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Characterization and Identification of *Bacillus thuringiensis* subsp. *tenebrionis* SR6 and SR8

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Abstract Physiological and molecular characteristics of *Bacillus thuringiensis* SR6 and SR8 were investigated, and phase contrast and electron microscopies revealed that a large rhomboidal crystal protein was present in the sporulating cells. SDS-PAGE and Western blot analyses showed that *B. thuringiensis* SR8 produced 70 kDa protein much more than other proteins, and that the 70 kDa protein could bind to the antibody of *B. thuringiensis* subsp. *tenebrionis*-crystal toxin protein, indicating that the crystal 70 kDa protein has an immunological homology with *B. thuringiensis* subsp. *tenebrionis*-crystal toxin protein. The DNA fragment of *B. thuringiensis* subsp. *tenebrionis*-toxin gene was detected in *B. thuringiensis* SR6 and SR8 by using PCR amplification analysis. Furthermore, the insect bioassay showed the insecticidal activity against Colorado potato beetle larvae. Based on the physiological and molecular similarities to *B. thuringiensis* subsp. *tenebrionis*, it is suggested that the *B. thuringiensis* SR6 and SR8 may be mutants of the *B. thuringiensis* subsp. *tenebrionis* strain overexpressing the crystal of 70 kDa toxin protein.

Key words: *Bacillus thuringiensis*, crystal protein, coleoptera

Bacillus thuringiensis (*B.t.*) is a Gram-positive, spore-forming bacterium characterized by parasporal crystalline protein inclusion. The crystal proteins are highly toxic to pests and specific in their activity [1, 12]. It captured attention of relatively few microbiologists over the first 70 years following its discovery at the beginning of this century. Nowadays, however, scientists from a wide range of disciplines are attempting to probe its secrets at the molecular, physiological, and ecological levels. Today, *B. thuringiensis* is not only the most successful commercial insecticide with worldwide application for protection of crops, forests, and human health, but it also started to replace conventional chemical insecticides in several areas of application. The basis for the current worldwide endeavor to develop a new generation of more effective and environmentally acceptable insect control products comes from relatively low development costs together with a high natural diversity of strains and toxins and good prospects for genetic manipulation.

From this point of view, we have identified and characterized two *B. thuringiensis* subsp. *tenebrionis* strains which were newly delivered from the University of Heidelberg, Germany, by using a molecular biological analysis method. The analysis identified that the two isolates may be the same strains or mutants of the *B. thuringiensis* subsp. *tenebrionis* strain.

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MATERIALS AND METHODS

Reagents and Enzymes

Restriction endonucleases, lysozyme, agarose, and *Pwo* DNA polymerase were purchased from Roche (Mannheim, Germany), nitrocellulose paper membranes from Schleicher & Schuell, and DNA labeling & detection kit were supplied by Boehringer Mannheim. The rest of the biochemicals were supplied by Sigma Chemical Co.

Strains and Media

B. thuringiensis SR6 and SR8 were delivered from the University of Heidelberg, Germany. They were grown at 30°C in LB agar plate and broth.

Microscopic Examination

The morphology of spores and parasporal inclusions of *B. thuringiensis* strain was observed by phase-contrast, transmission, and scanning electron microscopies. *B. thuringiensis* SR8 was grown at 30°C in LB medium for sporulation. For the observation by transmission electron

microscopy, the sample was fixed in 1% glutaraldehyde-1% paraformaldehyde in 0.1 M cacodylate buffer (pH 7.4) for 2-3 h. After several washes in 0.1 M cacodylate buffer, the pellet was post-fixed in 2% osmium tetraoxide (OsO_4) and dehydrated with ethanol and acetone as an intermediate medium. Pellet was then embedded in Epon812-araldite mixture, and hardened at 60°C for 2 days. Ultrathin section was cut on a Reichert Ultracut E, stained with 0.2% uranyl acetate and Reynolds lead citrate [3], and viewed and photographed by Zeiss EM109 transmission electron microscope (Germany) at 60 kV. For scanning electron microscopy, spore-crystal suspensions were air-dried and then coated with gold [9]. Spores and crystals were examined with a Hitachi S-2500 scanning electron microscope (Japan).

