Insecticidal Toxin Complex Proteins from Xenorhabdus nematophilus: Structure and Pore Formation

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Toxin complexes from Xenorhabdus or Photorhabdus spp. bacteria represent novel insecticidal proteins. We have purified a native toxin complex (toxin complex 1) from Xenorhabdus nematophilus. The toxin complex is composed of three different proteins; XptA2, XptB1, and XptC1 representing products from class A, B and C toxin complex genes, respectively. We show that recombinant XptA2, and co-produced recombinant XptB1 and XptC1 bind together with a 4:1:1 stoichiometry. XptA2 forms a tetramer of ~1,120 kDa that binds to solubilized insect brush border membranes, and induces pore formation in black lipid membranes. Co-expressed XptB1 and XptC1 form a tight 1:1 binary complex where XptC1 is C-terminally truncated resulting in a 77 kDa protein. The ~30 kDa C-terminal cleaved portion of XptC1 apparently only loosely associates with this binary complex. XptA2 has only modest oral toxicity against lepidopteran insects, but as a complex with co-produced XptB1 and XptC1 has high levels of insecticidal activity. Addition of co-expressed class B (TcdB2) and class C (TccC3) proteins from Photorhabdus luminescens to the Xenorhabdus XptA2 protein results in formation of a hybrid toxin complex protein with the same 4:1:1 stoichiometry as the native Xenorhabdus toxin complex 1. This hybrid toxin complex, like the native toxin complex, is highly active against insects.

Xenorhabdus and Photorhabdus spp. are two bacterial genera belonging to the family Enterobacteriaceae, known to be associated with entopathogenic nematodes (1-4) These bacteria represent potential sources for new genes encoding potent insecticidal toxins that could be put into plants as alternatives to Bacillus thuringiensis genes (5). Gene sequence analysis of Xenorhabdus and Photorhabdus bacteria show that these organisms contain a family of related toxin complex (tc) genes located at different loci (6-9). The toxin complexes are composed of three different classes of protein components, which, according to ffrench-Constant, can be categorized as class A, B, and C proteins based upon sequence similarity and size (10;11). Class A proteins are very large, having a molecular mass of ~280 kDa. Class B proteins are ~170 kDa, and class C proteins are ~110 kDa. There are many different varieties of class A, B, and C proteins in both gram negative and gram positive bacteria (12-15).

From earlier studies it has been suggested that class A proteins harbors the cytotoxic effects of the Tc toxins, whereas class B and C proteins rather modulate and enhance the toxicity of class A proteins (16). However, recently, we elucidated the molecular mechanism of the Photorhabdus luminescens Tc complex, which consists of the class A protein TcdA1, the class B protein TcdB2 and the class C proteins TccC3 or TccC5 (17). These studies revealed that the class C proteins harbor the biological activity. It was shown that TccC3 and TccC5 are ADP-riboosyltransferases, which target the actin cytoskeleton by modification of actin and Rho GTPases, respectively (17). Moreover, these studies suggested that the class A protein TcdA1 of P.
*P. luminescens* is most likely involved in the uptake of the enzyme component into target cells (18). Here we analyzed the structure and stoichiometrical composition of a toxin complex from *Xenorhabdus nematophilus*, which is largely related to the Tc complex from *P. luminescens* mentioned above. We found that native *Xenorhabdus* toxin complex 1 is composed of three proteins (XptA2, XptB1 and XptC1), representing class A, B, and C proteins combined in a respective 4:1:1 stoichiometry. Using individual purified recombinant protein components of the *Xenorhabdus* and *Photorhabdus* toxin complexes, we demonstrate that a fully active toxin complex requires the presence of all three class A, B and C proteins, and that class B and C proteins from *Photorhabdus* (TcdB2 and TccC3) can substitute for the B and C proteins from *Xenorhabdus* to form an active hybrid toxin complex that has greater insecticidal activity than the native toxin complex.

