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Biochemistry, Genetics, and Mode of Action of *Bacillus thuringiensis* δ-Endotoxins

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Strains within the *Bacillus thuringiensis* species produce protein δ-endotoxins that are toxic to a wide variety of lepidopteran and some dipteran larvae (35). These δ-endotoxins are synthesized during sporulation as parasporal protein inclusions (1, 43). Most of the 20 serotypes of *B. thuringiensis* are toxic to lepidopterans and synthesize a bipyramidal crystalline endotoxin that is refractile when viewed by phase-contrast microscopy. The *B. thuringiensis* subsp. *israelensis* δ-endotoxin is much more irregular in shape (25) and is extremely toxic to the larvae of mosquitoes and blackflies (5, 15). Partly because of their potential as biological insecticides, the study of these δ-endotoxins is of considerable scientific and commercial interest.

Recent studies (13, 16, 17, 20, 26, 27, 34, 49) have demonstrated the presence of a complex array of plasmids in most of the insecticidal *B. thuringiensis* strains. Mutant strains cured of one or more plasmids are frequently no longer able to synthesize the δ-endotoxin. Direct evidence for the existence of plasmid-borne toxin genes in *B. thuringiensis* strains HD-1, HD-73, HD-2, and HD-8 was obtained by analysis of a large number of acrystalliferous mutants (17, 20).

Reports of the isolation and expression of the δ-endotoxin gene of *B. thuringiensis* subsp. *kurstaki* (34, 42) and subsp. *berliner* (28) indicate that the δ-endotoxin gene may be variously located on plasmid DNA, chromosomal DNA, or both (18, 23, 28, 30, 42). For *B. thuringiensis* subsp. *israelensis*, two independent studies of strains cured of one or more plasmids concluded that δ-endotoxin synthesis is critically dependent on the presence of a 72- to 75-megadalton (MDa) plasmid (19, 49). Using the newly discovered capacity of *B. thuringiensis* for plasmid transfer by a conjugationlike mechanism, Gonzalez and Carlton (18) showed that one or more plasmids in several *B. thuringiensis* strains code for the δ-endotoxin structural gene. However, to date, transfer of plasmids from *B. thuringiensis* subsp. *israelensis* to other *B. thuringiensis* serotypes has not been achieved (18), and it therefore remains possible that the 72- to 75-MDa plasmid encodes a regulator of δ-endotoxin production, rather than the structural gene. Using the purified plasmid, we have attempted to resolve this question.

Despite the considerable scientific and industrial interest in these endotoxins, their mechanism of action at the molecular level has proved elusive. Native crystal δ-endotoxin is a water-insoluble protoxin which is solubilized in the alkaline conditions of the larval gut and activated by gut proteases. In vitro activation of the δ-endotoxin has been achieved by incubation of the crystals at high pH under reducing conditions and with insect gut proteases (14, 33). Histological studies in vivo have shown the primary target to be the larval midgut epithelial cells which swell and lyse, causing severe disruption of the gut wall (8, 10, 22, 32, 41). In vitro the activated toxin causes cytolysis of certain lepidopteran cell lines and larval midgut cells (6, 9, 38, 39, 45). Cytological effects have been reported as early as 1 min after exposure to the toxin (6, 12, 41) and are characterized by a rapid general breakdown of permeability barriers to small ions, dyes, and internal markers (8, 39).

Native crystals from different serotypes vary considerably in their polypeptide composition (Fig. 1; 7), and this is undoubtedly responsible at least in part for their differing insect specificity or potency, or both. Of the several polypeptides present in some crystals, it is possible that only one is the active δ-endotoxin; alternatively, each polypeptide may possess a distinct host range. The latter possibility was confirmed when two polypeptide species from the HD-1 strain of *B. thuringiensis* subsp. *kurstaki* were shown to be differentially active against mosquito and lepidopteran larvae (51). By analogy with other bacterial toxins, it is reasonable to argue that insect-specific factors, such as the individual gut environment and specific toxin receptors on the gut epithelial surfaces, play a role in defining the host range. As this report shows, the availability of susceptible insect cell lines has played a large part in allowing us to probe these aspects of δ-endotoxin structure and function.

EXPERIMENTAL PROCEDURES

Except where indicated, the sources of bacterial strains and insect cell lines, the growth and sporulation conditions, the purification and acti-

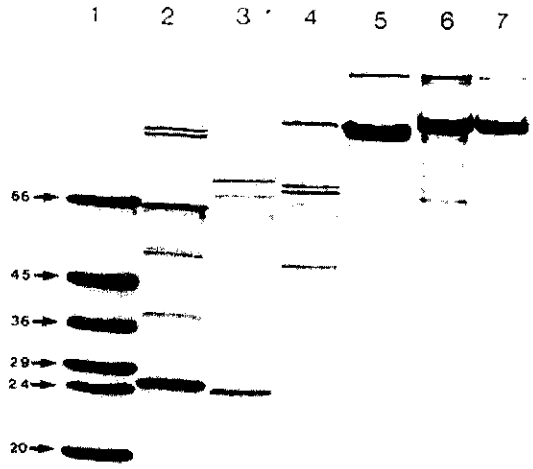

FIG. 1. SDS–10% polyacrylamide gel electrophoresis, Coomassie blue stained. Track 1, Molecular mass standards (kDa); tracks 2–7, native crystal δ-endotoxin from different strains of *B. thuringiensis* (track 2, subsp. *israelensis*; track 3, subsp. *kyushuensis*; track 4, subsp. *darmstadtiensis*; track 5, subsp. *colmeri*; track 6, subsp. *kurstaki*; track 7, subsp. *dakota*).

vation of the crystal δ-endotoxins, and the generation of cured strains have been described previously (29, 44–46, 49).

Preparation of larval gut extracts, activated toxin preparations, liposomes, cell lipids, and bacterial protoplasts was carried out by published methods (29, 45, 46) except where indicated.

In vivo and in vitro assays for toxicity and hemolytic assays were as described previously (45).

Except where indicated in the text, published methods (29, 45) were used for electrophoresis and protein and carbohydrate assays.

