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Caenorhabditis elegans Innate Immune Response Triggered by *Salmonella enterica* Requires Intact LPS and Is Mediated by a MAPK Signaling Pathway

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Summary

Compared to mammals, insects, and plants, relatively little is known about innate immune responses in the nematode *Caenorhabditis elegans*. Previous work showed that *Salmonella enterica* serovars cause a persistent infection in the *C. elegans* intestine [1, 2] that triggers gonadal programmed cell death (PCD) and that *C. elegans* cell death (*ced*) mutants are more susceptible to *Salmonella*-mediated killing [3]. To further dissect the role of PCD in *C. elegans* innate immunity, we identified both *C. elegans* and *S. enterica* factors that affect the elicitation of *Salmonella*-induced PCD. *Salmonella*-elicited PCD was shown to require the *C. elegans* homolog of the mammalian p38 mitogen-activated protein kinase (MAPK) encoded by the *pmk-1* gene. Inactivation of *pmk-1* by RNAi blocked *Salmonella*-elicited PCD, and epistasis analysis showed that CED-9 lies downstream of PMK-1. Wild-type *Salmonella* lipopolysaccharide (LPS) was also shown to be required for the elicitation of PCD, as well as for persistence of *Salmonella* in the *C. elegans* intestine. However, a presumptive *C. elegans* TOLL signaling pathway did not appear to be required for the PCD response to *Salmonella*. These results establish a PMK-1-dependant PCD pathway as a *C. elegans* innate immune response to *Salmonella*.

Results and Discussion

In recent work designed to determine if *C. elegans* responds to pathogen attack with an innate immune response, Mallo et al. [4] used transcriptional profiling analysis to identify a variety of pathogen-inducible genes. In addition, Kim et al. [5] carried out a forward genetic screen for *C. elegans* mutants with enhanced susceptibility to *Pseudomonas aeruginosa* and identified two genes that are part of a conserved p38 MAPK signaling pathway. In mammals, p38 MAPK is involved in mediating the immune response to bacterial LPS, raising the possibility that it may play a similar role in *C. elegans*.

The mammalian p38 MAPK signaling pathway is known to mediate stress responses and is activated by heat shock, ultraviolet (UV) light, the proinflammatory cytokines IL-1 or TNF-α, or bacterial LPS [6–8]. One consequence of p38 activation in mammalian cells is programmed cell death (PCD). In *C. elegans*, stress-activated PCD occurs in *C. elegans* gonadal cells, and a low level of gonadal programmed cell death occurs when worms feed on *E. coli*. In response to γ irradiation [9] or feeding on *Salmonella* [3], however, a much higher level of gonadal PCD is observed.

The MAPK-kinase and MAPK-kinase-kinase that function upstream of PMK-1, the *C. elegans* homolog of p38, are SEK-1 and NSY-1, respectively [10, 11]. Figure 1A shows that the enhanced level of gonadal PCD observed when wild-type worms feed on *Salmonella* is not observed in *sek-1* or *nsy-1* mutant worms or in worms in which *pmk-1* has been inactivated by RNAi. These results show that the enhanced level of PCD elicited by *Salmonella* feeding is PMK-1 dependent, but that the "basal" level of PCD observed when feeding on *E. coli* is PMK-1 independent.

Consistent with previous results showing that worms in which *Salmonella*-elicited cell death is blocked are hypersusceptible to *Salmonella*-mediated killing [3], RNAi *pmk-1* worms were also more susceptible to *Salmonella*-mediated killing compared to wild-type worms (Figure 1B).

