Caenorhabditis elegans saposin-like *spp-9* is involved in specific innate immune responses

Bhoomi Madhu¹ · Mohammed Farhan Lakdawala¹ · Neethu G. Issac¹ · Tina L. Gumienny¹

Received: 4 March 2020 / Revised: 20 July 2020 / Accepted: 24 July 2020 / Published online: 7 August 2020
© The Author(s), under exclusive licence to Springer Nature Limited 2020

Abstract

Animals counter specific environmental challenges with a combination of broad and tailored host responses. One protein family enlisted in the innate immune response includes the saposin-like antimicrobial proteins. We investigated the expression of a *Caenorhabditis elegans* saposin-like gene, *spp-9*, in response to different stresses. *spp-9* expression was detected in the intestine and six amphid neurons, including AWB and AWC. *spp-9* expression is increased in response to starvation stress. In addition, we discovered pathogen-specific regulation of *spp-9* that was not clearly demarcated by Gram nature of the bacterial challenge. Multiple molecular innate immune response pathways, including DBL-1/TGF- β -like, insulin-like, and p38/MAPK, regulate expression of *spp-9*. Our results suggest *spp-9* is involved in targeted responses to a variety of abiotic and bacterial challenges that are coordinated by multiple signaling pathways.

Introduction

All living organisms possess mechanisms to protect themselves from potentially harmful environments [1, 2]. Roundworms are a diverse group of organisms (comprising both free-living and parasitic members) that have adapted to various habitats ranging from terrestrial to marine environments [3]. In these environments, roundworms encounter a variety of challenges, including low food availability and pathogenic bacteria [4]. *Caenorhabditis elegans*, a free-living roundworm, is an established model organism to understand defense mechanisms nematodes use, including innate immune responses to bacteria [5]. It is important that these responses are well coordinated and appropriately regulated by the host [1, 2]. Aberrant activation of host immune responses could be a potential cellular stress for the host, making tight regulation of host immune responses important for host health in low-stress environments, as

well [6, 7]. How animals respond to the various challenges in their natural environment by mounting specific defenses is not well understood.

Several pathogens infect *C. elegans*, including bacterial strains *Pseudomonas aeruginosa*, *Staphylococcus aureus*, *Salmonella enterica* serovar Typhimurium, *Serratia marcescens*, *Enterococcus faecalis*, *Mycobacterium fortuitum*, *Bacillus megaterium*, *Microbacterium nematophilum*, and *Staphylococcus epidermidis*. *C. elegans* is also infected by fungi, including *Candida albicans* and *Drechmeria coniospora* [8–15]. MAPK signaling, insulin-like signaling, and DBL-1/TGF- β -like signaling pathways are induced upon infection by these pathogens [8–13, 16–20]. These pathways regulate an overlapping set of antimicrobial genes, which suggests crosstalk between the pathways upon infection [18, 21, 22]. These antimicrobial genes encode lipases, lysozymes, defensin-like proteins, and saposin-like proteins (SAPLIPs or caenopores).

C. elegans caenopores have structural similarities with the protozoan amoebapores and the mammalian peptides NK-lysin and granulysin [23, 24]. 28 genes are predicted to encode 33 SAPLIP-domain containing proteins, but antimicrobial activity of only a few saposins (SPPs) has been characterized. Functional analyses of SPP-3, SPP-5, and SPP-12 show that they display pore-forming activity, permeabilize the cytoplasmic membrane of bacteria, and kill bacteria [24–26]. SPP-1 is required for protection of *C. elegans* against *S. enterica* serovar Typhimurium and

Supplementary information The online version of this article (<https://doi.org/10.1038/s41435-020-0108-6>) contains supplementary material, which is available to authorized users.

✉ Tina L. Gumienny
tgumienny@twu.edu

¹ Department of Biology, Texas Woman's University, Denton, TX 76204-5799, USA

P. aeruginosa through the DAF-2/DAF-16 signaling pathway [18, 27]. Some *spp* genes are highly up- or down-regulated in response to bacterial challenges [18, 22, 24–27]. In addition, one caenopore gene, *spp-3*, has been shown to be induced in starved animals [24, 25]. Although some caenopores are strongly expressed in the intestine, some are also expressed in specific nerves [18, 24–28]. One caenopore, *spp-12*, is expressed only in NSML/R and I6 pharyngeal neurons [26].

To expand our understanding of the function of caenopores within the context of innate immunity and stress, we chose *spp-9*, one member of the caenopore family of antimicrobial proteins. Phylogenetic analyses show that *spp-9* is closely related to *spp-3*, whose role as an antimicrobial protein has been functionally characterized [24, 25]. *spp-9* is expressed in intestine, an organ on the front line of innate immunity [21]. *spp-9* expression is upregulated in response to loss of DKF-2/protein kinase D [29]. *spp-9* is also negatively regulated by the DBL-1 signaling pathway and is used as DBL-1 pathway reporter [21, 30]. While *spp-9* has been shown to be a DBL-1-responsive gene, here we characterized the role of *spp-9* in the larger context of starvation stress and innate immunity. We also determined additional molecular innate immune/stress pathways that regulate *spp-9* expression in *C. elegans*.

Materials and methods

Strains and maintenance

All *C. elegans* strains were maintained on EZ media plates at 20 °C except *daf-2* strains, which were maintained at 17 °C, unless specified otherwise [31]. Strains used were: wild-type N2, NU3 *dbl-1(nk3)* V, TLG182 *texIs100[dbl-1::dbl-1:gfp; ttx-3p::rfp]* IV, NL2099 *rff-3(pk1426)* II, TLG697 *texIs127[spp-9p::gfp]* X, LT998 *wkIs40[spp-9p::gfp]*, TLG707 *dbl-1(nk3)* V; *texIs127* X, TLG755 *oyIs44[odr-1::RFP; lin-15(+)]* V; *texIs127* X, TLG756 *daf-16(mu86)* I; *texIs127* X, TLG757 *pmk-1(km25)* IV; *texIs127* X, TLG758 *wkIs40; mut-2(r459)* I; *mek-1(pk97)* X, TLG759 *daf-2(e1370)* III; *texIs127* X, TLG760 *wkIs40; sek-1(km4)* X, TLG761 *wkIs40; tir-1(tm3036)* III. These strains were generated by standard genetic crosses and confirmed by PCR.

