Cell Host & Microbe
Article

Conditioning Protects *C. elegans* from Lethal Effects of Enteropathogenic *E. coli* by Activating Genes that Regulate Lifespan and Innate Immunity

Akwasi Anyanful,[1] Kirk A. Easley,[2] Guy M. Benian,[1] and Daniel Kalman[1,*]
[1]Department of Pathology and Laboratory Medicine
[2]Department of Biostatistics
Emory University, Atlanta, GA 30322, USA
*Correspondence: dkalman@emory.edu
DOI 10.1016/j.chom.2009.04.012

SUMMARY

Caenorhabditis elegans **exhibits 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 virulent or avirulent strains of the bacterial pathogen enteropathogenic *E. coli* (EPEC) "immunizes" *C. elegans* to survive a subsequent exposure that would otherwise prove lethal, a phenomenon we refer to as "conditioning." Conditioning requires dopaminergic neurons; 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 control innate immunity and lifespan may be regulated or "conditioned" by exposure to pathogens to allow survival in noxious environments.**

INTRODUCTION

In its natural habitat, the nematode *Caenorhabditis elegans* 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 (Darby et al., 1999; Tan et al., 1999a). Significantly, many virulence factors or toxins responsible for killing *C. elegans* also contribute to disease in plant and mammalian systems (Hendrickson et al., 2001; Tan et al., 1999b), suggesting conservation of virulence mechanisms among diverse eukaryotic hosts.

Studies of *C. elegans*-pathogen interactions indicate that nematodes have evolved behavioral mechanisms that facilitate survival. *C. elegans* can distinguish virulent from avirulent strains and undergo avoidance behavior (Zhang et al., 2005). *Serratia marcescens* produce a cyclic lipodepsipentapeptide, serrawettin W2, which is detected by AWB chemosensory neurons in *C. elegans* (Pradel et al., 2007) and causes animals to move away from bacteria. Moreover, *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 (5-HT) in the ADF sensory neurons and by 5-HT-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 receptor (IGFR)-1 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/IGFR-1 signaling pathway controls longevity and dauer formation in *C. elegans* (Kenyon et al., 1993). Activation of the IGFR homolog DAF-2 initiates a signaling cascade that negatively regulates the FOX-O family transcription factor DAF-16 (Ogg et al., 1997). DAF-16 upregulates expression of ~263 genes that promote longevity and downregulates expression of ~251 life-shortening genes (Hamilton et al., 2005; Murphy et al., 2003). Genes up- or downregulated 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 enterohemmorhagic *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* (Anyanful et al., 2005).

In contrast to the wealth of information on aversive behavioral responses to pathogens in *C. elegans* and on innate immune and

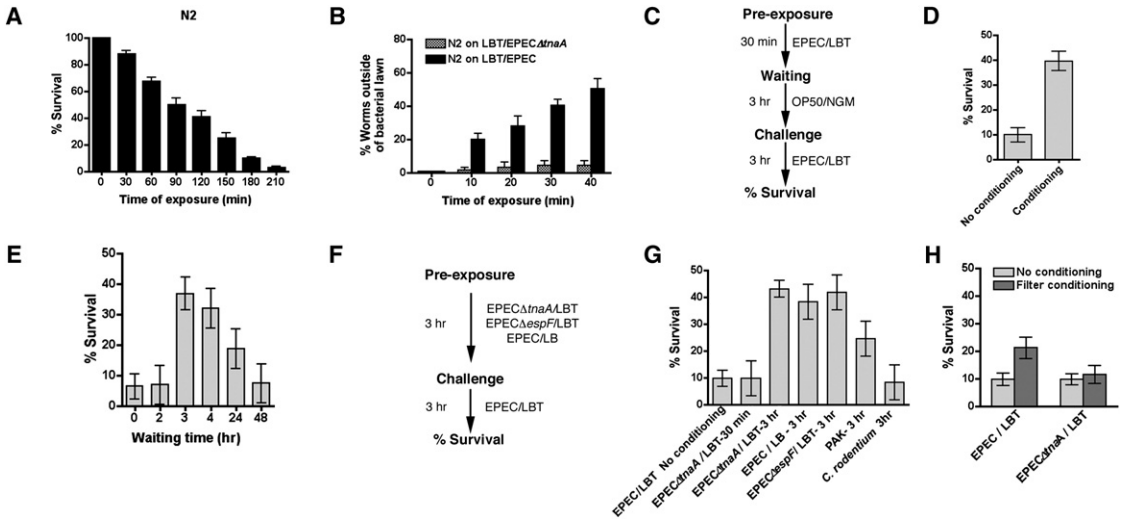

Figure 1. Conditioning of *C. elegans* by Pre-exposure to Virulent or Avirulent EPEC Strains

(A) Time course of killing of *C. elegans* by constitutive exposure to an EPEC lawn. The linear trend was highly significant ($p < 0.0001$).

(B) Time course of *C. elegans* movement away from an 8 mm diameter spot of EPEC or EPECΔ*tnaA*.

(C) Conditioning scheme.

(D) Conditioning of *C. elegans* using the scheme in C increases survival.

(E) A waiting period of 3 hr induces optimal survival, and by 48 hr no conditioning was evident.

(F) Alternative conditioning scheme with avirulent strains of EPEC or with EPEC grown on LB plates, which render the bacteria unable to kill.

(G) Exposure of *C. elegans* to avirulent EPEC strains, to EPEC grown on LB, to *P. aeruginosa* strain PAK1, or to *C. rodentium* using the conditioning scheme in (F).

(H) Effects of contact with EPEC or EPECΔ*tnaA* on conditioning. For (A) and (B), mean values ± 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.

aging genes that provide protection, information on how neuronal sensing of pathogens or behavior itself might regulate expression of protective genes is 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 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 min and subsequently death, scored as lack of movement and pharyngeal muscle activity (pumping) in worms 24 hr 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 wild-type worms (N2) within 3 hr (Figure 1A), whereas EPEC grown on *E. coli* direct (ECD) agar kills with the same efficacy but within ~8 hr (Anyanful et al., 2005). Killing upon exposure to EPEC/LBT was evident with similar kinetics using N2 strains from three sources (data not shown) and with *rol-6(su1006)* roller worms (Figure S1A).