**EXPERIMENTAL PROCEDURES**

**Purification and Characterization of Native *Xenorhabdus* Toxin Complexes** - Cell pellets obtained from a 2 liter culture after overnight incubation of the *Xenorhabdus nematophilus* bacteria were suspended in 50 mM Tris-HCl pH 8.0, 100 mM NaCl, 1mM DTT, 10% glycerol and lysozyme (0.6 mg/ml). A small amount of glass beads (0.5 mm dia.), was added and cells disrupted by sonication. Broken cells were then centrifuged at 48,000 x g for 60 min. at 4 °C, supernatant collected, a bacterial protease inhibitor cocktail added (Sigma, St. Louis), and the solution dialyzed against 25 mM Tris-HCl, pH 8.0 overnight. The protein was then loaded onto a Q Sepharose XL (1.6 x 10 cm) anion exchange column. Bound proteins were eluted using a linear 0 to 1 M NaCl gradient in 10 column volumes. The high molecular weight toxin complexes eluted in the early fractions and were concentrated and loaded onto a Superose 200 size exclusion column (1.6 x 60 cm), (Pharmacia) using 50 mM Tris-HCl, 100 mM NaCl, 5% glycerol, 0.05% Tween-20, pH 8.0. The large molecular weight proteins eluting from the column were brought to 1.5 M ammonium sulfate concentration and loaded onto a phenyl Superose (0.5 x 5 cm) hydrophobic-interaction column. Proteins were eluted using a decreasing linear gradient of 1.5 to 0 M ammonium sulfate in 25 mM Tris-HCl, pH 8.0 over 20 column volumes. The toxin complexes eluted together as a broad peak at low salt concentration. The proteins were dialyzed overnight against 25 mM Tris-HCl, and loaded onto a high resolution MonoQ (0.5 x 5 cm) anion exchange column. Two separate toxin complexes were resolved with baseline resolution using a linear gradient of 0 to 1 M NaCl in 25 mM Tris-HCl obtained in 20 column volumes (Figure 1). The proteins were identified by N-terminal amino acid sequencing and Matrix-Assisted Laser Desorption/Ionization Time-of-Flight (MALDI-TOF) mass spectrometry analysis. Purification of recombinant XptA2, and co-expressed XptB1 + XptC1, and TcdB2 + TccC3 were done using similar chromatographic procedures.

**Relative Mass Comparison by Size Exclusion Chromatography (SEC)** - XptA2 (0.5 mg/ml) was incubated overnight at 4°C with the following: 1) XptB1 + XptC1 (0.5 mg/ml each) in running buffer consisting of 25 mM Tris-HCl pH 8.0, 5% glycerol, 0.05% Tween-20, or 2) an equal volume of running buffer only (control). To subsequently separate XptA2 from the unbound B and C proteins, the mixtures were applied to a Superdex 200 10/30 gel filtration column (AP Biotech, Piscataway, NJ).

**Electrophoresis-** Analysis of proteins was done by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) (19) using 4-20% Tris-Glycine polyacrylamide gels (BioRad, Hercules, CA). Native-PAGE was conducted for the electrophoretic mobility shift assays (EMSA), using precast NuPAGE® Novex 3-8% Tris-Acetate gels (Invitrogen, Carlsbad, CA) and applying 150 volts for 3 h.

**Surface Plasmon Resonance** - The binding of proteins was measured by Surface Plasmon Resonance (SPR) using a BiaCore 3000 instrument. Briefly, the proteins were immobilized onto the surface of a dextran/gold CM-5 or CM-4 Biacore chip following the manufacturer’s recommended amine coupling procedure employing 1-ethyl-3-(3-dimethylaminopropyl)-carbodiimide hydrochloride (EDC) and N-hydroxysuccinimide (NHS). Remaining free reactive esters were blocked with ethanolamine. For the analysis, the buffer flow rate was 30 µl/min, using HPS-EP buffer (BiaCore). Association of XptB1 - XptC1 with the immobilized XptA2 was measured for 200 seconds, and dissociation also measured for 200 seconds by flowing buffer in the absence of XptB1-XptC1 protein over the immobilized XptA2. A “blank” surface was prepared using EDC and NHS and
blocking with ethanolamine using the same procedure as describe above, but without any protein. Signals from the “blank” surface were subtracted from the signal from the surface containing the immobilized proteins.

