The purpose of this study was to take advantage of the nematode *Caenorhabditis elegans* to perform a whole-animal chemical screen to identify potential immune activators that may confer protection against bacterial infections. We identified 45 marketed drugs, out of 1,120 studied compounds, that are capable of activating a conserved p38/PMK-1 mitogen-activated protein kinase pathway required for innate immunity. One of these drugs, the last-resort antibiotic colistin, protected against infections by the Gram-negative pathogens *Yersinia pestis* and *Pseudomonas aeruginosa* but not by the Gram-positive pathogens *Enterococcus faecalis* and *Staphylococcus aureus*. Protection was independent of the antibacterial activity of colistin, since the drug was administered prophylactically prior to the infections and it was also effective against antibiotic-resistant bacteria. Immune activation by colistin is mediated not only by the p38/PMK-1 pathway but also by the conserved FOXO transcription factor DAF-16 and the transcription factor SKN-1. Furthermore, p38/PMK-1 was found to be required in the intestine for immune activation by colistin. Enhanced p38/PMK-1-mediated immune responses by colistin did not reduce the bacterial burden, indicating that the pathway plays a role in the development of host tolerance to infections by Gram-negative bacteria.

**IMPORTANCE** The innate immune system represents the front line of our defenses against invading microorganisms. Given the ever-increasing resistance to antibiotics developed by bacterial pathogens, the possibility of boosting immune defenses represents an interesting, complementary approach to conventional antibiotic treatments. Here we report that the antibiotic colistin can protect against infections by a mechanism that is independent of its microbicidal activity. Prophylactic treatment with colistin activates a conserved p38/PMK-1 pathway in the intestine that helps the host better tolerate a bacterial infection. Since p38/PMK-1-mediated immune responses appear to be conserved from plants to mammals, colistin may also activate immunity in higher organisms, including humans. Antibiotics with immunomodulatory properties have the potential of improving the long-term outcome of patients with chronic infectious diseases.

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ver the past decade, the pharmaceutical industry has experienced increasing challenges in drug development since the process has become more expensive, lengthier, and riskier. This is highlighted by the fact that even though there has been a more than 10-fold increase in drug development spending during the last few years, there has been no growth in new drug approvals (1). The most important reason for this lack of growth is the difficulty of crossing the so-called “valley of death,” the gap between finding hits and increases the possibility of leads that can be more easily used to develop new therapeutic approaches to treat infections. Out of 1,120 studied compounds, 45 candidates that were capable...
of activating a conserved p38/PMK-1 mitogen-activated protein kinase (MAPK) pathway were identified. Among them, we unexpectedly found colistin, which is a last-resort antibiotic against multidrug-resistant Gram-negative bacterial strains (5). The results showed that colistin confers resistance to infections by the Gram-negative pathogens *Yersinia pestis* and *Pseudomonas aeruginosa* but not by the Gram-positive pathogens *Enterococcus faecalis* and *Staphylococcus aureus*. Further studies indicated that the immune activation by colistin is mediated by the p38 MAPK pathway in the intestine and that it requires the downstream transcription factor SKN-1. Immune activation induced by colistin also requires the conserved FOXO transcription factor DAF-16. Resistance did not correlate with a reduced bacterial burden, indicating that the immune activation mediated by colistin makes the animals more tolerant to infections.

## RESULTS

**C. elegans**-based screen for identification of drugs capable of activating innate immunity. We have used *C. elegans* as a whole-animal high-throughput system for a chemical screen for novel immunomodulators capable of controlling the conserved p38/PMK-1 pathway that is required for innate immunity (6–8). We focused on the p38/PMK-1 pathway because it comprises a NSY-1 (a MAPK kinase kinase [MAPKKK])/SEK-1 (a MAPKK)/PMK-1 (a p38 MAPK) cassette that is an evolutionarily conserved module used by mammals and nematodes in immune response against bacterial infections. In addition, it has been demonstrated that it is controlled not only at the cell-autonomous level but also at the organismal level by the nervous system (9, 10), increasing the number of putative drug targets. As a first step to identify compounds capable of modulating this conserved pathway, we screened the Prestwick chemical library. The Prestwick library has been designed to ensure maximal chemical and optimal therapeutic diversity. It contains 1,120 small molecules, 90% of which are compounds capable of modulating this conserved pathway, we decided to further study colistin because the identification of a drug with known antimicrobial properties as a putative activator of the p38/PMK-1 pathway was unexpected due to the control of the promoter of a reporter gene of p38/PMK-1 activity, F35E12.5, was used to screen 1,120 drugs. Out of the 45 drugs found to enhance GFP fluorescence on AY101 animals, 16 correspond to antimicrobial agents and 22 target the nervous system.

