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Enhanced Production of Insecticidal Proteins in *Bacillus thuringiensis* Strains Carrying an Additional Crystal Protein Gene in Their Chromosomes

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A two-step procedure was used to place a *cryIC* crystal protein gene from *Bacillus thuringiensis* subsp. *aizawai* into the chromosomes of two *B. thuringiensis* subsp. *kurstaki* strains containing multiple crystal protein genes. The *B. thuringiensis aizawai cryIC* gene, which encodes an insecticidal protein highly specific to *Spodoptera exigua* (beet armyworm), has not been found in any *B. thuringiensis* subsp. *kurstaki* strains. The *cryIC* gene was cloned into an integration vector which contained a *B. thuringiensis* chromosomal fragment encoding a phosphatidylinositol-specific phospholipase C, allowing the *B. thuringiensis* subsp. *aizawai cryIC* to be targeted to the homologous region of the *B. thuringiensis* subsp. *kurstaki* chromosome. First, to minimize the possibility of homologous recombination between *cryIC* and the resident crystal protein genes, *B. thuringiensis* subsp. *kurstaki* HD73, which contained only one crystal gene, was chosen as a recipient and transformed by electroporation. Second, a generalized transducing bacteriophage, CP-51, was used to transfer the integrated *cryIC* gene from HD73 to two other *B. thuringiensis* subsp. *kurstaki* strains. The integrated *cryIC* gene was expressed at a significant level in all three host strains, and the expression of *cryIC* did not appear to reduce the expression of the endogenous crystal protein genes. Because of the newly acquired ability to produce the CryIC protein, the recombinant strains showed a higher level of activity against *S. exigua* than did the parent strains. This two-step procedure should therefore be generally useful for the introduction of an additional crystal protein gene into *B. thuringiensis* strains which have multiple crystal protein genes and which show a low level of transformation efficiency.

Bacillus thuringiensis subsp. *kurstaki* HD1 contains at least five crystal protein genes, including *cryIA*(*a*), *cryIA*(*b*), *cryIA*(*c*), *cryIIA*, and *cryIIB* (14, 18). These *cryIA* and *cryII* genes are found in many *B. thuringiensis* subsp. *kurstaki* strains, and they encode proteins active against lepidopteran insect larvae such as *Heliothis virescens* and *Manduca sexta* (14). However, the CryIA and CryIIA proteins are not particularly active against *Spodoptera* species. In contrast, CryIC differs significantly from the CryIA and CryIIA proteins both in its amino acid sequence and insecticidal spectrum, particularly in its notable activity against *Spodoptera* species (33). To date, CryIC has only been found in *B. thuringiensis* subsp. *aizawai* and subsp. *entomocidus* (32).

We were interested in placing the *cryIC* gene, which encodes CryIC, into *B. thuringiensis* subsp. *kurstaki* strains to broaden their insecticidal spectra. We chose to integrate the *cryIC* gene into the chromosome because it has been found that crystal genes introduced on plasmids are not stably maintained in *B. thuringiensis*. For example, Crickmore et al. (8) used *Escherichia coli-B. thuringiensis* shuttle vectors made from *E. coli* and *Staphylococcus aureus* replicons to express three different *cry* (crystal protein) genes in several *B. thuringiensis* subspecies, but these vectors were found to undergo significant rearrangements. Shuttle vectors made from *B. thuringiensis* replicons joined to pUC vectors were found to have greater structural and segregational stability (3, 19), although in some cases, the presence of these vectors caused poor or delayed sporulation (19, 27). Furthermore, the maintenance of introduced plasmids generally requires the presence of antibiotic in the culture medium. To eliminate the need to maintain antibiotic selection and to increase plasmid stability, cloning vectors made exclusively of *Bacillus* DNA have been evaluated (4, 11). The use of *Bacillus* replicons was found to enhance stability, but these vectors sometimes displaced resident plasmids which carried important crystal protein genes.

To circumvent the problems associated with the introduction of recombinant plasmids, we instead used an integration vector to stably place the *cryIC* gene into the chromosome of *B. thuringiensis* HD73. The integration plasmid was introduced into HD73 via electroporation, and then generalized transduction was used to transfer the integrated *cryIC* gene from HD73 to two other *B. thuringiensis* subsp. *kurstaki* strains. The use of HD73 as an intermediate host for *cryIC* enabled us to introduce and stably maintain the *cryIC* gene in *B. thuringiensis* strains which contained multiple crystal protein genes. This approach can be generally applied to broaden the insecticidal spectrum of any *B. thuringiensis* strain which is not easily transformed.

