Isolation and Characterization of Intracellular Protein Inclusions Produced by the Entomopathogenic Bacterium Photorhabdus luminescens

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Cells of the entomopathogenic bacterium Photorhabdus luminescens contain two types of morphologically distinct crystalline inclusion proteins. The larger rectangular inclusion (type 1) and a smaller bipyramid-shaped inclusion (type 2) were purified from cell lysates by differential centrifugation and isopycnic density gradient centrifugation. Both structures are composed of protein and are readily soluble at pH 11 and 4 in 1% sodium dodecyl sulfate (SDS) and in 8 M urea. Electrophoretic analysis reveals that each inclusion is composed of a single protein subunit with a molecular mass of 11,000 Da. The proteins differ in amino acid composition, protease digestion pattern, and immunological cross-reactivity. The protein inclusions are first visible in the cells at the time of late exponential growth. Western blot analyses showed that the proteins appeared in cells during mid- to late exponential growth. When at maximum size in stationary-phase cells, the proteins constitute 40% of the total cellular protein. The protein inclusions are not used during long-term starvation of the cells and were not toxic when injected into or fed to Galleria mellonella larvae.

Photorhabdus luminescens is a bioluminescent gram-negative, rod-shaped bacterium that was first isolated from a light-emitting insect that had been infected by entomogenous nematodes of the family Heterorhabditidae (22, 29). Biochemical tests and analysis of the 16S rRNA revealed that P. luminescens is related to members of the Enterobacteriaceae in the gamma subdivision of purple bacteria (13, 31, 32).

The bacteria reside in the intestinal tract of the infective juvenile (IJ) stage of the nematode, which is the vector for transmission of the bacteria between insect prey. The IJ penetrates the insect, releasing the bacteria into the hemolymph. The bacteria multiply rapidly, killing the insect within 24 to 72 h, at which time the dead insect is visibly bioluminescent (23, 25, 29). A 50% lethal dose (LD₅₀) of fewer than 5 cells per insect has been reported for Galleria mellonella (wax moth) larvae (15). The bacterium produces potent insecticidal toxins during growth in the insect as well as in laboratory culture (9, 21). The nematode completes several rounds of reproduction while feeding on the bacteria in the insect carcass. Within 10 to 20 days several thousand IJ progeny, each carrying an inoculum of P. luminescens cells, migrate out of the cadaver in search of new insect prey.

Cells of P. luminescens growing in insect larvae and in culture medium produce phase-bright inclusion proteins within the cytoplasm (7, 23). Bacteria of the related genus Xenorhabdus, associated with entomogenous nematodes of the family Steinernematidae, also produce two cytoplasmic inclusion proteins (11). The genes encoding two inclusion proteins, cipA and cipB, of P. luminescens strain NC1 have been cloned and characterized (5). The genes are present at separate loci and show little nucleotide sequence similarity to each other. Blast searches using the nucleotide or amino acid sequences of the two genes reveal little evidence of homology to any known genes, including those encoding the insecticidal crystal proteins of Bacillus thuringiensis.

Cultures of P. luminescens exhibit a highly variable phenotype involving the spontaneous loss of many traits. The variants, termed secondary-phase cells, differ from the original primary phase in colony morphology, dye absorption, and biochemical utilization and show complete loss of or decrease in antibiotic production, pigmentation, bioluminescence, protease activity, lipase activity, hemolysin production, and the ability to support nematode growth (1, 2, 6, 12, 14, 27). The intracellular inclusion proteins are absent in the secondary phase cells (5).

The function of the inclusion proteins of Photorhabdus and Xenorhabdus is unknown. Because both genera of bacteria are entomopathogenic and are associated in a symbiosis with entomopathogenic nematodes, logical hypotheses are that the inclusion proteins are involved in the nematode association or in pathogenesis. The cost of producing the unusually large amounts of these proteins strongly suggests that the proteins must serve an important function for the bacteria.

This report describes the isolation and characterization of the two protein inclusions from P. luminescens NC1 and Hm and presents the results of attempts to define their function.

