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Isolation and Characterization of Strain of *Bacillus thuringiensis* subsp. *kenyae* Containing Two Novel *cry1*-Type Toxin Genes

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Abstract To identify novel crystal proteins, *Bacillus thuringiensis* 2385-1 was isolated from Korean soil samples and characterized. The H-serotype of 2385-1 was identical to that of subsp. *kenyae* (H4a4c), and its crystal toxin was bipyramidal-shaped. However, 2385-1 showed a much higher toxicity towards *Plutella xylostella* and *Spodoptera exigua* larvae than subsp. *kenyae*. In addition, the crystal protein profile and plasmid DNA pattern of 2385-1 differed from those of subsp. *kenyae*. To verify the crystal protein gene types of 2385-1, a PCR-RFLP analysis was performed, and the results revealed that 2385-1 contained two novel *cry1*-type crystal protein genes, *cry1-5* and *cry1-12*, in addition to the *cry1Ja1* gene. The deduced amino acid sequences of *cry1-5* and *cry1-12* showed a 97.9% and 75.7% sequence similarity with the Cry1Ab and Cry1Ja crystal proteins, respectively. Among the novel crystal proteins, Cry1-5 showed a high toxicity towards *P. xylostella* and *S. exigua* larvae. In conclusion, *B. thuringiensis* 2385-1 is a new isolate in terms of its gene types, and should be a promising source for an insecticide to control lepidopteran larvae.

Keywords: *Bacillus thuringiensis* 2385-1, PCR-RFLP, *cry1*-type gene, insecticidal activity, novel crystal protein

Bacillus thuringiensis is an aerobic, Gram-positive, spore-forming soil bacterium that produces proteinaceous parasporal inclusions during sporulation. These parasporal inclusions contain various types of insecticidal crystal protein (ICP). The crystal proteins from *B. thuringiensis* are among the most successful biological control agents for the suppression of agriculturally and medically important insect pests [28], being toxic to the larvae of lepidopteran, dipteran, and coleopteran insects [15], as well as certain hymenoptera, homoptera, and mallophaga, plus many nematodes, flatworms, and Sarcomastigophora [12, 37]. At present, more than 130 *B. thuringiensis* crystal proteins have been described based on their gene sequences and amino acid homologies [6].

B. thuringiensis isolates are distributed worldwide, and more than 60,000 have already been collected [24, 25] by various industries in an effort to obtain novel crystal proteins. In addition, *B. thuringiensis* isolates showing different crystal protein gene patterns from the reference strains have also been reported [23]. However, not many crystal protein genes have been identified that are effective against *Plutella xylostella* and *Spodoptera exigua* larvae. Accordingly, this study isolated and characterized a strain of *B. thuringiensis* subsp. *kenyae* encoding novel crystal protein genes with a high toxicity towards both *P. xylostella* and *S. exigua.*

Materials and Methods

Bacterial Strains and Growth Media

B. thuringiensis colonies were isolated from Korean soil samples using the method of Ohba and Aizawa [27]. The *B. thuringiensis* subsp. *kenyae* strain used as a reference was kindly provided by Dr. Ohba (Institute of Biological Control, Faculty of Agriculture, Kyushu University, Japan). GYS and SPY media were used for the preparation of the parasporal inclusions and analysis of the plasmid DNA, respectively.

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Preparation of H Antisera and H Agglutination

H antisera for 33 serotypes (H1 to H27) of reported *B. thuringiensis* strains were prepared as previously described [27] and the H antisera-antigen agglutinations performed as previously described using 96-well plates [21].

Morphological Observation and Protein Analysis

The parasporal inclusions were purified using the method of Thomas and Ellar [33] with a discontinuous 60%–85% sucrose gradient. The crystal morphology of the isolate was then examined using phase-contrast microscopy and scanning electron microscopy. The sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) was performed using the method of Li *et al.* [23].

Polymerase Chain Reaction-Restriction Fragment Length Polymorphism (PCR-RFLP)

A pair of universal oligonucleotide primers, K5un2 (5'-AGGACCAGGATTTACAGGAGG-3') and K3un2 (5'-GCTGTGACACGAAGGATATAGCCAC-3'), that was able to probe all possible *cry*-type genes was synthesized and used to perform the PCR using the method of Kuo and Chak [20]. After double digestion of the 1.6 kb PCR-amplified fragment with EcoRI/ClaI, EcoRI/PstI, or EcoRI/XbaI, the *cry*-type genes of the corresponding *B. thuringiensis* strains were identified by their RFLP patterns. The PCR amplification was performed using a DNA Thermal Cycler (Perkin Elmer Cetus, NY, U.S.A.).