MATERIALS AND METHODS

Bacterial strains and media. *E. coli* DH5α (Bethesda Research Laboratories) was used for cloning, and strain GM2163 (New England Biolabs) was used to produce DNA for *B. thuringiensis* transformations. *B. thuringiensis* HD73 and HD229 were obtained from the U.S. Department of Agriculture strain collection (Peoria, Ill.), and BT93 and BT94 were obtained from C. Y. Chen (Sandoz Agro, Inc.). CYS medium (34) was used for all studies unless otherwise noted. Modified CYS medium contained increased amounts of yeast extract, glucose, and KH_2PO_4 at 0.3, 0.7, and 0.5%, respectively. PA plates (30) were used for preparation of CP-51 transducing lysates. Prior to the isolation of *B. thuringiensis*

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TABLE 1. Oligonucleotides used in this study

Primer	Sequence	Gene or vector
cryIIA1	ACTATTTGTGATGCGTATAATGTA	*cryIIA*
cryIIA2	AATTCCCCATTCATCTGC	*cryIIA*
GALP1	CCACAGTTACAGTCTGTAGCTCAATTACC	*cryIC*
GALP2	CCGCTACTAATAGAACCTGCACCA	*cryIC*
KK14	AGCTTGCGGCCGCGTCGACCCCGGGCCATGGGGGCCCG	
KK14B	AATTCGGGCCCCCATGGCCCGGGGTCGACGCGGCCGCA	
NHS43	ATTTTGTATTAAACGG	*cryIIB*
NHS54	GAAATATTGCATAGAAGTAAAG	*plc*
NHS55	GGACGTTTAACAGATGATAATACG	*plc*
NHS56	CATGAAGTACCCATGTCATTCCG	*plc*
NHS62	GGCTATGAACAAAGAAAACCAAACGG	*plc*
NHS63	CGGAACATAATGGTGCAGGGCGCTGACTTCCGCG	pBR322
PHOS1	GGAACGCTACATACTAGTGATAGAGTAG	*plc*
PHOS4	GCTTGTACACCGCAACTGTTTTCGCATG	*plc*
SK29	GGAGAAGCCAGAGATGTTTGTATCTGGTGG	CP-51
SK29REV	CCTCTGGCTTACCTGCGTTGGCGAACACATC	CP-51
SKRAND	NNNNNNNNGC[a]	CP-51

[a] N represents any base.

plasmid DNA, strains were grown for 3 to 4 h at 37°C on SA plates (1× Spizizen salts [2], 1% Casamino Acids, 5% glucose, 5 μM $MnSO_4$). For spore counts, cultures were allowed to grow for 48 h after the transition from exponential growth into the stationary phase. A 1:10 dilution of the culture was heated at 65°C for 45 min, serial dilutions were plated, and colonies were counted after overnight incubation at 25°C.

DNA methods. Ligations, *E. coli* transformations, isolation of plasmid DNA, and isolation of DNA fragments were done by standard methods (28). The Sequenase version 2.0 DNA sequencing kit (United States Biochemical Corp.) was used for DNA sequencing according to the manufacturer's instructions. *B. thuringiensis* total DNA was isolated as described previously (16). *B. thuringiensis* plasmid DNA was isolated essentially as described by Birnboim and Doly (5), except that 3 M potassium acetate (pH 4.8) was used instead of 3 M sodium acetate. To isolate CP-51 phage DNA, a concentrated phage stock was heated to 67°C for 5 min and extracted once with phenol, twice with phenol-chloroform (1:1), and once with chloroform.

PCR was done by standard methods (29) unless otherwise noted. Chromosomal integration at the phospholipase C site was confirmed by PCR with total *B. thuringiensis* DNA as a template. *B. thuringiensis* cells were used as a template to determine crystal gene content. *B. thuringiensis* cells were grown overnight on solid medium and boiled for 10 min in 1× GeneAmp PCR buffer (Perkin-Elmer).

Cloning of the phosphatidylinositol-specific phospholipase C gene from HD73. The DNA sequence of a *B. thuringiensis* phospholipase C gene (referred to here as the *plc* gene) from strain ATCC 10792 (accession number X14178) was obtained from GenBank (20). Oligonucleotides PHOS1 and PHOS4 (Table 1) were designed on the basis of this sequence, and a 2.25-kb fragment was generated from strain HD73 by PCR. In strain 10792, this 2.25-kb fragment contained the 987-bp *plc* coding region, plus 450 bp upstream and 810 bp downstream. The *plc* fragment was cloned in pUC18, and the resulting plasmid was designated pSB139.

