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A Recombinase-Mediated System for Elimination of Antibiotic Resistance Gene Markers from Genetically Engineered *Bacillus thuringiensis* Strains

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A TnpI-mediated site-specific recombination system to construct genetically modified *Bacillus thuringiensis* strains was developed. Recombinant *B. thuringiensis* strains from which antibiotic resistance genes can be selectively eliminated were obtained in vivo with a new vector based on the specific resolution site of transposon Tn*4430*. For example, a *cryIC* gene, whose product is active against *Spodoptera littoralis*, was introduced into *B. thuringiensis* Kto harboring a *cryIA(c)* gene active against *Ostrinia nubilalis*. The resulting strain had a broader activity spectrum than that of the parental strain. It contained only *B. thuringiensis* DNA and was free of antibiotic resistance genes. This should facilitate regulatory approval for its development as a commercial biopesticide.

Increased public awareness of environmental safety, development of pest resistance to chemical insecticides, and increasing costs of discovering new insecticidal molecules have led to renewed interest in the development of alternative biological control techniques for crop protection.

Microbial pesticides, in particular, are promising for sustainable agriculture that is less reliant on chemical pesticides. Several biopesticides, toxic only to certain targeted insect pests, provide insect control as good as that of conventional insecticides (that are much more damaging to the environment) and are already in use for agricultural or medical pest management (7, 11). One example of such environmentally friendly biological control agents is *Bacillus thuringiensis*, a facultative aerobic, gram-positive, spore-forming bacterium found naturally in soil. *B. thuringiensis* produces several proteins (known as δ-endotoxins or Cry proteins) during the stationary phase that accumulate as parasporal crystalline inclusions (1) and are toxic and highly specific for a limited number of insect species (10). Due to its relative ease of production and lack of toxicity to nontarget organisms, *B. thuringiensis* has been used extensively for over 30 years and today accounts for more than 90% of the biopesticides used worldwide (11).

δ-Endotoxins form a large family of related proteins varying in amino acid composition, size, and toxic specificity. About 100 *cry* genes have now been isolated from various *B. thuringiensis* strains and classified on the basis of their molecular relatedness (10, 21). By identifying specific activities of individual cloned *cry* genes, it should be possible to construct new genetically modified *B. thuringiensis* strains that have either an optimized activity against a given insect pest or a broadened toxicity spectrum. The recent construction of *Escherichia coli-B. thuringiensis* shuttle vectors (2, 3) and the development of electroporation procedures to transform *B. thuringiensis* (5, 14) have made possible the construction of genetically engineered *B. thuringiensis* strains containing new combinations of toxin genes (6, 8, 12). However, the shuttle vectors utilized contain DNA sequences that are undesirable when seeking regulatory approval for the release of these recombinant strains and their eventual use as commercial biopesticides. Indeed, in addition to the DNA native to *B. thuringiensis*, these vectors generally harbor (i) a replicon functional in gram-negative bacteria to facilitate cloning in *E. coli* and (ii) antibiotic resistance genes for selection. Recently, site-specific recombination has been used with *B. thuringiensis* to remove DNA from plasmid vectors (4, 20). In one case, a vector that used the replication and stability functions of pHT1030, a natural plasmid of *B. thuringiensis* (2, 13), and harbored two copies of the internal resolution site (IRS) of transposon Tn*4430* (16) was constructed (20). The vector contains two IRSs, repeated in the same orientation, flanking the ColE1 replication determinants and a *bla* gene functional in *E. coli*. When introduced into *B. thuringiensis* hosts harboring Tn*4430*, the site-specific recombinase (TnpI) encoded by Tn*4430* catalyzed recombination between the two IRSs, resulting in excision of the intervening DNA. However, selection of the *B. thuringiensis* transformants requires the use of a *tet* gene not excised by the site-specific recombination (20). Since antibiotic resistance genes are not naturally present in native *B. thuringiensis* strains, it is unlikely that commercial insecticide formulations based on such recombinant *B. thuringiensis* strains will receive approval and acceptance for environmental release. This exemplifies a dilemma, namely, that antibiotic resistance genes are necessary for the selection of the transformants but unacceptable for release. To overcome this problem, we have developed a new strategy that allows the elimination of the resistance marker gene after introduction of the vector into the bacterium and selection of the transformants.

