Microbial Ecology

Specificity of Association between *Paenibacillus* spp. and the Entomopathogenic Nematodes, *Heterorhabditis* spp.

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Abstract

Endospore-forming bacteria, *Paenibacillus* spp., have recently been isolated in association with insect pathogenic nematodes *Heterorhabditis* spp. Sporangia adhere to nematode infective juveniles (J3) and are carried with them into insects. *Paenibacillus* proliferates in the killed insect along with *Heterorhabditis* and its obligate bacterial symbiont, *Photorhabdus*, despite the antibiotic production of the latter. Nematode infective juveniles leave the insect cadaver with *Paenibacillus* sporangia attached. The specificity of the relationship between *Paenibacillus* and *Heterorhabditis* was investigated. Sporangia of nematode-associated *Paenibacillus* adhered to infective juveniles (but not other stages) of all *Heterorhabditis* species tested, and to infective juveniles of vertebrate parasitic Strongylida species, but not to a variety of other soil nematodes tested. *Paenibacillus* species that were not isolated from nematodes, but were phylogenetically close to the nematode-associated strains, did not adhere to *Heterorhabditis*, and they were also sensitive to *Photorhabdus* antibiotics *in vitro*, whereas the nematode-associated strains were not. Unusual longevity of the sporangium and resistance to *Photorhabdus* antibiotics may represent specific adaptations of the nematode-associated *Paenibacillus* strains to allow them to coexist with and be transported by *Heterorhabditis*. Adaptation to specific *Heterorhabditis-Photorhabdus* strains is evident among the three nematode-associated *Paenibacillus* strains (each from a different nematode strain). *Paenibacillus* NEM1a and NEM3 each developed best in cadavers with the nematode from which it was isolated and not at all with the nematode associate of the other strain. Differences between nematode-associated *Paenibacillus* strains in cross-compatibility with the various *Heterorhabditis* strains in cadavers could not be explained by differential sensitivity to antibiotics produced by the nematodes' *Photorhabdus* symbionts *in vitro*.

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Introduction

Entomopathogenic nematodes of the genera *Heterorhabditis* and *Steinernema* are lethal pathogens of insects and are used as biological control agents of several insect pests [22]. Infective juveniles (IJs) of these nematodes are specialized third-stage juveniles that carry in their intestines symbiotic bacteria belonging to the genera *Photorhabdus* and *Xenorhabdus*, respectively [12]. Each nematode species is normally associated with a single species of bacterial symbiont, though a symbiont species may be associated with more than one nematode species [7]. Following entry into a suitable insect, the IJ releases the symbiont into the hemocoel, where it causes death of the host insect within 48 h [29]. The bacteria digest the insect tissues [13], and the nematodes feed on the bacteria and degraded insect tissue. *Photorhabdus* and *Xenorhabdus* produce a variety of antibiotics [13] which have a broad spectrum of activity [3] and prevent putrefaction of the insect cadaver [17]. Nematode reproduction is optimal when their symbiont dominates the microbiota of the insect cadaver [12]. Two to three generations of nematodes occur within the host, depending on available resources. As the nutritive status of the cadaver declines, the nematodes arrest at the IJ stage. Hundreds of thousands of such IJs may be produced in a single large insect. The nonfeeding IJs migrate from the cadaver into soil and search for new hosts [29]. *Heterorhabditis* spp. belong to the order Rhabditida, and their IJ stage is functionally and developmentally analogous to the dauer juvenile (dispersive and survival stage) of some free-living members of the order, including *Caenorhabditis elegans* [5].

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Monoxenicity is a notable feature of the *Heterorhabditis-Photorhabdus* and *Steinernema–Xenorhabdus* complexes [7, 8]. The symbiont is selectively retained within the intestine of the nematode infective juvenile by an as yet unknown mechanism and, once released into the insect hemocoel, antimicrobial compounds produced by the symbiont minimize the development of other bacterial species in the insect cadaver. However, there have been a number of reports of bacteria other than the symbiont being isolated from entomopathogenic nematodes, particularly from *Steinernema* species, and these are reviewed in [8]. Some of these contaminants are probably an artifact of prolonged laboratory culture in insects, where antibiotic-resistant contaminants are retained. The natural occurrence of many contaminants in *Steinernema scapterisci* is seen as an exception, which diminishes the nematode's potential for killing insects [8]. Entomopathogenic nematode IJs retain the molted cuticle of the previous juvenile stage as a protective sheath [20]; in *S. scapterisci* the contaminants are carried in the intercuticular space [8]. Fewer nonsymbiont bacteria have been reported from *Heterorhabditis*. *Providencia rettgeri* was isolated from several strains following prolonged laboratory culture [18], whereas *Ochrobactrum* spp. were found naturally associated with one third of newly isolated *H. indica* from the Caribbean [4]. In both of these studies the nematodes were surface sterilized, but not desheathed, prior to isolation of the bacteria [4, 18]; hence, the bacteria were carried either in the intestine or between the sheath and the living cuticle.

