A Conserved PMK-1/p38 MAPK Is Required in Caenorhabditis elegans Tissue-specific Immune Response to Yersinia pestis Infection

Yersinia pestis has acquired a variety of complex strategies that enable the bacterium to overcome defenses in different hosts and ensure its survival and successful transmission. A full-genome microarray analysis on Caenorhabditis elegans infected with Y. pestis shows enrichment in genes that are markers of innate immune responses and regulated by a conserved PMK-1/p38 MAPK. Consistent with a role in regulating expression of immune effectors, inhibition of PMK-1/p38 by mutation or RNA interference enhances susceptibility to Y. pestis. Further studies of mosaic animals where PMK-1/p38 is exclusively inhibited or overexpressed in a tissue-specific manner indicate that PMK-1/p38 controls expression of a CUB-like family of immune genes at the cell-autonomous level. Given the conserved nature of PMK-1/p38 MAPK-mediated signaling and innate immune responses, PMK-1/p38 MAPK may play a role in the immune response against Y. pestis in natural hosts.

Recently evolved from enteropathogenic Yersinia pseudotuberculosis, Yersinia pestis acts as a blood-borne pathogen capable of parasitizing insects and causing systemic disease in mammals (1, 2). Y. pestis has acquired a variety of complex strategies to overcome defense responses in different hosts to ensure its multiplication and survival (3–5). Although a number of virulence determinants contributing to Y. pestis persistence in mammals have been identified, interactions between Y. pestis and the host immune system remain poorly understood.

During its vector-mammal transmission cycle, Y. pestis must evade components of innate immunity in both insects and mammals. One of the initial immune responses against pathogens in vertebrates and invertebrates alike is the inducible humoral defense, including the production of antimicrobial peptides and reactive oxygen species (6, 7). These inducible effectors, possessing potent antimicrobial activity, are components of the phylogenetically ancient innate immune system that predates the origins of adaptive immunity. Evolutionary conservation among pathogen recognition receptors and signaling pathways contributing to the inducible immune response has permitted the use of alternative model hosts to study innate immunity.

In recent years, the nematode Caenorhabditis elegans has become an attractive alternative model host to study certain aspects of bacterial pathogenesis and innate immunity. Even though C. elegans lacks professional immune cells, it lives in soil environments where it is in contact with soil-borne microbes and has evolved physiological mechanisms to respond to different pathogens by activating the expression of innate immune response genes that are conserved across metazoans. Typically, C. elegans is grown in the laboratory by feeding them Escherichia coli. E. coli is effectively disrupted by the C. elegans pharyngeal grinder, and almost no intact bacterial cells can be found in the intestinal lumen. Once in the gut, however, pathogenic bacteria can overcome innate immune responses to proliferate and kill C. elegans. Infection of C. elegans with Y. pestis KIM5 leads to a persistent and lethal colonization of the nematode intestine (8). In addition, similar virulence factors are required for pathogenicity in nematodes and mice (8). Infectivity and persistence of Y. pestis KIM5 in the nematode makes C. elegans an attractive whole animal system for studying the host response to infection with the plague bacterium.

In this study, we examined the transcriptional response of C. elegans during infection with Y. pestis KIM5 to better understand conserved innate responses contributing to host defense during early stages of infection. Our results demonstrate a strong transcriptional response against Y. pestis highlighted by the induction of immune-related effectors that are predominantly regulated by PMK-1/p38. In C. elegans, as well as in other animals, PMK-1/p38 is likely expressed in a range of tissues where it can regulate immune responses at the cell-autonomous level or at the organismal level. Our studies indicate that PMK-1/p38 activity is required in the C. elegans intestine to regulate innate immunity at the cell-autonomous level.

