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Molecular Cloning of the 130-Kilodalton Mosquitocidal δ-Endotoxin Gene of *Bacillus thuringiensis* subsp. *israelensis* in *Bacillus sphaericus*

MAYUREE TRISRISOOK, SOMSAK PANTUWATANA, AMARET BHUMIRATANA, AND WATANALAI PANBANGRED*

Department of Microbiology, Mahidol University, Bangkok 10400, Thailand

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A 3.7-kilobase (kb) *Xba*I fragment harboring the *cryIVB* gene (L. Thorne, F. Garduno, T. Thompson, D. Decker, M. A. Zounes, M. Wild, A. M. Walfield, and T. J. Pollock, J. Bacteriol. 166:801–811, 1986) which encoded a 130-kilodalton (kDa) mosquitocidal toxin from a 110-kb plasmid of *Bacillus thuringiensis* subsp. *israelensis* 4Q2-72 was cloned into pUC12 and transformed into *Escherichia coli*. The clone with a recombinant plasmid (designated pBT8) was toxic to *Aedes aegypti* larvae. The fragment (3.7 kb) was ligated into pBC16 (tetracycline resistant [Tc^r]) and transformed by the method of protoplast transformation into *Bacillus sphaericus* 1593 and 2362, which were highly toxic to *Anopheles* and *Culex* mosquito larvae but less toxic to *Aedes* larvae. After cell regeneration on regeneration medium, the Tc^r plasmids from transformants (pBTC1) of both strains of *B. sphaericus* were prepared and analyzed. The 3.7-kb *Xba*I fragment from the *B. thuringiensis* subsp. *israelensis* plasmid was shown to be present by agarose gel electrophoresis and Southern blot hybridization. In addition, *B. sphaericus* transformants produced a 130-kDa mosquitocidal toxin which was detected by Western (immuno-) blot analysis with antibody prepared against *B. thuringiensis* subsp. *israelensis* 130-kDa mosquitocidal toxin. The 50% lethal concentrations of the transformants of strains 1593 and 2362 against *A. aegypti* larvae were 2.7×10^2 and 5.7×10^2 cells per ml, respectively. This level of toxicity was comparable to the 50% lethal concentration of *B. thuringiensis* subsp. *israelensis* but much higher than that of *B. sphaericus* 1593 and 2362 (4.7×10^4 cells per ml) against *A. aegypti* larvae. The transformants also retained high toxicity against *Anopheles* and *Culex* mosquito larvae. Finally, the recombinant plasmids were highly stable upon daily subculture for at least 4 weeks in medium without tetracycline.

Bacillus thuringiensis subsp. *israelensis* synthesizes an irregularly shaped parasporal crystal which is highly toxic to certain dipteran larvae (24). Studies of these parasporal bodies have shown that they are globular, are composed of different types of inclusions, and contain several proteins of molecular masses of approximately 25 to 28, 65, and 130 kilodaltons (kDa) (8, 28, 30, 31). Ibarra and Federici (9) reported that the 130-kDa protein is composed of at least two polypeptides of approximately 125 and 135 kDa; the genes encoding these polypeptides are located on a large plasmid of 72 MDa (6, 27). The genes *cytA*, *cryIVC*, and *cryIVB* (7), encoding the 28-kDa protein (3, 13, 26, 30), the 65-kDa protein (22), and the 130-kDa protein (1, 3, 20, 29, 32), respectively, have been cloned and sequenced (26, 28, 32), and the gene product, a 130-kDa protein, has been shown to be highly toxic to *Aedes aegypti* (1, 3, 20, 29). Despite the high toxicity of *B. thuringiensis* subsp. *israelensis* against *Aedes* larvae, the bacterium still has considerable disadvantages for use in extensive mosquito control programs. These disadvantages are due mainly to the nonpersistence of *B. thuringiensis* subsp. *israelensis* and its instability in the environment, where it often loses toxicity within a few days (5, 25).

Bacillus sphaericus, an endospore-forming bacterium, also produces toxin(s) which is toxic to larvae of a number of disease-transmitting mosquito species, such as *Culex* and *Anopheles* spp. (33). It has been reported that *B. sphaericus* not only persists and maintains the mosquito larvicidal toxin but also recycles in aquatic systems (16, 19). Because of these advantages it would be valuable to genetically improve strains of *B. sphaericus* in order to obtain strains with a broader spectrum of toxicity against mosquito larvae. One method of attaining an improved strain of *B. sphaericus* was to transfer *B. thuringiensis* subsp. *israelensis* toxin into *B. sphaericus* and observe the toxicity and persistence of both toxins in the same cells.

We report here the cloning of the gene encoding the 130-kDa protein (toxic to larvae of *A. aegypti*) from the 110-kilobase (kb) plasmid of *B. thuringiensis* subsp. *israelensis* 4Q2-72 into *B. sphaericus* 1593 and 2362 (33), which are toxic to larvae of *Culex* and *Anopheles* spp. The toxicities of these *B. sphaericus* transformants against three species of mosquito larvae and their stabilities were also determined.

