THE DBL-1/TGF-β SIGNALING PATHWAY REGULATES AN ARRAY OF BEHAVIORAL, MOLECULAR, AND PHYSIOLOGICAL DEFENSES IN *CAENORHABDITIS ELEGANS*

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DEDICATION

To my parents, grandparents, uncle and aunt, Het, Anuj, Smit, Manan, Chintan, and my friends.

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ABSTRACT

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THE DBL-1/TGF-β SIGNALING PATHWAY REGULATES AN ARRAY OF BEHAVIORAL, MOLECULAR, AND PHYSIOLOGICAL DEFENSES IN *CAENORHABDITIS ELEGANS*

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Organisms possess mechanisms to protect themselves from environmental threats. Animals have innate immune responses that include behavioral, molecular, and physiological components. A conserved cell-cell signaling pathway, transforming growth factor-beta (TGF-β) signaling pathway, is a major regulator of innate immune responses in animals. However, the requirement for this pathway in generating specific, robust responses to different bacterial challenges has not previously been well characterized. We used the roundworm *Caenorhabditis elegans*, which has conserved innate immune responses and TGF-β signaling pathways, to address how organisms use TGF-β signaling to tailor immune responses to different environmental threats. We tested the requirement for DBL-1/TGF-β signaling in a diverse array of immune responses to a selected panel of Gram-negative and Gram-positive bacteria. We showed that robust, protective, and specific responses to different bacteria require functional DBL-1 signaling. Animals lacking DBL-1 were more susceptible to all test bacteria. We discovered that canonical DBL-1 signaling is required to

suppress avoidance to Gram-negative bacteria, but non-canonical DBL-1 signaling represses the avoidance to Gram-positive bacteria. Furthermore, this work identified a novel role for SMA-4/co-Smad that is independent of the DBL-1 signaling pathway. This indicates that the DBL-1/TGF-β signaling pathway plays an important role in tailoring the animals' innate immune responses to the bacterial threat. Additionally, to investigate the role of saposin-like antimicrobial proteins in response to different environmental threats, we characterized the regulation of *spp-9* in response to the bacterial panel. We identified that *spp-9* is affected by both bacterial exposure and by starvation. We reported that the pathogen-specific regulation of *spp-9* expression was dependent on not only DBL-1/TGF-β signaling but also other innate immune signaling pathways including insulin-like and p38/MAP kinase. Lastly, to determine the role of DBL-1 signaling in regulating the epicuticle, a secreted lipid-rich barrier layer, we developed a method to isolate epicuticular lipids and characterized their composition. We showed that DBL-1 signaling regulates the composition and levels of both epicuticle and internal lipids. DBL-1 signaling also regulates expression of lipid metabolism genes. Collectively, these findings demonstrate bacteria-specific host immune responses regulated by the DBL-1/TGF-β signaling pathway.

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LIST OF ABBREVIATIONS

ANOVA	Analysis of variance
B. megaterium	*Bacillus megaterium*
C. elegans	*Caenorhabditis elegans*
DNA	Deoxyribonucleic acid
E. cloacae	*Enterobacter cloacae*
E. coli	*Escherichia coli*
E. faecalis	*Enterococcus faecalis*
FA	Fatty acids
FAMEs	Fatty acid methyl esters
FUdR	5-Fluorodeoxyuridine
GC	Gas chromatography
GC-MS	Gas chromatography-mass spectrometry
GFP	Green fluorescent protein
K. oxytoca	*Klebsiella oxytoca*
LPC	Lyso-phosphatidylcholine
MAPK	Mitogen-activated protein kinase
MAP2K	Mitogen-activated protein kinase kinase
MAP3K	Mitogen-activated protein kinase kinase kinase
M. luteus	*Micrococcus luteus*
mRNA	Messenger ribonucleic acid

PA	Phosphatidic acid
PC	Phosphatidylcholine
PCR	Polymerase chain reaction
PE	Phosphatidylethanolamine
PI	Phosphatidylinositol
P. aeruginosa	*Pseudomonas aeruginosa*
qRT-PCR	Quantitative real-time polymerase chain reaction
RFP	Red fluorescent protein
RNA	Ribonucleic acid
RNA-seq	RNA sequencing
S. enterica	*Salmonella enterica*
S. epidermidis	*Staphylococcus epidermidis*
S. marcescens	*Serratia marcescens*
TEM	Transmission electron microscopy
TGF-β	Transforming growth factor beta
TLC	Thin layer chromatography

CHAPTER I

INTRODUCTION

All living organisms possess mechanisms to protect themselves from environmental threats. Notably, animals employ coordinated immune responses against pathogenic bacteria. In humans, immune responses are mounted through the innate immune system and the adaptive immune system[1]. Innate immunity, which all animals have, is the first line of defense used by organisms to fight pathogens. Innate immunity also plays an important role in the regulation of adaptive immunity[2]. Some organisms use adaptive immunity to fight pathogens if the innate immune responses are not sufficient[3]. Adaptive immunity is long lasting and involves antigen-specific responses[4,5]. Innate immune responses are dynamically regulated. Living organisms utilize multiple cell signaling pathways to coordinate innate immune responses against pathogen exposure[1,4]. How are these innate immune responses specific upon exposure to different pathogenic bacteria? What are the underlying mechanisms of the dynamic interplay between host signaling and different pathogen exposure? These are some questions that need to be addressed to reduce the fundamental knowledge gap in understanding the mechanisms of appropriate coordination of protective host responses against specific bacterial challenges. Dissecting the network of specific protective mechanisms against different bacterial exposures will provide a deeper understanding of how hosts tailor protective responses. These findings

can be used to identify specific mechanisms that can be targeted to regulate host defense responses.

Studying such robust interactions if innate immune responses *in vivo* in higher organisms is challenging because of confounding issues of adaptive immunity and ethical and practical constraints[6]. To identify dynamic innate immune responses *in vivo,* we use the model organism *Caenorhabditis elegans*. *C. elegans* is a free-living roundworm and is an established system to dissect evolutionarily conserved innate immunity mechanisms[7]. 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[8,9]. In these environments, roundworms encounter bacteria, which can be beneficial or pathogenic to the roundworms[10]. Roundworms lack adaptive immunity and use their innate immune system of defenses to respond to pathogenic bacteria. To effectively fight infection, it is important that organisms appropriately coordinate and regulate innate immune responses[1]. How animals coordinate innate immune responses against the various challenges in their natural environment—to protect roundworms or other animals, including humans—is a fundamental knowledge gap in the field.

C. elegans naturally thrive in a soil environment and are in constant association with a diverse range of microbes that are food and possible threat to these roundworms[11,12]. Bacteria can infect *C. elegans* though multiple routes including intestine, cuticle, and tail (see Fig. 1.1)[13,14] . As such, *C. elegans* have

developed powerful protective mechanisms for their defense. The first line of defense is behavioral: avoidance behavior and reduced food (bacteria) intake[15–20]. *C. elegans* can sense and avoid pathogenic bacteria for protection against harmful environments[16,21,22].

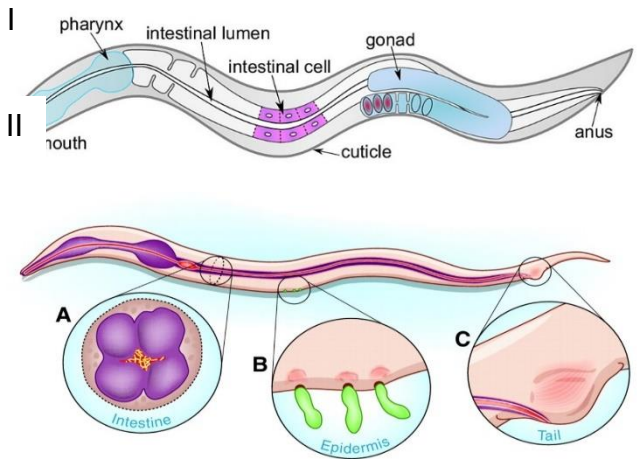

Fig. 1.1 Anatomy of *C. elegans* and different modes of infection. I) This figure represents the body anatomy of an adult hermaphrodite. II) Pathogens can either

The second defense mechanism is through physical means[12,23,24]. One way is through using the pharyngeal grinder and another is by means of cuticle.

Bacteria that *C. elegans* feed on are destroyed mechanically by the pharyngeal grinder. The pharyngeal grinder keeps live bacteria from passing to the gut and colonizing the intestinal lumen. Intact bacteria that escape the grinder colonize (infect) the nematode intestine[7]. It is reported that several pathogenic bacteria including *Serratia marcescens*, *Pseudomonas aeruginosa*, and *Enterococcus faecalis* colonize nematode intestine and reduce lifespan[25–27]. The cuticle (exoskeleton) is a physical barrier that protects the nematodes from their external environment (see Fig. 1.2). The cuticle is composed of multiple layers, including protein-rich inner layers, the lipid-rich epicuticle layer, and the outermost glycoprotein-rich surface coat (or glycocalyx). The protein composition of the cuticle is well characterized; however, its epicuticular lipid composition has not yet been dissected[28].

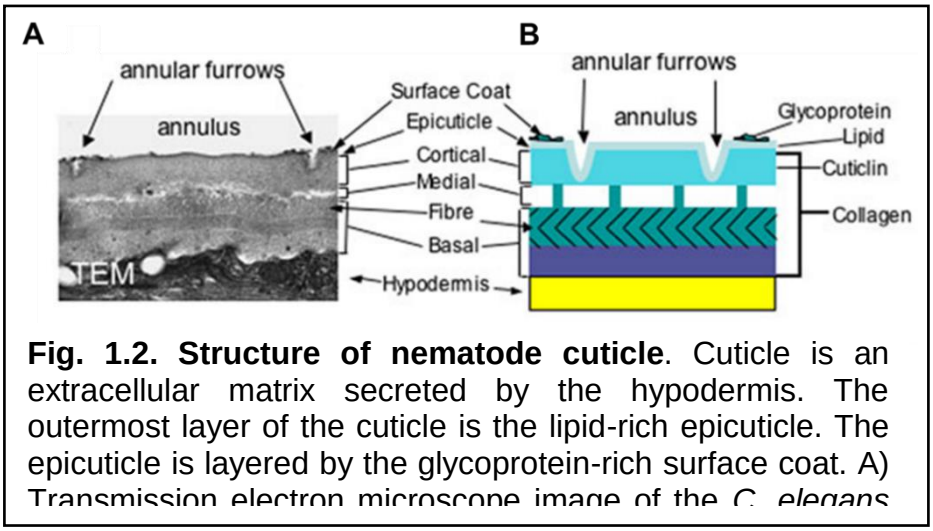

Fig. 1.2. Structure of nematode cuticle. Cuticle is an extracellular matrix secreted by the hypodermis. The outermost layer of the cuticle is the lipid-rich epicuticle. The epicuticle is layered by the glycoprotein-rich surface coat. A) Transmission electron microscope image of the *C. elegans*

The third mechanism is molecular: induction of antimicrobial genes upon infection. Several pathogens are reported to infect *C. elegans*, including bacterial

strains *P. aeruginosa*, *Salmonella enterica*, *S. marcescens*, *Bacillus megaterium*, *E. faecalis*, *Mycobacterium fortuitum*, *Staphylococcus aureus*, and *Staphylococcus epidermidis*, and the fungus *Drechmeria coniospora*[29–34]. Upon infection by these pathogens, Toll-like receptor, MAPK signaling, insulin-like signaling, and TGF-β signaling pathways are induced[29–36]. These pathways regulate an overlapping set of target genes, indicating cross-regulation of the pathways when *C. elegans* are exposed to infection. As in other animals, including humans, genes targeted by these signaling pathways encode antimicrobial proteins. However, it is unclear how the host coordinates appropriate defenses against a variety of Gram-negative and Gram-positive bacteria. While many studies have elucidated robust roles of MAPK and insulin-like signaling pathways in defense responses, the role of TGF-β signaling in regulating defense responses tailored to the pathogenic challenge is not well characterized[37–40].

The mammalian TGF-β signaling pathway functions and pathway components are highly conserved in *C. elegans*. DBL-1 is one of the five TGF-β superfamily ligands in *C. elegans*. DBL-1 is secreted from neurons and binds to its receptors SMA-6 and DAF-4, which are present on the cell membrane of the hypodermis, pharynx, and intestine. Upon ligand binding, the SMA-6/DAF-4 receptor complex is activated by auto-phosphorylation. The phosphorylated receptor complex in turn activates downstream transcription factor SMA-3/Smad by phosphorylation, which then binds other Smad transcription factors SMA-2

and SMA-4. This activated transcription factor complex translocates to the nucleus to regulate transcription of target genes (see Fig. 1.3)[41–43]. DBL-1 is known to play a role in innate immunity by regulating transcription of innate immunity genes and is also known to be involved in protecting animals from Gram-negative *S. marcescens* Db11[38]. Additionally, the DBL-1 signaling pathway is also involved in regulating body size, male tail development, and reproductive aging (see Table 1.1)[41,44–46]. The Gumienny lab reported another protective role of DBL-1/TGF-β signaling, regulating epicuticle lipid levels, in a dose-dependent manner[47]. Previous reports indicate that overexpression of *dbl-1* enriches expression of many immune response genes, including lectins, saposin-like, and lysozymes[32,48–51]. While the role of DBL-1 in defending nematodes from a few Gram-negative bacteria has been reported, its role in protection against Gram-positive bacterial infection is not well elucidated[25,38,52].

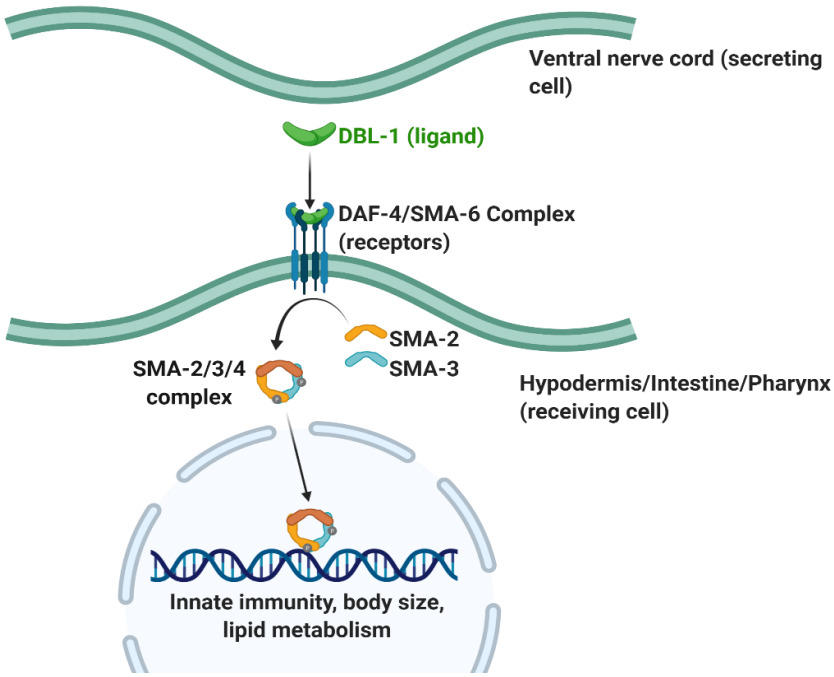

Fig. 1.3. DBL-1 signaling pathway. DBL-1 ligand is secreted from the ventral nerve cord and binds to its receptor complex present on the receiving cells that include hypodermis, intestine, and pharynx. The pathway signals through SMA-2, SMA-3, and SMA-4 transcription factors to regulate the expression of target genes.

Phenotypes	Wild-type (WT) *dbl-1*	Loss of *dbl-1*
body size	WT	small
control of innate immune response genes	yes	altered
male tail morphology	WT	fused rays crumpled spicules
epicuticle lipid levels	WT	decreased
permeability to drugs	WT	increased
lifespan	WT	decreased
reproductive aging	WT	reduced and extended

Table 1.1. DBL-1-associated phenotypes observed in *C. elegans*.

We hypothesize that *C. elegans* use the DBL-1/TGF-β signaling pathway to coordinate specific innate immune responses against a variety of Gram-negative and Gram-positive bacteria. These test bacteria were selected because they can cause opportunistic infections in humans. We tested the hypothesis by addressing if *C. elegans* use the DBL-1/TGF-β signaling pathway to coordinate specific innate immune behavioral responses (Aim 1), molecular responses (Aim 2), and physiological responses (Aim 3) against a panel of Gram-negative and Gram-positive bacteria that cause opportunistic infections in humans (see Fig. 1.4). Aim 1 is summarized in Chapters II and III, Aim 2 is summarized in Chapters II and IV, and Aim 3 is summarized in Chapter V. Our major findings summarized in Chapter II indicate that the DBL-1/TGF-β signaling pathway plays an important role in influencing the animals' perception of pathogenicity and in turn appropriately balances innate immune responses. Animals lacking DBL-1 signaling are more susceptible to the bacteria irrespective of the Gram nature. We also identified a correlation between reduced feeding (a protective response) and animal survival in response to select bacteria. However, animals with reduced canonical DBL-1 signaling displayed a strong avoidance response selectively to the Gram-negative bacteria. Additionally, we identified signature molecular responses regulated by DBL-1 in response to different Gram-negative and Gram-positive bacteria. Finally, we discovered that differential regulation of

DBL-1 signaling is part of the host's molecular response to Gram-negative and Gram-positive bacteria.

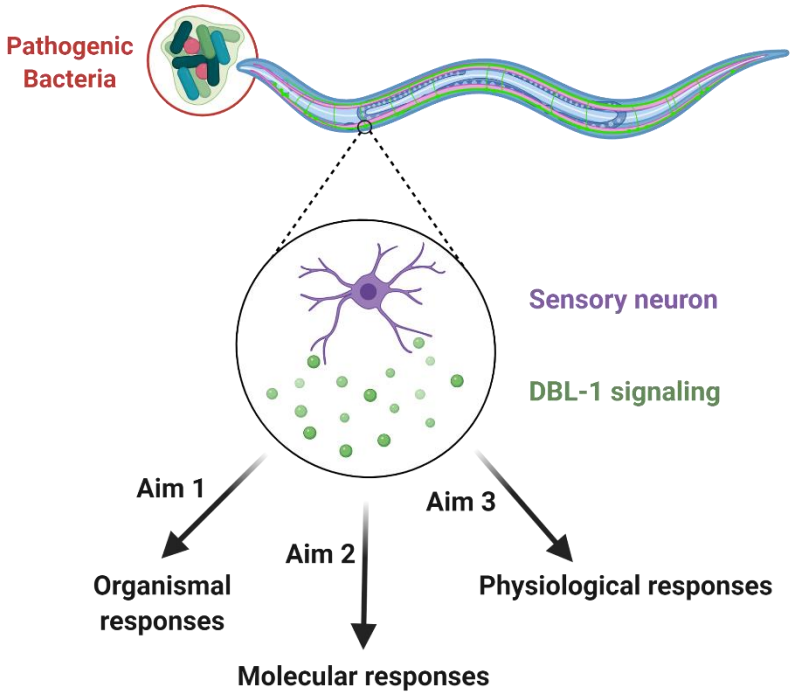

Fig. 1.4. Graphical representation of aims. This work reports findings determining if and how *C. elegans* use the DBL-1/TGF-β signaling pathway to coordinate specific innate immune

Chapter III summarizes the findings on the role of DBL-1 signaling in coordinating changes in egg-laying, which is a known protective response to pathogenic bacteria. We identified that loss of DBL-1 resulted in reduced brood size upon exposure to Gram-negative *S. marcescens* and Gram-positive *S. epidermidis*. We found that the brood size alterations were independently regulated by loss of DBL-1 signaling and exposure to the pathogenic bacteria.

Chapter IV summarizes the findings on bacteria-specific regulation of an antimicrobial gene, *spp-9*. We identified that expression of this antimicrobial gene is not only altered by selective pathogens but also by starvation. We reported that the pathogen-specific regulation of *spp-9* expression is dependent on DBL-1/TGF-β signaling. Additionally, we identified that other innate immunity signaling pathways, including insulin-like and p38/MAP kinase signaling, also regulate *spp-9* expression.

Chapter V provides a foundational understanding of the lipid composition of the *C. elegans* epicuticle and highlights the role of DBL-1/TGF-β signaling in regulating the epicuticle lipid composition. Additionally, we report that the DBL-1 signaling regulates the composition of internal lipids. We identified fatty acid metabolism genes regulated by DBL-1 signaling, and propose that changes in the expression of these genes results in the DBL-1-dependent lipid composition alterations.

Finally, Chapter VI provides a comprehensive model to summarize the role of DBL-1/TGF-β signaling in tailoring protective immune responses to a variety of Gram-negative and Gram-positive bacteria. This chapter culminates in questions that can be addressed using this work as a foundation to study differential host pathogen immune responses.

CHAPTER II

THE DBL-1/TGF-β SIGNALING PATHWAY REGULATES PATHOGEN-SPECIFIC INNATE IMMUNE RESPONSES IN *C. ELEGANS*

This chapter contains a preprint: Madhu, B. and Gumienny, T.L. (2021) The DBL-1/TGF-β signaling pathway regulates pathogen-specific innate immune responses in *C. elegans*. bioRxiv 2021.03.30.437693; doi: https://doi.org/10.1101/2021.03.30.437693

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Abstract

Innate immunity in animals is orchestrated by multiple cell signaling pathways, including the TGF-β superfamily pathway. While the role of TGF-β signaling in innate immunity has been clearly identified, the requirement for this pathway in generating specific, robust responses to different bacterial challenges has not been characterized. Here, we address the role of DBL-1/TGF-β in regulating signature host defense responses to a wide range of bacteria in *C. elegans*. This work reveals a role of DBL-1/TGF-β in animal survival, organismal behaviors, and molecular responses in different environments. Additionally, we identify a novel role for SMA-4/Smad that suggests both DBL-1/TGF-β-dependent and -independent functions in host avoidance responses. RNA-seq analyses and immunity reporter studies indicate DBL-1/TGF-β differentially regulates target gene expression upon exposure to different bacteria. Furthermore, the DBL-1/TGF-β pathway is itself differentially affected by the bacteria exposure. Collectively, these findings demonstrate bacteria-specific host immune responses regulated by the DBL-1/TGF-β signaling pathway.

Introduction

Living organisms recognize and respond to potential environmental insults by coordinating protective defenses[1]. Invertebrates and vertebrates both employ conserved innate immune response as immediate front-line protection from challenges including pathogenic bacteria[2–5]. These responses are tailored to the bacterial challenge. However, how these responses are specified and what the responses are to different pathogens remains a challenge[6].

The roundworm *Caenorhabditis elegans* is an established model system to study regulation of immune responses *in vivo*[7]. *C. elegans* naturally thrives in a soil environment where it feeds on bacteria and is in constant association with a diverse range of microbes that are both food and threat[3,7]. A limited number of pathogens are known to infect *C. elegans*, including mycobacterial, Gram-negative, and Gram-positive bacterial species, and fungi[8–15]. *C. elegans* has an innate immune system that confers protection through behavioral, physical (exoskeleton), and molecular mechanisms[7].

Infection in *C. elegans* induces molecular immune defenses coordinated by conserved Toll-like receptors, MAPK (mitogen-activated protein kinase) signaling, insulin-like signaling, and DBL-1/TGF-β (transforming growth factor β) signaling[8–13,16,17]. These pathways regulate an overlapping set of target defense genes, indicative of coordinated crosstalk between these signaling pathways. While the roles of Toll, MAPK, and insulin-like signaling pathways in immune responses to a wide variety of bacteria are well characterized in *C. elegans* and

other organisms, a role for DBL-1/TGF-β signaling in eliciting robust targeted immune responses to different bacterial challenges has been identified but is not well defined[16,18–22]. Previous reports indicate that overexpression of *dbl-1* enriches expression of many immune response genes including lectins, saposin-like proteins, and lysozymes[11,23–25]. While the role of DBL-1 in defending nematodes from a few Gram-negative bacteria is reported, its possible role in protection against Gram-positive bacterial infection has not been well characterized[20,26,27].