When wild-type *C. elegans* (N2) were placed within a spot of EPEC (8 mm in diameter), approximately 50% of the animals exited the spot within 30 min, and those remaining became paralyzed and later died (Figure 1B). By contrast, such avoidance behavior was not evident with EPECΔ*tnaA* (Figure 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 (Figure 1C) so as to more precisely control the time and degree of exposure to EPEC. Briefly, N2 worms were incubated with EPEC on LBT agar for a brief pre-exposure period (30 min), moved to NGM plates containing OP50 for a 3 hr waiting period, and then challenged with EPEC on LBT plates for an additional 3 hr. The duration of the pre-exposure period (30 min) was chosen so as to maximize survival and the duration of the challenge period (3 hr) to maximize lethality and minimize variance (Figure 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 (Figure 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) (Figure S1B). 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 30 min proved less effective in promoting survival (Figure S1C). Likewise, waiting periods of less than 3 hr or more than 4 hr proved less effective (Figure 1E), and though increased survival was still evident after 24 hr, none was evident after 48 hr (Figure 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 EPECΔ*espF* or EPECΔ*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 hr, the longest time tested; data not shown). Likewise, EPECΔ*espF* and EPECΔ*tnaA* are isogenic strains that contain mutations in *espF* and *tnaA*, respectively, and are avirulent when grown on LBT even with extended exposures (up to 48 hr for EPECΔ*espF* or 96 hr for EPECΔ*tnaA*, the longest times tested; data not shown). The capacity to kill *C. elegans* can be restored to EPECΔ*espF* and EPECΔ*tnaA* by complementation with plasmid- or chromosomally encoded *espF* or *tnaA*, respectively. Pre-exposure neither to EPEC grown under avirulent conditions nor to the avirulent strains EPECΔ*espF* and EPECΔ*tnaA* for 30 min (the protocol in Figure 1C) induced a statistically significant increase in survival (Figure 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 EPECΔ*espF* or EPECΔ*tnaA* grown on LBT for an extended period (3 hr) (Figures 1F and 1G) induces survival to an extent similar to pre-exposure to EPEC grown on LBT for 30 min (4.3-fold for N2 and 5.4-fold for *rol-6(su1006)*) (Figures 1D and S1B). Pre-exposure to EPEC grown on LBT for 30 min and then to EPECΔ*espF*, EPECΔ*tnaA*, or 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 (data 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 EPECΔ*tnaA* overnight atop 0.2 μm nitrocellulose filters. After removal of the filters, the plates were used to pre-expose the animals for 40 min to EPEC or for 3 hr to EPECΔ*tnaA*. After a waiting period of 3 hr, animals were challenged for 3 hr 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 EPECΔ*tnaA* on filters. These data suggest that survival induced by pre-exposure to EPEC did not require direct contact, whereas that induced by EPECΔ*tnaA* did (Figure 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 suggest 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 *P. aeruginosa* strains (e.g., PA01, PA14 [Darby et al., 1999; Tan et al., 1999a] and PAK1 [Laws et al., 2006]) kill *C. elegans*. However, PAK1 produced no detectable deleterious effects in *C. elegans*, even over extended periods on LBT plates (96 hr, the longest time tested; data not shown). Pre-exposure to PAK1 for 3 hr followed by exposure to EPEC for 3 hr induced a 2.7-fold increase in survival compared to animals exposed to OP50/NGM for 3 hr (Figure 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 (Figure 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 (Figure 2A).

We next assessed whether *daf-16* mutants exhibited increased survival upon pre-exposure to EPEC or EPECΔ*tnaA*. 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 EPECΔ*tnaA* or to EPEC (1.6- to 1.8-fold; Figure 2B with EPECΔ*tnaA*; Figure S2A 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 (EPECΔ*espF*) or by EPEC grown under nonpathogenic conditions (LB medium) also required *daf-16* (data not shown). Because results with pre-exposure to EPEC and EPECΔ*tnaA* were nearly identical, only results with EPECΔ*tnaA* are shown. Data with EPEC are presented in Supplemental Data 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.) (Figures 2B and S2A). Notably, the *daf-16* mutants and their integrated complemented strains still succumbed faster than N2 (Figure 2A), though *daf-16(mu86)* animals expressing DAF-16 under its own promoter as an Ex.A. had a slightly slower death rate (Figure 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)*

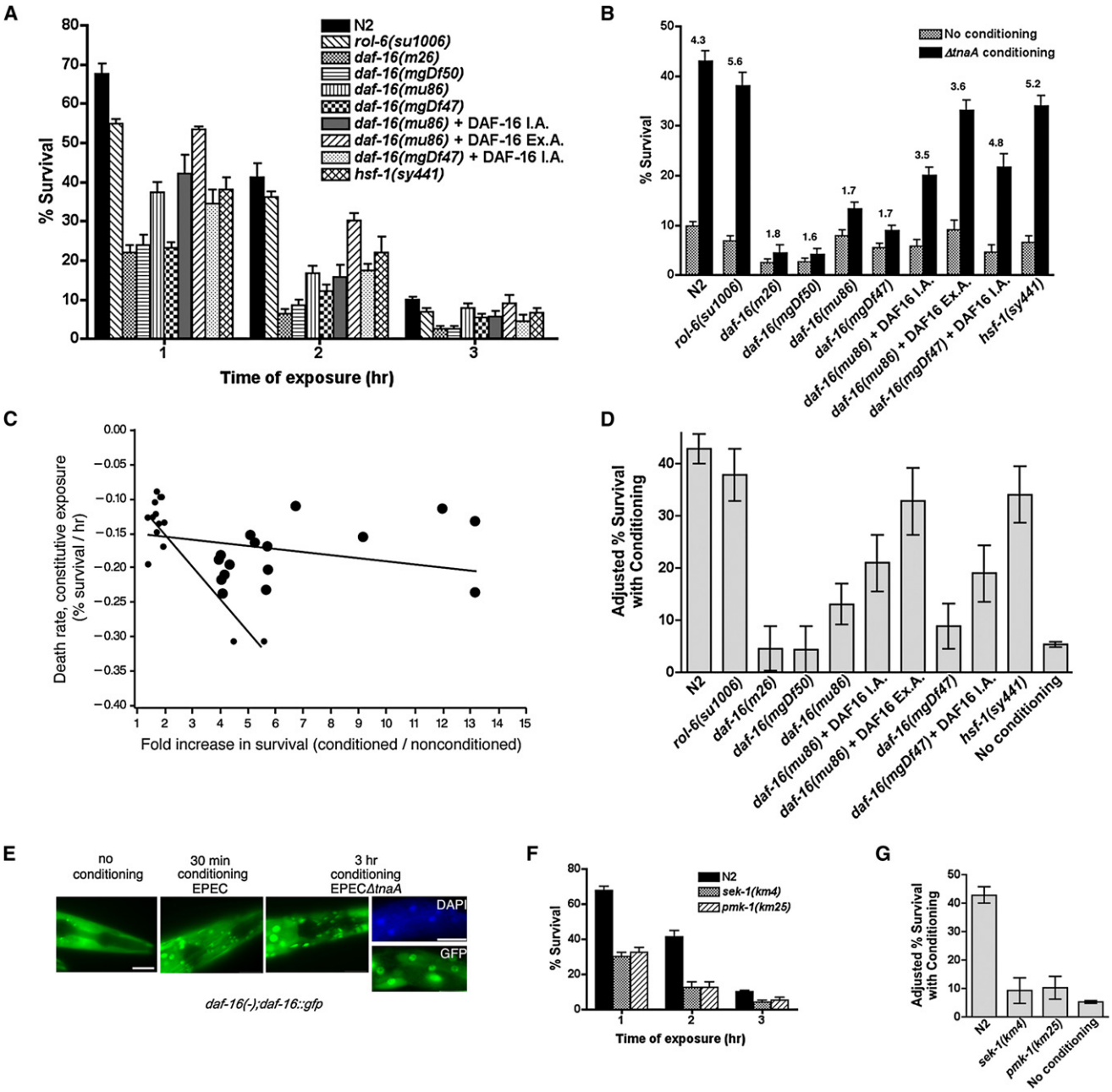

Figure 2. Genes that Promote Longevity and Innate Immunity Mediate Conditioning

(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) Effects of conditioning of *hsf-1(sy441)* and *daf-16* mutants by EPEC*ΔtnaA*. Mean survival ± SEM are shown, and fold differences relative to the unconditioned control are indicated.