**The antibiotic colistin can activate the p38/PMK-1 MAPK pathway.** We decided to further study colistin because the identification of a drug with known antimicrobial properties as a putative activator of the p38/PMK-1 pathway was unexpected and because even though it has been rediscovered as the last-line therapy for infections caused by Gram-negative “superbugs,” it has never been reported to target the host immune system. However, our studies indicate that in addition to its antimicrobial activity, it can activate the p38/PMK-1 pathway. Consistent with the initial findings described in Fig. 1, AY101 adult animals treated with colistin exhibited higher levels of pF35E12.5::gfp expression than untreated animals (Fig. 2A). As shown in Fig. 2A, the level of pF35E12.5::gfp expression induced by colistin seems comparable to that induced by *Y. pestis* infection, which is known to activate p38/PMK-1 and the expression of F35E12.5 (8). Consistent with visual observation, large-particle flow cytometry (Copas Biosort instrument) demonstrated that colistin-treated animals exhibit higher levels of pF35E12.5::gfp expression than the control and that the induction of pF35E12.5::gfp expression by colistin treatment was comparable to that elicited by *Y. pestis* infection (Fig. 2B).

We previously demonstrated that RNA interference (RNAi) inhibition of pmk-1 considerably reduced pF35E12.5::gfp expression in AY101 animals (8), indicating that inducible expression of F35E12.5 in response to *Y. pestis* infection was largely dependent upon PMK-1. Colistin may be able to induce F35E12.5 gene expression by targeting the PMK-1 pathway, similarly to *Y. pestis* infection, or it may activate a PMK-1-independent mechanism also capable of eliciting F35E12.5 expression. To distinguish between these two possibilities, we analyzed the fluorescence emit-
mals exhibited higher levels of active PMK-1 than control-treated animals (Fig. 2D). Taken together, these results show that colistin is capable of activating PMK-1, which results in the elicitation of its transcriptional activity.

**Colistin acts as an overall activator of immune pathways.** To investigate other possible effects of colistin on the activation of immune pathways, we utilized Agilent’s *C. elegans* gene expression microarray to find genes upregulated or down-regulated in response to the drug. Animals were synchronized by hypochlorite treatment and treated with control or 20 μg/ml colistin in liquid medium supplemented with heat-killed *E. coli* OP 50. After 24 h, the animals were collected and RNA extraction was performed. Overall, the microarray study revealed a change in the expression of 4,402 transcripts. Of these, 2,538 genes were upregulated and 1,864 genes were down-regulated more than 2-fold, with *P* value less than 0.05 (Fig. 3A; see also Tables S2 and S3 in the supplemental material). Interestingly, the cluster of genes upregulated by colistin is significantly enriched in genes upregulated in response to *Y. pestis* infection (Fig. 3B; see also Table S4, representation factor 4.8, *P*/H11021 1.05e*/H11002 25), indicating that the drug activates a transcriptional profile similar to that activated in response to *Y. pestis* infection.

Since the chemical screening was performed following the fluorescence emitted by GFP controlled by the promoter of gene *F35E12.5*, we expected to find this gene in the cluster of genes upregulated by colistin treatment. As shown in Fig. 3A (see also Table S2 in the supplemental material), *F35E12.5* showed a more than 5-fold increase in the colistin-treated group (*P*/H11021 0.001). To further confirm the role of colistin in the transcriptional activation of the PMK-1 pathway, we compared the 2,538 genes upregulated with colistin treatment with the transcripts positively regulated by PMK-1. As shown in Fig. 3B (see also Tables S5 and S6), there is a significant overlap between colistin-upregulated genes and genes that are positively induced by PMK-1 (representation factor 2.7; *P*/H11021 7.07e*/H11002 7).

