

# Tissue-Specific Activities of an Immune Signaling Module Regulate Physiological Responses to Pathogenic and Nutritional Bacteria in *C. elegans*

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DOI 10.1016/j.chom.2009.09.001

## SUMMARY

Microbes represent both an essential source of nutrition and a potential source of lethal infection to the nematode *Caenorhabditis elegans*. Immunity in *C. elegans* requires a signaling module comprised of orthologs of the mammalian Toll-interleukin-1 receptor (TIR) domain protein SARM, the mitogen-activated protein kinase kinase kinase (MAPKKK) ASK1, and MAPKK MKK3, which activates p38 MAPK. We determined that the SARM-ASK1-MKK3 module has dual tissue-specific roles in the *C. elegans* response to pathogens—in the cell-autonomous regulation of innate immunity and the neuroendocrine regulation of serotonin-dependent aversive behavior. SARM-ASK1-MKK3 signaling in the sensory nervous system also regulates egg-laying behavior that is dependent on bacteria provided as a nutrient source. Our data demonstrate that these physiological responses to bacteria share a common mechanism of signaling through the SARM-ASK1-MKK3 module and suggest the co-option of ancestral immune signaling pathways in the evolution of physiological responses to microbial pathogens and nutrients.

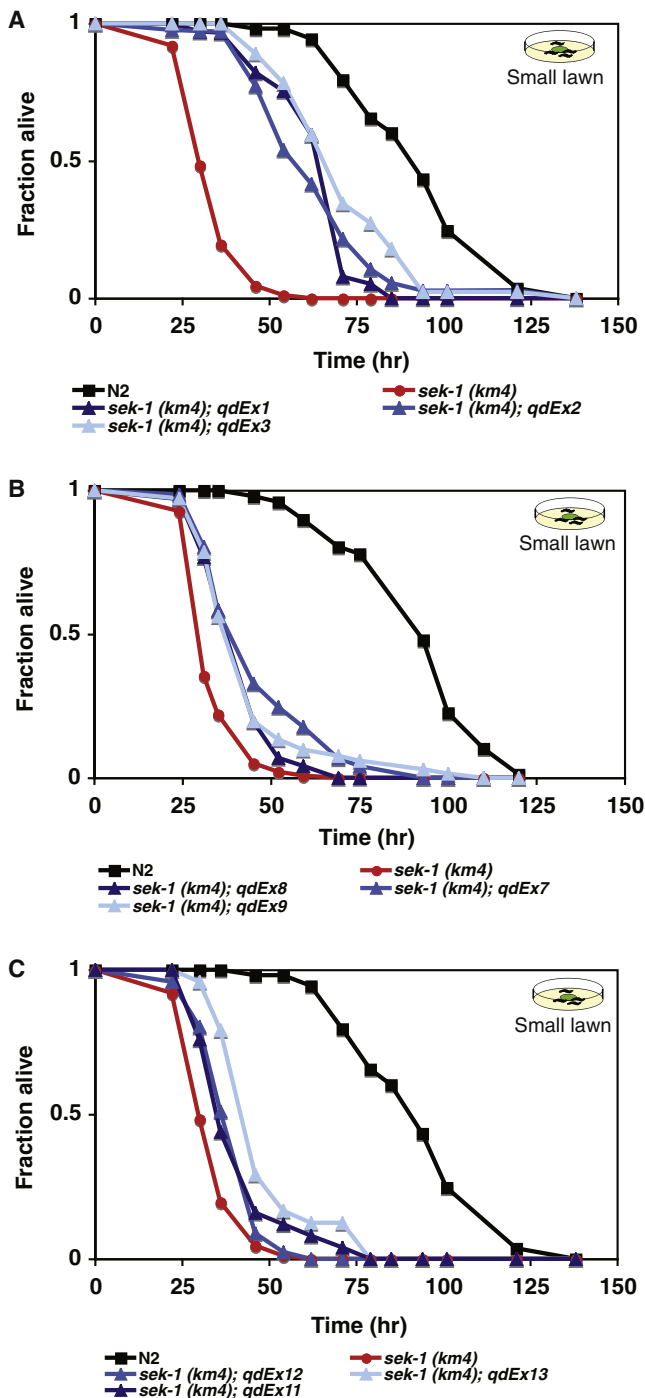
## INTRODUCTION

The microbial environment of multicellular organisms presents a complex challenge for the host to respond to both pathogenic and beneficial microbes with strategies that promote survival (Dethlefsen et al., 2007). Microbes represent both an essential source of nutrition and a potential source of lethal infection to the soil nematode *Caenorhabditis elegans*. Diverse modes of infection of *C. elegans* by microorganisms have been characterized (Couillault et al., 2004; Tan et al., 1999; Troemel et al., 2008), and conserved innate immune responses in *C. elegans* have been found to promote resistance to pathogens (Kim et al., 2002; Mallo et al., 2002; Nicholas and Hodgkin, 2004). Whereas innate immune signaling and the induction of local antimicrobial responses have been studied in a wide range of organisms (Hoffmann et al., 1999), the analysis of host behavioral responses

to bacteria have been less characterized. The relatively simple and well-characterized nervous system of *C. elegans* is well suited for such studies, and indeed, studies of *C. elegans* behavior in the presence of bacteria have revealed distinct responses to nonpathogenic bacteria provided as a nutrient source, including changes in locomotion, feeding, and egg-laying behaviors (Avery, 1993; Sawin et al., 2000; Trent et al., 1983). Pathogenic bacteria induce avoidance and aversive learning behavior (Pradel et al., 2007; Pujol et al., 2001; Reddy et al., 2009; Styer et al., 2008; Zhang et al., 2005). A common feature of innate immunity and behavioral responses to bacteria is the recognition of microbes and/or microbial infection and damage, raising the speculative possibility that common molecular mechanisms might be involved in these distinctly different physiological responses.

A small number of evolutionarily conserved core signaling pathways of innate immunity, such as Toll-like receptor signaling pathways and mitogen-activated protein kinase (MAPK) cascades, are utilized in host organisms to respond to microbial infection (Akira et al., 2006). Proteins containing the Toll-interleukin-1 receptor (TIR) domain are notably associated with innate immune signaling pathways and are present in the microbial response pathways of organisms from Dictyostelium (Chen et al., 2007) to humans (Akira et al., 2006; Hoffmann et al., 1999). In *C. elegans*, TIR-1, an ortholog of the mammalian TIR domain adaptor protein SARM (Mink et al., 2001), is required for pathogen resistance (Couillault et al., 2004; Liberati et al., 2004; Pujol et al., 2008), acting upstream of a conserved NSY-1-SEK-1-PMK-1 MAPK pathway that is orthologous to the mammalian ASK1-MKK3-p38 MAPK pathway (Kim et al., 2002). Genetic analysis in mice has demonstrated a requirement for ASK1-dependent p38 MAPK signaling in innate immunity, underscoring functional conservation of the p38 MAPK cascade in innate immunity (Matsuzawa et al., 2005). The function of SARM in mammalian immunity has been unclear, with conflicting data regarding a regulatory role for SARM in innate immune signaling (Carty et al., 2006; Kim et al., 2007). Interestingly, the predominant site of expression of SARM in mammals appears to be in the brain (Kim et al., 2007).

