Characterization of Mosquitocidal Activity of Bacillus thuringiensis subsp. fukuokaensis Crystal Proteins

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The mosquitocidal crystals of Bacillus thuringiensis subsp. fukuokaensis were isolated and bioassayed against fourth-instar larvae of two mosquito species. The 50% lethal concentration values of the crystals to Aedes aegypti and Culex quinquefasciatus were 4.1 and 2.9 μg/ml, respectively. In addition, the solubilized crystals had hemolytic activity; 50 μg/ml was the lowest detectable level. Sodium dodecyl sulfate-polyacrylamide gel electrophoresis analysis revealed that the crystals consisted of polypeptides of 90, 86, 82, 72, 50, 48, 37, and 27 kDa. When the solubilized inclusion was treated with C. quinquefasciatus midgut brush border membrane vesicles or Manduca sexta gut juice, only one major protein was detected. This protein retained mosquitocidal activity but had no detectable hemolytic activity. Immunological analysis of this subspecies and the subspecies israelensis, kyushuensis and darmstadiensis by using polyclonal antisera raised against the whole-crystal protein of B. thuringiensis subsp. fukuokaensis revealed that the proteins in subsp. fukuokaensis are distinct from proteins in the other subspecies because little cross-reaction was observed. Analysis of the plasmid pattern showed that the crystal protein genes are located on a plasmid of 130 MDa. Analysis of plasmid and chromosomal DNA from subsp. fukuokaensis showed little homology to the 72-kDa toxin gene (PG-14) of B. thuringiensis subsp. morrisoni. However, some of the proteins of B. thuringiensis subsp. fukuokaensis are homologous to other B. thuringiensis toxins because N-terminal amino acid analysis revealed that the 90-kDa protein is encoded by a cryIV gene type.

Bacillus thuringiensis, a gram-positive soil bacterium, is characterized by its ability to produce crystals during sporulation. Most B. thuringiensis strains are toxic towards lepidopteran insects; however, a few subspecies, such as israelensis (13), morrisoni PG-14 (28), darmstadiensis 73-E10-2 (27), kyushuensis (25), kurstaki HD-1 (38), and galleria (1), produce dipteran active toxins. Three of these strains, subsp. israelensis (12, 32), subsp. morrisoni PG-14 (11, 40), and subsp. darmstadiensis 73-E10-2 (6), also produce toxins that have cytolytic and hemolytic activity; however, the solubilized crystal proteins of subsp. kyushuensis are apparently not hemolytic (15). Because of these similar properties, a number of comparative studies on the immunological, biological, and genetic relationship between these dipteran active strains of B. thuringiensis have been published (8, 11, 18, 31).

The crystal protein genes of B. thuringiensis subsp. israelensis, kyushuensis, and morrisoni PG-14 are located on the 72-, 60-, and 94-MDa plasmids, respectively (8, 9, 14, 31). Even though their locations are on plasmids of different sizes, the protein toxins in these dipteran-active strains exhibit a high degree of homology. The greatest similarity is between the subspecies israelensis and morrisoni, in which the toxins appear to be immunologically similar (11). For example, the 72-kDa cytotoxins from these two subspecies differ by only one base and amino acid change (7, 9). Similarly, the amino acid sequence of the 72-kDa toxin of subsp. morrisoni (PG-14) is identical to that of the 72-kDa toxin of subsp. israelensis (8a). Further, Bourgouin et al. (2) recently showed that the plasmid DNA fragments in strain PG-14 hybridized with the 130-kDa protein gene of subsp. israelensis. In addition, the crystal toxin genes of subsp. kyushuensis and subsp. morrisoni PG-14 share significant homology to the crystal toxin gene of subsp. israelensis (31). More recently the monoclonal antibodies directed against the 68- and 135-kDa toxins of subsp. israelensis have been shown to cross-react with the 26- and 75- or 80-kDa proteins of subsp. kyushuensis, respectively (15).

In this article we describe the characterization of crystals produced from B. thuringiensis subsp. fukuokaensis which are toxic to larvae of mosquitoes and which in solubilized form are hemolytic. Further, by means of polyclonal antisera specific for crystal proteins of this strain we show that the crystal proteins of this strain do not cross-react with those of three other mosquitocidal strains. Further, we have determined that a large plasmid encodes for the crystal proteins of subsp. fukuokaensis.

