The influence of phase variants of *Xenorhabdus* spp. and *Escherichia coli* (Enterobacteriaceae) on the propagation of entomopathogenic nematodes of the genera *Steinernema* and *Heterorhabditis*

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SUMMARY

To quantify the assumed negative influence of phase II *Xenorhabdus* spp. on the propagation of entomopathogenic *Steinernema* and *Heterorhabditis* nematodes, the *in vitro* and *in vivo* reproduction potentials of five nematode species (strains) were determined. Bacteria-free *S. carpocapsae* (DD-136) were fed with phase variants (primary and secondary form) of *X. nematophilus* and with *Escherichia coli*. The growth profiles, determined by counting nematodes in three day intervals from cultures started with one female nematode, showed no significant differences between the reproduction potentials on phase I or II or *E. coli* cultures. Growth on *E. coli* resulted, however, in a delay of nematode development by about 6 days. Phase variants of *X. bovienii* and *X. poinarii* did not significantly alter the number of off-springs per female, final nematode yields, the generation time or the body length of the females of *S. bibionis* (OBS III) and *Steinernema* sp. (NC 513), respectively. The same results were obtained with *H. heliothidis* (NC-1 [HH]) and *Heterorhabditis* sp. (NZ). Phase II variants caused, however, a higher nematode mortality than phase I *X. luminescens*. The phase I bacterium of strain NZ stayed stable for 6 months. A stable phase II could only be obtained after prolonged subculturing for 11 months and resulted in an orange and a yellow pigmented clone. Nematode development was totally inhibited on the yellow pigmented variant. Percent mortality of last instar *Galleria mellonella* larvae did not differ when axenic or phase I or II bearing *S. carpocapsae* and *Steinernema* sp. (NC 513) dauer larvae were used. The propagation of phase II symbiont carrying nematodes was always lower. The reasons for the different nematode yields obtained from *in vitro* and *in vivo* cultures are discussed.

RÉSUMÉ

Influence des variants de phase de Xenorhabdus *spp. et* Escherichia coli *(Enterobacteriaceae) sur la propagation des nématodes entomopathogènes des genres* Steinernema *et* Heterorhabditis

Pour quantifier l'influence présumée négative des *Xenorhabdus* spp. en phase II sur la propagation des nématodes entomopathogènes *Steinernema* et *Heterorhabditis*, le potentiel reproductif, *in vitro* et *in vivo*, de cinq espèces (souches) de nématodes a été déterminé. Des *S. carpocapsae* (DD 136) sans bactéries ont été nourris avec des variants de phase (formes primaire et secondaire) de *X. nematophilus* et avec *Escherichia coli*. La croissance des élevages — déterminée en comptant les nématodes tous les trois jours à partir du début d'un élevage issu d'une seule femelle — ne montre pas de différences significatives dans le potentiel reproductif entre élevages sur phases I ou II ou sur *E. coli*. Cependant, la croissance sur *E. coli* provoque un retard d'environ six jours dans le développement du nématode. Des variants de phase de *X. bovieni* et *X. poinardii* ne modifient pas significativement le nombre de descendants par femelle, la récolte finale en nématodes, le temps de génération ou la longueur du corps des femelles appartenant, respectivement, à *S. bibionis* (OBS III) et *Steinernema* sp. (NC 513). Des résultats identiques ont été obtenus avec *H. heliotidis* (NC-1 [HH]) et *Heterorhabditis* sp. (NZ). Cependant, les variants en phase II de *X. luminescens* provoquent une mortalité plus élevée que celle causée par les variants en phase I. La bactérie en phase I de la souche NZ demeure stable pendant six mois. Des phases II stables ne peuvent être obtenues qu'après des repiquages prolongés pendant onze mois, et produisent un clône pigmenté en orange et un clône pigmenté en jaune. Le développement du nématode est totalement inhibé par le clône à pigment jaune. Le pourcentage de mortalité des larves de dernier stade de *Galleria mellonella* ne diffère pas que l'on utilise des larves (L3) de *S. carpocapsae* et *Steinernema* sp. (NC 513) axéniques ou associées à des phases I ou II de *Xenorhabdus*. La propagation des nématodes associés au symbionte de phase II est toujours plus faible. Les raisons des différences de récolte observées dans les élevages *in vivo* et *in vitro* sont discutées.

