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Cloning and expression of the crystal protein genes from *Bacillus thuringiensis* strain *berliner* 1715

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From a clone bank of the entire genome of *Bacillus thuringiensis*, one clone that contains a plasmid (pBT 15-88) harboring a sporulation gene was identified by molecular hybridization. This gene, identified as the crystal protein gene, occurs both on a large host plasmid DNA and in the chromosomal DNA in *B. thuringiensis* strain *berliner* 1715. The inserted sequence of pBT 15-88, which corresponds to the chromosomal sequence, was not expressed in *Escherichia coli*. In *B. thuringiensis* (*kurstaki*), the crystal gene was found only on a large host plasmid while in *B. thuringiensis* (*dendrolimus*), it is only on the chromosomal DNA. The plasmid crystal gene was cloned by ligation of a 14-kb *Bam*HI fragment of a host plasmid DNA of 42 megadaltons from strain *berliner* 1715 into the *Bam*HI site of the bifunctional vector pHV33. In *E. coli* and in sporulating *B. subtilis* the plasmid pBT 42-1 coded for a polypeptide, detected by antibodies against the crystal protein, with the same electrophoretic mobility as the crystal protein of *B. thuringiensis*. The crystal gene was not expressed in vegetative cells of *B. subtilis*, suggesting that the control at the transcriptional level is the same in *B. subtilis* and in *B. thuringiensis*. Protein extracts from the clones harboring the hybrid plasmid are toxic for the larvae of *Pierris brassicae* and the protein antigen forms cytoplasmic inclusion bodies in *E. coli* and *B. subtilis*, which are visible under the light microscope.

Key words: cloning/crystal genes/gene location/heterospecific expression/toxicity

Introduction

Bacterial sporulation is a simple form of cellular differentiation occurring in prokaryotes (Sonensheim and Campbell, 1978). During sporulation new RNA sequences are transcribed whereas some vegetative mRNAs cease to be synthesized (di Cioccio and Strauss, 1973), supporting the assumption that changes in RNA polymerase specificity are responsible for activating the sporulation genes (Haldenwang *et al.*, 1981).

The spore-forming bacterium *Bacillus thuringiensis* produces a parasporal proteinaceous crystal that represents a large part of the proteins synthesized after sporulation phase t_2. This organism is therefore particularly suitable for studying the activation of a specific sporulation gene. The mRNA coding for the crystal protein is relatively stable (half-life ~10 min) while during the vegetative phase only short-lived mRNAs (half-life ~2 min) are present (Glatron and Rapoport, 1972; Petit-Glatron and Rapoport, 1976). Two forms of RNA polymerase exist during *B. thuringiensis* sporulation; they differ from one another and from the vegetative enzyme in subunit composition and in some catalytic properties (Klier *et al.*, 1973, 1974; Rain-Guion *et al.*, 1976). The transcription products of one of them (form II) are largely homologous to the relatively stable mRNAs (Klier and Lecadet, 1976) and include the mRNA coding for the parasporal protein (Klier *et al.*, 1978).

A clone bank in *Escherichia coli* representative of the entire genome of *B. thuringiensis* has been constructed and used to map the ribosomal gene clusters (Klier *et al.*, 1979). Here we report identification of clones that contain hybrid plasmids carrying DNA sequences expressed only during the sporulation phase. One of them carries the chromosomal crystal gene, and the conditions of its transcription *in vivo* are reported. Schnepf and Whiteley (1981) have shown that the crystal protein gene is localized on a large host plasmid in strain *kurstaki*. Using the cloned chromosomal crystal gene as a probe, we have detected the crystal gene on a large host plasmid, ranging from 40 to 60 megadaltons (Md), in several strains of *B. thuringiensis*; however, in other strains (*berliner* 1715, *subtoxicus*) this gene is also located on the chromosomal DNA, and in one case (strain *dendrolimus*), only on the chromosomal DNA. The crystal protein gene derived from a large host plasmid of 42 Md from strain *berliner* 1715 was introduced into *E. coli* and *B. subtilis* using pHV33 as a cloning vector. The gene is subject to the same transcriptional control in *B. subtilis* and in *B. thuringiensis* during sporulation. The recombinant strains synthesize a polypeptide of 130 000 daltons which reacts specifically with antibodies directed against the crystal protein. The polypeptide forms cytoplasmic inclusion bodies in *E. coli* and *B. subtilis* and is toxic for the larvae of *Pierris brassicae*.

Results

Identification of hybrid plasmids carrying sporulation sequences

A bank of *E. coli* (SK1592) clones containing *B. thuringiensis* DNA was prepared using pBR322 as a cloning vector.

The clones, except those harboring *B. thuringiensis* rRNA genes, were pooled into groups of 50 and the plasmids extracted (Birnboim and Doly, 1979). A pre-hybridization step was carried out with an excess of non-labeled vegetative RNA. The nitrocellulose sheets were then hybridized with *in vitro* labeled RNAs from the *B. thuringiensis* mutant E_2, harvested during the sporulation phase (t_8), in the presence of non-labeled vegetative RNA. Twelve groups of clones gave a positive signal. The positive clones in each group were then identified by the procedure described by Grunstein and Hogness (1975). Twelve clones were identified containing sequences that begin to be transcribed during the first hours of sporulation. The plasmids extracted from the 12 positive clones were next hybridized with RNA fractions labeled *in vitro* and enriched in stable mRNA species. Four clones reacted strongly: they contained the plasmids pBT 3-47, pBT 15-88, pBT 22-84, and pBT 22-90.

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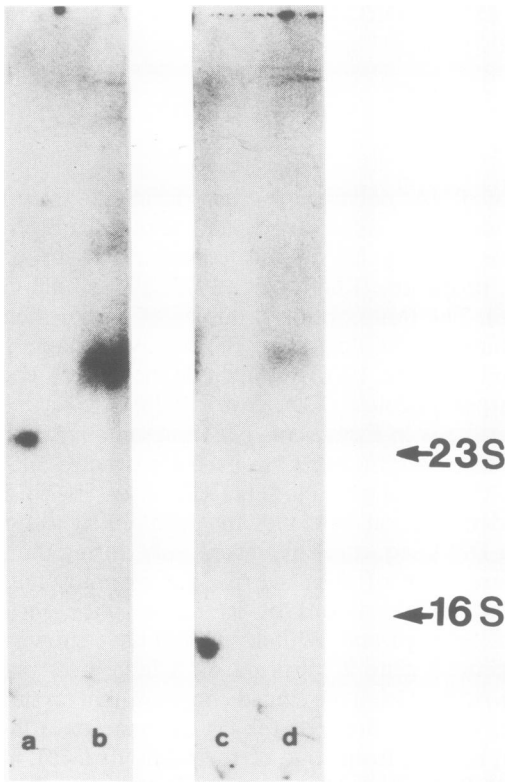

Fig. 1. Identification of the mRNA complementary to the inserted DNA sequence of pBT 15-88. 50 μg of RNA extracted from *B. thuringiensis* vegetative cells (**a**), sporulating cells harvested at t_8 (**b**) and t_{12} (**d**), and from the Sp^+ Cr^- mutant Cr_2 (**c**) were separated by electrophoresis, blotted onto DBM-paper, and hybridized with nick-translated pBT 15-88 DNA (7 x 10^6 c.p.m.). After extensive washing the filters were autoradiographed for 2 days.

