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Two Novel Strains of *Bacillus thuringiensis* Toxic to Coleopterans

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Two novel strains of *Bacillus thuringiensis* were isolated from native habitats by the use of genes coding for proteins toxic to coleopterans (*cryIII* genes) as hybridization probes. Strain EG2838 (isolated by the use of the *cryIIIA* probe) contained a *cryIIIA*-hybridizing plasmid of approximately 100 MDa and synthesized crystal proteins of approximately 200 (doublet), 74, 70, 32, and 28 kDa. Strain EG4961 (isolated by the use of a *cryIIIA*-related probe) contained a *cryIIIA*-hybridizing plasmid of approximately 95 MDa and synthesized crystal proteins of 74, 70, and 30 kDa. Structural relationships among the crystal proteins of strains EG2838 and EG4961 were detected; antibodies to the CryIIIA protein toxic to coleopterans reacted with the 74- and 70-kDa proteins of EG2838 and EG4961, antibodies to the 32-kDa plus 28-kDa proteins of EG2838 reacted with the 30-kDa protein of EG4961, and antibodies to the 200-kDa proteins of EG2838 reacted with the 28-kDa protein of EG2838. Experiments with *B. thuringiensis* flagella antibody reagents demonstrated that EG2838 belongs to H serotype 9 (reference strain *B. thuringiensis* subsp. *tolworthi*) and that EG4961 belongs to H serotype 18 (reference strain *B. thuringiensis* subsp. *kumamotoensis*). A mixture of spores plus crystal proteins of either EG2838 or EG4961 was toxic to the larvae of Colorado potato beetle (*Leptinotarsa decemlineata*), and significantly, the EG4961 mixture was also toxic to the larvae of southern corn rootworm (*Diabrotica undecimpunctata howardi*). DNA restriction blot analysis suggested that strains EG2838 and EG4961 each contained a unique gene coding for a protein toxic to coleopterans.

The insecticidal activity of *Bacillus thuringiensis* is primarily due to certain proteins that are synthesized in abundance during sporulation and that form crystals. The more common subspecies of *B. thuringiensis* (e.g., *kurstaki*, *aizawai*, and *tolworthi*) produce crystal proteins (CryI proteins) of approximately 130 kDa that are toxic to lepidopterans and that form bipyramidal crystals, and many varieties produce a crystal protein (CryIIA) of 71 kDa that is toxic to lepidopterans and dipterans (e.g., mosquitoes) and that forms cuboidal crystals. Krieg et al. (16) described the first strain of *B. thuringiensis* (strain BI 256-82 of *B. thuringiensis* subsp. *tenebrionis*) that is toxic to coleopterans which was shown to be toxic to *Leptinotarsa decemlineata* (Colorado potato beetle). *B. thuringiensis* subsp. *tenebrionis* produces flat, rhomboid crystals (16) composed of proteins of approximately 68 kDa (2). Herrnstadt et al. (14) reported the isolation of a strain of *B. thuringiensis* that is toxic to coleopterans, designated strain M7 of *B. thuringiensis* subsp. *san diego*, that appears to be identical to *B. thuringiensis* subsp. *tenebrionis* (17). Donovan et al. (6) reported the isolation of a strain of *B. thuringiensis* (strain EG2158) that was toxic to coleopterans and that differed from *B. thuringiensis* subsp. *tenebrionis*, as judged by plasmid array and by the production of certain crystal proteins. All three organisms (*B. thuringiensis* subsp. *tenebrionis*, *B. thuringiensis* subsp. *san diego*, and strain EG2158) contain an identical gene (*cryIIIA*) encoding a 73-kDa protein toxic to larvae of the Colorado potato beetle (6, 13, 15, 21, 25). Here we report the isolation and characterization of two novel strains that differ significantly from previously described strains that are toxic to coleopterans.

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MATERIALS AND METHODS

Bacterial strains. *B. thuringiensis* H serotype reference strains were obtained from the collection of Dulmage (7) or from the Entomopathogenic Bacteria collection of the Institut Pasteur, Paris, France.

Growth. For the production of crystal proteins, *B. thuringiensis* strains were grown as shake flask cultures in DSMG medium [0.4% (wt/vol) nutrient broth (Difco), 0.5% (wt/vol) glucose, 25 mM K_2HPO_4, 25 mM KH_2PO_4, 0.5 mM $Ca(NO_3)_2$, 0.5 mM $MgSO_4$, 10 μM $MnCl_2$, 10 μM $FeSO_4$] at room temperature (21 to 24°C) for 4 to 5 days until sporulation and cell lysis had occurred. For the preparation of flagella, *B. thuringiensis* strains were grown as static liquid cultures in nutrient broth (Difco) plus 0.1% (wt/vol) glucose.

Colony hybridization. A modified colony hybridization procedure of Grunstein and Hogness (12) was used. Environmental samples, consisting of soil, crop dust, and organic material, were suspended in spore buffer (0.06% [wt/vol] sodium phosphate [pH 7.0], 0.05% [wt/vol] potassium phosphate [pH 6.6], 0.005% [wt/vol] sodium dodecyl sulfate [SDS], 1 μl of 1-octanol per ml; final pH of buffer approximately 7) to a concentration of 1 g of material per 10 ml of buffer, heated for 30 min at 60°C, diluted in spore buffer, and spread on NSM (19) agar medium. After overnight growth at 25°C, colonies were blotted from the agar medium to nitrocellulose filters and treated with 0.5 M NaOH–1.5 M NaCl for 30 min and then with 1 M ammonium acetate–0.02 M NaOH for 30 min. The filters were probed at 65°C in a solution containing 3× SSC (1× SSC is 0.15 M NaCl plus 0.015 M sodium citrate), 10× Denhardt's solution (1× Denhardt's solution is 0.02% [wt/vol] bovine serum albumin, 0.02% [vol/vol] Ficoll, 0.02% [wt/vol] polyvinylpyrrolidone), 200 μg of heparin per ml, and 0.1% SDS with the 2.0-kb *Hin*dIII-*Xba*I *cryIIIA* fragment of strain EG2158 (6) or with

the 2.4-kb *Ssp*I *cryIIIA*-related fragment of strain EG2838 (6a) that had been radioactively labeled with [α-^{32}P]dATP by random primer extension (10). Filters were washed at 65°C in 3× SSC–0.1% SDS and exposed to X-ray film.

