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Transcriptional responses to pathogens in *Caenorhabditis elegans*

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Summary

Evolutionarily conserved signaling pathways, such as the p38 and ERK MAPK pathways, the TGF- β pathway, and the insulin signaling pathway are required for resistance to pathogens in *C. elegans*. Recent microarray expression profiling studies have identified both candidate immune effector genes which may recognize and eliminate microbial pathogens as well as uncharacterized gene classes that are broadly induced in response to pathogen. Comparative analysis of these microarray studies is suggestive of basal versus induced components of the ancient innate immune response in *C. elegans*. In particular, whereas the PMK-1 p38 MAPK pathway regulates genes that are induced by pathogen, the Forkhead family transcription factor DAF-16 confers pathogen resistance through the regulation of genes that are non-overlapping with pathogen-induced genes.

Introduction

C. elegans is a free-living soil nematode and thus encounters and feeds on a diverse microbial flora that presumably includes bacterial and fungal pathogens. The ecology of *C. elegans* and its microbial flora in the wild remain poorly understood, but in recent years, a number of investigators have developed experimental systems involving the intestinal infection of *C. elegans* with microbial pathogens of humans, including bacteria such as *Pseudomonas aeruginosa* [1–3], *Serratia marcescens* [4], *Salmonella enterica* [5,6], *Enterococcus faecalis* [7], and fungi such as *Cryptococcus neoformans* [8]. Moreover, the interaction between the *C. elegans* host and its naturally occurring extra-intestinal pathogens, such as *Microbacterium nematophilum* [9] and the fungus *Drechmeria coniospora* [10], which cause pathology through interactions with the cuticle and hypodermis of *C. elegans*, have also been studied using molecular genetics. Forward and reverse genetic approaches have lead to the identification and characterization of signaling pathways required for pathogen defense in *C. elegans*, and a number of reviews have focused on these studies [11–14]. Here, we review recent work aimed at defining the genes involved in the immune response.

Immune signaling in the *C. elegans* host

The genetic analysis of host defense has revealed that the signaling pathways of innate immunity have been conserved, notably parallels between Toll-like signaling in *Drosophila* and innate immune signaling in mammals [15]. Although there is a single Toll homolog in *C. elegans*, *tol-1*, the genes encoding critical components of the Toll signaling pathway

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components of mammalian innate immunity, most conspicuously the transcription factor NF- κ B, appear to be absent from the *C. elegans* genome. Genetic analysis of a possible role for *tol-1* in *C. elegans* immunity has been further complicated by the embryonic lethal phenotype of the *tol-1* null allele [16]. However, analysis of a second allele in which the cytoplasmic domain of TOL-1 is predicted to be deleted has revealed a potential role for TOL-1 in protecting pharyngeal tissue during infection with *Salmonella* [17].

The *C. elegans* genome has a single conserved member of the TIR domain adaptor protein family, TIR-1, which is a homolog of mammalian SARM, notably the only member of the TIR domain adaptor family that does not positively regulate Toll signaling. TIR-1 is required for immunity to *P. aeruginosa* [18] as well as *D. coniospora* [10], and functions upstream of a conserved PMK-1 p38 MAPK pathway that was identified from a forward genetic screen for mutants with enhanced susceptibility to *P. aeruginosa* [18,19]. Subsequent studies have shown that TIR-1-NSY-1-SEK-1-PMK-1 MAPK pathway, orthologous to the mammalian SARM-ASK1-MKK3-p38 MAPK pathway, is required for defense against intestinal pathogens [18, 19]. On the other hand, a second MAPK pathway referred to as the ERK pathway has been found to be required for the response to *M. nematophilum* infection in which bacterial cells adhere to the cuticle near the anus and cause swelling [12].

