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Bacillus thuringiensis crystal protein (δ-endotoxin) gene expression is independent of early sporulation specific functions

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Abstract. *Bacillus thuringiensis* produces a parasporal insecticidal crystal protein. The correlation between sporulation and crystal protein production in *Bacillus thuringiensis* var. *israelensis* was studied. The strain was made resistant'against streptomycin (St^R)-Acrystalliferous (Cry^-) cured derivatives and asporogenous acrystalliferous (Spo^- Cry^-) mutants blocked at an early stage of sporulation were isolated. Plasmid transfer experiments were performed between St^s Spo^+ Cry^+ (streptomycin sensitive sporogeneous crystalliferous) and St^R Spo^+ Cry^- and also between St^s Spo^+ Cry^+ and St^R Spo^- Cry^- strains. St^R colonies were selected. Insect toxicity was exhibited by the St^R isolates in both the cases. The process of crystal formation is, therefore, independent of early sporulative events.

Keywords. *Bacillus thuringiensis;* sporulation; crystal protein; δ-endotoxin; protease; DNAse.

1 Introduction

Bacillus thuringiensis (hereafter abbreviated BT), a gram-positive sporulating bacillus, produces a proteinaceous parasporal crystal (δ-endotoxin) which is toxic against a number of lepidopterans and dipterans (Burges 1982). The gene(s) coding for crystal protein in different species and subspecies of BT are plasmid encoded (Carlton and Gonzalez 1985) although examples of chromosomal location of the gene(s) have also been reported (Aronson *et al* 1986). The number of plasmids in different strains of BT varies from 2 to 12 with sizes ranging from *ca.* 1·5 to *ca.* 150 Md (Carlton and Gonzalez 1985). The crystal protein genes are mostly located in large plasmids (15–120 Md) (Lereclus *et al* 1982). Toxin activity appears to be mediated by binding of toxins to cell membrane phospholipids (Drobniewsky and Ellar 1987; Chow *et al* 1989). Several studies revealed that the expression of the crystal protein gene is functionally related to sporulation specific events (Monro 1961; Lecadet and Dedonder 1971) and it was also found that one of the subunits of spore coat protein shares some similarities with the crystal protein (Lecadet *et al* 1972; Meenakshi and Jayaraman 1979). However, desynchronization of sporulation and crystal formation could be achieved by short-term temperature shift-up (Arescaldins 1969) or addition of chloramphenicol (Meenakshi and Jayaraman 1979) or erythromycin (Arescaldins 1969; Somerville 1971). This latter finding evokes questions about the correlation between sporulation and crystal protein gene expression.

In sporulating bacteria, the entire process of sporulation can be divided into seven sequential morphologically defined stages distinguishable by biochemical markers (Bechtel and Bulla 1976; Losick *et al* 1986). Earlier studies (Arescaldins

1969; Somerville 1971; Meenakshi and Jayaraman 1979) regarding desynchronization of speculation and crystal production were informative, but they did not categorically mention whether sporulation was blocked at the early stage or at any latter stages.

The present study is undertaken to determine whether crystal protein synthesis is inextricably linked with sporulation. Asporogenous mutants, blocked at early stages of sporulation, have been isolated and evidence is presented that the process of crystal formation is not dependent on early sporulation specific functions.

2. **Materials and methods**

2.1 *Strain*

Bacillus thuringiensis var. *israelensis* was a kind gift from Prof. S K Majumdar, Jadavpur University, Calcutta.

2.2 *Media*

Strains were maintained in nutrient agar medium. Unless otherwise mentioned all experiments were performed in nutrient broth. Cells for protease assay and DNAse assay were grown in synthetic medium as described previously (Nickerson and Bulla 1974).

2.3 *Isolation of streptomycin resistant (St^R)* mutants

St^R colonies were isolated spontaneously by plating cells at 30° C in nutrient agar containing 50 unit/ml streptomycin.

2.4 *Isolation of asporogenous (Spo^-) mutants*

Spore suspension (4·5 ml) in distilled water (~ 10_8 spores/ml) was incubated at 37°C for 40 min with 0·5 ml of ethyl-methane-sulphonate (E Merck, Germany) (10% v/v). The spores were washed thrice in distilled water by centrifugation and resuspended in 5 ml of distilled water. Appropriate dilutions of the mutagenized spores were spread onto nutrient agar plates and incubated for 48 h at 30° C. Morphologically distinguishable colonies were picked up at random and grown in nutrient broth and monitored periodically for Spo - mutants by microscopic examination and finally by protease and DNAse assay.