MATERIALS AND METHODS

Bacteria and culture conditions. The inclusion proteins were purified from P. luminescens strains Hm (G. M. Thomas, University of California) and NC1 (Wayne Brooks, University of North Carolina). Stock cultures were maintained on 2% proteose peptone no. 3 (PP3) (Difco Laboratories, Detroit, Mich.) solidified with 1.5% Bacto agar (Difco). Cultures were incubated at 30°C for 72 h, stored at room temperature, and transferred at monthly intervals. Two stable secondary-phase variants were isolated from the primary-phase NC1. They are referred to as white secondary (nonpigmented) and yellow secondary (yellow pigmentation).
Microscopy. Phase-contrast micrographs were taken with a Zeiss photomicroscope. Cells on a coverslip were washed with saline (NaCl, KCl, MgCl₂, CaCl₂) and fixed in 2% glutaraldehyde in 100 mM phosphate buffer at pH 7.4, embedded in Durcupan (Sigma), then sectioned, and stained with lead citrate. The sectioned cells were viewed under a JEM-100CX electron microscope. For scanning electron microscopy, purified inclusions were suspended in sterile water (i.e., placed on double-stick tape on a steel post, dried, and coated with gold in vacuo. The samples were examined with a Hitachi S-570 scanning electron microscope.

Optimization of inclusion production. The conditions for optimum inclusion production in liquid culture (all culture media from Difco) were determined by growing cells in 5% yeast extract, 2% casitone, 2% proteose peptone no. 3, 2.5% nutrient broth, 10% peptone, or 2% Trypticase. Cells were examined after 72 h by phase-contrast microscopy.

Isolation of inclusions. A 2-ml suspension of P. luminiscens cells in 2% PPG broth was spread on 2% PPG agar in Pyrex glass baking dishes (18 by 30 cm). After 7 days of incubation at 28°C, 100 ml of SH₂O was added, and the cells were scraped from the agar surface with a bent glass rod. The cell suspension was centrifuged at 1,000 × g for 10 min. The resulting pellet was resuspended in sterile phosphate-buffered saline (PBS) consisting of (per liter) NaCl (8.0 g), KCl (0.20 g), Na₂HPO₄ (1.15 g), and KH₂PO₄ (0.2 g) (18) and centrifuged at 3,000 × g for 10 min. The cell pellets were resuspended in 10 ml of PBS and passed twice through a French press at 10,000 lb/ft². The cell lysate was diluted to 50 ml in PBS and centrifuged at 2,000 × g for 20 min. This step was repeated three times.

The chalky white pellets that were produced were resuspended in 9 ml of PBS, and three ml samples were applied to the top of discontinuous Percoll (Sigma Chemical Co., St. Louis, Mo.) density gradients (28). The Percoll solutions were prepared from a stock solution containing 1 part 2.5 M sucrose and 9 parts (vol/vol) Percoll. Step gradients consisted of a 5-ml layer of Percoll stock solution placed at the bottom of 30-ml Corex centrifuge tubes, followed by application of sequential layers of 95, 90, 80, and 70% dilutions of the stock solution. Following centrifugation (4 h at 5,000 × g) in a Sorvall type HS-4 swinging bucket rotor at 4°C, the two visually distinct inclusion-containing layers were removed separately and washed several times in SH₂O by centrifugation at 2,000 × g. Each fraction was then centrifuged through the gradients once more as described. The purified inclusions were stored as frozen pellets at −20°C, lyophilized, and stored under desiccation at room temperature or in SH₂O at 4°C.

Solubility of inclusions. A suspension of purified inclusions was made in SH₂O at an optical density at 600 nm (OD₆00) of 2.0. The pH of solutions was adjusted by slowly adding 1.0-mol amounts of 0.1 M HCl or 0.1 M NaOH, and the OD₆00 of the suspensions was monitored. To determine solubility in sodium dodecyl sulfate (SDS), 100 μl of 10% SDS (Calbiochem, San Diego, Calif.) was added to 900 μl of inclusion suspension. To determine solubility in urea or EDTA, inclusions were resuspended in 1 ml of 9 M urea or 100 mM EDTA at pH 8.0.

Gel electrophoresis and Western blot analyses. Gel electrophoresis and Western blot analyses were performed using a protocol designed for high resolution of proteins in the 5- to 30-kDa range (33). Proteins were stained with 0.1% Coomassie brilliant blue R-250. For Western blot analysis, proteins were electrophoresed onto nitrocellulose membranes in 25 mM Tris-192 mM glycine and 20% (vol/vol) methanol. The gels were electrophoresed for 1 h at 20 V constant voltage in a Genie Blotter (Ides Scientific, Minneapolis, Minn.). The AuroProbe BL plus and InterSEBL silver enhancement kit (Amersham Life Sciences, Arlington Heights, Ill.) were used according to the manufacturer's instructions to detect antigen on the blots. The primary antibody was used at a 1:1,000 dilution.

