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Inducible Antibacterial Defense System in *C. elegans*

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Summary

The term innate immunity refers to a number of evolutionary ancient mechanisms that serve to defend animals and plants against infection. Genetically tractable model organisms, especially *Drosophila*, have contributed greatly to advances in our understanding of mammalian innate immunity [1, 2]. Essentially, nothing is known about immune responses in the nematode *Caenorhabditis elegans* [3, 4]. Using high-density cDNA microarrays, we show here that infection of *C. elegans* by the Gram-negative bacterium *Serratia marcescens* provokes a marked upregulation of the expression of many genes. Among the most robustly induced are genes encoding lectins and lysozymes, known to be involved in immune responses in other organisms. Certain infection-inducible genes are under the control of the DBL-1/TGFβ pathway [5]. We found that *dbl-1* mutants exhibit increased susceptibility to infection. Conversely, overexpression of the lysozyme gene *lys-1* augments the resistance of *C. elegans* to *S. marcescens*. These results constitute the first demonstration of inducible antibacterial defenses in *C. elegans* and open new avenues for the investigation of evolutionary conserved mechanisms of innate immunity.

Results and Discussion

Animals and plants can respond to infection by the production of compounds that directly inhibit or kill invading pathogens. Examples include the induction of plant pathogenesis-related genes [6] and the expression of antimicrobial peptides by *Drosophila* [1, 2]. While both amoebapore- and defensin-like antimicrobial peptides have been described in *C. elegans* [7, 8], up until now, there have been no clear indications as to whether or not *C. elegans* responds to infection with the induction of defense genes. We sought to identify genes in *C. elegans* that were upregulated upon infection by *Serratia marcescens*, a Gram-negative bacterial pathogen with a broad host range [9]. When worms are fed on their standard laboratory diet, the *Escherichia coli* strain OP50, intact bacteria are not found within the intestinal lumen until 48 hr after the last larval stage (L4), and they do not provoke extensive tissue damage until between 4 and 6 days later [10], coincident with the first deaths. When worms at the L4 stage were transferred to *S. marcescens* strain Db11, after less than 6 hr, intact bacteria were found within the intestinal lumen where they proliferated rapidly. Apart from a progressive distension of the intestinal lumen, outwardly, worms showed relatively little sign of the infection for the first 24 hr, and their rate of egg laying, which reflects a worm's general state of health, was normal. There was then a progressive destruction of the worm's intestinal epithelium and of the germline, accompanied by a clear drop in the rate of egg laying after 48 hr (see the Supplementary Material available with this article online). Worms started to die after 72 hr of contact with Db11 [11]. We therefore decided to focus on gene expression patterns at 24 hr and 48 hr after transfer to Db11. The eggs that are laid by wild-type worms on Db11 hatch, and the early larval stages are resistant to infection (C.L.K., unpublished data). As a consequence, after 24 hr and 48 hr of infection, two generations of worms of mixed ages will be present on a plate. To avoid this, we decided to use the conditional sterile mutant *fer-15*, for which the time course of infection is essentially identical to that of wild-type worms (C.L.K., unpublished data).

We used nylon filters on which ~7,500 different cDNAs were arrayed [5] to compare the level of expression of the corresponding genes in synchronized populations of *fer-15* worms grown on OP50 with that seen in worms infected with Db11. For 5,845 of the genes represented on the filters, we obtained an analyzable signal under one or more of the test conditions from two independent experiments. The majority of these genes did not show a large variation in their expression after 24 hr or 48 hr of exposure to *S. marcescens* (Figures 1A and 1B; see the Supplementary Material). Seven genes showed a greater than 2-fold induction at both time points in both experiments (Table 1). The expression of five of these genes following infection was monitored by Northern analysis (Table 1, Figure 1D), and, in all cases, the induction of expression was confirmed. The correlation between the measured amplitude of the effect using the two methods was similar to that previously reported [5]. The differences seen between the two experiments by microarray reflects the intrinsic precision of the technique [5] as well as a limited quantitative variability in the response seen in independent infections (G.V.M. and

