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Corey J. Walther
University of Nebraska-Lincoln

Graham A. Couche
University of Nebraska-Lincoln

Mary Ann Pfannenstiel
University of Nebraska-Lincoln

Sara E. Egan
University of Nebraska-Lincoln

Lisa A. Bivin
University of Nebraska-Lincoln

See next page for additional authors

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Authors

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Analysis of Mosquito Larvicidal Potential Exhibited by Vegetative Cells of *Bacillus thuringiensis* subsp. *israelensis*

COREY J. WALTHER,† GRAHAM A. COUCHE,‡ MARY ANN PFANNENSTIEL, SARA E. EGAN, LISA A. BIVIN, AND KENNETH W. NICKERSON*

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

Vegetative *Bacillus thuringiensis* subsp. *israelensis* cells (6 × 10^5/ml) achieved 100% mortality of *Aedes aegypti* larvae within 24 h. This larvicidal potential was localized within the cells; the cell-free supernatants did not kill mosquito larvae. However, they did contain a heat-labile hemolysin which was immunologically distinct from the general cytolytic (hemolytic) factor released during solubilization of *B. thuringiensis* subsp. *israelensis* crystals. The larvicidal potential of the vegetative cells was not due to poly-β-hydroxybutyrate. Instead, it correlated with the ability of vegetative cells to sporulate during the bioassays. No toxicity was observed when bioassays were conducted in the presence of chloramphenicol or streptomycin. It is unlikely that the vegetative cells sporulate in the alkaline (pH 9.5 to 10.5) larval guts after ingestion. *B. thuringiensis* subsp. *israelensis* is not an alkalophile; we have been unable to grow it in culture at pH values of ≥9.5. Moreover, we have been unable to demonstrate formation of a protective capsule. However, bacteria may replicate in the gut fluids of dead or dying mosquito larvae because their alkaline gut pH values drop markedly after exposure to the *B. thuringiensis* subsp. *israelensis* crystal toxins.

The bacterium *Bacillus thuringiensis* subsp. *israelensis* produces a protein crystal upon sporulation that is toxic to the larval stage of many mosquito and black fly species. Purified crystals are highly toxic to mosquito larvae (25, 33), but it has not yet been established that the crystalline toxin constitutes the entirety of the larvicidal activity. We have been interested in the potential toxicity of vegetative *B. thuringiensis* subsp. *israelensis* cells. This interest derives from four characteristics of the entomopathogenic bacilli. (i) Many strains of the bacterium *B. sphaericus* are also toxic to mosquito larvae. However, depending on the strain, these *B. sphaericus* larvicidal toxins may be produced during sporulation or during both sporulation and vegetative growth (19). (ii) During vegetative growth, many strains of *B. thuringiensis* release a heat-stable adenosine nucleotide, known as the β-exotoxin (17), which is generally toxic to eucaryotic cells. (iii) During vegetative growth, many strains of *B. thuringiensis* release a 23-kilodalton extracellular phospholipase (23). Similarly, a 29-kilodalton phospholipase has been reported from the closely related bacterium *B. cereus* (15). (iv) Vegetative cells of *B. thuringiensis* subsp. *israelensis* are hemolytic (10, 11). When solubilized, crystals from *B. thuringiensis* subsp. *israelensis* also exhibit hemolytic activity (25, 30). This latter hemolytic activity resides in the 28-kilodalton subunit of the crystal (2, 10, 14, 31). Thus, there may be two separate hemolysins, one produced during vegetative growth and the other produced during sporulation. Alternatively, the 28-kilodalton hemolysin may be synthesized continuously but only packaged into the protein crystal during sporulation. To resolve these questions, we have now conducted a systematic analysis of the larvicidal and hemolytic potential of *B. thuringiensis* subsp. *israelensis* vegetative cells.

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* Corresponding author.

† Present address: College of Dentistry, University of Illinois-Chicago, Chicago, IL 60680.

‡ Present address: Biotechnology Australia, Roseville, NSW 2069, Australia.

