

# Mechanism underlying prolongevity induced by bifidobacteria in *Caenorhabditis elegans*

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12

13

1 **Abstract** Lactobacilli and bifidobacteria are probiotic bacteria that modify host defense  
2 systems and have the ability to extend the lifespan of the nematode *Caenorhabditis elegans*.  
3 Here, we attempted to elucidate the mechanism by which bifidobacteria prolong the lifespan  
4 of *C. elegans*. When the nematode was fed *Bifidobacterium infantis* (BI) mixed at various  
5 ratios with the standard food bacterium *Escherichia coli* strain OP50 (OP), the mean lifespan  
6 of worms was extended in a dose-dependent manner. Worms fed BI displayed higher  
7 locomotion and produced more offspring than control worms. The growth curves of  
8 nematodes were similar regardless of the amount of BI mixed with OP, suggesting that BI did  
9 not induce longevity effects through caloric restriction. Notably, feeding worms the cell  
10 wall fraction of BI alone was sufficient to promote longevity. The accumulation of protein  
11 carbonyls and lipofuscin, a biochemical marker of aging, was also lower in worms fed BI;  
12 however, the worms displayed similar susceptibility to heat, hydrogen peroxide, and paraquat,  
13 an inducer of free radicals, as the control worms. As a result of BI feeding, loss-of-function  
14 mutants of *daf-16*, *jnk-1*, *aak-2*, *tol-1*, and *tir-1* exhibited a longer lifespan than OP-fed  
15 control worms, but BI failed to extend the lifespan of *pmk-1*, *skn-1*, and *vhp-1* mutants. As  
16 *skn-1* induces phase 2 detoxification enzymes, our findings suggest that cell wall components  
17 of bifidobacteria increase the average lifespan of *C. elegans* via activation of *skn-1*, regulated  
18 by the p38 MAPK pathway, but not by general activation of the host defense system via  
19 DAF-16.

20

21 **Keywords** Longevity · Nematodes · Probiotics · Aging · Innate immunity

22

## 1 **Introduction**

2

3 Age at infection is one of the most important determinants of disease morbidity and mortality  
4 (Miller and Gay, 1997; Pop-Vicas and Gravenstein, 2011; Sligl and Majumdar, 2011).  
5 Senescence of the immune system, which is a result of aging-related functional and metabolic  
6 alterations in cells and tissues, leads to increased infections, malignancy, and autoimmunity  
7 (Grubeck-Loebenstein, 1997; Moulias et al., 1985; Pawelec and Larbi, 2008). Elderly also  
8 experience increased mortality from viral and bacterial infections (Bradley and Kauffman,  
9 1990). Slowing the senescence of immune function and decreasing mortality from infectious  
10 diseases may be possible through nutritional control; however, the difficulty of establishing a  
11 suitable animal model has limited studies on this topic. Although an increase of biomarkers  
12 related to immunological function has been observed in elderly with improved nutritional  
13 conditions (Bogden and Louria, 2004), few reports have shown beneficial influences of  
14 nutrition on immunity and the outcomes of experimental infection (Effros et al., 1991; Fulop  
15 et al., 2007; Hayek et al., 1997).

16 Probiotic bacteria are living microorganisms that exert beneficial effects on human health  
17 when ingested in sufficient numbers (Naidu et al., 1999). The concept of probiotic bacteria  
18 was first proposed in 1907 by Metchnikoff, who hypothesized that lactobacilli are important  
19 for promoting human health and longevity (Metchnikoff, 1907). Since this novel proposal  
20 over a century ago, lactic acid bacteria (LAB), including bifidobacteria, have been the most  
21 commonly used probiotic microorganisms. LAB have numerous physiological influences on  
22 their hosts, including antimicrobial, nutritional, anti-tumor, and immunomodulatory effects,  
23 microbial interference, and reduction of serum cholesterol and lipids. To date, however, few  
24 studies have examined the influence of LAB on longevity (Ottaviani et al., 2011).

25 *Caenorhabditis elegans* is a small, free-living soil nematode that feeds on bacteria and has

1 been extensively used as an experimental system for biological studies because of its  
2 morphological simplicity, transparent body, ease of cultivation, and suitability for genetic  
3 analysis (Riddle et al., 1997). In addition, the short and reproducible lifespan of *C. elegans* is  
4 particularly advantageous for aging studies (Finch and Ruvkun, 2001). We previously  
5 demonstrated that feeding nematodes LAB prolongs lifespan and enhances host defenses  
6 (Ikeda et al., 2007; Komura et al., 2012). Further, we showed that *Legionella pneumophila* is  
7 only pathogenic in older nematodes and that infection by this bacterium can be prevented by  
8 feeding worms *Bifidobacterium infantis* (BI) before the infectious challenge (Komura et al.,  
9 2010). However, neither the contributing bacterial factors nor the underlying mechanisms of  
10 these beneficial effects have been clarified.

11 Caloric restriction is well known to extend the lifespan of a wide range of organisms from  
12 yeasts to mammals (Ingram et al., 2006); however, it remains controversial whether caloric  
13 restriction increases the lifespan of primates (Austad, 2012). Here, we assessed whether  
14 bifidobacteria cause dietary or caloric restrictions in *C. elegans* based on the evaluation of  
15 worm biomarkers, including body and brood sizes (Pincus and Slack, 2010). While radical  
16 oxygen species (ROS) are recognized as key factors causing senescence because of their  
17 oxidative properties (Cannizzo et al., 2011), ROS are also considered to contribute to  
18 anti-senescence through the hormesis effect (Ristow and Schmeisser, 2011). Hence, we  
19 performed assays for muscle function, lipofuscin accumulation (gut autofluorescence), protein  
20 carbonyl content, stress resistance, and mitochondrial number to determine how bifidobacteria  
21 induce the numerous beneficial effects on physiological function and longevity. In addition,  
22 we evaluated whether the cell walls and protoplasts of BI contribute to the prolongevity of *C.*  
23 *elegans*. Finally, loss-of-function *C. elegans* mutants were fed BI to identify the genes  
24 involved in the probiotic effects in *C. elegans*.

25

1

## 2 **Materials and methods**

3

4 Nematode

5

6 *C. elegans* Bristol strain N2 and its derivative mutant strains were kindly provided by the  
7 Caenorhabditis Genetics Center, University of Minnesota. The mutations used in this study  
8 were IG10 *tol-1(nr2033)*, RB1085 *tir-1(ok1052)*, KU25 *pmk-1(km25)*, EU1 *skn-1(zu67)*,  
9 JT366 *vhp-1(sa366)*, VC8 *jnk-1(gk7)*, CF1038 *daf-16(mu86)*, CB1370 *daf-2(e1370)*, and  
10 RB754 *aak-2(ok524)*. Nematodes were maintained and propagated on nematode growth  
11 medium (NGM) according to standard techniques (Stiernagle, 1999). *E. coli* OP50 (OP) was  
12 used as the standard feed for nematode cultivation and was grown using tryptone soya agar  
13 (Nissui Pharmaceutical, Tokyo, Japan). Cultured bacteria (100 mg wet weight) were  
14 suspended in 0.5 ml M9 buffer, and 50 µl of the resulting bacterial suspension was then  
15 spread on peptone-free modified NGM (mNGM) in 5.0-cm diameter plates to feed worms.

16

17 Bacterial strains

18

19 The LAB *B. infantis* ATCC15697 (BI) used as a test food source for *C. elegans* and was  
20 cultured using GAM broth (Nissui) and TOS propionate agar (Yakult Pharmaceutical Industry,  
21 Tokyo, Japan).

22

23 Determination of *C. elegans* lifespan

24

25 Eggs were recovered from adult *C. elegans* worms after exposure to a sodium

1 hypochlorite/sodium hydroxide solution, as previously described (Sulston and Hodgkin,  
2 1988). The egg suspension was incubated overnight at 25°C to allow hatching, and the  
3 resulting suspension of L1 stage worms was centrifuged at 156 × g for 1 min. After removing  
4 the supernatant by aspiration, the remaining larvae were transferred onto fresh mNGM plates  
5 covered with OP and then incubated at 25°C. To synchronize pubescence, worms were  
6 allowed to feed on OP for two days until maturation, as it is well known that the reproductive  
7 system regulates aging in *C. elegans* (Hsin and Kenyon, 1999). Nematocidal assays were  
8 performed by adding 35 three-day-old adult worms to each mNGM plate covered with lawns  
9 of BI. The plates were incubated at 25°C, and the numbers of live and dead worms were  
10 scored every 24 h. At 25°C, worms produce progeny that develop into adults in 3 days and it  
11 is therefore difficult to identify the original worms. To avoid over-estimating the number of  
12 living worms, the original worms were transferred daily to fresh mNGM plates for 4 days  
13 until completing their egg-laying phase at 7 days of age. The worms were then transferred to  
14 fresh mNGM plates every second day. A worm was considered dead when it failed to respond  
15 to a gentle touch with a worm picker. Worms that died as a result of adhering to the wall of  
16 the plate were not included in the analysis. Nematocidal assays are generally performed using  
17 NGM agar plates containing peptone, which allows the overlaid bacteria to proliferate.  
18 However, the composition of NGM has been reported to influence the virulence of bacteria,  
19 and the *in-situ* production of metabolites by bacteria growing on the medium could also be  
20 fatal to nematodes (Anyanful et al., 2005). Thus, to exclude the possibility of  
21 bacterial-induced nematocidal effects from nutrients in the medium, the nematocidal assays  
22 were performed on mNGM plates lacking peptone. Each assay was carried out in duplicate  
23 and repeated twice unless otherwise stated.

