Optimization of Cry3A Yields in *Bacillus thuringiensis* by Use of Sporulation-Dependent Promoters in Combination with the STAB-SD mRNA Sequence

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The insecticidal activity of *Bacillus thuringiensis* strains toxic to coleopterous insects is due to Cry3 proteins assembled into small rectangular crystals. Toxin synthesis in these strains is dependent primarily upon a promoter that is active in the stationary phase and a STAB-SD sequence that stabilizes the *cry3* transcript-ribosome complex. Here we show that significantly higher yields of Cry3A can be obtained by using dual sporulation-dependent *cyt1Aa* promoters to drive the expression of *cry3Aa* when the STAB-SD sequence is included in the construct. The Cry3A yield per unit of culture medium obtained with this expression system was 12.7-fold greater than that produced by DSM 2803, the wild-type strain of *B. thuringiensis* from which Cry3Aa was originally described, and 1.4-fold greater than that produced by NB176, a mutant of the same strain containing two or three copies of *cry3Aa*, which is the active ingredient of the commercial product Novodor, used for control of beetle pests. The toxicities of Cry3A produced with this construct or the wild-type strain were similar when assayed against larvae of the cottonwood leaf beetle, *Chrysomela scripta*. The volume of Cry3A crystals produced with *cyt1Aa* promoters and the STAB-SD sequence was 1.3-fold that of typical bipyramidal Cry1 crystals toxic to lepidopterous insects. The dual-promoter/STAB-SD system offers an additional method for potentially improving the efficacy of insecticides based on *B. thuringiensis*.

The gram-positive bacterium *Bacillus thuringiensis* is a complex of subspecies, many of which produce large quantities of one or more insecticidal proteins (16). These proteins are typically produced during sporulation, with the expression of their encoding genes being driven by one or two sporulation-dependent promoters (30). Transcription from these promoters, referred to as BtI and BtII, is initiated by RNA polymerase complexes that contain, respectively, σ^E- and σ^K-like factors (3, 6, 30). After synthesis, the proteins accumulate in crystals, which compose as much as 25% of the cell dry weight (3, 5, 14).

The most common endotoxin proteins that occur in *B. thuringiensis* are those of the Cry1 class. These are actually 130- to 135-kDa protoxins, which form a single large bipyramidal crystal about the size of a spore in each cell. Examples include crystals formed by the Cry1Ac, Cry1B, and Cry1C proteins, which are toxic to lepidopterous insects (16). When ingested by a larva, the crystal dissolves and the 135-kDa molecule is activated by digestive enzymes, releasing a 60-kDa toxic fragment from the N terminus. Cry1 proteins are the active components of most commercial insecticides and insecticidal transgenic plants based on *B. thuringiensis* (27).

Whereas typical Cry1 proteins produce a large crystal, there are several other important Cry proteins that produce only a single, much smaller crystal per cell; this crystal is only 10 to 25% the size of a Cry1 crystal. These proteins include the Cry2A class (11, 16, 20, 21, 23, 29), toxic to lepidopterous and dipterous insects; the Cry3 class, toxic to coleopterous insects (8, 13, 26); and the Cry11 class, toxic to dipterous insects (12, 16). These proteins range from 60 to 67 kDa and are essentially naturally truncated versions of Cry1 molecules which contain a similar toxin core but lack the C terminus (16). The genetic basis for the comparatively small size of these Cry protein crystals is not known, but evidence suggests that it involves several factors including the existence of only a single promoter in some cases (6), poor mRNA stability (9, 10), and degradation of nascent polypeptides, the last possibly resulting from the lack of the large C-terminal domain that is thought to assist stability by facilitating crystallization (7, 14).

The amount of Cry protein produced in each cell, and per unit of culture medium, is of commercial interest because the higher the yield, the higher the potential toxicity per unit weight. This is particularly relevant to Cry3 proteins because these are the only proteins highly toxic to coleopterous insects. Moreover, because Cry3 proteins lack the large nontoxic C terminus of Cry1 proteins and produce only a single small crystal per cell (2), they provide a good model for attempting to increase toxin yields per cell. If net synthesis could be increased, the result should be larger crystals and correspondingly higher levels of toxicity. Thus, several strategies have been used to increase Cry3A synthesis, including expression of *cry3* genes in asporogenous mutants and *cry3* gene amplification. For example, the expression of *cry3* genes in asporogenous *B. thuringiensis* mutants yielded increases in Cry3A synthesis of two- to fivefold (21, 22, 25). Also, amplification of the *cry3A* gene copy number by gamma irradiation of *B. thuringiensis* subsp. *morrisoni* (strain tenebrionis) resulted in increases in the Cry3A yield of three- to fivefold (1). In other studies aimed at defining the genetic determinants of Cry3A production in wild-type isolates of *B. thuringiensis*, it was shown that synthesis was dependent primarily upon a σ^A-like promoter more than 558 bases upstream from the translational start site, which was active during vegetative growth and the stationary phase (2, 4, 9), and a Shine-Dalgarno sequence (STAB-SD) just downstream from the 5′ end of the major

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cry3A transcript (T-129). The latter significantly stabilized the transcript (4, 9). These elements were also shown to be important for synthesis of several other Cry3 proteins (3, 6) and probably account for the increased levels of Cry3 synthesis obtained in asporogenic mutants (21, 22, 25).

