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C. elegans pgp-5 IS INVOLVED IN RESISTANCE TO BACTERIAL INFECTION AND HEAVY METAL AND ITS REGULATION REQUIRES TIR-1 AND A p38 MAP KINASE CASCADE

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Abstract

Animals and plants respond to bacterial infections and environmental stresses by inducing overlapping repertoires of defense genes. How the signals associated with infection and abiotic stresses are differentially integrated within a whole organism remains to be fully addressed. We show that the transcription of a *C. elegans* ABC transporter, *pgp-5* is induced by both bacterial infection and heavy metal stress, but the magnitude and tissue distribution of its expression differs, depending on the type of stressor. PGP-5 contributes to resistance to bacterial infection and heavy metals. Using *pgp-5* transcription as a read-out, we show that signals from both biotic and abiotic stresses are integrated by TIR-1, a TIR-domain adaptor protein orthologous to human SARM, and a p38 MAP kinase signaling cassette. We further demonstrate that not all the TIR-1 isoforms are necessary for nematode resistance to infection, suggesting a molecular basis for the differential response to abiotic and biotic stress.

Keywords

C. elegans; *P. aeruginosa*; innate immunity; stress; cadmium; infection; P-glycoprotein

INTRODUCTION

In the soil, *Caenorhabditis elegans* contacts with natural toxins and must defend against potentially pathogenic microorganisms that constitute its food. To defend against pathogens, *C. elegans* uses an immune system that is regulated by several signaling pathways, including the TGF-β [1], insulin [2] and p38 MAPK pathways. [3]. Phosphorylation of the p38 MAPK, PMK-1 and nematode resistance to pathogens requires the evolutionarily conserved protein TIR-1 [4;5]. To resist environmental toxins, such as heavy metals, *C. elegans* utilizes strategies ranging from avoidance to detoxification. Evolutionary conserved signaling pathways, like

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JNK and p38 MAPK, regulate resistance to these stresses [6;7]. Interestingly, the p38 MAPK pathway is required for resistance to toxins and nematode immunity [6;8].

P-glycoproteins (PGP) represent an evolutionary well-conserved sub-group of ATP-binding cassette (ABC) transporters that protect cells by actively exporting drugs and toxins [9]. The *C. elegans* genome encodes 15 PGP proteins [10]. Athough present in almost every tissue [11], the functions of only 3 have been determined. *pgp-2* is expressed in the intestine and is required for acidification of lysosomes and lipid storage [12]. *pgp-1* and *pgp-3* are necessary to resist phenazine toxins secreted by the bacterial pathogen *Pseudomonas aeruginosa* [13], and contribute to heavy metals and drugs resistance [14;15].

Although signaling cascades necessary for the nematode resistance to infection and stresses have been identified and transcriptional responses to these stresses have been studied at the whole genome level [16;17;18;19], much remained to be elucidated about how specific downstream effectors are regulated. Moreover, it is not known whether the signaling modules regulating nematode immunity act independently or are part of a global regulation network that integrates the responses to pathogens and stress, as previously suggested [20].

We showed that the *C. elegans* P-glycoprotein gene *pgp-5* is induced upon exposure to heavy metals and bacterial pathogens and is necessary for full resistance to these treatments. By investigating the regulation of *pgp-5* expression, we found that the axis defined by TIR-1-p38 MAPK module plays a significant role in integrating the signals from both biotic and abiotic stresses.

MATERIALS AND METHODS

Infection and toxicity assays

Infection assays were performed as described [17]. For toxicity assays, three 1-day-old adult hermaphrodites were deposited on plates seeded with OP50 and allowed to lay eggs for 4–5 h. After the parents were removed, the eggs were counted and the plates incubated for 3 days at 20ºC. The percentage of adults was calculated as the total number of adults divided by the total number of eggs. Each test was performed at least twice with 4 replicates per condition.

RNAi and qRT-PCR experiments

RNAi and qRT-PCR experiments were performed as described [17], and detailed in Supplemental Materials and Methods. After 24 h treatment, animals were either collected for microscopy, COPAS analysis or RNA preparation. The identity of dsRNA-expressing bacteria (Geneservice, UK) was confirmed by sequencing or restriction digest.