EXPERIMENTAL PROCEDURES

Nematode and Bacterial Strains—C. elegans strains N2, KU25 pmk-1(km25), WM118 rde-1(ne300); nld9[myo-3::HA::rde-1, rol-6], NR222 rde-1(ne219); kzs9[pKK1260(pline-12:nls:gfp)], pKK1253(pline-26:rde-1), rol-6](9); and NR350 rde-1(ne219); kzs20[pDM715(pline-12:rde-1), pTG95(psar-5:nls:gfp), rol-6](9) were provided by the Caenorhabditis Genetics Center. Strain VP303 rde-1(ne219); kbls7[pnlix-2::rde-1, rol-6](10) was provided by Kevin Strange (Vanderbilt University). All strains were maintained at 20 °C on nematode growth medium
(NGM) and fed with *E. coli* OP50. The following bacterial strains were used for experiments: *E. coli* OP50 (11) and *Y. pestis* KIM5 (12). *E. coli* was cultured overnight in LB broth at 37 °C, and *Y. pestis* was cultured in LB broth at 25 °C.

**RNA Isolation**—Gravid adult N2 nematodes were lysed using a solution of sodium hydroxide and bleach and washed, and eggs were synchronized overnight in S basal liquid medium at room temperature. Synchronized L1 larval animals were seeded onto modified NGM plates with 0.35% peptone containing *E. coli* OP50 and incubated at 25 °C until the nematodes had reached the L4 larval stage. Animals were collected and washed with M9 buffer before transferring to modified NGM plates containing *Y. pestis* KIM5 or *E. coli* OP50 for 24 h. After 24 h, animals were collected and washed with M9 buffer, and RNA was extracted using TRIzol reagent (Invitrogen). Residual genomic DNA was removed by DNase treatment (Ambion, Austin, TX). Three independent RNA isolations were performed with each pathogen for microarray analysis, and two additional RNA isolations were performed for samples used for quantitative PCR.

**Microarray Analysis**—For each experimental condition, RNA was isolated from three biological replicate samples. cRNA was synthesized from 10 µg of total RNA, and samples were hybridized to the *C. elegans* GenChip (Affymetrix, Santa Clara, CA) by the Duke Microarray Facility. Microarray data were subjected to the robust multichip averaging algorithm using GeneSpring GX software (Agilent Technologies, Santa Clara, CA). Analysis of variance test and fold-change calculations were also performed using GeneSpring software. Transcripts showing a corrected *p* value of <0.05 were considered differentially expressed between *E. coli* OP50 and *Y. pestis* KIM5 experimental treatments. The microarray data have been deposited in the Gene Expression Omnibus, accession number GSE20053.

**Functional Enrichment Analysis**—Genes showing a significant change in expression by microarray analysis (*p* < 0.05) were analyzed using FatiGO software (13). Genes were compared against a 21,249 *C. elegans* gene database to identify over-represented Gene Ontology terms and Interpro motifs. Statistical analysis was performed by the FatiGO software using Fisher’s exact test and corrected for false discovery rate using the methods of Benjamini and Hochberg. Significant functional terms were defined as *p* < 0.05.

**Real Time Quantitative PCR**—cDNA was synthesized from 5 µg of total RNA using random hexamers and SuperScript II reverse transcriptase (Invitrogen). Real time PCR was performed using SYBR Advantage quantitative PCR premix (Clontech) and gene-specific oligonucleotide primers (supplemental Table 4) on the LightCycler (Roche Applied Science). Relative fold-changes for transcripts were calculated using the comparative *C*~T~ (2^−ΔΔC~T~) method (14) and normalization to pan-actin (*act-1*, *act-3*, or *act-4*) (15). Cycle thresholds of amplification were determined by LightCycler software (Roche Applied Science). All samples were run in triplicate.

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RNA Interference—*E. coli* HT115(DE3) bacterial strains expressing double-stranded RNA (16) were grown in LB broth containing ampicillin (100 µg/ml) at 37 °C 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. 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 4 h to synchronize a second generation RNAi population. Unc-22 RNAi was included as a positive control in all experiments to account for RNAi efficiency.

