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# Isolation and Identification of Novel Toxins from a New Mosquitocidal Isolate from Malaysia, *Bacillus thuringiensis* subsp. *jegathesan*

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**A new mosquitocidal *Bacillus thuringiensis* subsp., *jegathesan*, has recently been isolated from Malaysia. Parasporal crystal inclusions were purified from this strain and bioassayed against fourth-instar larvae of *Culex quinquefasciatus*, *Aedes aegypti*, *Aedes togoi*, *Aedes albopictus*, *Anopheles maculatus*, and *Mansonia uniformis*. The 50% lethal concentration of crystal inclusions for each species was 0.34, 8.08, 0.34, 17.59, 3.91, and 120 ng/ml, respectively. These values show that parasporal inclusions from this new subspecies have mosquitocidal toxicity comparable to that of inclusions isolated from *B. thuringiensis* subsp. *israelensis*. Solubilized and chymotrypsin-activated parasporal inclusions possessed low-level hemolytic activity. Sodium dodecyl sulfate-polyacrylamide gel electrophoresis revealed that the crystals were composed of polypeptides of 77, 74, 72, 68, 55, 38, 35, 27, and 23 kDa. Analysis by Western blotting (immunoblotting) with polyclonal antisera raised against toxins purified from *B. thuringiensis* subsp. *israelensis* reveals that proteins in parasporal inclusions of subsp. *jegathesan* are distinct, because little cross-reactivity was shown. Analysis of the plasmid content of *B. thuringiensis* subsp. *jegathesan* indicates that the genes for toxin production may be located on 105- to 120-kb plasmids. Cry$^-$ clones that have been cured of these plasmids are nontoxic. Southern blot analysis of plasmid and chromosomal DNA from subsp. *jegathesan* showed little or low homology to the genes coding for CryIVA, CryIVB, and CryIVD from *B. thuringiensis* subsp. *israelensis*.**

*Bacillus thuringiensis* is a gram-positive bacterium present in soil, water, and on plant surfaces. It produces characteristic protein inclusions during sporulation, which, when ingested, are toxic to a variety of insects (1, 7). To date, most strains of *B. thuringiensis* produce inclusions toxic to lepidopteran insects, though there are subspecies such as *israelensis* (10), *morrisoni* (21), *fukuokaensis* (31), *darmstadiensis* (20), and *kyushuensis* (18) which produce toxins that are predominantly toxic to dipteran insects. Subsp. *israelensis* was the first mosquitocidal subspecies isolated (10), and it produces the most highly toxic inclusions. Products with this strain have been successfully applied in the field as a biological control agent of mosquitoes and blackflies in locations throughout the world, and the strain has proven itself a practical alternative to traditional chemical control (15).

Numerous studies have explored the biological, immunological, and genetic similarities among different mosquitocidal *B. thuringiensis* subspecies (19, 21, 31). The genes for production of inclusions in *B. thuringiensis* subspecies *israelensis*, *fukuokaensis*, and *kyushuensis* are located on large plasmids of 110, 130, and 90 kb, respectively (11, 23, 31). The subsp. *israelensis* produces four proteins ranging from 135 to 27 kDa (8). In the subsp. *kyushuensis* proteins ranging from 140 to 25 kDa are observed (13); in subsp. *fukuokaensis* proteins of 90 to 27 kDa are observed (31); and in subsp. *medellin* proteins of 100 to 30 kDa are present (19). In all of these isolates a cytolytic toxin, generally 25 to 30 kDa, is also produced (4, 8, 9, 14, 31).

To date, there have been no reported cases of resistance development to *B. thuringiensis* subsp. *israelensis* in the field. In contrast, resistance to *B. thuringiensis* in field populations of lepidopteran pests that have been heavily sprayed with *B. thuringiensis* products has been reported (25). For example, Indian meal moth populations developed a 42-fold resistance to the *B. thuringiensis* subsp. *kurstaki* product Dipel (17). Further, as much as 33-fold resistance to Dipel was developed in populations of the diamondback moth, *Plutella xylostella*, located on watercress farms in Hawaii after heavy spraying from 1978 to 1982 (26). Laboratory selection of insects from this field population subsequently developed resistance by as much as 820-fold to Dipel after nine generations of selection. Resistance to Dipel has also been observed in the laboratory for the tobacco budworm (*Heliothis virescens*) (12). Diamondback moths that had acquired resistance to the CryIA(b) toxin from *B. thuringiensis* subsp. *kurstaki* had little cross-resistance to the CryIC toxin from subsp. *aizawai* (27). However, this CryIA(b)-resistant diamondback moth has high levels of cross-resistance to the CryIF toxin also from subsp. *aizawai* showing that cross-resistance to diverse toxins is possible (28).

