Toll-like receptor homolog TOL-1 regulates Bifidobacterium infantis-elicited longevity and behavior in Caenorhabditis elegans

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Bifidobacterium infantis, a Gram-positive bacterium, is one of the commonly used probiotics. We previously showed that B. infantis modified host defense systems and extended the lifespan of the nematode Caenorhabditis elegans. In the present study, we showed that the lifespan extension caused by B. infantis was enhanced in animals having a mutation in the tol-1 gene that encodes the sole C. elegans homolog of Toll-like receptors (TLRs). Meanwhile, lifespan increased by other probiotic bacteria, such as Bacillus subtilis or Clostridium butyricum, was not affected in the tol-1 mutant animals. A microarray analysis revealed that the expression of innate immune response-related genes was significantly increased in the tol-1 mutant. Worms with the tol-1 mutation exhibited reduced leaving behavior from the B. infantis lawn, while canonical downstream factors trf-1/TRAF and ikb-1/IκB appeared to not be involved. In conclusion, C. elegans tol-1/TLR regulates B. infantis-induced longevity and also regulates behavior against B. infantis.

Key words: Caenorhabditis elegans, Toll-like receptor, longevity, behavior, Bifidobacterium infantis

INTRODUCTION

Probiotic bacteria are defined as living microorganisms that exert beneficial effects on human health when ingested in adequate amounts [1]. Metchnikoff, who first proposed the concept of probiotic bacteria in 1907, hypothesized that lactic acid bacteria (LAB) were important for promoting human health and longevity. Bifidobacteria, which is one of the LAB in a broad sense, has been commonly used as a probiotic. Among them, Bifidobacterium longum subsp. infantis (B. infantis), an infant microbe, has been reported to exert a variety of probiotic properties, such as anti-microbial activities [2], immunomodulatory effects [3, 4], and symptom improvement in irritable bowel syndrome (IBS) [5].

Caenorhabditis elegans has been used extensively as an experimental animal model, especially for basic studies on senescence. However, the creature has also been used to study the influence of food and nutrition on senescence and host defense since we reported the beneficial effects of LAB on the worm [6]. The appeal of this organism is due to its ease of cultivation, abundance of genetic tools, and short and reproducible lifespan [7]. We have previously shown that feeding of B. infantis extends lifespan via the p38 MAPK pathway in C. elegans [8]. In the course of the mutant analyses, we noticed that the mutation in the tol-1 gene, encoding the C. elegans sole homolog of Toll-like receptors (TLRs), appeared to enhance the lifespan extension caused by B. infantis.

TLRs are pattern-recognition receptors that respond to diverse pathogen-associated molecular patterns (PAMPs) and initiate innate immune signals. The first Toll was discovered in Drosophila melanogaster [9–11] and was later found in diverse species from the nematode C. elegans to mammals, including humans [12, 13]. Canonical TLR signaling negatively regulates the transcriptional inhibitor IκB/Cactus and, in turn, NF-κB/Relish, a master regulator of the inflammatory response, and drives expression of pathogen-defense molecules, such as antimicrobial peptides in insects and interferons and cytokines in mammals [14]. In contrast to mammals and flies, C. elegans lacks NF-κB-like transcription factors [15]. Nevertheless, Tenor et al. demonstrated that TOL-1 has roles in defense responses to pathogenic Gram-negative and Gram-positive bacteria; tol-1 mutants showed increased susceptibility to certain Gram-negative bacteria, including Salmonella enterica, but increased resistance to Gram-positive bacteria such as Enterococcus faecalis [16]. Pujol et al. reported that TOL-1 is required for the avoidance of Serratia marcescens [17]. Subsequently, Brandt and Ringstad showed that TOL-1 in a pair of BAG chemosensory neurons is required for avoidance of S. marcescens [18]. The role of TOL-1 in response to probiotic bacteria, however, remains poorly understood. In the present study, we described the role of TOL-1 in longevity and behavior elicited by a probiotic, B. infantis.
MATERIALS AND METHODS

