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Caenorhabditis is a metazoan host for *Legionella*

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Abstract

We investigated whether nematodes contribute to the persistence, differentiation and amplification of *Legionella* species in soil, an emerging source for Legionnaires' disease. Here we show that *Legionella* spp. colonize the intestinal tracts of *Caenorhabditis* nematodes leading to worm death. Susceptibility to *Legionella* is influenced by innate immune responses governed by the p38 mitogen-activated protein kinase and insulin/insulin growth factor-1 receptor signaling pathways. We also show that *L. pneumophila* colonizes the intestinal tract of nematodes cultivated in soil. To distinguish between transient infection and persistence, plate-fed and soil-extracted nematodes fed fluorescent strains of *L. pneumophila* were analyzed. Bacteria replicated within the nematode intestinal tract, did not invade surrounding tissue, and were excreted as differentiated forms that were transmitted to offspring. Interestingly, the ultrastructural features of the differentiated bacterial forms were similar to cyst-like forms observed within protozoa, amoeba and mammalian cell lines. While intestinal colonization of *L. pneumophila dotA* and *icmT* mutant strains did not alter the survival rate of nematodes in comparison to wild-type strains, nematodes colonized with the *dot/icm* mutant strains exhibited significantly increased levels of germline apoptosis. Taken together, these studies show that nematodes may serve as natural hosts for these organisms and thereby contribute to their dissemination in the environment and suggest that the remarkable ability of *L. pneumophila* to subvert host cell signaling and evade mammalian immune responses evolved through the natural selection associated with cycling between protozoan and metazoan hosts.

Keywords

Legionella pneumophila; *Legionella longbeachae*; *Legionella*/pathogenicity; *Caenorhabditis elegans*; *Caenorhabditis briggsae*; Caenorhabditis/*microbiology; soil; soil microbiology; virulence; disease reservoir; commensalism

Introduction

Legionella pneumophila and related species are facultative intracellular parasites of aquatic protozoa, a lifestyle that is both highly successful and not dependent on human infection. In

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fact, human infection represents a dead end for these organisms, as they are not transmitted from person to person [for reviews see (Cianciotto, 2001, Steinert *et al*., 2002, Molofsky *et al*., 2004)]. *L. pneumophila*, like close relative *Coxiella burnetii*, possesses a developmental cycle in which vegetative replicating intracellular forms (RFs) differentiate late in infection into cyst-like planktonic extracellular forms (CFs) that are metabolically dormant, resilient and hyperinfectious (Cirillo *et al*., 1994, Garduno *et al*., 2002, Molofsky *et al*., 2004, Bachman *et al*., 2004). CFs feature distinct morphological characteristics, including thickened cell wall, intracytoplasmic membranes, and inclusions of poly-β-hydroxybutyrate, that are easily differentiated from RFs (Garduno *et al*., 2002). The developmental cycle is partly controlled by transcriptional regulators (RpoS, LetA/LetS, PmrAB, IhfAB and OxyR) that activate genes whose products participate in the remodeling of the cell envelope and the delivery of effector molecules to host cells by the Dot/Icm type IV secretion system (Ninio *et al*., 2007, LeBlanc *et al*., 2008, Sahr *et al*., 2009, Rasis *et al*., 2009, Morash *et al*., 2009, Dalebroux *et al*., 2009). The ability of the secreted products of the Dot/Icm system to subvert host cell defences and abrogate phagolysosomal fusion is a hallmark of Legionnaires' disease (Ninio *et al*., 2007, Isberg *et al*., 2009).

While *L. pneumophila* Philadelphia-1 serogroup 1 (sg1) strains are responsible for nearly 90% of cases of Legionnaires' disease reported worldwide (Benin *et al*., 2002), *Legionella longbeachae* is the leading case of Legionnaires' disease in Western Australia and Thailand and the third most common cause of Legionnaires' disease (and second most common cause of community-acquired legionellosis) in the United States (Cameron *et al*., 1991, Doyle *et al*., 1998, Benin *et al*., 2002, Phares *et al*., 2007). Interestingly, both sporadic cases and outbreaks of Legionnaires' disease due to *L. longbeachae* in Australia, Japan, the Netherlands, and the Pacific Northwest (USA) have been associated with exposure to potting soils or composted organic matter (Steele *et al*., 1990a, Koide *et al*., 1999, 2000, den Boer *et al*., 2007). A case of Legionnaires' disease due to *L. pneumophila* sg1 has similarly been associated with exposure to potting soil (Wallis *et al*., 2005). Recently, *L. longbeachae* was shown to be dimorphic in amoebae, with ultrastructural characteristics similar to those described with *L. pneumophila* (Montanaro *et al*., 2005). In most clinical cases where garden or potting soils were suspected, the causative strain was successfully isolated from the soil samples (Steele *et al*., 1990a, Koide *et al*., 1999, den Boer *et al*., 2007, Wallis *et al*., 2005). However, none of these soil investigations were pursued further, so little is known of the soil ecology that enables these organisms to persist or become sufficiently abundant to be transmitted to humans.

The community of organisms within fertile soil is biologically diverse and includes a wide array of microbes (bacteria, fungi, and protozoa) and predatory invertebrates (Young *et al*., 2004). Free-living protozoa in moist soils have generally been equated with the species commonly studied in aquatic systems, including the amoeboid protozoa *Hartmannella*, *Acanthamoeba* and *Naegleria*, and ciliate protozoa of the genus *Tetrahymena* (Steele *et al*., 1996, Fields, 1996, Shadrach *et al*., 2005). More recent studies with the free-living social amoeba *Dictyostelium discoideum*, commonly found in soils, may indicate a greater diversity in potential hosts for species of *Legionella* than previously considered (Hilbi *et al*., 2007). While *D. discoideum* is a predator of *Legionella*, it can also be prey of *C. elegans* and presumably other microbiovorous nematodes (Kessin *et al*., 1996). Given the complex assemblages of organisms found in food webs of terrestrial ecosystems, we have investigated the possibility that nematodes might in some capacity be associated with persistence of *Legionella* species in soils.

Foraging invertebrates have potent immune defense mechanisms to guard against the constant threat of harm by bacterial pathogens present within the natural environment. *C. elegans* defense mechanisms include constitutive and inducible innate immune responses

and pathogen avoidance behavior (Pujol *et al*., 2001, Zhang *et al*., 2005, Ewbank, 2006, Kim, 2008). Two evolutionarily conserved nematode signaling pathways – the NSY-1/SEK-1/PMK-1 p38 mitogen-activated protein kinase (MAPK) pathway and the DAF-2 insulin/IGF-1 receptor signaling pathway – play fundamental roles in innate immunity by acting in parallel to control expression of genes encoding a diverse array of antimicrobial products and, in the case of the p38 MAPK pathway, programmed cell death (PCD) of the germline during infection by *Salmonella enterica* serovar Typhimurium (Kim *et al*., 2002, Aballay *et al*., 2003, Sifri *et al*., 2003, Garsin *et al*., 2003, Troemel *et al*., 2006). Although *C. elegans* lacks known MyD88 and Rel/NFκB homologs, partial loss-of-function mutation of *tol-1*, the single Toll-like receptor (TLR) homolog, abrogates pathogen avoidance behavior towards *Serratia marcescens* and permits tissue invasion by *S*. Typhimurium (Pujol *et al*., 2001, Tenor *et al*., 2008).

While *C. elegans* can serve as a model system for a diverse array of human microbial pathogens, in few cases is the nematode considered a natural host or environmental reservoir (Anderson *et al*., 2006). Here we show that *L. pneumophila* can colonize and persist within the *C. elegans* digestive tract in laboratory assays and artificial soil environments without invading and replicating within host tissues. Nematodes colonized with *L. pneumophila* have a moderately shortened lifespan and do not exhibit pathogen avoidance behavior. Similar results were observed with *L. longbeachae*. Worm tolerance to infection was associated with innate immune responses, as PMK-1 p38 MAP kinase *C. elegans* mutants were more susceptible to *Legionella* killing, while stress resistant DAF-2 insulin signaling pathway mutants were less susceptible to infection. Our studies demonstrate that *Legionella* differentiate into highly infectious cyst forms within the nematode intestinal tract, which are then excreted by the worm. Furthermore, we show that nematodes colonized with *icmT* and *dotA* mutant strains have increased levels of germline apoptosis, suggesting the Dot/Icm type IV secretion system interacts with the *C. elegans* PCD pathway as it does with mammalian cell apoptosis pathways. We suggest that the unique association with metazoans may represent an intermediate in the evolution of host immune evasion traits attributed to the legionellae.

Results

While potting soils and composts have been shown to harbor species of *Legionella* (Steele *et al*., 1990a, Steele *et al*., 1990b, Hughes *et al*., 1994, Koide *et al*., 1999, 2000, den Boer *et al*., 2007, Wallis *et al*., 2005, Casati *et al*., 2009a, Casati *et al*., 2009b) and nutrient-rich organic matter appears to be a natural habitat for many *Caenorhabditis* species and other free-living nematodes (Barriere *et al*., 2005, Kiontke *et al*., 2006, Freckman, 1988), the role of nematodes as natural hosts for legionellae has not been investigated. With the objective of examining the predator-prey relationship between legionellae and a lower metazoan host, wild-type Bristol N2 (herein N2) *C. elegans* nematodes were fed lawns of *L. pneumophila* grown on BCYE agar plates. Over the course of 2-3 days, legionellae accumulated within (i.e. “colonized”) the nematode digestive tract but did not invade intestinal epithelial cells. To distinguish between a laboratory phenomenon and a biologically relevant relationship, we established several testable criteria that would define a *bona fide* host/parasite relationship: 1) bacterial infection shortens lifespan of the worm; 2) bacteria remain viable within the nematode; 3) bacteria alter their metabolism and/or cellular structure to adapt to the intraluminal environment; 4) bacteria are retained in the nematode when worms are fed other bacteria; 5) nematodes recycle bacteria in soils and transmit bacteria to offspring; and 6) the innate immune response of the nematode controls infection.

