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Pathogen Response Genes Mediate Caenorhabditis elegans Innate Immunity

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Description
Innate immunity is crucial in the response and defense against pathogens for invertebrates and vertebrates alike. The soil nematode Caenorhabditis elegans is a useful model to study the eukaryotic innate immune response to microbial pathogenesis. Prior research indicates that the protein receptor FSHR-1 plays an important role in the innate recognition of intestinal infection due to pathogen consumption. Determining what genes are controlled by FSHR-1 may uncover an unknown pathway that could increase not only the comprehension of the C. elegans immune system but also innate immunity generally. To characterize the function of FSHR-1, four candidate pathogen response genes that appear to be regulated by FSHR-1 were evaluated in worms infected with Pseudomonas aeruginosa. Although intestine specific RNA interference of these four genes did not show immunity phenotypes, quantitative PCR suggests that FSHR-1 regulates the basal and/or infection-induced expression of three of the four genes. To explore this FSHR-1-dependent transcriptional induction, fluorescent transgenic reporters were constructed for the three candidate FSHR-1 target genes. The spatial expression of one putative pathogen response gene was characterized in transgenic worms under both control and pathogenic conditions. RNA interference was performed to assess the FSHR-1 dependency of this expression pattern.

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Pathogen response genes mediate *Caenorhabditis elegans* innate immunity

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Abstract

Innate immunity is crucial in the response and defense against pathogens for invertebrates and vertebrates alike. The soil nematode *Caenorhabditis elegans* is a useful model to study the eukaryotic innate immune response to microbial pathogenesis. Prior research indicates that the protein receptor FSHR-1 plays an important role in the innate recognition of intestinal infection due to pathogen consumption (Powell et al. 2009). Determining which genes are controlled by FSHR-1 may uncover an unknown pathway that could increase not only the comprehension of the *C. elegans* immune system but also innate immunity generally. To characterize the function of FSHR-1, four candidate pathogen response genes that appear to be regulated by FSHR-1 were evaluated in worms infected with *Pseudomonas aeruginosa*. Although intestine-specific RNA interference of these four genes did not show immunity phenotypes, quantitative PCR suggests that FSHR-1 regulates the basal and/or infection-induced expression of three of the four genes. To explore this FSHR-1-dependent transcriptional induction, fluorescent transgenic reporters were constructed for the three candidate FSHR-1 target genes. The spatial expression of one putative pathogen response gene was characterized in transgenic worms under both infected and un-infected conditions. RNA interference was performed to assess the FSHR-1 dependency of this expression pattern.

Introduction

Host-pathogen interactions involve multiple molecular recognition events that are required both for effective infection by a pathogen and for resistance against infection by a host (Medzhitov 2007). Understanding this complex conversation between host and pathogen has become a major research focus in the biomedical sciences. The innate immune system is crucial in the response and defense against pathogens for invertebrate and vertebrate animals alike. The

innate immune response in *C. elegans* was originally described as a non-specific, first line of defense against infection, serving to detect pathogens and initiate a neutralizing response (Shivers *et al.* 2008; Akira *et al.* 2006). However, recent studies suggest a greater specificity than was initially anticipated, in that various gene classes such as lysozymes, CUB-domains, lectins, ShK-like toxins, and histones are transcribed at different levels depending on the pathogen (Shivers *et al.* 2008). In jawed vertebrates, innate immunity stimulates the adaptive immune system (Medzhitov 2007). Invasion of a pathogen is detected through host pattern recognition receptors (PRRs) which activate signaling pathways in order to upregulate antimicrobial molecules to combat infection.

Genetic analysis of pathogen resistance has revealed that immune mechanisms are highly conserved among diverse hosts. The nematode *Ceanorhabditis elegans* is an attractive model to study the effects of microbial pathogens on the eukaryotic innate immune system. In a natural environment, *C. elegans* lives in the soil and feeds on bacteria; due to this constant contact with soil microbes, it is hypothesized that *C. elegans* developed immune responses to combat potential pathogens. It is this close evolutionary relationship with soil microbes and the simplicity of the organism that makes *C. elegans* a useful model to study innate immunity (Alegado *et al.* 2006). *C. elegans* is easily maintained in the laboratory and, being transparent, can be viewed using simple microscopy (Schulenberg *et al.* 2004). Nematodes are propagated on lawns of attenuated *Escherichia coli* strains and survive for approximately three weeks in the laboratory. To assess pathogen virulence and host innate defense mechanisms, *C. elegans* can be fed pathogenic bacteria or fungi relevant to human infection (Kim 2006). The sequencing of the *C. elegans* genome has allowed for the recent development of molecular techniques important to the study of innate immunity. While there are many homologies between the innate immune

systems of invertebrates and vertebrates, the mechanism for the defense pathway in *C. elegans* is largely unknown.

Toll-like receptors (TLRs) function as transmembrane PRRs in mammals and Toll signaling mechanisms are evolutionarily conserved across species, from organisms such as *C. elegans* and *Drosophila* to mammals (Akira 2006). Interestingly, the ligand-binding domain of a majority of PRRs, including mammalian TLRs, is made up of multiple leucine-rich repeats (LRRs). While the role of the only *C. elegans* TLR homolog (TOL-1) as a PRR is unclear, Powell et al. (2009) has found that the LRR-containing G protein coupled receptor FSHR-1 functions in the *C. elegans* response to pathogens. Interestingly, GPCRs are known to be important for many aspects of development; however, few have been associated with infection recognition (Kudo *et al.* 2000). FSHR-1 is critical to the *C. elegans* innate immune response in the intestine, a major site of infection in worms due to pathogen consumption. FSHR-1 has been shown to act in parallel to both the insulin and p38 MAPK pathways known to contribute to the innate immune response in *C. elegans*. It is unclear what role FSHR-1 plays in innate immunity: whether FSHR-1 plays a regulatory role – by generally enhancing intestinal ability to respond to infection – or functions as a pathogen receptor responsible for sensing initial intestinal infection (Powell et al. 2009).

As a putative receptor for the *C. elegans* innate immune system, FSHR-1 may regulate the expression of pathogen response genes, or genes that give rise to antimicrobial molecules that work to neutralize infection. In order to define some of these pathogen response genes, microarray analysis experiments have been performed. A full-genome microarray analysis of *C. elegans* gene expression specific to *Pseudomonas aeruginosa* (PA14) infection demonstrated a robust transcriptional response. Specifically, 304 genes were upregulated and 114 genes were

downregulated more than two-fold in worms responding to *Pseudomonas* infection compared to worms fed non-pathogenic *E. coli* (OP50) (Troemel *et al.* 2006). To explore the targets of the infection receptor FSHR-1, Powell et al. (2009) performed *Pseudomonas*-specific microarrays on *fshr-1* null mutant worms compared to wild type (N2) worms. Interestingly, 179 genes were found to be upregulated in wild type worms but were not upregulated in *fshr-1* mutants, suggesting that these genes are induced by FSHR-1 in worms infected with *Pseudomonas* and are thus targets of the FSHR-1 pathway. Through the use of quantitative real-time polymerase chain reactions (qRT-PCR), Powell et al. (2009) were able to quantify the transcriptional induction of ten of these identified putative FSHR-1 mediated pathogen response genes.

Several gene families are overrepresented among known pathogen-response genes, including the CUB-like gene family. It has previously been shown in *C. elegans* that genes from the CUB-like gene family have induced expression in the presence of bacterial pathogens (Sharpira *et al.* 2006). The CUB domain, named based on the first three identified proteins of the family (complement C1r/C1s, Uegf, Bmp1), is a 110 amino acid domain that suggests an anti-parallel β-barrel structure similar to those of immunoglobins (Bork 1993). CUB is found in a number of extracellular and plasma membrane-associated proteins, many of which are proteases. CUB containing proteins are known to function in a multitude of processes, including development, cell communication and signaling, angiogenesis, fertilization, inflammation, neurotransmission, and tumor suppression (Blanc *et al.* 2007).

Interestingly, both the *Pseudomonas*- and *fshr-1*-specific microarrays identified a number of pathogen response genes that encode for proteins with a CUB-like domain, including paralogous genes C17H12.8 and C17H12.6. Powell et al. (2009) quantified the FSHR-1

mediated upregulation of CUB-like gene C17H12.8 and showed that its expression was 10-fold less in *fshr-1* mutant worms than wild type worms.

Of the pathogen response genes identified from the microarray, four genes were chosen for further study that had especially high induction levels on the microarray: C17H12.6 (CUB), F55G11.5 (dod-22), C05A9.1 (pgp-5) and T24B8.5 (ShK). The immune phenotypes of gene knockdowns were assessed through the use of RNA interference. Basal and induced expression levels of the four genes were determined through the use of quantitative PCR. To further examine the expression patterns of these candidate pathogen response genes, transcriptional reporters were constructed. Due to the observed transcriptional expression of CUB-like genes in infected worms, we have begun to characterize the expression of C17H12.6. The expression patterns of C17H12.6 were observed through the microinjection of C17H12.6pr::GFP into wild-type worms to produce transgenic lines. C17H12.6pr::GFP tissue specificity, basal expression and infection-induced expression patterns were observed via fluorescence microscopy in wild-type transgenic worms and in those lacking the FSHR-1 receptor. As there is strong evidence for the evolutionary conservation within the CUB-like gene family, characterizing the expression patterns of CUB-like C17H12.6 could lead to a greater understanding of the role CUB-like proteins play as antimicrobial effectors and to what extent they are regulated by FSHR-1. Uncovering the FSHR-1 pathway has the potential to increase the comprehension not only of the *C. elegans* immune system but also innate immunity generally.

Materials and Methods

Worm Synchronization

Worms were maintained at 20°C on nematode growth media (NGM), as described by Brenner (1974). Embryos were isolated by removing gravid hermaphrodites with 4 ml M9 buffer per 10 cm NGM plate, centrifuging for 30 seconds at 1000xg, and removing the supernatant for a total volume of 2 ml. Embryos were released from gravid hermaphrodites upon addition of NaOCl (400 µl) and 5N NaOH (100 µl). Embryos were washed 3 times with M9 buffer and re-suspended in 5 ml of M9 buffer for 24-48 hours while rotating. Larval stage 1 worms were dropped onto media + bacteria (~250 worms/plate) and incubated at 20°C for 96 hours. Larval stage 4 worms were used in induction assays.

RNA interference

To perform intestine-specific RNA interference, VP303 *rde-1 (ne219); kbIs7* worms were synchronized. Worms were fed non-pathogenic *E. coli* (RNAi strain HT115) expressing dsRNA of *fshr-1*, C17H12.6, F55G11.5, C05A9.1, or T24B8.5. Control worms were fed *E. coli* (RNAi strain HT115) expressing dsRNA of the empty plasmid L4440. All RNAi bacterial clones were obtained from the Ahringer RNAi library (Kamath et al. 2003). Bacteria were grown in LB + 50 µg/ml carbenicillin at 37°C with agitation, spread on 6 cm RNAi plates (nematode growth medium (NGM), 25 µg/ml carbenicillin, 5 mM isopropyl β-D-thiogalactopyranoside), and incubated at 25°C for 48 hours.

