Journal of Microbiology and Biotechnology
Vol. 4, No. 4, 290-295, 1994

Production and Characterization of Monoclonal Antibodies to *Bacillus thuringiensis* subsp. *canadensis*

JUNG, JAE-DEUK, JUNG-SUN PARK, YUNG-SOO JO, SOON-BOK HONG, HYUNG-HOAN LEE, AND MYUNG-HWAN CHO*

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

30 monoclonal antibodies (mAbs) were produced against *Bacillus thuringiensis* subsp. *canadensis*. Out of the these, 6 mAbs were selected for further studies. SDS-PAGE analyses of sonicated antigens of 10 *B. thuringiensis* strains showed that they generally had both predominant protein antigens of molecular weights of 45 kilodalton (kd) except for *shandogiensis* and *konkukian*, and 37kd except for *israelensis*, *tochigiensis*, and *shandogiensis*, respectively. These results indicate that 45kd and 37kd may be important for demonstrating common antigens except for a few strains of *B. thuringiensis*. In comparing the result of the western blot using mAbs with that of using polyclonal antibodies to *canadensis*, we found that immunoreactive proteins of 99 and 39 kd were identified as common antigens, which might act as antigenic determinants, and might be surface or flagella antigens. Reactivities of mAbs with 41 strains of *B. thuringiensis* demonstrated that mAbs of C-1, C-3, C-4, C-5 and C-6 except C-2 did not recognize epitopes of *thuringiensis*, but that all of the mAbs recognized epitopes of *galleriae*, *kurstaki*, *dakota*, *tohokuensis*, *silo*, *toguchini*, and *leesis*. The potential applications of the mAbs we produced would be useful tools for the clarification of taxonomy, investigation of antigenic relationship between *B. thuringiensis* strains, and localization of specific surface and flagella antigens.

Bacillus thuringiensis is a Gram-positive soil bacterium characterized by its ability to produce endotoxin crystals during stages *III* to *V* of sportulation(6), and one of biological agent with high potential as an alternative to chemical pesticides. The toxicity of the crystals to some insects varies according to the *B. thuringiensis* strains. They have a preferential toxicity to insects in the orders lepidoptera, diptera, and coleoptera (15). One to one mixtures of spores and crystals produced by *B. thuringiensisn* var. *aizawa*, *galleriae* and *wuhanensis* were highly potent in bioassay of *Galleria mellonella* (14). Endotoxin production is controlled by single genes and these can be manipulated, which opened the way to a new understanding of *B. thuringiensis's* mode of action and the mechanism involved in host specificity (5). *In vivo* activation of the protoxin and proteolytic activity of the toxin could cause any one of the pathological disorders that have been ascribed to the crystal (2).

Serological analysis of crystal proteins has suggested that differences in crystal antigenicity have some correlation to the host specificity of the toxin (11). Cry genes were classified into four groups based on insect specificity and nucleotide sequence similarities (7). Toxicity against five lepidopteran species, the molecular weight of the protoxin, toxin and monoclonal antibody's reactivity were used for the characterization of crystal proteins of *B. thuringiensis* strains (8). Efforts have recently been made to use a method based on polymerase chain reaction (PCR) (1).

To determine where the toxin acts and which sequences and/or conformation of the polypeptide are necessary for its action, monoclonal antibodies to crystal proteins have been used. Several monoclonal antibodies against activated δ-endotoxin from *B. thuringiensis* were obtained which reacted to at least four different antigenic sites on the δ-endotoxin (9). Inactivation antibodies may either bind to the active site or bind to another part of the protein and change its conformation (9). Because of the entomocidal activity, a great number of *B. thuringiensis* isolates have been identified and characterized. *B. thuringiensis* strains were classified into some 30 subspecies based on antigenic differences of the flagellar of the vegetative cells as well as a variety of biochemical tests (3).

Little is known about the characterization of *B. thuringiensis* antigens. This study was designed to produce monoclonal antibodies to the intact cell of *B. thuringiensis*

*Corresponding author
Key words: *Bacillus thuringiensis*, monoclonal antibody, epitope.

subsp. *canadensis* and examine their ability to cross-react with that of *B. thuringiensis* strains. Thereafter, protein profile patterns and western blot analysis in which monoclonal antibodies were used would reveal the antigenic relationship of *B. thuringiensis* strains.

