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# Isolation and Characterization of New *Bacillus thuringiensis* Strains with Insecticidal Activity to Difficult to Control Lepidopteran Pests

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Strains were isolated from soil samples collected from mountains and fields of Yeong–Dong and Ok–cheon, ChungBuk province in order to select *B. thuringiensis* strains with high insecticidal activities against lepidopteran pests. Eight *B. thuringiensis* strains producing spores and endotoxin proteins were selected from a total of 26 soil samples. Tests of biological activities in *Spodoptera litura*, *Spodoptera exigua*, *Plutella xylostella*, and *Aedes aegypti* were performed with 8 isolates of *B. thuringiensis*. CAB565 and CAB566 strains were selected, because such strains showed more than 80% of insecticidal activities against four kinds of pests. Selected two strains formed endotoxin proteins in a typical bipyramidal type. CAB565 and CAB566 strains were identified as *thuringiensis* subsp. *kurstaki*, and *thuringiensis* subsp. *aizawai*, respectively. $LC_{50}$ value of CAB566 strain against larvae of *S. litura* at the fourth stage was $7.7\times10^5$ (cfu/ml), which was the highest activity. $LC_{50}$ value of CAB565 strain against larvae of *S. exigua* at the fifth stage was $3.0\times10^6$ (cfu/ml), which was high activity. Through SDS–PAGE, CAB565 and CAB566 strains showed a band at 130 kDa. After it reacted with midgut juice of *S. litura* and *S. exigua*, a band of toxin protein was shown at 65 kDa. According to results of PCR analysis, it was found that *cry1Aa*, *cry1Ac* and *cry1I* genes were present in CAB565 strain and it was different from *B. thuringiensis* subsp. *kurstaki*. *cry1Aa*, *cry1C*, *cry1D* and *cry1I* genes were present in CAB566 strain and it was different from *B. thuringiensis* subsp. *aizawai*. As a result of plasmid DNA patterns, *B. thuringiensis* subsp. *kurstaki* KB099 strain and CAB565 strain had 5 and 10 plasmid DNAs, respectively. *B. thuringiensis* subsp. *aizawai* KB098 strain and CAB566 strain had 9 and 7 plasmid DNAs, respectively.

**Key words**: *Baciilus thuringiensis*, *Plutella xylostella*, *Spodoptera exigua*, *Spodoptera litura*

## INTRODUCTION

*Bacillus thuringiensis* was first discovered in silkworms with sotto disease by Ishiwata (1901) in Japan and discovered in flour moth larvae with Schlaffsucht disease by Berliner (1911) in Germany. It has been reported that *B. thuringiensis* commonly exists in natural ecosystems and it is isolated from the everyday habitats around us including soils (Ohba and Aizawa, 1978; Martin and Travers, 1989), insects (Carozzi *et al.*, 1991), leaves of deciduous and coniferous trees (Smith and Couche, 1991; Kaelin *et al.*, 1994), water (Hernández–Rodríguez and Ferre, 2008), powders of stored grains (Ejiofor and Johnson, 2002), and sericultural farm households (Ohba *et al.*, 1984).

Because an insecticidal protein of *B. thuringiensis* most commonly used as a microbial insecticide is safe to non–selected organisms, it is used as a main farming product for crop protection in eco–friendly agriculture and it accounts for 80–90% of biological pest control in the world. *B. thuringiensis* is an aerobic gram–positive *Bacillus*. It forms an endospore and produces insecticidal crystal proteins consisting of endotoxins. Endotoxins mainly consist of cry and cyt. It has been reported that cry proteins are toxic particularly to insects such as lepidoptera, diptera and coleoptera (Bravo *et al.*, 2005), and have activity against Hymenoptera, Hemiptera, Orthoptera, mites, nematodes and protozoa (Feitelson *et al.*, 1992; Schnepf *et al.*, 1998).

A crystal protein of *B. thuringiensis* has the insecticidal mechanism by forming small holes in the cell membrane and causing sepsis once it reacts with midgut juice of insects and it is decomposed into toxin proteins (Jurat–Fuentes and Adang, 2006; Bravo *et al.*, 2007). In addition, because pests must take it in order to activate the toxicity of *B. thuringiensis* (Schnepf *et al.*, 1998), it has drawbacks of giving effects on eggs, pupae and adults of pests while it kills larvae.

*Spodoptera litura* and *Spodoptera exigua* which are lepidopteran pests are the most common agricultural pests belonging to Lepidoptera and Noctuide. They are internationally well known and polyphagous pests which give damage to a variety of crops such as vegetables, flowers and fruit trees (Goh *et al.*, 1991; Bae *et al.*, 1997). As larvae of *S. litura* give very serious damages to economic crops by vigorous feeding activities and adults have a very high propagation rate by laying eggs covered in human hairs, a significant impact would be given to crop cultivation unless various selective pesticides are alternated and continuous control is made (Bae, 1999).

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As larvae of *S. exigua* may move a long distance and give the damage once entering the stem of the crop, it is less likely to be exposed to pesticides (Park and Goh, 1992; Kim *et al*., 2009a). *Plutella xylostella* belongs to Lepidoptera, hyponomeutidae and a major pest to cruciferous crops. Because annual number of generation is high and growth period is short, it is more likely to be exposed to pesticides and resistance is rapidly developed (Kim *et al*., 1990). With emergence of strains highly resistant against most of currently available chemical pesticides, it is difficult to control them. Moreover, because old larvae show a significantly low sensitivity to chemical pesticides (Choi *et al*., 1996; Bae *et al*., 2003), *B. thuringiensis* based microbial pesticides began to be used as one of effective methods to replace chemical pesticides (Schnepf *et al*., 1998).

The purpose of this paper is to isolate and identify *B. thuringiensis* from soils and select strains with excellent insecticidal activity through bioassay in order to control lepidopteran pests. We conducted the studies on insecticidal activities, morphological characteristics and molecular genetic characteristics such as endotoxin protein and plasmid DNA pattern analysis of selected strains.

