

University of Nebraska - Lincoln

DigitalCommons@University of Nebraska - Lincoln

Papers in Microbiology

Papers in the Biological Sciences

January 1990

Toxicity of Protease-Resistant Domains from the Delta-Endotoxin of *Bacillus thuringiensis* subsp. *israelensis* in *Culex quinquefasciatus* and *Aedes aegypti* Bioassays

Mary Ann Pfannenstiel
University of Nebraska-Lincoln

William C. Cray Jr.
University of Nebraska-Lincoln

Graham Couche
University of Nebraska-Lincoln

Kenneth W. Nickerson
University of Nebraska-Lincoln, knickerson1@unl.edu

Follow this and additional works at: <https://digitalcommons.unl.edu/bioscimicro>

 Part of the [Microbiology Commons](#)

Pfannenstiel, Mary Ann; Cray, William C. Jr.; Couche, Graham; and Nickerson, Kenneth W., "Toxicity of Protease-Resistant Domains from the Delta-Endotoxin of *Bacillus thuringiensis* subsp. *israelensis* in *Culex quinquefasciatus* and *Aedes aegypti* Bioassays" (1990). *Papers in Microbiology*. 26.
<https://digitalcommons.unl.edu/bioscimicro/26>

This Article is brought to you for free and open access by the Papers in the Biological Sciences at DigitalCommons@University of Nebraska - Lincoln. It has been accepted for inclusion in Papers in Microbiology by an authorized administrator of DigitalCommons@University of Nebraska - Lincoln.

Toxicity of Protease-Resistant Domains from the Delta-Endotoxin of *Bacillus thuringiensis* subsp. *israelensis* in *Culex quinquefasciatus* and *Aedes aegypti* Bioassays

MARY ANN PFANNENSTIEL, WILLIAM C. CRAY, JR., GRAHAM A. COUCHE,[†]
AND KENNETH W. NICKERSON*

School of Biological Sciences, University of Nebraska, Lincoln, Nebraska 68588-0343

Received 15 February 1989/Accepted 29 August 1989

The mosquitocidal glycoprotein endotoxin of *Bacillus thuringiensis* subsp. *israelensis* was digested with chymotrypsin to yield protease-resistant domains which were then separated from smaller protease digestion products by high-performance liquid chromatography. Once purified, the domains no longer bound wheat germ agglutinin, a lectin which binds *N*-acetylglucosamine (GlcNAc) and GlcNAc oligomers. Purified protease-resistant domains were as toxic for *Culex quinquefasciatus* larvae as intact solubilized toxin. In separate experiments, the toxicity of chymotrypsin-digested endotoxin for *Aedes aegypti* larvae was reduced fivefold or more. A model is presented in which GlcNAc-containing oligosaccharides are required for toxicity for *A. aegypti* larvae but not *C. quinquefasciatus* larvae.

Bacillus thuringiensis isolates produce crystalline proteins that are toxic for insect larvae. These delta-endotoxins are produced during sporulation. The crystal proteins are often synthesized as protoxins which require activation before they can express their entomocidal activity. In nature, crystals ingested by insect larvae are solubilized, and then the active toxins are released by the combined action of high pH and proteases in the gut. Solubilized protoxin may also be converted to active toxin in vitro. High-pH buffers are used to solubilize the crystals, and the protoxin is treated with proteases to produce an active toxin (1). The remaining protein entities are called protease-resistant domains. Protease-resistant domains have been obtained from the delta-endotoxins of *B. thuringiensis* subsp. *kurstaki* (4), *B. thuringiensis* subsp. *israelensis* (18), and *Bacillus sphaericus* (6).

The *B. thuringiensis* subsp. *israelensis* toxin is lethal for many mosquito and blackfly species, but it is not effective against lepidopteran insects (8). It is composed of multiple protein subunits and has both larvicidal activity and general cytolytic activity (3, 13, 20). A 28-kilodalton protein is responsible for the general cytolytic activity (2, 10, 18), but larvicidal activity is due to a synergistic combination of the 28-kilodalton protein and greater-molecular-mass proteins (18, 23, 27). The 28-, 70-, and 135-kilodalton proteins of the crystal are immunologically distinct and contain protease-resistant domains of 22, 38, and 65 kilodaltons, respectively (18). These domains have been characterized by size but not toxicity.

