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Conjugal Transfer of a Toxin-Coding Megaplasmid from *Bacillus thuringiensis* subsp. *israelensis* to Mosquitocidal Strains of *Bacillus sphaericus*

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Both *Bacillus sphaericus* and *Bacillus thuringiensis* subsp. *israelensis* produce mosquitocidal toxins during sporulation and are extensively used in the field for control of mosquito populations. All the known toxins of the latter organism are known to be encoded on a large plasmid, pBtoxis. In an attempt to combine the best properties of the two bacteria, an erythromycin resistance-marked pBtoxis plasmid was transferred to *B. sphaericus* by a mating technique. The resulting transconjugant bacteria were significantly more toxic to *Aedes aegypti* mosquitoes and were able to overcome resistance to *B. sphaericus* in a resistant colony of *Culex quinquefasciatus*, apparently due to the production of Cry11A but not Cry4A or Cry4B. The stability of the plasmid in the *B. sphaericus* host was moderate during vegetative growth, but segregational instability was observed, which led to substantial rates of plasmid loss during sporulation.

Mosquitoes are the vectors of many deadly and debilitating human diseases, such as malaria, filariasis, and arbovirus infections, including yellow fever and dengue fever. Control of vector mosquito populations often involves the use of insect-pathogenic bacteria which produce highly potent toxins that act specifically against mosquitoes and simulids and which have no effect on nontarget species. At present, only two types of mosquito-pathogenic bacteria are used in control programs in the field, *Bacillus sphaericus* and *Bacillus thuringiensis* subsp. *israelensis*. Both of these bacteria produce parasporal crystalline inclusion bodies composed of potent mosquito-toxic proteins. The two *Bacillus* species have different properties. *B. thuringiensis* subsp. *israelensis* is toxic as a result of the presence of a 128-kb plasmid, pBtoxis, that carries four *cry* (*cry4Aa*, *cry4Ba*, *cry10Aa*, and *cry11Aa*) and three *cyt* (*cyt1Aa*, *cyt2Ba*, and *cyt1Ca*) toxin genes. The resulting production of an arsenal of toxins appears to be sufficient to prevent the emergence of insect resistance (13). However, *B. thuringiensis* subsp. *israelensis* is more sensitive to UV radiation than *B. sphaericus*, and the duration of effective control is shorter, especially in polluted water (30). In contrast, *B. sphaericus* is less UV sensitive, survives well in polluted water (30, 42), recycles in the environment (11), and is toxic largely by virtue of a chromosomally encoded binary gut toxin (Bin) (4). However, *B. sphaericus* performs poorly against one major vector mosquito, *Aedes aegypti*, and although it is highly active against other important vectors, resistance has been reported in some mosquito species (31). It has been shown that the Cyt1Aa toxin from *B. thuringiensis* subsp. *israelensis* is highly synergistic with the Bin toxin of *B. sphaericus* (37, 38), and introduction of a *cyt1A* gene from another mosquitocidal *B. thuringiensis* strain has been shown to help overcome resistance to *B. sphaericus* (32). Thus, there is significant potential for strain improvement by combination of the additional toxins of *B. thuringiensis* subsp. *israelensis* with the better environmental performance (and extra crystal toxin) of *B. sphaericus*. Several groups have reported success in moving *B. thuringiensis* subsp. *israelensis* toxin genes, one or two at a time, into *B. sphaericus* and thereby enhancing its toxicity (3, 26, 27, 34). However, the use of single genes, while resulting in improved activity, may result in only a slight delay in the development of insect resistance, since Georghiou and Wirth (13) have shown that to prevent resistance to *B. thuringiensis* subsp. *israelensis* toxins, both Cry and Cyt toxins must be expressed. In addition, the strains produced in the previous studies are considered to be both genetically modified and recombinant by virtue of the methods by which the toxin genes were transferred to the *B. sphaericus* host. Optimum toxicity in *B. thuringiensis* subsp. *israelensis* arises from the presence of all of its toxins and is enhanced by the presence of at least two "helper proteins," P19 and P20, which may stabilize the toxins or aid in crystal formation (40). Since all the *B. thuringiensis* subsp. *israelensis* toxins and the two helper proteins are encoded on a single 128-kb plasmid (5), major enhancement of the mosquito-toxic properties of *B. sphaericus* might be attainable by transfer of this plasmid, which would combine the best properties of the two species in a single organism. Transfer of pBtoxis by the classical microbiological mating method could yield transconjugants that would not be considered genetically modified or recombinant. Conjugal transfer of pAMβ1 both within *B. sphaericus* species (7) and between species (23) has been accomplished using filter mating methods. Similarly, conjugation methods for both *B. thuringiensis* subsp. *israelensis* (15, 33) and *B. sphaericus* (7) in liquid media have also been reported, and transfer of a plasmid from *B. thuringiensis* to *B. sphaericus* using such a

