Development and Field Performance of a Broad-Spectrum Nonviable Asporogenic Recombinant Strain of *Bacillus thuringiensis* with Greater Potency and UV Resistance

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The main problems with *Bacillus thuringiensis* products for pest control are their often narrow activity spectrum, high sensitivity to UV degradation, and low cost effectiveness (high potency required). We constructed a sporulation-deficient SigK⁻ *B. thuringiensis* strain that expressed a chimeric *cry1C/Ab* gene, the product of which had high activity against various lepidopteran pests, including *Spodoptera littoralis* (Egyptian cotton leaf worm) and *Spodoptera exigua* (lesser [beet] armyworm), which are not readily controlled by other Cry δ-endotoxins. The SigK⁻ host strain carried the *cry1Ac* gene, the product of which is highly active against the larvae of the major pests *Ostrinia nubilalis* (European corn borer) and *Heliothis virescens* (tobacco budworm). This new strain had greater potency and a broader activity spectrum than the parent strain. The crystals produced by the asporogenic strain remained encapsulated within the cells, which protected them from UV degradation. The *cry1C/Ab* gene was introduced into the *B. thuringiensis* host via a site-specific recombination vector so that unwanted DNA was eliminated. Therefore, the final construct contained no sequences of non-*B. thuringiensis* origin. As the recombinant strain is a mutant blocked at late sporulation, it does not produce viable spores and therefore cannot compete with wild-type *B. thuringiensis* strains in the environment. It is thus a very safe biopesticide. In field trials, this new recombinant strain protected cabbage and broccoli against a pest complex under natural infestation conditions.

Every year, insect pests cause between a 15 and 25% loss of agricultural production worldwide. Yield losses vary widely between crops and geographic areas. Various strategies have been used to reduce or control this agricultural damage, the principal strategy being the use of chemical insecticides (23). The application of these synthetic compounds has resulted in the stabilizing or even increasing of agricultural yields. However, this strategy has now become one of the most costly aspects of agriculture. Moreover, the large-scale and indiscriminate use of nonspecific products, often toxic to mammals, birds, and fish, has resulted in contamination of the environment, destruction of nontarget organisms, and the development of pest resistance. Since the 1960s, biological pesticides have been seen as an environmentally benign, highly desirable alternative to chemicals and have therefore received considerable attention. Nevertheless, biopesticides have captured only a scant 2% of the pesticide market and have not significantly reduced chemical pesticide use (24).

The most widely used microbial pesticides worldwide are those based on preparations of the bacterium *Bacillus thurin-*

giensis (16, 24). *B. thuringiensis* is a spore-forming bacterium that produces highly specific insecticidal proteins, the δ-endotoxins, during sporulation. δ-Endotoxins accumulate as crystalline inclusions within the cell. At the end of sporulation, the cells lyse and the spores and crystals are liberated. If ingested by susceptible insects (usually the larvae), the crystals are dissolved and the δ-endotoxins, which are protoxin molecules, are specifically cleaved by insect gut proteases. The resulting activated toxins recognize specific receptors on the surfaces of the midgut epithelium cells and cause cell lysis and the death of insect larvae (10). Most of the δ-endotoxins are active against a small number of insect species. Commercial *B. thuringiensis* products generally consist of a mixture of spores and crystals, produced in large fermenters and applied as foliar sprays, much like synthetic insecticides.

Biopesticides containing *B. thuringiensis* are environmentally friendly and effective in a variety of situations. However, their performance is often considered to be poorer than that of chemicals in terms of reliability, spectrum of activity, speed of action, and cost effectiveness. *B. thuringiensis* products are not as potent or persistent in the field as chemical products: *B. thuringiensis* products act slowly, have a narrow activity spectrum (minimizing the size of their potential market), and are not stable in the environment after spraying because they are rapidly inactivated by exposure to sunlight (25) or other environmental factors. Consequently, the duration of pest control is often too short and its use on many crops is not cost-effective because too many applications are required (8). Therefore, the economic viability and acceptability of *B. thuringiensis* biopesticides depends on the potency and spectrum of activity of the

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TABLE 1. *B. thuringiensis* strains and plasmids

Strain or plasmid	Relevant genotype or plasmid characteristics	Reference(s)
407⁻	Acrystalliferous derivative of the wild-type *B. thuringiensis* strain 407 (H1 serotype) isolated by O. Arantes	3, 18
Kto (HD73)	Wild-type *B. thuringiensis* strain containing Tn*4430* and a *cry1Ac* gene	15, 31
Kto⁻	Acrystalliferous derivative of the wild-type *B. thuringiensis* strain Kto	31
407⁻(pHTF3-1C)	Cry1C δ-endotoxin produced in the 407⁻ background	This report
407⁻(pHTF3-1C/Ab)	Cry1C/Ab chimeric δ-endotoxin produced in the 407⁻ background	This report
AGRO2	Kto strain electrotransformed with plasmid pHTF3-1C/Ab-IRS-T and containing pHTF3-1C/Ab-IRS-T-Δ as a result of site-specific recombination in vivo	This report
Kto SigK⁻	Kto strain whose *sigK* gene was disrupted by the insertion (by homologous recombination) into the chromosome of a *sigK* gene interrupted by the *aphA3* kanamycin resistance gene (*sigK*::*aphA3*)	This report
AGRO1	Kto SigK⁻ strain containing pHTF3-1C/Ab-IRS-T-Δ as a result of site-specific recombination in vivo after electrotransformation of the host strain with pHTF3-1C/Ab-IRS-T	This report
pAB2	Plasmid containing the *sigK* gene of *B. thuringiensis* strain 407 disrupted by the *aphA3* gene of *E. faecalis*, cloned into pRN5101	7
pHT81	Plasmid containing a chimeric *cry1C/Ab* gene, consisting of the 5′ end region of the *cry1C* gene fused (at the *Kpn*I site) to the 3′ end region of the *cry1Ab* gene, cloned in pUC19	29
pHTF3-1C	Plasmid consisting of the coding sequence of the *cry1C* gene fused to the sporulation-independent *cry3A* promoter inserted into pHT315 (3)	30
pHTF3-1C/Ab	Plasmid consisting of the coding sequence of the chimeric *cry1C/Ab* gene fused to the *cry3A* promoter and inserted into pHT315 (3)	This report
pHTBS2	Plasmid containing the origin of replication of the *B. thuringiensis* resident plasmid pHT1030 and the tetracycline resistance gene carried by pBC16 of *B. cereus* cloned into pBluescript II KS(−)	30
pHT-IRS-BSK	Plasmid consisting of the pBluescript II KS(−), the *aphA3* gene of *E. faecalis*, and one copy of the IRS of Tn*4430*	31
pHTF3-1C/Ab-IRS-K and pHTF3-1C/Ab-IRS-T	Site-specific recombination vectors consisting of the pBluescript II KS(−) and the kanamycin resistance gene *aphA3* of *E. faecalis* (pHTF3-1C/Ab-IRS-K) or the tetracycline resistance gene carried by pBC16 of *B. cereus* (pHTF3-1C/Ab-IRS-T) between duplicated copies of the IRS of transposon Tn*4430* and segregated from the native *B. thuringiensis* plasmid components: the chimeric *cry1C/Ab* gene under the control of the *cry3A* promoter and the replication and stability regions of plasmid pHT1030	This report