A new strategy for selecting recombinant *B. thuringiensis* strains and eliminating antibiotic resistance genes. The recombination system of Tn*4430* can be used to eliminate in vivo unwanted DNA sequences from transforming vectors harboring two IRSs. Therefore, one approach is to position the antibiotic resistance gene to be eliminated between two IRSs and to select the transformants prior to excision. To examine the feasibility, a prototype vector, pHT-IRS-Km (Fig. 1), was constructed from plasmid pHT304 (2). It contains, in addition to the Em^r marker located on pHT304, the kanamycin resistance

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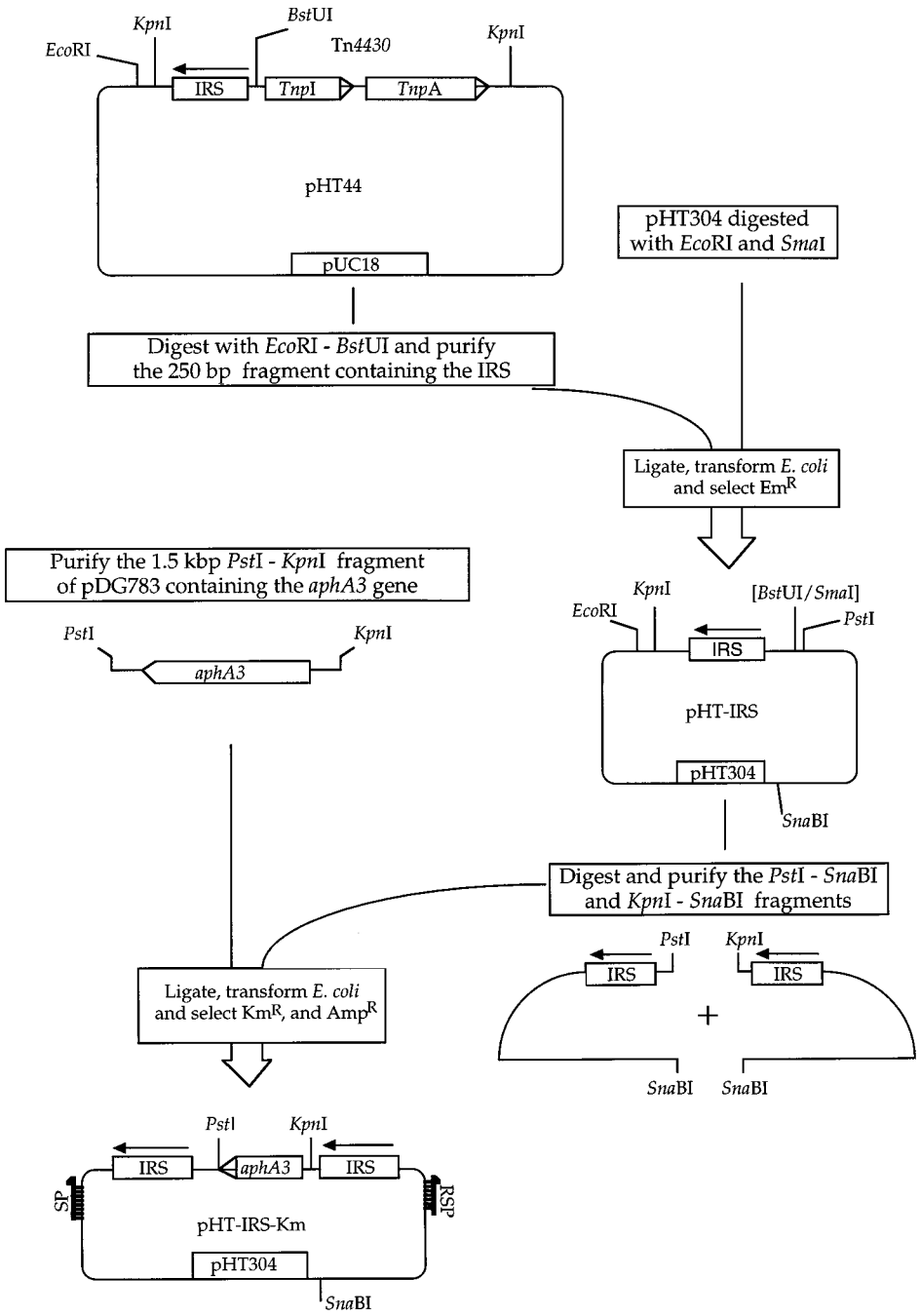