Pseudomonas Infection

Pseudomonas aeruginosa (strain PA14) and *E. coli* (strain OP50) were grown in LB for less than 14 hours at 37°C and seeded to SK plates as described by Powell and Ausubel (2008). Approximately 40 late stage 4 larvae were picked to SK plates seeded with pathogenic *P. aeruginosa* + 30µl FUDR for assays at 25°C.

Killing Assays

Worms were infected with *P. aeruginosa* for blind killing assays at 25°C. Plates were scored for sensitivity to the pathogen as previously described (Powell and Ausubel 2008).

RNA collection and isolation

Wild-type and *fshr-1* null mutant worms were maintained at 15°C on nematode growth media (NGM) described by Brenner (1974), synchronized and grown in parallel. Laval stage 1 worms were dropped onto 10 cm NGM plates with non-pathogenic *E. coli* and grown to laval stage 4. Worms were fed non-pathogenic *E. coli* or infected with *P. aeruginosa* at 25°C. After 4 hours, worms were removed and washed with M9 buffer. Worms were pelleted and all supernatant was removed. In a RNAse free area, 1ml RNAase free Tri-reagent was added to each pellet and frozen immediately at -80°C. Total RNA was extracted using TRI Reagent (Molecular Research Center, http://www.mrcgene.com) according to the manufacturer's instructions, as previously described by Troemel et al. (2006).

Quantitative RT-PCR

Quantitative RT-PCR was performed for three independent biological replicates, in triplicate, as previously described (Troemal et al., 2006). Primer sequences were designed for

each gene of interest (Table 1); F01D5.5 served as a control infection response gene. Statistical analysis was performed in Microsoft Excel. Induction, basal, and final expression values were normalized to *nhr-23*, a gene constitutively expressed in *C. elegans*. Values between conditions were compared using a 2-tail, paired t-test; SEM was calculated for each condition. Expression comparisons achieved statistical significance with $p<0.05$.

Table 1. Primer pairs used for qRT-PCR.

Primer Name	**Primer Sequence (5'-3')**
T24B8.5 Forward	TGTTAGACAATGCCATGATGAA
T24B8.5 Reverse	ATTGGCTGTGCAGTTGTACC
C05A9.1 Forward	TGTTCGAGCACTTAACATGGA
C05A9.1 Reverse	CCCTGAATTACAGCTCGTTTG
F55G11.5 Forward	ACTATGGCTCTCCAACGGTAG
F55G11.5 Reverse	CCCTTGGAAGTTTAGAGGTACG
C17H12.6 Forward	CTGAATGACTTGCAGGAGAACT
C17H12.6 Reverse	CATCCCACCCAGAACTAGC
F01D5.5 Forward	CTACCCCAGAACCACCAACT
F01D5.5 Reverse	AGAAGCAATTTGAGCAGAAGC
nhr-23 Forward	CGGATATTCTATAGCTGTTGC
nhr-23 Reverse	ACTTGTGGCGATGGGAAGC

Generating Promoter-GFP fusion constructs

Two-way fusion PCR was used in the generation of transcriptional reporters for C17H12.6, T24B8.5, and F55G11.5 in *C. elegans* (Figure 1). DNA segments containing the predicted promoter regions of T24B8.5, C17H12.6, and F55G11.5 were obtained by amplifying 4.3 Kb, 2.9 Kb, and 4.0 Kb regions upstream of the respective genes. Promoter regions were amplified in 25 µl PCR reactions using 1ng *C. elegans* genomic DNA for template and the corresponding Forward and Reverse primers for each gene (Table 2). Each reaction contained 0.25 µl Expand Long Template Enzyme Mix (Roche) and had a final concentration of 175 µM dNTP Mix, 0.5 µM forward primer, 0.5 µM reverse primer, and 1x Expand Long Template

Buffer 1 containing 17.5 mM $MgCl_2$. A Bio-Rad MyCycler performed the reactions with thermal cycling parameters of 1 minute initial denaturation at 95°C (1 cycle), 30 second denaturation at 95°C, 30 second annealing at specified temperature (Table 2), specified extension time (Table 2) at 68°C (30 cycles), 10 minutes final extension at 68°C (1 cycle), and unlimited cooling at 4°C. To determine successful amplification, 5 µl products were electrophoresed in a 1% Seakem LE agarose minigel in 1x TAE buffer.

Table 2. Primers for 2-way fusion PCR. Capitalized letters are those complementary to the DNA sequence of GFP.

Primer Name	**Primer Sequence (5'-3')**	**Annealing Temp. (°C)**	**Extension Time (sec)**
T24B8.5 Forward	AGTCGACCTGCAGGCATGCAAGCTtttggtgatacataaatgataactga	59	65
T24B8.5 Reverse	tcattgagcaaacagattgg		
T24B8.5 Nested Reverse	ggtcatattctgcaaatacagttg	60.8	80
C17H12.6 Forward	tgtgctagaatagatcatatcatcttg	59	65
C17H12.6 Reverse	AGTCGACCTGCAGGCATGCAAGCTtgtgctagaatagatcatatcatcttg		
C17H12.6 Nested Reverse (A)	acgatttaagtgtctgttttgctc	60.8	80
C17H12.6 Nested Reverse (B)	gcaaattgtgaggaacgagt	60.8	43
F55G11.5 Forward	gttaagcgtccacgcctgt	63	60
F55G11.5 Reverse	AGTCGACCTGCAGGCATGCAAGCTtttctagaaaaagtgaaaatcccg		
F55G11.5 Nested Forward	aagcggccgaaggttagt	63	61
GFP Forward	AGCTTGCATGCCTGCAGGTCGACT	56	60
GFP Reverse	AAGGGCCCGTACGGCCGACTAGTAGG		

As shown in Figure 1, fusions of predicted promoter regions to the DNA sequence for GFP were completed in a second PCR reaction. Promoter::GFP transcriptional reporter

constructs were generated for C17H12.6, T24B8.5, and F55G11.5. 1µl of each amplified product was diluted 40-fold for future use. Fusions were performed in 20µl reactions, containing 0.2 µl of Phusion® DNA Polymerase (New England Biolabs, Inc.), 200 µM Deoxynucleotide Mix, 0.5 µM Nested Forward primer, 0.5 µM Nested Reverse primer (Table 2), and 5x Phusion™ HF Buffer. Fusion reactions were performed in a Bio-Rad MyCycler with thermal cycling parameters of 30 second initial denaturation at 98°C (1 cycle), 35 cycles of a 5 second denaturation at 98°C, 10 second annealing at specified temperature (Table 2), and specified extension time at 72°C, 10 minutes final extension at 72°C (1 cycle), and unlimited cooling at 4°C. Successful fusion and amplification was determined by electrophoresing 5 µl of the 2-way fusion PCR products in a 1% Seakem LE agarose minigel in 1x TAE buffer. For the C17H12.6pr::GFP reporter (2.859 Kb), a concentration of 62.5ng/µl was approximated through gel electrophoresis of sample compared to 1 Kb ladder dilutions.

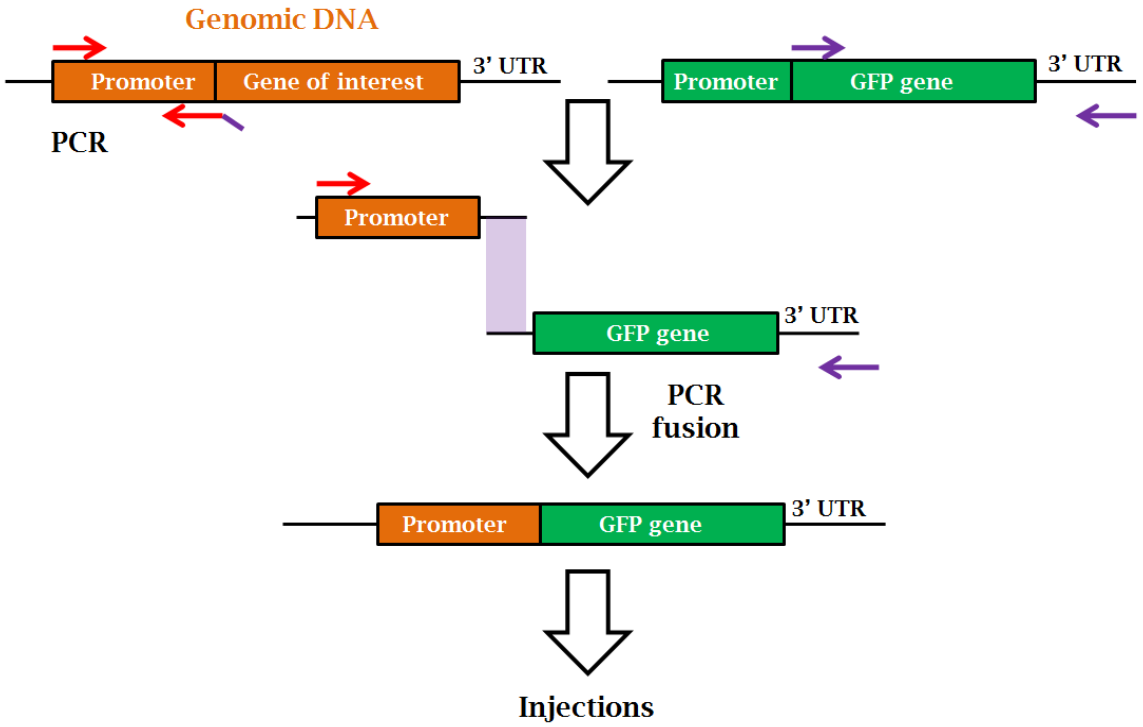

Figure 1. Schematic diagram of 2-way fusion PCR used to construct transcriptional GFP reporters for injection into wild-type *C. elegans*.

Generation of Transgenic Lines

An injection mixture consisting of 10ng/µl C17H12.6pr::GFP reporter construct as well as 50ng/µl co-injection marker *Pmyo-2*::mCherry and 40ng/µl salmon sperm carrier DNA was injected into the syncytial gonads of wild type worms. Using a Leica MZ16 F fluorescence stereomicroscope with a GFP filter, progeny of injected worms were examined for fluorescent expression. Two independent lines of C17H12.6pr::GFP transgenic *C. elegans* were isolated and found to transmit the extrachromosomal array to their offspring. A sample of each line was frozen and named: zvEx8 and zvEx9 (Table 3). The reporter array was integrated into the genome of strain JRP1016 by irradiation with 280 µJ/cm^2 of UV light using a Spectrolinker™ UV Crosslinker (Montreal Biotech Inc.). Transgenic strains were maintained at 20°C on nematode growth media (NGM) described by Brenner (1974).

