Proc. Natl. Acad. Sci. USA
Vol. 78, No. 5, pp. 2893–2897, May 1981
Biochemistry

Cloning and expression of the *Bacillus thuringiensis* crystal protein gene in *Escherichia coli*

(plasmids/radioimmunoassay/sporulation/insect toxicity)

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Communicated by Earl W. Davie, February 11, 1981

ABSTRACT ***Sau* 3A1 partial digestion fragments from *Bacillus thuringiensis* var. *kurstaki* HD-1 plasmid DNA were ligated into the *Bam*HI site of the cloning vector pBR322 and transformed into *Escherichia coli* strain HB101. Colonies presumed to contain recombinant plasmids were screened for production of an antigen that would react with antibody made against *B. thuringiensis* crystals. One strain, ES12, was isolated by using this procedure. ES12 contains a plasmid of M_r 11 × 10^6 that has DNA sequence homology with pBR322 as well as with M_r 30 × 10^6 and M_r 47 × 10^6 plasmids of *B. thuringiensis*. It makes a protein antigen, detected by antibodies to crystal, which has the same electrophoretic mobility as the *B. thuringiensis* crystal protein. Protein extracts of ES12 are toxic to larvae of the tobacco hornworm *Manduca sexta*.**

The intracellular protein crystals produced by sporulating cells of *Bacillus thuringiensis* account for 20–30% of the dry weight of sporulated cultures (1–3). The crystals are composed of repeating subunits of a M_r 130,000 polypeptide (4, 5) which is thought also to be a major component of the spore coat of crystal-producing strains (6, 7). This, plus the fact that some mutants blocked very early during sporulation fail to make crystals (ref. 8; unpublished data), suggests that the gene coding for this protein may be regulated in a manner similar to that of genes specifying other sporulation-specific polypeptides. A form of RNA polymerase isolated during sporulation in *B. subtilis* has recently been shown to transcribe *in vitro* a cloned *B. subtilis* DNA fragment that is transcribed during sporulation *in vivo* (9, 10). Several other forms of RNA polymerase have been detected during sporulation in *B. subtilis* (11, 12) and *B. thuringiensis* (13). The isolation of the crystal protein gene should permit detailed studies of the mechanisms regulating the expression of this gene and should allow testing of the proposal that this gene is transcribed by a form of RNA polymerase found only in sporulating cells (8).

The crystals made by *B. thuringiensis* are toxic to the larvae of a number of lepidopteran insects, and preparations containing crystals and spores are used commercially as a highly selective biological insecticide. Crystal-producing strains of *B. thuringiensis* frequently become acrystalliferous and simultaneously lose insecticidal activity; reversion of acrystalliferous strains to crystal production occurs rarely, if at all (14). Strains of *B. thuringiensis* harbor a large array of plasmids (15), and several reports (14, 16) have documented the loss of plasmid DNA concomitant with the generation of the acrystalliferous phenotype, suggesting that the gene encoding the crystal protein is on a plasmid.

Because DNA-mediated transformation is not well characterized in this organism we have approached the isolation of the gene for the crystal protein by recombinant DNA techniques. We report here the isolation of an *Escherichia coli* strain, ES12, harboring a recombinant plasmid, pES1, consisting of the cloning vector pBR322 and *B. thuringiensis* plasmid DNA. This recombinant strain synthesizes a polypeptide of M_r 130,000 which reacts specifically with antibody directed to the crystal protein. Cell extracts of this strain are toxic for larvae of the tobacco hornworm *Manduca sexta*.