Stability of inclusion proteins during growth and starvation. These were grown for 48 h at 30°C in 50 ml of 2% PPG broth in 500-ml flasks shaken at 250 rpm. Samples were removed at various times, and microscopic counts were determined using a Petroff-Hauser counting chamber. Viable-cell counts were determined by dilution of samples into fresh 2% PPG broth, plating on 2% PPG agar, and counting colonies after 5 days of incubation at 30°C. For starvation experiments, the 48-h PPG cultures were divided into two 25-ml portions. One sample was transferred to a 250-ml flask and incubated as above. The other sample was centrifuged at 2,000 × g for 5 min at room temperature. The cells were resuspended in 25 ml of SPBS and incubated as above. Samples were removed from the flasks, and microscopic counts and viable-cell counts were determined at various times.

Insect toxicity analyses. G. mellonella larvae were obtained from H. C. Coppel (Department of Entomology, University of Wisconsin–Madison) and grown by his method (26). Samples containing 25 μg of purified type 1 or 2 proteins in 10 μl of SH₂O were either fed to or injected into last instar G. mellonella larvae (9). The inclusion proteins were also solubilized with 10 mM HCl or 10 mM NaOH, filter sterilized with 0.2-μm-pore-sized membrane filters, and then fed or injected. Samples (cells plus broth) taken directly from 48-h PPG cultures were also fed to and injected into larvae. Freshly prepared (stored at 4°C) and frozen inclusion preparations were used for bioassays.

RESULTS

Production and isolation of inclusion proteins. A variety of liquid culture media were tested for their effect on inclusion production by both P. luminiscens strains NC1 and Hm. The inclusions were visibly evident using phase-contrast microscopy in most cells of both strains after 48 h of growth in 2.5% nutrient broth and 2% neopeptone. Approximately half the cells contained inclusions when grown in 2% Trypticase soya broth. The cells grew well but produced no visible inclusions when grown in 2% casitone, 5% yeast extract, or 10% peptone.

The best growth medium, in which more than 90% of the cells contained phase-bright inclusions, was 2% PPG. Photomicrographs of cells of strains NC1 and Hm grown on 2% PPG agar reveal the presence of phase-bright inclusions in the cells (Fig. 1A and 1B).

Transmission electron micrographs of thin-sectioned cells of NC1 and Hm show that both these strains contain two morphologically distinct inclusions (Fig. 2A and 2D). The protein...
inclusions released from cells by French pressure breakage became separated into two bands in the Percoll gradients. Scanning electron micrographs of Percoll-separated inclusions show the less dense type 1 inclusion to be rectangular (Fig. 2B and 2E). The morphology of the more dense type 2 inclusions of NC1 and Hm differs. The NC1 type 2 is bipyramidal (Fig. 2C), and the Hm type 2 has pointed ends and is elongated in the middle (Fig. 2F).

**Solubility, compositional analyses, and mass spectrometry.**

The purified type 1 and type 2 inclusions from both strains are insoluble in water and at neutral pH in PBS or Tris buffer. The effect of pH on the solubility of the inclusion structures was tested by slowly increasing and decreasing the pH of an aqueous suspension. The inclusion structures remained insoluble between pH 5 and 10. The OD$_{600}$ of the suspension decreased by more than 90% at pH 11 or 4; at both pHs, the inclusions become soluble. As the pH was slowly adjusted from 4 and 11 toward neutrality, the solutions became cloudy at pH 5 and pH 7, respectively, coincident with the formation of an amorphous precipitate. Both types of inclusions were soluble (greater than 90% OD$_{600}$ decrease) in 8 M urea and 1% SDS. The inclusions precipitate. Both types of inclusions were soluble by more than 90% at pH 11 or 4; at both pHs, the inclusions become soluble.

The type 1 protein inclusion contains 0 to 1% cysteine, 1 to 2% methionine, 20 to 24% leucine, and 4% lysine. The type 2 protein contains 4 to 5% cysteine, 13% methionine, 9 to 11% leucine, and 9 to 10% lysine. The type 1 protein contains approximately 47% hydrophobic amino acids, while the type 2 protein contains approximately 42% hydrophobic amino acid residues, with particularly high levels of valine, methionine, isoleucine, and leucine.

The amino acid compositional analysis obtained by acid hydrolysis of the proteins followed by high-pressure liquid chromatography (HPLC) showed four lysine residues in the type 1 inclusion of both NC1 and Hm. This value differs from the 14 lysine residues predicted by the Hm gene sequence (5). Most likely this difference is due to an unexplained error in the compositional analyses.

