Caenorhabditis elegans Immune Conditioning with the Probiotic Bacterium *Lactobacillus acidophilus* Strain NCFM Enhances Gram-Positive Immune Responses

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Although the immune response of *Caenorhabditis elegans* to microbial infections is well established, very little is known about the effects of health-promoting probiotic bacteria on evolutionarily conserved *C. elegans* host responses. We found that the probiotic Gram-positive bacterium *Lactobacillus acidophilus* NCFM is not harmful to *C. elegans* and that *L. acidophilus* NCFM is unable to colonize the *C. elegans* intestine. Conditioning with *L. acidophilus* NCFM significantly decreased the burden of a subsequent *Enterococcus faecalis* infection in the nematode intestine and prolonged the survival of nematodes exposed to pathogenic strains of *E. faecalis* and *Staphylococcus aureus*, including multidrug-resistant (MDR) isolates. Preexposure of nematodes to *Bacillus subtilis* did not provide any beneficial effects. Importantly, *L. acidophilus* NCFM activates key immune signaling pathways involved in *C. elegans* defenses against Gram-positive bacteria, including the p38 mitogen-activated protein kinase pathway (via TIR-1 and PMK-1) and the β-catenin signaling pathway (via BAR-1). Interestingly, conditioning with *L. acidophilus* NCFM had a minimal effect on Gram-negative infection with *Pseudomonas aeruginosa* or *Salmonella enterica* serovar Typhimurium and had no or a negative effect on defense genes associated with Gram-negative pathogens or general stress. In conclusion, we describe a new system for the study of probiotic immune agents and our findings demonstrate that probiotic conditioning with *L. acidophilus* NCFM modulates specific *C. elegans* immunity traits.

A number of groups have used the nematode *Caenorhabditis elegans* to identify and study evolutionarily preserved traits associated with host-pathogen interactions (36). In these *C. elegans* pathogenesis models, there is significant correlation between the microbial virulence traits required for mammalian infection and the killing of nematodes. In addition, recent studies have described certain evolutionarily conserved defense mechanisms in the nematode, including the insulin/insulin-like growth factor (IGF-1) pathway (28), the p38 mitogen-activated protein kinase (p38 MAPK) pathway (15), the transforming growth factor β (TGF-β) signaling pathway (48), and the β-catenin/HOX transcription factors (12). Therefore, *C. elegans* has been accepted as an alternative model host for the study of microbial infection and a simple model with which to study evolutionarily conserved aspects of innate immunity (21, 48).

Dietary resources, such as bacteria, play an important role in the control of the *C. elegans* life span (37), and nematodes exhibit a decreased life span when subjected to a diet of pathogens compared to that when given nonpathogenic laboratory food sources, such as auxotrophic strains of *Escherichia coli*. Interestingly, it is known that noxious exposure with bacterial pathogens changes *C. elegans* food preferences (47). In addition to this simple behavior, Anyanful and colleagues (3) showed that preexposure to some pathogenic bacteria can enhance protective responses of *C. elegans* to a subsequent infection of the pathogen via dopaminergic neurons that activate conserved signaling pathways, a phenomenon they refer to as "conditioning." However, the challenges with nonpathogenic or health-promoting bacteria and their molecular pathways involved in this phenomenon are poorly understood.

Probiotic bacteria provide functions that are of importance to the health and well-being of the host and contribute in a number of ways toward the functional improvement of foods (16, 45).

Interestingly, reports indicate that probiotic bacteria and several lactic acid bacteria (LAB) can enhance the host defense of *C. elegans* (11, 43). Here we report that the *Lactobacillus acidophilus* strain NCFM, one of the most widely recognized probiotics that is extensively used in the food and pharmaceutical industries (2), significantly enhances the host defense response of *C. elegans* to Gram-positive pathogens. The nematode response to conditioning with probiotic bacteria involves a number of previously described host immune responses and is mediated via the TIR-1, PMK-1, and BAR-1 signaling pathways.

MATERIALS AND METHODS

Nematode and pathogenic bacteria. *C. elegans* N2 Bristol wild-type, CF512 *fer-15(b26)II;fem-1(hc17)IV* (*fer-15;fem-1* worms), SS104 *glp-4(bn2)I,tir-1(qd2)* (*glp-4* worms), KU25 *pmk-1(km25)IV*, and EW15 *bar-1(ga80)X* strains were used in this study. *C. elegans* strains were routinely maintained on nematode growth medium (NGM) plates seeded with *E. coli* HB101 using standard procedures (5).

Bacillus subtilis ATCC 6633 (20) and *Salmonella enterica* serovar Typhimurium SL1344 (1) were cultured on Luria-Bertani (LB) medium at 37°C. *Enterococcus faecalis* MMH594 (27), *E. faecalis* V583 (vancomycin-

resistant [VRE] clinical isolate) (29), *Staphylococcus aureus* RN6390 (12), *S. aureus* MW2 (community-acquired [CA] methicillin-resistant [MRSA] isolate) (4), and *Pseudomonas aeruginosa* PA14 (15) were grown at 37°C using brain heart infusion (BHI; Difco, Detroit, MI) broth for the *E. faecalis* strains, tryptic soy broth (TSB; Difco) for the *S. aureus* strains, and King's broth for *P. aeruginosa* (38). For liquid killing assays, 50 μl of an overnight culture was spread onto 100 mm BHI agar (for *E. faecalis*), tryptic soy agar (TSA; for *S. aureus* assays), or NGM agar (for *S.* Typhimurium and *P. aeruginosa* assays) plates. The plates were incubated at 37°C for 18 h and cooled at room temperature (RT) for 1 h (*P. aeruginosa* for 24 h). For the solid killing assay, 10 μl of *E. faecalis* cultures was spotted in the middle of BHI agar plates (35 mm) including 80 μg/ml kanamycin, incubated at 37°C for 18 h, and cooled at room temperature for 1 h before beginning the experiment (35). For *S. aureus* (12), overnight cultures diluted 1:5 in fresh TSB were seeded on TSA plates (35 mm). In addition, *S.* Typhimurium (39) and *P. aeruginosa* (22) cultures were seeded on NGM agar plates (35 mm). The plates were incubated at 37°C for 18 h and then cooled at RT for 1 h (*P. aeruginosa* for 24 h) prior to use.