MATERIALS AND METHODS

Organisms and culture conditions. A single-colony isolate of *B. thuringiensis* subsp. *israelensis* taken from a Bactimos powder was grown on the GGYS medium described previously (24). Two transfers were made to allow existing spores to germinate. The cells were grown to 100 Klett₆₆ units (ca. 6 × 10^7 CFU/ml) and bioassayed with third-instar *Aedes aegypti* larvae (21). Each assay contained 10 larvae and was conducted in triplicate. Vegetative cells of *B. megaterium*, *B. cereus* T, *B. sphaericus* 1593, and *B. thuringiensis* subsp. *thuringiensis* (NRRL 4039) were prepared and bioassayed similarly.

The tests for capsule formation were conducted on cells grown on NBY agar containing 0.7% bicarbonate (12) and in two liquid media, both supplemented with 0.7% bicarbonate. The first was GGYS, while the second contained (per liter of distilled water): vitamin-free Casamino Acids, 1 g; K_2HPO_4, 5 g; $MnCl_2$, 0.4 g; and sodium lactate (60%), 10 ml; final pH 7.0. Cells were examined for capsules by an India ink-safranin staining procedure after 48 h of growth under defined air-carbon dioxide mixtures.

Extracellular proteins. To analyze extracellular proteins by gel electrophoresis, a GGYS-grown culture of ca. 100 Klett₆₆ units was harvested by centrifugation at 12,000 × *g*. Solid trichloroacetic acid was added to the cell-free supernatant until it was 10% (wt/vol) trichloroacetic acid. The trichloroacetic acid-containing supernatant was kept overnight at 4°C, heated at 100°C for 15 min, cooled to room temperature, and centrifuged at 12,000 × *g*. The pellet was washed once with 100% ethanol and twice with deionized water before being suspended in 200 µl of sodium dodecyl sulfate sample buffer (16). All other analyses of the cell-free supernatant from vegetative *B. thuringiensis* subsp. *israelensis* cells used proteins concentrated 40-fold with a 5,000-kilodalton-cutoff, hollow-fiber cartridge (Amicon Corp.), dialyzed overnight against 6 liters of distilled water and filter sterilized (210 µg of protein per ml).

Antiserum to the 28-kilodalton crystal protein. Purified *B. thuringiensis* subsp. *israelensis* crystal proteins (24) were resolved by electrophoresis (16) in 12.5% preparative gels. After staining with Coomassie blue, the protein band migrating at 28 kilodaltons was excised and frozen. The gel slice was subsequently macerated in a minimal volume of buffer (10 mM sodium phosphate, 150 mM sodium chloride, pH 7.2), emulsified with Freund adjuvant, and injected subcutaneously into New Zealand White rabbits in a series of five weekly injections. Collected serum was fractionated by ammonium sulfate precipitation, dialyzed, and passed through an affinity column containing purified crystal toxin coupled to cyanogen bromide-activated Sepharose 4B. Reactivity of the affinity-purified antiserum with test proteins was determined with a dot blot apparatus (Schleicher & Schuell). Solubilized *B. thuringiensis* subsp. *israelensis* crystal proteins and cell-free vegetative supernatant proteins were applied to nitrocellulose paper by vacuum filtration. Protein-antiserum binding was detected with a protein A-horseradish peroxidase conjugate (Bio-Rad Laboratories) at a 1:3,000 dilution. This procedure could detect as little as 10 ng of purified crystal protein. Incubation times, buffers, and development conditions were as recommended by Bio-Rad. The sensitivity of detection was determined by mixing known quantities of solubilized crystal toxin with the vegetative supernatant proteins.

