Xenorhabdus and Photorhabdus are gram-negative bacteria that are highly pathogenic to insects. We recently identified a novel 42-kDa protein from Xenorhabdus nematophila that was lethal to the larvae of insects such as Galleria mellonella and Helicoverpa armigera when it was injected at doses of 30 to 40 ng/g larvae. In the present work, the toxin gene txp40 was identified in another 59 strains of Xenorhabdus and Photorhabdus, indicating that it is both highly conserved and widespread among these bacteria. Recombinant toxin protein was shown to be active against a variety of insect species by direct injection into the larvae of the lepidopteran species H. armigera and Plodia interpunctella and the dipteran species Lucilia cuprina. The protein exhibited significant cytototoxicity against two dipteran cell lines and two lepidopteran cell lines but not against a mammalian cell line. Histological data from H. armigera larvae into which the toxin was injected suggested that the primary site of action of the toxin is the midgut, although some damage to the fat body was also observed.

Xenorhabdus and Photorhabdus are gram-negative bacteria that are highly pathogenic to insects (10). These bacteria live in symbiosis with rhabditid nematodes belonging to the genera Steinernema and Heterorhabditis. The nematodes and bacteria share a complex life cycle which involves both symbiotic and pathogenic stages. After nematode infection of an insect host, the nematodes release the bacteria into the insect hemocoel, where the bacteria grow to the stationary phase. The bacteria and nematodes work together to kill the insect host, although in most cases the bacteria alone are highly virulent once they are circulating in the insect hemocoel (16). The final stage of development is the reassocation of the bacteria and nematodes to form nonfeeding infective juveniles, which emerge from the insect carcass to find new hosts.

The bacterium-nematode associations have a wide insect host range (23) and are used for biological control of some lepidopteran, dipteran, and coleopteran pests of commercial crops (10). The susceptibility of a particular insect host to a bacterium-nematode association depends on a number of factors, including the species of bacteria or nematodes, the ease of finding and entering the insect host, the stage of the insect in the life cycle, the response of the insect immune system, and other biochemical and physiological responses to the various metabolites produced by the nematodes and bacteria (2). In particular, both the bacteria and the nematodes produce a range of toxins that are responsible for killing the insect host (2). Analysis of the genome of Photorhabdus luminescens resulted in identification of more putative toxin genes than have been found in any other bacterium sequenced to date (15). The only toxins that have been studied in detail from Xenorhabdus and Photorhabdus bacteria are the Tc toxins from P. luminescens strain W14 (7, 8, 17). The Tc toxins form a large protein complex consisting of about 10 polypeptides ranging in size from 30 to 200 kDa that is toxic to insects after either ingestion or injection (7, 8). Some work has also been performed with a 39-kDa toxin from Xenorhabdus nematophila (29), the large Xin toxin from X. nematophila strain BJ (27), and the 17-kDa pilin subunit from X. nematophila (20). We recently described a novel toxin, A24tox, from X. nematophila strain A24. This 42-kDa toxin is a secreted protein that is lethal to the larvae of insects such as Galleria mellonella and Helicoverpa armigera when it is injected at doses of 30 to 40 ng/g larvae (9).

The details of the modes of action of the various toxins of Xenorhabdus and Photorhabdus are still not known. Injection of the Tca complex of P. luminescens strain W14 or ingestion by Manduca sexta larvae damaged the midgut cells, resulting in shedding of the midgut epithelium into the gut lumen, followed by lysis of the epithelium (5). Injection of A24tox into lepidopteran larvae caused the larvae to cease feeding almost immediately, and preliminary histological studies indicated that the main site of action for the toxin was the insect midgut (9). Many other insecticidal toxins are known to act on the insect gut, including the well studied δ-endotoxins from Bacillus thuringiensis and cholesterol oxidase from Streptomyces (5).

In this study we used histology and cytotoxicity assays to obtain further insight into the mode of action of the novel insecticidal toxin A24tox. We also investigated the occurrence of genes closely related to the A24tox gene in 59 strains of Xenorhabdus and Photorhabdus bacteria that encompass most of the genetic diversity currently known for these two genera.