Cloning and sequencing of the region upstream of the *plc* gene. Southern blotting of the *plc* region revealed the presence of two *Eco*RI sites separated by 1.8 kb. One *Eco*RI site lay in the *plc* coding region, and the other lay upstream. An inverse PCR (24) was used to isolate this 1.8-kb *Eco*RI fragment. HD73 DNA was digested with *Eco*RI, and fragments in the 1- to 3-kb range were circularized by ligation. The ligation products were used as a PCR template with primers NHS54 and NHS55 (Table 1). The 1.4-kb PCR product was cloned in pUC18 to generate plasmid pSB043, and restriction mapping verified that pSB043 contained sequences upstream of the *plc* gene. Plasmid pSB043 was sequenced with primer NHS56 (Table 1) to obtain sequence upstream from the previously cloned *plc* fragment. This upstream sequence was used to design primer NHS62, which was used to verify integration in the *plc* region (see Fig. 2B).

Cloning of the *cryIC* gene. A subgenomic library of 6- to 9-kb *Eco*RI fragments from *B. thuringiensis* subsp. *aizawai* HD229 was constructed in the Lambda ZAP II cloning vector (Stratagene). Clones containing the *cryIC* gene were identified with a 984-bp *cryIC* probe generated by PCR from HD229 DNA with the primers GALP1 and GALP2 (Table 1). A pBluescript phagemid containing the *cryIC* gene on an 8-kb *Eco*RI fragment was excised from the Lambda vector and was designated pSB201. The *cryIC* gene, with its promoter and terminator, was removed from pSB201 in two pieces: the NH_2-terminal portion on a 2.7-kb *Hin*dIII-*Kpn*I fragment and the COOH-terminal portion on a 2.4-kb *Hin*dIII-*Eco*RI fragment. The two fragments were ligated into pTZ19R, which had been digested with *Eco*RI and *Hin*dIII. A PCR technique (7) was used to engineer an *Nco*I site at the translation start site and an *Eco*RI site immediately following the translation termination site. The *cryIC* coding region was excised with *Nco*I and *Eco*RI and transferred to an expression cassette in a pBluescript KS(+)-based plasmid, and this construct was designated pSB619 (Fig. 1A). The expression cassette carried the putative promoter region from *cryIC* (240 bp upstream of the translation start site) and 329 bp of the *cryIA*(*c*) terminator (nucleotides 3924 to 4253 in reference 1).

Construction of plasmid pSB210.2, used to place the *cryIC* gene in the *B. thuringiensis* chromosome. Plasmid pSB210.2 contained four functional DNA segments (Fig. 1B). It carried the *cryIC* gene, an erythromycin resistance gene (*ermC*) for selection in *B. thuringiensis*, a fragment of the *B. thuringiensis* chromosome encoding a phosphatidylinositol-specific phospholipase C (*plc*) for recombination between the plasmid and the homologous region of the chromosome, and the origin of replication and ampicillin resistance gene from the *E. coli* plasmid pBR322. The *cryIC* gene was placed under the control of its own promoter and the transcription terminator of the *cryIA*(*c*) gene from strain HD73. The *cryIA*(*c*) terminator was chosen because it contained dual regions of dyad symmetry. The erythromycin resistance gene, *ermC*, was isolated from *Bacillus subtilis* (22) and encodes a methylase that confers erythromycin resistance to *B. thuringiensis* cells.

Plasmid pSB210.2 was constructed as follows. The tetracycline resistance gene on a *Hin*dIII-*Ava*I fragment of pBR322 was replaced with the erythromycin resistance gene on a *Hin*dIII-*Sma*I fragment. The erythromycin resistance gene, *ermC*, cloned in pUC18 was obtained from Gamel and Piot (11). The *Apa*I, and *Not*I recognition sequences were introduced between the *Hin*dIII and *Eco*RI sites of the modified pBR322 with oligonucleotides KK14 and KK14B (Table 1). The *plc* gene, on a 2.25-kb *Kpn*I-*Bam*HI fragment from pSB139, was then inserted between the *Msc*I and *Bam*HI sites to create plasmid pSB210.1. The *Kpn*I end of the 2.25-kb *plc* fragment was made blunt prior to digestion with *Bam*HI. The *cryIC* gene, which had been excised from pSB619 with *Apa*I and *Not*I, was ligated into pSB210.1 to create pSB210.2.

