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LONGEVITY/FECUNDITY TRADEOFF IN *Caenorhabditis elegans* BY UNFAVORABLE BACTERIA *Microbacterium* sp. NEWLY ISOLATED FROM FOREST

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ABSTRACT

Bacteria are food sources for the *Caenorhabditis* nematodes. This prey-predator interaction becomes a model to investigate the effects of each bacterial strain on the nematodes at multiple levels (phenotypes to molecular regulations). To see how the interaction could be, we isolated many *Caenorhabditis* nematodes and the associated bacteria from the forests. Theoretically, they have been living and evolving, suggesting that they would have specific interaction. In this research, we report the identification of a new environmental bacteria associated with the nematode genus *Caenorhabditis* in Cat Tien National Park with the 16S rDNA. We identified the bacteria as *Microbacterium* sp. CFBb57.1. In the second part of our investigation, we investigated the impacts of the bacteria on the longevity and reproduction of the model nematode *Caenorhabditis elegans* using the control *Escherichia coli* OP50. The results showed that *C. elegans* living on CFBb57.1 had extended longevity and they had a reduction in reproduction, presenting a longevity/fecundity tradeoff. Last, in the bacterial preference test, we found their preference was modulated on CFBb57.1 in comparison with the control OP50, suggesting that the worms did not prefer CFBb57.1 over OP50. However, more CFBb57.1-acclimating worms were chemotactic to CFBb57.1, indicating that worms had genetic mechanism(s) to adapt to CFBb57.1. *C. elegans* responded differently to the *Microbacterium* bacteria in this study and previous reports. In this study, it first showed the tradeoff and acclimation. Thus, this report will facilitate the studies of how the *Caenorhabditis* nematodes could evolve the mechanism(s) of tradeoff that was also presented in other bacterial genera and the mechanism(s) of acclimation that would be epigenetic to neutralize the impacts of bacteria.

Keywords: Bacteria, longevity, nematodes, preference, reproduction.

INTRODUCTION

Caenorhabditis elegans, a nematode model for tracing numerous biological issues (Leung *et al.*, 2008), eats bacteria over its lifespan. Thus, the development of *C. elegans* depends on the nutrition of the bacteria. In favorable conditions with food, *C. elegans* grows and reproduces normally (Alvarez *et al.*, 2005; Mukhopadhyay &

Tissenbaum, 2007). In stressful conditions, however, it delays the development (Golden & Riddle, 1984; Angelo & Van Gilst, 2009) and behaves abnormally.

Although nutritional bacteria are beneficial, many others were found to be pathogenic bacteria and suppressed the development of *C. elegans*. They could be toxic and kill nematodes. Thus, in the wilderness, the *C. elegans*-bacteria communication is far more than a prey-predator intra-connection in the ecosystem (Dirksen *et al.*, 2020).

Different bacteria that would develop in a habitat of the nematodes are known as natural microbiota or microbiome. Nematodes can eat solely the microbiome throughout their life. In this scenario, the life history of the nematodes is entirely shaped by the bacteria. However, various bacteria may impact the nematodes differently (Le *et al.*, 2022). In this research, we deeply investigated the potential impacts of a new *Microbacterium* isolate on the *Caenorhabditis* nematodes.

As components of the natural microbiota of the nematode genus *Caenorhabditis* (Samuel *et al.*, 2016; Zimmermann *et al.*, 2020; Le *et al.*, 2022), bacteria within the genus *Microbacterium* are prevalent in various habitats from soil to human specimens, and share living spaces with the nematodes across continents (Funke *et al.*, 1995; Le *et al.*, 2022). The *Caenorhabditis* nematodes feed on the bacteria and have different genetic features. However, a few studies reported the effects of *Microbacterium* strains on *C. elegans*. For example, *Microbacterium nematophilum* CBX102/103, a case study canonical within the bacterial genus *Microbacterium*, was isolated in Europe. CBX102/103 is a pathogen adhering to and deforming the anal cuticle of *C. elegans*. In the efforts to characterize the responses of the host to the pathogen by screening mutants, several genes in *C. elegans* were found to be involved in the infection of the bacteria (Hodgkin *et al.*, 2000; Gravato-Nobre *et al.*, 2005; Nicholas & Hodgkin, 2009).