We hybridized nick-translated plasmid DNAs to total RNA from *B. thuringiensis* strain *berliner* 1715. Figure 1 shows the result of a typical experiment performed with nick-translated plasmid pBT 15-88. The only positive mRNA appears to be ~26S. This mRNA could code for a protein of ~150 000 daltons, which is close to the mol. wt. of the crystal subunit (130 000 daltons). No hybridization was found with RNA extracted from the vegetative cells or from the sporulating cells of the Cr^- mutant Cr_2. Two other plasmids, pBT 3-47 and pBT 22-90, gave the same results, and their inserted DNA sequences cross-hybridized with the inserted DNA of pBT 15-88. The fourth plasmid pBT 22-84 allowed the identification of a 16S mRNA also specific to sporulating cells.

To identify the stage of activation of these two sporulation genes, we studied the appearance of the 16S and the 26S mRNAs in RNA preparations extracted at different stages in the growth of *B. thuringiensis*. The 16S mRNA appears around t_2 while the 26S species appears around t_3 (stage III) and is synthesized until the end of the sporulation process. The 26S mRNA species is quite abundant around t_8 (data not shown). The kinetics of appearance and the relative amount of the 26S mRNA resemble those of the crystal protein mRNA (Glatron and Rapoport, 1972). Of the three plasmids that hybridized with the 26S mRNA, pBT 15-88 is ~20 kb corresponding to an inserted DNA of 15 kb. Most of the following work was done with this plasmid.

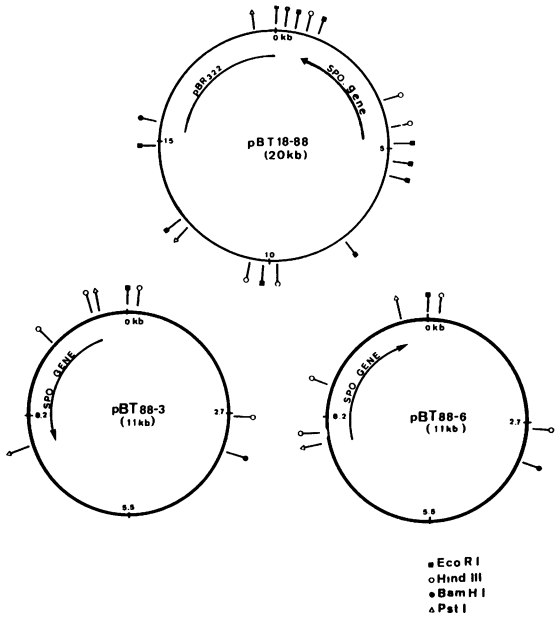

Fig. 2. Restriction maps of the recombinant plasmids pBT 15-88, pBT 88-3, and pBT 88-6. pBT 88-3 and pBT 88-6 have been prepared by insertion of the 4-kb *Eco*RI fragment of pBT 15-88 into the *Pst*I site of pHV33.

Characterization of the hybrid plasmid pBT 15-88

The restriction map of the plasmid pBT 15-88 (Figure 2) was established by the method of Smith and Birnstiel (1976). The coding sequence was localized by hybridization with RNAs extracted from sporulating cells and labeled *in vitro*. The sporulation gene is included in a 4-kb *Eco*RI fragment (Figure 2), whose size agrees well with that of the corresponding mRNA (26S). Two *Hind*III sites are located in this sequence, and we used a 0.15-kb fragment produced by digestion with *Hind*III and *Eco*RI to determine the orientation of transcription.

The strands of the 0.15-kb fragment were separated by acrylamide gel electrophoresis (Figure 3) and blotted to diazobenzyloxymethyl (DBM)-paper. Hybridization with labeled sporulating mRNAs revealed the slow migrating band as the DNA coding strand. The 0.15-kb fragment labeled only at the 5′ end, corresponding to the *Eco*RI site, was obtained by *Hind*III digestion of the 4-kb *Eco*RI fragment labeled at both 5′ ends by polynucleotide kinase. Figure 3 shows that the labeled strand was the fast migrating band, indicating that the ^{32}P-labeled 5′ end of this strand corresponds to the 5′ *Eco*RI segment of the 0.15-kb fragment; this establishes the direction of transcription as shown in Figure 2.

Identification of the B. thuringiensis sporulation gene

The loss of plasmid DNA correlates with the generation of the acrystalliferous phenotype (Gonzales *et al.*, 1981; Stahly *et al.*, 1978). Additionally, Schnepf and Whiteley (1981) have shown that the *B. thuringiensis* crystal protein gene is located on a large host plasmid in strain *kurstaki* HD-1. The 4-kb *Eco*RI fragment of the hybrid plasmid pBT 15-88 was used to determine the relationship between the cloned sporulation gene and the crystal gene (Figure 4). There was very strong hybridization between this fragment and large *B. thuringiensis* host plasmids from strains *berliner* and *kurstaki*, probably because the crystal proteins from both strains are closely

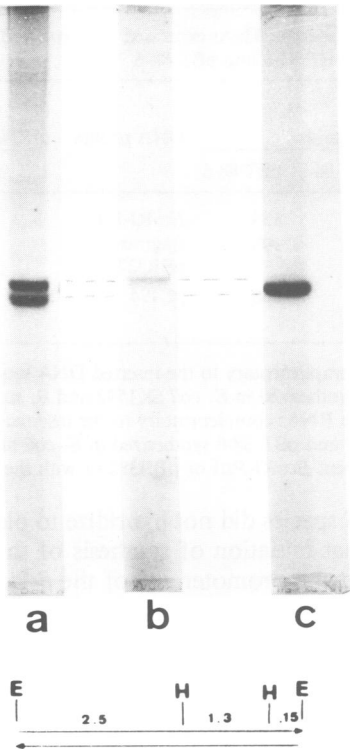

Fig. 3. Determination of the coding strand of the sporulation DNA sequence. The 0.15-kb fragment obtained by double digestion of pBT 15-88 by *Hind*III and *Eco*RI was labeled by nick-translation, denatured by sodium hydroxide, and submitted to 8% acrylamide gel electrophoresis (**a**). The same non-labeled fragment was submitted to the same treatment, then blotted onto DBM-paper and hybridized to [^{32}P]RNA extracted from the E_2 mutant harvested at t_8 (**b**). The 4-kb fragment obtained by digestion of pBT 15-88 by *Eco*RI, was labeled by polynucleotide kinase, then digested by *Hind*III. The resulting 0.15-kb fragment (**c**) was purified and submitted to the same denaturation treatment as (**a**).