For measurement of binding to insect gut binding proteins, brush border membrane vesicles were prepared by method of Wolfersberger (20) from last instar Heliothis zea insect larvae. The vesicles solubilized with CHAPS detergent (2% final) in 20 mM Tris, pH 7.5, 1 mM EDTA, 1 mM MgSO₄, 0.01% NaN₃, and 10% glycerol with protease inhibitors. Following 1-h gentle mixing at 4 °C, the mixture was centrifuged for 1 h at 100,000 x g at 4 °C, the supernatant collected, filtered through a 0.2 μm membrane and loaded onto a MonoQ (0.5 cm dia., 5 cm long) anion exchange column (Pharmacia) equilibrated in solubilization buffer containing 1% CHAPS. The proteins were eluted with a linear gradient from 0 to 500 mM KCl in solubilization buffer containing 1% CHAPS. Samples were kept at 4 °C and used the day of preparation. XptA2 was immobilized on a CM-5 chip and each fraction tested for binding. The fraction showing the strongest binding was then immobilized onto a CM-4 chip and various concentrations of XptA2 flowed over to measure binding.

**Insect Bioassays**—Proteins were tested for insecticidal activity in bioassays conducted with neonate lepidopteran larvae on artificial insect diet. Helicoverpa zea, Heliothis virescens, larvae came from a colony maintained by a commercial insectary (Benzon Research Inc. Carlisle, PA). The bioassays were conducted in 128-well plastic trays (C-D International, Pitman, NJ). Each well contained 1.0 mL of multi-species lepidoptera diet (Southland Products, AR). A 40 μL aliquot of sample was delivered by pipette onto the 1.5 cm² diet surface of each well (26.7 μL/cm²). Eight wells were treated for each insect, per replicate. Within a few hours of eclosion, individual larvae were deposited on the treated diet. The infested wells were then sealed and held under controlled environmental conditions (28°C, ~40% RH, 16:8 [L:D] photoperiod). After 5 days, the total number of insects exposed to each protein sample, the number of dead insects, and the weight of surviving insects were recorded. Average weight and standard error (95% confidence interval) for the insects were calculated from these data. A logistic two-parameter regression was used to fit growth inhibition to log-transformed protein concentration; GI50 (concentration required to reduce growth by 50%) values and 95% confidence intervals were estimated for each insect strain treated with each protein. All analyses were conducted in JMP 9.0 (SAS Institute Inc., Cary, North Carolina).

**Black Lipid Bilayer Experiments**—The method used for preparation of black lipid bilayers has been described previously (21;22). The experimental setup consisted of a Teflon chamber divided into two compartments by a thin wall and connected by a small circular hole with a surface area of about 0.4 mm². The aqueous solutions on both sides of the membrane were buffered with 10 mM MES-KOH to pH 6. Membranes were formed by spreading a 1% solution of diphytanoyl phosphatidylincholine dissolved in n-decane across the hole. After the membranes turned black, XptA1 was added to one side of the membrane, the cis side. The temperature was maintained at 20 °C during experiments. Membrane conductance was measured after application of a fixed membrane potential by using a pair of Ag/AgCl electrodes (with salt bridges) connected in series to a voltage source and a homemade current amplifier made with a Burr Brown operational amplifier. The amplified signal was monitored on a storage oscilloscope (OWON, Chorley, England) and recorded on a strip chart recorder (Rikadenki, Freiburg, Germany).

**RESULTS AND DISCUSSION**

**Purification of the native toxin complex**

Fractionation and purification of large molecular mass proteins from Xenorhabdus nematophilus cells resulted in the isolation of multiple proteins that eluted in two baseline separated peaks in the final anion exchange chromatography step (Figure 1A). Analysis of the proteins contained in the first eluting peak by SDS-PAGE showed it to consist of three major protein components, of 280, 170, and 77 kDa, and one minor protein component of approximately 30 kDa (Figure 1B). The 280 kDa band exhibited considerably greater staining intensity by Coomassie Blue dye compared to the other protein bands, even when taking into consideration its greater mass. Densiphotometric analysis of the three major bands, when adjusted for protein size, indicates that the ratio of the 280, to 170 to 77 kDa protein bands is 4:1:1.

Analysis of the individual protein bands by MALDI-TOF showed a complete match of the 280 kDa protein to XptA2, and a complete match of the
170 kDa protein to XptB1 (suppl. Figure S1). Tryptic digests of the 77 kDa band matched residues located in the N-terminal 2/3rd portion of XptC1, but no further, suggesting that the C-terminal 1/3rd portion of this protein was probably missing, which is consistent with the protein migrating on SDS-PAGE with an apparent molecular weight of 77 kDa (Figure 1B) as opposed to ~110 kDa for the full length gene product. The minor band migrating with an apparent molecular mass of ~30 kDa was analyzed by MALDI and determined to be the C-terminal fragment of XptC1. Thus it appears that XptC1 is proteolytically processed, either by enzymes located in Xenorhabdus nematophilus, or by the toxin complex protein itself, and that these three proteins associate together to form a large complex.