We study the forestry ecology in which we aim to uncover the interaction and further the underlying mechanisms between the *Caenorhabditis* nematodes and associated organisms. In this research, we report the isolation and identification of a new forestry *Microbacterium* sp. CFBb57.1 in Cat Tien National Park, southern Vietnam, where many *Caenorhabditis* nematodes live (Son *et al.*, 2023a; Son *et al.*, 2023b; Son *et al.*, 2024). We examined the effects of the bacteria on *C. elegans* and found that the bacteria affected the development and modulated the behavior of the nematode.

MATERIALS AND METHODS

Nematode strains

C. elegans var Bristol (N2) (lab number: CFBN22023) and *Escherichia coli* OP50 were gifted by colleagues. *Microbacterium* sp. CFBb57.1 was a new isolate of the environmental bacteria from a site (11º23’26”N; 107º21’1.8”E) in Cat Tien National Park, Vietnam, in this research.

Nematode growth media (NGM)

NGM was prepared as the protocol (Stiernagle, 2006). A mixture of 17 g of agar, 3 g of NaCl, 1 mL of 1M $CaCl_2$, 1 mL of 1M $MgSO_4$, 25 mL of 1M KPO4, and 5 g of peptone in 1 L of distilled water was mixed in a glass flask before autoclaving at 121ºC for 25 min. Soon after cooling down at 60ºC, 1 mL of 5 mg/mL cholesterol was added and

mixed thoroughly before pouring into petri dishes (Le *et al.*, 2021).

Luria-Bertani (LB) broth

Ten grams of peptone, 5 g of yeast extract, and 5 g of NaCl were added together in 1 L of distilled water in a flask. Next, the media was autoclaved at 121°C for 25 min.

Lifespan assay

The P0 worms developed on the bacteria-seeded NGM plates for three to five generations at 20°C in an incubator. They were transferred on a new plate to produce the F1 generation. Each group of nearly 60 F1 worms already acclimated to the bacteria was raised on the bacteria-seeded plate. They were transferred to a new plate every two or three days until they died. The bacteria were CFBb57.1 and OP50. A worm died on the agar surface if it did not move when gently touched by the picker. The average lifespan was the sum of the entire lifespan of all tested worms divided by the number of tested worms {Average lifespan (days) = the sum of all lifespans/ the number of tested worms} (Son le *et al.*, 2011; Stroustrup *et al.*, 2013). The lifespan comparison was statistically analyzed with the log-rank test in the code functions [Surv(stage,lifepan)] and [surdiff(sdt~bacteria)] (Le *et al.*, 2021).

Preparation of bacteria

The bacterial strain CFBb57 was previously described (Le *et al.*, 2022). In brief, to maintain the stock of CFBb57, the CFBb57 culture was kept cold in the LB broth at 10°C and monthly reactivated in the fresh LB broth at room temperature (RT; nearly 25°C) for around six months. Prior to the tests for the effects on *C. elegans*, the bacteria stock was grown again. A diluted bacterial culture was spread and incubated on an LB agar plate for three days at RT. Next, three single colonies on the plate were picked and cultured separately in the LB broth. One culture, known as CFBb57.1, was selected for further investigation. Following, the CFBb57.1 bacteria was sequenced for 16S rDNA. To identify the bacterial genus, the 16S rDNA sequence was compared with the DNA database of the National Center of Biotechnology Information (NCBI) (Le *et al.*, 2021).

