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## Conditioning protects *C. elegans* from lethal effects of enteropathogenic *E. coli* through activation of genes that regulate lifespan and innate immunity

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### SUMMARY

*Caenorhabditis elegans* exhibit avoidance behavior when presented with diverse bacterial pathogens. We hypothesized that exposure to pathogens might not only cause worms to move away but also simultaneously activate pathways that promote resistance to the pathogen. We show that brief exposure to the virulent or avirulent strains of the bacterial pathogen enteropathogenic *E. coli* (EPEC) “conditions” or “immunizes” *C. elegans* to survive a subsequent exposure that would otherwise prove lethal. Conditioning requires dopaminergic neurons. Conditioning also requires the p38 MAP Kinase pathway, which regulates innate immunity, and the insulin/IGFR pathway, which regulates lifespan. Our findings suggest that the molecular pathways that regulate innate immunity and lifespan and provide protection may, in nature, be regulated or “conditioned” by exposure to pathogens, and perhaps allow survival in noxious environments.

### Keywords

EPEC; *C. elegans*; pathogenesis; aging; model systems

### INTRODUCTION

In its natural habitat, the nematode *Caenorhabditis elegans* encounters diverse microorganisms. It utilizes odors or other chemical cues to detect bacteria on which it then feeds (Bargmann, 2006). However, *C. elegans* is susceptible to natural bacterial pathogens such as *Microbacterium nematophilum* (Hodgkin et al., 2000), as well as to a wide variety of gram-positive and gram-negative bacteria and even fungi that are pathogenic in humans (e.g. *Pseudomonas aeruginosa* (Darby et al., 1999; Tan, 1999b)). Studies of *C. elegans*-pathogen interactions indicate that some bacteria, such as *Salmonella typhimurium*, colonize and persistently infect *C. elegans* causing animals to succumb within days (Aballay et al., 2000), whereas others, such as *P. aeruginosa* and enteropathogenic *E. coli* (EPEC), secrete toxins that kill within hours (Anyanful et al., 2005; Mahajan-Miklos et al., 1999). Significantly, many virulence factors or toxins responsible for killing *C. elegans* also contribute to disease

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in plant and mammalian systems (Hendrickson et al., 2001; Tan, 1999a), suggesting conservation of virulence mechanisms among diverse eukaryotic hosts.

Studies of *C. elegans*-pathogen interactions also indicate that nematodes have evolved behavioral mechanisms that facilitate survival. *C. elegans* can distinguish virulent from avirulent strains and thereby undergo avoidance behavior (Zhang et al., 2005). For example, *Serratia marcescens* produce a cyclic lipodepsipeptide, serrawettin W2 that is detected by AWB chemosensory neurons in *C. elegans* (Pradel et al., 2007) and causes animals to move away from bacteria. Moreover, Bargmann and colleagues found that *C. elegans* modifies its olfactory preferences after exposure to pathogenic bacteria, so as to avoid noxious strains and prefer nonpathogenic ones (Zhang et al., 2005). This change in preference is mediated by serotonin in the ADF sensory neurons, and by serotonin-gated chloride channels in sensory interneurons (Zhang et al., 2005).

Besides aversive behavior, *C. elegans* have also evolved protective mechanisms against pathogens. These include the p38 Mitogen-Activated Protein (MAP) kinase (Kim et al., 2002) and the insulin/IGF-1 receptor (IGFR) signaling systems (Garsin et al., 2003). The p38 MAP kinase pathway, which includes NSY-1, SEK-1 and PMK-1 kinases, has been proposed to mediate pathogen-specific responses by regulating expression of ~86 protective immune response genes (Troemel et al., 2006). The insulin/IGF-1 signaling pathway controls longevity and dauer formation in *C. elegans* (Kenyon et al., 1993). Activation of the IGFR homologue DAF-2 initiates a signaling cascade that negatively regulates the FOX-O family transcription factor DAF-16 (Ogg et al., 1997). DAF-16 up-regulates expression of ~263 genes that promote longevity, and down-regulates expression of ~251 life-shortening genes (Hamilton et al., 2005; Murphy et al., 2003). Genes up- or down-regulated by DAF-16 include those likely to metabolize toxins (e.g. cytochrome p450s) or to destroy bacteria (e.g. lysozyme, saposins). It has been proposed that DAF-16 controls basal innate immune responses in *C. elegans*, whereas MAP kinase signaling regulates pathogen-induced responses (Troemel et al., 2006).

Using both *C. elegans* and mammalian systems, we have been studying EPEC (O127:H6 (Levine et al., 1985)) and the related pathogen enterohemorrhagic *E. coli* (EHEC O157:H7; (Frankel et al., 1998)). EPEC is a human gastrointestinal pathogen that is transmitted via contaminated food and water and causes severe diarrhea in humans, leading to high mortality particularly among infants in developing nations (Clarke et al., 2002). We have shown previously that EPEC and EHEC paralyze and kill *C. elegans* via a secreted toxin (Anyanful et al., 2005). Both activities depend on the presence of tryptophan in the growth media and on the bacterial tryptophanase gene (*tnaA*) as well as on genes encoding virulence factors (e.g. *espF*). Importantly, we have also identified the MAP kinase and aging pathways as mediators of a protective response to EPEC in *C. elegans*.

In contrast to the wealth of information on aversive behavioral responses to pathogens in *C. elegans* and on innate immune and aging genes that provide protection, information on how neuronal sensing of pathogens or behavior itself might regulate expression of protective genes, is much more limited. Kenyon and colleagues have shown that specific neurons within the chemosensory organs in the front of the animal, called amphids, can perceive and mediate environmental cues that regulate lifespan (Alcedo and Kenyon, 2004; Apfeld and Kenyon, 1999). These data raise the possibility that detection of pathogens by olfactory or other sensory cues might induce protracted changes in the expression of protective genes.

We report here that, as expected, the brief exposure to EPEC induces aversive behavior. The important advance in this work is that brief exposure to either EPEC or to avirulent EPEC strains conditions worms to survive a subsequent exposure to EPEC that would otherwise

prove lethal. Conditioning requires dopaminergic neurons as well as genes in the innate immunity and insulin/IGFR1 signaling pathways. Together, our findings suggest that *C. elegans* uses sensory mechanisms both to trigger aversive behavior and to induce expression of protective genes.

## RESULTS

### *C. elegans* avoid contact with EPEC

Exposure of *C. elegans* to EPEC grown in a lawn causes paralysis within 30 minutes and subsequently death, scored as lack of movement and pharyngeal muscle activity (pumping) in worms 24 hours after transfer to plates containing the nonpathogenic *E. coli* strain OP50, the laboratory food source (Anyanful et al., 2005). Paralysis and killing by EPEC depend on the presence of tryptophan in the media, and on the activity of bacterial tryptophanase (TnaA) and EPEC virulence factors (e.g. EspF) (Anyanful et al., 2005). The rate of killing depends in part on formulation of the agar media: EPEC grown on Luria-Bertani (LB) agar containing tryptophan (LBT) kills ~90% of wildtype worms (N2) within 3 hours (Fig. 1a), whereas EPEC grown on ECD media kills with the same efficacy but within ~8 hours (Anyanful et al., 2005). Killing upon exposure to EPEC/LBT was evident with similar kinetics using N2 strains from three different laboratories (not shown) and with *rol-6(su1006)* roller worms (Supp. Fig. 1a).

When wildtype *C. elegans* (N2) were placed within a spot of EPEC (8mm in diameter), approximately 50% of the animals exit the spot within thirty minutes, and those remaining became paralyzed and later died (Fig. 1b). By contrast, such avoidance behavior was not evident with EPEC $\Delta$ tnaA (Fig. 1b), a strain that does not cause paralysis or death (Anyanful et al., 2005). Thus, toxins produced by EPEC induce behavioral avoidance.

### Conditioning facilitates survival of *C. elegans* upon lethal exposure to EPEC

We next determined whether brief exposures to EPEC might activate protective responses within *C. elegans* and thus increase their capacity to survive a subsequent exposure that would otherwise prove lethal. To do this, we developed a “conditioning” protocol (Fig. 1c) so as to more precisely control the time and degree of exposure to EPEC. N2 worms were incubated with EPEC grown in a lawn on LBT agar for thirty minutes (“pre-exposure” period; Fig. 1c), and then moved to NGM plates containing OP50 for three hours (waiting period). Worms were then re-exposed to an EPEC lawn on LBT plates for an additional three hours (challenge) and the number of survivors scored. The EPEC lawn covered the 6cm plate so that worms could not exit during the pre-exposure or challenge period. The duration of the pre-exposure period was chosen so as to maximize survival, and the duration of the challenge period to maximize lethality and minimize variance (Fig. 1a). Pre-exposure of N2 worms to EPEC/LBT induced survival of ~40% of animals, compared to ~9.9% of animals without previous exposure, a ~4 fold increase in survival (Fig. 1d). Similarly, pre-exposure of *rol-6(su1006)* worms to EPEC induced a statistically significant increase in survival of ~4.9 fold (34% survival with pre-exposure compared to 7% without; Supp. Fig. 1b). Pre-exposure of N2 worms to LBT plates lacking bacteria was without effect (data not shown). Pre-exposure to EPEC for periods greater or less than thirty minutes proved less effective in promoting survival (Supp. Fig. 1c). Likewise, waiting periods of less than three hours or more than four hours proved less effective (Fig. 1e), and though increased survival was still evident after 24 hours, none was evident after 48 hours (Fig. 1e). These data suggest that pre-exposure to EPEC coupled with a waiting period promotes increased survival of *C. elegans*, and that the effect lasts for extended periods.