insecticidal toxins in the crystals and the ability of these products to control insect pests resistant to other insecticides, rather than on their low ecotoxicity and other ecological advantages. The environmental stability of the crystals after spraying is also important as it determines the duration of pest control and the number of applications needed.

We have shown that a recombinant *B. thuringiensis* strain expressing an additional *cry1* gene under the control of the *cry3A* gene expression system (30) yields more crystal protein than the wild-type strain. This is presumably because the expression systems of the *cry* genes differ and, therefore, do not compete for rate-limiting gene expression factors (2). Thus, it may be possible to increase the total amount of toxin produced in a *B. thuringiensis* strain. We have also shown that as much Cry1Aa protein is produced by a wild-type *cry1Aa* gene introduced by electroporation into a *B. thuringiensis* mutant, blocked at late sporulation by disruption of the chromosomal *sigK* gene, as is produced by the Spo⁺ strain (7). The toxins accumulated in the mother cell compartment to form crystal inclusions, which remained encapsulated within the cell wall.

We report herein the construction and field trials of a new recombinant *B. thuringiensis* strain that produces large amounts of two different crystal proteins and show that encapsulation of the toxins within the cell protects them from deactivation by UV radiation. The strain is a sporulation-deficient mutant that cannot survive in the environment, thereby minimizing any environmental effects arising from the dissemination of large numbers of viable spores.

MATERIALS AND METHODS

Bacterial strains, plasmids, and media. *B. thuringiensis* strains and plasmids used in this work are listed in Table 1. *Escherichia coli* SCS110 [*rpsL* (Str) *thr leu endA thi-1 lacY galK galT ara tonA tsx dam dcm supE44* Δ(*lac-proAB*) (F′ *traD36 proAB lacI*qZΔM15)] was used as the host for plasmid construction. *B. thuringiensis* strains were grown at 30°C with shaking in Luria broth (LB) or hydrolysate of casein tryptone (HCT) medium (17). *E. coli* was grown at 37°C in LB. Antibiotic concentrations for bacterial selection were as follows: ampicillin, 100 μg/ml⁻¹ (for *E. coli*); erythromycin and tetracycline, 10 μg/ml⁻¹ (for *B. thuringiensis*); and kanamycin, 200 μg/ml⁻¹ (for *B. thuringiensis*).

For greenhouse and field trials, strains AGRO1 and AGRO2 were grown in 200 liters of HCT medium in a 300-liter Biolafitte fermenter (Biolafitte, St. Germain-en-Laye, France) for 30 h. Biomass was collected by centrifugation at 14,000 × *g* in a Sharples AS16 centrifuge with a yield of 100 liters/h. The lysed AGRO2 spore-crystal biomass and AGRO1-cell-crystal biomass were washed once with 20 liters of 0.15 M NaCl and once with 20 liters of distilled water by centrifugation for 30 min at 14,000 × *g* in a Sharples AS16 centrifuge, with a yield of 60 liters/h. The pellets were freeze-dried. The yields were 1 g of freeze-dried powder per liter for AGRO2 and 2 g of freeze-dried powder per liter for AGRO1. These powders were used to prepare wettable powder (WP) spray formulations consisting of 50% AGRO1 or AGRO2 (20% of which was the active ingredient [crystal protein]), 2% wetting agent, 0.5% silica, and 47.5% clay.

pHTF3-1C/Ab was constructed by replacing the 3.4-kb *Bgl*II-*Eco*RI fragment of pHTF3-1C (which contains the 3′ end and downstream adjacent regions of the *cry1C* gene) with the 2.3-kb *Bgl*II-*Eco*RI DNA fragment of pHT81 (which contains the 3′ end and downstream adjacent region of the hybrid *cry1C/Ab* gene). The resulting plasmid consisted of the hybrid *cry1C/Ab* gene under the control of the *cry3A* promoter in pHT315 (3). The construction of pHTF3-1C/Ab-IRS-K and pHTF3-1C/Ab-IRS-T is described in Fig. 1.