MATERIALS AND METHODS

Bacterial Strains and Animals

The *B. thuringiensis* strains used in this study are listed in Table 1. Six-to seven-week-old BALB/c mice were used for immunization.

Preparation of Immunogens

B. thuringiensis subsp. *canadensis* was grown in broth medium with 0.7% pancreatic peptone (3), continuously shaken to give aeration. During early exponential growth, an aliquot was inoculated at the agar surface of the inner tube of a Craigie tube (9) filled with soft nutrient agar containing only 0.2% agar. The bacteria migrated down the inner tube and then up the outer container tube in about 20-24 h. This procedure was repeated in fresh Craigie tubes, until the migration was accelerated to about 14-16 h. A stock suspension of motile *B. thuringiensis* subsp. *canadensis* was grown by inoculating cells from the last Craigie tube into 200 ml of nutrient broth in a litre Erlenmeyer flask and shaking for 5-8 h at 30°. Growth was checked by turbidity until 10^9 cells/ml were obtained. Cells were harvested by Centrifugation and washed two times in phosphate buffered saline(PBS; pH 7.2). The cell concentration was adjusted to 1×10^7 cells/ml. At this stage, formalin was added to give a final concentration of 0.5% and the culture was stored at 4°C.

Immunization of Mice

Female BALB/c mice (6 to 7 weeks old) were immunized with whole cells. The formalin-fixed bacterial cells were emulsified with an equal volume of Freund's complete adjuvant (Gibco, Inc, Grand Island, N.Y., U. S. A) and 400 µl of the emulsion were injected intraperitoneally into the mice. The same amount of antigen with Freund's incomplete adjuvant (Gibco) was injected on day 15. Mice were tail-bled on day 22 to determine the titer of *B. thuringiensis* subsp. *canadensis* antibodies. A mouse with more than 1000 of serum titer was selected for fusion. A final injection of 100 µl of antigen containing 1×10^7 cells without adjuvant was administered on day 62 via tail vein. The mice were sacrificed on the 3rd day after the intravenous booster and their spleens were removed.

Fusion

About 1×10^8 spleen lymphocytes from a previously immunized mouse were fused with 1×10^7 cells of mouse myeloma cell line (SP2/0-Ag14) using 50% polyethyleneglycol 4000 (PEG; Gibco) as described by Köhler and Milstein (10). The fused cells were dispensed into 24 well tissue culture plates (Roskilde, Denmark) with RPMI hypoxanthine-aminopterin- thymine stock (HAT; Gibco)) and thymocyte-conditioned media and cultured at 37°C in a CO_2 incubator.

Selection and Cloning of Hybridoma

At about 10 to 14 days postfusion, colonies of growing hybridoma clones were visible in 24-well tissue culture plates. These visible colonies were transferred to 96-well tissue culture plates by sterile Pasteur pipettes and allowed to grow in RPMI base medium supplemented with 10% fetal bovine serum (FBS; Gibco), hypoxanthine-thymidine (HT; Gibco). Production of antibodies against *B. thuringiensis* subsp. *canadensis* was assayed by indirect immunofluoresence. Hybridomas producing monoclonal antibodies against *B. thuringiensis* subsp. *canadensis* were cloned by limiting dilution in 96 well plates to less than one cell per well (16).

Expanding and Freezing of Positive Clones

In the process of selection and cloning of hybridomas, positive wells had been identified and then the cells were transferred from the 24-well tissue culture to a 25 cm^2 flask and then to a 75cm^2 flask. At this stage, the hybridomas containing 5×10^6 to 5×10^7 cells/ml of the freezing medium were frozen in liquid nitrogen.

Indirect Immunofluorescence Assay(IFA)

Monoclonal antibodies were reacted to 41 strains of *B. thuringiensis* using IFA and observed the distribution of *B. thuringiensis* antigens according to the method of Tilahum and Stockdale (22). Smears on glass slides were prepared from the culture of *B. thuringiensis* subsp. *canadensis* and the other strains in PBS, air-dried, and fixed by heating. The smears were incubated for 30 min at 20°C with hybridoma supernatants, washed with PBS twice, incubated with 100 µl of FITC-conjugated goat antimouse IgG+IgM+IgA (Cappel, West Chester) diluted to 1/1000 in PBS for 20min. The cells were washed twice and resuspended in PBS containing 10% (vol/vol) glycerol and 100 µl of o-phenylenediamine per ml to prevent fading. Immunofluorescence was evaluated with a fluoresence microscope (Olympus BH-2, Japan, x1000)

