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Construction of Novel *Bacillus thuringiensis* Strains with Different Insecticidal Activities by Transduction and Transformation

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The shuttle vector pHT3101 and its derivative pHT408, bearing a copy of a *cryIA(a)* δ-endotoxin gene, were transferred into several *Bacillus thuringiensis* subspecies through phage CP-54Ber-mediated transduction, with frequencies ranging from 5×10^{-8} to 2×10^{-6} transductant per CFU, depending on the strain and on the plasmid. In Cry^- and Cry^+ native recipients, the introduction of the *cryIA(a)* gene resulted in the formation of large bipyramidal crystals that were active against the insect *Plutella xylostella* (order Lepidoptera). In both cases, high levels of gene expression were observed. Transductants displaying a dual specificity were constructed by using as recipients the new isolates LM63 and LM79, which have larvicidal activity against insects of the order Coleoptera. It was not possible, however, to introduce pHT7911 into *B. thuringiensis* subsp. *entomocidus*, *aizawai*, or *israelensis* by transduction. However, electrotransformation was successful, and transformants expressing the toxin gene *cryIIIA*, carried by pHT7911, were obtained. Again, high levels of expression of the cloned gene were observed. The results indicate that CP-54Ber-mediated transduction is a useful procedure for introducing cloned crystal protein genes into various *B. thuringiensis* recipients and thereby creating strains with new combinations of genes. Finally it was also shown that pHT3101 is a very good expression vector for the cloned δ-endotoxin genes in the different recipients.

The first genetic exchange system available in *Bacillus thuringiensis* was generalized transduction mediated by the *Bacillus cereus* phage CP-51, as reported by Thorne (33) in 1978. This system allows the transfer of chromosomal markers. Since then, various phage-based systems for mapping chromosomal genes have been developed by several groups (1, 17, 18). Using phage CP-54Ber, which is specific for *B. thuringiensis* subsp. *berliner* 1715 (18), we were able to order at least 18 markers that fall into four linkage groups (unpublished data). Unfortunately, for a long time the lack of an efficient transformation system prevented any further development of attempts to map the *B. thuringiensis* chromosome.

The second important advance in genetic exchange was the discovery by Gonzalez and Carlton (12) of a conjugation-like process involving plasmid transfer (11). This mating system can be used between a number of *B. thuringiensis* subspecies and sometimes between *B. thuringiensis* and other gram-positive species (24). It preferentially involves larger plasmids, particularly those harboring the crystal protein genes. The efficiency of transfer is variable, depending on the donor and recipient strains and on the size of the plasmids.

More recently, new transformation procedures (13), especially electroporation techniques, (5, 21, 26, 31), have provided invaluable tools to promote genetic transfer in *B. thuringiensis*. Electroporation is developing very rapidly and will allow the study of the expression of cloned δ-endotoxin genes in their native host.

Despite these new tools, there are limitations on the transfer of DNA, depending on the host strain and the origin of plasmid DNA (26, 27). This prompted us to assess the possibility of using transduction in *B. thuringiensis*. Bearing in mind a previous observation by Ruhfel et al. (29) that interspecies transduction of small plasmids (pBC16, pC194) can occur through CP-51 mediation, we decided to examine whether CP-54Ber can transfer recombinant plasmids bearing crystal protein genes into various *B. thuringiensis* strains, including Cry^- or Cry^+ native isolates. The plasmids used were the shuttle vector pHT3101 described by Lereclus et al. (21) and its derivatives pHT408 and pHT7911, which contain cloned δ-endotoxin genes of different origins. Many transductants expressing different insecticidal specificities were thus obtained.

In this paper we also report the results of transformation experiments leading to the construction of new strains expressing different combinations of δ-endotoxin genes with activity against insects belonging to different orders.

MATERIALS AND METHODS

Bacterial strains and growth conditions. *B. thuringiensis* subspp. *aizawai* 7-29, *entomocidus* 601, *israelensis* 1884, and *kurstaki* Kto were obtained from the World Health Organization Collaborating Center for Entomopathogen Bacilli (Institut Pasteur). Spontaneous mutants resistant to streptomycin (200 μg/ml) isolated from these strains in our laboratory were also used. *B. thuringiensis* subsp. *tenebrionis* DSM 2803 was purchased from the German Collection of Microorganisms. *B. thuringiensis* LM63 and LM79 were isolated from soil samples by Chaufaux et al. (6) and identified as coleopteran-active strains. *B. thuringiensis* 407 (H1 serotype) and its acrystalliferous derivative were isolated by O. Arantes as previously reported (21), and we are grateful to her for providing these strains. The *B. thuringiensis* subsp. *kurstaki* Cry^-B isolate was kindly provided

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TABLE 1. Plasmids used

Plasmid	Size (kb)	Phenotype	Relevant characteristics	Reference
pHT3101	6.6	Ap^r Em^r	Plasmid containing the replication region (2.9 kb) from the *B. thuringiensis* pHT1030 plasmid plus a 1.2-kb DNA fragment encoding resistance to erythromycin in gram-positive bacteria, both cloned in pUC18	Lereclus et al. (21)
pHT408	18.6	Ap^r Em^r	A 12-kb DNA fragment from plasmid pHT407, harboring a *cryIA(a)* gene[a] is cloned in pHT3101	Lereclus et al. (21)
pHT409 and pHT410	13.6	Ap^r Em^r	pHT408 derivatives in which a 7-kb DNA fragment harboring the same *cryI(a)* gene is cloned in pHT3101 in either orientation	Lereclus et al. (21)
pHT7911	12.6	Ap^r Em^r	A 6-kb DNA fragment from strain LM79, harboring a *cryIIIA* gene, is cloned in pHT3101	Chaufaux et al. (6)

[a] According to the classification proposed by Höfte and Whiteley (14).

by A. I. Aronson, and *B. cereus* NRRL 569 was provided by C. B. Thorne.

The *B. thuringiensis* cultures were grown at 30°C with shaking in BP or HCT medium (18) as previously described (23) until the liberation of spores and crystals was complete. Recipient cells for transduction were grown at 30°C in NBYS medium (18) inoculated with bacteria from isolated clones, until an optical density (at 600 nm) of 3 to 4 was reached. Recipient cells for transformation experiments were grown either in brain heart infusion (BHI) medium or in HCT medium to an optical density (at 600 nm) of 2.

