Interactions of Insecticidal Toxin Gene Products from *Xenorhabdus nematophilus* PMFI296

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Received 13 December 2002/Accepted 11 March 2003

Four genes on a genomic fragment from *Xenorhabdus nematophilus PMFI296* were shown to be involved in insecticidal activity towards three commercially important insect species. Each gene was expressed individually and in combinations in *Escherichia coli*, and the insecticidal activity of the lysates was determined. The combined four genes (xptA1, xptA2, xptB1, and xptC1), in *E. coli*, showed activity towards *Pieris brassicae*, *Pieris rapae*, and *Heliothis virescens*. The genes xptA1, xptB1, and xptC1 were involved in expressing activity towards *P. rapae* and *P. brassicae*, while the genes xptA2, xptB1, and xptC1 were needed for activity towards *H. virescens*. When each of these three genes was expressed individually in *E. coli* and the cell lysates were used in insect assays or mixed and then used, insecticidal activity was detected at a very low level. If the genes xptB1 and xptC1 were expressed in the same *E. coli* cell and this cell lysate was mixed with cells expressing xptA1, activity was restored to *P. rapae* and *P. brassicae*. Similarly mixing XptB1/C1 lysate with XptA2 lysate restored activity towards *H. virescens*. Individual gene disruptions in *X. nematophilus PMFI296* reduced activity to insects; this activity was restored by complementation with cells expressing either xptA1 or xptA2 for their respective disruptions or *E. coli* expressing both xptB1 and xptC1 for individual disruptions of either of these genes. The genes xptA2, xptC1, and xptB1 were expressed as an operon in PMFI296 and inactivation of xptA2 or xptC1 resulted in silencing of downstream gene(s), while xptA1 was expressed as a single gene. Therefore, the two three gene product combinations interact with each other to produce good insecticidal activity.

New insecticidal toxins with activity towards pests of commercial importance are needed for either the development of sprayable products or transgenic plants. We have previously described a group of toxins from *Xenorhabdus* species that kill lepidopteran insects. The genes responsible for this insecticidal activity were identified by screening a cosmid genomic library, expressed in *Escherichia coli*. One cosmid (CHRIM1) contained five genes related to insecticidal activity (12). One of these genes (xptA1) was central for insecticidal activity towards *Pieris brassicae*, and when expressed in *E. coli*, a low level of insecticidal activity was observed. However for full insecticidal activity a much larger region of DNA comprising xptA1 and at least two other genes, xptB1 and xptC1, was required. The precise genetic nature of toxin combinations resulting in insecticidal activity and their effect upon other insects had not been determined.

Genes homologous to those present on CHRIM1 have also been described in *Photorhabdus luminescens* (1, 10), a symbiont of entomopathogenic nematodes closely related to *Xenorhabdus* species. The requirement of three genes equivalent to xptA1, xptB1, and xptC1 in the expression of full insecticidal activity towards the model insect *Manduca sexta* (tobacco hornworm) was shown first in *P. luminescens* strain Hb (10) and later in *P. luminescens* strain W14 (13). Homologous genes to xptA1, xptB1, and xptC1 have also been identified in *Serratia entomophila* (7), where some species are the causative agent of the chronic Amber disease in the New Zealand grass grub (8). Three plasmid-encoded genes, sepA, sepB, and sepC, homologous to xptA1, xptB1, and xptC1, were all required to be expressed in the same *E. coli* cell to induce full disease symptoms (7). In this previous work individual clones of each gene expressed from *E. coli* promoters could not be obtained due to rearrangements within the constructs. In this study we have expressed four xpt genes individually in *E. coli*: xptA1 (tcdA/sepA-like; 7,841 bp; 287-kDa predicted protein), xptA2 (tcdA/sepA-like; 7,647 bp; 285-kDa predicted protein), xptB1 (iccC/sepC-like; 3,047 bp; 111-kDa predicted protein), and xptC1 (tcdB/sepC-like; 4,256 bp; 160-kDa predicted protein). We have also determined which combinations of these genes need to be expressed for activity against different insect species. Gene disruptions in *Xenorhabdus* strains have also been produced to determine if similar interactions of the gene products occur in the wild-type strain. These aspects of *Xenorhabdus* insect toxins have not been addressed in earlier work. In addition the effect of the toxin complexes on four commercial pests have been studied in this work. With the potential of these genes for use in insect control, it is important that these points are addressed. In order to achieve this each of the xpt genes from PMFI296 were cloned individually into *E. coli* under the control of the λ P1 promoter, and cloned in combinations on the same vector, or on different vectors using the P_LAC promoter in the same cell. Cells and cell lysates were used individually or mixed prior to inclusion into insect bioassays.