Crystal protein quantification. Sporulated cultures of *B. thuringiensis* were harvested by centrifugation, washed twice with TETX (10 mM Tris-HCl [pH 7.5], 1 mM EDTA, 0.005% [vol/vol] Triton X-100), suspended in TETX to a concentration of 100 mg (wet weight) (primarily spores plus crystal proteins) per ml, and stored at 4°C. Ten-microliter samples of a 100-mg/ml suspension of *B. thuringiensis* spores plus crystal proteins were injected, by use of a Hamilton syringe, into several volumes (usually 90 to 290 μl) of preheated (100°C) sample buffer (0.13 M Tris-HCl [pH 8.5], 2% SDS, 5% 2-mercaptoethanol, 0.05% bromphenol blue, 10% glycerol), incubated at 100°C for 7 min, vortexed for 10 s, and aliquots (usually 10- and 20-μl aliquots) of the mixture were loaded onto a polyacrylamide gel (18). Proteins were size fractionated by electrophoresis, stained with Coomassie blue dye, and quantified by densitometry with a Molecular Dynamics computing densitometer (model 300A), using known amounts of purified CryIIIA protein (quantified by the Bio-Rad protein assay kit; Bio-Rad Inc., Richmond, Calif.) as a standard.

Production of antibody reagents. Polyclonal antibodies were produced in mice, by the methods previously described by Sartorelli et al. (24), or in rabbits (4). EG2838 and EG2158 crystal protein antigens were purified by preparative SDS-polyacrylamide gel electrophoresis (PAGE), using the discontinuous system of Laemmli (18). Crude preparations of EG2838 and EG2158 spores and crystal proteins were washed twice with TETX and treated with protein buffer, and solubilized proteins were separated by SDS-PAGE (10% acrylamide resolving gel). Protein bands were visualized by treatment with 0.5 M KCl at 4°C and excised from gels with a sterile blade. Proteins were eluted from gel slices and emulsified with Freund's complete adjuvant for use as primary immunogens. Immunoglobulin G antibodies were purified from hyperimmune mouse ascitic fluids by protein A affinity chromatography (as described previously in reference 9, except that 3.0 M NaCl was added to the binding buffer). Antibodies to purified crystal proteins were screened for reaction with homologous antigens by SDS-PAGE or immunoblot analysis (see below).

Flagellar antibody reagents were made by using purified flagellar filaments or flagellated cells of *B. thuringiensis* H serotype reference strains (4) or strains EG2158, EG2838, and EG4961. For mouse antibody production, flagellar filaments were sheared from vegetative cells by vortexing, cells were removed by centrifugation, and flagellar filaments were collected from the supernatants on 0.2-μm-pore-size filters. Suspensions of purified flagellar filaments were used as immunogens for antibody production. Crude antisera were screened for specific agglutination of homologous cells.

SDS-PAGE or immunoblot analysis and flagellar serotyping assays. SDS-PAGE and immunoblot (Western blot) analysis was done essentially by the method of Towbin et al. (28). Nitrocellulose replicas of SDS-PAGE gels were blocked with 5% nonfat milk; antibody reagents were used at dilutions of 1/500 to 1/1,000 in Tris-buffered saline, pH 7.8; antibody wash solutions were Tris-buffered saline plus 0.05% Triton X-405 and 0.05% SDS. Bound antibody was detected by using an alkaline phosphatase-linked second antibody and was visualized with a 5-bromo-4-chloro-indolylphosphate/nitroblue tetrazolium substrate system.

Flagellar serotypes of strains EG2158, EG2838, and EG4961 were analyzed by antibody-mediated cell agglutination assays (4). Serial dilutions of mouse antisera were made in a round-bottomed 96-well microplate. Formalin-fixed cell suspensions were added to the wells, left undisturbed until cell masses were visible near well bottoms, and scored visually for cell agglutination from the bottom of the plate by using a magnifying mirror. Flagellar serotyping assays with rabbit antibody reagents were conducted as previously described (4).

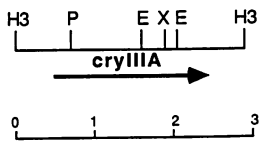

FIG. 1. Restriction map of *cryIIIA*, the gene which codes for a protein toxic to coleopterans, of strain EG2158. The *cryIIIA* gene was cloned as part of a 2.9-kb *Hin*dIII fragment (6). The 2.0-kb *Xba*I-*Hin*dIII fragment containing approximately three-fourths of the *cryIIIA* gene was subcloned and used as a hybridization probe. Numbers indicate length in kilobases. H3, *Hin*dIII; P, *Pst*I; E, *Eco*RI; X, *Xba*I.

Insect bioassays. First-instar larvae of *Diabrotica undecimpunctata howardi* (southern corn rootworm) were bioassayed via surface contamination of an artificial diet similar to the diet described by Marrone et al. (20) but without formalin. Each bioassay consisted of four serial aqueous dilutions with aliquots applied to the surface of the diet. After the diluent (an aqueous 0.005% Triton X-100 solution) had dried, larvae were placed on the diet and incubated at 28°C. Thirty-two larvae were tested per dose. First-instar larvae of *Leptinotarsa decemlineata* (Colorado potato beetle) were tested with similar techniques, except insect diet (catalog no. 9380; BioServe) with potato flakes added was used for the artificial diet. The most consistent bioassay results were obtained by scoring Colorado potato beetle mortality after 3 days and southern corn rootworm mortality after 7 days.