**MATERIALS AND METHODS**

Isolation of the txp40 toxin gene from P. luminescens strain V16. High-molecular-weight genomic DNA was isolated from P. luminescens strain V16 (31). The DNA was partially digested with Sau3AI and used to create a cosmid library, as described previously (9). This library was screened by hybridization using the previously discovered A24tox gene from X. nematophila strain A24 as a probe (9). Two hundred cosmid clones were grown overnight at 37°C on Luria-Bertani (LB) medium-ampicillin plates, transferred to nylon membrane disks (Colonies...
Plaque Screen; NEN DuPont), lysed in situ with treatment by 0.5 N NaOH, and neutralized with 1.0 M Tris–Cl (pH 7.5). After air drying, the filters were prehybridized in a solution consisting of 5× SSPE [1× SSPE is 50 mM sodium citrate, 20 mM sodium phosphate, pH 7.0, and 1 mM EDTA], 0.5% (wt/vol) skim milk, 0.5% (wt/vol) sodium dodecyl sulfate (SDS), and 0.2 mg/ml denatured salmon sperm DNA at 68°C for 3 h. A hybridization probe was prepared by radiolabeling approximately 100 ng of isolated A24tox gene with 50 nCi [32P]ATP by random primed synthesis using a Gigaprim DNA labeling kit (GPK-1; Bresatec). Filters were incubated with the A24tox probe in the same buffer that was used for the prehybridization step at 68°C overnight. The filters were rinsed briefly in 2× SSC (1× SSC is 150 mM NaCl plus 15 mM sodium citrate, pH 7.0) and washed once for 15 min at room temperature in 2× SSC containing 0.1% (wt/vol) SDS and once at 68°C for 30 min in 0.5× SSC containing 0.2% (wt/vol) SDS. After a final rinse in 0.5× SSC the filters were autoradiographed for 24 h at −80°C. For each clone that hybridized with the A24tox probe, cultures were grown and the cell lysates were assayed for toxicity using the G. mellonella injection bioassay (9). Clones of interest were analyzed by further restriction enzyme mapping (NotI, EcoRI, HindIII, EcoRV, and SmaI), cloned into the plasmid vector pBluescript II (KS+) (Stratagene), transformed into Escherichia coli strain DH10B (Stratagene), and tested with the G. mellonella injection bioassay. As described previously for the A24tox gene (9), both strands of the smallest deletion clone that retained insecticidal activity, the DNA immediately surrounding the toxin gene, and the DNA at the 5’ and 3’ ends of the cosmid clones and the intermediate deletion clones were sequenced using a combination of vector- and gene-specific primers. Similarity searches of the nonredundant nucleotide and protein databases (April 2005) were performed using the various BLAST programs available via NCBI (http://www.ncbi.nlm.nih.gov/BLAST/). Analysis of the genome was performed using the utilities available at PhotoList (http://genolist.pasteur.fr/PhotoList/index.html; release date, 25 September 2003). Similarity searches were also performed against the partially completed genome sequences of Xenorhabdus bowenii and X. nematophila strain ATCC 19061 (http://xenorhabdus.danforthcenter.org; release dates, 8 November 2004 and 21 March 2005, respectively), as well as PhotoList (http://www.sanger.ac.uk/Projects/ P.xenonhabdus/; release date, 14 March 2005).

Identification of the tsp40 toxin gene in other Xenorhabdus and Photorhabdus strains. Forty-seven Xenorhabdus strains and 17 Photorhabdus strains were tested for the occurrence of the toxin gene by PCR and Southern blot analysis. The strains were streaked from glycerol stocks onto LB agar plates and grown for 2 to 3 days at 28°C. Two-milliliter portions of LB medium were then inoculated with single colonies, and the cultures were grown overnight at 28°C and 200 rpm. The genomic DNA was isolated from all strains using the protocol for gram-negative bacteria of a DNeasy kit (QIAGEN). The concentration of DNA was determined using genomic DNA samples (200 μl) and the DNA-binding dye Hoechst 33258 (fluorescent DNA quantitation kit; Bio-Rad) in a 96-well plate (Maxisorp; Nunc). The fluorescence was measured by using a POLARstar fluoroescence plate reader (BMÖ Labtechnologies) with an excitation wavelength of 355 nm and an emission wavelength of 460 nm. The genomic DNA was analyzed using a combination of PCR and low-stringency Southern blot analysis. For Xenorhabdus strains, the A24tox clone originally constructed from X. nematophila strain A24 (9) was used to design two pairs of primers. The first pair of primers was designed to anneal to the 5’ and 3’ untranslated regions of the A24tox gene (AC15F [5’-ATTAGACCCCAATAATTTCGCG], 141 bp upstream of the A24tox gene start codon). If a PCR product was not observed with these primers, then the PCR was repeated using a second pair of primers that were internal to the A24tox gene (ToxF4 [5’-AGAGTACGCGTCCGGTAA], 54 bp downstream of the A24tox gene stop codon). For Photorhabdus strains, the primers were designed to anneal to the start codon (V16AC9F [5’-ATGGTTATACATACCAATACCTTGC], 141 bp upstream of the A24tox gene stop codon). The PCR products were further purified by visualization of fractions by SDS-polyacrylamide gel electrophoresis (PAGE) using zinc stain (Bio-Rad), excision of the toxin bands from the gel, and electroelution using an Electroeluter 422 (Bio-Rad). The protein was analyzed by SDS-PAGE and Western blotting (9), using an Ni-nitrilotriacetic acid agarose resin (QIAGEN) column. The protein was further purified by visualization of fractions by SDS-polyacrylamide gel electrophoresis (PAGE) using zinc stain (Bio-Rad). After excision of the toxin bands or the gel, and electroelution using an Electroeluter 422 (Bio-Rad), the protein was analyzed by SDS-PAGE and Western blotting (9), using an Ni-nitrilotriacetic acid horseradish peroxidase conjugate (QIAGEN) and the substrate 3-amino-9-ethylcarbazole. The final concentration of toxin was estimated by comparison to a bovine serum albumin standard using SDS-PAGE. The identity of the toxin was confirmed by tryptic digest mass spectroscopy as described previously (9). The His6-T16tox protein was used for purification of toxin antigen (9). The insect bioassays, insect rearing and bioassays with G. mellonella, H. armigera, and Lucilia cuprina were performed as described previously (9). Plodia interpunctella was reared at 25°C on an artificial diet containing 10 parts wheat bran, 2 parts wheat germ, 1 part yeast, and 2 parts glycerol. The activities of a recombinant protein corresponding to the toxin from P. luminescens strain V16 (Tsp40V16) were determined with G. mellonella, H. armigera, and L. cuprina; these analyses included determination of the 50% lethal doses (LD50) for G. mellonella and H. armigera. The activities of recombinant proteins corresponding to the toxins from X. nematophila strain Mex and P. luminescens strain HI were determined using the G. mellonella injection bioassay with doses of 10, 20, and 200 ng for Tsp40Mex and 50 and 500 ng for Tsp40HI. Recombinant Tsp40V16 was also assayed with P. interpunctella by using a dose of 100 ng.

