APPLIED AND ENVIRONMENTAL MICROBIOLOGY, July 1999, p. 3021–3026 Vol. 65, No. 7
0099-2240/99/$04.00+0

Production of Cry11A and Cry11Ba Toxins in *Bacillus sphaericus* Confers Toxicity towards *Aedes aegypti* and Resistant *Culex* Populations

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Received 4 February 1999/Accepted 4 May 1999

Cry11A from *Bacillus thuringiensis* subsp. *israelensis* and Cry11Ba from *Bacillus thuringiensis* subsp. *jegathesan* were introduced, separately and in combination, into the chromosome of *Bacillus sphaericus* 2297 by in vivo recombination. Two loci on the *B. sphaericus* chromosome were chosen as target sites for recombination: the binary toxin locus and the gene encoding the 36-kDa protease that may be responsible for the cleavage of the Mtx protein. Disruption of the protease gene did not increase the larvicidal activity of the recombinant strain against *Aedes aegypti* and *Culex pipiens*. Synthesis of the Cry11A and Cry11Ba toxins made the recombinant strains toxic to *A. aegypti* larvae to which the parental strain was not toxic. The strain containing Cry11Ba was more toxic than strains containing the added Cry11A or both Cry11A and Cry11Ba. The production of the two toxins together with the binary toxin did not significantly increase the toxicity of the recombinant strain to susceptible *C. pipiens* larvae. However, the production of Cry11A and/or Cry11Ba partially overcame the resistance of *C. pipiens* SPHAE and *Culex quinquefasciatus* GeoR to *B. sphaericus* strain 2297.

Mosquito and black fly populations, which transmit diseases such as malaria, filariasis, and onchocerciasis, have primarily been controlled with chemical pesticides. The emergence of resistant insect populations has led to increased interest in biological control, including utilization of naturally occurring entomopathogenic bacterial strains. Two bacterial strains, *Bacillus thuringiensis* subsp. *israelensis* and *Bacillus sphaericus*, have been used safely and efficiently to control black flies and *Culex* larvae, respectively. The entomopathogenic properties of these bacteria are due to their synthesis of protoxin crystals during sporulation. *B. thuringiensis* subsp. *israelensis* and *B. sphaericus* inclusions differ in toxin composition, activity spectra, and mode of action (reviewed in references 25–27).

The insecticidal activity of *B. thuringiensis* subsp. *israelensis* results from the synergistic action of four major proteins, of 125, 135, 68, and 27 kDa (6, 24), which are now referred to as Cry4Aa1, Cry4Ba1, Cry11Aa1, and Cyt1Aa1 (7), respectively. In this paper, the corresponding genes will be referred to as *cry4A*, *cry4B*, *cry11A*, and *cytA*, respectively. Two kinds of toxin (crystal toxins and Mtx toxins), differing in both composition and time of synthesis, seem to be responsible for the larvicidal activity of *B. sphaericus*. The crystal proteins (a binary toxin consisting of equimolar quantities of the 41.9-kDa and 51.4-kDa proteins) are present in all highly active strains and are produced during sporulation. The Mtx toxins, responsible for the activity of most strains with low activity, seem to be synthesized only during the vegetative phase (reviewed in references 3 and 5).

A number of other *B. thuringiensis* strains with mosquitocidal activity have been identified (28). *Bacillus thuringiensis* subsp. *jegathesan* and *Bacillus thuringiensis* subsp. *medellin* are the most promising of these, because both synthesize proteins, Cry11Ba1 and Cry11Bb1, respectively, that are 10 times more toxic to mosquitoes than the most active toxin of *B. thuringiensis* subsp. *israelensis* (9, 20). For simplicity, we will refer to the crystal toxins from *B. thuringiensis* subsp. *jegathesan* and *B. thuringiensis* subsp. *medellin* as Cry11Ba and Cry11Bb, respectively.

The simultaneous production of several larvicidal toxins in a single organism may broaden the host range of the strain and increase its toxicity. Combinations of toxins with overlapping targets but different modes of action might also delay the appearance of resistance in treated mosquito populations. *B. sphaericus* persists for longer in mosquito breeding sites than *B. thuringiensis* subsp. *israelensis* and can recycle in the larvae (4, 11, 21). It therefore appears to be the best host strain for combining multiple toxin genes.