(C) Death rate during constitutive exposure to EPEC is not correlated with fold increase in conditioned survival. Fold conditioning was calculated as a quotient (percent survival with conditioning / percent survival without). Death rates were estimated by regression for 30 mutants in this study. N2, *rol-6(su1006)*, daf-16, and innate immunity mutants only (small black dots) showed a strong negative correlation. No correlation was evident with all mutants (small and large black dots).

(D) Adjusted mean survival with conditioning for mutants in (B) using ANCOVA model. Nonoverlap of 95% confidence intervals indicates statistical significance.

(E) Representative images showing nuclear translocation of DAF-16::GFP in N2 animals upon exposure to EPEC for 30 min or EPEC*ΔtnaA* for 3 hr, but not in unexposed animals. Some animals were costained with DAPI to visualize nuclei. Scale, 30 μm.

(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*ΔtnaA* for strains in (F).

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 (Hajdu-Cronin et al., 2004) and die from internal hatching of young. We only used young adults, our experiments were completed within 7 hr, and no internal hatching was evident, suggesting that egg-laying defects play little if any role in phenotypes studied here. Both *hsf-1(RNAi)* (data not shown) and *hsf-1(sy441)* succumbed faster than N2 when exposed to EPEC, indicating a protective role (Figure 2A). However, *hsf-1 (sy441*) animals exhibited 5.2-fold more survival upon pre-exposure to EPECΔ*tnaA* compared to unconditioned animals, an increase comparable to that seen with N2 animals (Figures 2B and S2A 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 EPECΔ*tnaA*/LBT (as per protocol in Figure 1F) or EPEC (as per protocol in Figure 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 Figure 2C). However, when we considered these mutations, together with mutations in *hsf-1*, 5-HT signaling, and other genes (see below), we found a regression line with slope ~0 (small and large black dots in Figure 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, 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 Figures 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 Figures 4E and S4I). 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 Figure 2D and below for other mutants. For ease of comparing different mutants, we refer to the adjusted mean survival with pre-exposure as "conditioned survival" and present nonnormalized data with and without pre-exposure to EPEC or EPECΔ*tnaA* in the Supplemental Data.

The lack of overlap of confidence intervals indicates that all 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"; Figure 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 I.A. was not significantly different from the parental strain, *daf-16(mu86)*. However, conditioned survival of *daf-16(mu86)* rescued with an Ex.A. or *daf-16(mgDf47)* rescued with an I.A. 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 (Figure 2A). The observations that all four *daf-16* mutants 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 30 min or EPECΔ*tnaA* for 3 hr 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 min following exposure to EPEC and within 3 hr to EPECΔ*tnaA* (Figure 2E).

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 regulates 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 (Figure 2F) (Anyanful et al., 2005). Moreover, neither *sek-1(km4)* nor *pmk-1(km25)* animals exhibited as much conditioned survival as N2 upon pre-exposure to EPECΔ*tnaA* (Figures 2G and S2B) or EPEC (Figure S2C), and levels were similar those seen with *daf-16* mutations (Figure 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* 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 an RNAi 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 min, and greater than 60% survive after 3 hr (e.g., Figure 3A) (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 EPECΔ*tnaA*, no differences in survival were evident compared to *daf-2(e1370)* worms pre-exposed to OP50 (data not shown). To identify genes upregulated 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 4 hr exposure to EPEC (Figure 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 (data not shown). Inactivation of other DAF-16-inducible genes, such as *lys-8*, was without effect (Figure 3A).

To determine whether *spp-1* or *aqp-1* mediated conditioned survival in N2 animals, we fed N2 animals *E. coli* HT115 containing dsRNA for *spp-1*, *aqp-1*, or as controls, *lys-8* or the empty vector. Inactivation of *aqp-1* or *spp-1* in N2 animals increased the sensitivity to EPEC (Figure 3B), and knockdown of either *spp-1* or *aqp-1* resulted in no conditioned survival (Figures 3C and S3A). In accordance with RNAi results, the aquaporin mutant *aqp-1 (tm2309)* and the saposin mutant *spp-1(ok2703)* succumbed faster than N2 (Figure 3D), indicating a protective role, and *spp-1(ok2703)* was not conditionable, whereas *aqp-1(tm2309)* was only slightly conditionable relative to nonconditioned controls (Figure 3E). Rescue of *aqp-1(tm2309)* with wild-type *aqp-1* under its own promoter restored conditioned survival to levels comparable to those seen in N2 and *rol-6(su1006)*, and rescue of *spp-1(ok2703)* with *spp-1* partially restored conditioning (Figure 3E). Transgenic overexpression 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 (Figures 3F, S3C, and S3D). However, overexpression of *aqp-1* or *spp-1* in N2 did not significantly increase conditioned survival (data not shown), suggesting that an upper limit to conditioning exists using our protocols.

To determine whether pre-exposure induced changes in *aqp-1* or *spp-1* gene expression, we assessed mRNA levels. In N2 animals, but not in *daf-16(mu86)* or *pmk-1(km25)* animals, mRNA levels of *aqp-1* or *spp-1* increased ~1.5-fold upon pre-exposure to EPEC for 30 min followed by 3 hr waiting period on OP50/NGM (Figure 3G). The levels of *aqp-1* and *spp-1* were lower in conditioned compared to unconditioned *pmk-1(km25)* animals (Figure 3G), suggesting 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 in N2 (Figure S3E). 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

To determine how *C. elegans* detects bacterial factors that initiate conditioning, we first assessed whether *daf-16* expression in neurons or intestines was sufficient, using *daf-16(mu86)* worms containing DAF-16::GFP expressed in either neurons (with the *unc-119* promoter) or intestines (with 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 EPECΔ*tnaA* (Figures 4A and S4A). Similar results were obtained in *daf-16(mgDf47)* (data not shown). Thus, both neurons and intestinal cells mediate conditioned survival. Libina et al. (2003) reported that DAF-16 activity in neurons upregulates DAF-16 in specific responding tissues such as intestinal cells via insulin-like peptides. Thus, 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 (wormbase).

We have focused on how neuronal signaling regulates conditioning. To do this, we assessed conditioned survival in worm strains carrying mutations in the molecules that generate or regulate effects of serotonin (5-HT) or dopamine (DA), neurotransmitters implicated in responses to bacteria (Chase et al., 2004; Suo et al., 2003, 2004; Zhang et al., 2005). We first tested mutations in *cat-1*, which encodes a vesicular monoamine transporter that loads DA and 5-HT 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 (Figure S4B), but do not exhibit conditioning (Figures 4B and S4C). These data suggest that DA, 5-HT, or both mediate conditioning.

