Occurrence of Intracellular Inclusions and Plasmids in *Xenorhabdus* spp.

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Entomopathogenic bacteria of the genus *Xenorhabdus* produce crystalline inclusion bodies during *in vitro* culture. When cultured in liquid media, inclusions were present in primary forms but not secondary forms of *X. nematophilus*. In contrast, both primary and secondary forms of *X. luminescens* produced inclusion bodies during liquid culture. Two morphologically distinct forms of inclusion bodies were found in *X. nematophilus* subsp. *nematophilus* strain All. They were proteinaceous, and one of the proteins (IP-1) was present in seven strains of *X. nematophilus* subsp. *nematophilus*, but not in strains of *X. nematophilus* subsp. *bovienii*, *X. nematophilus* subsp. *poinarii*, *X. nematophilus* subsp. *beddingii* or *X. luminescens*. This protein may be useful as a taxonomic indicator. Plasmids were isolated from seven of ten strains of *Xenorhabdus*. They varied in size from 12 to 3.6 kb, and were present in both primary and secondary forms of *X. nematophilus* subsp. *nematophilus* and *X. nematophilus* subsp. *bovienii*.

INTRODUCTION

Insect pathogenic nematodes of the families *Heterorhabditidae* and *Steinernematidae* are symbiotically associated with bacteria of the genus *Xenorhabdus* (Thomas & Poinar, 1979; Akhurst, 1980). The bacterial symbiont is carried monoxenically within the intestine of non-feeding, infective-stage nematodes. Following invasion of an insect host, the infective nematode releases the bacteria, which contribute to killing the insect and enhance conditions for the reproduction of nematodes (Poinar & Thomas, 1966). *Xenorhabdus* species occur in two morphological forms (designated primary and secondary) which can be distinguished as differently coloured colonies on various bacteriological media and/or on the basis of antibiotic production (Akhurst, 1980, 1982). Both forms are pathogenic to insect larvae, but only the primary form is isolated from infective nematodes (Akhurst, 1980). Ultrastructural studies of various *Xenorhabdus* species have identified crystalline inclusions similar in appearance to the crystals of *Bacillus thuringiensis* (Boemare *et al.*, 1983; Grimont *et al.*, 1984).

Our investigations leading to the commercial development of entomogenous nematodes for use as insecticides have included studies on several aspects of the biology of *Xenorhabdus* bacteria. During investigations with *X. nematophilus* subsp. *nematophilus* (strain All), inclusions were observed in cells from primary form cultures. Two types of proteinaceous inclusion were isolated, large cigar-shaped inclusions containing a 26 kDa protein (IP-1) and small ovoid inclusions containing a 22 kDa protein (IP-2). The physico-chemical properties of these proteins and the kinetics of their synthesis will be described elsewhere (unpublished). In this report we describe the use of microscopic, electrophoretic and immunological techniques to examine a number of *Xenorhabdus* strains for inclusion formation during *in vitro* culture. Plasmid DNA profiles from these strains were prepared to examine the possible involvement of plasmid encoded functions in the formation of inclusion bodies and the transition from primary to secondary form.

Abbreviation: PMSF, phenylmethylsulphonyl fluoride.

Table 1. *Xenorhabdus* strains and their host nematodes

Bacterium		Nematode		Origin
Species	Strain	Species	Strain	
<i>X. nematophilus</i> subsp. <i>nematophilus</i>	All	<i>Steinernema feltiae</i>	All	Georgia, USA
	Mex		Mexican	Chihuahua, Mexico
	AN6		DD136	Virginia, USA
	BK		Breton	Brittany, France
	A24		Agriotos	Leningrad, USSR
	XnT		DD136	Virginia, USA
	Pi		Pieridarum	Poland
<i>X. nematophilus</i> subsp. <i>bovini</i>	T319	<i>S. bionis</i>	T319	Tasmania, Australia
	SK2	<i>S. kraussei</i>	SK/2	Czechoslovakia
	Dan 5	<i>S. affinis</i>	Dan 5	Denmark
<i>X. nematophilus</i> subsp. <i>poinarii</i>	NC513	<i>S. glaseri</i>	NC513	N Carolina, USA
<i>X. nematophilus</i> subsp. <i>beddingii</i>	Q385	<i>Steinernema</i> sp.	Q385	Queensland, Australia
<i>X. luminescens</i>	B1	<i>Heterorhabditis</i> <i>bacteriophora</i>	—	South Australia, Australia
	T327	<i>Heterorhabditis</i> sp.	—	Tasmania, Australia

METHODS

Bacterial strains. The strains of *Xenorhabdus* used in this study are listed in Table 1. All strains were kindly provided by Drs R. Bedding and R. Akhurst, CSIRO, Division of Entomology, Tasmania, Australia.

