Death and survival of spore-forming bacteria in the *Caenorhabditis elegans* intestine

Maria-Halima Laaberki and Jonathan Dworkin[*]

Department of Microbiology, College of Physicians and Surgeons, Columbia University, 701 W. 168th Street,
New York, NY 10032, USA, Tel. +1-212-342-3731, Fax. +1-212-305-1468, Email. jonathan.dworkin@columbia.edu

Abstract

The nematode *Caenorhabditis elegans* and spore-forming bacteria of the *Bacillus* genus are both common soil inhabitants. In this study, we investigated the interaction of the bacteriophagous *C. elegans* with these bacteria. We observed that vegetative forms of *Bacillus* species are highly sensitive to ingestion by *C. elegans*. In contrast, we found that spores are highly resistant to the worm's digestive process. Spore protective structures such as the spore coat likely hinder access of digestive enzymes such as lysozyme to sensitive structures like the cell wall and thus facilitate passage of the spore through the digestive tract. We developed methods to monitor the uptake of vegetative cells and spores and found that the spores have a particularly slow transit through the worm's intestine suggesting that the nematode may consider spores as inert particles rather than as food. Thus, the results of this study show that spores have features that enable them to stay longer times in the nematode intestine, suggesting that this transit of *Bacillus* sp. may have important implications for the evolution of resistance properties.

Keywords: *Caenorhabditis elegans, Bacillus subtilis, Bacillus anthracis, Bacillus cereus, Bacillus thuringiensis, Bacillus megaterium*, spores

1. Introduction

Caenorhabditis elegans is a soil nematode found commonly in compost heaps and in apparently close association with a variety of carrier invertebrates (Barriere and Felix, 2005; Caswell-Chen et al., 2005). *C. elegans* is bacteriophagous and appears to interact with soil bacteria like the spore forming Bacilli such as *Bacillus cereus* (Grewal, 1991). The specificity of this interaction is consistent with the presence of strains of *Bacillus thuringiensis*, a very close relative of *B. cereus*, that produce nematicidal toxins with a specificity and a potency against *C. elegans* (Griffitts et al., 2005). In addition, *C. elegans* exhibits different behavioral responses to different strains of *B. thuringiensis*, suggesting that this interaction has affected its ecological distribution (Schulenburg and Muller, 2004).

In the rhizosphere, Bacilli are found in two states depending on nutrient availability (Saile and Koehler, 2006). Under hospitable conditions and in the presence of sufficient nutrients, Bacilli can exist as metabolically active and dividing vegetative cells. These cells are considered to be a "good" source of food for *C. elegans* since they allow an increased life span of *C. elegans* N2 strain as compared to *E. coli*, their usual source of food in the laboratory (Garsin et al., 2003). When nutrient and/or environmental conditions change, the cells differentiate into dormant and resistant cells named spores. The spore is able to survive a wide range of conditions that are lethal to the vegetative bacterium like extreme temperatures, desiccation, and enzymatic and mechanical interventions (Nicholson and Setlow, 1990). Most Bacilli are found in the wild as spores (Nicholson, 2002) and the interaction of *C. elegans* and bacterial spores are likely to be a frequent occurrence in the rhizosphere given the high respective densities of each organism in the soil (for nematodes >100/g (Tate, 2000) and for spore-forming bacteria, 10^4–10^6/g (Martin and Travers, 1989)). Thus, nematodes likely carry *Bacillus*

[*]*The author to whom correspondence should be sent.*

species, especially in their spore state, at least some fraction of their life span. Surprisingly, there has been no systematic analysis of their interactions of *Bacillus* species cells with potential host like *C. elegans*. In this study, we investigate quantitatively and qualitatively the interaction of *C. elegans* with various *Bacillus* species.