Xenorhabdus spp., bacteria mutualistically associated with entomopathogenic nematodes of the genera *Steinernema** (syn. *Neoaplectana* Wouts *et al.*, 1982) and *Heterorhabditis*, tend to produce two phase variants, designated as primary form (phase I) and secondary form (phase II). The phase I represents the wild type and is usually isolated from nematode dauer larvae (Akhurst, 1980), which contain cells of their specific symbionts in a vesicle in the ventricular portion of the intestine (Bird & Akhurst, 1983). The wild type is unstable so that the secondary phase arises after several subcultures. The two phase variants can be distinguished by colony morphology, absorption of dyes from agar media and some biochemical characteristics (Akhurst, 1980; Boemare & Akhurst, 1988). The primary phase, but not the secondary, produces antibiotic compounds that inhibit the growth of a wide variety of micro-organisms (Paul *et al.*, 1981; Akhurst, 1982). Both phases are equally pathogenic when injected into the haemolymph of *Galleria mellonella* larvae (Akhurst, 1980, 1982; Dunphy & Webster, 1984; Boemare & Akhurst, 1988), although the reaction of the haemocytes towards the two forms is different. Both phases of *Xenorhabdus nematophilus* adhere to the haemocytes of *G. mellonella*, but addition of haemolymph increases the level of secondary form adhesion. Injection of the primary form causes a transient haemocytopenia followed by an increase of haemocytes, whereas the secondary form induces pronounced haemocytopenia and a sharp decline of haemocyte counts after a short period of increase (Dunphy & Webster, 1984). When either the primary or secondary phase of *X. nematophilus* is injected into *Galleria mellonella* larvae together with axenic *Steinernema carpocapsae*, the nematodes produce more off-springs in the presence of the primary phase. Nematodes mature and reproduce more rapidly and female nematodes are longer (Akhurst, 1980). The primary form is also claimed to be superior to the secondary form in its ability to support nematode propagation in monoxenic *in vitro* cultures for the mass-production of the nematodes (Bedding, 1981, 1984).

As the nematode-bacteria complex shows a great potential for the biological control of noxious insects in cryptic environments (*e.g.* Kaya, 1985), low'cost *in vitro* production is a prerequisite to expand the application of the antagonists in pest control. In order to improve nematode yields from monoxenic cultures, the present study examines the effect of the primary and secondary phase on nematode reproduction. It also considers a possible substitution of the symbiotic bacterium of *S. carpocapsae* by *Escherichia coli*, the closest relative of the nematode symbiont, as derived from a phylogenetic study for the genus *Xenorhabdus* by 16S rRNA cataloguing (Ehlers, Wyss & Stackebrandt, 1988).

* We use the generic name *Steinernema* but accept the recommendation of Dr. G. O. Poinar that the name *carpocapsae* is preferable to *feltiae* (Poinar, 1984).

Material and methods

Nematode and bacteria cultures

The nematode species and strains of associated bacteria species used are listed in Table 1. Bacterial symbionts (phase I) were obtained by streaking homogenates of surface-sterilized dauer larvae on diagnostic agar media (Akhurst, 1980). They were cultured in YS broth (Dye, 1968) at 29 °C and later stored in 15 % glycerol (v/v) at — 30 °C. Phase II symbionts were isolated from monoxenic *in vitro* cultures initially established with axenic nematodes and phase I symbionts. They were subcultured until they had totally converted into the secondary form. A phase was considered secondary when the phase I characters — absorption of bromothymol blue and neutral red from agar media (Akhurst, 1980) and growth inhibition of *Bacillus cereus* (Akhurst, 1982) — were not exhibited over a period of three subcultures.