In our laboratory a *Culex quinquefasciatus* colony has been selected for 72-fold resistance to CryIVD, one of the toxic proteins present in the subsp. *israelensis* toxin inclusion (2, 7). However, the level of resistance to the intact parasporal inclusion is low (5). Mosquito resistance to *B. thuringiensis* subsp. *israelensis* in the field has not been reported, but it may be possible to select for resistant mosquitoes if heavy continuous applications are used in the field. For example, resistance to the mosquitocidal *Bacillus sphaericus* has been reported after heavy field use in Brazil (6). Consequently, there is a good deal of interest in the isolation of new mosquitocidal strains of *B. thuringiensis* whose toxic properties differ significantly from those of *B. thuringiensis* subsp. *israelensis*. If new mosquitocidal *B. thuringiensis* products can be developed from strains producing novel toxins, it may be possible to apply these products either with or as an alternative to *B. thuringiensis* subsp. *israelensis* to lessen the chance of resistance development.

Here we describe the isolation and initial characterization of parasporal inclusions and proteins from a new isolate from

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Malaysia, *B. thuringiensis* subsp. *jegathesan* (serotype H-28a28c). Parasporal inclusions from this isolate have mosquitocidal activity as high as that of *B. thuringiensis* subsp. *israelensis*. Polyacrylamide gel electrophoresis (PAGE) and Western blotting (immunoblotting) indicate that the toxin proteins show little or no cross-reactivity to polyclonal antisera directed against *B. thuringiensis* subsp. *israelensis* toxins. Probes made from the cloned genes encoding CryIVA, CryIVB, and CryIVD do not hybridize to *B. thuringiensis* subsp. *jegathesan* DNA in Southern blots. We have evidence that the genes encoding the toxin proteins may be located on 105- to 120-kb plasmids present in the cell.

## MATERIALS AND METHODS

**Bacterial strains and culture conditions.** The *B. thuringiensis* subsp. *israelensis* strain was isolated from IPS82, which was obtained from the Pasteur Institute as a reference powder, and maintained in the laboratory. *B. thuringiensis* subsp. *jegathesan* (serotype H-28a28c) was isolated from Batu Gajah, Malaysia, and is part of the stock culture of the Institute for Medical Research, Kuala Lumpur, Malaysia. The YG-1 Cry$^-$ mutant of subsp. *israelensis* was prepared as previously described (31). *B. thuringiensis* strains were cultured on nutrient agar (Difco Inc.) at 30°C.

**Purification and solubilization of toxin inclusions.** After *B. thuringiensis* cells had undergone approximately 80% lysis on nutrient agar plates, the spore-inclusion mixture was suspended in 25 mM EDTA–2 mg of lysozyme per ml and incubated at 22°C for 30 min. Sodium chloride was added to 1 M, and the suspended lysate sonicated for 1 min. Inclusions were pelleted by centrifugation at 10,000 × *g* for 30 min in a Sorval SA600 rotor. The inclusions were thoroughly washed in 1 M NaCl–10 mM EDTA before purification on a 38 to 56% NaBr step gradient and centrifuged at 53,000 × *g* for 4 h in a Beckman SW28 rotor. The crystal inclusions were washed three times with 10 mM EDTA and stored at 4°C until needed. Crystal inclusions were solubilized in 50 mM $Na_2CO_3$–HCl (pH 10.0)–10 mM dithiothreitol at 37°C for 30 min.

**SDS-PAGE and Western blotting.** Sodium dodecyl sulfate (SDS)-PAGE was performed according to Laemmli (16) with 12% separating and 4% stacking gels. Following electrophoresis the gels were stained with 0.4% Coomassie blue R250. The molecular masses of proteins were determined by using protein standards (Sigma Chemical Co., St. Louis, Mo.). Protein concentrations were measured with the bicinchoninic acid protein assay kit from Pierce Chemical Co. (Rockford, Ill.) with bovine serum albumin as the standard.

Following SDS-PAGE, the resolved parasporal inclusion proteins were transferred to Immobilon-P nitrocellulose membrane (Millipore Corp., Bedford, Mass.) as previously described (29). The membrane was blocked with 5% skim milk in phosphate-buffered saline (PBS) for 1 h at 22°C and then incubated for 1 h at 4°C in 5% skim milk–PBS with polyclonal antibodies raised against *B. thuringiensis* subsp. *israelensis* toxin proteins. The antibodies used were previously developed in the laboratory to either intact parasporal inclusions or purified proteins. The nitrocellulose membrane was washed three times in PBS and then incubated with goat anti-rabbit immunoglobulin G–alkaline phosphatase in 5% skim milk–Tris-buffered saline for 1 h. The membrane was washed three times with Tris-buffered saline and then visualized with Nitro Blue Tetrazolium chloride and 5-bromo-4-chloro-3-indolyl phosphate as the substrate.