While all the above constructs yielded significant increases in Cry3A synthesis, ultrastructural studies of the crystals produced in the host cells showed they were not as large as typical Cry1 crystals (6, 21, 22, 25), suggesting that further increases in yield might be possible. In these constructs, the expression of *cry3* genes was primarily under the control of the single σ^A-like promoter that is active during the vegetative and stationary phases (1, 2, 21). This suggested that even higher yields of Cry3 proteins might be possible by placing *cry3* expression under the control of two strong sporulation-dependent promoters. Thus, in the present study, we expressed *cry3A*, including the STAB-SD mRNA-stabilizing sequence, under the control of the sporulation-dependent BtI and BtII promoters of the *cyt1Aa* gene (6, 28). Here we show that expression of this construct in *B. thuringiensis* increased the Cry3A yield by greater than 10-fold in comparison to the wild-type strain and by 1.4-fold in comparison to a commercial strain currently in use.

MATERIALS AND METHODS

Bacterial strains, genes, promoters, and plasmids. The *Bacillus* strains used in this study were DSM 2803, a wild-type isolate of *B. thuringiensis* subsp. *morrisoni* (strain tenebrionis); NB176, a mutant of this subspecies containing at least one additional copy of the *cry3Aa* gene (1); and 4Q7, an acrystalliferous strain of *B. thuringiensis* subsp. *israelensis*, obtained from the *Bacillus* Stock Center at Ohio State University, Columbus, Ohio. The source of the *cry3Aa* (referred to hereafter as *cry3A*) promoter was DSM 2803, and the original source of *cyt1Aa* (referred to hereafter as *cytA*) promoters and upstream region was the 125-kb plasmid of *B. thuringiensis* subsp. *morrisoni* PG-14 (15). Cloned genes and recombinant plasmid constructs were amplified in *Escherichia coli* DH5α [*supE44* ΔlacU169 (F80 *lacZ*ΔM15) *hsdR17 recA1 endA1 gyrA96 thi-1 relA1*]. The plasmid used to transform and express the *cry3A* constructs in *B. thuringiensis* 4Q7 was the *E. coli-B. thuringiensis* shuttle vector pHT3101 (20).

Expression vector construction. Our objective was to determine the relative effects of using the BtI and BtII *cytA* promoters, and the STAB-SD sequence (4), on Cry3A synthesis. To do this, we constructed vectors that expressed *cry3A* with (pPFT3As) and without (pPFT3A) the STAB-SD sequence, using *cytA* promoters to drive expression, and then compared the amount of Cry3A synthesized by using these vectors with the amounts produced by the wild-type DSM 2803 and mutant NB176 strains.

Plasmids pPFT3As and pPFT3A were constructed by cloning the *cry3A* gene with or without, respectively, the STAB-SD sequence into pHTCytA, a pHT3101 vector into which a 0.6-kb *Eco*RV fragment containing the *cytA* promoters had been cloned (Fig. 1). The *cry3A* gene and upstream STAB-SD sequence were isolated on a 2.98-kb *Hin*dIII fragment obtained from a *Hin*dIII digest of plasmids from strain DSM 2803. This fragment was cloned into pUC13, yielding plasmid pUC13Btt, and amplified in *E. coli* DH5α. The *cry3A* coding region with and without the STAB-SD sequence was obtained by PCR. For pPFT3As, *cry3A* with the STAB-SD sequence was obtained by using a 32-base oligomer, 5'-TC CCCCGGGATAATCTTGAAAGGAGGGATGCC-3', as the forward primer, and a 28-base oligomer, 5'-GAAGCTATAGAACGTTTAGAAAAACGTC-3', as the reverse primer. For pPFT3A, *cry3A* without the STAB sequence was obtained by using a 25-base oligomer, 5'-GGGAGGAAGAAAAATGAATCCGA AC-3', as the forward primer, and the same reverse primer used for the construct with the STAB-SD sequence. The 2.36- and 2.10-kb products obtained by PCR were treated with T4 DNA polymerase and T4 polynucleotide kinase to remove adenine residues, digested with *Sma*I, purified, and then ligated separately into the *Sma*I site of pHTCytA by using T4 DNA ligase, yielding, respectively, pPFT3As and pPFT3A (Fig. 1). Both plasmids were amplified in *E. coli* DH5α.

The recombinant plasmid constructs were confirmed by a combination of restriction enzyme analysis (24) and DNA sequencing. For the two most critical constructs, pPFT3A and pPFT3As, over 90% of the PCR products used to make these constructs, including all of the noncoding regulatory regions, were sequenced with M13-specific primers and a Li-Cor Automatic DNA Sequencer.

PCR. PCR were performed with the Expand Long Template PCR System (Boehringer GmbH, Mannheim, Germany) for 30 cycles as follows: 93°C for 1 min, 55°C for 1 min, and 72°C for 2 min.

Transformation of *B. thuringiensis* 4Q7. An overnight culture of 4Q7 was diluted 1:50 in 250 ml of brain heart infusion (BHI) with shaking at 30°C and grown until the optical density reached 0.7 at 600 nm. The cells were sedimented
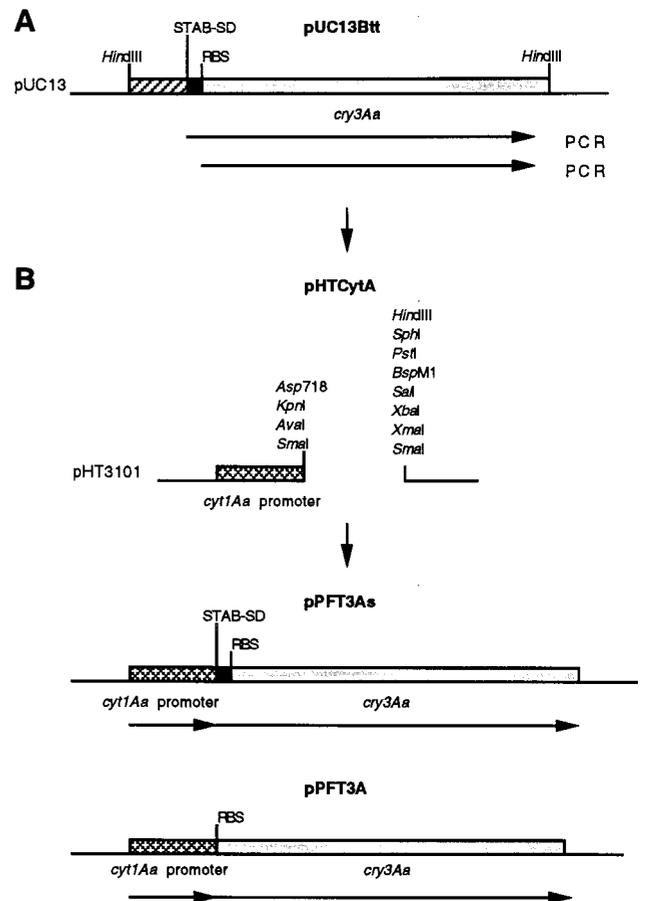