B. thuringiensis subsp. *israelensis* toxins are glycoproteins containing approximately 1.0% neutral sugars and 1.7% amino sugars (19). There are several reports suggesting that carbohydrates are involved in the insecticidal activity of *B. thuringiensis* toxins. (i) The toxicity of *B. thuringiensis* subsp. *kurstaki* toxin for a lepidopteran cell line was inhibited by *N*-acetylgalactosamine as well as by the lectins soybean agglutinin and wheat germ agglutinin (WGA) (14). (ii) The toxicity of trypsin-digested *B. thuringiensis* var.

aizawai toxin for another lepidopteran cell line was inhibited by preincubation with D-glucose (9). (iii) The toxicity of *B. thuringiensis* subsp. *israelensis* toxin for *A. aegypti* larvae was inhibited sevenfold by the presence of *N*-acetylglucosamine (GlcNAc) during the mosquito bioassays (16). These examples suggest a role for carbohydrates in the toxin-receptor interactions. However, there is still some disagreement about whether the relevant carbohydrates are located on the toxin (16, 19) or the insect receptor (9, 14) or both. This study examines the toxicity and lectin-binding abilities of protease-resistant domains of the delta-endotoxin from *B. thuringiensis* subsp. *israelensis* and identifies differences between *A. aegypti* and *C. quinquefasciatus* larvae in terms of the importance of the carbohydrate portion of the mosquitocidal toxins.

MATERIALS AND METHODS

Toxin preparation. Protein crystals from *B. thuringiensis* subsp. *israelensis* were purified as described previously (17), solubilized for 2 h at 37°C in 50 mM NaOH with 10 mM EDTA (pH 11.7), and centrifuged at 15,000 × *g* for 10 min. Amounts of solubilized toxin were determined from the A_{280} ($E_{1\text{ cm}}^{1\%} = 11.0$ [24]). Solubilized toxin was subjected to three slightly different protocols for proteolysis. In protocol A, solubilized toxin (1 mg) was adjusted to pH 8.0 with 2 M Tris hydrochloride (pH 7.4) and incubated with chymotrypsin (200 µg) in the presence of 5 mM CaCl₂ (final volume, 800 µl). After 20 h of incubation at 25°C, reactions were terminated by the addition of 6.4 µl of 100 mM phenylmethylsulfonyl fluoride and 6.4 µl of 200 mM EDTA (18). Protocol A was used to produce the protease-resistant domains referred to in Table 1 and Fig. 1 through 3. In protocol B, solubilized toxin (750 µg) in 50 mM Tris (pH 7.5) was incubated with 100 µg of chymotrypsin (final volume, 760 µl) for 2 h at 30°C before proteolysis was terminated by the addition of 3 µl of 100 mM phenylmethylsulfonyl fluoride. In a variation of protocol B, chymotrypsin was replaced by 100 µg of thermolysin at 38°C, and proteolysis was terminated by the addition of 6 µl of 200 mM EDTA. In protocol C, solubilized toxin (1 mg) in 50 mM Tris (pH 7.8) was incubated with 50 µg of chymotrypsin and 50 µg of trypsin (final volume, 1 ml) for

* Corresponding author.

[†] Present address: Abbott Laboratories, North Chicago, IL 60064.

24 h at 30°C. Protocol C was used to produce the protease-resistant domains referred to in Table 2. For each of the protocols, the protease-digested toxin was subjected to electrophoresis on sodium dodecyl sulfate–12.5% polyacrylamide gels by the discontinuous system of Laemmli (15), and the protein bands were visualized with either Coomassie blue or silver stain (Bio-Rad Laboratories, Richmond, Calif.).

