Journal of Food Safety

Journal of Food Safety ISSN 1745-4565

DETECTION OF EMETIC TOXIN GENES IN *BACILLUS CEREUS* ISOLATED FROM FOOD AND THEIR PRODUCTION OF CEREULIDE IN LIQUID CULTURE

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Received for Publication January 21, 2016
Accepted for Publication May 2, 2016

doi: 10.1111/jfs.12293

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ABSTRACT

Bacillus cereus is a Gram-positive that is ubiquitous in foods and the wider environment. The *B. cereus* emetic toxin, cereulide, synthesized by nonribosomal peptide synthetase (*NRPS*), can cause either gastrointestinal disorders and/or emetic form of food poisoning. In this study, 108 *B. cereus* isolates from foods were tested for a correlation between the presence of emetic toxin genes and cereulide production. Based on 16S rRNA sequence analysis, 107 isolates were identical to *B. cereus* spp. Further experiments showed that fourteen isolates exhibited 1,271, 2,200 and 188-bp bands, corresponding to amplification of the *ces*1, *ces*2 and *NRPS* gene, respectively. However, only 7 out of 13 strains possessing the *NRPS*, *ces*1, and *ces*2 genes produced cereulide in liquid culture. Strain number 93, isolated from fresh vegetables, produced the highest levels of cereulide (11.17 μg/mL); maximum cereulide levels were observed after 4 days of cultivation at 30C in LB broth. The amount of cereulide produced differed between each isolate.

PRACTICAL APPLICATIONS

The incident of *Bacillus cereus* in food could cause food poisoning. The results of our study on the presence of *B. cereus* in spoiled foods demonstrated that not all the food poisoning *B. cereus* spp. have the ability to synthesize the emetic toxin, cereulide. Nonribosomal peptide synthetase and cereulide synthetase genes could be used as biological markers for differentiation of cereulide producing and nonproducing *B. cereus.* Estimation of cereulide level reveals that the amounts of cereulide produced by some strains were very high and could be a threat to human health if consumed in contaminated foods. This study presents proper methods to select cereulide producing food-poisoning *B. cereus* spp. and gives more information on the condition of cereulide production to prevent future cases of emetic food poisoning.

INTRODUCTION

Bacillus cereus is a gram-positive, spore-forming, rod-shaped, and facultative anaerobe that is omnipresent in foods and the wider environment (Delbrassinne *et al.* 2015). *B. cereus* can cause two different forms of food poisoning which is diarrhea or emesis (Vangoitsenhoven *et al.* 2015), both of which are usually relatively mild and do not last more than 24 h. Nevertheless, some severe cases of *B. cereus* poisoning leading to death have also occurred (Dierick *et al.* 2005). Naranjo *et al.* (2011) reported a sudden death of a young adult resulted from *B. cereus* food poisoning. A *B. cereus* related emetic outbreak was also reported in a Belgian kindergarten which high levels of emetic *B. cereus* were detected in food (Delbrassinne *et al.* 2015).

The emetic form of *B. cereus* poisoning is caused by a small, hydrophobic, cyclic heat stable peptide toxin called cereulide (Frenzel *et al.* 2011), which is formed in food. Cereulide is a cyclic dodecadepsipeptide (D-O-Leu-D-Ala-L-O-

Val-L-Val)$_3$ and is stable over a wide pH range and resistant to proteolysis (Yamaguchi *et al.* 2013). Cereulide acts as an ionophore, transporting K^+ ions via the ion-carrier system into the mitochondria, downstream of the electrical and concentration gradients, similar to the mode of action of valinomycin (Hoornstra *et al.* 2003, 2013). Cell motility decreases due to the damaged mitochondria and subsequent failure of oxidoreductive functioning, causing changes in the macroscopic behavior of semen cells (Andersson *et al.* 2004). Therefore, the effects of cereulide potassium ionophore activity on mitochondrial function may lead to cell death (Vangoitsenhoven *et al.* 2014), resulting in emesis in the host. In addition, animal models also show that cereulide can cause cellular damage and inhibit human natural killer cells, and might, therefore, have an immunomodulating effect (Paananen *et al.* 2002; Rajkovic *et al.* 2008). Rajkovic *et al.* (2014) reported that low concentration of cereulide led to a significant decrease in energy managing and H_2O_2 detoxification proteins, increase in cell death markers, and may induce an altered enterocyte metabolism and membrane integrity. Cereulide also causes hepatotoxic effect and alters metabolism. Furthermore, other cell types such as sperm cells, beta cells, and human epithelial type 2 (Hep-2) cells also affected by cereulide toxicity (Vangoitsenhoven *et al.* 2015).