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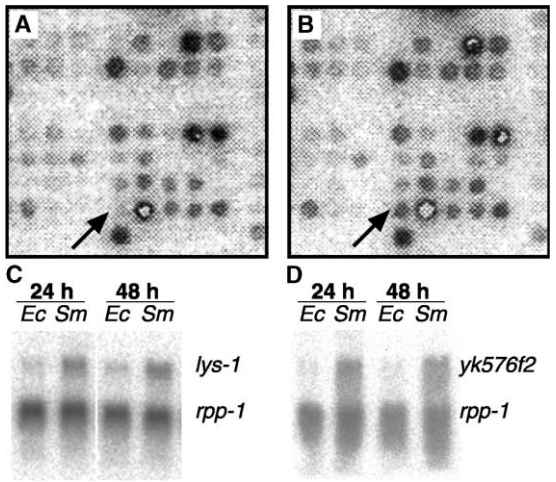

Figure 1. Specific Gene Induction Following *S. marcescens* Infection of *C. elegans*

(A and B) Sections of high-density filters hybridized with ^{33}P-labeled probes made from total RNA extracted from worms cultivated on (A) *E. coli* OP50 or in contact with (B) *S. marcescens* Db11 for 24 hr. The arrows highlight one clone, yk557g2, corresponding to the gene *lys-1*, which shows a clear induction following infection.

(C and D) Confirmation of gene induction by Northern blot. Northern blots made with total RNA extracted from worms cultivated on *E. coli* (*Ec*) or in contact with *S. marcescens* Db11 (*Sm*) for 24 hr or 48 hr were hybridized under stringent conditions with a ^{32}P-labeled probe corresponding to the ribosomal protein gene *rpp-1* (Y37E3.7; transcript size 0.4 kb), as a loading control, together with a probe corresponding to (C) *lys-1* or (D) yk576f2.

S.G., unpublished data; see the Supplementary Material).

Among the genes that are robustly induced, F55G11.4, R09B5.3, and W05E7.1 appear to be nematode specific. W05E7.1 is a member of the groundhog family and corresponds to the gene *grd-3*, but its function is as yet uncharacterized [12]. There is currently no known function either for F55G11.4 or R09B5.3. Only the gene ZK6.7 encodes a protein that has clear homology to proteins in other species, being similar overall to vertebrate gastric lipases. It has recently been shown that four lipase genes are induced following immune challenge in *Drosophila* [13]. Thus, lipases are likely to contribute to innate immune defenses, conceivably acting directly against invading microorganisms. The remaining three genes encode lectin domain-containing proteins but do not have clear homologs in other species. In both vertebrates [14] and invertebrates [15], certain lectins are known to play key roles in innate immunity. For example, in mammals, mannan binding lectin mediates the phagocytosis of pathogens [16], while, in the cockroach, endogenous serum lectins have been shown to act as nonself recognition molecules [17]. In *C. elegans*, the lectins represent a very large class of proteins, with at least 125 C-type lectins [18]. Although the sugar binding properties of certain nematode lectins have been studied in great detail (see [19], for example), nothing is known about their in vivo function. Our results suggest that a subset of nematode lectins might play a role in host defense. It will be of interest in the future to explore further the functions of the different genes, as well as of those induced only at 24 hr or 48 hr (see the Supplementary Material), to determine whether, for example, they are involved in the regulation of the observed response or whether they possess antimicrobial activity.

