogens, which have reciprocal relationship between their pathogenicity, and extended the nematode-trapping and killing strategy to the bacterial world (Hodgkin et al., 2013).

There are several distinctive mechanisms of worm killing that can be defined (Sifri et al., 2005): intestinal colonization and infection (human pathogens), persistent infection (*S. enterica*, *E. faecalis*), invasion (*D. coniospora*), biofilm formation (*M. nematophilum*, *Yersinia pestis*), and toxin-mediated killing (*P. aeruginosa*, *E. faecalis*, *Bacillus thuringiensis*). Obviously, some pathogens employ a combination of mechanisms, for instance *P. aeruginosa*, to ensure successful infection. While up today more than 40 different microbes (Tan and Shapira, 2011) have been shown to be pathogenic to *C. elegans*, there is a bias towards clinically relevant pathogens. Interactions with natural pathogens are also beginning to emerge from studies using *D. coniospora*, *B. thuringiensis*, Orsay virus, *N. parisii*, *M. nenatophilum*, *Leucobacter*. Such studies are of great importance since they expand the range of known interactions, leading to better understanding of host-pathogen relationships.

1.2.2.2 *C. elegans* innate immunity signaling pathways

C. elegans does not have an adaptive immune system and lacks specialized immune cells. Therefore, it relies exclusively on the innate immune system as the only line of defense against infections. This line of defense is represented by several conserved signaling pathways, which provide effective protection from diverse pathogens (Irazoqui et al., 2010; Schulenburg et al., 2004). Strikingly, *C. elegans* lacks the known Toll-NF-kB axis of insect and vertebrate immune systems. In vertebrates and *Drosophila*, Toll-like receptors are directly activated by pathogen-associated molecular patterns (PAMPs), which lead to the activation of nuclear factor-kB (NF-kB) and related transcription factors (Akira et al., 2006). This induces transcription of genes involved in innate immune response. The only *C. elegans* Toll-like receptor identified is *tol-1*, which, however, was not shown to play a role in the resistance to several pathogens (Pujol et al., 2001). Thus, in contrast to mammals and flies, TOL-1 is not a central part of the nematode innate immune response.

Mitogen-activated protein kinase (MAPK) pathways are ancient cascades involved in immunity. A genetic screen for mutants hypersensitive to *P. aeruginosa* infection revealed a conserved p38 MAPK pathway as a key component of *C. elegans* immune response, which was shown to protect the worm against multiple pathogens (Kim et al., 2002). This pathway involves several kinases (Table 1, Fig. 2), which function in a linear phosphotransfer cascade, leading to phosphorylation of p38 MAPK family member-1 (*pmk-1*). Phosphorylation of PMK-1 promotes its translocation into the nucleus, where it phosphorylates target transcription factors, for example *atf-7* (Shivers et al., 2010), to control gene expression.

Table 1. Summary of the major *C. elegans* innate immunity signaling pathways. Reprinted from Engelmann and Pujol 2010, with kind permission from Springer Science and Business Media. Copyright © 2010, Springer Science and Business Media.

Pathway	Tissue	Components	Homologues
p38 MAPK	Epidermis	GPA-12, RACK-1	G protein subunits
		EGL-8, PLC-3	Phospholipase C
		NIPI-3	Tribbles kinase
	Epidermis and intestine	TPA-1	Protein kinase C
		TIR-1	SARM
		NSY-1, SEK-1, PMK-1	MAP kinases
FSHR-1	Intestine	FSHR-1	G protein coupled receptor
ZIP-2	Intestine	ZIP-2	b-zip transcription factor
Insulin signalling	Nervous system	INS-7	Insulin-like peptide
	Intestine	DAF-2	Insulin receptor
		AGE-1	PI3 kinase
		AKT-1, AKT-2	Akt kinase
		DAF-16	FOXO transcription factor
TGF-β	Nervous system epidermis	DBL-1	TGF-β
		SMA-6	TGF-βreceptor
		SMA-3	SMAD protein
Wnt/Hox	Intestine/ Hindgut	BAR-1	β-catenin
		EGL-5	Hox transcription factor
ERK MAPK	Hindgut	LIN-45, MEK-2, MPK-1	ERK MAP kinase
		EGL-8	Phospholipase C
		SUR-2	Mediator component
UPR[1]	Intestine	XBP-1	X box protein
		HSP-4	Heat shock protein
	Pharynx	CED-1, C03F11.3	Scavenger receptor
Autophagy	Intestine	BEC-1, LGG-1	ATG proteins

Another MAPK cascade in *C. elegans* immunity is the extracellular signal-regulated kinase (ERK) pathway, which is involved it the defense against *M. nematophilum* (Nicholas and Hodgkin, 2004). Besides being involved in the response to bacterial infection in the *C. elegans* intestine, the PMK-1 pathway is required for the expression of antimicrobial genes (neuropeptide-like peptides) in the epidermis upon infection by fungal pathogen *D. coniospora* (Ziegler et al., 2009). However, in this case the upstream pathway leading to PMK-1 activation is different (Fig. 2). *C. elegans* response to fungal infection also requires a non-canonical transforming growth factor-β (TGF-β) signaling pathway (Fig. 2), composed of receptor subunits SMA-6 and DAF-4, which respond to DBL-1 signals from the nervous system (Zugasti and Ewbank, 2009). Thus, this pathway highlights the importance of the nervous system in the induction of host responses.

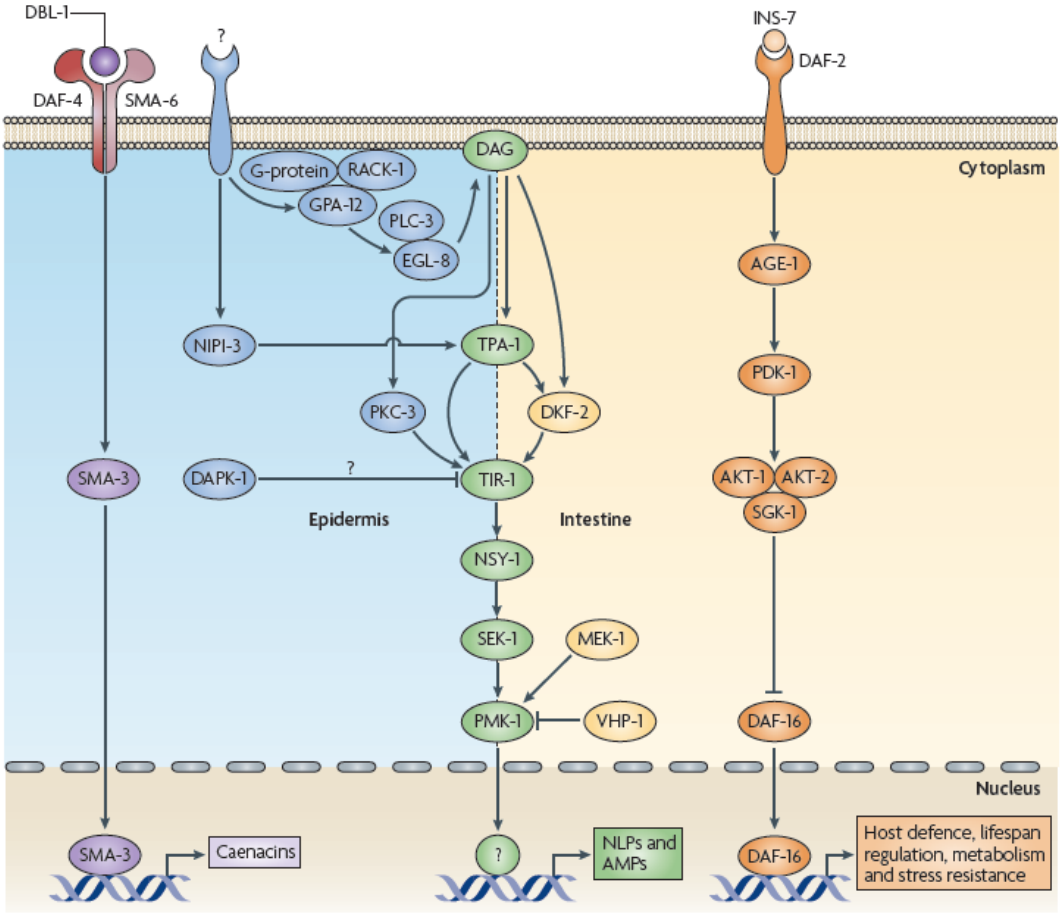

Figure 2. Scheme of *C. elegans* TGF-β (DAF-4/SMA-6), p38 MAPK (PMK-1) and DAF 2/DAF-16 (FOXO) insulin-like signaling pathways acting in parallel to promote host defense. Figure reprinted by permission from Macmillan Publishers Ltd: Irazoqui et al., Evolution of host innate defence: insights from *Caenorhabditis elegans* and primitive invertebrates. *Nature Reviews Immunology*, *10*, 47–58, copyright © 2010.

The DAF-2/DAF-16 (FOXO) insulin-like signaling pathway regulates different physiological processes in *C. elegans* (Wolkow et al., 2000), including innate immunity (Garsin et al., 2003). The insulin receptor DAF-2 inhibits the downstream transcription factor DAF-16 and retains it in the cytoplasm. Mutations in *daf-2* constitutively activate DAF-16, leading to its translocation to the nucleus, where DAF-16 regulates expression of detoxification, stress response, lifespan-promoting and antimicrobial genes (Murphy et al., 2003) (Fig. 2). Consequently, *daf-2* mutants are long-lived, stress and pathogen resistant, suggesting that DAF-16 is an important component of *C. elegans* defense against infection (Garsin et al., 2003). However, *daf-16* null mutants are not significantly more susceptible to infection than wild-type worms (Garsin et al., 2003; Irazoqui et al., 2008). Moreover, activation (nuclear translocation) of DAF-16 has not been detected upon pathogen challenge, suggesting that DAF-16 normally is not activated in *C. elegans* during pathogen infection (Irazoqui et al., 2008), although constitutive

activation of DAF-16 enhances pathogen resistance. It is therefore possible that the DAF-2/DAF-16 pathway is involved in the response to infection as part of general stress rather than a specific innate immune response.

β-catenin signaling and homeobox transcription factors, besides their well-established role in developmental regulation (Eisenmann et al., 1998), appeared to be crucial determinants of *C. elegans* response to *S. aureus* (Irazoqui et al., 2008) and *M. nematophilum* infection (Nicholas and Hodgkin, 2009). The role of β-catenin signaling and homeobox transcription factors in immunity is supported by their involvement in the regulation of the expression of antimicrobial peptides in humans and flies (Wang et al., 2005; Ryu et al., 2004). Other *C. elegans* pathways and transcription factors implicated in immune response are shown in Table 1 and were reviewed by Engelmann and Pujol, 2010. Thus, *C. elegans* evolved several innate immunity pathways, which are activated depending on the route of infection and the pathogen. These pathways often interact with each other to form signaling networks covering different tissues to provide an effective defense response against pathogen challenge.

1.2.2.3 Behavioural defenses against pathogens

C. elegans in its natural habitats is continuously exposed to diverse microbes. Although they are vitally important for the nematode as a food source, they can also be pathogenic. While *C. elegans* has a defense system against pathogens (see paragraph 1.2.2.2), the ability to discriminate between nutritious and pathogenic microbes is of highest importance for survival. Indeed, *C. elegans* has evolved behavioural mechanisms of protection against potential pathogens (Schulenburg and Ewbank, 2007). First of all, worms are able to distinguish the food quality of different bacteria, showing preference for strains with higher nutritional value (Shtonda and Avery, 2006). Additionally, nematodes try to minimize the general exposure to pathogens via avoidance behavior. When placed on a lawn of pathogenic *Serratia marcescens* Db11, worms were shown to strongly avoid the bacteria (Pradel et al., 2007). It appeared that cyclic lipopeptide serrawettin W2, produced by *S. marcescens* is sensed as an aversive stimulus by the worms (Pradel et al., 2007). Similarly, nematodes strongly avoided pathogenic *B. thuringiensis*, which was mediated by insulin signaling pathway (Hasshoff et al., 2007). Interestingly, *C. elegans* is also able to learn to avoid pathogens. Using *P. aeruginosa* and *S. marcescens*, it was shown that avoidance is significantly induced when animals are raised in the presence of respective pathogenic strains but not avirulent bacteria (Zhang et al., 2005). Thus, previous experience with pathogens enhances *C. elegans* avoidance and probability of survival. When there is no possibility to avoid the pathogen, *C. elegans* was shown to reduce the ingestion

rate or oral uptake when confronted with a number of pathogens, like *B. thuringiensis* (Hasshoff et al., 2007), *P. aeruginosa* (Tan et al., 1999), *S. enterica* (Abbalay et al., 2000). Such reactions aim to minimize the amount of consumed pathogens in order to increase chances of survival. All in all, behavioural defenses can be considered as an alternative and energy-saving way of defense. In contrast to costly innate immune response, pathogen avoidance has advantages in terms of minimizing the infection risk and saving animal's resources (Schulenburg and Ewbank, 2007).

1.2.2.4 Detection of infection

Multiple studies have shown that *C. elegans* responds to infection by strong expression of potential antimicrobials (Wong et al., 2007; Troemel et al., 2006; Shivers et al., 2008), indicating that worms can sense the presence of the pathogen. However, how nematodes recognize their pathogens is unknown. In vertebrates and flies, the innate immune system is equipped with numerous extracellular and intracellular sensors of microbes, including Toll-like receptors (TLRs) and NOD-like receptors (NLRs) (Ishii et al., 2008). These receptors, also called pattern recognition receptors (PRRs), recognize conserved microbial molecules called microorganism-associated molecular patterns (MAMPs), such as lipopolisacharide, peptidoglican, flagellin and others (Medzhitov, 2009). Detection of MAMPs by PRRs triggers immune response to infection. Given that *C. elegans* lacks PRR families (TLRs and NLRs) that have been identified in other animals, it was a mystery, how this nematode mounts a defense response against infection, and also how *C. elegans* differentiates between harmless and pathogenic bacteria. Several recent studies have shown that *C. elegans* detects harmful bacteria indirectly (McEwan et al., 2012; Dunbar et al., 2012). Using the *P. aeruginosa* infection model, it has been shown that Exotoxin A (ToxA), a *P. aeruginosa* toxin which acts as a translational inhibitor, upregulates the same genes normally induced by *P. aeruginosa*, suggesting that either ToxA itself or ToxA-mediated inhibition of translation induces an immune response (McEwan et al., 2012). Follow up experiments confirmed that ToxA-mediated inhibition of translation is responsible for the induction of immune genes, since inactive ToxA was not able to induce gene expression, and chemical inhibition of translation also resulted in the expression of immune response genes (McEwan et al., 2012). Another study has shown that not only inhibition of translation, but also disruption of the core cellular machineries triggers *C. elegans* aversion behavior and expression of genes associated with immune response (Melo and Ruvkun, 2012). Therefore, instead of sensing MAMPs directly, *C. elegans* recognizes alterations of cellular processes as danger-associated molecular patterns (DAMPs), which indirectly may indicate the presence of

pathogens (Vance et al., 2009). Thus, even in the absence of PRRs, *C. elegans* has evolved the capacity to mount a defense against pathogens by detecting the action of their virulence factors on core cellular processes.

1.2.2.5 Role of commensal and probiotic bacteria in *C. elegans* physiology

While *C. elegans* relies on bacteria as food source, numerous studies have shown that microbes play a much more important role in worm physiology (Cabreiro and Gems, 2013). For example, *Comamonas* bacteria, which were isolated form *C. elegans*-infested soil, accelerated worm development, but shortened the lifespan compared to *E. coli* diet (MacNeil et al., 2013). Combining gene expression analysis, forward and reverse genetic screens, a network of metabolic genes, that regulate *C. elegans* dietary responses and explain relationship between diet and development has been identified (Watson et al., 2013).

Bacterial diet has also an effect on worm longevity. When grown on *Bacillus subtilis*, worms live longer than on *E. coli* (Garsin et al., 2003). A recent study revealed the role of nitric oxide (NO), produced by *B. subtilis,* in mediating *C. elegans* increased longevity and also heat shock response (Gusarov et al., 2013). The fact that *C. elegans* lacks NO synthase but has enzymes that can be activated by NO, suggests the existence of a long-lasting crosstalk between host and microbial metabolisms.

E. coli was shown to have another sophisticated way of impairing *C. elegans* longevity. Worms fed with an *E. coli* strain, which expressed the noncoding RNA DsrA, exhibit reduced lifespan due to suppressed expression of the lipase gene F42G9.6 (Liu et al., 2012). The fact that DsrA-related noncoding RNA, which targets *daf-2* gene, implicated in longevity, was also found in *Bacillus mycoides*, suggests another possible mechanism of lifespan extension by *Bacillus* bacteria. This work also provides evidence for interspecies gene regulation crosstalk between host and microbes in addition to the well-established metabolic crosstalk.

Lactic acid bacteria *Lactobacillus* and *Bifidobacterium* have shown a beneficial effect on life and healthspan of nematodes (Ikeda et al., 2007; Komura et al., 2013). In addition to lifespan extension, probiotic bacteria have the potential to prime host defenses, leading to greater resistance to subsequent infection. Such priming effect was observed when *C. elegans* was fed with *Lactobacillus acidophilus* (Kim and Mylonakis, 2012). This led to increased resistance to Gram-positive pathogens *S. aureus* and *E. faecalis*, but not to Gram-negative *P. aeruginosa*. The protective effect of *L. acidophilus* was mediated by p38 MAPK and β-catenin signaling pathways (Kim and Mylonakis, 2012). While lactic acid bacteria have beneficial effects on nematodes, they are not known to be a part of the *C. elegans* natural diet, nor are they known to

live in association with the worms. Therefore, their effects on worm physiology might be completely artificial.

A more general and important question is the role of *C. elegans* natural microbiota? Answering this question was hampered by the fact that composition of the *C. elegans* natural gut microbial communities was a mystery for long time, and only now emerging studies try to shed light on this problem. A recent study described seventeen *C. elegans*-associated bacterial species from worms that were maintained in natural-like environment (Montalvo-Katz et al., 2013). *Bacillus* and *Pseudomonas* were the dominant species isolated from the nematodes. Two representatives from each of the main two groups of identified bacteria, *Bacillus megaterium* (BM) and *Pseudomonas mendocina* (PM), were shown to enhance *C. elegans* resistance to pathogens, although through different mechanisms. While the PM protective effect was a result of p38 MAPK-dependent priming of the immune system, BM led to increased resistance as a secondary consequence of compromised reproduction (Montalvo-Katz et al., 2013). This study also showed that, like in mammals, members of the *C. elegans* intestinal microbiota stimulate host defense responses and this has protective effect against infections. While this result is of great importance, it does not fully reflect the role of microbiota in host protection. Being exclusively host-centered, current studies have explained beneficial effects of microbes only considering their impact on the host, while completely ignoring the possibility that members of microbiota might protect the host by acting on the pathogens, e. g. directly inhibiting pathogens. Direct inhibition of the pathogens by antimicrobial effector molecules (bacteriocins, antibiotics) and competition for nutrients are two mechanisms of microbiota-mediated colonization resistance (CR), both of which act via the pathogen side and not the host (Buffie and Pamer, 2013). Whether similar mechanisms of microbiota-mediated CR are involved in *C. elegans* protection remains to be elucidated and is one of the aims of this thesis.

1.2.3 *Pristionchus pacificus* as a model for studying integrative evolutionary biology

C. elegans as a model organism has provided not only fundamental understanding of various biological processes, but also created a platform for comparative evolutionary studies with other nematodes (Sommer et al., 1996). One such nematode, *P. pacificus*, was successfully established as a comparative model to *C. elegans* (Hong and Sommer, 2006). While comparative studies of developmental processes with *C. elegans*, e. g. vulva development, formed the starting point for *P. pacificus* establishment as evo-devo model, gradually the *P. pacificus* system became a center of integrative evolutionary studies (Sommer and Mcgaughran, 2013), which unites evo-devo (macroevolution) with population genetics and ecology (microevolution) to

reveal how natural variation and changing conditions contribute to evolutionary processes. All these studies are facilitated by a variety of genetic tools available for *P. pacificus*, including forward and reverse genetic techniques (Hong and Sommer, 2006), a fully sequenced genome (Dieterich et al., 2008), fully characterized proteome (Borchert et al., 2010), microarray technology (Sinha et al., 2012) and transgenic techniques (Schlager et al., 2009). Additionally, the availability of hundreds of naturally isolated *P. pacificus* strains from all over the world (Morgan et al., 2012), as well as sister species and outgroups (Kanzaki et al., 2012) facilitates natural variation studies. While these studies have now a strong ecological context, trying to elucidate genetic determinants of different *P. pacificus* adaptive traits, early work in *P. pacificus* had a stronger evo-devo focus, studying vulva development (Kienle and Sommer, 2013; Wang and Sommer, 2012), dauer formation (Ogawa et al., 2009, 2011; Sinha et al., 2012), gonad development (Rudel et al., 2008), neurobiology (Bumbarger et al., 2013) and mouth form dimorphism (Bento et al., 2011; Ragsdale et al., 2013). The natural history and ecology of *P. pacificus* was also studied in detail, e. g. attraction to host beetle pheromones (Hong et al., 2008), host seeking and nictation behaviors (Brown et al., 2011), sensitivity to dauer pheromones (Mayer et al., 2011) and interactions with bacteria (Rae et al., 2008).

While *C. elegans* is considered a free-living nematode, commonly found in soil, compost heaps and decaying fruits (Félix and Duveau, 2012), *P. pacificus* has a different, but well-defined niche, namely it lives in a necromenic association with scarab beetles (Hermann et al., 2006). Necromeny is a type of associations, when nematodes reside on a living beetle in an arrested dauer stage and resume their development only after the death of the beetle, when microorganisms start to decompose the beetle carcass. In contrast to *C. elegans*, *P. pacificus* feeds not only on bacteria but also on fungi, protozoans and other nematodes. This omnivorous feeding gives a strong advantage to necromenic nematodes in the environment. Feeding on other nematodes, *P. pacificus* evolved predatory behavior, which is facilitated by novel teeth-like structures that are unknown from *C. elegans* (Ragsdale et al., 2013). Another morphological difference between *C. elegans* and *P. pacificus* is the absence of the grinder in the pharynx (Fig. 3) (Rae et al., 2008).

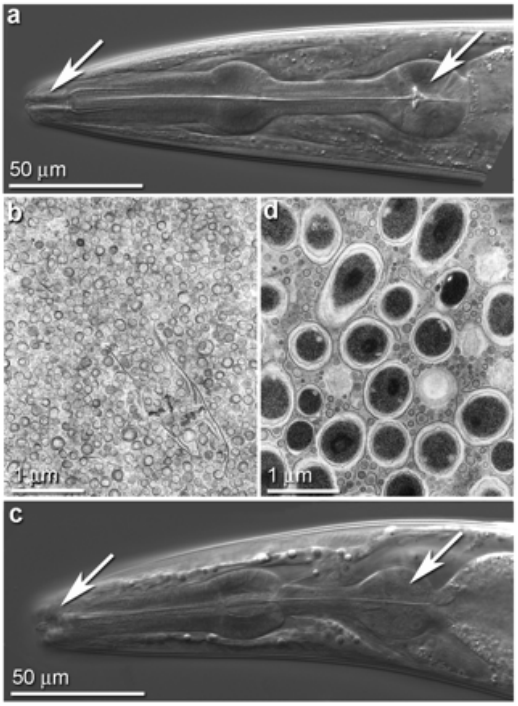

Figure 3. *C. elegans* pharynx (A) with grinder. *P. pacificus* pharynx (C) without grinder. *E. coli* OP50 crashed with the *C. elegans* grinder (B). *E. coli* OP50 cells are intact (D) after passage through *P. pacificus* pharynx. Figure taken from Rae et al., 2008.

The grinder is a structure known from *C. elegans* and other Rhabditidae, which is used to crash bacterial cells in order to get nutrients from them. Therefore, it is of absolute necessity for bacteriovorus nematodes. Without a grinder, *P. pacificus* ingests intact bacterial cells and can disseminate living bacteria to new areas (Chantanao and Jensen, 1969). However, how *Pristionchus* nematodes get nutrients from undisrupted cells awaits further analysis. Also, how strong ecological and morphological differences between *P. pacificus* and *C. elegans* affect their interactions with microbes remains to be elucidated.

1.2.4 *Pristionchus* immunity and interactions with bacteria

Interactions with microbes and immune response to infection are another aspects of comparative studies between *P. pacificus* and *C. elegans*. In spite of the wealth of knowledge about *C. elegans* innate immune response to infections (described above), little is known about what *C. elegans* actually feeds on in the wild and what microbes are associated with the worm. *P. pacificus*-associated bacteria were identified using metagenomic approaches (Rae et al., 2008). Interestingly, plant and animal pathogenic bacteria (*Pseudomonas*, *Enterobacter*, *Serratia*) were found among *P. pacificus*-associated bacteria. Subsequent survival experiments confirmed that *P. pacificus* is resistant to a number of human pathogens (*S. aureus*, *P.*

aeruginosa), that *C. elegans* is susceptible to (Rae et al., 2008). Thus, being constantly exposed to pathogens, *P. pacificus* has evolved mechanisms to combat infections. One such mechanism might be mediated via increased rate of detoxification of xenobiotic compounds (bacterial toxins), since the *P. pacificus* genome contains more cytochrome P450 and UDP-glucosyltransferase genes involved in detoxification, relative to *C. elegans* (Dieterich et al., 2008). The absence of grinder might also contribute to pathogen resistance of *P. pacificus*. It could be that if the pathogen cell is not disrupted, the lethal toxins will not be released. In support of this, *P. pacificus* die when fed pure Cry5B toxin, while nematodes are unaffected when fed *E. coli* cells that express the same toxin (Wei et al., 2003).

Using forward genetic approaches, two *P. pacificus* mutants were isolated that are hypersusceptible to Gram-positive pathogens (Rae at al., 2012a). They both have an *unc* (uncoordinated) phenotype and exhibit severe muscle defects, extended defecation cycle and, as a consequence, increased residence time of bacteria in the intestine. This prolonged exposition of the intestine to pathogens results in increased mortality. Similarly, *C. elegans* defecation mutants are also hypersusceptible to bacterial pathogens (Rae at al., 2012a). Thus, intact peristalsis is a crucial line of defense against pathogens, which prevents intestine from bacterial colonization.

Considering that germline elimination in *C. elegans* leads to increased longevity and pathogen resistance (Alper et al., 2010), Rae and co-workers wanted to study whether the same affect is observed in *P. pacificus,* and whether germline regulates lifespan and immunity via the same or different mechanisms (Rae et al., 2012b). Combining gonad ablation, pathogen exposure and whole genome microarrays, it was shown that germline ablation in *P. pacificus*, like in *C. elegans*, leads to extended longevity and increased resistance to pathogens (Rae et al., 2012b). This is regulated by numerous downstream effectors that affect many processes, like translation initiation, proteasome maintenance, insulin signaling and lipid metabolism, with DAF-16/FOXO transcription factor playing a key role. This study showed that longevity and immunity are regulated in a similar manner, and that the influence of the reproductive system on lifespan and innate immunity is conserved across evolution. While aforementioned studies provided important insights into *P. pacificus* defense mechanisms against infections, how *P. pacificus* responds to different pathogens on transcriptional level and whether this response differs from *C. elegans* is not well understood.

1.3 *Bacillus* and nematodes

Bacteria from the genus *Bacillus* are ubiquitously distributed in nature (Martin and Travers, 1989). Soil is, however, the most abundantly inhabited niche, where *Bacillus* bacteria

reach density of 10^4-10^6 per gram (Martin and Travers, 1989). Considering the diversity and abundance of other organisms in soil, *Bacillus* spp. are often involved in multitrophic interactions with other organisms. Nematodes, that can reach up to one million individuals per square meter in some soil systems (Floyd et al., 2002), are among *Bacillus* interacting partners. While bacteriovorus nematodes use many *Bacillus* species as food source (Laaberki and Dworkin, 2008a), they are also involved in dispersal of bacteria to new areas. First of all, this is facilitated by the fact that spores are resistant to digestion and pass the gut undisturbed (Laaberki and Dworkin, 2008b). Also, some nematodes, e. g. *Pristionchus* lack a grinder, which allows bacteria to stay alive in the intestine (Rae et al., 2008). Some *Bacillus* species exhibit virulence to nematodes, which was applied in biocontrol of plant- and animal-parasitic nematodes (Tian et al., 2007; Hu and Aroian, 2012). *Bacillus* bacteria use various strategies to kill nematodes. For example, *Brevibacillus laterosporus* G4 attaches to the nematode body, where it propagates and secretes extracellular proteases that damage nematode cuticle. Then, bacteria enter the body of the host, and use digested tissues as a source of nutrients (Huang et al., 2005). *B. nematocida* B16 evolved a sophisticated "trojan horse" mechanism of nematode killing-from-within (Niu et al., 2010). This bacterium actively attracts nematodes via production of volatile organic compounds. Once swallowed, the bacterium colonizes the intestine and produces proteases that damage intestinal tissues of the worm, leading to death. *B. thuringiensis* was also shown to produce a metalloproteinase Bmp1, which targets nematodes via intestinal damage (Luo et al., 2012). Interestingly, while Bmp1 alone is a nematicidal virulence factor, it can also increase lethality of Cry5B toxin to nematodes. Thus, *Bacillus* proteases function synergistically with Cry toxins.

Cry toxins or crystal toxin proteins are produced by *B. thuringiensis* during sporulation, and were extensively studied in terms of their insecticidal activity (Bravo et al., 2013). However, some families of Cry toxins, namely Cry5, Cry6, Cry12, Cry13, Cry14, Cry21, Cry55 (Wei et al., 2003; Guo et al., 2008), are active specifically against nematodes and not insects, suggesting that nematodes might be the primary targets of *B. thuringiensis* Cry toxins in the soil (Wei et al., 2003). Cry proteins are pore-forming toxins, which perforate membranes of host cells, disrupting membrane potential and cellular integrity (Soberon et al., 2010). However, considering diversity of pore-forming toxins produced by various pathogens, mechanisms of their action as well as cellular defense strategies against these virulence factors are poorly investigated. The *C. elegans* model has contributed intensively to the understanding of Cry toxin action. Research focusing on the *C. elegans* response to *B. thuringiensis* Cry5B toxin has revealed multiple pathways, like MAP kinases p38 and JNK (Kao et al., 2011), hypoxia (Bellier et al., 2009), unfolded protein response (Bischof et al., 2008), RAB-5 and RAB-11 dependent vesicle-trafficking pathways

(Los et al., 2011), involved in the defense against pore-forming toxin. Cry5B, like other Cry toxins, requires functional receptor for the binding, consequently *C. elegans* mutants, that lack receptors for Cry5B toxin, are extremely resistant to the toxin (Griffitts et al., 2005).

While studies of Cry5B - *C. elegans* interactions provided fundamental insights into nematode defense strategies against the toxin, little is known about the receptors and signaling pathways involved in *C. elegans* response to other nematicidal Cry toxins. Also, the full spectrum of nematicidal Cry toxins as well as their host targets is far away from completion. Which *Bacillus* species are pathogenic to nematodes, how abundant they are and what virulence mechanisms they use is also not known.

1.4 Aims of the thesis

The comparative method and the comparison of traits between different species is a very successful approach in evolutionary biology. For this reason, *P. pacificus* has been developed and extensively used for multifactorial comparative studies with *C. elegans*. Being different in terms of ecological habitat, pharynx morphology and feeding behavior, these two nematodes represent perfect candidates to study the evolution of pathogen response and interactions with bacteria. Therefore, the first aim of my thesis was to isolate and test natural *Bacillus* strains for nematicidal activity to find (a) suitable pathogens for innate immunity comparative studies and (b) to estimate how abundant and what type of nematicidal *Bacillus* strains there are. This screen revealed that most *Bacillus* strains are benign to nematodes, and *P. pacificus* is more resistant to pathogenic *Bacillus*. I selected the fast *C. elegans* killer *B. thuringiensis* DB27 for further analysis. The next aim was to characterize nematode defense mechanisms against *B. thuringiensis* DB27 using whole genome microarays and forward genetics. As a result, *C. elegans btr-1* and *btr-2* mutants resistant to *B. thuringiensis* DB27 were isolated and characterized. Additionally, I aimed to investigate *B. thuringiensis* DB27 nematicidal virulence factors to understand mechanisms of fast *C. elegans* killing. The final aim was to explore whether commensal *Bacillus* can protect *C. elegans* against bacterial infections and to reveal molecular mechanisms of protection.

2. RESULTS

2.1 A subset of naturally isolated *Bacillus* strains show extreme virulence to the free-living nematodes *Caenorhabditis elegans* and *Pristionchus pacificus*

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2.1.1 Synopsis

Bacteria from the genus *Bacillus* are among the most abundant microbes in the soil, reaching density of 10^4-10^6 per gram (Martin and Travers, 1989). While many *Bacillus* species serve as a benign food source for free-living bacteriovorous nematodes (Laaberki and Dworkin, 2008a), others evolved different strategies to kill the nematodes (Huang et al., 2005; Niu et al., 2010; Luo et al., 2012). However, which *Bacillus* species are pathogenic to nematodes, how abundant nematode-pathogenic strains are, and what virulence mechanisms they use was not known. To answer these questions, we used two genetically tractable model nematodes *C. elegans* and *P. pacificus*, which have striking ecological and morphological differences (Sommer and Mcgaughran, 2013), as hosts to carry out a systematic screen for nematode-pathogenic naturally isolated *Bacillus* strains. Most of 768 tested *Bacillus* strains appeared to be benign to nematodes and only 3% were pathogenic to *C. elegans* and *P. pacificus*, causing 70-100% mortality in five days. Additionally, pathogenic strains significantly affected development and brood size of nematodes. Interestingly, most of the nematode-pathogenic strains belong to *B. cereus* group (*B. cereus*, *B. mycoides*, *B. weihenstephanensis*). The most pathogenic strains (originally identified as *B. cereus*-like DB27) kill *C. elegans* in less than 24 hours, while *P. pacificus* is resistant. Interestingly, *C. elegans bre* mutants (resistant to *B. thuringiensis* Cry5B toxin (Griffitts et al., 2005)) and *daf-2* mutants (resistant to multiple pathogens (Garsin et al., 2003)) did not show increased resistance to the extreme killer DB27, suggesting that *C. elegans* requires novel mechanisms of defense against this pathogen. This study (I) reports a systematic screen for nematode-pathogenic naturally isolated *Bacillus* strains; (II) shows that most of the *Bacillus* strains are benign to nematodes; (III) leads to isolation of the DB27 strain, which is one of the most virulent *C. elegans* pathogens; (IV) provides a platform for the future comparative studies on nematode innate immune response and the pathogen virulence mechanisms, using DB27 strain.

2.1.2 Contributions

With Dr. Robbie Rae, I collected soil and beetle samples for bacterial isolation. I isolated *Bacillus* strains from these samples. With Hanh Witte, I extracted DNA from isolated *Bacillus* strains and performed 16S PCR and sequencing. I performed nematode survival and fungal inhibition assays, PCR screen for enterotoxins. Dr. Robbie Rae performed developmental assays and analyzed the data. I was involved in experimental design and preparation of the manuscript. In total, my contribution to this work was about 40%.

2.2 A system wide analysis of the evolution of innate immunity in the nematode model species *Caenorhabditis elegans* and *Pristionchus pacificus*

Amit Sinha, Robbie Rae, Igor Iatsenko, Ralf J. Sommer

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2.2.1 Synopsis

C. elegans has proven to be a very powerful model to study the mechanisms of innate immune response to pathogens. Several signaling pathways that protect the worm from pathogen attack have been discovered (Irazoqui et al., 2010). However, the role of these pathways in other nematodes is not known. *P. pacificus,* which has been extensively used for comparative studies with *C. elegans* (Sommer and Mcgaughran, 2013)*,* is a model of choice to address this question. Previous studies have shown, that *P. pacificus* lives in associations with numerous microbes in the wild (Rae et al., 2008) and differs from *C. elegans* in terms of survival, when exposed to the same pathogens (Rae et al., 2008). While some bacteria (*S. marcescens, X. nematophila*) are pathogenic to both nematodes, others (*B. thuringiensis* DB27, *S. aureus*) kill only *C. elegans* (Rae et al., 2008). The molecular basis of the differential response to the same pathogens of the two nematodes was not well studied. To gain insights into this differential response, we compared gene expression of *P. pacificus* and *C. elegans* after exposure to Gram-positive pathogens *B. thuringiensis* DB27 and *S. aureus* and Gram-negative pathogens *S. marcescens* and *X. nematophila*. First of all, this study identifies genes that might play an important role in the pathogen defense of the two nematode species. We also showed, that both nematodes mount a specific innate immune response to a given pathogen, and that two nematodes respond to the same pathogen in a different ways via distinct set of effector genes. Thus, our systems-wide analysis of the transcriptomic response of the two nematodes to bacterial pathogens tries to add a macroevolutionary level to the studies of the evolution of innate immune response, and shows that invertebrate innate immune systems are more complex and distinct than previously thought.

2.2.2 Contributions

I performed nematode survival assays on pathogens and collected infected *C. elegans* worms for microarray experiment. Dr. Rae collected infected *P. pacificus* worms for microarray experiment and was involved in the preparation of the manuscript. Dr. Sinha extracted RNA from infected worms and performed all microarray experiments. He also processed, analyzed and interpreted the microarray data. In total, my contribution to this work was about 20%.

2.3 New Role for DCR-1/Dicer in *Caenorhabditis elegans* Innate Immunity against the Highly Virulent Bacterium *Bacillus thuringiensis* DB27

Igor Iatsenko, Amit Sinha, Christian Rödelsperger, Ralf J. Sommer

Infection and Immunity 2013, 81(10), 3942 – 3957. doi:10.1128/IAI.00700-13.

2.3.1 Synopsis

Bacillus thuringiensis is an economically important pathogen of invertebrates, which is widely used for biological control of insect pests (Bravo et al., 2013). In our previous study (see 2.1) we isolated the *B. thuringiensis* strain DB27, which is extremely virulent to *C. elegans* but shows no toxicity to another model nematode *P. pacificus*. Interestingly, *C. elegans* mutants resistant to diverse pathogens (*daf-2*) (Garsin et al., 2003) or to *B. thuringiensis* toxin (*bre*) (Griffitts et al., 2005) are highly susceptible to *B. thuringiensis* DB27, indicating that potentially novel mechanisms of defense are required for *C. elegans* protection. To reveal these mechanisms of defense and to elucidate the mechanisms of the differential response of the two nematodes to *B. thuringiensis* DB27, we conducted a forward genetic screen for mutants of *C. elegans* resistant to *B. thuringiensis* DB27. One of the resistant mutants, which carries two missense mutations in the *nasp-1* (nuclear autogenic sperm protein) gene, was analyzed in detail.

Using genome-wide expression analysis, we revealed that the expression of many genes is downregulated in *nasp-1* mutant compared to wild type. Moreover, we found significant enrichment of *Dicer*-regulated genes among *nasp-1*-downregulated genes. Consistent with this, *Dicer* itself was repressed in *nasp-1* mutant, and both *Dicer* and *nasp-1* showed similar phenotypes, e. g. RNAi-deficiency and reduced longevity. These findings raise the possibility that *Dicer* is involved in the defense against *B. thuringiensis* DB27. In support of this, we showed that *Dicer* mutants are more resistant to *B. thuringiensis* DB27 in comparison with wild-type worms. However, the role of *Dicer* is allele-specific, since only microRNA-deficient, but not RNAi-deficient, *Dicer* alleles showed increased resistance to the pathogen. The fact that *Dicer* is repressed in *nasp-1* mutant and *Dicer* is resistant to *B. thuringiensis* DB27 suggests that reduced activity of *Dicer* might contribute to *nasp-1* resistance. In agreement with this, transgenic *Dicer* overexpression rescues *nasp-1* resistance to *B. thuringiensis* DB27. Additionally, we identified one of the downstream effectors of *nasp-1* and *Dicer*, which is represented by collagen-encoding gene *col-92*. Thereby, our study reveals novel *C. elegans* innate immunity regulator NASP-1 and uncovers an important role for *Dicer* in antibacterial immunity.

2.3.2 Contributions

Dr. Sinha extracted RNA from infected worms and performed all microarray experiments. He also processed and analyzed the microarray data. Dr. Rödelsperger analyzed whole genome sequencing data and helped to identify the causative mutations. I designed and performed all other experiments. I was also involved in data analysis and preparation of the manuscript. In total, my contribution to this work was about 80%.

2.4 Draft genome sequence of highly nematicidal *Bacillus thuringiensis* DB27

Igor Iatsenko, Craig Corton, Derek J. Pickard, Gordon Dougan, Ralf J. Sommer

Genome Announcements 2014, 2(1):e00101-14. doi:10.1128/genomeA.00101-14.

2.4.1 Synopsis

B. thuringiensis is a spore-forming bacterium which exhibits specific pathogenicity to variety of invertebrates (Bravo et al., 2013). Multiple virulence factors that contribute to *B. thuringiensis* pathogenic properties have been described (Raymond et al., 2010). Crystal proteins (Cry proteins) produced during the sporulation phase as parasporal inclusions are the most studied virulence factors of *B. thuringiensis* (Bravo et al., 2013). Cry proteins are pore-forming toxins that are highly specific to their target invertebrate hosts and are not toxic to vertebrates. Furthermore, being completely biodegradable, they are an important alternative for the control of agricultural pests (Bravo et al., 2011). While Cry toxins comprise at least 72 subgroups with numerous members (http://www.lifesci.sussex.ac.uk/home/Neil_Crickmore/Bt/toxins2.html), they show specific toxicity to certain insect orders and to nematodes. However, targeted insects and nematodes are known to evolve resistance to Cry toxins, which dramatically hampers the application of Cry toxins (Griffitts and Aroian, 2005). Therefore, novel toxins are constantly needed to overcome the resistant hosts and to delay the development of resistance. Naturally isolated *B. thuringiensis* strains represent an important reservoir of novel toxins. Consistent with this, we previously (see 2.1) isolated from dung beetles *B. thuringiensis* DB27, which showed remarkable toxicity to the nematode *C. elegans*. The aim of this work was to sequence the genome of *B. thuringiensis* DB27 to get insights into mechanisms of its virulence. The genome of *B. thuringiensis* DB27 is represented by a circular 5.7-Mb chromosome and seven plasmids of 4; 5.3; 6.5; 8; 44; 104; and 200 kb in size. The chromosome of *B. thuringiensis* DB27 has a G+C content of 35.2%, and the G+C content of the plasmids ranges from 31.5 to 34.4%. We found that there are 5851 predicted genes located on the chromosome and 451 genes located on the plasmids. Importantly, we identified three Cry-like genes on different plasmids (200 kb, 4 kb, 6 kb) with high similarity to nematicidal Cry21Ba1 toxin. This whole genome sequencing project revealed first and valuable insights into the potential nematicidal virulence factors of *B. thuringiensis* DB27.

2.4.2 Contributions

The genome sequencing project has been performed by the group of Prof. Dougan at Sanger Institute. Dr. Pickard prepared libraries for sequencing. Craig Corton analyzed the sequencing data. I extracted genomic DNA of *B. thuringiensis* DB27 and analyzed the data for the presence of Cry toxins. I initiated the project and was involved in the manuscript preparation and handling. In total, my contribution to this work was about 50%.

2.5 *Bacillus thuringiensis* DB27 produces two novel protoxins, Cry21Fa1 and Cry21Ha1, which act synergistically against nematodes

Igor Iatsenko, Iuliia Boichenko and Ralf J. Sommer

Applied and Environmental Microbiology, in press

Spotlight of the issue. Article of significant interest selected by the editors.

2.5.1 Synopsis

Bacillus thuringiensis is an economically important pathogen of multiple invertebrate hosts. While numerous virulence factors contribute to *B. thuringiensis* successful infection, Cry toxins are primarily responsible for host killing (Raymond et al., 2010). These toxins exhibit selective activity against a narrow range of invertebrate species (Bravo et al., 2011). Most of the described Cry toxins are active against different insect orders, however, the activity range of Cry toxins constantly increases (Bravo et al., 2013). Nematodes are also among the *B. thuringiensis* targeted hosts. Several families of nematicidal Cry proteins (Cry5, Cry6, Cry12, Cry13, Cry14, Cry21, Cry55) have been described (Wei et al., 2013). Some of these toxins have been used for biological control of animal- and plant-parasitic nematodes and also introduced into transgenic crops providing targeted way to control nematode pests. However, the mechanism of action of nematicidal Cry proteins is poorly investigated and is largely limited to Cry5B toxin (Griffitts et al., 2005), which does not fully reflect the mechanism of action of other nematicidal toxins. Also, the application of Cry toxins was always strongly hampered by host resistance (Griffitts and Aroian, 2005). Naturally isolated *B. thuringiensis* strains have proven to be an invaluable source of novel Cry toxins that help to overcome the resistant hosts. *B. thuringiensis* DB27, that we isolated previously (see 2.1), is strongly pathogenic to *C. elegans* wild-type worms and to *C. elegans* mutants resistant to Cry5B toxin, indicating that potentially novel nematicidal factors are produced by *B. thuringiensis* DB27. The aim of this work was to identify nematicidal factors of *B. thuringiensis* DB27. We found that *B. thuringiensis* DB27 in addition to *C. elegans* also kills other free-living and animal-parasitic nematodes. Using plasmid-cured variants of *B. thuringiensis* DB27, we showed that plasmid-encoded Cry toxins are the major virulence factors of *B. thuringiensis* DB27. Whole genome sequencing (see 2.4) identified three novel Cry protoxins (Cry21Fa1, Cry21Ga1, Cry21Ha1) located on plasmids. However, only Cry21Fa1 and Cry21Ha1 showed toxicity to *C. elegans,* when expressed in *E. coli*. While Cry21Fa1 and Cry21Ha1 are active as single protoxins, they also exhibit synergistic activity (synergism factor 1.9-2.6).

Thereby, we identified two novel proteins Cry21Fa1 and Cry21Ha1 with synergistic activity as the main nematicidal virulence factors of naturally isolated strain *B. thuringiensis* DB27.

2.5.2 Contributions

Iuliia Boichenko purified Cry21Fa1 and Cry21Ha1 proteins for quantitative assays. I designed and performed all other experiments and, together with Ralf J. Sommer, wrote the manuscript. In total, my contribution to this work was about 70%.

2.6 *Bacillus subtilis* GS67 protects *C. elegans* from Gram - positive pathogens via bacteriocin-mediated pathogen inhibition

Igor Iatsenko, Amit Sinha Joshua Jim, Frank Schroeder and Ralf J. Sommer

Manuscript in preparation

2.6.1 Introduction

C. elegans innate immunity studies have typically been conducted using *E. coli* OP50 as a standard food source before exposure of worms to the actual pathogen. However, *E. coli* is a very unlikely food source to be encountered by *C. elegans* in the wild. In the natural environment, *C. elegans* is exposed to and feeds on a variety of microbes, some of which are benign, while others are pathogenic. Multiple reports have shown that different bacteria used as a feeding source have dramatic effects on *C. elegans* physiology. For instance, *Bacillus subtilis* was shown to increase *C. elegans* stress resistance and longevity via NO production (Gusarov et al., 2013). Also lactic acid bacteria have shown protective effect against pathogens via induction of *C. elegans* immune responses (Kim and Mylonakis, 2012). Similarly, *C. elegans* exhibited dramatic changes of life-history traits via integration of metabolic and gene regulatory networks when fed the soil bacteria *Comamonas* (MacNeil et al., 2013; Watson et al., 2013). However, whether and how natural bacteria have an impact on worm immunity is currently poorly investigated. Therefore, we decided to address the question whether feeding on other non-pathogenic bacteria, which *C. elegans* is more likely to feed on in natural habitats, could affect survival on pathogens. Specifically, we concentrated on naturally isolated bacteria of the genus *Bacillus*. *Bacillus* bacteria are very abundant in the decomposting plant material (Siala et al., 1974), where *C. elegans* predominantly lives (Félix and Duveau, 2012) and therefore, may serve as a food source for nematodes in this environment. Additionally, *Bacillus* bacteria were abundantly isolated as a part of *C. elegans*-associated microbiota, suggesting that they may have symbiotic interactions with *C. elegans* and even provide protection against pathogens (Montalvo-Katz et al., 2013). We have previously tested the interaction of *C. elegans* with a large set of naturally isolated *Bacillus* strains and showed that most of them are benign to nematodes (see 2.1). One of the strains, *B. thuringiensis* DB27, exhibited extreme virulence to *C. elegans* and was studied in details. We have identified *C. elegans* defence mechanisms against this strain (see 2.3) as well as bacterial virulence factors (see 2.5), tackling both sides of this host-pathogen equation (Fig. 10). In the current study, we investigated the third side of the interactions, namely the effect of non-pathogenic *Bacillus* on *C. elegans* resistance to *B. thuringiensis* DB27.

2.6.2 Materials and methods

Worms and bacterial strains

All *C. elegans* strains were cultured on nematode growth media (NGM) plates and fed with the *E. coli* OP50. *S. marcescens* was isolated from La Reunion island, *S. aureus* Newman and *P. aeruginosa* PA14 were purchased from the Deutsche Sammlung von Mikroorganismen und Zellkulturen, Germany (DSMZ), *B. thuringiensis* DB27 was isolated from dung beetles. *B. subtilis* GS67 and other *Bacillus* used in this study were isolated from soil.

C. elegans survival assay

Each bacterium was grown overnight in a shaking incubator at 30°C in Luria-Bertani (LB) broth, except *S. aureus* and *P. aeruginosa*, which were grown at 37°C. 80 µl of the culture were spread to the edges of 6 cm NGM plates to prevent worms from escaping and incubated overnight. Three to six independent replicates of 20 adult worms per plate were exposed to pathogens and were monitored for survival. Survival assays were repeated multiple times and conducted at 25°C. Nematodes were transferred once a day to fresh plates and considered dead when they failed to respond to touch.

Bacillus feeding assay

Each *Bacillus* strain tested was grown in a shaking incubator at 30°C overnight in LB, 40 µl were spread onto 6 cm NGM plates and incubated overnight. *C. elegans* eggs obtained by bleaching were put onto these NGM plates with either *Bacillus* (experiment) or *E. coli* OP50 (control, normal conditions). Plates were incubated at 25°C until worms reached L4/young adult stage and used in a pathogen survival assays as described above.

Detection of antagonistic activity

The disc diffusion assay was used for the test of antagonistic activities of *B. subtilis* GS67 against other bacteria. Briefly, 50 µl of overnight cultures of test bacteria were spread on LB plates. Four paper discs (Whatman, 6 mm), three for the experiment and one for control were placed on top of the agar. 30 µl of the cell-free, filtered supernatant of *B. subtilis* GS67 grown for 48 hours in LB broth at 30°C were added to paper discs, LB broth was used as a negative control. The plates were incubated for 24 hours at 25°C and examined for clear inhibition zone around the discs.

Pathogen colonization assay

Nematodes were grown on different test bacteria (*B. subtilis* GS67, *B. subtilis* 1A699, *E.coli* OP50) till adult stage. Adults grown on different bacteria were infected with *B. thuringiensis* DB27 for three hours and were used for colony-forming units (CFUs) determination following the protocol of Kim and Mylonakis, 2012. Briefly, ten worms per each treatment were surface sterilized and mechanically homogenized, serial dilutions of the homogenate were spread on LB plates, and colony-forming units of *B. thuringiensis* DB27 were counted.

***C. elegans* rescue assay**

E. coli OP50-grown adult worms were infected with *B. thuringiensis* DB27 for four hours. Surface-sterilized worms were put on OP50 plates, which prior to worm transfer were treated with *B. subtilis* supernatant. Survival of the worms was scored after 24 hours incubation at 25°C. To prepare supernatant-treated plates, 300 µl of cell-free supernatant of overnight *B. subtilis* culture were added to the *E. coli* OP50 lawn on NGM plate. Plates were incubated at room temperature till all supernatant was absorbed.

2.6.3 Results

***B. subtilis* GS67 increases *C. elegans* resistance to Gram-positive pathogens**

To test whether non-pathogenic *Bacillus* bacteria have an effect on worm innate immunity, we developed a simple test assay. Bleached eggs from worms grown on OP50 plates were put on a plate seeded with monoxenic cultures of a non-pathogenic *Bacillus* sp., and worms were grown till adulthood. Then, adult worms were picked to plates with pathogenic *B. thuringiensis* DB27 and survival scores were compared with worms grown on *E. coli* OP50 (normal conditions). After testing of multiple strains of different *Bacillus* species (Fig. 4), we discovered that *B. subtilis* GS67 showed the strongest effect on *C. elegans* survival. Compared to control OP50-grown worms, which die after 16-24 hours on *B. thuringiensis* DB27, *B. subtilis* GS67-grown worms survive up to 72 hours (Fig. 5A). Due to strong protective effect *B. subtilis* GS67 was chosen for further investigations into the molecular mechanisms underlying its effects on *C. elegans*.