Detailed bioinformatic analyses of the Txp40V16 toxin, including similarity searches and motif and fold recognition, were also performed as described previously (9). Recombinant toxin was used in the insect bioassays, the cytotoxicity assays, and the histological experiments described below; E. coli maltose binding protein (MBP) prepared in the same manner with the IMpact system was used as a control. A six-histidine-tagged version of the toxin from P. luminescens strain V16 (His6Tsp40V16) was also purified. The EcoRV/Smal restriction fragment of the active cosmid clone from P. luminescens strain V16 was cloned into the N-terminal six-histidine-tag vector pQE-31 (QIAGEN) and transformed into E. coli XL1-Blue cells (Stratagene) by electroporation. The cells were grown, harvested, and lysed by sonication using standard methods, and the cell extract was purified on an Ni-nitrilotriacetic acid agarose resin (QIAGEN) column. The protein was further purified by visualization of fractions by SDS-polyacrylamide gel electrophoresis (PAGE) using zinc stain (Bio-Rad). After excision of the toxin bands from the gel, and electroelution using an Electroeluter 422 (Bio-Rad), the protein was analyzed by SDS-PAGE and Western blotting (9), using an Ni-nitrilotriacetic acid horseradish peroxidase conjugate (QIAGEN) and the substrate 3-amino-9-ethylcarbazole. The final concentration of toxin was estimated by comparison to a bovine serum albumin standard using SDS-PAGE. The identity of the toxin was confirmed by tryptic digest mass spectroscopy as described previously (9). The His6V16tox protein was used for preparation of toxin antigen (9).
TABLE 1. Forty-seven Xenorhabdus strains and 12 Photorhabdus strains which were shown to contain the txp40 gene using PCR or hybridization experiments

