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Selection and Characterization of *Bacillus thuringiensis* Strains with Biological Activity against *Lycoriella ingenua*

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In order to use *Lycoriella ingenua*, a major pest of mushroom cultivation, as a biological control agent, the biologic activity of *Bacillus thuringiensis* strain, which is toxic to diptera insects, was examined. The larvicidal activities of the strain against the larvae of *L. ingenua* and *Bradysia agrestis* as target agricultural pest species, and *Aedes albopictus, Aedes togoi*, and *Culex pipiens pallens* as sanitary pest species were examined. *B. thuringiensis* subsp. *darmstadiensis* and *B. thuringiensis* subsp. kyushuensis CAB464 strains that showed 90% or higher larvicidal activities against the five species of pests, of which the biologic activity was tested, were selected. Between the two selected strains, the *B. thuringiensis* subsp. *kyushuensis* CAB464 strain showed higher larvicidal activity against *L. ingenua* larvae, with an LC_{95} value of 1.6×10^6 (cfu/ml). In addition, the LC_{95} value of *B. thuringiensis* subsp. *darmstadiensis* strain was shown to be 3.5×10^6 (cfu/ml), which was higher larvicidal activity than the control strain *B. thuringiensis* subsp. *israelensis*. In an SDS–PAGE analysis of the endotoxin protein bands of the two *B. thuringiensis* strains that showed high toxicity against diptera pest, the two strains showed the same three bands at 150, 70, and 50 kDa, and in trypsin reactions, the two strains again showed the same pattern of being at approximately 65 and 60 kDa. In a PCR analysis for the crystal proteins of the two strains, it was identified that both strains had *cyt2Aa* gene. Meanwhile, in plasmid DNA patterns, five plasmid DNA bands were identified in *B. thuringiensis* subsp. *darmstadiensis* strain and nine plasmid DNA bands were identified in *B. thuringiensis* subsp. *kyushuensis* CAB464 strain.

Key words: *Bacillus thuringiensis*, Biological activity, *Lycoriella ingenua*

INTRODUCTION

Diverse pests species such as snails, nematodes, mites, and mushroom flies are reported to have occurred in mushroom cultivation farm, and among them, damage by mushroom flies is known to be the most serious (Lee *et al*., 2002). Seven species of mushroom flies comprising *Lycoriella ingenua*, *Bradysia difformis*, *Bradysia longimentula*, *Bradysia trispinifera*, *Leptosciarella subdentata*, *Scatopsciara camptospina*, and *Xylosciara inornata* have been reported occurring in mushroom houses, and among them, *L. ingenua* and *B. difformis* are known as dominant species (Shin *et al*., 2012, Kim *et al*., 2012). In particular, *L. ingenua* is an insect that belongs to order Diptera, family Sciaridae, and genus *Lycoriella*, and is known to cause reduction in the growth and yield of mushrooms and quality degradation by directly eating and causing damage to mushroom mycelium in mushroom cultivation farm, and to cause indirect damage by mediating various pathogens (Lee *et al*., 2002). In general, since the penetration of *L. ingenua* into mushroom cultivation farm begins when temperatures go down after completion of fermentation and occurs in all stages of mushroom cultivation ranging from seeding to harvest, the damage is serious (Lee *et al*., 2015a). Despite the fact that *L. ingenua* occurs in many mushroom growers and causes damage as such, the chemicals registered for control of these pest species are chemical insecticides such as benfuracarb, diflubenzuron, fenthion, and teflubenzuron (Kim *et al*., 2012, Kim *et al*., 2013). However, even those chemicals registered for control of *L. ingenu*a have many restrictions against use after mushrooms have been formed due to the nature of mushroom cultivation, so the control effects may be reduced, and problems such as resistance to those chemicals have been caused due to continuous use. Therefore, studies on and the development of control measures that can be stably used in the entire period of cultivation of mushrooms and have great effects are desperately needed (Kim *et al*., 2013). To satisfy such conditions, the necessity of studies on biological control such as *Bacillus thuringiensis* products and entomopathogenic nematodes (Kim *et al*., 2001) has been raised (Kim *et al*., 2012).

Bacillus thuringiensis, an insect pathogenic bacterium, is an aerobic gram–positive bacillus that forms endospores and produces an insecticidal protein composed of endotoxin proteins. The protein crystals with toxicity to insects such as Lepidoptera, Diptera, Coleoptera are composed of polypeptides called δ–endotoxin (Schnepf *et al*., 1998), and show the host specificity to have different pests toxicities depending on the type of endotoxin protein (Schnepf *et al*., 1995). Because of

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the insecticidal characteristics and molecular structures of more than 100 endotoxin proteins, the larvicidal activities of *B. thuringiensis* are classified into five types: for Lepidoptera (CryⅠ), for Lepidoptera and Diptera (CryⅡ), for Coleoptera (CryⅢ), for Diptera (CryⅣ), and for Lepidoptera and Coleoptera (CryV) (Crickmore *et al*., 1998). The development of microbial insecticides using *B. thuringiensis* has been carried out globally, and *B. thuringiensis* subsp. *israelensis* strain, which is highly toxic to diptera larvae, is mainly sold in the market as a product to control diptera pests (Seo *et al*., 2010). *B. thuringiensis*, currently marketed as a microbial insecticide in South Korea, has been used and studied for the control of larval pest in most cases (Lee *et al*., 2015b, Jin *et al*., 2015), and microbial insecticides registered to control diptera pest are insufficient. In addition, since the control value is shown to be low, studies are necessary to investigate effective use methods and treatment timing.