Preparation of Cell Lysates

B. thuringiensis strains were grown in 200 ml of Luria-bertani (LB; Difco, Detroit, Michigan, U.S.A) medium at 28°C for 18 h and shaken at 150 rpm to give aeration. The cultures were centrifuged for 20 min at 1,000xg, the cell pellets were washed twice in phosphate buffered saline (PBS; pH 7.2) and centrifuged again for 20 min at 1000xg after each wash. The final pellets were suspended in 5 ml of lysis buffer containing 1M NaCl and 0.01% Triton X-100 and kept at 4°C for 18 h. The suspended pellets were disrupted by ultrasonicator (Braun-sonic 1510, South Sanfrancisco, Cal., U.S.A) at an interval of 30s between bursts for cooling. At this stage, the bacterial suspension was maintained on ice. After centrifugation at 25,000 x g, 4°C for 30 min, the protein contents of the soluble fractions were estimated by the BCA kit (Pierece, Rockford, Ill., U.S.A) method. Soluble fractions were stored at -20°C until used.

SDS-PAGE Analysis of Sonicated *B. thuringiensis* Antigens

Sodium dodecyl sulfate polyacrylamide slab gel electrophoresis (SDS-PAGE) was carried out by the modification of the Laemmli method (12) in 10% separating gel and 5% stacking gel, using a micro slab gel electrophoresis system (SE 250-Might Small II, Hoefer, U. S. A). Antigens (30~50 μg) of cell lysates were dissolved in 60mM-Tris/HCl buffer (pH 6.8) containing 2% (w/v) SDS, 25%(v/v) glycerol, 14.4mM 2-mercaptoethanol, and 0.1% bromophenol blue by heating at 100°C for 4 to 5 min. After electrophoresis, the gels were stained with Coomassie blue R-250 (Sigma, St., Louis, U.S.A) for 20 min, or used for western transfer. The standard molecular markers (Bio-Rad, U.S.A) we used were myosin (200kd), β-galactosidase (116kd), bovine serum albumin (66kd), egg albumin (45kd), bovine carbonic anhydrase (31kd), trypsin inhibitor (21.5kd), egg lysozyme(14.4kd) and apotinin (6.5kd)

Antigenic Analysis by Western Blot Using mAbs

After electrophoresis, proteins were transferred to nitrocellulose sheets (Hoefer, Sanfrancisco, Cali., U.S.A) using an electro blotter (Hoefer, U.S.A) as described by Towbin (17). The buffer was 15.6 mM Tris (Sigma) and 120mM glycine (Sigma, pH 8.3) with a constant current applied for 90 min at 4°C. The gel was stained with Amido black (Sigma) to confirm complete transfer of the proteins. The nitrocellulose was blocked with 3% BSA in Tris buffered saline (TBS; 10mM Trizma base, 150 mM NaCl, pH 7.5) for 2 h at 37°C and washed three times with TBS. The nitrocellulose was incubated with hybridoma supernatants for l h at 37°C. Next, horse radish peroxidase conjugated to goat antimouse IgG+IgM (Jackson Immuno-Research Lab, Inc., West Grove, Pa., U.S.A) was diluted 1:1000 in 0.5% (w/v) BSA/TBS and incubated with the blots for 1 h 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 20 min at room temperature with a substrate solution consisting of 30 mg/ml chloronaphtol, 10 ml methanol, and 30 μl 30% H_2O_2 in 50 ml TBS. The developed blots were washed with distilled water and photographed.

RESULTS AND DISCUSSION

Production of Hybridoma

Of the 136 hybridomas obtained from the fusion of immune BALB/c splenocytes with myeloma cells (SP2/0-Ag14), only 30 hybridomas produced monoclonal antibodies that reacted specifically with whole-cell antigens of homologous *B. thuringiensis* subsp. *canadensis* when assayed by indirect immunofluorescence. The 6 mAbs were selected for the characterization and named as C-1 , C-2 ,C-3, C-4, C-5, and C-6, respectively.

SDS-PAGE Analysis of Sonicated *B. thuringiensis* Antigens

SDS-PAGE was used to determine whether the protein profiles of soluble protein extracts of *B. thuringiensis* strains are distinguished between strains. The 20 to 40 protein profiles of *B. thuringiensis* antigens revealed similarity among all the strains examined. However, some minor differences between strains were recognized, especially in contents and number of bands. As shown in Fig.1, *galleriae* had darkly-staining bands of 82, 61, 19 and 17kd, while *canadensis* had specific bands of 106, 97, 74, 58-47, 39, 37 and 36kd. de Barjac and Bonnefoi (4) had difficulty in serotyping the H-antigens between *galleriae* and *canadensis*, but we could easily distinguish between them.

