Journal of Microbiology and Biotechnology
Vol. 5. No. 6, 359-363, 1995

Immunological Analysis of Antigenic Variation of *Bacillus thuringiensis* subsp. *sotto* during Sporulation and Crystallization

CHO, JAE-MIN, GI-BUM NAM, SOON-BOK HONG AND MYUNG-HWAN CHO*

Department of Biology, Konkuk University, Seoul 133-701, Korea

The antigenic variation of *B. thuringiensis* subsp. *sotto* have been investigated for 120 hours during sporulation and crystallization by using SDS-PAGE and Western blot. Most antigens of a vegetative cell were found to disappear as it was in sporulation and crystallization, but protein antigens of 46, 29, 27,and 21 kDa continued to be expressed. The new protein bands of 293, 138, 119, 75, and 68 kDa appeared on days 2 through 5 in modified GYS medium. They were thought to be involved in sporulation and crystallization. The protein of 138 kDa was found to be a major protein of both crystal and spore. The expression patterns were immunologically analyzed by Western blot. The polyclonal antisera against the intact crystal showed strong immunoreactivity to proteins with molecular masses of 293, 138, 68, and 46 kDa. The polyclonal antisera against the spore recognized proteins of 293, 138, 68, and 46 kDa. Both crystals and spores appeared to express the common protein antigens.

Bacillus thuringiensis is a rod shaped, gram-positive soil, sporeforming bacterium which produces crystalline inclusions containing one or more proteinaceous insecticidal crystal proteins (ICPs) (17). It is an economically important microbial insecticide which differs from most other spore formers by synthesizing a discrete crystal in addition to the endospore (11). The sequence of events during spore development of *B. thuringiensis* is not different from those in related bacilli(4). An ovoid inclusion developed simultaneously into the crystal. More than one crystal can develop within a cell, but only one ovoid inclusion per sporangium is observed (1). The crystal is synthesized from amino acids formed by protein turnover within the observation so that crystal antigen is not found in the cell prior to sporulation (12). Vegetative cells are atoxic and although a small amount of toxic protein antigenically related to the crystal can be detected in the particulate fraction of a vegetative cell homogenate (10), there is good serological and biochemical evidence as given below that crystals are not formed by simple crystallization of a protein present in the vegetative cell or by assembly of a portion of the crystal formed in the vegetative phase and another part formed during sporulation (12). Incorporation of radioactive amino acids into crystal protein is the greatest during stages III and IV of sporulation (6, 9, 12, 15,).

Many studies about sporulation of *B. thuringiensis* have proceeded, but little is known about antigenic variation during sporulation and antigenic differences between sporulation and crystallization. This study was performed to investigate the antigenic variation of *B. thuringiensis* subsp. *sotto* during sporulation and crystallization by using SDS-PAGE and Western blot.

MATERIALS AND METHODS

Bacterial Strains

Bacillus thuringiensis subsp. *sotto* were obtained by the Institute of Genetic Engineering, Konkuk University.

Cultural Conditions

The bacterial cultures were grown in nutrient broth (NB; Difco, Detroit, Michigan, U.S.A.) medium at 28°C for 18 hours and shaken at 150 rpm to give aeration. Midexponential cells were transferred two times in the NB medium maintained at 28°C to obtain the synchronously dividing cells. And then, the 10 ml culture of NB medium was used to inoculate and the bacteria were grown in 250 ml modified Glucose Yeast Salt (GYS) medium contained in a 500 ml Erlenmeyer flask by rotary agitation at 150 rpm. The cultures were incubated over 72 hours until the sporangia had lysed and released free spores and crystals. Sporulation was monitored by phase-contrast

*Corresponding Author
Key words: antigenic variation, *Bacillus thuringiensis* sporulation, crystallization

microscopy (Olympus, Japan). After sporulation, the cultures were harvested by centrifugation at 3,000×g for 20 min, and the precipitate was washed three times with phosphate-buffered saline (PBS: pH 7.4).

Separation of Insecticidal Crystal Proteins and Spores

The crystals were separated from the spores by a discontinuous density gradient of 50, 65, 70, 75 and 80% (wt/vol) sucrose. After the spore-crystal suspension was layered on top of the gradient (30 ml), it was centrifuged at 100,000×g for 2 hours. The crystal bands were harvested with a Pasteur pipette and washed by centrifugation at 20,000×g for 20 min with distilled water. The purified crystal was stored at -20°C until used.

