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Engineering *Bacillus thuringiensis* Bioinsecticides with an Indigenous Site-Specific Recombination System

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The *cry* genes of *Bacillus thuringiensis* encode a diverse group of crystal-forming proteins that exhibit insecticidal activity, particularly against the larvae of lepidopteran, coleopteran, and dipteran insects. The efficacy of *B. thuringiensis*-based biopesticides may be improved through the genetic manipulation of these genes. A gene transfer system has been developed for the introduction and maintenance of cloned insecticidal *cry* genes on small plasmids in *B. thuringiensis*. This vector system combines a *B. thuringiensis* plasmid replicon and an indigenous site-specific recombination system that allows for the selective removal of ancillary or foreign DNA from the recombinant bacterium after introduction of the Cry-encoding plasmid. The site-specific recombination system is useful for engineering strains with unique combinations of *cry* genes, resulting in new active ingredients with improved insecticidal properties.

The gram-positive bacterium *Bacillus thuringiensis* is used worldwide as a biological pesticide for the control of various lepidopteran, dipteran, and coleopteran insect pests (32). The insecticidal activity of *B. thuringiensis* is due primarily to the proteinaceous crystals it produces during stationary and sporulation phase. Upon ingestion and solubilization of the crystals, the proteins contained therein are released into the insect midgut, where they act as stomach poisons, apparently by disrupting the osmotic regulation of insect midgut epithelial cells (26, 27). In commercial production, the spores and crystals obtained from fermentation are concentrated and formulated for spray-on application according to conventional agricultural practices.

The crystal (Cry) proteins of *B. thuringiensis*, most of which appear to be encoded on large plasmids (11, 31), comprise a remarkably diverse collection of active ingredients. The number of Cry proteins described in the scientific and patent literature has grown dramatically in recent years (42), including proteins that are active against nematodes (21) and aphids (45). An updated nomenclature system, based solely on amino acid sequence identity, has recently been proposed to accommodate this growth (16). In addition to sequence diversity, these proteins also exhibit significant differences in their spectrum of insecticidal activity (28) and, in some cases, may act synergistically with the spore (37, 38) or with each other (14, 15, 46).

Many of these active ingredients have yet to be exploited at the commercial level. Natural isolates of *B. thuringiensis*, though containing novel and useful Cry proteins may, nevertheless, exhibit inadequate activity for the particular market of interest and poor or inconsistent fermentation yields that preclude their use as cost-effective insecticides. Accordingly, the genetic manipulation of *cry* genes in *B. thuringiensis* is often desired to improve upon these characteristics. A conjugation-like system has been used to transfer Cry-encoding plasmids from one strain to another, resulting in transconjugant strains with improved insecticidal activity (25), but most *cry* genes are not readily transmissible via this process.

Cloned *cry* genes may be maintained in *B. thuringiensis* on recombinant plasmids or integrated into resident plasmids or the chromosome by homologous recombination (2, 4, 8, 12, 29, 33). With these approaches, combinations of Cry proteins can be designed rather than discovered and crystal protein production can be augmented by selecting host backgrounds suitable for large-scale fermentation. In addition, Cry proteins engineered for improved insecticidal activity, fermentation yield, or stability can be used as active ingredients, further expanding the possibilities for improving *B. thuringiensis*-based bioinsecticides.

Concerns have been raised about the ecological and health impact of disseminating genetically engineered microorganisms, particularly those containing antibiotic resistance genes (e.g., reference 24). In this paper, we describe a "homologous" expression vector system for introducing and expressing cloned *cry* genes in *B. thuringiensis*, using as an example the *cry3Bb* gene encoding the coleopteran-active crystal protein Cry3Bb (19). This vector system combines a *B. thuringiensis* plasmid replicon with an indigenous site-specific recombination system that allows for the selective removal of ancillary or foreign DNA from the recombinant bacterium after introduction of the Cry-encoding plasmid vector, thus eliminating the necessity of maintaining copies of an antibiotic resistance gene or any foreign DNA elements within the recombinant strain. Using this system, we have constructed a number of *B. thuringiensis* strains harboring unique combinations of crystal proteins not as yet found in nature, some of which exhibit improved insecticidal properties and/or improved crystal protein yield. As an example, we describe in this paper the construction of *Bacillus thuringiensis* subsp. *kurstaki* EG7673, a coleopteran-active strain which was approved as the active ingredient for Raven OF bioinsecticide by the U.S. Environmental Protection Agency in January 1995.

