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Studies on the extrachromosomal DNA of *Bacillus thuringiensis* var. *thuringiensis*

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Abstract

An extrachromosomal DNA was isolated from *B. thuringiensis* var. *thuringiensis* and characterized. Its molecular weight was found to be approx. 10×10^6. The covalently closed nature of the molecule was confirmed by CsCl-ethidium bromide centrifugation, agarose gel electrophoresis and electron microscopy. The extrachromosomal DNA was isolated at various stages of growth, under different growth conditions (with varying concentrations of cys). Two modes of existence for the extrachromosomal DNA were suggested: (*a*) an autonomous cytoplasmic form and (*b*) a cryptic form in which it presumably was integrated into the genomic DNA. It is suggested that some of the crystal protein and major spore coat protein could have been coded for by the extrachromosomal DNA of *B. thuringiensis* var. *thuringiensis* while it is in an extrachromosomal state. In the integrated state the extrachromosomal DNA is non-functional.

Key words: *B. thuringiensis*, extrachromosomal DNA, spore coat, crystal protein, inhibition, integration, cytoplasmic state.

1. Introduction

The presence of extrachromosomal DNA has been reported in several bacilli, like *B. subtilis* and *B. megaterium*[5–7,10,17,18]. Stahly *et al*[15] have reported the existence of multiple copies of such extrachromosomal DNA in *B. thuringiensis*. The functional role of these DNAs has not been clearly established. It was noticed that when *B. thuringiensis* strain 612 was transferred from a sporulating medium to a non-sporulating medium, no extrachromosomal DNA was found, and the crystal formation was also negligible[8]. Based on these results, it was suggested that there could be a relationship between the extrachromosomal DNA and the formation of the toxic crystalline inclusion in *B. thuringiensis*[8,15].

In the present study attempts were made to isolate and characterize the extrachromosomal DNA from *B. thuringiensis* var. *thuringiensis* at various stages of the growth of the organism, under different growth conditions, so as to examine its existence and

disappearance observed with respect to the nutritional status of the medium[8]. Cystine was used as a probe to study the relationship between the extrachromosomal DNA and the formation of the crystal toxin in *B. thuringiensis* var. *thuringiensis* and the results are discussed here.

2. Materials and methods

2.1. *Organism and culture conditions*

Bacillus thuringiensis var. *thuringiensis* serotype I was obtained from Prof. H. de Barjac, Institut Pasteur, Paris. Inoculum and the minimal medium containing mineral salts and 1% glucose were prepared as mentioned earlier[13]. For further studies the minimal medium was supplemented with low (0·05%), medium (0·15%) and high (0·25%) concentration of cys.

2.2. *Isolation and characterization of the extrachromosomal DNA of* B. thuringiensis *var.* thuringiensis

The extrachromosomal DNA was isolated from *B. thuringiensis* var. *thuringiensis* by acid-phenol extraction[19] and also by the lysozyme-EDTA technique of Gerry *et al.* The covalently closed nature of the extrachromosomal DNA was confirmed by CsCl-ethidium bromide centrifugation[2] as well as by agarose gel electrophoresis[11]. Fluorography of the agarose gels was carried out according to Bonner and Stedman[4]. The molecular weight of the extrachromosomal DNA was calculated by subjecting the sample to electrophoresis on 4% polyacrylamide gel, using λ-DNA, colitis bacteriophage, and colitis bacteriophage DNA as markers[14]. The cells were labelled with [^{3}H]-thymidine for 2 hr prior to the isolation of the extrachromosomal DNA. The bacteriophage λ was propagated in *E. coli* 621 as the host and the λ phage was purified on a linear sucrose density gradient (15–60% w/v, sucrose in 0·1 M tris-HCl buffer, pH 7·2). The colitis bacteriophage was propagated and purified as per the standard procedure[16]. DNA from the bacteriophage λ and the colitis bacteriophage DNA were isolated by the treatment of the phages with SDS (1%) and repeated phenol extraction (thrice) followed by extensive dialysis against 0·1 M NaCl. The λ-DNA was labelled with [^{3}H]-thymidine *i.e.*, the addition of radioactive material was made 10 min after the phage infection. The radioactive thymidine was used at a concentration of 0·1 mCi per 100 ml culture.

