Characterization of the Role of *acs-19* in *Caenorhabditis elegans* Bacterial Infection

Research Thesis

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By

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Abstract

The human body attracts a large number of bacterial pathogens, many of which have detrimental effects on the host and add a tremendous economic burden. *Caenorhabditis elegans* and its interaction with the bacterium *Microbacterium nematophilum* is a great model system to study various aspects of host-pathogen interactions including bacterial attachment, nematode surface antigen variation, host response to infection, and cellular morphogenesis. *M. nematophilum* adheres to the post-anal region of the nematode, causing it to display a deformed anal region (DAR) phenotype and experience severe constipation.

Previous research has shown that among the genes up regulated in *C. elegans* in response to *M. nematophilum* infection is *acs-19*, a gene encoding acyl-CoA synthetase, which plays a role in lipid metabolism. I have been investigating the specific function of ACS-19 and its role in this interaction. Specifically, I am studying whether its up regulation is dependent on the transcription factor EGL-38. So far, I have observed that the expression of *acs-19* goes down in *egl-38* mutants with respect to wild type. I am also using RNAi to knock down *acs-19* gene expression to investigate whether its up regulation in response to infection is involved in the host's defense against the pathogen, or if it is something necessary for pathogenicity. I have found that the upregulation of *acs-19* reported in the microarray experiments are part of the bacteria'a infection mechanism and that the DAR response is not dependent on *egl-38. In* the future, I will test the possibility of changes in ACS-19 protein localization in response to infection.

Introduction

Whether we are listening to the radio, going to the movies with friends, or ordering a meal, we are constantly surrounded by a torrent of microscopic organisms. We get yearly vaccinations for the flu, brush our teeth to remove bacterial plaque, and carry around bottles of hand sanitizers. The human body has always had a unique history of interactions with microorganisms, so it comes as no surprise that they, whether in the form of probiotic yogurt, cheese, alcohol, or serious infections, have found their way into our bodies and into our culture.

Pathogenic microorganisms can have detrimental effects on the host, and add a tremendous economic burden. Studying the interactions between host and pathogen in a simpler model system can allow us to better characterize the mechanisms that allow human pathogens to evade our immune defenses, establish a colony, and thrive. *Caenorhabditis elegans* is a soil roundworm whose relatively short generation time, transparency, and simple yet eukaryotic genetic system has made it an ideal model organism for developmental biology studies. However, it has also been established as a good model host system for studying infections by a wide variety of human pathogens, including *Pseudomonas aeruginosa, Yersinia pestis* and *Yersinia pseudotuberculosis* (Höflich et. al., 2004). Yet, because *C. elegans* is not the preferred host for these human pathogens, it would also be beneficial to study the relationship between *C. elegans* and a pathogen geared towards its species.

Microbacterium nematophilum is among the known pathogens that infect *C. elegans*. It adheres to the post-anal region of the worm, causing it to display a deformed anal region (DAR, Figure 1.) phenotype and experience severe constipation (Hodgkin et. al., 2000). This interaction allows for a great model system to study various aspects of host-pathogen interactions, including

bacterial attachment, nematode surface antigen variation, host response to infection, and cellular morphogenesis.

A recent microarray experiment identified genes whose transcriptions are altered in *C. elegans* in response to *M. nematophilum* infection (O'Rourke et al, 2006). Of the many genes identified, *acs-19*, a gene of unknown function that encodes an acyl-CoA synthetase, is unregulated in response to the infection (O'Rourke et al, 2006). Preliminary data from another microarray experiment have also revealed that acyl-CoA synthetase is down regulated in nematodes that have a mutation in the transcription factor gene *egl-38*, which suggests that the function of *egl-38* is to promote the expression of that gene. The results of these two microarray experiments are cross referenced in Table 1. *egl-38* encodes an orthologue of a small gene family called Pax genes in *C. elegans* . Pax genes encode a set of transcription factors, and are named for the paired box DNA binding domain that widespread in all family members. This family of transcription factors regulates genes that are important in cell proliferation, renewal and survival as well as genes that are important in biosynthetic pathways. Pax genes are developmentally crucial, and their knockdown results in cell apoptosis (Park et al. 2006 and Robson et al. 2006). In *C.* elegans, *egl-38* is important in the development of cells in the hindgut, the excretory system, and the egg-laying system, it and also promotes cell survival (Chamberlin et al., 1997). Acyl-CoA synthetase is a ligase that catalyzes the formation of acyl-CoA from fatty acids. It plays an important role in the metabolic pathways of the cell and has been shown to be part of the mechanism responsible for the increase in breast cancer cell proliferation, invasion and migration due to its function in the production of lipooxygenase and cyclooxygenase metabolites (Podestá et al. 2010). *acs-19* was chosen out of all the genes in Table 1 because our group is

studying *egl-38* as a simple model to understand Pax transcription factor function and the regulatory networks in which it participates.

