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A conserved Toll-like receptor is required for *Caenorhabditis elegans* innate immunity

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Pathogen recognition through Toll-like receptors (TLRs) is crucial in order to mount an appropriate immune response against microorganisms. On the basis of a lack of evidence indicating that *Caenorhabditis elegans* uses TLRs to elicit an immune response and on the absence of genes encoding Rel-like transcription factors in its genome, it is believed that TLR-mediated immunity arose after coelomates split from pseudocoelomates and acoelomates. Here, we show that *C. elegans tol-1*(*nr2033*) mutants are killed by the human pathogen *Salmonella enterica*, which causes a significant pharyngeal invasion in the absence of TOL-1-mediated immunity. We also show that TOL-1 is required for the correct expression of ABF-2, which is a defensin-like molecule expressed in the pharynx, and heat-shock protein 16.41, which is also expressed in the pharynx and is part of a HSP family of proteins required for *C. elegans* immunity. The results indicate that TOL-1 has a direct role in defence response to certain Gram-negative bacteria and indicate that part of the TLR-mediated immunity might be evolutionarily conserved.

Keywords: innate immunity; *C. elegans*; infection; TLR; Toll receptors

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INTRODUCTION

Although several conserved pathways and innate immune responses have been recently described in *Caenorhabditis elegans* (Kurz & Tan, 2004; Schulenburg *et al*, 2004; Gravato-Nobre & Hodgkin, 2005; Kim & Ausubel, 2005; Mylonakis & Aballay, 2005), it was believed that nematodes do not use Toll-like receptors (TLRs) for direct defence response (Pujol *et al*, 2001). Thus, a lack of evidence indicating that TOL-1 is required for immunity in *C. elegans* and the fact that *C. elegans* lacks NF-κB-like transcription factors have indicated that TLR-mediated immunity arose after coelomates split from pseudocoelomates and acoelomates (Kanzok *et al*, 2004).

Here, we show that TOL-1 is required to prevent *Salmonella enterica* invasion of the pharynx, which constitutes one of the first barriers against pathogens in *C. elegans*. We also show that TOL-1 is required for the correct expression of ABF-2, which is a defensin-like molecule expressed in the pharynx, and heat-shock protein 16.41 (HSP-16.41), which is also expressed in the pharynx and is part of a HSP family of proteins required for *C. elegans* immunity (Singh & Aballay, 2006a, b). Our results provide the first evidence, to our knowledge, that TOL-1 has a direct role in *C. elegans* defence against pathogens.

RESULTS AND DISCUSSION

TOL-1 is required for immunity to certain bacteria

The *C. elegans* genome appears to encode a single TLR, TOL-1, as well as single copies of TRF-1, PIK-1 and IKB-1, homologues of the mammalian downstream signal transduction components TNF receptor-associated factor 1 (TRAF1), interleukin 1 receptor associated kinase (IRAK) and inhibitor of κ B (IκB), respectively (Pujol *et al*, 2001). However, it was unclear whether TOL-1 has a direct role in defence to microbial infections, as it has only been associated with the avoidance of *Serratia marcescens* (Pujol *et al*, 2001; Pradel *et al*, 2007). Specifically, no difference was observed between wild type and the *tol-1*(*nr2033*) mutant in terms of (i) adhesion of *Drechmeria coniospora* spores to the animals and the course of infection, (ii) *Pseudomonas aeruginosa*-mediated killing and (iii) *Microbacterium nematophilum*-induced tail swelling (Pujol *et al*, 2001). Interestingly, *tol-1*(*nr2033*) mutants showed increased susceptibility to two *S. marcescens* strains. The increased susceptibility of *tol-1*(*nr2033*) mutants was, however, attributed to a reduced lifespan as *tol-1*(*nr2033*) mutants show a shortened lifespan when grown on live *Escherichia coli* (Pujol *et al*, 2001). The mutants *trf-1*(*nr2014*) and *ikb-1*(*nr2027*) also show a reduced lifespan in the presence of live *E. coli* (Pujol *et al*, 2001).