MATERIALS AND METHODS

Bacterial strains and plasmid vectors. *B. thuringiensis* subsp. *israelensis* 4Q2-72 harboring a 110-kb plasmid, isolated by D. Dean (Ohio State University, Columbus, Ohio), was a gift from S. Panyim, Mahidol University. *Escherichia coli* DH5α (Bethesda Research Laboratories, Inc.) was used as a primary host for cloning the *B. thuringiensis* subsp. *israelensis* toxin gene by using pUC12 (14) as a vector. *B. sphaericus* 1593, obtained from S. Singer (Department of Biological Science, Western Illinois University, Macomb, Ill.), and strain 2362, obtained from E. W. Davidson (Department of Zoology, Arizona State University, Tempe, Ariz.), were used as cloning hosts, and pBC16 from *Bacillus cereus* (17) was used as a plasmid vector.

Cloning of *B. thuringiensis* subsp. *israelensis* toxin into *E.*

* Corresponding author.

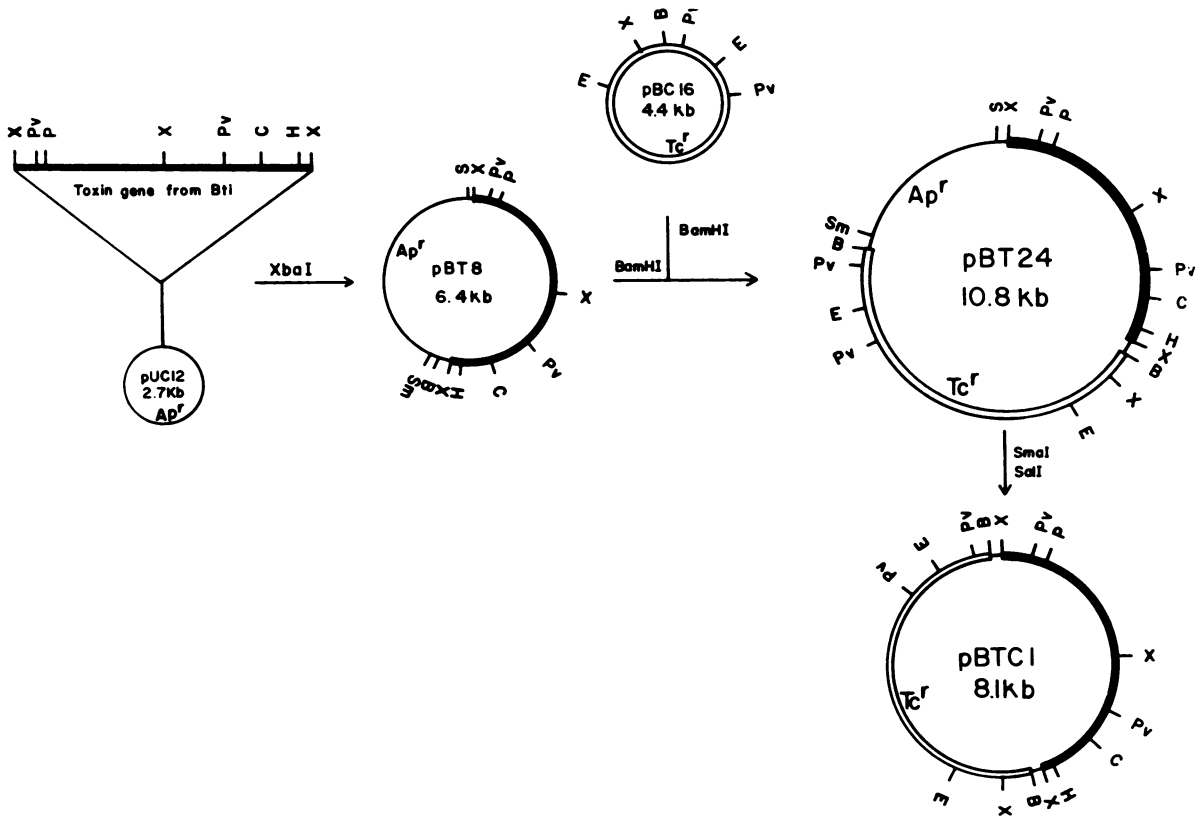

FIG. 1. Construction of plasmid pBTC1. A 3.7-kb *Xba*I fragment encoding a 130-kDa mosquitocidal toxin from a 110-kb plasmid (*B. thuringiensis* subsp. *israelensis* 4Q2-72) was cloned into pUC12 to create pBT8. pBC16 was joined to pBT8 at the *Bam*HI site and transformed into *E. coli* DH5α. This plasmid was designated pBT24. pBTC1 was constructed by digesting pBT24 with *Sal*I and making blunt ends by filling in with Klenow fragment and the linearized plasmid was then cut with *Sma*I to delete an approximately 2.6-kb *Sal*I-*Sma*I fragment of pUC12. The deleted plasmid was religated and transformed into *B. sphaericus*. Symbols: ■, toxin gene; ——, pUC12; ═══, pBC16. X, *Xba*I; Pv, *Pvu*II; P, *Pst*I; C, *Cla*I; E, *Eco*RI; H, *Hin*dIII; B, *Bam*HI; S, *Sal*I; Sm, *Sma*I; Bti, *B. thuringiensis* subsp. *israelensis*.

