APPLIED AND ENVIRONMENTAL MICROBIOLOGY, July 1997, p. 2792–2797 Vol. 63, No. 7
0099-2240/97/$04.00+0

Unique Regulation of Crystal Protein Production in *Bacillus thuringiensis* subsp. *yunnanensis* Is Mediated by the Cry Protein-Encoding 103-Megadalton Plasmid

G. SRINIVAS, S. JOHN VENNISON, S. N. SUDHA, P. BALASUBRAMANIAN, AND VAITHILINGAM SEKAR*

Department of Molecular Microbiology, School of Biotechnology, Madurai Kamaraj University, Madurai 625 021, India

Received 3 December 1996/Accepted 6 May 1997

In sporulating cultures of *Bacillus thuringiensis* subsp. *yunnanensis* HD977, two cell types are observed: cells forming only spores and cells forming only crystals. Curing analysis suggested that the crystal proteins are plasmid encoded. Through plasmid transfer experiments, it was established that a 103-MDa plasmid is involved in the crystal production. Conjugal transfer of this plasmid to Cry$^-$ recipient cells of *Bacillus thuringiensis* subsp. *kurstaki* HD73-26 conferred the ability to produce crystals exclusively on asporogenous cells of the recipient, indicating that the 103-MDa plasmid mediates the unique regulation of Cry protein production. When the dipteran-specific *cryIVB* gene was introduced into wild-type (Cry$^+$) and Cry$^-$ backgrounds of *B. thuringiensis* subsp. *yunnanensis* by phage CP51ts45-mediated transduction, similar to all other *B. thuringiensis* strains, irregular crystals of CryIVB protein were produced by spore-forming cells in both backgrounds. However, the synthesis of the bipyramidal inclusions of *B. thuringiensis* subsp. *yunnanensis* was still limited only to asporogenous cells of the transductant. Thus, it appears that the unique property of exclusive crystal formation in asporogenous cells of *B. thuringiensis* subsp. *yunnanensis* is associated with the crystal protein gene(s) per se or its *cis* acting elements. As the crystals in *B. thuringiensis* subsp. *yunnanensis* were formed only in asporogenous cells, attempts were made to find out whether crystal formation had any inhibitory effect on sporulation. It was observed that both Cry$^+$ and Cry$^-$ strains of *B. thuringiensis* subsp. *yunnanensis* (HD977 and HD977-1, respectively) exhibited comparable sporulation efficiencies. In addition, the Cry$^-$ *B. thuringiensis* subsp. *kurstaki* host (HD73-26) and its Cry$^+$ transconjugant (HD73-26-16), expressing the *B. thuringiensis* subsp. *yunnanensis* crystal protein, were also comparable in their sporulation efficiencies, indicating that production of the crystal proteins of *B. thuringiensis* subsp. *yunnanensis* does not affect the process of sporulation.

The sporulating soil bacterium *Bacillus thuringiensis* produces proteinaceous crystalline inclusions which are often toxic to lepidopteran, dipteran, and coleopteran insect larvae (10). Formation of crystalline inclusions in *B. thuringiensis* is, in general, a sporulation-dependent process and occurs in the mother cell compartment (19). Mutants blocked at early stages of sporulation are also acrystalliferous, suggesting the dependence of crystal production on sporulation-specific factors (20, 21). In general, developmental and spatial control of crystal protein gene (*cry* gene) expression in the mother cell compartment is achieved by the recognition of its promoter by sporulation-specific sigma factors (3). An exception is the *cryIIIA* gene, which is vegetatively expressed (1, 27). Most of the *cry* genes are located on low-copy-number, high-molecular-weight plasmids (megaplasmids), which are normally conjugative (4). However, cloning and expression of *cry* genes in high-copy-number plasmids in *Bacillus* spp. often results in the inhibition of sporulation (28). This is thought to be due to titration of sigma factors by the *cry* gene promoters, which are also required for the expression of sporulation-specific genes (*spo* genes). Thus, there exists a balanced expression of *cry* and *spo* genes in sporulating cells, leading to a perfect coordination between the two processes of crystal formation and sporulation.

* Corresponding author. Mailing address: Department of Molecular Microbiology, School of Biotechnology, Madurai Kamaraj University, Madurai 625 021, India. Phone: (91 452) 858684. Fax: (91 452) 859105. E-mail: sekar%bic-mku@dbt.ernet.in.

In *B. thuringiensis* subsp. *yunnanensis*, however, spore and crystal formation occur in separate cells (17). In a sporulating culture of *B. thuringiensis* subsp. *yunnanensis*, two types of cells are observed with respect to the formation of spores and crystals: cells forming only spores and cells forming only crystals. This unique regulation of crystal production in *B. thuringiensis* subsp. *yunnanensis* is maintained in each successive generation. It is not known at present how two genetically identical cells have different developmental fates.

In this report we present evidence to show that in *B. thuringiensis* subsp. *yunnanensis* the crystal proteins are encoded by a conjugative 103-MDa plasmid and that transfer of this plasmid to a well-studied Cry$^-$ *B. thuringiensis* subsp. *kurstaki* strain confers the ability to form crystals (like that of the donor) exclusively on asporogenous recipient cells. We have also established that introduction of the dipteran-specific *cryIVB* gene into Cry$^+$ and Cry$^-$ *B. thuringiensis* subsp. *yunnanensis* strains leads to the production of irregular CryIVB crystals in spore-forming cells of both strains. However, the synthesis of the bipyramidal *B. thuringiensis* subsp. *yunnanensis* crystals is still restricted to the asporogenous cells. Hence, it appears that the unique pattern of exclusive crystal formation in asporogenous cells of *B. thuringiensis* subsp. *yunnanensis* is associated with the *cry* gene(s) of this strain. In order to find out whether crystal production in *B. thuringiensis* subsp. *yunnanensis* is limited to asporogenous cells due to some inhibitory effect of the crystals on spore formation, we measured the sporulation efficiencies of Cry$^+$ and Cry$^-$ strains of *B. thuringiensis* subsp. *yunnanensis* as well as those of a Cry$^-$ *B. thuringiensis* subsp.

