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Isolation of New *Bacillus thuringiensis* Strains with Insecticidal Activities Against Diptera Insect Pests and Their Biological Characteristics

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Although chemical insecticides have been used to control Diptera agricultural pests, because of characteristics of the pests that invade into soil or into the roots of plants to do harm over most of their larva developmental period, it is difficult to effectively control the pests using chemical insecticides, and biological control methods are necessary due to increases in the production of eco–friendly agricultural products. Therefore, this study conducted the survay of new control agent. In order to isolate *Bacillus thuringiensis* strains that show insecticidal activities against Diptera, *B. thuringiensis* strains were selected from soils from the streams in Yangyang, Gangwon Province, Saryang–do, Chuja–do, and Jeju–do (islands), which are main habitats of Diptera. In bioassays, isolate S11 and C55 showed high insecticidal activities not lower than 90% in both *Lycoriella ingenua* and *Aedes albopictus* larvae, and the selected strain S11 showed insecticidal activities not lower than 50% in *Bradysia agrestis*. In investigation of the insecticidal activities against *L. ingenua* larvae, strains S11 and C55 showed higher insecticidal activities than the control strain *B. thuringiensis* subsp. *Israelensis* which is widely used as a pesticide against Diptera. SDS–PAGE was performed to compare the δ–endotoxin protein characteristics of the two selected strains. Strain S11 showed three main bands at 150 kDa, 75 kDa, and 65 kDa, while strain C55 showed three main bands at 150 kDa, 70 kDa, and 25 kDa. In the digestion reactions with *L. ingenua* triturated solution, the strain S11 was digested to 75 kDa and 25 kDa to show bands while strain C55 showed a band at 25 kDa. The results of PCR of cry genes of the two strains indicated that both strains have cyt2Aa genes. In the plasmid DNA patterns, S11 showed a total of six bands and C55 showed a total of two bands.

Key words: *Bacillus thuringiensis*, Biological activity, Diptera, Isolation

INTRODUCTION

Recently, various pathogens and insect pests have occurred in main fruit and vegetable growing regions in South Korea because optimum conditions for the survival of pathogens and insect pests have been formed in those regions. It has been reported that Diptera insect pests, which are important among diverse agricultural pests occurring in South Korea, are also widely distributed throughout the world (Jagdale *et al*., 2007; Vaughan *et al*., 2011). *Lycoriella ingenua* and *Bradysia agrestis* that belong to Diptera and Sciaridae, respectively, are known to be representative agricultural pests that cause severe economic damage because their larvae directly do harm to crops during their feeding activities (Shamshad, 2010). *L. ingenua* is an insect pest that does harm to mushrooms such as button mushrooms, oyster mushrooms, and shiitake mushrooms. It directly feeds on mushroom mycelia and does harm to fruiting bodies thereby reducing mushroom yields to cause damage to farmers (Erler *et al*., 2011; Kim *et al*., 2012). *B. agrestis* occurs in humid environments in temperate and tropical regions or in environments rich in organic matter in soil and does harm to the roots of fruits and vegetables such as onions, peppers, tomatoes, lettuce, and mushrooms to interfere with the growth and causes damage to crops with secondary infections by plant pathogens (Chambers *et al*., 1993; Cloyd, 2000).

Although chemical insecticides have been used to control *L. ingenua* and *B. agrestis* at domestic agricultural sites, effective control is difficult. The agricultural chemicals registered for *L. ingenua* should be applied after mushroom seeding or before and after covering up the seeds with soil and the agricultural pesticides applied at those times do not affect the insect pests. Since the use of agricultural chemicals in the early stage of the occurrence of insect pests is restricted due to the problem of pesticides residue, there are difficulties in controlling *L. ingenua* that occurs throughout the entire mushroom cultivation period (Lee *et al*., 2015a; Yoon *et al*., 2016). Meanwhile, difficulties in controlling *B. agrestis* come from the fact that *B. agrestis* larvae are present in soil during most of their developmental period before they become imagoes to do harm to the roots of plant bodies or invade inside the tissues of roots to do harm to the roots (Jeon *et al*., 2007). Although there are insecticides registered as agricultural chemicals such as teflubenzuron flowable, chlorphenapyr flowable, and acetamiprid wettable powder (KCPA, 2017), biological control methods are necessary due the issues such as the safety of agricultural products and environmental pollution.

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Bacillus thuringiensis is the most frequently used as a biological control agent. *B. thuringiensis* is an insect pathogenic Gram–positive aerobacillus and it is one of the important microorganisms used in biotic pesticides because it forms parasporal inclusions (Li and Bouwer, 2014; El Khoury *et al*., 2014). *B. thuringiensis*, an insect pathogenic bacterium, was first isolated from the body of diseased silkworm larvae in 1901 and identified to show high insecticidal activities in Lepidopteran larvae (Jin *et al*., 2009) and thereafter, in 1977 when *B. thuringiensis* subsp. *israelensis* was found, *B. thuringiensis* was reported to show insecticidal activities not only in Lepidoptera but also in Diptera (Goldberg and Margalit, 1997). As a subspecies of *B. thuringiensis* that shows high insecticidal activities in Diptera, *B. thuringiensis* subsp. *israelensis* (Balaraman *et al*., 1981; Shim *et al*., 1990; Abdel–Hameed *et al*., 1990), which shows high insecticidal activities in mosquitoes that are Diptera hygiene insect pests, was found (Balaraman *et al*., 1981; Shim *et al*., 1990; Abdel–Hameed *et al*., 1990). Thereafter, *B. thuringiensis* subsp. *Morrisoni* (Padua *et al*., 1984), *B. thuringiensis* subsp. *Medellin* (Orduz *et al*., 1992), etc. were isolated in various areas in the world but most of them were reported to have low toxicity to Diptera insect pests (Ishii and Ohba, 1993). Therefore, new *B. thuringiensis* species that show toxicity to Diptera insect pests should be isolated and their potential as biotic pesticides should be reviewed.

