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Author(s)	IIZUKA, Toshihiko; Faust, Robert M.
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**DETECTION AND CHARACTERIZATION OF
NATURALLY OCCURRING PLASMIDS
IN *BACILLUS CEREUS* ISOLATES
BY AGAROSE GEL ELECTROPHORESIS**

Toshihiko IZUKA

(Laboratory of Sericology, Faculty of Agriculture,
Hokkaido University, Sapporo, Japan)

Robert M. FAUST

(Insect Pathology Laboratory, USDA, ARS
Beltsville, Maryland, USA)

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Introduction

Plasmid deoxyribonucleic acid (DNA) has been found in sporeforming bacteria such as *Bacillus subtilis*^{5,20,30,31,42,43)}, *Bacillus pumilus*,^{28,29,31,32)} *Bacillus megaterium*,^{4,5,6,7,8,21,31)} *Bacillus cereus*,^{3,35)} *Bacillus thuringiensis*.^{9,10,12,14,15,23,24,34,35,36,40,41,45)} In the *Bacillus* the function of these plasmids is not clearly understood, although in a few *Bacillus* and other genera a variety of specific biochemical functions (fertility, resistance to antibiotics, production of bacteriocins, production of toxins, etc.) have been attributed to these genetic elements. Also, some evidence has been presented to indicate that plasmids may be involved in the synthesis of the parasporal crystal toxin responsible for pathogenicity of *Bacillus thuringiensis* to insects.^{16,38)}

Bacillus cereus is of special interest to us because of its close taxonomic relationship with the entomopathogen *B. thuringiensis* which makes *B. cereus* potentially useful as a plasmid recipient in studies on gene control of the δ -endotoxin in *B. thuringiensis*.¹¹⁾ The primary characteristic for the separation of *B. thuringiensis* from *B. cereus* has been the formation of the crystalline parasporal bodies,¹⁸⁾ which are associated with the toxicity of these bacilli for insects. Classification of the crystalliferous insect pathogens, reviewed and reported by de BARJAC and BONNEFOI,¹⁾ HEIMPEL,²²⁾ KRIEG,²⁷⁾ and ROGOFF,³⁷⁾ range from their actual separation into several species and varieties to the belief that these crystal-forming pathogens are so similar to *B. cereus* that they do not deserve even the varietal status proposed by

SMITH *et al.*³⁹⁾ Further, KRIEG²⁷⁾ after observing that the flagella antigens of strains of *B. cereus* and *B. thuringiensis* overlapped concluded that nontoxic acrySTALLIFEROUS mutant strains of *B. thuringiensis* had to be recognized as strains of *B. cereus*. Also, data of GORDON *et al.*¹⁷⁾ indicated that 15 strains isolated from diseased insects should not be separated from their strains of *B. cereus* and subsequently listed the strains as *B. cereus* var. *thuringiensis*. KRIEG,²⁸⁾ LYSENKO³³⁾ and VANKOVA⁴⁴⁾ have also suggested that crystal-formation as a taxonomic character is unreliable from observations of freshly prepared and stored cultures (some cultures had lost the ability to form crystals). Finally, several investigators have reported that loss of the ability to form crystals occurs at a much higher frequency than that ordinary due to spontaneous mutation.^{13,44)} Such instability of a phenotypic property is suggestive of plasmid inheritance. At the present time, Bergey's Manual of Determinative Bacteriology²⁾ lists the varieties of this organism as subspecies of *B. thuringiensis* based on the production of the crystalline parasporal body.

We have previously reported plasmid profiles in 26 *B. thuringiensis* strains.^{23,24)} This communication now reports the presence of naturally occurring plasmid DNA in various strains of the closely related acrySTALLIFEROUS *B. cereus* and compares the results with our earlier reports for *B. thuringiensis*.

Materials and Methods

Bacterial Strains

Table 1 lists the strains and sources of *B. cereus* used in these studies.

Cell Growth

Overnight cultures (16-18 hr) of the *B. cereus* strains were grown in 50 ml of Difco¹ nutrient broth at 30°C using 300 ml triple-baffled nephelo culture flasks. Then, 6 ml of the overnight culture was inoculated into 60 ml of Difco tryptose phosphate broth and reincubated (2-3 hr) until the synchronized culture had reached 10-15% optical transmission. 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 mM 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

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.