Immune signaling in vertebrates, as well as in insects to some degree, is carried out principally in specialized cells of the immune system. At the same time, nonimmune cell types, such as adipocytes and neurons, are also sites of inflammatory and



**Figure 1. SEK-1 Activities in the Intestine and the Chemosensory Neurons Contribute to Resistance to *P. aeruginosa* PA14**

Shown are survival curves on *P. aeruginosa* PA14 of L4 stage WT (N2) and the *sek-1(km4)* mutant, along with different *sek-1(km4)* mutant strains carrying transgenes expressing the *sek-1* cDNA fused at its C terminus to GFP, under the control of tissue-specific promoters. Pathogenesis assays were carried out under standard lawn conditions (bacteria seeded in a small spot in the center of the plate).

(A) Shown are survival curves for WT N2, *sek-1(km4)*, and three independent lines carrying the *Pges-1::sek-1::GFP* transgene in an extrachromosomal array, on the standard lawn.

innate immune signaling activities with pivotal roles in the pathogenesis of disease (Hotamisligil, 2006; Weiner and Selkoe, 2002), although the evolutionary origins of this signaling activity have been the subject of some speculation (Hotamisligil, 2006). *C. elegans* does not have cells that are dedicated to immune function, and tissues that are in contact with external or ingested microbes might be anticipated to serve the primary role in host defense. Immune signaling in the hypodermis has been shown to be required for the response to wounding and infection by the nematophagous fungus, *Drechmeria coniospora* (Pujol et al., 2008). In addition, the chemosensory neurons of *C. elegans* have ciliated projections that are exposed to the extrinsic environment (Bargmann, 2006), and the ADF chemosensory neuron pair has been shown to be involved in aversive learning in response to exposure to pathogenic bacteria (Zhang et al., 2005).

We investigated the cell types in *C. elegans* in which the TIR-1-NSY-1-SEK-1 module is required for resistance to the bacterial pathogen *Pseudomonas aeruginosa* strain PA14. We show that TIR-1-NSY-1-SEK-1 signaling has multiple tissue-specific activities in the regulation of immune and behavioral responses to bacteria. These data reveal an ancient commonality in TIR domain protein-dependent signaling at the intersection of innate immunity and behavioral responses to both pathogens and nutrient-providing, beneficial bacteria, and suggest that immune signaling in ostensibly nonimmune cell types may have origins in the interaction of microbes with different tissues in ancestral host organisms.

## RESULTS

### Cell-Autonomous Regulation of Intestinal Immunity by SEK-1

We investigated the cell types in which the TIR-1-NSY-1-SEK-1 signaling module is required for protective responses to the pathogenic bacterium *P. aeruginosa* PA14, which kills *C. elegans* by an intestinal infection (Tan et al., 1999). The *sek-1(km4)* mutant exhibits enhanced susceptibility to killing by PA14 relative to wild-type (WT), and no PMK-1 p38 MAPK activation is detectable in the *sek-1(km4)* mutant (Kim et al., 2002). The *sek-1* gene is expressed in the nervous system, uterine-vulval cells, and intestine of *C. elegans* (Tanaka-Hino et al., 2002). We generated strains carrying the *sek-1(km4)* mutation and a transgene engineered to express the *sek-1* cDNA fused at its C terminus to GFP, under the control of heterologous tissue-specific promoters. We first analyzed survival in the standard pathogenesis assay in which a small lawn of PA14 is spotted in the middle of the agar plate. Multiple independent lines of *sek-1(km4)* mutant worms carrying a *Pges-1::sek-1::GFP* transgene, which specifically directs expression in the intestinal cells (McGhee et al., 1990), were partially rescued with respect to the enhanced pathogen susceptibility phenotype of *sek-1(km4)* (Figure 1A).

(B) Shown are survival curves for WT N2, *sek-1(km4)*, and three independent lines carrying the *Punc-119::sek-1::GFP* transgene in an extrachromosomal array, on the standard lawn.  $p < 0.001$  for each transgenic line compared with *sek-1(km4)*.

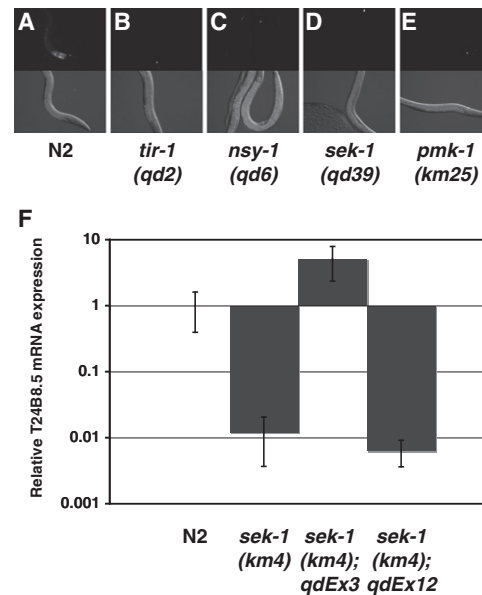
(C) Shown are survival curves for WT N2, *sek-1(km4)*, and three independent lines carrying the *Posm-5::sek-1::GFP* transgene in an extrachromosomal array, on the standard lawn.  $p < 0.001$  for transgenic lines compared with *sek-1(km4)*.

Based on gene expression data on transcriptional targets of the PMK-1 pathway (Troemel et al., 2006), we generated a green fluorescent protein (GFP) reporter strain that carries the GFP gene fused to the promoter of a transcriptional target of the PMK-1 pathway, *C. elegans* open reading frame T24B8.5, which is predicted to encode a short secreted peptide with homology to ShK toxin peptides. We observed that intestinal expression of this transgene, *agIs219*, is markedly diminished in the *pmk-1(km25)* mutant (Figures 2A and 2E). From a forward genetic screen for mutants with diminished GFP expression from *agIs219* and enhanced susceptibility to killing by PA14, we isolated multiple presumptive null alleles of genes encoding TIR-1-NSY-1-SEK-1-PMK-1 pathway components (R.P.S. and D.H.K., unpublished data), consistent with a role for the TIR-1-NSY-1-SEK-1-PMK-1 pathway in the regulation of intestinal gene expression. Representative mutants with diminished *agIs219* expression are shown in Figures 2C–2E. We also carried out quantitative analysis of endogenous T24B8.5 expression in WT and *sek-1(km4)* mutants that confirmed the strong regulation of T24B8.5 expression by the PMK-1 pathway (Figure 2F). In addition, we observed that intestine-specific expression of *sek-1* in the *sek-1(km4)* mutant conferred full rescue of T24B8.5 expression, demonstrating cell-autonomous regulation of putative immune effector gene expression in the intestine by SEK-1 (Figure 2F).