MATERIALS AND METHODS

Bacterial strains and culture conditions. The type strains of B. thuringiensis subsp. israelensis, fukuokaensis, kyushuensis, and darmstadiensis (strain 73-E10-2) were from Kyushu University, Fukuoka, Japan. Two Cry1 mutants used in this study, subsp. israelensis YG-1 and subsp. fukuokaensis YG-101, were prepared by curing the type strains as previously described (14). Growth of these strains was as described for B. thuringiensis subsp. israelensis (10).

Purification and solubilization of crystals. The spore-crystal mixture produced from B. thuringiensis was thoroughly washed with 1 M NaCl-10 mM EDTA before the purified crystals were obtained from a 40 to 70% Renografin density gradient centrifugation as previously described (40). The isolated crystals were washed three times with distilled water and stored at 20°C until needed. Solubilization of B. thuringiensis subsp. fukuokaensis crystals was performed in 50 mM Na2CO3 (pH 10.0)-10 mM dithiothreitol (DTT) at
37°C for 30 min. Activation of the solubilized toxin with *Manduca sexta* gut juice or with *Culex quinquefasciatus* brush border membrane vesicles (10:1 [vol/vol], toxin to protease) was performed at 37°C with a 2-h incubation. *M. sexta* gut juice and *C. quinquefasciatus* midgut brush border membrane vesicles were prepared as previously described (16, 40).

**SDS-PAGE.** Sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) was performed by the method of Laemmli (20) by using 10% running and 4% stacking gels, and the gels were stained with 0.4% Coomassie blue R250. The molecular mass of proteins was determined by using protein standards obtained from Sigma (St. Louis, Mo.). Protein concentrations were measured by the method of Lowry et al. (22) with bovine serum albumin as the standard.

**Antibody production and immunobots.** The whole crystals of *B. thuringiensis* subsp. *fukuokaensis* were mixed with Freud’s complete adjuvant and injected subcutaneously into a female New Zealand White rabbit. Three further immunizations were given at 7-day intervals in Freud’s incomplete adjuvant. A total of 1 mg of whole crystal protein was injected into the rabbit. The ability of the antisera developed against the whole crystal proteins of subsp. *fukuokaensis* to cross-react with protein toxins produced from four *B. thuringiensis* strains was determined by immunoblots. After SDS-PAGE, the resolved crystal proteins were transferred to nitrocellulose overnight as previously described (35). The nitrocellulose was then washed twice in phosphate-buffered saline (PBS; [in grams per liter] NaCl, 8; KCl, 0.2; KH₂PO₄, 0.2; and NaH₂PO₄, 2) containing 0.05% Tween 20 and then incubated for 2 h at room temperature with antibody developed against subsp. *fukuokaensis* crystal proteins in PBS–0.05% Tween 20. The nitrocellulose was then washed three times with PBS–0.05% Tween 20 and then incubated with goat anti-rabbit immunoglobulin G-alkaline phosphatase for 2 h. Visualization was performed with Nitro Blue Tetrazolium chloride and 5-bromo-4-chloro-3-indolyl phosphate as the substrate.

**Mosquitocidal activity.** The bioassays were performed according to World Health Organization standard protocols by using 100 ml of distilled water and 20 fourth-instar larvae of *Aedes aegypti* and *C. quinquefasciatus*. The assays were performed in triplicate, and mortality was scored after incubation for 24 h at room temperature. For bioassays with solubilized proteins second-instar larvae of *A. aegypti* were used, and these bioassays were performed with 20 larvae in 20 ml of water. Values of 50% lethal concentrations (LC₅₀) were determined by probit analysis as previously described (29).

**Hemolytic activity assay.** Sheep erythrocytes (Colorado Serum Co., Denver, Colo.) were washed twice with PBS (pH 7.4) and suspended to ca. 4 × 10⁸ cells/ml. Portions (100 μl) of this erythrocyte suspension and 100 μl of 50 mM Na₂CO₃ (pH 10.0)–10 mM DTT-solubilized toxin were then added in a 96-well microtiter plate. After incubation for 18 h at 37°C in a humidified 5% CO₂ chamber, the plate was centrifuged (1,500 × g for 10 min) and the supernatant was removed and read against appropriate blanks at 570 nm.

