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Protein Inclusions Produced by the Entomopathogenic Bacterium *Xenorhabdus nematophilus* subsp. *nematophilus*

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The entomopathogenic bacterium *Xenorhabdus nematophilus* subsp. *nematophilus* produces two types of intracellular inclusion bodies during in vitro culture. Large cigar-shaped inclusions (designated type 1) and smaller ovoid inclusions (designated type 2) were purified from cell lysates, using differential centrifugation in discontinuous glycerol gradients and isopycnic density gradient centrifugation in sodium diatrizoate. The inclusions, composed almost exclusively of protein, are readily soluble at high and low pH values and in the presence of cation chelators such as EDTA, anionic detergents (sodium dodecyl sulfate), or protein denaturants (urea, NaBr). Sodium dodecyl sulfate-polyacrylamide gel electrophoresis of purified inclusions revealed a single 26-kilodalton protein (IP-1) in type 1 inclusions and a 22-kilodalton protein (IP-2) in type 2 inclusions. Analysis of these proteins by isoelectric focusing in the presence of 8 M urea showed that IP-1 is acidic and IP-2 is neutral. Furthermore, each protein occurred in multiple forms differing slightly in isoelectric point. Other variations in peptides released by trypsin digestion, immunological properties, and amino acid composition revealed significant structural differences between IP-1 and IP-2. Kinetic studies using light microscopy, sodium dodecyl sulfate-polyacrylamide gel electrophoresis, and immunoblotting procedures showed that inclusion protein synthesis occurs only during the second half of exponential culture growth. Synthesis of inclusion proteins and their aggregation to form inclusions occurred concurrently. Possible functions for these abundant proteins are discussed.

Xenorhabdus species are entomopathogenic bacteria symbiotically associated with insect parasitic nematodes of the families Heterorhabditidae and Steinernematidae (1, 3, 30). The bacterial symbionts are asporogenous gram-negative rods, which are carried monoxenically in the intestine of nonfeeding infective-stage nematodes. On penetration of an insect host, the bacteria are released into the hemocoel. Colonization by the bacteria kills the host and establishes a suitable environment for reproduction of the nematodes by providing nutrients and inhibiting the growth of other microorganisms (1, 24).

When cultured in vitro, *Xenorhabdus* bacteria occur in two forms designated primary and secondary, which can be distinguished according to colony morphology and pigmentation on various bacteriological media, or on the basis of antibiotic production (1, 2). Both forms are pathogenic to insects, but only the primary form is isolated from infective nematodes (1). *Xenorhabdus* spp. will grow in a wide variety of artificial media which are rendered suitable for nematode reproduction, thus providing the basis for economical mass production of nematodes. Our investigations leading to the commercial development of entomogenous nematodes for use as insecticides have included studies on several aspects of *Xenorhabdus* biology. One area of interest relates to intracellular inclusion formation by *Xenorhabdus* spp.

During studies with *Xenorhabdus nematophilus* subsp. *nematophilus* (All strain), large inclusions were observed in cells from primary-form cultures. A subsequent survey of 11 strains of *X. nematophilus* and 2 strains of *Xenorhabdus luminescens* showed that all isolates were capable of producing inclusions when grown on solid media. In liquid media, inclusion formation occurred only in primary-form *X. nematophilus*, in contrast to *X. luminescens* in which both primary and secondary forms produced inclusions (10). Intracellular inclusion bodies have also been described in several ultrastructural studies of *Xenorhabdus* spp. (4, 7, 15, 18).

In this report, we describe the purification of two types of proteinaceous inclusion from *X. nematophilus* subsp. *nematophilus*, the physicochemical properties of the proteins from these inclusions, and the kinetics of their synthesis.

MATERIALS AND METHODS

Bacteria and culture conditions. Primary-form bacteria were isolated from cultures of *X. nematophilus* subsp. *nematophilus* (All strain; ATCC 53200) originally provided by R. J. Akhurst, Commonwealth Scientific and Industrial Research Organisation Division of Entomology, Canberra, Australia. The bacteria were maintained on nutrient agar (Oxoid Ltd.) supplemented with 0.004% (wt/vol) triphenyl tetrazolium chloride and 0.025% (wt/vol) bromothymol blue (1). 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 of medium aerated by rotary agitation on an orbital shaker operating at 160 rpm.

Isolation of inclusions. Inclusions were purified from cell lysates by differential centrifugation in discontinuous glycerol gradients and isopycnic centrifugation in sodium diatrizoate (Sigma Chemical Co.) density gradients as follows. In a typical purification, bacteria from 700 ml of stationary-phase culture (72 h) were harvested by centrifugation (10,000 × *g*, 15 min), suspended in 20 ml of buffered saline (15 mM K_2HPO_4, 85 mM NaCl, pH 7.3), and twice passed through a French press operated at 14,000 lb/in^2. The cell lysate was diluted with 80 ml of saline, and 10-ml aliquots were layered onto 25-ml cushions of 30% (wt/wt)

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glycerol in 50-ml plastic centrifuge tubes. Samples were centrifuged at 450 × *g* for 5 min. The material remaining in the saline or at the interface and the pellets containing intact cells and aggregates of debris were discarded. The glycerol fractions containing inclusions were pooled, and the inclusions were harvested by centrifugation (12,000 × *g*, 5 min). The pellets were taken up in 20 ml of saline, suspended by vortexing and sonication (10 min), and centrifuged into 30% (wt/wt) glycerol two more times. Resuspended inclusions (5-ml aliquots) were layered onto discontinuous gradients formed by successive layering of 10 ml each of 70, 60, and 30% (wt/wt) glycerol and centrifuged at 1,500 × *g* for 15 min. Inclusions were concentrated in the 60% (wt/wt) glycerol layers. The 60% (wt/wt) glycerol fractions were pooled and diluted (1:1) with saline, and the inclusions were harvested by centrifugation (12,000 × *g*, 5 min). The pellet was washed two times with saline, suspended in 20 ml of saline, and stored at −20°C until further purification.

Partially purified inclusions (5-ml aliquots) were layered onto 40 to 55% (wt/vol) sodium diatrizoate gradients (25 ml) in polyallomer centrifuge tubes. Tubes were centrifuged at 20,000 × *g* for 1 h in a Beckman SW28 swinging-head rotor. Two inclusion bands and a diffuse intermediate band of cell debris were harvested by aspiration and identified by microscopy. Homogeneous inclusion preparations were obtained after three successive density gradient purifications. Purified inclusions were extensively washed with deionized water and then lyophilized. Yields from such a purification were 80 to 100 mg of type 1 inclusions and 7 to 10 mg of type 2 inclusions.