In order to gain insights into the mechanisms of colistin-induced immune activation, we performed a biased gene enrichment analysis using several gene sets linked to immune or stress responses (Fig. 3C; see also Table S6 in the supplemental material). As expected, PMK-1-regulated genes showed the strongest overlap with colistin-induced genes among the selected gene sets.
We found that SKN-1- and DAF-16-regulated genes are also among the most highly enriched genes (Fig. 3C). SKN-1 is phosphorylated by PMK-1 and is required to protect *C. elegans* from reactive oxygen species (ROS) induced by bacterial pathogens (11). DAF-16 is a FOXO transcription factor that controls both longevity and innate immunity in *C. elegans* (12, 13). These results suggest that colistin not only induces a PMK-1-mediated immune response but also targets additional pathways that may control the expression of additional genes required for immunity against bacterial infections.

To identify related gene groups that are transcriptionally controlled by pathways targeted by colistin, we performed an unbiased gene enrichment analysis using the Database for Annotation, Visualization, and Integrated Discovery (DAVID) (http://david.abcc.ncifcrf.gov/). We separately analyzed 2,538 upregulated and 1,864 downregulated genes. The top 10 enriched annotation clusters of upregulated genes with *P* values of <0.05 correspond to genes encoding epidermal growth factor (EGF)-like domains, Apple-like domains, C-type lectins, Kunitz metazoan domains, homeobox, CUB-like domains, laminin G, neurotransmitter-gated ion-channel, Hedgehog/intein hint domains, and EGF-like calcium-binding domains (Fig. 4A). Among these gene groups found to be upregulated by colistin treatment, C-type lectins and CUB-like domain-containing proteins have been linked to antibacterial inducible defenses (7, 8, 14). The top 10 enriched annotation clusters of downregulated genes with *P* values of <0.05 correspond to genes encoding protein-tyrosine phosphatase, protein kinase, serine/threonine-specific protein phosphatase, protein kinase-ATP binding site, BRCT domains, histone core domains, transcription factor-fork head domains, SCP-like extracellular protein, glutamine synthetase, and zinc finger domains (Fig. 4B).

The major overlap between genes upregulated by *Y. pestis* infection and colistin treatment (Fig. 3B; see also Table S4 in the supplemental material) suggests that similar immune mechanisms are activated by the two stimuli and that similar gene groups should be upregulated. To test this hypothesis, we performed DAVID analysis on the clusters of genes misregulated in response to *Y. pestis* infection (see Fig. S1) and compared the results with those obtained by analyzing genes misregulated in response to colistin treatment. As shown in Fig. 4A, two of the three upregulated functional annotation clusters exhibiting the highest enrichment scores, EGF-like domains and C-type lectins, are induced by both *Y. pes-
tis* infection and colistin treatment.

**Prophylactic administration of colistin protects from infection by Gram-negative bacteria by inducing tolerance.** Since colistin treatment induces the expression of classes of transcripts that are also induced in response to *Y. pestis* (Fig. 3B and 4A), we hypothesized that colistin treatment may protect from bacterial infection. An advantage of using *Y. pestis* as a model pathogen is that it is naturally resistant to colistin (15). Our results also suggest that colistin does not affect the growth of *Y. pestis* at a 160-μg/ml concentration, which is much higher than the dosage used for treating the animals (see Fig. S2 in the supplemental material). Thus, the use of *Y. pestis* should allow us to distinguish the antimicrobial properties of colistin from its immunomodulatory properties. To further rule out the possibility that colistin may protect from bacterial infections due to any antimicrobial prop-

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**FIG 3** Colistin acts as a general immune activator that elicits an expression profile similar to that elicited by *Y. pestis* infection. (A) Microarray results shows 2,538 up- and 1,864 downregulated genes in response to colistin treatment (2-fold; *P* < 0.05). F35E12.5 showed a >5-fold increase in the colistin-treated group compared to expression in the untreated group (*P* < 0.001). (B) Venn diagrams of genes that are upregulated by colistin and positively induced by *Y. pestis* and PMK-1. (C) Representation factors (RFs) of gene sets linked to immune or stress responses. RF is the number of overlapping genes divided by the expected number of overlapping genes drawn from the corresponding gene sets. A representation factor below 1.0 indicates underrepresentation, whereas a value above 1.0 indicates overrepresentation.
mediated killing of *C. elegans* (see Fig. S4 in the supplemental material).