We next determined whether survival of *C. elegans* induced by pre-exposure required virulence factors expressed by EPEC. To do this, we pre-exposed *C. elegans* to EPEC grown on LB without added tryptophan, or to the EPEC strains EPEC $\Delta$ *espF* or EPEC $\Delta$ *tnaA* grown on LBT. Growth of EPEC on LB renders the bacteria avirulent, as no killing was observed even with extended exposure (up to 96 hrs, the longest time tested; not shown). Likewise, EPEC $\Delta$ *espF* and EPEC $\Delta$ *tnaA* contain isogenic mutations in *espF* and *tnaA* respectively, and are avirulent when grown on LBT even with extended exposures (up to 48 hrs for EPEC $\Delta$ *espF* or 96 hrs for EPEC $\Delta$ *tnaA*, the longest times tested; not shown). The capacity to kill *C. elegans* can be restored to EPEC $\Delta$ *espF* and EPEC $\Delta$ *tnaA* by complementation with plasmid- or chromosomally-encoded *espF* or *tnaA*, respectively (S. Bhatt and D.K. unpublished, and (Anyanful et al., 2005)). Neither pre-exposure to EPEC grown under avirulent conditions, nor to the avirulent strains EPEC $\Delta$ *espF* and EPEC $\Delta$ *tnaA* for thirty minutes (the protocol shown in Fig. 1c), induced a statistically significant increase in survival (Fig. 1g). Together, these data suggest that the transfer protocol itself did not induce enhanced survival, and that virulence factors or toxins produced by EPEC facilitate survival induced by pre-exposure.

### Avirulent EPEC strains and other pathogens induce conditioning upon extended exposure

Pre-exposure to EPEC grown on LB, or to either EPEC $\Delta$ *espF* or EPEC $\Delta$ *tnaA* grown on LBT for an extended period (three hours; Figs. 1f,g) induce survival to the same extent as pre-exposure to EPEC grown on LBT for 30 minutes (4.3 fold for N2 and 5.4 fold for *rol-6(su1006)*; Fig. 1d & Supp. Fig. 1b). Pre-exposure to EPEC grown on LBT for thirty minutes, and then to EPEC $\Delta$ *espF*, EPEC $\Delta$ *tnaA*, or to EPEC/LB during the waiting period, did not result in a significant difference in survival compared to exposure to OP50/NGM during the waiting period (not shown). Thus, survival can be enhanced by pre-exposure to EPEC under both virulent and avirulent conditions, as well as by avirulent EPEC strains. However, for all the conditioning protocols tested, no more than 40-50% of the animals survived.

We next grew EPEC or EPEC $\Delta$ *tnaA* overnight atop 0.2  $\mu$ m nitrocellulose filters. After removal of the filters, the plates were used to pre-expose the animals for forty minutes to EPEC, or for three hours to EPEC $\Delta$ *tnaA*. After a waiting period of three hours, animals were challenged for three hours with EPEC. We observed a significant increase in survival on plates pre-exposed to EPEC using this protocol, though the level was lower than that observed with direct contact. By contrast no such effects were evident on plates pre-exposed to EPEC $\Delta$ *tnaA* on filters. These data suggest that survival induced by pre-exposure to EPEC did not require direct contact whereas that induced by EPEC $\Delta$ *tnaA* did (Fig. 1h). The contact-dependence of pre-exposure and the difference in pre-exposure times required for EPEC grown under virulent or avirulent conditions or for avirulent strains, suggests that EPEC produces two “conditioning” factors; one is secreted, acts quickly, and depends on the toxin, whereas the other acts slowly, requires direct contact, and acts independently of the toxin.

We next assessed whether pre-exposure to other pathogenic bacteria could enhance survival upon exposure to EPEC. Some *Pseudomonas aeruginosa* strains (e.g. PA01, PA14; (Darby et al., 1999; Tan, 1999b) and PAK1 (Laws et al., 2006)) kill *C. elegans*. PAK1 produced no detectable deleterious effects in *C. elegans*, even over extended periods on LBT plates (96 hrs, the longest time tested; not shown). However, pre-exposure to PAK1 for 3 hours followed by exposure to EPEC for 3 hours induced a 2.7-fold increase in survival compared to animals exposed to OP50/NGM for 3 hours (Fig. 1g). By contrast, the rodent pathogen *Citrobacter rodentium*, which also does not kill *C. elegans*, did not induce a significant increase in survival to EPEC (Fig. 1g). These data suggest that some bacterial species can induce responses in *C. elegans* that are protective against other species.

## Conditioning depends on the insulin/IGFR-1 pathway

The insulin/IGFR-1 signaling pathway, which regulates lifespan (Kenyon, 2001; Kenyon et al., 1993; Lin et al., 1997; Ogg et al., 1997), also protects *C. elegans* from a variety of pathogens (Garsin et al., 2003), including EPEC (Anyanful et al., 2005). Worms with mutations in *daf-2* (e.g. *daf-2(e1370)*), which have extended lifespan (Kenyon et al., 1993), also are more resistant to EPEC, and mutations in *daf-16* abrogate such effects ((e.g. *daf-16(m26);daf-2(e1370)*), (Anyanful et al., 2005)). Thus, DAF-2 negatively regulates DAF-16. *daf-16* mutants also succumb more quickly than N2 upon constitutive exposure to EPEC (Fig. 2a).

We next assessed whether *daf-16* mutants exhibited increased survival upon pre-exposure to EPEC or EPEC $\Delta$ *tmaA*. In contrast to wild-type *C. elegans* (N2), neither *daf-16(m26)*, *daf-16(mgDf50)*, *daf-16(mu86)*, nor *daf-16(mgDf47)* animals exhibited survival upon pre-exposure to EPEC $\Delta$ *tmaA* or to EPEC (1.6 to 1.8 fold; Fig. 2b with EPEC $\Delta$ *tmaA*; Supp. Fig. 2a with EPEC) to the same extent as N2 (e.g. 4 to 4.3 fold for N2). Increased survival upon pre-exposure to other avirulent strains (EPEC $\Delta$ *espF*) or by EPEC grown under nonpathogenic conditions (LB medium) also required *daf-16* (not shown). Because results with pre-exposure to EPEC and EPEC $\Delta$ *tmaA* were nearly identical in our assays, subsequent experiments presented below show only results with EPEC $\Delta$ *tmaA*. Data with EPEC are presented in Supplementary Information or not shown. Enhanced survival upon pre-exposure was evident in transgenic *daf-16(mu86)* or *daf-16(mgDf47)* animals expressing DAF-16 under its own promoter either as an integrated array (I.A.; e.g. 3.5 to 4.8 fold for the rescued strains compared to 1.7 fold for the parental strains) or as an extrachromosomal array (Ex. A.; Fig. 2b; Supp. Fig. 2a). Notably, the *daf-16* mutants and their integrated complemented strains still succumbed faster than N2 (Fig. 2a), though *daf-16(mu86)* animals expressing DAF-16 under its own promoter as an extrachromosomal array had a slightly slower death rate (Fig. 2b).

To determine whether the effect of lifespan genes on conditioning was specific, we assessed whether enhanced survival upon pre-exposure required other genes that mediate protective responses to pathogens. HSF-1 activates a different subset of genes than DAF-16 (Singh and Aballay, 2006), and *hsf-1(RNAi)* worms have a shorter lifespan than N2 (Hsu et al., 2003). Moreover, *hsf-1* is required for innate immunity to a variety of bacterial pathogens (Singh and Aballay, 2006). *hsf-1(sy441)* suffer a severe egg laying defect (Hadju-ronin et al., 2004) and die from internal hatching of young. To minimize contributions from such defects in our assays, we only used young adults, and our experiments were completed within 7 hours. We did not observe the “bag of worms” phenotype associated with internal hatching suggesting that egg laying defects play little if any role in phenotypes we have studied. Both *hsf-1* RNAi (not shown) and *hsf-1(sy441)* succumbed faster than N2 when exposed to EPEC, indicating a protective role (Fig. 2a). However, *hsf-1(sy441)* animals exhibited 5.2 fold more survival upon pre-exposure to EPEC $\Delta$ *tmaA*, compared to unconditioned animals, an increase comparable to that seen with N2 animals (Fig. 2b; see also Supp. Fig. 2a for EPEC).

These data suggest that lack of conditioning is not due to increased susceptibility, and that conditioning is a specific response independent of other protective genes. Nevertheless, the observation that *daf-16* mutations cause animals to succumb more quickly raised the possibility that the apparent lack of conditioning of *daf-16* mutations results from an increased sensitivity to EPEC. To establish quantitatively whether a correlation existed between the rate of killing and the degree of conditioning, we first estimated the death rate of each mutant using regression techniques. We then regressed this rate against the fold conditioning, calculated as the fold increase in survival with pre-exposure to EPEC $\Delta$ *tmaA*/LBT (as per protocol in Fig. 1f) or EPEC (as per protocol in Fig. 1c) compared with pre-exposure only to OP50/NGM. When we considered only N2 and *rol-6(su1006)* together



with strains that showed little conditioning (e.g. *daf-16* mutations, mutations in innate immunity genes, and dopamine signaling mutants; see below) we found a statistically significant negative correlation between death rate and conditioning (small black dots in Fig. 2c). However, when we considered these mutations, together with mutations in *hsf-1*, serotonin signaling, and other genes (see below), we found a regression line with slope  $\sim 0$  (small and large black dots in Fig. 2c), indicating that no correlation existed between the rate of killing and the degree of conditioning. Thus, from a statistical standpoint, the apparent lack of conditioning in several mutant strains was not simply a reflection of a faster death rate.

The variability in rates of susceptibility to EPEC across a wide variety of strains raised questions of whether background mutations contribute to fold conditioning, complicating comparison of conditioning in different strains. To address this issue, we developed an analysis of covariance (ANCOVA) model to assess the contribution to conditioning of baseline survival without pre-exposure. Using ANCOVA, we quantitatively defined the contribution of extraneous variability that derives from pre-existing individual strain differences to the mean conditioned survival, insofar as those differences are reflected in the baseline survival. We then adjusted the mean conditioned survival to compensate for the fact that different mutants have different levels of survival without pre-exposure. The ANCOVA analysis accounts for procedural and strain differences because the baseline used for comparison of different mutants is the adjusted mean survival for all mutant animals considered. Notably, because the rate of death and degree of conditioning appeared highly uncorrelated, the ANCOVA correction was in most cases minimal (e.g. compare Figs. 2b and 2d), though differences between the corrected levels and fold conditioning were evident with particular mutants that showed very low basal levels of survival with pre-exposure (see Fig. 4e and Supp. Fig. 4i below). Adjusted mean survival with pre-exposure, together with 95% confidence intervals, are shown for *daf-16* mutants, the rescued strains, and *hsf-1(sy441)* in Fig. 2d, and below for other mutants. For ease of comparing different mutants, we hereafter refer to the adjusted mean survival with pre-exposure as “conditioned survival,” and present non-normalized data with and without pre-exposure to EPEC or EPEC $\Delta$ *tmaA* in Supplementary Information.