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MATERIALS AND METHODS

Bacterial strains and plasmids. *Escherichia coli* DH5α (Bethesda Research Laboratories), JM110, and Sure (Stratagene Corporation) and *B. thuringiensis* EG10368 were used as host strains for molecular cloning experiments. Strain EG10368, a HD73 derivative containing a cryptic 4.9-MDa plasmid, was kindly provided by William P. Donovan. Plasmid pEG911, containing the *cry3Bb* gene

TABLE 1. *B. thuringiensis* strains and plasmids

Strain or plasmid	Plasmid size (MDa)	Relevant characteristic	Reference or source
B. thuringiensis strains			
EG10368	4.9	Cry$^-$ host strain	W. P. Donovan
EG2424	130, 88[a], 60[b], 44[b], 43, 7.5, 5.4, 5.2, 5.0, 4.9		Ecogen, Inc.
EG7673	130, 88[a], 60[b], 44[b], 7.5, 5.4, 5.2, 5.0, 4.9, pEG930.9Δ (5.2)		This report
Plasmids			
p83		IRS plasmid	6, 7
pEG911		*cry3Bb* plasmid	5
pEG922		Tn*5401* vector	5
pEG928.9Δ		*cry1C-cry1Ac* plasmid	7
pEG930.9		*cry3Bb* plasmid	This report
pEG930.9Δ		*cry3Bb* plasmid	This report

[a] Contains a *cry3A* gene.
[b] Contains a *cry1Ac* gene.

(19) (formerly known as *cryIIIB2*), and plasmid p83 have been described previously (6). Other *B. thuringiensis* strains and plasmids are listed in Table 1.

DNA manipulations and analyses. Standard recombinant DNA procedures were performed essentially as described by Sambrook et al. (40). Transformation of *B. thuringiensis* was performed according to the electroporation procedure of Mettus and Macaluso (36). Plasmid DNAs were prepared from recombinant *B. thuringiensis* cultures grown in 1× brain heart infusion–0.5% glycerol (BHIG) at 30°C to mid-logarithmic phase by the alkaline lysis method (40). Plasmid DNAs were sequenced by the dideoxy-chain termination method (41) with the Sequenase 2.0 kit provided by U.S. Biochemical Corporation. Oligonucleotides were obtained from Integrated DNA Technologies, Inc. (Coralville, Iowa), or Ransom Hill Bioscience, Inc. (Ramona, Calif.). PCRs were performed in a Perkin-Elmer thermocycler (Perkin-Elmer Cetus) with either *Taq* polymerase (Perkin-Elmer Cetus) or Vent polymerase (New England Biolabs) and the following parameters: denaturation, 30 s at 94°C; annealing, 30 s at 46°C; and extension, 1 min at 70°C for 30 cycles. All ramp times were set at 1 s. Primer concentration in the PCR was 20 μM. Primer pr10 (5′-CTTCTTGAGATAAGCTAG-3′) corresponds to nucleotides 839 to 822 of Tn*5401* (5). Primer pr50 (5′-GCACTAAT AAGAGTATTTATAGGTG-3′) corresponds to nucleotides 4266 to 4290 within the *cry1F* 3′ region (13). Hybridization probes for Southern blot analysis were prepared by the random primer method of Feinburg and Vogelstein (20).

Construction of *ori43* copy-number variants. To isolate copy-number variants of *ori43*, the *B. thuringiensis-E. coli* shuttle vector pEG854 (8) was subjected to hydroxylamine mutagenesis as described by Smith et al. (44) and introduced into strain EG10368 via electroporation. Colonies were selected on Luria plates containing 50 μg of chloramphenicol per ml. Transformants were confirmed by restriction enzyme analysis of their plasmid DNAs. Copy-number variants were characterized by growing the transformants and the control strain EG10368/pEG854 to 150 Klett units (red filter) in BHIG at 30°C, extracting their plasmids, digesting the plasmid DNAs with *Sal*I, and resolving the DNA fragments on an agarose gel. A serial dilution of bacteriophage lambda DNA digested with *Hin*dIII was used as a standard to estimate relative plasmid copy number by densitometry using a model 300A Molecular Dynamics computing densitometer. Variant pEG854.9, containing *ori43.9*, exhibited a copy number two to three times that of pEG854 in strain EG10368. Sequence analysis of *ori43.9* revealed four transition mutations compared with the wild-type *ori43* sequence (top strand [9]): C56T, C529T, C611T, and G2749A.

