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Variation in pathogenicity of recombinations of *Heterorhabditis* and *Xenorhabdus luminescens* strains

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Summary – *Xenorhabdus luminescens* isolates were exchanged between four insect pathogenic nematode strains : two different *Heterorhabditis* isolates from the Netherlands and two different *Heterorhabditis bacteriophora* isolates. Of the sixteen possible combinations three combinations, of Dutch nematodes with bacteria of *H. bacteriophora,* were not able to grow and multiply. Of the thirteen combinations that could be propagated the four combinations of *H. bacteriophora* nematodes with bacteria from the Dutch nematodes were not pathogenic. When waxmoth larvae were injected with bacteria, the four non-pathogenic combinations were able to penetrate and multiply in these larvae. This showed that the lack of pathogenicity of these four combinations was due to the absence of bacteria. Both *H. bacteriophora* strains penetrated insect larvae in lower numbers than both Dutch *Heterorhabditis* strains, independent what bacterium they were carrying.

Résumé – ***Variabilité dans la pathogénie des combinaisons entre souches d'*Heterorhabditis *et de* Xenorhabdus luminescens** – Des isolats de *Xenorhabdus luminescens* ont été échangés entre quatre souches de nématodes entomopathogènes : deux isolats différents d'*Heterorhabditis* provenant des Pays-Bas et deux isolats différents d'*Heterorhabditis bacteriophora.* Parmi les seize combinaisons possibles, trois – nématodes néerlandais et bactéries provenant de *H. bacteriophora* – sont incapables de croître et de se multiplier. Parmi les treize combinaisons qui ont pu être élevées, les quatre combinaisons de nématodes provenant de *H. bacteriophora* et de bactéries provenant de nématodes néerlandais ne sont pas pathogènes. Si l'on injecte des bactéries dans les larves de *Galleria mellonella,* les quatre combinaisons non pathogènes sont capables de pénétrer à leur tour dans les larves et de s'y multiplier. Cela démontre que le manque de nocuité de ces quatre combinaisons est dû à l'absence de bactéries. Les deux souches de *H. bacteriophora* pénètrent en moins grand nombre dans les larves d'insectes que les deux souches néerlandaises d'*Heterorhabditis,* et ce indépendamment de la bactérie qu'elles transportent.

Key-words : Entomopathogenic nematodes, *Heterorhabditis, Xenorhabdus luminescens,* combination nematode-bacteria.

Insect pathogenic nematodes of the genera *Steinernema* and *Heterorhabditis* are symbiotically associated with bacteria of the genus *Xenorhabdus* (Thomas & Poinar, 1979). The infective dauerlarvae of the nematode carry the bacterial symbiont in the intestine. The nematode penetrates an insect host, moves into the haemocoel, and releases the bacterium. The bacterium starts multiplying and kills the host, helped by excretion products of the nematode that repress the immune system of the insect. *Xenorhabdus* further produces antibiotics to inhibit growth of other micro-organisms in the insect cadaver and provides nutrients utilized by the nematodes (Poinar & Thomas, 1966; Akhurst, 1982; Gerritsen *et al.,* 1992). Each *Steinernema* species is associated with its own *Xenorhabdus* species (Akhurst & Boemare, 1988). All *Heterorhabditis* spp. have *X. luminescens* as the symbiont, but the species *X. luminescens* is composed of several DNA homology groups which might also be considered separate species (Smits & Ehlers, 1991). Some of these DNA-homologous bacteria groups are associated with one *Heterorhabditis* species while other groups are associated with several *Heterorhabditis* species (Smits & Ehlers, 1991). Akhurst (1983) and Ehlers *et al.* (1990) showed that it is possible to grow *Steinernema* spp. on bacteria other than their own. Han *et al.* (1990) exchanged bacteria between *Heterorhabditis* spp. with the aim of separating *X. luminescens* subspecies. In our experiments the purpose was to make new combinations of *Heterorhabditis* and *Xenorhabdus* isolates and find possible differences in pathogenicity between these combinations. By combining the best nematode with the best bacterium the effectivity of the nematodes as biological control agent might be improved. This paper deals with the possibilities of exchanging bacteria between nematodes and the effect this has on the pathogenicity of these new combinations.