**C. elegans Survival Analysis**—*C. elegans* hermaphrodites treated with RNAi or a vector control were maintained at 20 °C until animals were young adults. Pathogen lawns for survival assays were prepared by inoculating modified NGM (in 6-cm Petri plates) with 60 µl of an overnight bacterial culture. Plates were incubated overnight at room temperature before animals were added. Young adult animals were transferred to modified NGM plates containing *Y. pestis* KIM5 bacterial lawns and incubated at 25 °C. Animals were scored every 12 or 24 h for survival and transferred to fresh pathogen lawns each of the first 5 days to avoid overgrowth by progeny. Animal survival was plotted using Kaplan-Meier survival curves and analyzed by log rank test using GraphPad Prism (GraphPad Software, Inc., La Jolla, CA). Survival curves resulting in *p* values of <0.05 relative to control were considered significantly different. The time for 50% of the nematodes to die (TD~50~) was calculated using nonlinear regression analysis of survival proportions (GraphPad Prism) utilizing the following equation: *Y* = bottom + (top − bottom)/(1 + (log(TD~50~ − 4))/Hill slope), where Top is set at 100, Bottom is set at 0; *X* is the time, and *Y* is the percentage of nematodes alive at time *X*. In this instance, TD~50~ is equivalent to EC~50~.

**Plasmid Constructs and Generation of Transgenic Lines**—A GFP transcriptional reporter plasmid, pDB09.1, was constructed by cloning 1974 bp of the F35E12.5 promoter region (position −15 to −1989 upstream of ATG) into pPD95.77 (Fire Lab *C. elegans* Vector Kit, Addgene, Cambridge, MA). Wild-type N2 nematodes were microinjected with pDB09.1 and pRF4 to generate the extrachromosomal array *acEx101*. Integration of the extrachromosomal array *acEx101* was achieved by UV irradiation, yielding strain AY101 (pDB09.1[pF35E12.5:gfp]; pRF4[rol-6(su1006)])]. Plasmid pDB09.2 was generated by cloning the cDNA sequence of *pmk-1* into pPD95.77 (Addgene), creating a translational fusion between PMK-1 and GFP. The *vha-6* promoter (17) was inserted upstream of the *pmk-1::gfp* coding sequence to confer intestine-restricted expression (18). *pmk-1*(km25) animals were microinjected with pDB09.2 and pRF4 to generate strain AY102 (*pmk-1*(km25) *acEx102*[pDB09.2[phva-6::*pmk-1::gfp]; pRF4[rol-6(su1006)])].

**Western Blot Analysis**—Western blots were prepared by separating total nematode lysates from 50 adult animals on a 10% SDS-polyacrylamide gel and transferring separated proteins to Immuno-Blot polyvinylidene difluoride membranes (Bio-Rad). Blots were incubated with peroxidase-conjugated anti-GFP-horseradish peroxidase (1:5000; Rockland Immunocichemicals) or anti-actin AC-40 (1:5000; Sigma) followed by horseradish

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2 The abbreviations used are: NGM, nematode growth medium; RNAi, RNA interference; MAPK, mitogen-activated protein kinase; GFP, green fluorescent protein.
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peroxidase-conjugated anti-mouse antibody (1:1000; Bio-Rad). Blot were developed using SuperSignal chemiluminescence substrate (Pierce).

COPAS Biosorter 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 for a minimum of 400 adult animals for each experimental sample. Plots were constructed using FlowJo flow cytometry analysis software (Tree Star, Inc., Ashland, OR).

RESULTS

Identification of Inducible Immune Responses to Y. pestis Infection—To investigate conserved innate immune mechanisms that play a role in early response to Y. pestis, we utilized Affymetrix GeneChip C. elegans Genome Arrays to find clusters of genes commonly up-regulated or down-regulated in response to Y. pestis infection. Infection of C. elegans was accomplished by transferring nematodes, propagated on E. coli OP50, onto bacterial lawns of Y. pestis KIM5. In previous studies, we have demonstrated that following ingestion Y. pestis establishes a persistent infection in the C. elegans intestine (8). Even though C. elegans is not known to be a natural host for Y. pestis, this persistent colonization of the nematode intestine by Y. pestis allows the host to properly recognize and respond to pathogen infection.