Preparation of conditioning plates with probiotic bacteria. To prepare NCFM conditioning plates, *L. acidophilus* NCFM bacteria (2) were grown in de Man, Rogosa, and Sharpe (MRS) medium (Difco, Detroit, MI) at 37°C for 18 h. Following five washes with M9 medium, 500 μl of each type of cells (ca. 2.0×10^9 CFU/ml) was spread on an NGM plate (100 mm) and dried for 3 h at room temperature. Conditioning plates were stored at 4°C and used within 1 week. *E. coli* strain HB101 and *Bacillus subtilis* ATCC 6633 were used as controls, and the same protocols were followed.

To employ heat-killed (HK) cells of the *L. acidophilus* NCFM strain, overnight cultures were washed twice with sterile M9 medium and resuspended with M9 medium (ca. 2.0×10^9 CFU/ml). Then cells were exposed to 80°C for 60 min, washed with M9 medium, and deposited onto NGM plates. HK cells were plated on MRS to ensure that no viable cells remained.

C. elegans killing assays. Liquid and solid killing assays were performed using published methods, with slight modifications (5, 35). For the liquid killing assay, L4/young adult worms were placed on conditioning plates with *L. acidophilus* NCFM at 25°C for 24 h. After exposure to NCFM for 24 h, worms were moved onto lawns of the pathogen and plates were incubated at 25°C for 5 h. Of note is that, as expected, there were no *E. coli* HB101 bacteria present in the *C. elegans* gut after they were moved onto an NCFM conditioning plate (data not shown). Then infected worms were washed twice with M9 medium and transferred into wells of a six-well microtiter dish (we used 40 worms per well). Each well contained 2 ml of the liquid assay medium used in previous *C. elegans* assays (20% BHI and 80% M9) (27). For the solid killing assay, we used 30 worms per plate and worms were transferred to prepared pathogen plates after conditioning on *L. acidophilus* NCFM plates for 24 h. After worms were placed on the plates with the pathogen, they were incubated at 25°C and examined at 24-h intervals for 15 days for viability using a Nikon SMZ645 dissecting microscope. Worms were counted as alive or dead worms by gentle touching with a platinum wire.

Measuring the bacterial burden in the C. elegans intestinal tract. The numbers of bacterial cells in the worm intestines were determined according to the modified methods as described previously (8). After conditioning for 24 h, *C. elegans* animals were moved onto lawns of *E. faecalis* and incubated at 25°C for 5 h. Worms were washed twice with M9 medium and then transferred into wells of a six-well microtiter dish (we used 40 worms per well). Each worm was removed from the liquid killing plates at 0, 1, 3, and 5 days. After removing surface bacteria, nematodes were washed five times with M9 medium on a BHI plate including gentamicin (25 μg/ml) (8), and worms were placed in new sterile tubes containing M9 medium with 1% Triton X-100 and were mechanically disrupted by using a pestle (Kontes, Vineland, NJ). Persistence was checked by diluting cells by 10^0 to 10^7 via 10-fold serial dilution steps in 0.85% NaCl solution, which was plated on BHI agar containing 80 μg/ml kanamycin for *E.*

faecalis or modified MRS (pH 4.5) agar for the *L. acidophilus* NCFM strain (7). Plates were incubated at 37°C for 24 h. For microscopic observation, at 5 days into the assay, nematodes were quickly placed on a pad of 2% agarose in a drop of 10 mM NaN₃ in M9 medium. The worms were examined at ×100 magnification with microscopy.

Induction of the green fluorescent protein (GFP) fusion. To elucidate the transcriptional host responses [*clec-60*::*GFP* in the N2 wild type or *clec-60*::*GFP* in the *bar-1*(*ga80*) mutant], nematodes were conditioned on plates seeded with *L. acidophilus* NCFM. After 12 or 24 h, animals were mounted on glass slides with 2% agarose pads, anesthetized with 10 mM NaN₃, and quickly visualized using an AxioImager Z1 fluorescence microscope (Zeiss) with A-Plan ×10 magnification. Images were taken using an AxioCam HR camera and analyzed with AxioVision 4.6 (Zeiss) software.

RNA isolation and qRT-PCR. Total RNA from worms was quickly isolated following the protocol of the TRIzol reagent (Invitrogen) and purified using the RNeasy minikit (Qiagen) including an on-column DNase digestion with RNase-free DNase (Qiagen). After RNA isolation, 50 ng of total RNA was used for a quantitative real-time PCR (qRT-PCR) using the SuperScript III Platinum SYBR green one-step qRT-PCR kit (Invitrogen). qRT-PCR was performed using the CHROMO4 real-time PCR system (MJ Research, Inc., Waltham, MA). Primers were designed using Primer3Input Software (v0.4.0) and are listed in Table S1 in the supplemental material. Relative expression levels were calculated using the $2^{-\Delta\Delta CT}$ threshold cycle method (23). The control gene *snb-1* (12) was used to normalize the genes' expression data.

Statistical analysis. *C. elegans* survival was examined by using the Kaplan-Meier method, and differences were determined by using the log-rank test (STATA6; STATA, College Station, TX). Differences in the bacterial numbers from CFU counting were determined by using the Student *t* test. Each experiment was performed with two different replicates. A *P* value of 0.05 in all replicate experiments was considered statistically significant.

RESULTS

Probiotic bacteria enhance the resistance of *C. elegans* to *E. faecalis* infection. First, we investigated whether the widely used probiotic bacterium *L. acidophilus* NCFM (2) influences the life span of *C. elegans*. As a control, we employed the Gram-positive bacterium *B. subtilis* strain ATCC 6633 (referred to as *B. subtilis* 6633), which is nonpathogenic to *C. elegans* (8). We found no significant difference in the viability of *C. elegans* exposed to *E. coli* HB101, *B. subtilis* 6633, and NCFM in liquid assays with *fer-15*; *fem-1* worms (data not shown) ($P = 0.6400$ for *B. subtilis* 6633 and $P = 0.2788$ for NCFM compared with worms feeding on HB101); hence, we concluded that NCFM is not harmful to *C. elegans*.