Several mosquitocidal toxin genes have been transferred into *B. sphaericus* (2, 23, 31, 33) by using shuttle vectors with antibiotic resistance genes which are undesirable in biopesticides. A method based on in vivo recombination, allowing the integration of heterologous genes into the chromosome of *B. sphaericus*, has been developed (22). Genes transferred in this way are stably maintained in the absence of antibiotic selective pressure and other foreign DNA. In this case, the expression of a single gene, *cry11A*, conferred activity against organisms of the genus *Aedes* on *B. sphaericus* and decreased the resistance of a resistant laboratory population of *Culex quinquefasciatus*.

We further increased toxicity to *Aedes* and resistant organisms of the genus *Culex* by introducing the highly mosquitocidal Cry11Ba from *B. thuringiensis* subsp. *jegathesan*, alone or in combination with Cry11A, into the chromosome of *B. sphaericus* 2297. We constructed a novel integrative vector to achieve this: the gene encoding the 36-kDa protease gene that may be involved in the cleavage of the Mtx protein (34) was used as the integration site for heterologous genes. The larvicidal activities of recombinant strains were determined by us-

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ing *Aedes aegypti* and *Culex pipiens* larvae. We also report the activities of the recombinant strains against *Culex* populations resistant to the binary toxin.

MATERIALS AND METHODS

Bacterial strains, media, and plasmids. *Escherichia coli* TG1 [K-12Δ(*lac-proAB*) *supE thi hsdD* F′(*traD36 proA*$^+$ *proB*$^+$ *lacI*^{q} *lacZ*ΔM15)] (14), used in cloning experiments, was grown in Luria broth (LB) containing ampicillin (100 μg/ml) or kanamycin (25 μg/ml). *B. sphaericus* 2297 from the IEBC Collection of the Laboratoire des Bactéries et Champignons Entomopathogènes (Institut Pasteur, Paris, France) and a recombinant strain of *B. sphaericus* 2297 containing the *cry11A* gene (*B. sphaericus* 2297*bin*::*cry11A*) (22) were used as recipient strains and were transformed by electroporation as previously described (23), except that the cells were grown in LB. Only unmethylated DNA isolated from *E. coli* ET12567 (17) was used for the transformation of *B. sphaericus*. Transformants were selected on erythromycin (20 μg/ml) or kanamycin (5 μg/ml). The plasmid vectors used, pRN5101, pHT560 (22), and pUC19 (35), have been described elsewhere.

DNA manipulations. Restriction enzymes, T4 DNA ligase, and the Klenow fragment of DNA polymerase I were used as recommended by the manufacturers. DNA polymerase, isolated from *Pyrococcus furiosus*, was used for PCR as recommended by the supplier (Stratagene, Cambridge, United Kingdom). Plasmid DNA was extracted and purified from *E. coli* with the Qiagen (Düsseldorf, Germany) plasmid kit. The procedure used for the extraction of genomic DNA from *B. sphaericus* has been described elsewhere (8). DNA was analyzed by electrophoresis in 0.8% agarose gels. Southern blot analysis and colony hybridization were carried out as described by Sambrook et al. (29) on Hybond N$^+$ filters (Amersham, Little Chalfont, Buckinghamshire, United Kingdom). DNA probes were labeled by using [α-^{32}P]dATP and a nick translation kit (Amersham). The nucleotide sequences of both strands were determined by using the Thermosequenase core sequencing kit with 7-deaza-dGTP (Pharmacia, Uppsala, Sweden) and a Vistra DNA sequencer 725.

Plasmid construction. (i) Disruption of the protease gene. Two internal, 550-bp fragments of the protease gene were amplified from *B. sphaericus* 2297 chromosomal DNA by PCR by using oligonucleotide primers PS304 and -305 (5′ATGTCGACGTTGGGAATTAACAGAGAACGG3′ and 5′ATGGATCCT GCTGCATGTCGAATAGCAGC3′) or PS306 and -307 (5′ATGGATCCTAG ATATCAAGCAAGCAACAGCTACTGGTACCAA 3′-5′ATAAGCTTATTG AACGCGAGCAAATCCAAATC 3′) based on the sequence of the gene encoding the subtilisin-like serine protease from *B. sphaericus*, SSII-1 (34). The 550-bp fragments were amplified and cloned into pUC19 *Bam*HI/*Sal*I sites for the 5′ end of the protease gene to yield pPS408 and into pUC19 *Bam*HI/*Hin*dIII sites for the 3′ end to yield pPS409. The coding sequences of the protease gene from *B. sphaericus* 2297 and SSII-1 strains differ at few positions. Introducing three enzyme restriction sites (*Bam*HI, *Eco*RV, and *Kpn*I) into oligonucleotides PS306 and -307 facilitated further plasmid construction. The integrative plasmid, pPS411, was constructed by insertion of the two 550-bp internal fragments (a *Sal*I/*Bam*HI fragment from pPS408 and a *Bam*HI/*Hin*dIII fragment from pPS409) into pRN5101 cut with *Sal*I/*Hin*dIII. The pPS424 plasmid was used to disrupt the protease gene by two crossover events. It was constructed by inserting the *Bgl*II/*Kpn*I fragment carrying the kanamycin resistance gene *aphA3* from *Enterococcus faecalis* (32) into pPS411 cut with *Bam*HI/*Kpn*I.

