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Cereulide, the emetic toxin of *Bacillus cereus*, is putatively a product of nonribosomal peptide synthesis

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ABSTRACT

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Aims: To determine if cereulide, the emetic toxin produced by *Bacillus cereus*, is produced by a nonribosomal peptide synthetase (NRPS).

Methods and Results: NC Y, an emetic strain of *Bacillus cereus*, was examined for a NRPS gene using PCR with primers recognizing a fragment of a NRPS gene from the cyanobacterium *Microcystis*. The amplicon was sequenced and compared with other gene sequences using BLAST analysis, which showed that the amplicon from strain NC Y was similar in sequence to peptide synthetase genes in other micro-organisms, including *Bacillus subtilis* and *B. brevis*, while no such sequence was found in the complete genome sequence of a nonemetic strain of *B. cereus*. Specific PCR primers were then designed and used to screen 40 *B. cereus* isolates previously implicated in outbreaks of foodborne illness. The isolates were also screened for toxin production using the MTT cell cytotoxicity assay. PCR and MTT assay screening of the *B. cereus* isolates revealed a high correlation between the presence of the NRPS gene and cereulide production.

Conclusions: The results indicate that cereulide is produced by a NRPS complex.

Significance and Impact of the Study: This is the first study to provide evidence identifying the mechanism of production of cereulide, the emetic toxin of *B. cereus*. The PCR primers developed in the study allow determination of the potential for cereulide production among isolates of *B. cereus*.

Keywords: *Bacillus cereus*, cereulide, genetics, intoxication.

INTRODUCTION

Bacillus cereus is an environmentally ubiquitous, Gram-positive, spore-forming organism recognized as a food spoilage hazard, and the causative agent of two distinct types of toxin-mediated foodborne illness known as the diarrhoeal and emetic syndromes (Drobniewski 1993). The diarrhoeal form of the disease is characterized by abdominal pain and diarrhoea which lasts for 12–24 h (Beattie and Williams 1999), and involves a proteinaceous heat-labile enterotoxin with a molecular weight of about 45 kDa (Shinagawa 1993). Emetic disease is caused by cereulide, a small, cyclic, heat-stable dodecadepsipeptide, which exhibits the absolute stereochemistry of [L-*o*-Val-L-Val-D-*o*-Leu-D-Ala]$_3$ (Agata *et al.* 1994; Isobe *et al.* 1995; Kuse *et al.* 2000). The highly heat, acid and protease-resistant toxin, typically preformed in farinaceous foodstuffs, predominantly rice, induces emesis within 1–5 h of consumption.

The significance of *B. cereus* as a foodborne pathogen has generated much research into the toxins they produce. Considerable data has been produced on the diarrhoeal enterotoxin, but comparatively limited information is available for the emetic toxin, cereulide, although this is rapidly changing. While the structure and properties of cereulide

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have been elucidated, there is no knowledge of the genes involved in the biosynthesis of cereulide, which would allow determination of the toxic potential of *B. cereus*.

One approach for detecting potentially toxigenic strains of *B. cereus* would be the amplification and identification of specific DNA sequences associated with production of the toxin. As yet, there is no gene-based system developed for the detection of *B. cereus* strains capable of producing emetic toxin. In elucidating a genetic target for the detection of cereulide-producing strains, the mechanism of toxin synthesis must be determined. We hypothesized that cereulide is produced nonribosomally by a large, multi-domain, enzyme complex. Cyclic structure and the presence of D-amino acids in peptides (Moffitt and Neilan 2000), and synthesis in late exponential to stationary phase (Schneider and Marahiel 1998; Finlay *et al.* 2002a,b; Häggblom *et al.* 2002), characteristics shared by cereulide, may indicate the involvement of a nonribosomal biosynthetic mechanism pathway.

Multi-domain enzymatic complexes serve as an alternative biosynthetic pathway to the well characterized ribosome-dependent systems. Characterized by a multi-domain structure, these complexes, known as nonribosomal peptide synthetases (NRPS), catalyse the synthesis of a structurally diverse family of bioactive peptides. They typically utilize a thio-template mechanism to activate, modify, and condense, via amide or ester bonds, the constituent amino acids of the peptide product (Stachelhaus and Marahiel 1995; Arment and Carmichael 1996).

