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Isolation and characterization of EG2158, a new strain of *Bacillus thuringiensis* toxic to coleopteran larvae, and nucleotide sequence of the toxin gene

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Summary. A novel strain of *Bacillus thuringiensis* was isolated from soybean grain dust from Kansas and found to be toxic to larvae of *Leptinotarsa decemlineata* (Colorado potato beetle). The strain (EG2158) synthesized two parasporal crystals: a rhomboid crystal composed of a 73116 dalton protein and a flat, diamond-shaped crystal composed of a protein of approximately 30 kDa. Plasmid transfer and gene cloning experiments demonstrated that the 73 kDa protein was encoded on an 88 MDa plasmid and that the protein was toxic to the larvae of Colorado potato beetle (CPB). The sequence of the 73 kDa protein, as deduced from the sequence of its gene (*cryC*), was found to have regions of similarity with several *B. thuringiensis* crystal proteins: the lepidopteran-toxic P1 proteins of var. *kurstaki* and *berliner*, the lepidopteran- and dipteran-toxic P2 (or CRYB1) protein of var. *kurstaki,* and the dipteran-toxic 130 kDa protein of var. *israelensis*. While *B. megaterium* cells harboring the *cryC* gene from EG2158 synthesized significant amounts of the 73 kDa CRYC protein, *Escherichia coli* cells did not. The *cryC*-containing *B. megaterium* cells produced rhomboid crystals that were toxic to CPB larvae.

Key words: *Bacillus thuringiensis* – Coleopteran-Toxic – Toxin gene cloning – Nucleotide sequencing – Plasmid transfer

Introduction

Strains of *Bacillus thuringiensis* synthesize large amounts of certain proteins during sporulation that aggregate to form parasporal crystals detectable by phase contrast microscopy. These crystal proteins are often highly toxic to specific insects, and their genes are located on large (>30 MDa) plasmids (for recent reviews see Whiteley and Schnepf 1986; Carlton and Gonzalez 1985). *B. thuringiensis* strains of var. *kurstaki,* such as HD-1, and also of many other varieties, synthesize a bipyramidal crystal which is composed of one or more related proteins (P1 proteins) of approximately 130 kDa. The P1 proteins are toxic to lepidopteran larvae. HD-1 also synthesizes a flattened cuboidal crystal composed of a 66 kDa protein (P2 protein) that is toxic to both lepidopteran and dipteran (mosquito) larvae (Yamamoto and McLaughlin 1981; Iizuka and Yamamoto 1983). Several genes encoding P1 proteins have been cloned and sequenced (Adang et al. 1985; Brizzard and Whiteley 1988; Geiser et al. 1986; Hefford et al. 1987; Hofte et al. 1986; Kondo et al. 1987; Oeda et al. 1987; Schnepf et al. 1985; Shibano et al. 1985; Thorne et al. 1986; Wabiko et al. 1986) and we have recently determined the sequence of the *cryBI* gene encoding the P2 (or CRYB1) protein (Donovan et al. 1988). The sequences of the various P1 proteins, as deduced from the sequences of their genes, are similar to one another and share limited sequence similarity with the P2 protein.

There have been no reports of coleopteran larvicidal activity associated with either the P1 or the P2 crystal proteins. Krieg et al. (1983) have described a strain of *B. thuringiensis* (var. *tenebrionis*), isolated in Germany, that was reported to be toxic to Colorado potato beetle (CPB) larvae. Bernhard (1986) reported that a crystal protein of approximately 68 kDa was responsible for the toxicity of var. *tenebrionis*. Herrnstadt et al. (1986) have reported a CPB-toxic strain of *B. thuringiensis* (var. *san diego*) that produces a toxic protein of approximately 65 kDa (Herrnstadt et al. 1987). The genes encoding the coleopteran-toxic proteins of *B. thuringiensis* var. *tenebrionis* and of *B. thuringiensis* var. *san diego* have recently been cloned and their nucleotide sequences reported (Herrnstadt et al. 1987; Hofte et al. 1987; Jahn et al. 1987; Sekar et al. 1987). Ecogen Inc is carrying out a systematic worldwide search for novel *B. thuringiensis* strains with unusual insecticidal activities, such as toxicity to coleopterans. The fine dust from crop storage areas, such as grain elevators, which was shown to abound in *B. thuringiensis* spores by DeLucca et al. (1982), is one of the materials in our search. We report the isolation and characterization of a new strain of *B. thuringiensis* toxic to CPB larvae: strain EG2158. A CPB toxin gene (*cryC*) encoding a 73 kDa crystal protein was cloned from EG2158. The expression of the cloned *cryC* gene in *Escherichia coli* and *B. megaterium* is described. The complete DNA sequence of the *cryC* gene, as well as the deduced amino acid sequence of the 73 kDa protein, is presented. The sequence of the *cryC* gene was compared to the reported sequences of several other *B. thuringiensis* toxin genes. Surprisingly, the *cryC* gene of EG2158 was apparently identical to a gene, as reported by Sekar et al. (1987) and by Hofte et al. (1987), of var. *tenebrionis,* and to a gene, as reported by Herrnstadt et al. (1987), of var. *san diego,* even though the strains differed in several ways.