The percentage of total cell protein attributable to the inclusions is approximately 40% in the 7-day-old cultures.

The density gradient-purified inclusions from both strains were analyzed by mass spectrometry (MALDI-TOF). The results show that the inclusions are composed almost entirely of a single-molecular-mass species. The molecular mass of the type 1 inclusion of the Hm strain is 11,323 Da, which is nearly identical to the predicted mass of 11,315 Da for the cipB gene product of strain Hm. The molecular mass of NC1 type 1 is 11,381 Da and is very close to the molecular mass of the Hm type 1 inclusion. The molecular masses of the Hm and NC1 type 2 inclusions are 11,711 and 11,697 Da, respectively, which is nearly identical to the predicted 11,700 Da of the cipA gene product.

The mass spectrometry data for the type 2 inclusions of both strains show a minor shoulder peak indicating components 42 mass units larger for the NC1 and 47 mass units larger for the Hm inclusions. This mass shift suggests that a small percentage of the protein may contain a posttranslational modification; most likely it is acetylated.

**SDS-PAGE analysis and proteolytic degradation.**

The results of SDS-PAGE analysis of the protein inclusions show that both type 1 and type 2 protein inclusions from strains NC1 and Hm are apparently composed of single proteins that each have a molecular mass of approximately 10 kDa (Fig. 3). This value correlates well with the mass estimated from amino acid analyses and mass estimates for the inclusion proteins.

The degree of proteolytic digestion of type 1 and type 2 inclusions of NC1 by four different proteases was analyzed by SDS-PAGE. The type 1 protein inclusion was extensively degraded by two different pronase preparations, was cleaved into two discrete fragments by trypsin, and was not hydrolyzed by the V8 protease. The type 2 protein inclusion was only slightly degraded by the pronase preparations, and most of the protein remained as a single intact band that was not degraded by trypsin or V8 protease (not shown). Inclusions present in cell lysates of both strains were also analyzed for degradation by indigenous cellular proteases. The cell lysates contained high levels of proteolytic activity, but degradation was not detected in suspensions of inclusions incubated in the lysates for as long as 1 week (not shown).

**Immunological analysis and temporal regulation of inclusion protein accumulation.**

Polyclonal antisera raised against type 1 and type 2 inclusion proteins of NC1 were used to
determine the time of inclusion protein production in growing cells. Western blot analyses (Fig. 4A and B) revealed that both type 1 and 2 proteins are first detected at 16 h (lanes 7, panels A and B). Both proteins reached high levels in 24-h cells (lanes 8). The inclusion protein detected at 0 h (lanes 1, A and B) resulted from the stationary-phase cells used as the inoculum. During the first 12 h of growth, the inclusion proteins were diluted relative to the total protein content of the cells. Microscopic examination of the cells confirmed that the protein inclusions were first visible at 16 h of growth. By 24 h, greater than 70% of the cells contained small inclusions (Fig. 5).

Two secondary-phase variants were isolated from the primary NC1 strain. The white secondary was deficient in all characteristics typical of primary-phase cells. The yellow secondary-phase variant was deficient in all characteristics except pigmentation and antibiotic production. Both secondary variants contained no visible inclusions (not shown). Western blot analyses did not detect inclusion proteins in the secondary-phase variants (Fig. 4C and 4D, lanes 1 and 2).

The type 1 and type 2 NC1 antisera were used to analyze the immunological cross-reactivity of the inclusion proteins from strains NC1 and Hm (Fig. 4C and 4D). Type 1 antiserum cross-reacts weakly with NC1 type 2 protein (Fig. 4C, lane 6) but strongly recognizes a 10-kDa band, and reacts more weakly with two proteins of approximately 20 and 30 kDa in the NC1 type 1 material (Fig. 4C, lane 5). Type 2 antiserum cross-reacts weakly with NC1 type 1 protein (Fig. 4D, lane 5) but strongly recognizes a 10-kDa band and more weakly a protein of ap-
proximately 20 kDa in the type 2 lane (Fig. 4D, lane 6). The cross-reactivity and banding patterns produced by the type 1 and 2 antisera with NC1 inclusion proteins were nearly identical to the results obtained for the Hm inclusion proteins (Fig. 4C and 4D, lanes 3 and 4). The higher-molecular-weight bands detected by the antisera may be multimers of the individual subunits of each inclusion type, since their migration distances are consistent with those of a dimer and a trimer of the individual proteins. If this conclusion is correct, these multimers occur even in the presence of SDS.

