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Effects of *Paenibacillus nematophilus* on the entomopathogenic nematode *Heterorhabditis megidis*

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Abstract

The insect parasitic nematodes *Heterorhabditis* spp. are mutualistically associated with entomopathogenic bacteria, *Photorhabdus* spp. A novel association has been detected between *H. megidis* isolate EU17 and the endospore-forming bacterium *Paenibacillus nematophilus*. *P. nematophilus* sporangia adhere to infective juveniles (IJs) of *H. megidis* and develop in insect hosts along with the nematodes and their symbiont. We tested the effects of *P. nematophilus* on *H. megidis*. The yield and quality (size, energy reserves, and storage survival) of IJs were not affected by co-culture in insects with *P. nematophilus*. Dispersal of IJs in sand and on agar was inhibited by adhering *P. nematophilus* sporangia: fewer than 2% of IJs with *P. nematophilus* sporangia reached the bottom of a sand column, compared to 30% of the control treatment. Sporangia significantly reduced infectivity of *H. megidis* for wax moth larvae in sand, but not in a close contact (filter paper) assay. The results suggest that *P. nematophilus* may reduce the transmission potential of *H. megidis* through impeding the motility of IJs.

Keywords: *Paenibacillus nematophilus*; *Heterorhabditis megidis*; Entomopathogenic nematode; Reproduction; Quality; Survival; Infectivity; Motility; Host-finding; Migration; Sporangia

1. Introduction

Heterorhabditis spp. nematodes are insect pathogens. Infective juveniles (IJs) carry in their intestine the symbiotic bacteria, *Photorhabdus* spp. (Forst et al., 1997). On infection of a suitable host, the symbiont is released into the hemocoel and death of the insect ensues within 48 h (Poinar, 1990). The nematodes develop and reproduce within the dead insect host, feeding on the bacteria and the digested insect tissues. The insect cadaver may support several nematode generations. As the nutritive status of the cadaver declines, non-feeding IJs develop and emerge into the soil. The IJs are adapted morphologically, physiologically, and behaviorally for dispersal, host finding, and infection, and for survival in the soil environment.

Heterorhabditis spp. have a strict nutritional requirement for *Photorhabdus*, and the degree of specificity is such that some species (including *H. megidis*) cannot utilize the symbiont of another *Heterorhabditis* species (Gerritsen and Smits, 1997; Han and Ehlers, 1998). The presence of non-symbiont bacteria may reduce *Heterorhabditis* yields in vitro (Ehlers, 2001) and in vivo (Poinar et al., 1990), while certain contaminant species may also induce morphological and behavioral abnormalities in *Heterorhabditis* (Poinar, 1988). Rapid growth rate and secretion of antibiotics by *Photorhabdus* prevent microbial contamination of the cadaver, mainly from the insect intestinal microflora (Jarosz, 1996; Webster et al., 2002). Shedding by infective juveniles of their protective sheath (the molted cuticle of the previous developmental stage) prior to entry into the host or host hemocoel

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(Bedding and Molyneux, 1982; Peters et al., 1997) may also help reduce the number of contaminants introduced into the hemocoel by the invading nematodes. Non-symbiont bacteria reported in association with *Heterorhabditis* include *Providencia rettgeri* (Jackson et al., 1995) and *Ochrobactrum* spp. (Babic et al., 2000). In both of these studies the IJs were surface sterilized, but not desheathed, prior to isolation of the bacteria, hence the bacteria were carried either in the intestine, or between the sheath and the living cuticle (Babic et al., 2000; Jackson et al., 1995). No adverse effects of either *Providencia* or *Ochrobactrum* on *Heterorhabditis* have been reported.

Associations between endospore-forming *Paenibacillus* spp. and *Heterorhabditis* spp. have recently been reported (Enright et al., 2003; Marti and Timper, 1999), including the newly described *Paenibacillus nematophilus*, isolated from *H. megidis* EU17 from Estonia (Enright et al., 2003). Spindle-shaped *Paenibacillus* sporangia adhere to the sheath of *Heterorhabditis* IJs and are carried by them into the insect where *Paenibacillus* proliferates despite the presence of *Photorhabdus*. *H. megidis* IJs emerging from cadavers co-infected with *P. nematophilus* can carry up to 100 sporangia each, but usually fewer than 50 (Enright, 2003; Griffin, unpublished data). *Paenibacillus–Heterorhabditis* associations are notable for the tendency of the sporangia to cause the *Heterorhabditis* IJs (but not most other nematodes) to clump in water; the nematodes adhere in large cross-linked groups (Enright and Griffin, 2004).