This project examines the role of *acs-19* in *M. nematophilum* infection and asks how this gene, which is known to be involved in metabolism, also involved in the pathogenesis of *M. nematophilum*. This is done in two parts, first by asking how the worms respond to *M. nematophilum* infection. This part of the project uses a *acs-19* transcriptional reporter, *acs-19::gfp*, to determine if the results from the two aforementioned microarray experiments can be replicated to the extent that the up regulation of *acs-19* is in response to *M. nematophilum* infection and down regulation of this same gene in *egl-38* mutants is observed with this reporter. If these two hold true, then if the DAR phenotype is observed on *egl-38* mutant animals grown on *M. nematophilum*, then the *egl-38* does not participate in the signal transduction pathway resulting in the DAR phenotype. If the expression of the *acs-19* transgene is the same in *egl-38* mutants grown on *M, nematophilum* as it is on these same animals grown on *E. coli*, then the pathway is mediated by *egl-38.*

The second part of this project aims to characterize both the normal function of *acs-19* and its role in *M. nematophilum* infection. This part of the project uses an RNAi feeding vector in *C.elegans* to knockdown *acs-19*. It was already mentioned that *acs-19* is up regulated in response to *M. nematophilum* infection. If the up-regulation of this gene is an immune response mounted by the host, then the knockdown of it will result in a more severe infection. If it is part of the mechanisms by which the bacteria infects its hosts, then the knockdown of *acs-19* will result in a less severe infection.

Figure 1. DAR Phenotype

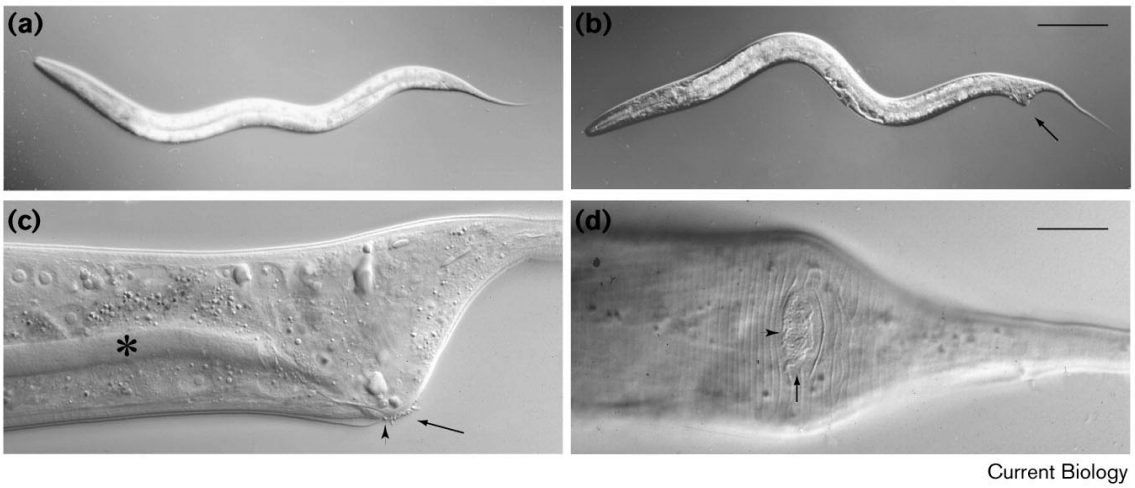


Image (a) shows an uninfected *C. elegans* worm while images (b), (c), and (d) all show infected worms. Image (d) is a later view of the epithelium of the tail.
Hodgkin 2002

Table 1. Genes Whose Transcription Are Altered in *C. elegans* in Response to *M. nematophilum* Infection

gene name	n578	infection ratio
C36A4.9 (*acs-19)*	0.466	2.04167
F43C11.3	0.196	2.05644
F46C5.1	0.101	2.16691
W07B8.4	0.225	2.39571
clec-61	0.098	2.43302
clec-82	0.123	2.35924
C05C10.3	1.88	0.45665
C17C3.1	1.706	0.43611
	1.63	
C35A5.3	3.394	0.49525
acdh-2	8.739	0.26597
dhs-25	2.044	0.4784
folt-2	2.694	0.39608
F22B7.9	0.306	2.64093