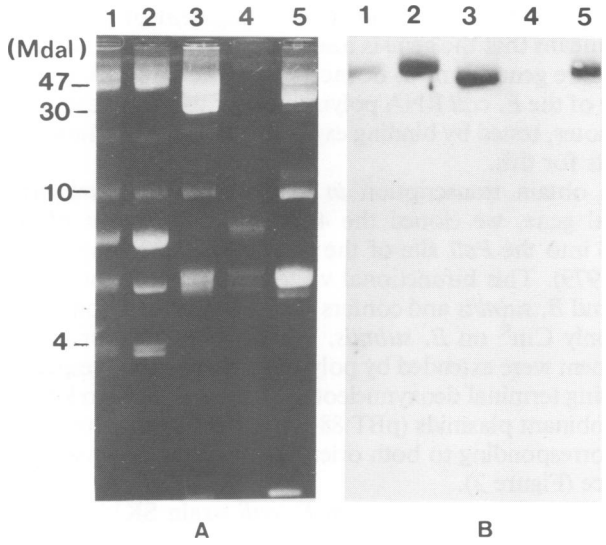

Fig. 4. Relationships between the isolated sporulation gene and the *B. thuringiensis* host plasmids. **A.** Electrophoretic patterns of *B. thuringiensis* plasmids extracted from the following strains: **lane 1**: *berliner* 1715 Cr$^-$ mutant; **lane 2**: *berliner* 1715 wild-type; **lane 3**: *kurstaki* HD_1 wild-type; **lane 4**: *kurstaki* HD_1 Cr$^-$ mutant; **lane 5**: *kurstaki* HD_1 wild-type. **B.** Hybridization of the corresponding transfer with the labeled 4-kb *Eco*RI fragment of pBT 15-88 used as a probe.

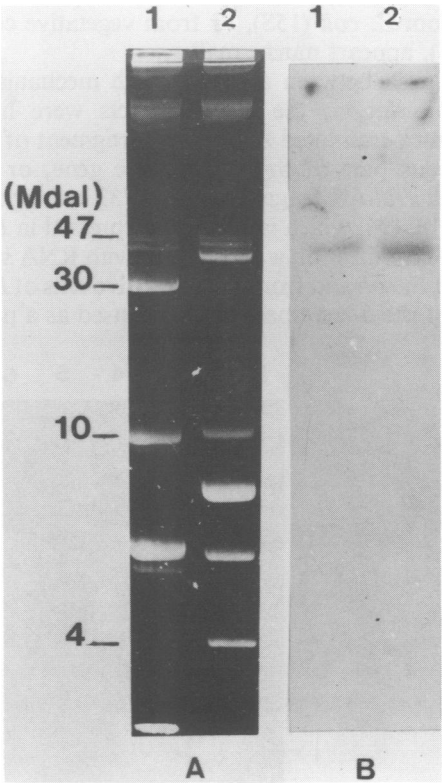

Fig. 5. Comparison of the plasmid from the *B. thuringiensis* strains *berliner* 1715 and *kurstaki* HD_1. **A.** Electrophoretic patterns of plasmids from strain *berliner* 1715 (**lane 1**) and *kurstaki* HD_1 (**lane 2**). **B.** Hybridization of the corresponding transfer with the labeled 4-kb *Eco*RI fragment of pBT 15-88 used as a probe.

related (Tyrell *et al.*, 1981). No hybridization was found with plasmids extracted from a Cr$^-$ mutant of strain *kurstaki*. In this case, a plasmid of 47 Md disappeared in the extract (Figure 4). The plasmid that hybridized with the 4-kb *Eco*RI fragment has a similar size in wild-type strains *berliner* 1715 (42 Md) and *kurstaki* (47 Md) (Figure 5). In a Cr$^-$ mutant of strain *berliner* 1715 (C_3), the Cr$^-$ phenotype was not associated with the loss of the 42-Md plasmid DNA, since the plasmid pattern was not changed, and a weak hybridization reaction was observed (Figure 4).

Similar experiments using the same probe suggest that in several other strains (*sotto, subtoxicus, tolworthi*) the crystal protein gene is also localized on a large plasmid ranging from 40 to 60 Md, but it seems that this is not the case for other strains (*aizawa, dendrolimus*) (data not shown). In reciprocal experiments with either the purified 47-Md plasmid DNA of strain *kurstaki* or the 42-Md plasmid DNA of strain *berliner* 1715 as probes, a specific and strong hybridization reaction was observed with the 4-kb *Eco*RI fragment of pBT 15-88, but not with the other regions of the inserted DNA (data not shown).

These results and those above provide good evidence that the cloned sporulation gene corresponds to the *B. thuringiensis* crystal gene.

In vivo expression of the B. thuringiensis crystal gene cloned in pBT 15-88

The plasmid pBT 15-88 was used to transform the minicell-producing strain *E. coli* AR1062; however, no polypeptide corresponding to the inserted DNA sequence was observed (not shown). Moreover, *in vivo*-labeled RNAs extracted from *E. coli* AR1062 transformed with the plasmid pBT 15-88 do

not hybridize with the 4-kb *Eco*RI fragment of pBT 15-88. This means that the gene is not expressed in *E. coli*, although the entire gene seems to be included in the insert. A poor affinity of the *E. coli* RNA polymerase for the sporulation gene promoter, tested by binding experiments (data not shown) accounts for this.

To obtain transcription *in vivo* of the *B. thuringiensis* crystal gene, we cloned the 4-kb *Eco*RI fragment of pBT 15-88 into the *Pst*I site of the plasmid pHV33 (Rapoport *et al.*, 1979). This bifunctional vector can replicate both in *E. coli* and *B. subtilis* and confers Ap^R Tc^R and Cm^R on *E. coli*, but only Cm^R on *B. subtilis*. The linearized vector and the fragment were extended by poly(dG) and poly(dC) respectively, using terminal deoxynucleotidyl transferase. Two kinds of recombinant plasmids (pBT 88-3 and pBT 88-6) were obtained, corresponding to both orientations of the sporulation sequence (Figure 2).

RNAs were extracted from *E. coli* strain SK1592 and *B. subtilis* strain QB666 after transformation with these two hybrid plasmids. They were hybridized to the nick-translated 4-kb *Pst*I fragment of pBT 88-3 (see Table Ia). The same gene sequence is transcribed in three different ways depending on the strains and the state of the cells. The size of the transcripts obtained is about the same with both plasmids. The mRNA which hybridizes to the probe in *B. subtilis* sporulating cells has the same size as that extracted from *B. thuringiensis* sporulating cells (26S). However, the corresponding mRNA extracted from *E. coli* (15S), or from vegetative cells of *B. subtilis* (19S), appears much smaller.