DNA hybridization analysis. Native plasmids of *B. thuringiensis* were size fractionated by electrophoresis through agarose gels (8, 11), treated with 0.5 M NaOH–1.5 M NaCl for 30 min and then with 1 M ammonium acetate–0.02 M NaOH for 30 min. Plasmids were transferred from the gel to a nitrocellulose filter by blotting (27), and the filter was probed with the radioactively labeled *cryIIIA* fragment as described above. DNA for restriction blot analysis was prepared by treating vegetative cells with a solution consisting of 4 mg of lysozyme per ml, 50 mM glucose, and 25 mM Tris hydrochloride (pH 8.0) at 37°C for 1 h and then adding 0.4% (wt/vol) SDS to a final concentration of 0.4% (wt/vol) to lyse cells. The mixture was extracted with phenol and chloroform, and total DNA was isolated by spooling with a glass rod.

RESULTS

Isolation of *B. thuringiensis* EG2838 and EG4961. We searched environmental samples for novel strains of *B. thuringiensis* that are toxic to coleopterans by using the cloned *cryIIIA* gene as a probe in colony hybridization experiments. The probe consisted of a 2.0-kb *Xba*I-*Hin*dIII fragment containing three-fourths of the *cryIIIA* gene (formerly called *cryC*) of *B. thuringiensis* EG2158 (6) (Fig. 1). Samples of soil, crop dust, and organic material were probed with the *cryIIIA* gene fragment. One sample from Illinois contained a *cryIIIA*-hybridizing colony of *B. thuringiensis*,

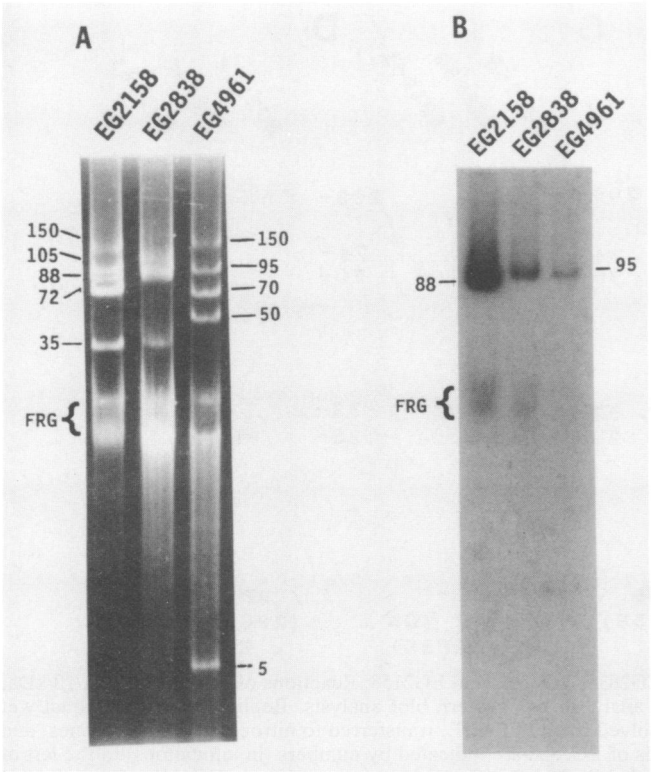

FIG. 2. Plasmid complements of strains EG2838 and EG4961. (A) Plasmids were size fractionated by electrophoresis (3 mA/1 h, 7 mA/0.5 h, and 28 mA/3 h [11]) through a 0.52% agarose gel and visualized by staining with ethidium bromide. Strain EG2158 is shown for comparison. Numbers to the left and right indicate the sizes (in megadaltons) of the plasmids of strains EG2158 and EG4961, respectively. FRG indicates fragments resulting from the breakdown of plasmid and chromosomal DNA. (B) The plasmids shown in panel A were blotted to a nitrocellulose filter, and the filter was hybridized with the radioactively labeled *cryIIIA* gene. Numbers to the right and left indicate the sizes (in megadaltons) of the *cryIIIA*-hybridizing plasmids of EG2158 and EG4961, respectively.

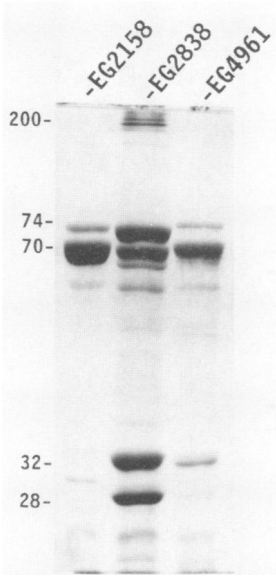

FIG. 3. Crystal proteins synthesized by strains EG2838 and EG4961. Mixtures of spores plus crystals of EG2838 and EG4961 were incubated in protein solubilization buffer and analyzed by SDS-PAGE (10% separating gel). For comparison, a mixture of spores plus crystals of EG2158 was treated in an identical manner. Each lane contains proteins solubilized from 100 μg (wet weight) of a mixture of spores plus crystals. Numbers to the left indicate sizes (in kilodaltons) of the crystal proteins of EG2838. The EG2838 200-kDa band is a protein doublet that is not clearly resolved in the photograph. Protein sizes were determined by comparison to molecular size standards (not shown), namely, carbonic anhydrase (29 kDa), egg albumin (45 kDa), bovine albumin (66 kDa), phosphorylase *b* (97.4 kDa), β-galactosidase (116 kDa), and myosin (205 kDa).

designated EG2838, that appeared to be unique, as judged by its plasmid array (see below). A 2.4-kb *cryIIIA*-hybridizing fragment of DNA was isolated from EG2838 (6a) and was used to probe environmental samples for novel strains of *B. thuringiensis* toxic to coleopterans. One sample contained a *B. thuringiensis* colony, designated EG4961, that specifically hybridized to the 2.4-kb fragment of EG2838 DNA.