24 Mean lifespan was estimated using the formula (Wu et al., 2006):

25 
$$\text{MLS} = \frac{1}{N} \sum_j \frac{x_j + x_{j+1}}{2} d_j$$

1 where  $d_j$  is the number of worms that died in the age interval  $(x_j, x_{j+1})$ , and  $N$  is the total  
2 number of worms. The standard error of the estimated mean lifespan was calculated using the  
3 equation:

$$4 \quad SE = \sqrt{\frac{1}{N(N-1)} \sum_j \left( \frac{x_j + x_{j+1}}{2} - \text{MLS} \right)^2 d_j.}$$

5 Maximum lifespan was calculated as the mean lifespan of the longest-living 15% of worms in  
6 each group.

7

8 Locomotory scoring of ageing nematodes

9

10 The motility of worms at different ages was examined using a scoring method described in  
11 previous reports (Gruber et al., 2011; Hosono et al., 1980). Briefly, worms were classified as  
12 class “A” worms when they showed spontaneous movement or vigorous locomotion in  
13 responding to prodding; class “B” worms were those that did not move unless prodded or  
14 appeared to have uncoordinated movement; and class “C” worms were those that moved only  
15 their head and/or tail in response to prodding. Dead worms were classified as class “D”. A  
16 minimum of 70 worms fed OP or BI were scored.

17

18 Lipofuscin

19

20 The autofluorescence of intestinal lipofuscin was analyzed as an indices of senescence from  
21 days 10 to 18 of adulthood. Briefly, randomly selected worms were washed five times in M9  
22 buffer and then placed onto fresh mNGM plates coated with 50 mM sodium azide to induce  
23 anesthesia. Lipofuscin autofluorescence was measured using a M165 FC fluorescence  
24 stereomicroscope (Leica Microsystems, Tokyo, Japan) equipped with a DsRED filter set



1 (excitation, 510-560 nm; emission, 590-650 nm) and a Leica DFC425 C digital microscope  
2 camera. The captured data was analyzed using Leica Application Suite imaging software  
3 (Version 3.7.0).

4

#### 5 Protein carbonyl formation

6

7 As biomarkers of oxidative stress, protein carbonyl groups were measured according to the  
8 method of Yang et al. (2007) with minor modifications. For each test group, 100  
9 seven-day-old adult worms fed OP or BI for 4 days were picked, washed 3 times in M9 buffer,  
10 resuspended in 3  $\mu$ l M9 buffer, and stored at -80°C until use. To lyse cells, 3  $\mu$ l lysis buffer  
11 (50 mM Tris-HCl buffer [pH 7.5], 150 mM NaCl, 0.1% SDS, 1% Triton X-100, 1 mM PMSE,  
12 and 2% 2-mercaptoethanol) and 4  $\mu$ l of 15% SDS were added to the samples, which were  
13 then subjected to repeated freeze-thawing to release proteins. After centrifugation at 15,490  $\times$   
14 g for 3 min, protein content was quantitatively measured using Bradford Ultra reagent  
15 (Expedeon Protein Solutions, Cambridge, UK). Detection of carbonyl groups was performed  
16 with the OxyBlot<sup>TM</sup> Protein Oxidation Detection Kit (Millipore, Billerica, USA). An equal  
17 volume of DNP solution (Oxyblot) or derivatization-control solution was added to samples,  
18 which were incubated at room temperature for 15 min. A volume of neutralization buffer  
19 (Oxyblot) equal to that of the DNP solution was then added to each sample. Samples  
20 containing one  $\mu$ g of total protein were then separated in 7.5% or 12% SDS-PAGE gels,  
21 transferred to PVDF membrane (GE Healthcare, Buckinghamshire, UK), and blocked with  
22 1% BSA for 1 h. The membrane was incubated with rabbit anti-DNP antibody (Oxyblot)  
23 (1:150) for 1 h, followed by goat anti-rabbit IgG (Oxyblot) (1:300) at room temperature for 1  
24 h. Membranes were incubated with the ECL Plus detection reagent (GE Healthcare) and then  
25 scanned using a Kodak Digital Science EDAS290SP and processed with Kodak 1D Image

1 Analysis Software. Band densities were analyzed using ImageJ software (developed by the  
2 National Institute of Health). Membranes were incubated with 15% hydrogen peroxide for 30  
3 min at room temperature and treated with anti-actin Clone C4 antibody (Millipore) to derive a  
4 density value for actin for each lane unless otherwise stated. The measured Oxyblot values  
5 were normalized to the density values determined for actin. Each assay was performed at least  
6 twice for each sample.

7

8 Measurement of body size

9

10 Three-day-old adult worms were placed on mNGM plates covered with lawns of BI. The  
11 plates were incubated at 25°C, and the body size of live worms were measured every 24 h  
12 until reaching 7 days of age. Images of adult nematodes were taken with a VCT-VBIT digital  
13 microscope (Shimadzu, Kyoto, Japan) and analyzed using ImageJ software. In this system,  
14 the area of a worm's projection was estimated automatically and used as an index of body  
15 size.

16

17 Brood size

18

19 Eggs isolated with a sodium hypochlorite/sodium hydroxide solution were allowed to develop  
20 to 3 days of age on mNGM plates coated with OP at 25°C. Three hermaphrodites were  
21 selected and transferred to an mNGM plate covered with a lawn of BI. The parental animals  
22 were transferred every 24 h to fresh mNGM plates until the end of the reproductive period.  
23 The resulting progeny were left to develop for 3 days and the progeny number was then  
24 determined. Each assay was performed with five plates and repeated twice.

25

## 1 Stress resistance assays

2

3 Worms were grown from 3 to 7 days of age on mNGM plates with OP or BI and then  
4 subjected to oxidative stress and heat shock assays. To conduct an oxidative stress assay,  
5 worms were transferred onto mNGM containing 1.0 or 2.5 mM paraquat or to M9 buffer plus  
6 0.1% cholesterol (5 mg ml<sup>-1</sup> in ethanol) containing 0.8 or 2.0 mM hydrogen peroxide,  
7 incubated at 25°C, and viability was then scored. To assess thermal tolerance, 7-day-old  
8 worms were placed on M9 buffer or onto mNGM at 35°C and then scored for viability. The  
9 survival of worms was determined by touch-provoked movement. Worms were scored as dead  
10 when they failed to respond to repeated touching with a worm picker. The assays were  
11 performed at least twice.

12

## 13 Quantification of mitochondria

14

15 Measurement of mitochondrial copy number was performed as described by Reinke et al.  
16 (2010) with partial modification. Briefly, 3-day-old worms were placed in a 400-μl of M9  
17 buffer containing 200 μg 2'-deoxy-5-fluorouridine (Tokyo Chemical Industry, Tokyo, Japan),  
18 which was used to block the embryonic development of progeny and avoid contamination  
19 from offspring. The suspension was then spotted on mNGM in 9.0-cm diameter plates.  
20 Worms were fed OP or BI for four days, recovered from the plates, and then washed five  
21 times with M9 buffer. The worms were placed in lysis buffer (0.2 M NaCl, 0.1 M Tris-HCl  
22 [pH 8.3], 50 mM EDTA, 0.5% SDS, and 100 μg ml<sup>-1</sup> proteinase K) and total DNA was then  
23 extracted using phenol/chloroform/isoamyl alcohol (25:24:1) and precipitated with ethanol.  
24 Quantification of mitochondrial genes was performed using an ABI PRISM<sup>®</sup> 7000 Sequence  
25 Detection System (Life Technologies, Carlsbad, CA, USA) and a QuantiTect SYBR Green

1 PCR Kit (Qiagen, Hilden, Germany) for 25- $\mu$ l reaction volumes containing 300 nM of each  
2 primer and 50 ng of DNA template. Reactions were initiated at 95°C for 15 min, followed by  
3 40 cycles of 94°C for 15 s, 60°C for 30 s, and 72°C for 30 s. For quantifying mtDNA, the  
4 *ctb-1* gene, which encodes cytochrome b of complex III, was targeted, while the *atp-2* gene,  
5 which encodes the b-subunit of the ATP synthetase complex, was selected for the nDNA  
6 control. The primer sequences were as follows: *ctb-1* forward,  
7 5'-TGAAGCTGACCCTATAATGAGGC-3' and *ctb-1* reverse,  
8 5'-CCCCTAAGACTTTATTTGGAATAGCAC-3'; and *atp-2*  
9 forward, 5'-GCAACGTTTCAGAAATGCGCT-3' and *atp-2* reverse  
10 5'-TGTTTGAGCTGAGGCGGACT-3'. Standard curves for mtDNA and nDNA were  
11 generated using 10-fold serial dilutions of DNA plasmids containing cloned *ctb-1* or *atp-2*  
12 genes.