Plasmid stability studies. The segregational stability of plasmid pEG930.9Δ in strain EG7673 was assessed in small-scale fermentation with a 1.25-liter BIOFLO III fermentor (New Brunswick Scientific, Edison, N.J.) and a soy flour-based medium. A fresh single colony isolated from a Luria plate was used to inoculate a 5-ml shake-flask culture containing BHIG. This culture was grown for 6 h at 30°C, and 1 ml of the turbid culture was used to inoculate a 500-ml shake-flask culture containing BHIG. After overnight growth at 30°C, 260 μl of the culture was used to inoculate a 1-liter fermentor. The fermentation of strain EG7673 was allowed to sporulate. The sporulated culture was then used as an inoculum for a second 1-liter fermentation. Following the second fermentation, diluted samples were heat treated at 65°C for 1 h to kill vegetative cells and plated onto nutrient agar plates to isolate single colonies arising from germinating spores. Colonies were patched onto nutrient agar plates and allowed to sporulate at 30°C. Colonies containing pEG930.9Δ could be distinguished either by restriction enzyme analysis of the plasmid DNA or by phase-contrast microscopy since the Cry3Bb protein encoded by plasmid pEG930.9Δ forms a distinctive oval-shaped inclusion (see Fig. 4). Colonies that had lost plasmid pEG930.9Δ displayed the relatively small rhomboid crystal characteristic of the Cry3A protein (10). Plasmid loss frequencies were estimated as described by Arantes and Lereclus (4).

Cry protein quantitation. *B. thuringiensis* cultures were grown in C2 medium (18) at 28 to 30°C for 3 days or until fully sporulated and lysed. The Cry proteins were resolved on sodium dodecyl sulfate (SDS)–10% polyacrylamide gels, visualized by Coomassie blue staining, and quantitated by densitometry using a model 300A Molecular Dynamics computing densitometer and purified Cry1Ac and Cry3A proteins or bovine serum albumin as a standard. Electron micrographs of sporulating EG7673 cells were prepared by the Electron Microscopy Center at Pennsylvania State University.

Insect bioassay. Activity against coleopteran larvae was determined via surface contamination of an artificial diet in a plastic feeding cup (175-mm^2 surface) as described by Rupar et al. (39). One neonate larva was placed in each cup and scored for mortality after 3 to 5 days (*Leptinotarsa decemlineata*) or 7 days (*Diabrotica undecempunctata*). Fifty percent lethal concentrations were determined by probit analysis as described by Daum (17) with Abbott's correction (1), employing an eight-dose testing procedure with 32 larvae per dose.

RESULTS

Site-specific recombination systems are useful tools for genome engineering since they can be used to catalyze deletions, insertions, and even chromosomal exchanges in vivo (30). The objective of this study was to develop such a system for engineering Cry-encoding *B. thuringiensis* plasmids in vivo with the site-specific recombination system encoded by the *B. thuringiensis* class II transposon Tn*5401* (5, 6). The TnpI recombinase of Tn*5401* encodes a 36-kDa protein that resolves cointegrate intermediates formed during Tn*5401* transposition. This recombination event occurs within a 111-bp region, referred to as an internal resolution site (IRS), located immediately upstream of the TnpI coding region (6). This site-specific recombination system, TnpI and its cognate IRS, was used to design a shuttle vector based on the *B. thuringiensis* plasmid replication origin *ori43* (9).

Construction of the *cry3Bb* plasmid pEG930.9. Plasmid p83 (6, 7) was used as the starting material for these cloning experiments, which are outlined in Fig. 1. Plasmid p83 contains two copies of the Tn*5401* IRS region, the copy located 3′ to the *tet* gene extending from nucleotides 107 to 768 of Tn*5401* and the copy located 5′ to the *tet* gene extending from nucleotides 219 to 926 of Tn*5401* (5). This plasmid has been shown to function as a substrate for TnpI-mediated recombination in vivo (6) and in vitro (4a). Two DNA fragments were isolated from p83: a ~3.5-kb *Sal*I-*Bln*I fragment containing the *B. thuringiensis* plasmid replication origin *ori43* and one copy of the IRS and a ~2.5-kb *Sst*I-*Sal*I fragment containing the *tet* gene and one copy of the IRS (Fig. 1). These fragments were subcloned together into the *Xba*I and *Sst*I sites of the *E. coli* cloning vector pTZ19u to yield plasmid p84. This cloning step placed the pTZ19u and *tet* gene segments between duplicate copies of the IRS. Plasmid p84 was cleaved with *Asp*718 and