Bioassays. The general cytolytic activity was determined by erythrocyte hemolysis (20). The mosquito larvicidal activity of protein preparations was determined by the latex bead bioassay described previously (5, 21). This assay system is necessary because mosquito larvae are filter feeders and selectively ingest particulate matter. Latex beads adsorb proteins nonspecifically. Couche et al. (5) used ^{14}C -labeled toxin to show that close-packed protein monolayers were formed on bead surfaces and that protein attachment was markedly insensitive to most chemical and physical variations. For this assay, second-instar larvae of *C. quinquefasciatus* and third-instar larvae of *A. aegypti* in 25 ml of deionized water were fed toxin preparations. The 50% lethal concentrations are the concentrations of toxin required to kill 50% of the larvae. Unless otherwise specified, insect mortality was determined after 24 h. All experiments included controls with solubilized toxin which had not been protease treated.

HPLC. Protease-resistant domains were concentrated approximately fourfold with a Centricon apparatus (Amicon Corp., Danvers, Mass.) containing a filter which allowed the passage of molecules of 10,000 daltons or smaller. The samples were not centrifuged to dryness, so not all of the smaller molecules were removed. The protease-resistant domains were separated from smaller peptide digestion products with high-performance liquid chromatography (HPLC) (model 2350; ISCO, Lincoln, Nebr.). Protease-digested toxin (570 μg) was injected into a 100- μl sample loop and run on a GP 100 gel filtration column (ISCO) at a flow rate of 1 ml/min. The running buffer was 10 mM sodium phosphate (pH 7.45)–150 mM NaCl (PBS). Protein peaks were identified and quantified by their A_{280} s and were analyzed by electrophoresis, toxicity bioassays, and lectin binding. Prior to electrophoresis, HPLC fractions were concentrated by precipitation in 10% trichloroacetic acid. After 30 min of incubation at 0°C, the samples were centrifuged at $15,000 \times g$ for 2 min. The pellet was washed two times with acetone, dried, suspended in sodium dodecyl sulfate sample buffer, boiled for 3 min, and run on gels as described above.

Lectin binding. The ability of protease-resistant domains to bind WGA was determined by applying proteins to nitrocellulose membranes by vacuum filtration with a dot blot apparatus (17). Subsequently, the membrane was incubated in 3% bovine serum albumin in PBS to block nonspecific binding sites, and the nitrocellulose was washed two times with PBS. The WGA-horseradish peroxidase conjugate (200 μg) (EY Laboratories, San Mateo, Calif.) was then added in 20 ml of PBS and 1.0% bovine serum albumin. The mixture was incubated for 2 h at 22°C and then washed four times in PBS and one time in 20 mM Tris hydrochloride–500 mM NaCl (pH 7.5). Horseradish peroxidase color development reagent (containing 4-chloro-1-naphthol) (Bio-Rad) was used to detect lectin binding. Positive and negative controls were ovalbumin and bovine serum albumin, respectively. Protein samples were 50 μg (determined by A_{280}), except for ovalbumin (2 or 10 μg).

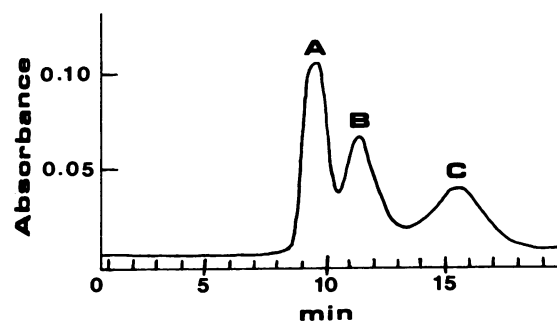


FIG. 1. HPLC of protease-digested *B. thuringiensis* subsp. *israelensis* toxin.

RESULTS

Protease-resistant domains. After digestion with chymotrypsin, the protease-resistant domains of *B. thuringiensis* subsp. *israelensis* toxin were visualized by gel electrophoresis. Domains with apparent molecular sizes of 22, 38, and 65 kilodaltons were present. No spontaneous breakdown products were observed; controls without added proteases maintained the same protein profiles as fresh intact crystals, even when incubated at 22 to 25°C for 24 h. The presence of EDTA during solubilization is necessary to inactivate contaminating metalloproteases (20). The sizes of these protease-resistant domains were the same as those we reported previously (18). Furthermore, no undigested 135- to 140-kilodalton proteins were observed following chymotrypsin digestion, even with the more sensitive silver stain. Thus, virtually all of the proteins used in subsequent analyses had been degraded to their protease-resistant domains.