We have found a novel endospore-forming bacterial species, *Paenibacillus nematophilus*, naturally associated with a strain of *Heterorhabditis megidis* [10]. The association between the bacterium and this nematode strain has been maintained through routine laboratory culture since their isolation from soil from Estonia in 1994 [14]. Spindle-shaped sporangia adhere to the nematode IJs and can be carried by them into an insect. *P. nematophilus* reproduces within the dead insect, despite *Photorhabdus*-produced antibiotics. Shortly before IJs emerge from the host, sporangia accumulate in the insect cadaver. When the IJs emerge, they carry sporangia adhering to the cuticle. Similar bacteria were naturally associated with an unidentified *Heterorhabditis* sp. from Georgia, USA [25], and with *Heterorhabditis indica* from southern India. Endospore-forming bacterial isolates from all three nematode sources were identified as *Paenibacillus* and found to share a higher degree of 16S rRNA gene sequence identity with each other than with any other named species within the genus [10]. The nematode-associated strains also share a high degree of phenotypic and chemotaxonomic similarity and are easily distinguished from related species, though DNA–DNA similarity studies revealed that they represent at least two, and possibly three, separate species [10]. *Paenibacillus macquariensis, Paenibacillus azoreducens*, and *Paenibacillus borealis* are among the species with highest sequence similarity to the nematode-associated strains.

The *Paenibacillus–Heterorhabditis* associations are notable for the tendency of the nematode IJs to clump in water (nematodes stick together in large cross-linked groups). Such clumping in water also occurs in other associations where bacteria adhere to a nematode (such as compatible *Rathayibacter–Anguina* combinations), and the degree of clumping can be used as a semiquantitative measure of the specificity of the adhesion [32]. *Paenibacillus*-induced clumping can be distinguished from the rosette aggregations, in which stored *Heterorhabditis* IJs adhere by their tails [31], by the adhesion of IJs at multiple and diverse points along their bodies, and by the onset of clumping immediately after emergence from a host.

As part of a broader study into the association, this work examines the specificity of the association between *Heterorhabditis* and *Paenibacillus* at two levels. At the higher taxonomic level, we ask on the one hand whether nematode-associated *Paenibacillus* strains adhere to nematodes other than *Heterorhabditis*, and on the other hand whether other species of *Paenibacillus* adhere to *Heterorhabditis* nematodes. We also compare the sensitivity of nematode and non-nematode-associated *Paenibacillus* species to *Photorhabdus* antibiotics in an *in vitro* assay. At a lower taxonomic level we address the specificity of association of the various nematode-associated *Paenibacillus* strains with the *Heterorhabditis* strain from which they were isolated. Cross-compatibility is assessed both in terms of adhesion to nematode IJs and multiplication in the insect cadaver. In an attempt to explain differential compatibility in cadavers, the sensitivity of nematode-associated *Paenibacillus* strains to various *Photorhabdus* spp. is investigated *in vitro*.

Methods

Nematodes and Bacteria. The nematode species and stages used are listed in Tables 1 and 2. *Heterorhabditis* and *Steinernema* spp. were cultured *in vivo* in late instar larvae of the wax moth, *Galleria mellonella*, at either 20°C or 26°C depending on the strain. Infective juveniles were harvested in water from the insect cadavers on White traps [21]. The IJs of *H. megidis* EU17, *H. indica* LN2, and *Heterorhabditis* Line 1 were treated with 0.5% sodium hypochlorite for 15 min (followed by washing with sterile distilled water) to remove *Paenibacillus* attached to the surface of the IJs. The treated IJs were used to infect *G. mellonella* larvae, thus establishing uncontaminated nematode cultures.

Table 1. Adhesion of sporangia of *Paenibacillus nematophilus* NEM1a to a variety of soil-dwelling nematode species and stages, including infective juveniles (IJs)

Nematode order and species	*Nematode stage tested*	*Life history*	*Adhesion*
Rhabditida			
Heterorhabditis megidis	Ensheathed IJ	Insect pathogen	+
	Exsheathed IJ		+
	Adult stage		−
H. downesi	Ensheathed IJ	Insect pathogen	+
H. bacteriophora	Ensheathed IJ	Insect pathogen	+
H. indica	Ensheathed IJ	Insect pathogen	+
H. argentiniensis[1]	Ensheathed IJ	Insect pathogen	+
Heterorhabditis sp. Line 1[2]	Ensheathed IJ	Insect pathogen	+
Steinernema feltiae	Ensheathed IJ	Insect pathogen	−
S. glaseri	Ensheathed IJ	Insect pathogen	−
S. carpocapsae	Ensheathed IJ	Insect pathogen	−
Phasmarhabditis hermaphrodita[3]	Ensheathed IJ	Slug parasite	−
Caenorhabditis elegans[4]	Mixed population	Free-living	−
	Dauer juveniles		−
C. briggsae[6]	Mixed population	Free-living	−
Rhabditis myriophila[6]	Mixed population	Free-living	−
R. blumi[6]	Mixed population	Free-living	−
Teratorhabditis palmarum[6]	Mixed population	Free-living	−
Rhabditella axei[6]	Mixed population	Free-living	−
Panagrellus redivivus[6]	Mixed population	Free-living	−
Pristionchus pacificus[6]	Mixed population	Free-living	−
Pellioditis typica[6]	Mixed population	Free-living	−
Mesorhabditis longespiculosa[6]	Mixed population	Free-living	−
Rhabditoides regina[6]	Mixed population	Free-living	−
Strongylida			
Haemonchus contortus[5]	Ensheathed IJ	Vertebrate parasite	+
Ostertagia circumcincta[5]	Ensheathed IJ	Vertebrate parasite	+
Trichostrongylus colubriformis[5]	Ensheathed IJ	Vertebrate parasite	+
Cooperia oncophora[5]	Ensheathed IJ	Vertebrate parasite	+
Tylenchida			
Meloidogyne sp.[7]	Second stage juveniles	Plant parasite	−
Globodera sp.[7]	Second stage juveniles	Plant parasite	−
Aphelenchida			
Aphelenchus avenae[4]	Mixed population	Free-living	−

Nematodes obtained from: [1] Byron Adams, University of Florida, USA; [2] Dick Marti, USDA, Tifton, Georgia, USA; [3] MicroBio Ltd, UK; [4] John Browne, NUI Maynooth, Ireland; [5] Gerald Cole, University of Bristol, UK; [6] *Caenorhabditis* Genetics Center, University of Minnesota, St. Paul, USA; [7] Tested by Dr. Keith Davies, IACR, Rothamsted, England.

