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Characterization of *Bacillus thuringiensis* Having Insecticidal Effects Against Larvae of *Musca domestica*

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Abstract The entomopathogenic bacterium *Bacillus thuringiensis* is the most widely used biopesticide. Insecticidal proteins, coded by genes located in plasmids, form typical parasporal, crystalline inclusions during sporulation. We isolated a *Bacillus thuringiensis* strain having insecticidal activity against larvae of the house fly (*M. domestica*) from the soils at a pig farm in Korea, and named it *Bacillus thuringiensis* SM. The culture filtrate from *Bacillus thuringiensis* SM showed strong lethality (83.3%) against *M. domestica* larvae. The parasporal crystal is enclosed within the spores' outermost envelope, as determined by transmission electron microscopy, and exhibited a bipyramidal form. The crystal proteins of strain SM consisted of five proteins with molecular weights of approximately ~130, ~80, ~68, ~42, and ~27 kDa on a 10% SDS-PAGE (major band, a size characteristic of Cry protein). Examination of antibiotic resistance revealed that the strain SM showed multiple resistant. The strain SM had at least three different plasmids with sizes of 6.6, 9.3, and 54 kb. Polymerase chain reactions (PCRs) revealed the presence of *cry1*, *cry4A2*, and *cry11A1* genes in the strain SM. The *cry1* gene profile of the strain SM appeared in the three respective products of 487 bp [*cry1A*(c)], 414 bp [*cry1D*], and 238 bp [*cry1A*(b)]. However, the strain SM has not shown the *cry4A2* and *cry11A1* genes. In *in vivo* toxicity assays, the strain SM showed high toxicity on fly larvae (*M. domestic*) [with LC_{50} of 4.2 mg/ml, LC_{90} of 8.2 mg/ml].

Key words: *Bacillus thuringiensis*, *M. domestica* larvae, crystal protein

The major characteristic of the Gram-positive, spore-forming bacterium *Bacillus thuringiensis* is the product of insecticidal crystal proteins during sporulation. The crystals kill certain insect larvae; therefore, the crystals and microorganisms are important for the development of microbial insecticidal pesticides [5]. Depending on their δ-endotoxin composition, the crystals have various forms, and a particular correlation between the structure and protein composition of the crystals has been established [22]. The morphology, size, and number of parasporal inclusions vary among different *B. thuringiensis* strains. However, five distinct morphologies are apparent: the typical bipyramidal crystals are related to Cry1 proteins [1]; cuboidal inclusions are related to Cry2 proteins and usually associated with bipyramidal crystals [32]; square, flat crystals are related to Cry3 proteins and Cyt proteins [16]; composite and amorphous crystals are related to Cry4 [14]; and bar-shaped inclusions are related to the Cry4D protein [18]. de Barjac and Bonnefoi [11] showed that the strain *Bacillus thuringiensis* can be distinguished from serotype based on their flagella (H) antigens. Thereafter, about 51 serotypes of *Bacillus thuringiensis* were reported [13].

Meadow [26] suggested that *Bacillus thuringiensis* would normally not germinate in soil but would wait for favorable conditions for growth to occur. Considering that insecticidal proteins are highly susceptible to UV light, and that crystals are deactivated within a few days under natural conditions, there is very little chance that a susceptible insect ingests enough active crystals, accompanied by spores, to cause its death. Nevertheless, the insecticidal activity of many proteins produced by *Bacillus thuringiensis* indicates that there is a coevolution of the spores, crystal proteins, and insects.

In this present work, we isolated a *Bacillus thuringiensis* strain, which shows biological activity against *M. domestica* (larvae), and we named the strain SM. We tested crystals purified from the strain SM, which showed high activity against *M. domestica* (larvae), and performed a preliminary characterization of the SM strain using a biochemical test, electron microscopy, antibiotic susceptibility, plasmid profile, multiplex PCR, and *in vivo* toxicity assays.