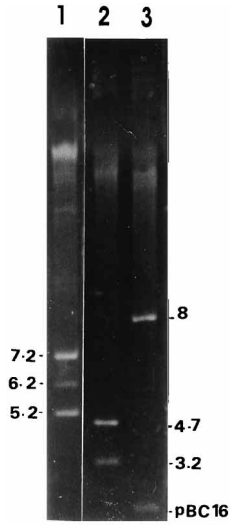

FIG. 1. Slot lysis electrophoretic analysis of *B. thuringiensis* subsp. *yunnanensis* and *B. thuringiensis* subsp. *yunnanensis* (pBC16). Lanes: 1, *B. thuringiensis* subsp. *thuringiensis* HD2 (marker strain); 2, HD977 wild type; 3, transductant *B. thuringiensis* subsp. *yunnanensis* (pBC16). The molecular masses (in MDa) of the small plasmids of strains HD2 and HD977 are shown on the left and right, respectively.

kurstaki host and its Cry^+ transconjugant, expressing the *B. thuringiensis* subsp. *yunnanensis* crystal proteins. It was observed that sporulation efficiencies of the Cry^+ and Cry^- strains of both backgrounds were comparable, indicating that the *B. thuringiensis* subsp. *yunnanensis* crystals do not inhibit the process of sporulation.

MATERIALS AND METHODS

Bacterial strains and growth conditions. *B. thuringiensis* subsp. *yunnanensis* HD977 was obtained from L. K. Nakamura, USDA Northern Regional Research Laboratory, Peoria, Ill. A Cry^- derivative of *B. thuringiensis* subsp. *kurstaki* HD73-26 was obtained from J. M. Gonzalez, Jr., University of Georgia, Athens. *Bacillus cereus* 569, containing phage CP51ts45, was a gift from C. B. Thorne, University of Massachusetts, Amherst. *Bacillus megaterium*(pGS1) was constructed essentially as described by Sekar and Carlton (25). Plasmid pGS1 contains the *cryIVB* gene from *B. thuringiensis* subsp. *israelensis* cloned in pBC16. This plasmid construct was introduced into *B. thuringiensis* subsp. *kurstaki* HD73-26 by electroporation. All *B. thuringiensis* strains were maintained on SCG (Spizizen's minimal agar medium [29] supplemented with vitamin-free Casamino Acids [acid-hydrolyzed casein] and glucose at 0.1 and 0.5% [wt/vol], respectively). The plates were incubated at 27°C for about 3 to 4 days until the cultures sporulated extensively and lysed. The production of crystal inclusions by various *B. thuringiensis* strains was observed by phase-contrast microscopy with a Nikon microscope as described by Bora et al. (2).

Isolation of acrystalliferous derivatives of *B. thuringiensis* subsp. *yunnanensis*. Cry^- derivatives were obtained by ethidium bromide treatment as described by Sekar (26) and were identified by phase-contrast microscopy. The plasmid profiles of Cry^- derivatives were analyzed by slot lysis electrophoresis with 0.6% vertical agarose gels (9).

Plasmid transfer by filter mating. Conjugal transfer of plasmids among various *B. thuringiensis* strains is a well-established phenomenon (8, 11). To monitor the plasmid transfer events, we used *B. thuringiensis* subsp. *yunnanensis* HD977 transduced with a 3.0-MDa Tet^r plasmid, pBC16, as the donor (18). The Cry^- Str^r strain HD73-26 (containing only a 4.8-MDa plasmid) was used as the recipient. Plasmid transfer from Cry^+ Tet^r Str^s HD977(pBC16) to Cry^- Tet^s Str^r HD 73-26 was performed by filter mating as described by Fisher et al. (7). The donor and recipient strains were grown separately in Luria broth in a shaker incubator for 12 to 14 h at 27°C. The stationary-phase cultures were diluted 1:100 in fresh Luria broth and incubated with shaking for 6 h. The donor and recipient ($\sim 10^8$ cells each) were mixed and collected by filtration on a Millipore HA membrane filter (0.45-μm pore size, 25-mm diameter). The filter was placed cell side up on a Luria agar plate and incubated overnight at 30°C. The cells were then removed from the filter by washing with 2 to 3 ml of Luria broth, concentrated to approximately 10^9 cells/ml, and plated on Luria agar containing streptomycin (150 μg/ml) and tetracycline (10 μg/ml). Str^r and Tet^r colonies were allowed to sporulate and were examined by phase-contrast microscopy for the presence of crystals. The plasmid profiles of the transconjugants were analyzed by slot lysis electrophoresis. To ensure that the selected strains were indeed transconjugants, flagellar serotyping of the putative transconjugants was performed with donor-specific (H20ab) and recipient-specific (H3a3b) flagellar antibodies according to the method of de Barjac (6). The H-antisera were a kind gift from M. Ohba of Kyushu University, Fukuoka, Japan.