coli **DH5α.** The purified 110-kb plasmid of *B. thuringiensis* subsp. *israelensis* (3, 27) was isolated as described previously (2). Partial *Xba*I-restricted 110-kb plasmid was ligated into *Xba*I-restricted pUC12, and the ligated product was used to transform *E. coli* DH5α. The ampicillin-resistant transformants were tested for toxicity against mosquito larvae of *A. aegypti* (second-instar larvae) by putting larvae directly into the bacterial suspension in microdilution plates (1 colony per 150 μl of 50 mM sodium phosphate buffer). Dead larvae were scored 48 h after feeding at room temperature.

Several clones with suspected recombinant plasmids which were toxic to *A. aegypti* larvae were selected, and the plasmids were analyzed. All clones were found to contain the 3.7-kb *Xba*I fragment (designated pBT8; Fig. 1) which was reported to encode the 130-kDa protein (32).

In order to construct a recombination plasmid capable of transforming *B. sphaericus*, pBC16 (4.4 kb) was joined to pBT8 at a *Bam*HI site and transformed into *E. coli* DH5α. Transformants resistant to ampicillin (50 μg/ml) and tetracycline (5 μg/ml) were selected and tested by restriction analysis for the presence of the *B. thuringiensis* subsp. *israelensis* toxin gene with pBC16 and pUC12. The ligated plasmid was designated pBT24 (Fig. 1). The toxicity of transformants was tested against *A. aegypti* larvae.

Transferring the pBTC1 plasmid into *B. sphaericus*. Recombinant plasmid pBT24 or its deleted plasmid pBTC1 (Fig. 1) was used to transform *B. sphaericus* 1593 and 2362. pBTC1 was constructed by digesting pBT24 with *Sal*I and making blunt ends by filling in with Klenow fragment (Bethesda Research Laboratories). The linearized plasmid was then cut with *Sma*I to delete an approximately 2.6-kb *Sal*I-*Sma*I fragment of pUC12. The deleted plasmid was religated and transformed into *B. sphaericus* by protoplast transformation by the method of Chang and Cohen (4), with some modifications. Specifically, the cells were converted into protoplast by treatment with lysozyme (4 mg/ml) and acromopeptidase (4 mg/ml). Plasmids at concentrations of 5 to 10 μg/ml were added to the protoplast suspension, and after polyethylene glycol treatment, the protoplasts were washed and plated onto DM3 regeneration medium (4) containing 0.5 M sucrose instead of succinic acid. The regeneration medium also contained 1% tryptone–0.5% NaCl and was supplemented with tetracycline (15 μg/ml). Transformants were observed within 5 to 10 days at 37°C.

Southern blot hybridization. DNAs from different strains of *E. coli* and *B. sphaericus* were digested with *Xba*I and *Pst*I, electrophoresed in agarose gel, and blotted onto nitrocellulose by the Southern transfer method (21). A 3.7-kb fragment (two 1.75-kb *Xba*I fragments) from pBT8 encoding the 130-kDa mosquitocidal toxin was recovered by low-melting-point agarose gel electrophoresis (11). The fragment was biotinylated by using a biotin nick translation kit (Bethesda Research Laboratories) and used as a probe. The hybridization was detected with a BluGENE kit (Bethesda Research Laboratories).

Western (immuno-) blot analysis. Proteins from *E. coli* cells were prepared by lysing cells directly in sodium dodecyl sulfate (SDS) reducing buffer (10). *B. sphaericus* and its transformants were grown until late exponential phase, washed, and suspended in 1/50 volume with 50 mM Tris hydrochloride, pH 8.0. The cells were ruptured by sonication (Soniprep 150) at 18 to 20 amplitude microns for 4 × 1 min. Cell debris was removed by centrifugation (12,000 × *g*, 10 min), and the supernatant was used for SDS-polyacrylamide gel electrophoresis (10% polyacrylamide). Proteins resolved by SDS-polyacrylamide gel electrophoresis were transferred electrophoretically to a nitrocellulose filter (23). The filter was soaked for 2 h in BLOTTO solution (5% skim milk powder, 16 mM Na_2HPO_4, 4 mM NaH_2PO_4, 120 mM NaCl [pH 7.5]) before being incubated with ^{125}I-protein A conjugated with rabbit antiserum raised against the 130-kDa crystal protein of *B. thuringiensis* subsp. *israelensis*, which was kindly given by S. Panyim (1). After a washing with the BLOTTO solution, the dry filter was autoradiographed (Fuji X-ray film).

Toxicity assays against mosquito larvae. *B. sphaericus* and *B. thuringiensis* subsp. *israelensis* were grown in NYSM broth (15) and nutrient broth, respectively, for 48 h. Ten milliliters of cell culture was added to sterile cups containing 90 ml of distilled water. A series of 10-fold dilutions were made from the bacterial suspension (10^{-1} to 10^{-8}). Ten second- or third-instar larvae of *A. aegypti*, *Anopheles dirus*, or *Culex quinquefasciatus* were added to each cup. The tests were done in duplicate. Ten larvae in distilled water were used as the control. The cups were kept at room temperature, and the numbers of surviving larvae were recorded in 2 days. Activities were calculated as 50% lethal concentrations according to the method described by Reed and Muench (18).

