

# Association with Soil Bacteria Enhances p38-Dependent Infection Resistance in *Caenorhabditis elegans*

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The importance of our inner microbial communities for proper immune responses against invading pathogens is now well accepted, but the mechanisms underlying this protection are largely unknown. In this study, we used *Caenorhabditis elegans* to investigate such mechanisms. Since very little is known about the microbes interacting with *C. elegans* in its natural environment, we began by taking the first steps to characterize the *C. elegans* microbiota. We established a natural-like environment in which initially germfree, wild-type larvae were grown on enriched soil. Bacterial members of the adult *C. elegans* microbiota were isolated by culture and identified using 16S rRNA gene sequencing. Using pure cultures of bacterial isolates as food, we identified two, *Bacillus megaterium* and *Pseudomonas mendocina*, that enhanced resistance to a subsequent infection with the Gram-negative pathogen *Pseudomonas aeruginosa*. Whereas protection by *B. megaterium* was linked to impaired egg laying, corresponding to a known trade-off between fecundity and resistance, the mechanism underlying protection conferred by *P. mendocina* depended on weak induction of immune genes regulated by the p38 MAPK pathway. Disruption of the p38 ortholog, *pmk-1*, abolished protection. *P. mendocina* enhanced resistance to *P. aeruginosa* but not to the Gram-positive pathogen *Enterococcus faecalis*. Furthermore, protection from *P. aeruginosa* was similarly induced by a *P. aeruginosa gacA* mutant with attenuated virulence but not by a different *C. elegans*-associated *Pseudomonas* sp. isolate. Our results support a pivotal role for the conserved p38 pathway in microbiota-initiated immune protection and suggest that similarity between microbiota members and pathogens may play a role in such protection.

The past decade has seen an explosion in the interest and in the understanding of the interactions between animals and their microbial communities. It is becoming increasingly clear that these large communities, which in the human gut include 10-fold more microbes than cells in the entire body, are strongly linked to healthy living. Gut microbes provide essential enzymes for digestion, contribute to proper intestinal development, and help control infection as well as immune responses (1–4).

By now, numerous studies have shown that density, composition, and complexity of the gut microbiota have strong effects on pathogen colonization, immune responses, and pathogen clearance (5, 6). Several mechanisms were shown to mediate this protection: direct inhibition of pathogens by members of the normal microbiota, competition for resources between the microbiota and invading pathogens, and stimulation of protective immune responses (reviewed in references 3 and 7). Of particular interest are mechanisms that suggest specificity in the ability of gut residents to prevent infection by certain pathogens, as these suggest a potential for pathogen-specific treatments. For example, commensal *Escherichia coli* strains were shown to effectively prevent gut colonization by strains with similar sugar utilization preferences (8); only a combination of such strains was able to prevent colonization by pathogenic O157:H7 *E. coli*. This suggests that the ability of commensals to prevent infection is dependent on niche overlap. Specificity is also demonstrated by the ability of particular bacterial classes to enhance immune responses to pathogens, against which responses are otherwise not strong enough to achieve clearance. For example, NOD1 stimulation by specific forms of peptidoglycan produced by members of the normal gut microbiota was shown to be essential for efficient activation of phagocytosis by neutrophils; addition of this peptidoglycan enhanced the killing of *Streptococcus pneumoniae* and *Staphylococcus aureus*, which typically express a peptidoglycan variant that is un-

able to induce their clearance (9). Similarly, secretion of the peptidoglycan-binding lectin RegIII $\gamma$  was shown to be induced downstream to TLR4 activation by normal-microbiota-derived lipopolysaccharide (LPS) and to be necessary for protection from Gram-positive vancomycin-resistant enterococci (10, 11). Such examples point to the important role of the composition of the microbiota in ensuring optimal protection from infection. Nevertheless, these examples probably represent only the tip of the iceberg, and additional modes through which commensals provide protection exist.

The model organism *Caenorhabditis elegans* is a bacterivorous soil nematode used extensively for studying host-pathogen interactions (reviewed in references 12–14). Even though very little is known about its natural pathogens or the interactions with natural populations of microbes (but see references 15 and 16), it has been useful in studying host-pathogen interactions involving various human pathogens. Such studies delineated a robust transcriptional immune response to the Gram-negative bacterium *Pseudomonas aeruginosa*, which is a prevalent constituent of soil and water environments but also a potent pathogen of both *C. elegans* and humans (17–19). The p38 mitogen-activated protein

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(MAP) kinase pathway plays an important role in inducing protective immune responses to *P. aeruginosa* infections (20, 21), similar to its pivotal roles in human innate immune responses (22).

