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Caenorhabditis elegans-Based Screen Identifies *Salmonella* Virulence Factors Required for Conserved Host-Pathogen Interactions

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Summary

A *Caenorhabditis elegans-Salmonella enterica* host-pathogen model was used to identify both novel and previously known *S. enterica* virulence factors (HilA, HilD, InvH, SptP, RhuM, Spi4-F, PipA, VsdA, RepC, Sb25, RfaL, GmhA, LeuO, CstA, and RecC), including several related to the type III secretion system (TTSS) encoded in *Salmonella* pathogenicity island 1 (SPI-1). Mutants corresponding to presumptive novel virulence-related genes exhibited diminished ability to invade epithelial cells and/or to induce polymorphonuclear leukocyte migration in a tissue culture model of mammalian enteropathogenesis. When expressed in *C. elegans* intestinal cells, the *S. enterica* TTSS-exported effector protein SptP inhibited a conserved p38 MAPK signaling pathway and suppressed the diminished pathogenicity phenotype of an *S. enterica sptP* mutant. These results show that *C. elegans* is an attractive model to study the interaction between *Salmonella* effector proteins and components of the innate immune response, in part because there is a remarkable overlap between *Salmonella* virulence factors required for human and nematode pathogenesis.

Results and Discussion

To identify *S. enterica* virulence-related genes, we sought to screen an *S. enterica* transposon mutant library for the ability of individual mutant clones to kill *C. elegans*. A technical difficulty in carrying out such a screen relates to the fact that the rate of *C. elegans* killing by *S. enterica* is slower than the generation time of the hermaphroditic *C. elegans* worms. Thus, during the first 4–5 days of the assay, hermaphrodite nematodes initially exposed to *S. enterica* need to be transferred each day to fresh plates to avoid losing track of these initial worms in the morass of progeny. This is tedious and time consuming, greatly reducing the number of killing assays that can be performed. We therefore tested whether temperature-sensitive *C. elegans glp-4* worms, in which germ cells are blocked in mitotic prophase and fail to proliferate and differentiate into gonads at the restrictive temperature of 25°C, could be substituted for wild-type N2 animals in the *S. enterica* killing assay. As shown in Figure 1A, *glp-4* worms exhibit a similar susceptibility to *Salmonella*-mediated killing when compared to wild-type animals grown at 25°C, the temperature used for the infection assays unless otherwise indicated.

A total of 960 *S. enterica* Tn*phoA* insertion mutants were individually screened for attenuated virulence using *glp-4* nematodes. Fifteen out of these 960 insertion mutants (Table 1) consistently showed a lower rate of *C. elegans* killing compared to killing by wild-type *S. enterica* and were chosen for further analysis. DNA blot analysis (not shown) showed that each of the mutant strains identified contained a single Tn*phoA* insertion that conferred a similar avirulent phenotype after each of the Tn*phoA* insertions were individually transduced into wild-type *S. enterica* strain SL1344 by phage P22 transduction. Seven of the 15 mutants contain Tn*phoA* insertions in genes which either had not been previously shown to be involved in virulence or for which a role in virulence had been presumed but not clearly demonstrated (*2A4, 3E4, 3E11, 3H7, 6D4, 5F3*, and *6E5*) (Table 1). The Tn*phoA* insertion in mutant *2A4* corresponds to the homolog of the *E. coli* carbon starvation protein coding gene *cstA*, mutant *3E4* corresponds to the *sb25* gene that encodes a fimbrial tail-like protein, mutant *3E11* corresponds to the *rhuM* gene which is located in SPI-3 [1], mutants *3H7* and *6D4* correspond to genes involved in wild-type expression of the O-antigen [2, 3], mutant *5F3* corresponds to the *leuO* gene which is involved in bacterial stringent response [4], and mutant *6E5* corresponds to gene *spi4-F* which is located in SPI-4 [5].

