Electrophoresis of Soluble Proteins from Two Species of *Xenorhabdus*, Bacteria Mutualistically Associated with the Nematodes *Steinernema* spp. and *Heterorhabditis* spp.

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Mutualistically associated bacteria were isolated from two genera of nematodes: *Xenorhabdus nematophilus* from four species of *Steinernema* (= *Neoaplectana*) and *X. luminescens* from two species of *Heterorhabditis*. Mutualistic bacteria were also isolated from two unidentified species of heterorhabditid nematodes and were partially characterized by selected standard bacteriological tests. The soluble proteins obtained from the primary and secondary forms of these bacteria were subjected to electrophoretic examination on acrylamide gels. Total protein profiles and certain isoenzyme electrophoregrams were compared. The results revealed distinct subspecific differences in profiles of *X. nematophilus* and suggested new subspecific groupings in *X. luminescens*.

INTRODUCTION

A symbiotic and specific association has been demonstrated between the bacterium *Xenorhabdus nematophilus* and species of the nematode *Steinernema* (= *Neoaplectana*), and between *X. luminescens* and *Heterorhabditis* spp. (Thomas & Poinar, 1979; Akhurst, 1983*b*). Each bacterial species occurs in at least two forms (designated as primary and secondary) which can be distinguished as differently pigmented colonies on various bacteriological media (Akhurst, 1980).

All bacteria associated with species of *Heterorhabditis* have been identified as *X. luminescens* with no subspecific designation (Akhurst, 1983*a*); *X. nematophilus* associated with *Steinernema* spp. has been given three subspecific designations based on host specificity, pigmentation and biochemical tests (Akhurst, 1983*a*). These subspecific groupings are: group I, *X. nematophilus* subsp. *nematophilus* (associated with *S. feltiae*); group II, *X. nematophilus* subsp. *bovienii* (associated with *S. bibionis*); and group III, *X. nematophilus* subsp. *poinarii* (associated with *S. glaseri*). Bacteria associated with *S. kraussei* and unidentified *Steinernema* species are currently in a group designated IV.

Electrophoretic techniques allow rapid and accurate identification of organisms. They are especially useful in clarifying relationships at subspecific and population levels when examining individual genetic variants (Kerstens & De Ley, 1980). We examined the primary and secondary forms of *Xenorhabdus* spp. associated with described species of *Steinernema* and *Heterorhabditis*. In addition, we characterized associated bacteria from two undescribed isolates of *Heterorhabditis* by electrophoretic and standard bacteriological methods.

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Abbreviations: 6PG, 6-phosphogluconate dehydrogenase; G6P, glucose-6-phosphate dehydrogenase; LDH, lactate dehydrogenase; MDH, malate dehydrogenase; PGM, phosphoglucomutase; PMS, phenazine methosulphate.

METHODS

Bacterial strains and growth conditions. Sources of bacteria and nematodes are listed in Table 1. Bacterial cultures were isolated from nematode colonies maintained in our laboratory as follows. *Galleria mellonella* larvae, infected with a single nematode strain, were dissected after 24, 48 or 72 h and haemolymph containing bacterial cells was streaked on an agar plate (see below).

To prevent clonal selection, bacteria from many colonies were used to streak new plates. These isolates were cultured and maintained on NBT agar (nutrient agar with 25 mg bromothymol blue l^{-1} and 30 mg 2,3,5-triphenyl-tetrazolium chloride l^{-1}) in the dark at 24 °C. For electrophoresis, bacteria from single colony isolates and multiple colony isolates were cultured in nutrient broth on a shaker at room temperature (25 ± 2 °C) for 24 h.