## MATERIALS AND METHODS

### Sample collection

Soil samples were collected from 26 sites in mountains and fields of Yeong–Dong and Ok–cheon, ChungBuk province. After soil was dug down a depth of 10 cm, it was collected using a sterile spatula. In mountains, the soil was collected under a tree. In fields, the soils were collected from the place in which biological pesticides were not applied. Soil samples were put in a sterile container. It was sealed and stored at 4˚C.

### Isolation and identification of *B. thuringiensis* strains

Isolation of *B. thuringiensis* strains was conducted according to the method of Ohba and Aizawa (1978). After 1 g of soil sample was added into a test tube, 9 ml of sterile water was added. It was vigorously stirred 3–4 times. It was heated in the water bath at 65˚C for 30 minutes in order to selectively remove strains which cannot form spores. After the heat treatment, the soil sample was allowed to settle down for 5 minutes. Supernatant was diluted in 10–3 and then it was evenly plated on the nutrient agar plate. This plate was incubated in the incubator at 27˚C for 3–4 days. Colony with morphological appearance similar to *B. thuringiensis* strain was selected among growing *Bacillus* colonies. Selected colonies were observed at 1,000 × magnification and strains forming endotoxin proteins were selected. Selected strains were diluted in sterile water and incubated as mentioned above. After that, samples were examined to see if autolysis occurred and then strains were harvested by centrifugation at 15,000 rpm for 15 minutes using a centrifuge (Avanti J–E, Beckman). Among 8 *B. thuringiensis* strains, 2 strains were sent to Institute of Microbial Ecology & Resources in Mokwon University in order to identify them by flagelin C gene.

### Phase contrast microscope and scanning electron microscope

Colonies were observed with a phase contrast microscopy (Olympus BX51) and scanning electron microscope (Quanta FEG MK2) in order to examine the morphology of spore crystal inclusion protein in *B. thuringiensis* strain which passed the spore forming phase as it was incubated on a nutrient agar plate. After a small amount of strain was dropped on a slide glass in order to be observed with a phase contrast microscope, the morphology of crystal inclusion protein was observed at 1,000 × magnification (Kim *et al*., 1995). Strains were sent to Korea Research Institute of Bioscience and Biotechnology in order to be observed with a scanning electron microscopy.

### Test insects

Artificial diet was used for lepidopteran pests such as *S. litura* and *S. exigua* used in this experiment (Gho *et al*., 1991) and a young radish was used as a host for *P. xylostella*. Adults were fed on 10% sugar water. *Aedes aegypti* belonging to Diptera was given by the insect physiology laboratory in Chungnam National University and it was fed on young fish meals added with yeast extract. All insects were cultured over successive generations in the biological pest control laboratory in Chungnam National University. Culture conditions are as follows: Temperature of 25 ± 2˚C, light conditions of 16L:8D and relative humidity of 50–60% (Kim *et al*., 2008).

### Test of biological activity and lethal concentration 50%

After two selected strains of *B. thuringiensis* were inoculated on nutrient agar plates and incubated at 27˚C for 5 days, the formation of crystal inclusion proteins was observed with a phase contrast microscope. When endotoxin proteins were formed, bacteria were harvested using a centrifugation. Supernatant was discarded and the remaining pellets were suspended in 20 ml of sterile water. Culture solutions of CAB565 and CAB566 corresponding to $1.0\times10^7$ (cfu/ml) and $1.7\times10^7$ (cfu/ml) were used to test the biological activity, respectively. *B. thuringiensis* subsp. *aizawai* KB098 strain and *B. thuringiensis* subsp. *kurstaki* KB099 strain in the laboratory were used as control strains to compare the insecticidal activity. It was compared with TB–WP product which had a serotype of aizawai and was being marketed as a biological pesticide.

*Toxicity test in S. litura and S. exigua*

For tests of biological activity in *S. litura* and *S. exigua*, 200 µl of culture solution was added to 1 g of artificial diet. Five larvae at the second stage were placed on a petri dish and it was repeated 6 times. Lethality rate was measured for 120 hours.

*Toxicity test* in *P. xylostella*

For test of biological activity in *P. xylostella*, a leaf dip bioassay using cabbage leaf (Tabashnik *et al*., 1990,

Shelton *et al.*, 1993) was performed with targets of larvae at the third through fifth stages. Leaf disk (diameter 5 cm) was dipped in 40 ml of culture solution and dried in the shade at room temperature for 1 hour. In a control group, leaf disk was treated with distilled water in the same conditions. After treated leaf disks were transferred onto a petri dish placed on the filter papers, 10 larvae were placed and it was repeated 3 times. Lethality rate was measured for 72 hours.

*Toxicity test in A. aegypti*

For test of biological activity in *A. aegypti*, the test was conducted by a modified test method of WHO (2005). 10 larvae at the second stage were put in 30 ml of water in a 90 ml flask cup, and 0.3 ml of diluted solution was inoculated. Lethality rate was measured for 72 hours and it was repeated three times.

*Lethal concentration 50% ($LC_{50}$)*

All tests were performed with 5 larvae 6 times. For dilution concentrations, the range of 5–7 concentrations from the death and to the survival of larvae was specified. Lethal concentration ($LC_{50}$) was calculated by using a PC program (Reymond, 1985) based on the probit analysis of Finney (1971) with measured extinction rate.

**SDS–PAGE**

Each strain used in tests was inoculated on the nutrient agar plate and incubated at 27˚C for 5 days. After it was examined to see if autolysis occurred with a phase contrast microscope, PBS buffer was added and it was centrifuged in a centrifuge tube at 15,000 rpm at 4˚C for 10 minutes. After centrifugation, the supernatant was discarded and it was washed with washing buffer I (500 mM NaCl, 2% Triton X–100) three times and washing buffer II (500 mM NaCl) twice, respectively. After sterile water was added, washed parasporal inclusion was stored at –20˚C. For SDS–PAGE, a gel was made with 12% separating gel and 5% stacking gel according to the modified method of Laemmli (1970). After the electrophoresis was finished, a gel was stained with 0.5% Coomassie Brilliant Blue.