Materials and methods

NEMATODE AND BACTERIA ISOLATES

Table 1 lists the sources of the nematodes and bacteria used. Bacteria were isolated from the haemocoel of waxmoth larvae *(Galleria mellonella)* infected by nematodes. The bacteria were cultured on nutrient agar (Lab

Lemco, Oxoid) at 25 °C. Shaking-cultures of the bacteria were made by inoculating single colonies into 50 ml nutrient both (0.8 % Lab Lemco Broth, Oxoid), shaken at 120 rpm at 25 °C in the dark. To make sure that all bacterium strains were in the primary form, uptake of bromothymol blue (BTB) by *Xenorhabdus* strains was tested on nutrient agar with 0.004 % (w/v) triphenyltetrazolium chloride and 0.025 % (w/v) bromothymol blue (NBTA) (Akhurst, 1982). To estimate the mean celle size of the bacteria the length of 100 cells from several colonies was measured using a microscop with an ocular micrometer.

Table 1. Origin of *Heterorhabditis* spp. and *Xenorhabdus luminescens* strains.

Nematode species or strain	RFLP * group	Nematode code	Bacterial code	Original place of isolation
Heterorhabditis NLH-E87.3	1	HE	XE	Eindhoven, The Netherlands
Heterorhabditis NLH-F85	1	HF	XF	Flevoland, The Netherlands
H. bacteriophora	2	Hbac	Xbac	Brecon, S-Australia
Heterorhabditis H-mol	2	Hmol	Xmol	Moldavia, GIS

* RFLP groups of *Heterorhabditis*: 1. North-West-European (NWE) group; 2. *H. bacteriophora* group (from Smits and Ehlers, 1991).

Protein analysis

Protein patterns of whole cell extracts of *X. luminescens* were made on a 12 % sodium dodecyl sulfate polyacrylamide gel, following electrophoresis methods described by Sambrook *et al.* (1989). The cells used for total protein analyses were collected from a 3 to 4 days old shaking-culture.

Injection of bacteria

Waxmoth larvae were injected with 3 µl of a suspension of *Xenorhabdus* in Ringer solution (Oxoid). 1000, 100, 10 or 0 (control) bacterium cells per larva were injected with a 5 microlitre syringe (S.G.E.). The number of cells in a bacterium suspension was determined by counting cells in a Bürker-Türk counting chamber. The larvae were incubated in Petri dishes at 25 °C. Each treatment contained fifteen larvae and the experiment was done twice. The larvae were checked for mortality after 4 days.

Monoxenization of nematodes

The methode for axenization of the nematodes was modified from Lunau *et al.* (1993). First-generation hermaphrodite nematodes with eggs were dissected from waxmoth larvae that were infected with *Heterorhabditis* juveniles and incubated for 7 days at 25 °C. Sixty to 100 hermaphrodites were put into an embryodish with 0.4 M NaOH. After 20 min the hermaphrodites started to disintegrate. The hermaphrodites were sucked up several times in a Pasteur-pipet to disintegrate them further. After the eggs were settled on the bottom of the dish, the NaOH with the remains of the hermaphrodites was siphoned off with a pipet and fresh NaOH was added. This was repeated three or four times, until the remains of the hermaphrodites were completely removed. Then, 90 min after starting the whole procedure, the eggs were washed twice with sterile Ringer solution (Oxoid) and then once with YS-broth (0.5 g/l $NH_4H_2PO_4$, 0.5 g/l K_2HPO_4, 0.2 g/l $MgSO_4 \cdot 7H_2O$, 5 g/l NaCl, 5 g/l yeast extract). The eggs in YS-broth were then put into a glass Petri dish (5 cm) with YS-broth and incubated on a gently rocking shaker at 25 °C. After two days the eggs hatched. Fifty to 100 L1 larvae were put on a 4-day-old bacterial lawn on lipid agar (16 g/l nutrient broth (Bacto), 5 g/l corn oil, 12 g/l agar (Wouts, 1981) in a 5 cm Petri dish. The Petri dishes were incubated at 25 °C. The YS-broth, from which the hatched larvae were taken, was shaken for 4 more days and observed for bacterial contamination as a proof that the nematodes used earlier were axenic. The infective juveniles produced on the Petri dishes were stored in sterilized tap water in tissue culture-flasks at 5 °C (HE and HF) or 10 °C (*H. bacteriophora*). In the bioassay tests only nematodes that had been stored for 3 to 5 days were used.