Biological activity of the toxin complex

The biological activity of toxin complex 1 was measured by applying 1, 10, 100, 1,000 or 3,000 ng/cm² of the purified protein onto the surface of insect diet and allowing the insects to feed for five days. The weight of insects was taken as a read out. (Figure 2). Toxin complex 1 was active against both insects, but with the greatest activity against Helicoverpa zea. Average weight of H. zea was significantly reduced with a toxin complex 1 concentration of 1 ng/cm², compared to the average weights of treated insects on the buffer-only control. The GI-50 of the toxin complex 1 was 0.3 ng/cm² for H. zea and 48.5 ng/cm² for H. virescens.

Pore-formation by XptA2 in black lipid membranes

Recently, it has been suggested that the XptA2-related toxin component TcdA1 from P. luminescens is involved in toxin uptake by forming pores in cell membranes (17). Therefore, we studied the effects of purified XptA1 in black lipid membranes. As shown in Figure 3, addition of the toxin component to the cis side of the membrane increased the membrane current in a stepwise manner. The single channel conductance in the presence of 0.15 and 1 M KCl was ~100 and ~400 pS, respectively. These data indicate that the XptA2 component exhibits strong membrane activity and forms pores.

Xenorhabdus Toxin Complex Binds to Solubilized Insect Gut Membranes.

Midgut membranes from Heliothis zea larvae were solubilized and immobilized onto a Biacore chip and the binding of native Xenorhabdus toxin measured by SPR. The results (Figure 4) show increased binding as increased concentrations of Xenorhabdus Toxin Complex-1 was flowed over the immobilized gut membrane protein fraction. When the binding curves are fitted to a Langmuir isotherm model for 1:1 interaction between the analyte and the immobilized ligand, a Kd value of 0.2 nM is obtained for the binding affinity.

Recombinant XptA2 forms tetramers

The gene xptA2, encoding the large class A protein, was cloned and expressed in E. coli. The resulting recombinant XptA2 protein was purified with similar techniques used to purify the native toxin complex proteins, resulting in a single protein band with an apparent molecular mass of 280 kDa when analyzed by SDS-PAGE (Figure 5B, lane 3). When analyzed by native-PAGE, XptA2 migrates significantly less distance in the gel than the highest molecular mass standard of 669 kDa, which is consistent with XptA2 existing as a high molecular mass oligomer (data not shown). The recombinant XptA2 was passed though a calibrated Superdex 200 size exclusion column (SEC) to determine its approximate molecular mass (Figure 5A). Recombinant XptA2 eluted from the SEC column at 9.57 ml, significantly earlier than the highest molecular weight standard (670 kDa) which eluted at 9.96 ml. Based upon an extrapolation of the calibration curve from the sizing column, the molecular mass of recombinant XptA2 was estimated to be approximately 1,200 kDa. This suggests that the XptA2 protein, in the absence of class B and C proteins, forms a tetramer, which would have a combined molecular weight of 1,120 kDa, closely agreeing with this result. The formation of a tetramer is consistent with a previously reported proposed tetramer structure for TcdA1 from Photorhabdus (16), and the more recently 3D structural characterization of XptA1, revealing a tetramer with a cage-like structure (23).

Complexes formed by recombinant toxin components

Next, recombinant XptB1 and XptC1 proteins were co-produced together in E. coli and purified to apparent homogeneity. When analyzed by SEC, XptB1 and XptC1 elute together as a single symmetrical peak at a volume corresponding to an apparent molecular size of approximately 250 kDa, the sum of the weights of the two proteins together (Figure 5A). On Native PAGE, the two proteins form
a single band, indicating these two proteins exist as a binary complex (data not shown). Similar to what was observed in the native toxin complex, when analyzed by SDS-PAGE, the recombinant class C protein XptC1 exists as a cleaved 77 kDa protein in this preparation, with a very small amount of the C-terminal 30 kDa fragment also present. N-terminal sequencing of the C-terminal fragment revealed that the cleavage site is identical to the site of cleavage of XptC1 when it is isolated from the native toxin complex 1 (R679-F680). The cleavage site is immediately after the site where the amino acid sequence similarities of different class C proteins from various organisms radically diverge.

