J. Microbiol. Biotechnol. (2015), 25(6), 872–879
http://dx.doi.org/10.4014/jmb.1502.02003

jmb

Various Enterotoxin and Other Virulence Factor Genes Widespread Among *Bacillus cereus* and *Bacillus thuringiensis* Strains

Min-Ju Kim[1], Jae-Kwang Han[1], Jong-Su Park[1], Jin-Sung Lee[2], Soon-Ho Lee[3], Joon-Il Cho[4], and Keun-Sung Kim[1]*

[1]*Department of Food Science and Technology, Chung-Ang University, Ansung 456-756, Republic of Korea*
[2]*Department of Biological Sciences, Kyonggi University, Suwon 442-760, Republic of Korea*
[3]*Nutrition Safety Policy Division, and* [4]*Division of Food Microbiology, The Korean Ministry of Food and Drug Safety, Cheongju 361-709, Republic of Korea*

Received: February 3, 2015
Revised: March 2, 2015
Accepted: March 15, 2015

First published online
March 20, 2015

**Corresponding author*
Phone: +82-31-670-3032;
Fax: +82-31-675-4853;
E-mail: keunsung@cau.ac.kr

pISSN 1017-7825, eISSN 1738-8872

Copyright© 2015 by
The Korean Society for Microbiology and Biotechnology

Many strains of *Bacillus cereus* cause gastrointestinal diseases, and the closely related insect pathogen *Bacillus thuringiensis* has also been involved in outbreaks of diarrhea. The diarrheal diseases are attributed to enterotoxins. Sixteen reference strains of *B. cereus* and nine commercial and 12 reference strains of *B. thuringiensis* were screened by PCR for the presence of 10 enterotoxigenic genes (*hblA*, *hblC*, *hblD*, *nheA*, *nheB*, *nheC*, *cytK*, *bceT*, *entFM*, and *entS*), one emetogenic gene (*ces*), seven hemolytic genes (*hlyA*, *hlyII*, *hlyIII*, *plcA*, *cerA*, *cerB*, and *cerO*), and a pleiotropic transcriptional activator gene (*plcR*). These genes encode various enterotoxins and other virulence factors thought to play a role in infections of mammals. Amplicons were successfully generated from the strains of *B. cereus* and *B. thuringiensis* for each of these sequences, except the *ces* gene. Intriguingly, the majority of these *B. cereus* enterotoxin genes and other virulence factor genes appeared to be widespread among *B. thuringiensis* strains as well as *B. cereus* strains.

Keywords: *Bacillus cereus*, *Bacillus thuringiensis*, enterotoxins, virulence factors, genes

Introduction

The *Bacillus cereus sensu lato* group contains six valid species, including *Bacillus cereus* and *Bacillus thuringiensis*, which show a high degree of phenotypic similarity [21]. The classification of these species is based on pathogenicity to mammals or insects, plasmid content, and gross morphological characteristics [30, 31]. The genetic similarity among members of the *B. cereus* group has been extensively studied by means of various molecular methods [17, 19, 28, 30].

B. cereus is an opportunistic endospore-forming bacterium involved in a range of intestinal and extraintestinal infections [5, 37]. It is a common soil inhabitant that is often implicated in food poisoning in humans [35, 37]. The virulence of this bacterium has been ascribed to different extracellular factors. Two of these virulence factors, hemolysin BL (HBL) [4] and nonhemolytic enterotoxin (NHE) [14], are tripartite protein complexes. Other enterotoxigenic factors are single gene products encoded by the cytotoxin K (*cytK*), enterotoxin FM (*entFM*), enterotoxin S (*entS*), and enterotoxin T (*bceT*) genes [2, 11, 18, 26]. The virulence of the emetic strains is related to cereulide, a thermostable cyclic dodecadepsipeptide synthesized by a nonribosomal peptide synthetase encoded by *ces* genes [10]. Products from other genes, such as hemolysin A (*hlyA*), hemolysin II (*hlyII*), hemolysin III (*hlyIII*), phosphatidylinositol-specific phospholipase C (*plcA*), cereolysin A or phospholipase C (*cerA*), cereolysin B or sphingomyelinase (*cerB*), cereolysin O (*cerO*), and their pleiotropic transcriptional activator (*plcR*), are also involved in the pathogenesis of *B. cereus* [3, 20, 32, 35, 37, 38]. *B. thuringiensis* is a typical endospore-forming bacterium distinguished by the accumulation of polypeptides that form a crystalline parasporal inclusion during sporulation [34]. These crystal proteins may be toxic to insects, leading to the extensive use of *B. thuringiensis* as a biological insecticide for crop protection [8, 34].

Recent studies have suggested that *B. cereus* and *B. thuringiensis* should be considered members of a single

species [19, 33, 42]. However, other studies have shown sufficient genetic differentiation of *B. cereus* and *B. thuringiensis* [9, 39]. Thus, at the present time, there is no consensus as to whether these two bacterial species should be considered separate species or specialized variants of a single species. Given such discordant taxonomic classification of *B. cereus* and *B. thuringiensis* strains and the introduction of high numbers of *B. thuringiensis* spores into the human food chain through vegetables treated with *B. thuringiensis*-based insecticides, we examined a panel of *B. cereus* and *B. thuringiensis* strains comprising mainly reference strains to assess the distribution of genes encoding the putative virulence factors mentioned above for *B. cereus*.