**Isolation and analysis of plasmid DNA.** *B. thuringiensis* strains were cultured overnight in Luria-Bertani broth, and plasmid DNA was purified by a modification of the method of Yu et al. (31). Following the precipitation of cell debris, the cell lysate was emulsified with one-half volume of Tris-equilibrated phenol, and then one-half volume of chloroform was added and emulsification continued. The lysate was centrifuged at 12,000 × *g* in a Sorval SA600 rotor to separate the aqueous phase, and 2 volumes of cold 100% ethanol was added to the aqueous phase to precipitate the DNA. The DNA was precipitated at −20°C for 2 h and then centrifuged at 12,000 × *g* for 20 min. The nucleic acid pellet was dissolved in Tris-EDTA buffer and stored at 4°C until needed. Plasmid DNA was analyzed by electrophoresis on a 0.5% agarose Tris-acetate-EDTA gel run at 3 V/cm for 5 h. Following electrophoresis and staining with ethidium bromide, plasmid DNA was transferred to a nylon membrane (Schleicher & Schuell, Keene, N.H.) by Southern blotting (24). Three DNA fragments encoding the proteins CryIVA, CryIVB, and CryIVD were gel purified with the Sephaglas bandprep kit (Pharmacia, Piscataway, N.J.) according to the manufacturer's instructions. The DNA fragments were labeled by random priming with digoxigenin-11-dUTP with the Genius DNA labeling and detection kit (Boehringer Mannheim Biochemicals, Indianapolis, Ind.). Labeled probes were hybridized to DNA bound on nylon membranes as described in the Genius kit. Posthybridization washes were performed at medium stringency (1.0× SSC [0.15 M NaCl plus 0.015 M sodium citrate] plus 0.1% SDS) at 55°C, and the bands were visualized with Nitro Blue Tetrazolium chloride and 5-bromo-4-chloro-3-indolyl phosphate as the substrate.

**Plasmid curing.** The 4B Cry$^-$ mutant of *B. thuringiensis* subsp. *jegathesan* was prepared by culturing overnight at 42°C as previously described (11), plating on nutrient agar, and selecting translucent colonies. Only Cry$^-$ colonies which still produced spores were selected for further study.

**Mosquitocidal activity.** Bioassays using purified intact parasporal inclusions were performed according to World Health Organization standard protocols with 150 ml of tap water and 25 fourth-instar larvae of *Culex quinquefasciatus*, *Aedes aegypti*, *Aedes togoi*, *Aedes albopictus*, *Anopheles maculatus*, and *Mansonia uniformis*. All colonies are maintained at the Institute for Medical Research except *M. uniformis*, which was collected from the field, and the F1 generations were used for testing. To facilitate comparison between the two bacterial subspecies, all assays were performed with the same populations of mosquito larvae. Assays were performed in triplicate, and mortality was scored after 24 and 48 h of incubation at 22°C. Values for 50 and 90% lethal concentrations ($LC_{50}$ and $LC_{90}$, respectively) were determined by probit analysis as previously described (22).

**Hemolytic activity.** Fresh human erythrocytes were washed three times with PBS and suspended in PBS at a concentration of $10^8$ cells per ml. The erythrocytes (125 μl) were mixed with equal volumes of solubilized toxin, previously activated with trypsin or chymotrypsin, and the mixture was then incubated at 37°C for 1 h. After centrifugation of cell debris for 5 min at 7,000 × *g*, the supernatant was removed and read against a blank at 570 nm. The maximum optical density for total releasable hemoglobin was determined by lysing cells with sterile distilled water. The toxin concentration that gave an optical density half that of the maximum was designated as the $LC_{50}$.

TABLE 1. Toxicity of parasporal inclusions from *B. thuringiensis* subsp. *israelensis* to fourth-instar larvae from mosquito species

| Species | $LC_{50}$ (ng/ml) (range) | $LC_{90}$ (ng/ml) (range) | Slope |
|---|---|---|---|
| *Aedes aegypti* | 1.03 (0.59–1.75) | 5.72 (1.58–25.24) | 1.73 |
| *Aedes albopictus* | 4.09 (2.81–7.08) | 32.0 (14.10–284.96) | 1.44 |
| *Aedes togoi* | 1.75 (1.19–2.29) | 10.8 (6.8–27.09) | 1.62 |
| *C. quinquefasciatus* | 0.70 (0.54–0.84) | 2.32 (1.80–3.49) | 2.46 |
| *Anopheles maculatus* | 1.62 (1.39–1.93) | 4.56 (3.34–8.17) | 2.87 |
| *M. uniformis* | 27.5 (18.86–38.0) | 327 (192.45–742.56) | 1.19 |

## RESULTS

**Parasporal inclusion morphology and biological activity.** Under light microscopy the parasporal inclusions isolated from *B. thuringiensis* subsp. *jegathesan* have a spherical morphology similar to that of the inclusions from subsp. *israelensis*. The inclusions from subsp. *jegathesan* have mosquitocidal activity comparable to that of parasporal inclusions from *B. thuringiensis* subsp. *israelensis*. This latter subspecies is more toxic than subsp. *jegathesan* to *Anopheles maculatus*, *Aedes aegypti*, and *Aedes albopictus* (Tables 1 and 2). However, subsp. *jegathesan* is more toxic towards *C. quinquefasciatus* and *Aedes togoi*. Both *B. thuringiensis* subsp. *israelensis* and subsp. *jegathesan* have lower toxicity towards *M. uniformis*, with subsp. *israelensis* being more toxic (Tables 1 and 2). The $LC_{50}$ for these two subspecies are 27.5 and 120 ng/ml, respectively. However, there was significant variability in the dose-response curve, particularly at high concentrations.

