Genetic and functional analysis of the cytK family of genes in *Bacillus cereus*

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CytK is a pore-forming toxin of *Bacillus cereus* that has been linked to a case of necrotic enteritis. PCR products of the expected size were generated with cytK primers in 13 of 29 strains. Six strains were PCR-positive for the related gene hly-II, which encodes haemolysin II, a protein that is 37% identical to the original CytK. Five of the strains were positive for both genes. The DNA sequences of putative cytK genes from three positive strains were determined, and the deduced amino acid sequences were 89% identical to that of the original CytK. The authors have designated this new cytK variant cytK-2, and refer to the original cytK as cytK-1. The CytK-2 proteins from these three strains were isolated, and their identity was verified by N-terminal sequencing. BLAST analysis using the cytK-2 gene sequences revealed very high homology with two cytK-2 sequences in the genomes of *B. cereus* strains ATCC 14579 and ATCC 10987. The differences between CytK-1 and the CytK-2 proteins were clustered to certain regions of the proteins. The isolated CytK-2 proteins were haemolytic and toxic towards human intestinal Caco-2 cells and Vero cells, although their toxicity was about 20% of that of CytK-1. Both native and recombinant CytK-2 proteins from *B. cereus* 1230-88 were able to form pores in planar lipid bilayers, but the majority of the channels observed were of lower conductance than those created by CytK-1. It is likely that CytK-2 toxins contribute to the enterotoxicity of several strains of *B. cereus*, although not all of the CytK-2 toxins may be as harmful as the CytK-1 originally isolated.

**INTRODUCTION**

Cytotoxin K (CytK) was initially isolated from a strain of *Bacillus cereus* that was responsible for a severe food poisoning outbreak that killed three people (Lund et al., 2000). This strain did not produce any of the known enterotoxins of *B. cereus* (Granum & Lund, 1997; Granum, 2001), and CytK was the only secreted protein that was toxic towards Vero cells. CytK, a protein of 34 kDa, is necrotic, haemolytic, and able to form pores in lipid bilayers. Furthermore, it has been shown that CytK is highly toxic towards human intestinal epithelial cells (Hardy et al., 2001). In sum, these properties suggested that CytK was responsible for the severe food poisoning and that it could cause necrotic enteritis (Hardy et al., 2001; Lund et al., 2000). Initial PCR analysis indicated that cytK was not widely distributed in *B. cereus* strains (Lund et al., 2000; Stenfors & Granum, 2001).

The amino acid sequence of CytK is similar (about 30% identity) to that of α-haemolysin (α-toxin, α-HL), leucocidins and γ-haemolysin of *Staphylococcus aureus*, and β-toxin of *Clostridium perfringens*, which all belong to the family of β-barrel pore-forming toxins (Song et al., 1996; Prevost, 1999; Steinthorsdottir et al., 2000). The capacity to form pores in planar bilayers (Hardy et al., 2001) is consistent with CytK being a member of this family of proteins with pore-forming ability.

Several proteins have been described as putative virulence factors in *B. cereus*. The extent to which they may belong to a family of pore-forming proteins is of interest, as many have been shown to possess haemolytic or cytotoxic activity. Beecher et al. (2000) partially characterized a novel haemolysin designated haemolysin IV. This toxin appeared to be one of the most rapidly acting or one of the most

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The GenBank accession numbers for the cytK-2 sequences are AJ318875, AJ318876 and AJ318877.
abundant haemolysins in crude culture supernatants from many *B. cereus* strains. Amino acid sequencing showed that 28 out of 30 amino acids in the N-terminal region were identical to those of CytK (Beecher *et al.*, 2000; Lund *et al.*, 2000).

A haemolysin of *B. cereus* designated haemolysin II (H-II) was first characterized by Coolbaugh & Williams (1978), although a similar haemolysin had been mentioned as early as 1963 (Fossum, 1963). A similar haemolysin has also been isolated from *Bacillus thuringiensis* (Pendleton *et al.*, 1973). A genetic determinant of *B. cereus*, hly-II, supposedly encoding haemolysin II, has been cloned and sequenced (Sinev *et al.*, 1993; Baida *et al.*, 1999). hly-II was, however, more characteristic of *B. thuringiensis* than *B. cereus* (Budarina *et al.*, 1994). The deduced amino acid sequence of hly-II (mature protein 42-3 kDa) showed similarity to known β-barrel pore-forming toxins of *S. aureus* (Baida *et al.*, 1999).

CytK of *B. cereus* is probably an important virulence factor in many *B. cereus* strains. We have therefore studied the occurrence of this toxin in different strains, and have identified a new cytK variant, which we designate as cytK-2, referring to the original cytK from *B. cereus* 391-98 as cytK-1. The haemolytic and cytotoxic activity of the CytK-2 proteins, and their pore-forming ability, were determined and compared with those of CytK-1. The relationship and relative importance of CytK-1, CytK-2, and haemolysin II as virulence factors are discussed.

