J. Biosci., Vol, 19, Number 2, June 1994, pp 145–153. © Printed in India.

Expression of parasporal crystal protein (δ−endotoxin) gene(s) of *Bacillus thuringiensis* var. *israelensis* in sporogenic and asporogenic mutant strains of *Bacillus cereus**

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MS received 6 October 1993; revised 27 January 1994

Abstract. The expression of parasporal crystal protein (δ-endotoxin) coding gene(s) of *Bacillus thuringlensis* var. *israelensis* and its association, if any, with sporulation was studied in sporogenic *Bacillus cereus* and its asporogenic mutant strains. Five asporogenous mutants of *Bacillus cereus* blocked at different stages of sporulation, were isolated from a streptomycin-resistant strain, The transconjugants isolated from the plasmid transfer experiments between *Bacillus thuringiensis* var. *israelensis* and streptomycin resistant *Bacillus cereus* and its asporogenous mutants, showed larvicidal activity. The crystal protein gene(s) are, therefore, expressed both in sporulating and in non-sporulating mutant strains of *Bacillus cereus* suggesting that the expression of crystal protein gene(s), is independent of sporulation specific functions in *Bacillus cereus*.

Keywords. *Bacillus thuringiensis; Bacillus cereus;* sporulation; δ-endotoxin; conjugation.

1. Introduction

The gram-positive spore-forming bacterium *Bacillus thuringiensis* (*B t*) produces parasporal proteinaceous insecticidal crystal protein (δ-endotoxin) toxic against larvae of a number of lepidopterans and dipterans (Burges 1982). *B t* var. *israelensis* (*B t i*) produces crystal protein toxic against mosquito and blackfly larvae (de Barjack 1978; Gonzalez *et al* 1982). Several investigators studied the relationship between crystal protein formation and sporulation (Monro 1961; Fitz–James 1962; Delafield *et al* 1968; Somerville 1968; Yousten and Rogoff 1969; Lecadet and Dedonder 1971; Bechtel and Bulla 1976); and suggested a close association between these two processes. However, contradictory reports documented non-synchronous formation of crystal protein and spore (Arscaldins 1969; Meenakshi and Jayaraman 1979). Isolation of Spo^{-} Cry^{+} (asporogenous crystalliferous) mutants blocked at early stage of sporulation and capable of producing normal crystal protein also suggests that crystal protein synthesis can occur in the absence of sporulation (Lecadet and Dedonder 1971; Somerville 1971, 1978).

Most of the *B t* crystal protein genes reside on plasmids although chromosomal location of the gene(s) have also been reported (Carlton and Gonzalez 1985). Different species and subspecies of *B t* harbour a number of plasmids, though all

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of them do not code for crystal protein (Aronson *et al* 1986) which is composed of multiple polypeptide components in *B t i* (Chilcott and Ellar 1988).

Crystal protein gene(s) of *B t i* have been cloned and expressed in *Escherichia coli* and the extracts of *E. coli* cells harbouring the recombinant crystal genes have been shown to be toxic to mosquito larvae (Waalwijck *et al* 1985; Ward and Ellar 986; Mclean and Whitely 1987; Adams *et al* 1989). Similar observations have been made for other *B t* strains (Schnepf and Whitely 1981; Wong *et al* 1983). *B t* crystal protein genes were also expressed in vegetative cells and in non-sporulating cells of *B. subtilis* (Shivakumar *et al* 1986), *B. megaterium* (Shivakumar *et al* 1989) and *Pseudomonas fluorescens* (Obukowicz *et al* 1986). Expression of plasmid borne δ-endotoxin gene of *B t i* were also observed in both wild type sporogenic and mutant asporogenic strains blocked at very early stage of sporulation of *B, subtilis* (Ward *et al* 1986). However, cry D and cry E genes of *B t* were found not to be expressed in *E. coli* (Donovan *et al* 1988).