DNA manipulation and transformation. Plasmid DNA for transformation was extracted from *E. coli* SCS110 by the standard alkaline lysis procedure and was further purified by using a Qiagen plasmid kit according to the manufacturer's instructions (Qiagen GmbH, Hilden, Germany). Restriction enzymes and T4
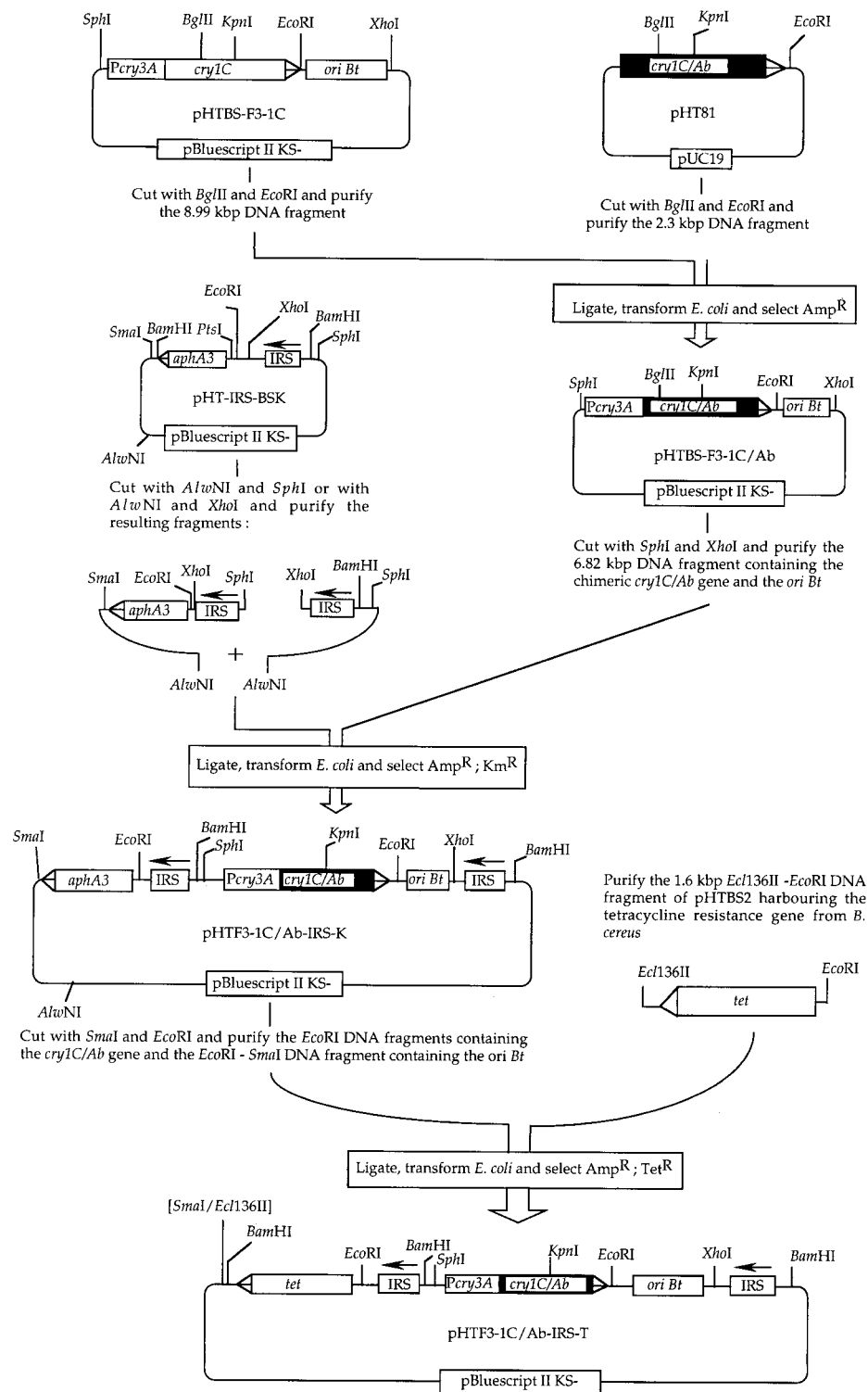
FIG. 1. Construction of plasmids pHTBS-F3-1C/Ab, pHTF3-1C/Ab-IRS-K, and pHTF3-1C/Ab-IRS-T. pHTBS-F3-1C/Ab was constructed as follows: the 2.3-kbp *Bgl*II-*Eco*RI DNA fragment of pHT81, which contains the 3' end and downstream adjacent regions of the *cry1C/Ab* chimeric gene, was purified and ligated with the 9-kbp *Bgl*II-*Eco*RI fragment of pHTBS-F3-1C. pHTF3-1C/Ab-IRS-K was obtained by ligating the *Sph*I-*Alw*NI and *Xho*I-*Alw*NI fragments of pHT-IRS-BSK (each carrying an IRS of Tn*4430*) to the 6.82-kbp DNA fragment of pHTBS-F3-1C/Ab that contains the origin of the replication of pHT1030 (*ori B. thuringiensis*) and the chimeric *cry1C/Ab* gene under the control of the promoter of the *cry3A* gene (*Pcry3A*). pHTF3-1C/Ab-IRS-T was constructed by a three-way ligation as follows: the 1.6-kbp *Ecl*136II-*Eco*RI DNA fragment of pHTBS2 (28) harboring a tetracycline resistance marker was purified and ligated with the *Sma*I-*Eco*RI and *Eco*RI-*Eco*RI fragments of pHTF3-1C/Ab-IRS-K. Restriction sites destroyed during the manipulation are indicated by square brackets, and only restriction sites relevant to the experimental design are shown. The various boxes representing genes or IRSs are not drawn to scale. *Bt, B. thuringiensis*.

DNA ligase were obtained from New England Biolabs (Beverly, Mass.), and DNA fragments used in cloning experiments were purified from agarose gels by using a Prep-A-Gene DNA purification matrix kit (Bio-Rad, Laboratories, Richmond, Calif.).

E. coli SCS110 was transformed by electroporation. Plasmid DNA was then isolated from this strain and used to electrotransform *B. thuringiensis* strains 407⁻, Kto, and its derivatives, as previously described (18). Plasmid DNA was extracted from *B. thuringiensis* by alkaline denaturation, and DNA was analyzed by electrophoresis in horizontal 0.8% agarose slab gels.

Irradiation of *B. thuringiensis* samples with a solar simulator. *B. thuringiensis* samples were irradiated with a solar simulator (Suntest) from Heraeus, which delivered a spectrum equivalent to that of sunlight passing through the earth's atmosphere. The products (0.5 mg of toxins) were sprayed onto 7- by 7-cm glass plates and freeze-dried. The coated plates were irradiated for 1 h by using the Suntest machine. The Suntest uses a xenon lamp emitting from 280 to 800 nm at 100 klx (10 times the intensity of light reaching the earth's surface in 1 h). During irradiation, the glass plates were cooled at 10°C. The irradiated toxins were recovered and bioassayed against *Heliothis virescens* as described below. For each irradiated sample, a control sample from the same batch was sprayed onto glass plates, freeze-dried, and recovered under the same conditions as for the irradiated plates.