Tests on pathogenicity and penetration of the combinations

Pathogenicity of the nematode/bacterium combinations was tested using a sand-column-bioassay. A cylindrical plastic container (diameter 36 mm, height 56 mm) with a waxmoth larvae *(Galleria mellonella)* at the bottom was filled with moist sand (heat-sterilized silver sand with 8 %, w/w, tap water). Thirty infective juveniles in 1 ml tap water were added to the sand surface; the container was capped and incubated at 25 °C. In a second series of experiments instead of 30 juveniles, one juvenile was put on the sand surface with a nematode fishing-rod (eyebrowhair glued to a bamboo stick). After 3 days the waxmoth larvae were removed from the sand and incubated separately at 25 °C for 4 days. Mortality was assessed. To assess the penetration rate of the nematodes the dead waxmoth larvae were dissected and the first-generation hermaphrodites were counted. Each treatment consisted of 30 containers and was done in three replicates.

To assess the penetration rate of the non-pathogenic combinations, the sand bioassay was also done with waxmoth larvae that were first injected with bacteria. The larvae were injected as described above. Per larva 1000 bacterium cells were injected, the *Xenorhabdus* strains injected corresponded with the *Xenorhabdus* strain in the combination tested (Table 5). Control larvae were injected with Ringer solution without bacteria.

Directly after injection the larvae were put into the plastic containers. The bioassay was carried out as described above, with 30 juveniles per container. As a control of the bacterial injection, containers without nematodes were incubated as well. Each treatment consisted of 30 containers and was done in two replicates. The results were statistically analyzed by analysis of variance. Pairwise comparisons were made by least-significant-difference-test (LSD).

Bacterial retention

To examine bacterial retention, 48 infective juveniles from a nematode/bacterium combination were surface sterilized (20 min, 0.4 % Hyamine 10X), individually transferred to a 24-wells tissue culture plate with 1 ml YS-broth per well, and incubated at 25 °C. After 21 days the wells were checked for bacterial growth. Only the combinations HEXE (combination of nematode HE and bacterium XE), HEXbac, HbacXbac, HbacXE, and HbacXF were tested.

Results

Bacteria

The four *Xenorhabdus* strains were all different from each other. Figure 1 shows that the four *Xenorhabdus* isolates differed in their protein pattern and differences were also found in RFLP-patterns of the four bacteria (Smits, unpubl.). There are more differences between the bacteria of the two nematode RFLP groups (Table 1) than within these groups. The strains differed in pigment production : XE and XF were red, Xbac was yellow and Xmol was orange. The length of the bacterium cells was measured : mean cell size of XE was 7.3 μm ± 2.6, of XF was 9.2 μm ± 4.2, of Xbac was 4.6 μm ± 1.3 and of Xmol was 7.3 μm ± 2.5. Only XF and Xbac differed significantly (LSD $\alpha = 0.01$). When injected into waxmoth larvae, the bacteria appeared equally pathogenic. All insect larvae were killed by as few as ten bacterial cells per larva. None of the control larvae, injected with Ringer solution, were killed.

Recombinants

The sixteen possible nematode/bacterium combinations can be separated into four groups (Tables 1, 2, 3, 4, 5); I : a group of NWE-nematodes with NWE-bacteria (HE and HF with XE and XF), II : a group of NWE-nematodes with *H. bacteriophora* bacteria (HE and HF with Xbac and Xmol), III : a group of *H. bacteriophora* nematodes with NWE-bacteria (Hbac and Hmol with XE and XF), and IV : a group of *H. bacteriophora* nematodes with *H. bacteriophora* bacteria (Hbac and Hmol with Xbac and Xmol). The combinations within these groups act mostly alike and in the remainder of this paper we shall refer to these groups. Three combinations in group II could not be made (tried five times or more). HE and HF were unable to develop on Xmol, and HF developed only once to females on Xbac but no infective juveniles developed (Table 2). The combination HEXbac is the exeption in group II, the only combination that can be made.

