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 Escherichia coli. The recombinant protein was refolded in vitro in the absence of its cognate chaperone by using a urea gradient. The protein oligomerized during in vitro refolding, forming multimers. Point mutations in the conserved N-terminal residues of the pilin protein greatly destabilized its oligomeric organization, demonstrating the importance of the 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 a conformation similar to that of the native protein. The 17-kDa pilin subunit was found to be orally toxic to fourth- or fifth-instar larvae of an important crop pest, H. armigera, causing extensive damage to the midgut epithelial membrane.

To our knowledge, this is first report describing an insecticidal pilin subunit of a bacterium.
folds. However, they lack the seventh C-terminal \( \beta \)-strand present in the canonical Ig fold. The absence of this strand produces a deep groove along the surface of the domain and exposes its hydrophobic core. The chaperone donates the missing strand to complement the incomplete Ig-like fold by transiently shielding the hydrophobic core and contributes to stabilization of the pilin subunit (8, 34). Assembly of subunits into the pilus fiber proceeds by a donor strand exchange mechanism in which the chaperone’s donor strand is replaced by the N-terminal of the next subunit (8). The structural basis of fiber formation has been revealed by high-resolution crystallography (33, 41).

To study the interaction of the 17-kDa pilin subunit of \( X. \) nematophila with the larval host, we cloned and expressed the protein in \( E. \) coli. The protein was produced as inclusion bodies, which were refolded in a biologically active, oligomeric form. Here we provide evidence that the recombiant structural subunit of the pilin of \( X. \) nematophila undergoes intermolecular donor strand complementation during in vitro folding and forms oligomers in the absence of its cognate chaperone. The 17-kDa protein showed oral larvicidal activity against \( H. \) armigera larvae. This is the first report demonstrating insecticidal activity in a pilin subunit.

### MATERIALS AND METHODS

#### Bacteria and growth conditions.
\( X. \) nematophila strain ATCC 19061 was obtained from the American Type Culture Collection (Rockville, Md.). The \( X. \) nematophila culture was streaked on nutrient agar supplemented with 0.004% (wt/vol) triphenyl tetrazolium chloride and 0.025% (wt/vol) bromothymol blue (4). Broth cultures were grown from a single blue colony in Luria-Bertani (LB) medium at 28°C with shaking at 150 rpm. \( E. \) coli K-12 was used as a reference strain.

#### Preparation and fractionation of OMV proteins of \( X. \) nematophila.
OMVs were prepared from the culture supernatant as described previously by Khandelwal and Bhatnagar (19). The OMV proteins were solubilized in TENs buffer (50 mM Tris-HCl [pH 7.2], 5 mM EDTA, 400 mM NaCl, 1.0% sodium dodecyl sulfate [SDS]) at 37°C overnight and applied to a Sephacryl S-300 column; then the proteins were eluted with TENs buffer, and the fractions were examined by SDS-polyacrylamide gel electrophoresis (PAGE).

#### Isolation and purification of native pilin protein from \( X. \) nematophila.
The native pilin protein was obtained from the surface of \( X. \) nematophila cells and purified by sucrose density gradient centrifugation as described by Korhonen et al. (23).

#### Preparation of polyclonal antiserum.
The purified native pilin protein was emulsified with Freund’s adjuvant and injected intramuscularly into a rabbit. Two booster doses were given at 2-week intervals. After 6 weeks, the rabbit was bled, and the antiserum was examined by Western blotting.

#### Phylogenetic analyses.
Phylogenetic neighbors of the \( X. \) nematophila pilin protein were determined using the program PSI-BLAST (http://www.ncbi.nlm.nih.gov/blast). Sequences were aligned by using the program CLUSTAL W (18). Phylogenetic analyses were carried out by using the PHYLIP (11) suite of programs, and the
program SEQBOOT was used to carry out 1,000-fold bootstrapping, which involved generation of 1,000 independent data sets by random sampling. Pairwise distances between sequences were calculated for each of these data sets by using the program PROTDIST. Phylogenetic trees were generated by the neighbor-joining method by using the program NEIGHBOR. The branch lengths were generated by the Fitch-Margoliash method (13). To generate a majority rule consensus tree from the 1,000 trees generated from the bootstrapped data, the program CONSENSE was used. The majority rule consensus method selected for monophyletic groups which occurred the maximum number of times in the consensus trees.