Media and culture conditions. *Xenorhabdus* strains were cultured and maintained on nutrient agar supplemented with 0.004% triphenyl tetrazolium chloride and 0.025% bromothymol blue (NBT agar; Akhurst, 1980). Where appropriate, NBT agar was used to distinguish primary and secondary forms as previously described (Akhurst, 1980; Hotchin & Kaya, 1984). However, primary and secondary forms of some strains (SK2, NC513, B1, T327) could not be distinguished on the basis of colony colour on NBT agar. Primary forms of these strains produce antibiotics, which were detected using overlay procedures with *Micrococcus luteus* as the test organism (Akhurst, 1982). Tryptone soya broth (Oxoid) was used as the complete growth medium for liquid cultures. Bacteria were cultured at 28 °C in 500 ml Erlenmeyer flasks containing 100 ml medium aerated by rotary agitation on an orbital shaker operating at 160 r.p.m.

Isolation of inclusions from *X. nematophilus* subsp. *nematophilus* (All). The bacteria were grown to stationary phase (48 h), harvested by centrifugation, resuspended in buffered saline (15 mM-K₂HPO₄, 85 mM-NaCl, pH 7.3), and disrupted by passage through a French press at 14000 lbf in⁻² (96.6 MPa). Inclusions were purified from the lysate by differential centrifugation (450 g, 5 min) in 30% (w/w) glycerol, followed by centrifugation (1500 g, 15 min) in discontinuous gradients formed by successive layering of 10 ml 75, 60 and 30% (w/w) glycerol. The inclusions banding in the 60% glycerol layer and at the 60/75% glycerol interface were further purified by isopycnic density gradient centrifugation (20000 g, 60 min) in linear 40–55% (w/v) sodium diatrizoate (Sigma) gradients. Inclusion bands were washed free of residual salt and lyophilized.

Preparation of cell lysates and protein estimation. Cells from 1 ml stationary-phase broth culture were suspended in 3 ml buffered saline containing 0.5 mM-PMSF and 5 mM-EDTA, then lysed by passage through a French press at 14000 lbf in⁻². Portions of lysate were solubilized in 2% (w/v) SDS for 30 min at room temperature, and total protein was estimated as described below from dot blots prepared using a Minifold microfiltration apparatus (Schleicher and Schuell). SDS-solubilized proteins were diluted to 250 µl with deionized H₂O and the proteins were precipitated by the addition of 50 µl 90% (v/v) TCA. Protein precipitates were collected on nitrocellulose, washed with 6% (w/v) TCA, stained for 10 min with 0.25% (w/v) naphthol blue black in 50% (v/v) methanol, 10% (v/v) acetic acid, and destained in 90% (v/v) methanol, 2.0% (v/v) acetic acid. Protein spots were excised, the dye was eluted in 1 ml 25 mM-NaOH, 50 mM-EDTA in 50% (v/v) ethanol, and the A₆₃₀ was determined. Protein content was estimated by comparison with IP-1 protein standards.

SDS-PAGE of cell lysates. Portions of lysate containing 100 µg total protein were solubilized in 2% (w/v) SDS and precipitated with TCA as described above. Precipitates were harvested by centrifugation (Beckman Microfuge), washed twice with 1 ml 100% ethanol, and resuspended in 50 µl SDS sample buffer. Samples were heated to 100 °C for 5 min and insoluble material was removed by centrifugation. Samples were analysed in 12.5%

(w/v) gels as described by Laemmli (1970). Electrophoresis was done in a Bio-Rad Miniprotein II electrophoresis cell following the manufacturer's instructions. Gels were stained with Coomassie blue R-250 or subjected to electrophoretic transfer blotting onto nitrocellulose.