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protocol has been achieved (36). In the case of *B. thuringiensis*, it has been demonstrated that in some subspecies the toxin-encoding plasmids themselves are able to conjugate between *B. thuringiensis* strains (14, 35). In this study we obtained proof of the mating transfer strategy concept using a "marked" derivative of pBtoxis, and the results indicated that plasmid transfer to *B. sphaericus* is possible and enhances the toxicity of the resulting transconjugants.

MATERIALS AND METHODS

Bacterial strains. *B. thuringiensis* subsp. *israelensis* was obtained from the commercial product VectoBac 12AS (Valent Biosciences Corporation), *B. thuringiensis* subsp. *israelensis* 4Q7 (also known as 4Q2-81) is a strain cured of all plasmids, and *B. thuringiensis* subsp. *israelensis* 4Q5 (also known as 4Q2-72) is a strain cured of all plasmids except pBtoxis. *B. thuringiensis* subsp. *israelensis* 4Q5::erm is a derivative of 4Q5 in which the *cyt1Aa* gene on pBtoxis was knocked out by in vivo recombination in order to insert the gene encoding erythromycin resistance (8), and it was kindly supplied by A. Delécluse of the Institut Pasteur, Paris, France. *B. sphaericus* strains 1593R$^-$ and 2362R$^-$ were kindly supplied by W. Burke, Arizona State University, and are restriction-negative variants of *B. sphaericus* 1593 and 2362, respectively, which were chosen for use so that transferred plasmid DNA would not be digested in the new *B. sphaericus* hosts. These strains are naturally resistant to chloramphenicol (1).

Transfer of pBtoxis::erm. The transfer strategy which we used relies on microbiological methods of mating rather than genetic engineering techniques and exploits the ability of the natural *B. thuringiensis* subsp. *israelensis* conjugative plasmid pXO16 to assist in the mobilization of other plasmids (17). Triparental mating was carried out using the wild-type VectoBac strain of *B. thuringiensis* subsp. *israelensis* that contains pXO16 to mobilize the pBtoxis::erm plasmid from strain 4Q5::erm. Fresh broth cultures of all strains were incubated at 30°C for 4 to 6 h. For mating, 100 μl of *B. thuringiensis* subsp. *israelensis* was spread onto LB agar plates, followed by 100 μl of the plasmid host *B. thuringiensis* subsp. *israelensis* 4Q5::erm and then 100 μl of the recipient *B. sphaericus* 1593R$^-$ or *B. sphaericus* 2362R$^-$. The plates were incubated overnight at 30°C, and the growth was harvested into 2 ml of quarter-strength Ringer's solution. The suspension was serially diluted in quarter- strength Ringer's solution, and 100 μl was spread onto LB agar containing chloramphenicol (5 μg/ml) and erythromycin (5 μg/ml) to select for *B. sphaericus* that had acquired pBtoxis::erm and then incubated at 30°C for 24 to 48 h. Controls consisting of donors and recipients were plated separately.