Cloning and Nucleotide Sequence Analysis

For cloning the *cry1*-type genes, including the N-terminal toxic fragment, the oligonucleotide primers ATG1-F (5'-ATGCAATGCGTACCTTACAATTGTTTAAGTAAT-3') and N400-R (5'-CCTTCCACGCCACTTGGCTTAGCTAC-3') were synthesized. The PCR amplification was performed using Pyrobest DNA Polymerase (TaKaRa Shuzo Co., Ltd., Japan) for 30 PCR thermal cycles (94°C for 1 min, 55°C for 2 min, and 72°C for 2 min). The amplified PCR products were then ligated to a pGEM-T Easy vector (Promega, U.S.A.) and their nucleotide sequences determined by Macrogen Co. (Korea). The phylogenetic analysis of the deduced amino acid sequences was performed using the maximum-parsimony (MP) method [9] incorporated in PAUP* (Phylogenetic Analysis Using Parsimony and Other Methods*) version 4 [31]. The plasmid DNA of the *B. thuringiensis* strains was isolated using a Qiagen midi prep kit (Qiagen Co., Germany) according to the manufacturer's instructions. The standard molecular and biochemical methods used have all been described previously [30]. The restriction enzymes were purchased from Roche Applied Science (Germany) and used according to the instructions from the manufacturer.

Expression of Cry1-type Toxins

To express Cry1-type toxins using a baculovirus expression system, the N-terminal toxic fragments of the *cry1*-type genes amplified using the oligonucleotide primers ATG1-F and N400-R were inserted into the transfer vector pOBI [13] under the control of the polyhedrin gene promoter of the *Autographa californica* nucleopolyhedrovirus (AcNPV). Recombinant AcNPVs expressing each Cry1-type toxin were then constructed by the cotransfection of each transfer vector with bApGOZA DNA into Sf9 cells [14].

Insect Bioassays

The insecticidal activities of *B. thuringiensis* 2385-1 and each Cry1-type toxin were measured using spore-parasporal inclusion suspensions of *B. thuringiensis* and the baculovirus occlusion bodies incorporated with each Cry1-type toxin, respectively. Serial dilutions of the purified samples were prepared in 0.01% (vol/vol) Triton X-100, and 100-µl aliquots of the serial dilutions were applied to the surface of a Chinese cabbage leaf (1×1 cm^2) along with 120 diamondback moth larvae, *P. xylostella* (third-instar larvae), or 120 beet armyworm larvae, *S. exigua* (second-instar larvae). The larval mortality was then recorded over 2 days for the *P. xylostella* assay and 3 days for the *S. exigua* assay. All the tests were conducted at 25°C in 60%–70% humidity with a 16 h:8 h light:dark cycle and repeated three times. The median lethal concentrations (LC_{50}) were calculated using probit analysis [29].

RESULTS

Characteristics and Insecticidal Activities of *B. thuringiensis* 2385-1

A serological study indicated that the H antigenic structure of isolate 2385-1 was identical to that of subsp. *kenyae* (H4a4c). *B. thuringiensis* 2385-1 produced typical bipyramidal

Table 1. Insecticidal activities of *B. thuringiensis* 2385-1 against *P. xylostella* and *S. exigua* larvae.

	B. thuringiensis 2385-1		*B. thuringiensis* subsp. *kenyae*	
	LC_{50} ($\times10^5$ CFU[a]/ml)	95% fiducial limits ($\times10^5$ CFU/ml)	LC_{50} ($\times10^5$ CFU/ml)	95% fiducial limits ($\times10^5$ CFU/ml)
P. xylostella	0.78	0.42–1.12	1.61	0.12–3.38
S. exigua	8.1	5.2–12.6	>100	–

[a]Colony-forming units.

parasporal inclusions, and no significant differences were found in the shapes and sizes of the vegetative cells, spores, and parasporal inclusions between 2385-1 and subsp. *kenyae* (data not shown).