Thus, in the present study, to use *L. ingenua*, a major pest in mushroom cultivation, as a biological control agent, *B. thuringiensis* strain, which is toxic to diptera insects, was selected. Thereafter, strains that showed high insecticidal effects against *L. ingenua* were selected and their larvicidal activity, morphological characteristics, endotoxin protein characteristics, and genes were analyzed.

MATERIALS AND METHODS

Selection and identification of *B. thuringiensis* strains

The strains used in the present experiment were *B. thuringiensis* strains stored in the biological pest control laboratory of Chungnam National University. The strains were cultured on a nutrient agar plate at 27°C for 3–4 days. Thereafter, the colonies were observed to select strains that formed spherical type endotoxin protein crystals. The selected strains were diluted in sterilized water and cultured in the same methods as above. After identifying that autolysis occurred, the bacteria were collected and centrifuged (Avanti J–E, Beckman) for 15 minutes at 15,000 rpm. Identification of *B. thuringiensis* strains was carried out by flagellin C gene at the Institute of Microbial Ecology and Resources, Mokwon University.

Morphological observation of *B. thuringiensis* strain

To determine the morphologies of the spores and endotoxin protein crystals of *B. thuringiensis* strain, the strain was streaking on nutrient agar plates and cultured at 27°C for 4–5 days. After the autolysis passed following proliferation on the medium, a small amount of *B. thuringiensis* strain was dropped on a slide glass for observation with a phase contrast microscope (Olympus BX51), and the morphology of endotoxin protein crystals was identified at ×1,000 magnification (Kim *et al*., 1995). To compare the larvicidal activity, the strain was compared and tested with *B. thuringiensis* subsp. *israelensis* strain, which has a high larvicidal activity against diptera and is widely used as a diptera pest insectide, as a control strain.

Test insect

The *L. ingenua* used in the experiment was collected from a button mushroom plantation in Songguk–ri, Chochon–myeon, and Buyeo–gun, Chungcheongnam–do. After collecting the adults using insect absorption tubes, the adults were immediately paralyzed by carbon dioxide and three each of male and female adults were put into a plastic petri dish (60×15 mm) containing water agar to induce oviposition. The oviposition was confirmed by microscopy and button mushrooms (*Agaricus bisporus*) were used as a feed of hatched larvae. Meanwhile, three species of hygienic insect pests: *Aedes albopictus*, *Aedes togoi*, and *Culex pipiens pallens*, were received from the Department of Disease Mediating Insects of the Korea Centers for Disease Control and Prevention and reared using a feed made by adding yeast extract to fry feed before being used in the experiment. The *Bradysia agrestis* used in the experiment was bred in the Biological Pest Control Laboratory in Chungnam National University and potatoes were used as a feed of larvae. The rearing conditions of all insects were kept at 25 ± 1°C, light condition 16L:8D, and relative humidity 50–60%.

Biologic activity assay

The biologic activity against *L. ingenua* was determined by diluting *B. thuringiensis* strain in sterilized water and adding 200 μl of the culture medium to 1 g of button mushrooms. Twenty larvae 3–4 days after hatching were put into a petri dish to examined the lethality for 168 hours and this was repeated three times.

The biologic activity against *B. agrestis* was determined by diluting *B. thuringiensis* strain in sterilized water and adding 200 μl of the culture medium to 1 g of potatoes. Twenty larvae 3–4 days after hatching were put into a petri dish to examined the lethality for 168 hours and this was repeated three times.

The biologic activity against mosquitoes was tested by partially modifying the experimental method of Larvicides (2005). Ten larvae 3–4 days after hatching were placed in a 90 ml plastic cup containing 30 ml of water, and 300 μl of the diluted bacterial solution was inoculated. The lethality was examined for 72 hours, and this experiment was repeated three times.

All experiments were repeated three times, and a lethal concentration 50 percent (LC_{50}) was calculated using the PC program based on Finney's probit calculation method (Finney, 1971) that investigated five to seven ranges from the concentration for complete death of the experimental larvae to the survival concentration (Raymond, 1985).

SDS–PAGE

Each strain used in the experiment was inoculated into nutrient agar medium and cultured at 27°C for 5 days. After identifying that autolysis occurred by phase

contrast microscopy, centrifugation was carried out at 15,000 rpm for 10 minutes at 4ºC using PBS buffer. After centrifugation, the supernatant was discarded and the pellets was washed three times with washing buffer I (500 mM NaCl, 2% Triton X–100) and twice with washing buffer Ⅱ (500 mM NaCl). The washed parasporal inclusion was stored at −20°C after adding sterile water. SDS–PAGE was conducted by partially modifying the method of Laemmli (1970) to use 12% separating gel and 5% stacking gel. After electrophoresis, the gel was stained with 0.5% Coomassie brilliant blue.

The parasporal inclusion of each strain was react with 50 mM NaOH solution for 5 min at room temperature, treated with 1 mg / ml trypsin at 10:1 (w / w), and incubated at 37°C for 30 min. Proteins that were solubilized by trypsin were identified by SDS–PAGE (Zhong *et al.*, 2000; Zouari and Samit, 1997).