METHODS

Isolation and Analysis of Plasmid DNAs. Plasmid pBR322 was isolated from *E. coli* strain HB101 (pBR322) as outlined by Blair *et al.* (17) and plasmids were obtained from *B. thuringiensis* var. *kurstaki* HD-1 (kindly provided by Lee A. Bulla, Jr.) according to the method for plasmid screening of White and Nester (18). All plasmid preparations were additionally purified by centrifugation in cesium chloride/ethidium bromide gradients. Samples (100 μg of DNA per gradient) for cloning experiments were fractionated by centrifugation through 5–25% sucrose gradients for 2.5 hr at 35,000 rpm in an International B-60 centrifuge using the SB-283 rotor. Electrophoresis in agarose gels was used to analyze plasmid DNAs (19) and fragments produced by digestion of DNA with restriction enzymes (20). Hybridization to plasmid DNAs was performed after partial depurination (21) and transfer of DNA from gels to nitrocellulose (22).

Enzymes and Radiolabeling Procedures. Restriction endonucleases *Sal* I, *Hin*dIII, *Bam*HI, and *Sau* 3A1 were used as recommended by the manufacturer (New England BioLabs). DNA fragments (3 μg of *B. thuringiensis* plasmid DNA and 0.15 μg of pBR322 DNA in a volume of 10 μl) were ligated as described for cohesive ends (23). *Staphylococcus* protein A (Pharmacia) was labeled with ^{125}I (Amersham) by using chloramine-T (24). The DNA polymerase I-catalyzed fill-in reaction (25) was used to label DNA fragments of *Sau* 3A1 digests with [α-^{32}P]dCTP (Amersham).

Transformation. Transformation was carried out as described by Cohen *et al.* (26); transformants were selected on media containing ampicillin at 100 μg/ml. Because cloning was performed by insertion of passenger DNA into the *Bam*HI site of pBR322, which is located in a gene coding for tetracycline resistance, ampicillin-resistant transformants were screened for sensitivity to tetracycline (25 μg/ml). Colonies resistant to ampicillin but sensitive to tetracycline were presumed to contain inserts (27).

Preparation of Antibodies. Crystals were purified from sporulated cultures of *B. thuringiensis* grown in modified G medium (28) by four successive centrifugations in Renograffin (Squibb) gradients (29). Contamination with spores was estimated as <0.1% by phase-contrast microscopy. Solubilized crystals (4) were electrophoresed on preparative 10% polyacrylamide slab

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Abbreviation: CHES, cyclohexylaminoethanesulfonic acid.

gels containing $NaDodSO_4$ (30), and the portion of the gel containing the major crystal protein polypeptide was sliced from the gel, crushed, mixed with an equal amount of Freund's complete adjuvant, and used to immunize rabbits. The IgG fraction was purified from the serum of the immunized rabbits by precipitation with ammonium sulfate and chromatography on DEAE-cellulose (31) and analyzed by Ouchterlony immunodiffusion (32).

Radioimmune Screening of Bacterial Colonies and Detection of Polypeptide Antigens. Colonies were transferred from agar plates to filter paper and denatured with phenol/chloroform/heptane and chloroform/methanol as described by Henning *et al.* (33). The filters were soaked in 1% bovine serum albumin (Sigma, fraction V) and incubated with antibody and ^{125}I-labeled protein A (^{125}I-protein A) (34); colonies containing material capable of reacting with the crystal protein antibodies were detected by autoradiography. The concentrations of antibody (dilutions, 10^{-3}–10^{-4}) and ^{125}I-protein A (0.2–1 × 10^6 cpm) were varied to obtain conditions permitting the detection of 5 ng of crystal protein in 1 μl spotted on a filter while colonies of *E. coli* HB101 (pBR322) were either unreactive or appeared light against a grey background. Protein samples were electrophoresed on 10% $NaDodSO_4$/polyacrylamide slab gels (30), transferred electrophoretically to nitrocellulose (35), and incubated with antibody (5 × 10^{-3} dilution) and ^{125}I-protein A (6 × 10^5 cpm). The reaction of transferred peptides with antibody and ^{125}I-protein A was detected by radioautography.