Isolation of plasmid DNA. pUC12 plasmid (37) was prepared from *Escherichia coli* JM101 (37) by a lysozyme-detergent lysis method (36) with the following modifications (J. Karn, personal communication): (i) Triton X-100 was used to a final concentration of 1% instead of sodium dodecyl sulfate (SDS), and (ii) NaCl was omitted after Triton addition. Total plasmid DNA from *B. thuringiensis* subsp. *israelensis* was prepared by the method of Casse et al. (3). The 72- to 75-MDa plasmid was subsequently purified from total plasmid DNA by preparative vertical electrophoresis on 0.5% low-gelling-temperature agarose slabs (Seaplaque, FMC Colloids Ltd.) 3 mm thick. Gel bands were visualized and excised as previously described (2, 50). Plasmid DNA from excised bands were purified by phenol-chloroform extraction and ethanol precipitation. Total covalently closed circular DNA for use in the in vitro transcription-translation system was purified on cesium chloride gradients as previously described (36).

Cloning of DNA. Vector (pUC12) and *B. thuringiensis* subsp. *israelensis* DNA (72- to 75-MDa plasmid) was digested to completion with *Hin*dIII (New England Biolabs). Amounts of 25 ng of restricted vector and 100 ng of restricted 72- to 75-MDa plasmid were ligated with T4 DNA ligase (New England Biolabs) at 15°C for 16 h. Portions of the ligation mix were used to transform *E. coli* JM101 (21), and transformants were selected on L agar (36) containing 170 μg of ampicillin per ml.

Preparation of antibodies. The protein δ-endotoxin from *B. thuringiensis* subsp. *israelensis* (26,000 M_r) was isolated from purified crystals by preparative gel electrophoresis (to be described elsewhere). Purified δ-endotoxin was mixed with Freund complete adjuvant, and antibodies were raised by subcutaneous injection of this material into New Zealand White rabbits.

Analysis of recombinants. Recombinant clones were analyzed by use of an in vitro transcription-translation system (24). DNA from individual recombinants or recombinant groups was extracted by a small-scale lysozyme-Triton plasmid preparation method (36; J. Karn, personal communication), and up to 5 μg of DNA was added to the *E. coli* system. Products from the in vitro system were analyzed by use of 13% acrylamide gels (31) and fluorography (4).

In vitro toxicity assays for cloned toxin. Recombinants were grown for 16 h in L broth (36) containing 100 μg of ampicillin per ml and were harvested by centrifugation. Pellets from 1-liter cultures were resuspended in 12 ml of 50 mM Na_2CO_3-HCl (pH 10.5) and disrupted by sonication. The resulting lysate was incubated at 37°C for 1 h, and then saturated ammonium sulfate was added to a final concentration of 30%. The precipitate was pelleted by centrifugation and suspended with 5 ml of 50 mM Na_2CO_3-HCl (pH 10.5). A 50- to 100-μl amount of this suspension was added to a 4-cm petri dish containing *Aedes albopictus* cells as previously described (45, 46).

In vivo toxicity assays of cloned toxin. Recombinants were assayed for in vivo toxicity by a modification of the method of Tyrell et al. (48). Recombinants were grown for 16 h at 37°C in L broth (36) containing 100 μg of ampicillin per ml and were harvested by centrifugation. Pellets from 100 ml of culture were resuspended in 6 ml of distilled water; 1.5 ml of this suspension was added to the cup containing 25 *A. aegypti* larvae.

RESULTS AND DISCUSSION

Mechanism of action of *B. thuringiensis* subsp. *israelensis* δ-endotoxin. The discovery of the

TABLE 1. Effect of various lipid preparations on toxin action in vivo and in vitro

Liposome composition[a]	Toxin/lipid ratio (wt/wt)	Cytopathic effect[b]	In vivo toxicity[c] (no. dead/no. used)
A. albopictus cell lipid	1:10	None	0/9
B. megaterium KM cell lipid	1:50	Lysis	—
Phosphatidylcholine	1:10	None	0/2
Phosphatidylcholine:cholesterol:stearylamine	1:10	None	0/11
Phosphatidylcholine:cholesterol:dicetylphosphate	1:10	None	0/11
Sphingomyelin:cholesterol:stearylamine	1:10	None	0/11
Sphingomyelin:cholesterol:dicetylphosphate	0:10	None	0/8
Soybean phosphatidylethanolamine:cholesterol:dicetylphosphate	0:10	None	0/10
Phosphatidylserine:cholesterol:dicetylphosphate	1:10	Lysis	—
Phosphatidylserine:cholesterol:dicetylphosphate	1:25	None	—
Cardiolipin:cholesterol:dicetylphosphate	1:25	Lysis	20/20
Phosphatidylinositol:cholesterol:dicetylphosphate	1:25	Lysis	4/4
Cerebroside:cholesterol:dicetylphosphate	1:50	Lysis	—

[a] Molar ratios were 2:1.5:0.5. Preparation, dispersion, and sonication of all lipid and liposome preparations were carried out at 30°C except in the case of cerebroside-containing liposomes, which were prepared at 60°C and subsequently assayed at 20°C.

[b] Cytopathology observed after exposure of *A. albopictus* cells to a solution of 5 μg of δ-endotoxin per ml after it had been incubated with the appropriate lipid.

[c] Subcutaneous inoculation of suckling mice; an equivalent of 25 μg of δ-endotoxin per g of mouse was used.

cellular target for *B. thuringiensis* subsp. *israelensis* δ-endotoxin had its origins in the observation (45) that a soluble preparation of subsp. *israelensis* toxin caused rapid lysis of insect and mammalian cells in vitro, but did not affect bacterial protoplasts. This toxin preparation was also hemolytic to a range of erythrocytes. These observations raised the possibility that the subsp. *israelensis* toxin causes lysis directly through interaction with a plasma membrane component.

Subsequent fractionation of mosquito cells to identify possible toxin receptors revealed that preincubation of the toxin with an excess of phospholipids purified from cultured *A. albopictus* cells rendered it inactive, but phospholipids from *B. megaterium* were ineffective even at high lipid-toxin ratios (Table 1). The surprisingly broad eucaryotic specificity of the solubilized subsp. *israelensis* endotoxin suggested that certain ubiquitous eucaryotic plasma membrane phospholipids or sterols might be the target. In contrast to insect and mammalian cells, the *Bacillus* membranes lack phosphatidyl choline, sphingomyelin, cholesterol, or significant amounts of unsaturated fatty acids, suggesting that one or more of these components is recognized by the toxin. This hypothesis was tested in a series of experiments in which sonicated lipid dispersions (liposomes) were examined for their ability to neutralize toxicity in subsequent in vitro and in vivo assays. The results (Table 1; 46) showed that the toxin binds avidly to liposomes containing phosphatidyl choline, sphingomyelin, or phosphatidyl ethanolamine, binds less strongly to phosphatidyl serine, and