Cloning a fragment of phage CP-51. DNA was isolated from phage CP-51 as described above. Fragments of the phage genome were amplified by PCR with a degenerate primer, SKRAND (Table 1). The thermal cycler was run at 94°C for 1 min, 37°C for 1 min, and 72°C for 2 min for 40 cycles with a primer concentration of 3.9 mM. The PCR fragments were cloned in pUC18, and one clone, pSB158, carrying an insert with a size of approximately 0.6 kb was sequenced at each end with the M13 universal and reverse sequencing primers (Pharmacia Biotech). Primers SK29 and SK29REV (Table 1) were designed to generate a 390-bp fragment from CP-51 DNA by PCR.

***B. thuringiensis* transformation.** To prepare competent cells, *B. thuringiensis* HD73 was grown in brain heart infusion medium (Difco) containing 0.5 M sucrose (BHIS) at 37°C with shaking until the culture reached an optical density at 600 nm of 0.2. The cells were harvested by centrifugation, washed once in 1 volume and then washed twice in 1/10th volume of 5 mM HEPES (*N*-2-hydroxyethylpiperazine-*N*′-2-ethanesulfonic acid [pH 7])–0.5 M sucrose. Electroporation of 100 μl of the cell suspension was performed in a 0.2-cm electrode gap cuvette with a Bio-Rad Gene Pulser set at 1.25 kV and 3 μF. Following electroporation, the cells were transferred to 5 ml of BHIS and grown for 3 h at 37°C and 250 rpm before being plated on Luria broth medium (LB) containing 10 μg of erythromycin per ml.

CP-51 transductions. Phage CP-51ts45, a temperature-sensitive phage CP-51 mutant, was generously provided by Curtis B. Thorne (University of Massachusetts, Amherst). To make the transducing lysate, the donor strain (SB110) was

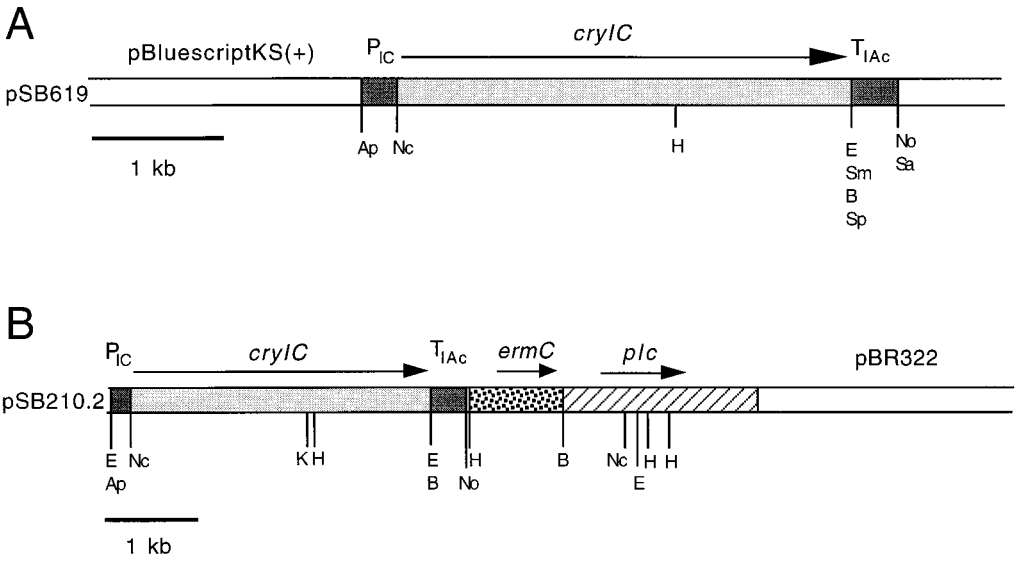

FIG. 1. Linear plasmid maps of plasmids pSB619 (A) and pSB210.2 (B). Vector DNA is indicated by open bars, and the vector name is written above the map. Gene positions are indicated by arrows. P_{IC} represents the *cryIC* promoter, and T_{IAc} represents the *cryIA*(*c*) terminator. Restriction site abbreviations: Ap, *Apa*I; B, *Bam*HI; E, *Eco*RI; H, *Hin*dIII; K, *Kpn*I; Nc, *Nco*I; No, *Not*I; Sa, *Sac*I; Sp, *Spe*I, Sm, *Sma*I.

grown in LB for 6 h and plated with CP-51 phage at multiplicities of infection of 4 and 10. After overnight incubation at 30°C, the top agar was macerated in 5 ml of PA and centrifuged to remove bacterial cells and cell debris, and the supernatant was filtered through a Millipore HA filter. The phage lysate was stored at 16°C. For transductions, recipient strains (BT93 and BT94) were grown in LB to a density of approximately 4×10^8 CFU/ml and mixed with the CP-51 phage lysate at a multiplicity of infection of 3. The phage-and-cell mixture was spread on an LB plate overlaid with an HA filter (Millipore) and incubated for 3 h at 37°C. The filters were then transferred to LB plates containing 10 μg of erythromycin per ml and incubated at 37°C for 36 h.