In this study, Gangwon–do, Chuja–do, Saryangdo, and Jeju–do were selected as these areas were expected to be habitats of Diptera insect pests because natural environments are well maintained, temperatures and humidity are high, and large amounts of organic matter exist in these areas. *B. thuringiensis* strains were isolated and identified from the soils obtained in these areas, strains with excellent activity were selected, and the strains' biological characteristics were studied to identify the strains' morphological characteristics and insecticidal activities, the characteristics of the insecticidal proteins of the strains, and plasmid DNA patterns of the strains.

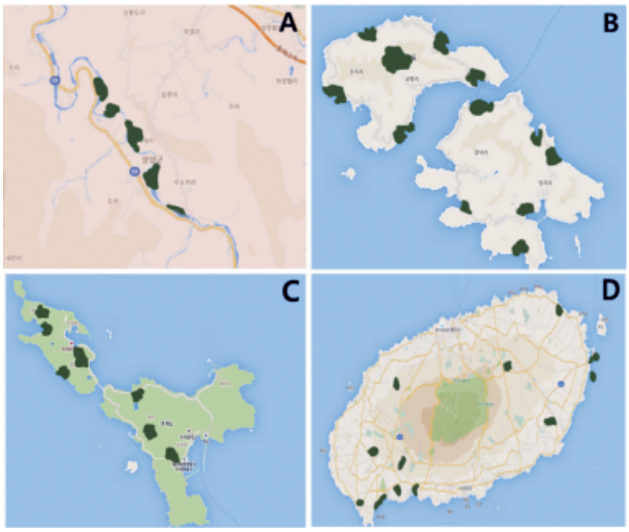

Fig. 1. Map of the locations in Korea where samples were collected for isolation of *B. thuringiensis* (areas in black). A: Gangwondo yangyang–namdaecheon; B: Saryang Island; C: Chuja Island; D: Jeju Island.

MATERIALS AND METHODS

Soil sampling

Soil samples were collected from various areas in Yangyang, Gangwon Province, Saryang–do (island), Chuja–do (island), and Jeju–do (island) (Fig. 1). A total of 477 soil samples were collected; 130 from Namdaecheon region in Yangyang, Gangwon Province, 94 from areas in Saryang–do, 131 from areas in Chuja–do, and 122 from areas in Jeju–do. As for the soil sampling method, 10 g each of soil at 10 cm depth from the surface of the earth was collected from each area using a sterilized reagent spoon. The collected samples were put into sterilized containers and the containers were sealed and stored at 4°C until the samples were used.

Isolation and morphological observation of *B. thuringiensis* strains

Bacillus strains were isolated using the method of Ohba and Aizawa (1978). 1 g of the collected soil and 10 ml of sterilized water were put into a test tube and stirred. In order to selectively remove those germs that form only nutrient cells and cannot form spores, the samples were heat treated in a constant–temperature water bath at 60°C for 30 minutes. After the heat treatment, the soil was allowed to settle, and the supernatant was diluted to 10^{-3} and was evenly spread on a nutrient agar plate with an inner diameter of 90 mm. The plate after spreading were cultured for 3–4 days at a 27°C incubator and colonies similar to *B. thuringiensis* strains were selected from among colonies formed. The selected colonies were streak–spread on an NA medium and cultured at 27°C for 4 to 5 days. Thereafter, the morphology of the δ–endotoxin protein crystals was checked with a phase contrast microscope of 1,000 magnifications. Strains in which δ–endotoxin protein crystals were identified were diluted in sterilized water, 2 ml each of the strain solution was spread on a 150–mm Nutrient agar plate, and the plate was incubated at 27°C for 5 days. After identifying that autolysis occurred, the strain solution was centrifuged for 14 minutes at 15,000 rpm with a centrifugal separator (Avanti JE, Beckman), and the bacilli were collected and used in biotic assays. For comparison of insecticidal activities, *B. thuringiensis* subsp. *israelensis* strain, widely known as a Diptera control agent was selected as a control strain.