Host response to pathogen challenge was assessed by measuring transcriptional activity of a synchronized population of nematodes exposed to Y. pestis KIM5. RNA was collected from animals following 24 h of pathogen exposure allowing sufficient time for bacterial accumulation within the intestine, yet prior to extensive morbidity of infected animals. Transcript expression levels were compared between Y. pestis-treated animals and nematodes maintained on the relatively nonpathogenic E. coli OP50 to identify factors involved in host response to Y. pestis. Overall, analysis using the Affymetrix C. elegans genome array revealed a change in expression of 258 transcripts (supplemental Table 1). Of these, 99 genes were up-regulated greater than 2-fold, demonstrating a robust inducible response to Y. pestis (supplemental Table 1). Conversely, 27 genes were down-regulated greater than 2-fold, representing transcripts directly suppressed in response to infection or reduced as a consequence of host pathology.

Several classes of transcripts that showed changes in expression in response to Y. pestis infection are markers of C. elegans immunity (Fig. 1A and Table 1). Members of gene families encoding CUB-like proteins, C-type lectins, and neuropeptide-like factors that are up-regulated in response to infection with several pathogens (15, 19–23) are similarly up-regulated in response to Y. pestis (Table 1). However, the overall transcriptional profile for genes belonging to these families was unique in comparison with the response to other pathogens. These results are in line with other studies that have reported shared and distinct inducible responses to pathogen exposure in C. elegans (22, 24).

Although only a limited number of factors encoded by the aforementioned gene families have been directly associated with antimicrobial activity (25) or resistance to pathogen-mediated killing (15, 19, 26), strong induction during infection supports that these factors participate in the nematode immune response. Changes in host transcript levels for nine representative genes were confirmed using quantitative real-time PCR as described under “Materials and Methods.” Relative expression levels of the indicated genes were determined using the comparative Ct method (14) with normalization to panactin (act-1, -3, -4) (15). Bar graphs correspond to expression levels relative to average expression following control treatment (E. coli). Error bars represent standard deviation among independent biological samples.

![Figure 1](image-url)
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**TABLE 1**

Summary of *C. elegans* transcripts showing greater than 2-fold change in expression during *Y. pestis* infection

| Group               | Gene          | Fold-change* | p value*   | Group               | Gene          | Fold-change* | p value*   |
|---------------------|---------------|--------------|------------|---------------------|---------------|--------------|------------|
| CUB-like domain     | F53E12.5      | 62.54        | 0.0062     | Proteolysis/hydrolysis | W03G1.7      | 13.55        | 0.0111     |
|                     | C32H11.10     | 12.43        | 0.0355     |                     | F53A9.1      | 9.37         | 0.0195     |
|                     | C32H11.12     | 9.97         | 0.0217     |                     | C05C10.4     | 5.48         | 0.0457     |
|                     | F55G11.5      | 6.82         | 0.0178     |                     | K10C2.3      | 5.12         | 0.0189     |
|                     | C32H11.4      | 6.52         | 0.0217     |                     | F21F8.4      | 4.45         | 0.0470     |
|                     | F35E12.6      | 6.25         | 0.0217     |                     | K17H12.2     | 3.36         | 0.0264     |
|                     | F08G5.6       | 6.21         | 0.0195     |                     | T10H4.12     | 2.57         | 0.0314     |
|                     | C17H12.8      | 4.94         | 0.032      |                     | F55F2.2      | 2.20         | 0.0195     |
|                     | F55G11.8      | 4.68         | 0.0219     |                     | F54F11.2     | 2.18         | 0.0256     |
|                     | K08D8.5       | 3.59         | 0.0256     |                     | F27E5.1      | 2.10         | 0.0195     |
|                     | ZK969.5       | 2.57         | 0.0149     |                     | Y16B4A.2     | 2.09         | 0.0460     |
|                     | H20E11.1      | 2.03         | 0.0298     |                     | CS5B7.3      | –2.48        | 0.0249     |
|                     | K10D11.5      | 2.00         | 0.0441     | Lipid metabolism    | Y56B4B.1     | 10.01        | 0.0249     |
| Sugar binding       | T05E12.6      | –4.52        | 0.0217     |                     | K03H6.2      | 8.91         | 0.0062     |
|                     | F56D6.15      | 10.09        | 0.0173     |                     | K03H6.2      | 8.91         | 0.0062     |
|                     | F56D6.2       | 9.24         | 0.0233     |                     | B0035.13     | 5.97         | 0.0227     |
|                     | ZK666.6       | 6.88         | 0.0438     |                     | F09C8.1      | 4.95         | 0.0382     |
|                     | F53S5.5       | 3.77         | 0.0282     |                     | F2B8H.7      | 3.31         | 0.0217     |
|                     | F53S5.9       | 3.59         | 0.0256     |                     | W06D12.3     | –4.66        | 0.0216     |
|                     | F40F4.6       | 3.42         | 0.0283     | Metabolic Process   | F41E6.5      | 4.71         | 0.0460     |
|                     | Y54G2A.14     | 2.79         | 0.0282     |                     | T23B9.7      | 2.03         | 0.0256     |
|                     | F38A5.3       | 2.74         | 0.0322     |                     | D18A5.8      | –2.55        | 0.0460     |
|                     | Y54G2A.8      | 2.58         | 0.0329     |                     | C17C12.12    | –3.33        | 0.0139     |
|                     | F21H7.4       | 2.47         | 0.0329     |                     | F47C10.6     | –3.83        | 0.0305     |
|                     | F53C5.8       | 2.00         | 0.0329     |                     | C50G12.2     | –4.08        | 0.0382     |
|                     | T09F5.9       | –4.19        | 0.0460     |                     | C04F5.7      | –4.33        | 0.0322     |
| Neuropeptide-like   | F45E8.4       | 7.78         | 0.0355     |                     | C55B7.4      | –8.32        | 0.0441     |
|                     | Y45F10A.5     | 2.63         | 0.0295     | Transport           | K09C8.1      | 4.26         | 0.0178     |
|                     | F37A8.4       | 2.58         | 0.0364     |                     | C18H9F.5     | –2.41        | 0.0012     |
|                     | M01D7.5       | 2.54         | 0.0371     |                     | C55S5.3      | –4.12        | 0.0219     |
|                     | T23E7.4       | 2.12         | 0.0280     |                     | T23B9.7      | –5.90        | 0.0280     |