Here, we describe the isolation of members of the *C. elegans* microbiota acquired in a natural-like environment of soil and rotting fruit. We found that growth on pure cultures of two isolates, *Bacillus megaterium* and *Pseudomonas mendocina*, conferred resistance to a subsequent infection with the pathogen *Pseudomonas aeruginosa*. Further, we found that the protection conferred by *P. mendocina* was associated with immune gene induction and depended on intact p38. This potentially suggests a new mode of infection protection provided by an organism's microbiota, namely, priming of p38-dependent immune protection.

## MATERIALS AND METHODS

**Strains.** *C. elegans* strains included Bristol N2 as the wild type, the *glp-4(bn2)* strain, and the *pmk-1(km25)* strain, all originally obtained from the *Caenorhabditis* Genetic Center (CGC). Bacterial strains used were *E. coli* OP50-1, obtained from the CGC, the Gram-positive pathogen *Enterococcus faecalis* (strain V583 [23]), the *P. aeruginosa* PA14 clinical isolate, and two derivatives of PA14, one expressing green fluorescent protein (GFP) and the other disrupted for *gacA* (17).

**Soils used.** Soil amended with compost (brewed in the lab according to standard practices; see <http://organicgardening.about.com/od/compost/ht/storagecompost.htm>) supported worm development to adulthood. Regular soils, such as that taken from a local oak knoll, were not able to support development and were therefore supplemented with either compost or rotting fruit, as described below. Experiments 1 and 2 used local soil (oak knoll) mixed with rotting pumpkins taken from a nearby farm; experiment 3 used rich garden soil; experiment 10 used local oak knoll soil supplemented with compost; experiment 14 used soil from a local apple orchard, including rotting apples; experiment 19 used rich garden soil supplemented with diced apples left to rot in soil for 2 weeks.

**Isolation of bacteria.** Worms were collected using a funnel lined with two layers of tissue paper, essentially as described elsewhere (24). To prevent worm death in the draining tube due to lack of oxygen, time of collection was limited to 2 h. Worms were collected into a 50-ml conical tube and subsequently transferred to 15-ml conical tubes in which they were washed six times in M9, leaving the worms suspended in volumes of 100 to 200  $\mu$ l in each wash, thus corresponding to dilution factors of 1:75 to 1:150, effectively removing cuticle-associated microbes. Separation of adult worms from undeveloped worms or progeny during these washes was based on gravity. Overall, worm harvesting took ca. 3 h, a time that should be sufficient for clearing bacteria not able to persist in the *C. elegans* intestine.

One hundred microliters of the last wash was spread on LB plates. Worms were ground using a motorized pestle in the remaining wash solution and similarly plated. Plates were incubated at room temperature for 1 day and overnight at 37°C, at the end of which dense microbial growth was apparent on plates with ground worms but no (or very few) colonies appeared on control plates with the last wash solution. Control experiments performed as described above but using soil to which *C. elegans* larvae were not added resulted in no or few colonies, similar to plates with the last wash solution. Worm-associated microbes were streaked from original plates to new plates to single colonies, and those were further streaked on separate plates, subsequently grown in liquid culture, and frozen.

**Bacterial colonization assay.** Bacteria specifically residing inside the worm gut were extracted following surface sterilization according to a modified version of a previously published protocol (25). Briefly, worms were washed off plates with M9, washed once to remove excess bacteria,

and plated on NGM plates supplemented with 100  $\mu$ g/ml gentamicin, on which worms were left to move about for an hour. Worms were then washed off plates in a solution containing 25 mM levamisole to contract shut their intestine and washed two more times (30 min at room temperature each) with M9 containing levamisole and gentamicin. Subsequently, worms were washed twice with M9 alone and ground using a motorized pestle, and debris, including released bacteria, was spread on LB plates to grow colonies; an equal volume of the last wash was similarly plated to verify killing of external bacteria.

**Identification of environmental isolates.** Initial identification was achieved using PCR amplification and sequencing of 16S rRNA genes. Conclusive identification of *Pseudomonas mendocina* and *Bacillus megaterium* was achieved by amplifying and sequencing *acsA*, *glyS*, *Rho*, and *aspA* for the former and *groEL*, *glyS*, and *dbpA* for the latter (see the primers described below).

**Egg laying measurements.** L4 worms were singled onto individual plates, and their progeny, both eggs and hatchlings, were counted (and removed) every 12 h.

**RNA extraction and quantitative reverse transcription-PCR (qRT-PCR).** RNA was extracted from worms using the TRIzol reagent (Invitrogen) from 100 to 200 worms per sample and treated with Turbo DNase (Ambion). RNA level measurements were normalized to actin and presented as fold changes over the levels of the appropriate control samples. Whenever shown, statistical significance was assessed with two-way analysis of variance (ANOVA) based on actin-normalized values.

