An insecticidal pilin subunit from insect pathogenic bacterium

Xenorhabdus nematophila

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Abstract

Xenorhabdus nematophila is an insect pathogen and produces protein toxins which kill
the larval host. Previously we characterized an orally toxic, large, outer membrane­
associated protein complex from the culture medium of X. nematophila. Here, we
describe the cloning, expression and characterization of a 17 kDa pilin subunit of X.
nematophila isolated from that protein complex. The gene was amplified by PCR, cloned
and expressed in E. coli. The recombinant protein was refolded in vitro in the absence
of its cognate chaperone by urea gradient. The protein oligomerized during in vitro
refolding, to form multimers. Point mutations in the conserved N- terminal residues of
the pilin protein greatly destabilized its oligomeric organization demonstrating the
importance of N-terminus in refolding and oligomerization of the pilin subunit by donor
strand complementation. The recombinant protein was cytotoxic to cultured Helicoverpa
armigera larval hemocytes causing agglutination and subsequent release of the
cytoplasmic enzyme lactate dehydrogenase. The agglutination of larval cells by the 17
kDa protein was inhibited by several sugar derivatives. The biological activity of the
purified recombinant protein indicated that it has acquired a conformation similar to the
native protein. The 17 kDa pilin subunit was found to be orally toxic to 4-5th instar larvae
of an important crop pest, H. armigera causing extensive damage to the mid gut epithelial
membrane. To our knowledge this is first report describing an insecticidal pilin subunit of
a bacterium.
Characterization of a cytotoxic pilin subunit of Xenorhabdus nematophila

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Abstract

Xenorhabdus nematophila is an insect pathogenic bacterium, known to produce protein toxins that kill the larval host. We have described a cytotoxic pilin subunit of X. nematophila, which is expressed on the cell surface and also secreted in the extracellular medium associated with outer membrane vesicles. A 17 kDa pilin subunit was isolated and purified from X. nematophila cell surface. The protein showed cytotoxicity to larval hemocytes of Helicoverpa armigera in an in vitro assay, causing agglutination of the cells, and releasing cytoplasmic enzyme lactate dehydrogenase in the medium. The pilin protein was able to bind to the surface of larval hemocytes. The binding and cytotoxicity of the purified 17 kDa protein to hemocytes was inhibited by antiserum raised against the pilin protein. The study demonstrates for the first time a cytotoxic structural subunit of pilin from an entomopathogenic bacterium X. nematophila that is excreted in the extracellular medium with outer membrane vesicles.

Keywords: Xenorhabdus; Hemocytes; Cytotoxic; Pilin subunit

The growing concern of development of resistance in the crop pests to crystal protein toxins of Bacillus thuringiensis has fuelled vigorous efforts to discover new orally active insecticidal proteins [1]. Xenorhabdus nematophila is a gram-negative bacterium belonging to the family Enterobacteriaceae [2,3]. It lives in symbiotic association with entomopathogenic nematodes of the family Steinernematidae [4]. X. nematophila is pathogenic to a wide range of insects [3,5,6] and is currently being used as a biological control agent against insects belonging to Lepidoptera [7], Coleoptera [8,9], and Diptera [10].

The biology and insecticidal potential of proteins produced by X. nematophila are being investigated in several laboratories. The infective juvenile larvae of the nematode host transport X. nematophila to the gut of the target insect [3,6] and release the bacterium in the insect hemocoel causing larval death within 48 h [4]. Bacterial multiplication and toxic protein secretion are the main causes of larval death [3]. The bacteria alone are sufficient to cause larval mortality following injection into the hemocoel [5], or orally when mixed in the diet [11,12].

The outer membrane and associated proteins exposed on the surface of pathogenic bacteria have been demonstrated to perform several critical functions under adverse environmental conditions such as recognition and interaction with the target host cells [13], transport of toxin proteins [14], and secretion of virulence factors and antibacterial proteins [15]. In X. nematophila, the outer membrane associated proteins may be required to perform functions that allow the bacterium to evade the insect host immune system and promote symbiotic association with the nematode host [3,16]. We have recently shown that outer membrane vesicles (OMVs) excreted in the medium by X. nematophila cells possess insecticidal activity [12]. The OMVs of X. nematophila showed larvicidal activity against an important crop pest, Helicoverpa armigera. The larvicidal protein complex contained several major and few minor polypeptides ranging from 15 to 300 kDa.

We undertook this study to isolate and characterize a major 17 kDa protein associated with the outer
membrane of *X. nematophila*. The N-terminal sequence of the 17 kDa band present in the OMV preparation [12] showed homology with the PapA subunit of P pilus of *Escherichia coli* [17]. The pil or fimbriae are hair-like appendages on the outer surface of gram-negative bacteria. For pathogenic bacteria, these organelles initiate pathogenesis by mediating the host cell recognition prior to invasion [18]. The fimbrial proteins have been shown to mediate recognition and subsequent interaction of several bacterial pathogens with the host cells and are considered as important virulence factors [19]. Moureau'x et al. [20] have described F1 fimbriae of *X. nematophila*, which caused mannose resistant agglutination of erythrocytes.

Presence of fimbriae exclusively on the surface of phase I cells is suggested to aid in the retention of the cells of *X. nematophila* by the nematode host [20].

In this study, we demonstrate binding and cytotoxic activity of a pilin subunit of the entomopathogenic bacterium *X. nematophila* to insect hemocytes, suggesting its involvement in the pathogenic process.