(ii) Cloning of the *cry11A* and *cry11Ba* genes into pPS411. The plasmid pHT643 (10), which carries the *p19*, *cry11A*, and *p20* genes from *B. thuringiensis* subsp. *israelensis* was used as a source of the *cry11A* gene. The plasmid pPS414 was obtained by inserting the 4.1-kb *Bam*HI/*Kpn*I fragment from pHT643 between the *Bam*HI and *Kpn*I sites of pPS411.

pPS416 was obtained by inserting the *cry11Ba* gene from pJEG80.2 into pPS411. pJEG80.2 was obtained by inserting the 2.9-kb *Nsi*I/*Pvu*II fragment from pJEG80.1 (9) between the *Sma*I and *Pst*I sites of pHT315 (1). The resulting 2.9-kb *Asp* 718/*Hin*dIII fragment of pJEG80.2, blunt-ended with the Klenow fragment of DNA polymerase I, was inserted into pPS411 digested with *Eco*RV. Both *cry11A* and *cry11Ba* genes were cloned with their own promoters.

(iii) Cloning of a *cry11A* fragment. An internal fragment of the *cry11A* gene was amplified by PCR by using oligonucleotides PS308 and -309 (5′ATGGATC CGAACCTACTATTGCGCCAGC3′ and 5′TAGCATGCGTATATAGGATG GACGCCACG3′) and inserted between the *Bam*HI and *Sph*I sites of pUC19 to yield pPS401. This fragment was used as a probe in Southern blot analysis.

In vivo recombination in *B. sphaericus*. *B. sphaericus* transformants were plated on selective medium containing erythromycin or kanamycin at a permissive temperature (30°C), for the replication of the pRN5101-derived plasmid. One transformant was grown in nonselective medium for about 20 generations at 37°C (nonpermissive temperature). At this temperature, the pRN5101-derived plasmid does not replicate in gram-positive bacteria. The cultures were plated on erythromycin plates: only cells resulting from integration via a single crossover event between the resident protease gene and the homologous fragment carried by the plasmid were able to grow. One clone obtained by a single-crossover event was grown at 30°C in LB without antibiotic selective pressure for 60 to 100 generations and was plated on LB plates without antibiotic (except for pPS424 transformants, which were plated on kanamycin). A second crossover event eliminated the vector carrying the erythromycin resistance gene. We selected a clone with a *cry* gene obtained via two crossover events, by colony hybridization with a specific probe using Erm^s cells.

Protein analysis. Wild-type and recombinant *B. sphaericus* strains were grown in MBS medium (15) at 30°C until cell lysis. Spore-crystal mixtures were then washed twice and suspended in 100 ml of ice-cold deionized water. Aliquots (10 ml) were frozen for bioassay experiments. The protein concentration of alkali-solubilized (30 min at 37°C in 0.05 N NaOH) bacterial suspensions was determined by Bradford assay (Bio-Rad, Munich, Germany). Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) was performed as previously described (16). The gel was stained with Coomassie brilliant blue, or proteins were transferred to Hybond-C super membrane (Amersham) and screened with the Amersham ECL Western blotting kit, according to the manufacturer's recommendations. Rabbit antisera directed against either total solubilized crystals from *B. thuringiensis* subsp. *israelensis* or purified Cry11Ba inclusions were used as primary antibodies.

Mosquitocidal activity assays. Bioassays were performed on fourth-instar larvae of *C. pipiens* populations either susceptible (strain Montpellier) or resistant to the binary toxin (strain Montpellier SPHAE), *A. aegypti* (Bora-Bora) and *C. quinquefasciatus* (GeoR) larvae resistant to the binary toxin (18, 19). Mosquitoes were reared in the laboratory at 26°C and 80% relative humidity with a 14-h day/10-h night photoperiod. Larvae were reared in dechlorinated water and were fed with commercial cat food. The crystal-spore mixtures of *B. sphaericus* strains were mixed with 10 ml of deionized water in petri dishes (diameter, 5.5 cm) and were tested in duplicate against 20 larvae each. Each bioassay was repeated at least three times. Larval mortality was recorded after 48 h, and 50% lethal concentrations (LC_{50}) were determined by Probit analysis (12) with a program designed by E. Frachon (Laboratoire des Bactéries et Champignons Entomopathogènes). In general, differences between log dose-Probit mortality lines were considered significant if there was no overlapping of fiducial limits of the lines at LC_{50}.