Phase II of *H. heliothidis* was the strain ATCC 29304 (American Type Culture Collection). The *E. coli* isolate was strain HB 101 (Bolivar *et al.*, 1977). It was obtained together with *B. cereus* from the Culture Collection of the Institut für Allgemeine Mikrobiologie, Kiel University. All *Xenorhabdus* isolates, wild type and phase II, could be assigned to described species of *Xenorhabdus* (Akhurst & Boemare, 1988) by species-specific DNA probes, developed for the type strains (Pütz *et al.*, 1990).

Production of monoxenic and axenic cultures

Rearing nematodes with different bacteria isolates requires axenization, preferably performed with eggs (Patel & McFadden, 1978), which were obtained from gravid giant females isolated from parasitized last instar larvae of *G. mellonella*. The eggs were surface-sterilized in 0.1 % merthiolate (w/v) for 4 h, rinsed twice in sterile Ringer's solution and then transferred to YPC agar (containing 2 g yeast extract, 10 g soy peptone, 25 g standard I agar [all from MERCK], and 0.2 g cholesterol [SIGMA], per 1 l distilled water). After an incubation for 48 h at 25 °C, plates free of micro-organisms were used for the initiation of axenic cultures of *Steinernema* spp. by adding slices of raw rat kidney or liver to the eggs and freshly hatched L_1 larvae.

Monoxenic cultures were obtained by adding the nematode specific phase variants from 24 h old broth cultures to axenic cultures of *Steinernema* spp. Only *S. carpocapsae* was cultured monoxenically on *E. coli*. As we were not yet able to rear *Heterorhabditis* spp. under

Table 1

Geographical origin of the nematodes and their associated bacterial symbionts

Nematode	Strain	Bacterial symbiont	Geographical origin
Steinernema sp.	NC 513	*X. poinarii*	North Carolina, USA
S. carpocapsae	DD-136	*X. nematophilus*	USA
S. bibionis	OBS III	*X. bovienii*	The Netherlands
Heterorhabditis sp.	NZ	*X. luminescens*	New Zealand
H. heliothidis	NC 1 (HH)	*X. luminescens*	North Carolina, USA

axenic conditions, monoxenic cultures were obtained by inoculation of phase I or II to surface-sterilized eggs and freshly hatched L_1 larvae.

TEST METHOD

To allow an adaptation to the monoxenic growth conditions, only nematodes of the F1-generation of cultures initiated with axenic nematodes and different phase variants or *E. coli* were used to inoculate the test cultures. A single preadult female was transferred together with two male nematodes to 24 h old bacteria slants on YPC agar (6 cm diameter Petri dishes) in the case of steinernematid nematodes. Test cultures for heterorhabditid nematodes were inoculated with a single preadult protandric hermaphrodite. Culture plates were incubated at 25 °C. Nematodes were extracted by melting the agar at 80 °C and passing the liquefied medium through a 15 µm sieve. All nematode stages remained on the sieve and were poured into subdivided culture plates for counting under the inverted microscope.

For the determination of the growth profiles of *S. carpocapsae* (DD-136) on both phase variants and on *E. coli*, all nematodes from ten cultures were counted at three day intervals. For the other nematode species the offsprings of the single female were counted on the third day after inoculation and the final yield was assessed on the fifteenth day for *Steinernema* spp. and on the eighteenth day for *Heterorhabditis* spp.

Total nematode counts were transformed to logarithms and yields obtained from phase I and phase II cultures were compared by Student's *t*-test. As the nematode development on *E. coli* was delayed by about six days, the data were compared with the six day younger data from primary and secondary form cultures. The daily population increase in the log-phase was calculated by regression analysis.