As with other mosquitocidal *B. thuringiensis* strains, subsp. *jegathesan* possesses low-level hemolytic activity. A dose-response curve in lysing human erythrocytes was observed (Fig.

TABLE 2. Toxicity of parasporal inclusions from *B. thuringiensis* subsp. *jegathesan* to fourth-instar larvae from mosquito species

| Species | $LC_{50}$ (ng/ml) (range) | $LC_{90}$ (ng/ml) (range) | Slope |
|---|---|---|---|
| *Aedes aegypti* | 8.08 (4.36–14.71) | 37.8 (9.05–198.68) | 1.91 |
| *Aedes albopictus* | 17.6 (13.51–22.52) | 40.3 (21.01–95.87) | 3.56 |
| *Aedes togoi* | 0.34 (0.1–1.17) | 11.2 (1.31–103.34) | 0.85 |
| *C. quinquefasciatus* | 0.34 (0.12–1.01) | 1.42 (0.19–11.74) | 2.06 |
| *Anopheles maculatus* | 3.91 (3.29–4.59) | 10.4 (8.36–14.33) | 3.01 |
| *M. uniformis* | 120 (high) | 13,800 (high) | 0.62 |

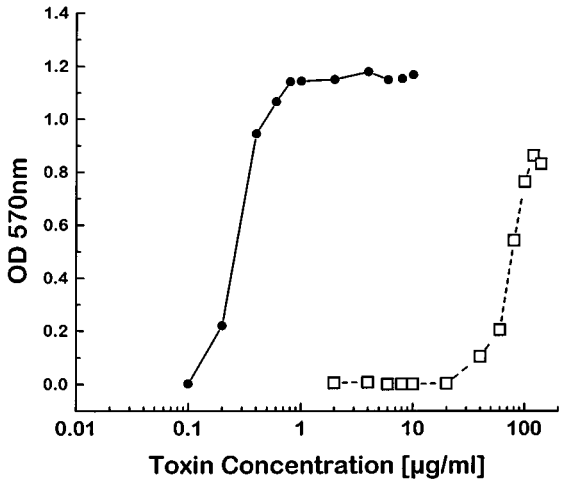


FIG. 1. A dose-response curve of the hemolytic activity of solubilized parasporal inclusions of *B. thuringiensis* subsp. *jegathesan* (open squares) and subsp. *israelensis* (closed circles). Data represent the average of duplicate assays. Similar results were obtained in replicate experiments. OD, optical density.

1), with an $LC_{50}$ of 80 μg/ml. Hemolytic activity was detected only with solubilized toxin that was activated with chymotrypsin. Untreated or trypsin-activated toxin displayed no activity. Significantly higher levels of hemolytic activity were detected in subsp. *israelensis*, either with or without prior proteolytic processing by trypsin ($LC_{50}$, 0.3 μg/ml).

**SDS-PAGE analysis and immunological similarity.** SDS-PAGE analysis of parasporal inclusions purified from *B. thuringiensis* subsp. *jegathesan* demonstrated significant differences in protein composition compared with that of *B. thuringiensis* subsp. *israelensis*. The parasporal inclusions of *B. thuringiensis* subsp. *jegathesan* contain a number of proteins (Fig. 2A, lane 2). Proteins with molecular masses of 77, 74, 72, 68, 55, 38, 35, 27, and 23 kDa were observed. This protein profile is significantly different from that for parasporal inclusions from *B. thuringiensis* subsp. *israelensis* (Fig. 2A, lane 1). The parasporal inclusions were isolated in the presence of ETDA to reduce metalloprotease activity. The use of phenylmethylsulfonyl fluoride did not change the protein profile. Nevertheless, some of the minor bands could arise from proteolytic processing.

Antigenic relationships among the crystal inclusion proteins of *B. thuringiensis* subsp. *jegathesan* and *B. thuringiensis* subsp. *israelensis* were examined by immunoblotting duplicate lanes of each subspecies with polyclonal antisera raised against whole parasporal inclusions of *B. thuringiensis* subsp. *israelensis*, the CryIVD toxin purified from *B. thuringiensis* subsp. *israelensis*, and purified CytA toxin from *B. thuringiensis* subsp. *israelensis*. Each of the three antisera reacted strongly with subsp. *israelensis* proteins but showed only slight cross-reactivity to *B. thuringiensis* subsp. *jegathesan* proteins (Fig. 2B through D). Antibodies against the whole parasporal inclusions of *B. thuringiensis* subsp. *israelensis* detected proteins of 105, 95, and 38 kDa in the parasporal inclusions of subsp. *jegathesan* (Fig. 2D, lane 2). The 105- and 95-kDa bands are very faint; they are more pronounced with longer color development. These proteins are, however, not visible on SDS-PAGE gels (Fig. 2A, lane 2). The CryIVD antibody had a low cross-reactivity to the 77-kDa protein from *B. thuringiensis* subsp. *jegathesan*. This was evident particularly when high protein concentrations were used and when color development on the immunoblot proceeded for a long period. The CytA antisera showed no apparent cross-reactivity to any of the subsp. *jegathesan* proteins (Fig. 2C).