**Inhibition of hemolytic activity.** The solubilized toxin proteins of *B. thuringiensis* subsp. *fukuokaensis* and subsp. *israelensis* were incubated for 30 min at 37°C with liposomes made from dioleoyl 1,2-dipalmitoylphosphatidylcholine as previously described (12). The liposome-treated toxins were then incubated with sheep erythrocytes in a final volume of 200 μl for 18 h at 37°C, and hemolysis was monitored as described above.

**Isolation and analysis of plasmid DNA.** *B. thuringiensis* was grown in LB broth, and plasmid DNA was prepared by using a modification of the method of Crosa and Falkow (5). Briefly, the pellet of a 50-ml culture was lysed with 8.5 ml of TE buffer (50 mM Tris, 20 mM EDTA [pH 8.5]) containing 2 mg of lysozyme per ml, 0.5 ml of 20% SDS solution, and 1.0 ml of a 5 mg/ml solution of protease (protease type XIV; Sigma) in TE buffer. After being mixed by gentle inversions, the cell suspension was incubated at 37°C for 30 min. Subsequently, 0.3 ml of 3 N NaOH was added to the suspension and mixed gently for 3 min. The suspension was neutralized by addition of 0.6 ml of 2 M Tris-HCl (pH 7.0) and mixed gently. One milliliter of 5 M NaCl was then added, and the suspension was mixed by inversions, placed on ice for 15 min, and then centrifuged at 12,000 × g for 15 min. The supernatant was transferred into a fresh centrifuge tube, and 2 volumes of ice-cold ethanol were added. The tube was kept at −20°C for 15 min and then centrifuged at 12,000 × g for 15 min. The supernatant was discarded, and the pellet was dried by inverting the tube over a paper towel for a few minutes. The pellet was dissolved in 0.5 ml of TE buffer and kept at −20°C until used. Plasmid DNA was analyzed by electrophoresis on 0.6% horizontal agarose slab gels.

**Detection of plasmid homology to the 72-kDa *B. thuringien-**sis subsp. *morrisoni* toxin gene. A small amount of immunological cross-reactivity that was probably nonspecific was observed with the 72-kDa protein of *B. thuringiensis* subsp. *israelensis* in immunoblots with antisera raised against crystal proteins of *B. thuringiensis* subsp. *fukuokaensis*. Therefore, to determine their homology, if any, at the DNA level, a 0.8-kb EcoRI fragment from plasmid pM1 (9), which contains the 72-kDa toxin gene of *B. thuringiensis* subsp. *morrisoni*, was used as a probe. The EcoRI fragment was gel purified using Geneclean (Bio 101, La Jolla, Calif.) following the manufacturer’s instructions. The DNA fragment was then labeled by random priming using digoxigenin-11-DUTP and the Genius DNA labeling and detection kit (Boehringer Mannheim Biochemicals, Indianapolis, Ind.). DNA separa-
ated on agarose gels was transferred to nylon membrane and hybridized with the labeled probe as described by the manufacturer of the detection kit. Posthybridization washes were performed at relatively low stringencies (0.5 × SSC [1× SSC is 0.15 M NaCl plus 15 mM sodium citrate] at 65°C) unless otherwise indicated.

**RESULTS**

*B. thuringiensis* subsp. *fukuokaensis* crystal toxin proteins possess both mosquitocidal and hemolytic activities. The intact crystals of subsp. *fukuokaensis* have significantly lower mosquitocidal activity than *B. thuringiensis* subsp. *israelensis*, with a LC₅₀ of 4.1 and 2.9 μg/ml to fourth-instar larvae of *A. aegypti* and *C. quinquefasciatus*, respectively (Table 1).

SDS-PAGE analysis of crystals purified from *B. thuringiensis* subsp. *fukuokaensis* and three other subspecies with mosquitocidal activity, *israelensis*, *kyushuensis*, and *darmstadtensis*, demonstrated that the protein composition of the subsp. *fukuokaensis* is distinctly different (Fig. 1A). The crystals of subsp. *fukuokaensis* contain proteins of 90, 86, 82, 72, 50, 48, 37, and 27 kDa. Thus, the intact crystals of all four subspecies of mosquitocidal *B. thuringiensis* have a protein with a molecular mass of 27 kDa.