Colony morphology and pigmentation. *Heterorhabditis* spp. obtained from the Netherlands (Simons, 1981) and Victorville, Calif., USA (Bowen, 1982) had associated luminescent bacteria presumed to be *X. luminescens* (which are henceforth designated as isolates of *X. luminescens*). Because these bacteria were uncharacterized, we did tests to obtain some basic information. The presumed primary and secondary forms were cultured, as described above, then observed for motility and stained for Gram reaction. Catalase activity was tested by flooding cultures with 10% (v/v) hydrogen peroxide. Three-day-old cultures were observed for bioluminescence, colony size and antibiotic activity (Akhurst, 1982) against strains of *X. nematophilus*. Pigmentation was also observed at 1 and 3 d on nutrient and MacConkey agar and at 3 d on NBT agar.

Preparation of soluble proteins. For electrophoresis, spheroplasts were prepared as follows. Bacterial cultures were centrifuged at 2500 g for 10 min. The pellets were resuspended in 33 mM-Tris/HCl pH 7.5, 50 mM-NaCl and recentrifuged. These pellets were resuspended in buffer (1 mM-EDTA, 10 mM-Tris/HCl pH 7.5, 0.5 M-sucrose) containing 50 µg lysozyme ml^{-1} and kept at 25 ± 2 °C for 30 min. Lysozyme was removed from the spheroplasts by centrifuging (2500 g for 15 min) twice in the buffer. Pellets of washed spheroplasts were resuspended in electrophoresis sample buffer [62.5 mM-Tris/HCl pH 6.8, 10% (v/v) glycerol, 0.001% (w/v) bromophenol blue] and sonicated using a Sonurgy Model G-15F (Fibra-Sonics Inc., Chicago, Ill., USA) on ice for 20 s.

Protein assay. The protein content of spheroplasts used for electrophoresis was determined by the method of Bradford (1976) using Coomassie Brilliant Blue G-250. Standard protein was prepared from a 24 h nutrient broth culture of the All primary strain of *X. nematophilus*. Cells were pelleted, resuspended in double distilled water and then passed three times through a French pressure cell press at 15000 lbf in^{-2} (103.5 MPa). Cell walls and membranes were removed by centrifugation at 15000 g for 30 min. The supernatant was dialysed in three changes of double distilled water for 24 h, lyophilized, and stored at -70 °C. All procedures were done on ice or at 10 °C.

Electrophoretic techniques. Gels and buffers were used as described by Davis (1964). The pH of the separating gel was 8.9 for protein profiles and lactate dehydrogenase (LDH) and 7.3 for glucose-6-phosphate dehydrogenase (G6P) and malate dehydrogenase (MDH). Soluble proteins (300–400 µg samples) were loaded on 1.25 mm thick, vertical slab gels (Model SE 500; Hoeffer Scientific Instruments, San Francisco, Calif., USA). Proteins were electrophoresed at 80 V through the 4% (w/v) acrylamide stacking gel and at 120 V through the 7% (w/v) acrylamide separating gel for about 5 cm. Gels were cooled during electrophoresis to 17 ± 1 °C with water circulating through an internal chamber.

Protein profiles were visualized by staining gels with 0.1% (w/v) Coomassie Brilliant Blue in 50% methanol/10% glacial acetic acid at 50 °C for 1 h. Gels were destained in 25% methanol/10% glacial acetic acid and stored in this solution.

To detect isoenzymes, gels were incubated at room temperature with (ml^{-1}) 5 mg of the appropriate substrate, 1 mg coenzyme, 0.1 mg phenazine methosulphate (PMS) and 1 mg 3-(4,5-dimethylthiazolyl-2)-2,5-diphenyl-tetrazolium bromide (MTT) in 100 mM-Tris/HCl. The substrates for MDH and LDH were L-malic acid and DL-lactic acid, respectively; the coenzyme used was β -nicotinamide adenine dinucleotide (NAD) in buffer at pH 7.0. The substrates for G6P and 6-phosphogluconate dehydrogenase (6PG) were D-glucose 6-phosphate and 6-phosphogluconate, respectively; the coenzyme used was β -nicotinamide adenine dinucleotide phosphate (NADP) in buffer at pH 7.0. Phosphoglucomutase (PGM) was stained with α -D-glucose 1-phosphate, 0.8 units G6P ml^{-1} , 2 mg $MgCl_2$ ml^{-1} , 0.5 mg EDTA ml^{-1} in buffer at pH 7.0 with PMS, NADP and MTT as above. After visualization of isoenzymes, gels were stored in 25% methanol/10% glacial acetic acid.