C45B2.2	3.027	0.312
F35E12.5	2.68	3.82298

Numbers indicate fold change in expression in the *egl-38* mutants compared to wild type

Background on *M. nematophilum*

M. nematophilum is a coryneform bacterium. Its genome is very GC rich, consisting of 2850 kb and a plasmid, pMN1, that is about 55kb long. Attempts to cure the bacteria of the plasmid have been unsuccessful, suggesting that it may contain essential genes. *M. nematophilum* is resistant to ampicillin, vanocomycin, kanamycin and spectinomycin, and sensitive to chloramphenicol, erythromycin, rifampicin and tetracycline, which is useful to know in terms of control mechanisms (Akimkina et al., 2006). Transformation to introduce more antibiotic resistance genes would allow for more systems of genetic study to be employed in this organism; for example, a plasmid encoding tetracycline resistance will need to be introduced in *M. nematophilum* in order to use RNA interference (RNAi) to test the response of *C. elegans* to infection by *M. nematophilum* in worms whose *acs-19* gene has been knocked down; however, no techniques for genetic manipulation have yet been reported for *Microbacterium* species (Akimkina et al., 2006). A non-virulent strain of *M. nematophilum,* UV336, fails to induce the swollen tail (DAR) phenotype in *C. elegans*, and often serves as a negative control in experiments (O'Rourke et al., 2006). *M. nematophilum* infection is lethal in most species of nematode, including *Caenorhabditis briggsae*; however, it was found that the bacteria only cause disease in *C. elegans*. This could be the result of infection and pathogenesis mechanisms that are geared towards the more sensitive species, but it could also reflect their lack of adequate defense mechanisms.

To establish an infection in *C. elegans*, the bacteria adhere to the rectal and post-anal cuticle of susceptible nematodes where they multiply and induce local swelling of hypodermal tissue. The swelling causes the infected worms to experience constipation and consequential slowed growth, otherwise, the infection is non-lethal. The infection and subsequent deformation of the post-anal region (DAR phenotype) appears to be an adapted survival strategy for the bacteria. *C. elegans* are soil dwellers, and they spend most of their time crawling around with open mouths feasting on bacteria, so they are *M. nematophilum*'s potential predators. By adhering to the post-anal region, the bacteria prevent the proper function of the defecation muscles of *C. elegans*, and almost all infected worms have "a distended gut indicative of constipation" even during early stages in the infection where there is very little post- anal swelling (Hodgkin et al., 2000). These constipated worms grow more slowly and feed less frequently, something that benefits the bacteria. Furthermore, what better hiding place than the tail of something that could possibly eat you (they would never find you)?

A signal cascade pathway mediates DAR phenotype caused by *M. nematophilum* infection. The infection induces an extracellular signal-regulated kinase (ERK) mitogen-activated protein (MAP) kinase cascade that mediates swelling of the post-anal tail, which protects *C. elegans* from sterility, and arrested development that can be caused by severe constipation. This response appears to be independent of factors that are upstream and downstream of the ERK-MAP kinase pathway. One such factor, the RAS LET-60 protein, usually activates the pathway in response to external signals to the cell. It was shown, however, that *let-60* mutants exhibit the wild type tail swelling, suggesting that the activation of the ERK-MAP kinase pathway in response to *M. nematophilum* infection is RAS independent (Nicholas and Hodgkin, 2004). The components of the ERK-MAP kinase pathway are the LIN-45 Raf, MEK-2, and MPK-1 kinases. Mutants in

these kinases, when infected with *M. nematophilum*, do not exhibit the swollen DAR phenotype that is seen in the wild type, and instead exhibited a bacterially unswollen (Bus) phenotype (Nicholas and Hodgkin, 2004). This involvement of the ERK-MAP Kinase pathway in pathogen defense is a new role for the pathway, as it has previously only been shown to be necessary for the division and differentiation of hypodermal cells that form the vulva and that reduction of this signaling results in a vulva-less phenotype (Vul) because the hypodermal cells fail to differentiate (Nicholas and Hodgkin, 2004). Conversely, constitutive activation of the ERK cascade gives rise to the multivulva (Muv) phenotype. In addition, this hyperactivity in some cases also gives rise to abnormal tail phenotype that is similar to the aforementioned DAR phenotype (Nicholas 2004).

