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The influence of *Photorhabdus luminescens* strains and form variants on the reproduction and bacterial retention of *Heterorhabditis megidis*

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Summary – The preference of nematodes for feeding on, and retention of strains and form variants of symbionts was tested. *Heterorhabditis megidis* strains DH-SH1 (= HSH) and NLH-E87.3 (= HE) could multiply on the primary forms of both symbionts, *Photorhabdus luminescens* strains PSH/1 and PE/1, respectively, and did not prefer one strain to the other. Both nematodes could not reproduce on the secondary form of their own symbiont; however, HSH could multiply on the secondary form PE/2. This suggests that the negative effect a secondary form bacterium has on nematode reproduction, is not a common factor in all secondary forms. When put on a plate with primary and secondary form bacteria, most infective juveniles preferred to feed on primary form bacteria. All nematodes were able to retain the bacteria they had been cultured on, including the secondary form. These results suggest that the nematodes prefer the primary form as a food source but the retention of bacteria is not as specific as suggested before in the literature.

Résumé – ***Influence de souches et de formes variantes de* Photorhabdus luminescens *sur la reproduction et la rétention bactérienne d'*Heterorhabditis megidis –** La préférence nutritionnelle et la rétention de souches et formes variantes de symbiotes ont été testées. Les souches symbiotes DH-SH1 (HSH) et NLH-E87.3 (= HE) peuvent se multiplier sur les formes primaires des deux symbiotes, *Photorhabdus luminescens* souche PSH/1 et PE/1, respectivement, et ne montrent aucune préférence envers l'une ou l'autre. Aucun des deux nématodes ne se reproduit en présence de la forme secondaire de son propre symbiote; toutefois, HSH peut se reproduire sur la forme secondaire PE/2. Ces observations suggèrent que l'effet négatif de la forme bactérienne secondaire sur la reproduction des nématodes ne constitue pas un facteur commun à toutes les formes secondaires. Lorsqu'ils sont placés en boîte de Petri, en présence de formes primaires et secondaires de la bactérie, la plupart des juvéniles infestants préfèrent la forme primaire comme source de nourriture. Tous les nématodes sont capables de retenir les bactéries sur lesquelles ils ont été élevés, y compris les formes secondaires. Ces résultats suggèrent que les nématodes préfèrent la forme primaire comme source de nourriture, mais que la rétention de la bactérie n'est pas aussi spécifique qu'il l'a été affirmé dans la littérature antérieure.

Key-words : Bacteria, entomopathogenic nematodes, *Heterorhabditis megidis,* insect pathology, phase variation, *Photorhabdus luminescens,* preference, reproduction, retention.

Photorhabdus luminescens (Boemare *et al.*, 1993) and *Xenorhabdus* spp. (Akhurst & Boemare, 1990) are insect pathogenic bacteria symbiotically associated with nematodes of the genera *Heterorhabditis* and *Steinernema,* respectively (Thomas & Poinar, 1979). The infective or dauer juvenile of the nematode carries the bacterial symbiont in its intestinal and pharyngeal lumen (Poinar, 1979; Bird & Akhurst, 1983; Endo & Nickle, 1991). The nematode penetrates an insect host, moves into the haemocoel, and releases the bacterium. The bacterium starts multiplying and kills the host, supported by excretion products of the nematode that repress the immune system of the insect (Götz *et al.*, 1981). *P. luminescens* and *Xenorhabdus* spp. further produce antibiotics to inhibit growth of other micro-organisms in the insect cadaver (Akhurst, 1982; Gerritsen *et al.*, 1992) and provide nutrients utilized by the nematodes (Poinar & Thomas, 1966).

Colony morphology and biochemical abilities of *Photorhabdus* and *Xenorhabdus* isolates are highly variable. Two extreme colony forms are characterized as phase 1 and phase 2, or primary and secondary forms, respectively (Akhurst, 1980; Bleakley & Nealson, 1988; Akhurst & Boemare, 1990), but several colony forms with intermediate properties have been described (Hurlbert *et al.*, 1989; Gerritsen *et al.*, 1992). The primary form has a unique colonial morphology, produces antibiotics, lipases, proteases and a pigment, absorbs certain dyes from agar media and, in the case of *P. luminescens,* is luminescent. The secondary form has lost all or some of these abilities, has a different colonial morphology and does not support growth of the nematode as well as the

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primary form does (Akhurst, 1980; Boemare & Akhurst, 1988; Akhurst & Boemare, 1990; Ehlers *et al.*, 1990). The primary form, which can be isolated from the infective juveniles, often converts into the secondary form when cultured *in vitro*.