TABLE 1. Sources of *Bacillus cereus* and Variety *mycoides* Strains Used in the Isolation and Molecular Weight Determinations of Indigenous Extra-chromosomal DNA Elements

Varietal epithet/strain		Source
<i>Bacillus cereus</i>	C-98	Laboratory of Sericology, Faculty of Agriculture, Hokkaido University; isolated from digestive juice of the silkworm.
<i>B. cereus</i>	AHU 1030	Laboratory of Culture Collection of Microorganisms, Faculty of Agriculture, Hokkaido University; isolated from soil.
	AHU 1357	Isolated from beet-top silage at Nakashibetsu, Japan.
	AHU 1555	Isolated from beet-top silage at Nakashibetsu, Japan.
	AHU 1560	Isolated from beet-top silage at Nakashibetsu, Japan.
	AHU 1563	Isolated from beet-top silage at Nakashibetsu, Japan.
	AHU 1567	Isolated from beet-top silage at Nakashibetsu, Japan.
	AHU 1572	Isolated from beet-top silage at Nakashibetsu, Japan.
<i>B. cereus</i> var. <i>mycoides</i>	AHU 1360	Isolated from beet-top silage at Bekkai, Japan.

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 μ l (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), 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 at 22°C 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 13,000 rpm (Sorvall RC2B), the supernatant fluid was carefully decanted, any remaining white flocculent material in the supernatant fluid was 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.

Agarose Gel Electrophoresis

Extrachromosomal DNA's were resolved on horizontal 0.7% agarose gels prepared by dissolving agarose [Agarose (ME) Miles Laboratories, Inc. (Marine Colloids), Elkhart, Indiana] 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 (Wakamori horizontal gel electrophoresis unit). For analytical gel electrophoresis, a slab gel 131 × 135 × 50 mm was prepared. The samples (100 μl) were adjusted with 20 μl of 50% glycerol (electrophoresis buffer) and made to 0.7% SDS. The DNA preparations were applied to the sample slots in 20-50 μl volumes. Also, samples containing λ DNA (Boehringer Mannheim, Indianapolis, IN) that had been digested with Eco RI and Hind III restriction endonucleases [molecular weights: 14.32, 3.40, 3.30, 2.82, 2.30, 1.31, 1.25, 1.05, 0.90, 0.62, 0.55, 0.37 and 0.09 megadaltons (Mdal), respectively] and *B. thuringiensis* var. *sotto* plasmid (IIZUKA *et al.*,²³ molecular weight: 107.15) 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 C 62, Ultraviolet Products, Inc., San Gabriel, CA) with Polaroid type high-speed No. 55 film and a Kodak No. 23 A red filter.

Standard Curve and Estimation of Extrachromosomal DNA Size

A standard curve (Fig. 1) was constructed from agarose gel electrophoresis data for the DNA standards whose range of molecular size varied from 0.37 to 107.15 Mdal on the gels. Molecular weights above 107.15 Mdal were linearly extrapolated from the standard curve of the selected agarose gel. The relative molecular weights of extrachromosomal DNA