Preparation of antisera. Purified inclusions were solubilized in SDS sample buffer and subjected to electrophoresis in 3 mm thick preparative SDS gels. Following electrophoresis, gels were incubated at 4 °C in 0.25 M-KCl for 30 min. White bands corresponding to precipitated potassium dodecyl sulphate-protein complexes were excised, divided into three equal portions and stored at -20 °C. Gel strips containing approximately 200 µg protein were thawed, macerated with a spatula, mixed with 1 ml 0.85% saline and 1.5 ml Montanide 888 adjuvant [Montanide 888 (SEPPIC) diluted 1 vol. in 9 vols Marcol 52 oil], and then homogenized using a Sorvall Omnimixer. The resulting emulsion was injected into New Zealand White rabbits in a series of three injections, 1 ml intramuscularly into each hip and 1 ml subcutaneously in the neck. Rabbits received a similar series of injections at monthly intervals for three months, and were bled 3 d after the final challenge. Crude serum was affinity purified on Protein A-Sepharose (Pharmacia) equilibrated with PBS (0.1 M-Na₂HPO₄ 0.17 M-NaCl, pH 8.0). Bound IgG was eluted with 0.1 M-glycine/HCl, pH 3.0. Fractions (4 ml) containing IgG were immediately mixed with 2 ml 2 M-Tris/HCl, pH 8.0, and then pooled. The resulting solution was extensively dialysed against 0.1 M-NaCl at 4 °C. The final IgG concentration was adjusted to 1 mg ml⁻¹ with 0.1 M-NaCl, and the solution was divided into 1 ml portions and stored at -20 °C.

Analysis of proteins by electrophoretic transfer blotting. Proteins from acrylamide gels were electrophoretically transferred to nitrocellulose (Towbin, 1979; Burnette, 1981) in a Hoefer Mighty Small Transphor unit. Non-specific binding sites on nitrocellulose filters were blocked by a 90 min incubation in Blotto, a solution of 10% (w/v) milk powder in 10 mM-Tris, 0.15 M-NaCl, pH 7.5 (Johnson *et al.*, 1984). The filters were rinsed twice in Tris/saline/Tween (1 min each; 12 mM-Tris, 0.12 M-NaCl, 0.05%, v/v, Tween 20, pH 8.0) and then incubated overnight at 4 °C in a 1:1 mixture of anti-IP-1 and anti-IP-2 IgG, final IgG concentration 1 µg ml⁻¹ in Tris/saline/Tween. Filters were successively washed three times each in Tris/saline (12 mM-Tris, 0.12 M-NaCl, pH 8.0), Tris/saline/Tween, and Tris/saline. Anti-rabbit IgG-alkaline phosphatase conjugate (Sigma) diluted 1/5000 with Tris/saline/Tween was added and the reaction was allowed to proceed at room temperature for 90 min. Unbound conjugate was removed by washing as described above. Bound IgG was revealed by addition of a substrate solution containing 0.01% (w/v) nitro-blue tetrazolium, 50 µg ml⁻¹ 5-bromo-4-chloro-3-indoxyl phosphate and 3 mM-MgCl₂ in 0.18 M-sodium barbitone/sodium acetate, pH 10.0.

Analysis of plasmid DNA. Plasmid DNA was isolated from cells by alkaline lysis as described by Maniatis *et al.* (1982). Procedures for the analysis of DNA by restriction endonucleases (*Eco*RI and *Bam*HI) and agarose gel electrophoresis were as reported previously (Lehrbach *et al.*, 1983).

RESULTS

Occurrence of inclusions

When *Xenorhabdus* was cultured on NBT agar for 72 h, inclusions were present in all isolates with the exception of secondary form *X. nematophilus* subsp. *nematophilus* strain All (Table 2). In shake flasks, cultures reached stationary phase in 48 h at 28 °C. At this time, inclusions were visible in cells from primary-form cultures of most strains, and in secondary forms of *X. luminescens* (B1 and T327). Neither primary- nor secondary-form cultures of *X. nematophilus* subsp. *poinarii* (strain NC513) contained inclusion-forming cells.

Inclusion morphology varied with the strain of *Xenorhabdus*. Small ovoid inclusions were present in all inclusion-forming strains, and were the only type of inclusion produced by strains SK2, Q385 and B1. Strains All, BK, A24, AN6, Mex, T319, Dan 5 and T327 produced inclusions of variable morphology and size, and commonly contained more than one inclusion (Fig. 1). The frequency of occurrence of inclusion-containing cells in TSB cultures was similarly variable. In cultures of strains All, BK, A24, Mex, AN6, T319 and T327, over 50% of cells contained one or more inclusions.