<table>
<thead>
<tr>
<th>Species or subspecies</th>
<th>Strain(s)</th>
<th>Gene product detected by&lt;sup&gt;a&lt;/sup&gt;</th>
<th>External primers</th>
<th>Internal primers</th>
<th>Hybridization</th>
</tr>
</thead>
<tbody>
<tr>
<td>X. nematophila</td>
<td>A24, All, AllD, AN6, BK, Ch, F1, Mex, Ohio, Pi, S. carp It</td>
<td>+</td>
<td>ND</td>
<td>ND</td>
<td></td>
</tr>
<tr>
<td>X. poinarti</td>
<td>ATCC 49121, ATCC 49122, NC32, NC33, NC40, NC513</td>
<td>-</td>
<td>+</td>
<td>ND</td>
<td></td>
</tr>
<tr>
<td>X. bovieni</td>
<td>F7, T363</td>
<td>+</td>
<td>ND</td>
<td>ND</td>
<td></td>
</tr>
<tr>
<td>X. beddungi</td>
<td>F3, F5, F9, Si, SK, T319</td>
<td>-</td>
<td>+</td>
<td>ND</td>
<td></td>
</tr>
<tr>
<td>X. japonica</td>
<td>Q58</td>
<td>-</td>
<td>-</td>
<td>+</td>
<td></td>
</tr>
<tr>
<td>Xenorhabdus sp.</td>
<td>ED3</td>
<td>+</td>
<td>ND</td>
<td>ND</td>
<td></td>
</tr>
<tr>
<td></td>
<td>C8401, C8503, CB 19, CB/2A/W, CB2B, CB-G, CB-W, EC1, K78, NC270, NC276, Q1, SaR, SaV, W1</td>
<td>-</td>
<td>+</td>
<td>ND</td>
<td></td>
</tr>
<tr>
<td></td>
<td>K77, W2/S.2</td>
<td>-</td>
<td>-</td>
<td>+</td>
<td></td>
</tr>
<tr>
<td>P. luminescens</td>
<td>C8406, HL, K80</td>
<td>ND</td>
<td>+</td>
<td>ND</td>
<td></td>
</tr>
<tr>
<td>P. luminescens subsp. luminescens</td>
<td>Hb, V16</td>
<td>ND</td>
<td>+</td>
<td>ND</td>
<td></td>
</tr>
<tr>
<td>P. luminescens subsp. laumondii</td>
<td>HP88</td>
<td>ND</td>
<td>+</td>
<td>ND</td>
<td></td>
</tr>
<tr>
<td>P. luminescens subsp. akhurstii</td>
<td>D1, Tetuan&lt;sup&gt;b&lt;/sup&gt;</td>
<td>ND</td>
<td>+</td>
<td>ND</td>
<td></td>
</tr>
<tr>
<td>P. temperata</td>
<td>C1, HW79, NZH3</td>
<td>ND</td>
<td>+</td>
<td>ND</td>
<td></td>
</tr>
<tr>
<td>Photorhabdus sp.</td>
<td>Q621</td>
<td>ND</td>
<td>+</td>
<td>ND</td>
<td></td>
</tr>
</tbody>
</table>

<sup>a</sup> The toxin gene in the following strains was sequenced: A24, All, AllD, AN6, BK, Ch, F1, Mex, F7, T363, F5, T319, Kushidai, C8401, CB-G, CB-W, SaV, HL, V16, Tetuan, and Q621.

<sup>b</sup> The primers for Xenorhabdus strains were designed from txp40<sub>A24</sub> or, and the primers for Photorhabdus were designed from txp40<sub>V16</sub> +, gene product detected; --, gene product not detected; ND, experiment not done.

<sup>c</sup> The PCR product observed for P. luminescens subsp. akhurstii strain D1 was obtained using the internal primers designed from the txp40<sub>A24</sub> gene.

RESULTS

Identification and sequencing of txp40 toxin gene. We used the recently identified toxin gene txp40<sub>A24</sub> (previously designated A24tx) (9) to identify a related toxin in a cosmid library of the bacterium P. luminescens strain V16. The Txp40<sub>V16</sub> toxin was 83% and 75% identical to Txp40<sub>A24</sub> at the nucleotide and protein levels, respectively. It also exhibited 98% identity to the hypothetical plu2326 protein encoded in the genome sequence of P. luminescens subsp. laumondii strain TT01. Further bioinformatic analyses of the Txp40<sub>V16</sub> Protein, including motif recognition or fold recognition, resulted in no significant matches to proteins with known structures or functions, as found for Txp40<sub>A24</sub> (9).

PCR and Southern blot analysis were then used to detect the presence of the toxin gene in 64 Xenorhabdus and Photorhabdus isolates. A PCR product that was the same size as the txp40<sub>A24</sub> or txp40<sub>V16</sub> control was detected in 43 Xenorhabdus strains and 12 Photorhabdus strains (Table 1). Note that the primary and secondary phases of X. nematophila strain Mex both produced a PCR product with the external primers. Genomic DNA from the four Xenorhabdus strains which did not produce a PCR product (strains K77, Q58, T228, and W2/5.2) were shown to hybridize to the txp40<sub>A24</sub> probe by low-stringency Southern blot analysis (data not shown). This indicated that 47 of the Xenorhabdus strains studied and 12 of
the *Photorhabdus* strains studied contained a gene related to *txp40*\(_{A24}\).

The toxin gene (*txp40*) was cloned from 17 *Xenorhabdus* strains and four *Photorhabdus* strains and sequenced. The predicted toxin proteins in all of these strains exhibited high levels of similarity to *T xp40* \(_{A24}\). Due to the high levels of similarity between the toxins from different strains, the diversity of the 21 sequences could be minimally represented by the eight sequences (see Fig. S1 in the supplemental material) revealed that there are three distinct forms of the toxin protein which differ by small insertions and deletions dispersed throughout the protein (Fig. 1).