Test insect

The diamondback moth (*Plutella xylostella*), a Lepidopteran insect pest used in this experiment, was bred for successive generations with young radishes as a host in the biotic insect pest control laboratory at Chungnam National University. *Spodeoptera litura* and *Spodoptera exigua* were bought from the National Institute of Agricultural Sciences of the Rural

Development Administration and those that were bred for successive generations using artificial feed were used. The imagoes were supplied with 10% sugar water as a feed. The *B. agrestis*, Diptera, used in this experiment was those that were bred for successive generations in the biotic insect pest control laboratory at Chungnam National University and the *L. ingenua* used in this experiment was those collected from a button mushroom farm located in Songgu–ri, Chochon–myeon, Buyeo–gun, Chungcheongnam Province and bred for successive generations. *Aedes albopictus* was purchased from the Insect Physiology Laboratory of Chungnam National University and those that were bred using fry feed were used. All insects were bred under at temperatures of 25 ± 1°C, a light condition of 16L:8D, relative humidity of 50–60%.

Bioactivity assay

The bioactivity of diamondback moth (*P. xylostella*) was tested using the leaf dipping method (Tabashnik *et al*., 1990) with some modification. Cabbage leaves cut to 5 cm×5 cm were dipped in 30 ml of *B. thuringiensis* diluted solution and dried thereafter at room temperature. Three repetitions of 10 third–instar larvae of diamondback moth (*P. xylostella*) were tested and the mortality was investigated for 72 hours. As for the bioactivity assay for *S. exigua*, artificial feed was cut to 1 g pieces and each piece was inoculated with 200 μl *B. thuringiensis* strain diluted solution, dried at room temperature, put into a Petri dish, six repetitions of five third–instar *S. exigua* larvae were tested, and mortality was investigated for 120 hours.

As for the bioactivity assay for *L. ingenua*, 1 g of king oyster mushroom was inoculated with 200 μl of *B. thuringiensis* strain diluted solution, dried at room temperature, and 10 *L. ingenua* 4–5 days old after hatching were put into a petri dish. Three repetitions of this test were carried out and the mortality was investigated for 168 hours.

As for the bioactivity assay for *B. agrestis*, 1 g of potato was inoculated with 200 μl of *B. thuringiensis* strain diluted solution, dried at room temperature, and 10 *B. agrestis* 3–4 days old after hatching were put into a petri dish. Three repetitions of this test were carried out and the mortality was investigated for 168 hours.

The biological activity assay *A. albopictus* was carried out using the experimental method of WHO (2005) with some modification. Ten larvae 3–4 days old after hatching were out into a 90 ml plastic cup containing 30 ml of water and 300 μl of *B. thuringiensis* strain diluted solution was put into the plastic cup. Three repetitions of this test were carried out and the mortality was investigated for 72 hours.

All experiments were carried out in three repetitions and the lethal concentration (LC_{50} and LC_{95}) was calculated with mortality rates obtained by investigating 5–7 ranges of concentrations from the concentration for deaths of all experimental larvae to the concentration for survival of all experimental larvae using the PC program based on Finney's (1971) probit calculation method (Raymond, 1985).

SDS–PAGE

The selected *B. thuringiensis* strain was inoculated on a nutrient agar medium and incubated at 27°C for 5 days. After identifying that autolysis occurred with a phase contrast microscope, the cells were centrifuged at 15,000 rpm and 4°C for 10 minutes using a PBS buffer. The supernatant was discarded and the remaining cells were washed three times with washing buffer I (500 mM NaCl, 2% Triton X–100) and twice with washing buffer Ⅱ (500 mM NaCl). Sterilized water was added to the washed parasporal inclusions and kept at –20°C until the parasporal inclusions were used (Lee *et al*., 2015b). The method of Laemmli (1970), was partially modified to use 12% separating gel and 5% stacking gel. After electrophoresis, the gel was stained with 0.5% Coomassie brilliant blue.

The triturated larva solution to be used for the digestion reaction by the triturated larva solution experiment was prepared by knocking the larvae cold by placing them at –20°C for 10 minutes, removing the attached limbs using a sterilized dissecting scissors, and disinfecting the surface of larva bodies, and triturating the larva bodies. The larva bodies were immersed in 1% $NaClO_3$ and 70% (w/w) ethanol for one minute for surface disinfection and washed with sterilized saline solution (9.32 g NaCl, 0.77 g KCl, 0.5 g $CaCl_2$, 0.18 g $NaHCO_3$, 0.01 g NaH_2PO_3, 1 l, pH7.4) to remove remaining ethanol. Thereafter, 200 μl of sterilized water was put into a 1.5 ml eppendorf tube, surface–disinfected larvae were put into the eppendorf tube one by one while triturating them with a sterilized insect trituration rod, the resultant triturated larva solution was centrifuged, and only the supernatant was taken. In the digestion reaction by triturated larva solution experiment, the insecticidal crystalline toxin protein was mixed with the triturated larva solution and the mixture was held for reaction at 37°C for 15 minutes. The protein dissolved by the triturated larva solution was identified through electrophoresis.

PCR analysis

Gene–specific primer sets were used to identify the Cry–type gene of *B. thuringiensis* strain δ–endotoxin genes (Table 1). PCR amplification was performed using Thermal Cycle C1000 ™ (BIO–RAD). The reaction solution was prepared by mixing 17 μl of distilled water, 1.0 μl of each primer set, and 1.0 μl of template DNA into a premix (Bioneer) containing dNTP and a buffer component and adjusting the final volume to 20 μl. PCR was conducted under conditions consisting of 30 cycles for 5 min at 95°C, 1 min at 95°C, annealing for 1 min at 60°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.