The lack of suitable and simple cereulide detection methods makes routine detection of this exotoxin in laboratory media and food samples difficult. Conventional methods, including bioassays of Hep-2 cell vacuolation and boar sperm immobilization, and high-performance liquid chromatography (HPLC) connected to ion-trap mass spectrometry (MS) are time consuming, laborious, and not precise (Forghani *et al.* 2015). Thus, several methods targeting toxin producing genes or species-specific genes have been developed (Forghani *et al.* 2016).

Although cereulide is produced by *B. cereus*, not all the *B. cereus* spp. found in foods have the ability to synthesize this toxin. Therefore, further experiments have to be performed to detect those strains that can produce cereulide. For this reason, many studies have been conducted to identify the specific genes coding for cereulide synthetase in cereulide-producing *B. cereus* strains. As a result, cereulide synthetase genes responsible for the nonribosomal production of cereulide (*ces* genes) have been identified and characterized. Furthermore, extensive molecular assays for the detection of emetic toxin producers have been described by Ehling-Schulz *et al.* (2005a,b, 2006a). Other studies have suggested that nonribosomal peptide synthetase (*NRPS*) may also be responsible for the production of cereulide (Horwood *et al.* 2004).

In this study, the correlation between the presence of emetic toxin genes (cereulide synthetase and *NRPS* genes) and the production of cereulide was analyzed. For this purpose, we examined a variety of foods in Korea for *B. cereus*, isolated and identified the specific contaminant strains. Identified *B. cereus* strains were then screened to detect emetic toxin genes (cereulide synthetase and *NRPS* genes). *B. cereus* strains with emetic toxin genes were then further investigated for the production of cereulide.

MATERIALS AND METHODS

B. cereus Isolates and Reference Strain

One hundred and eight *B. cereus* isolates were obtained from the Ministry of Food and Drug Safety in the Republic of Korea. These strains were isolated from Gochujang, Doenjang, Sashimi, fresh vegetables, and Gimbap available in Korea domestic markets. *B. cereus* strain F4810/72 was used as a reference strain for cereulide production and as a positive control.

Extraction of DNA

B. cereus isolates and control strain F4810/72 were cultured in LB agar (Becton, Dickinson and Company, MD, USA) for 1 day at 37C. DNA of *B. cereus* isolates and F4810/72 was extracted using the DNeasy Tissue Kit (Qiagen, VWR International AB, Sweden) according to the manufacturer's instructions. Extracted DNA was used for template DNA and stored at −20C.

16S rRNA Gene Sequencing

A total of 108 isolates were considered for further identification at the species level using 16S rRNA gene sequence characterization, as described by Fernádez-No *et al.* (2015). *B. cereus* isolates were identified by 16S rRNA gene sequencing. The 16S rRNA gene was amplified using two universal primer sets (8UA/907B and 774A/1492R) under the Polymerized Chain Reaction (PCR) conditions shown in Table 1 (Kim *et al.* 2010b). The PCR product was detected by agarose gel electrophoresis. Sequencing of the 16S rRNA gene was performed using the dideoxynucleotide-chain-terminating DNA sequencing method. Both strands of the amplified products were sequenced with an ABI 3730XL DNA Analyzer. Sequencing data for the 16S rRNA gene from each of the 108 isolates was analyzed by comparison of the accord sequence with the Genbank sequence, using the NCBI Basic Local Alignment Search Tool program.

Detection of Emetic Toxin Genes

All *B. cereus* isolates were screened to detect emetic toxin genes (*NRPS* and cereulide synthetase genes). The primer sets and PCR conditions used for amplification of the emetic toxin genes are shown in Table 1 (Horwood *et al.* 2004; Ehling-Schulz *et al.* 2005b). The final PCR mixture (20 μL)

TABLE 1. PCR PRIMER AND AMPLIFICATION CONDITIONS USED FOR 16S RRNA GENE ANALYSIS AND EMETIC TOXIN GENE DETECTION

			Amplication conditions[a]			
Target genes	*Pimer names*	*Primer sequences(5′→3′)*	D	A	E	*Products (bp)*
16S ribosomal RNA	8UA	AGT GTT TGA TCC TGG CTC AG	95C,30 s	45C,30 s	72C,1 min	1,484
	907B	CCG TCA ATT CMT TTR AGT TT				
	774A	GTA GTC CAC GCT GTA AAC GA				
	1492R	GGT TAC CTT ACG ACT T				
Nonribosomal peptide synthetase (NPRS)	CER1	ATC ATA AAG GTG CGA ACA AGA	94C,1 min	52C,1 min	72C,1 min	188
	EMT1	AAG ATC AAC CGA ATG CAA CTG				
Cereulide synthetase (ces1)	cesF1	GGT GAC ACA TTA TCA TAT AAG GTG	95C,1 min	58C,75 s	72C,50 s	1,271
	cesR2	GTA AGC GAA CCT GTC TGT AAC AAC A				
Cereulide synthetase (ces2)	cesF1	GGT GAC ACA TTA TCA TAT AAG GTG				2,200
	cesR1	GTT TTC TGG TAA CAG CGT TCT AC				