**Plasmid profile.** To determine whether the toxins produced by *B. thuringiensis* subsp. *jegathesan* are plasmid encoded, and to establish the molecular size(s) of the plasmid(s) that potentially codes for these toxins, mutant bacteria that do not pro-

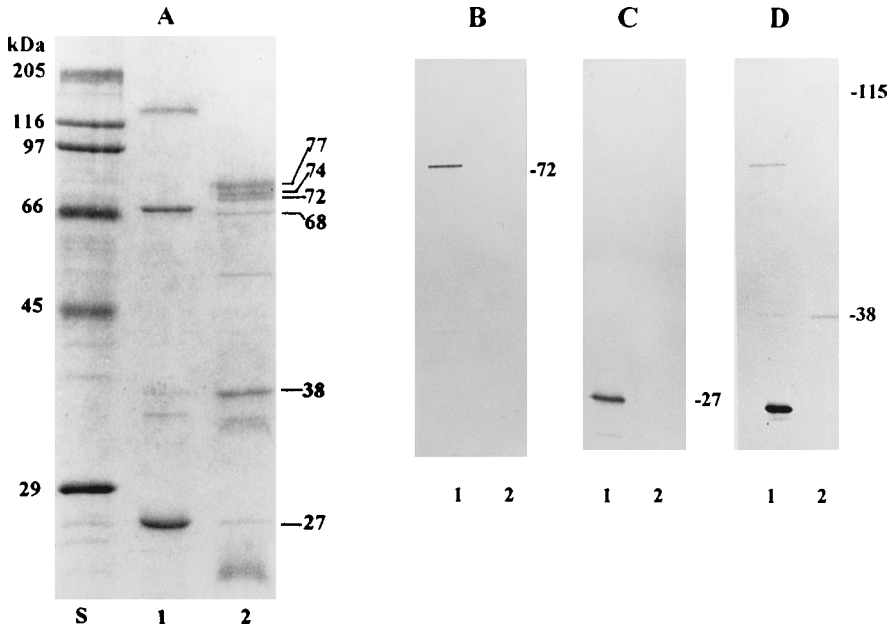


FIG. 2. SDS-PAGE (A) and immunoblots (B to D) of crystal inclusion proteins from mosquitocidal *B. thuringiensis*. Lanes 1, *B. thuringiensis* subsp. *israelensis*; lanes 2, *B. thuringiensis* subsp. *jegathesan*; lane S, standards as follows (in kilodaltons): carbonic anhydrase, 29; ovalbumin, 45; bovine serum albumin, 66; phosphorylase *b*, 97; β-galactosidase, 116; and myosin, 205. Immunoblots (B to D) were probed with antibodies raised against purified CryIVD, CytA, and whole *B. thuringiensis* subsp. *israelensis* crystal inclusion, respectively.

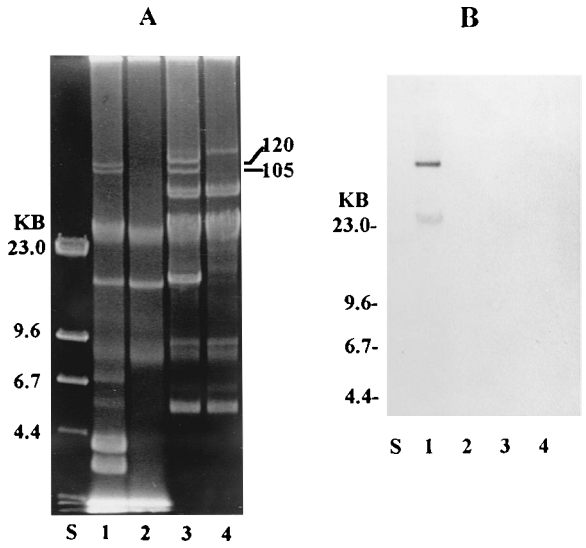


FIG. 3. Plasmid DNA of mosquitocidal *B. thuringiensis* strains. (A) Plasmid DNA from *B. thuringiensis* strains electrophoresed in a 0.5% agarose gel. (B) Southern hybridization of DNA from panel A with *cryIVB* gene. Molecular sizes (in kilobases) are shown. Lanes 1, plasmid DNA from subsp. *israelensis*; lanes 2, plasmid DNA from subsp. *israelensis* YG-1, a Cry$^-$ mutant; lanes 3, plasmid DNA from subsp. *jegathesan*; and lanes 4, plasmid DNA from subsp. *jegathesan* 4B, a Cry$^-$ mutant; lanes S, λ phage DNA digested with *Hin*dIII.

duce parasporal inclusions were isolated. Potential Cry$^-$ mutants were first identified on nutrient agar plates by their translucent appearance when viewed against a dark background. Mutants were subsequently confirmed by phase-contrast microscopy, plasmid isolation, and the lack of toxicity to *C. quinquefasciatus* larvae. Figure 3 shows the plasmid patterns of wild-type *B. thuringiensis* subsp. *israelensis* (panel A, lane 1) and wild-type *B. thuringiensis* subsp. *jegathesan* (panel A, lane 3). This new subspecies has at least nine plasmids of 135, 120, 105, 87, 85, 26, 17, 15, and 10 kb. Figure 3A, lane 2, shows the YG-1 Cry$^-$ mutant of *B. thuringiensis* subsp. *israelensis* which lacks the 110-kb plasmid encoding the CryIV and the CytA proteins, while lane 4 shows a Cry$^-$ mutant of *B. thuringiensis* subsp. *jegathesan*, 4B, lacking the 120-, 105-, and 26-kb plasmids. This 4B mutant, which still produces spores, lacks mosquitocidal activity.

