

# Cloning and Expression of an Insecticidal k-73 Type Crystal Protein Gene from *Bacillus thuringiensis* var. *kurstaki* into *Escherichia coli*

JAMES H. McLINDEN, JOSANNE R. SABOURIN, BURTON D. CLARK,<sup>†</sup> DIANA R. GENSLER, WESLEY E. WORKMAN, AND DONALD H. DEAN\*

Department of Microbiology, The Ohio State University, Columbus, Ohio 43210

Received 26 April 1985/Accepted 18 June 1985

A 75-kilobase plasmid from *Bacillus thuringiensis* var. *kurstaki* (HD-244) was associated with the k-73 type insecticidal crystal protein production by mating into *B. cereus* and subsequent curing of excess plasmids. This plasmid was partially digested with endonuclease R · *Sau3A* and the fragments were cloned into *Escherichia coli* (HB101) on vector pBR322. Candidate clones were screened for plasmid vectors which contained the expected insert size (at least 3 kilobases) and then with an enzyme-linked immunosorbent assay, using antisera prepared against electrophoretically purified, solubilized insecticidal crystal protein of 130,000 daltons. Several positive clones were isolated and were analyzed for expression, toxicity, and genetic content by restriction enzyme analysis. Electrophoretic transfer blots of proteins from a candidate *E. coli* clone, analyzed by enzyme-linked immunosorbent assay, demonstrated a predominant cross-reacting protein of about 140,000 daltons. Ouchterlony analysis also showed a single precipitin band. Extensive bioassays with *Manduca sexta* larvae revealed that the *E. coli* clones make toxin with a specific activity (50% lethal dose per microgram of cross-reacting protein) equivalent to that of the parental *B. thuringiensis* strain or a *B. cereus* transipient carrying the toxin-encoding, 75-kilobase plasmid.

*Bacillus thuringiensis* is a group of bacteria, closely related to *B. cereus*, which produces proteinaceous crystals during sporulation (1). In general, these crystals are insecticidal to certain caterpillars, and the bacteria are important commercial insecticides with low environmental impact on nontarget organisms. The strain used commercially in the United States is HD-1 (2), now recognized as the variety *kurstaki*. This strain produces an insecticidal crystal protein (ICP), characterized serologically as k-1 (11). Other isolates have been identified which have even greater toxicity than HD-1 for certain insects (2). One of these isolates, HD-244, has four times the potency of HD-1 to *Heliothis verescens* (tobacco budworm) (H. T. Dulmage, personal communication). HD-244 makes a crystal, designated k-73, which is serologically distinguishable from the HD-1 crystal (11, 24).

Recently two plasmids (75 and 225 kilobases [kb]) from HD-244 have been shown to share sequence homology with an intragenic fragment of the HD-1 ICP gene, suggesting that these plasmids encode toxin genes (10). Here we describe the cloning and expression of a k-73 ICP gene from HD-244, using the 75-kb plasmid as the source of DNA. In the process, the 75-kb plasmid was independently shown to contain an ICP gene which is distinct from that of HD-1.

## MATERIALS AND METHODS

**Bacterial strains.** The bacterial strains used in this study are listed in Table 1. *B. thuringiensis* var. *kurstaki* HD-1, HD-2, HD-244, and HD-73 were obtained from H. Dulmage (U.S. Department of Agriculture, Brownsville, Tex.) and are deposited in the *Bacillus* Genetic Stock Center (BGSC) as 4D1, 4A3, W4D, and 4D4, respectively. *Bacillus cereus* NRRL 569 was obtained from C. Thorne (University of Massachusetts, Amherst) and is deposited in the BGSC as 6A3. A spontaneous streptomycin-resistant mutant of 6A3

was isolated as BGSC 6A4. *Escherichia coli* HB101 and cloning vector pBR322 were originally obtained from H. Boyer (University of California Medical School, San Francisco). *E. coli* JM83 and cloning vector pUC8 were originally obtained from J. Messing (University of Minnesota, St. Paul). *E. coli* V517 (13) was used as the source of plasmid molecular weight standards.

**Enzymes.** Enzymes were purchased from Bethesda Research Laboratories, Inc. (Gaithersburg, Md.) except calf intestinal alkaline phosphatase, which was obtained from Boehringer GmbH (Mannheim, Federal Republic of Germany). All enzymes and buffers were used as recommended by the manufacturer.