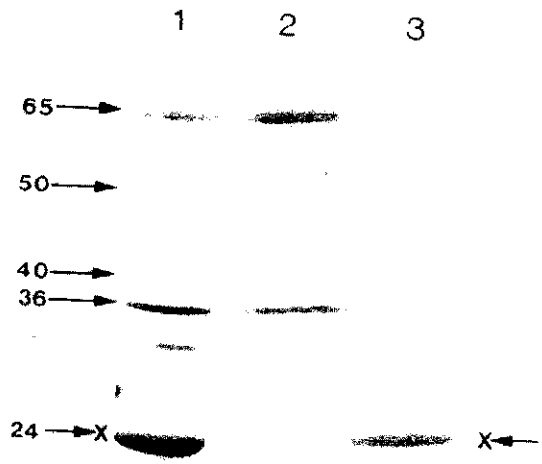

FIG. 2. SDS–13% polyacrylamide gel electrophoresis, Coomassie blue stained. Track 1, Soluble subsp. *israelensis* δ-endotoxin obtained by incubation of native purified crystal δ-endotoxin in 50 mM Na_2CO_3-HCl (pH 10.5); track 2, polypeptides remaining in the supernatant; track 3, polypeptides sedimenting with liposomes after centrifugation of a 1:10 (wt/wt) mixture of the soluble subsp. *israelensis* δ-endotoxin in track 1 with phosphatidyl choline (egg) liposomes that had been incubated at 37°C for 2 h. Endogenous proteolysis during the alkali solubilization of the native crystal in this experiment has resulted in degradation of the native *israelensis* 26,000 M_r toxin polypeptide to 24,000 M_r. Arrows denote molecular mass (kDa).

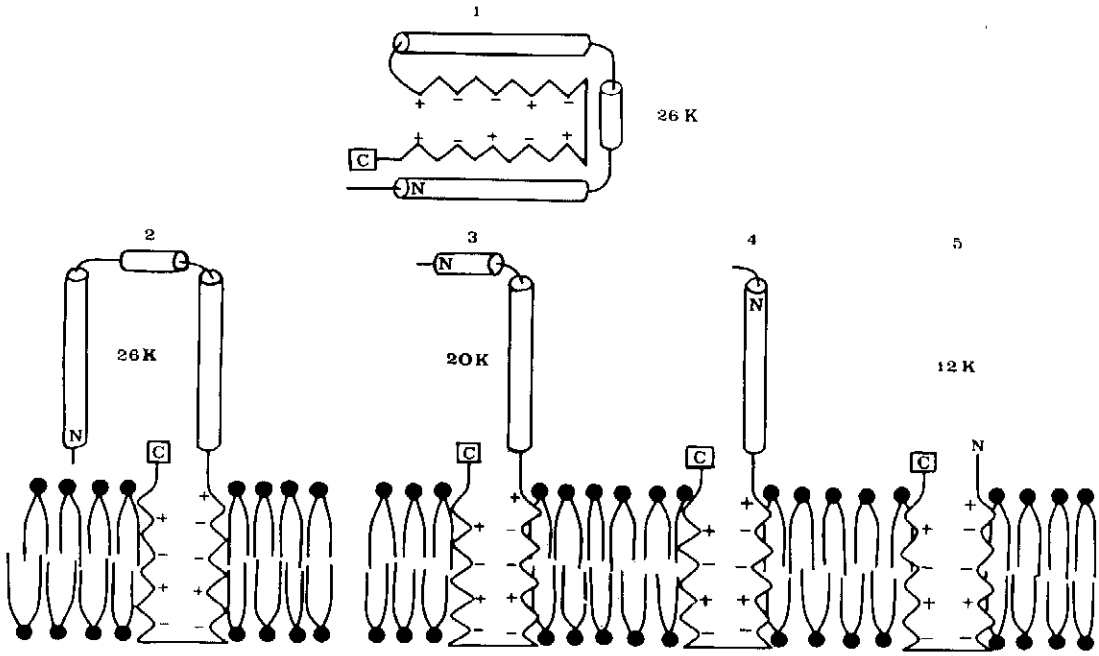

FIG. 3. Diagram summarizing the conformational changes occurring upon insertion of the 26,000 M_r alkali-soluble subsp. *israelensis* δ-endotoxin into lipid vesicles and the subsequent proteolytic processing after exposure of the vesicles to external trypsin.

shows no affinity for phosphatidyl inositol, cardiolipin, cerebroside glycolipid, or cholesterol. Confirmation of the strength of toxin binding came from the finding that, when phosphatidyl choline liposomes were centrifuged after incubation with the toxin, the supernatant was devoid of toxin activity (46). Gel electrophoresis of the liposome pellet and supernatant revealed that only one of the subsp. *israelensis* crystal polypeptides (26,000 M_r) had become inserted into the lipid bilayer (Fig. 2). These findings provided strong evidence for the identification of the 26,000 M_r component as the toxic polypeptide.

To determine the extent of insertion of the 26,000 M_r polypeptide into the membrane, we allowed a solubilized toxin preparation to bind to artificial membrane vesicles, removed unbound toxin, and then exposed the vesicles to trypsin to digest away any exposed portion of the 26,000 M_r polypeptide. When the vesicles were recovered and examined by electrophoresis and peptide mapping, a 12,000 M_r fragment of the δ-endotoxin was found to be protected from trypsin digestion by insertion into the lipid. Figure 3 summarizes these experiments. Upon contact with the membrane surface, the previously shielded hydrophobic terminal region partitions into the lipid bilayer, leaving the remaining half of the toxin exposed and unfolded where it is the substrate for a series of proteolytic processing steps that (depending on the protease specificity) can ultimately trim away all the exposed moiety.

