APPLIED AND ENVIRONMENTAL MICROBIOLOGY, Nov. 1997, p. 4413–4420 Vol. 63, No. 11
0099-2240/97/$04.00+0

Improvement of *Bacillus sphaericus* Toxicity against Dipteran Larvae by Integration, via Homologous Recombination, of the Cry11A Toxin Gene from *Bacillus thuringiensis* subsp. *israelensis*

SANDRINE PONCET,[1]* CÉCILE BERNARD,[1] ETIENNE DERVYN,[2] JANE CAYLEY,[3] ANDRÉ KLIER,[1] AND GEORGES RAPOPORT[1]

Unité de Biochimie Microbienne, URA 1300 du Centre National de la Recherche Scientifique, Institut Pasteur, 75724 Paris Cedex 15,[1] and Laboratoire de Génétique Microbienne, Institut National de la Recherche Agronomique, Domaine de Vilvert, 78352 Jouy en Josas Cedex,[2] France, and AgrEvo Environmental Health, Berkhamsted, Hertfordshire, HP4 2DY, United Kingdom[3]

Received 12 December 1996/Accepted 1 May 1997

Integrative plasmids were constructed to enable integration of foreign DNA into the chromosome of *Bacillus sphaericus* 2297 by in vivo recombination. Integration of the *aphA3* kanamycin resistance gene by a two-step procedure demonstrated that this strategy was applicable with antibiotic resistance selection. Hybridization experiments evidenced two copies of the operon encoding the binary toxin from *B. sphaericus* in the recipient strain. The *Bacillus thuringiensis* subsp. *israelensis cry11Aa1* gene (referred to as *cry11A*), encoding a δ-endotoxin with toxicity against *Culex*, *Aedes*, and *Anopheles* larvae, was integrated either by a single crossover event [strain 2297(::pHT5601), harboring the entire recombinant plasmid] or by two successive crossover events [strain 2297(::*cry11A*)]. The level of the Cry11A production in *B. sphaericus* was high; two crystalline inclusions were produced in strain 2297(::pHT5601). Synthesis of the Cry11A toxin conferred toxicity to the recombinant strains against *Aedes aegypti* larvae, for which the parental strain was not toxic. Interestingly, the level of larvicidal activity of strain 2297(::pHT5601) against *Anopheles stephensi* was as high as that of *B. thuringiensis* subsp. *israelensis* and suggested synergy between the *B. thuringiensis* and *B. sphaericus* toxins. The toxicities of parental and recombinant *B. sphaericus* strains against *Culex quinquefasciatus* were similar, but the recombinant strains killed the larvae more rapidly. The production of the Cry11A toxin in *B. sphaericus* also partially restored toxicity for *C. quinquefasciatus* larvae from a population resistant to *B. sphaericus* 1593. In vivo recombination therefore appears to be a promising approach to the creation of new *B. sphaericus* strains for vector control.

Efforts to reduce the populations of dipteran species which transmit tropical diseases such as malaria, filariasis, and onchocerciasis have mostly been based on chemical pesticides. As a consequence of the emergence of resistant insect populations, biological control, including utilization of naturally occurring entomopathogenic bacterial strains, has been receiving increased attention. *Bacillus thuringiensis* subsp. *israelensis* and *Bacillus sphaericus* are two gram-positive soil bacteria which have been successfully used for biological control of blackflies and *Culex* mosquitoes, respectively (for reviews, see references 30 to 32).

The entomocidal properties of both *B. thuringiensis* subsp. *israelensis* and *B. sphaericus* are due to protoxin crystals produced during sporulation and deposited alongside the spore or surrounded by the exosporium membrane, respectively. The proteins of these crystals are solubilized and activated in the insect midgut after ingestion by the larvae and cause host death following binding of the toxic fragment to specific receptors. *B. thuringiensis* subsp. *israelensis* and *B. sphaericus* crystals differ in their toxin compositions, modes of action, and insecticidal spectra. *B. thuringiensis* subsp. *israelensis* inclusions are made of at least four major polypeptides, now referred to as Cry4Aa1, Cry4Ba1, Cry11Aa1, and Cyt1Aa1 (formerly CryIVA, CryIVB, CryIVD, and CytA, respectively; for a review, see reference 18). The corresponding genes are referred to in this paper as *cry4A*, *cry4B*, *cry11A*, and *cyt1A* for simplicity. Each of these polypeptides has a particular specificity for dipteran larvae. Cyt1A is responsible for the nonspecific cytolytic activity of the dissolved crystals (12). The high toxicity observed for the whole inclusion results from synergistic interactions between the crystal components (1, 11, 13, 29). *B. sphaericus* inclusions are made of equimolar amounts of proteins of 52 and 42 kDa which act as a binary toxin (for a review, see reference 32). *B. thuringiensis* subsp. *israelensis* is toxic to *Aedes*, *Culex*, *Anopheles*, *Mansonia*, and *Simulium* larvae, whereas the *B. sphaericus* target spectrum is more restricted; most *B. sphaericus* strains are toxic for *Culex* and *Anopheles* larvae and are poorly or not toxic for *Aedes* larvae.

The development of biopesticides has been limited by some of the biological properties of the bacterial strains. *B. thuringiensis* subsp. *israelensis* does not persist for long periods in the environment. The crystals are rapidly inactivated by sunlight and other environmental degradative agents. Moreover, spore-crystal mixtures rapidly settle from the zone in which the larvae feed (near the surface); the bacterium does not recycle, and crystals thus become inaccessible to the targets. In contrast, *B. sphaericus* toxicity is more persistent than that of *B. thuringiensis* subsp. *israelensis*, and the spore-crystal mixture can persist in various larval habitats, including polluted waters. In addition, *B. sphaericus* can recycle under certain environmental conditions. *B. sphaericus* is therefore used to control *Culex*

* Corresponding author. Present address: Laboratoire de Génétique Microbienne, Institut National de la Recherche Agronomique, Domaine de Vilvert, 78352 Jouy en Josas Cedex, France. Phone: (33) 01 34 65 25 09. Fax: (33) 01 34 65 25 21. E-mail: sponcet@biotec.jouy.inra.fr.

larvae and is of potential value as an alternative tool for mosquito control (for reviews, see references 30 to 32). However, the major drawback of *B. sphaericus* as a biopesticide is its narrow host range specificity and the potential development of resistance to the binary toxin. High-level resistance against *B. sphaericus* 2362 or 1593 in *Culex quinquefasciatus* larvae can be obtained in the laboratory (resistance ratio of >100,000 [17]) or following intensive field treatments (resistance ratio of 10 [34] to 150 [33]), respectively. This phenomenon has not been observed for *B. thuringiensis* subsp. *israelensis*, even after years of intensive field treatments, probably because of the multicomponent structure of the crystals.

Genetic engineering has been used to combine *B. thuringiensis* subsp. *israelensis* and *B. sphaericus* crystal proteins in the same bacterial host, so as to enlarge the activity spectrum of *B. sphaericus*, to increase the toxicity and persistence of *B. thuringiensis* subsp. *israelensis*, and to help delay the appearance of resistant mosquitoes. Indeed, the receptors for *B. sphaericus* and *B. thuringiensis* subsp. *israelensis* toxins are different (25). The operon encoding the binary toxin of *B. sphaericus* 1593 has been cloned on a plasmid vector and transferred into *B. thuringiensis* subsp. *israelensis*, but the presence of an additional parasporal inclusion in the recipient cell conferred no enhancement of toxicity (9). *B. thuringiensis* subsp. *israelensis* toxin genes have also been transferred into *B. sphaericus* 2362. When this strain was transformed with replicative plasmids containing the *cry11A* gene, the transformants obtained showed various segregational or/and structural stabilities; some were moderately toxic to *Aedes aegypti* larvae (36). Bar et al. (2) have expressed the *cry4B* and *cyt1A* genes independently or in combination in strain 2362. The recombinants were more toxic than the parental strain for *A. aegypti* (2). We have obtained in our laboratory recombinant 2297 strains expressing either the *cry4B* or the *cry11A* gene from a shuttle vector. Both recombinant strains exhibited a weak toxicity against *A. aegypti*, whereas the parental strain was not toxic, and the level of toxicity obtained suggested that there was synergy between the Cry11A toxin and the toxic components of *B. sphaericus*, at least against *Aedes* larvae (28). In those studies, the expression level of *B. thuringiensis* subsp. *israelensis* toxins in the host cell was only moderate. Moreover, the *B. thuringiensis* subsp. *israelensis* toxin genes were on plasmids carrying antibiotic resistance genes, which are undesirable in biopesticides.

