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C. elegans Behavior of Preference Choice on Bacterial Food

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***Caenorhabditis elegans* is a free living soil nematode and thus in its natural habitat, *C. elegans* encounters many different species of soil bacteria. Although some soil bacteria may be excellent sources of nutrition for the worm, others may be pathogenic. Thus, we undertook a study to understand how *C. elegans* can identify their preferred food using a simple behavioral assay. We found that there are various species of soil bacteria that *C. elegans* prefers in comparison to the standard laboratory *E. coli* strain OP50. In particular, two bacterial strains, *Bacillus mycoides* and *Bacillus soli*, were preferred strains. Interestingly, the sole feeding of these bacteria to wild type animals results in extended lifespan through the activation of the autophagic process. Further studies will be required to understand the precise mechanism controlling the behavior of identification and selection of food in *C. elegans*.**

INTRODUCTION

All living organisms have the natural ability to acquire and select their food from a diverse range of available food, and this ability helps them to survive in their natural habitat. The first important step during food acquisition is to locate the food and then evaluate its nutritive quality. The analysis of food quality then helps in making the decision to either further continue the search for food or dwell on that particular food. When given a choice, it has been reported that animals select their food according to their environment and dietary requirements. Rats were able to discriminate between the imbalanced diet and a diet supplemented with a lacking nutrient (in the referred study, 0.1% of L-histidine-HC1) even when the texture was nearly identical (Sanahuja and Harper, 1962). This group has repeatedly demonstrated that rats avoid protein-free diets (Harper, 1967) in choosing between diets containing from 6 to 30% protein, but will choose a diet containing a low to moderate level of protein (5 to 30%) rather than a high-protein diet (50 to 80%) (Anderson and Li, 1987; Anderson et al., 1968; Leung et al., 1981). However, cats tend to consume less of a high soy-protein diet when it was offered one together with a moderate level of protein, possibly suggesting an attempt to limit the total protein intake (Cook et al., 1985). These various studies suggest that, if given an opportunity, all organisms have different requirements and choose their food accordingly.

Caenorhabditis elegans is a free living soil nematode., thus in its natural habitat, it may interact with various soil bacteria, a main food source source for worms. There are various types of soil bacteria present in natural conditions, which may vary from being pathogenic/harmful to beneficial and palatable for *C. elegans*. *E. coli*, normally present as normal flora in the lower intestine of warm-blooded animals, is used as the standard food for *C. elegans* in laboratory conditions even though it is an unnatural food source for nematodes in soil. Thus, we undertook a study to understand whether a soil nematode like *C. elegans* shows some preference towards natural soil bacteria in comparison to laboratory *E. coli*. We tested various soil bacteria and classified them into either positive or negative food sources based on their choice index assay.

Secondly, we studied the ability of *C. elegans* to eat the preferred bacteria by analyzing their pharyngeal pumping. Pharyngeal pumping can be described as the sequence of rapid and rhythmic muscle contractions of the pharynx that helps the worm to take food into the pharynx and to masticate food with the grinder before moving it into the intestine (Avery and Horvitz, 1989). Pharyngeal pumping is a coordinated and regulated process and the rate of pharyngeal pumping is tightly regulated under normal conditions and changes with the availability of food (Avery and Thomas, 1997). Finally, we correlated the food preference with lifespan on the given food source to estimate the initial decision of *C. elegans* in choosing a particular soil bacterium in comparison to the laboratory food *E. coli* strain OP50.

MATERIALS AND METHODS

C. elegans strains and culture conditions

Standard methods for maintaining *C. elegans* strains were used as described by Brenner (1974). Bristol strain N2 was

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used as the wild type and all animals were grown at 20°C unless otherwise stated. The following strains were obtained from the Caenorhabditis Genetics Center (CGC) at University of Minnesota, USA: Bristol type N2, *lim-4(ky403)* X, *ceh-36(ky646)* X. The wild type worms carrying the extrachromosomal array *GFP::LGG-1* were kindly provided by Alicia Melendez.