While not falling within the strict selection criteria applied to identify the seven genes shown in Table 1, the transcription of the nematode lysozyme gene *lys-1* was clearly induced following infection (Figures 1A–1C, Table 2). Lysozymes acting alone, or synergistically with bactericidins, have long been recognized as playing an important role in innate defense reactions. Unlike verte-

Table 1. Identity of Genes Induced upon *S. marcescens* Infection

				Induction[e]			
				Microarray		Northern	
cDNA[a]	Expresson[b]	Gene[c]	Domain[d]	24 hr	48 hr	24 hr	48 hr
yk66e9	seam cells	W05E7.1	None	6.9, 4.7	2.1, 2.4	ND	ND
yk263b10	intestine	ZK6.7	hydrolase (PF00561)	2.1, 3.0	5.1, 2.8	3.4	2.6
yk308a12	NE	R09B5.3	None	>10, >10	8.4, 3.7	ND	ND
yk377g8	intestine	R07B1.10	galactoside binding lectin (PF00337)	4.6, >10	3.6, >10	5.3	5.2
yk385e2	intestine	F55G11.4	DUF141 (PF02408)	>10, >10	>10, >10	4.8	>10
yk576f2	intestine	Y54G2A.6	C-type lectin (PF00059)	8.6, >10	7.5, 4.6	>10	>10
yk578h7	intestine	W04E12.8	C-type lectin (PF00059)	5.0, 2.9	3.5, 2.4	2.0	4.3

ND, not determined.

NE, not expressed at a sufficiently high level to give an analyzable result.

[a] Sequences and further information can be found at http://www.ddbj.nig.ac.jp/htmls/c-elegans/html/CE_INDEX.html. Precise selection criteria can be found in the Supplementary Material.

[b] The pattern for yk576f2 has been reported previously [5]. Expression was also seen at the anus and in the intestinal valve cells with yk377g8 and yk385e2, respectively.

[c] Wormpep57; http://www.sanger.ac.uk/Projects/C_elegans/wormpep/. R07B1.10 corresponds to the gene *lec-8*, and W05E7.1 responds to *grd-3*.

[d] Pfam 6.5; http://www.sanger.ac.uk/Software/Pfam/; Pfam accession numbers are given in parentheses. PF02408, DUF141 (domain of unknown function 141) is currently a worm-specific domain. W05E7.1 is said to contain Hog/Hint (PF01079) and Ground domains [12] but is thus not annotated in the Pfam database.

[e] The level of induction seen in two independent infections is shown together with the maximum obtained by Northern analysis from a number of independent infections.

Table 2. *C. elegans* Lysozyme Genes and Their Induction upon *S. marcescens* Infection

				Induction[d]			
				Microarray		Northern	
cDNA[a]	Expression[b]	Gene[c]	Gene Name	24 hr	48 hr	24 hr	48 hr
yk557g2	intestine	Y22F5A.4	*lys-1*	3.9, 1.3	3.9, 2,3	8.4	6.1
No cDNA		Y22F5A.5	*lys-2*				
yk550f6	NE	Y22F5A.6	*lys-3*	NE, NE	NE, NE	ND	ND
yk444e4	ND	F58B3.1	*lys-4*	NE, NE	1.0, NE	ND	ND
no cDNA		F58B3.2	*lys-5*				
yk94d3	NE	F58B3.3	*lys-6*	NE, NE	NE, NE	NE	NE
yk361g12	intestine	C02A12.4	*lys-7*	1.9, 6.6	6.1, 8.1	1.4	7.4
yk411h8	intestine	C17G10.5	*lys-8*	2.3, 2.8	1.9, 4.0	1.9	1.9
No cDNA		C54C8.6	*lys-9*				
No cDNA		F17E9.11	*lys-10*				

ND, not determined.
NE, not expressed at a sufficiently high level to give an analyzable result.
[a] Sequences and further information can be found at http://www.ddbj.nig.ac.jp/htmls/c-elegans/html/CE_INDEX.html.
[b] The pattern for yk411h8 was reported previously [5]. Expression was also seen at the terminal bulb cells and in the intestinal valve cells with yk411h8 and yk361g12, respectively.
[c] Wormpep57; http://www.sanger.ac.uk/Projects/C_elegans/wormpep/.
[d] The level of induction seen in two independent infections is shown together with the maximum obtained by Northern analysis from a number of independent infections.