Protein analysis. *B. thuringiensis* cultures for sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) were grown in modified CYS medium until spores and crystal were released into the medium (40 to 44 h). The harvested cultures were washed three times in 1 volume of 10 mM CAPS (3-[cyclohexylamino]-1-propanesulfonic acid)–NaOH (pH 10.5)–0.5 M NaCl–1 mM EDTA and resuspended in 1 volume of 10 mM Tris-HCl (pH 7.5)–10 mM EDTA. Western blotting (immunoblotting) was performed as described previously (16) with anti-CryIC polyclonal antiserum.

Bioassay. Cultures were grown in CYS medium for 72 h, and dilutions were incorporated into artificial insect diet. Each dilution was infected with 10 late-third-instar larvae, and the percentage of mortality was determined after a 4-day incubation at 28°C. The 50% lethal concentration was determined by probit analysis.

RESULTS

Integration of *cryIC* in strain HD73 and CP-51-mediated transduction. We chose to integrate the *B. thuringiensis* subsp. *aizawai cryIC* gene into several *B. thuringiensis* subsp. *kurstaki* strains to increase their activity against *Spodoptera exigua*. We cloned and sequenced the *cryIC* gene from *B. thuringiensis* subsp. *aizawai* HD229 and found it was identical to the *cryIC* previously isolated by Honée et al. from *B. thuringiensis* subsp. *entomocidus* (15[accession number M37242]). Furthermore, we demonstrated that the CryIC protein encoded by the HD229 *cryIC* gene was highly active against *S. exigua* (16).

B. thuringiensis subsp. *kurstaki* HD73 was chosen as the recipient for *cryIC* because it is relatively easy to transform and contains only one crystal toxin gene, *cryIA*(*c*). HD73 was transformed by electroporation with 9 μg of pSB210.2 DNA, and two isolates were obtained on a medium containing erythromycin. One isolate, SB110, was chosen to study expression of the integrated *cryIC* gene. To determine the transformation efficiency that led to integration, the competent HD73 cells were simultaneously transformed with a shuttle vector derived from pBC16-1 (17) and pTZ19R. Approximately 3.5×10^5 transformed *B. thuringiensis* cells were obtained per μg of shuttle vector DNA.

Two *B. thuringiensis* subsp. *kurstaki* strains which contained multiple crystal protein genes, BT93 and BT94, were chosen as recipient strains for the *cryIC* gene. The PCR was used to determine which *cry* genes were contained in these host strains. A mixture of primers that hybridized to *cryI*-type genes (16) showed that both BT93 and BT94 contained the *cryIA*(*a*), *cryIA*(*b*), and *cryIA*(*c*) genes. In addition, PCR with primer pairs cryIIA1 and cryIIA2 and NHS43 and cryIIA2 (Table 1) revealed that the *cryIIA* and *cryIIB* genes were also present in these strains.

Phage CP-51 (30) can carry out generalized transduction in *B. thuringiensis*, *Bacillus anthracis*, and *B. cereus* (31). A transducing lysate made from strain SB110 was used to transduce BT93 and BT94 to erythromycin resistance. The transduction frequencies were approximately 1×10^{-6} transductants per CFU for strain BT93 and 5×10^{-7} transductants per CFU for strain BT94. One BT93 transductant was designated SB136, and one BT94 transductant was designated SB137. PCR confirmed that strains SB110, SB136, and SB137 had acquired the *cryIC* gene while retaining their native *cry* genes. Consistent with this result, agarose gel electrophoresis showed that the plasmid profile of each recombinant strain was indistinguishable from that of the respective parent strain, indicating no plasmids were lost, gained, or altered in size as a result of transformation or transduction.

PCR also demonstrated the recombinant strains did not harbor any residual CP-51 phage, either as a lysogen or as a virulent phage. A piece of the CP-51 genome was cloned, and phage-specific PCR primers were used to identify sequences specific to phage CP-51. PCR with primers SK29 and SK29REV (Table 1) generated a 390-bp fragment from both CP-51 DNA and the CP-51 transducing lysate. However, no PCR fragments were amplified from cells or DNA isolated from strains HD73, BT93, BT94, SB110, SB136, and SB137.

