The *bceT* gene of *Bacillus cereus* encodes an enterotoxin protein

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A toxin gene (*bceT*) on a 2.9 kb DNA fragment of *Bacillus cereus* B-4ac was cloned and expressed in *Escherichia coli*, and its nucleotide sequence determined. The DNA fragment contained an open reading frame capable of encoding a polypeptide of 336 amino acids with a molecular mass of 41 039 Da. The translated product in *E. coli* exhibited Vero cell cytotoxicity, and was positive in a vascular permeability assay. It also caused fluid accumulation in a ligated mouse ileal loop and was lethal to mice upon injection. These biological activities are considered characteristic of diarrhoeal enterotoxins. We therefore conclude that this gene, designated *bceT*, encodes one of the enterotoxin proteins of *B. cereus* which cause food-borne diarrhoea.

Keywords: *Bacillus cereus*, food poisoning, enterotoxin protein, *bceT* gene

INTRODUCTION

The association of *Bacillus cereus* with food-borne illness has been comprehensively reported (Kramer & Gilbert, 1988; Turnbull *et al.*, 1979a). This organism causes two different types of human gastroenteritis (Turnbull, 1976; Turnbull *et al.*, 1979b). The ability of *B. cereus* to cause diarrhoea is attributed to the production of an enterotoxin (*B. cereus* diarrhoeal enterotoxin, designated bc-D-ENT in this study) (Gilbert & Kramer, 1984; Turnbull, 1981). bc-D-ENT is capable of causing fluid accumulation in ligated rabbit ileal loops (Punyashtiti & Finkelstein, 1971), altering the vascular permeability of guinea-pig skin (Glatz & Goefert, 1973), and showing cytotoxicity towards Vero cells (African Green monkey kidney cells) (Konowalchuk *et al.*, 1977). Purification of the fractions relevant to these biological properties has been attempted, and some components have been partially characterized (Bonventre, 1965; Shinagawa *et al.*, 1991; Spira & Goefert, 1975; Thompson *et al.*, 1984). These studies suggest that all these biological activities are due to a single entity. Thompson *et al.* (1984) suggested that the enterotoxin activity probably comprised more than one component, and Beecher & Macmillan (1991) reported that three components of haemolysin BL also composed the diarrhoeal enterotoxin. However, other studies, such as those of Turnbull *et al.* (1979b) and Shinagawa *et al.*

(1991), identified a single relatively unstable protein of 43–50 kDa which was responsible for the diarrhoeal-type *B. cereus* food poisoning. To resolve the contradiction among these results, we have cloned and sequenced the structural gene encoding a protein which has the biological activities of bc-D-ENT.

METHODS

Bacterial strains, antiserum, plasmids, and growth conditions. *Escherichia coli* strains for DNA manipulation were routinely cultured in LB medium (Miller, 1992) with aeration. *B. cereus* strains listed in Table 1 were grown in BHI broth (Difco). Antiserum against a crude preparation of *B. cereus* enterotoxin was prepared according to Spira & Goefert (1975). Plasmid pHSG299 was used for cloning, and bacteriophages M13 mp18 and mp19 for sequencing. pHSG299 is a kanamycin-resistant derivative of pUC19 and therefore carries the *lac* promoter-*lacZ* of pUC19 (Takeshita *et al.*, 1987). Expression of a DNA insert at the polycloning site should be under the control of the *lacZ* promoter. Kanamycin (Sigma) was added to liquid and solid media at 50 µg ml⁻¹ for selection of recombinant clones. In addition, to screen for the insertional inactivation of the *LacZ* peptide encoded by the vectors, 50 µM isopropyl β-D-thiogalactoside (IPTG; Sigma) and 0.01 % 5-bromo-4-chloro-3-indolyl β-galactoside (X-Gal; Sigma) were added to the media.

DNA manipulation. Extraction and purification of plasmid DNA and double-stranded phage DNA, and purification of single-strand phage DNA for sequencing were performed by published methods (Gilmore *et al.*, 1985; Maniatis *et al.*, 1982). Chromosomal DNA from *B. cereus* B-4ac was prepared by the method of Dubnau & Davidoff-Abelsen (1971). All other manipulations with DNA, such as enzyme digestion, ligation, and transformation of bacteria, were performed as described by Maniatis *et al.* (1982).

Abbreviation: bc-D-ENT, *Bacillus cereus* diarrhoeal enterotoxin.

The GenBank accession number for the nucleotide sequence reported in this paper is D17312.