[a]All PCR methods used an initial denaturation step at 95C for 5 min, followed by 35cycle at the temperatures and times indicated (D, denaturation; A, annealing; E, extension). A final extension step (5 min at 72C) was also included. PCR methods used an initial denaturation step at 95C for 10 min (NRPS) and 15 min (ces), followed by 35cycle at the temperatures and times indicated (D, denaturation; A, annealing; E, extension). A final extension step (5 min at 72C) was also included.

contained 5 μL of each template (as prepared above), 1U of Taq polymerase, 250 μM of dNTPs, 1.5 mM $MgCl_2$, 10 mM Tris-HCl (pH 9.0), 30 mM KCl, stabilizer and tracking dye (Bioneer, Korea). PCR amplifications were performed in a PTC-100TM Programmable Thermal Controller (MJ Research, Inc.). The amplification results were resolved by electrophoresis on a 1.5% agarose gel.

Extraction of Cereulide from Emetic Toxin-Producing Isolates

Cereulide was extracted following the method reported previously (Dybwad *et al.* 2012; Ueda *et al.* 2012) with a slight modification. *B. cereus* F4810/72 and isolates in which the *NRPS* and *ces* genes were detected were cultivated in LB agar for 1 day at 37C. Next, a single colony was selected, inoculated in 100 mL of LB broth in a 250 mL Erlenmeyer flask, and incubated at 30C with shaking at 150 rpm for 1 day. Cultures were then diluted in 200 mL of LB broth in a 500 mL Erlenmeyer flask to a final concentration of approximately 2×10^4 cfu/mL, and incubated at 30C with shaking at 150 rpm for 4 days. Biomass from liquid cultivation was collected using a centrifugal separator (900 × g for 15 min). Cells were lysed by three repeated freeze-thaw cycles and by using a French pressure cell press (1,000 psig) with 200 mL methanol. The resulting suspension was incubated for 24 h and then centrifuged at 900 × g for 15 min. The supernatant was collected in order to remove cell debris, evaporated to dryness, and the residue was resuspended in methanol (HPLC grade).

Analysis of Methanol Extract by HPLC

The methanol extract of emetic toxin-producing isolates and *B. cereus* F4810/72 were filtered through a PVDF filter (pore size, 0.22 μm; Advantec, MFS, Pleasanton, CA, USA) prior to HPLC analysis. The extracts were analyzed using a HPLC system (Agilent Technology 1200 series, USA) with a ZORBAX Eclipse XDB-C18 column (Agilent, 4.6 × 150 mm, 5 μm). HPLC was performed for 45 min at a constant flow rate of 1 mL/min with a UV-detector (Agilent Technology 1200 Diode Array Detector, USA) at 205 nm. The mobile phase consisted of acetonitrile, water, and trifluoroacetic acid (95:4.9:0.1, v/v).

Analysis of Cereulide by Liquid Chromatography-Tandem MS (LC-MS/MS)

The cereulide peak was separated from *B. cereus* F4810/72 methanol extraction using the Agilent HPLC system and the peak fraction (retention time of 22.38 min) was collected using a fraction collector. This fraction was then evaporated to dryness and the residue was resuspended in 1 mL of the mobile phase. LC-MS/MS was performed using a Thermo LTQ Velos (Accela HPLC, Thermo, USA). The analytical column was a ZORBAX Eclipse XDB-C18 (4.60 × 150 mm, Agilent, USA). The mobile phase was a mixture of acetonitrile, water and trifluoroacetic acid (95:4.9:0.1, v/v). Liquid chromatography was carried out under isocratic conditions with a flow rate of 1 mL per minute. A volume of 20 μL was injected for LC-MS/MS, and the samples molecular weight was compared with cereulide mass data. MS was performed in the positive electrospray ionization (ESI-) mode. For identification of molecular cereulide ions, the 1,153 ($M+H^+$ adduct) and 1,170 ($M+NH_4^+$ adduct) m/z values were detected. *B. cereus* F4810/72 was used as positive control.