Plasmid pSB210.2 integrated at the *plc* site. Since the integration plasmid did not contain an origin of replication functional in *B. thuringiensis*, erythromycin-resistant isolates were

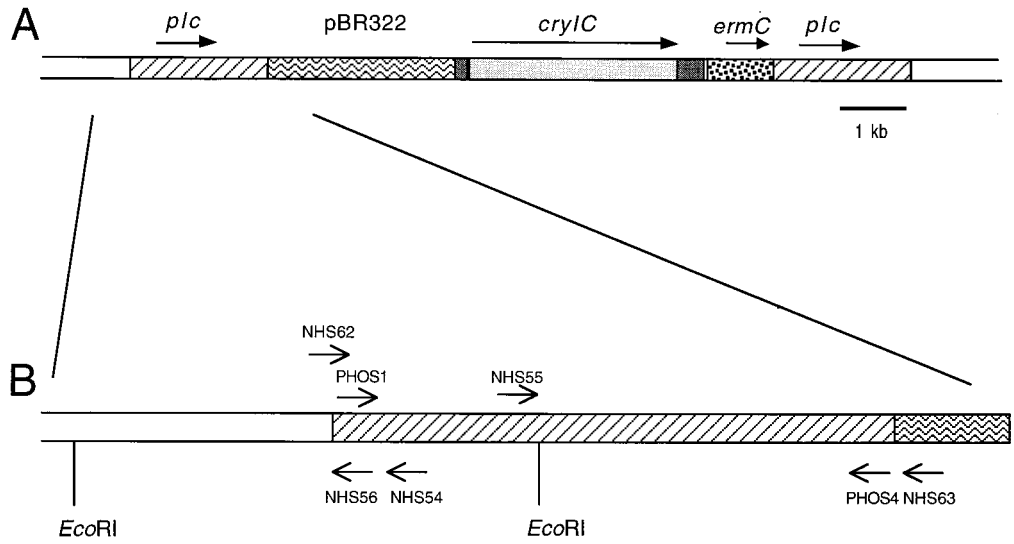

FIG. 2. (A) Expected arrangement of the phospholipase C region of the *B. thuringiensis* chromosome after integration of pSB210.2. Hatched bars represent the *plc* fragment cloned in pSB210.2, and open bars adjoining each *plc* fragment represent flanking chromosomal DNA. (B) Hybridization positions of oligonucleotide primers used for PCR and sequencing. PHOS1 and PHOS4 were used to isolate the 2.25-kb *plc* fragment. Primers NHS54 and NHS55 were used to generate the upstream region adjacent to the 2.25-kb *plc* fragment (plasmid pSB043) from a circularized chromosomal fragment. Primer NHS56 was used to sequence pSB043, and this sequence was used to design primer NHS62. Primers NHS62 and NHS63 were diagnostic for chromosomal integration at the *plc* site.

obtained only by integration of the entire plasmid into the chromosome by a single crossover at the phospholipase C region (Fig. 2A). To verify that plasmid pSB210.2 integrated in the phospholipase C region, PCR was used to generate a fragment between the flanking chromosomal DNA and the integrated plasmid. Primer NHS62 hybridized upstream of the *plc* gene, and primer NHS63 hybridized to the pBR322 portion of pSB210.2 (Fig. 2B). As a result, these primers amplified a 2.57-kb PCR fragment from DNA isolated from recombinant strains SB110, SB136, and SB137. *Xmn*I digestion of this 2.57-kb PCR product produced fragments of the expected sizes, confirming it was amplified from the *plc* region. No fragment was generated with DNA isolated from the parent strains, HD73, BT93, and BT94. DNA from all strains gave a PCR product with primers NHS62 and PHOS4, demonstrating that NHS62 hybridized upstream of the *plc* gene.