**Primers.** Primers for identifying bacteria are as follows: 16S forward (27f), AGAGTTTGATCCTGGCTCAG, annealing at 55°C; 16S reverse (1492r), TACGGCTACCTTGTTACGACTT; *acsA* forward, ACCTGGTG TACGCCTCGCTGAC, at 52°C; *acsA* reverse, GACATAGATGCCCTGC CCCTTGAT; *glyS*-PM forward, GCATGTACTGCTCGTTCAGG, at 55°C; *glyS*-PM reverse, TTCAAGCAGGACAAGAAGCA; *Rho* forward, GTTACTTCGCCCTGCTCAAG, at 55°C; *Rho* reverse, GCATCTCGGTC ACTTCTTCC; *aspA* forward, ACGTGAACATGGCGCAGT, at 55°C; *aspA* reverse, GGCAGGTTGATCTCGTTGAT; *glyS*-BM forward, TTAC AGAAGAATTCAAGTCCATTT, at 57°C; *glyS*-BM reverse, AAAAATT CATTCAAGAATCTCCAT; *groEL* forward, TTGGACCAAAAAGTCTGTT AAC, at 57°C; *groEL* reverse, GTTACCAACGCGTCCATT; *dbpA* forward, TGTAGCTGCGAGAGGAATTG, at 57°C; *dbpA* reverse, CCGC TCTAATTTCTTTTCTTTTCTTTT. Primers for qRT-PCR are as follows: F08G5.6 forward, CACAATGATTTCAATGCGAGA, at 60°C; F08G5.6 reverse, GTTTCGACCGAGAAATCGAG; *lys-2* forward, CCAATATCAA GCTGGCAAGG, at 60°C; *lys-2* reverse, GTTGGATTGTTTGGCCAGTT; F55G11.2 forward, TGGAAGCTAGTCGTTCCAAT, at 60°C; F55G11.2 reverse, TGTCCTCCGTTCAAAGTTC.

**Survival analyses.** Survival analyses were performed in triplicate with approximately 100 worms per group per experiment. Life span analyses were performed on NGM plates unless otherwise mentioned; infection experiments were performed on slow-killing plates (17). All experiments were performed at 25°C unless otherwise mentioned. Statistical significance was assessed by Kaplan-Meier survival analysis followed by a log rank test. Growth on *B. megaterium* prior to infection experiments typically began at the L2 stage, as worm development on these bacteria from the egg stage to L2 was significantly delayed while development from L2 to L4 was largely normal.

**GFP images.** GFP images were acquired using a Leica MZ16F equipped with a blue filter set for Leica and a MicroPublisher 5.0 camera. Identical settings were used for all images.

**Electron microscopy.** Wild-type worms were grown on *E. coli* or *P. mendocina* to the L4 stage, collected, and fixated by freeze substitution using a 1% OsO<sub>4</sub> and 0.1% uranyl acetate solution (26, 27). Following embedding in Epon resin, thin longitudinal sections were cut, stained with uranyl acetate and lead citrate, and viewed using a Jeol 1200EX transmission electron microscope (EM) at 80 kV.

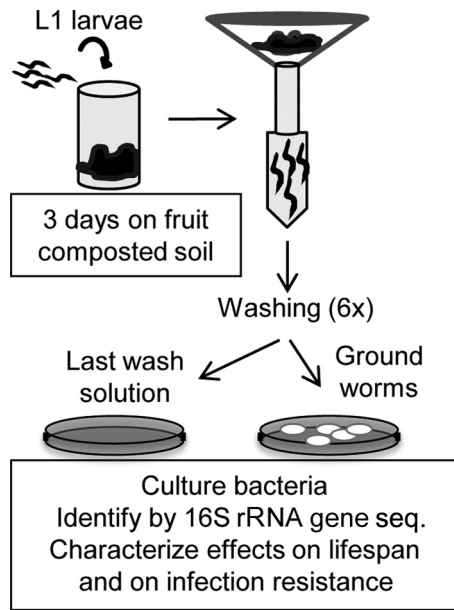


FIG 1 Isolation, identification, and characterization of *C. elegans* isolates. Worms used were of the lab N2 wild-type strain. Bacteria were identified from six separate experiments including together ca. 2,000 worms. Washes were performed in a volume of 15 ml, representing a dilution factor of ca. 1:100 for each wash. seq., sequencing.

## RESULTS

To facilitate characterization of the *C. elegans* microbiota in the lab, we reconstructed natural-like environments made of soil enriched with compost or rotting fruits, spanning a subrange of the habitats from which *C. elegans* has been isolated in the past (28). Sterile L1 larvae of the N2 wild-type lab strain were added to the different soils; following 3 days of growth at room temperature,

adult worms were harvested, washed extensively, and ground to release gut-residing bacteria, which were plated on LB agar and incubated at room temperature, followed by additional incubation at 37°C to facilitate microbial growth (Fig. 1). Only a few of these isolated microbes were fungi, judged by appearance (hyphae) and inability to amplify using primers specific for bacterial 16S rRNA genes.