Dopaminergic Neurons, Not Serotonergic Neurons, Mediate Conditioning

To distinguish between serotonergic and dopaminergic mechanisms, we assessed conditioning in animals with defects in *cat-2*, which encodes tyrosine hydroxylase (Lints and Emmons, 1999), the rate-limiting enzyme in DA biosynthesis; and in *dop-1*, *dop-2*, and *dop-3*, which encode DA receptors (Chase et al., 2004; Sugiura et al., 2005; Suo et al., 2002, 2003, 2004). We also assessed conditioning in animals with mutations in *goa-1*, which encodes an ortholog of a heterotrimeric G protein α subunit; *dgk-1*, which encodes an ortholog of diacylglycerol kinase; and *dat-1*, which encodes a DA 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*

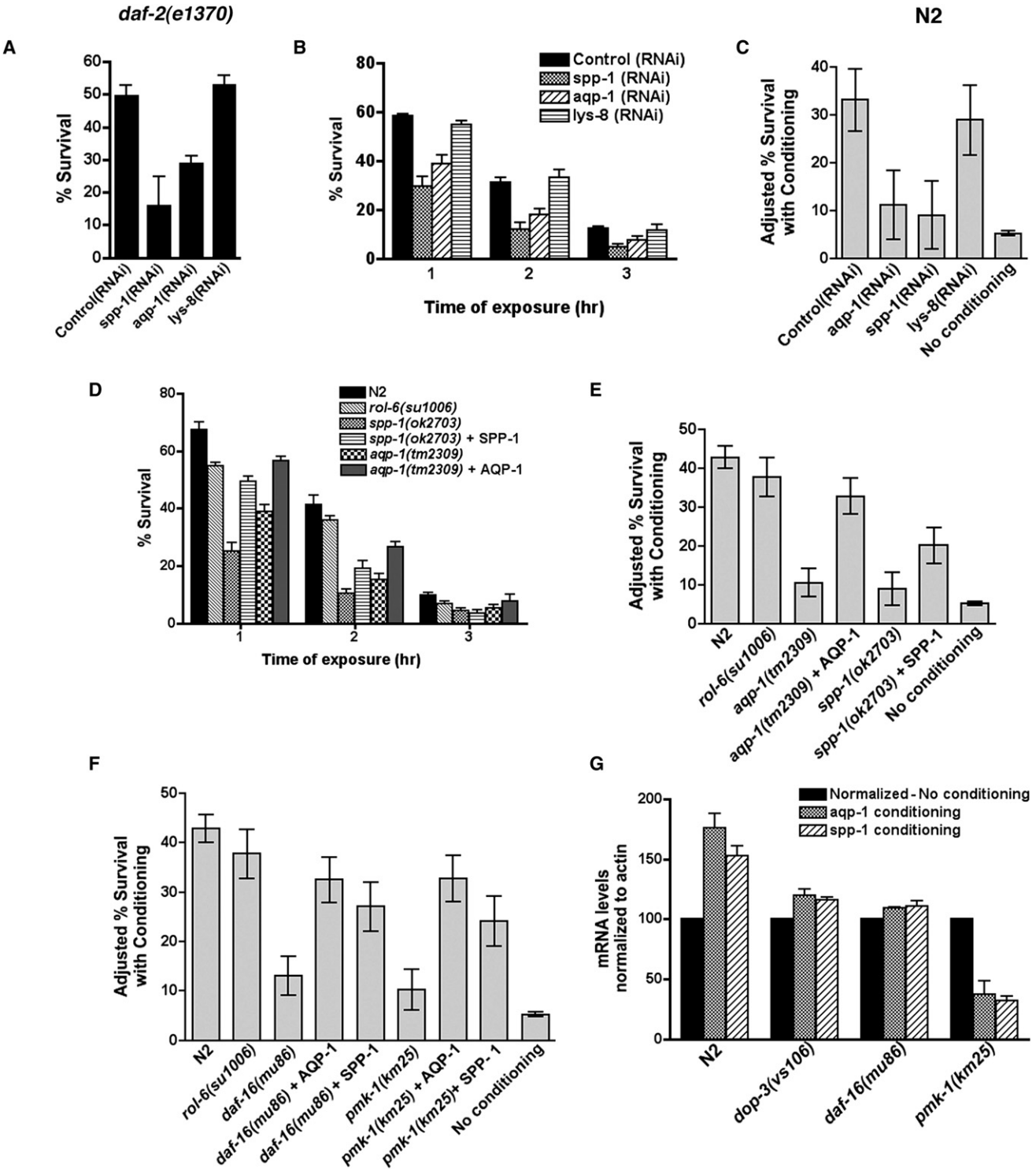

Figure 3. Selected Genes Regulated by DAF-16 and PMK-1 Mediate Conditioning

(A) Inactivation of *spp-1* and *aqp-1* but not *lys-8* by RNAi suppresses survival of *daf-2(e1370)* upon constitutive exposure to EPEC for 4 hr. The differences between the control and *spp-1* ($p < .001$) or *aqp-1* ($p = .006$), but not *lys-8* ($p = .50$), are significant.

(B) Effects of RNAi for genes in (A) on N2 animals upon exposure to EPEC.

(C) Effects of RNAi for genes in (A) on adjusted mean survival with conditioning of N2 animals by EPECΔ*tnaA*.

(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 EPECΔ*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 transgenic overexpression of *aqp-1* or *spp-1*.