To distinguish between a readthrough mechanism and a specific transcription, the same extracts were hybridized either with nick-translated *Eco*RI-*Pst*I fragment of pBR322, which contains part of the β-lactamase gene, or with the labeled small *Hind*III fragment of pHV33 corresponding to the plasmid pC194, which is normally expressed in *B. subtilis* (Table Ib). Hybridization was observed with RNA species extracted from *E. coli* and from the vegetative cells of *B. subtilis* when part of the β-lactamase gene was used as a probe, but

Table I. Properties of specific RNAs expressed in *E. coli* and *B. subtilis* upon transformation with pBT 83-3 and pBT 88-6

Cells	(a) Plasmids		DNA probes	(b) mRNA species		
	pBT 88-3	pBT 88-6		15S	19S	26S
E. coli	15S	15S	*Eco*RI-*Pst*I	+	+	–
B. subtilis vegetative cells	19S	19S	fragment of pBR322			
B. subtilis sporulating cells	26S	26S	pC194	–	–	–

[a]Size of the RNAs complementary to the inserted DNA sequences of pBT 88-3 and pBT 88-6 synthesized in *E. coli* SK1592 and *B. subtilis* QB666.
[b]Hybridization of the RNAs complementary to the inserted DNA sequences of pBT 88-3 and pBT 88-6 synthesized in *E. coli* and in *B. subtilis* with the DNA fragment *Eco*RI-*Pst*I of pBR322 or with the plasmid pC194.

the 15S and 19S species did not hybridize to plasmid pC194. This suggests that initiation of synthesis of these two RNA species occurs at the promoter site of the β-lactamase gene. To explain the size of the mRNAs observed we assume that transcription is prematurely terminated. No hybridization was found between either of the probes and the 26S mRNA from sporulating *B. subtilis*, indicating that initiation of transcription occurs at a specific promoter site in the sporulation gene. This means that a promoter site of a spore-forming bacterium can act, although with a low efficiency, in another sporulating bacterium. However, a polypeptide related to the crystal protein has not been found, suggesting that the mRNA is not translated.

Origin of the crystal gene cloned in pBT 15-88

The purified 4-kb *Eco*RI fragment of pBT 15-88 hybridizes with the chromosomal DNA of strains *berliner* and *subtoxicus* even after extensive washings in stringent conditions (0.01 SSC). This probe was then hybridized with *Hind*III digests of the host plasmids from strains *berliner* 1715 and *kurstaki* HD1 (Figure 6). Two reactive bands were detected in the

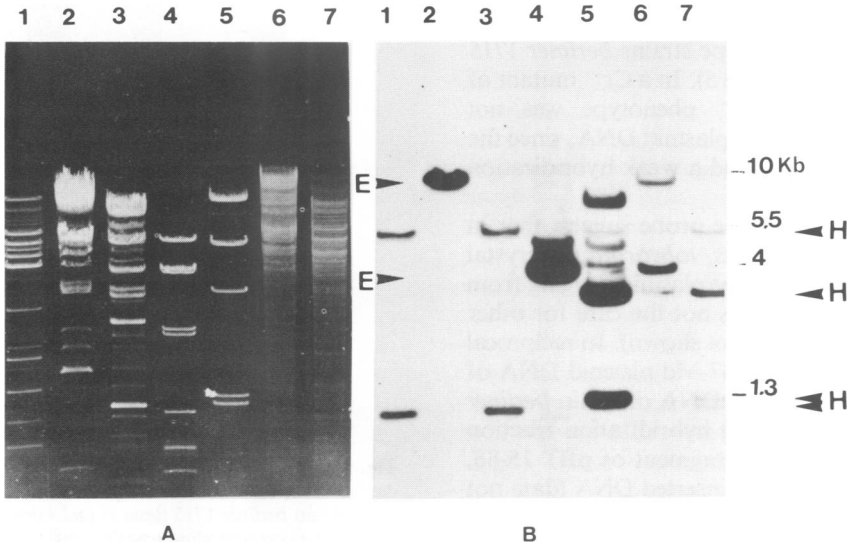

Fig. 6. Identification of the restriction fragments from plasmid and chromosomal DNAs harboring crystal DNA sequence. **A**: electrophoretic pattern of *Eco*RI or *Hind*III digests: **lane 1**: *B. thuringiensis* strain *kurstaki* plasmids (*Hind*III); **lane 2**: *B. thuringiensis* strain *berliner* 1715 plasmids (*Eco*RI); **lane 3**: *B. thuringiensis* strain *berliner* 1715 plasmids (*Hind*III); **lane 4**: recombinant plasmid pBT 15-88 (*Eco*RI); **lane 5**: recombinant plasmid pBT 15-88 (*Hind*I); **lane 6**: bulk DNA from *B. thuringiensis* strain *berliner* 1715 (*Eco*RI); **lane 7**: bulk DNA from *B. thuringiensis* strain *berliner* 1715 (*Hind*III); **B**: Hybridization of the corresponding transfer with the 4-kb *Eco*RI fragment of pBT 15-88 used as a probe. The arrows indicate the position of the *Eco*RI (E) or *Hind*III (H) fragments which are discussed in the text.

*Hind*III digest of the plasmid DNA from both strains (lanes 1 and 3), showing the same electrophoretic mobility. This strongly suggests that the gene sequences are closely related in both strains. Other experiments using the same probe were carried out with *Eco*RI or *Hind*III digests of the host plasmids, and of total DNA from strain *berliner* 1715, in parallel with digests of plasmid pBT 15-88 (Figure 6). The reactive bands of the *Eco*RI (lane 4) or *Hind*III (lane 5) digests of pBT 15-88 were not recovered in *Eco*RI (lane 2) or *Hind*III (lane 3) digests of the host plasmids of strain *berliner* 1715. The size of the detected bands was somewhat different. However, the two kinds of bands were found in the *Eco*RI (lane 6) or *Hind*III (lane 7) digests of strain *berliner* 1715 total DNA, which contains both chromosomal and plasmid DNAs.

We propose that the crystal gene is localized on a large host plasmid in most cases and that it is also present, at least in strains *berliner* 1715 and *subtoxicus* (not shown), on the chromosome. The restriction patterns suggest further that the inserted DNA of pBT 15-88 corresponds to the chromosomal sequence.

Cloning of the crystal protein gene from host plasmids of B. thuringiensis strain berliner 1715

We cloned the crystal gene derived from the host plasmid into the bifunctional vector pHV33. Using the 4-kb *Eco*RI fragment as a probe, we located the crystal gene on a 14-kb *Bam*HI DNA fragment from a 42-Md host plasmid in strain *berliner* 1715, and we cloned this fragment into the *Bam*HI site of pHV33. The ampicillin-resistant transformants of *E. coli* were screened for sensitivity to tetracycline and then for insertion of the 14-kb *Bam*HI DNA fragment, using the 4-kb *Eco*RI fragment as a probe. Only two colonies gave a positive result out of ~100 Tc^S clones. The plasmids isolated from these clones were shown to be identical by restriction digestions and were named pBT 42-1.

After digestion with *Bgl*II or *Sal*I, which cuts pBT 42-1 once, the linearized plasmid has a mobility corresponding to ~21 kb. Figure 7 shows the restriction map of the plasmid DNA. The different digests were blotted onto nitrocellulose filters and hybridized with the 4-kb *Eco*RI fragment. The related DNA sequence is located on a fragment delimited by two *Sst*I sites, and more precisely by a *Sst*I and *Pst*I site. The size of the latter DNA fragment (3.5 kb) agrees well with the size of the probe and with the size of the corresponding polypeptide chain (130 000 daltons).