In mammals, the pathophysiology of localized enteritis caused by *Salmonella* is characterized by movement of electrolytes and water as well as polymorphonuclear leukocytes (neutrophils) (PMN) into the intestinal mucosa and lumen from the underlying microvasculature. Therefore, the Tn*phoA* insertion mutants in novel genes required for full virulence in *C. elegans* were tested for their ability to induce PMN migration and invade epithelial cells [6–9]. Although *S. enterica* can enter intestinal epithelial cells, bacterial internalization is not required for the signaling mechanisms that induce PMN movement. The *pipA* mutant was used as a control since it has been shown to be required for enteropathogenesis [10]. Remarkably, all seven of these mutants showed a reduced ability to promote transepithelial migration of PMN compared to wild-type (Table 1). In contrast, at least three random Tn*phoA* insertion mutants that did not affect *C. elegans* killing were not distinguishable

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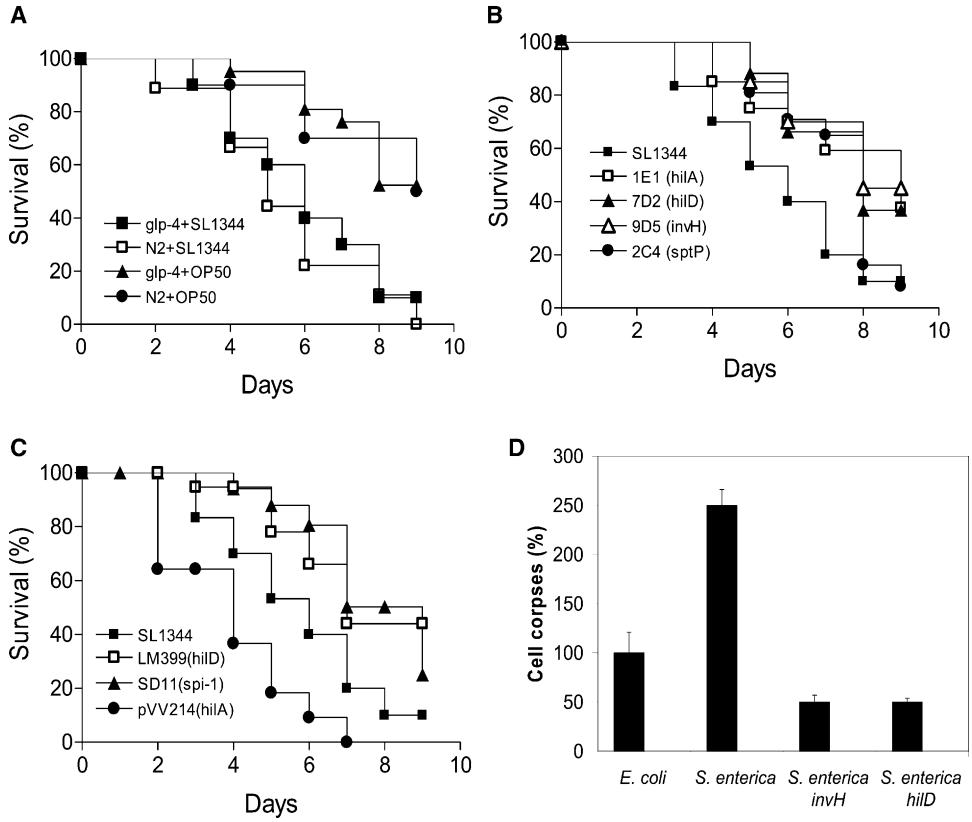

Figure 1. *C. elegans*-Based Screen Identifies *S. enterica* TTSS-Related Genes

(A) *C. elegans glp-4* young adult animals were exposed to *S. enterica* 1344 or *E. coli* OP50 ($p < 0.0002$), and *C. elegans* N2 (wild-type) young adult animals were exposed to *S. enterica* 1344 or *E. coli* OP50 ($p < 0.003$).

