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The construction of *Bacillus thuringiensis* strains expressing novel entomocidal δ-endotoxin combinations

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Using our recently reported method of electroporation to transform *Bacillus thuringiensis* [Bone & Ellar (1989) FEMS Microbiol. Lett. **58**, 171–178], cloned *B. thuringiensis* entomocidal δ-endotoxin genes have been introduced into several native *B. thuringiensis* strains. In many cases the resulting transformants expressed both their native toxins and the cloned toxin, producing strains with broader toxicity spectra. The introduction of the var. *tenebrionis* toxin gene into *B. thuringiensis* var. *israelensis* resulted in a strain with activity against *Pieris brassicae* (cabbage white butterfly), an activity which neither parent strain possesses. We discuss further the possibility of synergism and also the problems associated with introducing cloned DNA by this method.

INTRODUCTION

During sporulation the Gram-positive bacterium *Bacillus thuringiensis* synthesizes cytoplasmic crystalline inclusions composed of one or more entomocidal proteins (δ-endotoxins; Sommerville, 1978; Bulla *et al.*, 1980). The numerous strains of *B. thuringiensis* have been grouped into 34 serotypes on the basis of flagella antigens. Individual subspecies produce a range of different toxins with differing insect specificities and so can also be grouped into different pathotypes on the basis of their insect targets (Krieg *et al.*, 1983; Ellar *et al.*, 1986). Strains within a given pathotype often synthesize a characteristic pattern of polypeptides in the native crystal; those that are toxic to insects of the order Lepidoptera, for example, generally contain one or more 130 kDa toxins originally designated 'P1' (Yamamoto & McLaughlin, 1981) and now termed 'CryI', according to the classification scheme recently proposed by Hofte & Whitley (1989). Many strains contain more than one toxin in their crystal: *B. thuringiensis* var. *israelensis*, for example, contains polypeptides of 135, 130, 65 and 27 kDa as well as other minor species (Huber & Luthy, 1981; Thomas & Ellar, 1983). *B. thuringiensis* var. *israelensis* is specifically toxic to insects of the order Diptera, but it is unclear what role the individual polypeptides play in the overall toxicity. The genes for the individual polypeptides have been cloned and expressed in *Escherichia coli* (Ward *et al.*, 1984; Waalwijk *et al.*, 1985; Angsuthanasombat *et al.*, 1987; Donovan *et al.*, 1988; Ward & Ellar, 1988), and in all cases the individual polypeptides were toxic. There is also evidence of synergism between the individual polypeptides (Wu & Chang, 1985; Chilcott & Ellar, 1988).

The lack of an efficient transformation system for *B. thuringiensis* has hampered a more thorough study of expression and possible interaction between toxins within a crystal. Various methods have been reported in which toxin genes have been transferred between *B. thuringiensis* strains. Several workers made use of the observation that certain *B. thuringiensis* plasmids could be transferred between two strains grown in mixed culture (Gonzalez & Carlton, 1982). Gonzalez *et al.* (1982) reported the transfer of a toxin-encoding plasmid from a *B. thuringiensis* var. *kurstaki* strain to an acrystalliferous mutant of *B. thuringiensis* var. *thuringiensis*. Klier *et al.* (1983) later showed that a cloned toxin gene from *B. thuringiensis* var. *berliner*, transformed into *B. subtilis*, could be transferred to *B. thuringiensis* var. *israelensis* or to an acrystalliferous mutant of *B. thuringiensis* var. *kurstaki*. Attempts to transform *B. thuringiensis* have generally relied on the difficult and time-consuming method of protoplast formation and regeneration (Martin *et al.*, 1981; Miteva *et al.*, 1981; Fischer *et al.*, 1984; Rubinstein & Sanchez-Rivas, 1988). Heierson *et al.* (1987) developed a novel transformation procedure in which *B. thuringiensis*, grown in a rich medium, is made competent by treatment with a buffered 30%-(w/v)-sucrose solution. Bourgoin (1988) reported using this method to transform an acrystalliferous mutant of *B. thuringiensis* var. *israelensis* with a cloned toxin gene from *B. sphaericus*. We recently described the use of electroporation to transform *B. thuringiensis* (Bone & Ellar, 1989). This simple technique has allowed the transformation of a range of *B. thuringiensis* subspecies at frequencies up to 10^5 transformants/μg of DNA.

