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Investigation and Characteristics of Novel Biological Control Agents for Controlling Dipteran Pests

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An attempt was made to select new *Bacillus thuringiensis* strains in order to control dipteran pests that cause economic damage to agricultural product cultivation sites. To select *B. thuringiensis* strains, the insecticidal activities of *B. thuringiensis* strains against the larvae of *Lycoriella ingenua*, mushroom flies and *Bradysia agrestis*, fungus gnats which are major agricultural pests, and *Aedes albopictus*, which is a hygiene pest were investigated. From among the 5 *B. thuringiensis* strains of which the biological activity was investigated, one strain, *B. thuringiensis* subsp. *kyushuensis* CAB571, which exhibited at least 90% insecticidal activity against *L. ingenua* and *B. agrestis*, was selected. The LC_{95} values of the *B. thuringiensis* strain CAB571 against *B. agrestis* and *L. ingenua* were 8.7×10^7 (cfu/ml) and 3.5×10^6 (cfu/ml), respectively, indicating high insecticidal activities. The selected new *B. thuringiensis* strain was shown to have 5 major δ–endotoxin protein bands of about 150, 70, 65, 48 and 27 kDa on SDS–PAGE and 2 major endotoxin protein bands of about 65 kDa and 25 kDa in the reaction to trypsin treatment. This strain showed 8 plasmid DNA bands, and 4 genes, which are cry40A, cyt1C, cyt1D and cyt2Aa in PCR for crystal proteins.

Key words: *Bacillus thuringiensis*, spherical crystal, *Lycoriella ingenua*, *Bradysia agrestis*, biological activity

INTRODUCTION

Recently, eco–friendly organic farming techniques, which pursue the production of safe agricultural products while maintaining sustainable agricultural production environments, have been preferred. In addition, as the quality of life has become an important value following increases in national incomes, health functional foods and their consumption patterns have been also changing toward the pursuit of clearer, safer, and high–quality agricultural products (Kim *et al*., 2017). The occurrence of dipteran pests has been increasing every year in major domestic protected cultivation sites for the production of eco–friendly agricultural products, and the pests appear as hardly controllable problematic pests that cause the degradation of the quality and decreases in the yields of agricultural products. Such pests include *Bradysia agrestis* (Kim *et al*., 2019), *Lycoriella ingenua* (Lee *et al*., 2018), and *Delia antiqua*, which inflict economic damage to fruit vegetables such as cucumbers, strawberries, tomatoes, blueberries, and paprika, Liliaceae crops such as garlics, chives, green onions, and leeks, and mushrooms in protected cultivation sites (Lee *et al*., 2018). Such dipteran pests occur 2~4 times a year and cause significant damage directly or indirectly, but there is no particular eco–friendly control agent. The damage due to dipteran pests can be hardly observed with the naked eyes because the larvae gnaw the roots or stems of plants or invade into the inside of plants to cause damage (Kim *et al*., 2003). These larvae not only cause direct damage, but also transmit pathogens such as Fusarium and Pythium thereby causing the outbreak of plant diseases. In addition, the adult insects carry pathogens to other plants or facility houses by being smeared with hyphae on their body such as their belly, legs, and wings thereby causing damage (Ludwig and Oetting, 2001). In particular, since several kinds of dipteran pests occur simultaneously in one crop, their physiology and ecology are complex, and their generations ranging from eggs to adult insects are mixed when they occur, comprehensive methods using cultural, physical, and biological controls are necessary. As consumer demand for safe agricultural products increases, the scale of the eco–friendly agricultural products market is also increasing. Therefore, eco–friendly control technologies should be developed. Accordingly, eco–friendly agricultural materials are rapidly increasing and are being used diversely, but they show somewhat insufficient results in terms of effects (Kil *et al*., 2007). The development and studies of control measures that can be stably used throughout the entire period of cultivation of various eco–friendly agricultural products and have excellent effects are desperately needed (Kim *et al*., 2013). In order to meet such conditions, the need for studies of biological control using *Bacillus thuringiensis* and entomopathogenic nematodes (Kim *et al*., 2001) has been raised (Kim *et al*., 2012). *B. thuringiensis*, which is an entomopathogenic bacterium, is an aerobic Gram–positive bacillus that forms endospores and produces insecticidal toxin proteins consisting of