Materials and Methods

Bacterial Strains and Growth Conditions

Five commercial *B. thuringiensis* strains isolated from five different biopesticide products obtained from local retail establishments in rural areas of the Republic of Korea were screened in this study. A strain of each of the following commercial *B. thuringiensis* subspecies was also screened: *B. thuringiensis* subsp. *kurstaki*, *B. thuringiensis* subsp. *aizawai*, *B. thuringiensis* subsp. *israelensis*, and *B. thuringiensis* subsp. *tenebrionis*.

Twelve and 16 reference strains of *B. thuringiensis* and *B. cereus*, respectively, were screened in this study. *B. thuringiensis* ATCC 33679, *B. thuringiensis* ATCC 35646, *B. thuringiensis* ATCC 19266, *B. thuringiensis* ATCC 19268, *B. thuringiensis* ATCC 13367, *B. thuringiensis* ATCC 13366, *B. thuringiensis* ATCC 11429, *B. cereus* ATCC 21366, *B. cereus* ATCC 21768, *B. cereus* ATCC 10876, *B. cereus* ATCC 21772, *B. cereus* ATCC 11778, *B. cereus* ATCC 10702, *B. cereus* ATCC 13061, *B. cereus* ATCC 14579, *B. cereus* ATCC 21769, and *B. cereus* ATCC 21771 were obtained from the American Type Culture Collection (Manassas, VA, USA). *B. thuringiensis* KCTC 1508, *B. thuringiensis* KCTC 1510, *B. thuringiensis* KCTC 1511, *B. thuringiensis* KCTC 1512, *B. thuringiensis* KCTC 1513, and *B. cereus* KCTC 1094 were obtained from the Korean Collection for Type Cultures (Daejeon, Korea). *B. cereus* KFRI 181 was obtained from the Korea Food Research Institute (Sungnam, Korea). *B. cereus* IFO 3514, *B. cereus* IFO 3563, *B. cereus* IFO 3001, and *B. cereus* IFO 3003 were obtained from the Institute for Fermentation (Osaka, Japan). All bacterial strains were grown at 30°C on nutrient agar or in nutrient broth with shaking for preparation of template DNA for PCR screening.

PCR Detection of Genes for Enterotoxins, Emetic Toxin, and Other Virulence Factors

Preparation of total DNA. Template DNA for PCR screening was prepared by processing 5 ml of culture grown for 18 h at 30°C, using a QIAamp DNA Mini Kit (Qiagen, Inc., Chatsworth, CA, USA). The presence, concentration, and purity of total DNA in the prepared samples were detected by measuring the absorbance at 260 and 280 nm using an Ultraspec 3000 spectrophotometer (Pharmacia, Peapack, NJ, USA).

Target genes for PCR detection. PCR analyses were carried out to detect 10 enterotoxigenic genes (*hblA*, *hblC*, *hblD*, *nheA*, *nheB*, *nheC*, *cytK*, *bceT*, *entFM*, and *entS*), one emetogenic gene (*ces*), seven hemolytic genes (*hlyA*, *hlyII*, *hlyIII*, *plcA*, *cerA*, *cerB*, and *cerO*), and a pleiotropic transcriptional activator gene (*plcR*) among *B. cereus* and *B. thuringiensis* strains. Table 1 provides information concerning the primers used for the amplification of each gene in this study.

Conditions for PCR amplification. Twenty-five nanograms of DNA was used for each reaction. Ultrapure water (Invitrogen, Life Technologies, Seoul, Korea) was used in all negative control reactions and for the preparation of the PCR mixture. All reaction mixtures for amplification of sequences encoding toxins and putative virulence factors contained 5 µl of template DNA (25 ng), 10 mM Tris-HCl (pH 8.3), 10 mM KCl, 0.2 mM each deoxynucleoside triphosphate, 2.5 mM $MgCl_2$, 1 µM each primer, and 0.5 U of *Taq* DNA polymerase (Solgent Co., Daejeon, Korea). PCR amplification was conducted using a model PTC-100 Programmable Thermal Controller (MJ Research, Inc., MA, USA). The optimized PCR conditions were as follows: a single denaturation step of 3 min at 95°C; 35 cycles of denaturation at 94°C for 30 sec, annealing at 58°C for 45 sec, and extension at 72°C for 1.5 min; and a final extension at 72°C for 5 min.

To validate the results, all PCR amplifications were performed a minimum of three times. After DNA amplification, PCR fragments were analyzed by submarine gel electrophoresis, stained, and visualized under UV illumination. Suitable molecular size markers were included in each gel. To identify cases in which poor quality of template DNA caused amplification failure, the quality of any DNA extract that failed to amplify in a specific reaction was examined by attempting amplification with a pair of universal primers designed to amplify a region of the 16S rRNA gene. Negative controls were included with all amplifications. Suitable controls such as buffer, media, PCR mixtures, and template DNA were used to detect any false-positive or false-negative reactions.

Results

PCR Amplification of Virulence Genes Using Gene-Specific PCR Primers

The approach used in this study relied on PCR amplification of target virulence genes. Virulence genes targeted for PCR detection included a first set of genes encoding enterotoxins and emetic toxin and a second set of genes encoding extracellular hemolysins considered to be potential virulence factors of *B. cereus* and *B. thuringiensis*. The first set included genes encoding hemolytic (*hblA*, *hblC*, and *hblD*) and nonhemolytic (*nheA*, *nheB*, and *nheC*) enterotoxin complexes, cytotoxin K (*cytK*), various putative enterotoxins (*bceT*, *entFM*, and *entS*), and cereulide (*ces*) as shown in Fig. 1. The second set included genes encoding a

Table 1. Target genes, sequences, and amplicon sizes for PCR primers used in this study.