Bioassays of insecticidal activity. The quantity of protein in the preparations was estimated as follows. Sporulated and lysed cultures or cell-crystal suspensions were washed twice with 0.15 M NaCl and twice with distilled water. The protein concentration of the spore-crystal preparation or cell-crystal suspension was then assayed by using the Bio-Rad protein test. Ghost cells were briefly sonicated to liberate the crystals before protein assays were performed.

(i) Laboratory bioassays. Biological assays were conducted by using free ingestion techniques and neonates, the first and second instar larvae of several different insect species: *Spodoptera littoralis*, *Spodoptera frugiperda*, *H. virescens*, *Ostrinia nubilalis*, and *Plutella xylostella*. The activities against *S. littoralis*, *S. frugiperda*, and *O. nubilalis* were determined by contaminating an artificial diet dispensed into 50-well plates (165-mm² surface). Five concentrations of each preparation were applied uniformly over the food surface in the wells. One second (*S. littoralis* or *S. frugiperda*) or first (*O. nubilalis*) instar larva was placed in each of the 50 wells. Each concentration was tested two to four times. Mortality was scored after 5 days. *H. virescens* larvae were fed with an artificial diet in plastic feeding cups (175-mm² surface); one neonate was placed in each cup, and mortality was scored after 7 days. Sixteen neonates were challenged with each of five dilutions of the preparations (four independent experiments). Bioassays on *P. xylostella* were performed by surface contamination of cabbage leaf disks with a calibrated sprayer that uniformly delivered a known amount of toxin per square centimeter of leaf surface. Leaf disks (2.5-cm diameter) were cut out, treated, and placed in individual cups (five cups per dilution), and six second instar larvae were added to each cup. Mortality was assessed after 5 days. Mortality data were analyzed by using the log-probit program of Raymond et al. (26), which tests for the linearity of dose-mortality curves and calculates lethal concentrations and the slope of each curve. It also assesses whether two or more dose-mortality curves are parallel, calculates the ratios of different curves, and indicates whether the curves are significantly different ($P < 0.05$). Lethal concentrations are expressed per square centimeter of artificial diet or surface.

(ii) Greenhouse trials. The efficacy of the recombinant *B. thuringiensis* strains AGRO1 and AGRO2 against *P. xylostella* (diamondback moth) on cabbage was assessed in greenhouse conditions and compared with that of the standard *B. thuringiensis* products Delfin WG and Thuricide HP and the chemical insecticides Agrimek EC1.8, Decis EC2.5, and Hostathion EC40. The treatments involved applying AGRO1 and AGRO2 WP formulations at 10, 20, 40, 80, and 160 g of active ingredient per ha (ai/ha); Delfin WG at 125 to 1,000 g of ai/ha; Thuricide HP at 625 g of ai/ha; Agrimek EC1.8 at 18 g of ai/ha; Decis EC2.5 at 7.8 g of ai/ha; and Hostathion EC40 at 250 g of ai/ha to potted cabbage plants with a boom sprayer at a rate of 500 liters/ha. Four replicates per treatment were used in a complete randomized block design with a plot size of one plant. The treatment was applied 1 day after artificial infestation with 20 first and second instar larvae of *P. xylostella* per cabbage plant. One, 3, and 7 days after application, the numbers of dead and living larvae were counted on each plant and the percentage of dead larvae was calculated. The percent feeding damage by lepidopteran larvae was estimated, using Abbott's formula, for total leaf (plant) surface and was calculated plotwise (average of all treated plants for a given dose); treated plots were compared with those of untreated controls.

(iii) Field trials. The protection against natural infestation provided by AGRO1 and AGRO2 was assessed on cabbage and broccoli during the late cropping season of 1996 at the Western Field Research Station of AgrEvo in Fresno, Calif. AGRO1 and AGRO2 WP formulations at 20, 40, and 80 g of ai/ha and a control (the same as the WP formulation but without the *B. thuringiensis* extract) were applied to one broccoli and two cabbage plots. A commercial *B. thuringiensis* product, Mattch 126 SC (588 g of ai/ha), and the chemical insecticide Lannate 90 WP (1,121 g of ai/ha) were used as standards. Each trial was replicated three times and involved randomized blocks (plots) of 9.29 m², with five plants per plot and one plot per concentration. Two to four applications were sprayed at intervals of 4 to 7 days (depending on the trial) with a spray volume of 340 liters/ha. The total number of dead and alive larvae per plot (five plants) was counted before each new application and at the end of the study. The

TABLE 2. Activity of Cry1C and of a chimeric Cry1C/Ab toxin against different insect species

Insect species	LC₅₀ of indicated toxin[a]	
	Cry1C	Cry1C/Ab
S. littoralis	378 (189–754)	103 (80–130)
P. xylostella	174 (143–210)	4.6 (0.03–9)
O. nubilalis	3,200 (2,107–4,985)	822 (442–1,534)

[a] The LC_{50}s were calculated as nanograms of toxin per square centimeter by probit analysis, and the 95% confidence intervals are indicated in parentheses. Each value is the mean of results from three (*S. littoralis* and *P. xylostella*) or four (*O. nubilalis*) experiments.

percent damage due to insect feeding was assessed plotwise (average of all treated plants) by determining total leaf (plant) surface before treatment and 9 days after the last application; efficacy was calculated by using Abbott's formula.