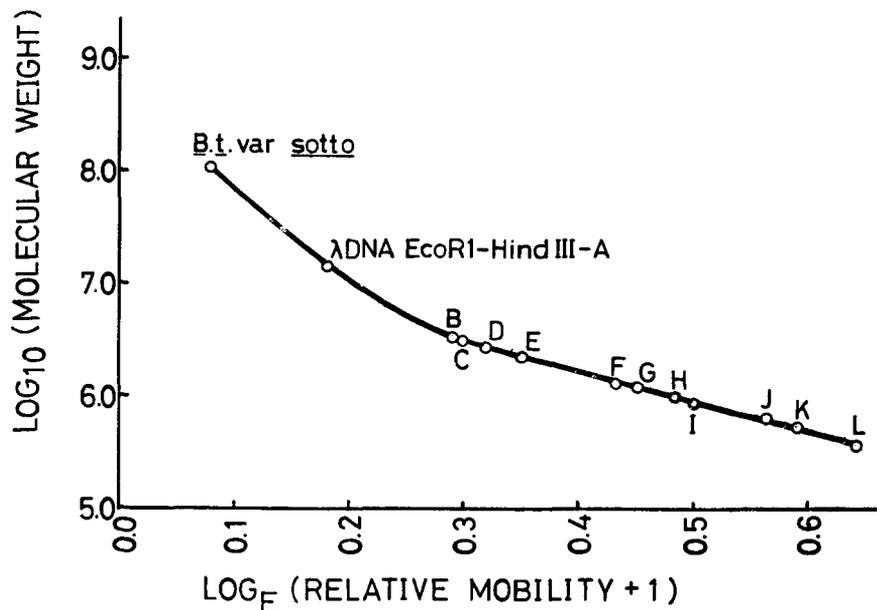


Fig. 1. Standard curve showing the relationship between relative mobility and molecular weight of different DNA — standard samples electrophorised in agarose gel. Twelve Eco R1-Hind III generated fragments of λ DNA and the *Bacillus thuringiensis* var. *sotto* plasmid were used as standards in estimating molecular weights of DNA molecules from strains of *Bacillus cereus* described in the text of this communication. Molecular weights of the DNA standards ranged from 0.09 to 107.15 megadaltons (Mdal). To give a positive logarithm, 1.0 was added to each relative mobility value. For sources of DNA standards, molecular weight data, and electrophoresis conditions see the materials and methods.

elements from the standard curve were determined essentially as described by ITO and KAWAMURA²⁵⁾ and HANSEN and OLSEN.¹⁰⁾

Results and Discussion

The plasmids of a variety of *B. cereus* strains, including one strain (AHU 1030) that produces parasporal-body like inclusions, range from more than 200 to approximately 1 Mdal in molecular mass. Typical results from a number of experiments are shown in Fig. 2, and estimates of the relative sizes of the extrachromosomal DNA elements are summarized in Table 2.

It is evident that each strain, including the one crystalliferous-like strain (AHU 1030), contains its own distinct array of extrachromosomal DNA

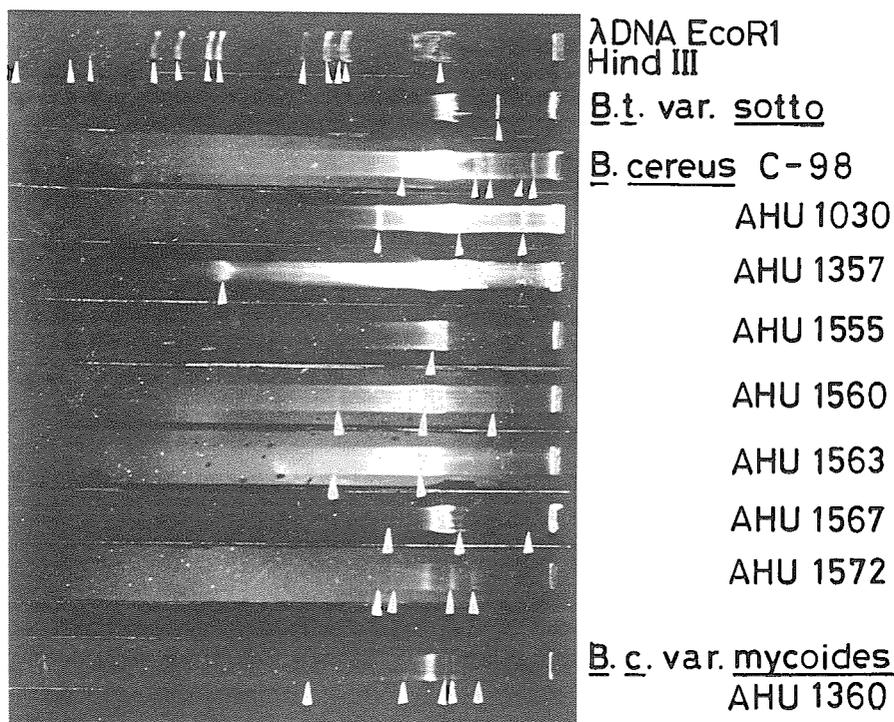


Fig. 2. Representative comparison of numbers of extrachromosomal DNA elements isolated from strains of *Bacillus cereus* on agarose gels subjected to electrophoresis. DNA preparations and standards were applied to the sample slots (20–50 μ l) of 0.7% agarose slab gel (Tris-EDTA-borate buffer at pH 8.0) 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 slots are chromosomal DNA.

