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Distribution of Toxin Genes and Enterotoxins in *Bacillus thuringiensis* Isolated from Microbial Insecticide Products

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Bacillus thuringiensis microbial insecticide products have been applied worldwide. Although a few cases of *B. thuringiensis* foodborne illness have been reported, little is known about the toxigenic properties of *B. thuringiensis* isolates. The aims of this study were to estimate the pathogenic potential of *B. thuringiensis* selected from microbial insecticide products, based on its possession of toxin genes and production of enterotoxins. Fifty-two *B. thuringiensis* strains selected from four kinds of microbial insecticide products were analyzed. PCR assay for detection of toxin genes and immunoassay for detection of enterotoxins were performed. The hemolysin BL complex as a major enterotoxin was produced by 17 (32.7%), whereas the non-hemolytic enterotoxin complex was detected in 1 (1.9%) of 52 *B. thuringiensis* strains. However, *cytK*, *entFM*, and *ces* genes were not detected in any of the tested *B. thuringiensis* strains. The potential risk of food poisoning by *B. thuringiensis* along with concerns over *B. thuringiensis* microbial insecticide products has gained attention recently. Thus, microbial insecticide products based on *B. thuringiensis* should be carefully controlled.

Keywords: *Bacillus thuringiensis*, microbial insecticide, enterotoxin, toxin gene

Introduction

Bacillus cereus has been recognized as the causative agent of diarrheal and emetic food poisoning associated with various categories of foods [5]. Food poisoning is induced by enterotoxins and emetic toxins such as hemolysin BL (HBL), non-hemolytic enterotoxin (NHE), enterotoxin FM (EntFM), cytotoxin K (CytK), and cereulide (emetic toxin) [34]. Concerns over *B. cereus* food poisoning are growing in the food industry [24]. *Bacillus thuringiensis* is commonly isolated from the environment, such as water, soil, insects, vegetables, and foods [1, 3, 12, 29, 39], and has been widely applied worldwide as a microbial insecticide to reduce amounts of chemical pesticides [22] based on its advantages, such as no harmful effects on humans or ecological environment, low impact on non-target organisms, and a narrow spectrum of lepidopteran targets [28].

B. cereus and *B. thuringiensis* cannot be discriminated by using genetic and phenotypic assays [25, 32], as they share high 16S rRNA gene sequence similarity (>99%) [31]. These reports suggest that these two strains are one species or at least originate from a common ancestor [21]. The distinguished characteristics between *B. cereus* and *B. thuringiensis* are the presence or absence of plasmid-encoded virulence properties, such as crystal proteins (δ-endotoxin) encoded by *cry* genes, which show insecticidal activity in *B. thuringiensis* [32]. Thus, an enumeration method for *B. cereus*, except *B. thuringiensis* cell counts, was established in the Korea Food Code.

On the other hand, *cry* genes encoded in the plasmid

show high potential for horizontal gene transfer between *B. thuringiensis* and *B. cereus* [20, 41]. *B. thuringiensis*, which lacks the *cry* gene, is indistinguishable from *B. cereus* [18]. These reports indicate that *B. thuringiensis* might be a food-poisoning bacterium, as a few cases of food poisoning caused by *B. thuringiensis* have been reported [19, 37]. Thus, it is necessary to evaluate the toxigenic potential of *B. thuringiensis* applied as microbial insecticides, even though little is known about the toxigenic properties of *B. thuringiensis* microbial insecticide products.

The objectives of this study were to identify *B. thuringiensis* selected from microbial insecticide products and to estimate its pathogenic potential based on possession of toxin genes and production of enterotoxins.

Materials and Methods

Samples

Four kinds of microbial insecticide products based on *B. thuringiensis* were purchased from Gyeonggi-do and the Internet from 2011 to 2013. Each microbial insecticide product was named as A, B, C, and D, respectively.

Biochemical Identification of *B. thuringiensis*

Each microbial insecticide product (1 g or 1 ml) was mixed with 10 ml of buffered peptone solution (Oxoid Ltd., Basingstoke, UK) and homogenized by a Vortex-Genie 2 mixer (Scientific Industries Inc., Bohemia, NY, USA) for 1 min. After vortexing, 1 ml of homogenate was serially diluted (10-fold) in 0.85% saline, and 100 µl of each diluent was spread onto Mannitol-Egg Yolk-Polymyxin agar (MYP; Difco, Detroit, MI, USA) and incubated at 30°C for 20 h. Thirteen colonies showing a pink color on MYP agar inoculated from each microbial insecticide product (total of 52 colonies) were randomly selected for further culture on tryptone soy agar (TSA; Oxoid Ltd) at 30°C for 20 h. Biochemical identification of the selected strains was carried out using the Vitek-II system with a BCL card (bioMérieux, Inc., Marcy l'Etoile, France) according to the manufacturer's directions.

