BACILLUS THURINGIENSIS SUBSP. *PAKISTANI* MUTANTS LOST THE BIPYRAMIDAL FORM CRYSTALS

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Introduction

Bacillus thuringiensis is an insect pathogenic bacterium which produces a inclusion body also called the crystal. The crystal is made of a protein toxic to many insect species. Numerous strains of *B. thuringiensis* have been isolated and classified into some 20 subspecies according to their flagellar antigen. Among those subspecies, subsp. *pakistani* was isolated in Pakistan by de BARJAC *et al.*.[2] *B. thuringiensis* subsp. *pakistani* is known to produce crystals in bipyramidal shape. Upon ingestion, the *pakistani* crystals are toxic to the larvae of *Carpocapsa pomonella, Pectinophora gossypiella* and *Pieris brassicae.* However, the silkworm, *Bombyx mori* is not sensitive to the *pakistani* crystal.[5]

In the past few years, several *B. thuringiensis* genes each coding for a crystal protein (CP) toxic to larvae of lepidopteran species have been isolated and their nucleotide sequence determined. To date, subsp. *sotto*[6] and *kurstaki* (strains HD-1[7] and HD-73[1]) have been chosen as cloning targets. Unlike subsp. *pakistani*, subspp. *sotto* and *kurstaki* are toxic to the silkworm. In order to understand the differences in the toxicity to the silkworm between subsp. *pakistani* and the other subspecies, one can clone and sequence the CP gene from *pakistani* and compare it with the other CP genes.

In the present study, we have attempted to locate the *pakistani* gene coding for CP. We have made some 30 mutants from a strain of subsp. *pakistani.* It was indicated that the 42.5 Mdal plasmid contains the gene coding for the bipyramidal crystal protein.

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Materials and Methods

Organisms. *B. thuringiensis* subsp. *pakistani* HD-395 (Shaikh 232/14) was obtained from Dr. Dulmage, USDA, Brownsville, Texas, and used.

The bacterium was maintained on nutrient agar (Difco) and cultured for 24 hours at 30°C. Mutants were created by using ethyl methane-sulfonate (EMS)[8,9]. Mutants were chosen on the basis of their crystal production from about 100 colonies. Plasmid patterns and observation of the crystals by scanning electron microscopy were used to confirm the mutation.

Electrophoresis. Extrachromosomal DNA was extracted from the cells and analyzed by electrophoresis in 0.7% agarose gel according to IIZUKA *et al.*[4].

Scanning electron microscopy (SEM). A mixture of crystals and spores was suspended in distilled water and air-dried on an alminum disk. After the sample was coated with carbon and gold, it was observed and photographed with a JEOL model JSM-S1 scanning electron microscope.

Bioassay. Fifth-instar larvae of the silkworm were used for bioassay. A mixture of spores and crystals was suspended in distilled water and the suspension was directly injected to a larval digestive tract with a microsyringe. After the sample was injected, the silkworm larvae were reared on an artificial diet at 25°C. ED_{50} (effective dose) was determined by log-concentration probit analysis.

Results and Discussion

Electron microscope observation revealed that HD-395 produces three crystal types. They are typical bipyramidal form, cylindrical form and small sized irregular form (Fig. 1). Based on our observation by SEM, mutants of HD-395 were classified into 4 morphological groups as listed in Table 1.

Subsp. *pakistani* HD-395 and the selected mutant strains, P3-1, P5-1, and P6-5 from group C (Table 1) were bioassayed against 5th-instar silkworm larvae. These mutants appeared to produce the spores, therefore the bioassay samples contained

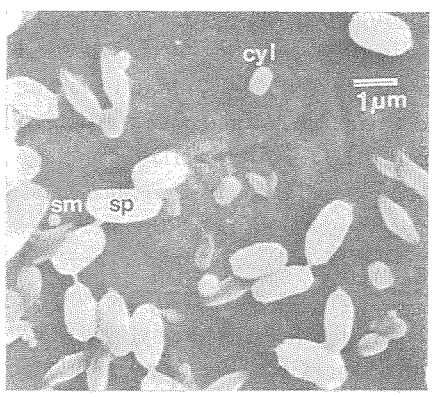

Fig. 1. Electron micrograph of *B. thuringiensis* subsp. *pakistani* HD-395. In the figure, sp indicates spore; cyl, cylindrical crystal; sm, small sized irregular crystals.

TABLE 1. Inducing mutants of *B thuringiensis* subsp. *pakistani* strain HD-395

	Crystal phenotype*				Total
	Group A Bp** + Cyl*** + Irr**** +	Group B Bp + Cyl − Irr −	Group C Bp − Cyl + Irr +	Group D Bp − Cyl − Irr −	
1) Spo +	84 (71.8)	1 (0.9)	18 (15.4)	11 (9.4)	114 (97.4%)
2) Spo −	0	0	1 (0.9)	2 (1.7)	3 (2.6)
Total	84	1	19	13	117

* Number of colonies and percentage in parenthesis.
** Bp: Bipyramidal.
*** Cyl: Cylindrical.
**** Irr: Irregular.