The time needed to develop infective juveniles (J3) differed between the NWE-group nematodes and the *H.*

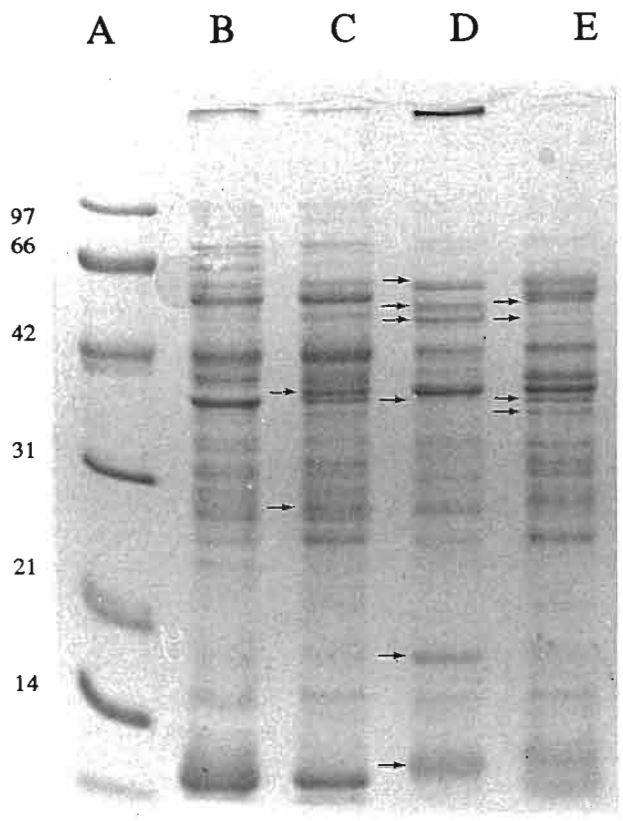

Fig. 1. Coomassie blue-stained SDS-polyacrylmide gel (12 %) of whole-cell extracts of *Xenorhabdus luminescens* isolates. A : Molecular weight standard with values in kD; B : XE; C : XF; D : Xbac; E : Xmol (Arrows mark position of differences in protein bands between isolates).

Table 2. Ability of *Heterorhabditis* spp. to grow and multiply on different *Xenorhabdus* isolates.

Nematode	Bacterium			
	XE	XF	Xbac	Xmol
HE	+	+	+	–
HF	+	+	–	–
Hbac	+	+	+	+
Hmol	+	+	+	+

+ : combination possible; – : combination not possible.

bacteriophora nematodes. All compatible HE and HF combinations (group I and HEXbac) produced infective juveniles after about 20 days. All Hbac and Hmol combinations (group III and IV), except for HbacXmol and HmolXmol, produced infective juveniles after about 14 days. Hbac and Hmol grown on Xmol produced juveniles after about 20-25 days, because only a few of the J1 or J3 juveniles put on Xmol bacterium plates developed into females, the others died. Also a lot of the second-generation juveniles, coming out of the eggs, died. Therefore several generations of HbacXmol and HmolXmol had to develop before the nematodes started to produce infective juveniles. When produced in waxmoth larvae all Hbac and Hmol combinations, also these with Xmol, produced J3 faster then the HE and HF combinations.

PATHOGENICITY

Table 3 shows the pathogenicity in the bioassays with one nematode per insect. The results show that one nematode by itself is not always able to find or kill the insect. The combinations in groups I, II and IV killed 20 % to 40 % of the insects. The differences between these combinations are not significant (LSD $\alpha = 0.01$). The four combinations of *H. bacteriophora* nematodes with bacteria of the NWE group (group III) were not able to kill insects at all. This is confirmed in the bioassay with 30 nematodes per waxmoth larvae (Table 4). Table 4 shows that 30 nematodes are almost always (90 %-100 %) able to kill an insect, but in the case of the four combinations in group III only 0 % to 4 % of the insects were killed. XE was isolated out of the four insects killed by HmolXE and XF was isolated out of the only insect killed by HbacXF. This means that these insects were actually killed by the combinations HmolXE and HbacXF and not by Hbac and Hmol " contaminated " with their own symbionts (e.i. HmolXmol or Hbac-Xbac). The bioassay was also done with 100 nematodes per larva and the four combinations in group III were still not able to kill more than 5 % of the insects (results not shown).