To determine whether strains EG4961 and EG2838 are novel, their plasmids were characterized by size fractionation on agarose gels and the plasmid arrays were compared with the plasmid array of the previously characterized strain EG2158 toxic to coleopterans. The results of this analysis (Fig. 2A) showed that the numbers and sizes of plasmids of EG2838 (100, 90, and 37 MDa) and of EG4961 (150, 95, 70, 50, 5, and 1.5 MDa) were different from the plasmids of EG2158 (150, 105, 88, 72, and 35 MDa) (the 100- and 90-MDa plasmids of EG2838 are not well resolved and the 1.5-MDa plasmid of EG4961 is not visible in the gel shown in Fig. 2A). EG2838 and EG4961 plasmids (Fig. 2A) were blotted to a nitrocellulose filter, and the filter was probed with the radioactively labeled *cryIIIA* gene. The *cryIIIA* probe hybridized strongly to the 88-MDa plasmid of EG2158, as expected since the 88-MDa plasmid contains the *cryIIIA* gene (6), and hybridized weakly, but specifically, to the 100-MDa plasmid and the 95-MDa plasmid of EG2838 and EG4961, respectively, suggesting that these plasmids contain *cryIIIA*-related genes (Fig. 2B). Although the 100-MDa plasmid and the 95-MDa plasmids appear similar in size in Fig. 2B, repeated gel electrophoresis experiments showed that the 95-MDa plasmid consistently migrated slightly faster than the 100-MDa plasmid.

Crystal proteins of strains EG2838 and EG4961. Phase-contrast microscopic examination showed that sporulated cultures of EG2838 and of EG4961 contained, in addition to phase-bright spores, phase-dark, extrasporal particles with irregular shapes. The appearance of the phase-dark particles suggested that they were composed of crystal proteins. EG2838 cultures also contained phase-dark, extrasporal, football-shaped crystallike particles. The spores and crystal proteins of EG2838 and EG4961 were solubilized (see Materials and Methods) and size fractionated by electrophoresis through a polyacrylamide gel. A mixture of spores plus crystals from EG2158 was treated in an identical manner for comparison. The mixture of spores plus crystals from EG2838 yielded major proteins of 200 (doublet), 74, 70, 32, and 28 kDa (Fig. 3). The 74-kDa protein of EG2838 appears as a major band on the gel shown in Fig. 3; however, on repeat experiments, the 74-kDa protein often appeared as a minor band (results not shown). The mixture of spores plus crystals from EG4961 yielded minor proteins of 74, 70, and 30 kDa (Fig. 3), and on repeat experiments, the 74-kDa protein occasionally appeared as a major protein band. The abundance of these proteins suggests that they make up the

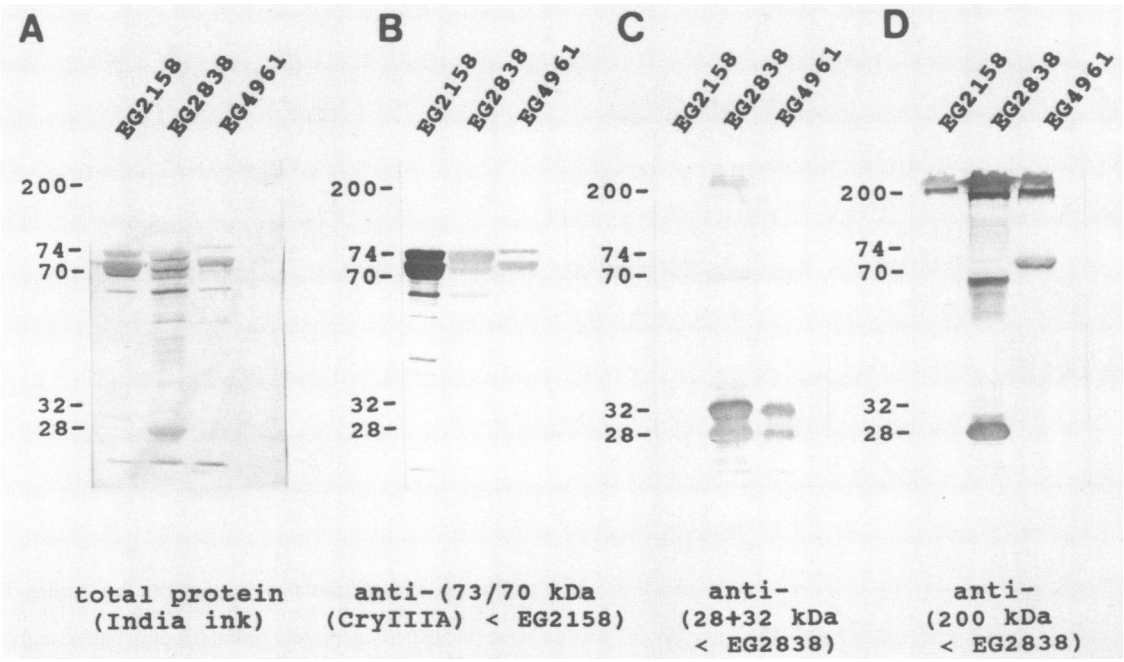

FIG. 4. Immunological relatedness of the crystal proteins of strains EG2838, EG4961, and EG2158. Reactions of EG2158 73-kDa/70-kDa, EG2838 32-kDa/28-kDa, and EG2838 200-kDa antibody reagents were analyzed by Western blot analysis. Replicate samples (equal wet weights) of EG2158, EG2838, and EG4961 spores plus crystals were resolved by SDS-PAGE, transferred to nitrocellulose membranes, and probed with each antibody reagent. Positions of the crystal protein bands of EG2838 are indicated by numbers (in kilodaltons) to the left of each panel. (A) Total protein stain (India ink); (B) antibodies against the 73-kDa/70-kDa (CryIIIA) proteins of strain EG2158; (C) antibodies against the 28-kDa and 32-kDa proteins of strain EG2838; (D) antibodies against the 200-kDa proteins of strain EG2838.

extrasporal crystals of strains EG2838 and EG4961. In comparison, strain EG2158 which is toxic to coleopterans produces crystal proteins of 73 and 70 kDa that appear very similar in size to the 74- and 70-kDa proteins of EG2838 and EG4961; EG2158 also produces minor crystal proteins of 31 and 29 kDa that are somewhat similar in size to the 32- and 28-kDa crystal proteins of EG2838 and to the 30-kDa crystal protein of EG4961 (Fig. 3).