Solubility experiments. Inclusions (300-μl aliquots; 500 μg/ml in deionized water) were mixed with 300 μl of a twice-concentrated stock solution of the appropriate solvent, and samples were incubated at 35°C for 1 h. Optical density recorded at 600 nm was used to indicate reductions in light scattering in the samples, and percent solubilization was estimated by comparison with appropriate controls. Solubility experiments were performed in triplicate except for urea and NaBr solubilizations, which had two replicates per treatment.

Trace element analysis. Trace element analysis was performed at the Australian Mineral Development Laboratories, Adelaide, South Australia. Purified inclusions (200 mg) were hydrolyzed in 1 ml of concentrated HNO_3, and the sample was diluted to 10 ml with deionized water. The sample was analyzed by simultaneous inductively coupled plasma atomic emission spectroscopy (6), using an ARL 3400 optical emission quantometer (Applied Research Laboratories), calibrated against synthetic reference standards. Elements assayed were Ca, Mn, Na, Mg, K, Ag, Fe, Cu, Pb, Zn, Al, Cd, Co, Ni, Cr, V, Ba, As, Mo, B, Sr, La, Sn, W, Ti, Y, P, SO_4, and SiO_2.

Carbohydrate analysis. Purified inclusions were analyzed for the presence of carbohydrates, using the phenol-sulfuric acid procedure as described by Herbert et al. (16). Carbohydrate content was estimated from the A_{490} by comparison with glucose standards.

Nucleic acid analysis. Nucleic acid content of inclusions was estimated by using a modification of the procedure described by Loffler and Labbe (20). Inclusions (500 μg) were taken up in 0.3 N $HClO_4$ (1 ml) at 4°C. Insoluble material was harvested by centrifugation (5,000 × *g*, 5 min), washed with 0.2 N $HClO_4$ (1 ml), air dried, and then hydrolyzed in 0.3 N KOH (0.5 ml) for 1 h at 37°C. After acidification with 0.6 N $HClO_4$ (0.5 ml), the mixture was centrifuged (5,000 × *g*, 5 min) and acid-soluble RNA hydrolysis products were determined from the A_{260} of the supernatant. The pellet was solubilized in 0.3 N KOH (1 ml) for 16 h at 37°C. $HClO_4$ (2 ml, 0.6 N) was added and the mixture was heated to 90°C for 10 min. Tubes were cooled and centrifuged (5,000 × *g*, 10 min), and acid-soluble DNA hydrolysis products were estimated from the A_{260} of the supernatant. Nucleic acid concentrations were calculated by using $E_{1\ cm}^{0.1\%} = 50$ for oligonucleotides (21).

SDS-PAGE. Unless otherwise stated, proteins subjected to polyacrylamide gel electrophoresis (PAGE) in the presence of sodium dodecyl sulfate (SDS) were analyzed in 12.5% gels as described by Laemmli (19). Electrophoresis was performed in a Bio-Rad Mini-protean II electrophoresis cell following the instructions of the manufacturer. Gels were stained with Coomassie blue (0.1% [wt/vol] Coomassie blue R-250, 50% [vol/vol] methanol, 10% [vol/vol] acetic acid) and destained in 50% (vol/vol) methanol–10% (vol/vol) acetic acid (15 min) followed by 5% (vol/vol) ethanol–7% (vol/vol) acetic acid.

Isoelectric focusing. Purified inclusions were solubilized in 8 M urea (Fluka) and subjected to isoelectric focusing in 0.4-mm-thick polyacrylamide slab gels (6% monomer, 1.5% cross-linker) containing 8 M urea and 2% Pharmalyte 3-10 (Pharmacia). After being prefocused at 0.5 W for 1 h, samples were applied and gels were focused at 1.0 Watt for 3 h (total, 3,600 V · h), using a Pharmacia electrophoresis system consisting of an FBE 3000 electrophoresis cell, an ECPS 3000/150 power supply, and a VH-1 volt-hour integrator. Coolant temperature was 18°C, the catholyte was 50 mM NaOH, and the anolyte was 25 mM H_3PO_4. After focusing, gels were fixed for 20 min in 5% (wt/vol) sulfosalicylic acid–10% (wt/vol) trichloroacetic acid (TCA), washed for 15 min in destaining solution (25% [vol/vol] methanol, 10% [vol/vol] acetic acid, 0.5% [wt/vol] $CuSO_4$), and stained for 20 min with Coomassie blue (0.05% [wt/vol] Coomassie blue G-250 in destaining solution). Gels were destained through several changes of destaining solution, and traces of $CuSO_4$ were removed by washing in 7% (vol/vol) methanol–5% (vol/vol) acetic acid. pH gradients were identified from the locations of proteins of known pI (broad-range calibration kit; Pharmacia).

Partial proteolysis of IP-1 and IP-2. Type 1 or 2 inclusions (1.5 mg) were solubilized in 1 ml of 2.5 mM NaOH, pH 11.25, for 1 h at 35°C. Insoluble material was removed by centrifugation (15,000 × *g*, 10 min.), and the soluble protein concentration of the supernatants was estimated from the A_{280} ($E_{1\ cm}^{1\%} = 10$ for IP-1; $E_{1\ cm}^{1\%} = 15$ for IP-2). The protein concentration was adjusted to 1 mg/ml with deionized water, and aliquots (approximately 50 μl) of 0.5 M Tris, pH 8.0, were added to give a final buffer concentration of 25 mM. The final pH was 8.1. Solubilized inclusion protein in 25 mM Tris, pH 8.1 (200 μl, 1 mg/ml), was mixed with 5 μl of trypsin (40 μg/ml in deionized water; Boehringer Mannheim Biochemicals), yielding a final enzyme concentration of 1 μg/ml and a protein/enzyme ratio of 1,000:1. Proteolysis was allowed to proceed at 30°C. At various times after addition of enzyme, 25-μl aliquots of digest were removed, mixed with 1 μl each of 250 mM EDTA and 50 mM phenylmethylsulfonyl fluoride and 25 μl of double-strength SDS sample buffer, and the samples were immediately heated to 100°C for 5 min. Samples were analyzed by SDS-PAGE in 18% Laemmli gels.

Amino acid analysis. Phenylisothiocyanate derivatives from acid hydrolysates of purified inclusions were prepared as described by Cohen et al. (9). Samples were analyzed by using a PICO-TAG amino acid analysis system (Waters Associates).

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 sulfate-protein complexes were excised, divided into three equal portions, and stored at −20°C. Gel strips containing approximately 200 μg of protein were thawed, macerated with a spatula, mixed with 1 ml of isotonic saline and 1.5 ml of Montanide 888 adjuvant (Montanide 888 [SEPPIC, Paris] diluted 1 part in 9 parts Marcol 52 oil), and then homogenized with a Sorvall Omnimixer. The resulting emulsion was injected into New Zealand White rabbits as 1-ml intramuscular injections into each hip and a 1-ml subcutaneous injection in the neck. Rabbits received a similar series of injections at monthly intervals for 3 months and were bled 3 days after the final challenge.