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Materials and methods

Media. C2 medium contains 1% glucose, 0.2% peptone, 0.5% NZ amine-A casein hydrolysate (Sheffield Products), 0.2% yeast extract, 15 mM $(NH_4)_2SO_4$, 23 mM KH_2PO_4, 27 mM K_2HPO_4, 1 mM $MgSO_4.7H_2O$, 600 µM $CaCl_2$, 250 µM $MnCl_2$ 17 µM $ZnSO_4.7H_2O$, 17 µM $CuSO_4.5H_2O$, 2 µM $FeSO_4.7H_2O$. LB agar medium contains 1% Difco tryptone, 0.5% Difco yeast extract, 0.5% NaCl, 1.5% agar, pH 7.0. DS agar medium contains 0.8% Difco nutrient broth, 13 mM KCl, 1 mM $Ca(NO_3)_2$, 0.5 mM $MgSO_4$, 10 µM $MnCl_2$, 10 µM $FeSO_4$, 1.5% agar, pH 7.0. M27 broth consisted of one-third strength NSM (Li and Yousten 1975), 140 mM NaCl, 0.1% glucose, 1% potassium phosphate buffer, pH 7.0.

Isolation of B. thuringiensis *strains.* One gram of grain dust was suspended by vortexing in 10 ml spore buffer (0.06% sodium phosphate, pH 7.0, 0.05% potassium phosphate, pH 6.6, 0.005% SDS, 1 µl/ml 1-octanol, final pH close to neutral) and pasteurized 30 min at 60° C to select for *Bacillus* spores. The suspension was revortexed, allowed to stand 5–15 min to allow the larger particles to settle out, and dilutions of the upper layer were spread on NSM agar (Li and Yousten 1975) and incubated at 30° C for 24–48 h. *B. thuringiensis* (e.g. crystal-producing) colonies were distinguished from the very similar but crystal-negative (Cry^-) *B. cereus* colonies by their synthesis of phase-bright parasporal inclusions (detected by phase contrast microscopy of individual colonies).

Plasmid transfer and analysis of recipient colonies. Transfer of plasmids from EG2158 into Str^rCry^- recipient strains was performed essentially as described previously (Gonzalez et al. 1982) except that M27 broth was used as the conjugation broth. Two Str^rCry^- recipient strains were used: HD73-26 and BC569-6. HD73-26 was derived from the Str^S, P1-producing wild-type strain, HD-73 (var. *kurstaki*) by growth at elevated temperatures; it is Str^rCry^-, and carries only one plasmid, 4.9 MDa in size (J.M. Gonzalez Jr., Ph.D. dissertation). BC569-6 was derived from the Str^S parental strain, *B. cereus* NRRL569, which contains two native plasmids, by growth at elevated temperatures; BC569-6 is Str^rCry^-, and plasmid free (J.M. Gonzalez Jr., unpublished results). Donor, recipient, and transconjugant plasmid arrays were visualized on agarose gels essentially as described in Gonzalez et al. (1982).

Purification of the coleopteran-toxic protein. Strain EG2158 was grown for 72 h at 30° C in C2 medium. Spores plus crystal proteins were harvested by centrifugation, washed twice with 1 M NaCl and twice with deionized water. Spores plus crystals were suspended in gel loading buffer (0.13 M Tris pH 6.8, 2% SDS, 5% 2-mercaptoethanol, 0.05% bromphenol blue, 10% glycerol) and incubated at 50° C for 7 min with occasional vortexing. Spores were pelleted by centrifugation and the supernatant loaded onto a preparative 10% polyacrylamide slab gel containing SDS for electrophoretic fractionation of proteins. The 68 kDa protein band was cut from Coomassie stained gels and the protein was separated from gel slices by electroelution as described by Hunkapiller et al. (1983). After acetone precipitation, approximately 200 µg (3 nmol) of the purified 68 kDa protein was resuspended in 100 µl of deionized water and the NH_2-terminal amino acid sequence was determined by Edman degradation as previously described (Donovan et al. 1988).

Plasmid library. Total DNA from strain EG2158 was digested with *Hind*III, electrophoresed through an agarose gel and gel slices containing size selected DNA fragments were excised with a razor blade. DNA fragments were separated from gel slices by electroelution. The fragments were ligated into plasmid pBR322 that had been digested with *Hin*dIII and treated with bacterial alkaline phosphatase. The resulting recombinant plasmids were transformed into *E. coli* strain HB101.

DNA sequencing. DNA fragments were cloned into the M13 vectors mp18 and mp19 as described in the text. A total of 21 *cryC*-specific 17mer primers (synthesized on an Applied Biosystems DNA synthesizer Model 380B) and one M13-specific primer (supplied by Bethesda Research Laboratories) were used to determine the complete DNA sequence of both strands of the *cryC* gene by the dideoxy method of Sanger et al. (1977).

Preparation of cell extracts for protein gels. Cells were grown for 48 h at 30° C on either LB agar or DS agar medium. The medium contained either 40 µg/ml ampicillin for growth of *E. coli*, 5 µg/ml tetracycline for growth of *B. megaterium* or no antibiotic for growth of *B. thuringiensis*. After growth, cells were removed with a spatula from the agar medium surface, washed once with 1 M NaCl and twice with deionized water and suspended in deionized water at 100 mg wet cell weight per ml. Fifty microliters of the cell suspension was added to 50 µl of 100 mM Tris pH 7.4, 20 mM EDTA, 10 mg/ml lysozyme in a 1.5 ml microtube and incubated at 37° C for 30 min. After incubation, SDS was added to a final concentration of 0.2% (w/v). The suspension was vortexed 30 s, centrifuged 5 min in a microfuge, and the pellet was suspended in 50 µl of 0.2% (w/v) SDS. Four microliters of the suspension (equivalent to 400 µg wet cells) was added to 36 µl of gel loading buffer (0.13 M Tris pH 6.8, 2% SDS, 5% 2-mercaptoethanol, 0.05% bromphenol blue, 10% glycerol) and incubated at 90° C for 7 min with occasional vortexing. Ten microliters of the mixture (equivalent to 100 µg wet cells) was immediately electrophoresed through a 12.5% polyacrylamide gel containing SDS.