Legionella kill N2 *C. elegans in vitro*

To investigate whether *Legionella* had a detrimental effect on nematode health, an optimized laboratory survival assay was developed. N2 hermaphrodites of the fourth larval stage (L4) were placed on live or heat-killed *Legionella*-spotted BCYE assay plates and their survival was observed on a daily basis. Both *L. pneumophila* and *L. longbeachae* were examined, as the former is the preeminent pathogen of the genus and the latter is especially linked to organic potting composts, a favored environment of *C. elegans* (Barriere *et al*., 2005, Kiontke *et al*., 2006). The median survival time (time in which 50% of the population is alive) was six days for *L. longbeachae* sg1 strain 33152 and seven days for *L. pneumophila* Philadelphia-1 and its derivative Lp02 strain, which was considerably shorter than the lifespan of nematodes fed heat-killed *Legionella* (Fig. 1A). The median survival time of nematodes fed *E. coli* OP50 on BCYE and on NGM was approximately 8 and 10 days, respectively (data not shown), once again demonstrating that live *E. coli* is pathogenic to nematodes when grown on rich culture medium rather than minimal culture medium (Garsin *et al*., 2001).

Multiple *Caenorhabditis* strains have shortened lifespans on *Legionella*

N2 is a wild-type, laboratory adapted *C. elegans* strain. To determine whether susceptibility to infection was restricted to the N2 nematode, several *Caenorhabditis* wild-type isolates from different regions around the world were examined in the survival assay. Four additional *C. elegans* strains and one *C. briggsae* strain also succumbed to infection by *L. pneumophila* and *L. longbeachae* (Fig. 2). *C. elegans* CB4555 and *C. briggsae* HK104 were moderately less susceptible to killing compared to the other strains surveyed. These results indicate that the detrimental effects of *Legionella* on worm survival are not limited to the N2 strain.

Pathology

Previous studies have shown that nontoxogenic killing of *C. elegans* is typically associated with colonization of the nematode digestive tract with live bacterial or fungal pathogens (Sifri *et al*., 2005). Similarly, we observed marked distension of the intestinal tracts of all *Caenorhabditis* strains fed *L. pneumophila* or *L. longbeachae* due to the accumulation of large numbers of intact bacteria (Fig. 1B and data not shown). Distension was not observed in animals fed UV-killed *L. pneumophila* and was delayed for several days in those fed *E. coli* OP50 (Fig. 1B). We enumerated *L. pneumophila* bacteria in the digestive tract following mechanical disruption and serial dilution plating over a period of several days (Fig. 1C). On day one, intestinal tract colonization of *L. pneumophila* numbered at ~10^2 CFU/nematode and increased to ~10^4 CFU/nematode by day two and ~10^5 CFU/nematode by day six.

Several additional attributes of *Legionella*-infected worms are consistent with a pathogenic process. While some nematode deaths were unremarkable, a significant portion of nematodes developed excessive fluid retention leading to extrusion of their viscera through the vulva. Others died with the well-described “bagging” phenotype, in which eggs of a gravid hermaphroditic adult hatch internally and consume the parent (Sifri *et al*., 2005). This event appears to occur with more frequency in nematodes fed *L. longbeachae* than in nematodes fed *L. pneumophila*.

Do legionellae persist in worms?

Previous studies have demonstrated that digestive tract colonization may either be transient or persistent, depending on pathogen and the “chase” bacteria (Sifri *et al*., 2005). To investigate whether *L. pneumophila* transiently or persistently colonizes the nematode

digestive tract, N2 nematodes were initially fed mCherry-producing *L. pneumophila* KB290 for three days ("pulsed") and then transferred to lawns of GFP-producing *L. pneumophila* KB130 for an additional five days ("chased"), and persistence of KB290 was tracked by fluorescence microscopy (Table 2). As summarized in Table 2, the small population of mCherry-*L. pneumophila* KB290 observed in the nematode digestive tract 24 hr post transfer (p.t.) substantially increased by 72 hr p.t. and remained at that level up to 120 hr p.t. Images of a representative nematode 72 hr p.t. are shown in Figure 3. Expansion of KB290 population appears to be the result of intraluminal replication of KB290 and not to overgrowth of KB290 on the transfer lawns, since mCherry-producing bacteria were observed in vanishingly small amounts in the lawn of the chase plates, as is evident by the presence of only GFP-producing *Legionella* (i.e. no red fluorescent bacteria) in the pharynx of nematodes p.t. (Fig. 3, arrowhead). Likewise, GFP-producing KB130 expanded in the nematode digestive tract after worms were initially fed KB130 and then transferred to mCherry-producing KB290, ruling out the possibility that a difference in bacterial fitness due to GFP vs. mCherry production accounts for selective retention and/or expansion of a bacterial strain within the digestive tract. Surprisingly, bacterial replication appears to occur exclusively within the lumen of the nematode digestive tract, since fluorescent KB130 and KB290 were only rarely observed outside the nematode intestinal tract in antemortem nematodes (≤5 bacteria/worm in <10% of worms examined).

By contrast, the number of KB290 or KB130 remained low but stable in nematodes initially exposed to the fluorescent *L. pneumophila* and then transferred to *E. coli* OP50. These results suggest that OP50, the laboratory food source for *C. elegans*, directly or indirectly alters the digestive tract environment to negatively influence *L. pneumophila* retention and/or replication. It should be noted that we found no evidence that OP50 was directly toxic to *L. pneumophila in vitro*. Other studies have also shown that feeding on OP50 can lead to the removal of most, but not all, of a persistent population of bacterial pathogens in the nematode digestive tract (Aballay *et al*., 2000).

Microscopic (fluorescence and TEM) analyses of *Legionella* within nematodes on BCYE assay plates indicate ultrastructural features similar to cyst-like forms

Fluorescent microscopic examination of nematodes fed KB130 or KB290 indicated that bacteria expelled from the anus via rhythmic peristalsis consisted of a mixture of motile short rods, characteristic of stationary phase forms, and motile coccoid forms, suggestive of terminally differentiated CFs. Of note, the fluorescent protein fusion gene fusion constructs of strains KB130 and KB290 employ the promoter of the *magA* gene, which is selectively induced during the later stages of intracellular growth of *L. pneumophila* (Hiltz *et al*., 2004). Since the *L. pneumophila* does not differentiate into CFs on BCYE agar (Garduno *et al*., 2002) we investigated the possibility that *Legionella* differentiation is induced within the worm intestinal tract. First, thin sections of BCYE agar grown *L. pneumophila* were prepared and analyzed by TEM. The bacteria exhibited a thin Gram negative cell wall structure and paucity of inclusion granules of poly-β-hydroxybutyrate (PHBA) characteristic of vegetative *Legionella* (Fig. 4A) (Garduno *et al*., 2002). By comparison, ingested *L. longbeachae*, viewed in a cross-section of the pharynx of a wild-type N2 nematode, is composed of a mixture of CFs, evident by abundant PHBA inclusions, as well as vegetative forms (Fig. 4B). TEM analysis of the intestinal tract of *C. elegans* fed *L. pneumophila* also revealed intraluminal bacteria with abundant PHBA granules (Fig. 4C-E). At higher magnification, the multiple laminar membranes observed in CFs that give the appearance of a thickened cell wall are clearly evident (Fig. 4F).

Legionella are viable for months in a soil environment populated by *C. elegans*

It is believed that legionellae are incapable of replicating extracellularly in the environment due to an obligate requirement for L-cysteine and other essential nutrients (Ewann *et al*., 2006). That these nutrients are not required for intracellular growth in protozoa or cell culture is *prima facie* evidence that *Legionella* acquires them from parasitized host cells. Morphologic differentiation and amplification of legionellae during plate-based *Caenorhabditis* assays, however, led us to hypothesize that the nematode digestive tract may be a previously unrecognized environmental niche for extracellular replication and development of *Legionella*. Indeed, the digestive tract of the nematode is both microaerobic and reducing (McGhee, 2007, Chavez *et al*., 2007), factors which will shift the equilibrium between cysteine and cystine and potentially make more cysteine available to the bacteria.

To replicate a more natural condition for nematode-*Legionella* interaction, we added mCherry-producing KB290 to hydrated autoclaved commercial potting soil without supplemental L-cysteine or other nutrients ('simulated soil'). Using the transgenic worm strains PS3729, which expresses the marker AJM-1::GFP brightly in the pharynx, and WS1904, which expresses YFP::actin predominantly in intestinal cells, to facilitate recovery of fluorescent worms from the soil, ~150 L4 stage nematodes and KB290 were used to inoculate ~2.5 gm of simulated soil. Of note, plate-based assays showed that PS3729 and WS1904 nematodes were similarly susceptible to *Legionella*-mediated killing as wild-type N2 animals (data not shown). Approximately 30 days after initial inoculation of mCherry-producing KB290, brightly fluorescent bacteria were observed in the pharynx region of recovered L2-L4 and adult stage nematodes (Fig. 5). As L4 stage nematodes were initially plated in the soil assay, the presence of extracted young larval nematodes demonstrates that the nematode life cycle has repeated itself within the soil assay. In addition, the presence of fluorescent bacteria within the pharynx indicates recent ingestion of the fluorescent bacteria, as shown in Figure 5A. The fluorescent bacteria are considered viable, as killed bacteria lose fluorescence (data not shown). Figure 5B depicts a young adult nematode with a number of mCherry-producing bacteria in the lower digestive tract. At 60 days post inoculation, some nematodes displayed anal swelling, which appeared to coincide with the accumulation of mCherry-producing bacteria in the lower digestive tract, as shown in Figure 5C. Some nematodes in the soil assays were infected to high densities with mCherry-producing bacteria consistent with bacterial accumulation and/or multiplication in the worm intestinal tract. As seen in Figure 5D, it appears that individual bacteria are undergoing cell division in the upper digestive tract (arrowhead). Even after 90 days (the duration of the experiment), simulated-soil recovered nematodes contained fluorescent *Legionella* within their upper and lower intestinal tracts. In no instance was *Legionella* invasion of worm tissues observed, echoing observations made in the plate-based assays. In addition, no protozoa were recovered throughout the course of the experiment. Taken together, these studies suggest that the nematode intestinal tract can be durably colonized with *Legionella* in a moist soil environment, even when presented another food source, leading to persistence and amplification of *L. pneumophila* as well as their transmission to nematode offspring.

TEM analysis of *Legionella* within the intestinal tract of soil-extracted nematodes demonstrates ultrastructural features similar to CFs

Since confocal microscopy demonstrated that nematodes recovered from simulated soil are colonized with *Legionella* that may be replicating within the digestive tract, TEM analyses were conducted to determine whether the bacteria underwent morphologic differentiation as was observed *in vitro*. As shown in Figures 6, TEM of PS3729 nematodes extracted from simulated soil 90 days after initial exposure to KB290 reveals a mixed population of *L. pneumophila* and *E. coli* OP50 with in the digestive tract. As was observed in plate-fed nematodes, the *L. pneumophila* in the soil-extracted nematodes had ultrastructural features

suggestive of CFs, including an electron-dense, multilaminar outer membrane and PHBA cytoplasmic inclusions.