Midgut juice of *S. exigua* and *S. litura* was prepared as follows. After larvae of 5 strains were placed at –4˚C for 10 seconds, only midguts were dissected by using a sterile dissecting blade and put in a centrifugation tube on ice. It was centrifuged at 13,000 rpm at 4 ˚C for 15 minutes. Only clear supernatant was placed in an eppendorf tube and stored at –20˚C. After pesticidal crystal toxin protein was mixed with 50 mM NaOH aqueous solution at room temperature for 5 minutes, it was centrifuged. Supernatant was discarded and pellets were mixed with midgut juice. After it was incubated at 37˚C for 15 minutes, it was checked (Zhong *et al.*, 2000; Zouari and Samit, 1997).

**PCR analysis**

Gene–specific primer set was used to identify Cry type gene of endotoxin genes in *B. thuringiensis* strain of two selected species (Table 1). PCR amplification was made by using Thermal Cycle C1000™ (BIO–RAD). 1.0 µl of template DNA, 1.0 µl of each primer set and 7 µl of distilled water were added into 10 µl of premix solution (Bioneer) containing buffer and dNTP. A final volume of 20 µl was used. PCR condition was as follows: Initial denaturation at 94˚C for 5 minutes. 30 cycles of denaturation at 94˚C for 1 minute, annealing at 57˚C for 1 minute and extension at 72˚C for 1 minute. Final extension at 72˚C for 5 minutes. These cycles yielded PCR product DNA. Electrophoresis was carried out in 1.5% agarose gel (Yang *et al.*, 2011, Abdullah *et al.*, 2009, Ye *et al.*, 2009).

**Plasmid DNA isolation**

Qiagen midi kit was used to isolate plasmid DNA from two selected species of *B. thuringiensis* strains according to a modified protocol. Strains were inoculated in 5 ml of LB media and incubated at 180 rpm at 27˚C for 8 hours. Culture solution was transferred into 50 ml of LB media and then incubated in the same condition for 16 hours. Bacterial cells were harvested by centrifugation at 6,000 g at 4˚C for 15 minutes. Supernatant was discarded and 4 ml of P1 buffer (50 mM Tris–HCl, pH 8.0,

**Table 1.** Crystal protein gene–specific primers for PCR analysis

| Cry gene | Primer sequences | Size of product (bp) |
|---|---|---|
| *cry1Aa* | 5’GAGCCAAGCGACTGGAGCAGTTTACACC3’ | 782 |
| *cry1Ab* | 5’TCGAATTGAATTTGTTCC3’ | 238 |
| *cry1Ac* | 5’GTCCAACCTTATGAGTCACCTGGGCTTC3’ | 550 |
| *cry1B* | 5’GTCCAACCTTATGAGTCACCTGGGCTTC3’ | 902 |
| *cry1C* | 5’CAACCCTATTTGGTGCAGGTTC3’ | 288 |
| *cry1D* | 5’GGTACATTTAGATGTTCACAGCCAC3’ | 465 |
| *cry1E* | 5’CTTAGGGATAAATGTAAGTACAG3’ | 961 |
| *cry1F* | 5’CCGGTGACCCATTAACATTCCAATC3’ | 383 |
| *cry13’* | 5’ATCACTGAGTCGCTTCGCATCTTTGACTTTCTC3’ | – |
| *cry1G5’* | 5’ATATGGAGTGAATAGGGGG3’ | 235 |
| *cry1G3’* | 5’TGAACGGCGATTACATGC3’ | – |
| *cry1I5’* | 5’GCTGTCTACCATGATTCGCTTG3’ | 1584 |
| *cry1I3’* | 5’CAGTGCAGTAACCTTCTCTTGC3’ | – |

10 mM EDTA, 50 µg/ml of RNase A) was added. Pellets were resuspended by vortexing. Four ml of P2 buffer (0.2 M NaOH, 1% SDS) was added into a tube and mixed by inverting a tube 4–6 times. It was incubated in an incubator at 15–25°C for 5 minutes. After 4 ml of chilled P3 buffer (4 M guanidine hydrochloride, 0.5 M potassium acetate, pH 4.2) was added and mixed by inverting a tube 4–6 times, it was incubated on ice for 15 minutes. After centrifugation at 15,000 rpm at 4°C for 30 minutes, supernatant was transferred into a new tube. While centrifugation at 15,000 rpm at 4°C for 15 minutes, Qiagen–tip 100 was placed horizontally and 4 ml of QBT buffer (50 mM NaCl, 50 mM MOPS, pH 7.0, 15% isopropanol, 0.15% Triton X–100) was applied into a column. Supernatant was applied to the equilibrated column. After it was allowed to enter the resin by gravity flow, Qiagen–tip 100 was washed with 10 ml of QC buffer twice. Five ml of QF buffer was applied to elute DNA. DNA was precipitated by adding 3.5 ml of room temperature isopropanol to the eluted DNA. It was mixed and centrifuged at 15,000 rpm at 4°C for 30 minutes. Supernatant was discarded and DNA pellet was washed with 2 ml of 70% ethanol and centrifuged at 15,000 rpm at 4°C for 10 minutes. Supernatant was discarded and the pellet was air dried for 5–10 minutes. Plasmid DNA was redissolved by adding 200 µl of distilled water. After plasmid DNA was mixed in distilled water, it was transferred into a sterile Eppendorf tube and stored at –20°C prior to use. After agarose was weighed to make 1% agarose gel and mixed in 1 × TAE buffer, it was melted by heating. It was poured in a gel tray and hardened for 20 minutes. 1 × TAE buffer was poured into the electrophoresis apparatus. The buffer depth over the gel should be 3–5 mm. After loading dye and loading star were mixed in 5:1, it was mixed with plasmid DNA sample in 1:5. It was loaded into each well and electrophoresis was performed at 50 V for 60 minutes. Band patterns were observed under UV light.