**Materials and methods**

**Bacteria and growth conditions.** The strain used in this study *X. nematophila* 19061 was obtained from ATCC (Rockville, MD). The *X. nematophila* culture was streaked on nutrient agar supplemented with 0.004% (w/vol.) triphenyl tetrazolium chloride and 0.025% (w/vol.) bromothymol blue (NBTA) [2]. Broth cultures were grown from a single blue colony in LB medium at 28°C with shaking at 150 rpm. *E. coli* K-12 was used as a reference strain.

**Preparation and purification of outer membrane vesicles from *X. nematophila*.** The outer membrane vesicles (OMVs) were prepared from the culture supernatant as described earlier by Khandelwal and Bhatnagar [12].

**Isolation and purification of pilin subunit from *X. nematophila*.** The pilin protein was prepared from *X. nematophila* cells as described by Korhonen et al. [21]. Briefly, *X. nematophila* cells from 24-h-old culture were plated on NBTA plates. The plates were incubated at 28°C for 48 h. Cells were scrapped and harvested in TEB buffer containing 5 mM Tris–HCl (pH 8.0), 1 mM EDTA (pH 8.0), and 1 mM benzamidine (Sigma). Fimbrial protein was removed from bacterial cell surface by homogenization in a homogenizer thrice for 5 min at 4°C. Cell mass was removed by centrifugation at 8000 rpm (45 min, 4°C) and the supernatant was precipitated with 20% ammonium sulphate. The precipitated proteins were centrifuged at 15,000 rpm (45 min, 4°C) and the pellet was resuspended in 1 ml TEB buffer. The fimbrial subunit protein was purified by sucrose gradient (10–60% in 5 mM Tris–HCl, pH 8.0) ultracentrifugation at 22,000 rpm (16 h, 15°C). Fractions containing purified protein were dialysed and concentrated with centrifcon (3 kDa cut off, Amicon). The protein concentration was estimated by Bio-Rad protein assay kit (Bio-Rad laboratories) with BSA as the standards.

**Preparation of antiserum.** The 17 kDa pilin subunit isolated from the surface of *X. nematophila* cells and purified by sucrose density gradient ultracentrifugation was injected in a rabbit for preparation of polyclonal antiserum. The antibody titre of the serum was determined by ELISA and Western blotting.

**Amino terminal protein sequencing.** The purified 17 kDa band in the SDS-PAGE was transferred on PVDF membrane under standard conditions, stained with amido black, and excised for N-terminal sequencing by Edman's method, using an automatic sequencer (ABI Model 492A).

**Electron Microscopy.** The purified 17 kDa protein was applied to formvar-coated copper grids for 1 min, washed with ultra pure grade water, and negatively stained with 2% uranyl acetate solution (15–30 s). Grids were examined with a Philips CM10 TEM under standard conditions at 60–80 kV.

**Hemagglutination of larval hemocytes.** Hemocytes were obtained from *H. armigera* larvae (fifth instar) in an anticoagulant buffer (96 mM NaOH, 186 mM NaCl, 17 mM EDTA, and 41 mM citric acid, pH 4.5). The hemocytes were centrifuged at 4000 rpm for 2 min, washed in 150 mM PBS, and resuspended in Grace’s insect cell medium without serum (Invitrogen). The hemocytes were plated in a 48-well tissue culture plate and allowed to settle for 30 min. The medium with the floating cells was removed by gentle aspiration and replaced with 200 μl serum free Grace’s insect medium. Two hundred microlitres of bacterial suspension (10⁷ cells/ml) or purified 17 kDa protein (100 μg) was serially 2-fold diluted, added to the wells, and incubated for 2 h at 28°C. Heat inactivated 17 kDa protein, pre-immune serum, PBS, Grace’s medium, BSA, and *E. coli* K12 strain were used as controls. Agglutination was observed with a light microscope. Agglutination titre was defined as reciprocal of the highest dilution of the protein that caused cell clumping within 2 h of incubation. Inhibition of agglutination was determined with anti-pilin antiserum using undiluted pre-immune serum as control. Different dilutions (1:10–1:1000) of the antiserum were mixed with 200 μl of 2-fold serially diluted purified protein (initial concentration 100 μg/ml) and incubated at room temperature for 1 h before adding to the hemocytes. Similarly, agglutination of erythrocytes from different sources (rabbit, sheep, and human) was also tested. All experiments were done in triplicate and performed more than three times.

**Cytotoxicity assay.** Larval hemocyte's cytotoxicity was determined on the basis of release of lactate dehydrogenase (LDH), a cytoplasmic enzyme generally excreted in the medium due to lysis or damage to the eukaryotic cells [22]. Hemocytes were obtained from *H. armigera* larval (fifth instar) in an anticoagulant buffer. The hemocytes were centrifuged, washed in 150 mM PBS, and resuspended in Grace's insect cell medium (without serum). Equal numbers of cells were plated in a 48-well tissue culture plate and allowed to adhere for 45 min. The medium and non-adherent cells were removed by gentle aspiration and replaced with 200 μl of serum free Grace's insect medium. Different concentrations of purified 17 kDa protein (5–25 μg/ml) were added to the wells and incubated for 4–5 h at 28°C. At different time points the medium was collected and centrifuged at 5000 rpm for 2 min. Supernatant was collected and LDH released was determined as described in Worthington Enzyme Manual [23]. Heat inactivated purified 17 kDa protein, PBS, and Grace’s medium were used as controls. Total LDH content of the cells was determined after complete lysis of the cells with 0.5% Triton X-100 in serum free Grace's medium. LDH activity was represented as mU/well. One unit is defined as “oxidation of 1 μmol of NADH per minute at 25°C and pH 7.3, under the specified conditions” and was calculated using the relation: U/mg = A/Abs × ml reaction mixture/6.22 × mg protein × minute. The absorbance was read at 340 nm using Jasco V-530 (UV/VIS) spectrophotometer and milli-units of LDH released was determined as explained above. Experiment was performed in triplicate.

