Development of *Heterorhabditis* spp. strains as characteristics of possible *Xenorhabdus luminescens* subspecies

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SUMMARY

Seven axenic *Heterorhabditis* strains were mutually recombined with their *Xenorhabdus luminescens* symbionts. Most strains, to a degree, developed successfully on four or five of the *X. luminescens* isolates to which they were exposed. None of the strains developed on all isolates. Those that developed successfully were maintained on Bedding's sponge medium and on *Galleria mellonella* larvae. On the basis of acceptability as food source by the different nematode populations the *X. luminescens* isolates could be placed in four groups : Group I contains the symbiont of *H. megidis,* which supports development of all *Heterorhabditis* strains tested; Group II with isolates supporting all nematode strains except *H. megidis,* Group III with isolates supporting some *Heterorhabditis* strains but not others and Group IV with isolates supporting only *Heterorhabditis* strains not supported by Group III isolates. In the successfully developing combinations, the percentage of infectives containing bacteria varied and was generally highest for bacteria belonging to the same group as its own symbiont.

RÉSUMÉ

*Le développement de souches d'*Heterorhabditis *spp.*
comme caractéristique d'éventuelles sous-espèces de Xenorhabdus luminescens

Sept souches axéniques d'*Heterorhabditis* ont été recombinées avec leur symbionte, *Xenorhabdus luminescens.* La plupart des souches se développent favorablement sur quatre ou cinq des isolats de *X. luminescens* auxquels elles ont été confrontées. Aucune des souches ne se développe sur tous les isolats. Celles ayant un développement satisfaisant ont été maintenues sur le milieu « éponge » de Bedding ou sur des larves de *Galleria mellonella.* En se fondant sur leur acceptabilité comme source nutritive par les différentes populations du nématode, les isolats de *X. luminescens* peuvent être classés en quatre groupes; le groupe I comprend le symbionte de *H. megidis* lequel permet le développement de toutes les souches de *Heterorhabditis* testées; le groupe II comprend les isolats compatibles avec toutes les souches de nématodes à l'exception de *H. megidis;* le groupe III comprend les isolats compatibles avec quelques souches d'*Heterorhabditis;* et le groupe IV les isolats compatibles avec les souches d'*Heterorhabditis* non compatibles avec les isolats du groupe III. Lors des recombinaisons réussies, le pourcentage de larves infestantes contenant des bactéries est variable; il est également plus élevé si la bactérie appartient au même groupe que ses propres symbiontes.

The symbiotic association between entomogenous nematodes of the families Heterorhabditidae and Steinernematidae and the bacteria of the genus *Xenorhabdus* (Enterobacteriaceae) in their intestines has been recognized by many workers (Poinar & Thomas, 1966; Akhurst, 1983*a;* Dunphy, Rutherford & Webster, 1985; Poinar, Jackson & Klein, 1987). The vectorial and protective role of the nematode (Thomas & Poinar, 1979; Gotz, Boman & Boman, 1981; Burman, 1982; Boemare, 1983) and the antibiotic (Poinar, Hess & Thomas, 1980; Paul *et al.,* 1981; Akhurst, 1982), pathogenic (Akhurst, 1980; Dunphy & Webster, 1988) and nutritive (Poinar & Thomas, 1966; Bedding, 1981; Wouts, 1981) functions of the bacteria show that this association plays an important part in the reproduction and pathogenicity of this group of nematodes.