To test whether or not the three recombinant proteins (XptA2, XptB1 and XptC1) together are capable of forming a larger complex, the three recombinant proteins were analyzed by SEC. When excess amounts of XptB1 + XptC1 were incubated with XptA2, and the mixture subsequently analyzed by SEC, two peaks elute from the column (Figure 5A). The first peak elutes at a volume less than where XptA2 alone elutes (8.99 ml vs 9.57 ml), indicating that these three proteins now associate together to form a larger complex. The size of this larger complex is difficult to determine since we do not have molecular mass standards this large to compare with, but apparently the molecular mass of the complex is now greater than the estimated size of 1,200 kDa for XptA2 alone since the newly formed complex elutes at a lower volume than XptA2 alone. Additionally, under these same conditions, the native toxin complex 1 elutes at nearly the same volume as the recombinant protein mixture (8.92 ml, chromatogram not shown), indicating the similar size of the recombinant and native complexes.

The two peaks that eluted from the SEC were then analyzed by SDS-PAGE to determine the content of each fraction (Figure 4B). The earlier eluting peak (8.99 ml) obtained after incubating XptA2 with XptB1 + XptC1 contained all three proteins (lane 2), which further indicated all three proteins are now associated together, as is the case with the native Xenorhabdus toxin complex 1 (Lane 1). Based upon the intensity of Coomassie Blue staining, XptA2 is the vastly predominant protein component. The level of staining of XptB1 and XptC1 showed both proteins to be present at an approximate 1:1 ratio taking into consideration the differences in molecular weight of these proteins (lanes 1, 2, and 4). Thus, XptB1 and XptC1 apparently bind together as a tightly associated 1:1 binary complex, and when added to XptA2, form a larger complex composed of all three proteins, but with XptA2 in greater quantity than either XptB1 or XptC1. The trailing peak from the SEC column, (11.55 ml) consisted of the excess XptB1 and XptC1 proteins which had not bound to the available XptA2 (lane 4).

Analysis of the toxin complex by native PAGEs

To further substantiate that the recombinant proteins XptA2, XptB1 and XptC1 bind together to form a tightly associated complex, electrophoretic mobility shift assays (EMSA) were conducted using these three proteins. When XptA2 is analyzed by native-PAGE, a single protein band of high apparent molecular mass is observed (Figure 6, lane 3), but this protein band migrates further in the gel than the Xenorhabdus native toxin complex 1 (Figure 6, lane 1). When XptA2 is mixed with the binary complex of XptB1 + XptC1, separated from the excess XptB1 + XptC1 by SEC, and then analyzed by Native-PAGE, a single high molecular weight band is observed that electrophoretically migrates to the same distance as native Xenorhabdus native toxin complex 1, and less than XptA2 by itself (Figure 6, lane 2). The observation that a single newly formed band is formed that migrates less distance in the gel compared to XptA2 by itself is consistent with the formation of a larger protein complex.

Surface Plasmon Resonance studies

To further characterize the interaction of XptB1 + XptC1 with XptA2, we measured the binding of these proteins by surface plasmon resonance (SPR). When XptA2 is immobilized onto the chip and the co-expressed purified XptB1 - XptC1 proteins are flowed over its surface, we observe initial rapid binding of the B-C proteins to the immobilized XptA2. This response is detected by an initial rapid increase in the resonance units (RU), followed by a slower rate of binding (Figure 7). We did not observe any measurable dissociation of XptB1 + XptC1 proteins when only running buffer was subsequently flowed over the immobilized XptA2 (Figure 7). Apparently, the binding of XptB1 + XptC1 proteins to XptA2 is exceedingly strong and the very low rate of dissociation prevents us from determining the K₅ for binding using this procedure. Binding of XptB1 + XptC1 to XptA2 protein is so tight that once bound they are essentially irreversibly associated with the immobilized XptA2, since we have not been able to
find conditions that dissociate these proteins that are not so harsh as to denature XptA2.