#### SEK-1 Activity in Chemosensory Neurons Is Required for a Protective Behavioral Avoidance Response to PA14

Motivated in part by the incomplete rescue of the pathogen susceptibility phenotype in *sek-1(km4)* mutant animals carrying the *Pges-1::sek-1::GFP* transgene, we investigated whether the expression of SEK-1 in the nervous system might also contribute to pathogen resistance. We generated *sek-1(km4)* mutant animals carrying the *Punc-119::sek-1::GFP* transgene, which directs expression of *sek-1* specifically in all neurons (Maduro and Pilgrim, 1995). We observed that on standard pathogenesis assay plates, panneuronal expression of *sek-1* also conferred a small but reproducible increase in survival for multiple independent transgenic lines (Figure 1B).

The *C. elegans* nervous system can detect environmental cues through the activities of ciliated sensory neurons, many of which have projections that extend through openings in the cuticle (Bargmann, 2006). Pathogenic bacteria represent a complex environmental challenge that can influence organism survival, and the ability of *C. elegans* to detect and avoid pathogens has been characterized (Pradel et al., 2007; Pujol et al., 2001; Zhang et al., 2005). We hypothesized that the neuronal function of SEK-1 in promoting resistance to PA14 might be through activity in the sensory nervous system, where SEK-1 could mediate detection of pathogen infection. Expression of a *Posm-5::sek-1::GFP* transgene, which specifically directs expression in the ciliated sensory neurons of *C. elegans* (Haycraft et al., 2001), resulted in a limited rescue of the *sek-1(km4)* enhanced pathogen susceptibility phenotype comparable to that observed for panneuronal expression of *sek-1* under the control of the *Punc-119* promoter (Figure 1C). These data suggest that SEK-1 activity in the sensory nervous system, in addition to a cell-autonomous role in the intestine, promotes resistance to PA14 infection.

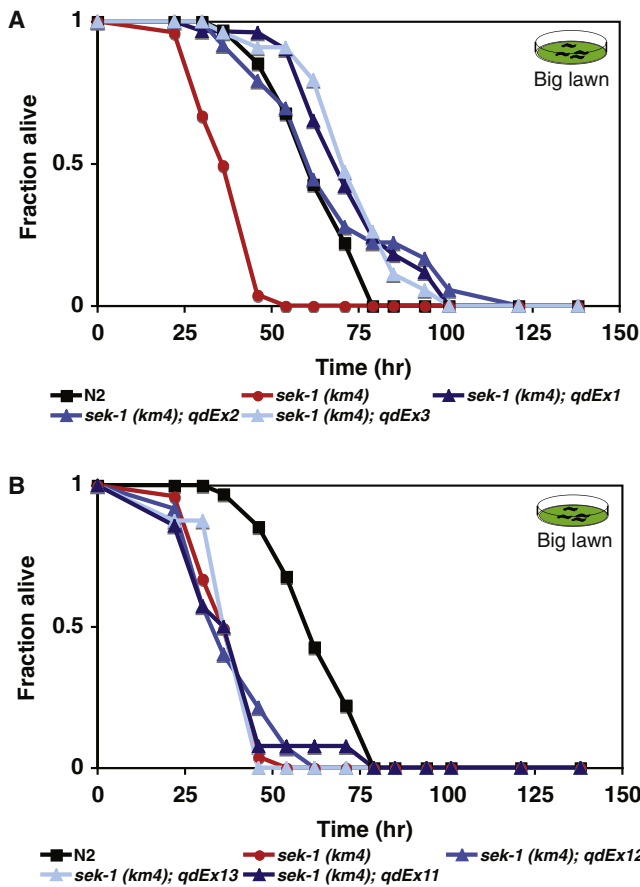


**Figure 2. Regulation of Intestinal Gene Expression by the TIR-1-NSY-1-SEK-1-PMK-1 Pathway**

(A–E) Shown are images of young adult animals carrying the integrated *agIs219* transgene, a GFP reporter of T24B8.5 transcriptional activity. T24B8.5 encodes a ShK toxin-like peptide that is expressed in the intestine. Strains are (A) WT (N2), (B) *tir-1(qd2)*, (C) *nsy-1(qd6)*, (D) *sek-1(qd39)*, and (E) *pmk-1(km25)*.

(F) Quantitative RT-PCR analysis of endogenous T24B8.5 expression in WT N2, *sek-1(km4)*, and *sek-1(km4); qdEx3 (Pges-1::sek-1::GFP)* and *sek-1(km4); qdEx12 (Posm-5::sek-1::GFP)* transgenic strains. Error bars represent the SEM between normalized biological replicates.

We have recently observed that behavioral avoidance of PA14 can manifest in survival differences under standard pathogenesis assay conditions, which involve a small lawn of PA14 spotted in the center of the agar assay plate, because avoidance of the pathogenic lawn during the course of the assay can confer survival benefit (Reddy et al., 2009). Differences in survival in the standard pathogenesis assay that can be attributed to avoidance of the pathogenic bacterial lawn are no longer observed in a modified “big lawn” assay in which PA14 is spread to cover the plate entirely. We hypothesized that the activity of SEK-1 in the chemosensory neurons of *C. elegans* is required for behavioral avoidance. Consistent with this hypothesis, we observed that the *Pges-1::sek-1::GFP* conferred full rescue of the enhanced pathogen susceptibility phenotype of the *sek-1(km4)* mutant in the big lawn assay (Figure 3A). In addition, the limited rescue conferred by the *Posm-5::sek-1::GFP* transgene in the standard assay (Figure 1C) was abrogated in the big lawn experiment (Figure 3B), as would be expected if SEK-1 activity in the chemosensory neurons promotes behavioral avoidance and concomitant survival on PA14. We observed that the tissue-specific expression of *sek-1* under the control of heterologous promoters did not compromise the survival of transgenic strains relative to *sek-1* on relatively nonpathogenic *Escherichia coli* OP50 (T.K., D.J.P., R.P.S., and D.H.K., unpublished data), although injection of *sek-1* at high concentrations of constructs directing expression of *sek-1* under neuron-specific promoters appeared to confer toxicity (R.P.S. and D.H.K., unpublished data).