Reproduction assay

We conducted two types of reproduction tests. First, the preliminary assays were to examine the possible effects of bacteria on the brood sizes of *C. elegans*. Three *C. elegans* worms (P_0) at the second larval stage (L_2) were grown on the CFBb57.1 or control OP50-seeded plate. The P_0 worm mothers reproduced the F_1 progeny in three days, and the F_1s reproduced the F_2 progeny in the next three days; both reproductions lasted for 10 days in the assay. The counts of the worm progenies on the test bacteria-seeded plate were estimated and compared with the OP50-seeded plate. The conclusions of the preliminary results were "worms on CFBb57.1 had more than, less than, or equal to the reproduction rate of the control OP50". The populations with extremely low progeny counts, i.e., CFBb57.1, were selected for the next experiments (brood size, lifespan, and bacterial preference).

In the second test for brood size, a group of three to five L_2 worms laid by the P_0 parents acclimated to the bacteria-seeded NGM for one or two generations (F_1 or F_2) was grown on one bacteria-seeded NGM plate. The

tested worms were daily transferred to a new plate until they stopped reproduction in two relentless days. The average brood size of a single worm in one group was the total brood size divided by the number of the grouped worms (i.e., three or five) {Average brood size (larvae) of a worm = the gross brood size of a plate/ the number of the grouped worms} (Le *et al.*, 2021). The statistical analysis of the brood sizes between the tested worm group and the control worm group was deployed with Dunnett's post-hoc test in the code function of [summary(glht(aov(linfct=mcp(ind="Dunnett")))] in the R program.

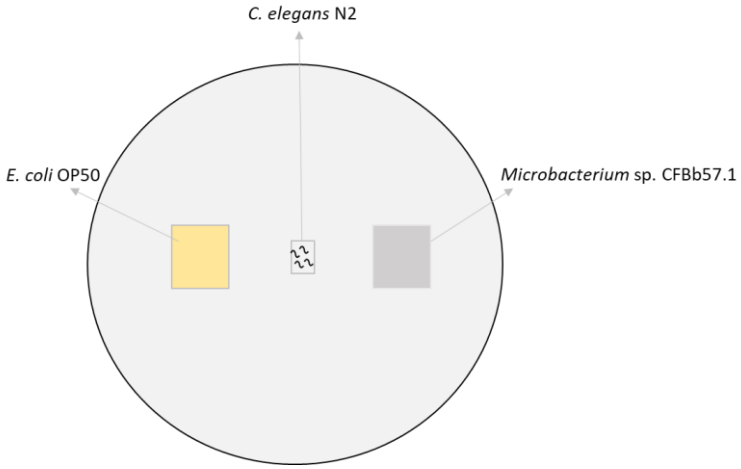

Figure 1. NGM plate for bacterial preference. *C. elegans* adult worms were gently placed in the middle spot between CFBb57.1 and OP50. They freely relocated to OP50 (yellow), CFBb57.1 (dark grey), no bacterial area (grey), or settled down in the middle spot.

Bacterial preference

In preparation of test plates, we marked two opposite spots that were 2 cm apart on each plain NGM plate (5 cm). Next, we seeded CFBb57.1 on one spot and OP50 on the other with a swapping blade. The plate was incubated for two days at room temperature (approx. 25°C). Twenty-nine to 32 reproducible worms were placed in the middle spot between the two bacterial spots. They were free to reach anywhere for five hours and 40 minutes on the surface of the test plate (Figure 1). Next, the worms were counted for their presence on four destination choices (two bacterial spots, the middle (Middle) spot, and the lost (Lost)) (Figure 1). The destination preference was quantified by the choice index (CI), which is the number of worms on each destination divided by the total tested worms {CI (%) of a destination = [the worm count on the destination/total tested worms] x 100%}. Statistical analyses for the effects of general destination on CIs were deployed with the 2-way ANOVA test in the function [summary (aov(value~Source * Destination, data))] and of pairwise destinations with the Dunnette's post-hoc test in the code function [summary(glht(aov,linfct=mcp(ind="Dunnett")))] in the R program.

Phylogeny tree construction

The qualified 16S rDNA sequences of the bacteria were aligned. Next, the phylogeny

tree of the 16S rDNA sequences was constructed using the Neighbour-Joining method in the MEGA11 software (Tamura *et al.*, 2021).