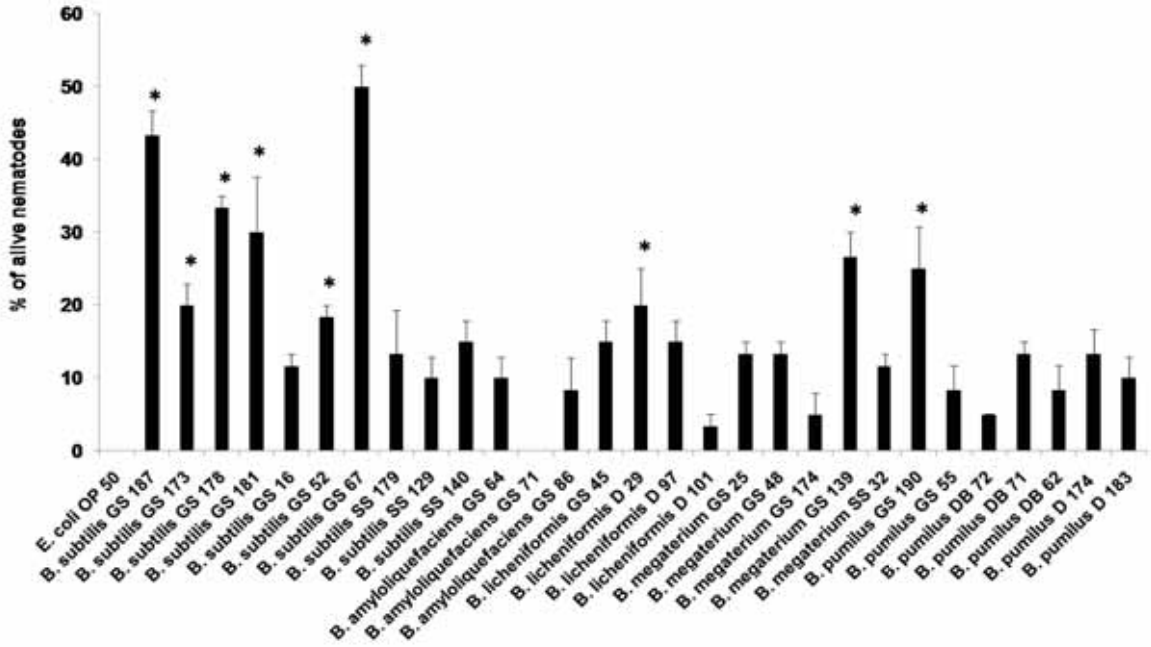

Figure 4. Survival of the nematodes on *B. thuringiensis* DB27 after 24 hours. Nematodes were grown on different *Bacillus* strains (shown on x-axis) till adult stage, exposed to *B. thuringiensis* DB27 for 24 hours and scored for survival. Asterisks indicate statistically significant difference compared to control (*E. coli* OP50).

Next, we asked if the observed effect of *B. subtilis* GS67 is specific to *B. thuringiensis* DB27 or if the nematodes acquire resistance to other pathogens as well. Upon exposure of worms pre-cultured on *B. subtilis* GS67 to another Gram-positive pathogen *Staphylococcus aureus*, we again observed significant ($p \leq 0.001$) increase of survival as compared to worms pre-cultured on *E. coli* OP50 (Fig. 5D). However, when we tested survival on two Gram-negative pathogens *S. marcescens* (Fig. 5B) and *P. aeruginosa* (Fig. 5C), we found no differences in survival between worms pre-cultured on either *B. subtilis* GS67 or *E. coli* OP50. Thus, *B. subtilis* GS67 increases *C. elegans* resistance to a group of Gram-positive pathogens, but has no effect on resistance to Gram-negative bacteria.

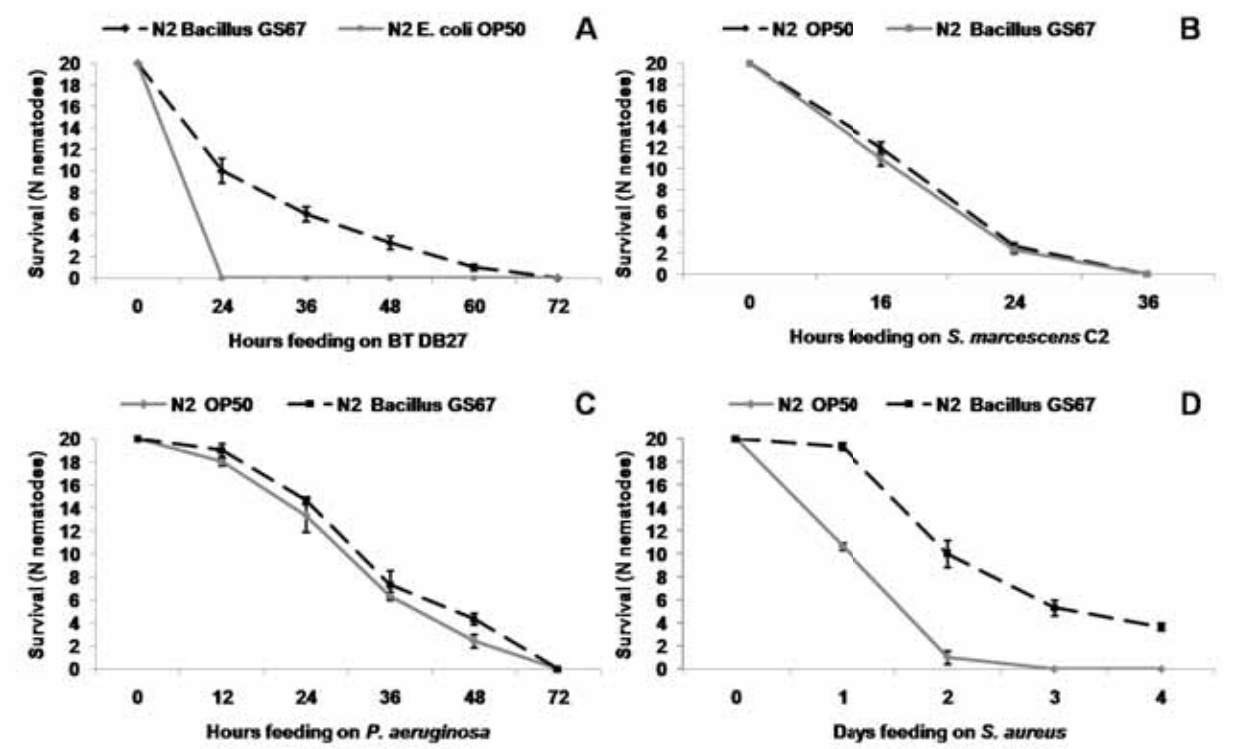

Figure 5. *C. elegans* grown on non-pathogenic *B. subtilis* GS67 is significantly more resistant to *B. thuringiensis* DB27 (A) and *S. aureus* (D) as compared to worms grown on *E. coli* OP50. Survival of *B. subtilis* GS67-grown worms on *S. marcescens* (B) and *P. aeruginosa* (C) is similar to *E. coli* OP50-grown worms.

B. subtilis GS67 inhibits *B. thuringiensis* DB27 and other Gram-positive bacteria

To gain insights into the underlying mechanisms of *B. subtilis* GS67-mediated protection, we decided to test the antibiosis hypothesis, assuming that *B. subtilis* GS67 might provide protection to *C. elegans* via direct inhibition of *B. thuringiensis* DB27 in a way similar to how host microbiota and probiotic bacteria inhibit growth of invading pathogens. To test this hypothesis, we employed a simple disc diffusion inhibition test. *B. thuringiensis* DB27 culture was spread on LB plate, then, paper discs soaked with the supernatant of *B. subtilis* GS67 were put on top of the *B. thuringiensis* DB27 lawn, and the assay LB plate was incubated for 24 hours. Strikingly, we found that supernatant of *B. subtilis* GS67 strongly inhibits growth of *B. thuringiensis* DB27 as illustrated by the presence of inhibition zones (Fig. 6). Interestingly, the spectrum of *B. subtilis* GS67 inhibitory activity seems to be restricted only to Gram-positive bacteria, while Gram-negative bacteria are not inhibited (Fig. 7). Specific antagonistic activity against Gram-positive bacteria might be the reason why the protective effect of *B. subtilis* GS67 is limited to Gram-positive pathogens. Thus, *B. subtilis* GS67-mediated inhibition of the pathogens might be one of the protective mechanisms against bacterial infection in *C. elegans*.

Figure 6. Overnight culture of *B. thuringiensis* DB27 was spread on LB plate. Cell-free supernatant of *B. subtilis* GS67 was applied to each of paper filter discs and plate was incubated for 24 hours at 25 °C. Clear inhibitory zones around paper discs are shown.

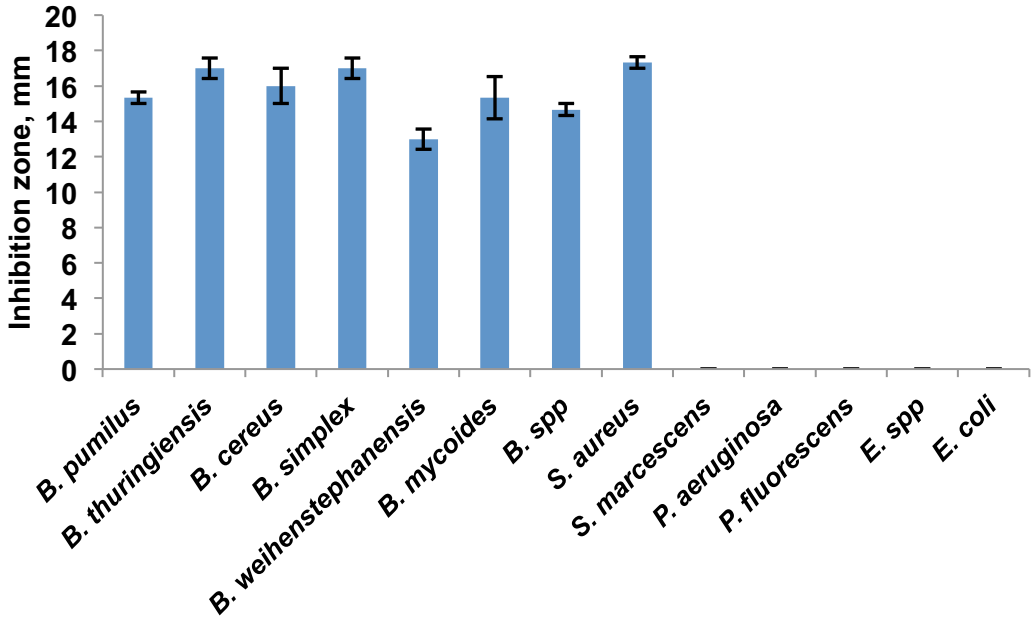

Figure 7. Spectrum of *B. subtilis* GS67 antagonistic activity. Indicated bacteria were subjected to disc diffusion test with *B. subtilis* GS67 supernatant. The size of the inhibition zones indicates the intensity of *B. subtilis* GS67 antagonism to a given bacterium. Inhibition zones were not observed in case of Gram-negative bacteria.

Antagonism contributes to protection *in vivo*

Next, we wanted to provide evidence that *B. subtilis* GS67 antagonistic properties observed *in vitro* contribute to *C. elegans* protection from pathogens *in vivo*. We found that *B. thuringiensis* DB27 colonization of *C. elegans* is strongly reduced in worms that were grown on *B. subtilis* GS67 when compared to worms grown on OP50 or on *B. subtilis* 1A699 (Fig. 8A), which lacks antagonistic activity. This indicates that *B. subtilis* GS67 might prevent colonization of the worm by the pathogen. Additionally, we found that *B. subtilis* GS67 can cure already infected worms from the pathogen. As shown in Fig. 8B, *B. thuringiensis* DB27-infected worms exhibit a much higher survival rate, when they are treated with the supernatant of *B. subtilis* GS67 compared to treatment with non-antagonistic *B. subtilis* 1A699 supernatant. These two experiments provide first evidence that the antagonistic properties of *B. subtilis* GS67 play an important role for *in vivo C. elegans* protection from the pathogen.

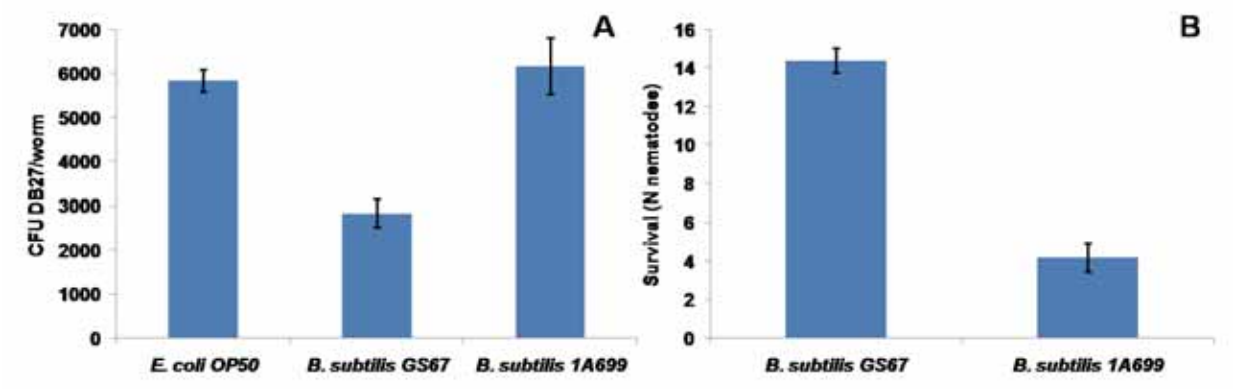

Figure 8. (A) *B. thuringiensis* DB27 colonization of *C. elegans*. Worms were grown on *E. coli* OP50, *B. subtilis* GS67 (antagonist of DB27), *B. subtilis* 1A699 (non-antagonist of DB27), infected with *B. thuringiensis* DB27 for three hours, and colony-forming units (CFUs) of *B. thuringiensis* DB27 were determined for each treatment. (B) Survival of *B. thuringiensis* DB27-infected worms after treatment with the supernatant of antagonistic strain (GS67) or non-antagonistic strain (1A699). GS67 compared to 1A699 significantly increases recovery of the infected worms.

A Lipoprotein is responsible for the antagonism

To obtain first insights into the biochemical and the molecular nature of the *B. subtilis* GS67 antagonistic properties, the supernatant of *B. subtilis* GS67 was treated with different enzymes and tested for activity. As shown in Fig. 9, treatment with lipase, trypsin and protease completely eliminated the antagonistic activity. These results suggest that the inhibitory molecule is a lipoprotein. The fact that this lipoprotein is heat stable and is active against closely related bacteria (Fig. 7), suggests that inhibitory molecule might act as a classical bacteriocin. Currently we are employing different techniques to reveal the exact structure of this bacteriocin.

Identification of the molecule is crucial because it will allow us to generate mutants deficient in the molecule production. Mutant *vs* wild type comparative studies on protective/antagonistic properties will allow us to convincingly show the importance of *B. subtilis* GS67 antagonism in *C. elegans* protection from *B. thuringiensis* DB27 infection. Therefore, the identification of the molecule is the major goal of our current studies.

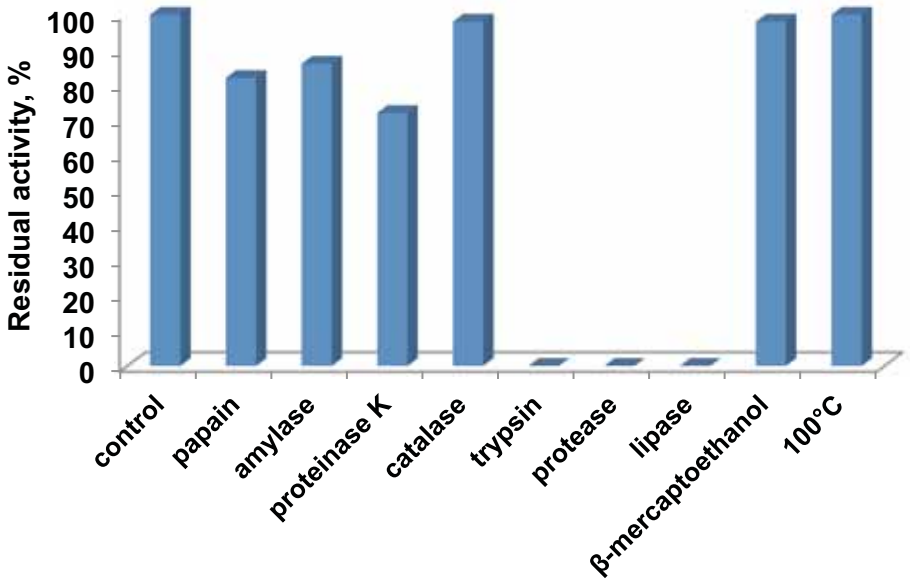

Figure 9. Cell-free supernatant of *B. subtilis* GS67 was treated with different enzymes (final concentration 1 mg/ml) for 1 hour at 37 °C, or 1% mercaptoethanol, or 100 °C for 1 hour. Control sample was treated for 1 hour at 37 °C without enzymes. Treated samples were subjected to disc diffusion assay. Size of the inhibition zone was used to calculate the residual activity.

2.6.4 Conclusion

Using a variety of molecular techniques and approaches, we successfully elucidated both sides of *C. elegans* - *B. thuringiensis* DB27 host-pathogen interactions (Fig. 10). From the host side, we discovered a novel innate immunity pathway, represented by *btr-1 (nasp-1)*, Dicer, miRNAs and *col-92*, which protects the worm from *B. thuringiensis* DB27 infection. The pathogen, in turn, produces two novel Cry21 toxins with synergistic activity to overcome host defense. In addition, we found that *C. elegans* commensal bacteria, like *B. subtilis* GS67, could change the outcome of host-pathogen interactions in favor of the host. In contrast to previous, host-centered studies, which considered the effect of commensal bacteria exclusively on the host, our results suggest that the *B. subtilis* GS67 protective effect is primarily mediated by its action on the pathogen. This result represents a novel aspect in *C. elegans*-pathogen interactions and highlights the importance of microbe-microbe interplay for the outcome of host-pathogen

interactions. Thus, the protective mechanisms of commensal bacteria against *C. elegans* pathogens are more complex than previously recognized and involve the commensal action on host and pathogen side, which has to be taken into account in future studies addressing the defensive role of commensals.

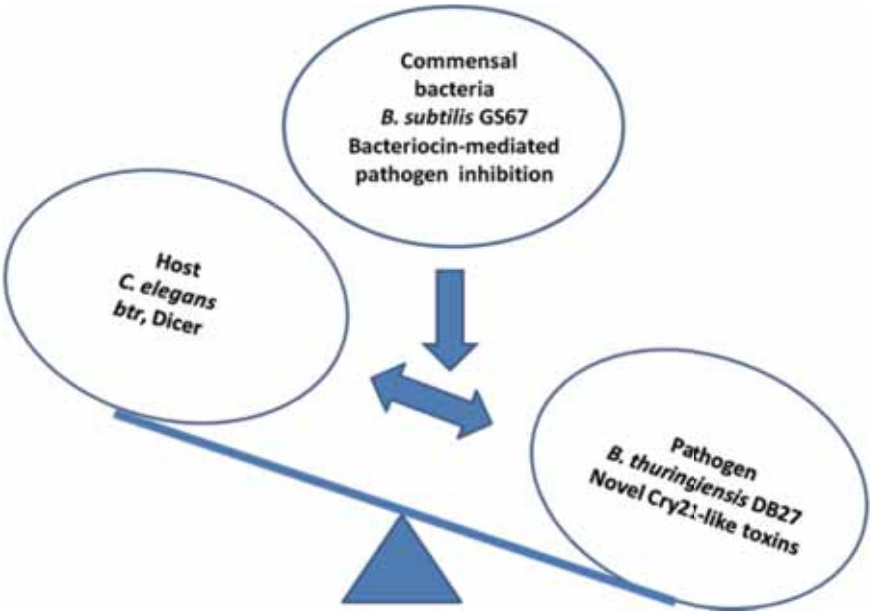

Figure 10. Schematic summary of the thesis. *C. elegans* - *B. thuringiensis* DB27 model system was used to study host-pathogen interactions. *C. elegans* defense genes (*btr*, Dicer) protect the host from *B. thuringiensis* DB27 infection, which is mediated by novel Cry21 toxins. *C. elegans* commensal *B. subtilis* GS67 protects the host from *B. thuringiensis* DB27 infection primarily via bacteriocin-mediated inhibition of the pathogen, shifting the host-pathogen balance in favor of the host.

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A subset of naturally isolated *Bacillus* strains show extreme virulence to the free-living nematodes *Caenorhabditis elegans* and *Pristionchus pacificus*

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Summary

The main food source of free-living nematodes in the soil environment is bacteria, which can affect nematode development, fecundity and survival. In order to occupy a reliable source of bacterial food, some nematodes have formed specific relationships with an array of invertebrate hosts (where bacteria proliferate once the hosts dies), thus forming a tritrophic system of nematode, bacteria and insect or other invertebrates. We isolated 768 *Bacillus* strains from soil (from Germany and the UK), horse dung and dung beetles and fed them to the genetically tractable free-living nematodes *Caenorhabditis elegans* and *Pristionchus pacificus* to isolate nematocidal strains. While *C. elegans* is a bacteriovorous soil nematode, *P. pacificus* is an omnivorous worm that is often found in association with scarab beetles. We found 20 *Bacillus* strains (consisting of *B. cereus*, *B. weihenstephanensis*, *B. mycoides* and *Bacillus* sp.) that were pathogenic to *C. elegans* and *P. pacificus* causing 70% to 100% mortality over 5 days and significantly affect development and brood size. The most pathogenic strains are three *B. cereus*-like strains isolated from dung beetles, which exhibit extreme virulence to *C. elegans* in less than 24 h, but *P. pacificus* remains resistant. *C. elegans* Bre mutants were also highly susceptible to the *B. cereus*-like strains indicating that their toxins use a different virulence mechanism than *B. thuringiensis* Cry 5B toxin. Also, mutations in the *daf-2*/*daf-16* insulin signaling pathway do not rescue survival. We profiled the toxin genes (*bcet*, *nhe* complex, *hbl* complex, *pcpl*, *sph*, *cytK*, *piplc*, *hly2*, *hly3*, *entFM* and *entS*) of these three *B. cereus*-like strains and showed presence of most toxin genes but absence of the *hbl* complex. Taken together, this study shows that the majority of naturally isolated *Bacillus* from soil, horse dung and *Geotrupes* beetles are benign to both *C. elegans* and *P. pacificus*. Among 20 pathogenic strains with distinct virulence patterns against the two nematodes, we selected three *B. cereus*-like strains to investigate resistance and susceptibility immune responses in nematodes.

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Introduction

Bacteria from the genus *Bacillus* are found in great abundance in the soil matrix, e.g. 10^4–10^6 per gram (Martin and Travers, 1989) as heat resistant spores (Nicholson, 2002). A subset of *Bacillus* are of medical and economic importance. For example, *Bacillus anthracis* is the causative agent of anthrax (Lew, 1995), *Paenibacillus larvae* is causative agent of American foulbrood disease in honeybees (de Maagd *et al*., 2001), which is responsible for large economic losses in the honey industry, and *B. cereus* is responsible for severe food poisoning (Granum and Lund, 1997). *Bacillus thuringiensis* (BT) is used as a commercial pesticide used to control insects in the orders Lepidoptera, Diptera and Coleoptera (Beegle and Yamamoto, 1992).

As well as insects, it has been speculated that nematodes may contribute to the evolution and/or spreading of *Bacillus* (in particular BT) (Wei *et al*., 2003), because nematodes are ubiquitous in the soil environment and many *Bacillus* species kill nematodes when fed spores or vegetative cells. For example, *B. firmus*, *B. amyloliquefaciens*, *B. cereus*, *B. licheniformis*, *B. megaterium*, *B. mycoides* and *B. pumilus* are toxic to plant parasitic nematodes (*Heterodera* sp. and *Meloidogyne* sp.) (Siddiqui and Mahmood, 1999; Terefe *et al*., 2009) and BT crystal proteins (Cry 6A, 5B, 14A and 21A) can kill free-living and animal parasitic nematodes (Borgonie *et al*., 1996; Wei *et al*., 2003) although some have no effect.

The free-living nematodes *Caenorhabditis elegans* and *Pristionchus pacificus* (Fig. 1A and B) have been developed as genetic model systems with a wealth of forward and reverse genetic tools and full genome sequence available (C. elegans Sequencing Consortium, 1998;

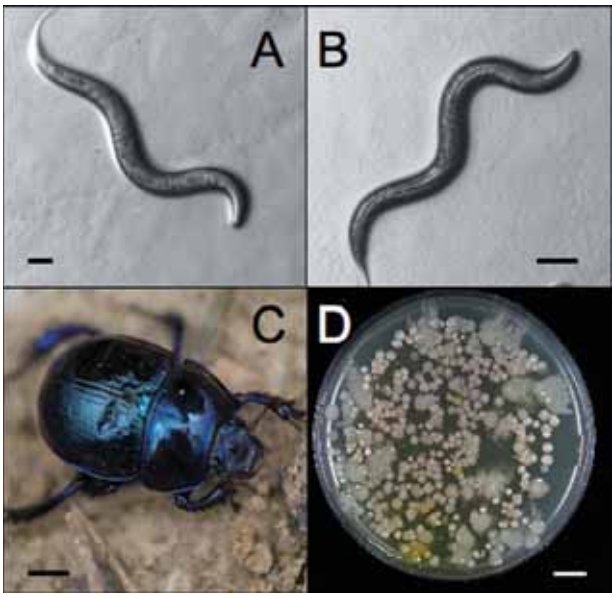

Fig. 1. Organisms used in this study. The nematodes *P. pacificus* (A) and *C. elegans* (B), *Geotrupes* sp. beetle (C) and *Bacillus* isolated from soil (D). Scale bars in A and B represent 100 μm, C represents 0.5 cm and D is 1 cm.

Dieterich *et al*., 2008). In nature, both these nematodes can be isolated from invertebrate hosts and soil samples from around the world, but they have a completely different ecology. *Caenorhabditis elegans* is a soil nematode that has occasionally been isolated from a range of invertebrates including isopods, slugs and snails (Mengert, 1953; Barriere and Felix, 2005; Caswell-Chen *et al*., 2005). In contrast, many *Pristionchus* species can be readily isolated from scarab beetles in an almost exclusive manner. For example, *P. pacificus* has been isolated from the oriental beetle in Japan and the USA (*Exomala orientalis*) (Herrmann *et al*., 2007), *P. maupasi* from cockchafers (*Melolontha* spp.), *P. entomophagus* from dung beetles (*Geotrupes* sp.) (Fig. 1C) in Europe (Herrmann *et al*., 2006a) and *P. uniformis* from the Colorado potato beetle (Herrmann *et al*., 2006b). These nematodes have a necromenic relationship with beetles whereby dauer stage nematodes infect a beetle host (Weller *et al*., 2010), wait for the beetle to die then resume growth and feed on microbes proliferating on the carcass. Once the food supply is depleted then the nematodes arrest in the dauer stage and move into the soil system to infect new adult beetles or soil dwelling grubs.

The usual food source under laboratory conditions for both *C. elegans* and *P. pacificus* is *Escherichia coli* strain OP50 (Brenner, 1974; Sommer *et al*., 1996). It remains unknown what bacteria *C. elegans* associates with in nature and only few studies have been carried out to date. Grewal (1991) isolated 10 bacteria species from the gut and cuticle of *C. elegans* including *Acinetobacter* sp., *Bacillus* sp., *Pseudomonas* sp. and *Enterobacter* sp. and showed varying levels of growth and reproduction when *C. elegans* was fed with each. In contrast, however, under natural conditions, *Pristionchus* species feed on a wealth of different pathogenic and non-pathogenic species isolated from both beetles and soil that can affect developmental rate, brood size, survival and behaviour (Rae *et al*., 2008). It has been shown that these two nematodes differ in their susceptibility to BT Cry 5B toxin (Wei *et al*., 2003) and the human pathogens *Staphylococcus aureus* and *Pseudomonas aeruginosa* (Rae *et al*., 2008). This could be due to the abundance of detoxification genes in *P. pacificus* compared with *C. elegans* (Dieterich *et al*., 2008) or differences in morphology. *Caenorhabditis elegans* has a grinder (used for crushing bacterial cells) at the base of the pharynx but *P. pacificus* does not (Rae *et al*., 2008). The lack of grinder means *Pristionchus* species can ingest whole bacterial cells and disseminate bacteria to new areas (Chantanao and Jensen, 1969).

Here, we assessed the pathogencity of 768 naturally isolated *Bacillus* strains (at vegetative cell stage) sampled from soil (from Germany and the UK), dung beetles (*Geotrupes* sp.) and horse dung to investigate whether these nematode species differ in their susceptibility and to discover what proportion of *Bacillus* are virulent to nematodes. As well as effects on nematode survival we recorded the effects on development and fecundity. We tested whether available *C. elegans* Bre mutants (BT toxin resistant) and Daf mutants (abnormal dauer formation) were resistant to the most toxic *Bacillus* strains observed in our survey, which consisted of a group of three *B. cereus*-like strains. Mutations in the gene *daf-2* in *C. elegans* extends lifespan as well as causing resistance to the bacterial pathogen *Enterococcus faecalis* (Garsin *et al*., 2003). Mutations in Bre genes in *C. elegans* cause defects in the production of carbohydrate structures on arthroseries glycolipids which bind Cry 5B and hence make these mutants resistance to BT Cry 5B toxin (Griffitts *et al*., 2005). We also sequenced the 16S rRNA gene from all 768 strains to determine species identity and profiled the toxin genes from the most pathogenic *Bacillus* strains.

From our survey of 768 *Bacillus* strains, only 20 showed severe pathogenicity to nematodes with the most toxic causing mortality to *C. elegans* in less than 24 h. This represents, to our knowledge, the most virulent nematocidal *Bacillus* bacteria described in the literature. These *Bacillus* strains are toxic only to *C. elegans*, whereas *P. pacificus* is resistant. Most interestingly, mutations in Bre and Daf genes do not alter resistance to the *B. cereus*-like strains, indicating the presence of novel virulence mechanisms.

Results

Survey and analysis of Bacillus *strains*

To study the pathogenicity of naturally isolated *Bacillus* strains on nematodes, we isolated 768 strains from soil (from Germany and UK), horse dung and *Geotrupes* beetles (Fig. 1C and D). *Bacillus* strains were designated with a code beginning with D, DB, GS or US for horse dung, dung beetles, soil from Germany or soil from UK, respectively. Each strain was fed to *C. elegans* and *P. pacificus* and the 16S rRNA gene of all 768 *Bacillus* isolates was sequenced and analyzed to determine species identity (for full list see Supporting Tables S1–S4). In summary, the most abundant three strains found in soil from Germany were *Bacillus* sp. CM-B72 (60 isolates), *Bacillus* sp. RA51 (43 isolates) and *Bacillus weihenstephanensis* (25 isolates). From soil collected from the UK, we found *Bacillus* sp. CM-B72 (62 isolates), *B. cereus* (49 isolates) and *Bacillus* sp. RA51 (39 isolates) the most prevalent. From *Bacillus* isolated from *Geotrupes* beetles *Bacillus* sp. HSCC (45 isolates), *B. longisporus* (43 isolates) and *Bacillus* sp. CM-B72 (22 isolates) were the most common. The most common *Bacillus* from horse dung were *Bacillus* sp. (44 isolates), *Bacillus* sp. CM-B72 (29 isolates) and *B. cereus* (28 isolates). We also isolated common soil *Bacillus* sp. such as *B. mycoides*, *B. pumilus*, *B. licheniformis*, *B. subtilis*, *B. simplex* and BT although in small amount.

Caenorhabditis elegans *and* P. pacificus *show distinct pathogenicity patterns to* Bacillus *strains*

We categorized a *Bacillus* strain as being pathogenic as after 5 day feeding 25% of nematodes or less were still alive. From soil collected from Germany, six *Bacillus* strains were pathogenic to *C. elegans* and *P. pacificus* which significantly affected survival after 5 day exposure compared with the *Escherichia coli* OP50 control ($P > 0.001$) (Fig. 2A and B, see Supporting Table S5 for complete list of nematocidal *Bacillus* strains). Generally, these strains were both toxic to *C. elegans* and *P. pacificus* although strain numbers GS108 only affected *C. elegans* and not *P. pacificus* and only strain number GS158 killed *P. pacificus* and not *C. elegans*. We did not identify any *Bacillus* strains isolated from soil from the UK that caused any mortality to either nematode species.

When fed 192 *Bacillus* strains isolated from horse dung three *Bacillus* strains severely affected survival of *C. elegans* (Fig. 2C) ($P > 0.001$) and four strains caused mortality to *P. pacificus* (Fig. 2D) ($P > 0.001$). Both strain numbers D5 and D60 killed both nematodes but only D112 affected *C. elegans* and only D106 and D149 affected *P. pacificus*.

Seven strains of *Bacillus* isolated from dung beetles killed *C. elegans* after 5 day exposure (Fig. 2E) ($P > 0.001$). In contrast to *C. elegans*, *P. pacificus* was largely unaffected by these seven strains and survival was only significantly affected by *Bacillus* strain DB35 (Fig. 2F) ($P > 0.001$), a strain that did not affect *C. elegans*. The most pathogenic of the *Bacillus* isolated from dung beetles (strain numbers DB7, DB27 and DB73) were significantly more toxic than all other strains and killed 100% *C. elegans* within 24 h ($P > 0.001$). From all 768 *Bacillus* strains tested these three were most virulent. Sequence analysis revealed that DB7, DB27 and DB73 are most similar to *B. cereus* and we therefore designate them as *B. cereus*-like strains throughout the analysis. These strains are currently investigated in greater detail in collaboration with Dr A. Hoffmaster (CDC, Athens, Georgia). We decided to concentrate on these three strains in further experiments (see below).

In summary, the majority of soil, horse dung and *Geotrupes* beetles are largely non-nematocidal. However, those *Bacillus* strains that do kill nematodes vary in pathogenicity (100% mortality caused in under 24 h to 5 days) and in what nematode species they can kill i.e. *P. pacificus* is largely unaffected by DB7, DB27 and DB73.

Effects of 20 pathogenic Bacillus strains on fecundity on C. elegans *and* P. pacificus

Pathogenic *Bacillus* strains can be used as food source, so we exposed single virgin hermaphrodite *C. elegans* and *P. pacificus* to each nematode pathogenic *Bacillus* and analyzed development and fecundity. After 3 day exposure, the mean number of *C. elegans* and *P. pacificus* juveniles produced on our 20 pathogenic *Bacillus* strains was significantly lower than the *E. coli* OP50 control ($P < 0.001$) (Fig. 3A and B). Surprisingly, young adult *C. elegans* sporadically managed to produce offspring on the *B. cereus*-like strains DB7, DB27 and DB73 albeit at very low levels. *Pristionchus pacificus*, however, managed to produce significantly more juveniles compared with *C. elegans* on strains DB7, DB27 and DB73 ($P < 0.05$). Taken together, these 20 *Bacillus* strains severely affect survival and fecundity of *C. elegans* and *P. pacificus.*

Bacillus cereus *like strains are toxic to* C. elegans *Bre and Daf mutants*

Given the strong virulence of the *B. cereus*-like strains DB7, DB27 and DB73 on *C. elegans*, we started to investigate the virulence mechanisms. Mutations in glycolipid receptors make *C. elegans* resistant to BT Cry 5B toxins (i.e. Bre mutants) (Griffitts *et al.*, 2005). We wanted to know if DB7, DB27 and DB73 utilized a similar mechanism as BT Cry 5B and would hence be resistant to these *Bacillus*

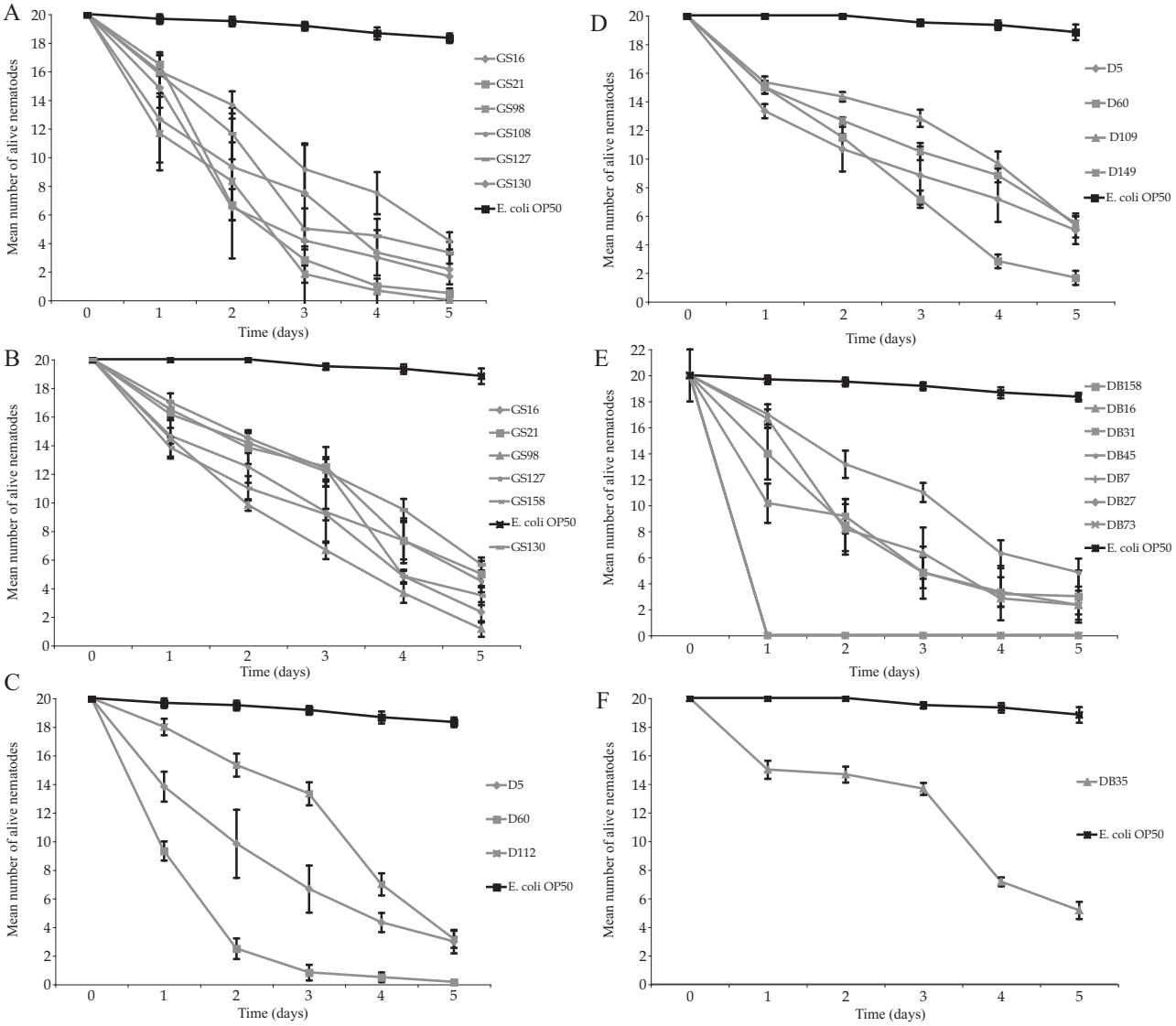

Fig. 2. Identification of nematode pathogenic *Bacillus* species. Mean number of alive *C. elegans* (A, C, E) and *P. pacificus* (B, D, F) exposed to *Bacillus* isolated from soil from Germany (A and B), horse dung (C and D) and *Geotrupes* sp. beetles (E and F) for 5 days. Bars represent ± one standard error.

strains. *Caenorhabditis elegans bre-1(ye4)*, *bre-2(ye31)* and *bre-3(ye26)* mutants exposed to DB7, DB27 and DB73 displayed the same survival dynamics as wild type *C. elegans* and 100% were dead after 24 h ($P < 0.001$, compared with *E. coli* OP50 control) (Fig. 4A and B, data not shown). Therefore, these *Bacillus* strains do not use the same pathogenic mechanism as BT Cry 5B toxin.

There are a number of *C. elegans* Daf mutants, e.g. *daf-2* that are resistant to bacterial pathogens (Garsin *et al*., 2003). We tested mutations in *daf-16(m27)*, *daf-2(e1368)*, *daf-12(m20)* and *age-1(hx546)* in the insulin signaling pathway that are known to affect longevity (Kenyon *et al*., 1993), dauer formation and stress response. *Caenorhabditis elegans daf-12*, although not known to have an effect on pathogenesis, was included because it represents the only Daf gene, for which there is a mutation available in *P. pacificus* (Ogawa *et al*., 2009). Interestingly, *daf-16(m27)* (Fig. 5B), *daf-2(e1368)* (Fig. 5C), *daf-12(m20)* (Fig. 5E) and *age-1(hx546)* (Fig. 5G) mutant worms are killed within 24 h of being fed DB7, DB27 and DB73 similar to *C. elegans* wild type (Fig. 5A) ($P > 0.05$, comparison of survival kinetics of mutants versus wild type). These results show that single gene mutations in the insulin-signaling pathway and *daf-12* have little effect on the survival on *C. elegans* when fed the three *B. cereus*-like strains. In contrast, *C. elegans daf-16(mg54), daf-2(e1370)* (Fig. 5D), *daf-12(m20), daf-2(m41)* (Fig. 5F), and *age-1(m333), daf-*

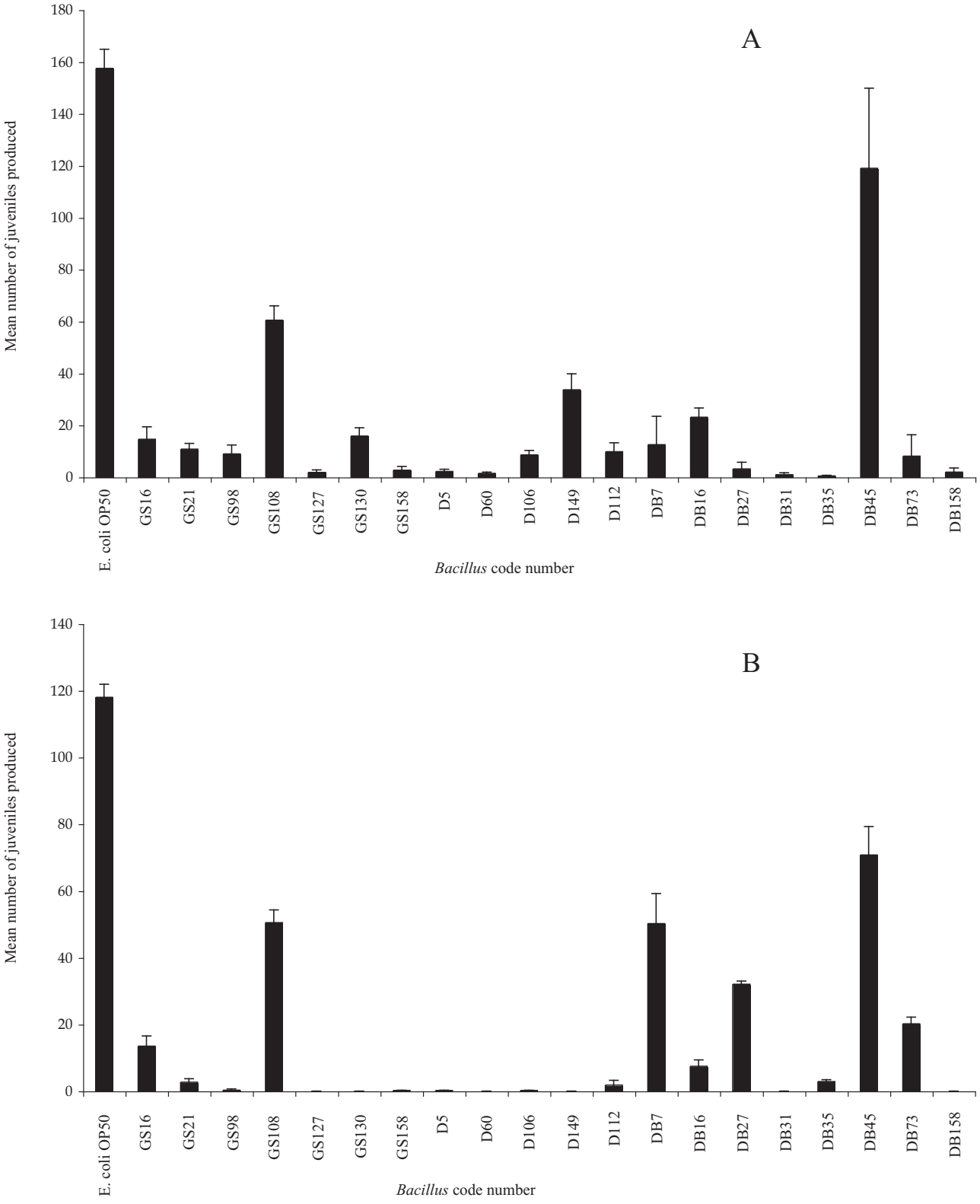

Fig. 3. Mean number of juveniles produced from single virgin hermaphrodite *C. elegans* (A) and *P. pacificus* (B) fed on 20 pathogenic *Bacillus* sp. and *E. coli* OP50 control for 3 days. Bars represent ± one standard error.

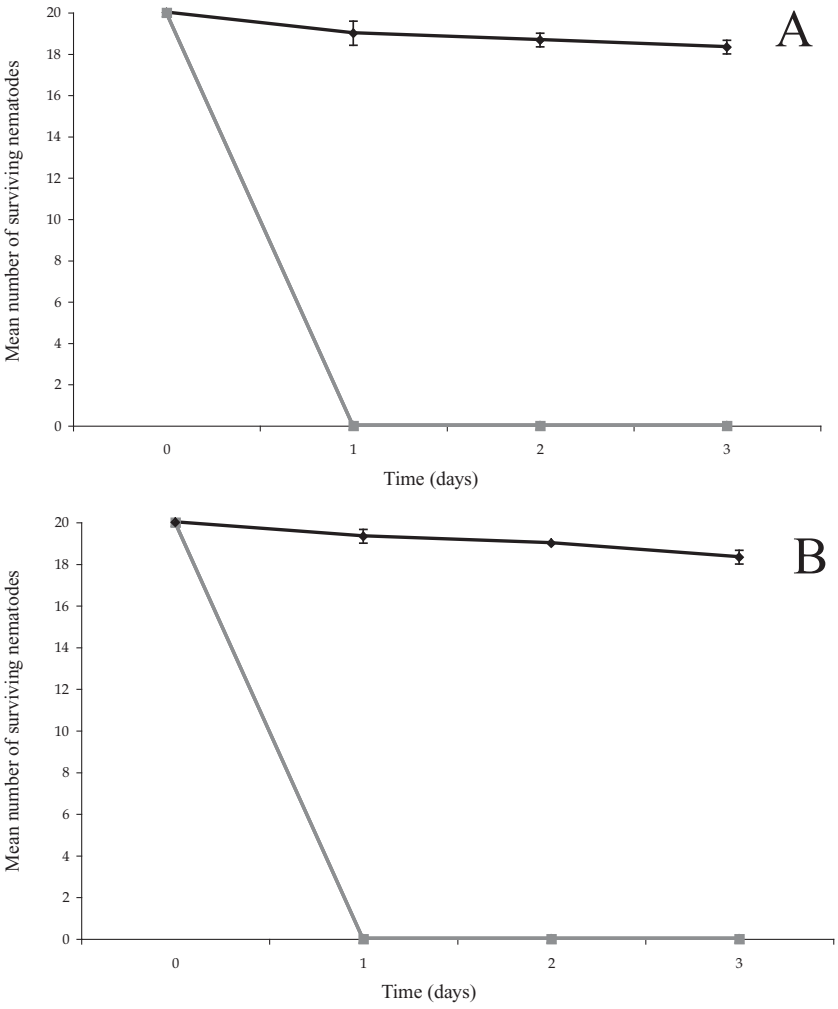

Fig. 4. Mean survival of *C. elegans* wild type (A) and *bre-1* (B) exposed to the most pathogenic *Bacillus* strains DB7 (dark grey squares) isolated from *Geotrupes* dung beetles and *E. coli* OP50 (black diamonds) for 3 days. Bars represent ± one standard error.

16(m26) (Fig. 5H) double mutants die, but have slightly lengthened survival dynamics compared with wild type. There are significant differences between wild type survival and *daf-16(mg54); daf-2(e1370)* on day 1 exposed to DB7 ($P < 0.05$), 27 ($P < 0.05$) but not DB73 ($P > 0.05$). This is also true for *age-1(m333); daf-16(m26)* on day 1 exposed to strain DB73 ($P < 0.05$) compared with wild type. Also, there are significantly more survivors of *daf-12(m20); daf-2(m41)* exposed to DB7 and DB73 ($P < 0.05$), compared with wild type on day 1. Although there are significant differences on day 1, these mutants all die and there are no survivors on day 3, so generally the effect is very minimal and is not comparable to published effects found on other bacteria, e.g. *E. faecalis* (Garsin *et al*., 2003).

Effect of 20 nematode pathogenic Bacillus strains *on* Beauveria bassiana

Pristionchus nematodes are known to feed on whole bacterial cells and to expel them up to 27 h after ingestion, potentially meaning that these nematodes can transport bacteria to new hosts and new areas (Chantanao and Jensen, 1969; Rae *et al*., 2008). We therefore wanted to investigate whether there were other potential antagonistic effects of the pathogenic bacteria against other organisms. One such candidate in soil ecosystems is the entomopathogenic fungus *Beauveria bassiana*. *Beauveria bassiana* has been found infecting *Melolontha* grubs with *Pristionchus* species present (Herrman, Rae and Sommer, unpubl. data), although the effect this fungus has on *Pristionchus* viability when growing on these infected beetles is unknown. We investigated the ability of each of the 20 nematocidal *Bacillus* strains to suppress growth of *B. bassiana* in an *in vitro* dual culture assay. As a control we also tested the effect of 20 randomly picked non-nematocidal *Bacillus* sp. on fungal suppression.

All nematocidal *Bacillus* strains inhibited growth of *B. bassiana* significantly compared with the control, including the strongest nematocidal *B. cereus*-like strains DB7, DB27 and DB73, but there was no significant difference between these three strains ($P > 0.05$) (Fig. 6A). The

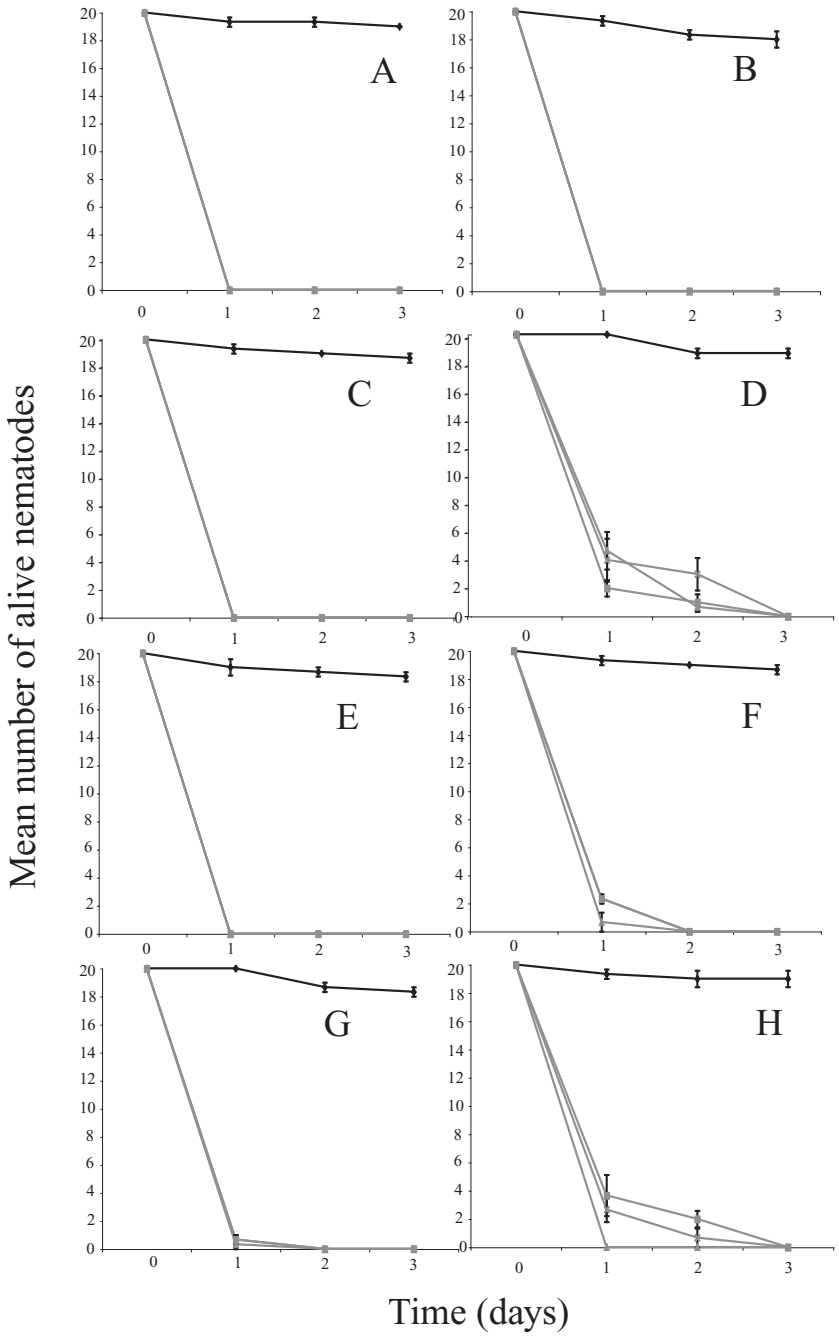

Fig. 5. Mean survival of *C. elegans* wild type (A), *daf-16* (B), *daf-2* (C), *daf-16*, *daf-2* (D), *daf-12* (E), *daf-12*, *daf-2* (F), *age-1* (G) and *age-1*, *daf-16* (H) exposed to the most pathogenic *Bacillus* strains DB7 (dark grey squares), DB27 (dark grey triangles) and DB73 (dark grey crosses) isolated from *Geotrupes* dung beetles and *E. coli* OP50 control (black diamonds) for 3 days. Bars represent ± one standard error.

strongest inhibitory effect (percentage inhibition > 60%) of *Bacillus* isolated from *Geotrupes* beetles was caused by DB31 (a strain of *B. cereus*).

The strength of fungal antagonism caused by nematocidal *Bacillus* strains differed significantly ($P < 0.05$) and so we classified this effect based on the percentage value of inhibition (see Supporting Table S6). The majority of strains belong to strong and very strong antagonists, while no weak antagonists were present. The highest percentage of strong and very strong antagonists was present in *Bacillus* isolated from *Geotrupes* beetles (87.5%) and *Bacillus* isolated from soil from Germany (85.7%), followed by horse dung *Bacillus* isolates (60%).