RESULTS

Larvicidal potential of vegetative bacilli. Whole-cell cultures of five *Bacillus* species were tested for their toxicity to *A. aegypti* larvae. Cultures were added to 25 ml of deionized water so that the viable count at the beginning of the bioassays was ca. 10^6 CFU/ml. Only phase-dark vegetative cells were present; no spores were detected visually or as heat-resistant CFUs. Twenty-four hours after the addition of vegetative cells, *B. thuringiensis* subsp. *israelensis* (6×10^5/ml) and *B. sphaericus* (2×10^6/ml) had caused 100% larval mortality. In contrast, no mortality was observed in larvae fed vegetative cells from *B. cereus*, *B. megaterium*, and a Lepidoptera-active *B. thuringiensis*, even after 48 h. The larvicidal potential of the *B. thuringiensis* subsp. *israelensis* culture was localized in the vegetative cells. Following centrifugation at $12,000 \times g$, the resuspended cell pellet contained all of the larvicidal activity. The undiluted cell-free supernatant was nontoxic to larvae for at least 48 h, while a solution of supernatant proteins which had been concentrated 40-fold permitted the emergence of adult mosquitoes.

Capsule formation. Many bacterial pathogens of plants and animals require the presence of a protective capsule to exert their pathogenicity. In *B. anthracis*, capsule production is plasmid coded (12) and requires growth in a CO_2-enriched atmosphere on media containing bicarbonate (32). *B. thuringiensis* subsp. *israelensis* is closely related to *B. anthracis*, as shown by the recently developed mating system for plasmid transfer between the two bacteria (3). However, no capsules were detected by negative staining of *B. thuringiensis* subsp. *israelensis* cells grown on solid or liquid media in 0.03, 5.0, 10.0, 15.0, or 20.0% CO_2. Each medium contained 0.7% $NaHCO_3$.

PHB. Like most sporeforming bacteria, *B. thuringiensis* accumulates large amounts of the storage product poly-β-hydroxybutyrate (PHB) in late exponential phase prior to sporulation (20, 22). The observed larvicidal activity of vegetative cells could be due to their subsequent production of toxic PHB. Two PHB preparations of differing density (membrane-bound and unbound granules) were tested. These PHB preparations from sporulated *B. thuringiensis* cultures had been purified on sodium bromide gradients (20). However, *A. aegypti* remained fully viable after 48 h in purified PHB. No toxicity was observed, even at 10 mg of PHB per ml. This level of PHB is 10^6 times greater than the 50% lethal concentration for purified *B. thuringiensis* subsp. *israelensis* crystals (25, 33). Microscopic examination of the larvae disclosed solid white engorged guts, demonstrating that the PHB granules were in fact ingested.

Sporulation. Vegetative *Bacillus* cells also have the capability of sporulating. Typically this differentiation takes 6 to 8 h. To determine whether sporulation was necessary for expression of the larvicidal activity of vegetative cells, the bioassays were conducted in the presence of two antibiotics (chloramphenicol and streptomycin) known to inhibit *B. thuringiensis* subsp. *israelensis* (unpublished data). Significantly, in the presence of either antibiotic (10 μg/ml), vegetative cells of *B. thuringiensis* subsp. *israelensis* exhibited no larvicidal activity within 24 h. In contrast, the same cell concentrations gave 100% mortality in the absence of added antibiotics. Neither chloramphenicol (100 μg/ml) nor streptomycin (100 μg/ml) affected the viability of control larvae.

It is well known that sporulation occurs when vegetative cells of the bacilli are suspended in distilled water (6, 13, 26) and, consequently, it is reasonable that sporulation could occur during a prolonged bioassay. This expectation was confirmed by microscopic examination and also by delaying the addition of mosquito larvae. When the larvae and bacteria were added simultaneously, larval death was observed after 10 to 12 h. When the vegetative *B. thuringiensis* subsp. *israelensis* cells had been added 24 h previously, this time was reduced to 4 to 6 h.

The preceding experiment demonstrates that bacterial sporulation occurs in the bioassay fluid. It is more difficult to determine whether sporulation can occur in the larval gut following ingestion of the vegetative cells. When larvae were allowed to feed on vegetative *B. thuringiensis* subsp. *israelensis* cells for 6 h and then transferred to distilled water without bacteria, no larval death was observed after an additional 24 h. The 6-h feeding was chosen to maximize the number of cells ingested while allowing insufficient time for sporulation to occur in the bioassay fluid.