Transduction of the *cryIVB* gene into *B. thuringiensis* subsp. *yunnanensis* strains with CP51ts45. Transduction was carried out as described by Ruhfel et al. (22). The recipients, *B. thuringiensis* subsp. *yunnanensis* HD977 Cry^+ and HD977-1 Cry^- strains, were grown in Luria broth containing 0.4% glycerol (vol/vol) for 16 to 18 h at 27°C. A 2.5-ml sample of the culture was transferred to 25 ml of fresh broth in a flask and incubated with shaking for 6 to 7 h at 27°C. Recipient cell culture (1 to 2 ml) containing 4×10^8 to 8×10^8 cells/ml was mixed with 0.5 to 1 ml of CP51ts45 lysate prepared on HD73-26(pGS1). The mixture was incubated on a shaker at 30°C for 1 h. The cells were pelleted and washed three times with a solution containing nutrient broth (8 g/ml) and yeast extract (3 g/ml) to remove the unbound phages. Samples (0.1 ml) were plated on Luria agar containing tetracycline (10 μg/ml) and incubated at 37°C for selection of transductants. The transductants were examined by phase-contrast microscopy for the presence of crystals and were also analyzed by slot lysis electrophoresis for the presence of transduced plasmid.

SDS-PAGE and immunoblotting. The transconjugants and transductants obtained were analyzed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and immunoblotting as described by Bora et al. (2). The *B. thuringiensis* strains were grown on SCG. Crystal protein was prepared from the plates as described previously (16). Protein concentrations in the preparations were determined by the method of Lowry et al. (14) after the protein crystals had been solubilized at 37°C for 3 h in 50 mM NaOH (pH 11.7) containing 10 mM EDTA. SDS-PAGE analysis was carried out with a MightySmall vertical-slab unit (Hoefer Scientific Instruments, San Francisco, Calif.) according to the method of Laemmli (12).

Proteins fractionated on SDS-PAGE gels were electrophoretically transferred onto a nitrocellulose membrane with a TE70 Semiphor semidry transfer unit (Hoefer Scientific Instruments). Immunoblotting was performed according to the method of Towbin et al. (31). Polyclonal rabbit antiserum directed against purified crystals of HD977 or CryIVB was used as the primary antibody. Goat anti-rabbit immunoglobulin conjugated to alkaline phosphatase served as the secondary antibody.

Determination of sporulation frequency. The sporulation frequency of *B. thuringiensis* strains grown in sporulation medium (24) was determined essentially as described by Shivakumar et al. (28).

RESULTS

Isolation of a Cry^- derivative of *B. thuringiensis* subsp. *yunnanensis* by plasmid curing. HD977 harbors a total of seven plasmids (4). Using the sizes of the plasmids of the standard *B. thuringiensis* subsp. *thuringiensis* strain, HD2, the sizes of the plasmids of *B. thuringiensis* subsp. *yunnanensis* were calculated

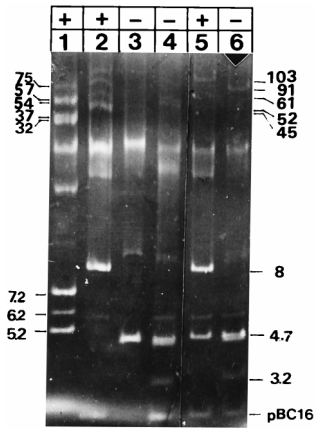

FIG. 2. Conjugal transfer of megaplasmids from HD977(pBC16) to HD73-26 was analyzed by slot lysis electrophoresis. Lanes: 1, *B. thuringiensis* subsp. *thuringiensis* HD2 (marker strain); 2, *B. thuringiensis* subsp. *yunnanensis* (pBC16), the donor; 3, HD73-26, the recipient; 4, 5, and 6, *B. thuringiensis* subsp. *kurstaki* transconjugants 73-26-13, 73-26-16, and 73-26-20, respectively. The molecular masses (in MDa) of plasmids of strains HD2 and HD977 are shown on the left and right, respectively. The presence or absence of crystals is indicated by + and –, respectively.

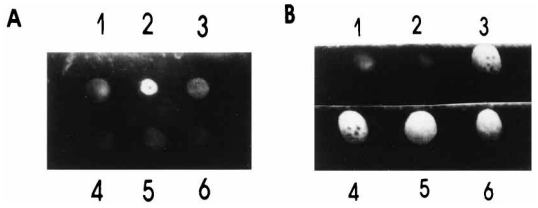

FIG. 3. Flagellar serotyping. (A) Using anti-*B. thuringiensis* subsp. *yunnanensis* flagellar antiserum (H-20ab). Sample 1, control cells [*B. thuringiensis* subsp. *yunnanensis* (pBC16)] without the addition of antiserum; samples 2 to 6, donor [*B. thuringiensis* subsp. *yunnanensis* (pBC16)], recipient (HD73-26), and transconjugant (HD73-26-13, HD73-26-16, and HD73-26-20) cells, respectively, mixed with the antiserum. A positive reaction was seen only in sample 2. (B) Using anti-*B. thuringiensis* subsp. *kurstaki* flagellar antiserum (H-3a3b). Samples are as in panel A. Positive reactions were seen only in samples 3 to 6.

to be 103, 91, 61, 52, 45, 4.7, and 3.2 MDa. The plasmid profile of one of the Cry$^-$ derivatives, strain HD977-1, showed the absence of 103-, 61-, and 4.7-MDa plasmids when compared to that of the wild type (data not shown). When subjected to SDS-PAGE analysis, the Cry$^-$ strain was found to be devoid of the four protein components of the crystals produced by the wild type (not shown). This indicated that one or more of these plasmids might harbor the crystal protein gene.

Plasmid transfer studies. In HD977(pBC16), the two small (3.2 and 4.7 MDa) plasmids of the wild type were absent. Instead, a larger plasmid of ~8 MDa appeared in this strain (Fig. 1, lane 3). This plasmid might be the cointegrate of the two small plasmids, as it exhibited considerable homology to both plasmids when tested by Southern hybridization (data not shown). The frequency of Str^r and Tet^r transconjugant colonies obtained was 5×10^{-5}. The transconjugants were allowed to sporulate on SCG and were monitored by phase-contrast microscopy. Of the 50 transconjugants analyzed, 7 showed the presence of bipyramidal crystals.