Plasmid DNA analysis

Plasmid DNA extraction was experimented with the protocol of the Qiagen midi kit with some modification.

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

Cry genes	Primer sequences	PCR product (bp)
cry1Aa	5' GAGCCAAGCGACTGGAGCAGTTTACACC	724
	3' ATCACTGAGTCGCTTCGCATGTTTGACTTTCTC	–
cry1Bc	5' CAGAAACAACAGAACGACC	921
	3' CACTTCCCCACCATCCAT	–
cry2A	5' CAGATACCCTTGCTGGTGTAA	1073
	3' ATAGGCCCGTGCTCCACCAGG	–
cry4Aa	5' CGAGGTGAAATTTGCTCC	1951
	3' ATGGCTTGTTTCGCTACATC	–
cry4Ba	5' GGTGCTTCCTATTCTTTGGC	347
	3' ATGGCTTGTTTCGCTACATC	–
cry10Aa	5' ACGCAAATAAGCCAAATCAACC	257
	3' CCAAACATTTGCAGGGTCAG	–
cry11Aa	5' TGCTGATAACAATGGCAATGAAA	254
	3' TGGTGGTATTGTTCCGGTTC	–
cry11Ba	5' GAATCAGCGCCTGAACAATG	615
	3' TAATGCGAGTTGCTGCGATA	–
cry19A	5' GGGTCCTGGTCATACAGGTG	664
	3' GCCATGCTTCACGTACATTG	–
cry20A	5' CCAGGCGAGTCAGGATTAGA	301
	3' CTTCTGAATCCACGGCTGTT	–
cry24C	5' CTGCAGCAGCGATAACTTCA	415
	3' GCGGCTTGTGTAAAGGTAGG	–
cry30A	5' AGCAAAACAGGCGGTAGATG	523
	3' TCGTACACGATCGCTTTCTG	–
cyt1Aa	5' GATGGGTGCTGTAGTGAGTT	221
	3' TAGCAGTTTCCTTGCCCCA	–
cyt2Aa	5' ACGGTTCCATCCAGTGATTTA	494
	3' TCAACATCCACAGAAACCTCA	–
cyt2Ba	5' GGCATATTGCATTAACAGTTCCA	401
	3' AGCTGATAAATTACGCCAAACAA	–

The selected *B. thuringiensis* strain was inoculated into 10ml of Luria–Bertani medium (Difco, USA) and cultured at 220 rpm at 30°C for 16–18 hours. Thereafter, 1 ml of the culture solution was inoculated into 100 ml of SPY medium (0.2% $(NH_4)_2SO_4$, 1.4% K_2HPO_4, 0.6% KH_2PO_4, 0.1% sodium citrate, 0.02% $MgSO_4 \cdot 7H_2O$, 0.2 yeast extract, 0.1% glucose), cultured for 4 hours, and centrifuged at 7,000 rpm at 4°C for 10 minutes. The supernatant was discarded, 200 μl of lysozyme (50 mg/ml) was added to 5 μl of P1 buffer, and the pellet was dissolved, and cultured at 37°C for 1 hour and 30 minutes. Thereafter, 5 μl of P2 buffer was added and mixed, and the mixture was centrifuged at 10,000 rpm at 4°C for 10 minutes and 5 μl of P3 buffer was added and centrifuged under the same conditions. After making 4 ml of QBT buffer pass through the Qiagen tip, the centrifuged supernatant and 20 ml of QC buffer were made to pass through the Qiagen tip in order of precedence. After completely passing the column, 5 ml of QF buffer was added to dissolve and isolate the DNA. After adding 3.5 ml of isopropanol to the isolated DNA solution and dividing the solution into Eppendorf tubes, the solution was centrifuged at 15,000 rpm at 4°C for 30 minutes. Thereafter, the supernatant was discarded, 1 ml of 70% ethanol was added, and the mixture was centrifuged for 10 minutes under the same conditions. After discarding the supernatant and drying the solution on a clean bench, 30–50 μl of triple distilled water was added to dissolve the DNA. The dissolved DNA was put into sterilized Eppendorf tubes and stored at –20°C until being used.

RESULTS AND DISCUSSION

Isolation of *B. thuringiensis strains*

Studies on isolation, identification, and characterization of the strains of *B. thuringiensis*, an insect pathogenic bacterium, have been actively carried out in various countries around the world. *B. thuringiensis* has been isolated at high rates; about 24% from soil samples

in Brazil (Bravo *et al*., 1998), about 60% from soil samples in Colombia (Armengol *et al*., 2007), and about 23% from soil samples in Spain (Quesada–Moraga *et al*., 2004). The differences as such seem to be attributable to the direct effects of differences in native microbes, pH, humidity, and organic matter concentrations among individual countries on the life cycle of the *B. thuringiensis* strains (Argôlo–Filho and Loguercio, 2013). Those *B. thuringiensis* strains that produces δ–endotoxin proteins were isolated at a rate of about 11%, that is, from 53 out of a total of 477 soil samples collected from Namdaecheon (stream) in Yangyang, Gangwon Province, Saryang–do and Chuja–do, Jeju– do which are areas rich in natural resources and biodiversity (Table 2).