Preparation of Lysates at Various Times after the Onset of Sporulation and Crystallization

At 24, 48, 72, 96 and 120 hours after the onset of sporulation and crystallization, 50 ml cultures in modified GYS media were taken out and harvested by centrifugation at 3,000×g for 20 min, and the precipitate was washed three times with phosphate-buffered saline (PBS: pH 7.4). After centrifugation of cultures, the suspended pellets were disrupted by ultrasonicator (Braun-sonic 1510, South Sanfrancisco, CA., U.S.A.) at an interval of 30 sec between bursts for cooling.

Preparation of Immunogens

Crystal proteins: The cultures of *B. thuringiensis* subsp. *sotto* were grown in modified GYS media at 28°C over 72 hours until the sporulation and were harvested by centrifugation at 3,000×g for 20 min. The pellet washed three times with PBS. The crystal proteins were separated from the pellet by a sucrose density gradient centrifugation. They were quantified by Bicinchoninic (BCA) assay (Pierce, Rockford, Ill., U.S.A.). Intact crystal proteins were used as immunogens, which was stored at -20°C until used.

Spore: The spores were separated by same method used in crystal proteins separation. The number of spores was counted by Pour Plate Counting Method after being centrifuged with being washed three times with PBS and diluted by Limit Dilution Method. The 10^8 spores were used as immunogens.

Lysates obtained in modified GYS medium: The lysates of *B. thuringiensis* subsp. *sotto* were produced in modified GYS media at 28°C over 72 hours and were harvested by centrifugation at 3,000×g for 20 min. The pellet washed three times with PBS. The spores among the suspended pellet was counted by Pour Plate Counting Method. Since Since the crystals and spores were both refractile and of roughly equal size, the quantities of the crystal were equally regarded as those of the spores (2). 5×10^7 spores were used as immunogens.

Production of Polyclonal Antibodies

Each female BALB/c mouse (6 weeks old) was immunized twice with crystal proteins, spores, lysates obtained in modified GYS medium, and vegetative cells of *B. thuringiensis* subsp. *sotto*. Each antigen was emulsified with an equal volume of Freund's complete adjuvant (Gibco, Inc, Grand Island, N. Y., U.S.A.), which formed a deposit protecting the antigen from rapid catabolism and stimulated the immune response non-specifically (7), and 500 µl of the emulsion was injected intraperitoneally into the mouse. The mice were boosted intraperitoneally with 500 µl of the same preparation in Freund's incomplete adjuvant (Gibco) after 21 days on primary injection. Ten days after secondary injection, the mice were sacrified and their blood was obtained. The blood was centrifuged at 1,000×g for 3 min and subsequently centrifuged at 15,000×g for 5 min. The supernatant was used as polyclonal antisera, which was stored at 4°C until used.

SDS-PAGE Analysis

Sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) was carried out by the modification of the Laemmli method (1970) in 10% separation gel and 5% stacking gel using a micro slab gel electrophoresis system (SE 250-Might Small II, Hoefer, U.S.A.). Antigens of crystal, spore and vegetative cell were dissolved in 60 mM Tris/HCl buffer (pH 6.8) containing 2% (w/v) SDS, 25% (v/v) glycerol, 14.4 mM 2-mercaptoethanol, and 0.1% bromophenol blue by heating at 100°C for 7 min. After electrophoresis, the gels were used for Western transfer, or stained for 20 min in 0.1% Coomassie blue R-250 (Sigma, St. Louis, U.S.A.) in methanol-acetic acid-water (9 : 2 : 9 v/v/v) and destained against several changes of methanol-acetic acid-water (1 : 1 : 8 v/v/v). The standard molecular markers (Bio-Rad, Richmond, CA., U.S.A.) used were myosin (200 kDa), β-galactosidase (116 kDa), rabbit muscle phosphrylase B (97.4 kDa), bovine serum albumin (66 kDa), hen egg white ovalbumin (45 kDa), bovine carbonic anhydrase (31 kDa), soybean trypsin inhibitor (21.5 kDa), hen egg white lysozyme (14.5 kDa) and bovine pancreases apotinin (6.5 kDa).