Two species were originally described in the genus *Xenorhabdus : X. nematophilus* (symbiotic with *Steinernema = Neoaplectana* species; Wouts *et al.,* 1982) and *X. luminescens* (symbiotic with *Heterorhabditis* species) (Thomas & Poinar, 1979). Pigment production, luminescence, and catalase production distinguish the two species. Each bacterial species occurs in two forms (designated as primary form and secondary form) which differ in differential pigmentation, absorption of bromothymol blue and neutral red from indicator agar media and the presence of lecithinase and antimicrobial activity (Akhurst, 1980, 1982, 1986; Akhurst & Boemare, 1988). The primary form further provides a better medium for the reproduction of nematodes (Akhurst, 1980; Bedding, 1981). Akhurst (1983*b,* 1986) recognized four subspecies of *Xenorhabdus nematophilus,* which he

named *nematophilus, bovienii, poinarii* and *beddingii*, for the symbionts of *S. feltiae* (syn. *N. carpocapsae;* Wouts *et al.*, 1982), *S. bibionis, S. glaseri* and two undescribed *Steinernema* species, respectively. Recently, these subspecies were elevated to species status (Akhurst & Boemare, 1988).

Xenorhabdus luminescens was first isolated by Khan and Brooks (1977) from *Heterorhabditis* (= *Chromonema*) *heliothidis* and by Poinar, Thomas and Hess (1977) from *H. bacteriophora.* Khan and Brooks (1977) described the colonies on nutrient agar (NA) as cream to orange-red in colour, and Poinar (1977) described them as yellow-brown. Both bacterial isolates produce bioluminescence and change the colour of the infected host to deep red. Wouts (1979) observed that the symbiont of a New Zealand population of *H. heliothidis* changes the colour of the infected host to yellow or pale orange. Akhurst and Boemare (1986) reported a non-luminescent strain of *X. luminescens* associated with *Heterorhabditis* sp. These differences reported between *Xenorhabdus luminescens* isolates, together with the isozyme results (Hotchkin & Kaya, 1984), DNA-DNA hybridization evidence (Grimont *et al.*, 1984) and phylogenetic distances (Ehlers, Wyss & Stackebrandt, 1988) suggest that like *X. nematophilus, X. luminescens* may represent several subspecies (or species).

To further study the existence of *X. luminescens* subspecies, axenic *Heterorhabditis* spp. strains were combined with each other's original *X. luminescens* symbiont to establish differences in nematode development of the resulting combinations as characters of such subspecies.

Materials and methods

Bacteria

Heterorhabditis populations from which bacterial strains of *X. luminescens* were isolated are listed in Table 1. The isolates were obtained from insect blood from the haemocoel of *Galleria mellonella* larvae 48 h infected with infective *Heterorhabditis* juveniles. Bacteria were cultured and tested at 20 °C. Stock cultures were maintained on NBTA (nutrient agar plates with 0.0025 % (w/v) bromothymol blue and 0.004 % (w/v) triphenyltetrazolium chloride; Akhurst, 1980*c*) at 12 °C and subcultured monthly. The primary form of the bacteria was obtained by selecting green or blue-green colonies on NBTA and repeated subculturing.

Table 1

Sources of nematodes and bacteria

Bacterium (*Xenorhabdus*)	*Heterorhabditis* host species and strain	Origin
X. luminescens H06 (X1H06)	*H.* sp. H06	Shandong, China (J. Wang)
X. luminescens H3 (X1H3)	*H.* sp. H3	Hailing, China (Y. Wang)
X. luminescens G12 (X1G12)	*H.* sp. G12	Hainan, China (R. Han)
X. luminescens Q6 (X1Q6)	*H.* sp. Q6	Fujian, China (J. Liu)
X. luminescens V16 (X1V16)	*H.* sp. V16	CSIRO, Australia (R. A. Bedding)
X. luminescens HNA (X1HNA)	*H. megidis* HNA	MAFTech, New Zealand (T. Jackson)
X. luminescens HNZ (X1HNZ)	*H. heliothidis* HNZ	DSIR, New Zealand (W. M. Wouts)

