

Detection and Purification of *Bacillus cereus* Enterotoxins

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Summary

Bacillus cereus causes two types of food poisoning, emetic and diarrheal. The emetic disease is caused by a small cyclic polypeptide (cereulide), and the diarrheal disease is caused by three different enterotoxins. Commercially available kits are used for detection of two of the enterotoxins. The enterotoxins are secreted by *B. cereus* in the early stationary phase and can be purified from the growth medium by chromatographic methods. The enterotoxins are membrane-active and the toxicity is tested on Vero cells, while the presence of the emetic toxin is detected using boar spermatozoa. Methods for detection and purification of enterotoxins are described, in addition to detection of the emetic toxin.

Key Words: *Bacillus cereus*; enterotoxins; emetic toxins; food poisoning.

1. Introduction

Bacillus cereus belongs to the taxonomically complex genus *Bacillus*. The bacteria belonging to this genus are aerobic, endospore-forming, Gram-positive rods commonly found in soil and water. The *Bacillus cereus* group comprises six separate species: *Bacillus cereus*, *Bacillus thuringiensis*, *Bacillus mycoides*, *Bacillus pseudomycoides*, *Bacillus weihenstephanensis*, and *Bacillus anthracis* (1–3). The group is genetically similar but phenotypically very diverse. *B. cereus* was first recognized as a food-borne pathogen in 1949, after an outbreak of diarrheal food poisoning at a hospital in Oslo, Norway (4), and it has been isolated from a variety of foods, including rice, spices, meat, eggs, milk, and milk products (5,6).

1.1. Identification

The six species are all lecithinase-positive mannitol-negative, and V-P-positive, and are facultative anaerobes. Aerotolerance tests should be performed to rule

Table 1
Criteria to Differentiate Between Members of *Bacillus cereus* Group

Species	Colony morphology	Hemolysis	Mobility	Susceptible to penicillin	Parasporal crystal inclusion
<i>B. cereus</i>	White	+	+	–	–
<i>B. anthracis</i>	White	–	–	+	–
<i>B. thuringiensis</i>	White/grey	+	+	–	+
<i>B. mycoides</i>	Rhizoid	(+)	–	–	–
<i>B. weihenstephanensis</i>	Separated from <i>B. cereus</i> by growth at <7°C and not at 43°C and can be identified rapidly using rDNA or cspA (cold shock protein A) targeted PCR (2).				
<i>B. pseudomycoides</i>	Not distinguishable from <i>B. mycoides</i> by physiological and morphological characteristics. Clearly separable based on fatty acid composition, and 16S RNA sequences (3).				

From ref. 6.

out anaerobic Gram-positive bacilli. BioMérieux recommends API CH50 used in conjunction with API 20E for identification of *B. cereus* (7). Criteria to differentiate among the members of the *B. cereus* group are listed in **Table 1**.

B. cereus is usually strongly β -hemolytic. *B. mycoides* are sometimes weakly β -hemolytic, with production of complete hemolysis only underneath the colonies. *B. anthracis* is usually nonhemolytic, but aging cultures may demonstrate weak γ -hemolysis. Proper precautions should be taken if a nonhemolytic colony is isolated. *B. cereus* can be differentiated from *B. anthracis* by penicillin resistance, distinct hemolysis on sheep blood agar, motility (at 35°C), rapid growth at 42°C, gelatine hydrolysis, and acid production from glucose, maltose, and salicin. Detection of the *B. anthracis* virulence genes by polymerase chain reaction (PCR) is recommended, although some strains may be negative (avirulent).

The genetic diversity of the *B. cereus* group has been studied using various methods, including multilocus enzyme electrophoresis (MEE), pulsed-field gel electrophoresis, and amplified fragment length polymorphism (8–10).

1.2. Isolation

B. cereus can be isolated from food by plating on blood agar and selective agar (see **Subheading 2.1.**). The selective agar contains mannitol and egg yolk medium in addition to a dye that changes color because of the lack of acid production from mannitol. Typical colonies of *B. cereus* will have a specific color

(blue or red, depending on the type of medium used), surrounded by an egg yolk reaction (lecithinase).

1.3. Sporulation

A number of Gram-positive genera—*Bacillus*, *Clostridium*, and *Sporosarcina*—are capable of developing dormant structures called endospores (11). These structures develop within vegetative cells and are extraordinarily resistant to environmental stress such as heat, ultraviolet radiation, chemical disinfectants, and desiccation. With unfavorable environmental conditions, endospores can remain dormant for many years. Spores of *B. cereus* are ellipsoidal, centrally located, and do not disseminate the cells (12). Generally, *B. cereus* strains will sporulate on most agar plates after 1 to 3 d incubation at 20 to 37°C. The percentage of sporulated cells is estimated using phase-contrast microscopy ($\times 1000$).