To avoid either segregational or structural instability and to eliminate the need for undesirable antibiotic resistance genes on plasmid vectors, we developed a novel method based on in vivo recombination. Foreign DNA was integrated into the chromosome of *B. sphaericus* 2297 by homologous recombination. Recombinant *B. sphaericus* strains harboring the *cry11A* gene integrated into the chromosomal DNA either by single or two-step crossover events were obtained. In this paper, we describe the toxicities of these strains against *A. aegypti*, *C. quinquefasciatus* and *Anopheles stephensi* larvae and report their activities against *C. quinquefasciatus* larvae resistant to *B. sphaericus* 1593.

MATERIALS AND METHODS

Bacterial strains, plasmid vectors, and transformation procedures. *Escherichia coli* JM83 [*ara* Δ(*lac-pro*) *strA* ϕ80 *lacZ*ΔM15] and TG1 [Δ(*lac-proAB*) *supE thi hdsD* F′(*traD36 proA*$^+$ *proB*$^+$ *lacI*^{q} *lacZ*ΔM15)] were used as recipient strains for subcloning experiments. Cells were transformed as previously described (22), and transformants were selected on ampicillin (100 μg/ml).

B. thuringiensis subsp. *israelensis* 4Q2-72 was kindly provided by D. H. Dean, Ohio State University, Columbus. *B. sphaericus* 2297 (19, 37) was used as the recipient strain and transformed as previously described (28). The plasmid vector pRN5101, kindly provided by A. Gruss (INRA, Jouy-en-Josas, France), was used for integration experiments. This plasmid is composed of both pBR322 and pE194ts. The pBR322 replication origin allows plasmid replication at 37°C in *E. coli*, whereas pRN5101 does not replicate in gram-positive hosts at temperatures higher than 30°C (8). pRN5101 carries an erythromycin resistance gene. Transformants were selected on erythromycin (20 μg/ml).

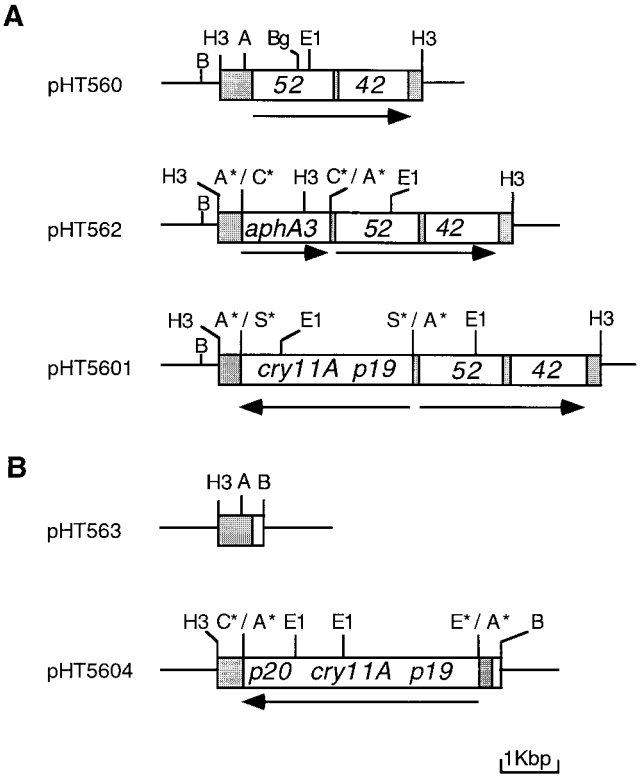

FIG. 1. Simplified restriction maps of plasmids used in this study. (A) Recombinant integrative plasmids containing both the 52- and 42-kDa toxin genes from *B. sphaericus* 1593 (plasmid pHT560). pHT562 and pHT5601 are derivatives of plasmid pHT560, harboring the *aphA3* gene and the Cry11A toxin operon, respectively, inserted at the single *Asp*718 restriction site in pHT560. (B) Integrative plasmids containing an 800-bp DNA fragment from the 5′ region of the *Hin*dIII fragment carrying the toxin genes from *B. sphaericus* 1593 (pHT563). pHT5604 is a derivative of pHT563 harboring the Cry11A toxin operon at the *Asp*718 restriction site mentioned above. The arrows indicate the positions and directions of transcription of the *B. sphaericus* toxin operon and of the *aphA3*, *p19*, *cry11A*, and *p20* genes. Asterisks indicate restriction sites that have been lost. The vector pRN5101 is represented as a black line. Abbreviations: A, *Asp*718; B, *Bam*HI; Bg, *Bgl*II; C, *Cla*I; E, *Eco*RV; E1, *Eco*RI; H3, *Hin*dIII; S, *Sac*I.

Construction of plasmids for integration. (i) Construction of the integrative plasmids pHT560 and pHT563. The integrative plasmid pHT560 was constructed by insertion of the toxin genes from *B. sphaericus* 1593, on a 3.5-kbp *Hin*dIII fragment from pGSP11 (9), into pRN5101 cut with *Hin*dIII. The 3.5-kbp *Hin*dIII fragment carries the entire operon encoding the 52- and 42-kDa toxins of *B. sphaericus* 1593, including the operon promoter and terminator sequences. The coding sequences of toxin operons from strains 1593 and 2297 differ at only seven positions. However, the *Hin*dIII fragments carrying the toxin genes from strains 1593 and 2297 differ at their 3′ ends: the *Hin*dIII DNA fragment carrying the operon from strain 1593 is about 3.5 kbp long, whereas the *Hin*dIII fragment from strain 2297 is 4.7 kbp (references 5 and 6 and this work). Plasmid pHT560 contains a single *Asp*718 site upstream from the promoter region of the toxin operon, which was used for further constructions (4, 10) (Fig. 1A).

A second integrative plasmid, pHT563, was constructed by inserting a *Hin*dIII-*Bam*HI fragment of about 0.8 kbp into pRN5101 digested with *Hin*dIII and *Bam*HI. This 0.8-kbp fragment was obtained by PCR, using the total DNA of strain 2297 as the template. The oligonucleotides used for PCR experiments corresponded to (i) the 5′ end of the known nucleotide sequence of the *Hin*dIII fragment carrying the toxin operon of strain 2297 (5′-CCCCAAGCTTGTCAA CATGTGAAG-3′) and (ii) a nucleotide sequence complementary to nucleotides 780 to 797 (5). Use of this second oligonucleotide (5′-GCGCGGATCCATCG TCTCTATCTGC-3′) created a *Bam*HI restriction site at the 3′ end of the PCR product. The PCR product thus corresponded to the 5′ end of the *Hin*dIII DNA

fragment carrying the toxin genes from strain 2297. The single *Asp*718 restriction site is flanked by *B. sphaericus* DNA fragments of about 400 bp (Fig. 1B).

(ii) Construction of pHT562. Plasmid pHT562 was obtained by insertion of a 1.5-kbp *Cla*I kanamycin resistance cassette (*aphA3* gene [35a]) into the *Asp*718 site in pHT560 (Fig. 1A); the *Cla*I and *Asp*718 ends were made blunt with Klenow fragment.