Western blot analysis. The lysate of *E. coli* cells was analysed by SDS-PAGE according to the method of Laemmli (1970). To prepare the lysate, *E. coli* cells were cultured for 15 h with constant shaking in 100 ml LB medium containing 0.01% IPTG and 50 µg kanamycin ml⁻¹, then harvested by centrifugation. The cell pellet was suspended in 5 ml 30 mM Tris buffer pH 8.0/50 mM NaCl containing 50 mg lysozyme (Sigma). After incubation at 37 °C for 1 h, the suspension was centrifuged for 15 min at 8000 *g* and the supernatant was used as the crude preparation. The proteins separated by 0.1% SDS/10% (w/v) polyacrylamide gel electrophoresis were transferred electrophoretically to nitrocellulose paper (Bio-Rad). The blots were probed with rabbit antiserum against diarrhoeal enterotoxin. The antiserum was pretreated with a lysate of *E. coli* JM109 to remove non-specific staining. Binding of the primary antibody was detected by using goat anti-rabbit immunoglobulin G conjugated to horseradish peroxidase (Cappel).

Sequencing. DNA fragments were subcloned into M13 mp18 or mp19 and used as templates for nucleotide sequencing by the dideoxynucleotide fluorescence labelling method (Applied Biosystems DNA Sequencer model 370A) using *Taq* DNA polymerase.

Assay for biological activities of the cell lysate. An IPTG-induced cell lysate of *E. coli* JM109 carrying a recombinant clone was used as a crude preparation of the gene product.

Mouse ileal loop (MIL) assay. The MIL technique was performed as described by Punyashthiti & Finkelstein, (1971). Although not ideal, the MIL model offers some advantages over the rabbit ileal loop test and is becoming more widely used (Shinagawa *et al.*, 1991). Briefly, ICR mice weighing 17–22 g were anaesthetized lightly with ether. The abdomen was opened and, from a point approximately 8 cm distal to the stomach, two 6 cm loops, separated from each other by an interloop of 1 cm, were isolated by silk ligatures, taking care to avoid blood vessels. Intraluminal injection of 0.2 ml of a sample was made into one test loop; the other loop was injected with a suitable control. The ileum was then replaced into the abdominal cavity, and the incision was sutured. After 6 h, the mice were killed, the abdominal cavities were opened immediately and the length of the excised loops and the fluid volume accumulated in the ligated ileal loops were measured. The results were judged positive when the ratio of fluid to length was 50 mg/cm or more.

Vascular permeability (VP) assay. VP activity was determined by the method of Glatz & Goefert (1973). Each sample (0.1 ml) was dialysed against phosphate buffer (0.02 M, pH 7.0), adjusted to 0.2 ml volume, and injected (0.05 ml) in duplicate into at least two female New Zealand White rabbits. After 3 h, each animal was injected intravenously with 2 ml of a 10% (w/v) solution of Evans blue prepared in 0.9% NaCl; reactions were read after an additional 1 h.

Mouse lethal (ML) activity. In the assay for ML activity (Glatz & Goefert, 1973), two mice were tested per sample. Death of mice within 30 min after injection of 0.5 ml of the test sample into the caudal vein was considered a positive reaction.

Cytotoxicity (CT) activity. CT assay to Vero cells was performed by the method of Konowalchuk *et al.* (1977) with several modifications. Cells were maintained in Dulbecco modified Eagle medium (Gibco Laboratories) supplemented with 3.5 mg glucose ml⁻¹ and 10% (v/v) foetal calf serum. Cells were split 1:6 into 24-well plates 3 d before test. Cell density for the test was 10⁵ cells cm⁻². Test samples (0.5 ml) were dialysed against phosphate buffer (0.02 M, pH 7.0), adjusted to 1 ml volume, and

passed through a 0.22 µm Millex (Nihon Millipore Kogyo K.K., Yonezawa, Japan); 100 µl was then added to each well. Cells were incubated in 5% (v/v) CO₂ at 36 °C. After 12 h, observations were made for the disruption of the cell monolayer.

Assay units. Activities in the MIL and VP assays were expressed as diarrhoeagenic units (DU) and VP factor units (PFU), respectively, and were calculated on the basis of the standard curves (Glatz *et al.*, 1974). ML dose (MLD) and CT dose were expressed as the reciprocal of the highest dilutions of the lysate to give positive reactions in these assays.