**Detection of binding of 17 kDa protein to larval hemocytes by immunofluorescence.** *Helicoverpa armigera* larval (fifth instar) hemocytes were seeded onto sterile coverslips, placed in a 6-well tissue culture plate. Different test proteins were added to the coverslips and incubated at 28°C for 1 h. The cells on the coverslips were fixed with 4% formaldehyde in PBS. The fixed cells were washed three times with PBS and incubated with pre-immune serum or polyclonal antiserum against 17 kDa protein, diluted 1:5000 in antibody dilution buffer (30 mM Tris–HCl, 15 mM NaCl, pH 7.5) containing 0.5% BSA in humidified conditions, at room
temperature, for 2 h. The coverslips were washed three times with PBS and incubated with fluorescein isothiocyanate (FITC)-conjugated goat anti-rabbit antibody (Sigma) diluted 1:200 in antibody dilution buffer containing 0.5% BSA in humidified conditions at room temperature for 1 h. The cells were washed with PBS three times and mounted in buffered glycerol (90% glycerol in 100 mM sodium phosphate buffer, pH 8.0) containing 1 mg/ml of 2,5-diphenyl-1,3,4-oxadiazole. The cells were observed in a fluorescence microscope and photographed.

Results

Purification and Identification of pilin subunit from X. nematophila

The 17 kDa pilin protein purified by ammonium sulphate precipitation and sucrose density gradient centrifugation migrated as a single band at 17 kDa in the

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**Fig. 1.** (A) SDS-PAGE profile of *X. nematophila* proteins during purification of 17 kDa pilin protein from cell surface. Surface proteins were extracted from the bacterial cells in TEB buffer (5 mM Tris, 1 mM EDTA, and 1 mM benzamidine), precipitated with 20% ammonium sulphate, and fractionated by sucrose gradient centrifugation. The proteins were resolved on a 12% SDS-PAGE and visualized by staining with Coomassie brilliant blue. Lane 1, outer membrane vesicle (OMV) proteins; lane 2, 20% ammonium sulphate precipitated proteins; and lane 3, purified 17 kDa pilin protein after sucrose gradient centrifugation. (B) SDS-PAGE of purified 17 kDa pilin protein boiled for 5 min with reducing and non-reducing dyes. Lane 1, 17 kDa protein with reducing dye; lane 2, 17 kDa protein with non-reducing dye. (C) Western blot of pilin protein in different fractions from *X. nematophila* cells. The proteins were blotted with rabbit polyclonal antiserum against purified 17 kDa pilin protein from *X. nematophila*. Lane 1, cell lysate of *X. nematophila*; lane 2, outer membrane vesicle (OMV) proteins from *X. nematophila*; lane 3, 20% ammonium sulphate precipitated proteins; and lane 4, purified pilin protein. (D) Electron micrograph showing 17 kDa pilin protein forming fibre like structures (magnification 24,500×), bar 0.1 μm.
SDS–PAGE (Fig. 1A, lane 3). The N-terminal sequence of the protein was found to be APTQGDGTVK, which was identical to the N-terminal sequence of the 17 kDa band present in the OMV preparation (Fig. 1A, lane1) and was 70% homologous to the PapA protein, the structural subunit of the P pilus of E. coli. The purified pilin subunit formed fibrous structures as seen by high-resolution electron microscopy (Fig. 1D). The pilin protein migrated as a monomer in both reducing and non-reducing SDS–PAGE indicating the absence of any intermolecular disulphide bonds in the protein (Fig. 1B). The antiserum against the pilin subunit reacted with the purified protein and the 17 kDa band present in the OMV preparation from the X. nematophila cells in Western blotting (Fig. 1C).

Agglutination assays

The purified 17 kDa pilin protein caused strong agglutination of H. armigera hemocytes in an in vitro assay (Fig. 2B). Agglutination of cells could be observed under the microscope as early as 30 min of incubation in the presence of the purified 17 kDa pilin protein (Fig. 2B). Purified 17 kDa protein agglutinated insect hemocytes at a concentration up to 3.5 µg/well. Heating the 17 kDa protein at 80°C for 15 min resulted in complete and irreversible loss of its agglutinating property (Fig. 2C). Antiserum against the pilin subunit inhibited agglutination of hemocytes by the protein (Fig. 2D). No agglutination was observed up to 1:500 dilution of the antiserum in the presence of the 17 kDa protein, and pre-immune serum had no effect on agglutination of hemocyte by the pilin protein (Table 1). E. coli K12 cells or OMVs prepared from it were not able to agglutinate larval hemocytes (Table 1). No clumping was seen in the buffer control (Fig. 2A). No effect was observed with erythrocytes of rabbit, sheep, and humans and on SF 21 cell line, derived from the ovary of Spodoptera frugiperda and macrophage J774A.1 cell line (data not shown).