Host surface molecules play an important role in the initial establishment of a bacterial infection because they mediate first contact with the pathogen. Eukaryotic cells are often heavily decorated with a variety of complex carbohydrates; therefore, many microbial pathogens exploit this, and use these molecules to adhere to the host surfaces to promote their own survival. Many higher organisms have evolved immune defenses, including innate defenses such as complement, antimicrobial peptides, and cell mediated adaptive immunity to counteract infection. Pathogenic nematodes often evade immune defenses by altering their surface antigenicity to avoid detection. This by altering of their surface antigenicity can also protect the nematodes from pathogens like *M. nematophilum*. s*rf-3* is a gene in *C. elegans* that encodes a multitransmembrane hydrophobic protein that has nucleotide sugar transportation activity. SRF-3 is only expressed in secretory cells, which suggests that it has a role in surface sugar modification (Höflich 2004). *M. nematophilum* is unable to colonize *C. elegans srf-3* mutants due to their altered sugar composition. Because both UDP- galactose and UDP-N-acetylglucosamine are substrates for Srf,

many surface complex carbohydrates could be affected, so it is not clear which ones are involved in the adherence of *M. nematophilum* to the cuticle of C. elegans; however, *bus-1*, whose product is predicted to act in the synthesis of core-1 type O-glycans sugars that decorate the outer membrane of *C. elegans,* mutants are also resistant to *M. nematophilum* infection. This observation implies that attachment of *M. nematophilum* to the cuticle is carbohydrate dependent, requiring the presence of host core-1 O-glycoconjugates (Palaima et al., 2010). However, it is not clear what factors are involved in the regulation of these carbohydrates on the cuticle.

Experimental Procedures

Strain Maintenance and Genetics

All *C. elegans* strains were grown and maintained at 20ºC on NGM media spotted with 1-2 mL of *E.coli* OP50. Transgenic animals used are as described: *acs-19::gfp* transcriptional reporter strain: CM 1858 *unc-119(e2498); guEx 1251*, *n578;acs-19::gfp egl-38* mutant strain: CM 2022 *egl-38(n578);unc-119(e2498); guEx 1251*, *acs-19::gfp protein* translational reporter strain: CM 2251 *unc-119(e2498); guEx 1382*, strain: *unc-119(e2498); guEx 1251*

M. nematophilum Assays

All experiments that indicate that *C. elegans* worms were grown in the presence of *M. nematophilum* were done so on NGM plates spotted with a 9:1 mixture of *E. coli* OP50 and *M. nematophilum* liquid culture.

Microscopy

All microscopy was preformed on a Zeiss Axioskop 2 microscope. All pictures included in this paper were taken at 100X magnification with DIC. All worms were evaluated on agar pads with 10 mM NaN_3.

Cloning of acs-19 Translation Fusion Reporter

The *acs-19* translation fusion reporter was constructed as described. The gene encoding ACS-19 and its upstream regulatory sequence (~3.7 kb) was replicated off of *C. elegans* genomic DNA using PCR with primers 3063 and 2500. This was then ligated to the *smaI* site of vector pPD95.79, which includes the coding sequences for GFP. This plasmid was then transformed into DH5α *E. coli*. Plasmid DNA was then isolated from the bacteria and purified (concentration 118.5 ug/ml) and then 6.6 ul of a concentration of 75 ng/ul was co injected into young adult staged RH10 *C. elegans* nematodes along with 15ng/ul *unc-119* DNA. Rescued animals were isolated and maintained as aforementioned.

Cloning of acs-19 RNAi Plasmid

The *acs-19* RNAi plasmid was constructed as follows. *C. elegans* cDNA was synthesized from *C. elegans* RNA and 50 to 250 ug of random primers. The gene for *acs-19* was then amplified off this cDNA using PCR with primers 2694 and 2693. The resulting product was inserted into vector pPD129.36 digested with NcoI and Kpn. The resulting plasmid was transformed into DH5α *E. coli*, and selected for using carbenicillin. The plasmid was then re-isolated and purified. This purified plasmid was then transformed into HT115 *E. coli*.