Stability of *B. sphaericus* transformants. *B. sphaericus* 1593 and 2362 carrying recombinant plasmids were grown in LB broth (Bethesda Research Laboratories; 1% tryptone, 0.5% NaCl, 0.5% yeast extract) with tetracycline (15 μg/ml) at 37°C overnight. The cultures were then subcultured daily in LB broth without tetracycline for 4 weeks. The presence of plasmid was determined every day for the first week and once a week for the second, third, and fourth weeks by diluting cells with 50 mM phosphate buffer (pH 7.0) and plating on LB agar (LA; same as LB but with 2.0% Bacto-Agar) with and without tetracycline.

RESULTS

Cloning of *B. thuringiensis* subsp. *israelensis* toxin gene (*cryIVB*) into *E. coli* DH5α and *B. sphaericus* 1593 and 2362. *E. coli* transformants harboring the recombinant plasmid pBT8 (i.e., a plasmid containing the 3.7-kb *Xba*I fragment inserted in 2.7-kb pUC12 at an *Xba*I site) were shown to be toxic to *A. aegypti* larvae. The inserted fragment gave the same restriction patterns reported elsewhere for the DNA encoding the 130-kDa toxin (1, 32). The gene was designated *cryIVB* (22).

To construct an intermediate plasmid carrying the *cryIVB* gene for transformation into *B. sphaericus*, pBT8 was joined to the *Bacillus* vector pBC16 (Tc^r) and transformed into *E. coli* DH5α and Ap^r Tc^r transformants were selected. A new shuttle vector (pBT24) capable of transforming both *E. coli* and *Bacillus* sp. was selected. This plasmid was used either immediately or after deletion of most of pUC12 to transform *B. sphaericus* 2362 and 1593. The deleted plasmid was designated pBTC1 (Fig. 1).

TABLE 1. Transformation of *B. sphaericus* 1593 and 2362 with plasmids pBT24 and pBTC1

B. sphaericus strain	Total transformants (CFU/ml) with:		Frequency[a]	
	pBT24	pBTC1	pBT24	pBTC1
1593	ND[b]	18	ND	3.6
2362	6	15	1.2	3

[a] Frequency of transformation was determined as number of transformants per microgram of DNA.
[b] ND, Not determined.

A total of 6 and 15 transformants were obtained from *B. sphaericus* 2362 by using pBT24 and pBTC1, respectively (Table 1). Similar results were obtained when *B. sphaericus* 1593 was used as the host organism. The transformants developed very slowly in modified DM3 regeneration medium, and some were detected as late as 10 days after initial plating. Many clones suffered plasmid deletion, resulting in plasmids smaller than expected.

Results of restriction analysis of pBT8, pBT24, and pBTC1 are shown in Fig. 2. *Xba*I complete digestion of pBT8 yielded two DNA fragments of 2.7 kb (pUC12) and two fragments of 1.85 kb (Fig. 2, lane 3). One of the 1.85-kb fragments split into two fragments of approximately 1.5 and 0.35 kb when subjected to further digestion with *Pst*I (Fig. 2, lane 4). In the same way, pBT24 digested with *Xba*I yielded one DNA fragment of approximately 7.1 kb (pUC12 linked to pBC16) and two fragments of 1.85 kb (Fig. 2, lane 6). When these fragments were further digested with *Pst*I, they gave DNA bands of 7.1, 1.85, 1.5, and 0.35 kb (Fig. 2, lane 7). Plasmid pBTC1 gave one fragment of 4.4 kb and two of 1.85 kb with *Xba*I (Fig. 2, lane 9) and one fragment each of 4.4, 1.85, 1.5, and 0.35 kb with *Xba*I followed by *Pst*I (Fig. 2, lane 10).

Southern blot hybridization. Biotin-labeled *B. thuringien-*

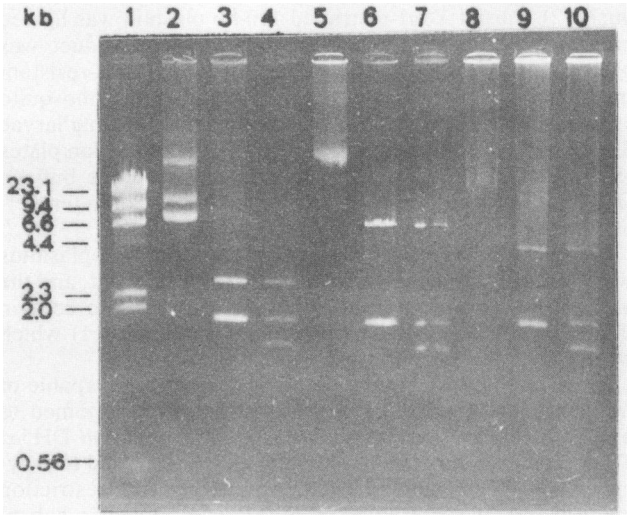

FIG. 2. Agarose gel electrophoresis of plasmids pBT8, pBT24, and pBTC1. Plasmids were electrophoresed in a 0.7% agarose gel. Lane 1, *Hin*dIII-digested λ DNA; lanes 2, 5, and 8, uncut pBT8, pBTC24, and pBTC1, respectively; lanes 3, 6, and 9, *Xba*I-digested pBT8, pBT24, and pBTC1, respectively; lanes 4, 7, and 10, *Xba*I-*Pst*I-digested pBT8, pBT24, and pBTC1, respectively. Fragment sizes (in kilobases) are shown at the left.