Observation of CUB expression

Approximately 40 larval stage 4 JRP1016 worms were fed non-pathogenic *E. coli* or infected with pathogenic *P. aeruginosa*. Worms were incubated at 25°C and scanned for basal or induced expression using a Leica MZ16 F fluorescence stereomicroscope. To visualize spatial C17H12.6pr::GFP expression, worms were anesthetized with 25 µM NaN_3 and photographed using a Nikon Eclipse 90i microscope with DIC and epi-fluorescence optics.

Visualization of C17H12.6pr::GFP expression in worms lacking FSHR-1

To observe the expression of C17H12.6pr::GFP in JRP1016 worms lacking FSHR-1, synchronized worms were fed *fshr-1* or L4440 RNAi bacteria. Late stage 4 larvae were infected with *P. aeruginosa*. To ensure that RNAi successfully knocked down *fshr-1*, killing assays were performed on one *P. aeruginosa* plate from each RNAi strain.

In silico Analysis of CUB-like proteins

Gene Selection

The presence of the CUB-like domain in the FSHR-1-regulated gene C17H12.6 piqued an interest in the CUB-like domain. A list of 59 *C. elegans* genes containing CUB-like domains were presented by wormbase.org. From that list, I searched for each gene in a condensed microarray data set provided by Dr. Powell. The microarray is specific to *Pseudomonas aeruginosa* infection, where wild type worms were exposed to the pathogen for four hours compared to worms on control, non-pathogenic *E. coli*. Of the CUB-like genes from the microarray, 15 genes were chosen (including C17H12.6) that had a broad range of *Pseudomonas*-induced expression levels in wild type worms. P values were also taken into consideration: genes with high or middle induction had small P values whereas most genes that had a low fold change had greater P values, thus illustrating even less significant induction. The selected genes were ordered by induction level and arranged into 3 categories: high, middle and low induction. Two of the 15 genes selected were genes containing a CUB domain. For each selected gene, I went to wormbase.org to obtain the spliced DNA sequence as well as the amino acid sequence.

Exploring sequence conservation

To explore whether the genes induced by *Pseudomonas* infection have conserved regions, multiple sequence alignments via UniProt ClustalW were constructed for analysis. Whole protein sequences were aligned for each of the three induction groups: high, middle, and low. The N terminus of C49G7.7 was removed due to the great length of the protein sequence that prevented the construction of a concise MSA. For the low induction group, the two CUB

genes were removed from the MSA because the sequences differed so greatly from the CUB-like sequences. In addition, an MSA was constructed from the amino acid sequences of all 13 genes with one CUB gene (R10H10.3) that served as the outgroup. A guide tree was also constructed using JalView to roughly compare the relationships between the CUB-like genes under study. To further explore the CUB-like domain within each of the 13 genes selected, a CUB-like domain sequence was taken from ncbi to serve as a representative CUB-like consensus sequence. Each of the three induction groups was aligned with this sequence in order to find the CUB-like domain for each of the genes. The sequences were then condensed to approximately 135 amino acids in length and aligned via UniProt ClustalW. A guide tree was constructed using JalView to assess possible relationships between the CUB-like domains of the genes under study. Jalview guide trees were labeled with expression levels taken from the original microarray data to best illustrate the connection between induction and sequence similarity.

Identifying evolutionary relationships

To best visualize the evolutionary relatedness and to investigate the possibility of conserved functions between the 13 selected CUB-like genes, PHYLIP phylogenetic trees were constructed. The PHYLIP program used the NJ algorithm to map the distance-based evolutionary relatedness for the genes' CUB-like domain sequences as well as for their entire amino acid sequences in a second tree. Trees were labeled with expression levels taken from the original microarray data to best illustrate the connection between pathogen induced transcription and evolutionary relatedness.

Results

FSHR-1 regulates a set of pathogen response genes

A series of *Pseudomonas*-specific microarrays defined a set of putative pathogen response genes that may be upregulated by *C. elegans* (Powell 2009). A subset of these *Pseudomonas*-response genes is thought to be dependent on the GPCR FSHR-1 for transcriptional induction in the presence of pathogen. The receptor FSHR-1 is expressed most strongly in the somatic tissues of the intestine and neurons (Sieburth et al. 2005). Given that intestinal tissue comes into direct contact with pathogen, FSHR-1 activity in the intestine has been shown to be necessary and sufficient for the innate immune response in *C. elegans* (Powell 2009). Of the 179 putative FSHR-1 dependent pathogen response genes identified, four were chosen for further study that had especially high induction levels on the microarray: C17H12.6 (CUB-like), F55G11.5 (dod-22), C05A9,1 (pgp-5) and T24B8.5 (ShK-like toxin).

To assess what effect each of the four selected target genes has on the overall innate immune response, the function of each candidate gene was removed via intestine-specific RNA interference (RNAi). The *C. elegans* strain VP303 *rde-1 (ne219); kbIs7* was used to exclusively target RNAi-mediated knockdown of gene expression to the intestine without affecting other tissues. VP303 has an intestine-specific promoter regulating the expression of *rde-1* in an *rde-1* mutant background; Argonaute RDE-1 slicer activity is required for the RNA interference mechanism. Killing assays were performed to assess the survivorship over time of RNAi worms infected with *Pseudomonas aeruginosa*. Worms fed an RNAi strain with an empty vector (L4440) served as a control. An immunocompromised phenotypic control was achieved via *fshr-1* RNAi. In comparison to the controls, the survivorship curves of intestine-specific RNAi for each of the 4 candidates mimicked L4440 RNAi curves and thus did not result in significant

pathogen sensitivity phenotypes (Figure 2). The lack of immune phenotypes for each candidate gene tested could be accounted for by the possibility that each antimicrobial effector plays a small role which contributes to a greater response against pathogen.

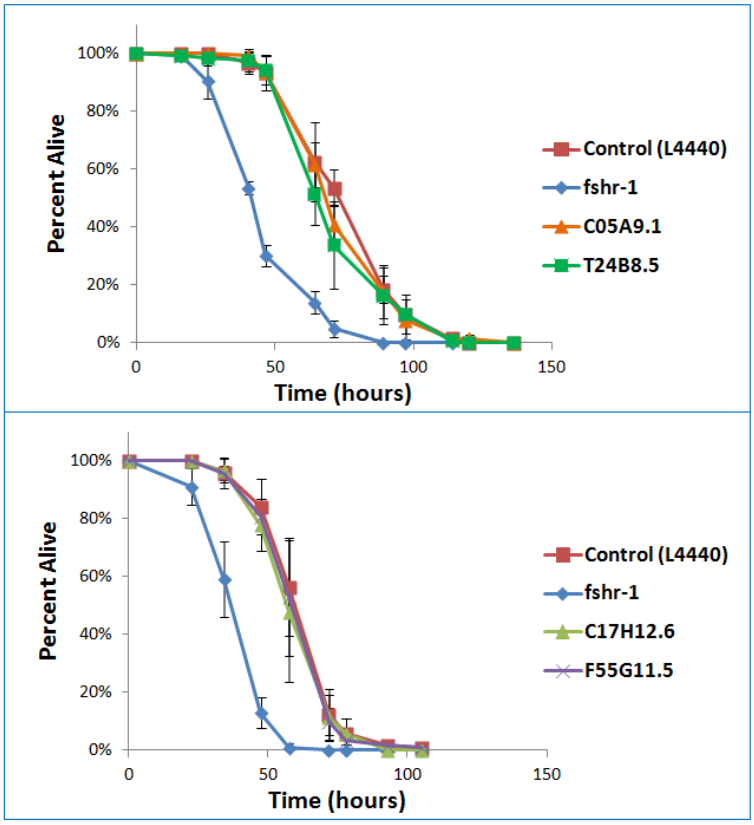

Figure 2. Survivorship curves of *C. elegans* (VP303 *rde-1 (ne219); kbIs7*) fed on pathogenic *P. aeruginosa*. Worms were fed on RNAi bacteria to knockdown *fshr-1*, C05A9.1, T24B8.5, C17H12.6 or F55G11.5 expression. Control worms were fed RNAi strain L4440 (empty vector). Error bars represent standard deviation.

Quantitative PCR (qRT-PCR) was used to quantify the relative expression levels of the four gene candidates. The relative amount of mRNA was quantified for the four genes in both healthy and *Pseudomonas* infected wild-type worms. For each gene, the mean induced expression – the ratio of gene expression in infected versus healthy worms – was determined for three independent RNA preparations and standardized based on the mean expression of a constitutively expressed gene, *nhr-23*. F01D5.5 served as a control due to its previously

determined FSHR-1-dependent induction (Powell et al., 2009). All qRT-PCR data is reported as a sample mean of three independent RNA preparations ± SEM. Paired, 2-tailed T-tests (*df* =2) were performed to identify statistically significant results. From this analysis, all candidate genes had over 10-fold greater expression in infected worms than in healthy worms (Figure 3). The CUB-like gene, C17H12.6, was induced 71.01±20.19 fold and F55G11.5, C05A9.1, and T24B8.5 were induced 48.56±24.93, 238.21±188.94, and 18.56±6.30, respectively.

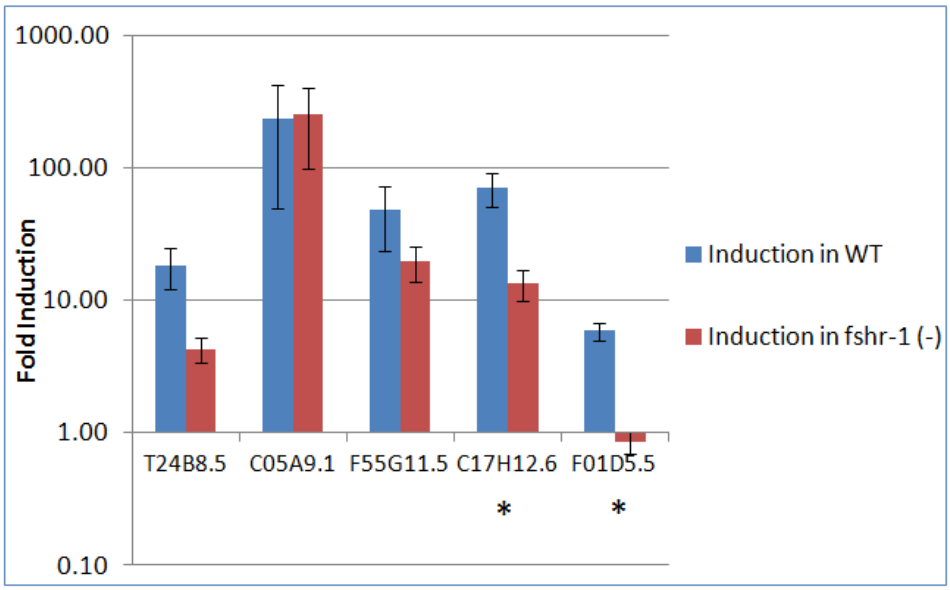

Figure 3. FSHR-1 regulates pathogen response genes. Induced expression of four candidate pathogen response genes measured via qRT-PCR. Fold induction is a ratio of expression on pathogenic *P. aeruginosa* to expression on non-pathogenic *E. coli* in wild-type and *fshr-1* mutant worms. F01D5.5 serves as a control infection response gene. Expression for all genes was normalized to *nhr-23* expression. Error bars represent SEM for three independent biological replicates. *Genes with reduction in *fshr-1(-)* induction relative to wild-type ($p<0.05$).