RNAi Experiment

Cultures of the empty vector, *acs-19* RNAi feeding vector, and *gfp* RNAi feeding vector bacteria were grown overnight at 37ºC. These cultures were then each spotted on 3 replicas of separate RNAi plates, and allowed to dry overnight. L4 stage *C. elegans acs-19;gfp protein* worms were then placed on the spotted plates. Three days were allowed to pass, and on the third day the offspring were analyzed under a fluorescent microscope for expression of GFP.

Transcriptional Reporter Experiment

L4 stage worms from each experimental condition- wild type grown on Op50, wild type grown on *M. nematophilum*, *n578; acs-19::gfp* nematodes grown on Op50, and *n578; acs-19::gfp* nematodes grown on *M. nematophilum* were separately picked in sets and analyzed under a florescent microscope for expression of *acs-19::gfp* in the cells U, K, K', and F in the hindgut. Data was recorded on which cells were expressed in each worm.

Viability Experiment

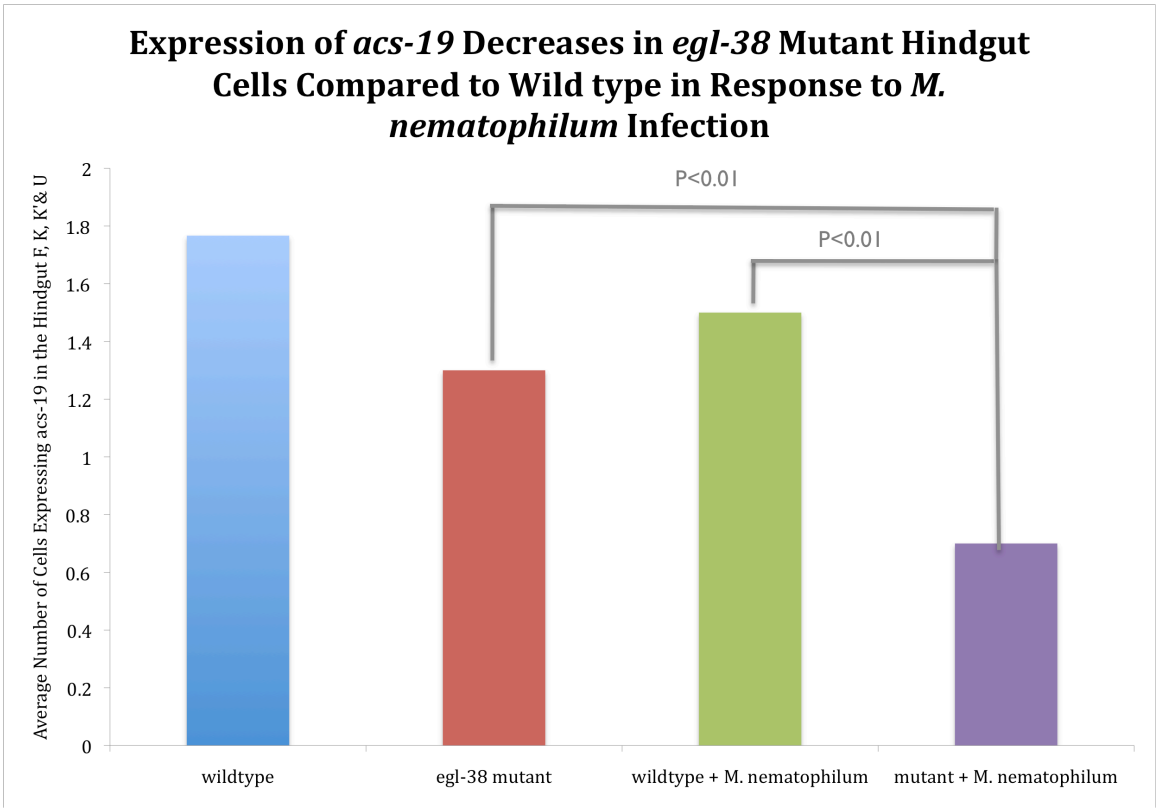
Cultures of the empty vector, *acs-19* RNAi strain, and *gfp* RNAi were grown overnight at 37ºC. These cultures were then each spotted on 3 replicas of separate RNAi plates, and allowed to dry overnight. L4 stage *C. elegans acs-19;gfp protein* worms were then placed on the spotted plates. The next day, this worm was removed from the plates, and the number of eggs laid were counted and recorded. Three days passed, and on the third day the number of worms staged above, below, and at L4 were counted.