Polymerase chain reaction (PCR). The oligonucleotide primers were synthesized on a commercial DNA synthesizer (391 DNA Synthesizer; ABI). The DNA sequences for the PCR primers used were as follows: a forward primer which corresponds to nucleotide positions 1354–1374 (5'-TTACATTACCAGGACGTGCTT-3'), and a reverse primer corresponding to the complement of positions 1761–1781 (5'-TGTTTGTGATTGTAATTCAGG-3'). Amplification was carried out in a DNA thermal cycler (Perkin-Elmer Cetus) for 25 reaction cycles of 1 min at 94 °C (denaturation), 2 min at 55 °C (annealing), and 2 min at 72 °C (extension-polymerization).

RESULTS

Cloning of an enterotoxin gene

B. cereus B-4ac is an isolate from a food poisoning outbreak. To identify the genes involved in biosynthesis of *B. cereus* diarrhoeal enterotoxins, a gene library of B-4ac was constructed. Chromosomal DNA from B-4ac was extracted, partially digested with *EcoRI* and *PstI*, and ligated to the *EcoRI*–*PstI* site of the plasmid vector pHSG299. From about 2000 transformants of *E. coli* JM109 screened by immunoblot analysis with anti-diarrhoeal enterotoxin rabbit antiserum, two antigenically positive clones were identified. By the preliminary assays for the biological activities of the diarrhoeal enterotoxin, only one clone, designated pAGA118, was found to be positive.

Nucleotide sequence determination

The size of the insert in the *EcoRI*–*PstI* site of pAGA118 was 2.9 kb. The physical map of the insert is shown in Fig. 1. DNA fragments resulting from digestion with appropriate restriction enzymes were subcloned into the same sites of M13 vectors mp18 and mp19. These subclones were sequenced by the dideoxynucleotide chain-termination method. For the portions where no suitable

Fig. 1. Restriction map and sequencing strategy for the *bceT* gene. The 2.9 kb *PstI*–*EcoRI* fragment from *B. cereus* B-4ac is on plasmid pAGA118.

restriction sites were available, synthetic oligonucleotide primers were synthesized. The complete nucleotide sequence was thus determined on both strands with full overlap (Fig. 2). As shown in Fig. 2, a search for open reading frames (ORFs) by computer analysis revealed an ORF coding for 336 amino acid residues (molecular mass 41039 Da). Tn5 insertions in the ORF using the Tn5 plasmid pCHR81 (Ohta *et al.*, 1991), although the precise positions of the insertion sites were not determined, abolished the enterotoxigenic activities (data not shown). This ORF was therefore designated *bceT*. Immediately upstream of the translation initiation codon, there is a putative ribosome-binding site CAGAA with weak homology to the 16S rRNA-binding site (Fig. 2). Immediately upstream of the putative translation initiation codon, there are three directly repeated sequences, each of which includes a stem-loop structure, a potential transcription terminator. A search of the GenBank and EMBL databases revealed no other genes with homologous nucleotide sequences. Homology searches for amino acid sequences in the NBRF database identified no proteins with significant homology either.

Detection of the *bceT* gene product by Western blot analysis

A cell lysate of *E. coli* JM109(pAGA118) was analysed by SDS-PAGE. A 41 kDa protein was detected on SDS-PAGE after IPTG induction (Fig. 3a, lane 2). This induced protein was also detected in Western blot analysis using antiserum against a crude enterotoxin preparation (Fig. 3b, lane 2). The apparent molecular size of this protein coincided with that calculated from the deduced amino acid sequence of the protein encoded by *bceT*. Without IPTG-induction, no protein band was observed at this position.

Biological activities of the *E. coli* lysate

The lysate of *E. coli* JM109(pAGA118) was assayed for biological activities which are characteristic of the diarrhoeal enterotoxin of *B. cereus*. The lysate of *E. coli* JM109(pAGA118) induced with IPTG exhibited 530 CTU, 1200 PFU, 75 MLU, and 12 DU (mg protein)⁻¹; lysates of JM109, or of JM109(pAGA118) not induced with IPTG, had none of these activities. This indicates that the gene was transcribed and translated in *E. coli*, and the gene product might be secreted into the periplasm. In conclusion, the *bceT* gene on pAGA118 was responsible for at least part of the activities of bc-D-ENT. However, the concentration of the active substance in the lysate of JM109(pAGA118) after induction with IPTG was apparently much lower than that in the culture supernatant of the parent *B. cereus* B-4ac.

Detection of the *bceT* gene in other *B. cereus* isolates

All 10 strains listed in Table 1, including four strains obtained from 'diarrhoeal syndrome' foodborne illness, three strains from 'emetic syndrome' foodborne illness

and three strains isolated from soil and from raw and cooked rice, were PCR-positive with amplified DNA bands at the same position (428 bp) after agarose gel electrophoresis (data not shown). This is consistent with the observation that the culture supernatants of all 10 strains, regardless of source, were positive for VP and CT activity (Table 1).