PCR analysis

A gene–specific primer set (Table 1) was used to identify the Cry–type gene of the endotoxin gene of the selected *B. thuringiensis* strain. PCR amplification was performed using Thermal Cycle C1000™ (Bio–Rad). The reaction solution was conducted by mixing 1.0 μl of

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

Genes		Primer sequences	PCR products (bp)
cry2A	5’	CAGATACCCTTGCTGGTGTAA	1073
	3’	ATAGGCCCGTGCTCCACCAGG	–
cry3A	5’	CCGAACAATCGAAGTGAA	1964
	3’	ATAGATGGTCCTACTT	–
cry3B	5’	CCGAACAATCGAAGTGAA	1359
	3’	GAATCCTGTGCACCTAA	–
cry3C	5’	CCTGAAAATTACAGGCC	1135
	3’	AATTGATCAATAGAATC	–
cry3D	5’	CCGAACAATCGAAGTGAA	1074
	3’	ATTGTTGCCGTCAACAA	–
cry4Aa	5’	GAACTGGGTATGGCACTCAAC	777
	3’	CTCACAACGATTAGACCCTTC	–
cry4Ba	5’	GCGAGGTTTCCCATGTCTAC	347
	3’	GTTGTAGGGTGGAATTGTTATC	–
cry5	5’	ATGGAAGATAGTTCTTTAGAT	2174
	3’	GGTAGATTTTAATTCTAC	–
cry9A	5’	ATCATGCCATGGATCAAAATAAACACGGAATTATTGG	571
	3’	CCGCTTCCAATAACATCTTTT	–
cry9B	5’	TCATTGGTATAAGAGTTGGTCTATAGAC	402
	3’	CCTCCTAGACACAGGGATGATTTCAATTC	–
cry9C	5’	CTGCTCCCTTTCAATCC	306
	3’	CCTCCTAGACACAGGGATGATTTCAATTC	–
cry9D	5’	CCGAGCTCTATGAATCGAAATAATCAAAATGAAT	1917
	3’	CCTCCTAGACACAGGGATGATTTCAATTC	–
cry10Aa	5’	ACGCAAATAAGCCAAATCAACC	257
	3’	CCAAACATTTGCAGGGTCAG	–
cry11Aa	5’	TGCTGATAACAATGGCAATGAAA	254
	3’	TGGTGGTATTGTTCCGGTTC	–
cry11Ba	5’	GAATCAGCGCCTGAACAATG	615
	3’	TAATGCGAGTTGCTGCGATA	–
cyt1Aa	5’	GATGGGTGCTGTAGTGAGTT	221
	3’	TAGCAGTTTCCTTGCCCCA	–
cyt2Aa	5’	ACGGTTCCATCCAGTGATTTA	494
	3’	TCAACATCCACAGAAACCTCA	–
cyt2Ba	5’	GGCATATTGCATTAACAGTTCCA	401
	3’	AGCTGATAAATTACGCCAAACAA	–

template DNA, 1.0 μl of each primer set, and 17 μl of distilled water into premix (Bioneer) containing the buffer component and dNTP, and adjusting the final volume to 20 μl. PCR was conducted under conditions consisting of 30 cycles of 95°C for 3 min, 95°C for 1 min, annealing at 57°C for 1 min, 72°C for 1 min and 72°C for 5 min to produce PCR product DNA, which was electrophoresed in 1% agarose gel (Ibarra *et al*., 2003).

Plasmid DNA analysis

The protocol of a Qiagen midi kit was used with some modification to extract plasmid DNA from *B. thuringiensis* strain. The strain was inoculated into 5 ml of LB medium and cultured at 27°C and 180 rpm for 8 hours. The culture was added to 50 ml of LB medium and incubated for 16 hours under the same conditions. The cultured bacteria were centrifuged at 6,000 g for 15 minutes at 4°C. The supernatant was discarded and 4 ml of P1 buffer (50 mM Tris–HCl, pH 8.0, 10 mM EDTA, 50 μg / ml of RNase A) was added, and the pellet was dissolved by vortexing. After adding 4 ml of P2 buffer (0.2 M NaOH, 1% SDS), the tube was inverted four to six times to mix the contents, and the contents were incubated at 15–25°C in an incubator for 5 minutes. Thereafter, 4 ml of chilled P3 buffer (4 M guanidine hydrochloride, 0.5 M potassium acetate, pH 4.2) was added and the tube was inverted four to six times to mix the contents, and the contents were incubated on ice for 15 minutes. After centrifugation at 15,000 rpm, 4°C for 30 minutes, the supernatant was moved to a new tube. After centrifugation at 15,000 rpm at 4°C for 15 minutes, Qiagen–tip 100 was laid horizontally to make 4 ml of QBT buffer (50 mM NaCl, 50 mM MOPS, pH 7.0, 15% isopropanol, 0.15% Triton X–100) pass through the column, and the centrifuged supernatant was put into the column. When the supernatant had completely passed through the column, the Qiagen–tip 100 was washed with 20 ml of QC buffer. The DNA was dissolved in 5 ml of QF buffer to separate the DNA, 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, the contents were centrifuged for 10 minutes at 15,000 rpm at 4°C. The supernatant was discarded, the pellet was air–dried for 5 to 10 minutes, and 200 μl of distilled water was added to dissolve the plasmid DNA again. The plasmid DNA was mixed with the third distilled water, put into a sterile Eppendorf tube, and stored at −20°C until the experiment. The electrophoresis was performed by mixing agarose gel in 1×TAE buffer at a ratio of 1%, dissolving by heating, and then pouring into a gel tray. The agarose gel was used after being hardened for 20 minutes. To the electrophoresis apparatus, 1×TAE buffer, which is the same as gel, was poured 3 to 5 mm above the gel, a 5:1 mixture of loading dye and loading star and plasmid DNA sample were mixed at a ratio of 1:5, the mixture was put into each well, electrophoresed at 50 V for 60 minutes, irradiated with UV, and the band patterns were confirmed.

