Journal of Nematology 37(1):18–25. 2005.


# Bionomics of a Phoretic Association Between *Paenibacillus* sp. and the Entomopathogenic Nematode *Steinernema diaprepesi*[1]

F. E. El-Borai,[2] I. W. Duncan,[2] and J. F. Preston[3]

*Abstract:* Spores of an unidentified bacterium were discovered adhering to cuticles of third-stage infective juvenile (IJ) *Steinernema diaprepesi* endemic in a central Florida citrus orchard. The spores were cup-shaped, 5 to 6 mm in length, and contained a central endospore. Based on 16S rDNA gene sequencing, the bacterium is closely related to the insect pathogens *Paenibacillus popilliae* and *P. lentimorbus*. However, unlike the latter bacteria, the *Paenibacillus* sp. is non-fastidious and grew readily on several standard media. The bacterium did not attach to cuticles of several entomopathogenic or plant-parasitic nematodes tested, suggesting host specificity to *S. diaprepesi*. Attachment of *Paenibacillus* sp. to the third-stage cuticle of *S. diaprepesi* differed from *Paenibacillus* spp. associated with heterorhabditid entomopathogenic nematodes, which attach to the IJ sheath (second-stage cuticle). The inability to detect endospores within the body of *S. diaprepesi* indicates that the bacterial association with the nematode is phoretic. The *Paenibacillus* sp. showed limited virulence to *Diaprepes abbreviatus*, requiring inoculation of larvae with $10^8$ spores to achieve death of the insect and reproduction of the bacterium. The effect of the bacterium on the nematode population biology was studied in 25-cm-long vertical sand columns. A single *D. abbreviatus* larva was confined below 15-cm depth, and the soil surface was inoculated with either spore-free or spore-encumbered IJ nematodes. After 7 days, the proportion of IJ below 5-cm depth was seven-fold greater for spore-free IJ than for spore-encumbered nematodes. Mortality of *D. abbreviatus* larvae was 72% greater ($P \leq 0.01$) for spore-free compared to spore-encumbered *S. diaprepesi*. More than 5 times as many progeny IJs ($P \leq 0.01$) were produced by spore-free compared to spore-encumbered nematodes. These data suggest that the bacterium is a component of the *D. abbreviatus* food web with some potential to regulate a natural enemy of the insect.

*Key words:* Antagonism, competition, *Diaprepes abbreviatus*, entomopathogenic nematode, *Paenibacillus*, phoresis, 16S rDNA, *Steinernema diaprepesi*, *Xenorhabdus* sp.

Entomopathogenic nematodes (EPN) are used widely in Florida citrus groves to help manage the citrus root weevil *Diaprepes abbreviatus* (Bullock et al., 1999; Duncan et al., 2000, 2003a,b; McCoy et al., 2000, 2002). In nature, enterobacteria in the genera *Photorhabdus* and *Xenorhabdus* are obligate symbionts of *Heterorhabditis* spp. and *Steinernema* spp., respectively (Akhurst and Boemare, 1990; Boemare et al., 1993; Forst and Nealson, 1996). These bacteria carried within the digestive tract of the EPN are released following infection of a host and are responsible for the death of the insect. Nematodes are able to complete several generations within the insect cadaver by feeding on the bacterial symbiont and on insect tissues conditioned by bacterial enzymes (Hu and Webster, 2000; Thaler et al., 1997). Antibiotics produced by the bacterial symbiont greatly reduce colonization of the insect cadaver by other microorganisms (Akhurst, 1982; Hu and Webster, 2000; Richardson et al., 1988).

Lysenko and Weiser (1974) reported a number of bacterial species other than *Xenorhabdus* spp. associated with the genus *Steinernema*, but none were highly pathogenic to *Galleria mellonella* larvae. Aguillera and Smart (1993) reported that *Steinernema scapterisci* carried bacterial species in addition to *Xenorhabdus* spp. inside the nematode. All of those bacteria species were capable of killing the insect host, although they were less virulent than *Xenorhabdus* sp.

Several *Paenibacillus* species have been reported associated with insects. Some are obligate insect pathogens such as *P. popilliae*, *P. lentimorbus*, and *P. larvae* (Pettersson et al., 1999), whereas others are facultative pathogens such as *P. apiarus* (Nakamura, 1996) and *P. alvei* (Krieg, 1981). *Paenibacillus popilliae* and *P. lentimorbus* (Walsh and Webster, 2003) are the causal agent of types A and B milky disease in Japanese beetle and other scarab larvae and have been used as biocontrol agents against these insects (Cappaert and Smitley, 2002; Klein and Kaya, 1995; Koppenhöfer et al., 2000). There are some reports of associations between EPN and *Paenibacillus* spp. These bacteria attach to the nematode cuticle and reproduce within insects infected by the nematode. Enright et al. (2001, 2003) described a phoretic association between *Heterorhabditis* spp. and *P. nematophilus*. Spores of the bacterium attached to the sheath (second-stage cuticle) of the third-stage juveniles of all tested *Heterorhabditis* species and some close relatives in the order Strongylida but not to several species of *Steinernema* or other soil-inhabiting nematodes. Marti and Timper (1999) reported an association between several *Heterorhabditis* sp. isolates and a putative *Bacillus* species that was morphologically similar to the *Paenibacillus* spp. reported by Enwright et al. (2003).

During surveys of the prevalence of endemic entomopathogenic nematodes in Florida orchards (Duncan and El-Borai, unpubl. Duncan et al., 2003b), spores morphologically similar to those reported by Enright et al. (2003) and Marti and Timper (1999) were fre-

Received for publication 12 July 2004.

[1] This research was supported by the Florida Agricultural Experiment Station, including CRIS projects LAL-03924 and MCS-03703, and by CRIS project MCS-03798 from USDA-CSREES NE-171 Regional Project, and a grant from the Florida Citrus Production Research Advisory Council, and approved for publication as Journal Series No. R-10331.

[2] Postdoctoral Research Scientist, Professor, University of Florida, IFAS, Citrus Research and Education Center, 700 Experiment Station Rd., Lake Alfred FL 33850

[3] Professor, Microbiology and Cell Science Dept., IFAS, University of Florida, P. O. Box 110700, Gainesville, FL 32611

The authors thank Guang Nong of the Department of Microbiology and Cell Science for assistance with DNA alignment and phylogenetic analysis.