Detection of Crystal Proteins

Microscopy observation was conducted to confirm the crystal proteins (δ-endotoxins; crystal shaped) produced by the selected *B. thuringiensis* strains. An optical microscope (Axioskop 2 plus; ZEISS, Jena, Germany) was used to observe the crystal proteins. The selected strains were cultured on TSA at 30°C for more than 120 h [9], after which cells were stained with TB carbol-fuchsin ZN (Difco) by using a simple staining procedure. The crystal proteins were observed with an oil immersion lens.

Immunoassay for Detection of Enterotoxins

The selected *B. thuringiensis* strains were cultured in tryptone soy broth (Oxoid Ltd) at 30°C for 24 h, after which 1 ml of each culture was centrifuged for 10 min at 10,000 ×*g*. The supernatants were applied for detection of enterotoxins, using immunoassay kits according to the manufacturer's directions. The hemolysin BL enterotoxin was determined using an enterotoxin-reversed passive latex agglutination (BCET-RPLA) kit (Oxoid), and non-hemolytic enterotoxin was detected with a *Bacillus* diarrheal enterotoxin visual immunoassay (BDE-VIA) kit (Tecra Diagnostics, Reading, UK). *B. cereus* ATCC 14579 and F4810/72 were used as reference strains.

DNA Extraction

All the *B. thuringiensis* strains selected, and the *B. cereus* ATCC 14579 and F4810/72 reference strains, were cultured in tryptone soy broth at 35°C for 18 h, after which 1 ml of the culture broth was centrifuged at 10,000 ×*g* for 10 min at 4°C (Mega 17R; Hanil Science Industrial, Incheon, Korea). The pellet was suspended in 500 µl of sterilized distilled water, centrifuged under the same conditions as before, and resuspended again in 500 µl of sterilized distilled water. The suspended pellet was boiled for 10 min and centrifuged at 10,000 ×*g* for 10 min at 4°C. The supernatants were stored at −20°C until analysis for templates.

PCR Assay for Detection of Toxin Genes

The primers for detection of toxin genes are presented in Table 1. PCR amplification with a final volume of 20 µl was conducted using a thermal cycler (Mastercycler Gradient S; Eppendorf, Germany) with the reaction mixtures (AccuPower PCR PreMix; Bioneer, Daejeon, Korea) containing 2 µl of template DNA. The PCR assay for detection of *cytK* was 95°C for 1 min, followed by 30 cycles of 95°C for 60 sec, 48°C for 60 sec, and 72°C for 60 sec, and a final extension at 72°C for 7 min. For the *entFM* gene, PCR assay was carried out at 95°C for 3 min, followed by 35 cycles of 95°C for 30 sec, 60°C for 30 sec, and 72°C for 60 sec, and a final extension at 72°C for 5 min. The PCR program for detection

Table 1. Primer sequences for detection of toxin genes.

Target gene	Product size (bp)	Primer sequence (5′–3′)	Reference
cytK	505	GTAACTTTCATTGATGATCC GAATACTAAATAATTGGTTTCC	[36]
entFM	596	AAAGAAATTAATGGACAAACTCAAACTCA GTATGTAGCTGGGCCTGTACGT	[35]
ces	1,271	GGTGACACATTATCATATAAGGTG GTAAGCGAACCTGTCTGTAACAACA	[7]

of the *ces* gene was 95°C for 15 min, followed by 25 cycles of denaturation at 95°C for 60 sec, extension at 72°C for 50 sec, and a final extension at 72°C for 5 min. The annealing condition was at 58°C for 75 sec. The amplified products were separated by an automated capillary electrophoresis system (QIAxcel; Qiagen, Hilden, Germany) with a QIAxcel DNA high resolution kit and Qiaxcel DNA size marker (100 bp). Electrophoresis was conducted by the OM400 method (sample injection voltage 5 kV for 10 sec, separation voltage 6 kV for 400 sec), and the gel image was visualized with BioCalculator 3.0 (Qiagen). *B. cereus* ATCC 14579 and F4810/72 were used as reference strains.