2. Materials and Methods

Care of bacterial strains and C. elegans

Bacillus subtilis strains used in this study were all isogenic derivatives of the *B. subtilis* PY79 strain (Youngman et al., 1984). The *B. subtilis* strain JDB1334 presents a chromosomal integration of P*spanc*::*gfp* and a spectinomycin resistance gene into the non-essential *amyE* locus. The P*spanc* promoter is a derivative of the P*spank* promoter that allows a constitutive high level of expression. The *B. subtilis* JDB623 strain is a derivative of the PY79 strain carrying an insertion of the *lacI* gene in the *amyE* gene and a kanamycin resistance gene. The *lacI* gene encodes the transcriptional repressor of the β-galactosidase utilization operon in *B. subtilis*. The *B. subtilis* JDB1071 strain contains an insertion of P$_{spoIIQ}$::*gfp* and a spectinomycin resistance gene in the *amyE* gene. P$_{spoIIQ}$ is induced upon *B. subtilis* sporulation (Londono-Vallejo et al., 1997). The *Bacillus anthracis* strain used is the attenuated capsule-deficient *B. anthracis* Sterne 34F2 strain (kindly provided by Adam Driks, Loyola University, Illinois). The *Bacillus thuringiensis* strain is the *B. thuringiensis* subspecies kurstaki HD73. The *Bacillus cereus* strain used is the *B. cereus* ATCC 14579 strain. Both *B. thuringiensis* and *B. cereus* strains were obtained from the *Bacillus* Genetic Stock Center, Ohio State University.

The *Escherichia coli* OP50 strain was obtained from Iva Greenwald, Columbia University, New York. The bacteria were maintained in LB-Lennox (10 g/l tryptone, 5 g/l yeast extract, 5 g/l NaCl) liquid or agar media. Spectinomycin resistance was selected on LB agar plates containing 10 µg/ml spectinomycin; kanamycin resistance was selected using a final concentration of 100 µg/ml kanamycin.

Sporulation was achieved by growing bacteria in liquid Difco Sporulation Medium and spores were purified as described (Henriques et al., 1995). Briefly, spores were purified using a 33% Diatrizoate meglumine – 8% Diatrizoate sodium solution and washed with cold water until 100% of phase bright spores are obtained. The spores were stored at 4°C in double distilled water and used within a week following the initial growth in DSM. *Caenorhabditis elegans* strain N2 (kind gift of Iva Greenwald, Columbia University, New York) was maintained under standard culturing conditions on Nematode Growth Medium agar (NGM) with *Escherichia*

coli strain OP50 as food source (Sulston and Hodgkin, 1988).

Mounting live animals

To visualize live worms, agar pads (2% agar in distilled water) were prepared on a microscope slide (Shaham, 2006). The worms were pipetted from a plate containing M9 1X with 0.1% Tween-20 and 30 mM NaN$_3$. For fluorescence pictures, the L2 stage of worms were chosen because the worms harbor less green auto-fluorescence than in older worms and are thus more suitable for fluorescence microscopy. Worms were placed on the agar pad and covered with a 18×18 mm coverslip. Observations were made with a Nikon Eclipse 90i microscope and pictures taken with a Hamamatsu ORCA-ER digital camera. Images were acquired with NIS software (Nikon) and post-processed with Adobe Photoshop.

Culturing bacteria from worms

N2 *C. elegans* eggs were isolated using a solution composed of 10% commercial bleach and 1 N NaOH followed by four washes with 1X M9 (Sulston and Hodgkin, 1988). 300 to 600 eggs were transferred to a 60 mm plate with Nematode Minimal Media (NMM) agar that contains 3 g/l NaCl, 17 g/l agar, 5 mg/ml Cholesterol (Sigma), 1 mM CaCl$_2$, 1 mM MgSO$_4$, 25 mM Potassium Phosphate (pH 6) and incubated overnight at 20°C to allow L1s to hatch. An overnight *E. coli* OP50 culture in LB was then added and larval development was allowed to continue until L4 (about 40 h at 20°C). The larva were washed three times in a large volume of 1X M9 and added to NMM plates. An equal quantity of spores or vegetative bacterial cells, based on their optical density, was added to the NMM plates (for spores) or NGM plates (vegetative Bacilli and *E. coli*) such that they formed a visible thick lawn. The worms and bacteria were incubated at room temperature for 3 h.