brates and other invertebrates, including *Drosophila* [20], *C. elegans* does not appear to possess chicken-type lysozymes, nor homologs of the other well-characterized lysozyme protein families, but rather has a family of lysozymes homologous to those of the amoeboid protozoon *Entamoeba histolytica* [21]. These have been suggested to act in synergy with *C. elegans*' amoebapore-like peptides to eliminate Gram-negative bacteria [21]. Six of the ten *C. elegans* lysozyme genes are represented on the filters. In addition to *lys-1*, two others, *lys-7* and *lys-8*, showed a significant induction following infection that was confirmed by Northern analysis (Table 2).

All genes represented on our filter are expected to be expressed constitutively, since the cDNA libraries used in its production were obtained from worms grown on OP50. To determine the site of constitutive expression of these different genes, we performed in situ hybridizations on uninfected worms using the corresponding cDNAs as probes. For *lys-1*, *lys-7*, and *lys-8*, expression was mainly detected in the intestinal cells, as was the case for almost all of the other genes (Tables 1 and 2; see the Supplementary Material). This is consistent with the fact that, during the infection, *S. marcescens* remains within the intestinal lumen (C.L.K., unpublished data). The relative level of constitutive expression along the length of the intestine was different for the different genes (see the Supplementary Material). In the case of *lys-8*, the expression domain has been reported to extend to the terminal bulb of the pharynx [5]. For *grd-3*, the expression in the seam cells was consistent with the pattern previously seen with a *lacZ* reporter construct [12].

Given its clear homology with amoeba lysozyme [21], with known antibacterial function, we decided to investigate in greater detail the expression and function of *lys-1*. In uninfected transgenic worms, the *lys-1* promoter drove expression of a *lys-1*::GFP fusion construct in the intestinal cells (Figures 2A and 2B) and in the six IL1 and six IL2 neurons as well as in a few unidentified neurons in the head ganglia (Figure 2C). The significance of the neuronal expression, assuming that it reflects accurately the expression of the endogenous gene, is currently unclear.

On a cellular level, the LYS-1::GFP fusion protein was found to have a vesicular localization (Figures 2C and 2D). In the intestinal cells, these vesicles are distinct from the auto-fluorescent secondary lysosomes and appear to be concentrated toward the apical surface (Figures 2E and 2F). It is tempting to speculate that this reflects trafficking of the protein toward the lumen, perhaps in a fashion analogous to granular exocytosis in *E. histolytica* [21] or to that seen for secretory lysosomes of cytotoxic T-lymphocytes [22]. In the future, it will be interesting to establish whether these LYS-1::GFP-containing vesicles correspond to the vesicles that are secreted into the intestinal lumen through holes in the terminal web observed by electron microscopy [23].

To establish whether *lys-1* overexpression increases the resistance of worms to *S. marcescens*, we followed the time course of infection of three independent lines of transgenic worms carrying the *lys-1*::GFP fusion construct (IG32, IG33, IG36). The longevity of these worms on *E. coli* and their survival in the presence of Db11 was not significantly different from that of control worms (IG66). The worms survived significantly longer than the controls, however, when placed in the presence of strain Db1140 [24], which, while being less virulent than Db11, is still pathogenic for worms [11] (Figure 3A; see the Supplementary Material). That the effect was specifically due to the expression of *lys-1*::GFP was confirmed by RNAi (see below and the Supplementary Material). Preliminary results indicate that abrogation of *lys-1* function in wild-type worms by RNAi treatment has little effect on survival in the presence of Db1140. Taken together, these results suggest that many factors help protect worms from infection and that altering the ex-