Nucleotide sequence accession number. The DNA sequence of the 36-kDa protease gene has been deposited in the EMBL Nucleotide Sequence Database under accession no. AJ238598.

RESULTS

Disruption of the protease gene by a double-crossover event. Poncet et al. (22) described an approach for introducing foreign DNA into the chromosome of *B. sphaericus* based on in vivo recombination. The *cry11A* gene of *B. thuringiensis* was integrated upstream from the *B. sphaericus* binary toxin operon by using the thermosensitive replicative plasmid pRN5101. A combination of heterologous *cry* genes was introduced by using a novel target locus of the *B. sphaericus* chromosome: the gene encoding the 36-kDa protease that may be responsible for the cleavage of the Mtx protein (34). The disruption of this gene may increase the production of the Mtx toxin during sporulation. Indeed, the product of the protease gene may be responsible for, or contribute to, the proteolysis of Mtx in *B. sphaericus* (34). We therefore cloned the protease gene from strain 2297 by PCR by using oligonucleotide primers derived from the known sequence of the SSII-1 protease gene. The DNA sequence was determined, and the amino acid sequence was deduced. The two proteases were 98% identical in amino acid sequence (data not shown). pPS424, which contains the kanamycin resistance gene (*aphA3*) introduced into the protease gene, was used to estimate double-crossover efficiency in *B. sphaericus* and to test the effect of disrupting the protease gene on larvicidal toxicity. In vivo recombination was performed as described in Materials and Methods. A second crossover event was obtained, eliminating the vector region, by culturing one clone resulting from a single-crossover event at 30°C without selective pressure for 60 to 100 generations. Only 3 to 6% of the cells were Erm^s. Of the Erm^s colonies that had lost the pRN5101 DNA by a second crossover event, 50 to 75% were Km^r. Similar values of double-crossover events were obtained with the *cry* genes (see next paragraph). The integration of *aphA3* into the protease locus of strain 2297 to yield the *B. sphaericus* 2297*pro*::*aphA3* strain was confirmed by Southern blot analysis (Fig. 1 and 2).

Integration of the *cry11A* and *cry11Ba* toxin genes into the chromosome. The strategy described above was used to inte-