Insect bioassay. Potato leaves were dipped in sporulated culture suspensions of the various *Bacillus* strains and placed in a petri dish with 5 neonate larvae of *Leptinotarsa decemlineata* (Colorado potato beetle; CPB) in the leaf dip bioassay system. Insect mortality was scored after 2 days. Diet cup bioassays were performed by topically applying 100 µl of sporulated culture suspensions of the various strains to 3 ml of an artificial diet (No. 9380, Bioserve). One CPB neonate larva was added per cup and mortality was scored after 2 days. At least 20 larvae were scored for each *Bacillus* strain.

Results

Isolation and characterization of strain EG2158

Suspensions of grain dust were heat treated, spread on NSM agar, and incubated at 30° C as described under Ma-

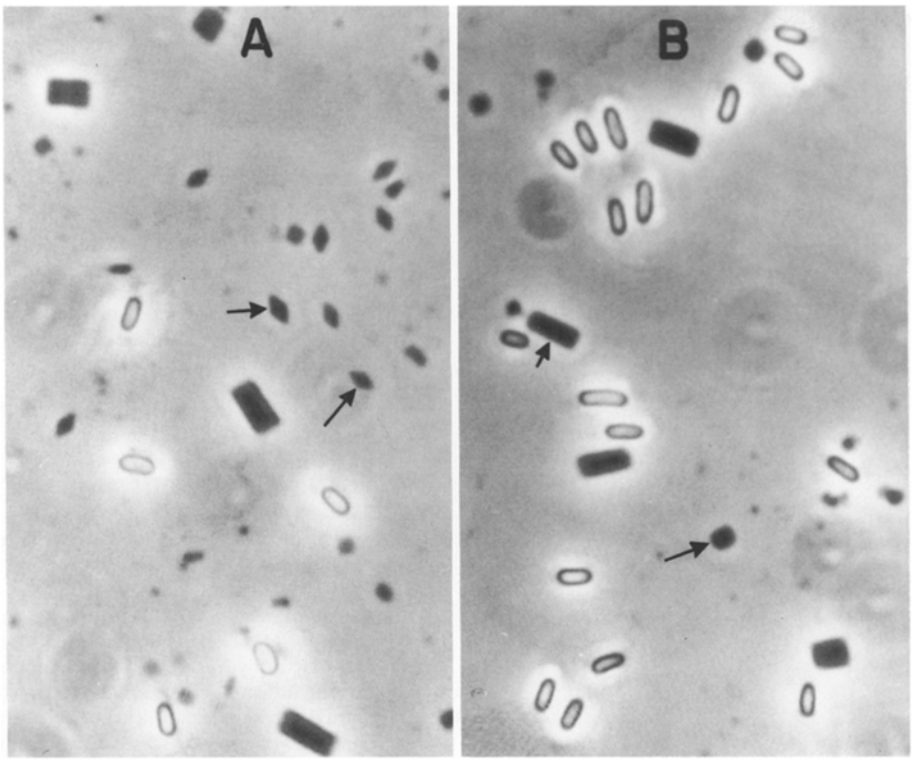

Fig. 1 A, B. Crystals synthesized by strains EG2158 (**A**) and EG2278 (**B**). *Arrows* indicate (**A**) flat, diamond-shaped crystals and (**B**) rhomboid crystals. Some of the rhomboid crystals are unusually elongated. Spores (white oval shapes) are approximately 1 μm in diameter

Table 1. Larvicidal activity of *Bacillus* strains

Strain	Plasmids[a]	Mortality[b](%) Colorado potato beetle larvae
B. thuringiensis		
EG2158	150, 105, 88, 72, 35	80
EG2159	105, 88, 72, 35	90
HD73-26	4.9	0
EG2278	88, 4.9	100
EG2279	105, 88, 4.9	70
B. cereus		
EG2725	88	90
B. megaterium		
EG1311	pBC16	6
EG1314	pEG213	98

[a] Numbers indicate plasmid size in MDa
[b] Mortality was determined by the leaf dip assay for *B. thuringiensis* and *B. cereus* and by the diet cup assay for *B. megaterium*.

terials and methods. *B. thuringiensis* colonies were identified by the presence of parasporal crystals. Strains that synthesized unusual crystals (e.g. crystals not of the common bipyramidal, lepidopteran-toxic type) were selected for screening against CPB larvae. One *B. thuringiensis* colony, from a soybean grain dust sample out of Kansas, synthesized two unusual parasporal crystals: a rhomboid crystal and a flat, diamond-shaped crystal (Fig. 1A). The strain, designated EG2158, was found to be highly toxic to CPB larvae (Table 1).