**Purification of plasmids.** Plasmids pBR322 and pUC8 were isolated from *E. coli* HB101 and JM83 cells, respectively. The 75-kb plasmid (originally from *B. thuringiensis* var. *kurstaki* HD-244) was isolated from *B. cereus* X9-6C2. All preparative purification of plasmids was done by the alkaline lysis method (14), with final purification by CsCl-ethidium bromide density gradients. A rapid, small-scale plasmid analysis was used for screening candidate *E. coli* clones for inserts as follows: a few milliliters of each culture were grown overnight in LB. A 1.5-ml sample was centrifuged (1 min) in a Beckman Microfuge B. The cell pellet was suspended in 50  $\mu$ l of TES (Tris-hydrochloride, 20 mM; NaCl, 100 mM; EDTA, 10 mM; pH 7.6), and 50  $\mu$ l of a water-saturated mixture of phenol, chloroform, and mercaptoethanol (500:500:1, vol/vol/vol) was added. The pH was adjusted to approximately 7.5 with 3 M NaOH, using pH paper (pHydron; Fisher Scientific Co., Pittsburgh, Pa.). The tubes were mixed 12 to 20 times by shaking and centrifuged again for 1 min in the Beckman Microfuge B. The aqueous upper phase was examined by agarose gel electrophoresis. Estimates of the insert size were made by comparison with an *E. coli* culture harboring the vector plasmid with *E. coli* V517 (13).

**Purification of ICP and spores.** Parasporal crystals of *B. thuringiensis* HD-73 and HD-244 were separated from

\* Corresponding author.

<sup>†</sup> Present address: Department of Health Science and Technology, Massachusetts Institute of Technology, Cambridge, MA 02139.

TABLE 1. Strains used in this study

Strain	Description	Origin
<i>B. thuringiensis</i>		
HD-1	k-1 crystal; commercial strain	H. Dulmage
HD-2	k-1 crystal; plasmid reference	H. Dulmage
HD-73	k-73 crystal	H. Dulmage
HD-244	k-73 crystal	H. Dulmage
<i>B. cereus</i>		
NRRL 569		C. Thorne
BGSC 6A4	Streptomycin-resistant mutant of NRRL 569	This study
X9-6C2	Cry <sup>+</sup> ; BGSC 6A4 × HD-244	This study
<i>E. coli</i>		
HB101	Cloning host	H. Boyer
JM83	Cloning host	J. Messing
V517	Plasmid reference	F. L. Macrina
<i>E. coli</i> clones		
87-158	CRP <sup>+</sup> <sup>a</sup>	This study
87-112	CRP <sup>+</sup> <sup>a</sup>	This study
87-18	CRP <sup>+</sup> <sup>a</sup>	This study
87-22	CRP <sup>+</sup> <sup>a</sup>	This study
87-165	CRP <sup>+</sup> <sup>a</sup>	This study
90-22	CRP <sup>+</sup> <sup>a</sup>	This study
12-87-158	CRP <sup>+</sup> <sup>b</sup>	This study
DS-87-158	CRP <sup>+</sup> <sup>c</sup>	This study

<sup>a</sup> Independent clone from *Sau3A* partial digest of 75-kb plasmid from X9-6C2 into HB101 via pBR322.

<sup>b</sup> Independent subclone from 87-158 into JM83 via pUC8.

<sup>c</sup> 87-158 moved into *E. coli* mini-cell line DS.

spores and cellular debris by flotation in gelatin (20) and buoyant density centrifugation in Renografin (E. R. Squibb & Sons, Princeton, N.J.) gradients (21). ICP was solubilized by boiling for 4 min in 6.5 mM sodium phosphate, pH 7.2, containing 1% sodium dodecyl sulfate–0.15% dithiothreitol. Spores were prepared from cells cultured (48 h, 30°C) on Schaeffer's sporulation medium agar plates (18). When a high percentage of released spores was evident, they were harvested by scraping cultures from the plates into distilled water and washed five times in distilled water.

**Immunological analysis.** Antisera against solubilized k-73 crystal from *B. thuringiensis* HD-73 were prepared as previously described (22). Sera were drawn from immunized rabbits (22) and the immunoglobulin G fraction was purified by DEAE–Affi-Gel Blue (Bio-Rad Laboratories, Richmond, Calif.) column chromatography. Immunoglobulin G was analyzed by double immunodiffusion precipitation (17) against solubilized crystals from *B. thuringiensis* HD-73 and yielded a single precipitin band.

In situ and dot-blot goat antirabbit-horseradish peroxidase enzyme-linked immunoblot assays (ELISA) and Western blots were performed as to the manufacturer's protocol (Bio-Rad Laboratories). Dot-blot ELISAs were performed on crude protein extracts from in situ ELISA-positive clones to quantitate crystal protein production, using *B. thuringiensis* HD-244 solubilized ICP dilutions as standards. The system was sensitive to detection of about 9 ng of the solubilized ICP.