Next we explored if NCFM can augment the *C. elegans* defense response to *E. faecalis*. We used both liquid (27) and agar-based solid (35) killing assays. Worms were conditioned by transferring young adult worms to NCFM lawns for 24 h and then transferring them to *E. faecalis* using liquid and solid killing assays with *fer-15*; *fem-1* worms. Probiotic conditioning with NCFM for 24 h did not affect *C. elegans* physiological characteristics, including body and brood size, compared to those of worms given the *E. coli* HB101 control (data not shown). As shown in Fig. 1A, NCFM-conditioned worms clearly exhibited less susceptibility to *E. faecalis* infection in the liquid killing assay than control worms that were preexposed to the nonpathogenic strain *E. coli* HB101 (the standard laboratory food source for *C. elegans*) or the Gram-positive strain *B. subtilis* ATCC 6633 ($P < 0.0001$ and $P = 0.5762$ for NCFM and *B. subtilis* 6633, respectively, compared to HB101). Consistent with the liquid killing assay, the viability of *C. elegans* was significantly enhanced when nematodes were conditioned on
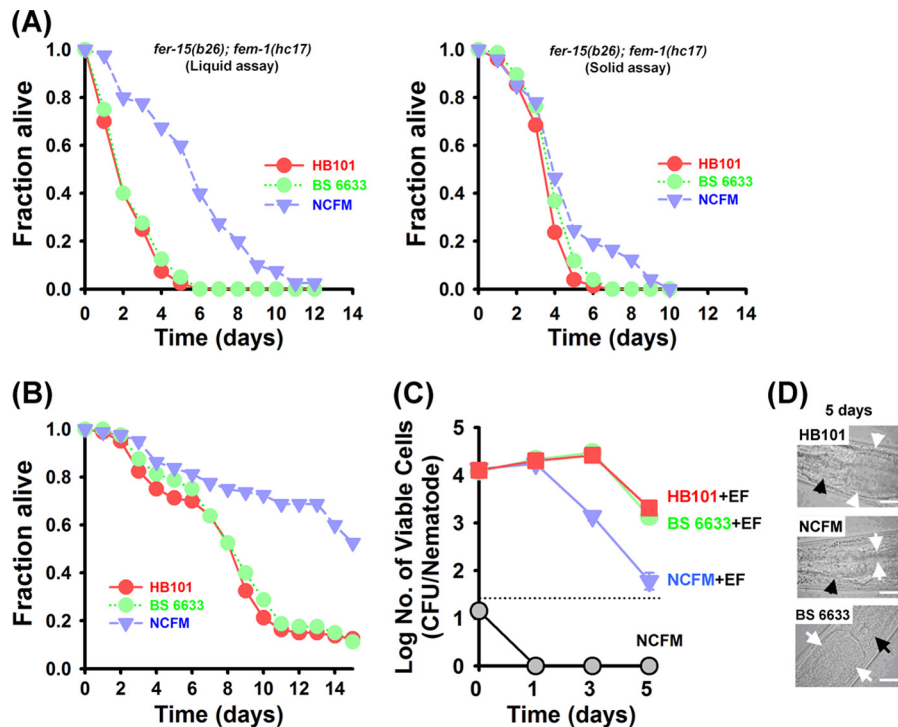
FIG 1 Immune conditioning with the probiotic bacterium *L. acidophilus* NCFM prolonged the survival of *C. elegans* nematodes infected with *E. faecalis* and decreased the enterococcal burden in the nematode intestine. (A) Liquid (left) (*n* = 40 per well) and solid (right) (*n* = 30 per plate) killing assays of *C. elegans* *fer-15;fem-1* worms infected with the *E. faecalis* MMH594 strain after conditioning with *L. acidophilus* NCFM or *B. subtilis* ATCC 6633 for 24 h. Survival statistics: liquid assays, *P* < 0.0001 and *P* = 0.5762 for NCFM and *B. subtilis* 6633, respectively, compared to worms preexposed to *E. coli* strain HB101; solid assays, *P* = 0.0200 and *P* = 0.0713 for NCFM and *B. subtilis* 6633, respectively, compared to worms preexposed to *E. coli* strain HB101. (B) Liquid killing assays of *C. elegans* (*n* = 40 per well) *glp-4* worms infected with the *E. faecalis* MMH594 strain after conditioning with *L. acidophilus* NCFM or *B. subtilis* ATCC 6633 for 24 h. Survival statistics: *P* < 0.0001 and *P* = 0.6629 for NCFM and *B. subtilis* 6633, respectively, compared to worms preexposed to *E. coli* strain HB101. These findings indicate that the conditioning effect of *L. acidophilus* NCFM was not specific to the strain of nematodes. (C) Studies evaluating colonization (numbers of CFU/nematode) of *L. acidophilus* NCFM (gray circles) in the nematode intestine. Also, we illustrate the bacterial burden (numbers of CFU/nematode) of *E. faecalis* in *fer-15;fem-1* nematodes after preexposure to *E. coli* HB101 (red squares) or conditioning with *L. acidophilus* NCFM (blue triangles) or *B. subtilis* ATCC 6633 (green circles). Conditioning with *L. acidophilus* NCFM significantly inhibited the concentration of *E. faecalis* in the nematode intestine at day 5. Each experiment was repeated with two independent biological replicates. Error bars show standard deviations. (D) Similar to our findings from the CFU assays, at day 5, the intestinal lumens of worms that had undergone conditioning with *L. acidophilus* NCFM were slim and without bacteria, while the intestinal lumens of control nematodes that were preexposed to *E. coli* HB101 or *B. subtilis* ATCC 6633 were distended by *E. faecalis* cells. White arrows indicate the borders of the intestinal lumen, and black arrows indicate the grinder organ. Scale bar, 25 μm.

lawns of NCFM and then exposed to *E. faecalis* infection in the solid killing conditions (*P* = 0.0002 and *P* = 0.0713 for NCFM- and *B. subtilis* 6633-conditioned nematodes, respectively, compared with worms feeding on HB101) (Fig. 1A). Thus, we concluded that conditioning with the probiotic strain NCFM makes *C. elegans* worms resistant to *E. faecalis* and that this beneficial effect was not associated with the preexposure of nematodes to other nonpathogenic bacteria (including the Gram-positive strain *B. subtilis* 6633).

Importantly, this effect was not specific to the strain of nematodes. For example, we employed the well-studied *C. elegans* *fer-15;fem-1* (28) and *glp-4* (18) mutants and found that conditioning of worms with NCFM prolonged nematode survival during *E. faecalis* infection in liquid assays (*P* < 0.0001 and *P* = 0.6629 for NCFM- and *B. subtilis* 6633-conditioned nematodes, respectively, compared with worms feeding on HB101) (Fig. 1B). Also, we performed studies using N2 wild-type nematodes that provided similar results in solid killing assays (data presented below). Unexpectedly, conditioning with heat-killed NCFM bacteria had no effect on resistance to *E. faecalis* (liquid or solid assays using *fer-15;fem-1* worms) (see Fig. S1 in the supplemental material).