Nematode-associated *Paenibacillus* spp. have no obvious pathological effects on *Heterorhabditis*, but may benefit by being transported by the nematodes (Enright et al., 2001; Marti and Timper, 1999). *Heterorhabditis*-associated *Paenibacillus* strains share two features that differ from other members of the genus and favor the maintenance of the association: resistance to *Photorhabdus* antibiotics, facilitating reproduction in infected cadavers, and retention of the sporangium, facilitating adhesion to *Heterorhabditis* spp. IJs (Enright and Griffin, 2004). Here we investigate whether the co-infection of hosts with *P. nematophilus* and *H. megidis* affects either the number of IJs produced (yield) or their quality (size, stored energy reserves, and survival). We also investigate whether dispersal and infectivity of IJs is affected when they are either produced in cadavers co-infected with *P. nematophilus*, or artificially encumbered with *P. nematophilus* sporangia.

2. Materials and methods

2.1. Nematodes and bacteria

Heterorhabditis megidis isolates UK211 and EU17 were routinely cultured at 20 °C in *Galleria mellonella* larvae (Mealworm Co. Sheffield, UK). Ten *G. mellonella* larvae were exposed to 1000 IJs on filter paper in a 9 cm diam. Petri dish. Infected cadavers were placed on White traps prior to IJ emergence (Kaya and Stock, 1997). Harvested IJs were washed three times by sedimentation in tap water. Nematodes were stored at 20 °C at a concentration of 1000 IJs/ml for up to 2 weeks before use. *P. nematophilus* NEM1a DSM 13559 was cultured on nutrient agar (Oxoid) at 30 °C.

Heterorhabditis megidis EU17 had been maintained in culture with *P. nematophilus* since its isolation in 1992. A culture free of *P. nematophilus* was established from surface sterilized IJs. IJs were treated with 0.5% sodium hypochlorite for 20 min, washed five times by sedimentation in sterile distilled water, and used to infect *G. mellonella* larvae. Samples from cadavers infected with putative spore-free IJs were heat treated and then plated onto nutrient agar, a selective isolation procedure for endospore-forming bacteria (Priest and Grigorova, 1990). These cadavers were shown to be free of *P. nematophilus* at all sampling times, while *P. nematophilus* was readily detected in cadavers infected with spore-encumbered IJs using this method (Enright, 2003). In addition, emerging IJs were confirmed to be free of sporangia by the absence of clumping and by microscopic examination. *P. nematophilus* was introduced into a culture of *H. megidis* UK211 by mixing sporangia with IJs and using the contaminated IJs to infect *G. mellonella* larvae. The emerging IJs carried sporangia and formed clumps. The uncontaminated EU17 and contaminated UK211were maintained in vivo as described above.

In some experiments, *H. megidis* IJs were mixed with sporangia and tested without subculture, in order to exclude possible effects of *P. nematophilus* acting during IJ development. A suspension of *P. nematophilus* NEM1a sporangia (10^8 sporangia/ml) was made by adding sporulated bacteria from a nutrient agar plate to sterile distilled water and vortex-mixing for 1 min. Sporangial suspension (undiluted or diluted 1/50) was added to IJ suspension at a ratio of 1:200 v/v, and caused the nematodes to clump. Added sporangia adhered at a rate of up to 50 per IJ, which is similar to the number adhering to IJs co-cultured with *P. nematophilus* in cadavers. Control nematodes received water. Suspensions were mixed by aspiration and stirring with a Pasteur pipette and incubated at 20 °C for up to 24 h before use.

2.2. Infective juvenile yield

Galleria mellonella larvae were exposed to IJs from the long-term *H. megidis* EU17–*P. nematophilus* culture, and to *H. megidis* EU17 from a culture free of *P. nematophilus* (control). There were three replicates of each treatment, with eight *G. mellonella* per replicate, exposed to 1000 IJs in a 90 mm diam. Petri dish. Six days post-infection, three cadavers from each replicate were dissected and the number of adult first generation

nematodes was counted. This number was taken to represent the number of IJs that had invaded the *G. mellonella* larvae, and did not differ between the two treatments (Mann–Whitney, $P > 0.05$). IJ yields were recorded from the remaining cadavers. IJs were harvested 3, 6, 9, and 15 days after emergence began and yield was assessed by counting the number of IJs in four replicate aliquots per harvest.