In this work, we examined the role of DBL-1/TGF-β signaling in regulating an array of microbe-specific immune responses. Using behavioral and molecular approaches, we identified DBL-1-dependent and -independent immune responses that are tailored to the specific bacterial exposure. We also identified a non-canonical role for the DBL-1 pathway transcription regulator SMA-4 in an avoidance response to specific bacteria. Additionally, we show that DBL-1 signaling is induced in response to Gram-negative bacteria but is repressed in response to Gram-positive bacteria. We propose that animals lacking DBL-1 signaling respond with heightened avoidance behaviors to selected bacterial environments because they perceive the environment as more hostile. Collectively, our findings highlight a central role for DBL-1 in regulating a suite of bacteria-specific host defenses and also demonstrate bacteria-responsive regulation of DBL-1 signaling.

Materials and Methods

Strains and Maintenance

C. elegans strains

All *C. elegans* strains were maintained on EZ media plates at 20°C[28]. *C. elegans* strains were maintained without contamination and starvation for at least five generations before every experiment. Supplementary Table 1 includes the list of all strains used in this study. These strains were generated by standard genetic crosses and confirmed by small body size phenotype and presence of fluorescence.

Bacterial strains

The bacterial strains used in this study include *Bacillus megaterium* (Carolina Biological Supply Company), *Escherichia coli* (OP50), *Enterobacter cloacae* (49141TM), *Enterococcus faecalis* (51299TM), *Klebsiella oxytoca* (49131TM), *Serratia marcescens* (Carolina Biological Supply Company), and *Staphylococcus epidermidis* (49134TM). *E. faecalis* in brain heart infusion media and all other bacteria in tryptic soy broth were grown for nine hours at 37°C as previously described[28]. Bacterial cells were pelleted at 5000 rpm for 15 minutes and concentrated twenty-fold. EZ media plates were freshly seeded with concentrated bacteria in full lawns. The plates were incubated at 37°C overnight before using for experiments.

Lifespan Assay

Lifespan assay was performed as previously described[29–31]. Concentrated bacterial cultures were spread on 6 cm diameter EZ media plates (full lawn plates) containing 50 µg/ml 5-fluorodeoxyuridine (FUdR) to cover the surface of the plates entirely. Wild-type and *dbl-1(-)* animals (n = at least 30) were fed on control and test bacteria on full lawn plates at the L4 stage in quadruplicate. The plates were scored for live and dead nematodes every 24 hours until all animals were dead. Animals were scored as dead if they did not respond to gentle touch with a sterilized platinum wire and were removed from the plate. At least three independent trials were performed. Worms that died by desiccating on the walls of the plates were censored from the analysis.

Pharyngeal Pumping Rate

Wild-type and *dbl-1(-)* L4 animals (n = 12) were fed on control and test bacteria on full lawn plates. The number of contractions of the pharyngeal bulb was counted for 20 seconds to calculate the pharyngeal pumping rate of animals. Two counts were made and averaged for each animal. Three independent trials were performed in triplicate[32].

Intestinal Barrier Function Assay

The intestinal barrier function assay was performed as previously described[33]. Wild-type and *dbl-1(-)* L4 animals were fed on control and test bacteria on full lawn plates. The assay was performed when about 50% of the population with the lowest mean lifespan remained alive. At least 15 animals

were sampled at the specified times to examine intestinal tissue integrity. The intestinal barrier integrity was assessed using a blue dye, eriogluacine disodium salt (5% wt/v), as an indicator of tissue integrity as the animals age. Leaking of this blue dye outside the intestinal lumen indicates reduced intestinal integrity. The animals were washed with S buffer and were incubated in erioglaucine disodium salt solution in a 1:1 ratio for 3 hours. The animals were then washed thrice with S buffer and were mounted on 2% agarose pads on glass slides. 10 µM levamisole was added to paralyze the animals. The animals were imaged on a Nikon DS-Ri2 camera mounted to a Nikon SMZ18 dissecting microscope. The leakiness of the intestine was assessed and scored as '1' for no leakage/no Smurf, '2' for mild leakage/mild Smurf, and '3' for severe leakage/severe Smurf phenotypes. The experiment was performed in at least three independent trials for each experimental condition.

Microbial Avoidance Assay

Microbial avoidance assays were performed as previously described[34]. 20 µl of the concentrated bacterial cultures were spotted on 6 cm diameter EZ media plates and incubated at 37°C overnight. Wild-type, *dbl-1(-)*, *sma-2(-)*, *sma-3(-)*, and *sma-4(-)* L4 hermaphrodites were placed on control and test bacterial lawn (n = 30 per condition/trial, performed in triplicate). The plates were scored for number of worms occupying the lawn at the indicated time points. Three independent trials were performed. The avoidance ratio (A) was calculated using the formula:

A = number of animals off the lawn/ total number of animals

Avoidance of populations was scored as mild if less than 40% of the population was not on the food source ($A < 0.4$), as moderate avoidance if $0.4 < A < 0.6$, and as strong avoidance if $A > 0.6$.

RNA Isolation

Animals were synchronized as embryos by bleaching mixed-stage populations[35]. Total RNA was extracted from animals at 48 hours after the L4 stage. Total RNA was extracted by the freeze cracking method as previously described[36].

Differential Expression Analysis by RNA Sequencing

RNA from wild-type and *dbl-1(-)* adult populations fed on control and test bacteria was extracted in three independent trials. Sequencing libraries from the extracted RNA were generated using the NEBNext® RNA Library Prep Kit for Illumina® (NEB, USA) following manufacturer's recommendations. 1 µg RNA of each sample was used as input material for the RNA sample preparations. Novogene performed RNA sequencing of samples. Differential expression analysis of wild-type compared to *dbl-1(-)* populations grown on different bacteria was performed using the DESeq R package (1.18.0)[37]. Genes with an adjusted *p*-value < 0.05 found by DESeq were assigned as differentially expressed.

cDNA Synthesis and qRT-PCR

After RNA isolation, cDNA was synthesized and quantitative real-time PCR was performed as previously described[38]. 2 µg of total RNA isolated 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 a QuantStudio3 system (Applied Biosystems by Thermo Fisher Scientific) using the PowerUP SYBR Green master mix (Applied Biosystems) according to manufacturer's instructions. Three independent biological trials were performed. Each biological trial was performed in three technical replicates for each condition. Primer sequences are available in Supplementary Table 2. QuantStudio Design and Analysis Software v1.5.1 was used to calculate raw C_t values (Applied Biosystems by Thermo Fisher Scientific). The C_t values for the target genes were normalized to the housekeeping gene *act-1* (actin). Fold change in gene expression between experimental sample and the control was determined by using the formula: $2^{(-\Delta\Delta C_t)}$.

Imaging

RAD-SMAD reporter strains were placed on full lawns of the control or test bacteria at the L4 stage. L2 progeny were mounted on 2% agarose pads and anesthetized by using 1 mM levamisole and fluorescence was captured by a Zeiss LSM 900 confocal microscope using a 40X oil objective. At least 15 animals with at least five hypodermal nuclei per worm in the focal plane were imaged per condition, giving a moderate effect size as determined by power analysis. The experiment was performed in three independent trials. The microscope conditions were optimized with respect to the control and test

conditions and kept consistent within each trial. Mean fluorescence intensities were measured as previously described using the Zeiss ZEN lite software[39].

The innate immune reporter strains were transferred to full lawns of the control and test bacteria at the L4 stage and were imaged after 48 hours of exposure. Fluorescence of the reporter strains was captured by a Nikon DS-Ri2 camera mounted on a Nikon SMZ18 dissecting microscope. Animals were mounted on 2% agarose pads and anesthetized with 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. Three independent trials were performed. Mean fluorescence intensities were measured using the Nikon NIS Elements AR v5.02 software.

Statistical Analyses

Lifespans of *C. elegans* populations were calculated by the Kaplan-Meier method and statistical analysis was performed using the two-tailed log-rank test. The average pharyngeal pumping rates were compared by the two-tailed unpaired *t*-test. The intestinal barrier function phenotypes were statistically analyzed by the Chi-square test. The avoidance ratio was compared by repeated measures ANOVA using Tukey's post-hoc test. qRT-PCR values and mean fluorescence intensities were evaluated using the two-tailed unpaired *t*-test. RNA

sequencing analysis was performed using the DESeq R package (1.18.0). The resulting *p*-values were adjusted using the Benjamini and Hochberg's approach for controlling the false discovery rate.

Results

Loss of DBL-1 Reduces Lifespan of Animals Fed on Specific Bacteria

To study the requirement for DBL-1 in specific responses to pathogens at behavioral, molecular, and physiological levels, we first established a panel of bacteria for innate immune studies in *C. elegans* that would facilitate genetic and molecular studies over time: previous studies have been limited in range of challenge and used a pathogen that killed animals in hours or a few days. The control bacteria chosen was Gram-negative *E. coli* OP50, a commonly used strain for laboratory culture of *C. elegans*. The panel of test bacteria comprises Gram-negative and -positive bacteria that are opportunistic pathogens in humans and are found in the natural habitat of *C. elegans*[40]. We selected three Gram-negative test strains, *S. marcescens*, *E. cloacae*, and *K. oxytoca*, and three Gram-positive test strains, *B. megaterium* and *E. faecalis*, and *S. epidermidis*.

We first asked if our panel of opportunistic bacteria affect lifespan of *C. elegans*. Wild-type animals on Gram-negative *S. marcescens* have lifespans comparable to *E. coli*-fed animals. Interestingly, we noted an extended lifespan of wild-type animals on the other two Gram-negative strains and all three Gram-positive strains (see Fig. 2.1, Supplementary Table 3).

Loss of DBL-1 has previously been shown to reduce lifespan of animals exposed to fungus *D. coniospora*, Gram-negative strains *E. coli* and *S. marcescens* (Db11), and Gram-positive *E. faecalis*[13,20,41]. To determine if DBL-1 is required in maintaining lifespan of animals subjected to our bacterial panel, we compared lifespans of *dbl-1(-)* animals exposed to the test or control bacteria to the wild type. In our conditions, loss of DBL-1 does not alter lifespan of animals fed on *E. coli* (see Fig. 2.1a). However, loss of *dbl-1* results in a significantly shortened lifespan on *S. marcescens*, consistent with a previous report that used the more virulent *S. marcescens* strain Db11 (see Fig. 2.1d, Supplementary Table 3)[20]. *dbl-1* mutant animals did not have the lifespan extension seen in wild-type populations on *E. cloacae* or *K. oxytoca*: lifespans of *dbl-1* mutant populations were the same on these two Gram-negative bacterial strains as on *E. coli* (see Fig. 2.1b, c, Supplementary Table 3). The lifespan of *dbl-1(-)* animals was extended upon exposure to Gram-positive *B. megaterium* and *E. faecalis* compared to the *E. coli*-fed population's lifespan, but was not as extended as the wild-type lifespan (see Fig. 2.1e, f, Supplementary Table 3). Lastly, *dbl-1* mutant animals displayed a significantly decreased lifespan on *S. epidermidis* (see Fig. 2.1g, Supplementary Table 3).

In conclusion, we have identified a panel of human opportunistic pathogens that can be used to interrogate genetic contributions to innate immune defenses. DBL-1 is required for normal lifespan responses regardless of bacterial Gram nature (see Fig. 2.1b–g). These results provide evidence that DBL-1

signaling normally confers protection against these bacteria and may play a role in the lifespan extension observed on most bacteria in the panel.

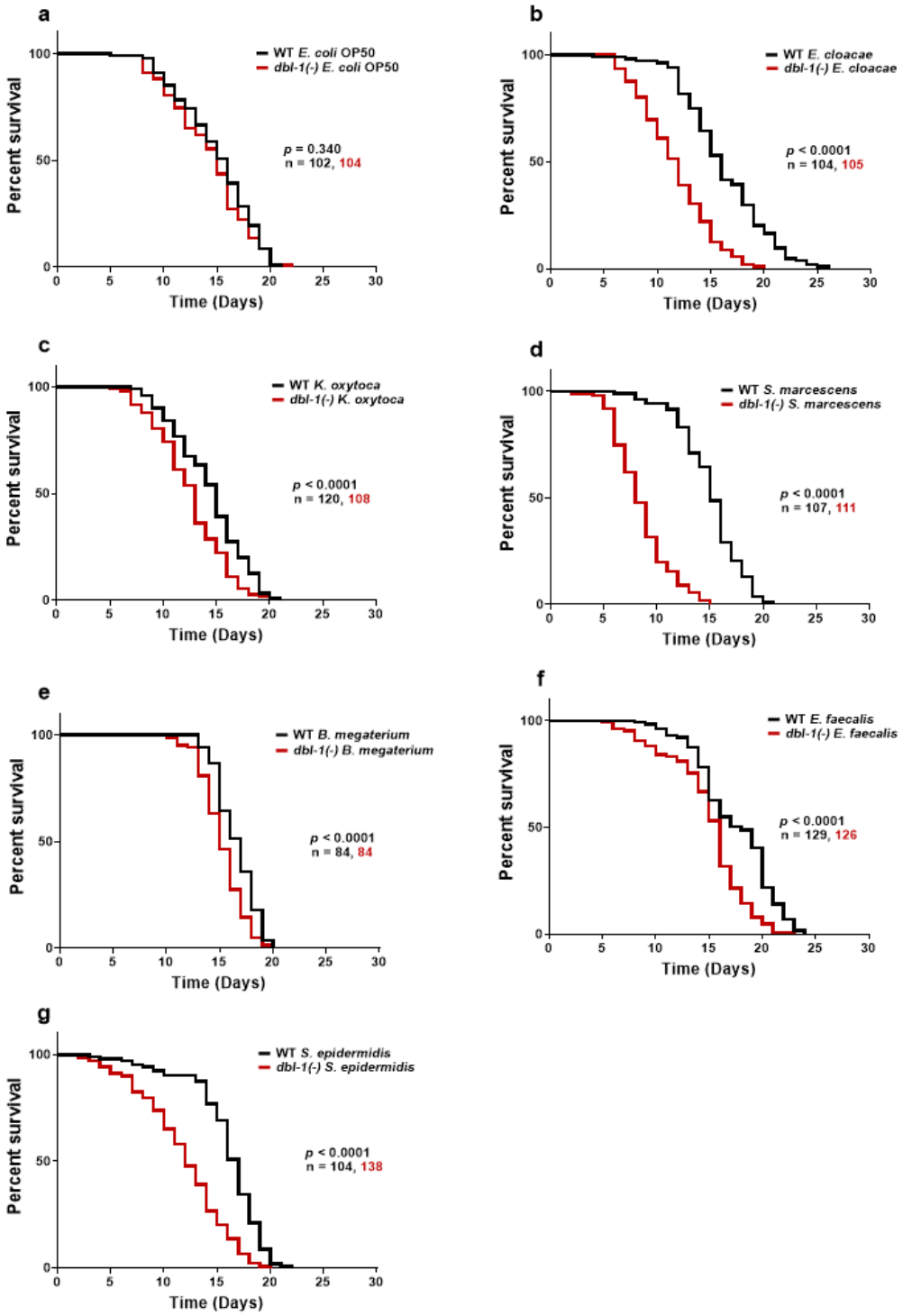
a
WT E. coli OP50
dbl-1(-) E. coli OP50
p = 0.340
n = 102, 104
Percent survival
Time (Days)
b
WT E. cloacae
dbl-1(-) E. cloacae
p < 0.0001
n = 104, 105
Percent survival
Time (Days)
c
WT K. oxytoca
dbl-1(-) K. oxytoca
p < 0.0001
n = 120, 108
Percent survival
Time (Days)
d
WT S. marcescens
dbl-1(-) S. marcescens
p < 0.0001
n = 107, 111
Percent survival
Time (Days)
e
WT B. megaterium
dbl-1(-) B. megaterium
p < 0.0001
n = 84, 84
Percent survival
Time (Days)
f
WT E. faecalis
dbl-1(-) E. faecalis
p < 0.0001
n = 129, 126
Percent survival
Time (Days)
g
WT S. epidermidis
dbl-1(-) S. epidermidis
p < 0.0001
n = 104, 138
Percent survival
Time (Days)

Figure 2.1. Loss of DBL-1 decreases lifespan of animals exposed to Gram-negative and Gram-positive bacteria. Wild-type and *dbl-1(-)* animals were scored for survival over time from the L4 stage (t = 0 hours) on the following bacteria: a) *E. coli* OP50 (control), b) *E. cloacae*, c) *K. oxytoca*, d) *S. marcescens*, e) *B. megaterium*, f) *E. faecalis*, and g) *S. epidermidis*. Survival fraction was calculated by the Kaplan-Meier method. *p*-values were calculated using log-rank test and $p < 0.01$ compared to wild-type animals exposed to the same bacteria was considered significant. One representative trial of at least three is presented.

Loss of DBL-1 and Exposure to Specific Bacteria Reduce Feeding

One reason for the increased lifespan of *C. elegans* on select bacteria could be that the animals experience dietary restriction because they reduce bacterial consumption[42,43]. To determine if animals reduce feeding on bacteria that increase lifespan, we measured and compared pharyngeal pumping rates of wild-type animals on control and test bacteria. Wild-type animals fed on *E. cloacae* exhibited a small but significant decrease in pharyngeal pumping. Pharyngeal pumping was not significantly reduced after exposure to *K. oxytoca*, which is not consistent with the mild lifespan extension. Wild-type animals fed on *S. marcescens* have the same pumping rate as on the control bacteria (see Fig. 2.2a). For wild-type animals fed on the three Gram-positive bacteria, though, the pumping rate was dramatically decreased, consistent with the lifespan extension these strains conferred to wild-type *C. elegans* (see Fig. 2.2b).

To determine whether DBL-1 affects feeding response on our panel of test bacteria, we compared the pharyngeal pumping rate of wild-type and *dbl-1(-)* animals on these bacteria. Loss of DBL-1 does not alter the pharyngeal pumping rate of animals fed on the control bacteria (see Fig. 2.2a, b). *dbl-1(-)* animals fed

on *E. cloacae* do not alter the pumping rate compared to either wild-type animals on *E. cloacae* or *dbl-1* animals on *E. coli* (control). Animals lacking DBL-1 show a mild but significant decrease when they are fed on *K. oxytoca* and *S. marcescens* (see Fig. 2.2a). The feeding rate of *dbl-1(-)* animals is further reduced from the wild-type rate on *E. faecalis* and *S. epidermidis* (see Fig. 2.2b). There is no reproducibly significant decrease in the pharyngeal pumping rate of *dbl-1(-)* animals fed on *B. megaterium* in comparison to that of the wild type (see Fig. 2.2b). These results collectively indicate that while the feeding reduction caused by exposure to these Gram-positive bacteria is independent of DBL-1, a stronger pharyngeal pumping depression in *dbl-1(-)* populations occurs in response to some bacteria (both Gram-negative and -positive bacteria), providing support to the idea that loss of DBL-1 sensitizes animals to certain pathogenic stressors. These findings suggest that even though the organismal responses (lifespan) requiring DBL-1 are similar, the underlying causes might be different, differences in animal feeding being one.

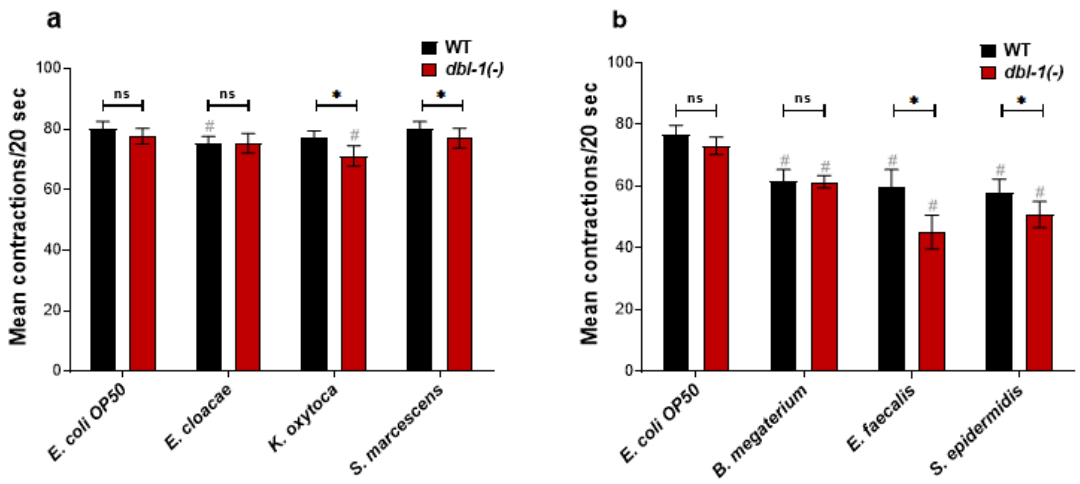

Figure 2.2. Loss of DBL-1 and exposure to specific bacteria results in decreased pharyngeal pumping. Wild-type and *dbl-1(-)* animals were exposed to the following bacteria: a, b) *E. coli* OP50 (control); a) *E. cloacae*, *K. oxytoca*, *S. marcescens*; b) *B. megaterium*, *E. faecalis*, or *S. epidermidis* at the L4 stage. After 48 hours of exposure, the number of pharyngeal pumps were counted twice per 20 seconds. The pharyngeal pumps were averaged for each animal. One representative trial of at least three is presented. Error bars represent standard deviation. n = 12 per condition. * $p < 0.01$, ns not significant, compared to wild-type animals exposed to the same bacteria, and # $p < 0.01$, respective genotype exposed to test bacteria in comparison to control bacteria by two-way ANOVA followed by unpaired *t*-test.

Intestinal Integrity of Animals Is Not Altered By Loss of *dbl-1* or Exposure to Specific Bacteria

The integrity of intestine can also be disrupted by exposure to pathogenic bacteria, but progressive loss of intestinal integrity is also a feature of aging[33,44]. To further examine the role of DBL-1 in the organismal responses to this panel of bacteria, we investigated the integrity of the intestinal barrier of animals fed on different bacteria. Using a cell-impermeable blue dye, we compared the intestinal barrier function of wild-type and *dbl-1(-)* animals exposed to control and test

bacteria when 50% of the population on the test bacteria was still alive. Consistent with previous reports, wild-type animals on *E. coli* exhibited an age-dependent reduction of intestinal integrity[44]. Animals lacking DBL-1 and grown on *E. coli* also displayed a significant decline in intestinal integrity similar to the wild type (see Supplementary Fig. 1a). Exposure of wild-type or *dbl-1* mutant animals to any of the Gram-negative and -positive bacteria in the panel did not result in further, reproducible decreases of intestinal integrity compared to *E. coli* (see Supplementary Fig. 1). Therefore, *dbl-1* is not required for intestinal integrity, nor its age-related decline. Furthermore, this result suggests the lifespan changes observed in *dbl-1* mutant populations are not caused by loss of intestinal integrity.

DBL-1 Signaling Is Required to Suppress Avoidance Against Gram-Negative Bacteria

C. elegans can sense and avoid pathogenic bacteria for protection against harmful environments [45–47]. To test how our panel of selected Gram-negative and -positive bacteria evokes an avoidance response in *C. elegans*, we performed the avoidance assay. We measured the avoidance response of wild-type animals fed on the test bacteria over the first two days of adulthood and compared it with the avoidance response on the control bacteria. Wild-type animals do not avoid *E. coli* (see Fig. 2.3a). We found that wild-type animals mildly avoid *S. marcescens*, but do not avoid *E. cloacae* and *K. oxytoca* (see Fig. 2.3b–d). Animals did not avoid the Gram-positive bacteria *E. faecalis* and *S. epidermidis*,

but had a moderate to strong response to *B. megaterium* (see Fig. 2.3e–g). This indicates that *S. marcescens*, though it did not extend lifespan, and *B. megaterium* are mildly pathogenic to animals.

To determine if DBL-1 regulates this avoidance phenotype, we tested avoidance behavior of *dbl-1(-)* exposed to the test bacteria and compared it with *dbl-1(-)* animals fed on the control bacteria. Loss of *dbl-1* usually results in mild but significantly higher avoidance to the control bacteria than the wild type (see Fig. 2.3a), in support of previous findings[48]. *dbl-1(-)* animals displayed a striking, strong avoidance response to all three tested Gram-negative bacteria and a mild response to *S. epidermidis* (see Fig. 2.3b–d, g). Interestingly, *dbl-1(-)* animals exhibited a similar avoidance behavior in response to *B. megaterium* and *E. faecalis* exposure (see Fig. 2.3e, f). This indicates that upon loss of DBL-1, animals perceive *E. cloacae*, *K. oxytoca*, *S. marcescens*, and *S. epidermidis* as more pathogenic. Avoidance responses to *B. megaterium* and *E. faecalis* is independent of DBL-1 levels. These results indicate that DBL-1 normally suppresses avoidance and loss of DBL-1 results in robust avoidance responses that depend on the type of bacterial exposure.