Because *E. faecalis* colonizes the nematode intestinal tract, forming a persistent lethal infection (8, 27), we evaluated the possibility that live NCFM cells colonize the *C. elegans* intestine, thereby decreasing the number of pathogenic bacteria. In order to investigate this possibility, we evaluated the presence of *Lactobacillus* and *E. faecalis* cells in the nematode intestine using CFU studies and microscopic observation. Collaborating with the killing assays, worms were conditioned with NCFM and then exposed to *E. faecalis* infection. In order to remove surface bacteria, extensive washes were performed on BHI plates containing gentamicin as described previously (8). However, no probiotic bacterial cells could be detected in the nematode intestine during the course of the experiments. In the NCFM-conditioned nematodes, although there was no difference in the number of *E. faecalis* cells that could be recovered from worms 24 h postconditioning, we found significantly fewer *E. faecalis* cells in the worm intestine at days 3 and 5 after conditioning than in nonconditioned or *B. subtilis*-conditioned worms (Fig. 1C). As noted above, we verified these findings using direct microscopic observation. As expected based on the CFU results, worms that were conditioned with NCFM exhibited thin intestinal lumens following infection with *E. faecalis*, whereas

the intestinal lumens of control nematodes preexposed to *E. coli* HB101 or *B. subtilis* ATCC 6633 were distended by *E. faecalis* cells (Fig. 1D). These CFU and microscopy studies confirm previous reports that probiotic bacteria are highly sensitive to passing through the nematode grinder (11). Taken in their totality, these data indicate that probiotic bacteria are digested by the nematode and do not impact the initial colonization of nematodes by *E. faecalis*. However, conditioning with NCFM significantly inhibited the concentration of *E. faecalis* in the nematode intestine at 5 days.

Enhanced resistance of *C. elegans* by conditioning with *L. acidophilus* NCFM is limited to Gram-positive pathogens. In the previous sections, we have shown that conditioning with live probiotic bacteria significantly prolongs the survival of *C. elegans* nematodes during *E. faecalis* infection and that even though nematodes start with a similar number of pathogenic bacteria, conditioned nematodes are able to decrease the bacterial burden over the course of the infection. These findings raise the hypothesis that conditioning with probiotic bacteria may upregulate nematode host defenses.

Irazoqui and his collaborators found that nematode immune responses to the Gram-negative pathogen *Pseudomonas aeruginosa* and the Gram-positive pathogen *Staphylococcus aureus* are distinct (13). More recently, our group found that the nematode responses are also different during fungal infection in whole-transcriptome analysis (33). In order to evaluate the hypothesis that Gram-positive probiotic strains induce species-specific immune responses in *C. elegans*, we evaluated the impact of conditioning with NCFM on nematode infection with a variety of other Gram-positive and Gram-negative bacteria. We found that, similarly to *E. faecalis* infection, conditioning with NCFM significantly prolonged the survival of *C. elegans* exposed to another Gram-positive pathogen, *S. aureus*, in liquid killing assays with *fer-15;fem-1* worms ($P = 0.0102$ and $P = 0.7030$ for NCFM and *B. subtilis* 6633, respectively, compared to nematodes preexposed to *E. coli* HB101) (Fig. 2A). Similar results were obtained in solid killing assays with N2 wild-type worms (see Fig. 4D). As shown in Fig. 2B, NCFM also prolonged the survival of *fer-15;fem-1* animals infected by other enterococcal and staphylococcal strains, such as vancomycin-resistant *Enterococcus* (VRE) ($P = 0.0022$ and $P = 0.1916$ for NCFM and *B. subtilis* 6633, respectively, compared to nematodes preexposed to *E. coli* HB101) and methicillin-resistant *S. aureus* (MRSA) ($P = 0.0005$ and $P = 0.6534$ for NCFM and *B. subtilis* 6633, respectively, compared to nematodes preexposed to *E. coli* HB101) strains that are among the most commonly recognized multidrug-resistant (MDR) pathogens. However, as noted above, preexposure to the Gram-positive control *B. subtilis* had no rescuing effect on the survival of nematodes exposed to pathogenic bacteria (Fig. 2A and B).

On the other hand, conditioning with NCFM had no effect on survival following infection with the Gram-negative pathogen *P. aeruginosa* in either liquid (Fig. 2C) ($P = 0.0585$ and $P = 0.2320$ for NCFM and *B. subtilis* 6633, respectively, compared with worms preexposed to HB101) or solid (data not shown) ($P = 0.5761$ and $P = 0.8285$ for NCFM and *B. subtilis* 6633, respectively, compared with worms fed HB101) killing assays. In addition, when challenged with another Gram-negative bacterium, *S. enterica* serovar Typhimurium, similar to challenge with *P. aeruginosa*, conditioning with NCFM had no statistically significant effect on the survival of *C. elegans* in either liquid (Fig. 2C) ($P =$
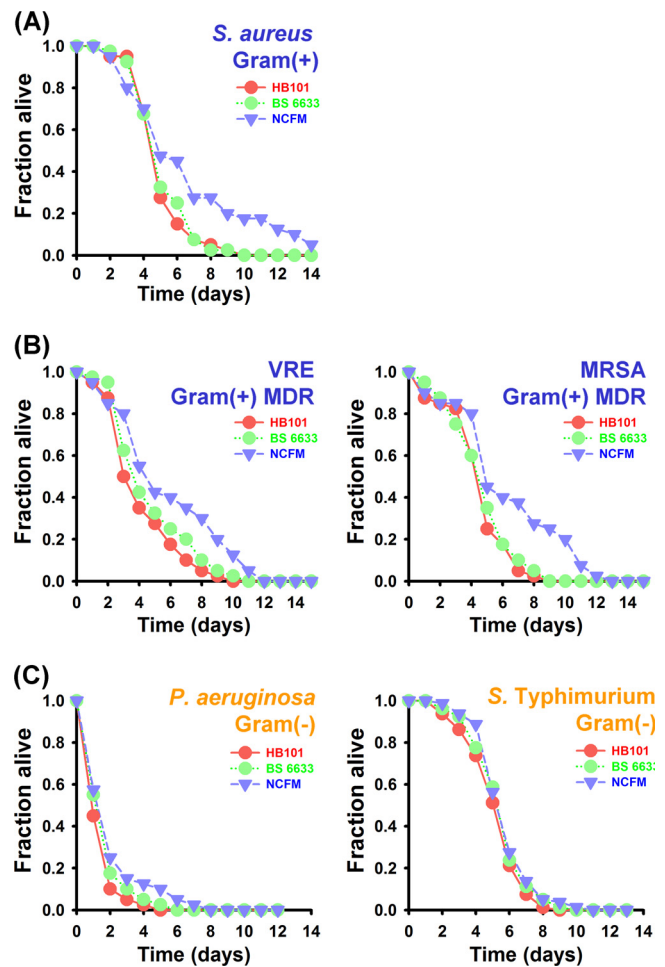