**Polyacrylamide gel electrophoresis.** Polyacrylamide gel electrophoresis for the preparation of purified ICP and Western blots was performed by a discontinuous buffer system (8), using 7.5% acrylamide.

**Molecular cloning.** Purified plasmid DNA from *B. cereus*

X9-6C2 was partially digested with *Sau3A* at 0.005 U/μg for 15 min, and 100-μg samples were sized on 5 to 20% NaCl gradients (36,000 rpm; Beckman SW41 rotor, 30°C, 4 h). Gradient fractions containing DNA fragments larger than 4 kb, as determined by 0.4% agarose slab gel electrophoresis, were pooled and ligated to *Bam*HI-digested, calf intestinal alkaline phosphatase-treated pBR322. Vector and passenger (1:3 mol/mol) were mixed at a concentration of 104 μg/ml in 50 μl, ligated with T4 DNA ligase for 18 h at 16°C, and transformed into *E. coli* HB101, using 100 ng of DNA per 100 μl of competent cells, according to the procedures of Morrison (16) and Maniatis et al. (14).

**Bacterial crude extracts.** Crude extracts from the genus *Bacillus* were from cells grown in nutrient broth (Difco Laboratories, Detroit, Mich.) for 48 h at 30°C. Cells were harvested by centrifugation, washed in 0.85% NaCl solution, suspended in a minimum amount of deionized water containing 10<sup>-3</sup> M EDTA–10<sup>-3</sup> M dithiothreitol, and sonicated in 30-s intervals for a total of 5 min. The sonicated cell extract was cleared by centrifugation at 50,000 × g for 1 h in a Beckman model JA-20 rotor. All crude extracts from *E. coli* were produced as described above with the exception that cells were grown in LB medium (tryptone [Difco], 10 g/liter; yeast extract [Difco], 5 g/liter; NaCl, 10 g/liter) and harvested at 16 h.

Crude bacterial extracts were brought to 30% (wt/vol) ammonium sulfate by the addition of solid (NH<sub>4</sub>)<sub>2</sub>SO<sub>4</sub>, allowed to stir at 4°C for 20 min, and then centrifuged at 50,000 × g for 30 min in a Beckman JA-20 rotor. The pellet was suspended in a minimal volume of 30 mM Tris-hydrochloride–50 mM NaCl–5 mM EDTA, pH 8.0 (TSE), and dialyzed thoroughly against TSE. The protein contents of bacterial extracts and crystal preparations were determined by the semimicro-biuret method (15).

**Bioassay.** *Manduca sexta* (tobacco hornworm) larvae were hatched from eggs received from the U.S. Department of Agriculture Southern Region Tobacco Research Lab, Oxford, N.C. Immediately upon arrival eggs were placed on diet (23) at room temperature, 40% humidity, and constant light, and allowed to hatch and develop. Healthy *M. sexta* larvae of second ( $\bar{x}$  = 10.9 ± 4.3 mg)- or third ( $\bar{x}$  = 25.4 ± 4.2 mg)- instar larvae were selected at day 8 or 9 after oviposition for bioassay experiments 1 and 2, respectively. Larvae were aliquoted to one per 1-ounce (30-ml) plastic chamber with lids perforated for air and starved for 4 h before treatment. Treatment dosages (10 μl) of ICP, purified spores, or crude protein extracts were applied uniformly to the surface of a 0.5-cm<sup>3</sup> disk of diet, allowed to air dry, and introduced to larvae at one treatment disk per chamber, with 15 larvae per treatment group. Larvae were maintained at room temperature, constant light, and 40% humidity, and additional food disks (untreated) were presented upon consumption of a diet disk to avoid starvation effects. Mortality, weight gain, and development (percent larvae to reach fifth instar) were monitored for 9 days after exposure to treatment disks. Lethal dosages, 50% (LC<sub>50</sub>), were calculated according to probit analysis (12).

## RESULTS

**Identification of plasmid which encodes an ICP gene.** The 75-kb plasmid from the highly toxic strain of *B. thuringiensis* var. *kurstaki*, HD-244, was identified as encoding an ICP gene by mating (4) into BGSC 6A4 which contains a 12-kb and a 225-kb plasmid (Fig. 1, lane 2). The resultant transcipts were screened by phase-contrast microscopy for the presence of crystals and for plasmids by the Eckhardt