To provide further insights into the mechanisms by which colistin confers protection against *Y. pestis* and *P. aeruginosa* infection, we analyzed the bacterial burden following the profile of accumulation of bacteria expressing GFP. Live bacteria in infected animals were quantified by gridding up the nematodes to recover live bacteria and counting the CFU after plating on the appropriate medium. As shown in Fig. 5C to H, colistin pretreatment did not reduce the bacterial burden. Taken together, these results indicate that while the animals cannot clear the infection, they are better equipped to tolerate it.

**Colistin protects from infection by Gram-negative bacteria in a PMK-1-dependent manner.** We studied whether the prophylactic effect of colistin requires PMK-1 by treating KU25 animals, which carry the *pmk-1(km25)* deletion allele, with colistin prior to *Y. pestis* or *P. aeruginosa* infection. Figure 6A and B shows that the survival of *pmk-1(km25)*-treated animals was comparable to that of untreated animals, indicating that the beneficial effects of colistin require a functional PMK-1 pathway.

Since PMK-1 functions in the intestine to confer resistance to both *Y. pestis* and *P. aeruginosa* infection (19, 20), we hypothesized that colistin may require intestinal PMK-1 for protection against infections. To evaluate the intestinal contribution of PMK-1 in response to colistin, we utilized a *C. elegans* strain capable of RNAi activity only in the intestine (strain MGH171). Enriched RNAi knockdown of *pmk-1* in the intestine of MGH171 animals completely abolished the protection conferred by colistin (Fig. 6C and D). This result indicates that intestine is the major site where colistin functions to modulate the immune response in *C. elegans*.

**Colistin-induced resistance to Gram-negative bacterium infections depends on the transcription factors SKN-1 and DAF-16.** It is known that ATF-7, an ortholog of mammalian ATF2/ATF7, is phosphorylated by PMK-1 and is responsible for the induction of PMK-1-regulated genes during pathogen infection (21). To study whether ATF-7 plays a role in the protective effect of colistin, we analyzed the effect of colistin on the survival of pathogen infection (19, 20), we hypothesized that colistin may require intestinal PMK-1 for protection against infections. To evaluate the intestinal contribution of PMK-1 in response to colistin, we utilized a *C. elegans* strain capable of RNAi activity only in the intestine (strain MGH171). Enriched RNAi knockdown of *pmk-1* in the intestine of MGH171 animals completely abolished the protection conferred by colistin (Fig. 6C and D). This result indicates that intestine is the major site where colistin functions to modulate the immune response in *C. elegans*.
Colistin protects *C. elegans* from infection by Gram-negative bacteria *Y. pestis* and *P. aeruginosa*. (A and B) L4 N2 animals treated with or without colistin for 24 h were transferred to plates containing *Y. pestis* or *P. aeruginosa* and scored for survival (*Y. pestis*, *P* < 0.01; *P. aeruginosa*, *P* < 0.001). Shown are representative assays of more than three independent experiments (*n* = 60 animals per treatment). WT, wild type. (C and D) Wild-type N2 animals treated with or without colistin (Col) for 24 h were exposed to *P. aeruginosa* expressing GFP for 48 h and then visualized using a fluorescence microscope. (E and F) Wild-type
resistance to pathogen infection induced by colistin is not dependent on ATF-7 activity.

The gene enrichment analysis indicates that there is a significant overlap between colistin-upregulated genes and genes positively regulated by SKN-1 and DAF-16 (Fig. 3C). Thus, we sought to investigate whether SKN-1 and DAF-16 are needed for colistin-induced immune activation. skn-1 RNAi completely abolished the beneficial effect of colistin (Fig. 7C and D). Colistin-induced resistance to infections was also found to be dependent on DAF-16, since daf-16 (mu86) mutation completely suppressed the enhanced resistance to both Y. pestis and P. aeruginosa upon colistin treatment (Fig. 7E and F). Taken together, these results indicate that colistin protection for the Gram-negative bacteria Y. pestis and P. aeruginosa depends on both SKN-1 and DAF-16.