Sequencing of bacterial 16S rRNA genes from isolated bacteria identified 17 species, some of which were isolated more than once in independent experiments (Table 1). These included several members of the *Firmicutes* phylum of Gram-positive bacteria, mostly *Bacillus* species but also the related *Paenibacillus*, *Lysinibacillus*, and *Staphylococcus* genera. Also well represented was the gammaproteobacteria genus *Pseudomonas*, of which four isolates were identified in two independent experiments. The identified bacteria were similar to bacteria previously reported to be associated with other rhabditid nematodes (29).

To begin characterizing the contribution of the *C. elegans*-associated microbiota to its host, we selected two representatives, one from each of the main two groups of identified bacteria, *Bacillus megaterium* and *Pseudomonas mendocina*. Multiple-gene-sequence-based identification showed them to represent new strains of the two species, named *C. elegans*-associated (CE)BM1 and CEP1, respectively (BM and PM, respectively, for short) (see Materials and Methods; see also Fig. S1 in the supplemental material). Worms grown on PM developed with the same time course as those grown on *E. coli*. On the other hand, growth with BM as food impeded early development, but past the L2 stage (reached with a 24-hour delay), BM was able to support a normal pace of development. This observation is in agreement with previous reports suggesting that *B. megaterium* is not an optimal food source for *C. elegans* due to its large size (30). In subsequent experiments assessing the effects of BM, worms were grown on *E. coli* until the L2 stage and transferred to BM for one additional day.

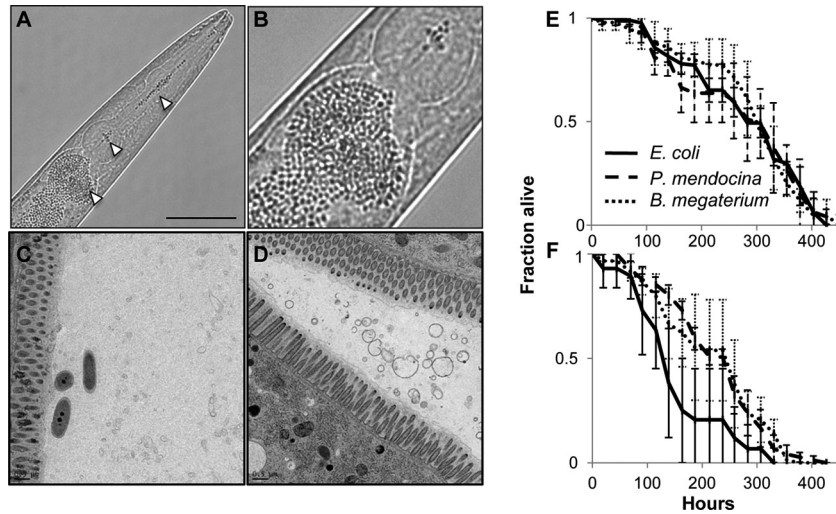
TABLE 1 Identified isolates

Top sequence hit(s) <sup>a</sup> (% identity)	Isolate(s) <sup>b</sup>
<i>Bacillus</i> sp. (99)	2.4, 2.5, 3.3 (90%), 19.3.9
<i>Bacillus megaterium</i> / <i>Bacillus</i> sp./ <i>Bacillus aryabhattai</i> / <i>Bacillus thuringiensis</i> (100)	<b>3.2</b>
<i>Bacillus foraminis</i> / <i>Bacillus asahii</i> (>97)	3.1
<i>Bacillus nealsonii</i> / <i>Bacillus circulans</i> (>98)	2.1
<i>Bacillus subtilis</i> (78*)	14.1
<i>Paenibacillus</i> sp./ <i>Paenibacillus lautus</i> / <i>Paenibacillus odorifer</i> (99)	3.7 (82%), 19.2.1, 19.1.4 (37°C)
<i>Lysinibacillus fusiformis</i> / <i>Lysinibacillus xylanilyticus</i> / <i>Lysinibacillus sphaericus</i> (99)	10.1, 10.3, 10.4, 10.6, 19.1.7
<i>Staphylococcus warneri</i> / <i>Staphylococcus pasteurii</i> (100)	2.6
<i>Bacillus</i> sp./ <i>Bacillus pumilus</i> (99)	14.3, 14.5
<i>Pseudomonas</i> sp./ <i>Pseudomonas oleovorans</i> / <i>Pseudomonas stutzeri</i> / <i>P. mendocina</i> / <i>Pseudomonas pseudoalcaligenes</i> / <i>P. aeruginosa</i> (99)	3.6
<i>P. aeruginosa</i> / <i>P. mendocina</i> / <i>Pseudomonas alcaligenes</i> (99)	2.2, <b>2.3</b>
<i>P. oleovorans</i> / <i>P. mendocina</i> / <i>P. pseudoalcaligenes</i> (100)	3.5
<i>Pseudomonas trivialis</i> / <i>Pseudomonas poae</i> (100)	19.1.4
<i>Pseudomonas fluorescens</i> / <i>Pseudomonas moraviensis</i> / <i>Pseudomonas koreensis</i> / <i>Pseudomonas putida</i> (99)	19.1.9, 19.3.5, 19.3.7, 19.1.8, 19.1.8, 19.3.12
<i>Arthrobacter</i> sp. (99)	3.4, 19.3.10
<i>Enterobacter</i> sp./ <i>Pasteurella aerogenes</i> (99)	19.3.1, 19.3.2
<i>Rahnella aquatilis</i> (>99)	19.3.3, 19.3.2 (37°C), 19.3.11
<i>Buttiauxella agrestis</i> / <i>Buttiauxella noackiae</i> (99)	19.3.8, 19.3.4