Strain EG2158 contained 5 plasmids of approximately 150, 105, 88, 72 and 35 MDa (Fig. 2A, lane 1), obtained by using HD-1 plasmids as size standards (not shown). The 150 MDa plasmid is not visible in the photograph. A major protein of approximately 68 kDa was observed on SDS-polyacrylamide gels when sporulated cultures of EG2158 were extracted at 50° C with protein solubilization buffer (Fig. 2B, lane 1). In addition, 2 minor proteins of approximately 30 kDa and 29 kDa were consistently observed in extracts of EG2158 cultures (Fig. 2B, lane 1). Conjugation experiments showed that both the 88 MDa and the 105 MDa plasmids of strain EG2158 could transfer into the Str^rCry^-, nearly plasmid-free *B. thuringiensis* recipient strain HD73-26. The transconjugants EG2278 (which acquired the 88 MDa plasmid alone, Fig. 2A, lane 4) and EG2279 (which acquired both the 88 MDa and 105 MDa plasmids, Fig. 2A, lane 5) both synthesized rhomboid crystals (shown for EG2278, Fig. 1B); these crystals appeared to be composed of the 68 kDa protein (Fig. 2B, lanes 4, 5). EG2158 also transferred the 88 MDa plasmid into the Str^rCry^-, plasmid-free, *B. cereus* recipient strain BC569-6, to give the transconjugant EG2725 (Fig. 2A, lane 7), which made rhomboid crystals (not shown) and the 68 kDa protein (Fig. 2B, lane 7). These transconjugants did not synthesize the flat, diamond-shaped crystal or the 30 kDa protein but did synthesize a minor protein of 29 kDa in addition to the major 68 kDa protein (Fig. 2B, lanes 4, 5, 7). EG2159, a derivative of EG2158 that had lost the 150 MDa plasmid (Fig. 2A, lane 2), no longer produced the flat, diamond-shaped crystals or the 30 kDa protein (Fig. 2B, lane 2). Significantly, EG2159, EG2278, EG2279 and EG2725, all of which synthesized rhomboid crystals but not flat diamond-shaped crystals, were all approximately as toxic to CPB larvae as EG2158 (Table 1).

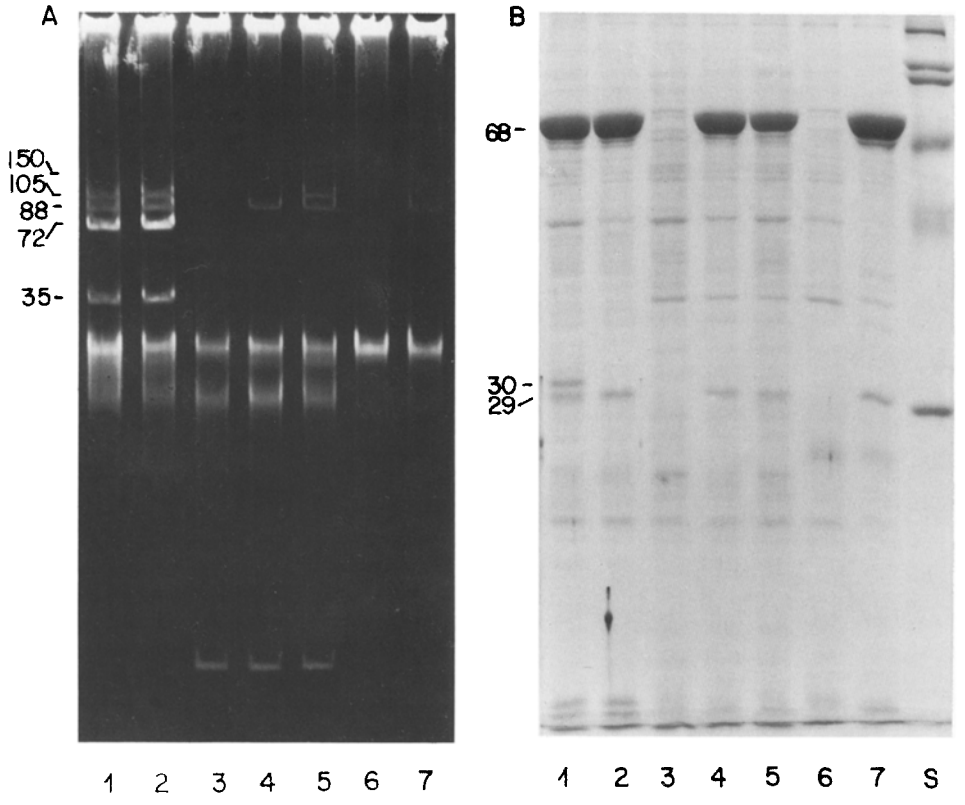

Fig. 2A, B. Plasmid (**A**) and protein (**B**) profiles of strain EG2158 and transconjugants. **A** and **B**: lane 1, EG2158; lane 2, EG2159; lane 3, HD73-26; lane 4, EG2278; lane 5, EG2279; lane 6, BC569-6; lane 7, EG2725; lane S, molecular weight standards: myosin, 205 kDa; β-galactosidase, 116 kDa; phosphorylase b, 97 kDa; bovine serum albumin, 66 kDa; egg albumin 45 kDa; carbonic anhydrase, 29 kDa. Numbers to the left indicate (**A**) plasmid size in MDa and (**B**) protein size in kDa. The agarose concentration in (**A**) was 0.52% (Sigma, Type I)

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      1                   5                  10
NH2- Asp Glu Ala Leu Thr Ser Ser Thr Asp Lys Asp Val Ile
 5'- GAT GAA GCA TTA ACA TCA TCA ACA GAT AAA GAT GTA ATT

          15                  20
     Gln Lys Gly Ile Ser Val Val Ile Asp Leu Leu - COOH
     CAA AAA GGA ATT TCA GTA GTA ATT GA - 3'
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Fig. 3. Partial NH_2-terminal amino acid sequence of the 68 kDa protein and sequence of the *cryC*-specific oligonucleotide probe

Cloning the cryC *gene*

The strategy used in cloning the gene that encoded the 68 kDa CPB toxin protein was to purify the protein, determine its NH_2-terminal amino acid sequence and synthesize a gene-specific oligonucleotide probe based on the amino acid sequence. Accordingly, proteins from EG2158 were solubilized, fractionated on SDS-polyacrylamide gels and the 68 kDa protein was electroeluted from gel slices. Sequential Edman degradation of the purified protein yielded the partial NH_2-terminal sequence shown in Fig. 3. The sequence did not contain an NH_2-terminal methionine.