The body length of first generation females from phase I and II cultures was measured and the generation time (time necessary for the development from the egg to an egg-laying female) was assessed. Before extracting the nematodes, bacteria probes were removed from the plates, diluted in Ringer's solution and plated on diagnostic agar media. After three days incubation at 29 °C the percentage of phase I colonies identified by dye absorption was estimated and compared with the phase I ratio obtained from control plates without nematodes.

IN VIVO ASSAY

To test the virulence and reproduction potential under *in vivo* conditions, last instar *G. mellonella* larvae (n = 30) were placed on moist filter paper in Petri dishes. Twenty axenic (DD-136 and NC 513) or either primary or secondary form bearing dauer larvae (DD-136, NC 513, HH) were added per insect. In addition ten insects were inoculated with 40 mono- or axenic *S. carpocapsae* (DD-136). Mortality of the insects was determined six days after inoculation. Dead insects were tranferred to a nematode trap (Dutky, Thompson & Cantwell, 1964) and the average number of dauer larvae emerging from every insect was assessed after twenty days.

Results

STEINERNEMA CARPOCAPSAE (DD-136)

The average number of nematodes per plate, the standard deviation and maximum and minimum yields obtained from cultures with phase variants of *X. nematophilus* and *E. coli* are shown in Table 2. The maximum yield was obtained on the fifteenth day and the highest average nematode number was recorded on the twelfth day, both on the secondary phase. Only stages between second stage juveniles to preadults were observed on the third day, thus the average number of off-springs per female is higher than 800 nematodes. When propagated with symbiotic bacteria, no further population increase was observed after the fifteenth day and only dauer larvae were present. A third generation did not develop. All nematodes reproduced on both variants of their symbiont. Of 70 plates inoculated with *E. coli*, propagation could, however, only be observed in 41 cultures and nematode development was delayed by approximately six days.

Table 2

Number of *Steinernema carpocapsae* in monoxenic cultures with phase I or II of *Xenorhabdus nematophilus* or *E. coli* at different days after inoculation of a single female

Days	Phase I[a]		Phase II[a]		*E. coli*	
	Mean s.d.[d]	Min. Max.	Mean s.d.	Min. Max.	Mean s.d.	Min. Max.
3	837	580	864	461	–[e]	–
	218	1 153	273	1 250	–	–
6	2 689	1 005	2 304	1 012	140[b]	65
	1 314	5 300	1 190	5 030	61	217
9	69 655	40 550	64 847	50 550	364[b]	177
	22 432	108 850	11 443	80 500	132	484
12	80 575	54 200	115 290	67 000	9 854[b]	4 220
	15 773	107 700	32 954	168 750	5 497	16 790
15	76 665	45 600	100 496	54 350	25 760[b]	7 720
	20 202	110 100	47 114	202 000	23 000	63 520
18	81 820	58 750	78 035	59 500	80 000[c]	–
	15 919	107 850	14 651	102 450	–	–
21	–	–	–	–	78 632[a]	36 600
	–	–	–	–	28 947	140 350
24	–	–	–	–	62 830[a]	25 300
	–	–	–	–	25 840	101 550

[a] N = 10; [b] N = 5; [c] N = 1; [d] Standard deviation; [e] Not determined.

The statistical comparison of the average data revealed no significant differences of nematode yields from primary or secondary form cultures or from cultures preinoculated with *E. coli* (P = 20 %). The growth profiles, based on the average number of nematodes per plate (Fig. 1) show a lag-, log-, stationary and dying phase. The average daily increase in the log-phase, obtained from data of both forms (day 6 and 9) and in addition of day 12 for phase II, was 19 244 nematodes (r = 0.92). The body length of the females in phase I or II cultures did not differ and was ≥ 10 mm. Only the first individuals of the F_1-generation developed to giant forms. The generation time at 25 °C was 3.5 days for both culture variants. Secondary form bacteria were stable in monoxenic cultures and control plates. The percentage of the primary form in monoxenic cultures decreased from 90 % on the third day to 30 % on the fifteenth and eighteenth day. In control plates the phase I symbiont stayed stable until the sixth day and the percentage never dropped below 95 % until the eighteenth day. Non-symbiotic bacteria were not found in any of the test cultures of all nematode species and strains examined.