FIG. 1. Construction of pHT-IRS-Km. A 250-bp *Eco*RI-*Bst*UI DNA fragment harboring the IRS of Tn*4430* was isolated from pHT44 (Tn*4430* cloned into vector pUC18) (15) and inserted between the *Eco*RI and *Sma*I sites of pHT304 (2) to give pHT-IRS. pHT-IRS-Km was constructed by a three-way ligation, as follows: the 1.5-kbp *Kpn*I-*Pst*I DNA fragment of plasmid pDG783 (9) harboring a kanamycin resistance marker was purified and ligated with the *Pst*I-*Sna*BI and *Kpn*I-*Sna*BI fragments of pHT-IRS (each carrying an IRS). The ligation mixtures were used to transform *E. coli* TG1, and the transformants were selected on LB plates containing the appropriate antibiotics at the following concentrations: ampicillin, 100 μg/ml; erythromycin, 10 μg/ml; and kanamycin, 200 μg/ml. The specific IRS of Tn*4430* is represented by open boxes designated IRS. The arrows above the IRS boxes indicate their respective orientations. Only relevant restriction sites are represented, and those destroyed during the manipulation are enclosed in brackets. pHT304 harbors two antibiotic resistance markers, an ampicillin resistance gene functional in *E. coli* and an erythromycin resistance gene functional in both *E. coli* and *B. thuringiensis* (2). The 24-mer sequencing primer (designated SP) and the 24-mer reverse sequencing primer (designated RSP; New England Biolabs) that are complementary to the pUC19 sequences of pHT-IRS-Km are represented by lines with arrows (indicating the 5′-to-3′ orientation) matching their complementary sequences in plasmid pHT-IRS-Km; they were used to amplify the DNA sequences positioned between the multiple-cloning site of the different pHT304 derivatives constructed in this work or obtained by in vivo site-specific recombination. The various boxes representing genes or IRSs are not drawn to scale.

gene *aphA3* (22) between the two IRSs of transposon Tn*4430*. In an appropriate host background, pHT-IRS-Km should undergo a controlled rearrangement resulting in the removal of the *aphA3* antibiotic marker. pHT-IRS-Km was used to test whether the rate of TnpI-mediated DNA excision would permit selection of transformants with Km^r activity.

B. thuringiensis Kto (a *B. thuringiensis* serovar kurstaki strain containing Tn*4430*) was transformed by electroporation with pHT-IRS-Km as described previously (14). The electroporated cells were added to 1.5 ml of Luria broth (LB) and incubated for 1 h at 37°C. Aliquots or appropriate dilutions (500 μl) were plated on LB medium with or without kanamycin (200 μg/ml) or erythromycin (10 μg/ml). Between 20 and 200 kanamycin-resistant transformants and between 2.5×10^6 and 5×10^6 erythromycin transformants were obtained. Thus, about 1 in 10^4 to 1 in 10^5 of the Em^r cells retained the *aphA3* gene between the two IRSs in pHT-IRS-Km.

Two types of Km^r colonies were obtained. About 20% were large colonies (designated L colonies); L colonies grew very well on the selective medium and were visible after 12 h of growth at 30°C. The colonies of the second type were smaller (designated S colonies); they grew much slower and were clearly visible only after 24 or 48 h of growth at 30°C. Four colonies (two of each type), i.e., L1, L2, S1, and S2, were reisolated on LB medium plates supplemented with kanamycin (200 μg/ml), and individual colonies were used to inoculate 2 ml of nonselective LB medium. After 8 h (about 10 generations) of growth, appropriate dilutions of the bacterial suspensions were plated on LB plates supplemented with erythromycin (10 μg/ml). About 25 Em^r colonies of L1, L2, S1, and S2 were transferred to LB plates supplemented with kanamycin (200 μg/ml), and the plates were incubated overnight at 30°C. All of the L1 and L2 cells retained the Km^r phenotype, whereas all of the S1 and S2 cells lost the Km^r phenotype.

