Characteristic of New Bacillus thuringiensis Strains with Insecticidal Activity Against Bradysia agrestis (Sciaridae: Diptera)

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Characteristic of New *Bacillus thuringiensis* Strains with Insecticidal Activity Against *Bradysia agrestis* (Sciaridae: Diptera)

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Recently, as the number of farms that cultivate crops using organic compost in greenhouses has been increasing in South Korea, the occurrence of *Bradysia agrestis* have been coming to the fore seriously. The larvae of *B. agrestis* appear in the roots of various crops because their host range is wide and they cause serious damage to the crops as they suppress the growth and defoliate the crops. Therefore, the search for new biological control agents was investigated.

To screening new *B. thuringiensis* with high insecticidal activities against Diptera, three isolates that form spherical type crystals were selected from domestic soils. The LC_{95} value of *B. thuringiensis* strain CAB452, which is the most toxic to the larvae of Diptera *B. agrestis* among those three strains, was shown to be 4.4×10^7 (cfu/ml). SDS–PAGE was performed to identify the δ–endotoxin protein band pattern of *B. thuringiensis* strain CAB452 and the result indicated one main protein band of about 150 kDa. In order to investigate the proteins of the active toxin of this strain, this strain was treated with trypsin, which is a digestive enzyme, or *B. agrestis* larva triturated solution and as a result, one main band of about 50 kDa was shown under each of the two conditions. PCR analysis of the crystal proteins of *B. thuringiensis* strain CAB452 revealed two genes, cry4A and cry9D.

Key words: *Bacillus thuringiensis*, Biological activity, *Bradysia agrestis*, Spherical crystal type

INTRODUCTION

Bradysia agrestis is an insect belonging to the genus *Bradysia* in the family Sciaridae in the order Diptera, which was first reported in watermelon seedlings in a greenhouse plantation in South Korea (Park *et al*., 1999). Diptera *Bradysia* occurs mainly in greenhouses, nurseries, and mushroom cultivation facilities and recently, the frequency of occurrences and damage have been rapidly increasing in seedling plantations for herbaceous ornamentals for garden plantation and home bonsai cultivation (Kim *et al*., 2001, 2012, 2013). In South Korea, 21 kinds of crops such as pepper, watermelon, melon, cucumber, tomato, strawberry, paprika, and chrysanthemum are recorded as host plants (Lee *et al*., 2001) and in foreign countries, 54 kinds such as begonia and carnation are recorded (Harris *et al*., 1995). In particular, the occurrences are severe in rockwool–cultured crops such as tomatoes and paprika and damage was identified in bulbs and tubers such as lilies and ginger, which are crops of which the roots are used. As such, the density of *B. agrestis* increases along with rich organic matter in soil and the humid greenhouse environments leading to severer damage (Lindquest *et al*., 1985). The symptoms of damage caused by larvae cannot be easily observed with the naked eye because the larvae gnaw the roots or stems of plants as if they peel the roots or stems or penetrate into roots through root caps to do harm (Binns, 1973). In addition to direct damage, the larvae cause diseases by transmitting pathogenic bacteria such as Fusarium, Phoma, Pythium and Vericillium and the imagoes carry mycelia attached to their body such as the belly, legs and wings to transfer pathogenic bacteria to other plants or greenhouses thereby causing damage, (Ludwig and Oetting, 2001). *B. agrestis* is an insect pest that cannot be easily controlled because once it has begun to occur in greenhouses where crops are cultivated using organic matter, its eggs, larvae, pupae, and imagoes coexist in a short period of time or all instars except for imagoes inhabit soil or root tissues (Lindquist *et al*., 1985). To control this insect pest, registered chemical insecticides such as benfuracarb, diflubenzuron, fenthion, and teflubenzuron have been used (Kim *et al*., 2012, Kim *et al*., 2013). However, the use of insecticides is very limited due to problems such as the safety of the product, the appearance of resistant individuals, damage to humans and livestock, and the induction of environmental pollution (Jagdale *et al*., 2004). Therefore, studies are necessary on biological control, which is an alternative method to effectively control *B. agrestis* while being safe to environments (Kim *et al*., 2013). Until recently, *B. thuringiensis* strains, insect pathogenic nematodes, and predatory mites have been used for biological control of Diptera in the genus Badysia (Jeon *et al*., 2007) but studies on biological control factors for Diptera are still insufficient.