Gut pH. One of the difficulties in evaluating bacterial growth in the larval gut is the fact that the parameters operative during pathogenesis are continually changing. Of these factors, pH is probably the most important. Midgut pH in healthy mosquito larvae ranges from 8.5 to 10.5, depending on the position in the gut (8, 27). Generally, the pH of the anterior two-thirds of the midgut is ≥ 9.5. However, these alkaline pH conditions drop markedly after exposure to *B. thuringiensis* subsp. *israelensis* toxin (Table 1). Within the time frame of larval pathogenesis, gut pH had dropped below 9.6 in a little over half the time necessary for larval death and had reached neutrality within 85% of the time necessary for larval death (Table 1). This rapid drop in gut pH is not surprising because maintenance of alkaline guts in mosquitoes is an active process (9), which ceases whenever the larvae become "traumatized" (9). Significantly, we have been unable to grow *B. thuringiensis* subsp. *israelensis* in culture at pH values of ≥ 9.5, regardless of whether the alkaline conditions were maintained by carbonate, Tris, or glycine buffer systems.

Hemolysin. Cell-free supernatants from vegetative *B. thuringiensis* subsp. *israelensis* cells were not larvicidal but

they did contain bacterial proteins (ca. 5 µg/ml), including a heat-labile (100°C for 5 min) hemolysin. To test the relationship between this vegetative hemolysin and the 28-kilodalton hemolysin obtained from solubilized *B. thuringiensis* subsp. *israelensis* crystals, we examined the protein composition of the cell-free vegetative supernatant by gel electrophoresis (Fig. 1). Numerous proteins were present in the concentrated supernatant (lane 2), but there was no apparent similarity to the proteins present in purified crystals (lane 1). In particular, no major bands were present corresponding to the 28-kilodalton crystal protein. However, minor bands were present at ca. 27 and 29 kilodaltons (lane 2). The conclusion that these minor bands constitute noncrystal proteins was confirmed by incubating the supernatant proteins with antiserum produced to the 28-kilodalton crystal protein. No antigen-antibody reaction was detected. If the 28-kilodalton crystal hemolysin is present in the vegetative supernatant, it must represent ≤0.25% of the total protein. Further evidence that the crystal hemolysin and the vegetative hemolysin are distinct entities was obtained from the hemolysis due to *B. thuringiensis* subsp. *israelensis* colonies growing on blood-agar plates. Scrapings from these plates contained ≤5 heat-resistant (80°C for 10 min) spores per 10^9 CFU.

DISCUSSION

Vegetative cells of *B. thuringiensis* subsp. *israelensis* are toxic to *A. aegypti* larvae in bioassays lasting 10 to 12 h or longer. This toxicity is specific for *B. thuringiensis* subsp. *israelensis* rather than being a general characteristic of Bacilli. The cells must sporulate to express their larvicidal activity, and they will sporulate even if the bioassays are conducted in distilled water. Thus, the 50% lethal concentrations observed for dilutions of whole broth cultures are due to the sum of preexisting spores and crystals and those vegetative cells still capable of sporulating. Extended (24- or 48-h) bioassays may easily overestimate the initial degree of sporulation in a culture. This problem is exacerbated if the bioassays contain nutrients such as glycerol which permit further cell division prior to sporulation.