2.3. *Isolation of the extrachromosomal DNA under different growth conditions*

The extrachromosomal DNA was isolated under varying growth conditions (*i.e.*, cells grown with 0·05%, 0·15% and 0·25% cys in a glucose-salts medium), at different stages of growth, starting from the mid-lag phase, with a regular interval of 30 min. Attention was paid to observe the appearance or the disappearance (if any) of the extrachromosomal DNA depending on the nutritional status of the medium as reported by Ermakova *et al.*[8]. Besides, excess cys was added at, and after the stationary phase to arrest the spore coat and the crystal formation, or the spore-crystal formation completely (unpublished

data), and the existence of the extrachromosomal DNA was studied. Reversal of sporulation-inhibition was carried out by the addition of glutamate or divalent metal ions or the intermediates of Krebs cycle (unpublished data) and the isolation of the extrachromosomal DNA was carried out under such conditions also.

2.4. *Electron microscopy*

Extrachromosomal DNA was analysed by electron microscopy as described by Bak *et al*[5]. The extrachromosomal DNA (2 μg per ml) in TES buffer (0·05 M NaCl + 0·05 M tris + 0·005 M EDTA, pH 8·0) was mixed with an equal volume of a 0·04% solution of cytochrome-*c* and twice the volume of 4 M ammonium acetate. This solution was spread on the surface of distilled water. The monolayer was transferred to 3 mm copper grids (300 mesh) covered with formvar. The preparation was fixed in absolute alcohol for 30 seconds, stained with 3% uranyl acetate (in absolute alcohol) and examined under Philips 301 electron microscope with 60 kV accelerating voltage using a magnification of approx. 7,500 ×. Contour lengths of the circular DNA were measured with a Hewlett–Packard digitizer (model 9107 A), after magnification (10 ×) of the negatives on to a white screen[6].

2.5. *Chemicals*

[^{3}H]-Thymidine was obtained from the Bhabha Atomic Research Centre, Bombay, India. Agarose, SDS, tris-(hydroxymethyl) aminomethane, lysozyme, CsCl, ethidium bromide, acrylamide, methylene bis-acrylamide, cytochrome-*c* and EDTA were obtained from Sigma Chemical Co., St. Louis, MO., USA. L-Cystine was obtained from E. Merck, Darmstadt. Formvar and uranyl acetate were obtained from Polaron Equipment Ltd., London, N3, UK. All the other chemicals were of analar or reagent grade.

2.6. *Instruments*

Growth of the culture was monitored by the absorbance at 600 nm in Bausch and Lomb Spectronic-20 colorimeter. Density gradient centrifugations were carried out in a Beckman L2–50 ultracentrifuge. Philips 301 transmission electron microscope was used for the electron microscopic studies of the extrachromosomal DNA. Radioactivity was measured by Beckman LS-100 liquid scintillation spectrometer.

2.7. *Other parameters*

Spore and crystal formation were monitored by observing a wet smear of the culture under phase microscope. Heat-stable spores were estimated by plating the sample on nutrient agar plates after exposing the spore suspension at 80° C for 15 min. Heat labile spores were estimated by plating the sample on nutrient agar plates after an appropriate dilution, without any heat treatment. Heat lability was confirmed by the lysozyme sensitivity of the spores[16]. Toxicity was checked by feeding the crude cell lysate to 2nd or 3rd instar larvae of *B. mori*. One mg protein was sprayed on mulberry leaves and fed to 20 worms of *B. mori*. After 4–5 hr the mortality was scored.

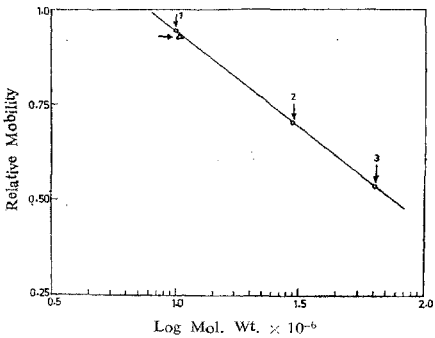


FIG. 1. Molecular weight determination of the extrachromosomal DNA by agarose gel electrophoresis; 1—colitis bacteriophage DNA (mol. wt. = 10×10^6) ; 2—colitis bacteriophage (mol. wt. = 20×10^6). 3—λ-DNA ; Δ—extrachromosomal DNA of *B. thuringiensis* var. *thuringiensis*.