2.3. Size and energy reserves of infective juveniles

Optical density per unit area, measured using image analysis, can be used to estimate neutral lipid levels, the major energy store of *Heterorhabditis* IJs (Fitters et al., 1997). In addition, total optical density of IJs correlates well with their total energy reserves (Qiu and Bedding, 1999). *H. megidis* EU17 and UK211 with and without *P. nematophilus* were cultured in *G. mellonella*. The method of Fitters et al. (1997) was used to measure optical density, area, and length of IJs (30–40 per treatment) from the first 4–5 days of emergence. The experiment was conducted three times with different culture batches of nematodes.

2.4. Survival of infective juveniles

The survival of *H. megidis* EU17 IJs that developed in cadavers with and without *P. nematophilus* was assessed during storage at 20 °C. IJs emerging from *G. mellonella* cadavers on day 4 and day 8–9 of emergence were harvested and tested separately. IJ suspension (200 IJs in 10 ml of tap water in 55 mm Petri dishes) was incubated at 20 °C. The number of live IJs per dish was determined at week 0 and after 1, 2, 3.5, 8, and 10 weeks. The experiment was conducted with IJs from three separate culture batches of each treatment.

2.5. Sand infectivity test

The effect of *P. nematophilus* on the ability of *H. megidis* IJs to locate and enter a *G. mellonella* larva following migration through 40 mm of sand was assessed using the method of Griffin (1996). One hundred IJs were applied to the top of a vial filled with moist sand (8% w/w water), and vials were incubated for 17.5 h at 20 °C. The number of nematodes established in the *G. mellonella* larvae was assessed by dissection 5 days later. Treatments were *H. megidis* EU17 and UK211, each raised with and without *P. nematophilus*, and controls (which received tap water only). There were 10 vials per treatment. The experiment was run three (EU17) or four (UK211) times.

2.6. Migration in sand columns

The effect of *P. nematophilus* on the migration of *H. megidis* EU17 IJs through a sand column in the presence of insect hosts was investigated. Each column (120 mm high × 45 mm internal diameter) was made up of three plastic rings (each 40 mm in height) packed with sand, and had three *G. mellonella* larvae at the bottom. Two thousand IJs in 1 ml were applied to the top of the column. There were three treatments of *H. megidis* EU17 IJs: without sporangia; with added *P. nematophilus* sporangia (undiluted), and with added *P. nematophilus* sporangia (1/50 dilution). There were three replicate columns for each treatment in randomized block design. After 4.5 h at 20 °C, IJs were eluted from the sand. The number of IJs present in each ring was estimated from counts of three replicate samples and expressed as a percentage of the total number recovered from the column. The *G. mellonella* larvae were rinsed to remove adhering IJs and were incubated at 20 °C. Insects that died were dissected after 6 days and the number of nematodes that had become established was counted.

2.7. Migration on agar in response to insect volatiles

The impact of *P. nematophilus* on the response of *H. megidis* UK211 IJs to volatile cues was assessed as described by Dempsey and Griffin (2002). Treatments were IJs without *P. nematophilus* (control), IJs cultured in vivo with *P. nematophilus*, and IJs with added *P. nematophilus* NEM1a sporangia. In summary, two pipette tips were inserted through the lid of a 90 mm diam. Petri dish containing agar. Tips were 35 mm from the center (i.e., 70 mm apart) and contained either two live late instar *G. mellonella* larvae (host tip) or nothing (no-host tip). Plates were incubated at 20 °C for 1 h to allow a volatile gradient to develop. Approximately 1000 IJs were introduced to the center of the agar plate and plates were incubated for a further 1 h at 20 °C. The number of IJs in each of the following sections was determined: a central inoculation zone (14 mm diam.); a 10 mm wide strip running across the Petri dish, perpendicular to the axis containing the pipette tips, but excluding the inoculation zone (neutral zone); a circle (14 mm diam.) directly underneath the host and no-host tips; the side of the dish containing the host (positive) and no-host (negative) tips and excluding the neutral zone. The number of IJs in each section was expressed as a proportion of the total number of IJs recovered, and as a proportion of the total number of IJs outside the inoculation zone (i.e., the dispersing IJs). The experiment was conducted three times with different culture batches of nematodes. In each experiment there were 4–5 replicate plates per treatment in randomized block design, with one of each of the three treatments per block.