1.4. *Bacillus cereus* Toxins

B. cereus produces toxins causing two different types of food poisoning: emetic and diarrheal syndromes (6). The diarrheal syndrome is caused by enterotoxins produced by the bacteria in the small intestine, which act on the epithelial cells, causing massive secretion of fluid into the intestinal lumen leading to diarrhea (13). *B. cereus* produces three different enterotoxins that are believed to be involved in food poisoning: hemolysin BL (Hbl), nonhemolytic enterotoxin (Nhe), and cytotoxin K (CytK) (6). Hbl and Nhe are both three-component enterotoxins, while CytK is composed of one single component. Hbl, originally believed to consist of one binding component, HblB, and two lytic components, HblL₁ and HblL₂, was the first *B. cereus* enterotoxin to be characterized (14,15). However, more recently, another model for the action of Hbl has been proposed, suggesting that the components of Hbl bind to target cells independently and then constitute a membrane attacking complex resulting in a colloid osmotic lysis mechanism (16). A 1:1:1 ratio of the three components seems to give the highest biological activity (17). Substantial heterogeneity has been observed in the components of Hbl, and individual strains produce various combinations of single or multiple variations of each component (18). This is probably due to multiple genes of *hbl* with sequence variation, but this must be established genetically. Hbl possesses a variety of biological effects such as dermonecrotic and vascular permeability activities, causes fluid accumulation in ligated rabbit ileal loops, and is a major contributor to *B. cereus* ocular virulence (18).

Nhe was characterized after an outbreak of food poisoning involving 152 people in Norway, caused by an *hbl*-negative strain (19). The three Nhe components, A, B, and C, differ from those of Hbl, although there are sequence sim-

ilarities. Nearly all tested *B. cereus* strains produce Nhe, while about 50% produce Hbl (20,21).

The newly discovered enterotoxin, cytotoxin K (CytK), is similar to the α -toxin of *Staphylococcus aureus* and the β -toxin of *Clostridium perfringens*, and was the cause of a severe outbreak of *B. cereus* food poisoning in France in 1998 resulting in three deaths (22). Two other enterotoxins have been proposed: enterotoxin T and enterotoxin FM (23,24). However, it was recently suggested that the *bceT* gene product does not possess biological activity and cannot contribute to food-borne diseases (25), and seems to be a cloning artifact (26). Nothing is known about the role of enterotoxin FM, but it has sequence homology to a cell wall hydrolase from *B. subtilis* (27), and is probably not an enterotoxin.

The emetic syndrome is caused by a cyclic dodecadepsipeptide, cereulide (28), which is heat-stable and resistant to proteolysis and extreme pH (29). The toxin is produced in food during vegetative growth, and after the toxin has been produced, no treatment can destroy this stable molecule, including stomach acid and the proteolytic enzymes of the intestinal tract (6,29). After release from the stomach into the duodenum, cereulide is bound to a 5-HT₃ receptor (30), and stimulation of the vagus afferent causes emesis (vomiting).

In addition to the enterotoxins and the emetic toxin, *B. cereus* produces a number of other membrane-damaging virulence factors. *B. cereus* produces at least three different phospholipase C proteins (31,32). Two of these, a sphingomyelinase and a phosphatidylcholin hydrolase, comprise the hemolysin cereolysin AB (31). Due to the presence of Ca²⁺ in the intestinal tract, phospholipase C is regarded as harmless to epithelial cells. In addition, three more hemolysins have been described (33–35).

2. Materials

2.1. Identification and Growth

1. *B. cereus* selective agar: *Bacillus cereus* selective agar base with *B. cereus* Selective Supplement from Oxoid, UK (blue colonies) or Bacto MYP Agar with Bacto Antimicrobial Vial P from Difco Laboratories, USA (pink colonies).
2. Blood agar plates: 7% bovine citrate blood in agar.
3. Brain heart infusion broth (BHI) (Oxoid, UK).
4. API CH50 and API 20E (BioMérieux, France).

2.2. DNA Isolation

1. SET buffer: 75 mM NaCl, 25 mM EDTA, 20 mM Tris-HCl, pH 7.5.
2. Lysozyme (lyophilized powder from chicken egg white, Sigma) in SET buffer.
3. RNase (Sigma).
4. Proteinase K (Sigma).