Preparation and Protease Digestion of *E. coli* Extracts. Cells grown for 16 hr in L broth containing ampicillin (100 μg/ml) were harvested by centrifugation, suspended in 0.1 M Tris, pH 7.0/1 mM EDTA containing 200 μg of phenylmethylsulfonyl fluoride per ml, and disrupted by sonication. Insoluble material, obtained by centrifugation of the sonicates at 100,000 × *g* for 30 min, was thoroughly suspended in 4 M urea/0.285 M 2-mercaptoethanol/0.05 M $NaHCO_3$, pH 9.5, and clarified by centrifugation. The supernatant fraction was dialyzed twice against 100 vol of 3 mM $NaHCO_3$/7 mM 2-mercaptoethanol, pH 9.5, for a total of 16 hr at 4°C and centrifuged at 100,000 × *g* for 30 min. Ammonium sulfate was added to the supernatant fraction to give 25% saturation, and the precipitated material was pelleted, dissolved in a small volume of 4 M urea/0.285 M 2-mercaptoethanol/0.05 M $NaHCO_3$, pH 9.5, and dialyzed against 0.05 M Tris buffer at pH 7.4 for insect toxicity assays or for electrophoretic analysis. For proteolysis with subtilisin or trypsin, the samples were prepared as above but with 0.05 M cyclohexylaminoethanesulfonic acid (CHES), pH 10.0/0.285 M 2-mercaptoethanol for the pH 9.5 urea buffer; final dialysis was against 0.01 M CHES buffer at pH 10.0. For trypsin digestion (2), 0.01 M CHES was used as the buffer and digestion was carried out at room temperature during dialysis against that buffer; digestion with subtilisin was performed according to Cleveland *et al.* (36). The reactions were stopped by boiling the samples in electrophoresis sample buffer (36), and polypeptides capable of reacting with antibody to the crystal protein were detected by using ^{125}I-Protein A and radioautography as described above. Protein concentrations were determined according to Bradford (37).

Insect Toxicity Assay. Neonate larvae of the tobacco hornworm were kindly supplied by J. Truman and L. Riddiford (Department of Zoology, University of Washington). Extracts were prepared as described above from 8 liters of the appropriate *E. coli* strains; 6–8 ml of extract was mixed with 50 ml of molten agar-based diet (38) and quickly poured to give a shallow layer. Strips of the solidified diet were placed in glass vials (3–4 ml per vial) with one neonate larva for 10 days at room temperature.

Containment Conditions. Recombinant DNA portions of these experiments were done in P2 physical containment with EK-1 biological containment.

RESULTS

Construction and Analysis of Recombinant Plasmid DNA. The plasmids of *B. thuringiensis* var. *kurstaki* HD-1 range from 47 to 1.32 megadaltons in molecular mass (lane 1 of Fig. 1 (14). (Occasionally a larger plasmid has been detected in this strain.) To enrich for the 47-megadalton plasmid and to decrease the complexity of the DNA to be inserted into the vector, plasmid DNA was sedimented on 5–25% sucrose gradients. Two fractions were obtained: a "large plasmid fraction" containing the 47- and 30-megadalton plasmids (lane 2 of Fig. 1), and a "small plasmid fraction" containing plasmids of 4.9, 5.2, 5.5, and 9.6 megadaltons (lane 3 of Fig. 1). Rough calculations of gene size indicated that the smallest plasmid seen in lane 1 of Fig. 1 (the M_r 1.32 × 10^6 plasmid) was too small to encode a polypeptide of M_r 130,000 and therefore no attempt was made to use this plasmid in cloning experiments.

Digestion of the two plasmid fractions was carried out with varying dilutions of *Sau* 3A1 and was monitored by agarose gel electrophoresis. To generate a source of inserts, the large plasmid fraction was digested with the minimal amount of enzyme needed to convert all the closed covalent circular molecules to the linear form; this resulted in fragments with an average size >10 megadaltons. For the small plasmid fraction, an amount of *Sau* 3A1 was used which left some closed covalent circular molecules but produced few linear fragments under M_r 2 × 10^6.