2.8. One-on-one infectivity assay

In this assay, one insect is challenged with one IJ on filter paper. Under these conditions, migration of IJs is

not required for infection, and IJ–IJ interference (by the formation of clumps) can also be discounted as a factor influencing infectivity. The assay was conducted as described by Glazer and Lewis (2000), with a single IJ and a single *G. mellonella* larva per well of a 24-well multiwell plate, incubated at 20 °C. Treatments were *H. megidis* EU17 IJs without *P. nematophilus* (control) and with added *P. nematophilus* NEM1a sporangia. IJ suspensions from which test IJs were taken were continuously agitated to prevent clumping. There were three experiments, with either 72 or 120 insects per treatment. In two of the experiments, insect mortality was assessed at intervals over the course of 6 days, and the time taken for 50% of the insects to die (LT_{50}) was calculated.

2.9. Statistics

More than two treatments were compared by one- or two-way analysis of variance. General linear models (GLM) were used for unbalanced data. Where significant differences were detected, means were separated using Tukey's post hoc test. Two treatments were compared by *t* test. Proportions were first subjected to arcsine transformation. Survival data were subjected to probit analysis to calculate ST_{50} (survival of infective juveniles) or LT_{50} (survival of insects in one-on-one assay). A significance level of $P < 0.05$ was used in all tests.

3. Results

3.1. Effect of P. nematophilus on H. megidis EU17 yield

The number of *H. megidis* IJs emerging from *G. mellonella* cadavers co-infected with *P. nematophilus* did not differ ($P > 0.05$) from the number emerging from control cadavers (with no *P. nematophilus*). Yields were 137,626 (±14,866) and 133,225 (±8134) (means ± SE) IJs per insect, respectively.

3.2. Effect of P. nematophilus on size and energy reserves of H. megidis EU17 and UK211 IJs

Heterorhabditis megidis IJs harvested from *G. mellonella* co-infected with *P. nematophilus* did not differ from IJs from control cadavers (no *P. nematophilus*) either in size (area, length) or in reserves (optical density and optical density per unit area) (Table 1). There was no effect due to either *P. nematophilus* or nematode isolate, for any of the four parameters ($P > 0.05$, two-way ANOVA or GLM).

3.3. Survival of H. megidis IJs raised in vivo with and without P. nematophilus

Heterorhabditis megidis IJs cultured in vivo with *P. nematophilus* survived as long as control IJs (cultured without *P. nematophilus*). The ST_{50} (50% survival time) values did not differ significantly, as evidenced by the overlap of fiducial limits (Table 2). This was true both for IJs that emerged on day 4 and for those which emerged later, on day 8–9. However, later emerging IJs did not survive as long (ST_{50} 4–5 weeks) as those of the day 4 emergence (ST_{50} 6–7 weeks), and the difference between harvest was significant for each culture type (Table 2). After 10 weeks (the last assessment date; data not shown), fewer than 5% of the day 8–9 emergence of each type remained alive, compared to over 20% of the day 4 emergence. A two-way ANOVA on these data showed that the presence of *Paenibacillus* did not affect the proportion of IJs alive at week 10 ($P = 0.79$) but that time of emergence did ($P < 0.001$).

3.4. Infectivity of H. megidis IJs in sand

Infective juveniles of *H. megidis* EU17 and UK211 raised in cadavers with *P. nematophilus* invaded

Table 2
Survival of infective juveniles (IJs) of *Heterorhabditis megidis* EU17 during storage in tap water at 20 °C

Emergence period	Culture conditions	ST_{50}[a] (weeks) with 95% fiducial limits
Day 4	*P. nematophilus*	6.93 (6.20–7.81)
	Control	6.55 (5.93–7.26)
Day 8–9	*P. nematophilus*	5.16 (4.54–5.85)
	Control	4.50 (3.93–5.11)

IJs had developed in *Galleria mellonella* cadavers co-inoculated with *Paenibacillus nematophilus*, or in cadavers without *P. nematophilus* (control) and emerged either 4 or 8–9 days after the start of IJ emergence.

[a] ST_{50}: time required for 50% of IJs to die.

Table 1
Area, length, optical density (OD), and optical density per unit area (OD/area) of *H. megidis* (EU17 and UK211) infective juveniles cultured in *Galleria mellonella* either with *Paenibacillus nematophilus* or without (control)

H. megidis isolate	Culture conditions	Area (μm²)	Length (μm)	OD (units)	OD/area (units/μm²)
EU17	*P. nematophilus*	24,510 ± 1503	849 ± 21	13,110 ± 62	0.5402 ± 0.033
	Control	23,747 ± 1002	831 ± 17	13,453 ± 382	0.5679 ± 0.008
UK211	*P. nematophilus*	23,202 ± 1003	814 ± 13	12,759 ± 189	0.5537 ± 0.026
	Control	23,199 ± 489	820 ± 6	13,033 ± 141	0.5631 ± 0.018

Mean (±SE) of three replicate experiments, except EU17 length, where data are from two experiments.