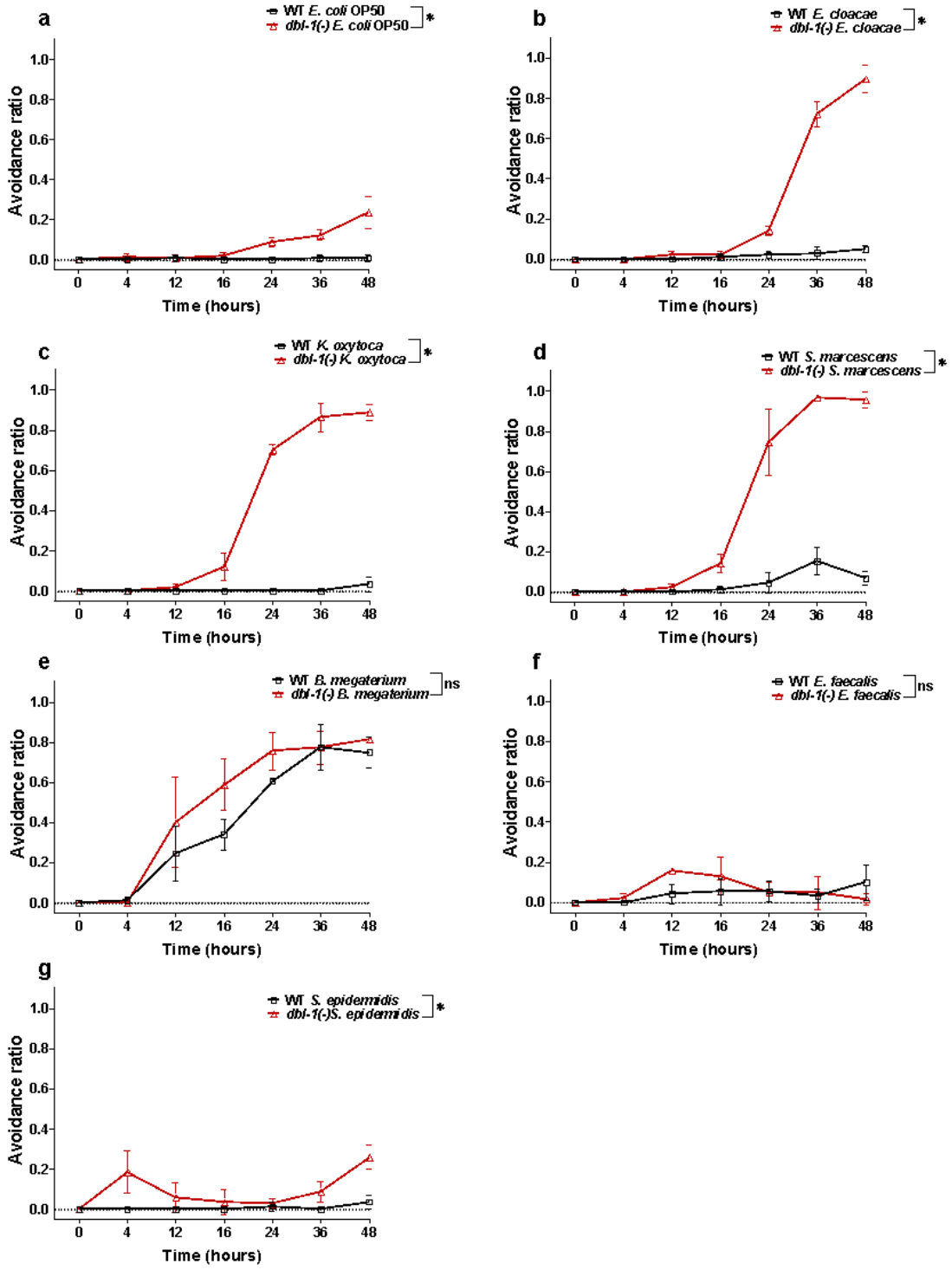

Figure 2.3. Avoidance to Gram-negative bacteria increases upon loss of DBL-1 signaling. Wild-type and *dbl-1(-)* animals at the L4 stage (t = 0 hours) were exposed to the following bacteria: a) *E. coli* OP50 (control), b) *E. cloacae*, c) *K.*

oxytoca, d) *S. marcescens*, e) *B. megaterium*, f) *E. faecalis*, or g) *S. epidermidis*. Avoidance of animals to the bacteria was monitored over time. The avoidance ratio was calculated and compared between wild-type and *dbl-1(-)* animals. One representative trial of three is presented. Error bars represent standard deviation. n = 30 per condition per trial. * $p < 0.05$, and ns not significant, compared to wild-type animals exposed to the same bacteria by repeated measures ANOVA using Tukey's post-hoc test.

SMA-4 Acts Independently of Other DBL-1 Core Signaling Components to Suppress Avoidance to Gram-Positive Bacteria

Because we observed strong bacteria-specific avoidance responses that were DBL-1-dependent, we next asked if the canonical DBL-1 signaling pathway is required to attenuate this response. Canonical signaling occurs by DBL-1 ligand binding to receptors SMA-6 and DAF-4, which activate downstream transcription factors SMA-2, SMA-3, and SMA-4[49]. A non-canonical DBL-1 pathway, which does not signal through SMA-2 and SMA-4, is required for *C. elegans* to respond to the fungus *D. coniospora*[13]. We measured the avoidance response of *sma-2(-)*, *sma-3(-)*, and *sma-4(-)* animals fed on the test Gram-negative or -positive bacteria and compared them with the avoidance response on the control bacteria. On the control bacteria, loss of *sma-3* did not result in significantly increased avoidance. However, populations lacking *sma-2* avoided the control bacteria mildly to moderately, and populations lacking *sma-4* showed a moderate to strong avoidance response (see Fig. 2.4a). *sma-2(-)*, *sma-3(-)*, and *sma-4(-)* populations displayed a strong, reproducible avoidance response to all Gram-negative bacterial strains tested that was comparable to the *dbl-1(-)* response (see Fig. 2.4b–d). In comparison, the response of Smad mutant

populations to the panel of Gram-positive bacteria was notably different. Similar to loss of *dbl-1*, loss of *sma-2* or *sma-3* did not increase avoidance responses to the three Gram-positive bacterial strains. However, loss of *sma-4* resulted in moderate to strong avoidance to all three Gram-positive strains (see Fig. 2.4e–g). Our findings indicate that while DBL-1 and SMA-3 do not play a major role in responding to *E. coli*, SMA-2 and SMA-4 are required to suppress avoidance to this standard lab food. Furthermore, our results support that canonical DBL-1 signaling plays a major role in suppressing animal avoidance responses to Gram-negative bacteria, but is not required for responding to Gram-positive bacteria. Interestingly, because of the strong effect loss of SMA-4 has on avoidance behavior, SMA-4 may act with another factor independent of the DBL-1 signaling pathway that heavily influences avoidance responses to Gram-positive bacteria.

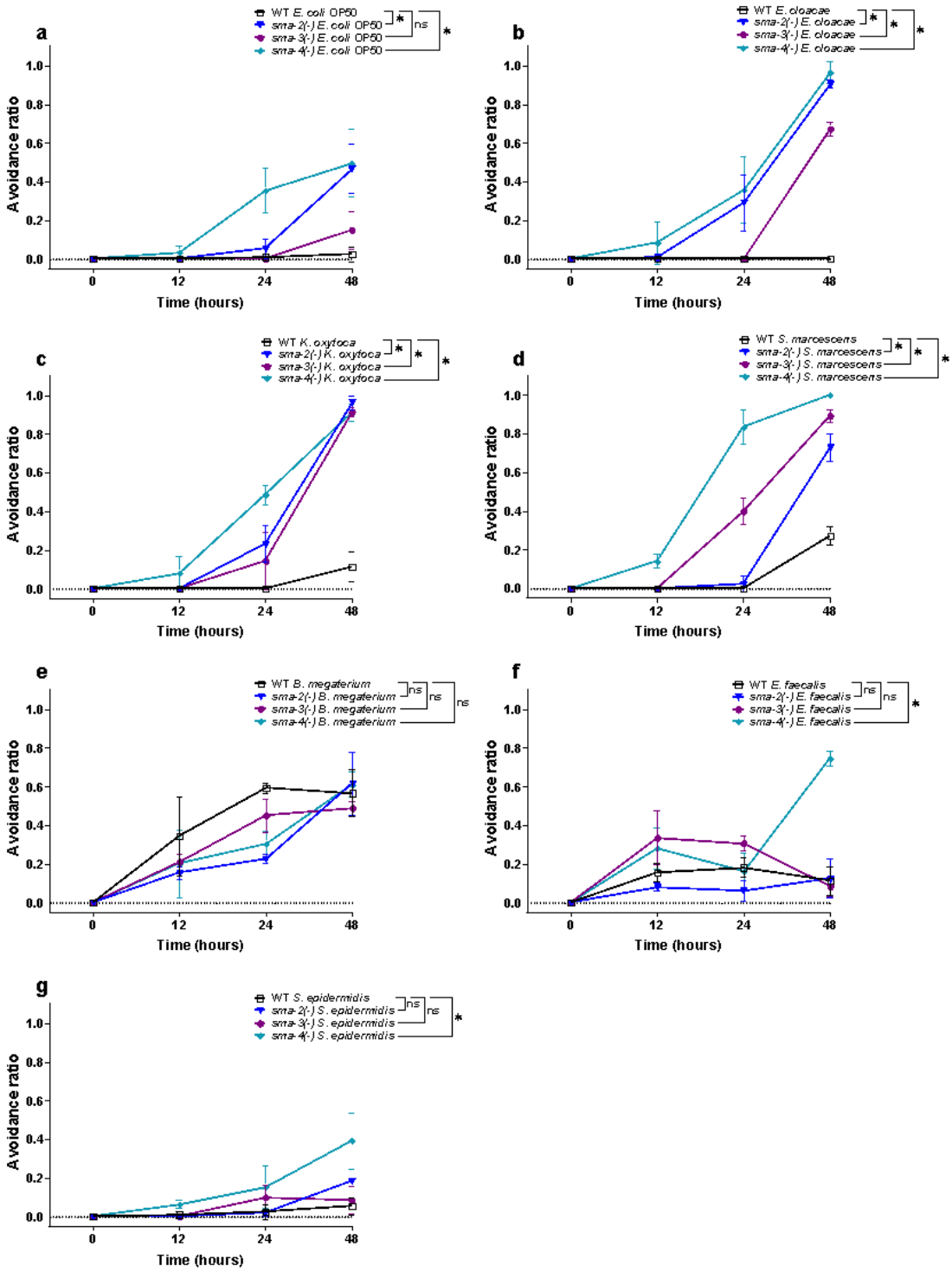

Figure 2.4. Avoidance to Gram-negative bacteria increases upon loss of canonical DBL-1 signaling. Wild-type, *sma-2(-)*, *sma-3(-)*, and *sma-4(-)* animals

at the L4 stage (t = 0 hours) were exposed to the following bacteria: a) *E. coli* OP50 (control), b) *E. cloacae*, c) *K. oxytoca*, d) *S. marcescens*, e) *B. megaterium*, f) *E. faecalis*, or g) *S. epidermidis*. Avoidance of animals to the bacteria was monitored over time. The avoidance ratio was calculated and compared between wild-type and Smad mutant animals. Avoidance ratio = number of animals off the bacterial lawn/ total number of animals. One representative trial of three is presented. Error bars represent standard deviation. n = 30 per condition per trial. * $p < 0.05$, ns not significant, compared to wild-type animals exposed to the same bacteria by repeated measures ANOVA using Tukey's post-hoc test.

sma-4 Expression is Specifically Induced in Response to Gram-Positive Bacteria

We next asked if the different avoidance responses to Gram-negative and -positive bacteria are associated with altered gene expression of the DBL-1 Smads. We tested gene expression levels of *sma-2*, *sma-3*, and *sma-4* in wild-type and *dbl-1* mutant backgrounds in response to our panel of bacteria. With the exception of *S. epidermidis* exposure, the relative levels of *sma-2* mRNA were consistently decreased in the *dbl-1* mutant background, but the test bacterial strains had no effect on *sma-2* expression (see Fig. 2.5a–d). The relative levels of *sma-3* were not reproducibly different between *dbl-1* and wild-type backgrounds, and the overall expression of *sma-3* in animals exposed to the test bacterial strains was not altered. *K. oxytoca* and *E. faecalis* conditions did result in significantly increased *sma*-3 levels that were not observed in the *dbl-1* background (see Fig. 2.5e–h). On control and Gram-negative bacteria, *sma-4* expression was similar in the *dbl-1* background as in the wild type. In addition, *sma-4* expression was not changed by exposure to the test Gram-negative

bacteria (see Fig. 2.5i, k). However, *sma-4* was significantly induced in response to all three Gram-positive bacteria in both wild-type and *dbl-1* mutant backgrounds, albeit less in the *dbl-1(-)* populations (see Fig. 2.5j, l). Together, these results suggest that the DBL-1 Smads are differently regulated at the level of gene expression by molecular pathways that are responsive to specific bacterial challenges. *sma-2*, but not *sma-3*, requires DBL-1 for full expression regardless of bacterial food source. Neither *sma-2* nor *sma-3* expression is largely affected by test bacteria. In contrast, *sma-4* expression is responsive to Gram-positive bacteria but not Gram-negative bacteria in the panel, possibly by both DBL-1 and DBL-1-independent mechanisms.

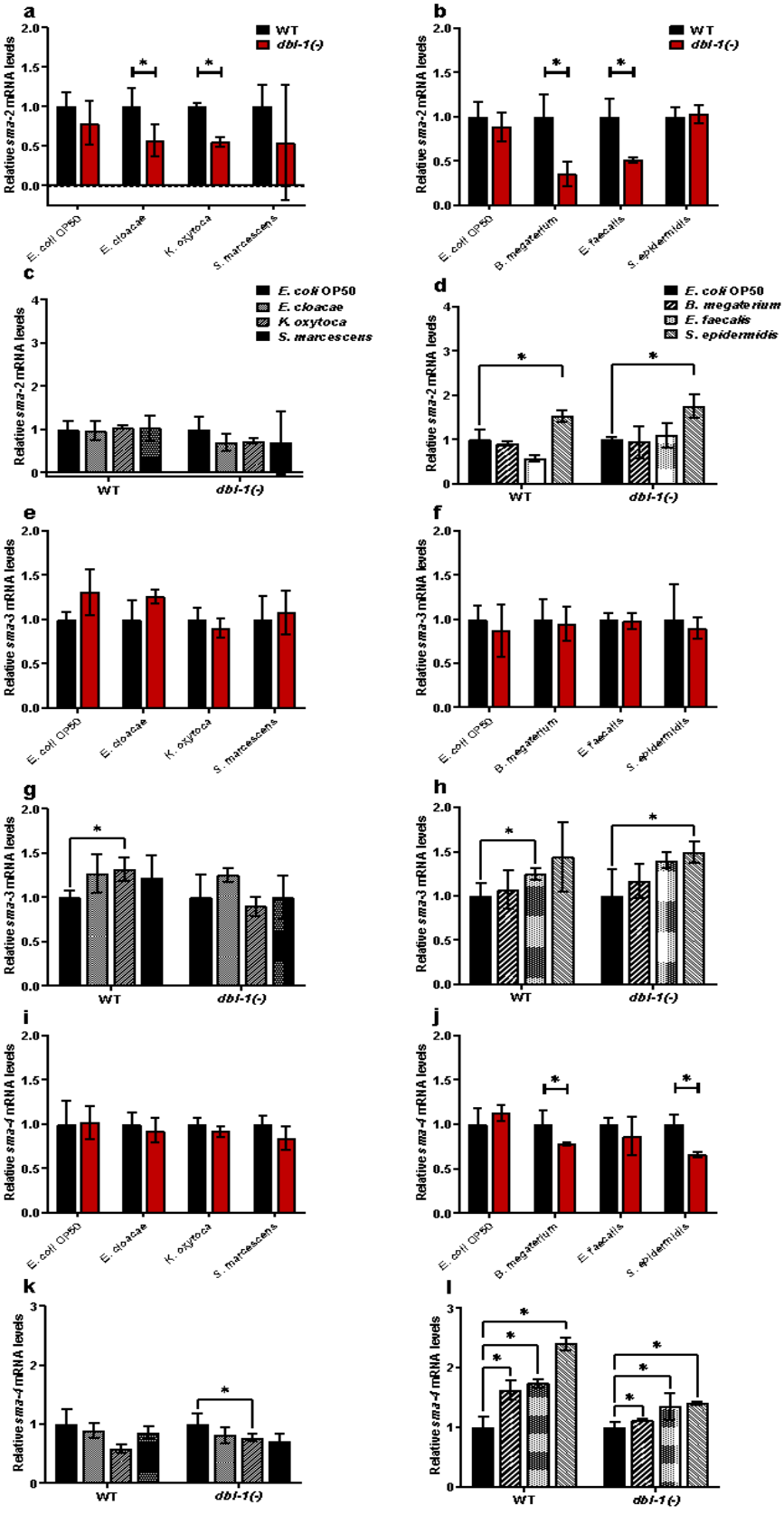
a
WT
dbl-1(-)
Relative sma-2 mRNA levels
E. coli OP50
E. cloacae
K. oxytoca
S. marcescens
b
WT
dbl-1(-)
Relative sma-2 mRNA levels
E. coli OP50
B. megaterium
E. faecalis
S. epidermidis
c
E. coli OP50
E. cloacae
K. oxytoca
S. marcescens
Relative sma-2 mRNA levels
WT
dbl-1(-)
d
E. coli OP50
B. megaterium
E. faecalis
S. epidermidis
Relative sma-2 mRNA levels
WT
dbl-1(-)
e
Relative sma-3 mRNA levels
E. coli OP50
E. cloacae
K. oxytoca
S. marcescens
f
Relative sma-3 mRNA levels
E. coli OP50
B. megaterium
E. faecalis
S. epidermidis
g
Relative sma-3 mRNA levels
WT
dbl-1(-)
h
Relative sma-3 mRNA levels
WT
dbl-1(-)
i
Relative sma-4 mRNA levels
E. coli OP50
E. cloacae
K. oxytoca
S. marcescens
j
Relative sma-4 mRNA levels
E. coli OP50
B. megaterium
E. faecalis
S. epidermidis
k
Relative sma-4 mRNA levels
WT
dbl-1(-)
l
Relative sma-4 mRNA levels
WT
dbl-1(-)

Figure 2.5. Smad transcription factors gene expression is altered by specific bacteria. Wild-type and *dbl-1(-)* animals at the L4 stage were exposed to *E. coli* OP50 (control), *E. cloacae*, *K. oxytoca*, *S. marcescens*, *B. megaterium*, *E. faecalis*, or *S. epidermidis* for 48 hours. mRNA expression levels of a–d) *sma-2*, e–h) *sma-3*, and i–l) *sma-4* were quantitated by real-time PCR. Experiments were performed in triplicate and in three independent trials. One representative trial of three is presented. Error bars represent standard deviation. * $p < 0.05$ in a, b, e, f, i, j, mRNA expression level in *dbl-1(-)* population compared to wild-type population exposed to the same bacteria by unpaired *t*-test. * $p < 0.05$ in c, d, g, h, k, l, mRNA expression level in respective genotype exposed to test bacteria compared to control bacteria by unpaired *t*-test.

DBL-1 Signaling Is Activated in Response to Gram-Negative Bacteria and Is Repressed in Response to Gram-Positive Bacteria

Because we observed changes in expression levels of the Smads, we asked if DBL-1 signaling activity is altered in response to the bacteria panel. To address this question, we challenged wild-type or *dbl-1(-)* animals expressing an integrated fluorescent DBL-1 pathway reporter to these bacteria and analyzing reporter fluorescence in L2 hypodermal nuclei. The expression of this reporter is robust in hypodermal nuclei at the L2 stage, and changes in DBL-1 affect hypodermal expression of RAD-SMAD[39]. This reporter (called RAD-SMAD) consists of the GFP gene under the control of multiple copies of a Smad binding element sequence[50]. In *dbl-1(-)* animals on *E. coli*, RAD-SMAD fluorescence was not detectable or very faint. In response to Gram-negative bacteria, reporter fluorescence in the wild-type background was significantly (two- to four-fold) increased compared to control bacterial conditions (see Fig. 2.6a–d, h). Reporter intensity in *dbl-1(-)* animals on all Gram-negative bacteria remained either

undetectable or very faint. In stark contrast, RAD-SMAD hypodermal fluorescence in wild-type animals was lost upon exposure to Gram-positive *S. epidermidis* (94%, n = 66 had no detectable expression) and *B. megaterium* (100%, n = 42). In about half of the wild-type population fed *E. faecalis*, hypodermal fluorescence was not detected (46%, n = 53), but fluorescence levels were wild type in those animals expressing RAD-SMAD in the hypoderm (see Fig. 2.6e–g, Supplementary Table 4). For animals lacking *dbl*-1 fed on any of the Gram-positive bacterial conditions, hypodermal RAD-SMAD fluorescence was undetectable or very faint. Exposure of *dbl-1(-)* animals to *S. epidermidis* and *B. megaterium* resulted in a stronger repression of RAD-SMAD activity than exposure to *E. faecalis*, similar to the wild-type responses to these Gram-positive bacteria (see Supplementary Table 4). In general, these results indicate that animals induce a DBL-1 signaling response to Gram-negative bacteria but repress DBL-1 signaling in response to Gram-positive bacteria. Furthermore, DBL-1 signaling levels appear to be modulated depending on the specific bacterial challenge that animals encounter.

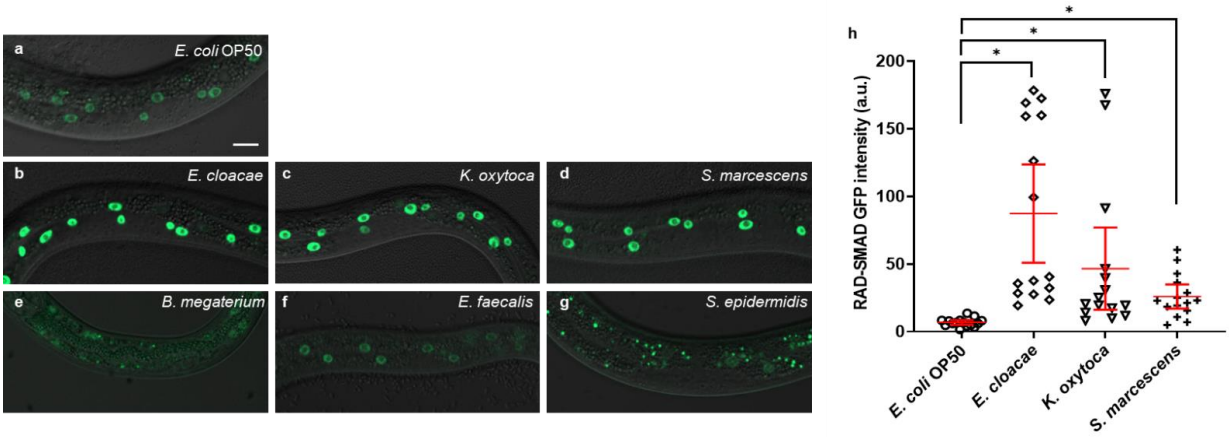

Figure 2.6. DBL-1 signaling is activated upon exposure to Gram-negative bacteria but is repressed in response to Gram-positive bacteria. L4 animals expressing the RAD-SMAD reporter in a wild-type background were exposed to a, h) *E. coli* OP50 (control), b, h) *E. cloacae*, c, h) *K. oxytoca*, d, h) *S. marcescens*, e) *B. megaterium*, f) *E. faecalis*, or g) *S. epidermidis* and L2-stage progeny were imaged. Mean RAD-SMAD fluorescence intensity of five hypodermal nuclei per animal was quantitated and compared. Experiments were performed in three independent trials. One representative trial is presented. Error bars represent 95% confidence intervals. n = 15 per condition in each trial. * $p < 0.05$, mean fluorescence intensity in wild-type background on test bacteria compared to control bacteria by unpaired *t*-test. Scale bar, 10 µm.