Against this background we attempted in other experiments to identify the structural features in the phospholipids that are responsible for endotoxin binding. The results (46) revealed two crucial determinants: (i) the presence of unsaturated fatty acid substituents and (ii) the correct

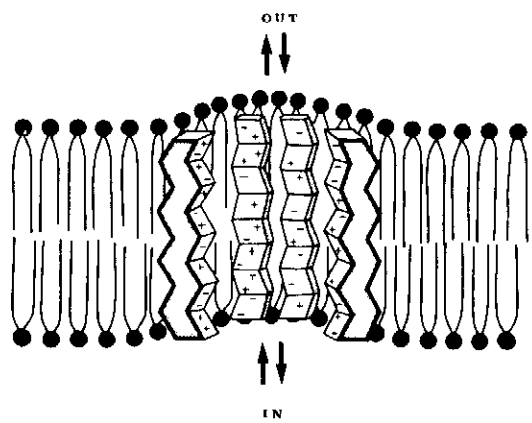

FIG. 4. Model illustrating a possible arrangement of transmembrane amphipathic helical polypeptide segments from one or more molecules of subsp. *israelensis* δ-endotoxin. (See text for details.)

phospholipid headgroup, exemplified by the dipolar ionic phosphatidyl choline or by phosphatidyl ethanolamine. Anionic phospholipids (even when highly unsaturated) were unable to bind the toxin. The broad eucaryotic specificity of the activated toxin is immediately explained by these results, since unsaturated phosphatidyl choline, sphingomyelin, and phosphatidyl ethanolamine are the major phospholipid species in most higher cells. We suggested (46) that in susceptible insects insertion of the δ-endotoxin into the plasma membranes of gut epithelial cells causes a detergentlike rearrangement of membrane lipids, leading to disruption of membrane integrity and eventually to cytolysis.

Further experiments are in progress to map the topography of the membrane lesion produced by toxin binding and to probe the stoichiometry of the toxin-phospholipid complexes. Figure 4 illustrates one speculative model combining the possibilities of toxin oligomerization with the protein structural feature known as the amphipathic helix (11). The latter can be formed by appropriate folding of a polypeptide with hydrophobic residues at every third or fourth position in such a way as to position all the hydrophobic residues on one helix face, while the other face is hydrophilic. This generates the amphipathic helices illustrated in Fig. 4, which have an affinity for both polar and nonpolar environments. Self-association of intramolecular or intermolecular helical segments can yield the transmembrane structure shown in the model, in which there is internal compensation of the charged helix face and maximal lipid solubility by virtue of the externally directed hydrophobic face.

Since the experiments summarized in Fig. 3 indicate that the 12,000 M_r toxin domain binds avidly to phospholipid, it is conceivable that the four amphipathic helical segments depicted in the model could be derived from one 12,000 M_r segment. Alternatively, the hydrophilic membrane channel may be created by the self-association of amphipathic helices from several 12,000 M_r fragments. Creation of a number of these hydrophilic channels by the toxin could prove cytolytic by subverting the selective permeability of the cell membrane. The model is a considerable oversimplification and completely neglects other potential toxic mechanisms, such as the possibility that a toxin in the form of an amphipathic helix could still seriously perturb critical membrane functions through an association with only the outer half of the lipid bilayer.

Molecular genetics of *B. thuringiensis* subsp. *israelensis* δ-endotoxin. In previous reports we (49) and others (19) demonstrated that δ-endotoxin synthesis in *B. thuringiensis* subsp. *israelensis* is critically dependent upon the presence of a 72- to 75-MDa plasmid. The simplest interpretation of this finding was that the endotoxin structural gene is located on this plasmid, although it was also possible that the plasmid merely encoded a regulator of δ-endotoxin synthesis. To distinguish between these two possibilities, we constructed recombinant plasmids by inserting *Hin*dIII restriction fragments of the subsp. *israelensis* plasmid into the *E. coli* vector pUC12.

Preliminary screening with restriction enzymes had shown that, when *Hin*dIII-digested *B. thuringiensis* subsp. *israelensis* total plasmid DNA was added to an *E. coli* in vitro transcription-translation system, a single novel polypeptide identical in molecular weight to the authentic subsp. *israelensis* toxin was precipitated by antiserum raised to the purified 26,000 M_r δ-endotoxin (Fig. 5). *Hin*dIII was therefore used to digest the purified 72- to 75-MDa plasmid, and the products were ligated into *Hin*dIII-digested pUC12. After transformation of *E. coli* JM101 with the ligation mixture, a library of 450 colonies was selected. Random analysis of this library showed that 77% were recombinants (data not shown).

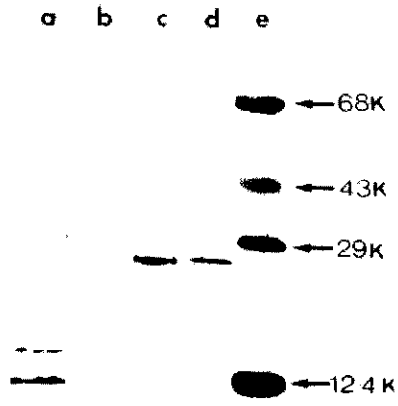

FIG. 5. Fluorographs of SDS–13% polyacrylamide gels of the ^{35}S-labeled polypeptides synthesized in the *E. coli* transcription-translation system primed with either *Hin*dIII-digested total *B. thuringiensis* subsp. *israelensis* plasmid DNA or plasmid pIP174 DNA and supplemented with L-[^{35}S]methionine: lane a, total products from pIP174 DNA; lane b, material precipitated from a by addition of preimmune serum; lane c, material precipitated from a by addition of antibody raised against the 26,000 M_r authentic *israelensis* δ-endotoxin; lane d, material precipitated by antibody against the 26,000 M_r authentic *israelensis* δ-endotoxin from the *E. coli* transcription-translation system primed with *Hin*dIII-digested total *B. thuringiensis* subsp. *israelensis* plasmid DNA; lane e, molecular weight standards.

FIG. 6. Agarose gel electrophoresis of covalently closed circular forms of pIP174, pUC12, and their *Hin*dIII digestion products. Lane a, pIP174 (covalently closed circular form); lane b, pUC12 (covalently closed circular form); lane c, *Hin*dIII-digested pIP174; lane d, *Hin*dIII-digested pUC12; lane e, *Hin*dIII-digested λ DNA with fragment sizes on the right margin (in kb).

Plasmid DNA was then extracted from the clone library and screened for toxin production by immunoprecipitation with the use of the in vitro transcription-translation system (49a). By using this approach, two toxin-coding recombinants, pIP173 and pIP174, were identified and used for subsequent analysis. Figure 6 shows the results of horizontal agarose gel electrophoresis of the covalently closed circular forms of pIP174 (lane a) and pUC12 (lane b) extracted from individual recombinants, together with their *Hin*dIII digestion products. Digestion of pIP174 yielded a 9.7-kilobase (kb) insert (lane c) in addition to the 2.7-kb fragment derived from pUC12 (lane d). Identical results were obtained with *Hin*dIII-digested pIP173 (data not shown).