Experiments using *Sst*I digests of host plasmids isolated from different *B. thuringiensis* strains (*berliner* 22105, *kurstaki* HD1, *subtoxicus*) suggest that the crystal gene is also included in a *Sst*I fragment of ~5–6 kb (data not shown).

Expression of the crystal protein gene in E. coli and B. subtilis

To prove that the 14-kb *Bam*HI DNA fragment contains the crystal gene, we tested extracts from clones containing the recombinant plasmid. Figure 8 shows the result of a typical double immuno-diffusion experiment using extracts of *E. coli* containing either the plasmid vector pHV33, or the plasmid pBT 42-1 and extracts from *B. thuringiensis* strain *berliner* 1715. A sample of purified crystal dissolved in thioglycollic acid was the control. An immunoprecipitate band was observed in all cases with rabbit antibodies directed against the crystal protein except with extracts from *E. coli* containing the plasmid vector alone. Clearly the *E. coli* cells harboring pBT 42-1 synthesized a polypeptide, related to the crystal

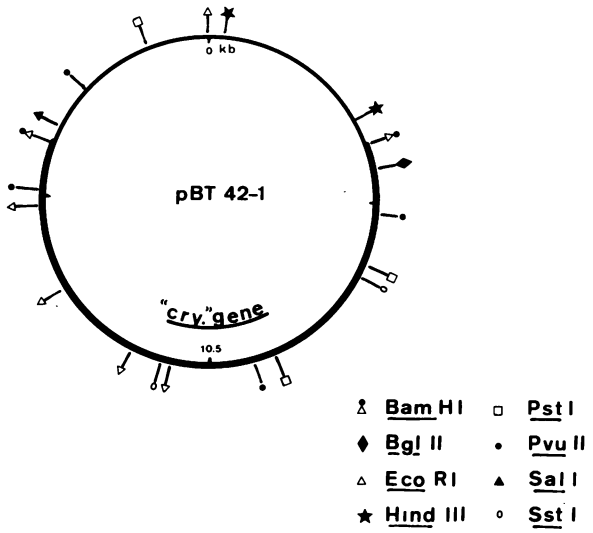

Fig. 7. Restriction map and localization of the coding sequence of the hybrid plasmid pBT 42-1.

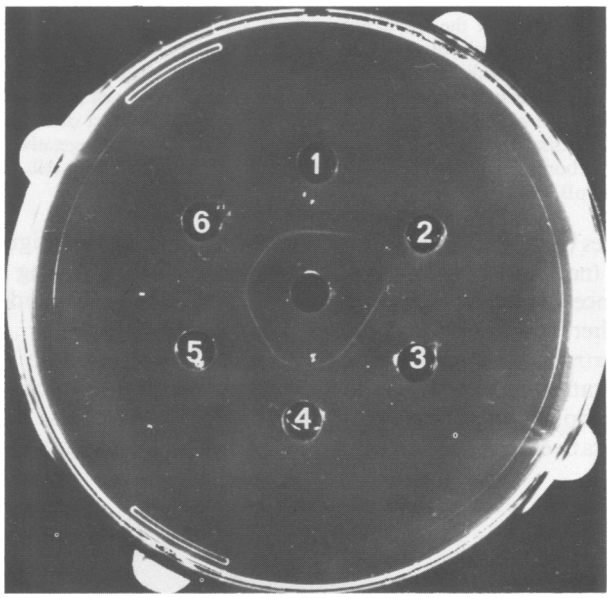

Fig. 8. Detection of the crystal antigen in *E. coli* HB101 by double immunodiffusion. The center well contains *B. thuringiensis* strain *berliner* 1715 crystal antibodies; **wells 1, 3,** and **5.** *B. thuringiensis* crystal antigens; **well 2:** extract of *E. coli* harboring pHV33; **well 4:** extract of *E. coli* harboring pBT 42-1; **well 6:** extract of *B. thuringiensis* strain *berliner* 1715.

protein, in amounts of ~10% of that synthesized by sporulating cells of *B. thuringiensis*.

Five-fold concentrated extract of *E. coli* harboring either pHV33 or pBT 42-1 or from *B. thuringiensis* strain *berliner* 1715 were spread on cabbage leaves. These leaves were fed *ad libitum* to the larvae of *P. brassicae*. Twenty-five larvae exposed to the extracts of *E. coli* containing pBT 42-1 showed symptoms that were indistinguishable in appearance and duration to those observed with a control group of larvae exposed to *B. thuringiensis* extracts. The larvae exposed to an equivalent extract from *E. coli* containing the vector pHV33 alone produced no deleterious effect on growth and development (Table II).

The plasmid pBT 42-1 was transformed into competent cells of *B. subtilis* strain QB666. The rate of transformation was relatively high (~10^5 transformants/μg DNA), but the 12

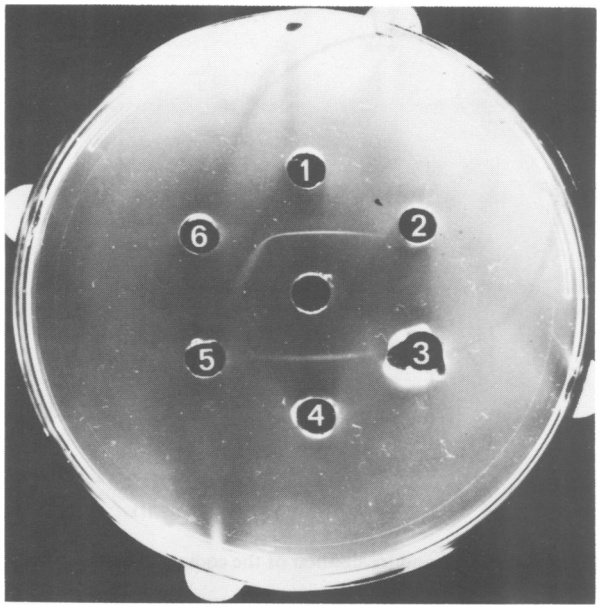

Fig. 9. Detection of the crystal antigen in *B. subtilis* QB666 by double immunodiffusion. The center well contains *B. thuringiensis* strain *berliner* 1715 crystal antibodies; **well 1**: *B. thuringiensis* crystal antigen; **well 2**: extract of *B. thuringiensis* vegetative cells harboring pHV33; **well 3**: extract of *B. subtilis* sporulating cells harboring pHV33; **well 4**: extract of *B. thuringiensis* strain *berliner* 1715; **well 5**: extract of *B. subtilis* vegetative cells harboring pBT 42-1; **well 6**: extract of *B. subtilis* sporulating cells harboring pBT 42-1.

clones examined contained plasmids smaller than the original one (not shown). Only four clones conserved the coding sequence of the crystal gene. One of them (BS_4) was studied further.