Bacterial strains and culture conditions

The soil bacteria were obtained from Korean Agricultural Collection Centre (KACC), Suwon, Republic of Korea: *Bacillus azotoformans* (KACC no. 12215), *B. coagulans* (KACC no. 10117), *B. firmus* (KACC no. 10897), *B. mycoides* (KACC no. 12063), *B. soli* (KACC no. 12115), *B. subtilis* (KACC no. 10111), *Cellulomonas flavigena* (KACC no. 11293), *Corynebacterium glutamicum* (KACC no. 10784), *Erwinia sp.* (KACC no. 10252), *Pseudomonas corrugata* (KACC no. 10141), *Mycoplana dimorpha* (KACC no. 11262), *Variovorax soli* (KACC no. 11579).

Behavioral assays

Binary choice assay

For the binary choice assay, bacteria were grown in LB broth at 25-30°C unless otherwise stated. The bacterial strains OP50 and *B. coagulans* were grown at 37°C. Assays were done on 90 mm plates containing NGM medium. Bacterial food was seeded at the distance of 1.5 cm from periphery with a diameter of ~0.5 cm (Fig. 1A) and then animals were put on the center of the plates and were allowed to migrate towards either control or tested bacterial lawns on opposite sides of a NGM plate. The number of worms on each bacterial lawn was counted after 3 h of incubation at 20°C. The choice index was calculated as follows-

$$\text{Choice index (CI)} = \frac{\text{No. of worms in tested bacteria - No. of worms in OP50}}{\text{Total number of used worms}}$$

If, CI = -1.0 represents complete preference for *Escherichia coli* OP50.
+1.0 represents complete preference for the test bacterium.
0.0 represents an equal distribution.

In this assay, a choice index of -1.0 represents complete preference for *Escherichia coli* OP50, an index of 1.0 represents complete preference for the test bacteria, and an index of 0 represents an equal distribution.

Pharyngeal pumping assays were performed on NGM agar plates with confluent lawns of soil bacteria or OP50 bacteria, the standard laboratory diet for *C. elegans* at 20°C using a Nikon SMZ1500 stereomicroscope (Avery and Horvitz, 1989). Briefly, L4 animals grown on OP50, after one day, were transferred to fresh plates of bacteria (either OP50 or soil bacteria) and grown for 30 min before counting the pharyngeal pumps. Pumping rate was recorded as the number of contractions in the terminal bulb of the pharynx in a 20 s period for an individual worm. The assay was performed at least twice with a total of 30 worms. Statistical analysis of data was done using unpaired, two-tailed *t*-test.

Lifespan analysis was conducted at 20°C as described previously unless stated otherwise (Kenyon et al., 1993). The different soil bacteria treatments were either performed till F1 or F3 generations. The wild type worms were grown at 20°C under optimal growth conditions for at least two generations before use in lifespan analysis. For this, young adults (P0) were added to plates seeded with the soil bacteria of interest and the feeding was continued in progenies and throughout the experiment. The lifespan analysis was performed in the F3 generation. In all experiments, the pre-fertile period of adulthood (L4 stage) was used as t = 0 for lifespan analysis. Kaplan-Meier survival analysis was used to compare the mean lifespan of different treatments, and *p* values were calculated using the Log-rank (Mantel-Cox method) to determine the relative effects of soil bacteria treatment versus OP50 on wild-type N2 animals. Each lifespan experiment was repeated at least two times with n ~50 animals per experimental group.

Autophagic events in N2 worms were assessed using an GFP::LGG-1 translational reporter characterized previously (Melèndez et al., 2003). Animals were raised at 20°C on desired soil/OP50 bacteria and the L3 stages of F2 generation were examined. GFP-positive puncta were counted (using 1000- fold magnification on a Zeiss Axioplan II microscope equipped with fluorescent optics) in the seam (lateral epidermal) cells of L3 transgenic animals. At least 3 to 10 seam cells were examined in each of 10-15 animals from at least two independent trials and averaged. Statistical analysis of data was done using unpaired, two-tailed *t*-test.

