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COMPARATIVE PROFILES OF EXTRACHROMOSOMAL DNA IN SINGLE AND MULTIPLE CRYSTALLIFEROUS STRAINS OF *BACILLUS THURINGIENSIS* VARIETY *KURSTAKI*

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Introduction

Bacillus thuringiensis is a spore forming soil bacterium that is pathogenic to mainly lepidopteran larvae. It has been studied intensively during the last 2 decades because of its promise for the biological control of insects. During sporulation a toxic protein parasporal crystal that is thermolabile and soluble in alkaline solutions is formed in the vegetative cells. Except in *B. thuringiensis* isolate BA-068[13,14] and *B. thuringiensis* var. *darmstadiensis*[8,9], which are bicrystalliferous, and one strain of *B. thuringiensis* var. *kurstaki* that has been recently isolated is multicrystalliferous (S. V. AMONKAR, personal communication), only 1 crystal/vegetative cell is usually produced. The temporal relationship of the formation of the parasporal crystal during sporulation sometimes varies among strains as does the site of synthesis within the cell.

Despite the fact that *B. thuringiensis* is used commercially in the United States and in some other countries on lettuce, cole crops, leafy vegetables, tobacco, and forests, either as an alternative to insecticides or in combination with them, its genetics is not well understood. However, there is some evidence that plasmid DNA may be involved in the synthesis of the parasporal crystals (*B. thuringiensis* δ-endotoxin) responsible for the pathogenicity of *B. thuringiensis* to insects[2,3,5,15]. For example, STAHLY *et al.*[16] examined crystalliferous and acrystalliferous strains of *B. thuringiensis* var. *kurstaki* and found that crystalliferous strains contained at least six extrachromosomal

DNA molecules. All nontoxic acrystalliferous mutants lacked the complete array present in the wild-type toxic strains, an indication of a relationship between presence of plasmid(s) and toxicity. Furthermore, the very high frequency of the acrystalliferous mutants found suggested involvement of an unstable genetic element such as a plasmid.

ERMAKOVA *et al.*[3] demonstrated that when *B. thuringiensis* var. *galleriae,* was grown in selective media that inhibited crystal formation, no extrachromosomal DNA could be isolated from the cells. Thus, these results suggested a correlation between the presence of plasmid DNA and the formation of crystals.

Also, the results of their medium-shift experiments support their hypothesis that plasmid DNA may have a chromosomal origin, that is, it may result from specific excision and amplification of certain chromosomal DNA segments. This explanation is also consistant with previously reported experiments with *B. megaterium* plasmids[1]. GALUSKA and AZIZBEKYAN[5] found that no extrachromosomal DNA elements could be detected after certain *B. thuringiensis* strains were cured with ethidium bromide or cultured under extreme conditions (increased temperature, alkaline pH of medium) and selected for acrystalline strains. DEBABOV *et al.*[2] obtained similar results.

The present study was undertaken in an attempt to correlate the presence of plasmids with the production of parasporal crystals by comparing the extrachromosomal DNA profiles of single and multiple crystalliferous strains of *Bacillus thuringiensis* var. *kurstaki* and to ascertain whether or not there is a correlation between plasmid profiles and strains of the same serotype.

Materials and Methods

Bacterial Strains

Single crystalliferous strains of *B. thuringiensis* var. *kurstaki* (HD-1, HD-73) were obtained from Dr. HOWARD T. DULMAGE, USDA, Brownsville, Texas, USA. The HD-1 commercial strain has a potency of 15,400 International Units/mg (IU/mg) against *Heliothis virescens* and 39,400 IU/mg against *Trichoplusia ni,* while the HD-73 strain has a potency of 34,500 IU/mg against *H. virescens* and 29,300 IU/mg against *T. ni* (H. T. DULMAGE, personal communication). The multicrystalliferous strain (2～5 parasporal crystals/vegetative cell) was obtained from Dr. S. T. AMONKAR, Bhabha Atomic Research Centre, Trombay, Bombay. Potency against *H. virescens* or *T. ni* has not been reported. All three strains belong to serotype 3 a, 3 b (Based on classification according to Dr. H. de BARJAC, Institut Pasteur,

Paris, France; personal communication).