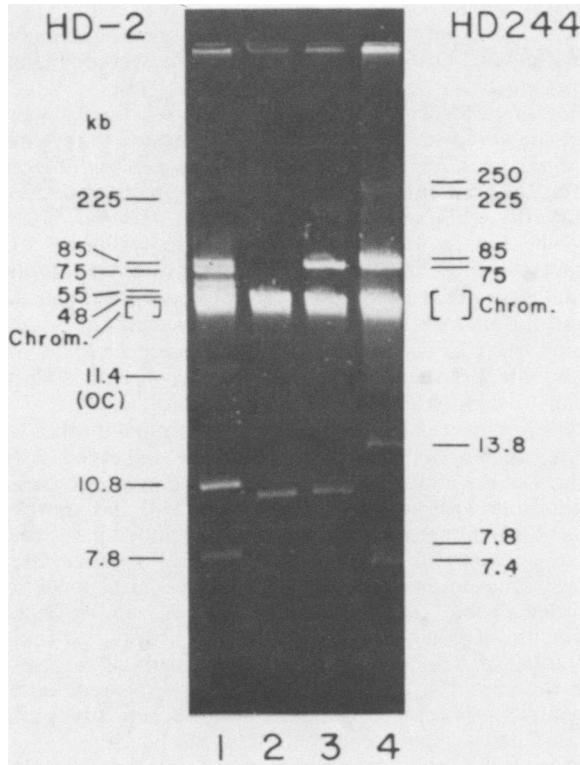


FIG. 1. Plasmid profiles of *B. thuringiensis* and *B. cereus* strains used in this study: Lanes: 1, *B. thuringiensis* HD-2 (plasmid molecular weight markers); 2, *B. cereus* BGSC 6A4; 3, *B. cereus* X9-6C2; 4, *B. thuringiensis* HD-244. OC, Open circular.

method (3). The 75-kb plasmid was present in all 20 crystal-producing (*Cry*<sup>+</sup>) colonies of the 48 streptomycin-selected colonies examined. Subsequent heat curing (growth at 42°C, 48 h) revealed that the 75-kb plasmid was absent in all eight of the acrySTALLIFEROUS (*Cry*<sup>-</sup>) cured progeny but remained in all of the *Cry*<sup>+</sup> cured progeny (52 of 60). One of the latter cultures contained only the 75-kb plasmid in addition to a 12-kb and a 225-kb resident plasmid (Fig. 1, lane 3) (present in NRRL 569 and BGSC 6A4; Fig. 1, lane 2). This culture was designated *B. cereus* X9-6C2 and was used as a source of plasmid for cloning. Figure 1 shows the plasmid patterns of HD-244 (lane 4), BGSC 6A4 (lane 2), and X9-6C2 (lane 3), with HD-2 (lane 1) as the plasmid standard.

**Cloning an ICP gene from HD-244.** Fragments of the 75-kb plasmid were cloned into *E. coli* HB101 on vector pBR322 (as described in Materials and Methods). From 23,000 ampicillin-resistant candidate clones, 1,656 were tetracycline sensitive, indicating an insertion into the *Bam*HI site of vector pBR322. Of these, 255 contained inserts of 4 kb or greater and 6 gave positive colony ELISAs as well as positive ELISAs on crude extracts. Figure 2 shows restriction enzyme analysis of three of the positive clones. Incomplete restriction mapping indicated that all of the six ELISA-positive clones had similar restriction patterns. One of these clones, 87-158, contained a plasmid, pOS1000, which was redigested with *Bam*HI and *Sal*I and subcloned into *E. coli* JM83 on vector pUC8. These subclones also were ELISA positive.

**Immunological evidence for expression in *E. coli*.** Since the candidate clones were screened with an ELISA, using antisera raised against electrophoretically purified, solubilized ICP, it was apparent that an antigenically similar protein was expressed by the clones. It remained to be demonstrated