Target gene	Primer name	Primer sequence (5′ → 3′)	Amplicon size (bp)	Reference
hblA (B subunit of HBL[a])	HBLA1	GTGCAGATGTTGATGCCGAT	301	[11]
	HBLA2	ATGCCACTGCGTGGACATAT		
hblC (L_2 subunit of HBL)	L2A	AATGGTCATCGGAACTCTAT	731	[11]
	L2B	CTCGCTGTTCTGCTGTTAAT		
hblD (L_1 subunit of HBL)	L1A	AATCAAGAGCTGTCACGAAT	411	[11]
	L1B	CACCAATTGACCATGCTAAT		
nheA (A subunit of NHE[b])	nheA 344 S	TACGCTAAGGAGGGGCA	480	[11]
	nheA 843 A	GTTTTTATTGCTTCATCGGCT		
nheB (B subunit of NHE)	nheB 1500 S	CTATCAGCACTTATGGCAG	754	[11]
	nheB 2269 A	ACTCCTAGCGGTGTTCC		
nheC (C subunit of NHE)	nheC 2820 S	CGGTAGTGATTGCTGGG	564	[11]
	nheC 3401 A	CAGCATTCGTACTTGCCAA		
cytK (cytotoxin K)	CK-F-1859	ACAGATATCGG(GT)CAAAATGC	809	[16]
	CK-R-2668	TCCAACCCAGTT(AT)(GC)CAGTTC		
entFM (enterotoxin FM)	ENTA	ATGAAAAAAGTAATTTGCAGG	1,269	[2]
	ENTB	TTAGTATGCTTTTGTGTAACC		
bceT (enterotoxin T)	bceT-f	GCTACGCAAAAACCGAGTGGTG	679	[1]
	bceT-r	AATGCTCCGGACTATGCTGACG		
entS (enterotoxin S)	TY123	GGTTTAGCAGCAGCTTCTGTAGCTGGCG	261	[2]
	TY124	CTTGTCCAACTACTTGTAGCACTTGGCC		
	TY123	GGTTTAGCAGCAGCTTCTGTAGCTGGCG	581	[2]
	TY125	GTTTCGTTAGATACAGCAGAACCACC		
	TY123	GGTTTAGCAGCAGCTTCTGTAGCTGGCG	857	[2]
	TY126	GTAACGTTATTGTTATTGTTATTGTTAACG		
	TY123	GGTTTAGCAGCAGCTTCTGTAGCTGGCG	1,222	[2]
	TY127	CAGAACTAATACGTACACCAGTTGCATCTG		
ces (cereulide)	cesF1	GGTGACACATTATCATATAAGGTG	2,200	[10]
	cesR1	GTTTTCTGGTAACAGCGTTCTAC		
hlyA (hemolysin A)	FHEMAF	CGTGAGAAAGCAAAACGC	725	[29]
	FHEMAR	TCAACAGAAACTGGAGAATGAT		
hlyII (hemolysin II)	FHLYII	GATTCTAAAGGAACTGTAG	868	[11]
	RHLYII	GGTTATCAAGAGTAACTTG		
hlyIII (hemolysin III)	bchem1	AATGACACGAATGACACAAT	605	[3]
	bchem4	TCATCGCACTACCACCTAAT		
	bchem2	ACACATGGTATCGGTGCCAT	395	[3]
	bchem3	ACGATTATGAGCCATCCCAT		
plcA (PI-specific phospholipase C)	phosC1	CGCTATCAAATGGACCATGG	569	[24]
	phosC2	GGACTATTCCATGCTGTACC		
cerA (cereolysin A)	cerA1	ACTGAGTTAGAGAACGGTAT	536	[13]
	cerA2	CGCTTACCTGTCATTGGTGT		
cerB (cereolysin B)	cerB1	TCGTAGTAGTGGAAGCGAAT	457	[13]
	cerB2	AGTCGCTGTATGTCCAGTAT		
cerO (cereolysin O)	cerOf	GTATCTACTTGGAATSAAAAG	659	[29]
	cerOr	TAATCTGTATTGTTATGAAC		
plcR (pleiotropic regulator, PlcR)	deg-PlcR-516-s	TGCA(AC)GCAGA(AG)AAATTAGGA	747	[25]
	deg-PlcR-1262	C(TC)A(AG)CTT(TC)(TC)(TC)TAGGCATTCA		

[a]HBL: Hemolysin BL

[b]NHE: Nonhemolytic enterotoxin

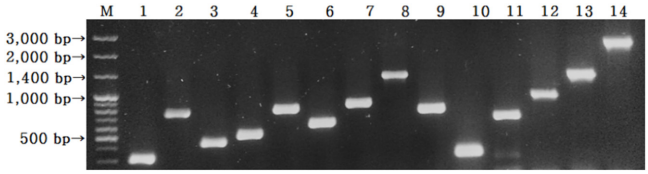

Fig. 1. Representative PCR amplicons to detect enterotoxigenic and emetogenic genes among *B. cereus and B. thuringiensis* strains.

Lane M, 100 bp DNA ladder; lane 1, *hblA* gene; lane 2, *hblC* gene; lane 3, *hblD* gene; lane 4, *nheA* gene; lane 5, *nheB* gene; lane 6, *nheC* gene; lane 7, *cytK* gene; lane 8, *entFM* gene; lane 9, *bceT* gene; lanes 10 to 13, *entS* gene (TY123/TY124, TY123/TY125, TY123/TY126, and TY123/TY127); and lane 14, *ces* gene.