**Nucleotide sequence surrounding the txp40 toxin gene in *P. luminescens* strain V16.** The genetic context of the *txp40* gene was investigated by limited sequencing of the DNA flanking this gene (9). The region around the toxin gene that was sequenced was 4,180 bp long (average G+ content, about 37%), including 1,693 bp upstream and 2,210 bp downstream of *txp40* \(_{V16}\), as well as some single sequences (approximately 450 to 550 bp) from the original cosmid clones or the intermediate deletion clones that were produced when *txp40* \(_{V16}\) was isolated. All of the sequences obtained were also present in the genome of *P. luminescens* subsp. laumondii strain TT01, with \(\geq 91\%\) identity. The sequences matching the 5’ and 3’ ends of the cosmid clone were approximately 44 kb apart in the *Photorhabdus* genome, a distance consistent with the size of the cosmid clone, and the plu2326 toxin gene was situated between the two end sequences. This suggests that the general arrangements of genes near the toxin gene are similar in *Photorhabdus* strains V16 and TT01. However, many of the open reading frames identified in this 44 kb of sequence in the *Photorhabdus* genome are annotated as open reading frames that encode unknown proteins, particularly downstream of the toxin gene (plu2327 to plu2346). The only annotated open reading frames in this region are upstream of the toxin gene and are a locus that includes eight genes annotated as a cluster that is very similar to the *Yersinia pestis* yersiniabactin cluster (plu2316 and plu2318 to plu2324). Similarity searches using all experimentally obtained sequences produced only three other convincing matches in more than 6,000 bp of sequence. The first match was an open reading frame that ended approximately 500 bp upstream of the *txp40* \(_{V16}\) gene and that was very similar to the *Bacillus subtilis* yvrK gene (blastx; gi:7445182; 948 bp; expect value, \(2 \times 10^{-87}\)). The second match was well upstream of *txp40* \(_{V16}\) and corresponded to DNA invertase transposon Tn5393 from *Erwinia amylovora* (blastx; gi:420963; 132 bp; expect value, \(8 \times 10^{-10}\)). The third match was downstream of the toxin gene and corresponded to the gene encoding the hypothetical protein PpoE, which is part of a flagellum operon (gi:AY422684) in *P. luminescens* (blastx; gi:AAQ97873; 135 bp; expect value, \(3 \times 10^{-11}\)).

Searches for proteins or DNA with similarity to the *Txp40* toxin or gene were also conducted with the partially completed genomes of *X. bovienii*, *X. nematopilia* strain ATCC 19061, and *P. asymbiotica*. The toxin gene was found in the genome of *X. nematopilia* strain ATCC 19061 and was shown to be identical to the gene encoding the toxin protein from *X. nematopilia* strain AN6. The sequence surrounding the toxin gene was also very similar to that obtained for *X. nematopilia* strain A24 (9), indicating that the toxin gene is in the same context in these two *X nematopilia* strains. The toxin gene was not found in the partially completed genomes of *X. bovienii* or *P. asymbiotica*, although in *P. asymbiotica* some matches to parts of the DNA sequence flanking the toxin gene were found.

**T xp40 toxin activity.** Recombinant proteins corresponding to the toxins from *X. nematopilia* strain Mex (Txp40\(_{Mex}\)). *P. aegypti* S2, Sf21, Sf9, and SP2 cells. Inhibition is expressed as a percentage relative to the growth of the control cells, which were treated with MBP. For each cell type, the inhibition with 0, 0.2, 2, 20, and 2,000 ng/ml of *Txp40*\(_{Mex}\) is shown. As there was no significant difference between the growth of the toxin-treated cells and the growth of the MBP-treated cells at doses of 0.002 and 0.02 ng/ml, the data for these concentrations were omitted for clarity.
**FIG. 3.** Histological images of toxin-treated *H. armigera* larvae, showing the effect of recombinant toxin on the midgut. All images (magnification, ×90) are images of longitudinal sections through the anterior region of the midguts of larvae inoculated with 100 ng of either MBP or Txp40A24 toxin. BM, basement membrane; E, midgut epithelium; L, midgut lumen; PM, peritrophic matrix. The open arrowheads indicate spaces between cells of the gut epithelium, the solid arrowheads indicate breakdown of the basement membrane, the asterisk indicates cells sloughed into the midgut lumen, and the multiplication signs indicate rafts of unidentified material in the midgut lumen. (a, c, and e) Larvae treated with MBP. (b, d, and f) Larvae treated with Txp40A24. Tissues were fixed at zero time (a and b) and at 12 h (c and d) and 18 h (e and f) after toxin injection.