Production of Cereulide

Production of Cereulide from Isolates. Thirteen isolates that possessed emetic toxin genes (*NRPS* and *ces* genes) were selected from 108 isolates and their cereulide

production was further investigated. Each isolate, and *B. cereus* F4810/72, were cultivated in LB agar for 1 day at 37C. A single colony was selected from the LB agar and inoculated in 100 mL of LB broth in a 250 mL flask for precultivation. Each isolate was then cultured in 200 mL of LB broth in a 500 mL Erlenmeyer flask to a final concentration of approximately 2×10^4 cfu/mL. Cultures were then incubated at 30C with shaking at 150 rpm for 4 days. Cells were harvested after 4 days of incubation and extracted with methanol as described above. Each sample of methanol extract was analyzed by HPLC as described above. The cereulide-confirmed peak (retention time of 22.38 min) was collected, concentrated in a vacuum evaporator and used as cereulide standard compound. HPLC analysis was performed by diluting the cereulide standard to concentrations of 500, 1,000, 2,500, 5,000 and 10,000 ppm; the area value of each concentration was then used to produce a cereulide calibration curve. Quantitative analysis of cereulide from each isolate in methanol extract was measured by HPLC using this calibration curve.

Cereulide Production During Cultivation. Strains that produce the highest level of cereulide were used to investigate the production of cereulide during cultivation. Two-hundred milliliters of LB broth were inoculated with an overnight culture and cultivated at 30C with shaking at 150 rpm. At each time point, cultivation was used to determine CFU and cereulide production. A serial dilution series was inoculated on LB agar for 24 h, and the colonies were counted to determine viable counts. Cereulide production was analyzed by HPLC using the cereulide calibration curve after extraction with methanol as described above.

RESULTS AND DISCUSSION

Identification of 108 *B. cereus* Isolates from Foods Sources

Approximately 1,400 bp of the 16S rRNA gene isolated from each food source was amplified and sequenced by PCR with overlapping primer pairs. Among the 108 isolates, the partial sequences of the 16S rRNA confirmed that 107 isolates are *B. cereus* spp. (99–100% similarity) and one isolate (stain No. 23) were confirmed to be *Bacillus cytotoxicus* spp. (Table 2). Therefore, further screening was done targeting the *ces*1, *ces*2 and *NRPS* genes.

In a study by Caamaño-Antelo *et al.* (2015), genetic discrimination of *Bacillus* spp. found interspecies sequence similarities of 92.06% for the 16S rRNA gene (1,538–1,555 bp). Apetroaie *et al.* (2005) also reported that the 16S rRNA gene sequences (1,442 or 1,521 bp) of 13 verified cereulide-producing strains were 100% identical over a continuous stretch of 1,435 bp, regardless of their origins. All these sequences were also identical to the database sequence of the cereulide-producing *B. cereus* F 4810/72 strain over a continuous stretch of 1,424 bp, and to the database sequences of *B. anthracis* strains Ames (AF155950) and Sterne (AF290552) over a continuous stretch of 1,435 bp. The 16S rRNA gene sequence of one (NC 08234-02) of the four virulent *B. anthracis* strains used as reference strains was also indistinguishable from those of the *B. cereus* cereulide producers.

TABLE 2. IDENTIFICATION AT SPECIES LEVEL OF 108 ISOLATES FROM FOODS BASED ON 16S RRNA GENE ANALYSIS

Sample No.	Sources	Number of isolates	16S rRNA gene (type strain)
1–15	Gochujang	15	*Bacillus cereus*(ATCC14579)
16–26	Doenjang	10	*Bacillus cereus*(ATCC14579)
		1	*Bacillus cytotoxicus*(NVH 391-98)
27–35	Sashimi	9	*Bacillus cereus*(ATCC14579)
36–42	Fresh vagetable	7	*Bacillus cereus*(ATCC14579)
43–72	Gimbap	30	*Bacillus cereus*(ATCC14579)
73–102	Fresh vagetable	30	*Bacillus cereus*(ATCC14579)
103–108	Doenjang	6	*Bacillus cereus*(ATCC14579)
Total		108	

Thus, in this study 108 isolates from food were further subjected to PCR using primers for *NRPS* and *ces* genes, to detected these cereulide-producing genes and distinguish cereulide-producing strains from other *B. cereus* strains.

Detection of Emetic Toxin Genes

Horwood *et al.* (2004) reported in their study that *NRPS* gene (a small 500-bp gene fragment which primer sequences are shown in Table 1) involves in cereulide production. Ehling-Schulz *et al.* (2005b) suggested that cereulide is synthesized nonribosomally by the peptide synthetase *Ces* (*ces*1 and *ces*2 which primer sequences are shown in Table 1). Therefore, in our study we decided to adopt primers for these three emetic toxin genes to detect potential cereulide-producing strains.