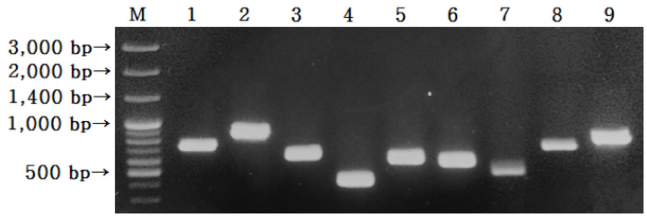

Fig. 2. Representative PCR amplicons to detect other putative virulence genes among *B. cereus and B. thuringiensis* strains.

Lane M, 100 bp DNA ladder; lane 1, *hlyA* gene; lane 2, *hlyII* gene; lanes 3 and 4, *hlyIII* gene (bchem1/bchem4 and bchem2/bchem3); lane 5, *plcA* gene; lane 6, *cerA* gene; lane 7, *cerB* gene; lane 8, *cerO* gene; and lane 9, *plcR* gene.

number of hemolytic factors (*hlyA*, *hlyII*, *hlyIII*, *plcA*, *cerA*, *cerB*, and *cerO*) and their pleiotropic transcriptional activator, PlcR (*plcR*), as shown in Fig. 2.

The gene-specific PCR primers successfully amplified each target virulence gene, except the *ces* gene, from total DNA of *B. cereus* and *B. thuringiensis* strains. All primers produced amplicons of the expected sizes from their respective target virulence genes, as representative PCR amplicons were shown in Figs. 1 and 2. Using three DNA templates independently prepared from each test strain as described above, the PCR amplification results in triplicate experiments were 100% reproducible for each target gene.

Distribution of *Bacillus* Enterotoxigenic and Emetogenic Genes Among *B. cereus* and *B. thuringiensis* Strains

The distribution of the genes encoding the two major *Bacillus* tripartite enterotoxins among the tested *B. cereus* and *B. thuringiensis* strains is shown in Table 2. Eight of the 12 *B. thuringiensis* reference strains possessed all three genes encoding the enterotoxic HBL complex (*hblA*, *hblC*, and *hblD*), whereas only two of the 16 *B. cereus* reference strains harbored all three genes. The remaining *B. thuringiensis* strains, except one commercial isolate and one reference strain, possessed at least one of the three genes encoding the complex, whereas four *B. cereus* reference strains had none and the remaining 10 *B. cereus* reference strains possessed at least one of the three genes. All three genes encoding the nonhemolytic enterotoxin (*nheA*, *nheB*, and *nheC*) were detected in 12 (57%) of the 21 *B. thuringiensis* strains, including all four known *B. thuringiensis* subspecies, and in nine (56%) of the 16 *B. cereus* reference strains.

Table 2. Occurrence of enterotoxigenic *hbl* and *nhe* genes in *B. cereus* and *B. thuringiensis*.

Strain	Frequencies of *hbl* genes (%)							
	hblA	*hblC*	*hblD*	*hblA* + *hblC*	*hblA* + *hblD*	*hblC* + *hblD*	*hblA* + *hblC* + *hblD*	None
B. thuringiensis								
Commercial (*n* = 9)	5 (56)	0 (0)	0 (0)	0 (0)	3 (33)	0 (0)	0 (0)	1 (11)
Reference (*n* = 12)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	3 (25)	8 (67)	1 (8)
Total (*n* = 21)	5 (24)	0 (0)	0 (0)	0 (0)	3 (14)	3 (14)	8 (38)	2 (10)
B. cereus								
Reference (*n* = 16)	1 (6)	1 (6)	4 (25)	0 (0)	3 (19)	1 (6)	2 (13)	4 (25)
	Frequencies of *nhe* genes (%)							
	nheA	*nheB*	*nheC*	*nheA* + *nheB*	*nheA* + *nheC*	*nheB* + *nheC*	*nheA* + *nheB* + *nheC*	None
B. thuringiensis								
Commercial (*n* = 9)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	2 (22)	7 (78)	0 (0)
Reference (*n* = 12)	0 (0)	0 (0)	0 (0)	3 (25)	1 (8)	3 (25)	5 (42)	0 (0)
Total (*n* = 21)	0 (0)	0 (0)	0 (0)	3 (14)	1 (5)	5 (24)	12 (57)	0 (0)
B. cereus								
Reference (*n* = 16)	1 (6)	0 (0)	1 (6)	1 (6)	0 (0)	2 (13)	9 (56)	2 (13)

Whereas all *B. thuringiensis* strains possessed at least two of the NHE genes, two *B. cereus* reference strains harbored only one gene and two other strains lacked all three genes.

The distribution of other *Bacillus* enterotoxigenic and emetogenic genes among the tested *B. cereus* and *B. thuringiensis* strains is shown in Table 3. The *cytK* gene was frequently detected in the *B. thuringiensis* (91%) and *B. cereus* strains (81%). The *bceT* gene was also frequently detected in the *B. thuringiensis* (95%) and *B. cereus* strains (88%). The *entFM* gene was detected in 10 (83%) of the 12 *B. thuringiensis* reference strains and 12 (75%) of the 16 *B. cereus* reference strains, but the gene was not detected in any of the five commercial *B. thuringiensis* isolates or the four known *B. thuringiensis* subspecies strains. PCR fragments were successfully amplified in all of the *B. thuringiensis* and *B. cereus* strains using at least one of the four primer sets targeting the *entS* gene (TY123/124, TY123/125, TY123/126, and TY123/127). The primer set targeting the *ces* gene (cesF1/cesR1) failed to amplify PCR fragments in any of the *B. thuringiensis* and *B. cereus* strains tested in this study.