**Figure 3. SEK-1 Activity in the Chemosensory Neurons Is Required for Behavioral Avoidance to *P. aeruginosa* PA14**

Shown are survival curves on *P. aeruginosa* PA14 of L4 stage WT (N2) and the *sek-1(km4)* mutant, along with different *sek-1(km4)* mutant strains carrying transgenes expressing the *sek-1* cDNA fused at its C terminus to GFP, under the control of tissue-specific promoters. Pathogenesis assays were carried out under big lawn (bacteria spread to cover the entire plate) conditions that, when compared to standard lawn assays (Figure 1), define contributions to survival caused by behavioral avoidance of the pathogenic lawn of bacteria.

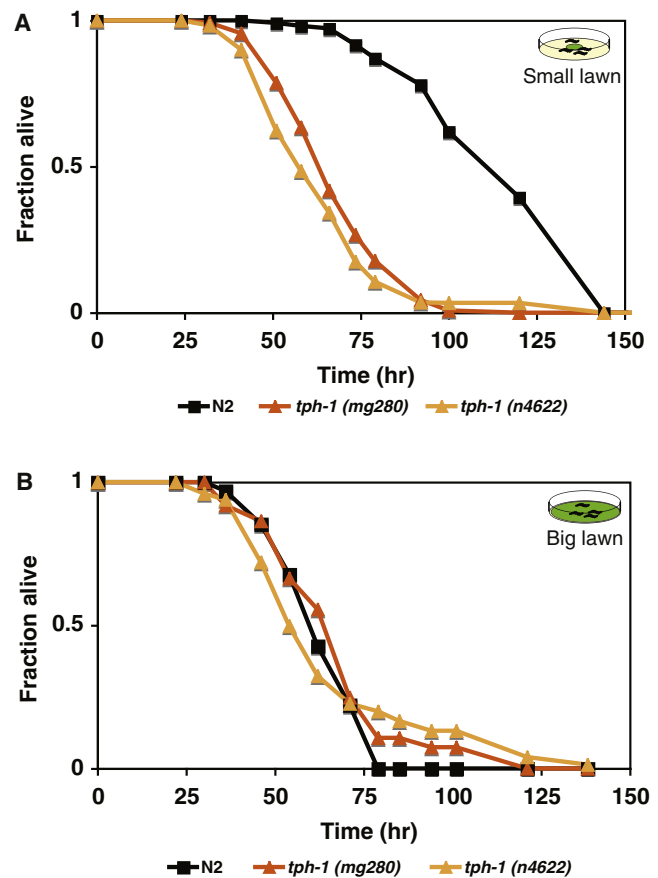
(A) Shown are survival curves for WT N2, *sek-1(km4)*, and three independent lines carrying the *Pges-1::sek-1::GFP* transgene in an extrachromosomal array, on the big lawn.

(B) Shown are survival curves for N2, *sek-1(km4)*, and three independent lines carrying the *Posm-5::sek-1::GFP* transgene in an extrachromosomal array, on the big lawn.

These data suggest that intestinal activity of *sek-1* is fully sufficient for innate immunity to PA14, whereas activity of *sek-1* in the chemosensory nervous system is required for a protective behavioral avoidance response. The lack of any rescue of T24B8.5 expression in the *sek-1(km4)* mutant in strains carrying the *Posm-5::sek-1::GFP* transgene (Figure 2F) also supports the model in which SEK-1 activity in the sensory nervous system regulates behavioral avoidance, as opposed to the direct neuronal modulation of intestinal immunity.

**Serotonin-Dependent Behavioral Avoidance Promotes Survival to PA14**

*C. elegans* has been shown to learn to avoid PA14 in a serotonin-dependent manner (Zhang et al., 2005). We hypothesized that



**Figure 4. Serotonin-Mediated Behavioral Avoidance Is Required for Resistance to Pathogens**

Shown are survival curves of L4 stage WT (N2), *tph-1(mg280)*, and *tph-1(n4622)* mutants on PA14, carried out under (A) standard pathogenesis assay conditions and (B) big lawn pathogenesis assay conditions.

defects in this behavioral response over the time course of the assay would be reflected in differential survival effects on the standard and big lawn assays, just as we observed for aerotaxis-dependent behavior mediated by *npr-1* (Reddy et al., 2009). The *tph-1* gene encodes tryptophan hydroxylase, the rate-limiting step in serotonin biosynthesis, and *C. elegans* *tph-1* mutants are deficient for serotonin production (Sze et al., 2000). We observed that the putative null mutant, *tph-1(mg280)*, exhibited enhanced susceptibility to PA14 relative to WT worms on the standard lawn assay (Figure 4A), but this difference in survival was abrogated on the big lawn assay (Figure 4B). The discrepancy between our findings on standard lawn assay conditions and prior analysis of pathogen susceptibility of the *tph-1(mg280)* mutant (Kawli and Tan, 2008; Zhang et al., 2005) may be due to strain background differences (see the Experimental Procedures) and/or the preparation of the pathogenesis plate assay, as our differential effects on standard and big lawn plates suggest that the result will be sensitive to the size of the PA14 lawn in the pathogenesis assay. We observed equivalent results using a second, independently derived deletion mutant, *tph-1(n4622)*, which is also a presumptive null allele (Figures 4A and 4B). In addition, we found that the standard pathogenesis



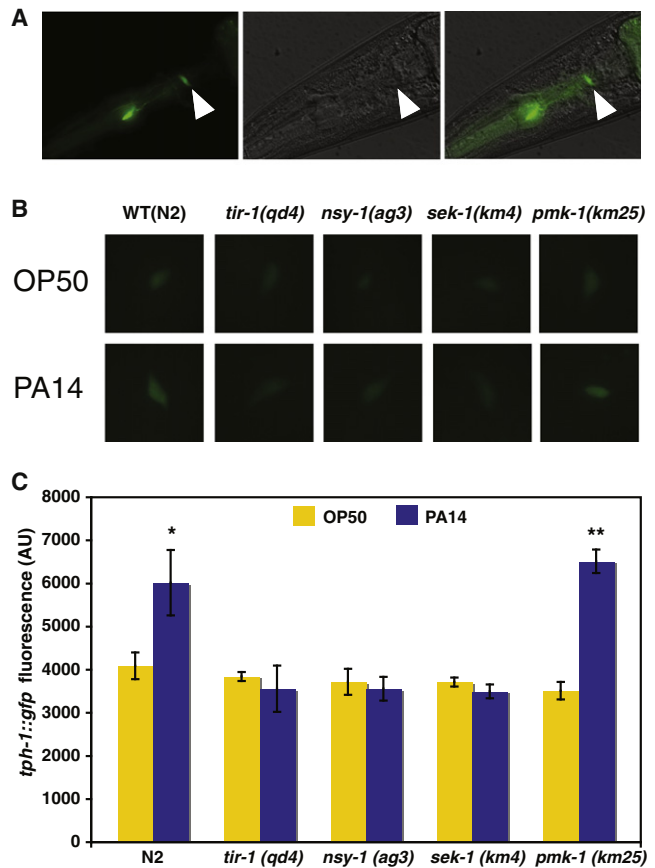
assay behavioral avoidance phenotype of *tph-1(mg280)* was not abrogated at lower oxygen concentration (10%) conditions (see Figure S1 available online), unlike the behavioral survival phenotype of the *npr-1* mutant we reported previously (Reddy et al., 2009; Styer et al., 2008), demonstrating that aerotaxis behavior alone cannot account for the behavioral survival phenotype of *tph-1* mutants.