Immunoblot Analysis

After electrophoresis, proteins were transferred to nitrocellulose sheets using an electro blotter (Hoefer, Sanfrancisco, CA., U.S.A.) as described by Towbin (1979). The buffer was 15.6 mM Tris (Sigma, St. Louis, U.S.A., pH 8.3) with a constant current applied for 90 min at 4°C. The gel was stained with Amido black (Sigma, St. Louis, U.S.A.) to confirm complete transfer of the proteins. The nitrocellulose was blocked with 3% BSA in Tris buffered saline (TBS; 10 mM Trizma

base, 150 mM NaCl, pH 7.5) for 2 hours at 37°C and washed three times with TBS. The nitrocellulose was incubated for 1 hour at 37°C with polyclonal antibodies diluted to 1 : 1000 in 0.5% (w/v) BSA/TBS. Horse radish peroxidase conjugated to goat anti-mouse IgG+IgM (Jackson Immuno-Research Lab, Inc., West Grove, Pa., U.S.A.) was diluted to 1 : 1000 in 0.5% (w/v) BSA/TBS and incubated with the blots for 1 hour at 37°C. Following each step, unbound reagents were removed with TBS by three washes for 30 min. The blots were finally treated for 10 to 30 min at room temperature with a substrate solution consisting of 30 mg/ml chloronaphtol, 10 ml methanol, and 30 µl H_2O_2 (30%) in 50 ml TBS. The developed blots were washed with distilled water and photographed.

RESULTS AND DISCUSSION

Separation of Crystal Proteins and Spores

One of the most striking aspects of *Bacillus thuringiensis* sporogenesis is the synthesis of the insecticidal crystal protein. Their synthesis depends on the ability of the cell to sporulate and on growth conditions which permit sporulation (14). A band of bright, reflective crystal proteins was formed at the interface of the 65 and 70% sucrose in relatively pure state with few spores. Other band of spores was formed from the interface of the 75 and 80% sucrose to the pellet (not shown). The crystal and the spore were judged by phase-contrast microscopy (Fig. 1). The crystal shape of *B. thuringiensis* subsp. *sotto* was bipyramidal.

SDS-PAGE Analysis

The new protein bands of 293, 138, 119, 75, and 68 kDa were found to appear after 24 hours during sporulation and crystallization. After 24 hours antigenic patterns were not changed (Fig. 2) because sporulation and crystallization ended entirely after 13 hours (1). Other cellular proteins became extensively degraded into small fragments after sporulation (Fig. 2; Lane 6), probably by various cellular proteases that commonly appear in bacilli during sporulation. Bechtel and Bulla (1976) described that the parasporal crystal of *B. thuringiensis* was observed first during engulfment (stage III, 8 hours) and possessed crystal lattice fringes at this early stage of development. The crystal was almost full-sized by the time the exosporium appears (stage IV, 9 hours). An ovoid inclusion developed simultaneously to crystal appearance. Both the parasporal crystal and ovoid inclusion were not consistently associated with a specific structure. The crystal was not connected to mesosomes or to membranes of the incipient forespore or exosporium (6). The parasporal crystal and forespore were always separated by cytoplasm (1). The components of the crystal protein and the spore account for most of the proteins that were synthesized after vegetative proliferation and during sporulation. The protein antigens of 46, 29, 27, and 21 kDa of vegetative cell continued to be expressed throughout all the stages of development. According to Luethy *et al.* (1975), a small amount of toxic protein antigenically related to the crystal could be detected in the par-

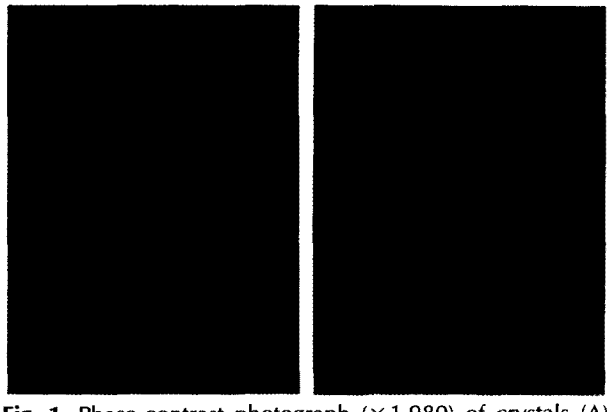

Fig. 1. Phase-contrast photograph (×1,980) of crystals (A) and spores (B) of *B. thuringiensis* subsp. *sotto* by 50~80% sucrose density gradient centrifugation.

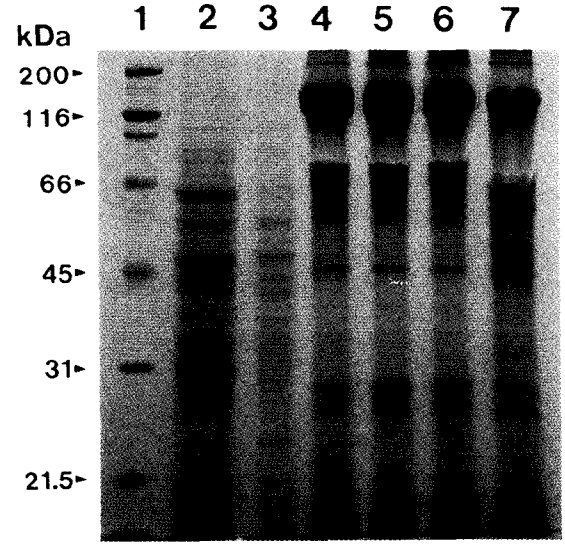

Fig. 2. SDS-PAGE analysis of *B. thuringiensis* subsp. *sotto* antigens during sporulation and crystallization.