FIG. 1. Major steps in the construction of vectors for expressing the *cry3A* gene with and without the STAB-SD sequence under the control of *cyt1A* promoters. (A) A *Hin*dIII fragment from *B. thuringiensis* subsp. *morrisoni* (strain tenebrionis) containing the *cry3A* gene was cloned into pUC13, generating pUC13-Btt. (B) Copies of *cry3A* with and without the STAB-SD sequence were generated by PCR and cloned into the *Sma*I site downstream from the dual *cytA* promoters of the *E. coli-B. thuringiensis* shuttle vector pHT3101. This yielded the expression vectors pPFT3As and pPFT3A for expressing, respectively, *cry3A* with and without the STAB-SD sequence under the control of the dual sporulation-dependent *cytA* promoters.

by centrifugation, washed twice with sterile distilled water, and suspended in 40% polyethylene glycol 6000 at a density 100 times that of the original culture. Then 200 μl of this cell suspension was mixed with 1 to 5 μg of plasmid DNA and held on ice for 10 min. Electroporation was performed with a 0.2-cm electroporation cuvette (Invitrogen) in a Bio-Rad gene pulser apparatus set at 400 W and 1.6 kV with the pulse controller at 25 μF. After the pulse, the culture was added to 2 ml of prewarmed BHI and incubated with gentle shaking for 3 h at 30°C. Transformed cells were plated on BHI supplemented with 25 μg of erythromycin per ml for growth and sporulation.

Selection of growth media. To select an optimal growth medium, growth and toxin yields for the nontransformed and transformed bacterial strains were initially compared by using the following four liquid media: glucose-yeast-salts (GYS) [0.1% glucose, 0.2% yeast extract, 0.05% K$_2$HPO$_4$, 0.2% (NH$_4$)$_2$SO$_4$, 0.002% MgSO$_4$, 0.005% MnSO$_4$, 0.008% CaCl$_2$], peptonized milk (1% peptonized milk [BBL Microbiology Systems], 1% dextrose, 0.2% yeast extract, 1.216 mM MgSO$_4$, 0.072 mM FeSO$_4$, 0.139 mM ZnSO$_4$, 0.118 mM MnSO$_4$), G-Tris [0.08% CaCl$_2$, 0.0025% FeSO$_4$, 0.005% CuSO$_4$, 0.005% ZnSO$_4$, 0.05% MnSO$_4$, 0.2% MgSO$_4$, 2% (NH$_4$)$_2$SO$_4$, 2% glucose, 0.5% 1 M Tris (pH 7.5), 0.15% yeast extract, 0.5% K$_2$HPO$_4$], and nutrient broth (0.8% nutrient broth medium [Difco]). Growth was measured by determination of the number of spores and CFU produced per ml, whereas toxins yields were determined by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE).

Estimation of spore yields. To estimate the number of spores formed per milliliter of culture medium, cells were grown in 50 ml of GYS, peptonized milk, G-Tris, or nutrient broth medium in 250-ml flasks shaken at 250 rpm for 5 days at 30°C. A 1-ml volume of culture broth was then placed in a 1.5-ml tube, heated

TABLE 1. Comparison of the growth efficiency of
B. thuringiensis strains in four media

Strain	No. of CFU/ml[a] in:			
	GYS[b]	Peptonized milk	G-Tris	Nutrient broth
4Q7	5.3×10^7 a	8.0×10^8	3.4×10^8	7.0×10^6
4Q7/pHT3101	4.3×10^7 b	5.9×10^8	3.1×10^8	2.2×10^6
DSM 2803	9.7×10^7 c	5.2×10^8	5.8×10^7	9.7×10^5
NB176	3.8×10^7 d	2.2×10^8	3.6×10^7	4.1×10^6
4Q7/pPFT3A	3.5×10^7 e	1.4×10^8	1.9×10^7	4.1×10^5
4Q7/pPFT3As	2.8×10^7 d	1.2×10^8	1.1×10^7	3.4×10^5

[a] Standard deviations ($\pm$) were as follows: GYS (4Q7, 2.5×10^6; 4Q7/pHT3101, 2×10^6; DSM 2803, 1.1×10^6; NB176, 2.9×10^6; 4Q7/pPFT3A, 4.6×10^6; 4Q7/pPFT3As, 2.0×10^6), peptonized milk (4Q7, 2.5×10^8; 4Q7/pHT3101, 1.8×10^7; DSM 2803, 1.9×10^7; NB176, 1.7×10^7; 4Q7/pPFT3A, 8.9×10^5; 4Q7/pPFT3As, 1.1×10^6), G-Tris (4Q7, 4.4×10^6; 4Q7/pHT3101, 1.9×10^6; DSM 2803, 4.5×10^6; NB176, 9.2×10^5; 4Q7/pPFT3A, 8.9×10^5; 4Q7/pPFT3As, 1.1×10^6), and nutrient broth (4Q7, 3×10^5; 4Q7/pHT3101, 1.3×10^5; DSM 2803, 1.4×10^5; NB176, 2.9×10^5; 4Q7/pPFT3A, 4.3×10^4; 4Q7/pPFT3As, 4.3×10^4).
[b] Values followed by different letters were significantly different at $P = 0.05$.

at 60°C for 20 min (1), diluted, and plated on nutrient agar. Colonies were counted after 12 h of growth at 30°C, and data were analyzed with the Super ANOVA program (Abacus Concepts, Berkeley, Calif.).