### METHODS

**Strains.** Twenty-nine strains of *B. cereus* were used in this study (Table 1). Several of these strains have previously been described (Granum *et al.*, 1993). The PHLS Food Hygiene Laboratory, London, UK, provided two clinical strains. Thirteen strains were isolates from foods, submitted to the National Reference Laboratory for *B. cereus* at the Norwegian School of Veterinary Science, Oslo, Norway. Strain 391-98, from which the original CytK-1 was isolated, was described by Lund *et al.* (2000). *Escherichia coli* JM109 (Stratagene) was used for construction of the pMS20 expression vector, and XL10-Gold cells (Stratagene) were used for expression of CytK-1 and CytK-2 in *E. coli*.

<table>
<thead>
<tr>
<th>Strain no.</th>
<th>Source</th>
<th>cytK primers (FcytK+RcytK)</th>
<th>hly-II primers (Fhly-II+Rhly-II)</th>
</tr>
</thead>
<tbody>
<tr>
<td>14</td>
<td>Milk</td>
<td>+</td>
<td>−</td>
</tr>
<tr>
<td>17</td>
<td>Cream</td>
<td>−</td>
<td>−</td>
</tr>
<tr>
<td>23</td>
<td>Cream</td>
<td>+</td>
<td>−</td>
</tr>
<tr>
<td>36</td>
<td>Cream</td>
<td>−</td>
<td>−</td>
</tr>
<tr>
<td>45</td>
<td>Cream</td>
<td>+</td>
<td>−</td>
</tr>
<tr>
<td>55</td>
<td>Cream</td>
<td>−</td>
<td>−</td>
</tr>
<tr>
<td>67</td>
<td>Cream</td>
<td>+</td>
<td>−</td>
</tr>
<tr>
<td>68</td>
<td>Butter</td>
<td>+</td>
<td>−</td>
</tr>
<tr>
<td>72</td>
<td>Semi-skimmed milk</td>
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<td>+</td>
</tr>
<tr>
<td>80</td>
<td>Cream</td>
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<td>87</td>
<td>Whipped cream</td>
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<td>−</td>
</tr>
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<td>96</td>
<td>Dairy product</td>
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<td>−</td>
</tr>
<tr>
<td>100</td>
<td>Stew</td>
<td>−</td>
<td>−</td>
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<tr>
<td>132</td>
<td>Milk</td>
<td>−</td>
<td>−</td>
</tr>
<tr>
<td>261-92</td>
<td>Meat</td>
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<td>−</td>
</tr>
<tr>
<td>453-92</td>
<td>Cream</td>
<td>−</td>
<td>−</td>
</tr>
<tr>
<td>F 4501-83</td>
<td>Clinical (PHLS)</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>15</td>
<td>Unknown (PHLS)</td>
<td>+</td>
<td>−</td>
</tr>
<tr>
<td>FM-1</td>
<td>Japan (food poisoning)</td>
<td>+</td>
<td>+</td>
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<tr>
<td>1230-88</td>
<td>Stew (food poisoning)</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>0075-95</td>
<td>Stew (food poisoning)</td>
<td>−</td>
<td>−</td>
</tr>
<tr>
<td>391-98</td>
<td>Vegetable purée</td>
<td>+</td>
<td>−</td>
</tr>
<tr>
<td>390-98</td>
<td>Scrambled egg</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>622-00</td>
<td>Madeira sauce</td>
<td>−</td>
<td>−</td>
</tr>
<tr>
<td>762-00</td>
<td>Ground cardamom</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>784-00</td>
<td>Ground beef</td>
<td>−</td>
<td>−</td>
</tr>
<tr>
<td>1038-00</td>
<td>Food plant</td>
<td>−</td>
<td>−</td>
</tr>
<tr>
<td>1549-00</td>
<td>Egg/tomato sandwich</td>
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<td>−</td>
</tr>
<tr>
<td>1694-00</td>
<td>Cream sauce</td>
<td>+</td>
<td>−</td>
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Isolation of DNA. Genomic DNA from B. cereus used for PCR screening and DNA sequencing was isolated using ADVAMAX beads (Advanced Genetics Technologies) or the Genomic tip-100 (Qiagen). The Genomic tip-100 procedure was essentially as described by the manufacturer but with some minor modifications in the lysis step, as follows. Thirty millilitres of a fresh culture was harvested at an OD_{600} of 0–5 (Beckman DU 650, path length 1 cm) by centrifugation at 10 000 g for 15 min. The pellet was resuspended in 7 ml buffer B1 containing 2 mM lysyzoyme ml^{-1}, 1-4 mg RNaseA ml^{-1} and 0-6 mg proteinase K ml^{-1}, and incubated at 37 °C for 2 h before buffer B2 was added and incubation continued at 55 °C for 60 min. Genomic DNA for DNA sequencing and for cloning of cytK-1 and cytK-2 into the pMS20 expression vector was isolated from B. cereus using the method of Pospiech & Neumann (1995). Plasmid DNA from E. coli was isolated using the Qiaquick Spin Miniprep Kit (Qiagen) or the Qiagen Plasmid Midi Kit (Qiagen). DNA was isolated from agarose gels using the QIAEX II Gel Extraction Kit (Qiagen).