Steinernema bibionis and *Steinernema* sp. (NC 513)

Highest average yields of *S. bibionis* and *Steinernema* sp. NC 513 were obtained from cultures preinoculated with the secondary phase *X. bovienii* and *X. poinarii*, respectively (Table 3), but the difference between the two forms was not significant (P = 20 %). The average number of off-springs per female was ≥ 200 for *S. bibionis* and ≥ 700 for NC 513. The generation time was 4 days for *S. bibionis* and 3.5 days for *Steinernema* sp. NC 513. The parental female nematodes were giant forms and their body length did not vary between

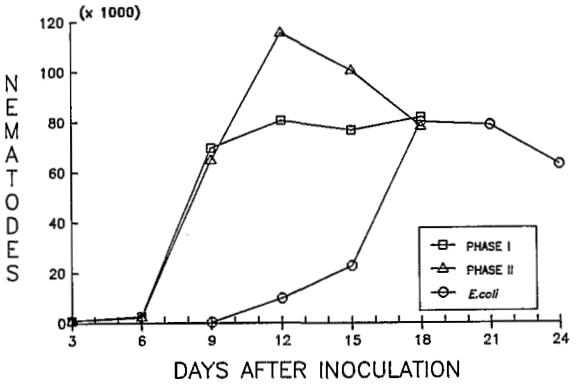

Fig. 1. Growth profiles of *S. carpocapsae* (DD-136) on phase variants of *X. nematophilus* (phase I and II) and *E. coli*.

Table 3

Number of nematodes of *S. bibionis* (OB), *Steinernema* sp. (NC), *H. heliothidis* (HH) and *Heterorhabditis* sp. (NZ) in monoxenic cultures with strain specific phase I or II of *Xenorhabdus spp.*

		Phase I			Phase II			
Strain N[a]	Days	Mean s.d.	Minima Maxima	*N* n[b]	Mean s.d.	Minima Maxima	N	t[c]
	3	197	80	*10*	209	76	*10*	0,02
OB		85	331	10	91	326	10	
20	15	47 805	30 400	*10*	52 730	41 000	*10*	0,02
		16 210	82 400	10	10 940	67 900	10	
	3	541	241	*10*	733	321	*10*	0,11
NC		231	878	10	260	1 178	10	
30	15	66 830	35 600	*20*	82 540	41 100	*20*	0,03
		18 448	109 100	20	48 646	237 700	20	
	3	389	201	*10*	289	141	*10*	0,10
HH		168	653	10	110	444	10	
50	18	102 125	40 900	*40*	57 819	35 750	*40*	0,14
		29 672	147 850	24	11 195	78 850	16	
	3	90	21	*10*	26	5	*10*	0,73
NZ		54	173	10	18	61	10	
70	18	136 889	31 600	*60*	90 400	45 200	*60*	0,05
		67 333	298 000	18	30 818	113 600	4	

[a] Number of plates per variant inoculated with a single female; [b] Number of plates with living off-springs; [c] Student's *t*-quantil, obtained by comparison of mean nematode numbers from phase I and II cultures.

Table 4

Percentage phase I colonies with bromothymol blue and neutral red absorption obtained from monoxenic nematode cultures inoculated with species specific phase I symbionts of *Xenorhabdus* spp. and from YPS agar without nematodes (control)

Strain	Days	Phase I ratio (%)	
		Monoxenic culture	Control
OB	3	90	100
	15	60	90
NC	3	80	100
	15	50	100
HH	3	80	100
	18	30	80
NZ	3	100	100
	18	95	100

phase I and II cultures. Secondary form symbionts were stable in monoxenic cultures and control plates. The ratio of phase I colonies decreased to a higher extent in monoxenic than in control cultures of the symbionts *X. bovienii* and *X. poinarii* (Table 4). In control plates of *X. poinarii* the primary form was stable until the last day measured.