FIG 2 Conditioning with *L. acidophilus* NCFM selectively influences the resistance of *C. elegans* worms infected by the Gram-positive bacterium *S. aureus* but not the Gram-negative pathogens *P. aeruginosa* and *S.* Typhimurium. Liquid killing assays with *C. elegans fer-15;fem-1* worms ($n = 40$ per well) infected with *S. aureus* strain RN6390 (A), *E. faecalis* strain V583 (VRE strain) or *S. aureus* strain MW2 (MRSA strain) (B), or *P. aeruginosa* strain PA14 or *S.* Typhimurium strain SL1344 (C) following conditioning with *L. acidophilus* NCFM for 24 h. Survival statistics: *S. aureus* RN6390, $P = 0.0102$ and $P = 0.7030$ for NCFM and *B. subtilis* 6633, respectively, compared with control worms preexposed to *E. coli* strain HB101; *E. faecalis* V583, $P = 0.0022$ and $P = 0.1916$ for NCFM and *B. subtilis* 6633, respectively, compared with control worms preexposed to *E. coli* strain HB101; *S. aureus* MW2, $P = 0.0005$ and $P = 0.6534$ for NCFM and *B. subtilis* 6633, respectively, compared with control worms preexposed to *E. coli* strain HB101; *P. aeruginosa* PA14, $P = 0.0585$ and $P = 0.2320$ for NCFM and *B. subtilis* 6633, respectively, compared with worms feeding on HB101; and *S.* Typhimurium SL1344, $P = 0.0668$ and $P = 0.2217$ for NCFM and *B. subtilis* 6633, respectively, compared with worms feeding on HB101.

0.0668 and $P = 0.2217$ for NCFM and *B. subtilis* 6633, respectively, compared with worms preexposed to HB101) or solid (data not shown) ($P = 0.0826$ and $P = 0.4811$ for NCFM and *B. subtilis* 6633, respectively, compared with worms preexposed to HB101) killing assays with *fer-15;fem-1* worms. These results suggest that the immune induction by the probiotic bacterium strain NCFM may be specific to Gram-positive pathogens.

***L. acidophilus* NCFM regulates specific gene transcriptions.** We next sought to determine the mechanism associated with *C. elegans* conditioning with NCFM. Based on our findings that pre-
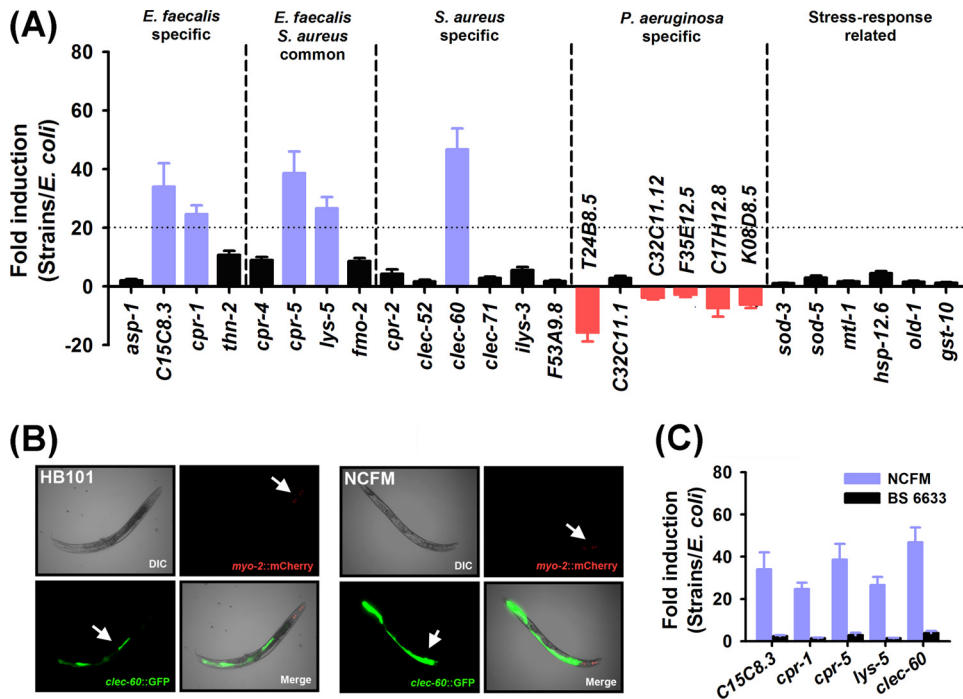
FIG 3 Conditioning with *L. acidophilus* NCFM regulates the transcription of immune genes. (A) qRT-PCR analysis evaluating the impact of conditioning on genes associated with nematode immune responses to *E. faecalis*, *S. aureus*, and *P. aeruginosa* as well as stress-related genes (nematodes "conditioned" with normal food, *E. coli* HB101, were used for comparison). Transcript levels were measured in young adult *fer-15;fem-1* worms conditioned with *L. acidophilus* NCFM for 24 h. (B) Induction of *clec-60*::GFP exposed to *L. acidophilus* NCFM for 24 h. *myo-2*::mCherry shows red pharyngeal expression as a marker for the presence of the transgene. Images were taken at the same time point with the same exposure time. DIC, differential interference contrast. (C) Transcripts of 5 selected genes after conditioning with *L. acidophilus* NCFM or *B. subtilis* ATCC 6633 for 24 h. Preexposure to *B. subtilis* had no impact on the regulation of five selected host defense genes.

exposure to the probiotic NCFM has an effect on Gram-positive bacteria but not *S. enterica* Typhimurium or *P. aeruginosa* (Gram-negative model pathogens that have been extensively studied in *C. elegans*), we postulated that conditioning with probiotic bacteria upregulates specific immune factors that are associated with *C. elegans* infection with *E. faecalis* or *S. aureus*. We mined the previous whole-transcriptome results linked to *C. elegans* infection with *E. faecalis* and *S. aureus* (12, 46), looking for upregulated genes with a >4.0-fold increase. We selected 14 target/candidate genes and categorized them in the following three groups: (i) *E. faecalis*-specific genes, such as *asp-1*, *C15C8.3* (encodes aspartyl proteases), *cpr-1* (encodes a cysteine protease), and *thn-2* (thaumatin/PR-5), (ii) genes upregulated during both *E. faecalis* and *S. aureus* infection, including *cpr-4* and *cpr-5* (encode cysteine proteases), *lys-5* (encodes a lysozyme), and *fmo-2* (encodes a flavin-containing monooxygenase), and (iii) *S. aureus*-specific genes like *cpr-2* (encodes a cysteine protease), *clec-52*, *clec-60*, and *clec-71* (that encode C-type lectins), *ilys-3* (encodes a invertebrate lysozyme), and *F53A9.8* (encodes a short His-rich protein). We investigated the regulation of these genes in *C. elegans* nematodes exposed to NCFM using quantitative real-time PCR (qRT-PCR). We found that almost all selected genes were upregulated by >5.0-fold by the strain NCFM. More specifically, our qRT-PCR results indicated that conditioning with NCFM dramatically induced the transcription of *C15C8.3* (34.1-fold ± 7.9-fold), *cpr-1* (24.6-fold ± 3.1-fold), *cpr-5* (38.6-fold ± 7.4-fold), *lys-5* (26.5-fold ± 3.9-fold), and *clec-60* (46.7-fold ± 7.2-fold) (Fig. 3A). These findings are consistent with our killing assay results that NCFM stim-