Paenibacillus nematophilus NEM1a, *Paenibacillus* sp. NEM2, and *Paenibacillus* sp. NEM3, isolated from the nematodes *H. megidis* EU17, *Heterorhabditis* sp. Line 1, and *H. indica* LN2, respectively [10], were routinely maintained on plates of nutrient agar (Oxoid) at 30°C.

Adhesion of Sporangia to Nematodes

Adhesion of Paenibacillus nematophilus NEM1a Sporangia to Various Species and Stages of Nematodes. Adhesion of *P. nematophilus* NEM1a sporangia to nematodes was detected both by their ability to cause clumping of nematodes in water and by microscopic examination. A

Table 2. Strains of *Heterorhabditis* used in experiments, with their natural *Photorhabdus* symbiont and associated *Paenibacillus* strain

Nematode species and strain	*Symbiont*	*Paenibacillus strain*	*Geographic source*
Heterorhabditis megidis EU17	*Photorhabdus temperata temperata* EU17	NEM1a	Estonia
H. downesi K122	*P. temperata*[a] K122	—	Ireland
H. bacteriophora EU222	*P. luminescens*[b] EU222	—	Hungary
H. bacteriophora MOL	*P. luminescens*[b] MOL	—	Moldavia
H. indica LN2	*P. luminescens akhurstii* LN2	NEM3	India
Heterorhabditis sp.[c] Line 1	*Photorhabdus* sp.[c] Line 1	NEM2	Georgia, USA

[a]Subspecies not named, but distinct from *P. temperata temperata* [11].
[b]Subspecies not identified.
[c]Unidentified species.

sporangial suspension with turbidity equivalent to McFarland standard 3 (bioMerieux, France) was added to an aqueous suspension of nematodes ($1–3 \times 10^3$ nematodes/mL) at a ratio of 1:200 v/v, in wells of 25-well plates. Nematodes used in these assays were not surface sterilized, to avoid potential alteration of the surface cuticle properties of any of the nematode species or stages tested. The experiment was conducted on different dates with groups of nematode species depending on availability. *Heterorhabditis megidis* IJs were included as a positive control on each date. Sterile distilled water was added to each nematode treatment as a negative control. There were three replicates of each treatment. Treatments were left to stand for ≥2 h and were then examined for clumping of nematodes. A sample of nematodes (≥20 per replicate) was then examined microscopically (400×), and the presence of adhering sporangia was noted. Nematodes were washed by sedimentation in sterile distilled water to remove free sporangia and a second sample was examined for sporangia. Adhesion to plant parasitic nematode species (*Meloidogyne* sp. and *Globodera* sp.) was tested by K. Davies (IARC, Rothamsted, England), using a similar method.

Numbers of sporangia adhering to nematodes were normally not counted, except in the comparison of ensheathed and exsheathed IJs. *Heterorhabditis* IJs spontaneously shed their sheath during storage. A mixture of exsheathed and ensheathed infective juveniles, obtained by incubating *H. megidis* UK211 IJs in water at 20°C for 8 weeks, was included in the assay, and the number of sporangia adhering to 10 individuals of each type per replicate was counted.

Some nematode species were tested as mixed cultures, which included various developmental stages. Clumping was scored for the mixture but individual stages were scored for the presence of sporangia. Species tested as mixed populations included all the free-living bactivorous rhabditid species received from the *Caenorhabditis* Genetics Center (Minnesota), which were tested without further subculture, as starved but viable cultures. This ensured the presence of a proportion of dauer juveniles in those species that form them.

Adhesion of Non-Nematode-Associated Paenibacillus and Bacillus Species to Heterorhabditis spp. Infective Juveniles. Sporulated cultures of nine *Paenibacillus* and *Bacillus* species were tested for the ability to clump *Heterorhabditis* spp. IJs. *Paenibacillus macerans* DSM 24^T, *P. macquariensis* DSM 2^T, *P. polymyxa* DSM 36^T, *P. alvei* DSM 29^T, *P. azotofixans* DSM 5976^T, *P. pabuli* DSM 3036^T, and *Bacillus thuringiensis* subsp. *galleriae* DSM 6110 were obtained from the German Collection of Microorganisms and Cell Cultures (DSMZ), Braunschweig, Germany. *Paenibacillus azoreducens* DSM 13822^T was obtained from G. McMullan (University of Ulster), and six *P. borealis* isolates, including the type strain DSM 13188^T, were obtained from S. Elo (University of Helsinki). *P. nematophilus* NEM1a DSM 13559^T served as a positive control. All strains were maintained on nutrient agar plates at 30°C except *P. macquariensis*, which was maintained at 20°C. Spore suspensions were made up to approximately McFarland standard 2, and 100 μL was added to 2.9 mL of *Heterorhabditis* IJ suspension (3000 IJs) in wells of 25-well plates. There were at least two replicates per treatment. These were left at room temperature and scored for clumping at intervals over 24 h. All bacterial strains were tested with *H. megidis* EU17, and some were also tested with *H. indica* LN2.

