Toxin gene profiling of enterotoxic and emetic *Bacillus cereus*

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Abstract

Very different toxins are responsible for the two types of gastrointestinal diseases caused by *Bacillus cereus*: the diarrhoeal syndrome is linked to nonhemolytic enterotoxin NHE, hemolytic enterotoxin HBL, and cytotoxin K, whereas emesis is caused by the action of the depsipeptide toxin cereulide. The recently identified cereulide synthetase genes permitted development of a molecular assay that targets all toxins known to be involved in food poisoning in a single reaction, using only four different sets of primers. The enterotoxin genes of 49 strains, belonging to different phylogenetic branches of the *B. cereus* group, were partially sequenced to encompass the molecular diversity of these genes. The sequence alignments illustrated the high molecular polymorphism of *B. cereus* enterotoxin genes, which is necessary to consider when establishing PCR systems. Primers directed towards the enterotoxin complex genes were located in different CDSs of the corresponding operons to target two toxin genes with one single set of primers. The specificity of the assay was assessed using a panel of *B. cereus* strains with known toxin profiles and was successfully applied to characterize strains from food and clinical diagnostic labs as well as for the toxin gene profiling of *B. cereus* isolated from silo tank populations.

Introduction

Toxin producing *Bacillus cereus* plays an important role as the causative agent of two types of food poisoning: diarrhea and emesis. The emetic syndrome is mainly characterized by vomiting a few hours after ingestion of the contaminated food. In the diarrhoeal syndrome, symptoms appear 8–16 h after ingestion, and include abdominal pain and diarrhea. In general, both types of food borne illness are relatively mild and self-limiting. Nevertheless, more severe cases have occasionally been reported involving hospitalization or even deaths (Mahler *et al.*, 1997; Lund *et al.*, 2000; Dierick *et al.*, 2005).

The two types of gastrointestinal disease caused by *B. cereus* are associated with very different types of toxins (Granum, 2001; Ehling-Schulz *et al.*, 2004a). The emetic syndrome is caused by a single heat stable peptide toxin called cereulide (Agata *et al.*, 1995), which is preformed in food. Cereulide has been shown to be toxic to mitochondria by acting as a potassium ionophore and has been reported to inhibit human natural killer cells (Paananen *et al.*, 2002). Recently, the peptide synthetase genes responsible for the nonribosomal production of cereulide (*ces* genes) have been identified and characterized, and the first molecular assays for the detection of emetic toxin producers have been described (Ehling-Schulz *et al.*, 2004b, 2005b, 2006). The diarrhoeal poisoning is caused by heat-labile enterotoxins produced during vegetative growth of *B. cereus* in the small intestine (Granum, 1994). At present, three different enterotoxins involved in food poisoning outbreaks are known: two protein complexes, hemolysin BL (HBL; Beecher *et al.*, 1995) and nonhemolytic enterotoxin (NHE; Granum *et al.*, 1999), and the single protein cytotoxin CytK (Lund *et al.*, 2000). HBL is a three-component hemolysin that consists of two lytic components (L2 and L1, encoded by *hbl*D and *hbl*C) and a binding protein B (encoded by *hblA*). NHE is also a three-component, but nonhemolytic, toxin that is encoded by three genes *nheA*, *nheB* and *nheC*. Both toxin complexes are organized in operons and the corresponding genes of the enterotoxin complex NHE have been shown to be transcribed together (Lindback *et al.*, 2004). Immunological assays are commercially available for the detection of NHE and HBL and monoclonal antibodies

targeting these enterotoxin complexes have been generated (Dietrich *et al.*, 1999, 2005) but no such tools are yet available for CytK or cereulide. Molecular assays for the detection of the different enterotoxin genes revealed a high degree of molecular diversity among the enterotoxin genes, which could lead to false negative results in PCR (Mäntynen & Lindström, 1998; Prüß *et al.*, 1999; Hansen & Hendriksen, 2001; Guinebretiere *et al.*, 2002).

The aim of this study was to develop a simple multiplex PCR system that allows the detection of all *B. cereus* toxins so far known to be involved in food poisoning, in a single reaction. Such an assay could improve diagnosis of gastrointestinal diseases caused by *B. cereus* and facilitate toxin gene profiling in population studies. Special emphasis was placed on the design of primers for enterotoxin genes since these toxins show great diversity at a molecular level while the cereulide synthetase genes are highly conserved in emetic *B. cereus* (Guinebretiere *et al.*, 2002; Ehling-Schulz *et al.*, 2005b).

Materials and methods

Bacterial strains

Forty-nine *Bacillus cereus* strains from diverse origins, belonging to different phylogenetic branches of the *B. cereus* group (Guinebretiere *et al.*, 2002, 2003) were used to assess the molecular diversity of enterotoxin genes (Fig. 1). Clinical strains and isolates from food with well-characterized toxin profiles were chosen for the development of the multiplex PCR assay. The reference set of strains comprised strains that carry the genetic loci of all three enterotoxins, namely LMG 17615 (F289/78), F3371/93, and 98HMPL63, and one strain, RIVM-BC91, that encode only the two enterotoxin complexes HBL and NHE. In addition, the original CytK strain NVH0391-98, which does not possess the genes encoding the enterotoxin complexes, and, as reference for the emetic toxin, the cereulide producing strain F4810/72 were added