DISCUSSION

We have shown here that prophylactic administration of colistin protects C. elegans from infection by Y. pestis and P. aeruginosa. This effect appears to involve conserved mechanisms that control immune responses in C. elegans, including those regulated by the p38/PMK-1 MAPK and DAF-16/SKN-1 pathways. Furthermore, our studies suggest that the enhancement of immune responses by colistin requires intestinal PMK-1 and that it does not protect the animals from the infection by reducing the bacterial burden but rather by making the animals more tolerant to the infection. Together, these findings identify an alternative mechanism by which the antibiotic colistin may protect from bacterial infections. Colistin is a rapid bactericide against Gram-negative bacteria, interacting with the lipid A moiety of lipopolysaccharide to cause disorganization of the outer membrane. Colistin was first introduced to the clinic in 1952, but due to reports of nephrotoxicity and neurotoxicity in the 1970s, it was largely replaced by other antibiotics. However, the increase in widespread multidrug resistance of Gram-negative pathogens during recent decades has led to the reconsideration of colistin as a therapeutic option. Although a large number of studies concerning colistin investigate its clinical use, antibacterial activity, and mechanism of microbial resistance, there is no information regarding specific immune pathways that may be targeted by the drug.

The results presented herein clearly show that colistin acts as an
activator of conserved immune pathways. *C. elegans* has a number of physical and enzymatic xenobiotic defenses, including various detoxification enzymes that can act as xenobiotic efflux pumps (22). However, the direct exposure of the intestinal cells of the animals to 20 μg/ml of colistin for 24 h appears to circumvent the aforementioned xenobiotic detoxification mechanisms, allowing colistin to reach intracellular concentrations appropriate to activate immune pathways and confer resistance to pathogen infections. Indeed, the expression profile study showed that colistin can induce a transcriptional response in *C. elegans* that is very similar to that induced by pathogen infection. In addition, colistin treatment results in the upregulation of a number of genes that are markers of immune activation, including those that encode EGF-like domains, C-type lectins, and CUB-like domains. The biased gene enrichment analyses highlighted pathways that are controlled by PMK-1, DAF-16, and SKN-1 (Fig. 3C). Both DAF-16 and SKN-1 are independently inhibited by the insulin receptor DAF-2 (23). DAF-16 is the sole ortholog of the FOXO family of transcription factors and responsible for being the primary transcription factor required for the profound life span extension and resistance to pathogen infection upon mutation of the insulin-like receptor DAF-2 (12, 24, 25). Consistent with previous findings demonstrating that PMK-1 plays a crucial role in immunity since it is critical for immunity even when the DAF-16 pathway is hy-

FIG 7 Colistin-induced resistance to the Gram-negative bacteria *Y. pestis* and *P. aeruginosa* depends on the transcription factors SKN-1 and DAF-16. (A and B) Wild-type N2 L4 animals fed with *E. coli* strain HT115 carrying a vector control or expressing dsRNA targeting *atf-7* were treated with or without colistin for 24 h, transferred to plates containing *Y. pestis* or *P. aeruginosa*, and scored for survival. (C and D) Wild-type N2 L4 animals fed with *E. coli* strain HT115 carrying a vector control or expressing dsRNA targeting *skn-1* were treated with or without colistin for 24 h, transferred to plates containing *Y. pestis* or *P. aeruginosa*, and scored for survival. (E and F) Wild-type N2 and *daf-16*(mu86) L4 animals treated with or without colistin for 24 h were transferred to plates containing *Y. pestis* or *P. aeruginosa* and scored for survival (n = 60 animals per treatment). Shown are representative results of at least two independent experiments.
peractivated by daf-2 mutation (7), we found that protection against bacterial infections induced by colistin is completely abolished by PMK-1 inhibition.

For decades, it has been noted that certain antibiotics have immunomodulatory properties, which can improve the long-term outcome of patients with some chronic diseases (26). The best-investigated family of antibiotics is macrolides. Several clinical trials have confirmed the beneficial effects of long-term treatment with macrolides such as azithromycin in patients with chronic inflammatory pulmonary diseases, such as diffuse panbronchiolitis (DBP) and cystic fibrosis (CF) (27, 28). The concept of taking advantage of the immunomodulatory properties of antibiotics to reduce the severity of pulmonary diseases has been so convincing that investigations have been expanded to other classes of antibiotics, such as tetracyclines and fluoroquinolones. However, a role for the polymycin antibiotic colistin in the control of immunity has not been reported. Here we showed that a short, nontoxic treatment with colistin can activate the innate immunity of C. elegans in a p38/PMK-1-dependent manner. Since p38/PMK-1-mediated immune responses appear to be conserved from plants to mammals, it is conceivable that colistin may also activate immunity in higher organisms, including humans.