The bacteria used in this study include *Bacillus megaterium* (Carolina Biological Supply Company), *Escherichia coli* (OP50), *Enterobacter cloacae* (49141TM), *Klebsiella oxytoca* (49131TM), *Serratia marcescens* (Carolina Biological Supply Company), and *Staphylococcus epidermidis* (49134TM). All bacteria were grown in tryptic soy broth for 9 h at 37 °C. Bacterial cells were pelleted at 5000 rpm for 15 min and concentrated 20-fold. EZ media plates were

freshly seeded with concentrated bacteria in full lawns. The plates were incubated at 37 °C overnight before they were used in experiments.

Dauer assay

daf-2(e1370) III; *texIs127* X and *texIs127* X populations were synchronized as embryos by bleaching [32]. Animals were transferred to 25 °C at the L2 stage. Dauered *daf-2(e1370)* III; *texIs127* X animals and L4 *texIs127* X controls were picked. Two days later, animals were imaged as dauer (*daf-2(e1370)* III; *texIs127* X) or adults (*texIs127* X). The experiment was performed in three independent trials.

Imaging

Fluorescence of the *spp-9* reporter strain at different developmental stages was captured by a Nikon A1 confocal system (Nikon Instruments, Melville, NY). Colocalization studies of *oyIs44* V; *texIs127* X animals were performed on a Nikon swept-field confocal system (Nikon Instruments, Melville, NY). Hermaphrodites were synchronized as embryos by bleaching and imaged 48 h after the L4 stage at 20 °C, unless otherwise noted [32]. *daf-16(mu86)* I; *texIs127* X animals and respective control animals (*texIs127* X) were grown at 25 °C and imaged 48 h after the L4 stage. Fluorescence of the *spp-9* reporter strains was captured by a Nikon DS-Ri2 camera mounted on a Nikon SMZ18 dissecting microscope (Nikon Instruments, Melville, NY). Animals were mounted on 2% agarose pads and anesthetized by using 1 mM levamisole. At least 15 animals were imaged per condition as determined by power analysis with a moderate effect size. The microscope conditions were optimized with respect to the control and test conditions and kept consistent within each trial. However, imaging exposure times were different between some trials to prevent saturation of signal in experimental conditions. All imaging experiments were performed in three independent trials. Mean fluorescence intensities were measured using the Nikon NIS Elements AR v5.02 software and were analyzed using the unpaired *t* test.

RNA isolation and qRT-PCR

Total RNA was extracted from animals at 48 h after the L4 stage. Animals were synchronized by bleaching [32]. Total RNA was extracted by the freeze cracking method as previously described [33]. After RNA isolation, 2 µg of total RNA was primed with oligo(dT) and reverse transcribed to yield cDNA using the SuperScript III reverse transcriptase kit as per manufacturer's protocol (Invitrogen). Real-time PCR was performed on QuantStudio3 (Applied Biosystems by Thermo Fisher Scientific) instrument using the PowerUP

Fig. 1 The *spp-9* reporter is expressed throughout larval and adult stages in intestine and head neurons. Confocal images show expression of *spp-9p::GFP* in (a) L1, (b) L2, (c) L3, (d) L4, and (e) 1-day adult in the intestine, with strong expression in the anterior and posterior ends of intestine. f Rotated swept-field confocal projection

reveals *spp-9p::GFP* is expressed in six head neurons, colocalizing with ODR-1::RFP in AWB and AWC head neurons of an adult animal (marked by filled and unfilled arrowheads, respectively). An asterisks mark a third neuron that expresses the *spp-9* reporter. Scale bar indicates a, b 50 μ m, c, d, e 100 μ m, and (f) 10 μ m.

SYBR Green master mix (Applied Biosystems) according to manufacturer's instructions. The experiment was performed in three technical replicates for each condition. Primer sequences for *dbl-1* are forward primer GCCATTCTCCACCTCTTCCT and reverse primer GGAACATCAATGCTCGGACC [34]. Primer sequences for *spp-9* are forward primer GTTCTCTTTCTGGTTGCGGT and reverse primer GCTCTACAAACATCTTCTGGTGCA. Primer sequences for *act-1* are forward primer CCATCATGAAGTGCGACATTG and reverse primer CATGGTTGATGGGGCAAGAG [13]. QuantStudio Design and Analysis Software v1.5.1 was used to calculate raw C_t values and to normalize the values for *dbl-1* and *spp-9* to the housekeeping gene *act-1* (actin) (Applied Biosystems by Thermo Fisher Scientific). Fold change in gene expression between experimental sample and the wild-type control was determined by this software using the formula: $2^{(-\Delta\Delta C_t)}$. Experimental ΔC_t values were compared with wild-type ΔC_t values using the unpaired *t* test.