To verify that the CED cell death pathway is acting downstream of PMK-1, we used worms carrying a loss-of-function mutation in the *ced-9* gene that encodes a negative regulator of the programmed cell death pathway [12]. Figure 1C shows that PMK-1 inhibition by RNAi in *ced-9* worms did not reduce the high level of spontaneous gonadal cell death observed when the worms were feeding on *E. coli* OP50. In our previously published work [3], we showed that, in a *ced-9* gain-of-function mutant, *Salmonella*-induced PCD was completely blocked, but that the level of spontaneous PCD observed when feeding on *E. coli* was not affected. This result indicates that *Salmonella*-elicited PCD is CED-9 dependent, and, in conjunction with the data in Figure 1C, we infer that the *Salmonella*-elicited CED cell death pathway lies downstream of PMK-1.

In vertebrates, in response to pathogen-associated molecular patterns (PAMPs), TLR receptors activate several signaling pathways, including the p38 MAPK pathway [13]. The *C. elegans* genome appears to encode a single TLR, TOL-1, as well as single copies of TRF-1, PIK-1, and IKB-1, homologs of the mammalian downstream signal transduction components TRAF1, IRAK, and IκB, respectively [14]. However, the *C. elegans* genome does not appear to encode Rel-like transcription factors, and it is not known whether *C. elegans* responds to bacterial-encoded PAMPs. Indeed, *C. elegans* TLR-associated signaling components appear to be associated with the avoidance of potential pathogens [14] rather than in the activation of host defense responses. To determine whether the TOLL receptor pathway is required for the PMK-1-dependant PCD pathway elic-

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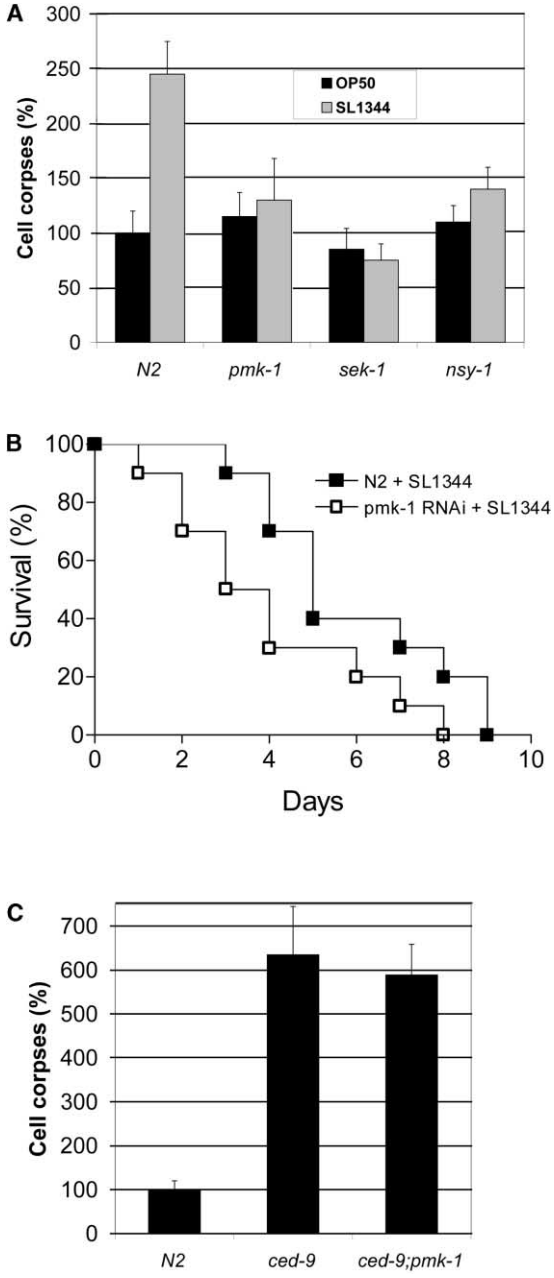

Figure 1. *S. enterica* Elicits a PMK-1-Dependent CED Cell Death Pathway in *C. elegans*

(A) N2, *nsy-1*, *sek-1*, or *pmk-1* RNAi young adult animals were exposed to *E. coli* OP50 or *S. enterica* SL1344, and cell corpses were counted 24 hr after the initial exposure, as previously described [31]. Data (mean ± SD) were from two independent experiments, and more than 15 animals were scored in each case.