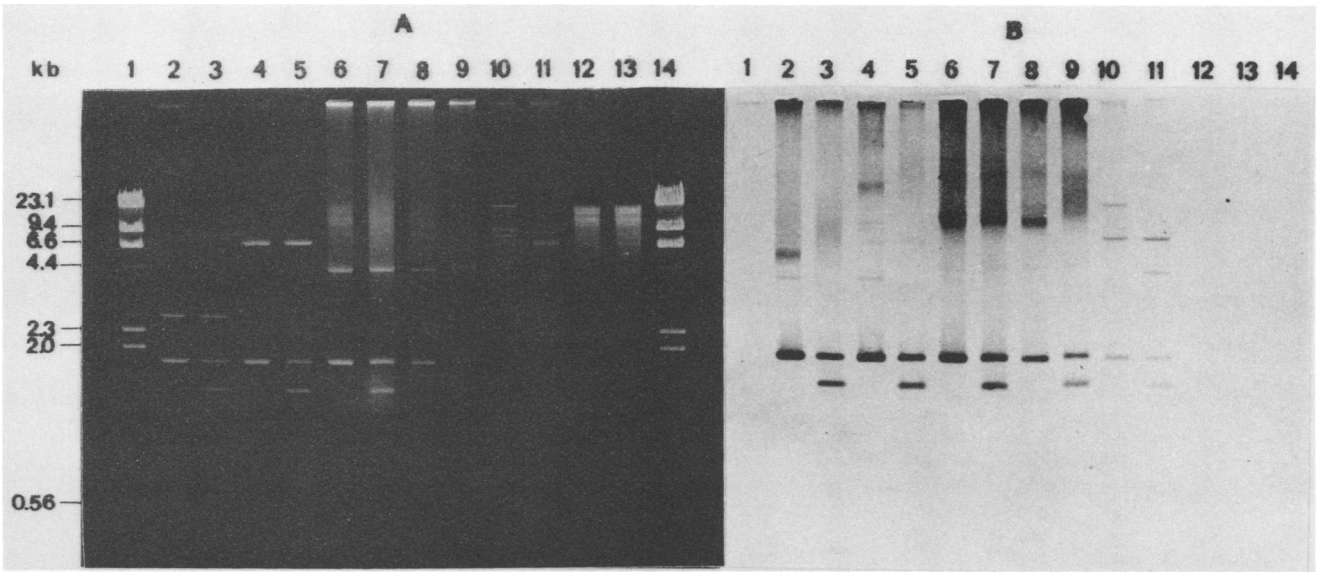

FIG. 3. Agarose gel and Southern blot analyses of various plasmid DNAs. Plasmid DNAs of several strains of *E. coli*, *B. sphaericus* transformants, and *B. thuringiensis* subsp. *israelensis* were separated on a 0.7% agarose gel (A). After being transferred to a nitrocellulose filter, the plasmids were probed with biotin-labeled *B. thuringiensis* subsp. *israelensis* toxin gene (3.7-kb *Xba*I fragment from pBT8) (B). Lanes 1 and 14, *Hin*dIII-digested λ DNA; lanes 2 and 3, pBT8 digested with *Xba*I and *Xba*I-*Pst*I, respectively; lanes 4 and 5, pBT24 digested with *Xba*I and *Xba*I-*Pst*I, respectively; lanes 6 and 7, 1593(pBTC1) digested with *Xba*I and *Xba*I-*Pst*I, respectively; lanes 8 and 9, 2362(pBTC1) digested with *Xba*I and *Xba*I-*Pst*I, respectively; lanes 10 and 11, 4Q2-72 digested with *Xba*I and *Xba*I-*Pst*I, respectively; lanes 12 and 13, *Xba*I-digested chromosomal DNA of strains 1593 and 2362, respectively. Fragment sizes are indicated at the left.

sis subsp. *israelensis* toxin gene (two 1.85-kb *Xba*I fragments from pBT8) was used as a probe to hybridize with restricted plasmid DNA from selected transformants. All plasmids from transformants of either strain 1593 or 2362 cross-hybridized with the probe (Fig. 3B, lanes 6 to 9). The hybridized fragments were 1.85-kb *Xba*I fragments and 1.5- and 0.35-kb *Xba*I-*Pst*I fragments. The other faint bands (Fig. 3B, lanes 2 to 12) probably resulted from partial digestion of the plasmid. The probe showed no homology with pUC12 (Fig. 3A, lanes 2 and 3, upper band), pBC16 (Fig. 3A, lanes 6 to 9, middle band), or chromosomal DNA from *B. sphaericus* 1593 and 2362 (Fig. 3A, lanes 12 and 13). The results from Southern hybridization confirm the presence of the 3.7-kb *B. thuringiensis* subsp. *israelensis* toxin gene. Restriction analysis and the hybridization experiments showed that the constructed plasmid, once established in the cells, remained unchanged after transformation, in terms of gene deletion or rearrangement.