The production of cereulide by a NRPS is further supported by the fact that other *Bacillus* species, such as *B. brevis*, *B. subtilis* and *B. licheniformis*, produce cyclic peptides using nonribosomal peptide synthesis (Stachelhaus and Marahiel 1995). More importantly, valinomycin, a close structural analogue of cereulide produced by *Streptomyces levoris*, is synthesized nonribosomally by a multi-enzyme complex consisting of several thio (-SH) template-containing polypeptides, or peptide synthetases (Perkins *et al.* 1990; Jack and Jung 1998).

The aim of this study was to determine the genetic basis of cereulide production, through PCR-based identification of the genes, and correlation with cereulide production, using the MTT cell cytotoxicity assay.

MATERIALS AND METHODS

B. cereus isolates and culture

Emetic *B. cereus* strains were obtained from Norio Agata (Nagoya City Public Health Research Institute, Nagoya, Japan), while those of Maria Andersson (University of Helsinki, Helsinki, Finland) were supplied by Paul Horwood (James Cook University, Townsville, Australia). Diarrhoeal *B. cereus* strains were obtained from Per Einar Granum (Department of Food Hygiene, Norwegian College of Veterinary Medicine, Oslo, Norway). The *B. cereus* strains were labelled as either emetic or diarrhoeal based on their isolation as causative agents of vomiting or diarrhoeal food poisoning episodes.

For screening assays (performed in duplicate) in the evaluation of toxic potential and toxin production, cultures of *B. cereus* were grown in 1·5 ml sterile tryptone soya broth (TSB; Oxoid, Melbourne, Australia) in 2 ml microfuge tubes for 48 h at 30°C with orbital shaking at 150 rev min^{-1}. Cells were pelleted by centrifugation for 10 min at 3000 *g*. The supernatant and pellet were retained for autoclaving and MTT analysis, and DNA extraction, respectively.

DNA extraction

DNA for PCR was prepared according to Neilan *et al.* (1993), while DNA required for sequencing reactions was extracted and purified using the methods of Neilan *et al.* (1995), modified by increasing incubation with lysozyme, to improve the yield of DNA from Gram-positive bacteria (US Dept. of Commerce, 1997).

Detection and analysis of putative cereulide biosynthesis genes

PCR amplification of putative peptide synthetase genes from NC Y, a known cereulide-producing strain of *B. cereus*, was first attempted using the degenerate primers MTF2 and MTR2, described previously for the identification of conserved functional regions of cyanobacterial NRPS adenylation domains (Neilan *et al.* 1999). DNA from *Microcystis* sp. PCC7806 served as a positive control.

The PCR reaction (total volume 30 μl) contained 3 μl 25 mM $MgCl_2$, 3 μl 10x buffer, 2 μl 2 mM dNTPs, 0·1 μl 5·5 U μl^{-1} *Taq* polymerase, 1 μl forward primer, 1 μl reverse primer, and 18 μl HPLC-grade water. DNA (2 μl, *ca* 200 ng) was included in the reaction volume instead of water. A negative control or blank was included in every run of the PCR and consisted of the PCR reaction mix with no DNA. The reaction conditions consisted of an initial denaturation step at 94°C for 2 min, followed by 30 cycles of denaturation at 94°C for 10 s, annealing at 50°C for 20 s, and extension at 72°C for 80 s, and a final extension step at 72°C for 7 min. PCR products were analysed by gel electrophoresis in 1% agarose gels in Tris-acetate buffer and visualized by staining with ethidium bromide.