MATERIALS AND METHODS

Bacterial strains and culture conditions. The strain *Xenorhabdus nematophilus PMFI296* was isolated from United Kingdom soil and has been described else-
where (12). The E. coli strain GI724 and the vector pLEX were obtained from Invitrogen (Groningen, The Netherlands). Regulation of gene expression on pLEX through the incorporation of tryptophan in the media has been described previously (12). Briefly, clones were maintained on RMG medium containing 100 μg of ampicillin ml⁻¹ (Invitrogen), and expression was obtained by growth in LB broth (Merck, Darmstadt, Germany) containing 100 μg of ampicillin ml⁻¹ and 100 μg of tryptophan ml⁻¹. pBBR1MCS (9) is a broad-host-range IncP plasmid that can be maintained in the same cell as plasmids that possess coE1-derived origins such as pLEX. The plasmid pBBR1MCS also contains a lac promoter upstream of the multiple cloning site that can be used for expression of cloned genes. Clones in pBBR1MCS were obtained on LB broth containing 50 μg of chloramphenicol ml⁻¹, and induction of the lac promoter was achieved by incorporating IPTG (isopropyl-β-D-thiogalactopyranoside) (1 mM) into the growth medium.

DNA purification and subcloning. Plasmid DNA was prepared by the Qiagen (Dorking, United Kingdom) midi and Qiawell 8 (Qiagen) systems. Restriction digests were performed using the manufacturers’ recommended conditions (Boehringer Mannheim, Lewes, United Kingdom; Life Technologies, Paisley, United Kingdom) and analyzed by agarose gel electrophoresis. After digestion, DNA was purified for cloning using the Qiagen PCR product clean-up system, following the manufacturer’s recommended conditions (Qiagen). Blunt end digestion of DNA was performed using 1 U of Klenow (Life Technologies) in recommended buffer with 0.015 mM concentrations of deoxyxynucleoside triphosphates for 15 min at room temperature. The Klenow enzyme was inactivated by heating at 70°C for 10 min. All subcloning was carried out in E. coli, and DNA was electrophoresed into strains (12.5 kV cm⁻¹) using a Bio-Rad GenePulser. Clones were selected on LB or RMG plates containing the appropriate antibiotics.

Cloning and expression of toxin genes. The construction of pLex-xptA1 in plasmid pHRI808 has been described elsewhere (12) and consists of the xptA1 gene placed downstream of the lac promoter in the E. coli strain GI724, where the cl element is present on the chromosome under the control of the trp operon (Invitrogen). The plasmid pHRI808 (P<sub>Lac-xptA1</sub>) was created by cloning an 8,788-bp KpnI/EcoRI fragment, containing the entire xptA2 gene from cosmid CHRIM1, into pLEX cut with KpnI/EcoRI. The plasmid pHRI803 (P<sub>Lac-xptB1/C1</sub>) was created from p338/2-AT2-191, an AT-2 transposon insertion mutation of clone 338/2 (12). The point of AT-2 insertion was identified as 171 bp upstream of the start codon of the xptC1 gene in the following sequence GGA GAG CCT A TAT C GAG CG CCG TAG-3) and the reverse primer (TAC CTG CAG AGT TCT TTA GGC CCG TAG-3) and the reverse primer (TAC CTG CAG AGT TCT TTA GGC CCG TAG-3). Amplification was carried out as follows: 50°C for 30 s; 35 cycles of 95°C for 1 min, 55°C for 1 min, and 72°C for 3 min; and a final extension of 72°C for 5 min. The amplification product was then cut with SalI and cloned into the SalI site of the suicide vector pHRI810 described previously (12). The suicide gene cassette was then inserted into the insecticidal gene present on the suicide plasmid. Figure 2 shows the enzyme sites used for the suicide cassette insertion in each gene. The E. coli strain ED6854 harboring PnJS000 (6) was then transformed with the appropriate gene disruption plasmid. Colonies were selected on LB agar containing kanamycin (50 μg ml⁻¹) and chloramphenicol (25 μg ml⁻¹).

