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Characterization of an Extracellular Protease from the Insect Pathogen *Xenorhabdus luminescens*

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Xenorhabdus luminescens **Hm cultured in gelatin broth produced a single extracellular protease. The protease was purified by a factor of 500 and characterized as a monomeric protein with an approximate molecular weight of 61,000. On the basis of inhibitor studies and its pH optimum, the protease was classified as an alkaline metalloprotease with a pH optimum near 8; the isoelectric point of the enzyme is 4.2 ± 0.2. The protease may be a major factor in the ecology of *X. luminescens*, which is carried as a symbiont of some parasitic nematodes.**

Most parasitic nematodes develop in their insect hosts without the assistance of bacteria. Notable exceptions to this occur within the families *Steinernematidae* and *Heterorhabditidae*, to which belong several genera of nematodes that harbor the *Xenorhabdus* bacteria as symbionts. The mutualistic relationship between the nematodes and the bacteria forms a remarkable insect-pathogen complex that is capable of infecting a variety of insects (22).

The infective, juvenile-stage nematode harbors the *Xenorhabdus* bacterium in its gut (24), and, after infecting an insect, the nematode burrows through the intestinal wall of the insect and voids the bacteria into the hemolymph (21). The bacteria avoid the immune response of the insect and proliferate rapidly so that within 48 h the insect is dead (24). When *Xenorhabdus luminescens* is involved in this infection, the insect cadaver is visibly luminous in darkness and has a brick red color in daylight. Both the luminescence and the pigmentation of the cadaver are the results of the bacterial population in the cadaver (24, 26). In addition, many researchers have noted the lack of putrefaction of the dead insect, presumably due to antibiotic(s) produced by the bacteria (2, 20, 26).

Axenic nematodes can still infect an insect, but they are much less virulent than *Xenorhabdus* sp.-carrying nematodes (22, 23). Furthermore, without the bacteria, the nematodes are hindered in the completion of their life cycle (3, 23). Both the virulence associated with the presence of the bacteria and the creation of suitable conditions for nematode growth and reproduction may be related to an extracellular protease produced by the *Xenorhabdus* bacteria.

Akhurst (3), Grimont et al. (10), and Boemare and Akhurst (5) have identified proteolytic activity in several *Xenorhabdus* strains. In this study, we report the purification and characterization of an extracellular protease produced by *X. luminescens* Hm, and we discuss its potential involvement in the bacterium-nematode-insect life cycle.

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MATERIALS AND METHODS

Organisms and growth conditions. *X. luminescens* Hm was kindly provided by J. Ensign (University of Wisconsin, Madison) and was used throughout this study. Stock cultures of strain Hm were maintained on agar plates containing 10 g of tryptone (Difco) per liter, 5 g of yeast extract per liter, 5 g of NaCl per liter, and 1.5% agar. Culture characteristics were monitored as previously described (B. Bleakley and K. H. Nealson, FEMS Microbial Ecol., in press) to ensure that the strain retained the properties of the primary form.

The protease was purified from cultures of strain Hm grown at 30°C in 2-liter flasks that each contained 750 ml of gelatin broth and that had been shaken at 300 rpm. A 5-ml inoculum culture, grown in gelatin broth, was used. The gelatin medium was a modification of those described by Bromke and Hammel (6), Kaska et al. (14), and Tyler et al. (27). It contained the following: 1.7 mM $MgSO_4$, 9.4 mM NH_4Cl, 68.0 μM $CaCl_2 \cdot 2H_2O$, 6.3 μM $FeCl_3 \cdot 6H_2O$, 0.15 μM $MnCl_2 \cdot 4H_2O$, 0.124 μM $Na_2MoO_4 \cdot 2H_2O$, 42.8 mM NaCl, 27.6 mM potassium phosphate (pH 6.7), and 2.0% gelatin (Difco). All components except the potassium phosphate (added aseptically from a sterile 0.276 M stock [pH 6.7]) were mixed and heated until the gelatin was completely dissolved.

Purification of protease. Cells were removed from the culture by centrifugation at 7,000 × *g* after 10, 17, or 30 days of incubation. Ammonium sulfate was added to the culture supernatant and stirred at 4°C for at least 3 h. The concentration of ammonium sulfate was increased in 10% increments from 20 to 90% saturation. The precipitate resulting from each step was harvested by centrifugation at 7,000 × *g*, dissolved in 50 mM Tris hydrochloride (pH 8.0), and dialyzed against the same buffer with Spectrapor membrane tubing with a molecular weight cutoff of 12,000 to 14,000. The dialyzed sample was then assayed for protease activity, as described below.