*Luminescens* strain V16 (Txp40V16), and *Photorhabdus* sp. strain HI (Txp40H1) were purified using the IMPACT system. The identities and purities of the proteins were confirmed by SDS-PAGE and mass spectroscopy (data not shown). LD$_{50}$s of 34 ng (95% confidence limits, 18 to 59 ng) and 64 ng (95% confidence limits, 36 to 114 ng) were determined for the interaction of recombinant Txp40V16 with *G. mellonella* and *H. armigera*, respectively (data not shown). Since the average larval mass at the time of injection was approximately 800 mg for *G. mellonella* and 140 mg for *H. armigera*, the LD$_{50}$s were 43 and 460 ng/g for *G. mellonella* and *H. armigera*, respectively. Qualitative results were also obtained for the activity of recombinant toxins against other insect species (data not shown). Txp40V16 was active against *L. cuprina* and *P. interpunctella*.
and Txp40Mex and Txp40HI were active against *G. mellonella*. The typical toxin dose required to cause \(\geq 50\%\) mortality was in the 5- to 100-ng range, although the dose required for Txp40HI was significantly higher (500 ng). These results show that the toxins are active against a variety of insect species, although the lethal dose varies with the toxin and the insect species.

A CyQUANT cell proliferation assay kit was used to determine the effect of 0.002 to 2,000 ng/ml of recombinant Txp40A24 on insect and mammalian cell lines (Fig. 2). Txp40A24 caused statistically significant reductions (up to 60%) in the number of cells (compared to the MBP control) for the *Spodoptera* cell lines Sf9 and Sf21 and the mosquito cell line (*A. aegypti*) at concentrations greater than 2 ng/ml and for the *D. melanogaster* cell line (S2) at the highest concentration tested (2,000 ng/ml). In contrast, the mammalian myeloma (SP2) cell line was not significantly affected by Txp40A24 at the range of toxin concentrations tested.

**Txp40 toxin histopathology.** *H. armigera* larvae treated with 10 ng or 100 ng of Txp40A24 were examined by microscopy (Fig. 3 to 5). The control larvae inoculated with 100 ng of MBP showed the histology expected for healthy *H. armigera* larvae (Fig. 3 to 5). At 6 h after injection of the toxin the only observable difference between the toxin-treated and control larvae was that the peritrophic matrix had started to disinte-
grate in the toxin-treated larvae. Twelve hours after injection with 100 ng of toxin, there were large gaps between the midgut cells, which were starting to separate from the basement membrane and each other (Fig. 3). The peritrophic matrix was also no longer visible (Fig. 3), a result that was confirmed by analysis of the citric acid- and heat-treated sections (Fig. 4). This treatment resulted in strong autofluorescence of the peritrophic matrix and revealed the presence and absence of the peritrophic matrix in the control and toxin-treated larvae, respectively. By 18 h with either concentration of toxin, the damage to the midgut was obvious, with numerous cells sloughed into the lumen and a large amount of unidentified material appearing in the lumen (Fig. 5). At the same time, the fat body showed signs of nuclear degradation, as shown by chromatin condensation (Fig. 5). By 24 h postinjection the midgut was quite disrupted and the basement membrane had started to disintegrate. The damage was evenly distributed throughout the midgut, and there was damage to the foregut or hindgut and no evidence of general cell lysis. All of these effects of the toxin were observed in at least two larvae from independent experiments.

DISCUSSION

Forty-seven Xenorhabdus strains and 12 Photorhabdus strains were shown to contain a gene closely related to txp40A24, and the toxin gene was also found in the genomes of X. nematophila strain ATCC 19061 and P. luminescens subsp. laumondii strain TT01. These results suggest that the toxin is ubiquitous among Xenorhabdus and Photorhabdus bacteria. The toxin gene was found in both phase 1 and phase 2 cells of X. nematophila strain Mex, which is consistent with evidence that there are no
differences at the DNA level (either chromosomal or plasmid) between the different phases of this bacterium (3, 6). For *Xenorhabdus*, the highest level of similarity to *txp40* (as shown by the production of a PCR product with primers external to *txp40*) was observed with strains of *X. nematophila* (Table 1). There was no apparent correlation between the geographic location of the *Xenorhabdus* strain and the method that was needed to detect the *txp40* homolog (Table 1; see Table S1 in the supplemental material).