Construction of a suicide plasmid for X. nematophilus. In order to insert the kanamycin resistance gene cassette into the X. nematophilus PMFI296 chromosome, an appropriate suicide vector was constructed using the suicide gene. The suicide gene codes for levan sucrose and is lethal in many gram-negative bacteria in the presence of 5% (wt/vol) sucrose (4). The gene was amplified from Bacillus subtilis 168 chromosomal DNA using the forward primer (5'-TAC CTG CAG ATG TCT TTA GGC CCG TAG-3'), which incorporated PstI digestion sites into the PCR product. Amplification was carried out as follows: 95°C for 30 s; 35 cycles of 95°C for 1 min, 55°C for 1 min and 7°C for 3 min; and a final extension of 72°C for 5 min. The amplified product was then cut with PstI and cloned into the PstI site of pBBR1MCS to form plasmid pHRI810. Transformants containing pHRI810 were selected on LB plates containing kanamycin (50 μg ml⁻¹) and chloramphenicol (25 μg ml⁻¹).

Gene disruptions in Xenorhabdus. The insecticidal genes of X. nematophilus PMFI296 (xptA1, xptA2, xptB1, and xptC1) were disrupted by insertion of a kanamycin resistance cassette (Pharmacia) into each gene. The first step in this process involved cloning the whole or part of the appropriate insecticidal gene into the multiple cloning site of the suicide vector pHRI810 described previously. The kanamycin resistance cassette was then inserted into the insecticidal gene present on the suicide plasmid. Figure 2 shows the enzyme sites used for the kanamycin cassette insertion in each gene. The E. coli strain ED6854 harboring PnJS000 (6) was then transformed with the appropriate gene disruption plasmid. Colonies were selected on LB agar containing kanamycin (50 μg ml⁻¹). Colonies were grown in LB medium containing kanamycin (50 μg ml⁻¹) at 37°C for 18 h
and diluted 1/100 into fresh medium. The cultures were incubated at 37°C until an optical density at 600 nm of 0.5 was reached. A 25-ml aliquot was centrifuged (4,000 × g for 10 min at 15°C), and the pellet was washed in 25 ml of LB. The recipient strain was prepared for mating in a similar way. X. nematophila PMF1296 was grown in 5 ml of LB at 30°C overnight. Cells were collected and washed with 25 ml of LB by centrifugation (4,000 × g for 10 min at 15°C). Both cell pellets were resuspended in 0.2 ml of LB. A subsample, 0.1 ml of each, was mixed and pipetted onto 1.4-cm diameter, 0.2-μm-pore-size nitrocellulose filters that had been placed on LB plates. Samples were incubated for 16 h at 30°C. Growth on the filter was scraped into 1 ml of LB and vortexed to resuspend the cells. Transconjugates were selected by plating these cells onto LB agar containing 100 μg of ampicillin ml⁻¹ and 50 μg of kanamycin ml⁻¹, taking advantage of Xenorhabdus species natural resistance to ampicillin. The X. nematophila PMF1296 strains containing each gene disruption plasmid were grown in 5 ml of LB containing 25 μg of kanamycin ml⁻¹ for 16 h at 30°C, and dilutions were plated on LB agar containing 25 μg of kanamycin ml⁻¹ and 5% (wt/vol) sucrose.

After incubation at 30°C for 48 h, colonies that grew through the sucrose selection were screened for plasmid loss by testing for chloramphenicol resistance. Colonies that were chloramphenicol sensitive were characterized by Southern blot analysis to confirm insertional inactivation within the correct gene. For the Southern blot, DNA was obtained from the strains using Qiagen chromosomal DNA kit, restricted with EcoRI and HindIII, and probed with digoxigenin-labeled PCR products approximately 500 bp in length that corresponded to the inactivated gene. The probes were chosen such that different size fragments would be highlighted for HindIII- and EcoRI-restricted DNA from the wild type, the insertion mutant, and the suicide plasmid. In each case the Southern blot revealed a single band corresponding to the correct predicted size for the insertion mutant (data not shown).