Statistical analysis

For survival analysis, StatView and Prism softwares were used to calculate the mean time to death (TD_{mean}) and the Kaplan-Meier Log rank test assessed the similarity between survival curves. Student's t-tests were calculated using Microsoft Excel. Values of $p < 0.05$ were considered statistically significant.

RESULTS

Transcription of pgp-5 is induced during infection and heavy metal stress

In whole-genome microarray experiments, expression of *pgp-5* was among the most highly induced in response to intestinal infection by *P. aeruginosa* strain PA14 (hereafter referred to as PA14) [17;18]. To confirm and extend these observations, we measured the mRNA levels of *pgp-5* and additional *pgp* genes upon PA14 infection and exposure to heavy metals by

quantitative RT-PCR (qRT-PCR). We included *pgp-1* and *pgp-3* for their known function in resistance to PA14 toxin [13], *pgp-4* because it is adjacent to *pgp-3*, and a cluster composed of *pgp-5*, *pgp-6*, *pgp-7* and *pgp-8* (Supplemental Fig. 1A). Following infection or exposure to cadmium, *pgp-5* was the only tested gene induced by at least 3-fold in both conditions (Fig. 1A and 1B). We chose *pgp-5* for further analyses because its robust induction by both infection and cadmium presents the opportunity to study how responses to biotic and abiotic stresses are integrated in a whole organism.

The magnitude and tissue distribution of pgp-5 expression in response to infection and environmental insults are different

To determine if the response of *pgp-5* to different forms of stresses differs in tissue expression, and to confirm the observed difference in magnitude, we monitored the expression of green fluorescence protein (GFP) in p*pgp-5::gfp* transgenic animals. The p*pgp-5::gfp* reporter strain contains an integrated transgene, p*pgp-5::gfp*, in which the GFP-encoding gene is under the control of the 300 bp upstream sequence of the *pgp-5* promoter (Supplemental Fig. 1B). Under standard growth conditions, green fluorescence was undetectable at low magnification (25X) and was detected at a very low level throughout the intestine (Supplemental Fig. 2A) at high magnification (200X) in the majority of adult animals. Consistent with microarray and qRT-PCR data, we observed a very strong increase of green fluorescence in the intestine of PA14-infected worms (Supplemental Fig 2B), and quantified fluorescence related to *pgp-5* induction with the COPAS worm sorter (Supplemental Fig 2D). *pgp-5* expression was also induced in the intestine of animals infected with pathogenic *Salmonella typhimurium*, *Serratia marcescens* or *Staphylococcus aureus*, but not in worms fed on the non-pathogenic *Bacillus subtilis* (Supplemental Table 2).

Heavy metals, such as cadmium robustly induced the *pgp-5* transcription in intestinal cells of adults (Supplemental Fig. 2C and D). In addition to the intestinal expression, majority of cadmium-treated animals expressed the transgene in the terminal bulb of the pharynx (80%, n = 59, Supplemental Fig. 2C). In contrast, only 14% (n = 74) of PA14-infected animals had detectable transgene expression in the pharynx. Copper, colchicine and zinc also potently induced *pgp-5* expression (Supplemental Table 2). In general, *pgp-5* expression was robustly induced in the intestinal cells of animals exposed to diverse pathogenic bacteria, but the induction was more moderate in animals exposed to several abiotic noxious molecules.

PGP-5 is necessary for full resistance to heavy metals and bacterial infections

The increase in *pgp-5* transcription in response to bacterial infections and toxic compounds suggested that PGP-5 could be involved in protection from these insults. We tested this possibility by analyzing the *pgp-5(ok856)* mutant, in which a 1 kb region, including exons 11 to 13 that encode the second transmembrane domain is deleted (Supplemental Fig. 1B). Under standard culture conditions, the *pgp-5(ok856)* mutant was phenotypically wild-type.