ulated the resistance of *C. elegans* to infection by Gram-positive bacteria (Fig. 1 and 2). We also studied the induction of *clec-60* using transgenic animals carrying the *clec-60*::GFP reporter line (12). Consistent with the qRT-PCR results, we noted strong induction of *clec-60*::GFP after conditioning with NCFM (Fig. 3B). Consistent with our survival assays (Fig. 1 and 2), preexposure to *B. subtilis* clearly did not influence the expression of any of the five selected host defense genes (Fig. 3C).

Since NCFM did not significantly protect nematodes from infection with *P. aeruginosa*, we also determined whether NCFM activates the expression of defense-related genes activated by *P. aeruginosa* infection. Consistent with our results from the killing assays (Fig. 2C), six genes (*T24B8.5*, *C32H11.1*, *C32H11.12*, *F35E12.5*, *C17H12.8*, and *K08D8.5*) predicted to be strongly induced by *P. aeruginosa* (31, 35, 41) were either unchanged (*C32H11.1*) or repressed (*T24B8.5*, *C32H11.12*, *F35E12.5*, *C17H12.8*, and *K08D8.5*) by NCFM conditioning (Fig. 3A). Notably, the transcription of *T24B8.5*, which encodes a ShK-like toxin peptide that is regulated by the PMK-1 signaling pathway and the ATF-7 transcription factor when worms are infected by *P. aeruginosa* (35), was decreased by 15.6-fold ± 3.0-fold. In addition, six general-stress-associated genes (10, 44), *sod-3*, *sod-5*, *mtl-1*, *hsp-16.2*, *old-1*, and *gst-10*, were not affected in the presence of NCFM (Fig. 3A). Taken in their totality, these results indicate that conditioning with the probiotic bacterium NCFM specifically stimulated the transcription of host defense genes associated with nematode responses to Gram-positive pathogens but had no or even a neg-
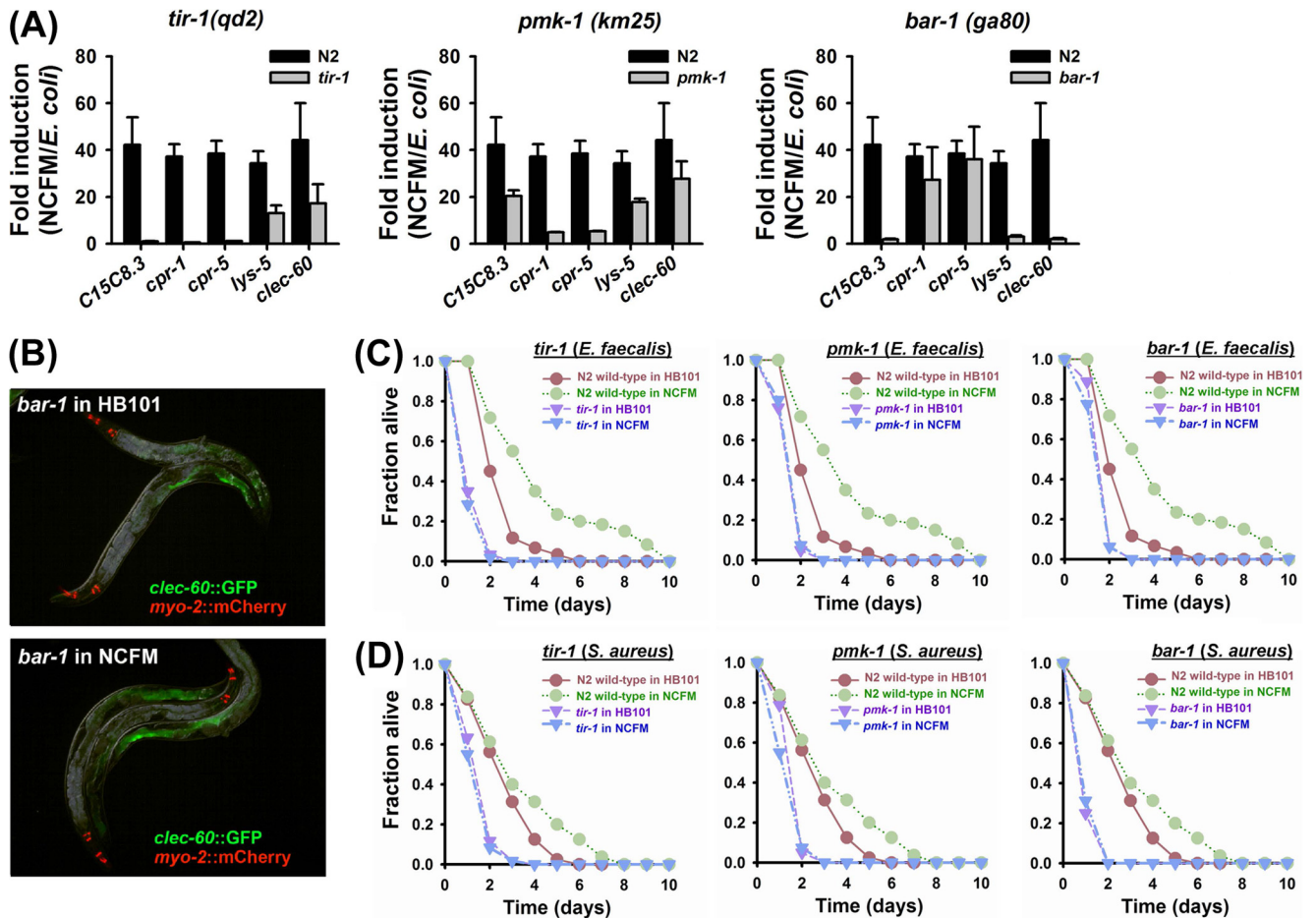
FIG 4 *tir-1*, *pmk-1*, and *bar-1* are required for the immune induction by *L. acidophilus* NCFM. (A) qRT-PCR analysis of five antimicrobial genes (*C15C8.3*, *cpr-1*, *cpr-5*, *lys-5*, and *clec-60*) in N2 wild-type and mutant animals after conditioning with *L. acidophilus* NCFM for 12 h. (B) Limited induction of *clec-60*::*GFP* by conditioning with *L. acidophilus* NCFM in the *bar-1* mutant. *clec-60*::*GFP* transgenic animals in the wild-type N2 (top) or *bar-1* mutant (bottom) background underwent conditioning with *L. acidophilus* NCFM for 12 h. Images were taken at the same time point with the same exposure time. *myo-2*::mCherry shows red pharyngeal expression as a marker for the presence of the transgene. (C) Solid killing of *C. elegans* strain N2 wild-type worms and *tir-1*, *pmk-1*, or *bar-1* loss-of-function mutants (*n* = 30 per plate) infected by *E. faecalis* MMH594. Survival statistics: *tir-1*, $P < 0.0001$ for *tir-1* control worms preexposed to *E. coli* HB101 and $P < 0.0001$ for *tir-1* worms conditioned with NCFM compared to N2 wild-type worms preexposed to *E. coli* HB101, $P = 0.3843$ for *tir-1* worms conditioned with NCFM compared to *tir-1* worms preexposed to *E. coli* HB101; *pmk-1*, $P < 0.0001$ for *pmk-1* worms preexposed to *E. coli* HB101 and $P < 0.0001$ for *pmk-1* worms conditioned with NCFM compared to N2 wild-type worms preexposed to HB101, $P = 0.4326$ for *pmk-1* worms conditioned with NCFM compared with *pmk-1* worms preexposed to HB101; and *bar-1*, $P < 0.0001$ for *bar-1* after preexposure to *E. coli* HB101 and $P < 0.0001$ for *bar-1* worms conditioned with NCFM compared to N2 wild-type worms preexposed to *E. coli* HB101, $P = 0.1403$ for *bar-1* worms conditioned with NCFM compared to *bar-1* worms preexposed to *E. coli* HB101. A 12-h conditioning protocol was used in all studies. (D) Solid killing of *C. elegans* strain N2 wild-type worms and *tir-1*, *pmk-1*, or *bar-1* loss-of-function mutants (*n* = 30 per plate) infected by *S. aureus* RN6390. Survival statistics: *tir-1*, $P < 0.0001$ for *tir-1* control worms preexposed to *E. coli* HB101 and $P < 0.0001$ for *tir-1* worms conditioned with NCFM compared to N2 wild-type worms preexposed to *E. coli* HB101, $P = 0.3636$ for *tir-1* worms conditioned with NCFM compared to *tir-1* worms preexposed to *E. coli* HB101; *pmk-1*, $P < 0.0001$ for *pmk-1* worms preexposed to *E. coli* HB101 and $P < 0.0001$ for *pmk-1* worms conditioned with NCFM compared to N2 wild-type worms conditioned with NCFM, $P = 0.0225$ for *pmk-1* worms conditioned with NCFM compared to *pmk-1* worms preexposed to *E. coli* HB101; and *bar-1*, $P < 0.0001$ for *bar-1* worms preexposed to *E. coli* HB101 and $P < 0.0001$ for *bar-1* worms conditioned with NCFM compared to N2 wild-type worms preexposed to *E. coli* HB101, $P = 0.4909$ for *bar-1* worms conditioned with NCFM compared to *bar-1* worms preexposed to *E. coli* HB101. A 12-h conditioning protocol was used in all studies.