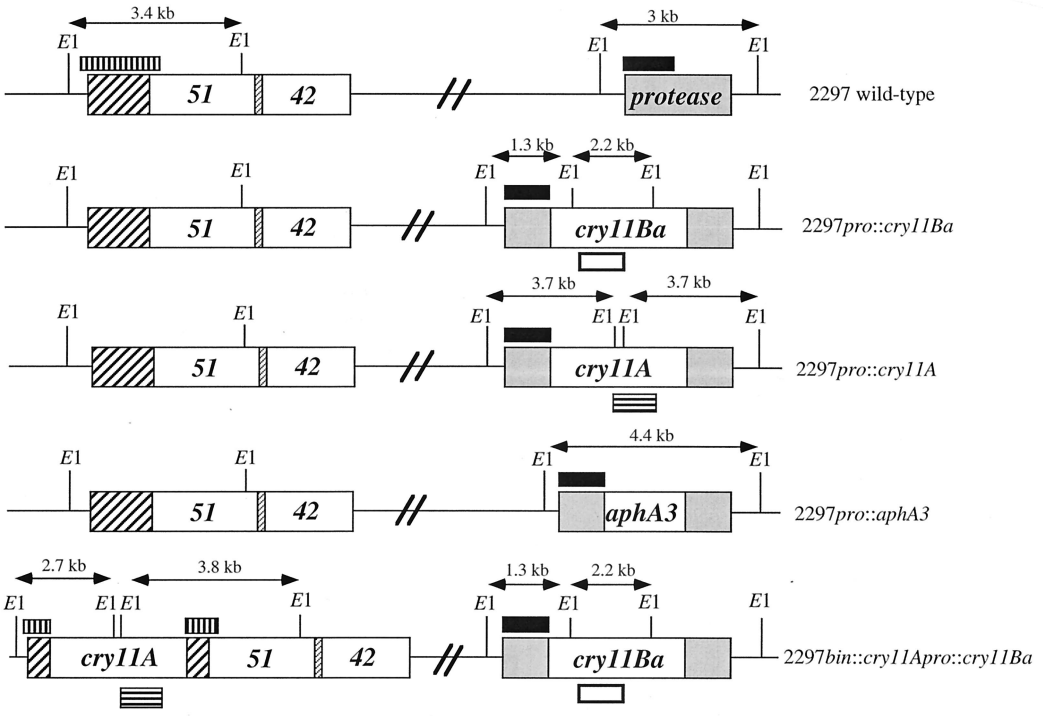

FIG. 1. Construction of the recombinant *B. sphaericus* strains by homologous recombination. The genes were introduced either upstream from the binary toxin (*cry11A*) or at the protease locus (*cry11A*, *cry11Ba*, and *aphA3*). *E*1, *Eco*RI. Probes used in Southern blot analysis were the 5′ end of the binary toxin (*Bam*HI/*Bgl*II fragment from pHT560) (▮▮▮▮), the 5′ end of the protease gene (*Bam*HI/*Sal*I fragment from pPS408) (▬), the internal part of *cry11Ba* (*Pst*I/*Hpa*I fragment from pJEG80.2) (▭), and the 5′ end of *cry11A* (*Bam*HI/*Sph*I fragment from pPS401) (▤).

grate the *cry11A* and *cry11Ba* genes into the chromosomal DNA of *B. sphaericus* 2297. The *cry11A* gene of *B. thuringiensis* subsp. *israelensis* was chosen because it encodes a polypeptide active against *A. aegypti*, *C. pipiens*, and *Anopheles stephensi* larvae (23). In addition, a recombinant strain of *B. sphaericus* 2297*bin*::*cry11A* (22), producing Cry11A, was available, making it possible to construct a recombinant strain containing both the *cry11A* and *cry11Ba* genes. The integration of the *cry11A* gene upstream from the toxin operon did not interfere with the sporulation process and did not abolish the synthesis of the binary toxin (see Fig. 3) (22). Plasmids pPS414 and pPS416 were constructed by insertion of the *cry11A* operon (encoding the P19 protein, Cry11A, and P20 polypeptide) and the *cry11Ba* gene, respectively, into pPS411. The *cry11Ba* gene was introduced into the protease loci of the wild-type strain *B. sphaericus* 2297 and *B. sphaericus bin*::*cry11A* to yield *B. sphaericus pro*::*cry11Ba* and *B. sphaericus bin*::*cry11A pro*::*cry11Ba*, respectively. The *cry11A* gene was also introduced into the protease gene of the parental strain, *B. sphaericus* 2297, to give the *B. sphaericus* 2297*pro*::*cry11A* recombinant strain. The various recombinant strains obtained were checked by Southern blot analysis by using four different probes (Fig. 1 and 2).

Protein analysis. Crystal proteins synthesized during sporulation by the parental strain 2297 and by the recombinant strains were compared. Cells were grown at 30°C in MBS medium with shaking until cell lysis (48 to 72 h). Aliquots of spore-crystal mixtures were analyzed by SDS-PAGE (Fig. 3). The parental strain 2297 and the recombinant strains produced the 42- and 51-kDa components of the binary toxin. The integration of a heterologous gene upstream from the binary toxin operon did not prevent the synthesis of the 42- and 51-kDa components. The recombinant strains also synthesized additional proteins: a 68-kDa component corresponding to the molecular weight of the Cry11A toxin from *B. thuringiensis* subsp. *israelensis* in strains 2297*bin*::*cry11A*, 2297*pro*::*cry11A*, and 2297*bin*::*cry11A pro*::*cry11Ba* and an 80-kDa protein corresponding to the molecular weight of the Cry11Ba toxin from *B. thuringiensis* subsp. *jegathesan* in strain 2297*pro*::*cry11Ba*. The presence of these proteins was confirmed by Western blot analysis, by using an antiserum directed against solubilized inclusions from *B. thuringiensis* subsp. *israelensis* or against