5. Chloroform, isoamylalcohol, NaCl, isopropanol (Merck).
6. Lauryl sulfate (SDS)(Sigma).

2.3. Detection of Enterotoxins

1. Bacillus Diarrhoeal Enterotoxin Visual Immunoassay (TECRA International Pty Ltd, Australia).
2. BCET-RPLA Toxin Detection Kit (Oxoid).
3. V-well microtiter plates (Greiner).

2.4. Purification of Enterotoxins

1. CGY: 2% casein hydrolysate (Merck), 0.4% glucose, 0.6% yeast extract, 0.2% $(\text{NH}_4)_2\text{SO}_4$, 1.8% K_2HPO_4 , 0.2% KH_2PO_4 , 0.1% sodium citrate, and 0.2% MgSO_4 .
2. DEAE Sephacel (Amersham Biosciences, UK).
3. XK 16/20 columns (Amersham Biosciences).
4. Bio-Gel HT Hydroxyapatite, hydrated (Bio-Rad).
5. Resource Q column (1 mL) (Amersham Biosciences).
6. Gradient mixer GM-1 (Amersham Biosciences).
7. Fraction collector FRAC-100 (Amersham Biosciences).
8. Peristaltic pump P1 (Amersham Biosciences).
9. Bis-Tris/HCl (Sigma).
10. Triethanolaminhydrochloride (Merck).

2.5. Cell Culture and Toxicity Test

1. Vero cells: Vero C 1008 (vero 76 cloneE6) ATCC number: CRL-1586.
2. Minimum essential medium (MEM), with Earle's salts, with L-glutamine (Gibco, UK). The medium is supplemented with 5% fetal calf serum and 1X penicillin/streptomycin (cat. no. P11-010, PAA Laboratories Ltd., UK).
3. Low-leucine medium: MEM Powder cat. no. 074-90494 (made on specification by Gibco). The box (91.6 g) is dissolved in 4 L H_2O . Add 100 mL 200 mM L-glutamine (cat. no. M11-004, PAA Laboratories) and 400 mL 0.5 M HEPES buffer (pH 7.7), and adjust to 10 L with H_2O . Sterilize by filtration in 500-mL bottles (pH 7.4–7.5).
4. Trichloroacetic acid (Merck)
5. Tissue Culture Plate, 24 well (Falcon, France).
6. L-[U- ^{14}C]Leucine, >300 mCi/mmol (Amersham Biosciences).
7. Scintillation cocktail (Ultima Gold, Packard BioScience, US).
8. Scintillation counter.

2.6. Testing for Emetic Activity

1. Boar spermatozoa: Porcine AI company in each country can supply boar spermatozoa.
2. Microscope ($\times 1000$) with heating block to keep the temperature at 37°C.

3. Methods

3.1. Isolation of DNA From *B. cereus*

This is a quick method for isolation of genomic DNA from Gram-positive bacteria (36). The DNA is suitable for cloning and PCR.

1. Grow bacteria at 37°C overnight in BHI.
2. Centrifuge 3.0 mL culture to pellet the cells.
3. Resuspend the cell pellet in 495 µL SET-buffer.
4. Add 50 µL freshly made lysozyme (10 mg/mL) and 10 µL RNase (10 µg/mL), and incubate with occasional inversion for 1 h at 37°C.
5. Add 50 µL 10% SDS and 5 µL proteinase K (25 mg/mL), and incubate for 2 h at 55°C.
6. Add 200 µL 5 M NaCl and 700 µL chloroform:isoamylalcohol (24:1) and incubate at room temperature with frequent inversions for 30 min.
7. Centrifuge for 30 min at 4500g and transfer the aqueous phase to a fresh tube.
8. Precipitate the DNA with an equal volume of isopropanol by centrifugation for 10 min at maximum speed in a tabletop centrifuge. Wash the precipitate with 70% ethanol. Let the pellet air-dry.
9. Resuspend the DNA in 100 µL H₂O.

3.2. Detection of Genes Encoding Enterotoxins

PCR is used for detection of genes encoding the *B. cereus* enterotoxins. At time of writing, the nucleotide sequence of three *B. cereus* strains and five *B. anthracis* strain genomes are available in public genomic databases (Genbank, EMBL DDBJ). The available nucleotide sequences are used to produce specific primers to identify the genes encoding the enterotoxins in other strains. Standard PCR programs—e.g., 95°C for 1 min, 30 cycles of 95°C for 1 min, 48–52°C for 1 min (annealing temperature according to the specific primers) and 72°C for 1 min, followed by a final extension step of 72°C for 7 min—are used to amplify the toxin genes. For both Nhe and Hbl, three different PCR reactions are necessary to ensure the presence of all three genes (Note 1).