A 62mer oligonucleotide probe, coding for amino acids 1–21 at the NH_2-terminus of the 68 kDa protein, was designed and synthesized (Fig. 3). At points of codon degeneracy, an adenine or thymine was always incorporated in the oligonucleotide because the *B. thuringiensis* genome is approximately 64% A + T (Laskin and Lechevalier 1981).

The oligonucleotide was radioactively labeled and hybridized at low stringency (30° C) to a DNA blot that contained total *Hin*dIII digested DNA from strain EG2158. The blot was alternately exposed to X-ray film and washed at temperatures increasing by 5° C until a washing temperature was reached (50° C) at which the oligonucleotide hybridized to a unique EG2158 *Hin*dIII restriction fragment of approximately 2.9 kb (data not shown). The radioactively labeled oligonucleotide was hybridized at 50° C with colony blots of *E. coli* cells that harbored a plasmid library of size selected EG2158 *Hin*dIII DNA fragments. One *E. coli* colony which hybridized strongly with the oligonucleotide was selected for further study. The colony contained a recombinant plasmid, designated pEG212 (Fig. 4), which consisted of pBR322 plus a 2.9 kb *Hin*dIII DNA fragment. The oligonucleotide hybridized specifically to a 1.0 kb *Pst*1–*Eco*R1 restriction fragment from plasmid pEG212. The 1.0 kb fragment was subcloned into the M13 vectors mp18 and mp19 and sequenced. A deduced amino acid sequence closely related to the one determined by Edman degradation for the 68 kDa protein was located 8 nucleotides from the *Pst*I site. Because of the accuracy of DNA sequencing, we assumed that the correct NH_2-terminal sequence of the

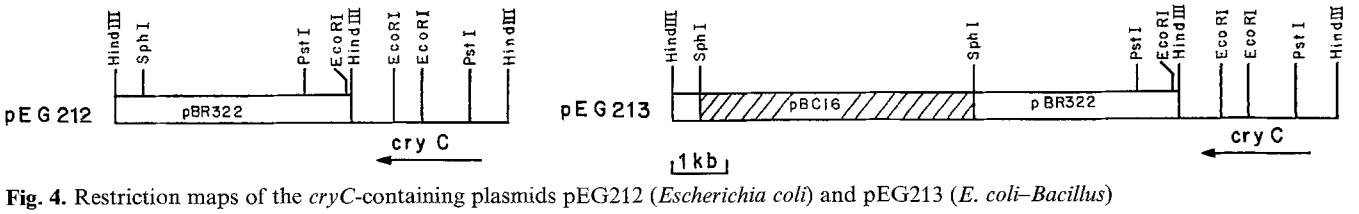

Fig. 4. Restriction maps of the *cryC*-containing plasmids pEG212 (*Escherichia coli*) and pEG213 (*E. coli–Bacillus*)