Electrophoretic analysis of cell lysates from *Xenorhabdus* strains

Cell lysates of 48 h cultures were analysed by SDS-PAGE to determine whether IP-1 and IP-2 were common to all strains of *Xenorhabdus* (Fig. 2). Bands appearing to correspond to IP-1 were present in extracts from primary-form cultures of *X. nematophilus* subsp. *nematophilus* (strains All, Mex and AN6; Fig. 2a, lanes 3, 4 and 5), while a band of similar mobility to IP-2 was found in extracts of strains All and Mex (Fig. 2a, lanes 3 and 4). IP-1 and IP-2 did not appear to be present in lysates of primary- or secondary-form *X. nematophilus* subsp. *bovienii* (T319, SK2

Fig. 1. Cells of *X. nematophilus* subsp. *nematophilus*, strain Mex. Dense inclusion bodies of variable morphology are present (arrows). Bar, 1 μ m.

Table 2. Occurrence of inclusions and plasmids in selected strains of *Xenorhabdus*

Strain	Form*	Presence of inclusions†		Inclusion morphology‡	Plasmid size (kb)§
		NBT agar	TSB		
All	1°	+	+	V	12, 3.6
	2°	—	—	—	12, 3.6
BK	1°	+	+	V	12, 3.6
	2°	+	+	—	12, 3.6
A24	1°	+	+	V	12, 3.6
	2°	+	—	—	12, 3.6
XnT	1°	ND	ND	ND	10
	2°	ND	ND	ND	10
Mex	1°	+	+	V	7
AN6	1°	+	+	V	—
T319	1°	+	+	V	7, 3.6
SK2	1°	+	+	O	5, 3.6
Dan 5	1°	+	+	V	—
	2°	+	—	—	—
NC513	1°	+	—	—	ND
	2°	+	—	—	ND
Q385	1°	+	+	O	—
	2°	+	—	—	—
B1	2°	+	+	O	ND
T327	2°	+	+	V	ND

ND, Not determined.

* Primary (1°) and secondary (2°) forms determined from colony pigmentation on NBT agar indicator plates, or from the presence of antibiotics as revealed by *Micrococcus* overlay procedures.

† NBT agar, nutrient agar containing bromothymol blue and triphenyl tetrazolium chloride; TSB, tryptone soya broth. +, Inclusions present; —, inclusions absent.

‡ Morphology of inclusions in TSB cultures: O, ovoid; V, variable.

§ Plasmid DNA size was determined by agarose gel electrophoresis of fragments obtained by cleavage with restriction endonucleases *Eco*RI and *Bam*HI and comparison with known DNA size markers. —, No plasmids detected.

and Dan 5), *X. nematophilus* subsp. *poinarii* (NC513), *X. nematophilus* subsp. *beddingii* (Q385), or *X. luminescens* (B1 and T327).

Analysis of cell lysates by electrophoretic transfer blotting

Immunological detection was used to identify IP-1 and IP-2 or other forms of these proteins in cell lysates (Fig. 3). Immunoblots confirmed the presence of IP-1 in lysates of *X. nematophilus* subsp. *nematophilus* strains All, Mex and AN6, and its absence in lysates from primary or

Fig. 2. SDS-PAGE of *Xenorhabdus* lysates. (a) Primary form: lane 1, molecular mass marker proteins (Bio-Rad); lanes 2 and 11, purified IP-1 and IP-2 (0.5 µg per band); lanes 3–10, strains All, Mex, AN6, SK2, T319, NC513, Dan 5 and Q385 respectively. (b) Secondary form: lane 1, molecular mass marker proteins; lanes 2 and 8, purified IP-1 and IP-2 (0.5 µg per band); lanes 3–7, strains T327, B1, NC513, Q385 and Dan5 respectively. Lysate samples contained approximately 8 µg protein.

Fig. 3. Immunoblots from SDS-PAGE gels of (a) primary form and (b) secondary form *Xenorhabdus* lysates. Molecular mass markers, inclusion protein standards, and strains are as described in Fig. 2. The lysate samples for SDS-PAGE contained approximately 4 µg protein; the inclusion protein standards contained 0.25 µg per band.

secondary cultures of ten other *Xenorhabdus* strains. Similarly, IP-2 was found in cultures of *X. nematophilus* subsp. *nematophilus* All and Mex, but was absent from the others. The anti-inclusion protein IgG further recognized a minor band of approximately 18 kDa in all lysates, and background reaction with higher molecular mass proteins was significant in samples from those strains that synthesized large quantities of IP-1 and IP-2 (Fig. 3a, lanes 3 and 4).

