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

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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 CP51s45-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 acrystitialiferous, 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.

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. yunnanensis.

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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 pBluescript. 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⁺ plasmid, pBluescript, as the donor (18). The Cry⁻ StrR strain HD73-26 (containing only a 4.8-MDa plasmid) was used as the recipient. Plasmid transfer from Cry⁻ Tet⁺ StrR HD977(pBluescript) to Cry⁻ Tet⁻ StrR 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 at 27°C, and the cells were pelleted and washed 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 (6 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.

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. yunnanensis strain, HD2, the sizes of the plasmids of B. thuringiensis subsp. yunnanensis were calculated...
to be 103, 91, 61, 52, 45, 4.7, and 3.2 MDa. The plasmid profile of one of the Cry2 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 Cry2 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 Strr and Tetr transconjugant colonies obtained was $5 \times 10^{-5}$. 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-
ramsial 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-
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\(^2\) 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\(^1\) 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...
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. *yunnanensis* or *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.

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