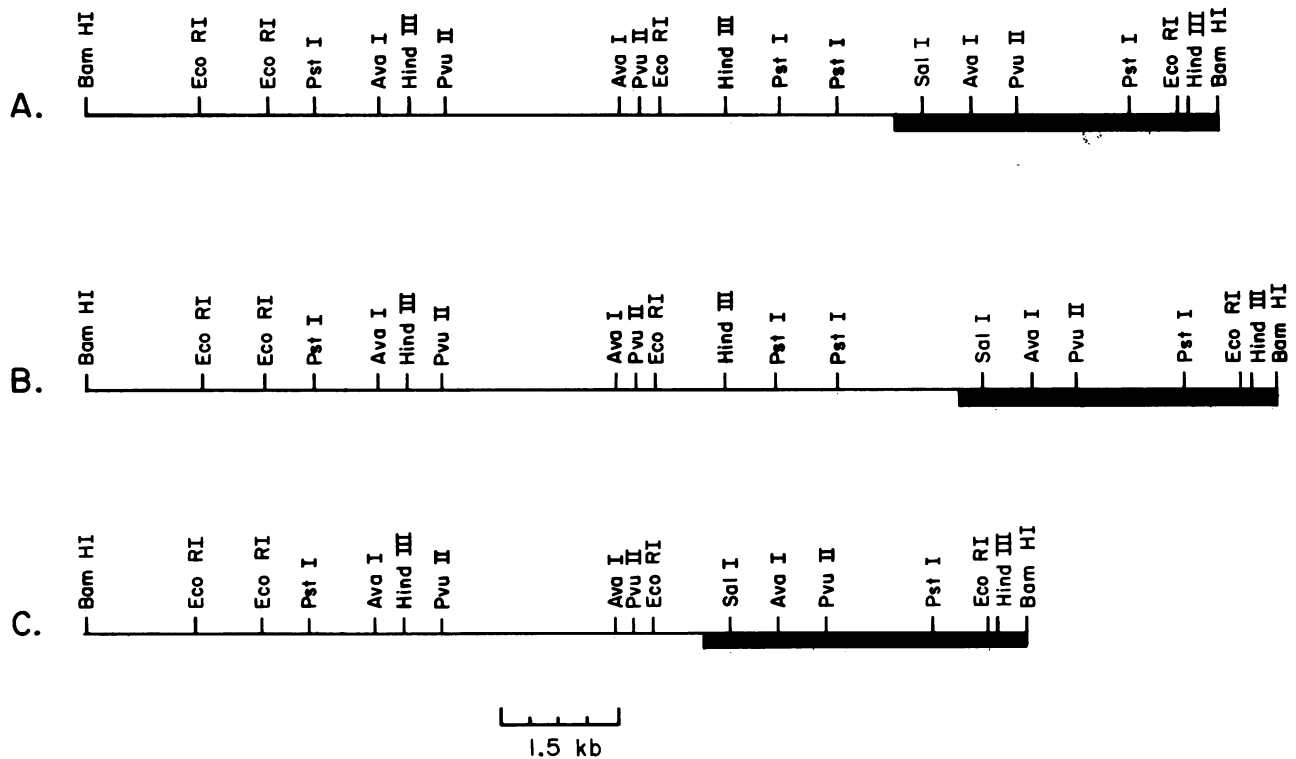


FIG. 2. Restriction maps of three independently derived clones of the 75-kb plasmid, using pBR322 into *E. coli* HB101. (A) pOS1000 from *E. coli* 87-158; (B) pOS1001 from *E. coli* 87-112; (C) pOS1002 from *E. coli* 87-22.

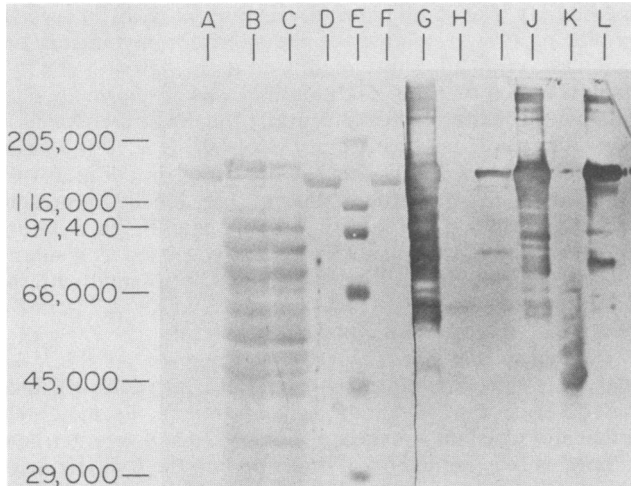


FIG. 3. Western blot analysis of protein from various sources, as described in the text. Lanes A to F are amido black-stained Western blot. Lanes G to L are labeled with a goat antirabbit horseradish peroxidase ELISA of a Western blot. Lanes A and G, *B. thuringiensis* HD-244 ICP (30 µg); lanes B and H, *E. coli* HB101 crude protein extract (100 µg); lanes C and I, *E. coli* 87-158 crystal clone crude protein extract (100 µg); lanes D and J, *B. thuringiensis* HD-73 ICP (30 µg); lanes E and K, molecular weight standards; lanes F and L, *B. thuringiensis* HD-1 ICP (30 µg).

that the protein being expressed was similar to ICP in other aspects. A double-diffusion precipitation assay (Ouchterlony) was performed and single precipitation bands, without spurs, were observed (data not shown).