(B) *C. elegans glp-4* young adult animals were exposed to *S. enterica*, SL1344, *1E1 (hilA)* ($p < 0.004$), *7D2 (hilD)* ($p < 0.005$), *9D5 (invH)* ($p < 0.001$), *2C4 (sptP)* ($p < 0.018$).

(C) *C. elegans* N2 young adult animals were exposed to *S. enterica*, SL1344, LM399 (*hilD)* ($p < 0.021$), SD11 (Δ*spi-1)* ($p < 0.012$), or pVV214 (*hilA*) ($p < 0.005$). Twenty animals were used in each case. Nematode survival was plotted using the PRISM (version 2.00) computer program. Survival curves are considered significantly different from a *S. enterica* control when p values are <0.05. Multiple experiments were done and the data presented are from a representative experiment.

(D) *C. elegans* N2 young adult animals were exposed to *E. coli* OP50 ($n = 24$), *S. enterica* SL1344 ($n = 20$), *S. enterica 9D5 (invH*) ($n = 20$), or *S. enterica 7D2 (hilD)* ($n = 15$) at 20°C for 24 hr. The number of apoptotic cells was scored after 24 hr of the initial exposure to *S. enterica* using a SYTO 12 (Molecular Probes, Eugene, OR) staining as previously described [28]. Data (mean ± SD) were from three independent experiments.

from wild-type *Salmonella* in epithelial cell invasion and the PMN transepithelial migration assays (data not shown). Although the *rhuM* mutant exhibited the least reduction in the PMN transepithelial migration assay of the seven mutants tested, it showed a significant decrease in epithelial cell invasion. The rest of the mutants tested showed significant reductions in both epithelial invasion and transepithelial migration, with the exception of the *leuO* mutant, which exhibited wild-type levels of epithelial invasion but highly reduced transepithelial migration of PMN. These results suggest that the *C. elegans* model can be readily used to identify novel *Salmonella* virulence factors capable of altering host-defense response signaling pathways in cells that are not in direct contact with *Salmonella*.

Many *Salmonella* virulence determinants are located in *Salmonella* pathogenicity islands, which are defined as large clusters of horizontally acquired virulence-related genes [11, 12]. SPI-1 and SPI-2 encode components of two TTSSs that direct the translocation of a variety of effectors that facilitate bacterial pathogenesis by modulating actin cytoskeleton dynamics, nuclear responses, and endocytic trafficking [13]. Our screen for *S. enterica* mutants that exhibit decreased ability to kill *C. elegans* led to the identification of four mutants that correspond to *Salmonella* TTSS-related genes (Table 1 and Figure 1B). *hilA* and *hilD* encode regulatory proteins that are part of a complex regulatory cascade required for SPI-1-encoded TTSS (SPI-1 TTSS) expression [14]. *invH* encodes a protein required for the proper assembly of the SPI-1 TTSS [15], and *sptP* encodes an effector protein that is translocated by the SPI-1 TTSS. The SptP carboxy-terminal domain has tyrosine phosphatase activity in vitro and displays amino acid sequence similarity to the *Yersinia* spp. tyrosine phosphatase YopH [16–18]. The amino-terminal domain of SptP has GTPase activating protein activity for Cdc42 and Rac and is similar to the bacterial cytotoxins YopE and ExoS [19–21]. Compe-

Table 1. Virulence Phenotypes of Tn*phoA* Insertion Mutants Isolated Based on Attenuated *C. elegans* Killing