**Are the protein inclusions nutrient reserves?** The possibility that the protein inclusions might serve as a reserve nutrient source for the bacteria was tested. The cells contained visible cytoplasmic protein inclusions at 16 h (Fig. 5). Growth in 2% PP3 broth reached maximum levels between 24 and 36 h (Fig. 6). The direct microscopic counts reached a maximum level of $5 \times 10^9$ cells/ml at 36 h. The viable-cell counts reached a maximum of $8 \times 10^8$ cells/ml at 24 h and decreased steadily until only about 1% of the cells ($10^7$/ml) were viable at 192 h. The cells starved in sPBS generally remained viable up to 192 h. The ratio of microscopic counts to viable plate counts remained nearly constant throughout the starvation period.

**TABLE 1. Amino acid composition of* P. luminescens* Hm and NC1 protein inclusions compared to the amino acid composition predicted from the Hm cipA and cipB gene sequences**

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<th>NC1 type 1</th>
<th>Hm type 2</th>
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<sup>a</sup>*, essential for* Neoplectana glaseri* (20).
<sup>b</sup>Based and amino acid composition analysis.
<sup>c</sup>Amino acid composition based on cipB gene sequence (5).
<sup>d</sup>Amino acid composition based on cipA gene sequence (5).
<sup>e</sup>ND, not determined.

Protein inclusions were still visible in most of the cells at 192 h. The ratio of microscopic counts to viable plate counts remained nearly constant throughout the starvation period.

**FIG. 3.** SDS-PAGE analysis (18% gel) of density gradient-purified protein inclusions from* P. luminescens* strains Hm and NC1. Lane Sta, molecular size markers (2 μg of protein per band); lane 1, Hm type 1 protein inclusions; lane 2, Hm type 2 protein inclusions; lane 3, NC1 type 2 protein inclusions; lane 4, NC1 type 1 protein inclusions. Each protein inclusion sample contained 3 μg of total protein.

**FIG. 4.** (A and B) Western blot analyses of time of appearance during growth of* P. luminescens* NC1 type 1 and 2 protein inclusions. (A) Cell lysates probed with type 1 inclusion antiserum. (B) Cell lysates probed with type 2 inclusion antiserum. Lanes 1, 0-h cells (inoculum); lanes 2, 6-h cells; lanes 3, 8-h cells; lanes 4, 10-h cells; lanes 5, 12-h cells; lanes 6, 14-h cells; lanes 7, 16-h cells; lanes 8, 24-h cells. (C and D) Western blot analyses of gradient-purified inclusions and cell lysates from secondary-phase cells. (C) Probed with type 1 inclusion antiserum. (D) Probed with type 2 inclusion antiserum. Lanes 1, 96-h NC1 yellow secondary cells; lanes 2, 96-h NC1 white secondary cells; lanes 3, type 1 protein inclusions from strain Hm; lanes 4, type 2 protein inclusions from strain Hm; lanes 5, type 1 protein inclusions from NC1; lanes 6, type 2 protein inclusions from NC1. Cell lysate lanes contain 2 mg of total protein. Purified protein inclusion lanes contain 0.1 mg of total protein.
and during this time the protein inclusions in the cells were not noticeably reduced in size (Fig. 5).

**Protein inclusions in dividing cells.** A possible explanation for the loss in viability of late-stationary-phase cells is that the large protein inclusions in the cytoplasm might interfere with cell division. This possibility is unlikely to be the case, because time-lapse phase-contrast micrographs clearly show that a cell with a large inclusion is capable of cell division (Fig. 7). The cell elongates and divides on either side of the inclusion. The inclusion protein remains visible inside the mother cell through several rounds of division. This result also shows that inclusion proteins are not detectably degraded and consumed by dividing cells.

**Toxicity of protein inclusions.** The intact and solubilized *P. luminescens* protein inclusions did not kill *G. mellonella* larvae. This was true for both frozen and freshly isolated inclusions.
Injection of larvae with several thousand viable *P. luminescens* cells from a 48-h culture killed the larvae in 24 h.

**DISCUSSION**

Cells of *P. luminescens* strains NC1 and Hm each contain two distinct intracellular protein inclusions that can constitute up to 40% of the total cell protein. The proteins are nearly identical in molecular size and solubility properties but differ significantly in amino acid content, susceptibility to protease digestion, and immunological cross-reactivity. The unusually high content of hydrophobic amino acids in the two protein classes (47% for type 1 and 42% for type 2) probably accounts for their insolubility at neutral pH and solubility at alkaline and acidic pH.