The majority of protease activity was found in precipitates from 40 to 70% saturated ammonium sulfate solutions. These samples were pooled and concentrated by pressure dialysis with an Amicon 20,000-molecular-weight-cutoff filter. The concentrated sample was applied to a flatbed isoelectric focusing apparatus with pH gradients of 3 to 10 or of 3.5 to 5.5. The band with the most activity from the isoelectric focusing gel was eluted with 10 mM KCl and concentrated with Amicon microconcentrators (molecular weight cutoff =

30,000). The final step in the purification employed a G-100 molecular sieve column equilibrated and eluted with 50 mM Tris hydrochloride (pH 8.0).

Protease assay. Protease activity was assayed by a modification of the method described by Himelbloom and Hassan (13). Assays during the purification and assays to determine inhibitor sensitivities were performed in Corning tissue culture plates (24 wells) incubated at 30°C while shaken at 50 rpm on a rotary shaker. Each well of the plate contained a reaction mixture (total volume, 1.25 ml) consisting of 7.5 mg of Hide Powder Azure (HPA) (Sigma) in 0.5 mM $CaCl_2 \cdot 2H_2O$ and 50 mM Tris hydrochloride (pH 8.0). Enzyme solutions (10 to 100 μl) were added to initiate the reaction. When used, inhibitors were added to the reaction mixture 20 min before the addition of the enzyme. The contents of each well were removed after a 2-h incubation and were centrifuged in an Eppendorf microcentrifuge to remove any undigested HPA. The optical density at 595 nm (OD_{595}) of the reaction supernatant was determined within 4 min by using an LKB 2010 spectrophotometer. One unit of enzyme activity was defined as the amount of enzyme that caused an increase in the OD_{595} of 0.01/min (13).

The pH optimum for the purified protease was determined by adding 50 μl of the purified enzyme preparation to a reaction mixture containing 25 μg of HPA, 500 μl of 5 mM $CaCl_2 \cdot H_2O$, 3.3 ml of double-distilled H_2O, and 1.125 ml of the appropriate pH buffer. Amounts (1 ml) were removed at 30-min intervals for 2 h and treated as described above. The following buffers were prepared as 200 mM stock solutions: sodium phosphate (pH 6), Tris hydrochloride (pH 7 to 9), and sodium carbonate (pH 10). The concentration of protein present in the assays was estimated by the method of Lowry et al. (17).

Electrophoresis. Protease activity was visualized in polyacrylamide gels by incorporating 0.1% gelatin into a sodium dodecyl sulfate (SDS)-polyacrylamide gel (12). Electrophoresis was performed at 4°C at a constant current of 8 mA. After electrophoresis was complete, the gel was removed and washed with gentle agitation for 2 h in 2.5% (vol/vol) Triton X-100 to remove SDS. The gels were then incubated at 30°C for 2 h in 0.1 M glycine adjusted to pH 8.0 with 0.1 M NaOH. After incubation, the gels were fixed and stained overnight in 0.1% amido black in methanol-acetic acid-water (30:10:60, vol/vol/vol). Gels were destained in methanol-acetic acid-water (30:10:60, vol/vol/vol). The background of the gel stained uniformly, while protein bands stained darker; any zones in which the gelatin was digested remained unstained.

The purity of each enzyme preparation was determined by staining 15% polyacrylamide gels (15) with Coomassie brilliant blue R.

RESULTS

The culture supernatant from *X. luminescens* Hm contained a single major proteolytic enzyme, which was visualized by electrophoresis in a gelatin-containing polyacrylamide activity gel (Fig. 1). This was the only protease seen in cultures that were incubated for 10, 17, or 30 days. Gelatin was the only substrate for a protease in the activity gel; therefore, it is possible that other proteases were present but remained undetected by this technique. The purified protease retained proteolytic activity and migrated to the same point as did the major proteolytic enzyme in the culture supernatant (Fig. 1).

Since the enzyme retained activity after being subjected to the denaturing conditions in SDS-polyacrylamide gel electrophoresis, it is likely that the active protease is a monomeric protein. This is supported by the fact that the relative molecular weight of the active protease was similar when determined by SDS-polyacrylamide gel electrophoresis (61,000) (Fig. 2) to that determined by column chromatography (60,000) on a G-100 molecular sieve column (Fig. 3).