Insecticidal assays. Initial bioassays to assess activity of E. coli clones against P. brassicae involved growing the strains in 50 ml of LB for 16 h. Cells were harvested by centrifugation of 1 ml of culture (13,000 × g for 2 min), the supernatant was removed, and the pellet was resuspended in 1 ml of PBS (10 mM phosphate buffer, pH 7.4; 2.7 mM KCl; 137 mM NaCl). The cells were lysed by sonication on ice for 20 s, and the potency of the lysate or a mixture of lysates was tested in incorporation assays. These were performed by spreading 50 μl of lysate onto agar based artificial diet (3) which contained streptomycin (20 μg ml⁻¹) and cefotaxime and tetracycline (each at 100 μg ml⁻¹) in plastic containers (diameter, 4.5 cm). After the surface had dried, 10 larvae were added and containers were incubated at 25°C (16-h day-length period) and relative humidity of 80%. Recordings of larval mortality were taken after 24 h. A positive result was scored if all larvae were dead, and a negative result if no larvae had died. In the assays negative controls included treatments with just buffer (PBS) and E. coli cells containing pLEX. More detailed bioassays to study the activity of X. nematophila PMF1296, disruption mutants, and E. coli clones against neonate P. brassicae, Heliotis virescens, and Plutella xylostella, as well as E. coli clones against Pieris rapae larvae were performed as follows. Cell samples of X. nematophila PMF1296 and the disruption mutants were each prepared from cultures grown for 72 h at 25°C on eight 9-cm-diameter petri plates containing L agar. Cells were harvested, suspended in 200 ml of PBS, washed by centrifugation at 6,000 × g for 10 min, and resuspended in 15 ml of 5% (wt/vol) lactose. Cell suspensions were then frozen at 70°C for 4 h and freeze-dried at −60°C for 48 h. For E. coli, cells were cultured in 200 ml of LB containing 50 μg of ampicillin ml⁻¹ for 40 h at 30°C. Cells were harvested by centrifugation, washed once in PBS, and suspended in 8 ml of PBS. The cells were then lysed by sonication using three bursts of 20 s at 18°C. To the cell lysate, 8 ml of 10% lactose (wt/vol) was added, and the resulting cell suspension was frozen and freeze-dried as described above. To measure the amount of total protein present in the freeze-dried samples the bichinchoninic acid protein assay kit (Pierce, Rockford, Ill.) was used, and the manufacturer’s instructions were followed. The amount of XptA1, XptA2, and XptC1 in the material was calculated by analyzing the samples by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and calculating the relative intensity of the appropriate toxin band to the sum of all the other bands using Phoretix 1D Analysis Software (version 4.01; BioGene). The same freeze-dried samples were used in all experiments to ensure the amount of toxin protein in the samples remained constant. To determine the potency of the bacterial samples each was tested in triplicate using multidose assays on artificial diet. These were performed using a series of five dilutions for each sample. For each dilution, 50-μl duplicates of bacterial suspension was tested against P. brassicae, P. rapae, and P. xylostella in spread assays as already explained. For H. virescens, four containers per dilution were used, to which five larvae were added. Recordings of P. brassicae, P. rapae, and P. xylostella larval mortality were taken after 6 days. For H. virescens, activity was measured as reduction in larval weight compared to that of untreated controls after 5 days of growth at 25°C. In all the assays, negative controls included treatments with just PBS. The results of the assays were evaluated by Logit transformation using Genstat (5th edition; VSN International Ltd.) to determine the 50% lethal concentration (LC50) and the concentration required to cause a 50% reduction in larval weight compared to an untreated control (EC50).

SDS-PAGE of cell proteins. E. coli clones were grown in induction media for 16 h and cells were harvested by centrifugation at 13,000 × g for 5 min. The cell pellet was resuspended in one-eighth of the original volume of PBS. Samples (5 μl) were added to equal volumes of 2× SDS-PAGE loading buffer (100 mM Tris HCl, pH 6.8; 1% mercaptoethanol; 4% SDS, 20% glycerol; 0.2% bromophenol blue) before being placed in a boiling water bath for 5 min. Samples were then loaded onto 5 to 8% gradient gels (Novex, San Diego, Calif.), which were run at 150 V for 1.5 h. Gels were stained with 0.25% (wt/vol) Coomassie brilliant blue in 40% (vol/vol) methanol-10% (vol/vol) acetic acid for 1 h and destained for 3 h.