RESULTS AND DISCUSSION

Selection and identification of *B. thuringiensis* strains

B. agrestis, which has been recently occurring in cultivation under structures, and *L. ingenua*, which has been occurring in mushroom growers, are diptera pests species, and no biological control agent for these species has been registered. However, farms is demands for environmentally friendly control for such pests species have seriously been coming to the fore. Therefore, 49 *B. thuringiensis* strains kept in the biological pest control laboratory of Chungnam National University to select those *B. thuringiensis* strains that have new insecticidal ranges were incubated in NA medium at 27°C and the parasporal inclusions produced by them were observed through a phase contrast microscope. Out of the 49 strains, 28 strains that form bipyramidal type parasporal inclusions and nine strains that form spherical type parasporal inclusions were identified. Among them, the nine strains that form spherical type parasporal inclusions were selected since spherical type parasporal inclusions are generally known to be effective on diptera. The activity of the nine strains that form spherical type parasporal inclusions against *L. ingenua*, which is in diptera, was tested (Table 2). Against *L. ingenua*, *B. thuringiensis* CAB464 strain showed high larvicidal activity not lower that 90%, CAB117, 459 and 469 strains showed at least 70% larvicidal activity, and CAB490 and 492 strains showed at least 50% larvicidal activity. In addition, against *B. agrestis* too, the strain showed high larvicidal activity not lower that 90%, CAB117 and 469 strains showed at least 70% larvicidal activity, and CAB459, 462, and 490 strains showed at least 50% larvicidal activity. However, CAB463 and 467 strains showed low larvicidal activity not higher than 50% against the two pest species. Therefore, CAB464 strain, which has high larvicidal activity not lower than 90%, was selected for

Table 2. Activity of *Bacillus thuringiensis* strains against *Lycoriella ingenua*

Strains	Tested insects	
	Lycoriella ingenua	*Bradysia agrestis*
Control	–	–
CAB117	++	++
CAB459	++	+
CAB462	–	+
CAB463	–	–
CAB464	+++	+++
CAB467	–	–
CAB469	++	++
CAB490	+	+
CAB492	+	–

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

two pest of *B. agrestis* and *L. ingenua*. The CAB464 strain, which exhibits high larvicidal activity, was identified by the flagellin C gene in the Institute of Microbial Ecology and Resources, Mokwon University, and as a result, it was classified into *B. thuringiensis* subsp. *kyushuensis*.

Morphological characteristics of *B. thuringiensis* strains

Since the forms of endotoxin proteins of *B. thuringiensis* strains provide information indicating close relationships with the range of insect hosts in which larvicidal activity is shown (Maeda *et al*., 2000), the observation of endotoxin protein morphology under phase contrast microscopy is provided as important information (Jin *et al*., 2015). The observation of *B. thuringiensis* subsp. *darmstadiensis* and *B. thuringiensis* subsp. *kyushuensis* CAB464 strains with high larvicidal activity against *L. ingenua* indicated that these strains form typical spherical type crystal proteins, known to show larvicidal activity against diptera pest. These proteins showed patterns similar to those of the spherical type endotoxin proteins of *B. thuringiensis* subsp. *israelensis*, which is a control strain with larvicidal activity against diptera (Fig. 1).

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

To test the host ranges and larvicidal activity of the nine *B. thuringiensis* strains with larvicidal activity against diptera pest and the selected *B. thuringiensis* subsp. *kyushuensis* CAB464 strain, the larvicidal activities of these strains were tested against the five pest species being reared in the laboratory: *L. ingenua*, *B. agrestis*, *A. albopictus*, *A. togoi*, and *C. pipiens pallens* (Table 3). With regard to the ranges of activity of the 10 strains, six strains showed high larvicidal activity exceeding 90% against *A. albopictus* and *A. togoi*, and seven strains showed high larvicidal activity exceeding 90% against *C. pipiens pallens*. In particular, *B. thuringiensis* subsp. *darmstadiensis* and *B. thuringiensis* subsp. *kyushuensis* CAB464 strains showed larvicidal activity exceeding 90% against *L. ingenua* and *B. agrestis*. Therefore, *B. thuringiensis* subsp. *darmsta-*