Western blot analysis of EG2838, EG4961, and EG2158 crystal proteins. Three major bands were visible on preparative SDS-PAGE gels of EG2838 spore and crystal samples used for purification of crystal protein antigens corresponding to about 200 kDa (an unresolved doublet), 74 kDa/70 kDa (consisting of the unresolved 74-kDa plus 70-kDa proteins), and 32 kDa/28 kDa (consisting of the unresolved 32-kDa plus 28-kDa proteins) (not shown). Antibody reagents were produced by using proteins eluted from the 200-kDa and 32-kDa/28-kDa bands. Similarly, an antibody reagent was prepared against the 73-kDa/70-kDa band (CryIIIA) that had been eluted from preparative SDS-PAGE gels of EG2158 spore and crystal samples.

Figure 4 shows specificities of antibody reagents prepared against EG2838 and EG2158 crystal protein antigens in Western blot analysis. Samples with identical wet weights of EG2158, EG2838, and EG4961 spores plus crystals were resolved by SDS-PAGE and transferred to nitrocellulose filters (Fig. 4). Figure 4A shows total proteins stained with India ink. The 32-kDa protein of strain EG2838 and the 30-kDa protein of strain EG4961 stain negatively with India ink and are not visible in Fig. 4A. The 200-kDa proteins of EG2838 are not visible probably as a result of poor transfer to the nitrocellulose or poor staining with India ink. The 200- and 32-kDa proteins of EG2838 and the 30-kDa protein of EG4961 are present on the filters, however, as seen in Fig. 4C and D. Relative physical amounts of individual protein bands for these samples are best shown in the Coomassie dye-stained gel shown in Fig. 3. Antibodies against SDS-PAGE-purified EG2158 73-kDa/70-kDa protein (CryIIIA) reacted with the 74-kDa/70-kDa crystal proteins of EG2838 and EG4961 and usually reacted with some smaller protein bands (probably breakdown products of the 74-kDa/70-kDa proteins) (Fig. 4B). This result demonstrates that the 73-kDa/70-kDa proteins of EG2158 are related to the 74-kDa/70-kDa proteins of EG2838 and EG4961. The antibodies against the 73-kDa/70-kDa proteins (CryIIIA) failed to react with either the 29-kDa crystal protein of EG2158, the 28- or 32-kDa crystal proteins of EG2838, or the 30-kDa crystal protein of EG4961 (Fig. 4B). This result strongly suggests that the 29-, 28-, 32-, and 30-kDa crystal proteins are not breakdown products of the 74-kDa/70-kDa proteins.

The EG2838 32-kDa/28-kDa antibody reagent reacted strongly with the 32-kDa and 28-kDa protein bands of strain EG2838 and with the 30-kDa crystal protein of strain EG4961 (Fig. 4C). This antibody reagent also reacted weakly with the 200-kDa bands of EG2838 but did not react with the 73-, 74-, or 70-kDa crystal proteins of EG2158, EG2838, and EG4961 (Fig. 4C). The EG2838 200-kDa antibody reagent reacted (as expected) with the 200-kDa doublet bands of EG2838 and also reacted with minor bands of 200 kDa for EG2158 and EG4961 (Fig. 4D). The EG2838 200-kDa antibody reacted strongly with the 28-kDa protein band of EG2838 and also reacted with somewhat diffuse bands located just below the 70-kDa protein bands of EG2838 and EG4961 (Fig. 4D). The presence of the 200-kDa proteins on SDS-PAGE was unaffected either by varying the concentration of 2-mercaptoethanol from 0 to 7.5% (vol/vol) in the protein solubilization

TABLE 1. Flagellar serotyping of strains and cell lines by antibody-mediated cell agglutination assay

Cell line or strain (subspecies)	Reaction with flagellar antibody reagent[a] (H serotype)									
	HD2 (1)	HD1 (3a, 3b)	HD5 (4a, 4c)	HD11 (7)	HD12 (8a, 8b)	HD13 (9)	3-71 (18)	EG2158	EG2838	EG4961
HD2 (*B. thuringiensis* subsp. *thuringiensis*)	+	−	−	−	−	−	−	−	−	−
HD1 (*B. thuringiensis* subsp. *kurstaki*)	−	+	−	−	−	−	−	−	−	−
HD5 (*B. thuringiensis* subsp. *kenyae*)	−	−	+	−	−	−	−	−	−	−
HD11 (*B. thuringiensis* subsp. *aizawai*)	−	−	−	+	−	−	−	−	−	−
HD12 (*B. thuringiensis* subsp. *morrisoni*)	−	−	−	−	+	−	−	+	−	−
HD13 (*B. thuringiensis* subsp. *tolworthi*)	−	−	−	−	−	+	−	−	+	−
3-71 (*B. thuringiensis* subsp. *kumamotoensis*)	−	−	−	−	−	−	+	ND	ND	+
EG2158	−	−	−	−	+	−	ND	+	−	−
EG2838	−	−	−	−	−	+	−	−	+	−
EG4961	−	−	−	−	−	−	+	−	−	+

[a] Serial dilutions of vegetative cells were incubated in the presence of flagellar antibody reagents and scored visually for cell agglutination. All flagellar antibody reagents were produced in mice, except for 3-71, which was produced in rabbits. +, positive reaction; −, no reaction; ND, not determined.

buffer or by varying the alkalinity of the protein solubilization buffer from pH 6.0 to pH 8.3 (data not shown), suggesting that the 200-kDa proteins do not result from aggregation of smaller proteins. In sum, these results demonstrate the following: (i) the 32-kDa or 28-kDa proteins or both of EG2838 are related to the 30-kDa protein of EG4961; (ii) the proteins of approximately 200 kDa of EG2838 are related to the 28-kDa protein of EG2838; (iii) the EG2838 32-kDa protein is distinct from the EG2838 28-kDa protein, since only the latter reacted with the 200-kDa antibody reagent; and (iv) EG2158 and EG4961 contain minor amounts of proteins of approximately 200 kDa that are related to the 200-kDa proteins of EG2838.