Given the fact that proliferating *E. coli* is a cause of death in *C. elegans* (Garigan *et al*, 2002), that *E. coli* grown on rich media kills *C. elegans* (Garsin *et al*, 2001) and that immunocompromised animals are killed and persistently colonized by *E. coli* (Kerry *et al*, 2006; Singh & Aballay, 2006b), the reduced lifespan of *tol-1*(*nr2033*) mutants grown on live *E. coli* is the expected phenotype of an

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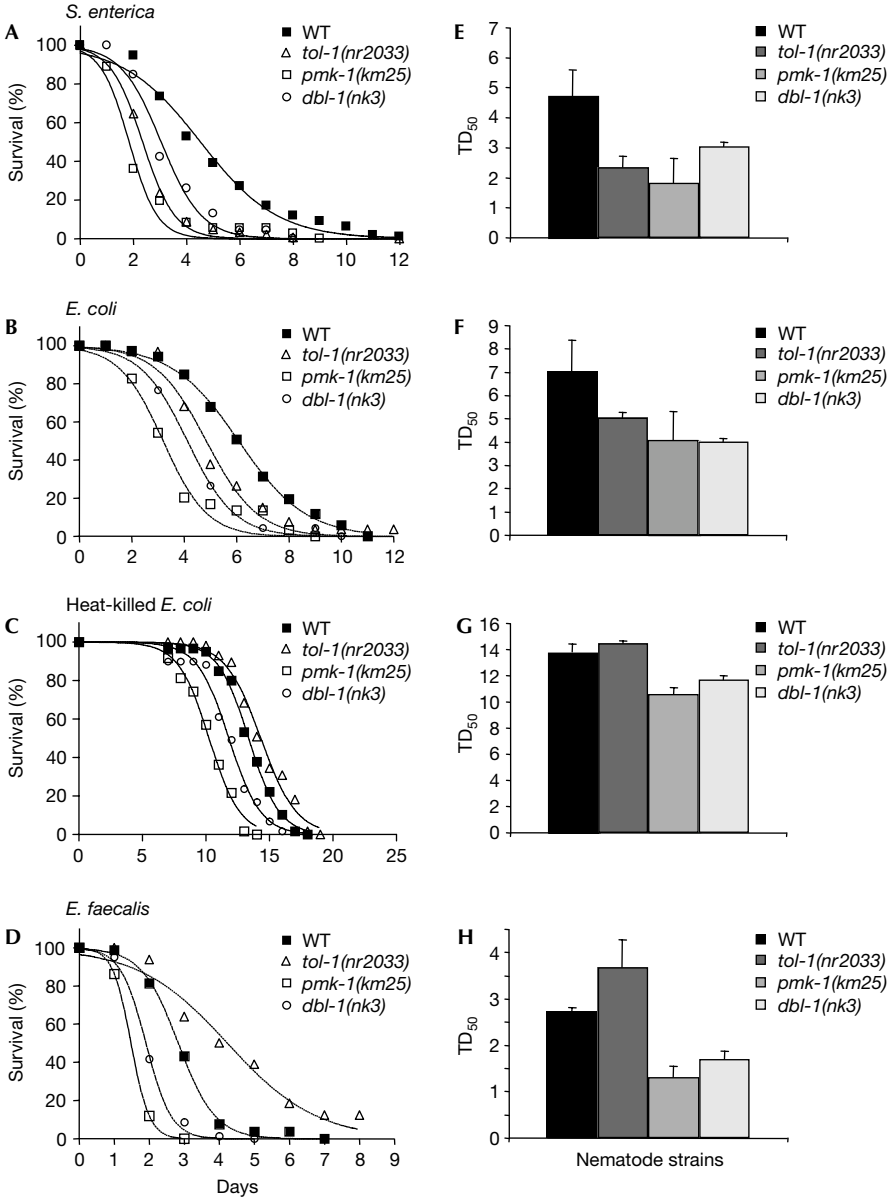

Fig 1 | TOL-1-mediated immunity is required for survival in the presence of live Gram-negative bacteria. (**A**) Wild-type N2 (WT), *tol-1*(*nr2033*) ($P<0.0001$), *pmk-1*(*km25*) ($P<0.0001$) and *dbl-1*(*nk3*) ($P<0.0001$) were exposed to *Salmonella enterica*. The graphs represent combined results of more than four independent experiments, each of which used 36–50 1-day-old adult hermaphrodites. (**B,C**) Wild-type N2, *tol-1*(*nr2033*), *pmk-1*(*km25*) and *dbl-1*(*nk3*) were exposed to live (*Caenorhabditis elegans* killing assay) or heat-killed *Escherichia coli* (lifespan assay). Although *tol-1*(*nr2033*) nematodes died faster than wild type on live *E. coli* ($P=0.0024$), their lifespan was similar to that of wild type ($P=0.3966$) when exposed to heat-killed *E. coli*. (**D**) *tol-1*(*nr2033*) lived longer on *E. faecalis* compared with wild-type ($P<0.0001$), whereas *pmk-1*(*km25*) ($P<0.0001$) and *dbl-1*(*nk3*) ($P<0.0001$) nematodes were highly susceptible. (**E–H**) The time for 50% of the nematodes to die (TD_{50}) was determined for each nematode strain exposed to different bacteria. The graphs are representative of at least three independent experiments using 36–50 1-day-old adult hermaphrodites, unless otherwise indicated; bars correspond to mean ± s.d.

organism showing a deficient immune response. To explore the possibility that TOL-1 is required for *C. elegans* immunity, we used *S. enterica*, which, like *S. marcescens*, is able to cause a persistent and lethal infection in nematodes (Aballay *et al*, 2000; Labrousse *et al*, 2000), using virulence factors that specifically target conserved signaling pathways (Tenor *et al*, 2004). As shown in Fig 1A,E, *tol-1*(*nr2033*) mutants died more quickly than wild-type nematodes when infected with *S. enterica*.