G. mellonella in sand at a lower rate than control IJs raised without the bacterium (Fig. 1). Presence of *P. nematophilus* had a significant effect on invasion (general linear model, $P = 0.023$) but nematode isolate did not ($P = 0.38$). Overall, the infectivity of *H. megidis* raised with *P. nematophilus* was less than half that of IJs raised without the bacterium (10.2 compared to 23.1 nematodes/insect).

3.5. Heterorhabditis megidis migration in sand columns

Addition of *P. nematophilus* sporangia to *H. megidis* EU17 IJs inhibited their vertical migration in the presence of insects (Fig. 2). After 4.5 h, 81–94% of IJs with added sporangia remained in the top ring of the sand column compared to 46% of control (sporangia-free) IJs. More control IJs (30%) reached the bottom ring of the column than IJs with sporangia added at either concentration (<2%). The number of nematodes infecting the insects at the bottom of the column was proportionately reduced: a total of 24 nematodes infected in the control compared to 1 and 2 nematodes in the undiluted and diluted sporangia treatments, respectively.

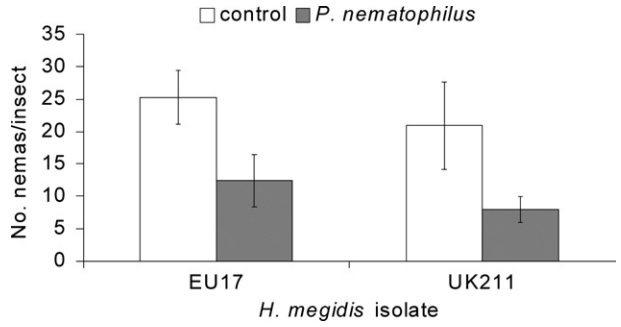

Fig. 1. The mean (±SE) number of *Heterorhabditis megidis* infective juveniles (out of 100) invading *Galleria mellonella* larvae in a sand infectivity assay. *H. megidis* EU17 and UK211 were either cultured with *Paenibacillus nematophilus* or without (control). Mean of three (EU17) or four (UK211) experiments.

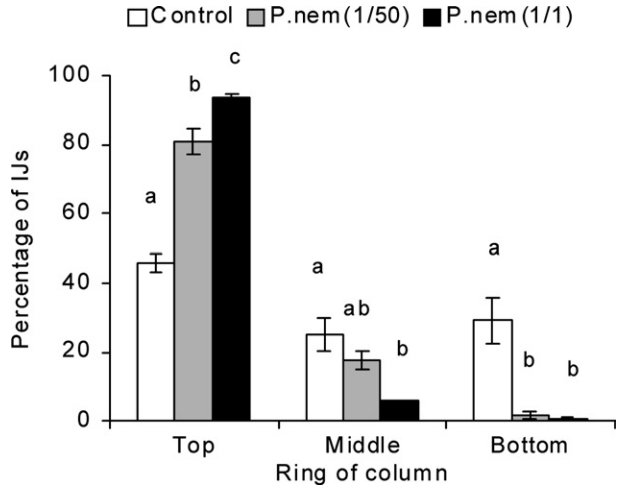

Fig. 2. The percentage of *Heterorhabditis megidis* EU17 infective juveniles (IJs) recovered from the top, middle and bottom rings in a sand column assay. Columns contained three *Galleria mellonella* larvae at the bottom, and IJs were inoculated at the top. *Paenibacillus nematophilus* NEM1a sporangia at two concentrations (undiluted and a 1/50 dilution) were mixed with IJs prior to inoculation, while control IJs had no sporangia. Each bar represents the mean (±SE) of three sand columns. Within a ring, treatments accompanied by the same letter are not significantly different (ANOVA, Tukey's test, $\alpha = 0.05$).

3.6. Migration of H. megidis IJs on agar in response to host volatiles

Paenibacillus nematophilus had a significant effect on the migration of *H. megidis* UK211 IJs on agar (one-way ANOVA, $P < 0.05$). The percentage of IJs that left the inoculation zone was lower in treatments with *P. nematophilus* (either added to or cultured with the IJs) than in the controls (Fig. 3).