The L colonies probably contained intact pHT-IRS-Km, and no site-specific recombination had occurred. This could have resulted from the absence of a functional site-specific recombination system due to loss of Tn*4430* or mutation of the *tnpI* gene. L colonies were not studied further. Conversely, it is likely that the small Km^r colonies are the consequence of the poor efficiency with which intact ($aphA3^+$) pHT-IRS-Km is segregated to daughter cells, causing a lower apparent growth rate in the presence of a kanamycin selection. To demonstrate that the loss of the Km^r phenotype in the small colonies, after growth in the absence of selective pressure, corresponds to the phenotypic detection of the precise in vivo excision of the Km^r marker, plasmid DNA preparations extracted from two kanamycin-sensitive S1 derivatives were used as templates for PCR amplification. The M13/pUC sequencing primer (−47) 24MER and the M13/pUC reverse sequencing primer (−48) 24MER (New England Biolabs, Beverly, Mass.) that are complementary to the pUC19 sequences of pHT-IRS-Km (represented by lines with arrows matching their complementary sequences in plasmid pHT-IRS-Km in Fig. 1) were used as primers. In each case, the amplified region consisted of a single DNA fragment of about 300 bp (data not shown) that could correspond to one copy of the IRS. This result is consistent with a site-specific recombination event occurring between the duplicate copies of the IRSs. These putative pHT-IRS-Km recombined plasmids were separated from the native plasmids of strain Kto by using the mixed plasmid preparations to transform *E. coli*. Ampicillin-resistant *E. coli* transformants were shown to carry a plasmid smaller than pHT-IRS-Km. Restriction enzyme analysis and DNA sequencing across the resolution site confirmed that recombination had occurred between the two IRSs, resulting in the generation of a plasmid consisting of pHT304 and one copy of the internal resolution site (data not shown). This recombined plasmid was designated pHT-IRS-Km-Δ. The single copy of the IRS that remains after the site-specific recombination event represents a vestigial piece of DNA that can serve as a definitive marker for the recombined plasmid when PCR techniques are used as described above.

The segregational stability of pHT-IRS-Km-Δ was examined under nonselective conditions by inoculating 20 ml of LB medium with a single S1 (pHT-IRS-Km-Δ) colony. After 8 h of growth at 30°C, bacterial suspensions were diluted 1,000-fold in 20 ml of fresh LB medium. This procedure was repeated twice such that cultures had grown at least 40 generations without antibiotic pressure after the initial inoculation. At each dilution step, samples were taken, diluted, and plated on LB medium and allowed to grow at 30°C for 24 h. Each time, 50 individual colonies were transferred from LB plates to LB plates supplemented with erythromycin (10 μg/ml) and the number of colonies that retained the plasmid were counted. All colonies (100%) were resistant to erythromycin, indicating that all cells had retained the plasmid even after 40 generations of growth on nonselective media. Thus, the segregational stability of pHT-IRS-Km-Δ is high, as is the case for the parental pHT1030 (13). Moreover, pHT1030 does not replicate by a rolling circle mechanism and is therefore also very stable structurally (13). Therefore, it is anticipated that a new class of vectors derived from pHT-IRS-Km can be constructed and used for the introduction and stable maintenance of *cry* genes into *B. thuringiensis* with the selective removal, after transformation, of the resistance marker genes used for selection.

Example of utilization: construction of a genetically engineered *B. thuringiensis* strain free of antibiotic resistance genes and of DNA foreign to *B. thuringiensis*. The strategy described above was used to construct a genetically engineered *B. thuringiensis* strain with improved insecticidal activity. *B. thuringiensis* Kto was chosen as the host strain because it naturally contains a *cryIA(c)* gene whose product is highly active against *Ostrinia nubilalis* (European corn borer) but has little activity against *Spodoptera littoralis* (Egyptian cotton leafworm). A cloned *cryIC* gene whose product shows good activity against *S. littoralis* (19) and little toxicity towards *O. nubilalis* (Table 1) was introduced into *B. thuringiensis* Kto, resulting in a *B. thuringiensis* recombinant strain exhibiting a wider activity spectrum against two important lepidopteran pests.

The previously described modified *cryIC* gene was used (20). It consists of the coding sequence of the sporulation-specific *cryIC* gene under the control of the sporulation-independent *cryIIIA* promoter. The use of the *cryIIIA* expression system, which differs from the expression system of the *cryIA(c)* gene already present in the Kto strain, was previously shown to be essential for creating the conditions for optimal expression of each gene in the recombinant strain (20). This *cryIC* gene was inserted into pHTF3-IC-IRS-Km (Fig. 2), a recombination site-specific shuttle vector containing two identical IRSs, in direct orientation flanking the *E. coli* plasmid pBluescript II KS^- and *aphA3*. The *cryIC* gene and the origin of replication and stability determinants of plasmid pHT1030 between the two IRSs are thus separated from the DNA not native to *B. thuringiensis*. pHTF3-IC-IRS-Km was introduced by electroporation into *B. thuringiensis* Kto, and transformants exhibiting kanamycin resistance were reisolated. The *aphA3* gene and *E. coli* replicon were then eliminated by growing individual Km^r colonies in nonselective medium for at least 10 generations as described above; after 10 generations of growth in the absence of kanamycin, 100% of the cells lost the Km^r phenotype. An analysis of plasmid DNA extracted from three independent