Strain	Gene Disrupted by Tn*phoA*	Description	*C. elegans* Killing	PMN Transmigration	Epithelial Cell Invasion
1E1	*hilA*[a]	transcriptional activator of SPI-1 invasion genes	45	ND	ND
7D2	*hilD*[a]	transcriptional regulator that de-represses *hilA*	50	ND	ND
9D5	*invH*[a]	required for TTSS proper assembly	45	ND	ND
2C4	*sptP*[a]	effector protein encoding gene	67	ND	ND
3E11	*rhuM*[b]	gene of unknown function located in SPI-3	55	81	56
6E5	*spi4-F*[c]	gene of unknown function located in SPI-4	67	15	61
8F4	*pipA*[d]	required for enteropathogenesis	45	11	63
2A6	*vsdA*[e]	required for virulence in several systems	50	ND	ND
4C4	*repC*[e]	involved in plasmid copy number control	67	ND	ND
3E4	*sb25*[f]	similar to fimbrial tail protein encoding gene	33	50	46
3H7	*rfaL*	essential for the expression of wild-type LPS	40	01	04
6D4	*gmhA*	essential for the expression of wild-type LPS	35	31	12
5F3	*leuO*	regulator involved in bacterial stringent response	67	37	107
2A4	*cstA*	carbon starvation protein encoding gene	67	39	54
7A2	*recC*	required for recombination	45	ND	ND

CEK was calculated 8 days after the infection. Data represent three independent experiments and are expressed as percentage of wild-type response.

[a] Genes located in *Salmonella* Pathogenicity Island-1.

[b] Genes located in *Salmonella* Pathogenicity Island-3.

[c] Genes located in *Salmonella* Pathogenicity Island-4.

[d] Genes located in *Salmonella* Pathogenicity Island-5.

[e] Genes located in virulence plasmids.

[f] Genes located in a prophage.

tition experiments indicate that *sptP* mutants fail to colonize the spleen of mice when coinoculated with wild-type *S. enterica* [16].

To confirm the role of SPI-1 encoded genes in *C. elegans* killing, we infected wild-type animals with an *S. enterica hilD* mutant and with a strain carrying a deletion of the entire SPI-1 cluster of genes. These two *S. enterica* mutants also exhibited reduced virulence compared to wild-type *S. enterica* (Figure 1C). We also examined whether overexpression of the SPI-1 regulatory gene *hilA* would enhance the rate of *C. elegans* killing. HilA, which functions directly downstream of HilD and HilC, is a transcriptional activator required for expression of several SPI-1 genes including *invF*, which also encodes a transcriptional activator that in turn activates several additional SPI-1 genes [22, 23]. Consistent with the involvement of TTSS in *C. elegans* killing, *S. enterica* overexpressing *hilA* from plasmid pVV214 resulted in faster *C. elegans* killing than that caused by wild-type *S. enterica* control (Figure 1C).

In most of the animals corresponding to the *Metazoan* phyla, which includes both vertebrates and invertebrates, the intestinal epithelium is protected with one or more barriers rich in carbohydrates and antimicrobial substances. Such membranes, termed peritrophic matrix (PM) in invertebrates, appear to be critical in protecting animals from bacterial infections. However, bacterial pathogens secrete toxins and enzymes that penetrate the PM and alter its permeability, allowing bacteria to access the midgut cells of invertebrates [24]. Although there is evidence of an undefined matrix lining the gut of *C. elegans*, this animal does not contain a highly structured PM [25]. In contrast to *E. coli* and various human pathogens known to kill *C. elegans*, *Salmonella* serovars are also capable of establishing a persistent infection in the *C. elegans* intestine [26, 27], suggesting that individual *S. enterica* cells have prolonged contact with the *C. elegans* gut, where TTSS-related genes may be activated and effector proteins translocated into the intestinal cells. The use of the *lacZ* reporter gene indicates that TTSS-related gene *hilA* is expressed in the intestinal lumen of *C. elegans* (see Supplemental Data).