We assayed the effects of different toxic compounds on the developmental rate of *pgp-5* mutant animals at a range of concentrations. In the absence of toxic molecules in the media, the N2 and *pgp-5* mutant worms developed at the same rate: close to 100% of the eggs developed into adults in 3 days at 20°C (Fig. 2A–B, the “0” columns). At concentrations of cadmium chloride and copper sulfate in which development of wild type animals was impaired, the proportions of *pgp-5* animals with developmental delay were significantly higher (Fig. 2A and B). No significant difference between wild type and *pgp-5* was observed on colchicine and sodium arsenite at all test concentrations (Supplemental Fig. 3A and B). We therefore conclude that *pgp-5* is required for *C. elegans’* full resistance to cadmium and copper.

We next asked if *pgp-5* is required to protect *C. elegans* from bacterial infections by determining the TD_{mean} of worms infected with PA14 or *S. typhimurium* SL1344. No significant difference in survival could be detected between wild type and *pgp-5* mutants on PA14 (TD_{mean} of 3.15 ± 0.05 days and 3.25 ± 0.05 days, respectively; Log rank test, $p > 0.11$) and SL1344 (TD_{mean} of 9.4 ± 0.6 days and 10.1 ± 0.5 days, respectively; Log rank test, $p > 0.07$) when the assays were carried out at 25°C. Because small differences in resistance to bacterial pathogens could be better resolved when infections are carried out at a lower temperature [21], we repeated the infection assays at 15°C. At 15°C, wild-type nematodes infected with PA14 had a TD_{mean} of 13.5 ± 1.7 days, whereas the TD_{mean} for *pgp-5* was 10.9 ± 1.0 days (Fig. 2C, Log rank test $p < 0.0001$). Similarly, *pgp-5* worms infected with SL1344 were significantly more susceptible than wild-type animals, with a TD_{mean} of 16.7 ± 1.0 days, and 19.3 ± 0.5 days, respectively (Fig. 2D, Log rank test, $p < 0.001$). Importantly, the lifespan of the *pgp-5* mutants cultivated on the innocuous *E. coli* strain OP50 was not different from that of N2 animals at 25°C (data not shown) nor at 15°C (Supplemental Fig. 3C). Together, the results indicate that PGP-5 has modest but detectable roles in providing full protection from bacterial infection and a subset of toxic chemicals.

The induction of pgp-5 in response to infection and heavy metal requires TIR-1 and the p38 MAP kinase signaling cascade

The *pgp-5* transgenic reporter strain allows us to visualize and quantify *pgp-5* induction. Combined with gene knock down by RNAi at later larval stages, thereby avoiding potentially confounding effects of developmentally important genes, this represents a powerful tool to identify *in vivo* regulatory elements that control *pgp-5* expression. Initially, we focused on the *nsy-1*/*sek-1*/*pmk-1* p38 MAPK cascade, because this module is important for defense against both infection and abiotic stresses [3;7], and on *tir-1*, an upstream component of the p38 MAPK pathway in *C. elegans* immunity [5]. With the exception of *tir-1*, RNAi knock down of *nsy-1*, *sek-1* and *pmk-1* did not significantly affect the basal expression of *pgp-5* (Fig. 3A). Increased fluorescence in *tir-1* RNAi-treated animals was due to a specific, but yet unexplained, increase in reporter gene expression in the pharynx (Supplemental Fig. 4). By contrast, knockdown of *tir-1* or each member of the *nsy-1*/*sek-1*/*pmk-1* module significantly reduced *pgp-5* induction in p*pgp-5::gfp* worms compared to control animals following cadmium exposure (Fig. 3B) and PA14 infection (Fig. 3C). We confirmed the requirement for *tir-1* and the *nsy-1*/*sek-1*/*pmk-1* module for *pgp-5* induction during PA14 infection by qRT-PCR (Fig. 3D).

Next we tested two other MAPK pathways, ERK and JNK. ERK contributes to resistance to rectal infection by *M. nematophilum* [22], and JNK is required for resistance to heavy metal stress [7]. dsRNA knockdown of *mpk-1* (ERK) and *mek-1* and *kgb-1* (JNK) did not significantly affect the basal expression nor the induction of *pgp-5* in response to infection or cadmium stress (Supplemental Fig. 5A–C). Two additional immunity pathways, the TGF-beta [1] and insulin pathways [2], also did not affect *pgp-5* induction upon infection (see Supplemental Results). Collectively, the results indicate that the *tir-1*-p38 MAPK pathway is important for the induction of *pgp-5* in response to infection and heavy metal stresses.