The number of IJs migrating to the host side, non-host side or central neutral zone of the plate was expressed as a percentage of the number leaving the point of inoculation (the dispersing IJs). The percentage of dispersing IJs that moved to the host side of the arena was lower in the two *P. nematophilus* treatments than in the control (Fig. 4). The difference between treatments was close to significance at $P < 0.05$ ($P = 0.061$; one-way ANOVA). When data for the two *P. nematophilus* treatments (co-cultured and added sporangia) were combined, the difference between the control and the combined *P. nematophilus* treatments was significant (*t* test, $P = 0.005$). The proportion of dispersing IJs moving to the host side of the plate was 54% in the control and 44% in the combined *P. nematophilus* treatments.

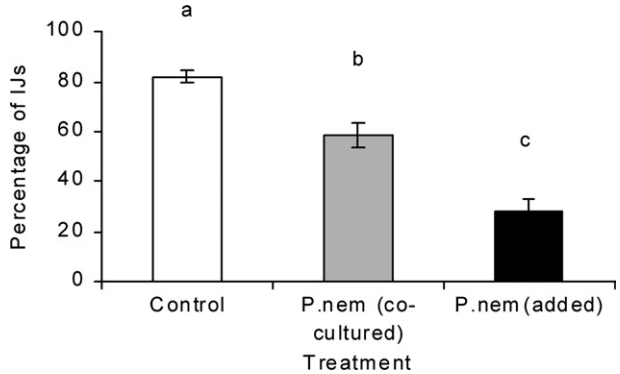

Fig. 3. The mean (±SE) percentage of *Heterorhabditis megidis* UK211 infective juveniles (IJs) dispersing from the point of inoculation in an agar plate assay. Treatments were IJs tested without *Paenibacillus nematophilus* (control), co-cultured with *P. nematophilus* and with added *P. nematophilus* NEM1a sporangia. Treatments accompanied by different letters are significantly different (ANOVA, Tukey's test, $\alpha = 0.05$). Mean of three experiments with 4–5 replicate plates per experiment.

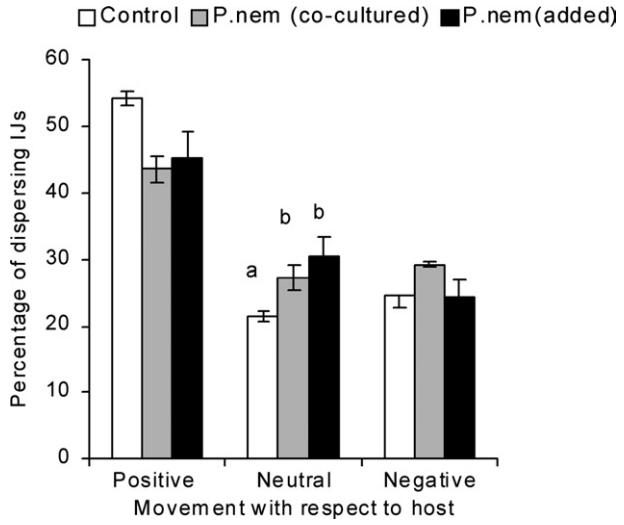

Fig. 4. The mean (±SE) percentage of *Heterorhabditis megidis* UK211 infective juveniles (IJs) which left the point of inoculation (the dispersing IJs), displaying positive (towards host), negative (away from host) and neutral movement on agar. Treatments were IJs tested without *Paenibacillus nematophilus* (control), cultured with *P. nematophilus* and with added *P. nematophilus* NEM1a sporangia. The percentage of IJs accumulating under the host and non-host tips are included in the positive and negative classes, respectively. Within a movement class, treatments with the same or no letter do not differ significantly (ANOVA, Tukey's test, $\alpha = 0.05$). Mean of three experiments with 4–5 replicate plates per experiment.

There was no significant difference between treatments in the proportion of dispersing IJs accumulating under the host tip, when analyzed either as three treatments or, as above, as two. On average 8.9% of IJs accumulated under the host tip compared to 1.9% under the non-host tip.

3.7. One-on-one infectivity

Single *H. megidis* IJs killed approximately half of the exposed insects in a one-on-one assay (Table 3). There was no significant difference in the proportion of insects killed by nematodes with or without *P. nematophilus* (*t* test, $P > 0.05$), nor was there a difference in the time taken to kill the hosts (LT_{50}), as judged by the overlap of fiducial limits (Table 3).

Table 3
Mortality of *Galleria mellonella* larvae in a one-on-one infectivity assay, and time required for 50% of insects to die (LT_{50})

Treatment	Mean (±SE) % of *G. mellonella* dead[a]	LT_{50} (h) with 95% fiducial limits[b]
H. megidis	52.9 (9.16)	97.6 (89.5–107.7)
H. megidis + *P.nematophilus*	48.9 (13.82)	89.0 (82.2–97.2)

Each insect was exposed to a single *Heterorhabditis megidis* EU17 infective juvenile either with *Paenibacillus nematophilus* NEM1a sporangia or without (control).