elements. The number of plasmid size classes per strain ranged from as few as 1 (in AHU 1357, AHU 1515) to as many as 5 (in C-98, var. *Mycooides* AHU 1360). Three strains (C-98, AHU 1030 and AHU 1567) harbored giant extrachromosomal DNA above molecular weight of 200 Mdal; whereas all the strains examined carried DNA elements less than 200 Mdal. The plasmid complements of most of these strains are of a complexity somewhat comparable to that of the *B. thuringiensis* complex plasmid system,²³⁾ which consists of 1 to 16 size classes. Indeed, several of the plasmids isolated from the *B. cereus* strains are similar in size to plasmids present in *B.*

TABLE 2. Relative Molecular Weights and Numbers of Extrachromosomal DNA in Strains of *Bacillus cereus* and var. *mycoides* as Determined by Agarose Gel Electrophoresis

Varietal epithet/strain	Estimated molecular weight ^a (Megadaltons)				Total DNA elements
	>200	<200~50	<50~10	10>	
<i>Bacillus cereus</i> C-98	281.00 200.00	70.80	44.60	6.60	5
<i>B. cereus</i> AHU 1030	200.00		21.40	5.02	3
AHU 1357				1.34	1
AHU 1555			13.90		1
AHU 1560		79.40	10.90	2.34	3
AHU 1563			10.90	2.34	2
AHU 1567	280.00		31.60	5.90	3
AHU 1572			44.60 23.40	6.60 5.02	4
<i>B. cereus</i> var. <i>mycoides</i> AHU 1360		56.20	24.40 15.90	7.37 2.52	5

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 KAWAMURA²⁵) and HANSEN and OLSEN¹⁹).

thuringiensis. For example, IIZUKA *et al.*,²⁸) examined 17 serotypes (24 strains) of *B. thuringiensis* for the presence of extrachromosomal DNA by agarose gel electrophoresis. The number of plasmid bands based on the electrophoresis profiles ranged from one for *B. thuringiensis* var. *sotto* and var. *thompsoni* to 16 for *B. thuringiensis* var. *kurstaki*. The reported plasmid profiles consisted of both CCC and OC forms with molecular weights of the CCC forms ranging from less than 1 Mdal to greater than 100 Mdal, depending on the strain. In serological identical varieties (*thuringiensis* Berliner and *thuringiensis* BA-068, serotype 1; *sotto* and *dendrolimus*, serotype 4 a, 4 b; *subtoxicus* and *entomocidus*, serotype 6), only serotype 6 strains showed similar extrachromosomal DNA profiles and while a number of strains of *B. thuringiensis* had some extrachromosomal DNA elements in common, distinct differences were observed in the DNA profiles between serotypes and even among strains of the same serotype.

A concomitant study by the same research team²⁴) compared the plasmid

profile of single and multiple crystalliferous strains of *B. thuringiensis* var. *kurstaki*. Plasmid DNA from the multicrystalliferous strain (2-5 crystals/cell) was compared to *B. thuringiensis* var. *kurstaki* (HD-1 and HD-73) in an attempt to correlate the presence of plasmids with the production of parasporal crystals and to ascertain whether or not there is a correlation between plasmid profiles and strains of the same serotype. An 18.62 Mdal plasmid was seen in the multicrystalliferous strain that was not detected in HD-1 or HD-73 strains of *B. thuringiensis*. However, other differences in the plasmid profile made it futile to associate plasmid DNA with crystal production.