PCR. PCR was carried out in an MJ Research Mastercycler or an Eppendorf Mastercycler Gradient thermal cycler. The primers used are listed in Table 2. Depending on the primers, the PCR programme used was: 95 °C for 1 min, 30 cycles of 95 °C for 1 min, 45–51 °C for 1 min and 72 °C for 1 min, and finally 72 °C for 7 min. PfuTurbo DNA Polymerase (Stratagene) was used for construction of pMS20 and cloning of cytK-1 and cytK-2 (from strain 1230-88) genes in pMS20, and DyNAzyme II DNA polymerase (Finnzymes) was used for all other PCRs.

DNA sequencing. DNA sequencing was performed using the ABI PRISM Big Dye Terminator Cycle Sequencing Ready Reaction Kit (PE Applied Biosystems) and a Perkin Elmer ABI Prism 377 automatic sequencer.

Sequence similarity search. A database search for similar protein sequences was carried out using the BLAST algorithm (Altschul et al., 1997). The deduced amino acid sequences were compared with the nonredundant protein databases, including GenBank CDS translations, PDB, SWISS-PROT, PUpdate and PIR. The values for percentage identity of DNA and amino acid sequences were obtained using the Smith–Waterman algorithm for local alignments (Smith & Waterman, 1981).

Culture medium and growth conditions. CGY culture medium containing 0-4 % glucose was used for production of enterotoxins in B. cereus (Beecher & Wong, 1994). The cultures were grown at 32 °C for 6 h; 1 mM EDTA was added at the time of harvest. Extracellular proteins were separated from the cells by centrifugation (10 000 g at 4 °C for 20 min). The supernatant proteins were precipitated with 70 % saturated (NH_{4})_{2}SO_{4}.

Purification of proteins. CytK-2 proteins from B. cereus strains 23, FM-1 and 1230-88 were isolated essentially as described by Lund & Granum (1996), including chromatography on a DEAE-Sephadex column (Amersham Biosciences) with Bistris/HCl buffer at pH 5-9, and chromatography on a column of Gel HT hydroxylapatite (HA) (Bio-Rad) with sodium phosphate buffer at pH 6-8. The last purification step was chromatography on a 1 ml Resource Q (ReQ) column (Amersham Biosciences) with 20 mM triethanolamine buffer at pH 8-1 and a linear 40 ml NaCl gradient from 0 to 0.2 M. The concentration of purified proteins was calculated from A_{280} measurements (Beckman DU 650, path length 1 cm).

Protein sequencing. Purified protein was sequenced from the N-terminus by Edman degradation using an Applied Biosystems 477 A automatic sequence analyser with an on-line 120 A phenylthiohydantoin amino acid analyser.

Electrophoresis. SDS-PAGE was carried out using a Bio-Rad Mini-Protein II Dual Slab Cell. The gels (12 % acrylamide) were silver stained according to Blum et al. (1987) and the molecular mass of the CytK-2 protein was estimated using the Bio-Rad Low Range SDS-PAGE standard.

Haemolysis assay. The CytK toxins were twofold serially diluted in PBS (pH 7.4), containing 1 mg BSA ml^{-1}. The diluted toxin was added to an equal volume of 2 % bovine red blood cells diluted in PBS, pH 7-4, and incubated for 1 h at room temperature. At the end of the incubation, the samples were centrifuged and the A_{450} of the supernatants was recorded (Beckman DU 650, path length 1 cm) to measure the release of haemoglobin. PBS was used as the blank.

Caco-2 and Vero cell assays. The human colon cancer cell line Caco-2 (Pinto et al., 1983) was cultivated in RPMI 1640 plus fetal calf serum, gentamicin and l-glutamine. Vero cells (derived from monkey kidney) were cultivated in MEM plus fetal calf serum, penicillin and streptomycin. The cells were grown in 24-well plates until confluence. Toxicity was determined using the inhibition of protein synthesis according to Sandvig & Olsnes (1982).