HETERORHABDITIS HELIOTHIDIS AND *HETERORHABDITIS* SP. NZ

Both heterorhabditid nematodes developed and reproduced best in phase I *X. luminescens* cultures, but without a significant difference to cultures with the phase II symbiont (Table 3).

From 50 cultures inoculated with *H. heliothidis* together with either phase I or II symbiont, 16 and 24, respectively, failed to develop and nematodes died within the first 7 days. The phase II symbionts were stable in monoxenic and control plates. The phase I ratio decreased to 30 % in monoxenic cultures (Table 4). Protandric females measured 4 mm and the generation

time was 3.5 days in both variant cultures. The number of nematodes after three days do not represent the number of off-springs per female, as heterorhabditid nematodes lived longer than steinernematids and still laid eggs when a second generation already reproduced. In the *in vitro* cultures both strains did not develop to giant forms as observed under *in vivo* conditions in *Galleria mellonella* larvae.

The NZ symbiont occurred in three variant forms : an orange phase I and an orange as well as a yellow pigmented phase II. The orange phase II variant was isolated from a month old monoxenic nematode culture originally started with the phase I symbiont. It appeared to be stable 7 months after isolation, after it had been subcultured twice a week, including a period of 6 weeks in YS broth 3 months after isolation. However, when the test cultures were to be inoculated, this phase again exhibited antibiotic activities. Further subcultures finally resulted in a loss of the property to produce antibiotic compounds 10 months after isolation. Besides the orange pigmented variant a yellow one was then isolated. The anthraquinone pigment of *X. luminescens* is pH-sensitive (Richardson *et al.*, 1988), however, an elevation of the pH in the yellow pigmented phase II culture did not change the colour. On this variant nematodes failed to develop when monoxenic cultures were initiated with eggs, so the test cultures had to be inoculated with nematodes from orange pigmented phase II plates. Of 70 cultures with the yellow variant 37 failed to support nematode development after 10 days and all nematodes died until the eighteenth day. On phase I and orange phase II (Table 3) all nematodes were still alive after three days, however, in 70 % of the phase I and 93 % of the orange phase II cultures nematodes died within 18 days.

IN VIVO ASSAY

Mortality of *G. mellonella* did not differ much when the last instar larvae were infected with either axenic or phase I or II symbiont carrying steinernematid dauer larvae (Fig. 2). A higher mortality was obtained with larvae of *H. heliothidis* carrying phase I symbionts. For strain DD-136 a higher inoculum dosis was more effective and also increased the final yields of dauer larvae (Fig. 3). At the lower dosage both *Steinernema* spp. with the phase II symbiont nearly failed to develop. Under *in vivo* conditions propagation of secondary form symbiont carrying larvae was always lower. A limited reproduction of axenic nematodes was only observed for *S. carpocapsae.*

Discussion

Detrimental effects of the phase II of *Xenorhabdus* spp. on the *in vitro* propagation of their symbiotic

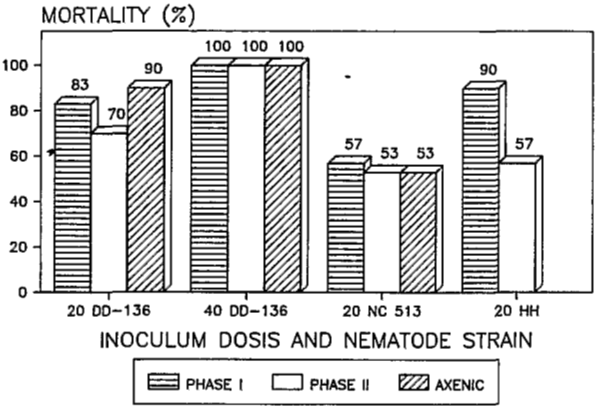

Fig. 2. Mortality (%) of last instar *G. mellonella* larvae infested with *S. carpocapsae* (DD-136), *Steinernema* sp. (NC 513) and *H. heliothidis* (HH) obtained from monoxenic cultures with strain specific phase I or II *Xenorhabdus* spp. and with two *Steinernema* spp. from axenic cultures.