The protease-resistant domains were subjected to HPLC to remove the small peptides generated during proteolysis (Fig. 1). The chromatography conditions did not separate the individual protein components of the toxin. These conditions were selected because the toxins act in a synergistic manner and the combined toxins possess the greatest larvicidal activity (18, 23, 27). Peak A (containing fractions 9 and 10) of the HPLC eluant (Fig. 1) contained all the major protease-resistant domains present after chymotrypsin digestion (Fig. 2), while peak B contained mainly a polypeptide of approximately 17 kilodaltons, probably autodigested chymotrypsin. No polypeptides were detected in peak C. Presumably, the peak C peptides were small enough that they ran at or ahead

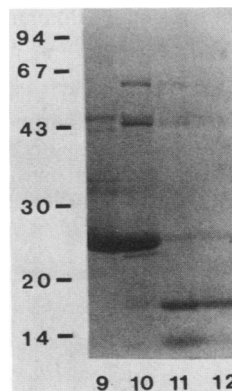


FIG. 2. Polyacrylamide gel electrophoresis of HPLC fractions. Fractions (9 through 12) from the digestion used for Fig. 1 were stained with Coomassie blue. The molecular size markers are in kilodaltons.

TABLE 1. Larvicidal activity of protease-resistant domains from *B. thuringiensis* subsp. *israelensis* against *C. quinquefasciatus*

Toxin sample	LC ₅₀ (ng/ml) ^a
Solubilized (no protease).....	62 ± 2
Chymotrypsin digested	79 ± 3
HPLC fraction: ^b	
9.....	66 ± 14
11.....	550 ± 50
13.....	— ^c

^a LC₅₀, 50% lethal concentration. Mean ± standard error.^b Fraction numbers refer to sample times in Fig. 1.^c —, No mortality was observed at the highest concentration tested (800 ng/ml).

of the dye front during gel electrophoresis or were not precipitated by the trichloroacetic acid.

***C. quinquefasciatus* bioassay.** *B. thuringiensis* subsp. *israelensis* toxin digested with chymotrypsin by protocol A was as toxic as undigested toxin (Table 1). Solubilized toxin at 62 ng/ml killed 50% of the *C. quinquefasciatus* larvae in 24 h, while chymotrypsin-digested toxin required 79 ng/ml. In addition, the HPLC-purified protease-resistant domains were tested for toxicity. The protein in peak A (fraction 9) was as toxic as undigested toxin, while peak B (fraction 11) was eightfold less toxic (Table 1). Peak C was not toxic at the highest concentration tested (800 ng/ml).

WGA binding. WGA is a lectin with binding specificity for GlcNAc and GlcNAc oligomers. The protease-digested *B. thuringiensis* subsp. *israelensis* toxin was tested for lectin binding with horseradish peroxidase-labeled WGA (Fig. 3). Solubilized toxin (dot 1) bound WGA, as did the protease-digested but unfractionated toxin (dot 2). Protease-digested toxin which had been concentrated fourfold by ultrafiltration (dot 3) bound less lectin than solubilized or unfractionated toxin did, while a minor amount of lectin-binding material was detected in the filtrate (dot 4). The presence of this material in dot 4 indicates that GlcNAc-containing peptides are present in the low-molecular-weight peptide digestion products. Significantly, even though the protein in peak A was as toxic for *C. quinquefasciatus* larvae as the solubilized toxin (Table 1), neither peak A (dot 5) nor peak B (dot 6) bound detectable levels of WGA (Fig. 3). Dots 5 and 6 contained 50 µg of protein (determined from A₂₈₀), while as little as 5 µg of solubilized toxin could be detected by WGA binding (16).