Although the immunoprecipitation of the in vitro-synthesized cloned product showed clearly that a 26,000 M_r polypeptide antigenically related to the subsp. *israelensis* δ-endotoxin was encoded by the 9.7-kb insert, additional experiments were needed to confirm that the polypeptide was biologically active. Lysates were therefore prepared from 1-liter cultures of colony 174 and control *E. coli* JM101 colonies containing pUC12 lacking any insert and were assayed for toxicity in vitro. Protein extracted from colony 174 caused cytolysis of *A. albopictus* cells indistinguishable from that previously described for authentic δ-endotoxin (45, 46, 49a). *Aedes* cells exposed to an equivalent protein extract from *E. coli* containing the vector pUC12 alone were unaffected, even after prolonged exposure (24 h). The authenticity of the pIP174-encoded polypeptide was further confirmed by demonstrating that the toxicity of the 174 lysate could be neutralized either by antiserum directed against the 26,000 M_r native δ-endotoxin or by preincubation of the lysate with sonicated preparations of those phospholipids previously shown to be the cell membrane receptors for authentic δ-endotoxin (46). Final confirmation of the biological authenticity of the cloned product was obtained from in vivo bioassays. Twenty-five second-instar *A. aegypti* larvae were killed in 4 h when fed an amount of *E. coli* containing pIP174 equivalent to 25 ml of original culture. In control experiments larvae fed equivalent amounts of *E. coli* JM101 containing pUC12 with no insert were unaffected.

Addition of a β-galactosidase inducer, isopropyl-β-D-thiogalactopyranoside, to cultures of *E. coli* JM101 containing pIP174 did not

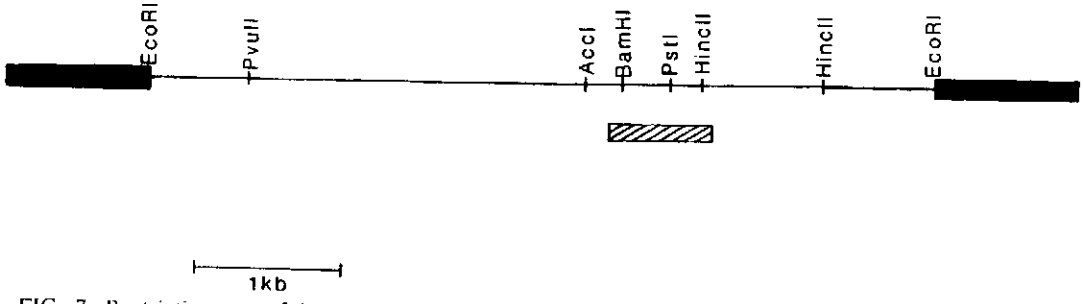

FIG. 7. Restriction map of the 5.4-kb insert of pIPEco5. Thick lines represent pUC12 DNA. The boxed area represents the location of the δ-endotoxin gene, determined by analysis of subclones of pIPEco5 in the *E. coli* in vitro system.

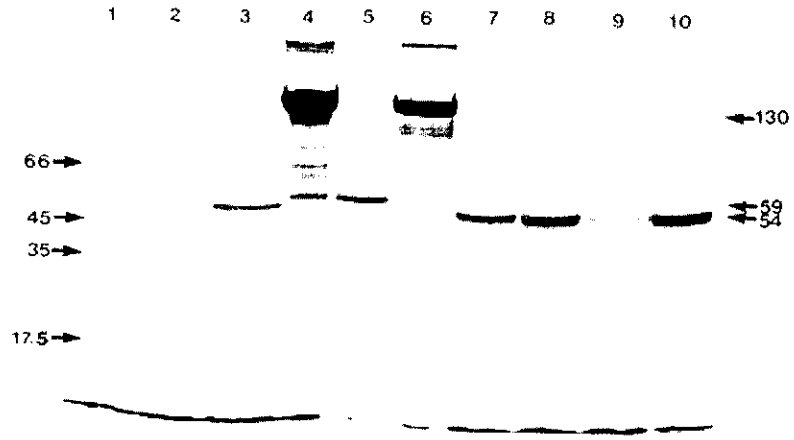

FIG. 8. SDS–10% polyacrylamide gel electrophoresis, Coomassie blue stained. Track 1, Molecular weight standards (kDa); track 2, 10 μl of *P. brassicae* gut extract; track 3, 50 μg of soluble subsp. *kurstaki* crystal protein incubated with 10 μl of *P. brassicae* gut extract for 15 min, 37°C; track 4, 50 μg of native subsp. *kurstaki* crystal δ-endotoxin; track 5, insoluble subsp. *kurstaki* crystal protein; track 6, soluble subsp. *kurstaki* crystal protein obtained by incubation of 50 μg of native crystal δ-endotoxin in 50 mM Na_2CO_3-HCl (pH 9.5) and 10 mM dithiothreitol; track 7, 50 μg of soluble subsp. *kurstaki* crystal protein after 20 days, 20°C; track 8, 50 μg of soluble crystal protein after 33 days, 20°C; track 9, 50 μg of soluble crystal protein after 45 days, 20°C; track 10, 50 μg of soluble crystal protein after 62 days, 20°C.

result in an increase in toxin production measured in vitro. This suggests that expression of the δ-endotoxin gene in *E. coli* is under the control of *B. thuringiensis* promoter sequences rather than the vector β-galactosidase promoter. More recent work has led to the isolation of a 5.4-kb *Eco*RI fragment derived from pIP173. A recombinant plasmid (pIPEco5) consisting of this 5.4-kb *Eco*RI insert in pUC12 directs the synthesis of the 26,000 M_r δ-endotoxin in the *E. coli* lysate. A preliminary restriction map of pIPEco5 is shown in Fig. 7. In subcloning experiments to date we have located the promoter region and almost all the structural gene on the smaller *Pvu*II-*Hin*cII fragment (3.1 kb) shown in Fig. 7. The availability of the δ-endotoxin primary sequence will be invaluable in assessing the various models for toxin-phospholipid interaction.