Southern blot analysis of the plasmids with a cloned 2.0-kb fragment of the gene of *B. thuringiensis* subsp. *israelensis* coding for CryIVB shows a strong hybridization to the 110-kb plasmid of *B. thuringiensis* subsp. *israelensis* (Fig. 3B, lane 1) but not to the YG-1 Cry$^-$ mutant of *B. thuringiensis* subsp. *israelensis* (Fig. 3B, lane 2). No hybridization signal was detected from any plasmid or chromosomal DNA with either the wild-type or the Cry$^-$ mutant of *B. thuringiensis* subsp. *jegathesan* (Fig. 3B, lanes 3 and 4). Two other blots were probed with cloned sequences of CryIVA and CryIVD, with similar results (data not shown). Since medium stringency conditions were used, it is possible, however, that the toxin genes from the new subspecies may have low levels of homology to the toxin genes from *B. thuringiensis* subsp. *israelensis*.

## DISCUSSION

In the search for potential alternatives to the application of *B. thuringiensis* subsp. *israelensis*, a new subspecies, *B. thuringiensis* subsp. *jegathesan* (belonging to serotype H-28a28c), that appears to possess novel mosquitocidal toxins has been isolated. In this study, we separated the parasporal inclusions from *B. thuringiensis* subsp. *jegathesan* and compared its insecticidal activity to those of six mosquito species from four genera, all of which are important vectors of human diseases in Southeast Asia.

The insecticidal activity of this new subspecies, *B. thuringiensis* subsp. *jegathesan*, against these six mosquito species is comparable to that of *B. thuringiensis* subsp. *israelensis*. The purified parasporal inclusions from both subspecies have $LC_{50}$s at the nanogram-per-ml level. Interestingly, parasporal inclusions of this new subspecies are slightly more toxic to some mosquito species, such as *C. quinquefasciatus* and *Aedes togoi*, while *B. thuringiensis* subsp. *israelensis* is slightly more toxic to *Aedes aegypti* and *Anopheles maculatus*. The mosquitocidal activity of this new strain is, however, much higher than the toxicity of other recently isolated mosquitocidal *B. thuringiensis* subspecies such as subsp. *darmstadiensis*, subsp. *fukuokaensis*, and subsp. *kyushuensis* (31).

Like other mosquitocidal *B. thuringiensis* isolates (3, 14, 19, 30, 31), subsp. *jegathesan* exhibits cytolytic activity following sodium carbonate-dithiothreitol solubilization and chymotrypsin activation. The activity of whole parasporal protein of subsp. *jegathesan* is, however, significantly lower than that of subsp. *israelensis*. Although the cytolytic toxin has yet to be identified, it is likely to be either the 23- or the 27-kDa protein and it probably is a new cytolytic toxin, CytD. The 23- and 27-kDa proteins are not present in high concentrations in the inclusion body, which may explain, in part, why the levels of hemolytic activity of intact inclusions of subsp. *jegathesan* are rather low. In contrast, the CytA protein is the predominant protein in subsp. *israelensis* crystal, hence the higher level of hemolytic activity observed with this subspecies.

Analysis by SDS-PAGE of the protein profile of the parasporal inclusions of *B. thuringiensis* subsp. *jegathesan* revealed at least 10 polypeptides with molecular masses of 77 to 23 kDa. The molecular masses of these proteins suggest there is no major overlap among any of these proteins with those of *B. thuringiensis* subsp. *israelensis* or with those of other mosquitocidal strains such as *B. thuringiensis* subsp. *medellin* (19), *B. thuringiensis* subsp. *fukuokaensis* (31), and *B. thuringiensis* subsp. *kyushuensis* (13).

Using antisera specific to *B. thuringiensis* subsp. *israelensis* toxins, we demonstrated that there is little immunological relationship among the parasporal inclusion proteins of *B. thuringiensis* subsp. *jegathesan* and *B. thuringiensis* subsp. *israelensis*. Antisera for whole *B. thuringiensis* subsp. *israelensis* parasporal inclusions only weakly cross-reacted to 105-, 95-, and 38-kDa protein bands of *B. thuringiensis* subsp. *jegathesan*. Potentially there are additional toxins of 105 and 95 kDa produced in low levels that are poorly visualized by SDS-PAGE but that cross-react with antisera raised against the whole parasporal inclusion of *B. thuringiensis* subsp. *israelensis*. Antisera specific for the CytA toxin of *B. thuringiensis* subsp. *israelensis* did not cross-react at all.