To investigate the antigenic relationships among the *B. thuringiensis* subsp. *fukuokaensis* crystal toxin and three
TABLE 1. Toxicity of crystal proteins from four strains of *B. thuringiensis* to fourth-instar larvae of *C. quinquefasciatus* and *A. aegypti*

<table>
<thead>
<tr>
<th>Subspecies</th>
<th><strong>LC$_{50}$ (µg/mL [fiducial limit])</strong></th>
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</thead>
<tbody>
<tr>
<td><em>israelensis</em></td>
<td>0.0049 (0.0042-0.0060)</td>
</tr>
<tr>
<td><em>fukuokaensis</em></td>
<td>4.1 (3.5-4.8)</td>
</tr>
<tr>
<td><em>kyushuensis</em></td>
<td>3.1 (2.3-3.9)</td>
</tr>
<tr>
<td><em>darmstadiensis</em></td>
<td>1.8 (1.6-2.1)</td>
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</tbody>
</table>

<table>
<thead>
<tr>
<th>Subspecies</th>
<th><strong>LC$_{50}$ (µg/mL [fiducial limit])</strong></th>
</tr>
</thead>
<tbody>
<tr>
<td><em>A. aegypti</em></td>
<td>0.0043 (0.0037-0.0052)</td>
</tr>
<tr>
<td><em>C. quinquefasciatus</em></td>
<td>2.9 (2.6-3.2)</td>
</tr>
<tr>
<td><em>C. morrisoni</em></td>
<td>3.2 (2.8-3.7)</td>
</tr>
<tr>
<td><em>S. fusca</em></td>
<td>1.2 (1.0-1.4)</td>
</tr>
</tbody>
</table>

* a 95% confidence level.

other subspecies with known mosquitocidal activity, a duplicate gel was immunoblotted with antiserum raised against the whole crystal of subspp. *fukuokaensis* (Fig. 1B). This antiserum cross-reacted strongly with subspp. *fukuokaensis* crystal proteins but showed little cross-reactivity against the three other *B. thuringiensis* subspecies.

The crystals of *B. thuringiensis* (subsp. *fukuokaensis*) dissolved readily in 50 mM $\text{Na}_2\text{CO}_3$-HCl (pH 10.0)-10 mM DTT (Fig. 2, lanes 3). This SDS-PAGE pattern of the solubilized preparation is similar to that of the intact crystal (Fig. 2, lane 1). However, there was little solubilization of intact crystals after incubation with *C. quinquefasciatus*-larvae brush border membrane vesicles or the gut juice of *M. sexta* larvae (Fig. 2, lanes 2 and 5). Only minor bands at 59 and 48 kDa were observed. However, treatment of crystals solubilized in 50 mM $\text{Na}_2\text{CO}_3$-HCl (pH 10.0)-10 mM DTT with gut membranes of dipteran (*C. quinquefasciatus*) larvae (Fig. 2, lane 4) or *M. sexta* larval gut juice (pH 10.0) (Fig. 2, lane 6) for 2 h at 37°C resulted in significant proteolysis. In the latter two conditions the same major protein band of 64 kDa was observed.

The protease-treated fraction has mosquitocidal activity with an **LC$_{50}$** of 259 µg/mL to second-instar larvae of *A. aegypti* (Table 2). This mosquitocidal activity is substantially lower than that of the crude solubilized fraction, which has an **LC$_{50}$** of 62 µg/mL.

To determine the plasmid that encodes the crystal proteins, wild-type and Cry$^-$ mutants of both *B. thuringiensis* subsp. *fukuokaensis* and *israelensis* were used for plasmid isolation. Cry$^-$ mutants were first observed by phase-contrast microscopy and subsequently confirmed by plasmid isolation. Figure 3 shows the plasmid patterns of subsp. *israelensis* (panel A, lane 1) and *fukuokaensis* (panel A, lane 2) wild-type strains and the plasmid patterns of Cry$^-$ mutants of subsp. *israelensis* YG-1 (panel A, lane 3) and subsp. *fukuokaensis* YG-101 (panel C, lane 4). The crystal-producing wild-type *B. thuringiensis* subsp. *israelensis* has a 72-MDa plasmid which is absent in the Cry$^-$ mutant. Similarly, the Cry$^-$ mutant of subsp. *fukuokaensis* lost a 130-MDa plasmid.