Quantification of Cry3A yields. The amount of Cry3A synthesized by each strain was quantified primarily by SDS-PAGE. In addition, electron microscopy was used to determine crystal dimensions and to calculate crystal volumes. For quantification by SDS-PAGE, each strain was grown at 30°C for 5 days in 50 ml of GYS medium in 250-ml flasks with shaking at 250 rpm. After sporulation and cell lysis, the spores, Cry3A crystals, and cellular debris were pelleted by sedimentation at $4,000 \times g$ for 15 min. Each pellet was suspended in 1 ml of double-distilled water, after which a 5-μl sample of each was disrupted in Laemmli sample buffer and boiled for 5 min until the pellet was completely dissolved. Proteins were separated by subjecting 5-μl samples to electrophoresis through a 7.5% gel as described by Laemmli (18). The gels were stained with 0.125% Coomassie blue R-250, destained, and dried, and the protein bands were scanned by the GAS 4000 gel documentation system (Evergene). The amount of Cry3A protein in each band was quantified with ImageQuant 4.1 densitometry software (Molecular Dynamics, Sunnyvale, Calif.). Ratios of toxin production were calculated by giving the amount of Cry3A produced by the wild-type DSM 2803 strain a value of 1 and then expressing the production of other strains as a multiple of this value. Samples from at least three different cultures of each strain were used to calculate Cry3A yields.

To validate the above comparative method of determining relative yield increases, a standard curve for quantifying the amount of Cry3A protein on SDS-containing gels was established by using Cry3A crystals from the DSM 2803 strain purified on a sucrose step gradient. After purification and washing, crystals were dissolved, a dilution series was made, and the amount of protein was quantified by the Bradford method (Bio-Rad). Samples containing different concentrations of Cry3A were then subjected to SDS-PAGE, and the resulting gel was scanned to establish a standard curve.

Microscopy. For light microscopy, sporulating cultures were monitored with a Zeiss Photomicroscope III, using a 100× oil immersion objective. For transmission electron microscopy, sporulated cells from liquid cultures were collected just before lysis, pelleted, fixed for 2 h in 3% phosphate-buffered glutaraldehyde, postfixed in 1% OsO_4, dehydrated in ethanol-propylene oxide, and embedded in Epon-Araldite (17). Ultrathin sections of sporulated cells and purified inclusions were examined and photographed in a Hitachi 600 electron microscope operating at an accelerating voltage of 75 kV. Crystal dimensions were measured on electron micrograph negatives and used to calculate volumes. For each strain, at least 15 crystals were measured for the determination of each dimension.

Bioassays. For bioassays, a 1-μl droplet of 22% sucrose with a known quantity of Cry3A solubilized from each test preparation was applied to a 4-mm-diameter hybrid poplar (*Populus* X *euramericana* 'Eugenii') leaf disc on top of 2% agar (Gelcarin) in 24-well tissue culture plates. Second instars from a laboratory colony of the cottonwood leaf beetle, *Chrysomela scripta*, were placed in each well, one per well, held for 24 h, and then transferred to fresh foilage. Cry3A was solubilized [in 10 mM $NH_4(CO_3)_2$] from lyophilized powders of sporulated preparations of the test strains and quantified by the Bradford method (Bio-Rad). Control buffers were either 50 mM Na_2CO_3–10 mM dithiothreitol (pH 10.5) or 10 mM $NH_4(CO_3)_2$–10 mM EDTA (pH 10.4). The 50% lethal concentrations (LC_{50}s) were calculated 96 h after treatment by using a minimum of 12 larvae per concentration and six dilutions per toxin, replicated three times.

Statistical analyses. Spore count data and the dimensions and volumes of crystals were analyzed by using Tukey's multiple comparison of means test with

the Super ANOVA program. For the determinations of Cry3A toxicity, maximum-likelihood estimates of LC_{50}s were calculated by probit analysis (POLO-PC; LeOra Software, Berkeley, Calif.).

RESULTS

Selection of growth medium. In the medium comparison tests, peptonized milk and GYS yielded the highest spore counts (Table 1). However, the yield of Cry3A for the transformed pPFT3As and NB176 strains was much higher with GYS medium than with peptonized milk (Fig. 2). Therefore, GYS was used for the remainder of these studies.

Effect of *cytA* promoters on the Cry3A yield. The yield of Cry3A obtained per milliliter of GYS with pPFT3A, which used *cytA* promoters to drive *cry3A* expression but lacked the STAB-SD sequence, was substantially higher than the yield obtained with the wild-type DSM 2803 strain but was not nearly as high as the yield obtained with the mutant NB176 strain (Fig. 3). By using the yield of Cry3A produced by DSM 2803 as a standard with a value of 1, analysis by SDS-PAGE showed that Cry3A production by the pPFT3A-transformed strain of *B. thuringiensis* 4Q7 was 2.3-fold that by DSM 2803 whereas Cry3A production by the NB176 strain was 8.8-fold that by DSM 2803 (Fig. 3). In sporulated cultures of DSM 2803, the Cry3A inclusions were comparatively small and difficult to detect until the cells had lysed (Fig. 4A). In 4Q7 cells transformed with pPFT3A, however, a distinct Cry3A crystal was easily observed, which in cross section generally appeared larger than the crystals in DSM 2803 cells (Fig. 4B). Crystals in the NB176 strain were also easily observed at or after cell lysis (Fig. 5A). Many cells in this strain did not, however, produce spores. By treating the Cry3A inclusion as a rectangular solid, the calculated mean crystal volumes produced by DSM 2803 and pPFT3A were 0.100 and 0.132 μm^3, respectively (Table 2), making the latter crystals approximately 1.3-fold larger than the wild-type crystals. The crystal volume for the NB176 strain was 0.236 μm^3, making these about 2.4-fold larger than the DSM 2803 crystals, and 1.8-fold larger than the crystals produced by the *cry3A* gene under the control of *cytA* promoters (Table 2).