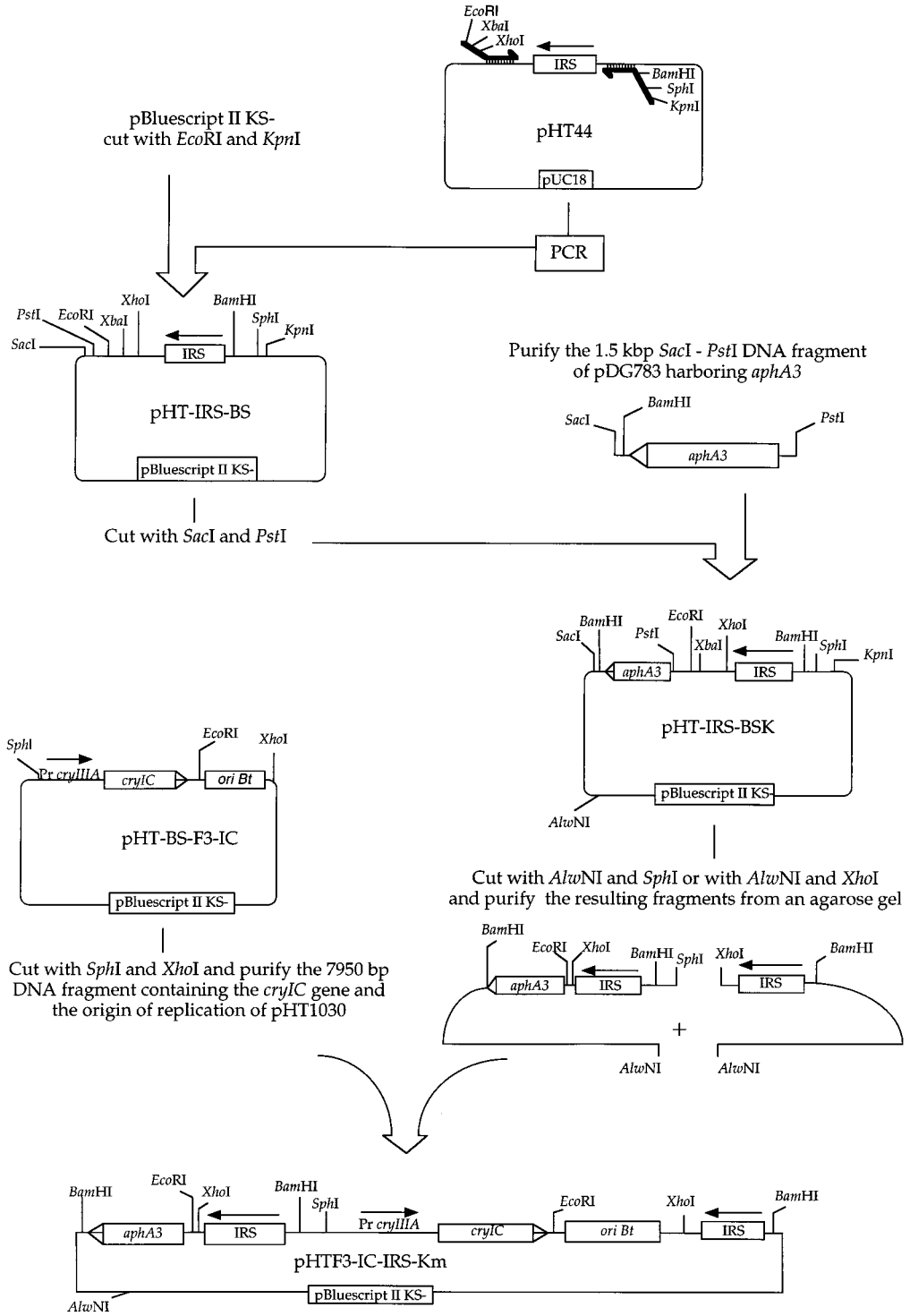

FIG. 2. Construction of pHTF3-IC-IRS-Km. A 250-bp DNA fragment containing the IRS of transposon Tn*4430* was amplified by PCR using two oligonucleotides synthesized by Genset (Paris, France): (i) 5′-CGGAATTCTCTAGACTCGAGCGTCTATAAATTGTGTTGCGT-3′ (oligo 3′ Res) and (ii) 5′-GGGGTACCGCAT GCGGATCCCATTATTCCAAGACACATACA-3′ (oligo 5′ Res). The sequences of the two oligonucleotides were chosen from the nucleotide sequence of Tn*4430* (16) with *Kpn*I, *Sph*I, and *Bam*HI sites (3′ Res) and *Xho*I, *Xba*I, and *Eco*RI (5′ Res) added at the 5′ ends. Oligonucleotides 5′ Res and 3′ Res are represented by lines with arrows (indicating the 5′-to-3′ orientation) matching their complementary sequences in plasmid pHT44 (15). The amplified DNA fragment containing the IRS was ligated between the *Eco*RI and *Kpn*I sites of the pBluescript II KS$^-$ to give pHT-IRS-BS. Plasmid pHT-IRS-BS was cut with *Sac*I and *Pst*I, purified, and ligated with the 1.5-kbp *Sac*I-*Pst*I DNA fragment from pDG783 (9) carrying the *aphA3* gene of *Enterococcus faecalis* (Km^r cassette) (22) to give pHT-IRS-BSK. Plasmid pHTF3-IC-IRS-Km was obtained by ligating the *Sph*I-*Alw*NI and *Xho*I-*Alw*NI fragments of pHT-IRS-BSK (each carrying an IRS) to the 7.95-kbp DNA fragment of pHTBS-F3-IC that contains a *cryIC* gene under the control of the promoter of the *cryIIIA* gene (Pr *cryIIIA*) and the origin of replication of pHT1030. The arrow above the *cryIIIA* promoter indicates its direction of transcription. Construction of pHTBS-F3-IC is described elsewhere (20). The various boxes representing genes or IRSs are not drawn to scale.

TABLE 1. Insecticidal activity of *B. thuringiensis* strains

Strain	Protein concn (mg/ml of crystal preparations)[a]	LC_{50}[b] (ng of protein/cm^2)	
		S. littoralis	*O. nubilalis*
Kto	1.88	981 (758–1,270)	1.7 (0.9–3)
Kto(pHTF3-IC-IRS-Km-Δ)	3.42	43 (34–56)	2 (0.2–25)
Kto$^-$(pHTIC)	3.12	67 (53–86)	>250[d]