The worms were harvested and washed with 1X M9 with 0.01% Tween-20 and 30 mM NaN$_3$ to induce paralysis. The surface of the worms was sterilized for 20 min with 1X M9 containing 3% commercial bleach and washed twice with 1X M9 containing 0.01% Tween-20. After the paralysis of the pharynx muscles, the worms are impermeable to any liquid allowing to sterilize only the outside of the worms. Indeed after washings, the worms are able to resume movement, indicating that sodium hypochlorite had not penetrated the gut of the worms.

After surface sterilization, the worms were resuspended in a final volume of 500 µl and counted in two 50 µl aliquots. The outside contamination was estimated by plating 100µl of the worm's solution. The worms were disrupted by vortexing with high-density 1 mm beads (Zirconia beads, BioSpec Products) and dilutions were
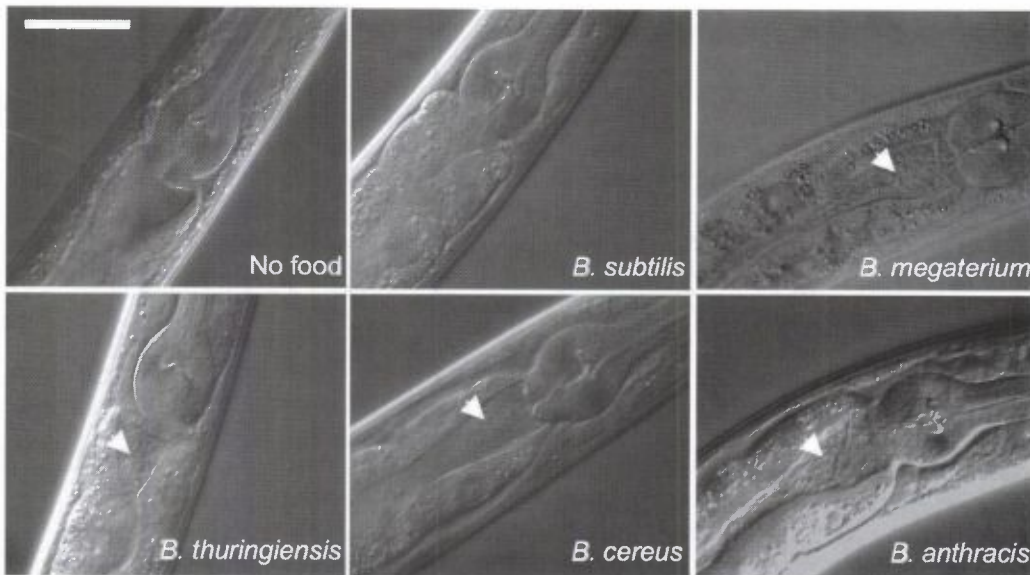
Figure 1. Digestibility of five *Bacillus* species by *C. elegans*. Representative DIC pictures of young adults of *C. elegans* N2 strain that were either starved ("No food") or fed with vegetative cells of *B. subtilis*, *B. megaterium*, *B. thuringiensis*, *B. cereus* or *B. anthracis*. Except for the "no food" control and *B. subtilis*, bacterial shapes are distinguishable in the intestine of the worms (arrows). Bar = 0.05 mm.

performed in T-base (15 mM NH_4SO_4, 80 mM K_2PO_4 (dibasic), 44 mM K_2PO_4 (monobasic), 3.5 mM Na Citrate), were plated on LB agar. The number of colony forming units that grew overnight at 37°C provided an estimation of the number of surviving bacteria in the disrupted worms. The heat resistance of the bacteria in the disrupted worms was tested by heating the samples 20 min at 80°C for *B. subtilis* and 65°C for 30 min for *B. anthracis*. To confirm that the disruption using beads did not affect the bacterial viability, we used gentler "guillotine" protocol to evaluate the number of CFU per individual worms. In this case, individual surface sterilized worms (at least 10 per sample) were dissected under a microscope and the content of each worm was plated on an LB agar plate (Martens and Goodrich-Blair, 2005). The figures obtained using the guillotine method (10 worms; 1501 +/– 142 bacteria/worm) were comparable to the bead-based method (~600 worms; 1527 bacteria/worm).