In the present paper we describe the transformation of several native *B. thuringiensis* strains with different cloned δ-endotoxins, producing a set of *B. thuringiensis* strains expressing novel δ-endotoxin combinations.

EXPERIMENTAL

Bacterial strains and growth conditions

The *E. coli* strain TG1 was used for all cloning experiments. *B. thuringiensis* strains var. *kurstaki* HD-1, var. *israelensis* IPS78, var. *tenebrionis* 1911, var. *sotto* SN913 and var. *aizawai* IC1 have been described previously (Ward *et al.*, 1984; Haider & Ellar, 1987; Carroll *et al.*, 1989; Ahmad & Ellar, 1990). *B. thuringiensis* strains were grown at 30 °C in LB (Luria–Bertani medium; Maniatis *et al.*, 1982) for transformation or plasmid preparation; and PWYE/CCY (peptone/water/yeast extract–casein/casein/yeast medium; Stewart *et al.*, 1981) for immunoblots or toxicity assays. All *E. coli* strains were grown at 37 °C in Luria–Bertani medium. The antibiotics chloramphenicol (5 μg/ml) and ampicillin (100 μg/ml) were added as necessary.

Transformation of *B. thuringiensis*

Transformation of *B. thuringiensis* was performed by electroporation as previously described (Bone & Ellar, 1989). Routinely, several loopfulls of an overnight culture on a Luria–Bertani plate were washed, and finally resuspended, in 800 μl of sucrose/phosphate buffer [272 mM-sucrose/7 mM-sodium phosphate buffer (pH 7.4)/1 mM-$MgCl_2$]. The cells were added to the DNA to be transformed (5 μl) in a 4 mm electroporation cuvette and given a single pulse at 25 μF and 2 kV (5000 V/cm). The electroporated cells were then added to 5 ml of Luria–Bertani medium and left for 1 h at 30 °C, after which they were plated on to selective media. Transformants appeared after overnight incubation at 30 °C.

DNA manipulation

Plasmid DNA was routinely prepared from both *B. thuringiensis* and *E. coli* by the alkaline-lysis method (Birnboim & Doly, 1979). All ligations, restriction–enzyme analyses and transformations of *E. coli* were performed as described by Maniatis *et al.* (1982).

PAGE and immunoblotting

SDS/PAGE was carried out by the method of Thomas & Ellar (1983). Transfer of proteins to nitrocellulose filters (Schleicher and Schuell) and immunoblotting was as described by Towbin *et al.* (1979). Horseradish-peroxidase-conjugated goat anti-rabbit immunoglobin was used to detect bound antibodies (Hawkes *et al.*, 1982). Antisera against *B. thuringiensis* var. *israelensis* 27 kDa toxin, var. *kurstaki* 130 kDa toxin and var. *tenebrionis* 73 kDa toxin were kindly provided by Dr. E. S. Ward, Dr. B. H. Knowles and Mr. T. Sawyer respectively of the Department of Biochemistry, University of Cambridge.

Toxicity assays

Synchronous cultures of the *B. thuringiensis* strains were obtained by inoculating heat-treated spores (70 °C, 30 min) into PWYE medium (Stewart *et al.*, 1981), incubating overnight, and then diluting into CCY medium (Stewart *et al.*, 1981). Synchronous cultures (1 dm^3) of the transformed and native strains were grown at 30 °C until 95% of the culture had lysed. The spore/crystal mix was harvested, washed in deionized water, and resuspended in 15 ml of 50 mM-Tris/HCl/10 mM-KCl, pH 7.5. For toxicity assays against *Pieris brassicae*, 10 μl of the spore/crystal mix was diluted to 200 μl in deionized water and spotted on to two 2 cm-diameter discs of cabbage leaf, prewashed in 0.02% Triton X-100. To these treated leaf discs five third-instar larvae were added and left at room temperature for 48 h, at which time mortality of the larvae and amount of cabbage leaf consumed were ascertained. For assay of toxicity towards the beetle *Phaedon cochleariae*, a 2 cm disc of turnip leaf was coated with 10 μl, diluted to 50 μl in water, of the spore/crystal mix and placed in a Petri dish containing moist Whatman 3MM paper. Eight first-instar *Phaedon* larvae were then added to the leaf. After 48 h incubation at room temperature, a second toxin-coated leaf was added. The amount of leaf consumed and the mortality of the larvae were scored after a further 72 h incubation. For toxicity assays against larvae of the mosquito *Aedes aegypti*, 10 μl of spore/crystal mix was diluted to 500 μl in tap water and 25 third-instar larvae added. Percentage mortality was ascertained over 24 h.