The association between nematodes and their symbionts is specific. Each *Steinernema* species is associated with its own *Xenorhabdus* species (Akhurst & Boemare, 1988). Currently all symbionts of *Heterorhabditis* spp. are classified as *P. luminescens*. However, studies have shown that *P. luminescens* may be a multispecies taxon representing four to five species (Grimont *et al.*, 1984; Smits & Ehlers, 1991; Akhurst *et al.*, 1992; Boemare *et al.*, 1993), each with several strains. The various strains of *P. luminescens* differ in their ability to support cultures of non-host *Heterorhabditis* spp. and in the ability of the nematode to retain the bacteria (Han *et al.*, 1990; Gerritsen & Smits, 1993).

In this study, the preference of two *H. megidis* strains for feeding on, and retention of symbiont strains and form variants was assessed.

Materials and methods

Nematode and bacterial isolates

Two *Heterorhabditis megidis* strains were used, strain NLH-E87.3 (HE) from The Netherlands and strain DH-SH1 (HSH) from Germany. *H. megidis* DH-SH1 was provided by R.-U. Ehlers, Kiel, Germany. The primary and secondary forms of the bacterial symbiont of strain HSH and the secondary form of the bacterial symbiont of HE were provided by K. C. Krasomil-Osterfeld, Kiel, Germany. The primary form bacteria, PSH/1 from HSH and PE/1 from HE, were isolated directly from infective juveniles, while the secondary form bacteria, PSH/2 from HSH and PE/2 from HE, were isolated after induction of the primary form under low osmotic conditions (Krasomil-Osterfeld, 1995). Form variants were identified by colony morphology, differential absorbtion of dye when grown on MacConkey agar (MacConkey broth [Merck], 1.5 % agar), antibiotic activity against *Micrococcus luteus* (Akhurst, 1982), and luminescence. Bacteria were grown on nutrient agar (0.8 % Lab Lemco Broth, Oxoid; 1.5 % agar) and incubated in the dark at 25 °C for 3 days.

Antiserum production

Four antisera were produced as described by Gerritsen *et al.* (1995). The antisera are polyclonal, produced in rabbits against live, whole cells, and cross absorbed to overcome cross reaction (Gerritsen *et al.*, 1995). Antiserum 9226 reacted to all four bacteria, PSH/1, PSH/2, PE/1, and PE/2. Antiserum 9351 was specific for PSH/1 and antiserum 9352 was specific for PSH/2. Antiserum 9353 was produced against a small colony variant of PE (XE-white, Gerritsen *et al.*, 1992) and reacted only to both PE forms, not to PSH forms.

Preference test

Bacteria were inoculated on lipid agar (16 g/l nutrient broth [Bacto], 5 g/l corn oil, 12 g/l agar; *cf.* Wouts, 1981) in a 5 cm Petri dish. The plate was divided into three sections and two of these sections were inoculated with bacteria. Each section received a different bacterium strain or form, or, as a control, both sections received the same bacterium (Fig. 1). The 5 cm Petri dishes, without lid, were put inside 9 cm Petri dishes. Plates were incubated in the dark at 25 °C. After one day, 100 infective juveniles (monoxenically cultured on their primary form, surface sterilized in 0.4 % Hyamine) of either HSH or HE were inoculated on the remaining section of the agar plate (Fig. 1). Plates were incubated in the dark at 25 °C and nematode growth and reproduction was monitored. As soon as infective juvenile production occurred in one of the treatments, all 9 cm Petri dishes were filled with sterilized tap water, leaving the 5 cm dish dry. The infective juveniles readily moved from the small dish into the water. The nematodes " trapped " in this water were transferred to tissue culture-flasks and stored at 5 °C. This test was repeated three times. The number of infective juveniles produced per agar plate was assessed.

To analyze the results statistically, the observed counts Y of infective juveniles of HSH and HE were fitted to loglinear models (McCullagh & Nelder, 1989), with replicates and treatments as explanatory variables.