Several reasons for the expression of cloned crystal protein gene(s) of *B t* in non-sporulating bacteria, sporogenic and mutant asporogenic bacteria have been given by investigators in further of the coupling of sporulation to crystal protein production. Either the regulatory region of the gene(s) coding for crystal protein might have undergone deletion during cloning so that the expression of the gene(s) do not respond to the regulatory signals due to lack of regulatory region and are expressed. Eventhough the gene including the regulatory region has been cloned, the non-sporulating bacteria do not produce the sporulation specific regulatory factors for regulating the expression of the crystal protein gene(s) of *B t* which might be under the control of sporulation specific functions in *B t.* In some cases the crystal protein gene(s) have been cloned in a multicopy plasmid (Ward and Ellar 1986; Ward *et al* 1986) which might have titrated the regulatory factors and resulted in its expression in sporulating bacteria, gene dosage effect (Shivakumar *et al* 1989). Alternatively, the expression of crystal protein gene of *B t* is independent of sporulation specific functions so that it is expressed in the vegetative form of the sporulating bacteria.

In order to overcome these limitations in studying the expression of cloned crystal protein genes of *B t,* the entire gene needs to be cloned in a single copy plasmid with all its regulatory elements.

With this in mind an attempt has been made in the present investigation to study the expression of the crystal protein gene as such by conjugation of *B t i* with a very closely related species of *B t,* viz, *B, cereus* (*B c*) without any *in vitro* manipulation.

B t and *B c* are taxonomically closely related species but the former differs from the latter by its ability to synthesize parasporal insecticidal crystal protein (Sneath 1986). Like other sporulating bacteria (Ohye and Murrel 1962; Ellar and Lundgren 1966; Decker and Maier 1975; Holt *et al* 1975; Losick *et al* 1986) the sporulative events in *B t* and *B c* can be divided into six morphologically defined stages which can be distinguished by sporulation stage specific diagnostic biochemical markers (Losick *et al* 1986) and in the seventh or final stage the mature spores are released through lysis of mother cell (Losick *et al* 1986).

In the present study several sporulation stage specific asporogenous mutants (Spo^-) of *B c* have been isolated. Plasmid transfer experiments were performed between sporogenous crystalliferous strain of *B t i* and sporogenous strain and

asporogenous mutant strains of *B c* to determine if crystal protein gene(s) of *B t i* are expressed in *B c,* whether expression is sporulation dependent or independent.

2. Materials and methods

2.1 *Strains*

The following strains were used:

(i) *Bacillus thuringiensis* var. *israelensis* H-14. (ii) *B. cereus* NCIB 9376. (iii) BC 1, a streptomycin resistant strain isolated from wild type *B c* strain, (iv) five asporogenous mutant strains BC 2 to BC 6 isolated from BC I, (v) six strains BC 7 to BC 12 isolated from the conjugation experiments between strain (i) and strain (iii) and strains from (iv).

2.2 *Media*

Strains were maintained in nutrient agar medium. All experiments were performed in nutrient broth unless otherwise mentioned. Cells for protease, alkaline phosphatase, glucose dehydrogenase and dipicolinic acid assay were grown in synthetic medium described elsewhere (Nickerson and Bulla 1974).

2.3 *Isolation of streptomycin resistant* (Str^R) *mutant of B c*

Str^R colonies resistant to 50 µg/ml of streptomycin were isolated spontaneously at 32°C in nutrient agar plates.

2.4 *Isolation of asporogenous (Spoo) mutants of B c*

Spo^- mutants were isolated by mutagenesis of the cells with N-methyl-N'-nitro-N-nitrosoguanidine (NTG) by a modification of the method described earlier (Hopwood 1970). Cells (~ $4{\cdot}5 \times 10^8$ cells/ml) from logarithmic phase of growth in nutrient broth were washed and treated with 100 µg/ml of NTG in 0·05 M Tris-maleic acid buffer (6·1 g Tris, 5·8 g maleic acid/1, adjusted to pH 6·0 with NaOH) for 20 min at 32°C. Prior to plating, cells were again washed, diluted in nutrient broth and plated onto nutrient agar medium and incubated at 32°C for 24 h. Colonies, morphologically different from wild type colonies in texture, opacity, etc., were picked at random, grown overnight in nutrient broth and tested for heat resistance. Most heat-sensitive mutants were tested for absence of spores by periodic microscopic examination and finally by assay of sporulation stage specific diagnostic biochemical markers to determine the stages at which sporulation was blocked in different mutants.