The mass spectrometry and SDS-PAGE analyses show that each inclusion type is composed of a single protein subunit. The mass spectrometry data also showed that the NC1 type 1 inclusion is approximately 66 mass units larger than the Hm type 1. This is probably due to minor differences in the amino acid composition of the proteins. All of these analyses combined with the amino acid composition analyses show that the type 1 inclusion is the *cipB* gene product and the type 2 inclusion is the *cipA* gene product (5).

The biological function of the inclusion proteins is not known. One possibility suggested by the interesting analogy to the parasporal insecticidal crystal proteins of *Bacillus thuringiensis* (3, 18, 19) is that the proteins are involved in insect toxicity of *P. luminescens*. Feeding and injection of *G. mellonella* larvae with both the native and solubilized inclusion proteins did not support this hypothesis. Insect larvae are highly susceptible to the intact bacterial cells; the injected lethal dose is 10 to 100 cells. Similarly, secondary-phase cells that contain no intracellular protein inclusions are equally virulent when injected into larvae (5).

Another plausible function for the proteins is involvement in the nematode symbiosis. The *Heterorhabditis* nematodes grow and multiply while feeding on the primary-inclusion-containing cells, but do not grow and multiply with the secondary-phase cells that lack inclusion proteins. The entomopathogenic nematode *Neoplectana* (*Steinernema*) *glaseri* requires 10 amino acids for growth (20), and these 10 amino acids account for more than 60% of the amino acids in the protein inclusions. The type 2 inclusion protein is especially rich in methionine, which constitutes 13% of the total amino acids. This level of methionine is unusual; the average methionine content of a collection of 207 proteins is 1.7% (22). Thus, intracellular protein inclusions might serve as a rich supply of essential amino acids for the nematode, although there is no known evidence for the nematodes’ obtaining these amino acids from the inclusions. If the protein inclusions are degraded by enzymes in the nematode intestine and are essential to nematode development, the nematodes would be expected to grow on killed cells. In preliminary studies, we found that the nematodes do not grow and reproduce on heat-, freeze-thaw-, or UV light-killed primary-stage cells that contain protein inclusions (unpublished observations). The requirement for living *P. luminescens* cells for nematode development indicates that the nature of the association between the two organisms is a complex interaction in which the inclusion proteins may be just one factor. Two mutants of *P. luminescens*, each missing just one of the inclusion proteins, did not support nematode growth (5). However, these mutants also acquired some secondary-phase characteristics, which could also explain the inability to support nematode growth. Further evidence that this symbiosis is a complex interaction is the report that a transposon-mediated mutation in a phosphopantetheinyl transferase gene of *P. luminescens* NC1 results in cells that no longer supported growth and reproduction of the nematodes (10). This mutant produced both the protein inclusions.

The observation that culture broth of *P. luminescens* NC1 contains bacteriocins and phage particles was the basis of speculation that they may be related to the cytoplasmic inclusions (4). The *P. luminescens* strain NC1 used in this study also produced both of these particles (S. Bintrim, unpublished observations). Western blot analyses using both type 1 and type 2 antisera did not detect any immunologically related material in the culture broth which contained these phage-like structures (unpublished observations).

The inclusions do not appear to be energy or amino acid reserves. The inclusion proteins were not degraded in starving cells (Fig. 6), and cells incubated on agar media or in broth media for several months retained the inclusions.

Another bacterium, *Xenorhabdus nematophilus*, is symbiotically associated with the entomopathogenic nematode *Steinernema carpocapsae* (30). This bacterium, which is related to *Photorhabdus* in some characteristics but clearly belongs to a different genus (8), also produces two intracellular crystal proteins (11). The sizes of these proteins, as estimated by SDS-PAGE analyses, were 22 and 26 kDa, which is twice the size of the *P. luminescens* proteins. The *X. nematophilus* protein inclusions are similar in some solubility characteristics to the *P. luminescens* protein inclusions; for example, they are insoluble at neutral pH but soluble at acidic and alkaline pH, but differ in being soluble in 5 mM EDTA, while both of the *P. luminescens* protein inclusions were insoluble at concentrations of up to 100 mM EDTA. Couche et al. suggested that the inclusion proteins of *X. nematophilus* might be associated with nematode growth and reproduction, but no supporting data were presented (11).

It is interesting that two different genera of bacteria involved in a symbiotic relationship with two different families of entomopathogenic nematodes both produce two intracellular protein inclusions. Because the proteins differ in size and other important aspects, it is likely that the two organisms developed this property independently, although perhaps for a common purpose.

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