2.5 *Curing experiments for isolation of acrystalliferous (Cry^-) cured derivatives*

Curing of plasmid(s) coding for crystal protein was carried out essentially as described by Bernhard *et al* (1978) or by Stahly *et al* (1978). An overnight culture was diluted in 1:100 nutrient broth containing 0·002% sodium dodecyl sulphate (SDS) and incubated with shaking at 30° C overnight. After appropriate dilutions, cells were plated on nutrient agar and incubated overnight at 30° C. Cells grown without SDS were also

plated and incubated. Morphologically different colonies of strain grown in the presence of SDS compared to cells grown in the absence of SDS were picked up at random and monitored by phase contrast microscopy and finally by insect bioassay for Cry^- cured derivatives or Cryy derivatives were isolated by heating spores at 80° C for 40 min, or at 80° C for two successive 20 min periods with a 20 min cooling interval. The heat-shocked spores were spread onto nutrient agar and incubated at 30°C for 48 h. Cry^- isolates were characterized as described above.

2.6 *Plasmid transfer experiments*

Plasmid transfer experiments were performed as described previously (Gonzalez *et al* 1982) with minor modification. Two sets of transfer experiments were performed: in one experiment a St^s Spo^+ Cry^+ cell was the donor and a St^R Spo^+ Cry^- was the recipient. In another set of experiment St^s Spo^+ Cry^+ acted as donor and St^R Spo^- Cry^- as recipient. A single pure colony of each strain was grown separately with shaking at 30° C in nutrient broth. Cells were then diluted (1:50) into 5 ml of nutrient broth and incubated with shaking for 6 h. Donor and recipient cells (0·1 ml) were mixed together in 5 ml of nutrient broth and incubated at 30° C with shaking for 8 to 96 h. At different time intervals 0·05 ml of mixed culture were spread on to nutrient agar containing streptomycin and incubated at 30° C overnight. St^R colonies were picked up at random and grown separately in 5 ml of nutrient broth and crystal production was examined by phase contrast microscopy and insect bioassay.

2.7 *Protease assay*

Extracellular protease activity was measured by a modification of the method described previously (Yasunubu and McConn 1970). Cell culture (2 ml) was centrifuged at 12,000 g for 10 min and 0·2 ml of supernatant was made 1 ml with 0·02 M NaCl solution. To the sample 5 ml of 1·0% casein solution (in 0·2 M Tris buffer, pH 8·4) was added, vigorously shaken and allowed to stand at 57° C for 10 min. Trichloroacetic acid (TCA) (5 ml) (TCA 0·1 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 15,000 *g* for 30 min. Absorbance of the supernatant was measured at 275 nm. For the blank, 5 ml of TCA solution was added to the diluted culture supernatant, kept at 57° C for 10 min and then 5 ml of casein solution was added, allowed to stand at room temperature for 30 min, and then proceeded exactly as for the sample. A unit of activity $[PU]^{casein}_{275nm}$ is defined as that enzyme activity which gives an extinction at 275 nm equivalent to 1 μg of tyrosin liberated in 1 min.

2.8 *Nuclease* (*DNAse*) *assay*

DNAse assay of excreted nuclease in the supernatant was carried out as described by Matsumoto *et al* (1978) with minor modification. Cells grown in synthetic medium were centrifuged at 12,000 *g* for 10 m and the supernatant was used for enzyme assay. The reaction mixture contained 1 ml of a 2 mg/ml concentration

of calf thymus DNA dissolved in 0·05 M $MgSO_4$, 1 ml of 0·2 M potassium phosphate buffer pH 7·4 and 1 ml of the supernatant. After incubation at 35°C for 1 h, 1 ml of 3·0 M HCl solution was added and the mixture left in an ice-water bath for 1 h. The mixture was centrifuged at 13,000 *g* for 30 min and absorbance was measured at 260 nm. One unit of enzyme activity was defined as the amount of enzyme yielding 1 μg of acid-soluble oligonucleotides formed from DNA at 35° C for 1 h.

2.9 *Bioassay of toxicity*

To determine toxicity, bioassay was performed on 3rd instar larvae of *Aedes aegypti* suspended in a total volume of 20 ml distilled water in petri plates. Routinely 3 plates of 25 larvae were tested for each two-fold dilution of bacterial culture with 10 plates each containing 25 larvae serving as control. Larval death was recorded at 8 h intervals for 24 h. Toxic activity is represented as LC_{50} values which is the amount of bacterial cells required to kill 50% of the larvae in 24 h at room temperature.