* Fold change represents the ratio of expression in *C. elegans* exposed to *Y. pestis* relative to expression on *E. coli*. Average expression levels from three samples were used to calculate fold change.

* p values represent the corrected p value for multiple comparisons using the Benjamini-Hochberg method with a cutoff of 0.05.

>2-fold by *Y. pestis* with transcripts regulated by PMK-1 and those regulated by the forkhead transcription factor DAF-16 (Fig. 2). Overall, there was significant overlap of genes up-regulated by *Y. pestis* infection with genes up-regulated by PMK-1 (Fig. 2, A and B, and Table 2). A lysozyme gene, *F58B3.2* (*lys-5*), down-regulated by PMK-1 was similarly down-regulated in response to *Y. pestis*. Genes up-regulated by *Y. pestis* infection also showed overlap with genes that were up-regulated by DAF-16 (Fig. 2, A and C); however, greater overlap of genes up-regulated in response to *Y. pestis* was observed with genes down-regulated by DAF-16 (Fig. 2D). Taken together, these data suggest that PMK-1 plays an important role in the control of genes that are up-regulated in response to *Y. pestis*.

*Genes That Are Markers of Response to Infection Contribute to *C. elegans* Resistance against *Y. pestis*-mediated Killing*—The expression profiling studies revealed that genes encoding CUB-like domains were among the most highly induced genes in response to *Y. pestis* (Table 1). The CUB domain, named for its founding members C1r/C1s, Uegf, and Bmp1, contains 110 amino acids found in extracellular and plasma membrane-associated proteins involved in a variety of different functions, including complement activation, development, tissue repair, tumor suppression, and inflammation (27–29). Mounting evidence supports the role of proteins carrying CUB-like domains in *C. elegans* immunity. Microarray expression analysis examining the response of *C. elegans* to several pathogens, including *Microbacterium nematophilum* (19), *Serratia marcescens* (21, 22), and *Pseudomonas aeruginosa* (15, 20, 22), have previously demonstrated induction of different CUB-like factors during infection. Moreover, distinct subsets of CUB-like genes are induced in response to different classes of pathogens (22), suggesting that different regulatory mechanisms may tightly control their expression.