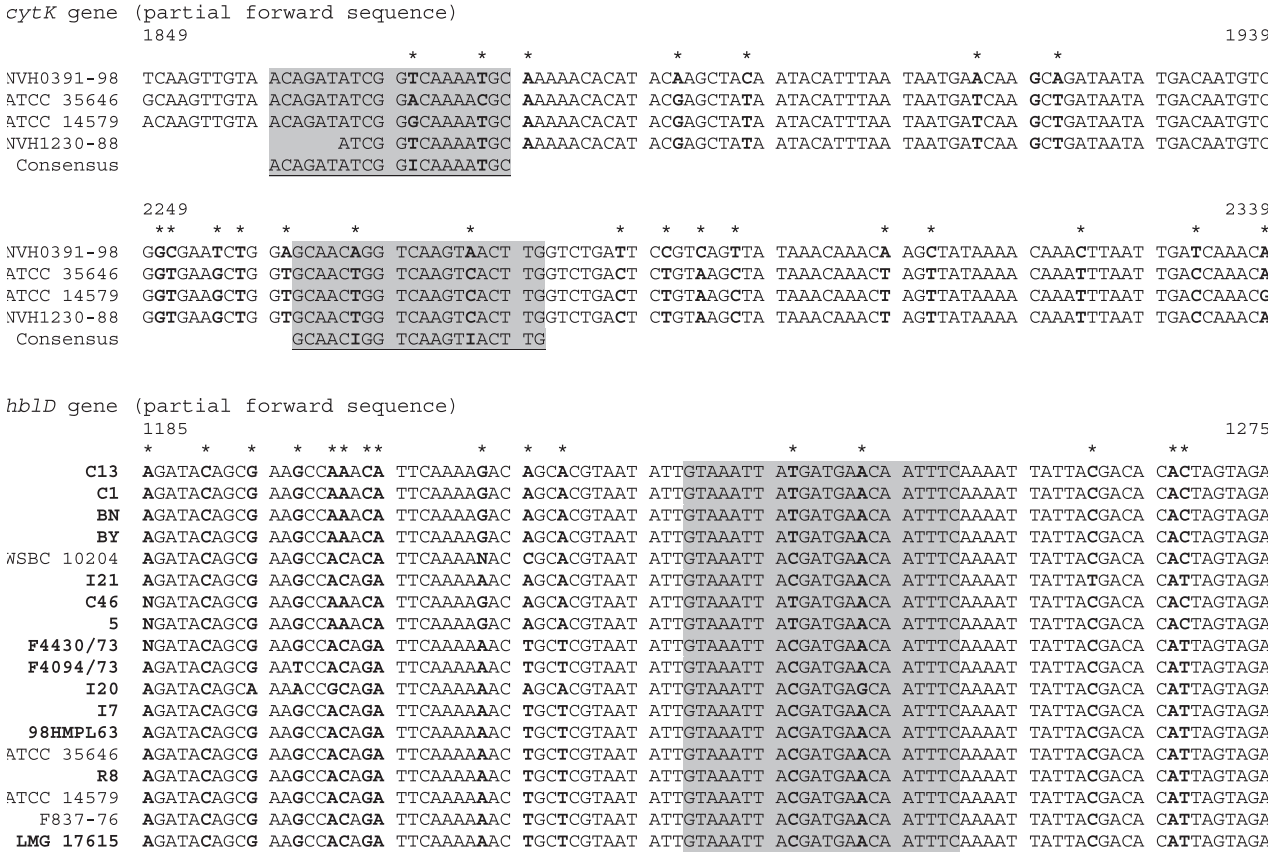

Fig. 1. Multiple sequence alignment of partial sequences of enterotoxin genes. Enterotoxin genes from strains of diverse origin were sequenced and aligned to database sequences to examine the molecular diversity of these genes. The detected point mutations are printed in bold face and marked by asterisk. The sequence sections for primer designation are shaded and designed primers, derived from consensus sequences, are underlined; 'I' refers to inosine substitutions. Positions of partial sequences were determined in reference to bank sequence with accession no. AJ277962 for *cytK*, AJ007794 for *hbl* and Y19005 for *nhe*. Sequences obtained in this study are printed in bold face (For strain descriptions, see Guinebretiere *et al.*, 2002). The other sequences were obtained from databanks.

```
        BC  AGATAAAGCG AAGCCACAGA TTCAAAAAAC AGCTCGTAAT ATTGTAAATT ACGATGAACA ATTTCAAAAT TATTACGACA CTTTAGTAGA
 F4635A-90  AGATAAAGCG AAGCCACAGA TTCAAAAAAC AGCTCGTAAT ATTGTAAATT ACGATGAACA ATTTCAAAAT TATTACGACA CTTTAGTAGA
CIP 103472  AGATAAAGCG AAGCCACAGA TTCAAAAAAC AGCTCGTAAT ATTGTAAATT ACGATGAACA ATTTCAAAAT TATTACGACA CTTTAGTAGA
        BL  AGATACAGCG AAGCCACAGN TTCAAAAAAC AGCACGTAAT ATTGTAAATT ACGATGAACA ATTTCAAAAT TATTATGACA CATTAGTAGA
 Consensus                                                GTAAATT AIGATGAICA ATTTC
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hblA gene (partial forward sequence)

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            2238                                                                                          2328
              **         * *         *         *                                        *         *       *     *
        BL  GAAGATGAAA GAAACCTTGC AAAAGGCCGG TTTATTTGCA AAATCTATGA ATGCCTATTC TTATATGTTA ATTAAAAATC CAGATGTGAA
 F4635A-90  GAGAATGAAA GAGACCTTGC AAAAAGCCGG GTTATTTGCA AAATCTATGA ATGCCTATTC TTATATGTTA ATTAAAAATC CAGATGTGAA
WSBC 10277  GAGAATGAAA GAGACCTTGC AAAAAGCCGG GTTATTTGCA AAATCTATGA ATGCCTATTC TTATATGTTA ATTAAAAATC CAGATGTGAA
WSBC 10256  GAGAATGAAA GAGACCTTGC AAAAAGCCGG GTTATTTGCA AAATCTATGA ATGCCTATTC TTATATGTTA ATTAAAAATC CAGATGTGAA
    WS2641  GAGAATGAAA GAGACCTTGC AAAAGGCCGG GTTATTTGCA AAATCTATGA ATGCCTATTC TTATATGTTA ATTAAAAATC CAGATGTGAA
WSBC 10360  GAGAATGAAA GAGACCTTGC AAAAAGCCGG GTTATTTGCA AAATCTATGA ATGCCTATTC TTATATGTTA ATTAAAAATC CAGATGTGAA
    WS3120  GAGAATGAAA GAGACCTTGC AAAAAGCCGG GTTATTTGCA AAATCTATGA ATGCCTATTC TTATATGTTA ATTAAAAATC CAGATGTGAA
WSBC 10202  GAGAATGAAA GAGACCTTGC AAAAAGCCGG GTTATTTGCA AAATCTATGA ATGCCTATTC TTATATGTTA ATTAAAAATC CAGATGTGAA
WSBC 10206  GAGAATGAAA GAGACCTTGC AAAAAGCCGG GTTATTTGCA AAATCTATGA ATGCCTATTC TTATATGTTA ATTAAAAATC CAGATGTGAA
    WS3119  GAGAATGAAA GAGACCTTGC AAAAGGCCGG GTTATTTGCA AAATCTATGA ATGCCTATTC TTATATGTTA ATTAAAAATC CAGATGTGAA
WSBC 10204  GAGGATGAAA GAAACCTTGC AAAAGGCCGG GTTATTTGCA AAATCTATGA ATGCCTATTC TTATATGTTA ATTAAAAATC CAGATGTGAA
    WS3118  GAGAATGAAA GAGACCTTGC AAAAGGCCGG GTTATTTGCA AAATCTATGA ATGCCTATTC TTATATGTTA ATTAAAAATC CAGATGTGAA
       C41  GAGGATGAAA GAAACTTTGC AAAAGGCAGG ATTATTTGCA AAATCTATGA ATGCCTATTC TTATATGTTA ATTAAGAATC CAGATGTGAA
    WS2629  GAGAATGAAA GAGACCTTGC AAAAGGCTGG ATTATTTGCA AAATCTATGA ATGCCTATTC TTATATGTTA ATTAAGAATC CTGATGTGAA
WSBC 10027  GAGAATGAAA GAGACCTTGC AAAAGGCTGG ATTATTTGCA AAATCTATGA ATGCCTATTC TTATATGTTA ATTAAGAATC CTGATGTGAA
WSBC 10028  GAGAATGAAA GAGACCTTGC AAAAGGCTGG ATTATTTGCA AAATCTATGA ATGCCTATTC TTATATGTTA ATTAAGAATC CTGATGTGAA
  F4094/73  GAGAATGAAA GAGACCTTGC AAAAGGCTGG ATTATTTGCA AAATCTATGA ATGCCTATTC TTATATGTTA ATTAAGAATC CTGATGTGAA
WSBC 10249  GAGAATGAAA GAGACCTTGC AAAAGGCTGG ATTATTTGCA AAATCTATGA ATGCCTATTC TTATATGTTA ATTAAGAATC CTGATGTGAA
  Bt 14007  GAGAATGAAA GAGACCTTGC AAAAGGCTGG ATTATTTGCA AAATCTATGA ATGCCTATTC TTATATGTTA ATTAAGAATC CTGATGTGAA
       R23  GAGAATGAAA GAGACCTTGC AAAAGGCTGG ATTATTTGCA AAATCTATGA ATGCCTATTC TTATATGTTA ATTAAGAATC CTGATGTGAA
ATCC 14579  GAGAATGAAA GAGACCTTGC AAAAGGCTGG ATTATTTGCA AAATCTATGA ATGCCTATTC TTATATGTTA ATTAAGAATC CTGATGTGAA
WSBC 28002  GAGAATGAAA GAGACCTTGC AAAAGGCTGG ATTATTTGCA AAATCTATGA ATGCCTATTC TTATATGTTA ATTAAGAATC CTGATGTGAA
    WS2734  GAGAATGAAA GAGACTTTGC AAAAGGCTGG ATTATTTGCA AAATCTATGA ATGCCTATTC TTATATGTTA ATTAAGAATC CTGATGTGAA