<sup>a</sup> Ambiguous identifications are marked as several possible species of the same genus; “sp.” represents a good hit to a yet-uncharacterized species, and “\*” represents weak similarity.

<sup>b</sup> Numbers indicate different isolates in independent experiments. See Materials and Methods for the soil used in each experiment. Isolates with lower similarity to species than that given in the first column are accompanied by the percent similarity in parentheses; isolates obtained by culturing at 37°C are marked. Boldface designates isolates selected for further investigation.





**FIG 2** Worms grown on *B. megaterium* or *P. mendocina* show internalized bacteria but a normal life span. (A) Young adult worm filled with *B. megaterium* spores (arrowheads), blown up in panel B; size bar, 50  $\mu\text{m}$ . (C and D) Electron micrographs of the intestine of worms grown on *P. mendocina* (C) or *E. coli* (D); size bar, 0.5  $\mu\text{m}$ . (E and F) Survival curves for worms grown on the designated bacteria. Curves present averages and standard deviations (SD) of 3 plates ( $n = 90$  to 93 worms for each group). (E) Growth on NGM plates (minimal medium); no significant differences. (F) Growth on LB plates (rich medium); worms grown on either *P. mendocina* or *B. megaterium* have a significantly longer life span than worms grown on *E. coli* ( $P < 0.0003$ , log rank test).

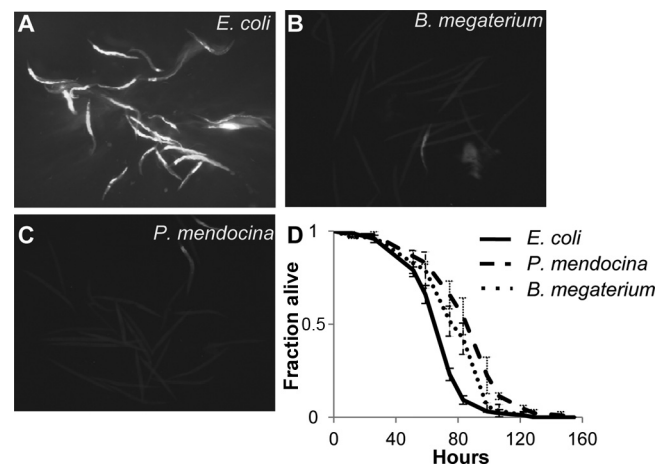
Light microscopy demonstrated accumulation of BM spores (appearing dark) in the worm intestine (Fig. 2A and B). This accumulation was not significantly persistent, as it was visibly cleared by defecation and disappeared within an hour following transfer to either *E. coli* or *P. aeruginosa* (but not when no other food was available). Presence of PM in the intestine was less apparent, but electron microscopy revealed it to be common (although in low numbers) (Fig. 2C). This finding was corroborated by experiments in which PM was cultured from worms following surface sterilization, demonstrating a prevalence of  $6.7 \pm 4.3$  CFU per worm, compared with  $1.2 \pm 0.2$  CFU/worm measured for *E. coli* (15 to 30 and 7 to 30 worms, respectively). EM analysis of worms grown on *E. coli* failed to show this sporadic presence of *E. coli* and revealed only bacterial “ghosts” inside the worm intestine, representing lysed bacteria (Fig. 2D).

The life span of worms grown on BM and PM under standard conditions (NGM plates, 25°C) was comparable to that of worms grown on the typical *E. coli* food strain OP50-1 (Fig. 2E). Since *E. coli* is known to become pathogenic when grown on rich medium, such as LB, we tested whether similar life span analyses performed on LB would expose pathogenicity. As expected, worms fed with *E. coli* grown on LB plates showed a compromised life span; however, worms fed with either BM or PM were affected only slightly by the rich medium, supporting their lack of pathogenic capabilities (Fig. 2F).