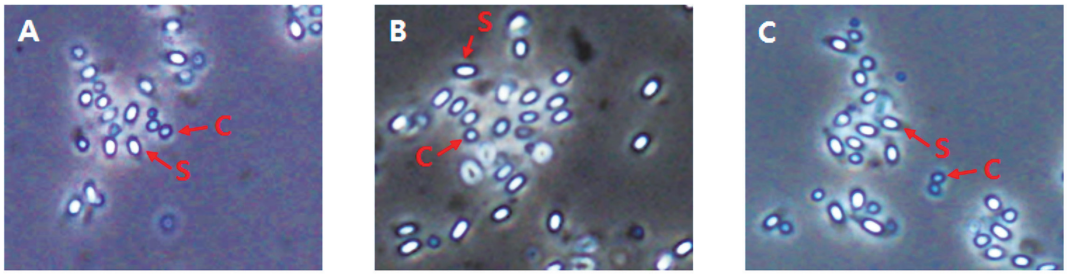

Fig. 1. Phase–contrast microscope photographs (x 1,000) of crystal shape of *B. thuringi–ensis*. (A): *B. thuringiensis* subsp. *darmstadiensis*; (B): *B. thuringiensis* subsp. *israelensis*; (C): *B. thuringiensis* subsp. *kyushuensis* CAB464.

Table 3. Insecticidal activities of *B. thuringiensis* isolates against Diptera larva

Strains	Insects tested				
	Lycoriella ingenua	*Bradysia agrestis*	*Aedes albopictus*	*Aedes togoi*	*Culex pipiens pallens*
Control	–	–	–	–	–
B. thuringiensis subsp. canadensis	++	++	+++	+++	+++
B. thuringiensis subsp. darmstadiensis	+++	+++	+++	+++	+++
B. thuringiensis subsp. fukuokaensis	++	++	–	–	++
B. thuringiensis subsp. higo	++	++	–	++	+++
B. thuringiensis subsp. israelensis	++	+	+++	+++	+++
B. thuringiensis subsp. japonensis	–	–	–	–	–
B. thuringiensis subsp. kyushuensis CAB464	+++	+++	++	+++	+++
B. thuringiensis subsp. morrisoni	+	+	+++	+++	+++
B. thuringiensis subsp. sotto	+	++	+++	++	–
B. thuringiensis subsp. tohokuensis	++	++	+++	+++	+++

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

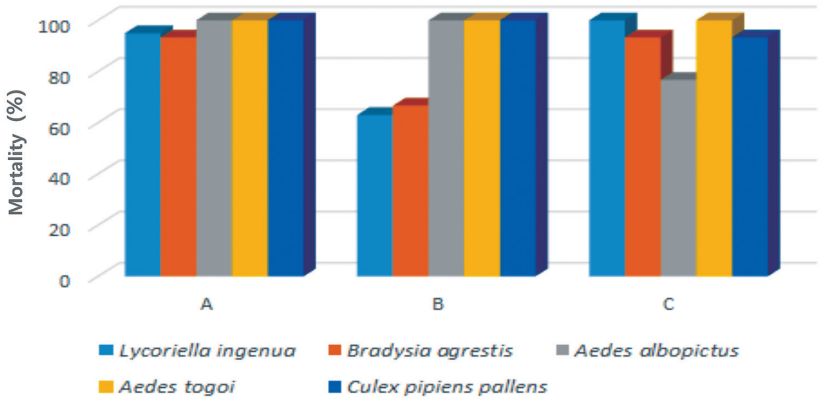

Fig. 2. Mortality of Diptera larva at the same concentrations (10^6 cfu/ml) of *Bacillus thuringiensis*. (A): *B. thuringiensis* subsp. *darmstadiensis*; (B): *B. thuringiensis* subsp. *israelensis*, (C): *B. thuringiensis* subsp. *kyushuensis* CAB464.

Table 4. Toxicity of *B. thuringiensis* subsp. *darmstadiensis* and *B. thuringiensis* subsp. *kyushuensis* CAB464 isolates against *Lycoriella ingenua* larvae

Strains	LC_{50} (cfu/ml)	LC_{95} (cfu/ml)
B. thuringiensis subsp. darmstadiensis	2.8×10^5	3.5×10^6
B. thuringiensis subsp. israelensis	3.1×10^6	9.8×10^7
B. thuringiensis subsp. kyushuensis CAB464	1.9×10^5	1.6×10^6

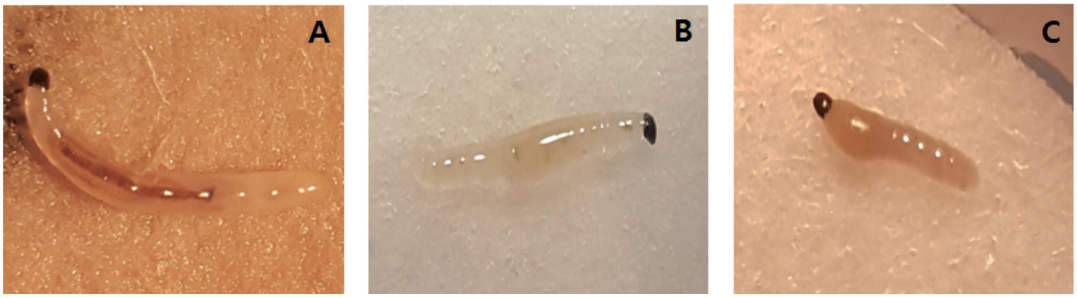

Fig. 3. The appearance of *Lycoriella ingenua* larva treated with *B. thuringiensis*. (A): control; (B) dead larva after 2 days, (C): dead larva after 5 days.