The *tir-1* mRNA can be spliced into at least five isoforms, namely *tir-1a-e* [23] (Supplemental Fig. 6A). The dsRNA construct used in the previous experiments targets all the isoforms (Supplemental Fig. 6A). To determine if different TIR-1 isoforms play distinct roles in discriminating between infection and heavy metal responses upstream of *pgp-5*, we generated the *tir-1b* and *tir-1a,c,e* isoform-specific dsRNA constructs (Supplemental Fig. 6A). Neither RNAi against the *tir-1a,c,e* nor the *tir-1b* isoforms reproduced the significant reduction as obtained by pan-isoform RNAi on the cadmium- and PA14-induced expression of p*pgp-5::gfp* (Supplemental Fig. 6C and D).

As only the pan-isoform *tir-1* RNAi significantly reduced the *pgp-5* induction by PA14 and heavy metals, we decided to directly test the role of the *tir-1* isoforms for resistance to infection. As reported [5], knock-down of all the *tir-1* isoforms markedly increased susceptibility to PA14 infection (Fig. 4A). Interestingly, RNAi against *tir-1a, c, e* did not affect resistance to PA14 (Log rank test, $p > 0.6$), whereas RNAi against *tir-1b* significantly reduced survival on PA14 compared to control animals (Fig. 4A). RNAi treatments did not significantly affect lifespan on *E. coli*: mean life spans for animals treated with vector control, *tir-1* RNAi and *tir-1b* RNAi were 9.7 ± 0.7, 10.0 ± 0.5 and 10.2 ± 0.2 days, respectively (Log rank test, $p > 0.8$ when compared to vector). The RNAi results were confirmed with *tir-1*(*ok1052)* animals, which has a deletion that only removes *tir-1a,c* and *e* isoforms [23]. The *tir-1*(*ok1052)* animals had an essentially wild-type resistance to PA14 (Fig. 4B), suggesting that *tir-1b,* and perhaps the *tir-1d* isoform, may be the most important *tir-1* isoforms for resistance to PA14.

DISCUSSION

P-glycoproteins protect many organisms from environmental insults. In *C. elegans*, the intestinal epithelial cells are the main sites of exchange with the external milieu and potential sites for bacterial colonization. Our results suggest that PGP-5 is part of the machinery necessary to protect *C. elegans* intestinal cells from certain biotic and abiotic toxins, as do other P-glycoproteins in *C. elegans* and mammals. Although PGP-5 is required for full resistance to cadmium, its role is minor compared to that of stress response regulators like KGB-1 or MEK-1 [7]. On the other hand, like the *C. elegans* metallothionein genes, the induction of *pgp-5* by heavy metals underscores its potential physiological role in heavy metal detoxification. PGP-5 is also contributes to resistance to bacterial infections and its expression is upregulated by infection. By analogy to PGP-1 and PGP-3, PGP-5 may be directly involved in depotentiating bacterial virulence factor(s) other than phenazines. *C. elegans pgp-5* is clustered with 3 highly similar genes, *pgp-6, -7* and *-8* whose expression are induced during PA14 infection and/or cadmium exposure (Fig. 1). The significant, though minor, effects of loss of *pgp-5* on sensitivity to pathogens and heavy metals may be due to partial compensation by these P-glycoproteins. The severely compromised resistance of *tir-1*(RNAi) (Fig. 4A, [4;5]), *nsy-1* and *sek-1* mutants to infection [3] is likely due to the deregulation of multiple downstream effectors. The modest effect of the *pgp-5* mutation relative to the loss of the TIR-1/p38 MAPK module on the nematode's capacity to resist bacterial infection indicates that PGP-5 is only one element of the worm's p38-dependent defense machinery.