C. elegans innate immune signaling controls *Legionella* infection

Recent studies using *C. elegans* as a surrogate model host for human microbial pathogens have identified several evolutionarily conserved signaling pathways of innate immunity that are shared across phyla. Prominent systems include the PMK-1 p38 MAPK and DAF-2 insulin/IGF-1 signaling pathways (Kim *et al*., 2002, Garsin *et al*., 2003, Troemel *et al*., 2006). These systems also play key roles in resistance to environmental stressors and natural nematode pathogens, including the nematophagous fungus *Drechmeria coniospora* and the nematocidal pore-forming toxin Cry5B of *Bacillus thuringiensis* (Birkenkamp *et al*., 2003, Huffman *et al*., 2004, Pujol *et al*., 2008). To explore whether *Legionella* elicits immune responses in *C. elegans*, nematodes with altered host defense function were exposed to *L. pneumophila* and *L. longbeachae*. As shown in Figure 7, loss-of-function *sek-1(km4)* and *nsy-1(ag-3)* mutants died more quickly than wild-type N2 nematodes when fed either *L. pneumophila* or *L. longbeachae*; by contrast, *nsy-1(ag3)* and *sek-1(ag1)* have normal lifespans on innocuous bacteria (Kim *et al*., 2002). NSY-1/MAPKKK and SEK-1/MAPKK are core components of the PMK-1 p38 MAPK pathway. Furthermore, stress and infection resistant loss-of-function *daf-2(e1370)* mutants were much less susceptible to *L. pneumophila* and *L. longbeachae* than wild-type nematodes (Fig. 8). While *daf-2* mutants have enhanced longevity and stress resistance in general, the extension of lifespan observed during *Legionella* exposure (~2.5-3.5 fold) is, like other pathogens, disproportionate to that observed with exposure to innocuous bacteria (~1 fold) (Garsin *et al*., 2003, Begun *et al*., 2007, Evans *et al*., 2008). Signaling through the DAF-2 insulin/IGF-1 receptor inhibits the activity of the forkhead transcription factor DAF-16 by hindering its translocation to the nucleus, and prior studies have shown that the pathogen-resistance of *daf-2(e1370)* mutants is *daf-16*-dependent (Garsin *et al*., 2003, Begun *et al*., 2007). As shown in Figure 8, the extended lifespan observed in *daf-2(e1370)* mutants exposed to *L. pneumophila* or *L. longbeachae* are abolished in *daf-2(e1370);daf-16(mgDf47)* nematodes. Together, these data demonstrate that well characterized *C. elegans* innate immune responses restrict *Legionella* infection *in vitro*.

TOL-1, the single Toll-like receptor ortholog of *C. elegans*, also plays an important role in innate immunity against certain Gram negative pathogens. Partial loss-of-function *tol-1(nr2033)* mutants are defective in pathogen avoidance when exposed to *S. marcescens* and are markedly more susceptible to invasion of pharyngeal tissues when exposed to *S.* Typhimurium, compared to wild-type nematodes (Pujol *et al*., 2001, Tenor *et al*., 2008). To determine whether *L. pneumophila* invaded worm tissues antemortem, young adult *tol-1(nr2033)* nematodes were exposed to GFP-expressing *L. pneumophila* KB130 or GFP-expressing *S.* Typhimurium SL1344 on BCYE plates and monitored daily by DIC and fluorescence microscopy. While invasion of pharyngeal tissues is seen with *S. enterica* fluorescent SL1344, invasion by *L. pneumophila* fluorescent Lp02 was not observed (data not shown).

L. pneumophila Dot/Icm type IV secretion system inhibits programmed cell death in the *C. elegans* germline

Apoptosis occurs at two times during the life of the *C. elegans* nematode: during embryonic and larval development, when 12% of all somatic cells die, and during germ cell morphogenesis, when half of all pachytene cells (pre-oocytes) die (Lettre *et al*., 2006). In the gonad, apoptotic cell death can be induced by a number of factors, including genotoxic, oxidative, osmotic, and heat shock stresses and during infection by *S.* Typhimurium (Aballay *et al*., 2001, Aballay *et al*., 2003). Interestingly, induction of apoptosis appears to

be pathogen-specific, as *Pseudomonas aeruginosa* does not elicit PCD within the *C. elegans* germline (Aballay *et al*., 2001). Since *L. pneumophila* induces caspase-3-mediated PCD in macrophages (Abu-Zant *et al*., 2007, Isberg *et al*., 2009), we investigated whether germline apoptosis was affected in nematodes colonized with *L. pneumophila*. We measured the number of gonadal apoptotic cells (i.e. corpse cells) in nematodes exposed to *L. pneumophila* Phildelphia-1, *L. longbeachae* ATCC 33462, *S.* Typhimurium SL1344 and *E. coli* OP50. As shown in Figure 9A, the number of apoptotic corpse cells observed in nematodes fed *L. pneumophila* and *L. longbeachae* were similar to those seen in nematodes fed *S*. Typhimurium and were significantly higher than those observed in OP50-fed nematodes.

While *L. pneumophila* induces apoptosis in macrophages, recent studies have also shown that PCD signaling is paradoxically inhibited early after phagocytosis of legionellae in a Dot/Icm-dependent manner (Santic *et al*., 2007). To investigate the contribution of the Dot/Icm system to disease in *C. elegans*, we assessed survival and germline apoptosis of nematodes colonized with *L. pneumophila dot/icm* mutant strains. We found that the *icmT* mutant strain GS3011 and the *dotA* mutant stain Lp03 were as virulent in the *C. elegans* plate-based survival assay as their isogenic parental strains JR32 and Lp02, respectively (Fig. 9B and data not shown). However, gonadal corpse cell counts for nematodes infected with GS3011 and Lp03 were significantly higher than those infected the respective parental strains (Fig. 9C). Moreover, germline apoptosis levels in nematodes colonized with the *dotA*-complemented strain Lp03(pKB9) were similar to those of the parental strain Lp02 (Fig. 9C). Taken together, these studies suggest that Dot/Icm type IV secretion system has an anti-apoptotic effect on PCD in *C. elegans*, mirroring observations of *L. pneumophila* in macrophage cells. How the Dot/Icm system inhibits apoptosis in *C. elegans* and whether this interaction influences the natural bacterium-host relationship remains to be determined.

Discussion

Legionella pneumophila and related species are considered obligate intracellular parasites of freshwater protozoa that can be grown *in vitro* on specialized complex medium supplemented with excess L-cysteine and ferric pyrophosphate (Ewann *et al*., 2006). This obligate nature is further underscored by the evolution of a complex type IV secretion system and family of secreted effector proteins that aid invasion and host cell remodeling to permit intracellular multiplication and differentiation of *Legionella* in protozoa, macrophages, *D. discoideum*, and a wide range of mammalian cell lines (Molmeret *et al*., 2004, Hilbi *et al*., 2007). Thus, our finding that *Legionella* spp. differentiate and multiply extracellularly within the intestinal tract of nematodes was unexpected. It follows that the nutrients necessary for *Legionella* survival and amplification must be available within the intestinal tract.

When fed *Legionella ad libitum* during plate-based survival assays, *Caenorhabditis* nematodes have a shortened lifespan that is dependent on both the species and strain of the bacterium and the nematode. Several observations suggest that this reduced longevity is the consequence of an infectious process. First, similar to many other microbial pathogens that kill *C. elegans*, large numbers of live *Legionella* colonize the digestive tract of nematodes during the course of infection, leading to marked intestinal distension and eventual worm death; however, colonization *per se* is not sufficient to kill worms (Garsin *et al*., 2001). Second, several distinctive abnormal phenotypes of nematode death – specifically bagging and the extrusion of viscera through the vulva – were observed during exposure to *Legionella*. While the former has been observed with other pathogens, to our knowledge the latter has not been reported in other *Caenorhabditis*-based infection models. Third, microscopic and ultrastructural analysis of intraluminal and defecated bacteria from plate-

fed nematodes display phenotypes of differentiated *Legionella*, demonstrating that at least some *Legionella* responses to the intestinal environment of *Caenorhabditis* parallel those observed within terminally-infected mammalian and protozoan cells (Cirillo *et al*., 1994, Byrne *et al*., 1998, Berk *et al*., 1998, Garduno *et al*., 2002, Berk *et al*., 2008). A final reason to conclude that *Legionella* is virulent towards *C. elegans* is that nematodes with altered immune systems exhibit similar phenotypes when exposed to *Legionella* as they do with other pathogens. The immunocompromised p38 MAP kinase mutants *nsy-1(ag3)* and *sek-1(ag1)* are more susceptible to killing by either *L. pneumophila* or *L. longbeachae*. Conversely, the insulin/IGF-1 receptor mutant *daf-2(e1370)* is markedly resistant to *Legionella* infection, and *daf-2(e1370)* resistance to infection is *daf-16* dependent. Based on these findings, we conclude that *Legionella* is pathogenic to *C. elegans* in plate-based survival assays. However, the virulence mechanisms that lead to demise of the nematode are not known at this time. Mutants of the Dot/Icm system fail to show differences in nematode survival *in vitro*, although they are significantly virulence-attenuated in host cell infection models (Molmeret *et al*., 2007, Isberg *et al*., 2009). These findings suggest that the factors mediating virulence within the intestinal tract of the nematode differ from those used to parasitize unicellular organisms and host cells. Further work will be needed to determine the role, if any, of other *L. pneumophila* virulence factors to disease in the nematode (as determined by plate-based survival assays), colonization of the nematode intestinal tract (as determined by soil-based co-culture experiments), and morphologic development (as determined by electron microscopy).

While a surfeit of *Legionella* is harmful to *C. elegans in vitro*, it is unlikely that free-living nematodes encounter *Legionella* in the amount or concentration present during plate-based assays in a natural aquatic or terrestrial environment. In natural freshwater habitats, *Legionella* concentrations have been reported to be 10^2 to 10^6 organisms per liter, depending on the specimen and detection method (Fliermans *et al*., 1981, Delgado-Viscogliosi *et al*., 2005, Declerck *et al*., 2007). Similarly, surveys of potting soils, organic composts and garden soils have recovered *Legionella* in concentrations of 10^3 to 10^8 CFU per gram soil (Steele *et al*., 1990b, Hughes *et al*., 1994, Casati *et al*., 2009a, Casati *et al*., 2009b). For this reason we developed simulated soil-based assays as a means to study *Legionella-Caenorhabditis* interactions in an environment that more closely approximates a natural condition.