Table 3. Mortality (% ± s.d.) of *Galleria mellonella* larvae exposed to different nematode/bacterium combinations in bioassays with one nematode per larva.

Nematode	Bacterium			
	XE	XF	Xbac	Xmol
HE	41 ± 10.3	19 ± 8.3	38 ± 4.0	–
HF	43 ± 23.0	22 ± 8.3	–	–
Hbac	0	0	32 ± 8.0	31 ± 14.0
Hmol	0	0	15 ± 5.0	21 ± 7.7

– : combination not possible.

Table 4. Mortality (% ± s.d.) of *Galleria mellonella* larvae exposed to different nematode/bacterium combinations in bioassays with 30 nematodes per larva.

Nematode	Bacterium			
	XE	XF	Xbac	Xmol
HE	100 ± 0	90 ± 14.6	100 ± 0	–
HF	100 ± 0	99 ± 1.9	–	–
Hbac	0 ± 0	1 ± 1.9	93 ± 6.6	97 ± 3.9
Hmol	4 ± 1.9	0 ± 0	88 ± 13.5	97 ± 5.8

– : combination not possible.

Table 5 shows the penetration rate of the nematodes. Since it was not possible to find the juveniles right after penetration of the insect, the penetration was assessed after the juveniles had developed into first generation hermaphrodites. Analysis of variance showed that the penetration rate of all *H. bacteriophora* combinations (groups III and IV) at 25 °C is significantly lower than the penetration rate of the HE and HF combinations (group I and HEXbac). LSD-test showed that the *H. bacteriophora* combinations did not significantly differ in penetration from each other and also the HE and HF combinations did not significantly differ from each other.

Table 5. Penetration of J3 juveniles (% J3 penetrating ± s.d.) of different nematode/bacterium combinations in *Galleria mellonella* larvae in bioassay with 30 nematodes per lava.

Nematode	Bacterium			
	XE	XF	Xbac	Xmol
HE	66^{a} * ± 23.0	61^{a} ± 20.0	56^{a} ± 20.2	–
HF	61^{a} ± 19.6	64^{a} ± 22.8	–	–
Hbac	?	(13) **	13^{b} ± 9.2	20^{b} ± 11.4
Hmol	(28)	?	25^{b} ± 25.2	25^{b} ± 14.9

* : LSD-test : a is significantly different from b (LSD-value : 28.4).
** : numbers between brackets are means of less than five insects.
– : combination not posible.
? : not able to assess penetration.

It was not possible to assess penetration of the non-pathogenic combinations (group III) this way, because they did not kill the insects and the nematodes could not develop into hermaphrodites in living insects. Only in the few insects that were killed (1 by HbacXF, 4 by HmolXE) the hermaphrodites were counted (Table 5). The theory that the non-pathogenic combinations were

able to penetrate the insect but could not kill the insect because they did not carry bacteria, was tested by injection of bacteria in waxmoth larvae before exposing the waxmoth larvae in the bioassay. Injection of the bacterium killed all insects, also in control containers without nematodes. In this way it was possible to assess penetration of the non-pathogenic combinations in these insects. Table 6 shows that the penetration of the non-pathogenic Hbac-combinations is equal to the penetration of the pathogenic Hbac combinations. The same experiment was done for the Hmol-combinations (only ten containers, no replicates) and showed the same penetration rate (about 15 %).

Table 6. Percentage J3 juveniles that have penetrated the *Galleria mellonella* larvae after injection with bacterium.