PCR with pBtoxis and 16S rRNA genes. To screen for the presence of pBtoxis in potentially transconjugant *B. sphaericus* strains, PCR screening for a number of plasmid genes was carried out. DNA for use in PCRs was isolated using a PUREGENE DNA purification kit for yeast and gram-positive bacteria (GENTRA Systems, Minneapolis, Minn.). Primers for pBtoxis coding sequences, distributed around the plasmid, were used. Primers Dip1A and Dip1B (CAAGCCGCAAATCTTGTGG and ATGGCTTGTTTCGCT ACATC, respectively) and primers Dip2A and Dip2B (GGTGCTTCCTAT TCTTTGGC and TGACCAGGTCCCTTGATTAC, respectively), designed by Carozzi et al. (6), were used to detect the presence of the Cry4A and Cry4B genes, respectively. In addition, the following primers were used: for pBt020, primers GAAGAACAAATTTTAGAGAAAGG and TAGTAGCTA TATTTATTAAATATGG; for pBt054, GCACTAGTACCTACTACATCC and GGTTTTGAATACTGTAAGCACG; for pBt084, TAATTGCCTTAG ATAGAACACC and GAGATTTTGGTTAAGTACTTTCC; for pBt136, TGT TCGAGATTATGGGTTATTTTG and CCATGCAACAGCTTGTGCTTTTA; and for pBt156,AAGAGGCTGATTTATTCGCAGG and GTTAATTTCATC AGGATCACCG. The PCRs were performed with an initial step consisting of 94°C for 5 min, followed by 35 cycles of 1 min of denaturation at 94°C, 1 min of primer annealing at 55°C, and 1 min of extension at 72°C and then a final 10-min step at 72°C. Products were analyzed by gel electrophoresis on a 1% agarose gel and visualized under UV light. Fragments of 16S rRNA genes were amplified using primers 63f (CAGGCCTAACACATGCAAGTC) and 1387r (GGGCGG AGTGTACAAGGC), which were designed by Marchesi et al. (20). The PCR was performed using the following conditions: 95°C for 5 min, followed by 30 cycles of 95°C for 1 min, 55°C for 1 min, and 72°C for 1 min and then a final 10-min extension at 72°C. The resulting amplicon was subjected to direct sequencing using the same primers in order to elucidate the amplified sequence. The origin of the sequence was verified by BLAST analysis (2) and by direct comparison to the equivalent sequences from *B. sphaericus* and *B. thuringiensis*.

Protein fingerprinting and Western blotting. Transconjugants and parental strains were analyzed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and Western blotting. *B. thuringiensis* subsp. *israelensis* strains were grown in a sporulation medium (nutrient broth, 8 g/liter; yeast extract, 1 g/liter; K_2HPO_4, 1 g/liter; $CaCO_3$, 0.1 g/liter; $MgSO_4 \cdot 7H_2O$, 0.1 g/liter; $FeSO_4 \cdot 7H_2O$, 0.01 g/liter; $MnSO_4 \cdot 7H_2O$, 0.01 g/liter; $ZnSO_4 \cdot 7H_2O$, 0.01 g/liter; pH 7.0).

Cultures were grown for 72 h (with >98% sporulation) and harvested, and samples were resuspended in SDS-PAGE loading buffer. Proteins were separated using the method of Laemmli (18) and a 12% acrylamide resolving gel at 200 V for 40 min.

Proteins from gels were transferred electrophoretically onto nitrocellulose membranes using a mini Protean II electrophoresis cell and a mini transblot module (Bio-Rad). Western blotting was carried out as described by Maniatis et al. (19). Cry proteins were detected with rabbit antisera against Cry4A and Cry4B crystal toxins (kindly provided by A. Delécluse, Institut Pasteur, Paris, France) together with an alkaline phosphatase-linked, anti-rabbit secondary antibody, followed by color development using a Bio-Rad AP conjugate substrate kit. Protein bands of interest that were identified in SDS-PAGE gels were blotted onto a polyvinylidene difluoride membrane from equivalent SDS-PAGE gels prepared with Tricine in place of glycine. Following rapid staining and destaining of the membrane, bands of interest were excised for N-terminal protein sequencing (Alta Bioscience, Birmingham, United Kingdom).

Mosquito colonies. The following *Culex quinquefasciatus* and *A. aegypti* colonies used in this work were maintained in the insectarium of the Centro de Pesquisas Aggeu Magalhães-FIOCRUZ, Brazil: (i) CqSF, a susceptible *C. quinquefasciatus* colony; (ii) CqRL1/2362, a colony derived from CqSF and selected with *B. sphaericus* strain 2362 under laboratory conditions, which had a stable and high level (resistance ratio, >162,000-fold) of resistance (22, 24); and (iii) AeLab, a susceptible *A. aegypti* colony. The insects from all colonies were reared at 26 ± 2°C with 70% relative humidity and with a photoperiod consisting of 12 h of light and 12 h of darkness. Larvae were reared in tap water and fed ground cat biscuits. The adults were maintained with a 10% sugar solution, and females were allowed to feed on chickens.