As TOL-1 is required for the avoidance of *S. marcescens* (Pujol *et al*, 2001), we studied whether the increased susceptibility to

S. enterica shown by *tol-1*(*nr2033*) mutants was due to the inability to avoid eating *S. enterica*. At 16 h after infection, wild-type and *tol-1*(*nr2033*) nematodes remained on the lawns of *S. enterica* (supplementary Fig S1 online), indicating that *S. enterica* does not elicit an avoidance behaviour and that TOL-1 is required for immunity to *S. enterica* rather than avoidance of this pathogen. Moreover, the increased susceptibility of *tol-1*(*nr2033*) mutants to *S. enterica* was comparable to that of *pmk-1*(*km25*) and *dbl-1*(*nk3*) mutants, which are immunocompromised owing to a lack of proper P38 mitogen-activated protein kinase (Kim *et al*, 2002, 2004; Aballay *et al*, 2003; Huffman *et al*, 2004) and transforming growth factor β (Mallo *et al*, 2002) signalling.

Similar to *pmk-1*(*km25*) and *dbl-1*(*nk3*) mutants, *tol-1*(*nr2033*) mutants also show a reduced lifespan when grown on plates containing *E. coli* (Fig 1B,F). The reduced lifespan of these mutants when grown on live *E. coli* is not surprising, as other immunocompromised animals are also killed by live *E. coli* (Kerry *et al*, 2006; Singh & Aballay, 2006b). However, when *tol-1*(*nr2033*) mutants are grown on heat-killed *E. coli*, their lifespan is comparable to that of wild-type animals (Fig 1C,G). These results indicate that *tol-1(nr2033)* animals are not capable of mounting a proper defence response and are killed by live bacteria.

To determine whether TOL-1 has a role in immunity to Gram-positive bacteria we used *Enterococcus faecalis*, which is capable of causing a persistent and lethal infection in the intestinal lumen of *C. elegans*, similar to the one caused by *S. enterica* (Garsin *et al*, 2001). As shown in Fig 1D,H, *tol-1*(*nr2033*) mutants are not more susceptible to *E. faecalis*-mediated killing than wild-type nematodes, indicating that TOL-1 does not produce universal immune protection against pathogens. Interestingly, *tol-1*(*nr2033*) mutants seem to be more resistant to *E. faecalis* than wild-type animals (Fig 1D,H); *tol-1*(*nr2033*) mutants are also more resistant to the Gram-positive bacterium *Streptococcus pneumoniae* than wild-type animals (supplementary Fig S4 online).

S. enterica rapidly invades the pharynx of *tol-1* mutants

To obtain an insight into the cause of death of *tol-1*(*nr2033*) nematodes, we tested whether the premature death correlated with the rapid accumulation of bacteria in the intestine. The profile of bacterial accumulation in the lumen of the intestine was examined by feeding nematodes *S. enterica* expressing green fluorescent protein (GFP) and following the accumulation of bacteria by direct observation under a fluorescence microscope after 48 h, as described previously (Aballay *et al*, 2000). In a typical infection, *S. enterica* accumulates in the anterior intestine of wild-type nematodes, which leads to a marked distension of the lumen (Aballay *et al*, 2000; Labrousse *et al*, 2000). As shown in Fig 2 and supplementary Fig S2 online, *S. enterica* was able to not only cause intestinal distension but also to invade the pharyngeal tissue of *tol-1*(*nr2033*) nematodes. The increased pharyngeal invasion of *S. enterica* is specifically observed in *tol-1*(*nr2033*) mutants (Fig 2B), as it is not seen in other immunocompromised animals such as *pmk-1*(*km25*) (Fig 2C) and *dbl-1*(*nk3*) (Fig 2D) mutants. By 48 h, 70% of the *tol-1*(*nr2033*) nematodes showed infected pharynxes. This phenotype was rescued by transformation with cosmid C07F11, which contains the entire *tol-1* gene (Fig 2G). The pharyngeal invasion observed in *tol-1*(*nr2033*) animals greatly contrasts with the limited pharyngeal invasion that was observed in wild-type, *pmk-1*(*km25*) and *dbl-1*(*nk3*) nematodes (Fig 2G).