3.3. Detection of Enterotoxins

Two different immunological tests, from Oxoid (UK) and TECRA (Australia), are commercially available for detection of the enterotoxins Hbl and Nhe of *B. cereus* (see Note 2). The kit from Oxoid uses antibodies reacting with the L₂ component of Hbl, while the kit produced by TECRA, *Bacillus* diarrheal enterotoxin (BDE) visual immunoassay (VIA), detects NheA (37,38).

For detection of enterotoxins using the TECRA kit, the bacteria should be cultured in BHI broth with 1% glucose for 6 to 8 h at 32°C with shaking. Cells are removed by centrifugation and the culture supernatants are added to wells coated with high-affinity antibodies against NheA. Captured enterotoxins are

detected with conjugate (enzyme-labeled antibodies) converting a colorless substrate into green.

The BCET-RPLA detection kit from Oxoid uses polystyrene latex particles sensitized with purified antiserum taken from rabbits immunized with purified *B. cereus* diarrheal enterotoxin. The test is performed in V-well microtiter plates. Dilutions of food extract or culture supernatants are made in wells and the latex particle suspension is added to each well. If *B. cereus* Nhe enterotoxin is present, agglutination occurs due to the formation of a lattice structure. After settling, this forms a diffuse layer on the base of the well. If *B. cereus* enterotoxin is absent or is at a concentration below the assay detection level, no such lattice structure can be formed, and a tight button will be observed. There is, at the time of this writing, no kit available for the detection of CytK.

3.4. Purification of Enterotoxins

The culture medium used for purification of enterotoxins is a modification of CGY medium (15,39) (see Notes 3 and 4).

1. 100 mL *B. cereus* overnight culture is used to inoculate 2 L CGY. The culture is grown with shaking at 32°C (see Note 5) for 6 to 7 h.
2. Extracellular proteins are separated from cells by centrifugation (10,000g at 4°C for 20 min).
3. The supernatant is concentrated by precipitation with 70% saturated (NH₄)₂SO₄ overnight at 4°C with mixing. The precipitated proteins are pelleted by centrifugation at 10,000g for 20 min at 4°C. The pellet is then resuspended in 25 mL H₂O, and dialyzed at 4°C against 25 mM Bis-Tris-HCl (pH 5.9)/1 mM EDTA.
4. The concentrated protein solution is applied to DEAE-Sephacel packed in a 1.6-cm diameter column (10 cm high) with peristaltic pump.
5. Proteins are eluted with a linear gradient of 0–0.5M NaCl in 25 mM Bis-Tris-HCl (pH 5.9) in 20 fractions over 200 mL.

When purifying Hbl and CytK, the fractions can be tested for hemolytic activity, while in purifying Nhe, the fractions must be tested for cytotoxic activity in a Vero cell assay. In addition, fractions can be visualized on silver-stained sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS-PAGE). Different Nhe and Hbl components will appear in different fractions, so combinations of fractions must be tested to obtain hemolytic or cytotoxic activity. When using a DEAE column to purify Nhe, NheB will elute at 25–75 mM NaCl, while NheA will elute at 200–300 mM NaCl. NheC has never been purified directly from *B. cereus* culture supernatant, probably because it interacts with either NheA or NheB (see Note 6). NheC is also produced in small amounts (1/10) compared to the production of NheB.

6. Following DEAE, selected fractions are pooled and applied directly to a column (1.6 cm diameter, 6 cm height) of Bio-Gel HT hydroxyapatite, equilibrated with 10 mM NaCl.
7. Proteins are eluted with a linear gradient of sodium phosphate buffer (pH 6.8) from 0 to 0.24 M in 20 fractions over 100 mL in 10 mM NaCl.
8. Selected fractions are dialyzed overnight at 4°C against 20 mM triethanolamine, containing 1 mM EDTA. The pH of the buffer is 8.1 for fractions containing NheB and CytK, and 7.8 for fractions containing the other proteins.
9. The dialyzed fraction is applied to a Resource Q column.
10. Proteins are eluted with a linear gradient of NaCl from 0 to 0.5 M in 20 fractions over 40 mL in 20 mM triethanolamine.