The *txp40* toxin gene was sequenced for 17 *Xenorhabdus* strains and four *Photorhabdus* strains. The toxins from five *Photorhabdus* strains were very similar to Txp40 differing by at most five amino acids. With the exception of toxin from *X. bovienii* strain T363, the toxin sequences from *Xenorhabdus* strains were all very similar to the sequence of Txp40, differing by a maximum of nine amino acids between residues 19 and 344 (Txp40 numbering). These results confirm that the toxin protein is highly conserved. The toxin from *X. bovienii* strain T363 was anomalous in that the sequence was more similar to the sequences of the *Photorhabdus* toxins, yet biochemical analysis (colony morphology, 16S RNA sequence, and negative catalase activity) clearly classified this organism as a *Xenorhabdus* strain. There appears to be heterogeneity within the species *X. bovienii* (1, 6), which may explain this result.

The schematic diagram of the toxin in Fig. 1 shows that there are three regions in the protein that can tolerate insertions or deletions, the N terminus, a 17-amino-acid region with low complexity (residues 254 to 270 for Txp40), and a single amino acid (residue 332 in Txp40). Based on these insertion-deletion regions, it is possible to classify the toxin proteins as either *Xenorhabdus*- or *Photorhabdus*-like, where a longer N terminus, the presence of the sequence with the low level of complexity, and the extra amino acid at position 332 classify the protein as *Xenorhabdus*-like. As mentioned above, *X. bovienii* strain T363 is anomalous according to this classification. Since toxins from both *Xenorhabdus* and *Photorhabdus* strains were shown to kill insects, the insertion-deletion regions cannot be essential for the lethality of the protein. This was confirmed by the observation that insertion of a myc epitope tag at the N terminus or at position 254 of Txp40 did not significantly affect the injection bioassay results (data not shown). Although the variable regions or the single amino acid substitutions are not essential for lethality, they may contribute to the differences in relative potency for particular insect species.

There were three interesting observations that could be made about the broader genetic context in which the *txp40* and *txp40* genes occur. First, for *txp40*, the sequence matches indicated that *P. luminescens* strain V16 had shared genetic material with other bacteria, that mobile genetic factors such as transposons were present, and that the G+C content of the DNA region containing the toxin (37%) was different from the G+C content estimated for the whole genome (43%) (15). This implied that the toxin gene was part of a genomic island involved in pathogenicity; as concluded previously for *txp40* (9). Other toxins in *Xenorhabdus* and *Photorhabdus*, including the Tc toxins and the mcf gene product, are also part of putative genomic islands involved in pathogenicity (36). Second, the analysis of the genetic context of the two *Photorhabdus* toxins, *txp40* and plu2326, resulted in identification of several proteins with functions associated with virulence. These proteins included the yersiniabactin-like cluster, which is responsible for iron acquisition using siderophores (11, 15, 37), the flagellum protein PpoE (28), and three pseudogene-encoded genes related to the TccB toxin (proteins encoded by plu2333 to plu2335) (15). The similar locations of different genes implicated in virulence are consistent with the idea that the *txp40* gene is part of a genomic island involved in pathogenicity. Third, despite the high degree of similarity between the *txp40* and *txp40* genes, the DNA surrounding the toxin genes exhibited no significant similarity, indicating that the context of the toxin genes in the two genera is quite different. This is consistent with the observation that the *txp40* gene product appears to be active as a single protein, unlike the Tc proteins, which are encoded by multiple genes arranged in multiple loci (35, 36). This is also another example of the molecular and biochemical differences between *Xenorhabdus* and *Photorhabdus* (16).

Recombinant Txp40 toxin was active against a range of lepidopteran species (*G. mellonella*, *H. armigera*, and *P. interpunctella*) and cell lines (*Spodoptera* cell lines Sf9 and Sf21), as well as dipteran species (*L. cuprina*) and cell lines (*A. aegypti* and *D. melanogaster* cell line S2). Khandelwal and colleagues also observed that an outer membrane vesicle preparation from *X. nematophila* was toxic to Sf21 cells (19). The broad insecticidal activity of the Txp40 toxin is consistent with observations that the nematode-bacterium association is active against a range of different insect species (23). It suggests that the toxin has a target that is common to many different insects.

The primary site of action of the Txp40 toxin appeared to be the insect midgut, as revealed by the histology of *H. armigera* larvae inoculated with the toxin (Fig. 3 to 5). The results are consistent with reports which described the colonization of *Spodoptera littoralis* by green fluorescent protein-labeled *X. nematophila* (32) and the colonization of *M. sexta* by *P. luminescens* (33). Both reports suggested that the bacteria occupy a niche in the extracellular matrix surrounding the midgut, so that the bacteria are associated closely with the midgut epithelial cells. Bacterial colonization appears to start in the anterior midgut and hemolymph, progress to the posterior midgut, and finally spread to other tissues, such as the fat body (32, 33). This is consistent with our observations which showed that there was damage to the midgut at the early stages of infection and damage to other tissues, such as the fat body, at the later stages of infection.