Bacteriophages and plasmids. Phage CP-54Ber was isolated and phage suspensions were prepared as previously described (18). Vegetative cells or spores of *B. thuringiensis* 407 Cry^- containing the plasmid vectors were constructed by electrotransformation (see below) and used as donors for phage propagation.

The plasmids used are listed in Table 1.

Transduction experiments. Samples of 1.6 to 1.8 ml of *B. thuringiensis* or *B. cereus* recipient cell culture in NBYS medium, containing 4×10^8 to 6×10^8 CFU/ml, were mixed with 0.2 to 0.4 ml of phage suspension (final volume, 2 ml) and incubated at 37°C for 30 min with gentle shaking. The cultures were then centrifuged at 6,000 × *g* for 6 min, and the pellets were resuspended in 2 ml of NBYS medium and incubated at 37°C for 30 min. Samples of 0.1 ml were plated on selective medium (BHI or HCT agar supplemented with 25 μg of erythromycin per ml). Transductants were scored after 24 to 48 h of incubation at 30°C. Nonselective agar plates were used to determine the number of recipient cells before transduction. In some experiments, diluted antiserum to phage CP-54Ber was spread together with transduction mixtures, but this did not increase the number of transductants.

Transformation experiments. Transformation of *B. thuringiensis* 407 Cry^- and of native *B. thuringiensis* strains of various subspecies was performed by electroporation as previously described (21). Aliquots of 200 μl of cultures (5×10^9 to 1×10^{10} CFU/ml) in cold 40% polyethylene glycol 6000 were mixed with 0.1 to 1 μg of plasmid DNA (10 μl in Tris-EDTA buffer) in 0.4-ml electroporation cuvettes, and a single pulse at 25 μF and 2.5 kV was given. The electroporated cells were added to 1.5 ml of BHI medium, incubated for 1 h at 37°C, plated on selective medium, and then incubated at 30°C for 24 to 48 h.

Plasmid extraction. Plasmid DNA for transformation was prepared from *Escherichia coli* JM83 by the alkaline lysis method (4) and further purified by using a Qiagen plasmid kit 500 as recommended by the manufacturer (Qiagen).

B. thuringiensis transductants or transformants were screened for plasmid DNA by the alkaline denaturation method (4) as modified by Lereclus et al. (23). The plasmids were submitted to electrophoresis with 0.6% agarose gels.

Analysis of crystal proteins. A rapid washing procedure was routinely applied to spore-crystal mixtures from transductants or transformants before protein characterization. Samples (2 ml) of lysed cultures were washed by centrifugation and resuspension once in 2 ml of 0.5 M NaCl and then twice in cold sterilized water containing 1 mM of the protease inhibitor phenylmethylsulfonyl fluoride. The resulting pellets were resuspended in 0.5 ml of 1 mM phenylmethylsulfonyl fluoride and then kept frozen at −25°C. The same procedure was followed for preparations used in the bioassays, except that phenylmethylsulfonyl fluoride was omitted.

The protein concentrations in the crystal preparations were estimated by the Lowry et al. procedure (25) after the crystals were dissolved in 0.1 M NaOH.

Aliquots (5 to 10 μl) of crystal preparations were heated (100°C, 10 min) in 2× sample buffer (16) and submitted to 10.5% (or 12%) sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) with an acrylamide/*N*,*N*-methylene bioacrylamide ratio of 100:1 as described by Thomas and Ellar (32).

Electrotransfer to nitrocellulose membranes and then immunodetection were performed as described by Towbin et al. (34) with goat anti-rabbit immunoglobulins conjugated with horseradish peroxidase as the second antibody. Rabbit antisera directed against purified active K-60 fractions (19) prepared from crystals of *B. thuringiensis* subsp. *tenebrionis* or of *B. thuringiensis* 407(pHT408) were used as the first antibody.

Bioassays of insecticidal activity. The toxicity of crystal preparations was estimated by using larvae of several different insect species: the Lepidoptera *Plutella xylostella* and *Spodoptera littoralis* and the Coleoptera *Phaedon cochleariae* and *Leptinotarsa decemlineata*. The technique of free ingestion (30) was used with groups of 30 second-instar larvae that were given cabbage leaves (*P. xylostella*), chinese cabbage leaves (*P. cochleariae*), or potato leaves (*L. decemlineata*) spread with dilutions of crystal suspensions. *S. littoralis* larvae were fed with an artificial diet (28) containing dilutions of the preparations to be tested. Larval death was monitored after 24, 48, and 72 h. Fifty percent lethal dose (LD_{50}) values, estimated after 72 h, are given in terms of nanograms per square centimeter of surface offered to the larvae.

To estimate the larvicidal activity against *Aedes aegypti* larvae, the crystal suspensions were diluted in 150 ml of distilled water. Groups of 25 second-instar larvae were placed in the assays, and mortalities were scored at 24 and 48

TABLE 2. Transduction of plasmids pHT3101 and pHT408[a]

Recipient	Transduction frequency (no. of Em^r colonies per CFU)	
	Plasmid pHT3101	Plasmid pHT408
B. thuringiensis Cry$^-$ strains		
subsp. *berliner* 1715 Cry2	5×10^{-8}	5×10^{-8} to 1.5×10^{-7}
407 Cry$^-$	2×10^{-8}	7×10^{-8} to 1.5×10^{-7}
subsp. *kurstaki* Cry$^-$B	0	0
B. cereus NRRL 569	ND	4×10^{-8}
B. thuringiensis Cry$^+$ strains		
subsp. *aizawai* 7-29	0	0
subsp. *entomocidus* 601	3×10^{-8}	4×10^{-7} to 1.4×10^{-6}
subsp. *tenebrionis* DSM 2803 (type strain)	1.6×10^{-8}	2.2×10^{-6}
LM 63	ND	2×10^{-7} to 5×10^{-7}
LM 79	ND	5×10^{-7} to 1×10^{-6}
subsp. *israelensis*	0	0

[a] The results are given as number of Em^r colonies per recipient cell and represent in most cases averages of three independent experiments. The number of CFU was approximately between 4×10^8 and 6×10^8/ml for each experiment. Multiplicities of infection ranging from 1 to 4 were used. ND, not determined.

h. The 50% lethal concentration (LC_{50}) values were estimated and are reported in nanograms per milliliter (9).