E-mail: lwdn@crec.ifas.ufl.edu

This manuscript was edited by S. Patricia Stock.

quently found attached to *H. indica* and *H. zealandica* emerging from cadavers of *D. abbreviatus* (Fig. 1A). Spores of unidentified and morphologically dissimilar bacteria were also found adhering in large numbers to the cuticle of third-stage infective juvenile (IJ) *S. diaprepesi* (Fig. 1B). In this paper, we report results of studies to (i) characterize the bacterium associated with *S. diaprepesi* based on fatty acid methyl ester (FAME) profiles and 16S rDNA gene sequencing; (ii) evaluate virulence of the bacterium to *D. abbreviatus* larvae; (iii) determine the effect of the bacterium on the motility, infectivity, and reproduction of *S. diaprepesi;* and (iv) evaluate the nematode vector specificity of the bacterium.

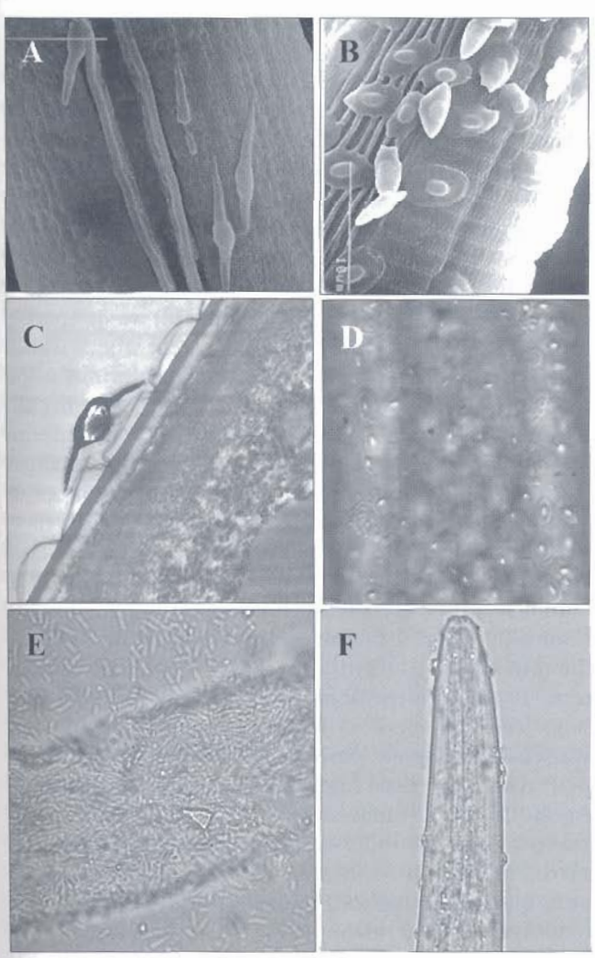


FIG. 1. A) Scanning electron micrographs of spindle-shaped spores attached to *Heterorhabditis zealandica* emerging from cadavers of *Diaprepes abbreviatus* (scale bar = 5 µm). B) Scanning electron micrographs (scale bar = 10 µm) of heavily spore-encumbered, exsheathed cuticle of IJ *Steinernema diaprepesi*. C) Transmission electron micrograph illustrates the attachment of the spores to IJ cuticle with no evidence of spore penetration tubes or development of the bacterium within the IJ. D, E, F) Compound microscope micrographs illustrate the *Paenibacillus* sp. spores adhering only to 3rd-stage cuticle of *S. diaprepesi* (nematode exsheathed) (D) (400X); cast cuticle of IJ *S. diaprepesi* filled with unidentified rod-shaped bacilli (E) (1000X); and cup-shaped unidentified bacterial spores adhering to the cuticle of IJ *S. glaseri* (F) (400X).

## MATERIALS AND METHODS

*Bacterium isolation:* A bacterium was isolated from infective third-stage *S. diaprepesi* that emerged from *D. abbreviatus* sentinel larvae recovered from the type locality of *S. diaprepesi* (Nguyen and Duncan, 2002). Spore-encumbered IJ were harvested, rinsed several times with sterile-distilled water, and placed in sterile Eppendorf tubes in a water bath (80 °C) for 15 minutes. The tubes were cooled and shaken, and 200-µl suspensions were streaked onto 1.5% nutrient agar (NA) (Sigma Chemical Company, St. Louis, MO) and incubated at room temperature (~ 25 °C). After 96 hours, single cell colonies were isolated on NA, grown for 72 hours, and sampled for microscopic observation (1000×). Colonies with combinations of rod-shaped bacteria and cup-shaped spores were re-isolated. The ability of spores to attach to *S. diaprepesi* was confirmed by surface-disinfesting spore-free nematodes with 0.2% streptomycin sulfate for 24 hours, rinsing repeatedly, and placing nematodes onto bacterial cultures. After 48 hours, the nematodes were rinsed repeatedly and examined microscopically. Colonies producing spores that attached to nematodes were maintained in vivo by exposing larvae of the weevil *D. abbreviatus* to spore-encumbered, infective third-stage *S. diaprepesi*, and in vitro on nutrient agar and Luria-Bertani (LB) agar (Fisher Scientific, Pittsburgh, PA).

*Nematode preparation:* Spore-encumbered *S. diaprepesi* used in all experiments were prepared by exposing surface-disinfested IJ, freshly harvested from *D. abbreviatus*, to 7-day-old colonies of the bacterium on NA or Luria-Bertani medium agar (LBA). Spore-free IJ were recovered from petri dishes of NA after equivalent exposure times.

*Bacterium characterization:* The bacteria were cultivated on either 1.5% agar (LBA) or broth (LB) at 30 °C (Davis et al., 1980). Stocks were maintained on LBA and transferred to 2-ml standing broth cultures to determine substrate requirements. For genus and species determination, LBA plates containing isolated colonies were sent to MIDI Labs (Newark, DE) for typing by FAME and 16S rDNA sequence analysis. The FAME analysis was also conducted on colonies obtained from tryptone soy agar in the diagnostic laboratory of the Department of Plant Pathology at the University of Florida. MIDI software and libraries were used for the assignment of similarity indices relative to other bacteria. The partial sequence of the 16S rDNA was determined for positions 1 to 536 (isolate Tc1) after PCR amplification from genomic DNA isolated from bacterial colonies. As noted in the protocol from MIDI Labs, the primers used for amplification corresponded to positions 005 and 531 for the gene encoding 16S rDNA in

*Escherichia coli*. Cycle sequencing was performed with AmpliTaq FS DNA polymerase and Rhodamine dye terminators and processed as described by MIDI Labs. The sequence comparisons were made with others archived in GenBank, and a phylogenetic tree was constructed with MEGA-2 (Kumar et al., 2001) using the Neighbor-Joining algorithm (Saitou and Nei, 1987).