 LMG 17605  GAGAATGAAA GAGACCTTGC AAAAGGCTGG ATTATTTGCA AAATCTATGA ATGCCTATTC TTATATGTTA ATTAAGAATC CTGATGTGAA
 LMG 17615  GAGAATGAAA GAGACCTTGC AAAAGGCTGG ATTATTTGCA AAATCTATGA ATGCCTATTC TTATATGTTA ATTAAGAATC CTGATGTGAA
   F837-76  GAAGATGAAA GAAACTTTGC AAAAGGCTGG ATTATTTGCA AAATCTATGA ATGCCTATTC TTATATGTTA ATTAAAAATC CTGATGTGAA
WSBC 10312  GAGAATGAAA GAGACCTTGC AAAAGGCTGG ATTATTTGCA AAATCTATGA ATGCCTATTC TTATATGTTA ATTAAGAATC CTGATGTGAA
       C35  GAAGATGAAA GAAACCTTGC AAAAGGCTGG ATTATTTGCA AAATCTATGA ATGCCTATTC TTATATGCTA ATTAAAAATC CAGATGTAAA
       SL'  GAAGATGAAA GAAACTTTGC AAAAGGCTGG GTTATTTGCA AAATCTATGA ATGCCTATTC TTATATGTTA ATTAAAAATC CAGATGTGAA
WSBC 10364  GAAGATGAAA GAAACTTTGC AAAAGGCTGG GTTATTTGCA AAATCTATGA ATGCCTATTC TTATATGTTA ATTAAAAATC CAGATGTGAA
        I3  GAAGATGAAA GAAACTTTGC AAAAGGCTGG GTTATTTGCA AAATCTATGA ATGCCTATTC TTATATGTTA ATTAAAAATC CAGATGTGAA
       I20  GAAGATGAAA GAAACTTTGC AAAAGGCTGG GTTATTTGCA AAATCTATGA ATGCCTATTC TTATATGTTA ATTAAAAATC CAGATGTGAA
 Consensus                                             AATCTATGA ATGCCTATTC T
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nheA gene (partial forward sequence)

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            575                                                                                           665
                             *   *       *           *      *           *       *      *           *  ** *  *             *
       I21  AAATTGTAAA TGCTGCAGAT AGTCAAACGA GGGAAGCAGC TCTTCGTATT CAGCAAAAGC AAAAAGAGCT ACTACCACTT ATTCAAAAGT
       C13  AAATTGTAAA TGCTGCAGAT AGCCAAACGA GAGAAGCAGC TCTTCGTATT CAGCAAAAAC AAAAAGAGCT ACTACCACTT ATTCAAAAGT
        SZ  AAATTGTAAA TGCAGCAGAT AGTCAAACGA GGGAAGCAGC TCTTCGTATT CAGCAAAAAC AAAAAGAGCT ACTACCACTT ATTCAAAAGT
         1  AAATTGTAAA TGCAGCAGAT AGTCAAACGA GGGAAGCAGC TCTTCGTATT CAGCAAAAAC AAAAAGAGCT ACTACCACTT ATTCAAAAGT
WSBC 10204  AAATTGTAAA TGCAGCAGAT AGTCAAACGA GGGAAGCAGC TCTTCGTATT CAGCAAAAAC AAAAAGAGCT ACTACCACTT ATTCAAAAGT
  F4815/94  AAATTGTAAA TGCTGCAGAT AGTCAAACGA GAGAAGCAGC TCTTCGCATT CAACAAAAGC AAAAAGAGTT ATTACCACTT ATTCAAAAGT
 CIP 53137  AAATTGTAAA TGCTGCAGAT AGTCAAACGA GAGAAGCAGC TCTTCGCATT CAACAAAAGC AAAAAGAGTT ATTACCACTT ATTCAAAAGT
ATCC 14579  AAATTGTAAA TGCTGCAGAT AGTCAAACGA GAGAAGCAGC TCTTCGCATT CAACAAAAGC AAAAAGAGTT ATTACCACTT ATTCAAAAGT
  F0285/78  AAATTGTAAA TGCTGCAGAT AGTCAAACGA GAGAAGCAGC TCTTCGCATT CAGCAAAAGC AAAAAGAGCT ACTACCACTT ATTCAAAAGT
NVH1230-88  AAATTGTAAA TGCTGCAGAT AGTCAAACGA GAGAAGCAGC TCTTCGCATT CAACAAAAGC AAAAAGAGTT ATTGCCACTT ATTCAAAAGT
ATCC 35646  AAATTGTAAA TGCTGCAGAT AGTCAAACGA GGGAAGCAGC TCTTCGCATT CAACAAAAGC AAAAGGAGTT ATTGCCACTT ATTCAAAAGT
        PF  AAATTGTAAA TGCTGCCGAT AGTCAAACGA GGGAAGCAGC TCTTCGCATT CAGCAAAAGC AAAAAGAGCT ATTGCCACTT ATTCAAAAGT
ATCC 10987  AAATTGTAAA TGCTGCAGAT AGCCAAACGA GAGAAGCAGC TCTTCGCATT CAGCAAAAGC AAAAAGAGCT ATTGCCACTT ATTCAAAAGT
        PA  AAATTGTAAA TGCTGCCGAT AGTCAAACGA GGGAAGCAGC TCTTCGTATT CAGCAAAAGC AAAAAGAGCT ATTGCCACTT ATTCAAAAAT
  BaKriger  AAATTGTAAA TGCTGCCGAT AGCCAAACGA GAGAAGCAGC TCTTCGCATT CAGCAAAAGC AAAAAGAGCT ATTACCACTT ATTCAAAAAT
 BaWesther  AAATTGTAAA TGCTGCCGAT AGTCAAACGA GAGAAGCAGC TCTTCGCATT CAGCAAAAGC AAAAAGAGCT ATTACCACTT ATTCAAAAAT
 BaStrAmes  AAATTGTAAA TGCTGCCGAT AGTCAAACGA GAGAAGCAGC TCTTCGCATT CAGCAAAAGC AAAAAGAGCT ATTACCACTT ATTCAAAAAT
   BaA2012  AAATTGTAAA TGCTGCCGAT AGTCAAACGA GAGAAGCAGC TCTTCGCATT CAGCAAAAGC AAAAAGAGCT ATTACCACTT ATTCAAAAAT
        I1  AAATTGTAAA TGCTGCTGAT AGTCAAACGA GGGAAGCAGC TCTTCGCATT CAGCAAAAGC AAAAAGAGTT ATTACCACTT ATTCAAAAGT
       C33  AAATTGTAAA TGCTGCTGAT AGTCAAACGA GAGAAGCAGC TCTTCGCATT CAGCAAAAGC AAAAAGAGCT ACTACCACTT ATTCAAAAGT
        BS  AAATTGTAAA TGCTGCAGAT AGCCAAACGA GAGAAGCAGC TCTTCGCATT CAGCAAAAGC AAAAAGAGCT ATTACCACTT ATTCAAAAGT
       C74  AAATTGTAAA TGCTGCAGAT AGTCAAACGA GGGAAGCTGC TCTTCGTATT CAACAAAAGC AAAAAGAGTT ATTACCGCTT ATTCAAAAGT
       C35  AAATTGTAAA TGCTGCAGAT AGTCAAACGA GGGAAGCAGC TCTTCGTATT CAACAAAAGC AAAAAGAGTT ATTACCACTT ATTCAAAAGT
 LMG 17615  AAATTGTAAA TGCTGCCGAT AGTCAAACGA GGGAAGCGGC TCTTCGTATT CAGCAAAAAC AAAAAGAGCT CCTACCACTT
       I15  AAATTGTAAA TGCTGCCGAT AGTCAAACGA GGGAAGCGGC TCTTCGTATT CAGCAAAAAC AAAAAGAGCT CCTACCACTT ATTCAAAAAT
 Consensus                                    AAGCIGC TCTTCGIATT C
```