Affinity purification of antisera. Crude serum was affinity purified on protein A-Sepharose (Pharmacia) equilibrated with phosphate-buffered saline (PBS; 0.1 M Na_2HPO_4, 0.17 M NaCl, pH 8.0). Bound immunoglobulin G (IgG) was eluted with 0.1 M glycine-HCl, pH 3.0. Fractions (4 ml) containing eluted protein (identified from the A_{280}) were immediately mixed with 2 ml of 2 M Tris hydrochloride, pH 8.0, and then pooled. The resulting solution was dialyzed against 0.1 M NaCl at 4°C. The final IgG concentration was adjusted to 1 mg/ml with 0.1 M NaCl, divided into 1-ml aliquots, and stored at −20°C.

Enzyme-linked immunosorbent assay procedures. Purified inclusion proteins (10 μg) in 100 μl of 2.5 mM NaOH–5mM EDTA were added to wells of a 96-well microtiter plate and incubated at 37°C overnight. The buffer was discarded and the plate was washed five times with PBS-Tween (50 mM NaH_2PO_4, pH 7.2, containing 0.17 M NaCl and 0.05% [vol/vol] Tween 20). Crude antiserum diluted with PBS, or PBS alone (100 μl), was added to the appropriate wells, and the plate was incubated at 37°C for 1 h. The plate was washed five times with PBS-Tween, and anti-rabbit IgG-horseradish peroxidase conjugate (Sigma) diluted 1/1,000 with PBS-Tween (100 μl) was added to each well. After incubation at 37°C for 1 h, unbound conjugate was removed by washing (five times) with PBS-Tween. Substrate solution [1 mg/ml; 2,2-azinobis (3-ethylbenzthiazoline sulfonic acid) [Sigma] in 0.1 M Na_2HPO_4-citric acid, pH 4.0, containing 1 μl of 32% (wt/wt) H_2O_2 per 10 ml of substrate solution; 100 μl per well] was added, and color development was allowed to proceed for 5 to 10 min. The reaction was stopped by addition of 1% (wt/vol) sodium azide (10 μl), and the results were recorded with a Titertek Multiscan plate reader (Flow Laboratories, Inc), using dual filters of 414 and 492 nm.

Protein estimation and preparation of SDS samples from cell lysates. Cells from 0.5- to 5-ml aliquots of tryptone soya broth cultures were harvested by centrifugation and suspended in 3 ml of buffered saline (15 mM K_2HPO_4, 85 mM NaCl, pH 7.3) containing 50 μM leupeptin–0.5 mM phenylmethylsulfonyl fluoride–5 mM EDTA. The cells were disrupted by passage through a French press operated at 14,000 lb/in^2. Aliquots of lysate (5 to 25 μl) were diluted to 220 μl with deionized water and then solubilized by the addition of 30 μl of 1 M Tris hydrochloride (pH 7.5) containing 2% (wt/vol) SDS. Proteins were precipitated by the addition of 50 μl of 90% (wt/vol) trichloroacetic acid (TCA). Protein precipitates were collected on nitrocellulose, using a Minifold microfiltration apparatus (Schleicher & Schuell). Precipitates were washed with 500 μl of 6% (wt/vol) TCA, stained for 10 min with 0.25% naphthol blue black in 50% (wt/vol) methanol–10% (vol/vol) acetic acid, and destained in 90% (vol/vol) methanol–2% (vol/vol) acetic acid. Protein spots were excised, the dye was eluted in 1 ml of 25 mM NaOH–50 mM EDTA in 50% (vol/vol) ethanol, and the A_{630} was determined. Protein content was estimated by comparison with inclusion protein standards. Aliquots of cell lysates containing 100 μg of total protein were made up to 750 μl with deionized water. Proteins were precipitated by addition of 150 μl of 90% (wt/vol) TCA, and the precipitate was harvested by centrifugation (15,000 × *g*, 5 min). Pellets were washed two times with 1 ml of ethanol and then solubilized for 10 min at 100°C in 50 μl of SDS sample buffer.

Electrophoretic blotting and immunological detection on nitrocellulose. Proteins from acrylamide gels were electrophoretically transferred to nitrocellulose (8, 32) in a Hoefer Mighty Small Transphor unit. Nonspecific binding sites on nitrocellulose filters were blocked by incubation for 90 min in Blotto, a solution of 10% (wt/vol) milk powder in 10 mM Tris–0.15 M NaCl, pH 7.5 (17). The filters were rinsed two times in Tris-saline-Tween (1 min each; 12 mM Tris, 0.12 M NaCl, 0.05% [vol/vol] Tween 20, pH 8.0) and then incubated overnight at 4°C in 1 μg of anti-inclusion protein IgG per ml 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 1/5,000 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% (wt/vol) Nitro Blue Tetrazolium, 50 μg of 5-bromo-4-chloro-3-indoxyl phosphate per ml, and 3 mM $MgCl_2$ in 0.18 M sodium barbitone-sodium acetate, pH 10.0.

RESULTS

Occurrence and purification of inclusions. *Xenorhabdus* cultures reached stationary phase in 2 to 3 days. Phase-contrast microscopy of 3-day cultures revealed large oval to rod-shaped cells 3 to 6 μm in length by 1 to 1.5 μm in width and occasional spheroplasts. Both rods and spheroplasts contained one or two phase-bright inclusions. A large cigar-shaped inclusion (type 1) occupying up to one-third of the cytoplasm was present in all cells. When a cell contained two inclusions, the second was always a smaller ovoid form (type 2; Fig. 1).

Differential centrifugation of cell lysates in glycerol gradients has been used successfully in the purification of polyhydroxybutyrate granules from *Bacillus megaterium* (29). Similar procedures employing discontinuous glycerol gradients provided a simple efficient means of separating *Xenorhabdus* inclusion bodies from cell debris as judged by microscopic examination and SDS-PAGE (Fig. 2). Isopycnic density gradient centrifugation of glycerol-purified inclusions in sodium diatrizoate gradients subsequently effected the purification to homogeneity of two types of inclusions with densities of 1.23 and 1.27 kg/liter. Microscopic examination identified these as type 1 and 2 inclusions, respectively (Fig. 1).