In our present study, three genes, *NRPS*, *ces*1 and *ces*2 genes, encoding the nonribosomal peptide synthetase gene and cereulide synthetase genes, were amplified by PCR for all isolates (Fig. 1). The *NRPS* gene (188 bp) was detected in 14 isolates, the *ces*1 gene (1,271 bp) was detected in 13 isolates, and the *ces*2 gene (2,200 bp) was detected in 13 isolates. Thirteen isolates possessed all three genes. One of 15 (7%) strains isolated from Gochujang, 1 of 17 (6%) strains from Doenjang, and 1 of 9 (11%) strains from Sashimi had all three emetic toxin genes. Moreover, 7 of 37 (19%) strains isolated from fresh vegetable contained all three emetic toxin genes. Of the strains isolated from Gimbap, three possessed all three toxin genes and one further strain had only the *NRPS* gene (Table 3). Therefore, 13 strains were considered to be possible cereulide-producing strains as they possessed

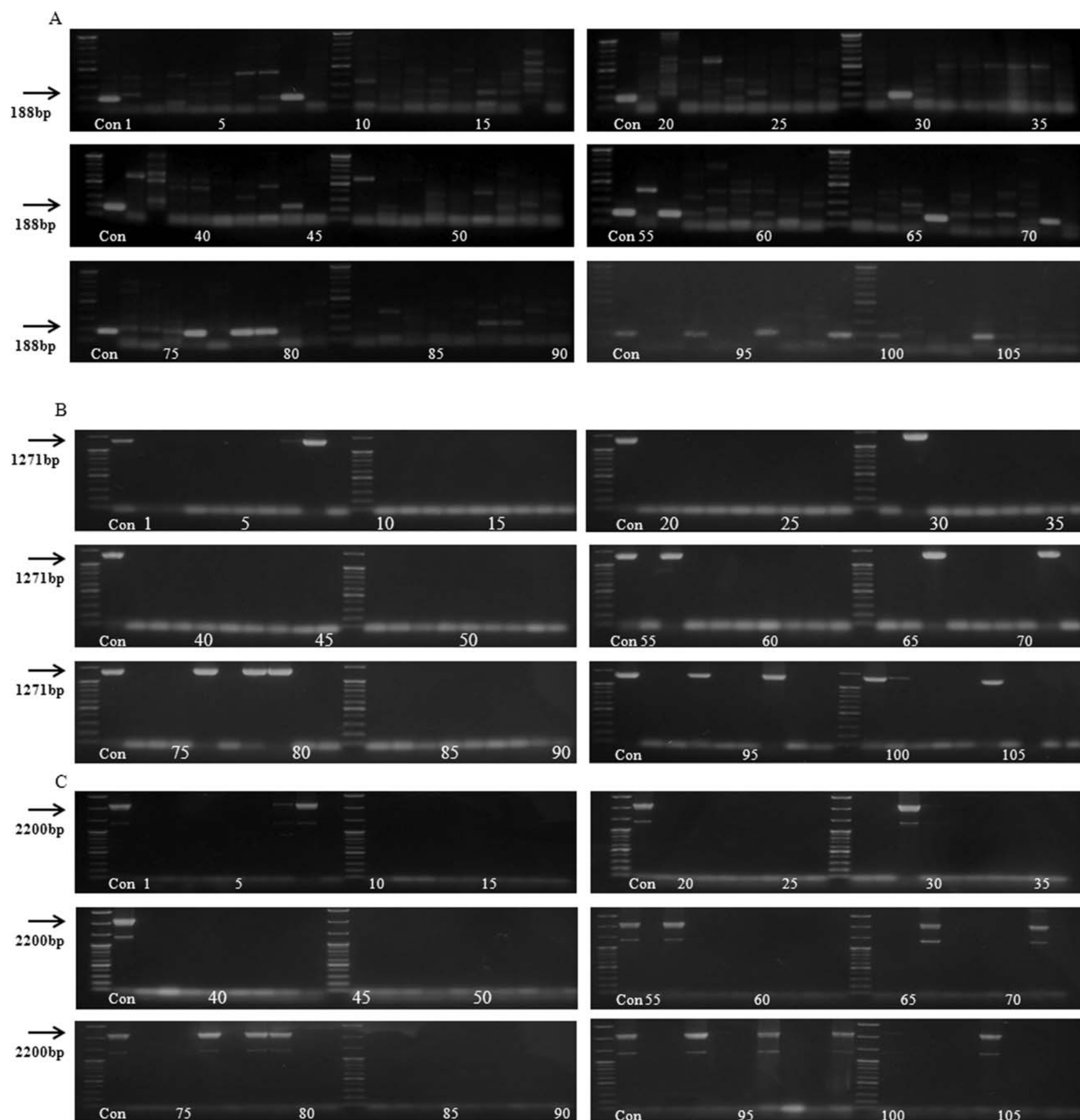

FIG. 1. PCR ANALYSIS OF (A) NONRIBOSOMAL PEPTIDE SYNTHETASE GENE (*NRPS*, 188 BP), (B) CEREULIDE SYNTHETASE GENES (*CES*1, 1,271 BP AND (C) *CES*2, 2,200 BP) OF108 ISOLATES

all three emetic toxin genes, and these strains were chosen for quantification of cereulide production.