Mechanism of action of *B. thuringiensis* subsp. *kurstaki* lepidopteran toxin. Crystals from the HD-1 strain of *B. thuringiensis* subsp. *kurstaki* contain at least two distinct toxic moieties, a lepodipteran-specific toxin (P1) and a "mosquito factor" (P2) toxic to both lepidoptera and diptera (51) that can be separated under alkaline reducing conditions (45). Figure 8 (tracks 5 and 6) shows the separation of the insoluble 63,000 M_r mosquito toxin (P2) from the alkali-soluble lepidopteran-specific P1 (45, 51). Activation of the 126,000 M_r P1 endotoxin by either larval gut proteases (track 3) or endogenous proteases (tracks 7 to 10) yielded a major polypeptide of 54,000 M_r. When activated by either procedure, the soluble toxin retained its lepidopteran specificity (29). Thus, the activated toxin was lethal to *Pieris brassicae* larvae in vivo, but no toxic effects were observed on injection of suckling mice. Similarly activated toxin at 50 μg/ml caused 50% lysis of CF1 cells in 60 min in vitro, as assessed by vital staining with trypan blue, but human erythrocytes were unaffected by 100 μg of toxin per ml after 3 h. Controls containing 50 mM Na_2CO_3-HCl (pH 9.5) and 10 mM dithiothreitol, with *P. brassicae* gut extract and fetal calf serum where appropriate, showed no toxic effects in vivo or in vitro (29).

As discussed above, the ubiquitous presence in eucaryotic plasma membranes of the phospholipids that serve as plasma membrane receptors for the potent mosquitocidal *B. thuringiensis* subsp. *israelensis* δ-endotoxin explains the finding that subsp. *israelensis* δ-endotoxin activated in vitro is cytolytic to a wide variety of cell types, including mammalian cells (45). In contrast, the observation that, even after activation, the *B. thuringiensis* subsp. *kurstaki* P1 δ-endotoxin affects only lepidopteran larvae and cell lines suggests that the membrane receptor for this toxin may be cell specific either in whole or in part. The identity of this proposed specific plasma membrane receptor was investigated initially by assessing the ability of various molecules to neutralize the cytolytic effect of activated P1 toxin against *Choristoneura fumiferana* CF1 cells in vitro (29).

Preincubation with various lipid preparations was used to investigate the possibility that the lepidopteran-specific subsp. *kurstaki* P1 toxin caused cytolysis by interaction with plasma

TABLE 2. Effect of preincubation with monosaccharides on toxicity of soluble δ-endotoxin in vitro

Monosaccharide[a]	Concn (mM)	Cytopathic effect[b]
N-Acetyl-D-glucosamine	400	Lysis
N-Acetyl-D-galactosamine	125	Complete protection
	25	Partial protection
N-Acetylneuraminic acid	125	Complete protection
	25	Partial protection
D-Galactose	250	Lysis
L-Fucose	250	Lysis
Muramic acid	75	Lysis
Galactosamine	250	Lysis
D-Glucose	250	Lysis
D-Mannose	250	Lysis

[a] Monosaccharides and activated soluble δ-endotoxin were incubated together for 60 min at 20°C before addition to cells.
[b] Cytopathology observed in CF1 cells at a δ-endotoxin concentration of 50 μg/ml.

membrane phospholipids, as observed for the *B. thuringiensis* subsp. *israelensis* mosquitocidal δ-endotoxin (46). The inability of any of the lipid preparations used (29) to neutralize toxicity against CF1 cells suggested that the specificity of toxin-membrane interactions was determined by a different membrane component. In a series of experiments designed to explore the possibility of a carbohydrate-containing P1 receptor, we tested the ability of various lectins and monosaccharides to neutralize the toxin. The results (Table 2) indicated that the toxin was completely inactivated by prior incubation with *N*-acetylgalactosamine and *N*-acetylneuraminic acid, but not by a range of other monosaccharides tested.

Of the lectins tested for toxin-neutralizing ability, only wheat germ agglutinin and soybean agglutinin partially protected CF1 cells from the toxin (Table 3). This effect was noted both with prior incubation of cells with lectin and of activated toxin with lectin. The ability of wheat germ agglutinin to inhibit toxicity was abolished when the lectin was incubated with 250 mM *N*-acetylglucosamine prior to incubation with cells or toxin.

Many other toxins and biological ligands bind to specific glycoprotein or glycolipid receptors in the plasma membrane. The above results with the P1 endotoxin led us to suggest (29) that binding of the lepidopteran-specific toxin to a specific glycoconjugate on the plasma membrane of susceptible cells is an essential feature of its cytolytic action. Since *N*-acetylgalactosamine completely neutralized the toxin, it seems likely to be part of the receptor. *N*-Acetylneuraminic acid is not likely to be involved since this monosaccharide is absent from all insects tested so far (29). Wheat germ agglutinin and soybean agglutinin both bind terminal *N*-acetylgalactosamine; therefore, their observed ability to protect CF1 cells from the toxin may be explained by competition between lectin and toxin for the same binding site on the cell surface. Specific binding of *N*-acetylgalactosamine to the carbohydrate recognition site of the endotoxin in the preincubation experiments blocks subsequent attachment to the cell surface receptor. The inability of *N*-acetylglucosamine to protect the cells (Table 2) is interesting in two ways. First, it rules out a nonspecific effect of amino sugars, and second, it indicates the extremely rigorous stereospecificity of the toxin, since, as Fig. 9 shows, the only difference between *N*-acetylgalactosamine and *N*-acetylglucosamine is the disposition of the hydroxyl group on carbon 4. *N*-Acetylneuraminic acid has no hydroxyl group in the analogous position.

Those toxins which must cross the plasma membrane, such as diphtheria toxin, generally show a time lag before the first symptoms of toxicity are observed. In contrast, the first effects of *B. thuringiensis* subsp. *kurstaki* δ-endotoxin occur within 1 min (6, 12, 41). The precise mechanism of action of the lepidopteran-specific subsp. *kurstaki* P1 toxin has yet to be elucidated, but it appears that after binding to a plasma membrane receptor the membrane is

TABLE 3. Effect of preincubation with lectins on toxicity of soluble δ-endotoxin in vitro

Lectin[a]	Sugar binding specificity	Cytopathic effect[b]
Concanavalin A	α-D-Mannose > α-D-glucose	Lysis
Peanut agglutinin	β-D-Galactose	Lysis
Soybean agglutinin	*N*-Acetylgalactosamine	Protection
Wheat germ agglutinin	*N*-Acetylglucosamine > *N*-acetylneuraminic acid > *N*-acetylgalactosamine	Protection
Ulex europaeus agglutinin	α-L-Fucose	Lysis

[a] Lectins and activated soluble δ-endotoxin were incubated together for 60 min at 20°C before addition to cells.
[b] Cytopathology observed in CF1 cells at a δ-endotoxin concentration of 50 μg/ml.

rapidly made leaky to small ions and larger molecules.