Solubility characteristics of type 1 and 2 inclusions. Lyophilized type 1 and 2 inclusions are insoluble in deionized water and common laboratory buffers at neutral pH, but readily disperse to form homogeneous suspensions. These properties were exploited to provide a simple means of assessing inclusion solubility based on the reduction in light scattering by inclusion suspensions. Relative solubilities of inclusions

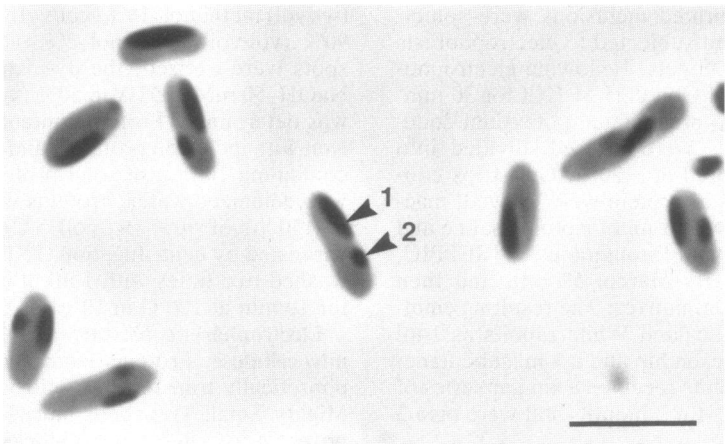

FIG. 1. Stationary-phase cells of *X. nematophilus* subsp. *nematophilus* All. Bacteria were harvested by centrifugation, suspended in 75% (wt/wt) glycerol, and photographed by using phase-contrast optics. The loss of refractility of normally phase-bright inclusions is caused by the glycerol mounting medium. Two morphological forms of inclusion are present, type 1 (1) and type 2 (2). Bar, 5 μm.

in a variety of solvents are listed in Table 1. In general, the solubility characteristics of both inclusion types were similar. They were readily soluble (>70%) in 5 mM EDTA, at high (>11.0) and low (<3.0) pH values, in the presence of the anionic detergent SDS and high concentrations of the denaturants urea and NaBr. Conversely, the inclusions were poorly soluble or insoluble between pH 4.0 and 9.0 and in the presence of nonionic and dipolar ionic detergents. Type 1 and 2 inclusions exhibit contrasting solubility characteristics in the cationic detergent tetradecyltrimethylammonium bromide, type 1 inclusions being 98% soluble and type 2 inclusions being insoluble.

Composition of type 1 and 2 inclusions. The ultrastructural morphology of *Xenorhabdus* inclusions is typical of bacterial protein crystals such as the parasporal bodies of various *Bacillus thuringiensis* strains (5, 7, 15). On the basis of staining characteristics with histological and ultrastructural stains, Boemare et al. (7) concluded that *Xenorhabdus* inclusions did not contain significant amounts of lipid or carbohydrate. Furthermore, attempted pronase digestion of inclusion material in thin sections gave negative results. In our initial studies, purified inclusions were solubilized in protein denaturants such as urea and SDS (Table 1). Solubilized inclusions showed a strong affinity for protein stains such as Coomassie blue and amido black, and UV absorption spectra displayed a major peak at 280 nm, confirming the presence of protein. Carbohydrate and nucleic acid analyses indicated that type 1 inclusions also contained a trace of DNA (0.5%, wt/wt). Type 2 inclusions contained minor amounts of carbohydrate (1.4%, wt/wt) and DNA (1.4% wt/wt) and a trace of RNA (0.5%, wt/wt). It is uncertain whether the carbohydrate and nucleic acid components are of structural or functional significance or simply represent contaminants trapped in the inclusion matrix. The role(s), if any, of these minor components was not examined further.

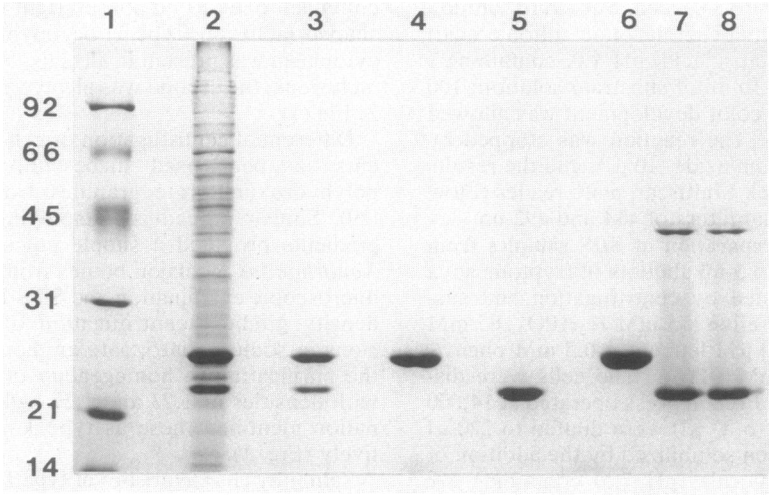

FIG. 2. SDS-PAGE of whole-cell proteins from *X. nematophilus* subsp. *nematophilus* All and inclusions at various stages of purification. Lanes: 1, protein standards; 2, TCA-precipitated whole-cell proteins; 3, inclusions purified by differential centrifugation in glycerol; 4, protein from type 1 inclusions (IP-1); 5, protein from type 2 inclusions (IP-2); 6, type 1 inclusions solubilized in nonreducing sample buffer; 7, type 2 inclusions solubilized in nonreducing sample buffer; 8, type 2 inclusions solubilized in nonreducing sample buffer containing 5 mM iodoacetic acid. Samples contained approximately 3 (lane 2) and 1 (lanes 3 to 8) μg of protein. Numbers on the left indicate size (10^3) of molecular weight marker proteins (Bio-Rad Laboratories).

The observation that both types of inclusion are highly soluble in dilute EDTA, and the apparent absence of significant disulfide cross-linking as a major structural feature of these inclusions (based on ready dissolution of inclusions in nonreducing conditions), implies that structural integrity of the inclusions is maintained by divalent or tetravalent cations. To identify and quantify cations present in type 1 inclusions (type 2 inclusions were not analyzed because sufficient inclusions were not available), purified inclusions were subjected to trace element analysis by simultaneous inductively coupled plasma atomic emission spectroscopy (6). Cations present in detectable amounts were sodium (904 mmol/mol), calcium (292 mmol/mol), and manganese (14 mmol/mol of protein), based on a subunit molecular mass of 26 kilodaltons (kDa) for IP-1 (Fig. 2). Other elements present in significant amounts were sulfur (22.7 mol/mol) and phosphorus (109 mmol/mol of protein). Quantities of all other elements assessed were <5 μmol/mol of IP-1. The relatively large quantities of calcium and manganese in type 1 inclusions suggest that these cations may have a role in maintaining inclusion stability. High sodium, sulfur, and phosphorus content probably reflects residual sodium diatrizoate from the purification procedure, methionine sulfur (see Table 2), and nucleic acid phosphate, respectively.