We demonstrate that successive generations of nematodes extracted from sterile soil seeded with *Legionella* and *C. elegans* several months earlier harbor viable *L. pneumophila* in their digestive tract. Confirming findings of the plate-based assays, microscopic analysis of soil-extracted nematodes suggests that *L. pneumophila* undergoes bacterial division within the digestive tract, leading to a concentration of bacteria in this environmental niche. How *Legionella* is retained in the intestinal tract and resists antimicrobial defenses is not clear at this time. Morphological differentiation of organisms to hardier cyst-like forms within the nematode intestinal tract, observed during both the plate- and soil-based assays, represents one potential mechanism. A second possibility is that *Legionella* subverts host signaling pathways to improve bacterial survival or replication within the nematode intestinal tract. Intracellular survival of *L. pneumophila* within alveolar macrophages is dependent upon subverting host cell functions including abrogation of the phagosome-lysosome fusion pathway, evasion of activated innate immune defenses, successful establishment of the replicative niche and promotion of host cell survival (Horwitz, 1983, Swanson *et al*., 1995, Isberg *et al*., 2009). For example, activation of p38 and SAPK/JNK pathways were found to be necessary for *L. pneumophila* intracellular replication (Welsh *et al*., 2004, Shin *et al*., 2008). However, these regulatory pathways are absent or obscure in protozoan genomes, raising the question of how *L. pneumophila* evolved mechanisms to modulate these signaling pathways when humans and probably all other mammals appear to be dead end

hosts (Katz *et al*., 1982). We show here that p38 MAP kinase and Daf pathways restrict *Legionella* infection *in vitro*, and induction of PCD within the nematode germline during *Legionella* infection is Dot/Icm-dependent. These results suggest a plausible evolutionary origin for the ability of *Legionella* to counteract and manipulate host apoptosis and immune signaling pathways in order to establish a replicative niche. Studies are currently underway to further define this paradigm.

In summary, we propose that the predator (host) – prey (pathogen) relationship between *Legionella* and *Caenorhabditis* modeled in these studies may reflect a natural ecological relationship between *Legionella* and free-living nematodes or other evolutionarily related bacteriovorus invertebrates in the environment. Consequently, predatory invertebrates may aid persistence, dissemination and amplification of *Legionella* in the natural environment, and thus may contribute to the acquisition of Legionnaires' disease. Field studies will be needed to determine whether *Legionella* are parasitic or commensal organisms of lower metazoans in natural terrestrial or aquatic ecosystems. Such interactions may have profound implications into the evolution, natural ecology, life cycle, environmental distribution, and intrinsic virulence of *Legionella*.

Experimental procedures

Bacterial and nematode strains, oligonucleotides, media and general methods

Strains, plasmids and oligonucleotides used in this study are listed in Table 1. All *Legionella* strains were grown on buffered charcoal yeast extract agar (BCYE) or in buffered yeast extract (BYE) broth as previously described (Feeley *et al*., 1978), supplemented with thymidine (100 μg/ml), streptomycin (100 μg/ml), or kanamycin (25 μg/ml) when required. *S*. Typhimurium and *E. coli* DH5α, (used as a host strain for cloning strategies) was grown at 37°C on Luria-Bertani (LB) agar or in LB broth supplemented with kanamycin (25 ug/ml) or ampicillin (100 μg/ml). Background plasmid-encoded DotA expression levels in Lp03 (pKB9) were sufficient and did not require induction with 1 mM IPTG (Roy et al., 1998). *Legionella*, in BYE broth containing 10% dimethyl sulfoxide, and *Salmonella* and *E. coli*, in LB broth containing 15% glycerol, were stored at -75°C. All reagents, chemicals and antibiotics were purchased from Sigma-Aldrich (St. Louis, MO) or Thermo Fisher Scientific (Waltham, MA), and oligonucleotides synthesized by Invitrogen (Carlsbad, CA). Enzymes and *Taq* polymerases were purchased from New England Biolabs (Ipswich, MA) and Qiagen (Germantown, MD), respectively. DNA manipulations followed general protocols (Ausubel *et al*., 2007).

C. elegans strains were maintained at 15°C on nematode growth medium (NGM) plates spread with live or UV-light treated *E. coli* strain OP50 as a food source and were manipulated using established techniques (Brenner, 1974, Lewis *et al*., 1995).

Nematode survival and germline apoptosis assays

A loopful of *Legionella* bacteria was harvested from freshly streaked 48-72 hr BCYE plates, resuspended in 500 μl of BYE, and 20 μl of the resuspension was spread onto 3.5-cm-diameter assay plates containing BCYE supplemented with thymidine (100 μg/ml), streptomycin (100 μg/ml), or kanamycin (25 μg/ml) when appropriate, and incubated overnight at 37°C.

For survival assays, approximately 15 hermaphrodite nematodes in the fourth larval stage (L4) were placed on the assay plates (cooled to room temperature), and their survival was monitored over time at 25°C. Nematodes were transferred to fresh assay plates on the days two through five in order to separate subjects from progeny. Experiments were conducted in triplicate and repeated at least three times. For experiments with heat-treated *Legionella*,

nematodes were transferred to fresh plates when necessary to ensure sufficient source of food. Nematodes were considered dead when they failed to respond to touch and pharyngeal pumping was no longer observed. Nematodes that died as a result of crawling off the plate were censored from the analysis. For each survival assay, nematode survival was calculated by the Kaplan-Meier method and survival differences were tested for significance using the log-rank test (GraphPad Prism, version 4.0).

For germline apoptosis assays, approximately 30 L4 hermaphrodite nematodes (N2 or WS2170) were placed on the assay plates and incubated at 25°C. For each time point, approximately 20 worms were removed and prepared for microscopic examination. Corpse cells in the gonad of N2 or WS2170 nematodes were enumerated using differential interference contrast or confocal fluorescence microscopy, respectively, as previously described (Kinchen *et al*. 2005).

For tissue invasion assays, approximately 15-20 young adult hermaphrodite *tol-1(nr2033)* nematodes were placed on the assay plates and incubated at 25°C. Nematodes were monitored on a daily basis for tissue invasion via fluorescent microscopic analysis.

Quantification of nematode colonization

For quantification of bacterial colonization of *C. elegans*, nematodes were allowed to feed on *L. pneumophila* under standard survival assay conditions. On a daily basis for a six day period, approximately 30 nematodes were transferred manually from the survival plates and divided into three Eppendorf tubes containing 100 μl of ice-cold M9 buffer (22 mM KH_2PO_4, 42 mM Na_2HPO_4, 86 mM NaCl, 1 mM $MgSO_4$). The nematodes were centrifuged in a microcentrifuge at 3000 rpm for 30 sec and decanted to remove residual loose bacteria. The nematodes were washed four more times with 500 μl of ice-cold M9 buffer and resuspended in 250 μl of ice-cold M9 buffer. Nematodes were homogenized by adding approximately 200 μl of 1 mm-diameter silicone carbide beads (BioSpec Products, Bartlesville, OK) and vortexing for 1 min. Serial decimal dilutions of the supernatant before and after vortexing were made to determine the net number of colony forming units (CFU) per nematode.

Persistence assays

Persistence of bacterial colonization of *C. elegans* was evaluated using fluorescent strains of *L. pneumophila* Lp02. Construction of the GFP-expressing strain KB130 has been described previously (Morash *et al*., 2009). mCherry (red fluorescent protein) – producing Lp02 was constructed by PCR-amplifying *mCherry* from plasmid pmCherry with primers PFMCHERRYXBAI (5'-GCGATATCTAGAATGGTGAGCAAGGGCGA-3') and PFMCHERRYSPHI (5'- GCGATAGCATGCTTACTTGTACAGCTCGTCCA-3'). These primers contain sequences for the restrictions enzymes XbaI and SphI (underlined). The XbaI and SphI-restricted amplicon was ligated into plasmid pKB127 that had been digested with the same restriction enzymes, replacing excised *gfpmut3*. The resulting plasmid pKB288 was then eletroporated into Lp02, yielding strain KB290.

To evaluate persistent bacterial colonization in agar plate-based assays, L4 nematodes were allowed to feed on the initial (pulse) strain of *L. pneumophila* for 72 hr and were then transferred to a second (chase) bacterial lawn of *L. pneumophila* or OP50. Each day prior to and after transfer, five nematodes were selected for fluorescent microscopic analysis and qualitatitve assessments of fluorescence intensity were made.

Soil assay

Commercial potting soil (Hyponex Corporation, Ohio) was sterilized by autoclaving, and an aliquot (~2.5 g) was placed in a 3.5-cm Petri plate. A loopful of mCherry-producing *L. pneumophila* KB290 was harvested from a freshly-streaked BCYE plate, washed three times in 1 mL sterile dH_2O, resuspended in 1 mL sterile dH_2O and added to the soil assay plate. Approximately 150 L4 stage nematodes (PS3729 or WS1904) were removed from a standard NGM propragation plate, washed three times and resuspended in M9 buffer and deposited onto the soil assay plate. These transgenic nematode strains produce green fluorescent protein (GFP), which aided in their re-isolation from the soil. Plates were kept in an aerobic humid chamber at 25°C. Overnight broth cultures of OP50 (3 ml) were concentrated, washed with sterile M9 buffer, and added to the soil every 1-2 weeks to ensure a sufficient source of food for maintenance of the nematode colony and progeny development. On a weekly basis, the nematodes were extracted for microscopic analysis and when necessary, additional M9 buffer was added to maintain moisture level. Nematodes were extracted from the soil using the NGM agar capture method, as previously described (Barriere *et al*., 2006, Sifri *et al*., 2008). Every several weeks soil samples (~0.1 gm) were resuspended in a 10x volume of PBS and aliquots were plated on BCYE agar plates supplemented with streptomycin and on LB agar plates. In each instance all recovered colonies of *L. pneumophila* remained fluorescent while recovered colonies of *E. coli* were nonfluorescent, demonstrating that pKB288 was retained by *L. pneumophila* and not transferred by conjugation or transformation to *E. coli*.

Differential interference contrast (DIC) and confocal microscopy

Nematodes were mounted on 2% agarose pads in M9, anesthetized with 3-5 mM levamisole, sandwiched under a coverslip, and examined by differential interference contrast microscopy with Nomarski optics using an Axiovert 200 microscope (Carl Zeiss AG, Oberkochen, Germany) equipped with a Qicam 12-bit Fast 1394 (QImaging Corp, Surrey, British Columbia, Canada) digital camera and fluorescence illuminator Xcite 120 (EXFO Life Sciences, Mississauga, Ontario, Canada). Images were captured with Image Pro Plus software (Media Cybernetics, Bethesda, MD). Confocal fluorescene microscopy was performed using a LSM 510 confocal microscope with accompanying LSM Image Browser software (Zeiss). Images were edited using Photoshop CS (Adobe).