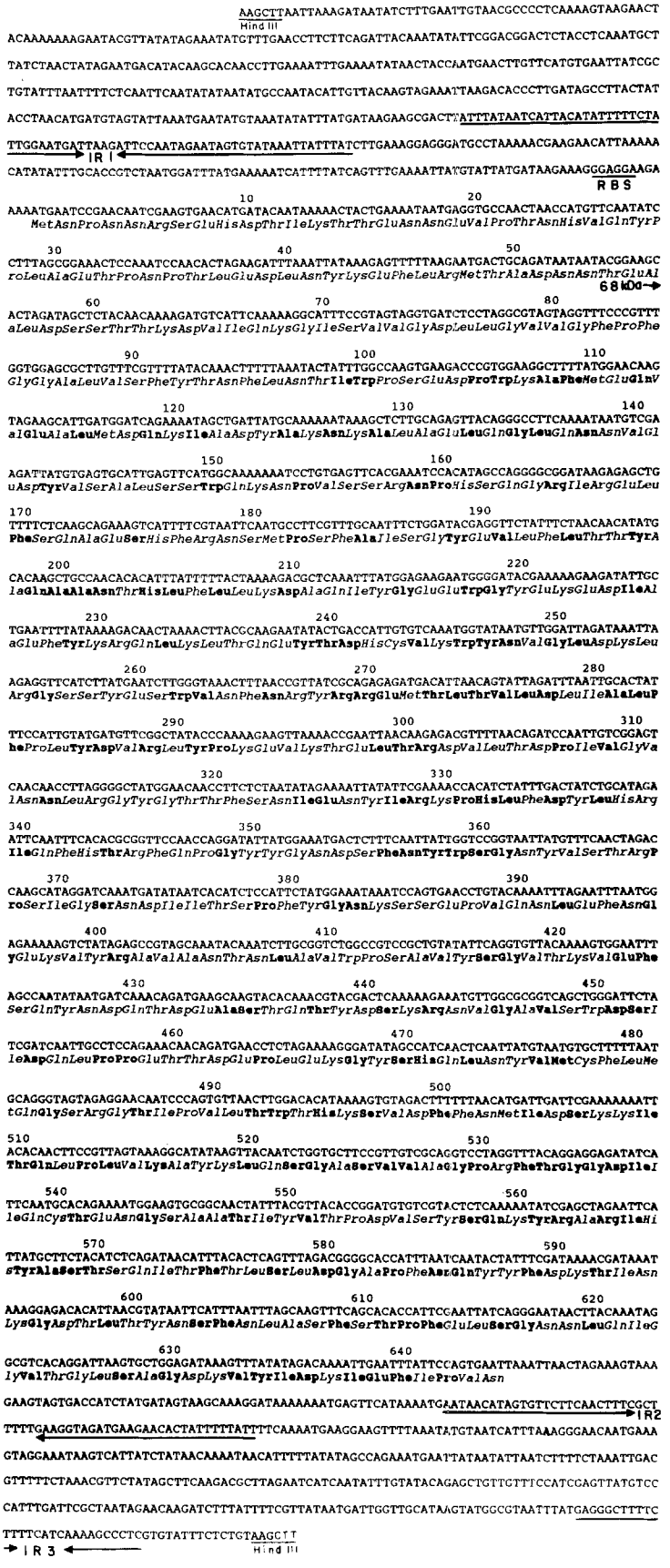

Fig. 5. DNA sequence of the *cryC* gene. *Arrows* with IR1, IR2 and IR3 indicate the locations of three imperfect inverted repeats having free energies of formation for stem-loop structures (as calculated by the rules of Tinoco et al. 1973) of –14.4, –12.6 and –15.2 kcal/mole, respectively. *Bold letters* indicate amino acids identical with those found in the NH_2-terminal half of one P1 protein (Schnepf et al. 1985). RBS: ribosome binding site

Table 2. Sequence similarities between the 73 kDa CRYC protein and other *Bacillus thuringiensis* crystal proteins

Proteins[a]	Extent of similarity[b]	Identity %
CRYC: P1-4.5	99–642: 64–605	37
CRYC: P1-5.3	99–642: 64–606	38
CRYC: P1-6.6	99–643: 64–608	35
CRYC: CRYB1	131–219:115–199	36
CRYC: 130 kDa BTi	23–642: 25–676	30

[a] Protein sequences were deduced from the sequences of their respective genes as reported by Schnepf et al. (1985; P1-4.5); Adang et al. (1985; P1-6.6); Hofte et al. (1986; P1-5.3); Donovan et al. (1988; CRYB1); and Ward and Ellar (1987; 130 kDa BTi)

[b] Numbers indicate the amino acid residues constituting the regions of similarity between the CRYC protein (left of colon) and other proteins (right of colon) indicated in the first column

68 kDa protein was the one deduced from the DNA sequence. The 1.0 kb fragment did not encode an NH_2-terminal methionine, therefore we reasoned that the translation start site for the 68 kDa protein began to the right of the *Pst*1 site (Fig. 4). To locate the translation start site, the 2.9 kb *Hin*dIII fragment of pEG212 was subcloned into mp18 and mp19 and sequenced. An open reading frame of 644 codons (73116 dalton) was located 569 nucleotides from one *Hin*dIII site (Fig. 5). The open reading frame began with an NH_2-terminal methionine codon and was immediately preceded by a sequence (GGAGGA) that most likely functions as a ribosome binding site (Fig. 5). The 68 kDa protein began 54 codons downstream from the NH_2-terminal methionine, indicating that the 68 kDa protein could be derived from the 73 kDa protein. The gene encoding the 73 kDa protein was designated *cryC*. Three imperfect inverted repeats capable of forming stable stem-loop structures were found adjacent to the *cryC* gene (Fig. 5).

Plasmid transfer experiments (described above) had indicated that the *cryC* gene was located on an 88 MDa plasmid. The 2.9 kb *Hin*dIII fragment containing the *cryC* gene was found specifically to hybridize, as expected, to the 88 MDa plasmid of EG2158 (data not shown).

Sequence similarities

The sequence analysis program of Queen and Korn (1984) was used to compare the sequence of the 73 kDa CRYC protein with the sequences reported for other *B. thuringiensis* crystal proteins. The CRYC protein contained sequences of 88, 543 and 619 amino acids that were similar to corresponding sequences in the CRYB1, P1 and 130 kDa BTi proteins, respectively (Table 2).

Expression of the cryC *gene in* E. coli *and in* B. megaterium

E. coli cells harboring plasmid pEG212 (*cryC*) did not contain detectable levels of either the 73 kDa or the 68 kDa CRYC protein (data not shown). The failure to detect the CRYC protein in *E. coli* suggests either that the protein was completely degraded after synthesis or, more likely, that *E. coli* lacks some factor(s) that is required for the efficient expression of the *cryC* gene. We reasoned that more of the CRYC protein might be synthesized by a *Bacillus* species containing the cloned *cryC* gene. Accordingly,