One candidate for a marker of an innate immune response that is observed in evolutionarily disparate species is programmed cell death (PCD). Interestingly, we found that *S. enterica* intestinal infection triggers somatic signals that induce programmed cell death in *C. elegans* germline cells [28]. Using a variety of *C. elegans* mutants in which cell death is blocked, it was shown that *S. enterica*-induced germline cell death is dependent on the well-characterized CED-9/CED-4/CED-3 pathway, homologous to the BCL2/APAF-1/CASPASE pathway in mammalian cells. Figure 1D shows that *S. enterica hilD* and *hilA* failed to elicit germline PCD, indicating that SPI-1 TTSS-related genes are required for the process. Two additional Tn*phoA* insertion mutants (*6E5* and *8F4*) that exhibit reduced virulence in *C. elegans* were not distinguishable from wild-type *Salmonella* in the elicitation of PCD (data not shown). Further studies will be required to understand which specific effector proteins and host signaling pathways are important in the modulation of the *S. enterica*-elicited PCD in nematodes.

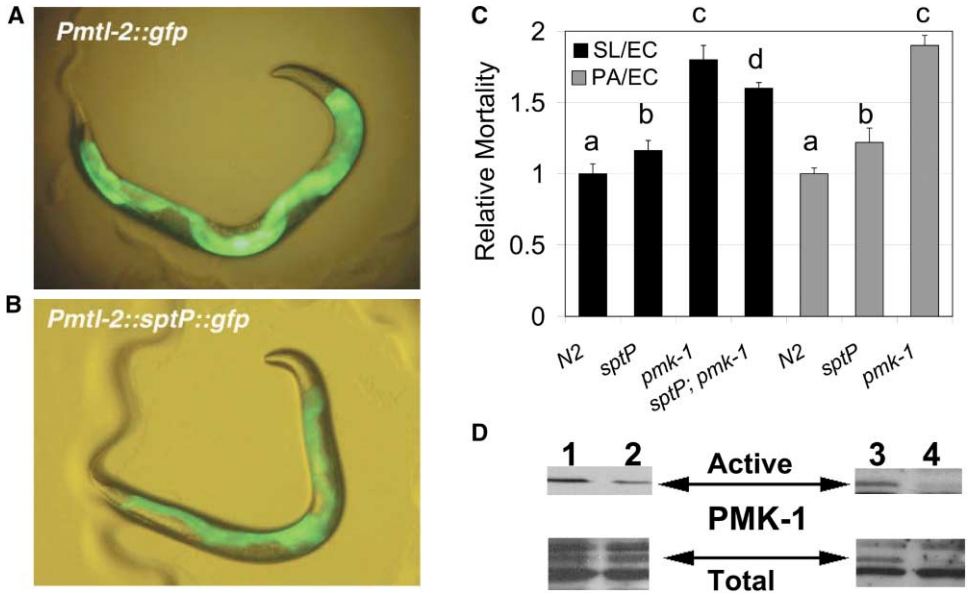

Figure 2. *S. enterica* SptP Targets a Conserved p38/MAPK Innate Immune Pathway in *C. elegans*

(A and B) Control animals expressing *Pmtl-2::gfp* (A) and *Pmtl-2::sptP::gfp* animals (B) were grown on *E. coli* until they reached adulthood.

(C) *C. elegans* N2, *Pmtl-2::sptP::gfp* (*sptP*), and *pmk-1* RNAi animals were exposed to control *E. coli*, *S. enterica* 1344, or *P. aeruginosa* PA14. The time for 50% of the nematodes to die (time to death 50, TD_{50}) was calculated with the PRISM (version 2.00) computer program using the equation: Y = Bottom + (Top − Bottom)/(1 + 10∧((LogEC50 − X)*HillSlope)), where X is the logarithm of days and Y is the average of dead worms. The relative mortality of *Pmtl-2::sptP::gfp* worms feeding on pathogens *S. enterica* (SL) or *P. aeruginosa* (PA) [defined as: (TD_{50} of N2 worms on pathogen/TD_{50} of *Pmtl-2::sptP::gfp* or *pmk-1* RNAi worms on pathogen)/(TD_{50} N2 worms on *E. coli*/TD_{50} of *Pmtl-2::sptP::gfp* or *pmk-1* RNAi worms on *E. coli*)] was calculated for two independent experiments in which more than 20 animals were used in each case. The relative mortality of *Pmtl-2::sptP::gfp; pmk-1* RNAi (*sptP; pmk-1*) animals was also calculated. Unpaired Student's t test indicates that differences among the groups are significantly different.

