Morphological and Functional Dimorphism in *Xenorhabdus* spp., Bacteria Symbiotically Associated with the Insect Pathogenic Nematodes *Neoaplectana* and *Heterorhabditis*

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Xenorhabdus spp., entomopathogenic bacteria symbiotically associated with the nematodes *Neoaplectana* and *Heterorhabditis*, occur in two forms. In general, only one form, designated the primary form, is transmitted into new hosts by the infective stage of the nematode. The significance of the relationship between the two forms has been examined with *X. nematophilus*, the symbiont of *N. feltiae*. The forms of *X. nematophilus* can be differentiated by their colony characteristics but by only two biochemical tests. The two forms of *X. nematophilus* are equally pathogenic when injected into the haemocoel of *Galleria* larvae. However, the primary form when injected into *Galleria* larvae with axenic nematodes provides better conditions for reproduction of the nematodes than the secondary form, for which a role has not been determined. Although the primary form readily converts to the secondary form *in vitro* and occasionally *in vivo*, the secondary form is usually stable. Possible causes of the instability have been investigated.

INTRODUCTION

Three genera of insect pathogenic nematodes are known to have specific symbiotic associations with bacteria: *Neoaplectana* spp. and *Heterorhabditis* spp. are associated with *Xenorhabdus* spp. (Thomas & Poinar, 1979) and *Steinernema kraussei* with a *Flavobacterium* sp. (Mráček, 1977). Each species of nematode is associated with a single bacterial species; all *Heterorhabditis* spp. examined have *Xenorhabdus luminescens* as the symbiont but each species of *Neoaplectana* is associated with a different species of bacterium (R. J. Akhurst, unpublished results). The bacterial symbiont is carried monoxenically in the intestine of the non-feeding infective stage of the nematode. The nematode penetrates an insect host and moves to the haemocoel where it voids the bacteria. The bacteria proliferate, kill the host and establish suitable conditions for reproduction of the nematodes by providing nutrients and inhibiting the growth of other bacteria (Poinar & Thomas, 1966). The symbiotic bacteria are also capable of rendering a wide variety of artificial media suitable for nematode reproduction, thus allowing the economical mass production of the nematodes (Bedding, 1976) necessary for the control of insect pests in the field.

During a study of the bacterial symbionts of many isolates of several species of *Neoaplectana* and *Heterorhabditis* from Australia, Europe, New Zealand and North America, I found that all may produce two forms of colony on agar media. The results of an investigation into the significance of and the relationships between the two forms of *X. nematophilus*, the symbiont of *N. feltiae*, are presented in this paper.

Table 1. *Nematodes from which bacteria were isolated for use in this study*

Nematode		Bacterium	Source
Species	Variety		
<i>Neoplectana feltiae</i>	Agriotos	<i>Xenorhabdus nematophilus</i>	G. O. Poinar, University of California, Berkeley, U.S.A.
	Nelson	<i>X. nematophilus</i>	<i>Vespula</i> sp., Tasmania, Australia
	Pieridarum	<i>X. nematophilus</i>	S. Stanuszek, Institute of Ecology, Warsaw, Poland
	P7	<i>X. nematophilus</i>	Soil*, Tasmania, Australia
	N55	<i>X. nematophilus</i>	Soil*, N.S.W., Australia
<i>N. bibionis</i>	NBP	<i>Xenorhabdus</i> sp.	Z. Mráček, Entomological Institute, CSAV, České, Budejovice, Czechoslovakia
	NZ	<i>Xenorhabdus</i> sp.	W. Wouts, DSIR, New Zealand
	V1	<i>Xenorhabdus</i> sp.	Soil*, Victoria, Australia
	5 isolates	<i>Xenorhabdus</i> sp.	Soils*, Tasmania, Australia
<i>N. glaseri</i>	—	<i>Xenorhabdus</i> sp.	H. Kaya, University of California, Davis, U.S.A.
<i>Heterorhabditis bacteriophora</i>	—	<i>X. luminescens</i>	W. Wouts, DSIR, New Zealand
<i>H. heliothidis</i>	NC	<i>X. luminescens</i>	W. Wouts, DSIR, New Zealand
	NZ	<i>X. luminescens</i>	W. Wouts, DSIR, New Zealand
<i>Heterorhabditis</i> spp.	D1	<i>X. luminescens</i>	Soil*, Northern Territory, Australia
	HP	<i>X. luminescens</i>	S. Stanuszek, Institute of Ecology, Warsaw, Poland
	T280	<i>X. luminescens</i>	Soil*, Tasmania, Australia
	T301	<i>X. luminescens</i>	Soil*, Tasmania, Australia

* Isolated by the method of Bedding & Akhurst (1975).