Hemocyte cytotoxicity and lactate dehydrogenase release

The interaction of pilin protein with the larval hemocytes was cytotoxic and resulted in the release of cytoplasmic enzyme, lactate dehydrogenase (LDH), in the medium (Fig. 3). The total LDH content of the hemocytes per well was 27.05 ± 2.0 mU. When the cells were incubated in the presence of buffer, 0.56 ± 0.2 mU of LDH was released in the medium. The LDH released by the cells ranged from 4.01 to 21.7 mU when larval hemocytes were incubated with different concentrations of pilin protein (5–25 µg). About 50% of the intracellular LDH was released when the cells were incubated in the presence of 15 µg of the pilin protein. Low LDH release (0.81 ± 0.12 mU) was detected in the presence of E. coli K12 cells or OMVs prepared from it. LDH released due to heat denatured or aggregated 17 kDa protein

Fig. 2. Agglutination of H. armigera fifth instar larval hemocytes by purified pilin protein. Hemocytes obtained from H. armigera were suspended in serum free Grace’s insect medium. Protein samples were added and the plate was incubated at 28°C for 2 h. Agglutination was observed with light microscope. (A), control, hemocytes in PBS; (B), 15 µg of purified 17 kDa pilin protein; (C), heat inactivated 17 kDa pilin protein; and (D), purified 17 kDa pilin protein premixed with polyclonal; (E), purified 17 kDa pilin protein premixed with pre-immune serum.
Table 1
Agglutination of rabbit erythrocytes and H. armigera fourth-fifth instar larval hemocytes by X. nematophila cells and pilin protein

<table>
<thead>
<tr>
<th>Sample</th>
<th>Agglutination titer</th>
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<tbody>
<tr>
<td>Buffer control</td>
<td></td>
</tr>
<tr>
<td>X. nematophila cells (10^7 cell/ml)</td>
<td>64</td>
</tr>
<tr>
<td>E. coli cells (10^7 cell/ml)</td>
<td>ND</td>
</tr>
<tr>
<td>17 kDa pilin protein from X. nematophila*</td>
<td>17</td>
</tr>
<tr>
<td>Heated 17 kDa pilin protein*</td>
<td>ND</td>
</tr>
<tr>
<td>Pre-immune serum (undiluted)</td>
<td>ND</td>
</tr>
<tr>
<td>17 kDa pilin protein + anti-17 kDa serum*</td>
<td></td>
</tr>
<tr>
<td>(1:100)</td>
<td>ND</td>
</tr>
<tr>
<td>(1:500)</td>
<td>ND</td>
</tr>
<tr>
<td>(1:1000)</td>
<td>ND</td>
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ND, not determined; --, no activity.

*Initial concentration of pilin protein was 100 μg/well.

Discussion

A 17 kDa protein constituting the structural subunit of pilin with cytotoxicity to insect larval hemocytes has been purified from the surface of X. nematophila cells. Hemocytes are the primary immunocompetent cells in insects, and interference in their normal functioning is known to affect insect viability adversely. Since X. nematophila whole cells have been shown to cause larval hemocyte clumping in an earlier study [20], we examined the activity of the pilin subunit in hemocyte agglutination assay. The pilin protein agglutinated hemocytes after binding to the surface of the cell (as shown by immunofluorescence). This interaction of hemocytes with the protein was cytotoxic to the cells as demonstrated by release of LDH, a cytoplasmic enzyme, in the extracellular medium. A properly folded 3D-structure appears to be essential for the hemocyte agglutination activity of the protein, as heating the protein resulted in irreversible loss in binding and cytotoxic activity.

The cytotoxic activity associated with the structural subunit of Xenorhabdus pilin is intriguing, as the structural subunits of the pilus rarely mediate host cell recognition [19]. Usually there is a lectin domain present in the adhesin subunit, at the tip of the pilus, which is primarily responsible for host cell recognition [24]. Recently a pilus operon of X. nematophila has been reported [AF 525420]. The 11.2 kb sequence contained homologues of all the essential genes including a putative adhesin gene encoding a protein of 30.8 kDa. However, our pilin protein...
Fig. 4. Immunofluorescence showing binding of 17 kDa pilin protein on H. armigera fifth instar larval hemocytes. Hemocyte monolayers were incubated with protein samples for 1 h and fixed with 0.4% formaldehyde in PBS. Binding of the pilin protein was detected using polyclonal antibodies against 17 kDa protein of X. nematophila followed by fluorescein labeled conjugate. (A), control, hemocytes with PBS buffer; (B), purified 17 kDa pilin protein; (C), heat inactivated 17 kDa protein; (D) purified 17 kDa pilin protein premixed with polyclonal antiserum against 17 kDa protein; (E), 17 kDa pilin protein detected with pre-immune serum; and (F), purified 17 kDa pilin protein premixed with pre-immune serum and detected with polyclonal antiserum against 17 kDa protein.

preparation does not show any other contaminating protein band indicating that the cytotoxic activity is indeed due to the 17 kDa pilin subunit. This suggests that the interaction of the structural subunit of Xenorhabdus pilus with the host cell is something unique and not reported before. Further, excretion of the protein in association with the outer membrane vesicles makes it more interesting in the context of toxin transport by pathogenic bacteria. Several recent reports have recognized outer membrane vesicles as a vehicle for transporting effector proteins outside the bacterial cell [12,13,25]. Apparently the pilin subunit recognized a specific molecule on the hemocyte surface, as no agglutination occurred in Sf 21 cells (another lepidopteran cell type) or erythrocytes from human, rabbit or sheep. No effect was observed on other phagocyte cells, macrophage cell line J774A.1 (data not shown). However, binding and toxicity of the pilus shaft protein is not inconceivable considering the two different ecological niches the Xenorhabdus cells are adapted to. In the nematode gut, where the bacterium resides in a symbiotic association, probably the usual adhesin is used for host cell recognition and colonization, while in the larval hemocoel, where the bacterium has to survive against the host immune onslaught, the surface exposed long pilus shafts made up of multiple structural subunits with toxicity may prove to be extremely useful. Work is in progress to understand the biological significance of the cytotoxic activity of the pilin protein in its natural insect host. At this stage we cannot comment on the nature of interaction, the 17 kDa pilin protein has with its target cells. Several sugars and derivatives like glucose, fructose, mannose, galactose, N-acetyl-lactosamine, and gastric mucin had no effect on the agglutination titre (data not shown). The immunofluorescence results suggest a possible binding event in the beginning of the entoxication process. Studies are in progress to determine nature of the above interactions.