13

#### 14 Cell wall fractionation of bifidobacteria

15

16 Cell walls were prepared as described by Tejada-Simon and Pestka (1999) with partial  
17 modification. Briefly, BI was anaerobically grown in GAM broth at 37°C for 48 h. Cells were  
18 harvested by centrifugation at 14,000  $\times$  g for 10 min at 4°C, washed once with sterile distilled  
19 water, and then centrifuged 3 times at 14,000  $\times$  g for 10 min. The cells were heated at 100°C  
20 for 50 min for use as heat-killed bacteria. For preparation of the cell wall fraction, bacteria  
21 were disrupted by sonication for 30 min on ice using an UH-50 Ultrasonic Homogenizer  
22 (SMT, Tokyo, Japan). After heating the suspension at 60°C for 15 min to inactivate autolytic  
23 enzymes, the suspension was centrifuged at 800  $\times$  g for 30 min at 4°C, and the pellet  
24 (unbroken cells) was removed. Cell walls were sedimented from the supernatant by  
25 centrifugation at 40,000  $\times$  g for 30 min at 4°C using an Optima<sup>TM</sup> MAX-E Ultracentrifuge

1 (Beckman, Brea, CA, USA). The pellet was further treated with PRONASE<sup>®</sup> Protease (0.78  
2 units mg<sup>-1</sup> of crude cell wall) (Merck, Darmstadt, Germany), ribonuclease A (250 µg ml<sup>-1</sup>)  
3 (MP Biomedicals, Illkirch, France), and DNase I (250 µg ml<sup>-1</sup>) (Wako, Osaka, Japan) in 0.1  
4 M Tris-HCl buffer (pH 7.4) at 37°C overnight to eliminate contaminating cytoplasmic  
5 material. The treated cell wall fraction was centrifuged at 800 × g for 30 min at 4°C followed  
6 by centrifugation at 40,000 × g for 30 min, weighed, heated to 90°C for 15 min, and then  
7 resuspended at a concentration of 200 mg ml<sup>-1</sup> in M9 buffer. The cell wall solutions were filter  
8 sterilized through a 0.45-µm filter and kept at -45°C until used as feed for *C. elegans*.

9

#### 10 Bifidobacteria protoplast formation

11

12 Protoplast formation was performed as described elsewhere with minor modifications  
13 (Lee-Wickner and Chassy, 1984). Briefly, protoplast formation buffer (20 mM HEPES  
14 (2-[4-(2-Hydroxyethyl)-1-piperazinyl] ethanesulfonic acid), 1 mM MgCl<sub>2</sub>, 0.5% gelatin, and  
15 500 mM lactose as a stabilizer; pH 7.0) was prepared and filter sterilized through a 0.45-µm  
16 filter. BI cells anaerobically grown on TOS propionate agar at 37°C for 48 h were harvested  
17 using a platinum loop, washed thrice in M9 buffer, and suspended at 10 mg/ml in the  
18 protoplast formation buffer supplemented with 25 µg ml<sup>-1</sup> N-acetylmuramidase SG  
19 (Seikagaku Biobusiness, Tokyo, Japan) and 1 mg ml<sup>-1</sup> lysozyme (MP Biomedicals). The  
20 resulting mixture was incubated at 37°C for 3 h, and the cells were then washed 3 times with  
21 protoplast formation buffer. Protoplast formation was confirmed by the Gram negative-like  
22 appearance of cells following Gram staining, the KOH method (Gregersen, 1978), and the  
23 quantification of peptidoglycan using the SLP Reagent Set (Wako).

24

#### 25 Statistical analysis

1

2 Nematode survival was calculated by the Kaplan-Meier method, and survival differences  
3 were tested for significance using the log-rank test. Differences in protein carbonyl levels and  
4 brood size were determined using the Student's *t*-test. Differences in lipofuscin levels were  
5 analyzed using the Mann-Whitney's U test. Differences in body sizes were analyzed using the  
6 Kruskal-Wallis test. The level of significance for survival analysis was set at  $P < 0.01$ , as  
7 indicated.

8

9

## 10 **Results**

11

### 12 Prolongevity effects of bifidobacteria

13

14 To ascertain whether the prolongevity effect of bifidobacteria on *C. elegans* is dose dependent,  
15 young adult worms fed OP for 2 days after hatching were transferred to plates containing BI  
16 mixed with OP at various ratios. Under these conditions, the lifespan of nematodes was  
17 significantly extended with increasing BI concentration (Table 1, Fig. 1).

18 In *C. elegans*, muscle function and accumulation of lipofuscin, as determined by gut  
19 autofluorescence, correlate with age, but vary between aged-matched individuals (Pincus and  
20 Slack, 2010). Here, locomotory ability was assayed as an indicator of muscle function, as  
21 locomotory class is predictive for the remaining lifespan of *C. elegans* worms after 8 days of  
22 age (Hosono et al., 1980). The ratio of worms displaying coordinated sinusoidal locomotion  
23 (class A) was clearly higher in the group fed BI than that of control worms (Fig. 2).

24 We also found that autofluorescence of lipofuscin, a lipid peroxidation product, was  
25 significantly lower among worms fed BI than that of control worms (Fig. 3a-c). For this

1 reason, the protein carbonyl content, which is a general biomarker of protein oxidation, was  
2 also measured in treated and control worms. The abundance of protein carbonyls was greater  
3 in the extracts from control worms than those from worms fed BI (Fig. 4a, b).

4 As mitochondria are involved in the oxidation of biomolecules, and because both  
5 lipofuscin and protein carbonyls were increased in control worms compared to the levels in  
6 worms fed BI, the copy number of mitochondria in 3- and 7-day-old worms was measured  
7 using real-time PCR. However, no significant differences in the copy number of the *ctb-1*  
8 gene, which resides on the mitochondrial chromosome, were detected between worms fed OP  
9 and those fed BI, irrespective of their ages (data not shown), indicating that the number of  
10 mitochondria was not affected by BI feeding.

11

## 12 Body and brood sizes

13

14 To examine whether the prolongevity effects of BI were a result of caloric reduction, the body  
15 and brood sizes of nematodes fed BI were compared with those of control worms fed OP. The  
16 feeding of nematodes BI did not alter the growth rate or body size of worms (Fig. 5).  
17 Although caloric restriction reportedly extends the lifespan of nematodes, (Bishop and  
18 Guarente, 2007), here, the mixed feeding of BI and OP not only extended the lifespan of *C.*  
19 *elegans*, but also increased the brood size of worms (Fig. 6).

20

## 21 Stress resistance assays

22

23 We previously reported that lactobacilli and bifidobacteria confer tolerance to bacterial  
24 infections in *C. elegans* (Ikeda et al., 2007; Komura et al., 2010). Here, the effects of feeding  
25 worms BI on resistance to physical and chemical stresses were examined. BI treatment

1 failed to improve the survival of worms against exposure to 1.0 and 2.5 mM paraquat, or  
2 35°C heat stress (data not shown). In addition, when 7-day-old worms were maintained in M9  
3 buffer containing either 0.8 or 2.0 mM hydrogen peroxide, the worms fed BI were more  
4 vulnerable than the controls, as exhibited by the shorter survival time of the former (Fig. 7a  
5 and 7b). Notably, BI-treated 3-day-old adult worms were killed more quickly on exposure to  
6 hydrogen peroxide than 7-day-old nematodes.