RESULTS

Cloning of the *B. thuringiensis* var. *tenebrionis* toxin gene

Total plasmid DNA from *B. thuringiensis* var. *tenebrionis*, prepared by the method of Gonzalez & Carlton (1980), was restricted with endonuclease *Hin*dIII and ligated into pUC18. The resulting library was probed with an oligonucleotide (5′TCTTGCGGTCTGGCCGTCCGCTGTA3′) of sequence identical with a section of the δ-endotoxin gene (Hofte *et al.*, 1987). One clone that gave a positive hybridization signal was restriction-mapped and found to be identical with published maps of the toxin gene (Herrnstadt *et al.*, 1987; Sekar *et al.*, 1987; McPherson *et al.*, 1988). An immunoblot of a lysate of an *E. coli* TG1 strain containing the clone showed a band of about 70 kDa that cross-reacted with antibodies to the toxin (results not shown).

Construction of *E. coli–B. thuringiensis* shuttle vectors

We have previously shown that the *Staphylococcus aureus* plasmid pC194 can be readily transformed into *B. thuringiensis* (Bone & Ellar, 1989) and that it replicates stably within this host. Table 1 shows a range of shuttle vectors that have been constructed between pC194 and the *E. coli* pUC vectors or pBR322. All of these vectors were stably maintained in *B. thuringiensis* in the presence of chloramphenicol (5 μg/ml).

Subcloning *B. thuringiensis* toxin genes into shuttle vectors

Table 1 also shows the various *B. thuringiensis* toxin genes, subcloned into the shuttle vectors described above, that were used in the present study. The plasmid camsot contains the *B. thuringiensis* var. *sotto cryIA*(a) toxin gene (Shibano *et al.*, 1985) on a *Bam*H1–partial *Kpn*1 fragment subcloned from pSE2 (Haider *et al.*, 1987) into compuc19. Camaiz contains a 130 kDa dual-specificity toxin gene from *B. thuringiensis* var. *aizawai* IC1 (Haider *et al.*, 1989) subcloned on a *Bam*H1–*Pst*1 fragment into campuc12. The plasmid camten contains the var. *tenebrionis* toxin-encoding *Hin*dIII fragment subcloned into pAK1205; pSVten contains the same fragment subcloned into pSV1.

Introduction of the cloned toxin genes into native *B. thuringiensis* strains

The *B. thuringiensis* strains var. *kurstaki*, var. *israelensis* and var. *tenebrionis* were transformed with the toxin-encoding plasmids listed in Table 1. Transformants were selected by their resistance to (5 μg/ml) chloramphenicol. In order to confirm structural stability of the introduced plasmid, the transformants were grown in Luria–Bertani medium to mid-exponential phase and the plasmid DNA was isolated. The introduced plasmid was separated from the native plasmids by transforming the mixed plasmid population into *E. coli* and selecting for ampicillin resistance (encoded by the shuttle vector). The structure of the plasmid was then confirmed by restriction mapping. In many cases there had been substantial rearrangement of the introduced plasmids. For example, the plasmid camten was unstable in all *B. thuringiensis* hosts used. Differences between host strains were also observed; var. *israelensis* was generally a better host than

Table 1. Construction of vectors

Vector	Method of Construction	Reference
Campuc12	pC194 and pUC12 joined at *Hin*dIII sites	Ward *et al.* (1986)
Campuc19	pC194 and pUC19 joined at *Hin*dIII sites	The present study
pAK1205	pC194 *Hin*dIII cut and end-repaired, ligated into *Sma*I site of pUC18	Ahmad *et al.* (1989)
pSV1	Large *Hin*dIII–*Pvu*II fragment of pC194, ligated into *Hin*dIII–*Pvu*II-cut pBR322	
Camsot	*cryIA*(a) toxin gene from var. *sotto* cloned in campuc19	The present study
Camaiz	*cryIA*(b) toxin gene from var. *aizawai* IC1 cloned in campuc 12	
Camten	*cryIIIA* toxin gene from var. *tenebrionis* cloned in pAK1205	
pSVten	*cryIIIA* toxin gene from var. *tenebrionis* cloned in pSV1	