## RESULTS AND DISCUSSION

### Isolation and identification of *Bacillus thuringiensis* strains

The soil is the major habitat of *B. thuringiensis*. It has been reported that about 60% or more of *B. thuringiensis* isolated from various soils exhibit toxicity to Lepidoptera and Diptera (Martin and Travers, 1989). Thus, we confirmed the possibility of isolation of new *B. thuringiensis* strains by collecting soils from 26 sites in mountains and fields of Yeong–Dong and Ok–cheon, ChungBuk province. Among 26 soil samples, *B. thuringiensis* strains were isolated from 6 soil samples, resulting in 23% which was a high percentage (Table 2).

Among 5,385 colonies formed on the nutrient agar plate, bacterial colonies with morphological appearance similar to *B. thuringiensis* colony were selected and observed with a phase contrast microscope to morphologically isolate *B. thuringiensis* strains. As a result of microscopic observation, 8 colonies were identified as *B. thuringiensis* colonies among 5,385 colonies (Table 3).

**Table 2.** Isolation of *B. thuringiensis* from soils at collecting areas

| Locality | Number of soil sample examined | Number of soil sample with *B.t* isolated |
|---|---|---|
| Young–dong | | |
| 1) Mountain | 5 | 1 |
| 2) Field | 9 | 2 |
| Ok–cheon | | |
| 1) Mountain | 4 | 2 |
| 2) Field | 8 | 1 |
| Total | 26 | 6 |

**Table 3.** Isolation of *B. thuringiensis* from bacterial colonies

| Locality | Number of colonies sample examined | Number of colonies sample with *B.t* isolated |
|---|---|---|
| Young–dong | | |
| 1) Mountain | 2,499 | 3 |
| 2) Field | 549 | 2 |
| Ok–cheon | | |
| 1) Mountain | 1,708 | 2 |
| 2) Field | 630 | 1 |
| Total | 5,386 | 8 |

Among 8 *B. thuringiensis* strains, 2 strains showing high insecticidal activity were sent to Institute of Microbial Ecology & Resources in Mokwon University in order to identify them with flagelin C gene. As a result, CAB565 and CAB566 strains were classified as *B. thuringiensis* subsp. *kurstaki*, and *B. thuringiensis* subsp. *aizawai*, respectively.

### Test of biological activity

To verify the host range and insecticidal activity of 8 selected *B. thuringiensis* strains, insecticidal activity was verified in four kinds of pests such as lepidopteran pests being cultured in the laboratory such as *S. litura*, *S. exigua*, *P. xylostella* and dipteran pest such as *A. aegypti* (Table 4). As a result, 6 strains belonging to the proper activity range were isolated in *P. xylostella*, which was the highest number. Among them, CAB565, CAB566 and CAB567 strains exhibited more than 90% insecticidal activity to pests. In addition, CAB565, CAB566 and CAB567 strains showed more than 90% insecticidal activity to *S. litura* and CAB565 and CAB566 strains showed more than 90% insecticidal activity to *S. exigua*. In addition, CAB565 and CAB566 strains showed more than 70% insecticidal activity which was lower than those isolated in Lepidoptera.

CAB565 and CAB566 strains showed high insecticidal activity and wide host range of 4 species of insects such as *S. litura*, *S. exigua*, *P. xylostella* and *A. aegypti*. Among 8 isolates, CAB559 and CAB560 strains were non–toxic strains without insecticidal activity. This result

**Table 4.** Toxicity of *B. thuringiensis* against lepidopteran and diptera larva

| Tested larvae | | Lepidoptera | | | Diptera |
|---|---|---|---|---|---|
| | | *Spodoptera litura* | *Spodoptera exigua* | *Plutella xylostella* | *Aedes aegypti* |
| Strain | CAB559 | – | – | – | – |
| | CAB560 | – | – | – | – |
| | CAB561 | + | + | ++ | + |
| | CAB562 | + | + | ++ | – |
| | CAB563 | – | – | + | – |
| | CAB565 | +++ | +++ | +++ | ++ |
| | CAB566 | +++ | +++ | +++ | ++ |
| | CAB567 | +++ | +++ | +++ | + |
| Total | | 5 | 5 | 6 | 4 |

+++: Highly effective, 90% lethality; ++: Effective, 70–89% lethality; +: Low effective, 50–69% lethality; –: not effective, 0–49%lethality

was similar to previous one in which endotoxin proteins were produced in about 30% *B. thuringiensis* strains isolated from domestic soils and non–toxic strains were reported (Kim *et al.*, 1995). *B. thuringiensis* subsp. *aizawai* KB098 strain and *B. thuringiensis* subsp. *kurstaki* KB099 strain in the laboratory were used as control strains to compare the insecticidal activity of CAB565 and CAB566 strains with high insecticidal activity. It was compared with TB–WP product which had a serotype of *aizawai* and was being marketed as a biological pesticide. It has been reported that *B. thuringiensis* subsp. *aizawai* KB098 strain has insecticidal activity to *S. litura*, *P. xylostella*, *Arete coerulea* and *S. exigua* (Kim *et al.*, 2009b). In addition, it has been reported that *B. thuringiensis* subsp. *kurstaki* KB099 strain has insecticidal activity to *S. litura*, *P. xylostella*, *Naranga aenescens* Moore, *Palpita indica* Saunders and *Culex pipiens molestus* (Choi *et al.*, 2008).