(B) *C. elegans* N2 or *pmk-1* RNAi young adult animals were exposed to *S. enterica* SL1344 ($p < 0.039$). A total of 20 animals were used in each case. Nematode survival was plotted with the PRISM (version 2.00) computer program. A p value that was <0.05 was considered significant.

(C) N2, *ced-9*, or *ced-9* RNAi *pmk-1* young adult animals were exposed to *S. enterica* SL1344, and cell corpses were counted 24 hr after the initial exposure. Data (mean ± SD) were from two independent experiments, and more than 15 animals were scored in each case.

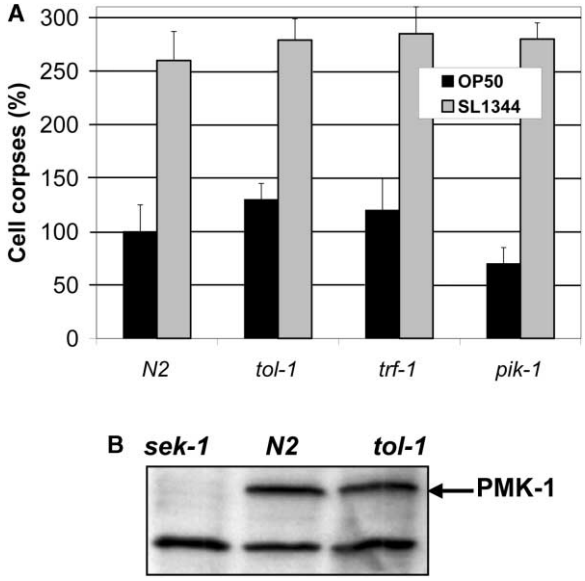

Figure 2. Toll-like Receptors Do Not Act Upstream of the MAPK-Dependant CED Cell Death Pathway in *C. elegans*

(A) N2, *tol-1*, *trf-1*, or *pik-1* young adult animals were exposed to *E. coli* OP50 or *S. enterica* SL1344, and cell corpses were counted 24 hr after the initial exposure. Data (mean ± SD) were from two independent experiments, and more than 15 animals were scored at each time point.

(B) Immunological detection of active PMK-1 in *sek-1*, N2, and *tol-1* young adult animals. Animals were grown at 20°C until young adult and whole-worm lysates were used to detect active PMK-1 by Western blotting using an anti-human p38 antibody from Promega.

ited by *Salmonella*, *C. elegans tol-1*, *trf-1*, *and pik-1* deletion mutants were fed on *Salmonella*, and gonadal cell death was monitored. As shown in Figure 2A, the amount of *Salmonella*-elicited PCD was not affected by any of these deletion mutants, indicating that TOL-1 is not the *C. elegans* receptor sensing the stimulatory signal for PMK-1 activation and that the presumptive *C. elegans* TOLL pathway in general is not required for *Salmonella*-elicited PCD. These results indicate that a receptor (or receptors) other than TOLL-like receptors is/are acting upstream of the MAPK-dependant CED cell death pathway in *C. elegans*. Consistent with this hypothesis, wild-type levels of activated PMK-1 were observed in *tol-1* mutants (Figure 2B).

In *Drosophila*, the Toll signaling pathway appears to mediate the activation of Dredd, a homolog of the *C. elegans* caspase CED-3, and it has been suggested that the Toll-mediated pathway of caspase activation is the evolutionary ancestor of the death receptor-mediated pathway for apoptosis induction in mammals [15]. However, our finding that *Salmonella*-mediated PCD and PMK-1 activation is not dependent on the single *C. elegans* Toll-like receptor suggests that another yet to be identified pathway distinct from the Toll signaling pathway may be the ancestor of the mammalian death receptor pathway. Moreover, given the fact that *C. elegans* does not appear to contain any Rel-like family transcription factors, it seems likely that both the upstream and downstream components of the MAPK cascade involved in the orchestration of host defense responses