Our results suggest that SDS-PAGE patterns demonstrate a useful method for identification. This supports the suggestion of Mulligan et al. (17) that both PAGE and serogrouping appear to be typing techniques that can be used to further define the epidemiology of *Clostridium difficile* induced disease.

Another interesting finding was that among the 10 strains examined, they generally have both predominant proteins of molecular weights (MW) of 45kd except for *shandogiensis* and *konkukian*, and 37 kd except for *is-*

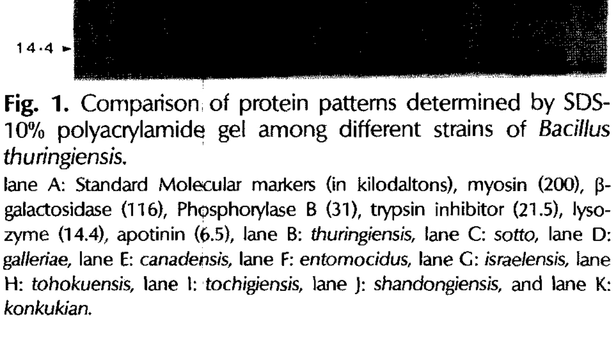

Fig. 1. Comparison of protein patterns determined by SDS-10% polyacrylamide gel among different strains of *Bacillus thuringiensis*.
lane A: Standard Molecular markers (in kilodaltons), myosin (200), β-galactosidase (116), Phosphorylase B (31), trypsin inhibitor (21.5), lysozyme (14.4), apotinin (6.5), lane B: *thuringiensis*, lane C: *sotto*, lane D: *galleriae*, lane E: *canadensis*, lane F: *entomocidus*, lane G: *israelensis*, lane H: *tohokuensis*, lane I: *tochigiensis*, lane J: *shandongiensis*, and lane K: *konkukian*.

raelensis, tochigiensis and *shandogiensis*, respectively. A common antigen of 45kd was somewhat similar to the result of Tabouret (21) who showed to be that of 42kd and 50kd in all *Listeria* species of Gram-positive rods. On the other hand, Smith et *al.* (20) reported that the PAGE patterns of the EDTA-soluble proteins from four species of Gram-positive anaerobic cocci had a single darkly-staining band with a narrow range of 76 to 78kd.

Our results indicate that proteins of 45kd and 37kd except for a few strains may be important for demonstrating a common antigenic site of the cell surface or flagella. Thus, polyacrylamide gel electrophoresis of soluble antigens could be a practical aid for the identification of serotyping.

Western Blot Analysis Using mAbs

Sonicated-protein patterns of SDS-PAGE showed that all of them had 13 major bands among 35 bands in the region between 24kd and 106kd. To determine the antigenic specificity of the mAbs, western blotting was performed with homologous antigens of *B. thuringiensis* subsp. *candensis*. Western blot analysis demonstrated that the mAbs' C-1 to C-3 of *canadensis* were specific for immunoreactive proteins with M.W of 56, 45 and 39kd, respectively, but that the rest of the mAbs recognized multiple proteins : C-4 specific for proteins of 99, 55, 42and 39kd; C-5 specific for those of 39and 42kd; C-6 specific for those of 82, 45 and 40kd (Fig. 2, Fig. 3).

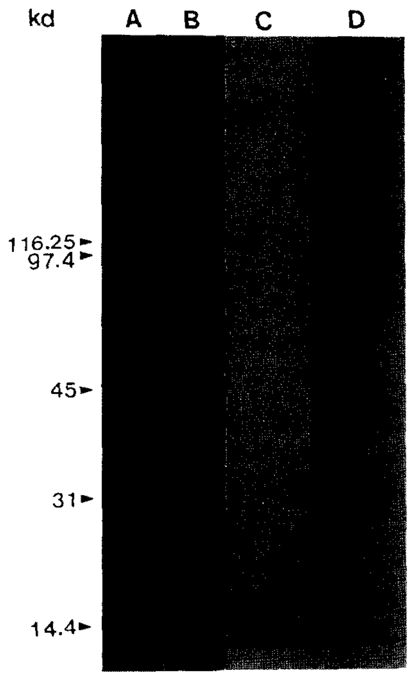

Fig. 2. Western blot analysis of *Bacillus thuringiensis* subsp. *canadensis* antigens reacted with monoclonal antibodies.
lane A: Standard molecular markers, lane B: amido black-stained antigens transferred to nitrocellulose, lane C: C-1, and lane D: C-2.