Fig. 1. Continued

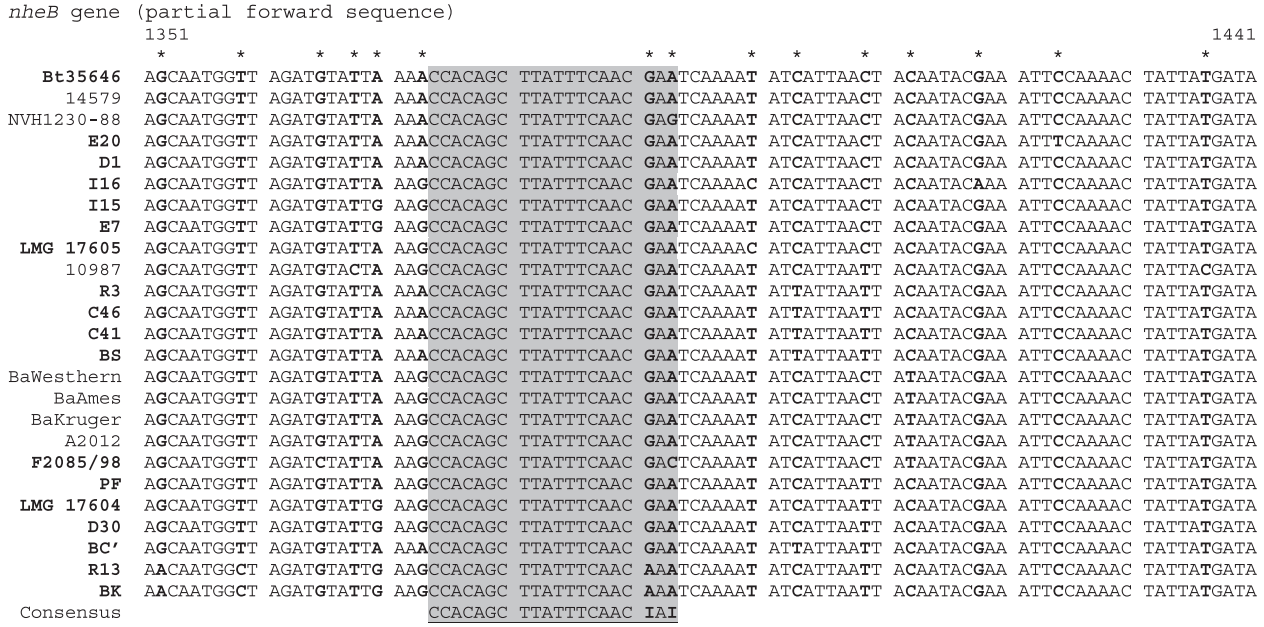

Fig. 1. Continued

to the reference set. Details of strain characteristics are provided elsewhere (Ehling-Schulz *et al.*, 2005a).

