# PCR–BASED DNA TESTS FOR DETECTION OF EMETIC *BACILLUS CEREUS* STRAINS PRODUCING CEREULIDE

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Two PCR-based methods for identification of emetic toxin producing *Bacillus cereus* strains were developed. The first set of primers cesBF and cesBR allowed for the amplification of *cesB*I 838 bp long fragment of cereulide biosynthesis operon for cereulide producing strains, or 421 bp long fragment of nonribosomal peptide synthetase (*nrps*) for emetic toxin non producing strains. Detection of both genes *cesB*I and *nrps* was possible in one PCR reaction. Among 24 strains of Cereus group tested, only one named 19W-cesB contained *cesB*I gene fragment. *Bacillus cereus* isolate 19W-cesB did not contain of any other genes of nonribosomal peptide synthetases responsible for the synthesis of other low molecular weight peptide toxins. The shdR and shdF second primer set allowed for specific amplification of the other 690 bp long fragment of *cesB*II gene. Only strain 19W-cesB allowed for the PCR synthesis of appropriate amplicon from all tested strains. Proposed methods may be fast and reliable techniques for detection of *Bacillus cereus* strains producing cereulide.

## INTRODUCTION

*Bacillus cereus* is a very common food borne pathogen responsible for two types of food poisonings. Diarrheal type is associated with production of diarrheal enterotoxins such as hemolysin BL, nonhemolytic enterotoxin NHE, cytotoxin K, enterotoxin BceT and other toxic proteins such as phospholipase C and lecithinase [Hansen & Hendriksen, 2001; Neil *et al.*, 2003; Ołtuszak-Walczak *et al.*, 2006]. Emetic type of food poisoning, characteristic for certain strains of *Bacillus cereus* is associated with the ability of them to produce cyclic depsipeptide, cereulide, consisting of triplicate repetitions of the following tetrapeptide D-O-Leu-D-Ala-L-O-Val-L-Val [Agata *et al.*, 1994; 1995]. Dierick *et al.* [2005], reported fatal food poisoning caused by cereulide producing *Bacillus cereus* contaminated food. Cyclic chemical structure of cereulide characterised by the alternating peptide and ester bonds as well as presence of D-amino acids, shows that it is synthesized nonribosomally by the enzymatic complex of nonribosomal peptide synthetase (NRPS). Ehling-Schulz *et al.* [2005], using degenerate PCR primers targeted to the known NRPS sequence motifs, discovered and sequenced large DNA fragment (5190 bp, AY691650) coding for partial sequence of nonribosomal peptide synthetase designated as *cesB*. Published sequence contained L-Valine activating, condensing and binding domains as well as thioesterase domain catalyzing cereulide cyclization. Complete cereulide biosynthesis gene cluster (24 000 bp, DQ360825) was recently sequenced by the same research group, and it revealed presence of seven genes that form an operon structure *cesHPT-*

*ABCD* [Ehling-Schulz *et al.*, 2006]. Genes responsible for cereulide biosynthesis from strain *Bacillus cereus* F4810/72 were located on megaplasmid pBCE4810 which was structurally similar to *Bacillus anthracis* toxin plasmid pXO1. Biochemical functions of nearly all genes involved in cereulide biosynthesis were also established. Gene *cesH* encode for hydrolase/acetyltransferase with unknown function whereas *cesP* encode for 4'-phosphopantetheinyl transferase, enzyme activating thiolation modules of nonribosomal peptide synthetases. Genes *cesA* and *cesB* encoding for cereulide specific nonribosomal peptide synthetase harbors two modules with the domain structure $A_i$-x-$A_{ii}$-T-C-A-T-E-C and $A_i$-x-$A_{ii}$-T-C--A-T-TE respectively. $A_i$ comprises the conserved core motifs A1-A8 while $A_{ii}$ contains the core motifs A9 and A10 of the amino acid activation module, and x refers to a region of unknown function. Genes *cesC* and *cesD* encode for the two--component ABC transporter possibly involved in cereulide secretion or cereulide resistance of producer cells.