Bioassays. Transconjugant strains were tested for toxicity to *A. aegypti* larvae and larvae from the binary toxin-sensitive and -resistant colonies of *C. quinquefasciatus* described above. Bioassays were performed with early fourth-instar larvae by using a standard method recommended by the World Health Organization (39). For all bioassays, larvae were exposed to serial dilutions of lyophilized spore-crystal powders of strains by placing groups of 20 larvae in 100 ml of distilled water in 125-ml plastic cups at 26 ± 2°C with the desired concentration of *B. sphaericus* spore powder (from cultures grown in NYSM medium [21]). At least five concentrations that resulted in levels of mortality between 2 and 98% were tested, and mortality was recorded after 48 h of exposure. A control group tested with only water was included in each experiment, and the bioassay was repeated two or three times on different days. One drop of larval food was added to each cup. The 50% lethal concentration, expressed in mg per liter, was determined using probit analysis (12) with the software SPSS 8.0.

Plasmid stability. To determine the stability of pBtoxis::erm in *B. sphaericus* vegetative transconjugants, cultures were grown in NYSM broth without antibiotics for 18 h at 30°C. A sample was taken and serially diluted up to a dilution of 10^{-5} in quarter-strength Ringer's solution, and 20-μl drops were plated onto both NYSM agar supplemented with chloramphenicol (10 μg/ml) to enumerate all *B. sphaericus* cells and NYSM agar supplemented with chloramphenicol (10 μg/ml) and erythromycin (1 μg/ml) to enumerate transconjugants. The plates were incubated at 30°C overnight, and then colonies were counted. Prior to plating cultures were checked by phase-contrast microscopy for the absence of spores. Fresh NYSM broth was inoculated using the previous culture, and the process was repeated.

To determine the stability of pBtoxis::erm in *B. sphaericus* transconjugants through sporulation, transconjugant strains were grown until the level of sporulation was at least 90% (4 days) in LB broth and heated at 70°C for 30 min to inactivate any vegetative cells. The cells and spores (1 ml) were centrifuged at 14,000 × *g* for 4 min and resuspended in 1 ml of quarter-strength Ringer's solution. The suspensions were then serially diluted up to a dilution of 10^{-5} in quarter-strength Ringer's solution, and 20-μl drops were plated as described above for analysis of total and transconjugant *B. sphaericus.* Successive subcultures were derived from colonies grown in the absence of antibiotics. Counts were obtained after 24 h of incubation at 30°C. Fresh LB broth was inoculated with 50 μl of an undiluted culture and incubated at 30°C, and the process was repeated serially.

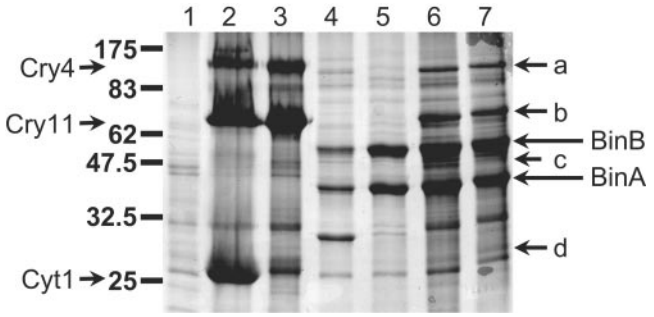

FIG. 1. Protein profiles of lysed sporulated cultures of transconjugant strains. Proteins from *B. thuringiensis* subsp. *israelensis* strains 4Q7 (lane 1), 4Q5 (lane 2), and 4Q5::erm (lane 3), *B. sphaericus* strains 1593R$^-$ (lane 4) and 2362R$^-$ (lane 5), and transconjugants 1593.1 (lane 6) and 2362.5 (lane 7) were examined. The positions of bands for Cry4A and Cry4B (Cry4), Cry11A, Cyt1A, Bin A, and Bin B are indicated, as are the positions of bands that were chosen for N-terminal sequencing, bands a to d; the positions of the molecular weight standards (in kDa) are indicated on the left.