Slot lysis electrophoretic analysis revealed the presence of the 4.8-MDa plasmid of HD73-26 and the 3.0-MDa pBC16 of the donor in all the selected transconjugants (Fig. 2, lanes 4 to 6). The Cry$^+$ transconjugant (HD73-26-16) received a megaplasmid of 103 MDa (Fig. 2, lane 5) along with an 8.0-MDa plasmid (the likely cointegrate of 4.7- and 3.2-MDa plasmids). The Cry$^-$ HD73-26-13 and HD73-26-20 strains were harboring the 52- and 45-MDa plasmids and the 91-MDa plasmid, respectively (Fig. 2, lanes 4 and 6). In these transconjugants, the 8-MDa plasmid was resolved into 4.7- and 3.2-MDa plasmids. A transconjugant that received the 61-MDa plasmid was also found to be acrystalliferous (data not shown). To ensure that the selected strains were indeed true transconju-

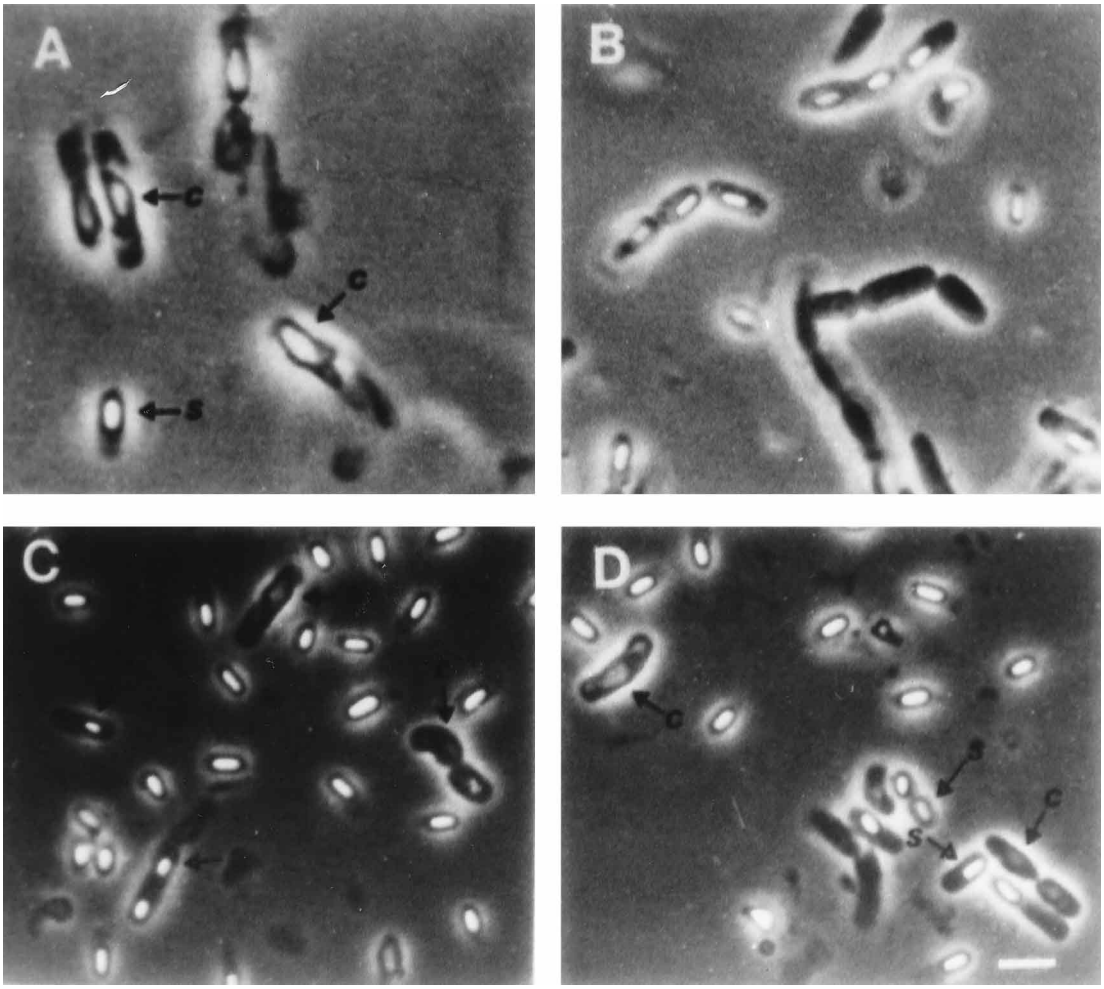

FIG. 4. Phase-contrast microscopic analysis of Cry$^+$ transconjugant. (A) HD977(pBC16), the donor. (B) HD73-26, the recipient. (C and D) HD73-26-16, the Cry$^+$ transconjugant. Arrows with labels C and S indicate bipyramidal crystals and spores, respectively. Bar, 5.0 μm.