Southern blot analysis of the plasmids with a 0.8-kb EcoRI fragment of *B. thuringiensis* subsp. *morrisoni* (PG-14) which encodes part of the 72-kDa protein confirms the absence of the 72-MDa plasmid in the Cry$^-$ mutants of subsp. *israelensis* (Fig. 3B, lane 3). In contrast there is a strong signal in the wild-type subsp. *israelensis* (Fig. 3B, lane 1). However, no hybridization to any plasmid or chromosomal DNA of strain YG-1 and subsp. *fukuokaensis* was observed (Fig. 3B, lane 2), suggesting that there is little homology between the 72-kDa protein gene of subsp. *morrisoni* and the genes of subsp. *fukuokaensis*. However, N-terminal sequencing of the 90-kDa protein shows that this protein is highly homol-

TABLE 2. Comparison of the toxicity of *B. thuringiensis* subsp. *fukuokaensis* and subsp. *israelensis* crystal proteins treated with gut protease to second-instar larvae of *A. aegypti*

<table>
<thead>
<tr>
<th>Subspecies</th>
<th>Treatment*</th>
<th><strong>LC$_{50}$ (µg/mL)</strong></th>
</tr>
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<tbody>
<tr>
<td><em>fukuokaensis</em></td>
<td>Solubilized</td>
<td>62</td>
</tr>
<tr>
<td></td>
<td>Solubilized and gut juice treated</td>
<td>259</td>
</tr>
<tr>
<td><em>israelensis</em></td>
<td>Solubilized</td>
<td>0.59</td>
</tr>
<tr>
<td></td>
<td>Solubilized and gut juice treated</td>
<td>0.82</td>
</tr>
</tbody>
</table>

* Solubilization was with 50 mM $\text{Na}_2\text{CO}_3$ buffer (pH 10.0) containing 10 mM DTT. Gut juice was obtained from *M. sexta* and used for protease treatment in a toxin-to-gut juice ratio of 10:1 (vol/vol).
of DNA from panel A. Lane 1, DNA from subsp. israelensis; lane 2, DNA from subsp. fukuokaensis; lane 3, DNA from subsp. israelensis strain YG-1, a Cry- mutant; lane 4, DNA from subsp. fukuokaensis strain YG-101, a Cry+ mutant.

FIG. 3. Plasmid DNA of mosquitocidal B. thuringiensis strains. The plasmid contents of the four B. thuringiensis strains were analyzed on a 0.6% agarose gel. (A and C) DNA separation of B. thuringiensis subsp. israelensis and fukuokaensis. (B) Southern blot hybridization of DNA from panel A. Lane 1, DNA from subsp. israelensis; lane 2, DNA from subsp. fukuokaensis; lane 3, DNA from subsp. israelensis strain YG-1, a Cry- mutant; lane 4, DNA from subsp. fukuokaensis strain YG-101, a Cry+ mutant.

A dose-response curve in lysing sheep erythrocytes was observed (Fig. 4); 50 μg/ml was the lowest level at which detectable hemolytic activity was observed. In contrast, significantly higher levels of hemolytic activity were detected in subsp. israelensis. The hemolytic activity of the solubilized subsp. fukuokaensis was lost after treatment with M. sexta gut juice. No hemolytic activity was observed at toxin concentrations of up to 600 μg/ml. Moreover, the hemolytic activity observed in subsp. fukuokaensis could be inhibited by prior incubation of the solubilized toxin with liposomes made of unsaturated phospholipids (Fig. 5). This lipid-mediated inhibition of toxicity appears similar to that observed with subsp. israelensis.

FIG. 4. A dose-response curve of the hemolytic activity of solubilized crystals of B. thuringiensis subsp. fukuokaensis and three other B. thuringiensis serotypes. The data given are means of three different assays performed. Symbols: ●, subsp. israelensis; ▲, subsp. fukuokaensis; ■, subsp. darmstadiensis; ○, subsp. kyushuensis.

FIG. 5. Inhibition of hemolytic activity by unsaturated phospholipids. The toxins were first mixed with liposomes of unsaturated phosphatidyl choline for 30 min prior to incubation with sheep erythrocytes for 18 h at 37°C. Symbols: ●, subsp. israelensis; ■, subsp. israelensis plus lip (5 mM); □, subsp. fukuokaensis; ■, subsp. fukuokaensis plus lip (17 mM).

DISCUSSION

In an effort to identify new mosquitocidal proteins we screened a variety of B. thuringiensis strains. In this study, we separated the mosquitocidal crystals of B. thuringiensis subsp. fukuokaensis, a new serotype isolated in Japan (26), and characterized its biological activity, immunological relationship, and plasmid pattern, and compared these with other mosquitocidal B. thuringiensis subspecies, viz., israelensis, kyushuensis, and darmstadiensis.