TABLE 1. Solubility characteristics of type 1 and 2 inclusions from *X. nematophilus* subsp. *nematophilus* All

Solvent	% Solubilized[a]	
	Type 1	Type 2
Deionized water	0	0
2% SDS–5 mM EDTA	100	100
5 mM EDTA	100	96
pH[b]		
11.5	88	97
11	15	91
10	8	49
9	11	0
4	0	19
3	76	73
2	98	95
Detergent[c]		
Nonionic		
NP-40	1	0
Triton X-100	0	0
Ionic		
SDS	98	98
TTAB	98	0
Dipolar ionic		
Zwittergent 3-16	13	0
Denaturants		
Urea		
2 M	23	10
4 M	37	51
8 M	96	93
NaBr		
2 M	81	41
4 M	97	90
8 M	88	93

[a] Based on the reduction in light scattering compared with inclusions in deionized water. Values are means of two or three replicates.

[b] Samples in alkaline (NaOH) or acid (HCl) water.

[c] 2% (wt/vol) SDS, tetradecyltrimethylammonium bromide (TTAB), and zwittergent 3-16. 2% (vol/vol) Nonidet P-40 (NP-40) and Triton X-100.

Analysis of purified inclusions by SDS-PAGE showed that each type of inclusion contained protein(s) of a single subunit size. Under reducing conditions, type 1 and 2 inclusions yield single bands with apparent molecular masses of 26 (IP-1) and 22 (IP-2) kDa, respectively (Fig. 2, lanes 4 and 5). These proteins correspond to two major proteins in whole-cell lysates, which, based on densitometer scans, may account for up to 55% of the SDS soluble cell protein (Fig. 2, lane 2). When analyzed by nonreducing SDS-PAGE, type 1 inclusions yield a single band of 26 kDa (Fig. 2, lane 6), indicating that the protein subunits of these inclusions neither are cross-linked by interchain disulfide bonds nor contain loop structures resulting from intrachain disulfide bonding. In contrast, type 2 inclusions contain a major protein of 22 kDa and a minor band of 44 kDa, the latter apparently being a disulfide-linked dimer of 22-kDa subunits (Fig. 2, lane 7). Based on densitometer scans, approximately 65% of IP-2 occurs in the monomer form, with 35% as a dimer. The occurrence of the 44-kDa protein is not influenced by the presence of iodoacetic acid, a sulfhydryl alkylating agent, in the solubilizing buffer (Fig. 2, lane 8). It is unlikely, therefore, that disulfide bond formation results from oxidation occurring during the solubilizing process.

Isoelectric focusing of Type 1 and 2 inclusion proteins in the presence of 8 M urea revealed multiple subunits with differing pIs (Fig. 3A). IP-1 subunits are acidic, typically focusing into two major proteins with pIs of 4.7 and 4.5 and a variable number of minor components with pIs between 5.0 and 4.3. IP-2 subunits have pIs around neutrality. Two subunits are present in significant quantities, a major subunit with a pI of 7.0 and a minor subunit with a pI of 6.6.

Alkali-solubilized IP-1 and IP-2 were subjected to partial proteolysis to identify any common structural domains. Peptides released after 0.5, 1, 2, 5, and 24 h of digestion with trypsin yielded characteristic patterns when analyzed by SDS-PAGE. Tryptic peptide fingerprints of IP-1 and IP-2 after 2 h of proteolysis are compared in Fig. 3B. Four protease-resistant domains with apparent molecular masses of 10.8, 7.5, 4.7, and 3.5 kDa were derived from IP-1 (Fig. 3B, lane 3). This pattern varied little throughout the 24-h digestion period, except for the disappearance of the 10.8-kDa band after 5 h of proteolysis and the appearance of increasing quantities of low-molecular-weight (<2,000) fragments that were poorly resolved in these gels. Peptide fingerprints from IP-2 differed from those of IP-1. Two prominent domains with molecular masses of 9.8 and 5.6 kDa (Fig. 3B, lane 4) were present throughout the 24-h digestion period. The absence of common structural domains in IP-1 and IP-2 indicates that these proteins differ in secondary structure.

Amino acid analyses of IP-1 and IP-2 are compared in Table 2. The compositions of both proteins are similar, each containing a high proportion of the hydrophobic residues alanine, valine, leucine, and isoleucine (30 to 40%) and significantly more aspartic acid/asparagine and glutamic acid/glutamine (20%) than arginine and lysine (8%). As these proteins have similar contents of basic residues, the observed differences in pI values between IP-1 and IP-2 (Fig. 3A) indicate that relatively more asparagine or glutamine or both is present in IP-2 than in IP-1. IP-1 but not IP-2 is rich in methionine, and no cysteine was detected in either protein. The failure to detect cysteine in IP-2, a protein that