Adhesion of Nematode-Associated Paenibacillus Strains to Various Heterorhabditis Species and Strains. Adhesion of the three nematode-associated *Paenibacillus* strains to each of six *Heterorhabditis* species/strains (*H. megidis* EU17, *H. downesi* K122, *H. bacteriophora* EU222, *H. bacteriophora* MOL, *Heterorhabditis* sp. Line 1, and *H. indica* LN2) was compared. For this purpose, the clumping assay described above was made semiquantitative. Three concentrations of sporangia were prepared to give a final concentration of 10, 100, and 1000 sporangia/IJ when added to the nematode suspension (4500 IJs in 3 mL water). All combinations were set up in duplicate. They were left at room temperature for 3 h, shaken gently for 30 s, and left to stand for a further 10 min, after which clumping was semiquantitatively assessed on a scale of 0 to 4: 0, no clumping; 1, >20 small clumps with numerous free nematodes; 2, 10–20 small clumps with some free nematodes; 3, 3–10 medium-sized clumps with some free nematodes; 4, 1–3 large clumps with few free nematodes.

Cross-Compatibility of Nematode-Associated Paenibacillus Strains with Various Heterorhabditis Species and Strains in Insect Cadavers. The compatability of various *Heterorhabditis* species and strains with each of the three nematode-associated *Paenibacillus* strains, in allowing their multiplication and transport, was investigated by infecting insects with various natural or novel *Paenibacillus–Heterorhabditis* combinations and assessing the number of nematode-borne *Paenibacillus* sporangia carried from the insect cadavers in which they had developed.

Nematode suspension (1500 IJs/mL) was mixed with *Paenibacillus* sporangial suspension of a known concentration (assessed by hemocytometer counts) to give a final ratio of 100 sporangia/IJ. Five *G. mellonella* larvae were exposed to 400 μL of this suspension on filter paper in 50-mm Petri dishes. *Galleria mellonella* were also exposed to 400 μL of a 1500 IJs/mL suspension of nematodes without added *Paenibacillus*. Infections involving nematode

strains from temperate regions (*H. megidis* EU17, *H. downesi* K122, *H. bacteriophora* MOL, and *H. bacteriophora* EU222) were incubated at 20°C, while those from semitropical/tropical climates (*H. indica* LN2 and *Heterorhabditis* sp. Line1) were incubated at both 20°C and 26°C. There were three replicates for each treatment. Cadavers were placed on White traps and IJs emerging into water were assessed for clumping (indicating that sporangia had been produced in the cadaver and were being carried by the nematodes). There were three experiments. Clumping was assessed in each experiment using a semi-quantitative scale. Numbers of sporangia were enumerated in one of the experiments. When nematodes ceased to emerge, all IJs harvested from a given White trap were pooled. The number of sporangia adhering to ten individual IJs was determined under 400× magnification. The total yield of IJs was assessed by counting replicate aliquots of nematode suspension. To aid counting, samples were treated with 0.5% sodium hypochlorite for 15 min to disperse clumps, then washed three times by sedimentation in water. The total number of sporangia carried by nematodes from cadavers was calculated by multiplying the total number of IJs that emerged by the average number of sporangia/IJ. For combinations in which no sporangia were carried by IJs, samples of cadaver material were examined microscopically 1 week after IJ emergence ceased (>4 weeks postinfection) for evidence of sporangia.

For combinations involving *P. nematophilus* NEM1a, the number of sporangia remaining in the cadavers was assessed 1 week after IJs stopped emerging. The five cadavers from each White trap were placed in 45 mL of sterile distilled water and disrupted by repeated vortexing. The number of sporangia was determined by hemocytometer counts of three replicate samples.

Sensitivity of Paenibacillus spp. to Photorhabdus spp. in Vitro. *Photorhabdus* was isolated from surface-sterilized [0.4% hyamine 2389 (BDH)] *Heterorhabditis* IJs macerated in sterile distilled water. *Photorhabdus* spp. exhibit phenotypic variation [12]; primary form (the normal wild-type form) was selected based on dye adsorbtion on NBTA agar [2]. Ten μL of overnight 30°C *Photorhabdus* nutrient broth culture was inoculated centrally on a nutrient agar plate (glass Petri dish; 90 mm). Plates were incubated at 20°C for 3 days. *Photorhabdus* was killed by inverting the plates and flooding the lids with chloroform, which was then allowed to evaporate. *Paenibacillus* sporangial suspension in sterile distilled water (equivalent to McFarland standard 3) was streaked in a straight line from the edge of the Petri dish to the edge of the killed *Photorhabdus* colony. After 1 week at 20°C, the distance between visible *Paenibacillus* growth and the *Photorhabdus* colony edge was measured.

In the first experiment, the three nematode-associated *Paenibacillus* strains (NEM1a, NEM2, and NEM3) and three non-nematode-associated species, *Paenibacillus polymyxa, P. macerans*, and *P. azoreducens*, were tested for sensitivity to two *Photorhabdus* strains, *Photorhabdus temperata temperata* EU17 and *Photorhabdus* sp. Line 1 (see Table 2). Each plate received four streaks, one of which was always *Paenibacillus macerans* to check for variation between replicate plates of a given *Photorhabdus* strain. Little variation was found in this control. The other three streaks were randomly chosen from among the six *Paenibacillus* strains. Each *Paenibacillus–Photorhabdus* combination was tested at least four times.