Although not excluded by our data, sporulation of ingested vegetative cells in the anterior portion of the larval midgut is unlikely for two reasons. (i) Even at neutral pH, sporulation of *B. thuringiensis* takes 6 to 8 h (4, 18), whereas the particle transit time through larvae of *Culex pipens* (7) and *A. aegypti* (our data) is only 3 to 4 h. (ii) *B. thuringiensis* subsp. *israelensis* does not qualify as an alkalophile. It does not grow at pH values of ≥9.5. However, the inhibitory effect of the alkaline gut pH is not a constant factor because

TABLE 1. Larval midgut pH during pathogenesis due to *B. thuringiensis* subsp. *israelensis* crystals[a]

pH	Change	Time (min)[b]
10–10.5 (8, 9)	Healthy	0
9.6 to 8.8	Blue to gray by thymol blue	66
8.8 to 8.0	Red to orange by cresol red	87
8.0 to 7.2	Orange to yellow by cresol red	99
≤7.2	Dead	116

[a] *A. aegypti* larvae were prelabeled for 1 h by feeding on a 0.1% solution of either thymol blue or cresol red and transferred to separate incubation tubes containing toxin in 5 ml of deionized water. The larval anterior midguts (their most alkaline region) were examined at intervals. Indicated times represent the average of 30 larvae observed in three separate experiments.

[b] Time following exposure to crystals. Control larvae (no crystals) maintained their gut pH of ≥9.6 for 24 h.
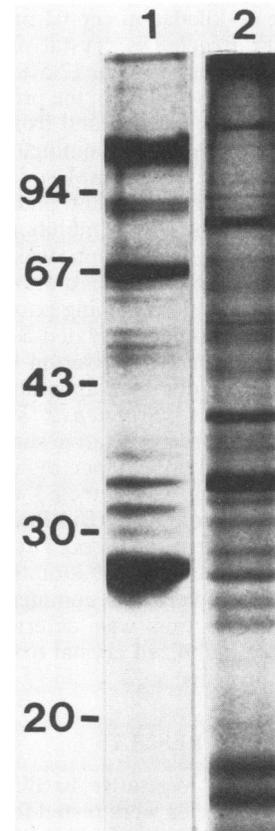

FIG. 1. Comparison of crystal proteins and cell-free supernatant proteins from *B. thuringiensis* subsp. *israelensis*. Crystal proteins (lane 1) and vegetative supernatant proteins (lane 2), ca. 25 µg of protein per lane. Numbers at left represent the molecular weight ($\times 10^3$) of the marker proteins (Pharmacia).

gut pH rapidly returns to neutrality with the onset of pathogenesis. The observations of Aly (1) on spore germination and vegetative growth in larvae of *A. aegypti* and *A. vexans* were conducted just prior to larval death. Thus, they concerned the ability of vegetative cells to exploit larval cadavers at neutral pH rather than to contribute to larval pathogenicity at alkaline pH.

Formation of PHB storage granules is a common step in the differentiation of vegetative cells into spores. The potential toxicity of PHB is illustrated by the observation of Nickerson et al. (22) that PHB was only 200 times less toxic to larvae of the tobacco hornworm *Manduca sexta* than were purified crystals. Similarly, Temeyer (29) reported a fraction from sporulated *B. thuringiensis* subsp. *israelensis* cultures which was toxic to larvae of the horn fly *Haematobia irritans*. Significantly, this otherwise uncharacterized fraction had been purified from density gradients at the position expected for PHB (20). However, our bioassays indicated that purified PHB was not toxic to *A. aegypti* larvae at 10 mg/ml, a concentration 10^6 times greater than the 50% lethal concentrations for purified *B. thuringiensis* subsp. *israelensis* crystals (25, 33). The larvicidal potential of *B. thuringiensis* subsp. *israelensis* vegetative cells is due to products made during sporulation (i.e., the protein crystal) rather than preceding sporulation.

B. thuringiensis subsp. *israelensis* cultures do not produce the heat-stable extracellular nucleotide known as the β-exotoxin (11) but supernatants from vegetative cells are still

hemolytic (11). However, this vegetative hemolysin is immunologically unrelated to the 28-kilodalton hemolysin obtained from solubilized B. thuringiensis subsp. israelensis crystals. The existence of two hemolysins synthesized at different growth stages is consistent with the report by Waalwijk et al. (34) that mRNA for the 28-kilodalton crystal protein is not present in vegetative cells. Transcription of the crystal hemolysin gene is limited to the early stages of sporulation.

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