The protease precipitated from the culture supernatant at ammonium sulfate concentrations between 40 and 70%. The

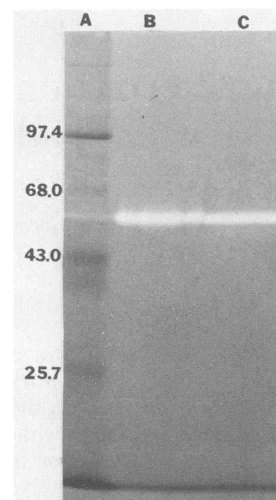

FIG. 1. Identification of protease activity in 11% polyacrylamide gels containing 0.01% gelatin. The gelatin background is uniformly stained with amido black. Lanes: A, standards (with relative molecular weights [in thousands] indicated) electrophoresed and stained darker than the background; B, sample of the culture supernatant from a 17-day-old culture; and C, purified protease. Lanes B and C have a single cleared zone where the gelatin was digested by proteolysis.

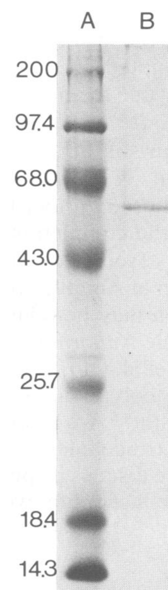

FIG. 2. Coomassie blue-stained polyacrylamide gel. Lanes: A, molecular weight markers; B, purified protease. Molecular weights (in thousands) are as follows: myosin, 200; phosphorylase *b*, 97.4; bovine serum albumin, 68; ovalbumin, 43; α-chymotrypsinogen, 25.7; β-lactoglobulin, 18.4; and lysozine, 14.3.

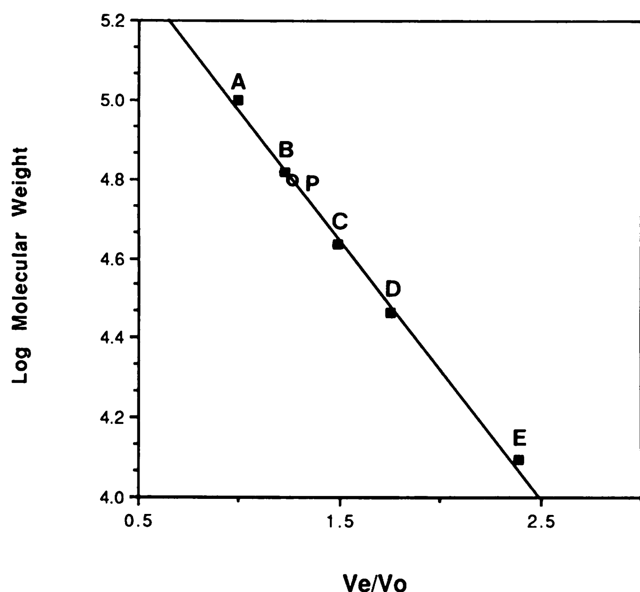

FIG. 3. Logarithm of the molecular weight of the protease (P) and protein standards versus the ratio of the elution volume (Ve) to the void volume (Vo) on a Sephadex G-100 column. The void volume (A) was determined with Blue Dextran (average molecular weight = 200,000). The molecular weights of the protein standards used were as follows: bovine serum albumin (B), 66,000; ovalbumin (C), 43,000; carbonic anhydrase (D), 29,000; and cytochrome *c* (E), 12,400.

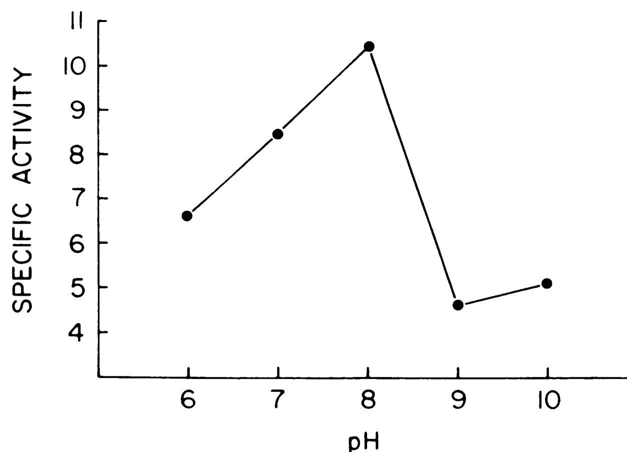

FIG. 4. Effect of pH on the activity of the protease from *X. luminescens* as measured by the release of Remazol Brilliant Blue dye (OD_{595}) from HPA. One unit of enzyme activity is arbitrarily defined as the amount of enzyme that causes an increase in OD_{595} equal to 0.01/min.

dialyzed protease from the ammonium sulfate precipitation focused at a pH of 4.2 ± 0.2 on flatbed isoelectric focusing gels and, after chromatography on the G-100 column, it was the major band visible on an SDS–15% polyacrylamide gel stained with Coomassie blue (Fig. 2). The specific activity of the protease increased by a factor of approximately 500 during the purification procedure (Table 1).