In summary, we for the first time demonstrate cytotoxic nature of the structural subunit of a pilin protein from a gram-negative bacterium. The fact that the cytotoxic protein was toxic to the hemocytes of an important crop pest, H. armigera, makes it all the more important as a potential biological control agent particularly when insect pests are becoming increasingly resistant to BT toxin.

Acknowledgments

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References

Insecticidal Activity Associated with the Outer Membrane Vesicles of *Xenorhabdus nematophilus*

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*Xenorhabdus nematophilus* secretes a large number of proteins into the culture supernatant as soluble proteins and also as large molecular complexes associated with the outer membrane. Transmission electron micrographs of *X. nematophilus* cells showed that there was blebbing of the outer membrane from the surface of the bacterium. The naturally secreted outer membrane vesicles (OMVs) were purified from the culture supernatant of *X. nematophilus* and analyzed. Electron microscopy revealed a vesicular organization of the large molecular complexes, whose diameters varied from 20 to 100 nm. A sodium dodecyl sulfate-polyacrylamide gel electrophoresis profile of the vesicles showed that in addition to outer membrane proteins, several other polypeptides were also present. The membrane vesicles contained lipopolysaccharide, which appeared to be of the smooth type. Live cells of *X. nematophilus* and the OMV proteins derived from them exhibited oral insecticidal activity against neonatal larvae of *Helicoverpa armigera*. The proteins present in the OMVs are apparently responsible for the biological activity of the OMVs. The soluble proteins left after removal of the OMVs and the outer membrane proteins also showed low levels of oral toxicity to *H. armigera* neonatal larvae. The OMV protein preparations were cytotoxic to SF-21 cells in an in vitro assay. The OMV proteins showed chitinase activity. This is the first report showing toxicity of outer membrane blebs secreted by the insect pathogen *X. nematophilus* into the extracellular medium.

*Xenorhabdus nematophilus* is a gram-negative bacterium belonging to family *Enterobacteriaceae* (7, 12). The bacteria reside as endosymbionts in the foreguts of soil nematodes belonging to the genus *Steinernema* (1). Bacteria are released from the gut upon invasion of the insect hemocoel by the nematode. Bacterial multiplication and secretion of toxic proteins are the primary causes of death of the insect host. The bacteria alone are known to be sufficient to cause larval mortality following injection into the hemocoel (4, 13) or following oral administration when they are mixed in the diet (27).

*Xenorhabdus* and *Photorhabdus* are two closely related genera of bacteria associated with soil nematodes. Members of both of these genera produce insecticidal proteins that are toxic to a wide variety of lepidopteran insects. The genes encoding larvicidal proteins in members of both of these bacterial genera have been identified and have been found to exhibit significant degrees of homology (10, 21). Large protein complexes containing several polypeptide species with larvicidal activity have been isolated from the culture supernatant of *Photorhabdus luminescens* (9, 15). However, there is no information on the activity profiles of the secreted proteins in *Xenorhabdus* culture media.

All gram-negative bacteria are known to produce spherical two-layer outer membrane (OM) blebs in culture media (5). The general importance of release of these blebs has only recently been recognized. OM vesicles (OMV) have been found emanating from bacteria growing under very different conditions, including in biofilms (6), on solid media or in liquid media (17), and in natural environments (6). Encapsulation of toxic proteins in the membrane vesicles protects the proteins from the degradative enzymes of the host (18) and also helps deliver the enclosed substances by facilitating fusion with lipid-rich host cell membranes (17). Several gram-negative pathogens have been shown to excrete their virulence factors enclosed in OMVs (5). *Pseudomonas aeruginosa*, *Proteus mirabilis*, and *Serratia marcescens* package phospholipase C, proteases, proelastases, and hemolysins in OMVs (5). *P. aeruginosa* is known to exhibit predatory activity towards other bacteria by secreting OMVs which fuse with the bacterial OM and subsequently release degradative enzymes (20).

The mutualistic mode of living of the entomopathogenic bacterium *X. nematophilus* in the nematode gut turns into a pathogenic mode when the bacterium enters the larval hemocoel. *X. nematophilus* is known to secrete highly potent protein toxins into the hemocoel, which rapidly kill the larval host. The insect carcass provides a rich nutrient source on which both the bacteria and the nematode feed, grow, and replicate. The OM of gram-negative bacteria performs many specialized functions under adverse environmental conditions (5), and protein secretion is one such mechanism used by bacterial pathogens when they are inside their hosts (23). Likewise, *X. nematophilus* also secretes many effector proteins to inactivate the host immune defense and establish itself in the host (12).

In this study we demonstrated the oral larvicidal activity of proteins secreted in association with OMVs into the extracellular medium by *X. nematophilus* 19061. We found that when the OMVs were incorporated into the diet, they were active against the common lepidopteran pest *Helicoverpa armigera.*
MATERIALS AND METHODS

Bacteria and growth conditions. X. nematophilus strain 19061 was obtained from the American Type Culture Collection (Rockville, Md.), and the culture was stored as stock preparations in Luria broth (LB) containing glycerol (1:1) at −80°C. The bacteria were plated on nutrient agar supplemented with 0.004% (w/v) triply triethyl tetrazolium chloride and 0.025% (w/v) bromothymol blue (7). Broth cultures were grown from a single blue colony obtained from the plated, described above in LB at 29°C with shaking. Escherichia coli K-12 was used as a reference strain.