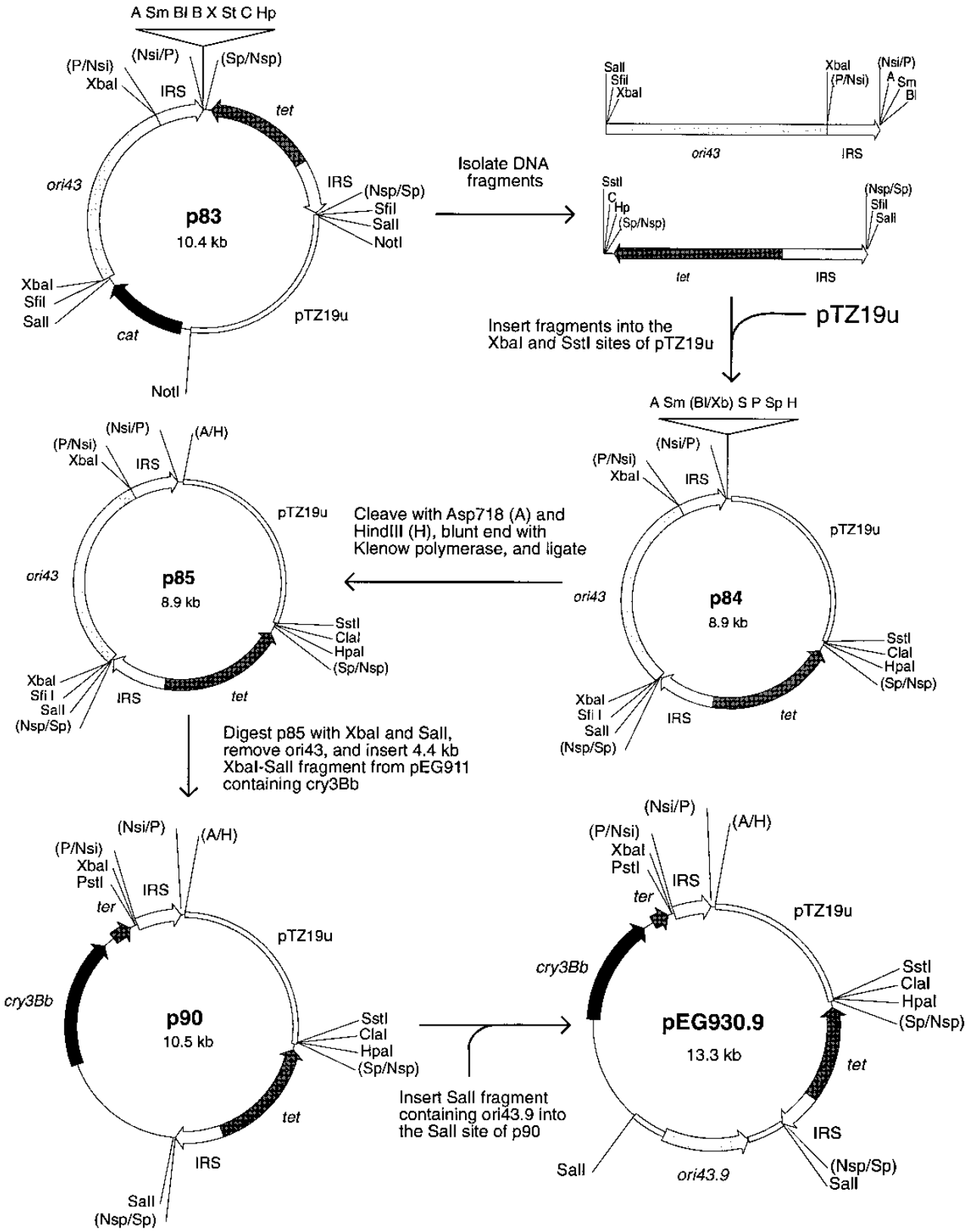

FIG. 1. Construction of the *cry3Bb* plasmid pEG930.9. Restriction endonuclease site abbreviations: A, *Asp718*; B, *Bam*HI; Bl, *Bln*I; C, *Cla*I; H, *Hin*dIII; Hp, *Hpa*I; Nsi, *Nsi*I; Nsp, *Nsp*I; P, *Pst*I; S, *Sal*I; Sm, *Sma*I; St, *Sst*I; Sp, *Sph*I; X, *Xho*I; Xb, *Xba*I. Other designations: *cat*, chloramphenicol acetyltransferase gene; *tet*, tetracycline resistance gene; *ori43*, *B. thuringiensis* plasmid replication origin; *ori43.9*, a copy-number variant of *ori43*; IRS, fragments containing the IRS of Tn*5401*; *ter*, *cry1F* transcription terminator.

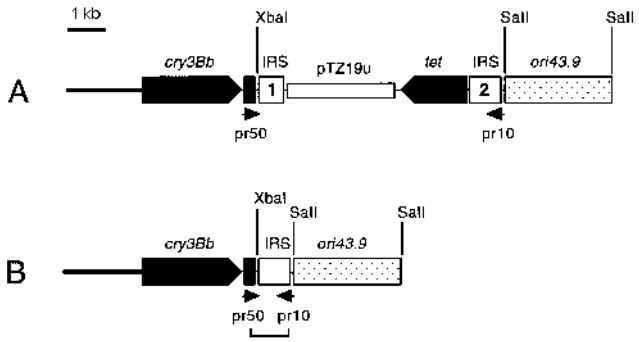

FIG. 2. Structural maps of the *cry3Bb* plasmids before (A) (pEG930.9) and after (B) (pEG930.9Δ) TnpI-mediated recombination. The approximate location and orientation of primers pr10 and pr50 are indicated by the arrows. Amplification of the IRS region of pEG930.9Δ with pr10 and pr50 should yield a 794-bp fragment. The two IRS regions of pEG930.9 are labeled 1 and 2. Other designations are described in the legend to Fig. 1.