METHODS

Isolation of bacteria. The symbiont of the DD136 strain of *Neoplectana feltiae* (syn. *carpocapsae*, Stanuszek 1974) was obtained from the American Type Culture Collection (ATCC 19061). The other symbiotic bacteria used in this study were isolated, directly or indirectly, from the nematodes listed in Table 1.

Some symbiotic bacteria were isolated directly from the infective stage nematode. 'Infectives' (about 50) were surface-sterilized by immersion in 0.1% (w/v) merthiolate (Eli Lilly & Co., Indianapolis, U.S.A.) for 2 to 3 h; after being washed three times in sterile water, they were suspended in YS broth ($\text{NH}_4\text{H}_2\text{PO}_4$, 0.5 g; K_2HPO_4 , 0.5 g; $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$, 0.2 g; NaCl , 5 g; yeast extract, 5 g; water, 1 l; Dye, 1968) and macerated in a tissue homogenizer. Samples of the macerate were spread on nutrient agar (NA) or nutrient agar with 0.004% (w/v) triphenyltetrazolium chloride and 0.025% (w/v) bromothymol blue (NBTA). Cultures of other symbiotic bacteria were obtained indirectly from the nematodes by sampling the haemocoel of *Galleria mellonella* (L.) larvae infected by nematodes or re-isolated from monoxenic cultures of nematodes and their symbiont on artificial media. These cultures were originally established with axenic nematodes obtained by the method of Poinar & Thomas (1966) and pure cultures of the symbiont.

Except where otherwise specified, all cultures were incubated in the dark at 28 °C.

Differentiation of the forms. Both forms of *X. nematophilus* isolated from the Agriotos strain of *N. feltiae* were examined for differences in gross cell morphology and in reaction in biochemical and antibiotic sensitivity tests. The tests (Table 2) were carried out using standard methods although the phenylalanine deaminase test was conducted at 5 instead of 2 d and for the lipase test the Tween 80 concentration was reduced to 0.2% (w/v).

The pathogenicity of the two forms of the *N. feltiae* Agriotos symbiont was compared by estimation of LD_{50} following intrahaemocoelic injection of *Galleria* larvae. The concentration of cells in shaken, 24 h YS broth cultures was estimated by use of a counting slide. Each culture was then serially diluted with sterile Ringer's solution (Cruickshank *et al.*, 1970) to give seven doses from approximately 0.05 to 50 cells μl^{-1} . For each dose, 20 *Galleria* larvae were injected using a 10 μl syringe with 0.5 mm diameter cannula. Another 20 larvae were injected with sterile Ringer's solution only. The larvae were incubated at 22 °C on dry filter paper for 3 d. LD_{50} was determined by probit analysis (Bliss, 1938). The LD_{50} values, transformed to logarithms, were compared by *t*-test.

Table 2. Tests used to compare the two forms of symbiotic bacteria

Acetoin	Gelatin hydrolysis
Acid from carbohydrates*	Indole
Aesculin hydrolysis	KCN
Antibiotic sensitivity†	Lecithinase
Bioluminescence	Lipase
Casein hydrolysis	Maximum growth temperature
Catalase	Methyl red
Chitinase	Motility
Cultural characteristics	NaCl tolerance
– nutrient agar	Nitrate reduction
– MacConkey agar	Oxidase
– tergitol-7 agar	Oxidation – fermentation
Triple sugar iron agar	Phenylalanine deaminase
Cytochrome oxidase	Protease
Decarboxylase – glutamic acid	Reducing substance from sucrose
– lysine	Starch hydrolysis
– ornithine	Tyrosinase
Dihydrolase – arginine	Urease
DNAase	Utilization of organic acids‡

* 30 carbohydrates tested.