Microscopic examination. Photomicrographs of sporulating cells were obtained by using an interferential contrast microscope with a Nomarski lens.

RESULTS

Transduction of plasmids pHT3101 and pHT408. The shuttle vector pHT3101 (6.6 kb) and its derivative pHT408 (18.6 kb), harboring a copy of a *cryIA(a)* gene (Table 1), were used to determine the conditions allowing transduction of plasmids into various Cry$^-$ isolates and native *B. thuringiensis* strains of different subspecies. Lysates were prepared from strain 407 Cry$^-$ containing either pHT3101 or pHT408 and used to transduce the recipient. The samples were plated on erythromycin (25 μg/ml). The transduction frequencies were scored and are summarized in Table 2. Phage CP-54Ber transferred resistance to erythromycin to most of the strains with frequencies that were high enough to be easily detectable. In the case of pHT3101, the transduction frequencies ranged from 1×10^{-8} to 5×10^{-8} transductant per CFU, whereas they appeared to be significantly higher for pHT408 transfer, particularly when Cry$^+$ native strains were used as recipients, reaching in some cases 1×10^{-6} transductant per CFU.

The extraction of plasmid DNA from selected representative transductants of *B. thuringiensis* subspp. *berliner* Cry2 and *entomocidus* 601 and *B. thuringiensis* LM79 and 407 followed by agarose gel electrophoresis indicated the possible presence of pHT3101 or pHT408 plasmids (Fig. 1). This was confirmed by transformation of competent *E. coli* JM83 cells with the same DNA extracts. The *E. coli* Ap^r Em^r transformants were shown to carry plasmids of the same size as pHT3101 or pHT408 (data not shown).

However, several Cry$^-$ or Cry$^+$ strains of *B. thuringiensis* subspp. *kurstaki*, *aizawai*, and *israelensis* did not take up the plasmids during transduction, despite displaying plating efficiencies (18) similar to those of the transducible strains.

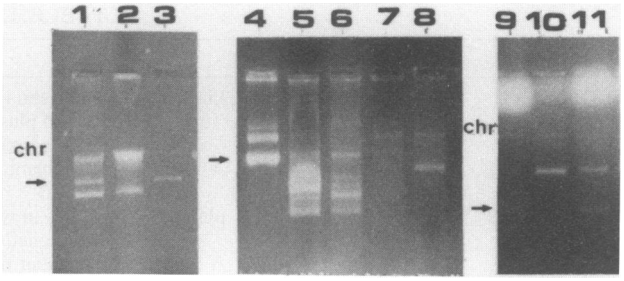

FIG. 1. Agarose gel electrophoresis of plasmid DNA from different *B. thuringiensis* transductants and the corresponding recipients. Lanes: 1, LM79(pHT408) transductant; 2, LM79 recipient; 3 and 4, donor plasmid pHT408 in two independent experiments; 5, *B. thuringiensis* subsp. *berliner* Cry2 recipient; 6, *B. thuringiensis* subsp. *berliner* Cry2(pHT408) transductant; 7, *B. thuringiensis* subsp. *entomocidus* 601 recipient; 8 and 9, *B. thuringiensis entomocidus* 601(pHT408) and 601(pHT3101) transductants, respectively; 10, LM79 recipient; 11, LM79(pHT3101) transductant. The arrows indicate the localization of the vector on each gel.

It should be noted that native *B. thuringiensis* subsp. *entomocidus* and the newly discovered strains *B. thuringiensis* LM63 and LM79 were good recipients for plasmid pHT408; the same was true when donor lysates were propagated on strain 407 Cry$^-$ containing pHT409 or pHT410 plasmids (Table 1). pHT409 and pHT410 are smaller (13.6-kb) plasmids that do not carry the 5-kb DNA sequence upstream of the *cryIA(a)* gene.

No transduction events were detected when the phage was propagated on the donor strain containing plasmid pHT7911 (Table 1), in spite of the fact that this plasmid is similar in size to plasmids pHT409 and pHT410 and the fact that the same donor strain was used. Thus, there are limitations to the use of the method that are possibly linked to the origin of the cloned DNA. However, for most of the recipients listed in Table 2, CP-54Ber-mediated transduction was more efficient (two- to fivefold [or more] higher frequencies) than electrotransformation for the transfer of plasmid pHT408 and its derivatives.

Analysis of crystal proteins produced by transductants. As expected, introducing plasmid pHT408 into different Cry$^-$ isolates resulted in the production of a 130-kDa protein constituting parasporal crystals. This was most striking for the recipient strain 407 Cry$^-$. The transductants produced very large diamond-shaped crystals that were larger and present in greater numbers than those produced in the parental strains. The total amount of crystal protein produced (Table 3) indicated a very high expression of the *cryIA(a)* gene in this recipient, to a level not previously obtained even after transformation (21).

In native *B. thuringiensis* subspecies producing one or several δ-endotoxins of the same gene family, such as *B. thuringiensis* subsp. *entomocidus*, the introduction of pHT408 did not significantly increase the total amount of crystal protein produced (data not shown). However, discrete changes in the morphology of crystals could be observed without any apparent modification of the protein pattern. The occurrence of slight changes in the relative amounts of the different gene expression products cannot be ruled out.

We focused on transductants obtained from *B. thuringiensis* subsp. *tenebrionis*. This subspecies produces crystals of a characteristic shape (3, 15). The introduction of the *cry-*

TABLE 3. Expression of *cry* genes in pHT408 transductants

B. thuringiensis strain	Expression of products[a] of:		Total amt of protein[b] (mg/ml)
	cryIA(a)	*cryIIIA*	
407 Cry$^-$	−	−	0.30
407(pHT408)	+	−	3.70[c]
LM63	−	+	0.83
LM63(pHT408)	+	+	1.85[c]
LM79	−	+	0.59
LM79(pHT408)	+	+	1.80[c]
Donor strain 407(pHT408)	+	−	2.30

[a] As detected after SDS-gel electrophoresis followed by Western blot analysis of spore-crystal mixtures.