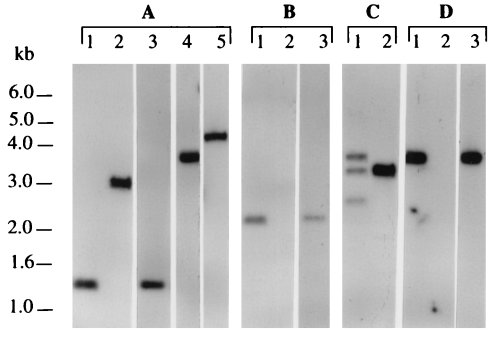

FIG. 2. Southern blot analysis of total DNA from the wild-type strain and recombinant *B. sphaericus* strains. Total DNA from strains 2297*bin*::*cry11A pro*::*cry11Ba* (lanes 1); 2297 (lanes 2); 2297*pro*::*cry11Ba* (lanes A3 and B3); 2297*pro*::*cry11A* (lanes A4 and D3); and 2297*pro*::*aphA3* (lane A5). DNA was digested with *Eco*RI and subjected to electrophoresis in a 0.7% agarose gel. DNA fragments were transferred onto Hybond N^+ membranes and hybridized with [α-^{32}P]dATP-labeled probes corresponding to the 5′ end of the protease gene (A), the internal part of *cry11Ba* (B), the 5′ end of the binary toxin operon (C), and the 5′ end of *cry11A* (D). The size of the reactive fragments was as expected. In part C, the existence of two copies of the binary toxin in strain 2297 led to the presence of the three bands observed in lane 1.

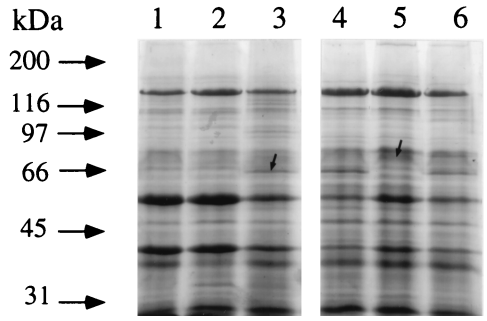

FIG. 3. Protein analysis of spore-crystal mixtures from wild-type and recombinant *B. sphaericus* strains producing the *B. thuringiensis* toxins. Spore-crystal suspensions (10 μg of protein) were subjected to SDS-PAGE (10% polyacrylamide) followed by staining with Coomassie blue. Lane 1, wild-type *B. sphaericus* 2297; lane 2, 2297*pro*::*aphA3*; lane 3, 2297*bin*::*cry11A*; lane 4, 2297*bin*::*cry11A pro*::*cry11Ba*; lane 5, 2297*pro*::*cry11Ba*; and lane 6, 2297*pro*::*cry11A*. Arrows indicate the positions of Cry11A (66 kDa) and Cry11Ba (80 kDa).

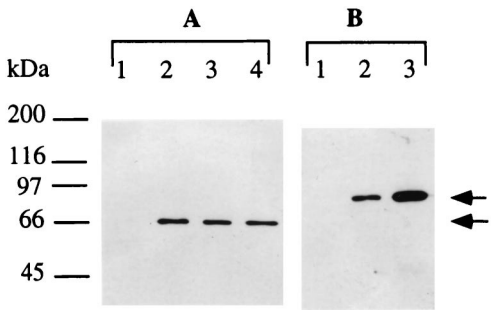

FIG. 4. Detection (indicated by arrows) of Cry11A (A) and Cry11Ba (B) in various *B. sphaericus* 2297 strains. Spore-crystal mixtures (1 μg of protein) were subjected to SDS-PAGE (10% polyacrylamide) and the proteins were transferred onto a nitrocellulose membrane. The membrane was incubated with antisera raised against either total solubilized crystals from *B. thuringiensis* subsp. *israelensis* (A) or solubilized Cry11Ba inclusions (B). Lanes 1, wild-type *B. sphaericus* 2297; lanes 2, *B. sphaericus* 2297*bin*::*cry11A pro*::*cry11Ba*; lane A3, *B. sphaericus* 2297*bin*::*cry11A*; lane B3, *B. sphaericus* 2297*pro*::*cry11Ba*; and lane A4, *B. sphaericus* 2297*pro*::*cry11A*. Immunoreactive polypeptides were detected with a peroxidase-conjugated secondary antibody.

Cry11Ba (Fig. 4). SDS-PAGE analysis showed that all recombinant strains except *B. sphaericus pro*::*aphA3* synthesized about half as much binary toxin as the wild-type strain. Cry11A was produced in all recombinants harboring the corresponding gene, in large enough amounts to be detected by gel staining. In contrast, only small amounts of the Cry11Ba toxin were detected in strain 2297*pro*::*cry11Ba* by Coomassie blue staining.