*Specificity and nature of bacterium-nematode symbiosis:* To investigate whether the association between the nematode and bacterium is pathogenic or phoretic, spore-encumbered nematodes that emerged from insect cadavers were maintained for 30 days on water and nutrient agar and periodically examined with light and transmission electron microscopy for evidence of development of the bacterium within the nematode. In addition, insect larvae were placed in individual petri dishes (60-mm-diam.) with 40 gm autoclaved fine sand (10% moisture) and 1 ml water with 400 IJ of *S. diaprepesi* that were either spore-free or encumbered with bacterial spores. After 72 hours exposure to nematodes, some insects were dissected to recover the cast cuticles of the colonizing IJ. Other insects were dissected daily for up to 1 week to recover all EPN life stages. All stages of the nematode were examined at 1000× magnification for evidence of infection and (or) development by the bacterium. Spore-encumbered nematodes were prepared for transmission electron microscopy using a method described by Giblin-Davis et al. (2001) with some modifications. The nematodes were heat-killed at 60 °C for 10 minutes, cut into three to five pieces each, transferred to a 1.5-ml Eppendorf tube, and centrifuged to pellet nematode pieces. Nematodes were chilled at 6 °C for 1 hour, then fixed in 2% (v/v) glutaraldehyde and 2% (v/v) formaldehyde in 0.1 M sodium cacodylate buffer (pH 7.2) overnight at 6 °C. Nematodes in the Eppendorf tube were embedded by pipeting 3% (w/v) agarose over them without disturbing the pellet, removed from the tube after gelling, and cut into 1- to 2-mm pieces. Glutaraldehyde was removed from the blocks of agarose with five rinses of 0.1 M sodium cacodylate buffer, after which 1 ml 0.1 M sodium cacodylate buffer with 2% (w/v) osmium tetroxide ($OsO_4$) and 1% (w/v) aqueous uranyl acetate was added. After 4 hours post-fix at room temperature, osmium solution was removed and agar pieces rinsed twice with 0.1 M cacodylate buffer. Nematodes were dehydrated 10 minutes each in an alcohol series up to 100% and then in acetone 3 times. After embedding in Spurr's epoxy resin, sections were cut using a diamond knife on an LKB Huxley microtome. Ten-micrometer-thick sections were stained with methylene blue azure A and then 0.5% Basic Fuchsin, dried, mounted, and observed under oil (Schneider, 1981). Thin sections were placed on a Formvar-coated (0.5%), 200-mesh copper grid, stained with 5% uranyl acetate for 15 minutes (Stempack and Ward, 1964), washed in distilled water 10 seconds, then stained with lead citrate 5 minutes (Reynolds, 1963). After washing in alternating 0.02 M NaOH and distilled water 3 times, grids were placed on filter paper to dry. Nematodes were observed in a Phillips 201 electron microscope and photographed with 3¼-×-4¼-inch plate film.

To evaluate the nematode vector specificity of the bacterium, *Rhabditis* sp. and different entomopathogenic (*S. carpocapsae, S. feltiae, S. riobrave, H. bacteriophora, H. indica,* and *H. zealandica*) and plant-parasitic nematodes (*Tylenchulus semipenetrans, Meloidogyne arenaria* race 1, *M. javanica, Pratylenchus* sp. *Radopholus* sp., and *Belonolaimus* sp.) were surface-disinfected with 0.2% streptomycin sulfate for 24 hours, rinsed repeatedly with sterile-distilled water (sdw), and transferred to 7-day-old colonies of the bacterium on NA. After 72 hours, the nematodes were rinsed repeatedly with sdw and examined microscopically to determine if spores of the bacterium adhered to the nematode cuticle.

*Bacterium pathogenicity and development in insects:* The isolated bacterium and *Xenorhabdus riobrave* were grown on LBA for 7 days, at which time the isolated bacterium had developed almost exclusively to the spore stage. Bacteria were washed from cultures in sdw, quantified with a hemacytometer (American Optical Co.), and a dilution series ($10^2$ to $10^8$ bacteria/10 µl) was prepared. Ten *G. mellonella* larvae (last instar) were injected with 10 µl of each concentration of each bacterium using Micro-fine IV syringes (0.36 mm × 13.0 mm; Becton Dickinson, Franklin Lakes, NJ). Larvae inoculated with 10 µl water served as controls. Larvae were maintained individually in petri dishes on moist filter paper at room temperature (23 ± 2 °C) for 52 hours when mortality was recorded. The experiment was conducted twice.

In a second experiment, rod-shaped stages from 96-hour-old colonies of the isolated bacterium were diluted in sdw to concentrations of $10^6$, $10^7$, and $10^8$ bacteria 10 $\mu l^{-1}$. Thirty *D. abbreviatus* larvae ($5^{th}$ to $8^{th}$ instar) were injected as described with 10 µl of each suspension. Larvae were maintained individually in petri dishes on moist filter paper at room temperature. At day 18, three cadavers from each dose were macerated in 5 ml sdw for 2 minutes with a minivortex and stirred rapidly for 5 minutes. The resulting suspensions were washed through a 74-µm sieve with 5 ml sdw for a total volume of 10 ml. Spores in these suspensions were quantified (2 counts) using a hemacytometer.

*Effect of the bacterium on infection of weevils by EPN:* Candler sand (97:2:1, sand:silt:clay, 10:100 wt water:dry wt soil) soil columns (5-cm-diam. × 20-cm-long) were established in PVC tubes consisting of four 5-cm-long sections secured with duct tape. A single *D. abbreviatus* larva was confined below 15-cm depth by placing a screen (2-mm openings) between the third and fourth sections of the column. *Steinernema diaprepesi* (20 IJ $cm^{-2}$) in 0.5 ml water were pipeted onto the soil surface of each column. Two treatments, replicated 40 times,

consisted of using either spore-encumbered or spore-free nematodes. The tops of the sand columns were covered with aluminum foil, and columns were maintained at room temperature (23 ± 2 °C). Seven days post-treatment, the PVC tubes were disassembled. Insect larvae and cadavers were removed from soil and placed onto moist filter paper in individual petri dishes (7-cm-diam.). The soil from each section of groups of five columns of the same treatments was combined, mixed, and 60 $cm^3$ subsamples were placed on Baermann funnels for 48 hours. Twenty-four hours after terminating the experiment, live insect larvae were discarded and cadavers were examined for up to 30 days for nematode emergence. Nematode reproduction was measured by macerating the cadaver and filter paper in 120 ml water in a blender. After settling, the suspension was aspirated to a volume of 50 ml from which nematodes in 1 ml were counted.