Following resolution by electrophoresis, amplicons were excised from the gel and the DNA purified using a QIAquick gel extraction kit (Qiagen, Doncaster, Victoria, Australia) and sequenced. For sequencing reactions, amplicons were purified by precipitation, using two volumes of

80% ethanol and 0·1 volume of 3 M sodium acetate. After standing for 15 min at room temperature, DNA was pelleted by centrifugation at 10 000 *g* for 15 min. The supernatant was discarded and residual liquid removed using a Speed-iVac vacuum centrifuge (Savant Instruments Inc., Holbrook, New York, USA). The dry pellet was resuspended in an appropriate volume of sterile Milli Q water. Linear amplification of fragments was performed in a 10-μl reaction volume containing 4 μl of sequencing mix, containing 1 μl Big Dye mix, 1·5 μl 5 CS buffer and 1·5 μl Milli Q water (Applied Biosystems Inc., Foster City CA, USA), 20–50 ng of target DNA, 5–10 pmol primer, and Milli Q water to 10 μl. The reaction conditions consisted of an initial denaturation step at 96°C for 3 min, followed by 30 cycles of denaturation at 96°C for 10 s, annealing at 50°C for 5 s, and extension at 60°C for 4 min. The labelled sequencing products were purified by precipitation, using 65 μl of 100% ethanol and 3 μl of 3 M sodium acetate. After standing for 20 min at room temperature, DNA was pelleted by centrifugation at 10 000 *g* for 20 min. The supernatant was discarded and residual liquid removed using a Speed-iVac vacuum centrifuge (Savant Instruments Inc.). The dry pellet was then submitted to the Sequencing Unit at UNSW for automated sequencing on a Model 373 Sequencer (Applied Biosystems). Sequence data were analysed using the Applied Biosystems Autoassembler software.

Sequences were compared with existing sequences in GenBank using the NCBI-BLAST (Basic Local Alignment Search Tool) program. Based on the aligned sequences, new specific primers were designed and synthesized (Genset, Lismore, Australia). Further sequence information, by walking upstream or downstream an unknown region of genomic DNA using PanHandle PCR previously described by Siebert *et al.* (1995) and Moffitt (2003). The resulting gene sequence, generated by the oligonucleotide primers CKC-F (5′-CAG CGT ACC AAA TCA CCC GT-3′), and CKC-R (5′-AGT TTT ACC ACA ATC TGA AT-3′) (Genset), was then used for comparative homology-based analysis against the genome of the nonemetic type strain ATCC 14579, using NCBI-BLAST.

MTT assay

The MTT colourimetric assay was performed as first described by Mosmann (1983), modified using protocols developed by Beattie and Williams (1999) and Finlay *et al.* (1999), specifically for the detection of cereulide. Autoclaved supernatants were dispensed in 100 μl volumes directly into separate wells of a 96-well microtitre plate. The negative control consisted of sterile TSB. All wells were then seeded with an equal volume of DMEM media (JRH Biosciences, Lenexa, KS, USA) supplemented with 10% FBS (Gibco-BRL Life Technologies, New York, NY, USA) containing 5×10^4 cells ml^{-1} of CHO-K1 cells, and incubated at 37°C for 72 h in a 5% CO_2/95% air incubator. After incubation, 1/10 volume of MTT (Sigma-Aldrich, Sydney, Australia) solution was added and plates incubated for a further 4 h for MTT cleavage. Media was then removed by plate inversion and gentle tapping of the plate, and 100% DMSO added at a solvent/reaction volume of 1 : 1, following by gentle agitation on a plate shaker for 30 min to dissolve formazan crystals. Results were read in an ELISA plate reader at 540 nm and also at 620 nm for reference. Samples were considered to be toxic if the optical density of the test well was ≥20% less than that detected in the negative control wells.

Purification of toxin for HPLC-MS analysis

Cereulide was extracted as described previously by Andersson *et al.* (1998), with slight modifications. From an overnight culture grown in TSB (Oxoid) at 30°C with shaking, 0·1 ml was spread plated onto TSA plates, which were incubated for a period of 48 h aerobically at 30°C. Following incubation, toxin was extracted from the lawn of bacterial cells with 100% methanol. The methanol extract was centrifuged at 10 000 *g* for 30 min at 4°C to remove cell debris, and the supernatant filtered through a 0·45-μm pore membrane (Advantec MFS Inc., Dublin, CA, USA) to separate residual cells. Methanol was removed by rotary evaporation. Methanol/water (90 : 10) was then added to re-dissolve the residue, and the sample loaded onto a Sep-pak C_{18} cartridge (Waters Australia, Rydalmere, NSW), washed with water/methanol (80 : 20), and eluted with 100% methanol. This eluate was used directly for toxin analysis by HPLC-MS. The negative control for HPLC-MS consisted of an extract from sterile TSA plates.