(D) Immunological detection of active and total PMK-1 in *Pmtl-2::gfp* (1) and *Pmtl-2::sptP::gfp* (2) animals. Active and total PMK-1 was also detected in control N2 animals (3) and *pmk-1* RNAi animals (4). Animals were grown at 20°C until 1-day-old adult and whole-worm lysates were used to detect active PMK-1 by Western blotting using an anti-human p38 antibody from Promega, Inc. The anti total PMK-1 antibody was kindly provided by Kunihiro Matsumoto, Nagoya University and CREST, Japan. Multiple experiments were done and the data presented are from a representative experiment.

In contrast to mammalian whole-organism models of *S. enterica* infection, we found that an *S. enterica sptP* mutant exhibited a significant avirulent phenotype in its ability to kill *C. elegans* (Figure 1B). We therefore determined whether we could use a variety of *C. elegans* genetic tools to investigate the molecular mechanism by which SptP functions as a virulence factor in a whole-animal model. It has been shown that intestinal Henle-407 cells infected with a *S. enterica sptP* mutant exhibit elevated levels of activated Erk MAPK compared to cells infected with *S. enterica* wild-type [29], suggesting that SptP tyrosine phosphatase activity blocks the *Salmonella*-induced activation of Erk. More recently, it has been shown that SptP inhibits the Erk MAPK signaling pathway in HeLa cells by preventing Raf translocation and subsequent phosphorylation through both its tyrosine phosphatase activity and GTPase-activating protein activity [30].

In previously published work, it was shown that a p38 MAPK signaling pathway is a conserved component of innate immunity in mammals and nematodes [31, 32]. Given the fact that, in mammalian cells, SptP acts as a GTPase-activating protein for Cdc42 and Rac, which are required for the activation of p38, we tested whether ectopic expression of SptP in *C. elegans* intestinal cells would enhance susceptibility of *C. elegans* to *S. enterica* and affect activation of the *C. elegans* p38 ortholog encoded by the *pmk-1* gene. To express *sptP* in *C. elegans* intestinal cells, we fused 444 bp of the 5′ UTR of *mtl-2* [33] to the *sptP* gene. To monitor expression of the *Pmtl-2::sptP* chimera, a sequence encoding the green fluorescent protein (GFP) reporter was subsequently fused to the 3′ end of *Pmtl-2::sptP* to generate the construct *Pmtl-2::sptP::gfp*. N2 wild-type animals were coinjected with the construct *Pmtl-2::sptP::gfp* and the plasmid pRF4, which carries the dominant collagen mutation *rol-6(su1006)* [34]. Figures 2A and 2B show that *Pmtl-2::sptP::gfp* animals reach adulthood and do not exhibit any gross visible morphological abnormality when grown on *E. coli* OP50 plates. We determined "relative mortality" of *Pmtl-2::sptP::gfp* animals compared to wild-type worms to assess whether *Pmtl-2::sptP::gfp* animals are more susceptible to *S. enterica*-mediated killing. This method has the advantage of normalizing any observed decrease in longevity of the *Pmtl-2::sptP::gfp* animals feeding on *Salmonella* to any decrease in longevity when feeding on *E. coli* and, therefore, takes into account a decrease in longevity due to a general defect in fitness rather than a specific defect in an innate immune response. Figure 2C shows that *Pmtl-2::sptP::gfp* animals exhibited a modest but highly significant increase in relative mortality to *S. enterica*