[b] Total crystal proteins were estimated from washed spore-crystal mixtures, as described in Materials and Methods, and refer to fourfold concentrated lysed cultures.

[c] Average value of four independent transductants.

IA(a) gene carried by pHT408 into strains LM63 and LM79 resulted in a two- or threefold increase in the total amount of crystal protein produced (Table 3). Under microscopic examination (Fig. 2b, c, and d), large bipyramidal inclusions were observed in sporulating transduced cells together with the flat rhomboid crystals characteristic of the recipient strain (Fig. 2a). SDS-PAGE (Fig. 3) indicated the production of two crystal proteins of 130 and 67 kDa. Electroblotting and testing with specific antisera demonstrated that the 130-kDa protein was labeled by antibodies directed against the *cryIA(a)* gene product, whereas the 67-kDa protein was labeled by antibodies specific for the *cryIIIA* gene product present in the parental recipient strain (Table 3). Thus it is clear that the two different genes are independently expressed in the same cell to produce both types of δ-endotoxin in similar amounts. Similar results were obtained when the type strain *B. thuringiensis* subsp. *tenebrionis* DSM 2803, first isolated by Krieg et al. (15), was used as the recipient. Heterologous crystal proteins were similarly expressed when pHT409 or pHT410 was used in place of pHT408.

Insecticidal activity of the transductants. The larvicidal activities of crystal preparations against the three insect species were determined as described in Materials and Methods. The results, in terms of LD_{50}s, are listed in Table 4. Crystals of the donor strain 407(pHT408) showed activity against the lepidopteran species *P. xylostella*, whereas they were inactive toward the two coleopteran species *P. cochleariae* and *L. decemlineata*. Crystals from the recipients LM63 and LM79 were only active against coleopteran species.

As expected, transductants obtained from these two strains were toxic to both Coleoptera and Lepidoptera insect species. The LD_{50}s of transductants for *P. cochleariae* were of the same order of magnitude as those of recipient strains; the apparently higher values in terms of protein were presumably due to the presence of two different endotoxins (hence twice as much crystal protein) as compared with the single protein present in the parental recipient. The same was true for the LD_{50}s for *L. decemlineata*.

The LD_{50}s of transductants of the type strain *B. thuringiensis* subsp. *tenebrionis* DSM 2803 were also determined (Table 4). The LD_{50} values of these transductants for *P. cochleariae* and *L. decemlineata* were of the same order as those of the LM63 and LM79 transductants. The native DSM 2803 recipient was significantly more active toward *L. decemlineata*, whereas crystals from LM63 were more active against *P. cochleariae*.

The activities of all the transductants against the lepidopteran *P. xylostella* were similar to that of the donor 407(pHT408), except that LM63 transductants were slightly more active.

Overall, the transductants with dual specificity retained the toxicity of the parent strain, while acquiring that of the newly introduced crystal protein gene. No significant synergistic effects were detected.

Introduction of cloned CryIIIA into strains active against lepidopterans by transformation. Plasmid pHT7911 carries a copy of a *cryIIIA* gene (Table 1). It would be of interest to examine the expression of this gene in native strains that are active against lepidopterans and to compare its fate with that of the *cryIA(a)* gene in the reciprocal situation examined above. However, all attempts to introduce pHT7911 by transduction into any of the recipient strains failed.

Electrotransformation was therefore used to construct the desired strains. Crickmore et al. (7) recently reported experiments of this type. pHT7911 was used to electrotransform the 407 Cry$^-$ isolate and a number of native strains of *B. thuringiensis* subsp. *kurstaki*, *entomocidus*, *aizawai*, and *israelensis*, with various transformation efficiencies (ranging from 0.2×10^2 to 5×10^3 transformants per μg of DNA), depending on the strain and on the plasmid. In strain 407(pHT7911), very large rhomboid crystals were seen (Fig. 2e, f, and g), indicating a high level expression of the *cryIIIA* gene. The estimated amount of crystal protein (Table 5) was larger than that produced by strain LM79 (Table 3), from which the gene was isolated. In all of the pHT7911 transformants, rhomboid crystals were produced, in addition to the bipyramidal or cubic inclusions present in the untransformed host. We focused on *B. thuringiensis* subspp. *aizawai* 7-29 and *entomocidus* 601 because of their particular specificity toward insects of the family Noctuidae and also on *B. thuringiensis* subsp. *israelensis* 1884. SDS-PAGE and Western immunoblot analysis were performed on the pHT7911 transformants of three strains. They all contained an additional polypeptide of 67 kDa (Fig. 4) that was not present in the parental hosts. This protein was labeled by antibodies directed against the anti-coleopteran toxin. The total amount of protein in crystal preparations from *B. thuringiensis* subsp. *aizawai* transformants (Table 5) was significantly higher than that in untransformed *B. thuringiensis* subsp. *aizawai*. The transformants contained all of the same crystal components (including the *cryIC* gene product, which is responsible for the activity against *S. littoralis*) and also the new product of the introduced gene. The same situation prevailed in several *B. thuringiensis* subsp. *entomocidus* transformants. However, the 130-kDa component present in the parental *B. thuringiensis* subsp. *entomocidus* strain was not expressed in some transformants (Fig. 4, lanes 11 and 14). *B. thuringiensis* subsp. *israelensis* strains containing pHT7911 produced much greater amounts of crystal than did the untransformed control, as assessed by PAGE (Fig. 5, lanes 2, 4, and 5), protein determination with crystal preparations (Table 5), and microscopic examination (Fig. 2i and j). The strain containing pHT409 or pHT410, carrying the *cryIA(a)* gene, also produced an additional protein of 130 kDa (Fig. 5, lanes 3 and 9); micrographs of such transformants showed large diamond-shaped crystals, which are characteristic of the *cryIA(a)* gene product (Fig. 2k and l).