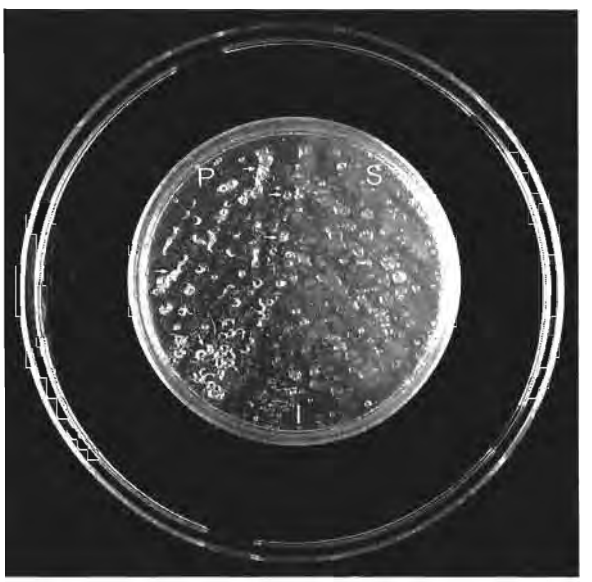

Fig. 1. *Preference test. Lipid agar plate with primary form bacterial lawn (P) and secondary form bacterial lawn (S) of* Photorhabdus luminescens *PSH* and Heterorhabditis megidis *strain HSH hermaphrodites (some pointed out by arrows). Infective juveniles were inoculated on I. Nematodes prefer to feed on the primary form.*

It was assumed that the dispersion of the data was greater than that predicted by a Poisson model. As an approximation, *var (Y) = s^2 mean (Y),* where s^2 denotes the dispersion parameter, was assumed. Treatment effects were assessed using the mean deviance ratio resulting in an approximate F-test. Pairwise differences between treatment means on the logarithm-scale were tested using a t-test. Differences between treatments were considered to be non-significant at $P \leq 0.05$. Analyses were performed using the Genstat (Anon., 1993) statistical program.

UPTAKE OF BACTERIA BY NEMATODES

Infective juveniles produced in the preference test were surface sterilized in 0.4 % Hyamine for 15 min, washed twice with sterile demineralized water and crushed in a Potter homogenizer. Of this suspension, 50 µl was inoculated on MacConkey agar plates, and 50 µl of a 1:10 dilution of this suspension in demineralized water was inoculated on nutrient agar plates. These undiluted and 1:10 diluted nematode-bacterium suspensions were used in indirect immunofluorescence cell-staining (IF). Indirect IF was performed according to Van Vuurde *et al.* (1983). Microscope slides with twenty-four 4 mm wells (Nutacon, 10-342-A) were used. Each well was filled with 5 µl of nematode-bacteria suspension. As a control, some wells were filled with 5 µl of a pure primary or secondary bacterial suspension of 10^5 to 10^7 cells/ml from nutrient agar plates.

The wells were air-dried and the bacteria were fixated for 10 min in 96 % ethanol. After washing with demineralized water, the slides were dried and each well was filled with 5 µl of one of the antisera 9226, 9353, 9351, or 9352, diluted to 1:300 in PBS (0.8 % NaCl, 0.27 % $Na_2HPO_4.12H_2O$, 0.04 % $H_2NaPO_4.2H_2O$, pH 7.2). All nematode-bacteria combinations were tested with the four antisera. The slides were incubated for 30 min under humid conditions in the dark. After washing with demineralized water, the slides were dried and incubated for 30 min with 5 µl anti-rabbit FITC-conjugated antibodies (Sigma) diluted 1:200 in PBS. After washing with demineralized water, the slides were dried and covered with 60 µl mounting buffer (2.1 % $Na_2HPO_4.12H_2O$, 0.1 % $H_2NaPO_4.2H_2O$, 33.3 % glycerol [Merck no. 4095], pH 7.6) and a coverglass. Slides were examined under a UV-microscope (objective 50 W/N.A.100) with incident blue fluorescent light (490 nm).

Results

PREFERENCE TEST

Table 1 shows the production of infective juveniles of HSH and HE on different bacteria combinations.

Single bacterium : Both nematodes could multiply on their own primary form symbiont and on the primary form bacterium of the other nematode. Both nematodes could not reproduce on the secondary form of their own bacterium. Although some infective juveniles, inoculated on the secondary form, developed to hermaphrodites, only some of these hermaphrodites produced eggs and all J1 hatched from these eggs died. HSH could multiply on secondary form PE/2, although the production of infective juveniles on these plates was less than on one of the primary forms. Only in the third experiment could HE multiply on secondary form PSH/2; in the other two experiments all nematodes died before they could produce a second generation.