Results

spp-9 localizes in the intestine and head neurons at all developmental stages

To determine where and when *spp-9* is expressed, we used a strain expressing an integrated transgene with the *spp-9* promoter driving expression of green fluorescent protein (*spp-9p::GFP*). Promoter activity of *spp-9* was observed in the entire intestine of animals, strongly in the anterior and posterior ends (Fig. 1a–e). *spp-9* transgene expression in the

intestine was visible at all developmental stages starting from the first larval stage up to adulthood. We also observed *spp-9* expression in six head neurons (Fig. 1f). We confirmed the expression of *spp-9* in the AWB and AWC neurons by showing colocalization with a fluorescent marker, *odr-1::RFP* (*oyIs44*), that is specifically expressed in the two AWB and two AWC sensory neurons [35, 36]. The *spp-9* reporter was also visible in a third amphid pair. VisCello for Visualization of Single Cell Data expression data suggests *spp-9* is expressed in the ASH neurons, which is consistent with the observed position of this third neuron pair [37]. Because robust intestinal expression of *spp-9* was observed only after L3, we quantitated *spp-9* reporter activity at 24 h, 36 h, and 48 h after staging animals at L4. We observed an increase in reporter activity over this time frame, with the highest level of expression detected in this time frame in two-day old adults (Fig. S1). These results indicate that *spp-9* is expressed majorly in the intestinal tissue and also in six head neurons throughout all the developmental stages of animals, with *spp-9* intestinal expression increasing from late larval to adult stages.

spp-9 expression is increased in starved animals

Animals can mount responses to a variety of environmental stresses. Loss of some *dpy* genes that encode cuticle collagens induce glycerol, osmotic, and detoxification responses, but do not change *spp-9* reporter expression [30, 38–41]. Starvation is another stress, and *spp-3* expression is induced in starved animals [24, 25]. However, *spp-5* expression, which is constitutively expressed and remains unchanged in pathogenic conditions, is also

Fig. 2 Starvation increases *spp-9* reporter activity. Fluorescence images show *spp-9p::GFP* intensities of (a) fed L4 animals and (b) age-matched animals starved for 8 h post L4 stage. Imaging conditions including exposure times were consistent. Scale bar indicates 100 μ m. c Comparison of *spp-9p::GFP* intensities of fed animals (control) with

starved animals indicates that *spp-9* reporter activity was significantly increased in starved animals in comparison to fed animals ($p = 0.0003$). One representative trial of three is presented. Error bars represent 95% confidence intervals. $n = 30$ per condition. $*p < 0.05$ compared with fed control by unpaired t test.

high in starved animals [24]. We asked if starvation affects expression of *spp-9*. By 8 h without food starting in the L4 stage, *C. elegans* show dramatic organismal responses, including a global reduction in protein synthesis and changes in stress response pathway gene expression [42]. L4 animals that were starved were imaged 8 h after starvation and compared with identically staged animals that remained fed during that time. *spp-9* expression was significantly increased when animals were deprived of food (Fig. 2). This suggests that expression of *spp-9* is upregulated in starved animals.

***spp-9* expression is altered in response to select Gram-positive and Gram-negative pathogens**

Because caenopores are predicted antimicrobial effector genes, a subset of this 23-member family has been tested for induction upon pathogen challenge. Expression of *spp-1* is induced upon infection with *S. enterica* serovar Typhimurium [43]. *spp-3* expression is induced upon confrontation with two Gram-positive bacterial species, *B. megaterium* and *M. luteus*, compared with normal lab food *E. coli* OP50, but was not induced on a different Gram-positive bacterium (*Lactobacillus lactis*) or any of the four Gram-negative species tested. Expression of *spp-5*, though, was consistent in animals exposed to different Gram-positive and Gram-negative bacteria, including *E. coli* [24]. To determine if expression of *spp-9* is responsive to exposure to pathogenic bacteria, we detected activity of the *spp-9p::gfp* reporter in response to a panel of Gram-positive and Gram-negative bacterial exposures. This panel included two Gram-positive (*B. megaterium* and *S. epidermidis*) and three Gram-negative strains (*E. cloacae*, *K. oxytoca*, and *S. marcescens*), plus the standard lab food, Gram-negative *E. coli* OP50. Animals expressing *spp-9p::gfp* were synchronized as L4s and fed on Gram-positive or Gram-negative bacteria for 48 h. Animals were tested at 48 h because by

that time, there was a robust response of the *spp-9* reporter on *E. coli* (Fig. S1). Basal expression was measured for *spp-9p::GFP* fluorescence in animals fed *E. coli* OP50 (Fig. 3a, e, h). We observed a significant increase in the *spp-9* reporter activity when animals were fed on select Gram-positive (*B. megaterium* and *S. epidermidis*), and Gram-negative (*K. oxytoca*) bacteria (Fig. 3b–d, g, j). On the other hand, exposure to *S. marcescens* caused a significant decrease in *spp-9* expression in two of the three independent trials (Fig. 3i, j). This reduced *spp-9* expression is consistent with previous studies showing exposure to *S. marcescens* induces the DBL-1 signaling pathway (which negatively regulates *spp-9*) [44]. Our results suggest that activity of *spp-9* is differentially regulated in response to specific Gram-positive and Gram-negative bacterial challenges.

***dbl-1* regulates endogenous *spp-9* expression levels**

Studies by our lab and others have shown that the activity of *spp-9* is highly regulated by DBL-1 in an inverse fashion. Animals overexpressing DBL-1 show reduced *spp-9p::GFP* activity and animals lacking DBL-1 display high reporter activity [21, 30]. We tested if DBL-1 regulates endogenous levels of *spp-9* expression by quantitative real-time PCR, comparing *spp-9* expression levels from *dbl-1* mutants to the wild-type control. Animals with the *dbl-1(nk3)* null allele, a deletion that deletes about 5 kb of the 3' end of the 7 kb open reading frame, have almost no *dbl-1* mRNA detected by qRT-PCR. Animals overexpressing *dbl-1* have about a 10-fold increase in *dbl-1* mRNA levels (Fig. S2a). We see an increase (about 2.6-fold) in the expression of *spp-9* in animals lacking *dbl-1*, whereas we see a decrease (about 0.5-fold), however not significant, in *spp-9* mRNA levels of animals overexpressing *dbl-1* (Fig. S2b). These qRT-PCR results support previously reported microarray results and the use of *spp-9p::GFP* as a reporter for DBL-1 pathway signaling [21, 30].