Bacterial strain and culture conditions

Escherichia coli OP50 was grown on tryptone soya broth (TSB) and tryptone soya agar (TSA) (Nissui Pharmaceutical, Tokyo, Japan) at 37°C. Similarly, Bacillus subtilis NBRC3134 and Staphylococcus aureus NBRC13276 were cultured using TSB and TSA. B. infantis ATCC15697 was cultured using GAM broth (Nissui) and TOS propionate agar (Yakult Pharmaceutical Industry, Tokyo, Japan). Clostridium butyricum MIYAIRI 588 (CBM 588), which was kindly provided by Miyarisan Pharmaceutical, was cultured using GAM broth and GAM agar (Nissui Pharmaceutical, Japan). Cultivated bacteria were scraped and weighed. Aliquots (100 mg wet weight) of bacteria were suspended in 0.5 ml of M9 buffer (5 mM potassium phosphate, 1 mM CaCl2, and 1 mM MgSO4) and used in the lifespan assays; aliquots at 66.7 mg/ml were used in the behavioral assays.

C. elegans strains and culture conditions

The wild-type C. elegans strain Bristol N2 and the following derivative mutant strains were obtained from the Caenorhabditis Genetics Center: IG10 tol-1(nr2033) I, NS2937 trf-1(nr2014) III, and NS3026 ikb-1(nr2027) I. C. elegans strains were maintained using standard techniques [19]. For gene expression analyses and lifespan assays, synchronized animals were prepared as follows: eggs were prepared from adult C. elegans by exposure to a sodium hypochlorite/sodium hydroxide solution. The egg suspension was incubated in M9 buffer for one day at 25°C to allow hatching and synchronization, and the resulting suspension of synchronized L1-stage worms was centrifuged at 156 × g for 1 min. After removing the supernatant by aspiration, the remaining larvae were transferred onto mNGM plates covered with 10 mg OP50. Microarray expression profiling was performed with the wild-type and tol-1 mutant worms. Approximately 100 worms in each group were collected by a worm picker and soaked in RNA later solution (Qiagen). Total RNA was isolated using an RNeasy Lipid Tissue kit (Qiagen).

DNA synthesis and microarray hybridization were performed by Kurabo Industries, Ltd. RNA quality (RNA integrity number (RIN) >7) was confirmed using the 2100 Bioanalyzer (Agilent Technologies, Santa Clara, CA, USA). A total of ~1 µg of RNA was used as the template for fluorescent labeling of cRNA. Labeled cRNAs were hybridized to the Affymetrix C. elegans Genome Array (containing 22,500 transcripts). Microarray data analyses were performed with MA5.0 (Microarray Suite statistical algorithm, Affymetrix). Differential expression was analyzed by the Comparison Analysis of MA5.0 using the Wilcoxon’s Signed Rank test. Each probe set on the experiment array (tol-1) was compared to its counterpart on the baseline array (wild type), and a ‘Change p-value’ was calculated. Probe sets that showed differential expression were assigned with ‘Change calls’ (increase, p<0.002; marginal increase, 0.002≤p<0.002667; marginal decrease, 0.997333≤p≤0.998; or decrease, p>0.998).

The ‘Signal Log Ratio’ was computed using a one-step Tukey’s Biweight method by taking a mean of the log ratios of probe pair intensities across the two arrays (wild-type vs. tol-1). The final data extraction was performed using DNA Microarray Viewer ver. 2 (Kurabo Industries, Ltd., Osaka, Japan).

Lawn-leaving assay

Assay plates were prepared as follows. Fifty microliters of bacteria suspension was placed at the center of 3.5-cm mNGM plates (1.7% (w/v) agar, 50 mM NaCl, 1 mM CaCl2, 5 µg/ml cholesterol, 25 mM KH2PO4, 1 mM MgSO4), and after the plates were allowed to dry for 20 min, a bacteria lawn approximately 1 cm in diameter appeared. Animals at the young adult stage were collected in microfuge tubes and washed 3 times with M9 buffer (5 mM potassium...
phosphate, 1 mM CaCl₂, 1 mM MgSO₄, and 0.5 g/l gelatin). Approximately 20 animals were placed in the center of the bacteria lawn. At 10 min after placing the animals, the number of animals that were outside of the lawn and the number of those that remained on the lawn were counted. The percentage of animals that were outside of the bacterial lawn was calculated as (number of animals that were outside of the lawn) / (total number of animals) × 100%.