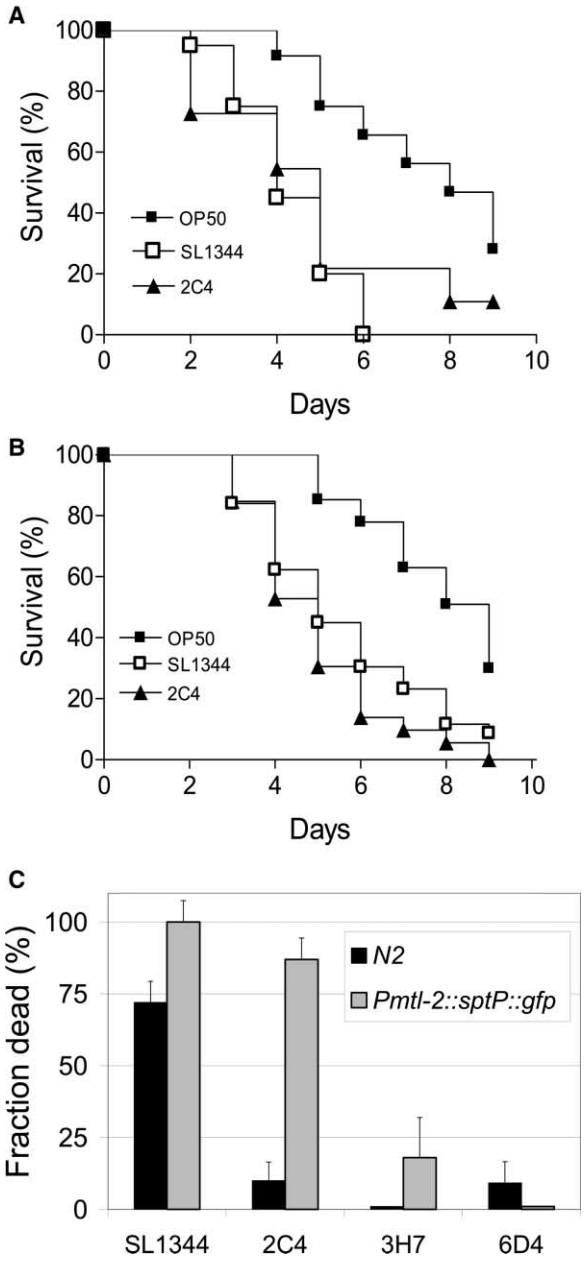

Figure 3. Inactivation of *ced-10/rac* and Intestinal Expression of *sptP* Suppress the Diminished Pathogenicity Phenotype of *S. enterica sptP* Mutant *2C4*

(A) *C. elegans ced-10(n3246)* 1-day-old animals were exposed to control *E. coli* OP50 ($p < 0.0001$), *S. enterica* 1344, and *S. enterica 2C4 (sptP)* ($p < 0.47$). The data presented are from three independent experiments. More than 20 animals were used in each case. Nematode survival was plotted using the PRISM (version 2.00) computer program. Survival curves are considered significantly different from a *S. enterica* 1344 when p values are <0.05.

(B) *C. elegans Pmtl-2::sptP::gfp* 1-day-old animals were exposed to control *E. coli* OP50 ($p < 0.0001$), *S. enterica* 1344, and *S. enterica 2C4 (sptP)* ($p < 0.189$). The data presented are from three independent experiments. Sixty animals were used in each case. Nematode survival was plotted using the PRISM (version 2.00) computer program. Survival curves are considered significantly different from a *S. enterica* 1344 when p values are <0.05.

(C) *C. elegans* N2 and *Pmtl-2::sptP::gfp* animals were exposed to *S. enterica* 1344 (SL1344), *S. enterica sptP* (2C4), *S. enterica rfaL* (3H7), or *S. enterica gmhA* (6D4). The fraction of dead animals was determined after 7 days on different bacterial strains. Error bars represent variation between two plates. Multiple experiments were done and the data presented are from a representative experiment. More than 20 animals were used in each case.

compared to wild-type N2 animals ($p < 0.0001$). *Pmtl-2::sptP::gfp* animals were also more susceptible to *P. aeruginosa* strain PA14 (Figure 2C), indicating that intestinal expression of SptP inhibits a defense response pathway that is not exclusive to *S. enterica*.