Statistical analysis

The statistical analyses were processed by autonomously coded functions in the R program (version 4.3.2). R was installed with the relevant functional packages. *P* values < 0.05 were statistically considered significant differences, and *P* values ≥ 0.05 were non-significant.

RESULTS

CFBb57.1 is a new *Microbacterium* sp.

To identify the taxa of CFBb57.1, the 16S rDNA sequence was compared with the nucleotide database with BLASTnt in NCBI. We revealed that CFBb57.1 was classified in the bacterial genus *Microbacterium*. CFBb57.1. It is 99.85% identical to the first three strains in China and India and 99.21% identical to *Microbacterium* sp. CFBb37. CFBb37 was previously isolated by our group at another site within the same ecosystem (11°27'5"N; 107°21'25.6"E) (Le *et al.*, 2022), indicating that CFBb57.1 is a new *Microbacterium* sp. (Figure 2). Next, we examined the effects of CFBb57.1 on *C. elegans*.

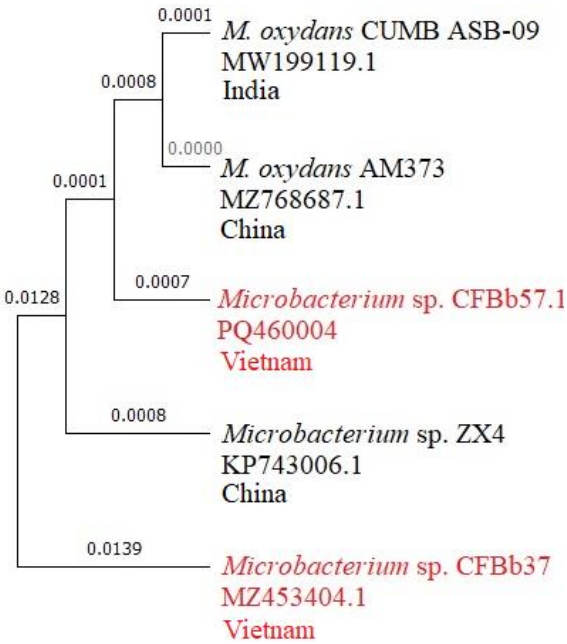

Figure 2. Phylogeny tree of *Microbacterium* sp. CFBb57.1. Each bacterium with the strain name (top), the GenBank accession number of 16S rDNA sequence provided by NCBI (middle), and the country of origin (bottom); our isolates (red). The phylogeny tree was constructed with bootstrap (500 replications) and Neighbour-Joining methods.

Longevity of *C. elegans* extended on CFBb57.1

The worms fed on CFBb57.1 had a longer average lifespan than the control OP50 (*P* = 0.03, log-rank test; Figure 3), indicating that CFBb57.1 extended the lifespan of worms. Among 187 tested worms, one individual survived the longest life for 50 days, nearly doubling the average lifespan, and another worm lived the shortest life for nine days.

