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Article

Toxin Gene Profiling of *Bacillus cereus* Food Isolates by PCR

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Seventy-one *Bacillus cereus* strains (12 references and 59 food isolates) were analyzed for the occurrence of five different enterotoxin genes (*nheABC*, *hblCDA*, *entFM*, *cytK*, and *bceT*) and one emetic toxin cereulide synthetase gene (*ces*) by PCR (polymerase chain reaction). PCR analysis revealed eight toxigenic patterns in all *B. cereus* strains tested; they all carried both *entFM* and *nheABC*. The presence of *hblCDA, cytK,* and *bceT* varied according to the enterotoxin-producing strains, among which *hblCDA* was the least frequently detected in the food-isolated strains. Only five *B. cereus* strains harbored *ces*, associated with the emetic type of food poisoning; however, these strains were devoid of *hblCDA*, *cytK*, and *bceT*.

Key words: *Bacillus cereus, emetic toxin, enterotoxin, PCR, toxigenic pattern*

Bacillus cereus is a Gram-positive, spore-forming food pathogen commonly found in soil, plants, and various kinds of foods [Kotiranta *et al.*, 2000; Rowan *et al.*, 2001; Stenfors Arnesen *et al.*, 2006]. Food poisoning caused by *B. cereus* can be classified into two types: diarrheal and emetic types. The diarrheal type of food poisoning causes abdominal pain and watery diarrhea after 8-16 h of the latent period [Rowan *et al.*, 2003]. The five different enterotoxins causing the diarrheal type of food poisoning are HBL, NHE, enterotoxin-T, cytotoxin-K, and enterotoxin FM [Sergeev *et al.*, 2005]. Among these, the HBL complex consists of three types of proteins encoded by the *hblC*, *hblD*, and *hblA*. NHE is also composed of three components encoded by the *nheA*, *nheB*, and *nheC*. Enterotoxin-T, cytotoxin-K, and enterotoxin FM consist of a single protein encoded by the *bceT*, *cytK*, and *entFM*, respectively.

The emetic type of food poisoning caused by *B. cereus* is characterized by vomiting and nausea [Altayer and Sutherland, 2006]. Emetic toxin causing the emetic type of food poisoning is a circular dodecadepsipeptide, known as cereulide. Cereulide does not lose its activity even at 121°C, has tolerance for a wide pH range of 2-11, and is stable after pepsin and trypsin treatments [Kotiranta *et al.*, 2000; Rajkowski and Smith, 2001; Ehiling-Schulz *et al.*, 2005a]. The symptoms of both types of food poisoning are relatively mild, although more severe cases have occasionally been reported, some even resulting in death [Dierick *et al.*, 2005].

B. cereus is commonly found in the food production environments due to the formation of endospores resistant to heat, desiccation, and disinfectants, and thereby contaminating many kinds of foods during the production stage [Pirttijärvi *et al.*, 2000; Ghelardi *et al.*, 2002; Stenfors Arensen *et al.*, 2008]. Traditionally, the common methods for detection and identification of the enterotoxin and the emetic toxin-producing *B. cereus* strains in contaminated foods rely on the culture techniques and the biochemical methods, both being time- and labor-intensive. Recently, PCR, based on DNA amplification, became recognized as one of the most important genetic tools in molecular diagnostics. The various toxins produced by *B. cereus* are the most popular targets for the identification of *B. cereus* strains [Hansen and Hendriksen, 2001; Sergeev *et al.*, 2005; Yang *et al.*, 2005; Lee *et al.*, 2008].

The objective of the present work was to determine the presence of enterotoxin genes and the emetic-specific sequences in 71 *B. cereus* strains (12 reference strains and 59 food-isolated strains) by PCR analysis and evaluate the distribution of toxin genes in the food-isolated strains.

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Abbreviations: *ces*, cereulide synthetase gene; HBL, hemolysin BL; LB, Luria-Bertani; MYP, mannitol-egg yolk-polymyxin; NHE, non-hemolytic enterotoxin; PCR, polymerase chain reaction