Extracts prepared from the BS_4 cells harvested in the vegetative or in sporulation phase were analyzed by double-diffusion immunoprecipitation (Figure 9). Extracts from vegetative cells did not contain a polypeptide recognized by

Table II. Insect toxicity assays

Extract	a	b	c
Dead larvae after 18 h	0	25	25

Five-fold concentrated extracts were prepared by sonication of 100 ml of stationary phase cultures of *E. coli* HB101 (pHV33) (a) or HB101 (pBT 42-1) (b) or *B. thuringiensis* strain *berliner* 1715 (c). About 1 ml was spread out on small cabbage leaves which were given to 25 larvae for each extract. The dead larvae were scored after 18 h free feeding.

antibodies directed against the crystal protein, whereas a related polypeptide was present in extracts of sporulating cells. The crystal gene is not expressed in vegetative cells and therefore is subjected in *B. subtilis* to the same control as in *B. thuringiensis*. The amount of crystal protein antigen produced by the sporulating cells of *B. subtilis* corresponds to ~10% of that synthesized by sporulating *B. thuringiensis*. With *B. subtilis* strain BD224, a Rec^- derivative of strain 168, only one transformed clone was obtained. Its plasmid was slightly deleted but contained the coding sequence of the crystal gene. Analysis of extracts from vegetative, or sporulating cells gave the same results as those obtained with the strain QB666.

The crystal protein and extracts of *E. coli* and *B. subtilis* cells harboring either pHV33 or pBT 42-1 were electrophoresed on a SDS-polyacrylamide gel, then transferred to nitrocellulose filters, treated with antibodies directed against the crystal protein and finally with [^{123}I]protein A (Figure 10). In extracts from *E. coli* and from sporulating cells of *B. subtilis* harboring pBT 42-1, several polypeptides gave a reaction with the antibodies and one had the same electrophoretic mobility (corresponding to a mol. wt. of 130 000 daltons) as the dissolved *B. thuringiensis* crystal. This polypeptide was missing in similar extracts from *E. coli* HB101 (pHV33), from *B. subtilis* BD224 (pHV33) vegetative or sporulating cells, and from *B. subtilis* BD224 (pBT 42-1) vegetative cells. Some

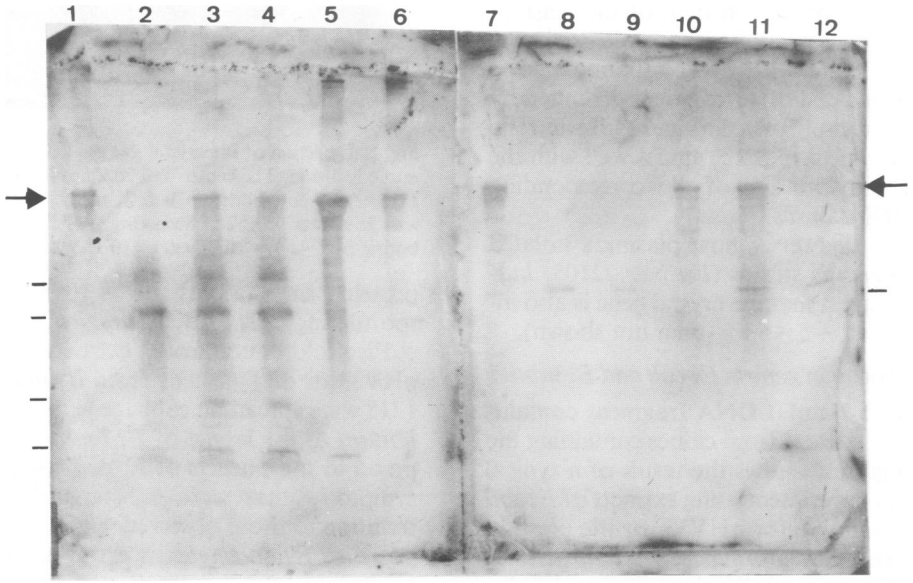

Fig. 10. Size of the polypeptide antigen. Autoradiogram of a solid phase radioimmunoassay for polypeptides reacting with crystal antibodies after SDS-polyacrylamide gel electrophoresis; **lanes: 1, 6,** and **7**: 1 μg of *B. thuringiensis berliner* 1715 crystal antigen; **lane 2**: 10 μg of *E. coli* HB101 (pHV33); **lanes 3** and **4**: 10 μg of *E. coli* HB101 (pBT 42-1); **lane 5**: 2 μg of *B. thuringiensis* sporulation cell extracts; **lane 8**: 12 μg of *B. subtilis* QB666 (pHV33) vegetative cells; **lane 9**: 12 μg of *B. subtilis* QB666 (pBT 42-1) vegetative cells; **lane 10**: 10 μg of *B. subtilis* QB666 (pBT 42-1) sporulating cells; **lane 11**: 15 μg of *B. subtilis* BD224 (pBT 42-1) sporulating cells; **lane 12**: 15 μg of *B. subtilis* BD224 (pBT 42-1) vegetative cells. The main arrows indicate the migration of the crystal subunit (130 000 daltons). Hyphens show the presence of non-specific polypeptides in *E. coli* and *B. subtilis* extracts.

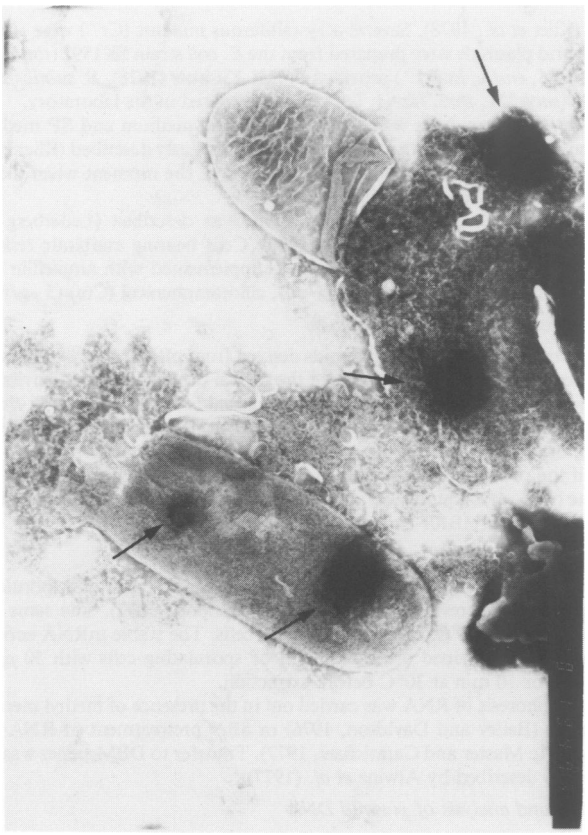

Fig. 11. Electron micrograph of *E. coli* producing the crystal antigen (negative staining of a sonicated late phase growth culture x 20 000). Typical inclusions are indicated by arrows.