Effect of *cytA* promoters combined with the STAB-SD sequence on Cry3A yield. Inclusion of the STAB-SD sequence in
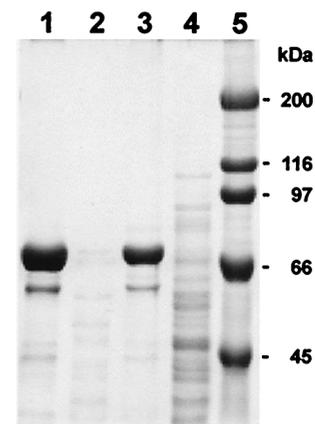

FIG. 2. Comparison of Cry3A yields by SDS-PAGE for two test media. Lanes: 1 and 2, *B. thuringiensis* 4Q7 transformed with pPFT3As grown, respectively, in GYS and peptonized milk; 3 and 4, NB176, the mutant strain of *B. thuringiensis* subsp. *morrisoni* (strain tenebrionis) with a higher *cry3A* copy number, grown, respectively in GYS and peptonized milk; 5, molecular mass markers. Pelleted patriculates from equal volumes of culture media (GYS or peptonized milk) in which test strains were grown were loaded in each lane.
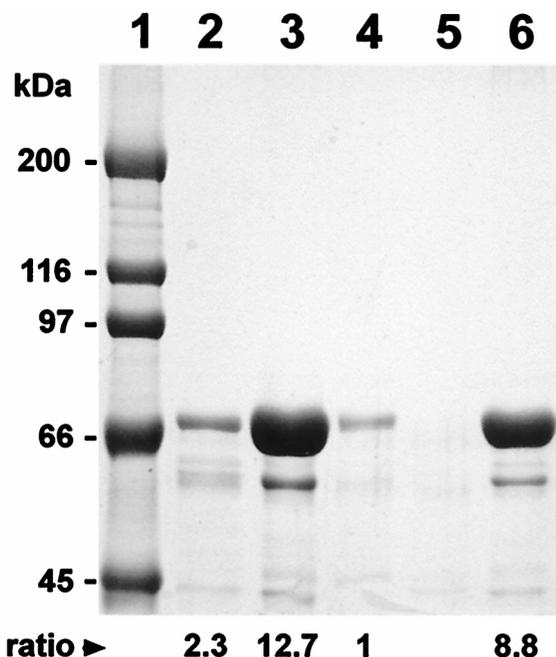
FIG. 3. Analysis of Cry3A production of wild-type, mutant, and engineered strains of *B. thuringiensis* by SDS-PAGE. Sedimented crystals, spores, and cellular debris obtained from equal volumes of culture medium at the end of sporulation were loaded into each lane. Lanes: 1, molecular mass markers; 2, *B. thuringiensis* 4Q7 transformed with pPFT3A (*cry3A* without the STAB-SD sequence under the control of *cytA* promoters); 3, 4Q7 transformed with pPFT3As (*cry3A* with the STAB-SD sequence under the control of *cytA* promoters); 4, wild-type *B. thuringiensis* subsp. *morrisoni* (strain tenebrionis) DSM 2803; 5, 4Q7 transformed with pHT3101; 6, NB176, the mutant strain of *B. thuringiensis* subsp. *morrisoni* (strain tenebrionis) with a higher *cry3A* copy number. The ratios at the bottom of the lanes were determined by densitometry scanning of the gel; they indicate the ratio of Cry3A per unit of GYS in comparison to that produced by the DSM 2803 strain.

the vector pPFT3As used to drive expression of *cry3A* with *cytA* promoters had a very marked effect on the yield of Cry3A (Fig. 3 to 5; Table 2). Analysis of Cry3A yields by SDS-PAGE showed that pPFT3As-transformed 4Q7 cells grown on GYS medium produced 12.7-fold as much Cry3A per unit of medium as the wild-type DSM 2803 strain did and 1.4-fold as much as the mutant NB176 strain did (Fig. 3). The high level of additional Cry3A production obtained by including the STAB-SD sequence in the construct resulted in much larger Cry3A crystals than those observed in DSM 2803, NB176, or 4Q7 cells transformed with pPFT3A (Fig. 4 and 5; Table 2). These large crystals were rectangular to rhomboidal and were easily observed in sporulated cells, where they occupied most of the cell (Fig. 4C and D). The Cry3A crystals in pPFT3As-transformed cells were so large that the spore was often dislocated to the cell periphery and the cell shape was essentially distorted to take on the shape of the enclosed crystal (Fig. 4C and D). In general, the Cry3A crystals produced by pPFT3As were much longer, wider, and thicker than those produced by DSM 2803 and thicker than those produced by NB176 (Fig. 5; Table 2). The calculated volume of Cry3A crystals produced by pPFT3As in these cells was 0.691 μm^3, making these crystals sevenfold larger than Cry3A crystals produced by the wild-type DSM 2803 strain and 1.9-fold larger than the crystals produced by the NB176 strain (Table 2).