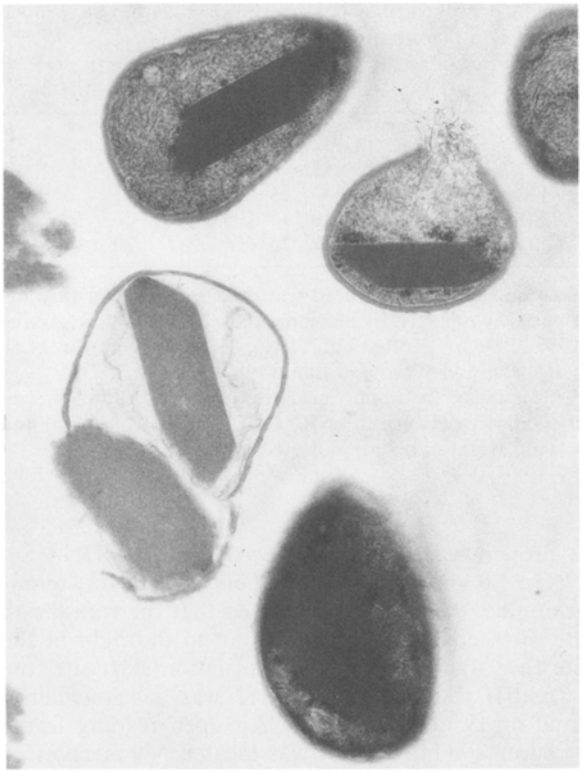

Fig. 6. Rhomboid crystals synthesized by *Bacillus megaterium* cells harboring pEG213 (*cryC*$^+$). The cells were grown on DS agar medium. Samples were prepared for electron microscopy as described by Shivakumar et al. (1986). The cells are approximately 2 μm in diameter

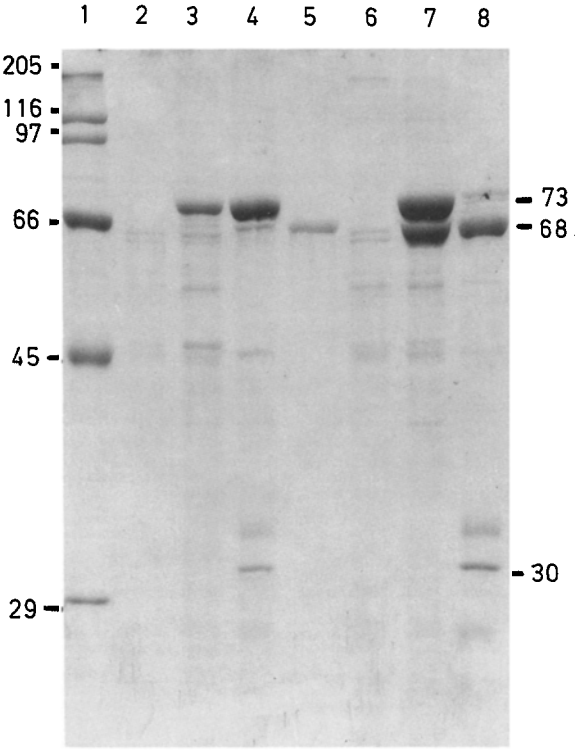

Fig. 7. Protein profiles of *Bacillus megaterium* cells harboring the cloned *cryC* gene. The cells were grown on LB agar medium (lanes 2–4) or on DS agar medium (lanes 6–8). Each lane, except 5, contains proteins extracted from 100 μg of wet cells. Lane 1, molecular weight standards; lanes 2 and 6, EG1311 [*B. megaterium* harboring pBC16 (Crg$^-$)]; lanes 3 and 7, EG1314 [*B. megaterium* harboring pEG213 (cryC$^+$)]; lanes 4 and 8, EG2158; lane 5, purified 68 kDa protein from EG2158. The 73 kDa and 68 kDa proteins in lanes 4 and 7 migrate slightly faster than the corresponding proteins in lanes 3, 5 and 8 due to the large amounts of the proteins that are present in lanes 4 and 7. Numbers to the left and right indicate protein sizes in kDa

the *B. cereus* plasmid, pBC16 (Bernhard et al. 1978), was inserted into the unique *Sph*1 site of plasmid pEG212 (*cryC*) and the resulting plasmid (pEG213 (*cryC*), Fig. 4) was transformed by the method of Von Tersch and Carlton (1984) into cells of the *B. megaterium* strain VT1660. *B. megaterium* cells harboring pEG213 (strain EG1314) synthesized rhomboid crystals; the cells were partially inhibited in their ability to form spores. After 48 h growth on DS agar sporulation medium, the *B. megaterium* (pEG213) culture contained roughly equal amounts of free spores and unlysed spore-free cells containing rhomboid crystals (Fig. 6), and the culture was highly toxic to CPB larvae (Table 1). Numerous free rhomboid crystals were also present (not shown). Two major proteins of 73 kDa and of 68 kDa were solubilized from a mixture of EG1314 cells plus crystals (Fig. 7, lane 7). *B. megaterium* cells containing the plasmid vector pBC16 alone (strain EG1311) sporulated normally on DS agar medium. EG1311 cells did not produce rhomboid crystals and were not toxic to CPB larvae (Table 1). EG2158 cells grown on the same medium produced free rhomboid crystals. A mixture of EG2158 cells plus crystals yielded minor amounts of the 73 kDa protein and major amounts of the 68 kDa protein (Fig. 7, lane 8). Cells grown on LB agar medium gave somewhat different protein patterns. *B. megaterium* (pEG213) cells grown to stationary phase on LB medium, which blocks sporulation in *B. megaterium*, contained rhomboid crystals inside unlysed cells (not shown), and a major protein of 73 kDa was solubilized from the cells (Fig. 7, lane 3). EG2158 cells sporulated normally on LB agar medium. The cells produced free rhomboid crystals and a major protein of 73 kDa was solubilized from a mixture of cells plus crystals (Fig. 7, lane 4).