The insecticidal activities of the crystal proteins produced by isolate 2385-1 were evaluated using *P. xylostella* and *S. exigua* larvae and compared with those of subsp. *kenyae* (Table 1). In the bioassay with *P. xylostella* larvae, the LC_{50} value for 2385-1 was two-fold lower than that for subsp. *kenyae*. In addition, 2385-1 showed a high toxicity towards *S. exigua* larvae, whereas subsp. *kenyae* was absolutely nontoxic to *S. exigua* larvae.

Crystal Protein and Plasmid Profiles of *B. thuringiensis* 2385-1

An SDS-PAGE analysis of the parasporal inclusion proteins revealed that the crystal protein profile of 2385-1 was different from that of subsp. *kenyae* (Fig. 1). Whereas only one major band with a molecular mass of about 130 kDa was present in the crystal protein profile of subsp. *kenyae*, two major bands with molecular masses of about 130 kDa and 150 kDa were present in the profile of 2385-1. In addition, when treated with the gut juice of *Bombyx mori*, the crystal proteins from 2385-1 were digested to about 50 kDa and 65 kDa, whereas the crystal proteins from subsp. *kenyae* were only digested to about 65 kDa. Furthermore, the plasmid DNA pattern for 2385-1 differed from that for subsp. *kenyae* (data not shown).

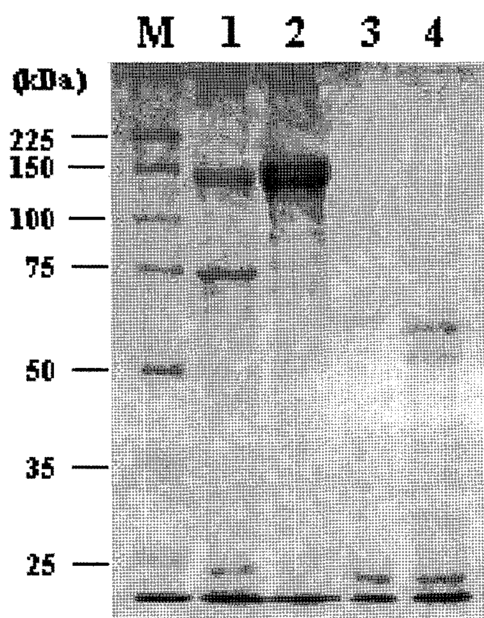

Fig. 1. SDS-PAGE analysis of parasporal inclusions of *B. thuringiensis* isolate 2385-1.
Lanes: M, molecular mass marker (Novagen, U.S.A.); 1, *B. thuringiensis* subsp. *kenyae*; 2, *B. thuringiensis* 2385-1; 3, *B. thuringiensis* subsp. *kenyae* treated with gut juice of *Bombyx mori*; 4, *B. thuringiensis* 2385-1 treated with gut juice of *Bombyx mori*.

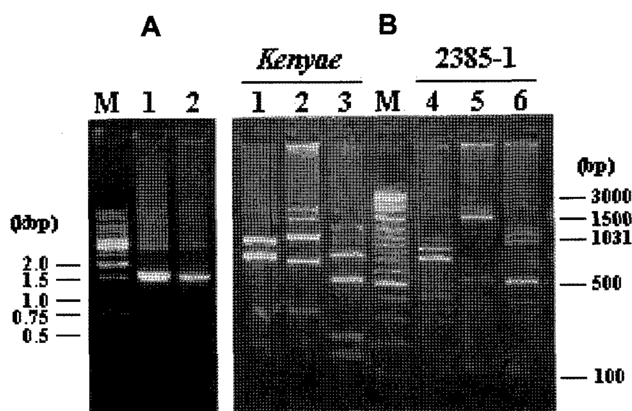

Fig. 2. PCR amplification with primers K5un2 and K3un2 (**A**) and RFLP pattern of PCR-amplified *cry1*-type genes (**B**) from *B. thuringiensis*.
A. Lanes: M, 1 kb DNA ladder; 1, *B. thuringiensis* subsp. *kenyae*; 2, *B. thuringiensis* 2385-1. **B.** Lanes: M, 100 bp DNA ladder (Fermentas, U.S.A.); 1 and 4, EcoRI and ClaI; 2 and 5, EcoRI and PstI; 3 and 6, EcoRI and XbaI; *Kenyae*, *B. thuringiensis* subsp. *kenyae*; 2385-1, *B. thuringiensis* 2385-1.