*Hin*dIII, the ends were rendered blunt with Klenow polymerase, and the ends were then ligated together with T4 ligase to yield plasmid p85. This step eliminated several unwanted restriction sites.

To construct plasmid pEG930.9, the *cry3Bb* gene from plasmid pEG911 (5) was inserted into plasmid p85 by cleaving p85 with *Xba*I and *Sal*I, removing the 2.8-kb *ori43* fragment, and inserting a 4.4-kb *Xba*I-*Sal*I fragment containing *cry3Bb* to yield plasmid p90. The *ori43.9* replication origin was isolated as a ~2.85-kb *Sal*I fragment from the shuttle vector pEG854.9 (see Materials and Methods) and inserted into the unique *Sal*I site of p90 as shown to yield plasmid pEG930.9 (Fig. 1 and 2). This copy-number variant of *ori43* was used to ensure high levels of Cry3Bb production in recombinant strains.

Plasmid pEG930.9 was introduced into the acrystalliferous strain EG10368 by an electroporation procedure (36) and selection for tetracycline-resistant colonies on Luria plates containing 20 μg of tetracycline per ml. The integrity of plasmid pEG930.9 was confirmed by restriction enzyme analysis of the plasmid DNAs isolated from the transformants. One transformant was designated EG10368/pEG930.9. Upon sporulation, this recombinant strain produced a large oval-shaped inclusion and yielded a ~65-kDa protein on SDS-polyacrylamide gels, as expected for the Cry3Bb protein (19).

Construction of strain EG7673. *B. thuringiensis* subsp. *kurstaki* EG2424, the active ingredient in Foil OF bioinsecticide, was chosen as the host strain for expression of Cry3Bb. Strain EG2424 is a transconjugant strain derived from strain HD263 that contains a Cry3A-encoding 88-MDa plasmid and Cry1Ac-encoding 44- and 60-MDa plasmids (25). Production by strain EG2424 of the coleopteran-active Cry3A protein in fermentation is poor, presumably because the copy number of the Cry3A-encoding plasmid is low (2). Because Cry3Bb and Cry3A show comparable insecticidal activity against the key target pest *L. decemlineata*, the Colorado potato beetle (unpublished data, this laboratory), the introduction of pEG930.9 into EG2424 should result in elevated levels of Cry3 protein production while maintaining insecticidal potency. Plasmid pEG930.9 was introduced into strain EG2424 by electroporation and selection for tetracycline-resistant colonies on Luria plates containing 20 μg of tetracycline per ml. EG2424 transformants containing pEG930.9 (designated EG2424/pEG930.9) were confirmed by restriction enzyme analysis of their plasmid DNAs. Strain EG2424/pEG930.9 produced a bipyramidal crystal, characteristic of the Cry1Ac protein, and a large oval-shaped inclusion indistinguishable from that produced by strain EG10368/pEG930.9.

A TnpI-mediated recombination event between the two copies of the IRS on plasmid pEG930.9 will result in deletion of the pTZ19u and *tet* gene sequences (5, 6). The expected product from this in vivo enzymatic reaction, designated pEG930.9Δ, is depicted in Fig. 2B. To accomplish this engineering step, strain EG2424/pEG930.9 was transformed with the thermosensitive Tn*5401* transposon vector pEG922 (5), selecting for chloramphenicol-resistant colonies. Transformants were patched onto Luria plates containing 3 μg of chloramphenicol per ml and grown overnight at 30°C. The TnpI protein produced by pEG922 catalyzed the recombination event between the duplicate IRS copies on pEG930.9, resulting in the loss of the pTZ19u and *tet* gene sequences and generating pEG930.9Δ in three of three transformants examined (see below). Subsequently, one transformant, designated EG2424/pEG930.9Δ/pEG922, was streaked out onto a Luria plate and incubated at 37°C, the restrictive temperature for pEG922 replication (5). Single colonies recovered from the plate grown at 37°C were patched onto a Luria plate and maintained at 30°C. Chloramphenicol- and tetracycline-sensitive isolates producing the Cry3Bb protein and harboring pEG930.9Δ were readily obtained. One such isolate was designated strain EG7673. Figure 3 shows a Southern blot analysis of the plasmids in strains EG2424 (lane A), EG2424/pEG930.9 (lane B), EG2424/pEG930.9Δ/pEG922 (lane C), and EG7673 (lane D), demonstrating the selective deletion of the pTZ19u and *tet* gene sequences from pEG930.9 and the curing of pEG922. The gel analysis shown in Fig. 3 further confirms the presence of the native *cry3A*- and *cry1Ac*-encoding plasmids.