2.5 *Assay of sporulation specific diagnostic biochemical parameters*

The following biochemical parameters, viz., protease, alkaline phosphatase, glucose dehydrogenase, dipicolinic acid, lysozyme sensitivity, heat resistance were studied.

Extracellular protease activity was measured by the method of Yasunobu and McConn (1970) with following modification. Bacterial cultures were centrifuged (10,000 *g,* 10 min) and 0·2 ml of culture suprenatant was made 1 ml with 0·02 M NaCl solution. Five ml of 1·0% casein solution (in 0·2 M Tris buffer, pH 8·4) was added to the sample, vigorously shaken and allowed to stand at 57° C for 10 min. Five ml of trichloroacetic acid (TCA) [TCA 0·11 M, sodium acetate 0·22 M and acetic acid 0·33 M] was added, shaken vigorously and allowed to stand at room temperature for 30 min centrifuged at 20,000 *g* for 30 min. Absorbance of the supernatant was measured at 275 nm. A unit of activity is defined as the enzyme activity which gives the extinction at 275 nm equivalent to 1 µg of tyrosine liberated in 1 min. Alkaline phosphatase assay was carried out as described previously (Somerville 1971). The activity is expressed as nmol substrate decomposed/h/mg protein. Glucose dehydrogenase was assayed spectrophotometrically as described previously (Warren 1968). The activity is expressed as nmol substrate decomposed/h/mg protein. For dipicolinic acid, 10 ml portions of the washed cell suspension was assayed by the method described previously (Janssen *et al* 1958). A suspension of vegetative cells was used as control. Dipicolinic acid activity is expressed as ng/mg protein. Lysozyme sensitivity was tested by the method of Stahly *et. al* (1978). Heat resistant spores were checked by heat-shocking the culture at 80°C for 30 min and plating the cells at different dilution on nutrient agar Plates.

2.6 *Protein*

This was estimated by the method of Lowry *et al* (1951).

2.7 *Reversion frequency from Spo⁻ to Spo⁺*

Cells from post exponential phase of growth were heated at 100°C for 10 min and serial 10-fold dilutions ranging from 10^{-5} to 10^{-8} were plated onto nutrient agar medium containing 50 µg/ml of streptomycin and incubated at 32°C for 48 h. Six plates were made for each dilution. Colonies were picked and grown in nutrient broth and sporulation was checked microscopically.

2.8 *Plasmid transfer experiment*

This was performed by a modification of the method described previously (Gonzalez *et al* 1982). Wild type *B t i* Str^S Spo^+ Cry^+ (streptomycin sensitive sporogenous crystalliferous) strain acted as donor and *B c* Str^R Spo^+ strain and *B c* Str^R Spo^- mutant strains were recipient. Overnight cell culture was diluted 1:50 into 5 ml of nutrient broth and incubated with shaking for 6 h. Recipient and donor cells (0·1 ml) were mixed together in 5 ml of nutrient broth and incubated at 30°C with mild shaking for 8 to 96 h. At different time interval 0·05 ml of mixed culture was spread onto nutrient agar containing streptomycin and incubated at 30°C for overnight. Str^R colonies were examined for crystal production by phase-contrast microscopy and finally by insect bioassay.