Isolation of DNA

Total DNA of bacteria was isolated using either the Aqua-Pure Genomic DNA Isolation Kit (Biorad, Germany) or the DNeasy Tissue kit (Qiagen, VWR International AB, Sweden) according to manufacturer's instructions. In addition, DNA was extracted using a simple boiling method. In brief, cells from one colony were suspended in sterile water, heated at 95 °C for 3 min and then placed on ice. After centrifugation the supernatant was used as template for PCR. Although the latter technique worked well for strains from culture collections, DNA prepared by this method from *B. cereus* isolated from food and clinical environments was not suitable for multiplex PCR.

PCR amplification of enterotoxin genes and sequence analysis

Fragments of the enterotoxin genes *nhe*A, *nhe*B, *hbl*D, and *hbl*A were amplified from diverse *B. cereus* strains and sequenced as described previously (Guinebretiere *et al.*, 2001, 2002). Primers used for amplification and sequencing are provided in Table 1. The resulting sequences and sequence data from *B. cereus* enterotoxin genes retrieved from databases were aligned using the software packages Clustal W (Thompson *et al.*, 1997) and the Multalign version 5.4.1 (Corpet, 1988). Positions of the partial sequences were determined with reference to GenBank nucleic acid sequence data; accession number AJ277962 for *cyt*K, AJ007794 for the *hbl* genes, and Y19005 for the *nhe* genes (Fig. 1).

Design of primers for multiplex PCR

Basic 18–20 bp oligonucleotide primers were designed using reference enterotoxin gene data (mentioned in Table 1) available from GenBank/EMBL databases and the Primer Designer software (Becker *et al.*, 1995). In a second step, sequence polymorphisms, previously identified by sequence alignments, were taken into account by substituting the bases at variable positions by inosine (Fig. 1). The resulting primers are presented in Table 1. Before setting up the final multiplex PCR assay, the primers were tested in singleplex PCR and in duplex PCRs using different combinations of primer pairs.

Multiplex PCR

The PCR conditions were optimized for primer and $MgCl_2$ concentrations. The final reaction mixture (50 μL) contained 0.2 mM of each dNTP, 3 mM $MgCl_2$, 0.2 μM of the oligonucleotide primers CesF1 and CesR2, 1 μM of HD2F and HA4R; 0.3 μM of NA2F and NB1R; 0.4 μM of CKF2 and CKR5; 1 U of ThermoStart *Taq* DNA polymerase (ABgene, Epsom, UK), 5 μL 10 × polymerase buffer and 1 μL template DNA. The PCR protocol started with a denaturation step for 15 min at 95 °C, followed by 30 cycles of 30 s at 95 °C, 30 s at 49 °C, and 1 min at 72 °C each, and ended with a final elongation step at 72 °C for 2 min. Sequences of all primers used are provided in Table 1.

Evaluation of the multiplex PCR assay

A panel of *B. cereus* group strains was compiled to evaluate the specificity of the multiplex PCR. The test panel included clinical strains and food strains with known toxin profiles (Ehling-Schulz *et al.*, 2005a). In addition, strains from other *B. cereus* group members: *B. anthracis* (ATCC6602, Cepanzo, CIP A2), *B. thuringiensis* (WS2734^T, WS28024, WSBC28009), *B. mycoides* (WS2641^T, WSBC10297, WSBC10293), *B. weihenstephanensis* (WSBC10204^T, INRA I20, INRA 1) and strains from other *Bacillus* species and non-*Bacillus* species were added to the test panel to assess the specificity of the assay (see Table 2).

Toxin gene profiling of *B. cereus* strains from diagnostic laboratories and environment

The evaluated multiplex PCR assay was used to type 60 clinical and food isolates provided by diagnostic laboratories (Technische Universität München, Freising; Landesanstalt für Verbraucherschutz, Halle; Technische Universität Dresden; private diagnostic labs; Institut f. Hygiene und Umwelt, Hamburg) and to determine the distribution and occurrence of toxin genes in *B. cereus* group populations from dairy silo tanks (Table 3). Details of the study on *B. cereus* populations in Swedish silo tanks are provided elsewhere (Svensson *et al.*, 2004).

Results and discussion

Development and evaluation of the multiplex PCR assay

Gene sequences from *de novo* sequenced enterotoxin genes of 49 *B. cereus* strains were aligned to enterotoxin sequences available from databases in order to design specific oligonucleotide primers. Our sequencing approach revealed high sequence polymorphisms of enterotoxin genes, which were not yet covered by the enterotoxin gene sequences available in databases (Fig. 1). These sequence polymorphisms might explain the false negative results observed in previously described PCR assays for the enterotoxins NHE and HBL (Mäntynen & Lindström, 1998; Hansen & Hendriksen, 2001; Guinebretiere *et al.*, 2002). The observed point mutations were taken into account when oligonucleotide primers were designed and inosine was inserted at variable positions (Table 1). The designed primers allowed the amplification of enterotoxin genes from strains, which were previously only detected by Southern blot analysis (Guinebretiere *et al.*, 2002). The forward and reverse primers were each located in two different genes of the corresponding operons, targeting two toxin genes in a single reaction. The forward primer, designed for the detection of the *nhe* complex, was located in *nheA* while the reverse primer was located in *nheB*, and primers for *hbl* were located in *hblD* and *hblA*, respectively (Table 1). Oligonucleotide primers for *cytK* were directed at highly conserved regions of the toxin gene so as to detect both forms of *cytK* (*cytK-1* and *cytk-2*), recently described (Fagerlund *et al.*, 2004), in a single reaction (Fig. 1). The primers for detection of emetic toxin producers were directed against a part of the cereulide synthetase essential for cereulide production. Disruption of this part of the *ces* genes by insertion mutagenesis led to a cereulide deficient phenotype (Ehling-Schulz *et al.*, 2005b).