Of 50 *C. elegans* genes encoding CUB-like factors, 16 showed a significant change in expression in response to *Y. pestis* (Table 3), with expression of several of these genes being highly induced (Table 1). This over-representation of CUB-like genes in the inducible response to *Y. pestis* suggests that they may play a role in host defense. To address whether CUB-like factors contribute to host defense against *Y. pestis*, we used RNAi to inhibit the expression of individual CUB-like genes and determined animal susceptibility to infection. Of nine *Y. pestis*-induced CUB-like genes that were evaluated, RNAi inhibition of *F08G5.6*, *C17H12.8*, and *C32H11.12* enhanced the susceptibility of the nematodes to *Y. pestis*-mediated killing (Table 4). The minor changes observed by inhibiting individual genes by RNAi could be attributed to incomplete RNAi or redundancy of function among members of the CUB-like family that are induced during infection. Consistent with our findings, RNAi of individual genes that are markers of inducible innate immunity has little effect on the resistance of *C. elegans* to different pathogens, maybe due to functional redundancy (15, 20, 21, 23).

PMK-1/p38 MAPK Plays a Key Role in the Regulation of CUB-like Genes in Response to *Y. pestis* Infection—The microarray analysis indicated that at least six CUB-like genes that are up-regulated in response to *Y. pestis* infection may be positively regulated by PMK-1 (Table 2). Thus, we examined whether increased expression of the most highly induced CUB-like gene in response to *Y. pestis* infection, *F35E12.5*, was de-
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Those genes whose expression significantly changed in response to \textit{Y. pestis} infection \((p < 0.05; \text{see supplemental Table 1})\) were compared with genes regulated by the PMK-1 pathway \((20)\) \((\text{reflecting relative levels of gene expression during infection})\). Genes up-regulated by PMK-1 \((20)\) and DAF-16 \((44)\) \((\text{overlap with factors induced in response to \textit{Y. pestis}})\) and genes down-regulated by DAF-16 \((44)\) \((\text{are shown})\).

TABLE 2
\textbf{Y. pestis} induces expression of transcripts similar to those regulated by PMK-1

| Description       | Sequence ID | \textit{Y. pestis} \(^{\text{a}}\) regulation \(^{\text{b}}\) | \textit{pmk-1} \(^{\text{a}}\) regulation \(^{\text{b}}\) |
|-------------------|-------------|---------------------------------|---------------------------------|
| CUB-like          | F35E12.5    | 62.54                           | 3.6                             |
|                   | C32H11.12   | 9.97                            | 5.0                             |
|                   | F35E11.5    | 6.21                            | 28.9                            |
|                   | C17H12.8    | 5.28                            | 8.3                             |
|                   | F55G11.8    | 4.68                            | 6.8                             |
|                   | K08D5.5     | 4.07                            | 3.5                             |
| C-type lectin     | F56D6.2     | 9.24                            | 5.0                             |
|                   | ZK666.6     | 6.88                            | \(-3.3\)                        |
| DUF274            | Y41D4B.16   | 7.96                            | \(3.3\)                         |
| Peptidase         | F35E12.5    | 5.12                            | 2.5                             |
| Peptidase         | K10C2.3     | 2.48                            | 3.3                             |
| Metridin-like Shk toxin | F01D5.3 | \(-2.3\)                        | \(-2.9\)                        |

\(^{\text{a}}\) Fold-change of transcript expression was following 24 h of \textit{Y. pestis} infection in wild-type N2 animals.

\(^{\text{b}}\) Fold-change of transcript expression in \textit{daf-2(e1368)} was compared with \textit{daf-2(e1368); pmk-1(km25)} animals, as described previously \((20)\).

TABLE 3
Over-represented functional terms in the response to \textit{Y. pestis}

| Term * | Domain name | Genes \(^{\text{c}}\) | \(p\) value \(^{\text{d}}\) |
|--------|-------------|----------------------|--------------------------|
| IPR003366 | CUB-like region | 16/50 | 1.6E-15 |
| IPR002055 | von Willebrand factor, type A | 8/52 | 5.6E-05 |
| IPR001461 | Peptidase A1 | 5/18 | 1.6E-03 |
| IPR001304 | C-type lectin | 15/260 | 4.8E-03 |
| IPR001969 | Peptidase aspartic, active site | 4/23 | 4.0E-02 |
| IPR009673 | Unknown function, DUF 1261 | 4/11 | 4.0E-02 |

\(*\) Interpro entry accession number is below.