2.9 *Insect bioassay*

B c Str^R Spo^- transconjugants and other strains were grown in nutrient broth at 32°C with shaking. After 48 h of growth, cells were centrifuged (10,000 *g,* 10 min), suspended in lysis buffer (1 mg of lysozyme/ml, 10 mM Tris HCl [pH 7·4] and 1 mM EDTA) and incubated at 37°C for 1 h. Sodium dodecyl sulphate (SDS) was added to a final concentration of 0·1 %, thoroughly mixed and the mixture was serially diluted. Two-fold dilutions were made. Dilutions were added to 20 ml of distilled water containing 20 *Aedes aegypti* 3rd instar larvae in petri plates with ten plates each containing 20 larvae as control. Three plates of larvae were tested for each dilution. Larval death was recorded at 8 h interval for 24 h. Experiments in which there is more than 10% mortality in the control plates were discarded. Toxic activity was represented as LC_{50} values which is the amount of lysed cells in suspension required to kill 50% of the larvae in 24 h at room temperature.

3. Results

The medium formulated by Nickerson and Bulla (1974) for the growth of *B t* strains was found to be equally good for the growth of *B c* strains (data not shown). The optimum temperature, concentration of and the period of treatment with NTG to mutagenise the *B c* strain were found to be 32°C, 100 µg/ml and 20 min respectively, with approximately 48 % killing.

Samples from the shake flasks were removed periodically at hourly interval beginning with t_0 over a period of t_{10} and different diagnostic biochemical changes occurring during different stages of sporulation were tested as described in §2. t_0 represents the on set of sporulation and this time-scale convention was maintained throughout the experiments. Fully matured spores appeared before t_{10}. Maximum values of different sporulation stage specific diagnostic biochemical parameters of the wild type strains and the mutant strains, blocked at different stages of sporulation in different strains and other properties are presented in table 1.

It appears from table 1 that development of resistance against streptomycin in *B c* (strain BC 1) seems to have no effect on the biochemical changes occurring during sporulation. The strain BC 2 exhibited a very low level of different biochemicals produced during sporulation. In strain BC 2, therefore, sporulation is blocked at early stages of sporulation, *i.e.*, at stages between 0 (*Spo* 0) and II (*Spo II*). The sporulation in strain BC 3 appears to be blocked between stage II (*Spo II*) and III (*Spo III*) of sporulation as it synthesized almost equal amount of protease compared to the parent strain but showed reduced level of other biochemical parameters. The mutant strain BC 4 produced almost equal amount of protease and alkaline phosphatase with respect to parent strain but showed low level of other parameters. It appears, therefore, that strain BC 4 is blocked at stage IV (*Spo IV*) of sporulation. The strain BC 5 produced protease, alkaline phosphatase and glucose dehydrogenase in a pattern similar to that of wild type strains but showed low level of dipicolinic acid and heat resistant spores. Therefore in strain BC 5 sporulation is seemed to be blocked at stage V (*Spo V*). Since in the isolate BC 6 the production of different biochemicals occurred at a similar level compared to the wild type strains except the number of heat resistant spores, in strain

Table 1. Diagnostic biochemical parameters of different strains.

Parameter	*B t i*	*B c*	BC 1	BC 2	BC 3	BC 4	BC 5	BC 6
Protease[a]	560	498	487	60·5	465·5	472	462	470
Alkaline phosphatase[b]	23	20	25	5	4	21	19	20
Glucose dehydrogenase[b]	57	55	60	15	12	9	48	45
Dipicolinic acid[c]	78	72	70	28	25	20	19	68
Lysozyme sensitivity	–	–	–	+	+	+	+	+
Heat resistant colonies/ml	$1{\cdot}8\times10^{8}$	$1{\cdot}4\times10^{8}$	$1{\cdot}5\times10^{8}$	$1{\cdot}7\times10^{1}$	$2{\cdot}1\times10^{1}$	$2{\cdot}7\times10^{1}$	$2{\cdot}9\times10^{1}$	$3{\cdot}5\times10^{2}$
Blocked at different stages of sporulation	–	–	–	0–II	II–III	IV	V	V–VI
Reversion frequency				10^{-6}	10^{-7}	10^{-8}	10^{-8}	10^{-8}
Streptomycin resistance	–	–	+	+	+	+	+	+
Toxicity against *Aedes aegypti* larvae	+	–	–	–	–	–	–	–

[a]Unit of activity is defined as the enzyme activity which gives the extinction at 275 nm equivalent to 1 µg or tyrosine liberated in 1 min.
[b]Activity is expressed as nmol substrate decomposed/h/mg protein.
[c]Activity is expressed as ng/mg protein.