Analysis by HPLC-MS

Methods for HPLC-MS analysis were based on protocols previously described by Häggblom *et al.* (2002). The column used for chromatographic separation was Sephasil C8 SC 2·1 mm × 100 mm, 5 μm (Amersham Biosciences, Sydney, Australia). Elution was isocratic, using 5% of 0·1% trifluroacetic acid in water and 95% of 0·075% trifluoroacetic acid in acetonitrile, at a flow rate of 100 μl min^{-1}. The injection volume was 10 μl for an extract and sample and 5 μl for the valinomycin, which served as a standard. Valinomycin (99% purity) was obtained commercially (Calbiochem, Merck, Kilsyth, Victoria, Australia). For detection, an absorbance of 215 nm was used. After separation by reversed phase chromatography, the sample was introduced into a Deca XP-Plus ESI ion trap mass analyser (ThermoFinnigan, San Jose, CA, USA). Source parameters for MS analysis were: capillary/dry temperature, 275°C; sheath gas flow/nebuliser,

20 lb/in^2; source/capillary voltage, 4·5 kV. The ion trap was operated in automatic gain control mode, with a maximum ion injection time of 200 ms. A mass range of m/z 500–1300 was collected for wide range ion monitoring, while a narrow mass range of 1128–1130 m/z was acquired for selected ion monitoring (SIM) for valinomycin (enabling greater sensitivity for calculation of a standard curve). The total ion chromatogram was smoothed with a Gaussian function and peak areas integrated with Xcalibur data analysis software. Sets of blank runs between samples, using 100% methanol, ensured there was no carry-over contamination or false-positive results.

RESULTS

Detection of putative NRPS genes

Using the degenerate primers MTF2 and MTR2, several amplicons were obtained from *B. cereus* NC Y, although one corresponded in size to that from *Microcystis* sp. PCC7806 (Fig. 1). BLAST analysis of the sequence from the NC Y amplicon (GenBank accession no. AY576054) against the GenBank nucleotide database showed it to be similar to the peptide synthetase genes from *B. subtilis* and *B. brevis*. Using the aligned sequences, the oligonucleotide primers BEF (5′-ACT TAG ATG ATG CAA GAC TG-3′), and BER (5′-TTC ATA GGA TTG ACG AAT TTT-3′) were designed, replacing the degenerate primers. BEF/BER proved specific for the putative peptide synthetase gene, yielding only one amplicon of *ca* 850 bp from *B. cereus* NC Y and no amplicon from *Microcystis* sp. PCC7806 (Fig. 1).

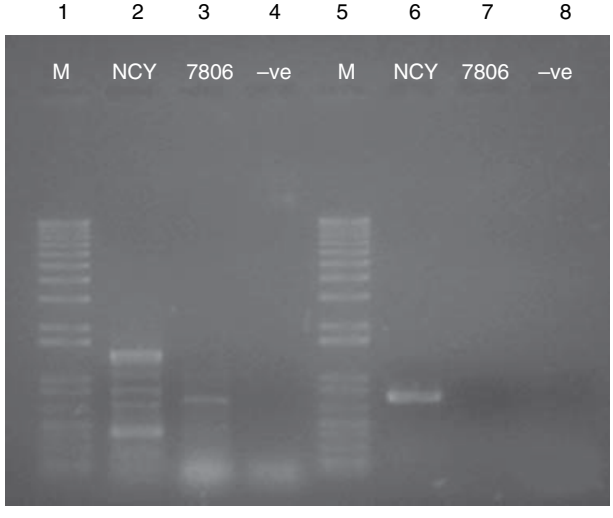

Fig. 1 Detection of putative NRPS genes in emetic *Bacillus cereus* NC Y: M, 1 kb Plus ladder; NC Y, *B. cereus* emetic NC Y; 7806, *Microcystis* PCC 7806; lanes 1–4, MTF2/MTR2 primers; lanes 5–8, BER/BEF-specific primers

Using the NCBI-BLAST program, comparative homology-based analysis of the NRPS gene sequence (generated by CKC-F and CKC-R primers) from *B. cereus* NC Y, against the completed genomes of *B. cereus* ATCC 14579 (a diarrhoeal strain), *B. anthracis* strain Ames, *B. halodurans*, and *B. subtilis* subsp. *subtilis* strain 168, showed no significant similarity. Although there are similarities between NPRS gene sequences in other *Bacillus* species, the NC Y NRPS gene sequence appears to be unique to the group of *B. cereus* organisms capable of producing cereulide.