DBL-1 Mediates Both Common and Specific Gene Expression Responses to Gram-Negative and -Positive Bacteria

We then asked if this differential modulation of DBL-1 signaling activity translated to bacterial-specific downstream transcriptional responses. To identify the role of DBL-1 in differentially regulating transcription of downstream genes, we performed RNA sequencing using wild-type and *dbl-1(-)* animals exposed to the control or test bacteria (Gram-negative *S. marcescens* or Gram-positive *E. faecalis*). The animals were synchronized as L4s and fed on the control or test

bacteria for 48 hours before analysis. In animals lacking DBL-1 and fed control bacteria, 83 genes were down-regulated and 49 genes were upregulated compared to the wild type ($p < 0.01$, see Supplementary Fig. 2a). Some genes and gene classes previously reported to be regulated by DBL-1 at different developmental stages were also regulated by DBL-1 in two-day adults[20,24,25,51]. In *dbl-1(-)* animals fed on *S. marcescens*, 102 genes were down-regulated and 117 genes were upregulated (see Supplementary Fig. 2b). In *dbl-1(-)* animals fed on *E. faecalis*, 63 genes were down-regulated and 64 genes were upregulated compared to the wild type (see Supplementary Fig. 2c). The lower number of highly regulated genes between wild-type and *dbl-1(-)* animals fed on *E. faecalis* is consistent with the reduced DBL-1 reporter fluorescence—and therefore DBL-1 pathway signaling—in wild-type animals on *E. faecalis* (see Fig. 2.6, Supplementary Fig. 2). Notably, some highly regulated genes were common in response to both pathogenic bacterial strains, but some genes that were differentially regulated by DBL-1 were unique in response to either *S. marcescens* or *E. faecalis* exposure. Using WormCat, gene enrichment analysis of genes regulated by DBL-1 in response to *S. marcescens* or *E. faecalis* exposure revealed differential regulation of sets of genes involved in pathogen response, stress response, lipid metabolism, and transmembrane transport (see Supplementary Data 1)[52].

We focused on the DBL-1-regulated genes that have known or putative roles in innate immunity. These genes were induced in wild-type animals upon

exposure to *S. marcescens* or *E. faecalis* and this induction was lost in *dbl-1(-)*. We also found some genes to be upregulated upon loss of *dbl-1* in response to *S. marcescens* or *E. faecalis*. Some gene classes are highly regulated in response to these bacteria, but the specific genes within these families differed, including lysozyme, aspartyl protease, saposin-like, and C-type lectin genes (see Fig. 2.7). These results suggest that DBL-1 is involved in regulating (positively and negatively) transcription of some innate immunity genes specific to the bacterial exposure and some genes that are commonly regulated upon exposure to different bacteria. This supports a role for DBL-1 signaling in differentially regulating host responses to bacteria.

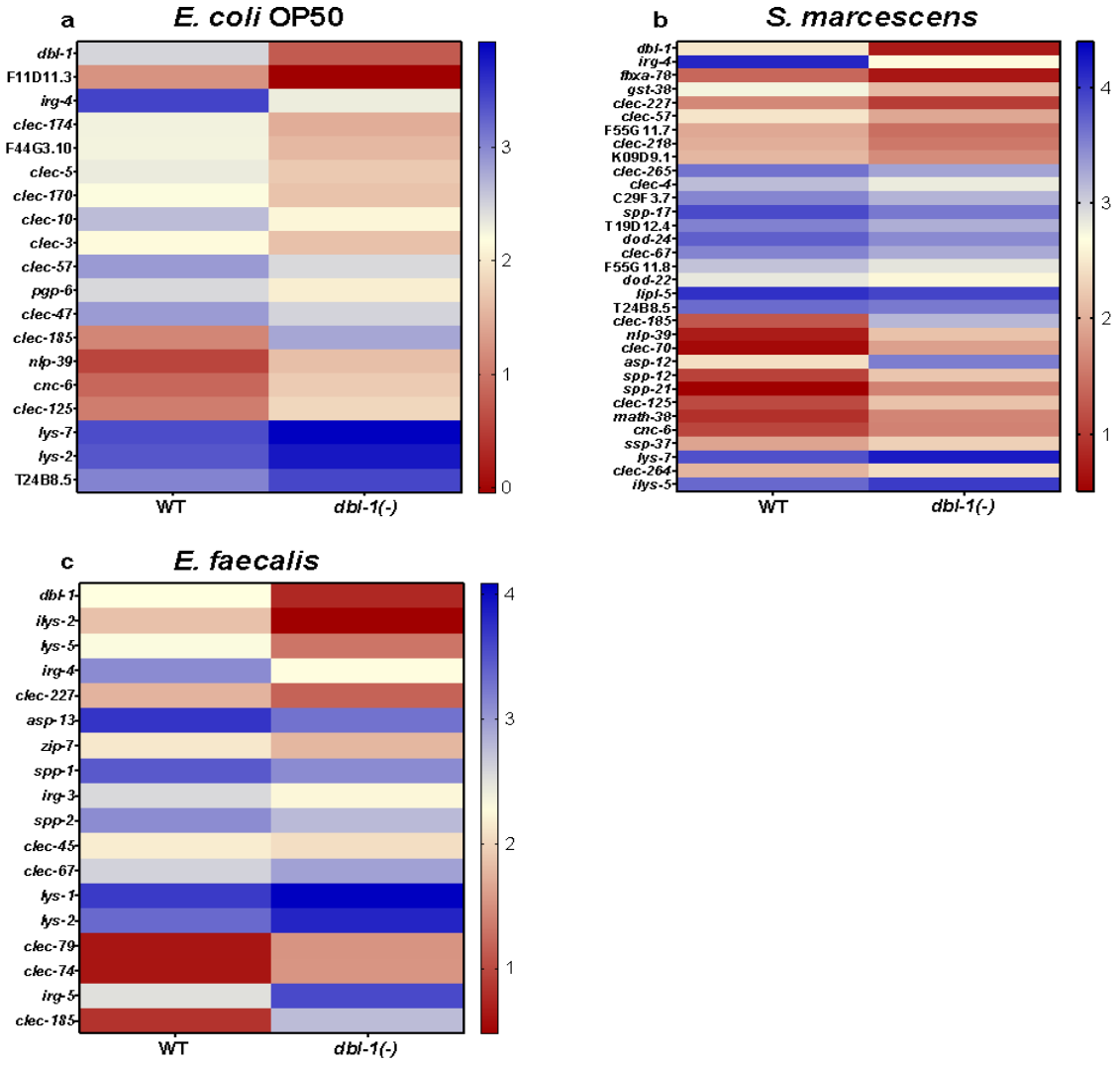

Figure 2.7. Expression of innate immunity genes is differentially regulated by DBL-1 signaling in different bacterial environments. L4 wild-type and *dbl-1(-)* animals were exposed for two days to *E. coli* OP50 (control), *S. marcescens*, or *E. faecalis*. RNA-seq analysis reveal significant changes (adjusted *p*-value < 0.01) in gene expression of animals lacking DBL-1. Heatmaps show differential innate immunity gene expression in animals lacking DBL-1 exposed to a) *E. coli* OP50, b) *S. marcescens*, and c) *E. faecalis* in comparison to wild-type animals exposed to the same bacteria. Average log FPKM values from three independent trials are represented.

DBL-1 Differentially Regulates Expression of Innate Immunity Genes Specific to the Gram Nature of Bacteria

From the RNA-sequencing results, we identified a panel of DBL-1 responsive innate immune genes that displayed differential responses to pathogen exposure. To determine if the expression of candidate target immunity genes is regulated by DBL-1 signaling in response to a wider variety of bacterial exposures, we used reporters of select immunity-related genes. Based on the RNA sequencing results, we selected *dod-22*, F55G11.7, *irg-4*, and *dod-24*. We also selected *ilys-3*, a known Gram-positive-responsive gene[9]. We tested expression of transcriptional reporters of these genes upon exposure to different Gram-negative and -positive bacteria. We measured and compared expression of these reporter genes in wild-type and *dbl-1(-)* backgrounds exposed to control or test bacteria. Animals were synchronized as L4s and fed on control, Gram-negative, or Gram-positive bacteria for 48 hours. Basal expression of these selected genes was measured in animals fed on the control *E. coli*.

dod-22 is a gene that is known to be regulated by the insulin-like signaling pathway transcription factor, DAF-16[21]. It is known to be involved in defense response to Gram-negative bacteria[16]. The *dod-22* reporter is induced in the wild-type background in the presence of all Gram-negative test bacteria compared to the control, but is not induced in response to the panel of Gram-positive bacteria. This induction of the *dod-22* reporter on Gram-negative bacteria is partly lost in the *dbl-1(-)* background, though loss of *dbl-1* does not affect expression levels on the

control *E. coli*. *B. megaterium* does not lead to a reproducible reduction of *dod-22* reporter fluorescence in the wild-type background, but a further reduction of fluorescence is observed in the *dbl-1(-)* background (see Fig. 2.8a, b). These results indicate that DBL-1 is not required for the basal expression of *dod-22*, but is required for *dod-22* induction on Gram-negative bacteria.

F55G11.7 is involved in innate immune responses to both Gram-negative and -positive bacteria in *C. elegans* and has been shown to be regulated by DAF-16/insulin, MAPK, and DBL-1 signaling pathways[16]. Expression of the F55G11.7 reporter did not change upon exposure to all test bacteria in the wild-type background except *B. megaterium*, where reporter expression was reduced (in two of three trials). We observed a significant reduction of F55G11.7 reporter activity in animals lacking DBL-1 except in response to *E. faecalis*. Additionally, a further reduction of F55G11.7 reporter fluorescence in *dbl-1(-)* animals was observed in response to Gram-positive *B. megaterium* and *S. epidermidis* compared to the response on control bacteria (see Fig. 2.8c, d). These findings indicate that expression of F55G11.7 is not altered upon exposure to most of the test bacteria but is generally down-regulated upon loss of DBL-1, in contrast with previous findings[16].

irg-4 is known to be involved in defense response to Gram-negative bacteria and has been shown to be regulated by DAF-16/insulin, MAPK, and DBL-1 signaling pathways[53–56]. In the wild-type background, we observed a visible, reproducible induction of *irg-4* reporter activity in response to Gram-

negative *S. marcescens*, but not to *K. oxytoca* or *E. cloacae*. Expression of the *irg-4* reporter in wild-type animals mildly increased in response to *S. epidermidis*, but not the other Gram-positive bacteria reproducibly. Loss of DBL-1 did not reproducibly alter *irg-4* reporter activity in control conditions. However, *irg-4* reporter induction in response to Gram-negative *S. marcescens* and Gram-positive *S. epidermidis* was lost in the *dbl-1(-)* background (see Fig. 2.8e, f). Indeed, *irg-4* reporter expression was reduced compared to the wild-type background on all bacteria except *E. cloacae* and *E. faecalis*. These results indicate that *irg-4* is responsive to a broad range of bacteria and is regulated in part by DBL-1 signaling.

dod-24, which is regulated by the insulin-like signaling transcription factor DAF-16, is involved in defense response to Gram-negative bacteria[53]. We observed robust expression of *dod-24* reporter activity in all tested Gram-negative bacteria, including the control, *E. cloacae*, *K. oxytoca*, and *S. marcescens*. Additionally, we observed further induction in response to *E. cloacae* and *S. marcescens*. We observed a striking decrease of *dod-24* reporter activity in wild-type animals exposed to all tested Gram-positive bacteria including *B. megaterium*, *E. faecalis*, and *S. epidermidis*. Loss of DBL-1 resulted in a significant reduction of *dod-24* reporter activity in control conditions. *dod-24* reporter activity was also drastically reduced in all Gram-negative bacterial conditions to levels significantly lower than the wild type (see Fig. 2.8g). In the three Gram-positive conditions, loss of DBL-1 resulted in a further decrease of

dod-24 reporter fluorescence relative to the wild type (see Fig. 2.8h). These results confirm that *dod-24* is differentially expressed in response to an array of Gram-negative and -positive bacteria and indicate that DBL-1 signaling plays a major role in regulating *dod-24* expression.

ilys-3 exhibits lysozyme activity and is involved in responding to Gram-positive bacteria[9]. *ilys-3* reporter activity in wild-type animals was unchanged or reduced in response to the tested Gram-negative bacteria (see Fig. 2.8i). We observed induction of *ilys-3* reporter activity upon exposure to all tested Gram-positive bacteria including *B. megaterium*, *E. faecalis*, and *S. epidermidis* (see Fig. 2.8j). Loss of DBL-1 did not alter *ilys-3* reporter activity in control conditions. The *ilys-3* reporter activity remained at relatively low levels in animals lacking DBL-1 exposed to Gram-negative bacteria (see Fig. 2.8i). However, *ilys-3* reporter activity also remained at relatively low levels upon loss of DBL-1 in response to Gram-positive bacteria *B. megaterium* and *S. epidermidis*, but was wild type in response to *E. faecalis* (see Fig. 2.8j). These results suggest that while DBL-1 is not required for basal levels of *ilys-3* expression, it is required for the induction of *ilys-3* expression in response to some Gram-positive bacteria.

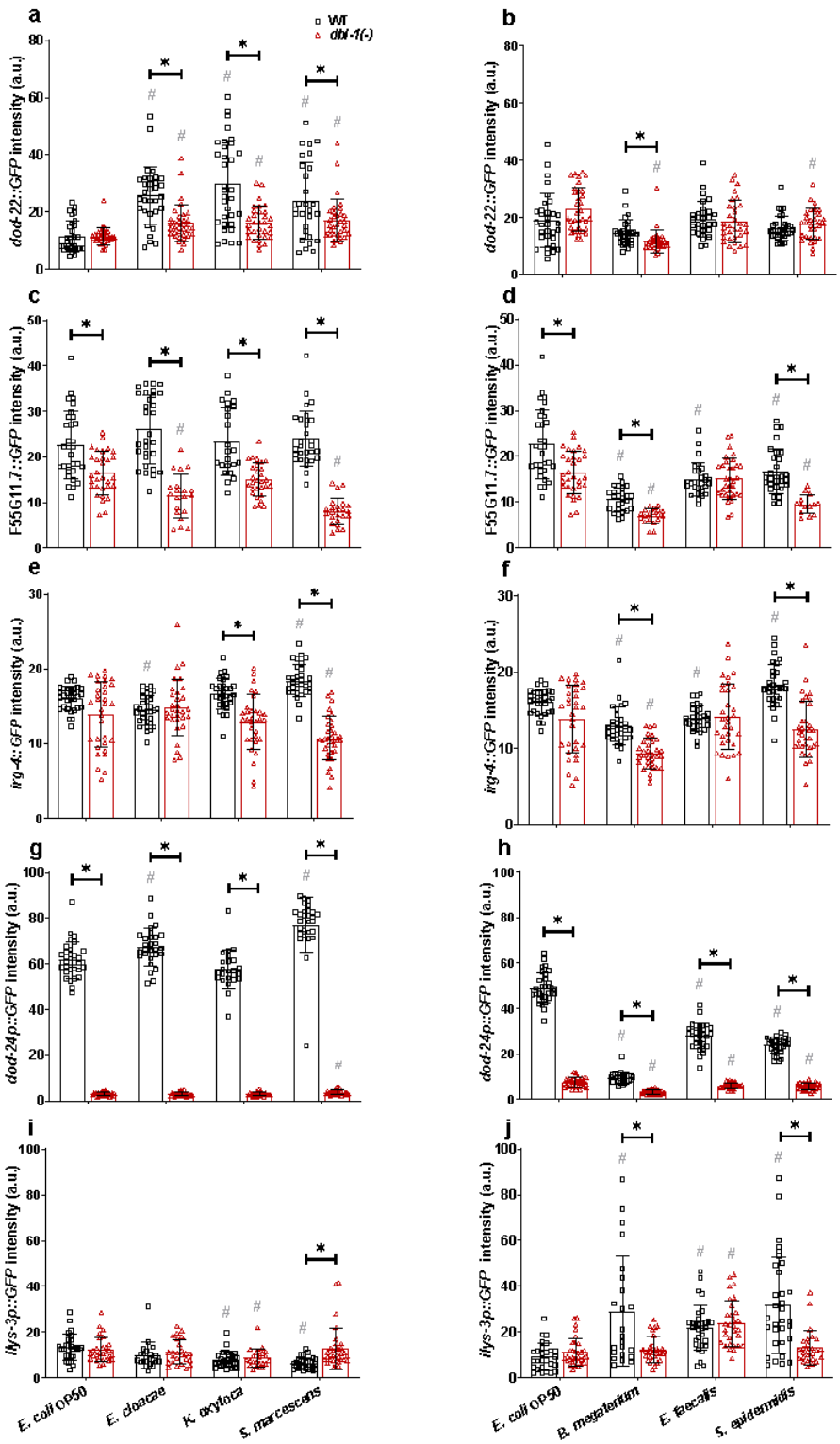
a
WT
dbl-1(-)
dod-22::GFP intensity (a.u.)
b
dod-22::GFP intensity (a.u.)
c
F55G11.7::GFP intensity (a.u.)
d
F55G11.7::GFP intensity (a.u.)
e
irg-4::GFP intensity (a.u.)
f
irg-4::GFP intensity (a.u.)
g
dod-24p::GFP intensity (a.u.)
h
dod-24p::GFP intensity (a.u.)
i
ilys-3p::GFP intensity (a.u.)
j
ilys-3p::GFP intensity (a.u.)
E. coli OP50
E. doacae
K. oxytoca
S. marcescens
E. coli OP50
B. megaterium
E. faecalis
S. epidermidis

Figure 2.8. Innate immune reporter activity is regulated by exposure to specific bacteria and by DBL-1 signaling. Comparison of a, b) *dod-22::*GFP, c, d) F55G11.7*::*GFP, e, f) *irg-4::*GFP, g, h) *dod-24p::*GFP, and i, j) *ilys-3p::*GFP intensities in adult wild-type and *dbl-1(-)* animals after a two-day exposure to the following bacteria; control *E. coli* OP50, *E. cloacae*, *K.* oxytoca, *S. marcescens, B. megaterium*, *E. faecalis*, or *S. epidermidis*. Imaging conditions including exposure times were consistent with respective control. Three independent trials were performed. One representative trial is shown. Error bars represent standard deviation. *n* = at least 15 per condition in each trial. * $p < 0.05$ compared to wild-type animals exposed to the same bacteria and # $p < 0.05$ respective genotype exposed to test bacteria in comparison to control bacteria, by two-way ANOVA followed by unpaired *t*-test.

These results collectively indicate the specific role of DBL-1 signaling in antimicrobial gene expression that helps tailor defense responses to specific bacterial challenges.

Discussion

Animals are subjected to a range of bacterial challenges, and how they respond is critical to the animals' health. Understanding how hosts respond to different pathogens is important for developing therapeutic strategies to help fight infections and prevent diseases. Dissecting the cell signaling pathways involved in host responses and their roles is critical. Our work expands the current understanding of how an organism integrates an arsenal of responses—from the molecular to the organismal—to different bacteria, and identifies role of the DBL-1/TGF-β pathway in robust host-specific responses to different types of bacteria. For this work, we established a panel of human opportunistic pathogens, including three Gram-negative and three Gram-positive strains, for the study of long-term innate immune responses in the roundworm *C. elegans*. The bacteria we selected for the panel elicit unique host response patterns that allowed us to

interrogate the role of the DBL-1 pathway in responding to different bacterial exposures. While DBL-1 has a known role in transcriptionally regulating innate immune gene expression, we show that the specific responses mediated by DBL-1 are not only molecular, but are also behavioral.

Our results support a model that the DBL-1 signaling pathway influences the organisms' perception of pathogenicity and helps keep the innate immune responses in check. Animals with reduced DBL-1 signaling—whether by downregulating signaling or by mutation—perceive the environment as more threatening and respond accordingly. DBL-1 pathway mutants display an outsized avoidance response to the Gram-negative bacteria. These animals also reduce intake of select Gram-negative bacteria as yet another way to reduce animals' interaction with the threat. Animals lacking DBL-1 also display a reduced lifespan in response to the Gram-negative bacteria that are sensed to be more pathogenic. There is also a correlation between the reduced feeding and extension of lifespan observed in animals exposed to select bacteria. Because exposure to the Gram-positive bacteria reduce the DBL-1 signaling activity, the avoidance response did not alter dramatically upon loss of DBL-1. The intake of the Gram-positive bacteria was reduced in both wild-type and *dbl-1(-)* populations, which correlated with extended lifespan observed for both populations on the Gram-positive bacteria. Damage to the intestine was not the underlying cause for the DBL-1-mediated lifespan alterations in response to the

tested bacteria. Determining how DBL-1 is involved in such behavioral modifications in response to different bacteria warrants future investigation.

DBL-1 signaling is also important for molecular responses to both Gram negative and Gram-positive bacteria. Regulation of DBL-1 signaling is part of the host's molecular response: the Gram-negative bacteria of our panel induced DBL-1 signaling while the Gram-positive bacteria repressed DBL-1 signaling. However, within these two bacterial groups, the host responses were tailored to the specific bacterial challenge. Our results show that DBL-1 signaling is also an important part of this molecular antimicrobial "fingerprint"[16]. Our RNA-sequencing analyses indicate both common and unique transcriptome-wide alterations mediated by DBL-1 after two days of exposure to Gram-negative and Gram-positive bacteria. We find that DBL-1 signaling is involved in activating as well as repressing innate immunity genes to maintain a balance of host immune responses (to possibly avoid overactivation of host immunity). In response to our panel of Gram-negative bacteria, DBL-1 signaling activity was induced and it further regulated expression of unique downstream innate immunity genes. In contrast, even though the DBL-1 signaling activity was repressed in response to the tested Gram-positive bacteria, DBL-1 was required to regulate expression of target immunity genes. While many gene classes differentially regulated by specific pathogens have been previously identified as important innate immune response genes, our work highlights the role that DBL-1 plays in tailoring the

molecular responses *C. elegans* engages against a range of pathogens[9,11,16,53,54,57].

Another major finding of this work is the differential requirement of the SMAD machinery to mediate avoidance responses to our panel of bacteria. Olofsson previously showed that loss of DBL-1, SMA-2, or SMA-4 increases avoidance of *E. coli*[48]. While our results with *sma-2* and *sma-4* are similar to theirs, we observe only mild avoidance of *E. coli* upon loss of DBL-1[48]. However, our work demonstrates that canonical DBL-1 signaling strongly suppresses avoidance to Gram-negative bacteria, but not to Gram-positive bacteria (see Fig. 2.3). *sma-4* mutants generally displayed stronger avoidance responses to both control and test bacteria suggesting a DBL-1-independent role for SMA-4 in suppressing avoidance responses. SMA-4 appears to play a double role in innate immune responses. Its starring role in immunity is with the DBL-1 pathway. DBL-1-independent induction of *sma-4* in response to Gram-positive bacterial conditions was also observed. This specificity of *sma-4* induction by the Gram-positive bacteria indicates specificity of innate immune responses. These results indicate that SMA-4 is not only recruited for defenses by something other than DBL-1, but also acts independently of the core DBL-1 pathway. PMK-1/MAPK signaling may be involved in regulating it as SMA-4 is predicted to genetically interact with PMK-1[58]. Interestingly, ATF-7, a transcription factor activated by PMK-1, is required for downregulation of *sma-4*—but not other DBL-1 pathway component genes—in wild-type animals exposed to Gram-negative *P.*

aeruginosa PA14[59]. It will be of interest to discover if SMA-4 plays a broader role in the innate immune response than acting in the DBL-1 pathway.

Overall, we propose that loss of DBL-1 signaling changes the animal's perception of the environment as more hostile, and this results in more robust protective responses that depend on the specific bacterial challenge. This may help explain the neuronal source of DBL-1 secretion that then targets hypoderm, intestine, and pharynx. DBL-1 secreted from the AVA interneurons activates DBL-1 signaling in the hypodermis to regulate aversive learning upon exposure to Gram-negative *P. aeruginosa*, but the neuronal circuit(s) used by DBL-1 to direct aversive behaviors remains to be identified[27]. SMA-4 plays a double role in innate immune responses, acting as part of the core DBL-1 signaling pathway but also acting in another additive way, suggesting crosstalk with other signaling pathways. Several studies report roles of signaling pathways including MAPK and insulin-like signaling pathways in mounting immune responses to many Gram-positive bacteria. Future work may identify the possible crosstalk mechanisms with the DBL-1 pathway in regulating organismal defense responses. In summary, these findings support a central role for DBL-1/TGF-β signaling not only in crafting tailored responses to unique bacterial challenges, from transcription of specific innate immunity genes to behavioral responses but also being modulated in response to bacteria.