***A. aegypti* bioassay.** The bioassay data (Table 1) which indicate that protease-resistant domains retain their full activity against *C. quinquefasciatus* larvae are in marked contrast to results with *A. aegypti* larvae. Two separate experiments were conducted, under slightly different proteolysis conditions. In the first experiment, proteolysis by protocol B caused the toxicity to decrease fivefold; 50% lethal concentrations for *A. aegypti* larvae increased from 80 to 400 ng/ml. Similar results were obtained with chymotryp-

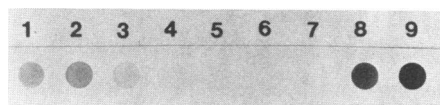


FIG. 3. Binding of WGA by *B. thuringiensis* subsp. *israelensis* toxin. Samples: 1, solubilized toxin; 2, protease-digested toxin; 3, protease-digested and concentrated toxin; 4, filtrate obtained during concentration; 5, fraction 9 (peak A of Fig. 1); 6, fraction 11 (peak B of Fig. 1); 7, bovine serum albumin; 8 and 9, ovalbumin.

TABLE 2. General cytolytic and larvicidal activities of protease-resistant domains from *B. thuringiensis* subsp. *israelensis* against *A. aegypti*

Protease digestion (h)	% Hemolysis ^a	% Larval mortality ^b after:	
		4 h	8 h
0 (no enzymes) ^c		72 ± 4	100 ± 0
0	61.3 ± 5.6	46 ± 5	66 ± 6
2	62.4 ± 3.2	20 ± 9	48 ± 10
5	72.2 ± 2.1	28 ± 10	58 ± 5
10	69.3 ± 4.6	16 ± 8	48 ± 8
24	62.3 ± 4.7	8 ± 4	38 ± 10

^a Percent erythrocyte lysis (20) of a 1:100 dilution of the toxin digest (± standard error). Final toxin concentration, 1.02 µg/ml. Controls of protease without toxin gave 14% lysis.^b Fixed toxin concentration, 400 ng/ml. Five replicates of 10 larvae each were used, with cumulative mortality (± standard error) determined 4 and 8 h after toxin addition. Three other experiments, i.e., 15 replicates (150 larvae), gave similar results.^c Differs from the time zero control in that no proteolytic enzymes were added to the solubilized toxin. In time zero samples, even following the 2,000-fold dilution for bioassay, some proteolysis would continue during the bioassay.

sin at 30°C or thermolysin at 38°C. In the second experiment (Table 2), proteolysis by protocol C caused larval mortality to decrease ninefold when measured after 4 h and threefold when measured after 8 h. Note that the bioassay data in Table 2 are based on the cumulative mortality of third-instar *A. aegypti* larvae at a fixed toxin concentration (400 ng/ml) rather than on 50% lethal concentrations. This proteolytic sensitivity of *A. aegypti* larvicidal activity differs from that of the other biological activity present in *B. thuringiensis* subsp. *israelensis* crystals. The general cytolytic activity (as measured by hemolysis) (Table 2) remained undiminished, even after 24 h of proteolytic digestion. This proteolysis insensitivity of the general cytolytic activity was previously noted by Armstrong et al. (2).

DISCUSSION

Protease-resistant domains from the *B. thuringiensis* subsp. *israelensis* toxin were obtained following digestion with chymotrypsin. Chymotrypsin was selected because it generates domains similar in size to those detected following prolonged digestion with gut enzymes from third-instar *A. aegypti* larvae (16). This report is the first to describe in detail the toxicity of protease-resistant domains from *B. thuringiensis* subsp. *israelensis* delta-endotoxins. When fed to *C. quinquefasciatus* larvae, the protease-resistant domains were as toxic as undigested toxin. This undiminished toxicity indicates that chymotrypsin does not cleave portions of the protein that are necessary for biological activity against *C. quinquefasciatus* larvae. Furthermore, removal of the peptide digestion products did not alter the toxicity of the protease-resistant domains for *C. quinquefasciatus* larvae.