The results of toxicity assays (Table 5) indicated that transformants displayed dual specificities, with levels of activity identical to those of the parental strains. Interest-

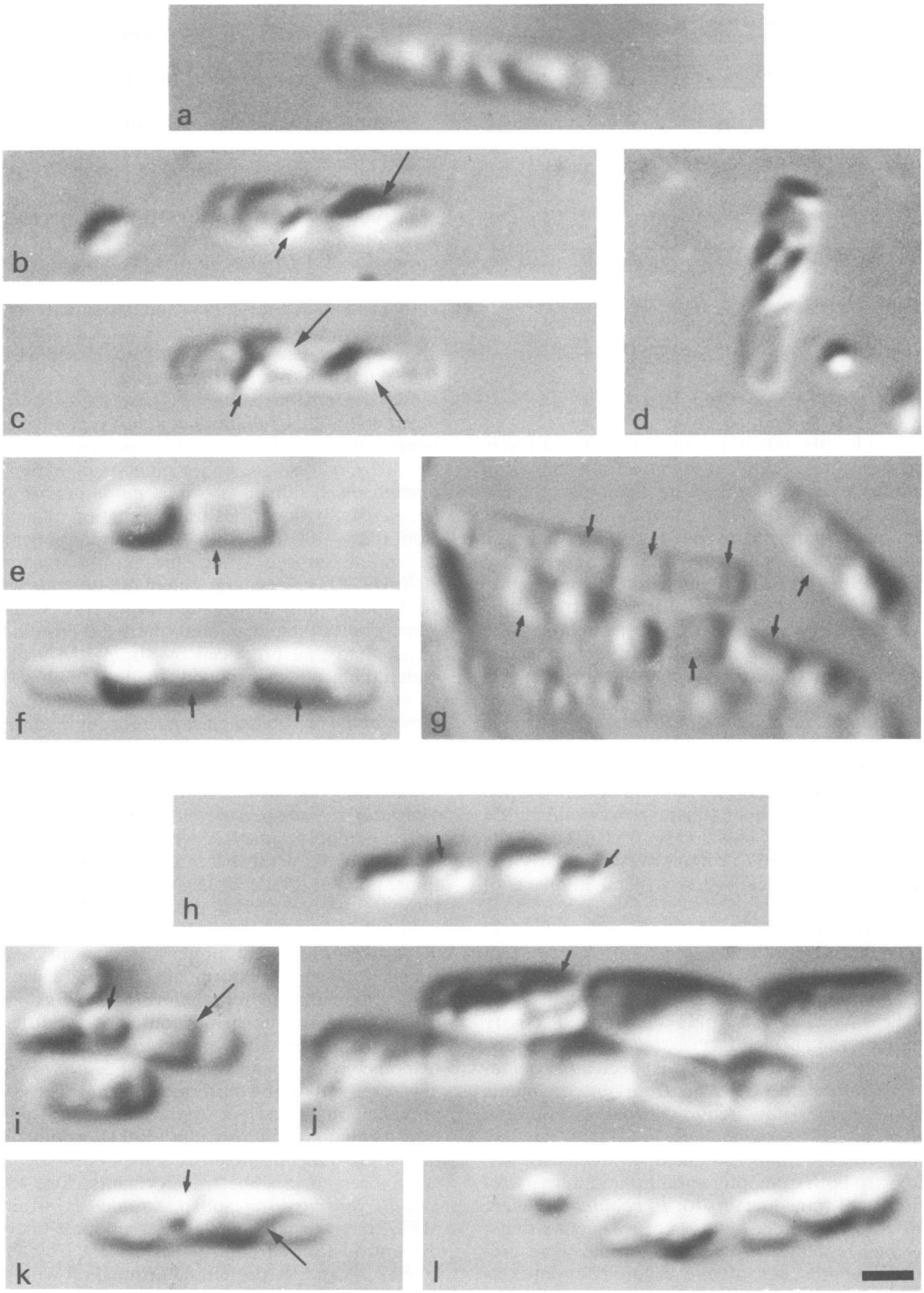

FIG. 2. Interferential contrast photomicrographs of *B. thuringiensis* transductants or transformants taken at the end of sporulation. (a) Control, LM79 wild-type cells with a spore and a flat rhomboid crystal; (b, c, and d) LM79(pHT408) cells containing one or several large bipyramidal crystals (long arrow) and a small rhomboid crystal (short arrow); (e, f, and g) 407(pHT7911) cells with large rhomboid crystals (short arrow) (in some of them the spore was no longer present); (h) control, *B. thuringiensis* subsp. *israelensis* 1884 wild-type cell with a typical small inclusion partially included in a second one (arrow); (i) *B. thuringiensis* subsp. *israelensis*(pHT7911) cells with a large rhomboid crystal (long arrow) close to small parental crystals (short arrow); (j) like panel i (the arrow indicates multiple inclusions); (k) *B. thuringiensis* subsp. *israelensis*(pHT410) cell with a large bipyramidal crystal (long arrow) close to a smaller parental inclusion (short arrow); (l) like k (multiple crystals are seen). The magnification was the same in all micrographs. Bar, 1 μm.

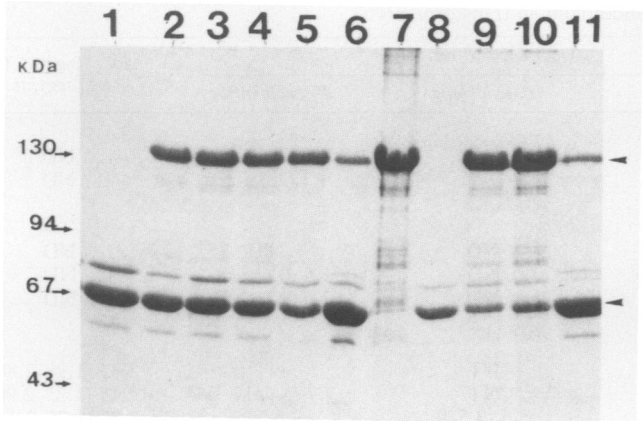

FIG. 3. Protein analysis of crystals synthesized by *B. thuringiensis* LM63(pHT408) and LM79(pHT408) transductants. Samples (8 to 15 μg) of crystal preparations were subjected to SDS-PAGE on 10.5% acrylamide gels and then stained with Coomassie blue. Lanes: 1, LM63 recipient; 2 to 6, isolated pHT408 transductants; 7, crystals from the donor strain 407(pHT408); 8, LM79 recipient; 9 to 11, isolated pHT408 transductants. The arrowheads indicate crystal protein components produced by the donor [*cryIA(a)* gene] and the recipient (*cryIIIA* gene).

ingly a class of *B. thuringiensis* subsp. *entomocidus* transformants (TfA) (which had lost a 130-kDa component) presented a very much reduced activity against *S. littoralis*, whereas the major class of transformants (TfC or TfB) produced crystals that were much more toxic to *S. littoralis*. The LC_{50}s of *B. thuringiensis* subsp. *israelensis* transformants toward *A. aegypti* were similar to that of the native strain 1884 in terms of total protein, thus indicating at least a twofold higher activity in terms of specific proteins. The LD_{50} values of each of the transformants toward *P. cochleriae* suggested that the newly introduced *cryIIIA* gene was strongly expressed in all transformants. This was confirmed by protein determinations of crystal proteins, as mentioned above.