Rationale for design of mutants. Pilin subunits of *E. coli* form multimers via head-to-tail interactions, in which the N-terminal segment (about 20 residues) of one subunit stabilizes the hydrophobic acceptor cleft in the carboxyl-terminal region of the preceding subunit by donor strand complementation (34). Sequence alignment of the N termini of several structural subunits known to participate in donor strand complementation showed a characteristic pattern of alternating hydrophobic residues (Fig. 1A), which are considered to be principal determinants of the specific interaction between two pilin subunits (33, 42). It is clear from Fig. 1A that the Val32, Phe34, and Ile38 in the N terminus of the protein to alanine separately and together (producing a triple mutant), and we tested structurally stable, oligomeric protein. Hence, we changed the three conserved residues, Val32, Phe34, and Ile38, in the N terminus of the protein to alanine separately and together (producing a triple mutant), and we tested the protein's resistance to disorganization by 1.5% SDS at room temperature (7, 37). To detect recombinant protein oligomers, the wild-type protein and the triple-mutant protein were incubated with 1.5% SDS in loading buffer at 25 or 95°C for 5 min and then resolved by SDS-PAGE and detected by Western blotting with anti-His antibodies (Clontech) or antisera against the native 17-kDa protein from *X. nematophila*. To check the presence of intermolecular disulfide bonds, the protein was heated in reducing and nonreducing buffers and resolved by SDS-PAGE.

Analytical gel chromatography. A Sephadex G-200 column (35 by 1.2 cm; Pharmacia) was equilibrated with 20 mM sodium phosphate buffer (pH 8.0). The column was calibrated by using globular molecular weight marker proteins (Sigma). The void volume was determined with blue dextran 2000. Recombinant wild-type protein in the buffer described above was applied to the column and eluted with the same buffer at a flow rate of 0.1 ml/min. Protein concentrations in the samples were determined by measuring the absorbance at 220 nm (0.1 U of absorbance at 220 nm was equivalent to 3.35 μg of protein/ml as determined by using the Bradford reagent with bovine serum albumin [BSA] as the standard).

CD spectroscopy. Circular dichroism (CD) spectra of the native and recombinant proteins were obtained with a JASCO J 715 spectropolarimeter at 20°C by using a 0.2-cm cell, wavelengths between 205 and 250 nm, and a scanning speed of 20 nm/min. All the proteins were dissolved in 10 mM sodium phosphate buffer (pH 8.0). The CD spectrum of the denatured protein was recorded after the protein was denatured in 8 M urea (Sigma). The void volume was determined with blue dextran 2000. Recombinant wild-type protein in the buffer described above was applied to the column and eluted with the same buffer at a flow rate of 0.1 ml/min. Protein concentrations in the samples were determined by measuring the absorbance at 220 nm (0.1 U of absorbance at 220 nm was equivalent to 3.35 μg of protein/ml as determined by using the Bradford reagent with bovine serum albumin [BSA] as the standard).

Hemagglutination and cytotoxicity assay of larval hemocytes. The hemagglutination and cytotoxicity for larval hemocytes of the recombinant protein were determined as described previously (20). Inhibition of agglutination was determined with anti-pilin antisera. To characterize the interaction between the 17-kDa protein and the hemocytes, various sugars and their derivatives were tested for the ability to inhibit the agglutination activity of the protein.