3. Results

3.1. *Existence of the extrachromosomal DNA under different growth conditions*

When the cells were provided with low concentration of cys (0·05%), spore-crystal formation was observed; under such conditions the extrachromosomal DNA was found in an 'extrachromosomal state', until 2 hr after the stationary phase. When the cells were provided with 0·15% cys, no spore coat and crystal formation was noticed (only heat labile spores were produced); whereas, when the cells were provided with high concentration of cys (0·25%) no spore and crystal formation was observed (unpublished data). Under the conditions of medium and high concentrations of cys, the extrachromosomal DNA could not be isolated from 60 min prior to the cessation of the vegetative growth (Table I); when the sporulation inhibition was reversed by the addition of reversal agents like glutamate, divalent metal ions or intermediates of Krebs cycle (unpublished data), the extrachromosomal DNA reappeared and the organism retained toxicity. The extrachromosomal DNA, present in the isolatable form until the addition of excess cys, disappeared as soon as the excess cys was added after the stationary phase. When the reversal agents were added 10 to 15 min prior to the addition of excess cys, the extrachromosomal DNA was in an isolatable form and there was spore and crystal formation (Table I).

3.2. *Characteristics of the extrachromosomal DNA*

The molecular weight of the extrachromosomal DNA was found to be approx. 10×10^6 (Fig. 1). The covalently closed nature of the molecule was confirmed by agarose gel

Table 1

Existence of the extrachromosomal DNA under different growth conditions

Organism	Growth medium	Cys %	Extrachromosomal DNA							
			midlog	−90	−60	0	+1	+2	+3	+12
Bacillus	Mineral	0·05	+	+	+	+	+	+	−	SC
thuringiensis var.	salts	0·15	+	+	−	−	−	−	−	HLS
thuringiensis	+1%		+	Reversal agents		+	+	+	−	SC
	glucose	0·25	+	+	−	−	−	−	−	−SC
			+	Reversal agents		+	+	+	−	SC
		0·05	+	+	+	0·15 or 0·2% cys	−	−	−	HLS or −SC
			+	+	+	Reversal agents	0·15 or 0·2% cys	+	−	SC

cys = cystine; Reversal agents = glutamate, divalent metal ions or intermediates of TCA cycle; SC = spore and crystal; HLS = heat labile spore; −SC = no spore and crystal; −90 = 90 *min prior* to stationary phase (SP); −60 = 60 min prior to SP; 0 = At SP; +1, +2, etc. = 1, 2, 3 ... hr after SP.

electrophoresis followed by fluorography (Fig. 3), and by CsCl-ethidium bromide centrifugation (Fig. 2*a*). Since the genomic DNA could not penetrate the gel, it remained at the top of the gel (0·7% agarose); once the covalently closed molecules linearise, they are susceptible to endonuclease digestion, which would result in no sharp band formation on agarose gel. Such digested molecules and the broken pieces of DNA, during the isolation procedure moved along the dye band. Hence the sharp band obtained on agarose gel electrophoresis was due to covalently closed molecules of *B. thuringiensis* var. *thuringiensis* (Fig. 3). As the intercalating dye ethidium bromide would relax the supercoiled nature of the extrachromosomal DNA, the density of the molecules would decrease on CsCl density gradient. Hence shift in the band density was observed when the centrifugation was carried out with ethidium bromide, as compared to the band obtained without ethidium bromide (Fig. 2*a*, *b*). For agarose gel electrophoresis and CsCl-ethidium bromide centrifugation, λ-DNA labelled with [^{3}H]-thymidine was used as marker (Figs. 2*b* and 3). Colitis bacteriophage and colitis bacteriophage DNA were used without radioactive labelling.

3.3. *Electron microscopy*

Electron microscopy also proved the covalently closed nature of the molecule (Fig. 4). The length of the extrachromosomal DNA was found to be 4·4 μm. The calculated molecular weight based on the length was found to be 8×10^6 (Table II). Some of the

Table II

Molecular weight determination of the extrachromosomal DNA based on the electron microscopic observation

Length of λ-DNA = 16·5 μm
= $16{\cdot}5 \times 10^4$ Å

Distance between bases = 3·5 Å

No. of base pairs = $(16{\cdot}5 \times 10^4)/3{\cdot}5$
= $4{\cdot}7 \times 10^4$

Mol. wt. of		
	A	135
	T	126
	G	151
	C	111

Take it for granted that AT : GC = 1 : 1

Mol. wt. of λ-DNA by electron microscopy = 26×10^6

Actual mol. wt. of λ-DNA = 30×10^6

Length of extrachromosomal DNA of *B. thuringiensis* var. *thuringiensis* = 4·4 μm = $4{\cdot}4 \times 10^4$ Å

No. of base pairs = $(4{\cdot}4 \times 10^4)/3{\cdot}5 = 1{\cdot}6 \times 10^4$

Mol. wt. obtained by electron microscopy = $8{\cdot}36 \times 10^6$

Mol. wt. obtained by agarose gel electrophoresis = 10×10^6.