**XptA2 forms a complex with Photorhabdus luminescens TcdB2-TccC3 fusion toxins**

To determine how receptive XptA2 is to bind class B and C proteins from different organisms, purified recombinant TcdB2 and TccC3 proteins from *Photorhabdus luminescens* strain W-14 were used to measure their interaction with XptA2. These represent two entirely different B and C proteins compared to XptB1 and XptC1, with less than 54% amino acid sequence identity. When *tcdB2* and *tccC3* are co-expressed together in *E. coli* and the proteins purified, we find that TccC3 is also cleaved into 77 kDa N-terminal and 30 kDa C-terminal products. The site of cleavage (L678-M679) is equivalent with that of XptC1. An electrophoretic mobility shift assay was conducted to measure the interaction of these proteins together by titrating XptA2 with TcdB2-TccC3 (Figure 8A). As TcdB2-TccC3 was added to XptA2, a higher molecular weight band having less electrophoretic migration in the native gel compared to XptA2 appeared. This newly formed band also correspondingly increased in amount as the concentration of TcdB2-TccC3 added to XptA2 was increased. We also observed a concomitant decrease in the amount of the band corresponding to XptA2 alone. This transitioning of banding patterns on the native-PAGE gel represents the formation of a hybrid toxin complex. Lane 5 of Figure 8A shows the condition in which all of the XptA2 is in the lesser migrating complex due to addition of TcdB2-TccC3 at saturating amounts, leaving no free XptA2. When this high molecular weight protein band is cut from the Native-PAGE gel, electroeluted, and analyzed by SDS-PAGE under denaturing conditions, we observed the presence of 3 different bands, corresponding to XptA2, TcdB2 and the N-terminal 2/3rd portion of TccC3 (Fig. 8B). Densiphotometry analysis of the staining intensity of these bands, taking into consideration of their differences in molecular weights, gives a 4:1:1 stoichiometry for the amount of XptA2 to TcdB2 to TccC3. Thus, there is a one to one ratio of TcdB2 and TccC3 which bind together, and this binary complex of proteins binds to four XptA2 proteins, as is observed for XptB1-XptC1 with XptA2 in the *Xenorhabdus* native toxin complex 1. The 30 kDa C-terminal fragment of TccC3, if present, was too low in concentration to detect by Coomassie blue staining.

The heterologous toxin complex of XptA2 and TcdB2-TccC3 possesses insecticidal activity

To demonstrate that the binding of TcdB2-TccC3 to XptA2 is a physiologically significant event, we tested the effects of the combination of toxin components in insects. As shown in Table 1, when fed to first instar *Helicoverpa zea* larvae in a top loaded artificial diet, purified recombinant XptA2 alone results primarily in stunting of larval growth. The maximum growth inhibition was 80% at the highest concentration tested (2,500 ng/cm²) and only 32% growth inhibition when tested at 500 ng/cm². In contrast, when *E. coli* lysate from bacteria producing TcdB2 and TccC3 was mixed with purified XptA2 and fed to the larvae in a top load diet, we obtain 100% larvae growth inhibition at XptA2 concentrations as low as 20 ng/cm², and with significant insect mortality at this and higher concentrations. Thus, in the presence of TcdB2 and TccC3, the insecticidal activity of XptA2 is increased by more than 100-fold. Feeding insect larvae the lysate containing TcdB2 and TccC3 without XptA2 resulted in only minor (16%) growth inhibition compared to that of non-transformed bacterial lysates, showing that these two proteins have little or no affect on the larvae by themselves. Thus these data are largely in agreement with our recent findings that the major toxicity of Tc toxins is attributed to component TccC3 (17;18) and suggest that XptA2 allows the uptake of the toxin component from *P. luminescens*. Moreover, comparing the activity of this hybrid toxin complex to the biological activity of the *Xenorhabdus* native toxin complex 1 (Figure 2 to Table 1), we obtained greater growth inhibition at 4 ng/cm² of XptA2 in the presence of TcdB2+TccC3 than what occurs with 100 ng/cm² of the complete *Xenorhabdus* native toxin complex 1, although both toxins are highly active. Thus, the ability to use different class B and C proteins from different bacterial sources to potentiate the biological activity of different class A proteins opens the possibility to greatly expand both spectrum and activity of this family of toxins.