diensis and *B. thuringiensis* subsp. *kyushuensis* CAB464 strains, which have the highest larvicidal activity against *L. ingenua*, which causes the greatest damage to mushroom plantations, and the control strain *B. thuringiensis* subsp. *israelensis* were selected to conduct biological assays (Fig. 2). In the results of the biologic activity tests of the selected two strains and the control strain at various concentrations, *B. thuringiensis* subsp. *kyushuensis* CAB464 strain showed the highest larvicidal activity with an LC_{95} value of 1.6×10^6 (cfu/ml) and *B. thuringiensis* subsp. *darmstadiensis* strain showed an LC_{95} value of 3.5×10^6 (cfu/ml), thereby showing higher larvicidal activity than the control strain *B. thuringiensis* subsp. *israelensis* (Table 4). The *L. ingenua* larvae killed by *B. thuringiensis* strain showed midgut regions that gradually melted to become pale so that the clear shapes of the midgut could not be identified, and showed gradual changes into dark colors (Fig. 3). To check the rapid activity of the selected strains and the control strain, the concentrations of the strains were adjusted to be identical and biological assays were conducted with *L. ingenua* larvae. In the results, *B. thuringiensis* subsp. *kyushuensis* CAB464 strain showed the highest toxicity and the rapid–acting insecticidal effect could be seen. *B. thuringiensis* subsp. *darmstadiensis* strain also showed higher toxicity and rapid–acting effects than the control strain, and the two strains showed a rapid increase in larvae lethality from the third day (Fig. 4). *B. thuringiensis* subsp. *darmstadiensis* and *B. thuringiensis* subsp. *kyushuensis* CAB464 strains were highly toxic to *L. ingenua* and *B. agrestis*, which are diptera pest, as well as mosquitoes, and can be expected to be *B. thuringiensis* strains useful for biological control in mushroom cultivation farms.

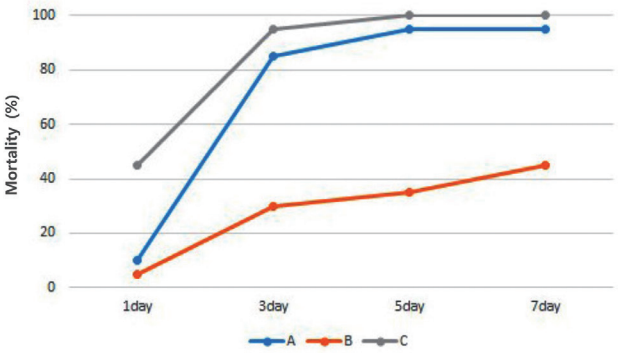

Fig. 4. Mortality of *Lycoriella ingenua* larva at various ages at the same concentrations (10^6 cfu/ml) of insecticide treatment. (A): *B. thuringiensis* subsp. *darmstadiensis*; (B): *B. thuringiensis* subsp. *israelensis*, (C): *B. thuringiensis* subsp. *kyushuensis* CAB464.

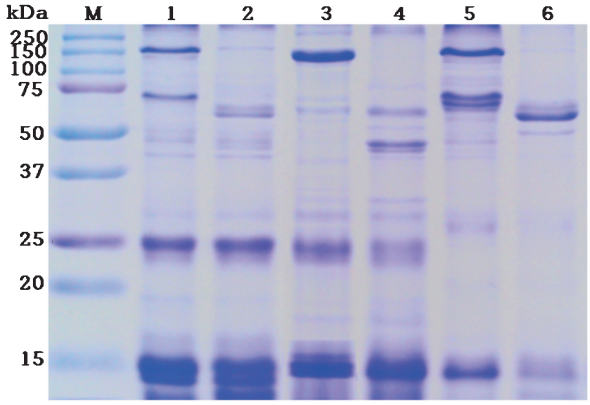

Fig. 5. SDS–PAGE analysis of parasporal inclusions of *B. thuringiensis* subsp. *darmstad–iensis* and *B. thuringiensis* subsp. *kyushuensis* CAB464. M; Broad range marker, Lane 1: *B. thuringiensis* subsp. *darmstadiensis* protoxin; Lane 2: *B. thuringiensis* subsp. *darmstadiensis* digested with trypsin; Lane 3: *B. thuringiensis* subsp. *israelensis* protoxin; Lane 4: *B. thuringiensis* subsp. *israelensis* digested with trypsin; Lane 5: *B. thuringiensis* subsp. *kyushuensis* CAB464 protoxin; Lane 6: *B. thuringiensis* subsp. *kyushuensis* CAB464 digested with trypsin.

SDS–PAGE

The molecular weight of the cry protein constituting the spherical endotoxin protein, which has larvicidal activity against diptera pest, is about 65 kDa, and the molecular weight of Cyt protein is reported to be about 22–30 kDa (Ibarra and Federici, 1986a, Nisnevitch *et al.*, 2013). In the case of *B. thuringiensis* subsp. *israelensis* strain, which shows larvicidal activity against diptera pest, when the parasporal body has been hydrolyzed in the larvae midgut, four major proteins with molecular weights of 27, 65, 128, and 135 kDa are formed (Manasherob *et al.*, 2001). Among them, the 135, 128 and 65 kDa proteins are Cry4 proteins and the 27 kDa protein is the cytolytic CytA. The toxicity is caused by the synergy between the 25 kDa protein, a 27 kDa protein degradation product, and one or more proteins with higher molecular weights (Lee *et al.*, 2003).