In contrast to the nematocidal strains, 20 randomly chosen non-nematocidal strains showed weaker or nearly no fungal antagonism (Fig. 6B). Specifically, non-nematocidal strains had a minimum and maximum inhibition of 5.92% ± 3.62% and 25.70% ± 6.51%, respectively, whereas nematocidal strains had a minimum and maximum inhibition of 19.84% ± 1.15% and 59.57% ± 0.43%, respectively. Also when the numbers of nematocidal and non-nematocidal *Bacillus* sp. that

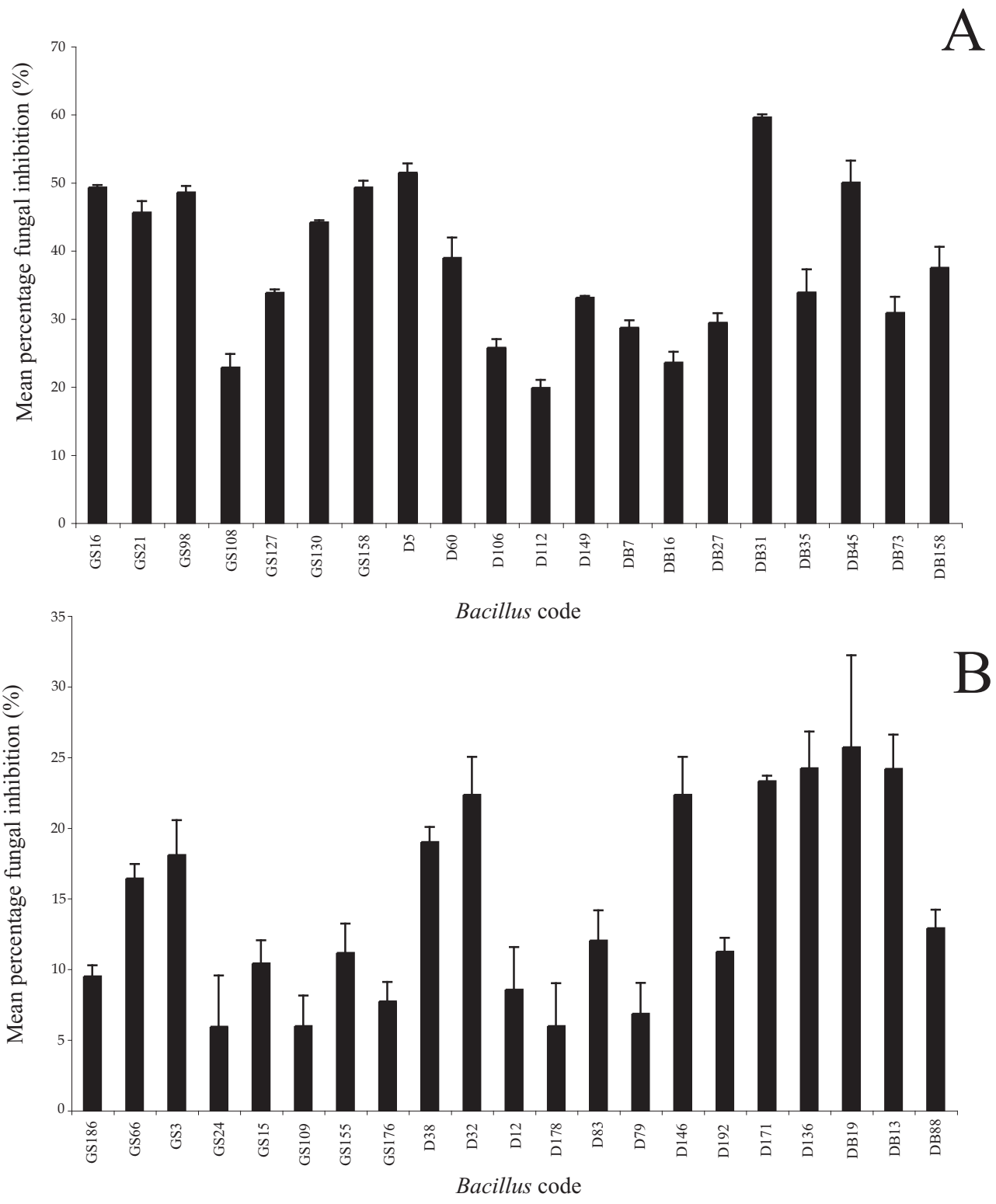

Fig. 6. Mean percentage inhibition of *B. bassiana* exposed to 20 nematocidal (A) and non-nematocidal (B) *Bacillus* sp. isolated from initial survey. Bars represent ± one standard error.

caused strong and very strong inhibitory effects on *B. bassiana* were compared, there were significantly more found in the nematocidal isolates ($P > 0.05$, see Supporting Table S6). Taken together, there is a strong overlap in nematode pathogenicity and in anti-fungal suppression.

Characterization of B. cereus *toxin genes and toxins*

We profiled the known toxin genes (see Fig. 7A) present in our nematocidal *Bacillus* isolates (Fig. 7B). The three *B. cereus*-like strains DB7, DB27 and DB73 are toxin gene rich and almost identical in profile even though they were isolated from separate *Geotrupes* beetles. All three strains tested positive for *nheA*, *nheB*, *nheC*, *pcpl* (*cerA*), *sph* (*cerB*), *piplc*, *cytK*, *hly3*, *entFM*, *entS* and four of five primer combinations for the *bceT* gene. We could only detect one component of the *hbl* complex (*hblC*) in DB7 and DB27 and none in DB73. Also DB7 and DB27 tested positive for *hly2* but DB73 did not. Although only six of 20 nematocidal strains are *B. cereus* (see Supporting Table S5) some of the other *Bacillus* strains have tested positive for specific *B. cereus* genes and therefore share same components of similar virulence mechanisms.

A

Gene	Gene code	Primer sequences (5'-3')	Reference
bceT	BCET 1	CGTATCGGTCGTTCACTCGG	Agata *et al* . (1995)
	BCET 2	AGCTTGGAGCGGAGCAGACT	
	BCET 3	GTTGATTTTCCGTAGCCTGGG	
	BCET 4	TTTCTTTCCCGCTTGCCTTT	
	BCET 5	TTACATTACCAGGACGTGCTT	
	BCET 6	TGTTTGTGATTGTAATTCAGG	
hblA (B)	HBLA1	GTGCAGATGTTGATGCCGAT	Hansen and Hendriksen (2001)
	HBLA2	ATGCCACTGCGTGGACATAT	
hblC (L2)	L2A	AATGGTCATCGGAACTCTAT	Hansen and Hendriksen (2001)
	L2B	CTCGCTGTTCTGCTGTTAAT	
hblD (L1)	L1A	AATCAAGAGCTGTCACGAAT	Hansen and Hendriksen (2001)
	L1B	CACCAATTGACCATGCTAAT	
nheA	nheA 344 S	TACGCTAAGGAGGGGCA	Hansen and Hendriksen (2001)
	nheA 843 A	GTTTTTATTGCTTCATCGGCT	
nheB	nheB 1500 S	CTATCAGCACTTATGGCAG	Hansen and Hendriksen (2001)
	nheB 2269 A	ACTCCTAGCGGTGTTCC	
nheC	nheC 2820 S	CGGTAGTGATTGCTGGG	Hansen and Hendriksen (2001)
	nheC 3401 A	CAGCATTCGTACTTGCCAA	
pcpl (cerA)	CERA 1	ACTGAGTTAGAGAACGGTAT	Hendriken *et al* . (2006)
	CERA 2	CGCTTACCTGTCATTGGTGT	
sph (cerB)	CERB 1	TCGTAGTAGTGGAAGCGAAT	Hendriken *et al* . (2006)
	CERB 2	AGTCGCTGTATGTCCAGTAT	
cytK	CK-F-1859	ACAGATATCGGKCAAAATGC	Guinebretiere *et al* . (2002)
	CK-R-2668	TCCAACCCAGTTWSCAGTTTC	
piplc	phosC 1	CGCTATCAAATGGACCATGG	Hansen *et al* . (1998)
	phosC 2	GGACTATTCCATGCTGTACC	
hly II	BcHlyII-S	AGAAGGAGTGGCTGTCTGTA	Hendriken *et al* . (2006)
	BcHlyII-A	TTCTTTCCAAGCAAAGCTAC	
hly III	BCHEM 1	AATGACACGAATGACACAAT	Hendriken *et al* . (2006)
	BCHEM 3	ACGATTATGAGCCATCCCAT	
entFM	ENTA F	ATGAAAAAAGTAATTTGCAGG	Minnaard *et al.* (2007)
	ENTA R	TTAGTATGCTTTTGTGTAACC	
entS	TY123 F	GGTTTAGCAGCAGCTTCTGTAGCTGGCG	Minnaard *et al.* (2007)
	TY125 R	GTTTCGTTAGATACAGCAGAACCACC	
hblB	hblB F	AAGCAATGGAATACAATGGG	Minnaard *et al.* (2007)
	hblB R	AATATGTCCCAGTACACCCG	

B

Toxin	Gene	Primer code	DB7	DB16	DB27	DB31	DB35	DB45	DB73	DB158	GS16	GS21	GS98	GS108	GS127	GS130	GS158	D5	D60	D109	D112	D149
B. cereus enterotoxin	*BCET*	1+3	+	+	+	-	-	+	+	+	+	+	+	-	+	+	+	+	+	+	+	+
	BCET	1+4	-	-	-	-	-	+	-	-	-	-	-	-	-	+	-	-	-	-	-	-
	BCET	2+3	+	+	+	+	-	+	+	-	+	+	+	-	+	+	+	+	+	+	-	+
	BCET	2+4	(+)	-	(+)	-	-	-	(+)	-	+	-	-	-	-	+	-	-	(+)	-	-	-
	BCET	5+6	+	+	+	-	-	+	+	-	-	+	+	-	-	+	-	-	-	+	-	-
Hemolysin BL	*hblA*	7+8	-	-	-	-	+	-	-	+	-	-	+	-	+	-	-	-	-	+	+	-
	hblB	35+36	-	-	-	+	+	-	-	-	-	-	+	-	+	-	-	-	-	+	+	-
	hblD	9+10	-	+	-	+	+	-	-	-	-	+	+	-	+	+	+	+	+	+	+	+
	hblC	11+12	+	-	+	+	+	-	-	+	-	+	+	-	+	+	+	+	+	+	+	+
Non-hemolytic enterotoxin	*nheA*	13+14	+	+	+	-	+	-	+	-	-	+	+	+	+	+	+	+	+	+	+	+
	nheB	15+16	+	+	+	-	+	+	+	+	+	+	+	-	+	+	+	+	+	+	+	+
	nheC	17+18	+	+	+	-	+	+	+	+	+	+	+	-	+	+	+	+	+	-	+	+
Phospholipases	*pcpl(cerA)*	19+20	+	+	+	+	+	+	+	+	-	+	+	-	+	+	+	+	+	+	+	-
	sph(cerB)	21+22	+	+	+	(+)	+	-	+	-	-	+	+	-	+	+	+	+	+	-	+	+
	piplc	25+26	+	+	+	-	+	+	+	-	-	+	+	-	+	-	+	-	+	+	+	-
Cytotoxin K	*cytK*	23+24	+	-	+	-	-	(+)	+	+	-	-	-	-	-	-	-	-	-	-	(+)	-
Hemolysin 2	*hly2*	27+28	+	+	+	-	+	-	-	-	-	+	-	-	+	-	-	-	-	+	+	+
Hemolysin 3	*hly3*	29+30	+	+	+	+	+	-	+	+	-	-	-	-	-	-	-	-	-	+	+	+
Enterotoxin FM	*ent FM*	31+32	+	+	+	-	+	-	+	+	-	+	-	-	+	+	-	-	+	+	+	-
Enterotoxin S	*ent S*	33+34	+	-	+	-	+	-	+	-	-	+	+	-	+	+	-	-	-	-	+	+

Fig. 7. *Bacillus cereus* primers used in this study (A). Presence (positive sign) or absence (negative sign) of each *B. cereus* toxin gene (B). Positive sign in brackets is where faint band was detected.

Discussion

There have been few studies on the effects naturally isolated *Bacillus* sp. have on free-living nematodes as most of the research has concentrated on animal parasitic nematodes (Kotze *et al*., 2005; Cappello *et al*., 2006) and plant parasitic nematodes (Li *et al*., 2007, 2008; Terefe *et al*., 2009). This is primarily to discover novel alternate biocontrol methods used to combat nematodes and rival current chemical nematocides. In our survey, the majority of *Bacillus* are not pathogenic to *C. elegans* and *P. pacificus* as we only identified 20 strains (out of 768 collected) that show pathogenicity towards these nematodes. Previous studies have concentrated on a number of *Bacillus* species that are virulent to nematodes, e.g. *B. firmus* (Terefe *et al*., 2009), *Brevibacillus laterosporus* (Huang *et al*., 2005), *B. nematocida* (Niu *et al*., 2006) and BT (Schulenburg and Muller, 2004). Huang and colleagues (2005) and Niu and colleagues (2006) showed that *B. nematocida* and *Br. laterosporus* protease production is the major factor in causing nematode death. In our case, however, the system is based on screening through isolates that cause death after food uptake through the intestine. We did not test the *Bacillus* spore stage, the life stage that is the most pathogenic to insects and is applied in pest control of insects. One reason for concentrating on vegetative cells rather than spores is that our previous work indicated that *Pristionchus* nematodes could successfully suppress spore germination in the intestine (Rae *et al*., 2008). Similarly, these nematodes can even use *Bacillus* spores as food source under laboratory conditions (R. Rae, unpubl. Obs.).

The three *B. cereus*-like strains DB7, DB27 and DB73 show remarkable pathogenicity towards *C. elegans* and begin to die after 8 h and are all dead after 16 h (I. Iatsenko, unpubl. obs.). From our knowledge this is one of the most toxic bacteria isolated so far when examined for pathogenicity to *C. elegans*, when grown under standard conditions using NGM medium. Other bacteria can exhibit the same fast killing dynamics but specifically have to been grown on other media to enhance this effect, e.g. *P. aeruginosa* grown on fast-killing medium kills *C. elegans* in under 24 h (see Tan *et al*., 1999). When fed *S. aureus*, *P. aeruginosa*, *Photorhabdus luminescens* or *Xenorhabdus nematophila C. elegans* begins to die rapidly after 24 h exposure (Tan *et al*., 1999; Begun *et al*., 2005; Rae *et al*., 2008) but with our *B. cereus*-like strains the pathogenicity process is much quicker.

We can also see from our study that virulence to nematodes varies between *B. cereus* strain and nematode species. In our study, we isolated 108 *B. cereus* strains, yet only six have a strong effect on *C. elegans* or *P. pacificus*. It is know that *B. cereus* induced food poisoning in humans varies from strain to strain (Granum, 1997) and this also seems to be the case with pathogenicity in nematodes as well.

The *B. cereus* group consists of six recognized species including *B. cereus*, BT, *B. anthracis*, *B. mycoides*, *B. pseudomycoides* and *B. weihenstephanensis* (Stenfors Arnesen *et al*., 2008). *Bacillus cereus* causes human food poisoning consisting of diarrhea and abdominal distress or nausea and vomiting and can cause a variety of infections including endophthalmitis, bacteremia, septicemia, endocarditis, salpingitis, cutaenous infections, pneumonia and meningitis (Drobniewski, 1993; Logan and Turnbull, 1999; Rasko *et al*., 2005). *Bacillus cereus* toxins include pore-forming cytoxins haemolysin BL (Hbl), non-haemolytic enterotoxin (Nhe) and cytotoxin K (CytK) (Beecher and MacMillan, 1991; Lund and Granum, 1996; Lund *et al*., 2000), which are activated by the transcriptional regulator PlcR (Lereclus *et al*., 1996; Gohar *et al*., 2002). The three *B. cereus*-like strains DB7, DB27 and DB73 contain a wealth of toxin genes, but we were unable to amplify the enterotoxin hemolysin BL (HBL) gene by standard Polymerase chain reaction (PCR) primers that work well for other strains. The presence of this gene seems to be variable among strains. For example, Hansen and Hendriksen (2001) only found the HBL complex present in 11 of 22 *B. cereus* strains tested and Mantynen and Lindstrom (1998) found *hblA* in 52% of *B. cereus* strains. It is not surprising that these genes from the HBL complex are present in our other nematocidal *Bacillus* as it has been reported that they have been identified in BT, *B. mycoides*, *B. weihenstephanensis*, *B. pseudomycoides* and *B. anthracis* (Ryan *et al*., 1997; Pruss *et al*., 1999; Hansen and Hendriksen, 2001). As we recorded presence of the majority of genes tested, it remains to be discovered which factors might be responsible for causing rapid nematode mortality.

Pristionchus pacificus differs in susceptibility to *S. aureus*, *P. aeruginosa* (Rae *et al*., 2008) and BT toxin Cry 5B (Wei *et al*., 2003) and in our study we were able to show that this is also true for a number of *Bacillus* species identified in our screening procedure. The main reasons for this are currently unknown but hyper susceptible mutants are being isolated to discover what genes are integral to *P. pacificus* immunity (Rae, unpubl. Obs.).

Mutations in *daf-2* create resistance to gram-negative and gram-positive pathogens such as *E. faecalis*, *P. aeruginosa* and *S. aureus* (Garsin *et al*., 2003). However, we have found that *C. elegans daf-2* does not enhance resistance to our *B. cereus* strains, therefore, although resistance to pathogens can be conferred through suppression of *daf-2* it strongly depends on bacterial species. Surprisingly, we found that *daf-2; daf-16* and *age-1; daf-16* double mutants showed a slight resistance to *Bacillus* DB27. One potential reason for this finding might

be that DAF-16 has pleiotropic effects and is part of several signaling systems involved in stress response (Kenyon, 2010). We also found that *C. elegans* Bre mutants were also susceptible to the *B. cereus*-like strains. This suggests that mutations in glycolipids that stop Cry 5B from binding to the intestinal cells does not stop the virulence process of *B. cereus*.

We have shown that some soil and dung derived *Bacillus* not only cause death to *C. elegans* and *P. pacificus* but also suppress *B. bassiana* using *in vitro* assays. In nature bacteria, nematodes and fungi share the same ecological niche in soil or on beetles. To survive in these 'microbial jungles' bacteria must protect themselves against predators (nematodes) as well as to suppress other competitors (fungi and other bacteria). Some bacteria have been shown to live in symbiosis with beetles and play and provide an important role in their life. Mutualistic associations with microorganisms are widespread in insects, and the microbes serve an array of functions for their insect hosts, including protective services (Lundgren *et al*., 2007). For instance, Colorado potato beetle isolates belonging to the genera *Pantoea* sp*., Enterobacter* sp*., Pseudomonas* sp. and *Bacillus* sp. inhibited growth of the entomopathogenic fungus *B. bassiana in vitro*. They have also been shown to protect the beetle against the entomopathogenic nematode *Heterorhabditis marelatus* by suppressing its bacterial symbiont *Photorhabdus temperata*, which is responsible for the killing of the beetle (Blackburn *et al*., 2008). In addition, some *Pseudomonas* sp*., Serratia* sp. and *Bacillus* sp. strains have been isolated from oral secretions of spruce beetles and when tested inhibited the growth of potentially pathogenic fungi associated with beetles (Cardoza *et al*., 2006). These bacteria also affect nematodes and it was therefore assumed that the bacteria might serve as a potential defense against nematodes and fungi. In our assays, we showed that nematocidal *Bacillus* strains also inhibit *B. bassiana*, which may point to the potential protective role of these bacteria.

Recently, Weller and colleagues (2010) profiled the nematode community from *Geotrupes* dung beetles sampled from the Schönbuch forest, Tübingen (location where we also sampled). They found that these beetles are infected with several different nematode species from the genera including *Pelodera*, *Koerneria*, *Strongyloidea* and *Spirurida* as well as *Pristionchus* species. When *Geotrupes* beetles die microorganisms, such as bacteria and fungi, proliferate on the beetle cadaver. It is at this point that resident nematodes classified as 'necromenic', e.g. *Pristionchus* and not *C. elegans*, can exit from the dauer stage and feed upon this feast for development, growth and nutrition. In order to survive these toxic conditions then nematodes must be able to tolerate a wealth of toxic bacteria. *Pristionchus pacificus* is a member of the Diplogastrid family and *C. elegans* is part of the Rhabditidae family and are thought to have diverged over 280–430 MYA (Dieterich *et al*., 2008). *Pristionchus pacificus* has evolved the ability to tolerate and digest pathogenic bacteria such as *P. aeruginosa*, *S. aureus* (Rae *et al*., 2008), BT Cry 5B (Wei *et al*., 2003) and three strongly nematocidal strains of *B. cereus* (from this study). The dramatic expansion of the detoxification machinery in the *P. pacificus* genome relative to *C. elegans* points to nematode adaptation possible due to digestion of bacteria without grinder and/or presence in hostile beetle host environments. By using forward and reverse genetic tools, both of which are available in *P. pacificus*, the molecular mechanisms associated with this tolerance can be identified in future studies.

Experimental procedures

Nematode, bacteria and fungal maintenance

Nematodes (*C. elegans* N2 Bristol strain and *P. pacificus* RS2333 strain) were maintained on NGM (Nematode Growing Media) agar plates seeded with 200–300 μl *E. coli* OP50 and stored at 20°C. Individual *Bacillus* strains were grown overnight in 5 ml LB at 30°C. *Beauveria bassiana* was isolated from an infected cock chafer (*Melolontha* spp.) from Kaferwald near Karlsruhe, Germany and maintained on potato dextrose agar (PDA) at room temperature. *Caenorhabditis elegans bre-1*(*ye4*), *bre-2* (*ye31*), *bre-3*(*ye26*)), *daf-2*(*e1368*, *daf-12*(*m20*), *daf-16*(*m27*) and *age-1*(*hx546*), as well as *daf-16*(*mg54*); *daf-2*(*e1370*), *daf-2*(*m41*); *daf-12* (*m20*) and *daf-16* (*m26*); *age-1* (*m333*) double mutants were obtained from the *Caenorhabditis* Genetic Centre (CGC), Minnesota.

Soil/horse dung and Geotrupes *sp. sampling regime*

Dung beetles (*Geotrupes* sp.) were collected from the Schönbuch forest (Tübingen, Germany). Fresh horse dung heaps were excavated thoroughly and any dung beetles found were placed in non-airtight plastic tubes and immediately transported back to the laboratory. Samples of horse dung were also taken at this location. Soil samples were collected from surrounding agricultural farmland, grassland, rhizosphere of clover (*Trifolium* sp.), moss (*Polytrichum commune*), mixed coniferous woodland (*Abies* and *Picea* sp.), and from leaf litter from deciduous forest floor (mainly Ash, *Fraxinus* sp.). Soil samples were treated similarly to *Geotrupes* sp. collection. One hundred soil samples were collected from the UK from farmland, grassland, coniferous and deciduous forest and coastal habitats.

Soil/horse dung and Geotrupes *sp.* Bacillus *isolation*

Soil and horse dung samples (approximately 10–30 g) were mixed vigorously with PBS (Phosphate Buffered Saline) for 2 min. One millilitre of soil/buffer mix was then heated to 80°C for 10 min to kill all resident bacteria apart from heat resistant

Bacillus spores. Samples (50–100 μl) were then spread on LB plates and incubated overnight at 25°C. One hundred collected *Geotrupes* sp. were washed in PBS for 5 min to remove any adhering horse dung and then immediately chopped into small pieces using sterile scissors and mixed with 1–2 ml PBS. The resultant solution was then heated and treated as described above. After an overnight period of growth single *Bacillus* colonies were streaked onto fresh LB plates and in the following days could be used in nematode feeding assays, long-term storage procedures and DNA extraction. In total, we picked 768 strains of *Bacillus* compromising of 192 from four sampling types (soil from UK and Germany, horse dung and *Geotrupes* beetles).

Assays for assessment of Bacillus effects on nematodes

To assess the pathogenicity of *Bacillus* strains to nematodes 80 μl of overnight *Bacillus* cultures were spread evenly over the surface of six NGM plates and incubated at 25°C overnight. The following morning 20 L4 stage *P. pacificus* and *C. elegans* were placed onto three separate plates and survival was recorded daily for 5 days. Every 2 days nematodes were transferred onto fresh *Bacillus* NGM plates to prevent confusion in differentiating between tested worms and their offspring. This procedure was repeated for *C. elegans* Bre and Daf mutants. The survival of nematodes fed *E. coli* OP50 was also tested using the same procedures as a control. To test the effect of *Bacillus* affecting brood size of each nematode species 30 μl of each *Bacillus* was added to the middle of six NGM plates and left to grow overnight at 25°C. Three single virgin *C. elegans* and *P. pacificus* hermaphrodites were placed in the centre of the bacterial spot and placed in an incubator at 25°C. The number of offspring produced by each hermaphrodite was then counted after 4 days. Experiments were repeated twice.

Bacillus DNA extraction, sequencing and toxin gene profiling

Each *Bacillus* strain was grown overnight at 30°C in 5 ml LB Broth. *Bacillus* DNA was extracted using the MasterPure gram-positive DNA purification kit (Epicentre, Madison, USA). The PCR amplification of bacterial 16S rRNA gene was carried out in 20 μl reactions using primer set 27f (5′ AGAGTTTGATCMTGGCTCAG 3′) and 1492r (5′ TACGGYTACCTTGTTACGACTT 3′) (Lane, 1991) and also internal primers (Forward 5′ CGTGCCAGCAGCCGCGGTAATA CGTA 3′ and Reverse 5′ ACTCCTACGGGAGGCAGCAGT 3′. Thermal cycling conditions were as follows: 3 mins at 95°C followed by 35 cycles of 15 s at 95°C, 30 s at 55°C and 1.5 min at 72°C there was then a final step of 8 min at 72°C. Reactions consisted of 2 μl 10 ×PCR Buffer, 2 μl 2 mM dNTPs, 1 μl 10 μM 27f, 1 μl 10 μM 1492r, one unit of *Taq* DNA polymerase, 12.8 μl H_2O and 1 μl of bacterial DNA. The PCR amplicons were visualized by standard agarose gel electrophoresis (Sambrook *et al*., 1989). Products of *Bacillus* 16S rRNA gene were diluted 10–20 fold and added to the Big Dye terminator sequencing mix (Applied Biosciences, USA), which contained the sequencing primers previously used for initial amplification. Sequencing reactions typically contained 0.4 units Big Dye, 2 μl 5 × Sequencing Buffer, 1 μl primer (10 μM), 1 μl DNA (previously diluted) and 5.6 μl H_2O. Thermal cycling condition were thus: 96°C for 30 s, followed by 50 cycles of 96°C for 10 s, 50°C for 5 s and 60°C for 4 min. Resultant gene sequences of *Bacillus* strains were aligned using Seqman (DNA Star) and compared with GenBank database sequences using Blastn searches using sequence similarity matches at 90%.

Also the toxin genes of our 20 toxic *Bacillus* strains isolated in our study were profiled. Specifically, we looked for the genes *bceT, nhe*, *hbl*, *pcpl*, *sph* (*cerB*), *cytK*, *piplc*, *pcpl* (*cerA*), *hlyII*, *hlyIII*, *entFM* and *entS*, which have been implicated in outbreaks of food poisoning (Guinebretiere *et al*., 2002) (see Fig. 7A for primers used). A typical reaction for each toxin gene consisted of 2 μl 10 ×PCR Buffer, 2 μl 2 mM dNTPs, 1 μl of each primer (10 μM), 0.3 μl *Taq* and 12.8 μl H_2O. PCR conditions were used according to Hendriksen *et al*., (2006). The PCR amplicons were visualized to determine presence or absence of each toxin gene.

All gene sequences from 768 *Bacillus* sequenced were submitted to GenBank and can be accessed using accession numbers HM566450–HM567157.

Fungi Antagonism study by dual-culture plate method

Methods to study antagonistic properties of nematode pathogenic *Bacillus* strains exposed to *B. bassiana* were followed by Swain and Ray (2009). One 10-mm disk of pure culture of *B. bassiana* was placed at the centre of a Petri plate (10 cm) containing PDA. A circular line made with a 6 cm diameter Petri plate dipped in a suspension of *Bacillus* strains was placed surrounding the fungal inoculum. In total, 20 nematocidal strains were tested and also compared with 20 randomly picked non-nematocidal strains. Plates were cultured for 144 h at 25°C and growth diameter of the fungus was measured and compared with control growth where the bacterial suspension was replaced by sterile distilled water. Each experiment was run in triplicate and repeated at least two times. Results are expressed as means % inhibition ± S.D. of the growth of *B. bassiana* in the presence of any of the *Bacillus* isolates. Percentage of inhibition was calculated using the following formula: % inhibition = (1 – (fungal growth in the presence of *Bacillus*/control growth)) × 100.

Statistical analysis

Raw counts of survival of nematodes (*P. pacificus, C. elegans* wild type, Bre and Daf mutants) fed each *Bacillus* isolates was analyzed using Two way Analysis of Variance (ANOVA). Nematode fecundity and fungal inhibition were analyzed using a One-Way ANOVA and differences between means were analyzed using Least Significant Difference (LSD) after corrected for comparing multiple comparisons using the Bonferroni method. Unpaired and paired student *t*-tests were used to compare number of juveniles in fecundity assays of *C. elegans* and *P. pacificus*,comparing survival of Daf mutants and wild type *C. elegans* and also counts of *Bacillus* that show fungal suppression from nematocidal and non-nematocidal strains.

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Supporting information

Additional Supporting Information may be found in the online version of this article:

Table S1. Analysis of 16S rRNA sequences of 192 *Bacillus* isolates from soil from UK.
Table S2. Analysis of 16S rRNA sequences of 192 *Bacillus* isolates from soil from Schönbuch, Tübingen, Germany.
Table S3. Analysis of 16S rRNA sequences of 192 *Bacillus* isolates from horse dung from Schönbuch, Tübingen, Germany.
Table S4. Analysis of 16S rRNA sequences of 192 *Bacillus* isolates from *Geotrupes* dung beetles soil from Schönbuch, Tübingen, Germany.
Table S5. Identification of nematocidal *Bacillus* strains from German soil, horse dung and *Geotrupes* beetles based on analysis of 16S rRNA gene.
Table S6. Classification of nematocidal and non-nematocidal *Bacillus* that inhibit growth of the entomopathogenic fungus *B. bassiana*.

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System Wide Analysis of the Evolution of Innate Immunity in the Nematode Model Species *Caenorhabditis elegans* and *Pristionchus pacificus*

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Abstract

The evolution of genetic mechanisms used to combat bacterial infections is critical for the survival of animals and plants, yet how these genes evolved to produce a robust defense system is poorly understood. Studies of the nematode *Caenorhabditis elegans* have uncovered a plethora of genetic regulators and effectors responsible for surviving pathogens. However, comparative studies utilizing other free-living nematodes and therefore providing an insight into the evolution of innate immunity have been lacking. Here, we take a systems biology approach and use whole genome microarrays to profile the transcriptional response of *C. elegans* and the necromenic nematode *Pristionchus pacificus* after exposure to the four different pathogens *Serratia marcescens, Xenorhabdus nematophila, Staphylococcus aureus* and *Bacillus thuringiensis* DB27. *C. elegans* is susceptible to all four pathogens whilst *P. pacificus* is only susceptible to *S. marcescens* and *X. nematophila*. We show an unexpected level of specificity in host responses to distinct pathogens within and across species, revealing an enormous complexity of effectors of innate immunity. Functional domains enriched in the transcriptomes on different pathogens are similar within a nematode species but different across them, suggesting differences in pathogen sensing and response networks. We find translation inhibition to be a potentially conserved response to gram-negative pathogens in both the nematodes. Further computational analysis indicates that both nematodes when fed on pathogens up-regulate genes known to be involved in other stress responses like heat shock, oxidative and osmotic stress, and genes regulated by DAF-16/FOXO and TGF-beta pathways. This study presents a platform for comparative systems analysis of two nematode model species, and a catalog of genes involved in the evolution of nematode immunity and identifies both pathogen specific and pan-pathogen responses. We discuss the potential effects of ecology on evolution of downstream effectors and upstream regulators on evolution of nematode innate immunity.

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Introduction

The struggle against infectious diseases caused by bacteria, viruses, fungi, protozoa and metazoan parasites is an important evolutionary agent [1] leading to rapid evolutionary changes responsible for much of the complexity found in the immune system of animals [2–4]. However, the molecular basis for the evolution of such host-pathogen interactions is only poorly understood. Over the past ten years studies of the nematode *Caenorhabditis elegans* have given insight into genes essential for host immunity [5,6] as well as identifying bacterial virulence mechanisms used by opportunistic mammalian pathogens [7,8]. These studies (and many others) have identified various signaling pathways critical for *C. elegans* survival when fed an array of bacterial and fungal pathogens e.g. ERK MAP kinase, p38 MAP kinase, TGF β, programmed cell death, DAF–2/DAF–16 insulin-like receptor signaling and JNK-like MAP kinase [6,9–13], as well as components such as the G-protein coupled receptor FSHR-1, bZIP transcription factor *zip−2* and beta-Catenin/*bar−1* which are required for an inducible pathogen response [14–16]. It remains to be discovered however, how important these pathways are in other nematode species and how these pathways contribute to the evolutionary trajectories of bacterial pathogenicity. A comparative approach with another nematode species would provide a first entry point to enhance our understanding of the evolutionary diversity of host (nematode) response to pathogens.

One nematode that has been used extensively for comparative studies with *C. elegans* is the diplogastrid species *Pristionchus pacificus* (see [17]). In addition to having a well characterized proteome and a fully sequenced genome [18,19], forward and reverse genetics [20], and transgenic techniques [21], full genome microarray technology has also been developed [22] allowing genomic analysis of many different traits. *P. pacificus* diverged from *C. elegans* 250–400 million years ago [18] and during this time there have been remarkable changes in vulva development [23,24], gonad morphogenesis [25], sex determination [26] and chemosensory behaviour [27] allowing for evolutionary and developmental comparisons with *C. elegans*. These two nematodes also differ in their ecological niches. *C. elegans* can be isolated from compost heaps, snails and rotten fruits [28], whereas *P. pacificus* is usually isolated from a range of scarab beetles [29–32]. *P. pacificus*, as well as other *Pristionchus* species live in a necromenic lifestyle,

that is feeding on microorganisms growing on the carcass of beetles once they die [29].

C. elegans and *P. pacificus* not only live in different ecological niches, but also differ in their susceptibility to bacterial pathogens. For example, *C. elegans* dies when fed the human opportunistic bacteria *Pseudomonas aeruginosa*, *Staphylococcus aureus* and insecticidal *Bacillus thuringiensis* Cry 5B toxin whereas *P. pacificus* is resistant [33,34]. More recently, a screen of about 1,400 naturally strains of *Bacillus* yielded three strains of *Bacillus thuringiensis* DB27 that are extremely toxic to *C. elegans* but *P. pacificus* remain resistant [35]. Anatomically, *C. elegans* and *P. pacificus* differ in that *C. elegans* contains a grinder in the posterior bulb of the pharynx that is involved in the physical lysis of bacterial food [36]. While the grinder is a typical structure of nematodes of the Rhabditidae family, no grinder exists in nematodes of the Diplogastridae family, to which *P. pacificus* belongs [35–37]. Given these strong differences in the ecology and anatomy of *C. elegans* and *P. pacificus*, these two species represent ideal candidates for studying the evolution of the genetic mechanisms of pathogen response in nematodes.

Here, we used a systems level approach by testing in parallel four different bacterial pathogens that cause distinct effects on the two nematodes. We analyzed whole genome gene expression of *C. elegans* and *P. pacificus* when fed four different pathogens (*Serratia marcescens*, *Xenorhabdus nematophila*, *B. thuringiensis* DB27 and *S. aureus*) and compared each nematode pathogen response to those fed on the control bacterium (the standard nematode lab food *Escherichia coli* OP50). *S. marcescens* is a broad host pathogen present in soil and insects that kills *C. elegans* [8,38] and *P. pacificus*. *X. nematophila* is a symbiotic bacteria of the entomopathogenic nematode *Steinernema carpocapsae*, which utilizes the bacteria to kill insects and feed on the resulting mass of proliferating bacteria [39], and also kills both the nematodes [34]. Our *B. thuringiensis* DB27 strain was isolated from a dung beetle (*Geotrupes* spp.) and seems to be one of the most pathogenic bacteria of *C. elegans* reported in the literature so far, which kills *C. elegans* in less than sixteen hours while *P. pacificus* is resistant [35]. We show an unexpected level of specificity in host responses to distinct pathogens within and across species, revealing an enormous complexity of effectors of innate immunity. This study presents (i) a platform for comparative systems biology of two nematode models, (ii) a catalog of genes involved in the evolution of nematode immunity and (iii) pathogen specific and pan-pathogen responses from both *C. elegans* and *P. pacificus*.

Results and Discussion

Survival of *C. elegans* and *P. pacificus* differs when fed gram-positive pathogens

To study the evolution of the genetic mechanisms involved in nematode resistance against bacteria, we fed the four bacterial pathogens *S. aureus*, *B. thuringiensis* DB27, *S. marcescens* and *X. nematophila* to the two nematode model species *C. elegans* and *P. pacificus* and assessed their effect on survival. When *C. elegans* is fed monoxenic lawns of each of the four pathogens, it dies within 2–5 days (Median survival time <5 days, Figure 1A). This is in stark contrast to *P. pacificus*, which is more resistant to the gram-positive pathogens *B. thuringiensis* DB27 and *S. aureus* and can survive for more than 7 days (Figure 1B and Figure S1, Median survival times $\sim$ 8 days). However, *P. pacificus*, like *C. elegans*, is susceptible to both *X. nematophila* and *S. marcescens* and 50% of the population dies within 2–3 days exposure (Figure 1B). We would like to note here that the difference in susceptibility of *P. pacificus* to gram-positive bacteria tested in this study is not simply an artifact of a longer life-span, as its wild-type life-span and developmental rate is comparable to that of *C. elegans* ([17,40] and our unpublished observations). Also, *P. pacificus* is highly susceptible (median survival $\sim$ 3.5 days) to a gram-positive *Bacillus* strain DB35 isolated from *Geotrupes sp*. beetles [35], indicating that it is not more resistant to gram-positive bacteria in general. We also note that *P. pacificus* is able to survive and reproduce on both *B. thuringiensis* and *S. aureus* indicating that its reduced susceptibility should not be due to reduced bacterial intake.

Significant transcriptional changes after exposure to different bacteria

To investigate the transcriptional response of the two nematodes upon exposure to these different bacterial pathogens, we identified differentially expressed genes using whole genome microarrays containing $\sim$43,000 probes for 20,149 *C. elegans* genes and $\sim$90,000 probes for 20,987 genes in *P. pacificus* respectively [22]. For each condition, total RNA was collected from four separate biological replicates of about 200 synchronized young adult worms exposed either to the pathogen or to the control *E. coli* (OP50) for 4 hours. The labeled cRNA produced from this total RNA was hybridized to species-specific microarrays according to manufacturer's protocols, and the raw data from scanned images was analyzed using the "limma" package in Bioconductor (see Methods for details). We observed that the exposure to pathogen resulted in a decrease of amount of total RNA produced per worm when compared to the relatively non-pathogenic *E. coli* strain (Figure S2). This global decrease in transcription is most likely a common feature of a core stress response, as it is also seen in case of dauer formation in both the species [22,41] or might be an effect of the various bacteria on efficieny of RNA extraction. Nonetheless, such global transcriptional changes call for optimization of parameters used in normalization of microarray data, without which the calculated fold-changes can be erroneous [22,42]. Our fold-change calculations take these factors into account (see Methods for details).

Although previous studies of pathogen response in *C. elegans* [9,43–47] have looked at the transcriptome at different time-points such as 4-hours, 8-hours or 24-hours after exposure, we chose to investigate one of the earliest time-point of 4 hours because we were interested in earliest transcriptional responses manifested in response to each of these pathogens. Pathogenesis related necrosis of host-tissue at later time-points is a common effect of many pathogens and such organism-wide necrosis might dominate the expression profile at later time points [46], masking the pathogen specific signatures. Also, pathogens like *Bacillus thuringiensis* DB27 kill *C. elegans* in less than 24 hours, making analysis of late time-points unfeasible [35].

Widely different numbers of genes are found to be up- or down-regulated in *C. elegans* or *P. pacificus* upon exposure to different pathogens (Table 1A and 1B), indicating both a pathogen-specific and a nematode-specific component to these responses. In this context it is interesting to note that just a 4-hour exposure to pathogen is sufficient to cause large transcriptional changes in both the species, suggesting rapid activation of innate immune response.

Changes in nematode gene expression depend on lethality and rate of killing

Based on the absolute number of differentially expressed genes under different conditions of survival, lethality and slower or faster killing rates of pathogens, some patterns can be discerned in our microarray data. First, the number of differentially expressed genes seems to be inversely correlated with the survival characteristics of the nematodes. For example, upon exposure to

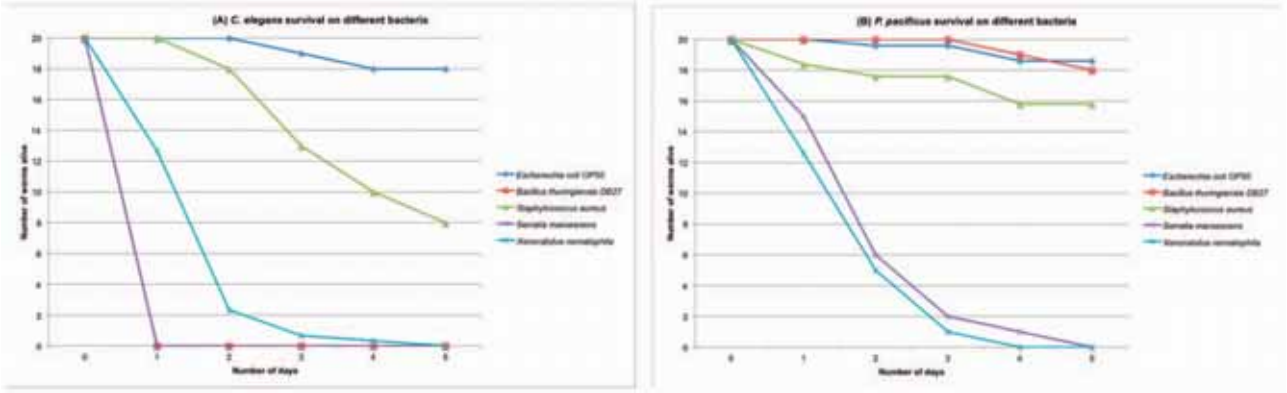

Figure 1. Differences in survival of *C. elegans* and *P. pacificus* upon exposure to different pathogenic bacteria. The survival of *C. elegans* and *P. pacificus* is different upon exposure to different bacteria. The survival curves for (A) *C. elegans* and (B) *P. pacificus* were obtained after exposure to the gram-positive bacteria *Bacillus thuringiensis* DB27 and *Staphylococcus aureus*, and the gram-negative bacteria *Serratia marcescens* and *Xenorhabdus nematophila*. Standard lab food *Escherichia coli* OP50 was used as a control for both nematodes. Both nematodes show reduced survival on *S. marcescens* and *X. nematophila*. *C. elegans* is also susceptible to *B. thuringiensis* DB27 and *S. aureus*, while *P. pacificus* shows higher resistance to these two bacteria.
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B. thuringiensis DB27, the pathogen most lethal to *C. elegans*, a remarkably large number of genes are affected in *C. elegans* (n = 5868, Table 1A), whereas much fewer genes (n = 217, Table 1B) are induced in *P. pacificus*, which is resistant to this pathogen. This can be attributed to the fact that while *P. pacificus* can use *B. thuringiensis* DB27 for food, *C. elegans* has to mount a robust response against a lethal pathogen.

For *C. elegans*, the number of differentially expressed genes is greater when exposed to faster-killing pathogens *B. thuringiensis* DB27, *X. nematophila* and *S. marcescens* as compared to that on *S. aureus*, where worms survive longer (Table 1A). Similarly, in *P. pacificus*, greater number of genes is differentially expressed on more lethal pathogens *X. nematophila* and *S. marcescens* (Table 1B) as compared to that on *B. thuringiensis* DB27 and *S. aureus* (Table 1B), to which *P. pacificus* is more resistant (Figure 1B).

Further, in *P. pacificus*, which is either more resistant to pathogens or shows slower mortality kinetics as compared to *C. elegans*, the expression profiles are observed to be usually smaller or just as large as that in *C. elegans*. We can rule out that these differences in profile size are due to potential differences in sensitivity of the two microarray platforms used, because we know from previous studies that our *P. pacificus* microarrays could detect differential expression of larger number of genes under different conditions such as dauer formation [22]. We also checked if changing the p-value cutoffs on microarray data abolishes the difference in profile sizes, but we find that the trend still holds (data not shown). Also, in *C. elegans* we have observed that when it is exposed to a non-pathogenic *Bacillus subtilis* strain for 4 hours, the number of genes differentially expressed is relatively low (~510 genes) [II, AS and RJS, unpublished observations]. Hence, the differences in profile size between *C. elegans* and *P. pacificus* are most likely biologically relevant and not just a technical artifact.

Gram-positive bacteria predominantly induce over-expression of genes while gram-negative bacteria cause transcriptional suppression

For both nematode species, the gram-positive bacteria tested induce up-regulation of relatively more genes as compared to down-regulation, while the reverse seems to be true for the gram-

Table 1. Widely different numbers of genes are differentially expressed in (A) *C. elegans* and (B) *P. pacificus* in response to the four bacteria.

(A) C. elegans	Up	Down	TOTAL	%Up	%Down
B. thuringiensis	5532	156	5688	97%	3%
S. aureus	181	68	249	73%	27%
S. marcescens	1465	4931	6396	23%	77%
X. nematophila	732	7884	8616	15%	85%
(B) P. pacificus	**Up**	**Down**	**TOTAL**	**%Up**	**%Down**
B. thuringiensis	156	61	217	72%	28%
S. aureus	178	140	318	56%	44%
S. marcescens	192	1007	1199	16%	84%
X. nematophila	848	4293	5141	16%	84%

The genes were called differentially expressed on microarrays if the FDR corrected p-value was less than 0.05 and the absolute value of fold changes was greater than 1.42 (corresponding to log2 fold change of 0.5 where log2(1.42) = 0.5).
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negative bacteria (Table 1). For example, in both the nematodes, exposure to the gram-positive pathogens *B. thuringiensis* DB27 and *S. aureus* causes induction of relatively more genes as compared to suppression (Figure 2, Table 1). On the other hand, exposure to the gram-negative pathogens *S. marcescens* and *X. nematophila* predominantly causes down-regulation of comparatively more genes than up-regulation (Figure 2, Table 1). Thus, the relative proportion of up-regulated versus down-regulated genes appears to depend upon some common factor(s) shared by either the gram-negative or the gram-positive bacteria, although more bacteria from both groups need to be tested to confirm this trend.

A bacterium-specific transcriptional response is mounted by both *C. elegans* and *P. pacificus*

Next, we investigated the intra-specific response of the two nematodes after exposure to different pathogens. Although *C. elegans* is unable to survive on any of the four pathogens, we find that the expression profiles on each of the pathogens are qualitatively quite different from each other and only a small fraction of genes are common between expression profiles obtained on different pathogens (Figure 3A). Specifically, only 102 genes change their expression upon exposure to each of the four pathogens (Figure 3A, genes with nCommon = 4 in Table S1) but the number slightly increases to about 687 genes when the criterion is relaxed to significant differential expression in more than one expression profile (genes with nCommon >1 in Table S1). A small number of overlap between multiple pathogen response profiles is a signature for highly specific pathogen response, and has also been observed before e.g. only 22 genes were reported to be common between profiles after 24 hour exposure to *Erwinia carotovara*, *Enterococcus faecalis*, and *Photorhabdus luminescens* [46]. Interestingly, the genes induced in *C. elegans* across all four pathogens include the transcription factors *pqm−1* and *zip−2* (Table S1). The stress responsive transcription factor *pqm−1* is also induced and required for defense in response to *P. aeruginosa* infection [43]. The bZIP transcription factor *zip−2* is a known to regulate a subset of *pmk-1* independent pathogen response genes on *P. aeruginosa* [15]. Similarly, the *P. pacificus* expression profiles also show a bacterium-specific signature, with only 18 genes being common across all the four profiles (n = 18, Figure 3B, genes with nCommon = 4 in Table S2) while 206 genes are common between the expression profiles on more than one pathogen (genes with nCommon >1 in Table S2).

This pathogen specific nature of expression profiles within a nematode species is further highlighted in an expression cluster analysis (see Methods), where we compare our data-sets with various published microarray studies of pathogen-response [12,43–50]. Based on the significance of overlaps between different microarray data sets (Table S3A and Table S4A for *C. elegans* and *P. pacificus* respectively), it is evident that only a small proportion of genes in each expression profile show an overlap with expression profiles on other pathogens.

We further evaluated the extent of similarities between different intra-specific expression profiles, by carrying out a two-dimensional hierarchical clustering [51] on log-fold change values of genes that were significantly differentially expressed on at least two bacterium (n = 687 genes in *C. elegans*, and n = 206 genes in *P. pacificus*, number of common 1:1 orthologs = 15, Table S1 and Table S2). Interestingly, in both *C. elegans* and *P. pacificus* heatmaps (Figure 4), the expression profile of *X. nematophila* response clusters separately from the profiles obtained in response to the other three bacteria, suggesting some differences in its pathogenicity mechanism(s) compared to other three bacteria. In summary, both *C. elegans* and *P. pacificus* show a bacterium-specific transcriptional response, with relatively few common genes being regulated across multiple bacteria in a given nematode.

Pfam domain enrichment analysis identifies a role for lipid metabolism and the detoxification machinery in pathogen response in *P. pacificus*

Identification of functional components in large gene-sets such as the pathogen response expression profiles can be better achieved by meta-analysis based on functional annotations rather than by searching for a gene-to-gene correspondence. Therefore, we tested for enrichment of various Pfam domains [52] in the proteins corresponding to the differentially expressed genes (see Methods for details) to see if common functional themes emerge despite only partially overlapping gene lists.