Results and Discussion

Confirmation of *B. thuringiensis*

Biochemical identification and microscopic observation were conducted to confirm the *B. thuringiensis*. The 13 strains randomly selected from each microbial insecticide product based on *B. thuringiensis* (total of 52 strains) were identified as *B. cereus*/*B. thuringiensis* using the Vitek-II system with a BCL card. The genetic and phenotypic properties between *B. thuringiensis* and *B. cereus* are rarely distinguishable [25, 32], and Helgason *et al.* [11] reported that these two strains might be one species. The Vitek-II system cannot discriminate between *B. cereus* and *B. thuringiensis*. The distinguished characteristics of *B. thuringiensis* are the presence of an insecticidal crystal protein (δ-endotoxins; crystal shaped) [28, 32]. Microscopic observation of crystal-shaped proteins was conducted to confirm the *B. thuringiensis*, strains. The results of crystal-shaped protein observation are shown in Table 2. Visible crystal-shaped proteins were detected in all strains selected from microbial insecticide products (Fig. 1). Thus, all strains selected from microbial insecticide products were identified as *B. thuringiensis*, based on biochemical identification and microscopic observation for crystal-shaped proteins.

Table 2. Toxin genes and enterotoxin production profiles of *Bacillus thuringiensis* strains selected from microbial insecticide products.

Selected strains	Microscope (crystal toxin)	Toxin genes			Enterotoxin production	
		cytK	*entFM*	*ces*	HBL[a]	NHE[b]
A1	+	-	-	-	+	-
A2	+	-	-	-	+	-
A7	+	-	-	-	+	-
A8	+	-	-	-	+	-
A10	+	-	-	-	+	-
A11	+	-	-	-	+	-
B3	+	-	-	-	+	-
B4	+	-	-	-	+	-
B5	+	-	-	-	+	-
B6	+	-	-	-	+	-
C2	+	-	-	-	+	-
C4	+	-	-	-	+	-
C8	+	-	-	-	+	-
C10	+	-	-	-	+	-
C11	+	-	-	-	+	-
C12	+	-	-	-	+	-
D1	+	-	-	-	+	+
ATCC 14579[c]	-	+	+	-	+	+
F4810/72[c]	-	-	+	+	-	+

[a]*B. cereus* enterotoxin-reversed passive latex agglutination (BCET-RPLA) kit was used to detect hemolysin BL (HBL).

[b]*Bacillus* diarrheal enterotoxin visual immunoassay (BDE-VIA) kit was used to detect non-hemolytic enterotoxin (NHE).

[c]*Bacillus cereus* 14579 as enterotoxic reference strain and *Bacillus cereus* F4810/72 as emetic reference strain.

Detection of Enterotoxins in *B. thuringiensis*

HBL and NHE are the major virulence factors among the HBL, NHE, CytK, EntFM, and cereulide (emetic toxin) produced by *B. cereus* [23, 37]. In order to estimate the potential risk of food poisoning by the *B. thuringiensis* strains selected from microbial insecticide products, we investigated for the production of HBL and NHE, and the results are shown in Table 2. The HBL and NHE enterotoxins were produced by 17 (32.7%) and 1 (1.9%) out of 52 *B. thuringiensis* selected strains, respectively. Only the

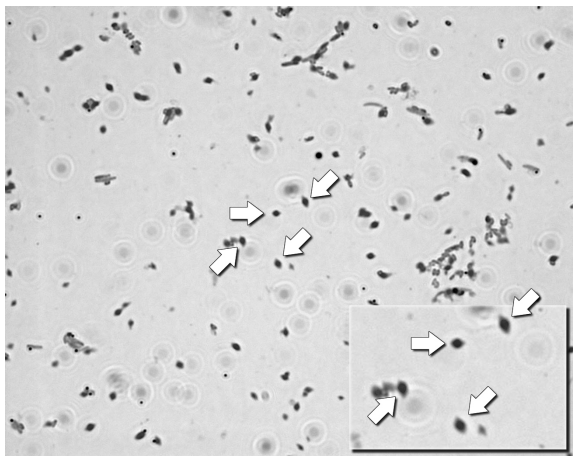

Fig. 1. Photomicrographs showing crystal proteins (δ-endotoxin) of *Bacillus thuringiensis* from microbial insecticide products.