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endotoxin proteins. This is because protein crystals toxic to insects such as Lepidoptera, Diptera, and Coleoptera consist of polypeptides called δ–endotoxin (Schnepf *et al.*, 1998), and have the host specificity to show toxicity only to target pests depending on the types of endotoxin proteins (Schnepf *et al.*, 1995). The insecticidal activities of *B. thuringiensis* strains are divided into five types, that is, those for Lepidoptera (Cry Ⅰ), Lepidoptera and Diptera (Cry Ⅱ), Coleoptera (Cry Ⅲ), Diptera (Cry Ⅳ), or Lepidoptera and Coleoptera (Cry Ⅴ) according to the insecticidal properties and molecular structures of their more than 100 types of endotoxin proteins (Crickmore *et al.*, 1998). Microbial insecticides have been developed using *B. thuringiensis* throughout the world, and the *B. thuringiensis* subsp. *israelensis* strains which are highly toxic to Diptera larvae, are sold as a product to control mosquito larvae (Seo *et al.*, 2010). Currently, *B. thuringiensis* agents, which microbial insecticides sold in South Korea, are developed in South Korea and are also imported for use in South Korea, and have been used and studied diversely to control Lepidoptera pests (Lee *et al.*, 2015, Jin *et al.*, 2015). However, no microbial insecticide has been registered for control of dipteran pests (Lee *et al.*, 2018).

Therefore, in this study, those strains that were showing excellent insecticidal effects on dipteran pests that occur in eco–friendly cultivation sites to inflict economic damage were selected and their insecticidal activities, morphological characteristics, endotoxin protein characteristics, and genes were analyzed.

MATERIALS AND METHODS

Selection and identification of *B. thuringiensis* strains

The strains used in this experiment were the *B. thuringiensis* strains isolated from domestic soil and stored in the laboratory for biological pest control at Chungnam National University in Korea. The *B. thuringiensis* strains were incubated in a nutrient agar medium (difco Detroit, MI, USA) at 27°C for 4 to 5 days and small quantities of them were dropped on slide glasses and the colonies of them were observed with a phase–contrast microscope (Olympus BX51) of 1,000 magnification to identify the morphology of endotoxin protein crystals thereby selecting those strains that were forming spherical type endotoxin protein crystals (Kim *et al.*, 1995). From among the selected strains, those *B. thuringiensis* strains that were exhibiting insecticidal activities against Diptera larvae were sent to the Institute of Microbial Ecology and Resources at Mokwon University and identified by the flagellin C gene.

Test insect

The *Lycoriella ingenua* and *Bradysia agrestis* used in this experiment were raised using button mushrooms and potatoes, respectively, as feeds. For the spawning of *L. ingenua* and *B. agrestis*, the adult insects were collected using aspirators, paralyzed using carbon dioxide, and three each of male and female adult insects were inoculated into a plastic Petri–dish (60×15 mm) containing a water agar medium to induce spawning. *Aedes albopictus* was purchased from the Department of Disease Mediating Insects of the Korea Centers for Disease Control and Prevention, and was raised with a feed made by adding yeast extract to fry feed before being used. All insects were raised under the raising conditions of a temperature of 25±1°C, a light condition of 16L:8D, and relative humidity of 50~60%.

Biologic activity assay

The bioactivity against *L. ingenua* was tested by applying 200 μl of the culture medium of each *B. thuringiensis* strain to the surface of 1 g of button mushrooms. The bioactivity of *B. thuringiensis* strain against *B. agrestis* was tested by applying 200 μl of the culture medium of each *B. thuringiensis* strain to the surface of 1 g of potato. Twenty each of the larvae of *L. ingenua* and *B. agrestis* 4~5 days after hatching were put into a petri dish and the fatality was investigated for 7 days. The bioactivity of the *B. thuringiensis* strain against *A. albopictus* was tested with the experimental method of WHO (2005) with some modification. Ten each of larvae 3~4 days after hatching were put into a 90 ml plastic cup containing 30 ml of water, and 300 μl of the diluted bacterial solution was inoculated to investigate the fatality for 72 hours. All experiments were carried out three times repeatedly and the lethal concentration (LC_{95}) was calculated with the mortality rates investigated with 5~7 ranges of concentrations ranging from the concentration at which all the larvae died and the concentration at which all the larvae survived using a PC program based on Finney's (1971) Probit calculation method (Raymond *et al.*, 1985).