For *C. elegans*, we find the domain enrichment profile to be similar for all bacteria except *S. aureus*. The common domains include those related to Proteasome function, ATPase activity (AAA domain), DNA helicases with DEAD box and Helicase_C

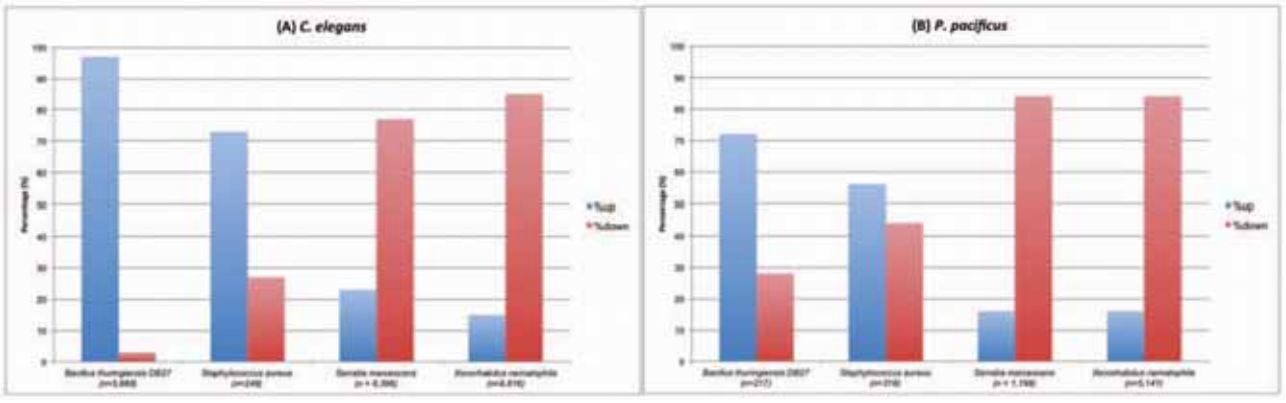

Figure 2. Gram-positive and gram-negative bacteria induce different proportions of up- versus down-regulated genes in *C. elegans* and *P. pacificus*. Despite the differences in number of genes differentially expressed on each of the pathogens in the two nematodes, the relative proportion of up-regulated genes is higher than that of down-regulated genes on gram-positive bacteria *B. thuringiensis* and *S. aureus* in both (A) *C. elegans* and (B) *P. pacificus*. On the other hand, exposure to the gram-negative bacteria *S. marcescens* and *X. nematophila* results in down-regulation of a greater fraction of genes as compared to the up-regulated genes in both (A) *C. elegans* and (B) *P. pacificus*.
doi:10.1371/journal.pone.0044255.g002

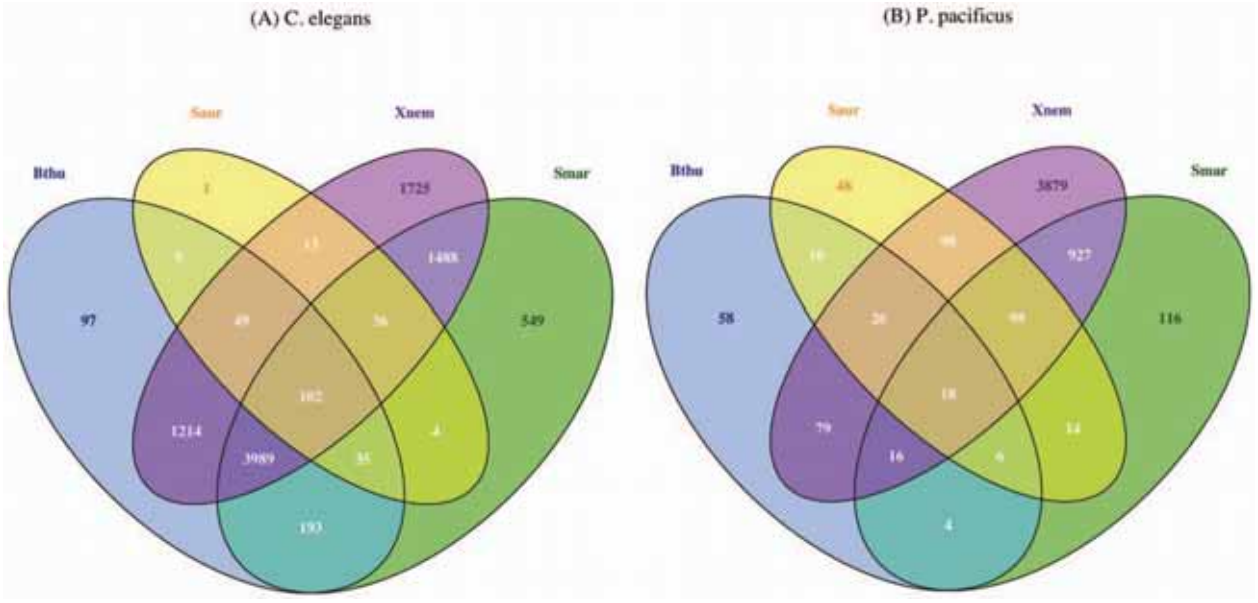

Figure 3. A pathogen-specific transcriptional response is mounted by both *C. elegans* and *P. pacificus* upon exposure to different bacteria. Overlap between the different genes differentially regulated in response to the four pathogens tested is represented as a Venn diagram for *C. elegans* and *P. pacificus*. Only 102 genes are found to be common between the expression profiles on all four pathogens in (A) *C. elegans*, while only 18 genes are common between the expression profiles corresponding to the four pathogens in (B) *P. pacificus*. This minimal overlap indicates the existence of a highly pathogen-specific immune response in both the nematodes. The abbreviations Bthu, Saur, Smar and Xnem refer to the bacteria *Bacillus thuringiensis*, *Staphylococcus aureus*, *Serratia marcescens* and *Xenorhabdus nematophila* respectively.
doi:10.1371/journal.pone.0044255.g003

domains, and the RRM_1 motif that is indicative of RNA binding protein activity (Table S5). Thus even though the gene-by-gene similarity is low between these expression profiles, we observe proteins with similar functional domains to be enriched in all of them. In *P. pacificus*, expression profiles for all four bacteria are enriched for various lipid metabolism related domains such as Lipase_GDSL, FA_desaturase, Acyl-CoA_dh_1 and Abhydro_lipase (Table S6), suggesting a role for lipid metabolism in *P. pacificus* immune response. Studies on *C. elegans* immune response have shown that the poly-unsaturated fatty acids gamma-linolenic acid and stearidonic acid are integral for immune response, acting via the p38 MAP Kinase pathway [53]. Lipases can potentially

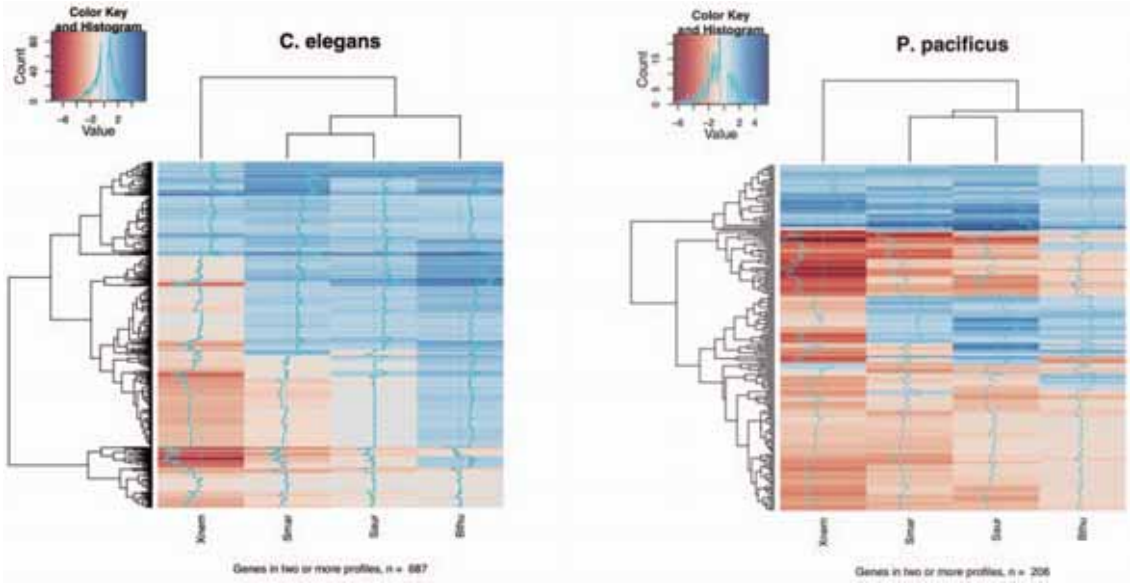

Figure 4. Hierarchical clustering of genes differentially expressed on more than one pathogen identifies clusters of co-regulated genes. The pathogen-response expression profiles for each nematode were clustered based on log2 of fold-changes for genes that were differentially expressed on at least two pathogens. In both (A) *C. elegans* and (B) *P. pacificus*, the expression profile in response to *X. nematophila* clusters separately from that in response to other pathogens, suggesting a difference in its mode of virulence from other pathogens.
doi:10.1371/journal.pone.0044255.g004

function as antagonists of invading pathogens [54] and are known to be induced in response to pathogens in both *C. elegans* [9] and *Drosophila melanogaster* [55,56]. We also see induction of lipase-like genes *lipl-1* and *lipl-3* across all pathogens in *C. elegans* (Table S1). Thus the enrichment of proteins containing lipase and related domains might contribute towards enhanced resistance of *P. pacificus* on some of the pathogens.

C- type lectins have been implicated in the *C. elegans* innate immune response [57] and in transcriptomic studies of exposure to *P. aeruginosa*, *M. nematophila* and *S. marcescens* [9,43–46,58]. Although C-type lectin domain encoding genes were differentially expressed when either *C. elegans or P. pacificus* was fed our four pathogens, the enrichment for the corresponding Pfam domain "Lectin_C" achieved statistical significance only in *P. pacificus* profiles on all pathogens except for that on *S. aureus* (Table S6).

The Pfam domains enriched in *P. pacificus* upon response to the relatively less pathogenic bacteria *S. aureus* as well as on exposure to the highly pathogenic *X. nematophila* also include various domains involved in detoxification and xenobiotic defense, such as Glucuronosyltransferase (UDPGT), Glutathione S-transferase (GST_C) and Cytochrome P450 domain (Table S6), which have been previously identified in expression studies of *C. elegans* exposed to xenobiotic compounds [59]. Interestingly, these domains have undergone an expansion in *P. pacificus* genome relative to the *C. elegans* genome and have been hypothesized to have adaptive significance in context of its necromenic lifestyle [18]. Here for the first time we show that the gene activity for the proteins containing these domains is enriched in a potentially pathogenic scenario and possibly confers an adaptive advantage.

Taken together, the Pfam domain analysis provides further insights into the pathogen response of the two nematodes. We observe similar Pfam domains to be enriched within a given nematode in response to different pathogens, but the set of enriched domains differs between *C. elegans* and *P. pacificus*, such that apart from the DNA helicase domain Helicase_C, we hardly find any other domains common between *C. elegans* and *P. pacificus*, even in response to the same bacteria.

Inhibition of translation machinery is a conserved effect of exposure to gram-negative pathogens in both *C. elegans* and *P. pacificus*

Since *C. elegans* and *P. pacificus* have very different survival behavior on the bacteria tested, we wanted to identify genes whose expression might be responsible for these differences. For comparing the expression profiles across the two nematode species, we restricted our analysis to the 6,126 1:1 orthologous pairs defined by the stringent best reverse BLAST method, for which the probes were present on both the microarrays (see Methods). Interestingly, we see different patterns of overlap between the expression profiles of the two nematodes depending on the bacteria tested. For the gram-positive bacteria *B. thuringiensis* DB27 and *S. aureus*, which kill *C. elegans* at a much higher rate than *P. pacificus*, we observe a very limited overlap in the expression profiles of the two species (Figure 5A and Figure 5B). It was *a priori* not clear whether the *P. pacificus* resistance to *B. thuringiensis* and *S. aureus* is due to induction of similar genes as in *C. elegans*, albeit at higher expression levels, or, if the activation of a totally different set of genes causes the resistance phenotype. The surprisingly small extent of overlap observed in our comparisons supports the second model. Since most of the genes induced in *P. pacificus* on gram-positive bacteria do not have a characterized function yet, future studies will shed light on their role in innate immunity.

In contrast, we observe a highly significant overlap between the *P. pacificus* and *C. elegans* expression profiles in response to the gram-negative bacteria, *S. marcescens* and *X. nematophila*, which are lethal to both the nematodes (Figure 5C and Figure 5D). This suggests that either these gram-negative bacteria induce a similar immune response in both the nematodes, or that late and secondary markers of pathogenesis dominate the expression profile related damages in both the nematode species.

Exposure to pathogens can be expected to affect germline development and reproduction, which might contribute to the set of differentially expressed genes. Consistent with this expectation, we see enrichment of oocyte and germline related expression clusters [60,61] in both *C. elegans* and *P. pacificus* profiles on various pathogens (see Tables S3 and S4, clusters "cgc6390:oogenesis-enriched", "WBPaper00037611:RNP-8-associated" and "WBPaper00037611:GLD-2-associated"). The overlap is strongest with the genes downregulated in response to the most lethal pathogens *S. marcescens* and *X. nematophila*.

To identify the conserved elements of the pathogen response in the two species, we focused on the gram-negative pathogens *S. marcescens* and *X. nematophila*, which are lethal to both the nematodes. Almost all the genes common between the two nematodes (Figure 5C and Figure 5D) show a downregulation in response to each of these bacteria (Tables S7A and S7B). We further found 410 genes to be common across both two nematodes on both the gram-negative pathogens, most of them being downregulated (Table S7C). Interestingly, the stress responsive transcription factor encoded by *pqm-1* was differentially expressed across all the four expression profiles (Table S7C), suggesting a potential and conserved role in innate immunity [43] across the two nematode species. Gene Ontology based analysis (Table S8) on any of these three lists shows an enrichment for biological processes related to "determination of adult lifespan", as well as processes related to protein translation such as "translational elongation", "translational initiation" and "ribosome biogenesis". The corresponding terms under the ontology molecular function include "structural constituent of ribosome", "translation elongation factor activity", and "translation initiation factor activity", and the enriched "cellular component" terms include "ribosome", "ribonucleoprotein complex" and "small ribosomal unit". Interestingly, it has recently been shown that inhibition of essential cellular processes like translation activates pathogen defense in *C. elegans* [62] and the gram-negative pathogen *Pseudomonas aeruginosa* response in *C. elegans* is activated by detecting its inhibitory effects on translation machinery [63,64]. Downregulation of components of translational machinery and ribosomes was also observed upon germline-ablation of *P. pacificus* that exhibited an increase in lifespan as well as resistance to the pathogen *S. marcescens* [66]. Hence our data together with these recent studies suggest that that downregulation of translation machinery could be a conserved response across the two nematodes, at least when exposed to gram-negative pathogens. We also find enrichment for genes involved in "proteasome complex" and "nuclear pore" complex, and these cellular components are known to have a potential role in immune response in *C. elegans* and *P. pacificus* longevity and immunity [62,65,66]. Other enriched processes commonly affected across the two nematodes include various processes related to metabolism, such as "glycolysis", "tricarboxylic acid cycle" and "fatty acid metabolic process" (Table S8) and cellular compartment GO terms such as "mitochondrial membrane" and "mitochondrial proton-transporting ATP synthase complex" (Table S8). These results suggest that exposure to a pathogens leads to similar changes in the metabolic activity of the two nematodes.

It should also be noted that apart from the overlap between *C. elegans* and *P. pacificus*, differential expression of a substantial number of 1:1 orthologs is specific to each of the nematode species.

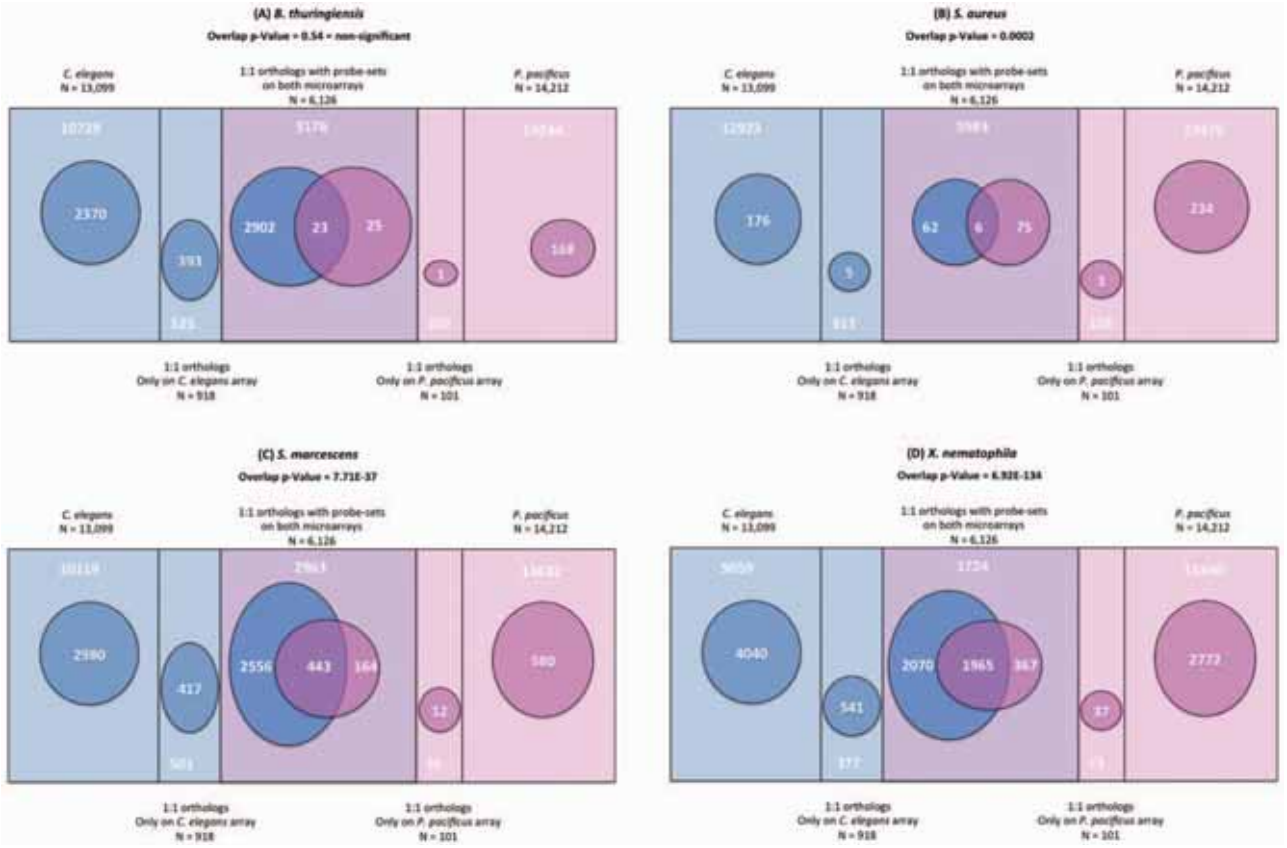

Figure 5. Overlap between *C. elegans* and *P. pacificus* expression profiles in response to different bacteria. The rectangular boxes represent the entire transcriptomes of *C. elegans* and *P. pacificus* genes assayed on our microarrays, and their area of overlap represents the set of 6,126 1:1 orthologs present on microarrays of both the nematodes. The ovals represent the fraction of differentially expressed genes in each of the subsets. For the 1:1 orthologs, we assessed the significance of overlap between the genes differentially expressed in response to a given pathogen using a 2×2 Fisher's exact test. Differences or similarities in survival characteristics of the two nematodes when exposed to the same bacteria are reflected in their respective transcriptional responses. (A) On *B. thuringiensis* DB27, which is highly lethal to *C. elegans* but not to *P. pacificus*, only 23 genes are common between the respective expression profiles of the two nematodes, this overlap being statistically not significant. (B) Similarly, *S. aureus* is more lethal to *C. elegans* than *P. pacificus*, and the overlap between the corresponding expression profiles is limited to just 6 orthologs. Although this overlap is statistically significant (p-value = 0.0002), the extent of overlap is too small to be biologically significant. (C) *S. marcescens* is lethal to both the nematodes and the extent and significance overlap between the orthologs differentially expressed in the corresponding expression profiles is also relatively high (443 common orthologs, p-value = 7.71E–37). (D) On the pathogen *X. nematophila*, the observed overlap between the expression profiles in the two nematodes is even higher, with 2,093 orthologs regulated in both nematodes (p-value = 6.92E–134).
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Additionally, the portion of the transcriptome with unresolved or no sequence similarity across the two species (Figure 5, rectangular areas specific to either *C. elegans* or *P. pacificus*) is also a significant contributor to the transcriptional response to the pathogens. Hence, based on these analysis of genes in the two nematodes exposed to the same gram-negative bacteria, it seems reasonable to conclude that some effectors of innate immunity are conserved across the two nematodes while some have diverged considerably during the last 250–300Mya separating the two nematode lineages.

Expression cluster based analysis identifies role for DAF-16, TGF-beta and p38 MAP Kinase pathways in pathogen response

To identify potential upstream regulators of immune response in the two nematodes, we tried using the existing knowledge from *C. elegans* to investigate what pathways appear to be mis-regulated. We therefore assessed the significance and the extent of overlap of our gene-sets with published microarray data sets available as "Expression Clusters" from WormBase [67] as well as with manually curated gene expression data from published microarray studies that were not available in WormBase (see Methods for details). These annotations were transferred to *P. pacificus* genes via the 1:1 orthology relations.

In agreement with the role of DAF-16 in innate immunity [68,69], we find DAF-16 targets to be enriched in genes up-regulated in *C. elegans* response to all pathogens except *B. thuringiensis* DB27 (cluster Murphy_etal_cgc5976_Class1 in Table S3B), while the DAF-16 repressed genes are over-represented in the set of down-regulated genes on *B. thuringiensis* and *S. aureus* (cluster Murphy_etal_cgc5976_Class2, Table S3B). Similarly, TGF-beta targets regulated by the ligand DBL-1 [70] are also enriched in many of our expression profiles (clusters "Roberts_etal_2010_DBL-1-UP" and "Roberts_etal_2010_DBL-1-DOWN", Table S3B), confirming an important role of TGF-beta pathway in response to specific pathogens [9,70].

Different MAP kinase pathways such as p38 MAPK and JNK pathways play a key role in *C. elegans* innate immunity and stress

response [12,49]. Consistent with this, we also see a significant overlap with genes regulated by the MAPKK SEK-1 and the JNK-like MAPK KGB-1, especially with down-regulated genes in all pathogen profiles (e.g cluster "Kao_etal2011_sek1_regulated", Table S3B). We also observe a robust induction of starvation response genes [71] (e.g. cluster "WBPaper00032948:StarveUp3", Table S3C) within just 4 hours of pathogen exposure, a reasonably short time not expected to induce actual starvation. This observation highlights the importance of metabolism related pathways in immune response [46,72–75]. We also observe enrichment of various dauer related gene clusters and other clusters regulated by stress such as heat shock and oxidative stress in some of the *C. elegans* profiles (Table S3C).

Somewhat similar patterns of overlaps with *C. elegans* expression clusters are also seen for *P. pacificus* pathogen response profiles (Table S4). Interestingly, unlike *C. elegans*, the clusters of genes regulated in response to Cry5B toxin and KGB-1 or SEK-1 MAP kinases show a significant overlap only with genes down-regulated upon exposure of *P. pacificus* to *S. marcescens* and *X. nematophila* (e.g. cluster "Kao_etal2011_sek1_regulated", Table S4B) but not with genes up-regulated in *P. pacificus*. This suggests potential differences either in targets of the MAPK pathways, or differences in mechanism of activation of these pathways, which might account for enhanced resistance of *P. pacificus*.

C. elegans DAF-16 targets are enriched in some *P. pacificus* profiles (cluster "Murphy_etal_cgc5976_Class2" in Table S4B), suggesting that DAF-16 might have a conserved role in innate response in both the species, at least on some pathogens. Interestingly, compared to *C. elegans* profiles, all *P. pacificus* profiles show a significant and more extensive overlap with genes involved in osmotic stress response [76] (e.g. cluster "WBPaper00035873:-osmotically_regulated", Table S4C), suggesting that osmotic regulation could be an important survival mechanism against potentially pathogenic bacteria [76].

Differential expression of *P. pacificus* pioneer genes

About 30% of the predicted transcriptome of *P. pacificus* is comprised of "pioneer genes", which do not show any detectable homology to the known protein universe [19] and whose functions are not known. We investigated their potential role in pathogen response by looking at their expression data. We indeed find 832 of these pioneer genes to be differentially expressed *P. pacificus* in a pathogen specific manner, with 160 genes being regulated on at least two pathogens (Figure S3 and Table S9). On each of the pathogens, the pioneer genes constitute 12% to 18% of the active transcriptome, significantly less than the expected proportion of about 30% (Fisher's 2×2 exact test p –values$<$0.001, Figure S4). Interestingly however, we find the differential expression levels of these pioneer genes to be significantly higher than the non-pioneer fraction of the respective transcriptomes (Figures S5A, S5B, S5C and S5D, Kolmogorov-Smirnov test p-values less than 2.00E–16 for all four pathogens), indicating specific increase in their expression levels after exposure to pathogens. These observations together suggest that some of these lineage specific genes might have been acquired for adaptation to a microenvironment populated by different set of bacteria, some of which might be pathogenic. We can thus ascribe a putative role for these pioneer genes in pathogen response, although further studies will be needed to test these predictions.

An ecological perspective on the evolution of effectors and regulators of nematode immunity

Our finding that *C. elegans* mounts a pathogen-specific transcriptional response is in agreement with the current understanding in the field [46,47,58]. We further show for the first time that the nematode *P. pacificus* can also activate a pathogen-specific response. Many evolutionary mechanisms contribute towards generating this specificity in invertebrates, which lack an adaptive immune system. These include high genetic diversity receptors and effectors involved in pathogen recognition [77], evolutionary diversification of innate immunity effectors e.g. C-type lectins [57], lysozymes [78] and *nlp*- family of antimicrobial effectors [79], natural variation in host susceptibility and virulence of the pathogen [35,38,80], and evolution of mechanisms such as recombination and sexual reproduction [81] or alternative splicing [82,83], all of which facilitate generation of genetic diversity.

The ecology of the organism is expected to be one of the key driving forces behind these changes, as the related species or even strains that occupy different ecological niches will be exposed to different set of non-pathogenic and pathogenic microbes and will need different strategies to survive. Due to these differences in selective pressures, the effectors of their immune systems can be expected to diverge rapidly and also affect the evolution of the host genomes.

The differences observed between response of *C. elegans* and *P. pacificus* can thus be best explained in the light of the distinct ecological niches occupied by both species. While *C. elegans* has recently been isolated from rotting fruit [28], *Pristionchus* nematodes and *P. pacificus* have a strong association with scarab beetles [29–32]. Once the beetle dies bacteria proliferate on the rotting carcass allowing mass growth of *Pristionchus* nematodes. Using a metagenomic approach we have previously shown that hundreds of plant and animal pathogenic bacteria occur on and in *Pristionchus* nematodes emerging from beetles [34]. Thus, *Pristionchus* is naturally exposed to a variety of bacteria and has evolved mechanisms to combat infections. Relative to *C. elegans*, the *P. pacificus* genome contains a larger set of genes encoding for cytochrome P450 and UDP-glucoronosyl/UDP-glucosyl transferases, which are required for coping with xenobiotic compounds [18], and we show here that differential activation of these gene families might contribute to its higher resistance to pathogens. The limited overlap on a gene-by-gene between expression profiles on different pathogens combined with the observation that similar Pfam domains are enriched within a nematode species, are consistent with evolutionary diversification and expansion of genes containing these functional domains.

Conclusions

This study provides a system wide analysis of the transcriptomic responses of the two nematode model species *C. elegans* and *P. pacificus* when feeding on four well-characterized bacterial pathogens. Studies on natural variation in the response of *C. elegans* to pathogens have contributed to micro-evolutionary studies of evolution of innate immunity. By adding studies on host-pathogen interactions in *P. pacificus*, we have tried to extend the evolution of innate immunity towards a macro-evolutionary perspective. We have studied nematode response as early as four hours after exposure to bacteria in order to capture initial events. While many previous studies have looked at various time points (4 h, 8 h, 24 h etc), it is known that by this time a common host necrotic response sets in [46]. The data generated for *P. pacificus* is the first of its kind, whereas our *C. elegans* dataset overlaps, in part, with previous studies. We performed these experiments *de novo*, rather than taking data from the literature, to rule out the effect of differences due to the microarray platform. Our *C. elegans* dataset is however, in strong agreement with existing datasets (e.g. [44,46,47]. This study fulfilled three major aims. First, it presents a platform for comparative systems biology analysis of two nematode model

species. Second, it generates a catalog of genes involved in the evolution of nematode immunity and finally, it identifies pathogen-specific as well as pan-pathogen, conserved responses in the two nematode species.

Research on *C. elegans* and its interactions with bacteria has lead to the identification of several pathways involved in innate immunity [84]. By using an alternative nematode model we have expanded on this knowledge and shown conservation as well as divergence in the transcripts regulated during an immune response when fed different pathogens. Our systematic comparisons of nematode survival and gene expression on multiple pathogens highlights the fact that substantial differences exist in the repertoire of genetic components deployed in response to varied pathogens between *C. elegans* and *P. pacificus*. The resulting catalogs of pathogen specific and pan-pathogen genes provide an entry point to study the mechanism and evolution of individual response genes in future studies. Using expression cluster analysis we could show that homologs of known targets of FOXO/DAF-16, TGF-beta and p38 MAP kinase pathways in *C. elegans* are also significantly enriched in *P. pacificus*, suggesting that the key signaling pathways might regulate innate immunity in both the species. Given the lack of corresponding mutants in the relatively new model system *P. pacificus*, future studies are needed to test this hypothesis.

Evolutionary studies will have to involve more closely related species and strains given the strong differences observed for *C. elegans* and *P. pacificus*. From a *P. pacificus* perspective, more careful analyses of individual genes in additional strains and closely related *Pristionchus* species will be necessary to obtain insight into the evolution of immunity-related gens. Finally, these results argue for the importance of a comparative approach towards uncovering mechanistic details of the genetic basis that accounts for the cross-species variation in susceptibility to a given pathogen.

Materials and Methods

Strains

C. elegans N2 and *P. pacificus* RS2333 were maintained on NGM plates seeded with *E. coli* OP50. *S. marcescens* was isolated from La Reunion, *S. aureus* Newman was purchased from the Deutsche Sammlung von Mikroorganismen und Zellkulturen, Germany (DSMZ), *X. nematophila* was a gift from Becker Underwood, U.K. *B. thuringiensis* DB27 was isolated from dung beetles [35] and was initially thought to be a strain of *B. cereus* but further sequence analysis has shown it is in fact a strain of closely related *B. thuringiensis*.

Assessing survival of *C. elegans* and *P. pacificus* exposed to pathogens

Each bacterium was grown in a shaking incubator at 30°C overnight in LB, apart from *S. aureus*, which was grown at 37°C. The following day 100 µl were spread onto previously dried 6 cm NGM plates and incubated overnight. Twenty adults of C. elegans or *P. pacificus* were separately placed onto 3 NGM plates per bacterium and stored at 25°C where survival was monitored daily for 7 days. The experiment was repeated twice. Pathogen survival was compared to worms cultured on *E. coli* control plates and differences in survival were analyzed using Kaplan Meier and logrank test.

RNA collection for microarray experiments

Synchronized populations of *C. elegans* or *P. pacificus* were obtained by hypochlorite treatment and allowed to grow to young adult stage on *E. coli* OP50 plates at 20°C. For each biological replicate, about 200 young adult hermaphrodites were picked onto pathogen plates for 4 hours of pathogen exposure, after which they were collected into 1mL of TRIzol (Invitrogen). Equal number of corresponding age-matched control worms were exposed to *E. coli* OP50 for 4 hours and transferred to 1ml TRIzol (Invitrogen). Four biological replicates were collected for each experimental and control condition. Total RNA was extracted using TRIzol reagent (Invitrogen) according to manufacturer's instructions. The isolated RNA was further purified using phenol: chloroform: isoamyl alcohol precipitation to remove trace left-overs of TRIzol etc. which might interfere with downstream reactions. The RNA pellet was suspended in RNAse free water and was assessed on a Nanodrop spectrophotometer for quantity and RNA quality. RNA samples were stored at −80°C until the microarray experiments.

Microarray experiments

A total of 32 microarray hybridizations were carried out for 8 conditions (2 nematode species x 4 pathogenic bacteria; 4 biological replicates per condition). Oligonucleotide microarrays for *C. elegans* containing ~43,000 unique probes for ~20,000 *C. elegans* genes were obtained from Agilent Technologies (NCBI GEO accession GPL10094). For *P. pacificus* experiments, we used our custom designed oligonucleotide microarrays manufactured by Agilent Technologies, which contain ~93,000 unique probes for the ~23,000 *P. pacificus* predicted genes (NCBI GEO accession GPL14372, see [22] for design details of custom microarrays). The *P. pacificus* gene sequences are available at http://www.pristionchus.org/download/ and the gene models can be seen in the genome browser at http://www.pristionchus.org/cgi-bin/genome.pl.

Equal amounts of total RNA (500 ng to 800 ng) from four biological replicates of each experimental and control samples was used to produce Cy5 or Cy3 dye labeled cRNA using Quick Amp Labelling Kit (Agilent Technologies Inc., USA) as per manufacturer's instructions. Depending upon the amount of total RNA used, appropriate amounts of positive control RNA (Spike Mix-A and Spike Mix-B, from Agilent Technologies) was added to the mix before reverse transcribing the total RNA, as per manufacturer's instructions. We used the *C. elegans* or *P. pacificus* microarrays in a two-color format where Cy5 and Cy3 dye labeled cRNA from experimental and control sample is co-hybridized on the same microarray. The four biological replicates per experiment included two dye-swaps experiments to account for differences in dye labeling. Hybridization and washing of the arrays was carried according to manufacturer-supplied protocol. The arrays were scanned on a GenePix 4000B Microarray Scanner, and raw data extracted using GenePix Pro sofware (version 6).

Microarray data analysis

We used the Bioconductor [85] package limma [86] for analysis of our microarray data. Array quality was checked for parameters such as uniform background and foreground intensities over the entire array. The raw signal was background corrected using the normexp method [87] and the arrays were then lowess normalized individually ("normalizeWithinArrays" option), with differential weights assigned to probes and to positive control spike-ins, which are expected to show no fold change [88]. This differential weighing of probes is particularly necessary to account for differences in differences in relative proportion of mRNA versus total RNA, and/or differences in the amount of RNA produced per worm under different experimental conditions. Without this differential weighing scheme, the fold change calculations can be erroneous [22,42]. The weight parameters were optimized based on MA-plots such that spike-in controls show their expected fold change values. lmFit function was used to fit a linear model to

probe intensities across arrays, differential expression was calculated by empirical Bayes method using the eBayes function [89], and control of FDR was employed as the multiple testing correction. MA-plots were also used as diagnostic to identify and remove outlier arrays before fold-change calculations, such that at least three biological replicates were used for each experiment. Genes with a FDR corrected p-value less or equal to than 0.05 and absolute log2 of fold change greater than 0.5 were called significantly differentially expressed. Further data analysis was carried out using custom scripts in Perl and the statistical package R. Venn diagrams were drawn using the R package VennDiagram [90]. Raw and processed data from all the experiments from this publication have been deposited in a MIAME compliant format [91] at NCBI's Gene Expression Omnibus database (http://www.ncbi.nlm.nih.gov/geo/). The accession numbers for *C. elegans* data are GSE36413, GSE36493, GSE36499, GSE36501, and the accession numbers for *P. pacificus* data are GSE36517, GSE36519, GSE36521 and GSE36523.

Identifying 1:1 orthologs between *C. elegans* and *P. pacificus*

We have previously used a pairwise best BLASTP strategy to identify 7,176 pairs of 1:1 orthologs between the *C. elegans* and *P. pacificus* [22]. Briefly, all protein sequences from *C. elegans* were run as query versus the database of *P. pacificus* gene predictions and vice versa. Only hits with a BLAST score $>=50$ bits were retained, and mutually best hits were identified as 1:1 orthologs. Probes for 6,126 of these gene pairs exist on microarrays of both the species.

Pfam domain annotation and enrichment analysis

Pfam domain annotations for *C. elegans* and *P. pacificus* proteomes (WS220 and predicted proteins respectively) were the same as described before [22]. Basically, hits with a p-value cut-off of 0.001 in HMM searches using HMMer 3.0 [92] on Pfam release V23/4 [52] were used as domain annotations. Only the domains, for which minimum 5 protein coding genes were represented on each microarray, were used for further enrichment analysis. Statistical significance of enrichment of Pfam domains in each expression profile determined using a 2×2 Fishers exact test, at a FDR corrected p-value cut-off of 0.05.

Expression cluster enrichment analysis

We have used "expression cluster" annotations from WormBase [67] in interpretations of other microarray expression profiles [22]. The list of microarray expression profiles in which a given *C. elegans* gene is known to be differentially expressed can be extracted from the section "Expression Cluster" from the WormBase gene summary page for each gene. We retrieved all available expression clusters for *C. elegans* genes from the WormBase web site. We also compiled data from other gene expression studies in *C. elegans* which are relevant to pathogen response but for which the corresponding expression clusters were not available in the WormBase (viz. [12,49,70]) and named these clusters with a prefix based on first author's last name and year of publication of the research article, and included them in our analysis. We inferred expression clusters for *P. pacificus* based on the set of 1:1 orthologs. P-values for expression cluster enrichment in each expression profile was computed with a 2×2 Fisher exact test. FDR corrected p-value cut-off of 0.05 was used as the significance threshold. The significance score was calculated as –log10 of the p-values and was set to zero to indicate non-significance when p-values was greater than 0.05.

Gene ontology analysis, prediction of signal peptide and antimicrobial activity

Gene ontology analysis (presented in Table S8) was done using Bioconductor tool topGO, using method "elimFisher" for calculating p-values [93]. For analyzing features of differentially expressed pioneer genes in *P. pacificus* (Table S9), SignalP tool was used to predict the presence of a signal peptide [94], and for genes coding for products smaller than 100 amino acids, CAMP tool [95] was used to predict whether they can act as potential Anti-Microbial Peptides (AMPs).

Supporting Information

Figure S1 Long-term survival curves for *P. pacificus* on various pathogens. *P. pacificus* has higher resistance than *C. elegans*, with longer median survival time of about 8 days on *B. thuringiensis* and *S. aureus*.
(PDF)

Figure S2 Global transcriptional suppression in response to pathogens. Exposure to pathogens resulted in a decrease of amount of total RNA produced per worm when compared to the non-pathogenic *E. coli* strain. This global decrease in transcription is seen in both (A) *C. elegans* and (B) *P. pacificus*.
(PDF)

Figure S3 Overlap between pioneer genes regulated in *P. pacificus* in response to the four pathogens. Of the 832 pioneer genes differentially expressed on any of the pathogens in *P. pacificus*, 160 genes are common between two or more than two expression profiles.
(PDF)

Figure S4 Relative proportions of pioneer genes versus non-pioneer genes in the active transcriptome of *P. pacificus* on each of the four pathogens. The *P. pacificus* genome contains about 30% pioneer genes. Compared to the random expectation of the same proportion of pioneer genes in different expression profiles, they are found to significantly under-represented (Fisher's test p-values$<$2E-16 for each comparison with whole-genome distribution.
(PDF)

Figure S5 Pioneer genes are expressed at higher levels than non-pioneer genes in each of the pathogen-induced expression profiles on *P. pacificus*. Cumulative distributions of fold-changes of pioneer genes (red curves) and non-pioneer genes (blue curves) for genes differentially expressed on (A) *B. thuringiensis* DB27 (B) *S. aureus* (C) *S. marcescens* and (D) *X. nematophila*. The Kolmogorov-Smirnov test p-values are less that 2E-16 in each case.
(PDF)

Table S1 Differential expression of genes in *C. elegans* exposed to different pathogens.
(XLSX)

Table S2 Differential expression of genes in *P. pacificus* exposed to different pathogens.
(XLSX)

Table S3 Expression clusters enriched in genes up- and down-regulated in *C. elegans* upon exposure to each of the four pathogens.
(XLSX)

Table S4 Expression clusters enriched in genes up- and down-regulated in *P. pacificus* upon exposure to each of the four pathogens.
(XLSX)

Table S5 Pfam domains enriched in *C. elegans* expression profiles in response to different pathogens.
(XLSX)

Table S6 Pfam domains enriched in *P. pacificus* expression profiles in response to different pathogens.
(XLSX)

Table S7 Genes common across expression profiles of *C. elegans* and *P. pacificus* upon exposure to the gram-negative bacteria (A) *S. marcescens* (B) *X. nematophila*, and (C) common to both *S. marcescens* and *X. nematophila* expression profiles.
(XLSX)

Table S8 GO enrichment analysis of genes common across both *C. elegans* and *P. pacificus* profiles obtained upon exposure to gram-negative bacteria *S. marcescens* and *X. nematophila*.
(XLSX)

Table S9 Features of pioneer genes differentially expressed on any of the four pathogens in *P. pacificus*.
(XLSX)

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Author Contributions

Conceived and designed the experiments: AS RR II RJS. Performed the experiments: AS RR II. Analyzed the data: AS. Contributed reagents/materials/analysis tools: AS RR II. Wrote the paper: AS RR RJS.

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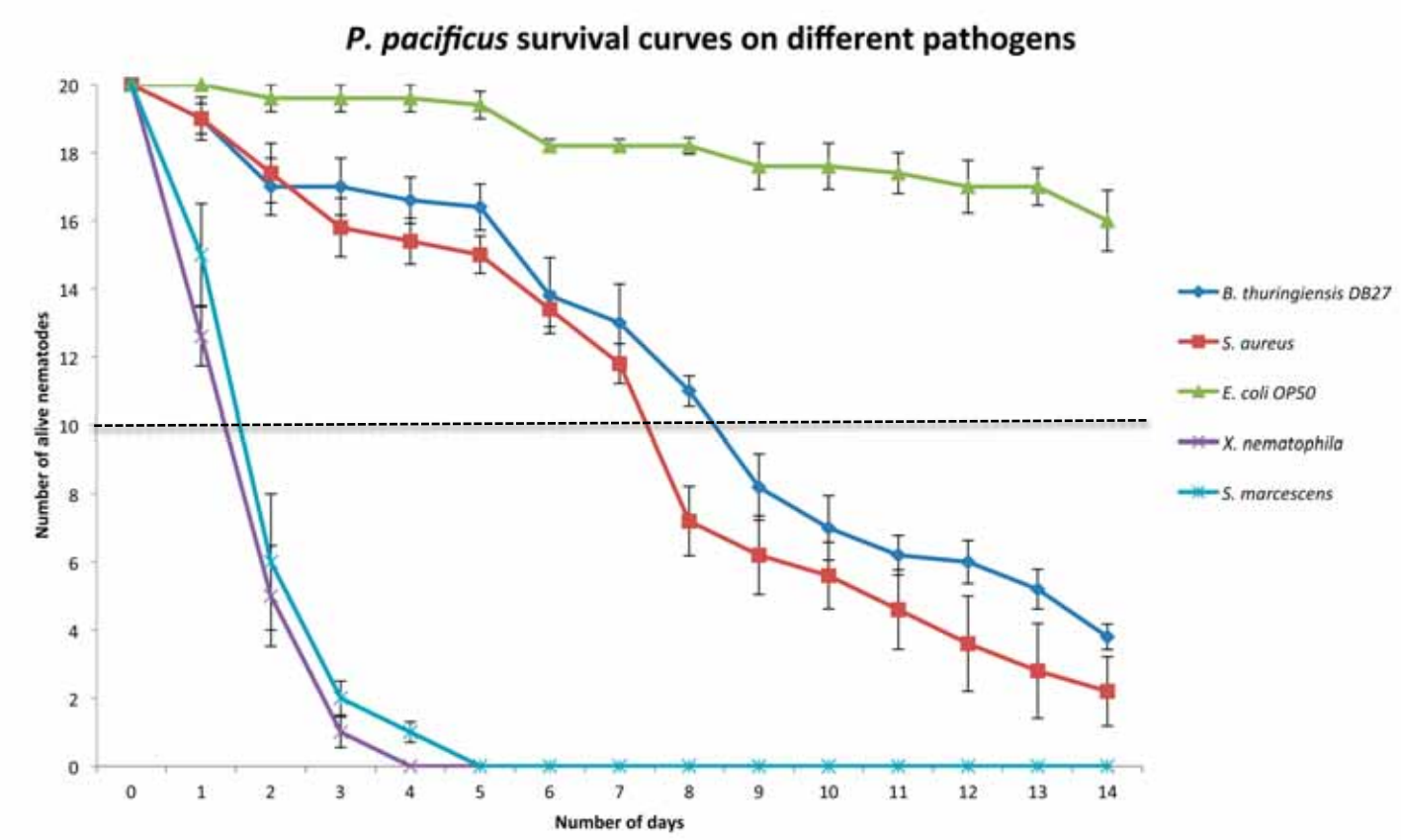
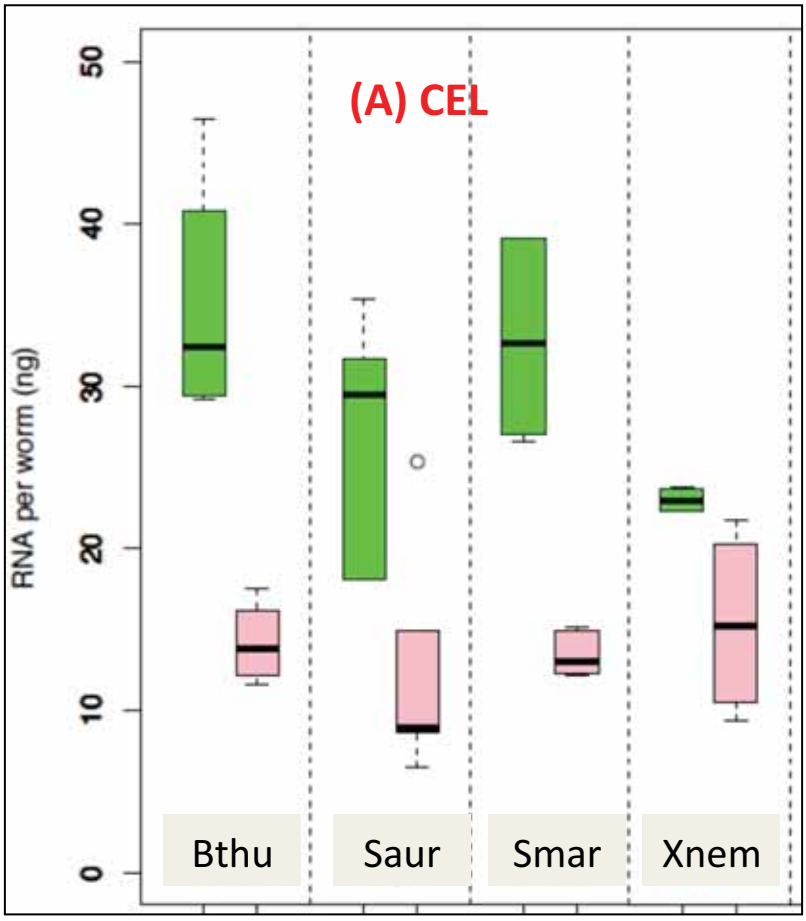
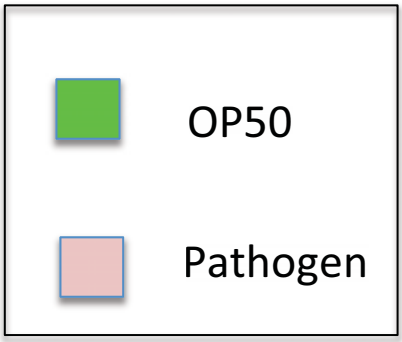
Supplementary Figure S1 : Long term survival curves for *P. pacificus* on various pathogens.

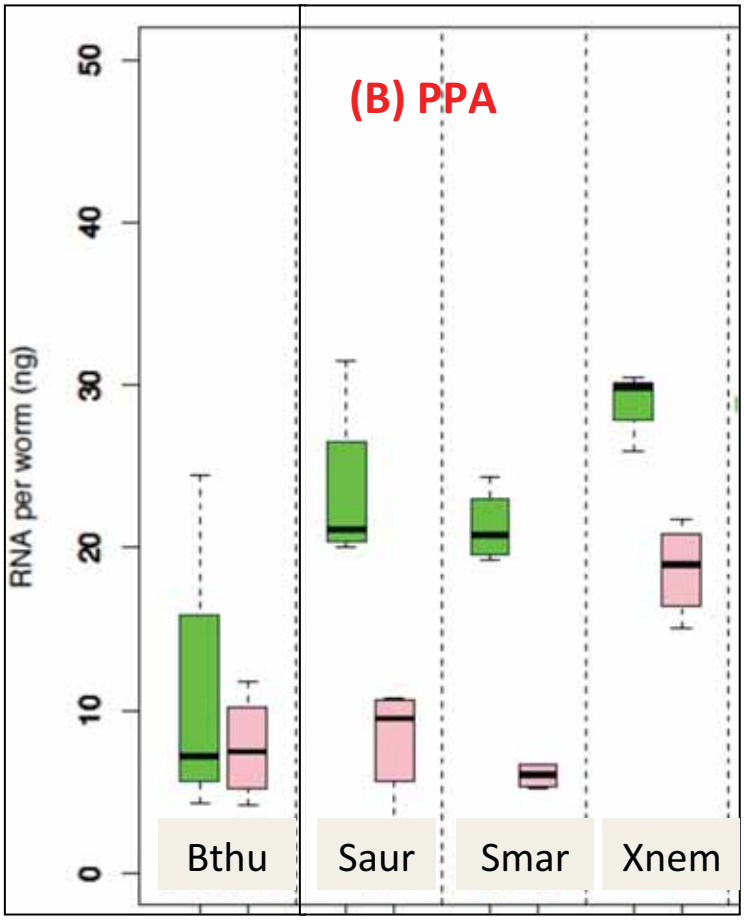
P. pacificus has higher resistance than *C. elegans* to *B. thuringiensis* and *S. aureus*, seen as longer median survival time of about 8 days on these pathogens



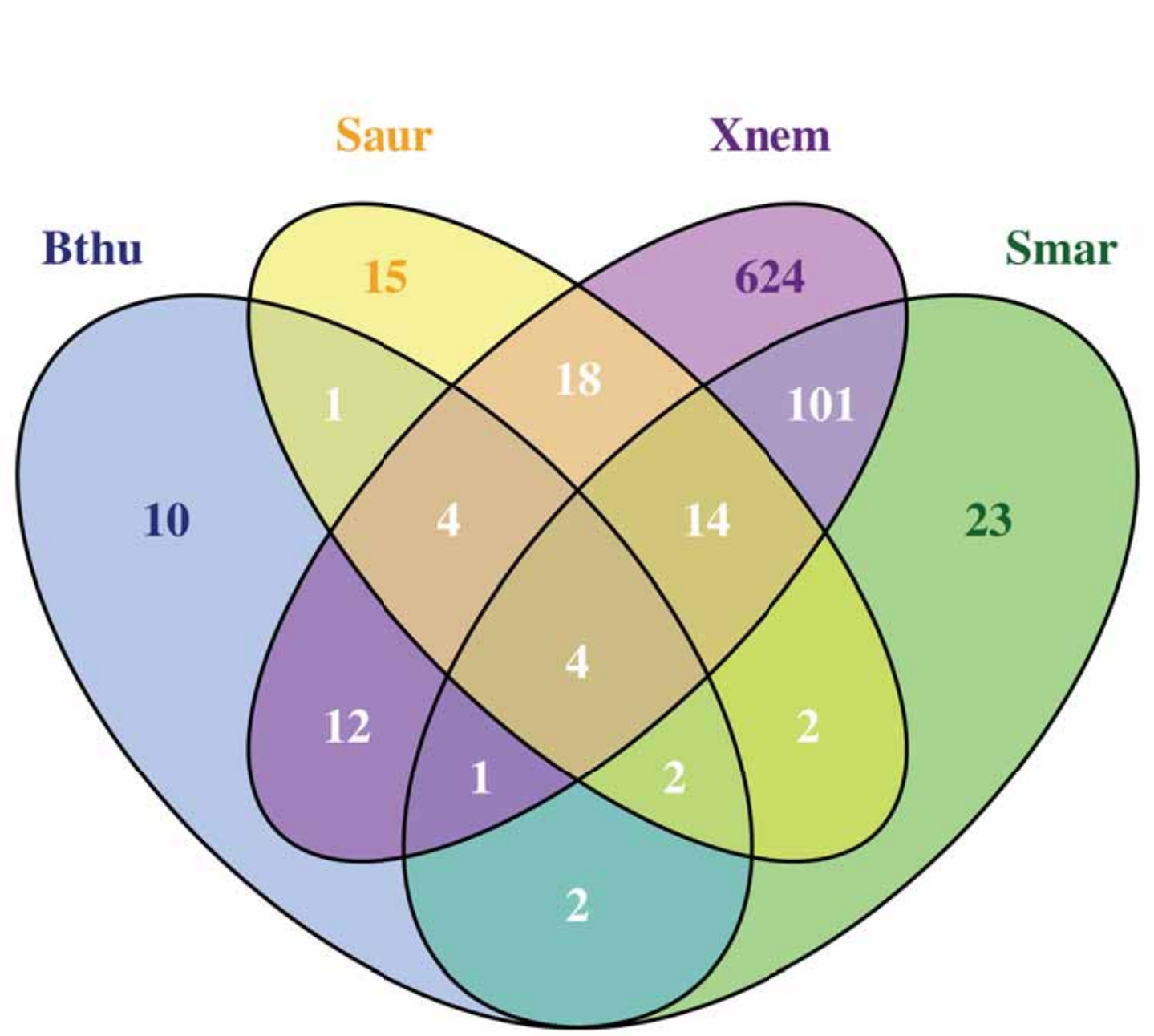
Supplementary Figure S2 : Global transcriptional suppression in response to pathogens in (A) *C. elegans* (B) *P. pacificus*





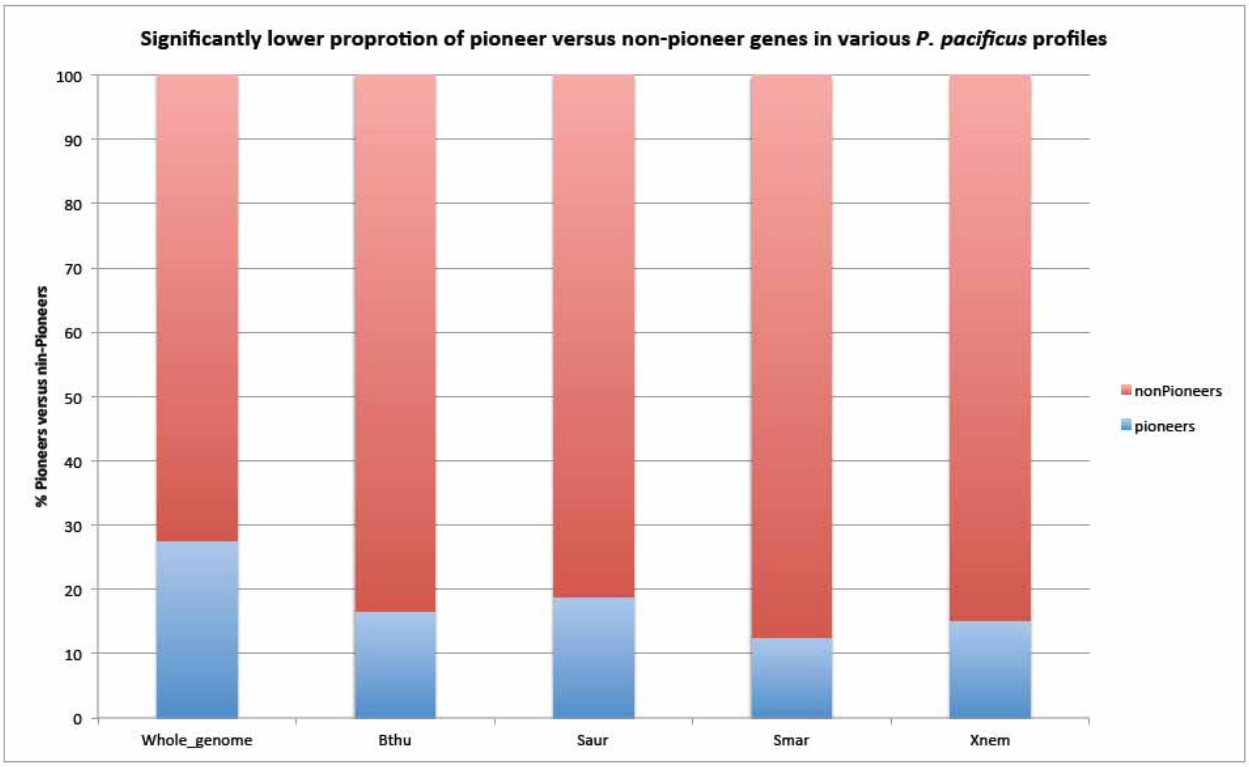


Supplementary Figure S3 : Overlap between pioneer genes regulated in *P. pacificus* in response to the four pathogens



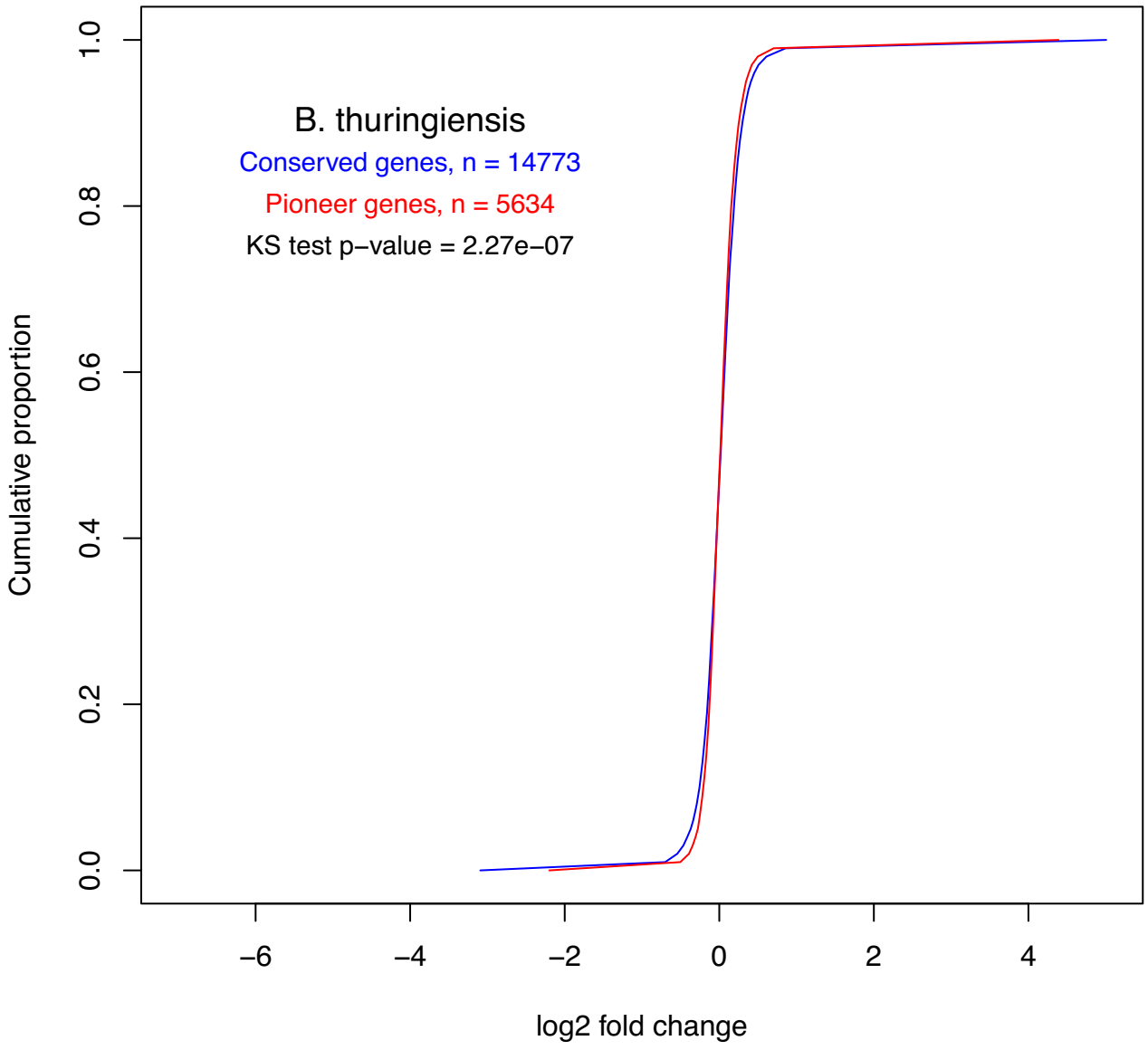
Supplementary Figure S4 : Relative proportions of pioneer genes versus non-pioneer genes in the active transcriptome of *P. pacificus* on each of the four pathogens.