Larvicidal toxicity of the recombinant strains. Spore-crystal mixtures of the wild-type strain and of each *B. sphaericus* recombinant strain were assayed for mosquitocidal activity against larvae of *A. aegypti* and *C. pipiens* (Table 1). Disruption of the protease gene with the *aphA3* gene did not significantly increase the larvicidal activity of the recombinant strain against *A. aegypti* or *C. pipiens*. The parental *B. sphaericus* strain and recombinants producing Cry11A were equally toxic to *C. pipiens*. In contrast, the strain producing Cry11Ba was twice as toxic as wild-type *B. sphaericus* 2297 to *C. pipiens*. Surprisingly, the presence of both the Cry11A and Cry11Ba toxins halved the toxicity to *C. pipiens*. Strains producing Cry11A and Cry11Ba, alone or in combination, with the binary toxin were toxic to *A. aegypti* larvae, whereas the parental strain 2297 was not. Strain 2297*pro*::*aphA3* was not toxic to *A. aegypti*, demonstrating that the toxicity was due to Cry11 toxins. The two recombinant strains that produced Cry11A (2297*pro*::*cry11A* and 2297*bin*::*cry11A*) were equally toxic to *A. aegypti*. Cry11Ba conferred the strongest toxicity against *A. aegypti*, the strain containing this toxin being twice as toxic as those containing Cry11A. As for bioassays performed on *Culex*, the simultaneous production of Cry11A, Cry11Ba, and the binary toxin did not increase larvicidal toxicity to *A. aegypti* over that of recombinant strains producing only one heterologous toxin (Cry11A or Cry11Ba): the toxicity was similar to that for the strain expressing Cry11A alone.

B. sphaericus recombinants were also tested on two *Culex* populations resistant to the binary toxin: *C. quinquefasciatus* GeoR, a strain highly resistant to *B. sphaericus* obtained by selection in the laboratory (18), and the highly resistant strain *C. pipiens* SPHAE, obtained from a field-collected population and further selected in the laboratory (19). Populations of *Culex* larvae resistant to *B. sphaericus* were 13,500 (for GeoR) to 20,000 (for SPHAE) times less susceptible than wild-type *C. pipiens* populations (Tables 1 and 2). The production of either Cry11A or Cry11Ba with binary toxin in *B. sphaericus* 2297 partially restored toxicity against both types of resistant larva. The strain 2297*pro*::*cry11Ba* (producing Cry11Ba) decreased the LC_{50} of *C. quinquefasciatus* GeoR by a factor of at least 1,500 and that of *C. pipiens* SPHAE by a factor of at least 3,000. Production of Cry11A increased toxicity by a factor of 250 for GeoR larvae and by a factor of 800 for SPHAE larvae. The toxicity of the strain producing both Cry11A and Cry11Ba toxins was also higher: it was 600 to 1,100 times more toxic than the parental *B. sphaericus* 2297 to GeoR and SPHAE larvae, respectively. Thus, all recombinant strains are more toxic than the wild type against SPHAE and GeoR larvae. The recombinant strain of *B. sphaericus* most active against *A. ae-*

TABLE 1. Mosquitocidal activities of spore-crystal mixtures from various *B. sphaericus* strains against fourth-instar larvae of *A. aegypti* and *C. pipiens*

B. sphaericus strain	Toxin components	LC_{50} (ng/ml)[a]	
		A. aegypti	*C. pipiens*
2297	Binary toxin	>5,000[b]	20.2 (18.6–21.9)
2297*pro*::*aphA3*	Binary toxin	>5,000[b]	15.8 (14.4–17.3)
2297*bin*::*cry11A*	Binary toxin, Cry11A	1,175 (965–1,446)	18.5 (16.2–20.9)
2297*pro*::*cry11A*	Binary toxin, Cry11A	912 (715–1,186)	18.6 (16.9–20.6)
2297*pro*::*cry11Ba*	Binary toxin, Cry11Ba	452 (386–530)	10.5 (9.2–12)
2297*bin*::*cry11A pro*::*cry11Ba*	Binary toxin, Cry11A, Cry11Ba	986 (792–1,249)	39.2 (36.2–42.8)

[a] LC_{50} expressed in nanograms of spore-crystal mixture per milliliter after 48 h. Means are from at least four independent assays. Numbers in parentheses are 95% confidence limits, as determined by Probit analysis.
[b] Less than 50% mortality observed.