Detection methods of cereulide producing strains of *Bacillus cereus* can be divided into four different groups.

First is based on the specific chemical properties of cereulide molecule for uncoupling of oxidative phosphorylation process and inhibition of ATP synthesis. It relies on reduction of respiration of rat liver mitochondria in the presence of cell extracts containing cereulide [Kawamura-Sato *et al.*, 2005].

Andersson *et al.* [1998], developed second sensitive, inexpensive, and rapid bioassay of the emetic toxin of *Bacillus cereus*. The assay was based on the loss of motility of boar spermatozoa upon 24 h of exposure to extracts from biomass of emetic *Bacillus cereus* strains or contaminated food.

Third method is based on HPLC analysis of methanol extracted biomass of emetic *Bacillus cereus* strains and may be a good choice for the detection of that kind toxin [Häggblom *et al.*, 2002].

Low molecular weight of cereulide results in a lack of its antigenic properties. Therefore it cannot be detected with immunological methods which were successfully applied for detection of diarrheal strains [Buchanan & Shultz, 1992, 1994; Beecher & Wong, 1994]. Low specificity of first three methods turned attention to PCR as an alternative for detection of emetic strains.

Horwood *et al.* [2004] published 497 bp fragment of DNA sequence (AY331260) that comprised part of cereulide synthetase gene. Similarly Toh *et al.* [2004] also sequenced 1661 bp DNA fragment (AY576054) being another part of cereulide synthetase gene. Both sequences were used for the development of detection methods of emetic *Bacillus cereus* strains based on PCR technique. *In silico* analysis of both sequences revealed 14 bp long overlapping sequence what suggested that they form common contig encoding cereulide synthetase fragment. Ehling-Schulz *et al.* [2005] also reported PCR detection method of *Bacillus cereus* emetic strains, with pair of primers used for the amplification of 1271 bp long DNA fragment, part of the 5190 bp AY691650 sequence. Computer alignment of sequence AY691650 with pair of sequences AY331260 and AY576054 did not revealed of any common parts what suggests that last two sequences belonged to different fragments of cereulide synthetase genes. Indeed, comparison of the whole sequence of cereulide gene cluster DQ360825 with sequences AY331260 and AY576054 showed that they are fragments of *cesA* gene whereas AY691650 comprises of *cesB* gene fragment. The aim of our work was to develop an alternative PCR-based method for the detection of cereulide producing emetic strains of *Bacillus cereus*.

## MATERIALS AND METHODS

**Bacterial strains.** Bacterial strains applied in this study (*Bacillus* sp. 1 – sp. 18) were isolated from different food products or environmental samples according to the method described in the Polish Standard [PN-EN: ISO 7932] using MYP selective medium (Oxoid). *Bacillus cereus* ŁOCK 0807, *Bacillus cereus* ATCC 11778, *Bacillus cereus* UL, *Bacillus thuringiensis* NCAIM 01262, *Bacillus mycoides* and *Bacillus pumilus* PL5 were used as reference strains. They originated from the Culture Collection of the Institute of Fermentation Technology and Microbiology, Technical University of Lodz.

**DNA preparation**. Chromosomal DNA was isolated according to the method of Marmur [1961] modified by additional treatment of lysozyme digested cell suspension with proteinase K.

**PCR amplification of *ces*BI and *ces*BII fragments**. Primer sequences for amplification of *cesB*I and *cesB*II fragments were derived from data records AY691650, AE016877 and DQ360825 using Vector NTI software, Invitrogen (Table 1). Amplification of *cesB*I fragment (838 bp) was performed in the following manner: 20 pmol of primer cesBF, 20 pmol of primer cesBR, 1.25 $\mu$L Red-Taq DNA polymerase (Sigma-Al-