Fig. 3 *spp-9* reporter activity is altered upon exposure to specific Gram-positive and Gram-negative bacteria. Fluorescence images show *spp-9p*::GFP intensities in adult animals after a two-day exposure to the following bacteria; (a, e, h) control *E. coli* OP50 ($n = 17$, 20, 20), (b) *B. megaterium* ($n = 17$), (c) *S. epidermidis* ($n = 20$), (f) *E. cloacae* ($n = 21$), (g) *K. oxytoca* ($n = 21$), or (i) *S. marcescens* ($n = 21$). Imaging conditions including exposure times were consistent with respective controls. Scale bar indicates 100 μm. **d** Comparison of *spp-9p*::GFP intensities of wild-type animals fed on *E. coli* OP50 (control) with animals fed on *B. megaterium* or *S. epidermidis*. *spp-9p*::GFP intensity was significantly increased when animals were exposed to *B.*

megaterium and *S. epidermidis* in comparison to the control ($p < 0.0001$). **j** Comparison of *spp-9p*::GFP intensities of wild-type animals fed on *E. coli* OP50 (control) with animals fed on *E. cloacae*, *K. oxytoca*, or *S. marcescens*. No significant change in *spp-9p*::GFP intensity was seen in animals fed on *E. cloacae* ($p = 0.1353$). Exposure to *K. oxytoca* caused a significant increase in the GFP intensity as compared with the control ($p < 0.0001$). On the other hand, exposure to *S. marcescens* caused a significant decrease in the GFP intensity in comparison to the control ($p = 0.0153$). One representative trial of three is presented. Error bars represent 95% confidence intervals. * $p < 0.05$ compared with *E. coli* OP50 by unpaired *t* test.

***spp-9* expression depends on DBL-1 and other innate immune response signaling pathways, depending on pathogen**

DBL-1 signaling pathway regulates expression of many innate immunity genes and is also involved in mounting protective immune responses against pathogens [13, 18, 44–48]. We showed that the DBL-1 pathway target gene *spp-9* is differentially regulated upon exposure to different types of Gram-positive and Gram-negative bacteria. Therefore, we asked if the differential regulation of the *spp-9* reporter activity in response to the panel of Gram-positive and Gram-negative bacteria is DBL-1 mediated. To test this, we exposed *spp-9p::gfp* and *dbl-1(-); spp-9p::gfp* animals to our panel of different bacteria at the L4 stage. These animals were imaged 48 h after L4 stage and

we measured and compared GFP intensities of the two genotypes fed on different bacteria. In three independent trials, the *dbl-1(-); spp-9p::gfp* animals when fed on standard lab food, *E. coli* strain OP50, showed a significantly increased fluorescence, consistent with previous reports (Fig. 4a, e) [21, 30]. We observed that animals lacking *dbl-1* when fed on *S. epidermidis*, *E. cloacae*, and *S. marcescens* showed a further increase in fluorescence in comparison to the control (*dbl-1(-); spp-9p::gfp* animals fed on *E. coli* OP50) (Fig. 4c, d, f, h, i). These findings indicate that loss of *dbl-1* increases the *spp-9* reporter activity and exposure to select Gram-positive (*S. epidermidis*) and Gram-negative (*E. cloacae* and *S. marcescens*) bacteria further increases the reporter activity when *dbl-1* is absent, suggestive of an additive effect on *spp-9* reporter activity, perhaps by other innate immune response pathways.

Fig. 4 *spp-9* reporter activity is altered in response to specific pathogens by DBL-1 and other innate immunity signaling pathways. Fluorescence images show *spp-9p::GFP* intensities in adult *dbl-1(-)* animals after a two-day exposure to the following bacteria; (a, e) control *E. coli* OP50 ($n = 20$), (b) *B. megaterium* ($n = 18$), (c) *S. epidermidis* ($n = 26$), (f) *E. cloacae* ($n = 19$), (g) *K. oxytoca* ($n = 22$), or (h) *S. marcescens* ($n = 21$). Imaging conditions including exposure times were consistent with respective controls. Scale bar indicates 100 μ m. **d** Comparison of *spp-9p::GFP* intensities in *dbl-1(-)* animals fed on *E. coli* OP50 (control) with animals fed on *B. megaterium* or *S. epidermidis*. No significant change in *spp-9p::GFP* intensity was seen

in animals fed on *B. megaterium* ($p = 0.3534$). The GFP intensity was significantly increased when animals were exposed to *S. epidermidis* in comparison to the control ($p = 0.0117$). **i** Comparison of *spp-9p::GFP* intensities in *dbl-1(-)* animals fed on *E. coli* OP50 (control) with animals fed on either *E. cloacae*, *K. oxytoca*, or *S. marcescens*. Exposure to *E. cloacae* ($p = 0.0001$) and *S. marcescens* ($p = 0.0385$) caused an increase in the GFP intensity as compared with the control. Exposure to *K. oxytoca* caused a decrease in the GFP intensity in comparison to the control ($p = 0.0001$). One representative trial of three is presented. Error bars represent 95% confidence intervals. * $p < 0.05$ compared with *E. coli* OP50 by unpaired *t* test.

On the other hand, *dbl-1(-); spp-9p::gfp* animals showed similar mean fluorescence intensities when fed on *B. megaterium* in comparison to *dbl-1(-); spp-9p::gfp* animals exposed to the *E. coli* OP50 control (Fig. 4b, d). This response is different from *spp-9* reporter activity in the wild type, which showed significantly increased fluorescence with *B. megaterium* exposure as compared with the control (Fig. 3b, d). This lack of further *spp-9* reporter induction in *dbl-1* mutants on *B. megaterium*, like was observed on *E. cloacae*, *S. marcescens*, and *S. epidermidis*, indicates that *spp-9* reporter activity induced on *B. megaterium* is DBL-1 mediated (Figs. 3 and 4).