3. Results

Two streptomycin resistant mutants were isolated, BT St^R 15 and Bt St^R 36. Asporogenous mutants and acrystalliferous cured derivatives were isolated from these two mutant strains: BT St^R 15 Spo^+ Cry^- (sporogenous acrystalliferous), BT St^R 15 Spo^- Cry^- (asporogenous acrystalliferous), BT St^R 36 Spo + Cry^- and BT St^R 36 Spo^- Cry^-. Figure 1 shows the growth curve of the wild type cells and the mutants in nutrient broth medium. The sporogenous acrystalliferous mutants in St^R 15 and St^R 36 backgrounds showed approximately identical growth rates, comparable to that of the parent strain. Similarly, the asporogenous acrystalliferous mutants in the St^R 15 and St^R 36 backgrounds showed identical growth rates, but lower compared to the wild type strain. Unlike asporogenous mutation, the Cry^- cured condition seems to have no effect on the growth rate of the mutant strains.

Samples from stationary phase were withdrawn at hourly intervals from shake flasks and were assayed for extracellular protease and DNAse activity. The maximum values from different strains are shown in table 1. The asporogenous mutants showed about 15-fold reduction in protease activity. The Cry^- cured condition has apparently no effect on protease production of the mutant strains. The mutant strains showed about 6-fold decrease in DNAse activity compared to wild type strains. Like protease activity, DNAse activity also seems to be unaffected by Cry^- cured condition.

The protease activity and the DNAse activity of the Spo^- mutant strains revealed that sporulation is blocked in between stage 0 (Spo 0) and stage II (Spo II) i.*e*., at the early stage of sporulation (Losick *et al* 1986). The St^R transconjugants from crosses of St_S Spo^+ Cry^+ and St_R Spo^+ Cry^- showing larvicidal activity (St^R 31 and St^R 42) showed similar level of protease and DNAse activity like wild type parent strain. The St^R transconjugants isolated from crosses of St^s Spo^+ Cry + and St^R Spo^- Cry^-, which exhibited larvicidal activity (St^R 45 and St^R 57) showed reduced level of protease and DNAse like the parent St^R Spo^- mutant strains. This

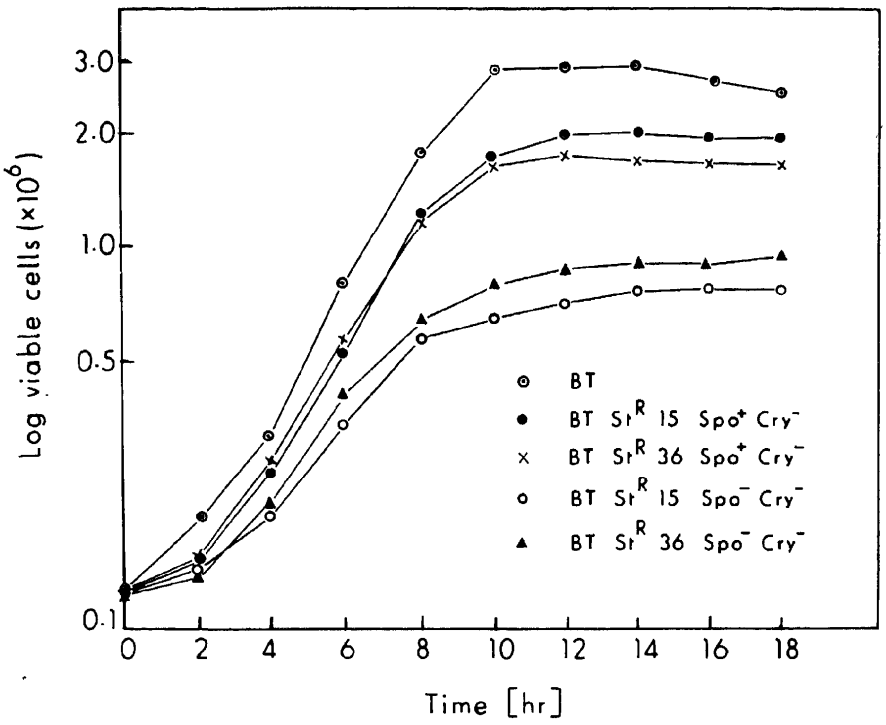

Figure 1. Growth curve of different BT strains.

Table 1. Maximum values of extracellular protease and DNAse activity of different BT strains.

Strains	Protease activity (U)	DNAse activity (U)
BT St^S Spo^+ Cry^+	586·9	85·0
BT St^R_{15} Spo^+ Cry^-	582·2	69·3
BT St^R_{36} Spo^+ Cry^-	555·7	71·4
BT St^R_{15} Spo^- Cry^-	43·4	13·5
BT St^R_{36} Spo^- Cry^-	36·4	12·9
BT St^R_{31}	540	80·5
BT St^R_{42}	560	78·2
BT St^R_{45}	38·5	10·2
BT St^R_{57}	40·2	12·5

indicates that even after conjugation and acquisition of Cry^+ characteristic, the St^R Spo^- transconjugants remains Spo^-.