Cell Growth

Overnight cultures (16~18 hr) of the *B. thuringiensis* strains were grown in 50 ml of Difco[1] nutrient broth at 30°C using 300 ml triple-baffled nephelo culture flasks. Then, 6 ml of the overnight culture were inoculated into 60 ml of Difco tryptose phosphate broth and reincubated (2~3 hr) until the synchronized culture had reached 10~15% optical transmission. This step is vital for obtaining full cell lysates of the relatively lysis resistant strains of *B. thuringiensis*. The cells are finally harvested by centrifugation at 700 g for 20 min; then the cells are washed once in 1/10 volume of 50 m Tris-HCl buffer (pH 8.0) and recentrifuged.

Cell Lysis and Extrachromosomal DNA Isolation

Cells were suspended in approximately 4 ml of 25% sucrose-0.1 M NaCl-50 mM Tris buffer (pH 8.0), 1 ml of lysozyme (3x cryst., Calbiochem, San Diego, California) (36 mg/ml in 0.1 M NaCl-8.9 mM boric acid -50 mM disodium EDTA -89 mM Tris buffer, pH 8.2) was added followed immediately by the addition of 500 μl (1 mg/ml in 0.4 M sodium acetate buffer, pH 4.0) RNase A (Worthington Biochemical Corporation, Freehold, New Jersey), preincubated at 98°C for 2 min, and the whole mixture was incubated for 45 min at 22°C. Pronase (grade B, Calbiochem, San Diego, California) in 178 mM boric acid -2.5 mM disodium EDTA -178 mM Tris buffer, pH 8.0 (electrophoresis buffer), and preincubated for 30 min at 37°C, was added to a final concentration of 1 mg/ml, and the mixture was incubated for 60 min at 22°C. Lysis was brought about by the addition of 20% SDS in electrophoresis buffer to a final concentration of 2%. An equal volume of 4 M NaCl was added to the cleared lysate, and the whole mixture was left overnight at 4°C. The mixture was centrifuged for 25 min at 13,000rpm (Sorvall RC2B), the supernatant fluid was carefully decanted, any remaining white flocculent material in the supernatant fluid removed with a Pasteur pipette, and 2 volumes of cold 95% ethanol (EtOH) were added. The mixture was stored overnight at -20°C, and then centrifuged at 13,000 rpm for 20 min; the supernatant fluid was discarded. The pellet was dissolved in 0.5~1.0 ml of electrophoresis buffer.

1 Mention of a proprietary product or a company name in this paper does not constitute an endorsement by Hokkaido University or the U. S. Department of Agriculture.

Agarose Gel Electrophoresis

Extrachromosomal DNA's were resolved on vertical 0.7% agarose gels [Agarose (ME), Miles Laboratories, Inc. (Marine Colloids), Elkhart, Indiana] prepared in electrophoresis buffer (178 mM Tris −2.5 mM disodium EDTA-178 mM boric acid, pH 8.0) and dissolving in a boiling water bath. After the homogenous solution was cooled to 60°C, it was poured into the slab gel apparatus. For analytical gel electrophoresis, a slab gel $12 \times 16 \times 0.3$ cm was prepared. The samples (200 μl) were prepared for gel electrophoresis by adding 40 μl of 0.7% bromophenol blue in 50% glycerol (electrophoresis buffer) and made to 0.7% SDS. The DNA preparations were applied to the sample slots in 20~120 μl volumes. Also, samples containing phage DNA (ϕ29, ITO *et al.*, 1976) that had been digested with Eco-R1 restriction endonuclease (molecular weights: 6.11, 3.89, 1.08, 0.54, and 0.36×10^6 daltons, respectively), samples containing a commercial preparation (Bethesda Research Laboratories, Rockville, MD) of *Staphylococcus aureus* plasmid pUB 110 (LACEY and CHOPRA[11], molecular weight: 3.0×10^6 daltons), *Agrobacterium tumefaciens* TI plasmid (WATSON *et al.*, 1975; molecular weight 120×10^6 daltons), λ phage (SZYBALSKI and SZYBALSKY[17]; molecular weight: 33.0×10^6 daltons), and λ DNA-Hind III fragments (SZYBALSKI and SZYBALSKI, 1979; molecular weights: 16.01, 6.42, 4.46, 2.90, 1.42, 1.28, and 0.40×10^6 daltons, respectively) were also applied and used as standards in the various experiments for estimation of relative molecular weights. Electrophoresis was carried out at 2 mA for 15 min followed by 40 mA for 5 hr. The gel was stained in electrophoresis buffer with 0.5 μg of ethidium bromide/ml for 30 min and photographed on a short wave transilluminator (type C61, Ultraviolet Products, Inc., San Gabriel, CA) with Polaroid type high-speed No. 55 film and a Kodak No. 23 A red filter. Relative molecular weights of extrachromosomal DNA elements was estimated as described by ITO and KAWAMURA[6] from a standard curve.