Knocking down *nsy-1*, *sek-1* or *pmk-1* function significantly reduced *pgp-5* induction by PA14 infection and cadmium. These results support a model in which defense pathways converge on the corresponding MAP3K and MAP2K and diverge downstream of their target p38 MAPK, PMK-1. TIR-1 could be one source of specificity in p38 signaling upstream of NSY-1 and SEK-1. At least 5 major isoforms of *tir-1* exist (Supplemental Fig. 6A). The *tir-1* locus is complex and the precise expression pattern of each *tir-1* isoform remains unknown. The *tir-1a,c,e* isoforms are involved in the activation of NSY-1 and SEK-1 in specific neurons during development [23]. Our results indicate that these isoforms are not important for resistance to pathogens, but implicate *tir-1b* in this function. The possibility that *tir-1d* is important for defense against intestinal infection must await the availability of isoform-specific mutants.

Although previous studies have identified genes upregulated by abiotic stress or bacterial infection in *C. elegans* [1;16;17;18;19], the p*pgp-5::gfp* reporter provides the first visual *in vivo* read-out for response against bacterial infections and abiotic stresses. The *pgp-5* transgenic strain should therefore serve as an important tool for systematic dissections of molecular networks responsible for the distinct but overlapping responses to the environment at the level of the whole organism.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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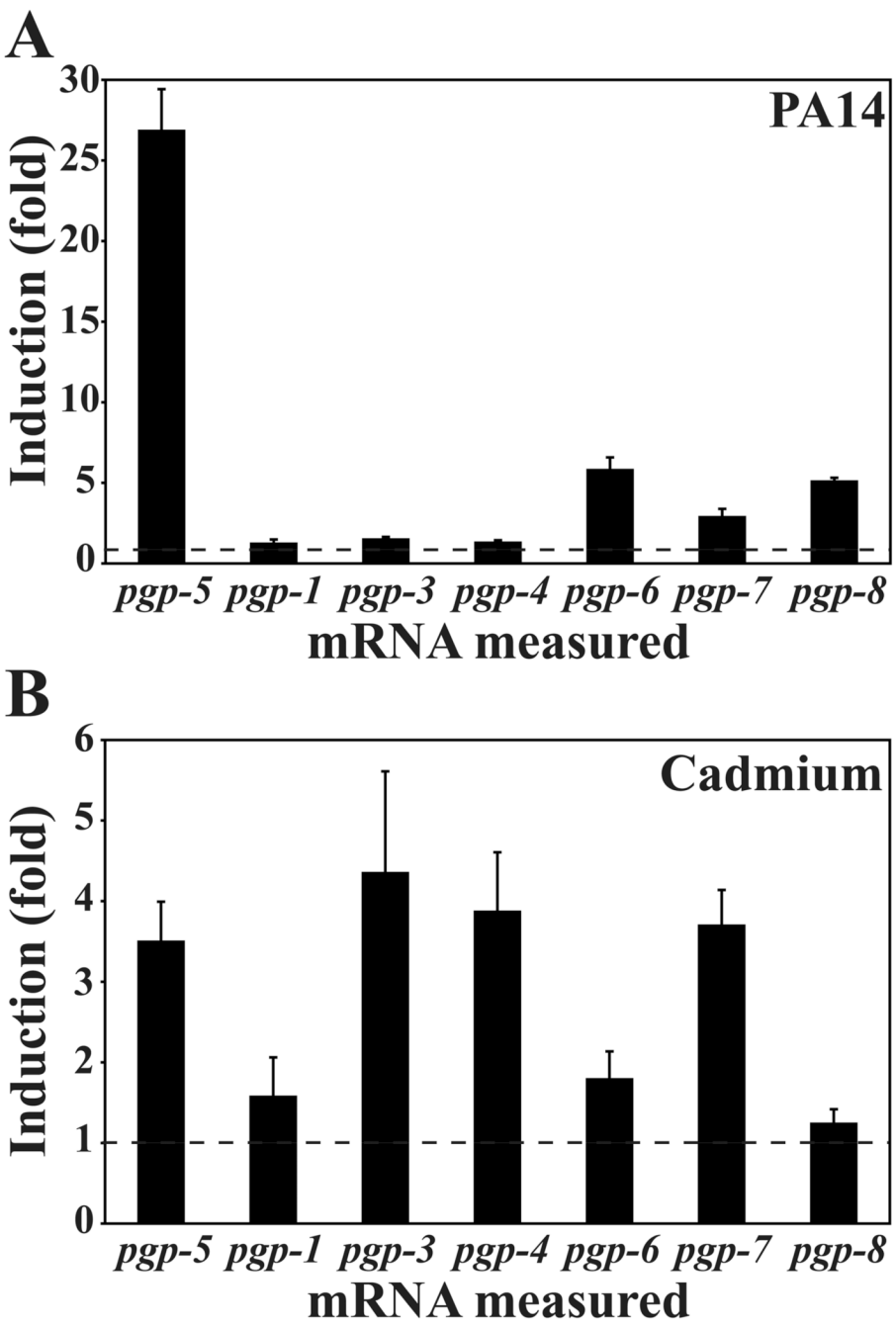