B. thuringiensis has been reported to have been

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isolated from every habitats around us, including soils, insects, leaves and conifer leaves, water, stored grain flour, and sericulture farms (Lee *et al*., 2014, 2015). *B. thuringiensis*, which is widely used as a microbial insecticide, is used as a major agricultural material in eco–friendly agriculture because it is safe to non–selective organisms (Lee *et al*., 2014, 2015). *B. thuringiensis* is an insect pathogenic bacterium, and aerobic gram–positive bacilli that forms endogenous spores and produce insecticidal proteins composed of δ–endotoxin proteins. These insecticidal crystal proteins (ICPs) exhibit host specificities that differ in their toxicity to various insects such as lepidoptera, Diptera, and Coleoptera (Schnepf, 1995). In addition, their activity has also been reported for Hymenoptera, Hemiptera, Orthoptera, mite, nematodes, and protozoans (Lee *et al*., 2014, 2015). Interest in *B. thuringiensis* as a biological control agent is increasing worldwide, and *B. thuringiensis subsp. israelensis* strains that have high toxicity to Diptera larvae are mainly marketed as products to control Diptera insect pests (Seo *et al*., 2010). However, other microbial insecticides registered in order to control other Diptera insect pests are insufficient.

Therefore, in this study, new *B. thuringiensis* strains, which are new biological control agents with insecticidal activities in *B. agrestis*, which is becoming problematic because the area of greenhouse cultivation where crops are cultivated using organic agent is rapidly increasing, were selected and their biological characteristics were analyzed.

MATERIALS AND METHODS

Isolation and identification of *Bacillus thuringiensis* isolate

A selection test was conducted with 50 strains which were searched and isolated from domestic soils and kept in the Biological Pest Control Laboratory of Chungnam National University. Those strains that form δ–endotoxin proteins were selected by observation under a phase contrast microscope. The selected strains were diluted in sterilized water and evenly spread on a nutrient agar plate. The plate was incubated in a 27°C incubator for 3 to 4 days and after identifying that autolysis had occurred, the plate was centrifuged at 15,000 rpm for 15 minutes using a centrifuge (Avanti J–E, Beckman) to collect bacteria. Among the 10 isolated strains of *B. thuringiensis*, one strain with the highest insecticidal activities in *Bradysia agrestis* was sent to Mokwon University Micro–Ecological Resources Research Institute to conduct identification of the strain with flagellin C gene.

Test insect

The Diptera *B. agrestis* used in this experiment was bred using potatoes as feed, and the *Aedes albopictus* was bred using yeast extract added to fry feed (Kim *et al*., 2008). The insects were bred for successive generations in the biological pest control laboratory of Chungnam National University under the conditions of temperature 25 ± 2°C, light condition 16L:8D, relative humidity 50–60% (Kim *et al*., 2008). *A. albopictus* imagoes were supplied with 10% sugar water as feed, and *B. agrestis* imagoes were bred with the method of Jang *et al*. (2018).

Bioactivity assay

The 10 selected strains of *B. thuringiensis* were inoculated into a nutrient agar medium and cultured at 27°C for 5–6 days. Thereafter, bacteria were collected while observing the formation of δ–endotoxin crystal proteins under a phase contrast microscope and the collected bacteria were centrifuged. The supernatant was discarded, and 20 ml of sterilized water was added to the remaining pellet for use in bioassay. In order to investigate insecticidal activities, experiments were conducted with a total of 11 strains, that is, 10 new strains held by the laboratory and *B. thuringiensis* subsp. *israelensis* strain.

The bioactivity test for *B. agrestis* in the family Sciaridae in the order Diptera was carried out by adding 200 μl of the culture solution to 1 g of potato and placing 20 each of 3–4 days old larvae after hatching in each petri dish. This experiment was carried out in three repetitions and the mortality rates were investigated for 168 hours.

The bioactivity test for *A. albopictus* in the family Culicidae in the order Diptera was conducted with the experimental method presented by WHO (2005) with some modification. Ten each of larvae 3–4 days old after hatching were put into each 90 ml plastic cup containing 30 ml of water, and 0.3 ml of the diluted bacterial solution was inoculated. The mortality rate was investigated for 72 hours, and the experiment was carried out in three repetitions.