Expression of cytK-1 and cytK-2 in E. coli. The kanamycin-resistance gene from pUC4K (Amersham Biosciences) was cloned into the Sacl/Ncol site of pGK12 (Kok et al., 1984). The strong, constitutive promoter p32 from plasmid pMG36e (van de Guchte et al., 1989) was cloned into the KpnI/MluI site of the resulting plasmid, and the final construct was designated pMS20. Primers with MluI restriction sites incorporated in their 5′ termini (Table 2) were used to PCR-amplify cytK-1 from B. cereus 391-98 (primers 391F and 391R) and cytK-2 from B. cereus 1230-88 (primers 1230F and 1230R). The PCR fragments were cloned into the MluI site of pMS20. The inserts were confirmed to be correct by DNA sequencing.

### Table 2. Primers

<table>
<thead>
<tr>
<th>Primer</th>
<th>Sequence (5′–3′)</th>
<th>Sequence position (5′–3′)/ GenBank accession no.</th>
</tr>
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<tbody>
<tr>
<td>FcytK</td>
<td>AACAGATATCGGTCAAAATGC</td>
<td>1858–1878/AJ277962</td>
</tr>
<tr>
<td>RcytK</td>
<td>CCAACCAGTATACAGCTCC</td>
<td>2670–2651/AJ277962</td>
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<tr>
<td>Fhly-II</td>
<td>GATTCTAAGGGAACGTAG</td>
<td>363–381/U94743</td>
</tr>
<tr>
<td>Rhly-II</td>
<td>GGGTTACTAGGAATCTTG</td>
<td>1230–1212/U94743</td>
</tr>
<tr>
<td>391F</td>
<td>CGACCGGTATCGCAAAAATAGGAAGTG</td>
<td>1713–1739/AJ277962</td>
</tr>
<tr>
<td>391R</td>
<td>CACGCGTACGGTTTGTCTGTTGTITT</td>
<td>2788–2761/AJ277962</td>
</tr>
<tr>
<td>1230F</td>
<td>TAAAGGCTATATATCGAACAAGAT</td>
<td>182992–182968/AE017001</td>
</tr>
<tr>
<td>1230R</td>
<td>CGACCGGTCTAAATATGTCCTTCTGT</td>
<td>181906–181930/AE017001</td>
</tr>
</tbody>
</table>
Preparation of concentrated supernatants and periplasmic 
extacts. Overnight cultures of E. coli XL10-Gold (Stratagene) 
containing the vector constructs pMS20-cytK-1, pMS20-cytK-2 
and pMS20 were diluted 1:100 in 400 ml BHI (brain heart 
infusion) medium containing 50 μg kanamycin ml⁻¹, and cultured 
at 200 r.p.m. and 37 °C until an OD₆₀₀ of 0.6–0.7 was reached 
(Shimadzu UV-160A, path length 1 cm). Then 10 mM Tris/HCl 
(pH 7–3) and 10 mM NaCl was added to the cultures, followed by 
icubation for a further 10 min. The cultures were centrifuged at 6000 g for 20 min. The supernatants were concentrated by precipita 
tion with 70 % saturated (NH₄)₂SO₄ followed by centrifugation at 
10000 g and 4 °C for 20 min and dialysis against 25 mM Bistris 
(pH 5-9) with 1 mM EDTA at 4 °C overnight. Periplasmic extracts 
were prepared from the bacterial cell pellets, using the following 
procedure. The pellets were resuspended in 10 ml 33 mM Tris/HCl 
(pH 7-3); 10 ml freshly prepared 33 mM Tris/HCl (pH 7.3) with 
40 % sucrose and 4 mM EDTA was added. The cells were gently 
mixed for 10 min at room temperature, followed by centrifugation at 
9000 g for 15 min. The pellets were gently resuspended in 2 ml 
icc-cold distilled H₂O. Ice-cold MgSO₄ was added to a concentration of 
5 mM, followed by incubation on ice for 10 min. The samples 
were centrifuged at 16000 g for 15 min, and the supernatants 
(periplasmic extracts) were collected and stored on ice.

Planar lipid bilayer recording. Planar lipid bilayer recordings 
were carried out using a system described by Williams (1995). 
Briefly, lipid bilayers were formed from a dispersion of 15 mg 
palmitoyloleoylphosphatidylethanolamine ml⁻¹ and 15 mg palmit 
Toyloleoylphosphatidylserine ml⁻¹ in n-decane, which was drawn 
across a 0.4 mm diameter hole in a polystyrene cup separating two 
solution-filled chambers, designated cis and trans. The cis chamber 
to which the toxin was added) was held at ground (0 mV), and the 
trans chamber was clamped to a range of potentials (GeneClamp 
500 patch-clamp amplifier, Axon Instruments). The sign of the 
membrane potential refers to the trans chamber, and currents are 
defined as ‘positive’ when cations flow from trans to cis. Trans 
membrane currents were low-pass filtered at 0.5–1 kHz (8 pole 
Bessel) digitized at 5 kHz. Membrane capacitance was measured by 
differentiating a triangulation wave input of 0-2 kHz. Only bilayers that 
had a conductance of less than 10 pS and initial capacitance of at 
least 300 pF were used. Unless otherwise stated, lipid bilayers were 
bathed in 250 mM NaCl containing 5 mM HEPE/S/NaOH (pH 7.0), 
and all recordings were made at room temperature (19–22 °C). The 
recordings were analysed off-line using 30–180 s recordings carried 
out for each holding potential and analysed using PAT and Win EDR 
software (Strathclyde Electrophysiology Software). Current steps 
yielding conductances over twice the size of CytK-1 (i.e. >220 pS) 
were excluded as simultaneous openings of more than one channel.