(iii) Cloning of the *cry11A* gene into pHT560 and pHT563. Plasmid pHT640 (27) was used as a source of the *cry11A* gene for construction of plasmid pHT5601. It was digested with *Sac*I, and a 3.7-kbp *Sac*I fragment carrying part of the *cry11A* operon (16) was inserted into the *Asp*718 site of pHT560, yielding plasmid pHT5601 (Fig. 1A). The *Sac*I and *Asp*718 sites were made blunt with T4 DNA polymerase and Klenow fragment, respectively, before ligation. pHT640 therefore harbors the gene encoding the P19 protein, *cry11A*, and the 5′ end of the gene encoding the 20-kDa polypeptide from *B. thuringiensis* subsp. *israelensis*.

Plasmid pHT5604 was obtained by insertion of the entire *cry11A* operon into pHT563 (Fig. 1B). A 4-kbp *Eco*RV-*Cla*I fragment from pEG217 (15, 16) was inserted into pHT563 hydrolyzed with *Asp*718, with the *Cla*I and *Asp*718 sites first having been made blunt ended with Klenow fragment.

DNA manipulation. Restriction enzymes, T4 DNA ligase, T4 DNA polymerase, and Klenow fragment were used as recommended by the manufacturers. *Thermus aquaticus* DNA polymerase was used for PCR as recommended by the supplier (Amersham, Little Chalfont, Buckinghamshire, United Kingdom). Plasmid DNA was extracted from *E. coli* by a standard alkaline lysis procedure (7). Total DNA was extracted from *B. sphaericus* strains as previously described for *B. thuringiensis* (12).

DNA was analyzed by electrophoresis on 0.8% horizontal agarose slab gels. Southern blot analysis and colony hybridization experiments were performed on Hybond-N^+ filters (Amersham). The DNA probes were labeled, and hybridization was carried out, with the enhanced chemiluminescence direct nucleic acid labeling and detection system, as described by the manufacturer (Amersham).

Protein analysis. Wild-type and recombinant *B. sphaericus* cells were grown in MBS medium (20) at 30°C until cells lysed. Cells of *B. thuringiensis* subsp. *israelensis* (strain 4Q2-72) were grown in HCT medium (21). Spore-crystal mixtures were then washed twice and resuspended in ice-cold deionized water. Crystal protein concentrations in the preparations were determined by the Bio-Rad assay; mixtures were solubilized in alkali (30 min at 37°C in 0.05 N NaOH), and the insoluble material was removed by centrifugation. Sodium dodecyl sulfate-polyacrylamide gel electrophoresis was performed as previously described (35); following electrophoresis, the gel was stained with Coomassie brilliant blue.

Electron microscopy examination. *B. sphaericus* 2297 and 2297(::pHT5601) were grown in MBS at 30°C and harvested after completion of sporulation by centrifugation for 5 min at 600 × *g*. Samples were fixed in Karnowsky's fixative (4% paraformaldehyde and 5% glutaraldehyde in 0.1 M phosphate buffer, pH 7.4) and postfixed in 1% OsO_4. Samples were dehydrated in solutions with ascending concentrations of ethanol (30 to 100%) and embedded in Spurr resin (Ladd Research Industries, Burlington, Vt.). Thin sections were cut on an LKB Nova ultramicrotome, collected on Formvar-coated nickel grids, and examined with a Philips CM20 electron microscope.

In vivo toxicity assays. *B. sphaericus* and *B. thuringiensis* subsp. *israelensis* strains were grown in sporulation medium for 48 to 72 h, and spore-crystal mixtures were prepared as described above and lyophilized. Lyophilized spore-crystal mixtures were diluted to give a series of concentrations in 0.005% Triton X-100. Test suspensions were assayed in duplicate against 10 third- to fourth-instar larvae of *A. aegypti*, *C. quinquefasciatus*, and *A. stephensi* in plastic cups. All tests were conducted with a final volume of 100 ml. Control experiments were performed to test the viability of the larvae in water and in 0.005% Triton X-100. Mosquitocidal assays against *C. quinquefasciatus* larvae from Kochi (India) were conducted with laboratory stock spore-crystal mixtures as previously described (29). Mortality was scored after 48 h at 28°C. Each sample was assayed at least four times, and the concentrations of crystal proteins giving 50% mortality (LC_{50}s) and the LC_{90}s were determined by log-probit analysis.

RESULTS

In vivo recombination in *B. sphaericus*. pHT560 and pHT562 (Fig. 1A) were used to demonstrate in vivo recombination in *B. sphaericus*. pHT560 carries the toxin genes of *B. sphaericus* 1593, which are believed to be located on the chromosome (24, 32). pHT562 was obtained by inserting a kanamycin resistance gene (*aphA3*) into pHT560, such that it is flanked by *B. sphaericus* chromosomal DNA sequences.

pHT562 was purified from *E. coli* and introduced into *B. sphaericus* 2297 by electroporation. Transformants were plated on selective medium (supplemented with erythromycin and kanamycin) at a permissive temperature (30°C), which allows the replication of the pRN5101-derived plasmid. About 4×10^2 transformants/μg of DNA were obtained, and one Km^r Erm^r transformant was chosen for further analysis. It was grown in nonselective medium for about 20 generations at a nonpermissive temperature (37°C). At this temperature, replication from the pE194ts origin is abolished. The cultures were plated on kanamycin and on nonselective plates; the frequency of integration (Km^r Erm^r cells/total cells) was estimated to be about 5×10^{-2}. Km^r Erm^r cells [corresponding to 2297(::pHT562)] resulted from integration via a single crossover event between the resident toxin operon of the host strain and the homologous DNA fragments flanking the kanamycin resistance gene (Fig. 1A and 2). A second crossover event between the homologous chromosomal DNA and the second region flanking the kanamycin resistance gene would eliminate the vector carrying the erythromycin determinant and the intact operon. One Km^r Erm^r colony, 2297(::pHT562), was chosen and grown at 37 and 30°C in Luria-Bertani medium supplemented with kanamycin. The culture was plated on kanamycin, and colonies were screened for sensitivity to erythromycin. At 37°C about 1% of the cells were Km^r Erm^s, whereas at 30°C 63% of the cells were Km^r Erm^s. Thus, in vivo recombination occurs in *B. sphaericus* and can be used to integrate foreign DNA into its genome. One Km^r Erm^s isolate, 2297(::*aphA3*), was chosen and used. It harbors a kanamycin resistance gene which has been integrated upstream from the *B. sphaericus* toxin operon via two successive crossover events.

Integration of foreign DNA into the chromosome from strain 2297 was confirmed by Southern blot analysis. Total DNAs from strains 2297, 2297(::pHT562), and 2297(::*aphA3*) were digested either with *Eco*RI or with *Hin*dIII. The resulting DNA fragments were separated by agarose gel electrophoresis, transferred onto a nylon membrane, and tested for hybridization with a 1.3-kbp *Bam*HI-*Bgl*II DNA fragment from pHT560 (Fig. 1A). The resulting hybridization patterns are shown in Fig. 3A. As expected (Fig. 2), one *Eco*RI fragment of 3.5 kbp and one *Hin*dIII fragment of 4.7 kbp were found in the wild-type strain 2297 (Fig. 3A, lanes 1 and 4, respectively). These hybridizing fragments were detected in strain 2297(::pHT562) (Fig. 3A, lanes 2 and 5), as expected from Fig. 2. Surprisingly, the same hybridizing 3.5-kbp *Eco*RI and 4.7-kbp *Hin*dIII fragments were detected in strain 2297(::*aphA3*) (Fig. 3A, lanes 3 and 6), suggesting that at least one additional copy of the intact operon was originally present in each recombinant strain. The probe hybridized with an additional 7.3-kbp *Eco*RI fragment (Fig. 3A, lane 2) and three additional *Hin*dIII fragments of about 4.5, 3.7, and 1.5 kbp (Fig. 3A, lane 5) in strain 2297 (::pHT562), which contains one copy of the intact operon from strain 2297 and one copy disrupted by the integrated pHT562 (Fig. 2). In strain 2297(::*aphA3*), one additional 4.7-kbp *Eco*RI fragment (Fig. 3A, lane 3) and two *Hin*dIII fragments of 4.5 and 1.5 kbp (Fig. 3A, lane 6) hybridized with the probe (Fig. 2).