<table>
<thead>
<tr>
<th>Primer</th>
<th>Sequence</th>
</tr>
</thead>
<tbody>
<tr>
<td>Degenerate primers</td>
<td></td>
</tr>
<tr>
<td>N terminal</td>
<td>5′ GCNCNACNCARGGNAGYGGNACN 3′</td>
</tr>
<tr>
<td>C terminal</td>
<td>5′ NARRTARTNRNGTARNTNGC 3′</td>
</tr>
<tr>
<td>Adapter sequence</td>
<td>5′ GATCCCTAAACCACCTACATAGGGCCGGCCGCCGGC 3′, 3′ GATATTGTTAGTGTATC CCGCCCGGGCGGCCG 5′</td>
</tr>
<tr>
<td>WPI</td>
<td>5′ GATCCTAATACCTACTACATAGGGCCGGCCGCCGGC 3′</td>
</tr>
<tr>
<td>Biotinylated primer (BPI)</td>
<td>5′ GTGTCAGGTTTTGATGTCACCACCTAC 3′</td>
</tr>
<tr>
<td>IP2</td>
<td>5′ GTCTCTGATGAAACGGCTGC 3′</td>
</tr>
<tr>
<td>Primers for amplifying 537-bp fragment</td>
<td></td>
</tr>
<tr>
<td>Forward primer</td>
<td>5′ GGAATCCCATGAACACCTACATAATTCG 3′</td>
</tr>
<tr>
<td>Reverse primer</td>
<td>5′ AAGCTTAAGGTTAGTTGAGTAGTATGGT 3′</td>
</tr>
<tr>
<td>Primers for point mutations</td>
<td></td>
</tr>
<tr>
<td>Val32Ala (forward)</td>
<td>5′ CTCAAGGGTGAGGGCCGAGCAAATTCACCCTGTTATATTAAATG 3′</td>
</tr>
<tr>
<td>Phe34Ala (forward)</td>
<td>5′ GTTACGGCGCCAGTTAAAGCACCCGTTCTATTATTTAGC 3′</td>
</tr>
<tr>
<td>Ile38Ala (forward)</td>
<td>5′ GCATTTAATTTACCGGTTTCTGCAATTATGTCACTGTTCAATC 3′</td>
</tr>
<tr>
<td>Triple mutation (forward)</td>
<td>5′ CTCAGGGTACGCCGGCAAGAGCAACCGGTTGCAAAATTCG 3′</td>
</tr>
<tr>
<td>(Val-Phe-Ile→Ala-Ala-Ala)</td>
<td>CAAG 3′</td>
</tr>
</tbody>
</table>

All the other strains were grown at 37°C in LB medium with 100 μg of ampicillin per ml. Expression was induced with 1 mM IPTG for 4 h at 37°C, and the cells were harvested by centrifugation at 6,000 × g for 10 min at 4°C. Each cell pellet was resuspended in 5 ml of denaturing buffer and incubated at 57°C for 1 h on a rotary shaker. The lysate was centrifuged at 12,000 × g for 45 min at room temperature, and the supernatant was loaded onto an Ni-nitritolactic acid (NTA) column previously equilibrated with the denaturing buffer. Recombinant protein bound to Ni-NTA was refolded by using a 8 to 0 M urea gradient in renaturation buffer (100 mM sodium phosphate, 10 mM Tris·Cl; pH 8.0). The proteins were eluted in the renaturation buffer with 250 mM imidazole. Fractions containing the recombinant protein were pooled and dialyzed against 10 mM Tris·Cl or 10 mM sodium phosphate (pH 8.0).

Identification of 17-kDa protein oligomers. In *E. coli*, the pilin subunits in the native pilus rod resist disorganization by 1.5% SDS at room temperature (7, 37). To detect recombinant protein oligomers, the wild-type protein and the triple-mutant protein were incubated with 1.5% SDS in loading buffer at 25 or 95°C for 5 min and then resolved by SDS-PAGE and detected by Western blotting with anti-His antibodies (Clontech) or antisera against the native 17-kDa protein from *X. nematophila*. To check the presence of intermolecular disulfide bonds, the protein was heated in reducing and nonreducing buffers and resolved by SDS-PAGE.
FIG. 1. (A) X-ray crystallographic structure of PapE with donor strand complementing N-terminal peptide segment of PapK (indicated by sticks). Conserved deeply buried residues are indicated by arrows. An alignment of N-terminal sequences of various structural pilin proteins is also shown; conserved hydrophobic residues are indicated by boldface type. Sequences were aligned with the CLUSTALW program, followed by manual adjustments to minimize gaps within secondary structures. (B) Neighbor-joining tree showing the branching pattern of different pilin protein amino acid sequences and the phylogenetic position of the *Xenorhabdus* pilin protein. Bootstrap resampling was done for 1,000 replicons. The numbers at the nodes in the consensus tree indicate the numbers of times that the subtree occurred in the 1,000 trees that were generated by NEIGHBOR. The designations of the proteins are followed by the accession numbers and names of the organisms in parentheses.
recombinant 17-kDa protein was incubated with various sugars for 1 h before it was added to larval hemocytes. Agglutination by wild-type 17-kDa protein was used as a positive control. Heat-inactivated wild-type 17-kDa protein, phosphate-buffered saline (PBS), and E. coli K-12 cells were used as controls.

Detection of binding of the 17-kDa protein to larval hemocytes by immunofluorescence. The binding of the protein to H. armigera larval (fourth- or fifth-instar) hemocytes was determined by binding of a fluorescent substrate on the surface of the cells, as described previously (20).