Figure 1. mRNA levels of pgp genes in PA14-infected and cadmium-exposed animals

mRNA level measured by qRT-PCR after exposure to PA14 (A) or OP50 with 50 μM $CdCl_2$ in the media (B) for 24 h at 25ºC. Fold induction corresponds to the ratio between mRNA levels from animals under test conditions and mRNA levels from animals on OP50. Error bars correspond to standard deviation from 3 replicates in the same experiment. Each graph is representative of at least two independent experiments. Dotted line represents the basal expression on OP50.

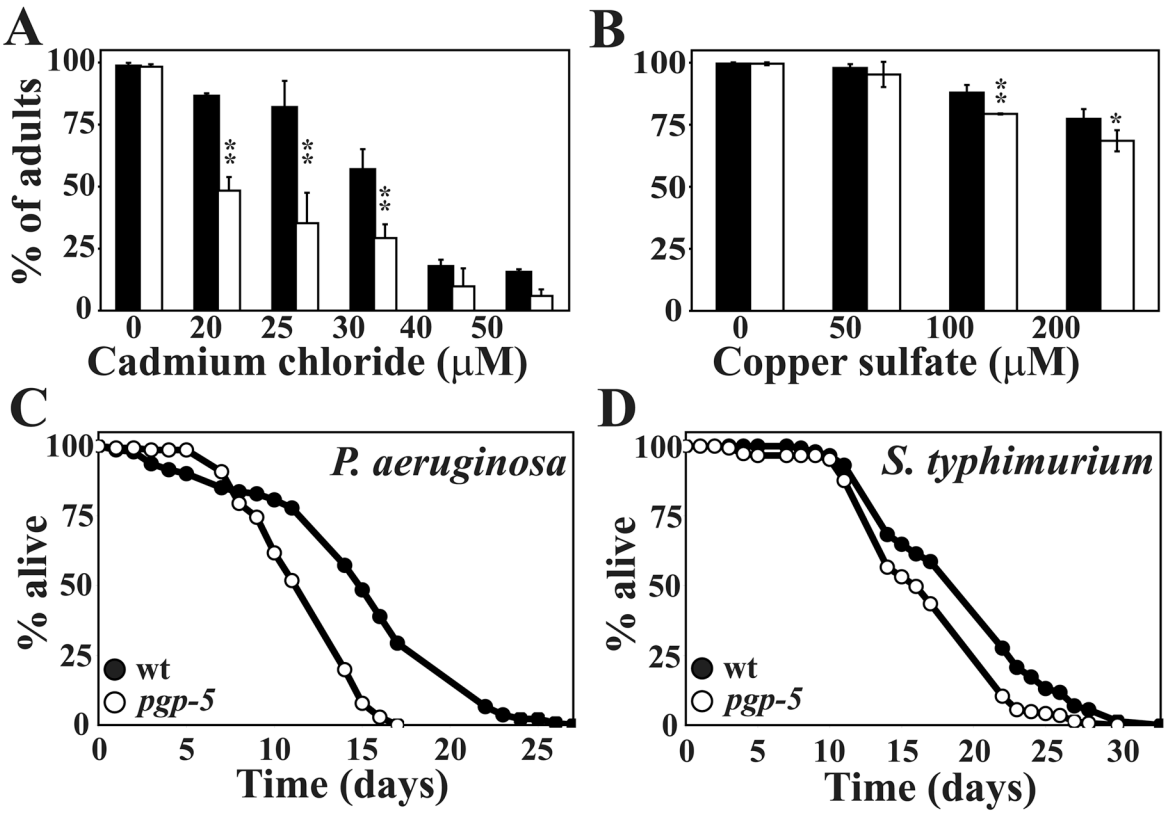

Figure 2. The pgp-5 mutant is susceptible to heavy metals and bacterial infection