A second experiment was conducted with modifications. The column length was increased to 25 cm and nematode-killed *D. abbreviatus*, rather than IJ in water, were placed on the surface of the column. To obtain cadavers, insect larvae were placed in individual petri dishes (60-mm-diam.) filled with autoclaved Candler fine sand (10% moisture) and 1 ml water with 400 IJ *S. diaprepesi* that were either spore-free or encumbered with bacterial spores. The dishes were sealed with parafilm and stored at room temperature (23 ± 2 °C). Cadavers were examined daily under a dissecting microscope for evidence of successful nematode reproduction. When nematode juveniles were clearly visible swarming beneath the insect cuticle, the cadaver was placed onto a soil column until 31 tubes each with spore-free or spore-encumbered nematodes were prepared. A live *D. abbreviatus* larva was secured in the bottom section of the column at the same time that cadavers were added to the soil surface. Experimental units were maintained and processed as described 7 days after the insects and cadavers were introduced (i.e., the termination dates varied). Insect mortality in each replicate was recorded, and 14 tubes were selected randomly for recovering and enumerating the numbers of IJ at each soil depth. In addition, the relative degree of spore encumbrance was estimated for 50 to 100 randomly selected specimens/sample using the following index: 1 = 0 to 10 spores, 2 = 11 to 100 spores, and 3 = >100 spores per nematode.

*Data analysis:* Data expressed as proportions were transformed (arcsin, square root) and means were compared with analysis of variance (Minitab Inc., Lancaster, PA). Untransformed means and standard errors are shown in figures. Means for proportion mortality were compared by calculating the standard normal variate (Z statistic, two tail) for binomial proportions (Bhattacharyya and Johnson, 1977). Probit analysis procedure using SAS software (SAS Institute, Cary, NC) was used to calculate the $LC_{50}$ and $LC_{90}$ for both bacteria.

## Results

*Specificity and nature of bacterium-nematode association: Heterorhabditis zealandica* and *H. indica* recovered from Florida citrus orchards by baiting with sentinel insects often reveal the presence of associated bacterial endospores. The scanning electron micrograph in Figure 1A shows spindle-shaped sporangia with central cores attached to the second-stage cuticle (sheath) of *H. zealandica* IJ emerging from cadavers of *D. abbreviatus*. A distinctive morphology defines bacterial endospores associated with the third-stage cuticles of *S. diaprepesi* IJ recovered from *D. abbreviatus* (Fig. 1B,C). Here the cores are discerned in the center of a sporangium resembling a pancake. The TEM in Figure 1C depicts an endospore associated with the exsheathed IJ cuticle, with electron-dense structures casting the endospore onto an exosporium extending over a slime layer presumably participating in adherence. Within 48 to 72 hours after penetration of *D. abbreviatus*, the cast IJ cuticles were filled with unidentified rod-shaped bacilli (Fig. 1E). No spores were detected on the cuticles or within the bodies of any life stages of the nematodes during the population growth phase within the cadaver. Following egression of IJ from insect cadavers, spores did not attach to the IJ sheath ($2^{nd}$-stage cuticle); however, most exsheathed IJ were heavily encumbered with spores (Fig. 1B,D). No spores were seen within the IJ body, and TEM presented no evidence of spore penetration tubes or development of the bacterium within the IJ. However, when IJs were placed on nutrient agar with the bacteria, some developed to adult stages in which spores were seen within the digestive tract and pseudocoelom of adult males and females. The spores did not attach to any of the other entomopathogenic or plant-parasitic nematode species tested. However, cup-shaped spores have been seen associated with other steinernematid species isolated from citrus orchards in Florida (i.e., *S. glaseri*) (Fig. 1F).

*Bacterium characterization:* The cultures of the bacterium on LBA medium all contained colonies that were opaque and cream colored. Sequential transfers and cultivation on LB broth provided cultures with decreasing turbidity as a function of the transfer. When plated on LB agar, these cultures showed two distinct colony types—one type opaque (Oc) and the other transparent (Tc). Each colony typed was picked from a culture inoculated into LB broth (2 ml) and incubated as standing cultures at room temperature (22 °C to 24 °C). The broth cultures were slightly turbid; cultures derived from the Oc isolates were noticeably more turbid than those from the Tc isolates. Samples from Oc cultures and Tc cultures were streaked on LB agar and incubated for 6 days. The colonies obtained a maximum diameter of 3 mm for Oc isolate and 5 mm for Tc isolates. Only the Oc isolate showed the differentiation into spore-like structures. Both the Tc and Oc isolates

contained Gram-negative and Gram-positive rods of approximately the same size, indicating gram stain-variation characteristic of many *Paenibacillus* spp. (Pettersson et al., 1999).

The partial 16S rDNA sequence (536 bp) was determined by MIDI Labs for PCR-generated products derived from both the Oc and Tc isolates. The sequences obtained for the two isolates showed identity values of 98.1%. The difficulty in generating PCR products from the Oc isolate may have contributed to inaccuracies in the determined sequence and, pending additional sequencing, the two isolates may be considered the same. The FAME analysis supports an identity or strong similarity between the two isolates.

The sequence for the Tc isolate was used for alignment and phylogenetic comparison with other endospore-forming bacteria. Figure 2 provides a phylogram that estimates the common evolutionary lineage with other *Paenibacillus* spp., particularly the insect pathogens *P. lentimorbus* and *P. popilliae*, as well as *P. thiaminolyticus*. More distantly related is *P. nematophilus*, a species for which endospores have been found attached to *Heterorhabditis megidis*. The sequence identity values for *Paenibacillus* sp. SdTc vs. *P. lentimorbus*, *P. thiaminolyticus*, *P. nematophilus*, and *P. popilliae* were 94.4%, 93.3%, 87.6%, and 83.3%, respectively.

*Bacterium pathogenicity and development in insects:* Larvae of *G. mellonella* were more susceptible to infection by *Xenorhabdus* than by *Paenibacillus* sp. The $LC_{50}$ and $LC_{90}$ of *Xenorhabdus* sp. were 54 and $3.0 \times 10^3$ bacteria, respectively, whereas $2.5 \times 10^5$ and $1.5 \times 10^6$ spores of *Paenibacillus* sp. were required to achieve similar results (Fig. 3A). *Paenibacillus* sp. exhibited a similar level of pathogenicity in *D. abbreviatus* larvae, with $LC_{50}$ estimated at $1.6 \times 10^5$ spores (data not shown).

All *D. abbreviatus* larvae that were injected with $10^6$ to $10^8$ vegetative rods of the bacterium died and contained only spores after 2 weeks. The final number of spores was similar for the three dosages (range = 1.2 to $4.4 \times 10^9$), suggesting an upper limit of several billion spores per cadaver under these conditions (Fig. 3B).