MATERIALS AND METHODS

Nematode and bacterial strains. C. elegans strains used in this study were N2 (Bristol, AL), W3510 (pgd-1), AY101 (pPD99.1-pf35E12.5::gfp), PR4 (rol-6 (su1006)) (8), KU25 (pmk-1 [km25]), CF1038 (daf-16D(mu86)), and MGH171 (sid-1[261]). All strains were maintained at 20°C on nematode growth medium (NGM) and fed with E. coli OP 50. Bacterial strains were used Escherichia coli OP 50 (30), Pseudomonas aeruginosa PA14 (31), Yersinia pestis KIMS (32), Enterococcus faecalis OG1RF (33), and Staphylococcus aureus MSSA476 (34). E. coli, P. aeruginosa, and S. aureus cultures were grown in Luria-Bertani (LB) broth overnight at 37°C. E. faecalis was grown in brain heart infusion (BHI) broth overnight at 37°C. Y. pestis culture was grown in LB broth at 25°C.

Chemical library screen. The Prestwick Library that comprises 1,120 small molecules was used (see Table S1 in the supplemental material). More than 95% of its compounds are marketed drugs (all off patent). Eggs containing S-basal were seeded with OP 50 and grown at 20°C until late L4 larval stage. L4 animals were harvested for RNA extractions, and treated with colistin as mentioned above. skn-1 RNAi started at the L1 stage and was performed by placing synchronized L1 animals on RNAi plates. unc-22 RNAi was included as a positive control in all experiments to account for RNAi efficiency.

Copol biossorter GFP analysis. Expression levels of the pf35E12.5::gfp reporter in AY101 transgenic animals were analyzed using the Copas Biosort instrument for large-particle flow cytometry (Union Biometrica, Holliston, MA). Synchronized animals treated with control vector or pmk-1 RNAi were exposed to E. coli or Y. pestis for 24 h and washed in M9 buffer prior to analysis. Fluorescence data were acquired from a minimum of 400 adult animals for each experimental sample. Plots were constructed using FlowJo flow cytometry analysis software (Tree Star, Inc., Ashland, OR).

Microarray analysis. RNA was extracted from three biological replicates using the TRIzol reagent (Invitrogen). Residual genomic DNA was removed by DNase treatment (Ambion, Austin, TX). Samples were hybridized to the C. elegans Gene Expression Microarray (Agilent Technologies) by the Duke Microarray Facility. Data were analyzed using Partek Genomics Suite software. Raw data were preprocessed, including background correction, normalization, and summarization using robust multivariable average analysis, and expression data were log2 transformed. Principal-component analysis (PCA) was performed to identify outliers and evaluate whether batch effects, ethnicity, or intrinsic subtype significantly affected the data. Gene lists were created using a cutoff P value of <0.05, 2-fold change (see Tables S2 and S3 in the supplemental material). For subsequent analyses, the list of up- and downregulated genes was curated by removing duplicates and using the WormBase Converter (http://wormbase.org/). For each experiment, a cutoff of P value of <0.05 was selected as significant. Probability calculations of enrichment were performed using an online hypergeometric probability tool to calculate the statistical significance of the overlap of gene groups (http://www.geneprof.org/GeneProf/tools/hypergeometric.jsp). The representation factor is the number of overlapping genes divided by the expected number of overlapping genes drawn from two independent groups. A representation factor below 1.0 indicates underrepresentation, whereas a value above 1.0 indicates overrepresentation.

C. elegans killing assays. The bacterial cultures were grown in LB broth containing ampicillin (100 μg/ml) at 37°C overnight and plated onto NGM containing 100 μg/ml ampicillin and 3 mM isopropyl 1-thio-β-D-galactopyranoside. RNAi-expressing bacteria were allowed to grow overnight at 37°C. L2 or L3 larval animals were placed on RNAi or vector control plates for 2 days at 20°C until nematodes became gravid. Gravid adults were then transferred to fresh RNAi-expressing bacterial lawns and allowed to lay eggs for 2 h to synchronize a second-generation RNAi population. For nematodes that were subjected to colistin pretreatment, L4 animals were harvested and treated with colistin as mentioned above. skn-1 RNAi started at the L1 stage and was performed by placing synchronized L1 animals on RNAi plates. unc-22 RNAi was included as a positive control in all experiments to account for RNAi efficiency.