TABLE 2. Toxic activities of *B. thuringiensis* subsp. *pakistani* strain HD-395 and its mutants against silkworm larvae

Strains	Symptoms	ED_{50}*
HD-395	paralysis	500 ng
P3-1	none	∞
P5-1	none	∞
P6-5	none	∞

* 50% effective dose.

not only the crystals but also the spores.

As listed in Table 2, the larvae developed a symptom of paralysis within 24 hours after the injection of HD-395 sample, but they recovered from the paralysis to a healthy condition. This indicates that the bipyramidal crystals of HD-395 are not lethal to the silkworm larvae. It is important to note that the bipyramidal crystal minus mutants did not cause any toxic effects to the silkworm larvae.

In order to locate the CP gene in particular one coding for the bipyramidal crystal, plasmid pattern of the wild type HD-395 was compared with these of its mutants. Plasmid patterns of P3-1. P5-1, and P6-5 are shown in Fig. 3. All bipyramidal crystal minus mutants lost 42.5 Mdal plasmids. This suggests that 42.5 Mdal plasmid contains the CP gene coding for the bipyramidal crystals.

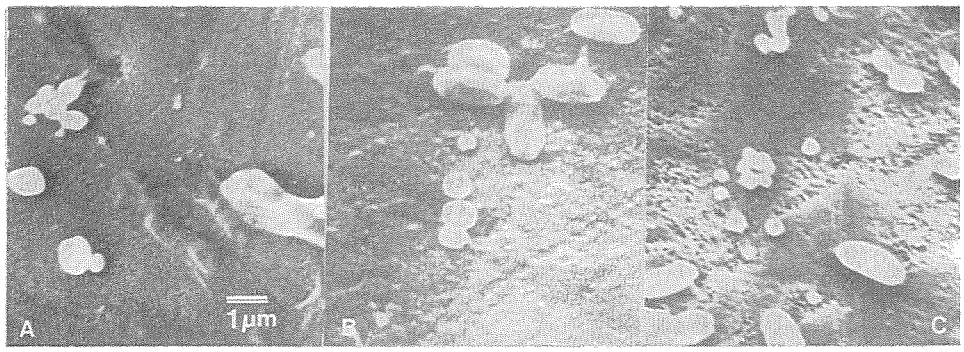

Fig. 2. Electron micrographs of mutant strains. A indicates P3-1; B, P5-1; C, P6-5.

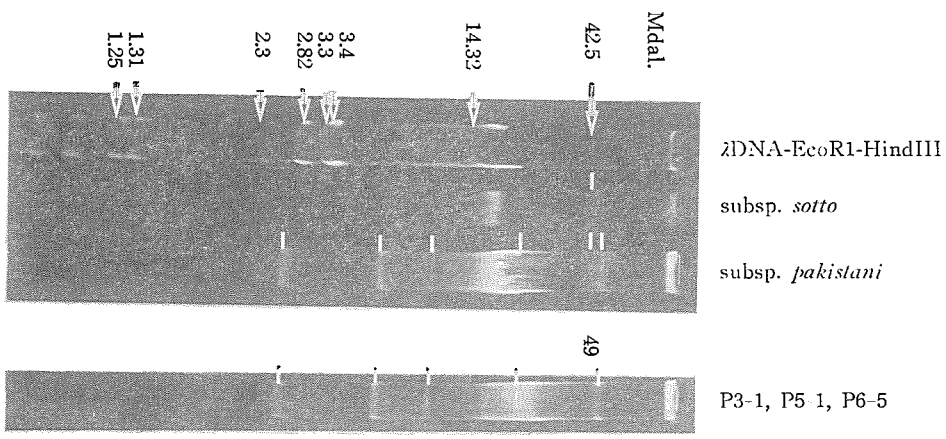

Fig. 3. Electrophoretic analysis of the plasmids of subsp. *pakistani* HD-395 and its mutants.

It has been known that HD-395 produces the crystals having no activity against silkworm larvae. However, the present study has demonstrated that the bipyramidal crystals of HD-395 cause the paralysis to the silkworm larvae. Our results further indicate that CP gene which might code for the bipyramidal crystals, is on the 42.5 Mdal plasmid.

Summary

It was attempted to demonstrate that the location of the gene coding for crystal protein (CP) in *B. thuringiensis* subsp. *pakistani* HD-395.

Thirty mutants which were lost in bipyramidal crystals, have been made from the parent strain and based on the analysis of plasmid pattern, 42.5

Mdal plasmid contains the gene coding for the bipyramidal crystal protein.

According to bioassay of HD-395 to the silkworm larvae, bipyramidal crystal protein causes a paralysis against silkworm larvae, but it is also demonstrated they have recovered.

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