Interestingly, while animals lacking *dbl-1* fed on *K. oxytoca* displayed increased fluorescence than *spp-9p::gfp* animals, that increase was significantly less in comparison to the same genotype fed on *E. coli* in two out of three trials (Figs. 3 and 4, data from both genotypes was collected in each trial). This suggests that some other signaling pathway

that negatively regulates *spp-9* activity is induced in *dbl-1(-)* animals upon exposure to *K. oxytoca*, resulting in decrease of *spp-9* activity. This suggests that the reporter activity response to *K. oxytoca* exposure depends on an innate immune response that is at least partially independent of *dbl-1*.

***spp-9* reporter activity is regulated by innate immunity/stress signaling pathways**

Because we see an additive effect of DBL-1 and infection on the activity of *spp-9*, and also DBL-1-independent but pathogen-dependent regulation of *spp-9*, we asked whether *spp-9* is also regulated by other signaling pathways in the context of immunity. Besides the DBL-1 signaling pathway, two major pathways that are required for animals to respond to pathogens are the insulin-like and p38/MAPK signaling pathways [19, 49, 50]. The insulin-like pathway is defined

Fig. 5 *spp-9* reporter activity is regulated by innate immunity signaling pathways. Fluorescence images show *spp-9p::GFP* intensities in two-day adults with (a, e, i, l) wild-type ($n = 24, 23, 19, 19$), (b) *daf-2*(-) ($n = 24$), (c) *daf-16*(-) ($n = 20$), (f) *sek-1*(-) ($n = 23$), (g) *tir-1*(-) ($n = 27$), (j) *pmk-1*(-) ($n = 20$), and (m) *mek-1*(-) ($n = 22$) backgrounds. Imaging conditions including exposure times were consistent with respective controls. Scale bar indicates 100 μ m. **d** Comparison of *spp-9p::GFP* intensities in wild-type (control), *daf-2*(-), and *daf-16*(-) backgrounds. Loss of *daf-2* caused a decrease in the GFP intensity ($p < 0.0001$), whereas loss of *daf-16* caused an increase in the GFP intensity ($p < 0.0001$) as compared with the control.

by the insulin receptor DAF-2 and the downstream master transcriptional regulator DAF-16. Loss of *daf-2* function not only causes animals to constitutively enter dauer but also increases resistance to pathogens. Loss of *daf-16* prevents animals from entering dauer and increases sensitivity to infection [12, 49, 51–54]. We measured reporter fluorescence intensities in *daf-2*(-) and *daf-16*(-) backgrounds and compared it to the reporter in the wild-type background. Interestingly, we observed a significant reduction in *daf-2*(-) mutants (Fig. 5b, d). As expected, animals lacking *daf-16*

showed increased *spp-9* reporter fluorescence (Fig. 5c, d). These findings suggest that *spp-9* is responsive to changes in insulin-like signaling: DAF-2 promotes, while DAF-16 normally represses, *spp-9* expression. The p38/MAPK signaling pathway includes TIR-1, TIR (Toll and Interleukin 1 Receptor) domain protein, which activates MAP3K, which activates MAP2K SEK-1, which activates MAPK PMK-1 [55–57]. We captured and measured GFP intensities of the reporter in *pmk-1*(-), *sek-1*(-), or *tir-1*(-) mutant backgrounds and compared them to the wild-

type reporter control. We observed a significant increase in the mean GFP intensities in *tir-1(-)* and *pmk-1(-)* mutants (Fig. 5g, h, j, k). However, loss of MAP2K gene *sek-1(-)* had no effect on the fluorescent intensities in comparison to the control (Fig. 5f, h). We then tested if MEK-1, a stress-responsive MAP2K that can also activate PMK-1 independent of TIR-1, affected *spp-9* reporter activity [56]. Loss of *mek-1*, like loss of *sek-1*, also had no effect on *spp-9* reporter fluorescence (Fig. 5m, n). These findings suggest that the MAPK pathway defined by *tir-1* and *pmk-1* normally suppresses *spp-9* activity, but does so using a MAP2K other than SEK-1 or MEK-1. Alternatively, these MAP2Ks act redundantly in the pathway that leads to expression of *spp-9*. Together, these results indicate *spp-9* is regulated by multiple signaling pathways.

Discussion

Here, we show that SPP-9 has a unique role within its large family of saposin-like, pore-forming proteins in *C. elegans*, and its expression is modulated to respond to specific pathogens and other stressors.

spp-9 is visibly expressed from the first larval stage, when animals are initially exposed to environmental conditions, including pathogens, and this expression is maintained throughout the life of the animal. This observation is consistent with previous RNAseq and microarray analyses that do not detect *spp-9* expression in embryos (WormBase.org). Basal expression of *spp-9* is observed in the intestine of animals fed normal lab food, *E. coli*. Other saposin family members play roles in the digestion of the roundworm's bacterial food. SPP-9 may also help digest bacteria and is upregulated in some environments with pathogenic bacteria. However, our work supports a role for *spp-9* in stress response, not just digestion. For one, *spp-9* expression is increased in starvation conditions (Fig. 2). *spp-3* and *spp-5* have also been shown to be expressed in starved adults [24]. Second, *spp-9* is expressed in the AWB and AWC sensory neuron pairs, which play roles in sensing numerous stimuli, including bacterial food sources [58–61]. *C. elegans* uses AWB to sense and avoid serrawettin W2, a chemical secreted by the pathogenic *S. marcescens* [62]. *spp-9* expression in amphid neurons might contribute to sensing different pathogens or other stresses. Some other *C. elegans* saposins are expressed not only in the intestine, but also or instead in a specific neuron or a few neurons. *spp-3* is expressed in the SDQR interneuron, *spp-12* is expressed in NSM/L and I6, which can sense bacteria, including pathogens, and *spp-7* is expressed in head neurons [25, 26]. Neurons in *C. elegans* and other species express saposins and other antimicrobial proteins, possibly to protect the

environmentally exposed neurons or surrounding tissues. Alternatively, the expression of different saposins in different environmentally exposed neurons could help specify distinct antimicrobial responses depending on the stimulus.