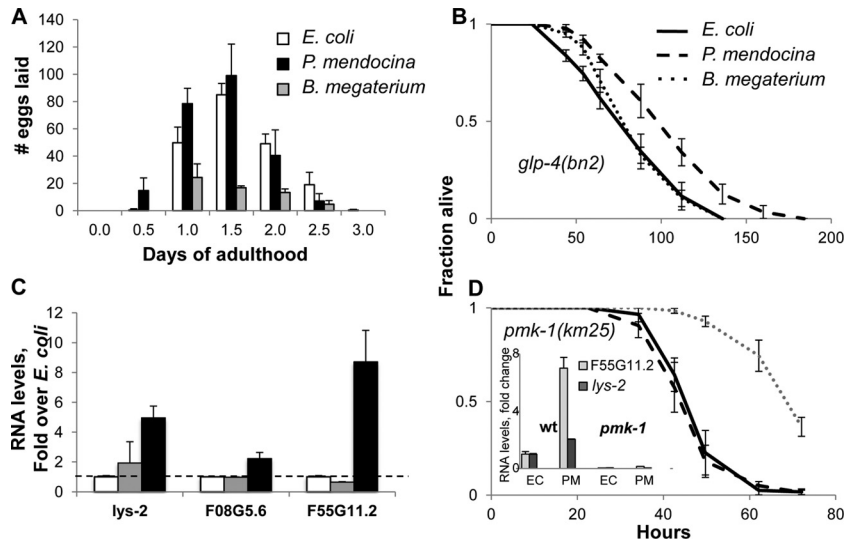
Infection of *C. elegans* in nature is likely to take place in the presence of its associated microbes, such as those identified here. We therefore examined the resistance of worms grown throughout their larval development on cultures of BM and PM to infection with the potent opportunistic pathogen *Pseudomonas aeruginosa* (strain PA14). Both were able to confer relative protection compared to worms initially grown on *E. coli* (Fig. 3). Protection was manifested as delayed colonization (Fig. 3B and C) as well as prolonged survival (Fig. 3D) and, in the case of PM, was conferred by as few as 4 h of exposure. An additional *C. elegans*-associated isolate, obtained through a separate set of experiments and iden-

tified by 16S rRNA gene sequences as *Acidovorax* sp. (99%), was unable to enhance resistance, suggesting that PM’s and BM’s ability to do so is not shared among all isolates (see Fig. S2 in the supplemental material). Interestingly, while PM enhanced resistance to the Gram-negative *P. aeruginosa*, it was unable to affect worm resistance to the Gram-positive pathogen *Enterococcus faecalis* (see Fig. S3 in the supplemental material).

Characterization of the modes through which BM and PM enhanced infection resistance demonstrated distinct mechanisms. Using BM as food not only delayed worm development but also



**FIG 3** Worm development in the presence of examined microbiota members enhances resistance to *P. aeruginosa* infection. (A to C) Representative fluorescent images showing accumulation of PA14 GFP in the intestine of worms grown initially on the designated bacteria; worms were exposed to the pathogen for 24 h starting at the L4 stage. (D) Survival curves on *P. aeruginosa* PA14; worms were grown until L4 on designated bacteria and subsequently exposed to the pathogen; both isolates conferred significant protection from PA14 compared to *E. coli* ( $n = 90$  to 97 worms;  $P < 0.0001$ , log rank test). Shown is a representative experiment out of 5 experiments with similar results.



**FIG 4** BM and PM increase infection resistance through distinct mechanisms. (A) Egg laying during early adulthood, shown for wild-type worms grown on the designated bacteria (averages and SD of 7 worms per group). One experiment out of two separate experiments with similar results is shown. (B) Survival curves of sterile *glp-4* mutants grown to L4 on designated bacteria and subsequently exposed to *P. aeruginosa* PA14 ( $n = 59$  to 91 worms per group;  $P$  [PM] < 0.0001,  $P$  [BM] = 0.65, log rank test). (C) qRT-PCR measurements of gene expression (genes designated on the x axis) in L4 worms fed with the designated bacteria, as in panel A. Shown are averages and SD of 2 experiments, each measured in duplicate. (D) Survival curves of *pmk-1(km25)* mutants grown to L4 on designated bacteria (marked as in panel B) and subsequently exposed to PA14 ( $n = 111$  to 155 worms per group). Infection was carried out at 20°C to improve resolution in the susceptible *pmk-1* strain; a similar lack of protective effects of PM was also observed at 25°C. (Inset) qRT-PCR measurements of immune gene expression in wild-type (wt) or *pmk-1* L4 larvae, shown as fold change compared to wt larvae grown on *E. coli* (EC). Shown is one experiment (averages and errors of duplicate measurements) out of 3 with similar results.