**RESULTS**

Expression of genes. Four genes present on the CHRIM1 cosmid, xptA1, xptA2, xptB1 and xptC1 were cloned into expression vectors. When gene expression was induced, the constructs PxptA1-xptA1, PxptA2-xptA2 and PpxptC1-xptC1 were expressed at high levels as judged by distinct bands on Coomassie blue stained SDS-polyacrylamide gels (Fig. 3). Each band was N-terminally sequenced and in each case the first 10 amino acids matched exactly with the predicted open reading frames (ORFs) of xptA1, xptA2, and xptC1 genes, respectively. The predicted size of xptB1 is 110 kDa, but no protein of this size (or any other size) could be detected when xptB1 was expressed alone or in the presence xptC1 (lanes 4 and 6). Although the XptC1 protein band visible in PpXptB1/xptC1 constructs (lane 6) was less intense than when xptC1 was expressed alone, it was confirmed to be xptC1 by N-terminal sequencing of the first 10 amino acids. The predicted XptB1 protein contains repeating peptide motifs termed YD repeats (14), which in certain proteins have been shown to bind to high molecular mass carbohydrates preventing the protein from being detected by SDS-PAGE (11). To see whether this was the case with xptB1, E. coli
cells expressing xptB1 alone and in conjunction with xptC1 were sonicated directly in 8 M urea and loaded onto SDS-polyacrylamide gels, but still no protein could be detected. However, when analyzing lysates from E. coli containing P<sub>L</sub>-xptB1/C1 or P<sub>L</sub>-xptB1/H11001 constructs using Mono-Q ion-exchange fast-performance liquid chromatography (Pharmacia), fractions where the visible XptC1 protein dominated contained a second prominent protein of approximately 65 kDa, whose N-terminal sequence (10 amino acids) matched that of the predicted XptB1 protein. Therefore, a truncated XptB1 protein is produced in these constructs which is difficult to detect. The P<sub>L</sub> clones showed expression patterns similar to those of the P<sub>L</sub> constructs when examined by SDS-PAGE, with both GI724(P<sub>L</sub>-xptB1/C1) and GI724(P<sub>L</sub>-xptB1/H11001) expressing XptC1, albeit at about 10-fold-lower levels due to the weaker P<sub>L</sub> promoter. As with the P<sub>L</sub> constructs, no XptB1 product could be detected by SDS-PAGE.

**Interaction of gene products for insecticidal activity.** Initial studies involved elucidating which genes were involved in activity against *P. brassicae* (Table 1). The *E. coli* strain GI724 (P<sub>L</sub>-xptA1 + P<sub>L</sub>-xptB1/C1) was shown to exhibit insecticidal activity toward *P. brassicae* (all larvae were dead after 48 h using undiluted lysate from overnight cultures), but individually P<sub>L</sub>-xptA1 and P<sub>L</sub>-xptB1/C1 showed no activity (no larvae dead after 48 h). Thus, interaction between the xptA1 and xptB1/C1 genes was essential for activity. This interaction need not occur in vivo as exhibited by the toxic activity of mixed lysates of P<sub>L</sub>-xptA1 and P<sub>L</sub>-xptB1/C1. In addition, mixing of the lysates from P<sub>L</sub>-xptA1 and either P<sub>L</sub>-xptC1 + P<sub>L</sub>-xptB1/H11001 or P<sub>L</sub>-xptB1/H11001 + P<sub>L</sub>-xptC1/H11001 also resulted in insecticidal activity. Therefore the xptC1 and xptB1 genes do not need to be colinear in order to interact. However, both genes do need to be expressed in the same cell, as shown by lack of insecticidal activity when xptB1 and xptC1 were expressed in separate cells (Table 1).

**Effect of gene products on different insect species.** Once the correct combination of genes required for toxicity had been elucidated more detailed studies on a range of Lepidoptera were performed. The effects of GI724(P<sub>L</sub>-xptA1) and GI724 (P<sub>L</sub>-xptA2) alone and in conjunction with P<sub>L</sub>-xptB1/C1 against different species of insect are shown in Table 2. The data show that *E. coli* strains expressing xptA1 when mixed with strains expressing xptB1 and xptC1 showed activity towards *P. brassicae* and *P. rapae*, whereas xptA2 in conjunction with xptB1 and xptC1 showed significant activity toward *H. virescens*. These results clearly illustrate that the xptA1 and xptA2 genes exhibit a different spectrum of activity, but both require the xptB1 and xptC1 gene products in order to produce full insecticidal activity.

**Activity of strains with gene disruptions.** Table 3 shows the results of insecticidal assays involving strains of *X. nematophila* PMF1296 with gene disruptions. A mutant with the xptA1 gene disrupted by a kanamycin cassette, PMF1296 (xptA1::kan), showed 10% of the activity toward *P. brassicae* than the PMF1296 wild type, but activity could be restored by adding XptA1 expressed in *E. coli*. This confirmed that XptA1 protein is essential for activity towards this pest. PMF1296 (xptB1::kan) and PMF1296 (xptC1::kan) strains also exhibited substantially less activity than the wild type to *P. brassicae*, but activity could not be restored by adding lysate from *E. coli* expressing either xptB1 or xptC1 singly. PMF1296 (xptB1::kan) and PMF1296 (xptC1::kan) strains could be complemented with *E. coli* strains expressing both xptC1 and xptB1 together. These results supported the earlier findings that the xptB1 and xptC1 genes need to be in the same *E. coli* cell in order to be able to complement xptA1. Similarly both genes have to be expressed within the same cell in *Xenorhabdus* strains.