As some level of pharyngeal invasion was observed in wild-type animals, we studied whether the infection of the pharynx was a common characteristic of an advanced course of infection in wild-type nematodes. Thus, we monitored nematodes individually for pharyngeal infection and death, and found that pharyngeal invasion correlates with death in the *tol-1*(*nr2033*) mutant ($r^2 = 1.00$), whereas for wild-type ($r^2 = 0.19$), *pmk-1*(*km25*)($r^2 = 0.27$) and *dbl-1*(*nk3*)($r^2 = 0.030$) nematodes the correlation was not significant (Fig 2H). These results indicate that TOL-1 is required to prevent *S. enterica* invasion of the pharynx and that the invasion might be a cause of death in the *tol-1*(*nr2033*) mutant. Also, these results indicate that only a small percentage of wild-type nematodes acquire infected pharynxes during the course of infection.

Consistent with a previous report showing that *tol-1*(*nr2033*) mutants are not hypersusceptible to *P. aeruginosa* (Pujol *et al*, 2001), we found no pharyngeal invasion by this pathogen in either wild-type or *tol-1*(*nr2033*) animals (Fig 2E,F). As shown in Fig 2I, most of the *S. enterica*-infected *tol-1*(*nr2033*) nematodes show infected pharynxes before death, whereas no pharyngeal invasion was observed in animals infected with *P. aeruginosa*. To ensure that the *S. enterica* invasion was not due to pharyngeal defects in *tol-1*(*nr2033*) mutants, we compared the pumping rates of *tol-1*(*nr2033*) to wild type and found no significant difference (supplementary Fig S3 online).

Study of candidate components of the TOL-1 pathway

Three putative TLR-associated signalling components, PIK-1, IKB-1 and TRF-1—homologues to mammalian IRAK, IκB and TRAF1, respectively—have been identified in *C. elegans* (Pujol *et al*, 2001). To address whether IKB-1 and TRF-1 have a role in the regulation of *C. elegans* innate immunity, *trf-1*(*nr2014*) and *ikb-1*(*nr2027*) animals containing putative null mutations (Pujol *et al*, 2001) were infected with *S. enterica*. As shown in Fig 3A,D, *trf-1*(*nr2014*) and *ikb-1*(*nr2027*) mutants were significantly more susceptible to *S. enterica*-mediated killing than wild type nematodes (Fig 3A,D), whereas their lifespans were comparable to that of wild type when grown on heat-killed *E. coli* (Fig 3B,E), indicating that they are immunocompromised animals killed by live bacteria. Consistent with the enhanced resistance to *E. faecalis* of *tol-1*(*nr2033*) animals, *trf-1*(*nr2014*) animals were more resistant to this pathogen than wild-type nematodes (Fig 3C,F).

Examination of *trf-1*(*nr2014*) and *ikb-1*(*nr2027*) by microscopy showed that *S. enterica* was able to infect the pharynxes of these nematodes. As shown in Fig 4A,B, *S. enterica* was able to invade the pharyngeal tissue of these mutants at early stages of the infection. Indeed, 48 h after infection, the number of *ikb-1*(*nr2027*) and *trf-1*(*nr2014*) nematodes exhibiting infected pharynxes was significantly higher than the number of wild-type animals infected in a similar way (Fig 4C). Although the percentage of infected pharynxes of *ikb-1*(*nr2027*) and *trf-1*(*nr2014*) mutants was higher than that of wild-type animals, it was smaller than that of *tol-1* (*nr2033*) mutants (Fig 4C), indicating that other adaptor proteins might mediate TOL-1 immune responses in a redundant manner. The increased susceptibility to *S. enterica* of *ikb-1*(*nr2027*) animals points to a different function for IKB-1 in the nematode, as the homologues in mammals and *Drosophila* act as inhibitors of TLR-mediated immunity. The results, however, are not surprising