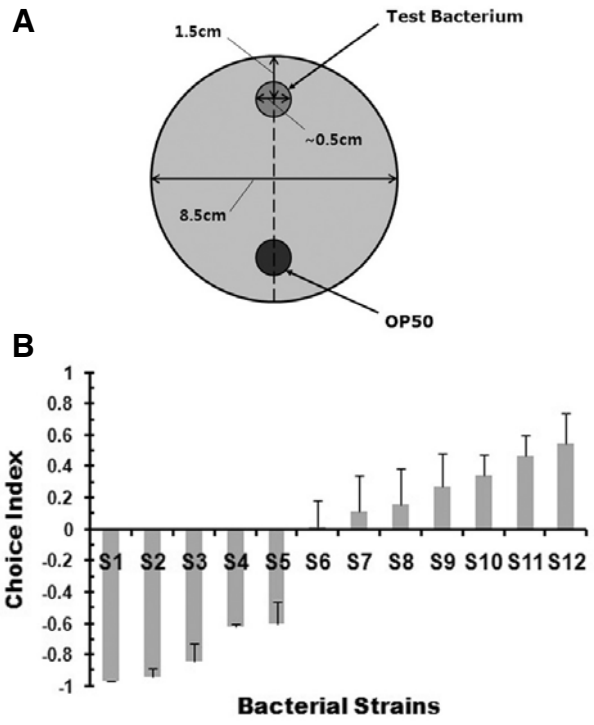

Fig. 1. Food preference assay between *E. coli* strain OP50 and different soil bacteria. (A) Schematic representation of assay plate used for studying the food preference and to calculate choice index (CI). (B) The food choice index of the soil bacteria when compared to OP50 and accordingly labeled from S1 (least preferred) to S12 (most preferred) for this study. S1: *Bacillus azotoformans*, S2: *Bacillus subtilis*, S3: *Bacillus coagulans*, S4: *Pseudomonas corrugata*, S5: *Cellulomonas flavigena*, S6: *Bacillus firmus*, S7: *Corynebacterium glutamicum*, S8: *Variovorax soli*, S9: *Mycoplana dimorpha*, S10: *Bacillus soli*, S11: *Bacillus mycoides*, S12: *Erwinia sp.*

RESULTS AND DISCUSSION

***C. elegans* exhibit food preference for various soil bacteria**

The nematode *C. elegans* showed clear preferences when

Table 1. List of soil bacteria used in this study with their morphological and microbiological characteristic properties

No.	Name of bacteria strain	KACC No.	Culture temperature	Gram stain	Shape	Arrangement	Motility	Choice index
S1	*Bacillus azotoformans*	11215	30°C	+	Rod	singles, pairs	Yes	-0.97
S2	*Bacillus subtilis*	10111	30°C	+	Rod	singles, chains	Yes	-0.94
S3	*Bacillus coagulans*	10117	37°C	+	Rod	single, pairs, chains	Yes	-0.84
S4	*Pseudomonas corrugata*	10141	26°C	-	Rod	singles	Yes	-0.62
S5	*Cellulomonas flavigena*	11293	30°C	+	Rod	-	Yes	-0.61
S6	*Bacillus firmus*	10897	30°C	+	Rod	short chain	Yes	+0.01
S7	*Corynebacterium glutamicum*	10784	30°C	+	Rod	Single, V-shaped pairs	No	+0.11
S8	*Variovorax soli*	11579	30°C	-	Rod	singles, pairs	Yes	+0.16
S9	*Mycoplana dimorpha*	11262	30°C	-	Rod	singles, pairs	Yes	+0.27
S10	*Bacillus soli*	12115	30°C	+	Rod	single, pairs, chains	Yes	+0.34
S11	*Bacillus mycoides*	12063	25°C	+	Rod	chains	No	+0.46
S12	*Erwinia sp.*	10252	28°C	-	Rod	single, pairs, chains	Yes	+0.55

given the choice between various soil bacteria and the laboratory bacterial strain OP50. The wild type worm showed positive food preference for at least three out of twelve tested soil bacteria. The choice index suggested that although worms preferred these bacteria but it was not a complete preference and a few worms were still observed near OP50 lawn. The maximum preference with respect to OP50, according to choice index, was shown by *Erwinia sp.* (S12) and *Bacillus mycoides* (S11). On the other hand, the two bacterial strains, *B. azotoformans* (S1) and *B. subtilis* (S2), showed a negative choice index and avoided these bacteria when compared with OP50 (Table 1, Fig. 1B). Bacterial strains preferred over OP50 or strains that were avoided by the worms showed no specificity based on microbiological characteristics such as shape, arrangement, motility or Gram staining. All the bacterial strains are rod shaped and one of the preferred bacteria (S12, *Erwinia sp.*) is Gram negative, where as the other preferred bacterial strain *B. mycoides* (S11) was positive in Gram staining (Table 1). Thus, we conclude that preference or avoidance towards a particular strain of bacteria is irrespective of their shape and/or cell wall structure.