To validate the findings of the microarray and to quantify the FSHR-1-dependent transcriptional induction of the candidate *Pseudomonas*-response genes, the relative amount of mRNA was also measured for the four genes in *fshr-1* mutants infected with *Pseudomonas aeruginosa* (Figure 3). The sample means for gene expression from three independent *fshr-1 (-)* RNA preparations were determined. The CUB-like gene, C17H12.6, was induced 13.48±3.47 fold and F55G11.5, C05A9.1, and T24B8.5 were induced 19.71±5.72, 254.64±155.18, and

4.33±0.90 fold, respectively. Of the four putative FSHR-1-dependent candidates, only the CUB-like gene, C17H12.6, had significantly higher expression levels induced by infection in wild-type worms than in *fshr-1* mutants (p=0.0484). While not statistically significant, F55G11.5 (p=0.1451) and T24B8.5 (0.0774) also had higher induced expression in wild-type worms in comparison to *fshr-1* mutants.

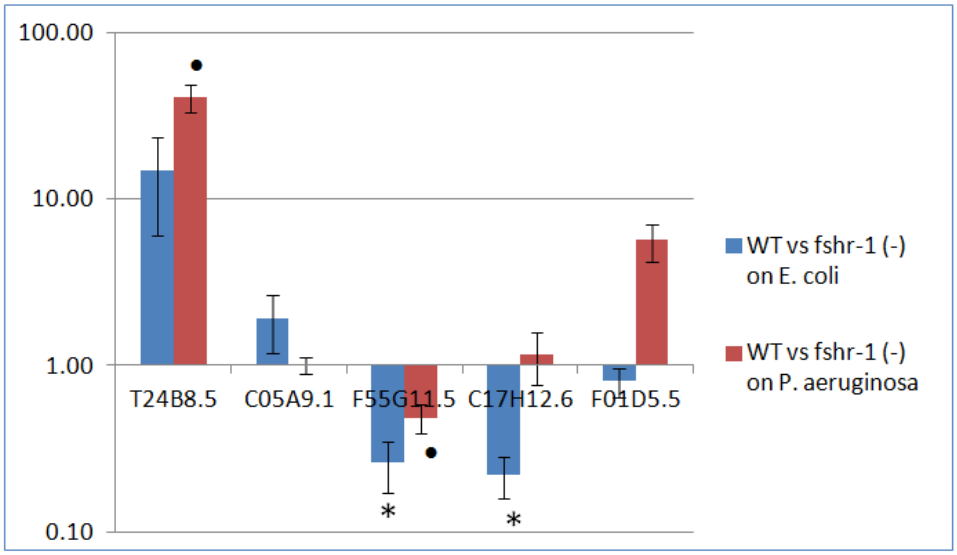

Figure 4. Fold change in gene expression levels measured via qRT-PCR is a ratio of wild-type to *fshr-1* mutant expression in worms fed non-pathogenic *E. coli* or pathogenic *P. aeruginosa.* F01D5.5 serves as a control infection response gene. Expression for all genes was normalized to *nhr-23* expression. Error bars represent SEM for three independent biological replicates. *Change in basal expression signifies a difference between wild-type and *fshr-1* mutants on *E. coli* ($p<0.05$). ●Change in final expression signifies difference between wild-type and *fshr-1* mutants on *P. aeruginosa* ($p<0.05$).

Additionally, qRT-PCR was used to measure basal expression of the four genes: the relative expression in wild-type to *fshr*-1 mutant worms fed exclusively on nonpathogenic *E. coli* (Figure 4). Interestingly, C17H12.6 and F55G11.5 are significantly down regulated by FSHR-1 for their basal expression, as expression in wild-type worms is significantly lower than *fshr-1* mutant worms: C17H12.6 at 0.22±0.06 fold (p=0.0063) and F55G11.5 at 0.26±0.09 fold (p=0.0144). Both C05A9.1 (1.91±0.73, p=0.3373) and T24B8.5 (14.81±8.72, p=0.2543) appear

to be upregulated by FSHR-1 for their basal expression, however these results did not achieve statistical significance. Further, the final expression of the 4 genes was determined in worms infected with *P. aerugoinsa* (Figure 4). On pathogen, the expression of T24B8.5 was significantly greater in wild-type worms than *fshr-1* mutants by 40.86±7.81 fold (p=0.036). Interestingly, F55G11.5 was still repressed in infected wild-type worms compared to *fshr-1* mutants at 0.49±0.09 (p=0.031), but at a lesser extent than in healthy worms. The final expression levels of C17H12.6 (1.17±0.41, p=0.717) and C05A9.1 (1.01±0.12, p=0.0953) did not achieve statistical significance.

C17H12.6 is induced upon *Pseudomonas* infection

In order to learn more about the tissue specificity and expression patterns of FSHR-1-dependent pathogen response genes, transcriptional reporters were constructed. Based on the qRT-PCR data, GFP reporter fusions were made for the three genes that appear to be partially dependent on FSHR-1 for induction (C17H12.6, T24B8.5, and F55G11.5). GFP reporter constructs were generated via 2-way fusion PCR. DNA segments containing the predicted promoter regions of T24B8.5, C17H12.6, and F55G11.5 were obtained by amplifying 4.3 Kb, 2.9 Kb, and 4.0 Kb regions upstream of the respective genes. Successful amplification of the promoter products was confirmed via agarose gel electrophoresis (Figure 5).

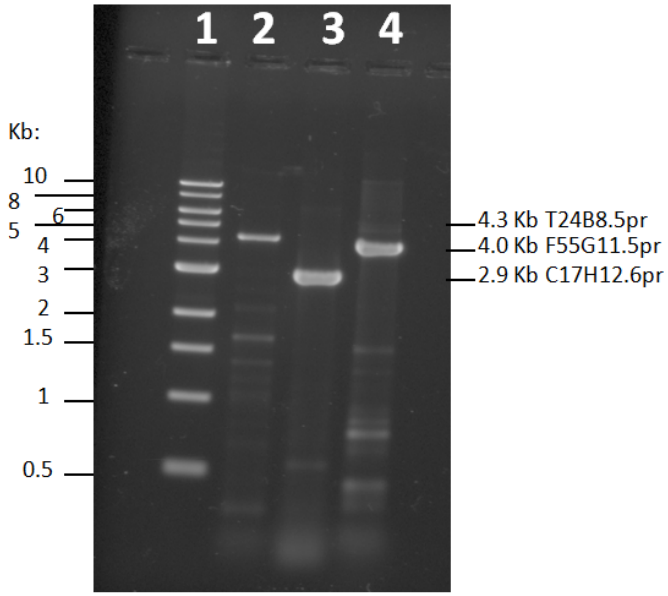

Figure 5. Amplification of the *C. elegans*T24B8.5, F55G11.5, and C17H12.6 predicted promoter regions. 5µl of each promoter product was electrophoresed at 80 volts in a 1% agarose minigel. Key to lanes: 1, Quick-load 1 Kb DNA ladder, 500 ng; 2, T24B8.5 promoter, 50 ng; 3, C17H12.6 promoter, 200 ng; 4, F55G11.5 promoter, 200 ng.

To serve as the reporter, the coding sequence of GFP (1.892 Kb) was also amplified by PCR. For the amplification of the promoter regions of the genes, reverse primers were designed with a marker tail sequence complementary to GFP in order to promote fusion of the two segments in a subsequent reaction. Agarose gel electrophoresis was used in the confirmation of the successful amplification of fusion PCR products: T24B8.5pr::GFP at 5.354 Kb, C17H12.6pr::GFP (A) 4.676 Kb and (B) 2.859 Kb, and F55G11.5 at 4.065 Kb (Figure 6).

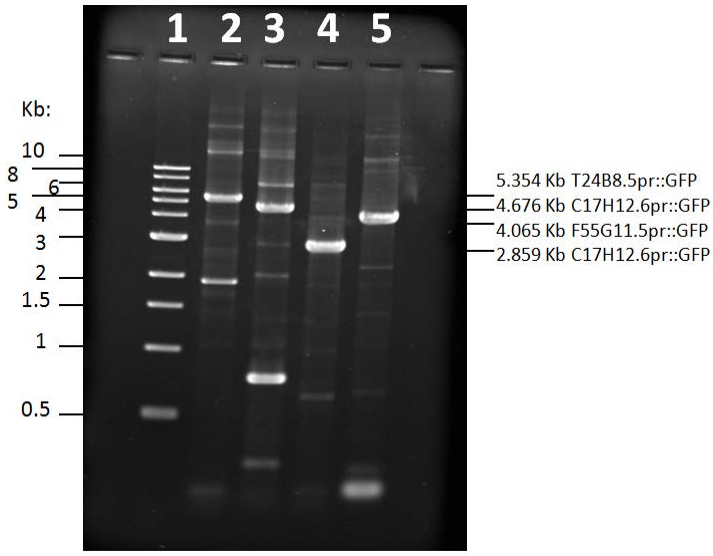

Figure 6. Amplification of the *C. elegans*T24B8.5pr::GFP, F55G11.5pr::GFP, and C17H12.6pr::GFP fusion constructs. 5µl of each 2-way fusion product was electrophoresed at 94 volts in a 1% agarose minigel. Key to lanes: 1, Quick-load 1 Kb DNA ladder, 500 ng; 2, T24B8.5pr::GFP, 5 µl; 3, C17H12.6pr::GFP (A), 5 µl; 4, C17H12.6pr::GFP (B), 5 µl; 5, F55G11.5pr::GFP, 5 µl.

Of the three genes that transcriptional markers were made, the CUB-like gene C17H12.6 had the most significant FSHR-1-regulation based on the qPCR data. Thus, a C17H12.6pr::GFP fusion was chosen for use in the first injection mixture. As two CUB reporter constructs were made, the shorter C17H12.6pr::GFP reporter (2.859 Kb) was chosen over the longer fusion due to the presence of another major product (<1 Kb) visualized on the GFP fusion product gel (Figure 6). A concentration of 62.5ng/µl for the 2.859 Kb product was approximated through agarose gel electrophoresis of sample compared to ladder dilutions; product band matches band at 3Kb from 250ng 1 Kb ladder in lane 3 (Figure 7).