Similar experiments were carried out to test the sensitivity of the three nematode-associated *Paenibacillus* strains to the *Photorhabdus* spp. strains listed in Table 2, at 20 and 30°C. Each plate was streaked with NEM1a, NEM2, and NEM3. There were five independent experiments (four at 20°C and one at 30°C) with a selection of *Photorhabdus* strains in each. There were at least five replicates for each *Photorhabdus–Paenibacillus* treatment at 20°C, and two to four replicates at 30°C.

Statistical Analysis. Data were tested for normality using the Anderson–Darling method, and homogeneity of variance was tested by Bartlett's method. The effect of sheath presence on numbers of sporangia adhering to nematode IJs was tested using a *t*-test, paired by sample, as each sample contained both ensheathed and exsheathed IJs. Comparison of three or more treatments was by one-way ANOVA; where a significant treatment effect was detected, means were separated using Tukey's multiple range test ($\alpha = 0.05$). Data for numbers of NEM1a sporangia per host were first subjected to square root transformation to homogenize variances. Proportion data were subjected to angle (arcsine square root) transformation. The Kruskal–Wallis test (conducted separately for each *Paenibacillus* strain) was used to investigate variation in numbers of sporangia carried from different nematode hosts. Correlation between parameters was tested using Pearson's correlation. All tests were carried out using Minitab 13.1 (Minitab Inc., Pennsylvania) and in each case significance was attained at $P < 0.05$.

Results

Adhesion of Sporangia to Nematodes

Adhesion of Paenibacillus nematophilus NEM1a Sporangia to Various Species and Stages of Nematodes. Results of tests for the adhesion of *P. nematophilus* NEM1a sporangia to various nematode species are shown in Table 1. Addition of NEM1a to ensheathed infective juveniles of all *Heterorhabditis* spp. tested resulted in clumping of these

nematodes, and the sporangia adhered along their entire surface. A few sporangia adhered to *H. megidis* IJs which had lost their sheath (1.0 ± 0.27 sporangia/IJ; mean ± SE), but to a lesser extent than to ensheathed individuals (17.2 ± 1.31 sporangia/IJ). The difference was significant (paired *t*-test, $P = 0.006$). There was no adhesion to *H. megidis* adults (hermaphrodites, males and females). Sporangia adhered in substantial numbers to ensheathed infective juveniles of all the animal parasitic Strongylida species tested but did not adhere to or clump ensheathed IJs of *Steinernema*, the other genus of entomopathogenic nematode. Sporangia failed to clump or adhere to any of the free-living bactivorous rhabditid species or any of the various other nematodes tested. Occasionally, single individuals of *Meloidogyne* sp. and *Globodera* sp. were found with a few sporangia adhering. However, the vast majority of individuals were free of sporangia and clumping did not occur.

Adhesion of Non-Nematode-Associated Paenibacillus and Bacillus Species to Heterorhabditis spp. Infective Juveniles. Neither *Bacillus thuringiensis* nor any of the eight non-nematode-associated species of *Paenibacillus* caused clumping at *Heterorhabditis* spp. IJs.

Adhesion of Nematode-Associated Paenibacillus Strains to Various Heterorhabditis Species and Strains. All three nematode-associated *Paenibacillus* strains adhered to and clumped all six *Heterorhabditis* strains at each of the three sporangial concentrations tested. In each case, there was an obvious decrease in the level of clumping as the sporangial concentration was decreased, from a score of 3 or 4 at the highest concentration to 1 (rarely 2) at the lowest. NEM3 tended to cause slightly more clumping than the other two strains. This was most evident at the highest sporangia/IJ concentration (where clumping was scored 4 for NEM3 and 3 for NEM1a and NEM2) and was observed for all of the nematode strains tested. There was no evidence of differences between *Heterorhabditis* strains in the degree of clumping caused by any of the nematode-associated *Paenibacillus* strains.

Cross-Compatibility of Nematode-Associated Paenibacillus Strains with Different Heterorhabditis Species and Strains in Cadavers. The number of *P. nematophilus* NEM1a sporangia produced varied depending on the nematode with which it was inoculated, ranging from 0 to 67×10^6 sporangia per *G. mellonella* cadaver (Table 3). The number of sporangia carried from the cadavers by nematode IJs was similarly nematode-dependent (Fig. 1). The number of NEM1a sporangia carried by nematode IJs from the *G. mellonella* cadavers is correlated with the number remaining within the cadaver (Pearson correlation $r = 0.98$, $P = 0.003$), showing that measuring the numbers of sporangia carried by nematodes from different host cadavers is a good indication of the relative suitability of the hosts for production of sporangia. Between 17% and 48% of the NEM1a sporangia produced were carried out of the cadaver by emerging nematodes, with a higher proportion carried where fewer sporangia were produced (Table 3).