Those transformed colonies presumed to carry *B. thuringiensis* DNA inserts were screened for the production of crystal protein antigen by using antibodies and ^{125}I-protein A. When the large plasmid fraction was used as a source of inserts, one colony was found that reacted specifically with antibodies to the

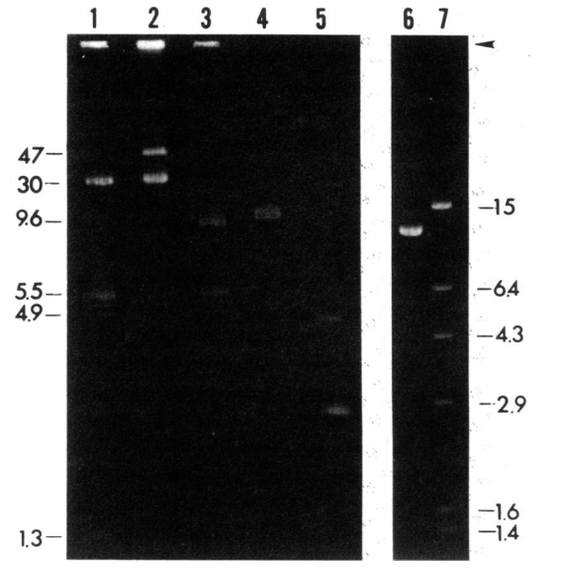

FIG. 1. Analysis of plasmid DNAs and marker DNA fragments, on 0.7% (lanes 1–5) and 0.35% (lanes 6 and 7) agarose slab gels stained with ethidium bromide. Lanes: 1, total plasmid complement of *B. thuringiensis* var. *kurstaki* HD-1; 2 and 3, "large plasmid fraction" and "small plasmid fraction" from *B. thuringiensis* (see text), respectively; 4, pES1; 5, pBR322; 6, pES1 digested with *Sal* I; 7, λ DNA digested with *Hin*dIII. M_rs (× 10^{-6}) on the left refer to the contour lengths observed for *B. thuringiensis* var. *kurstaki* HD-1 plasmid DNA (14). M_rs (× 10^{-6}) on the right refer to the *Hin*dIII digest of λ DNA (39). The arrowhead on the right marks the origin for lanes 6 and 7.

crystal protein. This strain was designated ES12. A plasmid, pES1, was isolated from this strain (lane 4 of Fig. 1). After digestion with *Sal* I, which cuts pES1 once, the linearized plasmid had a mobility corresponding to M_r *ca.* 11×10^6 compared to *Hin*dIII-digested λ DNA (lanes 6 and 7 of Fig. 1) (39). When *E. coli* strain HB101 was transformed with this plasmid, all ampicillin-resistant transformant colonies were found to react with antibody directed to the crystal protein, whereas ampicillin-resistant transformants containing plasmid pBR322 did not react with the antibody (data not shown).

Evidence that pES1 is a recombinant plasmid consisting of vector and target DNA is shown in Fig. 2. The plasmid vector pBR322, the total *B. thuringiensis* plasmid complement, and the large plasmid fraction described above were electrophoresed in adjacent lanes of a 0.7% vertical agarose slab gel. DNAs on three gels identical to this were transferred to nitrocellulose for hybridization with ^{32}P-labeled fragments. Labeled pBR322 hybridized to pBR322 DNA and did not hybridize to *B. thuringiensis* plasmid DNA. The recombinant plasmid hybridized with pBR322 DNA as well as with both the 47- and 30-megadalton plasmids of *B. thuringiensis*. Because trace amounts of linear plasmid DNA are present in the large plasmid fraction (shown with an arrow in lanes 3 and 9 of Fig. 2), hybridization to this portion of the filter was expected. When the total plasmid complement of *B. thuringiensis* was used as a probe, hybridization occurred with all of the *B. thuringiensis* plasmids but not with the vector plasmid. The recombinant plasmid pES1 therefore contains pBR322 DNA as well as DNA homologous to both the 30- and 47-megadalton plasmids of *B. thuringiensis*.