Effect of Cry3A synthesis on bacterial reproduction. To determine whether there was a relationship between increased levels of Cry3A synthesis and reproduction of the bacterial strains, spore counts were made for all strains. These tests revealed that bacterial reproduction on GYS was reduced by 60 to 70% in all strains which produced higher levels of Cry3A per cell than DSM 2803 (Table 1). However, most of this reduction was accounted for by strain differences and transformation, not Cry3A synthesis. For example, the DSM 2803 strain gave 9.7×10^7 CFU/ml whereas transformed strains that produced higher yields of Cry3A per cell gave in the range of
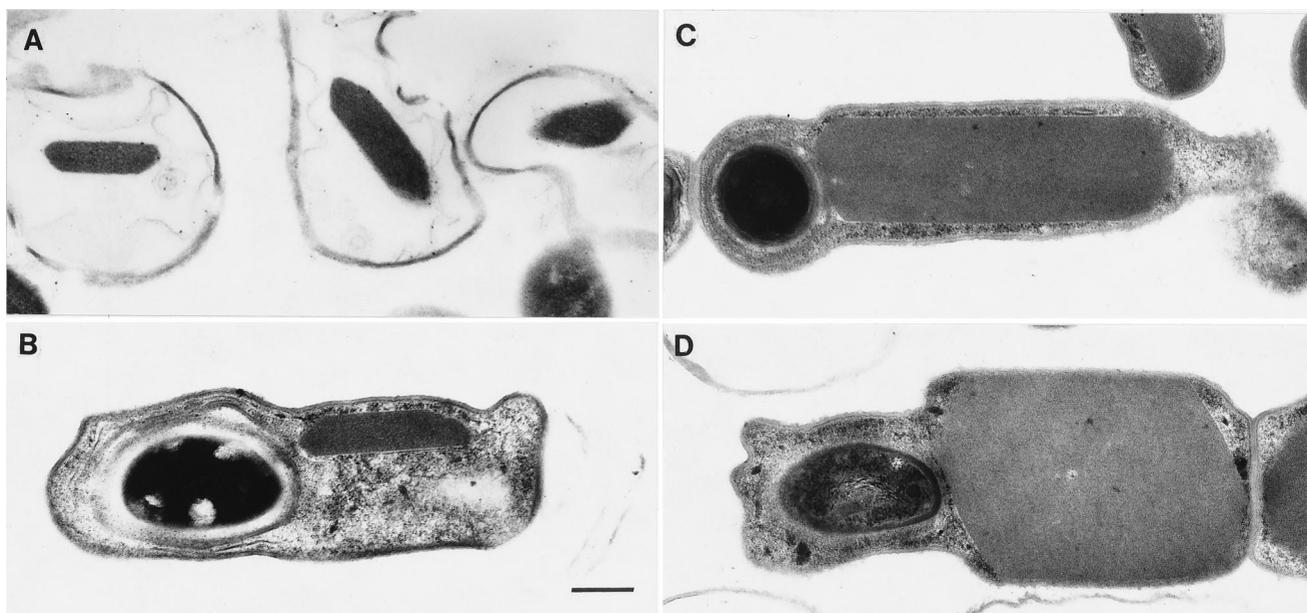

FIG. 4. Electron micrographs of sporulated wild-type and engineered strains of *B. thuringiensis*, illustrating crystals typical of these strains. (A) Wild-type *B. thuringiensis* subsp. *morrisoni* (strain tenebrionis) DSM 2803. (B) Acrystalliferous strain (4Q7) of *B. thuringiensis* subsp. *israelensis* transformed with pPFT3A (*cry3A* without the STAB-SD sequence under the control of *cytA* promoters). (C and D) Cross section (C) and sagittal section (D) through 4Q7 cells transformed with pPFT3As (*cry3A* with the STAB-SD sequence under the control of *cytA* promoters). All micrographs are at the same magnification. Bar, 300 nm.
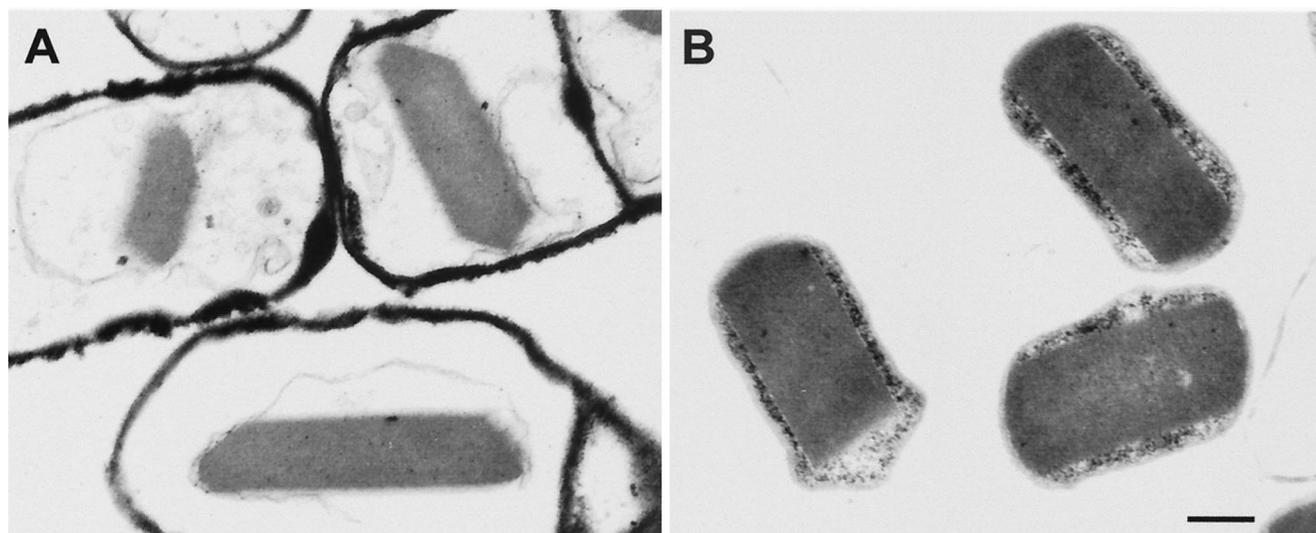
FIG. 5. Electron micrographs comparing the thickness of typical Cry3A crystals produced by NB176 and the acrystalliferous 4Q7 strain of *B. thuringiensis* subsp. *israelensis* transformed with pPFT3As. (A) NB176. (B) 4Q7/pPFT3As. Both micrographs are the same magnification. Bar, 300 nm.