Selection and morphological characteristics of *B. thuringiensis* strains

Among the 53 *B. thuringiensis* strains isolated, 13 were found to show at least 50% insecticidal activities in Diptera or Lepidoptera (Table 3). The types of δ–endotoxin proteins possessed by *B. thuringiensis* can be used as important information because they are related to the main insect orders that show insecticidal activities. The types of δ–endotoxin proteins in the 13 isolated *B. thuringiensis* strains were classified into 8 pyramidal types that show insecticidal activities in Lepidoptera and Diptera and 5 spherical types that show insecticidal activities in Diptera (Fig. 2).

Test of biological activity of *B. thuringiensis* strains

The 53 strains of *B. thuringiensis* isolated from soil were diluted to a concentration of 1.0×10^{7} (cfu/ml) each and their bioactivities in three Lepidoptera insect pest species, *diamondback moth* (*P. xylostella*), *S. exigua*, *S. litura* and three Diptera insect pest species, *L. ingenua*, *B. agrestis* and *A. albopictus* were tested in order to identify the host ranges of the strains. The results indicated that the strains that showed at least 50% insecticidal activities in Lepidoptera or Diptera were G53, S1, S2, S4, S6, S11, S43, C55, J9, J102, J114, J131, J145, J186, and J191, which are a total of 15 in number (Table 3). These results are similar to those reported for non–toxic strains that produce δ–endotoxin proteins but do not exhibit toxicity, which were at a rate of about 30% of the isolated strains (Kim *et al*., 1995). Among the isolated strains, those strains that

Table 2. Number of isolated *B. thuringiensis* from soil at collecting areas

Locality	Number of soil sample examined	Number of soil sample with *B. thuringiensis* isolated
Yangyang–namdaecheon	130	12
Saryang island	94	20
Chuja island	131	14
Jeju island	122	7
Total	477	53

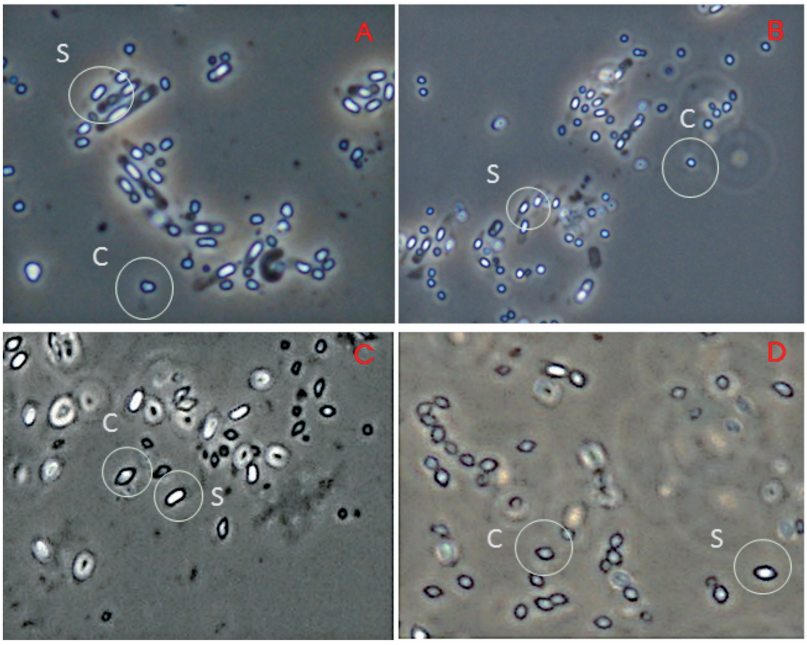

Fig. 2. Phase–contrast microscope photographs of crystal of *B. thuringiensis* isolates. A: *B. thuringiensis* S11; B: *B. thuringiensis* C55; C: *B. thuringiensis* J9; D: *B. thuringiensis* J145.

expressed insecticidal activities in Diptera were G53, S2, S6, S11, S43 and C55, which were a total of 6 in number and those strains that expressed insecticidal activities in both Lepidoptera and Diptera were J9, J102, J114, J131, J145, J186 and J191, which were a total of 7 in number. Among the 13 isolated *B. thuringiensis* strains, strains S11 and C55 showed at least 90% insecticidal activities in both of two species, *L. ingenua* and *A. albopictus*, and strain C55 showed at least 50% insecticidal activities in *B. agrestis*. Strains J9 and J145 showed at least 70% insecticidal activities in all three species of diamondback moth (*P. xylostella*), *S. exigua*, and *S. litura*, which are Lepidopteran insect pests while showing high insecticidal activities of at least 90% in *A. albopictus*, which is a Diptera insect pest (Table 4).