In comparing mAbs with polyclonal antibodies to *canadensis* (data not presented), we found two results. Firstly, immunoreactive proteins of 99 and 39kd were identified as common antigens. This result suggests that the immunoreactive proteins may act as immunodominant antigenic determinants and be surface or flagella antigens. Secondly, 61 and 32kd antigens were observed in the western blot analysis using polyclonal antibodies, but they did not appear in the analysis using mAbs. This indicates a possibility that mAbs are raised against a single epitope and the reaction of interest may be removed by the denaturing effect of the detergent such as SDS.

This result suggests that antigenic determinants may

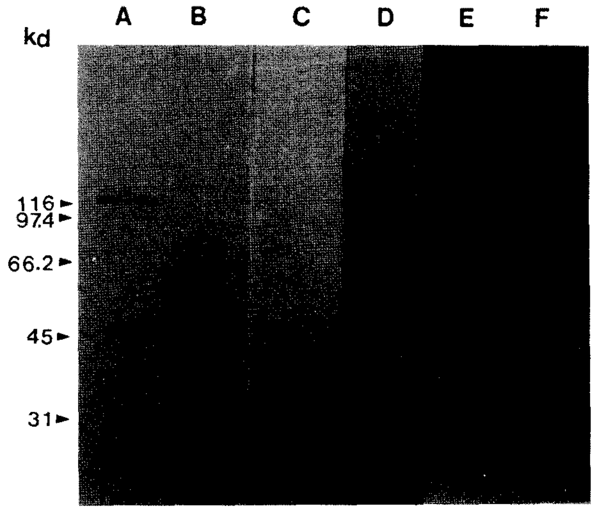

Fig. 3. Western blot analysis of Bacillus *thuringiensis* subsp. *canadensis* antigens reacted with monoclonal antibodies.
lane A: Standard molecular markers, lane B: amido black-stained antigens transferred to nitrocellulose, lane C: C-3, lane D: C-4, lane E: C-5, and lane F: C-6.

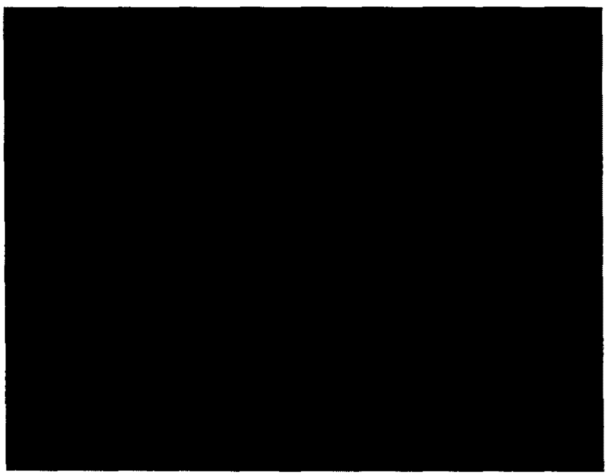

Fig. 4. Indirect immunofluorescent appearance of *Bacillus thuringiensis* subsp. *canadensis* reacted with monoclonal antibody C-1.

Table 1. Reactivities of monoclonal antibodies to *Bacillus thuringiensis* subsp. *canadensis* with strains of *B. thuringiensis* using indirect immunofluorescent assay.