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Materials and Methods

Bacterial strains. A total of 71 *B. cereus* strains were used in this study. *B. cereus* ATCC 11778, ATCC 12480, ATCC 13061, ATCC 14579, ATCC 21772, KCTC (Korean Collection for Type Cultures) 1013, KCTC 1014, KCTC 1092, KCTC 1094, KCTC 1526, and KFRI (Korea Food Research Institute) 181 were used as reference strains for the enterotoxin genes. Reference strain *B. cereus* F4810/72 was used for the evaluation of the emetic toxin-producing strain. Forty-nine *B. cereus* isolates (KFDA 202-213, KFDA 219-243, and KFDA 245-256) from food samples were obtained from the Department of Food Microbiology, Korea Food and Drug Administration. Ten *B. cereus* strains were isolated from *doenjang*, one of the traditional Korean soybean-fermented foods, using MYP agar as the selective medium and confirmed using the API 20E and 50CH kits (bioMerieux, Inc., Marcy l'Etoile, France). The non-target strains used in the present study were as follows: *Bacillus subtilis* KCTC 2213, *Bacillus amyloliquefaciens* KCTC 3002, *Campylobacter jejuni* ATCC 33238, *Clostridium perfringens* ATCC 3624, *Enterobacter sakazakii* ATCC 12868, *Escherichia coli* 0157:H7 KCTC 1039, *Listeria monocytogenes* ATCC 15313, *Salmonella enteritidis* KCCM 12021, *Salmonella typhimurium* ATCC 14028, *Shigella sonnei* KCTC 2518, *Staphylococcus aureus* ATCC 25923, *Vibrio parahemolyticus* ATCC 14547, and *Yersinia enterocolitica* KCCM 41657.

Primers and DNA extraction. The primers used in the present study for the detection of the toxin genes of *B. cereus* strains are shown in Table 1. All *Bacillus* strains were grown overnight in the LB broth at 37°C. The bacterial DNA was extracted from 1 mL of the overnight culture and purified using a Power Prep™ DNA Extraction Kit (Kogenebiotech, Seoul, Korea). The purified DNA was recovered in 100 µL of sterilized water. The DNA concentration was determined using a spectrophotometer at A_{260}.

PCR conditions. All PCR reactions were conducted in 25 µL volumes, each containing 50-100 ng of the template DNA, 10 pmol of each primer, 5 µL of 5×reaction buffer, 1 U of Taq polymerase, and deionized water. The PCR reactions were run on a PCR Express thermocycler (Hybaid, Waltham, MA) using the following program: 35 cycles of 30 s at 95°C, 30 s at 60°C, 1 min at 72°C, and finally 5 min at 72°C. Each reaction was conducted in triplicate. The PCR product (5 µL) was then loaded onto a 2% agarose gel containing ethidium bromide, resolved by electrophoresis, and visualized under UV illumination.

Table 1. Primers used in this study

Target gene	Primers	Primer sequence (5'→3')	Amplicon size (bp)	Ref.
bceT	BTF	GAC TAC ATT CAC GAT TAC GCA GAA	303	Lee *et al.* (2008)
	BTR	CTA TGC TGA CGA GCT ACA TCC ATA		
entFM	ENTFMF	AAA GAA ATT AAT GGA CAA ACT CAA ACT CA	596	Ghelardi *et al.* (2002)
	ENTFMR	GTA TGT AGC TGG GCC TGT ACG T		
cytK	Cyt-F	ATC GGK CAA AAT GCA AAA ACA CAT	800	Yang *et al.* (2005)
	Cyt-R	ACC CAG TTW SCA GTT CCG AAT GT		
hblA	FhblA	GCA AAA TCT ATG AAT GCC TA	884	Ngamwongsatit *et al.* (2008)
	RhblA	GCA TCT GTT CGT AAT GTT TT		
hblC	FHblC	CCT ATC AAT ACT CTC GCA A	695	
	RHblC	TTT CCT TTG TTA TAC GCT GC		
hblD	FHD	GAA ACA GGG TCT CAT ATT CT	1018	
	RHD2	CTG CAT CTT TAT GAA TAT CA		
nheA	NA-F1	ATT ACA GGG TTA TTG GTT ACA GCA GT	475	Yang *et al.* (2005)
	NA-R1	AAT CTT GCT CCA TACT CT CTT GGA TGC T		
nheB	NB-F1	GTG CAG CAG CTG TAG GCG GT	328	
	NB-R1	ATG TTT TTC CAG CTA TCT TTC GCA AT		
nheC	NC-F1	GCG GAT ATT GTA AAG AAT CAA AAT GAG GT	557	
	NC-R1	TTT CCA GCT ATC TTT CGC TGT ATG TAA AT		
ces	Ces3R	TTG TTG GAA TTG TCG CAG AG	405	Lee *et al.* (2008) Ehling-Schulz *et al.* (2006)
	CESR2	GTA AGC GAA CCT GTC TGT AAC AAC A		

Degenerate bases are designated as follows: K=G or T; W=T or A; and S=G or C.