drich), PCR reaction buffer 2.5 $\mu$L, dNTP Mix (200 mmol/L each, MBI Fermentas) 0.5 $\mu$L, were mixed together and supplemented with PCR grade water to a total volume of 24 $\mu$L. About 20 ng of *Bacillus cereus* chromosomal DNA in 1 $\mu$L of water was finally added as a template The amplification procedure consisted of one cycle of 2 min at 94$^\circ$C, followed by 35 cycles for 1 min at 94$^\circ$C, 1 min at 50$^\circ$C and 2 min at 72$^\circ$C with the final extension cycle for 2 min at 72$^\circ$C was performed using Uno II thermocycler, Biometra, with tube lid heating block set for 105$^\circ$C. The reaction mix for amplification of *cesB*II fragment (690 bp) was the same except of primers replaced by shdF and shdR in the concentration of 20 pmol each. The amplification procedure for *cesB*II fragment consisted of one cycle of 2 min at 94$^\circ$C, followed by 35 cycles for 1 min at 94$^\circ$C, 1min at 55$^\circ$C and 2min at 72$^\circ$C with final extension cycle for 2 min at 72$^\circ$C.

**Agarose gel analysis of PCR products**. Amplified PCR products of *cesB*I and *cesB*II fragments were analyzed on 1% (w/v) agarose gel in 0.5 TBE buffer containing 0.5 $\mu$g/mL ethidium bromide. Gels were run at 60 V for 3 h and photographed with digital camera through yellow filter.

**DNA sequencing.** Amplified PCR products obtained from reactions with cesBF and cesBR primers were purified with DNA cleanup kit "DNA Gdańsk" according to the manufacturer instruction prior to sequencing in the facilities provided by the "Laboratory of DNA sequencing and oligonucleotide synthesis", Institute of Biochemistry & Biophysics, PAS, Warsaw, Poland [http://oligo.ibb.waw.pl].

## RESULTS AND DISCUSSION

### Detection of cesBI fragment

Application of primers cesBF and cesBR allowed us for the detection of cesBI gene fragment (Figure 1) and obtained results showed that only one isolate of *Bacillus* sp. 3 assigned as W19-cesB contained cesB gene (about 840 bp amplification product, lane 9). PCR product characteristic for cesB gene has been sequenced in order to confirm its identity and obtained DNA sequence was deposited in GeneBank Database (Accession Number DQ238109). Its comparison with sequences of cesB gene (AY691650 and DQ360825) revealed 100% identity. Conceptual translation of amplified sequence into protein (ABB30175), showed presence of three regions: CaiC - being fragment of Acyl-CoA synthetases (AMP-forming)/AMP-acid ligase, PP-binding - Phosphopantetheine attachment site and Thioesterase domain of type I polyketide synthases or nonribosomal peptide synthetase. These findings fully confirmed that isolated strain *Bacillus cereus* 19W-cesB contained cesB gene and most probably was able to synthesize cereulide. Low intensity band of the same size (about 840 bp) was observed also for strain *Bacillus cereus* UL. PCR products of about 420 bp were observed for other strains of cereus group (lanes 5 - 8, 10, 12, 13-20 and 22-24). All of them had the same size but differed in band intensity. PCR product from strain Bacillus sp. 8 (lane 14) has been partially sequenced and obtained DNA sequence was compared to the NCBI GeneBank Database using BLASTN ver. 2.2.15, Oct-15-2006 [Altschul *et al.*, 1997]. Results showed its
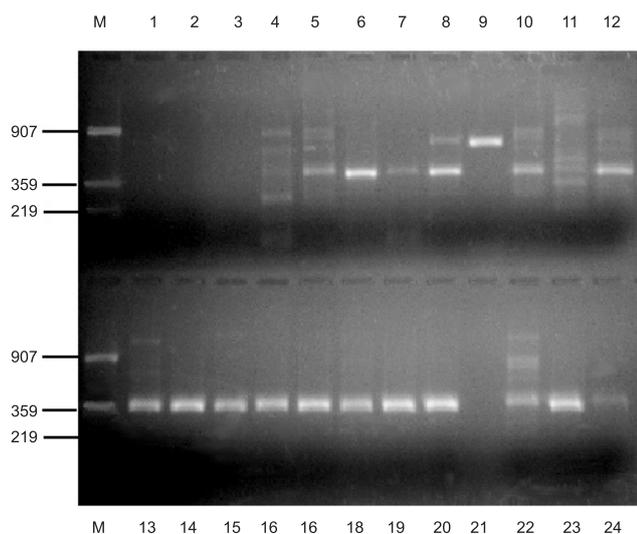
FIGURE 1. Electrophoregram of amplicons obtained from tested strains with cesBF and cesBR primers (*cesB*I fragment). W- DNA size marker; lanes: 1 - *Bacillus cereus* ŁOCK 0807; 2 - *Bacillus cereus* ATCC 11778; 3 - *Bacillus* sp. 1; 4 - *Bacillus* sp. 2; 5 - *Bacillus mycoides*; 6 - *Bacillus thuringiensis* NCAIM 01262; 7 - *Bacillus pumilus* PL5; 8 - *Bacillus cereus* UL; 9 - *Bacillus* sp. 3; 10 - *Bacillus* sp. 4; 11 - *Bacillus* sp. 5; 12-*Bacillus* sp. 6; 13-*Bacillus* sp. 7; 14 - *Bacillus* sp. 8; 15 - *Bacillus* sp. 9; 16 - *Bacillus* sp. 10; 17 - *Bacillus* sp. 11; 18 - *Bacillus* sp. 12; 19 - *Bacillus* sp. 13; 20 - *Bacillus* sp. 14; 21 - *Bacillus* sp. 15; 22 - *Bacillus* sp. 16, 23 - *Bacillus* sp. 17; 24 - *Bacillus* sp. 18.