The addition of 0.5 mM calcium chloride (which is known to stabilize other metalloproteases [4]) to the protease reaction mixture increased the activity of the protease by 35% and so was always included in assays of the enzyme. The proteolytic cleavage of HPA was linear for at least 2 h under the conditions tested. By using the slope of the curves resulting from the HPA assay at various pH values, the pH optimum was determined to be near pH 8 (Fig. 4).

The enzyme was inhibited by the metal chelators EDTA and 1,10-*o*-phenanthroline and showed a dose-dependent response to these inhibitors at concentrations between 0.02 and 2.0 mM (Fig. 5). The protease was not significantly inhibited by phenylmethylsulfonyl fluoride, an inhibitor of serine proteases, and was inhibited to only a small extent by *p*-chloromercuribenzoate, an inhibitor of thiol proteases. The concentrations of these protease inhibitors used were well in excess of the amount usually required (25).

DISCUSSION

The entomopathogenic bacterium *X. luminescens* is carried as a symbiont by some parasitic nematodes and is thought to be the primary pathogen of insects infected by the nematodes (22). The bacterium is also required to provide suitable conditions for nematode reproduction in the insect carcass (1). Both the pathogenicity and the creation of a suitable environment for nematode reproduction may be due in part to the production of an extracellular protease by *X. luminescens*.

In gelatin broth cultures, *X. luminescens* Hm produces a single extracellular protease. According to the classification scheme of Hartley (11), this protease can be classified as a metalloprotease because it is inhibited by the metal-chelating compounds EDTA and 1,10-*o*-phenanthroline. Inhibitors of serine and thiol proteases are ineffective against the *Xenorhabdus* protease, and the slightly alkaline pH optimum for the enzyme excludes it from classification as an acid protease.

Metalloproteases can be further subdivided into neutral or alkaline proteases on the basis of their pH optima (4, 19). The protease from strain Hm may be classified as an alkaline metalloprotease since the pH optimum for the enzyme is near 8. This class of proteases is produced by several species

TABLE 1. Purification of the extracellular protease from cultures of *X. luminescens* Hm

Fraction	Volume (ml)	Protein		Protease		
		[Conc (mg/ml)]	% Recovery	Total activity (units)	Sp act (units/mg of protein)[a]	% Recovery
Culture	1,350	30.8	100	1,316	0.04	100
$(NH_4)_2SO_4$	5.6	133.0	1.8	256	0.43	19
IEF[b]	3.6	87.0	0.8	65	0.26	5
G-100	0.9	1.6	0.004	26	22.39	2

[a] One unit of enzyme activity is arbitrarily defined as the amount of enzyme that causes an increase in OD_{595} equal to 0.01/min (13).
[b] IEF, Isolelectric focusing.

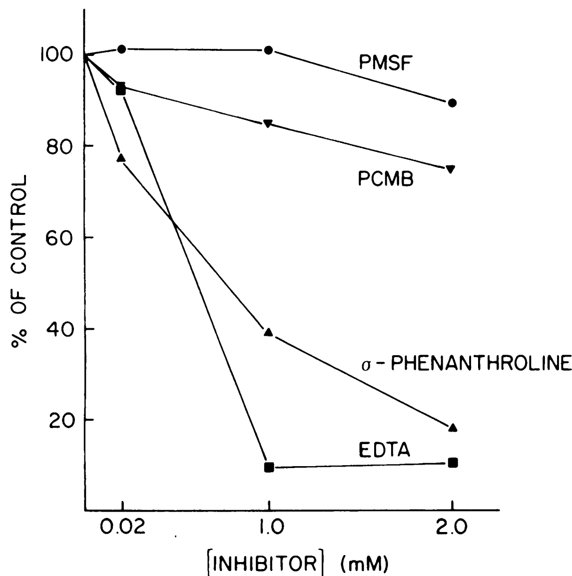

FIG. 5. Effect of inhibitors on protease activity. Each point is the average of duplicate samples. The control rate was 13.67 units mg/(protein) per min. PCMB, *p*-Chloromercuribenzoate; PMSF, phenylmethylsulfonyl fluoride.