The results from PMF1296 (xptA2::kan, xptC1::kan, or xptB1::kan) mutants confirmed, as expected, that the contiguous genes xptA2, xptC1 and xptB1 were expressed as a single polycistronic operon within the wild-type strain. The kanamy-

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<th>Gene(s)</th>
<th>LC&lt;sub&gt;50&lt;/sub&gt;/ng of toxin for:</th>
<th>ED&lt;sub&gt;50&lt;/sub&gt;/ng of toxin for</th>
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<td></td>
<td><em>P. brassicae</em></td>
<td><em>P. rapae</em></td>
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<tr>
<td>xptA1&lt;sup&gt;a&lt;/sup&gt;</td>
<td>1,170 (740–1,850)&lt;sup&gt;b&lt;/sup&gt;</td>
<td>&gt;5,000</td>
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<td>xptA2&lt;sup&gt;a&lt;/sup&gt;</td>
<td>&gt;5,000</td>
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<td>xptB1&lt;sup&gt;a&lt;/sup&gt;</td>
<td>&gt;5,000</td>
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<tr>
<td>xptA1 + xptB/C&lt;sup&gt;c&lt;/sup&gt;</td>
<td>3.20 (2.13–5.76)</td>
<td>45.1 (31.5–64.4)</td>
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<tr>
<td>xptA2 + xptB/C&lt;sup&gt;c&lt;/sup&gt;</td>
<td>306 (195–481)</td>
<td>&gt;5,000</td>
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<tr>
<td>xptA1 + xptA2 + xptB/C&lt;sup&gt;c&lt;/sup&gt;</td>
<td>5.85 (3.90–9.36)</td>
<td>93.5 (65.5–133)</td>
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<sup>a</sup> Some reduction in size.

<sup>b</sup> 95% confidence limits are given in parentheses.

<sup>c</sup> Data reported as LC<sub>50</sub> or ED<sub>50</sub> per ng of toxin (XptA1, XptA2, or XptC1) present in the lysate. The amount of XptA1, XptA2, and XptC1 in each lysate as a percentage of total protein was protein was 4.7, 4.6, and 2.8, respectively. XptB1 was below detectable levels, and therefore its concentration was not used in the calculations.

<sup>d</sup> Data reported as LC<sub>50</sub> or ED<sub>50</sub> per ng of combined toxins present in the lysate. The ratio of XptA2/A1 to XptC1 in the mixed lysate was approximately 1.6:1, and that for XptA1 to XptA2 to XptC1 was approximately 1.6:1.6:1.
cin cassette was constructed with a transcriptional terminator, which when inserted into a gene would result in truncation of a polycistronic transcript. In this approach it would lead to transcription of downstream genes. As such, an xptA2 insertion would silence xptC1 and xptB1, while an xptC1 insertion would silence xptB1. PMFI296 (xptA2::kan) mutants showed reduced activity toward P. brassicae, but results obtained from E. coli expressing xptA2 (Table 2) showed this gene was not involved in activity toward this insect. The addition of XptC1/B1, but not XptA2 to PMFI (xptA2::kan) strains restored activity against P. brassicae to wild-type levels. These results confirmed that xptA2, xptC1 and xptB1 were expressed as a polycistronic transcript.

PMFI (xptA1::kan) mutants showed activity comparable to the wild type against H. virescens, confirming that this gene was not involved in activity towards this insect, and that lack of expression of XptA1 did not change expression or activity of xptA2, xptC1, and xptB1. However xptA2 and xptB1xptC1 disruptions reduced significantly the activity towards H. virescens. Addition of XptA2 to PMFI296(xptA2::kan) strains did not restore insecticidal activity to H. virescens confirming previous results that xptC1 and xptB1 were also silenced. Insecticidal activity for strains with xptC1 and xptB1 gene disruptions could be complemented with XptC1/XptB1 produced in the same E. coli strain. However, insecticidal activity could not be restored by adding lysate from E. coli expressing singly either xptB1 or xptC1, for their respective gene disruptions. These results follow the same pattern that was observed for activity of PMFI xpt disruption mutants against P. brassicae.