Transmission electron microscopy

Worms exposed to *Legionella* on BCYE assay plates or extracted from soil were washed three times in sterile PBS and resuspended in 200 μl of 4% glutaraldehyde in PBS and stored at 4°C until processing. Intact *Legionella*-exposed worms were fixed overnight at 4°C in modified Karnovsky's fixative (0.1 M phosphate buffer (PB) containing 4% (w/v) paraformaldehyde and 2.5% (w/v) glutaraldehyde, pH 7.4). All subsequent processing was carried out at room temperature unless otherwise noted. Following primary fixation, the worms were washed three times for a total of 45 min with PB and post-fixed for 1 hr in 1.0% osmium tetroxide. The worms were then washed in distilled water, dehydrated employing a graded ethanol series, embedded in Embed 812 epoxy resin (Electron Microscopy Sciences, Hatfield, PA), and polymerized for two days at 60°C. Ultrathin sections approximately 70 nm in thickness, obtained with a diamond knife (Diatome, Hatfield, PA) on a Ultracut UCT ultramicrotome (Leica, Richmond, IL), were collected on 200 mesh copper grids, contrast stained with uranyl acetate and lead citrate according to routine procedures, and examined in a JEM-1230 transmission electron microscope (JEOL, Tokyo, Japan). Images were acquired using an SIA L3-C digital camera (Scientific Instruments and Applications, Duluth, GA).

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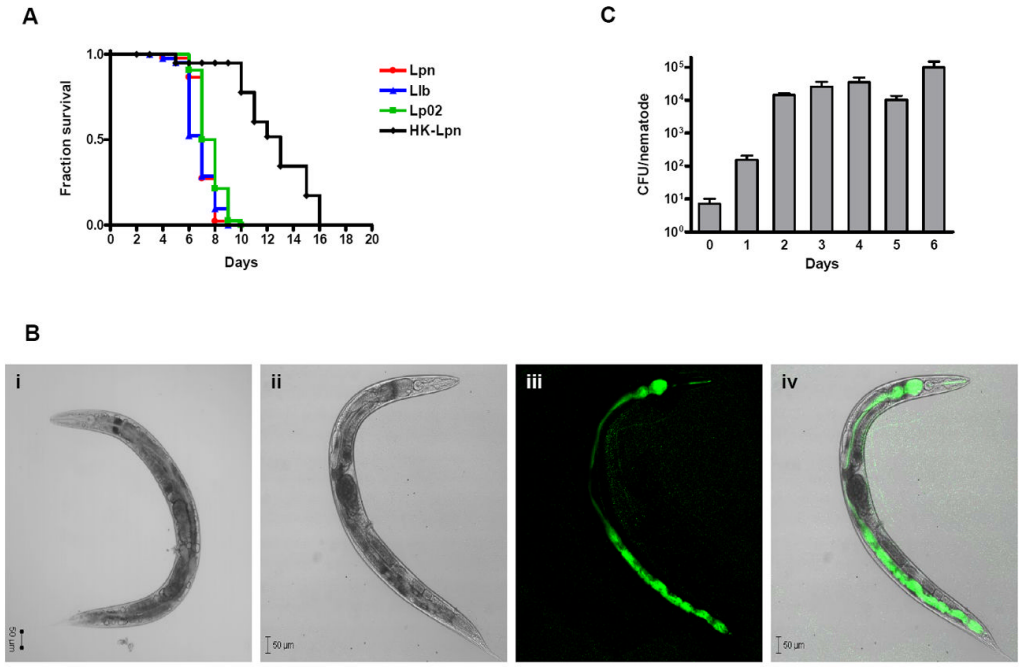

Figure 1.

Survival of *C. elegans* fed on *Legionella*. (A) Kaplan-Meier survival plots of N2 nematodes fed *L. pneumophila* Philadelphia-1 (Lpn; circles, n=45), *L. longbeachae* ATCC 33462 (Llb; triangles, n=43), *L. pneumophila* Philadelphia-1 derivative Lp02 strain (squares; n=44), and heat-killed *L. pneumophila* Philadelphia-1 (HK-Lpn; diamonds, n=28). Nematode loss was not a significant consideration in the survival assay as the nematodes remained mostly within the bacterial lawn. $P < 0.0001$ by pairwise comparison by the log-rank test of the each of the strains (Lpn, Llb, Lp02) versus heat-killed. $P \leq 0.01$ by pairwise comparison by the log-rank test for each of the strains Lpn and Llb vs. Lp02. Data is representative of one of three independent experiments. (B) Confocal microscopy of *Legionella* colonization and persistence in wild-type N2 nematodes. DIC images of wild-type N2 nematodes fed (i) heat-killed or (ii-iv) live GFP-expressed *L. pneumophila* KB130 for five days. (ii) Bright-field image using DIC optics; (iii) KB130 producing GFP colonizing the length of intestinal tract; and (iv) merged image of ii and iii. (C) Bacterial intestinal load of *L. pneumophila* KB130 within wild-type N2 nematodes (CFU/nematode) over a period of six days. Bacterial loads were determined in triplicate from approximately ten disrupted worms. Data represent mean ± SEM.

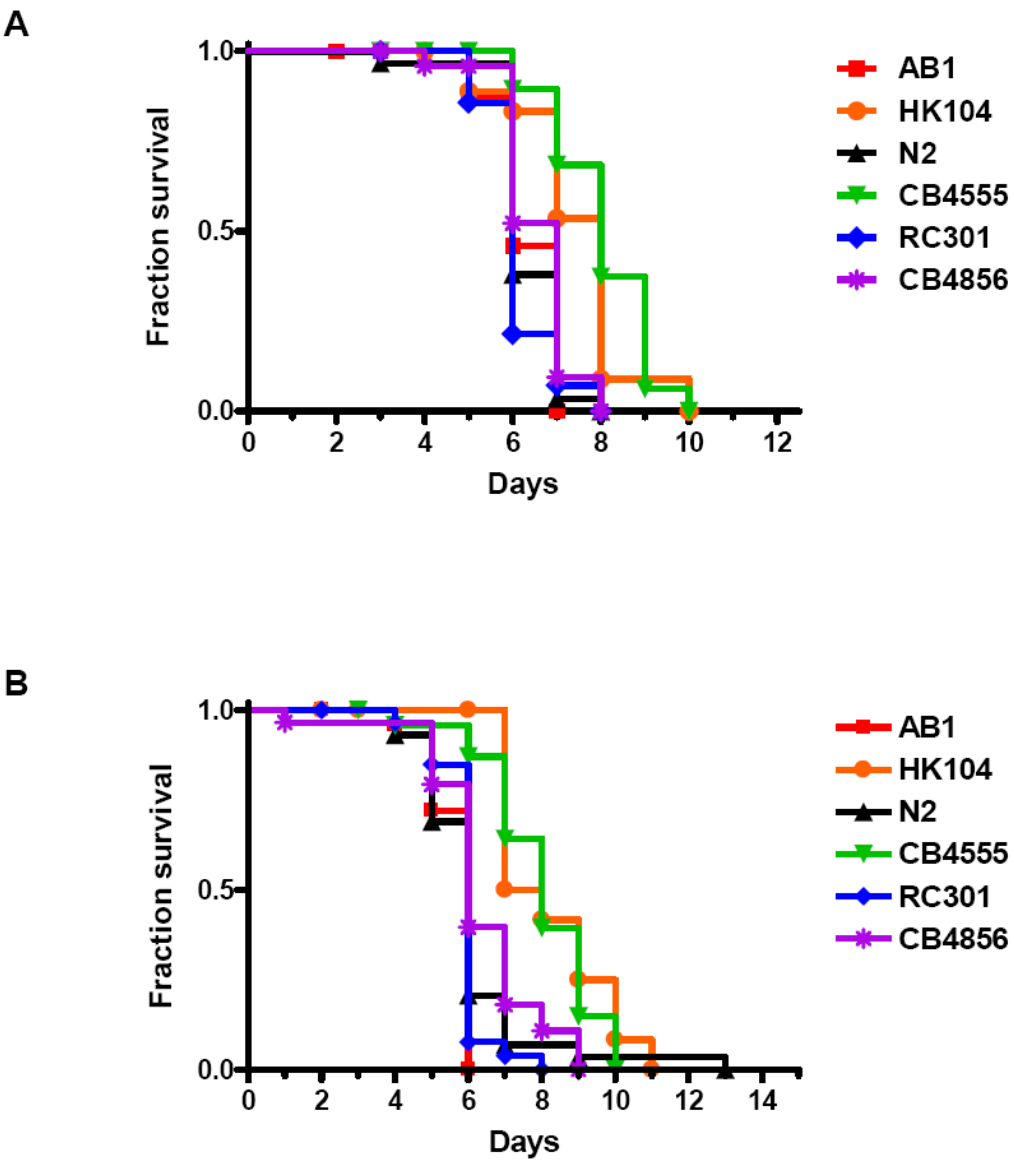

Figure 2.

Survival of multiple *Caenorhabditis* nematodes fed *Legionella*. (A) Kaplan-Meier survival plot of *C. elegans* AB1 (squares, n=30), *C. briggsae* HK104 (circles, n=31), *C. elegans* N2 (triangles, n=30), *C. elegans* CB4555 (inverted triangles, n=24), *C. elegans* RC301 (diamonds, n=15), and *C. elegans* CB4856 (asterisks, n=26) fed *L. pneumophila* Philadelphia-1 type strain. (B) Kaplan-Meier survival plot of *C. elegans* AB1 (squares, n=26), *C. briggsae* HK104 (circles, n=27), *C. elegans* N2 (triangles, n=29), *C. elegans* CB4555 (inverted triangles, n=25), *C. elegans* RC301 (diamonds, n=30), and *C. elegans* CB4856 (asterisks, n=29) fed *L. longbeachae* ATCC 33462. $P \leq 0.0002$ by pairwise comparison by the log-rank test of each of the nematode strains (HK104 and CB4555) versus wild-type N2 for both *Legionella* strains. All other nematode strain pairwise comparisons versus wild-type N2 were found to be statistically insignificant. Data is representative of one of three independent experiments.