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Author Contributions

B.M. and T.L.G. conceptualized and designed experiments, interpreted data, and contributed to writing; B.M. performed experiments.

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Competing Interests

The authors declare no competing interests.

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Supplementary Material

Supplementary Table 1. List of strains

Strains used include:

Strain	Genotype
N2	Wild type
NU3	*dbl-1(nk3)* V (referred to as *dbl-1(-)* in this work)
CB502	*sma-2(e502)* III (referred to as *sma-2(-)* in this work)
CB491	*sma-3(e502)* III (referred to as *sma-3(-)* in this work)
DR1369	*sma-4(e729)* III (referred to as *sma-4(-)* in this work)
LW2436	*jjIs2277[pCXT51(5*RLR::pes-10p(deleted)::GFP) + LiuFD61(mec-7p::RFP)]* I or IV (RAD-SMAD)
CB6710	*eEx650[ilys-3p::GFP + unc-119(+)]*
CF3556	*agIs6[dod-24p::GFP]*
SAL139	*denEx17[dod-22::GFP + unc-119(+)]*
SAL143	*denEx21[*F55G11.7*::GFP + unc-119(+)]*
SAL148	*denEx26[irg-4::GFP + unc-119(+)]*

Strains created for this work include:

Strain	Genotype
TLG803	*dbl-1(nk3)* V; *agIs6[dod-24p::GFP]*
TLG804	*dbl-1(nk3)* V; *eEx650[ilys-3p::GFP + unc-119(+)]*
TLG805	*dbl-1(nk3)* V; *denEx26[irg-4::GFP + unc-119(+)]*
TLG806	*dbl-1(nk3)* V; *denEx21[*F55G11.7*::GFP + unc-119(+)]*
TLG807	*dbl-1(nk3)* V; *denEx17[dod-22::GFP + unc-119(+)]*
TLG810	*jjIs2277[pCXT51(5*RLR::pes-10p(deleted)::GFP) + LiuFD61(mec-7p::RFP)]* I or IV; *dbl-1(nk3)* V

Supplementary Table 2. List of primers for qRT-PCR

Target gene	Forward primer (5'->3')	Reverse primer (5'->3')
sma-2	TCCACCAGGAGTTCCAACAT	ACCTGTTCTCCGACTCTTGT
sma-3	GAGAACACACGGATGCATATTGG	ACTGTGCGGTGGTATTCGG
sma-4	GATGCTCCGACGTTCTCGAT	CGCATCCTGTCAACTCCACT
act-1	GCCGGAATCCACGAGACTTC	TCTGGTGGGGCGATGATCTT

Supplementary Table 3. Survival assay summary

Supplementary Table 3. Survival assay summary													
Bacteria	Nematode strain	Trial 1				Trial 2				Trial 3			
		test mean lifespan ± SE	n	control mean lifespan ± SE	n	test mean lifespan ± SE	n	control mean lifespan ± SE	n	test mean lifespan ± SE	n	control mean lifespan ± SE	n
E. cloacae	WT	16.22±0.39	104	12.3±0.37	110	15.58±0.30	105	14.98±0.35	102	16.68±0.52	71	13.84±0.40	75
	dbl-1(-)	11.64±0.33 *	105	11.87±0.38	111	13.67±0.41 ns	112	14.36±0.36	104	11.66±0.47 *	58	14.17±0.40	58
K. oxytoca	WT	14.34±0.31	120	12.3±0.37	110	16.16±0.27	110	14.98±0.35	102	16.41±0.30	75	13.84±0.40	75
	dbl-1(-)	12.55±0.32 *	108	11.87±0.38	111	14.87±0.31 ns	100	14.36±0.36	104	9.31±0.55 *	62	14.17±0.40	58
S. marcescens	WT	15.09±0.28	107	15.19±0.23	111	12.40±0.38	115	12.3±0.37	110	13.43±0.39	113	14.98±0.35	102
	dbl-1(-)	8.56±0.25 *	111	14.58±0.22	97	6.58±0.29 *	108	11.87±0.38	111	6.21±0.30 *	105	14.36±0.36	104
B. megaterium	WT	16.60±0.21	101	13.44±0.21	115	16.54±0.21	84	14.06±0.24	95	12.86±0.46	63	9.07±0.55	71
	dbl-1(-)	13.82±0.19 *	119	14.2±0.18	116	15.25±0.22 *	84	13.99±0.22	84	13.47±0.27 ns	73	8.61±0.37	77
E. faecalis	WT	17.77±0.28	121	13.82±0.25	121	16.54±0.29	119	13.20±0.22	123	17.46±0.32	129	13.32±0.24	121
	dbl-1(-)	15.83±0.34 *	75	13.20±0.29	99	13.25±0.39 *	142	13.72±0.28	129	14.97±0.33 *	126	13.58±0.23	122
S. epidermidis	WT	17.92±0.21	122	13.82±0.25	121	15.97±0.34	104	13.20±0.22	123	16.60±0.20	115	13.32±0.24	121
	dbl-1(-)	8.84±0.42 *	138	13.20±0.29	99	11.88±0.35 *	138	13.72±0.28	129	13.32±0.39 *	143	13.58±0.23	122

* log rank test $p<0.0001$, ns log rank test $p>0.01$, comparing mean *dbl-1(-)* lifespan with wild-type lifespan on each bacteria

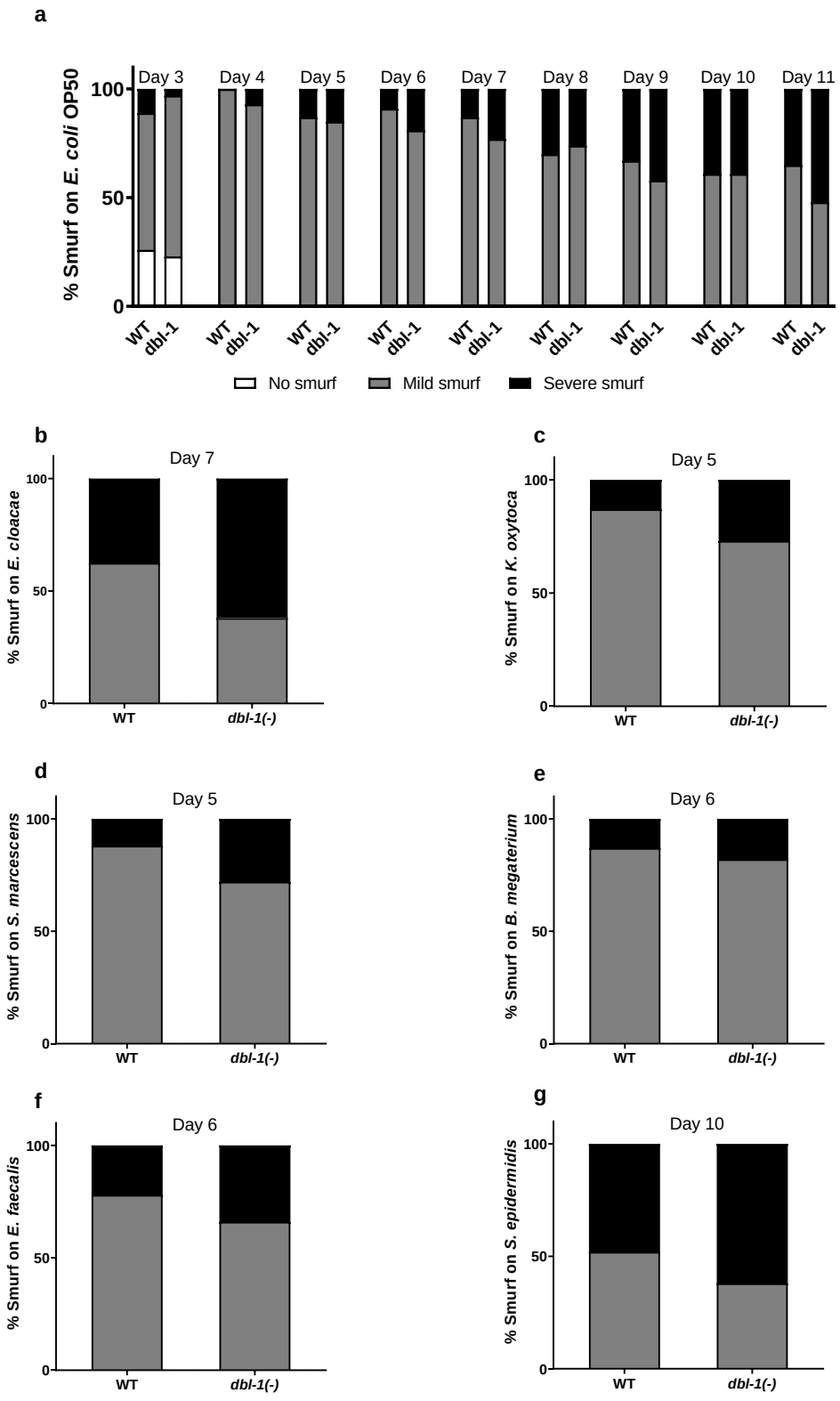
Supplementary Table 4. RAD-SMAD reporter activity in response to Gram-positive bacteria

Bacteria	Nematode strain	% animals with no detectable fluorescence		
		Trial 1	Trial 2	Trial 3
B. megaterium	WT	100%	100%	100%
	dbl-1(-)	100%	100%	100%
E. faecalis	WT	95%	22%	21%
	dbl-1(-)	62%	40%	65%
S. epidermidis	WT	95%	95%	91%
	dbl-1(-)	100%	95%	78%

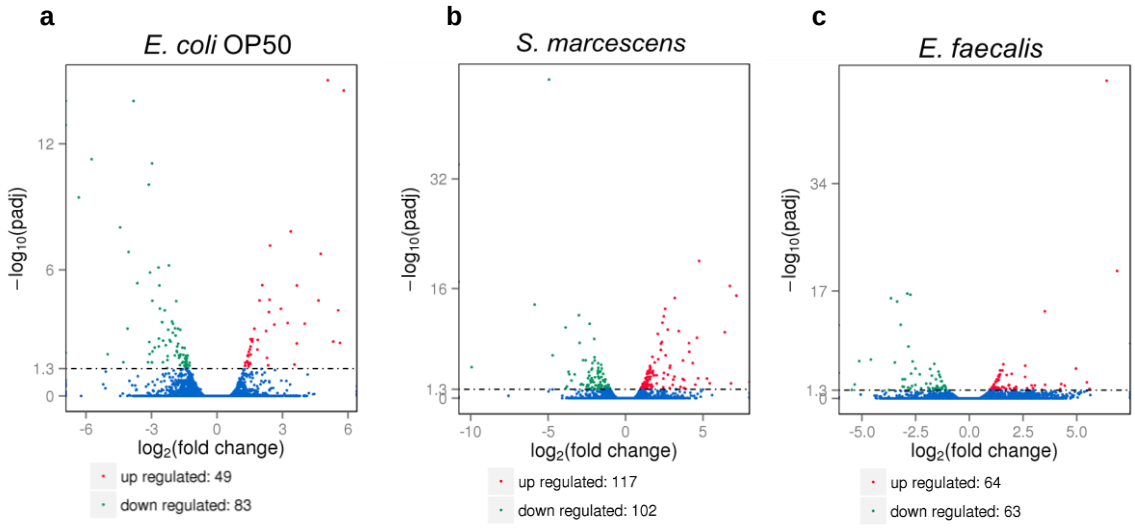
Supplementary Data 1. Gene enrichment analysis by WormCat of differentially expressed genes between wild-type and *dbl-1(-)* populations exposed to a) *S. marcescens* and b) *E. faecalis*

a) http://www.wormcat.com/static/dynamic/RGS_Mar-25-2021-05_56_38/sunburst.html

b) http://www.wormcat.com/static/dynamic/RGS_Mar-25-2021-05_58_42/sunburst.html


a
% Smurf on E. coli OP50
100
50
0
Day 3
Day 4
Day 5
Day 6
Day 7
Day 8
Day 9
Day 10
Day 11
WT
dbl-1
No smurf
Mild smurf
Severe smurf
b
Day 7
% Smurf on E. cloacae
WT
dbl-1(-)
c
Day 5
% Smurf on K. oxytoca
d
Day 5
% Smurf on S. marcescens
e
Day 6
% Smurf on B. megaterium
f
Day 6
% Smurf on E. faecalis
g
Day 10
% Smurf on S. epidermidis

Supplementary Figure 1. Loss of DBL-1 does not affect intestinal integrity upon exposure to specific bacteria. Wild-type and *dbl-1(-)* animals at the L4 stage were exposed to the following bacteria a) *E. coli* OP50 (control), b) *E. cloacae*, c) *K. oxytoca*, d) *S. marcescens*, e) *B. megaterium*, f) *E. faecalis*, or g) *S. epidermidis*. Intestinal barrier function was assessed using erioglaucine disodium salt a) over time or b–g) when *dbl-1(-)* populations neared their half lifespan. The leakiness of the intestine was assessed and scored as '1' for no leakage/no Smurf, '2' for mild leakage/mild Smurf, and '3' for severe leakage/severe Smurf phenotypes. The fraction of animals indicating these phenotypes was calculated. One representative trial of at least three is presented. n = at least 10 per condition.



Supplementary Figure 2. DBL-1 regulates differential gene expression in response to Gram-negative and Gram-positive bacteria. Wild-type and *dbl-1(-)* animals were exposed to *E. coli* OP50 (control), *S. marcescens*, or *E. faecalis* at the L4 stage for two days. RNA-seq analysis volcano plots show differential gene expression in animals lacking DBL-1 exposed to a) *E. coli* OP50, b) *S. marcescens*, and c) *E. faecalis* in comparison to wild-type animals exposed to the same bacteria (adjusted *p*-value < 0.01). Genes down-regulated in *dbl-1(-)* animals are represented in green, genes up-regulated in *dbl-1(-)* animals are represented in red, and genes with no change in expression are represented in blue.

CHAPTER III

CAENORHABDITIS ELEGANS BROOD SIZE AND EGG-LAYING RESPONSES TO *SERRATIA MARCESCENS* AND *STAPHYLOCOCCUS EPIDERMIDIS* ARE INDEPENDENT OF DBL-1 SIGNALING

This chapter contains a publication: Madhu, BJ; Salazar, AE; Gumienny, TL (2019). *Caenorhabditis elegans* egg-laying and brood-size changes upon exposure to *Serratia marcescens* and *Staphylococcus epidermidis* are independent of DBL-1 signaling. microPublication Biology. https://doi.org/10.17912/2r51-b476.

Description

Caenorhabditis elegans naturally thrives in a soil environment where they feed on bacteria and are in constant association with a diverse range of microbes (Barker et al. 1994). *C. elegans* egg laying is delayed or reduced when animals are infected with *Burkholderia pseudomallei*, *Burkholderia thailandensis*, *Staphylococcus aureus*, and *Serratia marcescens* (Irazoqui et al. 2010; Mallo et al. 2002; O'Quinn et al. 2001). These changes in egg laying may be a protective response to pathogenic bacteria. Mutants of TGF-β-like DBL-1 signaling pathway also display reduced brood size (Luo et al. 2009; Roberts et al. 2010). While the peak of egg-laying activity seen in normal animals between days 2 and 4 is depressed in *dbl-1* pathway mutants, the reproductive span of these *dbl-1* pathway mutants is increased to up to 13 days (Luo et al. 2009). To determine if the egg-laying observed during infection is DBL-1 pathway-dependent, we tested the effect of the DBL-1 signaling pathway on egg

laying when *C. elegans* were fed on representative Gram-negative (*S. marcescens*) and Gram-positive bacteria (*Staphylococcus epidermidis*).

Similar to previously published reports, we found that loss of DBL-1 pathway signaling decreases brood size and increases reproductive span in normal laboratory conditions (*E. coli* strain OP50 and 20°C incubation; see Figure 3.1A and B; Luo et al. 2009; Roberts et al. 2010).

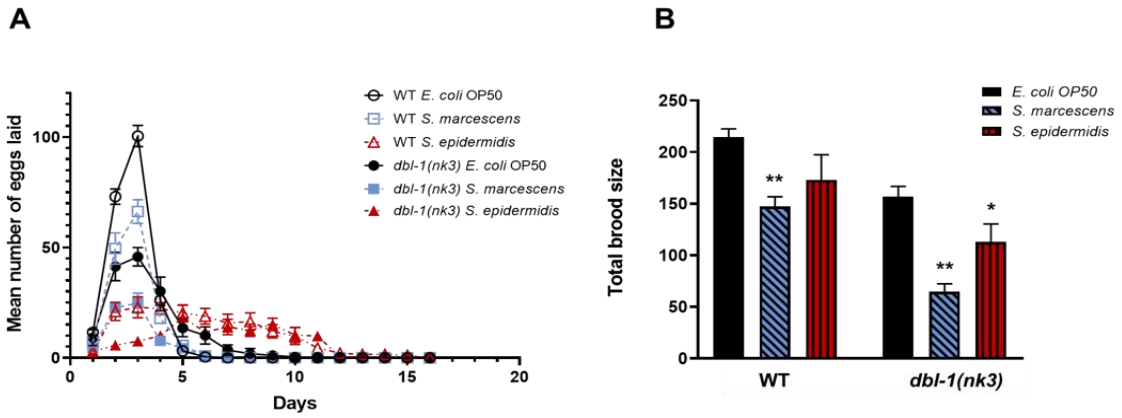


Figure 3.1. Effects of *S. marcescens* and *S. epidermidis* on egg laying and brood size in wild-type and *dbl-1(nk3)* populations. (A) Comparison of mean eggs laid between wild-type (WT) and *dbl-1(nk3)* populations fed on *E. coli* OP50, *S. marcescens*, or *S. epidermidis*. The counts of number of eggs laid were reported only for plates with live animals. Error bars represent SEM. n = 7–10 surviving animals each. (B) Comparison of total brood size between wild-type and *dbl-1(nk3)* populations fed on *E. coli* OP50, *S. marcescens*, or *S. epidermidis*. Total brood sizes of animals that desiccated were censored. Error bars represent SEM. n = 7–10 surviving animals each. Within each genetic background, significant differences between the *E. coli* OP50-fed control and the pathogen-fed populations are marked with asterisks (*, $p < 0.05$, **, $p < 0.001$).

Here, we report three new results. First, brood size reductions caused by infection and by loss of DBL-1 signaling are independent (see Figure 3.1A). Wild-type and *dbl-1(nk3)* animals both significantly decrease their brood size when

grown on *S. marcescens* ($p = 0.005$ and $p < 0.001$, respectively). *dbl-1* mutant animals laid even fewer eggs than the wild-type animals on *S. marcescens*, suggesting that the reduced brood size phenotype is independently affected by both *S. marcescens* exposure and by loss of DBL-1 ($p < 0.001$). While the decrease in brood size of wild-type animals on *S. epidermidis* was not significant ($p = 0.115$), the decreased brood size of *dbl-1(nk3)* animals was significant on this pathogenic bacterial strain ($p = 0.045$). Indeed, the decreases in brood size upon infection with either *S. marcescens* or *S. epidermidis* in both wild-type and *dbl-1(nk3)* populations are similar ($p = 0.57$), suggesting the pathogenic bacteria affect brood size independent of DBL-1. Because *dbl-1(nk3)* populations display a further reduced brood size upon infection by either pathogen compared to the wild type, the negative effects of pathogen exposure and loss of DBL-1 signaling on brood size appear to be additive ($p < 0.05$).

Second, while the wild-type population on *S. marcescens* survived until all animals ceased laying eggs, all *dbl-1(nk3)* animals died on *S. marcescens* by Day 5. These results explain why the extended reproductive span normally seen in *dbl-1(nk3)* populations was not observed on *S. marcescens*. These results also support previous reports of decreased viability of *dbl-1* mutant animals on another variety of *S. marcescens*, Db11 (Mallo et al. 2002).

Third, *S. epidermidis* affects egg-laying patterns similar to loss of *dbl-1* function. Initially, both wild-type and *dbl-1(nk3)* strains on *S. epidermidis* have reduced eggs laid in the first four days compared to strains grown on the *E.*

coli control. Loss of DBL-1 further reduced the number of eggs laid during each of these days, suggesting that this phenotype is independently affected by both *S. epidermidis* exposure and by loss of DBL-1 ($p = 0.004$). Then, both wild-type and *dbl-1(nk3)* strains on *S. epidermidis* have similar extended reproductive spans, extending to at least Day 13 (one tenacious wild-type hermaphrodite laid embryos until Day 15). This *S. epidermidis*-induced reproductive span extension appears to be independent of DBL-1 signaling, because the numbers of eggs laid by both wild-type and *dbl-1(nk3)* populations between Days 5 and 16 were similar at these time points ($p = 0.509$).

Methods

Animals were age-synchronized by hypochlorite treatment (Stiernagle, 2006) and grown on plates seeded with *Escherichia coli* OP50. Ten L4 animals were manually transferred to individual plates seeded with *S. marcescens* or *S. epidermidis*. Plates were completely covered by bacteria to prevent animals from avoiding the bacteria. Adults were daily transferred to new plates and the number of eggs laid on each plate was counted every 24 hours until no more eggs were laid. Statistical analyses were performed using repeated measures ANOVA and Tukey's post-hoc test.

Reagents

Strains were maintained on EZ media plates at 20°C (0.55 g Tris-Cl, 0.24 g Tris base, 3.1 g BD Bacto™ Peptone, 8 mg cholesterol, 2 g sodium chloride, 20 g agar, in water to 1 L (E. Lambie, personal communication). The *C.*

elegans strains used were N2 and NU3 *dbl-1(nk3).* The Gram-negative bacterial strains used were *Escherichia coli* OP50 (CGC) and *Serratia marcescens* (Carolina Biological Supply Company). The Gram-positive bacterial strain used was *Staphylococcus epidermidis* (ATCC 49134). *S. marcescens* and *S. epidermidis* were provided by A. J. Hammett, TWU. All bacterial strains were grown for 9 hours in tryptic soy broth at 37°C before plating on EZ media plates.

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CHAPTER IV

CAENORHABDITIS ELEGANS SAPOSIN-LIKE *SPP-9* IS INVOLVED IN SPECIFIC INNATE IMMUNE RESPONSES

This chapter contains a publication: Madhu, B., Lakdawala, M.F., Issac, N. G., Gumienny, T.L. (2020) *Caenorhabditis elegans* saposin-like *spp-9* is involved in specific innate immune responses. Genes & Immunity. https://doi.org/10.1038/s41435-020-0108-6

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Key words: saposin-like proteins, *spp-9*, *dbl-1*, innate immune responses, TGF-β superfamily signaling

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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 anti-microbial 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 *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 *rrf-3(pk1426) II*, TLG697 *texIs127[spp-9p::gfp] X*, LT998 *wkIs40[spp-9p::gfp]*, TLG707 *dbl-1(nk3) V; texIs127 X*, TLG755 *oyIs44 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 nine hours at 37°C. Bacterial cells were pelleted at 5000 rpm for 15 minutes and concentrated twenty-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 hours 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 hours 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 hours 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 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 actin gene *act-1* (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 to wild-type ΔC_t values using the unpaired *t*-test.

Data and Reagent Availability

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

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 (see Figure 4.1A–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 (see Figure 4.1F). We confirmed the expression of *spp-9* in the AWB and AWC neurons by showing co-localization 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 hours, 36 hours, and 48 hours 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 (see Figure 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.