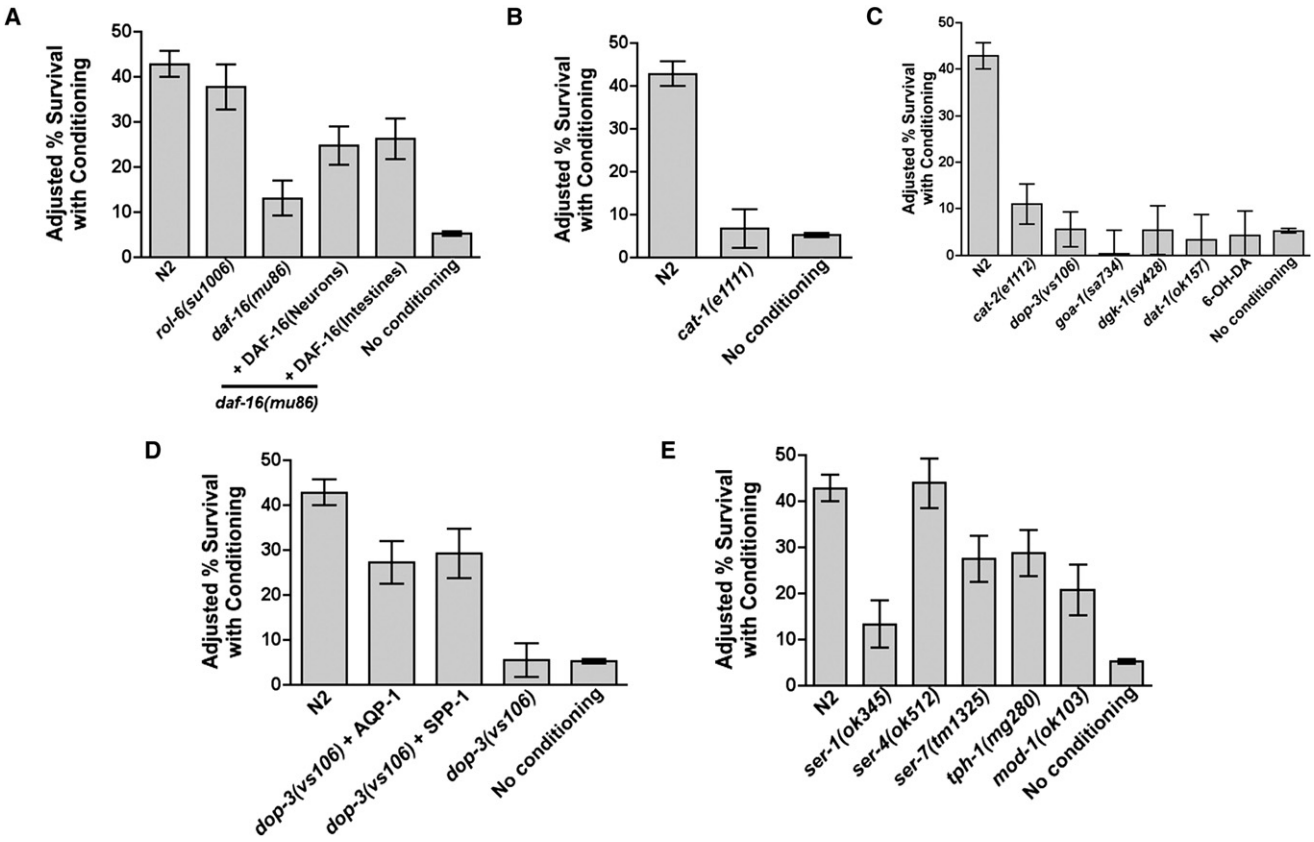

Figure 4. Dopaminergic Signaling Mediates Conditioned Survival

(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 eliminates conditioned survival, except minimal conditioning of *cat-2(e1112)* was evident.

(D) Rescue of conditioned survival in *dop-3(vs106)* animals overexpressing *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 was particularly low (Figure S4I).

(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., 2003, 2004).

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 (Figure S4D). Notably, conditioned survival by EPEC or by EPECΔ*tna* was significantly lower in *dop-3(vs106)* mutants compared to N2, and none was evident in any of the other DA signaling mutants (Figure 4C; Figure S4E). Additionally, *dop-1(vs100)* and *dop-2(vs105)* were only partially conditionable (data not shown). Overexpression 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 (Figures 4D, S4F, and S4G). To further verify a role for DA signaling in conditioning, we treated *C. elegans* with 6-hydroxy-DA (6-OH-DA), which selectively kills dopaminergic neurons (Nass et al., 2002). *C. elegans* previously exposed to 6-OH-DA were not conditionable (Figure 4C). Finally, we found that mRNA for *aqp-1* and *spp-1* were not induced in *dop-3 (vs106)* following exposure to EPEC (Figure 3G). These data provide evidence that DA 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 5-HT biosynthesis (Sze et al., 2000); in *mod-1*, which encodes a 5-HT-gated chloride channel (Ranganathan et al., 2000); and in three 5-HT 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 *P. aeruginosa* PA14 (Zhang et al., 2005). Similar to *hsf-1(sy441)*, all 5-HT mutants succumb faster than N2 in response to EPEC, indicating that 5-HT signaling is protective upon constitutive exposure to EPEC

(G) Quantitative RT-PCR 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).

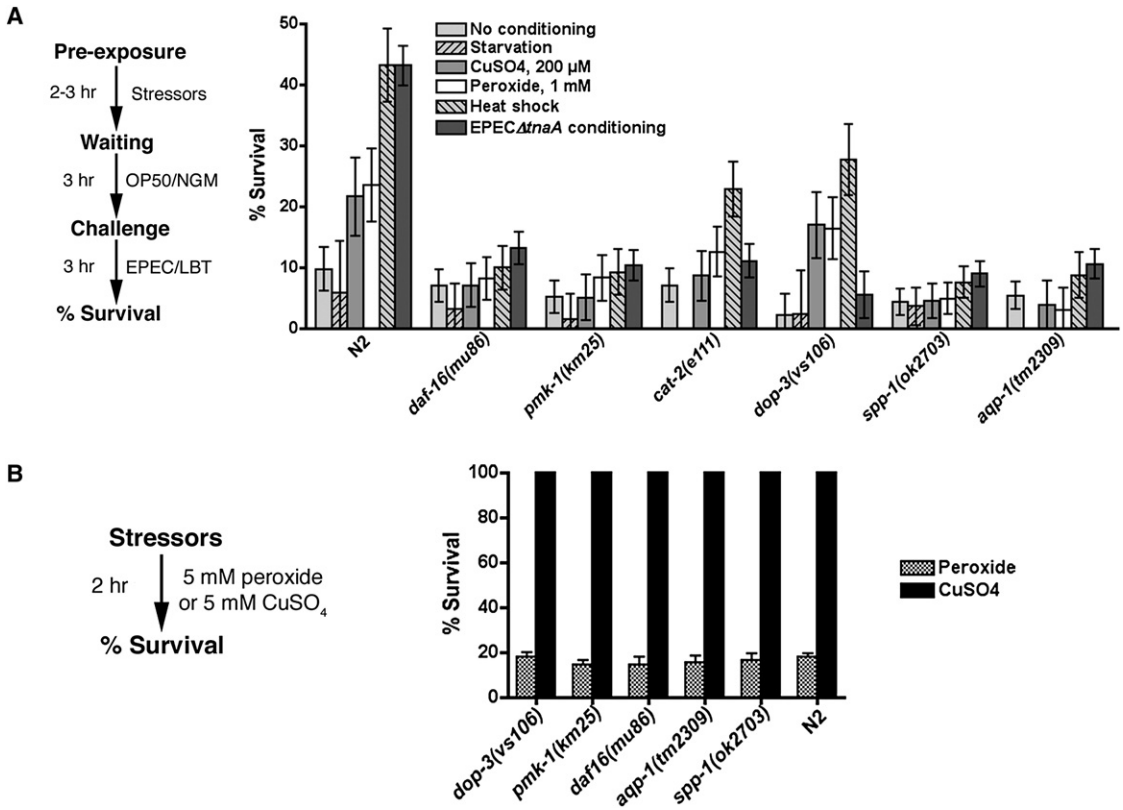

Figure 5. Stressors Condition *C. elegans* against EPEC by Bypassing Neuronal Signaling

(A) $CuSO_4$, H_2O_2 (1 mM), and heat shock, but not starvation, induce conditioned survival against EPEC in N2, *dop-3(vs106)*, and *cat-2(e1112)* animals, but not other animals. Conditioning schema is shown at left. Bars indicate 95% confidence intervals.