SDS-PAGE and Immunological Binding Analysis

A loopful of sporulated cultures, grown on LB agar plate, was cultured at 30°C in 100 ml LB broth for 96 h, until the sporulation and the synthesis of toxin crystal protein were completed. The spore-crystal mixtures from 1 ml of culture were washed twice with 1 M NaCl and distilled water by centrifugation at 12,000 ×*g* for 10 min. The pellet was suspended again in distilled water and sonicated (2×5 min) in ice. After centrifugation at 12,000 ×*g* for 5 min, the over phase was divided in aliquots of 100 µg total protein after the Bradford protein determination method [2]. A few aliquots were directly applied to bioassays, and the rest were resuspended in 5× sample buffer [60 mM Tris-Cl (pH 7.5), 2% (w/v) SDS, 0.1% (w/v) bromophenolblue, 25% (v/v) glycerol] and denatured at 100°C for 10 min for SDS-PAGE. The SDS-PAGE samples were analyzed on 10% SDS-polyacryamide gel by the method of Laemmli [8]. The gel was stained with Coomassie Brilliant Blue R 250 (Sigma Chemical Co., St. Louis, U.S.A.). Immunoblot analysis [11] was carried out after the transfer of the protein onto the nitrocellulose paper with a monospecific *B. thuringiensis* subsp. *tenebrionis*-toxin antibody and a conjugated alkaline phosphatase as previously described [10].

Isolation of Chromosomal DNA and PCR

Isolation of chromosomal DNA from the *B. thuringiensis* SR6 and SR8, *Bacillus thuringiensis* subsp. *kurstaki*, and *B. thuringiensis* subsp. *tenebrionis* strains were performed by the method of Dubnau and Abenson [5]. PCR was done in 50 µl containing 100 pmol each of primer specific to *B. thuringiensis* subsp. *tenebrionis*-toxin gene (SR6, 5'-GGATCCATGAATCCGAACAA-3'; SR6-1, 5'-AAGCT-TTTAATTCACTGGAA-3'), 2.5 U *Pwo* DNA polymerase, 100 ng each of total DNA, and 0.2 mM dNTPs (TAKARA BIO INC., Otsu, Japan) in the 10× buffer, provided by Roche. Amplification was carried out in an Applied Biosystems GeneAmp PCR System 2400 Thermal cycler in 30 cycles [4, 7]. Reaction was done with denaturation, 94°C (in the first cycle for 5 min and in subsequent cycle for 30 sec elongated), annealing in 45 sec at 45°C, and polymerization in 1 min 30 sec, with 2 sec elongation time every cycle. Following the amplification, the PCR products were analyzed by 0.8% agarose gel electrophoresis.

Insect Bioassay

The bioassay samples of *B. thuringiensis* SR6 and SR8 were spread on each potato leaf of 10 cm^2 excised from a single plant. Eight Colorado potato beetle larvae (*Leptinotarsa decemlineata*; coleoptera) were applied to each leaf placed on water-saturated filter paper in a petri dish. Insecticidal activity was checked 2 days later.

RESULTS AND DISCUSSION

Morphology of Parasporal Inclusions

B. thuringiensis SR8 was incubated for 72 h to form the spore or protein crystal. The morphology of spores and crystal proteins of *B. thuringiensis* SR8 was observed first by phase-contrast microscopy. The microscopic observation showed that the spores and crystal proteins were produced (Fig. 1). The spores and crystal proteins were further prepared for morphological analysis by the electron microscope. The transmission electron microscope analysis confirmed that the spore has a spherical shape (Figs. 2A and 2B), and that the strain produced one or more crystal proteins in the cells (Fig. 2B). The size of the large crystal protein was 1.5-2.5 µm in length and 0.2-0.5 µm in width, and the small one was 0.3-0.5 µm in length and 0.2-0.5 µm in width. The crystal proteins were rhomboidal and produced in a single sporulating cell (Figs. 3A-3C). Based on the microscopic analysis, it was presumed that the strain SR8 started to develop spores concurrently with

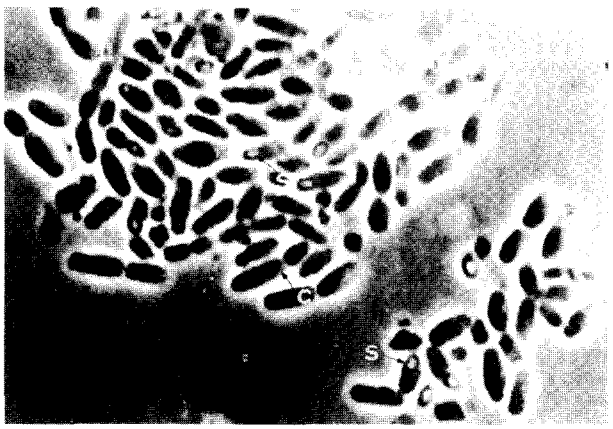

Fig. 1. Phase-contrast micrographs showing formation of spores and crystal proteins of *B. thuringiensis* SR8.
B. thuringiensis SR8 was grown for 72 h at 30°C. 'S', spore; 'C', crystal.