SDS–PAGE

The strains used in the experiment were inoculated into a nutrient agar medium and cultured at 27°C for 5 days and the occurrence of autolysis was identified with a phase–contrast microscope. Thereafter, the culture medium was put into a centrifuge tube and centrifuged for 10 minutes at 15,000 rpm and 4°C to collect the bacteria. Thereafter, the supernatant was discarded and the tube was washed 3 times with washing buffer I (500 mM NaCl, 2% Triton X–100) and 2 times with washing buffer Ⅱ (500 mM NaCl). The collected parasporal inclusions were added with sterile water and stored at –20°C. The SDS–PAGE was carried out using a 12% separating gel and a 5% stacking gel by partially modifying the method of Laemmli (1970). After electrophoresis, the gel was stained with 0.5% Coomassie brilliant blue. The parasporal inclusions of individual strains were made to react with 50 mM NaOH aqueous solution at room temperature for 5 minutes, treated with 1 mg/ml trypsin at 10:1 (w/w), and incubated for 30 minutes at 37°C. The proteins dissolved by trypsin were analyzed using SDS–PAGE to identify the result (Zouari and Samit, 1997).

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, pH 8.0, 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, pH 4.2) 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, pH 7.0, 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 50V for 60 minutes, and irradiated with UV to identify the band pattern.

PCR analysis

A gene–specific primer set was used to identify the Cry–type genes among the endotoxin genes of the selected *B. thuringiensis* strains. Thermal Cycle C1000TM (BIO–RAD) was used for PCR amplification. The reaction solution used was made by mixing 1.0 μl of template DNA, 1.0 μl of the primer set, and 17 μl of distilled water with a premix (Bioneer) containing a buffer component and dNTP to make the final volume into 20 μl. PCR product DNA was produced under the PCR conditions of 3 minutes at 95°C, a total of 30 cycles of 1 minute at 95°C, annealing for 1 minute at 57°C, 1 minute at 72°C, and 5 minutes at 72°C, and subjected to electrophoresis in 1% agarose gel (Yang *et al.*, 2011).

RESULTS AND DISCUSSION

Selection and identification of *B. thuringiensis* strains

No microbial pesticide for controlling dipteran pests that occur in and cause damage to domestic facility cultivation and mushroom cultivation farms has been registered yet, and even although there are applicable chemical pesticides, there are difficulties in control because there are many restrictions on the time of use due to the problem of pesticide residues. Due to this problem, the development of a biological control agent that does not affect the growth of crops, is safe, and can be selectively used only for dipteran pests is desperately needed. Therefore, in order to select *B. thuringiensis* strains having new insecticidal ranges, *B. thuringiensis* strains stored in the biological pest control laboratory of Chungnam National University were cultured in NA medium at 27°C, and the toxin protein crystals (parasporal inclusions) produced by them were observed through a phase–contrast microscope. Since spherical type toxin protein crystals are generally known to be effective on Diptera, five strains that form spherical type toxin protein crystals were selected from among them. The insecticidal activities of the selected five strains against the agricultural pests *B. agrestis* and *L. ingenua* and the hygienic pest *A. albopictus* were tested (Table 1). *B. thuringiensis* strain CAB571 showed high insecticidal activities not lower than 90%, strains CAB517 and CAB544 showed insecticidal activities not lower than 70%, and strain CAB592 showed insecticidal activities not lower than 50% against *L. ingenua*. In addition, *B. thuringiensis* strain CAB571 showed high insecticidal activities not lower than 90%, strain CAB544 showed insecticidal activities not lower than 70%, and strains CAB517 and CAB592 showed insecticidal activities not lower than 50% against *B. agrestis*. However, the strain CAB581 showed low insecticidal activities not exceeding 50% against both pests. On the other hand, no strain showed high insecticidal activities not lower than 90% against *A. albopictus*, which is a hygiene pest. Therefore, strain CAB571 that showed high insecticidal activities not lower than 90% against *B. agrestis* and *L. ingenua* was selected. Strain CAB571, which exhibits high insecticidal activity against dipteran pest, was tested for the flagellin C gene at Mokwon University Microbial Ecological Resources Research Institute and it was identified as *B. thuringiensis* subsp. *kyushuensis* (Fig. 1).