Table 2. Toxin expression

Immunoreactivity is graded as follows: ++, strong reaction; +, weak reaction; −, no reaction. Toxicity is graded as follows:

Pieris	++	0–5% of leaf consumed; 50–100% mortality
	+	5–50% of leaf consumed
	−	> 50% of leaf consumed
Aedes	++	100% mortality within 1 h
	+	50–100% mortality within 24 h
	−	No mortality within 24 h
Phaedon	++	0–50% of leaf consumed; > 25% mortality
	+	50–80% of leaf consumed
	−	80–100% of leaf consumed

Strain of *B. thuringiensis*	Immunoreactivity			Toxicity		
	P1	27 kDa	73 kDa	*Pieris*	*Aedes*	*Phaedon*
Var. *sotto* (native)	++	−	−	++	−	−
Var. *aizawai* IC1 (native)	++	−	−	++	+	−
Var. *israelensis*						
Native	−	++	−	−	++	−
Camsot	++	++	−	++	++	−
Camaiz	++	++	−	++	++	−
pSVten	−	++	++	+	++	++
Var. *kurstaki*						
Native	++	−	−	++	+	−
pSVten	++	−	++	++	+	++
Var. *tenebrionis*						
Native	−	−	++	−	−	++
Camaiz	++	−	+	++	+	+

either var. *tenebrionis* or var. *kurstaki*. Table 2 lists some cases in which stable transformants were obtained and shows the results of a series of immunoblotting experiments designed to investigate the expression of both the introduced toxin gene and of the native toxin genes. A spore/crystal mix of the transformant, grown in CCY medium, was precipitated at 4 °C with 12.5% (w/v) trichloroacetic acid, run on an SDS/PAGE gel, transferred to a nitrocellulose filter and blotted with antibodies raised against purified toxins. In each case it can be seen that the introduced toxin is efficiently expressed; however, in the case of var. *tenebrionis* (camaiz), expression of the native toxin was significantly affected.

Insecticidal activities of the transformants

The toxicities of the transformants, and of the parent strains, against *Pieris brassicae*, *Aedes aegypti* and *Phaedon cochleariae* were determined as described in the Experimental section. The results are summarized in Table 2. In all cases the toxicity spectrum of the parent strain was maintained, although in the case of var. *tenebrionis* (camaiz) the toxicity of the transformant against *Phaedon* was markedly reduced compared with the native var. *tenebrionis* strain. This correlates well with the reduced expression of the 73 kDa toxin described in the previous section. The introduction of the var. *sotto* CryIA(a) toxin or the var. *aizawai* IC1 dual-specificity toxin into var. *israelensis* conferred lepidopteran toxicity on the strain, which was otherwise absent. Under the conditions of the assay, no change could be seen in the activity of var. *israelensis* against *Aedes* upon introduction of the dual-specificity *aizawai* toxin. Introduction of the var. *tenebrionis* toxin gene conferred coleopteran toxicity on both var. *israelensis* and var. *kurstaki* without obviously diminishing their respective toxicities towards *Aedes* and *Pieris*. Interestingly though, the var. *israelensis* (pSVten) transformant resulted in a significant feeding inhibition of *Pieris* larvae, which was not observed with either native var. *israelensis*, native var. *tenebrionis* or an equal mixture of the two.

DISCUSSION

In the present paper we have described the use of shuttle vectors to introduce cloned *B. thuringiensis* toxin genes back into various *B. thuringiensis* strains. A major problem with this approach has been structural instability of the plasmid within *B. thuringiensis*. Such problems are well known in *B. subtilis*, where deletions are known to occur between regions of direct or indirect repeats (Peeters *et al.*, 1988); other deletions are known to be associated with the formation of single-stranded intermediates during plasmid replication (Ballester *et al.*, 1989). Furthermore, the end points of these various deletions often appear randomly distributed (Peijnenburg *et al.*, 1988). We have recently been investigating the structural stability of various plasmids in *B. thuringiensis* in an attempt to define regions of DNA that might be responsible for this instability. The clone camten, in which the *tenebrionis* toxin gene was subcloned on to a shuttle vector containing pUC18 and pC194, proved to be unstable in all *B. thuringiensis* hosts used. However, when the same gene-encoding fragment was subcloned into a different shuttle vector (pSV1, containing pBR322 and pC194), the resulting plasmid, pSVten, was stably maintained, Thus we believe that sequences unique to camten may be the cause of the instability problem. We have also found that some plasmids are stable in some *B. thuringiensis* strains, but not in others; camsot was stably maintained in var. *israelensis*, for example, but was unstable in var. *tenebrionis*. Stable transformants in var. *tenebrionis* and var. *kurstaki* were difficult to isolate; the var. *tenebrionis* (camaiz) transformant discussed here represents the only 130 kDa-expressing clone we isolated. Although the restriction map of the recovered plasmid seemed identical with the original construct, it remains a possibility that it may have undergone slight rearrangement. An alternative possibility is that the host strain may have acquired some mutation, preventing deletion of the plasmid.