[a] Mean of three experiments with 72 or 120 insects per experiment.

[b] Mean of two experiments.

4. Discussion

There was no evidence of a negative effect of *P. nematophilus* on *H. megidis* development: the yield, size, stored energy reserves (estimated by optical density), and survival of IJs from hosts co-infected with *P. nematophilus* were the same as those from hosts infected with nematodes only. This may be a general feature of *Paenibacillus–Heterorhabditis* associations, as IJ yield was also unaffected in two other natural *Paenibacillus–Heterorhabditis* associations, and in all but one of the 15 novel *Paenibacillus–Heterorhabditis* associations tested (Enright and Griffin, 2004). *P. nematophilus* is relatively slow-growing in vitro (Enright et al., 2003) and, despite being tolerant of *Photorhabdus* antibiotics, produced only 10^7 sporangia per *G. mellonella* cadaver (Enright and Griffin, 2004). Either the impact of *P. nematophilus* on *Photorhabdus* growth was too slight to have a measurable effect on nematode nutrition, or the nematodes may actually have fed on *P. nematophilus*. *Heterorhabditis* spp. are fastidious in their nutritional requirements, developing only when *Photorhabdus* (and frequently only a narrow range of strains) is present (Ehlers, 2001; Gerritsen and Smits, 1997; Han and Ehlers, 1998). However, it is not known whether the developing nematodes can utilize non-symbiont microbes when they constitute a small proportion of the microbial flora dominated by a nutritionally suitable strain of symbiont. Even if *Paenibacillus* itself is not utilized as food by the nematodes, it may enhance the nutritional quality of the cadaver for *Heterorhabditis* by contributing to the breakdown of the host tissues, as suggested by Thurston et al. (1993) for *H. bacteriophora* developing with *Paenibacillus popilliae*.

IJs emerging late from *H. megidis*-infected cadavers survived less well in storage than those emerging earlier. This presumably reflects the deteriorating cadaver conditions under which they developed. Nguyen and Smart (1995) found a negative linear relationship between IJ length and time of emergence from the host cadaver, and other physiological and behavioral effects of emergence time have also been attributed to changes in the quality of the cadaver resource (O'Leary et al., 1998; Ryder and Griffin, 2003).

Paenibacillus nematophilus sporangia significantly reduced the motility of *H. megidis* IJs. The admixture of *P. nematophilus* sporangia more than halved the proportion of IJs leaving the top ring of a sand column, and the proportion of IJs leaving the inoculation zone on an agar plate was similarly reduced. Only 28% of IJs with added *P. nematophilus* left the zone compared to 82% in the control. Adhesion of other microorganisms is known to inhibit the motility of nematodes. For example, motility and infection of root-knot nematodes (*Meloidogyne* spp.) is reduced when they are encumbered with spores of the parasite *Pasteuria penetrans* (Davies et al., 1991). The effect of microorganisms on nematode motility

might be caused by the adhering propagules interfering with undulatory movement of individual nematodes (Adiko and Gowen, 1999), or by the propagules causing groups of nematodes to clump together. Microorganisms can induce tight clumps of nematodes, which may be difficult to break apart (Riley and McKay, 1990). *P. nematophilus* induces clumping in *H. megidis* IJs in water. Although observations suggest that clumped IJs can break apart from each other in sand, the time spent by IJs in breaking apart from clumps, together with possible re-clumping of individuals as they migrate through soil, may lead to a reduction in dispersal.

The infectivity of *H. megidis* for wax moth larvae in sand was reduced by more than 50%. It is likely that this result is due largely to the reduction of IJ motility in sand discussed above, rather than to interference with the nematode's ability to recognize or invade the insect. *P. nematophilus* had no effect on infectivity in close contact assays conducted on filter paper, in either the one-on-one assays reported here or in other assays with multiple IJs per host (Enright, 2003). However, interference by *P. nematophilus* with the IJs' ability to detect host volatiles may contribute to the reduction in the infection rate. *H. megidis* IJs are considered to be "cruise foraging" nematodes (Downes and Griffin, 1996). Such nematodes actively search the soil environment, responding to host-produced volatiles but switching to more localized searching after contact with an insect's "active space" (Lewis et al., 1992). In the agar assay, *P. nematophilus* reduced the proportion of IJs moving to the host side of the dish, but not to the non-host side. This raises the possibility that chemotaxis of IJs in response to host volatiles is inhibited by *P. nematophilus* or by substances, such as lectins, associated with it. Lectins may inhibit nematode chemoreception by binding to carbohydrate moieties in the amphids (Lewis et al., 1996), and there is evidence for the involvement of a sialic acid–lectin interaction in the adhesion of *P. nematophilus* sporangia to *H. megidis* IJs (unpublished data). However, it is unknown whether the lectin or carbohydrate moiety is on the sporangium.