Spore pulse-chase experiment

A large population of *C. elegans* N2 (L3-L4 larval stage) was synchronized as described above, washed and placed on a 100 mm NMM agar plate containing a lawn of spores of the strain JDB1334. After 2 h at 20°C, the worms were washed five times with 1X M9 supplemented with 0.01% Tween-20. 500–600 worms were then added to 60 mm NMM plates containing no bacteria ("no food") or contained a lawn of *B. subtilis* JDB623 spores or an overnight LB culture of *E. coli* OP50. After the indicated time of interaction, the number of Spec[R] and/or Kan[R] bacteria per worm was determined by plating on appropriate selective media.

3. Results and Discussion

We analyzed the fate of Bacilli in *C. elegans* by testing vegetative cells of five prototypic species: *B. subtilis*, *B. anthracis*, *B. cereus*, *B. thuringiensis* and *B. megaterium* in adult *C. elegans*. These five species have distinguishable morphological differences and belong to three different *Bacillus* groups. *B. anthracis*, *B. cereus* and *B. thuringiensis* belong to the *Bacillus cereus* group (Helgason et al., 2000). *B. subtilis* and *B. megaterium* are non-pathogenic species of the *Bacillus* genus that persist in or transiently associate with the complex animal gut ecosystem (Barbosa et al., 2005). On the contrary, *B. anthracis* and *cereus* are mammalian pathogens whereas *B. thurigiensis* is an invertebrate pathogen (Helgason et al., 2000). We tested the ability of *C. elegans* to digest Bacilli that present differences in their shape and cell wall structure (e.g. the presence of extracellular structures such as an S-layer, (Mesnage et al., 2001)). The worms were placed on plates containing no bacteria ("no food") or plates seeded with vegetative bacteria. There was no noticeable modification observed in the feeding behavior of the worms exposed to the different bacterial lawns.

After 3 h, the worms were paralyzed and observed under the microscope (Fig. 1). No bacterial shapes were apparent in the intestine of worms either placed on the "no food" plate or on the plate seeded with *B. subtilis* vegetative cells. In contrast, bacterial shapes were clearly seen in the intestine of the worms fed with *B. anthracis*, *B. cereus*, *B. thuringiensis* and *B. megaterium*. Thus, vegetative *B. subtilis* are efficiently digested by *C. elegans*, consistent with previous observations (Garsin et al., 2001). On the other hand, *B. megaterium* had been shown to be a less

attractive food in comparison to *E. coli* OP50 (Shtonda and Avery, 2006). The apparent lower digestibility of *B. megaterium* by *C. elegans* could explain the weak attraction of *C. elegans* for this bacterium (Shtonda and Avery, 2006). It is noteworthy that *B. anthracis*, *B. cereus* and *B. thuringiensis* belong to the same phylogenetic group suggesting that common properties of this group could explain the apparent higher resistance of these species to *C. elegans* digestion.

To determine if the forms seen in the intestine were those of viable bacteria, we measured the number of surviving bacteria after disruption of the worms. For this purpose, L4 stage *C. elegans* were fed either with vegetative cells of *B. subtilis*, *B. anthracis* or with cells of *E. coli* OP50. The number of colony forming units per worms (CFU/worm) was on average <1 for worms fed with *B. subtilis* or *B. anthracis* and relatively higher (~30 per worm) for worms fed with *E. coli* (Fig. 2A). We also found that the CFU per worm obtained were <10 for *B. cereus* and *B. megaterium* in the intestine of adult *C. elegans* (data not shown). From these experiments, we conclude that vegetative cells of *Bacillus* species are efficiently killed during *C. elegans* ingestion under the laboratory conditions tested and that the shapes seen in the worm intestine (Fig. 1) were most probably the remains of the ingested bacteria. The partial resistance of *E. coli* to *C. elegans* digestion (Garigan et al., 2002) is consistent with our observation that *E. coli* survives *C. elegans* ingestion better than vegetative Bacilli.