Distribution of Other Putative *Bacillus* Virulence Factor Genes Among *B. cereus* and *B. thuringiensis* Strains

The distribution of other putative *Bacillus* virulence factor genes among the tested *B. cereus* and *B. thuringiensis* strains is shown in Table 4. The *hlyA* gene was detected in 19 (90%) of the 21 *B. thuringiensis* strains, including all five commercial *B. thuringiensis* isolates and the four known *B. thuringiensis* subspecies strains, and in all (100%) of the 16 *B. cereus* reference strains. The *hlyII* gene was detected in three (60%) of the five commercial *B. thuringiensis* isolates and two (50%) of the four known *B. thuringiensis* subspecies strains but only one (8%) of the 12 *B. thuringiensis* reference strains. This gene was also detected in four (25%) of the 16 *B. cereus* reference strains. Both primer sets targeting the *hlyIII* gene (bchem1/4 and bchem2/3) failed to amplify PCR fragments in three (8%) of the tested strains: one commercial *B. thuringiensis* isolate and two known *B. thuringiensis* subspecies strains. At least one of the two primer sets successfully amplified a PCR fragment in the remaining *B. thuringiensis* and *B. cereus* strains. The *plcA* gene was detected in 20 (95%) of the 21 *B. thuringiensis* strains, including all five commercial *B. thuringiensis* isolates and the four known *B. thuringiensis* subspecies strains, and in 12 (75%) of the 16 *B. cereus* reference strains. The *cerA* gene was detected in all (100%) of the five commercial *B. thuringiensis* isolates and three (75%) of the four known *B. thuringiensis* subspecies strains but only six (50%) of the 12 *B. thuringiensis* reference strains. This gene was also detected in six (38%) of the 16 *B. cereus* reference strains.

Table 3. Occurrence of other enterotoxigenic and emetogenic genes in *B. cereus* and *B. thuringiensis*.

Strain	Cytotoxin K (*cytK*)	Enterotoxin T (*bceT*)	Enterotoxin FM (*entFM*)	Enterotoxin S (*entS*)				Cereulide (*ces*)
				TY123/TY124	TY123/TY125	TY123/TY126	TY123/TY127	
B. thuringiensis								
Commercial (n = 9)	7 (78)	8 (89)	0 (0)	9 (100)	4 (44)	0 (0)	0 (0)	0 (0)
Reference (n = 12)	12 (100)	12 (100)	10 (83)	10 (83)	9 (75)	1 (8)	12 (100)	0 (0)
Total (n = 21)	19 (91)	20 (95)	10 (48)	19 (91)	13 (62)	1 (5)	12 (57)	0 (0)
B. cereus								
Reference (n = 16)	13 (81)	14 (88)	12 (75)	13 (81)	9 (56)	0 (0)	15 (94)	0 (0)

Table 4. Occurrence of hemolytic genes and pleiotropic transcriptional activator gene in *B. cereus* and *B. thuringiensis*.

	Hemolysin A (*hlyA*)	Hemolysin II (*hlyII*)	Hemolysin III (*hlyIII*)		Phosphatidylinositol -specific phospholipase C (*plcA*)	Cereolysin AB		Cereolysin O (*cerO*)	Pleiotropic transcriptional activator (*plcR*)
			bchem1/4	bchem2/3		*cerA*	*cerB*		
B. thuringiensis									
Commercial (n = 9)	9 (100)	5 (56)	4 (44)	5 (56)	9 (100)	8 (89)	7 (78)	7 (78)	9 (100)
Reference (n = 12)	10 (83)	1 (8)	12 (100)	11 (92)	11 (92)	6 (50)	11 (92)	9 (75)	12 (100)
Total (n = 21)	19 (90)	6 (29)	16 (76)	16 (76)	20 (95)	14 (67)	18 (86)	16 (76)	21 (100)
B. cereus									
Reference (n = 16)	16 (100)	4 (25)	14 (88)	15 (94)	12 (75)	6 (38)	9 (56)	8 (50)	16 (100)

The *cerB* gene was detected in 18 (86%) of the 21 *B. thuringiensis* strains and in nine (56%) of the 16 *B. cereus* reference strains. The *cerO* gene was detected in 16 (76%) of the 21 *B. thuringiensis* strains, including all five commercial *B. thuringiensis* isolates, and in eight (50%) of the 16 *B. cereus* reference strains.

Discussion

B. cereus is known to produce a number of toxins that are involved in its ability to cause gastrointestinal and somatic diseases. These toxins include at least two enterotoxins, one emetic toxin, one cytotoxin, two hemolysins, and three enzymes involved in the degradation of phospholipids [20]. *B. thuringiensis*, like the foodborne and opportunistic human pathogen *B. cereus*, belongs to the *B. cereus sensu lato* group. *B. thuringiensis* and *B. cereus* are closely related and are indistinguishable phenotypically and genetically, except that the former harbors insecticidal plasmids and produces crystal toxin inclusions during sporulation [37]. Thus, measuring the toxins and monitoring the toxin-producing capabilities of a strain may be more important than identifying the species using other criteria. The virulence factors produced by members of the *B. cereus sensu lato* group include enterotoxins, phospholipases, and delta endotoxins.