SDS–PAGE

Each strain was inoculated on a nutrient agar medium and cultured at 27°C for 5 days. After identifying that autolysis had occurred with a phase contrast microscope, the bacteria were collected and centrifuged at 15,000 rpm at 4°C for 10 minutes. After the centrifugation, the supernatant was discarded and the bacteria were washed twice with washing buffer I (500 mM NaCl, 2% Triton X–100) and three times with washing buffer Ⅱ (500 mM NaCl) to remove some resistant spores while collecting δ–endotoxin crystalline proteins. Thereafter, sterile water was added to the washed parasporal inclusions and 1 ml each of the mixture was put into each 1.5 ml and kept at –20°C. SDS–PAGE was conducted in the method of Laemmli (1970) with some modification using 12% separating gel and 5% stacking gel. After electrophoresis, the gel was stained with 0.5% Coomassie brilliant blue, decolored for 12 hours, and the results were identified.

Plasmid DNA analysis

The protocol of Qiagen midi kit was used after some modification to extract plasmid DNAs from the selected *B. thuringiensis* strains. Each of the strains was inocu-

lated into 5 ml of LB medium and cultured at 27°C and 180 rpm for 8 hours. The culture solution was put into 50 ml of LB medium and incubated for 16 hours under the same conditions. The cultured bacteria were centrifuged under the conditions of 6,000 g, 15 minutes, and 4°C. The supernatant was discarded, 4 ml of P1 buffer (50 mM Tris–HCl, pH8.0, 10 mM EDTA, 50 μg / ml of RNase A) was added to the pellet, and the pellet was dissolved by vortexing. Thereafter, 4 ml of P2 buffer (0.2 M NaOH, 1% SDS) was added, the tube was turned upside down 4–6 times to mix the content, and the content was incubated in a 15–25°C incubator for 5 minutes. Then, 4 ml of chilled P3 buffer (4 M guanidine hydrochloride, 0.5 M potassium acetate, pH4.2) was added, the tube was turned upside down 4–6 times to mix the content, and the content was incubated in ice for 15 minutes. The content was centrifuged at 15,000 rpm, 4°C for 30 minutes, and the supernatant was transferred to a new tube. After centrifugation at 15,000 rpm at 4°C for 15 minutes, a Qiagen–tip 100 was placed horizontally, 4 ml of QBT buffer (50 mM NaCl, 50 mM MOPS, pH7.0, 15% isopropanol, 0.15% Triton X–100) was put into the Qiagen–tip 100 to pass through it, and the centrifuged supernatant was put into the column. When the supernatant completely passed through the column, the Qiagen–tip 100 was washed twice with 10 ml of QC buffer. The DNA was dissolved and isolated with 5 ml of QF buffer and 3.5 ml of isopropanol at room temperature was added to precipitate the DNA and the solution was centrifuged at 15,000 rpm and 4°C for 30 minutes. The supernatant was discarded, 2 ml of 70% ethanol was added to wash the DNA pellet, and the solution was centrifuged for 10 minutes at 15,000 rpm at 4°C. The supernatant was discarded, the DNA pellet was air–dried for 5 to 10 minutes, and 200 μl of triple distilled water was added to dissolve the plasmid DNA again. The plasmid DNA was mixed with the triple distilled water, placed in a sterile Eppendorf tube, and stored at –20°C until being used in the experiment. The electrophoresis was performed using the agarose gel made by mixing agarose gel in 1 × TAE buffer at a ratio of 1%, dissolving the gel by heating, and then pouring the gel into a gel tray, and allowing the gel to be hardened for 20 minutes. Thereafter, 1 × TAE buffer, which is identical to the gel, was poured on the electrophoresis apparatus so that it came up about 3–5 mm above the gel, a mixed fluid made by mixing the loading dye and loading star at a ratio of 5:1 was mixed with the plasmid DNA sample at a ratio of 1:5. The mixture was put into individual wells, electrophoresed at 50 V for 60 minutes, and irradiated with UV to identify the band pattern.