RESULTS

Activity of a chimeric Cry1C/Ab toxin against several lepidopteran insects. We have reported the construction of a chimeric δ-endotoxin gene, *cry1C/Ab*, in pHT81 (29) (Table 1 and Fig. 1). This hybrid toxin gene comprised the 2,194 5′ nucleotides of the coding region of the *cry1C* gene and the 1,295 3′ nucleotides of the coding region of the *cry1Ab* gene, both from *B. thuringiensis aizawai* 7-29 (HD137) (28). The fusion was made by using the unique *Kpn*I site present in a region conserved between the two δ-endotoxin genes. This conserved carboxy-terminal half of the δ-endotoxin molecule (or protoxin segment) is not essential for toxicity and is cleaved during activation in the midgut of host lepidopteran larvae. Thus, the resulting protease-resistant active amino-terminal fragment of the chimeric Cry1C/Ab toxin is that of Cry1C. However, preliminary toxicity data indicated that the hybrid Cry1C/Ab toxin was more toxic than the Cry1C toxin to *S. littoralis*. We therefore compared the activities of the Cry1C and Cry1C/Ab toxins against various major agricultural pests. The acrystalliferous *B. thuringiensis* strain 407⁻ was transformed with pHTF3-1C and pHTF3-1C/Ab, similar constructs harboring the native *cry1C* and hybrid *cry1C/Ab* genes, respectively (Table 1) (see Materials and Methods for construction details). Transformants were selected for resistance to erythromycin and grown in HCT sporulation medium for 48 to 72 h. The production of crystals was monitored by phase-contrast microscopy. Both 407⁻(pHTF3-1C) and 407⁻(pHTF3-1C/Ab) produced large bipyramidal inclusions. The insecticidal activities of chimeric Cry1C/Ab and native Cry1C crystal preparations were determined against *S. littoralis*, *O. nubilalis*, and *P. xylostella* (Table 2): Cry1C/Ab was 3, 4, and 35 times more active than Cry1C, respectively. The 50% lethal concentrations (LC_{50}s) and slopes of the dose-mortality curves for each toxin against the three insect species were compared by using the log-probit program of Raymond et al. (26). In each case, the difference in activity between the two toxins was significant ($P < 0.05$). We therefore used the Cry1C/Ab toxin gene to construct *B. thuringiensis* strains with better pest control properties.

Construction of a site-specific recombinant plasmid harboring *cry1C/Ab*. Site-specific recombination vectors for introducing *cry* genes into *B. thuringiensis* strains have been developed (6, 30, 31), facilitating the construction of *B. thuringiensis* recombinant strains free of antibiotic resistance markers and other non-*B. thuringiensis* DNA sequences. These vectors harbor two internal resolution sites (IRSs) of the *B. thuringiensis* class II transposons, Tn*4430* (20) and Tn*5401* (5), that flank the DNA sequences not native to *B. thuringiensis*. In an appro-
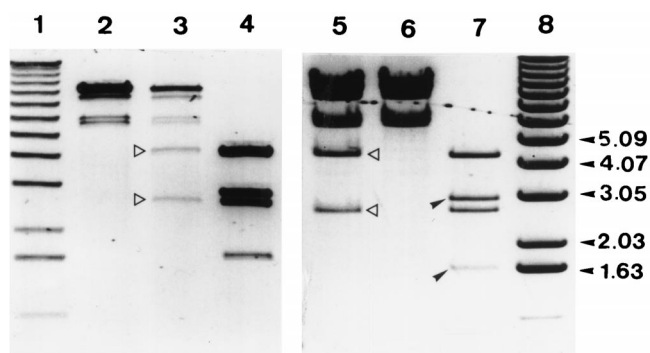
FIG. 2. Agarose gel electrophoresis of *Bam*HI-*Xho*I-*Eco*RI-digested plasmid DNA from native and transformed *B. thuringiensis* Kto and Kto SigK⁻ strains. Lanes: 1 and 8, 1-kb DNA ladder; 2, Kto recipient; 3, Kto (pHTF3-1C/Ab-IRS-T-Δ); 4 and 7, pHTF3-1C/Ab-IRS-T isolated from *E. coli*; 5, Kto SigK⁻ (pHTF3-1C/Ab-IRS-T-Δ); 6, Kto SigK⁻ recipient. The 2.9-kbp *Bam*HI DNA fragment corresponding to the pBluescript II KS(−) and the 1.6-kbp *Bam*HI-*Eco*RI DNA fragment corresponding to the *tet* gene of *B. cereus* are indicated by black arrows (lane 7). These DNA fragments are absent from the Kto and Kto SigK⁻ transformants harboring pHTF3-1C/Ab-IRS-T-Δ. Only the 2.6-kbp *Eco*RI-*Xho*I and 4.3-kbp *Bam*HI-*Eco*RI DNA fragments corresponding to the origin of replication of pHT1030 and the *cry1C/Ab* gene, respectively, remain (indicated by open triangles in lanes 3 and 5) after the site-specific recombination that removes the *tet* gene and pBluescript II KS(−). The sizes (in kilobase pairs) of the 1-kb ladder are shown on the right.

TABLE 3. UV stability of *B. thuringiensis* AGRO1 and AGRO2

| Strain | LC_{50} for the indicated test[a] | | % Loss (P) |
	None	Sun test (for 1 h)	
AGRO1	13.5 (11–17)	11 (8.5–13.5)	0 (0.74)
AGRO2	2 (0.1–7.5)	32 (25–40)	94 (0.003)
aizawai	5.5 (4.5–7)	25 (20–31)	78 (0.00001)

[a] Activity was determined in nanograms of toxin per square centimeter against *H. virescens*. The control yielded 0% ± 0% mortality. The percent loss is calculated as $[(LC_{50})_{sun\ 1\ h} - (LC_{50})_{no\ sun}]/(LC_{50})_{sun\ 1\ h}$. Ninety-five percent confidence intervals are indicated in parentheses.