RESULTS AND DISCUSSION

Transconjugant production. Following the on-plate mating described above, a number of colonies were selected on plates containing chloramphenicol (to select for *B. sphaericus* since the parental *B. sphaericus* 1593R$^-$ and 2362R$^-$ strains were chloramphenicol resistant) and erythromycin (to select for the presence of pBtoxis::erm). Two such colonies were selected for further study, and they were designated transconjugant strains 1593.1 and 2362.5. The presence of plasmids in these transconjugants was confirmed by PCR. All pBtoxis primer pairs described above produced amplicons of the expected sizes (results not shown), confirming not only the identity of the plasmid but also, due to the distribution of the primers, that it was likely to be substantially intact. The host bacteria were confirmed to be *B. sphaericus* by their ability to germinate on LB agar plates containing 0.25 M sodium acetate, by their binary toxin production, as judged by SDS-PAGE (see below), and by amplification and sequencing of a portion of the 16S rRNA gene.

Toxin production. Production of the *B. sphaericus* binary toxin and production of the *B. thuringiensis* subsp. *israelensis* Cry toxins were analyzed by SDS-PAGE. Figure 1 shows the protein profiles of lysed sporulated cultures of *B. thuringiensis* subsp. *israelensis* 4Q7, 4Q5, and 4Q::erm, the parental *B. sphaericus* strains, and transconjugants. There are several protein bands in the profiles of the transconjugant strains that are absent from the profiles of the parental *B. sphaericus* strains and appear to match bands in the profile of the *B. thuringiensis* subsp. *israelensis* 4Q5::erm donor. Bands a, b, c, and d in the strain 1593.1 profile (Fig. 1) were chosen for N-terminal sequencing. Band a was present in the profiles of the parental *B. sphaericus* strains but, in Tricine gels used for sequencing, resolved as a doublet with sizes consistent with the sizes of the Cry4Aa and Cry4B toxins (125 kDa and 135 kDa, respectively). However, both bands yielded the same N-terminal sequence, AQLND. No such sequence is encoded by pBtoxis, and the presence of equivalent bands in the parental *B. sphaericus* strains confirmed that this molecule was a host protein. Thus, it appeared that no Cry4 toxins were produced by the transconjugant strains. Band b produced the sequence MEDSS, corresponding to the N terminus of the Cry11Aa toxin, while the faint band c had an ambiguous N-terminal residue that may have been Ala, Met, or Lys, followed by the sequence QEID. The sequence IQEID occurs in the Cry10A sequence encoded by pBtoxis, and Q is residue 128 in this protein. Given the ambiguous first residue of our N-terminal sequence, band c may correspond to a proteolytic product of Cry10A or may be a *B. sphaericus* protein produced under the influence of pBtoxis. This plasmid encodes putative transcriptional regulators (5), some of which have been shown to be transcribed in *B. thuringiensis* subsp. *israelensis* (31a). Band d was another faint band and produced the sequence ALNAQ, corresponding to the sequence encoded by coding sequence Bt075 in the pBtoxis sequence (5) but lacking its initiator methionine residue. This sequence was annotated as a hypothetical protein (with a low level of similarity to a hypothetical protein from *Yersinia pestis*), and this is the first demonstration that this or any other pBtoxis protein not linked to toxicity (i.e., toxin proteins or the P19 and P20 accessory proteins) is actually produced. As indicated above, protein bands (e.g., band a in Fig. 1) in transconjugant strains whose sizes were consistent with the sizes of Cry4A and Cry4B were actually unrelated to these proteins. Western blotting using antisera raised against these proteins also failed to detect Cry4 toxins in transconjugant strains (results not shown). This apparent failure of the transconjugants to express the Cry4A toxin reflects the poor synthesis of this protein when it is encoded on a low-copy-number plasmid in a crystal-minus strain of *B. thuringiensis* subsp. *israelensis* (9). In their study, Delécluse et al. demonstrated that this was not due to low levels of transcription from the Cry4A promoter. In the same system, however, Cry4B did accumulate in *B. thuringiensis* subsp. *israelensis* to high levels (9), in contrast to the results presented here for the *B. sphaericus* transconjugants. Previous studies in which recombinant approaches were used to transfer the *cry4B* or *cry11A* gene to *B. sphaericus* showed that there was expression of both proteins in the recombinant strains (27, 34). However, Cry4B was reported to account for less than 2% of the total protein, while the production of Cry11A could not be assessed since it was reported only from Western blotting experiments (27). Cry11A production was significant, however, when the gene was integrated into the *B. sphaericus* genome by homologous recombination (26). The toxicity of Cry11A is intermediate between the toxicity of Cry4A and the toxicity of Cry4B against both *A. aegypti* and *Anopheles stephensi* (28), but a *cry11A* knockout strain seemed to indicate that it has a particular role in increasing the toxicity of *B. thuringiensis* subsp. *israelensis* against *Culex pipiens* and *A. stephensi* (25). The *cry4A*, *cry4B*, and *cry11A* genes are all transcriptionally controlled by σ^E, while *cry4A* and *cry11A* are additionally controlled by σ^H and σ^K, respectively (10, 41). Perhaps the availability of related transcription factors in *B. sphaericus* influences the different levels of transcription of these genes in *B. sphaericus* compared to *B. thuringiensis* subsp. *israelensis.* Plasmid pBtoxis itself carries a coding sequence (Bt108) that appears to be related to σ^E (5), but its role, if any, in toxin production is still unclear. While the level of production of the Cry toxins appears to be substantially lower in the transconjugants than in *B. thuringiensis* subsp. *israelensis*, the presence of pBtoxis apparently does not decrease the levels of Bin toxin that accumulate in the transconjugants.