Replacement of an intact toxin operon copy by a modified copy in strain 2297(::*aphA3*) did not interfere with the sporulation process and, in particular, did not abolish the synthesis of the binary toxin (data not shown). Moreover, the *aphA3* gene was stably maintained for more than 100 generations without antibiotic selection pressure (data not shown).

Integration of the *cry11A* toxin gene. Chromosomal integration of a heterologous toxin gene by homologous recombination could lead to new *B. sphaericus* strains with enlarged insecticidal spectra and/or higher toxicities. To assess this possibility, the strategy described above was used to integrate the *cry11A* gene from *B. thuringiensis* subsp. *israelensis* into the chromosomal DNA of *B. sphaericus* 2297. The Cry11A toxin gene was chosen because it encodes a polypeptide active against *A. aegypti*, *Culex pipiens*, and *A. stephensi* larvae (29). pHT5601 was constructed by insertion of a copy of the *cry11A* operon, encoding the P19 protein, Cry11A, and a truncated

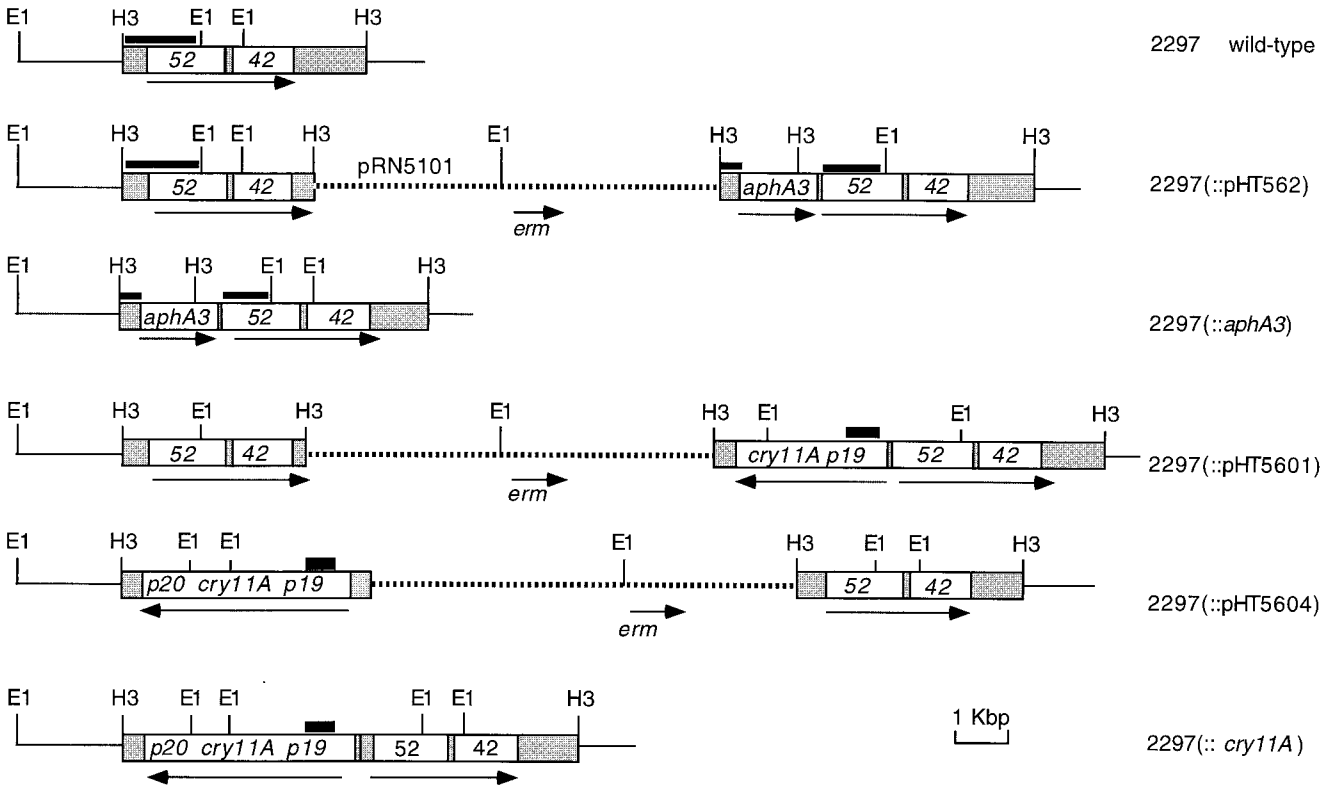

FIG. 2. Construction of the recombinant *B. sphaericus* strains by homologous recombination. Simplified restriction maps of selected chromosomal DNA regions of *B. sphaericus* 2297 after integration, via one or two successive crossover events, of the various plasmids used in this study are represented. Black bars represent the fragments hybridizing with the probes used in Southern blot experiments whose results are shown in Fig. 3. Restriction site abbreviations are as described in the legend to Fig. 1. The arrows indicate the positions and directions of transcription of the *B. sphaericus* toxin operon and of the *aphA3* and *cry11A* genes. The vector pRN5101 is represented by a broken line, with the black line representing chromosomal DNA from *B. sphaericus* 2297. Southern blot analysis suggested that two copies of the operon encoding the binary toxin may be present in the wild-type strain 2297. The second copy of the toxin operon is not represented here, but in each recombinant strain described here, one intact operon (corresponding to the wild-type strain 2297 operon) is present.

P20 polypeptide, into pHT560 (Fig. 1A). pHT5601 was used to transform *B. sphaericus* 2297. Integration by a single crossover event of the entire recombinant plasmid, including both the *cry11A* and erythromycin resistance genes, was obtained at the nonpermissive temperature. An Erm^r clone, 2297(::pHT5601), was chosen and was cultured at both 37 and 30°C in nonselective medium. Erm^s colonies, which had lost the pRN5101 DNA by a second crossover event, were screened for the presence of the *cry11A* gene by colony hybridization with a fragment carrying the *cry11A* operon promoter region and the *p19* gene as

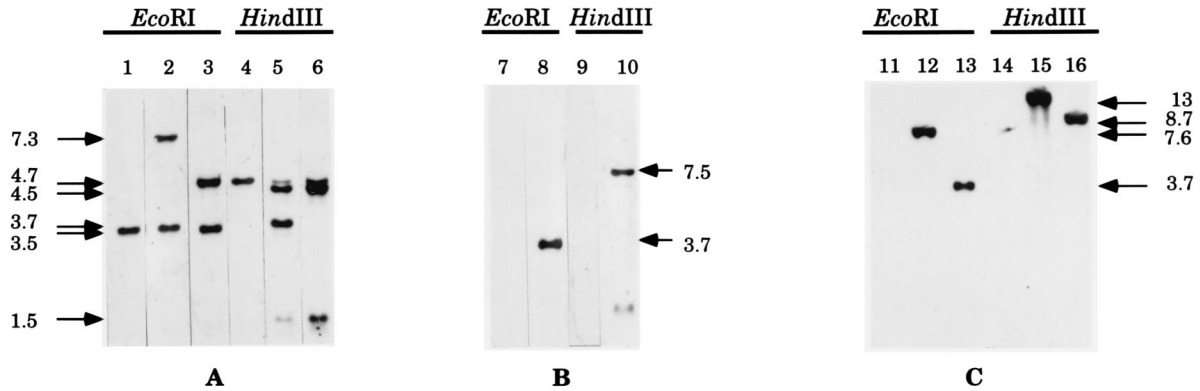

FIG. 3. Southern blot analysis of total DNA from wild-type and recombinant *B. sphaericus* strains. (A) Total DNA (4 μg) from strains 2297, 2297(::pHT562), and 2297(::*aphA3*). (B) Total DNA (4 μg) from strains 2297 and 2297(::pHT5601). (C) Total DNA (4 μg) from strains 2297, 2297(::pHT5604), and 2297(::*cry11A*). Total DNA was hydrolyzed with *Eco*RI or *Hin*dIII and subjected to electrophoresis in 0.8% agarose gels. DNA fragments were transferred onto Hybond-N^+ membranes (Amersham) and hybridized with labeled probes corresponding to the *Bam*HI-*Bgl*II fragment from pHT560 (described in Fig. 1A) (A) and to the 5′ end of the *cry11A* operon (see Fig. 2) (B and C). Lanes 1, 4, 7, 9, 11, and 14, strain 2297 (wild type); lanes 2 and 5, 2297(::pHT562); lanes 3 and 6, 2297(::*aphA3*); lanes 8 and 10, 2297 (::pHT5601); lanes 12 and 15, 2297(::pHT5604); lanes 13 and 16, 2297(::*cry11A*). The sizes of hybridizing DNA fragments (in kilobase pairs) are indicated in the margins.