A crude extract of clone 87-158 was compared with ICP and a crude extract of *E. coli* HB101 by polyacrylamide gel

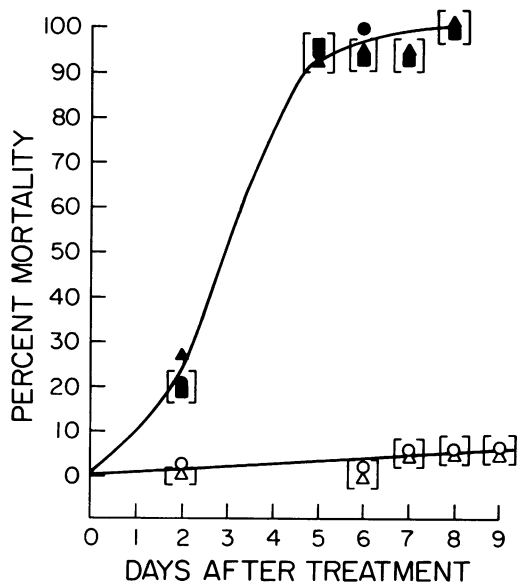


FIG. 4. Comparison of *M. sexta* larvae mortality after acute exposure to dosages of crude protein extracts from *Bacillus* sp. and *E. coli* clones. Symbols: (▲) *B. thuringiensis* HD-244 (3 mg of protein or 22 µg of CRP per treatment); (■) *B. cereus* X9-6C2 (3 mg of protein or 17 µg of CRP per treatment); (●) *E. coli* 87-158 (6 mg of protein or 14 µg of CRP per treatment); (○) *E. coli* HB101 (4 mg of protein per treatment); (△) distilled water.

electrophoresis (Fig. 3, lanes A to F). The gel was electroblotted onto a nitrocellulose membrane and analyzed by ELISA, as described in Materials and Methods (Fig. 3, lanes G to L).

**Bioassay evidence for expression in *E. coli*.** Bioassays with *M. sexta* were used to demonstrate expression of the crystal protein gene in *E. coli* and to compare the levels of expression of the gene in *B. cereus* X9-6C2 (by mating) or *E. coli* 87-158 (by cloning) with the parental *B. thuringiensis*, HD-244. Figure 4 compares percent mortality of crude extracts of these three species at equivalent amounts of crystal cross-reacting protein (CRP) per treatment. The same mortality curve was observed for each species. The cloning host, *E. coli* HB-101 (pBR322), and distilled water had no effect. Other ELISA-positive *E. coli* clones showed similar to slightly less mortality (Fig. 5).

Bioassays were also performed to determine if other toxic effects, typical for the ICP, would be observed. Crude extracts of the above three species were compared with one another and with purified crystals (HD-244) and spores (*B. cereus* 6A4). Other *E. coli* clones were included in the study. These samples were fed to second- or third-instar larvae, and the weight gain and percent to reach the fifth instar were recorded (Table 2). *E. coli* clones and *B. cereus* X9-6C2, bearing the ICP gene, consistently demonstrated all toxicity symptoms of *B. thuringiensis*. It was observed that spores from the parent strain of *B. cereus* 6A4 cross-reacted with the antisera prepared against electrophoretically purified ICP and had a slight toxicity to *M. sexta* larvae.

To measure larvicidal activity more precisely, we determined the LC<sub>50</sub> values of purified crystals of three *B.*

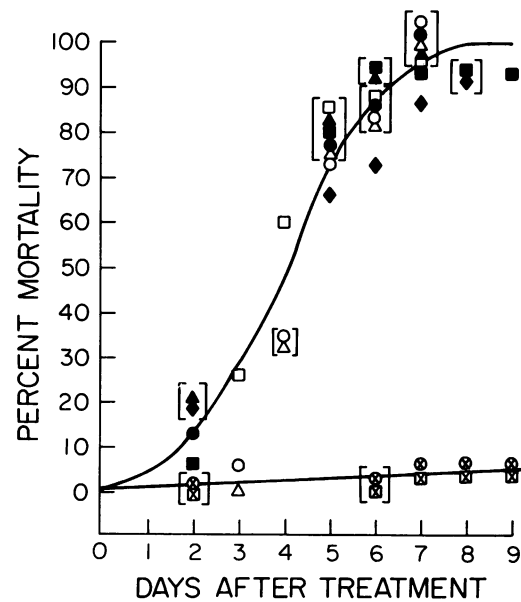


FIG. 5. Comparison of *M. sexta* larvae mortality after acute exposure to dosages of crude protein extracts from various sources. Symbols: (■) *E. coli* 87-18 (4 mg of protein or 7 µg of CRP per treatment); (●) *E. coli* 87-22 (6 mg of protein or 7 µg of CRP per treatment); (▲) *E. coli* 87-112 (5 mg of protein or 6 µg of CRP per treatment); (◆) *E. coli* 87-165 (5 mg of protein or 4 µg of CRP per treatment); (○) *E. coli* 90-22 (4 mg of protein or 10 µg of CRP per treatment); (□) *E. coli* DS-87-158 (3 mg of protein or 16 µg of CRP per treatment); (◇) *E. coli* 12-87-158 (4 mg of protein or 12 µg of CRP per treatment); (⊠) *E. coli* HB101 (4 mg of protein per treatment); (△) distilled water.