TABLE 1. Larvicidal activity of *B. sphaericus* strains against *C. quinquefasciatus* larvae from a susceptible colony (CqSF) and from a colony resistant to *B. sphaericus* 2362 (CqRL1/2362) and against larvae from an *A. aegypti* colony (AeLab) after 48 h of exposure

Colony	Expt	50% lethal concn (mg/liter)[a]	
		B. sphaericus 2362R$^-$	*B. sphaericus* 2362.5
CqSF	1	0.013 (0.0045–0.019)[b]	0.012 (0.006–0.022)
	2	0.011 (0.0041–0.031)	0.014 (0.011–0.019)
	3	0.009 (0.0039–0.016)	
	Mean	0.011 (0.0042–0.022)	0.013 (0.008–0.021)
CqRL1/2362	1	>270	0.49 (0.36–0.75)
	2		0.41 (0.27–0.65)
	Mean		0.45 (0.32–0.70)
AeLab	1	>10	1.3 (1.1–1.6)
	2		0.70 (0.51–0.90)
	3		0.47 (0.32–0.61)
	Mean		0.83 (0.63–1.1)

[a] The 50% lethal concentrations were calculated using the software SPSS 8.0 for Windows.
[b] The values in parentheses are limits.

Toxicity. Transconjugant strain 2362.5 and its parent strain, 2362R$^-$, were bioassayed with susceptible (CqSF) and resistant (CqRL1/2362) laboratory colonies of *C. quinquefasciatus* and with *A. aegypti* (Table 1). There was not a significant difference in toxicity for the susceptible *C. quinquefasciatus* strain, but this could have been expected since *B. sphaericus* 2362R$^-$ is highly toxic to this strain, through the activity of the Bin toxin. Previous studies in which the *cry11A* gene was introduced into the chromosome of *B. sphaericus* strain 2297 showed that there were no (29) or slight (26) increases in toxicity. In both studies, the 2297(::*cry11A*) strains exhibited greatly enhanced toxicity (>100-fold) for *A. aegypti* (which naturally has a low level of susceptibility to *B. sphaericus*). For transconjugant 2362.5 there was also a clear increase in toxicity for this insect. This effect was probably due to the production of Cry11A by the transconjugant since in *B. thuringiensis* subsp. *israelensis* this protein is known to contribute significant mosquitocidal activity against both *A. aegypti* and *C. quinquefasciatus* (25). This effect against *C. quinquefasciatus* was seen most clearly with the Bin-resistant colony, which was much more sensitive to transconjugant 2362.5 than to the parental strain 2362R$^-$, confirming previous studies (29) and indicating that expression of Cry11A in *B. sphaericus* is a viable strategy for combating mosquito resistance.

Plasmid maintenance. Transconjugants of strains 1593R$^-$ and 2362R$^-$ containing pBtoxis::erm were tested to determine the stability of the introduced plasmid by growing them in the presence and absence of selective antibiotics. When strain 2362.5 was used, the pBtoxis plasmid seemed to be relatively stably maintained in vegetative culture without selection (Table 2). However, in spores the plasmid was apparently substantially lost during the initial subculture but appeared to be retained in later rounds (Table 2); thus, with some strain development it might be possible to select strains with more stable maintenance of the plasmid.