*Effect of the bacterium on infection of weevils by EPN:* In the first experiment, more spore-free than spore-encumbered IJ were recovered on average from the sand columns ($4.15 \pm 0.75/cm^3$ vs. $2.35 \pm 0.25/cm^3$, mean and standard error; $P < 0.05$). Fewer spore-encumbered IJ of *S. diaprepesi* moved downward in sand columns compared to spore-free IJ (Fig. 4A). The proportion of spore-free IJ in a column recovered at depths below 5 cm was 7-fold that of spore-encumbered IJ ($P < 0.01$). The proportion of the population recovered at 15–20 cm was 39 times greater for spore-free compared to spore-encumbered IJ ($P < 0.01$). Spore-free IJ killed nearly twice as many as *Diaprepes* larvae (Fig. 4B) and produced nearly 5-fold more progeny than did spore-encumbered nematodes ($P \leq 0.01$) (Fig. 4C). The number of progeny per cadaver did not differ ($P > 0.05$) between spore-encumbered (4,178 ± 3,056; mean and standard deviation) and spore-free populations (9,623 ± 7,866).

Results of the second experiment were similar, but the effect of the bacterium on nematode migration was less pronounced using nematodes that emerged natu-

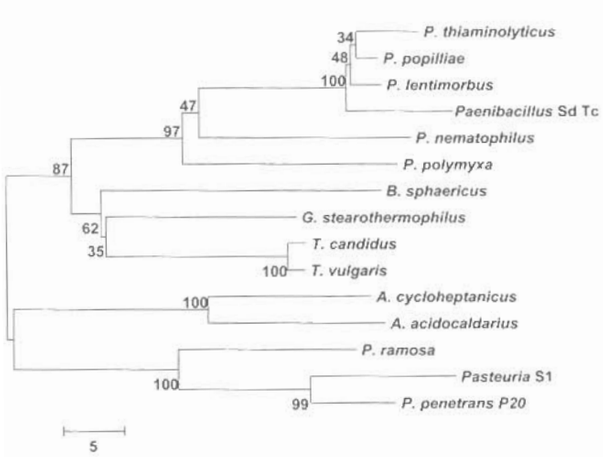


FIG. 2. Phylogenetic relationship of *Paenibacillus* sp. SdTc1 to other endospore-forming bacteria based upon nucleotide sequences of genes encoding 16S rDNA. The phylogenetic tree was constructed from rDNA sequences registered with GenBank, using MEGA2 (Kumar et al., 2001) with the Neighbor-Joining method (Saitou and Nei, 1987). The scale represents a relative evolutionary distance, and the whole numbers are bootstrap values for 1,000 analyses. The sequences for the defined species were obtained from GenBank with the following accession numbers: *P. thiaminolyticus*, D78475; *P. popilliae*, X60633; *P. lentimorbus*, AB110988; *P. nematophilus*, AF 480937; *Paenibacillus polymyxa*, AF355463; *Bacillus sphaericus*, AJ311893; *Geobacillus stearothermophilus*, AB021196; *Thermoactinomyces candidus*, M77490; *T. vulgaris*, M77491; *Alicyclobacillus cycloheptanicus*, X51928; *A. acidocaldarius*, X60742; *Pasteuria ramosa*; U34688; *Pasteuria* S1, AF254387; *P. penetrans* P20, AF077672.

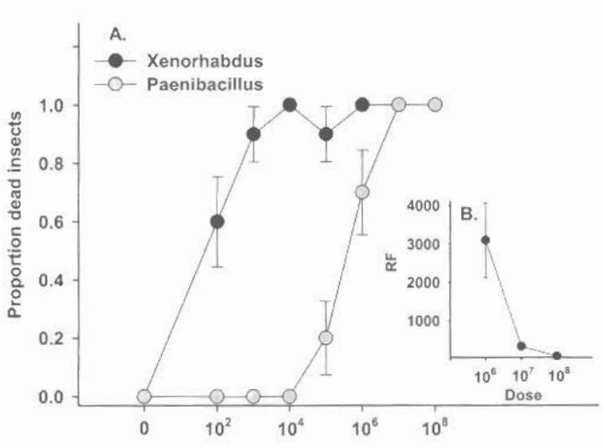


FIG. 3. Virulence of *Xenorhabdus* rods and *Paenibacillus* sp. spores injected into 5th instar larvae of *Galleria mellonella* (A) and reproduction factor (RF = final population density/initial population density) of the vegetative rod-shaped stages of *Paenibacillus* sp. to mature spores inside 5th to 8th instar *D. abbreviatus* larvae (B). Error bars = standard error of the means for $N = 10$ (A) and $N = 3$ (B).

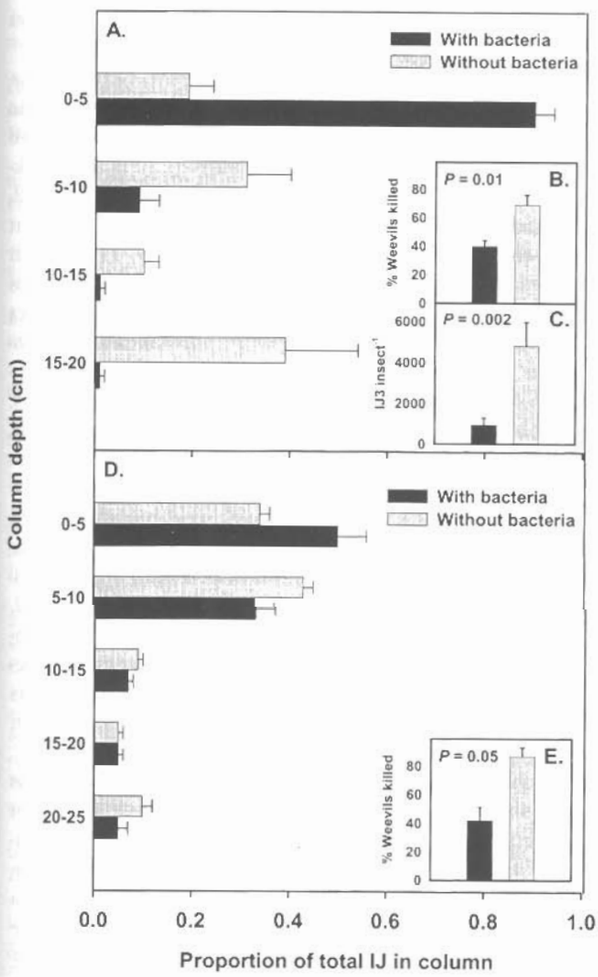


FIG. 4. Effect of *Paenibacillus* sp. spores on movement and infection of *Diaprepes abbreviatus* by *Steinernema diaprepesi* when spore attachment to the IJ occurs in vitro (top panel) and in vivo (bottom panel); (A, D) Proportion of spore-encumbered and spore-free IJ recovered at each soil depth (bars = standard error of the mean, $N = 8$); (B, E) Mortality of *D. abbreviatus* larvae; (C) reproduction of *S. diaprepesi*.