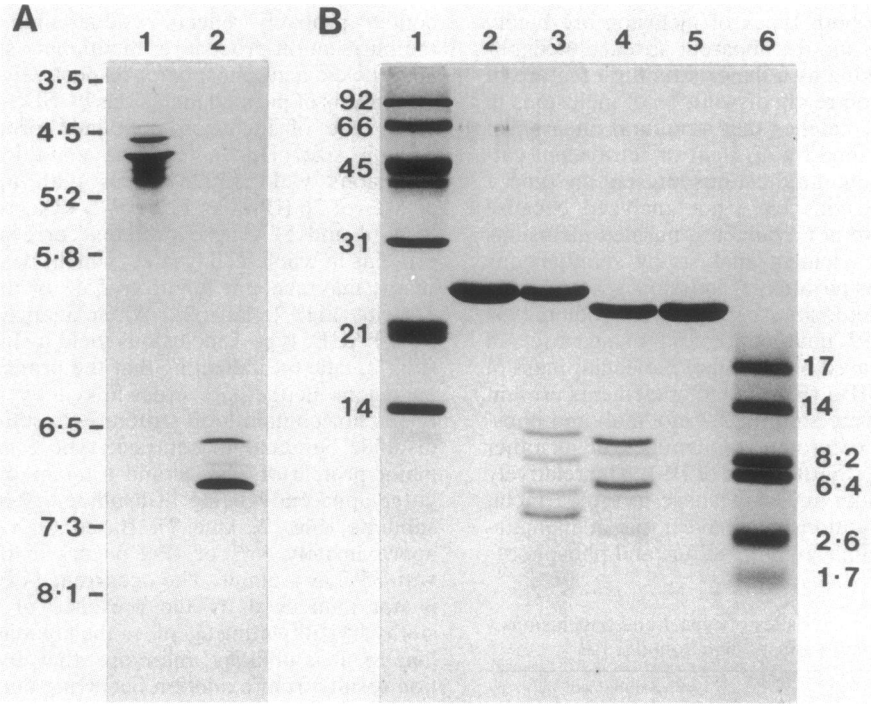

FIG. 3. Comparison of IP-1 and IP-2 by isoelectric focusing and partial proteolysis. (A) Purified type 1 and 2 inclusions were solubilized and subjected to isoelectric focusing in the presence of 8 M urea as described in Materials and Methods. Lane 1, IP-1; lane 2, IP-2 (approximately 5 μg of protein per lane). Numbers to the left indicate the pH gradient, identified from the location of pI marker proteins (broad-range calibration kit; Pharmacia). (B) Alkali-solubilized IP-1 and IP-2 were taken up in 25 mM Tris (pH 8.1), digested with trypsin for 2 h at 30°C, and prepared for SDS-PAGE as described in Materials and Methods. Samples (2 μg of protein) were analyzed in 18% gels. Lane 1, Molecular weight marker proteins (Pharmacia); lane 2, IP-1; lane 3, tryptic digest of IP-1; lane 4, tryptic digest of IP-2; lane 5, IP-2; lane 6, polypeptide molecular weight markers (Pharmacia). Numbers to the left and right indicate the molecular weight (10^3) of marker proteins or polypeptides.

occurs as a disulfide cross-linked dimer (Fig. 2), is not surprising as this residue is known to be susceptible to degradation during acid hydrolysis prior to phenylisothiocyanate derivatization (35).

TABLE 2. Amino acid analysis of proteins from type 1 and 2 inclusions produced by *X. nematophilus* subsp. *nematophilus* All

Amino acid	mol/mol of protein	
	IP-1[a]	IP-2[b]
Asp + Asn	36	27
Glu + Gln	12	18
Ser	19	28
Gly	16	24
His	3	6
Arg	6	4
Thr	12	10
Ala	29	15
Pro	12	10
Tyr	3	3
Val	30	21
Met	21	3
Cys	0	0
Ile	18	13
Leu	20	17
Phe	0	2
Lys	12	12
Trp	ND[c]	ND

[a] IP-1 theoretical molecular weight = 26,102.
[b] IP-2 theoretical molecular weight = 21,837.
[c] ND, Not determined.

Immunological characteristics of IP-1 and IP-2. Polyclonal antisera produced in response to denatured IP-1 or IP-2 were prepared in rabbits and used to assess the immunological relatedness of these proteins. The specificity of reaction of the resulting antisera with native and SDS-denatured inclusion proteins is illustrated in Fig. 4. Both IP-1 and IP-2 were strongly antigenic, each protein eliciting a significant antibody response in vaccinated rabbits. When antisera were assayed against nonhomologous native inclusion protein, no cross-reaction was observed (Fig. 4A).

Anti-inclusion protein IgG isolated by affinity chromatography on protein A-Sepharose was used for immunoblots of SDS-denatured inclusion proteins (Fig. 4B). In contrast to the results obtained with native proteins, significant cross-reaction was observed between anti-IP-1 IgG and the nonhomologous protein, IP-2. Experiments involving electrophoretic transfer of IP-2 tryptic peptides from SDS-PAGE gels to nitrocellulose and immunological detection with anti-IP-1 IgG have demonstrated that the cross-reactivity is associated with the 5.6-kDa domain of IP-2 (unpublished data). In contrast, no cross-reaction was observed between anti-IP-2 IgG and IP-1. The one-way cross-reactivity of inclusion protein antisera may indicate the presence of a common antigenic determinant that is numerous in IP-1 (yielding a strong specific IgG response) but rare in IP-2 (yielding a weak specific IgG response). If the antibody response to such a determinant is very low in the IP-2-vaccinated rabbit, the extent of cross-reaction with IP-1 may be less than that required for detection in immunoblots. In dot blots, the lower detection limits for IP-1 and IP-2 with

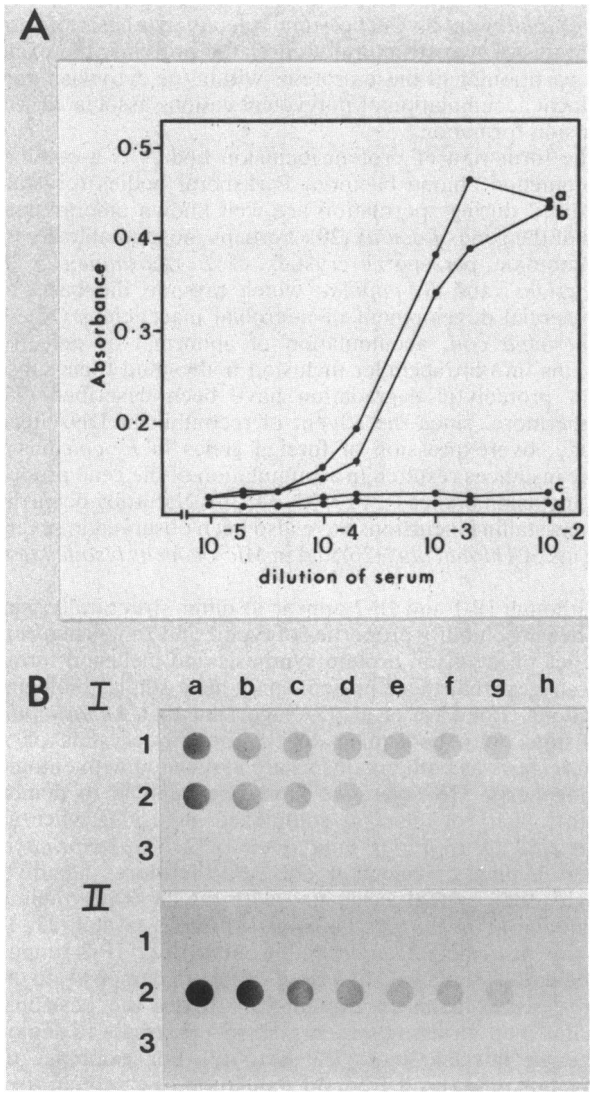

FIG. 4. Immunological characteristics of type 1 and 2 inclusion proteins. (A) Titration of crude sera and cross-reactivity with solubilized native proteins: a, reaction of IP-2 serum with IP-2; b, reaction of IP-1 serum with IP-1; c, reaction of IP-2 serum with IP-1; d, reaction of IP-1 serum with IP-2. Reaction of anti-inclusion protein IgG with 10 μg of alkali-solubilized inclusion protein was detected by the anti-rabbit IgG-horseradish peroxidase method as described in Materials and Methods. (B) Sensitivity of alkaline phosphatase immunodetection procedure, using protein A–affinity-purified antisera, and cross-reactivity against SDS-denatured inclusion proteins. Purified inclusions were solubilized in 5 mM EDTA. Aliquots containing 1 μg (a) and 500 (b), 250 (c), 125 (d), 62 (e), 31 (f), 15 (g), and 8 (h) ng of protein were made up to 220 μl with deionized water and denatured by addition of 30 μl of 2% SDS in 1.0 M Tris (pH 7.5), and the protein was precipitated with 50 μl of 90% (wt/vol) TCA. Protein precipitates were collected on nitrocellulose filters with a microfiltration apparatus. Immunological detection was performed as described in Materials and Methods, using an anti-rabbit IgG-alkaline phosphatase conjugate. Reaction with anti-IP-1 IgG (I) and anti-IP-2 IgG (II): 1, IP-1; 2, IP-2; 3, buffer control.

anti-IP-1 IgG were 16 n and 62 ng of protein, respectively. The lower detection limit for IP-2 with anti-IP-2 IgG was 8 to 16 ng of protein (Fig. 4B).