Based on the results of biological activity tests, four strains, strains S11, C55, J9 and J145 were selected and their LC_{50} and LC_{95} values were compared with those of *B. thuringiensis* subsp. *israelensis* strain CAB199, which is known to have high insecticidal activities in the Diptera insect pest mosquito (Table 5). As a result, strain the S11 showed the highest insecticidal activities with an LC_{50} value of 1.14×10^4 in *L. ingenua* larvae while the comparison strain CAB199 showed the highest LC_{50} value of 1.2×10^6 in *B. agrestis* larvae. As for the LC_{50} values in *A. albopictus* larvae, the comparison strain CAB199 showed the highest insecticidal activities with an LC_{50} value of 1.37×10^4. The LC_{95} values were calculated and in the results, as with LC_{50} values, strain S11 showed the highest LC_{95} value indicating the highest insecticidal activities in *L. ingenua* larvae and the comparison strain CAB199 showed the highest insecticidal activities in two Diptera insect pest species, *B. agrestis* larvae and *A. albopictus* larvae.

Table 3. Number of insecticidal *B. thuringiensis*

Crystal type	Number of insecticidal *B. thuringiensis*
Bipyramidal	8
Spherical	5
Total	13

Table 5. Toxicity of *B. thuringiensis* S11, C55 isolates against Diptera

Tested larvae	strains	LC_{50} (cfu/ml)	LC_{95} (cfu/ml)
L. ingenua	S11	1.14×10^4	2.73×10^7
	C55	1.88×10^5	5.14×10^7
	CAB199	7.92×10^6	2.28×10^9
B. arestis	S11	1.28×10^8	2.8×10^9
	C55	3.77×10^6	1.75×10^{10}
	CAB199	1.27×10^6	1.26×10^9
A. albopictus	S11	4.36×10^6	1.99×10^8
	C55	9.00×10^5	1.8×10^7
	J9	1.5×10^5	3.4×10^6
	J145	3.5×110^5	7.87×10^6
	CAB199	1.37×10^4	4.37×10^5

Based on the results of the tests of the strains' insect pest lethal concentrations in Diptera, strain S11 were found to have high toxicity to the Diptera insect pest *L. ingenua*, indicating potential as a biological control agent.

SDS–PAGE

The δ–endotoxin protein patterns of strains S11, C55, J9 and J145 that showed high insecticidal activities on Diptera insect pests in bioactivity tests were checked through SDS–PAGE. Electrophoresis was conducted and according to the results, strain S11 that showed

Table 4. Insecticidal activities of isolated *B. thuringiensis* against Lepidopteran and Dipteran larva

Tested larvae		Lepidopteran			Dipteran		
		Yponomenutidae	Noctuidae		Sciaridae		Culicidae
		P. xylostella	*S. exigua*	*S. litura*	*L. ingeaus*	*B. arestis*	*A. albopictus*
Isolates	G53	−	−	−	+	−	−
	S2	−	−	−	+++	−	+
	S6	−	−	−	++	−	−
	S11	−	−	−	+++	−	+++
	S43	−	−	−	+++	−	++
	C55	−	−	−	+++	+	+++
	J9	+++	+++	+++	−	−	+++
	J102	+++	+++	+++	−	−	++
	J114	+++	+++	++	−	−	++
	J131	+++	+++	−	−	−	+
	J145	+++	+++	+++	−	−	+++
	J186	+++	+++	+++	−	−	++
	J191	+++	+	−	−	−	+++

+++: Highly effective, >90% lethality, ++: Effective, 70~89% lethality, +: Low effective, 50~69% lethality, −: not effective, 0~49% lethality.

insecticidal activity only in the Diptera insect pests showed three bands, at 150 kDa, 75 kDa and 65 kDa, and strain C55 showed three main bands, at 150 kDa, 70 kDa and 25 kDa (Fig. 3). The comparison strain, *B. thuringiensis subsp. israelensis* strain CAB199 showed bands at 130 kDa, 70 kDa, 28 kDa and 22 kDa thereby showing differences from the two strains. In order to identify the active toxins of strains S11 and C55, the parasporal inclusions of the two strains were made to react with *L. ingenua* triturated solution and electrophoresis was performed. As a result, the 150 kDa band of strain S11 in was decomposed by the *L. ingenua* triturated solution so that bands were identified at 50 kDa and 25 kDa (Fig. 4) and the 150 kDa and 75 kDa bands of strain C55 strain were decomposed by the *L. ingenua* triturated solution so that a band was shown at 60 kDa, 45 kDa, 40 kDa, and 25 kDa (Fig. 5).

In the case of strains J9 and J145, which exhibited insecticidal activities in Diptera as well as in Lepidoptera, a band about 130 kDa was identified identically to *B. thuringiensis* subsp. *aizawai* strain KB098, which is known to show insecticidal activities in Lepidoptera and Diptera. To identify the active toxins of the three strains, the parasporal inclusions of the two strains were made to react with *L. ingenua* mid–gut

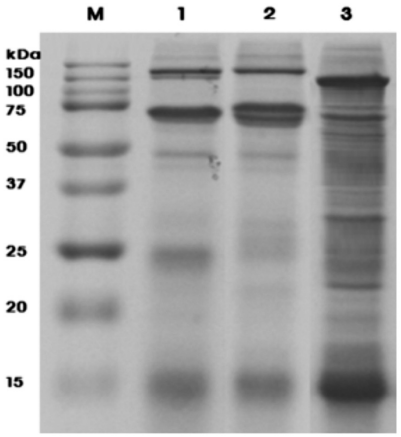

Fig. 3. SDS–PAGE analysis of parasporal inclusion of *B. thuringiensis* C55, S11, and CAB199. M: Standard marker; lane 1: *B. thuringiensis* C55; lane 2: *B. thuringiensis* S11; lane 3: *B. thuringiensis* subsp. *israelensis* CAB199.