Third, a strong induction of *spp-9* was observed in animals exposed to some bacterial challenges, but not all, suggesting specificity of recruiting SPP-9 in a response that is not demarcated by Gram nature (Figs. 3 and 4). Furthermore, we discovered that *spp-9* is specifically upregulated by loss of specific innate immune/stress response pathways: the DBL-1/TGF- β -like pathway, the DAF-2/DAF-16 insulin-like pathway, and the p38/MAPK defined by TIR-1 and PMK-1, but independent of MAPKKs SEK-1 and MEK-1 (Figs. 4 and 5). This may explain why we see an additive induction of *spp-9* in animals lacking DBL-1 signaling and challenged with *E. cloacae*, *S. marcescens*, or *S. epidermidis*: other innate immune response pathways may be further inducing *spp-9* expression in this context. However, the response to *B. megaterium*, in which the *spp-9* induction depends on functional DBL-1 signaling, suggests that the animals use DBL-1 but not other innate immune pathways to induce a *spp-9* response to *B. megaterium*. However, the reduced *spp-9* induction upon exposure to *K. oxytoca* in *dbl-1* mutant animals suggests that other signaling pathways are dampening this response. These findings also support that DBL-1, insulin-like, and MAPK pathways act independently but converge to regulate *spp-9* expression. These results suggest that *spp-9* contributes to the “antimicrobial fingerprint” proposed by Alper et al., in which distinct molecular responses are generated against specific pathogen exposures [18].

spp-9 was identified as a highly regulated gene by the DBL-1 signaling pathway and a *spp-9* promoter-GFP transgene has been used as a reporter for this pathway [21, 30]. The changes observed in the *spp-9* transcriptional reporter are consistent with the changes in endogenous *spp-9* expression levels in different *dbl-1* backgrounds, indicating that the reporter activity is representative of endogenous *spp-9* expression levels. Our findings support that *spp-9* is a valid reporter of DBL-1 signaling, with the caveat that the experimental environment must be closely controlled.

In summary, we discovered that *spp-9* expression is induced by starvation, specific bacteria, and by not only DBL-1, but other innate immune/stress pathways. It will be of interest to determine the molecular role of SPP-9 in replete and stress conditions, and to identify how multiple signaling pathways coordinate *spp-9* expression. This work revealed further insights into the environmental responsiveness of the saposin family and how organisms generate complex, targeted molecular responses to physiological challenges.

Data availability

Strains are available upon request. Supplementary figures are available in Figshare.

Acknowledgements We thank Paul Yeatts and TWU CRDA for assistance with statistical analyses. The bacterial strains were provided by Amy Jo Hammett. We thank Robert Waterston for ASH neuron information. We thank Mehul Vora and Richard Padgett for *wkIs40* and Maxwell Heiman for *oys44*. Some strains were obtained from the *Caenorhabditis* Genetics Center (CGC), which is funded by NIH Office of Research Infrastructure Programs (P40 OD010440). We thank WormBase. We thank Laura Hanson, Yasar Arfat Kasu, and members of the Gumienny lab for constructive feedback. This work was supported by NIH R01GM097591 to TLG, a TWU Research Enhancement Program grant to TLG, internal funding by Texas Woman's University, a TWU Experiential Learning Scholar Award to BM, and a TWU Teaching and Research Grant for Equipment and Technology. This work is dedicated to Chimanlal Madhu and Darshan Madhu.

Compliance with ethical standards

Conflict of interest TLG work was funded by the NIH. The other authors declare no conflict of interest.

Publisher's note Springer Nature remains neutral with regard to jurisdictional claims in published maps and institutional affiliations.