The histopathological effects on lepidopteran larvae of three other toxins from *Xenorhabdus* or *Photorhabdus* have been examined previously (5, 13, 20). All three toxins (the Tc toxins, the mcf gene product, and a 17-kDa pilin subunit) also damage the midgut. The Tc toxins from *Photorhabdus* cause shedding of the midgut epithelium of *M. sexta* larvae into the gut lumen and lysis of the epithelial cells (5). The Tc toxin has been reported to cause no damage to other tissues of *M. sexta* larvae (5). The mcf gene from *P. luminescens* was cloned into *E. coli*, and the bacteria were injected into *M. sexta* larvae, resulting in shedding of the insect midgut epithelium and destructive blebbing of hemocytes (13). The peritrophic matrix was intact 24 h after ingestion of the bacteria (13). *E. coli* expressing the mcf gene product was also shown to trigger apoptosis in mammalian cells (14). The damage to the lepidopteran midgut and other tissues caused by the Tc toxins and...
the mcf gene product is different than the damage seen with the Txp40 toxin. The third toxin studied, the 17-kDa pilin subunit from *X. nematophila*, was administered orally to *H. armigera* larvae and was shown to break down the midgut epithelial lining and basement membrane and to cause sloughing of cell debris into the midgut lumen (20). The limited analysis of the previously published microscopy images that was possible suggested that the histopathology is somewhat similar to that seen with the Txp40 toxin. This indicates that there are now at least four toxin proteins from *Xenorhabdus* or *Photorhabdus* that are known to cause significant damage to the insect midgut.

The Txp40 toxin also caused damage to the fat body (Fig. 5). No damage has been reported to tissues other than the midgut for the other three toxins from *Xenorhabdus* or *Photorhabdus* that have been studied. The lack of reported damage to the fat body may be due to the fact that large numbers of *Photorhabdus* bacteria are not recovered from the *M. sexta* fat body until &gt;48 h after bacterial infection (33), suggesting that damage to the fat body occurs late in the infection process. One study reported that *Xenorhabdus* bacteria disrupt the fat body, silk glands, hyphoderm, and epithelia (18). Proteins or extracts from *Xenorhabdus* and *Photorhabdus* have also been shown to damage insect hemocytes, which is consistent with observations that the bacteria manage to evade the cellular immune response of the insect (4). However, in general, reports have focused on the damage that *Xenorhabdus* or *Photorhabdus* bacteria and their toxins cause to the insect midgut.

Due to the limited amount of work on the histopathology of *Xenorhabdus* and *Photorhabdus* toxins, it is informative to examine the larger body of work for *B. thuringiensis* and its toxins. The Txp40 toxin caused a significant decrease in midgut intercellular adhesion, degradation of the peritrophic matrix lining the midgut cells, and degradation of the fat body nuclei (Fig. 3 to 5). All of these tissues have also been reported to be damaged by *B. thuringiensis* or isolated *B. thuringiensis* toxins, although the data are somewhat sparse and sometimes contradictory. Several reports indicate that treatment of insects with *B. thuringiensis* or its toxins disrupts the peritrophic matrix (12, 30). The *B. thuringiensis* toxins have been reported to damage the intercellular junctions of midgut cells of *A. aegypti* (12) and *M. sexta* (22) but not *Pieris brassicae* (24). The fat body of *H. armigera* larvae was shown to be damaged by *B. thuringiensis* (30). Thus, although many known insect toxins appear to target the insect midgut, it is clear that bacteria such as *Xenorhabdus* and *B. thuringiensis* and their toxins can also cause significant damage to other insect tissues.

The results suggest that the Txp40 toxin has multiple target sites in *H. armigera* and that the primary site of action is the midgut, although there is also damage to other tissues, such as the fat body. It is intriguing to consider how the toxin can act at several different sites yet leave sites such as the foregut and hindgut unaffected. Such tissue specificity is accompanied by activity against a range of insects but not against a mammalian cell line. An attractive hypothesis to explain these results is that the toxin targets a protein that is found in smooth septate (continuous) intercellular junctions, which are found in the insect midgut but not in mammals or other insect tissues, such as the foregut and hindgut (21). Regardless of speculation about the possible mode of action of the toxin, it is clear that Txp40 is a widely occurring and highly conserved toxin in *Xenorhabdus* and *Photorhabdus* bacteria. Txp40 is important for the broad insecticidal activity of these bacteria and is a significant component of the extensive array of toxins that the bacteria and nematodes use to destroy their insect hosts.

REFERENCES