**CONCLUSIONS**

Based upon the results presented in this study, a model of the structure for the Toxin Complex is proposed (Figure 8). In this model, the class A protein (XptA2) forms a 1,140 kDa tetramer. We have drawn these proteins simply as ovals grouped...
together where they line up in a parallel fashion forming a tube with two contacts between adjacent proteins. This arrangement approximates the 25 Å resolution structure observed from studies using transmission electron microscopy of negatively stained XptA2 (23). The class B and C proteins (XptB1 and XptC1) form a strongly associated binary complex with the C-terminal portion of XptC1 truncated and only weakly associated with the complex. It is believed that this complex of full length XptB1 and both sections of XptC1 then strongly bind to the tetrameric XptA2 to form the complete and fully active toxin complex.

The class C protein components from *Photorhabdus luminescens* have been shown to be ADP ribosyltranferases that modify actin and RhoA proteins resulting clustering of actin (17,18). We have not determined the activity of class C proteins from *Xenorhabdus* toxin complexes. Our studies of XptA2 show this component to strongly bind solubilized insect midgut membrane fractions and is able to form pores in artificial membranes. It is likely that this component is involved in translocation of the class C toxin component in the intoxication process. The site on the tetrameric class A protein where the B-C proteins bind is not known, but it is tempting to assume that the site is in the middle of the tetramer where there would presumably exist a symmetrical channel.

The finding that the class A components are capable of delivery of Tc toxins from different types of bacteria will largely facilitate studies on this family of toxins. Moreover, the interchangeability of the toxins might be of great benefit for the development of novel biological insecticides.

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Reference List


Table 1. Biological activity of XptA2 against corn earworm larvae in the presence or absence of E.coli lysate expressing TcdB2-TccC3. Numbers in column under XptA2 represent the concentration of this protein in ng/cm² that is placed on top of the diet prior to addition of insects. The + or – symbol in the column under TcdB2+TccC3 lysate indicate if a 1:10 dilution of an E.coli cell lysate co-expressing these two proteins was added to the diet in addition to the indicated concentration of XptA2. Number of dead larvae is from n=8. Percent growth inhibition is the mean weight of eight treated larvae compared to the mean weight of 8 larvae receiving only water as the diet treatment.

FIGURE LEGENDS

Figure 1. Final chromatographic resolution of Xenorhabdus toxin complexes. A. High resolution anion exchange chromatogram (MonoQ 0.5 x 5 cm) of high molecular weight fractions off the hydrophobic-interaction column. Blue line is O.D. at 280 nm, and brown line is conductance (mS/cm). Peak 1 is Xenorhabdus Toxin Complex 1 and Peak 2 is Xenorhabdus Toxin Complex 2. Analysis of the second peak revealed a second toxin complex of completely different structure which will be described in more detail in a future report. B. SDS-PAGE of purified native Toxin Complex 1 (XTC-1) from Xenorhabdus nematophilus under reducing and denaturing conditions. Numbers represent molecular weight from BenchMark™ markers. Relative staining intensity of the three major bands, when adjusted for protein size, is 4:1:1 for XptA2, XptB1 and XptC1, respectively.

Figure 2. Biological activity of Xenorhabdus native toxin complex-1. The ability of the toxins to inhibit growth of two different insect larvae Helicoverpa zea Boddie (open bars), and Heliothis virescens (solid bars), over a 5 day time period when the amount of purified toxin complex protein in ng/cm² applied to the top surface of artificial insect diet. Weights are compared to insects receiving protein.
buffer alone. N=24-36 insects tested per dose. Error bars represent standard error (95% confidence interval).

**Figure 3. Current recordings of diphytanoyl phosphatidylcholine/n-decane membranes of channels formed by XptA1.** 5 min after the start of the recordings XptA2 was added to the cis side of different membranes. The aqueous phase contained 0.15 M KCl (A) or 1.0 M KCl (B) and 10 mM MES-KOH (pH 6). The applied membrane potential was 20 mV; T = 20 °C.

**Figure 4. Binding of XptA2 to Solubilized Brush Border Membrane Fraction from *H. zea* larvae.** XptA2 added at 2-fold increasing concentrations ranging from 1.5 to 100 nM to solubilized insect midgut membrane fractions immobilized onto a CM4 chip. Binding measured by SPR.