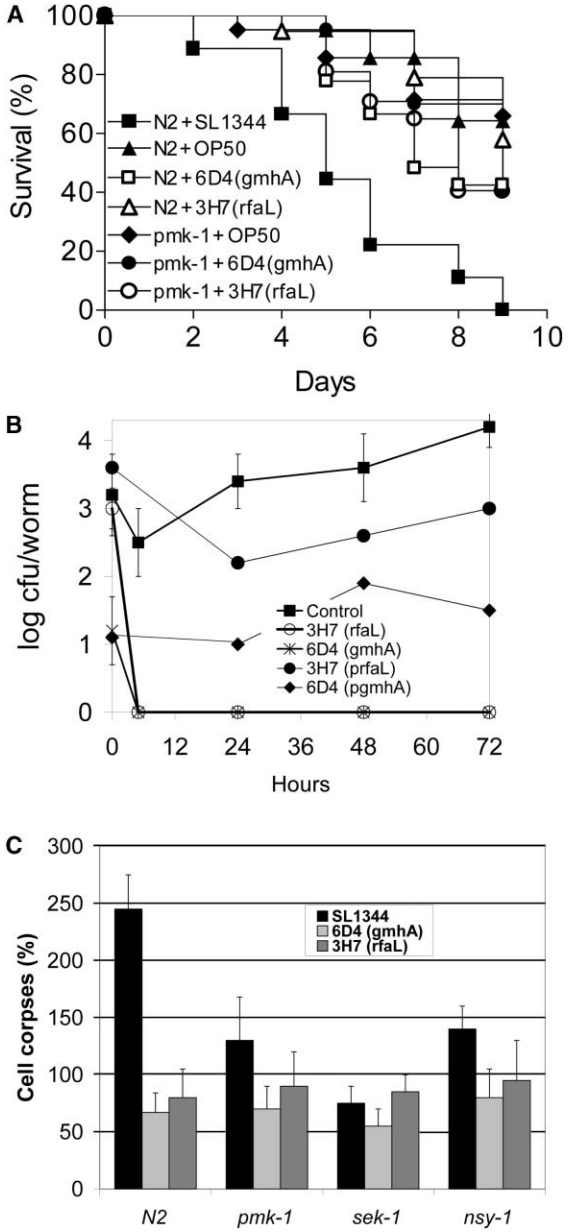

Figure 3. Intact *Salmonella* LPS Is Required for Both a Persistent Infection and Induction of SEK-1-Dependant Host Innate Immune Responses

(A) *C. elegans* N2 young adult animals were exposed to *S. enterica* SL1344, *E. coli* OP50 ($p < 0.0001$ compared to SL1344), *S. enterica 6D4 (gmhA)* ($p < 0.005$), and *S. enterica 3H7 (rfaL)* ($p < 0.0001$). Also, *pmk-1* RNAi young adult animals were exposed to *E. coli* OP50 ($p < 0.0001$), *S. enterica 6D4 (gmhA)* ($p < 0.0007$), and *S. enterica 3H7 (rfaL)* ($p < 0.004$). A total of 20 animals were used in each case. Nematode survival was plotted with the PRISM (version 2.00) computer program. Survival curves were considered significantly different from *S. enterica* SL1344 when p values were <0.05.

(B) A total of 100–120 1-day-old adult hermaphrodite worms were seeded on bacterial lawns and were allowed to feed on *S. enterica* SL1344 (control), *S. enterica 6D4 (gmhA)*, *S. enterica 3H7 (rfaL)*, *S. enterica 6D4* expressing *gmhA (6D4/pgmhA)*, and *S. enterica 3H7* expressing *rfaL (3H7/prfaL)*. After 20 hr, the worms were transferred to plates containing *E. coli* OP50 for 15 min and were then transferred to new plates containing OP50. The worms were transferred to new plates every 24 hr, and the number of *S. enterica* cells associated with individual worms was determined.

against Gram-negative pathogens are not conserved between mammals and *C. elegans*; this finding suggests that the p38 MAPK cascade may be the most ancient conserved feature of the host defense response against Gram-negative pathogens in metazoans.