7

#### 8 Prolongevity-associated components of bifidobacteria

9

10 To determine whether the lifespan-extending factor of bifidobacteria is located in the cell wall,  
11 worms were fed the cell walls or protoplasts, which were negative for peptidoglycan, of BI.  
12 However, the lifespan of worms was prolonged similarly by cell walls and protoplasts (Fig. 8a  
13 and 8b).

14

#### 15 Prolongevity effects in mutants

16

17 The PMK-1 (p38 mitogen-activated protein kinase; p38 MAPK) and DAF-2/DAF-16  
18 insulin-like signaling pathways contribute to host defense against pathogens. To investigate  
19 whether these two pathways are involved in the prolongevity effects of BI, the lifespan of  
20 several *C. elegans* loss-of-function mutants fed BI was compared with that of control worms  
21 fed OP (Figs. 9 and 10). BI failed to prolong the lifespans of *pmk-1* and *skn-1* mutants. As  
22 these results suggested the importance of the p38 MAPK pathway for the prolongevity effect  
23 of BI, mutants of genes (*tol-1*, *tir-1*, *jnk-1*, and *vhp-1*) that are possibly associated with the  
24 p38 MAPK pathway were also examined. BI prolonged the mean lifespans of the *tol-1*, *tir-1*,  
25 and *jnk-1* mutants by 113% to 155%, whereas that of the *vhp-1* mutant was not extended by



1 BI treatment. Concerning the insulin/IGF-1 signaling (IIS) pathway, BI treatment extended  
2 the lifespan of the *daf-16* *C. elegans* mutant; however, the lifespan of the *daf-2* mutant was  
3 not markedly affected. A mutant of *aak-2*, which encodes the low-energy sensing  
4 AMP-activated protein kinase AMPK, was also used to investigate the contribution of caloric  
5 restriction to the longevity effect of BI. Upon BI feeding, the lifespan of the *aak-2* mutant  
6 was clearly extended.

7

8

## 9 **Discussion**

10

11 Based on the observed longevity of Bulgarians who consumed large quantities of yogurt,  
12 Metchnikoff hypothesized over a century ago that lactobacilli are important for human health  
13 (Metchnikoff, 1907). Previously, we demonstrated that lactobacilli and bifidobacteria modify  
14 the host defense and prolong the lifespan of nematodes (Ikeda et al., 2007; Komura et al.,  
15 2012; Komura et al., 2010); however, the mechanism by which these probiotic bacteria affect  
16 the longevity of bacteriovorous nematodes remained unclear. In the present study, we have  
17 shown that bifidobacteria can increase the average lifespan of *C. elegans* in a dose-dependent  
18 manner due to components present in the bacterial cell wall. Our findings suggest that *C.*  
19 *elegans* is a useful model for evaluating the potential of probiotic bacteria as biological  
20 response modifiers in higher eukaryotes, because LAB, including bifidobacteria, have  
21 developed symbiotic relationships with a variety of animals prior to the appearance of humans  
22 on earth.

23 When BI mixed with OP was fed to *C. elegans*, the lifespan of worms was extended in a  
24 BI dose-dependent manner. The worms fed BI clearly displayed more locomotion than  
25 age-matched control worms. The typical protein modification associated with oxidative stress

1 is the addition of carbonyl groups to amino acid side chains. Here, the accumulation of  
2 lipofuscin, a marker of aging, and the levels of protein carbonyls were lowest in worms fed  
3 bifidobacteria. The total amount of ROS leaking from mitochondria is thought to be small and  
4 result in lower lipofuscin and protein carbonyl levels in BI-fed worms (Cannizzo et al., 2011;  
5 Pincus and Slack, 2010). Although the concept that caloric restriction upregulates  
6 mitochondrial biogenesis and may confer anti-aging effects is generally accepted (Guarente,  
7 2008), we did not observe a difference in the number of mitochondria in response to BI  
8 feeding.

9 Caloric restriction is well recognized as a method to extend longevity and has been shown  
10 to slow aging not only in numerous non-mammalian taxa, but also in mammals (Ingram et al.,  
11 2006). However, it is unlikely that bifidobacteria have lower caloric energy or are simply  
12 more indigestible than OP because the growth curves of nematodes were similar regardless of  
13 the amount of BI mixed with OP. Furthermore, the BI-fed nematodes produced more  
14 offspring than the control worms, a finding that is in contrast to a study by Bishop and  
15 Guarente (2007), who showed that although dietary restriction can extend the life of worms,  
16 their brood sizes are smaller than worms fed a normal diet (Bishop and Guarente, 2007).

17 We previously observed that LAB and bifidobacteria suppress the age-associated increase  
18 in sensitivity of *C. elegans* to bacterial infection (Ikeda et al., 2007; Komura et al., 2010).  
19 Consequently, we anticipated that the host defense mechanisms to a variety of stresses would  
20 be enhanced in worms fed bifidobacteria. The lower lipofuscin and protein carbonyl levels in  
21 BI-fed worms were taken as evidence of enhanced antioxidant systems. However, the  
22 BI-induced prolongevity was unlikely due to the non-specific enhancement of host defense  
23 systems, as the worms were as vulnerable to heat and paraquat as the control worms. In  
24 addition, the BI-fed worms had lower resistance to the oxidative stress induced by hydrogen  
25 peroxide than the control worms; however, this finding may reflect the degree of aging of

1 BI-fed worms, as 7-day-old BI-fed worms exhibited similar susceptibility to hydrogen  
2 peroxide as 3-day-old control worms. If age was not an influential factor, this phenomenon  
3 would be consistent with the trade-off of host defense reported by Marsh et al. (2011), who  
4 observed that deletion of the protist-type lysozyme LYS-7 in nematodes enhanced tolerance  
5 to *Salmonella*, but increased susceptibility to *Cryptococcus neoformans*.

6 BI failed to prolong the lifespan of the *C. elegans pmk-1* and *skn-1* mutants, suggesting  
7 that the p38 MAPK pathway contributes to the longevity effect of bifidobacteria in  
8 nematodes (Fig. 11). BI appears to promote the induction of phase 2 detoxification enzymes  
9 in *C. elegans* through activation of SKN-1, an ortholog of mammalian Nrf transcription  
10 factors (Inoue et al., 2005). The p38 MAPK pathway elicits an immune response distinct from  
11 that associated with DAF-16 (Troemel et al., 2006) via the activation of *skn-1* (Inoue et al.,  
12 2005; Van der Hoeven et al., 2011). Recently, *Lactobacillus acidophilus* was also reported to  
13 function as an immunomodifier in *C. elegans* via signals transmitted by the p38 MAPK  
14 pathway (Kim and Mylonakis, 2012). As we found that BI prolonged the lifespans of *tol-1*  
15 and *tir-1* mutants of *C. elegans*, the pattern recognition system in innate immunity is not  
16 likely involved in the activation of p38 MAPK by BI. PMK-1 is inactivated by the MAPK  
17 phosphatase VHP-1, whose suppression reportedly increases PMK-1 phosphorylation and  
18 increases resistance to pathogens (Kim et al., 2004). It is likely that the longevity effect of  
19 BI was reduced in the *vhp-1* mutant because *pmk-1* was upregulated irrespective of BI  
20 treatment in the absence of VHP-1-mediated suppression. It also seems likely that BI induces  
21 longevity in *C. elegans* via suppression of *vhp-1*, a speculation that is consistent with the  
22 fact that the BI factor associated with longevity would not interact with the MAPK  
23 phosphatase VHP-1 in the *vhp-1* mutant. In addition, the comparatively shorter lifespan of the  
24 *vhp-1* mutants is consistent with the adverse effects of excessive SKN-1 activity in *C. elegans*  
25 (Papp et al., 2012).

1        Although *C. elegans* is bacteriophagous, bacteria typically consumed as a food source can  
2 be pathogenic to older nematodes (Garigan et al., 2002). Therefore, the activation of host  
3 defense systems via *pmk-1* through the ingestion of BI may have contributed to the observed  
4 longevity effects. However, the increased lifespan of the BI-fed worms is unlikely  
5 explained solely by the upregulation of antimicrobial defense systems, as these worms lived  
6 longer than those fed heat-killed OP (Ikeda et al., 2007). As no differences in the number of  
7 pathogens recovered after infection with *Salmonella* or *Legionella* were detected between BI-  
8 and OP-fed worms in our previous studies (Ikeda et al., 2007; Komura et al., 2010), it is also  
9 unlikely that the enhanced survival of worms observed here was due to increased expression  
10 of antibacterial factors induced by BI. Through experiments using *Drosophila melanogaster*  
11 as a model host for *Salmonella* infection, Shinzawa et al. (2009) also reported that p38 MAPK  
12 is involved in host tolerance to this pathogen. As SKN-1 appears to be involved in damage  
13 control and stress resistance (Oliveira et al., 2009), endogenous antioxidant systems  
14 upregulated via activated SKN-1 may account for the enhanced tolerance to pathogens,  
15 extension of lifespan, and reduced accumulation of lipofuscin and protein carbonyls in worms  
16 fed BI. Notably, however, the activity of these antioxidant systems was insufficient to protect  
17 cells from exogenous oxidants. Oliveira et al. (2009) reported that most genes controlled by  
18 SKN-1 under normal conditions are not upregulated in response to chemical stress caused by  
19 sodium arsenite or butyl hydrogen peroxide; thus, SKN-1 appears to act together with other  
20 regulators involved in specialized responses to exogenous stresses. Future experiments  
21 comparing transcriptional changes in *pmk-1* mutant and wild-type nematodes fed BI are  
22 expected to reveal which genes under the control of *pmk-1* contribute to longevity in *C.*  
23 *elegans*.