In our study, genes encoding enterotoxins were more frequently found in the *B. thuringiensis* strains than in the *B. cereus* strains. The presence of enterotoxin-encoding genes in commercial *B. thuringiensis* strains was also found in previous studies [12, 18, 19, 23, 27]. Since all commercial *B. thuringiensis* strains harbor genes for three known enterotoxins, HBL, NHE, and CytK, there is a risk that high levels of these organisms may cause human disease. Taking this enterotoxigenic potential into account, as well as the fact that *B. thuringiensis* cannot be separated from *B. cereus* at the chromosomal level, vegetable producers and authorities responsible for food safety should consider the amount of *B. thuringiensis* insecticide residue left on products after harvest. The European Food Safety Authority has recommended that processors ensure that the numbers of *B. cereus* do not reach 10^3 to 10^5 per gram at the stage of consumption. The Korean Ministry of Food and Drug Safety has specified that processed foods should contain fewer than 10^3 to 10^4 *B. cereus* per gram, depending on the food type. We suggest that this guideline should also apply to residues of commercial, enterotoxin-encoding *B. thuringiensis* strains.

B. thuringiensis is one of the leading biological insecticides for use on crops. *B. thuringiensis* insecticidal sprays, which contain mixtures of spores and insecticidal crystals, are chosen by organic farmers to meet guidelines for using strictly nonsynthetic materials. Many Korean local governments and the Korean Ministry of Agriculture, Food and Rural Affairs do not oppose the use of biological insecticides to produce organic agricultural products. *B. thuringiensis* spore/crystal formulations must be safe and effective, must be easy to use, and should have a long shelf-life. The spore/crystal mixture in commercial formulations is more effective and is cheaper to obtain than the crystals alone. Because some strains of *B. thuringiensis* have the potential during their vegetative growth to produce various toxins that may produce symptoms in mammals, the production process must be closely controlled and monitored to ensure that these exotoxins are not present in the spore/crystal formulations at levels that can cause significant adverse health effects. Some features of *B. thuringiensis* spore/crystal-based biopesticides limit their use in insect control. First, the biopesticides must be ingested by the target insect. Second, the timing of *B. thuringiensis* sprays is critical to attaining economic levels of insect control. *B. thuringiensis* is usually applied when early instar larvae are present, as older larvae are more tolerant. Third, *B. thuringiensis* sprays persist for only a few days on the leaf surface. Both leaf surface proteases and sunlight contribute to the degradation of crystal proteins.

In *B. thuringiensis* strains harboring toxin-encoding genes, vegetative cells can produce various toxins if they proliferate to the point of toxin production. However, the spores of such strains cannot produce toxins at all, as long as they remain dormant. Although *B. thuringiensis* spores can germinate in rat gastrointestinal tracts, their growth can be inhibited by indigenous gastrointestinal microbial communities. Thus, the resulting vegetative cells cannot proliferate to the point of toxin production [6, 7, 40, 41]. Here, the indigenous gastrointestinal microbial communities are important to inhibit the outgrowth of germinated *B. thuringiensis* spores. To date, the health effects of *B. thuringiensis* spore/crystal-based biopesticides have not been demonstrated in mammals in any infectivity or pathogenicity study. In field trials, no outbreaks have been reported in humans, although additional investigations are needed to determine whether *B. thuringiensis* toxin-encoding genes are expressed in the human gut after ingestion of the spores. Despite the ubiquitous presence of genes encoding enterotoxigenic and other virulence factor proteins and their expression in vegetative cells *in vitro* and *in vivo*, *B. thuringiensis* spore/crystal-based biopesticides do not

appear to pose a real food poisoning risk. This is supported by public health studies that have failed to link exposure to the spore/crystal spray products with an increased incidence of gastroenteritis, even in urban areas after large-scale aerial application or under intense exposure conditions in greenhouses [15, 22, 36]. Nevertheless, the presence of genes encoding enterotoxigenic and other virulence factor proteins continues to fuel negative public perceptions and could ultimately lead to restricted use of *B. thuringiensis* in pest control.

In summary, we detected the distribution of various enterotoxin genes and other virulence factor genes considered unique to *B. cereus* among strains of *B. cereus* and *B. thuringiensis*. These two species are members of the *B. cereus sensu lato* group and are indistinguishable phenotypically and genetically, except that *B. thuringiensis* produces crystal toxins during sporulation. *B. cereus* enterotoxins and other virulence factors produced during vegetative growth have been implicated in numerous food-poisoning outbreaks in humans, while *B. thuringiensis* spore/crystal complexes have been extensively used for crop protection against insects. *B. cereus* enterotoxin genes and other virulence factor genes appear to be widespread among *B. thuringiensis* strains as well as *B. cereus* strains.

Acknowledgments

This research was supported by the Korean Ministry of Food and Drug Safety in 2010 (10162food poisoning056) and the Chung-Ang University Research Scholarship Grants in 2013.