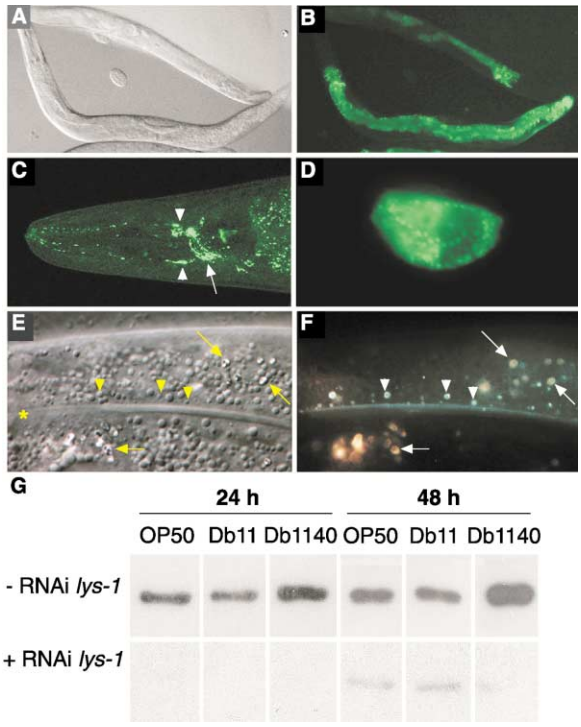

Figure 2. Expression of a *lys-1*::GFP Reporter Construct

(A–F) Adult transgenic worms carrying a *lys-1*::GFP reporter construct (strain IG36) were observed by (A and E) Nomarski or (B–D and F) fluorescence microscopy. (A and B) Worms showing expression of *lys-1*::GFP throughout the intestine. (C) A confocal image of the head of a worm showing *lys-1*::GFP in the IL1 and IL2 neurons (arrowheads) and the nerve ring (arrow). (D) Concentration of *lys-1*::GFP in vesicles in a single posterior intestinal cell. (E and F) A section of the intestine; the intestinal lumen is marked by an asterisk in (E). (F) *lys-1*::GFP-containing vesicles, shown in blue, are concentrated on the apical side of an intestinal cell. They are distinct from the auto-fluorescent vesicles shown in orange (F). Certain vesicles of the two classes are indicated with arrowheads and arrows. (G) LYS-1::GFP levels determined by Western blotting with an anti-GFP antibody in IG36 worms cultivated on *E. coli* OP50 or in contact with *S. marcescens* (Db11 or Db1140) for 24 hr or 48 hr, (upper panels) or in IG36 worms pretreated with *lys-1* RNAi (lower panels). The samples are all exactly equivalent in terms of the amount of total worm extract loaded, but, in the lower panels, the signal has been amplified more than 5-fold relative to the upper panels to reveal the faint bands.

pression of one such effector does not have dramatic consequences on survival.

No obvious change in the domain of expression or in the subcellular localization of the GFP fusion protein was observed by fluorescence microscopy during the course of infection with Db11, or with Db1140. Since the level of quantitative resolution attainable was relatively limited, we also assayed the levels of the fusion protein in transgenic animals by Western blotting using an anti-GFP antibody. Despite the augmentation of expression of *lys-1* at the transcriptional level seen upon infection with Db11, the level of LYS-1::GFP was not observed to increase after 24 hr and 48 hr of infection. Db11 is known to secrete extracellular proteases that degrade bactericidal proteins in insects. Db1140, on the other hand, is a protease-deficient derivative of Db11 [24]. While infection with Db1140 provoked only a slight increase in the level of *lys-1* mRNA, as judged by Northern analysis (unpublished data), we observed a clear and reproducible augmentation in the level of LYS-1::GFP (Figure 2G). We further demonstrated that the constitutive expression of LYS-1::GFP, and its induction, could be efficiently abolished by RNAi against *lys-1*, at least up until 48 hr postinfection (Figure 2G). Taken together, our findings would tend to support the hypothesis that LYS-1 is secreted into the intestinal lumen, that there it is capable of partially protecting worms from Db1140, but that, in the case of infection by Db11, this effect is countered by the secretion of bacterial proteases.