Crystal Protein Gene Profile of *B. thuringiensis* 2385-1

To study the crystal protein gene profile of isolate 2385-1, a PCR-RFLP analysis was conducted using the primers K5un2 and K3un2 (Fig. 2). The RFLP pattern for 2385-1 was completely different from that for the reference strain subsp. *kenyae* and did not a match any previously reported crystal protein genes.

To investigate the *cry1*-type genes contained in 2385-1, crystal protein gene fragments of about 2.4 kb, including the N-terminal toxic region, were PCR-amplified using the primers ATG1-F and N400-R. The amplified PCR products were then cloned into a pGEM-T Easy vector, resulting in

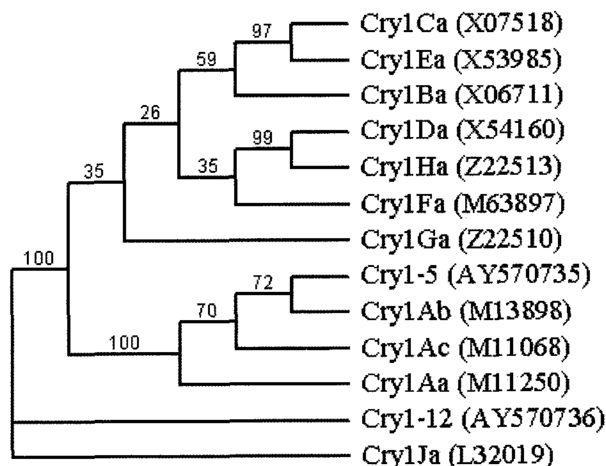

Fig. 3. Phylogenetic relationships among amino acid sequences of novel and previously reported crystal proteins.
The GenBank accession number for each crystal protein gene is shown within brackets. The MP tree was obtained using a heuristic search. The numbers on the branches represent bootstrap values for 1,000 replicates.

Table 2. Insecticidal activities of Cry1-5 and Cry1-12 crystal proteins against *P. xylostella* and *S. exigua* larvae.

	P. xylostella		*S. exigua*	
	LC_{50} (ng/ml)	95% fiducial limits (ng/ml)	LC_{50} (ng/ml)	95% fiducial limits (ng/ml)
Cry1Ac	20.8	10.5–39.4	> 2,000	–
Cry1C	204.6	68.9–1,560.6	454.4	311.2–702.1
Cry1Ja	>500	–	> 2,000	–
Cry1-5	5.2	2.6–9.2	480.5	361.0–646.5
Cry1-12	134.5	65.8–345.1	> 2,000	–

three distinct PCR product clones. Nucleotide sequence determination of the three clones revealed that one was the *cry1Ja* gene (GenBank Accession No. L32019). However, the other two clones did not match any previously reported crystal protein genes, and thus were named *cry1-5* (GenBank Accession No. AY570735) and *cry1-12* (GenBank Accession No. AY570736). Notwithstanding, the deduced amino acid sequences of *cry1-5* and *cry1-12* showed a 97.9% and 75.7% sequence similarity with the Cry1Ab and Cry1Ja crystal proteins, respectively (Fig. 3).

Insecticidal Activities of Novel Cry1-type Toxins from *B. thuringiensis* 2385-1

To investigate the insecticidal activities of the novel toxins, Cry1-5, Cry1-12, Cry1Ac, and Cry1C were all expressed using the recombinant polyhedra technique [5]. According to this method, each crystal protein was expressed as a fusion protein with the polyhedrin of AcNPV, and the fusion protein occluded into the polyhedra of AcNPV. The recombinant polyhedra containing each crystal protein were then used to compare the insecticidal activities of Cry1-5 and Cry1-12 with those of Cry1Ac and Cry1C towards *P. xylostella* and *S. exigua* larvae (Table 2). In the bioassay with the *P. xylostella* larvae, the LC_{50} value for Cry1-5 was three-fold lower than that for Cry1Ac. Additionally, Cry1-5 showed a higher toxicity than Cry1C towards the *S. exigua* larvae. However, Cry1-12 showed a very low toxicity to both the *P. xylostella* and *S. exigua* larvae.