Plasmid DNA analysis

The protocol of Qiagen midi kit was used after some modification to extract plasmid DNAs from the selected *B. thuringiensis* strains. Each of the strains was inoculated into 5 ml of LB medium and cultured at 27°C and 180 rpm for 8 hours. The culture solution was put into 50 ml of LB medium and incubated for 16 hours under the same conditions. The cultured bacteria were centrifuged under the conditions of 6,000 g, 15 minutes, and 4°C. The supernatant was discarded, 4 ml of P1 buffer (50 mM Tris–HCl, pH8.0, 10 mM EDTA, 50 μg / ml of RNase A) was added to the pellet, and the pellet was dissolved by vortex. Thereafter, 4 ml of P2 buffer (0.2 M NaOH, 1% SDS) was added, the tube was turned upside down 4–6 times to mix the content, and the content was incubated in a 15–25°C incubator for 5 minutes. Then, 4 ml of chilled P3 buffer (4 M guanidine hydrochloride, 0.5 M potassium acetate, pH4.2) was added, the tube was turned upside down 4–6 times to mix the content, and the content was incubated in ice for 15 minutes. The content was centrifuged at 15,000 rpm, 4°C for 30 minutes, and the supernatant was transferred to a new tube. After centrifugation at 15,000 rpm at 4°C for 15 minutes, a Qiagen–tip 100 was placed horizontally, 4 ml of QBT buffer (50 mM NaCl, 50 mM MOPS, pH7.0, 15% isopropanol, 0.15% Triton X–100) was put into the Qiagen–tip 100 to pass through it, and the centrifuged supernatant was put into the column. When the supernatant completely passed through the column, the Qiagen–tip 100 was washed twice with 10 ml of QC buffer. The DNA was dissolved and isolated with 5 ml of QF buffer and 3.5 ml of isopropanol at room temperature was added to precipitate the DNA and the solution was centrifuged at 15,000 rpm and 4°C for 30 minutes. The supernatant was discarded, 2 ml of 70% ethanol was added to wash the DNA pellet, and the solution was centrifuged for 10 minutes at 15,000 rpm at 4°C. The supernatant was discarded, the DNA pellet was air–dried for 5 to 10 minutes, and 200 μl of triple distilled water was added to dissolve the plasmid DNA again. The plasmid DNA was mixed with the triple distilled water, placed in a sterile Eppendorf tube, and stored at –20°C until being used in the experiment. The electrophoresis was performed using the agarose gel made by mixing agarose gel in 1 × TAE buffer at a ratio of 1%, dissolving the gel by heating, and then pouring the gel into a gel tray, and allowing the gel to be hardened for 20 minutes. Thereafter, 1 × TAE buffer, which is identical to the gel, was poured on the electrophoresis apparatus so that it came up about 3–5 mm above the gel, a mixed fluid made by mixing the loading dye and loading star at a ratio of 5:1 was mixed with the plasmid DNA sample at a ratio of 1:5. The mixture was put into individual wells, electrophoresed at 50 V for 60 minutes, and irradiated with UV to identify the band pattern.

PCR analysis

Gene–specific primer sets known to have activity in Diptera were used to identify the Cry–type gene among the δ–endotoxin genes of the selected *B. thuringiensis* strains. PCR amplification was performed using Thermal Cycle C 1,000 ™ (BIO–RAD). The reaction solution was prepared by mixing 17 μl of distilled water, 1.0 μl of template DNA, and 1.0 μl of each primer set into a premix (Bioneer) containing a buffer component and dNTP and adjusting the final volume to 20 μl. PCR was conducted under conditions consisting of 30 cycles for 3 min at 95°C, 1 min at 95°C, annealing for 1 min at 57°C and for

5 min at 72°C to produce PCR product DNA. The product DNA produced as such was electrophoresed on 1.0% agarose gel (Yang *et al*., 2011).