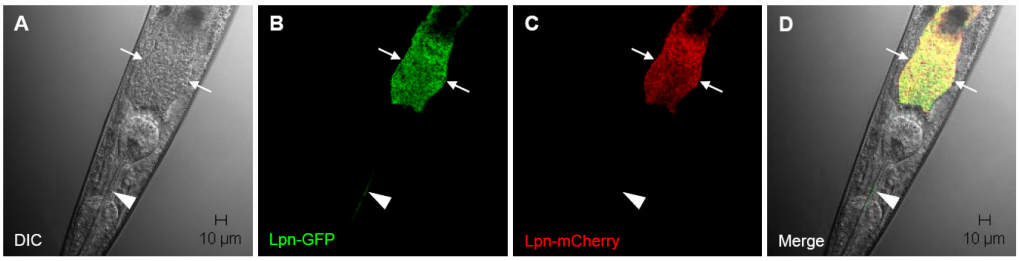

Figure 3.

Confocal microscopy of *Legionella* colonization and persistence in wild-type N2 nematodes. Wild-type worms were "pulsed" with mCherry-producing KB290 for three days and "chased" with GFP-producing KB130. Representative images after three days of exposure to KB130 are shown. (A) Bright-field image using DIC optics; (B) KB130 producing GPF in the intestinal and pharyngeal lumens; (C) KB290 producing mCherry in the intestinal lumen; and (D) merged images of A, B and C. Arrows demarcate the intestinal tract lumen; arrowhead indicates intact KB130 producing GFP in the pharyngeal lumen.

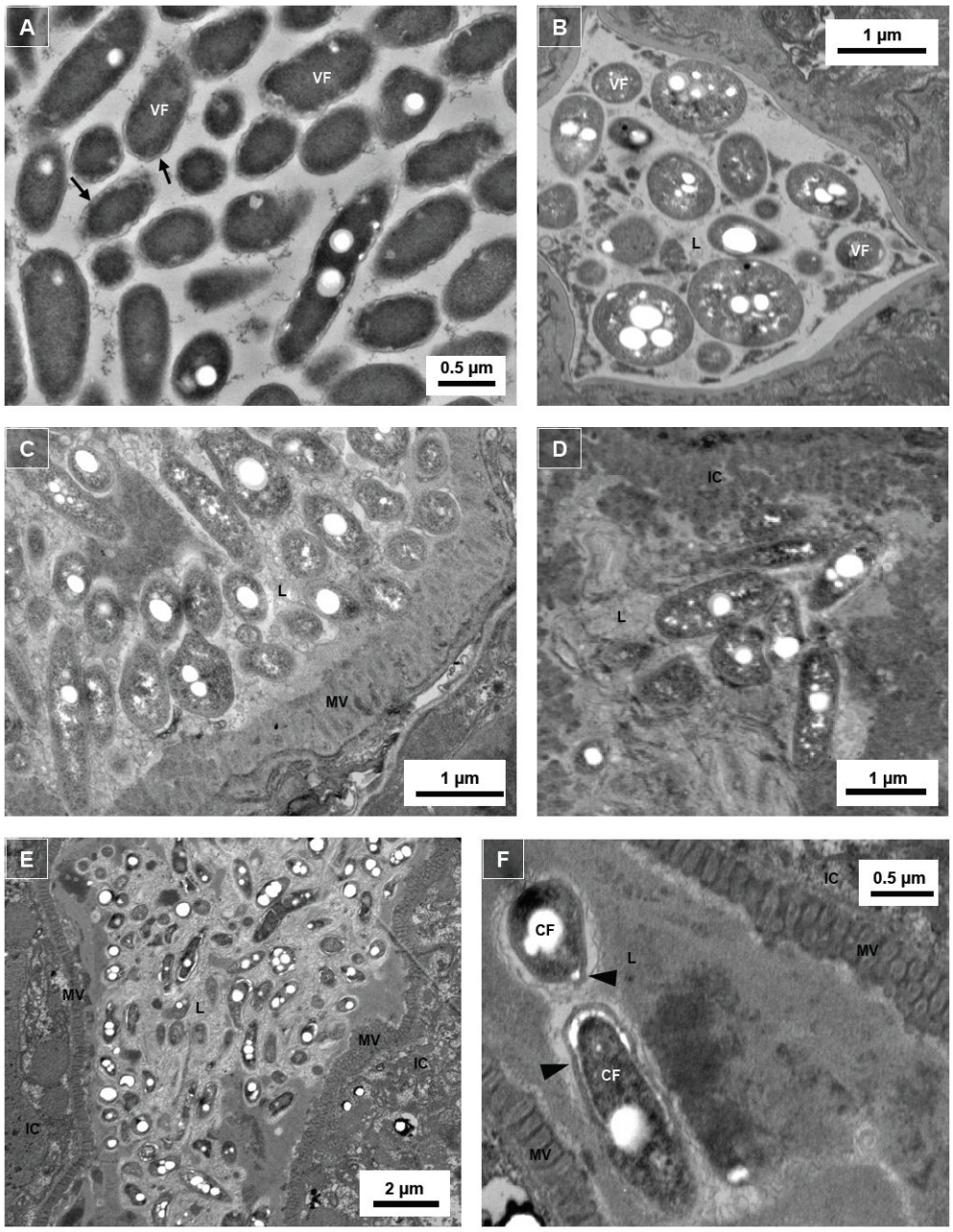

Figure 4.

Intraluminal *Legionella* exhibit morphologically differentiated forms. Transmission electron microscopic images of (A) *Legionella* harvested from 6-day old assay plate culture without the presence of N2 nematodes; (B) cross-cut pharynx section of N2 nematodes colonized with *L. longbeachae* ATCC 33462 (note triangular shape of the grinder); (C) and (D) mid-body cross-cut digestive tract sections of N2 nematodes colonized with *L. pneumophila* Philadelphia-1 type strain; and (E) and (F) mid-body transverse-cut digestive tract sections of nematodes colonized with *L. pneumophila* SVir. Note that the thickened cell walls and the white circular spaces within the *Legionella* bacteria are poly-β-hydroxybutyrate (PHBA) granules. VF, vegetative form; CF, cyst-like form; L, intestinal lumen; IC, intestinal cell;

MV, microvilli; arrow, thin cell wall of VF *Legionella*; arrowhead, thick cell wall of CF *Legionella*.

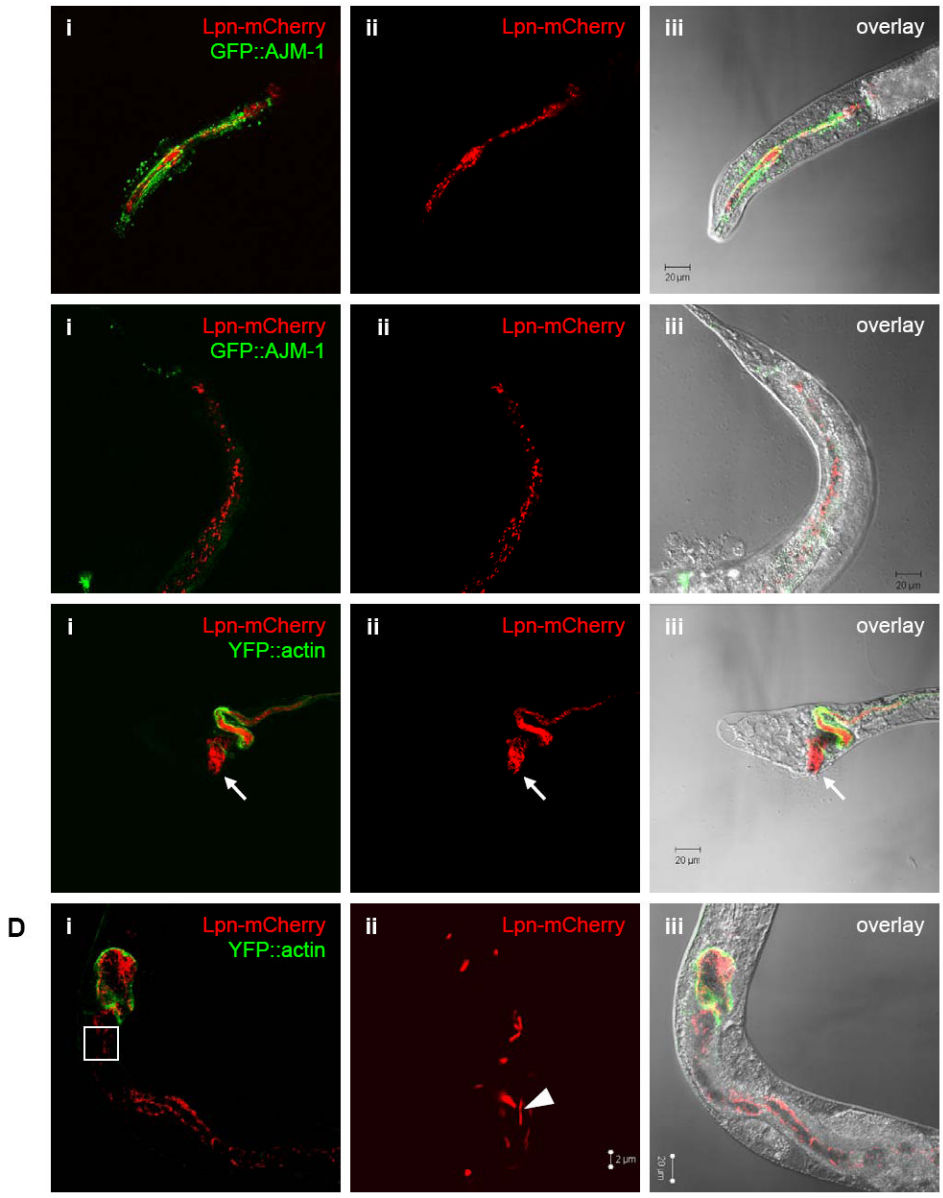

Figure 5.

Presence of *Legionella* in nematodes cultivated in a soil environment. Confocal microscopic images of mCherry-producing *L. pneumophila* KB290 in *C. elegans* PS3729 (A) head of a L2 stage and (B) tail of a young adult 30 days after initial inoculation, and in WS1904 (C) tail of a L3 stage and (D) mid-body of an adult views 60 days after initial inoculation. Panels i-iii correspond (i) red and green channels showing fluorescent KB290 and nematode intestinal cells, (ii) red channel alone showing only KB290,and (iii) overlayed red channel, green channel, and DIC bright-field images. Green fluorescence indicates the apical tight junctions within the pharynx structure in PS3729 and outlines the intestinal tract in WS1904. Note that image (Dii) is at higher magnification and corresponds to the outlined inset box in (Di). Arrows denote a heavily colonized anus leading to anal swelling; arrowhead demonstrates bacilli that appear to have recently divided.