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Bacterial Strains and Media

The SM strain was isolated from the soils at a pig farm in Korea. Two other bacterial strains, *Bacillus thuringiensis* subsp. *kurstaki* HD-1 and *Bacillus thuringiensis* subsp. *israelensis* HD-522, were used. These two bacterial strains were obtained from KCTC (Korean Collection for Type Cultures). All strains were cultured in nutrient broth medium (NB medium, 0.3% beef extract, 0.5% peptone) at 30°C in a rotary shaker for plasmid isolation, MIC test of antibiotics, and multiplex PCR. GYS medium (2 g yeast extract, 1 g glucose, 2 g $(NH_4)_2SO_4$, 0.5 g K_2HPO_4, 0.41 g $MgSO_4$-$7H_2O$, 0.0008 g $CaCl_2$, 0.0007 g $MnSO_4$-$5H_2O$ in 1 l dH_2O) was used for crystal formation and insect bioassay.

Isolation and Selection of *Bacillus thuringiensis* Strain

Bacillus thuringiensis strains were isolated as described by Travers *et al.* [30] with some modifications. All the colonies with the morphological characteristics similar to those of known *Bacillus thuringiensis* were identified and examined by phase contrast microscopy for the presence of spores and crystals. The presence of crystals in the cells was taken as presumptive evidence that the culture was *Bacillus thuringiensis*. Isolated strains were subcultured onto nutrient agar and tested for further identification.

M. domestica (larvae) were obtained from a pig farm near Chungju city (Korea). One or two colonies of *Bacillus thuringiensis* were inoculated in 10 ml of NB and then cultured at 37°C at 200 rpm overnight. One ml of the culture was transferred into 100 ml of GYS medium and cultured again at 30°C for 3 days (10^7 to 10^8 spores/ml). After harvesting the culture at 10,000 ×*g* for 20 min at 4°C, the supernatant was decanted and the pellet was washed three times with sterilized saline by centrifugation at 10,000 ×*g* for 20 min. The pellet was diluted with sterilized saline at dilutions of 1×10^{-1} and 1×10^{-2}. Then, 3 ml of the diluted sample and supernatant was added to the surface of the pig feces (3 g) in a petri dish covered with a filter paper. Ten fly larvae, the third-instar larvae, were placed on the pig feces in a petri dish. Larval mortality was recorded at room temperature (25±2°C) with a constant humidity of 40–60% for 72 h using 10^5 to 10^6 spores/ml. Bioassay was performed in triplicates.

Morphological and Biochemical Characteristics of *Bacillus thuringiensis* SM

Bioassays were performed with instar *M. domestica* larvae using spore-crystal mixtures from the five *Bacillus thuringiensis* isolates. Among five strains, the supernatant of one strain showed a strong lethality of 83.3% against *M. domestica* (larvae). Pellet suspension of the strain showed a lethality of 65% against *M. domestica* (larvae). We named the strain SM. Other strains showed a weak lethality against *M. domestica* (larvae) or did not show lethality at all. The ultrastructure of the crystals from the SM strain was obtained by transmission electron microscopy. As shown in Fig. 1, bacterial cells have a bastonete shape containing the exosporium and a crystal. The crystal shape produced by the strain had a typical bipyramidal form (Fig. 1A). The biochemical characteristics of strain SM were examined. The biochemical characteristics revealed that the isolate had general properties similar to the already known serotypes of *Bacillus thuringiensis* strains [11, 12, 21, 28].

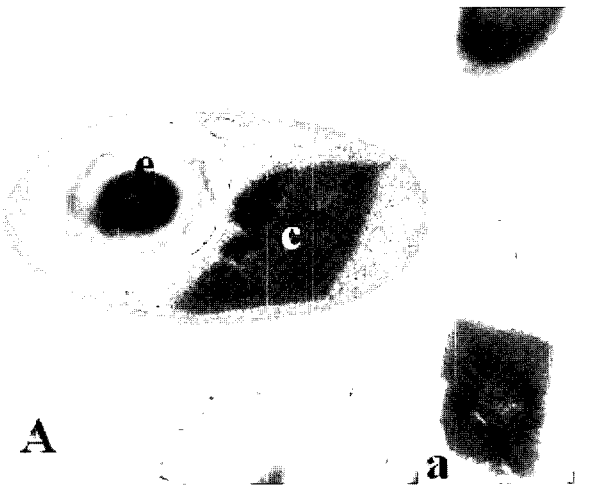

Fig. 1. Transmission electron micrographs of sporulating of *Bacillus thuringiensis* isolate SM, amplified 25,000× (A). Parasporal crystal (c) and spore (e) are enclosed within the exosporium as indicated. The inset (a) shows further details of the crystals.