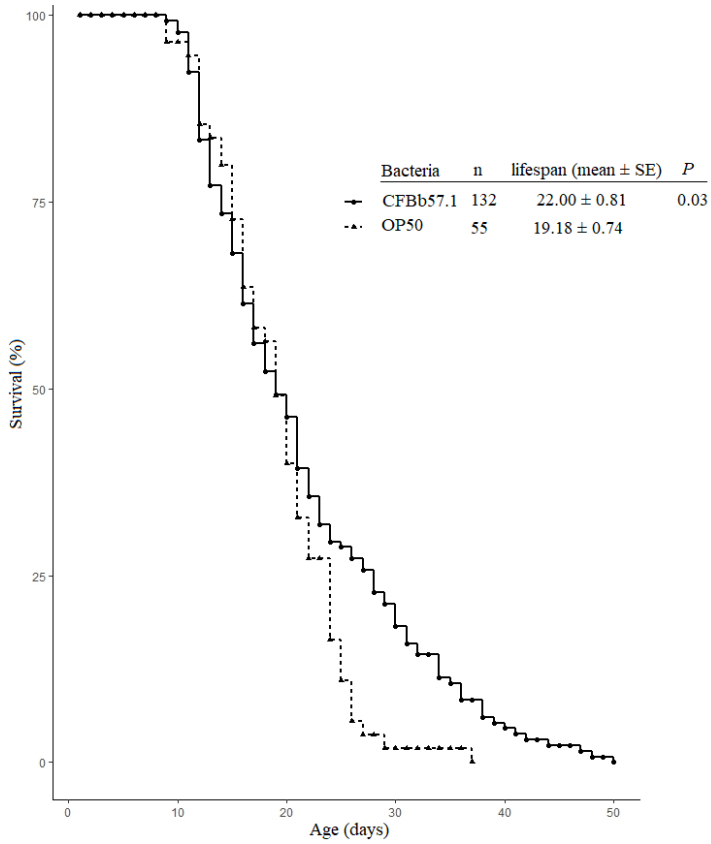

Figure 3. Survival rate over the entire lifespan. n is the number of tested hermaphrodites.

Brood size of *C. elegans* reduced on CFBb57.1

Brood size (or the count of larvae) is another parameter to evaluate the reproduction ability of *C. elegans*. Worms were fed on either CFBb57.1 or OP50.The worms were transferred to new plates daily and they laid eggs (embryos) on the same plate. The transfer was continued for four to five days until the worms did not produce offspring. The eggs hatched larvae that were counted for the brood size data. Statistically analyzing the brood sizes, we found that the brood size of worms on CFBb57.1 significantly differs from that of worms on the control OP50 ($P < 0.001$, Dunnette's post-hoc; Table 1), indicating that CFBb57.1 suppressed the reproduction of *C. elegans*.

Table 1. Brood sizes in the second tests.

Bacteria	N/n	Brood size (mean ± SD)	*P* value
Microbacterium sp. CFBb57.1	10/50	101.68 ± 25.57	*P* < 2e-16
E. coli OP50 (control)	14/42	161.83 ± 25.58	

N – biological replicate; n – the number of tested hermaphrodites.

C. elegans did not prefer CFBb57.1

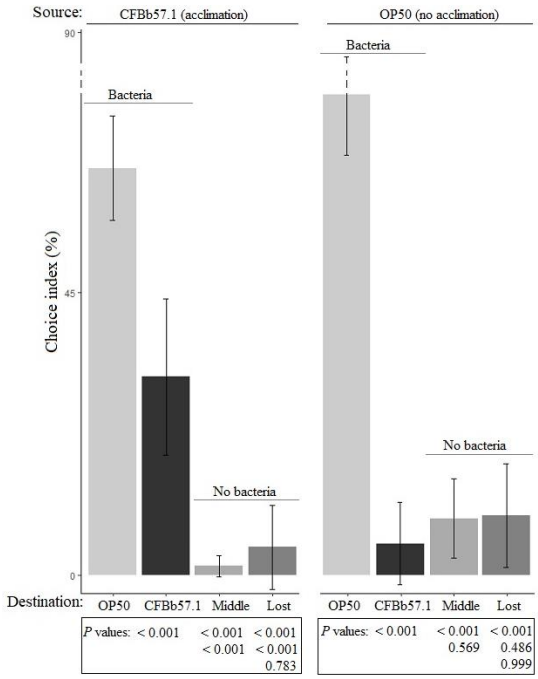

Figure 4. Choice index of preference. Worms acclimated (left) and not acclimated to CFBb57.1 (right). The *P* values in one row represent the pairwise comparisons from the first left to the right destinations.