RESULTS

The tol-1 mutation enhanced the prolongevity caused by B. infantis

In our previous work, the mean lifespan of C. elegans increased by approximately 40% when the tol-1(nr2033) mutant worms were fed B. infantis; the same regimen increased the lifespan of the wild-type worms by approximately 20% [8]. From these observations, we hypothesized that the tol-1 mutation may enhance lifespan extension caused by B. infantis. To test this hypothesis, we determined the significant differences between the lifespans of the wild-type and tol-1 mutant animals. The lifespan of the tol-1 mutant was significantly longer than that of the wild-type animal after feeding with B. infantis (Fig. 1, Table 1). In contrast, the lifespan of the tol-1 mutant was similar to that of the wild-type animal after feeding with the standard food, E. coli OP50 (Fig. 1, Table 1). These results suggest that the mutation in the tol-1 gene enhanced the longevity caused by B. infantis. Notably, enhancement of B. infantis-elicited longevity in the tol-1 mutant animals was observed after 20 days (Fig. 1). Previously, Zhao et al. identified two classes of death: early deaths with a swollen pharynx, which is mainly caused by bacterial infection and damage to the pharynx (“early damage”), and later deaths with an atrophied pharynx (“later intrinsic causes”) [22]. Taken together with our results, tol-1 might suppress the effect of B. infantis on the reduction of later deaths due to intrinsic causes.

The tol-1 mutation affected lifespan modulated by certain Gram-positive bacteria

Tenor et al. showed that tol-1 mutants were more resistant to Gram-positive bacteria such as E. faecalis compared with wild-type worms [16]. We confirmed that tol-1 mutants exhibited increased resistance to Staphylococcus aureus, which is a Gram-positive bacterium known to shorten the lifespan of the worms [23] (Fig. 2A, Table 1). The lifespan of the animals fed B. subtilis was reported to be significantly longer than that of the animals fed E. coli OP50 [24], and more recently, we showed that feeding with C. butyricum extended the lifespan of C. elegans [25]. Therefore, we examined whether the tol-1 mutation enhanced the lifespan extension caused by the Gram-positive probiotic bacteria B. subtilis and

Fig. 1. C. elegans longevity caused by feeding with B. infantis was enhanced in the tol-1 mutants.

Survival curves of the wild-type (WT) and tol-1 mutant animals fed E. coli OP50 (OP) or B. infantis (BI). The lifespan of the tol-1 mutant fed BI (black dash line) was increased compared with that of the wild-type fed BI (black solid line), while in the feeding of a standard food, E. coli OP50, the lifespan of the tol-1 mutant (gray dash line) was similar to that of the wild-type animal (gray solid line). ***p<0.001, log-rank test. NS: not significant, log-rank test. Detailed lifespan data and statistics are provided in Table 1.

Table 1. Summary of lifespan experiments

| Strain/Food          | Mean lifespan (days) ± SE (days) | Maximum lifespan (days) ± SE (days) | Number of animals (n) | p-value |
|----------------------|----------------------------------|-------------------------------------|-----------------------|---------|
| N2/E. coli OP50 (OP) | 16.79 ± 0.36                     | 23.96 ± 0.21                       | 257                   | 0.57924 |
| tol-1/E. coli OP50 (OP) | 17.35 ± 0.31                   | 23.44 ± 0.23                       | 243                   |         |
| N2/B. infantis (BI)  | 24.11 ± 0.38                     | 30.41 ± 0.36                       | 212                   | 0.00002 |
| tol-1/B. infantis (BI) | 25.24 ± 0.47                   | 34.03 ± 0.30                       | 238                   |         |
| N2/S. aureus         | 7.04 ± 0.14                      | 8.75 ± 0.37                        | 52                    | 0.04922 |
| tol-1/S. aureus      | 7.69 ± 0.14                      | 9.63 ± 0.23                        | 54                    |         |
| N2/B. subtilis       | 20.40 ± 0.67                     | 27.70 ± 0.47                       | 68                    | 0.23265 |
| tol-1/B. subtilis    | 20.08 ± 0.92                     | 29.00 ± 0.43                       | 67                    |         |
| N2/C. butyricum      | 23.00 ± 0.45                     | 28.44 ± 0.30                       | 112                   | 0.75936 |
| tol-1/C. butyricum   | 22.67 ± 0.50                     | 28.97 ± 0.49                       | 124                   |         |
| N2/astaxanthin       | 19.55 ± 0.64                     | 25.61 ± 0.63                       | 59                    |         |
| tol-1/astaxanthin    | 20.63 ± 0.62                     | 26.72 ± 0.40                       | 60                    | 0.24357 |