Whole-Animal Screen for Immune System Activators

Western blot analysis. Whole-worm lysates were prepared in the presence of protease and phosphorylase inhibitors. Active PMK-1 was detected using an Anti-Active p38 polyclonal antibody (pAb), rabbit (pT-GpY), from Promega, Inc. β-Actin was detected using a monoclonal anti-β-actin antibody produced in mouse from Sigma. Blots were developed using SuperSignal chemiluminescence substrate (Pierce). Functional gene enrichment analysis for all differentially expressed genes. DAVID (Database for Annotation Visualization and Integrated Discovery; http://david.abcc.ncifcrf.gov/7) was used for functional annotation bioinformatics microarray analysis to determine the functional enrichment and Gene Ontology annotation; clusters with P values of <0.05 were selected as significant. Probability calculations of enrichment were performed using an online hypergeometric probability tool to calculate the statistical significance of the overlap of gene groups (http://www.geneprof.org/GeneProf/tools/hypergeometric.jsp). The representation factor is the number of overlapping genes divided by the expected number of overlapping genes drawn from two independent groups. A representation factor below 1.0 indicates underrepresentation, whereas a value above 1.0 indicates overrepresentation.

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target gene. E. coli HT115(DE3) was grown in LB broth containing ampicillin (100 μg/ml) at 37°C overnight and plated onto NGM containing 100 μg/ml ampicillin and 3 mM isopropyl 1-thio-β-D-galactopyranoside. RNAi-expressing bacteria were allowed to grow overnight at 37°C. L2 or L3 larval animals were placed on RNAi or vector control plates for 2 days at 20°C until nematodes became gravid. Gravid adults were then transferred to fresh RNAi-expressing bacterial lawns and allowed to lay eggs for 2 h to synchronize a second-generation RNAi population. For nematodes that were subjected to colistin pretreatment, L4 animals were harvested and treated with colistin as mentioned above. skn-1 RNAi started at the L1 stage and was performed by placing synchronized L1 animals on RNAi plates. unc-22 RNAi was included as a positive control in all experiments to account for RNAi efficiency.

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the times indicated and were considered dead when they failed to respond to touch. Animal survival was plotted as a nonlinear regression curve using the PRISM (version 4.00) computer program. Survival curves were considered different when P values were <0.05. PRISM uses the product limit or Kaplan-Meier method to calculate survival fractions and the log rank test, which is equivalent to the Mantel-Haenszel test, to compare survival curves.

Profile of bacterial accumulation in the nematode intestine. After pretreatment with colistin for 24 h at 20°C, N2 animals were then transferred to plates seeded with either P. aeruginosa/GFP for 48 h or Y. pestis/GFP for 96 h at 25°C. Animals were transferred to fresh pathogen lawns every day. At 48 h or 96 h, animals were transferred to an NGM plate seeded with E. coli for 15 min and transferred again to a new NGM plate seeded with E. coli for 30 min to eliminate P. aeruginosa/GFP or Y. pestis/GFP adhered to the body of the nematodes. Animals were then visualized using a fluorescence stereomicroscope.

Quantification of intestinal bacterial loads. After exposure to P. aeruginosa/GFP for 48 h or Y. pestis/GFP for 96 h, animals were transferred to a new NGM plate seeded with E. coli for 30 min to eliminate external P. aeruginosa/GFP or Y. pestis/GFP. Ten nematodes per condition were transferred into 50 l of phosphate-buffered saline (PBS) containing 0.1% Triton and homogenized. Serial dilutions of the lysates (10⁻¹, 10⁻², 10⁻³, and 10⁻⁴) were plated onto LB-kanamycin plates and grown for 24 h at 37°C and 48 h at 25°C to select for P. aeruginosa/GFP or Y. pestis/GFP cells, respectively.

SUPPLEMENTAL MATERIAL
Supplemental material for this article may be found at http://mbio.asm.orglookup/suppl/doi:10.1128/mBio.01235-14/-/DCSupplemental.

Table S1, PDF file, 1.2 MB.
Table S2, PDF file, 0.3 MB.
Table S3, PDF file, 0.5 MB.
Figure S3, PDF file, 0.6 MB.
Figure S4, PDF file, 0.3 MB.

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