Western blot analysis. SDS-polyacrylamide gels stained with Coomassie brilliant blue R-250 showed that extracts from *B. sphaericus* 1593 and 2362 and from *E. coli* transformants harboring the *B. thuringiensis* subsp. *israelensis* toxin gene contained extra protein bands with electrophoretic mobilities the same as that of the *B. thuringiensis* subsp. *israelensis* toxin (Fig. 4, lanes 2 and 5). No extra band was observed from *E. coli* harboring pBT8 compared with *E. coli* extract. This may be due to low expression of the *B. thuringiensis* subsp. *israelensis* toxin gene in *E. coli* and resulted in an inadequate amount of protein to be seen by SDS-polyacrylamide gel electrophoresis. The presence of the toxin in *E. coli*(pBT8) was later confirmed by Western blot analysis.

To immunologically detect the crystal protein from transformants, proteins resolved in SDS-polyacrylamide gel electrophoresis were transferred to nitrocellulose sheets and Western blot analysis was performed, using purified rabbit anti-*B. thuringiensis* subsp. *israelensis* toxin. The transformants produced a 130-kDa protein which reacted with ^{125}I-protein A conjugated to the antibody against 130-kDa toxin (Fig. 5, lanes 1, 4, and 6). Such proteins could not be detected in host cells (Fig. 5, lanes 2, 5, and 7). The many protein bands detected in lane 3 (crystal toxin) were probably degraded forms of the toxin. The various bands from

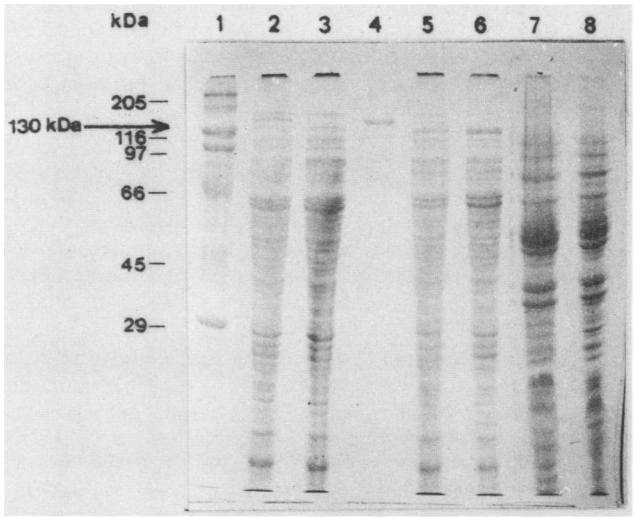

FIG. 4. SDS-polyacrylamide gel of the protein extracts from *E. coli* and *B. sphaericus* transformant cells. Proteins were electrophoresed through an SDS–10% polyacrylamide gel and then stained with Coomassie brilliant blue R-250. Lane 1, Protein size marker; lane 2, extract from 1593(pBTC1); lane 3, 1593; lane 4, 130-kDa crystal protein; lane 5, 2362(pBTC1); lane 6, 2362; lane 7, *E. coli*(pBT8); lane 8, *E. coli*. The arrow indicates the position of the 130-kDa crystal protein. Fragment sizes are indicated at the left.