Further investigation of plasmid stability in the other transconjugant (1593.1) during successive rounds of subculture overnight with no antibiotic selection showed that there was a general trend toward rapid loss of erythromycin resistance, presumably indicating that there was a loss of pBtoxis in vegetative cells. Overnight cultures grown in the presence of antibiotics to select for pBtoxis::erm generally behaved as expected; i.e., they retained a high level of antibiotic resistance, although for strain 1593.1 less than 1% of the cells seemed to retain erythromycin resistance compared to the total counts.

The variability of the retention of antibiotic resistance and, by implication, plasmid stability suggests that pBtoxis is not just segregationally unstable but also genetically unstable. A further study of genetic changes that may have occurred in pBtoxis in various transconjugants may be required. At a practical level, if such strains are to be used for mosquito control in jurisdictions where genetically manipulated organisms are accepted, it would be best if relatively stable strains could be used and if antibiotic selection could be retained for all stages prior to inoculation of the production culture. With wild-type, unmarked plasmids, monitoring for the presence of plasmids at all stages may be necessary.

B. thuringiensis subsp. *israelensis* plasmid pXO16 has been shown previously to be capable of mobilizing small plasmids that lack both the *mob* gene and the *oriT* site (16). In this work, we showed for the first time that very large plasmids, such as pBtoxis::erm (128 kb), can be mobilized for transfer to distantly related bacteria by using this system. Despite plasmid stability issues, this work provides the first demonstration that toxin-coding plasmids are able to replicate and can be sustained for any period outside the *Bacillus cereus* group of

TABLE 2. Vegetative and spore counts obtained as described in Materials and Methods using *B. sphaericus* transconjugant strain 2362.5

Cells	Subculture	Total cell count (CFU ml^{-1})[a]	Transconjugant cell count (CFU ml^{-1})[a]	% Cells retaining pBtoxis
Vegetative	First	$2.9 \times 10^8 \pm 3.1 \times 10^7$	$3.2 \times 10^8 \pm 2.0 \times 10^7$	111.4
	Second	$5.9 \times 10^9 \pm 5.0 \times 10^8$	$5.9 \times 10^9 \pm 4.1 \times 10^8$	100
	Third	$2.3 \times 10^8 \pm 3.1 \times 10^7$	$2.5 \times 10^8 \pm 2.7 \times 10^7$	106.9
	Fourth	$3.0 \times 10^8 \pm 2.1 \times 10^7$	$3.1 \times 10^8 \pm 2.9 \times 10^7$	103.0
Spores	First	$1.7 \times 10^8 \pm 2.5 \times 10^7$	$8.3 \times 10^7 \pm 2.3 \times 10^6$	48.6
	Second	$1.4 \times 10^8 \pm 1.3 \times 10^7$	$9.7 \times 10^7 \pm 1.3 \times 10^7$	71.1
	Third	$2.1 \times 10^8 \pm 2.2 \times 10^7$	$1.2 \times 10^8 \pm 1.0 \times 10^7$	50.1
	Fourth	$8.5 \times 10^7 \pm 1.9 \times 10^7$	$1.4 \times 10^8 \pm 2.8 \times 10^7$	16.2
	Fifth	$1.4 \times 10^8 \pm 1.8 \times 10^7$	$2.8 \times 10^6 \pm 7.6 \times 10^5$	2.0

[a] The values are means ± standard deviations ($n = 6$).

bacteria (which includes *B. thuringiensis*). This offers the potential to transfer the toxin-coding plasmids to other species that may have useful environmental properties for enhanced insect control. The existence of Cry toxins in non-*Bacillus* hosts, such as *Clostridium bifermentans* subsp. *malaysia* (25), could perhaps be linked to the natural transfer of such a plasmid during the evolution of the strain. The production of the *B. sphaericus* transconjugants without in vitro intervention using only microbiological mating techniques means that resulting strains that were not marked with antibiotic resistance genes would not be classified as "genetically modified" or "recombinant." This is likely to be very important in regulatory terms in many countries, if a commercializable strain is developed.

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