A set of reference strains, carrying different combination of toxin genes, was compiled and used for the development of the multiplex assay (see Materials and methods for strain details). After optimization of $MgCl_2$ concentration and adjustment of primer concentrations, the PCR system was evaluated using a panel of 50 *B. cereus* strains with known toxin profiles. Closely related members of the *B. cereus* group, other *Bacilli* and known food pathogens were added to the test panel, to assess the specificity of the established assay (Table 2). The toxin gene profiles revealed by the novel multiplex assay were in accordance with the typing results of all strains obtained previously by singleplex PCR and/or Southern blotting (data not shown). Enterotoxin genes from strains, which were previously detected only by Southern blot analysis (Guinebretiere *et al.*, 2002; Ehling-Schulz *et al.*, 2005a) could now be identified by the novel multiplex assay (Fig. 2). None of the non-*B. cereus* group species isolates cross-reacted with the primer system (data not shown). In addition, the system turned out to be, in principle, suitable for the detection of enterotoxin genes in other members of the *B. cereus* group (Fig. 2, see also Table 3). The specificity and robustness of the assay was tested in two independent labs on two different cycler systems.

Toxin gene profiling of strains from diagnostic laboratories and from environment

A total of 60 *B. cereus* strains from different food and clinical diagnostic labs were typed by the established multiplex PCR system and the system was successfully applied to obtain toxin gene profiles of 80 *B. cereus* group strains, which had been collected during a population study from dairy silo tanks (Svensson *et al.*, 2004). Specific toxin gene profiles turned out to be more common than others. Only five of the seven toxin gene profiles described previously (Ehling-Schulz *et al.*, 2005a), were detected in our survey, which covered a total of 125 food isolates and 15 clinical isolates. The population of the silo tanks was dominated by strains with the toxin profile 'C' (*nhe*$^+$, *hbl*$^+$, *cytK*$^-$, *ces*$^-$) and 'F' (*nhe* $^+$, *hbl*$^-$, *cytK*$^-$, *ces*$^-$), whereas the prevalence of the toxin profile 'C' was much lower in isolates from diagnostic labs (Table 3). The most common toxin profile found among the latter isolates was toxin profile 'F'. However, NHE could

Table 1. Characteristics of oligonucleotide primers used in this study

Primer	Gene	Amplified fragment size (bp)	Sequence (5′ → 3′)	Position*	Sequence reference or strain	EMBL/ Genbank Accession no.	Source
Primers for sequencing							
HD F	*hblD*	829	ACC GGT AAC ACT ATT CAT GC	970	*B. cereus*	AJ007794	Guinebretiere
HD R			GAG TCC ATA TGC TTA GAT GC	1799	ATCC 14579^T		*et al*. (2002)
L1A	*hblD*	429	AAT CAA GAG CTG TCA CGA AT	2854	*B. cereus*	U63928	Hansen &
L1B			CAC CAA TTG ACC ATG CTA AT	3283	F837/76		Hendriksen
HD F3	*hblD*	571	ATT (AG)GC TGA AAC AGG (AG)TC (CT)C	1064	*B. cereus*	AJ007794	(2001)
HD R1			C(AG)A TCC ACC ACC (AG)AT TGA CC	1635	ATCC 14579^T		This work
HA F	*hblA*	1154	AAG CAA TGG AAT ACA ATG GG	1951			Guinebretiere
HA R			AGA ATC TAA ATC ATG CCA CTG C	3105			*et al*. (2002)
NA F	*nheA*	755	GTTAGGATCACAATCACCGC	430	*B. cereus*	Y19005	Guinebretiere
NA R			ACGAATGTAATTTGAGTCGC	1185	NVH1230/88		*et al*. (2002)
NA F2	*nheA*	551	GAA TGT (AG) CG AGA (AG)TG GAT TG	543			This work
NA R2			GC(CT) GCT TC(CT) CTC GTT TG(AG) CT	1095			
NB F	*nheB*	743	TTTAGTAGTGGATCTGTACGC	1682			This work
NB R			TTAATGTTCGTTAATCCTGC	2425			
Primers for multiplex PCR							
HD2 F	*hbl*	1091	GTA AAT TA**I** GAT GA**I** CAA TT**T**C	1188	*B. cereus*	AJ007794	This work
HA4 R			AGA ATA GGC ATT CAT AGA TT	2279	ATCC 14579^T		
NA2 F	*nhe*	766	AAG C**I**G CTC TTC G**I**A TTC	608	*B. cereus*	Y19005	This work
NB1 R			**ITI** GTT GAA ATA AGC TGT GG	1374	NVH1230/88		
CK F2	*cytK*	421	ACA GAT ATC GG**I** CAA AAT GC	1859	*B. cereus*	AJ277962	This work
CK R5			CAA GT**I** ACT TGA CC**I** GTT GC	2280	NVH0391/98		
CesF1	*ces*	1271	GGTGACACATTATCATATAAGGTG	21 816	*B. cereus*	DQ360825	Ehling-Schulz
CesR2			GTAAGCGAACCTGTCTGTAACAACA	23 087	F4810/72		*et al*. (2005a)

*Primer position relative to sequence reference.

F, forward primer; R, reverse primer; *B. cereus*, *Bacillus cereus*.

contribute substantially to the enterotoxic activity of a strain *in vitro* (Moravek *et al*., 2006). Further research will therefore be necessary to elucidate the exact role and importance of NHE in diarrhoeal food poisoning.

The incidence of emetic strains was generally low, and emetic strains carrying *cytK* seem to be quite rare, nevertheless emetic strains were found in all environments sampled in this study, in diverse foods, including baby foods and dry food products, as well as in clinical settings and in the silo tank environments. These findings are in accordance with recently published results on the occurrence of emetic strains in soil, dairy plants and farms (Yang *et al*., 2005; Altayar & Sutherland, 2006; Svensson *et al*., 2006). *cytK* was mainly found in combination with the two other enterotoxin genes, none of the tested isolates carried only *cytK*. From this study and our previous work (Guinebretiere *et al*., 2002; Ehling-Schulz *et al*., 2005a) one could assume that the occurrence of strains that possess only *cytK* is quite limited, nevertheless such strains could be highly toxic (Lund *et al*., 2000).