Our data on the pathogen susceptibility of *tph-1* mutants on standard and big lawn plates corroborate a role for serotonin in behavioral pathogen avoidance shown by Zhang et al. (2005). In addition, we show that this serotonin-dependent behavioral avoidance phenotype is manifest in differences in survival upon exposure to PA14 under assay conditions in which the animals can avoid the lawn of pathogenic bacteria.

### The TIR-1-NSY-1-SEK-1 Module Is Required for Pathogen-Induced Expression of the Serotonin Biosynthetic Enzyme TPH-1

Serotonin signaling in the ADF chemosensory neuron pair has been implicated in aversive learning to pathogens (Zhang et al., 2005). Exposure of *C. elegans* to pathogenic PA14 induces the increased expression of *tph-1* in the ADF pair of chemosensory neurons relative to the basal level of *tph-1* expression observed when worms are propagated on relatively nonpathogenic *E. coli* OP50. PA14 induction of *tph-1* expression in the ADF neurons can be monitored using strains carrying a *Ptph-1::GFP* transgene, which shows basal expression in the ADF, NSM, and HSN neurons (Zhang et al., 2005). Because we observed a requirement for SEK-1 in the sensory nervous system in behavioral avoidance to pathogens, we investigated whether signaling through the TIR-1-NSY-1-SEK-1-PMK-1 pathway is required for the induction of *tph-1* expression in ADF neurons by PA14. We confirmed that exposure to PA14 induces the expression of a *Ptph-1::GFP* transgene relative to propagation on OP50 (Figures 5B and 5C), as reported previously (Zhang et al., 2005). However, we did not observe induction of *Ptph-1::GFP* expression by PA14 in the *sek-1(km4)* and *nsy-1(ky397)* mutant backgrounds (Figures 5B and 5C), consistent with a neuronal role for SEK-1 and NSY-1 in pathogen avoidance. We did observe induction of *Ptph-1::GFP* expression by exposure to PA14 in the *pmk-1(km25)* mutant background (Figures 5B and 5C), suggestive that a different MAPK is activated downstream of the TIR-1-NSY-1-SEK-1 module in the nervous system. We speculate that *pmk-2*, a p38 MAPK ortholog with a high degree of sequence identity to *pmk-1*, may serve as the downstream MAPK, though RNAi experiments were inconclusive, possibly due to the limited efficacy of RNAi in neurons (T.K. and D.H.K., unpublished data).

In order to investigate in detail the role of *tir-1* in *C. elegans* immunity and behavioral avoidance, we isolated and characterized a *tir-1(qd4)* deletion allele that is a putative null allele similar to a recently reported *tm3036* allele (Pujol et al., 2008). We also isolated an additional mutant allele, *tir-1(qd2)*, which has a missense mutation affecting all isoforms of *tir-1* (Figure S2A). The *tir-1(qd4)* and *tir-1(qd2)* mutants exhibited a strong enhanced pathogen susceptibility phenotype to PA14 (Figure S2B), and we observed that the intestinal expression of the PMK-1 transcriptional target, T24B8.5, is markedly diminished in the *tir-1(qd2)* mutant (Figure 2E). A previously isolated *tir-1(tm1111)* allele



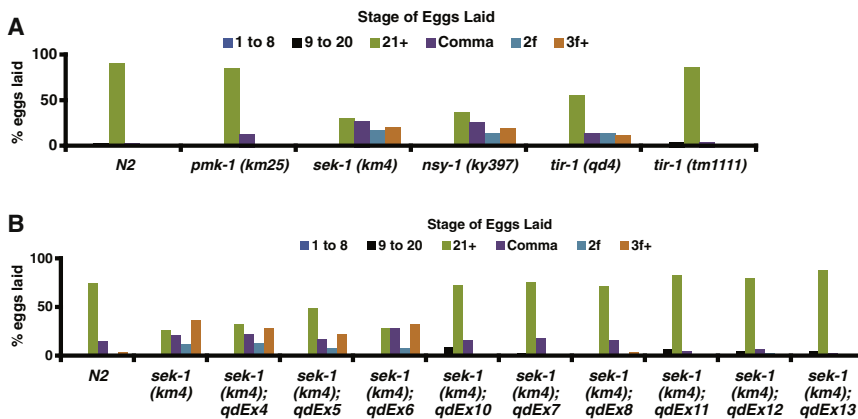
**Figure 5. The SARM-ASK1-MKK3 Pathway in *C. elegans* Is Required for Pathogen Induction of Serotonin Biosynthesis in the ADF Chemosensory Neuron Pair**

(A) Representation of *Ptph-1::gfp* expression in *C. elegans* WT worms under GFP channel, DIC, and merged channels. The white arrowhead points to an ADF neuron.

(B) Images show representative fluorescence microscopy images of one ADF neuron of L4 stage worms carrying an integrated *Ptph-1::GFP* transgene, *nls145*, propagated on *E. coli* OP50 and after 6 hr exposure to *P. aeruginosa* PA14 in the following strains: WT(N2), *tir-1(qd4)*, *nsy-1(ag3)*, *sek-1(km4)*, and *pmk-1(km25)*.

(C) Quantitation of *tph-1::GFP* fluorescence in the ADF neurons of WT (N2), *tir-1(qd4)*, *nsy-1(ag3)*, *sek-1(km4)*, and *pmk-1(km25)* worms propagated on OP50, both without (yellow) and after 6 hr of exposure to PA14 (blue). Error bars reflect standard error of the mean (SEM). Asterisks indicate the results of Student's t test comparing values of fluorescence on OP50 versus PA14 for indicated strains: \* $p < 0.01$ , \*\* $p < 0.05$ .