Antigenic Analysis of the Recombinant *E. coli* Strain. Lanes 1–3 of Fig. 3 show that the antigen made by ES12, which reacted with crystal protein antibodies, had the same electrophoretic mobility as the *B. thuringiensis* crystal protein. Dissolved *B. thuringiensis* crystals and cell extracts of HB101 (pBR322) and ES12 were electrophoresed on a $NaDodSO_4$/polyacrylamide gel and treated with anti-crystal antibody and ^{125}I-protein A after transfer to nitrocellulose. The ES12 extract contained a polypeptide antigen having the same (or very similar) electrophoretic mobility as the dissolved *B. thuringiensis* crystals. This polypeptide antigen was missing from a similar extract of HB101 (pBR322) and was not detected when preimmune serum was substituted for anti-crystal antibody or when ^{125}I-protein A was used without prior antibody treatment (data not shown). Comparison of the bands shown in Fig. 3 (1 μg of crystal protein in lane 1) with the total amount of protein applied to lane 3 (100 μg) indicates that the crystal protein antigen accounts for a small amount of the protein (1% or less) in ES12. When the radioimmune detection of polypeptides was used to monitor the fractionation of ES12 extracts, it was found that a reducing agent plus a denaturant or an alkaline pH was required to solublize the crystal protein antigen; these conditions were also required to solublize *B. thuringiensis* crystals (40).

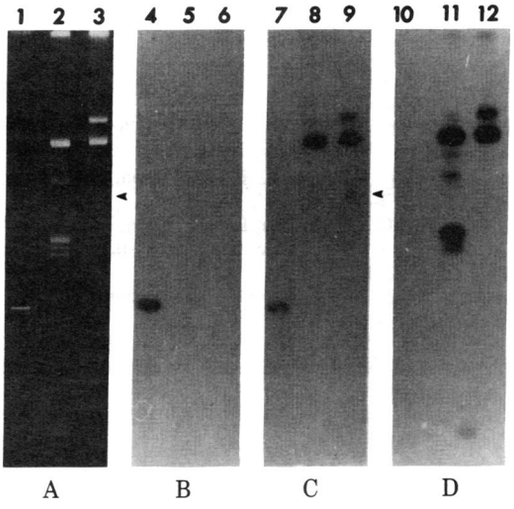

FIG. 2. Hybridization analysis of plasmid DNAs transferred to nitrocellulose. (*A*) Ethidium bromide-stained 0.7% agarose gel. Lanes: 1, *Bam*HI-digested pBR322 DNA; 2, total plasmid complement of *B. thuringiensis*; 3, "large plasmid fraction" from *B. thuringiensis*. (*B*) Autoradiogram of ^{32}P-labeled pBR322 DNA hybridized as follows. Lanes: 4, *Bam*HI-digested pBR322 DNA; 5, total plasmid complement; 6, "large plasmid fraction" from *B. thuringiensis*. (*C*) Autoradiogram of ^{32}P-labeled pES1 DNA hybridized as follows. Lanes: 7, *Bam*HI-digested pBR322; 8, total plasmid complement; 9, "large plasmid fraction" from *B. thuringiensis*. (*D*) Autoradiogram of ^{32}P-labeled total *B. thuringiensis* plasmid DNA hybridized as follows. Lanes: 10, *Bam*HI-digested pBR322 DNA; 11, total plasmid complement; 12, "large plasmid fraction" from *B. thuringiensis*.