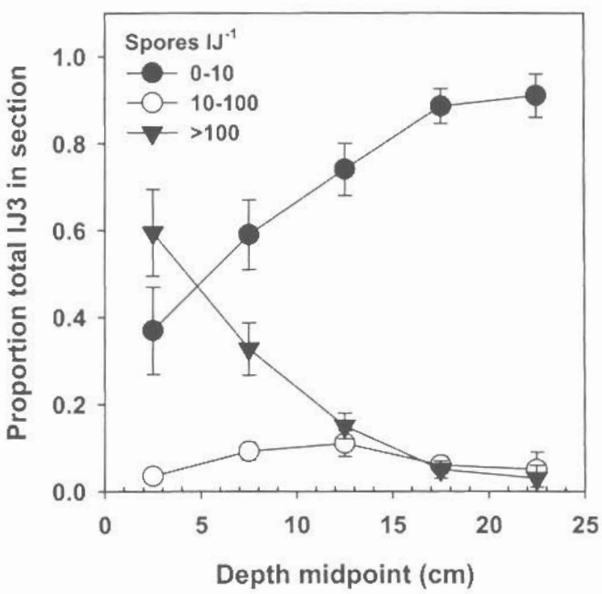


FIG. 5. The relationship between the level of spore encumbrance and depth in soil columns at which IJ were recovered. Error bars = standard error of the means for 50 to 100 randomly selected specimens/sample using the following index: 1 = 0 to 10 spores, 2 = 11 to 100 spores, and 3 = > 100 spores per nematode.

rally from the insect cadavers. Fewer IJ ($P \leq 0.05$) were recovered from sand columns in the spore-encumbered treatment (226 ± 58/cm$^3$) compared to the spore-free treatment (365 ± 46/cm$^3$). A greater percentage ($P \leq 0.01$) of IJ in the spore-free treatment were recovered below 5 cm (66%) compared to the spore-encumbered treatment (50%) (Fig. 4D). Infective juveniles in the spore-free treatment killed more than twice as many weevils ($P \leq 0.001$) compared to IJ in the spore-encumbered treatment (0.87 ± 0.06 vs. 0.41 ± 0.09) (Fig. 4E). There was an inverse relationship between the degree of spore encumbrance and depth at which IJ were recovered (Fig. 5). The average proportion of nematodes in each encumbrance class (± standard error) recovered per column was 0.54 ± 0.08 (0–10), 0.08 ± 0.01 (10–100), and 0.38 ± 0.07 (> 100).

## DISCUSSION

This is the first report of a symbiotic association between a *Paenibacillus* sp. and nematodes in the genus *Steinernema*. This form of bacterial association with the infective nematode stage appears to be adaptive because we commonly observe bacterial spores adhering to the cuticle of IJ *S. glaseri* and to the sheath of IJ *Heterorhabditis indica* and *H. zealandica* recovered from Florida citrus orchards. *Steinernema riobrave* is the only EPN isolated from these soils without evidence of associated Paenibacillus, perhaps because the nematode is thought to be exotic to Florida, having been introduced to manage *D. abbreviatus*. The spores attached to heterorhabditids in Florida are spindle shaped as described from heterorhabditids in Georgia (Marti and Timper, 1999) and in Estonia and India (Enright et al., 2001, 2003). Although the core of the endospore of the *Paenibacillus* sp. from *S. diaprepesi* appears similar in size and shape to those associated with heterorhabditids, the sporangium is not spindle shaped but rather ovoid, completely encircling the core. The bacterium from *S. diaprepesi* also exhibited a greater degree of nematode specificity than reported for bacteria isolated from heterorhabditid species. *Paenibacillus nematophilus* attaches to several parasitic nematodes in the genus *Heterorhabditis* and the order Strongylida, but not to other soilborne nematodes tested (Enright et al., 2001), whereas the *Paenibacillus* sp. from *S. diaprepesi* attached to none of the other heterorhabditid, steinernematid, or tylenchid species tested in this study. A further difference between *Paenibacillus* sp. from *S. diaprepesi* and those

from heterorhabditids is that spores attach to the third-stage cuticle for the former isolate and to the sheath formed by the second-stage cuticle for the latter groups. The selection for this difference may derive from a greater tendency of heterorhabditids to retain the sheath when moving through soil compared to steinernematids (Campbell and Gaugler, 1991; Patel et al., 1997; Timper and Kaya, 1989).

As with other *Paenibacillus* species associated with entomopathogenic nematodes, the functional relationship between *Paenibacillus* sp. and *S. diaprepesi* appears to be phoretic. The bacterium is able to enter the insect with the nematode and then reproduce while feeding on insect tissues. Although bacterial spores were observed in the body cavity of adult male and female nematodes, their effect on nematode biology is unknown. The bacterium did not require the presence of nematodes to reproduce within the insect cadaver, nor did the presence of the bacterium prevent nematode population growth in the insect. Nevertheless, additional research is needed to establish if *Paenibacillus* sp. affects nematode population growth rate within the cadaver. Moreover, the coexistence of *Paenibacillus* sp. and *Xenorhabdus* sp. within the cadaver merits study because *Xenorhabdus* sp. produces potent inhibitors to the growth of *Paenibacillus* sp. in vitro and reduces population growth of *Paenibacillus* sp. in vivo (El-Borai, unpubl.). *Enterococus* sp. was shown to temporarily avoid antibiotic inhibition by *Xenorhabdus* sp. by completing its growth phase prior to that of the nematode symbiont (Issacson and Webster, 2002; Walsh and Webster, 2003). If sporangia of *Paenibacillus* sp. are resistant to effects of antibiotics and other metabolites produced by *Xenorhabdus* sp., growth and sporulation by *Paenibacillus* sp. either before or after that of *Xenorhabdus* sp. may permit the bacteria to coexist. The known *Paenibacillus* spp. associated with heterorhabidtid nematodes differ from *Paenibacillus* sp. from *S. diaprepesi* in that they are insensitive to antibiotics produced by the *Photorhabditis* spp. symbionts of the nematodes (Enright and Griffin, 2004).