Table 1. Insecticidal activities of *B. thuringiensis* isolates against dipteran larvae of 3 different species

Strains	Tested insects		
	Lycoriella ingenua	*Bradysia agrestis*	*Aedes albopictus*
B. thuringiensis CAB517	++	+	++
B. thuringiensis CAB544	++	++	–
B. thuringiensis CAB571	+++	+++	+
B. thuringiensis CAB585	–	–	–
B. thuringiensis CAB592	+	+	+
Control	–	–	–

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

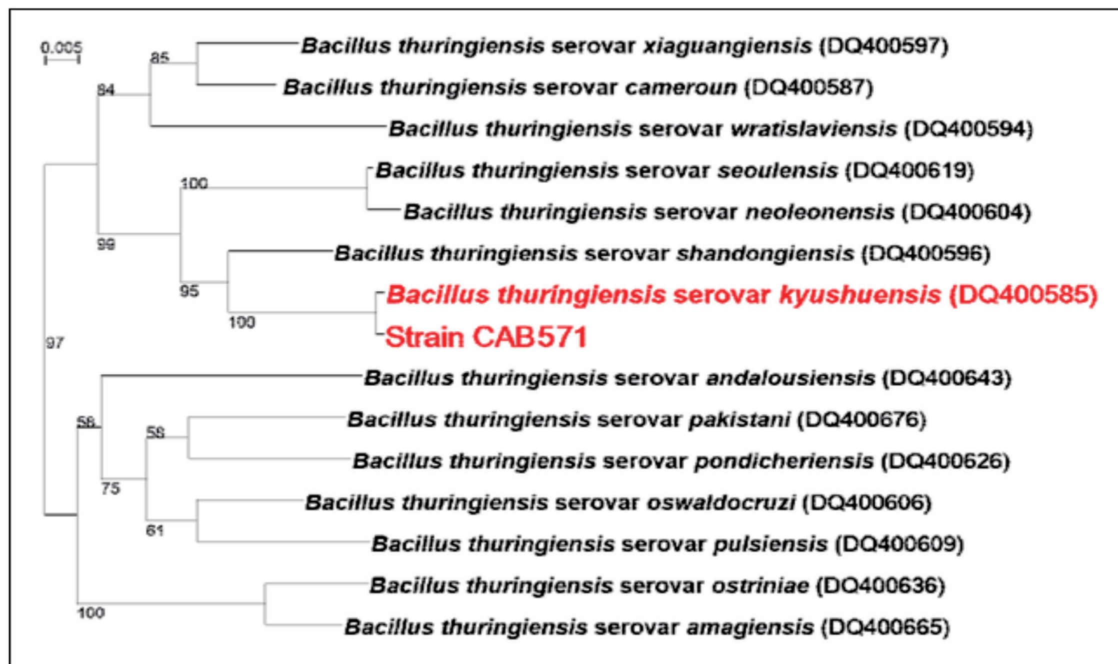

Fig. 1. Phylogenetic tree based on flagellin C gene sequences showing the position of strain *B. thuringiensis* CAB571 and related bacterial taxa.

Morphological characteristics of *B. thuringiensis* strains

The morphologies of δ–endotoxin proteins of *B. thuringiensis* strains are provided as important information for identifying the target pests and strains because they give information indicating close relationships with the insect host ranges that exhibit insecticidal activities (Maeda *et al*., 2000). In general, bipyramidal type crystals have been reported to have high insecticidal activities against lepidopteran pests (Armengol *et al*., 2007; Donovan *et al*., 1988). *B. thuringiensis* subsp. *kyushuensis* strain CAB571, which exhibits high insecticidal activities against *B. agrestis* and *L. ingenua*, was observed through a phase contrast microscope, and the result indicated that strain CAB571 forms typical spherical type crystal proteins known to exhibit insecticidal activity against dipteran pests. The typical spherical type crystal proteins showed similar patterns to those of the typical spherical type endotoxin proteins of *B. thuringiensis* subsp. *israelensis*, which shows high insecticidal activity against mosquitoes (Fig. 2).