Table 3. The total number of *Paenibacillus nematophilus* NEM1a sporangia produced per *Galleria mellonella* infected with different species of *Heterorhabditis*, and the number and proportion of sporangia remaining within the insect cadaver after nematode emergence ceased

	Number of sporangia $\times 10^6$		*Proportion of sporangia remaining in cadaver*
Nematode	*Total*[1]	*In cadaver*	
H. megidis EU17	66.7 a[2]	53.3 a[2]	0.79 ab[3]
H. downesi K122	39.4 ab	32.7 a	0.83 a
H. bacteriophora EU222	13.6 bc	8.4 b	0.62 bc
H. bacteriophora MOL	3.6 c	1.9 b	0.52 c
H. indica LN2	0	0	–

[1]Sporangia remaining within the cadaver + sporangia carried by nematode IJs.
[2,3]Within a column, treatments with the same letter are not significantly different (Tukey's test; $\alpha = 0.05$, on square-root[2] or angle[3] transformed data).
Data are untransformed means.

There were differences between the *Paenibacillus* strains in their compatibility with the different nematode strains, as judged both by the degree of clumping of IJs (data not shown) and numbers of sporangia carried by the IJs (Fig. 1). For each of the three nematode-associated *Paenibacillus* strains the number of sporangia carried from insect cadavers was significantly (Kruskall–Wallis, $P < 0.05$) affected by the nematode with which the insect was inoculated. NEM2 and NEM3 shared a similar spectrum of compatibility: for both strains, the highest number of sporangia was carried from the cadaver by *H. indica* LN2 and none were carried by *H. megidis* EU17 or *H. downesi* K122. *P. nematophilus* NEM1a showed the opposite trend, with large numbers of sporangia being carried by *H. megidis* EU17 and *H. downesi* K122 and none by *H. indica* LN2 (Fig. 1). Where no sporangia were carried by IJs, none were found by microscopic examination of the cadavers. Incubations were performed at a temperature optimal for the nematode associate (20 or 26°C); when the warm-adapted nematodes, *H. indica* and *Heterorhabditis* sp. Line 1, were incubated at 20°C, results were similar to those at 26°C: sporangia were produced by NEM2 and NEM3 but not by NEM1a.

NEM1a and NEM3 were each carried in highest number by the nematode from which they were originally isolated, whereas NEM2 was carried in higher number by *H. indica* LN2 (the natural associate of NEM3) than by *Heterorhabditis* sp. Line 1, its own natural associate (Fig. 1).

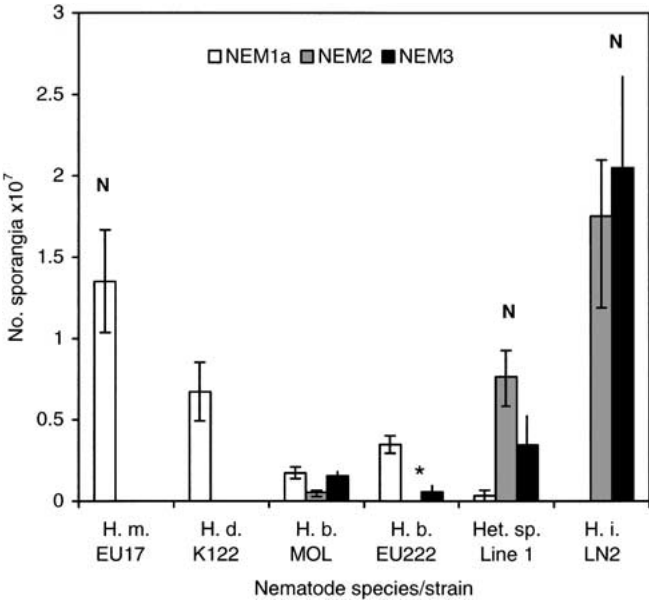

Figure 1. The number of sporangia carried by *Heterorhabditis* spp. infective juveniles per *Galleria mellonella* cadaver for each of three *Paenibacillus* strains: *P. nematophilus* NEM1a, *Paenibacillus* sp. NEM2, and *Paenibacillus* sp. NEM3. The *Heterorhabditis* species tested were *H. megidis* (H. m.), *H. downesi* (H. d.), *H. bacteriophora* (H. b.), *Heterorhabditis* sp. (Het. sp.), and *H. indica* (H. i.). *Not tested. N denotes the natural *Paenibacillus–Heterorhabditis* association. Data are means (±SE) of three replicates of five cadavers each.

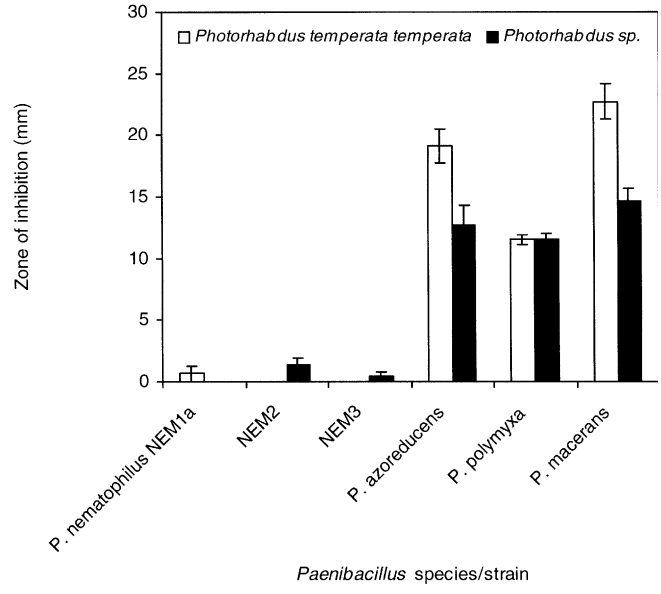

Figure 2. Inhibition of growth in an agar plate assay of nematode-associated (NEM1a, NEM2, NEM3) and non-nematode-associated *Paenibacillus* species by *Photorhabdus temperata temperata* EU17 and *Photorhabdus* sp. Line 1. Data are means (±SE) of four to 11 replicates per combination.