We then explored the possibility of odor sensation (olfactory behavior) in selecting a particular type of bacteria and we observed that the tested mutants *lim-4(ky403)*, required for the differentiation of AWB chemosensory neurons, and *ceh-36(ky646)*, required for the specification of AWC olfactory neurons, showed similar trends in the preference assay yet the choice index was considerably different from wild type worms (Fig. 2). The reduced value of choice index in these mutants suggests that chemosensation may be required for the initial detection of food in *C. elegans*. However, to determine the exact role of chemosensation, additional studies are required using chemotaxis assay with bacterial extracts or supernatant.

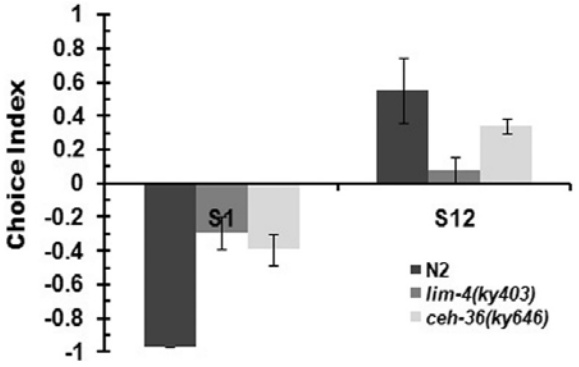

Fig. 2. Food preference assay for *ceh-36* and *lim-4* mutants. The choice index in wild type worms and mutants with defective chemosensory neurons was calculated after feeding the bacteria showing minimum CI in wild type worms (S1: *B. azotoformans*) and bacteria showing maximum CI in wild type worms (S12: *Erwinia sp.*).

Food preference and eating behavior are independent process

C. elegans nematodes ingest bacteria by rhythmic contractions of the pharynx muscles. Therefore, the contraction of pharyngeal muscles, known as pharyngeal pumping, can be a reliable index of food ingestion by the worm. Thus, we measured the pharyngeal pumps of wild type worms feeding on representative bacteria from the group showing less preference than OP50, namely. *B. subtilis* (S2), *B. coagulans* (S3) and *P. corrugata* (S4). The two bacterial strains showing similar choice as OP50 were *B. firmus* (S6) and *C. glutamicum* (S7), and the third group showing increased preference over OP50 consists of bacterial strains *B. soli* (S10) and *B. mycoides* (S11). We observed that the pharyngeal pumping in four bacteria out of seven tested bacteria were comparable to the pharyngeal pumping rate of worms feeding on OP50 bacteria (Fig. 3). However, we observed significant decrease in pharyngeal pumps on *P. corrugata,* S4 (preference was less than OP50) and *B. mycoides*, S11, (preference was higher than OP50). On the other hand, we found a significant increase in pharyngeal pumping when the wild type worms were fed only with *B. soli*, S10, which was preferred over OP50 (Fig. 3). These results suggest that even if the worm favors some bacteria and shows preference choice over OP50, when they are solely on those bacteria, they do not necessarily display increased pumping compared to feeding on OP50. Thus, our pharyngeal pumping data suggests that the food preference does not strongly correlate with eating behavior.

Food preference influence the lifespan of *C. elegans*

Lifespan analysis gives us an idea of normal physiological processes occurring in an individual. Thus, we studied the lifespan of wild type worms when fed with bacteria showing signifycant differences in pharyngeal pumping. The lifespan of wild

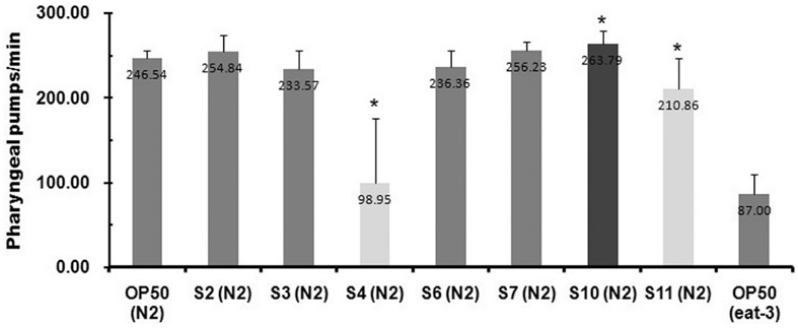

Fig. 3. Food eating behavior for different soil bacteria. The pharyngeal pumping/min in wild type worms was measured for different soil bacteria. The *eat-3* mutant worms were included as the feeding defective control. *, indicates the significant value with $p \leq 0.00002$ when compared to the pumping rate while eating OP50.