Results and Discussion

Detection of various toxin genes using PCR. The specificities of the nine primer sets of five different enterotoxin genes and one primer set for the emetic-specific sequence directed against *ces* of *B. cereus* strains were evaluated in 12 reference strains, 59 food isolates, and 13 non-target strains by PCR (Tables 2 and 3). The nine primer sets mentioned above were also used to verify the presence of enterotoxin genes in the closely related *B. cereus* group strains comprising *B. cereus*, *B. thuringiensis*, *B. mycoides*, *B. anthracis*, and *B. weihenstephanensis*. None of the 13 non-target strains cross-reacted with the 10 primer sets tested (data not shown).

The occurrence of enterotoxin genes and emetic-specific sequences of *B. cereus* reference strains are shown in Table 2. All *B. cereus* reference strains carried genes *nheA, nheB*, *nheC,* and *entFM*, as reported by previous studies [Hsieh *et al.*, 1999; Guinebretière *et al.*, 2002; Yang *et al.*, 2005; Ngamwongsatit *et al.*, 2008]. The presence of *hblC, hblD, hblA, cytK,* and *bceT* genes varied according to the enterotoxin-producing strains. The enterotoxin- and the emetic toxin-producing strains can be differentiated by the occurrence of *ces* in the emetic toxin producers [Ehling-Schulz *et al.*, 2005b].

Table 2. PCR analysis of *B. cereus* reference strains

Bacterium	Strain	Target gene									
		nheA	*nheB*	*nheC*	*cytK*	*bceT*	*entFM*	*hblA*	*hblC*	*hblD*	*ces*
Bacillus cereus	ATCC 11778	+	+	+	+	+	+	+	+	+	-
	ATCC 21772	+	+	+	-	-	+	-	-	-	-
	ATCC 13061	+	+	+	-	-	+	-	-	-	-
	ATCC 14579	+	+	+	+	+	+	+	+	+	-
	ATCC 12480	+	+	+	+	+	+	+	+	+	-
	KFRI 181	+	+	+	+	+	+	-	-	-	-
	KCTC 1013	+	+	+	+	-	+	+	+	+	-
	KCTC 1014	+	+	+	-	-	+	-	-	-	-
	KCTC 1092	+	+	+	+	+	+	+	+	+	-
	KCTC 1094	+	+	+	+	+	+	+	+	+	-
	KCTC 1526	+	+	+	+	+	+	+	+	+	-
	F4810/72	+	+	+	-	-	+	-	-	-	+

+: PCR product of the expected size was observed.
-: No PCR product was observed.

Table 3. PCR analysis of *B. cereus* food isolates

Bacterium	No. of Strains	Target gene									
		nheA	*nheB*	*nheC*	*cytK*	*bceT*	*entFM*	*hblA*	*hblC*	*hblD*	*ces*
KFDA Isolates (total 49)	11	+	+	+	+	+	+	-	-	-	-
	10	+	+	+	+	+	+	+	+	+	-
	10	+	+	+	-	+	+	-	-	-	-
	7	+	+	+	-	-	+	-	-	-	-
	5	+	+	+	-	+	+	+	+	+	-
	2	+	+	+	+	-	+	-	-	-	-
	1	+	+	+	+	-	+	+	+	+	-
	1	+	+	-	-	-	+	-	-	-	-
	2	+	+	+	-	-	+	-	-	-	+
Doenjang Isolates (total 10)	3	+	+	+	+	+	+	+	+	+	-
	2	+	+	+	-	-	+	-	-	-	-
	1	+	+	+	+	+	+	-	-	-	-
	1	+	+	+	+	-	+	-	-	-	-
	1	+	+	+	-	+	+	-	-	-	-
	2	+	+	+	-	-	+	-	-	-	+

Table 4. Different toxigenic patterns of *B. cereus* strains used in this study

Pattern	*nheABC*	*cytK*	*bceT*	*entFM*	*hblCDA*	*ces*	No.(%) of *B. cereus* strain	
I	+	+	+	+	+	-	19	(26.8)
II	+	+	+	+	-	-	13	(18.3)
III	+	-	-	+	-	-	13	(18.3)
IV	+	-	+	+	-	-	11	(15.5)
V	+	-	+	+	+	-	5	(7.0)
VI	+	+	-	+	-	-	3	(4.2)
VII	+	+	-	+	+	-	2	(2.8)
VIII	+	-	-	+	-	+	5	(7.0)

Among the reference strains, only the F4810/72 strain, known as an emetic toxin-producing reference strain, was positive for the *ces*-specific primers.