On each of the pathogens, the pioneer genes constitute 12% to 18% of the active transcriptome, significantly less than the expected proportion of about 30% from the distribution in the entire transcriptome. One-sided Fisher's 2x2 exact test p –values are (a) 9.72E-05 on *B. thuringiensis* (b) 1.01E-04 on *S. aureus* (c) 8.75E-39 on *S. marcescens*, and (d) 8.01E-128 on *X. nematophila*



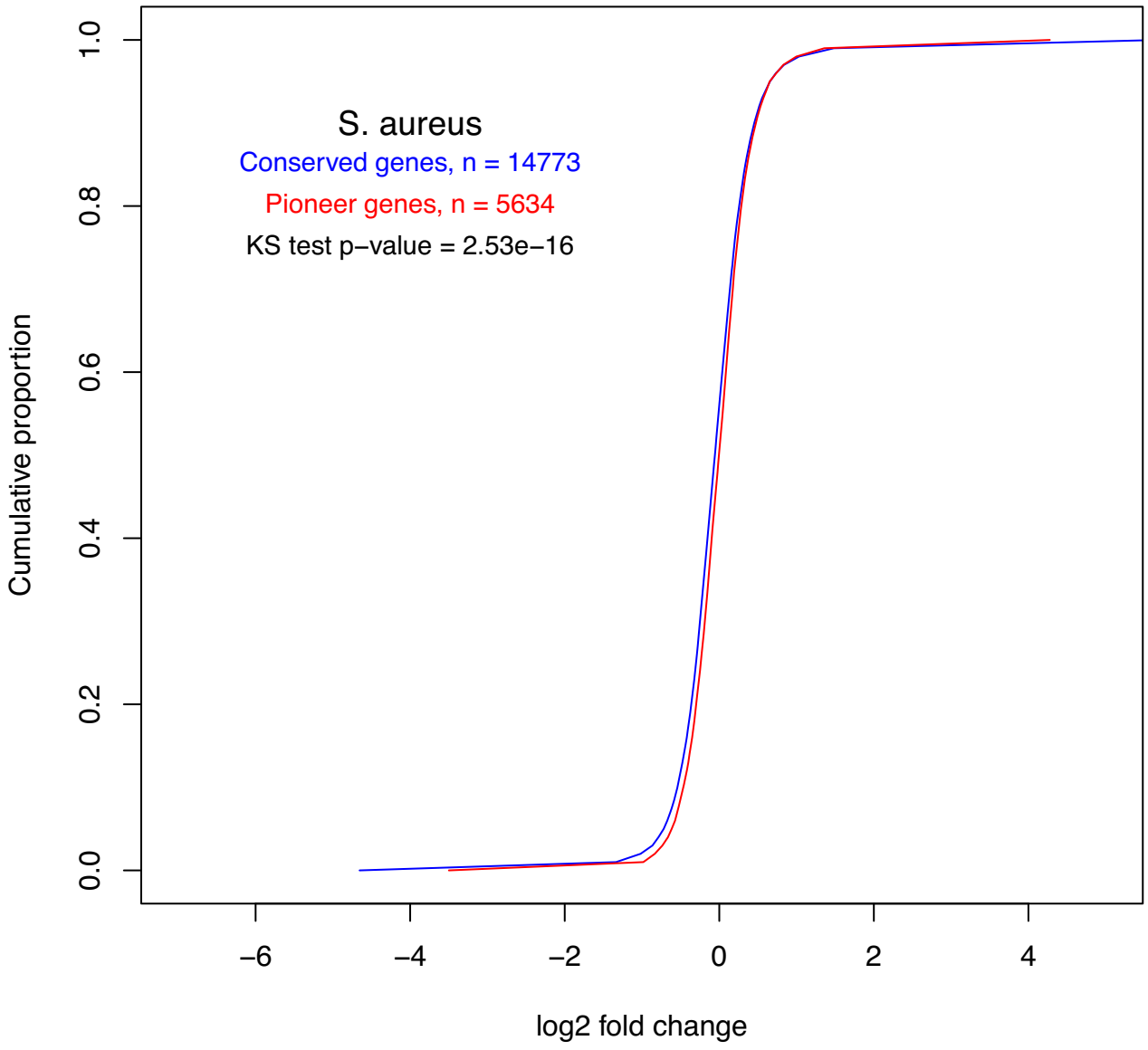
Supplementary Figure S5 .Pioneer genes are expressed at higher levels than non-pioneer genes in each of the pathogen-induced expression profiles on *P. pacificus*.

(A) *B. thuringiensis*



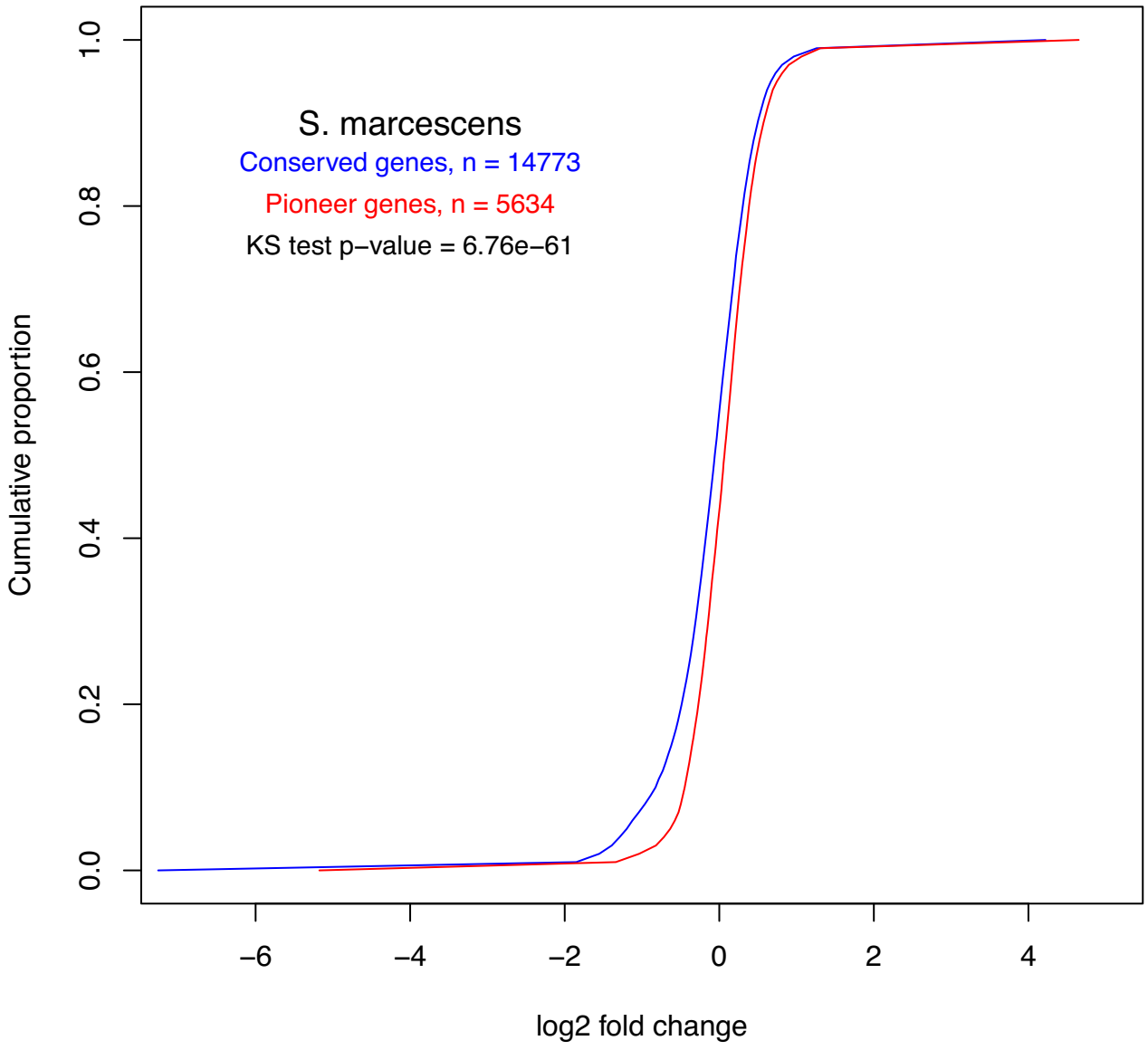
Supplementary Figure S5 .Pioneer genes are expressed at higher levels than non-pioneer genes in each of the pathogen-induced expression profiles on *P. pacificus*.

(B) : *S. aureus*



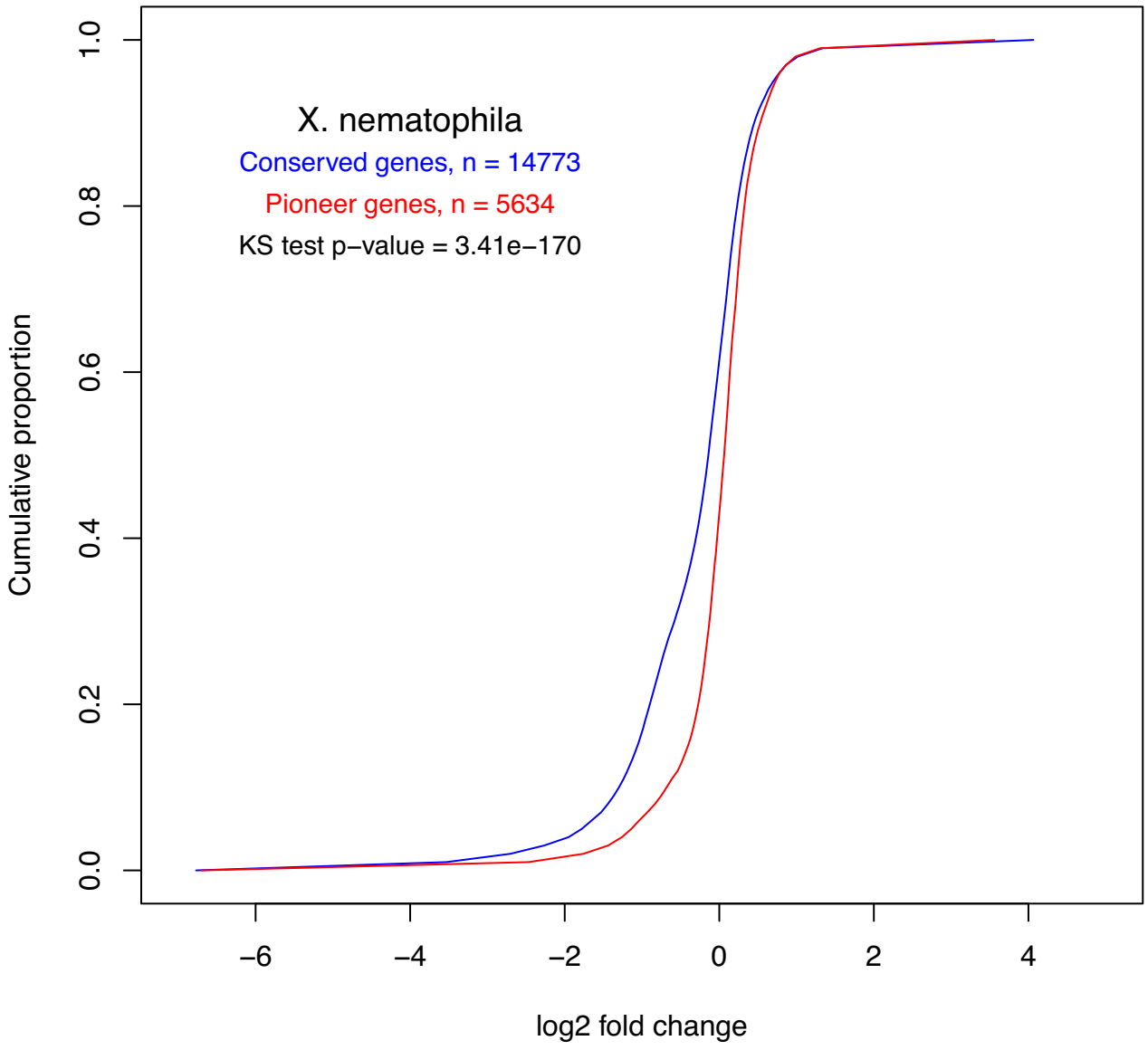
Supplementary Figure S5 .Pioneer genes are expressed at higher levels than non-pioneer genes in each of the pathogen-induced expression profiles on *P. pacificus*.

(C) : *S. marcescens*



Supplementary Figure S5 .Pioneer genes are expressed at higher levels than non-pioneer genes in each of the pathogen-induced expression profiles on *P. pacificus*.

(D) : *X. nematophila*



New Role for DCR-1/Dicer in Caenorhabditis elegans Innate Immunity against the Highly Virulent Bacterium Bacillus thuringiensis DB27

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New Role for DCR-1/Dicer in *Caenorhabditis elegans* Innate Immunity against the Highly Virulent Bacterium *Bacillus thuringiensis* DB27

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Bacillus thuringiensis **produces toxins that target invertebrates, including *Caenorhabditis elegans*. Virulence of *Bacillus* strains is often highly specific, such that *B. thuringiensis* strain DB27 is highly pathogenic to *C. elegans* but shows no virulence for another model nematode, *Pristionchus pacificus*. To uncover the underlying mechanisms of the differential responses of the two nematodes to *B. thuringiensis* DB27 and to reveal the *C. elegans* defense mechanisms against this pathogen, we conducted a genetic screen for *C. elegans* mutants resistant to *B. thuringiensis* DB27. Here, we describe a *B. thuringiensis* DB27-resistant *C. elegans* mutant that is identical to *nasp-1*, which encodes the *C. elegans* homolog of the nuclear-autoantigenic-sperm protein. Gene expression analysis indicated a substantial overlap between the genes downregulated in the *nasp-1* mutant and targets of *C. elegans dcr-1*/Dicer, suggesting that *dcr-1* is repressed in *nasp-1* mutants, which was confirmed by quantitative PCR. Consistent with this, the *nasp-1* mutant exhibits RNA interference (RNAi) deficiency and reduced longevity similar to those of a *dcr-1* mutant. Building on these surprising findings, we further explored a potential role for *dcr-1* in *C. elegans* innate immunity. We show that *dcr-1* mutant alleles deficient in microRNA (miRNA) processing, but not those deficient only in RNAi, are resistant to *B. thuringiensis* DB27. Furthermore, *dcr-1* overexpression rescues the *nasp-1* mutant's resistance, suggesting that repression of *dcr-1* determines the *nasp-1* mutant's resistance. Additionally, we identified the collagen-encoding gene *col-92* as one of the downstream effectors of *nasp-1* that play an important role in resistance to DB27. Taken together, these results uncover a previously unknown role for DCR-1/Dicer in *C. elegans* antibacterial immunity that is largely associated with miRNA processing.**

Genetically tractable model organisms, including the nematode *Caenorhabditis elegans*, have provided detailed insights into the origin and fundamental principles of immunity. More than a decade of research on *C. elegans* has yielded a wealth of knowledge about its innate immune response to various pathogens, uncovering multiple signaling pathways critical to *C. elegans* survival after exposure to pathogens (for reviews, see references 1 to 7). Once activated, these pathways induce the expression of an array of antimicrobial effectors that differ, depending on the pathogen present (8–11). A variety of microbes, including bacteria (12), fungi (13), viruses (14), and microsporidia (15), have been used in *C. elegans* infection studies, with the main focus on etiological agents of human diseases. In some cases, work on *C. elegans* has led to the identification of conserved virulence factors of human pathogens (16, 17).

Among the natural pathogens of *C. elegans*, *Bacillus thuringiensis* has been extensively studied and *B. thuringiensis* likely coexists and coevolves with its host in the natural environment (18). *B. thuringiensis* produces a variety of pore-forming toxins (PFTs), called Cry toxins, that target the intestinal cells of insects (19) and nematodes (20). The ability of some Cry toxins to target *C. elegans* has been exploited to use this nematode as an *in vivo* system for studying PFTs (21), and several *C. elegans* defense mechanisms against PFTs have been discovered (22–27).

Although the *C. elegans* model has provided invaluable insight into the immune defense against pathogens, a comparative approach with other nematodes would enhance our understanding of the host response to pathogens. One nematode that has been used extensively for comparative studies with *C. elegans* is *Pristionchus pacificus*. In contrast to *C. elegans*, which is often found on compost heaps and rotten fruit (28), *P. pacificus* is found in tight association with scarab beetles (29). Not surprisingly, therefore, these two nematodes show different resistance patterns (30–32) and transcriptional responses to pathogens (11). For example, *C. elegans* dies when fed the human opportunistic bacteria *Pseudomonas aeruginosa* and *Staphylococcus aureus* and *B. thuringiensis* Cry5B toxin, whereas *P. pacificus* is resistant (20, 30). Moreover, we have previously shown (31) that out of 768 naturally isolated *Bacillus* strains, only 20 were pathogenic and show distinct patterns of virulence for *C. elegans* and *P. pacificus*. The most extreme difference was seen with regard to *B. thuringiensis* strain DB27. DB27 kills *C. elegans* in less than 24 h, representing one of the fastest killers of *C. elegans* known to date, whereas *P. pacificus* is resistant to this bacterial strain (31). Our previous study (31) also indicated that the determinants of *B. thuringiensis* DB27 virulence for *C. elegans* and also the mechanisms of *C. elegans* resistance to this pathogen are unknown. Specifically, we have shown that the *bre* and *daf-2* mutants, which are known to be resistant to *B. thuringiensis* PFT (22) and other bacterial pathogens (33), respectively, are as susceptible to *B. thuringiensis* DB27 as wild-type worms are (31). These findings suggest that potentially novel mechanisms are required to provide defense against this highly virulent *Bacillus* strain.

Here, we report a genetic screen for *B. thuringiensis* DB27-

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resistant mutants of *C. elegans* and show that a gene represented by two alleles is identical to the *nasp-1* gene C09H10.6. Further characterization revealed that the *nasp-1* mutant shows a reduced life span and reduced fecundity and is RNA interference (RNAi) deficient. Microarray analysis uncovered an unexpected enrichment of *dcr-1*-regulated genes in *nasp-1* mutants. We explored a potential role for *dcr-1* in *C. elegans* innate immunity and show that *dcr-1* allele mutants deficient in microRNA (miRNA) processing, but not those that are only deficient in RNAi, are resistant to *B. thuringiensis* DB27. Transgenic expression of *dcr-1* in *nasp-1* mutants can rescue the resistance phenotype of *nasp-1* mutants. We identified the collagen-encoding gene *col-92* as one of the downstream effectors of *nasp-1* and *dcr-1*. These findings describe NASP-1 as a novel *C. elegans* innate immunity regulator and identify a previously unknown role for DCR-1 in the *C. elegans* antibacterial immune response.

MATERIALS AND METHODS

Nematodes and bacterial strains. *C. elegans* strains were maintained on nematode growth medium (NGM) agar plates with *Escherichia coli* OP50 as a food source. The following strains were kindly provided by the *Caenorhabditis* Genetics Center (University of Minnesota): wild-type Bristol (N2), Hawaiian mapping strain (CB4856), DA453 *eat-2*(*ad453*), WM49 *rde-4*(*ne301*), WM27 *rde-1*(*ne219*), CB189 *unc-32*(*e189*), BB1 *dcr-1*(*ok247*)/*unc-32*(*e189*) *III*, YY11 *dcr-1*(*mg375*), VC1138 *drsh-1*(*ok369*), VC446 *alg-1*(*gk214*), BA1 *fer-1*(*hc1*), and BA15 *fer-15*(*hc15*). *btr-1*/*C09H10.9*(*tu439*) and *btr-1*(*tu440*) mutant worms were generated in this study and backcrossed to wild-type worms four times before analysis. Double mutants were generated by using standard procedures (34). *Serratia marcescens* C2 was isolated from Reunion Island, *S. aureus* Newman and *P. aeruginosa* PA14 were purchased from the Deutsche Sammlung von Mikroorganismen und Zellkulturen, and *B. thuringiensis* DB27 was isolated from dung beetles (31).

Genetic screen for *B. thuringiensis* DB27-resistant nematodes. After ethyl methanesulfonate (EMS) mutagenesis (50 mM for 4 h at 20°C), single parental (P_0) mothers were separated onto NGM OP50 plates and allowed to lay 10 to 30 eggs. F_1 mothers were allowed to lay F_2 progeny that, upon reaching the L4 stage, were placed onto plates with pathogenic *B. thuringiensis* DB27. After 24 h of incubation at 25°C, single worms that survived pathogen exposure were transferred to NGM OP50 plates and allowed to reproduce. These candidates were retested several times in survival assay to confirm the resistance phenotype.

***C. elegans* killing assays.** Each bacterium was grown overnight in a shaking incubator at 30°C in Luria-Bertani (LB) broth, except *S. aureus* and *P. aeruginosa*, which were grown at 37°C. A 100-μl volume of the culture was spread to the edges of a 6-cm NGM plate to prevent worms from escaping and incubated overnight. Three to six independent replicates of 20 adult worms per plate were exposed to pathogens and monitored for survival. Survival assays were repeated multiple times and conducted at 25°C. Nematodes were transferred once a day to fresh plates and considered dead when they failed to respond to touch. We note that the exact nature of the killing assay (inoculum size etc.) might have an influence on the quantitative killing results reported in this study.

Complementation test, mapping, sequencing, and rescue of *tu439*. Complementation tests were performed by testing F_1 progeny from the crosses between *btr-2*(*tu438*) mutant males and *dpy-5*;*tu439* or *dpy-5*;*tu440* mutant hermaphrodites. To test if *tu439* and *tu440* are allelic, F_1 progeny from a cross between *tu439* mutant males and *dpy-5*;*tu440* mutant hermaphrodites were tested. For mapping, the *btr-1* mutant was crossed to Hawaiian strain CB4856 and approximately 400 F_2 cross progeny were isolated and screened for *B. thuringiensis* DB27 resistance. DNA was isolated from a total of 96 *tu439*-positive recombinant progeny and used for restriction fragment length polymorphism (RFLP)–single-nucleotide polymorphism (SNP) mapping (35). SNP data from the most informative recombinants identified a 160-kb region on chromosome II between the markers F38A3 and F37H8. Whole-genome sequencing of *btr-1* mutants revealed that both *btr-1* alleles contain two identical mutations in the mapping interval, which were found in the gene C09H10.6. For transformation rescue, a 2.3-kb PCR fragment, covering upstream and downstream regions of the C09H10.6 transcript, was amplified from wild-type genomic DNA and injected together with a *sur-5*::*gfp* plasmid as a coinjection marker into a *btr-1* mutant as described elsewhere (36).

For *dcr-1*, *dpy-5*, and *col-92* overexpression, respective transcripts with presumptive regulatory regions were PCR amplified from wild-type genomic DNA, purified, and used for injection together with the *sur-5*::*gfp* plasmid as a coinjection marker. For intestine-specific expression, a 3.5-kb *cpr-1* promoter fragment was amplified from wild-type DNA and fused with the *dcr-1* transcript by using Phusion High-Fidelity DNA polymerase (NEB) according to the manufacturer's instructions. The resulting construct was used for *btr-1* microinjection. Reporter gene constructs for gene expression analysis were created by the PCR fusion method as described previously (37). A *nasp-1*::GFP (green fluorescent protein) translational reporter was obtained by PCR amplification of the *nasp-1* transcript with the presumptive promoter region from wild-type genomic DNA, which in the next step was PCR fused to GFP, which was amplified from the pPD95.75 plasmid. A *col-92* transcriptional reporter was generated in the same way, with the exception that only a 1.4-kb *col-92* promoter region was fused to GFP. The resulting constructs were purified and used for injection together with the pRF4 plasmid (*rol-6*). Several independent lines were obtained and tested for all of the transgenic experiments described here.

Mutant identification by whole-genome sequencing. We sequenced a genomic library of the *btr-1*(*tu439*) mutant on the Illumina Genome Analyzer II platform and obtained 7,398,278 read pairs (2 × 100 bp). Raw reads were aligned with the *C. elegans* genome assembly (WS235) by using bwa (version 0.5.9-r16) (38). After the removal of duplicated reads to exclude false variant calls due to PCR amplification biases, 98.8% of the *C. elegans* assembly was covered by at least one read, with a genome-wide mean coverage of 15×. Variants were called by using SAMtools (version 0.1.18-r982:295) (39). In total, we identified 2,092 homozygous base substitutions with a SAMtools quality score of at least 20, of which six variants were located within the 160-kb mapping interval on chromosome II. Filtering for variants with a potential deleterious effect (nonsynonymous substitution, premature stop codons, and mutations near splice sites) could exclude three variants as intergenic and intronic, respectively. Two of the remaining three nonsynonymous substitutions were found to affect the *nasp-1* gene (amino acid changes, L53S and D307N), and one substitution at genomic position II:11069603, G to A (WS235), was found to cause an S-to-F amino acid exchange in various alternative transcripts of *unc-53*. A rescue experiment confirmed that mutations responsible for the phenotype occurred in the *nasp-1* gene.

Pumping rate measurement. Pharyngeal pumping was counted for 1 min starting on the first day of adulthood. Worms were placed on *B. thuringiensis* DB27 plates at 25°C and left undisturbed for 3 h before measurement. All worms remained on food during the period of observation. The *eat-2*(*ad453*) mutant, known to have low pumping, was used as a control.

Brood size measurement. Brood size was determined by picking L4 worms (one per plate) and transferring them to fresh plates every 24 h until egg laying ceased. The offspring were counted 2 days after the mothers were removed from the plates. Ten worms were used per strain per experiment, and experiments were repeated twice.

Life span assay. Life span assays were performed on NGM plates containing 0.1-mg/ml 5′-fluorodeoxyuridine (FUDR) to prevent progeny from hatching at 20°C. Plates were seeded with concentrated OP50 and allowed to dry overnight. A synchronized population of L4 worms grown on OP50 was placed onto the plates and scored every 48 h. For *col-92* RNAi life span assays, NGM plates containing 50 μg/ml carbenicillin, 1 mM isopropyl-β-D-thiogalactoside (IPTG), and 0.1 mg/ml FUDR were seeded

with *E. coli* HT115 expressing *col-92* double-stranded RNA (dsRNA) (experiment) or *E. coli* HT115 harboring the empty vector (control). A synchronized population of L4 worms grown on *col-92* RNAi bacteria or vector-containing bacteria were placed onto the RNAi plates with FUDR and scored every 48 h. Upon depletion of food, worms were transferred to fresh plates. Worms were considered dead when no response to touch was observed. Approximately 100 worms were used per strain per experiment, and experiments were repeated twice.

RNAi. To generate specific gene knockdowns, we used RNAi by feeding nematodes with *E. coli* expressing dsRNA that is homologous to a target gene. *E. coli* harboring the empty vector was used as a control. *E. coli* strain HT115 harboring the appropriate vector was grown in LB broth containing 100 μg/ml ampicillin at 37°C overnight. Bacteria were plated onto NGM plates containing 50 μg/ml carbenicillin and 1 mM IPTG and allowed to grow overnight at 37°C. Three adult hermaphrodites were placed on each RNAi plate and allowed to lay eggs. These eggs were grown on RNAi plates for 3 days at 22°C to the adult stage before being transferred to plates with *B. thuringiensis* DB27 for pathogenicity assay. All of the RNAi clones used in this study are part of the RNAi library obtained from Source BioScience UK Limited (Nottingham, United Kingdom). *cdc-25* RNAi plates were prepared as described above. However, worms were grown at 25°C to the adult stage. *cdc-25.1* encodes a CDC25 phosphatase homolog that affects embryonic viability and is necessary for cell proliferation in the germ line. In brief, gravid worms were laid on *cdc-25.1* RNAi plates for 4 h and then transferred to similar plates for an additional 4 h of egg laying. After that, gravid hermaphrodites were removed and eggs were left to hatch and grow in the presence of *cdc-25.1* RNAi to produce sterile worms. *cdc-25* RNAi by injection was performed as described below. For *mom-2* RNAi, three adult hermaphrodites were placed on *mom-2* RNAi plates and allowed to lay eggs. These eggs were grown on RNAi plates at 20°C to the adult stage. Single adults were subsequently placed onto freshly prepared *mom-2* RNAi plates and allowed to lay eggs for 24 h and then removed from the plates. After 16 h, the total amounts of eggs and hatched larvae were scored and viability was expressed as follows: (number of hatched larvae/total number of eggs) × 100. The *mom-2* coinjection experiments were conducted at 20°C as described previously (40).

dsRNA for injection experiments was prepared by *in vitro* transcription. DNA templates were PCR amplified from plasmids corresponding to the RNAi clones with T7 primers or PCR amplified from *C. elegans* genomic DNA with gene-specific primers with attached T7 promoters in case the gene is not present in the available RNAi library. PCR products were used for dsRNA synthesis in a single reaction with the TranscriptAid T7 high-yield transcription kit (Thermo Scientific). Concentrations of dsRNAs were determined with a spectrophotometer, and the quality and size of dsRNAs were assessed by gel electrophoresis. Young hermaphrodites were injected in the gonads with 300 to 600 ng/μl of dsRNA of the candidate gene. Given that *dpy-5* RNAi produces a clear morphological phenotype but has no effect on survival, *dpy-5* dsRNA was used as a positive control for injection and as a negative control in survival assays. Adult F_1 worms were used in the pathogen survival assay.

RNA collection for microarray experiments. Synchronized populations of wild-type and *tu439* mutant *C. elegans* worms were obtained by hypochlorite treatment and allowed to grow to the young adult stage on *E. coli* OP50 at 20°C. Worms were exposed to *B. thuringiensis* DB27 for 4 h and then picked into TRIzol reagent for RNA extraction. Four biological replicates were collected for each experimental condition and the control condition. For each biological replicate, about 200 young adult hermaphrodites were picked into 1 ml of TRIzol (Invitrogen) and total RNA was extracted according to the manufacturer's instructions and purified further by phenol-chloroform-isoamyl alcohol precipitation. The RNA pellet was suspended in RNase-free water and assessed on a NanoDrop spectrophotometer for quantity and RNA quality.

Microarray experiments and data analysis. Oligonucleotide microarrays for *C. elegans* containing ~43,000 unique probes for ~20,000 *C. elegans* genes were obtained from Agilent Technologies (NCBI GEO accession number GPL10094). All experiments are in a two-color format, where Cy5 and Cy3 dye-labeled cRNAs from experimental and control samples are cohybridized on the same microarray. The four biological replicates per experiment included two dye swap experiments to account for differences in dye labeling. Experimental procedures and data analysis were essentially the same as described before (41). Microarrays corresponding to three of the four biological replicates passed the quality criteria and were used in the final analysis. Genes with a false-discovery rate (FDR)-corrected *P* value of ≤0.05 and an absolute fold change greater than or equal to a cutoff of 1.5 on a $\log_2$ scale were called differentially expressed.

Expression cluster enrichment analysis. The list of microarray expression profiles in which a given *C. elegans* gene is known to be differentially expressed can be extracted from the Expression Cluster section of the WormBase (42) gene summary page for each gene. We retrieved all of the available expression clusters for *C. elegans* genes from the WormBase website (www.wormbase.org) and used them to identify expression clusters enriched in our expression profile, an approach that we have successfully used previously to analyze genome-wide trends in expression profiles (11, 41). *P* values for expression cluster enrichment in each expression profile were computed with a two-by-two Fisher exact test. An FDR-corrected *P* value cutoff of 0.05 was used as the significance threshold.

qRT-PCR. Synchronized *C. elegans* worms at the L4 stage were exposed to *B. thuringiensis* DB27 for 4 h before harvesting. Nematodes were collected by washing plates with M9 buffer, and RNA was extracted with TRIzol reagent. cDNA was synthesized with the SuperScript III kit (Invitrogen). Quantitative real-time PCR (qRT-PCR) was conducted with the LightCycler 480 SYBR green I master kit (Roche) on a LightCycler 480 real-time PCR instrument (Roche) in a 96-well format. Relative fold changes in transcripts were calculated by the comparative cycle threshold (C_T) method after normalization to *snb-1*, which has been used previously in qRT-PCR studies of *C. elegans* innate immunity (43–45). C_Ts were determined by the Roche LightCycler software. All samples were run in triplicate. The sequences of the primers used are available upon request.

Statistical analysis. Kaplan-Meier nonparametric comparisons and a log rank test were used for statistical analysis of *C. elegans* survival in the presence of pathogens and for life span analysis. In each case, curves represent the combined data from at least three independent experiments. Statistical analysis of the difference between two values was performed with Student's *t* test. Statistical significance was set at $P \leq 0.05$.

Microarray data accession number. Raw and processed data from all of the experiments described here are available under accession number GSE43905 in the NCBI Gene Expression Omnibus database (http://www.ncbi.nlm.nih.gov/geo/).

RESULTS

Forward genetic screen for *B. thuringiensis* DB27-resistant (*btr*) mutants. *B. thuringiensis* DB27 was isolated from a dung beetle (31) and was selected for detailed investigation because of its high virulence for *C. elegans*. This strain is 100% lethal in just 16 h under standard assay conditions (Fig. 1A). Our previous studies indicated that all of the *bre* and *daf-2* mutants tested were as susceptible to *B. thuringiensis* DB27 as wild-type worms are (31). In contrast to the observed susceptibility of *C. elegans*, the second model nematode organism, *P. pacificus*, is fully resistant to *B. thuringiensis* DB27 (31). To uncover a potential resistance mechanism and to better understand the specificity of *B. thuringiensis* virulence for certain nematode species, we conducted a forward genetic screen for mutants of *C. elegans* with strong resistance to *B. thuringiensis* DB27. Specifically, we screened for mutants that would extend *C. elegans* survival on monoxenic cultures of *B. thuringiensis* DB27 by at least a factor of 3, in order to select for major effectors. From 1,800,000 EMS-mutagenized gametes, we isolated

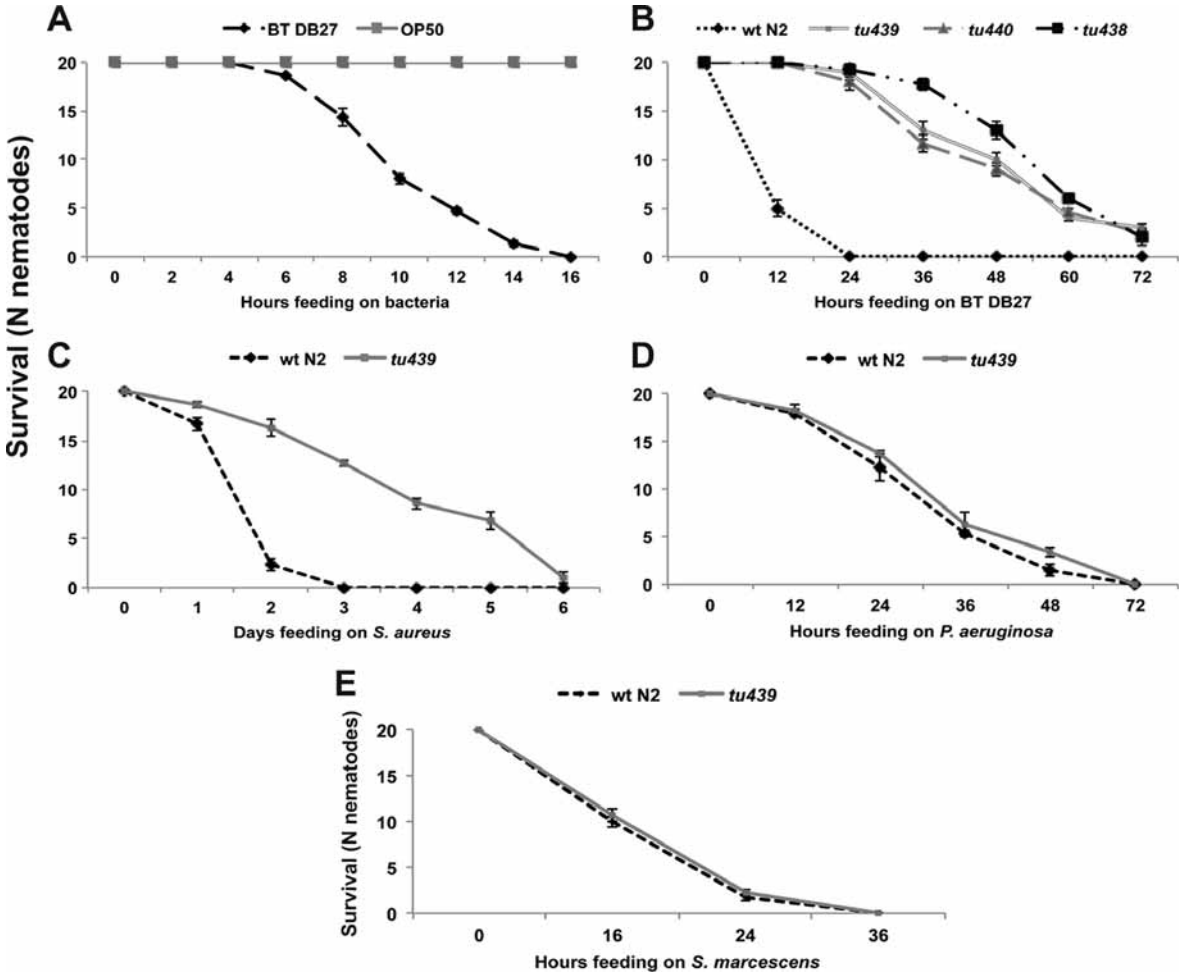

FIG 1 Differences in the survival of wild-type and *btr* mutant worms upon exposure to different pathogenic bacteria. (A) *C. elegans* wild-type (wt) N2 is highly susceptible to *B. thuringiensis* (BT) DB27, in contrast to standard *E. coli* OP50, which was used as a control ($P < 0.0001$). (B) Survival of *btr* mutants on monoxenic cultures of *B. thuringiensis* DB27. All alleles showed significantly ($P < 0.0001$) enhanced survival compared to that of the wild type. (C) *tu439* mutants show increased resistance to *S. aureus* compared to that of the wild type ($P < 0.0001$). (D, E) *tu439* mutants are as susceptible as wild-type worms to *P. aeruginosa* (D, $P > 0.05$) and to *S. marcescens* (E, $P > 0.05$). For survival curves, the numbers of live versus dead worms were scored over time. The number of worms alive (N nematodes) is plotted as a function of time. The data shown are means ± the standard errors of the means. For each condition in the survival assay, 60 to 80 worms (20 per plate) were used for each of multiple independent biological replicates.

three *btr* (*B. thuringiensis*-resistant) mutants that exhibited strong resistance to *B. thuringiensis*-mediated killing (Fig. 1B). Complementation tests revealed that these mutants fall into two complementation groups (data not shown) with two alleles (*tu439* and *tu440*) of a locus provisionally named *btr-1* and a single allele (*tu438*) of a second locus called *btr-2*. Here we further characterize *btr-1*.

***btr-1*(*tu439*) mutants are resistant to additional pathogens and have a reduced life span.** Considering that several pathogen-resistant *C. elegans* mutants are resistant to multiple pathogens (33), we exposed *btr-1*(*tu439*) mutant worms to additional pathogens to further characterize the specificity of the mutant. We found that *tu439* mutants exhibited stronger resistance to the Gram-positive pathogen *S. aureus* than wild-type worms did (Fig. 1C). In contrast, *tu439* mutants showed wild-type survival on the Gram-negative pathogens *P. aeruginosa* and *S. marcescens* (Fig. 1D and E). These results indicate that *tu439* confers resistance to two, but not all, of the pathogens tested.

To explore additional functions, we tested several physiological parameters of *tu439* mutant worms. First, we estimated the pumping rate as an indirect measurement of bacterial uptake to elucidate bacterial consumption. The pumping rate of the *tu439* mutant on DB27 is similar to that of the wild type, whereas the control *eat-2*(*ad453*) mutant shows the expected reduction of pumping in our assay system (Fig. 2A). Thus, pharyngeal pumping is unaffected, suggesting that pathogen consumption is not reduced in mutant worms. To rule out the possibility that bacteria might persist in the intestines of wild-type but not mutant worms, we fed worms with DB27 for 4 h, which is sufficient to observe accumulation of bacteria in the gut (see Fig. S1A in the supplemental material). We then shifted the worms to OP50 for 2 h and measured the DB27 CFU. Neither the wild-type nor the mutant worms showed a difference in their DB27 CFU counts after the shift to OP50, suggesting that the persistence of DB27 is not extended in wild-type worms (see Fig. S1B). Second, we observed that *tu439* mutants exhibit reduced fertility (see Fig. S1C). A similar phenotype was also annotated for other pathogen-resistant mu-

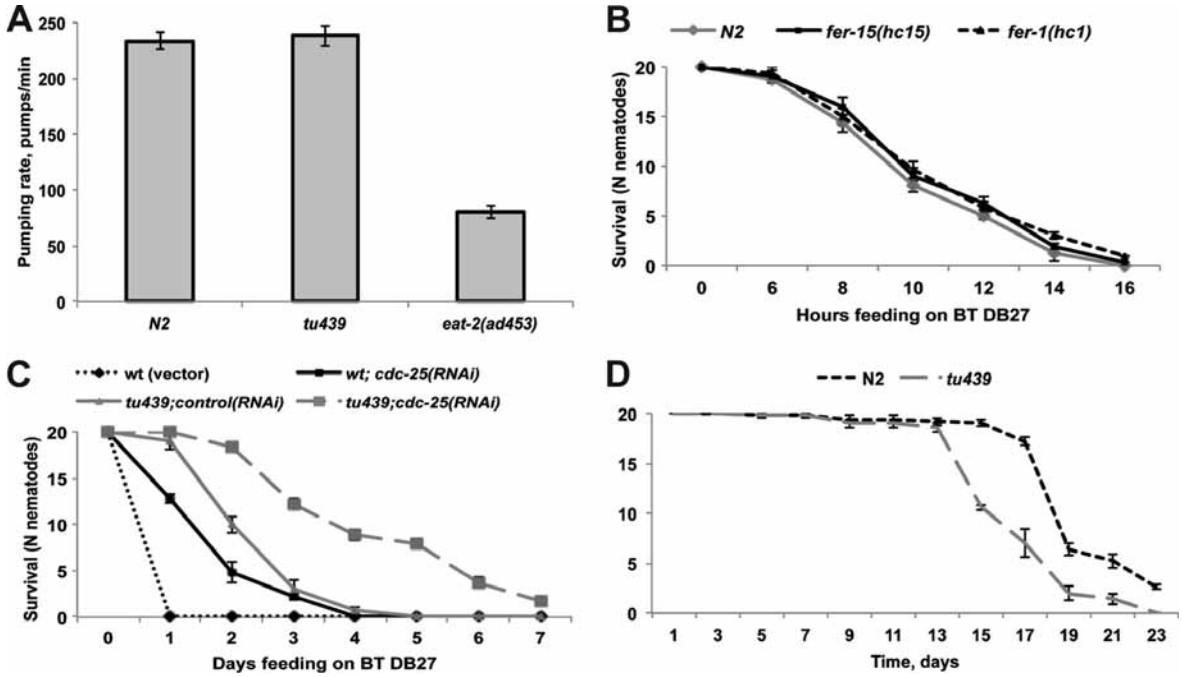

FIG 2 *tu439* mutants exhibit increased resistance to DB27 independently of reduced fertility and longevity. (A) The pumping rate of *tu439* mutants shows no difference from that of the wild type ($P > 0.05$). The control *eat-2* mutant strain shows the expected lower pumping rate than that of the wild type ($P < 0.0001$). At least 10 worms were scored per strain. The data shown are means ± the standard errors of the means. (B) *fer-1* and *fer-15* mutants show no difference ($P > 0.05$) from wild-type worms on a lawn of *B. thuringiensis* (BT) DB27. The data shown are means ± the standard errors of the means. For each condition in survival assays, 60 to 80 worms (20 per plate) were used for each of multiple independent biological replicates. (C) Wild-type and *tu439* nematodes were injected with *cdc-25* interfering dsRNA to obtain sterile worms or with control interfering dsRNA (see Materials and Methods) and exposed to DB27. *cdc-25* RNAi significantly increases the resistance of wild-type [$P < 0.0001$ for wt (vector) versus wt;*cdc-25* worms] and *tu439* mutant ($P < 0.0001$ for *tu439*;control versus *tu439*;*cdc-25* worms) worms. The survival of *tu439*;*cdc-25* mutant worms is significantly higher ($P < 0.0001$) than that of *tu439* mutant or wt;*cdc-25* worms alone. (D) The life span of *tu439* mutants is significantly ($P < 0.001$) lower than that of wild-type worms on standard food (*E. coli* OP50). At least 100 worms were used per strain per experiment, and each experiment was done twice. The data shown are means ± the standard errors of the means.

tants (46–48) and suggests that there is a tradeoff between reproduction and an elevated immune response. This raises the possibility that *tu439* confers increased resistance to *B. thuringiensis* DB27 because of diminished fecundity.

To elucidate this further, we assessed the survival of sterile mutants exposed to *B. thuringiensis* DB27. First, we investigated fecundity in combination with matricidal internal hatching of eggs by comparing the survival of wild-type worms to that of *fer-1* and *fer-15* mutants (47). *fer* mutants have a germ line but do not suffer from matricidal effects due to the lack of fertilization since their sperm production is affected (49). Both *fer* mutants are as susceptible to *B. thuringiensis* DB27 as wild-type worms are, suggesting that matricidal hatching of eggs does not contribute to *B. thuringiensis* DB27 killing and that increased resistance of *tu439* to DB27 is not due to a lack of matricide (Fig. 2B). This finding is further supported by the fact that males are susceptible to DB27 (not shown).

In a second set of experiments, we used *cdc-25* RNAi, which also causes sterility. *cdc-25* RNAi led to enhanced survival of wild-type worms exposed to *B. thuringiensis* DB27 (Fig. 2C). However, c*dc-25* RNAi in a *tu439* background resulted in a significant increase in resistance to DB27 that was stronger than the resistance of single *tu439* mutant or wild-type *cdc-25* RNAi worms (Fig. 2C). The cumulative effect of the two mutations suggests that the underlying molecular mechanisms are independent and that *tu439*-mediated resistance to *B. thuringiensis* DB27 is not due simply to reduced fecundity. Additionally, in contrast to sterile mutants, the *tu439* mutant is not resistant to *S. marcescens* and *P. aeruginosa* (Fig. 1D and E). Finally, we measured the life span of *tu439* mutants, given that life span extension often correlates with increased pathogen resistance in *C. elegans* (33, 47). Interestingly, we found that *tu439* mutants fed on *E. coli* OP50 show a slight but significant life span reduction in comparison to wild-type worms (Fig. 2D). These results show a novel relationship between life span and immunity and suggest that *tu439* mutant worm resistance to bacterial infection is not a simple consequence of extended longevity. Taken together, *tu439* mutant worms are resistant to several Gram-positive bacteria, have a reduced life span and fecundity, and show that immunity can be mechanistically uncoupled from aging.

***btr-1* is identical to the *C. elegans nasp-1* gene.** To clone *tu439*, we used RFLP-SNP mapping (35) and next-generation sequencing (50). In brief, we mapped *btr-1* to an approximately 160-kb region on chromosome II between the markers F38A3 and F37H8. Whole-genome sequencing of both alleles revealed mutations in the C09H10.6 gene, which has been described as the *C. elegans* homolog of the gene for human nuclear-autoantigenic-sperm protein (*nasp-1*) (Fig. 3A). *nasp-1* has previously been implicated in *C. elegans* female development (51).

Interestingly, the two alleles appear to contain two identical mutations, which might be due to the fact that they were derived from a single mother. The first mutation is a T-to-C substitution

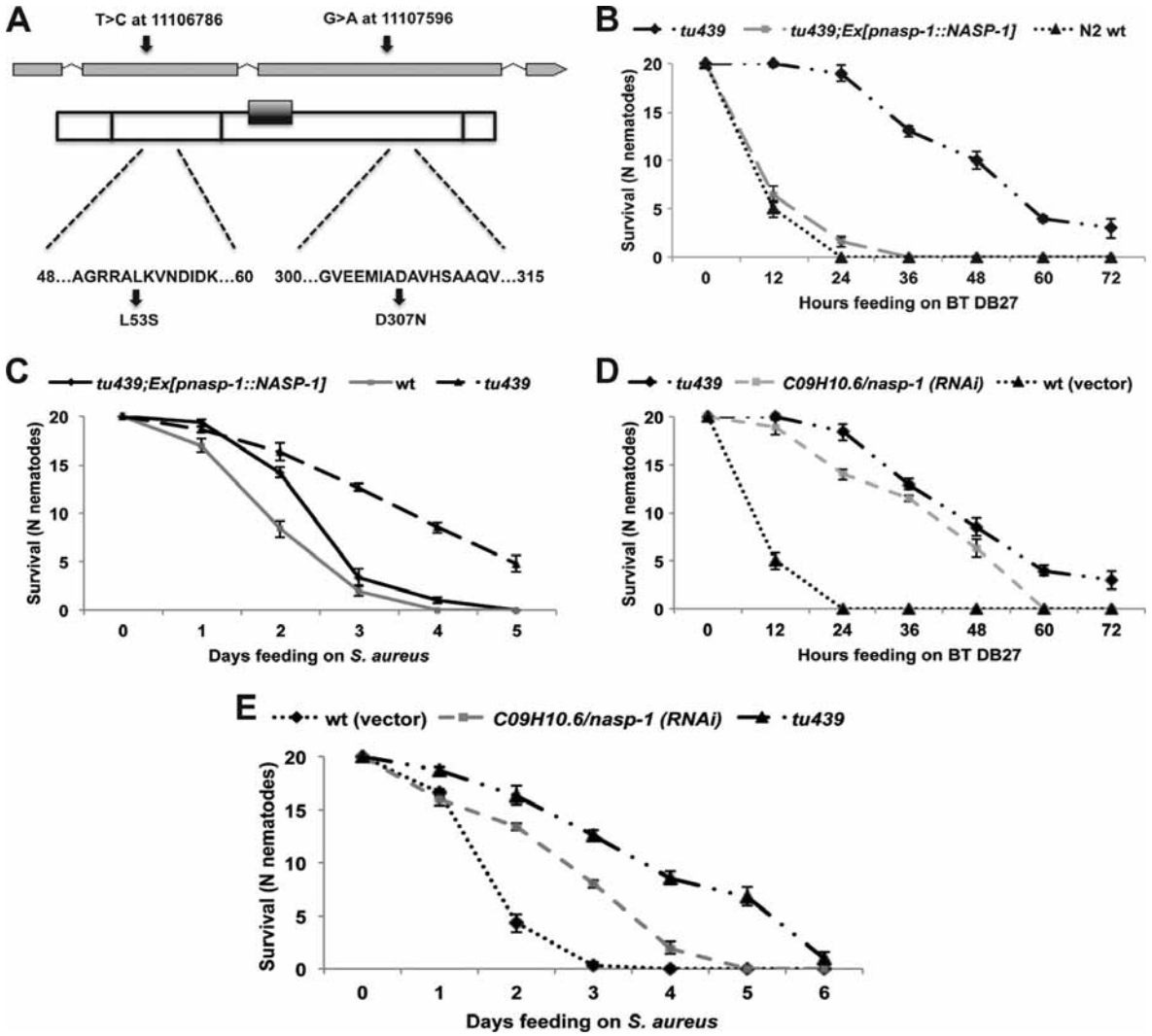

FIG 3 Identification of the *tu439* allele. (A, top) Exon-intron structure of the C09H10.9 gene, adapted from WormBase (WS235), with the positions of mutations indicated by arrows. Numbers indicate positions on chromosome II where single-nucleotide mutations occurred. (A, bottom) Schematic domain structure of the C09H10.9 protein. The black box shows the location of the TPR domain, which is not affected in either of the *btr-1* mutants. The dashed lines highlight the amino acid sequences flanking the mutation sites. The black arrows show the position and type of amino acid substitution. (B) Rescue experiment. Transgenic expression of *nasp-1* under the control of its endogenous promoter restores wild-type (wt) susceptibility to *B. thuringiensis* (BT) DB27 to the *tu439* mutant. (C) *tu439* mutant resistance to *S. aureus* is rescued by transgenic expression of *nasp-1* under the control of its endogenous promoter ($P > 0.05$ for the wild type versus the transgenic line). The data shown are means ± the standard errors of the means. (D) The survival on a lawn of *B. thuringiensis* DB27 of wild-type worms grown on dsRNA for C09H10.6, which corresponds to *tu439*, is significantly ($P < 0.0001$) greater than that of worms grown on dsRNA as a vector control. (E) The survival on a lawn of *S. aureus* of wild-type worms grown on dsRNA for C09H10.6, which corresponds to *tu439*, is significantly ($P < 0.001$) greater than that of worms grown on dsRNA as a vector control. The data shown are means ± the standard errors of the means. For each condition in survival assays, 60 to 80 worms (20 per plate) were used for each of multiple independent biological replicates.

in the second exon of *C. elegans nasp-1* resulting in a leucine-to-serine substitution at amino acid position 53, whereas the second mutation (a G-to-A change in exon 3) leads to a substitution from aspartic acid to asparagine at amino acid position 307 (Fig. 3A). To confirm that the resistance phenotype of *tu439* is due to these mutations in C09H10.6, we performed rescue and RNAi experiments. First, transgenic expression of a wild-type copy of C09H10.6 under the control of its endogenous promoter in the *tu439* mutant background resulted in almost complete restoration of wild-type susceptibility to *B. thuringiensis* DB27 (Fig. 3B) and *S. aureus* (Fig. 3C). Additionally, transgenic expression of C09H10.6 partially rescued the diminished reproduction of the *tu439* mutant (not shown). Second, C09H10.6 RNAi increases nematode resistance to *B. thuringiensis* DB27 (Fig. 3D) and *S. aureus* (Fig. 3E) in a way similar to that of *tu439*, providing additional evidence that the mutant is allelic to C09H10.6. Also, we noticed that *nasp-1* RNAi worms have slower growth and less fecundity at 25°C than vector-treated worms. The high conservation of the amino acids that were mutated (see Fig. S2 in the supplemental material), especially D307, suggests that our *nasp-1* mutant is a strong reduction-of-function allele. Also, the similarity between the *nasp-1* RNAi and the mutant phenotype suggests that the isolated alleles represent reduction-of-function rather than neomorphic mutations. In summary, we conclude that *btr-1*(*tu439*) is identical to the *nasp-1*-like gene C09H10.6. Following standard *C. elegans* nomenclature rules, we rename *btr-1* as *nasp-1*.