Results

Expression of acs-19 Decreases in egl-38 Mutant Hindgut Cells Compared to Wild type in Response to M. nematophilum Infection

egl-38 mutant *C. elegans* worms show a decrease in expression of *acs-19* in hindgut cells F, K, K', and U compared to wild type in the presence of *M. nematophilum* (Figure 2). This indicates that *egl-38* may be needed to regulate the expression of *acs-19* in the presence of the bacterium. These results also show that the results of the two aforementioned microarray experiments are not replicated here with the transcriptional reporter used. No increase in expression of *acs-19* was observed in wild type grown on *M. nematophilum* compared to wild type grown on *E. coli*, and although a decrease in expression of this gene was observed between *egl-38* mutants compared to wildtype, this decrease was not statistically significant.

Figure 2. Average Number of Cells Expressing *acs-19* in Response to *M. nematophilum* Infection

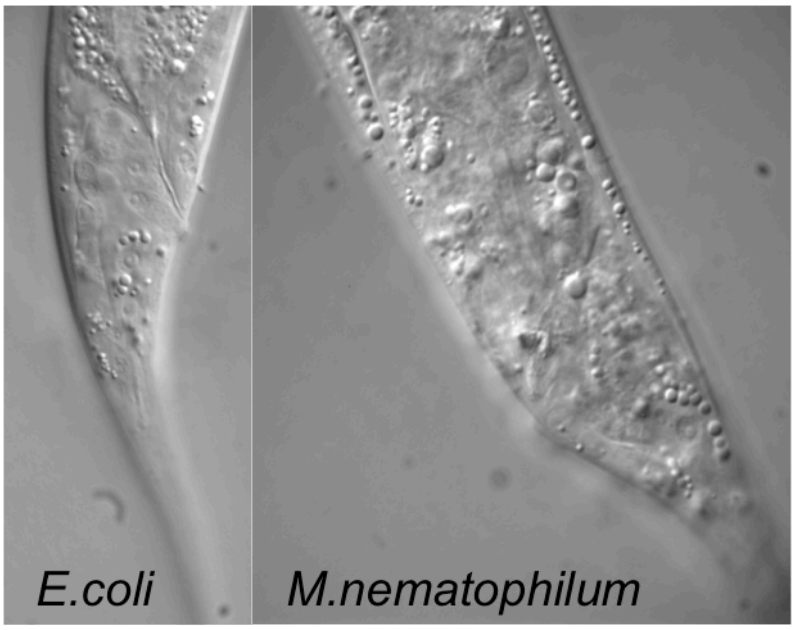


Statistical analysis was preformed using the Z Test. All other comparisons were shown to be insignificant. Each condition has a sample size of 30 worms.

egl-38 Mutants Exhibit a Slight DAR Phenotype

egl-38 mutant *C. elegans* worms grown in the presence of *M. nematophilum* are observed to exhibit a slightly less severe DAR phenotype (Figure 3.), indicating that *egl-38* is not responsible for the signal transduction pathway leading to the formation of this phenotype.