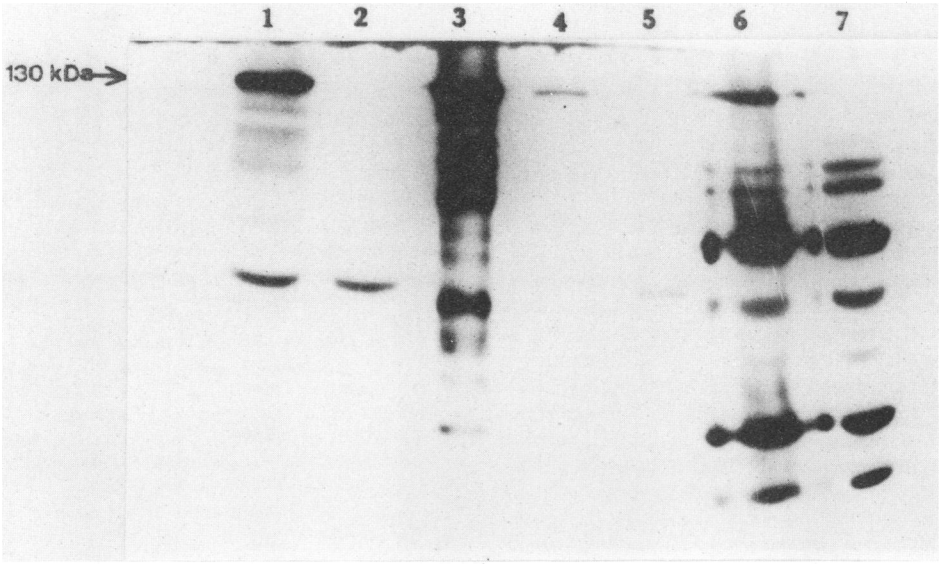

FIG. 5. Immunoblot analysis of protein extracts from *E. coli* and *B. sphaericus* transformant cells. Proteins were transferred onto nitrocellulose after electrophoresis by SDS-polyacrylamide gel electrophoresis. They were incubated with anti-130-kDa protein antiserum. Lane 1, extract from strain 1593(pBTC1); lane 2, 1593; lane 3, 130-kDa crystal protein; lane 4, 2362(pBTC1); lane 5, 2362; lane 6, *E. coli*(pBT8); lane 7, *E. coli*. The arrow indicates the position of the 130-kDa crystal protein.

crude extracts representing proteins smaller than 130 kDa were caused by cross-hybridization of the antibody, since the antibody used was polyclonal and was used without adsorption. Although no quantitative interpretation can be made from these data, the results from the immunoblot experiment confirm the presence of the 130-kDa *B. thuringiensis* subsp. *israelensis* toxic protein in the *E. coli* and *B. sphaericus* transformants.

Mosquito larva bioassay. *B. sphaericus* 1593 and 2362 are highly toxic to *Anopheles* and *Culex* mosquitoes but less toxic to *Aedes* larvae. Cells containing recombinant plasmids were tested for toxicity toward the dipteran mosquito larvae of *A. aegypti*, *Anopheles dirus*, and *C. quinquefasciatus*. In this experiment, the *B. sphaericus* 1593 and 2362 transformant clones containing recombinant plasmids encoding the 130-kDa toxin were 100 times more toxic to *A. aegypti* larvae than were the parent strains (1593 and 2362) (Table 2). At the same time, they retained high toxicity to *Anopheles* and *Culex* larvae (Table 2). The high toxicity to *A. aegypti* larvae remained unchanged even after several passages of the transformants under nonselectable conditions.

Stability of recombinant plasmids in *B. sphaericus* transformants. Cells of *B. sphaericus* 1593 and 2362 containing recombinant plasmids were subcultured daily for 4 weeks in medium without tetracycline. The number of cells from drug-free culture broth were determined on LA and LA supplemented with tetracycline (15 μg/ml). The cell numbers on the two agar media were the same (Fig. 6). The results demonstrated the stability of the plasmid without the presence of selective pressure. However, *B. sphaericus* 1593(pBTC1) showed a slight decrease in the number of cells harboring plasmid. A few clones from LA medium were randomly selected, analyzed for the presence of plasmid, and tested for toxicity and could be shown to retain intact pBTC1 plasmids.

DISCUSSION

McDonald and Burke (12) reported the transformation of pBC16 and pUB110 into *B. sphaericus* 1593 by the protoplast transformation method. The frequency of transformation was very low (less than 1 transformant per μg of DNA). In this report, a hybrid plasmid harboring the *B. thuringiensis* subsp. *israelensis* toxin gene (*cryIVC* gene) gave a frequency of about 3.6 and 3 transformants per μg of DNA for strains 1593 and 2362, respectively. These were the averages of results of two separate transformation experiments. Transformation with pBC16 gave a frequency of 4 transformants per μg of DNA for both strains 1593 and 2362. Therefore, our modified DM3 regeneration medium gave slightly higher transformation frequencies than the usual DM3 regeneration medium. In our experiments, no transfor-

TABLE 2. Toxicities of *B. sphaericus*, *B. sphaericus* transformants, and *B. thuringiensis* subsp. *israelensis* against mosquito larvae

Strain	LC_{50}[a] against mosquito larvae		
	A. aegypti	*Anopheles dirus*	*C. quinquefasciatus*
B. sphaericus			
1593	4.8×10^4	1.3×10^4	1.3×10^3
2362	4.7×10^4	9.7×10^3	1.2×10^4
1593(pBTC1)	5.8×10^2	1.4×10^4–8×10^4	4.6×10^3
2362(pBT24)	9.5×10^3	ND[b]	ND
2362(pBTC1)	2.7×10^2	1.0×10^4	4.5×10^3
B. thuringiensis subsp. *israelensis*			
4Q2-72	1.2×10^2	2.4×10^5	5.5×10^3

[a] LC_{50}, 50% lethal concentration (expressed as number of cells per ml that killed 50% of mosquito larvae). These numbers represent the average of two independent experiments.
[b] ND, Not determined.