Flagellar serotyping of strains EG2158, EG2838, and EG4961. EG2158, EG2838, and EG4961 cells were analyzed in a cell agglutination assay by using a panel of antibody reagents raised in mice against purified flagellar filaments from six *B. thuringiensis* H serotype reference strains plus strains EG2158, EG2838, and EG4961. Cells of each *B. thuringiensis* reference strain were included as positive-control samples. Results of this experiment are given in Table 1. Cells of HD1 (*B. thuringiensis* subsp. *kurstaki*; the reference strain for H serotype 3a, 3b, 3c), HD2 (*B. thuringiensis* subsp. *thuringiensis*; the reference strain for H serotype 1), HD5 (*B. thuringiensis* subsp. *kenyae*; the reference strain for H serotype 4a, 4c), HD11 (*B. thuringiensis* subsp. *aizawai*; the reference strain for H serotype 7), HD12 (*B. thuringiensis* subsp. *morrisoni*; the reference strain for H serotype 8a, 8b), and HD13 (*B. thuringiensis* subsp. *tolworthi*; the reference strain for H serotype 9) reacted strongly and specifically with their respective flagellar antibody reagents, demonstrating that these are valid antibody reagents for cell agglutination assays of unknown *B. thuringiensis* strains. EG2158 cells gave a positive reaction with the H serotype 8a, 8b (*B. thuringiensis* subsp. *morrisoni*) antibody reagent, and HD12 cells (*B. thuringiensis* subsp. *morrisoni*) gave a positive reaction with EG2158 antibody reagent (Table 1), demonstrating that EG2158 is *B. thuringiensis* subsp. *morrisoni*. EG2838 cells gave a positive reaction with the H serotype 9 (*B. thuringiensis* subsp. *tolworthi*) antibody reagent, and HD12 cells (*B. thuringiensis* subsp. *tolworthi*) cells gave a strong positive reaction (agglutination rate 12,800) only with EG2838 antibody reagent (Table 1), demonstrating that EG2838 is *B. thuringiensis* subsp. *tolworthi*. EG4961 did not give a positive reaction with any of the six reference strain mouse antibody reagents (Table 1). EG4961 cells gave a weak but positive reaction with the homologous EG4961 flagellar antibody reagent, confirming the presence of flagella on the EG4961 cells tested. These results demonstrate that the flagellar serotype of EG4961 is different from any of the six reference strains tested.

To further characterize the flagellar serotype of EG4961, rabbit flagellar antisera directed against the 32 already known reference strains of *B. thuringiensis* subspecies (de Barjac and Frachon [5]; for serotypes H28 to H32, de Barjac [4a]) were used. Only one, serotype H18 (*B. thuringiensis* subsp. *kumamotoensis*; agglutination rate, 1,600) gave a positive reaction with EG4961 (Table 1). In addition, agglutination tests with the 32 reference strains of *B. thuringiensis* with a mouse antiserum against flagella of EG4961 gave a weak positive result only with cells of the reference strain 3-71 of H serotype 18 (agglutination rate, 400). This low agglutination rate is in agreement with the fact that EG4961 is not very motile, as observed with the phase-contrast microscope, and with the very low homologous titer observed (agglutination rate, 200), using a vegetative cell suspension of EG4961. Despite the low flagellar titer, these results clearly show that EG4961 is H serotype 18 (*B. thuringiensis* subsp. *kumamotoensis*).

Insecticidal specificity of strains EG2838 and EG4961. Mixtures of spores plus crystals from EG2838 and from EG4961 were assayed for toxicity to first-instar larvae of Colorado potato beetle and southern corn rootworm. For purposes of comparison, a mixture of spores plus crystals from strain EG2158 that is toxic to coleopterans was also assayed. Both EG2838 and EG4961 were highly toxic to the larvae of Colorado potato beetle. On the basis of sample weight (wet weight), the mixture of spores plus crystals from EG4961 was more toxic to Colorado potato beetle larvae than the mixture of spores plus crystals from EG2838, and the mixture of spores plus crystals from EG2158 was somewhat more toxic to Colorado potato beetle larvae than the mixture from either EG4961 or EG2838 (Table 2). It should be noted that washed, sporulated cultures of strains EG2838 and EG4961 were used in these bioassays. The use of washed cultures reduced the chance that toxicity was due to some metabolite present in the medium. Furthermore, unwashed, sporulated cultures of EG2838 and EG4961 were not toxic to flies (*Musca domestica*) (data not shown), indicating that these strains did not secrete a β-exotoxin-like toxic metabolite. A significant difference in the three strains with regard to their toxicity to southern corn rootworm larvae was

TABLE 2. Insecticidal activities of strains EG2838, EG4961, and EG2158 against Colorado potato beetle

Strain	Dose[a]	% Mortality[b] of Colorado potato beetles
EG2838	100	86
	33	45
	11	17
	4	11
EG4961	100	100
	33	100
	11	82
	4	38
EG2158	100	100
	33	100
	11	97
	4	93

[a] Dose is micrograms of the spore and crystal preparation per insect diet cup (175-mm^2 surface area).
[b] Mortality is for first-instar larvae (32 larvae per dose) scored after 3 days. The percent mortality was corrected for control death (approximately 10%) by the method of Abbott (1), with the control consisting of the diluent alone.