SE: standard error.
Neither the lifespan prolonged by *Bacillus subtilis* nor that prolonged by *Clostridium butyricum* was extended in the *tol-1* mutant animals.

Survival curves of animals fed several Gram-positive bacteria or astaxanthin. There was a significant difference between the wild-type (WT) and *tol-1* mutant animals when they were fed with *S. aureus* (A) but not when they were fed with *B. subtilis* (B), *C. butyricum* (C), or astaxanthin (D). *p*<0.05, log-rank test. NS: not significant, log-rank test. Detailed lifespan data and statistics are provided in Table 1.

Table 2. Gene Ontology (GO) enrichment for genes upregulated in the *tol-1* mutants

| GO terms                                           | p-values          |
|---------------------------------------------------|-------------------|
| Innate immune response                            | 1.4 × 10^{-4}     |
| Lipid storage                                     | 2.2 × 10^{-4}     |
| Endoplasmic reticulum unfolded protein response   | 2.7 × 10^{-4}     |
| Protein ubiquitination                            | 1.8 × 10^{-3}     |
| Positive regulation of smooth muscle contraction  | 4.0 × 10^{-3}     |

Table 3. Lysozyme genes upregulated in the *tol-1* mutants

| Gene symbol (Ensembl name) | Log ratio | p-values          |
|---------------------------|-----------|-------------------|
| lys-3 (Y22F5A.6)          | 2.3       | 2.0 × 10^{-5}     |
| ilys-2 (C45G7.2)          | 1         | 5.5 × 10^{-4}     |
| ilys-3 (C45G7.3)          | 0.7       | 9.7 × 10^{-4}     |
| ilys-4 (C55F2.2)          | 0.6       | 3.9 × 10^{-4}     |

*C. butyricum*. Neither the lifespan prolongation caused by *B. subtilis* nor that caused by *C. butyricum* was enhanced in the *tol-1* mutant animals. Finally, we tested whether the lifespan increase due to antioxidative compounds was extended further in the *tol-1* mutants. We previously showed that oral supplementation with astaxanthin prolonged the lifespan of the worms [20]. Again, the prolonged lifespan caused by astaxanthin was not changed by the *tol-1* mutation. Taken together, the *tol-1* mutation appears to affect lifespan modulated by several, but not all, Gram-positive bacteria.

Expression of innate immune response-related genes was significantly upregulated in the *tol-1* mutant

To investigate the TOL-1-dependent molecular mechanism(s) in *C. elegans*, we performed a microarray analysis. Comparing the transcriptional profiles of the wild-type and *tol-1* mutant worms revealed genes that were upregulated and downregulated in the *tol-1* mutant. Genes with | Signal Log Ratio | ≥ 1 were subjected to the enriched gene ontology (GO) terms using DAVID (https://david.ncifcrf.gov/summary.jsp). Notably, the top-ranked GO term in the upregulated genes was ‘innate immune response’ (p=1.4 × 10^{-4}) (Table 2). We also found that the lys-3, ilys-2, ilys-3, and ilys-4 genes, which encode lysozyme, were induced in the *tol-1* mutants (Table 3). *ilys-2* and *ilys-3* have been reported to be specifically induced by Gram-positive bacteria [26], although *lys-3* is upregulated by both Gram-positive and Gram-negative bacteria and by fungi [26]. Because lysozyme catalyzes the...
Taken together, effective digestion of the crucial component(s) for longevity are included in the cell wall of *B. infantis*. We previously reported that the cell-wall fraction of the *B. infantis* lawn tol-1 mutants showed reduced leaving behavior from the wild-type lawn. The leaving assay using *B. infantis* lawn due to low preference [27] despite its beneficial effects, such as lifespan extension. The lawn-leaving assay using *B. infantis* revealed that the tol-1 mutants displayed significantly reduced leaving behavior from the *B. infantis* lawn when compared with the wild-type animals (Fig. 3A, B). Brandt and Ringstad showed that the canonical TLR signaling factors *trf-1/TRAf* and *ikb-1/IxB* were also required for avoidance of *S. marcescens* [18]. The *trf-1* and *ikb-1* mutants, however, showed normal leaving behavior from the *B. infantis* lawn (Fig. 3C). Thus, the mechanisms underlying the leaving behavior from the *B. infantis* lawn are at least partially distinguishable from those underlying the leaving behavior from the *S. marcescens* lawn.