The crystal protein profile of B. thuringiensis subsp. fukuokaensis revealed polypeptides of 90, 86, 82, 72, 50, 48, 37, and 27 kDa. The intact crystals of subsp. fukuokaensis and the other mosquitocidal B. thuringiensis strains each contained a major protein with a molecular mass of 27 kDa. However, there is no overlap in the profiles of the other crystal proteins with those of these four B. thuringiensis subspecies.

Using antisera specific to B. thuringiensis subsp. fukuokaensis, we have demonstrated that there is little immunological relationship between the crystal proteins of subsp. fukuokaensis and the other mosquitocidal B. thuringiensis. These results suggest that the crystal proteins of subsp. fukuokaensis are evidently distinct from toxin proteins of the other three B. thuringiensis subspecies. Antiserum prepared against the whole crystal proteins of subsp. fukuokaensis cross-reacted with all major crystal proteins of this strain. However, other than small amounts of probably nonspecific reactivity, this antibody did not cross-react with the crystal
proteins of any of the three other strains. A similar lack of immunological cross-reactivity between proteins of mosquitoicidal strains has been observed previously. For example, the toxin proteins of *B. thuringiensis* subsp. *darmstadensis* have been shown to be immunologically distinct from that of *B. thuringiensis* subsp. *israelensis* (6, 18). However, when monoclonal antibodies are used, there is usually some cross-reactivity, showing that some of these proteins may share common epitopes and thus protein structure. For example, Held et al. (15) showed that monoclonal antibodies directed against the 68- and 135-kDa proteins of subsp. *israelensis* cross-react with the 26- and 75- or 80-kDa proteins of *B. thuringiensis* subsp. *kyushuensis*, respectively.

The plasmid pattern of *B. thuringiensis* subsp. *fukuoakensis* is substantially different from that of subsp. *israelensis*. Only a single, large plasmid of 130 MDa was observed. This plasmid is apparently required for toxin production (Fig. 3) because curing of the strain of this plasmid as observed with a spontaneous Cry- mutant, YG-101, resulted in a loss of crystal production and mosquitoicidal activity. Moreover, there appears to be little homology between the 130-MDa plasmid and chromosomal DNA of subsp. *fukuoakensis* and the 72-kDa protein gene in both subsp. *israelensis* and *morrisoni* PG-14. However, there is significant amino acid homology in the N-terminal sequence of the 90-kDa protein of subsp. *fukuoakensis* and the proteins encoded by the cryIVA and cryIVC genes of subsp. *israelensis* (33, 36). It appears that these mosquitoicidal proteins therefore have similar origins, but the 90-kDa protein in subsp. *fukuoakensis* is naturally truncated, as is the protein encoded by the cryIVC gene (33).

The mosquitoicidal activity of this isolate is substantially less than that of *B. thuringiensis* subsp. *israelensis* or subsp. *morrisoni* PG-14, both of which are mosquitoicidal at the nanogram per milliliter level. However, the mosquitoicidal activity of subsp. *fukuoakensis* is on the same order of magnitude as that of other mosquitoicidal isolates, viz., subsp. *darmstadensis* 73-E10-2 and subsp. *kyushuensis*. The crystals are toxic to both *A. aegypti* and *C. quinquemaculatus*; however, the latter mosquito species is more sensitive. The toxicity of crystals of subsp. *kyushuensis* is significantly less than that reported by Earp et al. (8) but higher than that reported by Held et al. (15).

Solubilization of the crystals was readily achieved in 50 mM Na2CO3 (pH 10.0) containing 10 mM DTT. The solubilized fraction which shows a similar SDS-PAGE profile (Fig. 2) is, however, substantially less mosquitoicidal than the crystals. This decrease in mosquitoicidal activity of solubilized toxins is expected because of the filter feeding behavior of mosquitoes. A similar decrease in mosquitoicidal activity has been reported by Schnell et al. (30), among others, for solubilized *B. thuringiensis* toxins. However, immobilization of the solubilized toxins either by adsorption to latex beads (30) or by precipitation (3) results in a recovery of this decrease in mosquitoicidal activity, demonstrating that the inherent mosquitoicidal activity is not significantly lost by solubilization of the crystals.