Kim *et al.* (2014) profiled the enterotoxin and emetic toxin genes of *B. cereus* and *B. thuringiensis* in Korean rice and reported that one isolate from 37 white rice samples possessed the *ces* (1,271 bp) gene. Ehling-Schulz *et al.* (2005b) identified *B. cereus* isolates derived from food poisoning cases. Two out of the 30 food isolates tested carried the *ces* gene and produced the emetic toxin cereulide. In another study, the cereulide synthetase gene, *ces* (1,271 bp), was detected in 18 out of 40 *B. cereus* strains isolated from food (Ehling-Schulz *et al.* 2006b). PCR targeting the *ces* gene was also performed to detect the cereulide synthetase gene in *B. cereus* in a study by Kim *et al.* (2010a). Three isolates and the emetic-type reference stain showed 1,271 bp bands amplified from the *ces* gene. However, Ankolekar *et al.*

TABLE 3. DISTRIBUTION OF EMETIC TOXIC GENES FROM 108 ISOLATES *B. CEREUS* STRAINS IN FOODS

		Target genes			
Sources	Number of isolates	*NRPS*	*ces*1	*ces*2	Ratio(%)
Gochujang	15	1	1	1	7
Doenjang	17	1	1	1	6
Sashimi	9	1	1	1	11
Fresh vegetable	37	7	7	7	19
Gimbap	30	4	3	3	10
Total	108	14	13	13	12

(2009) reported that none of *Bacillus* isolates possessed *ces* gene, except the positive control strain which a PCR product of 1,271 bp was detected.

Analysis of *B. cereus* Methanol Extract by HPLC

Methanol extract from *B. cereus* F4810/72 was analyzed by HPLC using C18 column with a diode array detector (DAD) at 205 nm. A mobile phase made up of 95% acetonitrile, 4.9% water and 0.1% trifluoroacetic acid was applied isocratically. The HPLC chromatogram (Fig. 2) showed one peak at a retention time of 22.38 min. This peak was collected for identification by LC-MS/MS to verify that it was cereulide.

Previously, HPLC analysis of cereulide has been performed using different methods. Various mobile phase combinations were used and the combination which yield the most satisfied result was acetonitrile: water: trifluoroacetic acid. C18 column was commonly applied for cereulide detection (Jääskeläinen *et al.* 2004; Hoornstra *et al.* 2013; Rønning *et al.* 2015). However, due to different HPLC conditions, the retention time of cereulide eluted from column were differed from one another.

Analysis of Cereulide by LC-MS/MS

The HPLC purified peak extracted from *B. cereus* 4810/72 with methanol was analyzed by LC-MS/MS. The LC-MS electrospray ionization (ESI-) mass spectrum for the cereulide peak is shown in Fig. 3, and includes protonated molecular ions at m/z 1,153.73 and 1,170.85. This spectrum indicated that the chromatogram (time versus relative abundance) of the cereulide $[H^+]$ adducts (A), and cereulide $[NH_4^+]$ adducts (B), showed the same mass spectrum as that of reference strain (*B. cereus* F4810/72) and that of previously reported studies by Yamaguchi *et al.* (2013). The mass/mass spectrum of the ion $[M+NH_4^+]$ at m/z 1,170.86 from the collected peak corresponding to mass ion $[M+NH_4^+]$ at m/z 1,170.86 of cereulide is shown in Fig. 3. A study by Biesta-Peters *et al.* (2010) found that the NH_4^+ adducts of cereulide had a molecular weight of 1,170.6, a value very similar to the result to that obtained in our study. Figure 3 also shows that the fragmentation patterns of a series mass ions retrieved from the precursor ion of cereulide correspond to the known sequence of hydroxy and amino acids in cereulide: O-Val–Val–O-Leu–Ala–O-Val–Val–O-Leu–Ala and O-Leu–Ala–O-Val–Val–O-Leu–Ala–O-Val–Val. Thus, it can be concluded that collected peak is cereulide.

Production of Cereulide

Production of Cereulide from *B. cereus* Isolates. A calibration curve for cereulide was obtained from a series of dilutions by HPLC. The correlation value of the calibration curve was 0.99969. The cereulide products from 13 *B. cereus* isolates were quantified using this calibration curve. LB