References

- Medzhitov R, Janeway CA Jr. An ancient system of host defense. *Curr Opin Immunol*. 1998;10:12–5.
- Cui J, Chen Y, Wang HY, Wang RF. Mechanisms and pathways of innate immune activation and regulation in health and cancer. *Hum Vaccin Immunother*. 2014;10:3270–85.
- McSorley R. Adaptations of nematodes to environmental extremes. *Fla Entomologist*. 2003;86:138–42.
- Murfin KE, Dillman AR, Foster JM, Bulgheresi S, Slatko BE, Sternberg PW, et al. Nematode-bacterium symbioses-cooperation and conflict revealed in the “omics” age. *Biol Bull*. 2012;223:85–102.
- Engelmann I, Pujol N. Innate immunity in *C. elegans*. *Adv Exp Med Biol*. 2010;708:105–21.
- Cheesman HK, Feinbaum RL, Thekkiniath J, Dowen RH, Conery AL, Pukkila-Worley R. Aberrant activation of p38 MAP kinase-dependent innate immune responses is toxic to *Caenorhabditis elegans*. *G3*. 2016;G3:541–9.
- Pukkila-Worley R. Surveillance immunity: an emerging paradigm of innate defense activation in *Caenorhabditis elegans*. *PLoS Pathog*. 2016;12:e1005795.
- Couillault C, Ewbank JJ. Diverse bacteria are pathogens of *Caenorhabditis elegans*. *Infect Immun*. 2002;70:4705–7.
- Gravato-Nobre MJ, Nicholas HR, Nijland R, O'Rourke D, Whittington DE, Yook KJ, et al. Multiple genes affect sensitivity of *Caenorhabditis elegans* to the bacterial pathogen *Microbacterium nematophilum*. *Genetics*. 2005;171:1033–45.
- Begun J, Gaiani JM, Rohde H, Mack D, Calderwood SB, Ausubel FM, et al. Staphylococcal biofilm exopolysaccharide protects against *Caenorhabditis elegans* immune defenses. *PLoS Pathog*. 2007;3:e57.
- Wong D, Bazopoulou D, Pujol N, Tavernarakis N, Ewbank JJ. Genome-wide investigation reveals pathogen-specific and shared signatures in the response of *Caenorhabditis elegans* to infection. *Genome Biol*. 2007;8:R194.
- Singh V, Aballay A. Regulation of DAF-16-mediated innate immunity in *Caenorhabditis elegans*. *J Biol Chem*. 2009;284:35580–7.
- Zugasti O, Ewbank JJ. Neuroimmune regulation of antimicrobial peptide expression by a noncanonical TGF- β signaling pathway in *Caenorhabditis elegans* epidermis. *Nat Immunol*. 2009;10:249–56.
- Pukkila-Worley R, Ausubel FM, Mylonakis E. *Candida albicans* infection of *Caenorhabditis elegans* induces antifungal immune defenses. *PLoS Pathog*. 2011;7:e1002074.
- Dzakah EE, Waqas A, Wei S, Yu B, Wang X, Fu T, et al. Loss of miR-83 extends lifespan and affects target gene expression in an age-dependent manner in *Caenorhabditis elegans*. *J Genet Genomics*. 2018;45:651–62.
- Aballay A, Yorgey P, Ausubel FM. *Salmonella typhimurium* proliferates and establishes a persistent infection in the intestine of *Caenorhabditis elegans*. *Curr Biol*. 2000;10:1539–42.
- Garsin DA, Sifri CD, Mylonakis E, Qin X, Singh KV, Murray BE, et al. A simple model host for identifying Gram-positive virulence factors. *Proc Natl Acad Sci USA*. 2001;98:10892–7.
- Alper S, McBride SJ, Lackford B, Freedman JH, Schwartz DA. Specificity and complexity of the *Caenorhabditis elegans* innate immune response. *Mol Cell Biol*. 2007;27:5544–53.
- Kim DH, Ewbank JJ. Signaling in the innate immune response. *WormBook*. Pasadena, CA, 2018,1–35.
- Sun L, Zhi L, Shakoar S, Liao K, Wang D. microRNAs involved in the control of innate immunity in *Candida* infected *Caenorhabditis elegans*. *Sci Rep*. 2016;6:36036.
- Roberts AF, Gumienny TL, Gleason RJ, Wang H, Padgett RW. Regulation of genes affecting body size and innate immunity by the DBL-1/BMP-like pathway in *Caenorhabditis elegans*. *BMC Dev Biol*. 2010;10:61.
- Dierking K, Yang W, Schulenburg H. Antimicrobial effectors in the nematode *Caenorhabditis elegans*: an outgroup to the Arthropoda. *Philos Trans R Soc Lond B Biol Sci*. 2016;371:20150299.
- Banyai L, Patthy L. Amoebapore homologs of *Caenorhabditis elegans*. *Biochim Biophys Acta*. 1998;1429:259–64.
- Roeder T, Stanisak M, Gelhaus C, Bruchhaus I, Grotzinger J, Leippe M. Caenopores are antimicrobial peptides in the nematode *Caenorhabditis elegans* instrumental in nutrition and immunity. *Dev Comp Immunol*. 2010;34:203–9.
- Hoeckendorf A, Leippe M. SPP-3, a saposin-like protein of *Caenorhabditis elegans*, displays antimicrobial and pore-forming activity and is located in the intestine and in one head neuron. *Dev Comp Immunol*. 2012;38:181–6.
- Hoeckendorf A, Stanisak M, Leippe M. The saposin-like protein SPP-12 is an antimicrobial polypeptide in the pharyngeal neurons of *Caenorhabditis elegans* and participates in defence against a natural bacterial pathogen. *Biochem J*. 2012;445:205–12.
- Evans EA, Kawli T, Tan MW. *Pseudomonas aeruginosa* suppresses host immunity by activating the DAF-2 insulin-like signaling pathway in *Caenorhabditis elegans*. *PLoS Pathog*. 2008;4:e1000175.
- Murphy CT, McCarroll SA, Bargmann CI, Fraser A, Kamath RS, Ahringer J, et al. Genes that act downstream of DAF-16 to influence the lifespan of *Caenorhabditis elegans*. *Nature*. 2003;424:277–83.
- Ren M, Feng H, Fu Y, Land M, Rubin CS. Protein kinase D is an essential regulator of *C. elegans* innate immunity. *Immunity*. 2009;30:521–32.
- Lakdawala MF, Madhu B, Faure L, Vora M, Padgett RW, Gumienny TL. Genetic interactions between the DBL-1/BMP-like pathway and *dpy* body size-associated genes in *Caenorhabditis elegans*. *Mol Biol Cell*. 2019;30:3151–60.