Additional conserved signaling pathways are implicated in pathogen resistance in *C. elegans*. The insulin-signaling pathway plays a pivotal role in development, metabolism, stress resistance and aging in *C. elegans* [20]. Reduction-of-function mutations in *daf-2*, encoding an insulin receptor homolog, confer dramatic resistance to killing by several types of pathogens in a manner dependent on the downstream Forkhead family transcription factor DAF-16 [21], suggestive that the transcriptional targets of DAF-16 promote pathogen resistance and longevity in *C. elegans*. Another conserved signaling pathway that plays important roles in development and metabolism, the TGF- β pathway, has also been implicated in the regulation of immunity in *C. elegans* [4].

Pathogen-specific transcriptional signatures induced by infection in *C. elegans*

Recent studies from multiple groups have focused on the transcriptional response to pathogen infection (Table 1). In an initial study of genes upregulated in response to pathogen in *C. elegans*, Mallo *et al.* identified 6 genes that were induced by *S. marcescens*. Several of these genes represent members of gene classes subsequently found to be expressed upon exposure to pathogen. For example, two genes that encoded C-type lectin domain (CTLCD) containing proteins and one gene that encoded a protein with a CUB-like domain (formerly DUF141 domain) were identified. C-type lectin domain containing proteins (CTLCDs) contain a putative carbohydrate recognition domain which in mammals has been shown to bind sugar moieties on the surface of pathogens and stimulate phagocytosis [22], as well as to function in anti-microbial killing [23,24]. A second class of genes induced by *S. marcescens* and other pathogens encode proteins containing CUB-like domains. First identified in human complement proteins C1r/C1s, CUB domains form a β -barrel similar to those found in immunoglobulins [25,26]. CUB-domain containing proteins function in a diverse number of roles in protein-protein interactions, including physiological roles such as inflammation and tissue repair [27].

Recently a number of microarray-based transcriptional profiling studies aimed at utilizing a genome-wide approach to identifying changes in gene expression upon infection in *C. elegans* have been conducted in which gene expression is compared in the presence and absence of pathogen. In two such studies, Shapira *et al.* [28] utilized a cDNA-based microarray that included approximately 8000 genes, and Troemel *et al.* [29] utilized the Affymetrix-based full-

genome microarray to compare the transcriptional response of young adult *C. elegans* to infection by *P. aeruginosa* PA14 and the nonpathogenic *E. coli* OP50, the standard laboratory food source for *C. elegans*. Since *C. elegans* begin to die within approximately 50 hours after exposure to *P. aeruginosa* strain PA14, to focus on the initial response to infection both Shapiro *et al.* and Troemel *et al.* analyzed the transcriptional response at time points early in the infection process (4–24 hours post infection, Table 1). Troemel *et al.* also analyzed the response to the *P. aeruginosa* PA14 *gacA* mutant, which is markedly attenuated in virulence. Shapira *et al.* identified 232 genes for which expression changed during the course of infection (197 up-regulated and 35 down-regulated in response to infection). Troemel *et al.* identified 418 genes that showed altered expression in response at 4 hours after bacterial exposure (304 up-regulated and 114 down-regulated). The transcriptional response to *P. aeruginosa* included genes encoding several members of specific protein families, such as the previously identified CTLD proteins, CUB-like domain proteins, as well as lysozymes, ShK-like toxin proteins, saponins and proteins that contain a DUF274 domain. Most of these genes are predicted to be secreted, and a number, such as lysozymes and CTLDs, have an identity that is consistent with a role in antimicrobial defense [23,24,30]. Specifically, lysozymes are enzymes that cleave the sugar backbone present the cell wall of bacteria and have been shown to have antimicrobial activity [31]. ShK-like toxins are proteins that share similarity to a toxin produced by sea anemones that block potassium channels yet lack a crucial lysine residue necessary responsible for this function [32,33]. Thus their precise role in the response to infection remains unclear. Saponins are secondary metabolites found predominantly in plants that form a glycoside steroid that contains antimicrobial activity and has been shown to disrupt plasma membranes [34]. DUF274 encodes a domain found in *Caenorhabditis* proteins whose function is currently unknown.