For the tests of biological activity in *S. litura*, insecticidal activity was verified with larvae from the second larvae stage to the fourth larvae stage. As a result, $LC_{50}$ value of larvae at the second stage in CAB565 strain was $1.9\times10^3$ (cfu/ml) which was significantly higher insecticidal activity than that of *B. thuringiensis* subsp. *kurstaki* KB099 which was a control strain and TB–WP product (Table 5, Fig. 1). On the other hand, $LC_{50}$ value of larvae at the third stage in CAB566 strain was $4.6\times10^5$ (cfu/ml), which was the highest insecticidal activity. $LC_{50}$ value of larvae at the fourth stage was $7.7\times10^5$ (cfu/ml), which was significantly higher insecticidal activity than that of *B. thuringiensis* subsp. *aizawai* KB098 which was a control strain and TB–WP product. It has been reported that it is difficult to control it because *S. litura* has strong resistance and larvae at the third or later stage have low sensitivity to drugs. (Bae *et al.*, 2003). Based on these results, it is thought to be an effective method to control *S. litura* at the early larvae stage which is the initial development stage. For tests of biological activity in *S. exigua*, insecticidal activity was verified with larvae from the second larvae stages to the fifth larvae stage. $LC_{50}$ value of larvae from the second

**Table 5.** Toxicity ($LC_{50}$) of *B. thuringiensis* against *S. litura*

| Strain | 2nd (cfu/ml) | 3rd (cfu/ml) | 4th (cfu/ml) |
|---|---|---|---|
| KB098 | $9.8\times10^5$ | $5.2\times10^7$ | $>10^7$ |
| KB099 | $2.7\times10^5$ | $3.8\times10^6$ | $5.0\times10^7$ |
| CAB530 | $2.3\times10^5$ | $4.4\times10^6$ | $>10^7$ |
| CAB565 | $1.9\times10^3$ | $9.0\times10^6$ | $>10^7$ |
| CAB566 | $3.9\times10^3$ | $4.6\times10^5$ | $7.7\times10^5$ |
| TB–WP | $1.4\times10^5$ | $5.8\times10^7$ | $>10^7$ |

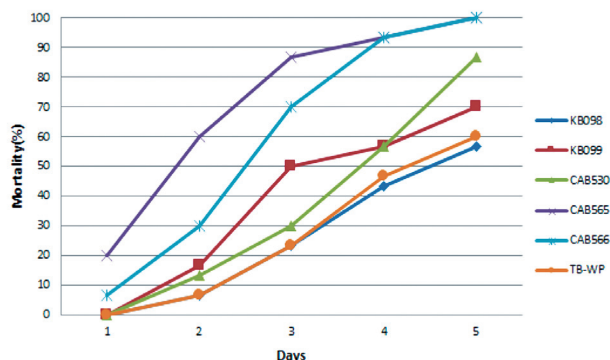


**Fig. 1.** Mortality of *S. litura* 2nd larva at the same concentrations ($10^5$ cfu/ml) of insecticide treatment.

stage to the fifth stage in CAB566 strain was $2.5\times10^4$ (cfu/ml), $3.0\times10^5$ (cfu/ml), $4.6\times10^5$ (cfu/ml) and $9.7\times10^5$ (cfu/ml), respectively. These values showed significantly higher insecticidal activity than that of *B. thuringiensis* subsp. *kurstaki* KB098 which was a control strain and TB–WP product (Table 6, Fig. 2). The value at the second stage was the most sensitive, but other $LC_{50}$ values from the third stage to the fifth stage were not significantly different.

To compare tests of biological activities and rapid actions of CAB565, CAB566 strains, *B. thuringiensis* subsp. *aizawai* KB098, *B. thuringiensis* subsp. *kurs-*

*taki* KB099 strain and TB–WP product against *P. xylostella*, it was treated with larvae from the third stage to the fifth stage with the same concentration. As a result, CAB565 and CAB566 strains showed higher toxicity than that of other strains and the product. More rapid insecticidal activities were shown (Fig. 3). In the case of CAB565 and CAB566 strains, the mortality was dramatically increased from 36 hours. CAB565 strain showed 100% of mortality from 48 hours. CAB566 strain showed 100% of mortality from 60 hours. It was shown that CAB565 and CAB566 strains had high toxicity against lepidopteran pests. Thus, it is expected that it could be a good *B. thuringiensis* strain to be used in biological control.

**Table 6.** Toxicity ($LC_{50}$) of *B. thuringiensis* against *S. exigua*

| Strain | 2nd (cfu/ml) | 3rd (cfu/ml) | 4th (cfu/ml) | 5th (cfu/ml) |
|---|---|---|---|---|
| KB098 | $1.3\times10^{6}$ | $8.7\times10^{6}$ | $9.8\times10^{6}$ | $3.3\times10^{7}$ |
| KB099 | $8.1\times10^{5}$ | $2.3\times10^{6}$ | $2.7\times10^{6}$ | $1.2\times10^{7}$ |
| CAB530 | $2.3\times10^{5}$ | $2.5\times10^{6}$ | $2.7\times10^{6}$ | $9.8\times10^{6}$ |
| CAB565 | $4.9\times10^{4}$ | $3.2\times10^{5}$ | $5.6\times10^{5}$ | $3.0\times10^{6}$ |
| CAB566 | $2.5\times10^{4}$ | $3.0\times10^{5}$ | $4.6\times10^{5}$ | $9.7\times10^{5}$ |
| TB–WP | $1.0\times10^{6}$ | $3.2\times10^{6}$ | $4.9\times10^{7}$ | $3.8\times10^{7}$ |

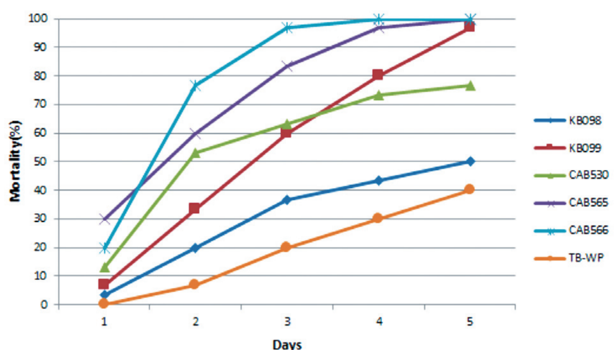


**Fig. 2.** Mortality of *S. exigua* 2nd larva at the same concentrations ($10^{6}$ cfu/ml) of insecticide treatment.

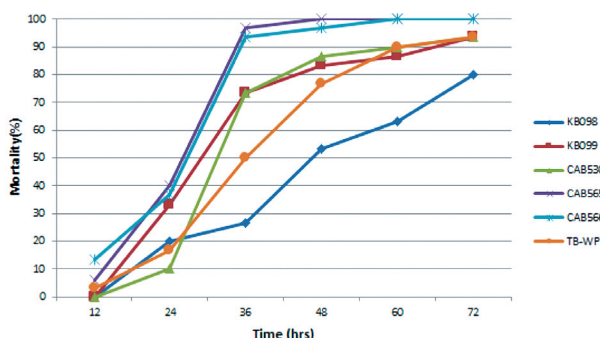


**Fig. 3.** Mortality of *P. xylostella* various age larva at the same concentrations ($10^{3}$ cfu/ml) of insecticide treatment.