of insect pathogens, including *Pseudomonas aeruginosa*, *Proteus mirabilis*, and *Serratia marcescens* (14, 19). These proteases are thought to be fatal to infected insects by specifically precipitating hemolymph proteins and causing damage to the hemocytes (18). The protease produced by *S. marcescens* may also be the extracellular factor that inactivates the immune system of *Cecropia* spp. (8, 9).

In addition to the potential toxicity of the protease, the products of proteolysis may provide nutrients for the bacteria and the developing nematodes. In tests with *Galleria mellonella* infected by nematodes carrying *X. luminescens*, proteolysis of the dead insect was evidenced by the ammoniacal smell of the dead carcasses after several days (Bleakley and Schmidt, unpublished results). Proteolysis of the insect carcass should also cause an alkaline shift in the pH of the carcass. This shift occurs, as determined by the change in color of the carcass from yellow to red. This change is due to a pigment produced by the bacteria that undergoes a pH-sensitive color shift from yellow at near-neutral pH to red near pH 9 (26). The initial pH of the insect hemolymph is 6.5 (7).

Alkaline metalloproteases generally have a broad range of peptide and protein substrates; no regularity in specificity has been observed (19). Despite the broad substrate range, the *X. luminescens*-carrying nematodes go through a complete life cycle within the insect carcass and presumably are exposed to the bacterial protease. Juvenile and adult nematode cuticles contain protein (16, 22); thus, the nematodes must tolerate the action of the extracellular protease produced by *X. luminescens*.

When plated on a solid substrate, two *Xenorhabdus* colony types are observed (1, 5; Bleakley and Nealson, in press), the predominant, wild-type form and a natural variant which has been termed the secondary form (1). The secondary form of strain Hm has several characteristics that distinguish it from the primary form, including lack of protease production, decreased luminescence, and lack of antibiotic and pigment production (Bleakley and Nealson, in press). These characteristics change in concert in strain Hm (Bleakley and Nealson, in press), but these traits are not necessarily linked to one another in the conversion of primary forms to secondary forms in other strains (5). The role of this phase variation in *X. luminescens* in nature is not currently understood, but it is the primary form of *X. luminescens* that is necessary for the nematodes to complete their life cycle in parasitized insects (1).