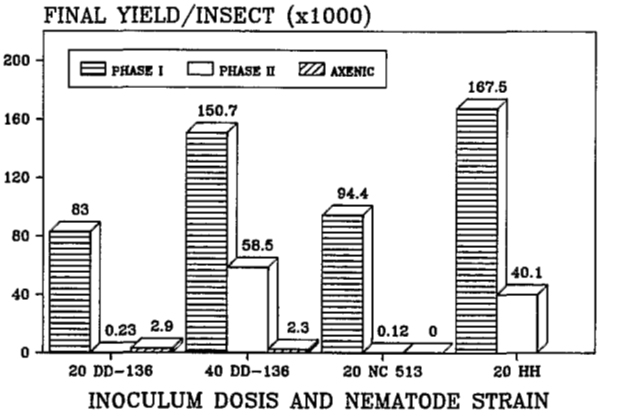

Fig. 3. Nematode yields per dead last instar *G. mellonella* larvae infested with *S. carpocapsae* (DD-136), *Steinernema* sp. (NC 513) and *H. heliothidis* (HH) obtained from monoxenic cultures with strain specific phase I or II *Xenorhabdus* spp. and with two *Steinernema* spp. from axenic cultures.

nematode (Bedding, 1981, 1984) can obviously not be correlated to the different phase characters used to separate the variants (Akhurst, 1980, 1982). The presented results show no negative effects of the secondary forms of *X. nematophilus, X. bovienii* and *X. poinarii* on nematode propagation. The primary form of these *Xenorhabdus* spp. partially converts into the secondary form after a single passage through a monoxenic nematode *in vitro* culture. Further selection applying phase II characters results in stable secondary form symbionts after a few subcultures. Once the phase I characters are lost they are not recovered again. In our studies stable phase II symbiotic bacteria of steinernematids were established after 4 months by continuous selection for phase II characters. If negative influences on nematode

propagation and development in the *in vitro* cultures should be caused by aging bacteria symbionts of *Steinernema* spp., these effects can occur at the earliest 4 months after culturing in artificial media.

The *X. luminescens* isolate of *H. heliothidis* takes longer to convert. Therefore the ATCC strain was applied, which had no contact with its symbiotic nematode since its deposition in the culture collection, but still supported nematode reproduction. The isolate NZ stayed stable in the primary form for a long time and could only be " forced " into a secondary form. When subcultured biweekly for one year and selected for phase I characters, it did not convert into the secondary form (unpubl.). Both phase II variants had been subcultured and selected for 11 months when they finally exhibited stable phase II characters, although the yellow pigmented form again produced orange pigments after storage at - 30 °C. During the process of repeated subcultures the NZ bacterium strain often jumped back to phase I characters.

A negative influence of *E. coli* or *Xenorhabdus* variants on the potential of nematode reproduction did not become evident. However, obvious criteria for detrimental effects on the nematodes were a delay in the development (= longer generation time), observed for *S. carpocapsae* when cultured on *E. coli* and a higher nematode mortality of *Heterorhabditis* spp. In this respect a negative correlation to the age of bacteria cultures became evident for *X. luminescens*, but not for steinernematid symbionts, at least not within the period tested.