Studies with other *B. thuringiensis* strains. Progress of the type described above has provided a platform for us to begin a larger comparative study of a number of *B. thuringiensis* strains from different serotypes with reportedly differing insect specificity. This approach makes use of antibodies to purified toxin polypeptides and cloned toxin genes as probes in conjunction with the in vitro cell assay. As an example of the results, we have found (F. Drobniewski, T. Sawyer, and D. J. Ellar, unpublished data) that, although the δ-endotoxin from a derivative of a known serotype 10 strain (40) with potent mosquitocidal activity closely resembles serotype 14 subsp. *israelensis* in its mechanism of action, it does not cross-react with antisera to the purified subsp. *israelensis* δ-endotoxin. Similar studies have shown that, among other mosquitocidal toxins that we have purified, some (from *kurstaki* P2 and *colmeri*) are immunologically unrelated to that of *israelensis*, whereas others (from *kyushuensis*) are weakly cross-reactive. These and other data on the mechanism of action of these different endotoxins indicate that several mosquitocidal strategies are exploited by *B. thuringiensis*. The availability of primary sequence data for many of these toxins, especially those which are immunologically similar but show different 50% lethal concentrations, should provide valuable data on the toxin active site residues.

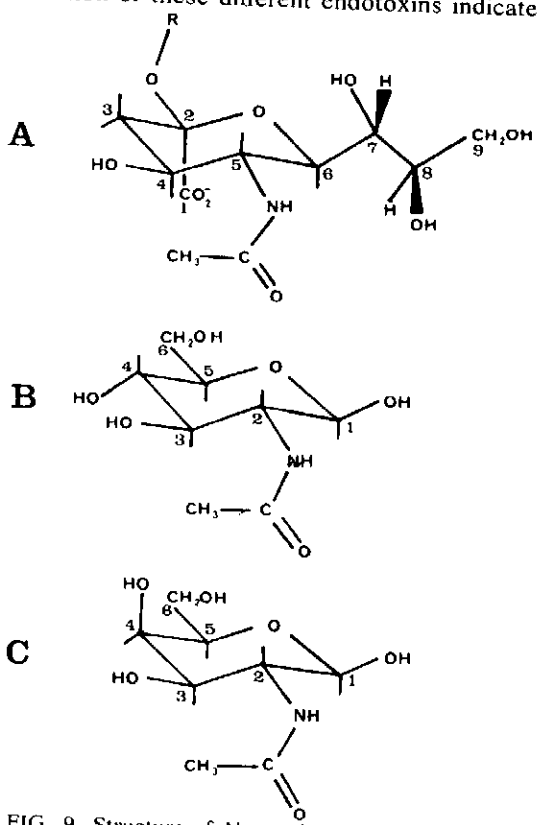

FIG. 9. Structure of *N*-acetylneuraminic acid (A), *N*-acetylglucosamine (B), and *N*-acetylgalactosamine (C).

Other workers (7) have reported that a number of *B. thuringiensis* strains produce apparently normal δ-endotoxin crystals that have not proved to be toxic to any insect species so far tested in vivo. In some cases (*dakota*) we have been able to activate the protoxin in vitro and demonstrate activity in the in vitro cell assay (D. Last, B. Knowles, C. Nichols, and D. J. Ellar, unpublished data). It thus appears that, in some cases at least, absence of in vivo toxicity in the test insects may reflect a failure in protoxin processing in the gut.

For some of the lepidopteran toxins, such as *thuringiensis* HD-2 and *kurstaki* P1 toxins, analysis of glycoproteins and glycolipids in plasma membranes from susceptible insect cells is likely to uncover the membrane receptor(s). Toxins like those of *kurstaki* P2 and *colmeri* that are active against mosquitoes and lepidopterans both in vivo and in vitro are an especially interesting group by comparison with the *israelensis* toxin, which, despite its ubiquitous phospholipid target, is inactive in the lepidopteran gut although rapidly lethal when injected into the hemocoel. The latter observation highlights the possibility that individual insect gut environments may in part be an explanation for the differing insecticidal potency of *B. thuringiensis* strains. The observation (W. E. Thomas, B. Knowles, J. Lewis, and D. J. Ellar, unpublished data) that freshly isolated lepidopteran gut cells display a degree of resistance to the *israelensis* toxin, whereas cultured lepidopteran cell lines are rapidly lysed (45), suggests that resistance in this case may be determined by some feature of the gut cell plasma membrane. In view of the need for the *israelensis* endotoxin to make close contact with the lipid bilayer for toxin insertion, it is conceivable that steric hindrance or charge interactions caused by epithelial cell surface components may play a role in resistance. These results also illustrate the need for caution in interpreting the results from in vitro assays performed with stable insect cell lines which may lack features normally present in the differentiated native gut cells.

SUMMARY

Specific phospholipids have been identified as the membrane receptors for the *B. thuringiensis* subsp. *israelensis* insecticidal δ-endotoxin. Experiments with artificial membranes show that the toxin binds to phosphatidyl choline, sphin-

gomyelin, and phosphatidyl ethanolamine, provided these lipids contain unsaturated fatty acyl substituents. A possible insecticidal mechanism is proposed in which toxin insertion into the epithelial cell membrane creates a nonspecific hydrophilic leak channel, leading to a breakdown of membrane integrity and rapid cytolysis. *Hin*dIII fragments of the 72- to 75-MDa plasmid containing the *israelensis* δ-endotoxin gene have been cloned in pUC12. Two recombinants (pIP174 and pIP173) producing the 26,000 M_r δ-endotoxin have been identified by screening a clone library in an *E. coli* in vitro transcription-translation system. The 26,000 M_r polypeptide synthesized in vivo from pIP174 transformed into *E. coli* JM101 was lethal to mosquito larvae and cytotoxic to mosquito cells in vitro. An investigation of the in vitro-activated lepidopteran-specific P1 protoxin from *B. thuringiensis* subsp. *kurstaki* showed that its toxicity towards *C. fumiferana* CF1 cells was specifically inhibited by preincubation with *N*-acetylgalactosamine and by the lectins soybean agglutinin and wheat germ agglutinin, which bind *N*-acetylgalactosamine. These results suggest that the lepidopteran membrane receptor for this P1 toxin is a glycoconjugate containing terminal *N*-acetylgalactosamine.