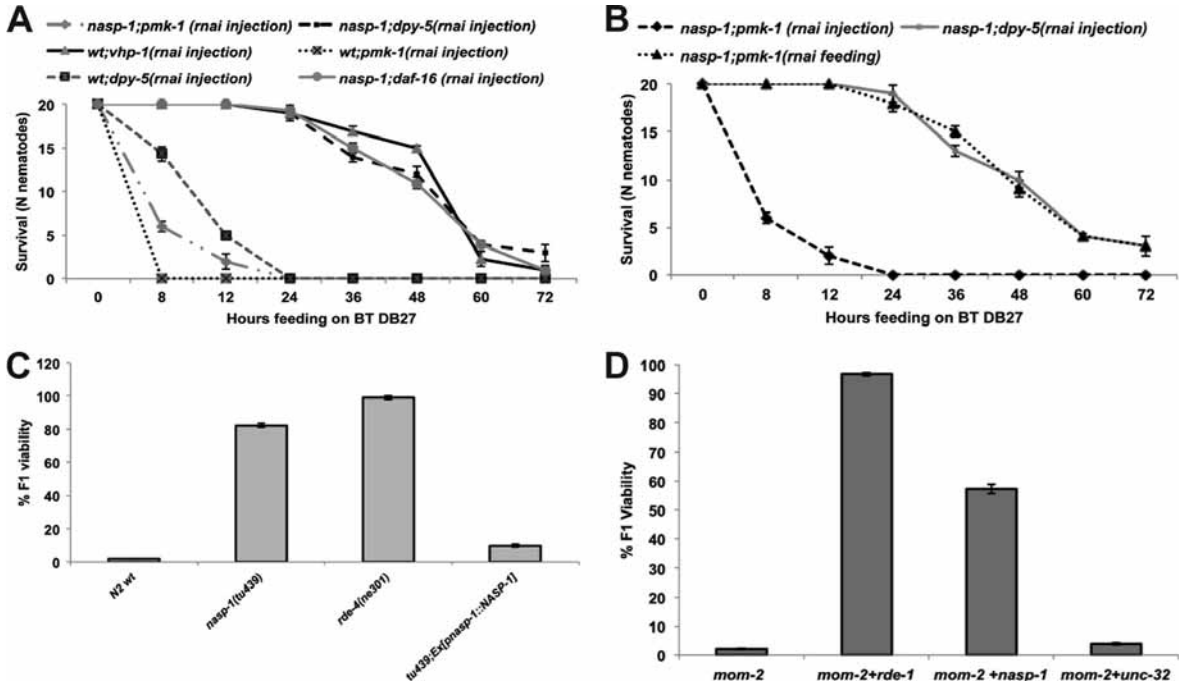

FIG 4 *nasp-1* mutant exhibits RNAi deficiency and requires PMK-1 but not DAF-16 for DB27 resistance. (A) *pmk-1* knockdown upon RNAi by injection leads to hypersusceptibility in wild-type (wt) worms ($P < 0.0001$ for wt;*dpy-5* versus wt;*pmk-1* worms) and abolishes the resistance of the *nasp-1* mutant ($P < 0.0001$ for *nasp-1*;*dpy-5* versus *nasp-1*;*pmk-1* mutant worms) to *B. thuringiensis* (BT) DB27 below the level of wild-type survival ($P < 0.05$ for *nasp-1*;*pmk-1* versus wt worms). *daf-16* knockdown upon RNAi by injection has no effect on *nasp-1* mutant ($P > 0.05$ for *nasp-1*;*dpy-5* versus *nasp-1*;*daf-16* mutant worms) resistance to *B. thuringiensis* DB27. *vhp-1* RNAi significantly ($P < 0.0001$) increases the survival of wild-type worms on a lawn of *B. thuringiensis* DB27 compared to control *dpy-5* RNAi, which was used as a positive control for injection and as a negative control in a survival assay (see Materials and Methods). (B) *pmk-1* knockdown upon RNAi by feeding has no effect on *nasp-1*-mediated resistance to *B. thuringiensis* DB27 ($P > 0.05$), while *pmk-1* knockdown upon RNAi by injection abolishes the resistance of the *nasp-1* mutant ($P < 0.0001$). The data shown are means ± the standard errors of the means. For each condition in survival assays, 60 to 80 worms (20 per plate) were used for each of multiple independent biological replicates. (C) *nasp-1* mutant is RNAi deficient. Wild-type and *nasp-1* and *rde-4* (positive control) mutant worms and *nasp-1* rescuing line worms were fed *E. coli* expressing dsRNA of *mom-2*, which is essential for viability. Viability was assessed as the proportion of hatched larvae relative to the total number of eggs laid. Both *rde-4* and *nasp-1* mutants show significantly ($P < 0.0001$) higher survival of progeny than the wild type, indicating that both mutants are resistant to RNAi-mediated *mom-2* lethality. *nasp-1* overexpression restores the *nasp-1*(*tu439*) mutant's susceptibility to *mom-2*-mediated lethality. The data shown are means ± the standard errors of the means. (D) Wild-type hermaphrodites were coinjected with each candidate dsRNA and *mom-2* dsRNA. *rde-1* RNAi was used as a positive control, while *unc-32* RNAi was used as a negative control. *nasp-1* RNAi significantly increases F_1 viability ($P < 0.0001$) relative to that of the negative control or *mom-2* RNAi alone. The data shown are means ± the standard errors of the means.

The p38 MAPK pathway is required for the *nasp-1* mutant's resistance. Next, we searched for potential interactions of *nasp-1* with known pathogen response regulators to further explore its biological role. First, we tested the p38 mitogen-activated protein kinase (MAPK) pathway (52) by performing injection of *pmk-1* dsRNA into a *nasp-1* mutant background. After *pmk-1* depletion, the resistance of the *nasp-1* mutant to *B. thuringiensis* DB27 is completely abolished, even below the wild-type level (Fig. 4A). Similarly, overactivation of PMK-1 by *vph-1* RNAi (53) strongly increased the survival of *C. elegans* on *B. thuringiensis* DB27 (Fig. 4A). In contrast, injection of *dpy-5* as a control dsRNA had no effect on the survival of wild-type and *nasp-1* mutant worms, although it did result in a dpy phenotype (Fig. 4A). Together, these results suggest that the effects of *pmk-1* and *vhp-1* on survival are gene specific and that *nasp-1* potentially may function in parallel with the p38 MAPK pathway. In contrast, RNAi of *daf-16*, another *C. elegans* innate immunity regulator (33), had no effect on the *nasp-1* mutant's resistance to *B. thuringiensis* DB27 (Fig. 4A), indicating that *nasp-1* does not confer resistance to *B. thuringiensis* DB27 by acting through *daf-16*/FOXO. This result also provides an additional argument that the *nasp-1* mutant's resistance is independent of reproduction, because increased pathogen resistance of most of the sterile mutants requires *daf-16* (47).

***nasp-1* mutants are deficient for RNAi by feeding.** When performing the RNAi experiments described above, we noticed that a knockdown of genes by RNAi in a *nasp-1* mutant background was only possible by injection of dsRNA, suggesting that the *nasp-1* mutant exhibits a sid phenotype (54). In contrast, RNAi by feeding in the *nasp-1* mutant background did not have the same effect (Fig. 4B). Specifically, *pmk-1* RNAi by feeding did not change the *nasp-1* mutant's resistance phenotype, whereas *pmk-1* RNAi by injection abolished the *nasp-1* mutant's resistance to *B. thuringiensis* DB27 completely (Fig. 4B). Similarly, we observed that *dpy-5* RNAi by injection resulted in a dpy phenotype in a *nasp-1* mutant background, whereas *dpy-5* RNAi by feeding did not (data not shown). These observations provide first evidence that *nasp-1* mutants might be defective in their RNAi response; however, they do not exhibit a complete rde phenotype but rather show similarity to sid mutants.

To further explore this phenotype, we fed *nasp-1* mutants with *E. coli* expressing dsRNA against *mom-2*, a gene essential for viability (40, 55). Almost no viable progeny were obtained from wild-

type worms, whereas nearly 80% of the *nasp-1* mutant progeny survived *mom-2* RNAi by feeding (Fig. 4C). Transgenic expression of *nasp-1* under the control of its endogenous promoter in the *tu439* mutant background resulted in almost complete restoration of the wild-type response to *mom-2*-mediated lethality. *rde-4* mutants, which are known to be RNAi deficient, were used as a control and showed nearly 100% viability (Fig. 4C). Additionally, we coinjected wild-type worms with dsRNA of *mom-2* and/or dsRNA of *nasp-1* or control dsRNA and scored the viability of the progeny. The survival of progeny indicates that RNAi against *nasp-1* enables worms to overcome the lethality of *mom-2* RNAi (Fig. 4D). Worms coinjected with dsRNA of a positive-control gene, *rde-1*, showed 97% survival, indicating strong protection from *mom-2* lethality by *rde-1* RNAi. Coinjection with dsRNA of *unc-32* or other genes dispensable for RNAi resulted in a very low survival rate. In contrast, coinjection of *nasp-1* dsRNA significantly rescued the lethality caused by *mom-2* dsRNA. Thus, in contrast to RNAi mutants, such as *rde-1* RNAi mutants, that are resistant to RNAi independently of the delivery method (56), *nasp-1* mutants are still able to perform RNAi when dsRNA is injected into the germ line. In this, they are similar to sid mutants (54), suggesting that they function primarily in the uptake or spreading of dsRNA rather than in modulation of the core RNAi machinery.

To provide further insight into this phenotype, we generated transgenic worms expressing a *nasp-1*-encoded translational protein fusion with GFP to study the site of action of *nasp-1*. Interestingly, expression was localized primarily in the pharynx (see Fig. S3 in the supplemental material). Expression was seen in the metacorpus and terminal bulb of the pharynx with a clear intracellular localization. Given that the pharynx is exposed to pathogens, this observation further supports the idea that *nasp-1* functions in innate immunity; however, the link to the sid phenotype remains unknown. Taken together, these results support the observation that *nasp-1* is required for a robust RNAi response.

The transcriptional response of *nasp-1* mutants to *B. thuringiensis* DB27 reveals a strong overlap with *dcr-1* mutants. To identify genes differentially regulated in *nasp-1* mutants when they are exposed to *B. thuringiensis* DB27, we used whole-genome microarrays on an Agilent platform. Microarray analysis revealed that differential expression in *nasp-1* mutants mostly resulted in downregulation (247 genes), whereas only 31 genes were more strongly expressed in *nasp-1* mutants than in wild-type worms (see Data Set S1 in the supplemental material, FDR-corrected *P* value of $\leq$0.05, fold change cutoff of 1.5 on a $\log_2$ scale). We validated these results by selecting a total of 12 down- and upregulated genes and confirmed their expression by qRT-PCR (Fig. 5A). Interestingly, 58 of the 247 downregulated genes were previously identified as genes that are induced by *B. thuringiensis* DB27, in comparison to OP50, in wild-type worms (11) (Fig. 5B). These results, together with the finding that most expression changes in *nasp-1* mutants result in transcriptional repression, suggest that NASP-1 normally acts as an activator of transcription.

Surprisingly, further characterization of downregulated genes in *nasp-1* mutants revealed a highly significant overlap with genes regulated by different components of the RNAi machinery (57), such as *rde-4*, *rde-3*, and *rrf-1* (see Fig. S4 in the supplemental material). The most significant enrichment was found for genes previously annotated as targets of the only *C. elegans* dicer gene, *dcr-1*. Specifically, 116 of the 247 *nasp-1*-repressed genes were previously identified as *dcr-1*-regulated genes (Fig. 5C). This strong overlap is further supported by quantitative PCR experiments showing that *dcr-1* expression is itself reduced in *nasp-1* mutants compared to that in the wild type (Fig. 5A). These findings are consistent with the reduced life span and RNAi deficiency of *nasp-1* mutants (see above). Thus, an intriguing possibility is that repression of DCR-1 function is responsible for the increased pathogen resistance observed in *nasp-1* mutants.

***dcr-1* is involved in innate immunity through its role in miRNA processing.** The unexpected correlation between downregulated genes in *nasp-1* and *dcr-1* mutants encouraged us to look for further interactions between these two loci. We hypothesized that if reduced DCR-1 function in *nasp-1* mutants were responsible for their increased resistance to *B. thuringiensis* DB27, *dcr-1* mutants should also be more resistant to the pathogen. Indeed, when we tested several *dcr-1* alleles, we found that the mutant for presumptive null allele *dcr-1*(*ok247*) showed greater survival on *B. thuringiensis* DB27 than the wild type (Fig. 6A). This is unlikely to be due to altered reproduction because the *dcr-1*(*ok247*) mutant is not resistant to *S. marcescens* (see Fig. S5A in the supplemental material) and *P. aeruginosa* (see Fig. S5B) and exhibits a reduced life span (58), in contrast to sterile mutants (59, 60). As the *dcr-1*(*ok247*) deletion allele was marked with a recessive allele of *unc-32* to facilitate the isolation of *dcr-1*(*ok247*) homozygous worms (57) and considering that the *unc-32* mutant was shown to have increased resistance to *Erwinia carotovora* and *Photorhabdus luminescens* (8), we also tested the survival of *unc-32* mutants on *B. thuringiensis* DB27 and found that they have a wild-type response to the pathogen (Fig. 6A). Furthermore, transgenic expression of *dcr-1* in a *dcr-1*(*ok247*) mutant restored wild-type susceptibility to *B. thuringiensis* DB27 (Fig. 6A). Together, these findings indicate a novel role for *dcr-1* in innate immunity to the pathogen *B. thuringiensis* DB27.

Interestingly, the effect of *dcr-1* on pathogen resistance is allele specific. We found that survival on *B. thuringiensis* DB27 was unchanged in the mutant with the activating *dcr-1*(*mg375*) allele (Fig. 6A). The *dcr-1*(*mg375*) allele has an impaired helicase domain and small interfering RNA (siRNA) production and, consequently, enhanced RNAi, whereas miRNA synthesis is intact (61). To further test the idea that resistance to *B. thuringiensis* DB27 is associated with altered miRNA processing, we investigated the effects of the *rde-4* and *rde-1* mutations on pathogen survival. *rde-1* and *rde-4* play a role in RNAi but not in miRNA processing (57), and they were shown to produce a reduced life span but normal survival on *S. marcescens*, although the immune response is upregulated (57). Indeed, *rde-1* and *rde-4* mutants did not show increased resistance to *B. thuringiensis* DB27 (Fig. 6B). In contrast, mutants with changes in the *drsh-1* and *alg-1* genes, which are involved in miRNA processing (62, 63), but not in RNAi, exhibited increased resistance to *B. thuringiensis* DB27 (Fig. 6C). To prove that the resistance of *drsh-1* and *alg-1* mutants is not due to impaired germ line development and fertility, we rendered both mutants sterile via *cdc-25* RNAi and compared their survival to that of control vector-treated worms. The survival of *alg-1* mutants was significantly increased after *cdc-25* RNAi compared to that of vector-treated *alg-1* mutants and compared to wild-type worms treated with either the empty vector or *cdc-25* RNAi (Fig. 6D), indicating that *alg-1* and *cdc-25* mutants exhibit resistance via distinct mechanisms and that *alg-1*-mediated resistance to *B. thuringiensis* DB27 is not simply a consequence of altered fertility. However, we did not observe any differences in survival between

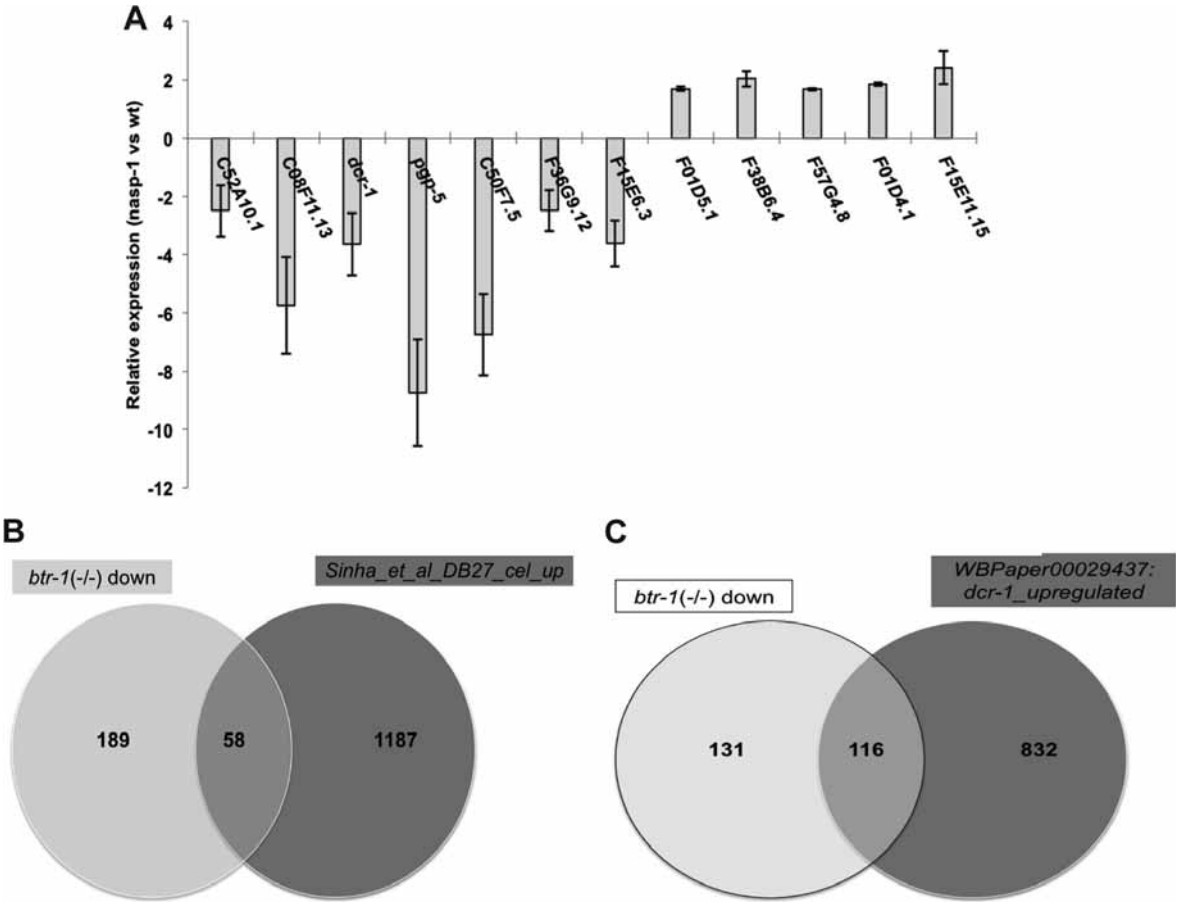

FIG 5 Transcriptional response of *nasp-1* mutant worms to *B. thuringiensis* DB27 infection. (A) qRT-PCR confirmation of microarray results. Wild-type (wt) and *nasp-1* mutant worms were exposed to *B. thuringiensis* DB27 for 4 h and used for RNA isolation. The data shown are the average of two independent RNA isolations. Each transcript was measured in triplicate and normalized to a control gene. Error bars show the standard errors of the means. (B) Venn diagram of the genes downregulated in the *nasp-1* mutant and upregulated in wild-type worms upon exposure to *B. thuringiensis* DB27 (11). The overlap is significant ($P = 1.11E-15$). (C) Venn diagram showing the overlap between genes downregulated in the *nasp-1* mutant and genes upregulated in the *dcr-1* mutant (47). The overlap is significant ($P = 3.70E-79$).

drsh-1 vector- and *drsh-1 cdc-25* RNAi-treated worms (Fig. 6E), suggesting that the increased resistance of *drsh-1* mutants to *B. thuringiensis* DB27 is indeed a secondary effect of sterility. Taken together, these results suggest that the impairment of DCR-1 function in miRNA processing confers resistance to *B. thuringiensis* DB27. Furthermore, these data, together with the downregulation of *dcr-1* expression in *nasp-1* mutants, support the conclusion that the resistance of *nasp-1* mutants is due to reduced DCR-1 activity.

***nasp-1* acts through DCR-1/Dicer.** The experiments described above are consistent with the hypothesis that *dcr-1* is genetically downstream of *nasp-1*. This conclusion is further supported by previous expression profiling of *dcr-1* mutants, which did not show any change in the expression of *nasp-1* in *dcr-1* mutants (57). To further test this hypothesis, we investigated whether overexpression of *dcr-1* would abolish the increased resistance of the *nasp-1* mutant to *B. thuringiensis* DB27. Indeed, *dcr-1* overexpression under the control of its endogenous promoter significantly reduced the survival of *nasp-1* mutant worms on *B. thuringiensis* DB27 (Fig. 6F). Additionally, *dcr-1* overexpression restored the reduced life span of *nasp-1* mutants (see Fig. S6 in the supplemental material). Given that *C. elegans dcr-1* is expressed at high levels in the intestine (58), which is also the major site of infection by ingested pathogens, we investigated whether upregulation of *dcr-1* specifically in the intestine could affect the *nasp-1* mutant's pathogen resistance. When we overexpressed *dcr-1* under the control of the intestine-specific *cpr-1* promoter, we noticed a significant decrease in the *nasp-1* mutant's survival on *B. thuringiensis* DB27 (Fig. 6F). Thus, the previously unknown role of DCR-1 in *C. elegans* innate immunity is genetically downstream of *nasp-1* and acts through the *C. elegans* intestine.

NASP-1 and Dicer resistance to *S. aureus* is due to impaired fecundity. Considering that the *nasp-1* mutant shows increased resistance to *S. aureus*, we investigated whether it is also mediated by the same mechanism that is employed in the defense against *B. thuringiensis* DB27. Given that the *nasp-1* mutant has reduced fecundity, a common feature of *S. aureus*-resistant mutants (47), we first tested if the *nasp-1* mutant's resistance to *S. aureus* depends on reproduction. As shown in Fig. 7A, *nasp-1* mutants injected with *cdc-25* interfering dsRNA survived *S. aureus* exposure longer than control-injected worms. However, their survival was similar to that of wild-type worms injected with *cdc-25* interfering dsRNA (Fig. 7A). Thus, *nasp-1* mutants do not have an additive effect with *cdc-25* RNAi for *S. aureus* resistance, as is the case for *B.*

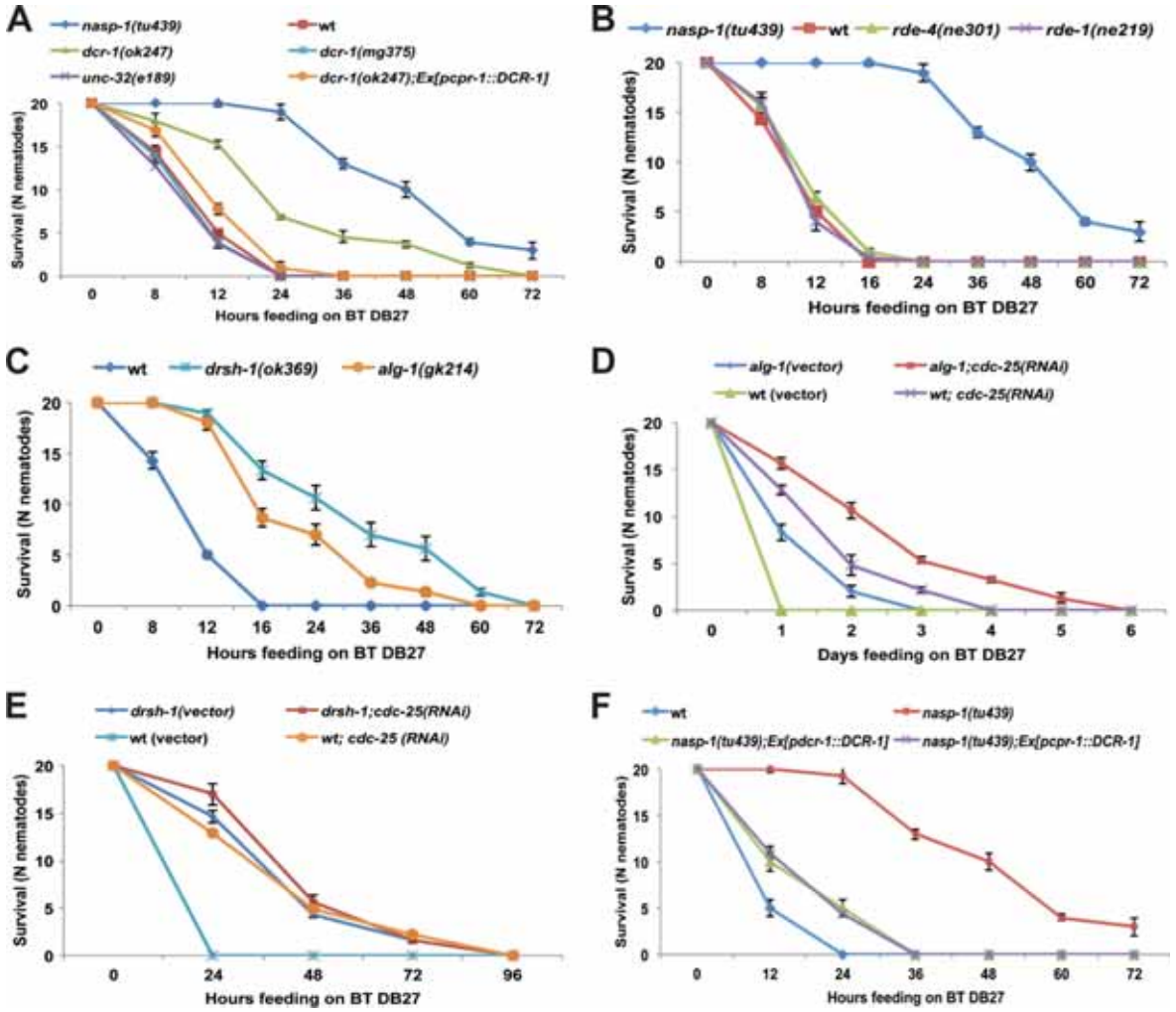

FIG 6 Repression of DCR-1 activity in miRNA processing but not in RNAi results in increased resistance to *B. thuringiensis* DB27. (A) The survival of different *dcr-1* alleles on a lawn of *B. thuringiensis* (BT) DB27 in comparison with wild-type (wt) and *nasp-1* mutant worms. *dcr-1*(*ok247*) mutant worms are significantly ($P < 0.0001$) more resistant to *B. thuringiensis* DB27 than wild-type worms are. *dcr-1*(*mg375*) mutant worms show wild-type susceptibility to the pathogen ($P > 0.05$). Transgenic expression of *dcr-1* rescues the increased resistance of the *dcr-1*(*ok247*) mutant to *B. thuringiensis* DB27. (B) RNAi-defective *rde-4*(*ne301*) and *rde-1*(*ne219*) mutants show survival comparable to that of wild-type worms ($P > 0.05$) upon exposure to *B. thuringiensis* DB27. (C) Survival of miRNA-deficient mutants upon exposure to *B. thuringiensis* DB27. *alg-1*(*gk214*) and *drsh-1*(*ok369*) mutants show resistance to DB27 greater than that of wild-type worms ($P < 0.0001$). (D, E) Wild-type, *alg-1* mutant (D), and *drsh-1* mutant (E) nematodes were fed with *cdc-25* RNAi vector to obtain sterile worms or with empty RNAi vector and exposed to DB27. *cdc-25* RNAi significantly increases the resistance of wild-type ($P < 0.0001$ for wt;vector versus wt;*cdc-25* worms) and *alg-1* (D) ($P = 0.002$ for *alg-1*;vector versus *alg-1*;*cdc-25*) worms but not *drsh-1* mutants (E) ($P > 0.05$ for *drsh-1*;vector versus *drsh-1*;*cdc-25* worms). The survival of *alg-1*;*cdc-25* mutant worms is significantly different ($P < 0.05$) from that of wt;*cdc-25* worms. The data shown are means ± the standard errors of the means. (F) Transgenic overexpression of *dcr-1* abolishes the resistance of the *nasp-1* mutant to *B. thuringiensis* DB27. The resistance of *nasp-1* mutants with transgenically expressed *dcr-1* under the control of its endogenous promoter (p*dcr-1*) or an intestine-specific promoter (p*cpr-1*) is significantly lower than that of *nasp-1* mutants ($P < 0.0001$). For each condition in survival assays, 60 to 80 worms (20 per plate) were used for each of multiple independent biological replicates. The data shown are means ± the standard errors of the means.

thuringiensis DB27 (Fig. 2C). These findings suggest that *nasp-1* and *cdc-25* RNAi increase resistance to *S. aureus* via the same mechanism and the *nasp-1* mutant's resistance to *S. aureus* is very likely due to partial sterility. In contrast, *nasp-1* and *cdc-25* RNAi function in independent pathways for *B. thuringiensis* DB27 resistance (Fig. 2).

This difference between *S. aureus* and *B. thuringiensis* DB27 was further explored by studying the allele specificity of Dicer mutants. There was no allele specificity in terms of Dicer resistance to *S. aureus* since both *dcr-1*(*ok247*) and *dcr-1*(*mg375*) mutant worms were more resistant to *S. aureus* than wild-type worms were (Fig. 7B). Also, *dcr-1* mutant resistance can be rescued by transgenic overexpression of *dcr-1* under the control of its endogenous promoter (Fig. 7B), indicating that Dicer might play a role in the *C. elegans* defense against *S. aureus*. Considering the reproductive defects of *dcr-1* mutants, which may contribute to increased resistance to *S. aureus*, we used *cdc-25* RNAi to sterilize *dcr-1*(*mg375*) mutant worms. The survival of *mg375* mutant worms was greater than that of vector-treated worms but was not significantly different from that of wild-type worms treated with *cdc-25* RNAi, indicating the absence of a cumulative effect of *dcr-1* and *cdc-25* (Fig. 7C). Thus, the *dcr-1* mutant's resistance to *S. aureus* is due to diminished reproduction, as in the case of the *nasp-1* mutant. We also assessed the survival of RNAi- and

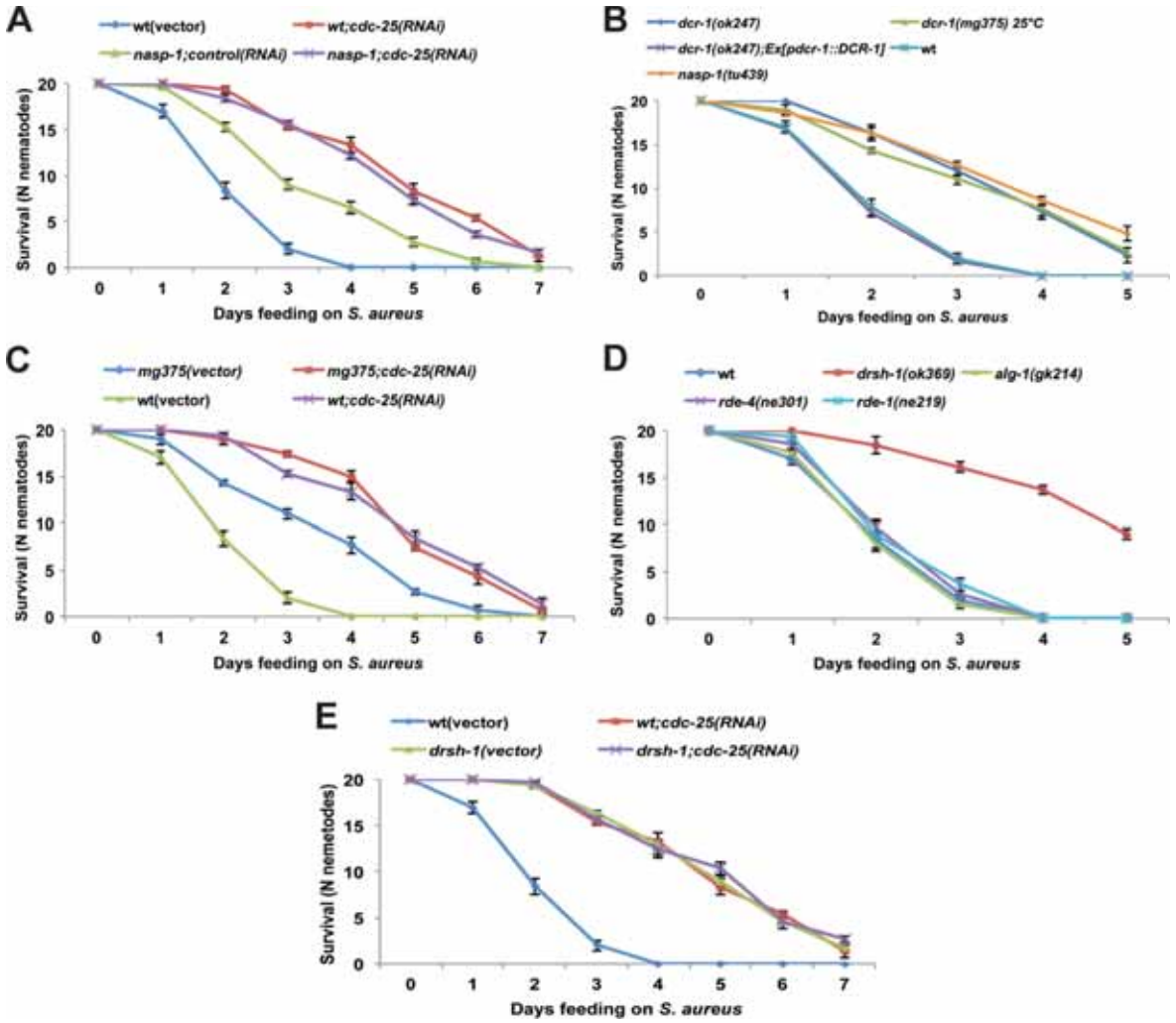

FIG 7 *nasp-1*- and *dcr-1*-mediated resistance to *S. aureus* is due to partial sterility. (A) Wild-type (wt) and *nasp-1* mutant nematodes were injected with *cdc-25* interfering dsRNA to obtain sterile worms or with control dsRNA (see Materials and Methods) and exposed to *S. aureus*. *cdc-25* RNAi significantly increases the resistance of wild-type ($P < 0.0001$ for wt;vector versus wt;*cdc-25* worms) and *nasp-1* mutant ($P < 0.0001$ for *nasp-1*;control versus *nasp-1*;*cdc-25* worms) worms. The survival of *nasp-1*;*cdc-25* mutant worms is not significantly different ($P > 0.05$) from wt;*cdc-25* worms. The data shown are means ± the standard errors of the means. (B) Both *dcr-1* mutants *ok247* and *mg375* grown at 25°C to induce sterility exhibit increased resistance to *S. aureus* ($P < 0.0001$ for *ok247* mutant versus wt worms; $P < 0.001$ for *mg375* mutant versus wt worms), which can be rescued by transgenic expression of *dcr-1*. The *ok247* and *mg375* mutants do not differ in the level of resistance ($P > 0.05$). The data shown are means ± the standard errors of the means. (C) Wild-type and *dcr-1*(*mg375*) mutant nematodes were fed with *cdc-25* RNAi vector to obtain sterile worms or with empty RNAi vector (see Materials and Methods) and exposed to *S. aureus*. *cdc-25* RNAi significantly increases the resistance of wild-type ($P < 0.0001$ for wt;vector versus wt;*cdc-25* worms) and *mg375* ($P = 0.018$ for *mg375*;vector versus *mg375*;*cdc-25* mutant worms) worms. The survival of *mg375*;*cdc-25* mutant worms is not significantly different ($P > 0.05$) from that of wt;*cdc-25* worms. The data shown are means ± the standard errors of the means. (D) Survival of wild-type worms and RNAi- and miRNA-deficient mutants upon exposure to *S. aureus*. Only *drsh-1*(*ok369*) mutant worms showed greater *S. aureus* resistance than wild-type worms ($P < 0.0001$). The data shown are means ± the standard errors of the means. (E) Wild-type and *drsh-1* mutant nematodes were fed with *cdc-25* RNAi vector to obtain sterile worms or with empty RNAi vector and exposed to *S. aureus*. *cdc-25* RNAi significantly increases the resistance of wild-type ($P < 0.0001$ wt;vector versus wt;*cdc-25* worms) but not *drsh-1* mutant ($P > 0.05$ *drsh-1*;vector versus *drsh-1*;*cdc-25* mutant worms) worms. The survival of *drsh-1*;*cdc-25* mutant worms is not significantly different ($P > 0.05$) from that of wt;*cdc-25* worms. For each condition in survival assays, 60 to 80 worms (20 per plate) were used for each of multiple independent biological replicates. The data shown are means ± the standard errors of the means.

miRNA-deficient mutants on *S. aureus*. Both *rde-1* and *rde-4* mutants showed a wild-type response to *S. aureus* (Fig. 7D). The *alg-1* mutant, which has increased survival on *B. thuringiensis* DB27, exhibited survival similar to that of the wild type on *S. aureus* (Fig. 7D), while only the *drsh-1* mutant was more resistant to *S. aureus* infection (Fig. 7D). However, as in the case of *B. thuringiensis* DB27, the increased resistance seen is the consequence of sterility since *cdc-25* RNAi did not further increase the survival of *drsh-1* mutants (Fig. 7E). Taken together, these results show that the *nasp-1* mutant's resistance to *S. aureus* is very likely due to impaired fecundity and at this stage it is impossible to disconnect immune and reproductive mechanisms that confer the *nasp-1* mutant's resistance to *S. aureus*.

The collagen gene *col-92* acts downstream of *nasp-1* and confers resistance to DB27. Finally, we wanted to uncover the molecular mechanisms of *nasp-1*- and *dcr-1*-mediated resistance to *B. thuringiensis* DB27. For this, we hypothesized that the repression of *dcr-1* activity in *nasp-1* mutants leads to overactivation of effec-

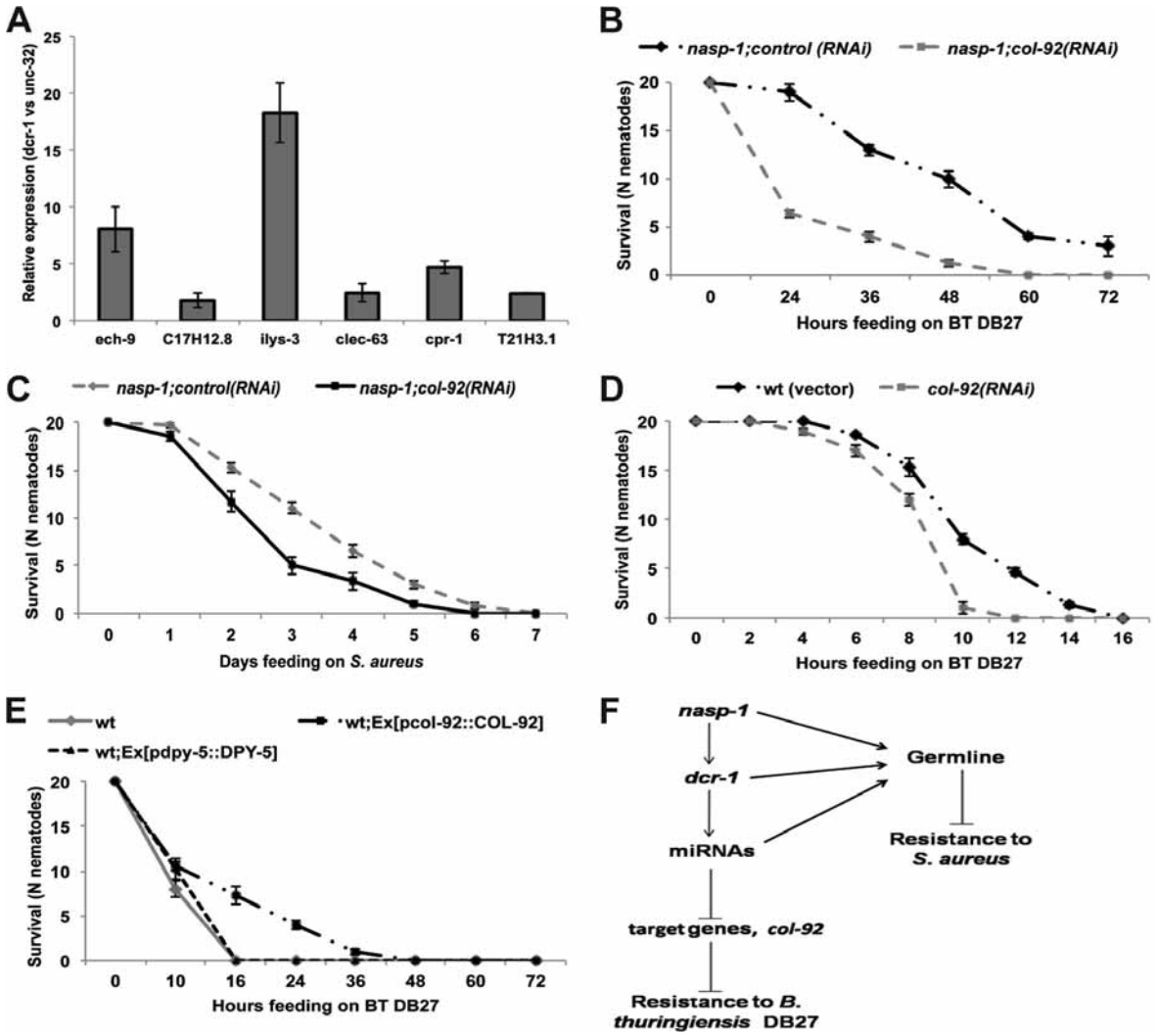

FIG 8 Collagen gene *col-92* acts downstream of *nasp-1* and is required for resistance to *B. thuringiensis* (BT) DB27. (A) Some of the DB27 response transcripts (selected from reference 11) show high expression in *dcr-1* mutant worms as determined by qRT-PCR. The data shown are averages of two independent RNA isolations. Each transcript was measured in triplicate and normalized to a control gene. Error bars show the standard errors of the means. (B) Injection of *col-92* interfering dsRNA significantly ($P < 0.001$) suppresses the *nasp-1* mutant's resistance to *B. thuringiensis* DB27 compared to the injection of a control gene (*dpy-5*). The data shown are means ± the standard errors of the means. (C) Injection of *col-92* interfering dsRNA significantly ($P = 0.021$) decreases the *nasp-1* mutant's resistance to *S. aureus* compared to the injection of a control gene (*dpy-5*). The data shown are means ± the standard errors of the means. (D) RNAi knockdown of *col-92* increases the susceptibility of wild-type (wt) worms to *B. thuringiensis* DB27 infection ($P < 0.01$). The data shown are means ± the standard errors of the means. (E) Transgenic expression of *col-92* but not of *dpy-5* significantly ($P < 0.02$) increases the survival of worms exposed to *B. thuringiensis* DB27. The data shown are means ± the standard errors of the means. (F) Mechanism by which *nasp-1* may regulate the innate immune response to *B. thuringiensis* DB27.

tor genes, which make *C. elegans* more resistant to infection. Consistent with this idea, we found that several *B. thuringiensis* DB27-responsive genes are more strongly expressed in *nasp-1* and *dcr-1* mutants than in the wild type (Fig. 8A). To test this hypothesis further, we used RNAi to knock down *nasp-1*-upregulated genes individually (see Data Set S1 in the supplemental material) in the *nasp-1* mutant background. While the knockdown of the majority of these genes resulted in no obvious effect on *nasp-1*-mediated resistance (see Fig. S7 in the supplemental material), *col-92* RNAi produced significant suppression of *nasp-1*-mediated resistance to DB27 (Fig. 8B; see Fig. S7) and *S. aureus* (Fig. 8C). Furthermore, knockdown of *col-92* in wild-type worms makes them hypersusceptible to *B. thuringiensis* DB27 (Fig. 8D), *S. aureus*, and *P. aeruginosa* (see Fig. S8A and B) but not to *S. marcescens* (see Fig. S8C). However, the life span of *col-92* RNAi worms was not affected (see Fig. S8D), suggesting that its hypersusceptibility to bacterial infection is not a result of impaired fitness. In contrast, *col-92* overexpression under the control of its endogenous promoter increases resistance to *B. thuringiensis* DB27 (Fig. 8E) and *S. aureus* (see Fig. S8E) infections. At the same time, overexpression of *dpy-5*, used as a control, resulted in no obvious effects on resistance to *B. thuringiensis* DB27 (Fig. 8E) and *S. aureus* (see Fig. S8E). Moreover, *dpy-5* RNAi and *col-93* RNAi had no effect on *C. elegans* survival upon exposure to *B. thuringiensis* DB27, indicating that the effect of *col-92* is specific and is unlikely to be due only to simple alteration of cuticle structure.

To shed more light on the immune function of *col-92*, we studied the expression of the gene. It should be noted that COL-92 is a

member of a nematode-specific family of collagens, characterized by a cuticle collagen N-terminal domain. Transgenic lines harboring a *col-92*–GFP transcriptional fusion construct showed that the gene is expressed predominantly in the hypodermis (see Fig. S9 in the supplemental material). Therefore, although *col-92* is a downstream target of NASP-1, its immune function awaits further characterization and might be associated with cuticle. Thus, the previously uncharacterized collagen gene *col-92* plays a pivotal role in the *C. elegans* defense against bacterial infection and represents a downstream target of NASP-1 and DCR-1.

DISCUSSION

In this study, we isolated a *B. thuringiensis* DB27-resistant mutant in *C. elegans* that we showed to be identical to *nasp-1*, a gene that has previously been implicated in female development (51). Both *nasp-1* alleles are reduction-of-function but not null alleles. Further characterization of *nasp-1* helped to discover a previously unknown function of *dcr-1*/Dicer in the immune response of *C. elegans*. Mammalian NASP1 is a tetratricopeptide (TPR) repeat domain-containing H1 linker histone binding protein that is part of a multichaperone complex implicated in nucleosome remodeling (64, 65). NASP1 is highly expressed in dividing cells and is required for normal cell cycle progression (65). A role for *C. elegans nasp-1* in chromatin remodeling is supported by its interactions with a variety of chromatin-remodeling proteins like linker H1 histones and histone deacetylase (51). We hypothesize that *nasp-1* might regulate the pathogen response of *C. elegans* via transcription and chromatin-remodeling mechanisms. Strikingly, it was shown that changes in NASP1 expression lead to the misregulation of a variety of genes in HeLa cells, including genes involved in the immune responses (66).

The RNAi deficiency of *C. elegans nasp-1* is similar to that of other chromatin-remodeling genes (40) and provided the entry point for the discovery of the function of *dcr-1* in *C. elegans* innate immunity, the most unexpected finding of this study. DCR-1 is an RNase III endoribonuclease whose primary function is the cleavage of dsRNA into smaller fragments that mediate RNAi (67). DCR-1 is required for miRNA processing and the synthesis of endo-siRNAs implicated in gene silencing and epigenetic regulation (67–69). Previous studies have shown a role for DCR-1 and the RNAi machinery in the *C. elegans* response to viral infections (14, 70). However, our results point toward a broader function of Dicer in innate immunity. To our knowledge, this study is the first to show a direct role for DCR-1 in antibacterial immunity. Results obtained by using different mutant alleles of *C. elegans dcr-1* support the conclusion that its function in pathogen defense is mediated primarily by impaired miRNA biogenesis. Further evidence for this conclusion comes from the analysis of other genes that allow discrimination between miRNA processing and RNAi. Consistent with our conclusion is a recent report showing that miRNA activity in the *C. elegans* intestine is largely dedicated to attenuating the activity of pathogen response genes (48). Specifically, Kudlow et al. (48) have shown that *ain-1* mutants, which have altered miRNA-induced silencing complex activity, are more resistant to *P. aeruginosa* than wild-type worms are. Additionally, those authors were able to identify specific miRNA families that are involved in damping of the immune response in the absence of infection. Together, these findings suggest that intestinal miRNAs act as negative regulators of infection response genes. However, we have been unable to identify a miRNA or siRNA that is likely to be a part of the mechanisms of *nasp-1*-mediated resistance to *B. thuringiensis* DB27. Such studies might be complicated by the fact that redundancy among small RNAs is a well-known phenomenon in *C. elegans*.

The increased resistance of *dcr-1* mutants to *B. thuringiensis* DB27 might be due to the upregulation of pathogen response genes. Indeed, our RT-PCR experiment showed induction of some of the *B. thuringiensis* DB27-responsive genes in *dcr-1* mutants (Fig. 8A), indicating that DCR-1 may function as a suppressor of the *C. elegans* immune response to *B. thuringiensis* DB27 via suppression of miRNA synthesis. By using a reverse genetic approach, we found that one of the *nasp-1*-upregulated genes, namely, *col-92*, is an important determinant of the *nasp-1* mutant's resistance that acts downstream of *nasp-1* and *dcr-1*. Collagens are differentially expressed in response to a variety of microbes (8, 71), suggesting that they may represent an essential part of the immune response. Nematodes, including *C. elegans*, are known for the unusually high number of collagens encoded in their genomes, and collagens have been implicated in life span and immunity (71–74). A potential role for collagens in the immune response might be in part responsible for the high number of nematode collagens, although direct proof of this assumption is hard to provide, even in the age of reverse genetics. One phenomenon that might hinder the identification of specific functions of collagens is redundancy, which is often seen among structurally related genes (73).

Our findings on the role of DCR-1 in *C. elegans* innate immunity strongly correlate with the identification of Dicer as a regulator of the mammalian immune response. Tang and coworkers found that the inhibition of Dicer in human cells upregulates major histocompatibility complex class I-related molecules A and B, which are innate immune system ligands for the NKG2D receptor expressed by natural killer cells (75). Those authors proposed further that a Dicer knockdown is indirectly linked to human innate immunity via the DNA damaging pathway. Whether a similar DNA-damaging pathway is involved in the upregulation of the *C. elegans* innate immune response awaits further analysis. Additionally, various miRNAs have been implicated in the regulation of diverse aspects of human innate immunity (76), including direct microbial killing. For example, *miR-223*-deficient neutrophils were shown to kill *Candida albicans* more effectively then wild-type cells do (77). A role for miRNAs in the human immune system is further supported by evidence that many immunological diseases are caused by alteration of miRNA function (76). Therefore, Dicer and miRNAs might play a conserved role as negative regulators in immunity.

Although alterations of *dcr-1* or miRNA functions are beneficial to *C. elegans* under pathogenic conditions because of immune response activation, this can be deleterious under normal growth conditions. Consistent with this, *nasp-1* mutants showed compromised reproduction, like many other pathogen-resistant mutants (47), indicating that the immune system might compete with the reproductive system for resources. Generally, chronic immune activation has been linked to several pathological disorders, like inflammatory bowel disease in humans (78). Therefore, it is crucial to any organism to have mechanisms of innate immune suppression in the absence of pathogens.

On the basis of our results, we propose the following mechanism for the function of *nasp-1* in the regulation of *C. elegans* innate immunity (Fig. 8F). First, *nasp-1* is a regulator of *dcr-1*. In

nasp-1 mutants, *dcr-1* function is repressed because of chromatin remodeling or related mechanisms, which leads to, besides RNAi deficiency, the inhibition of *dcr-1*-mediated miRNA processing. Consequently, certain miRNAs are not processed and, in turn, target genes, including *col-92*, are more strongly expressed than normal. As a consequence, mutant worms are more resistant to *B. thuringiensis* DB27 infection. Additionally, *nasp-1*, *dcr-1*, and *drsh-1* mutants exhibit increased resistance to *S. aureus* as a secondary consequence of altered reproduction likely because of germ line defects. In general, germ line defects and fecundity might play a role in the observed resistance phenotypes of *nasp-1* and *dcr-1* mutants. It is important to note, however, that *nasp-1 dcr-1* double mutants with *cdc-25* RNAi gave different results for resistance to *B. thuringiensis* DB27 and *S. aureus*, respectively. These findings indicate complex interactions between fecundity and nematode immunity.