MTT assay and PCR screening of *B. cereus* strains

Using the BEF/BER primer pair, 40 *B. cereus* isolates were screened for presence of the NRPS gene sequence (Table 1). All isolates recognized as emetic strains yielded an amplicon, and also produced cereulide according to the MTT assay. Of the 10 isolates recognized as diarrhoeal strains, four were negative by both PCR and MTT assay. Three diarrhoeal strains yielded an amplicon and, of these, two were found to produce cereulide by the MTT assay. Three further diarrhoeal strains were positive for toxin production by the MTT assay, but yielded no amplicon.

To allay concerns regarding the correlation between a positive result from PCR and cereulide production as determined by the MTT assay, an extract of a culture of *B. cereus* N1230-88, a recognized diarrhoeal strain, was analysed using HPLC-MS. Separation by HPLC identified a peak with a retention time of 8·38 min (Fig. 2a) very similar to the retention time of 8·32 for the peak from a recognized emetic strain, NC 7401 (Fig. 2b) for cereulide. MS analysis yielded an identical ion (m/z, 1170·5; NH_4^+ adduct). By comparison, valinomycin (Fig. 2c) gave a retention time of 8·42 min and an ion of 1128·5 (NH_4^+ adduct).

Using HPLC-MS in SIM mode, a standard curve was constructed for valinomycin, comparing concentration to peak area (Fig. 3). Based on the close structural relationship and similar HPLC-MS response between valinomycin and cereulide, the standard curve was used to estimate the amount of cereulide produced by different isolates of *B. cereus*. Under identical cultural conditions, both *B. cereus* NC 7401 (emetic) and N1230-88 (diarrhoeal) produced cereulide, although the estimated concentrations were substantially different (86·4 and 0·12 μg ml^{-1}, respectively).

DISCUSSION

This is the first study to provide strong evidence that cereulide, the emetic toxin produced by *Bacillus cereus*, is synthesized nonribosomally by a peptide synthetase.

Table 1 *Bacillus cereus* isolates used in this study

Strains*	Origins	Screening	
		MTT	PCR
NC F	Emetic, food poisoning, SH−	+	+
NC G	Emetic, food poisoning, SH−	+	+
NC Y	Emetic, food poisoning, SH−	+	+
NC 88F	Emetic, faeces, SH−	+	+
NC 90T	Emetic, faeces, SH−	+	+
NC 954	Emetic, food poisoning, SH−	+	+
NC 1044	Emetic, food poisoning, SH−	+	+
NC 1078	Emetic, food poisoning, SH−	+	+
NC 1090	Emetic, food, SH−	+	+
NC 1128	Emetic, soil, SH−	+	+
NC 1149	Emetic, vomitus, SH−	+	+
NC 1154	Emetic, vomitus, SH−	+	+
NC 1184	Emetic, vomitus, SH−	+	+
NC 1204	Emetic, faeces, SH−	+	+
NC 1219	Emetic, faeces, SH−	+	+
NC 1237	Emetic, food, SH−	+	+
NC 1240	Emetic, food, SH−	+	+
NC 1245	Emetic, food, SH−	+	+
NC 1246	Emetic, soil, SH−	+	+
NC 1249	Emetic, soil, SH−	+	+
NC 1260	Emetic, food, SH−	+	+
NC 1287	Emetic, faeces, SH−	+	+
NC 1291	Emetic, food, SH−	+	+
NC 1310	Emetic, faeces, SH−	+	+
NC 1315	Emetic, food, SH−	+	+
NC 7401	Emetic, faeces, SH−	+	+
F 47	Emetic, no case history, SH−	+	+
F 4426	Emetic, no case history, SH−	+	+
F 4810/72	Emetic, no case history, SH−	+	+
F 5881	Emetic, no case history, SH−	+	+
N1230-88	Diarrhoeal, food poisoning – oriental stew, SH+	+	+
N1649-99	Diarrhoeal, food poisoning – mashed turnip, SH+	+	−
N1694-00	Diarrhoeal, food poisoning – cream sauce, SH+	−	−
N1651-00	Diarrhoeal, food poisoning – caramel pudding, SH+	+	+
NVH200	Diarrhoeal, food poisoning – dish with rice, SH+	−	−
N0075-95	Diarrhoeal, food poisoning – vegetable stew, SH+	+	−
N0248-00	Diarrhoeal, food poisoning – lasagna, SH+	−	−
N0674-98	Diarrhoeal, food poisoning – scrambled eggs, SH+	−	−
N1104-98	Diarrhoeal, food poisoning – fish soup, SH+	−	+
N1113-01	Diarrhoeal, food poisoning – roast veal, SH+	+	−