\(^{\text{c}}\) Number of genes that show a significant change in expression during \textit{Y. pestis} infection relative to the total number of \textit{C. elegans} genes for the indicated domain.

\(^{\text{d}}\) \(p\) values were calculated by FatiGO software using Fisher’s exact test and adjusted for multiple comparisons using the Benjamini and Hochberg method.

TABLE 4
Effect of RNAi inhibition of CUB-like genes on susceptibility to \textit{Y. pestis} infection

| RNAi treatment | Survival ratio \((\text{No. of animals})^{\text{a}}\) | Experiment 1 | Experiment 2 | \(p\) value\(^{\text{b}}\) |
|----------------|-------------------------------------|--------------|--------------|--------------------------|
| Vector control | 1.00 \((89)\) | 1.00 \((91)\) | 0.8636 |
| F35E12.5 | 1.02 \((82)\) | 0.98 \((90)\) | 0.0899 |
| C32H11.12 | 0.90 \((85)\) | 0.88 \((92)\) | 0.0018 |
| F35E12.5 | 0.82 \((82)\) | 0.98 \((84)\) | 0.4396 |
| F55G11.5 | 0.99 \((88)\) | 0.81 \((84)\) | 0.1417 |
| C32H11.4 | 0.90 \((84)\) | 1.00 \((87)\) | 0.0006 |
| F35E12.6 | 0.86 \((86)\) | 0.88 \((83)\) | 0.0806 |
| F08G5.6 | 0.81 \((86)\) | 0.88 \((83)\) | 0.0017 |
| C17H12.8 | 0.86 \((90)\) | 0.85 \((92)\) | 0.0006 |
| K08D5.5 | 0.92 \((94)\) | 0.89 \((89)\) | 0.8363 |

\(^{\text{a}}\) Represents the ratio of the TD50 for animals given the designated RNAi treatment relative to the TD50 of animals given control vector treatment. TD50 values were calculated using nonlinear regression analysis (GraphPad Prism). The number of genes scored as dead in each individual experiment are indicated in parentheses.

\(^{\text{b}}\) Data from two independent experiments are shown.

\(^{\text{c}}\) Statistical analyses were conducted on survival data pooled from two independent experiments. \(p\) values were determined using the log rank test. \(p\) values < 0.05 were considered significant and are denoted by an asterisk.
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FIGURE 3. PMK-1 MAPK contributes to the induction of C. elegans genes in response to Y. pestis. A, AY101 pmk-1(pmgf::pRF4);rol-6(su1006)) animals containing a transcriptional reporter for pRF4 were exposed to Y. pestis strain KU25 for 24 h and imaged using fluorescence microscopy. An asterisk indicates the head of the animals. DIC, differential interference contrast; B, Western blot analysis of GFP expression levels in AY101 animals. AY101 transgenic animals were treated with a control vector or pmk-1-specific RNAi. Total nematode lysates were collected from 50 adult animals following 24 h of exposure to E. coli or Y. pestis. C and D, analysis of GFP fluorescence intensity in AY101 animals using the COPAS BIOSORT instrument (Union Biometrica, Holliston, MA) (45). C, AY101 animals treated with a control RNAi vector were exposed to Y. pestis or E. coli for 24 h. D, AY101 animals treated with control vector or pmk-1 RNAi were exposed to Y. pestis. GFP fluorescence intensity (FLU-1) was plotted against adult animal size, measured as time of flight (TOF). Each dot represents an individual nematode. All results are representative of three or more independent experiments.