References

1. Agata N, Ohta M, Arakawa Y, Mori M. 1995. The *bceT* gene of *Bacillus cereus* encodes an enterotoxic protein. *Microbiology* **141:** 983-988.
2. Asano SI, Nukumizu Y, Bando H, Iizuka T, Yamamoto T. 1997. Cloning of novel enterotoxin genes from *Bacillus cereus* and *Bacillus thuringiensis*. *Appl. Environ. Microbiol.* **63:** 1054-1057.
3. Baida GE, Kuzmin NP. 1995. Cloning and primary structure of a new hemolysin gene from *Bacillus cereus*. *Biochim. Biophys. Acta* **1264:** 151-154.
4. Beecher DJ, Wong AC. 2000. Tripartite haemolysin BL: isolation and characterization of two distinct homologous sets of components from a single *Bacillus cereus* isolate. *Microbiology* **146:** 1371-1380.
5. Bottone EJ. 2010. *Bacillus cereus*, a volatile human pathogen. *Clin. Microbiol. Rev.* **23:** 382-398.
6. Ceuppens S, Uyttendaele M, Drieskens K, Heyndrickx M, Rajkovic A, Boon N, Van de Wiele T. 2012. Survival and germination of *Bacillus cereus* spores without outgrowth or enterotoxin production during *in vitro* simulation of gastrointestinal transit. *Appl. Environ. Microbiol.* **78:** 7698-7705.
7. Ceuppens S, Van de Wiele T, Rajkovic A, Ferrer-Cabaceran T, Heyndrickx M, Boon N, Uyttendaele M. 2012. Impact of intestinal microbiota and gastrointestinal conditions on the *in vitro* survival and growth of *Bacillus cereus*. *Int. J. Food Microbiol.* **155:** 241-246.
8. Chattopadhyay A, Bhatnagar NB, Bhatnagar R. 2004. Bacterial insecticidal toxins. *Crit. Rev. Microbiol.* **30:** 33-54.
9. Cherif A, Brusetti L, Borin S, Rizzi A, Boudabous A, Khyami-Horani H, Daffonchio D. 2003. Genetic relationship in the *'Bacillus cereus* group' by rep-PCR fingerprinting and sequencing of a *Bacillus anthracis*-specific rep-PCR fragment. *J. Appl. Microbiol.* **94:** 1108-1119.
10. Ehling-Schulz M, Vukov N, Schulz A, Shaheen R, Andersson M, Märtlbauer E, Scherer S. 2005. Identification and partial characterization of the nonribosomal peptide synthetase gene responsible for cereulide production in emetic *Bacillus cereus*. *Appl. Environ. Microbiol.* **71:** 105-113.
11. Fagerlund A, Ween O, Lund T, Hardy SP, Granum PE. 2004. Genetic and functional analysis of the *cytK* family of genes in *Bacillus cereus*. *Microbiology* **150:** 2689-2697.
12. Frederiksen K, Rosenquist H, Jørgensen K, Wilcks A. 2006. Occurrence of natural *Bacillus thuringiensis* contaminants and residues of *Bacillus thuringiensis*-based insecticides on fresh fruits and vegetables. *Appl. Environ. Microbiol.* **72:** 3435-3440.
13. Gilmore MS, Cruz-Rodz AL, Leimeister-Wachter M, Kreft J, Goebel W. 1989. A *Bacillus cereus* cytolytic determinant, cereolysin AB, which comprises the phospholipase C and sphingomyelinase genes: nucleotide sequence and genetic linkage. *J. Bacteriol.* **171:** 744-753.
14. Granum PE, O'Sullivan K, Lund T. 1999. The sequence of the non-haemolytic enterotoxin operon from *Bacillus cereus*. *FEMS Microbiol. Lett.* **177:** 225-229.
15. Green M, Heumann M, Sokolow R, Foster LR, Bryant R, Skeels M. 1990. Public health implications of the microbial pesticide *Bacillus thuringiensis*: an epidemiological study, Oregon, 1985-86. *Am. J. Public Health* **80:** 848-852.
16. Guinebretiere MH, Broussolle V, Nguyen-The C. 2002. Enterotoxigenic profiles of food-poisoning and food-borne *Bacillus cereus* strains. *J. Clin. Microbiol.* **40:** 3053-3056.
17. Guinebretiere MH, Thompson FL, Sorokin A, Normand P, Dawyndt P, Ehling-Schulz M, *et al.* 2008. Ecological diversification in the *Bacillus cereus* group. *Environ. Microbiol.* **10:** 851-865.
18. Hansen BM, Hendriksen NB. 2001. Detection of enterotoxic *Bacillus cereus* and *Bacillus thuringiensis* strains by PCR analysis. *Appl. Environ. Microbiol.* **67:** 185-189.
19. Helgason E, Økstad OA, Caugant DA, Johansen HA, Fouet A, Mock M, *et al.* 2000. *Bacillus anthracis*, *Bacillus cereus*, and *Bacillus thuringiensis* – one species on the basis of genetic evidence. *Appl. Environ. Microbiol.* **66:** 2627-2630.