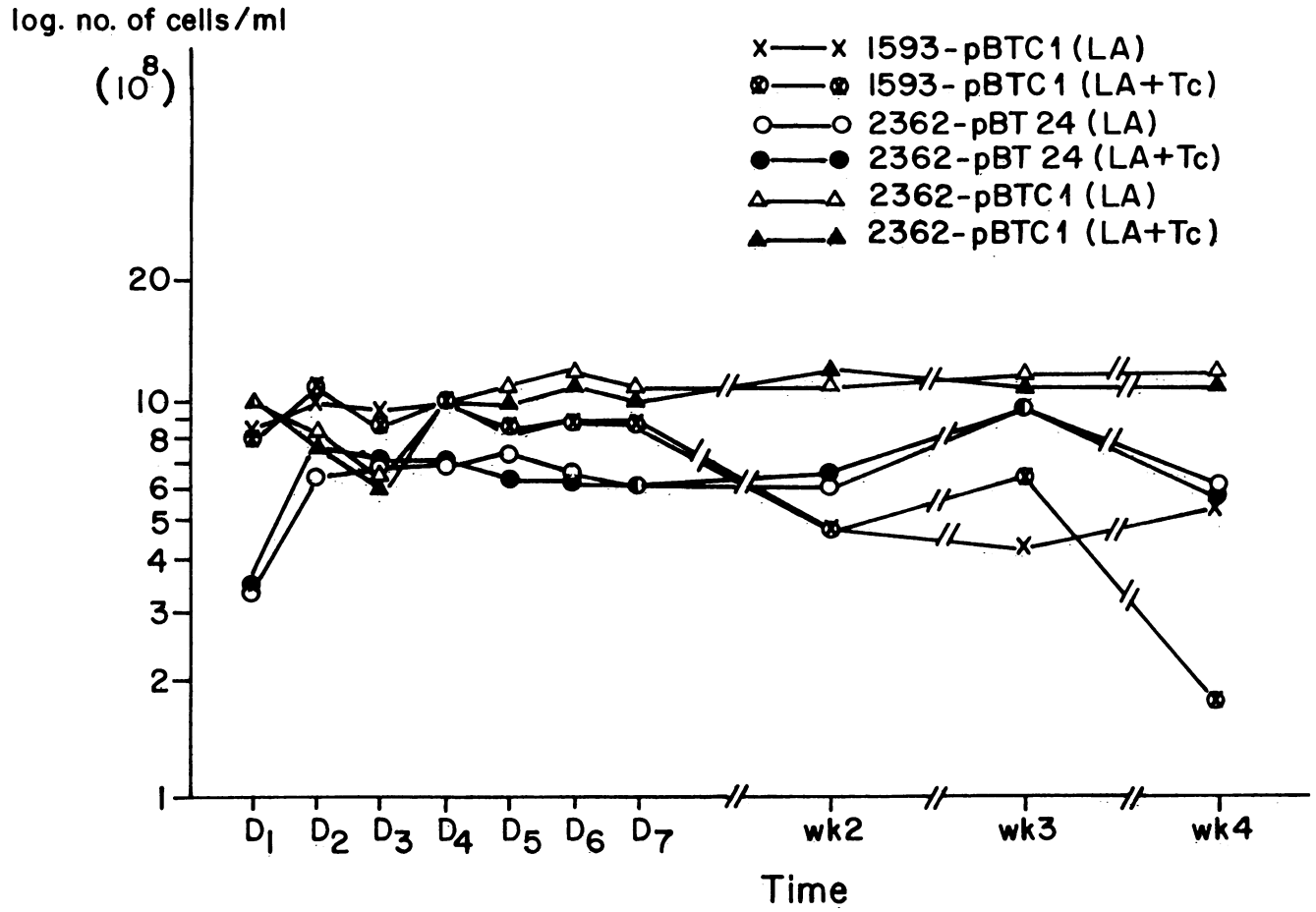

FIG. 6. Stability of recombinant plasmid in *B. sphaericus* 1593 and 2362. Cells were subcultured daily on LB broth without tetracycline (15 μg/ml) and plate counted on LA with and without tetracycline. The numbers of bacteria were determined every day for the first week and once a week for the second, third, and fourth weeks. D, Day.

mants could be obtained in normal DM3 medium. However, only 5 transformants each from *B. sphaericus* 1593 (total, 18 transformants) and 2362 (total, 15 transformants) retained plasmid pBTC1 encoding the intact *B. thuringiensis* subsp. *israelensis* toxin gene (Table 1). The other transformants suffered from DNA deletions which included the *B. thuringiensis* subsp. *israelensis* toxin gene and parts of pBC16. Nevertheless, transformants still harbored tetracycline resistance, as shown by their growth on agar medium supplemented with tetracycline. The deletion might have occurred during the transformation process.

There seemed to be no synergistic effect derived from the presence of *B. thuringiensis* subsp. *israelensis* toxin (from *cryIVB* gene) together with the *B. sphaericus* toxins in a single strain, since the 50% lethal concentrations were no greater than those in the control experiments using *B. thuringiensis* subsp. *israelensis* or *B. sphaericus* alone against *A. aegypti* and *Anopheles* and *Culex* spp. (Table 2).

The data presented above show that the *B. thuringiensis* subsp. *israelensis* toxin gene can be transferred and expressed in either *B. sphaericus* 1593 or 2362. The gene retains its toxicity and stability in the transformants. This information may help in future attempts to construct suitable strains for controlling mosquito vectors.

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