Although the results described above as well as the data published by Pujol et al. [14] suggest that the *C. elegans* Toll-like signal transduction pathway is not involved in the recognition of bacterial PAMPs such as LPS, it seems likely that *C. elegans* is nevertheless responding to *Salmonella*-encoded signals in the activation of gonadal PCD. To identify putative *Salmonella*-encoded PAMPs, we screened a set of 15 *S. enterica* Tn*phoA* transposon insertion mutants that exhibited reduced killing of *C. elegans* (see the Experimental Procedures) for ones that also failed to elicit PCD. The rationale for this experiment was based on our previous observation that a *S. enterica* mutant in the PhoP/PhoQ regulatory system that is highly attenuated in virulence also failed to induce PCD as well as establish a persistent infection in the *C. elegans* intestine [3]. Mutations in the *S. enterica phoP/phoQ* genes are known to affect a variety of virulence-related factors, including the synthesis of LPS [16]. By identifying other mutants that exhibited a similar phenotype in the *C. elegans* model, we hoped to be able to determine which of the many phenotypes of *phoP/phoQ* mutants were relevant to the induction of gonadal PCD.

Two of the 15 *S. enterica* mutants that exhibited reduced levels of *C. elegans* killing, *6D4* and *3H7* (Figure 3A), failed to establish a persistent infection in the *C. elegans* intestine. Figure 3B shows that, as early as 5 hr after a shift from feeding on *Salmonella* to feeding on *E. coli*, the *6D4* and *3H7* mutants could not be detected in worms, whereas a Tn*phoA* control insertion in a gene not relevant for pathogenesis persisted and proliferated in the *C. elegans* intestine for at least 3 days.

Mutant *6D4* encodes phosphoheptose isomerase (*gmhA*), a conserved protein that catalyzes the first step of the biosynthesis of ADP-L-glycero-D-manno heptose [17]. *gmhA* mutants produce a short LPS core that lacks glyceromannoheptose and terminal hexoses [18]. Mutant *3H7* encodes O-antigen ligase (*rfaL*) [19], which is involved in LPS core modification prior to attachment of O antigen [20, 21]. Figure 3B shows that the defective persistent infection phenotype of the mutants was partially or mostly compensated when the *gmhA* or *rfaL* genes under the control of the constitutive *lacZ* promoter were expressed in strains *6D4* or *3H7*, respectively. Similarly, the failure of the two mutants to kill *C. elegans* was also partially or mostly compensated by the cloned *gmhA* or *rfaL* genes, respectively (data not shown). The fact that the cloned *rfaL* gene complemented mutant *3H7* better than the cloned *gmhA* gene complemented *6D4* is consistent with the facts that the gene downstream of *rfaL* is transcribed in the opposite

(C) N2, *nsy-1*, *sek-1*, or *pmk-1* RNAi young adult animals were exposed to *S. enterica* SL1344, *S. enterica 6D4 (gmhA)*, or *S. enterica 3H7 (rfaL)*, and cell corpses were counted 24 hr after the initial exposure. Data (mean ± SD) were from two independent experiments, and more than 15 animals were scored in each case.

direction to *rfaL* [22], whereas the gene downstream of *gmhA* is transcribed in the same direction as *gmhA* and appears to be in the same operon. Thus, it is not likely that the transposon insertion in *3H7* exerts a polar effect on a downstream gene, whereas the opposite may be true of the insertion in *6D4*.