Kinetics of inclusion protein synthesis and inclusion formation. The kinetics of inclusion protein synthesis and inclusion formation were investigated by using light microscopy, SDS-PAGE, and immunoblotting procedures. To avoid carry-over of inclusion proteins from the original inoculum, a single colony from a streak plate was cultured to early exponential growth (optical density at 600 nm, 0.5 to 1.0) and then subcultured in a similar fashion a further five times. Typical growth characteristics of the final cultures are illustrated in Fig. 5A. Microscopic examination of bacteria at various times throughout culture growth revealed the first appearance of inclusions during the second half of exponential growth (28 h of culture). The inclusions were small ovoid structures and were present in about 15% of the cells. By 36 h, inclusions were present in 90% of the bacteria. Small cigar-shaped inclusions (type 1) appeared at this time. Inclusion formation was completed by 40 h, when all cells contained one or two inclusions.

To determine the relationship between inclusion protein synthesis and inclusion formation, bacteria were sampled at 4-h intervals during culture growth, and SDS-soluble whole-cell protein was analyzed by SDS-PAGE. A protein band that appeared to correspond to IP-1 was present in all of the cell lysates from 16 h onwards. The amount of IP-1 in lysates increased rapidly between 28 and 40 h of culture, after which there was little change in the size of the IP-1 band. IP-2 first appeared in 28-h cells. Thereafter, accumulation of this protein mirrored that of IP-1, appearing to be maximal in 36- to 40-h cells (Fig. 5B). Immunoblots were used to confirm the identity of the relevant bands as IP-1 and IP-2 and to indicate more accurately the time at which inclusion protein synthesis is initiated. In contrast to the observations from Coomassie blue-stained gels, IP-1 was first detected in lysates from 28-h cultures (Fig. 5C), indicating that 26-kDa protein present in younger cultures and detected by Coomassie blue is an unrelated protein that comigrates with IP-1. IP-2 appeared slightly earlier than IP-1, being present in cultures after 24 h of growth (Fig. 5C).

DISCUSSION

We have described the purification of two types of proteinaceous intracellular inclusion from stationary-phase *X. nematophilus* subsp. *nematophilus* All. Although each type of inclusion contains proteins of a single subunit size, multiple forms differing slightly in pI were detected by isoelectric focusing. Such differences may reflect minor variations in the relative abundance of aspartic acid and glutamic acid residues and their amide homologs asparagine and glutamine. The ratio of acidic residues/amide residues apparently accounts for the major differences in pI between IP-1 and IP-2, as the content of basic residues in these proteins is similar. Further variations in immunological properties, peptide maps, and amino acid composition have revealed significant structural differences between IP-1 and IP-2.

Results of kinetic studies of inclusion protein synthesis and inclusion formation were similar for both type 1 and 2 inclusions. The appearance of inclusion proteins and inclusions after 24 to 28 h of culture and the close correlation between increasing rates of inclusion protein synthesis and increasing inclusion size suggest that inclusion protein synthesis and inclusion formation are concurrent events. Whether aggregation of inclusion protein occurs spontaneously as intracellular protein concentration increases or the process is regulated by the bacterium cannot be determined from these studies. Precise cellular regulation might be expected as inclusion formation in *X. nematophilus* subsp.