FIGURE 4. PMK-1 MAPK is necessary for host defense against Y. pestis in the nematode C. elegans. Kaplan-Meier survival analysis of C. elegans strains challenged with Y. pestis KIM5. N2 wild-type animals were treated with either a control vector or pmk-1 RNAi. Strain KU25 contains the pmk-1(km25) deletion allele. Survival assays were performed as described under “Materials and Methods,” and animal survival was monitored every 12–24 h. Log rank analysis confirmed a significant decrease in survival following RNAi knockdown of pmk-1 (p < 0.0001) and a significant decrease in survival in strain KU25 (p < 0.0001) when compared with N2 animals.
other tissues. Alternatively, despite phosphorylation of PMK-1::GFP, the fusion protein might not retain complete functionality in comparison with wild-type PMK-1. Importantly, the increased resistance to infection in AY102 animals was specific to expression of intestinal PMK-1::GFP as the enhanced resistance to \textit{Y. pestis} was abolished following treatment with \textit{pmk-1} RNAi (Fig. 6D).

**DISCUSSION**

Successful transmission of the vector-borne pathogen \textit{Y. pestis} requires that the bacterium be able to rapidly adapt to...
diverse host environments. Following transmission to a mammalian host, plague bacteria that withstand early innate defenses can persist in the extracellular environment through expression of factors that enable increased resistance to serum and phagocytosis, induce apoptosis of immune cells, and suppress the inflammatory response (30–38). Thus, for the host, efficient recognition and elimination of the pathogen by the innate immune response during early stage infection is critical in defense against *Y. pestis*. Using the invertebrate host *C. elegans* to model early stage infection with *Y. pestis*, we demonstrate a robust inducible response during infection and identify the PMK-1/p38 MAPK pathway as a central component in protective immunity.

PMK-1/p38 MAPK regulates the expression of several classes of genes induced during pathogen infection such as those encoding CUB-like domains, C-type lectins, and ShK toxins (20). Factors including the CUB-like genes *F08G5.6*, *F20G2.5*, and *F35E12.7* (15, 26) and the C-type lectin genes *ZK666.6*, *E03H4.10*, and *C54D1.2* (19) have been demonstrated to affect the host response during infection with either *P. aeruginosa* or *M. nematophilum*, supporting a function in immunity for these factors. Consistent with these observations, a significant number of PMK-1-regulated factors were overrepresented in the inducible response to *Y. pestis* (Fig. 2 and Table 2). Our findings also reveal a role for the CUB-like genes *F08G5.6*, *C17H112.8*, and *C32H111.12* in host defense to *Y. pestis*. Although knockdown of several CUB-like factors induced in response to *Y. pestis* failed to alter pathogen susceptibility, possibly due to redundancy, enhanced susceptibility of animals failed to alter pathogen susceptibility, possibly due to redundancy, enhanced susceptibility of animals lacking pmk-1 demonstrates a convincing role overall for PMK-1/p38-dependent factors in the host response to *Y. pestis*. Similar studies show a lack of a phenotype when candidate immune effector genes are inhibited by RNAi but show strong phenotypes when upstream regulators of their expression are altered (15, 20, 23).

In *C. elegans*, PMK-1/p38 MAPK signaling is involved in the response to diverse physiological stimuli and environmental stresses. Cell-autonomous activity of PMK-1/p38 MAPK signaling in *C. elegans* has been described during oxidative stress in the intestine (39) and in the localized response to *Drechmeria coniospora* infection in the epidermis (40). Our findings highlight a cell-autonomous role of PMK-1/p38 MAPK in the intestinal response to pathogen infection. This response of PMK-1/p38 MAPK in the intestine is consistent with the localization of *Y. pestis* colonization (8) and is also the major site of expression for markers of *C. elegans* immunity (Fig. 3) (22).

In summary, our findings reveal that *C. elegans* mounts a striking inducible response to *Y. pestis* consisting of several factors that are prominent markers of nematode immunity. This inducible response is largely regulated by the cell-autonomous activity of intestinal PMK-1/p38. Although cell-autonomous function of PMK-1/p38 MAPK is shown to regulate intestinal host defense in the present study, recent observations from our laboratory indicate that the nematode nervous system can also influence the p38 MAPK intestinal response to pathogens (41). Additional examples of non-cell-autonomous regulation of inducible immunity have been demonstrated for the DAF-16 signaling in the intestine (42) and the transforming growth factor-β-mediated response in the epidermis (43). Together, these observations indicate that mechanisms underlying the regulation of PMK-1/p38 MAPK immunity can be quite complex and likely involve both a localized response to pathogen infection while jointly integrating cues from the rest of the organism.

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