SDS-PAGE was carried out in 10% separating gel and 5% stacking gel using a micro slab gel electrophoresis system. The gel was stained with Coomassie blue R-250 for 40 min, and then destained at 18 hours. Lanes: 1, Standard molecular markers; 2, Vegetative cell grown in nutrient broth medium; 3, Lysates obtained in modified GYS medium after 24 hours; 4, after 48 hours; 5, after 72 hours; 6, after 96 hours; 7, after 120 hours.

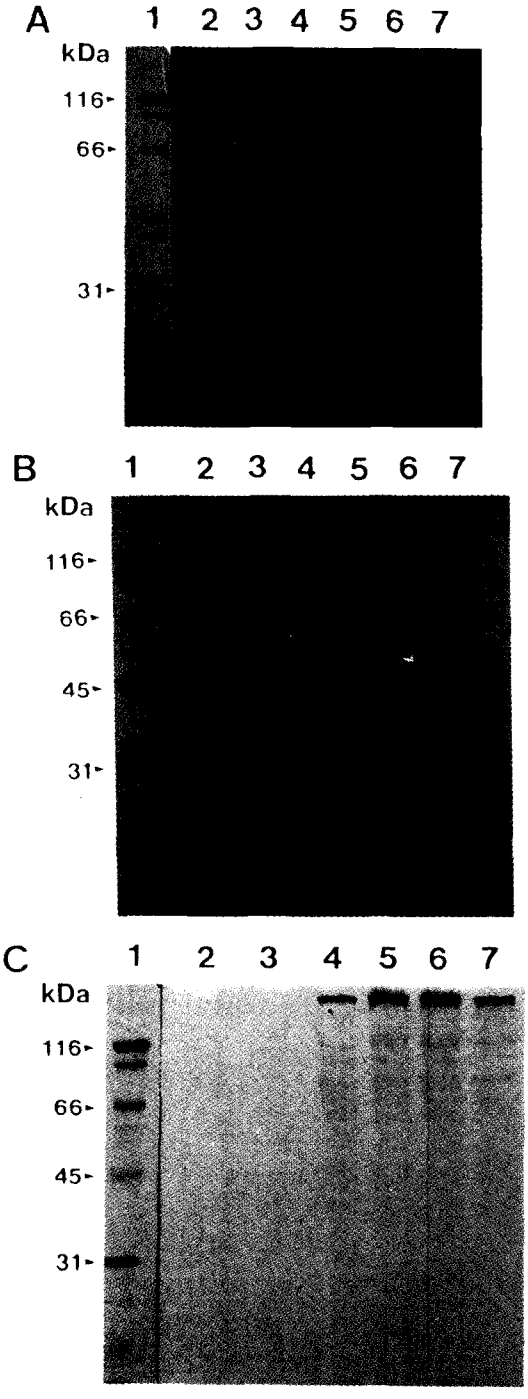

Fig. 3. Western blot analysis of antigens using polyclonal antisera against crystals (A), spores (B), and lysates (C) obtained in modified GYS medium.

After SDS-PAGE, proteins were transferred to nitrocellulose sheets using an electro blotter. Polyclonal antisera were diluted to 1 : 1,000 in 0.5% (w/v) BSA/TBS solution. Horse radish peroxidase conjugated goat anti-mouse IgG+IgM was diluted to 1 : 4,000 in 0.5% (w/v) BSA/TBS. Lanes: 1, Standard molecular markers; 2, Lysates obtained in nutrient broth medium; 3, Lysates obtained in modified GYS medium after 24 hours; 4, after 48 hours; 5, after 72 hours; 6, after 96 hours; 7, after 120 hours.

ticulate fraction of a vegetative cell homogenate. Therefore, it is considered that the new five proteins and the existing four proteins are associated with sporulation and crystallization.