Treatment of the solubilized fraction with either the gut membranes of *C. quinquemaculatus* or the gut juice of *S. sexta* results in the formation of one major band of 64 kDa on SDS-PAGE. The gut juice from lepidopteran larvae generally has chymotrypsin- and trypsinlike activities. For example, Lecadet and Dedonder (21) showed that the gut juice protease purified from *Pieris brassicae* larvae had the ability to dissolve the crystals and had protease activity similar to those of trypsin and chymotrypsin. The brush border mem

brane vesicles of *C. quinquemaculatus* also cause similar chymotrypsin- and trypsinlike cleavage of solubilized proteins of *B. thuringiensis* subsp. *israelensis* (5a). Muthukumar and Nickerson (24) also demonstrated that chymotrypsin generates domains similar in size to those detected following prolonged digestion with gut enzymes from third-instar *A. aegypti* larvae. Also, Tojo and Aizawa (34) reported that the gut juice protease was essential for crystal dissolution at a pH of between 10 and 11. Thus, chymotrypsinlike and/or trypsinlike action on the solubilized crystal proteins essentially results in the formation of a major protein of 64 kDa.

The formation of only one major protein of 64 kDa on treatment with proteases suggests either that the proteins of 72 to 90 kDa are substantially similar and have the same protease-resistant core or that only one of these proteins contains a protease-resistant core. This 64-kDa protein has mosquitoicidal activity; however, it is substantially lower than that of the crude solubilized crystals. This suggests that other proteins in the solubilized crystals are probably also mosquitoicidal. Potentially, the 27-, 37-, 48-, and 50-kDa proteins that disappear on solubilization and protease treatment could be mosquitoicidal.

The Na2CO3-DTT-solubilized proteins of *B. thuringiensis* subsp. *fukuoakensis* also exhibit hemolytic activity that is considerably lower than that of subsp. *israelensis*. A similar level of hemolytic activity was observed with the solubilized crystal of subsp. *kyushuensis*. These results differ from those of a previously reported study (14) in which no hemolytic activity was observed in subsp. *kyushuensis*. *B. thuringiensis* subsp. *darmstadensis* 73-E10-2 had negligible levels of hemolytic activity when sheep erythrocytes were used as the target. However, when human erythrocytes were used, subsp. *darmstadensis* had higher levels of hemolytic activity (6) than subsp. *fukuoakensis*, which has the same level of hemolytic activity with either human or sheep erythrocytes (data not shown).

The ability of solubilized proteins from *B. thuringiensis* subsp. *fukuoakensis* to cause hemolysis suggests that most mosquitoicidal strains of *B. thuringiensis* identified to date contain a cytolytic protein. The reason for this is not apparent. However, it has been demonstrated that the cytolytic toxin from subsp. *israelensis* causes the synergism of both the 72- and 130-kDa toxins (3, 17, 37, 39). In subsp. *israelensis* and *morrisoni* (PG-14) high levels of a 27-kDa cytolytic protein are observed. Potentially, therefore, greater mosquitoicidal activity may be obtained with increased levels of a cytolytic toxin. Although we have no conclusive evidence, the 27-kDa protein is likely to be the cytolytic toxin in subsp. *fukuoakensis*. Furthermore, since there is little cross-reactivity between these cytolytic toxins it appears that a series of cytolytic toxins, all of about 27kDa, are present in mosquitoicidal *B. thuringiensis*.

The hemolytic activity of *B. thuringiensis* subsp. *fukuoakensis* can be inhibited by prior incubation of the toxin with unsaturated phosphatidyl choline. The mode of action of all *B. thuringiensis* cytolytic toxins therefore appears to be disruption of the cell membrane by insertion of the toxin into the membrane by using unsaturated phospholipids as the target (6, 12, 32). Following insertion, the toxin undergoes aggregation on the cell membrane, subsequently causing cell lysis (4, 23). The aggregation apparently results in the formation of pores (19) that facilitate cell lysis.

The hemolytic activity of the solubilized *B. thuringiensis* subsp. *fukuoakensis* was lost after protease treatment. In contrast, when solubilized subsp. *morrisoni* (PG-14) crystals are incubated with gut proteases of lepidopteran larvae, a
24-kDa peptide is observed and the hemolytic activity associated with it is retained (40).

In conclusion, we report here the partial characterization of a new *B. thuringiensis* serotype which has mosquitocidal and cytolytic activities. The biological activity in this serotype is derived from proteins that are immunologically distinct from the known *B. thuringiensis* mosquitocidal and cytolytic proteins.

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