Insect bioassay. The larvicidal activity of the pilin protein was determined as described previously (19). The test protein preparations were diluted in 10 mM sodium phosphate buffer (pH 8.0) and mixed into the artificial diet. Each group contained 24 larvae that were placed individually on the surface of the diet. Mortality and larval weight were recorded periodically over the entire larval period. A dose of protein was the amount of protein mixed into the diet and was not always the actual amount consumed by the larvae. The bioassay was performed more than three times, and the data presented below are the data from one representative experiment. Heat-inactivated 17-kDa recombinant protein, BSA, E. coli K-12 cells, and buffer were used as controls. The 50% lethal dose (LD50) was determined by Probit analysis (12).

Histopathology. Six-day-old insect larvae were fixed with 4% formaldehyde in PBS; several holes were punctured into the cuticle to allow penetration of the fixative. The larvae were embedded in paraffin wax, and 5-μm sections were cut. The sections were stained with cosin and hematoxylin and mounted with glycerol, and images were obtained.

Nucleotide sequence accession number. The nucleotide sequence reported in this paper has been deposited in the GenBank database under accession number AY140909.

RESULTS

Isolation of 17-kDa protein from OMVs of X. nematophila. The OMVs were isolated from the culture filtrate of X. nematophila. When the OMVs were subjected to Sephacryl S-300 column chromatography, a 17-kDa polypeptide eluted in the void volume fractions (Fig. 2, lanes 1 and 2), indicating that the protein was likely present in an oligomeric form even after exposure to 1% SDS at 37°C. The N- and C-terminal sequences of the 17-kDa protein were found to be APTQGD GAVK and TGEFTAIANFTLNYL, respectively. The N-terminal sequence was same as that of the pilin subunit described previously (20).

FIG. 2. Purification of 17-kDa pilin protein from OMVs of X. nematophila. OMV proteins were solubilized in TENS buffer and applied to a Sephacryl S-300 column. The proteins were eluted with TENS buffer. Peak fractions were pooled and examined. Lanes 1 and 2, void volume fractions; lanes 3 to 7, subsequent column fractions. The proteins were resolved on an SDS—12% PAGE gel and were visualized by staining with Coomassie brilliant blue. Numbers on the right are molecular weight markers (in thousands).

Cloning, expression, and purification of recombinant 17-kDa protein. The 537-bp DNA fragment encoding the pilin subunit was amplified by PCR and cloned as described in Materials and Methods. Cells harboring the DNA produced a protein with an apparent molecular mass of 17 kDa as determined by SDS-PAGE (Fig. 3A). The gene showed 35% identity and 65% similarity at the amino acid level with the PapA subunit of the P pili of E. coli.

No recombinant protein was detected when a 468-bp DNA fragment (without the leader peptide sequence) was expressed in several commonly used expression vectors (data not shown). However, when the gene was expressed together with its signal sequence, the protein was recovered as inclusion bodies. The N-terminal sequence of the purified recombinant protein from both of the constructs used (PK4 and PK 5) showed that the signal sequence was processed in the E. coli host. Thus, the recombinant 17-kDa protein expressed by strain PK4 contained no histidine tag (cleaved with the signal sequence) in the N terminus and was obtained in substantially pure form after DEAE column chromatography (Fig. 3A, lane 1). Preliminary experiments were performed with this protein.

The recombinant 17-kDa protein with a six-His tag in the C terminus was obtained from strain PK5 (Fig. 3A, lane 2). The protein that eluted from the Ni-NTA column at the end of an 8 to 0 M urea gradient was found to be modestly stable in solution (it remained in solution at concentrations up to 0.5 mg/ml, but it slowly precipitated at concentrations above this concentration). A 25 μM solution of the protein showed no visible precipitation even after dialysis in a buffer without urea.