The integrated *cryIC* gene was expressed from the *B. thuringiensis* chromosome. Strains SB110, SB136, and SB137 and their wild-type parent strains were grown in modified CYS medium, and their crystal proteins were analyzed by SDS-PAGE. CryIC has a predicted molecular mass of 134.8 kDa, slightly greater than the predicted molecular masses of CryIA(a), CryIA(b), and CryIA(c), which are 133.2, 131, and 133.3 kDa, respectively. The CryIC protein appeared as a band with the expected size above the CryIA proteins of the three recombinant strains (Fig. 3A, lanes 2, 4, and 6). Furthermore, an immunoblot showed that anti-CryIC antiserum reacted with proteins from strains SB110, SB136, and SB137 as well as purified CryIC (Fig. 3B). No reaction was seen with proteins from strains HD73, BT93, and BT94. In addition, the expression of CryIC caused an increase in the total amount of CryI (CryIA plus CryIC) protein produced by the recombinant strains. Densitometric scanning of the total CryI protein on several polyacrylamide gels indicated that SB136 and SB137 produced approximately twofold more CryI protein than the parental strains.

Growth, sporulation, and stability of the integrated sequences. During exponential growth in CYS medium, there was no difference in doubling time between each recombinant

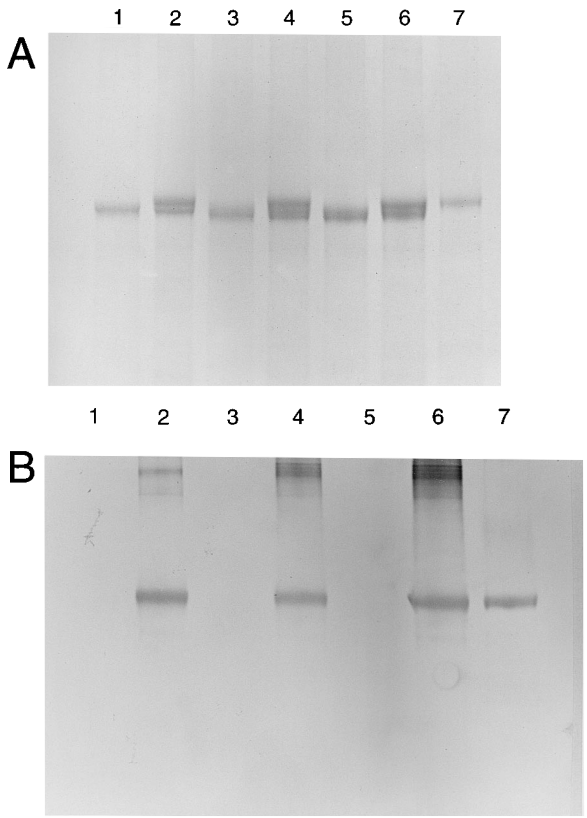

FIG. 3. SDS–10% polyacrylamide gel (A) and Western blot (immunoblot [B]) of crystal proteins produced by recombinant and parental strains. Lanes: 1, HD73; 2, SB110; 3, BT93; 4, SB136; 5, BT94; 6, SB137; 7, purified CryIC (0.5 μg). To purify CryIC, the *cryIC* gene was expressed in an acrystalliferous *B. thuringiensis* strain on a shuttle vector composed of plasmids pBC16-1 and pBluescript KS(+). The CryIC protein was purified by Sephacryl S300 column chromatography as described previously (34).

TABLE 2. Insecticidal activity of *B. thuringiensis* strains against *S. exigua*

Strain	LC[a]	
	50%	90%
BT93	20.0	36.1
SB136	3.6	6.9
BT94	26.9	62.9[b]
SB137	4.2	7.7

[a] 50% and 90%, 50 and 90% lethal concentrations (LC), respectively. Values are expressed as microliters of fermentation broth in 1 g of insect diet and represent the average of three tests except as noted.
[b] One test only.

strain and its respective parent strain. In addition, all of the recombinant and parent strains sporulated with similar efficiencies. To determine the stability of the integrated sequences, 100 colonies from germinated spores of each recombinant strain (SB110, SB136, and SB137) were transferred onto a medium containing erythromycin. All colonies were erythromycin resistant, indicating the integrated plasmid was stably maintained throughout sporulation and germination.

Insecticidal activity of strains SB136 and SB137. Bioassay clearly confirmed that the expression of *cryIC* in strains SB136 and SB137 caused enhanced activity against *S. exigua*. In the average of three tests, the 50% lethal concentrations of SB136 and SB137 were approximately sixfold lower than those of the respective parent strains, BT93 and BT94 (Table 2).