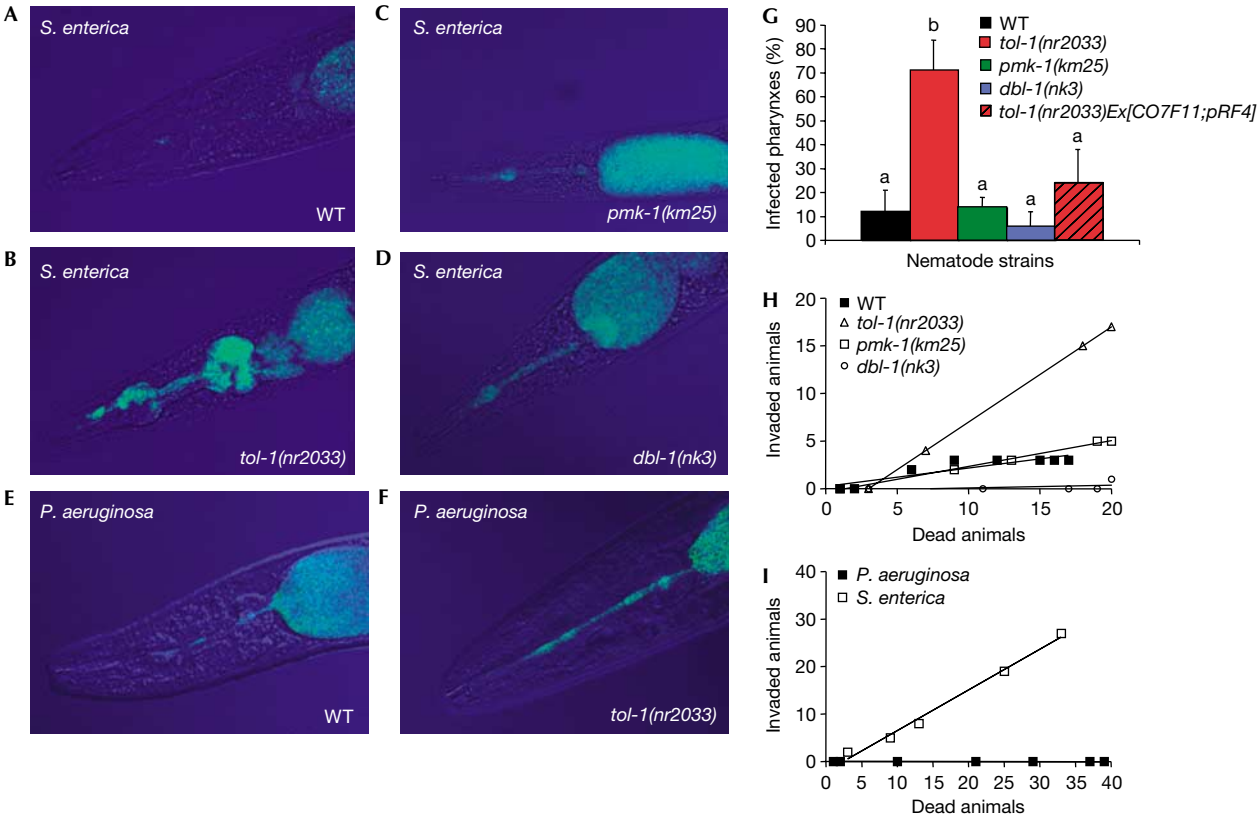

Fig 2 | *Salmonella enterica* invades the pharynx of *tol-1*(*nr2033*). (**A–D**) Confocal images show the pharynxes of wild-type N2 (WT), *pmk-1*(*km25*), *dbl-1*(*nk3*) and *tol-1*(*nr2033*) nematodes exposed for 48 h to *S. enterica* expressing green fluorescent protein (GFP). (**E,F**) Confocal images show the pharynxes of wild-type and *tol-1*(*nr2033*) nematodes exposed for 48 h to *Pseudomonas aeruginosa* expressing GFP. (**G**) The percentage of nematodes with infected pharynxes was determined for wild type, *tol-1*(*nr2033*), *pmk-1*(*km25*), *dbl-1*(*nk3*) and *tol-1*(*nr2033*)*Ex[CO7F11;pRF4]*; bars correspond to mean ± s.d. The Mann–Whitney test indicates that differences among the groups are significantly different; $n = 6–11$. (**H**) Linear regression was used to analyse the relationship between pharyngeal infection and death for wild-type, *pmk-1*(*km25*), *dbl-1*(*nk3*) and *tol-1*(*nr2033*) nematodes exposed to *S. enterica* expressing GFP. (**I**) Linear regression was used to analyse the relationship between pharyngeal infection and death for *tol-1*(*nr2033*) nematodes exposed to *P. aeruginosa* or *S. enterica* expressing GFP. Individual nematodes were monitored daily for pharyngeal infection (24 h before death) and mortality. Images and graphics are representative of 3–11 independent experiments. For each experiment, 20–50 1-day-old adult hermaphrodites were used.

as the strong, lethal *tol-1*(*nr2013*) allele is not suppressed by the *ikb-1*(*nr2025*) allele (Pujol *et al*, 2001).