Since all the strains of *B. cereus* examined harbor plasmids that have different physical properties, these strains are indeed independent isolates. A frequent occurrence of plasmids of different sizes has also been observed in *B. cereus* strains by BERNHARD *et al.*³¹ These investigators examined 15 isolates and found that 12 out of 15 strains contained one or two plasmids. One strain harbored three plasmids. Their molecular weights ranged from 1.6 to 105 Mdal. Bacteriocin production could be attributed to a 45 Mdal plasmid (pBC 7) from *B. cereus* DSM 336, and tetracycline resistance to a 2.8×10^6 plasmid (pBC 16) from *B. cereus* GP 7. MITEVA³⁵ also reported the presence of plasmid DNA in 3 out of 6 strains of *B. cereus* examined. Unfortunately, the molecular weights and numbers of DNA elements were not indicated in the study.

Comparison of the plasmid patterns in Fig. 2 shows that the crystalliferouslike strain (AHU 1030) contains a plasmid system similar in complexity to that of the related acrySTALLIFEROUS strains. Other laboratories have reported that the loss of parasporal crystal toxin production in *B. thuringiensis* is accompanied by the loss of all extrachromosomal DNA.^{9,14,41} MITEVA,³⁵ however, revealed that acrySTALLIFEROUS strains may still possess a complex array of plasmid molecules similar to our results reported here.

Recently, the question of whether production of the parasporal crystal toxin is associated with a specific plasmid has been investigated by GONZALEZ *et al.*¹⁶ Plasmid patterns of both crystal formers (Cry⁺) and acrySTALLIFEROUS mutants (Cry⁻) were analyzed. Loss of crystal production was associated with the loss of a 75 Mdal plasmid in *B. thuringiensis* var. *thuringiensis*. A 50-Mdal plasmid of *B. thuringiensis* var. *kurstaki* (HD-73) was lost in the Cry⁻ mutants. Crystal production in *B. thuringiensis* var. *alesti* appeared to be associated with a plasmid about 105 Mdal in size. In *B. thuringiensis* var. *kurstaki* (HD-1), a smaller plasmid (29 Mdal in size) seemed to be involved. In *B. thuringiensis* var. *galleriae*, a large plasmid (~130 Mdal in

size) was implicated in crystal production. The evidence presented by these investigators suggests that in each strain only a single plasmid is involved, although the size of the implicated plasmid varies among strains.

Obviously, conclusive proof of the postulated association of *B. t.* parasporal crystal toxin determinants with plasmids require the demonstration that acrySTALLIFEROUS *Bacillus* can produce parasporal crystal toxin by genetic transformation of presumed toxin-coding plasmid. We are now in a position to undertake a definitive investigation of plasmid DNA involved in toxin production. Preliminary results with an acrySTALLIFEROUS derivative of *B. thuringiensis* var. *israelensis* show the consistent loss of a plasmid DNA element and will be described elsewhere. Transformation of this plasmid into strains of *B. cereus* investigated here will be carried out to clarify the toxin-plasmid relationship.

Summary

Extrachromosomal DNA molecules of 9 different *B. cereus* strains were consistently isolated and partially characterized on agarose gels. The results obtained can be summarized as follows:

1. Nine strains of *B. cereus* contained extrachromosomal DNA molecules of various sizes that were readily visualized with agarose gel electrophoresis and that ranged from >200 to approximately 1 megadalton (Mdal), depending on the strain.

2. A determination of the numbers of extrachromosomal DNA molecules based on agarose gel profiles revealed that there were 1 for strains AHU 1357 and AHU 1555, 2 for strain AHU 1563, 3 for strains AHU 1030, AHU 1560, and AHU 1567, 4 for strain AHU 1572, and 5 for strains C-98, and var. *mycoides* AHU 1360. The plasmid complements of most of these strains are of a complexity somewhat comparable to that of the closely related *B. thuringiensis* and its complex plasmid system reported by other investigators.

3. Each *B. cereus* strain examined, including the one crystalliferous-like strain (AHU 1030), contains its own distinct array of extrachromosomal DNA elements, indicating that each strain is an independent isolate. The results show that the lack of parasporal-body like inclusion production is not necessarily accompanied by the absence of all plasmids as has been reported in acrySTALLIFEROUS mutant strains of *B. thuringiensis*.

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