Furthermore the specific toxicity of 407(pHT7911) and other pHT7911 transformants was higher than that of strain LM79, from which the *cryIIIA* gene was isolated (Table 4).

In the same way, pHT409 or pHT410 transformants expressing the *cryIA(a)* gene acquired toxicity toward *P. xylostella* (Table 5).

Plasmid stability in transductants and transformants. To investigate the stability of the recombinant plasmids in the various recipient cells, cultures of transductants and transformants were grown in Luria broth for about 45 generations in the absence of selective pressure. Simultaneously, a second set of cultures was developed in BP medium, allowing a good sporulation, in order to estimate the plasmid stability in heat-resistant spores. The results presented in Table 6 indicated the percentages of vegetative cells and of spores resistant to erythromycin (25 μg/ml) after 18 and 45 generations in the absence of antibiotics.

In recipient strain 407 Cry^-, the shuttle vector pHT3101 and the plasmid pHT7911 appeared stably maintained. Plasmid pHT408 was less stable in one transductant, whereas in a second transductant the plasmid appeared much more stable. In the other recipients the stability of pHT408 ranged between 90 and 100% after 45 generations, depending on the host strain.

Plasmid pHT7911 appeared quite stable in the different Cry^+ recipients, except in *B. thuringiensis* subsp. *kurstaki* Kto.

The BP cultures were also examined for crystal production after 45 generations in the absence of selective pressure. The level of crystal protein synthesis by cells in culture without antibiotics was similar to that of the same constructs in cultures with the antibiotic, with the exception of transductant 407(pHT408) (transductant b in Table 6), which produced less protein in the absence of antibiotic. Moreover around 25 to 30% of the cells in cultures of this transductant did not contain crystals.

The ability of the recombinant strains to sporulate in the usual growth conditions and in the presence of antibiotic was investigated. Cultures of 407 Cry^- transformed with pHT3101, pHT408, pHT409, pHT410, or pHT7911 and an untransformed control were grown until the liberation of spores. The numbers of heat-resistant spores, scored at intervals between 24 and 48 h in each sample, were in all cases similar to those of the controls (data not shown). However, after 40 h the viable spore count in the 407(pHT7911) samples fell to about 10%, but the crystals were larger. This trend continued after 64 h. It appears, therefore, that a part of the spore population degenerates, whereas crystals continue to grow. Similar observations have been reported by Donovan et al. (10) for *Bacillus megaterium* cells harboring plasmid pEG213, in which a *cryIIIA* gene isolated from *B. thuringiensis* subsp. *tenebrionis* EG2158 was cloned.

Similar experiments were performed with pHT7911 transformants of *B. thuringiensis* subsp. *entomocidus* or *israelen-*

TABLE 4. Insecticidal activity of pHT408 transductants

B. thuringiensis strain	LD_{50} (ng/cm²)[a] for:		
	P. xylostella	*P. cochleariae*	*L. decemlineata*
407(pHT408)	8.9 (1.5–10.2)	NT[b]	NT
LM63	NT	12.2 (8.2–18.0)	17.0 (12.0–23.0)
LM63(pHT408)[c]	5.1 (2.9–15.8)	23.0 (15.2–35.0)	21.0 (15.0–42.6)
LM79	NT	21.2 (11.5–38.3)	16.2 (10.6–24.6)
LM79(pHT408)[c]	16.9 (8.4–25.0)	50.2 (27–95.2)	29.0 (20.5–41.3)
DSM 2803	807.0 (93.0–7,320)	23.1 (17.7–30.6)	5.8 (3.5–11.0)
DSM 2803(pHT408)[c]	22.2 (19.4–32.2)	48.0 (31.2–46.7)	21.5 (14.3–30.3)
407(pHT7911)	1,110 (404–3,051)	3.1 (2.0–5.1)	0.93 (0.07–32.1)

[a] LD_{50}s (with confidence intervals within parentheses) represent the crystal protein amounts at which 50% of the larvae were killed (after 72 h). The values were deduced from the protein concentration in dilutions of the different crystal preparations added to the food.

[b] NT, no significant mortality with undiluted extracts.

[c] Data show average values of independent transductants.

TABLE 5. Expression of δ-endotoxins in transformants[a]

B. thuringiensis strain	Crystal protein (mg/ml)	LD_{50} (ng/cm^2)[b] for:			LC_{50} (ng/ml)[c] for *A. aegypti*
		S. littoralis	*P. xylostella*	*P. cochleariae*	
subsp. *aizawai* 7-29					
Native	2.30	275 (97–780)	ND[d]	NT[e]	ND
(pHT7911)	2.86[f]	305 (147–619)[f]	ND	4.3 (2.3–6.6)[f]	ND
subsp. *entomocidus* 601					
Native	0.9	950 (680–1,360)	ND	NT	ND
(pHT7911) TfA	0.65	5,170 (760–34,910)	ND	1.5 (0.6–3.6)	ND
(pHT7911) TfC	1.3	140 (88–290)	ND	2.6 (1.0–7.5)	ND
subsp. *israelensis* 1884					
Native	0.9	NT	NT	NT	2.43 (1.98–2.88)
(pHT7911)	1.93[f]	NT	NT	7.7 (3.6–17.3)	2.17 (1.35–2.90)[f]
(pHT409)	1.71	NT	5.6 (3.5–9)	NT	2.36 (1.75–3.15)
Donor strains					
407(pHT7911)	2.59	NT	1,100 (404–3,056)	3.1 (3.6–5.1)	NT
407(pHT408)	2.27	NT	8.9 (1.5–12.0)	NT	NT