found: high doses of EG2158 and EG2838 spores plus crystals failed to kill significant amounts of southern corn rootworm larvae, whereas similar or lower amounts of the spores plus crystals of EG4961 killed a relatively high percentage of larvae (Table 3). For example, the top dose of 5,000 μg of spores plus crystals of either EG2158 or EG2838 failed to kill more than 7% of the southern corn rootworm larvae. In contrast, a dose of 1,667 μg of spores plus crystals of EG4961 killed 43% of the southern corn rootworm larvae (Table 3). The fact that high doses (5,000 μg) of spores plus crystals of either EG2158 or EG2838 failed to kill more than a low percentage of southern corn rootworm larvae demonstrates that the killing observed with EG4961 is specific and is not due simply to a general effect of spores or crystals of *B. thuringiensis*.

Sporulated cultures of strains EG2838 and EG4961 were used in bioassay experiments to determine their toxicity to

TABLE 3. Insecticidal activities of strains EG2838, EG4961, and EG2158 against southern corn rootworm

Strain	Dose[a]	% Mortality[b] of southern corn rootworm
EG2838	5,000	7
	1,667	7
	556	0
	185	4
EG4961	5,000	65
	1,667	43
	556	39
	185	4
EG2158	5,000	4
	1,667	7
	556	0
	185	0

[a] Dose is micrograms of the spore and crystal preparation per insect diet cup (175-mm^2 surface area).
[b] Mortality is for first-instar larvae (32 larvae per dose) scored after 7 days. The percent mortality was corrected for control death (approximately 10%) by the method of Abbott (1), with the control consisting of the diluent alone.

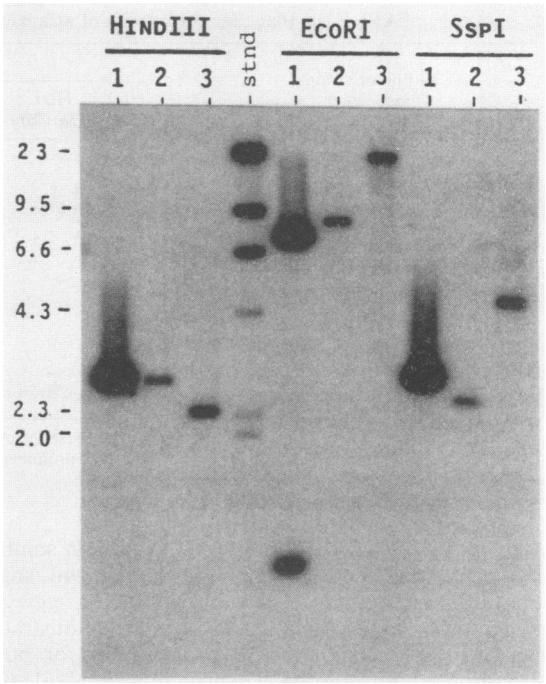

FIG. 5. Comparison of *cryIIIA*-hybridizing restriction fragments of strains EG2838, EG4961, and EG2158. The DNA from strain EG2158 (lanes 1), EG2838 (lanes 2), and EG4961 (lanes 3) was digested with *Hin*dIII, *Eco*RI, or *Ssp*I restriction enzymes as indicated and probed with the radioactively labeled *cryIIIA* gene. Numbers to the left indicate sizes (in kilobases) of the *Hin*dIII restriction fragments of radioactively labeled lambda phage DNA (stnd).

the larvae of selected Lepidoptera. No significant toxicity to lepidopteran larvae (tobacco budworm [*Heliothis virescens*], beet armyworm [*Spodoptera exigua*], and cabbage looper [*Trichoplusia ni*]) was observed with either EG2838 or EG4961 (data not shown).

Hybridization blot analysis of EG2838 and EG4961 DNA. The *cryIIIA* probe hybridized to native plasmids of 100 and 95 MDa from strains EG2838 and EG4961, respectively, as well as to the 88-MDa *cryIIIA*-containing plasmid of EG2158 (Fig. 2). To determine whether the *cryIIIA*-hybridizing plasmids might contain different *cryIIIA*-related sequences, total DNA was extracted from strains EG2838, EG4961, and EG2158 and analyzed by restriction blot hybridization. As shown in Fig. 5, each of the strains displayed a unique pattern of *cryIIIA*-hybridizing restriction fragments. For example, the *cryIIIA* probe hybridized to 7.5- and 0.6-kb *Eco*RI restriction fragments of EG2158 DNA, to an 8.6-kb *Eco*RI fragment of EG2838 DNA, and to a 21.0-kb *Eco*RI fragment of EG4961 DNA (Fig. 5). The *cryIIIA* gene hybridized more strongly to itself (EG2158 DNA) than to the *cryIIIA*-related sequences of either EG2838 or EG4961 DNA (Fig. 5). This difference in hybridization intensity is not due to differences in gene copy number, since the *cryIIIA* gene and *cryIIIA*-related sequences are carried on plasmids of approximately 88, 100, and 95 MDa in strains EG2158, EG2838, and EG4961, respectively, and the cellular concentration of each plasmid is roughly the same (Fig. 2A and B). Altogether, these results suggest that the 100- and 95-MDa plasmids of EG2838 and EG4961, respectively, contain *cryIIIA*-related sequences that differ from one another and

that also differ from the *cryIIIA* gene contained on the 88-MDa plasmid of EG2158.