A previous study, using the same high-density cDNA filters that were used here, sought to identify genes that are under the control of the transforming growth factor-β (TGFβ)-related gene *dbl-1*. The DBL-1 signal was found to positively regulate the expression of 22 genes, and the expression of these genes is strongly downregulated in *dbl-1* mutants [5]. Among the 22 genes, 2, namely, *lys-8* and F46F2.3, were also identified in the current screen. For two others, corresponding to yk557a8 and yk405b4, a reproducible induction of their expression at 48 hr was observed (see the Supplementary Material). We therefore tested the *dbl-1* mutant for its resistance to infection by *S. marcescens*.

The *dbl-1* mutants exhibited a dramatically reduced survival in the presence of Db11 and Db1140 (Figure 3B) relative to wild-type worms. They also showed a reduced longevity when grown on OP50 (Figure 3C), raising the possibility that this bacterium might have a detrimental effect on the mutants. It has already been shown that, under certain culture conditions, OP50 is pathogenic for *C. elegans* [25], and it has been proposed that, under the standard culture conditions used here, old *C. elegans* are killed by live *E. coli* by means of infection [26]. We therefore measured the survival of the worms in the presence of heat-killed OP50. While the survival of wild-type worms was somewhat increased under these conditions, that of *dbl-1* mutants was lengthened very significantly (Figure 3C), suggesting that the mutants are indeed more susceptible to infection by OP50 than wild-type worms. Consistent with this, *dbl-1* mutants were visibly sick when grown on live OP50, but not when they were grown on the heat-killed bacteria. Further, the survival of *dbl-1* mutants was very similar to that of wild-type worms when grown on OP50 plates containing 5-fluoro-2′deoxyuridine, which blocks DNA synthesis and prevents bacterial replication (see the Supplementary Material).

Thus, *dbl-1* mutants are more susceptible to infection than wild-type worms and are vulnerable to the otherwise relatively innocuous OP50. This would be consistent with a reduction in the expression of several genes required by *C. elegans* to counter infection. Remarkably, *C. elegans* mutants from the same TGFβ pathway were isolated in screens for worms hypersensitive to infection with *Pseudomonas aeruginosa* (M.-W. Tan, personal communication). Thus, it would appear likely that these two Gram-negative pathogens are capable of inducing overlapping if not identical defense responses. Establishing the exact specificity of the antibacterial response in wild-type worms remains a challenge for the future. While a *dbl-1* cDNA is not present on our filters, its