To study whether *tol-1*, *trf-1* and *ikb-1* might be part of a unique pathway required for immunity, we analysed double mutants. As shown in Fig 4C, the percentage of infected pharynxes of *tol-1*(*nr2033*) was comparable with that of *tol-1*(*nr2033*); *trf-1*(*nr2014*). *tol-1* overexpression rescued the increased pharyngeal invasion phenotype of *tol-1* (Fig 2G), whereas it did not confer extra protection to wild-type, *trf-1* or *ikb-1* animals (Fig 4C). In addition, the susceptibility to *S. enterica* infection of *tol-1*(*nr2033*) was comparable with that of *tol-1*(*nr2033*); *trf-1*(*nr2014*) and *tol-1* overexpression did not protect wild type, *trf-1*(*nr2014*) or *ikb-1*(*nr2027*) from *S. enterica*-mediated killing (Fig 4D,E). Interestingly, *tol-1*(*nr2033*);*ikb-1*(*nr2027*) and *trf-1*(*nr2014*);*ikb-1*(*nr2027*) mutants were synthetically lethal, indicating that *ikb-1* is part of a different pathway. This result is consistent with the different response of *tol-1*(*nr2033*) and *ikb-1*(*nr2027*) animals to *E. faecalis* infection (Figs 1D,3C). Together, these results indicate that TRF-1, but not IKB-1, might be required for the effects of TOL-1 in immunity and that there might be other downstream components that regulate TOL-1-mediated immunity in a redundant manner.

To study further the role of TOL-1 in preventing *S. enterica* killing and invasion of the pharynx, we analysed whether it is required for the correct expression of candidate immunity genes *abf-2* and *hsp-16.41*. ABF-2 is an insect-like defensin that shows pharyngeal expression and has proven antimicrobial properties against Gram-negative and Gram-positive bacteria (Kato *et al*, 2002). HSP-16.41 is highly expressed in the pharynx in response to stress (Hong *et al*, 2004) and it belongs to a family of heat-shock proteins recently found to be required for *C. elegans* immunity (Singh & Aballay, 2006a,b). As shown in Fig 4F, quantitative reverse transcription–PCR expression analysis shows that the expression of *abf-2* and *hsp-16.41* is decreased in *tol-1*(*nr2033*) animals, indicating that TOL-1 is required for their correct expression in the presence of *S. enterica*.