impaired adult reproduction, causing a 70 to 90% decrease in the total number of eggs laid compared to that by *E. coli*-fed worms (Fig. 4A). Since sterility is correlated with infection resistance (31), we tested whether enhanced infection resistance in BM-fed worms depended on the concomitant decrease in reproduction. To this end, we used *glp-4* mutants, which are sterile at 25°C. Unlike wild-type animals, BM-fed *glp-4* mutants were not more resistant to infection than *glp-4* animals grown on *E. coli*. In contrast, PM-fed *glp-4* animals presented enhanced infection resistance compared to their *E. coli*-fed siblings, similar to the trend observed in wild-type animals (Fig. 4B). This suggested that the ability of BM to enhance infection resistance was linked to its effects on reproduction, possibly as an indirect consequence of being a nonoptimal food source. On the other hand, the protection provided by PM was independent of reproduction.

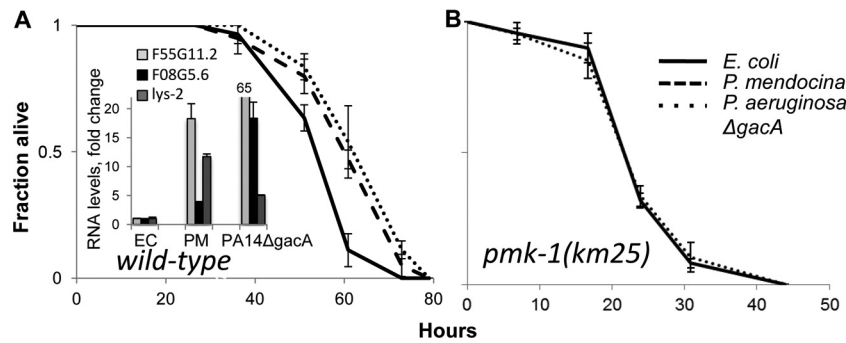
Focusing on immune gene expression downstream to PM exposure provided a clue regarding how PM may enhance infection resistance. RNA levels of three genes known to respond to PA14 infection—two of which provide immune protection and one (F55G11.2) with a yet-unknown function (18, 32)—were elevated prior to exposure to the PA14 pathogen in PM-fed worms compared to RNA levels in BM- or *E. coli*-fed animals (Fig. 3C). All three genes are known to be regulated by the p38 mitogen-activated protein kinase (MAPK) pathway (21, 33). This suggested that exposure to PM may have provided protection from a subsequent PA14 infection by priming protective, p38-dependent immune responses. To test this hypothesis, we examined worms disrupted for the p38 homolog gene *pmk-1*. Similar to wild-type animals, BM-fed *pmk-1* mutants were better protected from infection than their siblings grown on *E. coli*. However, the enhanced resistance conferred by growth on PM was lost in *pmk-1* mutants, concomitantly with impaired immune gene expression

(Fig. 4D). These results support the hypothesis that PM-initiated protection was *pmk-1* dependent.

What makes PM able to induce p38-dependent immune responses? One possibility is that similarity to the pathogen, both being of the same genus, triggered similar immune responses, thus providing heightened preparedness. If this is true, additional *Pseudomonas* species may similarly enhance resistance to *P. aeruginosa*. Furthermore, attenuated *Pseudomonas aeruginosa* mutants should be well suited to enhance resistance to the wild-type pathogen. We first tested isolate 19.3.7, which showed 99% and 98% similarity in the sequences of its 16S rRNA gene and *aspA* gene, respectively, to those of *Pseudomonas fluorescens* (Table 1 and results not shown). While supporting worm development and reproduction similarly to PM, this isolate did not enhance resistance to a subsequent infection with PA14 (see Fig. S4 in the supplemental material). In contrast, PA14 mutants disrupted for *gacA*, a master regulator of virulence genes, were able to enhance resistance to wild-type PA14 as effectively as PM (Fig. 5A) (34), and as with PM, this protection was *pmk-1* dependent (Fig. 5B). While these results do not corroborate the possibility that being a member of the *Pseudomonas* genus is sufficient to enhance resistance to the pathogenic *P. aeruginosa*, they demonstrate that similarity to the pathogen may enhance resistance in a p38-dependent manner.

## DISCUSSION

Although *C. elegans* has been used as a model organism for many years, including the past decade, in which it became a valuable system for studying host-pathogen interactions, very little is known about its interactions with microbes in its natural environment, as it is typically grown with *E. coli* as the sole source of food. It is likely that studying *C. elegans* in a more natural context would