ative effect on genes associated with Gram-negative bacteria or genes involved in the response to general stress.

The nematode host response to conditioning with NCFM is multifactorial and involves the TIR-1, PMK-1, and BAR-1 signaling pathways. To elucidate the immune pathways stimulated by NCFM, we employed loss-of-function *tir-1*, *pmk-1*, or *bar-1 C. elegans* immune-related mutants and investigated the regulation of the selected genes, including *C15C8.3*, *cpr-1*, *cpr-5*, *lys-5*, and *clec-60*, in these mutant strains after conditioning with NCFM. The *bar-1* loss-of-function mutation strongly affected the fold in-

duction of *clec-60*, whereas either the *tir-1* or *pmk-1* loss-of-function mutation significantly attenuated the upregulation of *cpr-1* and *cpr-5* (Fig. 4A). Moreover, we observed that *clec-60*::*GFP* was not induced by conditioning of the *bar-1* mutant with NCFM (Fig. 4B), in contrast to conditioning of the wild type (Fig. 3B). Interestingly, we found that mRNA levels of *C15C8.3* and *lys-5* were significantly affected by the deletion of *tir-1*, *pmk-1*, or *bar-1* when they were exposed to NCFM (Fig. 4A); hence, we suggest that the TIR-1, PMK-1, and BAR-1 signaling pathways participate in the regulation of *C15C8.3* and *lys-5* by NCFM exposure.

Taken in their totality, our results described above support the hypothesis that the TIR-1, PMK-1, and BAR-1 signaling pathways are involved in immune stimulation by conditioning with NCFM. In order to further investigate this hypothesis, we carried out solid killing assays with mutants or N2 wild-type worms to confirm that the TIR-1, PMK-1, and BAR-1 signaling pathways are directly involved in *C. elegans* by conditioning with NCFM. Surprisingly, worms with deletion of either *tir-1*, *bar-1*, or *pmk-1* were highly susceptible to *E. faecalis* and *S. aureus*. Similar to our results with other nematode strains (Fig. 1 and 2), NCFM significantly induced the resistance of wild-type nematodes (N2 strain) infected by *E. faecalis* and *S. aureus* ($P < 0.0001$ and $P = 0.0041$, respectively, compared with worms feeding on HB101). However, consistent with qRT-PCR results, worms lacking *tir-1*, *pmk-1*, or *bar-1* were still susceptible to *E. faecalis* (Fig. 4C) and *S. aureus* (Fig. 4D) infections even though they were conditioned with NCFM. Taken together, our results indicate that the TIR-1, PMK-1, and BAR-1 pathways all play an important role in immune conditioning with these probiotic bacteria.