TABLE 2. Effect of insecticidal protein on development of *M. sexta* larvae

Treatment <sup>a</sup>	Dosage		Surviving larvae at 6 days after treatment		
	mg of protein per treatment	µg of CRP per treatment	% to reach 5th instar	Avg wt (mg)/larvae	% wt reduction from control
Water			92.8	64.6 <sup>b</sup>	
Water			86.7	446.7 <sup>c</sup>	
<i>E. coli</i> HB101 (CE)	4	0	85.7	51.2	20.7 <sup>b</sup>
<i>B. cereus</i> 6A4 (SP)	4	46	60.0	248.4	44.5 <sup>c</sup>
<i>B. thuringiensis</i> HD-244 (CY)	0.04	40	0	22.6	94.9 <sup>c</sup>
<i>B. thuringiensis</i> HD-244 (CE)	3	22	0	11.9	81.5 <sup>b</sup>
<i>B. cereus</i> X9-6C2 (CE)	3	17	0	8.7	86.5 <sup>b</sup>
<i>E. coli</i> 87-158 (CE)	6	14	0	— <sup>d</sup>	— <sup>d</sup>
<i>E. coli</i> 87-18 (CE)	4	7	0	9.5	85.3 <sup>b</sup>
<i>E. coli</i> 87-112 (CE)	5	6	0	8.6	86.7 <sup>b</sup>
<i>E. coli</i> 87-22 (CE)	6	7	0	9.2	85.8 <sup>b</sup>
<i>E. coli</i> 87-165 (CE)	5	4	0	8.6	86.7 <sup>b</sup>
<i>E. coli</i> 90-22 (CE)	4	10	0	— <sup>e</sup>	—
<i>E. coli</i> 12-87-158 (CE)	4	12	0	—	—
<i>E. coli</i> DS-87-158 (CE)	3	16	0	—	—

<sup>a</sup> CE, Crude extract; CY, solubilized ICP; SP, spores.

<sup>b</sup> *M. sexta* of second instar exposed to treatment, experiment 1.

<sup>c</sup> *M. sexta* of third instar exposed to treatment, experiment 2.

<sup>d</sup> —, 100% mortality at 6 days after treatment.

<sup>e</sup> —, data not available.

*thuringiensis* strains and from crude extracts of an *E. coli* clone, 87-158, and the parental *B. thuringiensis*, HD-244. *B. cereus* spores were also evaluated (Table 3). The results among the crystal types were comparable by probit analysis (not shown). The results for the *E. coli* clone and the parental *B. thuringiensis* strain (crude extract) were comparable. HD244 crystals were seen to be twofold more potent than crude extracts of clone 87-158 (LC<sub>50</sub> per microgram of CRP), whereas *B. cereus* spores were two orders of magnitude less potent.

### DISCUSSION

Two publications have reported cloning the ICP genes from *B. thuringiensis* var. *kurstaki* HD-1 (6, 19) and one has reported cloning the ICP genes from *B. thuringiensis* strain Berliner 1715 (9). These publications support other work demonstrating that toxin genes are borne on plasmids (5). Two of these publications show that chromosomal sequences hybridize with plasmids (6, 9), and it has been claimed that the chromosomal sequences represent a nonexpressed chromosomal gene (6, 9).

TABLE 3. Lethal dosages of insecticidal protein samples to *M. sexta* larvae

Treatment <sup>a</sup>	Dosage LC <sub>50</sub> (total mg of protein per treatment)	µg of CRP per mg of protein	Dosage LC <sub>50</sub> (µg of CRP per treatment)
<i>B. thuringiensis</i> HD-1 (CY)	0.00085	915	0.778
<i>B. thuringiensis</i> HD-73 (CY)	0.0000835	910	0.076
<i>B. thuringiensis</i> HD-244 (CY)	0.0005	928	0.464
<i>B. thuringiensis</i> HD-244 (CE)	0.4129	7.1	2.934
<i>E. coli</i> 87-158 (CE)	0.4222	2.4	1.021
<i>B. cereus</i> W6A1 (SP)	13.248	12.4	164.666

<sup>a</sup> CE, Crude extracts; CY, crystal protein; SP, spores.