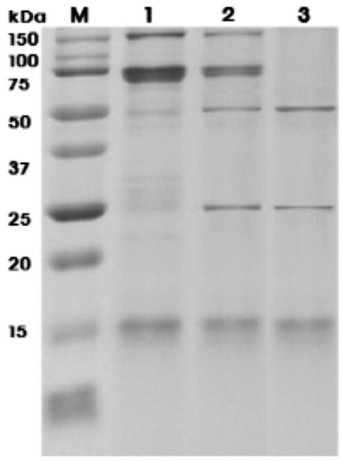

Fig. 4. SDS–PAGE analysis of *B. thuringiensis* S11. M: Standard marker; lane 1: *B. thuringiensis* S11; lane 2: *B. thuringiensis* S11 digested with larval homogenate for 15min; lane 3: *B. thuringiensis* S11 digested with larval homogenate for 30min.

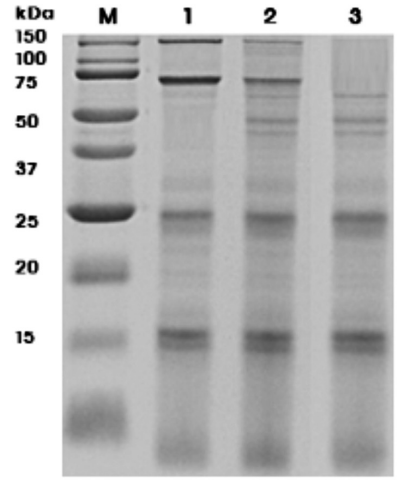

Fig. 5. SDS–PAGE analysis of *B. thuringiensis* C55. M: Standard marker; lane 1: *B. thuringiensis* C55; lane 2: *B. thuringiensis* C55 digested with larval homogenate for 15min; lane 3: *B. thuringiensis* C55 digested with larval homogenate for 30 min.

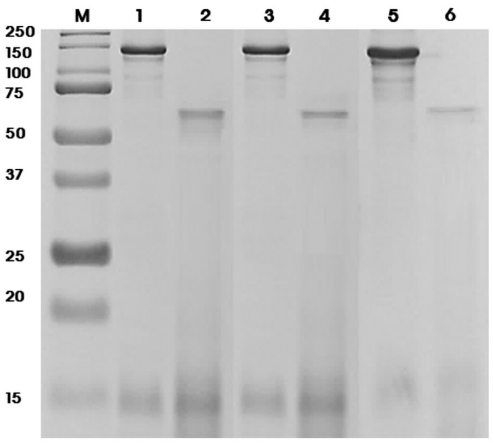

Fig. 6. SDS–PAGE analysis of parasporal inclusion of *B. thuringiensis* subsp. *aizawai* KB098, J9, and J145. M: Standard marker; lane 1: *B. thuringiensis* subsp. *aizawai* KB098; lane 2: *B. thuringiensis* subsp. *aizawai* KB098 digested with larval homogenate for 30 min; lane 3: *B. thuringiensis* J9; lane 4: *B. thuringiensis* J9 digested with larval homogenate for 30 min; lane 5: *B. thuringiensis* J145; lane 6: *B. thuringiensis* J145 digested with larval homogenate for 30 min.

fluid and electrophoresis was performed. As a result, the total toxin at 130 kDa of the three strains were decomposed and toxin bands with insecticidal activities in the Diptera could be identified at about 60–65 kDa. The sizes of δ–endotoxin proteins and the active toxin band patterns after the reaction with *S. exigua* mid–gut fluid were shown to be similar in all of the three strains and it can be expected that strains J9 and J145 are *B. thuringiensis subsp. aizawai* strains (Fig. 6).

Cry–type gene identification

Strains S11 and C55, which showed insecticidal activities in at least two species of Diptera insect pests, were selected and the cry genes of those strains were analyzed. The representative cry genes with insecticidal activities in Diptera were reported as a total of 15 cry genes, which are cry1Aa, cry1Bc, cry2A, cry4Aa, cry4Ba, cry10Aa, cry11Aa, cry11Ba, cry19A, cry20A, cry24C, cry30A, cyt1Aa, cyt2Aa, and cyt2Ba including a total of six possessed by *B. thuringiensis* subsp. *israelensis*, which are cry4Aa, cry4Ba, cry10Aa, cry11Aa, cyt1Aa, cyt2Ba, and cyt2Aa (Van Frankenhuyzen, 2009). To analyze the cry genes possessed by the isolated *B. thuringiensis* strains S11 and C55, the representative 15 cry gene primers with insecticidal activities in Diptera were used to check the presence of each gene.

As a result, a band of 494 bp size appeared in strains S11 and C55 indicating that these strains have cyt2A gene, which was not consistent with other cry genes possessed by *B. thuringiensis* subsp. *israelensis*, and no other gene appeared except for cyt2Aa (Fig. 7A and 7B). This result can be expected that there are unknown cry genes for having activities in Diptera except the tested cry genes (Table 6).