The lack of overlap of confidence intervals indicates that all the mutations in *daf-16* tested cause a statistically significant decrease in conditioned survival compared to N2. All but one of these mutants, *daf-16(mu86)*, did not show significant conditioned survival compared to the baseline adjusted mean survival without conditioning (“no conditioning”; Fig 2d). All the *daf-16* strains rescued with overexpressed DAF-16 show a statistically significant increase compared to the baseline. Conditioned survival of *daf-16(mu86)* rescued with DAF-16 in an integrated array was not significantly different from the parental strain, *daf-16(mu86)*. However, conditioned survival of *daf-16(mu86)* rescued with an extrachromosomal array or *daf-16(mgDf47)* rescued with an integrated array were statistically different from their respective parental strains. These data suggest that the rescue of both mutants was incomplete, in accordance with the observation that the rate of death for both mutants was not significantly different from their parental strains, and different from N2 (Fig. 2A). It is not without precedent that rescue by integrated or extrachromosomal arrays is not always complete. Transgenic arrays contain many copies of the wild type gene and thus there is overexpression, and the complete up- or downstream regulatory sequences may not be included in the rescue construct, thus gene regulation may not be “native” (Mello, 1995). Nevertheless, the observations that all four *daf-16* mutants tested exhibited significantly lower conditioning levels compared to N2, and that conditioned survival of two of the rescued *daf-16* mutant strains was significantly higher than baseline levels or levels seen with the parental strains, suggest a role for *daf-16* in mediating conditioning.

DAF-16 protein translocates into the nucleus upon activation by environmental stimuli to promote longevity (Henderson and Johnson, 2001). To determine whether exposure to EPEC likewise induces DAF-16 translocation, we assessed nuclear localization of DAF-16 in N2 worms carrying an integrated DAF-16::GFP transgene (N2; zls356[ExDAF-16::DAF-16-GFP, *rol-6(su1006)*]) that were exposed to EPEC for thirty minutes, or EPEC $\Delta$ *tnaA* for three hours, or left unexposed. Without exposure, GFP fluorescence was predominantly cytoplasmic. DAF-16::GFP translocation into the nucleus, identified by DAPI staining, was evident within 30 minutes following exposure to EPEC and within 3 hrs to EPEC $\Delta$ *tnaA* (Fig. 2e). Together, these data suggest that DAF-16 translocates into the nucleus upon pre-exposure to bacteria.

### Conditioning also depends on the MAP kinase pathway

Whereas genes activated by DAF-2/DAF-16 pathway have been proposed to confer basal resistance to pathogens, the SEK-1/PMK-1 pathway mediates expression of several immune response genes upon exposure to pathogens and regulate both innate immunity and longevity in *C. elegans* (Kim et al., 2002; Troemel et al., 2006). *sek-1* and *pmk-1* encode MAP kinase kinase and MAP kinase orthologs respectively. Both *sek-1(km4)* and *pmk-1(km25)* succumbed faster than N2 when exposed to EPEC indicating that these genes mediate protection against the pathogen (Fig. 2f and (Anyanful et al., 2005)). Moreover, neither *sek-1(km4)* nor *pmk-1(km25)* animals exhibited as much conditioned survival as N2 upon pre-exposure with EPEC $\Delta$ *tnaA* (Fig. 2g; Supp. Fig. 2b) or EPEC (Supp. Fig. 2c), and levels were similar those seen with *daf-16* mutations (Fig. 2d). Together, these experiments indicate that *daf-16*, *sek-1* and *pmk-1* mediate not only protection against EPEC but also conditioning.

### The DAF-16-regulated genes *spp-1* and *aqp-1* genes mediate conditioning

DAF-16 regulates genes that promote longevity, including factors that have antibacterial activity (e.g. lysozyme), inactivate toxins (e.g. cytochrome p450s), and facilitate responses to stress (Hamilton et al., 2005; Murphy et al., 2003). Murphy et al. (2003) found that DAF-16 upregulates ~263 genes that promote longevity and downregulates ~251 life-shortening genes. We reasoned that a subset of these genes might regulate susceptibility to EPEC by providing protection when activated by *daf-16* during pre-exposure. We first determined which of the upregulated genes are responsible for increasing resistance to EPEC. To do this, we devised a screen in which *E. coli* HT115 bacteria expressing dsRNA for 85 selected genes of the ~263 upregulated genes described by Murphy et al. (2003) were fed to *daf-2(e1370)* worms. *daf-2(e1370)* mutants are not paralyzed by EPEC after 30 minutes and greater than 60% survive after 3 hours (Anyanful et al., 2005). Moreover, these animals appear to exhibit maximal conditioning constitutively, likely due to nuclear translocation of DAF-16. Thus, upon pre-exposure to EPEC or EPEC $\Delta$ *tnaA*, we could detect no differences in survival compared to *daf-2(e1370)* worms pre-exposed to OP50 (not shown). To identify genes up-regulated by DAF-16 that mediate survival in response to EPEC, we challenged *daf-2(e1370)* worms fed with various dsRNAs with EPEC, and scored for animals that readily succumbed.

We identified two genes, *spp-1* and *aqp-1*, which, when inactivated, suppressed the capacity of *daf-2(e1370)* worms to survive a four hour exposure to EPEC (Fig. 3a). When inactivated, several other genes partially abrogated *daf-2*-mediated survival but not to the same extent as *spp-1* and *aqp-1*, and were not pursued further (not shown). Inactivation of other DAF-16-inducible genes, such as *lys-8*, was without effect (Fig. 3a).

We next determined whether *spp-1* or *aqp-1* mediated conditioned survival in N2 animals. To do this we fed N2 animals *E. coli* HT115 containing dsRNA for *spp-1*, *aqp-1* or, as

controls, *lys-8*, or the empty vector. As shown in Fig. 3b, inactivation of *aqp-1* or *spp-1* in N2 animals increased the sensitivity to EPEC. Moreover, knockdown of either *spp-1*, or *aqp-1* resulted in no conditioned survival (Fig. 3c; see also Supp. Fig. 3a). In accordance with the RNAi results, the aquaporin mutant *aqp-1(tm2309)* and the saposin mutant *spp-1(ok2703)* succumbed faster than N2 (Fig. 3d), indicating a protective role, and *spp-1(ok2703)* was not conditionable whereas *aqp-1(tm2309)* was only slightly conditionable relative to non conditioned controls (Fig. 3e).

We next set out to rescue *aqp-1(tm2309)* or *spp-1(ok2703)* with the wildtype gene under its own promoter using an extrachromosomal array. Rescue of *aqp-1(tm2309)* with *aqp-1*, restored conditioned survival to levels comparable to those seen in N2 and *rol-6(su1006)*, and partial restoration of conditioning was achieved upon rescue of *spp-1(ok2703)* with *spp-1* (Fig. 3e). Expression of *aqp-1* or *spp-1* in both *daf-16(mu86)* and *pmk-1(km25)* animals also significantly increased conditioned survival, suggesting that each is sufficient (Fig 3f; Supp Fig 3c,d). However, expression of *aqp-1* or *spp-1* in N2 did not result in a significant increase in conditioned survival lending support to the idea that an upper limit to conditioning exists using our protocols (not shown).

To determine whether pre-exposure induced changes in *aqp-1* or *spp-1* gene expression, we next assessed RNA levels. We found that in N2 animals, but not in *daf-16(mu86)* or *pmk-1(km25)* animals, mRNA levels of *aqp-1* or *spp-1* increased by ~1.5 fold upon pre-exposure to EPEC for thirty minutes followed by three hour waiting period on OP50/NGM (Fig. 3g). Notably, the levels of *aqp-1* and *spp-1* were lower in conditioned compared to unconditioned *pmk-1(km25)* animals (Fig. 3g), raising the possibility that PMK-1 regulates basal levels of expression and DAF-16 regulates induced levels. In *daf-2(e1370)* animals, in which DAF-16-regulated genes are constitutively activated, basal mRNA levels of *aqp-1* or *spp-1* were significantly higher than those seen in N2 (Supp. Fig. 3e). Together these data suggest that both *aqp-1* and *spp-1* mediate conditioned responses to EPEC upon activation of DAF-16 and PMK-1 signaling.

### A neuronal circuit mediates conditioning

We next set out to determine how *C. elegans* detects bacterial factors that initiate conditioning. Previous reports indicate that pathogenic bacteria induce changes in olfactory neurons that cause aversion of *C. elegans* to pathogenic strains, and increase the preference for nonpathogenic ones (Zhang et al., 2005). Moreover, specific chemosensory neurons within the amphids perceive and integrate environmental cues that regulate lifespan (Alcedo and Kenyon, 2004; Apfeld and Kenyon, 1999). Finally, *daf-16* expression in neurons and intestines has been shown to regulate lifespan (Libina et al., 2003). Therefore, we assessed whether *daf-16* expression in neurons or intestines was sufficient for conditioning. We used *daf-16(mu86)* worms containing DAF-16::GFP expressed in either neurons (using the *unc-119* promoter) or intestines (using the *ges-1* promoter) (Libina et al., 2003). Expression of *daf-16* in either cell type was sufficient to permit enhanced conditioned survival in animals pre-exposed to EPEC $\Delta$ *tnaA* (Fig. 4a; see also Supp. Fig. 4a). Similar results were obtained in *daf-16(mgDf47)* (data not shown). The sufficiency of *daf-16* expression in neurons and intestines suggests the involvement of both neurons and intestinal cells in conditioned survival. Kenyon and colleagues (Libina et al., 2003) have reported that DAF-16 activity in neurons can up-regulate DAF-16 in specific responding tissues such as intestinal cells via insulin-like peptides. These data raised the possibility that a neuronal circuit might sense bacterial conditioning factors and communicate the signal to other tissues such as the intestines, where expression of *aqp-1* and *spp-1* are prominent. In this paper, we have focused on the role of neuronal signaling in regulating conditioned survival.