The isolates of the conjugation experiments and the insect toxicity of the strains are presented in table 2. Since there is no direct selection marker for Cry^+ characteristics of the transconjugants, the St^R colonies were picked up at random and larvicidal activity was determined. The percentage of transconjugants (per cent St^R colonies that exhibited larvicidal activity) is presented in table 2. Spo^+ Cry^- and Spo- Cry- strains exhibited no toxic effect. BT StR 31 and BT StR 45 showed

Table 2. The isolates of the conjugation experiment and the insect toxicity of different strains.

Conjugation	Strains	Transconjugant[a] (%)	LC_{50} (μg/ml)
	BT St^S Spo^+ Cry^+		0·45
	BT ST^R_{15} Spo^+ Cry^-		–
	BT St^R_{36} Spo^+ Cry^-		–
	BT St^R_{15} Spo^- Cry^-		–
	BT St^R_{36} Spo^- Cry^-		–
BT St^S Spo^+ Cry^+ × BT St^R_{15} Spo^+ Cry^-	BT St^R_{31}	67	0·40
BT St^S Spo^+ Cry^+ × BT St^R_{15} Spo^- Cryk	BT St^R_{45}	59	0·47
BT St^S Spo^+ Cry^+ × BT St^R_{36} Spo^+ Cry^-	BT St^R_{42}	58	0·70
BT St^S Spo^+ Cry^+ × BT St^R_{36} Spo^- Cry^-	BT St^R_{57}	63	0·65

[a] % StR colonies showed lavecidal activity.
' - ', No toxicity

almost identical toxicity with respect to parent strain. But BT St^R 42 and BT St^R 57 showed a little higher LC_{50} value. The result of the conjugation experiment shows that Spo^+ Cry^- or Spo^- Cry^- when conjugated with Spo^+ Cry^+ the resultant isolates exhibited insecticidal activity. The crystal production was, therefore, not dependent on early sporulation specific events.

4. Discussion

The synthesis and assembly of crystal proteins occurred during sporulation (Drobniewsky and Ellar 1987) and the crystal protein was found to be a component of the spore coat of crystal producing strains (Lecadet, *et al* 1972). Addition of inhibitors of sporulation affected both sporulation and crystal formation (Yousten and Rogoft 1969). These suggested that the genes for crystal protein were under sporulation control. However, the process of sporulation and crystal formation could be desynchronized by the addition of chloramphenicol (Meenakshi and Jayaraman 1979), erythromycin (Arescaldins 1969), growth at suboptimal temperature (Smiraoft 1963). These suggest that the process of sporulation and that of crystal formation could be delinked.

The sporulation specific events in sporulating bacteria can be morphologically devided into seven stages (Bechtel and Bulla 1976). The synthesis of crystal protein, if dependent on sporulation, either could be concomitant with the very onset of sporulation or its synthesis could be initiated at some later stages of sporulation. Despite the wealth of information concerning desynchronization of sporulation and crystal protein production, earlier studies (Smirnoft 1963; Arescaldins 1969;

Meenakshi and Jayaraman 1979) did not specifically reveal exactly at what stages the sporulation was blocked. The present study clearly demonstrates the stage at which sporulation was blocked in the Spo$^-$ mutants.

The Spo$^-$ mutants isolated were found to be protease negative and nuclease (DNAse) negative. Therefore, in these Spo$^-$ mutants sporulation was blocked at the early stage of sporulation, *i. e.,* between stage 0 (Spo 0) and sgate II (Spo II) (Losick *et al* 1986). The St^R Spo$^-$ transconjugants which exhibited larvicidal activity showed reduced level of protease and DNAse activities compared to the wild type parent strain. This reveals that even after the acquisition of Cry$^+$ characteristic from a Cry$^-$ cured condition (by conjugation) the transconjugants retained their Spo$^+$ identity. The St^R isolates from both the crosses of St^s Spo$^+$ Cry$^+$ and St^R Spo$^+$ Cry$^-$ and St^s Spo$^+$ Cry$^+$ and St^R Spo$^-$ Cry$^-$ showed toxicity against larvae. Therefore, sporulation specific functions, even at the early stage of sporulation, are not functionally related to the process of formation of crystal proteins. This study clearly indicates that the synthesis and assembly of crystal protein can proceed to completion without expression of early spore specific functions.

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