Combination of primary forms : Both nematodes multiplied very well on a combination of primary form bacteria, and both bacterial lawns had been eaten completely at the end of the experiment.

Combination of secondary forms : HE did multiply on both secondary forms in the first but not in the other two experiments. HSH could not multiply on a combination of secondary forms.

Combination of primary and secondary forms : Both nematodes could multiply on a combination of primary and secondary form bacteria. The production of infective juveniles on these plates was less than on plates with pure primary form (Table 1). When put on a plate with primary and secondary form bacteria, most infective juveniles preferred the primary form bacteria (Fig. 1). Some wandered through the secondary form bacterial lawn but soon settled in the primary form lawn. Second generation nematodes wandered through the whole plate but at the end of the experiment the primary form bacterial lawn had been eaten by the nematodes while most of the secondary form bacterial lawn was still

Table 1. *Production of infective juveniles of* Heterorhabditis *strains HSH and HE on different bacteria forms and combinations of bacteria. (Total number of infective juveniles produced on one lipid agar plate, data are mean of three experiments).*

Bacteria	Nematode	
	HSH	HE
PSH/1	22 442 *a*	26 642 *ab*
PSH/2	0+	900*+
PE/1	17 400 *ab*	35 700 *a*
PE/2	7 338 *bc*	0+
PSH/1 + PE/1	22 777 *a*	31 633 *a*
PSH/2 + PE/2	0+	1 100*+
PSH/1 + PSH/2	6 863 *b*	12 413 *c*
PE/1 + PE/2	8 967 *b*	15 998 *bc*

Values with the same letter do not differ significantly on a log-scale. HSH and HE were analyzed separately.
* = Result of one experiment, other experiment no infective juvenile production.
+ = These values were not used in the statistical analysis.

present. Also HSH, which could reproduce on PE/2, preferred primary form PE/1 to secondary form PE/2, when there was a choice. However, HSH ate both PE/1 and PE/2 bacterial lawn in the end.

Although the infective juveniles carried their own symbiont when put on the agar plates with other bacteria, only a few plates contained some colonies of these bacteria (discriminated by colony pigmentation) and the infective juveniles produced on these plates carried only the bacteria on which they where cultured.

RETENTION OF BACTERIA BY NEMATODES

All nematodes were able to take up the bacteria they had been cultured on. In IF, PSH, and PE could be discriminated and PSH/1 and PSH/2 could be discriminated but PE/1 and PE/2 could not. On plates, all four bacteria could be discriminated by color and colony morphology, but plating efficiency was very low and in some cases no bacteria developed.

When cultured on one bacterium only, the infective juveniles carried this bacterium in their guts, in all cases. Even HSH infective juveniles cultured on PE/2 carried many bacteria in their guts.

When cultured on a mixture of bacteria, nematodes contained both bacteria in their guts. When cultured on both primary forms, HSH and HE contained both bacteria in their guts in equal amounts. Only in one experiment did HE reproduce on a combination of PSH/2 and PE/2, and the infective juveniles produced on this plate contained both bacteria in equal amounts.

HSH and HE cultured on a mixture of their primary and secondary forms were able to take up both forms in their guts. IF results showed that the nematodes contained more PSH/1 primary cells than PSH/2 secondary cells. Since antibodies able to distinguish between PE/1 and PE/2 were not available, the difference between these forms was not visible in IF; also, on plates, the plating efficiency was so low that it was not possible to draw a conclusion on the uptake of a mixture of PE/1 and PE/2 by the nematodes.

Discussion

Heterorhabditis spp. need bacteria of the species *Photorhabdus luminescens* to provide them with essential nutrients. In contrast to *Steinernema* spp., *Heterorhabditis* spp. cannot be cultured on media without symbiotic bacteria (Akhurst, 1986; Ehlers *et al.,* 1990; Lunau *et al.,* 1993). Not every *P. luminescens* strain can be used as a food source for *Heterorhabditis* spp. Han *et al.* (1990, 1991) and Gerritsen and Smits (1993) showed that some *Heterorhabditis* spp. are unable to reproduce on *P. luminescens* strains of other *Heterorhabditis* spp. The results presented here show that *H. megidis* strains HSH and HE are able to reproduce on each others primary form symbiont. When the nematodes have a choice to feed on their own symbiont or on the symbiont of the other nematode, they have no preference. They feed on both bacteria and also retain both bacteria in the guts of the infective juveniles. This implies that when these two nematode strains co-infect an insect, the infective juveniles produced in the insect can leave the cadaver with a mixture of symbionts. Most nematode strains carry a specific symbiont strain (Gerritsen *et al.,* 1995), suggesting that co-infection hardly ever occurs in nature.