(B) *aqp-1(tm2309)*, *spp-1(ok2703)*, and *dop-3(vs106)* were as sensitive as N2 to 5 mM H_2O_2 and as resistant as N2 to 5 mM $CuSO_4$. Bars in (B) represent SEM.

(Figure S4H). 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 (Figure 4E). Notably, all the 5-HT 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 (Figure S4I). 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 conclude that 5-HT signaling is not required for conditioning (Figure S4I), and that some serotonergic genes mediate only aversive responses, while dopaminergic genes mediate both aversion 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 conditioned N2 or various mutants with stressors such as starvation, heat shock, heavy metals, or oxidative stress (H_2O_2) and then exposed animals to EPEC. 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 (Figure 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 (Figure S4D), and pre-exposure to EPEC or EPECΔ*tnaA* does not significantly increase conditioned survival (e.g., Figure 4C). However, pre-exposure to heat shock significantly increases survival of *dop-3(vs106)* and, to a lesser extent, *cat-2 (e1112)* (Figure 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 DA signaling. Moreover, *aqp-1(tm2309)*, *spp-1 (ok2703)*, and *dop-3(vs106)* were as sensitive as N2 to H_2O_2 and as resistant as N2 to heavy metals, at least under the conditions tested (Figure 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 DA signaling pathways.

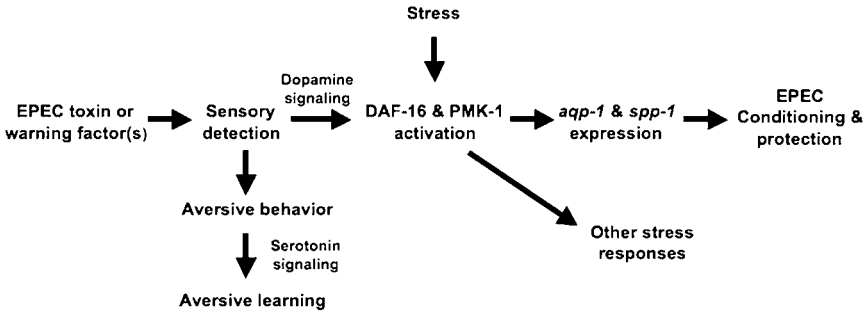

Figure 6. Model of *C. elegans* Conditioning by Virulent or Avirulent EPEC Strains

EPEC toxins or factors on avirulent EPEC strains or other pathogens trigger a neuronal circuit mediated by dopaminergic neurons that activates DAF-16 and PMK-1 signaling, which in turn activate *aqp-1* and *spp-1* to protect *C. elegans*. Stressors such as heavy metals, peroxide, or heat shock also activate DAF-16 and PMK-1 independent of neuronal signaling and protect *C. elegans* against both EPEC and other stressors. Exposure to EPEC induces *aqp-1* and *spp-1*, which are specifically required for survival against a subsequent lethal exposure to EPEC, but the response elicited is protective against a variety of stressors, including other pathogens.

DISCUSSION

Our observations suggest that *C. elegans* detect EPEC directly or indirectly via dopaminergic neurons. Once secreted, DA 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, lasts for approximately 24 hr, 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 Figure 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)* (Figure 5A). Thus, despite the sensitivity of these mutants, protective mechanisms can be activated by bypassing DA signaling. Second, no correlation was evident between susceptibility and fold conditioning (Figure 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 (Figure 2D). A similar result was obtained with the 5-HT mutants (Figures 4E and S4H).

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, EPECΔ*tnaA*, 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 IGFR-1/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 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 has only 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 other pathogens (e.g., *Pseudomonas*) and stressors (Figures 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 (Figure 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, 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 in intestinal cells and downregulation of some DAF-16 targets (*thn-2*, *spp-1*, and *lys-7*), but not others (*abf-2*, *lys-2*, and F08G5.6). These changes depend 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. 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 increased sensitivity of *daf-2(e1370)* RNAized worms upon EPEC exposure and no effect on conditioning. Together, these results suggest that particular bacterial factors, together with the timing or dynamics of its presentation to the worm, may affect 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 *Entoamoeba histolytica*, 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* also appears to confer protection by previously unrecognized means, because EPEC-mediated killing does not involve direct contact. Data presented here suggest that *aqp-1* and *spp-1* mediate conditioning, but understanding the roles of these genes at the molecular level will require a definition of the cellular basis for conditioning.

EXPERIMENTAL PROCEDURES

Killing and Conditioning Assays

Experiments were carried out with enteropathogenic *E. coli* serotype 0127:H6 strain E2348/69 and *E. coli* OP50 or various mutants as described (Anyanful et al., 2005). All *C. elegans* strains were maintained on Nematode Growth Medium (NGM) under standard culturing conditions with *E. coli* OP50 as food source (Sulston and Hodgkin, 1988). Strains are described in the Supplemental Data. All assays were performed at 25°C. Worm-killing assays were carried out essentially as described previously (Anyanful et al., 2005), though times of exposure and the media were altered slightly. Briefly, EPEC, EPECΔ*tnaA*, EPECΔ*espF*, PAK1, or *C. rodentium* was cultured in LB broth overnight to an OD_{600} of 0.8–1.0 and 170 μl spread on 6 cm LB agar (Fisher; Pittsburgh, PA) plates containing 2 mg/ml tryptophan (LBT plates). After incubation for 20 hr at 37°C, the plates were cooled for 1 hr in 25°C incubators. Young adult animals maintained at 20°C were transferred to each plate, and at least 180 worms per strain were tested for each experiment. For killing assays, worms were exposed to EPEC for 3 hr at 25°C before being transferred to OP50 on NGM plates. The EPEC lawn covered the 6 cm plate so that worms could not exit during the exposure period. After 24 hr, 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.

For conditioning assays, young adult worms were exposed to LBT/EPEC for 30 min (pre-exposure), transferred to OP50 on NGM plates for 3 hr (waiting period), and then again to fresh LBT/EPEC for 3 hr (challenge). Worms were transferred to OP50/NGM plates and scored after 24 hr. For nonpathogenic conditioning assays, worms were exposed for 3 hr to EPECΔ*tnaA*, EPECΔ*espF*, *P. aeruginosa* (PAK1), or *C. rodentium* on LBT plates or EPEC on LB plates before being transferred to LBT/EPEC plates for 3 hr and subsequent recovery on NGM/OP50 for 24 hr. For most of the experiments, EPECΔ*tnaA*/LBT was used. For experiments with 0.2 μm nitrocellulose filters (Figure 1H), we confirmed that bacteria did not penetrate the filters, because bacteria were never observed on sample plates cultured at 37° for 48 hr following removal of filters.

Stressor and Other Assays

Worms were subjected to heat shock at 32°C for 2 hr, exposed to 200 μM $CuSO_4$ for 2 hr, or exposed to 1 mM H_2O_2 or starvation conditions for 2 hr. Afterwards, the worms were transferred to NGM/OP50 for 3 hr before being exposed to EPEC/LBT for 3 hr. Worms were then transferred to NGM/OP50, and survival was assessed 24 hr later. For killing assays, worms were exposed to either 5 mM H_2O_2 or 5 mM $CuSO_4$ for 2 hr and then transferred to NGM/OP50 and assessed 24 hr later. Standard methodologies were used for DAF-16::GFP nuclear localization assays, real-time PCR, RNAi, 6-OH-DA experiments, and construction of transgenic worms. Detailed methods can be found in the Supplemental Data.