The LPS produced by both the *gmhA* and the *rfaL* mutants contains lipid A, the component of LPS that is recognized in mammals by TLR4 and triggers a variety of host defense mechanisms, including programmed cell death [23, 24]. Figure 3C shows that, despite the fact that mutants *6D4* and *3H7* synthesize lipid A, *Salmonella*-elicited germline cell death was not elicited in *C. elegans* feeding on either the *6D4 (gmhA)* or *3H7 (rfaL)* mutants, whereas the other 13 Tn*phoA* mutants that were tested all elicited the same level of PCD as wild-type *Salmonella*. This result suggests that intact LPS is required for the *Salmonella*-mediated induction of gonadal cell death. Moreover, the level of gonadal PCD in PMK-1 RNAi worms feeding on the *gmhA* or *rfaL* mutants was the same as wild-type worms feeding on *E. coli*, suggesting that the basal level of gonadal PCD observed when worms are fed *E. coli* is not triggered by bacterial LPS. Consistent with these results, the same basal level of gonadal PCD was also observed in worms fed *E. coli* deficient in LPS biosynthesis or nonpathogenic Gram-positive bacteria (data not shown).

It is important to point out that, although *6D4 (gmhA)* and *3H7 (rfaL)* did not cause a persistent infection, they nevertheless accumulated to high titers in the intestinal lumen (Figure 3B), and these high levels rule out the trivial possibility that the *6D4 (gmhA)* and *3H7 (rfaL)* mutants failed to activate PCD because they failed to accumulate. The failure of the *gmhA* and *rfaL* mutants to cause a persistent infection suggests that intact LPS may be required for *Salmonella* to adhere to receptors present on *C. elegans* intestinal cells. Consistent with this conclusion, virulence in *6D4 (gmhA)* and *3H7 (rfaL)* mutants could not be restored by the exogenous application of purified *S. enterica* LPS (data not shown). The virulence of *gmhA* and *rfaL* mutants was also tested in RNAi *pmk-1* worms, and values comparable to the ones observed in wild-type worms were obtained, showing that the avirulent phenotype of the LPS mutants is "epistatic" to the hypersusceptibility phenotype of the RNAi *pmk-1* worms (Figure 3A). Figure 3A also shows that, when feeding on *E. coli*, the life span of RNAi *pmk-1* worms was comparable to that of wild-type, showing that the shortened life span of the RNAi *pmk-1* worms feeding on *Salmonella* is not simply a consequence of being sickly.

By showing that PCD is downstream of the p38 signaling cascade, the experiments reported in this paper link PMK-1 signaling with PCD. The data also show that *Salmonella*-activated PCD depends on intact LPS, and it is tempting to conclude that LPS signaling is also mediated by PMK-1 signaling. If this were the case, the homology of the mammalian and *C. elegans* innate immune responses would involve not only p38, a key signaling component in innate immunity, but also its specific role in mediating the response to LPS as a pathogen-associated molecular pattern (PAMP) molecule. However, initial attempts to show that purified LPS activates p38 or that *Salmonella* or *E. coli* LPS mutants fail to activate p38 have proved inconclusive (data not shown). This is not surprising given the wide number of potential roles that p38 MAPK could play in the organism. LPS, for example, may lead to p38 (PMK-1) activation in only a subset of *C. elegans* cells that cannot be detected in whole-animal lysates. It is also possible that LPS activates a signaling pathway in parallel to the p38 pathway that leads to PCD. Another possibility is that two independent signals, one of which is LPS, are required to activate the PCD pathway, thereby allowing *C. elegans* to discriminate between Gram-negative bacteria in general and Gram-negative pathogens. A similar situation may exist in *Drosophila*, where it has been suggested that a peptidoglycan-recognition protein (PGRP-LC) binds both peptidoglycan and LPS, or cooperates with a parallel signaling pathway involving other pattern-recognition receptors that bind LPS directly [25]. Finally, the component of LPS recognized by PAMP receptors in mammals is lipid A, a structure that is intact in both *6D4 (gmhA)* and *3H7 (rfaL)* mutants. Thus, our results suggest that *C. elegans* recognizes modifications in the *Salmonella* LPS outer core. Interestingly, *S. enteritidis* 1047, which synthesizes a different LPS outer core than *S. enterica*, also elicited PCD to levels comparable to the ones observed in *C. elegans* infected with *S. enterica* (data not shown).