Results and Discussion

The possibility that the *B. t.* δ-endotoxin (parasporal crystal) gene instructions may be plasmid-borne led to the screening and comparision of extrachromosomal DNA elements in three strains of *B. thuringiensis* var. *kurstaki*, each of which elicits different toxicities to susceptible insects and/or produces either single or multiple parasporal crystals per vegetative cell. Typical results from a number of experiments are shown in Fig. 1, and estimates of the relative sizes of the extrachromosomal DNA elements are summarized in Table 1. A total of 16 DNA bands that ranged from 0.69

to $>200\times10^6$ daltons appeared on the agarose gels of *B. thuringiensis* var. *kurstaki* (HD-1). This strain appears to have four giant DNA elements that band above the chromosomal DNA. Below the chromosomal DNA twelve bands can be seen corresponding to extrachromosomal DNA sizes found in other *Bacillus* species. Likewise, strains HD-73 and the multi-crystalliferous strain have four giant DNA elements that band above the chromosomal DNA; however, only eleven DNA elements can be seen below the chromosomal DNA in contrast to the twelve identified in the HD-1 strain. The extrachromosomal DNA profile of the HD-1 strain is similar to the results reported in a earlier communication[4], and by MARTIN and DEAN[12], and STAHLY *et al.*,[15] except for the additional elements isolated as a result of the improved lysis procedure described here. Unfortunately, the data does not lend itself to make a definitive statement concerning plasmid

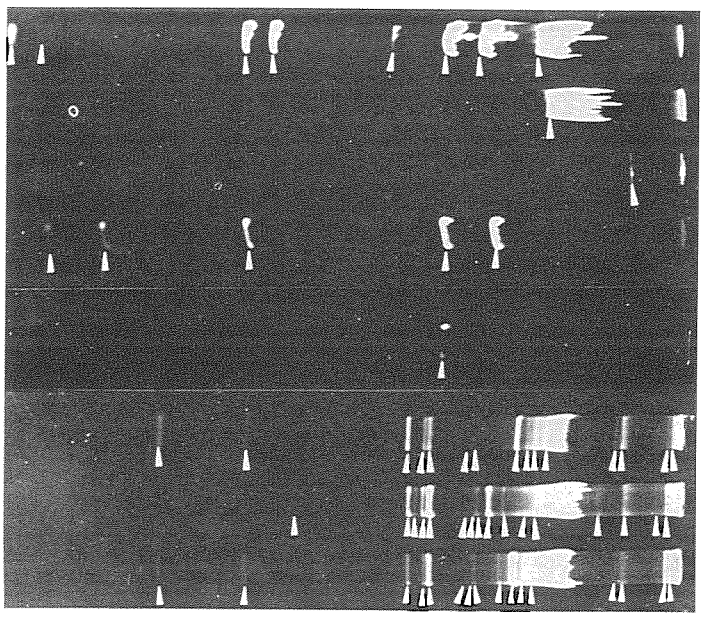

Fig. 1. Representative comparison of numbers of extrachromosomal DNA elements isolated from single and multiple crystalliferous strains of *Bacillus thuringiensis* var. *kurstaki* on agarose gels subjected to electrophoresis. DNA preparations and standards were applied to the sample slots (20-120 μl) of 0.7% agarose slab gel (Tris-EDTA-borate buffer at pH 8.0) and electrophoresis was carried out at 2 mA for 15 min followed by 40 mA for 5 hr. Direction of migration is from the right to the left. White pointers indicate the positions of extrachromosomal DNA elements as revealed on the original photographic negatives. The unmarked thick white bandings in the *kurstaki* slots are chromosomal DNA.