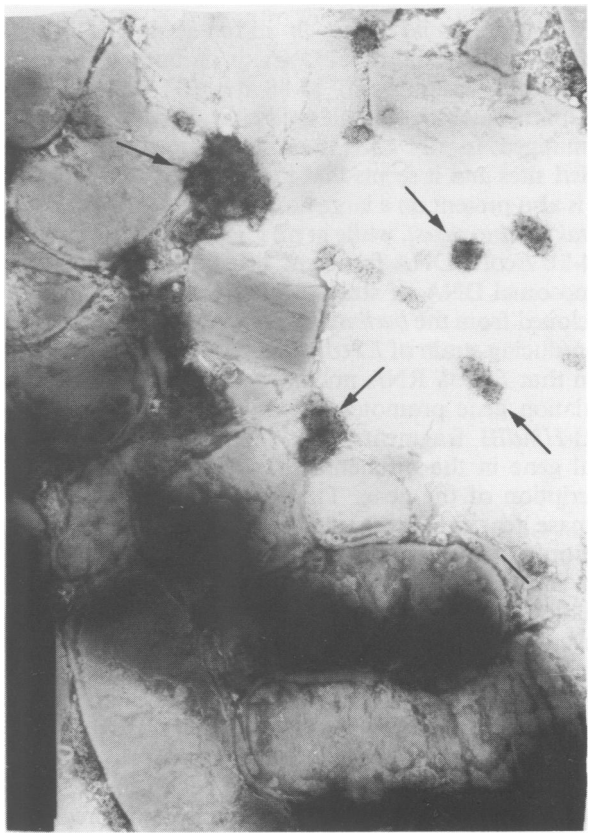

Fig. 12. Electron micrograph of *B. subtilis* sporulating cells producing the crystal protein (negative staining of a sonicated sporulating culture x 20 000). Typical inclusions are indicated by arrows.

other polypeptides of lower mol. wt. reacted with the antibodies, but these bands were also present in the extracts from strains containing the plasmid vector pHV33 alone. It should be noted that these polypeptides did not give precipitation lines in Ouchterlony tests, indicating that their affinity for the specific antibodies is low.

In *E. coli* containing pBT 42-1, light microscopy revealed a small dark body located often at one end of the cells, especially during the stationary phase. In *B. subtilis* cells harboring the plasmid, these inclusions were much smaller. Sonicated extracts from *E. coli* and from *B. subtilis* were examined by electron microscopy. Inclusion bodies of ~0.3 μm and 0.1 μm respectively were observed (Figures 11 and 12). About one body per cell was present in *E. coli*, whereas several were detected in *B. subtilis*. They did not have a crystal-like structure or regular organization. The inclusion bodies were purified from sonicated extracts of *E. coli*, by centrifugation in sucrose gradient (10–30%). Dissolved in thioglycollic acid they were tested with the specific antibodies. A strong immunoprecipitation band was detected by the Ouchterlony test, suggesting that the particles contain the polypeptide antigen coded by the plasmid pBT 42-1.

Discussion

From 2900 clones of *E. coli* containing inserted fragments of *B. thuringiensis* DNA in the vector pBR322, we have isolated a recombinant plasmid (pBT 15-88) harboring a sporulation-specific gene. This gene is expressed from stage t_3 until the end of sporulation. The corresponding mRNA has a size of ~26S and is absent from a Sp^+ Cr^- mutant. The sporulation gene hybridizes with a cloned DNA fragment derived from the 42-Md host plasmid of strain *berliner* 1715, which codes for the crystal protein. The cloned sequence of pBT 15-88, which corresponds to the crystal gene also hybridizes with one of the large host plasmids present in *B. thuringiensis* (strains *kurstaki, sotto, subtoxicus*). In some Cr^- mutants, no hybridization was detected between the cloned DNA sequence and the plasmid DNA fraction, and this correlated with the disappearance of the large plasmid containing the crystal gene. In strain *berliner* 1715, we also detected hybridization between the cloned sequence of pBT 15-88 and chromosomal DNA. Comparison of the restriction patterns, which are somewhat different for the plasmid and for the chromosomal sequences, indicates that the crystal gene is carried on the plasmid and the chromosome. Such a situation has already been reported for the exfoliative toxin of *Staphylococcus aureus*, which is coded for by two genes both of which are located on a plasmid or on the chromosomal DNA (Rogolsky, 1979). Comparison of restriction fragments suggests that the two crystal genes are closely related and in this connection it is interesting to note that in strain *berliner* 1715 it is difficult to isolate spontaneous Cr^- mutants. Similar evidence concerning the dual location of the crystal gene has also been obtained in strain *subtoxicus*. In other strains such as *kurstaki, sotto*, and *tolworthi*, the crystal gene is apparently present only on plasmid DNA, while in strain *dendrolimus* the gene is only found on the chromosome. These results make the suggestion that the crystal gene is included in a transposon-like structure attractive; if that is the

case, the gene could occur in different strains either on plasmid or on chromosomal DNA or on both.

The hybrid plasmids pBT 15-88 and pBT 42-1, containing the chromosomal and the plasmid crystal genes, respectively, were mapped. In pBT 42-1, the coding sequence is flanked by two *Sst*I sites and it seems that the resulting 6-kb DNA fragment is also present on a large host plasmid from other strains (*kurstaki, subtoxicus*), while in pBT 15-88 the gene is included in a 4-kb *Eco*RI DNA fragment, which is also found in the chromosomal DNA of strain *subtoxicus*. The chromosomal gene cloned from the *berliner* strain is not expressed in a mini-cell-producing strain of *E. coli*, and binding experiments have shown that *E. coli* RNA polymerase does not recognize the sporulation gene promoter, which is included in a 0.15-kb *Eco*RI-*Hind*III fragment. Subcloning of the chromosomal crystal gene in the bifunctional vector pHV33 allowed the transcription of the gene. Transcription began from the β-lactamase gene in *E. coli*, while in vegetative *B. subtilis* cells initiation of transcription probably takes place at the promoter site of the crystal gene. However, in no case has a translation product been detected.

Nevertheless, extracts of cells of *E. coli* containing the recombinant plasmid pBT 42-1 harboring the plasmid crystal gene were shown to be toxic for the larvae of *P. brassicae*. This result confirms that the DNA insert of pBT 42-1 codes for a polypeptide possessing properties similar to those of the *B. thuringiensis* crystal protein. The gene product was expressed in a biologically active form in *E. coli* and represented ~10% of that produced by *B. thuringiensis*. This amount was, however, sufficient to produce small inclusions that are visible under the light microscope but are not crystalline. The crystal protein is relatively insoluble and the formation of inclusions may result from simple precipitation and aggregation. A recent paper reported the presence of cytoplasmic inclusion bodies in *E. coli* cells synthesizing human insulin polypeptides (Williams *et al.*, 1982).

The hybrid plasmid pBT 42-1 was transformed into *B. subtilis* Rec^+ and Rec^- derivatives. The crystal protein gene is not expressed in vegetative cells but is in sporulating cells. In this case inclusions (smaller than those obtained in *E. coli*) were also found inside the cells. This suggests a similar control of transcription in *B. subtilis* and *B. thuringiensis*. The crystal gene is a sporulation gene which is transcribed only by a specific sporulation RNA polymerase in *B. thuringiensis* (Klier *et al.*, 1978). Therefore, it is tempting to conclude that the promoter sequence and the σ sporulation factor are related in both these sporulating bacteria. Although the gene is transcribed in *E. coli*, it is not known whether the transcription starts at the specific promoter of the gene, or at another promoter site in the vector recognized by the *E. coli* RNA polymerase. In both *E. coli* and *B. subtilis* the polypeptide chain is ~130 000 daltons, suggesting that, if the transcription starts in *E. coli* at a plasmid promoter, translation is correctly initiated. We do not know, however, why only the crystal gene of plasmid origin is translated in *E. coli* and *B. subtilis*. Further work is needed to determine how the two genes are differently regulated.