RESULTS AND DISCUSSION

Selection and characterization of *B. thuringiensis* strains

B. agrestis, which occurs in domestic greenhouses, causes serious damage by directly doing harm to the roots of crops or ornamental plants grown using organic agent on culture media or soil (Jagdale *et al*., 2004). Twenty one kinds of crops have been reported as host plants in South Korea (Lee *et al*., 2001) and 54 kinds have been recorded in foreign countries (Harris *et al*, 1995). Sticky traps are used at forms to control the imagoes that cause damage to the upper parts of crops but the population of *B. agrestis* cannot be completely controlled without controling the larvae that cause damage to roots. Therefore, to select new *B. thuringiensis* strains, which are biological control agents, effective for control of *B. agrestis* larvae, 50 strains that had been kept in the Biological Pest Control Laboratory of Chungnam National University were selected. The morphology of the toxin protein crystals of the cultured strains was examined under a phase contrast microscope. Of the strains, 12 did not form any toxin protein crystal and 28 that formed bipyramidal type toxin protein crystals were investigated. In addition, the remaining 10 strains were found to form spherical type toxin protein crystals known to have activity in Diptera. The 10 strains that form spherical type toxin protein crystals were selected and their insecticidal activities in *B. agrestis* in the family Sciaridae in the order Diptera and *Aedes albopictus* in the family Culicidae in the order Diptera were tested. According to the results, among the 10 strains, *B. thuringiensis* strains CAB463 and CAB467 had almost no effect in *B. agrestis*. In *B. agrestis*, *B. thuringiensis* strain CAB452 showed high insecticidal activities over 90%, *B. thuringiensis* strains CAB117, CAB469 and CAB599 showed insecticidal activities over 70%, and *B. thuringiensis subsp. israelensis* strain and *B. thuringiensis* strains CAB459, CAB462, CAB490 and CAB492 showed insecticidal activities of at least 50%. In *A. albopictus*, *B. thuringiensis subsp. israelensis* strain and *B. thuringiensis* strains CAB117, CAB452 and CAB599 showed high insecticidal activities of over 90%. Therefore, *B. thuringiensis* strains CAB117, CAB452, CAB599 that showed insecticidal activities of at least 70% in both Diptera *B. agrestis* and *A. albopictus* were selected.

Bioactivity assay

B. thuringiensis strains CAB117, CAB452, CAB599 that showed insecticidal activities of at least 70% in both Diptera *B. agrestis* and *A. albopictus* were selected and the bioactivity of these strains were tested using *B. thuringiensis* subsp. *israelensis* strain held in the laboratory as a control (Table 1). According to the results, the LC_{95} values of *B. thuringiensis* strains CAB117, CAB599, and *B. thuringiensis* subsp. *israelensis* strain were at least 10^8 (cfu/ml) and that of LC_{95} value of *israelensis* strain was over 10^8 (cfu/ml) and the LC_{95} value of *B. thuringiensis* strain CAB452 was 4.4×10^7 (cfu/ml). In *A. albopictus*, the LC_{95} value of *B. thuringiensis* strain CAB452 was 4.2×10^6 (cfu/ml) that of *B. thuringiensis* strain CAB599 was 3.9×10^7 (cfu/ml) that of *B. thuringiensis* strain CAB117 was 2.6×10^7 (cfu/ml), and that of *B. thuringiensis* subsp. *israelensis* strain was 2.2×10^5 (cfu/ml). Whereas *B. thuring-*

Table 1. Toxicity (LC_{95}*) of *B. thuringiensis* strain against *B. agrestis* and *A. albopictus*

Strains	Diptera	
	B. agrestis (cfu/ml)	*A. albopicus* (cfu/ml)
CAB452	4.4×10^7	4.2×10^6
CAB117	$> 10^8$	2.6×10^7
CAB599	$> 10^8$	3.9×10^7
B. thuringiensis israelensis	$> 10^8$	2.2×10^5
Control	0	0

* LC_{95} (lethal concentration 95%): Concentration of toxic substances in which 95% of test insect die.