Presence of plasmids in Xenorhabdus strains

The presence of plasmid DNA in ten strains of *Xenorhabdus* was determined using agarose gel electrophoresis. Plasmids were detected in primary-form cultures of seven of the ten strains

examined (Table 2), with three of these (All, BK and A24) having identical plasmid patterns. Where both forms were analysed, no differences in plasmid content were detected between primary and secondary cultures.

DISCUSSION

Ultrastructural studies of cells from various *Xenorhabdus* strains have revealed the presence of crystalline inclusion bodies similar in morphology to the crystals of *B. thuringiensis* (Boemare *et al.*, 1983; Grimont *et al.*, 1984). These inclusions can be readily observed under the light microscope using phase contrast optics, where they appear as phase bright structures occupying up to a third of the cytoplasm. We have examined various strains of *Xenorhabdus* for the formation of intracellular inclusions during *in vitro* culture. These strains were selected to represent the major taxonomic groups described by Akhurst (1983, 1986).

The ability to form inclusions under appropriate culture conditions appears to be a general characteristic of *Xenorhabdus* bacteria, as inclusions were observed in most of the cultures grown on nutrient agar. In rich liquid media such as tryptone soya broth, inclusion formation by *X. nematophilus* strains is dependent on the morphological and presumably physiological form of the bacteria. Inclusions were present only in primary-form cultures except for strain NC513 where neither primary nor secondary forms produced inclusions. In contrast, both primary forms (Boemare *et al.*, 1983) and secondary forms of *X. luminescens* produced inclusions in liquid media.

Two morphologically distinct types of inclusion were formed in primary cultures of *X. nematophilus* subsp. *nematophilus*, strain All. SDS-PAGE analysis of purified inclusions from these bacteria has revealed that both types of inclusion are proteinaceous, and that each is composed of a single protein subunit (IP-1 or IP-2). Electrophoretic blotting of proteins from SDS-PAGE gels onto nitrocellulose, followed by immunological detection with polyclonal antisera prepared against IP-1 and IP-2, has demonstrated that these proteins are subspecies specific, being found only in lysates of *X. nematophilus* subsp. *nematophilus*. Protein IP-1 which has been identified in lysates from seven strains of *X. nematophilus* subsp. *nematophilus* (All, Mex, AN6, Fig. 3a; BK, A24, XnT and Pi, unpublished data), may be useful as a taxonomic indicator.

The composition of inclusions formed by strains of *X. nematophilus* subsp. *bovienii*, *X. nematophilus* subsp. *poinarii*, *X. nematophilus* subsp. *beddingii* and *X. luminescens* is not known. If proteinaceous, the lack of immunological relatedness to IP-1 and/or IP-2 (Fig. 3) suggests that they may also be structurally unrelated to these proteins.

The function(s) of inclusions produced by *Xenorhabdus* is likely to be significant, as this phenomenon appears in all strains. Boemare *et al.* (1983) suggested that *Xenorhabdus* inclusions may be similar to the crystalline protein toxins of *B. thuringiensis*, as both bacteria are associated with entomopathogenesis. However, the subunit sizes of IP-1 and IP-2 are smaller than most of the *B. thuringiensis* toxins, except for the 28 kDa cytolytic toxin of *B. thuringiensis* subsp. *israelensis* (Davidson & Yamamoto, 1984; Hurley *et al.*, 1985). Furthermore, no toxicity was detected when IP-1 and/or IP-2 were administered by injection or feeding to insects such as *Heliothis punctiger* and *Lucilia cuprina*. Haemolytic activity such as that associated with the 28 kDa protein of the *B. thuringiensis* subsp. *israelensis* crystal (Thomas & Ellar, 1983; Pfannenstiel *et al.*, 1984) has not been detected in solubilized IP-1 and/or IP-2 preparations. The role(s) of inclusion proteins is therefore unclear. The possibility that these proteins serve as a nutrient and/or energy source while the host nematode is dormant in the soil is being investigated further.

Plasmids have not previously been detected in *Xenorhabdus* strains (Akhurst, 1980); however we have identified one or more plasmids in many of the strains examined. Plasmid content was similar in primary- and secondary-form cultures, suggesting that plasmid loss is not involved in the transition from primary to secondary form. The question of whether plasmid-encoded functions are involved in inclusion body formation requires further investigation. This is apparently not so in certain strains (AN6, Dan 5 and Q385), as these strains form inclusions but lack plasmids.

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