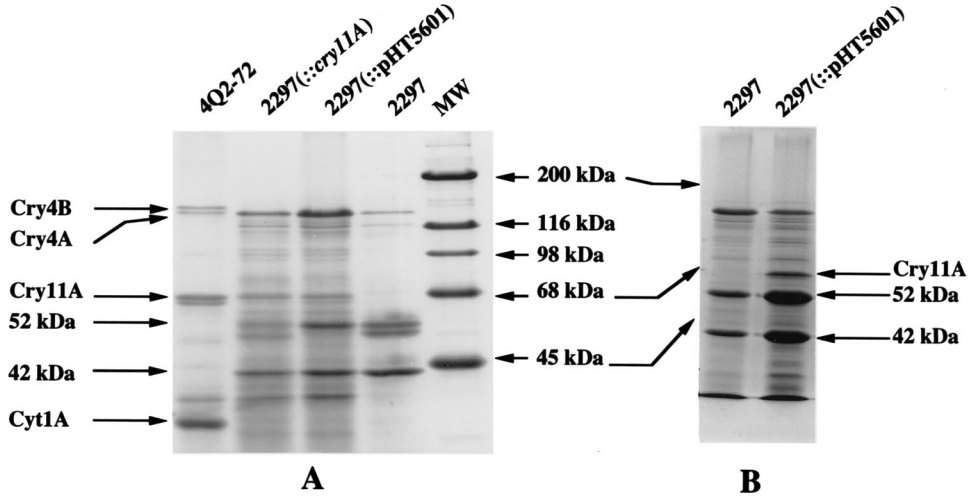

FIG. 4. Analysis of proteins in wild-type and recombinant *B. sphaericus* strains 2297, 2297(::pHT5601), and 2297(::*cry11A*). *B. sphaericus* strains were grown in MBS, and *B. thuringiensis* subsp. *israelensis* 4Q2-72 was grown in HCT, at 30°C until cell lysis. Spore-crystal mixtures containing approximately 15 μg of protein (A) and identical sample volumes of spore-crystal mixtures, corresponding to 100 μl of crude extracts (B), were subjected to electrophoresis on a sodium dodecyl sulfate–10% polyacrylamide gel and stained with Coomassie brilliant blue. Lane MW, standard protein markers.

a probe (15). This fragment carries the *cry11A* gene. Despite screening of a large number of isolates, no colonies containing the appropriate gene were found. Erm^s clones therefore presumably resulted from the loss of both the *cry11A* operon and the erythromycin resistance gene by a second crossover event in the upstream 3.2-kbp flanking region (Fig. 1A), leading to the excision of all the pHT5601 DNA.

A new attempt was made with the integrative plasmid pHT5604, using the strategy described above. pHT5604 is derived from pHT563 and harbors the entire *cry11A* operon flanked by 400-bp DNA fragments from *B. sphaericus* 2297 (Fig. 1B). After integration of the entire plasmid at the nonpermissive temperature [leading to strain 2297(::pHT5604)], Erm^r recombinant cells were grown at 30°C without antibiotic selection. Colony hybridization with Erm^s cells, using the *p19* probe, indicated that the second crossover event occurred with the same frequency in the upstream and downstream flanking regions. One Erm^s clone, 2297(::*cry11A*), was chosen for further analysis.

Integration of foreign DNA into the chromosomes of strains 2297(::pHT5601) (Fig. 3B) and 2297(::pHT5604) and 2297 (::*cry11A*) (Fig. 3C) was verified by Southern blot hybridization, using a DNA fragment corresponding to the promoter region and to the *p19* gene from the *cry11A* operon as a probe (Fig. 2) (15). As expected, no hybridization was observed with the wild-type 2297 DNA digested with *Eco*RI or *Hin*dIII (Fig. 3B, lanes 7 and 9, and Fig. 3C, lanes 11 and 14). In 2297 (::pHT5601) the crossover event had occurred in the largest flanking region, located upstream from the *cry11A* operon (Fig. 2). The 3.7-kbp *Eco*RI fragment hybridizing with the probe (Fig. 3B, lane 8) corresponded to the *Eco*RI fragment of pHT5601 [Fig. 2, strain 2297(::pHT5601)], whereas the 7.5-kbp *Hin*dIII fragment (Fig. 3B, lane 10) corresponded to DNA from pHT5601 including the *cry11A* operon [Fig. 2, strain 2297 (::pHT5601)]. As expected (Fig. 2), the probe hybridized with a 7.6-kbp *Eco*RI fragment and a 13-kbp *Hin*dIII DNA fragment from strain 2297(::pHT5604) (Fig. 3C, lanes 12 and 15, respectively). The same probe hybridized with a 3.7-kbp *Eco*RI fragment and a 8.7-kbp *Hin*dIII DNA fragment from strain 2297(::*cry11A*) (Fig. 2 and Fig. 3C, lanes 13 and 16, respectively). Hybridization experiments with a probe corresponding to the cloning vector pRN5101 confirmed that all DNA sequences from the vector, including the erythromycin resistance gene, had been eliminated from the chromosomal DNA in strain 2297(::*cry11A*) after the second crossover event (data not shown).

Protein analysis. Crystal proteins synthesized by strains 4Q2-72, 2297 (wild type), 2297(::pHT5601), and 2297(::*cry11A*) were analyzed and compared. Cells were grown in appropriate sporulation media at 30°C until cell lysis (48 to 72 h). Aliquots of spore-crystal mixtures containing about 15 μg of crystal proteins were analyzed as described in Materials and Methods (Fig. 4A). Parental strain 2297 and the recombinant strains 2297(::pHT5601) and 2297(::*cry11A*) produced the 42- and 52-kDa components of the binary toxin. The recombinants also synthesized an additional 68-kDa protein corresponding to the Cry11A toxin from *B. thuringiensis* subsp. *israelensis*. The presence of the Cry11A polypeptide in spore-crystal mixtures from recombinant strains was confirmed by Western blot analysis, using an antiserum raised against dissolved inclusions from *B. thuringiensis* subsp. *israelensis* (data not shown). However, the recombinant strain 2297(::pHT5601) synthesized about 2.5 times more binary toxin than did the wild-type strain (Fig. 4B). As assessed by densitometric analysis performed on the Coomassie blue-stained gel, the Cry11A toxin accounted for about 10 to 20% of crystal proteins in strain 2297(::pHT5601).

Ultrathin sections of sporulated cultures from 2297 (::pHT5601) were examined under a transmission electron microscope (Fig. 5). Two inclusions were observed inside the cellular membrane in strain 2297(::pHT5601).