This evidence that small peptides do not contribute to toxicity for *C. quinquefasciatus* is important, because it appears that GlcNAc-containing oligosaccharide portions of the *B. thuringiensis* subsp. *israelensis* toxins occur predominantly in those regions which are removed by extensive proteolysis. Protease-resistant domains no longer bound WGA. This conclusion is based on the information in Fig. 3, in which the chymotrypsin-treated but unfractionated toxin (dot 2) bound WGA but the HPLC-purified toxin (dot 5) did not. Furthermore, the low-molecular-weight peptide digestion products (dot 4) also bound WGA. Presumably, the

regions removed during proteolysis are located at the N and C termini.

Assuming that protein N glycosylation in bacteria follows the patterns observed in eucaryotes, examination of the amino acid sequences for each of the three immunologically distinct (18) crystal components confirms the presence of Asn-X-Ser/Thr glycosylation sites close to the N and C termini. The 28-kilodalton protein subunit is composed of 249 amino acids. It has four possible glycosylation sites, including an Asn-X-Thr tripeptide at positions 246 to 248 (25). The 72-kilodalton protein subunit is composed of 643 amino acids. It has five possible glycosylation sites, including Asn-X-Thr tripeptides at positions 13 to 15 and 22 to 24 (7). The 135-kilodalton protein subunit is composed of 1,180 amino acids. It has 18 possible glycosylation sites, including Asn-X-Ser tripeptides at positions 14 to 16 and 21 to 23 and an Asn-X-Thr tripeptide at positions 25 to 27 (26).

The undiminished toxicity of chymotrypsin-digested crystals for *C. quinquefasciatus* larvae is in marked contrast to the approximately fivefold reduction in toxicity of the crystals for *A. aegypti* larvae. This difference may be related to the concurrent removal of GlcNAc residues (WGA-binding sites) during proteolysis. This suggestion is compatible with our previous findings (16) on the importance of covalently attached amino sugars and leads to the conclusion that the *B. thuringiensis* subsp. *israelensis* delta-endotoxin requires GlcNAc-containing oligosaccharides for high toxicity against *A. aegypti* larvae (16) but not *C. quinquefasciatus* larvae. It is likely that this difference in relative toxicity for mosquito larvae reflects differences in the relative binding specificities of their respective toxin receptors. This situation would be analogous to the different receptor binding sites found by Hofmann et al. (12) in brush border membranes from the larval midgut of the tobacco hornworm *Manduca sexta* and the cabbage butterfly *Pieris brassicae* (12).

On a more speculative note, it is attractive to extrapolate from *B. thuringiensis* subsp. *israelensis* to *B. sphaericus*. These two organisms are the most important bacterial pathogens of mosquitoes. Both are sporeformers which produce mosquitocidal parasporal protein crystals. Both are highly toxic for *C. quinquefasciatus* larvae. However, *B. sphaericus* is virtually nontoxic for *A. aegypti* larvae (22). Our data (M. Notohamiprodjo, M.S. thesis, University of Nebraska, Lincoln, 1987) indicate that *B. sphaericus* 2362 is 2.8×10^4 times less toxic for *A. aegypti* larvae than for *C. quinquefasciatus* larvae. By analogy with our *B. thuringiensis* subsp. *israelensis* data, one explanation for the restricted host range of *B. sphaericus* toxin is that the bacterial cells lack the glycosylating enzymes necessary to attach the GlcNAc-containing oligosaccharides. The amino acid sequence of the 41.9-kilodalton toxin from *B. sphaericus* has recently been deduced by Hindley and Berry (11) from the DNA sequence of its cloned gene. The sequences from strains 1593 and 2362 were identical. They contained six Asn-X-Ser/Thr sites that could be N-glycosylated. The suggestion that *B. sphaericus* and *B. thuringiensis* differ in their capacities for posttranslational protein glycosylation leads to three readily tested predictions. (i) *B. sphaericus* toxins do not normally have GlcNAc-enriched oligosaccharides or WGA-binding sites. (ii) If the gene for a normally glycosylated protein, such as the 28-kilodalton toxin from *B. thuringiensis* subsp. *israelensis*, is transformed into *B. sphaericus*, the gene product will not exhibit the strong WGA binding it did when expressed in *B. thuringiensis*. (iii) If the gene for the 42-kilodalton *B. sphaericus* toxin is expressed in *B. thuringien-*

sis or another gram-positive bacterium capable of protein glycosylation, that gene product will exhibit WGA binding and toxicity for both *C. quinquefasciatus* and *A. aegypti* larvae. This approach raises the possibility of extending the *B. sphaericus* host range to include *A. aegypti* mosquitoes.