The presence of *Paenibacillus* did not affect the number of nematode IJs produced relative to the control in five of the six *Heterorhabditis* strains (data not shown; ANOVA, $P > 0.05$ for each nematode strain). Yield of *H. bacteriophora* MOL varied between treatments (ANOVA, $P < 0.05$); the number of IJs produced in the presence of *Paenibacillus* NEM3 (2.6×10^5 IJs/cadaver) differed (Tukey's test, $\alpha = 0.05$) from the control (3.5×10^5 IJs/cadaver), whereas numbers of NEM1a and NEM2 did not.

Sensitivity of Nematode-Associated Paenibacillus Strains and Related Species to Photorhabdus spp. Antibiotics. Each of the three nematode-associated *Paenibacillus* strains was inhibited to a much lesser extent than each of the other three species of *Paenibacillus* tested (Fig. 2). All three nematode-associated strains had average inhibition zones of 0–2 mm, while the other species had inhibition zones ranging from 12 to 23 mm.

In experiments involving additional *Photorhabdus* strains, there was little or no inhibition of nematode-associated *Paenibacillus* (inhibition zones normally <4 mm). The largest inhibition zone (6 mm) was recorded for *P. nematophilus* NEM1a with *Photorhabdus luminescens akhurstii* LN2 (with which it was not compatible in cadavers). However, NEM1a tended to be the most sensitive of the three nematode-associated strains, no matter which *Photorhabdus* was used—even that with which it is naturally associated.

Discussion

Of all the nematode species tested, *Paenibacillus nematophilus* NEM1a adhered only to IJs of *Heterorhabditis* and Strongylida species. Adhesion to the latter probably reflects the close phylogenetic relatedness of *Heterorhabditis* with members of the Strongylida [6]. Like *Heterorhabditis* spp., the infective juvenile stage of many Strongylida species retains the molted second-stage cuticle as a sheath. However, possession of a sheath is not the only factor determining whether sporangia will adhere. Infective juveniles of another rhabditid nematode, the slug parasitic *Phasmarhabditis hermaphrodita*, also possess a sheath [34], as do IJs of *Steinernema* spp. and a number of the free-living rhabditid species tested. However, there was no adhesion of NEM1a sporangia to ensheathed juveniles of these nematodes. The lack of adhesion of NEM1a sporangia to species of the other genus of entomopathogenic nematodes, *Steinernema*, is not inconsistent. Despite sharing a similar life history, including an association with mutualistic bacteria belonging to the Enterobacteriaceae, *Heterorhabditis* and *Steinernema* are not very closely related [6, 24] and are assumed to have arrived at these similarities by convergent evolution [30].

The fact that all three nematode-associated *Paenibacillus* strains adhered to all *Heterorhabditis* species and strains tested suggests a broad conservation of the relevant surface moieties involved in adhesion across the nematode genus. The genus *Heterorhabditis* is morphologically conservative [15] and contains relatively little phylogenetic diversity [24]. The possible existence of subtle quantitative differences between *Heterorhabditis* and/or *Paenibacillus* strains with respect to these surface moieties cannot be discounted, because of the relative insensitivity of the semiquantitative clumping assay employed. However, the broad conservation of binding moieties is further supported by the finding (unpublished data) that sporangia-induced nematode clumping in all three natural *Heterorhabditis–Paenibacillus* associations was inhibited by the same carbohydrate, *N*-acetylneuraminic acid (sialic acid). This finding may point to the involvement of a sialic acid–binding lectin in sporangial adhesion. Such broad conservation of binding moieties across the *Heterorhabditis* genus would differ markedly from the strain-specific adhesion of the parasite *Pasteuria penetrans* to its nematode hosts [33], and of *Rathayibacter* spp. to their nematode vectors, *Anguina* spp. [26]. Nematode-associated *Paenibacillus* strains cause no obvious pathology to their *Heterorhabditis* associates and have no effects on their development, though adhesion of *P. nematophilus* sporangia impedes the dispersal of IJs [9]. However, either the effects are not strong enough under natural conditions or these associations are not common enough to exert evolutionary pressure on *Heterorhabditis* to vary their surface receptors to evade *Paenibacillus* attachment.

From the results of tests involving a range of *Paenibacillus* species, it appears that the ability to adhere to and clump *Heterorhabditis* IJs is a feature peculiar to the nematode-associated strains. A morphological characteristic shared by these nematode-associated strains is that the sporangia remain intact even following long periods of storage [10], whereas those of other *Paenibacillus* spp. tested tend to disintegrate and release the bacterial endospore. Such longevity of the sporangium is not a common feature within the genus *Paenibacillus*, or in Gram-positive aerobic endospore formers in general. The adhesion of the sporangium to certain nematode species may be an important trait and could represent a specific adaptation allowing these bacteria access to a niche dependent on transport by nematodes.