SH, starch hydrolysis.

*NC, supplied by Norio Agata, Nagoya City Public Health Research Institute, Nagoya, Japan; F, supplied by Paul Horwood, James Cook University, Townsville, Australia; and N, supplied by Per Einar Granum, Department of Food Hygiene, Norwegian College of Veterinary Medicine, Oslo, Norway.

Screening showed all strains recognized as emetic to be positive in both the MTT and PCR assays. These results are not surprising as the strains were derived from outbreaks of emetic food poisoning. The MTT assay (Beattie and Williams 1999; Finlay *et al.* 1999) has been accepted and used widely as a relatively simple screening method for cereulide production, and thus serves as a useful correlate against which to compare the results of the PCR assay.

Of the 10 diarrhoeal strains, four were negative for the putative emetic gene and in the MTT assay. However, three strains recognized as diarrhoeal were PCR-positive, of which two gave positive reactions in the MTT assay. While emetic

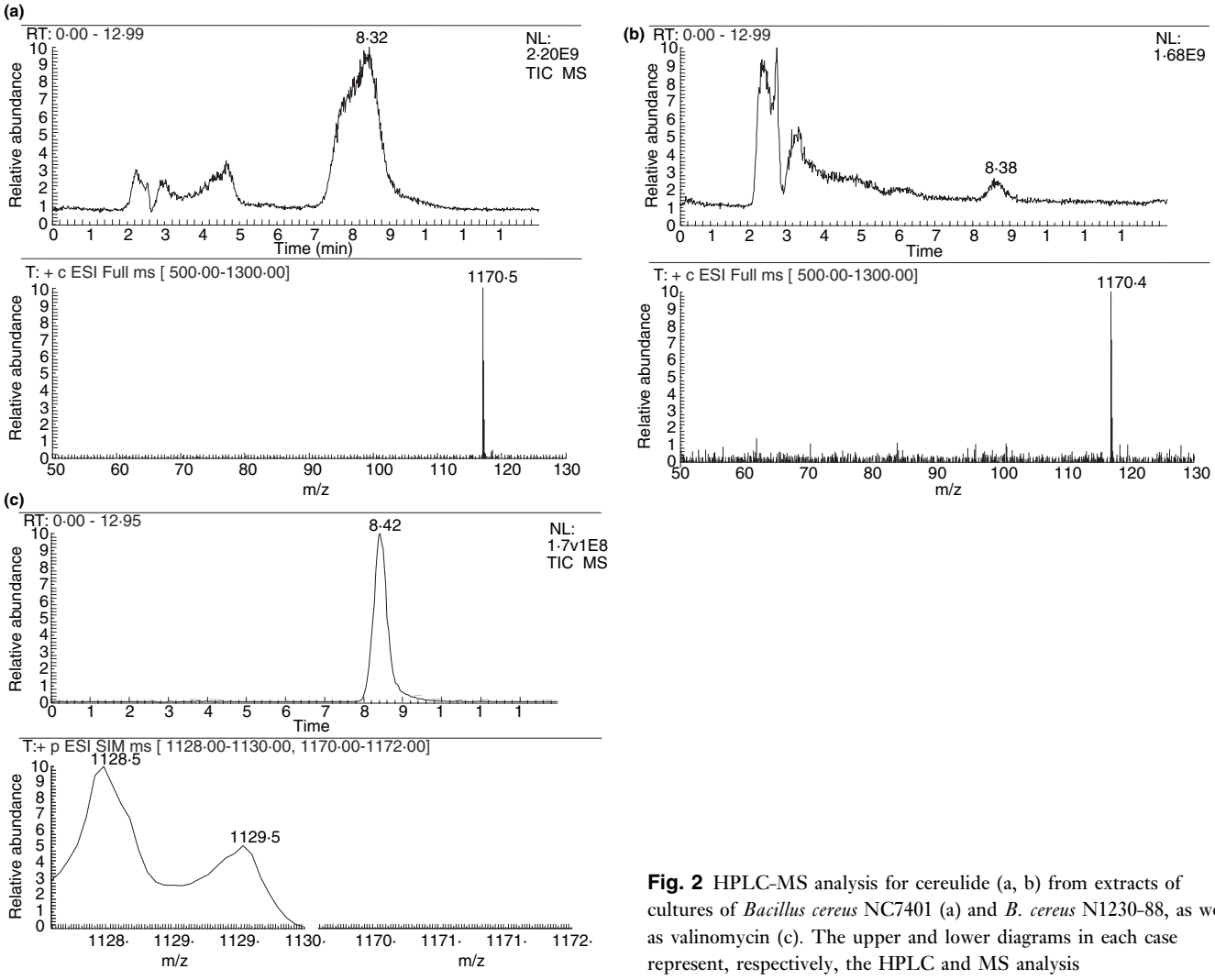

Fig. 2 HPLC-MS analysis for cereulide (a, b) from extracts of cultures of *Bacillus cereus* NC7401 (a) and *B. cereus* N1230-88, as well as valinomycin (c). The upper and lower diagrams in each case represent, respectively, the HPLC and MS analysis

and diarrhoeal strains appear to be distinct, producing one type of toxin or another (Shinagawa 1990), there is evidence of exceptions. Reports of some cases of food poisoning involving *B. cereus* indicate observation of both vomiting and diarrhoea, probably because of the production of both types of toxins (Granum and Lund 1997). If a strain is capable of producing both types