24        Downstream of the insulin/IGF-1 signaling (IIS) pathway, which has a conserved role in  
25 modulating lifespan, the forkhead family transcription factor DAF-16 regulates genes that

1 promote stress resistance and extend the lifespan of nematodes (Lee et al., 2003). Heat-shock  
2 transcription factor (HSF)-1 is one such gene under the regulation of DAF-16 also plays an  
3 important role for host defense (McColl et al., 2010; Singh and Aballay, 2006). Oh et al.  
4 (2005) found that the c-Jun N-terminal kinase (JNK) family, a subgroup of the MAPK  
5 superfamily, is a positive regulator of DAF-16. As JNK is part of a signal transduction  
6 cascade in mammals that is activated by cytokines, including TNF and IL-1, it is possible that  
7 bifidobacteria enhance host defense systems and extend the lifespan of nematodes by  
8 activating DAF-16 via the JNK pathway. However, we found that neither the *daf-16* nor *jnk-1*  
9 genes were necessary for the longevity effect of BI in *C. elegans*. Although worms with  
10 activated DAF-16 should have upregulated *sod-3* expression and exhibit resistance to heat or  
11 chemical oxidants (Murphy et al., 2003), BI feeding did not increase the resistance of worms  
12 to either heat or oxidants in the present study. As the signal induction systems involved in host  
13 defense should respond to stressors and modulate stress responses to minimize damage to the  
14 host, it is logical that the longevity induced by BI feeding does not necessarily lead to  
15 nonspecific resistance to chemical oxidants. [

16 BI failed to prolong the lifespan of the *daf-2* mutant. At first glance, this result appears  
17 contradictory to the longevity effects of BI observed for the *daf-16* mutant; however, this  
18 discrepancy is likely because not only the p38 MAPK, but also the IIS pathway, for which  
19 DAF-2 is the only insulin/IGF-1 receptor, regulates SKN-1, which modulates both oxidative  
20 and xenobiotic stress responses in *C. elegans* (Inoue et al., 2005; Tullet et al., 2008). It is  
21 possible that the *skn-1* gene was no longer under control of DAF-2 signaling and was  
22 sufficiently activated in the *daf-2* mutant to enhance host resistance, with the addition of BI  
23 having little additional effect on the activation of SKN-1. Once the *skn-1* gene is no longer  
24 suppressed by the IIS pathway, SKN-1 seems to act against the *daf-28* and *ins-39* genes,  
25 which encode insulin-like substances, suggesting that SKN-1 could cause a positive feedback,

1 thereby mitigating the enhancement of SKN-1 by BI (Okuyama et al., 2010). Although the  
2 lack of the prolongevity effect of BI in *daf-2* is indicative of caloric restriction, dietary  
3 restriction has been shown to extend the lifespan of *daf-2 C. elegans* mutants (Bishop and  
4 Guarente, 2007). Furthermore, successful prolongation of the lifespan of the *aak-2* mutant fed  
5 BI also negates the possibility that BI induces longevity via caloric restriction, as AAK-2  
6 plays an important role in prolongevity in response to caloric restriction (Apfeld et al., 2004).

7 The cell walls of LAB are thought to be primarily responsible for immunostimulation, and  
8 differences in the cell wall composition of probiotic organisms may account for the various  
9 levels of immune activation observed in hosts (Rutherford-Markwick and Gill, 2004). Here,  
10 the lifespan of worms fed OP together with the cell wall fraction of BI was clearly extended.  
11 Hence, we assumed that the protoplast of BI does not induce longevity in worms. Surprisingly,  
12 however, the feeding of BI protoplasts to *C. elegans* extended the mean lifespan of worms.  
13 Among the possible component(s) that are present in both protoplasts and cell walls, we  
14 speculate that lipoteichoic acid might be the responsible factor, as it may have remained on  
15 the bacterial cell membrane even after the formation of protoplasts by enzymatic treatment.

16 In conclusion, the findings from the present study suggest that bifidobacteria extend the  
17 lifespan of *C. elegans*, presumably via modulation of the p38 MAPK pathway, but not via  
18 caloric restriction. Notably, we found that the cell wall of bifidobacteria plays a key role in  
19 increasing the longevity of nematodes. After we previously reported that *C. elegans* is an  
20 appropriate model to screen for useful probiotic strains, two groups succeeded in selecting  
21 potentially therapeutic strains using this model (Lee et al., 2011; Wang et al., 2011). However,  
22 time-consuming lifespan analyses need to be performed with these strains, as the mechanisms  
23 involved in the prolongevity effects remain to be clarified. Our present findings suggest that a  
24 gene involved in the p38 MAPK pathway can be utilized as an indicator of prolongevity  
25 induced by bifidobacteria. If this *C. elegans* gene is identified and labeled with GFP, it would

- 1 be possible to screen for probiotic strains by observing GFP expression in worms after the
- 2 ingestion of candidate bacteria.
- 3

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2

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## Figure legends

**Fig. 1.** Survival curves of *C. elegans* fed OP mixed with BI at different ratios as compared with the lifespan of control worms fed only OP. Each plate contained 10 mg (wet weight) of bacteria. Worms were 3-days-old on Day 0. The table summarizes the data obtained from all experiments, and the curve was drawn based on the results of a representative experiment.

**Fig. 2.** Locomotory activity of *C. elegans* fed BI. Young adult worms fed OP for 2 days after hatching were transferred to plates containing 10 mg of either OP or BI on the surface. Worms were 3-days-old on Day 0. Animals were classified into four classes based on their locomotion: class A, robust, coordinated sinusoidal locomotion (blue bars); class B, uncoordinated and/or sluggish movement (yellow bars); class C, no forward or backward movement, but head movements or shuddering in response to prodding (red bars); and class D, dead animals (black bars). The rates of each class at the indicated time point are indicated.

**Fig. 3.** Lipofuscin accumulation in the intestine of nematodes. (a) Intestinal autofluorescence from lipofuscin in age-synchronized worms fed OP was apparent on day 14, but was weaker in worms fed bifidobacteria (b). (c) Fluorescence on days 14, 16, and 18 of adulthood was quantified using ImageJ software to determine the lipofuscin levels. Each bar represents the average values of ten worms, except for the bar corresponding day 18, for which six OP-fed worms. The bar graph depicts the percent difference of the mean value in arbitrary units relative to that of control worms fed OP on day 14. Lipofuscin fluorescence was weaker among worms fed BI compared with control worms fed OP. \*\* indicates a statistically significant difference from control worms fed OP at a  $p$  value of  $< 0.01$ . Error bars represent the SE.

**Fig. 4.** Influence of BI-feeding on protein oxidation evaluated using an OxyBlot kit. Non-derivatized proteins were used as negative controls for each sample. (A) Proteins recovered from lysed worms fed BI or OP were labeled with DNP solution (Oxyblot) to show the presence of protein carbonyls and were separated on SDS-PAGE gels (lanes OP and BI). Derivatization-control proteins were also included (lanes BI-neg and OP-neg). After electrophoresis, the proteins were transferred to a PVDF membrane and visualized using a chemiluminescence method. The membrane was re-probed with anti-actin antibody to correct for loading variation. (B) Band densities were quantified to determine the protein carbonyl levels using ImageJ software. The bar graph depicts the percent difference of the mean value of five independent experiments (error bars represent the S.E.) in arbitrary units relative to that of control worms fed OP. Actin or coloration of CBB-stained bands was used to compensate for loading variation. \* indicates a statistically significant difference from control worms fed OP at a  $p$  value of 0.037.

**Fig. 5.** Growth curve of worms fed OP mixed with BI at ratios of 3:7 or 1:4. Images of adult nematodes were taken with a digital microscope, and the area of the worm's projection was measured and used as an index of body size. The body sizes of the worms fed BI at all ratios were similar to those of the control worms fed only OP. No value reached the significance limit of  $p < 0.05$  compared with control worms. All results are presented as means  $\pm$  SD.