(Chuang and Bargmann, 2005), which is predicted to affect only the isoforms of *tir-1* that include the N-terminal HEAT/Armadillo repeats (Figure S2A), exhibited little or no pathogen susceptibility phenotype (Figure S2B) and did not affect T24B8.5 gene expression (Figure S2C), in contrast to that observed for the putative *tir-1* null alleles, suggestive that the HEAT/Armadillo repeats are not required for TIR-1 activity in innate immunity. Consistent with a role for TIR-1 upstream of NSY-1 and SEK-1 in the regulation of behavioral avoidance to pathogens, no induction of *Ptph-1::GFP* expression upon exposure to PA14 was observed in the *tir-1(qd4)* mutant (Figures 5B and 5C).



**Figure 6. The SARM-ASK1-MKK3 Pathway Functions in the Sensory Nervous System of *C. elegans* to Promote Egg-Laying Behavior**

(A) Percentage of eggs laid by synchronized adult animals for WT (N2), and *tir-1(qd4)*, *nsy-1(ag3)*, *sek-1(km4)*, and *pmk-1(km25)* mutant animals at each of the following stages over a 1 hr period: 1–8 cells, 9–20 cells, 21+ cells, comma, 2-fold, 3-fold. The egg-laying defective phenotype is manifest by mutant worms laying eggs with more advanced developmental stage relative to WT worms.

(B) Same assay carried out for WT (N2) and *sek-1(km4)* mutant animals along with *sek-1(km4)* strains carrying extrachromosomal arrays expressing the *sek-1::GFP* transgene under the control of tissue-specific promoters, *Pges-1* (intestine) (*qdEx4*, *qdEx5*, *qdEx6*), *Punc-119* (panneuronal) (*qdEx7*, *qdEx8*, *qdEx10*), and *Posm-5* (ciliated sensory neurons) (*qdEx11*, *qdEx12*, *qdEx13*). Three independent transgenic lines for each tissue-specific promoter are presented.

These data are consistent with a role for TIR-1-NSY-1-SEK-1, but not PMK-1, in the serotonin-dependent neuroendocrine response to PA14 that is required for protective behavioral avoidance. The TIR-1-NSY-1-SEK-1 pathway has been shown to be required for an asymmetric cell fate decision in the AWC neurons (Chuang and Bargmann, 2005; Sagasti et al., 2001). The *tir-1(tm1111)* mutant has a pronounced loss-of-function phenotype in this assay (Chuang and Bargmann, 2005), but little or no pathogen susceptibility phenotype (Figure S2B), suggestive that the roles of TIR-1-NSY-1-SEK-1 in AWC neuronal development and in the modulation of survival in response to pathogens are distinct. Furthermore, these data suggest that the distinct isoforms of *tir-1* that encode the N-terminal HEAT/Armadillo repeats are required for neuronal development but are not required for the behavioral and innate immune responses to pathogen infection.

### The TIR-1-NSY-1-SEK-1 Signaling in the Chemosensory Neurons Is Required for Reproductive Egg-Laying Behavior that Is Stimulated by Nutritional Bacteria

We and others have previously noted that *nsy-1* and *sek-1* mutants are defective in egg laying (Kim et al., 2002; Sagasti et al., 2001; Tanaka-Hino et al., 2002), but the phenotype has not been examined in detail. Egg-laying behavior shares similarities with pathogen avoidance behavior in that both represent serotonin-dependent behaviors that are stimulated by bacteria (Trent et al., 1983; Zhang et al., 2005). We sought to determine whether TIR-1-NSY-1-SEK-1 signaling might also have a role in the detection of the bacterial signal that induces egg laying. Using an assay that groups the embryos that are laid by gravid animals during a fixed period of time by developmental stage (Ringstad and Horvitz, 2008), we observed that loss-of-function mutations in the TIR-1-NSY-1-SEK-1 pathway, but not PMK-1, conferred a defective egg-laying phenotype (Figure 6A), as noted qualitatively previously. We observed that this phenotype could be rescued by the addition of exogenous serotonin (data not shown), suggestive of a role for TIR-1-NSY-1-SEK-1 upstream of the neuromuscular execution step of the egg-laying process.

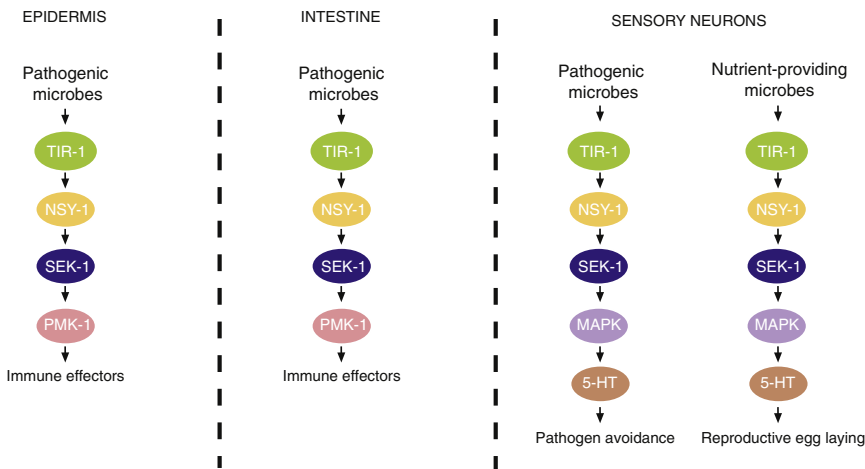
We defined the tissues in which *sek-1* activity is required for egg-laying behavior using *sek-1(km4)* mutant strains carrying transgenes directing tissue-specific expression of *sek-1*. We determined that whereas intestinal expression had no effect on the egg-laying defect, neuronal expression, and more specifically, expression of *sek-1* in the chemosensory neurons, resulted in rescue of the egg-laying defective phenotype (Figure 6B). As in the case of the pathogen avoidance phenotype, PMK-1 is not the downstream MAPK involved in egg laying, and the *tir-1(tm1111)* allele is unaffected, suggestive that the N-terminal HEAT/Armadillo repeats are not required for egg-laying behavior. These data establish a role for TIR-1-NSY-1-SEK-1 in the sensory nervous system in the egg-laying process, a site of activity that is consistent with a role for TIR-1-NSY-1-SEK-1 in the detection of bacteria that stimulates egg-laying behavior.

## DISCUSSION

### A Commonality in Signaling Mechanism for Innate Immunity and Behavioral Avoidance in the *C. elegans* Response to Pathogens

Our data show that *C. elegans* innate immunity and behavioral avoidance have in common the tissue-specific activities of an ancient TIR domain-dependent MAPK activation module, TIR-1-NSY-1-SEK-1 (Figure 7), that is orthologous to mammalian SARM-ASK1-MKK3. We have shown that TIR-1-NSY-1-SEK-1 functions in a cell-autonomous manner to activate PMK-1 in the intestine, paralleling the role of this pathway in epidermal immunity (Pujol et al., 2008). Recent studies also indicate that intestinal and epidermal PMK-1 pathways have in common signaling through the conserved protein kinase C delta isoform TPA-1 upstream of TIR-1 (Ren et al., 2009; Ziegler et al., 2009).