Figure 3. DAR Phenotype in *egl-38* Mutants

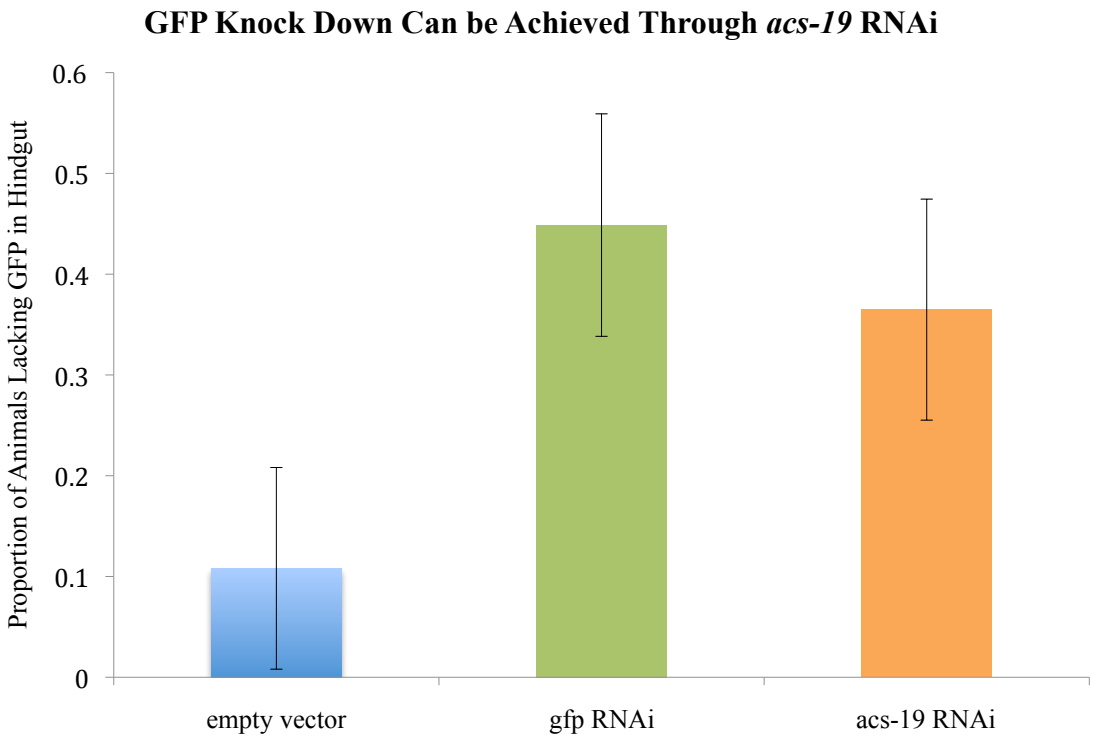


egl-38 worms grown in the presence of the bacteria listed. Pictures are taken are 100X magnification.

RNAi Can Be Used to Knock Down acs-19

A translational reporter of *acs-19* was constructed to observe the expression and localization of ACS-19 protein when tagged with GFP. This strain was also used to evaluate the effectiveness of RNAi knockdown of *acs-19* using RNAi. Because the protein is tagged with GFP, it is anticipated that RNAi treatment against acs-19 or gfp sequence should target the reporter transgene. No significant difference between knock down was observed in wild type *C. elegans* worms grown with the *acs-19* RNAi feeding vector and these same worms grown on a *gfp* RNAi feeding vector control Figure 4.), indicating that RNAi can be used to knock down *acs-19*.

Figure 4. *acs-19* RNAi Knock Down

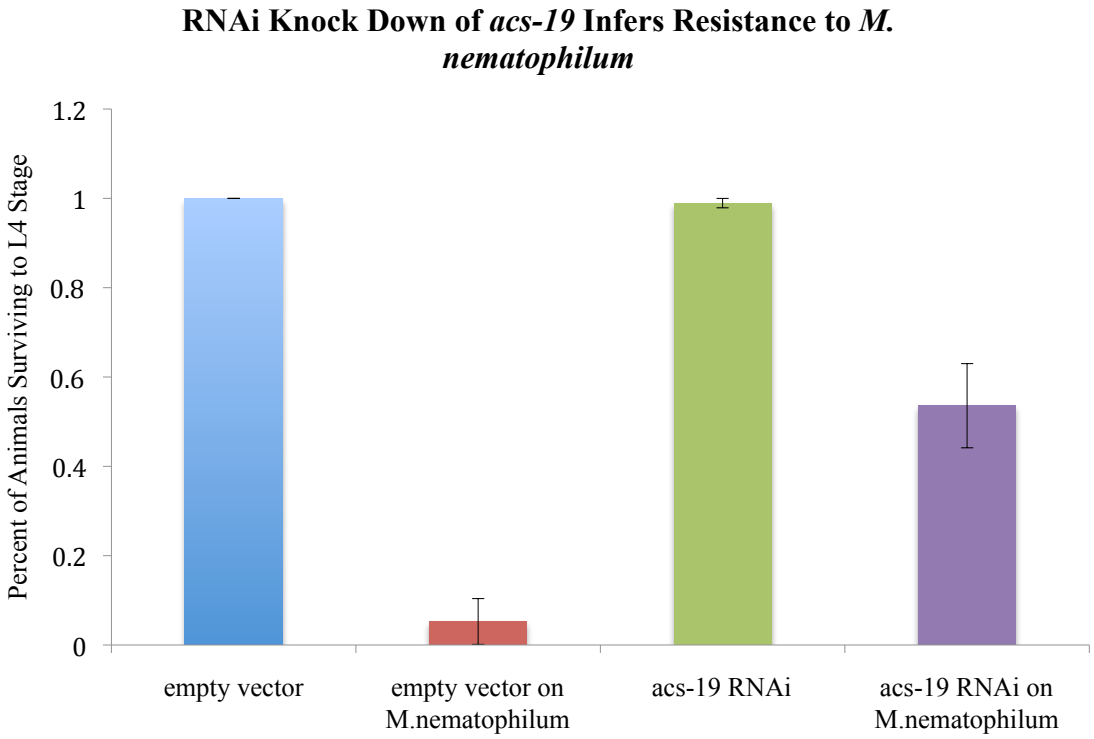


Error bars represent 95% confidence interval.