Isolation of OMVs and soluble proteins from the culture medium. The OMVs naturally secreted into the medium were collected from 20- to 24-h-old culture supernatant as described by Kadorugamou and Beveridge (17). Briefly, the cells were pelleted by centrifugation at 10,000 × g for 20 min, and the supernatant was filtered through a 0.45-µm-pore-size filter to remove the remaining bacterial cells. The OMVs were obtained by centrifugation at 150,000 × g for 2 h in a Ti 45 rotor with a Beckman ultracentrifuge, washed and resuspended in 50 mM Tris-HCl buffer (pH 7.5), and stored at −20°C. The supernatant obtained after the high-speed centrifugation was concentrated with a Centricon 3000 and used as a source of extracellular soluble proteins.

Preparation of OMVs from X. nematophilus cells. OMVs were prepared as described by Leisman et al. (19). A bacterial pellet was washed once in LB and resuspended in 20 mM sodium phosphate buffer (pH 7.0) on ice by using 10-l/min pulses at 140 W. The cell lysate was centrifuged at 10,000 × g for 15 min, and the clear supernatant was subjected to ultracentrifugation at 150,000 × g for 15 min in a Ti 50 rotor. The crude membrane pellet was suspended in 50 mM sodium phosphate buffer for 1 h to solubilize the cytoplasmic membrane components. The suspension was centrifuged at 300,000 × g for 15 min. The pellet was washed once in sodium phosphate buffer and resuspended in the same buffer.

Isolation of LPS from Xenorhabdus cells. Lipopolysaccharide (LPS) was isolated from the Xenorhabdus cells by phenol-water extraction as described by Apicella et al. (2). To determine the LPS profile of the OMVs, a known amount of protein was denatured by boiling with sodium dodecyl sulfate (SDS)-containing sample buffer for 5 min, followed by treatment with protease K for 1 h at 56°C. The protease-treated samples were electrophoresed on SDS-polyacrylamide gel electrophoresis (PAGE) gels and visualized by silver staining.

Protease and chitinase activities. Protease activity was determined by using Hide Powder Azure (Sigma) as described by Schmidt et al. (26). Chitinase activity was determined as described by Holst et al. (16) by using 4-naphthyl-N,N',N''-triacetylchitotrioside as the substrate. Release of free methylumbelliferyl was measured with a fluorescence spectrophotometer by using excitation at 360 nm and emission at 450 nm.

Electron microscopy. Xenorhabdus cells that were grown overnight were washed once and fixed in 0.1% glutaraldehyde–2% formaldehyde in phosphate-buffered saline (PBS) for 20 min at room temperature. The cells were collected by centrifugation at 12,000 × g for 5 min at 4°C, washed twice in PBS, sequentially dehydrated in 50%, 70%, and 100% ethanol, and embedded in EY 2112 araldite. Thin sections were cut with an ultramicrotome, and the sections were stained with alcoholic uranyl acetate and lead acetate (10 min each). The grids were examined by transmission electron microscopy. The OMVs collected from the culture supernatant were visualized by negative staining with a 2% uranyl acetate solution on a Formvar-coated copper grid, rinsed, and examined with a Philips CM10 transmission electron microscope under standard conditions at 60 to 80 kV.

Insect bioassay. X. nematophilus was grown in LB overnight, washed once with sterile PBS, diluted in the same buffer, and added to the diet. The test protein preparations were diluted in 20 to 100 µl of water and mixed with the artificial diet. Each group contained 10 to 20 necrotic larvae placed on the surface of the diet, and the plate was sealed and placed in a humidified growth chamber at 28°C. Mortality was scored for 4 to 5 days. Each dose was used in triplicate to establish the toxin potency. The experiments were repeated three times. The 50% lethal dose (LD₅₀) was defined as the protein concentration that resulted in 50% mortality, as determined by Probit analysis (11).

Cytotoxicity assay with cultured SF-21 cells. The cytotoxicity assay used was based on the procedure described by Armstrong et al. (3). The insect cells (SF-21 cell line) were cultured at pH 6.2 in Grace's insect medium (Invitrogen) supplemented with yeast extract and lactalbumin containing 10% fetal bovine serum and 10 µg of genticin per ml. Each experiment was performed in a 48-well tissue culture plate. Approximately 10⁶ cells in serum-free medium were added to each well and allowed to attach for 1 to 2 h at 28°C. The medium was removed, different concentrations of sterile OMVs preparations in 0.25 ml of serum-free medium were added to the wells, and the plate was incubated at 28°C. Heinz-triacetylated OMVs from X. nematophilus, OMVs from E. coli K-12, and serum-free medium were used as controls. Each plate was monitored at regular intervals, and the experiment was terminated after 6 to 7 h. The supernatant was removed, and 200 µl of 3-(4,5-dimethylthiazolyl)-2-yl)-2,5-diphenyl tetrazolium bromide dissolved in serum-free medium was added to each well to a final concentration of 0.5 mg ml⁻¹. The plate was incubated at 28°C for 2 h. The supernatant was decanted, and the plate was air dried. The resulting precipitate was solubilized in a solution of 25 mM HCl in 90% isopropl alcohol containing 0.5% SDS, and the optical density at 620 nm (OD₆₂₀) was measured. The percentage of viability was calculated as follows: (OD₆₂₀ of treated cells/OD₆₂₀ of control cells) × 100. The assay was performed three times in triplicate, and the results shown below are representative of one experiment.