To determine whether specific sets of neurons mediate conditioning, we assessed conditioned survival in several worm strains carrying mutations in the molecules that generate or regulate effects of serotonin or dopamine, neurotransmitters implicated in responses to bacteria (Chase et al., 2004; Suo et al., 2004; Suo et al., 2003; Zhang et al., 2005). We first tested mutations in *cat-1*, which encodes a vesicular monoamine transporter that loads dopamine and serotonin into presynaptic vesicles (Duerr et al., 1999). *cat-1(e1111)* animals also have defects in associative learning (Zhang et al., 2005) and detection of bacteria on lawns (Sawin et al., 2000). *cat-1(e1111)* animals succumb at a faster rate than N2 upon constitutive exposure (Supp. Fig. 4b), but do not exhibit conditioning (Fig. 4b; Supp. Fig. 4c). These data raise the possibility that dopamine or serotonin or both might mediate conditioning.

### Dopaminergic neurons, but not serotonergic neurons mediate conditioning

To distinguish between serotonergic and dopaminergic mechanisms, we first assessed conditioning in animals with defects in *cat-2*, which encodes tyrosine hydroxylase (Lints and Emmons, 1999), the rate limiting enzyme in dopamine biosynthesis, and in *dop-1*, *dop-2*, and *dop-3*, which encode dopamine receptors (Chase et al., 2004; Sugiura et al., 2005; Suo et al., 2004; Suo et al., 2002; Suo et al., 2003). We also assessed conditioning in animals with mutations in *goa-1*, which encodes an ortholog of a heterotrimeric G protein  $\alpha$ -subunit, *dgk-1*, which encodes an ortholog of diacylglycerol kinase, and *dat-1*, which encodes a dopamine reuptake transporter (Nass et al., 2005). Both *goa-1* and *dgk-1* mediate signaling distal to DOP-3 receptor (Chase et al., 2004). Notably, like *cat-1(e1111)*, *dop-3(vs106)*, and to a lesser extent *dop-1(vs100)*, and *dop-2(vs105)*, have been shown to be defective in detecting bacteria on lawns (Chase et al., 2004; Suo et al., 2004; Suo et al., 2003).

We found that *cat-2(e1112)*, *dop-3(vs106)*, *goa-1(sa734)*, *dat-1(ok157)* and *dgk-1(sy428)* all succumbed faster than N2 upon constitutive exposure to EPEC (Supp. Fig. 4d). Notably, conditioned survival by EPEC or by EPEC $\Delta$ ma was significantly lower in *dop-3(vs106)* mutants compared to N2, and none was evident in any of the other dopamine signaling mutants (Fig. 4c; Supp. Fig. 4e). Additionally, we found that *dop-1(vs100)* and *dop-2(vs105)* were only partially conditionable (not shown). Expression of *aqp-1* or *spp-1* in *dop-3(vs106)* under control of their own promoters restored conditioned survival to levels similar to those seen in N2 animals (Fig. 4d; Supp. Fig. 4f,g). To further verify a role for dopaminergic signaling in conditioning, we treated *C. elegans* with 6-hydroxy-dopamine (6-OH-DA), which selectively kills dopaminergic neurons (Nass et al., 2002). *C. elegans* previously exposed to 6-OH-DA were not conditionable (Fig. 4c). Finally, we found that mRNA for *aqp-1* and *spp-1* were not induced in *dop-3(vs106)* following exposure to EPEC (Fig. 3g). Together, these data provide evidence that dopaminergic signaling in neurons mediates conditioning upon exposure to EPEC.

We next assessed conditioned survival in animals with mutations in *tph-1*, which encodes tryptophan hydroxylase, the rate limiting enzyme in serotonin biosynthesis (Sze et al., 2000), in *mod-1*, which encodes a serotonin-gated chloride channel (Ranganathan et al., 2000), and in three serotonin receptors (*ser-1(ok345)*, *ser-4(ok512)*, and *ser-7(tm1325)*). *mod-1(ok103)* and *tph-1(mg280)* have defects in associative learning in response to *Pseudomonas aeruginosa* PA14 (Zhang et al., 2005). Similar to *hsf-1(sy441)*, all serotonin mutants succumb more quickly than N2 in response to EPEC indicating that serotonergic signaling is protective upon constitutive exposure to EPEC (Supp. Fig. 4h). However, all exhibited conditioned survival levels significantly greater than unconditioned animals, with *ser-4(ok512)* having levels comparable to those seen in N2, and *ser-7(tm1325)*, *tph-1(mg280)*, and *mod-1(ok103)* somewhat less than N2. Only *ser-1(ok345)* exhibited conditioned survival that was only slightly above baseline levels (Fig. 4e). Notably, all the

serotonin mutants had significantly lower baseline levels of conditioning, with *ser-1(ok345)* having the lowest. Moreover, fold increases over the baseline were either greater than or comparable to those seen with N2 (Supp. Fig. 4I). Thus, although *ser-1(ok345)* appeared to have little conditioning relative to all the mutants, the fold conditioning was nonetheless comparable to levels seen with N2. We therefore conclude that serotonergic signaling is not required for conditioning (Supp. Fig. 4i). These data suggest that some genes mediate only protective responses, whereas others mediate both protection and conditioning.

### Dopaminergic signaling, *aqp-1* and *spp-1* are required for conditioning by EPEC but not by stressors

We next determined whether conditioned survival in EPEC is a specific response that utilizes a particular signaling pathway or a general stress response. To do this, we exposed N2 or various mutants to stressors such as starvation (3 hours), heat shock (32°C, 2 hours), heavy metals (CuSO<sub>4</sub>, 2 hours), or oxidative stress (H<sub>2</sub>O<sub>2</sub>; 2 hours). After a three hour waiting period on OP50, animals were then exposed to EPEC for three hours. Heat shock and, to a lesser extent, copper and peroxide, induced conditioned survival in a manner that depended on *daf-16*, *aqp-1*, *spp-1*, and *pmk-1* whereas starvation did not (Fig. 5a). These data are in accordance with previous reports suggesting that these stressors activate *daf-16* signaling (Liang et al., 2006). As shown above, *dop-3(vs106)* and *cat-2(e1112)* succumb more rapidly than N2 when exposed to EPEC (Supp. Fig. 4d) and pre-exposure to EPEC or EPEC $\Delta$ *tmaA* does not significantly increase conditioned survival (e.g. Fig. 4c). However, pre-exposure to heat shock significantly increases survival of *dop-3(vs106)* and to a lesser extent *cat-2(e1112)* (Fig 5a). Heavy metal and peroxide induced more modest levels of conditioned survival in *dop-3(vs106)*. The effect of these stressors was not evident in *cat-2(e1112)*, perhaps because these animals had higher levels of basal conditioning compared to *dop-3(vs106)*. Together these data indicate that survival induced by pre-exposure to bacteria specifically depends on dopaminergic signaling. Moreover, *aqp-1(tm2309)*, *spp-1(ok2703)*, and *dop-3(vs106)* were as sensitive as N2 to H<sub>2</sub>O<sub>2</sub>, and as resistant as N2 to heavy metals, at least under the conditions tested (Fig. 5b), suggesting that the effects of *aqp-1*, *spp-1*, and *dop-3* were specific to EPEC, and that these genes do not nonspecifically mediate responses to all stressors. Thus, whereas many stressors can activate *daf-16* and downstream effectors (e.g. *aqp-1* and *spp-1*) that protect against EPEC, bacteria activate these effectors via dopaminergic signaling pathways.

## Discussion

Our observations suggest that *C. elegans* detect EPEC directly or indirectly via dopaminergic neurons. Once secreted, dopamine may act postsynaptically on metabotropic DOP-3 receptors to induce sustained increases in the levels of a subset of DAF-16 dependent genes that control longevity (e.g. *aqp-1*) and allow *C. elegans* to survive subsequent lethal exposure. Protection develops over a period of hours and lasts for approximately 24 hours, and involves a different set of genes than those mediating protection upon acute exposure. Thus, some genes mediate only protection (e.g. *ser-1*, *hsf-1*), whereas others mediate both protection and conditioning (e.g. *dop-3*, *daf-16*, *pmk-1*). Conditioning requires master regulators of longevity (e.g. *daf-16*) and innate immunity (e.g. *pmk-1*), which respond to many environmental challenges, including pathogens (Garsin et al., 2003; Troemel et al., 2006). Sensory inputs into these pathways activate a specific subset of responses that can protect against some environmental insults and not others. Thus, *C. elegans* appear to utilize sensory receptors and neuronal signaling networks in conjunction with innate immunity and aging pathways to generate a protective immune response that is specific, adaptive, and persistent. These observations are summarized in Fig. 6.

Several lines of evidence argue against the idea that mutants may not appear conditionable simply because they are more sensitive to EPEC. First, heat shock and other stressors markedly increase conditioned survival of *dop-3(vs106)* and *cat-2(e1112)* (Fig. 5a). Thus, despite the sensitivity of these mutants, protective mechanisms can be activated by bypassing dopamine signaling. Second, no correlation was evident between susceptibility and fold conditioning (Fig. 2c). As an example, *hsf-1* mutants die as fast as *daf-16* mutants, but whereas *hsf-1* mutants are as conditionable as N2, *daf-16* mutants are not (Figs. 2d). A similar result was obtained with the serotonin mutants (Supp. Fig. 4h, Fig. 4e).