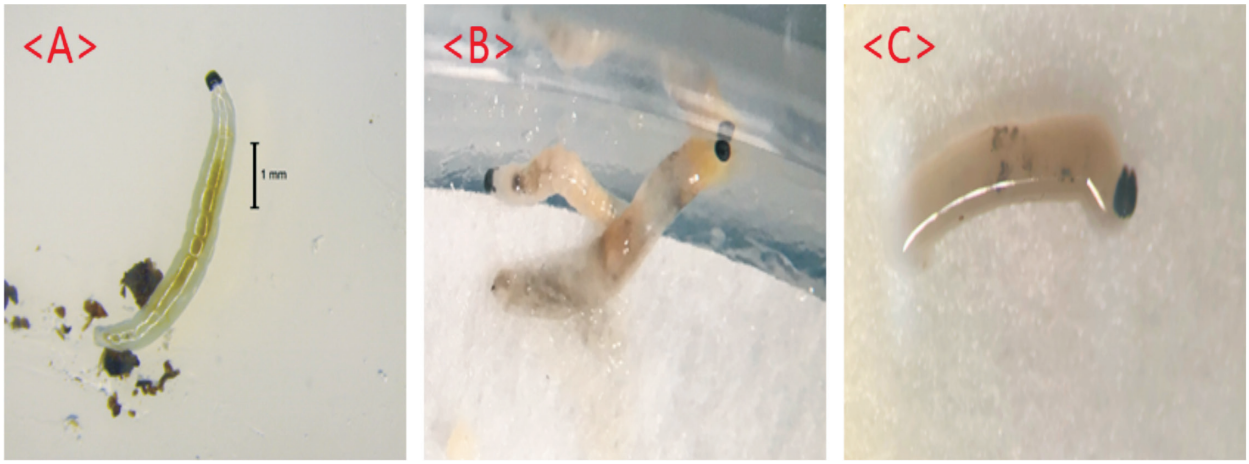

Fig. 1. The appearance of *B. agrestis* larva treated with *B. thuringiensis* CAB452. <A>: control; <B>: 3 days of dead larva; <C>: 5 days of dead larva.

iensis strain CAB117 and *B. thuringiensis* subsp. *israelensis* strain showed low LC_{95} values in *A. albopictus*, strain CAB452 showed high insecticidal activities in both *B. agrestis*, which is in Diptera Sciaridae, and *A. albopictus*, which is in Diptera Culicidae. Therefore, *B. thuringiensis* strain CAB452 can be expected to be suitable as a biological control agent. It could be seen that whereas the larvae not treated with *B. thuringiensis* strain CAB452 had clearly visible mid–gut and transparent body, the larvae treated with *B. thuringiensis* strain CAB452 were infected with the train and the mid–gut became blurred and partially black and turbid at the third day after treatment (Fig. 1). On the seventh day after treatment with *B. thuringiensis* strain CAB452, the mid–gut was not visible and the larvae died while the whole body was turned black. In addition, although no experimental data was provided, the toxicity of *B. thuringiensis* strain CAB452 to *Spodoptera exigua and Spodoptera litura* was tested in order to identify the range of activity of *B. thuringiensis* strain CAB452 and the insects did not die in the tests.

Morphological characteristics of *B. thuringiensis* strains

To identify the crystal proteins of *B. thuringiensis* strain CAB452 with high insecticidal activities in Diptera insect pests, the strain was observed under a phase contrast microscope. According to the result, spherical type crystals, which are known to have insecticidal activities in Diptera pests, were formed. When compared with *B. thuringiensis* subsp. *israelensis* is strain, which shows activity in mosquitos, the spherical type crystals formed were quite similar. In addition, the crystals of *B. thuringiensis* strain CAB452 and *B. thuringiensis* subsp. *israelensis* strain were observed under a scanning electron microscope. Morphologically, spherical type crystals, known to have insecticidal activities in Diptera, were identified as with the observation under the phase contrast microscope (Fig. 2).

SDS–PAGE

In general, according to findings reported thus far, the crystal proteins produced by B. *thuringiensis* strains have molecular weights between 25 and 140 kDa and are composed of δ–endotoxin. Among the three types of crystal proteins formed by *B. thuringiensis*, the spherical type reported to have activity in Diptera pests has been reported to have molecular weights of – 65 kDa (Ibarra and Federici, 1986a, 1986b; Ibarra *et al.*, 2003).

SDS–PAGE was performed to compare the characteristics of the δ–endotoxin proteins of *B. thuringiensis* strain CAB452 that shows high insecticidal activities against Diptera *B. agrestis* and *A. albopictus* and *B. thuringiensis* subsp. *israelensis* strain known to have activity in Diptera. *B. thuringiensis* strain CAB452 showed a main protein band of about 150 kDa in size. To identify the active toxin patterns of the parasporal inclu-