TABLE 2. Mosquitocidal activities of spore-crystal mixtures from various *B. sphaericus* strains against resistant strains of *Culux*

B. sphaericus strain	Toxin components	LC_{50} (ng/ml)[a]	
		C. quinquefasciatus GeoR	*C. pipiens* SPHAE
2297	Binary toxin	>270,000[b]	>400,000[b]
2297*bin*::*cry11A*	Binary toxin, Cry11A	965 (873–1,066)	469 (419–518)
2297*pro*::*cry11Ba*	Binary toxin, Cry11Ba	182 (163–201)	120 (134–146)
2297*bin*::*cry11A pro*::*cry11Ba*	Binary toxin, Cry11A, Cry11Ba	435 (479–527)	329 (293–368)

[a] LC_{50} expressed in nanograms of spore-crystal mixture per milliliter after 48 h. Means are from at least three independent assays. Numbers in parentheses are 95% confidence limits, as determined by Probit analysis.
[b] Less than 50% mortality observed.

gypti and both susceptible and resistant *Culex* populations was the strain producing the Cry11Ba toxin with the binary toxin.

DISCUSSION

We constructed recombinant *B. sphaericus* strains by developing a new integration vector, facilitating the replacement of a 36-kDa protease gene by a heterologous toxin gene. The percentage of double crossover with this system was lower (6%) than previously reported (63%) if the integration occurred upstream from the binary toxin gene (22), but the frequency of gene insertion versus gene excision was higher than previously reported (75%). The efficiency was similar if the DNA from a strain already containing an integration at the binary toxin locus was used. This system is therefore valuable for the construction of recombinants containing combinations of toxin genes.

By using this novel vector, recombinant *B. sphaericus* strains containing Cry11A and Cry11Ba, alone or in combination, were constructed. All recombinants were bioassayed against *Aedes* and both susceptible and resistant *Culex* populations. Despite the low level of *cry11Ba* expression in the recombinant producing Cry11Ba, the strain 2297*pro*::*cry11Ba* was the most toxic for all mosquito species tested. This is due to the activity of Cry11Ba, which has been shown to be the most active mosquitocidal toxin (9). However, none of the recombinants was as toxic as *B. thuringiensis* subsp. *israelensis* to this species: 10 to 20 ng of crystals was enough to provide the LC_{50} under the same conditions. The overexpression of *cry11Ba* might increase activity against *A. aegypti*. However, it is more likely that the combination with Cry11Ba of other mosquitocidal polypeptides which act synergically would increase activity. The presence of several polypeptides in a single host would also delay the appearance of insect resistance, as previously demonstrated (13).

Surprisingly, the combination of Cry11A and Cry11Ba was less toxic than Cry11Ba alone. Although Cry11Ba and Cry11A are similar, they differ in many amino acids whose role in toxicity is not known. One explanation for these results is the possibility that the two toxins bind to similar receptors, resulting in competition. Another possible explanation concerns the relative amounts of each toxin contained in spore-crystal mixtures of the different strains. The production of Cry11Ba is lower in *B. sphaericus* 2297*bin*::*cry11A pro*::*cry11Ba* than in *B. sphaericus* 2297*bin*::*cry11Ba*. Consequently, the global activity in the strain containing both toxins will be lower than the global activity in the strain containing Cry11Ba alone.

In conclusion, our results demonstrated that in vivo recombination is a valuable tool for the construction of new *B. sphaericus* strains with enlarged activity spectra and higher toxicities. Such recombinants would be of value in terms of vector control and environmental-risk limitation, because they contain no antibiotic resistance genes or other foreign DNA, except the toxin genes of interest. Moreover, since the heterologous genes are integrated into the chromosome, the risk of transfer to other microorganisms is low, and no selective pressure is needed. It would be of interest to show that recombinant *B. sphaericus* strains are also able to delay the emergence of resistance among treated mosquito populations. The utilization of two sites of integration into the chromosomal DNA of *B. sphaericus* (the binary toxin locus and the 36-kDa protease gene) allows the creation of a combination of appropriate toxin genes in *B. sphaericus*. This technique permits not only the introduction of heterologous genes into the chromosome but also the disruption of specific genes. For instance, the major protease genes can be deleted in order to increase toxin stability as previously demonstrated by Thanabalu and Porter (30). It may be also noted that in vivo recombination could be used to engineer toxin genes by, for example, the introduction of strong promoters upstream of toxin genes.

ACKNOWLEDGMENTS

We thank Christina Nielsen-LeRoux for rearing *Culex* resistant larvae and André Klier for his constant interest in this work.

This investigation received financial support from the United Nations Development/World Bank/World Health Organization Special Programme for Research and Training in Tropical Diseases, the Institut Pasteur, the Centre National de la Recherche Scientifique, AgrEvo, and University Paris 7.

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