priate host background (*B. thuringiensis* strains containing Tn*4430* or Tn*5401*), site-specific recombination between the duplicate IRSs, catalyzed by the TnpI recombinase of Tn*4430* or Tn*5401*, eliminates the intervening DNA (6, 30, 31). We used this type of vector to introduce the *cry1C/Ab* gene into *B. thuringiensis* under the control of the promoter of the *cry3A* δ-endotoxin gene of *B. thuringiensis* subspecies *tenebrionis* (Fig. 1). *cry1* δ-endotoxin genes are transcribed from specific sporulation promoters, whereas the *cry3A* gene is transcribed from a promoter which resembles vegetative promoters (1). This makes it possible to produce this toxin (19) or other Cry1 toxins (30) at high levels in sporulation-deficient or wild-type backgrounds. The first construct, pHTF3-1C/Ab-IRS-K, consisted of the origin of the replication of pHT1030 and the chimeric *cry1C/Ab* gene under the control of the *cry3A* promoter between two identical IRSs in direct orientation flanking *E. coli* pBluescript II KS(−) and the *aphA3* gene of *Enterococcus faecalis*. The DNA native to *B. thuringiensis* was thus separated from all other sequences by the IRSs (Fig. 1). pHTF3-1C/Ab-IRS-T, harboring the *tet* gene from *Bacillus cereus* in place of the kanamycin cassette in pHTF3-1C/Ab-IRS-K, was also constructed (Fig. 1) to be used in a *B. thuringiensis sigK*::*kan* background (see below).

Construction of recombinant *B. thuringiensis* strains. *B. thuringiensis* strains were constructed from a wild-type strain of *B. thuringiensis* serovar *kurstaki*, strain Kto, initially isolated in France by Kurstak (15). The Kto strain contains, on a 75-kb plasmid, a copy of transposon Tn*4430* and a *cry1Ac* gene, the product of which is highly active against the larvae of two major insect pests: *O. nubilalis* (European corn borer) and *H. virescens* (tobacco budworm). A nonsporulating derivative of strain Kto was obtained by disrupting the chromosomal *sigK* gene, which encodes the σ²⁸ sporulation-specific sigma factor, by in vivo homologous recombination with a disrupted copy of *sigK*::*kan*, as described by Bravo et al. (7), using the integrative thermosensitive vector pAB2. Briefly, pAB2 was used to transform *B. thuringiensis* Kto by electroporation, and transformants were selected at a nonpermissive temperature for the integra-

tion of the vector into the chromosome at the *sigK* locus. A second recombination event was then selected such that the vector was eliminated. The *B. thuringiensis* Kto SigK⁻ mutant, unlike the wild-type *B. thuringiensis* strain Kto, did not produce mature-phase refractile spores at the end of sporulation. Instead, it produced large parasporal inclusions (composed of Cry1Ac) that remained encapsulated in the cells, which did not lyse. Under the light microscope, SigK⁻ cells grown to stationary phase looked like ghost cells. The insecticidal activities of spore-crystal or cell-crystal preparations of the Kto and Kto SigK⁻ strains were analyzed against *H. virescens*. The LC_{50}s were 2.5 ng/cm² for Kto and 12 ng/cm² for Kto SigK⁻. The lower activity of the Kto SigK⁻ strain may have been due to the crystals being tightly encapsulated and therefore less available to the insect. A second asporogenic recombinant strain (designated AGRO1) was constructed by introducing the *cry1C/Ab* gene, the product of which is active against spodopteran pests such as *S. littoralis* (Egyptian cotton leafworm) and *Spodoptera exigua* (lesser [beet] armyworm), into the *B. thuringiensis* Kto SigK⁻ background. pHTF3-1C/Ab-IRS-T, carrying the *cry1C/Ab* toxin gene, was introduced into the *B. thuringiensis* Kto SigK⁻ strain by electroporation, and tetracycline-resistant colonies were selected on LB plates. The tetracycline resistance gene and the *E. coli* DNA present on pHTF3-1C/Ab-IRS-T were eliminated (to yield the recombined plasmid pHTF3-1C/Ab-IRS-T-Δ) by growing the Tet^r transformants in nonselective medium as previously described (31). The presence of pHTF3-1C/Ab-IRS-T-Δ in AGRO1 was confirmed by restriction enzyme analysis (Fig. 2). A third recombinant strain, AGRO2, consisting of the Kto strain expressing both the *cry1Ac* and *cry1C/Ab* genes, was also constructed. AGRO2 was morphologically indistinguishable from its parental strain, differing only in the presence of the recombined plasmid, pHTF3-1C/Ab-IRS-T-Δ, containing the *cry1C/Ab* gene and the *ori B. thuringiensis* (Fig. 2). It was expected to have the same activity spectrum as AGRO1 but to produce viable spores that might germinate and multiply in favorable nutritional conditions, such as those in the insect's gut. Its survival in the environment was expected to be similar to that of a natural *B. thuringiensis* strain.

Effects of crystal encapsulation on residual insecticidal activity after UV irradiation. Strains AGRO1 and AGRO2 produced identical crystals. However, AGRO1 crystals remained encapsulated in the cells, whereas those of AGRO2 were released. These strains and a natural *aizawai* strain, the active ingredient of Xentari WDG (Abbott), were used to determine whether encapsulation protects δ-endotoxins from sunlight-mediated degradation. Spore-crystal preparations of AGRO2, the reference *aizawai* strain, and cell-crystal suspensions of AGRO1 were irradiated for 1 h under controlled conditions (see Materials and Methods). The toxicities of irradiated and control samples from the same batch were tested against *H.*

TABLE 4. Biological activity of *B. thuringiensis* AGRO1 and
AGRO2 against different insect pests

Insect species	LC_{50} of indicated toxin[a]		
	AGRO1	AGRO2	*aizawai*
H. virescens	11 (9–14)	5 (3–7)	8 (3–24)
S. littoralis	207 (123–346)	103 (72–109)	84 (39–117)
S. frugiperda	113 (73–171)	66 (43–97)	ND
O. nubilalis	57 (40–94)	10 (7–13)	ND
P. xylostella	0.7 (0.2–1.2)	0.9 (0.1–2)	ND

[a] The LC_{50}s were calculated as nanograms of toxin per square centimeter, and 95% confidence intervals are indicated in parentheses. Each value is the mean of results from at least two independent experiments. ND, not done.

virescens larvae (Table 3). LC_{50}s were significantly higher after irradiation ($P < 0.005$) for AGRO2 and the *aizawai* reference preparation than for the nonirradiated samples, whereas the LC_{50}s for AGRO1 did not differ significantly ($P > 0.5$) before and after irradiation.