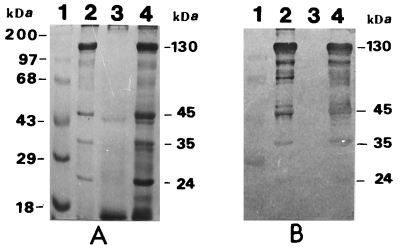

FIG. 5. (A) SDS-PAGE of crystals produced by the Cry^+ transconjugant. Lanes: 1, prestained protein molecular mass standards (top to bottom: myosin, phosphorylase b, bovine serum albumin, ovalbumin, carbonic anhydrase, and B-lactoglobulin); 2, HD977(pBC16), the donor; 3, HD73-26, the recipient; 4, HD73-26-16, the Cry^+ transconjugant. Molecular masses (in kDa) of the protein standards and the protein components of bipyramidal crystals of HD977(pBC16) are shown on the left and right, respectively. (B) Immunoblot analysis of SDS-PAGE gel with polyclonal antisera directed against purified crystals of HD977.

gants, the presence of a recipient-specific 4.8-MDa plasmid was ascertained in these strains. In addition, the flagellar serology of the selected transconjugants was also confirmed as H-serotype 3a3b (Fig. 3). Phase-contrast microscopic observation of sporulating cultures of the transconjugant HD73-26-16 indicated that, as was found in the donor strain, HD977(pBC16) (Fig. 4A), crystal formation occurred only in asporogenous cells (Fig. 4C and D). SDS-PAGE analysis of crystal inclusions produced by the transconjugant showed the presence of four protein components (Fig. 5A, lane 4) which cross-reacted with the antiserum prepared against purified crystals of HD977 (Fig. 5B). These results suggest that the 103-MDa plasmid is likely to harbor the genes encoding all four proteins of the bipyramidal crystals.

Introduction of the *cryIVB* gene into wild-type and Cry^- strains of *B. thuringiensis* subsp. *yunnanensis* by CP51ts45-mediated transduction. Since transformation and conjugation methods were unsuccessful for introducing other *cry* genes into *B. thuringiensis* subsp. *yunnanensis* strains, transduction procedures were adopted. Phage CP51ts45 has been demonstrated to be a useful vehicle to introduce small plasmids containing antibiotic markers in various *B. thuringiensis* strains (13, 22). To study the formation of heterologous crystals in *B. thuringiensis* subsp. *yunnanensis*, we selected irregular CryIVB crystals (25) for transduction experiments, as they are easily distinguishable from the bipyramidal crystals of *B. thuringiensis* subsp. *yunnanensis.*

CP51ts45 propagated in HD73-26(pGS1) was used to transduce the plasmid pGS1, containing the *cryIVB* gene, into Cry^+ and Cry^- strains of *B. thuringiensis* subsp. *yunnanensis*. Plasmid profiles of the transductants [HD977(pGS1) and HD977-1 (pGS1)] indicated the presence of pGS1 (Fig. 6, lanes 3 and 5, respectively). The presence of this plasmid in these strains was also confirmed by Southern hybridization with pGS1 as a probe (data not shown).

In HD977-1(pGS1), similar to HD73-26(pGS1) (Fig. 7A), irregular crystals of CryIVB protein were observed in spore-forming cells (Fig. 7C). However, in the case of HD977(pGS1), while spore-forming cells showed the presence of irregular crystals of CryIVB protein, only the asporogenous cells showed the formation of bipyramidal crystals (Fig. 7D). Coomassie blue staining of the SDS-PAGE-fractionated proteins of HD977-1 (pGS1) revealed the presence of the 130-kDa protein of the CryIVB crystals (Fig. 8A, lane 4). The four constituent proteins of the bipyramidal crystal of strain HD977 were also found in HD977(pGS1) (Fig. 8A, lane 5). However, it is difficult to distinguish the 130-kDa proteins of irregular and bipyramidal crystals. To find out whether a part of the 130-kDa protein component is indeed the CryIVB protein, we carried out immunoblotting with anti-CryIVB antiserum. Immunocross-reactive 130-kDa protein was found to be produced by both transductants (Fig. 8B). Immunoblotting with antiserum raised against crystal inclusions of *B. thuringiensis* subsp. *yunnanensis* revealed that all four proteins, including the 130-kDa protein of *B. thuringiensis* subsp. *yunnanensis*, were present in HD977(pGS1). However, no cross-reaction was observed with the 130-kDa protein of the irregular crystals of HD977-1 (pGS1) and HD73-26(pGS1) (data not shown). This clearly shows that both types of crystals are formed in HD977(pGS1). The irregular crystals produced by these transductants were toxic to dipteran insect larvae of *Aedes aegypti* (data not shown).

Effect of crystal production on sporulation. To study the effect of crystal production on sporulation, we established the efficiency of sporulation in strains HD977 (Cry^+) and HD977-1 (Cry^-). The sporulation frequencies of these strains remained the same (39% for HD977 and 39.5% for HD977-1). Similarly, the extent of sporulation in the Cry^- HD73-26 host and its transconjugant (HD73-26-16), expressing the *B. thuringiensis* subsp. *yunnanensis* crystal proteins, was measured. The sporulation efficiencies of these two strains were very similar (66% for HD73-26 and 72% for HD73-26-16).

DISCUSSION

It is generally known that crystal protein genes are expressed during the process of sporulation in *B. thuringiensis*, as their promoters are recognized by sporulation-specific sigma factors (10). However, the crystal production in *B. thuringiensis* subsp. *yunnanensis* is unusual in that stationary-phase cultures of this strain undergo either spore formation or crystal formation. To analyze the unique behavior of crystal formation in *B. thuringiensis* subsp. *yunnanensis*, we took two approaches. (i) If the phenomenon observed is caused by an altered phenotype of the bacterium due to a possible mutation in the chromosome (23) and not by the crystal protein gene, introduction of a well-studied crystal protein gene, such as *cryIVB* (which forms crystals in sporulating cells), into a Cry^- derivative of *B. thuringiensis* subsp. *yunnanensis* should produce crystals in as-

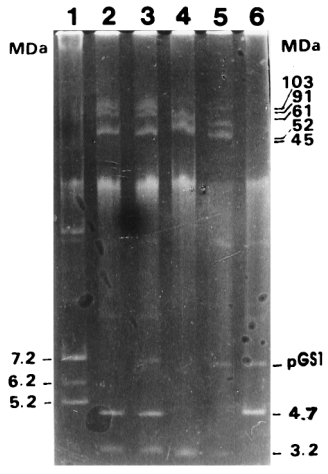

FIG. 6. Analysis of CP51ts45-mediated transduction of pGS1 into HD977 and HD977-1 by slot lysis electrophoresis. Lanes: 1, *B. thuringiensis* HD2 (marker strain); 2, HD977; 3, HD977 transduced with pGS1; 4, HD977-1; 5, HD977-1 transduced with pGS1; 6, HD73-26 transformed with pGS1. The molecular masses (in MDa) of plasmids of strains HD2 and HD977 are shown on the left and right, respectively.