[a] The amount of specific crystal protein contained in the crystal preparations that were used to determine the larvicidal activity of the different strains was estimated as follows. One-hundred-milliliter cultures of the different strains were grown at 30°C in HCT medium until the liberation of spores and crystals was complete, as described previously (20). The spore-crystal mixtures were then washed twice with 0.15 M NaCl and twice with distilled water and then concentrated fivefold. The crystal protein concentration in the spore-crystal preparation was assayed (for 1-ml samples) by the Bio-Rad (Munich, Germany) protein assay after the crystals were dissolved in 0.1 N NaOH.

[b] LC_{50}, concentration of protein required to kill 50% of second-instar larvae after 5 days. The LC_{50}s were calculated by probit analysis, and the 95% confidence intervals are indicated in parentheses. Each value is the mean of three (*S. littoralis*) or two (*O. nubilalis*) experiments. The toxicities of the preparations were determined by a free ingestion technique using *S. littoralis* and *O. nubilalis* larvae as described before (18) and an artificial diet (17).

[c] Kto$^-$(pHTIC) is an acrystalliferous derivative of strain Kto that was transformed with plasmid pHTIC (20), which harbors only the *cryIC* gene.

[d] An LC_{50} value has not been obtained; a dose of 236 ng/cm^2 causes 6% mortality.

kanamycin-sensitive Kto transformants is presented in Fig. 3. The DNA fragments corresponding to the *cryIC* gene (5.35 kbp) and to the origin of replication from *B. thuringiensis* replicon pHT1030 (2.6 kbp) were present in the plasmid preparations extracted from the three transformants examined, but the DNA fragments corresponding to pBluescript II KS$^-$ (2.9 kbp) and the *aphA3* gene (1.5 kbp) were absent. This confirmed that recombination had occurred between the two IRSs, generating a plasmid consisting of a *cryIC* gene, a *B. thuringiensis* origin of replication, and one copy of the IRS (250 bp) (data not shown in Fig. 3). This plasmid was designated pHTF3-IC-IRS-Km-Δ.