Plasmid DNA analysis

The cry genes of *B. thuringiensis* are involved in the formation of the size, shape, and activity of δ–endotoxin proteins and are present on the plasmid DNA. The sizes and numbers of Plasmid DNAs vary from strain to strain even among strains in the same subspecies so that they can be important data that can be used to obtain information regarding the cry genes that encode endotoxins (McDowell and Mann, 1991, Sarrafzadah *et al.*, 2007, Loeza–Laraa *et al.*, 2005).

The Plasmid DNAs of the selected strains S11 and C55 were extracted and electrophoresed. As a result, strain S11 showed a total six bands, three above

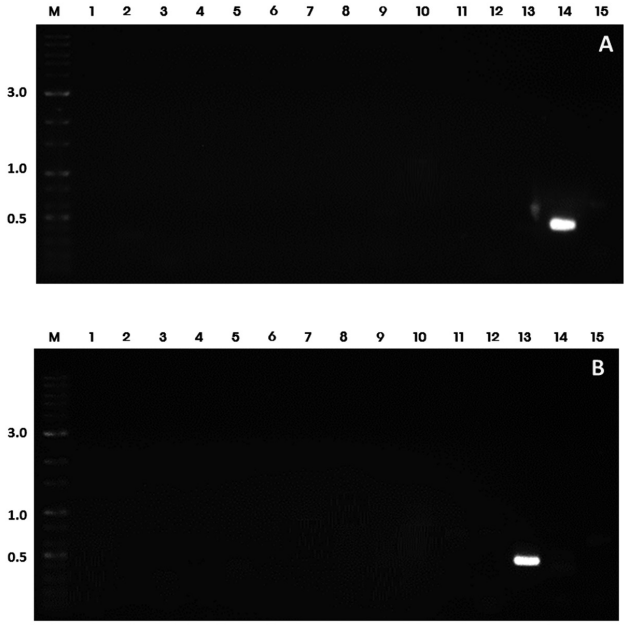

Fig. 7. Agarose gel (1%) electrophoresis of PCR products obtained with specific primers for the cry genes.
A: *B. thuringiensis* S11. M: 100 bp DNA ladder marker; lane 1: cry 1Aa, lane 2: cry 1Bc; lane 3: cry 2A; lane 4: cry 4Aa; lane 5: cry 4Ba; lane 6: cry 10Aa; lane 7: cry 11Aa; lane 8: cry 11Ba; lane 9: cry 19A; lane 10: cry 20A; lane 11: cry 24C, lane 12: cry 30A; lane 13: cyt 1Aa; lane 14: cyt 2Aa; lane 15: cyt 2Ba.
B: *B. thuringiensis* C55. M: 100 bp DNA ladder marker; lane 1: cry 1Aa, lane 2: cry 1Bc; lane 3: cry 2A; lane 4: cry 4A; lane 5: cry 4Ba; lane 6: cry 10Aa; lane 7: cry 11Aa; lane 8: cry 11Ba; lane 9: cry 19A; lane 10: cry 20A; lane 11: cry 24C, lane 12: cry 30A; lane 13: cyt 2Aa; lane 14: cyt 1Aa; lane 15: cyt 2Ba.

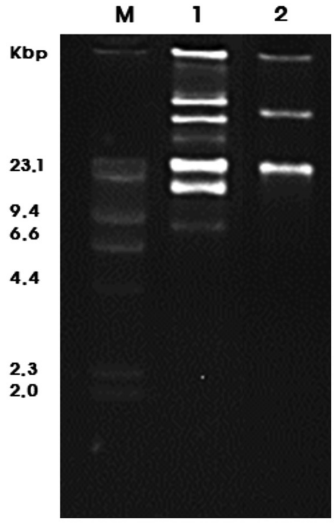

Fig. 8. Plasmid DNA patterns of *B. thuringiensis* isolates. M: Lamda DNA HindIII marker; lane 1: *B. thuringiensis* S11; lane 2: *B. thuringiensis* C55.

Table 6. Profiles of cry genes in *B. thuringiensis* strains

Strains	*cry* genes
B. thuringiensis S11	cyt2Aa
B. thuringiensis C55	cyt2Aa
B. thuringiensis subsp. *israelensis*	cry4Aa, cry4Ba, cry10Aa, cry11Aa, cyt1Aa, cyt2Ba

23.1 Kbp and three below 23.1 Kbp. Strain C55 showed a total of two bands, one above 23.1 Kbp and one below 23.1 Kbp (Fig. 8).

From the findings of this study, it was identified that strains S11 and C55 were different from the comparison strain *B. thuringiensis* subsp. *israelensis* with excellent insecticidal activities in Diptera insect pests in morphological characteristics, δ–endotoxin protein characteristics, and plasmid DNA patterns and differences were identified between *B. thuringiensis* strains S11 and C55 too. Based on the above results, genetic studies on *B. thuringiensis* strains S11and C55 that show insecticidal effects on Diptera and studies on their potential as microbial insecticides should be continued.

AUTHOR CONTRIBUTIONS

Young A Hur designed the study, performed the comprehensive experiments, analyzed the data and wrote the paper. You Kyoung Lee 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 on 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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