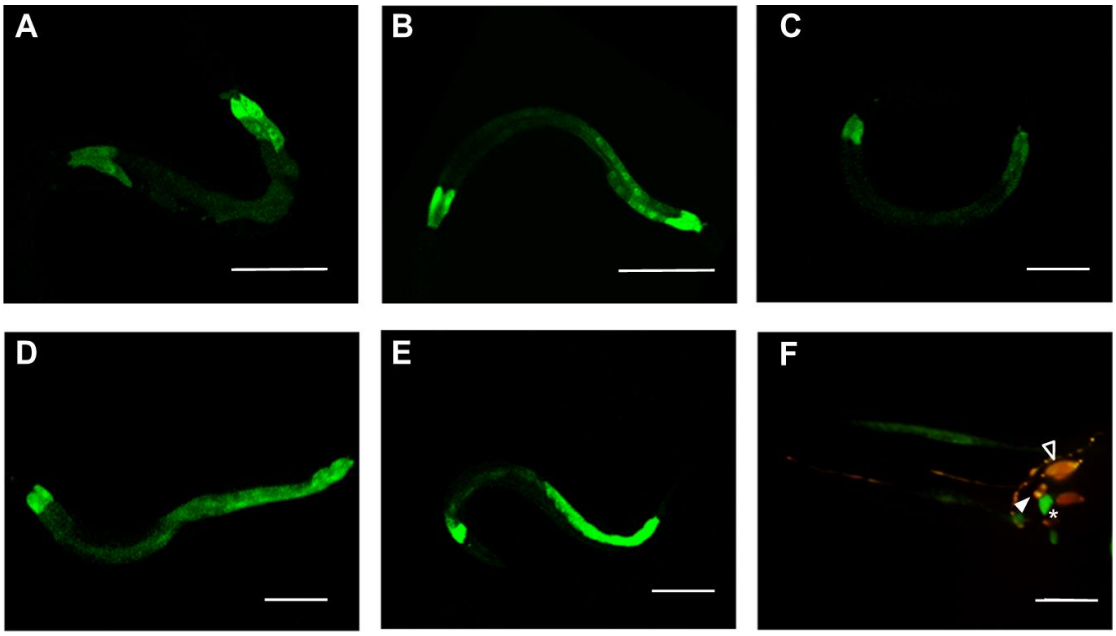

Figure 4.1. The *spp-9* reporter is expressed throughout larval and adult stages in intestine and head neurons. Confocal images show expression of *spp-9p::*GFP in A) L1, B) L2, C) L3, D) L4, and E) 1-day adult in the intestine, with strong expression in the anterior and posterior ends of intestine. 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). Asterisks mark a third neuron that expresses the *spp-9* reporter. Scale bar indicates A, B) 50 µm, C, D, E) 100 µm, and F) 10 µm.

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 high in starved animals (24). We asked if starvation affects expression of *spp-9*. By 8 hours 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 hours after starvation and compared to identically staged animals that remained fed during that time. *spp-9* expression was significantly increased when animals were deprived of food (see Figure 4.2). This suggests that expression of *spp-9* is upregulated in starved animals.

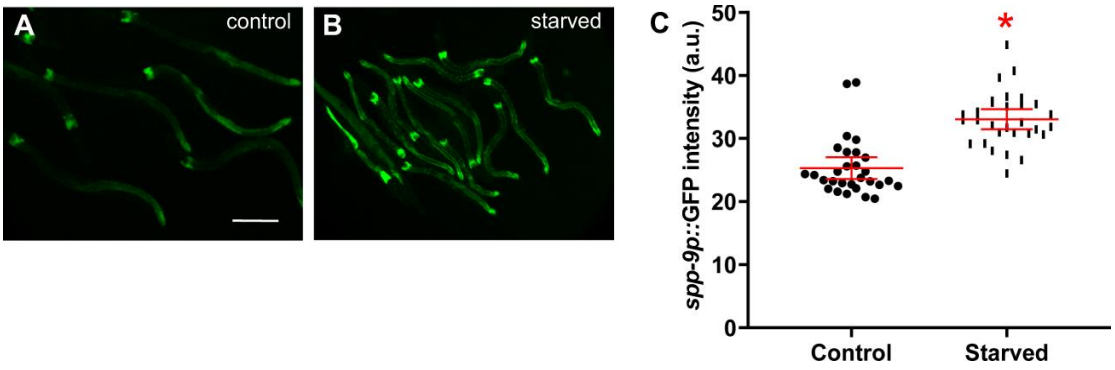

Figure 4.2. Starvation increases *spp-9* reporter activity. Fluorescence images show *spp-9p::*GFP intensities of A) fed L4 animals and B) age-matched animals starved for 8 hours post L4 stage. Imaging conditions including exposure times were consistent. Scale bar indicates 100 µm. C) Comparison of *spp-9p::*GFP 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 to fed control by unpaired *t*-test.

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 to 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 hours. Animals were tested at 48 hours because by that time, there was a robust response of the *spp-9* reporter on *E. coli* (see Figure S1). Basal expression was measured for *spp-9p*::GFP fluorescence in animals fed *E. coli* OP50 (see Figure 4.3A, 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 (see Figure 4.3B, C, 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 (see Figure 4.3I, 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.

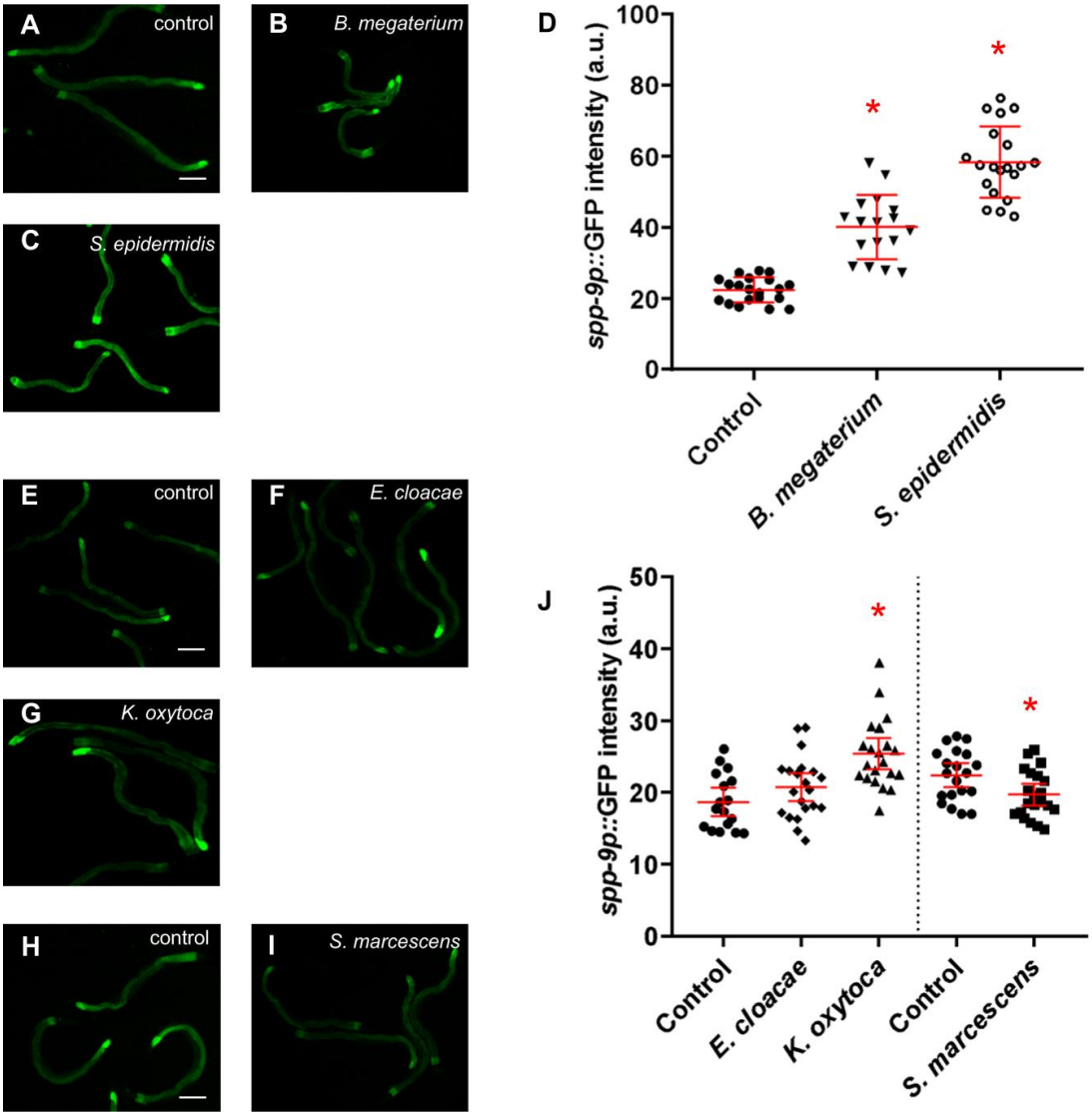

Figure 4.3. *spp-9* reporter activity is altered upon exposure to specific Gram-positive and Gram-negative bacteria. Fluorescence images show *spp-9p::*GFP intensities in adult animals after a two-day exposure to the following bacteria; A, E, H) control *E. coli* OP50 (*n* = 17, 20, 20), B) *B. megaterium* (*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 to 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 to *E. coli* OP50 by unpaired *t*-test.

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 (see Figure 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* (see Figure 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).

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 hours 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 (see Figure 4.4A, 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) (see Figure 4.4C, 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.

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 (see Figure 4.4B, 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 to the control (see Figure 4.3B, 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 (see Figures 4.3 and 4.4).

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 (see Figures 4.3 and 4.4, 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*.

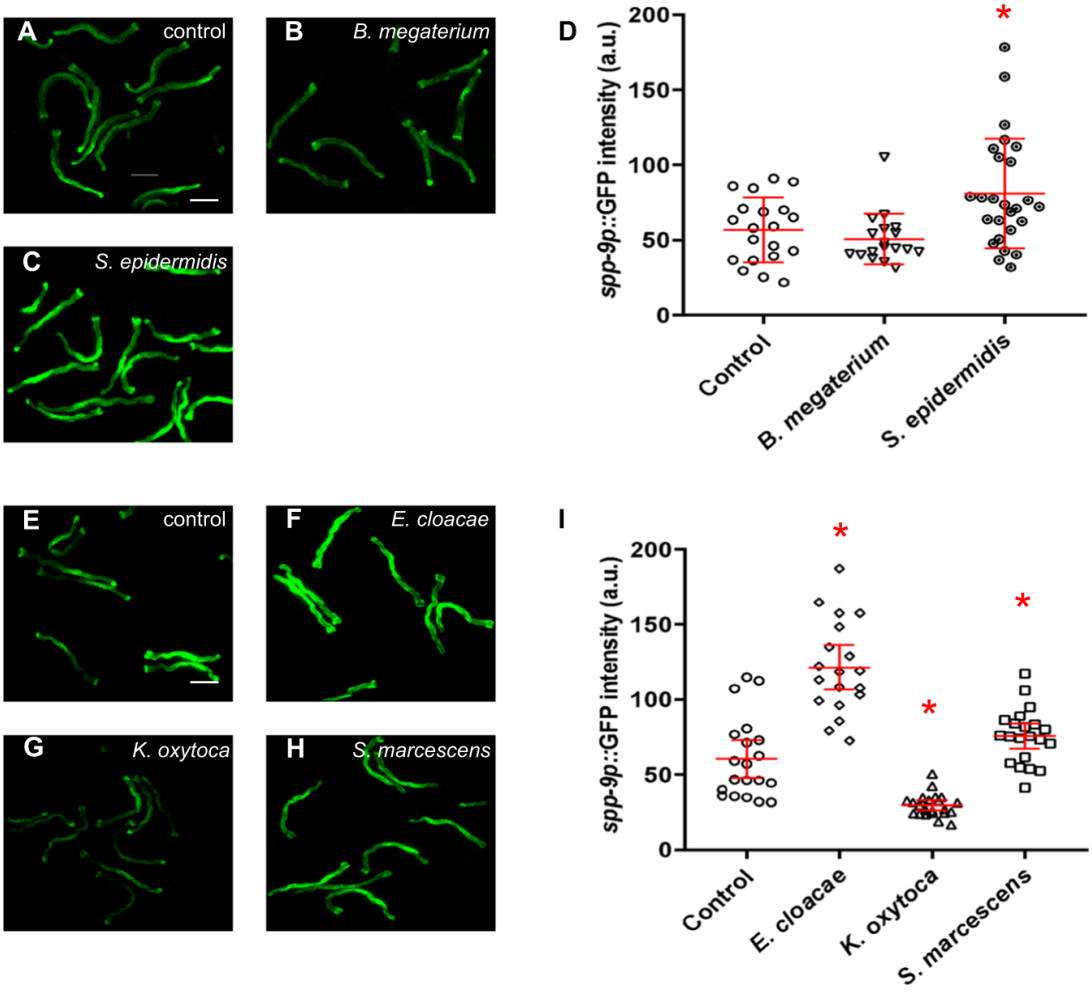

Figure 4.4. *spp-9* reporter activity is altered in response to specific pathogens by DBL-1 and other innate immunity signaling pathways. Fluorescence images show *spp-9p::*GFP intensities in adult *dbl-1(-)* animals after a two-day exposure to the following bacteria; A, E) control *E. coli* OP50 (*n* = 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 µm. 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 to 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 to *E. coli* OP50 by unpaired *t*-test.

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 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 (see Figure 4.5B, D). As expected, animals lacking *daf-16* showed increased *spp-9* reporter fluorescence (see Figure 4.5C, 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 (see Figure 4.5G, H, J, K). However, loss of MAP2K gene *sek-1(-)* had no effect on the fluorescent intensities in comparison to the control (see Figure 4.5F, 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 (see Figure 4.5M, 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.

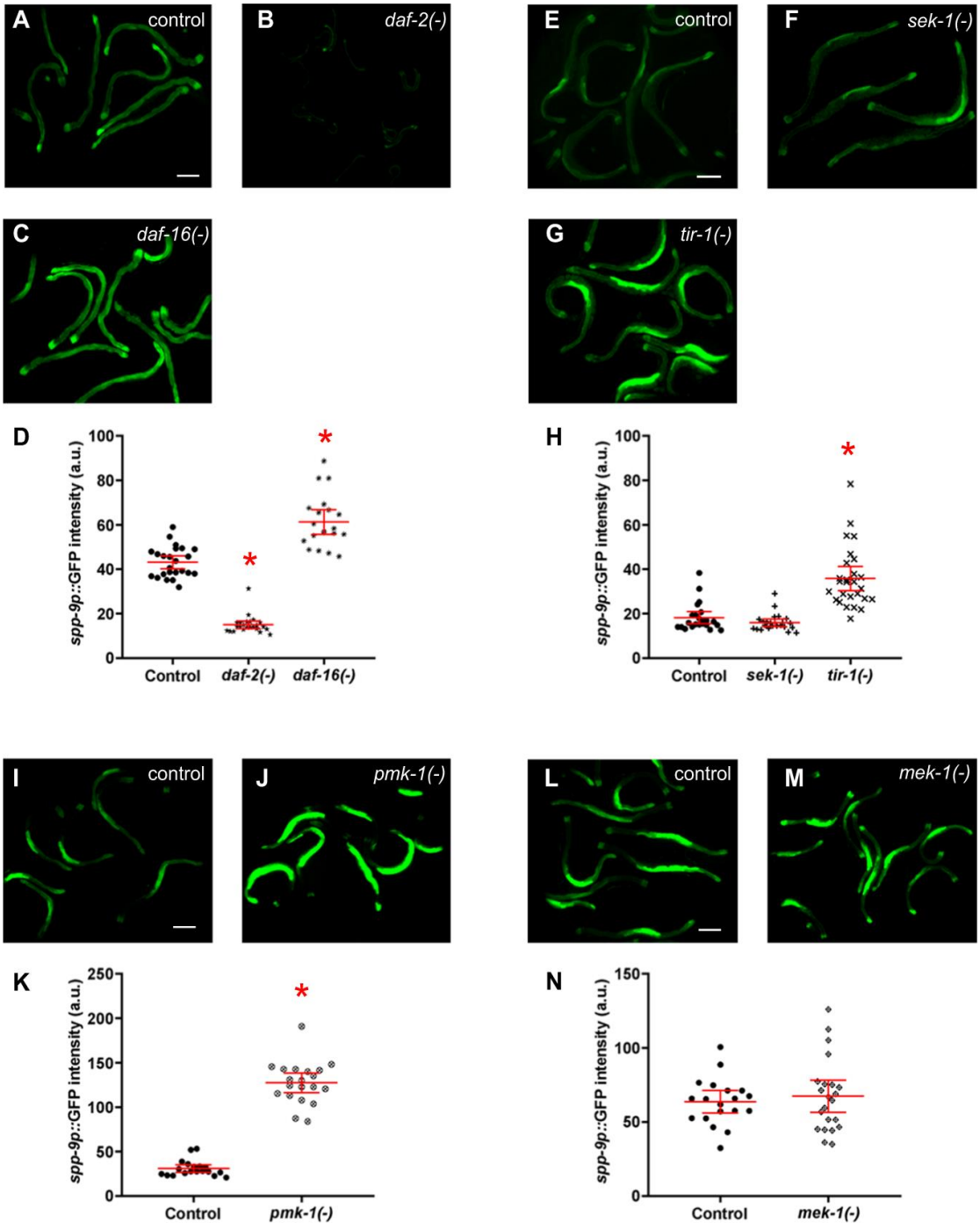

Figure 4.5. *spp-9* reporter activity is regulated by innate immunity signaling pathways. Fluorescence images show *spp-9p::*GFP intensities in two-day adults with A, E, I, L) wild-type (*n* = 24, 23, 19, 19), B) *daf-2(-)* (*n* = 24), C) *daf-16(-)* (*n* = 20), F) *sek-1(-)* (*n* = 23), G) *tir-1(-)* (*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 µm. 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 to the control. H) Comparison of *spp-9p::*GFP intensities in wild-type (control), *sek-1(-)*, and *tir-1(-)* backgrounds. Loss of *tir-1* caused an increase in the reporter activity in comparison to the control ($p < 0.0001$). K) Comparison of *spp-9p::*GFP intensities in wild-type (control) and *pmk-1(-)* backgrounds. The GFP intensity increased in animals lacking *pmk-1* ($p < 0.0001$). N) Comparison of *spp-9p::*GFP intensities in wild-type (control) and *mek-1(-)* backgrounds. No significant change in *spp-9p*::GFP intensity was seen in *mek-1(-)* animals ($p = 0.5674$). One representative trial of three is presented. Error bars represent 95% confidence intervals. *, $p < 0.05$ compared to *spp-9p::*GFP in wild-type background by unpaired *t*-test.

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 (see Figure 4.2). *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 (see Figures 4.3 and 4.4). 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 (see Figures 4.4 and 4.5). 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.

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Conflict of Interest

The authors declare no conflict of interest.

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Supplementary Information

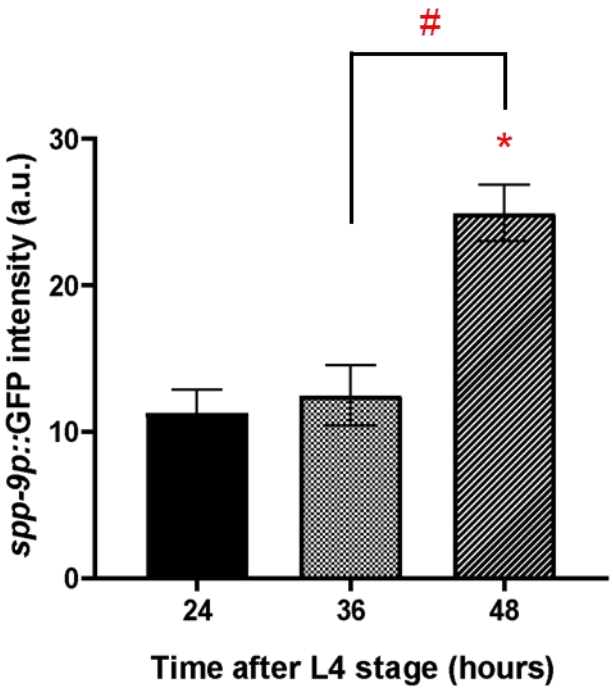


Figure S1. **Expression of the *spp-9* reporter increases over time.** Comparison of the *spp-9p::*GFP reporter fluorescence over time indicates that *spp-9* expression increased by 48 (*n* = 13) hours post L4 stage in comparison to 24 (*n* = 10) and 36 hours (*n* = 14) post L4 stage ($p < 0.0001$). No significant difference in *spp-9p*::GFP intensity was observed between 24 hours after L4 and 36 hours after L4 stage ($p = 0.297$). Imaging conditions including exposure times were consistent with control. Error bars indicate standard deviation. *, $p < 0.05$ compared to *spp-9p::*GFP reporter fluorescence 24 hours after L4 by one-way ANOVA with Tukey HSD post-hoc test. #, $p < 0.05$ compared to *spp-9p::*GFP reporter fluorescence 36 hours after L4 by one-way ANOVA with Tukey HSD post-hoc test.

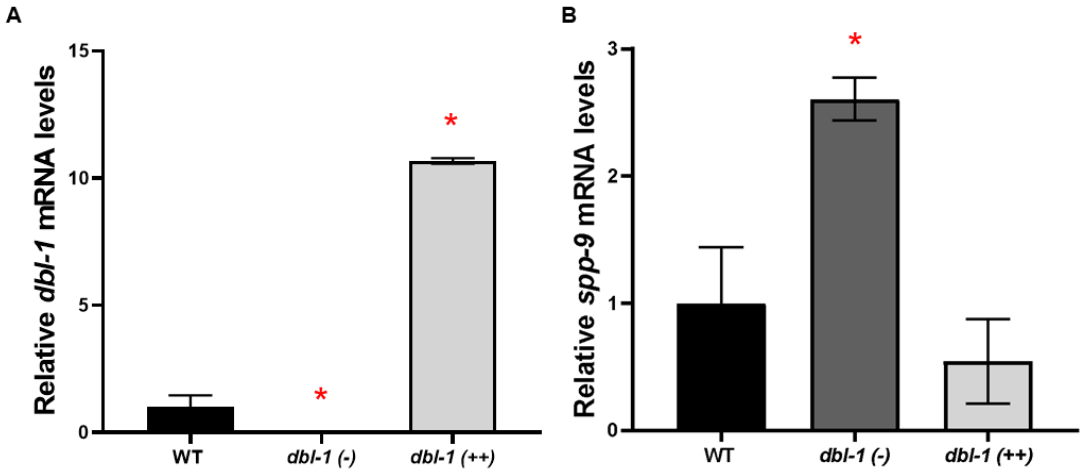


Figure S2. ***dbl-1* regulates endogenous *spp-9* expression levels.** qRT-PCR analysis of A) *dbl-1* and B) *spp-9* gene expression in 48-hour post-L4 wild-type (WT, strain N2), *dbl-1* loss-of-function (*dbl-1(-)*, strain NU3), and DBL-1-overexpressing (*dbl-1(++)*, strain TLG182) strains. *dbl-1* expression was absent in *dbl-1(-)* animals ($p = 0.0001$) and increased 10-fold in *dbl-1(++)* animals ($p = 0.0002$). In *dbl-1(-)* animals, *spp-9* expression was increased 2.6-fold ($p = 0.0287$), whereas *spp-9* expression in *dbl-1(++)* animals is not significantly decreased ($p = 0.1409$). Experiment was performed in triplicate. Error bars represent standard deviation. *, $p < 0.05$ compared to wild-type by unpaired *t*-test.

CHAPTER V

DBL-1 REGULATES THE COMPOSITION OF THE PROTECTIVE SURFACE BARRIER OF *C. ELEGANS*

Introduction

All living organisms have external structures that protect the organisms against the environment including microbe infections. Roundworms (nematodes) also possess such protective structures, which make up the first line of defense against harmful organisms. The cuticle (exoskeleton) is a physical barrier that protects the nematodes from their external environment. Nematodes use the cuticle not only to protect themselves from the harmful environments but also help parasitic nematodes evade their hosts' responses[1,2]. The cuticle is primarily made up of proteins including collagens and cuticlins. The outermost layer of the cuticle, the epicuticle, is rich in lipids. This layer is covered by a glycoprotein-rich layer called the surface coat (or glycocalyx). The protein composition of the protective cuticle is well characterized[3]. While the general lipid content has been profiled and conserved lipid metabolism pathways/enzymes have been identified in this organism, its epicuticular lipid composition has not yet been dissected[4–6].

It has been previously reported that the DBL-1/TGF-β signaling pathway regulates the cuticle barrier function. Loss of the ligand DBL-1 increases cuticular permeability to many drugs while overexpression of DBL-1 reduces permeability to drugs. The report also showed that the DBL-1/TGF-β signaling regulates

epicuticular lipid levels and loss of signaling alters cuticular layer organization[7]. Another study showed that altered DBL-1 levels reduce lipid stores and lipid droplet count and regulate lipid metabolism via the insulin-like signaling pathway[8]. Many transcriptional datasets indicate that DBL-1 signaling regulates several genes involved in lipid metabolism such as lipases, hydrolases, and esterases[9–11]. The DBL-1/TGF-β signaling also is known to regulate expression of many cuticle collagens that comprise the protein-rich layer[11,12].