For bacteria to survive and reproduce in a *Heterorhabditis*-infected cadaver they need to tolerate the conditions produced by the *Photorhabdus* sp. symbiont. Strains of *Providencia rettgeri* and some (but not all) of the *Ochrobactrum* spp. strains which were found associated with *Heterorhabditis* spp. were resistant to *Photorhabdus* antibiotics [4, 18]. When tested *in vitro*, the three nematode-associated *Paenibacillus* strains were shown to be resistant to *Photorhabdus*, unlike other species of *Paenibacillus* including the closely related *P. azoreducens*. This suggests that resistance to antibiotics by these nematode-associated strains has been selected for during association with *Photorhabdus*. We cannot exclude the possibility that some further selection occurred during *in vivo* laboratory culture of the associations. However, *H. megidis* EU17 IJs clumped strongly (indicating the presence of abundant sporangia) from the time of their first isolation, suggesting that *P. nematophilus* NEM1a was already resistant before laboratory culture.

The differential ability of the various strains of *Heterorhabditis* (with their associated *Photorhabdus*) to support growth of the three nematode-associated *Paenibacillus* strains may be partially understood in reference to *Paenibacillus* and *Heterorhabditis*/*Photorhabdus* taxonomic relationships. *Paenibacillus* NEM2 and NEM3 were compatible with a similar spectrum of *Heterorhabditis* strains, different from that of *P. nematophilus* NEM1a, and it has been shown that these two strains share higher DNA-DNA similarity (60%) than either shares with NEM1a (both ~40%) [10]. The close relatedness between *H. megidis* and *H. downesi* [1] and that of their symbionts (both *Photorhabdus temperata*) may help explain why both species performed similarly in allowing development of NEM1a but not of either NEM2 or NEM3. Cross-compatibility results suggest that, in theory, *Paenibacillus* bacteria could be exchanged between some (but not all) species of *Heterorhabditis* following direct or indirect contact in soil; for example, all three nematode-associated *Paenibacillus* strains were compatible with *H. bacteriophora*, with which *Paenibacillus* has so far not been found in the wild.

Differential compatibility of the nematode-associated *Paenibacillus* strains with various *Heterorhabditis* strains in cadavers could not be explained by the results of *in vitro* assays testing their sensitivity to the nematodes' *Photorhabdus* symbionts. In these assays, all three nematode-associated strains were relatively insensitive to all of the *Photorhabdus* strains tested; thus, differences between nematode-associated strains were small, but showed a trend for NEM1a to be always the most, and NEM3 the least inhibited, irrespective of *Photorhabdus* strain used. The failure of these assays to explain the differential compatibility in cadavers may be due to secondary metabolite production by *Photorhabdus* being impoverished on agar relative to that in insect cadavers [16]. Alternatively, factors other than antimicrobials may be responsible for rendering some cadavers more hostile than others to nonadapted microbes; for example, it has been proposed [19] that *Photorhabdus*'s competitive growth rates are more important than antimicrobials in suppressing competing microorganisms. Alternatively, the nematodes themselves may play an as-yet-unknown

role in modifying the cadaver environment for microorganisms.

Although the bacteria of which NEM1a and NEM3 are representative were each recovered from soil only once, NEM2 is representative of a more widespread association: sporangia-encumbered nematodes were isolated at four of the eight sites sampled in Georgia [25]. In addition to the three associations included in the present study, sporangia morphologically similar to those of *P. nematophilus* are routinely found associated with *H. zealandica* and *H. indica* in Florida (pers. comm., L.W. Duncan). Associations between *Heterorhabditis* spp. and *Paenibacillus* similar to those of this study may turn out to be of widespread occurrence. They have already been found in both temperate and tropical regions, in three continents (Europe, Asia, and North America), and involve at least three species of nematode and two closely related genetic clusters of *Paenibacillus* [10]. Such associations are not restricted to *Heterorhabditis*: a bacterium which is tentatively identified as a *Paenibacillus* close to *P. popilliae* is frequently found adhering to IJs of *Steinernema diaprepesi*, apparently in a phoretic relationship (FE Borai, LW Duncan, JF Preston, D Dunn. A phoretic association between a putative *Paenibacillus* sp. and the entomopathogenic nematode *Steinernema diaprepesi*, Abstract, Society of Nematologists Annual Meeting 2003).

The genus *Paenibacillus* contains a number of species that are associated with dead insects in one capacity or another. These include obligate insect pathogens such as *P. popilliae, P. lentimorbus*, and *P. larvae* [28], as well as non-pathogens or facultative pathogens such as *P. apiarus* [27] and *P. alvei* [23]. Although the *Heterorhabditis*-associated strains lie phylogenetically closer to species with no known association with insects, such as *P. macquariensis* and *P. azoreducens* [10], they provide an interesting addition to this list. As yet there is no evidence that nematode-associated *Paenibacillus* strains are entomopathogens, but only limited investigations have been carried out [9]. The evidence so far suggests that these *Paenibacillus* strains utilize *Heterorhabditis* for transport and introduction into the nutrient-rich environment of an insect cadaver. Adaptations to this way of life may include the unusual longevity of the sporangia allowing adhesion to the nematodes, and resistance to *Photorhabdus* antibiotics. The fact that the *Paenibacillus* strains tended to be most compatible with their natural *Heterorhabditis–Photorhabdus* associate indicates that a degree of adaptation to local species or strains has occurred. Although relatively few sporangia are produced per insect host (7×10^7 for *P. nematophilus* NEM1a with its natural associate), active dissemination of propagules by tens of thousands of mobile infective juveniles may compensate for this.

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