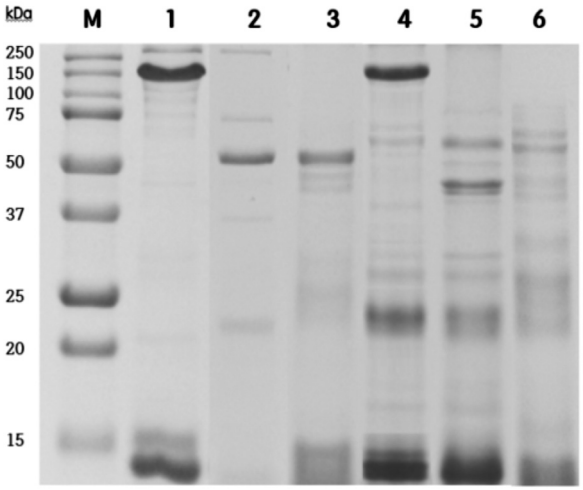

Fig. 3. SDS–PAGE analysis of parasporal incubation of *B. thuringiensis* isolates digested with trypsin. M: Standard Marker; lane 1: *B. thuringiensis* CAB452; lane 2: *B. thuringiensis* CAB452 digested with trypsin; lane 3: *B. thuringiensis* CAB452 digested with gut juice of *B. agrestis*; lane 4: *B. thuringiensis* subsp. *israelensis*; lane 5: *B. thuringiensis* subsp. *israelensis* digested with trypsin; lane 3: *B. thuringiensis* subsp. *israelensis* digested with gut juice of *B. agrestis*.

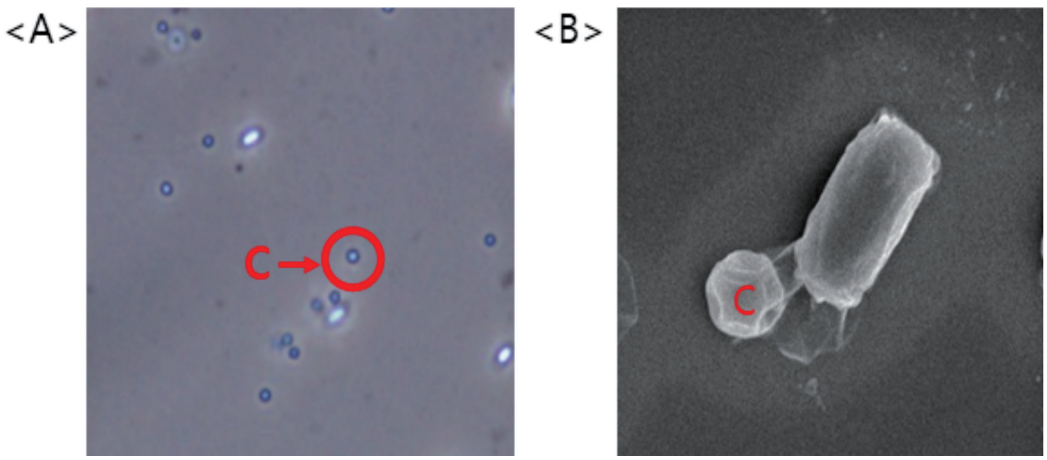

Fig. 2. <A>: Phase–contrast microscope photographs (× 1,000) of crystal (C) shape of *B. thringiensis* CAB452. <B>: Scanning eletron microscopy of spore–crystal (C) mixtures of *B. thringiensis* CAB452.

sions produced by the two strains, the strains were treated with trypsin, which is a digestion enzyme, and triturated solution of the entire *B. agrestis* larvae (since the gut cannot be separated as the larvae are too small) and the two treatments were compared (Fig. 3). It was shown that the 150 kDa sized one main band of *B. thuringiensis* strain CAB452 was digested by trypsin or *B. agrestis* larvae triturated solution and only one 50 kDa sized main protein band was formed. This is completely different from the protein molecular weights produced by other *B. thuringiensis* strains that shows toxicity to Lepidoptera or mosquito larvae reported thus far.

Identification of Cry–type gene

B. thuringiensis strain CAB452, which was selected because it was judged to be different from general *B. thuringiensis* strains based on the results of tests of insecticidal activities in Diptera and comparison of protein patterns, was sent to Mokwon University Micro–Ecological Resources Research Institute to conduct identification of the strain. The strain was analyzed with molecular genetic analysis based on the flagellin C gene, a specific gene in *B. thuringiensis* with H serotype, and as a result, the strain was classified into new subspecies of *B. thuringiensis* (Fig. 4).