Bioassay tests against mosquito larvae. Lyophilized spore-crystal mixtures from strains 4Q2-72, 2297, 2297(::pHT5601), and 2297(::*cry11A*) were assayed for mosquitocidal activity on larvae of *A. aegypti*, *C. quinquefasciatus*, and *A. stephensi*. Both recombinant strains 2297(::pHT5601) and 2297(::*cry11A*) were active against *A. aegypti* larvae, whereas the parental strain 2297 was not, even at high doses (Table 1). The toxicities of strains 2297 and 2297(::*cry11A*) against *A. stephensi* were almost identical. Strain 2297(::pHT5601) was about six times more active against *A. stephensi* than the wild-type strain 2297 and was as active as *B. thuringiensis* subsp. *israelensis* (Table 1). After 48 h of exposure, the toxicities against *C. quinquefascia-*

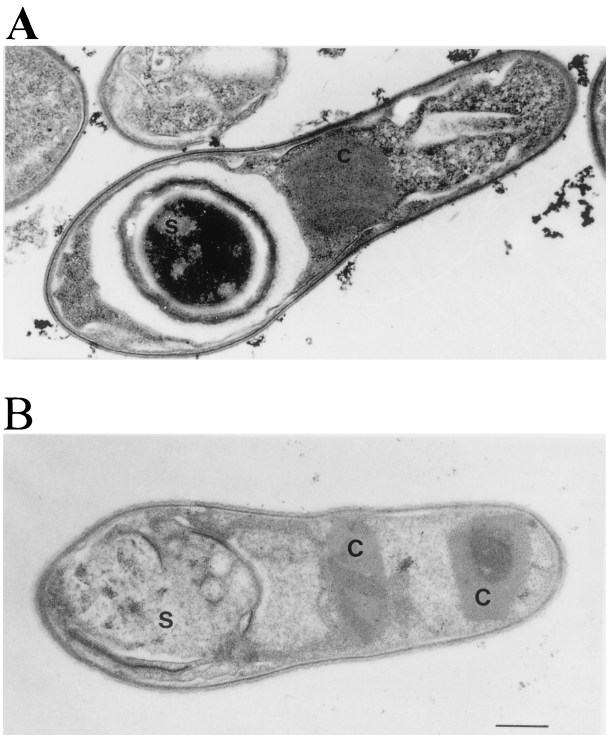

FIG. 5. Electron micrographs of ultrathin sections of *B. sphaericus* 2297 (wild type) (A) and 2297(::pHT5601) (B). Cells were grown at 30°C in MBS medium for 48 h. S, spore; C, crystalline inclusion. Bar, 0.2 μm.

tus larvae of all the strains used were similar. Strain 2297 (::*cry11A*) was tested against a resistant laboratory *C. quinquefasciatus* population selected from a population from Kochi, India. This population was first collected from an area which had been treated for 2 years with preparations of *B. sphaericus* 1593 and then reared under laboratory conditions (33). In bioassays, larvae from Kochi were less sensitive to *B. sphaericus* 2297 than a susceptible laboratory strain of *C. quinquefasciatus* (compare Tables 1 and 2). However, expression of the Cry11A toxin in 2297(::*cry11A*) conferred a low, but significant, toxicity against these larvae (Table 2).

DISCUSSION

We describe a novel approach for expressing *B. thuringiensis* subsp. *israelensis* toxin genes in *B. sphaericus*. This method can be used to obtain new *B. sphaericus* strains with improved potency for vector control.

In vivo recombination was used to introduce foreign DNA into the chromosome of *B. sphaericus* 2297. To our knowledge, this technique has never previously been used with *B. sphaericus*, although toxin genes have been transferred into *B. sphaericus* by using replicative plasmid vectors (2, 28, 36). For *B. thuringiensis* subsp. *israelensis*, homologous recombination has already been used for gene disruption in one of its plasmids to study the role of crystal components in the overall toxicity of the inclusions; these studies involved the utilization of suicide vectors (12, 27). Our system is based on a thermosensitive plasmid, pRN5101. Derivatives of this plasmid have already been used for gene inactivation or replacement in gram-positive bacteria, including *Lactococcus lactis* (8) and *Streptococcus pyogenes* (26). In addition, pRN5101-derived plasmids have been used to enlarge the activity spectrum of *B. thuringiensis* subsp. *kurstaki* HD73 (23). The use of a thermosensitive replicon allowed the transformation step to be uncoupled from the first crossover event.

A first set of pRN5101-derived plasmids was constructed and used as delivery vectors; the regions of homology flanking the *aphA3* gene or the *cry11A* gene (plasmids pHT562 and pHT5601, respectively) were not equivalent in length. The results showed that integration occurred preferentially in the larger region of homology, which was about 10 times larger than the other flanking region. By using kanamycin as a selective marker, it was possible to obtain a second crossover event in the shorter homologous fragment. This led to the excision of the plasmid vector DNA [strain 2297(::*aphA3*)]. When kanamycin selection was used, the vector was excised in 63% of the cells at 30°C as compared to 1% at 37°C, the temperature at which the integrated thermosensitive plasmid cannot replicate. These results suggest that activation of the rolling-circle replication of pE194ts stimulates the second step of a double crossover event in *B. sphaericus*, as in *L. lactis* (8). With pHT5601, it was not possible to obtain the insertion of the *cry11A* gene by a two-step crossover event, even at 30°C. A second crossover event in the largest region of homology led to the excision of pHT5601, including the Cry11A toxin gene (data not shown). A pRN5101-derived plasmid harboring the operon encoding the *cry11A* toxin gene flanked by homologous DNA regions equivalent in size was therefore constructed. After integration by a single crossover event into the chromosomal DNA in strain 2297 [strain 2297(::pHT5604) (Fig. 2)], this plasmid allowed the insertion of the *cry11A* gene with the same frequency as gene excision [leading to strains 2297 (::*cry11A*) and 2297, respectively (Fig. 2)], without antibiotic selection pressure. It appears, therefore, that in *B. sphaericus*, as in *L. lactis* (8), the homologous recombination frequency is a function of the length of homology between the recipient-cell DNA and the regions flanking the gene to be integrated. Hy-

TABLE 1. Mosquitocidal activities of lyophilized spore-crystal mixtures from various strains used in this study

Species and strain	Mosquitocidal activity[a] against:					
	A. aegypti		*A. stephensi*		*C. quinquefasciatus*	
	LC_{50}	LC_{90}	LC_{50}	LC_{90}	LC_{50}	LC_{90}
B. thuringiensis 4Q2-72	0.5 (0.3–0.8)	1.2 (0.8–1.8)	3.9 (2.6–5.8)	8.3 (5.6–12.2)	0.3 (0.2–0.4)	0.7 (0.6–1.0)
B. sphaericus						
2297	>2,400	ND[b]	17.0 (10.1–28.1)	44.0 (26.6–74.2)	0.3 (0.1–0.7)	2.2 (0.9–5.3)
2297(::pHT5601)	15.0 (7.5–28.6)	58.0 (28.0–122.0)	3.1 (1.9–5.2)	8.9 (5.4–14.7)	0.2 (0.1–0.3)	0.9 (0.4–1.8)
2297(::*cry11A*)	23.0 (16.0–30.0)	67.0 (46.0–97.0)	15.0 (6.9–33.0)	33.0 (15.0–72.0)	0.10 (0.03–0.13)	0.5 (0.2–1.1)

[a] Expressed as nanograms of crystal protein per milliliter after 48 h. Numbers represent the averages from at least four independent assays (see Materials and Methods). Numbers in parenthesis are 95% confidence limits, as determined by probit analysis.
[b] ND, not determined.

TABLE 2. Mosquitocidal activities of spore-crystal mixtures from strains 2297 and 2297(::*cry11A*) against *C. quinquefasciatus* larvae from Kochi, India

B. sphaericus strain	Mosquitocidal activity[a] (10^3)	
	LC_{50}	LC_{90}
2297	>12[b]	ND[c]
2297(::*cry11A*)	2.3 (2.1–2.6)	9.0 (7.0–11.5)

[a] See Table 1, footnote *a*.
[b] At this concentration, less than 50% mortality was observed.
[c] ND, not determined.

bridization experiments demonstrated that the genetically modified strain 2297(::*cry11A*) harbors no foreign DNA other than the Cry11A toxin operon. Since this gene is integrated into the chromosomal DNA of the host cell, the risk of its transferring into heterologous microorganisms is small. This is important for a recombinant organism which will be deliberately released into the environment.