BC 6 sporulation is supposed to be blocked at stages between V (*Spo V*) and VI (*Spo VI*).

The larvicidal activity of transconjugants and other strains are presented in table 2. Since there is no direct selection marker for streptomycin resistant transconjugants showing larvicidal activity, the resistant colonies were picked at random and larvicidal activity was tested. The per cent transconjugants (% streptomycin resistant colonies exhibiting larvicidal activity) is presented in table 2. Strains *B c* and BC 1 showed no toxicity against *A. aegypti* larvae. The isolates BC 7 to BC 12 showed toxicity against the larvae. The streptomycin resistant transconjugants showing

Table 2. The larvicidal activity of the transconjugants and other strains.

Conjugation	Transconjugants (%)[a]	Strains	LC_{50} (µg/ml)[b]
–	–	*B t i*	0·040
–	–	*B c*	> 50
–	–	BC 1	> 50
B t i × BC 1	55	BC 7	0·045
B t i × BC 2	48	BC 8	0·050
B t i × BC 3	52	BC 9	0·055
B t i × BC 4	58	BC 10	0·060
B t i × BC 5	54	BC 11	0·055
B t i × BC 6	50	BC 12	0·060

[a]% Str^R colonies having larvicidal activity.
[b]Amount of lysed cells in suspension require to kill 50% of the larvae in 24 h at room temperature.

larvicidal activity remains Spo^- as determined by the assay of sporulation stage specific diagnostic biochemical markers (data not shown). This indicate that even after acquisition of Cry^+ character, the transconjugants showing larvicidal activity retains their Spo^- identity.

These results show that the crystal protein coding gene(s) have been successfully transfered from *B t i* into *B c* Str^R Spo^+ strain and also into Str^R Spo^- mutant strains of *B c* blocked at different stages of sporulation. Moreover the blocking of sporulation at any stages did not prevent crystal protein gene(s) from being expressed in the Spo^- mutant strains of *B c*.

4. Discussion

Chilcott and Ellar (1988) purified different crystal protein components from *B t i* and found all of them to be toxic against *A. aegypti* larvae. Several investigators cloned and found the crystal protein gene(s) of *B t* and *B t i* to be expressed in non-sporulating bacteria (Schnepf and Whitely 1981; Wong *et al* 1983; Waalwijck *et al* 1985; Ward and Ellar 1986; Mclean and Whitely 1987; Adams *et al* 1989), in vegetative cells and in asporogenic mutant cells of sporulating bacteria (Ward *et al* 1986; Calegero *et al* 1989; Shivakumar *et al* 1989). However, some crystal protein gene(s) of *B t i* were found not to be expressed in *E. coli* (Donovan *et al* 1988). These findings do suggest that at least some of the crystal protein genes of *B t,* may be expressed in the absence of expression of sporulation specific functions.

In the present investigation five asporogenous mutants blocked at different stages of sporulation, were isolated from streptomycin resistant *B c* strain based on the studies of sporulation stage specific diagnostic biochemical characteristics.

The transconjugants (BC 7 to BC 12) resistant against streptomycin, isolated from conjugation of wild type *B t i* Spo^+ Cry^+ with streptomycin resistant BC 1 strain and five asporogenous mutant strains derived from BC 1, were found to be toxic against *A. aegypti* larvae (table 2).

This study, therefore shows that the gene(s) coding for crystal protein in *B t i* are expressed in both the sporulating *B c* strain and its asporogenous mutant strains irrespective of blocking at different stages of sporulation suggesting that sporulative events are not functionally related to the process of crystal protein synthesis in *B c* strain.

Thus the synthesis of crystal protein components, of *B t i* are not dependent on sporulative events in *B c* and the synthesis and assembly of the crystal proteins can occur without the expression of sporulation specific events in *B c*.

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