To further study the resistance and sensitivity of *Bacillus* species to *C. elegans* predation, we tested whether the nematode could digest spores of prototypical *Bacillus* species. We observed L2 worms fed with spores of a strain of *B. subtilis* carrying a $P_{spoIIQ}::gfp$ fusion (Fig. 2B). In this strain, the expression of GFP is under the control of a promoter active only during the sporulation of the bacteria. Ovoid objects can be seen within the worm pharynx and upper intestine and co-localize with the green fluorescence indicating that they are spores (Fig. 2B). To more thoroughly assess the survival of spores in *C. elegans*, L4 worms were fed with *B. subtilis* or *B. anthracis* spores. As above, we determined the number of viable spores after 3 h of incubation, finding that a reproducible number of about 1300 CFU/worms was found for both species (Fig. 2A) and that at least 90% of the CFU survived heat treatment before being plated on rich media (data not shown). Since heat resistance is characteristic of spores but not of vegetative cells (Nicholson and Setlow, 1990), these results indicate that spores are resistant to worm ingestion and that they are the only forms of Bacilli that survive ingestion.

When the worms were fed with spores of *B. subtilis*, we noticed that the defecation rate of the worms seemed slower than for the worms fed with vegetative cells or *E. coli* (not shown). The intestinal transit in *C. elegans* depends notably on the presence of food and the nematode shows a slower

A.

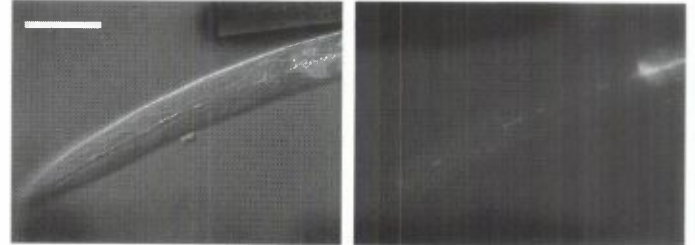| Strain	CFU/worm	SD
B. subtilis vegetative cells	0.05	0.02
B. anthracis vegetative cells	0.75	0.4
E. coli OP50	32	1.3
B. subtilis spores	1349	158
B. anthracis spores	1246	55

B.

Figure 2. *Bacillus* spores but not vegetative cells survive *C. elegans* digestion. A. The survival of either vegetative cells of wild type *B. subtilis* and *B. anthracis*, cells of *E. coli* OP50 and spores of *B. subtilis* and *B. anthracis* were assayed as described. The numbers of colony forming units recovered per L4 worms (CFU/worm) were calculated from duplicate samples, and the standard deviation is indicated. The experiments were performed at least twice and a representative experiment is shown. B. Larval stage 2 of *C. elegans* were fed with spores of a *B. subtilis* strain that expresses GFP under the control of a sporulation specific promoter ($P_{spoIIQ}::gfp$). The DIC picture (left) and fluorescence picture (right panel) show a representative sample.
Bar = 0.05 mm.

rate of defecation when food is scarce (Liu and Thomas, 1994). A slower defecation rate of the worms feeding on spores could indicate that the spores are not recognized as food by the worms. To assess this possibility, we performed a pulse-chase experiment with *B. subtilis* spores. We mixed *C. elegans* L4 with spores from a spectinomycin resistant (Spec^R) strain. After 3 h of interaction, we shifted the nematodes to a plate seeded with spores of a strain of a kanamycin resistant (Kan^R) strain. We then determined the number of CFU per worm with spectinomycin or kanamycin resistance at different times subsequent to the shift, finding that the number of Spec^R CFU per worm decreased by more than half within the first 20 minutes following the shift whereas the number of Kan^R CFU increased with time (Fig. 3A). Interestingly, the number of Kan^R spores was higher than the initial number of Spec^R spores. This higher number can be explained by the fact that the worms are 3 hours older than before the shift and their growing intestine might accumulate more spores than at the beginning of the experiment. After 3 h, only about 100 of the initial spores (Spec^R) remained in the intestine of the worms (Fig. 3A). The residence time for *E. coli* in the intestine of *C. elegans* is <2 minutes and more than 40% of the intestinal volume is expelled at each defecation cycle every 45 sec (Ghafouri and McGhee, 2007).
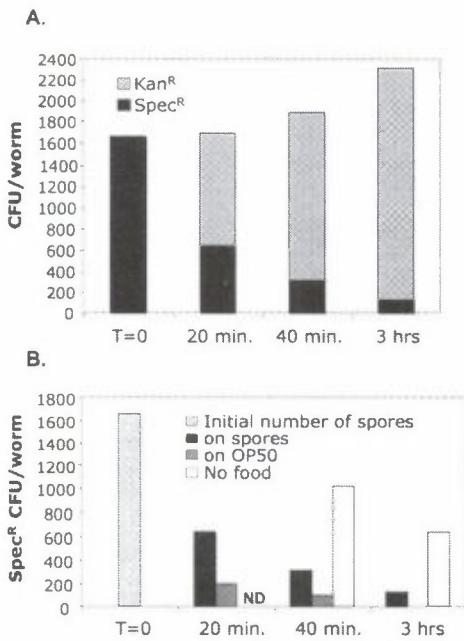
A.