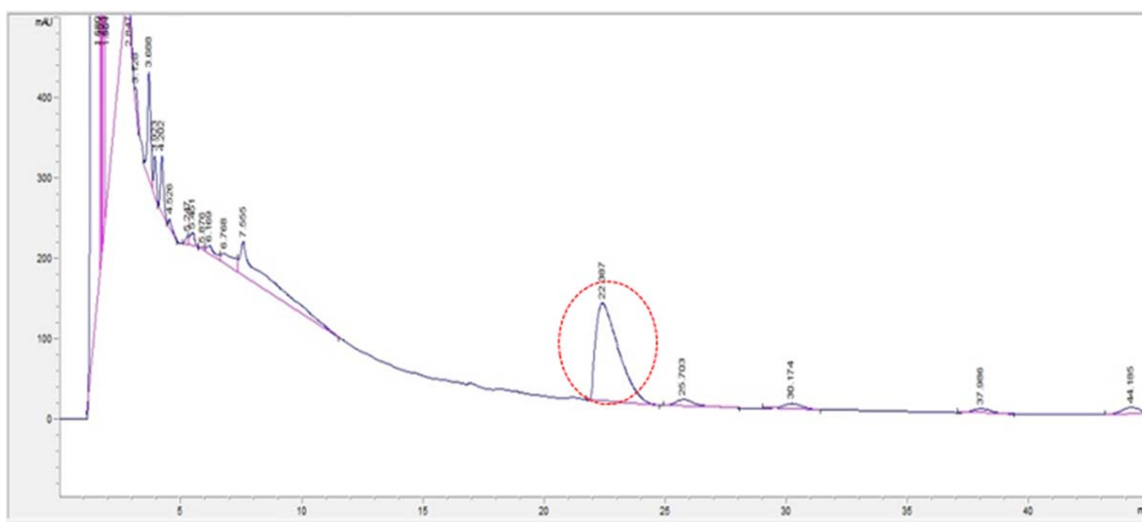

FIG. 2. HPLC CHROMATOGRAM FOR THE EXTRACT OF *B. CEREUS* F4810/72

FIG. 3. (A) MASS SPECTRUM OF CEREULIDE: A 1153.73 [M+H+] ION RANGE(M/Z), B 1170.86 [M+NH_4^+] ION RANGE(M/Z) AND (B) FRAGMENTATION PATTERNS OF THE SERIES MASS IONS FROM THE PRECURSOR ION AT M/Z 1170.85 AND CORRESPONDING AMINO AND HYDOX ACID SEQUENCES OF CEREULIDE

broth was used to determine the production of cereulide in our study as it is the commonly used liquid medium for cereulide production in various studies (Lücking *et al.* 2009; Frenzel *et al.* 2011; Lücking *et al.* 2015; Marxen *et al.* 2015). The isolate of strain number 93 (from fresh vegetables) produced the highest level of cereulide (11.17 μg/mL) which is 1.68 μg/mL higher than that of the reference strain, *B. cereus* F4810/72. The amount of cereulide produced by other isolates varied considerably (Table 4). Stark *et al.* (2013) analyzed the amount of cereulide produced *B. cereus* isolated from vomit or faeces from intoxicated patients. They successfully discriminate a total of 78 *B. cereus* strains carrying the *ces* genes into no/low, medium and high producers of the emetic toxin cereulide. In our present study, six of the isolates did not produce cereulide, despite possessing the *NRPS*, *ces*1 and *ces*2 genes. This could be because the level of expression of these genes was too low for cereulide production, or because these genes were not transcribed to mRNA. According to Lücking *et al.* (2015), cereulide biosynthesis is a complex network and undergoes multiple levels of toxin

TABLE 4. QUANTIFICATION OF CEREULIDE PRODUCTION FROM ISOLATED THIRTEEN *B. CEREUS* STRAINS IN FOODS

Source	Strain No.	Amount(μg/mL)
Standard strain	F4810/72	9.49
Gochujang	No. 8	1.78
Sashimi	No. 29	1.71
Gimbap	No. 56	2.32
Gimbap	No. 66	ND
Gimbap	No. 71	1.33
Fresh vegetable	No. 76	ND
Fresh vegetable	No. 78	ND
Fresh vegetable	No. 79	ND
Fresh vegetable	No. 93	11.17
Fresh vegetable	No. 96	9.57
Fresh vegetable	No. 99	0.77
Fresh vegetable	No. 100	ND
Doenjang	No. 104	ND

ND: Not decteted.

formation control. Therefore, further studied of the correlation of cereulide production with cereulide-producing gene expression at the transcriptional level should be conducted.

Häggblom *et al.* (2002) reported that strain NC 7401 accumulated up to 25 μg/mL of cereulide in liquid medium at room temperature (21C) in 1–3 days; this was higher than the level of cereulide production from the isolates identified in our study. Apetroaie-Constantin *et al.* (2008) found that *B. cereus* stain NS 58 produced large amounts of cereulide: up to 200–250 ng/mg on oatmeal agar inoculated at 37C for 48 h. Cereulide with a concentration of 539 ng/g in inoculated pasta and 1,351 ng/g in rice were detected by Rønning *et al.* (2015). During the outbreak in Belgian, Delbrassinne *et al.* (2015) quantified the level of cereulide in the leftovers and detected the level of cereulide varied between 3.1 and 4.2 μg/g in food samples. Cereulide production in infant food has been reported by Shaheen *et al.* (2006). When infant foods were inoculated with $>10^5$ cfu/mL of cereulide-producing *B. cereus*, 2–200 μg of cereulide per 100 mL of food accumulated during 24 h of nonrefrigerated storage (Shaheen *et al.* 2006). This result was lower than the level of cereulide production from the isolates in our study.