Antibiotics Susceptibility of *Bacillus thuringiensis* SM

The test was performed with the use of serial 2-fold dilutions of each antibiotic as described by Cleeland and Grunberg [6]. The resistance of *Bacillus thuringiensis* SM against antibiotics was examined after cultivation for 18 h at 37°C. The antibiotic susceptibilities of the SM strain were different from those of already known serotypes of

Table 1. Determination of minimum inhibitory concentration of various antibiotics against *B. thuringiensis* strain SM, *B. thuringiensis* subsp. *kurstaki* (HD-1), and *B. thuringiensis* subsp. *israelensis* (HD-522).

Antibiotics	MICs (μg/ml)		
	Strain SM	*Kurstaki* (HD-1)	*Israelensis* (HD-522)
Ampicillin	62.5	100	100
Kanamycin	<1.95	12.5	3.9
Oxacillin	62.5	250	7.8
Colistin	31.3	125	62.5
Tetracycline	7.9	6.25	3.9
Penicillin G	125	>1000	>1000
Erythromycin	250	<1.95	<1.95
Neomycin	7.9	3.9	3.9

Bacillus thuringiensis strains [19]. As shown in Table 1, the strain showed higher sensitivity to kanamycin, tetracycline, and neomycin. In the case of ampicillin, oxacillin, colisin, penicillin G, and erythromycin, the strain SM exhibited higher resistance. The strain SM had shown multiple resistance to many antibiotics.

Parasporal Crystal Protein Composition

Purification of crystals was carried out by discontinuous sodium bromide (NaBr) gradients of 30 to 70%. The major proteins in the crystals were characterized with respect to size by SDS-PAGE. When the protein composition was analyzed by SDS-PAGE, crystals of the SM strain showed bands with molecular masses ~130, ~80, ~68, ~42, and ~27 kDa (Fig. 2, lane 2). Their estimated molecular masses (~130, ~80, ~68, ~42, and ~27 kDa) corresponded to those of typical Cry proteins [19, 27, 28]. The crystal from *kurstaki* HD-1 contained two crystal proteins with apparent masses of ~130 and 65 kDa (Fig. 2. lane 1) [33]. Yamato and Mclaughlin [32] reported that the mosquitocidal activity of *kurstaki* HD-1 is attributable to the 65 kDa protein. But the crystal protein of *israelensis* HD-522 showed biological activity against horn fly larvae [29]. *Israelensis* HD-522 contained four crystal proteins with apparent masses of ~130, ~68, ~42, and ~30 kDa (Fig. 2, lane 3). The molecular mass ~130 kDa protein corresponded to those of typical Cry4B proteins, and their masses of ~68 and ~42 kDa corresponded in size to Cry11A [6, 26]. Cry4 and Cry11 proteins also have biological activity against horn fly larvae. Also, these proteins showed comigration with the strain SM (Fig. 2, lanes 2, 3). The strain SM has a specific protein of ~27 kDa but this was not observed in other *Bacillus thuringiensis* strains. The molecular mass of the

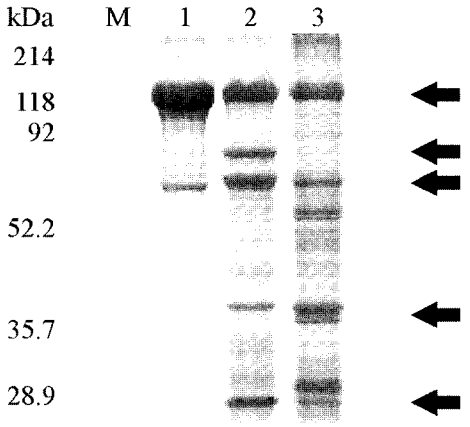

Fig. 2. Analysis of purified crystal proteins of *Bacillus thuringiensis* subsp. *kurstaki* (HD-1) (lane 1), strain SM (lane 2), and *israelensis* (HD-522) (lane 3).
M, molecular marker; arrows indicate Cry proteins of ~130, ~80, ~68, ~42, and ~27 kDa.