2.8×10^7 to 3.5×10^7 CFU/ml. However, the values for the nontransformed acrystalliferous 4Q7 strain and for the 4Q7 strain transformed with the pHT3101 vector alone, i.e., without the *cry3A* gene, were not much higher, being in the range of 5.3×10^7 and 4.3×10^7 CFU/ml, respectively (Table 1). When the effect of increased Cry3A synthesis was examined within the same bacterial strain, a high level of Cry3A synthesis reduced the bacterial count by about 20%. More specifically, 4Q7 transformed with pPFT3A yielded 3.5×10^7 CFU/ml and the same strain transformed with pPFT3As yielded 2.8×10^7 CFU/ml (Table 1).

Toxicity of Cry3A crystals. In bioassays of Cry3A solubilized from lyophilized powders, the LC_{50}s against second instars of the cottonwood leaf beetle were 3.4 ng of solubilized protein per mm^2 of leaf disc for DSM 2803 and 4.8 ng/mm^2 for 4Q7 cells transformed with pPFT3As (Table 3). These values were not significantly different statistically, demonstrating that the quality of the Cry3A produced in the highest yielding strain was similar to that of the wild-type strain. The amount of Cry3A obtained by solubilization of the powder, however, was much larger for the protein from the pPFT3As strain than from DSM 2803 (Table 3), probably due to the larger amount of toxin produced per cell.

DISCUSSION

Previous studies have shown that substantial increases in Cry3A yields are obtained by gene amplification and expression of *cry3* genes in asporogenous mutants. By introducing the wild-type *cry3A* gene into an asporogenic *B. thuringiensis* mutant, increases in the yield of as much as fivefold were obtained (21). In this expression system, the vector used to transform the nonsporeforming *B. thuringiensis* strain was pHT315, a plasmid which typically has a copy number of 10 to 15 copies per cell (19), a copy number severalfold higher than that of most plasmids bearing toxin genes. Since expression of the *cry3A* gene in wild-type strains generally produces small crystals and there was no direct alteration of the gene to enhance expression, the increased yield obtained by using pHT315 in an asporogenic strain was probably due to increases in the *cry3A* copy number and to the absence of the spore. The latter would

make amino acids and energy sources that normally went into spore production available for additional Cry3A synthesis and would provide a prolonged stationary phase that could further increase yields (22). A gene amplification mechanism was also responsible for the two- to threefold and five- to sevenfold increases obtained, respectively, with an irradiated strain of *B. thuringiensis* and the same strain in which an additional copy of *cry3A* was introduced into the chromosome (1). In the studies reported here, yields similar to and higher than those resulting from expression of *cry3* genes in asporogenous mutants and gene amplification were obtained by using the wild-type gene containing the STAB-SD sequence in combination with dual *cytA* promoters to drive *cry3A* expression. Evaluation of the constructs with and without the STAB-SD sequence showed that this sequence was essential to obtaining the marked increase in Cry3A yield (Fig. 3 and 4). The increased levels of Cry3A synthesis would therefore appear to be due primarily to increased stabilization of the ribosome-transcript complex (4). The use of dual *cytA* promoters, in combination with the STAB-SD sequence and *cry3A*, provides a direct and alternative method for producing very high yields of Cry3A.

The Cry3A crystals produced by combining dual sporulation-dependent promoters with the STAB-SD sequence are of

TABLE 2. Dimensions and volumes of Cry3A crystals produced by different strains[a]

Strain	Length (μm)	Width (μm)	Thickness (μm)	Vol[b] (μm³)
DSM 2803	0.701 ± 0.143	0.701 ± 0.143	0.187 ± 0.031	0.100 ± 0.054 a
NB176	0.941 ± 0.171	0.941 ± 0.171	0.280 ± 0.060	0.236 ± 0.138 b
4Q7/pPFT3A	0.817 ± 0.121	0.817 ± 0.121	0.193 ± 0.026	0.132 ± 0.047 a
4Q7/pPFT3As	1.555 ± 0.273	1.024 ± 0.098	0.434 ± 0.092	0.691 ± 0.242 c

[a] Means for each crystal dimension were calculated separately, whereas the volumes for those of DSM 2803 ($n = 17$), NB176 ($n = 20$), and 4Q7/pPFT3A ($n = 19$), were calculated for individual crystals by assuming that the length and width of each crystal were equal. Therefore, multiplying the mean dimensions for these three strains will yield values slightly different from those presented in the Volume column. The length and width of crystals produced by 4Q7/pPFT3As, however, were not equal, and thus the volume value is the product of the mean dimensions.

[b] Values followed by different letters were significantly different at $P = 0.05$.