At least two factors from EPEC induce conditioning: one is secreted and diffusible, whereas the other requires direct contact with the worm. That some bacterial strains (e.g. EPEC, EPEC $\Delta$ *tmaA*, PAK1), virulent or not, can induce protection against EPEC, but others cannot (*C. rodentium*, OP50), suggests that conditioning may be a general strategy to discriminate classes of bacteria as beneficial or deleterious food sources. Noxious stimuli from bacterial pathogens induce olfactory learning that changes olfactory preferences and trains *C. elegans* to avoid the pathogen (Zhang et al., 2005). Our data suggest that in addition to behavioral avoidance, such stimuli may also upregulate protective responses that allow the animal to survive subsequent exposure to toxins, and perhaps even establish a feeding niche in an otherwise noxious environment. Thus, aversive behavior may provide a window for protective responses to develop. Notably, whereas olfactory learning is mediated by serotonergic circuits (Zhang et al., 2005), our data suggest that dopaminergic neurons mediate induction of long term protective responses. Our current efforts are focused on defining EPEC factors that stimulate conditioning, and the sensory detectors of such factors in *C. elegans*. Moreover, because the signaling pathways regulating longevity and innate immunity are highly conserved from *C. elegans* to humans, we hypothesize that such detection mechanisms are coupled to protective responses mediated by analogous pathways in mammalian systems.

Previous work based on identification of *C. elegans* mutants that are resistant to various pathogens or toxins raised the possibility that the degree of host susceptibility was based on basal expression of protective genes, mediated by IGF-1R/DAF-16 signaling, and induced expression of protective genes, mediated by the MAP kinase signaling (Troemel et al., 2006). Our data provide a refinement of this idea and raise the possibility that when encountering pathogens, *C. elegans* use a neuronal circuit to regulate both aversive behavior and induction of protective responses via aging and innate immune signaling pathways. As noted above, different circuits appear to mediate particular functions. Thus, whereas serotonergic signaling is required for aversive behavioral responses, dopaminergic signaling is required for long-term protective responses. Moreover, both circuits are plastic and modifiable by previous exposure or experience. Therefore, aversive behavior and neuronal-mediated induction of protective genes evident in ancient organisms such as *C. elegans* serve analogous functions to the innate and adaptive immune systems found in vertebrates.

The question arises as to whether acquired resistance to EPEC is likely to represent a specific response to bacteria versus a generalized stress response. Our data suggest that the response comprises elements of both. As noted above, the capacity to discriminate pathogens from food sources is in part achieved by coupling of neuronal detection and signaling systems to either behavioral responses or to master stress regulators such as PMK-1 and DAF-16. Effectors that regulate responses to aging or stress include factors that inactivate toxins or have antimicrobial activity. However, effectors are not restricted to antibacterial functions, likely because the stimuli that activate these pathways are so diverse and enhanced longevity requires survival from challenges other than just infection. It is interesting to note that the contribution of *daf-16*-regulated genes as a group to longevity has

been proposed to be cumulative. Thus, loss of function of particular life-shortening or life-extending genes only has an incremental effect on lifespan (Kenyon, 2005). Our data suggest that two genes, *aqp-1* and *spp-1*, are major contributors to protection against EPEC induced by not only EPEC toxins and warning factors but also by other pathogens (e.g. *Pseudomonas*) and stressors (Figs. 1, 3 and 5). These genes are specific for protection against bacteria; *aqp-1* and *spp-1* are not important for survival from heavy metals (Fig. 5b). Together these data suggest that neuronal activation of aging and innate immunity pathways likely results in activation of a broad range of protective genes, including *aqp-1* and *spp-1*. Should the dopaminergic signaling pathway we have identified transduce signals from diverse sensory receptors, then we expect that the conditioning paradigm identified here may be activated in response to a wide variety of pathogens.

Using a different exposure protocol than ours, Evans *et al.* (Evans et al., 2008) report that infection of *C. elegans* with *P. aeruginosa* activates DAF-2 signaling, leading to translocation of DAF-16 protein from nuclei to cytosol of intestinal cells, and down-regulation of some DAF-16 targets (*thn-2*, *spp-1* and *lys-7*), but not others (*abf-2*, *lys-2* and F08G5.6). These changes depended upon the bacterial two-component regulator *gacA* and the quorum-sensing regulators *lasR* and *rhlR*. In contrast, exposure to *S. typhimurium* or *E. faecalis* induced all six genes. Evans et al. (Evans et al., 2008) conclude that the down-regulation is characteristic of *P. aeruginosa* infections and is not applicable to *S. typhimurium* and *E. faecalis*. Our data likewise suggest that EPEC induces different responses than *P. aeruginosa*. Accordingly, we knocked down *lys-7* and *thn-2* by RNAi and found no increase sensitivity of *daf-2(e1370)* RNAized worms upon EPEC exposure and no effect of these genes on conditioning. Together, these results suggest that particular bacterial factor, together with the timing or dynamics of its presentation to the worm may have significant effect on the nature or extent of the protective response engendered.

*aqp-1* encodes an aquaglyceroporin (Huang et al., 2007), which facilitates transport of water and glycerol across cell membranes and in addition regulates lifespan (Murphy et al., 2003). We surmise that such channels may prove important in uptake of EPEC toxins or warning factors. *spp-1* encodes a peptide similar to amoebaphores produced by *Entamoeba histolytica* and is thought to function by forming pores in the membranes of bacterial target cells, and limits *Salmonella* infection (Alegado and Tan, 2008; Banyai and Patthy, 1998). *spp-1* may confer protection by a novel means because EPEC-mediated killing does not involve direct contact. Data presented here suggests that *aqp-1* and *spp-1* mediate conditioning, but understanding the roles of these genes at the molecular level will require that a definition of the cellular basis for conditioning.

## Materials and Methods

### Bacterial and nematode strains

Experiments were mainly carried out with *enteropathogenic E. coli* serotype 0127:H6 strain E2348/69 (Levine et al., 1985) and *E. coli* OP50 (Brenner, 1974). EPEC $\Delta$ *tmaA*, EPEC $\Delta$ *espF*, *Pseudomonas aeruginosa* strain PAK1 and *Citrobacter rodentium* have been described previously (Anyanful et al., 2005; McNamara and Donnenberg, 1998). The following *C. elegans* mutants were obtained from the *Caenorhabditis* Genetics Center: *daf-16(m26)*, *daf-16(mgDf50)*, *daf-16(mu86)*, *daf-2(e1370)*, *hsf-1(sy441)*, *sek-1(km4)*, *pmk-1(km25)*, *spp-1(ok2703)*, *cat-1(e1111)*, *cat-2(e1112)*, *dop-1(vs100)*, *dop-1(vs101)*, *dop-2(vs105)*, *dop3(vs106)*, *goa-1(sa734)*, *dgk-1(sy428)*, *dat-1(ok157)*, *ser-1(ok345)*, *ser-4(ok512)*, *ser-7(tm1325)*, *tph-1(mg280)*, *mod-1(ok103)*, *daf-16(mgDf47);xrls87[daf-16a::gfp::DAF-16B, rol-6(su1006)]* (strain GR1352), *daf-16(mu86);muls61[daf-16::GFP, rol-6(su1006)]* (strain CF1139), N2;zls356[Ex DAF-16-GFP, *rol-6(su1006)*] (strain TJ356), *rol-6(su1006)* and wild-type Bristol strain N2.

*aqp-1(tm2309)* and *daf-16(mgDf47)* and additional N2 worms were generously provided by Shohei Mitani, Siu Silvia Lee, Shoichiro Ono and Guy Benian respectively. All *C. elegans* strains were maintained on Nematode Growth Medium (NGM) under standard culturing conditions with *E. coli* OP50 as food source (Sulston, 1988). All assays were performed at 25° C.

### Worm killing assays

Worm killing assays were carried out essentially as described previously (Anyanful et al., 2005), though times of exposure and the media were altered. Briefly, EPEC, EPEC $\Delta$ *tnaA*, EPEC $\Delta$ *espF*, PAK1 or *C. rodentium* were cultured in Luria-Bertani (LB) broth overnight to an OD<sub>600</sub> of 0.8-1.0 and 170 $\mu$ L spread on 6 cm LB agar (Fisher) plates containing 2mg/mL tryptophan (LBT plates). After incubation for 20 hours at 37°C, the plates were cooled in 25°C incubators for an hour. Young adult worms maintained at 20°C were transferred to each plate, and at least 180 worms/strain were tested for each experiment. For killing assays, worms were exposed to EPEC for 3 hours at 25°C before being transferred to OP50 on NGM plates. After 24 hours worms were gently prodded with a platinum wire and considered dead if they failed to respond to touch and showed no indication of pharyngeal pumping.

### Worm conditioning assays

For EPEC conditioning assays, young adult worms were exposed to LBT/EPEC for 30 minutes, transferred to OP50 on NGM plates for 3 hours and then again to fresh LBT/EPEC for 3 hours. Worms were then transferred to OP50 on NGM plates and scored after 24 hours. For non-pathogenic conditioning assays, worms were exposed for 3 hours to either EPEC $\Delta$ *tnaA*, EPEC $\Delta$ *espF*, *Pseudomonas aeruginosa* (PAK1) or *Citrobacter rodentium* on LBT plates, or EPEC on LB plates before being transferred to LBT/EPEC plates for 3 hours and subsequent recovery on NGM/OP50 for 24 hours. For most of the experiments we used EPEC $\Delta$ *tnaA*/LBT for the conditioning. For experiments with 0.2  $\mu$ m nitrocellulose filters (Fig. 1h), we confirmed that bacteria did not penetrate the filters by culturing sample plates at 37° for 48 hours following removal of filters. We never observed bacterial growth on plates overlaid with filters.

### DAF-16::GFP nuclear localization assay

Young adult N2; zls356 [*daf-16::DAF-16::GFP*, *rol-6(su1006)*] (strain TJ356) worms were left unexposed or exposed to EPEC for 30 minutes or EPEC $\Delta$ *tnaA* for 3 hours. GFP fluorescence was monitored using a Zeiss 200M inverted microscope with a 63x N.A.1.4 lens. For some experiments, worms were stained with DAPI to visualize nuclei.