Percent of N2 (closed bars) and *pgp-5(ok856)* mutant (open bars) to develop from eggs to adults in the presence of (A) $CdCl_2$ and (B) $CuSO_4$. The number of animals quantified from left to right were in (A) 351, 521, 715, 607, 641, 578, 444, 634, 769, 529, 325, 491, and in (B) 162, 135, 500, 470, 446, 449, 485, 493. Representatives of at least two independent experiments are shown. * represents $p < 0.05$ and ** represents $p < 0.0001$ by Student's t-test. (C and D) Survival of N2 (closed circles) and *pgp-5(ok856)* mutant (open circles) infected with *P. aeruginosa* PA14 (C) or *S. typhimurium* SL1344 (D).

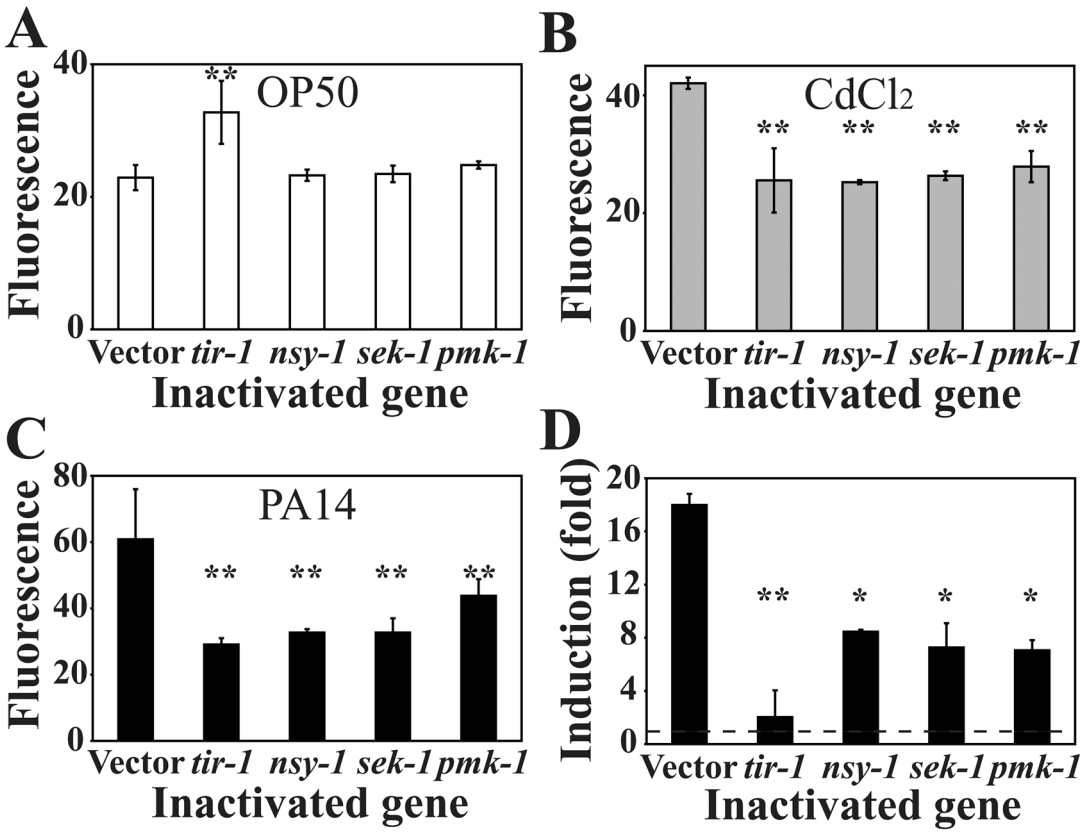

Figure 3. The p38 MAPK signaling cascade and TIR-1 are required for pgp-5 induction upon bacterial infection and cadmium exposure