of toxin then, ecologically, the involvement of a single strain in the sequential production of both syndromes in a single host is possible. Growth in a farinaceous food encourages production of cereulide which, when ingested, elicits an emetic response. A proportion of the bacilli ingested survive passage through the stomach, arriving in the large intestine where they develop and cause an infective diarrhoeal intoxication. Further, cereulide-positive strains have been shown by ELISA to produce diarrhoeal enterotoxin (Pirttijarvi *et al.* 1999). Another cell cytotoxicity assay using McCoy cells, which utilizes a staining procedure coupled with visual examination for detection of diarrhoeal enterotoxin, also found emetic strains capable of producing the enterotoxin (Fletcher and Logan 1999). Finally, one of the diarrhoeal strains in this study, N1230-88, was shown conclusively, by HPLC-MS, to produce cereulide, the MS revealing the ion specific for cereulide (Häggblom *et al.* 2002). Although this strain produced cereulide, the amount produced was considerably less than that produced by NC 7401, under cultural conditions.

One strain recognized as diarrhoeal, N1104-98, was PCR-positive but MTT-negative. The detection of a fragment of a gene, while suggesting the ability to produce toxin, may not necessarily mean toxin production will occur. As an example, Prüß *et al.* (1999) carried out both molecular and immunological tests for the presence of the enterotoxin gene *hblA* amongst members of the *Bacillus cereus* group of organisms. While the gene was found to be broadly distributed amongst members of the group, in certain strains of *B. thuringiensis*