Consistent with the hypothesis that SptP inhibits the MAPK pathway required for *C. elegans* defense response by preventing the phosphorylation of PMK-1/p38, PMK-1 activation is reduced at least 2-fold in *Pmtl-2::sptP::gfp* animals compared to control animals (Figure 2D), showing that the intestinal expression of SptP partially blocks the *C. elegans* defense response by inhibiting the PMK-1/MAPK pathway. These results are also consistent with the relatively modest increase in susceptibility of *Pmtl-2::sptP::gfp* worms to *S. enterica* and *P. aeruginosa* compared to worms in which PMK-1 was knocked down by RNAi (Figure 2C). Figure 2C also shows that the modest increased susceptibility to *S. enterica*-mediated killing of *Pmtl-2::sptP::gfp* animals, which is probably caused by a reduction in the level of active PMK-1, is greatly increased by complete inhibition of active PMK-1 by RNAi (*sptP;pmk-1* animals).

Since SptP acts as a GTPase-activating protein involved in the inactivation of Rac, we tested whether *ced-10/rac* nematodes carrying a Gly 60 to Arg mutation in a conserved motif implicated in GTP binding [35] would exhibit enhanced susceptibility to *S. enterica*-mediated killing. The time required for 50% of *ced-10(n3246)* animals to die (TD_{50}) when fed at 25°C on *S. enterica* was 3.25 ± 0.6 days compared to 5.6 ± 0.7 days for wild-type animals, indicating that *ced-10(n3246)* animals are hypersusceptible to *S. enterica*-mediated killing. We also tested whether the reduced virulence of the *S. enterica sptP* mutant *2C4* could be reversed by the inactivation of CED-10. Figure 3A shows that the virulence of the *sptP* mutant was not significantly different than the virulence of wild-type *S. enterica* in *ced-10(n3246)* animals, indicating that the reduced virulence phenotype of the *sptP* mutant could be suppressed by the inactivation of CED-10. Similarly, Figure 3B shows that the reduced virulence of the *S. enterica sptP* mutant *2C4* could be reversed (complemented) by the expression of *sptP* in *C. elegans* intestinal cells. Figure 3B shows that, in contrast to the *sptP* mutant, intestinal expression of *sptP* in *C. elegans* intestinal cells did not suppress the avirulent phenotypes of *S. enterica* mutants *3H7* and *6D4*, eliminating the possibility that transgenic animals expressing SptP are simply hypersusceptible to any *Salmonella* strain. To further explore both the suppression of the virulence phenotype of the *sptP* mutant in *Pmtl-2::sptP::gfp* animals and the mechanism by which *Pmtl-2::sptP::gfp* animals are more susceptible to *Salmonella*, we used a persistent colonization assay described before [26]. The results indicate that *S. enterica 2C4* persistently colonizes the intestine of *Pmtl-2::sptP::gfp* animals 4-fold more efficiently than

the intestine of *Pmtl-2::gfp* control animals 96 hr after the initial exposure (370,000 cfu/*Pmtl-2::sptP::gfp* animal versus 84,000 cfu/*Pmtl-2::gfp* control animal).

In this work, we showed that the *S. enterica* TTSS-associated genes *sptP*, *hilD*, *hilA*, and *invH* are required for maximum *S. enterica*-mediated killing of *C. elegans*. We also showed that SptP acts as a virulence factor, in part, by targeting a conserved innate immune pathway. These results are particularly interesting because they open the possibility of using the highly sophisticated *C. elegans* genetic system to study the interaction between effector proteins and the targeted host signaling pathways. A genome-wide screen may identify additional novel virulence factors that modulate innate immunity.

Supplemental Data

Supplemental Data including a figure and Experimental Procedures are available http://www.current-biology.com/cgi/content/full/14/11/1018/DC1.

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