† 15 antibiotics tested.

‡ 13 organic acids tested.

Effect of bacterial forms on nematode reproduction. The two forms of the *N. feltiae* Agriotos symbiont were cultured separately in YS broth shaken at 25 °C for 24 h and diluted with sterile Ringer's solution to about 10^5 cells ml⁻¹. *Galleria* larvae were injected with 10 axenic *N. feltiae* Agriotos infectives 24 h after injection of 10^8 cells of one or other form of the symbiont (the interval between the injections avoiding the effect of the live insect host on nematode growth). The *Galleria* larvae were incubated at 22 °C. After 5 and 8 d some of the larvae were dissected and the lengths of nematode females were measured, using an eyepiece micrometer. When nematode reproduction was completed, the remaining 20 *Galleria* cadavers were dissected in water and the number of nematodes was estimated by counting the nematodes in three samples taken from an agitated suspension.

Stability. The stability (i.e. the ability to resist change to the alternative form) of the forms was examined under a variety of conditions. Monoxenic cultures of nematodes and their symbionts on artificial media and cultures of either form of symbiont in peptone water and in YS broth, both shaken and stationary, were tested for a change of form over 30 d by streaking on NA or NBTA. Pure cultures of either form were also incubated, aerobically and anaerobically, on NA. These were subcultured three times at intervals of 3 d, and at these times samples were streaked on to NBTA and incubated aerobically. *Galleria* infected by *N. feltiae* infectives while buried in sand, or injected with either form of the symbiont, with or without *N. feltiae* infectives, were also sampled. Each *Galleria* cadaver was dipped in ethanol, ignited and plunged into sterile Ringer's solution; the haemocoel was exposed and irrigated with the Ringer's solution. The liquid was serially diluted and 0.1 ml samples were spread on NBTA. Infective nematodes, harvested from injected or naturally infected *Galleria* and stored in water from 1 to 90 d, were macerated as before to determine the form of the symbiont carried.

Deep-freezing and freeze-drying were tested as methods of storing pure cultures of the forms over long periods. A loopful of bacteria from a 24 h YS agar culture was dispersed in 5 ml nutrient broth containing 17 % (v/v) glycerol and immediately deep-frozen (–18 °C). Such cultures were revived by rapid thawing in a 56 °C water bath and subculturing on YS agar or in YS broth. For freeze-drying, the bacteria were washed from 48 h YS broth cultures with 5 % (w/v) peptone/3 % (w/v) sucrose and freeze-dried in a Dynavac FD16 high vacuum freeze-drying unit at 10^{-3} Torr (about 0.1 Pa) and –70 °C.

Cause of form change. The symbiont of *N. feltiae* Agriotos was investigated to determine whether the change of form may have been mediated by bacteriophage or plasmid. The treatments used were u.v.-irradiation, culture in the presence of a chemical curing agent (acridine orange, ethidium bromide, sodium dodecyl sulphate) and culture near the maximum temperature for growth.

RESULTS

All isolates of symbiotic bacteria from *N. feltiae* (6 strains), *N. bibionis* (9), *N. glaseri* (1), *H. bacteriophora* (1), *H. heliothidis* (2) and unidentified *Heterorhabditis* spp. (6) occurred in two forms. Infective stage nematodes contained only one of the forms, with the exception

Table 3. *Pathogenicity of primary and secondary forms of the symbiont of N. feltiae Agriotos following intrahaemocoelic injection of Galleria larvae*

LD₅₀ was determined by probit analysis and the values of log LD₅₀ were compared by a *t*-test.

	Primary form	Secondary form	
LD ₅₀	3.0	2.0	<i>t</i> = 1.45 (not significant)
S.E.	0.6	0.4	

Table 4. *Effect of primary and secondary form bacteria on the size and yield of N. feltiae following injection of bacteria and axenic infectives into Galleria larvae*

Data on the length of adult females was analysed by analysis of variance and that on yield by a *t*-test.