RESULTS

Results of the standard bacteriological tests on the Netherlands and Victorville isolates of *X. luminescens* are summarized in Table 2. Colonies of the Victorville isolate of *X. luminescens* on NBT agar were typical of those described for the other isolates in the species. The primary form was convex, circular with a slightly irregular margin, green with a red centre, and with a clear zone in the agar around the colony; the secondary form was flatter, with a wider circle, which had a more irregular margin, blue with a red centre, and with no clear zone in the agar. The primary form of the Netherlands isolate was similar to those previously described: convex,

Table 1. Sources of isolates of *Xenorhabdus*

Species or subspecies	Isolate	Form*	Associated nematode	Country (state)	Source†
<i>X. nematophilus</i> subsp. <i>nematophilus</i>	All1	P	<i>Steinernema feltiae</i>	USA (Georgia)	1
	All2	S	<i>S. feltiae</i>	USA (Georgia)	1
	A24	P	<i>S. feltiae</i>	USSR	2
	A25	S	<i>S. feltiae</i>	USSR	2
	Agro	P	<i>S. feltiae</i>	USSR	2
	Bret	P	<i>S. feltiae</i>	France	3
	Ital	P	<i>S. feltiae</i>	Italy	4
	Mex1	P	<i>S. feltiae</i>	Mexico	5
	Mex2	S	<i>S. feltiae</i>	Mexico	5
<i>X. nematophilus</i> subsp. <i>borienii</i>	Bib1	P	<i>S. bibionis</i>	Australia	2
	Bib2	S	<i>S. bibionis</i>	Australia	2
	T319/1	P	<i>S. bibionis</i>	Australia	2
	T319/2	S	<i>S. bibionis</i>	Australia	2
<i>X. nematophilus</i> subsp. <i>poinarii</i>	Glas	S	<i>S. glaseri</i>	USA (N. Carolina)	6
	G6	S	<i>S. glaseri</i>	USA (N. Carolina)	2
<i>Xenorhabdus</i> sp.	SK3/1	P	<i>S. kraussei</i>	Czechoslovakia	2
	SK3/2	S	<i>S. kraussei</i>	Czechoslovakia	2
	Q58/1	P	<i>Steinernema</i> sp.	Australia	2
	Q58/2	S	<i>Steinernema</i> sp.	Australia	2
<i>X. luminescens</i>	B1	P	<i>Heterorhabditis bacteriophora</i>	Australia	2
	B2	S	<i>H. bacteriophora</i>	Australia	2
	C1	P	<i>H. heliothidis</i>	Australia	2
	C2	S	<i>H. heliothidis</i>	Australia	2
	H1	P	<i>H. heliothidis</i>	USA (N. Carolina)	6
	H2	S	<i>H. heliothidis</i>	USA (N. Carolina)	6
	N1	P	<i>Heterorhabditis</i> sp.	Netherlands	7
	N2	S	<i>Heterorhabditis</i> sp.	Netherlands	7
	V1	P	<i>Heterorhabditis</i> sp.	USA (California)	8
	V2	S	<i>Heterorhabditis</i> sp.	USA (California)	8

* P, primary; S, secondary.