DISCUSSION

Previous studies from various laboratories have presented different results on the number of components responsible for the activities of bc-D-ENT and on the molecular mass of each component. Turnbull *et al.* (1979b) reported that the intestinonecrotic toxin, which was considered to be the same as the diarrhoeal enterotoxin, was a single relatively unstable protein with an approximate molecular mass of 50 kDa and an isoelectric point of about 4.9. On the other hand, Thompson *et al.* (1984) found experimental evidence for a probable multicomponent or subunit enterotoxin structure. Three antigenic components were identified by chromatographic separation of extracellular proteins produced by *B. cereus* B-4ac. These components were estimated to be proteins of 38, 39.5 and 43 kDa. Fractions which contained only one or two of the three components exhibited no toxic activity. When the two nontoxic fractions were combined, activities in all of the biological assays recovered. Beecher & Macmillan (1991) also reported that three components (35, 36 and 45 kDa, respectively) were necessary to produce oedema and blueing in a rabbit vascular permeability assay, and these activities were ascribed to the bc-D-ENT. These three proteins were originally identified as the components of the haemolysin BL of *B. cereus*. Granum & Nissen (1993) determined the sequence of the first 14–15 amino acids of their identified three components. The N-terminal amino acid sequence of bc-D-ENT protein that we deduced from the DNA sequence has no homology with these proteins. Shinagawa *et al.* (1991) reported that the purified enterotoxin was a single, electrophoretically homogeneous protein with a molecular mass of 45 kDa and an isoelectric point of about 5.5.

Our cloned *bceT* gene encodes a single protein of calculated molecular mass 41039 Da, and *E. coli* bearing this clone produced a biologically active substance which exhibited cytotoxic activity, positive vascular permeability reaction, mouse lethal activity and fluid accumulation in a ligated mouse ileal loop. All these activities fulfil the criteria of the diarrhoeal enterotoxins. We therefore conclude that bc-D-ENT is one of the enterotoxin proteins of *B. cereus*. Although the biological activities of bc-D-ENT were detected in the cell lysate of JM109(pAGA118), the level of the activities was much lower than that in the culture supernatant of the parent *B. cereus* strain. The *bceT* gene should be transcribed from the *lacZ* promoter of pHSG299, since without addition of IPTG, the level of the activities of the cell lysate was far lower than that of the IPTG-induced cell lysate. The presence of potential terminators in the three directly repeated sequences upstream of the ORF sequence may

Fig. 2. Nucleotide sequence of the *B. cereus bceT* gene and the flanking region; the deduced amino acid sequence is shown above the nucleotide sequence. A putative ribosome-binding site (SD) and direct repeats are underlined. An asterisk denotes the termination codon and arrows indicate stem-loop structures.

Fig. 3. SDS-polyacrylamide gel (a), and Western blot analysis (b) of a cell lysate of *E. coli* JM109(pAGA118). The arrow indicates the expected 41 kDa protein. Lanes 1, total protein from uninduced JM109 lysate; lanes 2, total protein from induced JM109(pAGA118) lysate.

Table 1. *B. cereus* strains used in this study

Strain*	Properties†				Source
	MIL	ML	VP	CT	
B-4ac	++	++	++	++	'Diarrhoeal syndrome' food poisoning
D-1	++	++	++	++	
D-2	++	++	++	++	
D-3	++	++	++	++	
E-1	—	—	++	++	'Emetic syndrome' food poisoning
E-2	—	—	++	+	
E-3	—	—	++	++	
N-1	++	++	++	++	Raw rice
N-2	—	—	++	++	Cooked rice
N-3	+	++	++	++	Soil

* All strains were phenotypically the same in motility, indole production, nitrate reduction, Voges-Proskauer reaction and urease production. B-4ac, D-1, D-2, D-3, N-1 and N-3 were positive in the starch hydrolysis test; the other strains were negative.

† Assays for enterotoxin activity: MIL, mouse ileal loop; ML, mouse lethal; VP, vascular permeability; CT, cytotoxicity. ++, Strongly positive, +, weakly positive, —, negative.

interfere with the transcription of the gene from the *lac* promoter in *E. coli*. The *bceT* gene product is a unique protein: no homologous protein has been reported previously. However, the DNA sequence corresponding to this gene was detected by PCR in all of the *B. cereus* strains examined. Regardless of source, the culture supernatants of all these strains were positive in some of

the enterotoxin activities. It is therefore possible that isolates from food or soil may be candidates for causing diarrhoea-type food poisoning.

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