**DISCUSSION**

This study shows that for full insecticidal activity, as for homologous genes in S. entomophila and P. luminescens, interactions between the products of three xpt genes (xptA, xptB, and xptC) are required. Also, for the first time, xptA- and xptC-like genes have been individually expressed in E. coli and an xptB1-like protein fragment has been detected. Previous attempts to express xptA-like genes from P. luminescens in E. coli have proved unsuccessful. Plasmids containing only the tcdB gene from P. luminescens could not be constructed due to rearrangement (13) and plasmids containing the sepA from S. entomophila were unstable in E. coli, suggesting that the SepA protein is detrimental to the growth of the host bacterium (7). The successful cloning of xptA1 and xptA2 in these studies may be due to different properties of these genes compared to sepA and tcdB, or more likely it is due to the tight regulation of their expression in the pLEX constructs. If the XptA1 and XptA2 proteins are deleterious to the host cell, then the fact that they are not expressed under regulated growth conditions in the pLEX system, would remove the pressure for rearrangements.

Also, importantly, the effect of these genes on two commercial pests, P. rapae and H. virescens, has been elucidated, and it has been found that different spectrums of activity can be achieved by substituting different xptA genes with the same xptB1-xptC1 construct. This is important because to date, the effect of the toxin genes of Photorhabdus and Serratia have only been studied in detail on the model insect M. sexta and on Costelytra zealandica, which is a pest of New Zealand grasslands. Further adding to our understanding of xpt like genes are the findings that the interaction between XptA1 and XptB1/C1 can occur in vitro by mixing cell lysate, but the interaction of the xptB1 and xptC1 genes requires their expression in the same bacterial cell.

Interestingly, disruption of the xptC1, xptB1, or xptA2 genes reduced the activity of PMFI296 mutants against P. xylostella by more than 30-fold (Table 3). However, when lysate from an E. coli expressing xptA2 was mixed with a lysate of E. coli expressing xptB1 and xptC1 no activity towards P. xylostella was observed (Table 2). Therefore, there is possibly another unidentified insecticidal toxin gene in PMFI296, which requires XptB1/XptC1 proteins for activity and is responsible for activity towards P. xylostella. Alternatively the xptA2 gene may be responsible for such activity but is inactive in E. coli due to lower levels of expression or other factors present in Xenorhabdus but lacking in E. coli. The latter is more likely since xptA2 is highly expressed in P1-xptA2 constructs.

Previous work on XptA-like toxins in P. luminescens showed that single purified XptA1 like proteins were sufficient for toxicity (5), but when the genes coding for these proteins were expressed in E. coli, insect activity was not conferred (1). In light of the present work and other research (13), one explanation for this could be that tiny undetectable amounts of XptC1 and XptB1 like proteins may have contaminated the protein preparation in these protein studies, and contributed to the toxicity observed. Alternatively, processing of the XptA1 protein by XptC1 and XptB1 like proteins may have occurred in the Photorhabdus cell before purification. Therefore, the proteins purified represent the processed or active forms of XptA1.

From the results presented here, processing of an inactive XptA type protein by the XptB1/C1 complex to produce an active XptA protein remains a possible theory. The fact that

**TABLE 3.** The activity of *X. nematophilus* gene knockout mutants and cloned toxin genes found to supplement insect activity

<table>
<thead>
<tr>
<th>Strain or mutant</th>
<th>LC50 (µg of protein per cm² of diet) vs:</th>
<th>ED50 (µg of protein per cm² of diet) vs:</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td><em>P. brassicae</em></td>
<td><em>P. brassicae</em> + suppl.*</td>
</tr>
<tr>
<td>PMFI296</td>
<td>0.04 (0.02–0.05)</td>
<td>0.96 (0.62–1.38)</td>
</tr>
<tr>
<td>A1::Kan</td>
<td>0.54 (0.39–0.83)</td>
<td>0.06 (0.05–0.09) [A1]</td>
</tr>
<tr>
<td>A2::Kan</td>
<td>0.65 (0.49–0.87)</td>
<td>0.04 (0.29–0.06) [B/C]</td>
</tr>
<tr>
<td>C1::Kan</td>
<td>1.65 (1.27–1.93)</td>
<td>0.13 (0.09–0.17) [C/B]</td>
</tr>
<tr>
<td>B1::Kan</td>
<td>2.05 (1.64–2.54)</td>
<td>0.15 (0.13–0.19) [B/C]</td>
</tr>
</tbody>
</table>