DISCUSSION

A significant finding of this research is the discovery that at least three distinct types of *B. thuringiensis* toxic to coleopterans exist in nature. These three types of *B. thuringiensis* can be distinguished by the following properties: (i) flagellar serotype, (ii) native plasmid complement, (iii) sizes of *cryIIIA*-hybridizing restriction fragments, (iv) crystal proteins, and (v) insecticidal activity. In this report, we have shown that strain EG2158 that is toxic to coleopterans (6) belongs to H serotypes 8a, 8b (*B. thuringiensis* subsp. *morrisoni*). Previously described strains toxic to coleopterans (i.e., *B. thuringiensis* subsp. *san diego* and *B. thuringiensis* subsp. *tenebrionis*) also belong to H serotype 8a, 8b (16). In contrast, we have found that the new strains toxic to coleopterans, EG2838 and EG4961, belong to H serotype 9 (*B. thuringiensis* subsp. *tolworthi*) and H serotype 18 (*B. thuringiensis* subsp. *kumamotoensis*), respectively. The original reference strains of *B. thuringiensis* subsp. *tolworthi* (22) and *B. thuringiensis* subsp. *kumamotoensis* (23) have not been reported as being toxic to coleopterans. In fact, the reference strains for *B. thuringiensis* subspecies *tolworthi* (HD13) and *kumamotoensis* (HD867) produce CryI-type crystal proteins toxic to lepidopterans (10a).

The *cryIIIA* gene coding for a protein toxic to coleopterans of strain EG2158 hybridized to the 100- and 95-MDa plasmids of EG2838 and EG4961, respectively, and antibodies to the CryIIIA protein (73 kDa/70 kDa) toxic to coleopterans of EG2158 reacted with the 74- and 70-kDa crystal proteins of EG2838 and EG4961. These findings suggest that the 100- and 95-MDa plasmids of EG2838 and EG4961, respectively, contain *cryIIIA*-related genes whose products are the 74-kDa/70-kDa crystal proteins. The putative *cryIIIA*-related genes of EG2838 and EG4961 appear to be different from the *cryIIIA* gene of EG2158 that is toxic to coleopterans, as evidenced by the fact that strains EG2838 and EG4961 each yielded unique patterns of *cryIIIA*-hybridizing restriction fragments which differed from that of EG2158.

Sick et al. (26) have reported the sequence of a gene toxic to coleopterans (*cryIIIB*) isolated from a strain of *B. thuringiensis* reported to be H serotype 9 (*B. thuringiensis* subsp. *tolworthi*). No data regarding plasmid complement, insecticidal activity, or crystal proteins of this *B. thuringiensis* strain were presented, and thus we cannot compare the strain reported by Sick et al. (26) to the H serotype 9 strain (EG2838) of this report.

We noticed in repeated experiments that when a mixture of spores plus crystals from either EG2838 or EG4961 was analyzed by SDS-PAGE, an inverse relationship of the amounts of the 74- and 70-kDa proteins was observed, i.e., when more of the 74-kDa protein was observed, correspondingly less of the 70-kDa protein was seen, and vice versa. This observation indicates that during solubilization or SDS-PAGE analysis, the 74-kDa crystal proteins of EG2838 and EG4961 are processed to proteins of 70 kDa. A similar situation has been observed for the 73-kDa EG2158 CryIIIA protein toxic to coleopterans: the protein appears to be rapidly processed upon solubilization to 70 kDa (6). Carroll et al. (3) found that when protease inhibitors were added to the growth medium during sporulation of *B. thuringiensis* subsp. *tenebrionis*, more of the 73-kDa CryIIIA protein was observed and correspondingly less of an approximately 70-kDa protein was seen, indicating that the approximately 70-kDa protein resulted from proteolytic processing of the 73-kDa CryIIIA protein. On the other hand, McPherson et al. (21) found that recombinant *cryIIIA*-containing *Pseudomonas fluorescens* cells utilized different translation initiation sites to produce the 73- and 70-kDa forms of the CryIIIA protein; the 70-kDa protein was synthesized from a translation initiation site located 48 codons downstream from the translation initiation site for the 73-kDa protein. Although our results suggest that the 70-kDa CryIIIA-related proteins of EG2838 and EG4961 arise by processing of the respective 74-kDa CryIIIA-related proteins, we have not ruled out the possibility that certain amounts of the 70-kDa proteins are produced by the use of alternate, downstream translation initiation sites.

In addition to the 74-kDa/70-kDa CryIIIA-related crystal proteins, strain EG2838 synthesized crystal proteins of 200 (doublet), 32, and 28 kDa, and strain EG4961 synthesized a crystal protein of 30 kDa. These proteins were not found to be related to the 74-kDa/70-kDa crystal proteins by Western blot analysis (Fig. 3). Surprisingly, antibodies against the EG2838 200-kDa proteins reacted with the EG2838 28-kDa protein, indicating structural relatedness between these proteins. Whether the 200-kDa proteins are multimeric aggregates of the 28-kDa protein is not known; however, the 200-kDa proteins were observed after treatment which normally eliminates aggregation (i.e., 100°C, 5% 2-mercaptoethanol, 2% SDS). If the EG2838 200-kDa proteins were monomers, they would be the largest crystal proteins reported for a *B. thuringiensis* strain.

Sporulated cultures of EG2838 and of EG4961 were found to be highly toxic to larvae of the Colorado potato beetle, with EG4961 being somewhat more toxic than EG2838. It is likely that the 74-kDa/70-kDa crystal proteins of these strains are the primary factors responsible for this toxicity because of the following: (i) the 74-kDa/70-kDa proteins are immunologically related to the 73-kDa/70-kDa CryIIIA protein of EG2158 that is toxic to the Colorado potato beetle and (ii) the 74-kDa/70-kDa proteins are the most abundant crystal proteins synthesized by each strain. A significant finding of this work was that a sporulated culture of EG4961 was toxic to larvae of the southern corn rootworm. In contrast, neither EG2158 nor EG2838 displayed such toxicity. Nevertheless, the toxicity of EG4961 to southern corn rootworm larvae was roughly 2 orders of magnitude less than its toxicity to Colorado potato beetle larvae. Work is in progress to characterize the gene(s) responsible for the toxicity of strains EG2838 and EG4961 to coleopterans.

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