### Real time PCR assays

Synchronized young adults of N2, *dop-3(vs106)*, *pmk-1(km25)*, *daf-16(mu86)* and *daf-2(e1370)* were exposed to EPEC for 30 minutes and then transferred to OP50 on NGM for three hours. Worms were washed free of bacteria with M9 buffer and frozen at -80°C. Unexposed worms grown on NGM/OP50 for control were collected at the young adult stage and frozen. Total RNA from a ~250 $\mu$ L worm pellet was extracted using Trizol (Life Technologies) following manufacturer's protocol and quantitated by UV absorbance. cDNAs were synthesized with Oligo (dT) Primers using a RETROscript kit (Ambion). Real time PCR was performed in 50 $\mu$ L volume using SYBR-green detection in a 96 well plate on an iCycler machine (Biorad). Duplicates for each sample were included for each reaction and three independent reactions were performed. Primer pairs and product lengths for *act-1* and *sod-3* have been described (Li et al., 2007). Primers for *spp-1* and *aqp-1* are shown below. The PCR reaction was initiated at 95°C for 3 minutes followed by 15s at 95°C, 60s at



60°C and 30s at 68°C for 40 cycles. The real time PCR experiments were repeated three times using independently derived RNA preparations. The mRNA levels of the *aqp-1* and *spp-1* from N2, *dop-3(vs106)*, *daf-16(mu86)* and *pmk-1(km25)* were expressed relative to that of actin by calculation using the cycle threshold (Ct). The following equation described by Maeda et al., (2006) was used: mRNA expression level (%) =  $2^{(Ct \text{ pre-exposure}) - (Ct \text{ post-exposure})} \times 100$ . Ct post-exposure = [Ct values of *aqp-1* and *spp-1* in N2, *dop-3*, *daf-16* and *pmk-1* post-exposure - Ct values of *act-1* in N2, *dop-3*, *daf-16* and *pmk-1* post-exposure] and Ct pre-exposure = [Ct values of *aqp-1* and *spp-1* in N2, *dop-3*, *daf-16* and *pmk-1* pre-exposure - Ct values of *act-1* in N2, *dop-3*, *daf-16* and *pmk-1* pre-exposure].

*spp-1*

5'-GATGATCTCGATGCATGGCTTGATG-3' (forward)

5'-CCTTGCACGCCTTGTCTGGAGAATCC-3' (reverse).

*aqp-1*

5'-GGAGGTAATCGCACAATCCTCGGAGC-3' (forward)

5'-GTATGCGAGAACTCCGGTACCGACGC-3' (reverse)

### RNA interference (RNAi) assays

We performed RNAi using the *E. coli* strain HT115(DE3) carrying the RNAi vector L4440 or L4440-derived plasmids engineered to express double stranded RNA (dsRNA) targeting genes of interest, obtained from the *C. elegans* RNAi library (Kamath et al., 2003). Briefly, specific HT115(DE3) bacteria from the library were cultured overnight at 37°C on LB plates containing 100µg/mL ampicillin and 15µg/mL tetracycline. Single colonies were then cultured overnight at 37°C in LB broth containing 50µg/mL ampicillin and spotted onto 10 cm plates containing NGM agar supplemented with 5mM IPTG and 25µg/ml carbenicillin. The plates were incubated overnight at room temperature to induce the dsRNA expression. 15 N2 or *daf-2(e1370)* worms at the L3 stage were transferred onto the plates and cultured at 20°C to purge any eggs. After 48 hours, 12 worms were then transferred to fresh plates and cultured at 20°C. After removing the parents 48 hours later, F1 worms grown to the young adult stage were subject to EPEC killing and conditioning assays.

### 6-hydroxydopamine experiments

Synchronized L4 to young adult worms were collected and washed with M9 buffer and added to a mixture of 1mL 10mM ascorbic acid and 50mM 6-hydroxydopamine (Nass et al., 2002) for 1 hour at 24°C, mixing gently every 15 minutes. Worms were washed thoroughly with M9 buffer and transferred to NGM/OP50 plates. After 15 hours, worms were subjected to killing and conditioning assays.

### Construction of transgenic worms

To generate AQP-1::GFP, a 6036bp genomic fragment including 3635bp of putative promoter sequence and 2401bp of total coding sequence was amplified using platinum taq DNA polymerase (Invitrogen) and primers AQP-1F and AQP-1R (see below). The fragment was cloned in-frame into the BamHI site of the expression vector pPD95.75 (a gift from Andy Fire, Stanford University). To generate SPP-1::GFP, a 2466bp genomic fragment including 2094bp promoter sequence and 373bp of total coding sequence was amplified similarly with primers SPP-1F and SPP-1R. The fragment was cloned into the BamHI site of pPD95.75. For rescue experiments, these constructs were microinjected into *aqp-1(tm2309)* and *spp-1(ok2703)* respectively, using 20µg/mL of vector construct and 80µg/mL of pRF4 as marker. Transgenic F2 progeny were selected by their roller phenotype and expression of

GFP in the specific tissues confirmed. For over-expression experiments, the constructs were injected into *daf-16(mu86)*, *pmk-1(km25)* and *dop-3(vs106)* and transgenic worms selected as above. In all cases two independent lines were used for analysis. To generate *daf-16* tissue-specific promoter fusions, we used a PCR fusion-based approach as described (Hobert, 2002). We first generated the *gfp* coding sequence plus the 3' untranslated region from vector pPD95.75 using primers C and D. We then generated DAF-16 from N2 cDNA using primers A and B. Primer B had a 24bp overlap with primer C. Finally, we used primers UNC-119F & UNC-119R and GES-1F & GES-1R to generate the promoter regions of *unc-119* and *ges-1* respectively from genomic DNA. Primers UNC-119R and GES-1R have a 33bp overlap with DAF-16 cDNA. To perform fusion PCR, we used 10ng of each fragment and Nested D primer as reverse and Nested UNC-119F and Nested GES-1F primers as forward. We used Clontech polymerase for all fusion PCR reactions. After sequence confirmation, we injected and selected transgenics as above. Using the same procedure, we generated *Pdaf-16::DAF-16::GFP* to confirm the rescue of the *daf-16* mutation with our construct.

<b>AQP-1F</b>	CTTCGGATCCGAAAAGCGGCCGCCACATTTCG
<b>AQP-1R</b>	CAACGGATCCAGCTTGAAGCAATTTTGTGCTCTAC
<b>SPP-1F</b>	CAACGGATCCGCCCTGAAGCGGAATCTGCTCCGC
<b>SPP-1R</b>	CAACGGATCCCGCACAAATCAACATCCTTGCACGC
<b>Primer D</b>	AAGGGCCCGTACGGCCGACTAGTAGG
<b>Nested D</b>	GGAAACAGTTATGTTTGGTATATTGGG
<b>Primer C</b>	AGCTTGCATGCCTGCAGGTCGACT
<b>Primer B</b>	AGTCGACCTGCAGGCATGCAAGCTCAAATCAAATGAATATGCTG CCCTCC
<b>Primer A</b>	ATG AAC GAC TCA ATA GAC GAC G
<b>UNC-119R1</b>	CGGCGGAAAATCGTCGTCTATTGAGTCGTTTCATATATGCTGTT GT AGCTGAAAATTTTGG
<b>UNC-119F</b>	CTAGGCCATCGGTGACGTCATTACTC
<b>Nested UNC-119F</b>	CATGGCATTGCCTATGCCATTTTCAC
<b>GES-1R1</b>	CGGCGGAAAATCGTCGTCTATTGAGTCGTTTCATCTGAATCAA AG ATAAGATATGTAATAG
<b>GES-1F</b>	CTACTGGAATCCGCCAAATTGTCGACAAC
<b>Nested GES-1F</b>	CTTGAGTGTTAGCGGCGTCTTACC
<b>DAF-16-R1</b>	CGGCGGAAAATCGTCGTCTATTGAGTCGTTTCATTCTGGAAGCT GTGCTCCTCCGAAGGGG
<b>DAF-16-FO</b>	CAACGGATCCAGGGATAAGGG AGATTCCGAACAG
<b>Nested DAF-16-FO</b>	CAACGGATCCCTAGTCATAAGTAGTCAGGCAGGC

### Stressor conditioning and killing assays

For conditioning assays, worms were subjected to heat shock by incubating them at 32°C for 2 hours, or exposed to 200µM CuSO<sub>4</sub> for 2 hours, or exposed to 1mM H<sub>2</sub>O<sub>2</sub> or starvation conditions for two hours. Afterwards, the worms were transferred to NGM/OP50 for 3 hours before being exposed to EPEC/LBT for 3 hours. For starvation experiments, no incubation with NGM/OP50 was done. Worms were transferred to NGM/OP50 after EPEC exposure and % survival determined 24 hours later. For killing assays, worms were exposed to either 5mM H<sub>2</sub>O<sub>2</sub> or 5mM CuSO<sub>4</sub> for two hours. They were then transferred to NGM/OP50 and % survival determined after 24 hours.

### Statistical analysis

**ANOVA analysis and test for trend**—In Figure 1a, we used an ANOVA and a test for linear trend in survival over time. For this experiment, a linear trend test over the seven time points was significant ( $p < 0.0001$ ). For Figures 1d, 1e, 1g, and 1h, and for Figure 5,

survival was compared using ANOVA. The comparisons were highly significant ( $p < 0.0001$ ). 95% confidence intervals are shown for each.