3.5. Test for Hemolytic Activity

Add 10 µL of the sample and 100 µl 2% bovine citrate blood (in 0.9% NaCl) to each well of a microtiter plate, and observe. No hemolytic activity will result in a tight button of blood cells at the bottom of the well.

3.6. Toxicity Test Using Vero Cells

Vero cells are grown in MEM medium supplemented with 5% fetal calf serum. Cells are seeded into 24-well plates 2 to 3 d before testing. Before use, check that the growth of the Vero cells is confluent. If so, remove the medium and wash the cells once with 1 mL preheated (37°C) MEM medium.

1. Add 1 mL preheated (37°C) low-leucine medium to each well and then add the toxin to be tested (max 100 µL).
2. Incubate the cells for 2 h at 37°C.
3. Remove the low-leucine medium with the toxin, wash each well once with 1 mL preheated (37°C) low-leucine medium. Mix 8 mL (enough for 24 wells) preheated low-leucine with 16 µL ¹⁴C-leucine and add 300 µL of this mixture to each well.
4. Incubate the cells for 1 h at 37°C.
5. Remove the radioactive medium and add 1 mL 5% trichloroacetic acid (TCA) to each well, and incubate at room temperature for 10 min.
6. Remove the TCA, and wash the wells twice with 1 mL 5% TCA.
7. After removing the TCA, add 300 µL 0.1 M KOH and incubate at room temperature for 10 min. Transfer the content of each well to liquid scintillation tubes with 2 mL liquid scintillation cocktail. Vortex the tubes, and count the radioactivity in a scintillation counter for 1 min.
8. Percentage inhibition of protein synthesis is calculated using the following formula (see Note 7):

$$[(\text{Neg. ctrl} - \text{sample}) / \text{Neg. ctrl}] \times 100$$

The negative control is Vero cells from wells without addition of sample.

3.7. Testing for Emetic Activity

Boar sperm motility is inhibited by exposure to cereulide (emetic toxin), and boar sperm is useful for detecting cereulide concentrations toxic to humans. The threshold concentration of cereulide provoking visible damage in boar sperm *in vitro* is 2 ng cereulide/mL boar sperm (40). Exact concentration of the emetic toxin can be measured by LC-MS (41).

1. Spread the bacteria on an agar plate and incubate 1 to 3 d at 22°C.
2. Pick three colonies and dissolve in 200 μ L methanol (use glass equipment with tight capsule).
3. Boil for 10 min in a water bath, and cool to room temperature.
4. Preheat boar sperm, pipet tips, and microscope slides to 37°C.
5. Add 5 to 10 μ L cooled extract to 200 μ L boar sperm.
6. Incubate for 10 min at 37°C.
7. The motility of the exposed sperm cells is estimated using phase-contrast microscopy at 37°C.

4. Notes

1. All three genes encoding the three components of Nhe or Hbl must be present for production of active enterotoxins. Even so, with positive PCR results there might be strains that are enterotoxic-negative resulting from lack of or mutation in the PlcR regulator, or mutation in the toxin genes.
2. The two commercially available immunological kits for enterotoxin detection test for only one out of three components in the enterotoxin complex, while all three components must be present for biological activity. A positive TECRA or Oxoid test does not necessarily mean that active enterotoxin is produced.
3. When purifying proteins from culture supernatants, the use of a strain producing only one of the enterotoxins is highly preferable. The properties of the toxin components in the two different three-component enterotoxin complexes are similar so they will copurify in most cases. Positive strain, NVH 0075-95, produces exclusively Nhe and NVH 0391-98 produces exclusively CytK and may be requested from the authors.
4. For purification of enterotoxins from culture supernatant, CGY is chosen, as CGY contains fewer large proteins than BHI.
5. Expression profiles of the enterotoxins will vary for each strain at different growth temperatures. The growth temperature for optimal enterotoxin expression has to be established for each strain.
6. Small amounts of NheC will often be purified together with NheA and NheB, as NheC seems to be associated with NheA and NheB in the culture supernatant. To obtain NheA and NheB absolutely pure of NheC, they should be expressed recombinantly.
7. The correlation between percentage inhibition of protein synthesis in Vero cells and concentration of toxin are linear in the range from about 30 to 75%, so minimum or maximum toxicity measurements should be kept within this range.

Acknowledgment

This work was supported by the European Commission (QLK-CT-2001-00854).

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Food-Borne Pathogens

Methods and Protocols

Adley, C. (Ed.)

2006, XII, 268 p., Hardcover

ISBN: 978-1-58829-465-4

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