Given the comparative framework of our work, what about the specificity of *nasp-1* and the resistance of *P. pacificus* to *B. thuringiensis* DB27? While *nasp-1* is clearly conserved in sequence, the amino acid positions that are changed in the two *C. elegans nasp-1* mutants are not. It is therefore possible that the exact molecular mechanisms of *nasp-1* and its role in nematode immunity did change during the course of evolution. More generally, our parallel work on innate immunity in *P. pacificus* has recently revealed that more general anatomical and physiological parameters are in part responsible for the observed differences between the two species. *P. pacificus* is not equipped with a grinder, as is typical of members of the family Diplogastridae, and so does not lyse its bacterial food in the pharynx. *P. pacificus* mutants hypersusceptible to bacterial pathogens are often Unc mutants with an abnormal defecation cycle, indicating that the regulation of the defecation cycle is crucial for innate immunity (32). Together, this comparative work on innate nematode immunity points toward the correlated action of anatomical, physiological, and molecular mechanisms that control nematode-bacterial interactions and regulate the evolution of innate nematode immunity.

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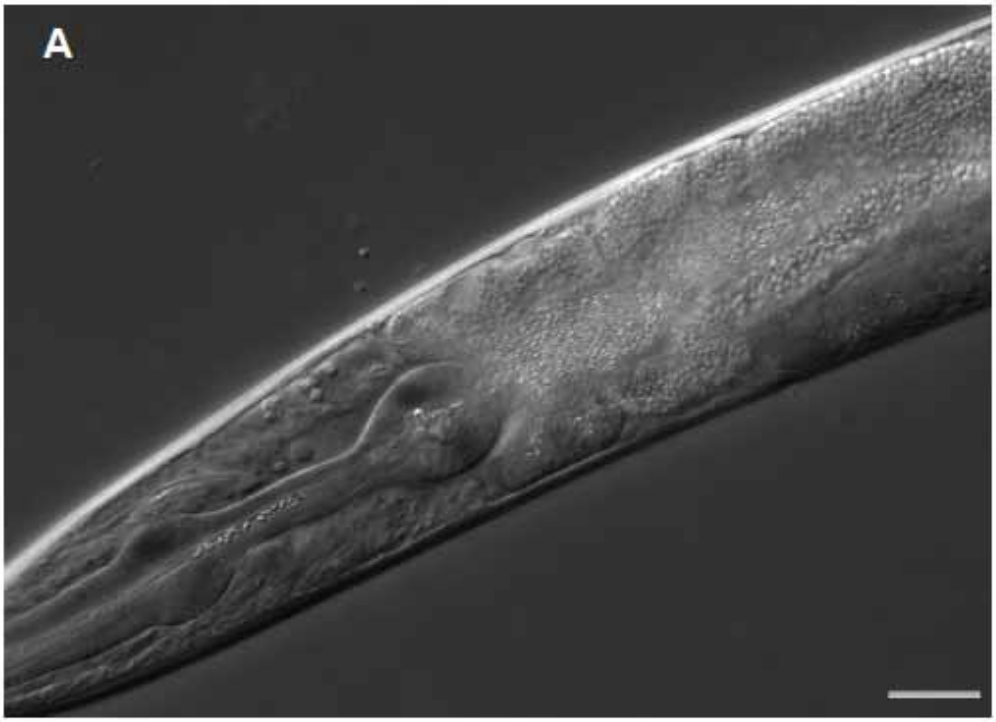
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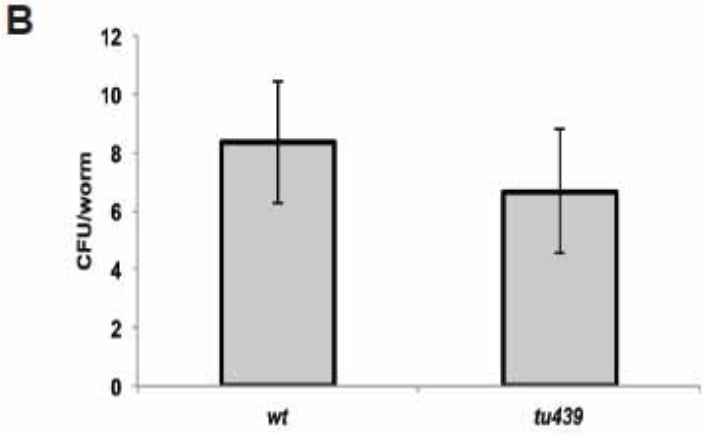
B
CFU/worm
12
10
8
6
4
2
0
wt
tu439

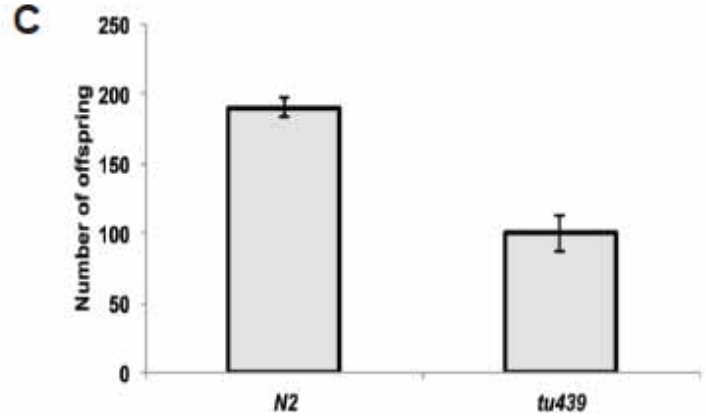
C
Number of offspring
250
200
150
100
50
0
N2
tu439

Fig S1. (A) Wild-type worms fed with DB27 for four hours show accumulation of bacteria in intestine. Scale bar is appr. 20 μm. (B) Wild-type and tu439 mutant were fed with DB27 for four hours and then shifted to OP50 for two hours before measuring CFUs (colony-forming units). Wild-type and tu439 mutant show no differences in the number of DB27 CFUs per worm. CFUs were astimated as described in Garsin et al., 2001. Ten worms were used per each of three replicates and experiment was repeated twice. Data represent mean±SEM. (C) tu439 mutants have reduced fertility compared to wild-type ($p < 0.001$) as determined by counting the number of viable offspring per worm (n=10). Data represent mean±SEM.

Garsin DA, Sifri CD, Mylonakis E, Qin X, Singh KV, Murray BE, Calderwood SB, Ausubel FM. A simple model host for identifying Gram-positive virulence factors. Proc Natl Acad Sci USA. 2001;98(19):10892–10897.

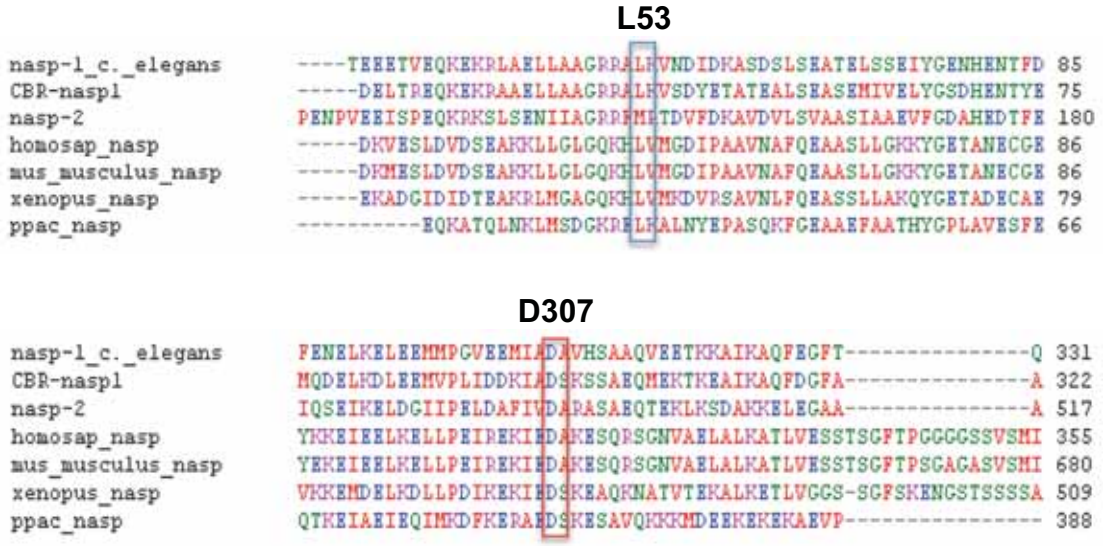

Fig S2. ClustalW multiple sequence alignments of the *C. elegans* NASP-1 and NASP-2 proteins in comparison with closest homologues from other organisms. Only parts of the sequences where the mutations occurred are shown. Both amino acids that were mutated in *nasp-1(tu439)* mutant are marked**.**

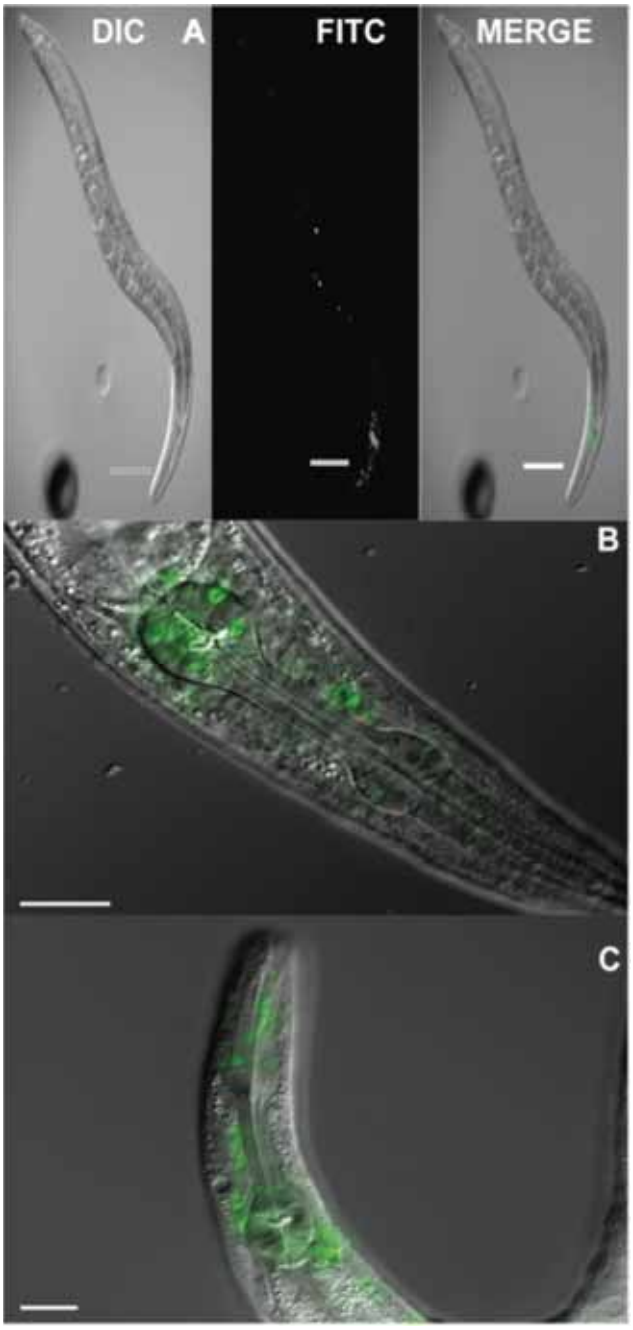

Fig S3. Images of transgenic animals in which GFP was fused with the entire NASP-1 protein. A. (DIC) Nomarski image of the animal, (FITC) Fluorescent image of the same animal, showing NASP-1 expression in the pharynx, (MERGE) Merged image. B. Merged image, showing NASP-1 expression in the terminal bulb of the pharynx. C. Merged image, showing expression in metacoprus and terminal bulb. Scale bar is 100 μm (A) and 20 μm (B, C).

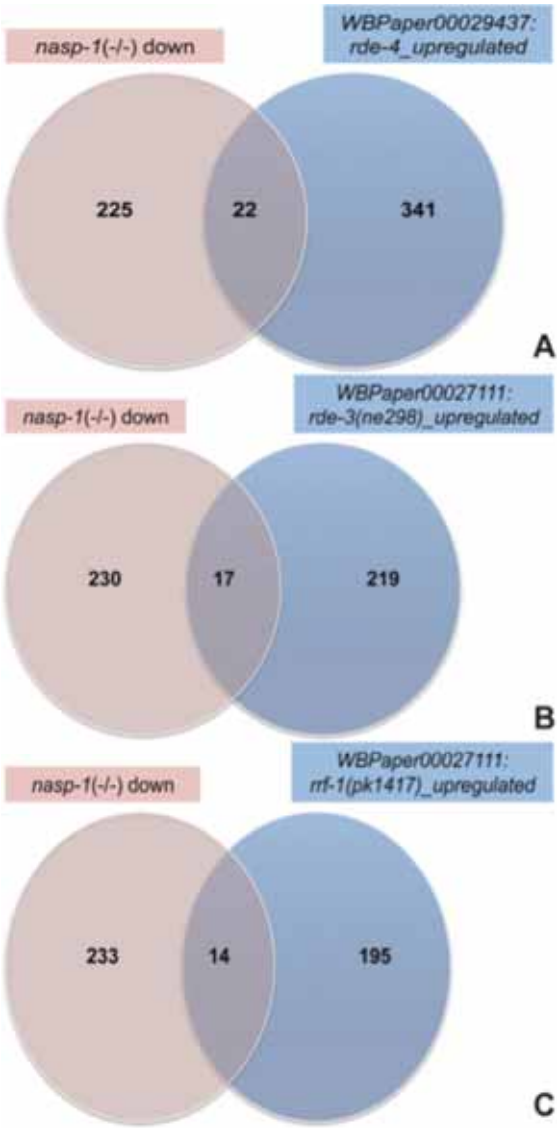

Fig S4. Venn diagrams showing overlap between *nasp-1* down-regulated genes and genes up-regulated in *rde-4* (A, p-value = 1.90E-08), *rde-3* (B, p-value = 7.13E-08) and *rrf-1* (C, p-value = 2.47E-06) mutants.

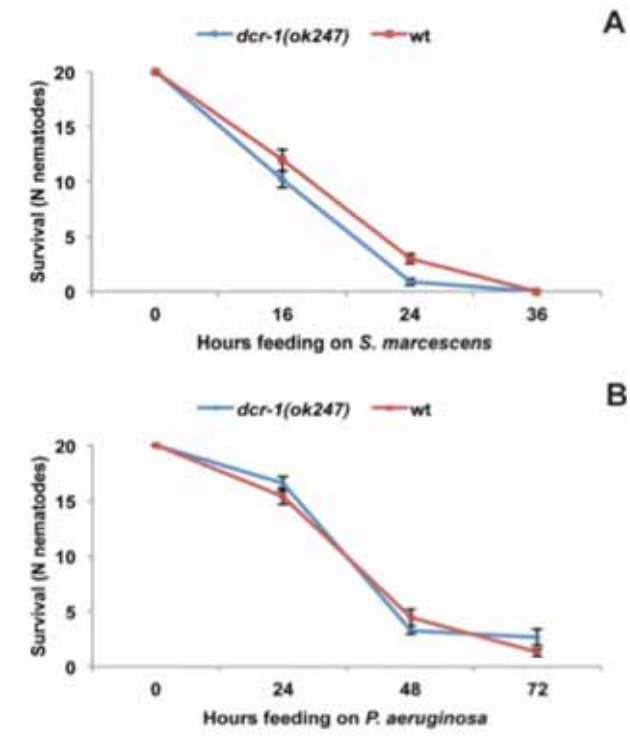

Fig S5. Survival of *dcr-1(ok247)* mutants exposed to *S. marcescens* (A) and *P. aeruginosa* (B) is not different from wild-type survival ($p > 0.05$). Data represent mean±SEM.

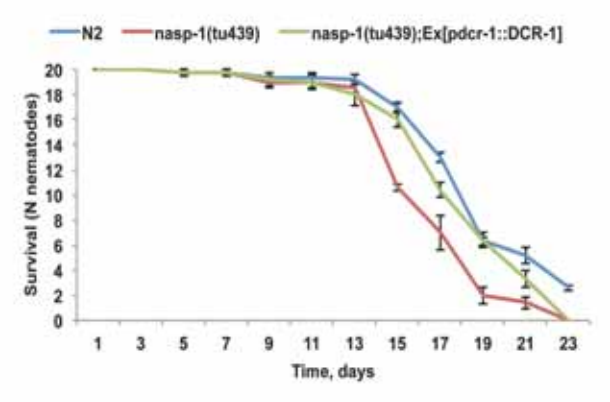

Fig S6. Reduced lifespan of *nasp-1* is rescued by *dcr-1* expression. Transgenic overexpression of *dcr-1* rescues reduced longevity of *nasp-1* mutant on standard food *E. coli* OP50 (wt vs. *nasp-1* transgenic line $p>0.05$; *nasp-1* vs. *nasp-1* transgenic line $p<0.05$). Data represent mean±SEM.

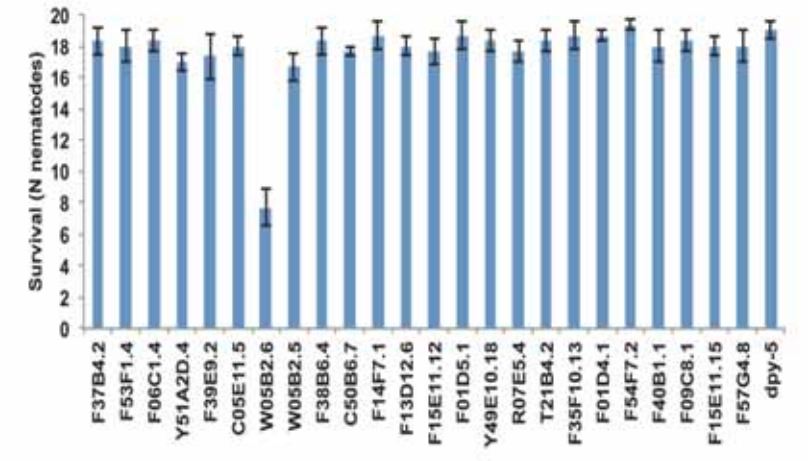

Fig S7. Effect of RNAi knockdown of individual *nasp-1*-up-regulated genes on *nasp-1* survival on the lawn of *B. thuringiensis* DB27. *nasp-1* mutants were injected with dsRNA against candidate genes and survival of F1 progeny was assessed after 24 hours of exposition to *B. thuringiensis* DB27. Animals injected with *dpy-5* dsRNA were used as a control. Only injection of *W05B2.6 (col-92)* dsRNA resulted in significant ($p<0.001$) suppression of *nasp-1* survival compared to control injection. At least 6 animals were injected per each of candidate genes. For survival assay 80-120 F1 animals (20 animals per single plate) were used. Data represent mean±SEM.

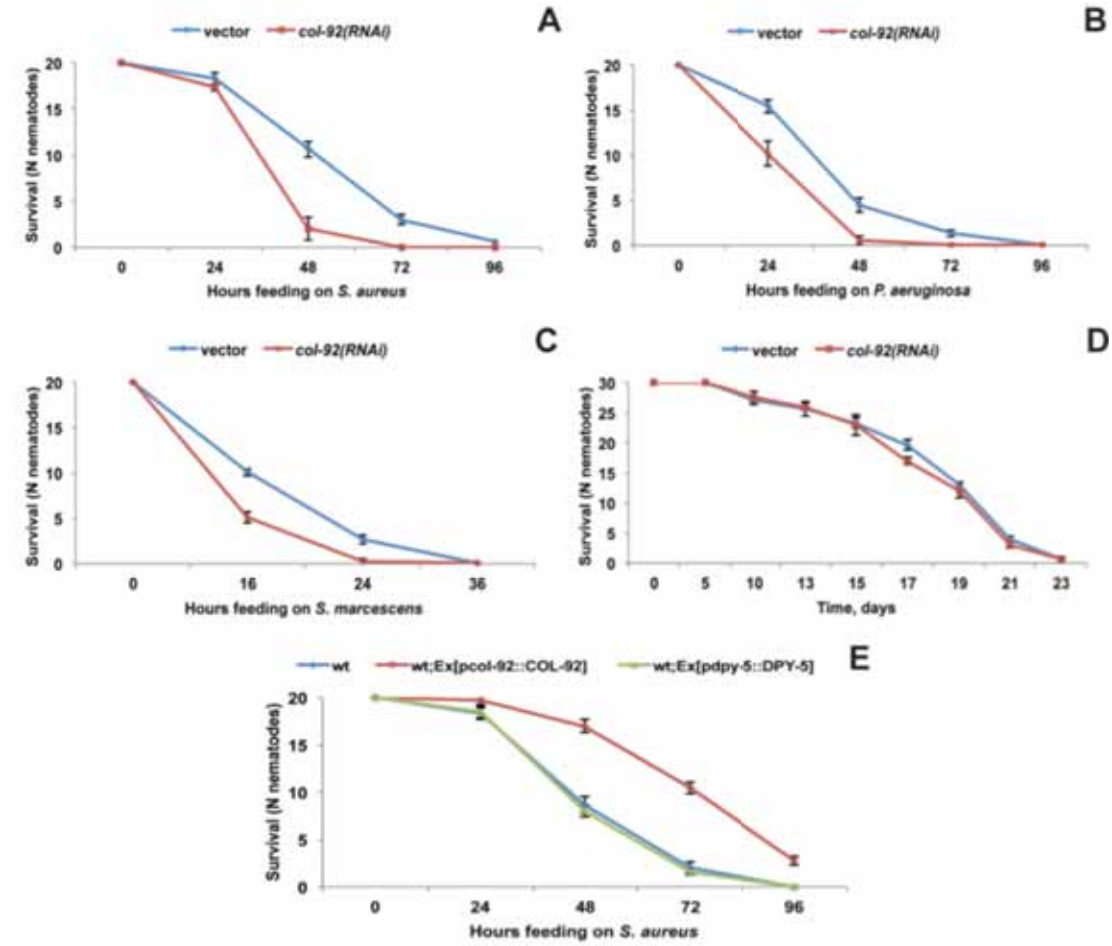

Fig S8. *col-92* (RNAi) significantly increases susceptibility of the worms to *S. aureus* (A) ($p = 0.021$) and *P. aeruginosa* (B) ($p = 0.034$), while on *S. marcescens* (C) effect is not significant ($p > 0.05$). Lifespan of the *col-92* (RNAi) worms (D) is similar to vector-treated worms ($p > 0.05$). (E). Transgenic expression of *col-92* but not of *dpy-5* significantly ($p < 0.001$) increases survival of worms exposed to *S. aureus.* Data represent mean±SEM.

Fig S9. Images of transgenic animals in which GFP was fused with the promoter of COL-92. Upper panel. Nomarski images. Lower panel. Fluorescent images of the same worms. Strong expression is observed in the hypodermis. Scale bar is approx. 100 μm.

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Draft Genome Sequence of Highly Nematicidal *Bacillus thuringiensis* DB27

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Here, we report the genome sequence of nematicidal *Bacillus thuringiensis* DB27, which provides first insights into the genetic determinants of its pathogenicity to nematodes. The genome consists of a 5.7-Mb chromosome and seven plasmids, three of which contain genes encoding nematicidal proteins.

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Bacillus thuringiensis is a Gram-positive bacterium pathogenic to a number of invertebrate hosts (1). The pathogenicity of *B. thuringiensis* is largely mediated by crystal (Cry) toxin proteins produced during sporulation (2). Due to the insecticidal properties of the Cry toxins, which are not toxic to vertebrates, *B. thuringiensis* has been extensively used as a biopesticide (1, 2).

Whole-genome sequencing has proven to be a useful tool for the discovery of novel Cry toxins (3). While several *B. thuringiensis* strains have been sequenced (4–9), there is lack of whole-genome sequence data for nematicidal *B. thuringiensis* strains. Obviously, such information is of great importance, since it helps to reveal unknown aspects of *B. thuringiensis* pathogenic mechanisms, e.g., pathogenicity against animals other than insects. One potential target is nematodes, which often live in the soil and were previously shown to interact with *Bacillus* Cry toxins (10).

We previously isolated the nematicidal strain *B. thuringiensis* DB27 from dung beetles, and this strain exhibits strong virulence to the nematode *Caenorhabditis elegans* (11). While the mechanisms of *C. elegans* resistance (12) and transcriptional response (13) to this pathogen have been described, the nematicidal virulence determinants of *B. thuringiensis* DB27 are currently unknown. To provide first insights into *B. thuringiensis* DB27 virulence mechanisms, we sequenced its whole genome.

Genomic DNA was isolated from *B. thuringiensis* DB27 using the MasterPure Gram-positive DNA isolation kit (Epicenter). Whole-genome sequencing was performed using Roche and Illumina platforms with a GS FLX Titanium 8-kb paired-end library and an Illumina 250-bp paired-end library, respectively. Approximately 2.4 million 150-bp Illumina reads were assembled using Velvet version 1.1.06 (14). The resulting Velvet assembly was combined with ~280,000 454 reads, with an average length of 308 bases, using Newbler *de novo* version 2.6 assembler, generating a total of 387 contigs, 260 of which were contained in 33 scaffolds representing 98.4% of the total genome assembly. The combined assembly was then improved using computational and manual methods: (i) IMAGE (15) was used for the Newbler-generated scaffold information and Illumina reads were used to reduce the number of sequence gaps, (ii) ICORN (16) used Illumina data to correct base errors introduced by 454 sequencing, and (iii) the sequence was manually edited in Gap4 (17). The final assembly is represented by 235 contigs, of which 156 contigs are in 7 scaffolds representing the main chromosome, 49 contigs are unplaced, and 30 contigs are in 7 scaffolds identified as plasmids. The open reading frames (ORFs) were identified using Prodigal version 2.6 (18).

The genome of *B. thuringiensis* DB27 consists of a 5.7-Mb chromosome and seven plasmids ranging in size from 4 to 200 kb. The G+C content of the chromosome is 35.2%, and that of the plasmids ranges from 31.5% to 34.4%. The total number of predicted genes is 6,302, with 5,851 genes located on the chromosome and 451 genes on the plasmids. Toxin genes were identified using BtToxinScanner (3). In total, 3 Cry-like genes belonging to the Cry21 nematicidal family were identified as being carried by 200-kb, 8-kb, and 6-kb plasmids.

Nucleotide sequence accession numbers. The draft of the whole-genome sequencing project has been included in the European Nucleotide Archive at EMBL-EBI under accession no. CBXL010000001 to CBXL010000235.

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Bacillus thuringiensis DB27 Produces Two Novel Protoxins, Cry21Fa1 and Cry21Ha1, Which Act Synergistically against Nematodes

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Bacillus thuringiensis **(BT) has been widely used as a biopesticide, primarily for the control of insect pests, but some *B. thuringiensis* strains specifically target nematodes. However, nematicidal virulence factors of *B. thuringiensis* are poorly investigated. Here, we describe virulence factors of nematicidal *B. thuringiensis* DB27 using *Caenorhabditis elegans* as a model. We show that *B. thuringiensis* DB27 kills a number of free-living and animal-parasitic nematodes via intestinal damage. Its virulence factors are plasmid-encoded Cry protoxins, since plasmid-cured derivatives do not produce Cry proteins and are not toxic to nematodes. Whole-genome sequencing of *B. thuringiensis* DB27 revealed multiple potential nematicidal factors, including several Cry-like proteins encoded by different plasmids. Two of these proteins appear to be novel and show high similarity to Cry21Ba1. Named Cry21Fa1 and Cry21Ha1, they were expressed in *Escherichia coli* and fed to *C. elegans*, resulting in intoxication, intestinal damage, and death of nematodes. Interestingly, the effects of the two protoxins on *C. elegans* are synergistic (synergism factor, 1.8 to 2.5). Using purified proteins, we determined the 50% lethal concentrations (LC_{50}s) for Cry21Fa1 and Cry21Ha1 to be 13.6 μg/ml and 23.9 μg/ml, respectively, which are comparable to the LC_{50} of nematicidal Cry5B. Finally, we found that signaling pathways which protect *C. elegans* against Cry5B toxin are also required for protection against Cry21Fa1. Thus, *B. thuringiensis* DB27 produces novel nematicidal protoxins Cry21Fa1 and Cry21Ha1 with synergistic action, which highlights the importance of naturally isolated strains as a source of novel toxins.**

Bacillus thuringiensis is a Gram-positive, spore-forming bacterium which is extensively used for biological control of insects and nematodes (1, 2). *B. thuringiensis* produces an array of virulence factors that contribute to its pathogenic effect. These virulence factors include exotoxins, extracellular proteases, enhancins, chitinase, and collagenase, which breach the epithelial cells of the insect gut (3). However, the major virulence factors of *B. thuringiensis* (4) are pesticidal proteins called Cry and Cyt produced during sporulation as a crystal inclusions.

These crystal proteins are pore-forming toxins lethal to insects and nematodes but nontoxic to vertebrates, which makes *B. thuringiensis* a safe and an effective pesticide that has been successfully used for many years (1, 4). While annotated Cry toxins are quite AQ: A diverse in terms of sequence similarity (http://www.lifesci.sussex.ac.uk/home/Neil_Crickmore/B. thuringiensis), they exhibit specific activity against insects of the orders Lepidoptera, Diptera, Coleoptera, Hymenoptera, Homoptera, Orthoptera, and Mallophaga (4), and they are also toxic to nematodes (5, 6). In contrast, Cyt toxins have shown mainly dipteran specificity, being able to kill mosquitoes and black flies (7). However, considering the diversity and amount of nematode species in soil, which is also ubiquitously inhabited by *B. thuringiensis*, nematodes also have to be considered the target for *B. thuringiensis* and its toxins (5). In support of this, several families of Cry proteins (Cry5, Cry6, Cry12, Cry13, Cry14, Cry21, and Cry55) were shown to be toxic to a number of free-living and parasitic nematodes (5, 8), but the full spectrum of nematicidal Cry toxins as well as their host targets is far from completion. Also, considering the growing problem of pest resistance to existing toxins, there is a high demand for new toxins (9).

Naturally isolated *Bacillus* strains have often been used as a source of new Cry toxins (see, for example, reference 10). Given that *B. thuringiensis* and nematodes coexist and coevolve in the natural environment, they undergo reciprocal changes, one of which is increased pathogen virulence (11). Therefore, naturally isolated *B. thuringiensis* strains may serve as a reservoir of novel Cry toxins, some of which may be used for the production of biological pesticides. In support of this, we have previously isolated several nematicidal *Bacillus* strains from environmental samples (12). One of the strains, *B. thuringiensis* DB27, was isolated from dung beetles and exhibits extreme toxicity to the model nematode *Caenorhabditis elegans* (12). Previously, we investigated *C. elegans* transcriptional response to *B. thuringiensis* DB27 infection (13) and elucidated the mechanisms of *C. elegans* resistance (14). However, the molecular mechanisms of the high virulence of *B. thuringiensis* DB27 for *C. elegans* are still unknown.

Here, we show that *B. thuringiensis* DB27 produces novel plasmid-encoded Cry protoxins (Cry21Fa1 and Cry21Ha1), which act synergistically to kill free-living and animal-parasitic nematodes via intestinal damage. We determined the 50% lethal concentrations (LC_{50}s) for Cry21Fa1 (13.6 μg/ml) and Cry21Ha1 (23.9 μg/ml) and found that they are comparable to the LC_{50} of nematicidal Cry5B. Additionally, we show that *C. elegans* conserved pathways provide protection against multiple pore-forming toxins.

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MATERIALS AND METHODS

Bacterial and nematode strains. The following strains were provided by the *Caenorhabditis* Genetics Center (University of Minnesota): *C. elegans* wild-type Bristol (N2), *Oscheius carolinensis*, *Pelodera strongyloides*, *Panagrellus redivivus*, *pmk-1*(*km25*), *jun-1*(*gk551*), *kgb-1*(*um3*), *xbp-1*(*zc12*), *bre-2*(*ye31*), and *bre-3*(*ye26*). Nematodes were maintained on nematode growing medium (NGM) agar plates with *Escherichia coli* OP50 as a food source and stored at 20°C. *Strongyloides papillosus* was maintained as described previously (15) and was kindly provided by Adrian Streit. *B. thuringiensis* DB27 was isolated by our group (12). Its plasmid-cured strain was generated in the current study. *E. coli* JM103 and protein expression vector pQE9 were provided by Raffi Aroian.

Nematode killing assays. (i) Vegetative cells. *B. thuringiensis* DB27 was grown overnight in a shaking incubator at 30°C in Luria-Bertani (LB) broth. A 40-μl volume of the culture was spread to the edges of 6-cm-diameter NGM plates, and plates were incubated for 12 to 14 h at 25°C before the assay. A total of 20 adult worms were placed into each plate in three to six independent replicates and were monitored for survival. Every 6 h (before bacteria on a plate started sporulation), worms were transferred to freshly prepared plates to ensure exposure to vegetative cells. Microscopy was used to monitor the state of bacteria on a plate.

(ii) Mixture of vegetative cells and spores. *B. thuringiensis* DB27 was grown overnight in a shaking incubator at 30°C in Luria-Bertani (LB) broth. An 80-μl volume of the culture was spread to the edges of 6-cm-diameter NGM plates, and plates were incubated for 24 h at 25°C before the assay. A total of 20 adult worms were placed into each plate in three to six independent replicates and were monitored for survival.

(iii) Spores. *B. thuringiensis* DB27 was grown overnight in a shaking incubator at 30°C in Luria-Bertani (LB) broth. An 80-μl volume of the culture was spread to the edges of 6-cm-diameter NGM plates, and plates were incubated for 24 h at 25°C before the assay. A total of 20 adult worms were placed into each plate in three to six independent replicates and were monitored for survival. Survival assays were repeated multiple times and conducted at 25°C.

Chemotaxis assays. Chemotaxis assays were modified from previous studies (16). Briefly, 25 μl of overnight *B. thuringiensis* DB27 suspension was placed 0.5 cm away from the edge of a 6-cm-diameter petri dish filled with NGM. The same amount of *E. coli* OP50 was placed on the opposing side and acted as the counterattractant. Approximately 50 to 200 J4/adult-stage *C. elegans* individuals were placed between the two bacterial spots. All nematodes used were previously fed on *E. coli* OP50. Plates were then sealed with Parafilm and stored at room temperature in the dark. After defined periods, the number of nematodes found in each bacterial spot was recorded. A chemotaxis index was used to score the response of the nematodes and was calculated as follows: number of nematodes in the test bacteria − number of nematodes in control bacteria/total number of nematodes counted (16). This gave a chemotaxis score ranging from −1.0 (total repulsion from test bacteria) to 1.0 (total attraction toward test bacteria). A score of around 0 means there were equal numbers of nematodes in all bacterial spots. Five plates were used per replicate, and the procedure was repeated five times.

Pulse-chase experiment. Pulse-chase experiments were conducted to find the minimum time required for *B. thuringiensis* DB27 to establish infection in *C. elegans*. Plates for pulse-chase experiments were prepared in the same way as for the nematode killing assay (mixture of vegetative cells and spores). Larval stage 4 (L4)-synchronized worms were exposed to *B. thuringiensis* DB27 for a defined period of time, washed five times with phosphate-buffered saline (PBS) buffer to remove surface bacteria, and then shifted to *E. coli* OP50 plates. Survival was scored after 24 h.

Plasmid isolation, gel electrophoresis, and plasmid curing. Plasmids of *B. thuringiensis* DB27 were extracted following the protocol of Reyes-Ramírez and Ibarra (17). Plasmids were resolved using 0.7% agarose gel, following a previously published protocol (18). Plasmid curing was performed by growing *B. thuringiensis* DB27 at 42°C with small amounts (0.0002%) of SDS in culture medium. Plasmid-cured derivatives were selected based on changes in colony morphology after plating on LB plates and verified by plasmid profiling.

Coomassie stain and EM of crystals. *B. thuringiensis* sporulation medium (the recipe can be found at http://www.bgsc.org) was used to produce large amounts of spore-crystal mixtures. *B. thuringiensis* DB27 spore-crystal mixtures were collected by centrifugation and washed three times with 1 M NaCl and ice-cold distilled water. The washed spore-crystal mixtures were resuspended in 1 ml of distilled water, and 10 μl of each sample was dropped onto a glass slide. Samples were fixed in 1% OsO_4, air-dried overnight, and then coated with gold. The scanning electron microscopy (SEM) observation was conducted on a Hitachi S-800 microscope (Hitachi, Japan), following the instructions for the device. For light microscopy, spore-crystal mixtures were spread on a glass slide, heat fixed, stained with Coomassie blue (0.133% Coomassie blue–50% acetic acid), and observed under ×100 magnification using immersion oil.

Solubilization and SDS-PAGE profiling of crystal proteins. Spore-crystal mixtures were collected and washed as described above. The spore-crystal pellet was resuspended in solubilization buffer (50 mM Na_2CO_3, 25 mM dithiothreitol [DTT], pH 10.5) and incubated at 37°C for 2 h. Insoluble remainings were removed by centrifugation, and the solubilized proteins from the supernatant were analyzed using SDS-PAGE.

Toxin cloning, protein expression, killing assay, and synergism assays. The Cry21 genes were PCR amplified from genomic DNA of *B. thuringiensis* DB27, digested with BamHI restriction enzyme, and ligated into the BamHI site of expression vector pQE9, generating pQE9(Cry21) plasmids for expression of His-tagged proteins. Recombinant plasmids were electroporated into *E. coli* JM103, which is used for Cry toxin expression (5). Bacteria were grown at 37°C to midlog phase (optical density at 600 nm [OD_{600}] = 0.6 to 0.8), and expression of Cry21 was induced with 1 mM IPTG (isopropyl-β-D-thiogalactopyranoside). After induction, bacteria were grown at 30°C for 4 h and then 30 μl of bacterial culture was spread in the center of enriched nematode growth (ENG) plates (19) containing 1 mM IPTG (ENG-IC plates). Plates were incubated overnight at 25°C and then used for nematode toxicity assays. Synchronized nematodes at the adult stage (20 per plate) were transferred to toxicity plates and monitored for survival and intoxication. Nematodes were transferred to fresh plates every day and were considered dead when they failed to respond to touch. *E. coli* JM103 transformed with empty pQE9 vector was used as a control. The expression of Cry21 protoxin was verified by SDS-PAGE.

For synergism assays, *E. coli* protoxin-expressing cultures were grown and induced as described above. After induction, the OD_{600} of each culture, including the empty vector control, was measured and adjusted to 2.0. To determine synergism between two proteins, the respective protoxin-expressing *E. coli* cultures were mixed in equal amounts (100 μl plus 100 μl) and 30 μl of bacterial culture was spread in the center of ENG plates and used for toxicity assays. Given that the final amount of protoxin-expressing *E. coli* in synergism plates was 50%, empty-vector *E. coli* culture mixed with each of the protoxin-expressing *E. coli* cultures (Cry21 plus vector) was used for comparison and each was termed a single protoxin treatment. When all three protoxins were used, three *E. coli* cultures were mixed in equal amounts.

Protein purification. Bacteria were grown at 37°C to midlog phase (OD_{600} = 0.6 to 0.8), and then IPTG was added at 1 mM final concentration to induce the expression of Cry21Fa1. Since Cry21Fa1 yield is higher at lower temperatures, after IPTG was added, the temperature was reduced to 25°C. Expression was carried out during a 10-h time period, and then cells were harvested by centrifugation (6,328 × *g* for 20 min). Cry21Ha1 was expressed similarly with the exception that expression was carried out during a 6-h time period at 30°C. The bacterial pellet was resuspended in buffer (20 mM Tris-HCl [pH 8.0], 500 mM NaCl, 0.5 mM β-mercaptoethanol, 4 mM $MgCl_2$, a protease inhibitor mix [Roche], phenylmethylsulfonyl fluoride [PMSF], DNase I), sonicated, and centrifuged at 35,000 rpm for 1 h at 4°C using an ultracentrifuge (Beckman). The supernatant was applied onto a nickel-nitrilotriacetic acid (Ni-NTA)

column (GE Healthcare). Bound protein was eluted from the column with a linear gradient of 0 to 0.5 M imidazole in a buffer (20 mM Tris-HCl [pH 8.0], 500 mM NaCl, 0.5 mM β-mercaptoethanol). Fractions were analyzed by SDS-PAGE and Western blotting using anti-His antibodies. Cry21-containing fractions were pooled, dialyzed against buffer (20 mM Tris-HCl, 10 mM NaCl, 0.5 mM β-mercaptoethanol), and loaded on an anion exchange column (Mono Q; GE Healthcare). A linear gradient of 0 to 3 M KCl in the loading buffer was used to elute bound Cry21. The Cry21 fractions were identified by SDS-PAGE and Western blotting, pooled, and dialyzed against 20 mM Tris-HCl–150 mM NaCl–0.5 mM β-mercaptoethanol buffer. The final step was size exclusion chromatography (Superdex 200; GE Healthcare) performed using 20 mM Tris-HCl–150 mM NaCl–0.5 mM β-mercaptoethanol. Fractions were analyzed by SDS-PAGE and Western blotting, pooled, and concentrated. Western blotting was performed with standard procedures as described elsewhere (20). Briefly, protein samples were separated in 8% SDS-PAGE gels and then transferred to nitrocellulose membranes (GE Healthcare). The membrane was incubated overnight with a 1:1,000 dilution of anti-His antibodies. Horseradish peroxidase-conjugated secondary antibody was used at 1:5,000. The signal was visualized with enhanced chemiluminescence (Bio-Rad).

Liquid assay with purified Cry21 protoxins. Purified protoxins were used for *C. elegans* single-well toxicity assays as described in reference 19 to determine the effect of known concentrations of protoxin on single nematodes. L4 nematodes were individually placed into the wells of 96-well microtiter plates. Each well contained S medium, 3 μl of a saturated OP50 culture as a standard food, the desired dose of purified Cry21 protoxin, and 2 μl of 8 mM FUDR (5-fluorodeoxyuridine). The final volume in each well was 120 μl. Wells containing buffer instead of protoxin were used as a control. To calculate LC_{50} values, L4 *C. elegans* hermaphrodites were subjected to a single-well assay and incubated in a humid chamber for 5 days at 25°C. Nematodes that did not show movement when touched were considered dead. Experiments were repeated at least three times per dose, and representative data were used to generate a semilog plot. The fraction of dead worms was plotted as a function of protoxin concentration using a semilog plot. Dead or intoxicated nematodes were not observed in buffer-containing control wells. The data were fitted to a line by the least-squares method, and LC_{50} was calculated from the line fit. Probit analysis (Minitab) was used to calculate 95% fiducial limits. The data (total number dead/number tested) are as follows: for Cry21Fa1, 22/24 at 58 μg/ml, 20/30 at 29 μg/ml, 20/48 at 14.5 μg/ml, 10/30 at 7.25 μg/ml, 6/36 at 3.1 μg/ml, and 3/36 at 1.6 μg/ml; and for Cry21Ha1, 22/24 at 80 μg/ml, 16/26 at 40 μg/ml, 10/28 at 20 μg/ml, 8/32 at 10 μg/ml, and 4/32 at 5 μg/ml.

To determine the potential synergism between Cry21Ha1 and Cry21Fa1 protoxins, nematodes were exposed to different protein ratios (1:1, 1:2, and 2:1) of Cry21Ha1/Cry21Fa1 mixtures in a single-well assay and observed LC_{50} values were calculated as described above. The theoretical (expected) LC_{50} values were calculated according to Tabashnik's equation (21), assuming a simple additive effect. The theoretical LC_{50} value is the harmonic mean of the intrinsic LC_{50} values of the components weighted by the ratio used in the mixture. The synergism factor (SF) was calculated by dividing the expected toxicity by the observed toxicity of the mixture. SF values greater than 1 indicate synergism.

Statistical analysis. Kaplan-Meier nonparametric comparison and a log-rank test (Minitab) were used for statistical analysis of survival curves based on the number of survivors at the sampled time points. In cases where multiple replicates were examined in one experiment, the average survival rate at each time point was determined. Bonferroni correction was applied when multiple comparisons were performed. Statistical significance was set at $P \leq 0.05$. Log-rank statistical analysis of survival curves is shown in Table S1 in the supplemental material.

Nucleotide sequence accession numbers. The nucleotide sequences for Cry21Fa1, Cry21Ga1, and Cry21Ha1 in strain DB27 have been deposited in the GenBank database (accession no. KF701307, KF771885, and KF771886, respectively) and designated Cry21Fa1, Cry21Ga1, and Cry21Ha1 by the *Bacillus thuringiensis* Toxin Nomenclature Committee.

RESULTS

***B. thuringiensis* DB27 kills *C. elegans* via intestinal damage.** The *B. thuringiensis* DB27 strain was isolated previously and potentially uses novel virulence factors, since *C. elegans bre* mutants resistant to *B. thuringiensis* Cry5B toxin are susceptible to *B. thuringiensis* DB27 (12). *B. thuringiensis* DB27 showed remarkable toxicity for *C. elegans*, killing 100% of worms in just 16 h (Fig. 1A) (12, 14). Microscopic examination of the bacterial culture used in this assay revealed the presence of a mixture of vegetative cells and spores (see Fig. S1A in the supplemental material). To elucidate which stages of *B. thuringiensis* DB27 are virulent to nematodes, the assay plates were prepared with pure vegetative cells or spores for verification by microscopy (see Fig. S1B and C). Interestingly, survival of the worms was not affected on pure vegetative cells (Fig. 1A) even after 36 h, while pure spores showed toxicity even higher than that seen with the mixture of spores and vegetative cells (Fig. 1A). These findings suggest that virulence factors are produced during sporulation but not by vegetative cells. Although the spores were the most virulent, we used in all subsequent assays mixed cultures of spores and vegetative cells to prevent starvation (22), which might affect the outcome of the assay.

Additionally, we noticed that *C. elegans* did not avoid *B. thuringiensis* DB27 in the chemotaxis assay, showing equal preferences for OP50 and *B. thuringiensis* DB27 (Fig. 1B).

To study whether *B. thuringiensis* DB27 is able to establish infection in *C. elegans*, we performed pulse-chase experiments, where worms were exposed to *B. thuringiensis* DB27 for a defined period of time and then shifted to *E. coli* OP50. We found that a 6-h pulse was sufficient to establish a lethal infection in nearly 100% of worms (Fig. 1C). After 3 h, nearly 40% of worms were already infected, suggesting that *B. thuringiensis* DB27 relies on an active infection process as part of its virulence mechanism. This is further supported by our microscopy observations. Compared to control worms on *E. coli* OP50 (Fig. 1D), *B. thuringiensis* DB27-infected worms exhibited strong intestinal changes, namely, intestinal shrinkage and dissociation from body walls (Fig. 1E). Furthermore, we could also show a dramatic accumulation of *B. thuringiensis* DB27 spores and cells in the *C. elegans* intestine (Fig. 1F), which is consistent with the pulse-chase experiments and confirms that *B. thuringiensis* DB27 relies on an active infection process. Similar levels of intestinal destruction and accumulation of spores in the intestine were reported previously for another nematicidal *B. thuringiensis* isolate (23). Together, these results show that *B. thuringiensis* DB27 infects the *C. elegans* intestine, leading to intestinal damage and subsequent death of the nematode.

***B. thuringiensis* DB27 is pathogenic to diverse nematodes.** To determine the specificity of *B. thuringiensis* DB27 virulence, we exposed several other nematodes to the pathogen. As shown in Fig. 1G and H, the nematodes *Oscheius carolinensis*, *Pelodera strongyloides*, and *Panagrellus redivivus* were also killed by *B. thuringiensis* DB27, although they showed different degrees of susceptibility. While *O. carolinensis* and *P. strongyloides* were more resistant to *B. thuringiensis* DB27 than *C. elegans* (Fig. 1G), *P. redivivus* was significantly more susceptible (Fig. 1H). In addition, we found that the free-living stage of the animal-parasitic nematode *Strongiloides papillosus* was also highly susceptible to *B. thurin-*

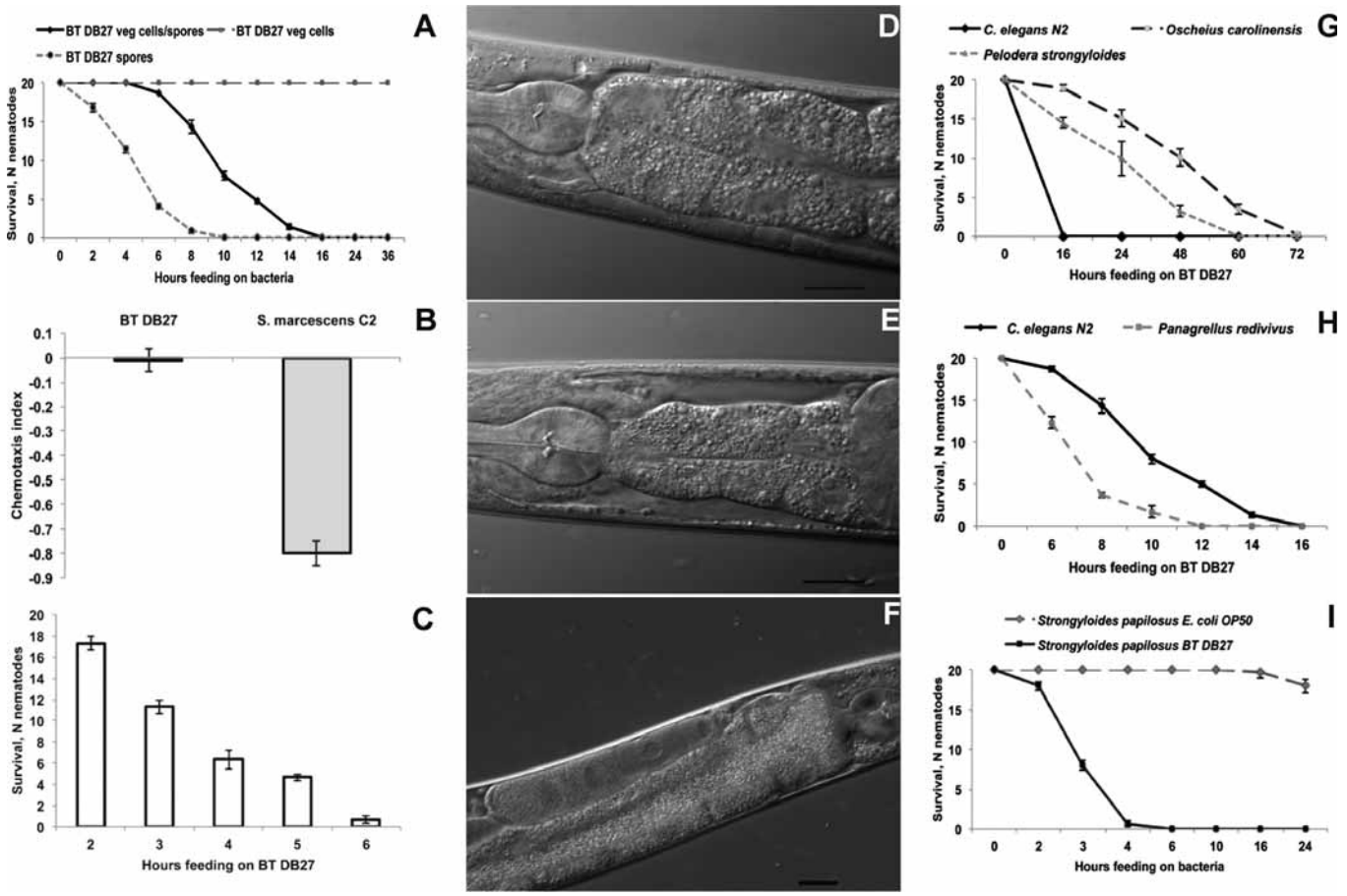

FIG 1 *B. thuringiensis* DB27 (BT DB27) kills diverse nematodes via intestinal infection. (A) *C. elegans* survival on monoxenic cultures of *B. thuringiensis* DB27. Pure spores of *B. thuringiensis* DB27 are significantly ($P < 0.0001$) more toxic to *C. elegans* than a mixture of spores and vegetative cells. Vegetative (veg) cells alone are not virulent. (B) *B. thuringiensis* DB27 did not repel *C. elegans* in a chemotaxis assay, in contrast to *Serratia marcescens*, used as a positive control. (C) *C. elegans* survival after a short exposure to *B. thuringiensis* DB27 (pulse-chase). The *x* axis shows the pulse (time of exposure to the pathogen). The *y* axis shows the number of nematodes (scored 24 h postinfection) that recovered after a given pulse. (D to F) *C. elegans* intestinal changes caused by *B. thuringiensis* DB27. Compared to control worms on OP50 (D), worms exposed to *B. thuringiensis* DB27 exhibit intestinal shrinkage (E) and accumulation of bacteria in the gut (F). Bar, 20 μm. (G to I) Other nematodes show levels of susceptibility that differ from that of *B. thuringiensis* DB27. (G) *O. carolinensis* and *P. strongyloides* are more resistant ($P < 0.0001$) to *B. thuringiensis* DB27-mediated killing than *C. elegans*. (H) *P. redivivus* is significantly ($P < 0.001$) more susceptible than *C. elegans*. (I) Animal-parasitic nematode *S. papillosus* is killed rapidly by *B. thuringiensis* DB27 compared to control *E. coli* OP50. For survival curves, the number of worms alive (N nematodes) is plotted as a function of time. The data shown are means ± standard errors of the means.

giensis DB27, being killed in as little as 4 h (Fig. 1I). Thus, *B. thuringiensis* DB27 is toxic to a number of free-living and also animal-parasitic nematodes, indicating its potential application as a nematicidal agent.