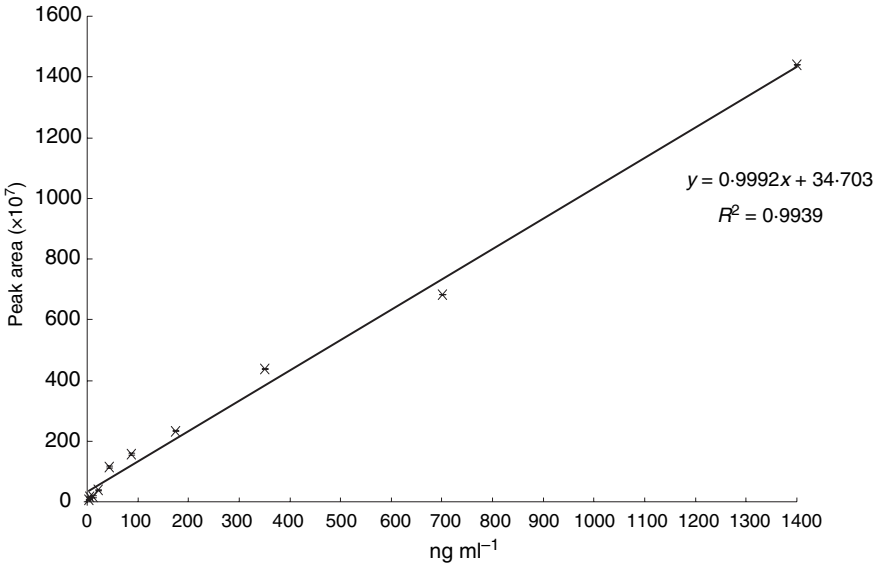

Fig. 3 Standard curve for valinomycin correlating peak area, as determined by HPLC-MS, with concentration

and *B. mycoides* the gene was either incomplete or silent, as these strains did not demonstrate cytotoxic effects, a characteristic induced by the enterotoxin gene product. Expression of the gene may occur, even in species considered nontoxigenic; *B. thuringiensis* has previously been found to be involved in outbreaks of diarrhoea (Lund 1990).

The final three strains recognized as diarrhoeal, N0075-95, N1649-99 and N1113-01, were PCR-negative, but MTT-positive. PCR-based assays designed to detect toxin genes may not always produce a positive signal when in fact the toxin is synthesized, as shown by some diarrhoeal strains in this study. This may be a result of a mutation in the binding site of one of the primers (Prüß *et al.* 1999). Alternatively, the negative result in the PCR may indeed reflect the absence of the NPRS gene. The reactions observed in the MTT assay may have been due to a nonspecific reaction rather than a reaction because of cereulide. For example, it is possible that significant cell damage caused by enterotoxin may have led to the positive MTT results.

The inability to hydrolyse starch has previously been considered a correlate with cereulide production (Shinagawa 1993; Agata *et al.* 1996). This study has shown that strains recognized as diarrhoeal, and capable of starch hydrolysis are in fact able to produce cereulide. The inability to hydrolyse starch is considered to constrain the growth of *B. cereus* strains traditionally recognized as emetic, in starchy foods, inducing the production of cereulide. Thus, starch-hydrolysing strains, traditionally recognized as diarrhoeal, are unlikely to produce cereulide in starchy foods, even if they possess the ability to do so.

Comparison of the gene sequence obtained in this study, from a strain known to induce emesis, against a known non-emetic toxin producer, further supports the nonribosomal production of cereulide by a peptide synthetase. Recently, the genome of the nonemetic strain, *B. cereus* ATCC 14579, was sequenced (Ivanova *et al.* 2003). No sequences corresponding to that described in the present study could be identified. While a cluster of four genes with similarity to peptide synthetase subunits was found in the genome, none of them had the racemase domain. This suggested that the cluster was unlikely to code for the biosynthesis of cereulide, which contains D-O-Leu and D-Ala (Ivanova *et al.* 2003).

In conclusion, among 30 isolates of *B. cereus* recognized as emetic, and capable of producing cereulide, as demonstrated by the MTT assay, all contained the NPRS gene fragment as shown by PCR screening. This, coupled with the composition and structure of the toxin, properties suggestive of a molecule synthesized nonribosomally, provide strong evidence that cereulide is indeed produced by a nonribosomal peptide synthetase. The specific PCR described in this study, or a DNA probe based on the amplicon could serve as a useful means of discrimination between pathogenic and nonpathogenic strains. Further, the sequence identified in this study may provide a useful epidemiological marker to determine relationships among *B. cereus* isolates obtained during the course of an outbreak investigation (Nishikawa *et al.* 1996). When the gene is detected, strains can be regarded as potential toxin producers (In't Veld *et al.* 2001). The method can also act as a corroborating diagnostic tool for confirmation of emetic toxin producers.

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