In conclusion, this work shows that an important feature of innate immune signaling pathways, the activation of PCD downstream of a p38 MAPK signaling cascade, has been conserved between nematodes and mammals. Thus, this work provides important new insights into the evolutionary origins of innate immunity. This work also demonstrates that *C. elegans* may respond to a component of LPS, but whether this component is a highly conserved PAMP remains to be determined. In any case, it appears that *C. elegans* recognizes a different component of LPS than that recognized by mammalian TLRs. Finally, the *Salmonella* LPS signal that activates the *C. elegans* PCD response apparently functions independently of a TOLL-like pathway. Although we have not ruled out the possibility that the lipid A component of LPS is recognized by the single *C. elegans* TLR, it seems likely that one of the most prominent features of insect and mammalian innate immunity, the involvement of TLRs in LPS signaling, may not be conserved in nematodes.

Experimental Procedures

Bacterial Strains and Growth Conditions

E. coli OP50 [26] and *S. enterica* SL1344 [27] have been described. Bacterial cultures were grown in Luria-Bertani (LB) broth or M63 [28] minimal medium with appropriate antibiotics at 37°C. Bacterial lawns used for *C. elegans* killing assays were prepared by spreading 10 μl of an overnight culture of the bacterial strains on modified NG agar medium (0.35% instead of 0.25% peptone). Plates were incubated at 37°C for 12 hr and were then allowed to equilibrate to room temperature for 3 hr before seeding with worms.

A screen of 960 *S. enterica* strain SL1344 Tn*phoA* transposon insertion lines was carried out to identify mutants that exhibited reduced killing of *C. elegans*, and two mutants, *6D4 (gmhA)* and *3H7 (rfaL)*, were chosen for further analysis. The *6D4* and *3H7* Tn*phoA* insertion mutants grew at the same rate as the wild-type strain in minimal (M63), rich (LB), or NG media (not shown). DNA blot analysis

showed that both *6D4* and *3H7* contained a single Tn*phoA* insertion that conferred a similar avirulent phenotype after the Tn*phoA* insertions in *6D4* and *3H7* were individually transduced into wild-type *S. enterica* strain SL1344 by phage P22 transduction.

Construction of *rfaL*- and *gmhA*-Complementing Clones

The *rfaL* and *gmhA* genes were amplified from *S. enterica* SL1344 chromosomal DNA by using primers (rfaL1) 5′-TCGTATCGGTTGA TACCGGC-3′ and (rfaL2) 5′-GAACCTATGTCGAGCGACAG-3′ and (gmhA1) 5′-CTGACCACTTGTGATGATTA-3′ and (gmhA2) 5′-AGCA GATATCCGTCGGCACA-3′, respectively. The amplified products were first cloned into the pCR 2.1-TOPO cloning vector (Invitrogen). Subsequently, a 1.68-kb EcoRI *rfaL*-containing fragment, which only contains an intact *rfaL* ORF, and a 1.07-kb EcoRI *gmhA*-containing fragment, which only contains an intact *gmhA* ORF, were subcloned into the corresponding sites of pUCP19 and pUCP18, respectively, such that expression of both genes was driven by the plasmid *lacZ* promoter.

C. elegans Strains

sek-1 and *nsy-1* were from our laboratory [5]; *tol-1*, *trf-1*, and *pik-1* were from Cambria Biosciences LLC [14]. RNAi *pmk-1* worms were obtained by growing the nematodes as described [29, 30]. Progeny of these worms were transferred to modified NG agar medium containing 1:1 mixtures of *S. enterica* and *E. coli* carrying an L4440-derived vector with a sequence specific to *pmk-1*.

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