COPAS biosort quantification of green fluorescence in p*pgp-5::gfp* animals treated with a given dsRNA followed by exposure to either OP50 (A), 100 μM $CdCl_2$ (B) or PA14 (C). Column represents mean ± SD of green fluorescence in arbitrary, but constant units. For each bar from left to right, the total number of animals tested (n) from two independent experiments were: (A), n = 645, 290, 1213, 918 and 460, in (B), n = 548, 784, 449, 403, and 813, and in (C), n = 346, 598, 784, 410 and 390. (D) Effect of dsRNA-treatment (x-axis) on fold induction of *pgp-5* by PA14 infection (y-axis) as determined by qRT-PCR. Column represents mean ± SD of fold induction, which is the ratio of mRNA levels between animals infected with PA14 and animals fed with OP50. Representative of 2 independent experiments, each with 3 replicates, is shown. Dotted line in (D) represents the basal expression level on OP50. * represents $p < 0.01$ and ** represents $p < 0.0001$ by Student's t-test.

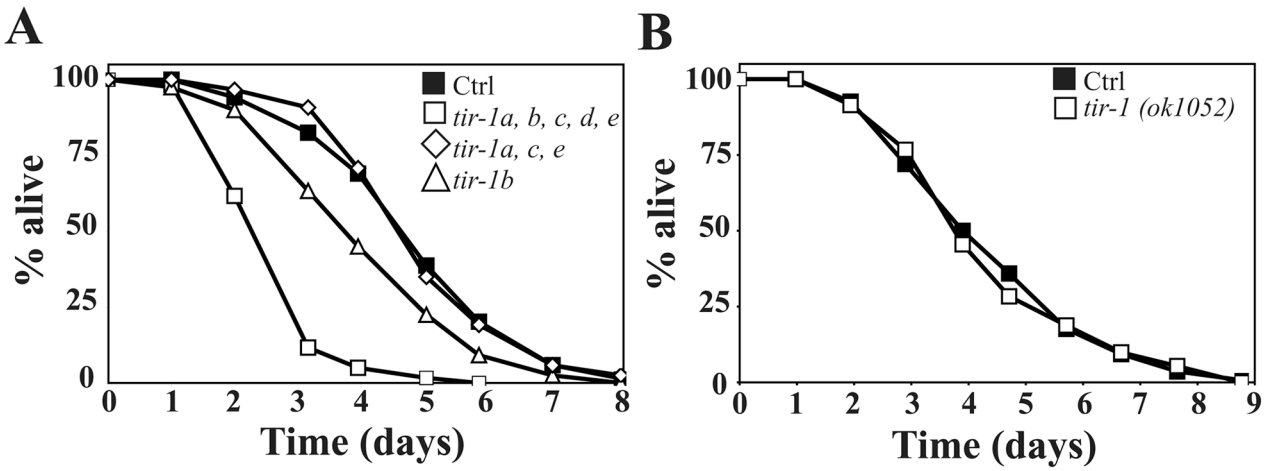

Figure 4. TIR-1b, but not TIR-1ac,e is required for the C. elegans resistance to the PA14 infection

(A) Survival of animals treated with either control (closed squares, TD_{mean}=115 ± 8.5 hours), *tir-1* (open squares, TD_{mean}=67 ± 10 hours), *tir-*, TD_{mea}*1b* (triangle, TD_{mean}=88 ± 8 hours) or *tir-1 a,c,e* (open squares, TD_{mean} of 109 ± 11 hours) dsRNA at the L1 stage for 48 h then exposed to PA14 at 25°C. (B) Survival curves of *tir-1(ok1052)* (open squares) and N2 animals (closed squares) exposed to PA14 at 25°C.