ACKNOWLEDGMENTS

This research was supported by Public Health Service grants AI16538 and AI21021 (to M.A.P.) from the National Institutes of Health, grant DMB-85-21800 from the National Science Foundation, and grant 86-CRCR-1-1973 from the U.S. Department of Agriculture.

LITERATURE CITED

1. Andrews, R. E., Jr., M. M. Bibilos, and L. A. Bulla, Jr. 1985. Protease activation of the entomocidal protoxin of *Bacillus thuringiensis* subsp. *kurstaki*. Appl. Environ. Microbiol. 50: 737-742.
2. Armstrong, J. L., G. F. Rohrmann, and G. S. Beaudreau. 1985. Delta endotoxin of *Bacillus thuringiensis* subsp. *israelensis*. J. Bacteriol. 161:39-46.
3. Bourguoin, C., A. Klier, and G. Rapoport. 1986. Characterization of the genes encoding the haemolytic toxin and the mosquitocidal delta-endotoxin of *Bacillus thuringiensis israelensis*. Mol. Gen. Genet. 205:390-397.
4. Chestukhina, G. G., L. I. Kostina, A. L. Mikhailova, S. A. Tyurin, F. S. Klepikova, and V. M. Stepanov. 1982. The main features of *Bacillus thuringiensis* delta-endotoxin molecular structure. Arch. Microbiol. 132:159-162.
5. Couche, G. A., M. A. Pfannenstiel, and K. W. Nickerson. 1986. Parameters affecting attachment of *Bacillus thuringiensis* var. *israelensis* toxin to latex beads. Appl. Microbiol. Biotechnol. 24:128-133.
6. Davidson, E. W. 1982. Purification and properties of soluble cytoplasmic toxin from the mosquito pathogen *Bacillus sphaericus* strain 1593. J. Invertebr. Pathol. 36:6-9.
7. Donovan, W. P., C. Dankocsik, and M. P. Gilbert. 1988. Molecular characterization of a gene encoding a 72-kilodalton mosquito-toxic crystal protein from *Bacillus thuringiensis* subsp. *israelensis*. J. Bacteriol. 170:4732-4738.
8. Goldberg, L. J., and J. Margalit. 1977. A bacterial spore demonstrating rapid larvicidal activity against *Anopheles sergenti*, *Uranotaenia unguiculata*, *Culex inquitatus*, *Aedes aegypti* and *Culex pipiens*. Mosq. News 37:355-358.
9. Haider, M. Z., and D. J. Ellar. 1987. Analysis of the molecular basis of insecticidal specificity of *Bacillus thuringiensis* crystal-endotoxin. Biochem. J. 248:197-201.
10. Held, G. A., Y.-S. Huang, and C. Y. Kawanishi. 1986. Effect of removal of the cytolytic factor of *Bacillus thuringiensis* subsp. *israelensis* on mosquito toxicity. Biochem. Biophys. Res. Commun. 141:937-941.
11. Hindley, J., and C. Berry. 1987. Identification, cloning and sequence analysis of the *Bacillus sphaericus* 1593 41.9 kD larvicidal toxin gene. Mol. Microbiol. 1:187-194.
12. Hofmann, C., H. Vanderbruggen, H. Höfte, J. Van Rie, S. Jansens, and H. Van Mellaert. 1988. Specificity of *Bacillus thuringiensis* δ -endotoxins is correlated with the presence of high-affinity binding sites in the brush border membrane of target insect midguts. Proc. Natl. Acad. Sci. USA 85:7844-7848.
13. Hurley, J. M., S. G. Lee, R. E. Andrews, Jr., M. J. Klowden, and L. A. Bulla, Jr. 1985. Separation of the cytolytic and mosquitocidal proteins of *Bacillus thuringiensis* subsp. *israelensis*. Biochem. Biophys. Res. Commun. 126:961-965.
14. Knowles, B. H., W. E. Thomas, and D. J. Ellar. 1984. Lectin-like binding of *Bacillus thuringiensis* var. *kurstaki* lepidopteran-specific toxin is an initial step in insecticidal action. FEBS Lett. 168:197-202.
15. Laemmli, U. K. 1970. Cleavage of structural proteins during the assembly of the head of bacteriophage T4. Nature (London) 227:680-685.
16. Muthukumar, G., and K. W. Nickerson. 1987. The glycoprotein toxin of *Bacillus thuringiensis* subsp. *israelensis* indicates a