Development of successful nematode/bacterium associations

To obtain monoxenic *Heterorhabditis* cultures, infective juveniles collected from infected *G. mellonella,* were gravity washed three times with sterile M9-buffer (Brenner, 1974), surface-disinfected in 0.1 % merthiolate for 2 h, washed with sterile M9-buffer, and placed on 24 h old bacterial lawns on nutrient agar (NA) plates. Adult females developed within 5 days, they were washed twice with sterile M9-buffer, surface-disinfected in 0.1 % merthiolate for 30 min, submerged for 3 h in antibiotic solution containing about 7000 units streptomycin, ampicillin and penicillin per millilitre distilled water, washed in sterile M9-buffer, transferred to 0.1 % merthiolate for 30 min and washed in sterile M9-buffer. Of each strain about five to ten axenic females were placed on bacterial lawns on fortified lipid agar (1.6 % nutrient broth, 1.2 % agar and 1 % corn oil; Wouts, 1981) of each of the symbionts tested. Control plates had no bacterial lawn. Gravid females developing on the bacterial lawns were transformed to fresh plates. There were six replicates of each nematode/bacterium combination.

The combination of a *Heterorhabditis* population with a *Xenorhabdus* strain was considered a successful nematode/bacterium association if nematode development continued after three biweekly subcultures onto new bacterial lawns of the same *Xenorhabdus* strain on fortified lipid agar. When a nematode/bacterium combination did not develop successfully, at least four repeat attempts were made. Before and after each subculture, monoxeny and bacterial form were tested on NBTA, NA and fortified lipid agar plates. Successful nematode/bacterium associations were also reared in sponge cultures (Bedding, 1981; Wouts, 1981) with the medium consisting of 1 % peptone, 1 % beef extract, 20 % eggs, 15 % soya flour, 5 % corn oil, 8 % polyether polyurethane sponge, and 50 % distilled water (w/w) (modified from Li, Liang and Zhu, 1987) and in *G. mellonella* larvae. For production in *G. mellonella* larvae, six instars from Biosuppliers, Auckland, New Zealand, weighing 179 ± 7.8 mg, were infected by exposure to infective juveniles from fortified lipid agar cultures, on damp filter paper. Developing infectives were extracted from the cadaver on a White water trap.

BACTERIAL RETENTION

To examine bacterial retention approximately 80 infectives (two subsamples) from each successful nematode/bacterium combination reared in *Galleria* larvae were surface-sterilized, individually transferred to a drop of sterile water on a microcospe slide, and the cuticle cut near the anterior end with an oculist's scalpel. After extrusion of the foregut and treatment with 0.5 % methyl violet 6 B, the proportion of the infectives containing bacteria was determined. The data were analysed by Chi-square test.

Results

ESTABLISHMENT OF NEMATODE/BACTERIUM COMBINATIONS

Nematode/bacterium combinations tested are shown in Table 2. Four groups were clearly delineated in *X. luminescens* isolates. Group I contains the symbiont of *H. megidis* HNA (Poinar, Jackson & Klein, 1987). It seems to be the most universally acceptable medium for nematode development. All strains tested were able to reproduce on it. Group II contains X1H06 and supports all nematode strains except *H. megidis* HNA. Group III consists of symbionts that support each other's nematode strain and includes X1G12 and X1H3 and Group IV contains X1HNZ, X1Q6 and X1V16 symbionts that support all nematode strains except those of Group III.

Table 2

Monoxenic *in vitro* cultures of various combinations of *Heterorhabditis* spp. and *Xenorhabdus luminescens*

Nematode	Combinations maintained in lipid agar							
	Bacterium							
	X1HNA	X1H06	X1G12	X1H3	X1HNZ	X1Q6	X1V16	None
H. megidis HNA	+	–	–	–	+	+	+	–
Heterorhabditis								
sp. H06	+	+	–	–	+	+	+	–
sp. G12	+	+	+	+	–	–	–	–
sp. H3	+	+	+	+	–	–	–	–
H. heliothidis								
HNZ	+	+	–	–	+	+	+	–
Heterorhabditis								
sp. Q6	+	+	–	–	+	+	+	–
sp. V16	+	+	–	–	+	+	+	–

+ = Nematode developed in this combination.
– = Nematode did not develop in this combination.