RESULTS AND DISCUSSION

The cell walls of gram-negative bacteria are dynamic in that OMVs are constantly sloughed off from the surfaces of the cells during growth. During this discharge process a number of periplasmic components are entrapped and are exported out of the cell enclosed in the OM covering. Several pathogenic bacterial species have been shown to release OMVs containing toxic proteins (5) and infectious DNA (18, 29) into the medium during in vitro growth. Greiner and Maryland (14) demonstrated that Bacteroides gingivalis vesicles adhered to the epithelial cells of a host during toxicity tests under in vitro conditions.

Like other gram-negative bacteria, X. nematophilus also secreted OM blebs into the growth medium. Electron micrographs of Xenorhabdus cells clearly showed that there was blebbing of the OM at several places (Fig. 1A). The diameters of the membrane vesicles varied from 20 to 100 nm (Fig. 1B), although the possibility that there were some modifications in size during vesicle isolation cannot be ruled out. Many of vesicles contained electron-dense contents, which stained dark. The OMVs contained a number of proteins in addition to the OM proteins, as revealed by a comparison of the SDS-PAGE profiles of the OM of X. nematophilus and proteins isolated from the medium (Fig. 2A).

The porins OmpP (as determined by the N-terminal sequence) and OmpA (52 kDa; with the N-terminus sequence AEIFNKGDKNLDRY) (19), were the predominant proteins in the OMVs, as well as in OM preparations (Fig. 2A, lanes 2 and 4). Another major OM protein identified in the OM preparation was a 17-kDa protein whose N-terminal sequence (APTOGDDTVK) was very similar to the N-terminal sequence of the P pilin protein of a uropathogenic strain of E. coli (25) (Fig. 2A). Moura et al. (22) described a fimbrial protein having a similar molecular weight in X. nematophilus. The N-terminal sequence of a high-molecular-mass band at about 300 kDa (ALPRKLKLYN) exhibited 90% homology with the sequences of phage tail-like proteins. Xenorhabdus species have been reported to produce phage tail-like bacteriophages in the culture medium in a highly oligomeric form (8, 28). The OMVs produced additional protein bands at 22 to 27 and 60 to 75 kDa and several bands at molecular masses greater than 100 kDa, while the OM preparation contained almost no proteins having molecular masses greater than 100 kDa. The soluble protein fraction obtained after separation of the OMVs from the culture supernatant contained a large number of polypeptide species, as revealed by SDS-PAGE (Fig. 2A, lane 3), and many of them appeared to have
electrophoretic mobilities similar to those of the OMV-associated proteins. The presence of LPS in the OMV preparation confirmed its OM origin (Fig. 2B, lanes 1 and 2). The LPS of Xenorhabdus was visualized by silver staining of the SDS-PAGE gels (Fig. 2B). The presence of repeating saccharide units forming O side chains of different lengths produced the typical ladder-like structure of the smooth phenotype.

When *X. nematophilus* 19061 was grown in LB to saturation and tested for larvicidal activity, it exhibited oral toxicity against *H. armigera* neonatal larvae. A dose-dependent toxic effect was observed. Incorporation of $10^2$ cells/g of diet resulted in the death of $10\% \pm 2\%$ (mean ± standard deviation) of the larvae of *H. armigera* after 48 h of exposure, and the value increased to $82\% \pm 2\%$ at a concentration of $10^6$ cells/g. The $LD_{50}$ was calculated to be $4 \times 10^4$ cells for *H. armigera* larvae (Table 1). At cell densities greater than the $LD_{50}$ the larvae that remained alive were small and moribund. These results indicated that *Xenorhabdus* cells were insecticidal and that growth in the nematode host was not necessary to produce the toxin proteins.

The OMVs also showed larvicidal activity when they were incorporated into the diet of neonatal larvae of *H. armigera* (Table 2). At a concentration of 10 $\mu$g of OMV protein/g of diet, $12\% \pm 5\%$ larval death was recorded, and the death rate increased to $40\% \pm 0\%$ when the concentration of the proteins was increased to 50 $\mu$g/g. The maximum mortality ($98\% \pm 2\%$) was observed when the protein concentration was 100 $\mu$g/g.
and this occurred within 48 h. The LD$_{50}$ was calculated to be 37 µg/g of diet. Protein levels greater than 25 µg/g were feeding detergents, and the larvae that did not die within 48 h remained thin and small. The major part of the insecticidal activity of the excreted proteins was present in the particulate fraction obtained after high-speed centrifugation. The soluble proteins remaining in the supernatant were also tested with the bioassay. The proteins in the latter fraction were found to be less active, causing 10% ± 7% and 30% ± 11% mortality when the protein concentrations were 25 and 100 µg/g of diet, respectively (Table 2). The identity of the insecticidal protein(s) in the soluble fraction is not known; there could be a distinct set of proteins, or proteins could be produced by disintegration of OMVs. Purified LPS, the endotoxin of gram-negative bacteria, which is typically active at very low concentrations, showed no oral toxicity to the insect larvae even at levels of 10 to 20 µg/g of diet (Table 2). The OM preparations also showed low-level oral toxicity to the Helicoverpa larvae, killing 20% ± 4% of the larvae at a concentration of 50 µg/g. Heating OMVs at 80°C for 15 min completely inactivated the insecticidal activity (Table 2). Furthermore, OMVs isolated from E. coli culture supernatant had negligible toxicity. Since the OM preparations alone showed low-level toxicity in the bioassay, the more potent insecticidal factors may be enclosed in the lumina of OMVs or associated with the surfaces of the OMVs. The results described above demonstrate the insecticidal potential of Xenorhabdus cells, as well as the OMVs. When the OMVs were treated with protease K, 80 to 90% of the proteins were degraded, as determined by SDS-PAGE (data not shown), and the digested OMVs were found to be inactive in the insecticidal assay. The active factors in the OMVs are proteinaceous, as suggested by their heat and protease sensitivities. The requirement for larger doses of OM and OMV preparations for toxicity in these experiments compared to the previously reported values for purified proteins (9, 15) could be due to the presence of a number of protein species in the OMV preparations, and the major OM proteins present in large excess may not have any role in toxicity. In addition, larval age and insect species are also important factors determining susceptibility to external toxins. It is also possible that other proteins are needed for synergistic action of the OMV preparations, as has been suggested previously for a cloned toxin of X. nematophilus (21). Our efforts to determine the toxicity of individual proteins present in the OMV mixture proved to be unsuccessful. Purification of the proteins was attempted after detergent solubilization. Different detergents, including Triton X-100, octyl glucoside, 3-[3-cholamidopropyl]-dimethylammonio]-1-propanesulfonate (CHAPS), and SDS, were used to solubilize the OMV proteins, and only SDS was able to solubilize the protein complex efficiently. However, treatment with SDS resulted in total loss of the biological activity of the proteins.