Role of plasmids in *B. thuringiensis* DB27 virulence. Given that *B. thuringiensis* major virulence factors are often encoded by plasmids (24), we next explored the role of *B. thuringiensis* DB27 plasmids in pathogenicity against nematodes. First, we extracted and gel-separated plasmids of *B. thuringiensis* DB27 and found that it harbored seven plasmids, ranging in size from 3 to above 16 kilobases (Fig. 2A). Next, we generated several plasmid-cured variants of *B. thuringiensis* DB27, some of which lost all seven plasmids (Fig. 2A). When we exposed *C. elegans* to those plasmid-cured variants, we found that they lost their virulence completely (Fig. 2B), suggesting that nematicidal factors are encoded by plasmids.

B. thuringiensis Cry toxins are almost exclusively encoded by plasmids (24) and are therefore potential nematicidal candidates produced by *B. thuringiensis* DB27. To explore that idea further, we tested whether *B. thuringiensis* DB27 produces Cry toxins. Light microscopy of Coomassie-stained spore-crystal mixtures indicated the presence of Cry protein crystals in *B. thuringiensis* DB27 (Fig. 2C) but not in a plasmid-cured derivative (Fig. 2D). Interestingly, crystals showed a strong association with spores, the phenotype previously described as spore-crystal association (SCA) (25). SEM images confirmed this phenotype (Fig. 2E) and showed striking similarity to the SCA of a rare filamentous *B. thuringiensis* strain (26). In contrast to wild-type *B. thuringiensis* DB27, the plasmid-cured variant did not produce any Cry protein crystals, as shown in light microscopy (Fig. 2D) and scanning EM images (Fig. 2F). To further confirm this phenotype, we solubilized proteins from spore-crystal mixtures of *B. thuringiensis* DB27 and its plasmid-cured variant and resolved them in an SDS-PAGE gel. While the *B. thuringiensis* DB27 profile revealed a dominant protein of around 130 kDa (see Fig. S2 in the supplemental material), which corresponds to the size of some Cry protoxins, the profile of the plasmid-cured variant showed no proteins at all (see Fig. S2). These findings confirm our microscopy results show-

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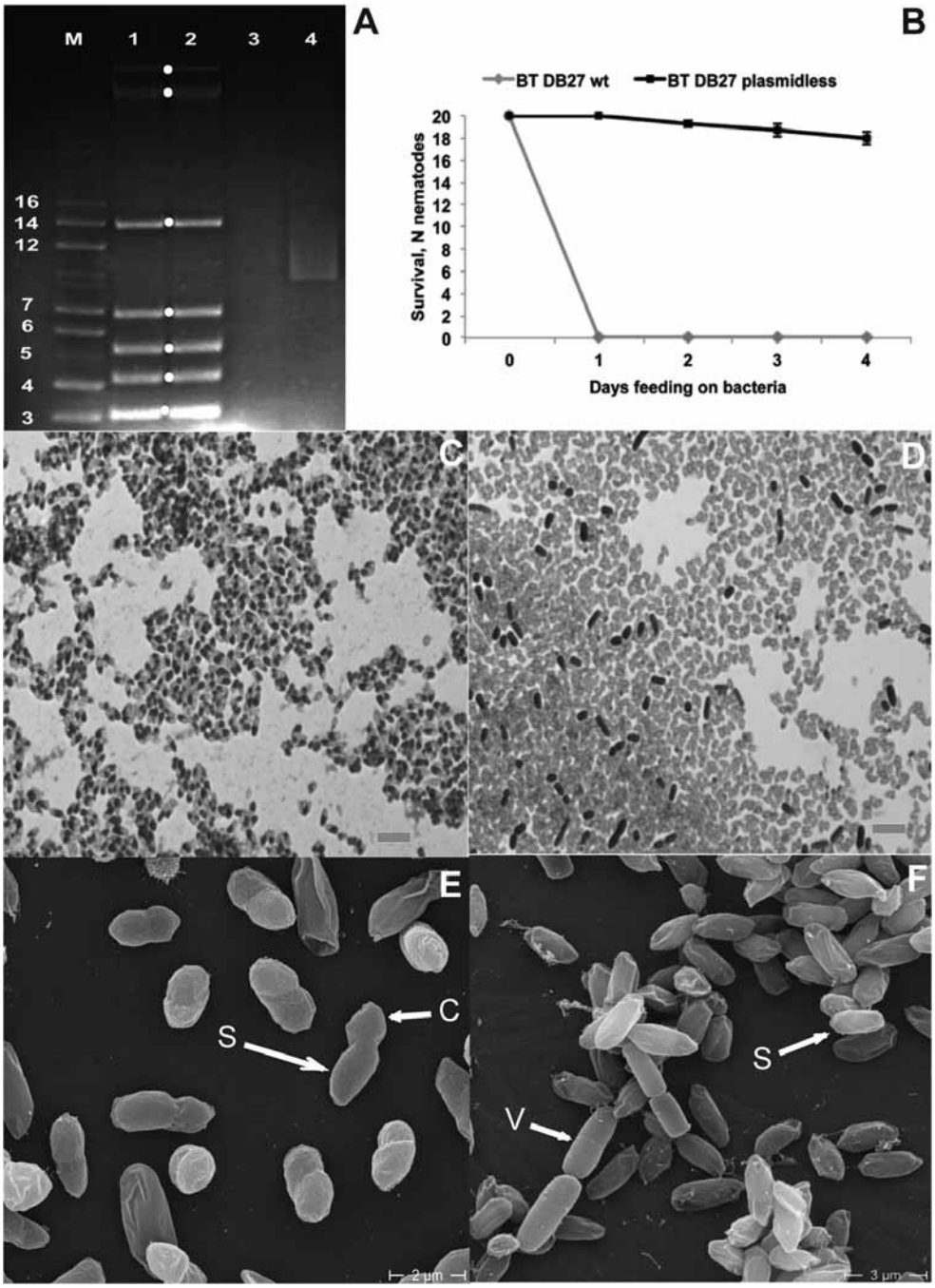

FIG 2 *B. thuringiensis* DB27 virulence factors are plasmid-associated Cry protoxins. (A) Plasmid profile of *B. thuringiensis* DB27 (lanes 1 and 2) and its plasmid-cured derivative (lanes 3 and 4). *B. thuringiensis* DB27 has seven plasmids (marked with dots), while the plasmid-cured variant lost all of them. M, marker (kb). Considering that the marker is represented by linear DNA fragments and the state of the plasmids (linear, circular, or supercoiled) is not known, it cannot be used as a determinant of the exact plasmid size. (B) *C. elegans* survival on the *B. thuringiensis* DB27 plasmid-cured variant is not affected ($P < 0.0001$) compared to that of the wild-type (wt) strain. (C and D) Light microscopy images (100×) of Coomassie-stained spore-crystal mixtures of wild-type *B. thuringiensis* DB27 (C) and its plasmidless derivative (D). Crystals (stained in black in panel C) show a strong association with the spore (SCA phenotype). No crystal inclusions are formed by plasmid-cured derivative. Rod-like black structures not attached to spores are vegetative cells. Bars, 100 μm. (E and F) Scanning electron microscopy images of spore-crystal mixtures of wild-type *B. thuringiensis* DB27 (E) and its plasmidless derivative (F). Arrows point to spores (S), crystals (C), and vegetative cells (V).

ing that the plasmid-cured variant does not produce Cry protoxins. Together, these results indicate that *B. thuringiensis* DB27 virulence factors are plasmid-encoded Cry protoxins, which also agrees with the fact that sporulating cultures show high virulence activity (Fig. 1A). Consistent with this, vegetative cells that did not kill nematodes (Fig. 1A) did not produce any Cry proteins, as verified by SDS-PAGE (see Fig. S3); spores that showed the highest virulence also yielded the largest amount of Cry proteins (see Fig. S3); and spore/vegetative cell mixtures showed an intermediate level of killing and an intermediate amount of Cry proteins (see

Fig. S3). Thus, there is a correlation between the amounts of Cry protoxins produced by different stages and the levels of nematode lethality.

Candidate virulence factors identified by genome sequencing. To gain further insight into *B. thuringiensis* DB27 virulence factors, we sequenced the genome of this bacterium (27) and analyzed the genome sequence for the presence of potential virulence factors. Multiple proteases, enterotoxins, cytotoxins, collagenase, and chitinase were found (see Table S2 in the supplemental material). However, neither Cyt nor Vip (vegetative insecticidal protein) toxins implicated in *B. thuringiensis* insecticidal activity (7, 28) were found in the *B. thuringiensis* DB27 genome. Besides the circular chromosome, whole-genome sequencing revealed the presence of seven plasmids, ranging in size from 4 to 200 kb (See Table S3 in the supplemental material), which agrees with the observed plasmid profile (Fig. 2A). Given that our results suggest the nematicidal factors to be plasmid-encoded Cry protoxins, we concentrated specifically on plasmid-encoded factors. Indeed, we found several Cry-like toxins belonging to nematicidal families to be located on plasmids (See Table S3). Specifically, the 200-kb plasmid harbored a Cry-like toxin which showed similarity to the Cry21Ba1 toxin. In addition, the 8-kb and 6-kb plasmids also carried Cry-like toxins, both of which are similar to Cry21Ba1. Although a BLAST search identified sequence similarity of all three toxins to Cry21Ba1 toxin, more-detailed sequence comparisons revealed that all three proteins were different and that only the C-terminal part was conserved (see Fig. S4). Considering the low degree of sequence similarity to known Cry toxins, all three proteins were identified by the Cry toxin nomenclature committee as novel and were assigned new official names (See Table S3). Based on their sequence similarity, the newly identified Cry21 proteins are potentially nematicidal. However, they probably differ in the sensitivity spectrum of nematodes and the extent of toxicity.

Novel Cry21 protoxins show synergistic nematicidal activity. To test the potential role of these proteins in toxicity to *C. elegans*, we cloned them individually into the unique BamHI restriction site of the pQE9 *E. coli* protein expression vector. *E. coli* transformed with pQE9-Cry21 plasmids produced a Cry protein of above 130 kDa (not shown), which corresponds to the size of some Cry protoxins. This also suggests that the 130-kDa protein from the *B. thuringiensis* DB27 spore-crystal mixture corresponds to Cry21 protoxins, given the similarities in size.

When we fed *C. elegans* with *E. coli* expressing the three protoxins individually, we observed different degrees of intoxication (Fig. 3A). Cry21Fa1 was the most effective protoxin, killing worms in around 48 h. Cry21Ha1 showed moderate toxicity and caused 100% lethality in 5 days. In contrast, Cry21Ga1 did not show any obvious toxicity to *C. elegans* even after 5 days (Fig. 3A). Note that longer exposure times were not feasible in these experiments since even *E. coli* with empty vector is toxic to *C. elegans* on rich medium such as ENG. Worms fed with Cry21Fa1 or Cry21Ha1 protoxin exhibited classical intoxication phenotypes, such as slow movement, pale appearance, reduction in body size, and, finally, death of the worms. Additionally, in contrast to nematodes fed on *E. coli* with an empty vector (Fig. 3B), worms exposed to the *E. coli*-expressed protoxin exhibited intestinal shrinkage and damage (Fig. 3C), in similarity to worms fed with *B. thuringiensis* DB27 (Fig. 1E).

Given that Cry toxins often show synergistic action (29), we next fed *C. elegans* with mixtures of *E. coli* clones expressing different protoxins. As shown in Fig. 3D, combining Cry21Fa1 and Cry21Ha1 protoxins resulted in significantly higher *C. elegans* mortality compared to that seen with the single protoxins, suggesting that the two protoxins might act synergistically. A Cry21Ga1 combination with Cry21Fa1 and/or Cry21Ha1 did not increase *C. elegans* lethality compared to single protoxins (Fig. 3D), indicating that Cry21Ga1 (alone or in combination with other protoxins) is not involved in *C. elegans* killing. Interestingly, the *C. elegans nasp-1*(*tu439*) mutant, which is resistant to *B. thuringiensis* DB27-mediated killing, also exhibits increased resistance to the Cry21Fa1 and Cry21Ha1 protoxins (Fig. 3E). Thus, Cry21Fa1 and Cry21Ha1 are important nematicidal factors produced by *B. thuringiensis* DB27 that show potential synergistic action.

Quantitative effect of Cry21Fa1 and Cry21Ha1 on *C. elegans*. To quantify Cry21 protoxin actions, Cry21Fa1 and Cry21Ha1, which showed toxicity to *C. elegans* in a feeding experiment (Fig. 3A), were purified as a His-tagged proteins using affinity chromatography, verified by Western blotting (see Fig. S5 in the supplemental material), and used to intoxicate *C. elegans* in liquid assays. The purified protoxins showed clear dose-dependent action (Fig. 3F and G and Materials and Methods). While 58 μg/ml of Cry21Fa1 protoxin was sufficient to kill 100% of worms in 5 days, the estimated concentration of Cry21Fa1 that kills 50% of worms is 13.6 μg/ml (calculated LC_{50}). For Cry21Ha1, the LC_{50} is 23.9 μg/ml. Thus, Cry21Fa1 is almost twice as toxic as Cry21Ha1, which agrees with the results of the feeding experiment (Fig. 3A). While these values are very close to the LC_{50} of another nematicidal toxin, Cry5B (12.6 μg/ml) (30), direct comparison is not possible due to experimental differences in the toxin purification. When Cry21Ha1 and Cry21Fa1 protoxins were combined at different ratios (Table 1), all the observed LC_{50} values of protoxin mixtures were lower than the expected LC_{50} values and the mixtures exhibited clear synergistic activity. Specifically, the Cry21Fa1/Cry21Ha1 combination with a ratio of 2:1 exhibited the best synergistic activity (LC_{50} 6.1 μg/ml), representing a 2.6 reduction in the LC_{50} value compared to the expected LC_{50} (15.88 μg/ml) (Table 1).

***C. elegans* requires conserved defense pathways against pore-forming toxins.** Considering that multiple *C. elegans* pathways involved in the defense against nematicidal Cry5B toxin have been previously described (31–33), we wanted to test whether they are important for the defense against Cry21 protoxins. First, we tested *C. elegans bre* mutants that lack the receptor for Cry5B (32). Interestingly, those mutants showed slightly increased but not significantly different survival compared to wild-type animals when exposed to *E. coli*-expressed Cry21 protoxins (Fig. 4A). This result is consistent with previous studies and agrees with the fact that *bre* mutants do not show cross-resistance to multiple Cry toxins (32). This finding also indicates that Cry21Fa1 and Cry21Ha1 protoxins may require a different and/or additional receptor or epitope in comparison to the Cry5B toxin. The p38 mitogen-activated protein kinase (MAPK) pathway and the c-Jun N-terminal kinase (JNK) MAPK pathway were previously shown to play a central role in the *C. elegans* response to Cry5B pore-forming toxin (33). Consistent with this, we found that animals carrying mutations in the p38 MAPK pathway (*pmk-1*) or in a downstream transcription factor of JNK MAPK pathway *jun-1* were all hypersusceptible to Cry21Fa1 protoxin (Fig. 4B). JNK MAPK pathway mutant *kgb-1* was slightly, but not significantly, more susceptible to Cry21Fa1

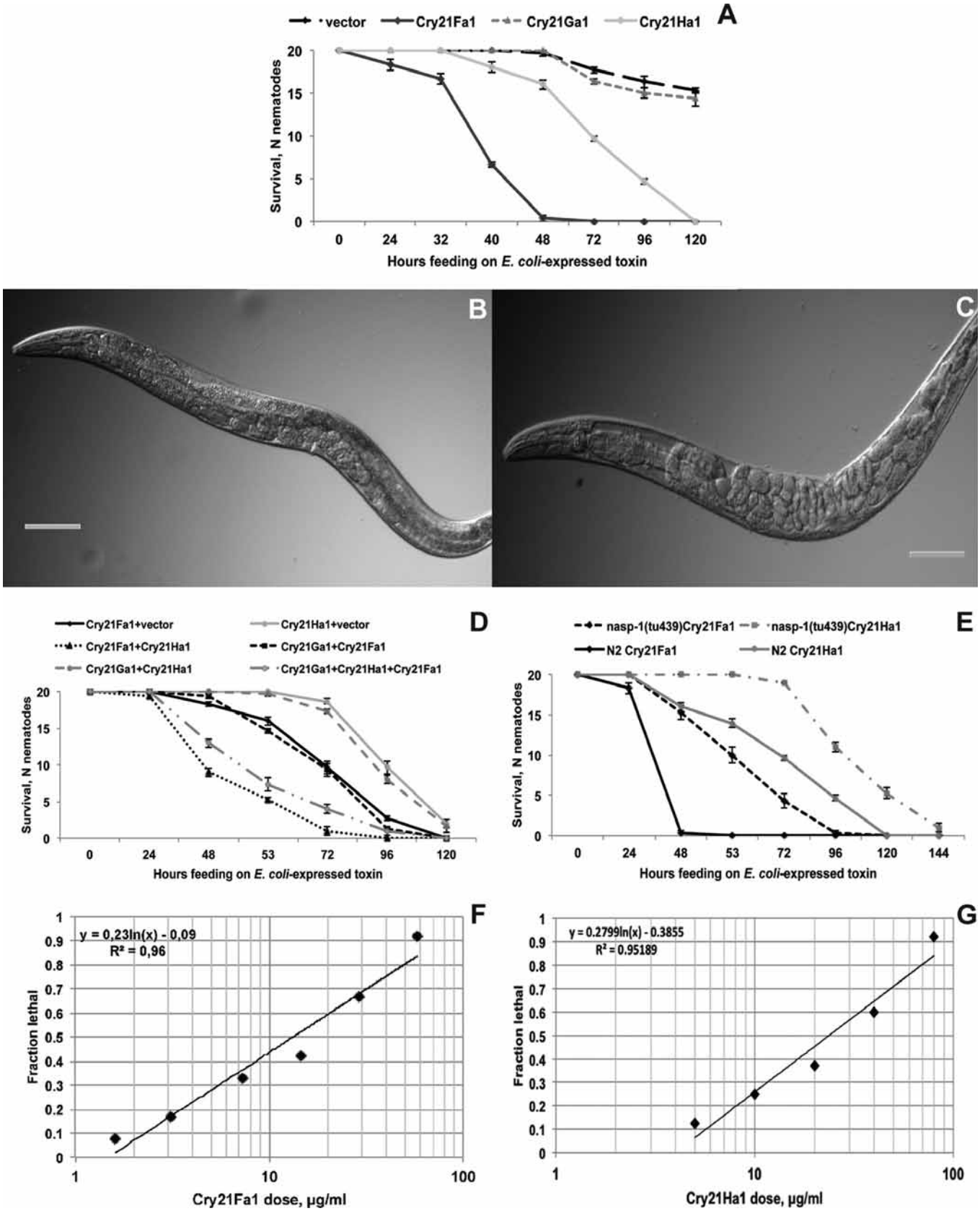

FIG 3 Cry21 protoxins are the nematicidal virulence factors of *B. thuringiensis* DB27. (A) *C. elegans* survival on *E. coli* which expresses individual Cry21 protoxins. Cry21Fa1 and Cry21Ha1 significantly ($P < 0.0001$) reduce *C. elegans* survival compared to the vector control. The Cry21Ga1 effect is similar ($P > 0.05$) to that of the vector control. (B and C) *C. elegans* fed with *E. coli*-expressed Cry21Fa1 protoxin exhibits dramatic intestinal shrinkage and destruction (C)

TABLE 1 Toxicity to *C. elegans* of Cry21Fa1 and Cry21Ha1 single protoxins and their mixtures

Cry21Fa1/ Cry21Ha1 ratio	LC_{50} (μg/ml)		Synergism factor[c]
	Observed[a]	Expected[b]	
1:0	13.6 (10.07–19.6)		
0:1	23.9 (18.07–33.25)		
1:1	8.7 (6.21–11.98)	17.33	1.99
1:2	7.98 (4.75–12.76)	19.08	2.39
2:1	6.1 (3.88–8.56)	15.88	2.6

[a] LC_{50}s were determined experimentally; 95% fiducial limits determined by Probit analysis are given in parentheses.
[b] Theoretical LC_{50}s were calculated by using Tabashnik's equation and assuming a simple additive effect.
[c] Synergism factors were calculated by dividing the expected LC_{50} by the observed LC_{50}.

AQ: H protoxin (Fig. 4B). This surprising result suggests that the role of *kgb-1* in *C. elegans* defense against pore-forming toxins might be toxin dependent, and more-detailed investigation will be needed to elucidate the molecular mechanisms of the differentially protective role of *kgb-1*. Additionally, we found that *xbp-1* mutants, which are sensitive to Cry5B (31), are also more susceptible to Cry21Fa1 than wild-type worms (Fig. 4B), confirming the protective role of the *xbp-1* pathway against pore-forming toxins. Thus, *C. elegans* requires some conserved pathways for defenses against multiple Cry pore-forming toxins.

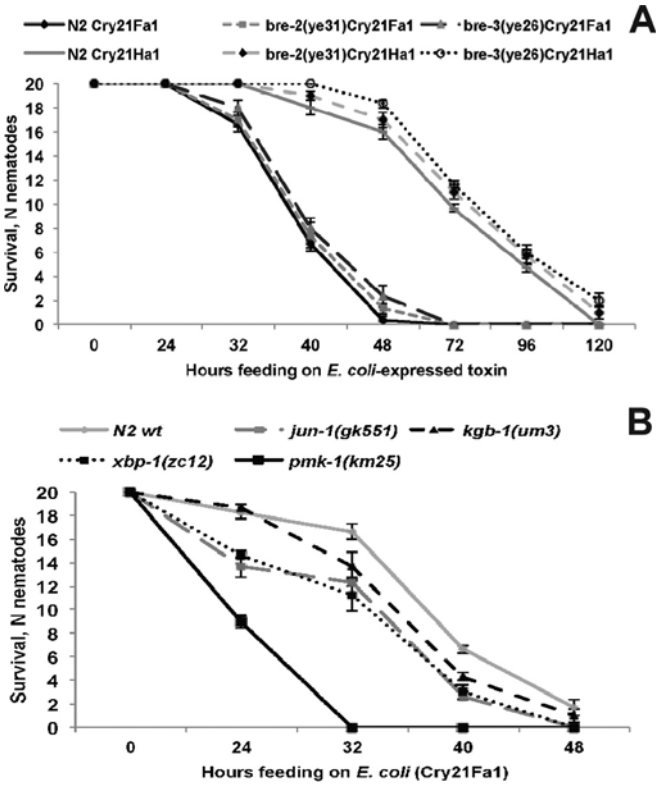

FIG 4 (A) *bre-2* and *bre-3* mutants are as susceptible to Cry21Fa1 and Cry21Ha1 protoxins as wild-type worms. (B) *C. elegans jun-1* ($P < 0.05$), *xbp-1* ($P < 0.05$), and *pmk-1* ($P < 0.0001$) mutants are hypersensitive to Cry21Fa1 protoxin compared to wild-type worms. Survival of the *kgb-1* mutant is not significantly ($P > 0.05$) different from wild-type survival. The data shown are means ± standard errors of the means.

DISCUSSION

In the present study, we characterized novel virulence factors of the highly nematicidal *B. thuringiensis* DB27 strain. Combining plasmid curing, whole-genome sequencing, and a candidate gene approach, we identified three proteins related to Cry21Ba1 toxin as potential nematicidal factors. Previous methods of Cry toxin identification are clearly dominated by PCR-based techniques (34). While these methods proved to be useful, they have certain limitations. Therefore, whole-genome sequencing and genome mining become reasonable alternatives for the identification of novel Cry toxins (8, 35). Applying these techniques, we found that three different plasmids encode three Cry-like proteins. All three proteins appeared to be novel Cry protoxins and were assigned new official names.

In contrast to previous reports showing that the genes encoding Cry toxins are located on large plasmids (24), which is also true for Cry21Fa1 protoxin produced by *B. thuringiensis* DB27, we found that two other protoxins, Cry21Ga1 and Cry21Ha1, are encoded by small 8- and 6-kb plasmids, respectively. Interestingly, another nematicidal toxin, Cry55Aa1, was also encoded by a relatively small 17.7-kb plasmid (8). Whether this unusual location has any functional consequences is not clear yet and remains to be elucidated.

While all three novel proteins belong to the Cry21 family of nematicidal toxins, only two, Cry21Fa1 and Cry21Ha1, showed activity against *C. elegans*. Whereas they are potent as single protoxins, they also showed synergistic activity against *C. elegans*. Synergism between Cry toxins and Cyt toxins has been described previously (7, 29). Additionally, enzymes such as chitinase and collagenase and different proteases have been shown to have an enhancing effect on Cry toxin efficiency (3, 36). Given that whole-genome sequencing of *B. thuringiensis* DB27 revealed the presence of multiple enzymes with potential enhancing properties, we do not exclude their involvement in *B. thuringiensis* DB27 virulence, but their role awaits further investigation. At this stage, it is not known why Cry21Ga1 is not active against *C. elegans*. It is possible that Cry21Ga1 functions in combination with other toxins and/or enzymes. In addition to this, there are many other examples in which Cry toxins do not show pesticidal activity (37). Thus, the exact target host and molecular function of Cry21Ga1 in *B. thuringiensis* DB27 pathogenicity will require further investigation.

B. thuringiensis strains very often carry multiple plasmids with different Cry toxins. *B. thuringiensis* DB27 carries seven plasmids,

compared to vector-fed control worms (B). Bars, 100 μm. (D) *C. elegans* survival upon exposure to combinations of *E. coli* clones that express different Cry21 protoxins. *C. elegans* survival is significantly ($P < 0.0001$) reduced when worms are exposed to the combination of Cry21Fa1 and Cry21Ha1 protoxins compared to exposure to each toxin individually. Combining Cry21Ga1 with either Cry21Fa1 or Cry21Ha1 does not significantly ($P > 0.05$) change the survival compared to that seen with the individual protoxins. The combination of all three proteins is almost as toxic as the combination of Cry21Fa1 and Cry21Ha1, suggesting that Cry21Ga1 has no synergistic effect. (E) The *nasp-1* mutant is significantly more resistant to Cry21Fa1 ($P < 0.0001$) and Cry21Ha1 ($P < 0.001$) protoxins than the wild-type strain. The data shown are means ± standard errors of the means. (F and G) *C. elegans* dose-dependent lethality to purified Cry21Fa1 (F) and Cry21Ha1 (G) protoxins in a liquid assay. A semilog plot of animals that died versus the concentration of toxin is shown. The data were fitted to a line by the least-squares method. The LC_{50}s for Cry21Fa1 (13.6 μg/ml) and for Cry21Ha1 (23.9 μg/ml) were calculated from the line fits. The data can be found in Materials and Methods.

two of which harbor nematicidal Cry21 protoxins with synergistic activity. This trait may provide a strong selective advantage to the pathogen. First, loss of one of the toxins does not completely eliminate its virulence. Second, the synergistic action of two toxins facilitates faster host killing than is seen with strains with a single toxin. Third, the presence of multiple toxins drastically reduces the probability of the targeted host developing resistance (38). Consistent with this, several rounds of mutagenesis were needed in order to isolate a *C. elegans nasp-1* mutant resistant to *B. thuringiensis* DB27 (12), while multiple alleles of five *bre* mutants resistant to Cry5B toxins were isolated in a single mutagenesis screen (30).

Previous studies have shown that *C. elegans* exhibits avoidance behavior when challenged with different pathogens (39). *B. thuringiensis* was one of the pathogens that *C. elegans* strongly avoided (40). Other nematode-pathogenic *Bacillus* spp., such as *B. nematocida*, evolved strategies to attract nematodes (41). Interestingly, in the case of *B. thuringiensis* DB27, worms showed neither repulsion from nor attraction to the bacteria. The absence of host-aversive behavior very likely benefits the pathogen via increasing the chances of successful infection.

Interestingly, EM and light microscopy revealed that *B. thuringiensis* DB27 spores and crystals have strong associations. Crystals are normally located outside the exosporium and are separated from spores after lysis of the mother cell. However, in a few strains, such as *B. thuringiensis* subsp. *finitimus* strains (42) and *B. thuringiensis* subsp. *oyamensis* strain LBIT-113 (43), the parasporal crystals are located between the exosporium and the spore coat and continue to adhere to the spore after mother cell lysis. This phenotype has been previously described as spore-crystal association (SCA) (25). While many studies have concentrated on identification of genes responsible for this phenotype (25, 26, 44), the functional significance of SCA is not yet clear. Considering that Cry proteins are not stable in the environment, the exosporium may be used as a protective membrane. At the same time, SCA may be used as a secure strategy to deliver the spores together with toxins to the host gut, which is not guaranteed when crystals are separated from spores. Given that *B. thuringiensis* spores (45) and vegetative cells (46) have been shown to enhance the toxicity of Cry proteins, SCA thus ensures that the two components are always present together to achieve fast killing of the host. Therefore, *B. thuringiensis* DB27 SCA may be an important advantageous strategy combined with the production of several novel Cry21 protoxins acting synergistically to ensure efficient host killing.

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Fig S1 Light microscopy images (100X) of *B. thuringiensis* DB27 cultures used in *C. elegans* killing assays. A. Culture which kills nematodes in 16 hours and marked "BT DB27 veg cells/spores" in Fig 1A. Mixture of vegetative cells (often represented by chains of cells) and spores is shown. B. Culture which kills worms in 10 hours and marked "BT DB27 spores" in Fig 1A. Pure spores are shown. C. Culture which is not virulent to nematodes and marked "BT DB27 veg cells" in Fig 1A. Pure vegetative cells that form long chains are shown. Scale bar is 20 μm.

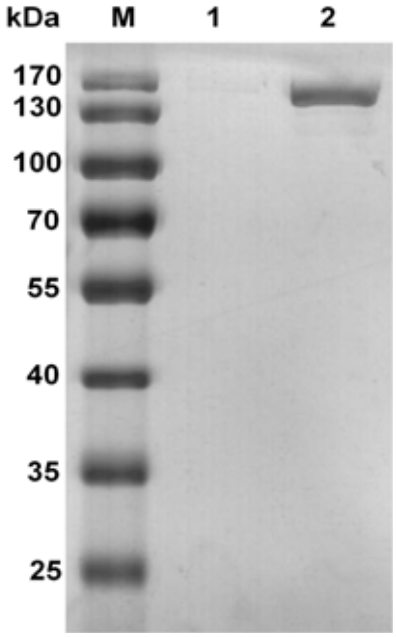

Fig S2 SDS-PAGE confirmation of Cry toxin production. Spore-crystal mixtures of *B. thuringiensis* DB27 (lane 2) and of plasmidless strain (lane 1) grown in BT sporulation medium were treated with alkaline solubilization buffer. Obtained proteins were resolved using SDS-PAGE, stained with Coomassie Briliant Blue.

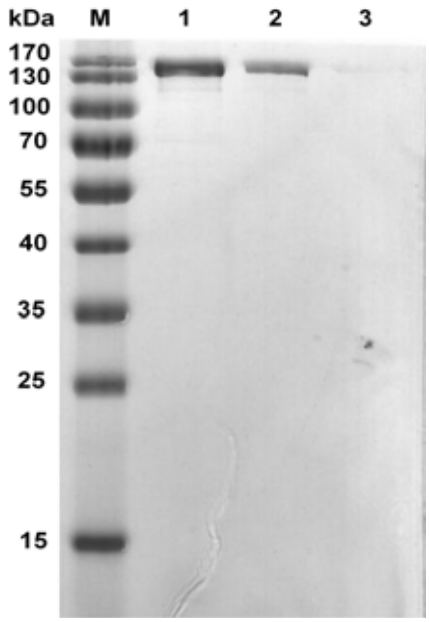

Fig S3 SDS-PAGE image of Cry proteins isolated from *B. thuringiensis* DB27 pure spores (lane 1), mixed culture of spores and vegetative cells (lane 2), pure vegetative cells (lane 3). Analyzed samples were normalized to total protein content. Vegetative cells do not produce Cry proteins, spores showed the highest production.

Fig S4 Multiple sequence alignment (Clustal Omega) of three novel Cry21 protoxins discovered in this study in comparison with Cry21Ba1 toxin, which shows the highest similarity to all three proteins

*Fast, scalable generation of high-quality protein multiple sequence alignments using Clustal Omega*Sievers F, Wilm A, Dineen DG, Gibson TJ, Karplus K, Li W, Lopez R, McWilliam H, Remmert M, Söding J, Thompson JD, Higgins DMolecular Systems Biology 7 Article number: 539doi:10.1038/msb.2011.75

An * (asterisk) indicates positions which have a single, fully conserved residue.
A : (colon) indicates conservation between groups of strongly similar properties.
A . (period) indicates conservation between groups of weakly similar properties.

Colours mark the residues according to their physicochemical properties:

RED – small and hydrophobic
BLUE – acidic
MAGENTA – basic
GREEN - Hydroxyl + sulfhydryl + amine

```
Cry21Ga1      MADLSNIYPIPYNTVSSQYFYQNQLDVDPNGENNPLTKNEQLIEDFKKTLKEKPGNLLTA      60
Cry21Ha1      MVVLNNIYKGPYNVLATPTF----LDTQEGSFDDLITDLQSAWDNFNKT-----GAFS--      49
Cry21Ba1      MADLTELYPSYHNVLARPIR----LDSIFDPFIDIFNALKGGWEEFAKT-----GYKD-P      50
Cry21Fa1      MVILNDIYKRPYNVLANPPIIVE-EGPTPGSFMDIFEDIKKAFEEFQKT-----GNLQ--      52
              *. *.::*   :*.::                 : :   :   ::* **     *

Cry21Ga1      GADIFKDIYNAI-DKQEVDYLSLTTSILGLVSIFVPEIGFVAPLLGLFYRAMG---TGNT      116
Cry21Ha1      -TEVLNQAYKMYENGGSFDYLALFKAGITVVGSVFPEIAPAVPFITMIANFIFPHLFGGT      108
Cry21Ba1      LEQHLKIAWNAS-QNGTIDYLALTKASISFIGL-IPDADAVVPFINMFVDFIFPKLFGEG      108
Cry21Fa1      -TTALQQAWNAY-QGGTIDYLALLKSSLSLVGLLIPGGEAAVPFIGMFLDFVFPKLFGAS      110
                  ::  ::   :   .***:* .: : .:.  .*    ..*:: ::   :     *

Cry21Ga1      TSDPNMNDIFEALKPKIEEMIDSKLTQEQEDFLNKTVEGLQDNLSNYRNAVRTFTIAKQS      176
Cry21Ha1      SDN--KQTIINIIDDEVNRLLNERLEQDKKDELQGYLNGMGNNIKDFGQKIVDTLFNS--      164
Cry21Ba1      SQQNSQAQFFELIIEKVKEIVDQEFRNFTLNTLLNDLDGMQTTLEHFQNDVQIAICQGEQ      168
Cry21Fa1      GSN--SDNVFEIIIKEVKQWTNQQFENFTLNSLNNTLIGIQSNISSFNEMIQIAICQEET      168
               .:     .:: :  :::.  :..: :   : *   : *:  .:. : : :

Cry21Ga1      KNTDKI--------------------KAAKTFLQRTIDIIDQIFTNQLAHLLSSVHVKL      215
Cry21Ha1      --------NKKPLIPNSHSLHDVYQSYSGFIGNVNTVIDQFR-----------LKSYEKM      205
Cry21Ba1      PGLILD-EKHPPCTPTKNHLVSVKESFKNARTSIETVLPHFKNPMTNNKTPDFNSDTVLL      227
Cry21Fa1      PGDDKSSTPSPLCTPTAEHLKNVWTQFQIARTQIEASLPYFKNPMQLDASADFQSNYIML      228
                                        .     ::  :  :             .    :

Cry21Ga1      SLPYYAMMGTLYLALLKDTITNGVEWGYEDVVL----------NTKKYELREKIKTNTER      265
Cry21Ha1      SLPYYCLAVTLVLNVYRDFIRYGKKWIYTITDETDYTTYENYINTAIKNMNQLTSKATKY      265
Cry21Ba1      TLPMYTTAATLNLILHQGYIQFVERWKSVDYDEAFI-------NQTKADLQHRIQEYSTT      280
Cry21Fa1      TLPLYTMAATLNLTLYQSFIQFADKHKDVYYDLGTM-------EQTKANHRKNIKSYTAT      281
              :** *    ** * : :  *    .                   :    : ..  .  :
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Cry21Ga1 ALAVFKSQLQTKLTPLETSDYNKQVDYITGATVNCLDIIRLWPTFDPLDYPIEID--LEL 323
Cry21Ha1 VLNTFKTYL-PTYNYNSKSQLNTYNSYIRSMTISCLDYVAIWPTLNPIDYPIPTQ--LDK 322
Cry21Ba1 VSTTFEKFK-PTLSN-KKSSINTYNKYVRSMTLNCLDIAATWPTLDNVNYPSNVEIQLDQ 338
Cry21Fa1 VRSTVINFQ-PTGDRSNKDKANLFNQYMRVMTVQCLDFVAIWPTLDPFDYPIQTE--LNP 338
Cry21Ga1 TRVLRNGIYGDQNITPPQQPFPTDT----WKADSTVDINDLQTGGIGVPIYGNKNLSGIT 379
Cry21Ha1 TRIIFQDIVGPTGKDIHFDLFDIYGK--KLP-------N----NDVFRYSYGGFQLSSAR 369
Cry21Ba1 TRLVFSNLVGPFEGNDDISTYTRRSIMNYSKGDTPGDVN----SAIQSLRYPRLELSKVQ 394
Cry21Fa1 TRIILGDIVGPVEGVGDLQFSYIDADKNPLKNNDPLN----------YFYNGYQLSFAS 387
Cry21Ga1 IHQYSDSSNWFSGPDGITQQYTDGSIISYGKTDQQ----------ASL-----TPIAID 423
Cry21Ha1 F---LTSSY---HPSDIKHCIFSGFTAENINYKSEVTQQNEYDLQNGSLYADSSWSNTIT 423
Cry21Ba1 F---Y---THDQRSNGVRHCYTSGFNLTFNDNSSM-----SAKQ-DESATA---DSPPLT 439
Cry21Fa1 M---NYENIKDNGGRGREHIFPKSLHTSYSNHDGTT---LTFDS-DNSSYN---GSFSFP 437
Cry21Ga1 NPLFGINFRYP------WPKAFIDLIFFKNQDN----------YYYGNFRYLANHKIHAV 467
Cry21Ha1 SPMYLFNMASQNTMYLDYSALYNSDKYFTIAGCYPLNDNSTGKGYHSNVTSTANHKIQAI 483
Cry21Ba1 APIKNMNANSQNSQYYDYSSINIDN---QGGGGCS---AFP--SYQSNNPILPNQKINVF 491
Cry21Fa1 MPLTAINFASMYNSNEDV--VAVYA---NPGGRMLEGNYYLSYTDKSGNQVIGNQKVQAI 492
Cry21Ga1 YPAFLPGDPIPTH----------AVGASFIPLELFPQNILGMPDVNGNIN---AKGFPFE 514
Cry21Ha1 YAMHNNPDV-------EQDGRKRGILASLVPFEVTPTNIIGQTDQNENQK---IQAFPIE 533
Cry21Ba1 YPYGSSAHPIDPHTTDPDTWFKLGYVSSHIPYDLTPQNVIGEIDQDTKQPSLILKGFPAE 551
Cry21Fa1 YPVTSYQNQVWGNG------TKYGLIYTLIPATTSPTIIIKKD-QI--------TTFSAE 537
Cry21Ga1 KSSKN-----SSVRLEWINGSNPVYMKIYDSNLIMNITNITTTNYKIRFRIATNTDNETL 569
Cry21Ha1 KINHL---DYNKIKVERSNGANSISMETG-DVYSIYFTNVLHQNYQIRVRAATSSNNIQI 589
Cry21Ba1 KG--YG--GSIEYVSEPLNGANAAKLTLN-QILYMQVTNLTTQKYQIRLRYATKNDTTAS 606
Cry21Fa1 NTSSFKNSSTNNHKYELINGASAIQLNTK-EEVTYKLTIQDPGRYQIRVRASTNTDSQTL 596
Cry21Ga1 TISIPNLLSPTTIPLPNTSTMTNEF------NDPDTAHAVGPKGEVNSQYVLVDGP---- 619
Cry21Ha1 KGSIYNEDLNGTY----VASFQPSLSNTSILNTENPELNNLIEGINGDIYKLFSSSEETL 645
Cry21Ba1 VW-FHIIGPNNQDIINHSPDIPPRSNNKMFVQGEN----------GKYV-LDTLVDSI 652
Cry21Fa1 SFQIKELGFTNSLTLPNTSKYN--NDSQKGLSGEQTTYILIPESKHDGNFI-YQTPTENI 653
Cry21Ga1 AITIPSGSIELNIE--VNSDDPIYLDRIEFIPIPNQIKFKRIN-----IKGDQVYEIWKD 672
Cry21Ha1 NLTLSPGNYRLEF-SLQTGDTPVILDRVEFIPIPSQIKPQNLN-----IQDNKSYEIWRD 699
Cry21Ba1 E--LPSGQLTILIQNINP-DQDLFLDRIEFVPIPTLPTNPNISIPKTDTSPKDSKVLWEA 709
Cry21Fa1 VNFNRSGTFTLTLQNTNSQPSPIVLDRIEFLSLPPTEFE----IPILSGVENTIYEIWKN 709
Cry21Ga1 NDYY----SSYLSGTVIS--IINPAYDNPTVTFEYFDGETSLGTTSKTFNQPEFY----- 721
Cry21Ha1 NNNN---YASYLSGTVVP--IFNPTTDNPIVTFEYFDGEESLGTIPKKYSNVGFD----- 749
Cry21Ba1 SPDIPIANTITLTGSVY---------DFADITFELYKNGNMVTSYPIKGPGPIPHRSHGN 760
Cry21Fa1 DIYY----SKYLSGTIDLFQPWDTENSKINLTFEYFDGNTSLGTITQKYSGPDANKVHD- 764
Cry21Ga1 ------GIPKWN------GKKFNRVTVTTSN--FTYSATSTLVFDSLTINDITSKPSQFT 767
Cry21Ha1 ------DIPKWL------GKKFNRVTVTVSN--FTFPPGSGITLANLIIQDIKNKYSQFT 795
Cry21Ba1 YVSCSQGILSYNYENKPVLDGFDQLRININSDPSFYDSNSGCDTKNQYSAEI-KINPNLS 819
Cry21Fa1 -------IEKWT------GKNFNRVTVKISD----LDDSGTIYLENLIVNELTNQPSQFT 807
Cry21Ga1 APEDLEKITNQANQLFTSSSQTELANTVTDYRIDQVVLKVNALSDDKFGVEKKALRKLVN 827
Cry21Ha1 TPEDLEKITNQVNQLFTSSSQTELTPTVTDYGIDQMVLKVDALSDDVFGMEKKTLRKLVN 855
Cry21Ba1 ATTDLEKITNQVNQLFTSSSQTELANTITDYRIDQIVMKVDALSNNVFGVEKKALRKLVN 879
Cry21Fa1 APEDLEKITNQVNQLFSSSSQTELAHTVSDYKIDQVVLKVNALSDDVFGVEKKALRKLVN 867

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Cry21Ga1      QAKQLSKARNVLVGGNFEKGHKWVLGRKATMVANHDLFKGDHLLLPPPTLYPSYAYQKID      887
Cry21Ha1      QAKQLSKARNVLVGGNFEKGHEWVLGRKATMVANHDLFKGDHLLLPPPTLYPSYAYQKID      915
Cry21Ba1      QAKQLSKARNVLAGGNFEKGHEWVLGREATMIANHELFKGDHLLLPPPTLYPSYAYQKID      939
Cry21Fa1      QAKQLSKTRNVLVGGNFEKGHEWALGREATMVANHELFKGDHLLLPPPSLYPSYAYQKID      927
              *******:****.********:*.***:***:***:***********:**********

Cry21Ga1      ESKLQSNTRYTVSGFIAQSEHVEVVVSRYGKEVHDMLDVPYEEALPISSDESPNCCKPAA      947
Cry21Ha1      ESKLQPNTRYTVSGFVAQSEHLEVVVSRYGKEVHDMLDVPYEEALPISSDESPNCCKPAA      975
Cry21Ba1      ESKLKSNTRYTVSGFIAQSEHLEVIVSRYGKEVHDMLDVPYEEALPISSDESPNCCKPAT      999
Cry21Fa1      ESKLKSNTRYTVSGFIAQSEHVEVIVSRYGKEVHDMLDVPYEEALPISSDESPNCCKPAA      987
              ****: *********:*****:**:************************************:

Cry21Ga1      CQCQSCDGSKRDSHFFSYSIDVGSVQSDVNLGIELGLRITKPNGFAKISNLEIKEDRPLT      1007
Cry21Ha1      CQCSSCDGSQPDSHFFSYSIDVGSLQSDVNLGIEFGLRIAKPNGFAKISNLEIKEDRPLT      1035
Cry21Ba1      CQCPSCDGSQPDSHFFSYSIDVGSVQSDVNLGIEFGLRIAKPNGFAKISNLEIKEDRPLT      1059
Cry21Fa1      CQCSSCDGSQPDSHFFSYNIDVGSVQSDVNLGIEFGLRIAKPNGFAKISNLEIKEDRPLT      1047
              *** *****: *******.*****:*********:****:*******************

Cry21Ga1      EKEIKKVQRKEQKWKKAFDQEQTEVTARLQPTLDQINALYQNEDWNGSLHPHVTYQDLSA      1067
Cry21Ha1      EKEIKKVQRKEQKWKKAFDQEQAEVAATLQPTLDQINALYQNEDWNGSLHPHVTYQHLSA      1095
Cry21Ba1      DQEIKKIQRKEQKWKKAFDQEQAEVAATFQPTLDQINALYQNEDWNGSLHPHVTYQHLSA      1119
Cry21Fa1      EKEIKKVQRKEQKWKKAFDQEQAELATTLQPTLDQINALYQNEDWNSSLHSHVTYQHLSA      1107
              ::*****:****************:*::: :*****************.*** *****.***

Cry21Ga1      VVVPTLPKQRHWFMEDRKGEHSGLTQQFQQALDRAFQQIEEQNLIHNGSFTNGLTDWTVT      1127
Cry21Ha1      VVLPTLPKQRHWFMEDREGEHYSVTQQFQQALDRAFQQIEEQNLIHNGSFANGLTDWTVT      1155
Cry21Ba1      VVLPTLPKQRHWFMEDREGEHYGVTQQFQQALDRGFQQIEEQNLIHNGSFANGLTDWTVT      1179
Cry21Fa1      VVLPALPKQRHWFMEDREGEHYGVTQQFQQALDRAFQQIEEQNLIHNGSFANGLTDWTVT      1167
              **:*:************:*** .:**********.***************:*********

Cry21Ga1      GDAQLTIFDEDPVLELAHWDASVSQTIEIMDFEEETEYKLRVRGKGKGTVTVQHGEEELE      1187
Cry21Ha1      GDAQLTIFDEDPVLELAHWDASVSQTIEIMDFEEDTEYKLRVRGKGKGTVTVQHGEEELE      1215
Cry21Ba1      GDAQLTIFDEDPVLELAHWDASVSQTIEIMDFEEETEYKLRVRGKGKGTVTVQHGEEELE      1239
Cry21Fa1      GDAQLTIFDEDPVLELAHWDASVSQTIEIMDFEEDTEYKLRVRGKGKGTVTVQHGEEELE      1227
              ************************************:************************

Cry21Ga1      TMTFNTTSFTTQEQTFYFEGKTVDVHVQSENNTFLVDSVELIEVVEE-  1234
Cry21Ha1      TMTFNTTSFTTQEQTFYFEGDTVDVHVQSENNTFLVDSVELIEVVEEE  1263
Cry21Ba1      TMTFNTTSFTTQEQTFYFEGDTVDVHVQSENNTFLVDSVELIEVVEE-  1286
Cry21Fa1      TMTFNTTSFTTQEQTFYFEGNTVDVHVQSENNTFLVDSAELIEVVEEE  1275
              ********************.*****************.********
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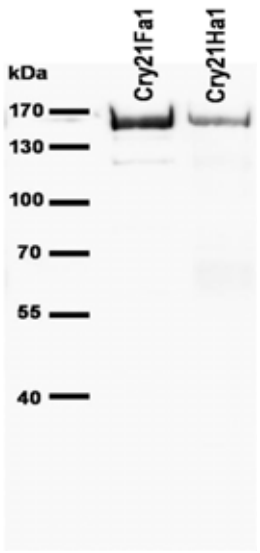

Fig S5 Representative western blot image of purified Cry21Fa1 and Cry21Ha1 proteins. Western blot was used to confirm that size-selected proteins in chromatography-separated fractions are proteins of interest. Anti-His primary antibodies were used in 1:1000 dilution. Horseradish peroxidase-conjugated secondary antibody (1:5000) and enhanced chemiluminescence were used to visualize the signal.

Table S1 Log-rank statistical analysis of survival curves

Figures	Treatment[a]	Mean survival±SD[b]	Comparison[c]	P value[d]
1A	DB27 veg. cells	20.0±0.0[3]	Veg. cells vs mix cells/spores	<0.00001
	DB27 mix cells/spores	10.7±0.6[3]	Veg. cells vs spores	<0.00001
	DB27 spores	5.4±0.48[3]	Mix cells/spores vs spores	<0.0001
1G	*C. elegans* N2	16.0±0.0[3]	*C. elegans* N2 vs *P. strongyloides*	<0.0001
	P. strongyloides	35.8±3.79[3]		
	O. carolinensis	50.0±4.1[3]	*C. elegans* N2 vs *O. carolinensis*	<0.0001
1H	*C. elegans* N2	10.7±0.6[3]	*C. elegans* N2 vs *P. redivivus*	<0.001
	P. redivivus	7.8±0.43[3]		
3A	Vector	n. a.[*]	Vector vs Fa1	<0.00001[***]
	Cry21Fa1	42.0±2.23[2]	Vector vs Ga1	0.82[**]
	Cry21Ga1	n. a.[*]	Vector vs Ha1	<0.00001[**]
	Cry21Ha1	84.4±6.18[2]	Fa1 vs Ha1	<0.0001
3D	Cry21Fa1+vector	83.3±5.19[2]	Fa1+v vs Fa1+Ha1	<0.0001
	Cry21Ha1+vector	109.2±4.07[2]	Ha1+v vs Fa1+Ha1	<0.0001
	Cry21Fa1+Cry21Ha1	55.0±3.37[2]	Fa1+v vs Ha1+v	<0.001
	Cry21Ha1+Cry21Ga1	104.4±4.69[2]	Fa1+v vs Fa1+Ga1	0.612
	Cry21Ga1+Cry21Fa1	80.2±4.63[2]	Fa1+v vs Fa1+Ha1+Ga1	0.014
	Cry21Ha1+Cry21Fa1+ Cry21Ga1	63.9±4.84[2]	Ha1+v vs Ha1+Ga1	0.525
			Ha1+v vs Fa1+Ha1+Ga1	<0.0001
3E	N2 Cry21Fa1	47.05±1.23[2]	N2 vs *nasp-1* Cry21Fa1	<0.0001
	N2 Cry21Ha1	83.3±6.26[2]	N2 vs *nasp-1* Cry21Ha1	<0.001
	nasp-1 cry21Fa1	67.25±4.66[2]		
	nasp-1 cry21Ha1	114.0±4.88[2]		
4A	N2 Cry21Fa1	42.8±1.95[2]	N2 vs *bre-2* Fa1	0.804
	N2 Cry21Ha1	84.4±6.18[2]	N2 vs *bre-2* Ha1	0.532
	bre-2 Cry21Fa1	44.0±2.43[2]	N2 vs *bre-3* Fa1	0.552
	bre-2 Cry21Ha1	89.6±6.4[2]	N2 vs *bre-3* Ha1	0.303
	bre-3 Cry21Fa1	44.8±2.35[2]		
	bre-3 Cry21Ha1	93.6±6,25[2]		
4B	N2 wt	41.6±1.89[2]	N2 vs *xbp-1*	0.006
	xbp-1	34.4±1.75[2]	N2 vs *jun-1*	0.012
	jun-1	34.4±1.93[2]	N2 vs *pmk-1*	<0.0001
	pmk-1	27.6±0.91[2]	N2 vs *kgb-1*	0.223
	kgb-1	38.8±1.77[2]		

Mean survival and statistical significance were calculated for each experiment as detailed in Materials and Methods.

[a] Treatment indicates which nematode species, mutant genotype and/or toxins were analysed.

[b] Mean survival and standard error for indicated treatment. Each treatment was tested in at least three replicates and repeated at least two times. Superscript indicates the number of repetitions of that experiment. The reported mean is based on the average survival curve of the replicates of the representative experiment.

[c] Comparison shows which treatments are statistically compared.

[d] P value for a given comparison.

n. a. * survival curves for these treatments are not completed, therefore it is not possible to calculate mean survival time

** P values for time point 120 hours

*** P value for time point 48 hours

Table S2 Potential virulence factors of *B. thuringiensis* DB27 detected by whole genome sequencing

Potential virulence factor	Function	Quantity	Location
Microbial collagenase	Peptidase M9	1	plasmid
Hemolysin BL	enterotoxin	3	plasmid
NheA, NheB, NheC	non-hemolytic enterotoxin	1	chromosome
Phospholipase (pipls, cerA, cerB)	lipase	3	chromosome
Collagenase	Peptidase M9	2	chromosome
Immune inhibitor A	InhA peptidase M6 superfamily	3	chromosome
Chitinase	chitinase	2	chromosome
Lipase	lipase	9	chromosome
Bacillolysin	Neutral protease	4	chromosome
Proteases	protease	>40	chromosome
Enhancin	mettaloprotease	1	chromosome
Hemolysin(CytK, Hly3)	cytotoxins	3	chromosome

Table S3 Features of *B. thuringiensis* DB27 plasmids and Cry toxins detected by whole genome sequencing

Plasmid name	Plasmid size, bp	Detected Cry toxins	% of protein similarity to known toxins	Designated new names	GenBank accession number
pDB27210	201029	Cry21Ba1-like	54	Cry21Fa1	KF701307
pDB27104	104550	-			
pDB2743	43904	-			
pDB278	8003	Cry21Ba1-like	49	Cry21Ga1	KF771885
pDB276	6525	Cry21Ba1-like	50	Cry21Ha1	KF771886
pDB275	5336	-			
pDB274	4121	-			