**Fig. 6.** Brood size of BI-fed worms. Total brood size was determined from 30 animals. \* indicates statistical significance at  $p < 0.05$  compared to the control worms fed OP only. Error bars represent the SE.



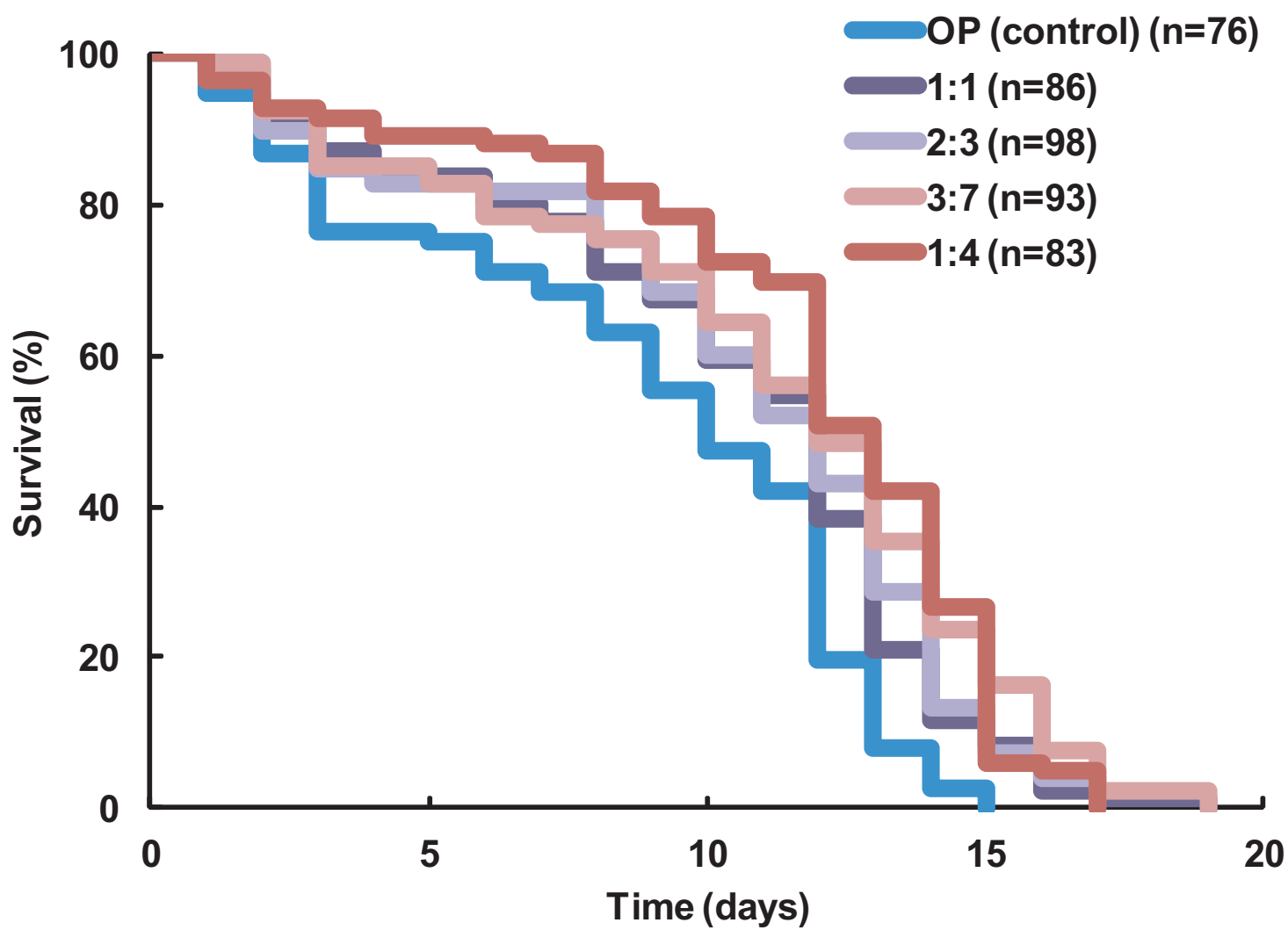
**Fig. 7.** Influence of BI-feeding on susceptibility of worms to oxidative stress caused by hydrogen peroxide. Worms were fed BI for 4 days beginning from 3 days of age and then incubated with (a) 0.8 mM or (b) 2 mM hydrogen peroxide.  $^{\S} p = 1.1\text{E-}10$ ,  $^{\dagger} p = 1.73\text{E-}13$ ,  $^{\Psi} p = 0.002176$ , and  $^{\ddagger} p = 2.83\text{E-}06$ , as compared to the control worms exposed to hydrogen peroxide from 7 days of age.

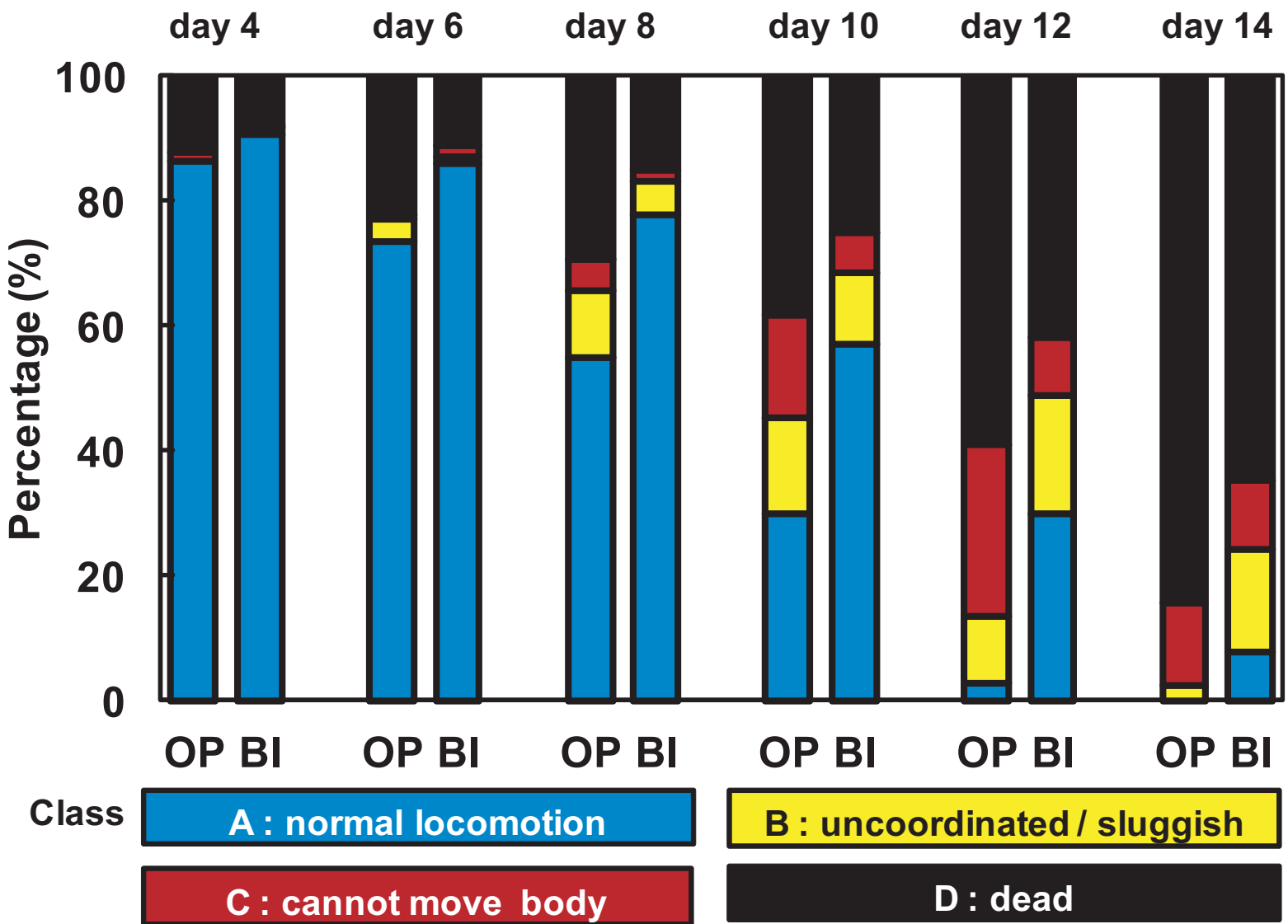
**Fig. 8.** Survival curves of *C. elegans* fed the protoplasts or cell walls of bifidobacteria mixed with OP. Adult worms fed OP for 3 days after hatching were placed onto plates containing OP (2 mg) mixed with the cell wall (8 mg) or protoplasts of BI as a food source. Worms were 3 days of age at day 0. The lifespan of worms was prolonged similarly by cell walls (a) and protoplasts (b). The table shows the data obtained from all experiments, and the curves were drawn based on representative experiments.