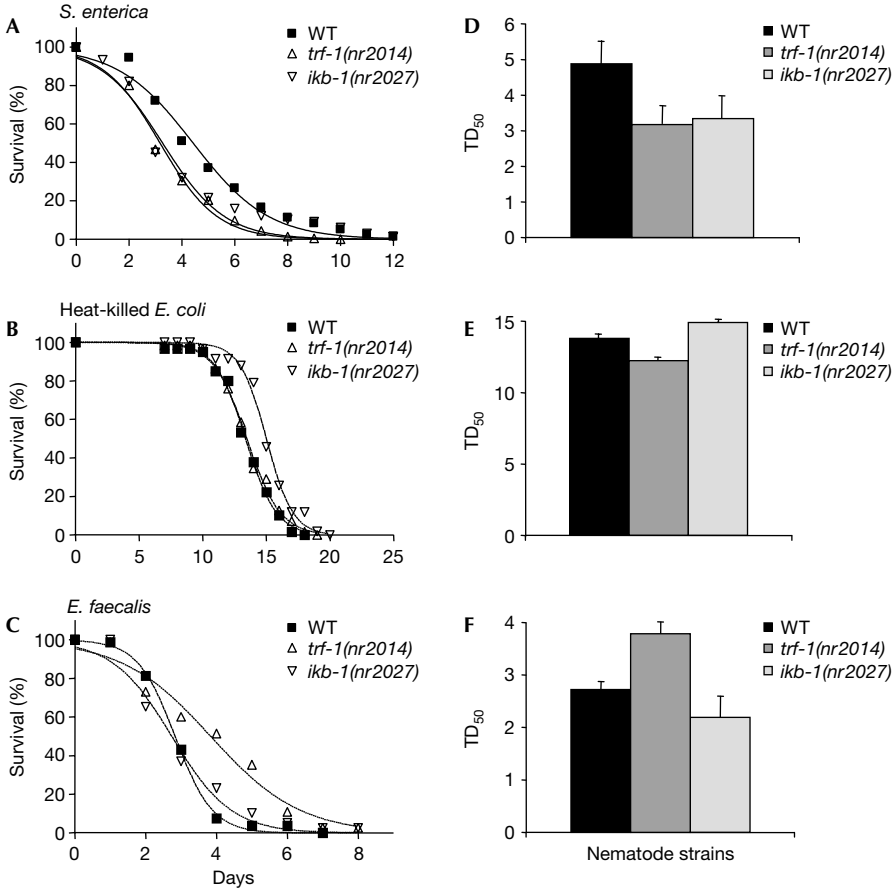

Fig 3 | Analysis of the role of candidate downstream components of the TOL-1 pathway in immunity to *Salmonella enterica*. (**A**) Wild-type N2 (WT), *trf-1*(*nr2014*) ($P = 0.0001$) and *ikb-1*(*nr2027*) ($P = 0.0001$) were exposed to *S. enterica*. The graphs represent combined results of more than four independent experiments, each of which used 36–50 1-day-old adult hermaphrodites. (**B**) The lifespan of the *ikb-1*(*nr2027*) is similar to that of wild type ($P = 0.3236$), whereas the lifespan of *trf-1*(*nr2014*) is slightly longer than wild type ($P = 0.0197$). (**C**) *trf-1*(*nr2014*) are more resistant to *Enterococcus faecalis*-mediated killing than wild type ($P < 0.0064$), whereas *ikb-1*(*nr2027*) ($P < 0.6730$) shows a susceptibility similar to that of wild type. (**D–F**) The time for 50% of the nematodes to die (TD_{50}) was determined for each nematode strain exposed to different bacteria. The graphs are representative of at least three independent experiments, each of which used 36–50 1-day-old adult hermaphrodites; bars correspond to mean ± s.d., unless otherwise indicated.

CONCLUSION

Until now, TOL-1 has been thought to function in the *C. elegans* nervous system to mediate the recognition and avoidance of certain pathogens (Pujol *et al*, 2001). It seems that the nematode nervous system is crucial for pathogen recognition and elicitation of immune response. The insulin/IGF-1-like receptor DAF-2, which is a crucial regulator of ageing and immunity in *C. elegans*, functions in neurons (Apfeld & Kenyon, 1998; Wolkow *et al*, 2000; Kodama *et al*, 2006) from where it blocks the activation of the FOXO transcription factor DAF-16 in distant tissues and regulates genes that promote longevity and immune effectors (Murphy *et al*, 2003; Troemel *et al*, 2006). The regulation by TOL-1 of immune effectors in the pharynx and other tissues is consistent with the regulation of immunity by DAF-2 and with the fact that TOL-1 is expressed in several neural cells including URY neurons, the endings of which reach the anterior end of the pharynx (Pujol *et al*, 2001).

Our results indicate that TOL-1 is required for proper innate immunity to certain Gram-negative bacteria and for the correct expression of ABF-2 and HSP-16.41. As *tol-1*(*nr2033*) animals are not more susceptible to Gram-positive *E. faecalis* (Fig 1D) or *S. pneumoniae* (supplementary Fig S4 online), TOL-1 might be more important in innate immunity to Gram-negative bacteria than to Gram-positive bacteria. However, ABF-2 has strong activity against both Gram-negative and Gram-positive bacteria (Kato *et al*, 2002) and the heat-shock transcription factor 1 pathway, which regulates HSPs, is required for immunity to Gram-negative and Gram-positive bacteria (Singh & Aballay, 2006b). The strong resistance of *tol-1*(*nr2033*) animals to the Gram-positive pathogens studied here indicates that *C. elegans* might have evolved a TOL-1-dependent mechanism to elicit immunity to certain pathogens while inhibiting immunity to others. It would be interesting to study whether the natural habits of *C. elegans* are enriched in certain types of microorganisms and whether the