Statistical Analysis

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, 1H, and 5, survival was compared using ANOVA. The comparisons were highly significant ($p < 0.0001$). Ninety-five percent confidence intervals are shown for each. Fold increases in survival with conditioning were calculated as the quotient of the percent of animals surviving following procedure in Figure 1C (EPEC/LBT pre-exposure) or Figure 1F (EPECΔ*tnaA*/LB pre-exposure) divided by the percent surviving following procedure in Figure 1C (OP50/LB pre-exposure) or Figure 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 hr. The estimated rates of survival were then regressed against fold increase in survival with conditioning (Figure 2D). Linear regression of survival rate (the dependent variable) on fold change (the independent variable) for all mutants yielded the following relationship: Survival Rate = −.146 − (.00444 × Fold Change); Intercept = −.146 (SEM = .01729); slope = −.00444 (SEM = .00316). When comparing the estimated slope to zero using a t test, a value of $p = .17$ 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 = −.0573 − (.04686 × Fold Change); Intercept = −.0573 (SEM = .0207); slope = −.04686 (SEM = .0089); $p < .0001$ when comparing the estimated slope to zero using a t test; SD of the regression = .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.

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% ± 0.243% (mean ± SEM), and the slope estimate was 0.05585. For N2, the adjusted mean survival with conditioning, Y, was 0.42595 + .05585 × mean survival without conditioning: Y = .42595 + (.05585 × .05335) = .429 or 42.9%.

SUPPLEMENTAL DATA

Supplemental Data include Supplemental Experimental Procedures, Supplemental References, and four figures and can be found online at http://www.cell.com/cell-host-microbe/supplemental/S1931-3128(09)00140-1.

ACKNOWLEDGMENTS

The authors thank H. Lu, V. Brezina, D. Kalman, P. O'Lague, M. Sherman, and S. Bhatt for comments and discussion; A. Swimm for preparing figures; and C. McKenzie for assistance. Most worm strains were provided by the *Caenorhabditis* Genetics Center, which is supported by NIH. Supported by NIDDK R01DK074731 and NIAID R01AI056067 to D.K.

Received: September 3, 2008
Revised: December 18, 2008
Accepted: April 6, 2009
Published: May 20, 2009

REFERENCES

Alcedo, J., and Kenyon, C. (2004). Regulation of C. elegans longevity by specific gustatory and olfactory neurons. Neuron *41*, 45–55.

Alegado, R.A., and Tan, M.W. (2008). Resistance to antimicrobial peptides contributes to persistence of Salmonella typhimurium in the C. elegans intestine. Cell. Microbiol. *10*, 1259–1273.

Anyanful, A., Dolan-Livengood, J., Lewis, T., Sheth, S., DeZalia, M.N., Sherman, M., Kalman, L.V., Benian, G.M., and Kalman, D. (2005). Paralysis and killing of C. elegans by enteropathogenic E. coli requires the bacterial tryptophanase gene. Mol. Microbiol. *57*, 988–1007.

Apfeld, J., and Kenyon, C. (1999). Regulation of lifespan by sensory perception in Caenorhabditis elegans. Nature *402*, 804–809.

Banyai, L., and Patthy, L. (1998). Amoebapore homologs of Caenorhabditis elegans. Biochim. Biophys. Acta *1429*, 259–264.

Bargmann, C. I. (2006). Chemosensation in C. elegans. In WormBook, The *C. elegans* Research Community, ed. doi/10.1895/wormbook.1.123.1, http://www.wormbook.org.

Chase, D.L., Pepper, J.S., and Koelle, M.R. (2004). Mechanism of extrasynaptic dopamine signaling in Caenorhabditis elegans. Nat. Neurosci. *7*, 1096–1103.

Clarke, S.C., Haigh, R.D., Freestone, P.P., and Williams, P.H. (2002). Enteropathogenic Escherichia coli infection: history and clinical aspects. Br. J. Biomed. Sci. *59*, 123–127.

Darby, C., Cosma, C.L., Thomas, J.H., and Manoil, C. (1999). Lethal paralysis of Caenorhabditis elegans by Pseudomonas aeruginosa. Proc. Natl. Acad. Sci. USA *96*, 15202–15207.

Duerr, J.S., Frisby, D.L., Gaskin, J., Duke, A., Asermely, K., Huddleston, D., Eiden, L.E., and Rand, J.B. (1999). The cat-1 gene of Caenorhabditis elegans encodes a vesicular monoamine transporter required for specific monoamine-dependent behaviors. J. Neurosci. *19*, 72–84.

Evans, E.A., Kawli, T., and Tan, M.W. (2008). Pseudomonas aeruginosa suppresses host immunity by activating the DAF-2 insulin-like signaling pathway in Caenorhabditis elegans. PLoS Pathog. *4*, e1000175.

Frankel, G., Phillips, A.D., Rosenshine, I., Dougan, G., Kaper, J.B., and Knutton, S. (1998). Enteropathogenic and enterohaemorrhagic Escherichia coli: more subversive elements. Mol. Microbiol. *30*, 911–921.

Garsin, D.A., Villanueva, J.M., Begun, J., Kim, D.H., Sifri, C.D., Calderwood, S.B., Ruvkun, G., and Ausubel, F.M. (2003). Long-lived C. elegans daf-2 mutants are resistant to bacterial pathogens. Science *300*, 1921.

Hajdu-Cronin, Y.M., Chen, W.J., and Sternberg, P.W. (2004). The L-type cyclin CYL-1 and the heat-shock-factor HSF-1 are required for heat-shock-induced protein expression in Caenorhabditis elegans. Genetics *168*, 1937–1949.

Hamilton, B., Dong, Y., Shindo, M., Liu, W., Odell, I., Ruvkun, G., and Lee, S.S. (2005). A systematic RNAi screen for longevity genes in C. elegans. Genes Dev. *19*, 1544–1555.

Henderson, S.T., and Johnson, T.E. (2001). daf-16 integrates developmental and environmental inputs to mediate aging in the nematode Caenorhabditis elegans. Curr. Biol. *11*, 1975–1980.

Hendrickson, E.L., Plotnikova, J., Mahajan-Miklos, S., Rahme, L.G., and Ausubel, F.M. (2001). Differential roles of the Pseudomonas aeruginosa PA14 rpoN gene in pathogenicity in plants, nematodes, insects, and mice. J. Bacteriol. *183*, 7126–7134.

Hodgkin, J., Kuwabara, P.E., and Corneliussen, B. (2000). A novel bacterial pathogen, Microbacterium nematophilum, induces morphological change in the nematode C. elegans. Curr. Biol. *10*, 1615–1618.

Hsu, A.L., Murphy, C.T., and Kenyon, C. (2003). Regulation of aging and age-related disease by DAF-16 and heat-shock factor. Science *300*, 1142–1145.