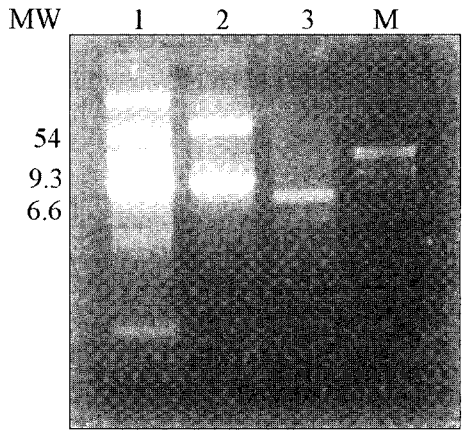

Fig. 3. Plasmid patterns of *Bacillus thuringiensis* subsp. *kurstaki* (HD-1) (lane 1), strain SM (lane 2), and *Bacillus thuringiensis* subsp. *israelensis* (HD-522) (lane 3).
MW, estimated molecular size (in kilobases) of covalently closed circles of SM. **M**, Three fragments of λDNA digested by *Hin*dIII (23.1 kb, 9.4 kb, and 6.5 kb).

~27 kDa protein corresponds to that of typical Cyt1Aa proteins [4, 8]. Cyt1Aa proteins have activity against Diptera [9]. Therefore, it is considered that the strain SM may be a different strain from the other known strains, *Bacillus thuringiensis* subsp. *kurstaki* HD-1 and *israelensis* HD-522. Based on these findings, it appears that the ~130, 80, 68, 42, and ~27 kDa proteins from the strain SM may be responsible for the significant toxicity to *M. domestica* (larvae).

Analysis of Plasmid DNA Profile

Analysis of plasmid DNA profile is one of many identification methods for *Bacillus thuringiensis*. Plasmid DNA in *Bacillus thuringiensis* strains were isolated by the protoplastalkaline lysis (PAL) procedure as described by Voskuil and Chambliss [31] except that the bacterial pellet was incubated with 10 mg/ml of lysozyme at 37°C for 1 h. Plasmid DNA patterns of the strain SM, *Bacillus thuringiensis* subsp. *kurstaki* HD-1, and *Bacillus thuringiensis* subsp. *israelensis* HD-522 were compared on a 0.7% agarose gel electrophoresis in TBE buffer. Plasmid DNA patterns of *Bacillus thuringiensis* strains may vary from strain to strain. All of known *Bacillus thuringiensis* strains harbor a set of plasmids, ranging from 2 to 17 in number [15]. Their variations in pattern are representatively divergent. Likewise, the strain SM contains an unusual set of plasmids, small and large. Figure 4 shows the typical plasmid pattern of the strain SM compared with the control strains *kurstaki* HD-1 and *israelensis* HD-522. The strain SM had 3 commonly different plasmid DNA elements in sizes of 6.6, 9.3, and 54 kb. One of them can be considered a mega plasmid, which is similar to the plasmids of *kurstaki* HD-1. In addition, the plasmid DNA pattern of the new isolate was distinguishable from those of known serotypes in size and number [24].

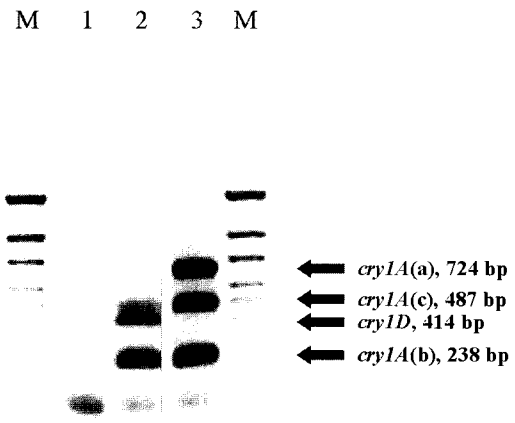

Fig. 4. PCR analysis of *Bacillus thuringiensis* subsp. *israelensis* HD-522 (lane 1), *Bacillus thuringiensis* SM (lane 2), and *Bacillus thuringiensis* subsp. *kurstaki* HD-1 (lane 3) for *cry1* contents.
Total DNA samples from the three *Bacillus thuringiensis* strains were analyzed by PCR with a mixture of *cry1* specific primers. **M:** bio basic 100 bp- 1 kb ladder.