D1 strain produced both HBL and NHE simultaneously. These results indicate that the strains tested in this study were from different microbial insecticidal products such as A, B, C, and D. The production of HBL enterotoxin composed of B, L2, and L1 subunits depends on the expression of all three genes; namely, *hblC*, *hblD*, and *hblA* [30]. The NHE enterotoxin complex comprises proteins NheA, NheB, and NheC encoded by *nheA*, *nheB*, and *nheC* [33]. The NHE is only produced when all three NHE enterotoxin complexes are present [30]. Rosenquist *et al.* [29] reported that HBL and NHE are expressed in 36 (90.0%) and 40 (100%) out of 40 *B. cereus*-like organisms, respectively, and 81–94% of *B. cereus* isolated from clinical and food samples [4, 15]. The much lower results of HBL (32.7%) and NHE (1.9%) production rates of *B. thuringiensis* are in contrast to previous studies where HBL-positive rates were present in 24 (85.7%) of 28 *B. cereus*-like organisms that possessed crystal protein [29], and all of the 59 *B. thuringiensis* strains (100%) were HBL positive [42]. NHE production rates were reported in 4 (30.7%) out of 13 *B. thuringiensis* isolates from rice products and 15 (36.6%) out of 41 *B. thuringiensis* isolates [10, 24]. *B. thuringiensis* from human fecal samples produced enterotoxins and presented similar DNA fingerprints as microbial insecticide products based on *B. thuringiensis* without gastrointestinal symptoms [13]. This report indicates that human illness is not directly related with microbial insecticide products based on *B. thuringiensis*. However, there are concerns over *B. thuringiensis* microbial insecticide products based on the HBL enterotoxin production in the present study. Thus, we should ensure that *B. thuringiensis* strains in microbial insecticides cannot produce enterotoxins. The enumeration method for *B. cereus* in the Korea Food Code, which excludes *B. thuringiensis* cell counts, will be reconsidered to reduce food safety concerns.

Detection of Toxin Genes in *B. thuringiensis*

The distributions of *cytK*, *entFM*, and *ces* genes among *B. thuringiensis* strains selected from microbial insecticide products are presented in Table 2. The *cytK*, *entFM*, and *ces* genes were not detected in any of the *B. thuringiensis* strains. The *cytK* gene-encoded enterotoxin causing cytotoxic disease has been implicated in three deaths in France and is associated with severe foodborne outbreak of hemolysis [17, 21]. Oh *et al.* [24] reported the *cytK* gene in 5 out of 13 (38.5%) *B. thuringiensis* strains from rice products. The distribution of *cytK* gene ranges from 0% to 4.7% in *B. thuringiensis* isolated from cooked rice (0 out of 20 strains), milk, and soil (1 out of 21 strains) [2, 14]. The variable distribution of *cytK* gene is in the range of 13% to 73% of *B. cereus* isolated from the food, environment, and patients [8, 15, 36]. The *entFM* gene-encoded enterotoxin is thought to be a cell wall peptidase that participates in biofilm formation, adhesion, and virulence [40]. Prabhakar and Bishop [26] demonstrated that all nine Antarctic *B. thuringiensis* isolates carry the *entFM* gene. Kim *et al.* [15] demonstrated that *B. cereus* isolated in Korea carries the *entFM* gene (65%), and another study also detected the *entFM* gene in all *B. cereus* isolates from *Sunsik* [4]. The emetic toxin cereulide has a molecular mass of 1.2 kDa and is an acid-stable cyclic peptide ([D-*O*-Leu-D-Ala-L-*O*-Val-L-Val]$_3$) [6]. Cereulide also resists different proteolytic enzymes and has remarkable heat stability [27]. The *ces* gene encoding cereulide synthetase was not detected in any of the strains tested in this study, which is consistent with previous reports of the *ces* gene in only 0.05% of *B. cereus* isolated in a dairy production chain [38] as well as reports that emetic strains are rare [16]. The *cytK*, *entFM*, and *ces* genes were not detected in *B. thuringiensis* in this study, which suggests that HBL enterotoxin was the most frequent toxin.

In conclusion, these results indicate that there is a potential risk of food poisoning by *B. thuringiensis*, raising concerns over *B. thuringiensis* microbial insecticide products. Thus, more study into the toxigenic properties of different *B. thuringiensis* strains is needed, and microbial insecticide products based on *B. thuringiensis* should be carefully controlled.

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