Both of the studies described above used RNAi to functionally validate genes shown by microarray analysis to be induced by infection. While Shapira *et al.* demonstrated that reducing the activity of some induced genes by RNAi caused enhanced susceptibility to infection, in similar experiments Troemel *et al.* did not observe any effects of RNAi on single induced genes on pathogen susceptibility. The latter result may be explained by functional redundancy, particularly given the large number of genes for some families of induced genes.

Another recent microarray experiment examined *C. elegans* genes induced by the bacterial pathogen *M. nematophilum* [35]. Through Affymetrix full-genome microarray analysis, O'Rourke *et al.* found altered expression of 68 genes in response to a 6 h infection of L2/L3 larval stage worms. These 68 induced genes coded for protein classes that included CTLD and CUB-domain proteins, DUF274 proteins, and lysozymes. Functional analysis of genes induced by *M. nematophilum* infection was carried out by RNAi and established the involvement of 11 of the genes in the response to infection. While the classes of genes induced by *M. nematophilum* were similar to those induced by *P. aeruginosa*, the specific genes induced did not show much overlap. Interestingly, O'Rourke *et al.* noted a strong chromosomal clustering of genes involved in the response to *M. nematophilum*. Of particular interest in this regard, Thomas has suggested that genes in homologous clusters that he identified as undergoing concerted evolution may represent antimicrobial effectors [36].

An additional microarray study by Wong *et al.* compared the transcriptional response to a several different pathogens, including both a Gram-positive bacterium (*Enterococcus faecalis*) and Gram-negative bacteria (*Serratia marcescens*, *Erwinia carotovora* and *Photobacterium luminescens*) that cause intestinal infections [37]. From the transcriptional analysis, Wong *et al.* observed the found that approximately 2000 genes were up-regulated in response to at least one pathogen tested; again, several gene classes are over represented in the genes that are induced, including antimicrobial factors such as CTLDs. However, when the specific genes are compared only 254 genes are upregulated in response to more than one

pathogen. Wong *et al.* suggest that *C. elegans* is capable of inducing a specific transcriptional profile in response to each pathogen—that is, the transcriptional response to infection has a pathogen-specific component, even as common classes of genes are induced by infection.

The identification of pathogen-induced genes and the annotations of some of the gene classes represented, such as CTLDs, which are suggestive of antimicrobial function, provide an initial overview of the candidate immune effector mechanisms in *C. elegans* immunity. While some of the candidate immune effectors have also been characterized for their role in pathogen susceptibility *in vivo* using RNAi, detailed understanding of the function of these effectors, particularly the CTLDs, await further biochemical analysis. To date only a subset of antimicrobial peptides encoded by the *C. elegans* genome has been characterized for *in vitro* microbicidal activity [10,38].

Regulation of basal/constitutive and pathogen-induced immune responses in *C. elegans*

The genome-scale identification of genes induced by infection of wild-type *C. elegans* can be compared with accumulating data on the genes regulated by signaling pathways that have been shown to play a role in *C. elegans* immunity. Alper *et al.* analyzed the expression patterns and regulation of putative antimicrobial proteins in *C. elegans*, observing that all three of the established immune signaling pathways (PMK-1 p38 MAPK, TGF- β , and insulin signaling) regulate distinct sets of antimicrobial genes that are partially overlapping [39]. From an analysis of regulatory elements in pathogen-induced genes, Shapira *et al.* identified the GATA family transcription factor, ELT-2, which is involved in the expression of all intestinally-expressed genes, as being required for the pathogen-induced intestinal immune response [28]. Troemel *et al.* observed on a full-genome scale that approximately 25% of genes identified as being regulated by the PMK-1 p38 MAPK pathway also showed pathogen induction as assessed by microarray analysis [29]. These latter data are suggestive of an important role for the PMK-1 MAPK pathway in a pathogen-induced response to *P. aeruginosa*.