**Fig. 9.** Effects of bifidobacteria on the lifespan of *C. elegans* mutants. Survival curves of (a) *pmk-1(km25)*, (b) *skn-1(zu67)*, (c) *vhp-1(sa366)*, (d) *tol-1(nr2033)*, and (e) *tir-1(ok1052)* mutant hermaphrodites fed with or without BI from 3 days of age are shown. The worms were 3 days of age at day 0. The table summarizes the data obtained from all experiments, and the curves were drawn based on representative experiments.

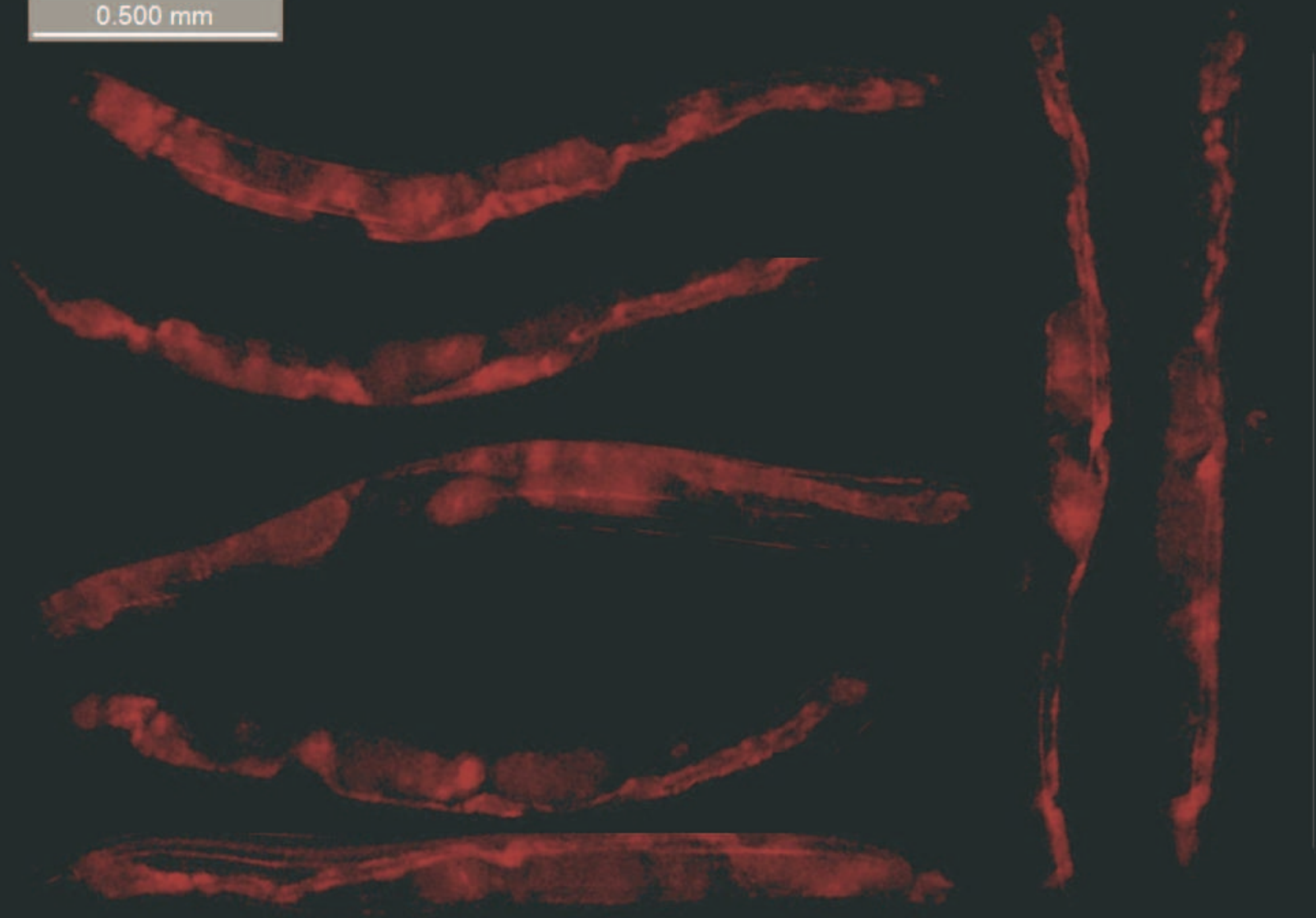
**Fig. 10.** Effects of bifidobacteria on the lifespan of *C. elegans* mutants. Survival curves of (a) *daf-16(mu86)*, (b) *daf-2(e1370)*, (c) *jnk-1(gk-7)*, and (d) *aak-2(ok524)* mutant hermaphrodites fed with or without BI from 3 days of age are shown. The worms were 3 days of age at day 0; however, the *daf-2* mutants were 7 days of age at day 0 due to their slow growth. The tables summarize the data of all experiments, and the curves were drawn based on a representative experiment.

**Fig. 11.** Pathways predicted to be involved in the prolongevity effect of bifidobacteria. Red lines indicate signals that upregulate or activate genes, and black lines indicate signals involved in suppression. Established pathways are shown by solid lines. Dotted lines indicate signals or genes whose contribution to the prolongevity effects of BI was estimated to be minimal. Dashed lines suggest hypothetical pathways that remain to be elucidated.