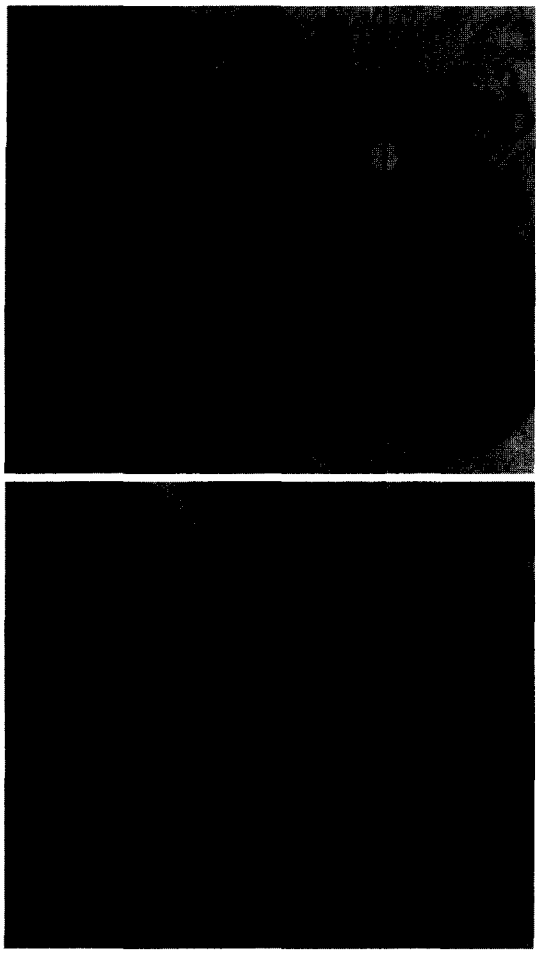

Fig. 2. Transmission electron micrographs displaying the thin section of sporangia of *B. thuringiensis* SR8 and longitudinal section of the sporulating cell with the forespore and rhomboidal crystal protein in cross-section.
B. thuringiensis SR8 was grown for 48 h at 30°C (A), and 72 h at 30°C (B). [Bar: 0.23 μm]. 'S', Forespore; 'C', Rhomboidal crystal protein.

production of crystal proteins by at least 48 h of incubation. After the incubation for 96 h, the cells were completely disrupted, and then spores and crystal proteins were released.

SDS-PAGE and Western Blot Analysis

SDS-PAGE was carried out to analyze the compositions of proteins produced in *B. thuringiensis* SR8. Thus, the cell culture and preparation of the samples for SDS-PAGE were carried out as described above. As seen in Fig. 4, lane 1, gel electrophoresis revealed that *B. thuringiensis* SR8 produced mainly a 70 kDa protein, compared to other proteins. The protein of 70 kDa was also produced by *B. thuringiensis* subsp. *tenebrionis* (Fig. 4, lane 6). However, the strain SR8 protein was produced much more than that of *B. thuringiensis* subsp. *tenebrionis*. The other strains did not produce the 70 kDa protein significantly (Fig. 4, lanes