Following the demonstration that the *C. elegans* nervous system is able to mediate the detection and behavioral response to pathogenic bacteria (Pradel et al., 2007; Zhang et al., 2005), a number of recent studies point to multiple mechanisms by which the nervous system may be involved in protective responses to microbial pathogens (Zhang and Zhang, 2009). Neuronal signaling influences survival on pathogenic bacteria



**Figure 7. Tissue-Specific Activities of the TIR-1-NSY-1-SEK-1 Module in *C. elegans* Responses to Bacteria**

In response to pathogen infection, the TIR-1-NSY-1-SEK-1 module is required in the intestinal and epidermal cells for the PMK-1 p38 MAPK-dependent, cell-autonomous activation of innate immunity. In addition, the TIR-1-NSY-1-SEK-1 module is required in the sensory nervous system for serotonin-dependent neuroendocrine signaling from the ADF chemosensory neurons, which is required for behavioral avoidance to pathogens. Serotonin-dependent neuroendocrine pathways also mediate the behavioral reproductive egg-laying response to nutritional bacteria through the TIR-1-NSY-1-SEK-1 signaling module.

through behavioral avoidance mechanisms (Reddy et al., 2009; Styer et al., 2008), or through the direct modulation of the epidermal or intestinal immune response through neuroendocrine signaling, as has been recently reported for neuronal TGF- $\beta$  (Zugasti and Ewbank, 2009) and insulin (Kawli and Tan, 2008) signaling pathways, and in the context of enhanced resistance subsequent to pre-exposure of pathogen (Anyanful et al., 2009; Zhang and Zhang, 2009).

We have identified a requirement for TIR-1-NSY-1-SEK-1 in the neuroendocrine regulation of serotonin-dependent pathogen avoidance behavior. SEK-1 signaling in the chemosensory nervous system promotes survival to *P. aeruginosa* in a manner that is dependent on assay conditions in which *C. elegans* can avoid the pathogenic lawn. We observe that the TIR-1-NSY-1-SEK-1 module is required for the induction of *tph-1* expression in the ADF chemosensory neuron pair in response to pathogenic bacteria. Thus, the TIR-1-NSY-1-SEK-1 module points to a commonality in signaling mechanism between the distinctly different behavioral and innate immune responses to pathogenic infection (Figure 7).

The involvement of TIR domain protein signaling in the microbial recognition mechanisms of the immune system has been strikingly conserved from Dictyostelium (Chen et al., 2007) to humans (Akira et al., 2006). The tissue-specific activities of this TIR domain protein-containing signaling module for triggering dual responses to pathogen infection are suggestive that the involvement of TIR domain immune signaling in pathogen defenses may have been co-opted as different cell types, including neurons, in ancestral multicellular organisms adopted diverse roles in microbial recognition. Consistent with the idea that common pathogen detection mechanisms may be conserved in both immune and neuronal processes are intriguing recent reports of the presence of formylpeptide receptors, which can detect formylated peptides derived from bacteria and function in leukocyte chemotaxis (Migeotte et al., 2006), in the vomeronasal organ of mammals (Liberles et al., 2009; Riviere et al., 2009). Studies in additional organisms may help identify other candidate instances of the evolutionary co-option of TIR domain protein-dependent and other immune signaling pathways in nonimmune responses to microbes that may have had their origins in the bacterial recognition processes of ancestral organisms.

### An Ancient Role for Immune Signaling in a Behavioral Response to Nutrient-Providing, Beneficial Bacteria

In addition to the activities of TIR-1-NSY-1-SEK-1 in the chemosensory neurons and intestinal cells in protective responses to pathogenic bacteria, we have defined a role for TIR-1-NSY-1-SEK-1 in reproductive egg laying, a serotonin-dependent behavior that is dependent on bacteria provided as a nutrient source. The requirement for TIR-1-NSY-1-SEK-1 signaling in the chemosensory neurons for egg laying is consistent with a role for the TIR-1-NSY-1-SEK-1 module in the detection of nutrient and/or bacterial signals. These data further point to the multiple tissue-specific roles for TIR-1-NSY-1-SEK-1 in regulating not only responses to pathogenic bacteria but also behavioral responses to beneficial bacteria that provide essential nutrients. The role for this ancient TIR domain-dependent signaling module in these processes underscores the challenges posed to the host organism by the duality of bacteria, which may represent a source of nutrition, or a source of potentially lethal infection. These data are intriguing in view of the emerging causative role for inflammatory and immune signaling mediated by stress-activated MAPKs such as JNK in nonimmune endocrine cells in the pathogenesis of metabolic disease, where the response to what is essentially “nutrient overload” mimics what is observed in the response to infection (Hotamisligil, 2006; Hotamisligil and Erbay, 2008). The evolutionary origins of such signaling activity have been the subject of speculation (Hotamisligil, 2006; Hotamisligil and Erbay, 2008). Our data suggest that inflammatory and immune signaling in the pathogenesis of metabolic disease may have origins in the tissue-specific responses of ancestral host organisms to the pathogen/food duality presented by a world of microbial diversity.

### EXPERIMENTAL PROCEDURES

#### Strains

*C. elegans* was cultured on the bacterial strain *E. coli* OP50 as described (Brenner, 1974). N2 was obtained from the Caenorhabditis Genetics Center. The *nsy-1(ag3)*, *pmk-1(km25)*, *sek-1(km4)* mutants were described previously (Kim et al., 2002, 2004; Tanaka-Hino et al., 2002). The strain carrying *aglS219* was constructed by PCR amplification of the 5 kb region upstream of the start codon of the T24B8.5 gene, PCR-mediated ligation with *GFP::unc-54-3'UTR* (Hobert, 2002), and transformation of N2 with the coinjection marker *Pttx-3::GFP::unc-54-3'UTR* using standard methods (Mello et al., 1991). A resulting