Time after injection of nematodes (d)	Character	Bacterial form		<i>P</i>
		Primary	Secondary	
5	No. of females measured	11	17	< 0.01
	Mean length (mm)	12.2	4.1	
	S.E.	1.4	2.0	
8	No. of females measured	15	10	< 0.001
	Mean length (mm)	11.7	4.9	
	S.E.	0.2	3.5	
20	Estimated no. of infectives produced from 20 <i>Galleria</i> larvae	2.14 × 10 ⁶	0.30 × 10 ⁶	< 0.001
	S.E.	0.02 × 10 ⁶	0.013 × 10 ⁶	

of the NZ strain of *H. heliothidis* and under some experimental conditions. This form was designated primary and the other secondary. The secondary form occurred, with the primary form, in monoxenic cultures initiated with the primary form and occasionally in the haemocoel of infected *Galleria* larvae. Infective stage nematodes only carried secondary form bacteria when the nematodes were produced in the absence of the primary form.

Differentiation of the forms

On NA, colonies of the primary form of *X. nematophilus*, the *N. feltiae* symbiont, were white, opaque, convex and circular with an irregular margin; colonies of the secondary form were translucent, flatter and of greater diameter than the primary form. The difference in the colonies showed up more clearly on agars containing bromothymol blue and triphenyltetrazolium chloride (TTC), e.g. NBTA. On these agars, the primary form absorbed bromothymol blue and reduced TTC, producing colonies which had a red core overlaid by dark blue and were surrounded after 3 to 5 d by a clear zone; the secondary form reduced TTC but did not absorb bromothymol blue and so produced red colonies, which sometimes appeared maroon because of the blue agar, with no clear zone.

Xenorhabdus nematophilus produced some colonies that were intermediate between the two forms. On NBTA these had segments of blue and red, or were maroon rather than blue or red. Pure cultures of either form could rarely be obtained by subculturing from these intermediate colonies.

The two forms of the *N. feltiae* Agriotos symbiont did not differ in antibiotic resistance or reaction in most biochemical tests. However, the primary form of this bacterium gave negative results for phenylalanine deaminase and lipase activity while the secondary form was positive. When samples of cultures containing both forms of the *N. feltiae* Agriotos symbiont were spread on NA, NBTA and tergitol-7 plus TTC, the highest proportion of colonies of the secondary form were on NA and the lowest on tergitol-7 plus TTC.

Fig. 1. Proportion of *X. nematophilus* in the primary form in *Galleria* larvae incubated at 22 °C after natural infection by Agriotos (○) and Nelson (●) strains of *N. feltiae*. Two larvae were sampled at each time; serial dilutions of dissected larvae were spread on NA, incubated at 28 °C and assessed after 4 to 6 d. Experiments were terminated when the recovery of *X. nematophilus* from the larvae was too low to give a realistic estimate of the proportion of *X. nematophilus* in the primary form. For both the Agriotos and Nelson varieties, the estimate of proportion at the last sampling was based on the result from one cadaver only. Mean and s.e. were calculated on arcsin $\sqrt{\text{percentage transformed data}}$. Vertical lines represent s.e.

There was no significant difference in the pathogenicity of the primary and secondary forms of the *N. feltiae* Agriotos symbiont (Table 3).

Effects of bacterial forms on nematode reproduction

The effects of primary and secondary form bacteria on the size of adult female *N. feltiae* Agriotos in *Galleria* injected with axenic infectives and either form of the bacterium are shown in Table 4. In each case the difference between the primary and secondary forms was highly significant.

The nematodes matured and reproduced more quickly in the presence of the primary form. The yield from *Galleria* injected with primary form *X. nematophilus* and axenic infectives was greater than from *Galleria* injected with secondary form and axenic infectives (Table 4).

Stability

The primary form of *X. nematophilus* was unstable but the degree of instability was very variable. The symbionts of the Agriotos and Nelson strains of *N. feltiae* converted from primary to secondary form much more readily than those of the DD136 and Pieridarum strains.