RESULTS

PCR screening for the presence of cytK and 
hly-II in B. cereus strains

To investigate the presence of cytK genes in different strains 
of B. cereus, PCR using the primers FcytK and RcytK 
(Table 2) was performed with DNA from 29 different strains 
that were shown to be toxic against Vero cells. Previously, 
PCR with a set of primers constructed from the cytK-1 
sequence of B. cereus strain 391-98 had given negative 
results (Lund et al., 2000). In that study, one of the primers 
targeted a region of CytK-1 showing low homology to 
other β-barrel pore-forming toxins. In this work, however, 
positive results were obtained for 13 strains (Table 1). The 
presence of the hly-II gene was confirmed in six of the 
strains (Table 1) by PCR with the primers Fhly-II and Rhly-II 
(Table 2), constructed from the hly-II gene sequence 
(Baida et al., 1999). Both genes were present in five strains.

Sequence analysis and comparison of the cytK 
genes

The cytK PCR products of B. cereus strains 1230-88, FM-1 and 
23 were DNA sequenced directly, thus obtaining 76 % of the 
gene sequences. Alignments of the deduced amino acid 
sequences revealed conserved parts of the protein. 
Furthermore, the identity between the obtained amino 
acid sequences from strains 1230-88, FM-1 and 23 was 97– 
99 %, while the identity between these sequences and the 
original CytK-1 from strain 391-98 was only 89 %. We have 
named this new CytK-variant CytK-2. The CytK-2 
sequences obtained were used to search the completed 
genomes of B. cereus ATCC 14579 and B. cereus ATCC 
10987 using the BLAST algorithm (Altschul et al., 1997). A 
cytK-2 gene was identified in both strains (Fig. 1).

In order to obtain the remaining DNA sequences of the 
cytK-2 genes, PCRs were performed using primers 
constructed based on the ATCC 14579 cytK-2 DNA sequence. 
Using this approach, we obtained the full-length sequence 
of cytK-2 from strain 1230-88, and the 5’ termini of cytK-2 
from strains FM-1 and 23. In addition, the promoter 
sequence ~200 bp upstream of the cytK-2 start codons 
was obtained for strains 1230-88 and 23. Inverse PCR was 
used to obtain the sequence of the 3’ terminus of cytK-2 
in B. cereus strain FM-1. Genomic DNA was digested with 
EcoRV, followed by intramolecular ligation of the individual 
restriction fragments. The circularized DNA was then 
used as a template in PCR, thus obtaining the sequence 
downstream of the cytK-2 gene. This sequence was used to 
design primers that were used to PCR-amplify the 3’ 
termminus of the cytK-2 gene in strain 23 as well.

The mature CytK-2 proteins showed about 89 % identity 
to the CytK-1 from strain 391-98. The differences between 
the CytK-2 sequences and CytK-1 were clustered in specific 
regions (Fig. 1). The identities between the mature CytK-2 
sequences of strains ATCC 14579, ATCC 10987, 1230-88, 
FM-1 and 23 were between 96 % and 100 %. The FM-1 
CytK-2 had an additional amino acid at the C-terminal end as 
compared with the other CytK proteins, while the mature 
sequences of CytK-2 from strains ATCC 10987 and 
23 were 100 % identical.