Huang, C.G., Lamitina, T., Agre, P., and Strange, K. (2007). Functional analysis of the aquaporin gene family in Caenorhabditis elegans. Am. J. Physiol. Cell Physiol. *292*, C1867–C1873.

Kenyon, C. (2001). A conserved regulatory system for aging. Cell *105*, 165–168.

Kenyon, C. (2005). The plasticity of aging: insights from long-lived mutants. Cell *120*, 449–460.

Kenyon, C., Chang, J., Gensch, E., Rudner, A., and Tabtiang, R. (1993). A C. elegans mutant that lives twice as long as wild type. Nature *366*, 461–464.

Kim, D.H., Feinbaum, R., Alloing, G., Emerson, F.E., Garsin, D.A., Inoue, H., Tanaka-Hino, M., Hisamoto, N., Matsumoto, K., Tan, M.W., and Ausubel, F.M. (2002). A conserved p38 MAP kinase pathway in Caenorhabditis elegans innate immunity. Science *297*, 623–626.

Laws, T.R., Atkins, H.S., Atkins, T.P., and Titball, R.W. (2006). The pathogen Pseudomonas aeruginosa negatively affects the attraction response of the nematode Caenorhabditis elegans to bacteria. Microb. Pathog. *40*, 293–297.

Levine, M.M., Nataro, J.P., Karch, H., Baldini, M.M., Kaper, J.B., Black, R.E., Clements, M.L., and O'Brien, A.D. (1985). The diarrheal response of humans to some classic serotypes of enteropathogenic Escherichia coli is dependent on a plasmid encoding an enteroadhesiveness factor. J. Infect. Dis. *152*, 550–559.

Liang, B., Moussaif, M., Kuan, C.J., Gargus, J.J., and Sze, J.Y. (2006). Serotonin targets the DAF-16/FOXO signaling pathway to modulate stress responses. Cell Metab. *4*, 429–440.

Libina, N., Berman, J.R., and Kenyon, C. (2003). Tissue-specific activities of C. elegans DAF-16 in the regulation of lifespan. Cell *115*, 489–502.

Lin, K., Dorman, J.B., Rodan, A., and Kenyon, C. (1997). daf-16: An HNF-3/forkhead family member that can function to double the life-span of Caenorhabditis elegans. Science *278*, 1319–1322.

Lints, R., and Emmons, S.W. (1999). Patterning of dopaminergic neurotransmitter identity among Caenorhabditis elegans ray sensory neurons by a TGFbeta family signaling pathway and a Hox gene. Development *126*, 5819–5831.

Murphy, C.T., McCarroll, S.A., Bargmann, C.I., Fraser, A., Kamath, R.S., Ahringer, J., Li, H., and Kenyon, C. (2003). Genes that act downstream of DAF-16 to influence the lifespan of Caenorhabditis elegans. Nature *424*, 277–283.

Nass, R., Hall, D.H., Miller, D.M., 3rd, and Blakely, R.D. (2002). Neurotoxin-induced degeneration of dopamine neurons in Caenorhabditis elegans. Proc. Natl. Acad. Sci. USA *99*, 3264–3269.

Nass, R., Hahn, M.K., Jessen, T., McDonald, P.W., Carvelli, L., and Blakely, R.D. (2005). A genetic screen in Caenorhabditis elegans for dopamine neuron insensitivity to 6-hydroxydopamine identifies dopamine transporter

mutants impacting transporter biosynthesis and trafficking. J. Neurochem. *94*, 774–785.

Ogg, S., Paradis, S., Gottlieb, S., Patterson, G.I., Lee, L., Tissenbaum, H.A., and Ruvkun, G. (1997). The Fork head transcription factor DAF-16 transduces insulin-like metabolic and longevity signals in C. elegans. Nature *389*, 994–999.

Pradel, E., Zhang, Y., Pujol, N., Matsuyama, T., Bargmann, C.I., and Ewbank, J.J. (2007). Detection and avoidance of a natural product from the pathogenic bacterium Serratia marcescens by Caenorhabditis elegans. Proc. Natl. Acad. Sci. USA *104*, 2295–2300.

Ranganathan, R., Cannon, S.C., and Horvitz, H.R. (2000). MOD-1 is a serotonin-gated chloride channel that modulates locomotory behaviour in C. elegans. Nature *408*, 470–475.

Sawin, E.R., Ranganathan, R., and Horvitz, H.R. (2000). C. elegans locomotory rate is modulated by the environment through a dopaminergic pathway and by experience through a serotonergic pathway. Neuron *26*, 619–631.

Singh, V., and Aballay, A. (2006). Heat-shock transcription factor (HSF)-1 pathway required for Caenorhabditis elegans immunity. Proc. Natl. Acad. Sci. USA *103*, 13092–13097.

Sugiura, M., Fuke, S., Suo, S., Sasagawa, N., Van Tol, H.H., and Ishiura, S. (2005). Characterization of a novel D2-like dopamine receptor with a truncated splice variant and a D1-like dopamine receptor unique to invertebrates from Caenorhabditis elegans. J. Neurochem. *94*, 1146–1157.

Sulston, J., and Hodgkin, J. (1988). Methods. In The Nematode Caenorhabditis elegans, W.B. Wood, ed. (Cold Spring Harbor, NY: Cold Spring Harbor Laboratory Press), pp. 587–606.

Suo, S., Sasagawa, N., and Ishiura, S. (2002). Identification of a dopamine receptor from Caenorhabditis elegans. Neurosci. Lett. *319*, 13–16.

Suo, S., Sasagawa, N., and Ishiura, S. (2003). Cloning and characterization of a Caenorhabditis elegans D2-like dopamine receptor. J. Neurochem. *86*, 869–878.

Suo, S., Ishiura, S., and Van Tol, H.H. (2004). Dopamine receptors in C. elegans. Eur. J. Pharmacol. *500*, 159–166.

Sze, J.Y., Victor, M., Loer, C., Shi, Y., and Ruvkun, G. (2000). Food and metabolic signalling defects in a Caenorhabditis elegans serotonin-synthesis mutant. Nature *403*, 560–564.

Tan, M.-W., Mahajan-Miklos, S., and Ausubel, F.M. (1999a). Killing of Caenorhabditis elegans by Pseudomonas aeruginosa used to model mammalian bactreial pathogenesis. Proc. Natl. Acad. Sci. USA *96*, 715–720.

Tan, M.-W., Rahme, L.G., Sternberg, J.A., Tompkins, R.G., and Ausubel, F.M. (1999b). Pseudomonas aeruginosa killing of Caenorhabditis elegans used to identify P. aeruginosa virulence factors. Proc. Natl. Acad. Sci. USA *96*, 2408–2413.

Troemel, E.R., Chu, S.W., Reinke, V., Lee, S.S., Ausubel, F.M., and Kim, D.H. (2006). p38 MAPK regulates expression of immune response genes and contributes to longevity in C. elegans. PLoS Genet. *2*, e183.

Zhang, Y., Lu, H., and Bargmann, C.I. (2005). Pathogenic bacteria induce aversive olfactory learning in Caenorhabditis elegans. Nature *438*, 179–184.