Cloning, expression, and purification of recombinant 17-kDa protein. The proteins were resolved on an SDS—12% PAGE gel and were visualized by staining with Coomassie brilliant blue or blotted with antiserum. Lane 1, purified recombinant 17-kDa protein without histidine tag after DEAE ion-exchange purification; lane 2, Ni-NTA affinity column-purified 17-kDa protein; lane 3, Ni-NTA affinity column-purified triple-mutant protein (Val-Phe-Ile → Ala-Ala-Ala). Numbers on the left are molecular weight markers (in thousands). (B) Western blot of recombinant 17-kDa pilin protein of X. nematophila. Lanes 1, 2, and 3 were developed with monoclonal antibodies against the native 17-kDa protein of X. nematophila. Lane 1, cell lysate of uninduced culture of strain PK5; lane 2, cell lysate of induced culture of strain PK5; lanes 3 and 5, Ni-NTA-purified 17-kDa protein; lane 4, DEAE column-purified 17-kDa protein (without tag); lane 6, Ni-NTA-purified triple-mutant protein (Val-Phe-Ile → Ala-Ala-Ala). Numbers on the left are prestained markers.

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The protein obtained from the column was more than 95% pure, as shown by SDS-PAGE (Fig. 3A, lane 2). The purified recombinant 17-kDa protein from PK5 reacted with both anti-His tag antibody and antiserum against the 17-kDa protein (Fig. 3B, lanes 2, 3, and 5). The recombinant 17-kDa protein with the six-His tag was used for the detailed structural and biological studies.

The yield of the purified wild-type protein was 4 mg per 100 ml of culture, and the yields of mutant proteins were 0.5, 2.5, 3.0, and 0.4 mg per 100 ml of culture for the Val32Ala, Phe34Ala, and Ile38Ala mutants and the triple mutant (Val-Phe-Ile → Ala-Ala-Ala), respectively.

Detection of protein oligomers. When the recombinant 17-kDa protein was incubated in the presence of 1.5% SDS at 25°C for 5 min and then subjected to SDS-PAGE and blotted with antibodies for the six-His tag, bands corresponding to higher oligomers were observed (Fig. 4A, lane 1). However, when the protein was heated at 95°C in 1.5% SDS, only the...
monomer was seen, as expected (Fig. 4A, lane 2). The recombinant protein without the histidine tag behaved just like the tagged protein, as shown by blotting with anti-pilin serum (Fig. 4B, lanes 5 and 6). Similar experiments performed with the triple mutant (Val-Phe-Ile→Ala-Ala-Ala) did not show any oligomers, and the protein migrated as monomers only (Fig. 4A, lane 3, and Fig. 4B, lane 3). The recombinant protein migrated as a monomer when the samples were boiled in the reducing or nonreducing loading dye before SDS-PAGE (data not shown), indicating that the oligomerization was not due to intermolecular disulfide bridges.

**Analytical gel chromatography.** Gel filtration was performed to determine the degree of oligomerization of the recombinant 17-kDa protein. The elution profile of the protein obtained with a Sephadex G-200 column is shown in Fig. 4C. The protein recovery estimates showed that about 22% of the recombinant protein was irreversibly adsorbed to the column, possibly due to the formation of large insoluble aggregates, and was not eluted, as has been shown previously (39). A small fraction of the protein eluted in the void volume as large multimers (whose molecular organization was not determined), while the major portion eluted as oligomers of different sizes; the molecular mass of the largest population was in the range from 50 to 160 kDa. In contrast, 65% of the triple-mutant protein was irreversibly adsorbed on the column, and the amount of the eluted protein was barely within detection limits (data not shown).

**CD spectroscopy.** The CD spectra of the native pilin protein, the recombinant wild-type protein, and the mutant proteins are shown in Fig. 5A and C. The recombinant 17-kDa proteins

![Graph A](image1.png)

![Graph B](image2.png)

![Graph C](image3.png)

**FIG. 5.** Comparison of far-UV CD spectra of the native and recombinant 17-kDa pilin proteins. Twenty spectra were averaged, and CD signals were converted to molar ellipticity. (A) CD spectra of both the native and recombinant 17-kDa pilin proteins at a concentration of 20 μM. Solid line, recombinant 17-kDa protein with six-His tag; dotted line, recombinant 17-kDa protein without tag; dotted and dashed line, purified native pilin protein. (B) CD spectra for different concentrations of recombinant 17-kDa protein. Solid line, 20 μM; dashed line with single dots, 30 μM; dashed line, 40 μM; dashed line with double dots, 20 μM recombinant 17-kDa pilin protein denatured with 8 M urea. (C) CD spectra at a protein concentration of 20 μM, showing relative CD signal intensities. Solid line, wild-type 17-kDa protein; dashed line with single dots, Val32Ala mutant; dashed lines with double dots, Phe34Ala and Ile38Ala mutants; dashed line, triple mutant (Val-Phe-Ile→Ala-Ala-Ala).
buffer control. About 8.5 ± 0.32 and 14.2 ± 1.2 mU of the intracellular LDH per well were released when the cells were incubated in the presence of 10 and 15 μg of the recombinant 17-kDa protein, respectively (Fig. 7). Only 1.01 ± 0.12 mU of LDH per well was detected in the presence of E. coli K-12 cells. Heat-inactivated recombinant wild-type protein resulted in the release of 0.61 ± 0.22 mU of LDH per well. The protein that precipitated at a higher concentration was not toxic to cells.