**DISCUSSION**

Our results suggest that the mutation in the *tol-1* gene enhanced the increased lifespan caused by *B. infantis* but not by other probiotic bacteria, such as *B. subtilis* or *C. butyricum*. In addition, Tenor *et al.* and the present study showed that *tol-1* mutants exhibited increased resistance to Gram-positive bacteria, including *E. faecalis* and *S. aureus*, but decreased resistance to Gram-negative bacteria [16]. Together, the *tol-1* mutation appears to affect lifespan modulated by several, but not all, Gram-positive bacteria. Although Tenor *et al.* discussed that TOL-1 might be more important in innate immunity to Gram-negative bacteria than in innate immunity to Gram-positive bacteria and that *C. elegans* might have evolved a TOL-1-dependent mechanism to enhance immunity to certain pathogens while inhibiting immunity to others [16], the molecular mechanisms that account for this have not been elucidated. Our microarray results showed that the expression of innate immune response-related genes was induced in the *tol-1* mutant and that lysozyme genes were also upregulated. The increased expression of these genes might be one of the mechanisms that enhance resistance to *B. subtilis* and *C. butyricum*. Kim *et al.* reported that there were some similarities and differences in the expression patterns of defense-related genes that were induced by Gram-positive bacteria; e.g., clec-60, which encodes a C-type lectin and is known to be upregulated by *S. aureus* infection, was induced by *Lactobacillus acidophilus* NFCM, but not by *B. subtilis* ATCC 6633 [28]. Identification of genes affected by *B. infantis* but not by *B. subtilis* or other probiotic bacteria.
butyricum may be fruitful in exploring the mechanism(s) by which TOL-1 specifically modulates the lifespan of worms in the presence of B. infantis.

We demonstrated that tol-1 mutants showed reduced leaving behavior from the B. infantis lawn. Recent findings suggest that mammalian TLRs play essential roles not only in immune responses but also in nervous system development, neurodegeneration, and diseases [29]. As shown in mammals, the C. elegans TLR is also expressed in neurons and plays roles in pathogen-related avoidance behavior [17, 18]. Considering that the tol-1 gene has been reported to be expressed in the four URY neurons, six mechanosensory cells (ALML/R, AVM, PLML/R, and PVM), and six interneurons (ALNL/R, AVDL/R, and two neurons in the retroganglionic ganglion that remain to be identified) [3, 17], TOL-1 might act in the neurons to regulate B. infantis-related longevity and/or behavior. In the present study, it remains to be determined whether the reduced lawn-leaving behavior was responsible for the enhancement of the prolonged lifespan caused by B. infantis. However, we previously reported that daf-16/FOXO isoform b plays a positive role in leaving behavior from the B. infantis lawn [27], and determining whether B. infantis-induced lifespan extension is enhanced by the daf-16 mutation, may give a hint.

In conclusion, the C. elegans TLR, TOL-1, regulates B. infantis-induced longevity and also regulates leaving behavior from the B. infantis lawn. TLRs are highly conserved among animal species, including humans, and B. infantis is distributed in the human gut and is also widely used as a probiotic. It would be worth pursuing the role of TLRs in innate immunity and the nervous system as well as B. infantis-induced beneficial effects, including prolongevity.

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