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LITERATURE CITED

1. **Akhurst, R. J.** 1980. Morphological and functional dimorphism in *Xenorhabdus* spp., bacteria symbiotically associated with the insect pathogenic nematodes *Neoaplectana* and *Heterorhabditis*. J. Gen. Microbiol. **121**:303–309.
2. **Akhurst, R. J.** 1982. Antibiotic activity of *Xenorhabdus* spp., bacteria symbiotically associated with insect pathogenic nematodes of the families Heterorhabditidae and Steinernematidae. J. Gen. Microbiol. **128**:3061–3065.
3. **Akhurst, R. J.** 1983. Taxonomic study of *Xenorhabdus*, a genus of bacteria symbiotically associated with insect pathogenic nematodes. Int. J. Syst. Bacteriol. **33**:38–45.
4. **Barrett, A. J.** 1986. An introduction to proteinases, p. 3–22. *In* A. J. Barrett and G. Salvensen (ed.), Proteinase inhibitors. Elsevier Biomedical Press, Amsterdam.
5. **Boemare, N. E., and R. J. Akhurst.** 1988. Biochemical and physiological characterization of colony form variants in *Xenorhabdus* spp. (Enterobacteriaceae). J. Gen. Microbiol. **134**:751–761.
6. **Bromke, B. J., and J. M. Hammel.** 1979. Regulation of extracellular protease formation by *Serratia marcescens*. Can. J. Microbiol. **25**:47–52.
7. **Dunphy, G. B., D. B. Morton, A. Kvopinski, and J. M. Chadwick.** 1986. Pathogenicity of lipopolysaccharide mutants of *Pseudomonas aeruginosa* for larvae of *Galleria mellonella*: bacterial properties associated with virulence. J. Invertebr. Pathol. **47**:48–55.
8. **Flyg, C., K. Keene, and H. G. Boman.** 1980. Insect pathogenic properties of *Serratia marcescens*: phage-resistant mutants with a decreased resistance to *Cecropia* immunity and a decreased virulence to *Drosophila*. J. Gen. Microbiol. **120**:173–181.
9. **Flyg, C., and K. G. Xanthopoulos.** 1983. Insect pathogenic properties of *Serratia marcescens*. Passive and active resistance to insect immunity studied with protease-deficient and phage-resistant mutants. J. Gen. Microbiol. **129**:453–464.
10. **Grimont, P. A. D., A. G. Steigerwalt, N. Boemare, F. W. Hickman-Brenner, C. Deval, F. Grimont, and D. J. Brenner.** 1984. Deoxyribonucleic acid relatedness and phenotypic study of the genus *Xenorhabdus*. Int. J. Syst. Bacteriol. **34**:378–388.
11. **Hartley, B. S.** 1960. Proteolytic enzymes. Annu. Rev. Biochem. **29**:45–72.
12. **Heussen, C., and E. B. Dowdle.** 1980. Electrophoretic analysis of plasminogen activators in polyacrylamide gels containing sodium dodecyl sulfate and copolymerized substrates. Anal. Biochem. **102**:196–202.
13. **Himelbloom, B. H., and H. M. Hassan.** 1985. Optimization of the hide powder azure assay for quantitating the protease of *Pseudomonas fluorescens*. J. Microbiol. Methods **4**:59–66.
14. **Kaska, M., O. Lysenko, and J. Chaloupka.** 1976. Exocellular proteases of *Serratia marcescens* and their toxicity to larvae of *Galleria mellonella*. Folia Microbiol. **21**:465–473.
15. **Laemmli, U. K.** 1970. Cleavage of structural proteins during the assembly of the head of bacteriophage T4. Nature (London) **227**:680–685.
16. **Lee, D. L., and H. J. Atkinson.** 1977. Physiology of nematodes, 2nd ed. Columbia University Press, New York.
17. **Lowry, O. H., N. J. Rosebrough, A. L. Farr, and R. J. Randall.** 1951. Protein measurement with the Folin phenol reagent. J.

Biol. Chem. **193**:265–275.

18. **Lysenko, O.** 1985. Non-spore forming bacteria pathogenic to insects: incidence and mechanisms. Annu. Rev. Microbiol. **39**: 673–695.
19. **Morihara, K.** 1974. Comparative specificity of microbial proteinases. Adv. Enzymol. Relat. Areas Mol. Biol. **41**:179–243.
20. **Paul, V. J., S. Frautschy, W. Fenical, and K. H. Nealson.** 1981. Antibiotics in microbial ecology: isolation and structure assignment of several new antibacterial compounds for the insect-symbiotic bacteria *Xenorhabdus* spp. J. Chem. Ecol. **7**:589–597.
21. **Poinar, G. O.** 1966. The presence of *Achromobacter nematophilus* in the infective stage of a *Neoaplectana* sp. (Steinernematidae: Nematoda). Nematologica **12**:105–108.
22. **Poinar, G. O.** 1983. The natural history of nematodes. Prentice-Hall, Inc., Englewood Cliffs, N.J.
23. **Poinar, G. O., and G. M. Thomas.** 1966. Significance of *Achromobacter nematophilus* Poinar & Thomas (Achromobacteriaceae: Eubacteriales) in the development of the nematode, DD136 (*Neoaplectana* sp. Steinernematidae). Parasitology **56**: 385–390.
24. **Poinar, G. O., G. M. Thomas, M. Haygood, and K. H. Nealson.** 1980. Growth and luminescence of the symbiotic bacteria associated with the terrestrial nematode *Heterorhabditis bacteriophora*. Soil Biol. Biochem. **12**:5–10.
25. **Powers, J. C., and J. W. Harper.** 1986. Inhibitors of metalloproteases, p. 219–298. *In* A. J. Barrett and G. Salvensen (ed.), Proteinase inhibitors. Elsevier Biomedical Press, Amsterdam.
26. **Richardson, W. H., T. M. Schmidt, and K. H. Nealson.** 1988. Identification of an anthraquinone pigment and a hydroxystilbene antibiotic from *Xenorhabdus luminescens*. Appl. Environ. Microbiol. **54**:1602–1605.
27. **Tyler, M. E., J. R. Milam, R. L. Smith, S. C. Schank, and D. A. Zuberer.** 1979. Isolation of *Azospirillum* from diverse geographic regions. Can. J. Microbiol. **25**:693–697.