Multiplex PCR for Rapid Determination of *cry1*, *cry4*, and *cry11* Gene Profiles

The 10 primers contained eight forward primers and two reverse primers for *cry1* gene determination as used in this study. The respective sequences were published by Kalman *et al.* [20]. To identify *cry4* and *cry11*, two forward and two reverse primers for each *cry4*, *cry11* (selected from their highly variable regions) were used together in one reaction. Two specific primers were designed for *cry4A2* and *cry11A1*. The sequences and match positions of specific primers and expected sizes of their PCR products are displayed in the paper published by Ben-Dov *et al.* [3]. They were kindly synthesized by COSMO GeneTech Co., Korea. The eight forward primers are specific to the eight *cry1* genes indicated below. One of the reverse primers is amplified to *cry1A(b)*. Another reverse primer is the universal primer for the other seven *cry1* genes. The two forward primers and two reverse primers are amplified to *cry4A2* and *cry11A1*. Amplification was carried out in a Takara PCR thermal cycler for 30 reaction cycles (1 min of denaturation at 95°C, 2 min of annealing at 52°C, 3 min of extension at 72°C) with 2 min of predenaturation at 95°C, and 7 min of extra extension at 72°C. PCR products were analyzed by a 1% agarose gel electrophoresis in TBE buffer.

The extended multiplex PCR screening is a rapid method for detecting and differentiating (by their PCR product profiles) *Bacillus thuringiensis* field strains and for predicting their insecticidal activities in order to direct them for subsequent toxicity assays against Lepidoptera, Coleoptera, and Diptera. This method enriches existing PCR strategies for screening the most currently known *cry* genes by improving and developing the expanded PCR set of specific primers. The amplified DNA products by multiplex PCR were separated clearly and distinct bands appeared in the agarose gel, confirming the specificity of the primers. One set of eight primer pairs was used to detect, in one reaction, eight published *cry1*-type genes, from *cry1A*(a) to *cry1F* [20]. Two genes, *cry4* and *cry11*, which encode Diptera-specific polypeptides and are located on the same plasmid in *Bacillus thuringiensis* subsp. *israelensis* [2], were identified together by one set of specific primers [3]. These two specific primers were designed for *cry4A2* and *cry11A1* [3]. PCR with these primers and total DNA template generated a uniquely sized fragment for each *cry1* type gene, *cry4A2* and *cry11A1*. Therefore, the sizes of PCR products indicated the presence of particular crystal protein genes. Bourque *et al.* [4] claimed that multiplex PCR can be used to monitor the presence of specific commercial strains in the field after application. We applied this method in order to compare the *cry1*, *cry4A2*, and *cry11A1* gene profiles of the strain SM with the reference strains. HD-1 and HD-522 are the reference strains used in this study. HD-1, which is known to contain all three *cry1A* genes, [17, 25] produced the expected profile containing the three respective products of 724 bp, 238 bp, and 487 bp (Fig. 3, lane 3). HD-522 did not show *cry1* genes by multiplex PCR (Fig. 3, lane 1). The *cry1* gene profile of the strain SM appeared in the three respective products of 487 bp [*cry1A*(c)], 414 bp [*cry1D*], and 238 bp [*cry1A*(b)] (Fig. 3, lane 2). HD-1 did not show *cry4A2* and *cry11A1* genes (data not shown). HD-522, which is known to contain *cry4A2* and *cry11A1* genes [3], produced the expected profile containing the two respective products of 1,529 bp [*cry4A2*] and 445 bp [*cry11A1*] (data not shown). The *cry4A2* and *cry11A1* genes of the strain SM did not appear on 1% agarose gel (data not shown). These results indicate that the strain SM had specific genetic characteristics different from the already known *cry1*, *cry4A2*, and *cry11A1* gene profiles of *Bacillus thuringiensis* [4, 25].