The PCR products obtained using the primers Fhly-II and 
Rhly-II (Table 2) with DNA from strains 1230-88 and 
FM-1 were sequenced. The sequences were 99 % identical 
to that deduced from the original hly-II (results not shown). 
This sequence was 37-5 % identical to CytK-1, and 39 % 
identical to CytK-2 (all containing 6 % gaps). The identity 
between the CytK proteins and z-haemolysin of S. aureus 
was 29–30 %, with the differences between the proteins 
distributed throughout the amino acid sequences.
PlcR is a pleiotropic regulator involved in the control of extracellular virulence factor expression in pathogenic Bacillus spp. (Agaisse et al., 1999). It has previously been shown that the expression of CytK-2 was abolished in a plcR-deficient mutant of B. cereus ATCC 14579 (Gohar et al., 2002). The recognition site for the positive transcriptional regulator PlcR in the promoter region of cytK-1 from strain 391-98 was 5′-TATGCAATTTGGCATA-3′, thus containing a one base mismatch (C11) compared to the PlcR binding site consensus sequence 5′-TATGNAN4TN-CATA-3′ (Agaisse et al., 1999). In contrast, the PlcR boxes upstream from the cytK-2 genes in strains ATCC 14579, ATCC 10987, 1230-88 and 23 were identical to the highly conserved regulatory sequence. The location of the centre of the palindromic sequence of the PlcR box was at position 286 for cytK-1 from strain 391-98 and at position 289 for the cytK-2 genes, relative to the start codon. In comparing the promoter regions extending ten nucleotides upstream from the PlcR box through to the start codon, the identity between the cytK-1 promoter region in strain 391-98 and the corresponding cytK-2 promoter sequences in strains ATCC 14579, ATCC 10987, 1230-88 and 23 was 66–73%, containing 4-5% gaps. The identity between the promoter regions of the four strains containing cytK-2 was 92–99%. The differences in the promoter regions explain the negative results obtained when PCRs were run with DNA from strains 1230-88, 68 and 23 using a primer made from the promoter region of cytK-1 from strain 391-98 (Lund et al., 2000).

Protein isolation and comparison of the toxic activity of CytK-1 and CytK-2

To confirm that B. cereus strains 1230-88, FM-1 and 23 actually produce the CytK-2 toxins, supernatant proteins were precipitated with ammonium sulphate and purified on columns of DEAE, HA and ReQ. The CytK-2 proteins were eluted mainly in the void volume from the DEAE column at pH 5.9 and at approximately 100 mM sodium phosphate from the HA column (as for the original CytK-1). However, the original CytK-1 was eluted from ReQ at pH 8.1 at 20 mM NaCl, while the CytK-2 proteins were eluted at lower NaCl concentrations and not in a sharp peak. The CytK-2 protein of FM-1 seemed to be pure after fractionation on the HA column (Fig. 2), CytK-2 from strain 23 was pure after fractionation on the ReQ column, while the CytK-2 protein of strain 1230-88 (Fig. 2) was still slightly contaminated with another protein after the last step on the ReQ column. The mobility of CytK-1 and the CytK-2 proteins on SDS-PAGE seemed to be identical, indicating that the size of the proteins is similar. The 20 first amino acid residues from the N-terminal ends of the CytK-2 proteins from strains 1230-88 and 23 were determined by amino acid sequencing, confirming the identity of the proteins.
The toxicity of the CytK-2 proteins of strains 1230-88, FM-1 and 23 against Caco-2 cells and lysis of bovine erythrocytes were measured as described for CytK-1 (Lund et al., 2000; Hardy et al., 2001). Table 3 shows that the CytK-2 proteins were toxic against both cell types, but not to the same extent as the original CytK-1. The CytK-2 proteins lost activity during storage and freeze–thaw cycles, so the toxicity tests had to be carried out within a few days after the purification of the proteins from each strain. We also observed that the cytotoxic activity decreased through the chromatography steps in the isolation procedure, presumably due to aggregation as the protein sample became increasingly pure. The haemolytic activity, however, seemed to be retained. In contrast to CytK-1 samples, pure fractions of CytK-2 were not shown to be toxic towards Vero cells. It has previously been shown that the amount of protein necessary for 50% inhibition of protein synthesis in ~5 × 10^4 Vero cells was five times the amount necessary for the same effect using the Caco-2 cell line (Hardy et al., 2001), so the lack of Vero cell cytotoxicity could be due to low assay sensitivity.

As it was difficult to retain the activity of CytK-2 through the purification steps and during storage, cytK-1 from B. cereus 391-98 and cytK-2 from B. cereus 1230-88 were cloned into the pMS20 vector and expressed in E. coli. A control containing the pMS20 empty vector was also prepared. Cultures were grown to late exponential phase, and concentrated supernatants and periplasmic extracts were prepared for all three clones. CytK-1 and CytK-2 were thus produced under the same conditions, and furthermore, aggregates should not be expected to form, as the CytK toxins were present in solution with other secreted or periplasmic proteins. The active CytK proteins were isolated from the periplasm of E. coli, and in contrast to pure CytK-2 proteins purified from B. cereus culture supernatant, the periplasmic extract containing CytK-2 from strain 1230-88 was toxic towards Vero cells. The supernatants from all three cultures and the periplasmic extract from the culture containing E. coli harbouring the pMS20 empty vector were neither haemolytic towards bovine erythrocytes nor cytotoxic towards Vero cells.