Strain	Monoclonal antibody					
	C-1	C-2	C-3	C-4	C-5	C-6
B. t. var. *canadensis*	+	+	+	+	+	+
B. t. var. *thuringiensis*	−	−	−	−	−	−
B. t. var. *alesti*	−	+	+	+	−	+
B. t. var. *kurstaki*	+	+	+	+	+	+
B. t. var. *sotto*	+	+	−	−	−	−
B. t. var. *kenyae*	+	−	+	+	+	+
B. t. var. *galleriae*	+	+	+	+	+	+
B. t. var. *finitimus*	+	−	+	+	−	+
B. t. var. *entomocidus*	−	−	+	+	−	+
B. t. var. *aizawai*	+	−	+	+	−	+
B. t. var. *morrisoni*	+	−	−	+	−	+
B. t. var. *ostriniae*	−	+	+	−	−	+
B. t. var. *tolworthi*	+	+	+	+	−	−
B. t. var. *thompsoni*	+	−	+	+	+	−
B. t. var. *pakistani*	−	−	+	+	+	+
B. t. var. *israelensis*	−	−	+	+	+	+
B. t. var. *dakota*	+	+	+	+	+	+
B. t. var. *indiana*	+	−	−	+	−	−
B. t. var. *tohokuensis*	+	+	+	+	+	+
B. t. var. *kumanotoensis*	−	−	+	−	+	+
B. t. var. *tochigiensis*	+	+	+	−	−	+
B. t. var. *yunnanensis*	−	−	+	−	+	+
B. t. var. *colmeri*	−	+	+	+	+	+
B. t. var. *shandongiensis*	+	−	+	+	+	+
B. t. var. *japonensis*	−	+	+	+	+	+
B. t. var. *neolenoensis*	+	+	−	+	+	+
B. t. var. *coreanensis*	+	−	+	+	+	+
B. t. var. *silo*	+	+	+	+	+	+
B. t. var. *mexicanensis*	+	−	+	+	+	−
B. t. var. *monterray*	+	−	+	+	+	+
B. t. var. *amagiensis*	+	−	+	+	+	+
B. t. var. *medellin*	+	+	+	−	+	−
B. t. var. *toguchini*	+	+	+	+	+	+
B. t. var. *cameroun*	−	+	+	+	−	+
B. t. var. *leesis*	+	+	+	+	+	+
B. t. var. *konkukian*	+	−	+	+	+	+
B. t. var. *sumiyoshiensis*	−	−	+	−	+	+
B. t. var. *fukuokaensis*	+	−	+	−	−	+
B. t. var. *nigeriensis*	+	+	−	+	+	−
B. t. var. *darmstadiensis*	−	+	−	+	−	+
B. t. var. *toumanoffi*	−	+	−	+	+	−
B. t. var. *pondicheriensis*	−	−	+	−	−	+

+ : Positive reaction of monoclonal antibody to *B. t* strains, − : Negative reaction of monoclonal antibody to *B. t* strains.

be exposed on the surface of the protein and a limited number of them are detected. Lövgren *et al.* (13) reported that purified flagellin from *B. thuringiensis* subsp. *alesti* was 32kd, whereas Murakami *et al.* (18) proved that a common epitope of *B. cereus* and *B. thuringiensis* alesti was 61kd protein of the flagellin by immunoblot analysis using H15A5 mAb against *B. cereus*. According to Murakami *et al.* (18), the result of H15A5mAb suggested that the similar properties of H antigen and toxin were observed between *B. cereus* and *B. thuringiensis*. On the basis of this, mAbs obtained will make it possible to study relationships between whole cells and toxins of *B. thuringiensis*, and be of use in localizing the epitopes by an immunogold microscope assay using mAbs.

Reactivities of Monoclonal Antibodies to 41 Strains of *B. thuringiensis*

Monodonal antibodies were examined in indirect immunofluorescent assay for the reactivities with *B. thuringiensis* strains (Fig. 4). Selected mAbs to *canadensis* reacted with intact cells of 41 *B. thuringiensis* strains to investigate the antigenic relationship among strains of *B. thuringiensis* (Table 1). These results showed that mAbs cross-reacted with 21 to 34 strains of *B. thuringiensis*, indicating that they shared common antigens among strains of *B. thuringiensis*.

It is of interest that mAbs of C-1, C-3, C-4, C-5 and C-6 did not react with *thuringiensis*, while all of the mAbs reacted with *gallleriae*. These results indicated that all of the mAbs except C-2 did not recognize antigenic determinants of *thuringiensis*, but that all of the mAbs recognized epitopes of *galleriae, kurstaki, dakota, tohokuensis, silo, toguchini,* and *leesis*. The latter result confirms that the antigenic structure of *B. thuringiensis* sub. *canadensis* is almost identical to *galleriae* (4). Therefore, the potential applications of the mAbs produced would be useful tools for the clarification of taxonomy, investigation of antigenic relationships between *B. thuringiensis* stains, and localization of specific surface and flagella antigens.

Acknowledgement

This work (92-0400-014-2) was supported by Korea Science and Engineering Foundation.

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(Received June 23, 1994)