31. Madhu BJ, Salazar AE, Gumienny TL. *Caenorhabditis elegans* egg-laying and brood-size changes upon exposure to *Serratia marcescens* and *Staphylococcus epidermidis* are independent of DBL-1 signaling. *microPublication Biology*. 2019. <https://doi.org/10.17912/2r51-b476>.
32. Stiernagle T. Maintenance of *C. elegans*. *WormBook*. Pasadena, CA, 2006;1–11.
33. Portman DS. Profiling *C. elegans* gene expression with DNA microarrays. *WormBook*. Pasadena, CA, 2006;1–11.
34. Kwon G, Lee J, Lim YH. Dairy Propionibacterium extends the mean lifespan of *Caenorhabditis elegans* via activation of the innate immune system. *Sci Rep*. 2016;6:31713.
35. Kim K, Kim R, Sengupta P. The HMX/NKX homeodomain protein MLS-2 specifies the identity of the AWC sensory neuron type via regulation of the *ceh-36* Otx gene in *C. elegans*. *Development*. 2010;137:963–74.
36. Low IIC, Williams CR, Chong MK, McLachlan IG, Wierbowski BM, Kolotuev I, et al. Morphogenesis of neurons and glia within an epithelium. *Development*. 2019;146:dev171124.
37. Packer JS, Zhu Q, Huynh C, Sivaramakrishnan P, Preston E, Dueck H, et al. A lineage-resolved molecular atlas of *C. elegans* embryogenesis at single-cell resolution. *Science*. 2019;365:eaax1971.
38. Wheeler JM, Thomas JH. Identification of a novel gene family involved in osmotic stress response in *Caenorhabditis elegans*. *Genetics* 2006;174:1327–36.
39. Lamitina T, Huang CG, Strange K. Genome-wide RNAi screening identifies protein damage as a regulator of osmoprotective gene expression. *Proc Natl Acad Sci USA*. 2006;103:12173–8.
40. Zugasti O, Thakur N, Belougne J, Squiban B, Kurz CL, Soule J, et al. A quantitative genome-wide RNAi screen in *C. elegans* for antifungal innate immunity genes. *BMC Biol*. 2016;14:35.
41. Dodd W, Tang L, Lone JC, Wimberly K, Wu CW, Consalvo C, et al. A damage sensor associated with the cuticle coordinates three core environmental stress responses in *Caenorhabditis elegans*. *Genetics* 2018;208:1467–82.
42. Larance M, Pourkarimi E, Wang B, Brenes Murillo A, Kent R, Lamond AI, et al. Global proteomics analysis of the response to starvation in *C. elegans*. *Mol Cell Proteom*. 2015;14:1989–2001.
43. Alegado RA, Tan MW. Resistance to antimicrobial peptides contributes to persistence of *Salmonella typhimurium* in the *C. elegans* intestine. *Cell Microbiol* 2008;10:1259–73.
44. Mallo GV, Kurz CL, Couillault C, Pujol N, Granjeaud S, Kohara Y, et al. Inducible antibacterial defense system in *C. elegans*. *Curr Biol*. 2002;12:1209–14.
45. Mochii M, Yoshida S, Morita K, Kohara Y, Ueno N. Identification of transforming growth factor- β -regulated genes in *Caenorhabditis elegans* by differential hybridization of arrayed cDNAs. *Proc Natl Acad Sci USA* 1999;96:15020–5.
46. Liang J, Yu L, Yin J, Savage-Dunn C. Transcriptional repressor and activator activities of SMA-9 contribute differentially to BMP-related signaling outputs. *Dev Biol*. 2007;305:714–25.
47. Zhang X, Zhang Y. DBL-1, a TGF- β , is essential for *Caenorhabditis elegans* aversive olfactory learning. *Proc Natl Acad Sci USA*. 2012;109:17081–6.
48. Berg M, Monnin D, Cho J, Nelson L, Crits-Christoph A, Shapira M. TGF- β /BMP immune signaling affects abundance and function of *C. elegans* gut commensals. *Nat Commun*. 2019;10:604.
49. Garsin DA, Villanueva JM, Begun J, Kim DH, Sifri CD, Calderwood SB, et al. Long-lived *C. elegans daf-2* mutants are resistant to bacterial pathogens. *Science* 2003;300:1921.
50. Troemel ER, Chu SW, Reinke V, Lee SS, Ausubel FM, Kim DH. p38 MAPK regulates expression of immune response genes and contributes to longevity in *C. elegans*. *PLoS Genet*. 2006;2:e183.
51. Kenyon C, Chang J, Gensch E, Rudner A, Tabtiang RA. *C. elegans* mutant that lives twice as long as wild type. *Nature*. 1993;366:461–4.
52. Kimura KD, Tissenbaum HA, Liu Y, Ruvkun G. *daf-2*, an insulin receptor-like gene that regulates longevity and diapause in *Caenorhabditis elegans*. *Science*. 1997;277:942–6.
53. Zhao L, Zhao Y, Liu R, Zheng X, Zhang M, Guo H, et al. The transcription factor DAF-16 is essential for increased longevity in *C. elegans* exposed to *Bifidobacterium longum* BB68. *Sci Rep*. 2017;7:7408.
54. Zhou M, Liu X, Yu H, Yin X, Nie SP, Xie MY, et al. Cell signaling of *Caenorhabditis elegans* in response to enterotoxigenic *Escherichia coli* infection and *Lactobacillus zeae* protection. *Front Immunol*. 2018;9:1745.
55. Kim DH, Feinbaum R, Alloing G, Emerson FE, Garsin DA, Inoue H, et al. A conserved p38 MAP kinase pathway in *Caenorhabditis elegans* innate immunity. *Science*. 2002;297:623–6.
56. Kim DH, Liberati NT, Mizuno T, Inoue H, Hisamoto N, Matsumoto K, et al. Integration of *Caenorhabditis elegans* MAPK pathways mediating immunity and stress resistance by MEK-1 MAPK kinase and VHP-1 MAPK phosphatase. *Proc Natl Acad Sci USA*. 2004;101:10990–4.
57. Liberati NT, Fitzgerald KA, Kim DH, Feinbaum R, Golenbock DT, Ausubel FM. Requirement for a conserved Toll/interleukin-1 resistance domain protein in the *Caenorhabditis elegans* immune response. *Proc Natl Acad Sci USA*. 2004;101:6593–8.
58. Bargmann CI, Hartwig E, Horvitz HR. Odorant-selective genes and neurons mediate olfaction in *C. elegans*. *Cell*. 1993;74:515–27.
59. Troemel ER, Kimmel BE, Bargmann CI. Reprogramming chemotaxis responses: sensory neurons define olfactory preferences in *C. elegans*. *Cell*. 1997;91:161–9.
60. Sagasti A, Hobert O, Troemel ER, Ruvkun G, Bargmann CI. Alternative olfactory neuron fates are specified by the LIM homeobox gene *lim-4*. *Genes Dev*. 1999;13:1794–806.
61. Olofsson B. The olfactory neuron AWC promotes avoidance of normally palatable food following chronic dietary restriction. *J Exp Biol*. 2014;217:1790–8.
62. Pradel E, Zhang Y, Pujol N, Matsuyama T, Bargmann CI, Ewbank JJ. Detection and avoidance of a natural product from the pathogenic bacterium *Serratia marcescens* by *Caenorhabditis elegans*. *Proc Natl Acad Sci USA*. 2007;104:2295–300.