Insecticidal activity of strains AGRO1 and AGRO2. (i) Laboratory bioassays. The insecticidal activity of strains AGRO1 and AGRO2 was assayed on larvae of *H. virescens*, *S. littoralis*, *S. frugiperda*, *P. xylostella*, and *O. nubilalis* (LC_{50}s are listed in Table 4). The LC_{50}s of the two crystal preparations did not differ significantly ($P < 0.05$) for *S. littoralis*, *S. frugiperda*, and *P. xylostella*. However, the LC_{50}s for *H. virescens* and *O. nubilalis* were significantly higher for AGRO1 cell-crystal preparations than for AGRO2 free spore-crystal preparations. This effect on *H. virescens* and *O. nubilalis* may be due to the slower

TABLE 5. Effects of *B. thuringiensis* AGRO1 and AGRO2 on the
mortality of *P. xylostella* larvae and on the damage inflicted on
cabbage, compared with the effects of other *B. thuringiensis*
and chemical insecticidal products[a]

Treatment	Rate (g of ai/ha)	% Larval mortality at:			% Visual crop damage at 7 DAA
		1 DAA	3 DAA	7 DAA	
Untreated		0	4	5	42
AGRO1 WP 10	10	35	84	84	6
	20	37	79	90	8
	40	33	92	97	5
	80	62	95	100	3
AGRO2 WP 10	10	57	73	83	14
	20	58	90	100	7
	40	52	97	100	5
	80	88	98	100	4
Delfin WG	125	0	28	42	21
	250	23	61	78	20
	500	19	62	60	18
	1,000	37	69	83	16
Thuricide HP	625	19	54	81	30
Agrimek EC1.8	18	36	54	66	31
Decis EC2.5	7.8	28	42	50	38
Hostathion EC40	250	17	41	47	38

[a] Each plant was artificially infested with 20 first and second instar larvae of *P. xylostella* 1 day prior to the application of a single treatment. Each value is the mean of results from three assays, each with one potted cabbage plant. DAA, day(s) after application.

TABLE 6. Field performances of *B. thuringiensis* AGRO1 and
AGRO2 against a natural infestation of worms on
cabbage and broccoli[a]

Treatment	Rate (g of ai/ha)	% Crop damage at 9 days after last application[b]		
		Trial no. 1 (crop, cabbage; applications, 2; interval, 7 days)	Trial no. 2 (crop, broccoli; applications, 3; intervals, 5 and 7 days)	Trial no. 3 (crop, cabbage; applications, 4; interval, 4 days)
Untreated	0	67	48	37
AGRO1 WP 10	20	47	37	12
	40	43	33	10
	80	27	18	7
AGRO2 WP 10	20	50	38	12
	40	35	25	8
	80	27	15	6
Mattch 126 SC	588	25	18	6
Lannate 90 WP	1,121	18	3	6

[a] Location: Western Field Research Station, Fresno, Calif. Dates of trials: August to September 1996. There were three replications per trial.

[b] The crop used, the number of treatment applications, and the interval(s) of days between applications are given for each field trial in parentheses.

release and/or dissolution of the encapsulated AGRO1 crystal proteins in the gut of these insects. Alternatively, the higher toxicity of strain AGRO2 may be due to synergy with infectious live spores (9). We therefore compared, in the laboratory, the insecticidal activity of the AGRO1 strain with that of a combination of AGRO1 and 10^4 spores of an acrystalliferous derivative of strain Kto (Kto⁻) (31) against *O. nubilalis* and *P. xylostella*. Two independent experiments indicated that, for *O. nubilalis*, the LC_{50} of the combined spore-crystal preparation was significantly lower ($P < 0.05$) than that of AGRO1 {LC_{50}s of 4 (95% confidence interval [CI], 3 to 5) ng/cm² and 20 (95% CI, 17 to 26) ng/cm², respectively}, indicating that spores increased the toxicity of AGRO1 crystals to the European corn borer. In contrast, for *P. xylostella* (three experiments), the difference in activity between the two preparations was not significant ($P > 0.05$) (LC_{50}s of 1.5 [95% CI, 0.9 to 2.1] ng/cm² and 2.4 [95% CI, 0.6 to 8.7] ng/cm², respectively).

(ii) Greenhouse trials. AGRO1 and AGRO2 preparations were tested against *P. xylostella* (diamondback moth) on cabbage in greenhouse conditions (Table 5). At the standard application rates of 40 and 80 g of ai/ha, both strains caused 97 to 100% larval mortality 7 days after application, with no significant difference between the two preparations. The minimum dose providing acceptable control of the pest (prevention of feeding damage) was around 20 g of ai/ha for both. The two recombinant strains controlled the diamondback moth larvae infesting the cabbage with an efficacy matching or exceeding that of the chemical standards and *B. thuringiensis* products tested (Table 5).

(iii) Field trials. Field trials were conducted with cabbage and broccoli to assess the value of AGRO1 and AGRO2 in field conditions against various lepidopteran pests in natural infestation conditions. The AGRO1 and AGRO2 preparations, the commercial *B. thuringiensis* product Mattch 126 SC, and the chemical insecticide Lannate 90 WP were applied by spraying two to four times, and the damage to cabbage and broccoli was assessed 9 days after the last application (Table 6). AGRO1 and AGRO2 treatments significantly and dose dependently reduced insect damage. Untreated larvae of a natural pest complex consisting of *Trichoplusia ni*, *Artogeia rapae*, and *P. xylostella* caused 37, 48, and 67% damage, respectively, in three independent trials, whereas losses in AGRO1-

or AGRO2-treated plots at the maximum dose of 80 g of ai/ha were 7, 18, and 27% (AGRO1), respectively, or 6, 15, and 27% (AGRO2), respectively. Both strains applied at 80 g of ai/ha were as effective as the reference materials Mattch (588 g of ai/ha) and Lannate (1,121 g of ai/ha) against the cabbage pest complex.