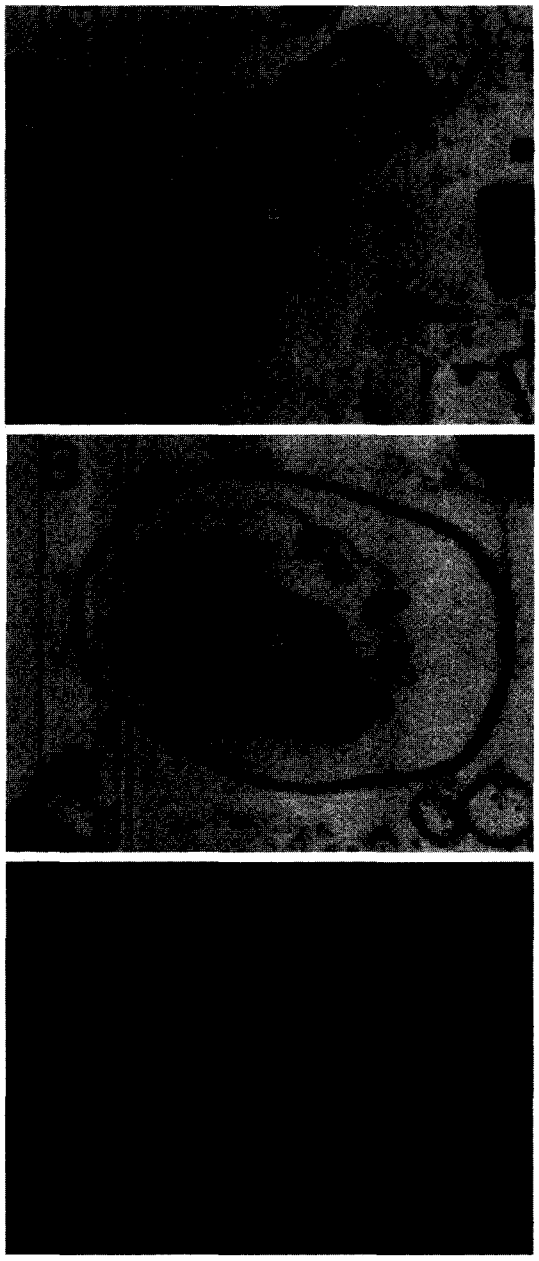

Fig. 3. Rhomboidal crystals synthesized by *B. thuringiensis* SR8.
Longitudinal section of the rhomboidal crystal protein in cross-section (A), plane section (B), and scanning electron microscope (C). The crystals are approximately 0.5–2.5 μm in length and 0.2–0.5 μm in width [Bar: 0.23 μm].

2–5). The 70 kDa protein of *B. thuringiensis* subsp. *tenebrionis*, a rhomboidal crystal protein, is important for the insecticidal toxin activity which is specific to coleopteran larvae and belongs to the group CryIII [6]. To confirm whether the 70 kDa protein of *B. thuringiensis* SR8 belongs to the group CryIII, Western blot analysis was performed with *B. thuringiensis* subsp. *tenebrionis*-toxin antibody. The blot analysis showed that the crystal

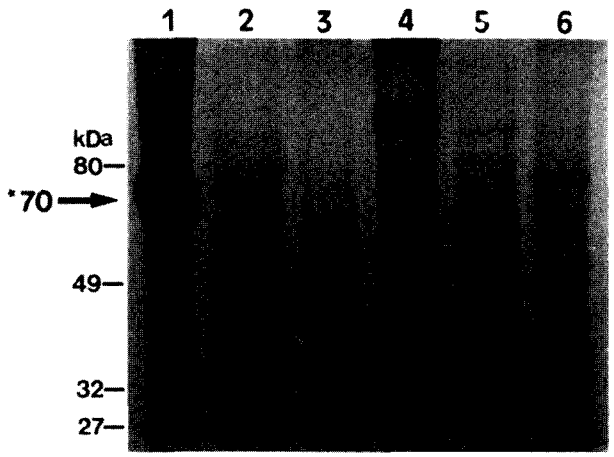

Fig. 4. Analysis of proteins produced in various *B. thuringiensis* strains on SDS-PAGE.
Lanes 1, *B. thuringiensis* SR8; 2, *B. thuringiensis* subsp. *israelensis*; 3, *B. thuringiensis* subsp. *kurstaki*; 4, *B. thuringiensis* subsp. *morrisoni*; 5, *B. thuringiensis* subsp. *thompsoni*; 6, *B. thuringiensis* subsp. *tenebrionis*. Protein sizes are indicated in kDa. The polyacrylamide concentration was 10%.

protein of 70 kDa was bound to the antibody of *B. thuringiensis* subsp. *tenebrionis*-toxin and produced more in cultivation time (Fig. 5). The immunological binding indicated that the 70 kDa protein of *B. thuringiensis* SR8 was identical to the *B. thuringiensis* subsp. *tenebrionis*-toxin protein. The results of SDS-PAGE and Western blot analyses suggest that the overexpression of the 70 kDa crystal protein in *B. thuringiensis* SR8 may be due to early formation and release of the crystal proteins in the culture medium. For such a process, *B. thuringiensis* SR8 may develop its own toxin gene expression system. From this point of view, it may be interesting to investigate the gene expression system for the overproduction of the crystal protein in *B. thuringiensis* SR8.