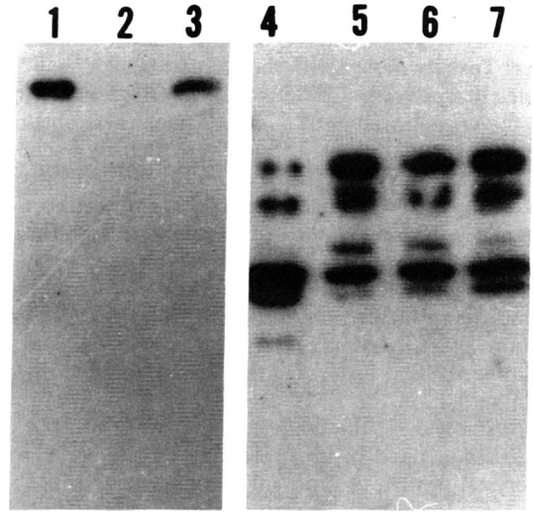

FIG. 3. Radioimmunoassay of crystal protein and proteins produced by ES12 before and after digestion with trypsin. Autoradiogram of a solid-phase radioimmunoassay for polypeptides (lanes 1–3) and tryptic polypeptide fragments (lanes 4–7) which react with anticrystal antibody after $NaDodSO_4$/polyacrylamide gel electrophoresis. Lanes: 1, 1 μg of *B. thuringiensis* crystal protein; 2, 100 μg of *E. coli* HB101 (pBR322) protein extract; 3, 100 μg of ES12 protein extract; 4, dissolved *B. thuringiensis* crystals treated with 5% (wt/wt) trypsin at pH 10 for 3 hr at room temperature; 5, 6, and 7, ES12 extract treated with 2% (wt/wt) trypsin (lane 5), 4% (wt/wt) trypsin (lane 6), or 6% (wt/wt) trypsin (lane 7) at pH 10 for 3 hr at room temperature.

It has been reported that the crystal protein can be digested by a number of proteases at pH 10 to produce primarily a single polypeptide (2). Lanes 4–7 of Fig. 3 show the results of an experiment in which dissolved *B. thuringiensis* crystals and an eluate from the particulate fraction of ES12 were subjected to partial digestion at pH 10 with the indicated amounts of trypsin. Digestion of the ES12 extract with increasing amounts of trypsin yielded a pattern that was similar to the pattern produced by trypsin digestion of the crystal protein of *B. thuringiensis*. Qualitatively, the patterns of the bands generated from the two preparations were similar. The quantitative (and minor qualitative) differences may reflect less-efficient digestion of the crystal protein antigen in ES12 extracts due to the presence of numerous other polypeptide species. The larger number of polypeptides produced by trypsin digestion of the crystal protein in these experiments, as opposed to the number reported by Lilley *et al.* (2), may be due to differences in the conditions of trypsin treatment. Similar experiments using subtilisin also showed agreement in the electrophoretic mobilities of the bands pro-

duced from the crystal protein of *B. thuringiensis* and the bands produced by digesting extracts of ES12 (data not shown).

Insect Toxicity Assays with the Recombinant *E. coli* Strain. Evidence that extracts of ES12 contain a biological activity similar to the crystal protein of *B. thuringiensis* was obtained by assaying for insect toxicity. Extracts of particulate fractions obtained from *E. coli* HB101 (pBR322) and ES12 were mixed with feed meal supplied to neonate caterpillars of tobacco hornworm. The results indicated that the extracts of the recombinant strain were toxic to caterpillars. The 15 larvae exposed to the ES12 extracts showed symptoms which in appearance and duration were indistinguishable from those of a group of larvae exposed to *B. thuringiensis* crystal protein at 17 μg/ml: all died before completing the first instar. On the assumption that the crystal protein antigen in the ES12 extracts was 0.5–1% of the total protein (see above), the feed meal prepared with the extract from ES12 contained 12–25 μg of crystal protein per ml whereas pure crystal protein at 2 μg/ml is sufficient to achieve 100% killing of the larvae (38). The minimal amount of extract of ES12 required to kill the larvae has not yet been determined. Larvae exposed to an equivalent amount of extract from *E. coli* HB101 (pBR322) showed no deleterious effects on growth or development through at least the third instar compared with larvae grown on feed meal without any bacterial extract added. Identical results were obtained when this experiment was repeated with another set of extracts from ES12 and *E. coli* HB101 (pBR322).