The recombinant strains produced a large amount of Cry11A during the sporulation process. In addition, the binary toxin was present, indicating that expression of heterologous toxin genes did not abolish the accumulation of the endogenous toxin in *B. sphaericus*. The recombinant strain 2297 (::pHT5601) produced more binary toxin than did the parental strain. It harbored a parental copy and a modified copy of the 2297 toxin operon, the *cry11A* operon, and the toxin operon from strain 1593 (Fig. 2). Hybridization experiments suggested that there had been no amplification in strain 2297(::pHT5601) (data not shown). The additional copy of the 1593 toxin operon carried by the integrated pHT5601 may therefore explain the higher rate of synthesis of the 52- and 42-kDa toxins in strain 2297(::pHT5601). Recombinant strain 2297(::pHT5601) (Fig. 5B) synthesized an additional inclusion compared to the case for the wild-type strain 2297 (Fig. 5A). However, the inclusions showed a regular crystalline lattice. Further studies are necessary to elucidate the exact composition of these inclusions, to assess if these inclusions are located inside the exosporium membrane or deposited in the mother-cell compartment, and to determine whether the Cry11A toxin is assembled separately from the other toxins in recombinant *B. sphaericus* cells.

Insect bioassays showed that the spectra of activity of strains 2297(::pHT5601) and 2297(::*cry11A*) against *A. aegypti* larvae were increased (Table 1); however, these recombinants were about 30-fold less toxic than *B. thuringiensis* subsp. *israelensis* 4Q2-72. This ratio is the same as that calculated for purified Cry11A inclusions and wild-type inclusions containing all the crystal components from strain 4Q2-72 (29). Since Cry11A represents only a fraction of the total crystal protein in recombinant *B. sphaericus* cells (about 15%), this toxicity may result from synergistic interactions. Strain 2297(::pHT5601) was about sixfold more toxic than the parental strain 2297 and as toxic as strain 4Q2-72 against *A. stephensi* larvae (Table 1). This increased larvicidal activity was not observed for strain 2297(::*cry11A*). It is not clear whether this increase in toxicity was due to the synthesis of the Cry11A toxin or to the binary toxin from strain 1593, encoded by the integrated plasmid. Bioassay results suggested that there is synergy, at least against *A. stephensi* and *A. aegypti* larvae, between Cry11A and either the binary toxin, another toxic component, or the spores from strain 2297. Synergy between the Cry toxins and the toxins of *B. sphaericus* has already been suggested (28). No improvement of toxicity against *C. quinquefasciatus* larvae was evidenced (Table 1). Nevertheless, by comparison of toxicities at 24 and 48 h, there was a striking difference in the speed of killing by strain 2297(::pHT5601) or 2297(::*cry11A*) and the wild-type strain 2297. Strain 4Q2-72 was the most rapid, with no increase in toxicity between 24 and 48 h; there was a 10-fold difference in toxicity between 24 and 48 h for strain 2297, whereas 2297(::pHT5601) was intermediate with about a 3- to 4-fold difference (data not shown). Thus, the production of *B. thuringiensis* subsp. *israelensis* Cry11A toxin in *B. sphaericus* speeds up the larval killing by the host cell. Strain 2297 (::*cry11A*) was tested against a *B. sphaericus*-resistant *C. quinquefasciatus* Kochi population. The larvae were cross resistant to *B. sphaericus* 2297. However, the Kochi larvae were still sensitive to *B. thuringiensis* (data not shown), which is not unexpected since it is known that toxins from *B. thuringiensis* subsp. *israelensis* and *B. sphaericus* have different receptors (25). Interestingly, the presence of a heterologous toxin in strain 2297(::*cry11A*) partially restored activity against Kochi larvae, although not to the level of that of strain 4Q2-72. These results suggest that whatever the mechanism involved in resistance is, it does not abolish the Cry11A effect.

The work presented here demonstrates that in vivo recombination is a promising approach to introduce heterologous toxin genes into the chromosomal DNA of *B. sphaericus*. The genes are stably maintained without selection for antibiotic resistance markers, which is desirable for environmental applications in disease vector control. Integration of the Cry11A toxin gene allowed the production of new *B. sphaericus* strains with enlarged activity spectra, improved larvicidal activities, and faster kinetics of toxicity. Moreover, such strains are active against a *Culex* population resistant to currently used *B. sphaericus* formulations. The appearance of resistance against *B. sphaericus* in treated mosquito populations is of growing significance. Associating toxins with different modes of action to the binary toxin in recombinant *B. sphaericus* strains may be a strategy for resistance management. We have already demonstrated synergy between the Cry4A and Cry4B and between the Cry4A and Cry11A toxins from *B. thuringiensis* subsp. *israelensis* (29). Work is in progress to create new *B. sphaericus* strains by homologous recombination of combinations of *B. thuringiensis* genes. Integration of newly characterized toxin genes (3, 14) may also enhance the entomopathogenic properties of *B. sphaericus*.

ACKNOWLEDGMENTS

We thank Christina Nielsen-Leroux (Unité des Bactéries Entomopathogènes, Institut Pasteur, Paris) for the gift of pRN5101 and Alexandra Gruss for rearing *C. quinquefasciatus* Kochi larvae. We are grateful to Christine Dugast for typing the manuscript and to Alex Edelman for help with the English language. We gratefully acknowledge Hélène Ohayon and Brigitte Chavinier-Jove (Station Centrale de Microscopie Electronique, Institut Pasteur) for electron microscopy.

This work was supported by grants from the Institut Pasteur, Centre National de la Recherche Scientifique, AgrEvo, the United Nations Development-World Health Organization Special Programme for Research and Training in Tropical Diseases, and University Paris 7 Denis Diderot.

REFERENCES

1. **Angsuthanasombat, C., N. Crickmore, and D. J. Ellar.** 1992. Comparison of *Bacillus thuringiensis* subsp. *israelensis* CryIVA and CryIVB cloned toxins reveals synergism *in vivo*. FEMS Microbiol. Lett. **94:**63–68.
2. **Bar, E., J. Lieman-Hurwitz, E. Rahamim, A. Keynan, and N. Sandler.** 1991. Cloning and expression of *Bacillus thuringiensis israelensis* δ-endotoxin DNA in *B. sphaericus*. J. Invertebr. Pathol. **57:**149–158.
3. **Barloy, F., A. Delécluse, L. Nicolas, and M.-M. Lecadet.** 1996. Cloning and expression of the first anaerobic toxin gene from *Clostridium bifermentans* subsp. *malaysia*, encoding a new mosquitocidal protein with homologies to *Bacillus thuringiensis* delta-endotoxins. J. Bacteriol. **178:**3099–3105.
4. **Baumann, L., and P. Baumann.** 1989. Expression in *Bacillus subtilis* of the