96.8% identity with BC2456 gene fragment sequence (AE017005) of nonribosomal peptide synthetase operon of Bacillus cereus ATCC 14579 [Ivanova *et al.*, 2003], (Figure 2). *In silico* sequence analysis of BC2456 gene revealed that primers cesBF and cesBR

are compatible in 95.7% and 68.4% respectively to the analyzed fragment. Theoretical PCR product (421 bp) was similar in size to that obtained experimentally. Application of cesBF and cesBR primers allowed for detection in one PCR reaction of cesB gene, responsible for cereulide synthesis, as well as nonribosomal peptide synthetase genes nrps coding for the synthesis of other cyclic peptides. However, no strains in which two amplicons present simultaneously were found what may suggest that cereulide producing strains does not contain other nrps genes.

### Detection of cesBII fragment

Structural similarity between cereulide synthetase genes and other *nrps* genes usually present in *Bacillus cereus* genomes, showed that primers compatible to the activation, condensation and thiolation domains of cereulide and *nrps* genes are not so specific. Since *cesA* and *cesB* genes are characterized with unusual structures of first activation domains which are interrupted between A8 and A9 core motifs with region X coding for short chain dehydrogenase *shd* and unknown function, another set of primers shdF and shdR based on the analysis of $X_a$ and $X_b$ regions was designed for the detection of cereulide synthetase genes Table 1. They allowed for the amplification of 690 bp fragment of c*esB* gene located within sequence $X_b$. Complementary copy of primer shdR codes for TGGLGGIG amino acid sequence of protein CesB and TG-GLGGLG of CesA and are 100% compatible with *cesB* gene. Corresponding region of cesA gene is only 75% compatible with shdR primer. Again, shdF primer is 100% compatible to *cesB* gene and only 66.7% to *cesA* gene. In the latter case unspecific binding of both primers to the corresponding regions of *cesA* gene would result in amplification of 768 bp fragments. Among 20 *Bacillus cereus* isolates as well as 4 type strains from culture collection, only 19W-cesB gave positive



FIGURE 2. Alignment of 188 bp long fragments of *nrps* DNA sequence from fully sequenced chromosome of *Bacillus cereus* ATCC14579 and PCR product obtained from strain *Bacillus cereus* sp. 8 with pair of primers cesBF and cesBR.