- lectinlike receptor in the larval mosquito gut. Appl. Environ. Microbiol. **53**:2650–2655.
17. Pfannenstiel, M. A., G. A. Couche, G. Muthukumar, and K. W. Nickerson. 1985. Stability of the larvicidal activity of *Bacillus thuringiensis* subsp. *israelensis*: amino acid modification and denaturants. Appl. Environ. Microbiol. **50**:1196–1199.
 18. Pfannenstiel, M. A., G. A. Couche, E. J. Ross, and K. W. Nickerson. 1986. Immunological relationships among proteins making up the *Bacillus thuringiensis* subsp. *israelensis* crystalline toxin. Appl. Environ. Microbiol. **52**:644–649.
 19. Pfannenstiel, M. A., G. Muthukumar, G. A. Couche, and K. W. Nickerson. 1987. Amino sugars in the glycoprotein toxin from *Bacillus thuringiensis* subsp. *israelensis*. J. Bacteriol. **169**:796–801.
 20. Pfannenstiel, M. A., E. J. Ross, V. C. Kramer, and K. W. Nickerson. 1984. Toxicity and composition of protease-inhibited *Bacillus thuringiensis* var. *israelensis* crystals. FEMS Microbiol. Lett. **21**:39–42.
 21. Schnell, D. J., M. A. Pfannenstiel, and K. W. Nickerson. 1984. Bioassay of solubilized *Bacillus thuringiensis* var. *israelensis* crystals by attachment to latex beads. Science **223**:1191–1193.
 22. Singer, S. 1980. *Bacillus sphaericus* for the control of mosquitoes. Biotechnol. Bioeng. **22**:1335–1355.
 23. Thiery, I. 1987. Similarities between crystal protein subunits of *Bacillus thuringiensis* strains 1884 serotype H14 and PG14 serotype H8a,8b, and their relationship with mosquitocidal activity. Ann. Inst. Pasteur/Microbiol. **138**:457–470.
 24. Tyrell, D. J., L. A. Bulla, Jr., R. E. Andrews, Jr., K. J. Kramer, L. I. Davidson, and P. Nordin. 1981. Comparative biochemistry of entomocidal parasporal crystals of selected *Bacillus thuringiensis* strains. J. Bacteriol. **145**:1052–1062.
 25. Waalwijk, C., A. M. Dulleman, M. E. S. van Workum, and B. Visser. 1985. Molecular cloning and the nucleotide sequence of the Mr 28,000 crystal protein gene of *Bacillus thuringiensis* subsp. *israelensis*. Nucleic Acids Res. **13**:8207–8217.
 26. Ward, E. S., and D. J. Ellar. 1987. Nucleotide sequence of a *Bacillus thuringiensis* var. *israelensis* gene encoding a 130 kDa delta-endotoxin. Nucleic Acids Res. **15**:7195.
 27. Wu, D., and F. N. Chang. 1985. Synergism in mosquitocidal activity of 26 and 65 kDa proteins from *Bacillus thuringiensis* subsp. *israelensis* crystal. FEBS Lett. **190**:232–236.