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Table 2. Characteristics of *Xenorhabdus* isolated from *Heterorhabditis* collected in the Netherlands and Victorville, USA

Characteristic	Netherlands		Victorville	
	Primary	Secondary	Primary	Secondary
No. of isolates	8	8	8	9
Cell morphology	rod	rod	rod	rod
Motility	+	+	+	+
Gram stain	—	—	—	—
Colony diameter (mm)	2.0–2.5	2.5–3.0	3.0–3.5	3.5–4.0
Pigmentation in:				
nutrient agar	orange	orange	orange	buff
MacConkey agar	red	orange	red	pink
Catalase	—	—	—	—
Bioluminescence	+	+	+	+
Antibiotic activity against isolates:				
A25, A25	+	+	+	+
T319/1, T319/2	+	+	+	+
G6	+	+	+	+
SK3/1, SK3/2	+	+	+	+

Fig. 1. Protein profiles of *Xenorhabdus luminescens*. See Table 1 for abbreviations of bacteria.

Fig. 2. Protein profiles of *Xenorhabdus nematophilus*. See Table 1 for abbreviations of bacteria. B, Bib; G, Glas; Ag, Agro; A, All; Br, Bret; I, Ital; M, Mex; Q, Q58; S, SK3; T, T319.

circular with an almost smooth margin, green with a large green-brown centre, and with a clear zone in the agar surrounding the colonies. The secondary form, however, was different from those previously described. Colonies were convex, circular with an irregular margin, brick red throughout, and imparted a red colour to the NBT agar.

Electrophoretic separation of *X. nematophilus* and *X. luminescens* is shown in Figs 1 and 2. Protein profiles were virtually identical for primary and secondary forms and within most subspecies of *X. nematophilus* from different geographical locations. Further, the different subspecies were electrophoretically distinct. Very little qualitative difference was observed between the protein profiles of *X. nematophilus* subsp. *nematophilus* and *X. nematophilus* subsp. *bovienii*. The former isolates had an additional band in the low molecular weight proteins as compared to the latter (isolates Bib and T319). The two *X. nematophilus* subsp. *poinarii* isolates were notably different in protein mobility. The group IV isolates were very similar in profile to that of *X. nematophilus* subsp. *bovienii* isolates SK3/1 and SK3/2.

Fig. 3. Isoenzyme stains of *Xenorhabdus luminescens*. See Table 1 for abbreviations of bacteria. G6P, glucose-6-phosphate dehydrogenase; LDH, lactate dehydrogenase; MDH, malate dehydrogenase; PGM, phosphoglucumutase.

The protein profiles of the primary and secondary forms of the two *H. heliothidis* isolates and the one *H. bacteriophora* isolate of *X. luminescens* were identical. However, the profile of the Netherlands and Victorville isolates differed in their primary and secondary forms from each other and from the other isolates. Further, the primary and secondary forms of the Victorville isolate differed from each other. Thus the primary and secondary forms of the Netherlands and Victorville isolates gave the appearance of being two separate strains.

Results from isoenzymic reactions substantiated the differences observed in the protein profiles (Figs 3 and 4). All isolates of *X. luminescens*, except for the secondary form of Netherlands and the primary form of Victorville, had identical mobility for all isoenzymes. The enzyme bands of primary Victorville forms consistently migrated faster than those of secondary Netherlands forms, which, in turn, migrated faster than enzyme bands of other isolates. Three isoenzyme reactions (MDH, LDH and PGM) revealed only one band and one isoenzyme reaction (G6P) had two or three bands.

The *X. nematophilus* isolates showed more isoenzymic variability than the *X. luminescens* isolates. Geographically distinct isolates had similar banding patterns except for isolate SK3, which differed from other *X. nematophilus* subsp. *bovienii* isolates (Bib and T319) in G6P and MDH reactions, and G6 and Glas which differed in G6P, LDH and MDH reactions. Group IV (Q58) was similar to *X. nematophilus* subsp. *bovienii* in 6PG reactions and to *X. nematophilus* subsp. *nematophilus* in LDH reaction. The MDH pattern of *X. nematophilus* subsp. *nematophilus* revealed numerous bands whereas other species had only one or two bands. All *X. nematophilus* extracts were prepared at the same time and under the same conditions and it seems unlikely therefore that these multiple MDH bands represent breakdown products. Alternatively, the MDH proteins of *X. nematophilus* subsp. *nematophilus* may be very heat labile and, despite cooling of the electrophoretic system, heating may have caused some denaturation of protein.