Two lines of evidence suggest that the role of PMK-1 in host defense is not limited to its regulation of pathogen-induced genes. First, not all pathogen-induced genes are regulated by PMK-1. Indeed, Troemel *et al.* found that the majority of pathogen-induced genes are not regulated by PMK-1, suggestive that PMK-1-independent pathways may contribute to the induced immune response in *C. elegans* [29]. Second, the expression levels of a number of genes regulated by PMK-1 do not change with pathogen infection, suggestive that PMK-1 may regulate other genes without a role in pathogen defense or genes that are involved in a basal/constitutive pathogen response. A potential caveat to this conclusion is that although the control or background for microarray studies has been the relatively non-pathogenic strain of *E. coli* OP50, it is conceivable that *E. coli* could nevertheless induce the expression of immune-related genes that might result in such genes not appearing to be induced by microbial infection. *C. elegans* fed heat-killed *E. coli* live longer than animals cultured on live bacteria, suggesting that OP50 has the potential to become an opportunistic pathogen [40,41]. Consistent with this possibility, *E. coli* becomes packed within the intestine of older worms where it may cause a lethal infection [41].

Role of DAF-16 in basal host defense

The overlap between PMK-1-regulated genes and pathogen-induced genes corroborates the genetic studies of the role of the PMK-1 pathway in *C. elegans* immunity. In contrast, recent extensive characterization of downstream targets of the insulin signaling pathway suggests that DAF-16 regulates genes independently of the presence of pathogen. Studies comparing gene expression levels in wild type and *daf-16* mutants have shown that DAF-16 acts as a

transcriptional regulator, binding to specific cis-regulatory elements that either stimulate or repress gene expression [42–44]. Among the diverse classes of genes regulated by DAF-16 are putative pathogen recognition molecules and immune effectors. However, minimal overlap is observed between genes positively regulated by DAF-16 and those identified as being induced by infection. Indeed, Troemel *et al.* found that many of the genes that are positively regulated by the PMK-1 p38 MAPK pathway are in fact negatively regulated by DAF-16 [29], including CTLDs and CUB-like proteins. While there is the possibility that DAF-16 may regulate some genes only under conditions of infection, that the putative antimicrobial genes regulated by DAF-16 fail to be induced by pathogen is suggestive of a role for DAF-16 in the regulation of a basal, or constitutively expressed response to microbes.

DAF-16 positively regulates the expression of genes encoding two classes of proteins that are important for host defense: antimicrobial immune effectors and stress resistance proteins. Many of the putative immune effectors regulated by DAF-16, including the lysozymes LYS-7 and LYS-8, the saposins SPP-1, SPP-9 and SPP-12, and the thaumatins (similar to the PR-5 family of plant pathogenesis related [PR] proteins) F28D1.3, F28D1.5, and F49A5.6 disrupt microbial cell membranes. Another gene, T20G5.7, is somewhat similar to an antimicrobial peptide found in plants, and thus may eliminate pathogenic fungi [12]. RNAi treatment against T20G5.7, as well as *lys-7* and *spp-1*, partially suppress the enhanced longevity phenotype of *daf-2* mutants [42]. Considering evidence supporting a tissue-specific requirement for DAF-16 in the intestine (a frequent site of infection) to extend the lifespan of *daf-2* worms [45], these observations suggest that antimicrobial activity of these immune effector targets of DAF-16 may contribute to longevity of the worm.

Conclusions and Future Directions

Recent studies discussed in this review have started to shed light on the downstream effector mechanisms involved in the *C. elegans* immune response. Because of redundancy among the classes of candidate effector genes expressed, the identification of these genes are more amenable to microarray-based approaches instead of traditional forward genetic approaches, which have been better suited to identifying the core signaling pathways required for immune defense. The studies to date are suggestive of a basal or constitutively expressed component to immunity that is partially regulated by DAF-16, as well as a pathogen-induced component that has both PMK-1 p38 MAPK-dependent and independent components. Furthermore, transcriptional responses to diverse pathogens are suggestive of pathogen specificity in the *C. elegans* immune response (summarized in Figure 1).