[a] In these experiments, *B. thuringiensis* subspp. *aizawai* 7-29 (Sm^r) and *entomocidus* 601 (Sm^r) were used as recipients.
[b] The LD_{50} is as defined in footnote *a* of Table 4. The values were deduced from the protein concentration in dilutions of each crystal preparation added to the food.
[c] For mosquito bioassays, LC_{50}s (with confidence intervals within parentheses) represent the crystal protein concentrations at which 50% of the larvae were killed (48 h). The values were deduced from the dilutions of each crystal preparation in which larvae are grown.
[d] ND, not determined.
[e] NT, no significant mortality with undiluted extracts.
[f] Average value of independent transductants.

sis. The number of heat-resistant spores also fell to about 15 to 20% of that in the parental strains after 40 h. Transformation of *B. thuringiensis* subsp. *aizawai* with pHT7911 did not affect the number of heat-resistant spores. It should be noted that in transductants or transformants the development of heat resistance seemed to be delayed (1 to 3 h) as compared with that in the recipient 407 Cry$^-$ (data not shown).

Finally, the introduction of the recombinant plasmids harboring a crystal protein gene, particularly the *cryIIIA* gene, induced in several recipients modifications in the timing of appearance of heat-resistant spores and also in their viability. Nevertheless, the percentage of plasmid-containing spores after 45 generations without selective pressure was the same as that for vegetative cells (Table 6), thus indicating that plasmids pHT3101, pHT408, and pHT7911 are stably maintained during sporulation.

DISCUSSION

The results presented in this report demonstrate that phage CP-54Ber, a generalized transducing phage, can mediate the transfer of recombinant plasmids between *B. thuringiensis* strains at detectable frequencies, thus providing an additional tool for genetic exchange in this bacteria. This is of particular value for introducing cloned crystal protein genes into various *B. thuringiensis* subspecies, including

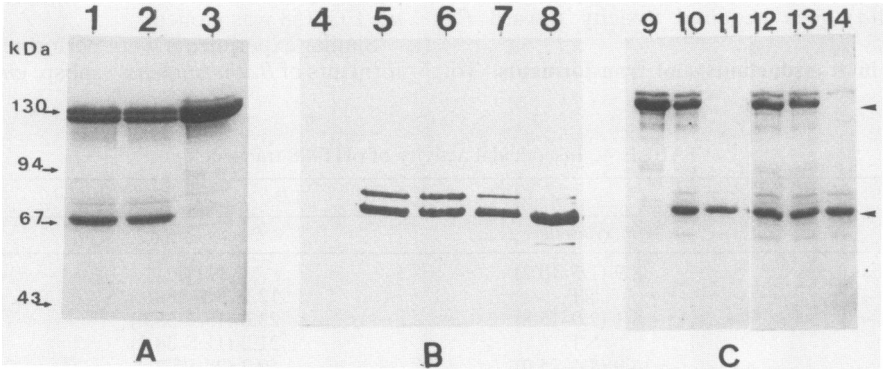

FIG. 4. SDS-PAGE and immunodetection of crystal proteins in transformants obtained from *B. thuringiensis* subspp. *aizaiwai* 7-29 and *entomocidus* 601. Samples (15 to 25 μg [A], 12 to 20 μg [B], or 8 to 14 μg [C]) of crystal preparations were run on 10.5% acrylamide gels. (A and C) Staining with Coomassie blue; (B) Western blot analysis with antiserum specific for the *cryIIIA* gene product. Lanes: 1, 2, 5, and 6, *B. thuringiensis* subsp. *aizawai*(pHT7911) transformants; 3 and 4, native *B. thuringiensis* subsp. *aizawai* 7-29 recipient; 8, crystals from the donor strain 407(pHT7911); 9, native *B. thuringiensis* subsp. *entomocidus* recipient; 7, 10, 11, 12, 13, and 14, *B. thuringiensis* subsp. *entomocidus*(pHT7911) transformants TfC, TfF, TfE, TfC, TfB, and TfA, respectively. The arrowheads indicate the 130- and 67-kDa crystal components.

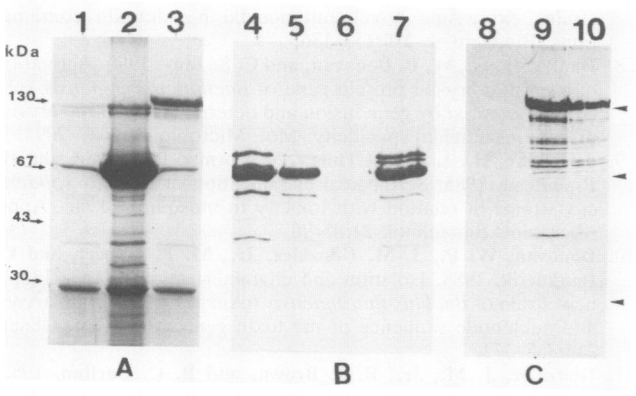

FIG. 5. SDS-PAGE and immunodetection of crystal proteins in transformants obtained from *B. thuringiensis* subsp. *israelensis* 1884. Samples (14 to 22 μg) of crystal preparations were run on 12% acrylamide gels. (A) Staining with Coomassie blue; (B and C) Western blot analysis with antiserum specific for the *cryIIIA* and *cryIA(a)* gene products, respectively. Lanes: 1, 6, and 8, native *B. thuringiensis* subsp. *israelensis* 1884 recipient; 2, 4, and 5, *B. thuringiensis* subsp. *israelensis*(pHT7911) transformants; 7, crystals from the donor strain 407(pHT7911); 3 and 9, *B. thuringiensis* subsp. *israelensis*(pHT409) transformant; 10, crystal from the donor strain 407(pHT409). The arrowheads indicate the 130-, 68-, and 28-kDa components of the crystals.

both Cry^- and Cry^+ strains. The transduction frequencies were relatively low, lower than those obtained for transduction of chromosomal markers (18), particularly with the native vector pHT3101 and when Cry^- recipients were used. In addition, some strains did not accept plasmid DNA from other subspecies. This contrasts with the transduction of plasmids pBC16 and pC194 with the closely related phage CP-51, which allows interspecies transduction (29).