Fig. 4. Isoenzyme stains of *Xenorhabdus nematophilus*. See Table 1 for abbreviations of bacteria. G6P, glucose-6-phosphate dehydrogenase; LDH, lactate dehydrogenase; MDH, malate dehydrogenase; 6PG, 6-phosphogluconate dehydrogenase.

DISCUSSION

Electrophoretic techniques for identification and classification are widely utilized and are particularly useful for organisms that are difficult to distinguish by other means. Electrophoretic differences between bacterial strains can serve as a powerful tool for separation of species and subspecies because the same set of proteins is always produced by bacteria grown in standardized conditions (Kerstens & De Ley, 1980). However, with physiological and biochemical tests, the results may differ for identical species because of subjective interpretation of test results. These differences in interpretation can be overcome by electrophoretic techniques. Hence, isolates collected world-wide can be electrophoretically compared to one another for absolute identification of species (subspecies or strain) status.

Protein profiles of subspecies within the *X. nematophilus* group were similar but easily distinguished from one another and essentially substantiated previous subspecific designations. Subspecific isolates from the USA, Europe and Australia had virtually identical protein profiles in both the primary and secondary forms. Although isolate G6 differed from isolate Glas, this was probably due to the occurrence of two secondary forms of *X. nematophilus* subsp. *poinarii* (Akhurst, 1982). One can only speculate as to why *S. glaseri* has two secondary forms of bacteria. The profile of isolate SK3 bears a stronger resemblance to that of isolate Q58 than to the other two isolates of *X. nematophilus* subsp. *bovienii* (Bib and T319).

Protein profiles of isolates of *X. luminescens* from *H. bacteriophora* and *H. heliothidis* were identical, but differed from both Victorville and Netherlands isolates. The primary and secondary profiles of Victorville isolates differed from each other. This evidence suggests that there may be at least two subspecies of *X. luminescens*.

Since proteins with different primary structure may produce identical electrophoretic profiles, it is important to corroborate similarities by other methods, such as staining for specific enzymes or isoenzymes, as we have done. Coyne (1982) demonstrated that isoenzymes with apparently identical mobilities can be separated by changing electrophoretic conditions (pH of the separating gel and gel concentration), thus revealing cryptic protein variations. Although we expected no differences in isoenzyme profiles from isolates of the same subspecies, when we did look for cryptic differences in two enzymic reactions, we found the isoenzyme patterns to be unchanged.

Isoenzymic reactions may be used as further phenotypic evidence to confirm differences between subspecies, as seen when gels were stained for total proteins. The MDH, LDH and 6PG results revealed isolate SK3 to be close but not identical to the other *X. nematophilus* subsp. *bovienii* isolates (Bib and T319). However, the G6P results clearly separated the SK3 isolates. The Q58 isolates appear to be a different subspecies as overall isoenzymic reactions were not similar to any one subspecies.

Isoenzyme patterns of *X. luminescens* isolates reveal further differences between primary and secondary forms of Netherlands and Victorville isolates. The primary form of Netherlands and the secondary form of Victorville were always consistent with the other *X. luminescens* isolates, although the protein profile of secondary Victorville was clearly different from the other profiles. The phenotypic evidence from the protein profiles and isoenzyme patterns, taken with the gross morphology of the colonies of the Netherlands and Victorville isolates, suggests that these bacteria do not entirely conform to the description of *X. luminescens*. The cumulative evidence suggests several possibilities: that *X. luminescens* is composed of more than one primary or secondary form (analogous to the situation with *X. nematophilus* subsp. *poinarii*), that it is comprised of more than one subspecies, or that there is more than one species of *Xenorhabdus* associated with heterorhabditid nematodes.

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