In this study, we characterized the lipid composition of the *C. elegans* epicuticle (surface-enriched) and propose a model in which DBL-1/TGF-β signaling transcriptionally affects epicuticle lipid composition. We identified that the relative levels of specific surface-enriched lipids are reduced by loss of DBL-1. We also observed a difference in the acyl moieties of the internal lipids of *dbl-1* knockout populations compared with wild-type populations. Furthermore, we demonstrate that loss of DBL-1 decreased expression of fatty acid synthase, *fasn-1*, and fatty acid desaturases, *fat-5* and *fat-7*. This study identified the specific lipid composition of the *C. elegans* surface barrier of both wild-type and *dbl-1* mutant animals helping to characterize the underlying molecular mechanisms for synthesis of surface barrier lipids regulated by DBL-1.

Materials and Methods

Strains and Maintenance

All *C. elegans* strains were maintained at 20°C on EZ media plates (0.55 g Tris-Cl, 0.24 g Tris base, 3.1 g BD Bacto™ Peptone, 8 mg cholesterol, 2 g

sodium chloride, 20 g agar, in water to 1 L)[13]. The *C. elegans* strains were maintained on *E. coli* OP50 without contamination or starvation for at least five generations before every experiment. The *C. elegans* strains used were N2 (wild-type) and NU3 *dbl-1(nk3)* V (referred to as *dbl-1(-)* in this work).

Lipid Extraction

Asynchronized, *E. coli* OP50-fed populations of wild-type and *dbl-1(-)* animals at a concentration of about 250 animals/µl were harvested with ultrapure water. The animals were subjected to low speed centrifugation to form a pellet of approximately 0.5 ml. The pellet was resuspended in 0.5 ml water and 2 ml chloroform/methanol (2:1, v/v). The animals were vortexed for about 6–7 seconds, then centrifuged at low speed for 25 seconds. After centrifugation, the organic phase containing the surface-enriched lipid fraction was collected. The pellet was resuspended in 1 ml of chloroform. The surface-enriched lipid fraction (organic phase) was added to the first fraction. Following the extraction of surface-enriched lipids, 0.5 ml water and 2 ml of chloroform/methanol (2:1, v/v) was added to the tube containing the animal pellet. The pellet was crushed to extract remaining lipids using a Dounce homogenizer. The organic phase containing the internal lipid fraction was collected in a separate tube. To the original tube containing the animal pellet, 1 ml of chloroform was added and the internal lipid fraction was again collected. Heptadecanoic acid (2.5 µg) was added to the extracted lipids as the standard. The extracted lipids were then

used for thin-layer chromatography or were transmethylated for analysis by gas chromatography mass spectrometry (GC-MS).

Fatty Acid Methyl Ester Derivatization

Transmethylation was performed as previously described[14]. Briefly, 1 ml of 2.5% sulfuric acid in methanol was added to the extracted lipids and the mix was incubated at 80°C for 1 hour to transmethylate fatty acids. After the incubation, the tubes were cooled on ice and then 1 ml water and 0.5 ml hexane were added. The fatty acid methyl esters (FAMEs) were then extracted in the hexane layer after brief vortexing followed by centrifugation at low speed. The hexane layer was collected in gas chromatography (GC) vials without transferring the aqueous phase.

Gas Chromatography Mass Spectrometry (GC-MS) Injection

FAMEs were analyzed using an Agilent Intuvo 9000 gas chromatographer coupled with a 30m x 0.25mm x 0.25µm Agilent Intuvo GC column with helium as the carrier gas at a rate of 1.2 ml/min. The gas chromatogram was set at in initial temperature of 50°C for 0.3 min, followed by an increase of 250°C/min to 175°C, followed by an increase of 8°C/min to 190°C, with a 0.5 min hold time. This was followed by an increase of 10°C/min to 240°C with a 2 min hold time.

Thin Layer Chromatography (TLC)

Lipids were separated on silica gel plates using 25:25:25:10:9 (v/v) chloroform/methylacetate/1-propanol/methanol/0.25% potassium chloride solvent mix. Lipids were visualized on the silica plates by using 0.001% primuline dye

dissolved in acetone. A phospholipid mix containing phosphatidic acid, phosphatidylethanolamine, phosphatidylinositol, phosphatidylcholine, and lyso-phosphosphatidylcholine was used as the standard (soy phospholipid mixture, Avanti Polar Lipids, Alabama).

RNA Isolation

Animals were synchronized by bleaching[15]. Total RNA was extracted from animals at 48 hours after the L4 stage. Total RNA was extracted by the freeze cracking method as previously described[16].

Quantitative Reverse Transcription PCR

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 SYBR Green master mix (Applied Biosystems) according to manufacturer's instructions. The experiment was performed in three technical replicates for each condition in at least three independent biological trials. QuantStudio Design and Analysis Software v1.5.1 was used to calculate raw C_t values and to normalize the values for the candidate genes to the housekeeping actin gene *act-1* (Applied Biosystems by Thermo Fisher Scientific). Fold change in gene expression between experimental sample and the wild-type control was determined with this software using the formula: $2^{(-\Delta\Delta C_t)}$. Experimental ΔC_t values

were compared with wild-type ΔC_t values using an unpaired *t*-test. Table 5.1 contains the list of all primer sequences.

Target gene	Forward primer	Reverse primer
fasn-1	GGAAGCTGCCGTTCATTCGA	ATTCCATGCTCCTTGTGCCC
fat-5	AGAGATCACCGGTGCCATCA	CGACTGGAATGAAGGTGGGC
fat-7	ATGGGATGGCTTCTTGTGCG	CCAAGTGGCGTGAAGTGTGA
act-1	GCCGGAATCCACGAGACTTC	TCTGGTGGGGCGATGATCTT

Table 5.1. Primer sequences for real-time PCR.

Differential Expression Analysis by RNA Sequencing

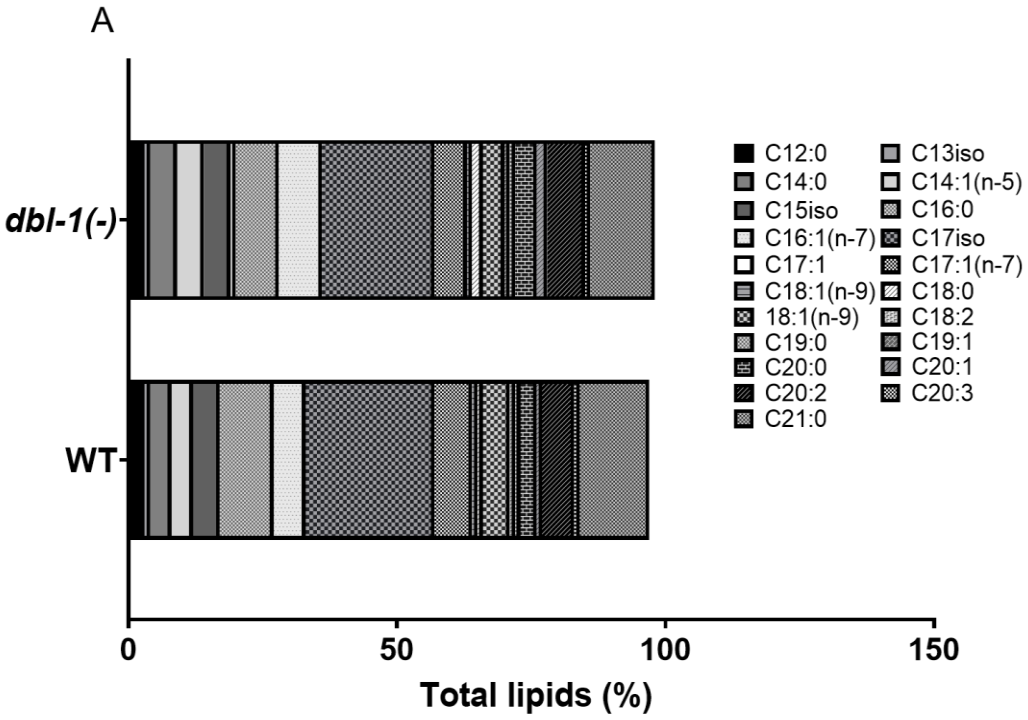
Sequencing libraries were generated from 1 µg of wild-type and *dbl-1(-)* adult populations' RNA using NEBNext® RNA Library Prep Kit for Illumina® (NEB, USA) following manufacturer's recommendations. Novogene performed RNA sequencing of samples. Differential expression analysis of WT compared to *dbl-1(-)* populations grown on *E. coli* OP50 was performed using the DESeq R package (1.18.0). The resulting *p*-values were adjusted using the Benjamini and Hochberg's approach for controlling the false discovery rate[17]. Genes with an adjusted *p*-value < 0.05 found by DESeq were assigned as differentially expressed.

Results

Loss of DBL-1 Does Not Affect Total Lipid Varieties but Reduces Unsaturated Fatty Acid Quantity

Loss of DBL-1 reduced lipid levels of the *C. elegans* epicuticle[7]. To determine if loss of DBL-1 alters the global lipid (total lipid) composition (the types of lipids) in *C. elegans*, we compared fatty acid types and quantities of wild-type and *dbl-1(-)* populations. To quantitate the proportions/levels of and compare fatty acids of wild-type and *dbl-1(-)* animals, we extracted, transmethylated, and identified lipids by GC-MS. We observed fatty acids ranging from 12 to 21 carbon atoms in length in both wild-type and *dbl-1(-)* animals (see Fig. 5.1A). Despite the similar composition of fatty acids in both the populations and preparing similar masses of animal sample from both strains, we found that

the amount of total lipids of *dbl-1(-)* animals was reduced by 27% on average in comparison to the total lipids of wild-type animals. We further analyzed the difference between the saturated and unsaturated fatty acid levels of wild-type and *dbl-1(-)* strains. We found that there was a small, non-significant decrease in the level of *dbl-1(-)* saturated fatty acids. However, we observed a significant reduction in the quantity of unsaturated fatty acids in *dbl-1(-)* animals in comparison to wild-type animals (see Fig. 5.1B). These results indicate that loss of DBL-1 causes a reduction in the total amount of lipids, largely by reduction of unsaturated fatty acid species.



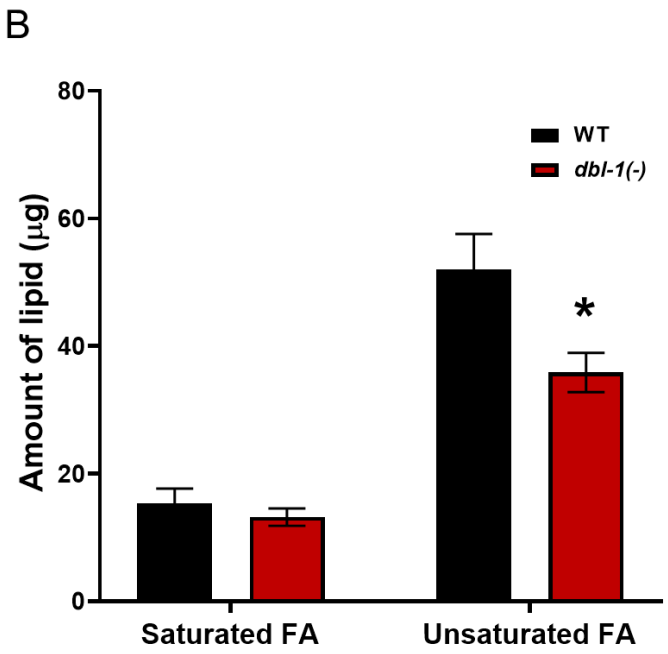


Fig. 5.1. Loss of DBL-1 results in reduction of unsaturated fatty acid levels but does not alter total lipid composition. Total lipids of wild-type and *dbl-1(-)* animals were extracted. The extracted lipids were transmethylated to obtain fatty acid methyl esters (FAMEs) and injected on the Agilent Intuvo 9000 GC-MS. A) Wild-type and *dbl-1(-)* populations total lipids are composed of fatty acids ranging from 12 to 21 carbons. The lipid composition of both the populations is similar. B) The fatty acids (FA) analyzed from total lipids of both the populations were sorted by their saturation levels. The amount of saturated fatty acids in wild-type and *dbl-1(-)* animals is similar. In *dbl-1(-)* animals, there is a significant reduction in the levels of unsaturated fatty acids. Comparison of saturated and unsaturated fatty acids between WT and *dbl-1(-)* populations is mean of at least 6 independent trials. Error bars represent standard deviation. *, $p < 0.05$, unpaired *t*-test.

C. elegans Surface Lipids Consist of Saturated and Unsaturated Fatty Acids

To identify lipids of the *C. elegans* surface coat, we developed a protocol to sequentially isolate surface-enriched lipids and internal lipids. The lipids that were represented in higher amounts in the surface-enriched lipid fraction in

comparison to the internal lipid fraction were identified by GC-MS analysis and categorized as surface-enriched lipids. We found the presence of both saturated and unsaturated fatty acids in the surface coat of wild-type animals. These fatty acids ranged from 12 to 19 carbon atoms in length (see Fig. 5.2B). In conclusion, the protocol was effective at isolating different lipid populations in measurable quantities.

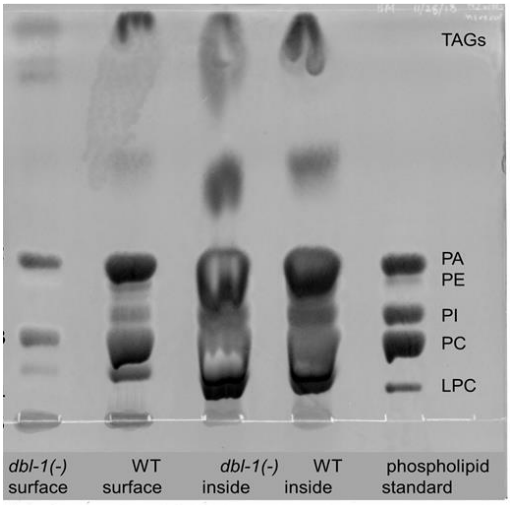
DBL-1 Affects Both Surface-Enriched and Internal Lipid Levels, Including Saturated and Unsaturated Fatty Acids

To determine if DBL-1 affects lipid composition of the surface coat, we performed thin layer chromatography using the surface-enriched and internal lipid fractions of wild-type and *dbl-1(-)* animals (see Fig. 5.2A). We observed similar lipid species in the surface-enriched fraction of wild-type and *dbl-1(-)* animals. Triacylglycerols, phosphatidic acid, and phosphatidylcholine were the major lipid species in the surface-enriched lipids of both wild-type and *dbl-1(-)* animals. These lipid species were also major components of the internal lipid fraction of both the populations. However, phosphatidylethanolamine, phosphatidylinositol, and lyso-phosphosphatidylcholine were also major lipid species in both wild-type and *dbl-1(-)* internal lipids, but not surface-enriched lipids. These results expand on previous reports that the epicuticular lipid is a subset of total lipid distinct from the lipids within the body[4]. Furthermore, these results indicate that DBL-1 does not alter the variety of lipid species of the surface coat or the internal lipids.

We then identified and compared the fatty acid methyl esters (FAMEs) of surface-enriched and internal lipid fractions of the wild-type and *dbl-1(-)* lipids by GC-MS (see Fig. 5.2B). While dodecanoic acid (12:0) levels remained unchanged between wild-type and *dbl-1(-)* surface-enriched lipid fractions, unsaturated fatty acid levels were significantly reduced in *dbl-1(-)* populations compared to the wild type. This indicates that loss of DBL-1 specifically reduces unsaturated fatty acid levels in the surface coat of *C. elegans*.

Additionally, in the internal lipid fraction of animals lacking DBL-1, there was a decrease in some fatty acids, not only monounsaturated and polyunsaturated fatty acids but also saturated fatty acids. These results indicate that loss of DBL-1 results in reduction in the amount of some internal fatty acids of animals (see Fig. 5.2C). These results collectively indicate that while DBL-1 does not alter the overall lipid types in the surface coat and internal lipids of animals, it does affect the proportions of unsaturated fatty acids comprising both the surface coat and the internal lipids of animals and saturated fatty acids of the internal lipids of animals.

A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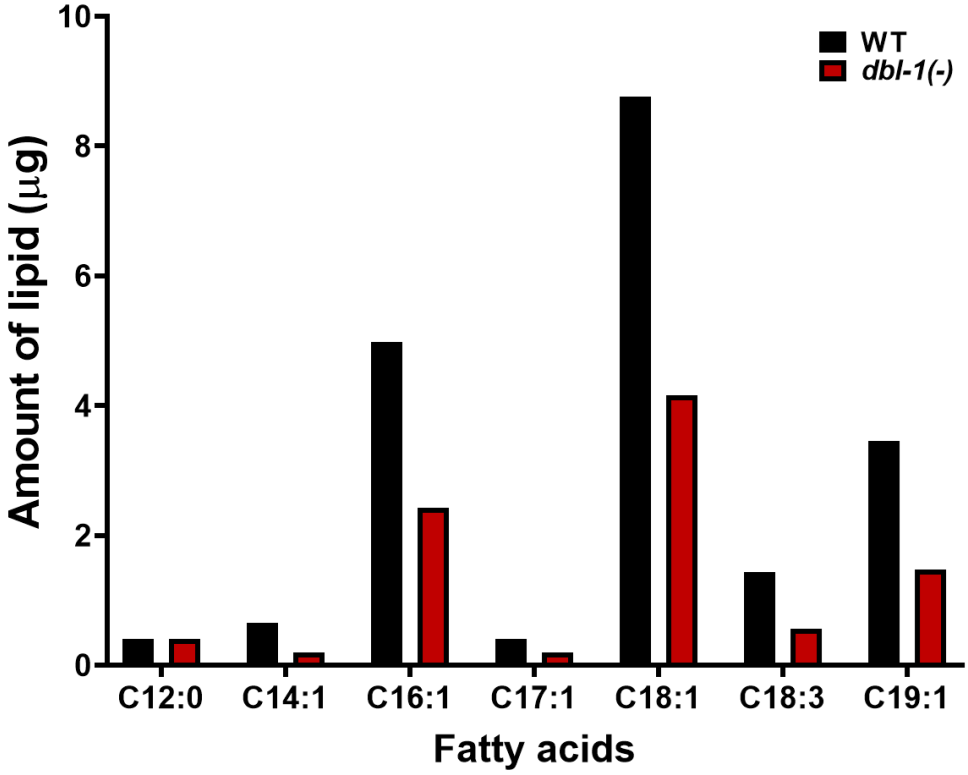
PI
PC
LPC
dbl-1(-) surface
WT surface
dbl-1(-) inside
WT inside
phospholipid standard

B


WT
dbl-1(-)
Amount of lipid (μg)
0
2
4
6
8
10
C12:0
C14:1
C16:1
C17:1
C18:1
C18:3
C19:1
Fatty acids

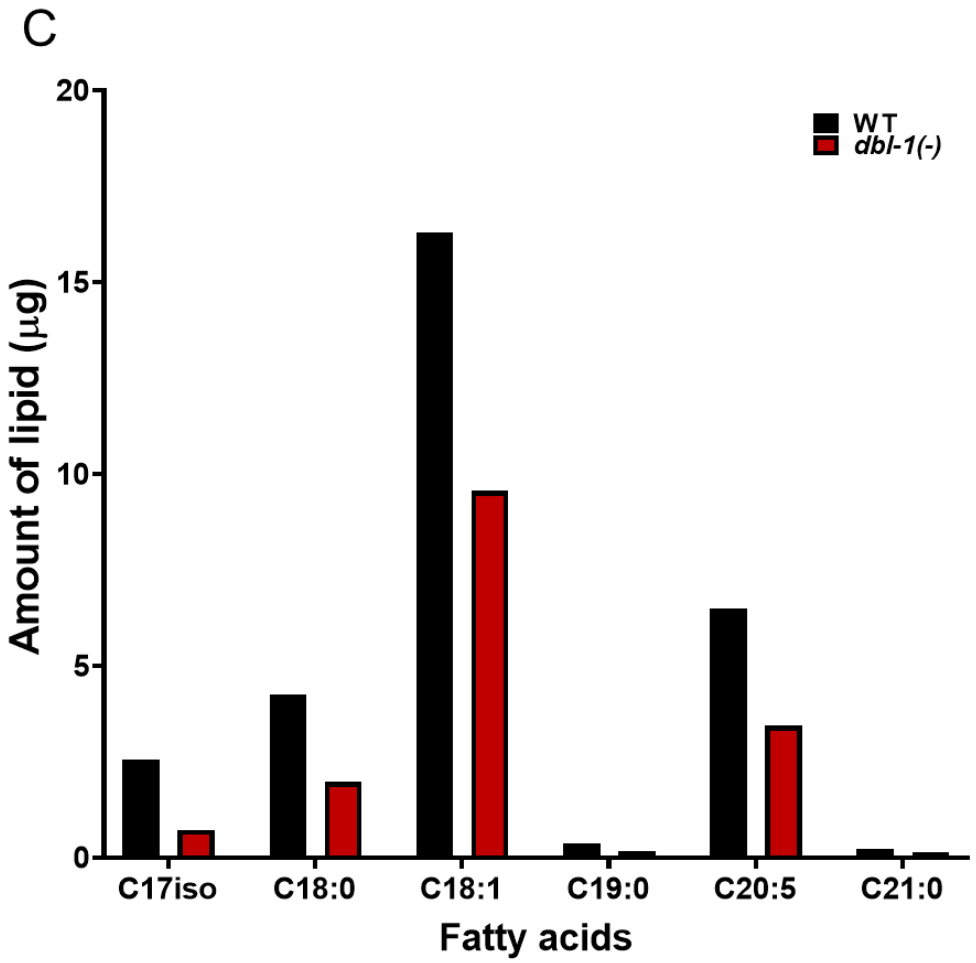


Fig. 5.2. Identification of surface-enriched and internal lipids of wild-type and *dbl-1(-)* animals. A) Lipid species were separated by thin layer chromatography (TLC). Surface-enriched lipids and internal lipids were loaded on silica plates. Separation of lipid classes of total lipids by TLC indicates presence of similar lipid species in wild-type and *dbl-1(-)* populations. The phospholipid standard was used as a reference. This standard contains phosphatidic acid (PA), phosphatidylethanolamine (PE), phosphatidylinositol (PI), phosphatidylcholine (PC), and lyso-phosphosphatidylcholine (LPC). B) The composition of surface-enriched fatty acids of the wild-type and *dbl-1(-)* animals. C) The composition of internal fatty acids of the wild-type and *dbl-1(-)* animals. One representative trial of surface-enriched and internal lipids is shown.

DBL-1 Regulates Expression of Lipid Metabolism Genes

Because we identified reduction in levels of fatty acids upon loss of DBL-1, we determined if DBL-1 regulates lipid metabolism in adult animals by performing RNA sequencing (RNA-seq) and assessing changes in the transcription of lipid metabolism genes in wild-type and *dbl-1(-)* strains. Wild-type and *dbl-1(-)* populations were synchronized and harvested as two-day adults for RNA sequencing. We found reduction in the expression levels of lipid metabolism genes *fasn-1*, *fat-5*, *fat-7*, and *F10D11.6* (see Table 5.2). *fasn-1* is a fatty acid synthase gene required for the synthesis of long-chain saturated fatty acids[18]. *fat-5* and *fat-7* are Δ^9 fatty acid desaturases, which are required for the conversion of saturated fatty acids to unsaturated fatty acids[19,20]. *F10D11.6* is an ortholog of a human phospholipid transfer protein, but its function is uncharacterized[21]. Additionally, we found an increase in the expression levels of some lipid metabolism genes in animals lacking DBL-1, including *oac-5*, *far-6*, and *acs-2* (see Table 5.2). *acs-2* is fatty acid CoA synthetase, which is involved in the breakdown of fatty acids[18]. *far-6* is a nematode-specific protein that can bind lipids, but the cellular function of FAR-6 is unknown[22]. *oac-5* has O-acyl transferase activity and is predicted to add acyl groups to lipids (WormBase.org). To validate these findings, we performed real-time PCR to compare expression of select lipid metabolism genes in wild-type and *dbl-1(-)* animals. Consistent with RNA-seq results, expression of *fasn-1*, *fat-5*, and *fat-7* was significantly decreased in animals lacking DBL-1 (see Fig. 5.3). These results indicate that

DBL-1 regulates expression of lipid metabolism genes, including *fasn-1*, *fat-5*, and *fat-7*.