The volume of periplasmic extracts containing CytK-1 and CytK-2 necessary to obtain 50% haemolytic activity towards bovine erythrocytes was determined. To normalize the two samples, their volumes were adjusted to contain the same amount of haemolytic activity. Then, the volume of the normalized periplasmic extracts needed to obtain 50% inhibition of protein synthesis in Vero cells was determined. It was necessary to add five times the volume of periplasmic extract containing CytK-2 as compared to periplasmic extract containing CytK-1 to obtain the same degree of protein inhibition in Vero cells (mean values of duplicate experiments). Thus, relative to the haemolytic activity, CytK-1 from strain 391-98 was about five times more toxic against Vero cells than CytK-2 from strain 1230-88.

**Analysis of the pore-forming ability of CytK-1 and CytK-2 using planar lipid bilayer recordings**

The difference in activity between batches of purified CytK-1 and CytK-2 proteins was examined using planar lipid bilayers to test for pore formation. Five preparations were examined and the results are summarized in Fig. 3. For CytK-1 isolated from B. cereus 391-98 supernatant, two samples were tested: one active preparation, and one sample that had lost cytotoxicity upon purification and storage. Addition of up to 10 μg protein of the active CytK-1 protein to the cis bilayer chamber resulted in the

<table>
<thead>
<tr>
<th>Toxin</th>
<th>Amount of protein (ng) for:</th>
</tr>
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<tbody>
<tr>
<td></td>
<td>Haemolysis</td>
</tr>
<tr>
<td>CytK-1, strain 391-98*</td>
<td>15–30</td>
</tr>
<tr>
<td>CytK-2, strain 23</td>
<td>42</td>
</tr>
<tr>
<td>CytK-2, strain 1230-88</td>
<td>34</td>
</tr>
<tr>
<td>CytK-2, strain FM-1</td>
<td>54</td>
</tr>
</tbody>
</table>

*The values for CytK-1 are from Lund et al. (2000) and Hardy et al. (2001).
appearance of rectangular current fluctuations. The time taken for single-channel current steps to appear was generally less than 15 min. The active CytK-1 protein purified from *B. cereus* 391-98 (i.e. haemolytic and cytotoxic) yielded single-channel events with a mean single-channel conductance of $143 \pm 3 \text{ pS}$ (Fig. 3a), similar to that described previously (Hardy *et al.*, 2001). The pore-forming ability of CytK-2 proteins was examined using CytK-2 purified from *B. cereus* 1230-88 supernatant. The purified protein sample was haemolytic but not cytotoxic towards Vero cells. As shown in Fig. 3(d), the CytK-2 protein was able to form pores in lipid bilayers but the distribution of pore sizes was distinct from that of the active CytK-1 protein, with 63% of the channels having a conductance less than 100 pS. Recombinant CytK-1 and CytK-2 proteins from the same strains isolated from the periplast of *E. coli* were examined for channel conductance. Fig. 3(b) shows the distribution of channel conductances obtained from recombinant CytK-1 (r391-98), showing that 75% of the channels were between 101 and 220 pS, and thus were of similar size as the channels formed by the active CytK-1 protein isolated from *B. cereus*. Recombinant CytK-2 protein from strain 1230-88 (Fig. 3e) yielded a spread of channel conductances, but the predominant conductance values were less than 100 pS.

The batch of CytK-1 isolated from *B. cereus* 391-98 supernatant that was not cytotoxic towards Vero cells was able to produce single-channel events in the lipid bilayers, but the distribution of channel conductances was different from that of the cytotoxic batch. Fig. 3 shows that the predominant channel sizes formed by the non-cytotoxic CytK-1 were smaller than those formed by the cytotoxic protein. Less than 30% of the total number of single-channel events observed with the non-cytotoxic CytK-1 had channel conductances comparable to that of the active protein (Fig. 3a versus 3c), while the majority of pores formed instead had lower conductances. The same pattern was observed with the non-cytotoxic batch of CytK-2 from strain 1230-88, which showed a predominance (63%) of pores of lower conductance than the cytotoxic CytK-1 protein (Fig. 3d). These results indicate that cytotoxicity but not haemolytic activity of CytK-1 and CytK-2 proteins correlates with the ability to form pores of conductances greater than 100 pS in 250 mM NaCl. It is interesting to note that the properties of the haemolytic but non-cytotoxic batch of CytK-1 from strain 391-98 yielded a pattern of channel conductances similar to that of the CytK-2 protein from strain 1230-88.