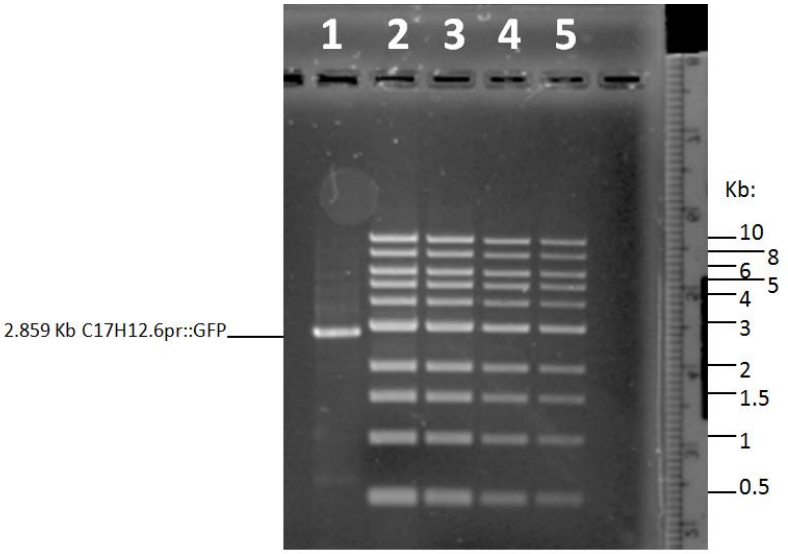

Figure 7. Concentration of C17H12.6pr::GFP fusion construct. 5µl of 2-way fusion product was electrophoresed with series of ladder dilutions at 90 volts in a 1% agarose minigel. Key to lanes: 1, C17H12.6pr::GFP (B), 1 µl; 2, Quick-load 1 Kb DNA ladder, 500ng; 3, Quick-load 1 Kb DNA ladder, 250ng; 4, Quick-load 1 Kb DNA ladder, 100ng; 5, Quick-load 1 Kb DNA ladder, 50ng.

An injection mixture consisting of the fusion construct as well as co-injection marker *Pmyo-2*::mCherry was injected into the syncytial gonads of wild type worms. Via fluorescence microscopy, two independent lines of C17H12.6pr::GFP transgenic worms were isolated and found to transmit the array to their offspring (Table 3). Both lines had very similar C17H12.6pr::GFP basal and infection- induced expression patterns. The array in strain JRP1015 was integrated into the genome to generate strain JRP1016 with the zvIs7 genotype, whose expression patterns were further characterized.

Table 3. Transgenic *C. elegans* strains containing a C17H12.6 transcriptional reporter.

***C. elegans* strain**	**Genotype**	**Source**
JRP1013	zvEx8 – C17H12.6pr::GFP Extrachromosomal Array	Powell Lab
JRP1015	zvEx9 – C17H12.6pr::GFP Extrachromosomal Array	Powell Lab
JRP1016	zvIs7 – C17H12.6pr::GFP Integrated	Powell Lab

Through the use of fluorescent microscopy, the spatial and temporal expression of CUB-like C17H12.6 in healthy JRP1016 worms fed non-pathogenic *E.coli* was assessed (Figure 8). C17H12.6pr::GFP expression is limited to the posterior intestinal cells, as no expression was observed in any other tissues. Seen in Figure 8, as worms age, C17H12.6pr::GFP expression becomes more concentrated in the posterior intestine and has minimal anterior extension.

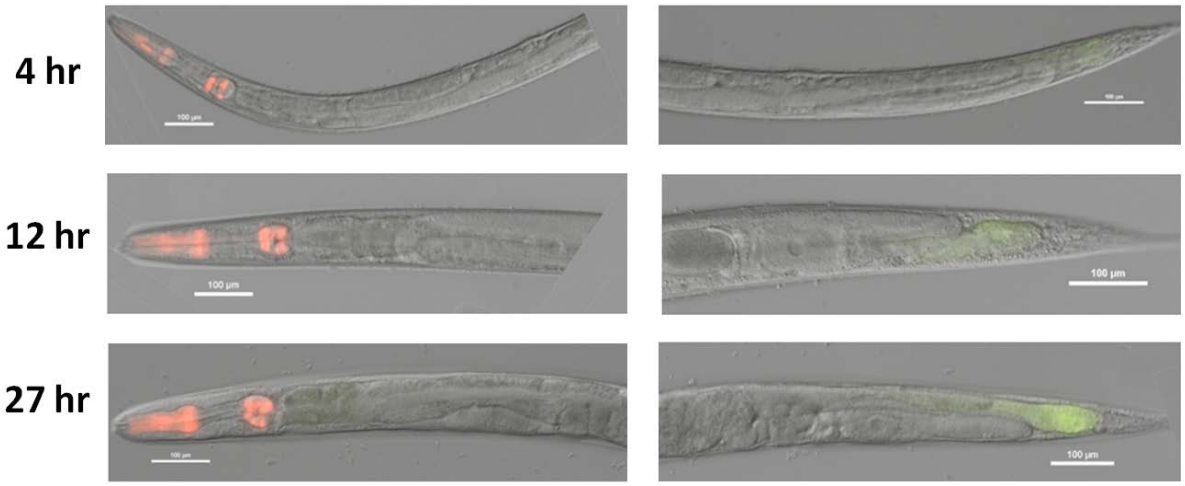

Figure 8. Basal expression of GFP in uninfected worms carrying an integrated C17H12.6pr::GFP transcriptional reporter. Worms at the fourth larval stage were fed on non-pathogenic *E. coli* at 25°C for 4, 12 and 27 hours and photographed at optimal exposure DIC, 75ms FITC, and 15ms Rhodamine. Scale bars, 100 µm.

The transcriptional induction of C17H12.6 was observed in worms fed on pathogenic *P. aeruginosa*. JRP1013 displays clear induced expression of C17H12.6pr::GFP when worms are infected with pathogen. In comparison to healthy worms, most worms placed on *Pseudomonas* have C17H12.6pr::GFP expression that is brighter and extends from the posterior end of the intestine to the vulva, which marks the midsection of the worm (Figure 9). Induced expression of C17H12.6pr::GFP was observed as early as 4 hours but was best observed 24-27 hours post initial pathogen exposure.

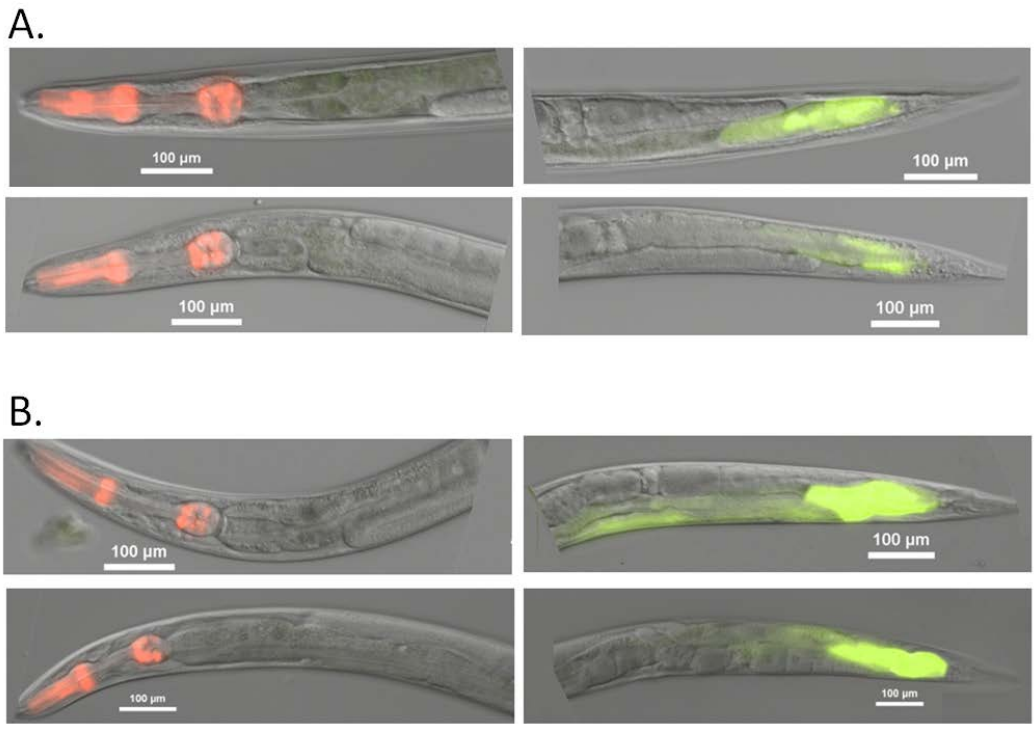

Figure 9. Expression of GFP in healthy and infected worms carrying an integrated C17H12.6pr::GFP transcriptional reporter. Worms at the fourth larval stage were fed on (A) non-pathogenic *E. coli* or (B) pathogenic *P. aeruginosa* at 25°C for 27 hours and photographed at optimal exposure DIC, 75ms FITC, and 15ms Rhodamine. Scale bars, 100 µm.

RNA interference was performed to assess the FSHR-1 dependency of this CUB expression patterns. The induction seen in both control and *fshr-1* knockdowns appeared to be at approximately equal levels. In most worms, infection-induced C17H12.6pr::GFP expression was limited to the posterior intestine and was visualized at 12 hours post initial pathogen exposure. Interestingly, C17H12.6pr::GFP induction in both control (L4440) and fshr-1 RNAi worms was less concentrated than that of induced-expression in wild-type worms in the previous induction assays. This change in expression could be attributed to feeding larval worms on the RNAi *E. coli* strain (HT115) versus *E. coli* (OP50) before placement on pathogenic *Pseudomonas*.