In conclusion, the assay we developed allows the detection of all genes, so far known, to be connected to

Table 2. Bacterial species used to test the specificity of PCR assay

Bacterial species (no. of species)	No. of strains tested
Bacillus cereus group (5)	
*Bacillus cereus**	50
Bacillus anthracis	3
Bacillus thuringiensis	3
Bacillus mycoides	3
Bacillus weihenstephanensis	3
Other *Bacillus* sp. (3)	
Bacillus subtilis	1
Bacillus licheniformis	3
Bacillus amyloliquefaciens	1
Other non-*Bacillus* species (7)	
Staphylococcus aureus	3
Staphylococcus equorum	1
Clostridium perfringens	3
Listeria monocytogenes	3
Campylobacter sp.	3
Escherichia coli (incl. serovar O157)	3
Salmonella sp.	3

*Including 40 clinical isolates and isolates from food remnants connected to food poisoning, 10 isolates from food and environment with known toxin profiles (Ehling-Schulz *et al*., 2005b).

Table 3. Toxin gene profiles of *Bacillus cereus* isolates obtained from clinical and food environments and selected isolates belonging to the *B. cereus* group

Source/species	Toxin profile							
	A (nhe$^+$, hbl$^+$, cytK$^+$)	B (nhe$^+$, cytK$^+$, ces$^+$)	C (nhe$^+$, hbl$^+$)	D (nhe$^+$, cytK$^+$)	E (nhe$^+$, ces$^+$)	F (nhe$^+$)	G (cytK$^+$)	Number of isolates
Test panel*								
B. cereus strains from food	8	–	1	–	1	2	–	12
B. cereus strains from food poisoning and clinical settings	9	2†	2	3	18†	5	1	40
B. anthracis	–	–	–	–	–	3	–	3
B. thuringiensis	1	–	2	–	–	–	–	3
B. mycoides	–	–	2	1	–	–	–	3
B. weihenstephanensis	–	–	3	–	–	–	–	3
Isolates from diagnostic labs and silo tank populations‡								
Food isolates	6	–	6	7	5	**21**	–	45
Clinical isolates	1	–	1	3	5§	**5**	–	15
Silo tank isolates	12	–	**34**	3	1	30	–	80

*Compiled from a set of strains with known toxin profiles (Ehling-Schulz *et al.*, 2005b).
†Emetic outbreaks.
‡Most prevalent toxin profiles are printed in bold.
§Including four isolates from emetic food poisoning.

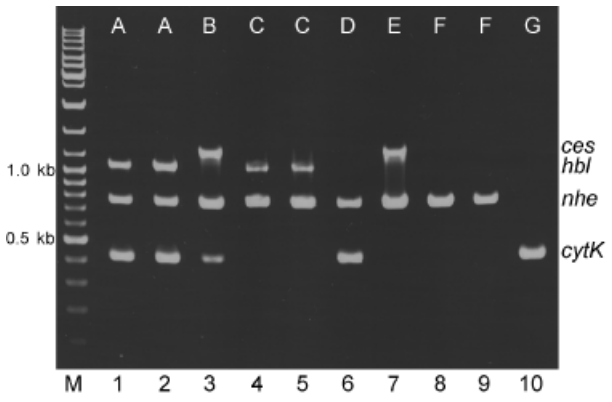

Fig. 2. Toxin gene profiling by PCR. Gel electrophoresis of PCR products from purified DNA of selected *Bacillus cereus* group strains amplified with the four pair of primers targeting the cereulide (emetic toxin) synthetase genes (*ces*, 1271 bp amplicon), the enterotoxin complexes HBL (*hbl*, 1091 bp amplicon) and NHE (*nhe*, 766 bp amplicon), and the cytotoxin K (*cytK*, 421 bp amplicon). Lane 1, *B. thuringiensis* israelensis; lane 2, clinical *B. cereus* isolate derived from wound infection; lane 3, clinical *B. cereus* isolate (feces) connected to emetic syndrome; lane 4, *B. cereus* isolated from cooked food; lane 5, *B. cereus* isolated from silo tank; lane 6, *B. cereus* isolated from milk powder; lane 7, emetic reference strain *B. cereus* F4810/72 derived from patient vomitus (food poisoning); lane 8, *B. anthracis* ATCC6602 (pXO1$^-$/pXO2$^+$); lane 9, *B. cereus* strain isolated from food remnants connected to diarrhoeal food poisoning; lane 10, original CytK strain *B. cereus* NVH 0391-98 (food poisoning); M: Marker 100 bp ladder (Promega). Toxin profiles are depicted in the upper part of the gel image: A: (*nhe*$^+$, *hbl*$^+$, *cytK*$^+$); B: (*nhe*$^+$, *cytK*$^+$, *ces*$^+$); C: (*nhe*$^+$, *hbl*$^+$); D: (*nhe*$^+$, *cytK*$^+$); E: (*nhe*$^+$, *ces*$^+$); F: (*nhe*$^+$); G: (*cytK*$^+$) (for details see text and Table 3).

gastrointestinal diseases caused by *B. cereus* in a one-step PCR. Improved primers, taking the discovered sequence polymorphism in enterotoxin genes into consideration, allowed the detection of enterotoxin genes previously missed by PCR. The described assay can facilitate diagnostics and could provide a powerful tool for toxin gene profiling of *B. cereus* in population studies. Such studies could provide new insights into the occurrence and distribution of toxin genes in different environments and could contribute to developing a better understanding of the epidemiology of toxic *B. cereus*. More detailed analysis will be necessary to examine if specific toxin gene pattern correlate with specific environments or genotypes as has been shown recently for emetic strains (Ehling-Schulz *et al.*, 2005a).

Note added in proof

The Genbank accession numbers for the sequences of the internal enterotoxin gene fragments reported in this paper are AJ937140-AJ937208.

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References

Agata N, Ohta M, Mori M & Isobe M (1995) A novel dodecadepsipeptide, cereulide, is an emetic toxin of *Bacillus cereus*. *FEMS Microbiol Lett* **129**: 17–20.

Altayar M & Sutherland AD (2006) *Bacillus cereus* is common in the environment but emetic toxin producing isolates are rare. *J Appl Microbiol* **100**: 7–14.

Becker A, Napiwotzki J & Damain MS (1995) Primer Design – a new program to choose PCR primers and oligonucleotide probes. *Med Genetik* **7**: A-158.