Nematode	Bacterium	Injection	% J3 penetrated ± s.d.
Hbac	Xbac	Xbac	12.8 ± 10.1
Hbac	Xbac	Ringer	13.3 ± 8.8
Hbac	XE	XE	14.4 ± 10.3
Hbac	XE	Ringer	(15) *
Hbac	XF	XF	21.1 ± 11.4
Hbac	XF	Ringer	(14) *

* : numbers between brackets are means of less than five insects.

Bacterial retention

Bacterial retention of two combinations of group III was tested. As controls one nematode/bacterium-combination of each of the other three groups was also tested. The results are shown in Table 7. The wells with the nematodes Hbac and HE with their own *Xenorhabdus* symbiont (HbacXbac, group IV and HEXE, group I), showed about 90 % bacterial growth. Only 48 % of the wells with the combination HEXbac (group II) showed bacterial growth. The non-pathogenic combinations of Hbac nematodes with XE or XF bacteria (group III) did not release any bacteria. An interesting observation was that the nematodes did not release the bacteria immediately after they had been transferred to the YS-broth. The first bacteria started to grow after 3 days but in some wells bacterial growth was seen only after 21 days. In all wells with bacterial growth the nematodes were dead. It was not clear whether the nematodes died because of the bacterial growth (lack of oxygen) or that the bacteria were only released after the nematode had died. The experiment was stopped after 21 days when most wells without bacteria contained dead nematodes with a hyaline intestine.

Table 7. Percentage of infective juveniles of different nematode/bacterium combinations that release their bacteria.

Nematode	Bacterium		
	XE	XF	Xbac
HE	85.4	n	47.9
Hbac	0	0	91.7

n = not tested.

Discussion

The results of the experiments show that it is possible to make new nematode/bacterium combinations by exchanging *Xenorhabdus* strains between *Heterorhabditis* strains. However, not all combinations could be made. *Heterorhabditis* strains HE and HF are not able to grow on Xmol and HF is not able to grow on Xbac. This confirms the results of Han *et al.* (1990). When Han *et al.* (1990) exchanged bacteria between *Heterorhabditis* strains, they also found that some combinations were not successful. *H. megidis*, which is related to the nematodes of the NWE group (Smits *et al.*, 1991), was able to develop on some Chinese *Xenorhabdus* strains but not on others. All the Chinese *Heterorhabditis* strains, on the other hand, were able to develop on the *H. megidis* bacterium. This suggests that the bacteria of *H. megidis* and the NWE-group seem to be a " universal " food source on which all other nematodes can grow. The nematodes of the *H. megidis* and NWE-group on the other hand, are very specific in their choice of bacteria.

Both *H. bacteriophora* nematodes develop very slowly on agar plates with Xmol. In *G. mellonella* larvae, however, HbacXmol and HmolXmol develop equally to other Hbac and Hmol combinations. This suggests that Xmol is not able to produce optimal nutrients for the nematode on lipid-agar plates, and may be the reason why HE and HF are unable to develop on Xmol.

The bacteria differ in several characters and in their protein patterns (Fig. 1). Smits and Ehlers (1991) show that the bacteria are also genetically different. They show differences in hybridisation of XE, XF and Xmol with oligonucleotide probes. DNA-fingerprints of the four isolates also shows differences (Smits, unpubl.). The bacteria, although they differ in protein and DNA pattern, did not differ in pathogenicity towards *G. mellonella*. Therefore it is not possible to see what part of the pathogenicity is due to the bacterium and what part to the nematode. In these experiments there is no significant difference in pathogenicity between the combinations that are pathogenic. Han *et al.* (1991) exchanged bacteria between *Heterorhabditis* strains that do differ in pathogenicity. They show that by exchanging bacteria between *Heterorhabditis* strains the pathogenicity of

these strains can increase or decrease dependant on the bacteria they have developed on.

Although the *H. bacteriophora* combinations penetrate the insect with a lower frequency than the HE and HF combinations, the pathogenicity is not affected by this. This is probably due to the high susceptibility of the waxmoth larvae. One nematode is able to kill a waxmoth larva on its own (Table 3). Commercially these nematodes are used to control insects like black vine weevil and grubs (Klein, 1990). These insects are less susceptible than waxmoth larvae and may need more then one nematode to kill them (Smits, 1992). The difference in penetration-rate may have an effect on the pathogenicity of the nematodes against these other insects.