In Vivo Toxicity Assays

The *Bacillus thuringiensis* strain SM was grown in sporulation medium at 48°C for 72 h, and spore-crystal mixtures were prepared as described above and lyophilized. Lyophilized spore-crystal mixtures were diluted to give a series of concentrations in water. Test suspensions were assayed in duplicate against 10 third- to fourth-instar larvae of *M. domestica* in plastic cups. All tests were conducted with a final volume of 100 ml. Control experiments were performed to test the viability of the larvae in water. *In vivo* assays against *M. domestica* larvae from Korea were conducted with laboratory stock spore-crystal mixtures as previously described [26]. Mortality was scored after 48 h at 28°C. Each sample was assayed at least four times, and the concentrations of crystal proteins giving 50% mortality

(LC_{50}) and 90% mortality (LC_{90}) were determined. The strain SM was toxic to house fly larvae of *M. domestica* (LC_{50} 4.2 mg/ml, LC_{90} 8.2 mg/ml). Jung *et al.* [19] have reported that the toxins of *kurstaki* HD-1 have high activity against Lepidoptera, but these toxins are not active against Diptera. The toxins of *Israelensis* HD-522 have shown activity against a variety of different species of Diptera [29]. These toxins are Cry4 and Cry11 proteins with high activity against fly larvae. Cry4 and Cry11 proteins are typically crystal proteins with a molecular mass of 134 kDa [Cry4B], 125 kDa [Cry4A], and 66 kDa [Cry11A] [7, 26]. The corresponding strain of SM does not have Cry4A2 and Cry11A. Although the strain SM does not have Cry4A2 and Cry11A1, toxins of the strain SM were significantly toxic to the larvae of flies (*M. domestica*). These findings suggest that the SM strain contains other crystal proteins in addition to Cry1 protein. Ben-Dov *et al.* have reported that Cry2Aa and Cry2Ab toxins are very toxic against Diptera [3], and Cyt1Aa has shown high activity against Diptera [9]. It appears to be a strong possibility that the strain SM possesses the Cyt1A protein because it has a molecular mass of ~27 kDa Cry protein (Fig. 2, lane 3) which is typical of the mass of Cyt1 proteins. Further research therefore needs to be carried out to characterize the Cry proteins of the strain SM.

The characterization of the strain SM has demonstrated its uniqueness among other *Bacillus thuringiensis* strains. Generally, *Bacillus thuringiensis*, which has a *cry1* gene and Cry1 protein, shows biological activity against lepidopterans. *Bacillus thuringiensis kurstaki* HD-1, which acts specifically against lepidopterans, is a good example of this [29]. Most Cry1 proteins are active against Lepidoptera, but some Cry1 proteins [Cry1A(b), Cry1B(a), and Cry1C(a)] are active against Diptera [10]. The *Bacillus thuringiensis* strains having the *cry4* and *cry11* genes show specific activity against fly larvae [3, 23]. Lopez and Hofte *et al.* [17, 23] have reported that the *Bacillus thuringiensis* strains having the *cry4A*, *cry11*, and *cytA* genes show specific activity against fly larvae. The strain SM does not have the *cry4* and *cry11* genes. Nevertheless, the strain SM has a specific biological activity against fly larvae (*M. domestica*). The strain SM has the *cry1* gene [*cry1A*(b), *cry1D*, *cry1A*(c)], Cry1 protein, and other Cry proteins. Furthermore, the plasmid profile and *cry1*, the gene type of the strain SM, are different from other reported cases of *Bacillus thuringiensis,* which show biological activity against fly larvae. According to these results, the strain SM is a new strain which has the *cry1* gene other *cry* genes, and specific activity against fly larvae (*M. domestica*). This study shows that the strain SM can be utilized as a biopesticide against fly larvae. Livestock farming fly control is the most important problem for farmers. The availability of a biological agent to use against this insect could solve many of the toxicological environmental problems caused by insecticides. This study indicates that it is possible to find natural strains of *Bacillus thuringiensis* which are toxic to fly larvae and which could contain novel insecticidal proteins.

Acknowledgments

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