**Morphological characteristics**

To characterize the morphology of crystals of CAB565 and CAB566 strains with high insecticidal activity against lepidopteran pests, crystal and spore were observed with a phase contrast microscope. As a result of observation with a phase contrast microscope, it was found that CAB565 and CAB566 strains had crystal with a general bipyramidal shape (Fig. 4A & B). It has been reported that crystal with a bipyramidal shape has cry1 toxin protein and activity against Lepidoptera (Armengol *et al.*, 2007; Donovan *et al.*, 1988).

It was found that CAB565 strain had crystal with a bipyramidal shape similar to *B. thuringiensis* subsp. *kurstaki* KB099 strain, and CAB566 strain had crystal with a bipyramidal shape similar to *B. thuringiensis* subsp. *kurstaki* KB098 strain.

In addition, a scanning electron microscope was used to compare crystals of CAB565 and CAB566 strains. Like observation with a phase contrast microscope, it was confirmed that it had crystal with a bipyramidal shape (Fig. 4C & D). As both strains had crystals with bypyramidal shape and activities against lepidopteran pests, we can assume that both strains have cry1 toxin protein gene.

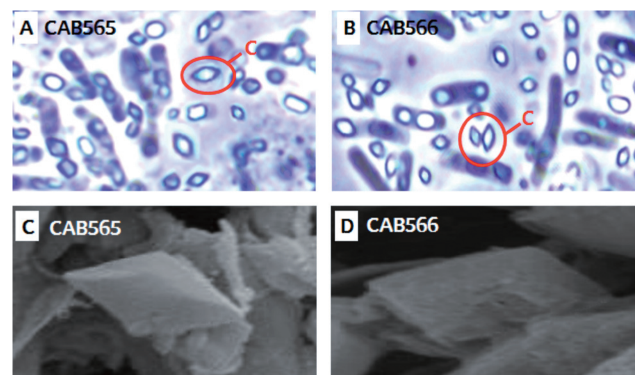


**Fig. 4.** (A) and (B): Phase–contrast microscope photographs (×1000) of crystal shape of *B. thuringiensis* isolates. (C) and (D): Scanning electron microscopy of spore–crystal mixtures of *B. thuringiensis* strains.

**SDS–PAGE**

Crystal protein has a molecular weight of 25–140 kDa and consists of $\delta$–endotoxin. Among three kinds of crystal proteins produced in *B. thuringiensis*, a protein with a molecular weight of 130 kDa–145 kDa in bipyramidal shape has activity mainly against lepidopteran pests. A protein with a molecular weight of 100 kDa in cuboidal shape has activity mainly against coleoptera (Bravo *et al.*, 1998). A protein with a molecular weight of 65 kDa in spherical shape has activity mainly against diptera (Ibarra and Federici, 1986).

Protein patterns of CAB565 and CAB566 strains showing high insecticidal activity against lepidopteran pests were examined. As a result of electrophoresis with 12% separating gel, it was found that CAB565 strain had a band at 130 kDa which was shown in *B. thuringiensis* subsp. *kurstaki* KB099 strain. Because it showed a very similar pattern, it was assumed that it was *kurstaki* strain.

It was found that CAB566 strain had a band at 130 kDa which was shown in *B. thuringiensis* subsp. *kurstaki* KB098 strain. In addition, two similar bands with the same size were observed (Fig. 5).

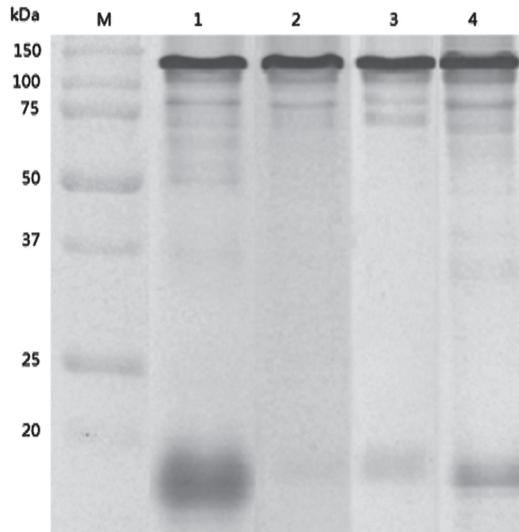


**Fig. 5.** SDS–PAGE analysis of each strain crystal. M: Broad range marker, Lane 1: *B. thuringiensis* subsp. *aizawai* KB098; Lane 2: *B. thuringiensis* subsp. *kurstaki* KB099; Lane 3: *B. thuringiensis* subsp. *kurstaki* CAB565; Lane 4: *B. thuringiensis* subsp. *aizawai* CAB566.

To determine the aspects of active toxin of parasporal inclusion of two strains, midgut juice of *S. litura* and *S. exigua* was extracted and allowed to react with each strain at 37°C. First, when midgut juice of *S. litura* was treated, protoxin of 130 kDa in CAB565 and CAB566 strains was digested by midgut juice and a band of toxin was observed at about 65 kDa (Fig. 6). When midgut juice of *S. exigua* was treated, protoxin of 130 kDa of CAB565 and CAB566 strains was digested by midgut juice and a band of toxin was observed at about 65 kDa as well (Fig. 7).