BACTERIAL RETENTION

Percentage of infectives containing bacteria, for each nematode population tested, varied with different bacterial groups ($p < 0.01$) but showed no significant difference between natural combinations ($\chi^2 = 5.005$, $p > 0.05$) (Tab. 3).

The first test requires that there is no heterogeneity in the data. This can be checked by totalling the Chi-square statistics for the two subsamples for each combination, except those in which almost none or almost 100 % of the infectives contain bacteria. For the six degrees of freedom in the test $\chi^2 = 3.2$. A value greater than 6 would be required to expect heterogeneity.

Table 3

The percentage of the infectives with bacteria in *in vivo* culture of various combinations of *Heterorhabditis* spp. and *Xenorhabdus luminescens*

Nematode strain	Bacterium				
	X1HNA	X1H06	X1G12	X1H3	X1HNZ
H. megidis	94 (3.9)[a]	–[b]	–	–	41 (3.4)
Heterorhabditis					
sp. H06	0	98 (1.4)	–	–	83 (4.0)
sp. G12	11 (1.1)	0	92 (1.3)	98 (0.1)	–
sp. H3	27 (1.8)	n[c]	94 (3.9)	94 (0.9)	–
H. heliothidis					
HNZ	1 (0.01)	88 (2.3)	–	–	93 (2.3)

[a] Percentage with (SE).
[b] Not successful combination. See Table 2.
[c] n = No infectives available.

Discussion

Seven *X. luminescens* strains tested in this study showed difference in their suitability as a medium for a particular nematode population. Without accompanying bacteria the nematode was unable to reproduce. It is worth noting that the non-luminescent strain X1H3 and pale pigment-producing strain X1G12 of *X. luminescens* behave as one and the same isolate both being compatible with the same nematode strains and showing the same bacterial retention by infectives.

Steinernema spp. cannot reproduce on *X. luminescens* (Akhurst, 1983 *a*), but they can reproduce on a variety of bacterial species not belonging to the genus *Xenorhabdus* (Poinar & Thomas, 1966; Boemare, 1983; Boemare *et al.*, 1983; Ehlers & Stoessel, 1988). The association between *Heterorhabdus* spp. and *X. luminescens* is much more strict. On the basis of *Heterorhabditis* specificity, *X. luminescens* isolates can be distinguished into

four groups. Group I isolate supports all *Heterorhabditis* strains tested, Group II isolate supports all strains except *H. megidis* HNA, Group III isolates support some strains but not others and Group IV supports strains not supported by bacteria of Group III. These data, in conjunction with the spectrum of antibiotic activity of *X. luminescens* isolates (Han, unpubl.), indicate that *X. luminescens* subspecies may exist. Further research is in progress to compare these data with a bacteriological study of these symbionts.

Except for X1HNA+H06 (Strain H06 nematodes with X1HNA bacteria) and X1H06+H3 (Strain H3 nematodes with X1H06 bacteria), combinations maintained successfully in *Galleria* larvae through several subcultures did not lose viability, which indicates that artificial nematode-bacterium combinations will be active in nature and can survive in the field.

For each nematode population, the percentage of infectives containing bacteria in *in vivo* cultures varied with the *X. luminescens* group. The infectives are much better able to carry bacterial isolates from the group their own symbiont belongs to than from other bacterial group. Akhurst and Boemare (1988) observed a close relationship between the taxonomic grouping of the bacteria and that of their nematode hosts. Whether the different groups of *X. luminescens* also reflect a specific difference in nematode strains requires further study.

The mechanism that determines the nematode-bacterium association has not been established. It seems that the metabolic products of the bacteria, the nutrients transformed by them in the culture media, as well as the evolution of nematode-bacterium relationship are involved. The understanding of how nematodes retain their specific bacteria would be very useful for selecting nematode-bacterium combination with high growth and bacterial retention rates that would be more successful in mass production and more effective against an insect pest.

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