* Knockouts mutants of *X. nematophilus* mixed with extracts of E. coli containing cloned toxin genes (in brackets), found to supplement insect activity. A1, XptA1; A2, XptA2; B/C, XptB1/C1.
* Values in parentheses are 95% confidence limits.
XptA1 shows slight toxic effects on its own, alongside the fact that purified homologues from \textit{P. luminescens} W-14 are active supports this theory. The different spectrum of activities exhibited by XptA1 and XptA2 is also consistent with this theory. If this is the case then it seems likely that the XptB1/C1 complex is capable of activating a range of XptA-like molecules with differing spectrums of activity. Such activation of any XptA1 like proteins may not be easy to detect because SDS-PAGE and matrix-assisted laser desorption-ionization time-of-flight analysis showed that the size of the active purified TcaA and TcaB proteins were equal to the size predicted by their ORFs (5). In our experiments no differences in the protein pattern of XptA1 was observed when expressed alone, or in the presence of XptB1/C1. Therefore, protease activity or any covalent modification by the XptB1/C1 complex that increases or decreases the size of the XptA1 protein significantly, is unlikely.

Coexpression of \textit{tdcA} (xptA-like) and \textit{tdcB} (xptC-like) in \textit{E. coli} resulted in formation of a phage-like structure, visible in toxic particulate preparations. Expressing \textit{tccC} (xptB-like) in the same cell as \textit{tdcA} and \textit{tdcB} does not alter these structures but renders them orally toxic (13). We have looked at crude lysates from recombinant \textit{E. coli} and have been unable to observe these structures. However, our expression constructs, unlike those used for expressing \textit{tdcA} and \textit{tdcB}, do not contain phage-like genes such as \textit{lysR}. In addition, our cultures are also not induced with UV, which may result in expression of endogenous prophage proteins. Both of these factors may aid in the formation of phage-like structures seen with recombinant \textit{tdcA} and \textit{tdcB}. If formation of these phage-like structures is essential for toxicity, then such formation would have to take place outside the cell since xptA1/xptA2 and xptB1/C1 lysates can be mixed in vitro to produce insect activity.

In this study the interactions seen with \textit{E. coli} expressed proteins was reflected in those expressed in the wild type \textit{Xenorhabdus} strain. This moves the interactions recorded away from transcriptional and translational interactions, to posttranscriptional modifications or protein interactions. These interactions are not additive. This study showed that xptA2, xptC1, and xptB1 were expressed as a single polycistronic message, and xptA1 was expressed independently. Disruptions in xptA2 using a trimethoprim gene cassette with no transcriptional terminator allowed the expression of the downstream genes xptC1 and xptB1, and maintenance of activity against \textit{P. brassicae} (12). In this study, disruption of xptA2 with a kanamycin resistance gene cassette with a transcriptional terminator, prevented downstream expression of xptC1 and xptB1 and loss of insecticidal activity. Therefore in \textit{Xenorhabdus}, the genes xptA2, xptA1, and xptC1 are colocalized and are expressed on a polycistronic message from promoters that also function in \textit{E. coli}. In \textit{P. luminescens} W14, the genes \textit{tcb}, \textit{tdc}, \textit{tca}, and \textit{tca} are clearly different in this regard, since they are distributed separately over the chromosome, and expressed individually from inducible phage-like promoters (2). They are also expressed in an uncoordinated fashion at different times during growth. In \textit{Serratia} although the \textit{sep} genes are more tightly grouped, an ORF still separates \textit{sepB} and \textit{sepC} and there is no xptA1-like gene equivalent in the arrangement. Thus, the arrangement of these genes in \textit{Xenorhabdus} may represent a more evolved structure, where over time distal mobile genes which are able to interact for a beneficial effect, i.e., insect toxicity, have, through numerous integration events, formed a tightly integrated unit.

Clearly questions remain relating to how xpt gene products interact to form an active toxin. Since XptA1 and XptA2 individually and XptB1 and XptC1 combined show a low level of insecticidal activity, they may all be active toxins. In this way the interaction of the toxins, either before they come into contact with the target cell, or after this interaction, could result in an effect which kill insects quicker. However, the fact that we now have individual proteins expressed at high levels in \textit{E. coli} and some combinations are active when mixed in vitro, this will help answer some of these questions. Further studies on purified proteins expressed in \textit{E. coli} and their interactions are needed.

### REFERENCES