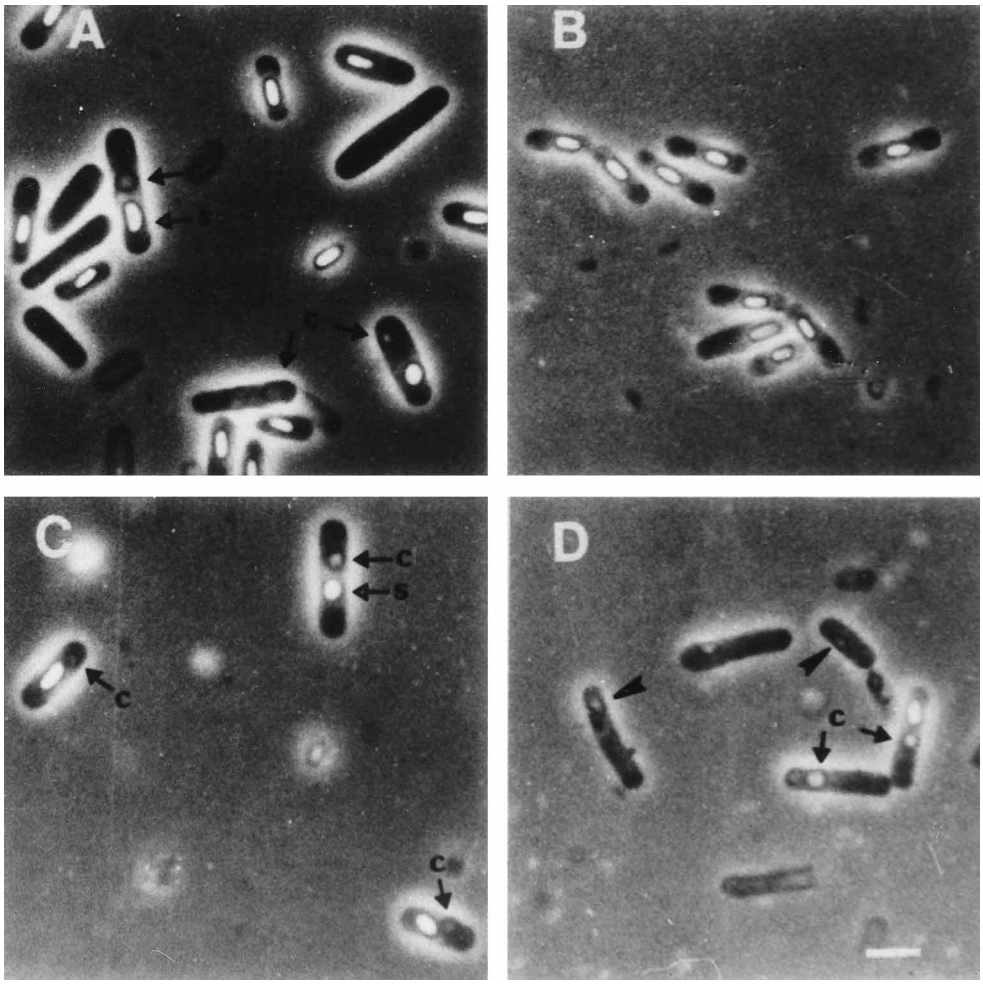

FIG. 7. Phase-contrast microscopic analysis of the transductants. (A) HD73-26 transformed with pGS1. (B) HD977-1 Cry$^-$ strain. (C) HD977-1 transduced with pGS1. (D) HD977 transduced with pGS1. Arrows with labels C and S indicate irregular crystals of CryIVB protein and spores, respectively. The bipyramidal crystals of *B. thuringiensis* subsp. *yunnanensis* are shown by arrowheads. Bar, 5.0 μm.

porogenous cells only. Our results, however, show that the crystals are formed in sporulating cells and not in asporogenous cells, suggesting that there is no change in the phenotype or physiology of the *B. thuringiensis* subsp. *yunnanensis* strain compared with other strains of *B. thuringiensis*. (ii) To test the possibility that the crystal protein gene itself is responsible for the unique behavior of crystal formation, we conjugally transferred the crystal-encoding 103-MDa plasmid into a Cry$^-$ HD73-26 strain. Our results clearly show that the transfer of the 103-MDa plasmid to HD73-26 confers the ability to produce crystals only on asporogenous cells. In the case of *B. thuringiensis* subsp. *kurstaki* HD1-9, it has been shown that several apparently cryptic plasmids (110, 29, and 4.9 MDa) are involved in a complex mechanism of conditionally temperature-regulating crystal protein synthesis (15). To rule out the possibility of sequences other than crystal protein genes of the 103-MDa plasmid playing a role, we introduced the *cryIVB* gene into the Cry$^+$ strain HD977 by transduction. The resulting transductant, HD977(pGS1), formed irregular crystals of CryIVB protein in sporulating cells and formed bipyramidal crystals of *B. thuringiensis* subsp. *yunnanensis* exclusively in asporogenous cells. Hence, it appears that the unique regulation of crystal production observed in *B. thuringiensis* subsp. *yunnanensis* is the property of the *cry* genes and/or their *cis*-acting elements.