Hybridization studies that used an internal *EcoRI* restriction enzyme fragment of the HD-1 "Dipel" ICP gene as a probe (10) showed that two plasmids from HD-244 hybridized with the probe: a 75-kb plasmid and a larger one estimated to be 165 to 225 kb. The larger plasmid was implicated as encoding a second ICP gene. Our plasmid-mating transfers resulted in Cry<sup>+</sup> recipients which had only the 75-kb plasmid. In no case did a Cry<sup>+</sup> transipient contain only a 165 to 225-kb plasmid.

Molecular cloning of a 75-kb plasmid resulted in a number of Cry<sup>+</sup> *E. coli* clones. The restriction patterns of the hybrid plasmids from these clones reveal a physical map (Fig. 2) which is significantly different from that of the HD-1, k-1 ICP gene (19). The consistency of restriction patterns in the clones indicates the fidelity of the selection methods, and the consistency of the expression upon recloning into another vector and an *E. coli* strain indicates that it is the cloned DNA rather than some unusual attribute of the cell which resulted in the positive ELISA. Immunological evidence supports the conclusion that the gene expressed in *E. coli* makes ICP (albeit not in crystalline form) of the same serological determinants and molecular weight (Fig. 3) as the ICP made by *B. thuringiensis* HD-244.

The bioassay data reveal two significant points for discussion. The first is that when a *B. cereus* strain (which has presumably never expressed a crystal protein toxin) received by mating a single plasmid of 75 kb from *B. thuringiensis* HD-244, as in the case of X9-6C2, it gained larvicidal activity resulting in 100% mortality of *M. sexta* at comparable levels of CRP (micrograms) as HD-244 ICP (Fig. 4; Table 2). The simplest interpretation of this result is that all of the ICP-coding capacity resides on the 75-kb plasmid. If this is true, other data based on hybridization would be interpreted as sequences with some homology but no functional contribution in either toxicity or regulation. Other possibilities exist. For example, the ICP gene on the 75-kb plasmid may be expressed more efficiently (without competition) in a background without other ICP genes.

The second point is that an *E. coli* clone carrying the ICP gene produces a noncrystalline protein in crude extracts that is equivalent in larvicidal activity, on a total milligrams of protein basis, to crude extracts of *B. thuringiensis* HD-244 that contain only noncrystalline toxin proteins (crystals are removed by centrifugation during the production of the crude extract) (Table 3). In addition, the toxic proteins produced by *E. coli* are roughly equivalent in toxic activity to those found in crude extracts or purified crystals of *B. thuringiensis* HD-244, in micrograms of CRP (LC<sub>50</sub>). When *E. coli* clone 87-158 is compared in this manner to HD-244 ICP, it is approximately one-half as toxic as ICP and about three times as toxic as crude extracts of *B. thuringiensis* HD-244 cells. The decreased activity of crude extracts of *B. thuringiensis* HD-244 as compared with toxicity per microgram of CRP can be explained by the presence of cross-reacting spore coat proteins, which are much less toxic. This is supported by the presence of CRP observed in *B. cereus* 6A4 spores.

Although the toxin protein produced by *E. coli* clones is relatively comparable in toxic activity per microgram of CRP to that produced in *B. thuringiensis* HD-244, the quantity produced is much lower in *E. coli*. If one assumes that the quantity of ICP produced by *B. thuringiensis* during sporulation ranges from 10% to as high as 25% of the total cellular protein (6), the level of toxin protein produced in *E. coli* can be compared with that produced in *B. thuringiensis* HD-244. In table 3, we can compare the micrograms of CRP per milligrams of total protein and calculate the percent CRP to be 0.24% of the total protein in crude extracts of *E. coli* 87-158. This in turn corresponds to about 1 to 3% of that produced in *B. thuringiensis*. The latter finding is in agreement with previous reports (6, 19).

We have also examined the relative toxicity of ICP from *B. thuringiensis* strains HD-1, HD-244, and HD-73 for *M. sexta* (Table 3). HD-244 is found to be 1.7 times more toxic (LC<sub>50</sub> per microgram of CRP) for *M. sexta* than HD-1. Since our antisera were prepared against k-73 ICP, the difference in specific activities must be even greater than this. Dulmage et al. (2) had previously reported that HD-244 was four times more toxic (LC<sub>50</sub>/1) than HD-1 for *H. verescens*. This points to the differences in insect specificity, further illustrated in Table 3 in that HD-73 ICPs are 10 times more toxic (LC<sub>50</sub> per microgram of CRP) to *M. sexta* than that of HD-1 and 6 times more toxic than that of HD-244. HD-73 is, however, relatively less toxic to *H. verescens* (Dulmage, personal communication). These marked differences in toxicity among different *B. thuringiensis* isolates, even within the same crystal serotype, suggests that important differences exist in these proteins at the molecular level.

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