The plasmid profile of *B. thuringiensis* subsp. *jegathesan* is distinctly different from that of *B. thuringiensis* subsp. *israelensis*, with plasmids ranging from 4.2 to 135 kb observed. The 120- and/or 105-kb plasmid is apparently required for production of parasporal inclusions, because isolates cured of these plasmids became Cry$^-$ and lost their mosquitocidal activity.

The unique SDS-PAGE profile, the lack of high levels of immunological similarity, and the lack of DNA hybridization to the CryIVA, -B, and -D toxin genes suggest that most of the toxins found in the parasporal inclusions of *B. thuringiensis* subsp. *jegathesan* are likely to be unique mosquitocidal toxins. However, some of the toxins, in particular the 77-kDa toxin,

could have low homology to the CryIVD toxin, since low-level immunological cross-reactivity was observed with antibodies raised to the CryIVD toxin. Moreover, if the 105- and 95-kDa proteins observed in immunoblots are indeed toxins, then these proteins are likely to have some homology with *B. thuringiensis* subsp. *israelensis* toxins.

The mosquitocidal activity observed in the bioassay using three *Aedes* species shows that different species within this genus can have different sensitivities to a particular toxin, as is evident with *B. thuringiensis* subsp. *jegathesan*. Hence, it is critical that data generated against *Aedes aegypti* not be used as indicative of the toxicity towards other *Aedes* species. It is highly probable that even greater differences will be observed in *Anopheles* and other mosquito genera.

The lower activity of both *B. thuringiensis* subsp. *israelensis* and *B. thuringiensis* subsp. *jegathesan* to *M. uniformis*, a vector of filariasis in Southeast Asia, could in part be due to the different types of larval behavior of this mosquito species. If these low activities are indeed reflective of the biological responses of this mosquito species, this study further illustrates the need to seek new *B. thuringiensis* strains with increased mosquitocidal activities towards this species. Moreover, it is critical that an evaluation of mosquitocidal activities of new *B. thuringiensis* isolates utilize mosquito species that are important vectors of diseases in developing countries.

In conclusion, we have isolated and begun to characterize a new mosquitocidal *B. thuringiensis* serotype whose toxin proteins are significantly different from those of *B. thuringiensis* subsp. *israelensis*. Although different, *B. thuringiensis* subsp. *jegathesan* is apparently as toxic to a variety of mosquito species as *B. thuringiensis* subsp. *israelensis* and may potentially be an alternative or supplement to the application of *B. thuringiensis* subsp. *israelensis* for the biological control of mosquitoes. An ability to use this new subspecies or toxins from this new species will require further studies to determine if these toxins indeed act at the same or alternative target sites.