SDS–PAGE was performed to compare the characteristics of the endotoxin proteins of *B. thuringiensis* subsp. darmstadiensis and *B. thuringiensis* subsp. *kyushuensis* CAB464 strains, which showed high larvicidal activity in the assay of biologic activity against *L. ingenua* and the control strain *B. thuringiensis* subsp. *israelensis*. Major protein band patterns were identified with sizes of about 150, 70, 66, 50, and 27 kDa in *B. thuringiensis* subsp. *darmstadiensis* strain and about 150, 70, 66, 50, and 27 kDa in *B. thuringiensis* subsp. *kyushuensis* CAB464 strain (Fig. 5). To identify the active toxin patterns of the parasporal inclusions generated by the two strains, the two strains were treated with trypsin, which is a digestive enzyme. In the case of *B. thuringiensis* subsp. *darmstadiensis* strain, the protein of about 150 kDa was degraded by trypsin to form new protein bands of about 65 and 60 kDa, and in the case of *B. thuringiensis* subsp. *kyushuensis* CAB 464 strain too, the protein of about 150 kDa was degraded by trypsin to form new protein bands of about 65, 60, and 50 kDa.

Identification of Cry–type gene

B. thuringiensis strain produces two kinds of insecticidal proteins including 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 a high larvicidal activity against diptera pest, has *cry4Aa, cry4Ba, cry10, cry11Aa* and *cyt1Aa* and *cyt2Ba* genes (Bravo *et al.*, 2007), and *B. thuringiensis* subsp. *morrisoni* strain has been reported to have *cry4A, cry4B, cry10A, cry11A* and *cyt1A* genes (Ibarra and Federici, 1986b, Padua *et al.*, 1984). Many *B. thuringiensis* strains with larvicidal activity against mosquito larvae have been shown to have genes and proteins similar to *cyt1A* (Ragni *et al.*, 1996). Cyt toxins are a protein group produced by some strains of *B. thuringiensis*, and are classified into three types: cyt1, cyt2, and cyt3, based on amino acid homology (Crickmore *et al.*, 1998). These proteins have a lethal activity on the larvae of diptera pest and have been reported to have cytolytic activity on a wide range of cells including red blood cells in vitro (Thomas *et al.*, 1983). Various cyt toxins have been found in the *B. thuringiensis* strain, which has larvicidal activity against mosquito larvae, and the gene encoding this toxin has been identified and sequenced (Crickmore *et al.*, 1998).

The cry genes of *B. thuringiensis* subsp. *darmstadiensis* strain and *B. thuringiensis* subsp. *kyushuensis* CAB464 strain selected in the present study were confirmed, and according to the results, it could be seen that the two strains have a *cyt2Aa* gene that was not identified in *B. thuringiensis* subsp. *israelensis* strain (Table 5). The *cyt2Aa* gene is found in *B. thuringiensis* subsp. *darmstadiensis* strain and *B. thuringiensis* subsp. *kyushuensis* CAB464 strain and has been reported to be the only protein that causes larvicidal activity against mosquito larvae (Ishii and Ohba, 1994). Since the two strains have a *cyt2Aa* gene not identified

Table 5. Profiles of crystal protein genes in *Bacillus thuringiensis* strains

Strain	Genes
B. thuringiensis subsp. *darmstadiensis*	*cyt2Aa*
B. thuringiensis subsp. *israelensis*	*cry4Aa, cry4Ba, cry10Aa, cry11Aa, cyt1Aa, cyt2Ba*
B. thuringiensis subsp. *kyushuensis* CAB464	*cyt2Aa*