With the exception of var. *tenebrionis* (camaiz), introduction of the cloned toxin gene did not seem to affect expression of the native toxins. It is believed that several of the toxin genes, including the var. *sotto* 130 kDa and the var. *israelensis* 27 kDa genes, are all transcribed from a homologous promoter recognized by a particular sigma factor (Brown & Whiteley, 1988). If the amount of sigma factor is limiting for toxin expression, then one might expect that the introduction of that promoter on a multicopy plasmid would titrate out the sigma factor, resulting in reduced expression of the native genes. Such an effect has been observed in *B. subtilis*, where the introduction of a *spo*VG promoter on a multicopy plasmid reduces expression of the chromosomal *spo*VG gene (Banner *et al.*, 1983). The introduction of camsot into *B. thuringiensis* var. *israelensis*, however, did not seem to reduce expression of any of the native toxins, including the 27 kDa gene, which is believed to share the same promoter. Thus, although it seems probable that toxin-gene expression is activated by the appearance of a novel sigma factor, continued expression does not appear to be limited by the amount of sigma factor present.

Several workers have reported the possibility of synergism between individual toxins within a crystal (Wu & Chang, 1985; Chilcott & Ellar, 1988). The mechanism of this synergistic action remains unknown. The expression of novel δ-endotoxin combinations within a strain is an effective strategy for investigating possible synergisms. We have found two possible cases where such an effect may exist; the transformant var. *tenebrionis* (camaiz) was more toxic towards *Aedes* larvae than either native var. *tenebrionis* or var. *aizawai*. Some activity against *Aedes* might have been expected, since the *aizawai* gene product has been reported to show dual specificity towards both lepidopteran and dipteran species (Haider *et al.*, 1989). When, however, this gene was expressed in an acrystalliferous mutant of var. *israelensis*, bipyramidal crystals were produced that were toxic to *Pieris*, but showed no activity towards *Aedes*. The other possible case of synergism was the feeding inhibitory action of var. *israelensis* (pSVten) on *Pieris* larvae. The possibility exists, then, that novel combinations of δ-endotoxins might result in activities against insects not affected by the individual components. A report by Karamata & Piot (1989) draws similar conclusions. These workers introduced, by conjugation, the toxin gene from HD73 into var. *tenebrionis*. They reported that hybrid strains, as well as being toxic to both lepidopteran (*Trichoplusia ni*, the cabbage looper) and coleopteran (*Phaedon cochleariae*) species were also toxic to *Spodoptera littoralis* (Egyptian cotton leafworm). Neither the parent strains, nor a mixture of the two, were active against this *Spodoptera* species. We have retested these strains and have found, initially, that they appear to be unstable, readily losing the plasmid containing the HD73 toxin gene. However, when a spore/crystal mixture was prepared from isolates still containing both genes, we were unable to detect any activity against *Spodoptera littoralis*.

In order to confirm our initial observations indicating that the var. *israelensis* (pSVten) construct had toxicity against *Pieris*, the assays were repeated using purified inclusions. The results confirmed our initial findings. However, a new isolate of var. *israelensis* (pSVten) was also tested and found to have no activity towards *Pieris*. The possibility of contamination of the original isolate was eliminated when immunoblots of the purified inclusions with antibodies against CryI and CryII proteins proved negative. Further experiments showed that when either the original var. *israelensis* (pSVten) or var. *tenebrionis* (camaiz) transformants were regrown to provide more spore/crystal mix, their respective activities towards *Pieris* and *Aedes* had disappeared. We can only conclude that the observed novel activities are transiently expressed, and this could also be true of the var. *tenebrionis*-HD73 transconjugants.

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