**DISCUSSION**

CytK-2 proteins have been isolated from three different strains of *B. cereus*. The identity between the deduced amino acid sequences of these proteins was close to 100%, while the identity of these sequences to the corresponding sequence of the original CytK-1 from *B. cereus* 391-98 was 89%. Differences in sequence between CytK-1 and the CytK-2 proteins were mainly localized to the same residues and clustered in specific regions (Fig. 1). The CytK-2 proteins were haemolytic, able to form pores in planar lipid bilayers, and toxic towards human intestinal cells, although not to the same extent as CytK-1 from strain 391-98. When expressed in *E. coli*, the cytotoxic activity (relative to the haemolytic activity) of the CytK-2 protein from *B. cereus* 1230-88 was five times lower than that of the original CytK-1. Relatively small differences in sequence seem to change the toxicity of these proteins. The extent to which the reduction of toxicity is related to pore size is at present speculative, although the data provided indicate that CytK-2 proteins have smaller pore size than the pores formed by the original CytK-1. Nevertheless, it is reasonable to assume that CytK-2 toxins from several strains of *B. cereus* may contribute to enterotoxicity.
There are differences between CytK-1 and the CytK-2 proteins in six positions in the putative pore-forming region (Fig. 1). The substituted residues are clustered in the region that constitutes the base of the stem, and are predicted to line the exterior of the membrane channel. However, the amino acid substitutions were mainly conservative, indicating that the pore of the CytK-2 proteins will resemble that of the original CytK-1 protein. Nonetheless, the possibility that the differences in this region could have biological effect cannot be excluded.

The reduction in cytotoxic activity in the CytK-1 and CytK-2 proteins upon purification and storage may be attributed to spontaneous oligomerization of monomers. Chattopadhyay & Banerjee (2003) reported that *Vibrio cholerae* haemolysin A (HlyA), a β-barrel channel-forming toxin with 22% identity to the CytK proteins, spontaneously and irreversibly transformed from the haemolytically active monomer to a haemolytically inactive heptamer when kept in water for days.

The pleiotropic transcriptional activator PlcR is involved in the control of extracellular virulence factor expression in *B. cereus* (Agaisse et al., 1999). The recognition site for PlcR, a conserved palindromic sequence (the PlcR box; Agaisse et al., 1999), was found upstream of the cytK-2 genes, strongly indicating that PlcR regulates their expression. Gohar et al. (2002) examined the extracellular proteome of the wild-type *B. cereus* strain ATCC 14579 and a mutant strain with disrupted plcR. CytK-2 was one of the proteins identified in the culture supernatant of the wild-type strain, which was not present in the mutant strain. It is interesting to note that the recognition site for PlcR in the promoter of cytK-1 from strain 391-98 was atypical, displaying a one-nucleotide mismatch (C11 versus T11) compared with the original sequence reported by Agaisse et al. (1999). A functional PlcR box that differs from the consensus sequence has previously only been described for the zinc-requiring metalloproteinase InhA-2, found in both *B. thuringiensis* and *B. cereus* (Fedhila et al., 2003).

The CytK-2 proteins show relatively high sequence similarity (39% identity) to the deduced product of *hly-II* (Baida et al., 1999). However, in aligning the deduced sequences of cytK and *hly-II*, 6% gaps are present, whereas none are present when comparing CytK-1 and the CytK-2 proteins (with the exception that the FM-1 CytK-2 was one amino acid longer than the other CytK proteins). The presence of a lysine residue, K125, at the end of the possible transmembrane loop in the product of *hly-II* may cause cleavage of this protein by trypsin and thus result in the protein being inactivated in the small intestine. Its contribution to the toxicity of *B. cereus* strains is therefore probably not of any significant importance. The overlapping part of the sequence of haemolysin IV (only the 30 N-terminal amino acids were determined; Beecher et al., 2000) and the CytK-2 protein of *B. cereus* ATCC 14579 are identical. It is therefore reasonable to include haemolysin IV, which is suggested to be an important virulence factor, in the CytK group of toxins. In summary, there seem to exist two subclasses of β-barrel pore-forming toxins in *B. cereus* and *B. thuringiensis*. The product of *hly-II* sequenced by Baida et al. (1999) occurs widely in *B. thuringiensis* strains, while the other subclass, CytK toxins (including haemolysin IV), seems to be common in *B. cereus* strains.

The enterotoxin complexes Nhe (Lund & Granum, 1996) and Hbl (Beecher & Wong, 1994), or the genes encoding them, have been shown to be present in some of the strains containing CytK-2 proteins (Lund & Granum, 1997; Stenfors & Granum, 2001). For instance, *B. cereus* 1230-88, which caused a severe food poisoning (although without bloody diarrhoea), expresses a CytK-2 toxin, Nhe and Hbl, and contains *hly-II*. Thus, several toxins may contribute to the enterotoxicity of certain *B. cereus* strains. In contrast, strain 391-98 only expresses CytK-1. It will be of interest to test the hypothesis that the CytK toxins form ulcerative and haemorrhagic lesions in the intestine whereas the other enterotoxin genes produce watery diarrhoea.

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