The Up Regulation of acs-19 in Response to M. nematophilum Infection is Part of the Bacteria's Infection Mechanism

RNAi knockdown was used to evaluate the function of *acs-19* in the response to *M. nematophilum* infection. A higher proportion of *C. elegans acs-19::gfp protein* translational reporter animals grown in the presence of the *acs-19* RNAi feeding vector than with the empty vector survived to the L4 stage when exposed to *M. nematophilum* (Figure 5.), indicating that the up regulation of *acs-19* reported in the aforementioned microarray experiment is part of the mechanism by which *M. nematophilum* infects its host.

Figure 5. Growth Assay



Discussion

We show in this study that the *acs-19* transcriptional reporter does not replicate the results of the two aforementioned microarray experiments. One possible explanation is that the transcriptional reporter used in this study does not include all of the gene's possible regulatory sequences- it only includes some upstream regulatory sequence. In contrast, the *acs-19;gfp protein* translational reporter includes more possible regulatory sequences, including the gene's introns and exons, though it contains no additional upstream and no downstream sequences. Thus, future experiments could test whether this reporter has the potential to replicate the results of the microarray experiments. The other explanation is that the results of the microarray experiments are not replicable.

This study also suggests that part of the function of *egl-38* is to promote the expression of *acs-19*, as the expression of that gene decreases in observed hindgut cells in its absence. *Egl-38* encodes an orthologue of Pax 2, 5, and 8 subgroup transcription factors, a group that is responsible for key developmental processes including cell proliferation, stem-cell renewal, resistance to apoptosis, migration and invasion. Because *acs-19* encodes an acyl-CoA synthetase, an enzyme involved in acly-CoA biosynthesis, this finding has the potential to link this activity to a specific set of transcription factors.

egl-38 has not been previously shown to participate in the signal cascade pathway that results in the DAR phenotype. This result was more or less confirmed in the Nicholas et al. 2004 paper where the authors established that the DAR phenotype was mediated by a RAS-independent ERK-MAP kinase cascade, and the participation of *egl-38* in the cascade was contingent upon replication of the results of the microarray experiments.

An RNAi feeding vector can be successfully used to knock down *acs-19*, indicating that the gene is not essential. Upon knockdown of this gene, a higher proportion of worms survive to the L4 stage when exposed to *M. nematophilum* compared to wild type, indicating that the presence of the protein product is detrimental to the worms. This suggests that the up regulation of *acs-19* reported in the aforementioned microarray experiments is part of the mechanism by which *M. nematophilum* infects its host. It was mentioned before that the attachment of *M. nematophilum* to the cuticle of *C. elegans* worms is carbohydrate dependent, and requires the presence of host core-1 O-glycoconjugates (Palaima 2010). Thus, it could be that *acs-19* is involved in regulating the carbohydrate composition of the cuticle surface.

This work, along with previous research, has many possible therapeutic applications. Acyl-CoA synthetase is a ligase that catalyzes the formation of acyl-CoA from fatty acids, the

first step in fatty acid metabolism. It plays an important role in the metabolic pathways of the cell, and has been shown to be part of the mechanism responsible for the increase in breast cancer cell proliferation, invasion and migration due to its function in the production of lipooxygenase and cyclooxygenase metabolites (Podestá et al. 2010). Furthermore, its knockdown has been associated with cell apoptosis in the aggressive phenotype (Podestá et al. 2010). Studying its function, regulation, and localization using *C. elegans* as a model system can help to elucidate its role in breast cancer progression. In addition, studying the interaction between *M. nematophilum* not only elucidates many aspects of host pathogen interaction including bacterial attachment, antigen variation, and cellular morphogenesis, but this work also suggests a novel biocontrol agent that could be active against nematodes of economic importance, including those in the genera *Steinernema* and *Heterorhabditi.*

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