Nematode reproduction is also influenced by form variation in *P. luminescens*. It is often stated that the secondary form does not support growth and reproduction of nematodes as well as the primary form does (Akhurst, 1980; Bedding, 1981; Akhurst & Boemare, 1990; Ehlers *et al.,* 1990). Results presented in this paper also show that both *H. megidis* strains are not able to reproduce on the secondary form of their own symbiont. Ehlers *et al.* (1990) suggested that the secondary form of *P. luminescens* produces a toxin that kills nematodes. They found a negative effect of *P. luminescens* secondary form on *Heterorhabditis* spp. but *Xenorhabdus* secondary form had no negative effect on *Steinernema* spp. Akhurst (1980) showed a negative effect of both *Xenorhabdus* and *Photorhabdus* secondary forms on nematode reproduction and he suggested a difference in nutrient supply by the two forms (Akhurst, 1993), arguing that Ehlers *et al.* (1990) used a rich medium that was nutritious enough to support *Steinernema* reproduction, thus masking any differences in nutrient supply by the bacteria. The results presented here show that the nematodes, when cultured on a mixture of primary and secondary form bacteria, survive, even when they are close to the secondary form bacterial lawn (Fig. 1). If the secondary form would produce a toxin, the nematodes would not survive right next to the secondary form colonies. Therefore, it is more plausible that the lack of nematode reproduction on secondary form bacteria is nutrient related. The nematodes feed on nutrients produced by the bacteria but also on the bacterial cells. The secondary form either does not produce the right nutrients or the nematode cannot use the secondary form cells as a food source. When the infective juveniles leave the agar plates at the end of the experiment, the bacterial lawn of the primary form bacteria is totally consumed. In a mixture of primary and secondary forms, only the primary form is eaten while most of the secondary form bacterial lawn is still there. It might be that the nematodes are not able to digest the secondary form cells. When the secondary form cells cannot be digested, the nematodes cannot use them as a food source and therefore cannot survive on a plate with just a secondary form bacterial lawn.

The low support of nematode reproduction is mentioned as a common characteristic of all secondary forms (Akhurst & Boemare, 1990). The results presented here show that, although both *H. megidis* strains are unable to reproduce on the secondary form of their own symbiont, *H. megidis* strain HSH is able to multiply on secondary form PE/2 of strain HE. This suggests that the negative effect of a secondary form bacterium on

nematode reproduction is not a common factor in all secondary forms. This can be explained if the secondary form is an adaptation of the symbiont to avoid digestion by its own nematode host. As a result, the nematodes cannot eat all the bacterial cells and leave the insect without the symbiont. Also, digestion of the symbiont inside the infective juvenile has to be avoided. This specific interaction between symbiont and host was developed during years of co-evolution, and might not be effective in another nematode host.

The nematodes retain the bacteria on which they have been cultured. When nematodes are cultured on a mixture of bacteria and when both bacteria are a good food source, then the nematodes carry both bacteria in equal amounts and they show no preference for their own symbiont. When cultured on both primary and secondary form, the infective juveniles carry both bacteria but not in equal amounts, the nematodes retain more primary than secondary form bacteria. Akhurst (1980) stated that *Steinernema* and *Heterorhabditis* spp. preferentially take up the primary form when cultured on a mixture of primary and secondary form, with the exception of one *H. heliothidis* strain. The results presented here show that, although the nematodes carry more primary form cells, they also carry secondary cells. It is likely that the nematodes do not distinguish between primary and secondary cells when they take up cells. Because the nematodes prefer the primary form as a food source they get in contact with primary form cells more and therefore carry more of these cells. HSH cultured on PE/2 did carry this secondary form in as large amounts as a primary form. This suggests that there is no preference for retaining a primary form, and the nematodes retain all bacteria they have been feeding on.

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