Discussion

We have isolated a CPB-toxic strain of *B. thuringiensis* (EG2158) that synthesizes rhomboid crystals and flat diamond-shaped crystals. EG2158 contains native plasmids of 150, 105, 88, 72 and 35 MDa. Transfer of the 88 MDa plasmid into both *B. thuringiensis* and *B. cereus* Cry$^-$ recipients converted them into CPB-toxic, rhomboid crystal producers, demonstrating that the 88 MDa plasmid encodes the rhomboid crystals and that these crystals are CPB-toxic. Plasmid curing experiments demonstrated that the non-transmissible 150 MDa plasmid encoded the flat diamond-shaped crystals. These crystals were composed of a 30 kDa protein which was not necessary for CPB toxicity.

We have cloned and sequenced a gene (*cryC*) from EG2158 that encodes a protein of 73116 dalton. *B. megaterium* cells harboring the cloned *cryC* gene synthesized rhomboid, CPB-toxic crystals when the cells were grown on a medium (DS) that promoted sporulation as well as on a medium (LB) that blocked sporulation. We had previously

found that *B. megaterium* cells harboring the cloned *cryB1* gene (encoding the 66 kDa lepidopteran- and dipteran-toxic crystal protein of var. *kurstaki*) synthesized significant amounts of the CRYB1 protein when grown on DS medium, but failed to synthesize detectable levels of the protein when grown on LB medium (Donovan et al. 1988). This suggests that the regulation of the *cryC* gene differs from that of the *cryB1* gene.

Preparations of cells plus rhomboid crystals of strain EG2158 and of *B. megaterium* harboring the cloned *cryC* gene yielded a major protein of 73 kDa and/or a major protein of 68 kDa, depending upon the conditions under which the cells were grown and upon the conditions used to solubilize the crystals. More of the 68 kDa protein was observed when the cells were grown on DS agar sporulation medium or when the crystals were solubilized at 50° C, whereas growth of cells on LB agar medium and solubilization of crystals at 90° C favored the appearance of the 73 kDa protein. We interpret these results to mean that the rhomboid crystal is composed of a 73 kDa protein. Upon solubilization of the protein, 53 NH_2-terminal amino acids are removed to yield the 68 kDa protein. We speculate that native proteases, susceptible to heat inactivation at 90° C, are responsible for this processing and that more of these proteases are produced when the cells are grown on a relatively nutrient-poor medium, such as DS agar, than on a richer medium like LB agar. McPherson et al. (1988) have shown that *E. coli* and *Pseudomonas fluorescens* cells containing a cloned coleopteran-toxic gene (whose reported sequence is identical to that of the *cryC* gene) from var. *tenebrionis,* utilize two functional translation initiation codons in the same reading frame to produce both a full length 73 kDa protein and an N-terminal truncated 67 kDa protein. Although our results indicate that the 68 kDa protein can arise by processing of the 73 kDa protein, we have not ruled out the possibility that EG2158 cells, when grown on certain media (e.g. DS agar), utilize separate translation initiation sites on the *cryC* gene to produce the 73 kDa protein and the 68 kDa protein.

Three imperfect inverted repeats (capable of forming stable stem-loop structures) were located adjacent to the *cryC* gene: one inverted repeat upstream and two inverted repeats downstream from the gene. Inverted repeats are located downstream from the crystal protein genes that encode the P1 and CRYB1 proteins (Wong and Chang 1986; Donovan et al. 1988). Wong and Chang (1986) showed that when the P1 gene inverted repeat was placed downstream from either the *penP* gene of *B. licheniformis* or the interleukin 2 cDNA from the human Jurkat cell line, the expression of those genes in *E. coli* and *B. subtilis* was enhanced, probably by mRNA stabilization.

The sequence of the *cryC* gene was apparently identical to that reported for cloned genes from *B. thuringiensis* var. *tenebrionis* (Hofte et al. 1987; Sekar et al. 1987) and from *B. thuringiensis* var. *san diego* (Herrnstadt et al. 1987). *B. thuringiensis* var. *san diego* has been reported to be identical with var. *tenebrionis* (Krieg et al. 1987). Strain EG2158 and var. *tenebrionis* differed in several ways. The strains were isolated from widely separated locations: EG2158 from a soybean grain dust sample out of Kansas, USA, var. *tenebrionis* from a coleopteran (*Tenebrio molitor*) pupa in Darmstadt, Germany (Krieg et al. 1983). The flat, diamond-shaped crystals that are synthesized by EG2158 have no reported counterpart in var. *tenebrionis*. Strain EG2158 contained large plasmids of 150, 72 and 35 MDa that are apparently not present in var. *tenebrionis* (Sekar et al. 1987). It is remarkable that these distinct strains should have identical nucleotide sequences for what is probably a non-essential gene.

The 73 kDa coleopteran-toxic protein of EG2158 was found to contain regions of similarity with three distinct *B. thuringiensis* crystal proteins: the 66 kDa lepidopteran- and dipteran-toxic CRYB1 protein of var. *kurstaki,* the 130 kDa lepidopteran-toxic P1 proteins of var. *kurstaki* and *berliner,* and the 130 kDa dipteran-toxic protein of var. *israelensis*. Interestingly, the region of similarity with the P1 proteins corresponded closely to the portions of the P1 proteins previously shown to be necessary and sufficient for lepidopteran toxicity (Hofte et al. 1986; Schnepf and Whiteley 1985). These similarities indicate that four crystal-protein genes having distinct entomocidal activities evolved from a common ancestor.

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