RNA sequencing was performed with samples extracted from two-day adult populations of wild-type and *dbl-1(-)* in three independent trials. DBL-1 differentially regulates expression of lipid metabolism genes. Adjusted $p < 0.05$ with Benjamini and Hochberg's approach for controlling the false discovery rate.

Gene name	**Function**	**Read count WT**	**Read count *dbl-1(-)***	**Log fold change *dbl-1(-)*/WT**
fasn-1	Fatty acid synthesis	12104.75	7319.67	-0.73
fat-5	Fatty acid biosynthesis	934.87	360.00	-1.38
fat-7	Fatty acid biosynthesis	610.95	77.78	-2.97
F10D11.6	Ortholog of human phospholipid transfer protein	2126.86	772.81	-1.46
acs-2	β- oxidation	1942.28	3773.98	0.96
far-6	Lipid binding activity	1014.29	2677.87	1.40
oac-5	Acyl transferase activity	1.93	31.33	4.02

Table 5.2. DBL-1-regulated lipid metabolism and putative transport genes.

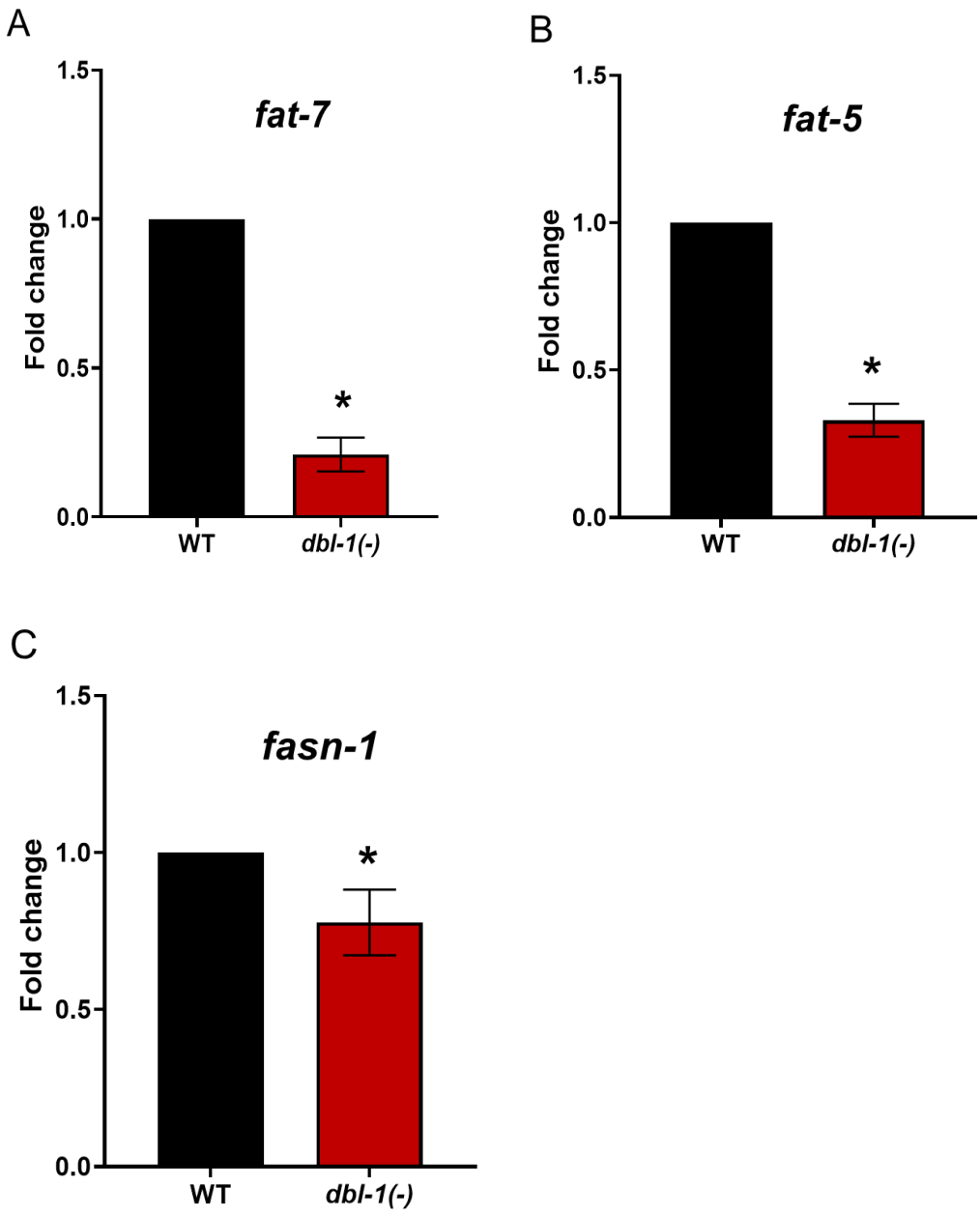


Fig. 5.3. DBL-1 positively regulates expression of some lipid metabolism genes. Relative gene expression levels of A) *fat-7*, B) *fat-5*, and C) *fasn-1* in wild-type and *dbl-1(-)* animals were compared. Data represents the average of at least three independent trials. Error bars represent standard deviation. *, $p < 0.05$ compared to wild-type by unpaired *t*-test.

Discussion

Roundworms protect themselves from their environments, in part, by secreting a lipid-rich epicuticle. In this study, we isolated and characterized the lipids enriched in the *C. elegans* epicuticle. We identified that the epicuticle is composed of triglycerides, phosphatidylethanolamine, phosphatidylcholine, and phosphatidic acid, adding to previous reports[4]. Additionally, we found that the DBL-1/TGF-β signaling pathway regulates levels and composition of lipids, but not the lipid classes, for both secreted (epicuticular lipids) and inside the body (internal lipids), affecting primarily the unsaturated fatty acids. These results provide an unprecedented level of detail to previous findings that demonstrate DBL-1 signaling has important roles in epicuticle barrier integrity and lipid homeostasis[7,8].

We also identified a possible mechanism for how DBL-1 signaling affects lipid levels and composition: DBL-1 pathway signaling may regulate expression of lipid metabolism genes in the hypodermis, which makes and secretes epicuticle lipids. Specifically, expression of fatty acid synthase gene *fasn-1* and fatty acid desaturase genes *fat-5* and *fat-7*, at least, was significantly reduced in populations lacking DBL-1. The DBL-1-dependent reduction of the unsaturated fatty acids in the epicuticle can be attributed to the decrease in expression of fatty acid desaturases and fatty acid synthase in animals lacking DBL-1. These findings reveal molecular mechanisms by which DBL-1 may regulate the protective surface barrier in *C. elegans* (modelled in Fig. 5.4). Furthermore, RNA-

seq analysis revealed that expression of F10D11.6, an ortholog of human phospholipid transfer protein, was reduced in absence of DBL-1. F10D11.6 is predicted to enable lipid binding activity and may play a role in the transport of lipids to the cuticle surface[21]. Determining if F10D11.6 plays a role in regulating *C. elegans* lipid composition could lead to novel insights into how the epicuticle is formed and how DBL-1 signaling protects animals from environmental threats.

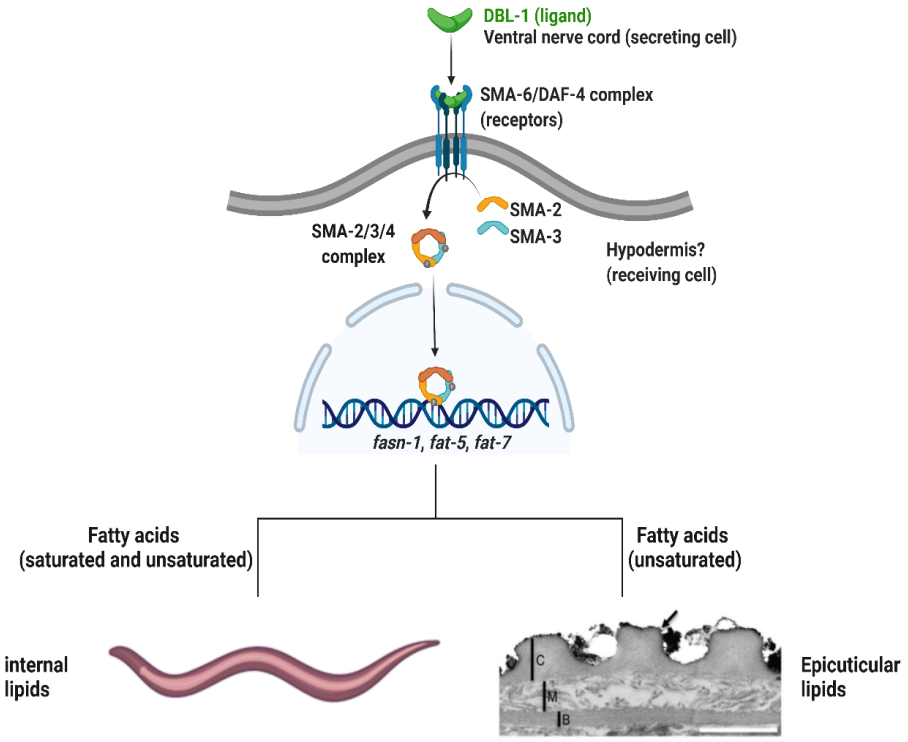


Fig. 5.4. Model of DBL-1 pathway-mediated regulation of both internal and epicuticular lipids. The model suggests a mechanism for the DBL-1-dependent lipid composition alterations. The changes in unsaturated fatty acids of the epicuticle and both saturated and unsaturated fatty acids of internal lipids may be due to DBL-1-mediated regulation of lipid metabolism genes including *fasn-1*, *fat-5*, and *fat-7*.

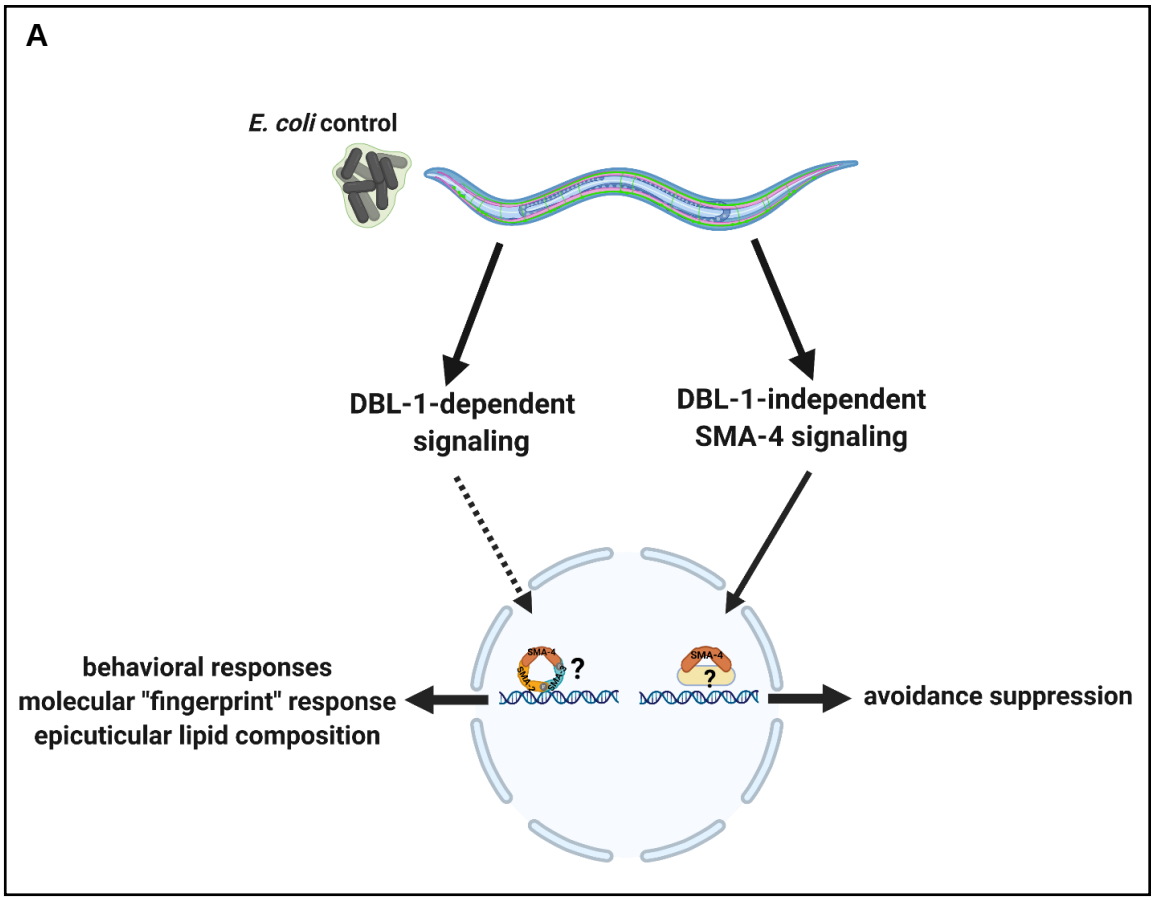
This work also suggests a mechanism for the observations that DBL-1 signaling is required for normal barrier function of the cuticle. Animals lacking DBL-1 have increased cuticle permeability to some anesthetics and animals with increased DBL-1 pathway signaling are more resistant to anesthetics, which correlates with transmission electron microscopy analyses that show dose-dependent effects of DBL-1 on surface lipid levels, cuticle structure, and cuticle organization[7]. Our findings indicate that DBL-1 signaling regulates transcription

of lipid metabolism genes including *fasn-1*, *fat-5*, and *fat-7* and we proposed that reduced expression of these target genes in animals lacking DBL-1 contributes to decreased unsaturated fatty acids of the epicuticle, reducing surface lipid level, altering cuticle structure, and increasing cuticle permeability. Future studies may identify other targets of DBL-1 signaling that contribute to surface barrier function and confer protection to animals against different pathogens and environmental challenges.

CHAPTER VI

CONCLUSIONS AND FUTURE DIRECTIONS

Collectively, the work presented in this dissertation supports a model in which the DBL-1/TGF-β signaling pathway plays an important role in protective host responses against a variety of bacteria (see Fig. 6.1). We propose that DBL-1/TGF-β signaling is involved in sensing pathogens and tailoring immune responses at behavioral, molecular, and physiological levels.



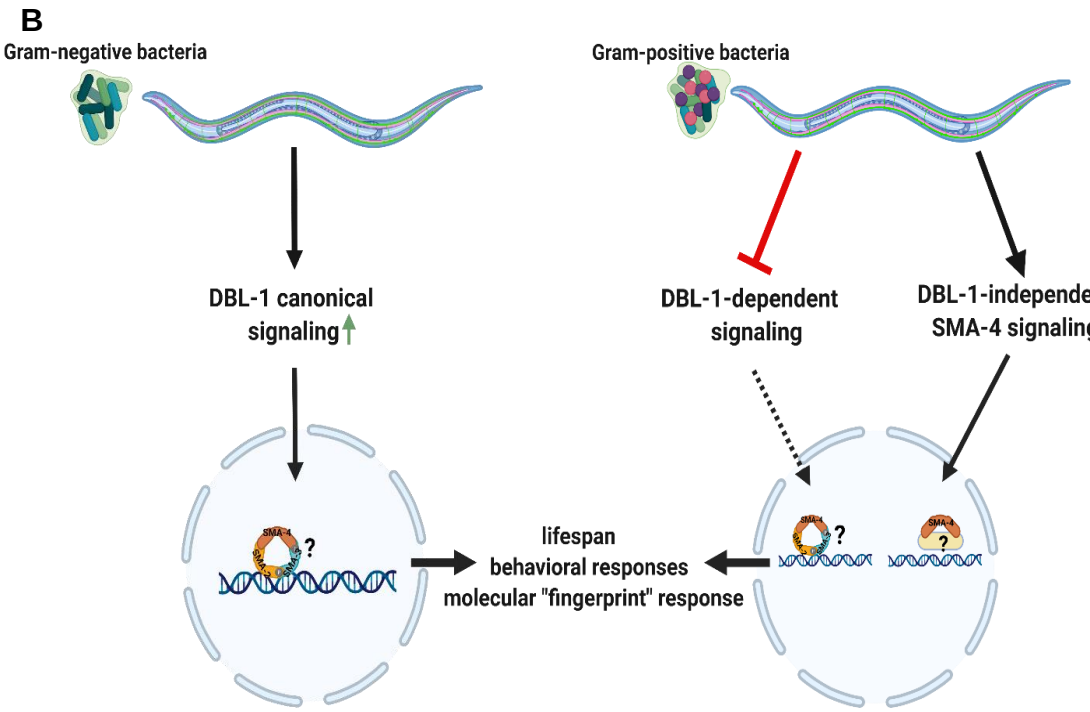


Fig. 6.1. Model figure indicating involvement of DBL-1 in regulating pathogen-specific response. A) The DBL-1/TGF-β signaling pathway plays a major role in regulating behavioral and molecular responses and the epicuticular lipid composition in control lab conditions. B) The DBL-1/TGF-β signaling pathway is differentially regulated in response to Gram-negative and Gram-positive bacteria and in turn tailors specific host responses that affect organism lifespan, behavior, and molecular responses to different bacteria.

The findings of this work indicate that DBL-1/TGF-β signaling coordinates differential host responses at a behavioral level (Aim 1). Work reported in Chapters II and III expands the current understanding of how *C. elegans* coordinate organismal defense responses upon sensing harmful environments. Our findings indicate that DBL-1 is a major player in orchestrating these robust, protective, and specific responses to different types of bacteria. We identified that DBL-1 signaling is required to suppress avoidance to the tested Gram-negative,

but not Gram-positive bacteria. This finding broadens our understanding of the known role of the DBL-1 pathway signaling in suppressing avoidance of *E. coli*[1]. Animals lacking DBL-1 were susceptible to both Gram-negative and Gram-positive bacteria. We reported that damage to the intestine was not the underlying cause of this susceptibility. Reduced bacterial intake was dependent on both loss of DBL-1 and exposure to select bacteria. Reduced brood size was also dependent on both loss of DBL-1 and exposure to select bacteria. It would be of interest to identify how DBL-1 is involved in such differential behavioral responses. Previous studies have reported that DBL-1 secretion from the AVA interneurons activates DBL-1 signaling in the hypodermal tissue to mediate aversive learning in response to pathogenic *P. aeruginosa*[2]. The neuronal source of DBL-1 might explain its role in sensing the pathogenic environments. However, the downstream physiological targets of this circuit that result in the different DBL-1-mediated responses remain to be identified.

A major finding of this work is the differential requirement of the SMAD machinery to coordinate the avoidance response to test bacteria. Previous studies report the requirement of SMA-3 but not SMA-2 and SMA-4 to regulate transcription of antimicrobial genes against a fungal pathogen, *D. coniospora*[3]. We identified that canonical DBL-1 signaling suppresses avoidance to Gram-negative bacteria but not to Gram-positive bacteria. We identified a novel role of SMA-4 in suppressing avoidance independent of DBL-1. This is indicative of SMA-4 partnering with a different transcription factor, hinting to a potential

crosstalk with other immune signaling pathways (see Fig. 6.1B). PMK-1/MAPK signaling may be involved in regulating it as SMA-4 is predicted to genetically interact with PMK-1[4]. Interestingly, ATF-7, a transcription factor activated by PMK-1, is required for downregulation of *sma-4*—but not other DBL-1 pathway component genes—in wild-type animals exposed to Gram-negative *P. aeruginosa* PA14[5]. It will be of interest to discover if SMA-4 plays a broader role in the innate immune response than acting in the DBL-1 pathway. In the future, studies focusing on the interplay between multiple signaling pathways to regulate specific immune responses will be important to understand the dynamic immune responses of organisms.

Furthermore, this work reports that DBL-1 differential tailors host responses at a molecular level (Aim 2). Work reported in Chapters II and IV expands the current understanding of how *C. elegans* coordinate molecular defenses in response to environmental challenges[6,7]. This work reveals signature molecular responses regulated by DBL-1/TGF-β in response to a variety of bacterial pathogens. Our findings support and add to the “antimicrobial fingerprint” proposed by Alper[8]. The results indicate that DBL-1 tailors antimicrobial gene expression depending on the type of bacterial exposure. These findings strengthen our model that canonical DBL-1/TGF-β signaling is activated in response to Gram-negative bacteria, thereby coordinating specific organismal behaviors and expression of innate immunity genes. On the other hand, canonical DBL-1/TGF-β signaling is repressed upon exposure to Gram-

positive bacteria. Interestingly, our results indicate that SMA-4 potentially cross-talks with other immune signaling pathways. This suggests that exposure to Gram-positive bacteria results in DBL-1-independent or SMA-4-dependent organismal behaviors and regulation of innate immunity gene expression. Future work will identify if ATF-7 or other transcription factors partner with SMA-4 to regulate these immune responses. It will be of interest to determine the bacterial components resulting in these differential host responses.

Our findings summarized in Chapter IV provide a snapshot of the dynamic crosstalk between multiple immune signaling pathways converging on the genetic regulation of a caenopore, *spp-9*[9]. This work revealed further insights into the environmental responsiveness of the caenopore family and how organisms generate dynamic, complex, targeted molecular responses to physiological challenges. We identified not only that *spp-9* is regulated by DBL-1/TGF-β depending on the environment, but also that insulin-like and p38/MAP kinase signaling pathways regulate *spp-9* expression. The DBL-1-independent regulation of *spp-9* in response to select test bacteria may require SMA-4, as suggested by our finding reported in Chapter II. Our results support a role for *spp-9* as part of the distinct “antimicrobial fingerprint” response upon exposure to different pathogens and suggest it also plays a role in stress response. Expression of *spp-9* in some head sensory neurons suggests a role in sensing different bacterial stimuli that may be independent of DBL-1 because DBL-1 receptors are not expressed on these cells. Identifying the role of *spp-9* in this

neuro-immune circuit will provide novel insights into communication along the gut-brain axis within the context of immunity.

Finally, this work reports the role of DBL-1 in regulating host defenses at a physiological level (Aim 3). The findings of Chapter V reveal the lipid composition of the *C. elegans* protective epicuticle. We identified that DBL-1/TGF-β signaling regulates epicuticular lipid composition, specifically the unsaturated fatty acids. Additionally, the total lipid composition—but not the general lipid classes—was regulated by DBL-1/TGF-β signaling. These findings also reveal a mechanism by which DBL-1 regulates the protective surface barrier in *C. elegans*. The DBL-1-dependent reduction in the unsaturated fatty acids of the epicuticle can be partly attributed to the decrease in expression of fatty acid synthase (*fasn-1*) and fatty acid desaturases (*fat-5* and *fat-7*) in animals lacking DBL-1. These results build the foundation to understanding the biochemistry of the protective epicuticle of the free-living nematode *C. elegans* and how DBL-1/TGF-β signaling confer protection to animals from different pathogenic bacteria by means of surface barrier (epicuticle). Overall, these findings indicate an important role of DBL-1 in regulating lipid metabolism in congruence with previous work[10]. Previous reports demonstrate that alterations in lipid metabolism modifies innate immune responses in *C. elegans*[11–13]. These studies and our collective findings hint to a link between lipid metabolism and innate immune regulation controlled by the DBL-1 signaling pathway. Future work will determine if DBL-1-mediated tailored

host defenses—behavioral, molecular, and physiological—require DBL-1-dependent lipid metabolism changes.

In conclusion, this work provides novel insights into how DBL-1/TGF-β signaling is altered in response to different pathogens and in turn appropriately coordinates expression of antimicrobial genes to fight pathogens and keep host immune responses in check. We propose a model in which animals use DBL-1/TGF-β signaling to tailor specific organismal responses upon sensing different pathogenic environments (see Fig. 6.1). DBL-1/TGF-β signaling is altered in response to different pathogens and in turn appropriately coordinates expression of antimicrobial genes to fight pathogens and keep host immune responses in check. Our work establishes a strong system in which to characterize dynamic and differential host immune responses *in vivo*. These findings lay the foundation to study the role of TGF-β signaling coordinated with other immune signaling pathways in orchestrating differential host responses against pathogenic bacteria.

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