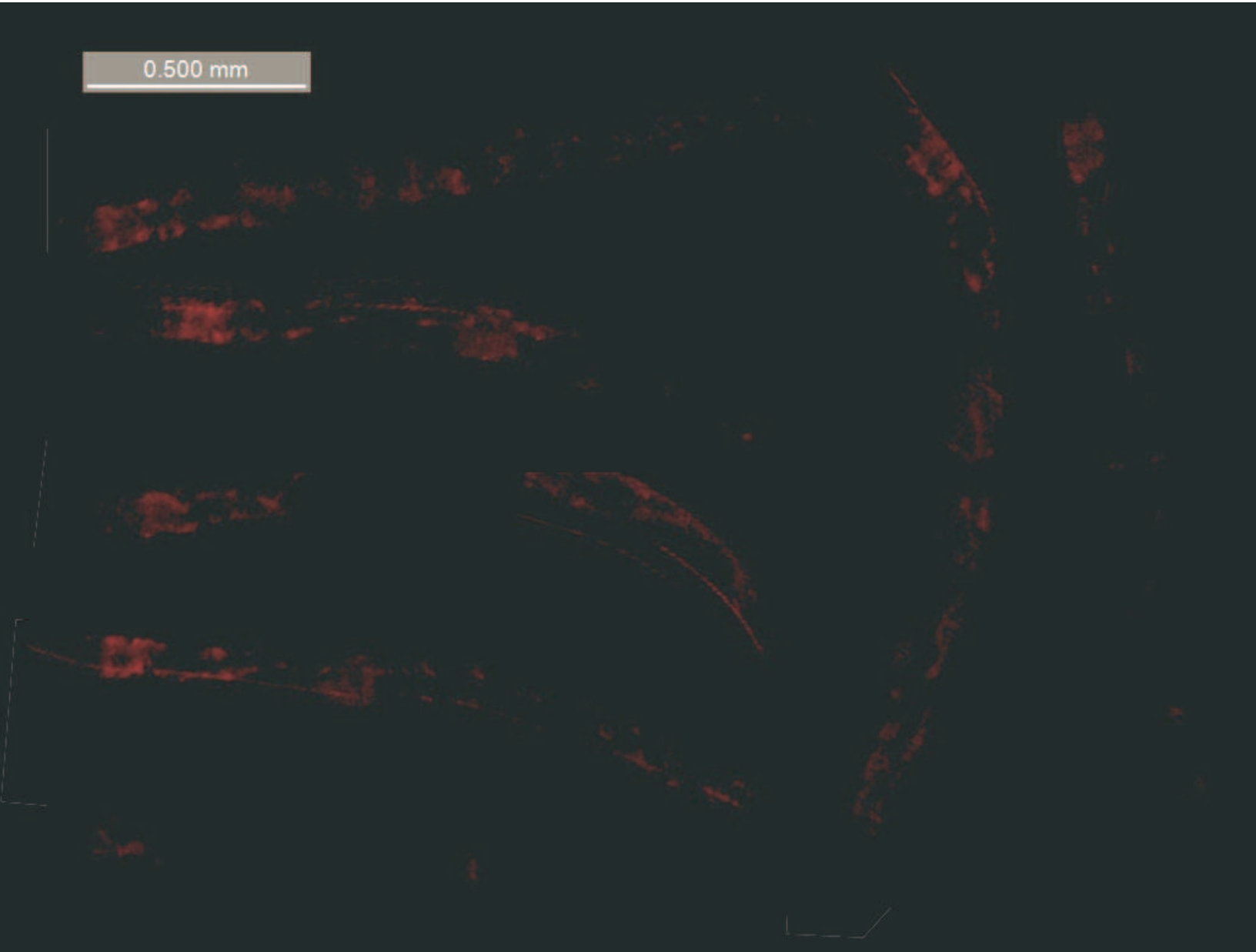


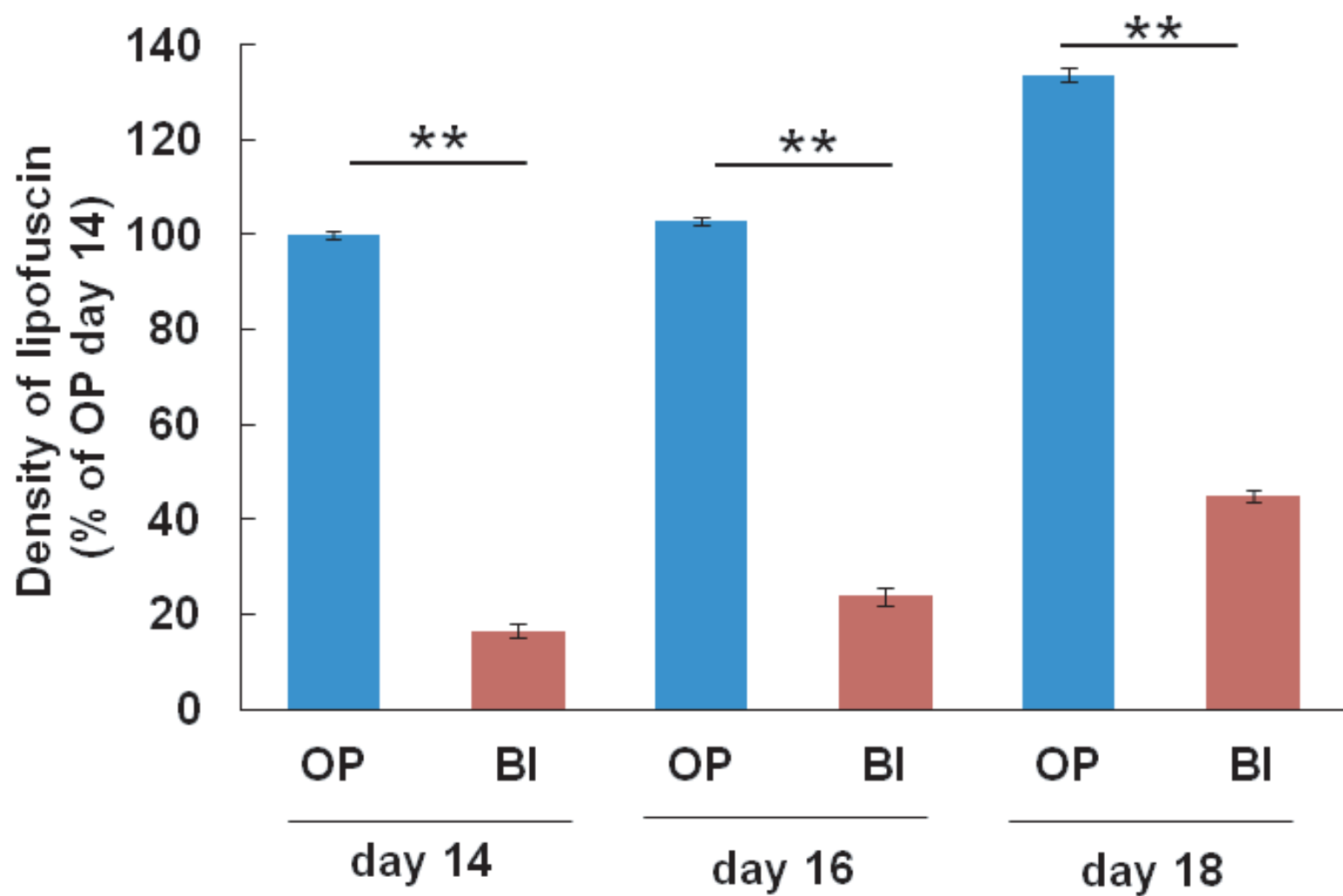


0.500 mm



0.500 mm



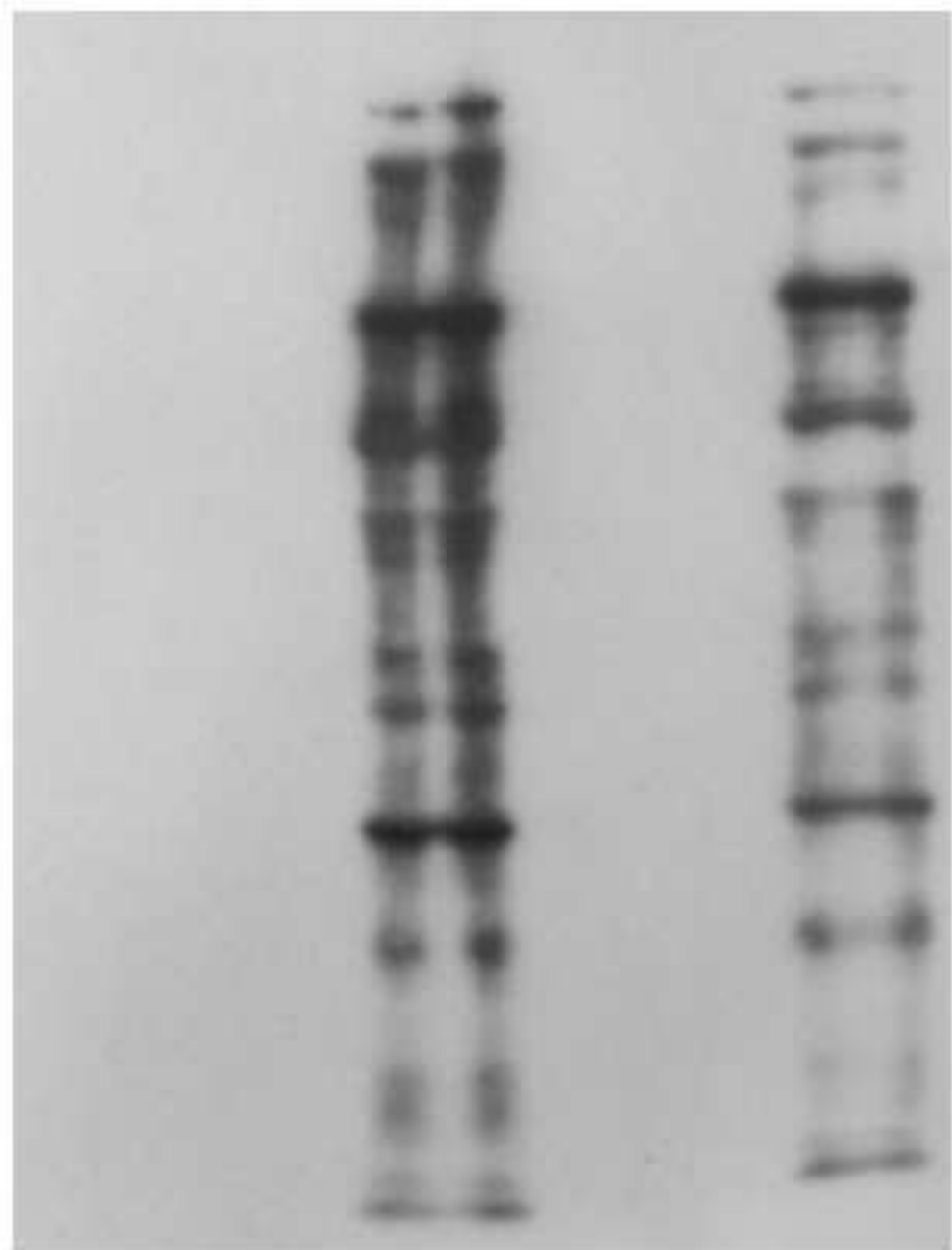


OP neg

OP

BI neg

BI



**Actin**