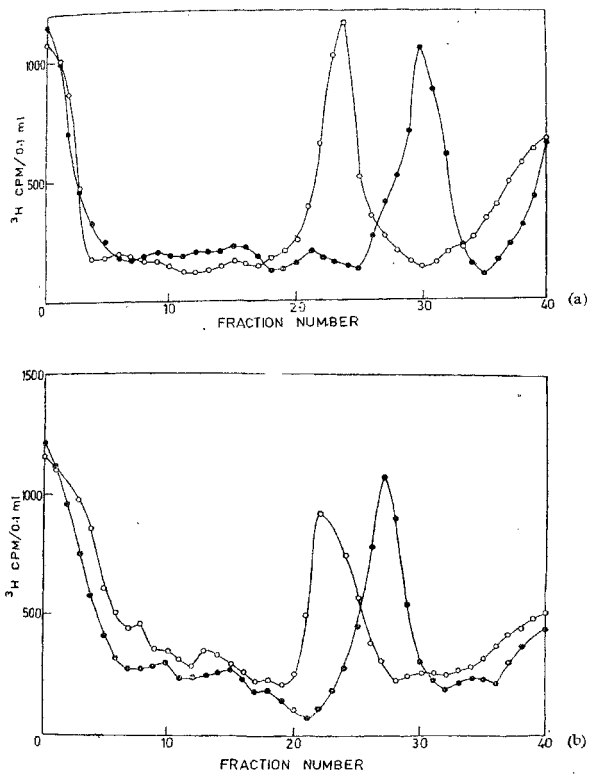


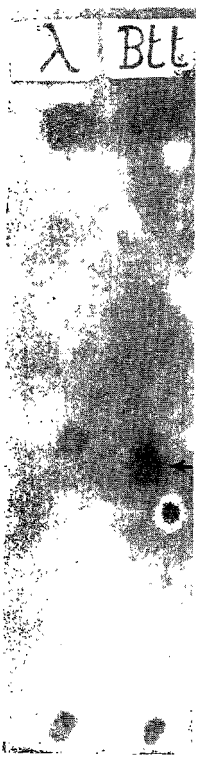
FIG. 2. CsCl–ethidium bromide centrifugation of (2*a*) extrachromosomal DNA of *B. thuringiensis* and (2*b*) λ-DNA. The extrachromosomal DNA and the λ-DNA were purified on a 5–20% sucrose linear gradient containing 0·1 M NaCl, 0·005 M EDTA and 0·05 M phosphate buffer pH 7·5. To study the ethidium bromide binding, 1·654 g per ml CsCl, 15 μg of ethidium bromide per ml of the buffer and 2 μg per ml of DNA were used. The centrifugation was carried out at 15° C in a Beckman SW 50 rotor for 24 hr (44,000 rpm). The tubes were punctured, 0·1 ml fractions were collected and the radioactivity was measured. O—O without ethidium bromide; ●—● with ethidium bromide. Fractions 1 to 40 correspond to the fractions from bottom to top.

covalently closed molecules had a length of 8·8 μm (Fig. 5); and some such dimers had '8-shaped' structure (Figs. 6, 7 and 8). These '8-shaped' molecules could be either replicating molecules of the extrachromosal DNA. They could also have formed due to recombination between the molecules of the extrachromosomal DNA[3,12]. Thus the existence of the '8-shaped' molecules proves the possibility of the presence of more than one copy of the extrachromosomal DNA per cell of *B. thuringiensis* var. *thuringiensis*.