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## REFERENCES

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. **Dai, S.-M.** 1992. *Bacillus thuringiensis* subsp. *israelensis* CryIVD toxin: resistance development, proteolysis and binding characteristics in *Culex quinquefasciatus*. University of California, Riverside.
3. **Drobniewski, F. A., and D. J. Ellar.** 1989. Purification and properties of a 28-kilodalton hemolytic and mosquitocidal protein toxin of *Bacillus thuringiensis* subsp. *darmstadiensis* 73-E10-2. J. Bacteriol. **171:**3060–3067.
4. **Drobniewski, F. A., B. H. Knowles, and D. J. Ellar.** 1987. Nonspecific ionic effects on the cytolytic and hemolytic properties of *Bacillus thuringiensis* delta-endotoxins. Curr. Microbiol. **15:**295–299.
5. **Georghiou, G. P.** 1990. Resistance potential to biopesticides and consideration of countermeasures, p. 409–420. *In* J. E. Casida (ed.), Pesticides and alternatives. Elsevier Sciences, Amsterdam.
6. **Georghiou, G. P.** 1994. Mechanisms and microbial characteristics of invertebrate resistance to bacterial toxins, p. 48–50. *In* Proceedings of the VIth International Colloquium on Invertebrate Pathology and Microbial Control, vol. 1. Society of Invertebrate Pathology, Montpellier, France.
7. **Gill, S. S., E. A. Cowles, and P. V. Pietrantonio.** 1992. The mode of action of *Bacillus thuringiensis* endotoxins. Annu. Rev. Entomol. **37:**615–636.
8. **Gill, S. S., J. M. Hornung, J. E. Ibarra, G. J. P. Singh, and B. A. Federici.** 1987. Cytolytic activity and immunological similarity of the *Bacillus thuringiensis* subsp. *israelensis* and *Bacillus thuringiensis* subsp. *morrisoni* isolate PG-14 toxins. Appl. Environ. Microbiol. **53:**1251–1256.
9. **Gill, S. S., G. J. P. Singh, and J. M. Hornung.** 1987. Cell membrane interaction of *Bacillus thuringiensis* subsp. *israelensis* cytolytic toxins. Infect. Immun. **55:**1300–1308.
10. **Goldberg, L. J., and J. Margalit.** 1977. A bacterial spore demonstrating rapid larvicidal activity against *Anopheles sergentii*, *Uranotaenia unguiculata*, *Culex univitattus*, *Aedes aegypti* and *Culex pipiens*. Mosquito News **37:**355–358.
11. **Gonzalez, J. M., Jr., and B. C. Carlton.** 1984. A large transmissible plasmid is required for crystal toxin production in *Bacillus thuringiensis* variety *israelensis*. Plasmid **11:**28–38.
12. **Gould, F., A. Martinez-Ramirez, A. Anderson, J. Ferre, F. J. Silva, and W. J. Moar.** 1992. Broad-spectrum resistance to *Bacillus thuringiensis* toxins in *Heliothis virescens*. Proc. Natl. Acad. Sci. USA **89:**7986–7990.
13. **Knowles, B. H., P. J. White, C. N. Nicholls, and D. J. Ellar.** 1992. A broad-spectrum cytolytic toxin from *Bacillus thuringiensis* var. *kyushuensis*. Proc. R. Soc. Lond. B **248:**1–7.
14. **Koni, P. A., and D. J. Ellar.** 1993. Cloning and characterization of a novel *Bacillus thuringiensis* cytolytic delta-endotoxin. J. Mol. Biol. **229:**319–327.
15. **Kurtak, D. C., C. Back, A. Chalifour, J. Doannio, J. Dossou-Yovo, J. Duval, P. Guillet, R. Meyer, M. Ocran, and B. Wahle.** 1989. Impact of *B. t. israelensis* on blackfly control in the Onchocerciasis Control Programme in West Africa. Isr. J. Entomol. **23:**21–28.
16. **Laemmli, U. K.** 1970. Cleavage of structural proteins during the assembly of the head of bacteriophage T4. Nature (London) **227:**680–685.
17. **McGaughey, W. H.** 1985. Insect resistance to the biological insecticide *Bacillus thuringiensis*. Science **229:**193–196.
18. **Ohba, M., and K. Aizawa.** 1979. A new subspecies of *Bacillus thuringiensis* possessing 11a:11c flagellar antigenic structure: *Bacillus thuringiensis* subsp. *kyushuensis*. J. Invertebr. Pathol. **33:**387–388.
19. **Orduz, S., T. Diaz, I. Thiery, J. F. Charles, and W. Rojas.** 1994. Crystal proteins from *Bacillus thuringiensis* serovar *medellin*. Appl. Microbiol. Biotechnol. **40:**794–799.
20. **Padua, L. E., M. Ohba, and K. Aizawa.** 1980. The isolates of *Bacillus thuringiensis* serotype 10 with a highly preferential toxicity to mosquito larvae. J. Invertebr. Pathol. **36:**180–186.
21. **Padua, L. E., M. Ohba, and K. Aizawa.** 1984. Isolation of a *Bacillus thuringiensis* strain (serotype 8a:8b) highly and selectively toxic against mosquito larvae. J. Invertebr. Pathol. **44:**12–17.
22. **Raymond, M.** 1985. Presentation d'un programme basic d'analyse log-probit pour micro-orinateur. Cah. O.R.S.T.O.M. Ser. Entomol. Med. Parasitol. **23:**117–121.
23. **Sekar, V.** 1987. DNA homology between the crystal toxin genes of several mosquito pathogenic *Bacillus thuringiensis* strains. Curr. Microbiol. **15:**287–289.
24. **Southern, E. M.** 1975. Detection of specific sequences among DNA fragments separated by gel electrophoresis. J. Mol. Biol. **98:**503–528.
25. **Tabashnik, B. E.** 1994. Evolution of resistance to *Bacillus thuringiensis*. Annu. Rev. Entomol. **39:**47–79.
26. **Tabashnik, B. E., N. L. Cushing, N. Finson, and M. W. and Johnson.** 1990. Field development of resistance to *Bacillus thuringiensis* in diamondback moth (Lepidoptera: Plutellidae). J. Econ. Entomol. **83:**1671–1676.
27. **Tabashnik, B. E., N. Finson, F. R. Groeters, W. J. Moar, M. W. Johnson, K. Luo, and M. J. Adang.** 1994. Reversal of resistance to *Bacillus thuringiensis* in *Plutella xylostella*. Proc. Natl. Acad. Sci. USA **91:**4120–4124.
28. **Tabashnik, B. E., N. Finson, M. W. Johnson, and D. G. Heckel.** 1994. Cross-resistance to *Bacillus thuringiensis* toxin CryIF in the diamondback moth (*Plutella xylostella*). Appl. Environ. Microbiol. **60:**4627–4629.
29. **Towbin, H., T. Staehelin, and J. Gordon.** 1979. Electrophoretic transfer of proteins from polyacrylamide gels to nitrocellulose sheets: procedures and some applications. Proc. Natl. Acad. Sci. USA **76:**4350–4354.
30. **Waalwijk, C., A. M. Dullemans, M. E. S. 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.
31. **Yu, Y.-M., M. Ohba, and S. S. Gill.** 1991. Characterization of mosquitocidal activity of *Bacillus thuringiensis* subsp. *fukuokaensis* crystal proteins. Appl. Environ. Microbiol. **57:**1075–1081.