DISCUSSION

Since Metchnikoff's work (26), a wealth of data have shown that probiotic bacteria are involved in host immune responses in humans (9, 24). Probiotic bacteria constantly exist in the human intestinal tract (34), and accumulating evidence indicates that they are involved in the regulation of the host immune system by modulating key signaling pathways (including MAPK, Akt/phosphoinositide 3-kinase [PI3K], and peroxisome proliferator-activated receptor gamma [PPARγ]) and by influencing downstream pathways (40). Because some of these signaling pathways are highly conserved from *C. elegans* to humans, the question that arises is whether probiotic bacteria have a similar effect in the immunity of *C. elegans* (14). In this work, we employed a conditioning concept to study the effects of probiotic bacteria in the immune response of *C. elegans*. Our findings demonstrate that nematode responses can undergo conditioning by probiotic bacteria, making them more resistant to virulent pathogens. We demonstrate that conditioning with the probiotic bacterium *L. acidophilus* strain NCFM stimulates *C. elegans* immune responses, making nematodes more resistant to infection by the Gram-positive pathogenic bacteria *E. faecalis* (Fig. 1) and *S. aureus* (Fig. 2A), including even antibiotic-resistant strains (Fig. 2B). Importantly, probiotic NCFM bacteria significantly inhibited the burden of *E. faecalis* in the nematode intestine even though they are completely digested by the nematode (Fig. 1C). In addition, based on *C. elegans* survival rates and qRT-PCR results, preexposure to the Gram-positive bacterium *B. subtilis* (Fig. 1, 2, and 3C) did not have the similar beneficial effects, indicating that the conditioning effect we found using the probiotic bacterium NCFM is, at least to some degree, specific.

Our findings on the ability of *C. elegans* nematodes to undergo conditioning are supported by the work of Anyanful and coworkers (3). These workers demonstrated that preexposure to the avirulent enteropathogenic *E. coli* (EPEC) Δ*tnaA* mutant (that is lacking the activity of tryptophanase) can induce responses associated with *C. elegans* infection with virulent EPEC and that these effects depend on dopaminergic neuronal signaling that activates the insulin/IGF-1 and p38 MAPK pathways (3). Notably, it has been known that cell wall molecules (CWMs) may be key probiotic ligands that interact with host receptors and induce signaling path-

ways, resulting in immune-stimulating effects (19). Taken along with our findings, it is reasonable to assume that virulent, avirulent, and probiotic microbes share common features, including pathogen- and microbe-associated molecular patterns (PAMPs/MAMPs) such as peptidoglycan, lipoteichoic acid, and polysaccharides (19, 25), and regulate similar nematode immune responses. Equally importantly, the additional possibility is that probiotic bacteria may lead to release or exposure of damage-associated molecular patterns (DAMPs). DAMPs are host-derived molecules that can activate immune responses (42). It is possible that NCFM conditioning triggers the production of DAMPs that mediate responses to Gram-positive bacteria. This hypothesis is also supported by our finding that the immune responses are specific and that NCFM conditioning had no effect on or even resulted in downregulation of genes associated with the nematode responses to the Gram-negative pathogens *P. aeruginosa* and *S.* Typhimurium. Of note is that some cross-responses to different DAMPs might exist, as suggested by the experiments by Ikeda et al. in which conditioning with lactic acid bacteria prolonged the survival of nematodes exposed to *Salmonella* spp. (11). In addition, probiotic effects in human health are multifactorial and they are dependent on the species and strain (19, 45). Ikeda and colleagues (11) employed different probiotic strains, including *Bifidobacterium*, *Lactobacillus helveticus*, and *Lactobacillus plantarum*, in *C. elegans* studies, whereas we used *L. acidophilus* NCFM, one of the most widely recognized probiotic bacteria that is extensively used in the food and pharmaceutical industries (2). Thus, probiotic specificity may result in a unique immune-stimulating effect in *C. elegans*. Indeed, a number of immune-stimulating molecules, including peptidoglycan (19), S-layer protein (18), and exopolysaccharide (EPS) (16, 17), were identified from the probiotic cell surface. Taken together, we conclude that activation of *C. elegans* host immunity by probiotic bacteria can be mediated by either exogenous (heat-labile PAMPs/MAMPs) or endogenous (DAMPs) features (or both) and that further work is required to elucidate which scenarios/mechanisms are directly involved in probiotic bacterium-mediated host response.

Several immune response signaling pathways have been identified in *C. elegans* (12, 15, 28, 31, 32, 48). Importantly, we found that *C. elegans* conditioning with the NCFM probiotic involves the p38 MAPK (via PMK-1) pathways, including the Toll/interleukin-1 receptor (TIR-1) pathway as well as the β-catenin (via BAR-1) signaling pathway (Fig. 4). PMK-1 and BAR-1 are key features of *C. elegans* innate immunity (14). The previous challengers indicated that either the *pmk-1* or *bar-1* signaling pathway is strongly involved in infection with the Gram-positive pathogen *S. aureus* (12). Also, the Toll-like receptor (TLR) signaling pathway is required for the *C. elegans* immune response to microbial pathogen infection (32). In the *C. elegans* genome, the following two genes encode TLR-related proteins: (i) *tir-1*, which encodes a conserved TIR domain protein that is homologous to the mammalian gene known as the sterile-α and Armadillo motif (SARM) (22), and (ii) *tol-1*, which encodes a potential Toll receptor homologue (32). *tir-1* functions upstream of a conserved p38 MAPK cascade and is a positive regulator of PMK-1 (6). Consistent with our results (Fig. 4), inactivation of *tir-1* results in enhanced susceptibility of *C. elegans* to infection with the Gram-positive pathogen *E. faecalis* (22). Interestingly, this pathway is also part of the mammalian immune response to probiotics (30). Notably, although *tol-1* appears to play a role in the nematode response to the

Gram-negative bacterium *S.* Typhimurium, it is not involved in the immune response to the Gram-positive pathogens *S. aureus* and *E. faecalis* (13, 39) and *C. elegans* lacks myeloid differentiation primary response protein 88 (MYD88) and a nuclear factor-κB (NF-κB)-like transcription factor (14). Therefore, we propose that NCFM-mediated conditioning of *C. elegans* is mediated by multiple pathways, including signaling through PMK-1/TIR-1 and BAR-1.

Taken in their totality, the signaling pathways through PMK-1 (with TIR-1) and BAR-1 are key components of the *C. elegans* immune conditioning with the probiotic bacterium NCFM and these pathways appear to act in parallel in order to promote immunity. Our working model (see Fig. S2 in the supplemental material) proposes a novel approach to probiotic-mediated immune conditioning in *C. elegans*. These findings demonstrate that *C. elegans* immunity can undergo conditioning and that Gram-positive pathogens and probiotic bacteria share key immune response signaling pathways.

ACKNOWLEDGMENTS

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