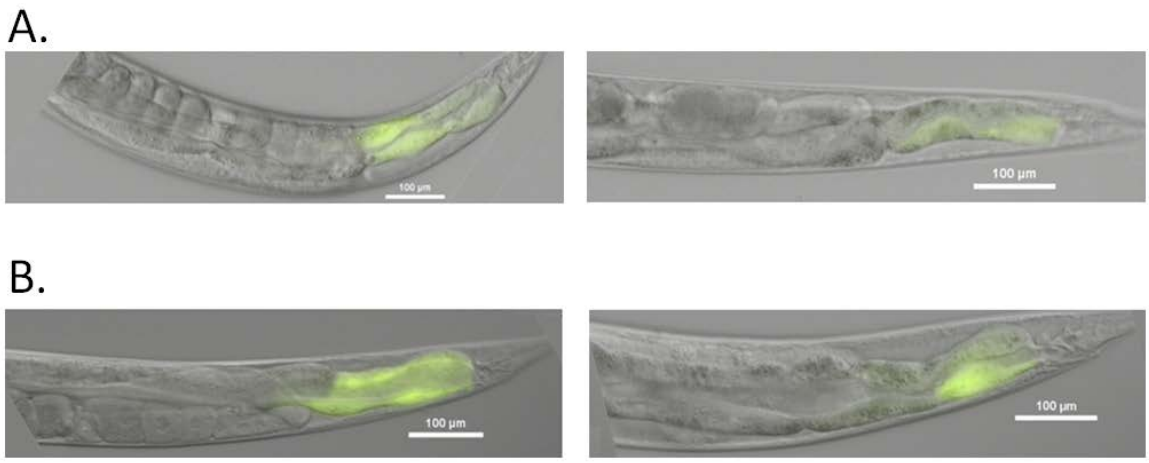

Figure 10. Expression of GFP in infected (A) control (L4440) and (B) *fshr-1* RNAi worms carrying an integrated C17H12.6pr::GFP transcriptional reporter. Worms at the fourth larval stage were fed on pathogenic *P. aeruginosa* at 25°C for 26 hours and photographed at optimal exposure DIC and 300 ms FITC. Scale bars, 100 µm.

Shared *Pseudomonas*-induced expression is suggestive of evolutionary conservation among CUB-like genes

Given that the qRT-PCR data as well as induction assays using JRP1013 worms indicate that the expression of CUB-like gene C17H12.6 is induced by *P. aeruginosa* infection, bioinformatics analysis was employed to investigate the evolutionary conservation and function of related CUB-like genes. Prior *Pseudomonas*-specific microarray data shows a gradient of expression of CUB-like genes in *C. elegans* in response to infection. The microarray identified C17H12.6 as highly upregulated (18.477 fold) in wild type worms exposed to *Pseudomonas* infection compared to worms fed non pathogenic *E. coli.* It was hypothesized that CUB-like genes with the highest induced expression levels may be more closely related than the CUB-like genes with little or no *Pseudomonas*- induced transcription. Table 4 contains the 13 CUB-like genes, including C17H12.6, and 2 true CUB genes selected for analysis, arranged by *Pseudomonas*-specific transcription expression levels.

Table 4. Genes selected based on microarray fold change expression levels in wild type *C. elegans* when exposed to pathogenic *P. aeruginosa* versus non-pathogenic *E. coli.* Fold change values are relative to the microarray.

High induction level		
Annotation	**Expression fold change**	**t test p value**
C17H12.8	7.421	0.008044047
C49G7.7	8.497	0.01819496
F55G11.5 (dod-22)	11.188	0.01050894
F55G11.4	12.819	0.003948842
C17H12.6	18.477	0.007656637
Middle induction level		
Annotation	**Expression fold change**	**t test p value**
C29F3.7a	3.229	0.004632528
F35E12.9a	5.044	0.005668923
F53C11.1	5.402	0.008237721
C32H11.1	5.723	0.06055998
K08D8.5	5.829	0.004448748
Low induction level		
Annotation	**Expression fold change**	**t test p value**
T05E12.6	0.361	0.007059164
F35E12.2	0.97	0.7646276
ZK896.1	0.976	0.7651929
CUB Domains:		
Annotation	**Expression fold change**	**t test p value**
K05C4.11	0.993	0.8627944
R10H10.3	1.256	0.2137785

To investigate the possibility that *Pseudomonas*-response genes containing a CUB-like domain may share conserved regions for antimicrobial function, multiple sequence alignments were constructed for genes with high, middle, and low induction levels respectively. As represented by Figure 11, the MSA for highly induced putative pathogen-response genes shows conserved regions within and outside of the N-terminal CUB-like domain. The MSA's for middle and low induction gene subsets also reveal conservation within and outside of the CUB-like regions (Appendix). However, there is greater overall sequence similarity observed for the

three low induction genes than the five middle induction genes. The greater overall similarity in the low induction subset could be the result of only three sequences within that alignment.

```
CLUSTAL 2.1 multiple sequence alignment

C49G7.7  FEMFEKPLLLLAVIVAVSAVGNGCQLGKAIYKPVIDGTPVYWPPSWTETQPAPQLETEQS 61
F55G11.4 --MFQKLLAFLTVVVFVSAAGNSCKIGKVINKPVIDGTPVYWPASWNETQPAPQLEKEQS 59
F55G11.5 --MLSKVVLFLAVLSSASAS--VCKTGNLINKPVND-SPVIWPATWDMKFAPPKLDKGQT 56
C17H12.8 ----MIVAFWLLAL---VTVSTALECTQIP-EPIIAGEFVTIPAGANDTVQIPPN---FS 50
C17H12.6 ----MSATPLIISLGLLVGVTAALDCTQIPNNAIIPLSYTTIPAGATGLVEIPPN---FN 54
                  :  :          .  :   ::     .  *.        *      .

C49G7.7  CSWIVTIPRGYYAKLIISGKTTDKDSRFQTVDTAGNLVQTTQEN---MEPYYFPSPKFTI 118
F55G11.4 CSWYVTIPRGYYAKLIISGKTTDKDSRFQTVDSAGNLIQTTHEK---MVPYYFPASKFTL 116
F55G11.5 CSWTVTVPDGFYAKLVISAKAMDRDTYFQTIDSAGNLAKTGNEK---MKPYYFVGPKFTI 113
C17H12.8 CTYNVNAPPMIYAHVTLENGLNGNNDMITVIDEQLTRTIVSSRSAKFVHFYIFPNTTTKF 110
C17H12.6 CVYNVKVPKMVYARVRLENGLKGYNDLITVQDQQGTYTRVSSRSPSVLNFYVFPNTTTTF 114
         * : *. *   **:: :.    . :  : . *   .   .  ..   :  * *  .. .:

C49G7.7  AVSNTGS---ATFAFKVVWFPLPYLDDSIR-VGPLAKVMNATSTVNYNNYWDANSNSLTL 174
F55G11.4 AVSNEGS---ATFAFKVVWWPLPTEKYVDI-VASIGQVINVTETVVAMEY--AAPGGITL 170
F55G11.5 ALSSNAP---AAFGFKIIWLPFPNIDIGYSGVTEAAEVLNATGIIYKQSIY--SRGGIHL 168
C17H12.8 QVITKSVNMHSSFRIVVHFERMLNTTVTHLGNPDMKYFMLNDLRVNSYRTPQTMISNERI 170
C17H12.6 QVVTKSVDMHSSFRMIVFYQPMYTETVTHLGNPDMKYFMLNDLQENSYKTPQTIISNEKI 174
          : . .    ::* : : :  :               .:               ..  :

C49G7.7  MTFPADTKNYYSLRSTLVFDGPGLSSGCYISNLYQLYQTTNQWT-SSQKSIIVLNLEASS 233
F55G11.4 LTFPEDLKNYNSLRSTLIYDGSSLTSATYVSNLFLLNQSKKQWT-SSQDGIVVVNVEASR 229
F55G11.5 LPFPQDPTNYFSLRSALVFEGGSFPGCNYVGNLYQMYRSKKPYSFSSEGSIVVFNLAASG 228
C17H12.8 SLSIAHSGWYSDIFDNYFVIEGYIENPKAVYRMSKFVYQGYISTGNILTVVGLDNRVSES 230
C17H12.6 SVSMASSGWDADIFENYFIIKGDLNNTKSVSRLSRFVGQGYISTGSSLTVVGLDNRVSES 234
                  .: .  .     : .   : .:  :       : .    : : *  :.

C49G7.7  SNDKLLFLSSKYLAGIGEMVKLQPQANSIYNGTVNG--LMSSLVAAS------------ 278
F55G11.4 SMNKLLIQGSVYLAGIDEIVELHPQPNSIYNGTVNAGAHMSSLVAVSDLELQMIDVQMKD 289
F55G11.5 NSDKLLIQDTEYVQDIAQFVELYPEIKTSYTETINGGKLKSSLVSVSGANFKLTKVKMDD 288
C17H12.8 SVTFVPLSQAQQYDSYTAFSTYFQANQLDMDSTVGDKKKAVTVISMEDIVLVIDVQKTN 290
C17H12.6 SVTFVPFSQAQQYDSYTTFSTYFEANQLKIDATVGNKKKAVTVISMKNIVTVLDVQRVT 294
         .   : :  :    .   :       :     *:.     :  *

C49G7.7  --TVTVYNGSPAVYALNKTYTGTQLKNALPLSFSGPVVQFVVSSGKAVFTFKS------ 329 *
F55G11.4 DSTVSVYYGSPDAFTLDKTYTGAELKKALPLPFGGYFVQFVVSSGKAVFTFKS------ 342
F55G11.5 EATMAVYYGSPTVGTIVKNYTALEINKAVPLNFQGEVLQFVVSSGKADFTFDGWK---- 343
C17H12.8 EPNCVLRAVQYPPTPSSEVYLDFTAVYDYPRNITHKSFCIVAENCAATFKLVSPYPEQN 349
C17H12.6 ------------------------------------------------------------
```

Figure 11. Multiple sequence alignment of protein products from highly induced genes expressed in *C. elegans* during *P. aeruginosa* infection. Area highlighted in yellow denotes approximate CUB-like domain region. Area highlighted in green represents an interesting extra-CUB-like domain region. *Due to the extended length of G49G7.7, the N-terminal end was removed for a more precise alignment.