The sugar galactose and the sugar derivatives xylitol, glucuronic acid, and gluconic acid inhibited hemocyte agglutination by the protein, as reflected by values for inhibition of LDH release ranging from 50 to 80% (Table 4). Interestingly, when whole cells of X. nematophila were used as a source of agglutinating protein, in addition to all the compounds mentioned above, N-acetyl-lactosamine and arabinoose.

### TABLE 3. Agglutination of rabbit erythrocytes and H. armigera fourth- or fifth-instar larval hemocytes by X. nematophila cells and recombinant 17-kDa protein

<table>
<thead>
<tr>
<th>Sample</th>
<th>Agglutination titer&lt;sup&gt;a&lt;/sup&gt;</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Erythrocytes</td>
</tr>
<tr>
<td>Buffer control</td>
<td>NA</td>
</tr>
<tr>
<td>X. nematophila cells (10&lt;sup&gt;7&lt;/sup&gt; cells/ml)</td>
<td>64</td>
</tr>
<tr>
<td>E. coli cells (10&lt;sup&gt;7&lt;/sup&gt; cells/ml)</td>
<td>ND</td>
</tr>
<tr>
<td>Wild-type 17-kDa protein with six-His tag</td>
<td>NA</td>
</tr>
<tr>
<td>Wild-type 17-kDa protein without tag</td>
<td>NA</td>
</tr>
<tr>
<td>Heat-inactivated 17-kDa protein with six-His tag</td>
<td>NA</td>
</tr>
<tr>
<td>Triple mutant with six-His tag</td>
<td>NA</td>
</tr>
<tr>
<td>E. coli K-12 purified pilin protein</td>
<td>ND</td>
</tr>
<tr>
<td>Preimmune serum (undiluted)</td>
<td>NA</td>
</tr>
<tr>
<td>Wild-type 17-kDa protein + preimmune serum</td>
<td>NA</td>
</tr>
<tr>
<td>Wild-type 17-kDa protein + anti-17-kDa serum</td>
<td>ND</td>
</tr>
</tbody>
</table>

<sup>a</sup> The initial amount of protein was 100 μg.
<sup>b</sup> NA, no activity; ND, not determined.

*FIG. 7. Cytotoxicity for larval hemocytes of recombinant 17-kDa proteins (His tagged). The cells were incubated with the test proteins at 28°C for 4 to 5 h. The supernatants were separated, and LDH activity was determined.*
also inhibited hemocyte agglutination (Table 4). These results suggest that there are two distinct binding specificities present on the cells of *X. nematophila*.

**Binding of recombinant 17-kDa protein to larval hemocytes.** Binding of the recombinant 17-kDa protein to the larval hemocytes was reflected by strong fluorescence on the cell surface (Fig. 8B). Preincubation of the protein with antiserum against the 17-kDa protein inhibited binding of the recombinant 17-kDa protein to the hemocytes (Fig. 8C), while incubation with preimmune serum had no effect on binding of the protein as the intensity of emission from the cells remained unchanged (Fig. 8D). The binding of proteins to the hemocyte surface was consistent with cell clumping activity.

**Insecticidal activity of the toxin.** The native pilin and the purified recombinant 17-kDa pilin subunit of *X. nematophila* showed oral larvicidal activity when they were incorporated into the diet of neonatal *H. armigera* (Fig. 9). A dose-dependent effect on larval mortality was observed throughout the larval period (Fig. 9). The LD₉₀s were 3.3 and 3.6 μg/cm³ for the native and recombinant wild-type 17-kDa proteins, respectively. A corresponding decrease in the larval weight was observed at all of the concentrations of the proteins (Fig. 9). Heating the protein at 80°C reduced the larvicidal activity substantially; this treatment resulted in 5.1% ± 2% larval death at a protein dose of 10 μg/cm³ of the diet. Furthermore, *E. coli* whole cells, BSA at a concentration of 20 μg/cm³ of the diet, and the buffer used to resuspend the proteins showed no larval toxicity (Fig. 9A).