The benefit to *Paenibacillus* sp. from its association with an organism that effectively transports it to a food source comes at a cost to the nematode of reduced host-finding and subsequent population growth. This effect occurred for nematodes that were infested in vitro and for those that egressed naturally from insect cadavers containing the bacterium. Approximately half of the IJ that emerged from bacteria-infested cadavers retained 10 or fewer spores when recovered from the soil, and these were virtually the only nematodes detected in the deepest portion of the sand columns. Insect mortality in this treatment was 48% of that for the non-encumbered nematodes. Such a marked effect on finding a host and nematode population growth suggests that *Paenibacillus* sp. may be a component of the *Diaprepes* food web with the potential for density-dependent regulation of a natural enemy of this insect pest.

A phoretic and commensal relationship between *Paenibacillus* spp. and EPN is in contrast to the parasitic relationship that species of the genus *Pasteuria* have with their respective nematode hosts (Chen and Dickson, 1998; Preston et al., 2003). As noted in Figure 2, *Pasteuria* spp. are in a clade phylogenetically distant from the clade including *Paenibacillus* spp. The known *Paenibacillus* species that exist in phoretic relationships with nematodes are non-fastidious, in contrast to insect pathogens in the genus such as *P. popilliae* and *P. lentimorbus*. Although there are no reports on the entomopathogenic nature of other nematode-phoretic species, the *Paenibacillus* sp. from *S. diaprepesi* is avirulent to *D. abbreviatus* and *G. mellonella* compared to other insect pathogenic bacteria. The intimate association between steinernematids and the symbiotic and highly virulent *Xenorhabdus* spp. provides the basis for their propagation in their insect hosts, obviating the need for virulence in these *Paenibacillus* species. Thurston et al. (1993, 1994) reported that heterorhabditid and steinernematid nematodes are able to infect insects and reproduce concomitantly with *P. popilliae*, which suggests a scenario for the evolution of associations between EPN and phoretic, non-virulent *Paenibacillus* sp.

Since its recent definition, the genus *Paenibacillus* has been shown to include a large number of species in different soil ecosystems. The identification of a number of these as having an apparent comensal relationship with nematodes (rhabditids) and others having a parasitic relationship with insect (scarab beetle) larvae suggests a process of divergent evolution in this ecosystem that is dependent upon invertebrate fauna. Further physiological and phylogenetic comparisons of *Paenibacillus* spp. associated with the motile juvenile stages of nematodes, including EPN, may provide useful insight into the evolutionary transition between commensal and parasitic relationships. Such relationships may then be exploited for the biocontrol of agronomic pests.

## Literature Cited

Akhurst, R. J. 1982. Antibiotic activity of *Xenorhabdus* spp. bacteria symbiotically associated with insect pathogenic nematodes of the families Heterorhabditidae and Steinernematidae. Journal of General Microbiology 128:3061–3065.

Akhurst, R. J., and N. E. Boemare. 1990. Biology and taxonomy of *Xenorhabdus*. Pp. 75–90 *in* R. Gaugler and H. K. Kaya eds. Entomopathogenic nematodes in biological control. Boca Raton, FL: CREC Press.

Aguillera, M., and G. Smart. 1993. Development, reproduction and pathogenicity of *Steinernema scapterisci* in monoxenic culture with different species of bacteria. Journal of Invertebrate Pathology 62:289–294.

Bhattacharyya, G. K., and R. A. Johnson. 1977. Statistical concepts and methods. New York: John Wiley & Sons.

Boemare, N. E., R. J. Akhurst, and R. J. Mourant. 1993. DNA relatedness between *Xenorhabdus* spp. (Enterobacteriaceae), symbiotic bacteria of entomopathogenic nematodes, and a proposal to transfer

*Xenorhabdus luminescens* to a new genus, *Photorhabdus* gen. nov. International Journal of Systematic Bacteriology 43:249–255.

Bullock, R. C., R. R. Pelosi, and F. E. Killer. 1999. Management of citrus root weevils (Coleoptera: Curculionidae) on Florida citrus with soil-applied entomopathogenic nematodes (Nematoda: Rhabditida). Florida Entomologist 82:1–7.

Campbell, J. R., and R. Gaugler. 1991. Mechanisms for exsheathment of entomopathogenic nematodes. International Journal for Parasitology 21:219–224.

Cappaert, D. L., and D. R. Smitley. 2002. Parasitoids and pathogens of Japanese beetle (Coleoptera: Scarabaeidae) in southern Michigan. Environmental Entomology 31:573–580.

Chen, Z. X., and D. W. Dickson. 1998. Review of *Pasteuria penetrans*: Biology, ecology, and biological control potential. Journal of Nematology 30:313–340.

Davis, R. W., D. Botstein, and J. Roth. 1980. Advanced bacterial genetics, Appendix 1, New York: Cold Spring Harbor Laboratory.

Duncan, L. W., D. C. Dunn, G. Bague, and K. Nguyen. 2003a. Competition between entomopathogenic and free-living bactivorous nematodes in larvae of the weevil *Diaprepes abbreviatus*. Journal of Nematology 35:187–193.

Duncan, L. W., J. H. Graham, D. C. Dunn, J. Zellers, C. W. McCoy, and K. Nguyen. 2003b. Incidence of endemic entomopathogenic nematodes following application of *Steinernema riobrave* for control of *Diaprepes abbreviatus*. Journal of Nematology 35:178–186.

Duncan, L. W., C. W. McCoy, J. H. Graham, and D. I. Shapiro. 2000. Entomopathogenic nematodes as a component of citrus root weevil IPM. Diaprepes Short Course, Citrus Research & Education Center, Lake Alfred, FL, March 22, 2000. Pp. 77–89.

Enright, M. R., M. M. Finnegan, J. O. McInerney, M. O'Laighleis, and C. T. Griffin. 2001. Specific adherence of sporangia of a *Paenibacillus* sp. bacterium to *Heterorhabditis* spp. nematodes. Hitching a ride to lunch? Pp. 301–306 *in* C. T. Griffin, A. M. Burnell, M. J. Downes, and R. Mulder, eds. Development of entomopathogenic nematode/bacteria research. Brussels: European Commission.

Enright, M. R., and C. T. Griffin. 2004. Specificity of association between *Paenibacillus* spp. and the entomopathogenic nematodes, *Heterorhabditis* spp. Microbial Ecology 48:412–421.

Enright, M. R., J. O. McInerney, and C. T. Griffin. 2003. Characterization of endospore-forming bacteria associated with entomopathogenic nematodes, *Heterorhabditis* spp., and description of *Paenibacillus nematophilus* sp. nov. International Journal of Systematic and Evolutionary Microbiology 53:435–441.