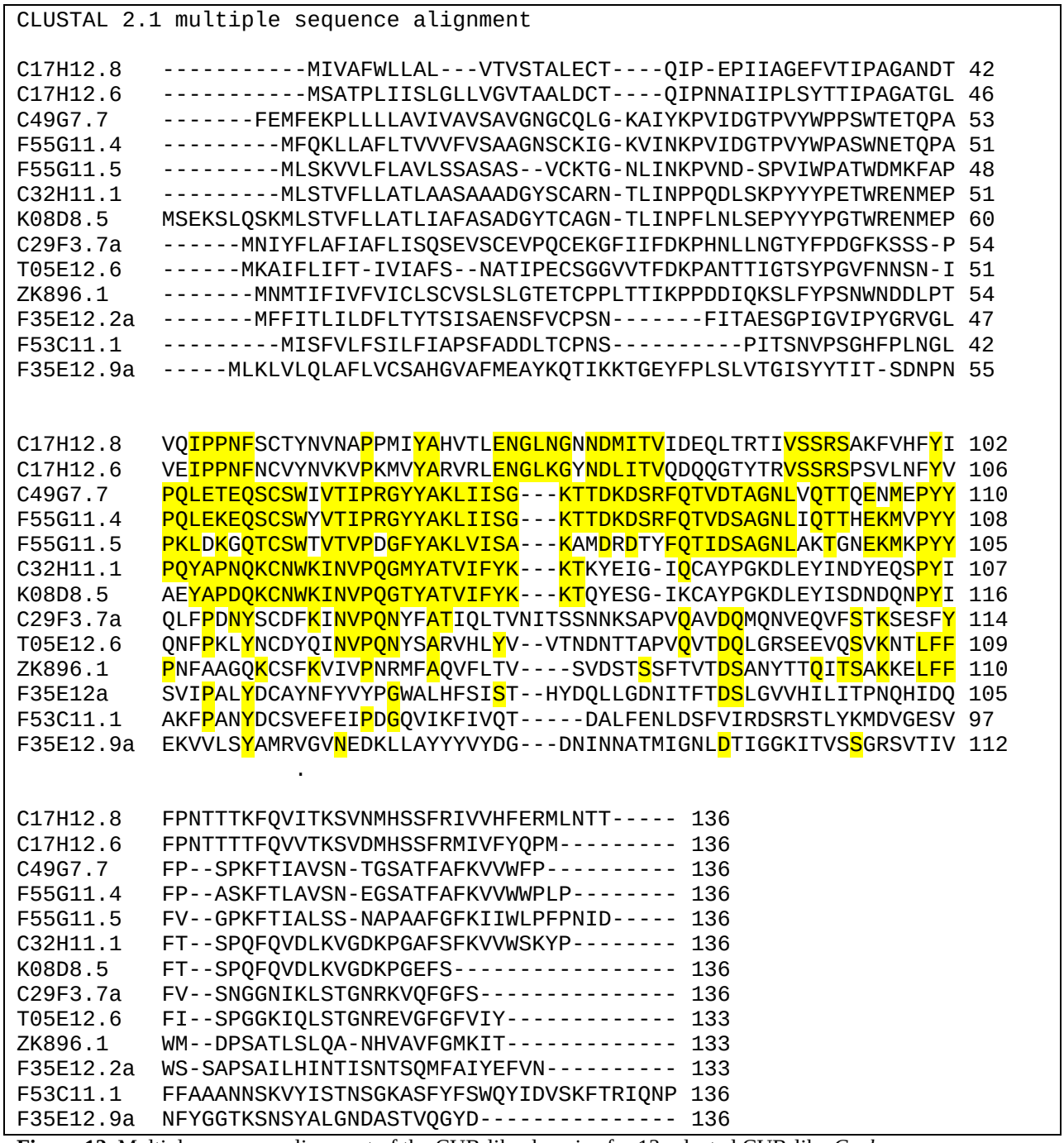

Figure 12. Multiple sequence alignment of the CUB-like domains for 13 selected CUB-like *C. elegans* genes varying in *Pseudomonas*-induction levels. Yellow highlighting in the second row depicts examples of visually identified conserved regions not highlighted by the ClustalW program.

To visualize sequence conservation within the CUB-like domain, a MSA was constructed for the CUB-like domains from the 13 genes under study (Figure 12). While the MSA only shows one amino acid marked for significant conservation across all 13sequences, a visual analysis of

the aligned sequences reveals the abundant sequence conservation within the domain. The guide tree from JalView for this alignment showed some interesting relationships that were further investigated by the construction of a PHYLIP phylogenetic tree via the NJ algorithm. The phylogenetic tree in Figure 13 shows the evolutionary relationships between the CUB-like protein domains of the 13 genes from the alignment in Figure 13, with a CUB gene R10H10 that serves as the outgroup. Interestingly, there are two clades that house two (C17H12.6 and C14H12.8) and three (C49G7.7, F55G11.5, F55G11.4) high induction genes respectively. In addition, there is one clade of two genes (C32H11.1 and K08D8.5) from the middle induction subset. The rest of CUB-like domains from the middle and low induction subsets are grouped together.

The relationships in Figure 13 show greatest sequence conservation of CUB-like domains from genes more highly transcribed in response to *Pseudomonas* infection. This is suggestive of conserved antimicrobial function within the CUB-like domain of the high induction gene subset. Also intriguing is the pairing of genes with similar names, for example C17H12.6 and C17H12.8. The names imply that they are neighboring genes on the DNA strand. The close proximity that the genes have to each other coupled with tight sequence similarity within their functional domains suggests that the genes are paralogous: one of the genes, C17H12.6 for example, was probably duplicated to give rise to its paralog, C17H12.8. Two other possible paralogous gene couples– F55G11.4 with F55G11.5 and F35E12.2a with F35E12.9a – were identified by their names and grouping in Figure 13. Unlike the C17H12 genes that appear to have recently diverged, the F35E12 genes have an earlier point of divergence which can explain lesser similarity within their CUB-like sequences.

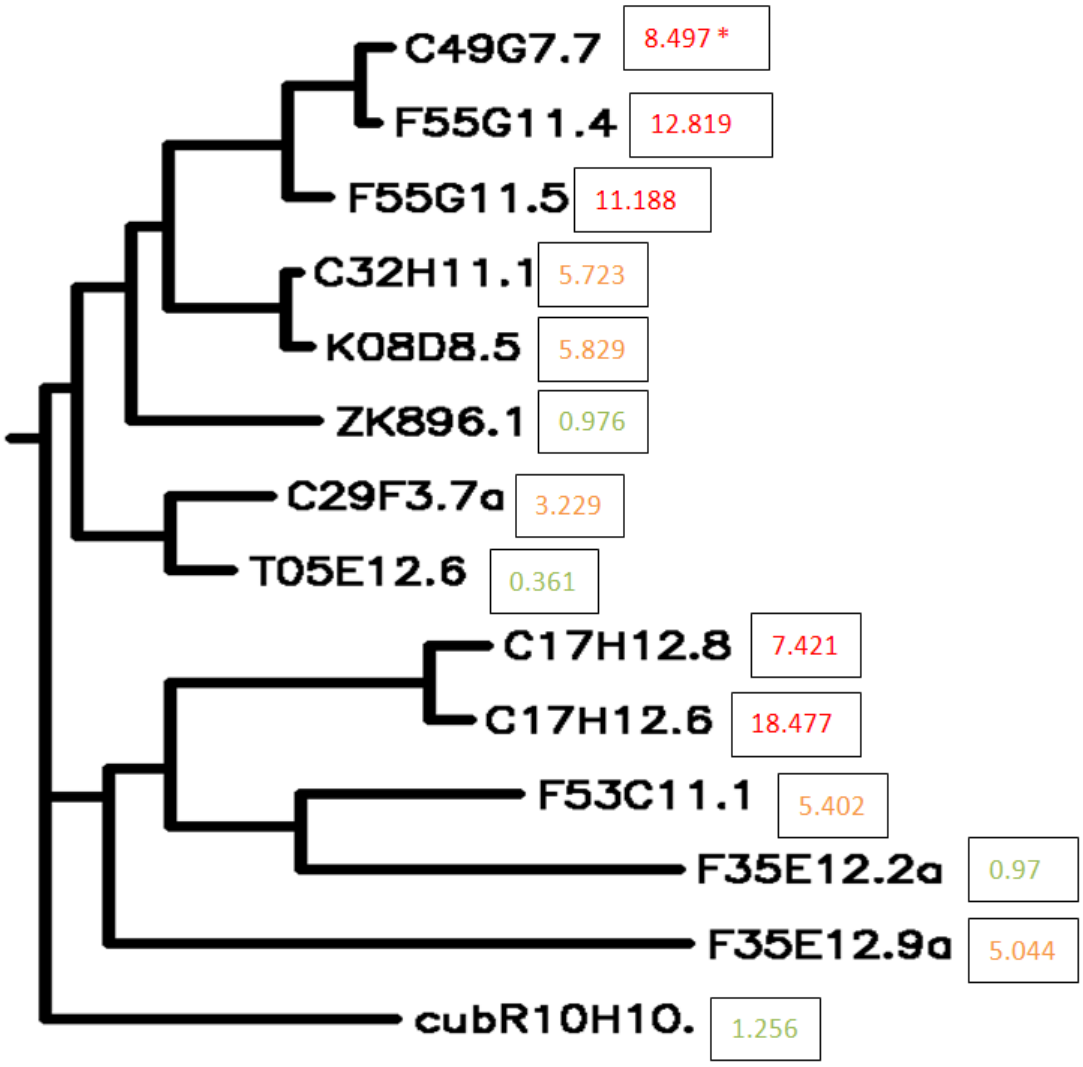

Figure 13. PHYLIP generated phylogenetic tree via the NJ algorithm illustrating relationships between 13 CUB-like domains from selected pathogen response genes ranging in induced expression levels in *C. elegans* during *P. aeruginosa* infection. CUB R10H10.3 serves as the outgroup. Induction levels are labeled red, orange, and green for high, middle, and low induction levels respectively. *C49G7.7 was shortened at the N terminus.

To determine whether the extra-CUB-like domain sequences of the genes may also function in antimicrobial protection, the whole amino acid protein sequences were mapped through PHYLIP via the NJ algorithm. Figure 14 depicts the phylogenetic tree produced for which a true CUB gene R10H10 serves as the outgroup. Intriguingly, a clade of all five highly

induced genes, including CUB-like C17H12.6, is observed; the strong evolutionary relationship between this group is suggestive of conserved functional regions outside of the CUB-like domain as well as within it. Further, strong relationships were seen within the two-member clade of C32H11.1 and K08D8.5 that share middle induction values. A three-member clade containing two middle and one low induction genes (F53C11.1, F35E12.9a and F35E12.2a) also show strong evolutionary conservation.