C. elegans had two opposite responses: The lifespan was longer and the brood size was smaller on CFBb57.1. Next, we investigated how the worms preferred the bacteria. In the assay, two groups of worms were cultured in different conditions. One group of worms was raised on the CFBb57.1 source for generations. Thus, the worms acclimated to CFBb57.1 but never to the control OP50. Similarly, the second group of worms grew on the control *E. coli* OP50, and did not experience CFBb57.1. The results showed that both groups of worms had significant preference biases (general *P*s < 0.001, ANOVA).

Table 2. Choice index on bacteria.

Source	**CFBb57.1 (N = 9; n = 282)**				**OP50 (N = 9; n = 276)**			
Destination	OP50	CFBb57.1	Middle	Lost	OP50	CFBb57.1	Middle	Lost
Average (mean ± SE)	64.77 ± 2.77	31.55 ± 4.14	1.43 ± 0.57	4.24 ± 2.22	76.47 ± 3.22	5.01 ± 2.19	9.03 ± 2.12	9.49 ± 2.74

N – biological replicate; n – the number of tested hermaphrodites.

Next, we analyzed the choice indices for each of the two worm groups (acclimation and no acclimation). The CFBb57.1-acclimated worms preferred the bacteria differently, more with the OP50 and less with CFBb57.1, to the non-bacteria (Ps < 0.001; Table 2; Figure 4, left). In contrast, the OP50-acclimated worms preferred the control OP50 to the other destinations (Ps < 0.001, Dunnette's post-hoc; Figure 4, right) and had no significant difference between the other three destinations (Ps = 0.486 to 0.999; Table 2; Figure 4, right).

DISCUSSION

CFBb57.1 is a new *Microbacterium* sp.

In this research, we isolated a new *Microbacterium* sp. CFBb57.1 at a site in Cat Tien National Park. Previously, we characterized the *Microbacterium* sp. CFBb37 in a different site near the CFBb57.1 site (Le *et al.*, 2022). The two bacteria shared the proximal habitats and they did not have identical 16S rDNA sequences. Regarding the reproductive span, which was studied in this research, *C. elegans* on CFBb57.1 had a short reproductive span (approximately 4 days), but the worms on CFBb37 nearly doubled the span (approximately 8 days) (Le *et al.*, 2022). To us, these indicate they would be different strains from the same or different species.

Notably, *M. nematophilum* CBX102/10, which was isolated possibly in Germany, Europe, has been the only bacteria known to infect the anus of *C. elegans* and form tail swelling so far (Hodgkin *et al.*, 2000). However, CFBb57.1 and the previously reported CFBb37 did not deform the tail of *C. elegans*. These indicate that these two *Microbacterium* strains are not within the same species as *M. nematophilum* CBX102/10.

Unfavorable CFBb57.1 causes a longevity/fecundity tradeoff in *C. elegans*

Compared with the control *E. coli* OP50, the longevity of *C. elegans* on CFBb57.1 increased by 114% (22.00/19.18). In contrast, the reproduction ability was reduced by 62.83% (101.68/161.83).

Given the longevity and reproduction results, *C. elegans* on the two *Microbacterium* sp. isolates in Cat Tien National Park, CFBb57.1 in this research and CFBb37 in our previous report (Le *et al.*, 2022) showed longevity/fecundity tradeoffs in which life is increased and reproduction is decreased. To our knowledge, these *Microbacterium* strains were first found to regulate the longevity/fecundity tradeoff in *C. elegans*. However, other bacteria, i.e., *B. amyloliquefaciens* and *B. megaterium*, were unfavorable food and caused the same tradeoffs in *C. elegans* (Yu *et al.*, 2015).

In the preference assays, worms did not prefer CFBb57.1 to the control OP50, indicating that CFBb57.1 is unfavorable food for *C. elegans*. However, if worms experience CFBb57.1 over generations, they could be chemotactic to survive CFBb57.1, indicating that worms had a molecular mechanism that evolved and was already encoded in the genome and worked epigenetically (Rankin, 2015; Baugh & Day, 2020) to neutralize CFBb57.1.

CONFLICT OF INTEREST

The authors declare that there is no conflict of interest.

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