### Calculation of fold survival with conditioning and survival rates

Fold increases in survival with conditioning were calculated as the quotient of the percent of animals surviving following procedure in Fig. 1c (EPEC/LBT pre-exposure) or Fig. 1f (EPEC $\Delta$ tnaA/LB pre-exposure) divided by the percent surviving following procedure in Fig. 1c (OP50/LB pre-exposure) or Fig. 1f (OP50/LB pre-exposure). For Figure 2d, the survival rate for each mutant was estimated by linear regression using the percentage of animals surviving following exposure to EPEC for 0, 1, 2, or 3 hours. The estimated rates of survival rate were then regressed against fold increase in survival with conditioning (Fig. 2d). Linear regression of survival rate (the dependent variable) on fold change (the independent variable) for all mutants yielded the following relationship: Survival Rate =  $-0.146 - (0.00444 \times \text{Fold Change})$ ; Intercept =  $-0.146$  (standard error =  $0.01729$ ); slope =  $-0.00444$  (standard error =  $0.00316$ ); When comparing the estimated slope to zero using a t-test, a value of  $p = 0.17$  was obtained indicating the difference was not statistically significant. Linear regression of survival rate on fold change for only *daf-16* mutants, N2 and *rol-6(su1006)* yielded: Survival Rate =  $-0.0573 - (0.04686 \times \text{Fold Change})$ ; Intercept =  $-0.0573$  (standard error =  $0.0207$ ); slope =  $-0.04686$  (standard error =  $0.0089$ );  $p < 0.0001$  when comparing the estimated slope to zero using a t-test; SD of the regression =  $0.04476$ , mean square error =  $0.002$ . Note that a significant negative correlation was evident when considering only a subset of mutants, but not with all mutants

### ANCOVA

We used ANCOVA to remove the effects of pre-existing mutant differences, and ensure that mutants are starting out approximately equal, on average, with respect to all factors that might be pertinent to how well they are likely to respond to the conditioning paradigms. Such a correction is useful because individual differences in conditioning displayed by a particular mutant could potentially be correlated with survival rate. The mean survival and 95% confidence intervals for all 39 mutants without conditioning was  $0.05335$  or  $5.3\% \pm 0.243\%$  (mean  $\pm$  sem), and the slope estimate was  $0.05585$ . For N2, the adjusted mean survival with conditioning,  $Y$ , was  $0.42595 + 0.05585 \times \text{mean survival without conditioning}$ :  $Y = 0.42595 + (0.05585 \times 0.05335) = 0.429$  or  $42.9\%$ .

### Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

### Acknowledgments

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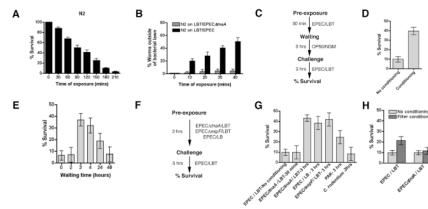
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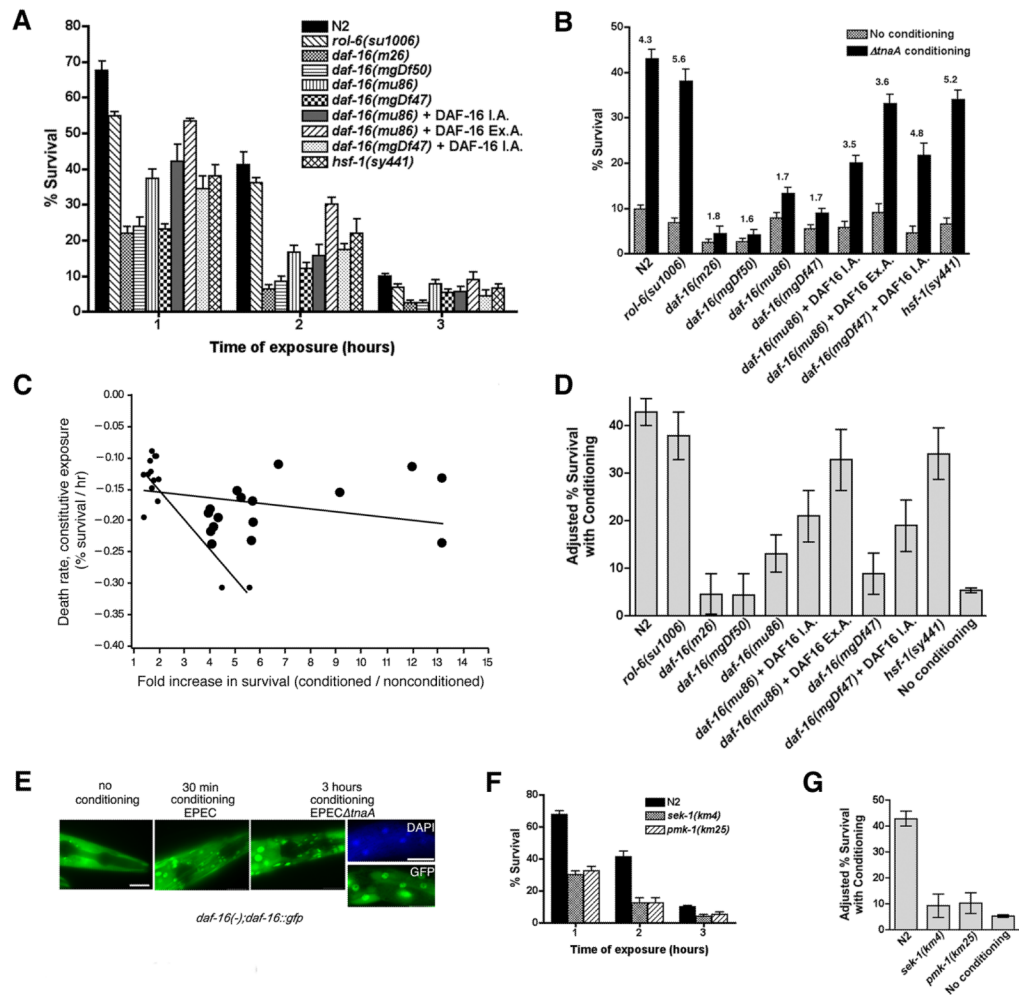
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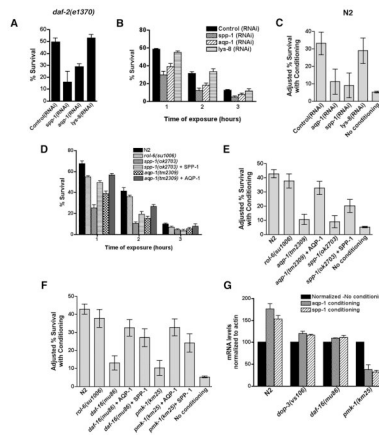
**Figure 1. Conditioning of *C. elegans* by pre-exposure to virulent or avirulent EPEC strains**  
 For A, and B, mean values  $\pm$  SEM are presented. For D, E, G, and H, statistically significant differences, calculated by ANOVA, of mean survival values are evident as a lack of overlap of 95% confidence intervals. **A.** Time course of killing of *C. elegans* by constitutive exposure to an EPEC lawn. N2 worms were exposed to EPEC grown on LBT agar for the times indicated and then removed to OP50 grown on NGM agar. After 24 hours, survival was scored. Conditioning and challenge times used were 0.5 and 3 hrs respectively. The linear trend was highly significant ( $p < 0.0001$ ). **B.** Time course of *C. elegans* movement away from an 8mm diameter spot of EPEC or EPEC $\Delta$ maA. The percentage of worms outside the spot was scored at each time point. **C.** Conditioning scheme. Conditioned exposure of *C. elegans* to EPEC lawns on LBT plates for 30 minutes (conditioning period), followed by exposure to OP50 on NGM plates for 3 hours (waiting period), and then exposure to EPEC on LBT plates for 3 hours (challenge). **D.** Conditioning of *C. elegans* using the scheme in C increases survival. **E.** A waiting period of 3 hrs induces optimal survival, and by 48 hours no conditioning was evident. **F.** Alternative conditioning scheme with avirulent strains of EPEC (EPEC $\Delta$ maA, EPEC $\Delta$ espF) or with EPEC grown in lawns on LB plates, which renders the bacteria unable to kill. An exposure time of 3 hours induces optimal survival. **G.** Exposure of *C. elegans* to avirulent EPEC strains or to EPEC grown in lawns on LB, to *Pseudomonas aeruginosa* strain PAK1, or to *C. rodentium* using the conditioning scheme in F. Survival of *C. elegans* conditioned on EPEC as in C is shown for comparison. **H.** EPEC or EPEC $\Delta$ maA were grown on filters on top of LBT agar overnight, the filters discarded, and the animals pre-exposed on the plates for 40 minutes for EPEC or 3 hours for EPEC $\Delta$ maA. Animals were then exposed to OP50 for 3 hours (waiting), and then to EPEC for 3 hours (challenge).



**Figure 2. Genes that promote longevity and innate immunity mediate conditioning**

**A.** *daf-16* and *hsf-1* mutants succumb more quickly than N2 upon constitutive exposure to EPEC, suggesting a protective role for these genes. **B.** *hsf-1(sy441)*, but not *daf-16* mutants, are as conditionable as N2 by EPEC $\Delta$ *tmaA*. *daf-16* mutants carrying an extrachromosomal array are partially rescued for conditioning. Mean survival  $\pm$  SEM are presented, and fold differences relative to the unconditioned control are indicated above the bar. For statistical analysis see C and D. **C.** Death rate during constitutive exposure to EPEC is not correlated with fold increase in conditioned survival. Death rates are plotted as a function of fold-increase in survival with conditioning. Fold conditioning was calculated as a quotient (percent survival with conditioning divided by the percent survival without), and death rates were estimated by regression for 30 mutants described in this study. Regression analysis for only N2, *rol-6(su1006)* and *daf-16*, and innate immunity mutants (small black dots) showed a strong negative correlation. Regression analysis for all mutants (small and large black dots) showed no correlation. **D.** Adjusted mean survival with conditioning for mutants shown in B using an ANCOVA model (see Methods). For this panel and G below, nonoverlap of 95% confidence intervals indicates statistical significance. No significant differences exist between control strains N2 or *rol-6(su1006)*, and *hsf-1(sy441)*. However, statistically significant differences are evident between N2 and all *daf-16* mutants, between the *daf-16* parental strains and strains rescued with the integrated array (I.A.) and the extrachromosomal array (Ex.A.). There was no difference between *daf-16* mutants and the