Test of the biological activity of *B. thuringiensis* strains

The LC_{95} values of *B. thuringiensis* subsp. *kyushuensis* strain CAB571B, which shows high insecticidal activity against dipteran pests, and those of strain CAB199 identified as *B. thuringiensis* subsp. *israelensis*, which shows high insecticidal activity against mosquitoes and is widely used worldwide, against the agricultural pests *B. agrestis* and *L. ingenua*s were compared (Table 2). As a result, the LC_{95} values of *B. thuringiensis* strain CAB571 against *B. agrestis* and *L. ingenua* were shown to be 8.7×10^7 (cfu/ml) and 3.5×10^6 (cfu/ml), respectively, indicating high insecticidal activities, and the LC_{95} values of *B. thuringiensis* strain CAB199 were shown to be at least 10^8 (cfu/ml). On the other hand, the LC_{95} values of *B. thuringiensis* strain CAB571 against *A. albopictus*, a hygiene pest, was shown to be 1.1×10^8 (cfu/ml), which is relatively lower

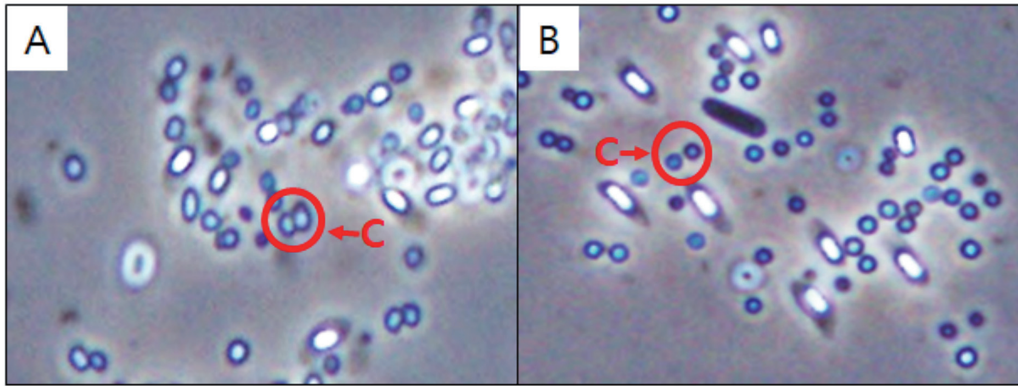

Fig. 2. Phase–contrast microscope photographs (×1,000) of crystal shape of *B. thuringiensis*. (A): *B. thuringiensis* subsp. *kyushuensis* CAB571; (B): *B. thuringiensis* subsp. *israelensis* CAB199.

Table 2. Toxicity (LC_{95}) of *B. thuringiensis strain* against *Lycoriella ingenua*, *Bradysia agrestis* and *Aedes albopictus*

Strains	*Lycoriella ingenua*	*Bradysia agrestis*	*Aedes albopictus*
B. thuringiensis subsp. *kyushuensis* CAB571	4.5×10^6	9.2×10^6	1.1×10^8
B. thuringiensis subsp. *israelensis* CAB199	$>10^8$	$>10^8$	3.9×10^4

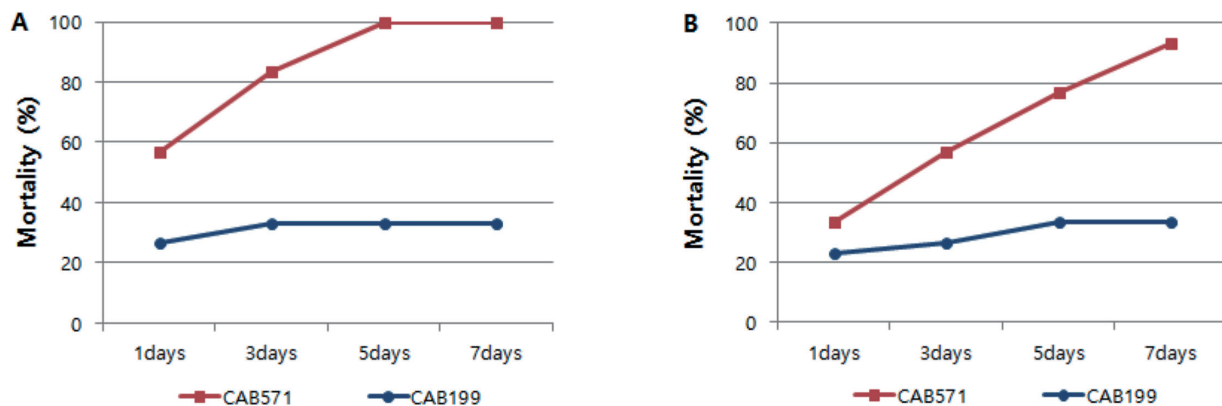

Fig. 3. S Mortality of (A) *L. ingenua* and (B) *B. agrestis* various age larva at the same concentrations (10^6 cfu/ml) of insecticide treatment.