As crystals are produced only in asporogenous cells of *B. thuringiensis* subsp. *yunnanensis* and are quite large compared

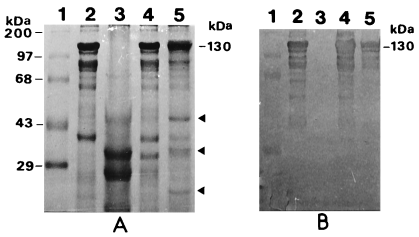

FIG. 8. (A) SDS-PAGE of crystals produced by *B. thuringiensis* subsp. *yunnanensis* strains transduced with pGS1. Lanes: 1, prestained protein molecular mass standards (same as Fig. 5); 2, HD73-26 transformed with pGS1; 3, HD977-1; 4, HD977-1 transduced with pGS1; 5, HD977 transduced with pGS1. Molecular masses (in kDa) of the protein standards and CryIVB protein are shown on the left and right, respectively. Arrows indicate the positions of the protein components of bipyramidal crystals of HD977. (B) Immunoblot analysis of the SDS-polyacrylamide gel with polyclonal antisera directed against purified CryIVB crystals.

to those of other *B. thuringiensis* strains (30), we wanted to establish whether crystal formation is inhibitory to sporulation. The effect of crystal formation on the production of spores was determined by measuring the sporulation frequencies of Cry^+ and Cry^- strains of *B. thuringiensis* subsp. *yunnanensis*. There was no significant difference in sporulation between the Cry^+ and Cry^- strains (39 and 39.5%, respectively). We have generally observed that the strains HD73 and HD73-26 sporulate much more efficiently (about 70%) than strain HD977. Introduction of crystal protein genes of *B. thuringiensis* subsp. *yunnanensis* into the Cry^- strain HD73-26 did not affect its sporulation. The sporulation frequency of the Cry^+ *B. thuringiensis* subsp. *kurstaki* transconjugant (HD73-26-16) is considerably higher (~72%) than that of the *B. thuringiensis* subsp. *yunnanensis* strain (~39%). Thus, it is clear that formation of *B. thuringiensis* subsp. *yunnanensis* crystals does not affect the sporulation of *B. thuringiensis* subsp. *yunnanensis* or *B. thuringiensis* subsp. *kurstaki* strains. However, whether sporulation has any deleterious effects on crystal production is not known at present.

Chung et al. (5) have measured the expression of *spo* genes in single cells of *Bacillus subtilis* by using *spo-lacZ* fusions. Through fluorescence microscopy they determined that mutations which decrease the amount of activated Spo0A transcription factor cause a decrease in the expression of early developmental genes in a subpopulation of cells. The size of this subpopulation correlates well with the fraction of cells that do not produce spores. On the basis of these results they concluded that a threshold level of activated Spo0A must accumulate to activate sporulation-specific gene expression. Whether such a mechanism is operational in *B. thuringiensis* subsp. *yunnanensis* is not known at present. Although it is clear from the current work that the unique regulation of crystal production in *B. thuringiensis* subsp. *yunnanensis* is mediated by a 103-MDa plasmid, further studies are needed to establish the exact molecular basis of this interesting phenomenon.

ACKNOWLEDGMENTS

This work is supported by grants from The Department of Science and Technology (SP/SO/D65/89) and The Department of Biotechnology (BT/TF/03/23/002/88), Government of India. G.S., S.N.S., and P.B. thank the Council of Scientific and Industrial Research, New Delhi, India, for the award of Senior Research Fellowships.

We are grateful to M. Ohba, Kyushu University, Fukuoka, Japan, for the generous gift of the flagellar antisera. We thank A. K. Gupta, K. Veluthambi, and Anup Kumar for help with photography. The assistance provided by A. Pandiarajan in the preparation of the manuscript is acknowledged.