**Histopathology.** The midgut membrane of a 6-day-old *H. armigera* larva showed typical cellular morphology of the epithelial lining, limited by a basement membrane (Fig. 10A). The membrane integrity was not affected in an *E. coli*-fed larva (Fig. 10B). However, when a larva was fed the 17-kDa pilin protein, breakdown of the epithelial lining was observed. Both the basement membrane and the cellular lining were disorganized, and extensive damage to the integrity of the gut lining was observed at later stages, resulting in sloughing of cell debris in the lumen (Fig. 10C).

### DISCUSSION

In this study we investigated the insecticidal potential of a 17-kDa protein present in the large protein complex secreted by *X. nematophila* as OMVs (19). The purified protein was cytotoxic to the larval hemocytes of *H. armigera* (20). To gain insight into the basis of the toxicity of the 17-kDa protein in the host, we cloned and expressed the gene encoding the pilin subunit of *X. nematophila*. Phylogenetic comparison of the deduced amino acid sequence of the 17-kDa protein with other fimbrial sequences identified by PSI-BLAST demonstrated that the *Xenorhabdus* protein forms a distinct tight cluster with the structural subunits of mannos-resistant fimbrial subunits, including MrpA of *P. mirabilis* and SfmA of *Serratia marcescens* (Fig. 1B). Several *E. coli* fimbrial proteins, like PrsA, KS71A, F7-2, and PapA, the major structural proteins of the P pilus, also belong to this cluster. The bootstrap value based on 1,000 random trials at the node separating the cluster from the remaining fimbrial proteins was as high as 970, showing that the cluster can be reliably considered to be distinct.

The high level of sequence similarity of the *Xenorhabdus* fimbrial gene with the *papA* gene, which encodes the structural subunit of the P pilus of *E. coli*, provided the opportunity to include the wealth of structural information available for the
PapA protein and on this basis verify structural characteristics of the recombinant Xenorhabdus protein. It was necessary to ensure that the protein had attained a native-like structure, as the recombinant protein was refolded under in vitro conditions in the absence of the cellular machinery thought to be essential for pilin assembly.

The recombinant protein was expressed in E. coli in an insoluble form. Analysis of the recombinant 17-kDa protein showed that the protein was expressed as it is in native Xenorhabdus, without its leader sequence or a tag at the N terminus. Upon refolding, the recombinant 17-kDa protein (no tag) had an ordered conformation (as demonstrated by its CD spectrum [Fig. 5A]) and was organized into small, native-like, SDS-resistant oligomers showing biological activity in the in vitro assay. However, since the yields of the purified protein were low (1 to 2 mg/liter), we expressed the protein in the pET23a vector with a C-terminal His tag. When the structural and functional properties of the histidine-tagged protein were compared with those of the recombinant (no histidine tag) and native proteins, it was evident that a significant portion of the recombinant protein had refolded and had a structure similar to that of the native protein; hence, we performed all further investigations with the protein containing a six-His tag at the C terminus.

Elution of the recombinant 17-kDa pilin subunit as a broad peak at molecular masses ranging from 50 to 160 kDa from the gel filtration column and the CD spectra together indicated that the oligomeric protein consisting of monomers with an ordered structure was formed during in vitro refolding. The oligomers were similar to those formed by the interaction of monomers in the mature, native pilin, as 1.5% SDS at 25°C could not destabilize them (Fig. 4A, lane 1). Although the degree of oligomerization of the native protein was substantially higher than that of the recombinant wild-type proteins, the fact that even small oligomers were produced in the absence of the chaperone is significant. Like the Xenorhabdus protein, the recombinant PapA protein of E. coli was also shown to produce only small oligomers, even when it was expressed in the presence of the cognate chaperone (7, 37).

Furthermore, the oligomers were produced by the interaction between neighboring subunits through the conserved N-termi-
nal donor strand, as point mutations in the conserved N-terminal region of the protein substantially reduced this interaction, causing structural defects in the protein. Taken together, the results described above reinforce the role of the N-terminal region in the assembly of a stable, biologically active pilin protein of *X. nematophila*.