than that of *B. thuringiensis* subsp. *israelensis* strains known to have insecticidal activity against mosquitoes. In order to identify the fast–acting properties of the strains selected against the agricultural pests *B. agrestis* and *L. ingenua*, the concentrations were adjusted to be equal at 10^6 (cfu/ml) and bioassays were conducted on the larvae of *B. agrestis* and *L. ingenua*. According to the results, it was identified that in the case of *B. thuringiensis* strain CAB571, the mortality of *L. ingenua* rapidly increased from the 3 day and was shown to be 100% on the 7 day (Fig. 3A), and the mortality of *B. agrestis* continuously increased from the 3 day to the 7 day (Fig. 3B). Therefore, *B. thuringiensis* strain CAB571 was shown to have high toxicity to the agricultural pests *L. ingenua* and *B. agrestis*, and was shown to have the potential as a new microbial insecticide that can be used for biological control at domestic facility cultivation and mushroom cultivation farms.

SDS–PAGE

The crystal proteins produced by the *B. thuringiensis* strains have molecular weights between 25 and 140 kDa, and are composed of δ–endotoxin. The molecular weights of Cry protein, which constitutes the bipyramidal type δ–endotoxin proteins with insecticidal activity against Lepidoptera, is about 130 to 140 kDa, and is digested into a toxin protein of about 60 to 65 kDa by the high alkalinity and proteolytic enzyme in the mid–intestine of Lepidoptera to show insecticidal effects (Aroson *et al.*, 1991). The molecular weight of the cry protein constituting the spherical type endotoxin protein having insecticidal activity against dipteran pests is about 65 kDa, and the molecular weight of the cyt protein is reported to be about 22 to 30 kDa (Ibarra and Federici, 1986a, Nisnevitch *et al.*, 2013). SDS–PAGE was performed to compare the properties of the endotoxin proteins of *B. thuringiensis* strain CAB571 and *B. thuringiensis* strain CAB199 that exhibit insecticidal

activities against the dipteran pests *B. agrestis* and *L. ingenua*. In the case of *B. thuringiensis* strain CAB571, five major protein band patterns of sizes about 150, 70, 65, 48 and 27 kDa were identified, and in the case of *B. thuringiensis* strain CAB199, four protein band patterns of sizes about 135, 75, 37 and 27 kDa were identified (Fig. 4). *B. thuringiensis* strain CAB571 was treated with the digestive enzyme trypsin to identify the active toxin pattern of the parasporal inclusion produced by *B. thuringiensis* strain CAB571. It could be seen that in *B. thuringiensis* strain CAB571, the protein of about 150 kDa was degraded by trypsin to form protein bands of about 65, 60, and 25 kDa. *B. thuringiensis* strain CAB571 generally has 2 major active toxin bands, a protein of a molecular weight of about 65 kDa, which is the a molecular weight of Cry protein, a spherical type endotoxin protein, and a protein of a molecular weight of 25 kDa, which is the a molecular weight of cyt protein that generally show activity against mosquitoes, a hygiene pest.