REFERENCES

1. **Agaisse, H., and D. Lereclus.** 1994. Structural and functional analysis of the promoter region involved in full expression of the *cryIII* toxin gene of *Bacillus thuringiensis*. Mol. Microbiol. **13:**97–107.
2. **Bora, R. S., M. G. Murty, R. Shenbagarathai, and V. Sekar.** 1994. Introduction of a lepidopteran-specific insecticidal crystal protein gene of *Bacillus thuringiensis* subsp. *kurstaki* by conjugal transfer into a *Bacillus megaterium* strain that persists in the cotton phyllosphere. Appl. Environ. Microbiol. **60:**214–222.
3. **Brown, K. L., and H. R. Whiteley.** 1988. Isolation of a *Bacillus thuringiensis* RNA polymerase capable of transcribing a crystal protein gene. Proc. Natl. Acad. Sci. USA **85:**4166–4170.
4. **Carlton, B. C., and J. M. Gonzalez.** 1985. Plasmids and delta-endotoxin production in different subspecies of *Bacillus thuringiensis*, p. 246–252. *In* J. A. Hoch and P. Setlow (ed.), Molecular biology of microbial differentiation. American Society for Microbiology, Washington, D.C.
5. **Chung, J. D., G. Stephanopoulos, K. Ireton, and A. D. Grossman.** 1994. Gene expression in single cells of *Bacillus subtilis*: evidence that a threshold mechanism controls the initiation of sporulation. J. Bacteriol. **176:**1977–1984.
6. **de Barjac, H.** 1981. Identification of H-serotypes of *Bacillus thuringiensis*, p. 35–43. *In* H. D. Burges (ed.), Microbial control of pests and plant diseases, 1970–1980. Academic Press, London, England.
7. **Fisher, H. M., P. Luthy, and S. Schweitzer.** 1984. Introduction of plasmid pC194 in *Bacillus thuringiensis* by protoplast transformation and plasmid transfer. Arch. Microbiol. **139:**213–217.
8. **Gonzalez, J. M., B. J. Brown, and B. C. Carlton.** 1982. Transfer of *Bacillus thuringiensis* plasmids coding for delta-endotoxin among strains of *Bacillus thuringiensis* and *Bacillus cereus*. Proc. Natl. Acad. Sci. USA **79:**6951–6955.
9. **Gonzalez, J. M., and B. C. Carlton.** 1984. A large transmissible plasmid is required for crystal toxin production in *Bacillus thuringiensis* variety *israelensis*. Plasmid **11:**28–38.
10. **Hofte, H., and H. R. Whiteley.** 1989. Insecticidal proteins of *Bacillus thuringiensis*. Microbiol. Rev. **53:**242–255.
11. **Jensen, G. B., A. Wilcks, S. S. Petersen, J. Damgaard, J. A. Baum, and L. Andrup.** 1995. The genetic basis of the aggregation system in *Bacillus thuringiensis* subsp. *israelensis* is located on the large conjugative plasmid pXO16. J. Bacteriol. **177:**2914–2917.
12. **Laemmli, U. K.** 1970. Cleavage of structural proteins during the assembly of the head of bacteriophage T4. Nature (London) **227:**680–685.
13. **Lecadet, M.-M., J. Chaufaux, J. Didier, and D. Lereclus.** 1992. Construction of novel *Bacillus thuringiensis* strains with different insecticidal specificities by transduction and transformation. Appl. Environ. Microbiol. **58:**840–849.
14. **Lowry, O. H., N. J. Rosebrough, A. L. Farr, and R. J. Randall.** 1951. Protein measurement with the Folin phenol reagent. J. Biol. Chem. **193:**265–275.
15. **Minnich, S. A., and A. I. Aronson.** 1984. Regulation of protoxin synthesis in *Bacillus thuringiensis*. J. Bacteriol. **158:**447–454.
16. **Murty, M. G., G. Srinivas, R. S. Bora, and V. Sekar.** 1994. A simple method for separation of the protein crystals from *Bacillus thuringiensis* using carboxymethyl cellulose column chromatography. J. Microbiol. Methods **19:**103–110.
17. **Ohba, M., and K. Aizawa.** 1986. Crystals of *Bacillus thuringiensis* subsp. *yunnanensis* are produced only in asporogenous cells. J. Invertebr. Pathol. **48:**254–256.
18. **Reddy, A., L. Battisti, and C. B. Thorne.** 1987. Identification of self-transmissible plasmids in four *Bacillus thuringiensis* subspecies. J. Bacteriol. **169:**5263–5270.
19. **Ribier, J., and M.-M. Lecadet.** 1973. Etude ultrastructure et cinetique de la sporulation de *Bacillus thuringiensis* var. *berliner* 1715. Remarques sur la formation de inclusion parasporale. Ann. Inst. Pasteur. **124A:**311–314.
20. **Ribier, J., and M.-M. Lecadet.** 1981. *Bacillus thuringiensis* var. *berliner* 1715. Isolement et characterisation de mutants de sporulation. C. R. Acad. Sci. Ser. III.
21. **Rubikas, J., D. Androsiuniene, G. Chestukina, T. Smirnova, O. Kapitonova, and V. Stepanov.** 1987. Crystal protein formed by *Bacillus subtilis* cells. J. Bacteriol. **169:**5258–5262.
22. **Ruhfel, R. E., N. J. Robillard, and C. B. Thorne.** 1984. Interspecies transduction of plasmids among *Bacillus anthracis*, *B. cereus*, and *B. thuringiensis*. J. Bacteriol. **157:**708–711.
23. **Santo, L. Y., and R. H. Doi.** 1973. Crystal formation by a ribonucleic acid polymerase mutant of *Bacillus subtilis*. J. Bacteriol. **116:**479–482.
24. **Schaeffer, P., J. Millet, and J. Aubert.** 1965. Catabolite repression of bacterial sporulation. Proc. Natl. Acad. Sci. USA **54:**704–711.
25. **Sekar, V., and B. C. Carlton.** 1985. Molecular cloning of the delta-endotoxin gene of *Bacillus thuringiensis* var. *israelensis*. Gene **33:**151–158.
26. **Sekar, V.** 1987. Location of the crystal toxin gene of *Bacillus thuringiensis* var. *aizawai*. Curr. Microbiol. **14:**2893–2897.
27. **Sekar, V.** 1988. The insecticidal crystal protein gene is expressed in vegetative cells of *Bacillus thuringiensis* var. *tenebrionis*. Curr. Microbiol. **17:**347–349.
28. **Shivakumar, A. G., R. I. Vanags, D. R. Wilcox, L. K. Katz, P. S. Vary, and J. Lawrence.** 1989. Gene dosage effect on the expression of delta-endotoxin genes of *Bacillus thuringiensis* subsp. *kurstaki* in *Bacillus subtilis* and *Bacillus megaterium*. Gene **79:**21–31.
29. **Spizizen, J.** 1958. Transformation of biochemically deficient strains of *Bacillus subtilis* by deoxyribonuclease. Proc. Natl. Acad. Sci. USA **44:**1072–1078.
30. **Srinivas, G., M. G. Murty, and V. Sekar.** 1995. Variation in the crystal morphology of *Bacillus thuringiensis* ssp. *yunnanensis* induced by medium composition. Microbios **81:**147–154.
31. **Towbin, H., T. Staehelin, and J. Gordon.** 1979. Electrophoretic transfer of proteins from polyacrylamide gels to nitrocellulose sheets: procedure and some applications. Proc. Natl. Acad. Sci. USA **76:**4350–4354.