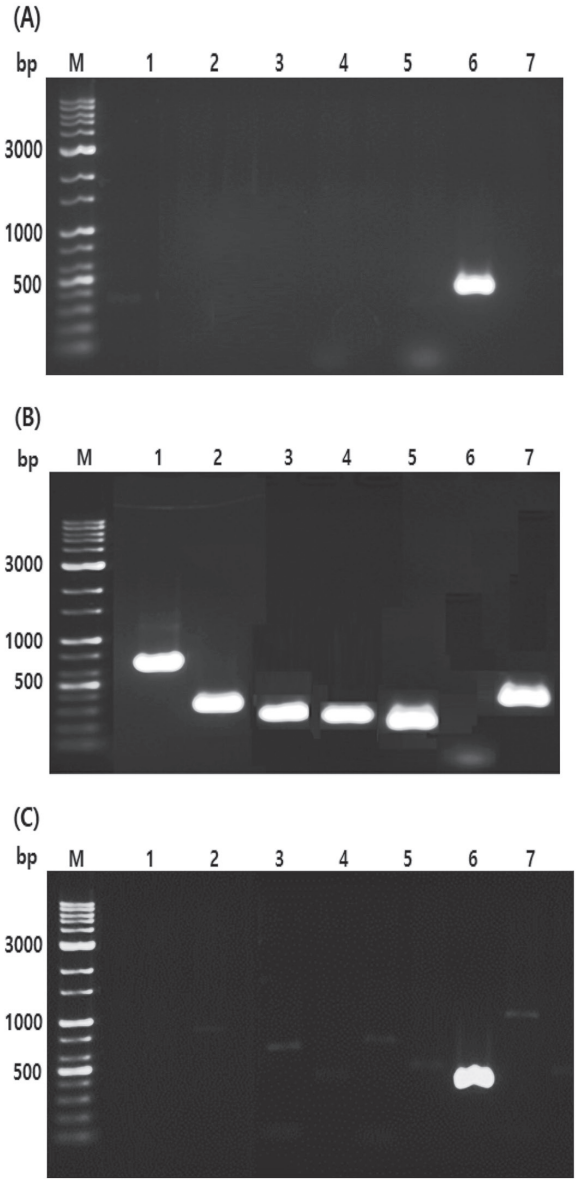

Fig. 6. Agarose gel (1%) electrophoresis of PCR products obtained with specific primers for the cry genes. (A): *B. thuringiensis* subsp. *darmstadiensis*; (B): *B. thuringiensis* subsp. *israelensis* (C): *B. thuringiensis* subsp. *kyushuensis* CAB464. M: 100 bp ladder marker; Lane 1: *cry4Aa*; Lane 2: *cry4Ba*; Lane 3: *cry10Aa*; Lane 4: *cry11Aa*; Lane 5: *cyt1Aa*; Lane 6: *cyt2Aa*; Lane 7: *cyt2Ba*.

in *B. thuringiensis* subsp. *israelensis* strain, it can be assumed that these two strains have high larvicidal activity against *L. ingenua* (Fig. 6).

Plasmid DNA analysis

The *cry* gene, which is involved in the formation of

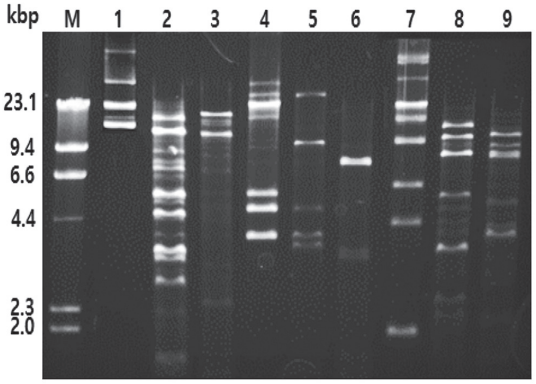

Fig. 7. Plasmid DNA patterns of *Bacillus thuringiensis*. M: Lamda HindⅢ marker; Lane 1: *B. thuringiensis* subsp. *darmstadiensis*; Lane 4: *B. thuringiensis* subsp. *israelensis*; Lane 9: *B. thuringiensis* subsp. *kyushuensis* CAB464, Lane 2, 5, 8: *B. thuringiensis* digested with EcoRI, Lane 3, 6, 9: *B. thuringiensis* digested with HindⅢ.

crystal proteins with larvicidal activity by *B. thuringiensis* strain, mainly exists on plasmid DNA. The plasmid DNAs existing in *B. thuringiensis* strain reach up to 17 types, and the lengths are about 2–250 kbp (Sarrafzadeh *et al.*, 2007). The numbers and lengths of plasmid DNAs owned by individual strains have been reported to be diverse. Likewise, the *cry* gene that forms crystal proteins does not always exist on the plasmid DNA of the same size in all strains, but exists on different plasmid DNAs by strain (Kronstad *et al.*, 1983; Aronson *et al.*, 1986). Plasmid DNA patterns of *B. thuringiensis* subsp. *darmstadiensis* strain and *B. thuringiensis* subsp. *kyushuensis* CAB464 strain were confirmed using *B. thuringiensis* subsp. *israelensis* strain as a control strain (Fig. 7). In the case of *B. thuringiensis* subsp. *darmstadiensis* strain, five plasmid DNA bands could be identified, including two plasmid DNA bands larger than 23.1 kb. In the case of *B. thuringiensis* subsp. *kyushuensis* CAB464 strain, nine plasmid DNA bands could be identified, including two plasmid DNA bands larger than 23.1 kb. In addition, it could be seen that *B. thuringiensis* subsp. *darmstadiensis* strain and *B. thuringiensis* subsp. *kyushuensis* CAB464 strain showed three identical bands, while only the 23.1 kb band was identical with that of the control strain *B. thuringiensis* subsp. *israelensis*.

In the present study, *B. thuringiensis* subsp. *darmstadiensis* strain and *B. thuringiensis* subsp. *kyushuensis* CAB464 strain could not be distinguished from the control strain *B. thuringiensis* subsp. *israelensis* strain based on only configurational characteristics or protein characteristics, but the differences

were identified through biological assays and molecular biological experiments. Since *B. thuringiensis* subsp. *darmstadiensis* strain and *B. thuringiensis* subsp. *kyushuensis* CAB464 strain showed high insecticidal activity against *L. ingenua*, they are expected be actively used by button mushroom cultivation farms as new microbial insecticides that can control *L. ingenua*.

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. Young Nam Youn edited the paper. Chisa Yasunaga–Aoki participated in the design of the study and discussed the experiments and the results. Yong 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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