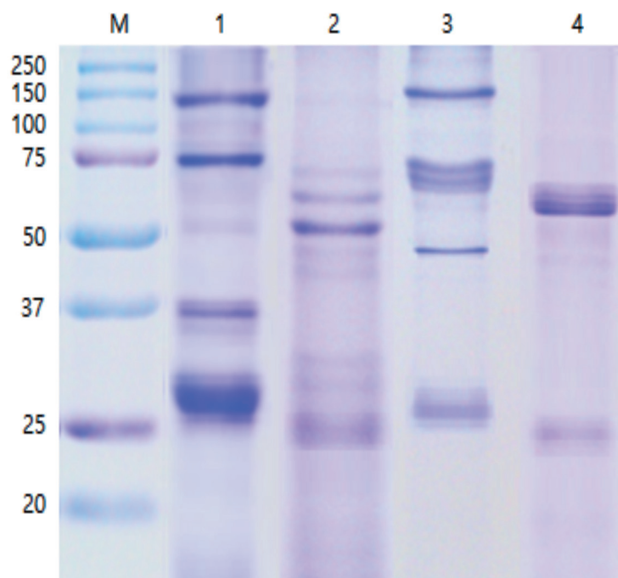

Fig. 4. SDS–PAGE analysis of parasporal inclusions of *B. thuringiensis*. M; Broad range marker, Lane 1: *B. thuringiensis* subsp. *israelensis* CAB199; Lane 2: *B. thuringiensis* subsp. *israelensis* CAB199 digested with trypsin; Lane 3: *B. thuringiensis* subsp. *kyushuensis* CAB571; Lane 4: *B. thuringiensis* subsp. *kyushuensis* CAB571 digested with trypsin.

Plasmid DNA analysis

The cry gene, which is involved in forming the activity, size and shape of the δ–endotoxin proteins formed by *B. thuringiensis* strains, is present on the plasmid DNA. The numbers and lengths of the plasmid DNAs possessed by individual subspecies of each *B. thuringiensis* strain are reported to be diverse, and the plasmid DNAs present in a strain consist of up to 17 ones and the lengths are about 2 to 250 kbp (Sarrafzadeh *et al*., 2007). Likewise, the cry gene that forms crystal proteins does not always exist in the plasmid DNA of the same size in all strains, but exists differently by subspecies (Kronstad *et al*., 1983; Aronson *et al*., 1986). The plasmid DNA patterns of *B. thuringiensis* strain CAB571 and *B. thuringiensis* strain CAB199 were identified (Fig. 5). In the case of *B. thuringiensis* strain CAB571, 8 plasmid DNA bands including 2 plasmid DNA bands larger than 23.1 kb could be identified. In addition, the two larger plasmid DNA bands including the 23.1 kb band of *B. thuringiensis* strain CAB571 were shown to be identical to those of *B. thuringiensis* strain CAB199.

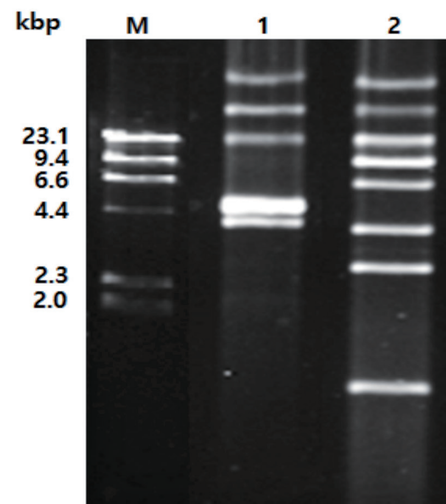

Fig. 5. Plasmid DNA patterns of *Bacillus thuringiensis*. M: Lamda Hind Ⅲ marker; Lane 1: *B. thuringiensis* subsp. *israelensis* CAB199; Lane 2: *B. thuringiensis* subsp. *kyushuensis* CAB571.

Identification of Cry–type gene

Those *B. thuringiensis* strains that exhibit specific activity against dipteran mosquitoes, which have been reported thus far, produce two types of insecticidal endotoxin proteins including Cry (Crystal protein) and Cyt (cytolytic toxin) proteins in the sporulation stage (Höfte and Whitely, 1989). Individual endotoxin proteins produced by *B. thuringiensis* strains exhibit toxicity to different insect species such as Lepidoptera, Diptera, and Coleoptera, and most of *B. thuringiensis* strains carrying Cyt gene show toxicity to dipteran pests (Soberón *et al*., 2013). Cyt toxins are a group of proteins produced by some strains of *B. thuringiensis*, and are classified into three types: cyt1, cyt2, and cyt3 based on amino acid homogeneity (Crickmore *et al*., 1998). These proteins have lethal activity against the larvae of dipteran pests and have been reported to have cytolytic activity against a wide range of cells including red blood cells in vitro (Thomas and Ellar, 1983). Diverse cyt toxins were found in those *B. thuringiensis* strains that have insecticidal activity against dipteran pests, and the genic values encoding these toxins were identified and sequenced (Crickmore *et al*., 1998). *B. thuringiensis* subsp. *israelensis* strains known to have high insecticidal activity against mosquitoes among dipteran pests have been reported to have cry4Aa, cry4Ba, cry10,