The construction of pEG930.9Δ was confirmed by restriction enzyme analysis of EG2424/pEG930.9 and EG7673 plasmid DNAs with the restriction endonucleases *Xba*I and *Sal*I (Fig. 2). To confirm the fidelity of the recombination step, opposing primers (pr10 and pr50) predicted to flank the single IRS region on plasmid pEG930.9Δ were used to amplify this region with EG7673 plasmid DNA as a template. This PCR yielded a ~800-bp fragment, consistent with the predicted recombination event. Sequence analysis of the amplified fragment revealed the expected sequence for the Tn*5401* IRS region (data not shown).

Plasmid stability. The segregational stability of plasmid pEG930.9Δ in strain EG7673 was evaluated in small-scale fermentation (see Materials and Methods). A plasmid loss frequency of 5×10^{-4} per generation was calculated from these experiments, or the equivalent of a 2.5% loss of the plasmid after 50 generations or cell divisions. For comparison, these analyses were extended to pEG928.9Δ, a related plasmid containing a chimeric *cry1C-cry1Ac* gene and plasmid replicon *ori43.9* (7). *B. thuringiensis* strains harboring this plasmid showed plasmid loss frequencies of $<4 \times 10^{-4}$ per generation in shake-flask cultures containing BHIG. These frequencies of plasmid loss are similar to those reported by Arantes and Lereclus for copy-number variants of the pHT1030 plasmid replicon (4).

Cry protein production. Strain EG7673 exhibits normal sporulation and lysis and is indistinguishable from strain EG2424 in its growth characteristics. Both EG2424 and EG7673 produce a bipyramidal Cry1Ac crystal upon sporulation but differ with respect to their Cry3 crystals. The Cry3A protein accumulates as a relatively small rhomboid-shaped crystal (e.g., reference 10) while Cry3Bb accumulates as a large oval-shaped inclusion. The Cry3 parasporal body formed by strain EG7673 (Fig. 4) is indistinguishable from the oval-shaped Cry3Bb inclusion formed by strain EG10368/pEG930.9. Strains EG2424 and

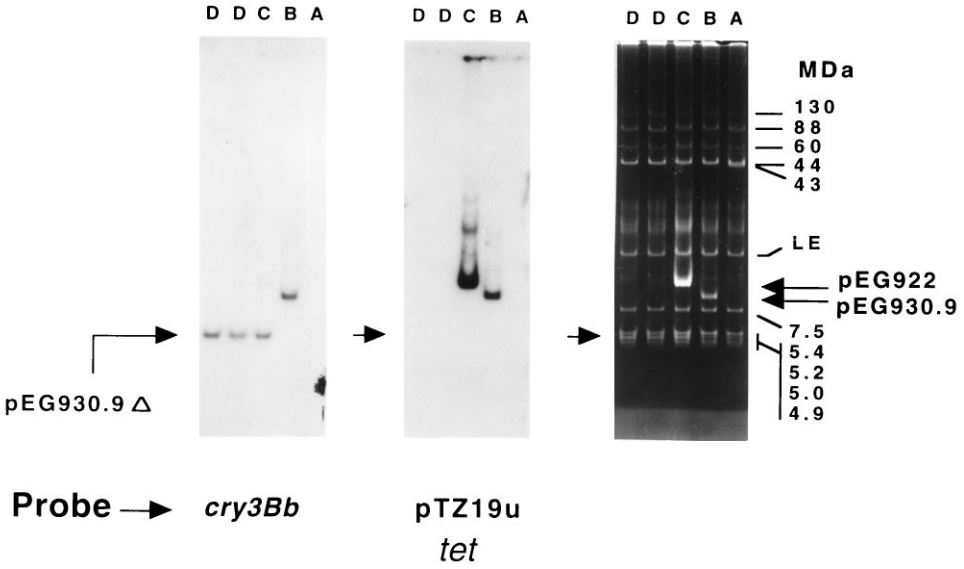

FIG. 3. Southern blot analysis of plasmids in strain EG7673 and progenitor strains. Plasmids from EG2424 (A), EG2424/pEG930.9 (B), EG2424/pEG930.9Δ/pEG922 (C), and EG7673 (D) (duplicate loadings) were resolved on a 0.52% vertical agarose gel and blotted to a nitrocellulose membrane for Southern blot analysis. A photograph of the ethidium bromide-stained gel is shown in the right panel. The membranes were hybridized to ^{32}P-labeled DNA fragments containing either *cry3Bb* (left panel) or pTZ19u and *tet* (center panel). Native plasmids are designated by their molecular mass in megadaltons. Plasmid pEG930.9Δ (arrow) migrates as a 5.2-MDa plasmid. LE, linear DNA element.