While revealing many types of immune effectors, whole genome transcriptional profiling studies of the response of *C. elegans* to infection have failed to uncover homologues of pattern recognition receptors shown to play a pivotal role in detecting microbial pathogens in other systems, including *D. melanogaster* and mammals. This raises the possibility of a supplementary and perhaps more ancient mechanism of pathogen recognition in the worm. Perhaps instead of directly detecting microbial pathogens, *C. elegans* may sense endogenous cues triggered by the stress and tissue damage associated with infection. Supporting this possibility, using complete *C. elegans* transcriptome microarrays to identify genes induced by the cytolytic pore-forming toxin Cry5B of *B. thuringiensis*, Huffman *et al.* discovered a role for MAPK signaling in defense against such toxins [46]. Accordingly, Troemel *et al.* found substantial overlap between genes that are induced by Cry5B and genes upregulated in response to *P. aeruginosa* [29], suggesting a response to intestinal damage resulting from infection. Along with the PMK-1 pathway, insulin signaling has also been shown to play a protective role against many types of stress, including heavy metals [47], heat, hypoxia, and UV [48]. Indicative of likely overlap in the mechanisms by which DAF-16 mediates survival on pathogens and responses to stress, Chavez *et al.* demonstrated that genes required for oxidative

stress resistance, including *sod-3* (encoding superoxide dismutase), along with *ctl-1* and *ctl-2* (each encoding catalase enzymes), are required for the enhanced resistance to pathogen of *daf-2* mutants [49]. Thus the well-established role of DAF-16 in responses to abiotic stresses may also contribute in part to its role in resistance to pathogen-induced killing. The nature of endogenous stress signals and their potential role in eliciting the response to infection in *C. elegans*, however, await further studies.

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Figure 1. Regulation of pathogen defense in *C. elegans*

Recent studies are suggestive of both pathogen-induced and basal or constitutively-expressed components of the *C. elegans* pathogen defense. Microarray studies are also suggestive of distinct responses to different pathogens. The PMK-1 p38 MAPK pathway regulates both pathogen-induced and constitutively expressed genes, and pathogen-induced genes are also regulated by PMK-1-independent mechanisms. The set of genes regulated by the DAF-16 transcription factor appears to overlap minimally with pathogen-induced genes, suggestive of a role for DAF-16 in the regulation of basal or constitutively-expressed antimicrobial genes.

Table 1
Microarray studies of the transcriptional response to pathogens in *C. elegans*

Authors	Pathogen Exposed	Time of Exposure	Microarray	# of genes induced	# of genes repressed	Validation of candidates	Classes of genes induced
Mallo <i>et al.</i>	<i>S. marcescens</i>	24 and 48 hours	Nylon membranes spotted with cDNAs	7	ND	Northern blots	Lectins (3) CUB-domain (1) Hydrolase (1) Lysozyme
Shapira <i>et al.</i>	<i>P. aeruginosa</i>	4, 12 and 24 hours	PCR spotted arrays	197	35	qRT-PCR, 49 of 52 targets confirmed RNAi, 21 tested had Esp phenotype	CUB-domain(17) Lysozymes (2) Lectins (11) DUF274 (9) GST (11) Histones (7)
Troemel <i>et al.</i>	<i>P. aeruginosa</i>	4 and 8 hours	Affymetrix GeneChips for <i>C. elegans</i>	304(4 hours) 261(8 hours)	114(4hours) 216(8hours)	qRT-PCR, 7 of 7 targets confirmed	CUB-domain(38) Lectins (17) ShK-like Toxin(8) DUF274(9) GST (10)
O'Rourke <i>et al.</i>	<i>M. nematophilum</i>	6 hours	Affymetrix GeneChips for <i>C. elegans</i>	68		RNAi, 11 targets tested affect response to pathogen	Lectins (11) CUB-domain(8) DUF274 (2) ShK-like Toxin(1) Lysozyme (3)
Wong <i>et al.</i>	<i>S. marcescens</i> <i>E. faecalis</i> <i>E. carotovora</i> <i>P. luminescens</i>	24 hours	UltraGAPS oligo spotted arrays	2,171 (one pathogen) 254 (multiple pathogens)	2,025 (one pathogen) 266 (multiple pathogen)	qRT-PCR, 6 common response genes verified	Lysozymes Lectins GST