**Figure 5. Analytical Size Exclusion Chromatography (SEC) comparison of elution volumes for XptA2 alone (red) and XptA2+XptB1xwi-XptC1xwi (blue).** A. The earlier elution of XptA2 to which XptB1-XptC1 was added (compared with XptA2 alone) from the Superdex 200 10/30 column shows that incubation of XptB1-XptC1 with XptA2 results in a size increase of the complex and indicates that the XptB1-XptC1 complex is binding to XptA2. B. **SDS-PAGE of SEC samples.** Lane 1: native *Xenorhabdus* toxin complex-1 control having all three proteins, XptA2, XptB1, and XptC1; Lane 2: XptA2 + XptC1-XptC1, 8.99 ml eluting peak in Figure 3A; Lane 3: XptA2, 9.57 ml eluting peak in Figure 3A. Lane 4: XptB1-XptC1, 11.55 ml eluting peak in Figure 3A.

**Figure 6. Native-PAGE of electrophoretic mobility shift assay (EMSA) of XptC1-XptB1 binding to XptA2.** Lane 1: Native XTC-1; Lane 2: XptA2 + XptB1-XptC1; Lane 3: XptA2; Lane 4: XptB1-XptC1. Incubation XptA2 with XptB1-XptC1 causes decreased migration of XptA2 into the native gel, indicating a change in size and/or pI has occurred. Gel stained with Coomassie blue.
**Figure 7.** SPR sensorgram of XptC1-XptB1 binding to XptA2 surface. Fifty microliters of a 1.7 mg/ml solution of XptB1-XptC1 was injected over an XptA2 surface of approximately 7100 RU (7.1 ng). The flow rate during injection was 10 µl/min. Running buffer was composed of 50 mM Tris-Cl, 100 mM NaCl, 10% glycerol, 0.05% Tween 20, pH 8. The sensorgram represents blank-subtracted data.

**Figure 8.** A) Native PAGE of a titration of XptA2 with TcdB2-TccC3. Lanes 1 thru 5 represent 0, 0.1, 0.25, 1.0, and 4.0 µM TcdB2-TccC3 added to 1 µM XptA2, respectively. B) SDS-PAGE under denaturing and reducing conditions of high molecular weight toxin complex after being cut from the native gel (Lane 5 panel A), and electroeluted. Densiphotometry readings of the bands give a 4:1:1 ratio of XptA2 to TcdB2 to TccC3, when taking into account the difference in molecular weight of the bands.

**Figure 9.** Putative model of the complete native toxin complex 1 from *Xenorhabdus nematophilus*. The large 285 kDa XptA2 protein forms a homo-tetramere resulting in a 1,140 kDa complex. The XptB1 and XptC1 proteins bind together to form a 1:1 dimer. Either after they bind together, or just prior to binding together, the 112 kDa XptC1 protein is cleaved into a N-terminal 80 kDa fragment, and a 32 kDa C-terminal fragment, both still bound to XptB1. The mechanism of cleavage is not known. This complex then binds to the tetrameric XptA2 to form the complete 1,402 kDa Toxin Complex. The exact location where XptB1 and XptC1 proteins bind to XptA2 is not known but is positioned on top of the complex for illustrative purposes.
<table>
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<th>XptA2 (ng/cm²)</th>
<th>TcdB2+TccC3 Lysate</th>
<th># Dead</th>
<th>% Growth Inhibition</th>
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**FIGURES**

**A**

![Graph A](image)

*Figure 1.*

**B**

![Graph B](image)

*Figure 2.*
Figure 3. Kinetic analysis of native Xenorhabdus nematophilus toxin complex binding to Solubilized CEW BBMV Fraction. Concentrations of native Xenorhabdus nematophilus toxin complex added ranged from 1.5 - 100 nM conducted in duplicate. $K_d = 0.2$ nM.

Figure 4.
Figure 5.

Figure 6.
Figure 7.

![Graph showing response over time with injection and dissociation points marked.]

Figure 8.

![Native-PAGE gel with MW markers and bands labeled for XptA2, TcdB2 + TccC3, XptA2, TcdB2, and TccC3.]

**A**

**B**
Figure 9.
Insecticidal toxin complex proteins from Xenorhabdus nematophilus: Structure and pore formation
Joel J. Sheets, Tim D. Hey, Kristin J. Fencil, Stephanie L Burton, Weiting Ni, Alexander E. Lang, Roland Benz and Klaus Aktories

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