All 59 food-isolated strains tested contained *nheA, nheB*, *nheC, and entFM*, similar to the reference strains (Table 3). The presence of *hblCDA, cytK,* and *bceT* in the food-isolated strains also varied. Food isolates KFDA 229 and KFDA 250 were previously reported as emetic toxin-producing strains [Lee *et al.*, 2008]. Two strains isolated from *doenjang* were also judged as emetic toxin-producing strains. All emetic toxin-producing strains tested in the present study harbored the emetic-specific sequence and the enterotoxin genes, *nheABC* and *entFM*. The results of some of the previously reported emetic toxin-producing strains were identical to ours, yet in some other cases *cytK* was also detected [Ehling-Schulz *et al.*, 2005b; Yang *et al.*, 2005]. The presence of an additional enterotoxin gene in the emetic toxin-producing strains suggests that the symptoms of emetic-type food poisoning may occasionally include diarrhea after vomiting [Yang *et al.*, 2005]. Thus, it can be deduced that the emetic toxin-producing strain emerged recently with the acquirement of *ces* by some enterotoxin-producing strains [Ehling-Schulz *et al.*, 2005b].

Toxigenic patterns of *B. cereus* strains. A total 71 *B. cereus* strains including the reference strains and the food isolates were divided into eight different toxigenic patterns according to the presence of the five enterotoxin genes, *nheABC*, *cytK*, *bceT, hblCDA*, and *entFM*, and the emetic-specific sequences (Table 4). The toxigenic patterns of enterotoxin-producing strains were classified into I to VII, and VIII was further classified into the emetic toxin-producing strain. In pattern I (19 strains, 26.8%), all five enterotoxin genes occurred simultaneously, while pattern II (13 strains or 18.3%) was devoid of *hblCDA*. Pattern III (13 strains, 18.3%) carried only two enterotoxin genes, *nheABC* and *entFM*. Pattern IV (11 strains, 15.5%) lacked both *hblCDA* and *cytK*. The percentages of the other patterns (V-VIII) were below 7%. In pattern VIII, five strains (7%) carried *ces*, and these strains were devoid of *hblCDA, cytK,* and *bceT*.

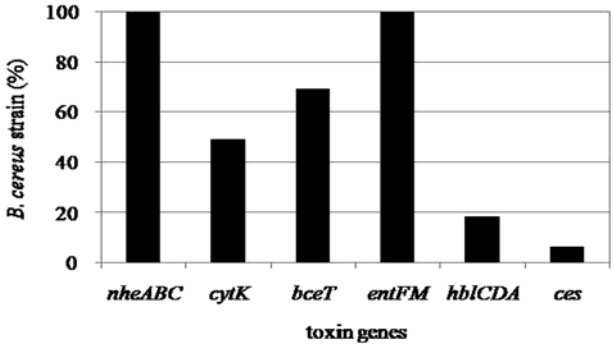

Fig. 1. Distribution of enterotoxin genes and emetic-specific sequence among 59 *B. cereus* food isolates.

Distribution of toxin genes in food-isolated strains. Figure 1 shows the comparison of the distributions of the five enterotoxin genes and the emetic-specific sequences of 59 food-isolated *B. cereus* strains. All food-isolated strains harbored enterotoxin genes, *nheABC* and *entFM*. Both genes were suitable targets for the rapid detection of the toxigenic *B. cereus* strains in foods by PCR. The occurrences of *bceT* and *cytK* in the food-isolated strains were 68 and 48%, respectively. The *hblCDA* genes were detected in 18% of the food-isolated strains, showing the lowest percentage out of all five enterotoxins; yet there were cases in which almost half of the food-poisoning strains contained *hblCDA* [Yang *et al.*, 2005]. The incidences of the enterotoxin genes in the food-poisoning strains are known to be higher than that of the food-isolated strains [Guinebretière *et al.*, 2002; Yang *et al.*, 2005]. This high occurrence of *hblCDA* in the food-poisoning strains revealed that the HBL, NHE, and cytotoxin-K proteins were the primary virulence factors in the diarrheal type of food poisoning [Granum *et al.*, 1999; Ngamwongsatit *et al.*, 2008]. The distribution of *ces*, whose detection confirms the presence an emetic toxin-producing strain, was found to be relatively low in

the food-isolated *B. cereus* strains. Compared to the ubiquitous presence of the enterotoxin-producing strains, the incidence of the emetic toxin producers was rare; emetic outbreaks have mainly been associated with such farinaceous foods as rice and noodles [Ghelardi *et al*., 2002; Ehling-Schulz *et al*., 2004; Ehling-Schulz *et al*., 2006]. Recently, *ces* has been used for the detection and the identification of the emetic *B. cereus* strains through the PCR-based assay [Ehling-Schulz *et al*., 2005a; Ehling-Schulz *et al*., 2006; Lee *et al*., 2008].

In the present study, the toxigenic patterns and distribution of five enterotoxin genes and the emetic-specific sequences of 71 *B. cereus* strains were analyzed. The results suggest that a PCR assay based on the amplification of various toxin genes may be useful for the rapid detection and differentiation of the *B. cereus* strains in the food products.

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