TABLE 1. Primers used for PCR amplification of *cesB*I and *cesB*II fragments.

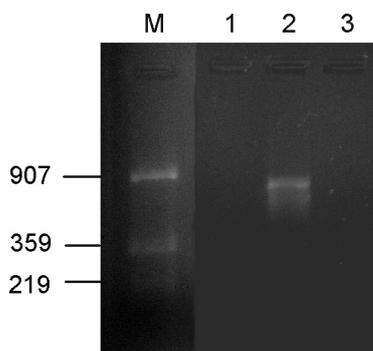| Gene fragment | Name | Primer | Sequence source |
|---|---|---|---|
| *cesB*I | CesBF<br>CesBR | 5'-CAAGTGAAAATTCGTGGATTCC-3'<br>5'-CCCCTAAGGAGTGGCCACC-3' | AY691650, AE016877 |
| *cesB*II | shdF<br>shdR | 5'-AATGACAGAACAATTCCTGCTT -3'<br>5'-TCCGATTCCACCTAAACCACCT-3' | DQ360825 |



FIGURE 3. Electrophoregram of three example amplicons obtained from tested strains with shdF and shdR primers (*cesB*II fragment). M- DNA size marker; lanes: 1 - *Bacillus cereus* ATCC 11778; 2 - *Bacillus* sp*.* 3 - (19W-cesB); 3 - *Bacillus cereus* sp. 4.

PCR reaction with new pair of primers resulted in amplification of 690 bp fragment (Figure 3). Such primers ensured high selectivity and specificity to the *cesB*II gene fragment.

**CONCLUSIONS**

A rapid and reliable method of distinguishing between cereulide positive and negative strains is very important to ensure that food products are safe. Proposed methods of identification based on PCR technique are the most accurate, fast and reliable and therefore may fulfill the technical gap in laboratory practice concerning detection of cereulide producing strains. Simultaneous detection of *ces*B gene and *nrps* genes in one PCR reaction with use of *ces*BF and *ces*BR primers introduces new possibility of strain characterization with respect to their ability for production of other than cereulide cyclic peptides with positive or negative biological activity. Alternatively the use of the second pair of primers shdR and shdF allows for specific detection of cereulide producing strains of *Bacillus cereus*.

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## WYKRYWANIE ZA POMOCĄ TESTÓW PCR WYMIOTNYCH SZCZEPÓW *BACILLUS CEREUS* WYTWARZAJĄCYCH CEREULID

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Opracowano dwie metody identyfikacji szczepów *Bacillus cereus* wytwarzających toksynę wymiotną cereulid, bazujące na reakcji PCR. Pierwszy zestaw starterów cesBF i cesBR pozwalał na amplifikację fragmentu genu *cesB*I o długości 838 pz dla szczepów zawierających operon biosyntezy cereulidu lub fragmentu genów nierybosomalnej syntetazy peptydowej (*nrps*) o długości 421 pz dla szczepów nie wytwarzających toksyny wymiotnej. W jednej reakcji PCR możliwa była detekcja genów biosyntezy cereulidu jak i genów biosyntezy innych cyklicznych peptydów (*nrps*). Spośród 24 przebadanych szczepów grupy Cereus wykazano jedynie u szczepu 19W-cesB obecność genu *cesB*. Stwierdzono również, że szczep wytwarzający toksynę wymiotną nie zawierał innych genów nierybosomalnych syntetaz peptydowych. Drugi zestaw primerów, shdR i shdF pozwalał na bardzo specyficzną amplifikację innego fragmentu genu *cesB*II o długości 690 pz gdyż spośród 24 szczepów, jedynie dla szczepu 19W-cesB otrzymano charakterystyczny amplikon. Zaproponowane metody pozwalają na szybką i prostą diagnostykę szczepów potencjalnych producentów toksyny wymiotnej.