Paenibacillus nematophilus had detrimental effects on *H. megidis* IJs irrespective of how the IJs became encumbered, either naturally during co-culture in insects from which they emerged with attached sporangia (sand infectivity test; agar assay) or artificially, by mixing sporangia with emerged IJs (sand column test; agar assay). The "added sporangia" treatment demonstrates the large effect played by the presence of the bacteria at the time of testing. We cannot rule out the possibility that, in addition, the presence of *P. nematophilus* during development of the nematodes affected their subsequent behavior as IJs. Under natural conditions, by interfering with their ability to actively disperse in search of hosts, *P. nematophilus* may ultimately affect the transmission success of nematode strains with which it is associated.

To date, we know of three natural associations of *Paenibacillus* with *Heterorhabditis* spp. (*H. megidis* from Estonia, *H. indica* from India, and *Heterorhabditis* sp. from Georgia, USA; Enright et al., 2003). The nematode-associated *Paenibacillus* from India and Georgia are closely related to *P. nematophilus* (Enright et al., 2003). There are two other similar associations, involving *H. zealandica* and *H. indica* in Florida, where the identity of the bacterium has yet to be confirmed (L.W. Duncan, University of Florida, pers. comm.). In relation to the number of *Heterorhabditis* isolations worldwide, this represents a rather low incidence of association with *Paenibacillus*. The Estonian and Indian associations are each known from a single isolation, but more frequent isolations of *Heterorhabditis–Paenibacillus* in Georgia (Marti and Timper, 1999) and Florida (Duncan, pers. comm.) suggest that they may be ecologically important locally. A similar association between *Steinernema diaprepesi* and a putative *Paenibacillus* has recently been reported, but features such as spore morphology and sequence data suggest that it is not closely related to the *Heterorhabditis* associates (El-Borai et al., 2002, 2003; Duncan, pers. comm.). While *Heterorhabditis*-associated *Paenibacillus* adhere preferentially to the IJ sheath (Enright and Griffin, 2004), the *S. diaprepesi* associate adheres to the IJ cuticle, and not to the sheath (El-Borai et al., 2003). Each of these strategies seems well adapted to exploit the respective nematode associate for transport, since *Steinernema* IJs lose the sheath readily in soil, while *Heterorhabditis* IJs do not (Campbell and Gaugler, 1991). In our experience, *P. nematophilus* develops in virtually every *G. mellonella* exposed to *Heterorhabditis* IJs bearing sporangia. As the sporangia adhere only to the IJ sheath, this indicates that at least some IJs enter the host without exsheathing.

The genus *Paenibacillus* contains a number of entomopathogenic species, such as *P. popilliae* and *P. lentimorbus*. Enright (2003) investigated the pathogenicity of *P. nematophilus* sporangia injected into larvae of *G. mellonella* and the garden chafer *Phyllopertha horticola*. *P. horticola* is a natural host of *H. megidis* in Europe (Smits et al., 1994). *G. mellonella* injected with up to 10^5 sporangia did not die, while an LD_{50} of 1.5×10^5 sporangia/insect was recorded for *P. horticola* (Enright, 2003). This exceeds the 10^4 cells/insect below which a bacterium should be considered an entomopathogen (Bucher, 1960). These limited investigations cannot fully ascertain the pathogenicity of *P. nematophilus* for insects, since members of this genus typically have narrow host ranges.

The relationship between *P. nematophilus* and *H. megidis* appears to be largely one-sided. We have found no clear benefits to *H. megidis* from its association with *P. nematophilus* and a definite potential for a reduction in *H. megidis* transmission. On the other hand,

P. nematophilus may benefit by being transported to nutrient-rich resources. In an established transport relationship, we might expect that *Paenibacillus* will be under selection pressure to minimize its impact on nematode transmission, since it also benefits from colonizing additional hosts. Perhaps the fusiform shape of the sporangium is an adaptation to minimize effects on nematode migration, which would otherwise be even greater than reported here.

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