Forst, S., and K. Nealson. 1996. Molecular biology of the symbiotic pathogenic bacteria *Xenorhabdus* spp. and *Photorhabdus* spp. Microbiology Review 60:21–34.

Giblin-Davis, R. M., D. S. Williams, W. P. Wergin, D. W. Dickson, T. E. Hewlett, S. Bekal, and J. O. Becker. 2001. Ultrastructure and development of *Pasteuria* sp. (S-1 strain), an obligate endoparasite of *Belonolaimus longicaudatus* (Nemata: Tylenchida). Journal of Nematology 33:227–238.

Hu, K., and J. M. Webster. 2000. Antibiotic production in relation to bacterial growth and nematode development in *Photorhabdus*-*Heterorhabditis* infected *Galleria mellonella* larvae. FEMS Microbiology Letters 189:219–223.

Isaacson, P. J., and J. M. Webster. 2002. Antimicrobial activity of *Xenorhabdus* sp. RIO (Enterobacteriaceae), symbiont of the entomopathogenic nematode, *Steinernema riobrave* (Rhabditida: Steinernematidae). Journal of Invertebrate Pathology 79:146–153.

Klein, M., and H. K. Kava. 1995. *Bacillus* and *Serratia* species for scarab control. Memórias do Instituto Oswaldo Cruz Rio de Janeiro 90:87–95.

Koppenhöfer, A. M., M. Wilson, I. Brown, H. K. Kava, and R. Gaugler. 2000. Biological control agents for white grubs (Coleoptera: Scarabaeidae) in anticipation of the establishment of the Japanese beetle in California. Journal of Economic Entomology 93:71–80.

Krieg, A. 1981. The genus *Bacillus*: Insect pathogens. Pp. 1743–1755 *in* M. P. Starr, H. Stolp, H. G. Truper, A. Balows, and H. G. Schlegel, eds. The prokaryotes. Berlin: Springer.

Kumar, S., K. Tamura, I. B. Jakobsen, and M. Nei. 2001. MEGA2: Molecular evolutionary genetics analysis software. Bioinformatics 17: 1244–1245.

Lysenko, O., and J. Weiser. 1974. Bacteria associated with nematode *Neoaplectana carpocapsae* and the pathogenicity of this complex for *Galleria mellonella* larvae. Journal of Invertebrate Pathology 24: 332–336.

Marti, O. G., Jr., and P. Timper. 1999. Phoretic relationship between a *Bacillus* sp. and the entomopathogenic nematode, *Heterorhabditis* sp. Journal of Nematology 31:553 (Abstr.).

McCoy, C. W., D. I. Shapiro, L. W. Duncan, and K. Nguyen. 2000. Entomopathogenic nematodes and other natural enemies as mortality factors for larvae of *Diaprepes abbreviatus* (Coleoptera: Curculionidae). Biological Control 19:182–190.

McCoy, C. W., R. J. Stuart, L. W. Duncan, and K. Nguyen. 2002. Field efficacy of two commercial preparations of entomopathogenic nematodes against larvae of *Diaprepes abbreviatus* (Coleoptera: Curculionidae) in Spodosol type soil. Florida Entomologist 85:537–544.

Nakamura, L. K. 1996. *Paenibacillus apiarius* sp. nov. International Journal of Systematic Bacteriology 46:688–693.

Nguyen, K. B., and L. W. Duncan. 2002. *Steinernema diaprepesi* n. sp. (Rhabditida: Steinernematidae), a parasite of the citrus root weevil *Diaprepes abbreviatus* (L.) (Coleoptera: Curculionidae). Journal of Nematology 34:159–170.

Patel, M. N., R. N. Perry, and D. J. Wright. 1997. Desiccation survival and water contents of entomopathogenic nematodes, *Steinernema* spp. (Rhabditida: Steinernematidae). International Journal for Parasitology 27:61–70.

Pettersson, B., K. E. Rippere, A. A. Yousten, and F. G. Priest. 1999. Transfer of *Bacillus lentimorbus* and *Bacillus popilliae* to the genus *Paenibacillus* with amended descriptions of *Paenibacillus lentimorbus* comb. nov. and *Paenibacillus popilliae* comb. nov. International Journal of Systematic Bacteriology 49:531–540.

Preston, J. K., D. W. Dickson, J. E. Maruniak, G. Nong, J. A. Brito, L. M. Schmidt, and R. M. Giblin-Davis. 2003. *Pasteuria* spp.: Systematics and phylogeny of these bacterial parasites of phytopathogenic nematodes. Journal of Nematology 35:198–207.

Reynolds, E. S. 1963. The use of lead citrate at high pH as an electron-opaque stain in EM. Journal of Cell Biology 17:208.

Richardson, W. H., T. M. Schmidt, and K. H. Nealson. 1988. Identification of an anthraquinone pigment and hydroxystilbene antibiotic from *Xenorhabdus luminescens*. Applied and Environmental Microbiology 54:1602–1609.

Saitou, N., and M. Nei. 1987. The neighbor-joining method: A new method for reconstructing phylogenetic trees. Molecular Biology and Evolution 4:406–425.

Schneider, H. 1981. Plant anatomy and general botany. Pp. 315–373 *in* G. Clark, ed. Staining procedures for biological stain commission, 4th ed. Baltimore: Williams and Wilkins.

Stempack, J. G., and R. T. Ward. 1964. An improved staining method for electron microscopy. Journal of Cell Biology 22:697–701.

Thaler, J. O., M. H. Boyer Giglio, and N. E. Boemare. 1997. New antimicrobial barriers produced by *Xenorhabdus* spp. and *Photorhabdus* spp. to secure the monoxenic development of entomopathogenic nematodes. Symbiosis 22:205–215.

Thurston, S. G., H. K. Kaya, T. M. Burlando, and R. E. Harrison. 1993. Milky disease bacterium as a stressor to increase susceptibility of Scarabaeid larvae to an entomopathogenic nematode. Journal of Invertebrate Pathology 61:167–172.

Thurston, S. G., H. K. Kava, and R. Gaugler. 1994. Characterizing the enhanced susceptibility of milky disease-infected Scarabaeid grubs to entomopathogenic nematodes. Biological Control 4:67–73.

Timper, P., and H. K. Kaya. 1989. Role of the second-stage cuticle of entomogenous nematodes in preventing infection by nematophagous fungi. Journal of Invertebrate Pathology 54:314–321.

Walsh, K. T., and J. M. Webster. 2003. Interaction of microbial populations in *Steinernema* (Steinernematidae, Nematoda) infected *Galleria mellonella* larvae. Journal of Invertebrate Pathology 83:118–126.