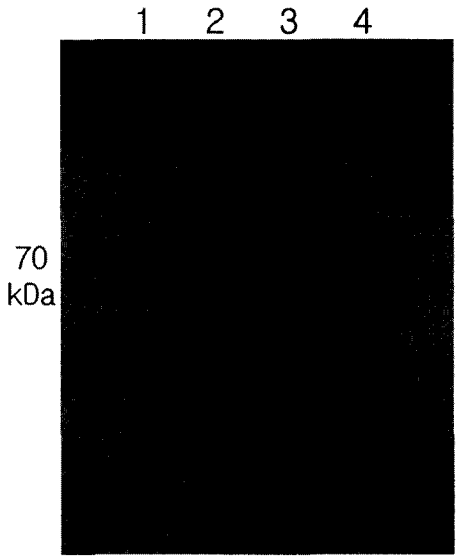

Fig. 5. Western blot analysis of *B.t.*-toxins produced in *B. thuringiensis* SR8 as incubation time.
Lanes 1, 24 h; 2, 48 h; 3, 72 h; 4, 96 h. Arrow indicates the *B.t.*-toxins of 70 kDa.

Detection of CryIII-Toxin Gene in *B. thuringiensis* SR6 and SR8

It was of interest to investigate whether the cryIII toxin gene is located in *B. thuringiensis* SR6 and SR8. In order to detect the toxin gene, PCR amplification was performed with the specific primers of *B. thuringiensis* subsp. *tenebrionis*-toxin gene as described above. After the PCR amplification, the amplified DNA fragments were separated on the agarose gel. DNA fragments from *B. thuringiensis* SR6 and SR8 appeared on the position at about 2.0 kb identical with that from *B. thuringiensis* subsp. *tenebrionis* (Fig. 6, lanes 2, 4, and 5). In contrast, no DNA fragment was detected from *B. thuringiensis* subsp. *kurstaki* (Fig. 6, lane 3). The results of PCR amplification suggest that the crystal toxin genes from both *B. thuringiensis* SR6 and SR8 are identical, or similar to that of *B. thuringiensis* subsp. *tenebrionis*. The sequence analysis of DNA fragments is now underway.

Insect Bioassay

From the morphological, physiological, and molecular biological data described above, it seemed obvious that the *B. thuringiensis* SR6 and SR8 may be the mutants of *B. thuringiensis* subsp. *tenebrionis* strain and overexpress

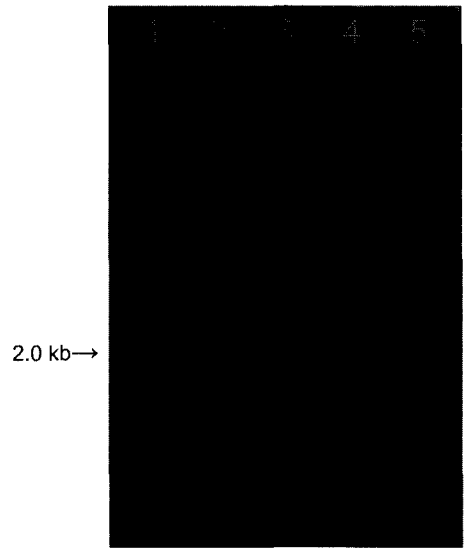

Fig. 6. Detection of DNA fragments of *B. thuringiensis* subsp. *tenebrionis*-toxin gene amplified by PCR on the agarose gel.
Lanes 1, λ/DNA *Hin*dIII size marker; 2, *B. thuringiensis* subsp. *tenebrionis*-toxin gene (positive control); 3, *B. thuringiensis* subsp. *kurstaki*; 4, *B. thuringiensis* SR8; 5, *B. thuringiensis* SR6. Arrow indicates amplified 2.0 kb fragments.

Table 1. Bioassay with the cell extracts of *B.t.* strains against eight potato beetle larvae, *L. decemlineata*. The size of leaf was 10 cm^2.

B. thuringiensis strains	Mortality	Leaf damage
B. thuringiensis subsp. *tenebrionis*	8/8 (100%)	3%
B. thuringiensis SR6	3/8 (38%)	15%
B. thuringiensis SR8	4/8 (50%)	10%
Control	0/8 (0%)	60%

the 70 kDa crystal toxin protein. It was of interest to find whether the toxin proteins produced in the two strains possess insecticidal toxic activities against the same insect larvae. Therefore, the bioassay with the cell extracts prepared from *B. thuringiensis* SR6 and SR8 was carried out against the larvae of Colorado potato beetle, *L. decemlineata*. Eight Colorado potato beetle larvae were tested on the potato leaf spreaded with 100 μg protein of the cell extracts. Mortality of the larvae was recorded in 2 days after feeding. The mortality rates, when treated with the cell extracts from *B. thuringiensis* subsp. *tenebrionis*, and *B. thuringiensis* SR6 and SR8, were 100%, 50%, and 38%, respectively. In contrast, about 60% of the leaf treated with only distilled water were consumed by the larvae, but no mortality was found. The results of the bioassays are summarized in Table 1.

Acknowledgments

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