51- and 42-kilodalton mosquitocidal toxin gene of *Bacillus sphaericus*. Appl. Environ. Microbiol. **55:**252–253.
5. **Baumann, L., A. H. Broadwell, and P. Baumann.** 1988. Sequence analysis of the mosquitocidal toxin genes encoding 51.4- and 41.9-kilodalton proteins from *Bacillus sphaericus* 2362 and 2297. J. Bacteriol. **170:**2045–2050.
6. **Berry, C., J. Jackson-Yap, C. Oei, and J. Hindley.** 1989. Nucleotide sequence of two toxin genes from *Bacillus sphaericus* IAB59: sequence comparisons between five highly toxinogenic strains. Nucleic Acids Res. **17:**7516.
7. **Birnboim, H. C., and J. Doly.** 1979. A rapid alkaline extraction procedure for screening recombinant plasmid DNA. Nucleic Acids Res. **7:**1513–1523.
8. **Biswas, I., A. Gruss, S. D. Ehrlich, and E. Maguin.** 1993. High-efficiency gene inactivation and replacement system for gram-positive bacteria. J. Bacteriol. **175:**3628–3635.
9. **Bourgouin, C., A. Delécluse, F. de la Torre, and J. Szulmajster.** 1990. Transfer of the toxin protein genes of *Bacillus sphaericus* into *Bacillus thuringiensis* subsp. *israelensis* and their expression. Appl. Environ. Microbiol. **56:**340–344.
10. **Broadwell, A. H., L. Baumann, and P. Baumann.** 1990. The 42- and 51-kilodalton mosquitocidal proteins of *Bacillus sphaericus* 2362: construction of recombinants with enhanced expression and in vivo studies of processing and toxicity. J. Bacteriol. **172:**2217–2223.
11. **Chang, C., Y.-M. Yu, S.-M. Dai, S. K. Law, and S. S. Gill.** 1993. High-level *cryIVD* and *cytA* gene expression in *Bacillus thuringiensis* does not require the 20-kilodalton protein, and the coexpressed gene products are synergistic in their toxicity to mosquitoes. Appl. Environ. Microbiol. **59:**815–821.
12. **Delécluse, A., J.-F. Charles, A. Klier, and G. Rapoport.** 1991. Deletion by in vivo recombination shows that the 28-kilodalton cytolytic polypeptide from *Bacillus thuringiensis* subsp. *israelensis* is not essential for mosquitocidal activity. J. Bacteriol. **173:**3374–3381.
13. **Delécluse, A., S. Poncet, A. Klier, and G. Rapoport.** 1993. Expression of *cryIVA* and *cryIVB* genes, independently or in combination, in a crystal-minus strain of *Bacillus thuringiensis* subsp. *israelensis*. Appl. Environ. Microbiol. **59:**3922–3927.
14. **Delécluse, A., M. Rosso, and A. Ragni.** 1995. Cloning and expression of a novel toxin gene from *Bacillus thuringiensis* subsp. *jegathesan* encoding a highly mosquitocidal protein. Appl. Environ. Microbiol. **61:**4230–4235.
15. **Dervyn, E., S. Poncet, A. Klier, and G. Rapoport.** 1995. Transcriptional regulation of *Bacillus thuringiensis* subsp. *israelensis cryIVD* gene operon. J. Bacteriol. **177:**2283–2291.
16. **Donovan, W. P., C. Dankocsik, and M. P. Gilbert.** 1988. Molecular characterization of a gene encoding a 72-kilodalton mosquito-toxic crystal protein from *Bacillus thuringiensis* subsp. *israelensis*. J. Bacteriol. **170:**4732–4738.
17. **Georghiou, G. P., J. I. Malik, M. Wirth, and K. Sainato.** 1992. Characterization of resistance of *Culex quinquefasciatus* to the insecticidal toxins of *Bacillus sphaericus* (strain 2362), p. 34–35. *In* Annual report on mosquito control research. University of California.
18. **Höfte, H., and H. R. Whiteley.** 1989. Insecticidal crystal proteins of *Bacillus thuringiensis*. Microbiol. Rev. **53:**242–255.
19. **Kalfon, A., J.-F. Charles, C. Bourgouin, and H. deBarjac.** 1984. Sporulation of *Bacillus sphaericus* 2297: an electron microscope study of crystal-like inclusion biogenesis and toxicity to mosquito larvae. J. Gen. Microbiol. **130:**893–900.
20. **Kalfon, A., I. Larget-Thiery, J.-F. Charles, and H. deBarjac.** 1983. Growth, sporulation and larvicidal activity of *Bacillus sphaericus*. Eur. J. Appl. Microbiol. Biotechnol. **18:**168–173.
21. **Lecadet, M.-M., M.-O. Blondel, and J. Ribier.** 1980. Generalized transduction in *Bacillus thuringiensis* var. *berliner* 1715, using bacteriophage CP54 Ber. J. Gen. Microbiol. **121:**202–212.
22. **Lederberg, E. M., and S. N. Cohen.** 1974. Transformation of *Salmonella typhimurium* by plasmid deoxyribonucleic acid. J. Bacteriol. **119:**1072–1074.
23. **Lereclus, D., M. Vallade, J. Chaufaux, O. Arantes, and S. Rambaud.** 1992. Expansion of insecticidal host range of *Bacillus thuringiensis* by *in vivo* genetic recombination. Bio/Technology **10:**418–421.
24. **Liu, J.-W., J. Hindley, A. G. Porter, and F. G. Priest.** 1993. New high-toxicity mosquitocidal strains of *Bacillus sphaericus* lacking a 100-kilodalton toxin gene. Appl. Environ. Microbiol. **59:**3470–3473.
25. **Nielson-Leroux, C., J.-F. Charles, I. Thiéry, and G. P. Georghiou.** 1995. Resistance in a laboratory population of *Culex quinquefasciatus* (*Diptera*: *Culicidae*) to *Bacillus sphaericus* binary toxin is due to a change in the receptor on midgut-brush border membranes. Eur. J. Biochem. **228:**206–210.
26. **Perez-Casal, J., J. Ayres-Price, E. Maguin, and J. R. Scott.** 1993. An M protein with a single C repeat prevents phagocytosis of *Streptococcus pyogenes*: use of a temperature sensitive shuttle vector to deliver homologous sequences to the chromosomes of *S. pyogenes*. Mol. Microbiol. **8:**809–819.
27. **Poncet, S., G. Anello, A. Delécluse, A. Klier, and G. Rapoport.** 1993. Role of the CryIVD polypeptide in the overall toxicity of *Bacillus thuringiensis* subsp. *israelensis*. Appl. Environ. Microbiol. **59:**3928–3930.
28. **Poncet, S., A. Delécluse, G. Anello, A. Klier, and G. Rapoport.** 1994. Transfer and expression of the *cryIVB* and *cryIVD* genes of *Bacillus thuringiensis* subsp. *israelensis* in *Bacillus sphaericus* 2297. FEMS Microbiol. Lett. **117:**91–96.
29. **Poncet, S., A. Delécluse, A. Klier, and G. Rapoport.** 1994. Evaluation of synergistic interactions between the CryIVA, CryIVB and CryIVD toxic components of *Bacillus thuringiensis* subsp. *israelensis* crystals. J. Invertebr. Pathol. **66:**131–135.
30. **Porter, A. G.** 1996. Mosquitocidal toxins, genes and bacteria: the hit squad. Parasitol. Today **12:**175–179.
31. **Porter, A. G., E. W. Davidson, and J. W. Liu.** 1993. Mosquitocidal toxins of bacilli and their genetic manipulation for effective biological control of mosquitoes. Microbiol. Rev. **57:**838–861.
32. **Priest, F. G.** 1992. Biological control of mosquitoes and other biting flies by *Bacillus sphaericus* and *Bacillus thuringiensis*. J. Appl. Bacteriol. **72:**357–369.
33. **Rao, D. R., T. R. Mani, R. Rajendran, A. S. Joseph, A. Gajanana, and R. Reuben.** 1995. Development of a high level of resistance to *Bacillus sphaericus* in a field population of *Culex quinquefasciatus* from Kochi, India. J. Am. Mosq. Control Assoc. **11:**1–5.
34. **Silva-Filha, M.-H., L. Regis, C. Nielsen-Leroux, and J.-F. Charles.** 1994. Low-level resistance to *Bacillus sphaericus* in a field-treated population of *Culex quinquefasciatus* (Diptera: Culicidae). J. Econ. Entomol. **88:**525–530.
35. **Thomas, W. E., and D. J. Ellar.** 1983. *Bacillus thuringiensis* var. *israelensis* crystal δ-endotoxin: effects on insect and mammalian cells *in vitro* and *in vivo*. J. Cell. Sci. **60:**181–197.
35a. **Trieu-Cuot, P., A. Klier, and P. Courvalin.** 1985. DNA sequences specifying the transcription of the streptococcal kanamycin resistance gene in *Escherichia coli* and *Bacillus subtilis*. Mol. Gen. Genet. **198:**348–352.
36. **Trisrisook, M., S. Pantuwatana, A. Bhumiratana, and W. Panbangred.** 1990. Molecular cloning of the 130-kilodalton mosquitocidal δ-endotoxin gene of *Bacillus thuringiensis* subsp. *israelensis* in *Bacillus sphaericus*. Appl. Environ. Microbiol. **56:**1710–1716.
37. **Wickremesinghe, R. S. B., and C. L. Mendis.** 1980. *Bacillus sphaericus* spore from Sri-Lanka demonstrating rapid larvicidal activity on *Culex quinquefasciatus*. Mosq. News **40:**387–389.