DISCUSSION

Chromosomal integration of plasmid DNA has been used extensively in *B. subtilis* for many purposes, including analysis of gene fusions, mapping cloned genes, definition of transcriptional units, and mutagenesis (10, 13, 23, 25). Calogero et al. (6) used an integration vector to express a crystal protein gene from *B. thuringiensis* in *B. subtilis*. They placed the *cryIA*(*c*) gene from strain HD73 into the *B. subtilis* chromosome, and after amplification of the inserted sequences, their recombinant strain produced bipyramidal crystals. So far, no reports describing chromosomal integration in *B. thuringiensis* have been published. However, homologous recombination has been used in *B. thuringiensis* to integrate DNA fragments into resident plasmids, both for mutagenesis and for heterologous gene expression. Delécluse et al. (9) used homologous recombination to disrupt the plasmid-encoded *cytA* gene to determine the contribution of the CytA protein to the dipteran activity of *B. thuringiensis* subsp. *israelensis*. Lereclus et al. (21) used recombination to place the *cry*(III)*A* gene in an insertion sequence on the 75-kb plasmid in strain HD73. They cloned *cryIIIA* on a temperature-sensitive replicon, within the insertion sequence (IS*232*), and transformed strain HD73. Integration of *cryIIIA* into the 75-kb plasmid occurred when they raised the temperature to a point at which the introduced plasmid could no longer replicate.

Chromosomal integration appeared to occur much less readily in *B. thuringiensis* than in *B. subtilis*. We found it was necessary to achieve relatively high transformation efficiencies for chromosomal integration to occur. In our experiments, integrants were obtained only when the transformation efficiency was greater than 3×10^5 transformants per μg of DNA. Lereclus et al. (21) hypothesized that recombination does not occur efficiently in *B. thuringiensis*. They state that the low frequency of recombination prompted them to design experiments (utilizing the temperature-sensitive replicon) in which the recombination step is separated from the transformation step, especially for strains that are not transformed at high levels of efficiency. Because crystal toxin genes constitute a large family of highly related genes, it seems that recombination must occur in *B. thuringiensis*. It was interesting to find that chromosomal integration is truly difficult in this organism.

B. thuringiensis subsp. *kurstaki* BT93 and BT94 produce bipyramidal and cuboidal crystals which are toxic to various lepidopteran and dipteran larvae, including *Trichoplusia ni*, *H. virescens*, and *Aedes aegypti*. PCR analysis showed that strains BT93 and BT94 contain at least five *cry* genes, and the insecticidal spectra of these strains indicate that many of these genes are highly expressed. When *cryIC* was placed into the chromosomes of these strains, it was expressed and the total amount of *cryI* protein increased substantially. We found it noteworthy that high-yielding *B. thuringiensis* strains, such as BT93 and BT94, were able to produce an additional insecticidal protein at a significant level.

After *cryIC* was integrated in strain HD73, generalized transduction was used to transfer *cryIC* to two additional *B. thuringiensis* strains. Transduction provided an efficient means for transfer of the integrated *cryIC* gene while bypassing the need for highly efficient transformation. Additionally, the use of transduction ensured that the *cryIC* gene would integrate at the desired chromosomal locus in BT93 and BT94. Although the NH_2-terminal coding region of *cryIC* differs substantially from those of the *cryIA* genes, they are approximately 90% identical in a 1.7-kb portion of the COOH-terminal coding region. Such a high level of homology could cause recombination between *cryIC* and the plasmid-encoded *cryIA* genes present in multiple copies to occur. Because of this, a strain such as HD73, which carries a single crystal gene (or a *cry*-minus strain), makes a good transformation recipient for integration of a crystal protein gene.

It is well accepted that plasmid-encoded crystal genes are transferred between *B. thuringiensis* strains in nature by a conjugationlike process (12). However, plasmid movement is limited by certain factors, and these limitations may be responsible for the relationship between subspecies and crystal gene content. For instance, no *B. thuringiensis* subsp. *kurstaki* strains that contain *cryIC* have been identified; the *cryIC* gene has only been found in *B. thuringiensis* subsp. *aizawai* and subsp. *entomocidus* (32). However, we successfully transferred the *cryIC* gene from a *B. thuringiensis* subsp. *aizawai* strain to *B. thuringiensis* subsp. *kurstaki* and found that the *cryIC* gene was expressed efficiently. In a related experiment, J. Christophe Piot used conjugation to transfer *cryIC* from *B. thuringiensis* subsp. *entomocidus* to BT93 (26). He found that the *B. thuringiensis* subsp. *entomocidus* plasmid which carried *cryIC* was not easily mobilized. This may at least partially explain the fact that CryIC has only been found in *B. thuringiensis* subsp. *entomocidus* and subsp. *aizawai*. The confinement of CryIC to these subspecies may be due primarily to properties of the plasmid which carries the *cryIC* gene rather than properties of *cryIC* itself.

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