Among *B. thuringiensis* strains, those strains that exhibit activity in Diptera produce important two kinds of insecticidal proteins including the Cry (Crystal protein) and Cyt (cytolytic toxin) proteins in the spore formation stage (Höfte and Whitely, 1989). *B. thuringiensis* subsp. *israelensis* strain, which is known to have high insecticidal activities in Diptera pests had six genes, which are cry4Aa, cry4Ba, cry10, cry11Aa, cyt1Aa, and cyt2Ba (Bravo *et al*., 2007). However, when the cry genes of *B. thuringiensis* strain CAB452, which was selected as it showed activity in Diptera, were checked, it could be seen that it had cry9D gene, which did not appear in *B. thuringiensis* subsp. *israelensis* strain that shows activity in mosquitos. In addition, it had cry4A gene, which is known to have activity in Diptera (Table 2). In addition, the production of cry9D and a new protein cry9 was reported in *B. thuringiensis* subsp. *japonensis* strain, reported to have activity in mosquito larvae (Wasano *et al*., 1998; Wasano *et al*., 2001). This gene is known to have insecticidal activities in Coleoptera. In this study, cry9D and cry4A gene bands were identified in *B. thuringiensis* strain CAB452 with insecticidal activities in Diptera.

B. thuringiensis strain CAB452 that shows activity in Diptera and *B. thuringiensis* subsp. *israelensis* strain could not be easily distinguished when observed through two types of microscopes because there was no difference in appearances but these strains showed clear differences in proteins and plasmid DNA patterns. *B. thuringiensis* strain CAB452 was reviewed as a new strain expressing insecticidal activities in *B agrestis* and its potential as a new microbial insecticide could be identified.

Table 2. Profiles of cry genes in *B. thuringiensis strains*

Strain	Crystal protein
B. thuringiensis subsp. CAB452	*cry9D, cry4A*
B. thuringiensis subsp. *israelensis*	*cry4Aa, cry4Ba, cry10Aa, cry11Aa, cyt1Aa, cyt2Ba*

AUTHOR CONTRIBUTIONS

Hee Ji Kim designed the study, performed the isolation of new *B. thuringiensis* strains, the comprehensive experiments, analyzed the data and wrote the paper. You Kyung Lee participated in the pest control experiments. Young Nam Youn edited the paper. Chisa Yasunaga–Aoki participated in the design of the study and discussed on the experiments and the results. Yong

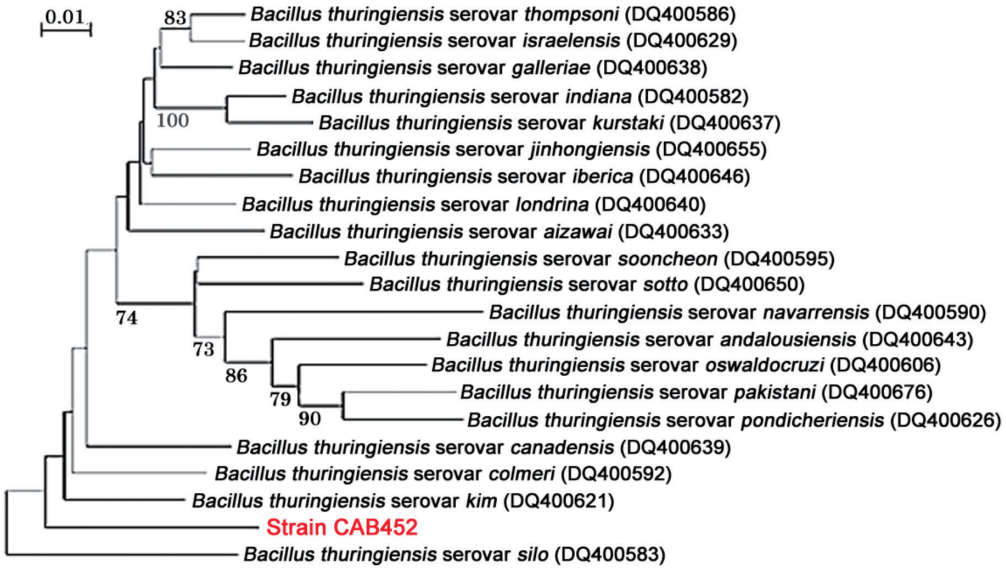

Fig. 4. Phylogenetic tree based on flagellin C gene sequences showing the position of Strain *B. thuringiensis* CAB452 and related bacterial taxa.

Man Yu supervised the work and wrote the paper. All authors assisted in editing of the manuscript and approved the final version.

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