The cytotoxicity of the OMV proteins for the Sf-21 cells of insect origin also reflects the insecticidal potential of the secreted toxins of X. nematophilus. Figure 3 shows that in the presence of 1 µg of the OMV proteins (per well), the number of viable cells was reduced to 43% of the original number after 6 to 7 h and that the toxicity increased in a dose-dependent manner. The number of live cells was reduced to 20% of the original number in the presence of 10 µg of protein per well. Heating the proteins at 80°C for 15 min substantially reduced the toxicity, and OMV preparations from E. coli had no effect on the Sf-21 cells (Fig. 3). Similar to their low oral toxicity for H. armigera neonates, the toxicity of the OMV proteins for the Sf-21 cells was also marginal. At a protein concentration of 25 µg per well, 88% of the cells remained viable after 6 to 7 h (Fig. 3).

### Table 1. Toxicity of X. nematophilus 19061 cells for neonatal larvae of H. armigera

<table>
<thead>
<tr>
<th>Sample</th>
<th>Protein conc (µg of diet)</th>
<th>% Mortality</th>
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<tbody>
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<td>Control</td>
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<td>0</td>
</tr>
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<tr>
<td>OMVs from E. coli</td>
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* Each group contained 10 neonatal larvae, and mortality was determined after 48 h.
* Mean ± standard deviation.

### Table 2. Toxicity of secreted proteins of X. nematophilus for neonatal larvae of H. armigera

<table>
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<tr>
<th>Sample</th>
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* Each group contained 10 neonatal larvae, and mortality was determined after 48 h.
* Mean ± standard deviation.
OMVs were also prepared from a <i>Photorhabdus</i> strain (24) and tested in the oral toxicity assays. These OMVs were found to be biochemically active against neonatal <i>H. armigera</i> larvae (data not shown), although the specific activity of the OMVs isolated from the <i>Photorhabdus</i> strain was lower than the specific activity of the OMVs isolated from the <i>Xenorhabdus</i> strain used in this study. SDS-PAGE analysis of the OMV proteins (Fig. 2A, lane 1) showed that there were a number of proteins in the higher-molecular-weight range, as determined by electrophoretic mobility, similar to the proteins found in the larvicidal, large polypeptide complexes isolated from the culture supernatant of <i>P. luminescens</i> (9, 15). In this context it is tempting to speculate that the large protein complexes isolated from the culture supernatants in the previous study (15) could be organized like the <i>Xenorhabdus</i> OMV proteins.

The characteristics of the individual active factors are currently being investigated. A low level of protease activity (data not shown) was detected in the OMV preparations; however, it does not appear to be enough to account for the level of activity observed in the larvicidal assay, suggesting that there is more than one active moiety in the OMV preparation. Association of strong chitinase activity with the OM and OMV fractions (Fig. 4) indicates that the chitinase is probably an OM protein. We do not know the insecticidal efficacy of this chitinase at this stage; however, its presence in the OMVs further strengthens the pathogenic potential of the secreted protein complex. Recently, Morgan et al. (21) described a DNA region of <i>X. nematophila</i> encoding an insecticidal protein together with other attributes of pathogenicity, including a chitinase gene, organized as a pathogenicity island, suggesting the multicomponent nature of the <i>Xenorhabdus</i> toxins. Similarly, the toxin complex secreted by <i>P. luminescens</i> into the culture medium has also been shown to contain multiple polypeptides with larvicidal activity (9, 15).

In conclusion, this study demonstrated for the first time the insecticidal potential of the OM-associated proteins that are secreted as OMVs by <i>X. nematophila</i> into its growth medium. The presence of chitinase activity together with bacteriocin, adhesin protein, and pore-forming proteins in the insecticidal multiprotein complex supports the role of this complex in pathogenicity, as these proteins are known to mediate host-pathogen interactions in other pathogenic bacteria. The membrane vesicles provide an efficient mechanism to transport effector molecules to the larval host. Work is in progress to characterize the individual members of the protein complex, which should throw more light on the virulence mechanisms of the bacteria which have symbiotic relationships with insects.

**REFERENCES**


LOCUS  AY140909
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VERSION  AY140909.1  GI:27461950
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REFERENCE 1 (bases 1 to 537)  Khandelwal, P. and Nirupama, B.B.  Submitted (14-Aug-2002) Insect Resistance, ICGEB, Aruna Asaf Ali Marg, New Delhi, Delhi 110067, India
REFERENCE 2 (bases 1 to 537)  Khandelwal, P. and Nirupama, B.B.  Direct Submission
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