All four combinations of *H. bacteriophora* nematodes with bacteria of the NWE-group are not pathogenic. It is apparent that these nematodes are able to penetrate the insect but the insect is not killed (Table 6). The penetration of the four non-pathogenic combinations (group III) is equal to the penetration of the other Hbac and Hmol combinations (group IV). When the bacterium is injected into the insect prior to the penetration of the nematode, the latter is able to grow and multiply in the insect. This shows that the bacterium is the limiting factor in the pathogenicity of these four combinations. The nematodes either do not carry the bacterium in their guts or they are not able to release the bacterium. The results of the retention experiment confirm this conclusion; the combinations of Hbac with the bacteria XE and XF do not release any bacteria to the medium. There can be several reasons for the incompatibility of the nematode to take-up or release bacteria. The difference in cell size of the bacteria seemed to be a possible reason for not taking-up the bacterium since the infective juveniles of the NWE-group are bigger than the infective juveniles of *H. bacteriophora*. However, Xmol and XE are the same size, so the cell size of the bacterium can not be the reason why the nematodes are not able to take up the bacteria. It is possible that the bacteria are not able to attach to the guts of the nematode or the signals between the nematode and bacterium to either take up or release the bacterium are not compatible. Of the pathogenic combination HEXbac less than 50 % of the nematodes carried a symbiont, showing that the combination with this *Xenorhabdus* isolate is not as compatible as the combination with its own symbiont. The few insects that are killed by HbacXF and HmolXE were probably killed because one of the nematodes carried some bacteria by chance, either in their guts or in between the double skin of the nematode. Han *et al.* (1990) show that some *Heterorhabditis* spp. grown on the symbiont of *H. megidis* do not carry bacteria, while normally over 90 % of these nematodes carry their symbiont. These nematodes without bacteria are also not pathogenic (Han *et al.*, 1991). Akhurst (1983) and Dunphy *et al.* (1985) show that *Steinernema* spp. that are able to grow on *Xenorhabdus* symbionts of other *Steinernema* spp. often do not take up these bacteria in their intestine. This shows that the uptake of bacteria by *Heterorhabditis* and *Steinernema* is more specific than development on these bacteria. Although *Heterorhabditis* spp. do not have a vesicle like *Steinernema* spp. (Bird & Akhurst, 1983) they are able to discriminate between bacteria, and only take up their own symbiont or a related *Xenorhabdus* strain.

The pathogenicity of axenic *S. glaseri* and *S. glaseri* on *X. bovienii* is less than *S. glaseri* with its own symbiont (Akhurst, 1986; Dunphy *et al.*, 1985). Ehlers *et al.* (1990) show that axenic and monoxenic *S. carpocapsae* and *Steinernema* spp. NC513 are equally pathogenic. *H. bacteriophora* on the other hand, is not pathogenic at all without *Xenorhabdus*.

It has not been possible to make axenic *Heterorhabditis* nematodes the way axenic steinernematid nematodes are produced (Lunau *et al.*, 1993). Since *H. bacteriophora* infective juveniles do not carry bacteria in their guts when grown on NWE-group bacteria, these nematodes are axenic after surface sterilization. With these nematodes it is possible to do tests with axenic *Heterorhabditis* strains. Here, for the first time, it is shown that *Heterorhabditis* spp. are not able to kill an insect without their *Xenorhabdus* symbiont. These axenic nematodes also offer possibilities for separate cryopreservation of axenic juveniles and their symbionts. This way nematodes and bacteria are stored separately and can be recombined whenever they are needed.

The new combinations made in these experiments did not show an increased pathogenicity. This is probably due to the lack of difference in pathogenicity between the original strains and the choice of waxmoth larvae as target insect. To improve the efficacy, selections should be made of nematode strains and bacterium strains. The strains can be selected for specific characters, for instance cold activity or high pathogenicity to specific insects in specific habitats. Combining these selected nematodes and bacteria might give new and improved combinations that are better suited for biological control of insects.

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