Table 3. Profiles of cry genes in *Bacillus thuringiensis* strains

Strain	*cry* genes
B. thuringiensis subsp. *kyushuensis* CAB571	cry40A, cyt1C, cyt1D, cyt2Aa
B. thuringiensis subsp. *israelensis* CAB199	cry4Aa, cry4Ba, cry11Aa, cyt1Aa, cyt1C

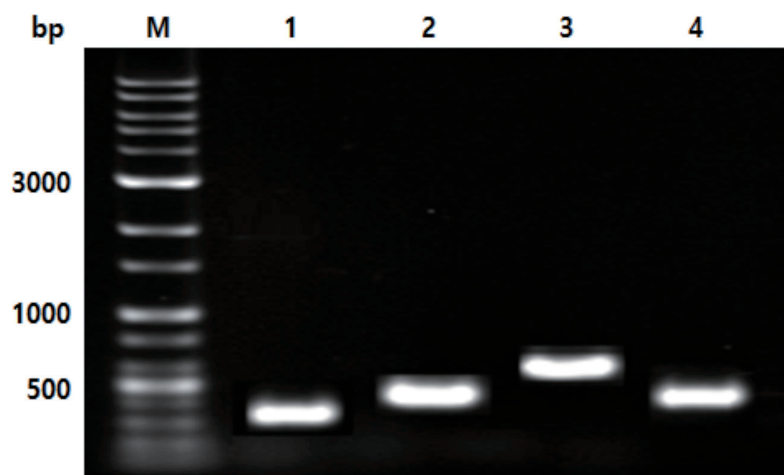

Fig. 6. Agarose gel (1%) electrophoresis of PCR products obtained with specific primers for the genes of *B. thuringiensis* subsp. *kyushuensis* CAB571. M: 100 bp ladder marker; Lane 1: cry40A; Lane 2: cyt1C; Lane 3: cyt1D; Lane 4: cyt2Aa.

cry11Aa, cyt1Aa, and cyt2Ba genes (Bravo *et al.*, 2007) and *B. thuringiensis* subsp. *morrisoni* strains have been reported to have cry4A, cry4B, cry10A, cry11A, and cyt1A genes (Ibarra and Federici, 1986b; Padua *et al.*, 1984). The cry genes of the *B. thuringiensis* strain CAB571 selected in this study were checked and as a result, whereas cry4Aa, cry4Ba, cry11Aa, cyt1Aa, and cyt1C genes were identified in *B. thuringiensis* strain CAB199, cry40A, cyt1D, and cyt2A genes not identified in *B. thuringiensis* strain CAB199 were identified in *B. thuringiensis* strain CAB571 (Table 3 and Fig. 6). The cyt1C gene identified in both strains is found in *B. thuringiensis* subsp. *israelensis* strains, does not dissolve red blood cells, and has not been reported to have any other biological functions against mosquito larvae. Although Cyt genes alone exhibit low toxicity, it has been reported that when combined with cry genes, these genes show synergistic effects thereby inhibiting the emergence of resistance in pests (Ben–Dov, 2014). Through these results, cyt toxin, which is closely related to the insecticidal activity on dipteran pests, is considered to be an element necessary to improve the insecticidal activity of *B. thuringiensis* strains and to control a wide range of dipteran pests.

In this study, *B. thuringiensis* subsp. *kyushuensis* strain CAB571 and *B. thuringiensis* subsp. *israelensis* strain CAB199 could be hardly distinguished from each other based on their appearance or protein characteristics only, and it was identified that they were different through bioassays and molecular biological experiments. Since *B. thuringiensis* subsp. *kyushuensis* strain CAB571 shows high insecticidal effects on the larvae of *L. ingenua* and *B. agrestis*, it is considered to be usable in deriving the candidates of new microbial insecticides that can control dipteran pests that cause economic damage to eco–friendly agricultural product cultivation sites.

AUTHOR CONTRIBUTIONS

You Kyoung Lee designed the study, performed the comprehensive experiments, analyzed the data and wrote the paper. Na Young Jin participated in the pest control experiments. Hee Ji Kim performed the isolation of *B. thuringiensis* strains. Yong Man Yu edited the paper. Chisa Yasunaga–Aoki participated in the design of the study and discussed on the experiments and the results. Young Nam Youn supervised the work and wrote the paper. All authors assisted in editing of the manuscript and approved the final version.

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