Cereulide Production During Cultivation. Cereulide concentration and *B. cereus* viability in relation to cultivation time is shown in Fig. 4. The log phase of *B. cereus* was between 3 and 6 h followed by a stationery phase 20–73 h and reached a phase of decline henceforth. However, the production of cereulide increased over prolonged cultivation time. The production of cereulide started after 12 h of inoculation and continued to increase significantly, even after the viability of *B. cereus* began to decrease. Cereulide production reached its maximum level at 4 days with a concentration of 9.02 μg/mL. In a study by Häggblom *et al.* (2002) that quantitatively analyzed cereulide production under various conditions, it was reported that the culture reached stationary phase within 20 h, with a cell density of 2×10^8 to 6×10^8 cfu/mL and cereulide production did not commence until after the end of logarithmic growth, approximately 24 h into the stationary phase. Although this finding differs from that of our study, similar trends in cereulide production were observed. According to Delbrassinne *et al.* (2011), cereulide was not detected at the time of inoculation, indicating that cereulide production may require a sufficient amount of bacterial cells. After 1 day, intoxicating levels of cereulide were found, and after this time the amount of cereulide produced steadily increased during cultivation period. Agata *et al.* (2002) reported that the production of cereulide

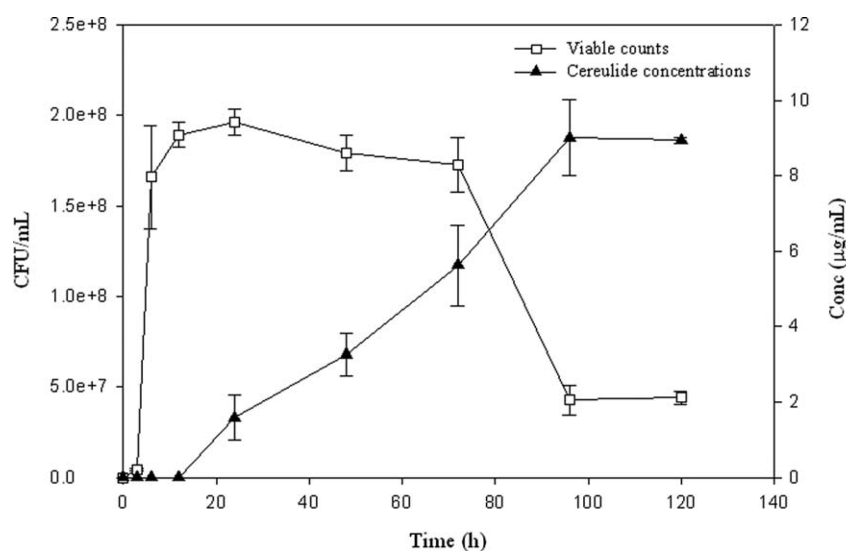

FIG. 4. CEREULIDE PRODUCTION DURING CULTIVATION

tended to increase with prolonged storage time in foods contaminated by *B. cereus*. This finding was also in accordance with the results of our study.

CONCLUSION

In this study, foods contaminated *B. cereus* isolates were identified, screened for emetic toxin genes, and the amount of cereulide produced by the isolated strains was quantified. The results showed that among 108 stains isolated, only 13 possessed emetic toxin genes. When the levels of cereulide produced by these strains were quantified, only 7 of 13 strains possessing the *NRPS*, *ces*1 and *ces*2 genes produced cereulide. Consequently, we found that the presence of emetic toxin genes (cereulide sythetase genes and the *NRPS* gene) is weakly correlated with cereulide production, owning to the fact that not all the strains with emetic toxin genes can produce cereulide. Therefore, future studies are needed to develop a more detailed understanding of the genes involved in cereulide production. The level of cereulide produced by each strain varied widely. The levels of cereulide were very high in some strains, and could be a threat to human health if consumed in contaminated foods. Thus, more analysis of the mechanisms of food poisoning caused by *B. cereus* is needed to help prevent future cases of emetic food poisoning.

ACKNOWLEDGMENT

This research was supported by the Basic Science Research Program through the National Research Foundation of Korea (NRF) funded by the Ministry of Education, Science, and Technology (2012R1A2A2A01003635). This research was also supported by the Chung-Ang University Graduate Research Scholarship in 2015.

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