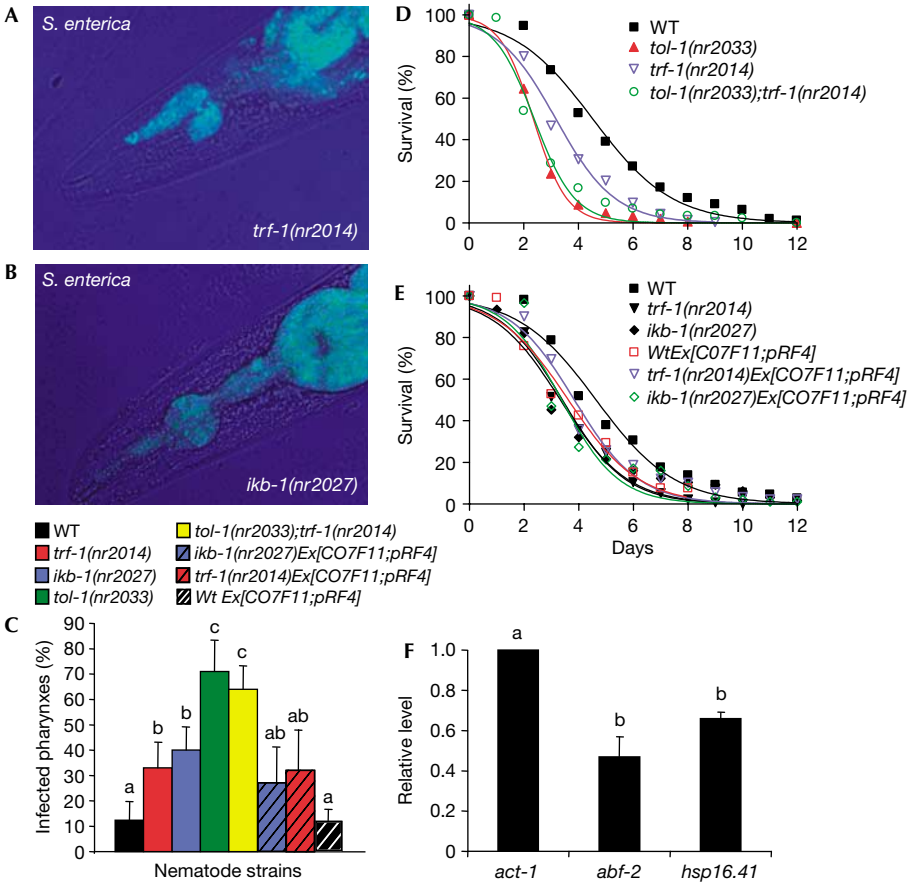

Fig 4 | Characterization of downstream components of the TOL-1 pathway. (**A,B**) Confocal images show the pharynxes of *trf-1*(*nr2014*) and *ikb-1*(*nr2027*) nematodes exposed for 48 h to *Salmonella enterica* expressing green fluorescent protein (GFP). GFP-expressing bacteria were detected in the pharynx and intestinal lumen of both mutants. (**C**) The percentage of nematodes with infected pharynxes was determined for wild type (WT), *trf-1*(*nr2014*), *ikb-1*(*nr2027*), *tol-1*(*nr2033*), *tol-1*(*nr2033*); *trf-1*(*nr2014*), *trf-1*(*nr2014*) *Ex[CO7F11;pRF4]* and *ikb-1*(*nr2027*)*Ex[CO7F11;pRF4]*. For each experiment, 20–50 1-day-old adult hermaphrodites were used. The Mann–Whitney test indicates that differences among the groups are significantly different; $n = 3$–11. (**D,E**) Wild-type N2, *tol-1*(*nr2033*) ($P < 0.0001$), *trf-1*(*nr2014*) ($P = 0.0013$), *tol-1*(*nr2033*);*trf-1*(*nr2014*) ($P < 0.0001$), wild-type *Ex[CO7F11;pRF4]* ($P < 0.0024$), *trf-1*(*nr2014*)*Ex[CO7F11;pRF4]* ($P < 0.0015$) and *ikb-1*(*nr2027*)*Ex[CO7F11;pRF4]* ($P < 0.002$) were exposed to *S. enterica*. The graphs represent combined results of more than four independent experiments, each of which used 36–50 1-day-old adult hermaphrodites. (**F**) Quantitative reverse transcription–PCR analysis of *act-1*, *abf-2* and *hsp-16.41* expression in *tol-1*(*nr2033*) relative to wild-type nematodes grown on *S. enterica*. Data were analysed by relative quantitation using the comparative cycle threshold method and normalization to *act-1*. Student's exact *t*-test indicates that differences among the groups are significantly different; $n = 3$; bars correspond to mean ± s.d.

microbial composition of different habitats has shaped TOL-1 immunity. Further studies should shed light on the understanding of the specificity and evolution of TOL-1-mediated immunity.

METHODS

Bacterial and nematode strains. The following bacterial strains were used: *E. coli* OP50 (Brenner, 1974), *S. enterica* serovar *Typhimurium* SL1344 (Wray & Sojka, 1978), *P. aeruginosa* PA14 (Tan *et al*, 1999) and *E. faecalis* OG1RF (Murray *et al*, 1993).

The strains of *C. elegans* that were used were wild-type N2 Bristol, *dbl-1*(*nk3*), *ikb-1*(*nr2027*), *trf-1*(*2014*), *tol-1*(*nr2033*) and *pmk-1*(*km25*). These strains were originally obtained from the *Caenorhabditis* Genetics Center and then maintained in our laboratory. Double mutants and transgenic animals were generated as described in the supplementary information online.

Growth conditions. Nematodes were maintained on nematode growth medium (NGM) medium containing a lawn of *E. coli* OP50 at 20 °C. Synchronous populations were acquired by placing gravid adults on NGM plates containing *E. coli* OP50 for 5 h at 25 °C. The gravid adults were removed, leaving the eggs to hatch and develop into 1-day-old hermaphroditic adults at 20 °C for use in the different assays. All experiments were carried out at 25 °C as described in the supplementary information online. **Supplementary information** is available at *EMBO reports* online (http://www.emboreports.org).

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