TABLE 3. Toxicity of Cry3A produced by DSM 2803 and 4Q7/pPFT3As to *C. scripta*[a]

Strain	Mean LC$_{50}$ (ng/mm^2)[b] (95% fiducial limits)	Slope (± SE)	Amt of Cry3A (% of solubilized protein)[c]
DSM 2803	3.4 (1.75–5.65)	1.88 ± 0.37	7.7
4Q7/pPFT3As	4.8 (3.25–6.83)	1.82 ± 0.25	52.9

[a] Cry3A was solubilized from lyophilized powders of each strain grown on GYS medium and assayed against second instars feeding on leaf discs.
[b] These values are not significantly different.
[c] This value represents the proportion that Cry3A constituted of the total protein solubilized from the lyophilized powders of these strains.

TABLE 4. Calculated crystal yields per unit of culture medium for different Cry3A strains

Strain	Bacterial count (CFU/ml)[a]	Crystal vol/cell (μm^3)	Total crystal vol/ml (μm^3)	Ratio
DSM 2803	9.71×10^7	0.100	9.7	1.0
4Q7/pPFT3A	2.84×10^7	0.132	3.7	0.4
4Q7/pPFT3As	3.54×10^7	0.691	24.6	2.6
NB176	3.75×10^7	0.236	8.9	1.0

[a] Most of the colonies obtained resulted from viable spores.

interest because these crystals are similar in size to, or even larger than, the bypyramidal crystals formed by Cry1 proteins of *B. thuringiensis*. This can be demonstrated by comparing the calculated volumes of Cry3A crystals produced by pPFT3As with Cry1A crystals (19). An average measurement for bipyramidal crystals is 2 μm in length by 0.9 μm in width at the widest point. By considering the widest point as the base (*B*) of a square pyramid, with the height (*H*) being half the length of the crystal, the volume (*V*) of the bipyramidal crystal is equal to $2B^2H/3$. This yields a volume of 0.54 μm^3. By comparison, if the volume of a Cry3A crystal produced by pPFT3As, such as those illustrated in Fig. 4 and 5, is considered to be the volume of a rectangular solid, then $V = $ length (*L*) × width (*W*) × thickness (*T*). Using the average values for the Cry3A crystals obtained with pPFTAs, the volume is 1.555 (*L*) × 1.024 (*W*) × 0.434 (*T*) μm, which is 0.691 μm^3, a volume 1.28-fold that of the Cry1A bipyramidal crystal. Variation in the size of bipyramidal crystals occurs with the same strain grown on different media, as well as among different strains and subspecies of *B. thuringiensis*. Thus, these calculations are made only to show that the amount of endotoxin protein obtained per cell with the *cytA*/STAB-SD expression system is comparable to, if not greater than, that produced by *B. thuringiensis* strains that form typical bipyramidal crystals. A critical difference, however, is that the Cry3A crystals are almost pure toxin, since the Cry3A molecule lacks the nontoxic C terminus of Cry1 molecules, whereas only approximately 50% of the typical Cry1 molecule is toxin. Large Cry3A crystals, comparable in size to those produced by pPFT3As, have also been reported in two previous studies (8, 13), but the genetic basis for their large size was not determined.

Another observation made in the present study that is relevant to potential improvement in the insecticidal efficacy of Cry3A strains was that although the toxin yields were higher per unit volume of medium, especially with pPFT3As (Fig. 1), the number of bacteria per milliliter, due primarily to spores, was much smaller than that obtained with the DSM 2803 and slightly smaller than those obtained with NB176 (Table 1). This results in a higher endotoxin-to-spore ratio, which should further increase the toxicity per unit weight of fermentation medium. Although this has not been tested directly, evidence for such an effect comes from our bioassays. The toxicity of Cry3A obtained from 4Q7 transformed with pPFT3As was comparable to that of DSM 2803, but the yield of solubilized protein, most of which was Cry3A (data not shown), per weight of lyophilized powder obtained with the transformed strain was much higher (Table 3). Thus, if the *cytA*/STAB-SD expression system is shown to be commercially viable for the production of *B. thuringiensis* products, added value should come from the increased efficacy brought about by the higher toxin-to-spore ratios, assuming that the crystals fully dissolve, which may not

always be the case, and by the reduced application of spores to the environment, over which there remains some concern.

The use of *cytA* promoters in combination with the STAB-SD sequence clearly increased the Cry3A yield in comparison to that for the DSM 2803 strain, by about sevenfold with respect to crystal size (Fig. 3; Table 2) and 12-fold with respect to Cry3A that could be solubilized (Fig. 2). However, there were discrepancies between these values, especially between the SDS-PAGE values and the calculated increases in Cry3A yield per milliliter of GYS medium based on CFU counts and measurements of crystal size. For example, estimating the crystal volume produced per milliliter by multiplying the crystal volume by the number of CFU per milliliter suggested only a 2.6-fold increase in the amount of toxin produced by pPFT3As in comparison to DSM 2803 and a calculated yield for the pPFT3A-transformed strain that was less than half that for DSM 2803 (Table 4). A similar discrepancy was found between DSM 2803 and the NB176 strain. Possible reasons for these discrepancies include differences in the solubility characteristics of the Cry3A crystals among these strains and lack of correlation between CFU counts and the actual number of crystals produced. For example, as noted above, the NB176 strain produced many cells that lacked spores but contained large Cry3A crystals. We did not specifically test the viability of the spores produced by 4Q7 cells transformed with pPFT3A or pPFT3As. However, in previous studies it has been shown that high levels of Cry3A synthesis are associated with decreases of 60 to 90% in spore counts and the numbers of viable spores (8, 13, 19). Nevertheless, even if the results reported here translated into only a 30 to 50% increase in the toxicity of technical materials produced during fermentation, they may still be of commercial value.

It is not known whether the combination of dual sporulation-dependent promoters and the STAB-SD sequence can be used to obtain high yields of other naturally truncated *B. thuringiensis* proteins such as Cry2A and Cry11A and truncated Cry1-type proteins, but this possibility is currently under evaluation.

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