20. Hendriksen NB, Hansen BM, Johansen JE. 2006. Occurrence and pathogenic potential of *Bacillus cereus* group bacteria in a sandy loam. *Antonie Van Leeuwenhoek* **89:** 239-249.
21. Jensen GB, Hansen BM, Eilenberg J, Mahillon J. 2003. The hidden lifestyles of *Bacillus cereus* and relatives. *Environ. Microbiol.* **5:** 631-640
22. Jensen GB, Larsen P, Jacobsen BL, Madsen B, Smidt L, Andrup L. 2002. *Bacillus thuringiensis* in fecal samples from greenhouse workers after exposure to *B. thuringiensis*-based pesticides. *Appl. Environ. Microbiol.* **68:** 4900-4905.
23. Kyei-Poku G, Gauthier D, Pang A, van Frankenhuyzen K. 2007. Detection of *Bacillus cereus* virulence factors in commercial products of *Bacillus thuringiensis* and expression of diarrheal enterotoxins in a target insect. *Can. J. Microbiol.* **53:** 1283-1290.
24. Lechner M, Kupke T, Stefanovic S, Götz F. 1989. Molecular characterization and sequence of phosphatidylinositol-specific phospholipase C of *Bacillus thuringiensis*. *Mol. Microbiol.* **3:** 621-626.
25. Lereclus D, Agaisse H, Gominet M, Salamitou S, Sanchis V. 1996. Identification of a *Bacillus thuringiensis* gene that positively regulates transcription of the phosphatidylinositol-specific phospholipase C gene at the onset on the stationary phase. *J. Bacteriol.* **178:** 2853-2860.
26. Lund T, de Buyser ML, Granum PE. 2000. A new cytotoxin from *Bacillus cereus* that may cause necrotic enteritis. *Mol. Microbiol.* **38:** 254-261.
27. Ngamwongsatit P, Buasri W, Pianariyanon P, Pulsrikarn C, Ohba M, Assavanig A, Panbangred W. 2008. Broad distribution of enterotoxin genes (*hblCDA*, *nheABC*, *cytK*, and *entFM*) among *Bacillus thuringiensis* and *Bacillus cereus* as shown by novel primers. *Int. J. Food Microbiol.* **121:** 352-356.
28. Priest FG, Barker M, Baillie LWJ, Holmes EC, Maiden MCJ. 2004. Population structure and evolution of the *Bacillus cereus* group. *J. Bacteriol.* **186:** 7959-7970.
29. Raddadi N, Belaouis A, Tamagnini I, Hansen BM, Hendriksen NB, Boudabous A, *et al*. 2009. Characterization of polyvalent and safe *Bacillus thuringiensis* strains with potential use for biocontrol. *J. Basic Microbiol.* **49:** 293-303.
30. Rasko DA, Altherr MR, Han CS, Ravel J. 2005. Genomics of the *Bacillus cereus* group of organisms. *FEMS Microbiol. Rev.* **29:** 303-329.
31. Rasko DA, Rosovitz MJ, Økstad OA, Fouts DE, Jiang L, Cer RZ, *et al*. 2007. Complete sequence analysis of novel plasmids from emetic and periodontal *Bacillus cereus* isolates reveals a common evolutionary history among the *B. cereus*-group plasmids, including *Bacillus anthracis* pXO1. *J. Bacteriol.* **189:** 52-64
32. Salamitou S, Ramisse F, Brehélin M, Bourguet D, Gilois N, Gominet M, *et al*. 2000. The *plcR* regulon is involved in the opportunistic properties of *Bacillus thuringiensis* and *Bacillus cereus* in mice and insects. *Microbiology* **146:** 2825-2832.
33. Schmidt TR, Scott EJ II, Dyer DW. 2011. Whole-genome phylogenies of the family Bacillaceae and expansion of the sigma factor gene family in the *Bacillus cereus* species-group. *BMC Genomics* **12:** 430.
34. Schnepf E, Crickmore N, Van Rie J, Lereclus D, Baum J, Feitelson J, *et al*. 1998. *Bacillus thuringiensis* and its pesticidal crystal proteins. *Microbiol. Mol. Biol. Rev.* **62:** 775-806.
35. Schoeni JL, Wong AC. 2005. *Bacillus cereus* food poisoning and its toxins. *J. Food Prot.* **68:** 636-648.
36. Siegel JP. 2001. The mammalian safety of *Bacillus thuringiensis*-based insecticides. *J. Invertebr. Pathol.* **77:** 13-21.
37. Stenfors Arnesen LP, Fagerlund A, Granum PE. 2008. From soil to gut: *Bacillus cereus* and its food poisoning toxins. *FEMS Microbiol. Rev.* **32:** 579-606.
38. Tran SL, Guillemet E, Ngo-Camus M, Clybouw C, Puhar A, Moris A, *et al*. 2011. Haemolysin II is a *Bacillus cereus* virulence factor that induces apoptosis of macrophages. *Cell. Microbiol.* **13:** 92-108.
39. Vilas-Boas G, Sanchis V, Lereclus D, Lemos MV, Bourguet D. 2002. Genetic differentiation between sympatric populations of *Bacillus cereus* and *Bacillus thuringiensis*. *Appl. Environ. Microbiol.* **68:** 1414-1424.
40. Wilcks A, Hansen BM, Hendriksen NB, Licht TR. 2006. Fate and effect of ingested *Bacillus cereus* spores and vegetative cells in the intestinal tract of human-flora-associated rats. *FEMS. Immunol. Med. Microbiol.* **46:** 70-77.
41. Wilcks A, Hansen BM, Hendriksen NB, Licht TR. 2006. Persistence of *Bacillus thuringiensis* bioinsecticides in the gut of human-flora-associated rats. *FEMS. Immunol. Med. Microbiol.* **48:** 410-418.
42. Zahner V, Cabral DA, Régua-Mangia AH, Rabinovitch L, Moreau G, McIntosh D. 2005. Distribution of genes encoding putative virulence factors and fragment length polymorphisms in the *vrrA* gene among Brazilian isolates of *Bacillus cereus* and *Bacillus thuringiensis*. *Appl. Environ. Microbiol.* **71:** 8107-8114.