DISCUSSION

A strain of *E. coli*, ES12, has been isolated which carries a recombinant plasmid and produces a protein antigen that reacts with antibodies specific for the crystal protein of *B. thuringiensis*. The recombinant plasmid isolated from this strain, pES1, simultaneously transformed cultures of *E. coli* strain HB101 to both ampicillin resistance and the production of the crystal protein antigen, whereas the plasmid vector pBR322 transformed HB101 to ampicillin and tetracycline resistance, but no crystal antigen could be detected. When identified with anti-crystal antibodies, the intact polypeptide and the tryptic peptide fragments from ES12 had electrophoretic mobilities similar to those of the crystal protein and tryptic fragments of the crystal protein. Extracts of the recombinant strain, but not extracts of *E. coli* containing the cloning vector, were toxic to neonate larvae of tobacco hornworm. The results suggest that the DNA insert of the recombinant plasmid pES1 encodes the ability to make a polypeptide possessing properties similar to those of the crystal protein of *B. thuringiensis*. *In toto* these results suggest strongly that the DNA insert of pES1 contains the gene for the crystal protein of *B. thuringiensis* and that this gene is expressed in a biologically active form in *E. coli*. Because the DNA insert of pES1 consists of *B. thuringiensis* plasmid DNA, our results provide positive evidence that the gene encoding the crystal protein of *B. thuringiensis* var. *kurstaki* HD-1 is located on a large plasmid.

The recombinant plasmid pES1 consists of the plasmid vector pBR322 and DNA homologous to both the 30- and 47-megadalton plasmids of *B. thuringiensis*. One explanation for this result is that two *Sau* 3A1 partial digest fragments, one from each of the large *B. thuringiensis* plasmids, were ligated together and then jointly ligated into the vector. An alternate explanation is that the 30- and 47-megadalton plasmids share a region of homology and that this region was included in a single, large *Sau* 3AI partial digestion fragment containing the gene for the crystal protein. In preliminary studies, when restriction digests of pES1 and the 30-megadalton plasmid of *B. thuringiensis* were compared, at least one fragment was found having a common electrophoretic mobility (unpublished data) whereas another restriction fragment from pES1 DNA hybridized exclusively to the 47-megadalton plasmid (J. W. Kronstad, personal communication). The location of the crystal protein gene both within the insert and within the *B. thuringiensis* plasmid population will be described elsewhere.

It has been proposed that the gene for the crystal protein is under sporulation control and that it is transcribed by a form of RNA polymerase not found in vegetative cells of *B. thuringiensis* (13). Yet we have shown that this gene is expressed in *E. coli*, for which no similar RNA polymerase modification is expected. However, it is not known whether transcription in *E. coli* originates at the promoter for the crystal protein gene (which may require a modified polymerase in *B. thuringiensis*) or at an adventitiously placed promoter either in the inserted DNA or on the plasmid vector, which can be recognized by the *E. coli* RNA polymerase. The fact that ES12 makes only small amounts of the crystal protein, compared to the amount made by *B. thuringiensis*, indicates that these organisms may regulate the production of this protein differently.

Subcloning and deletion mapping of the recombinant plasmid would be expected to provide a means of investigating which promoter is being used for transcription of the gene in the recombinant strain as well as to produce a DNA fragment containing the authentic crystal protein gene promoter so that the "modified polymerase hypothesis" can be tested.

We thank Dr. Arthur Aronson for helpful discussions and Dr. Michael Lovett for his useful advice on the radioimmunologic techniques. This research was supported by U.S. Public Health Service Grant GM-20784 from the National Institute of General Medical Science. H.E.S. was supported by Developmental Biology Training Program Grant HDF-002666 from the National Institute of Child Health and Human Development; H.R.W. is a recipient of Research Career Award K6-GM-443 from the National Institute of General Medical Sciences.

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