EG7673 were grown in triplicate in shake-flask cultures containing C2 medium and assessed for crystal protein production by SDS-polyacrylamide gel electrophoresis analysis (Fig. 5). Strain EG2424 produced 0.48 g of Cry3 protein per liter (standard deviation [SD] = 0.11) while strain EG7673 produced 2.10 g of Cry3 protein per liter (SD = 0.17). Both strains produced comparable amounts of Cry1Ac protein: 0.95 g/liter (SD = 0.37) for strain EG2424 and 1.03 g/liter (SD = 0.10) for strain EG7673. Thus, strain EG7673 produced approximately four times more Cry3 protein than did the progenitor strain EG2424.

Insecticidal activity of strain EG7673. Spore-crystal preparations of strain EG2424 and strain EG7673 were compared in quantitative eight-dose bioassays to assess the effect of Cry3 overproduction on insecticidal activity (Table 2). Strain EG7673 was considerably more toxic than strain EG2424 to the Southern corn rootworm (*Diabrotica undecempunctata howardi*) because of production of the Cry3Bb protein (19). Bioassays with purified Cry3Bb protein yielded comparable 50% lethal concentration values against this *Diabrotica* species (data not shown). Bioassays against Colorado potato beetle larvae (*L. decemlineata*), the target pest which strain EG2424 was originally designed to control, demonstrated that strains EG2424 and EG7673 exhibit comparable levels of insecticidal activity per nanogram of Cry3 protein (Table 2). Thus, the Cry3 yield gains achieved by introduction of the multicopy *cry3Bb* plasmid pEG930.9Δ into strain EG2424 may be used to develop more-concentrated formulations at a lower cost.

DISCUSSION

The genetic manipulation of *B. thuringiensis* and its insecticidal crystal protein genes provides a means of improving the efficacy of this biopesticide as well as its cost of production. To this end, several strategies have been used to introduce and stably maintain cloned insecticidal *cry* genes in *B. thuringiensis*. In addition to the use of conventional shuttle vectors, some employing *B. thuringiensis* plasmid replicons (4, 8), integrational vectors have been used to insert cloned *cry* genes into resident plasmids (2, 33) as well as into the chromosome (29) by homologous recombination. The gene transfer system described in this paper combines a native *B. thuringiensis* plasmid replicon with an indigenous site-specific recombination system that allows for the selective removal of foreign DNA from the recombinant bacterium after introduction of the Cry-encoding plasmid vector. Unlike chromosomal integration, which typically relies on amplification of both the *cry* gene and an antibiotic resistance gene marker to increase gene copy number

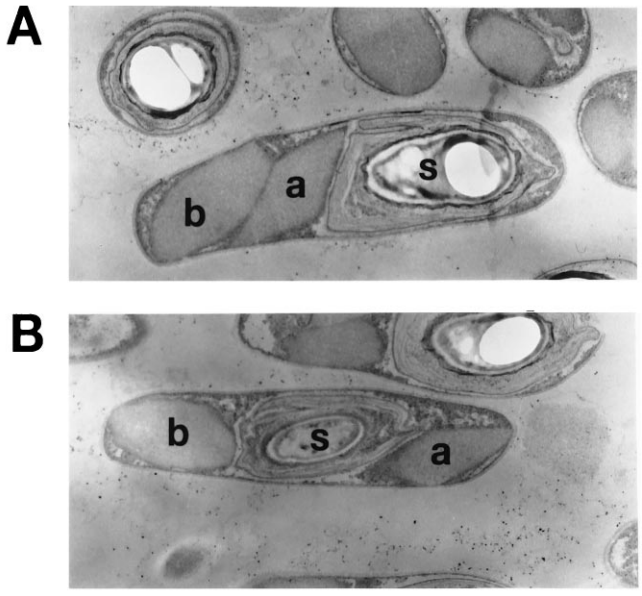

FIG. 4. Electron micrograph of sporulating cells of strain EG7673. Designations: a, Cry1Ac crystal; b, Cry3 crystal; S, spore. The Cry1Ac crystal (a) shown in panel A is approximately 1.4 μm in length.

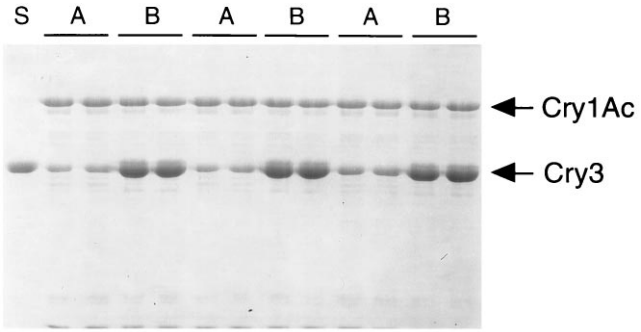

FIG. 5. SDS-polyacrylamide gel electrophoresis of crystal proteins from EG2424 (A) and EG7673 (B). Equal volumes (2.5 μl) of sporulated C2 broth cultures, grown in triplicate, were loaded in duplicate onto a 10% gel. Crystal protein yields were determined by densitometry using bovine serum albumin (S) as a standard.