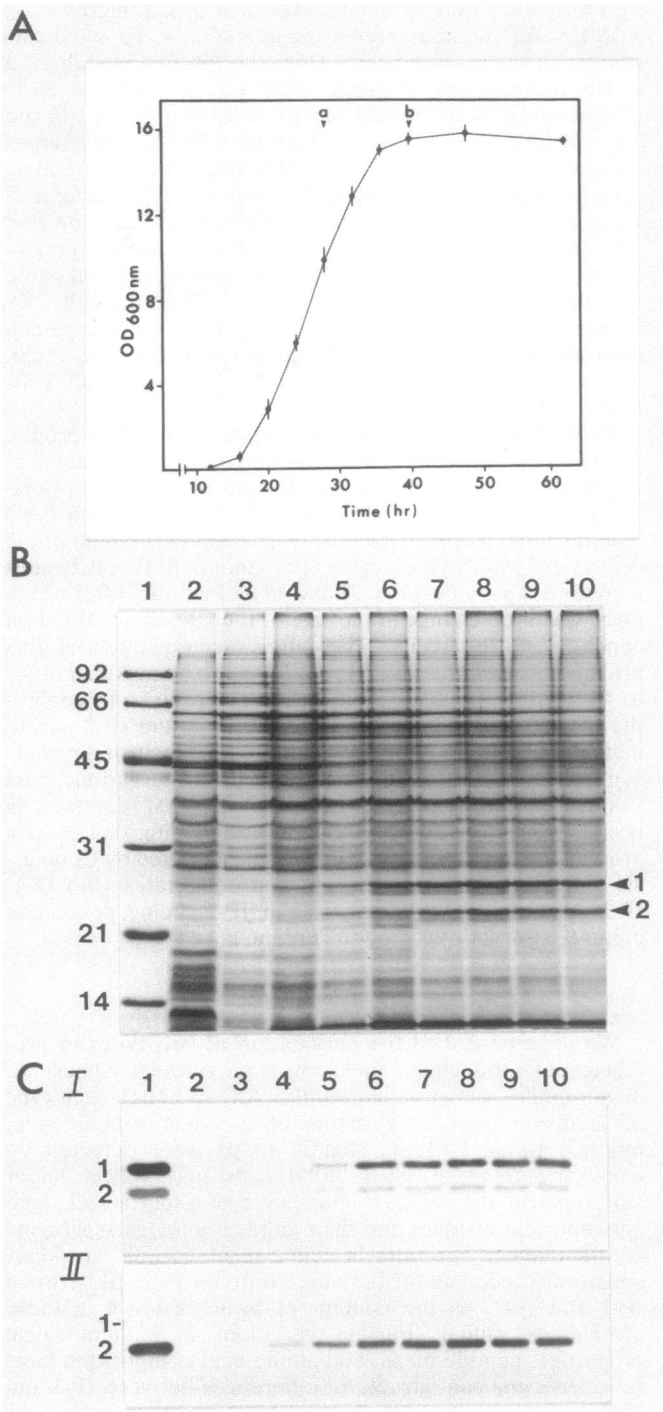

FIG. 5. Growth of *X. nematophilus* subsp. *nematophilus* All in liquid culture and kinetics of inclusion protein synthesis. (A) Optical density at 600 nm (OD_{600nm}) of *X. nematophilus* subsp. *nematophilus* culture at various times during culture growth. Each datum point is the mean of recordings from three similar cultures. The vertical bars indicate the standard error of the mean. The arrow at (a) indicates the time at which inclusions first appeared in cultures. The arrow at (b) indicates the time at which all cells contained inclusions. (B) SDS-PAGE of lysates of cells sampled at various times during culture growth. SDS samples were prepared as described in Materials and Methods. There was approximately 8 μg of protein per lane. Lanes: 1, molecular weight markers; 2, 16-h cells; 3, 20-h cells; 4, 24-h cells; 5, 28-h cells; 6, 32-h cells; 7, 36-h cells; 8, 40-h cells; 9, 48-h cells; 10, 62-h cells. Numbers on the left indicate the molecular weight (10^3) of marker proteins; arrows 1 and 2 on the right indicate IP-1 and IP-2, respectively. (C) Immunodetection of IP-1 and IP-2 in lysates of cells sampled at various times during culture growth. Proteins analyzed by SDS-PAGE were transferred electrophoretically to nitrocellulose. Inclusion proteins were detected by using anti-inclusion protein IgG and anti-rabbit IgG-alkaline phosphatase conjugate as described in Materials and Methods. Cell lysate samples applied to SDS gels contained approximately 2 μg of protein. Lanes: 1, purified inclusion proteins; 2 to 10, same as for (B). Numbers to the left indicate IP-1 (1) and IP-2 (2). I, Reaction with anti-IP-1 IgG; II, reaction with anti-IP-2 IgG.

nematophilus involves the simultaneous synthesis of large quantities of two structurally unrelated proteins, the exclusive partitioning of these proteins within the cytoplasm, and the local accumulation of polyvalent cations associated with inclusion formation.

The formation of protein inclusion bodies is a common phenomenon among bacteria. Parasporal bodies (crystals) produced during sporulation are well known among members of the genus *Bacillus* (28). Perhaps most notable are the entomotoxic parasporal crystals of *B. thuringiensis*, *B. sphaericus*, and *B. popillae* which provide the basis for commercial development of microbial insecticides (12). In *Escherichia coli*, accumulation of abnormal or defective proteins into intracellular inclusion bodies and their subsequent proteolytic degradation have been described (25). Furthermore, since the advent of recombinant DNA technology, overexpression of foreign genes in *E. coli* has in many instances resulted in accumulation of the gene product into inclusion bodies (13, 27, 33, 34, 36). Naturally occurring paracrystalline inclusions have also been observed in several species of *Thiobacillus* (26) and in *Micrococcus lysodeikticus* (14).

Although IP-1 and IP-2 appear to differ structurally, similarities in solubility properties of type 1 and 2 inclusions and kinetics of inclusion protein synthesis and inclusion formation suggest that these proteins may have similar biological functions. Boemare et al. (7) suggested that *Xenorhabdus* inclusions may be similar to the protein crystals of *B. thuringiensis* as both organisms are associated with entomopathogenesis. However, we have been unable to demonstrate toxicity of intact or solubilized inclusions when administered to *Heliothis punctigera* or *Lucilia cuprina* by either feeding or injection (10). Furthermore, hemolytic activity such as that associated with the 28-kDa protein of the *B. thuringiensis* var. *israelensis* protein crystal (23, 31) has not been detected in solubilized IP-1 or IP-2 (unpublished data). While we have been unable to demonstrate that *Xenorhabdus* inclusion proteins are toxic, the possibility remains that our assay systems are inappropriate to demonstrate an intrinsic biological activity. For example, the inclusions may result from accumulation of a protein toxin that is normally secreted during pathogenesis and is overproduced under conditions of nutrient limitation, as colonization of the host advances. An analogous situation has been described in *Clostridium perfringens* in which overproduction of enterotoxin as cultures approach stationary phase leads to accumulation of the protein in intracellular inclusion bodies (20).

Alternatively, structural characteristics of *Xenorhabdus* inclusions suggest that these structures may be readily amenable to mobilization of their protein constituents, as might be expected of storage protein reserves. Inclusion

bodies such as those formed from defective or overexpressed foreign proteins (13, 27, 33) or the toxic protein crystals of *B. thuringiensis* (11, 22) and enterotoxin inclusion bodies of *C. perfringens* (20) are chemically refractile, often requiring harsh conditions such as high pH (>11.5) or denaturants (guanidine hydrochloride, urea, SDS) and the presence of reducing agents to effect solubilization of the component polypeptide chains. At least in part, the poor solubility of these inclusions results from disulfide cross-links between adjacent protein subunits. In contrast, disulfide cross-links are not significant structural features of *Xenorhabdus* inclusions. The high cation content (292 mmol of calcium and 14 mmol of manganese per mol of protein) and the ready dissolution of inclusions in the presence of EDTA suggest that protein interactions with these cations has an important role in stabilizing *Xenorhabdus* inclusions. Inclusion structure based on ionic interactions may facilitate release of protein from inclusion bodies in response to endogenous chelating agents such as citrate or dipicolinate, a Ca^{2+} chelator commonly found in bacterial spores (28). We propose that, once dissociated from the mass of the inclusion, released protein may be readily metabolized as required by the cell.

The ability to synthesize IP-1 and IP-2 and the formation of their respective inclusions appear to be subspecies specific, occurring in seven strains of *X. nematophilus* subsp. *nematophilus* but not in other subspecies or species of *Xenorhabdus* (10). As *X. nematophilus* subsp. *nematophilus* strains are the specific natural symbionts from isolates of *Steinernema feltiae*, the function(s) of these proteins may be related to the host nematode. Recent observations that *Xenorhabdus* inclusions are metabolized in vivo in the presence of larval nematodes support the hypotheses that type 1 and 2 inclusions are storage protein reserves and that mobilization of these reserves is related to the presence of host nematodes. Further research to define the role of inclusion proteins in the *Xenorhabdus*-nematode symbiosis is in progress.

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