**FIG 5** An attenuated *P. aeruginosa* mutant enhances resistance to the wild-type pathogen in a p38-dependent manner. (A) Survival curves for wild-type (wt) worms grown to L4 on *E. coli* (EC), PM, or a *P. aeruginosa* PA14  $\Delta$ *gacA* mutant, all subsequently exposed at 0 h to wild-type PA14. Shown are averages and SD of 3 plates ( $n = 80$  to 86 worms per group;  $P < 0.0001$  for both *P. mendocina* and PA14  $\Delta$ *gacA*). (Inset) qRT-PCR measurements of immune gene expression in wild-type animals grown to L4 on designated bacteria, shown as fold change (averages and errors of duplicate measurements) compared to animals grown on *E. coli* (EC). (B) Survival assays performed with *pmk-1(km25)* mutants; worm mutants were shifted to PA14  $\Delta$ *gacA* 6 h before transfer to wild-type PA14, an exposure time which is sufficient for  $\Delta$ *gacA* mutants to enhance protection in wild-type worms (not shown). Both panels show representative experiments of  $\geq 2$  experiments with similar results.

expose new aspects of its physiology. This is demonstrated in the study described here, showing a role for members of the *C. elegans* microbiota in enhancing its resistance to pathogens.

This study is an initial exploration of the natural *C. elegans*-associated microbiota. To facilitate isolation of microbiota members, we reconstructed in the lab natural-like environments representing several locales from which *C. elegans* has been isolated in the past. This allowed work with the genetically characterized wild-type strain and with a large number of worms. We isolated 17 bacterial species from worms, divided mostly between the *Bacillus* (as well as *Paenibacillus* and *Lysinibacillus*) and *Pseudomonas* genera. Identified bacteria most likely represent species that can persist in the worm gut, as was shown for BM and PM, but it is also possible that some may be strongly adhering cuticle-associated bacteria. Furthermore, it is likely that additional unidentified, nonculturable bacteria are part of this microbiota. Repeated isolation of members of the same genera and even of specific species in different experiments, in which soil came from different sources, suggests a more-than-coincidental association between *C. elegans* and these bacteria. That this composition may also represent the microbiota(s) of *C. elegans* in the wild is supported by previous isolation of similar bacteria from other rhabditid species in nature (29).

Two species representing the two main genera, *Bacillus megaterium* and *Pseudomonas mendocina*, both common soil inhabitants, were tested for their effects on infection resistance. When used as food during development, both enhanced worm resistance to infection with the potent pathogen *P. aeruginosa* compared to worms grown on *E. coli* without any discernible effect on life span. However, the mechanisms through which this protection was conferred differed between the two bacteria. For BM, enhanced resistance was linked to decreased reproduction, similar to the case of numerous mutants with compromised fecundity (31). For PM, enhanced resistance was independent of reproduction; instead, it was found to be associated with *pmk-1*-dependent immune gene expression and was abolished by *pmk-1* disruption. The results are in line with the hypothesis that PM primes the worm immune system, resulting in better preparedness to a pathogenic challenge. This was useful against the phylogenetically related *P. aeruginosa* PA14 but not against a much different Gram-

positive pathogen. What directs the selective protection is not clear, but an attractive hypothesis is that similarity between PM and PA14 is at the source. This hypothesis is supported by the ability of an attenuated PA14 mutant to enhance resistance to the wild-type PA14 but is weakened by the inability of a different *Pseudomonas* isolate to achieve this. It is possible that the latter species is just not similar enough to PA14, which may share relevant features with some members of the genus but not with others; however, it might be that a different mechanism altogether is responsible for PM's ability to prime p38-dependent resistance to PA14.

A recent study presents certain parallels between the contribution of PM to immune resistance and that of the human probiotic Gram-positive bacterium *Lactobacillus acidophilus* strain NCFM. Exposure to the latter enhanced resistance to two Gram-positive pathogens but not to Gram-negative pathogens (35). This occurred without substantial accumulation of the probiotic bacteria in the worm intestine. Resistance was associated with induction of genes which are part of the *C. elegans* immune response specifically against Gram-positive pathogens and was dependent on the p38 pathway. This report corroborates our results by suggesting that activation of the p38 pathway by microbes in its environment can initiate preemptive immune activation. That different microbes initiate different responses through activation of the p38 pathway suggests that signals upstream of this pathway determine its mode of activation and its downstream targets. What these signals are, whether they rely on recognition of microbes on the outside of the worm or the few microbes that find their way into the intestine, is yet unknown.

Stimulation of innate immunity by members of the intestinal microbiota, typically assessed by levels of antimicrobial peptides, has been previously observed in mammals and has been shown to limit intestinal infections (reviewed in reference 3). Such innate immune responses were shown to require various signaling modules, including TLR-MyD signaling or the extracellular signal-regulated kinase (ERK) MAP kinase pathway. However, the p38 pathway, otherwise a pivotal innate immune signaling pathway, was not found to date to take part in microbiota-dependent protective responses. Our experiments in *C. elegans* demonstrate a role for the p38 pathway in microbiota-induced protective im-



mune responses. As the p38 pathway is one of the more functionally conserved signaling modules in animals, it is likely that similar interactions occur in mammals.

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