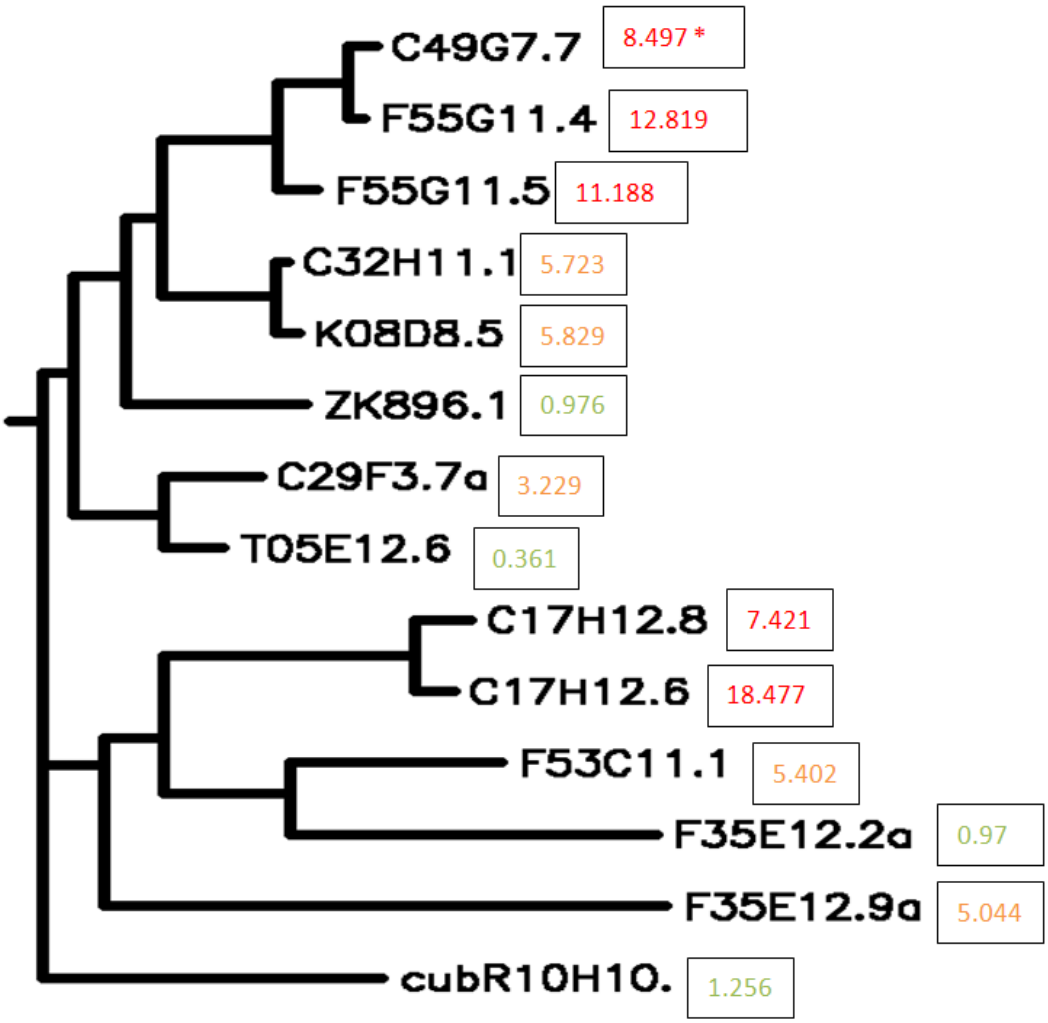

Figure 4. PHYLIP generated phylogenetic tree via the NJ algorithm illustrating relationships between whole protein products from selected CUB-like pathogen response genes ranging in induced expression levels in *C. elegans* during *P. aeruginosa* infection. CUB R10H10.3 serves as the outgroup. Induction levels are labeled red, orange, and green for high, middle, and low induction levels respectively. *C49G7.7 was shortened at the N terminus.

Discussion

To characterize the function of the receptor FSHR-1 in the context of innate immunity, four candidate FSHR-1-dependent pathogen response genes (C17H12.6, F55G11.5, C05A9.1 and T24B8.5) were evaluated in worms infected with *Pseudomonas aeruginosa.* Intestine-specific RNA interference of each of the four candidate genes in infected worms did not yield significant pathogen sensitivity phenotypes. The lack of immune phenotype for each putative infection response gene tested could be explained by the possibility that each alone plays a small role but the culmination of all FSHR-1-dependent effectors results in an effective response against pathogen.

Through quantification of gene expression via qRT-PCR, all candidate response genes had over 10-fold induced expression, suggesting antimicrobial activity in response to *P. aeruginosa* infection. Three (T24B8.5, F55G11.5 and C17H12.6) of the 4 candidates tested appear to be partially dependent on FSHR-1 for induction, however, only C17H12.6 was statistically significant ($p=0.0484$). Powell et al. (2009) found that a closely related CUB-like gene C17H12.8 was significantly dependent on FSHR-1 for its induction, as its expression was 10-fold less in *fshr-1* mutant worms than wild type worms. Interestingly, Troemel et al. (2006) reported that the same CUB-like gene, C17H12.8, is upregulated 17 fold by PMK-1 of the p38 MAPK pathway. As C17H12.6 and C17H12.8 share such great homology, it is possible that C17H12.6 also relies on PMK-1 for induction.

Basally, FSHR-1 significantly down regulates C17H12.6 expression in healthy worms ($p=0.0063$) while the receptor is responsible for C17H12.6 upregulation in response to infection. Similarly, Powell et al. (2009) reported that C17H12.8 may be repressed by FSHR-1 in the

absence of pathogen because it was expressed at significantly lower levels in wild-type worms than *fhsr-1* mutants ($p<0.01$). While another candidate T24B8.5 did not reach statistical significance, it appears to be marginally dependent upon FSHR-1 for upregulation in response to infection. Thus, FSHR-1 appears to play a role in *C. elegans* response to infection because it directly contributes to the induction of some genes. Importantly, T24B8.5 is also PMK-1 dependent, as it is upregulated by PMK-1 7-fold in response to *P. aeruginosa* infection ($p=0.0001$) (Troemel et al. 2006). As FSHR-1 and p38MAPK signal in parallel to each other, these results support the hypothesis that these pathways converge on a common set of target effector genes in response to attack by pathogenic *P. aeruginosa* (Powell et al. 2009). Additionally, from its apparent role in constitutive (basal) gene expression, FSHR-1 may contribute to the balance of immune efficacy and energy conservation: FSHR-1activates at least one gene, T24B8.5, and represses the transcription of at least two others, F55G11.5 and C17H12.6. It is possible that the constitutive expression of some antimicrobial effectors such as T24B8.5 provides an immediate defense against pathogen while the repression of other antimicrobial effectors may facilitate energy conservation in *C. elegans*.

To learn more about the expression patterns of FSHR-1 regulated effectors, the expression pattern of C17H12.6 was closely examined. C17H12.6pr::GFP is expressed in the posterior intestinal cells under both healthy and infectious conditions. Intestine limited expression of C17H12.6 supports its proposed antimicrobial effector identity, given that *C. elegans* intestinal tissue comes into direct contact with pathogen. In healthy worms, C17H12.6 is transcribed in the absence of infection and expression is limited to the very posterior intestinal region. Intriguingly, C17H12.6pr::GFP expression becomes more concentrated in the posterior intestine and has minimal anterior extension. The low C17H12.6pr::GFP expression in later stage

4 larval worms compared to visibly higher expression in 1 day old adults could be explained by a change in gene expression program; McCarroll et al. (2004) found that the transcriptional responses of both *C. elegans* and *D. melanogaster* to stress were significantly correlated with early adult transcriptional programs in both organisms, suggesting that transcriptional regulation is developmentally timed in young adults.

Based on the expression of C17H12.6pr::GFP, C17H12.6 appears to be a true pathogen response gene; it is induced upon infection with *Pseudomonas aeruginosa*: in the posterior intestine as its expression becomes visibly saturated. Further, expression extends anteriorly to midsection of infected worms, reaching the vulva. The concentrated posterior-intestinal expression of C17H12.6 may explained by the ability of bacteria to infiltrate the rectum via the anus. Irazoqui et al. (2010) reported that gram positive *Staphylococcus aureus* accumulates in the *C. elegans* posterior intestine and the rectum after only 4 hours, with less accumulation in the midsection of the intestinal lumen; however, while marked deformation occurs in the anal region due to *S. aureus* infection, no deformed anal phenotype was observed due to *P. aeruginosa* infection. Another gram positive pathogen, *Microbacterium nematophilum*, is known to adhere to the rectal and post-anal cuticle of *C. elegans*, causing slowed growth, constipation, and a defensive swelling response of rectal hypodermal cells (O'Rourke et al. 2006). Given the posterior-intestinal expression of C17H12.6, it would be interesting to compare C17H12.6 expression patterns in bacterial pathogens like *S. aureus* and *M. nematophilum* known to specifically target the posterior intestine and anus for infection. Moreover, due to the apparent antimicrobial activity of C17H12.6, it would be interesting to use JRP1016 as a tool for doing RNAi screens to identify the intermediate signaling molecules in regulating antimicrobial effectors.

Contrary to the qRT-PCR results, there was no visible decrease in C17H12.6pr::GFP expression in infected *fshr-1* RNAi worms compared to infected wild-type worms. This could suggest that C17H12.6 does not require FSHR-1 for its induction; it is possible that the mRNA isolated from wild-type or *fshr-1* mutant worms was not representative of true C17H12.6 transcription. Another explanation is that the reduction in induction of C17H12.6 in the absence of FSHR-1, as revealed by qRT-PCR, may not be detectable for C17H12.6pr::GFP in visual assays. However, based on the qRT-PCR data, it is most probable that the predicted C17H12.6 promoter region amplified to make the GFP reporter construct does not encompass the regions necessary for FSHR-1 regulation. To further investigate the apparent FSHR-1-dependency of C17H12.6, given the average length between *C. elegans* genes is ~5 Kb (Kent and Zahler, 2000), a new transcriptional reporter could be constructed that simply incorporates a larger part of the C17H12.6 promoter region than the 2.9 Kb segment used for making C17H12.6pr::GFP used in this study. As a heterologous 3' UTR was used in the current C17H12.6pr::GFP construct, it could be important to construct a promoter using the endogenous C17H12.6 3'UTR. However, a reporter in which the C17H12.6 protein is fused with GFP would best ensure the incorporation of the necessary FSHR-1 regulatory regions.

Bioinformatics analysis revealed that the transcriptional responses to *Pseudomonas aeruginosa* infection are reflective of the tight evolutionary conservation for genes within the CUB-like class. The strong evidence for the functional conservation of highly induced CUB-like genes could further the understanding of the *C. elegans* innate immune response. While RNAi of CUB-like C17H12.6 did not have a pathogen sensitivity phenotype, it is possible that the ablation of all 5 identified highly induced CUB-like genes may produce an immune phenotype.

Further, it would be interesting to both measure and visualize the expression of the five highly induced CUB-like genes, including C17H12.6, on other intestinal pathogens to investigate whether expression is specific to *Pseudomonas* infection or is constitutive in the response to all types of intestinal pathogenesis. Microbes that are known to cause intestinal infections in *C. elegans* such as *Serratia marcescens* and *Salmonella enteric* as well as fungi such as *Cryptococcus neoformans* in addition to *Staphylococcus aureus* and *Microbacterium nematophilum* could be pathogens used in such experiments (Shivers et al. 2008; O'Rourke et al. 2006; Irazoqui et al. 2010). With regard to the overarching question of the function of FSHR-1 in innate immunity, a continuation of the investigation of C17H12.6 and other closely related CUB-like gene homologs may increase the understanding of FSHR-1 regulation. A comprehensive study of the FSHR-1 dependency of a subset of effectors containing a potential antimicrobial functional domain like CUB could contribute substantial information pertaining to innate immunity generally and lead towards a greater understanding of the FSHR-1 mechanism.

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