B.

Figure 3. Transit of *B. subtilis* spores in the *C. elegans* intestine. A. L4 *C. elegans* were fed with spores of a *B. subtilis* strain carrying a spectinomycin resistance gene (Spec^R) and then transferred to a lawn of spores of *B. subtilis* strain resistant to kanamycin (Kan^R). The number of CFU per worm that were spectinomycin and kanamycin resistance were determined before the transfer (T=0) and 20 minutes, 40 minutes and 3 hours following the transfer. The data shown are representative of three experiments. B. L4 larva were fed Spec^R *B. subtilis* spores and transferred to plates containing either a lawn of spectinomycin sensitive spores (spores), a lawn of *E. coli* OP50 (OP50) or without any source of food (no food). The number of Spec^R CFU per worm were determined before the transfer (T=0) and 20 minutes, 40 minutes and 3 hours following the transfer on each plates. The data shown are representative of three experiments.

In contrast, we found that 60% of the initial intestinal volume filled with *B. subtilis* wild type spore is expelled after 20 minutes. Thus, the residence time of spores in the *C. elegans* intestine appears to be longer than for metabolically active bacteria (*E. coli*), and may reflect the quality (i.e., the 'digestibility') of the food.

We further examined the influence of the food source on the rate of clearance of spores. We shifted worms fed with Spec^R spores to either an empty plate ("no food") or to a plate containing a lawn of spectinomycin sensitive *B. subtilis* spores (Spec^S) or *E. coli* OP50 (respectively "on spores" and "OP50"; Fig. 3B). The clearance of spores within 20 min when the worms were shifted to a plate seeded with OP50 (12%) was more efficient as compared to a shift to a plate seeded with spores (40%) (Fig. 3B). In fact, no Spec^R CFUs were identified in worms fed with OP50 after 3 h. The number of Spec^R CFU decreased more slowly when the worms were placed on a plate without any

bacteria, with 60% of the initial number of Spec^R CFU/worm remained after 40 min and almost 40% after 3 h (Fig. 3B). Thus, the clearance of spores was accelerated in the presence of the readily digestible *E. coli* and retarded in the absence of any bacterial food source. This result is consistent with apparent dependence of intestinal transit in *C. elegans* on food availability, with a slower rate of defecation when food is scarce (Liu and Thomas, 1994).

It has become increasingly clear in organisms such as earthworms that metabolic activity of gut bacteria is important for intestinal function. This activity, consisting of enzymes produced by bacteria, could enhance the degradation of ingested organic matter and increase the assimilation of nutrients (Drake and Horn, 2007). Thus, the interaction between *C. elegans* and spore-forming bacteria may result in a symbiotic relationship between the organisms in the nematode gut with the bacteria capable of generating useful metabolic activities. Additionally, it has been hypothesized that spore-forming bacteria only undergo replication inside of hosts and not in the soil (Jensen et al., 2003), so the nematode gut may provide an environment hospitable for bacterial replication. *Sensuo stricto*, we do not know if the interaction between *C. elegans* and spore-forming bacteria is symbiotic, but future research will use the methodologies described here to examine this possibility in detail.

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