Materials and methods

Bacterial strains and transformation procedure

B. thuringiensis strains *berliner* 1715 and 22105, *kurstaki* (HD1), *subtoxicus, dendrolimus, tolworthi,* and *sotto* were used to prepare bulk and plasmid DNAs and rRNA. The asporogenous mutant E_2 (blocked at the end of stage III), which expresses the phenotype $Sp^- Cr^{++}$, was used to prepare total RNA (Klier *et al.*, 1978). Several acrystalliferous mutants (Cr^-) were also used. Hybrid plasmids were prepared from the *E. coli* strain SK1592 (*ton*A, *gal, thi, sbc*B15, *end*A, *hsd*M^+) provided by S.R.Kushner (1978). *B. subtilis* strain QB666 (*sac*A321, *thr*5, *his*A1, *leu*A8) was prepared in the laboratory.

E. coli and *B. subtilis* were grown in L liquid medium and SP medium, respectively, and *B. thuringiensis* in medium previously described (Klier *et al.*, 1973). Time zero of sporulation (t_0) is defined as the moment when the cell growth ceases to be exponential.

E. coli and *B. subtilis* were transformed as described (Lederberg and Cohen, 1974; Niaudet and Ehrlich, 1979). Cells bearing antibiotic resistant plasmids were selected on L-agar plates supplemented with ampicillin (Ap) (100 μg/ml), tetracycline (Tc) (15 μg/ml), chloramphenicol (Cm) (5 μg/ml).

Preparation of recombinant plasmids

The preparation of hybrid plasmids derived from pBR322 has been described (Klier *et al.*, 1979). The cloning of the crystal protein gene was carried out as follows: 2 μg of a *Bam*HI digest of the plasmids extracted from *B. thuringiensis* strain *berliner* 1715 and 1 μg of the *Bam*HI linearized vector pHV33 (Rapoport *et al.*, 1979) were incubated for 1 h at 20°C and then for 16 h at 8°C in the presence of 0.25 unit of T4 DNA ligase (Boehringer) in a final volume of 25 μl. Aliquots (2 μl) of the ligation mixture were used to transform the *E. coli* strain HB101 (r_k^- m_k^- $recA^-$).

Extraction of RNA

Total RNA was extracted from *B. thuringiensis* vegetative and sporulating cells as reported previously (Glatron and Rapoport, 1972). The same procedure was used for *E. coli* and *B. subtilis* cells. The stable mRNA enriched fractions were obtained after treatment of sporulating cells with 50 μg/ml rifampin for 10 min at 30°C before extraction.

Electrophoresis of RNA was carried out in the presence of methyl mercuric hydroxide (Bailey and Davidson, 1976) or after pretreatment of RNA with glyoxal (Mc Master and Carmichael, 1977). Transfer to DBM-paper was performed as described by Alwine *et al.* (1977).

Isolation and analysis of plasmid DNA

E. coli plasmid DNA was purified either from large volumes (1–2 l) of culture on hydroxyapatite according to Colman *et al.* (1978), or from small volumes according to Birnboim and Doly (1979). The *B. thuringiensis* plasmid DNA was also purified according to the rapid procedure with the following modification: sucrose (20%) was added to the lysis buffer to generate spheroplasts. The cell suspensions were incubated in a 37°C water bath for 60 min and the appearance of spheroplasts was monitored by phase contrast microscopy. The plasmids were further purified by CsCl gradient.

DNA fractions were analyzed by electrophoresis on horizontal 0.7 or 0.5% agarose slab gels. Electrophoresis was carried out in 89 mM Tris-89 mM boric acid 2.5 mM Na_2 EDTA, pH 8.3 for 16 h at 3 V/cm. Gels were stained with 1 μg/ml ethidium bromide and photographed under u.v. irradiation. Small DNA fragments were separated by vertical 8% acrylamide slab gel electrophoresis using the same buffer.

Good recovery of restriction fragments after electrophoresis was obtained using low melting agarose (Seaplaque, Marine Colloids Inc.). The heated gel slices were passed through a hydroxyapatite column and DNA was recovered after phosphate elution. Electro-elution of the DNA fragment in a dialysis bag was also used with a good yield.

Radioactive probes

The different RNA species were *in vitro* labeled using [γ-^{32}P]ATP (NEN 3000 Ci/mmol) and T4 polynucleotide kinase (Boehringer) as described by Maizels *et al.* (1976). Freshly prepared probe had a specific activity of ~5 x 10^6 c.p.m./μg RNA. DNA was labeled by nick-translation using *E. coli* DNA polymerase (B.R.L.) in the presence of [α-^{32}P]dCTP and [α-^{32}P]dATP (NEN 400 Ci/mmol) as described by Rigby *et al.* (1977). Labeling of the DNA at both 5′ ends was carried out according to Maxam and Gilbert (1980).

Colony hybridization

E. coli clones containing *B. thuringiensis* genes were detected as described by Grunstein and Hogness (1975). *In situ* hybridization reactions were performed as described (Klier *et al.*, 1979).

Blotting, hybridization, and autoradiography

The technique used was essentially that of Southern (1975) using Schleicher and Schull nitrocellulose filters BA85. Hybridization containing 40% formamide, 6 x SSC and 0.1% SDS. When denatured DNA probes were used, SDS was omitted but 2 x Denhardt's reagent (1966) was added. After hybridization the filters were extensively washed and autoradiographed (Kodak X Omat R).

SDS-polyacrylamide gel electrophoresis and immunodetection

The separation of the polypeptides was performed on a 7.5–15% exponential gradient gel according to O'Farrell (1975). Transfer of polypeptides

on nitrocellulose filters was done as described by Bowen *et al.* (1980). Reaction with crystal antibodies was carried out as follows: the filters were soaked in a solution containing the specific rabbit serum diluted 50-fold in 50 ml of 0.1 M Tris-HCl pH 7.5, 0.15 M NaCl, 0.25% bovine serum albumin (TSB) and incubated for 2 h at 37°C in the presence of 10 μCi of [^{125}I]protein A from *S. aureus* (NEN 86 μCi/μg) in 50 ml of TSB. After three washings with 100 ml of TSB and drying, the filters were autoradiographed as indicated above.

Double diffusion analyses were performed in 1.2% agarose gel, 0.01 M Tris-HCl pH 7.5, and 1 mM EDTA. Antigen and antiserum were placed into the gels and allowed to incubate overnight at room temperature. Precipitin bands were visible within 12–14 h.

Insect toxicity assays

P. brassicae were taken at the third instar stage. Five concentrated extracts were prepared by sonication of 100 ml of the appropriate *E. coli* or *B. thuringiensis* strains. About 1 ml was spread out on small cabbage leaves and the leaves were given to the larvae. The dead larvae were scored after 18 h of free feeding at room temperature.

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