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LITERATURE CITED

1. **Bulla, L. A., Jr., D. B. Bechtel, K. J. Kramer, Y. I. Shethna, A. I. Aronson, and P. C. Fitz-James.** 1980. Ultrastructure, physiology and biochemistry of *Bacillus thuringiensis*. Crit. Rev. Microbiol. **8**:147–204.
2. **Burns, D. M., and I. R. Beacham.** 1983. A method for the ligation of DNA following isolation from low melting temperature agarose. Anal. Biochem. **135**:48–51.
3. **Casse, F., C. Boucher, J. S. Juliott, M. Michel, and J. Dénairié.** 1979. Identification and characterization of large plasmids in *Rhizobium meliloti* using agarose gel electrophoresis. J. Gen. Microbiol. **113**:229–242.
4. **Chamberlain, J. P.** 1979. Fluorographic detection of radioactivity in polyacrylamide gels with water-soluble fluor, sodium salicylate. Anal. Biochem. **98**:132–135.
5. **De Barjac, H.** 1978. Une nouvelle variété de *Bacillus thuringiensis* trés toxique pour les moustiques: *Bacillus thuringiensis* var. *israeliensis* sérotype 14. C. R. Acad. Sci. Ser. D **286**:797–800.
6. **De Lello, E. E., W. K. Hanton, S. T. Bishoff, and D. W. Misch.** 1984. Histopathological effects of *Bacillus thuringiensis* on the midgut of tobacco hornworm larvae (*Manduca sexta*): low doses compared with fasting. J. Invertebr. Pathol. **43**:169–181.
7. **De Lucca, A. J., M. S. Palmgren, and H. De Barjac.** 1984. A new serovar of *Bacillus thuringiensis* from grain dust: *Bacillus thuringiensis* serovar comeri (Serovar 21). J. Invertebr. Pathol. **43**:437–438.
8. **Ebersold, H. R., P. Lüthy, P. Geiser, and L. Ettlinger.** 1978. The action of the δ-endotoxin of *Bacillus thuringiensis*: an electron microscopic study. Experientia **34**:1672.
9. **Ebersold, H. R., P. Lüthy, and H. E. Huber.** 1980. Membrane damaging effect of the δ-endotoxin of *Bacillus thuringiensis*. Experientia **36**:495.
10. **Endo, Y., and J. Nishiitsutsuji-Uwo.** 1980. Mode of action of *Bacillus thuringiensis* δ-endotoxin: histopathological changes in the silkworm midgut. J. Invertebr. Pathol. **36**:90–103.
11. **Epand, R. M.** 1983. The amphipathic helix: its possible role in the interaction of glucagon and other peptide hormones with membrane receptor sites. Trends Biochem. Sci. **8**:205–207.
12. **Fast, P. G., and T. P. Donaghue.** 1971. The δ-endotoxin of *Bacillus thuringiensis*. II. On the mode of action. J. Invertebr. Pathol. **18**:135–138.
13. **Faust, R. M., K. Abe, G. A. Held, T. Iizuka, L. A. Bulla, and C. L. Meyers.** 1983. Evidence for plasmid-associated crystal toxin production of *Bacillus thuringiensis* subsp. *israeliensis*. Plasmid **9**:98–103.
14. **Faust, R. M., G. M. Hallam, and R. S. Travers.** 1974. Degradation of the parasporal crystal produced by *Bacillus thuringiensis* var. *kurstaki*. J. Invertebr. Pathol. **24**:365–373.
15. **Goldberg, L. J., and J. Margalitt.** 1977. A bacterial spore demonstrating rapid larvicidal activity against *Anopheles sergentii*, *Uranotaenia unguiculata*, *Culex univittatus*, *Aedes aegypti* and *Culex pipiens*. Mosq. News **37**:355–358.
16. **Gonzalez, J. M., Jr., B. J. Brown, and B. C. Carlton.** 1982. Transfer of *Bacillus thuringiensis* plasmids coding for δ-endotoxin among strains of *B. thuringiensis* and *B. cereus*. Proc. Natl. Acad. Sci. U.S.A. **79**:6951–6955.
17. **Gonzalez, J. M., Jr., and B. C. Carlton.** 1980. Patterns of plasmid in crystalliferous and acrystalliferous strains of *Bacillus thuringiensis*. Plasmid **3**:92–98.
18. **Gonzalez, J. M., Jr., and B. C. Carlton.** 1982. Plasmid transfer in *Bacillus thuringiensis*, p. 85–95. *In* U. N. Streips, S. H. Goodgal, W. R. Guild, and G. A. Wilson (ed.), Genetic exchange, a celebration and a new generation. Marcel Dekker, New York.
19. **Gonzalez, J. M., Jr., and B. C. Carlton.** 1984. A large transmissible plasmid is required for crystal toxin production in *Bacillus thuringiensis* variety *israeliensis*. Plasmid **11**:28–38.
20. **Gonzalez, J. M., Jr., H. T. Dulmage, and B. C. Carlton.** 1981. Correlation between specific plasmids and δ-endotoxin production in *Bacillus thuringiensis*. Plasmid **5**:351–365.
21. **Hanahan, D.** 1983. Studies on transformation of *Escherichia coli* with plasmids. J. Mol. Biol. **166**:557–580.
22. **Heimpel, A. M., and T. A. Angus.** 1960. Bacterial insecticides. Bacteriol. Rev. **24**:266–288.
23. **Held, G. A., L. A. Bulla, Jr., E. Ferrari, J. Hoch, A. I. Aronson, and S. A. Minnich.** 1982. Cloning and localization of the lepidopteran protoxin gene of *Bacillus thuringiensis* subsp. *kurstaki*. Proc. Natl. Acad. Sci. U.S.A. **79**:6065–6069.
24. **Howe, C. J., C. M. Bowman, T. A. Dyer, and J. C. Gray.** 1982. Localization of wheat chloroplast genes for the beta and epsilon subunits of ATP synthase. Mol. Gen. Genet. **186**:525–530.
25. **Huber, H. E., and P. Lüthy.** 1981. *Bacillus thuringiensis* delta-endotoxin: composition and activation, p. 209–234. *In* E. W. Davidson (ed.), Pathogenesis of invertebrate microbial diseases. Allanheld Osmun & Co., Totowa, N.J.
26. **Jarrett, P.** 1983. Comparison of plasmids from twelve isolates of *Bacillus thuringiensis* H-serotype 7. FEMS Microbiol. Lett. **16**:55–60.
27. **Kamdar, H., and K. Jayaraman.** 1983. Spontaneous loss of a high molecular weight plasmid and the biocide of *Bacillus thuringiensis* var. *israeliensis*. Biochem. Bi-