DISCUSSION

To date, about 60,000 *B. thuringiensis* isolates lethal to lepidopterans, dipterans, coleopterans, and nematodes have been maintained in culture collections throughout the world [3]. Although *B. thuringiensis* subsp. *aizawai*, subsp. *entomocidus*, subsp. *kenyae*, and subsp. *galleriae*, which contain the *cry1C*, *cry1D*, *cry1E*, or *cry1F* genes, have been reported as significantly active against *Spodoptera* species, very few are toxic to the diamondback moth *P. xylostella* [4, 26, 34, 35]. However, the *B. thuringiensis* 2385-1 isolated in this study showed a high toxicity towards both *S. exigua* and *P. xylostella*, suggesting that this isolate could be developed as a novel microbial insecticide.

B. thuringiensis 2385-1 was found to belong to subsp. *kenyae* as a result of H agglutination, yet the insecticidal activities and crystal gene profiles of 2385-1 were absolutely different from those of subsp. *kenyae*. The reference strain, *B. thuringiensis* subsp. *kenyae*, has been reported to contain the *cry1Ab*, *cry1Ac*, and *cry1E* genes, which are not active or only minimally active against *S. exigua* larvae [3, 26, 34, 36]. In contrast, *B. thuringiensis* 2385-1 was found to contain the *cry1Ja* gene and two novel genes, which were named *cry1-5* and *cry1-12*.

Since *cry1Ja* has already been reported to have little or no toxicity towards *P. xylostella* and *S. exigua* larvae [11, 32], the high toxicity exhibited by isolate 2385-1 was expected to be due to the novel crystal protein genes, and a bioassay using each crystal proteins separately revealed that Cry1-5 was responsible for the high toxicity of isolate 2385-1 against both the *P. xylostella* and *S. exigua* larvae. Consequently, these results suggest that the *cry1-5* gene could be useful in the construction of transgenic plants and development of microbial insecticides based on recombinant *B. thuringiensis*.

Cry1-5 showed a high level of similarity to Cry1Ab, which is active against *P. xylostella* yet shows very low activity against *S. exigua* [8]. Previously, Haider and Ellar [10] reported on a natural mutant Cry1Ab protein in *B. thuringiensis* subsp. *aizawai* isolate IC1, which only differs from the holotype in three amino acid residues, yet unlike the holotype, Cry1Ab protein shows a dual specificity against lepidopteran and dipteran insects. Crystal proteins consist of three structural domains: domain I, which is involved in pore formation; domain II, which contains the primary determinants that specify the receptor binding on the midgut brush border; and domain III, which is implicated in toxin stability and binding specificity [2, 22]. Toxins sharing a high homology in the loops of domain II often share a midgut binding site [1, 7], which also suggests that mutation in the domain II region could affect the receptor binding affinity, resulting in an alteration of the insecticidal activity of the toxin. Cry1-5 differed from Cry1Ab in eleven amino acid residues within the N-terminal toxic region, thereby implying that these eleven amino acid residues may have affected the expansion of the host spectrum of this toxin. However, further investigation, such as site-directed mutagenesis, is needed to verify the

amino acid residues responsible for the dual specificity of the Cry1-5 protein.

Over the last few years, several PCR-based methodologies, including a PCR using specific primers, multiplex PCR, and PCR-RFLP, have been proposed for the detection of novel *cry* genes from *B. thuringiensis* isolates [16, 17, 20]. Nonetheless, since the PCR-amplified fragments resulting from these methods did not correspond to the N-terminal toxic region, a pair of universal oligonucleotide primers, ATG1-F and N400-R, were designed to amplify the N-terminal toxic fragments of both known and novel *cry1*-type genes. As a result, the pair of primers enabled effective screening of the active regions of *cry1*-type genes from massive plasmid sources of various *B. thuringiensis* isolates. Furthermore, the fragments amplified using these primers could be directly applied to investigate the insecticidal activity by introducing the amplified fragments to a suitable expression system. Previously, the present authors developed a recombinant polyhedra system in which a foreign protein is incorporated into the occlusion body of a baculovirus [13, 18, 19]. When a crystal protein of *B. thuringiensis* is expressed using this system, the insecticidal protein is then delivered to the gut of the insect by simple *per os* administration of the recombinant polyhedra [5]. Moreover, the insecticidal activities of different crystal proteins can also be compared using the recombinant polyhedra system, as the quantity of the target protein incorporated into the polyhedra can be estimated. Therefore, a comparison of the insecticidal activities of *cry1*-type genes corresponding to the N-terminal toxic domain using recombinant polyhedra is an additional novel approach for the screening of effective insecticidal genes.

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