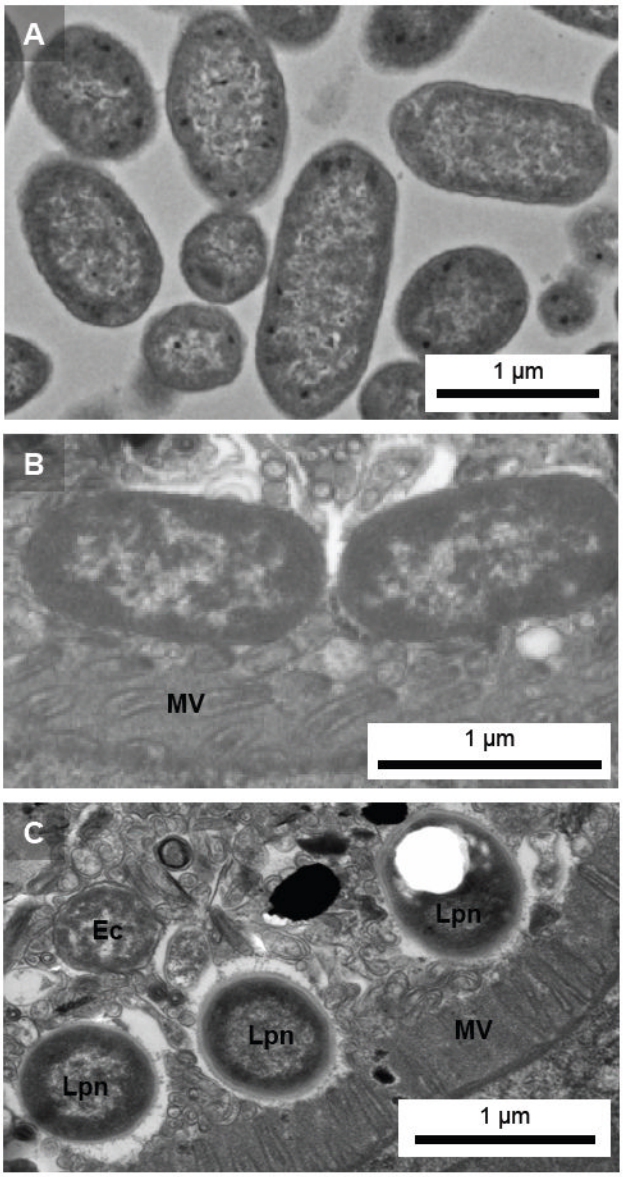

Figure 6.

Ultrastructural analysis of bacteria within *C. elegans* digestive tract. Transmission electron microscopic images of (A) plate grown *E. coli* OP50, and transverse-cut sections of the digestive tracts of *C. elegans* PS3729 nematodes extracted from a soil environment 90 days after initial inoculation with intermittent supplementation of *E. coli* OP50 detail bacteria embedded within intestinal lumen lining the digestive tract; (B) *E. coli* OP50, and (C) mixed population of OP50 and *Legionella*. Note ultrastructural differences between OP50 and *Legionella*, in particular the thickened cell walls and the white spaces indicating the presence of PHBA in *Legionella*. Lpn, *L. pneumophila*; Ec, *E. coli* OP50; MV, microvilli.

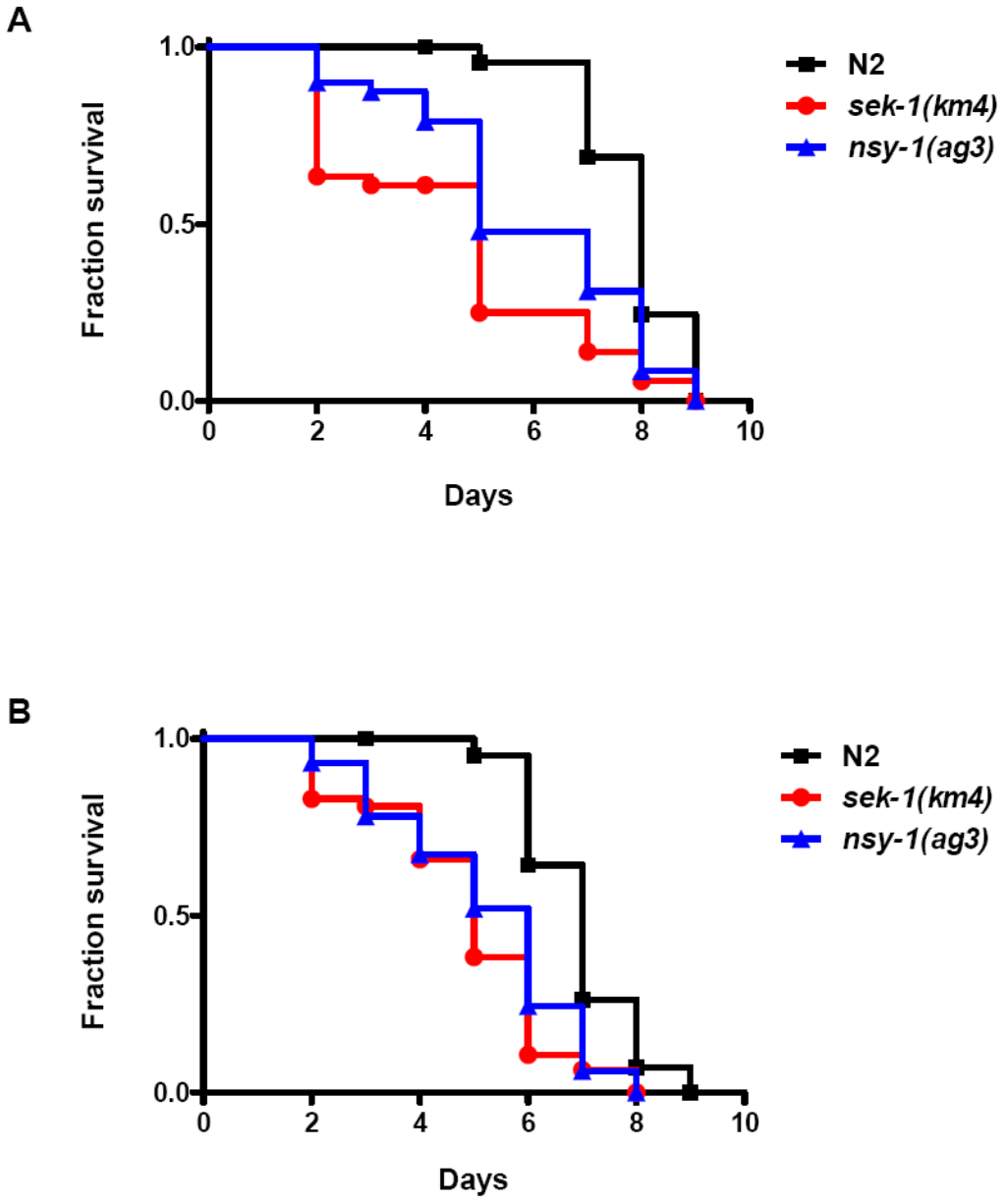

Figure 7.

Survival of immunocompromised *C. elegans* fed *Legionella*. (A) Kaplan-Meier survival plot of *C. elegans* N2 (squares, n=45), *sek-1(km4)* (circles, n=44), and *nsy-1(ag3)* (triangles, n=45) fed *L. pneumophila* Philadelphia-1 type strain. (B) Kaplan-Meier survival plot of *C. elegans* N2 (squares, n=45), *sek-1(km4)* (circles, n=46), and *nsy-1(ag3)* (triangles, n=44) fed *L. longbeachae* type strain. $P < 0.0001$ by pairwise comparison by the log-rank test of each of the nematode strains [*sek-1(km4)* and *nsy-1(ag3)]* versus wild-type N2 for both *Legionella* strains. Data are representative of one of three independent experiments.

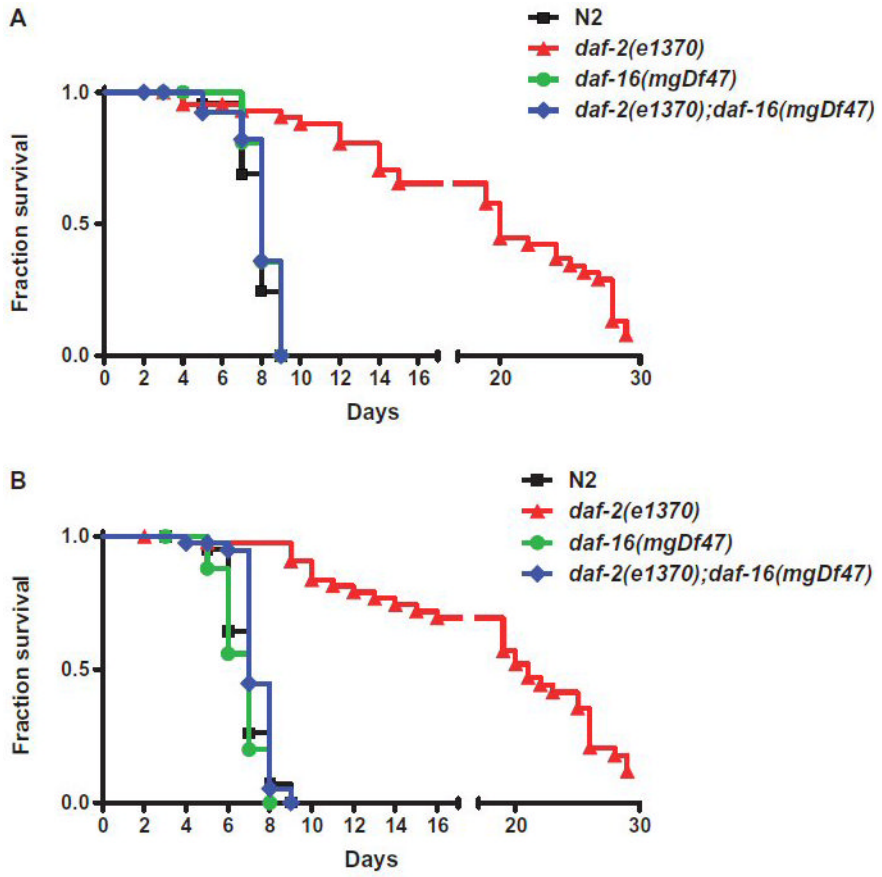

Figure 8.