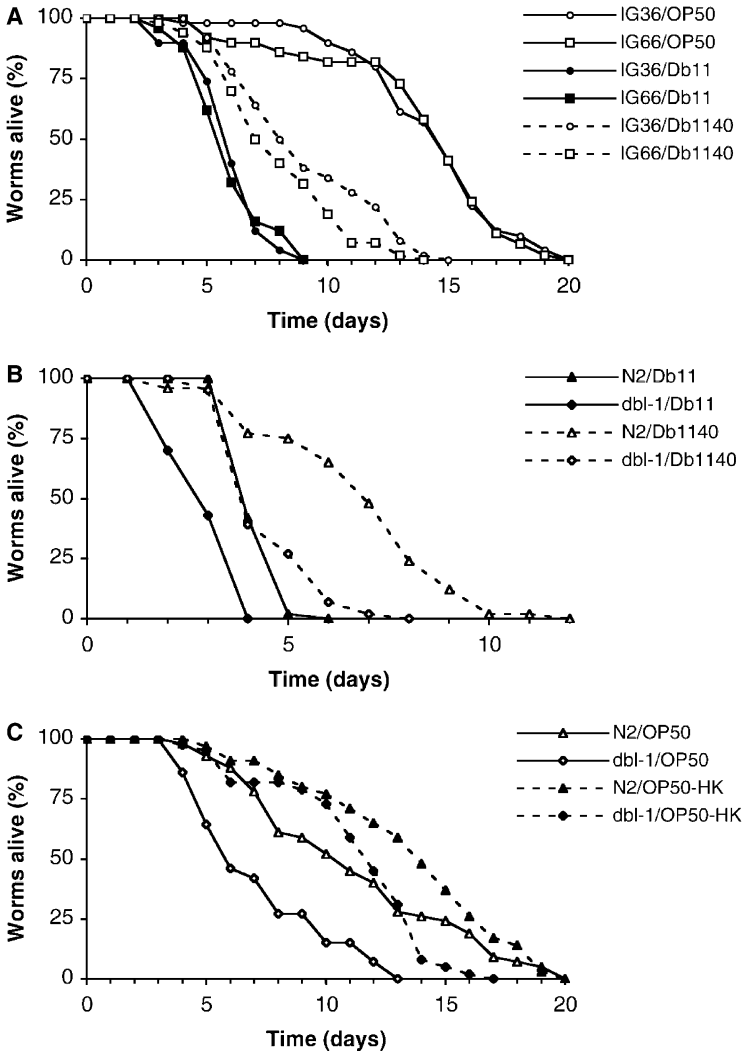

Figure 3. Modulation of the Resistance of *C. elegans* to Infection

(A) Transgenic worms expressing *lys-1*::GFP resist infection by *S. marcescens*. Representative time courses of the survival of control worms (IG66; squares) or worms expressing *lys-1*::GFP (IG36; circles) in the presence of *E. coli* OP50 (open symbols) and *S. marcescens* strains Db11 (closed symbols) and Db1140 (open symbols, dashed lines). The increase in the survival of IG36 relative to IG66 on Db1140 is very significant ($p = 0.009$, one-sided rank log test).

(B and C) *dbl-1* mutants are vulnerable to infection. Representative time courses of the survival of wild-type N2 worms (triangles) and *dbl-1(nk3)* mutants (diamonds) in the presence of (B) *S. marcescens* Db11 (closed symbols) and Db1140 (open symbols, dashed lines) and (C) *E. coli* OP50 (open symbols) or heat-killed OP50 (closed symbols, dashed lines). While the difference between the survival of wild-type worms on OP50 and on heat-killed OP50 is not extremely significant, that for *dbl-1* mutants is ($p = 0.05$ and <0.0001, respectively). All tests were repeated at least three times, starting with 50 worms under each condition per test.

D. melanogaster homolog, Decapentaplegic (dpp), has recently been shown to be upregulated following immune challenge with the Gram-positive bacterium *Micrococcus luteus* [27], suggesting a role for the TGFβ pathway in fly defense also.

Conclusions

In order to establish the existence of inducible defenses in *C. elegans*, we looked for genes that are robustly induced upon infection by the Gram-negative bacterium *Serratia marcescens*. Using high-density cDNA arrays, we have identified such genes, including a subset that is under the control of a TGFβ pathway. By gain-of-function and loss-of-function experiments, we obtained evidence that supports the hypothesis that these genes are indeed important for defense against infection. Thus, 30 years after the demonstration of an inducible immune response in *Drosophila* [28], we are able to conclude that *C. elegans* does have an inducible system of antibacterial defenses that rests at least in part on a TGFβ pathway. The further study of nematode defenses should contribute to a better understanding of vertebrate innate immunity.

Supplementary Material

Supplementary Material including the Experimental Procedures is available at http://images.cellpress.com/supmat/supmatin.htm. Additional information including the results of egg-laying tests, the raw data from the microarray experiments together with clone selection criteria, the results of in situ hybridizations, and additional curves is available at http://www.ciml.univ-mrs.fr/EWBANK_jonathan/SuppMat/Microarray/Mallo.html.

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