4. Discussion

A number of bacilli have been reported to contain the extrachromosomal DNA[3,7,8,10,17,18]. Stahly *et al*[15] have confirmed the presence of multiple copies of extrachromosomal DNA in *B. thuringiensis*. Ermakova *et al*[8] have isolated three plasmids of molecular weight of $5{\cdot}9 \times 10^6$, 10×10^6 and $10{\cdot}9 \times 10^6$ from *B. thuringiensis* var. *galleriae* strain 612. The functional role of these plasmid DNAs has not been clearly established. It was noticed that when *B. thuringiensis* strain 612 was grown in a liquid or solid medium, there was spore and crystal formation; the existence of the plasmid DNA in those cells was also established[8]; when those cells were transferred to Spizizen salts medium (SM medium) or basal salts medium (BM medium) supplemented with 0·2% w/v sodium citrate, no extrachromosomal DNA was found and the crystal formation was also negligible[8]. In the present study, disappearance of the extrachromosomal DNA was observed depending on cys concentration. The presence of the extrachromosomal DNA was related to the spore coat and the crystal formation (Table I). The spore coat formation and the crystal protein synthesis were observed only when the extrachromosomal DNA was present in an 'extrachromosomal state' (Table I). When the sporulation inhibition was reversed by various means (unpublished data), the extrachromosomal DNA was found to reappear. These results suggest that there could be a correlation between the presence of the extrachromosomal DNA and the formation of the spore coat and the crystal protein. It could be possible that under certain physiological conditions, like high concentration of cys or transferring of the culture to a non-sporulating medium as performed by Ermakova *et al*[5], the extrachromosomal DNA gets integrated into the genomic DNA, so that it is not isolatable (Table I). Further, it is conceivable that the genes of the extrachromosomal DNA could be expressed only when it is in the extrachromosomal state, and not in an integrated state. These results show that there is a clear relationship between the presence of the extrachromosomal DNA and the production of the crystal and the spore coat in *B. thuringiensis* var. *thuringiensis* (Table I). Probably, the crystal and some of the major spore coat protein are synthesized from similar species of mRNA coded for by the extrachromosomal DNA of *B. thuringiensis* var. *thuringiensis*.

The calculated molecular weight of the extrachromosomal DNA based on the studies on electron microscopy was found to be less than that obtained by agarose gel electrophoresis. Such an altered molecular weight was obtained for λ-DNA also (Table II). Multiple copies of extrachromosomal DNA have been isolated from *B. thuringiensis* by Stahly *et al*[15]. The results obtained suggest that only one copy of extrachromosomal



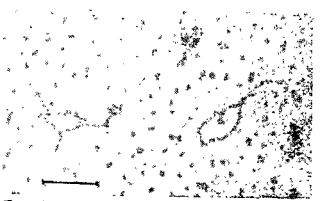


FIG. 4. Electron micrograph of the extrachromosomal DNA. Bar represents 1 μm.

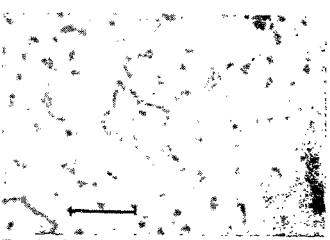


FIG. 5 The dimer molecule. Bar represents 1μm.

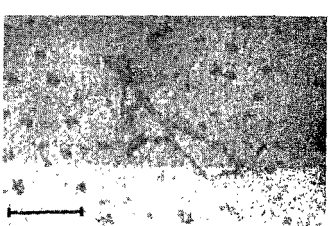


FIG. 6. The '8-shaped' molecule. Bar represents 1μm.

IG. 3. Fluorographic pattern of the extrachromosomal DNA. Btt—*B. thuringiensis* var. *thuringiensis* ; – λ DNA. The top spot corresponds to the contamination from the host DNAs. The bottom spot s the broken pieces of the DNA which move along with the dye band, *i.e.*, bromophenol blue. The niddle spot corresponds to the extrachromosomal DNA of *B. thuringiensis* var. *thuringiensis* and the -DNA.

IGS. 7 and 8. The '8-shaped' molecule. One of the circles of '8-shaped' structure is broken, robably during isolation. Bar represents 1 μm.

)NA was isolated in *B. thuringiensis* var. *thuringiensis*. This could be due to the fact hat the culture was maintained routinely on nutrient agar and extensively subcultured. During such process the other copies of the extrachromosomal DNAs, which would have been present earlier, could have lost, leaving the molecules of molecular weight 10×10^6. The presence of the dimer molecules and the '8-shaped' molecules suggest the presence of more than one copy of the extrachromosomal DNA per bacillus, since the presence of the '8-shaped' molecules has been recognised as intermediate molecules during enetic recombination[3,12].

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