Immunoblot Analysis

The polyclonal antisera against the crystal proteins and against the spore identically recognized the same antigens of 293, 138, 68 and 46 kDa (Fig. 3; A, B). The polyclonal antisera against the lysates obtained in modified GYS medium recognized five protein antigens of 293, 138, 119, 75, and 68 kDa (Fig. 3; C). Particularly, the polyclonal antisera against the spore strongly reacted with the protein antigen of 293 kDa, which was very immunodominant. In this study, the antigenic variation was demonstrated to exist in the developmental stages of *Bacillus thuringiensis* subsp. *sotto.*

Acknowledgement

This work was in part supported by Korea Science and Engineering Foundation.

REFERENCES

1. Bechtel, D. B. and L. A. Bulla, Jr. 1976. Electron microscope study of sporulation and parasporal crystal formation in *Bacillus thuringiensis*. *J. Bacteriol.* **127**: 1472-1481.
2. Bourque, S. N., J. R. Valero, M. C. Lavoie, and R. C. Levesque. 1995. Comparative analysis of the 16S to 23S ribosomal intergenic spacer sequences of *Bacillus thuringiensis* strains and subspecies and of closely related species. *Appl. Envir. Microbiol.* **61**: 1623-1626.
3. Delafield, F. P., H. J. Somerville, and S. C. Rittenberg. 1968. Immunological homology between crystal and spore protein of *Bacillus thuringiensis*. *J. Bacteriol.* **96**(3): 713-720.
4. Dulmage, H. T. and cooperators. 1981. Insecticidal activity of isolates of *Bacillus thuringiensis* and their potential for pest control. p. 193-248. *In* H. D. Burgess (ed.) *Microbial control of pests and plant diseases 1970-1980.* Academic Press, Inc., London.
5. Ghosh, B. K. 1974. The mesosome clue to the evolution of the plasma membrane. Sub-Cell. *Biochem.* **3**: 311-367.
6. Glatron, M. F. and G. Rapoport. 1972. Biosynthesis of the parasporal inclusion of *Bacillus thuringiensis*: half-life of its corresponding messenger RNA. *Biochemie.* **54**: 1291-1301.
7. Harlow, E., and D. Lane. 1988. *Antibodies; A laboratory manual.* p. 53-138. Cold Spring Harbor Laboratory. New York.
8. Laemmli, U. K. 1970. Cleavage of structural proteins during the assembly of the head of bacteriophage T4. *Nature* **227**: 680-685.

9. Lecadet, M. M. and R. Dedonder. 1971. Biogenesis of the crystalline inclusion of *Bacillus thuringiensis* during sporulation. *Eur. J. Biochem.* **23**: 282-294.
10. Luethy, P. 1975. Zur bakteriologischen Schaedlingsbekae mpfung: die entomo-pathogenen *Bacillus*-Arten, *Bacillus thuringiensis* und *Bacillus popillae*. *Vjsch. Naturf. Ges. Zuerich.* **120**: 81-163.
11. Mettus, A. M. and A. Macaluso. 1990. Expression of *Bacillus thuringiensis* δ-endotoxin genes during vegetative growth. *Appl. Envir. Microbiol.* **56**: 1128-1134.
12. Monro, R. E. 1961. Protein turnover and the formation of protein inclusions during sporulation of *Bacillus thuringiensis*. *Biochem. J.* **81**: 225-232.
13. Pang, A. S. D. 1994. Detection of *Choristoneura fumiferana* brush border membrane-binding molecules specific to *Bacillus thuringiensis* δ-endotoxin by crossed affinity immunoelectrophoresis. *Biochem. and Biophys. Res. Commun.* **199**: 1194-1199.
14. Salama, H. S., M. S. Foda, H. T. Dulmage, and A. El-Sharaby. 1983. Novel fermentation media for production of δ-endotoxins from *Bacillus thuringiensis*. *J. Invertebr. Pathol.* **4**: 8-19.
15. Somerville, H. J. 1971. Formation of the parasporal inclusion of *Bacillus thuringiensis*. *Eur. J. Biochem.* **18**: 226-237.
16. Tapp, H. and G. Stotzky. 1995. Insecticidal Activity of the Toxins *Bacillus thuringiensis subspecies kurstaki* and *tenebrionis* Adsorbed and Bound on Pure and Soil Clays. *Appl. Envir. Microbiol.* **61**: 1786-1790.
17. Towbin, H., T. Staeelin, and J. Gordon. 1979. Electrophoretic transfer of proteins from polyacrylamide gels to nitrocellulose sheets: Procedure and some applications. *Proc. Nat. Acad. Sci.* USA. **76**: 4350-4354.
18. Whiteley, H. R. and H. E. Schnepf. 1986. The molecular biology of parasporal crystal body formation in *Bacillus thuringiensis*. *Annu. Rev. Microbiol.* **40**: 549-576.

(Received July 20, 1995)