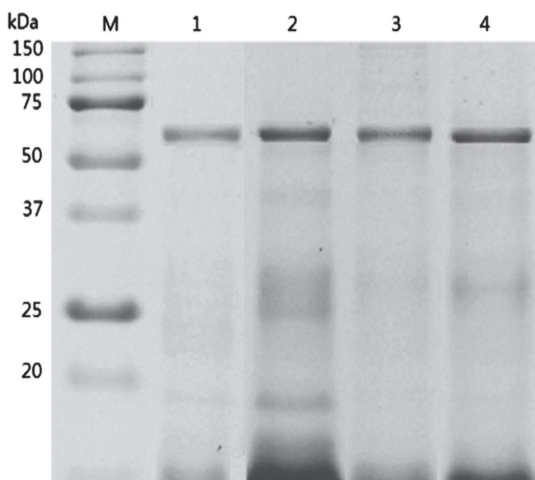


**Fig. 6.** Effect of protoxin digested by *S. litura* midgut juice. M: Broad range marker, Lane 1: *B. thuringiensis* subsp. *aizawai* KB098; Lane 2: *B. thuringiensis* subsp. *kurstaki* KB099; Lane 3: *B. thuringiensis* subsp. *kurstaki* CAB565; Lane 4: *B. thuringiensis* subsp. *aizawai* CAB566.

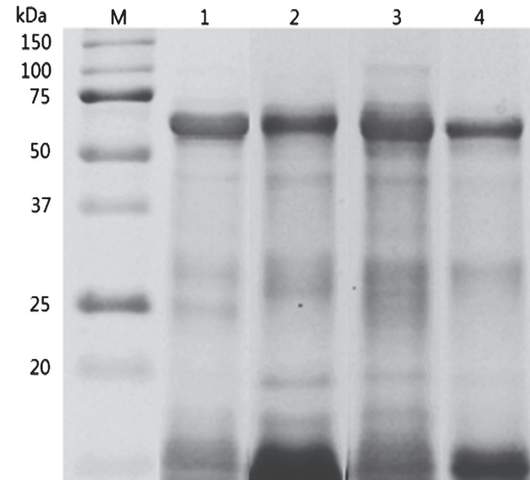


**Fig. 7.** Effect of protoxin digested by *S. exigua* midgut juice. M: Broad range marker, Lane 1: *B. thuringiensis* subsp. *aizawai* KB098; Lane 2: *B. thuringiensis* subsp. *kurstaki* KB099; Lane 3: *B. thuringiensis* subsp. *kurstaki* CAB565; Lane 4: *B. thuringiensis* CAB566.

**Identification of Cry–type genes**

PCR analysis was used to identify crystal proteins in *B. thuringiensis*. It has been reported that *cry1B* had activity against Lepidoptera, Coleoptera and Diptera (Zhong *et al*., 2000), *cry1I* had activity against Lepidoptera and Coleoptera (Tailor *et al*., 1992), and *cry2* had activity against Lepidoptera and Diptera (Donovan *et al*., 1988). It has been reported that *B. thuringiensis* subsp. *kurstaki* KB099 strain has 5 kinds of genes such as *cry1Aa*, *cry1Ab*, *cry1C*, *cry1D* and *cry1I* (Jung *et al*., 2010). It has been reported that *B. thuringiensis* subsp. *aizawai* series have four kinds of genes such as *cry1Aa*, *cry1Ab*, *cry1C* and *cry1D* (Aronson *et al*., 1991; Höfte and Whitely, 1989). *B. thuringiensis* subsp. *aizawai* KB098 strain showed activity against *S. exigua* in the previous experiment and it had *cry1Aa*, *cry1Ab*, *cry1C* and *cry1D* genes. *B. thuringiensis* subsp. *kurstaki* KB099 strain showed activity against *Spodoptera litura* and it had *cry1Aa*, *cry1Ab*, *cry1C*, *cry1D* and *cry1I* genes (Table 7). It was found that CAB565 strain isolated and identified in this experiment had *cry1Aa*, *cry1Ac* and *cry1I* genes and it had the patterns of crystal genes similar to those of *B. thuringiensis* subsp. *kurstaki* KB099 strain (Fig. 8A). In addition, it was found that CAB566 strain had *cry1Aa*, *cry1C*, *cry1D* and *cry1I* genes, showing the same patterns of those in *B. thuringiensis* subsp. *aizawai* KB098 strain (Fig. 8B).

It has been known that *cry1C* gene has high activity against *S. litura* and *S. exigua* and *cry1D* gene has toxicity to *S. litura* (Porcar *et al*., 2000). Because CAB566 strain shows high activity against *S. litura* and *S. exigua*, we can assume that this strain has high activity against both pests due to the presence of *cry1C* and *cry1D* genes in this strain.

**Table 7.** Profiles of cry genes in *B. thuringiensis* strains

| Strain | Cry genes |
|---|---|
| *B. thuringiensis* subsp. *kustaki* CAB565 | *cry1Aa, cry1Ac, cry1I* |
| *B. thuringiensis* subsp. *kustaki* KB099 | *cry1Aa, cry1Ab, cry1C, cry1D, cry1I* |
| *B. thuringiensis* subsp. *aizawai* CAB566 | *cry1Aa, cry1C, cry1D, cry1I* |
| *B. thuringiensis* subsp. *aizawai* KB098 | *cry1Aa, cry1Ab, cry1C, cry1D* |