strain, carrying the *PT24B8.5::GFP::unc-54-3'UTR* and *Pttx-3::GFP::unc-54-3'UTR* transgenes in an extrachromosomal array, was irradiated, and isolates carrying the integrated array were isolated. The *agls219* was mapped to the left arm of LG III. The *nsy-1(qd6)*, *tir-1(qd2)*, and *sek-1(qd39)* isolates were isolated from an ethylmethanesulfonate (EMS)-based screen of mutagenized *agls219* animals for mutants with diminished GFP expression and enhanced susceptibility to PA14 (R.P.S. and D.H.K., unpublished data). The *nsy-1(qd6)* allele carries a mutation that causes a W391Stop. The *tir-1(qd2)* allele carries a A451V mutation with respect to TIR-1B isoform (as shown in Figure S2A). The *sek-1(qd39)* allele carries a mutation that causes a W143Stop. The *tir-1(qd4)* deletion allele (as shown in Figure S2A) was isolated from screening of a deletion library of *C. elegans* clones generated by EMS (S. McGonagle and H.R. Horvitz). The *tir-1(qd4)* deletion spans a 1078 bp region from base 1504 to base 2582 with respect to cosmid F13B10. *tph-1(mg280)* (Sze et al., 2000) was obtained as an outcrossed strain recovered by M. Alkema and H.R. Horvitz. The *tph-1(n4622)* deletion allele was isolated from screening of a deletion library of *C. elegans* clones generated by EMS. The *tph-1(n4622)* deletion allele spans a 2,117 bp region from base 25,956 to base 28,072 with respect to cosmid ZK1290 (N. Ringstad, D. Omura, and H.R. Horvitz, personal communication). The strain carrying the integrated array *nls145*, expressing *Ptph-1::GFP*, was constructed by E. Miska using the plasmid previously described by Sze et al. (2000).

#### Construction of *sek-1* Tissue-Specific Rescuing Constructs

*Pges-1::sek-1::GFP* was generated by subcloning the 2.9 kb promoter region upstream of the *ges-1* gene (McGhee et al., 1990) into a pPD95.75 (A. Fire)-derived vector into which the *sek-1* cDNA was fused at its 3' end in-frame with GFP and the 3'UTR of *unc-54*. *Punc-119::sek-1::GFP* was generated by subcloning the 3.07 kb promoter region upstream of the *unc-119* gene (Maduro and Pilgrim, 1995) in front of *sek-1::GFP::unc-54-3'UTR* in a pPD95.75-derived vector. *Posm-5::ges-1::GFP* was generated by subcloning a 384 bp promoter region upstream of the *osm-5* gene (Haycraft et al., 2001) in front of *sek-1::GFP::unc-54-3'UTR* in a pPD95.75-derived vector. Young adult worms were injected with the tissue-specific constructs at the following concentrations: *Pges-1::sek-1::GFP* (25 ng/μl), *Punc-119::sek-1::GFP* (5 ng/μl), and *Posm-5::ges-1::GFP* (12 ng/μl). All injections were done with *myo-2::mStrawberry* (25 ng/μl) as a coinjection marker, with the exception of *qdEx1*, *qdEx2*, and *qdEx3*, which contained no coinjection marker, and pBluescript plasmid DNA as carrier to make a final concentration of DNA of 100 ng/μl. Appropriate tissue-specific *sek-1::GFP* expression patterns were confirmed in transgenic worms prior to each pathogenesis assay.

#### Quantitative RT-PCR

Synchronized populations of worms were prepared by hypochlorite treatment followed by dropping eggs onto NGM plates seeded with OP50. RNA was harvested from at least 800 predominantly L4 worms per strain using TRI reagent and reverse transcribed using the Retroscript kit (Ambion). For experiments involving strains carrying extrachromosomal arrays, transgenic worms were singly selected. The cDNA was analyzed by qRT-PCR on a Mastercycler Realplex (Eppendorf) with SYBR Green detection. *T24B8.5* levels were normalized to the control gene *snb-1*, and primers for both *T24B8.5* and *snb-1* were the same as reported previously (Troemel et al., 2006). Fold change was calculated using the Pfaffl method (Pfaffl, 2001). Standardization between two biological replicates was performed as described in Willems et al. (2008).

#### *P. aeruginosa* PA14 Pathogenesis Assays

The standard slow killing assay was performed as described (Tan et al., 1999), modified with the addition of 50 μg/ml 5-fluorodeoxyuridine (FdUR) (Miyata et al., 2008; Reddy et al., 2009). For the standard and modified killing assays using PA14, a minimum of 60 worms for each genotype were assayed in at least two independent trials. The big lawn killing assay used an agar plate composition identical to the slow killing assay, but 5 μl of PA14 was spotted onto each plate and spread to cover the entire agar surface as described previously (Reddy et al., 2009). The 10% oxygen killing assay was done on standard plates at room temperature in an acrylic chamber as described previously (Reddy et al., 2009). A mass flow controller (Sierra Instruments, Monterey, CA) was used to regulate gas flow into the chamber. Where indicated, statis-

tical analysis comparing survival curves was carried out using the SPSS statistical program (SPSS Inc.) and p values derived from the log-rank test.

#### *Ptph-1::GFP* Imaging and Quantifying of GFP Fluorescence

L4 worms were transferred to agar plates seeded with OP50 and incubated at 25°C for 12 hr, at which time worms were transferred to plates containing either OP50 or PA14. The worms were incubated at 25°C for 6 hr after transfer, mounted in 10 mM sodium azide on 2% agarose microscope slides. The focal plane with the strongest GFP signal in the ADF neuron was used to take each picture. Images were analyzed using the line tool in Axiovision (v.4.5). The pixel intensity values along this line were examined, and the pixel intensities within the highest 1 μm of the peak of the signal were averaged to obtain the *Ptph-1::GFP* fluorescence value for the worm. Averages and standard error of mean were calculated for at least three worms under each condition. Statistical analysis comparing *Ptph-1::GFP* fluorescence on OP50 versus PA14 was carried out using Student's t test.

#### Quantifying Egg-Laying Defects in *sek-1* Mutant Worms

Egg-laying assays were performed as previously described (Ringstad and Horvitz, 2008). Briefly, L4 worms were transferred to agar plates seeded with OP50 and incubated at 25°C for 12 hr, at which time five to ten worms were transferred to new plates for 1 hr and then removed. The eggs were counted and the stage of each egg laid was determined by visual examination on a dissecting microscope.

#### SUPPLEMENTAL DATA

Supplemental Data include two figures and can be found with this article online at [http://www.cell.com/cell-host-microbe/supplemental/S1931-3128\(09\)00310-2](http://www.cell.com/cell-host-microbe/supplemental/S1931-3128(09)00310-2).

#### ACKNOWLEDGMENTS

We thank N. Ringstad, P. Reddien, and members of the Kim lab for advice and discussions. We acknowledge O. Kamanzi and S. McGonagle for technical assistance. We thank the Caenorhabditis Genetics Center for strains. We thank M. Alkema, E. Miska, D. Omura, N. Ringstad, and H.R. Horvitz for providing unpublished strains. T.K. was partially supported by undergraduate research funding provided by the Howard Hughes Medical Institute. This work was supported by a grant from the National Institutes of Health (NIH) (GM084477), a Burroughs Wellcome Fund Career Award in the Biomedical Sciences, and an MIT Swanson Career Development Professor Award to D.H.K.

Received: April 1, 2009

Revised: June 9, 2009

Accepted: September 2, 2009

Published: October 21, 2009

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