The reasons for the observed cases of mortality of *Heterorhabditis* spp. on primary and secondary phase cultures are not known. The addition of enzyme inhibitors to nematode cultures (Despommier & Jackson, 1972; Jackson & Platzer, 1974) retards development and growth as well as reproduction, but does not cause mortality. The same effects arise by a lack of essential nutritional factors in *S. glaseri* cultures (Jackson, 1962; Jackson & Siddiqui, 1965). These factors are unlikely to be responsible for the higher death rates in our experiments. Nematode mortality is caused by the addition of chemicals like piperazine (Jackson, 1961) or by an accumulation of bacterial toxins (Bolla, 1987). *Xenorhabdus* spp. are known to synthesize metabolites with various antibiotic activities (Rhodes *et al.*, 1984; Gregson & McInerney, 1986). Although it is difficult to imagine that a mutualistic bacterium may produce toxins affecting its symbiont, it remains to be examined whether these metabolites could also have detrimental effects on *Heterorhabditis* spp. It may be speculated, that the nematodes are equipped to metabolize these toxins. A detoxication would then depend on a certain population ratio nematode : bacterium, which is sometimes not reached in cultures inoculated with a single *Heterorhabditis* sp. female. In our experiments possibly only those nematode cultures survived, which developed rapidly to a population threshold with the capacity to detoxicate the bacterial metabolites. The even higher mortality in aged symbiont cultures may have been caused by an increase of toxin production of phase II *X. luminescens.*

Nematode yields from infected *G. mellonella* confirm the results of Akhurst (1980) that nematode numbers are lower, when the insect is injected with the secondary form. Considering our *in vitro* results, this effect can only indirectly be influenced by the phase II bacteria. A successful development of the nematodes is inhibited by the presence of non-symbiotic micro-organisms (Boemare *et al.*, 1983). The loss of antibiotic activity (Akhurst, 1982) and a severe damage of the haemocytes by the secondary form (Dunphy & Webster, 1984) will lead to an earlier contamination of the haemocoel with non-symbiotic bacteria, thus causing lower nematode yields. If these bacteria are absent (like under monoxenic *in vitro* conditions) the effects on reproduction are excluded.

Akhurst (1980) reported a longer generation time and shorter female nematodes when the secondary form was present. This does not correspond with our results from the *in vitro* cultures with *Xenorhabdus* spp. The nematode size depends on the amount of available nourishment and nematode density (Poinar *et al.*, 1972). So it must be concluded, that the secondary form *X. nematophilus* can equally well support giant female formation like the primary form. The shorter size of female nematodes under *in vivo* conditions was probably influenced by detrimental effects caused by non-symbiotic bacteria. A much lower increase of the secondary form symbiont in the haemolymph compared to the primary form, as observed by Dunphy and Webster (1984), may also have contributed to unfavourable trophic conditions. A low inoculum of *S. carpocapsae* and consequently also a low phase II *X. nematophilus* inoculum may have disabled the symbiont to suppress contaminating bacteria and thus nematode propagation may have been maintained by non-symbiotic bacteria. The limited propagation of axenic nematodes was probably supported by non-symbiotic bacteria inhabiting the insect intestine.

For the *in vitro* production of *Steinernema* spp. and *Heterorhabditis* spp. it is advisable to use bacteria variants that still produce antibiotic compounds. Mass-production methods (Bedding, 1981, 1984) describe the inoculation of cultures with surface-sterilized dauer larvae. Some non-symbiotic bacteria always survive a surface-sterilization of dauer larvae (unpubl.), thus a contamination of the media cannot be prevented. Under these polyxenic conditions the preinoculation with phase I bacteria may suppress the growth of non-symbiotic micro-organisms, thus supporting nematode propagation better than the secondary phase.

Industrial mass production requires scaling-up for which the monoxenic state of starting cultures must be guaranteed. The described initiation of monoxenic

cultures ensures an early check for the absence of non-symbiotic micro-organisms, thus providing a more reliable method to start scaling-up procedures.

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