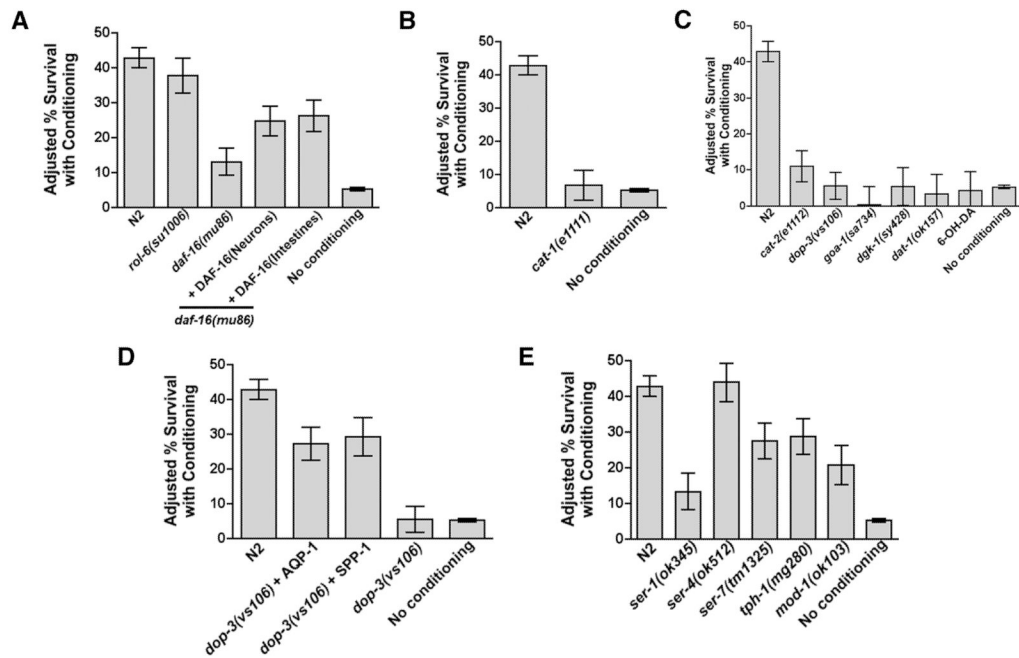
baseline unconditioned control, with the exception of *daf-16(mu86)*, which exhibited slight conditioning. **E.** Representative images showing nuclear translocation of DAF-16::GFP in N2 animals upon exposure to EPEC for 30 minutes or EPEC $\Delta$ *tmaA* for 3 hours, but not in unexposed animals. In some experiments, *C. elegans* were co-stained with DAPI to visualize nuclei. Note that GFP-fluorescence colocalizes with DAPI staining in images of *C. elegans* exposed to EPEC $\Delta$ *tmaA*. All images were acquired and fluorescence adjusted in an identical manner. Scale bar, 30  $\mu$ m. **F.** Time course of survival of *sek-1(km4)* and *pmk-1(km25)* animals following constitutive exposure to EPEC. **G.** Adjusted mean survival with conditioning upon pre-exposure to EPEC $\Delta$ *tmaA* for N2, *sek-1(km4)* and *pmk-1(km25)* animals .



**Figure 3. Genes regulated by DAF-16 and PMK-1 mediate protective responses to EPEC**

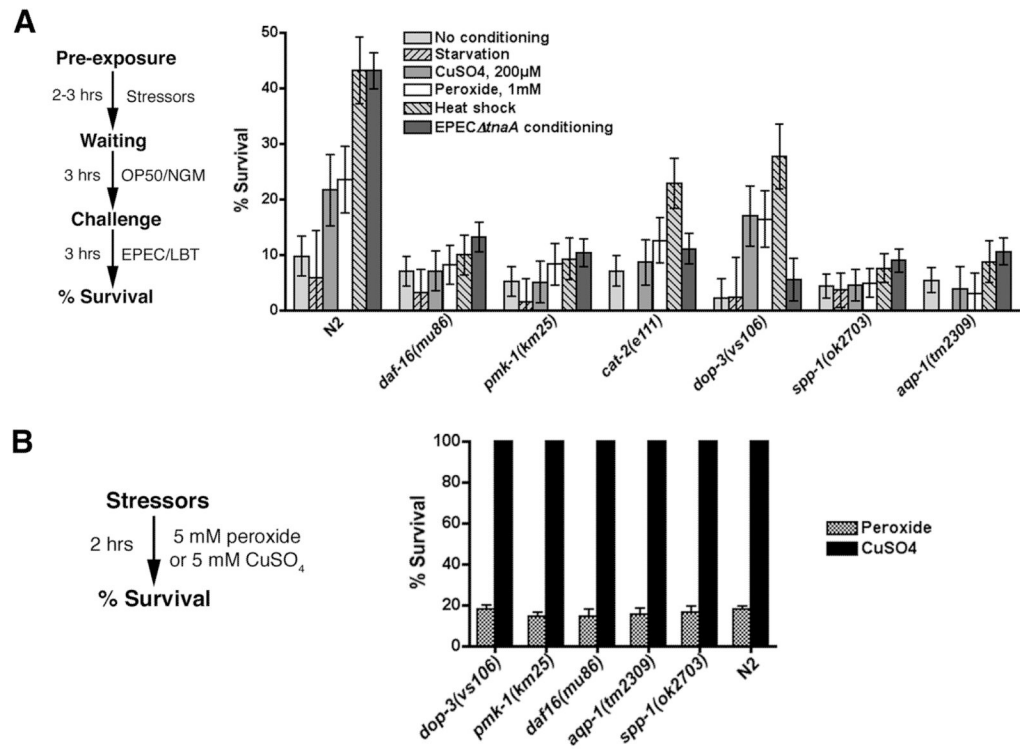
**A.** Inactivation of *spp-1* and *aqp-1* but not *lys-8* by RNAi suppress survival of *daf-2(e1370)* upon constitutive exposure to EPEC for 4 hrs. The differences between the control and *spp-1* ( $p < .001$ ) or *aqp-1* ( $p = .006$ ), but not *lys-8* ( $p = .50$ ) are statistically significant. **B.** Time course of survival upon exposure to EPEC of N2 animals, or N2 animals in which *spp-1*, *aqp-1*, or *lys-8* were inactivated by RNAi. **C.** Adjusted mean survival with conditioning for N2 animals, or N2 animals in which *spp-1*, *aqp-1*, or *lys-8* were inactivated by RNAi. **D.** Time course of survival of N2, *rol-6(su1006)*, *aqp-1(tm2309)*, *spp-1(ok2703)* and rescued strains following exposure to EPEC. **E.** Adjusted mean survival with conditioning by pre-exposure to EPEC $\Delta tnaA$  of N2, *rol-6(su1006)*, *aqp-1(tm2309)*, *spp-1(ok2703)* and rescued strains. **F.** Rescue of conditioned survival in *daf-16(mu86)* or *pmk-1(km25)* by extrachromosomal arrays of *aqp-1* or *spp-1*. **G.** Quantitative RTPCR of *aqp-1* or *spp-1* mRNA in N2, *dop-3(vs106)*, *daf-16(mu86)*, or *pmk-1(km25)* worms before and after conditioning. For each worm strain, the mRNA levels of *aqp-1* and *spp-1* before and after conditioning were normalized to those of *act-1*. Fold differences in the mRNA levels of *aqp-1* and *spp-1* were calculated relative to pre-exposure levels, which were arbitrarily set to a value of 100 for each (black bars).



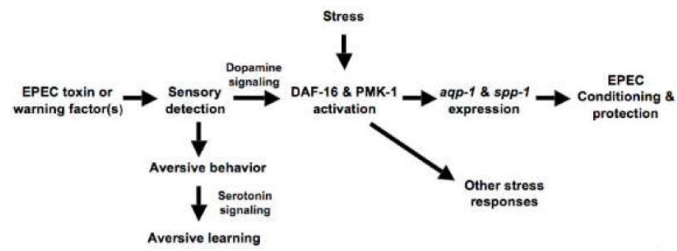


**Figure 4. Dopaminergic neuron signaling mediates conditioned survival from EPEC**

**A.** Adjusted mean survival with conditioning for N2, *rol-6(su1006)*, or *daf-16(mu86)* animals expressing DAF-16::GFP in neurons (*unc-119* promoter) or intestinal cells (*ges-1* promoter). **B.** Adjusted mean survival with conditioning of *cat-1(e1111)*. **C.** Dopaminergic signaling mutants or N2 animals treated with 6-OH-DA, which kills dopaminergic neurons, eliminates conditioned survival, except minimal conditioning of *cat-2(e1112)* was evident. **D.** Rescue of conditioned survival in *dop-3(vs106)* animals expressing *aqp-1* or *spp-1*. **E.** Serotonergic signaling mutants exhibit conditioned survival. *ser-1(ok345)* exhibited far less conditioning than other mutants, but baseline survival of this mutant were particularly low (see Supp Fig. 4I).



**Figure 5. Stressors condition *C. elegans* against EPEC by bypassing neuronal signaling**  
**A.** CuSO<sub>4</sub>, 1 mM H<sub>2</sub>O<sub>2</sub> (peroxide), and heat shock, but not starvation induce conditioned survival against EPEC in N2, *dop-3(vs106)*, and *cat-2(e1112)* animals, but not *pmk-1(km25)*, *daf-16(mu86)*, *aqp-1(tm2309)*, or *spp-1(ok2703)* animals. Conditioning schema is shown to the left. Bars in A indicate 95% confidence intervals derived from ANOVA analysis. **B.** *aqp-1(tm2309)*, *spp-1(ok2703)*, and *dop-3(vs106)* were as sensitive as N2 to 5 mM H<sub>2</sub>O<sub>2</sub>, and as resistant as N2 to heavy metals (e.g. 5 mM CuSO<sub>4</sub>). Bars in B represent standard errors of the mean.



**Figure 6. Model of *C. elegans* conditioning by virulent or avirulent EPEC strains**

Conditioning initiated by toxin or by factors on avirulent EPEC strains or other pathogens triggers a neuronal circuit mediated by dopaminergic neurons that activates DAF-16 and PMK-1 signaling. These mediators of longevity and innate immunity in turn activate *aqp-1* and *spp-1*, which protect *C. elegans*. Note that stressors such as heavy metals, oxidative stress, or heat shock, but not starvation, can also activate DAF-16 and PMK-1 in a manner that does not depend on neuronal signaling, and which protect *C. elegans* against both EPEC and other stressors. Likewise, although exposure to EPEC induces genes such as *aqp-1* and *spp-1* that are specifically required for protection against subsequent lethal exposure to EPEC, the response elicited is protective against a variety of stressors, including other pathogens.