Survival of pathogen and stress-resistant *C. elegans* fed *Legionella*. (A) Kaplan-Meier survival plot of *C. elegans* N2 (squares, n=45), *daf-2(e1370)* (triangles, n=46), *daf-16(mgDf47)* (circles, n=46), and *daf-2(e1370)/daf-16(mgDf47)* (diamonds, n=45) fed *L. pneumophila* Philadelphia-1 type strain. (B) Kaplan-Meier survival plot of *C. elegans* N2 (squares, n=45), *daf-2(e1370)* (triangles, n=45), *daf-16(mgDf47)* (circles, n=27), and *daf-2(e1370)/daf-16(mgDf47)* (diamonds, n=41) fed on *L. longbeachae* type strain. $P < 0.0001$ by pairwise comparison by the log-rank test of *daf-2* versus wild-type N2 for both *Legionella* strains. All other nematode strain pairwise comparisons versus wild-type N2 were found to be statistically insignificant. Data is representative of one of three independent experiments.

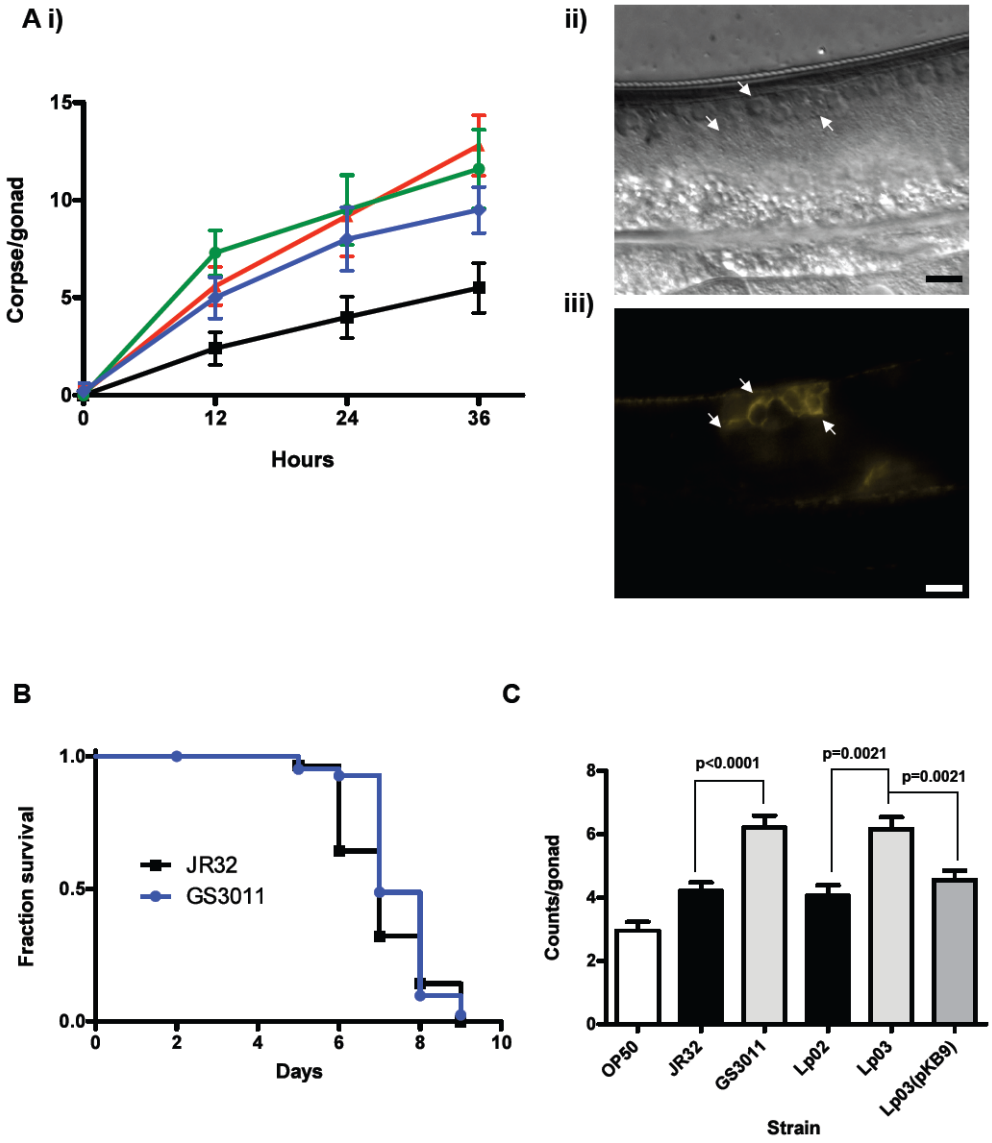

Figure 9.

Elevated levels of germline apoptosis in *C. elegans*. (A) Germline apoptosis in *C. elegans* fed *Legionella*: (i) Number of corpse cells counted per gonad (n=10) plotted over time at 0, 12, 24, 36 hr in *C. elegans* N2 fed on *E. coli* OP50 (squares), *L. pneumophila* Philadelphia-1 type strain (triangles), *L. longbeachae* type strain (circles) and *S.* Typhimurium SL1344 type strain (diamonds); (ii) DIC image of corpse cells (indicated by arrows) in a gonad of a transgenic *C. elegans* WS2170 fed *L. longbeachae* for 36 hr with (iii) corresponding image of fluorescent actin filaments associated with corpse cells. Size marker is 10 μm. (B) Kaplan-Meier survival plot of *C. elegans* N2 fed *L. pneumophila* JR32 (squares, n=30) and the isogenic *icmT* mutant GS3011 (circles, n=44). P > 0.05 by pairwise comparison by the log-rank test. Data representative of three independent experiments. (C) Bar graph of mean corpse cell counts per gonad (n=20) in transgenic *C. elegans* WS2170 nematodes fed on the designated bacterial strain for 24 hr. P = 0.002 and P = 0.015 by pairwise comparison by the log-rank test of OP50 versus JR32 and Lp02, respectively.

Table 1

Strains and plasmids used in this study.

Strain or plasmid	Description (genotype and/or phenotype)	Reference or source[a]
Strains		
Legionella pneumophila		
Phildelphia-1	Type strain (ATCC 33152)	CDC, Atlanta
SVir	Spontaneous *L. pneumophila* Philaldephia-1 Sm^r mutant	(Hoffman *et al*., 1989)
Lp02	Philadelphia-1 derivative, *rpsL hsdR thyA* (Sm^r) mutant	(Berger *et al*., 1993)
KB130	Lp02 (pKB127), Thy^+	(Morash *et al*., 2009)
KB290	Lp02 (pKB288), Thy^+	This work
Lp03	*dotA*03, *thyA, hsdR, rpsL*	(Berger *et al*., 1994)
Lp02(pKB9)	*dotA* complement	This study
JR32	NaCl-sensitive isolate of *L. pneumophila* AM511	(Sadosky *et al*., 1993)
GS3011	JR32 *icmT3011*::Km	(Segal *et al*., 1998)
Legionella longbeachae		
ATCC 33462	Type strain	Yousef Abu Kwaik
Escherichia coli		
DH5α	F^- Φ80d*lacZ*ΔM15 Δ(*lacZYA-argF*)*U169 endA1 recA1 hsdR17 deoR thi-1 supE44 gyrA96 relA1*	Clonetech
OP50	Uracil auxotrophy	CGC, (Brenner, 1974)
***Salmonella enterica* serovar Typhimurium**		
SL1344	Type strain	Lab collection
SM022	SL1344(pGP704:*rpsM*::*gfp*)	Alejandro Aballay, (Vazquez-Torres *et al*., 1999)
Caenorhabditis briggsae		
HK104	Wild isolate from Okayama, Japan	CGC
Caenorhabditis elegans		
Bristol N2	Wild-type isolate from Bristol, England	CGC, (Brenner, 1974)
AB1	Wild isolate from Adelaide, Australia	CGC
CB4555	Wild isolate from Pasadena, California, USA	CGC
RC301	Wild isolate from Freiburg, Germany	CGC
CB4856	Wild isolate from Hawaii, USA	CGC
KU4	*sek-1(km4)*	(Tanaka-Hino *et al*., 2002)

Strain or plasmid	Description (genotype and/or phenotype)	Reference or source[a]
AU3	*nsy-1(ag3)*	(Kim *et al.*, 2002)
CB1370	*daf-2(e1370)*	(Garsin *et al.*, 2003)
	daf-16(mgDf47)	(Garsin *et al.*, 2003)
GR1309	*daf-2(e1370); daf-16(mgDf47)*	(Garsin *et al.*, 2003)
	tol-1(nr2033)	CGC
PS3729	*unc-119(ed4); syIs78[ajm-1::gfp + unc-119(+)]*	CGC
WS1904	*unc-119(ed3); opEx813[P_{ced-10}::yfp::actin + unc-119(+)]*	Kinchen and Hengartner, unpublished
WS2170	*unc-119(ed3);opIs110[P_{ced-1}::YFP::act-5;unc-119(+)]*	(Kinchen *et al.*, 2005)
Plasmid		
pKB9	*dotA* expression plasmid	(Roy *et al.*, 1998)
pBH6119	RSF1010 ori, promoterless, *gfpmut3 tdΔi* ; Thy^+, Amp^r	(Hammer *et al.*, 1999)
pKB127	pBH6119::*magA* 245-bp promoter region cloned into BamHI and XbaI sites	(Morash *et al.*, 2009)
pmCherry	pUC19::*mCherry*, Amp^r	Roger Y. Tsien, (Shaner *et al.*, 2004)
pKB288	PCR-amplified *mCherry* inserted into XbaI and SphI sites of pKB127 replacing *gfpmut3*	This work

[a] ATCC, American Type Culture Collection; CDC, Centers for Disease Control and Prevention; CGC, *Caenorhabditis* Genetics Center.

Table 2

Persistence of mCherry-producing *L. pneumophila* KB290 in N2 nematodes “pulsed” with KB290 for three days before placement on “chase” bacterial strains for an additional five days.

	Number of hours post-exposure (h)				
Bacterial strain	**24**[a]	**48**	**72**	**96**	**120**
E. coli OP50	+++[b]	+++	++	+	+
GFP-*L. pneumophila* KB130	+	+	+++	+++	+++

[a] Five worms were randomly selected from each bacterial strain for fluorescent microscopic analysis.

[b] Qualitative observations of the fluorescence intensity level with + representing the least intensity and +++ representing the most intensity.