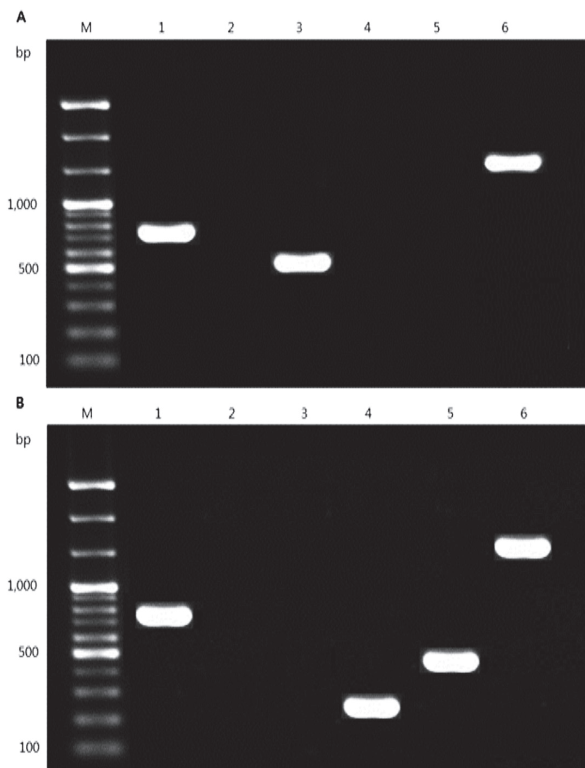


**Fig. 8.** Agarose gel (1.5%) electrophoresis of PCR products obtained with specific primers for the cry genes. (A) *B. thuringiensis* subsp. *kurstaki* CAB565. Lane 1: *cry1Aa*; Lane 2: *cry1Ab*; Lane 3: *cry1Ac*; Lane 4: *cry1C*; Lane 5: *cry1D*; Lane 6: *cry1I*. (B) *B. thuringiensis* subsp. *aizawai* CAB566. Lane 1: *cry1Aa*; Lane 2: *cry1Ab*; Lane 3: *cry1Ac*; Lane 4: *cry1C*; Lane 5: *cry1D*; Lane 6: *cry1*.

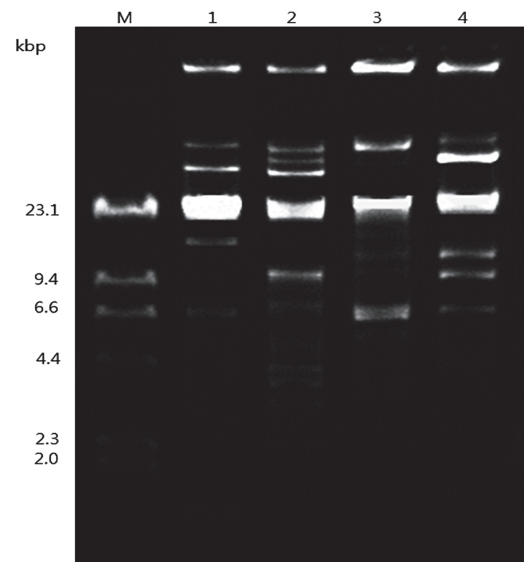


**Fig. 9.** Plasmid DNA patterns of *B. thuringiensis*. M. Lamda Hind Ⅲ marker. Lane 1: *B. thuringiensis* subsp. *kurstaki* KB099; Lane 2: *B. thuringiensis* subsp. *kurstaki* CAB565; Lane 3: *B. thuringiensis* subsp. *aizawai* KB098; Lane 4: *B. thuringiensis* subsp. *aizawai* CAB566.

## Plasmid DNA analysis

Cry gene involved in the production of crystal protein showing insecticidal activity against *B. thuringiensis* target pests is present mainly in plasmid DNA. Plasmid DNA present in *B. thuringiensis* consists of up to 17 DNAs and the length is 2–250 kb (Sarrafzadeh *et al.*, 2007). Number and length of plasmid DNA vary in each strain. Similarly, cry gene is not present in plasmid DNA with the same size in all strains but present in plasmid DNA with different locations (Kronstad *et al.*, 1983; Aronson *et al.*, 1986).

CAB565 strain and *B. thuringiensis* subsp. *kurstaki* KB099 strain were used as control strains. CAB566 strain and *B. thuringiensis* subsp. *aizawai* KB098 strain were used to identify plasmid DNA patterns as control strains (Fig. 9). As a result of entire plasmid DNA analysis of CAB565 strain, it had different plasmid DNA patterns from that of *B. thuringiensis* subsp. *kurstaki* KB099 strain despite the same serotype. It was found that CAB565 strain and *B. thuringiensis* subsp. *kurstaki* KB099 strain contained 10 and 5 plasmid DNAs, respectively. In addition, it was confirmed that CAB565 strain and *B. thuringiensis* subsp. *kurstaki* KB099 strain had 3 and 2 plasmid DNAs which were bigger than 23.1 kb, respectively. It was found that CAB565 strain had bands of 9.4 kb and 4.4 kb which were not present in *B. thuringiensis* subsp. *kurstaki* KB099 strain. Like previous results, as a result of entire plasmid DNA analysis of CAB566 strain, it had different plasmid DNA patterns from that of *B. thuringiensis* subsp. *aizawai* KB098 strain despite the same serotype. It was found that CAB566 strain and *B. thuringiensis* subsp. *aizawai* KB098 strain contained 7 and 9 plasmid DNAs, respectively. It was confirmed that both CAB566 strain and *B. thuringiensis* subsp. *aizawai* KB098 strain had 2 plasmid DNAs which were bigger than 23.1 kb and a band of 6.6 kb was additionally observed. It was found that CAB566 strain had bands of 9.4 kb and 4.4 kb which were not present in *B. thuringiensis* subsp. *aizawai* KB098 strain. Bands of smaller than 4.4 kb were not shown. Thus, the difference between two strains was confirmed by the presence of bands of 4.4 kb or low.

In previous results, it was difficult to distinguish CAB565 and CAB566 strains from *B. thuringiensis*

subsp. *kurstaki* KB099 and *B. thuringiensis* subsp. *aizawai* KB098 strains as controls only with morphological characteristics and protein properties. It was confirmed that both strains were different through results of plasmid DNA analysis. CAB565 and CAB566 strains had the same serotype of strains being used to control the pests in the existing microbial agents, but they had different patterns of gene expression. On the basis of above results, we would like to confirm that those strains are molecular biologically new strains through Cry DNA analysis and review the possibility of a new microbial insecticide at the same time.

It is found that both strains are molecular biologically different and new strains even if both had the same serotype as *B. thuringiensis* subsp. *aizawai* and *B. thuringiensis* subsp. *kurstaki*.

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