Pure cultures of the primary form of all isolates of *X. nematophilus* could be maintained by freeze-drying, deep-freezing or by frequent subculture. However, when broth cultures, shaken or stationary, and cultures on agar media incubated at 28 °C for more than 7 d were subcultured, some colonies of the secondary form were evident. *In vitro* monoxenic cultures of nematodes and primary form bacteria also produced the secondary form, though sometimes not for several weeks and after several subcultures. The primary form of most isolates was stable in *Galleria* larvae, whether or not nematodes were present. However, the

primary form of the symbionts of the Agriotos and Nelson strains of *N. feltiae*, though stable if nematodes were absent, was unstable in cadavers in the presence of nematodes (Fig. 1).

The secondary form of the *N. feltiae* symbiont was stable on agar media incubated aerobically, in monoxenic culture with nematodes and in *Galleria*, with or without nematodes. However, it was unstable in unshaken broth media and in anaerobic culture on NA. Infective nematodes harvested from *Galleria* injected with axenic *N. feltiae* Agriotos and secondary form bacteria contained only stable secondary form bacteria.

Cause of form change

There was no evidence for the involvement of bacteriophage or plasmid in mediating the change from one form to the other.

DISCUSSION

The existence of two forms of each bacterial species symbiotically associated with the insect pathogenic nematodes *Neoplectana* and *Heterorhabditis* is undoubtedly significant as all such bacteria examined exhibit this phenomenon, as does the *Flavobacterium* sp. symbiont of a similar nematode, *Steinernema kraussei* (R. J. Akhurst, unpublished results). In *N. feltiae* (and other species of *Neoplectana* and *Heterorhabditis*; R. J. Akhurst, unpublished results) the primary form is preferentially retained in the intestine of the infective stage of the nematode, allows faster and significantly greater reproduction by the nematodes, and is less stable than the secondary form. It is therefore difficult to envisage the role of the secondary form. This form may be the original symbiont from which has developed another, unstable, form that better suits the nematodes' requirements and has gained a selective advantage in being retained preferentially by the infective stage. The secondary form certainly has significance in *in vitro* monoxenic culture of the nematodes, where it is detrimental to the final yield.

Infective *N. feltiae* produced in insects or *in vitro* culture in the presence of both forms of *X. nematophilus* contain only the primary form, even though the proportion of *X. nematophilus* in the primary form within the insect or culture may be only 20 %. However, these nematodes are capable of carrying the secondary form internally as this occurs when infectives are produced where only the secondary form is present. The mechanism which ensures that infective *N. feltiae* preferentially carry the primary form, which is superior for their reproductive requirements, without losing the capacity to carry the secondary form, has not been determined. This mechanism must operate within the nematode, either during the feeding, pre-infective stages or the non-feeding infective stage, since there is no transmission of bacteria through the egg (R. J. Akhurst, unpublished results).

Only the symbionts of the Agriotos and Nelson strains of *N. feltiae* produced the secondary form when introduced into *Galleria* larvae with axenic nematodes and these differed in the rate at which the secondary form was produced (Fig. 1). It appears that different isolates of *Xenorhabdus* exhibit different levels of stability with the symbiont of *N. feltiae* Agriotos being the most unstable of those examined.

In characterizing the genus *Xenorhabdus*, Thomas & Poinar (1979) have apparently used only primary form bacteria, though the cultures of Hb, Hb-M and Hm may have contained some secondary form bacteria. The characterization of the genus may need to be amended to allow for the inability of the secondary form colonies to absorb bromothymol blue and that of the species *X. nematophilus* to allow for the lipase and phenylalanine deaminase activity of the secondary form.

Neoplectana feltiae Agriotos produced 7 times as many infectives from *Galleria* larvae when the primary, rather than secondary, form was injected into the insects with axenic infectives. R. A. Bedding (personal communication) found that mass cultures of *N. feltiae* Agriotos and *H. heliothidis* NZ with the primary form of their symbionts yielded 1.6 and 2.9 times, respectively, as many nematodes as cultures with the secondary form. The

difference in the ratio for *N. feltiae* Agriotos in *Galleria* and in monoxenic cultures is probably due to the contamination by the secondary form of monoxenic cultures of nematode and primary form (and vice versa) that is unavoidable when Bedding's method is used (unpublished results).

Apart from the obvious biological interest of this phenomenon, dimorphism in *Xenorhabdus* spp. is of considerable practical importance in the mass production of *Neoaplectana* and *Heterorhabditis* for pest insect treatment.

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