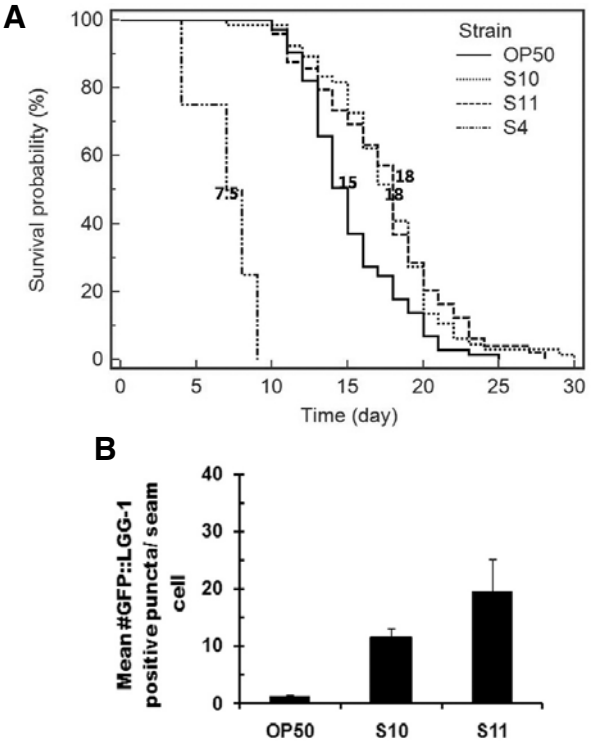

Fig. 4. Lifespan and autophagic process for preferred bacteria. (A) A Kaplan-Meier survival curve showing the lifespan curve of F2 generation N2 wild type worms fed with soil bacteria either S4: *Pseudomonas corrugate,* S10: *Bacillus soli* or S11: *Bacillus mycoides*. (B) Quantification of GFP::LGG-1 positive puncta in seam cells of F2 generation N2 wild type worms fed with either S10: *Bacillus soli* or S11: *Bacillus mycoides*.

type worms after feeding *P. corrugata* (S4) was significantly reduced with a mean lifespan of 7.5 days as compared to 15 days when fed with OP50 (p value < 0.0001). This considerable shortening of lifespan indicates that *P. corrugata* may be potentially harmful to the worms and thus decreased the lifespan to almost half of the normal lifespan. On the contrary two other bacteria tested for lifespan showed a significant increase in wild type worms. Both *B. soli* (S10) and *B. mycoides* (S11) increased the mean lifespan to 18 days (Fig. 4A). The *daf-2* and *daf-16* mutants, mutants well-characterized in their effect on lifespan, also exhibited similar increase in their lifespan (data not shown). Thus, there may be a correlation between preferred food choices and lifespan of the animal dwelling on those bacterial strains.

Since, we observed an increase in lifespan in preferred bacterial strains, we wanted to understand what effect these bacteria may be having on the animal to increase lifespan. We were interested in studying the process of autophagy (Dwivedi and Ahnn, 2009) as many of genetic pathways known to influence lifespan depend on autophagic processes to increase lifespan. Interestingly, we found significant increase in the autophagic puncta in the worms feeding on both bacteria (Fig. 4B). This observation indicates that extended lifespan observed in wild type worms after feeding two bacteria, S10 and S11, may be due to the activation of autophagic process.

CONCLUSIONS

In this study we conclude that *C. elegans* wild type animals indeed show a clear preference towards soil bacteria, their probable natural food, when compared to the *E. coli* strain OP50, an artificial food source in laboratories. The food preference for a particular bacterial strain does not correlate well with the eating behavior of the same bacteria. However, food preference and choice of the bacteria influence the lifespan of worms and we suggest induced autophagy as one of the mechanism for extended lifespan. The genetic mechanism leading to successful identification of food, its ingestion and assimilation need to be further elucidated.

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