Beecher DJ, Schoeni JL & Wong AC (1995) Enterotoxic activity of hemolysin BL from *Bacillus cereus*. *Infect Immun* **63**: 4423–4428.

Corpet F (1988) Multiple sequence alignment with hierarchical clustering. *Nucleic Acids Res* **16**: 10881–10890.

Dierick K, Van Coillie E, Swiecicka I, Meyfroidt G, Devlieger H, Meulemans A, Hoedemaekers G, Fourie L, Heyndrickx M & Mahillon J (2005) Fatal family outbreak of *Bacillus cereus*-associated food poisoning. *J Clin Microbiol* **43**: 4277–4279.

Dietrich R, Fella C, Strich S & Märtlbauer E (1999) Production and characterization of monoclonal antibodies against the hemolysin BL enterotoxin complex produced by *Bacillus cereus*. *Appl Environ Microbiol* **65**: 4470–4474.

Dietrich R, Moravek M, Burk C, Granum PE & Märtlbauer E (2005) Production and characterization of antibodies against each of the three subunits of the *Bacillus cereus* nonhemolytic enterotoxin complex. *Appl Environ Microbiol* **71**: 8214–8220.

Ehling-Schulz M, Fricker M & Scherer S (2004a) *Bacillus cereus* the causative agent of an emetic type of food-borne illness. *Mol Nutr Food Res* **48**: 479–487.

Ehling-Schulz M, Fricker M & Scherer S (2004b) Identification of emetic toxin producing *Bacillus cereus* strains by a novel molecular assay. *FEMS Microbiol Lett* **232**: 189–195.

Ehling-Schulz M, Svensson B, Guinebretiere MH, *et al.* (2005a) Emetic toxin formation of *Bacillus cereus* is restricted to a single evolutionary lineage of closely related strains. *Microbiology* **151**: 183–197.

Ehling-Schulz M, Vukov N, Schulz A, Shaheen R, Andersson M, Märtlbauer E & Scherer S (2005b) Identification and partial characterization of the nonribosomal peptide synthetase gene responsible for cereulide production in emetic *Bacillus cereus*. *Appl Environ Microbiol* **71**: 105–113.

Ehling-Schulz M, Fricker M, Grallert H, Riek P, Wagner M & Scherer S (2006) Cereulide synthetase gene cluster from emetic *Bacillus cereus*: structure and location on a mega virulence plasmid related to *Bacillus anthracis* toxin plasmid pXO1. *BMC Microbiol* **6**: 20.

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.

Granum PE (1994) *Bacillus cereus* and its toxins. *J Appl Bacteriol Symp* **76**: (Suppl): 61S–66S.

Granum PE (2001) *Bacillus cereus Food Microbiology: Fundamentals and Frontiers*. (Doyle MP, *et al.*, eds), pp. 373–381. ASM Press, Washington, DC.

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.

Guinebretiere MH, Berge O, Normand P, Morris C, Carlin F & Nguyen-The C (2001) Identification of bacteria in pasteurized zucchini purees stored at different temperatures and comparison with those found in other pasteurized vegetable purees. *Appl Environ Microbiol* **67**: 4520–4530.

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.

Guinebretiere MH, Girardin H, Dargaignaratz C, Carlin F & Nguyen-The C (2003) Contamination flows of *Bacillus cereus* and spore-forming aerobic bacteria in a cooked, pasteurized and chilled zucchini purée processing line. *Int J Food Microbiol* **82**: 223–232.

Hansen BM & Hendriksen NB (2001) Detection of enterotoxic *Bacillus cereus* and *Bacillus thuringiensis* strains by PCR analysis. *Appl Environ Microbiol* **67**: 185–189.

Lindback T, Fagerlund A, Rodland MS & Granum PE (2004) Characterization of the *Bacillus cereus* Nhe enterotoxin. *Microbiology* **150**: 3959–3967.

Lund T, De Buyser ML & Granum PE (2000) A new cytotoxin from *Bacillus cereus* that may cause necrotic enteritis. *Mol Microbiol* **38**: 254–261.

Mahler H, Pasi A, Kramer JM, Schulte P, Scoging AC, Bar W & Krahenbuhl S (1997) Fulminant liver failure in association with the emetic toxin of *Bacillus cereus*. *N Engl J Med* **336**: 1142–1148.

Mäntynen V & Lindström K (1998) A rapid PCR-based DNA test for enterotoxic *Bacillus cereus*. *Appl Environ Microbiol* **64**: 1634–1639.

Moravek M, Dietrich R, Buerk C, Broussolle V, Guinebretiere MH, Granum PE, Nguyen-The C & Märtlbauer E (2006) Determination of the toxic potential of *Bacillus cereus* isolates by quantitative enterotoxin analyses. *FEMS Microbiol Lett* **257**: 293–298.

Paananen A, Mikkola R, Sareneva T, Matikainen S, Hess M, Andersson M, Julkunen I, Salkinoja-Salonen MS & Timonen T (2002) Inhibition of human natural killer cell activity by cereulide, an emetic toxin from *Bacillus cereus*. *Clin Exp Immunol* **129**: 420–428.

Prüß BM, Dietrich R, Nibler B, Martlbauer E & Scherer S (1999) The hemolytic enterotoxin HBL is broadly distributed among species of the *Bacillus cereus* group. *Appl Environ Microbiol* **65**: 5436–5442.

Svensson B, Ekelund K, Ogura H & Christiansson A (2004) Characterisation of *Bacillus cereus* isolated from milk silo tanks at eight different dairy plants. *Int Dairy J* **14**: 17–27.

Svensson B, Monthan A, Shaheen R, Andersson MA, Salkinoja-Salonen M & Christiansson A (2006) Occurrence of emetic toxin producing *Bacillus cereus* in the dairy production chain. *Int Dairy J* **16**: 740–749.

Thompson JD, Gibson TJ, Plewniak F, Jeanmougin F & Higgins DG (1997) The CLUSTAL_X windows interface: flexible strategies for multiple sequence alignment aided by quality analysis tools. *Nucleic Acids Res* **25**: 4876–4882.

Yang IC, Shih DY, Huang TP, Huang YP, Wang JY & Pan TM (2005) Establishment of a novel multiplex PCR assay and detection of toxigenic strains of the species in the *Bacillus cereus* group. *J Food Prot* **68**: 2123–2130.