TABLE 1. Relative Molecular Weights (Daltons $\times 10^6$)[a] and Numbers of Extrachromosomal DNA in Single and Multiple Crystalliferous Strains of *Bacillus thuringiensis* var *kurstaki*

kurstaki (HD-1)	*kurstaki* (HD-73)	*kurstaki* (Multicrystalliferous)
275.42	—	281.84
263.03	263.03	263.03
—	194.98	—
95.50	95.50	97.72
87.10	—	85.11
—	53.70	—
—	—	18.62
13.49	14.79	15.13
10.96	12.02	12.02
9.55	—	9.77
7.58	7.76, 6.02	—
5.37, 5.25	5.49, 5.37	5.37
4.90	4.90	4.90
3.71	3.71	3.71
3.63	3.55, 3.47	3.63
3.16	3.31	3.23
1.17	—	1.17
0.69	—	0.69

a Estimates of molecular weights were determined from a standard curve (log molecular weights vs. log relative mobility) by mobilities relative (R_f) to the DNA standards included in the agarose gels as described by ITO and KAWAMURA (1976).

profile and single versus multicrystalliferous production of parasporal crystals. One major difference between the multicrystalliferous strain and the two single crystalliferous strains that can be noted is the presence of the 18.62×10^6 dalton DNA band in the former strain. Other differences can be noted among all three strains examined that make any attempt at correlation between toxicity, extrachromosomal DNA profile, and parasporal crystal production exceedingly difficult.

We originally thought that extrachromosomal DNA patterns of different strains may be correlated with serotype. The data, however, demonstrate that while strains of the same serotype had some extrachromosomal DNA elements in common, differences do exist in the DNA profiles of strains

belonging to the same serotype. In fact, KRYWIENCZYK *et al.*,[10] has distinguished on the basis of serology two groups within *B. thuringiensis* var. *kurstaki* (serotype 3 a, 3 b). The two groups were DULMAGE's isolates HD-1 and HD-73. It was suggested that serotype 3 a, 3 b be separated into two subgroups designated as K-1 and K-73 to correspond with the HD-1 and HD-73 crystal serotypes, respectively. We can also distinguish between these two isolates based on their comparative extrachromosomal DNA profiles. In contrast MARTIN and DEAN[12] reported that extrachromosomal DNA from two different *B. thuringiensis* isolates (serotype 1) had identical patterns, even though the strains were obtained from different sources. Also, after examining two *kurstaki* HD-1 cultures having widely different histories, MARTIN and DEAN[12] found the plasmid patterns also essentially identical. However, a third HD-1 culture (derivative of Abbott's Dipel strain grown at 37°C) that was selected as a double mutant resistant to streptomycin and sodium azide showed a change in plasmid pattern that reflected the sensitivity of this strain to ampicillin present in the parent strain. The sensitivity to ampicillin was correlated with the loss of a 9.64×10^6 dalton plasmid. This strain still produced a crystal and was as toxic to *Trichoplusia ni* larvae as the parent strain.

Further genetic and biochemical studies are obviously necessary to determine the biological functions of extrachromosomal DNA elements in *B. thuringiensis* and to definitely determine which are indeed autonomous replicons that may be useful in genetic manipulation to improve effectiveness of this bacterium. DNA-mediated transformation experiments of individual extrachromosomal DNA elements that prove to be autonomous replicons into acrystalliferous strains to elucidate the gene(s) controlling synthesis of the *B. t.* δ-endotoxin should prove useful in these efforts. Further investigations in this area are continuing.

Summary

Extrachoromosomal DNA from single and multiple crystalliferous strains of *Bacillus thuringiensis* var. *kurstaki* (serotyp 3 a, 3 b) were consistently isolated using a procedure which optimizes lysis of the normally lysozyme resistant cells. The results obtained can be summarized as follows:

1. A variety of large and small extrachromosomal DNA elements were readily visualized with agarose gel electrophoresis that generally included nine between 0.69 and <9.0 megadaltons (Mdal) in size, three between 0.9 and <50 Mdal, two between 50 and 200 Mdal, and two that were larger than 200 Mdal.

2. The HD-1 strain contained as many as 16 extrachromosomal DNA elements while only 15 were isolated from the HD-73 and the multicrystalliferous strains.

3. Only one major difference between the multicrystalliferous and the two single crystalliferous strains that could be noted was the absence of an 18.62 Mdal DNA element in the single crystalliferous strains, although a number of other differences in the extrachromosomal DNA profiles was also noted among all three strains which makes it futile to attempt to correlate the production of parasporal crystals with any particular extrachromosomal DNA element(s).

4. Although strains of the same serotype have a number of extrachromosomal DNA elements in common, the data demonstrate that there can be differences in extrachromosomal DNA patterns between strains of the same serotype.

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