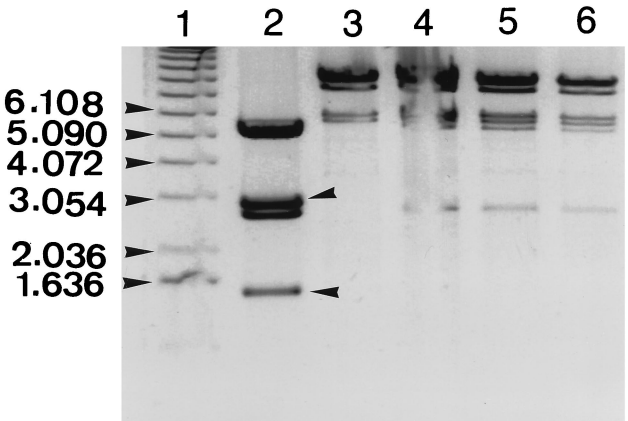

FIG. 3. Agarose gel electrophoresis of *Bam*HI-*Xho*I-*Eco*RI-digested plasmid DNA from native and transformed *B. thuringiensis* Kto. Lanes: 1, 1-kb DNA ladder; 2, pHTF3-IC-IRS-Km isolated from *E. coli*; 3, Kto recipient; 4, 5, and 6, three independent Kto(pHTF3-IC-IRS-Km-Δ) clones. The 2.9-kbp *Bam*HI DNA fragment corresponding to the pBluescript II KS$^-$ and the 1.5-kbp *Bam*HI-*Eco*RI DNA fragment corresponding to *aphA3* are indicated by arrows (lane 2). These DNA fragments are absent from the Kto transformants harboring pHTF3-IC-IRS-Km-Δ. The 2.6-kbp *Eco*RI-*Xho*I and 5.35-kbp *Bam*HI-*Eco*RI DNA fragments corresponding to the origin of replication of pHT1030 and *cryIC* gene, respectively, are present (lanes 4, 5, and 6). The sizes (in kilobase pairs) of the 1kb ladder are shown on the left.

The toxicities of the transformant and of the parent strain against *S. littoralis* and *O. nubilalis* were determined (Table 1). *B. thuringiensis* Kto(pHTF3-IC-IRS-Km-Δ) showed good activity against both insects, whereas the parental recipient strain was active only against *O. nubilalis* and *B. thuringiensis* Kto$^-$(pHTIC) was toxic only to *S. littoralis*. Introduction of the recombinant *cryIC* gene carried by pHTF3-IC-IRS-Km-Δ into strain Kto resulted in a twofold increase in the total amount of crystal protein produced (Table 1). This is consistent with the findings of Lecadet et al. (12), who observed that introduction of a *cryIA(a)* gene into a strain harboring a *cryIIIA* gene resulted in an increase of the total amount of crystal protein produced. We previously reported (20) this type of effect and have proposed that the expression of these two genes is simply summed in the recombinant strain, presumably because they do not compete for rate-limiting elements of the gene expression since the expression systems of the two genes are different.

In conclusion, using a new strategy and a site-specific recombination vector with high inheritable stability, we were able to construct a *B. thuringiensis* recombinant strain containing a unique combination of *cryI* genes conferring a wider activity spectrum than that of the parental strain. This allows simultaneous protection against a broader range of pests. The new site-specific recombination vector described here was designed specifically for selectively removing antibiotic resistance markers from *B. thuringiensis* after introduction of the plasmid into the host strain. Therefore, the recombinant *B. thuringiensis* strain obtained by this procedure and vector is free of non-*B. thuringiensis* DNA and does not harbor any antibiotic resistance genes. Moreover, the origin of replication from *B. thuringiensis* replicon pHT1030 in this new vector has no conjugative function, and its mobilization by the conjugative 75-kb plasmid of strain Kto has never been observed in laboratory conditions (unpublished data). Therefore, the ability of the recombinant plasmid to be transferred to another *B. thuringiensis* strain is presumably low. The use of this vector to express cloned *cry* genes should thus have an ecological impact similar to that of using a natural strain of *B. thuringiensis*. As a consequence, problems of regulatory approval for field trials or development of a *B. thuringiensis* commercial product based on a recombinant strain with this vector or its derivatives are minimized.

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