DISCUSSION

We report the construction of two new recombinant *B. thuringiensis* strains, AGRO1 and AGRO2, their use to test the effect of encapsulation on crystal toxicity, and their residual activity after UV exposure. Sunlight-mediated inactivation of *B. thuringiensis* crystals is often cited as the major factor affecting the performance and economic viability of *B. thuringiensis* products. Irradiation destroys up to 35% of tryptophan residues in purified *B. thuringiensis* subspecies HD1 and HD73 crystals, resulting in a loss of toxicity (25). Encapsulated crystals produced by AGRO1 were better protected against UV than unencapsulated crystals produced by AGRO2 or by the natural *aizawai* strain that constitutes the active ingredient of Xentari (Table 3). Therefore, formulations in which mature crystals are produced and encapsulated in *B. thuringiensis* ghost cells show promise for reducing the loss of biological activity due to sunlight in the field. We also show increased activity of the Cry1C toxin against certain insect pests by replacing part of its carboxy-terminal protoxin region with the equivalent region from the Cry1Ab δ-endotoxin (Table 2). This increase in toxicity was presumably due to the fusion protein containing a portion of the carboxy-terminal half of Cry1Ab, which lacks 26 amino acids, including four cysteine residues likely to be involved in the formation of the protease-resistant crystal structure via disulfide bond formation. This may increase the solubility of the chimeric protein relative to that of native Cry1C, accounting for differences in toxicity between the crystals. Toxicity requires the δ-endotoxins to be solubilized and activated in the gut of insect larvae. Hence, the efficacy of a particular δ-endotoxin is partly dependent on its solubility and proteolytic cleavage into toxins. This has been shown for the crystal produced by *B. thuringiensis* subsp. *aizawai* (HD133) (4). We assessed the efficacy of encapsulated and nonencapsulated chimeric crystal proteins in field trials. Both AGRO1 and AGRO2 strains effectively controlled various lepidopteran pests on cabbage in artificial and natural infestation conditions (Tables 4 to 6). AGRO2 was slightly more biologically active than AGRO1 in laboratory bioassays (Table 4); this difference in activity was significant ($P < 0.05$) for *H. virescens* and *O. nubilalis* but not for *S. littoralis*, *S. frugiperda*, and *P. xylostella* ($P > 0.05$). The addition of spores to the AGRO1 preparation did not change its activity against *P. xylostella* but reduced its LC_{50} against *O. nubilalis* by a factor of 5. This is consistent with the difference in activity between AGRO1 and AGRO2 toward *O. nubilalis* (Table 4). It suggests that the lower potency of AGRO1 than AGRO2, in laboratory bioassays, against *O. nubilalis* reflects the contribution of spores to toxicity in this insect species rather than encapsulated crystals being less available to insects. The spores of *B. thuringiensis* subsp. *kurstaki* HD1 have been described as having a similar effect against *Plodia interpunctella* (Indian meal moth) larvae. In this species, spore germination and the resulting septicemia in larval hemolymph synergized the toxicity of purified Cry1Ab and Cry1C crystal proteins (13, 14). In greenhouse and field conditions, the efficacy of AGRO1 and AGRO2 strains, applied at 80 g of ai/ha, for the control of lepidopteran pests was equivalent to that of the reference products Mattch and Lannate (Tables 5 and 6). However, these trials involved comparing nonopti-

mized formulations of AGRO1 and AGRO2 with commercial formulations of chemical and other *B. thuringiensis* products. The efficacy of these new recombinant strains could still be greatly increased by formulation.

Another important feature of the AGRO1 recombinant strain is that it is a mutant blocked at late sporulation that does not produce viable spores. Sporogenic *B. thuringiensis* strains have been used for more than 40 years and have a good safety record for vertebrates and other nontarget organisms. However, in rare cases, *B. thuringiensis* has been shown to be responsible for infections in man (11, 27). A case of severe war wounds becoming infected by *B. thuringiensis* serotype H34 has been described, and this strain was found to be pathogenic in a mouse model of cutaneous infection (12). *B. thuringiensis* spores also frequently infect larvae of the mulberry silkworm, *Bombyx mori*, in silkworm-rearing areas (21), and a recent study has also reported adverse effects of a *B. thuringiensis* formulation on a beneficial organism commonly used to control lepidopteran pests (22). Biological safety and environmental impact are therefore important considerations, of increasing public concern, that must be taken into account when developing new genetically engineered biopesticides. The recombinant AGRO1 strain is asporogenic and is therefore at a considerable competitive disadvantage compared to wild-type *B. thuringiensis* strains present in the environment and sporogenic commercial and recombinant *B. thuringiensis* strains. We found that a culture of AGRO1 grown for 30 h at 30°C in liquid HCT medium and plated onto nutrient agar plates gave no colonies (data not shown), indicating that at this stage (after the sporulation-deficient cells have produced crystal inclusions) the cells are no longer viable. Consequently, the ecological impact of disseminating AGRO1 or other asporogenic *B. thuringiensis* strains should be extremely low, as their viability is much lower than that of natural strains.

Toxin encapsulation with asporogenic strains, therefore, has two potential advantages: (i) improved biological and environmental safety (the strains are nonviable and do not contain foreign DNA—except the *kan* cassette in the chromosome, but this gene can be removed—minimizing the risks of accidental infection and DNA transfer and dissemination) and (ii) UV light resistance (the δ-endotoxins are better protected from degradation than the nonencapsulated δ-endotoxins). A possible disadvantage is the absence of spores that sinergistically increase toxicity for certain insects. However, in greenhouse and field tests, the potency and efficacy of the encapsulated asporogenic strain were similar to those of the *B. thuringiensis*-based and synthetic insecticides tested. Therefore, this mutant could be used to design and construct a new generation of recombinant *B. thuringiensis* strains that are more environmentally friendly than the existing sporogenic commercial strains. In addition, costs associated with the γ irradiation of *B. thuringiensis* formulations to kill the spores before sale (when required) and with adding UV protectants to *B. thuringiensis* products (to avoid the rapid loss of their biological activity after spraying) are avoided with asporogenic mutants.

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