The novel system described here allows the transfer of larger plasmids (10 to 20 kb), such as pHT408, bearing genes encoding various insecticidal proteins. In most cases, transductants expressing the cloned *cryIA(a)* gene to a high level were obtained; the level of expression was particularly high in transductant 407(pHT408). There are two points to note. (i) The shuttle vector pHT3101, in which *cryIA(a)* was subcloned, carries the replication region of a *B. thuringiensis* cryptic plasmid (22) and is known to be a good expression vector in *B. thuringiensis* (21). (ii) The 407 Cry^- (H1 serotype) strain was the most efficient Cry^- recipient, allowing the expression of cloned crystal protein genes. The cloned *cryIIIA* gene was also highly expressed in this 407 Cry^- recipient after being introduced through transformation.

TABLE 6. Plasmid stability in *B. thuringiensis* recombinants[a]

Recombinant *B. thuringiensis* strain	% of Em^r cells after:	
	18 generations	45 generations
407(pHT3101)	100 (100)	100 (100)
407(pHT408)		
Transformant a[a]	96 (99)	97 (84)
Transductant b[b]	88 (68)	52 (45)
Transductant c[b]	93 (97)	93 (78)
407(pHT7911)	93 (99)	98 (90)
LM63(pHT408)	95 (97)	96 (90)
LM79(pHT408)	96 (98)	98 (90)
subsp. *entomocidus*(pHT408)	100 (99)	100 (100)
subsp. *entomocidus*(pHT7911)	100 (99)	100 (97)
subsp. *kurstaki* Kto(pHT7911)	86 (100)	81 (83)
subsp. *aizawai* 7-29(pHT7911)	98 (100)	94 (100)
subsp. *israelensis* 1884(pHT7911)	100 (99)	99 (100)

[a] Samples of cells grown successively for 18 and 45 generations in LB medium without antibiotics were plated on unselective media after appropriate dilutions. About 130 colonies from each sample were transferred with toothpicks to agar plates containing erythromycin (25 μg/ml) to determine the percentage of plasmid-containing bacteria. The numbers in parentheses represent the percentages estimated with heat resistant spores resulting of cultures grown in BP medium (convenient for sporulation) under the conditions described above.

[a] Strain obtained by transformation.

[b] Independent transductant.

The results also show that plasmid transduction works efficiently when certain native Cry^+ strains, such as *B. thuringiensis* subsp. *entomocidus* or *tenebrionis*, were used as recipients. Strains with increased specificities were thereby obtained. In the resulting transductants, the *cryIIIA* gene determining activity against coleopterans and the introduced *cryIA(a)* gene specific for lepidopterans are simultaneously and independently expressed; again, the expression of the cloned *cryIA(a)* gene carried by pHT408 was strong. However when the introduced gene was the same as or homologous to a gene in the recipient, the presence of the introduced gene did not increase total crystal protein production. The relative expression of the two genes in the transductants was variable. This is consistent with the findings of Baum et al. (2), who worked with the *cryIA(c)* gene.

The failure in transducing plasmids into several recipients, including *B. thuringiensis* subspp. *aizawai* 7-29, *kurstaki* Kto, and *israelensis* 1884, suggested the involvement of host factors. We previously observed (18) a decreased transduction efficiency of chromosomal markers when crosses involved strains belonging to different subspecies, in spite of the fact that such strains did allow phage propagation as assessed by plating efficiency.

Transformation can be used to introduce plasmids into these recipients. In some of them, particularly *B. thuringiensis* subsp. *aizawai* 7-29, even this procedure was poorly efficient (transformation frequencies of $\leq 1 \times 10^{-8}$ transformant per CFU). Plasmid pHT7911 carrying the *cryIIIA* gene was introduced into these transduction-defective native strains, resulting in transformants with new specificities without affecting the production of original toxins. Crickmore et al. (7) used different gene combinations and other vectors in similar experiments.

The results obtained with transformants confirmed and added to those obtained from the analysis of transductants. Introduced genes were expressed jointly with a normal expression of the δ-endotoxin genes harbored by *B. thuringiensis* subsp. *aizawai*, *entomocidus*, and *israelensis* recipients. Consequently, transformants with dual specificities were obtained. In *B. thuringiensis* subsp. *israelensis* transformants, there was a large increase in the total amount of crystal protein produced. It is known that the expression of the cloned *cryIIIA* gene is generally high (10); as suggested by Dankocsik et al. (8), this could be due to a strong promoter or to adjacent regulatory sequences located upstream of the gene.

The level of expression of the cloned genes introduced into different backgrounds by transformation varied depending on the host and the vector. In most cases the pHT7911 transformants displayed a 10-fold higher activity toward *P. cochleariae* than toward the strain from which the gene was cloned, thus indicating that negative regulatory mechanisms

could be present in the natural host. This also suggests that the *cryIIIA* gene was poorly expressed in the parental strain or diluted among other *cry* genes encoding polypeptides with different toxic specificities. This high activity was in most cases linked to the high production of the cloned toxins rather than to synergistic effects, and this study does not provide any evidence for such effects. Nevertheless the activity against *S. littoralis* of a particular class of *B. thuringiensis* subsp. *entomocidus* transformants (TfC; Table 5), as compared with that of native *B. thuringiensis* subsp. *entomocidus*, is such that this possibility cannot be ruled out.

The stability of the introduced plasmids depended mainly on the host. Surprisingly, transformants or transductants of native strains, particularly *B. thuringiensis* subspp. *aizawai* and *israelensis* transformed with pHT7911 harboring the *cryIIIA* gene, were among the most stable. The results are in good agreement with observations reported by Lereclus and Arantes (20) demonstrating the high stability of vectors harboring the 2.9-kb replication region of the *B. thuringiensis* plasmid pHT1030 (22). In spite of the difficulties of using transformed strains in the field, the stability of a number of the recombinant strains is encouraging for potential development.

In summary, new strains displaying plurality in their host specificities have been obtained by transduction as well as by transformation. This is the first example of transfer by transduction of plasmids carrying crystal protein genes. Either procedure can be used, depending on the strains and on the vectors, and they are complementary tools.

The use of shuttle vectors such as pHT3101 and others (2, 7, 8) with different replication regions functional in *B. thuringiensis* will facilitate the construction of new strains that have multiple activities and can be more effective against insect populations in which new forms of resistance are emerging.

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