and ensure adequate Cry protein production, the *cry* plasmids resulting from TnpI-mediated recombination are already present in multiple copies and require no resistance gene marker. Although integrational vectors targeted to resident plasmids can be adapted to achieve the same end result by a two-step recombination procedure (33), this method is, in our experience, relatively cumbersome and inefficient. By employing the method described in this paper, plasmid copy-number variants, generated in vitro, can also be used to further manipulate *cry* gene expression in *B. thuringiensis*, as was done in the case of strain EG7673. The nontransgenic or native character of the recombinant strains generated by this gene transfer system may facilitate their commercialization as bioinsecticides.

The site-specific recombination system was used to enhance the production of coleopteran-toxic crystal protein by strain EG2424, the active ingredient in Foil bioinsecticide, marketed for the control of the Colorado potato beetle (25). In shake-flask cultures and in small-scale (1-liter) fermentation, strain EG7673 consistently produced three to four times more Cry3 protein than the progenitor strain EG2424. Assuming additive contributions by *cry3A* and *cry3Bb* to Cry3 production in strain EG7673, between two-thirds and three-fourths of the Cry3 protein produced by this strain would be Cry3Bb. The morphology of the Cry3 inclusion in strain EG7673 resembles the Cry3 inclusion produced by EG10368 recombinant strains containing only the *cry3Bb* gene, suggesting that a large fraction of the Cry3 protein in strain EG7673 is Cry3Bb. Although the small rhomboid Cry3A crystal is not present in strain EG7673, Cry3A could still be present in the oval-shaped inclusion since some Cry proteins can form heterogeneous crystals.

Cry3 production had little effect on Cry1Ac production in strain EG7673 (Fig. 5). In contrast, the introduction of *cry1Aa* on a recombinant plasmid has been shown to reduce the production of Cry1Ac protein encoded on native plasmids, and vice versa (25). Unlike *cry1Aa* and *cry1Ac*, which are transcribed from mother cell-specific promoters, both *cry3A* and *cry3Bb* appear to be transcribed from σA-like (vegetative) promoters and, in fact, are not sporulation dependent (3, 10, 34, 35, 43). This independent regulation of *cry1A* and *cry3* genes may account for the effective production of Cry1Ac protein by strain EG7673.

TABLE 2. Activity of EG7673 and EG2424 against *L. decemlineata* and *D. undecempunctata howardi*

Strain	*L. decemlineata*		*D. undecempunctata*	
	pLC50[a]	95% CI[b]	pLC50[c]	95% CI[b]
EG2424	104	86–123	1,081	766–1,794
EG7673	92	75–111	67	54–83

[a] pLC50, 50% lethal concentration of the protein. Nanograms of Cry3 protein per well in replicated assays.
[b] CI, confidence interval.
[c] Micrograms of Cry3 protein per well in replicated assays.

The overproduction of Cry3 protein by strain EG7673 was also observed in large-scale (≥50,000-liter) fermentation (data not shown). This enhanced yield of coleopteran-toxic protein by strain EG7673 represents a significant reduction in the cost of production compared with strain EG2424. Plasmid analyses of spores recovered from EG7673 production fermentation broths did not reveal any problems with plasmid stability (data not shown), consistent with the excellent Cry3 protein yields obtained and corroborating earlier plasmid stability studies (see Results). The recombinant plasmid in strain EG7673, pEG930.9Δ, does show progressive loss over repeated rounds of sporulation, but this is of little consequence in production since the strain does not sporulate until the end of the fermentation run. This segregational loss makes it probable that the recombinant plasmid will eventually disappear from the soil population following product application.

In the future, *B. thuringiensis* strains may be engineered not only for improved toxicity or more efficient crystal protein production but also for use in managing insect resistance to other insecticides. The Colorado potato beetle has readily developed resistance to every chemical insecticide deployed against it (23), suggesting that resistance to transgenic plants expressing Cry3A or new broad-spectrum insecticides (e.g., imidacloprids) may rapidly develop in the absence of an effective resistance management program (22). Since the Cry3Bb protein shows minimal cross-resistance with Cry3A when compared in bioassays with a Cry3A-resistant Colorado potato beetle colony (45a), strain EG7673 could be a useful addition to such a program given its unique coleopteran-active toxin.

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ADDENDUM

The gene transfer system described here has been used to engineer a number of *B. thuringiensis* strains, including EG7841, a broad-spectrum lepidopteran-toxic strain that was approved as the active ingredient for CRYMAX WDG bioinsecticide by the U.S. Environmental Protection Agency in February 1996.

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