It is important that refolding and oligomerization of the *Xenorhabdus* protein occurred in the absence of the cognate chaperone and produced stable oligomers. This is rather surprising, because the presence of the chaperone is considered indispensable during pilin assembly (34), as the chaperone binds to the newly synthesized pilin monomers and protects them from premature aggregation and degradation by proteases (24). So far, there has been only one report demonstrating expression and oligomerization of pilin subunits in vitro in the absence of the chaperone. Vetch et al. (39) showed that refolding of the FimH adhesin protein of *E. coli* occurred in vitro in the absence of its cognate chaperone, FimC; however, the stability of the refolded pilin domain was low. In the case of the *Xenorhabdus* protein, the role of the chaperone is presumably partially taken over by the N-terminal overhang of the monomers, which act as a chaperone for a neighboring subunit, albeit with reduced efficiency. In the process of in vitro refolding in the presence of a relatively high concentration of the recombinant protein, there is ample opportunity for the molecules to visit various conformational states. At least some of these states can be involved in productive interactions with the N-terminal extension of another molecule, forming stable multimeric structures.

The inhibition of cytotoxicity for cultured hemocytes by the sugar derivatives points to a specific recognition event between the pilin protein and the insect cells. At this stage we do not know if the pilin protein has any role in the recognition of the nematode host. Interestingly, the two compounds which inhibited the interaction of whole bacterial cells with the hemocytes, N-acetyl-lactosamine and arabinose, showed no inhibition when they were added to the hemocytes in the presence of the 17-kDa protein, suggesting that there is an additional molecule with a distinct binding specificity on the bacterial surface. This could be the adhesin subunit, which is normally responsible for host cell recognition in other gram-negative bacteria. Recently, the *mrx* fimbral operon of *X. nematophila* has been reported, and this operon contains an open reading frame corresponding to the adhesin subunit (17); however, no information concerning the characteristics of the adhesin protein of *X. nematophila* is available. Moureaux et al. (27) also described inhibition of agglutination of sheep erythrocytes by arabinose and N-acetyl-lactosamine in the presence of *X. nematophila* cells. So far, there is no direct evidence showing biological activity of the structural subunit of pilin. Our results demonstrate for the first time sugar-specific recognition of host cells by a structural subunit of a pilin.

The most significant finding of this study is the association of oral larvicidal activity and concomitant damage of the gut membrane with the structural subunit of the pilin protein. Several protein toxins of *Xenorhabdus* and the related genus *Photorhabdus* have been described previously. The LD<sub>90</sub> of the individual Xpt toxins isolated from *X. nematophila* were shown to range from 2 to 5 μg for different species of *Pieris* and *Heliothis* (36); these values are comparable to the values for the pilin protein studied here. The high-molecular-weight TcA protein isolated from *Photorhabdus* had a lower LD<sub>90</sub> (870 ng) for *Galleria* larvae (5). At this stage we cannot comment on the contribution of hemocyte cytotoxicity to the overall insecticidal activity of the protein.

*X. nematophila* is a successful endosymbiont of the soil nematode *S. carpocapsae*, but it switches to an aggressive mode inside an insect larva. Recent sequencing of several endosymbiotic bacterial genomes has revealed that obligate host-associated bacteria have some of the smallest genomes known in nature (25). Although the whole genome of *Xenorhabdus* is not available yet, it should be mentioned that the recently published *mrx* operon of *X. nematophila* is considered to be the smallest fimbral operon studied to date (17). In this context it is tempting to speculate that in *X. nematophila*, synthesis of proteins with dual functions may be a mechanism to minimize the genome size in order to get a competitive edge in the natural habitat. Thus, the presence of abundant pilin protein on the surface of the cells, which is primarily used for host recognition, also serves as a convenient tool to intoxicate the insect larva rapidly. Furthermore, OMVs, which have been recognized as a means of effector protein secretion in several gram-negative pathogens (40), provide a ready vehicle for transportation of the protein outside the cell if necessary.

In conclusion, this is a first report demonstrating the larvicidal nature of a structural subunit of *X. nematophila* pilin. Our results also show that the recombinant protein can be refolded and oligomerized in vitro without the cognate chaperone, which has not been reported previously. It can acquire an oligomeric organization similar to that of the native protein through donor strand complementation of the N-terminal extension, between successive monomers. The finding that the protein can assume a structure competent for biological activity without the assistance of any other factor demonstrates its potential as a biological control agent, particularly in the context of an urgent need for discovering novel biopesticides.

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