Insertional inactivation of *hblC* encoding the L₂ component of *Bacillus cereus* ATCC 14579 haemolysin BL strongly reduces enterotoxigenic activity, but not the haemolytic activity against human erythrocytes

Toril Lindbäck, Ole Andreas Økstad, Anne-Lise Rishovd and Anne-Brit Kolstø

Author for correspondence: Anne-Brit Kolstø. Tel: +47 22958460. Fax: +47 22694130.
e-mail: annebko@biotek.uio.no

Biotechnology Centre of Oslo and School of Pharmacy, Department of Microbiology, University of Oslo, PO Box 1125 Blindern, N-0349 Oslo, Norway

Haemolysin BL (HBL) is a *Bacillus cereus* toxin composed of a binding component, B, and two lytic components, L₁ and L₂. HBL is also the enterotoxin responsible for the diarrhoeal food poisoning syndrome caused by several strains of *B. cereus*. The three genes encoding the HBL components constitute an operon and are transcribed from a promoter 608 bp upstream of the *hblC* translational start site. The first gene of the *hbl* operon, *hblC*, in the *B. cereus* type strain, ATCC 14579, was inactivated in this study. Inactivation of *hblC* strongly reduced both the enterotoxigenic activity of *B. cereus* ATCC 14579 and the haemolytic activity against sheep erythrocytes, while maintaining full haemolytic activity against human erythrocytes.

Keywords: *Bacillus cereus*, enterotoxin, haemolysin, HBL

INTRODUCTION

Bacillus cereus is a Gram-positive, spore-forming, motile, aerobic rod, commonly found in soil and water. *B. cereus* has also been isolated from a variety of foods, including rice, spices, meat, eggs and dairy products (Kramer & Gilbert, 1989), and from drugs including both topical and oral pharmaceutical products (Garcia-Arribas *et al.*, 1988). *B. cereus* is the causative agent of two distinct food poisoning syndromes, the emetic and the diarrhoeal syndromes (Turnbull *et al.*, 1979). Both forms of the illness are toxin-mediated. The emetic toxin, a cereulide, has been isolated and described by Agata *et al.* (1994, 1995b).

At least three diarrhoeal toxins have been isolated from *B. cereus* strains, the best characterized being the enterotoxic haemolysin BL (HBL) (Beecher & Macmillan, 1991). HBL is composed of three components, B, L₁ and L₂, forming a protein complex possessing haemolytic, cytotoxic, dermonecrotic and enterotoxigenic activities (Beecher & Wong, 1994a; Beecher *et al.*, 1995). In addition to its haemolytic and enterotoxic

activities, *B. cereus* is considered one of the most destructive organisms to affect the eye (O'Day *et al.*, 1981; Pflugfelder & Flynn, 1992). *B. cereus* ocular virulence is multifactorial and HBL is one of the major factors contributing to this virulence (Beecher *et al.*, 1995).

The genes encoding the B, L₁ and L₂ components, *hblA*, *hblD* and *hblC*, respectively, have previously been cloned and sequenced from the *B. cereus* strain F837/76 (Heinrichs *et al.*, 1993; Ryan *et al.*, 1997). Beecher & Macmillan (1991) have shown that a combination of all three components, B + L₁ + L₂, is required for complete lysis of sheep erythrocytes. Neither B + L₁ nor any of the components alone was haemolytic. Using mAbs against the B component, it was shown by immunofluorescence staining of erythrocytes that protein B binds to the cell surface before the L components can act to cause cell lysis (Beecher & Macmillan, 1991). The potency of HBL has been compared to that of cholera toxin, and HBL has therefore been suggested to be the primary virulence factor in diarrhoea caused by *B. cereus* (Beecher *et al.*, 1995).

Studies by Agata *et al.* (1995a) and Lund & Granum (1996) have identified other proteins responsible for a

Abbreviation: HBL, haemolysin BL.

diarrhoeal-type of *B. cereus* food poisoning. Agata *et al.* (1995a) cloned a gene encoding a 41 kDa protein, bc-D-ENT, identified in 10 strains isolated from food or outbreaks of foodborne illness. The amino acid sequence of the protein showed no homology to the amino acid sequence of the HBL components, did not possess an N-terminal sequence characteristic of *Bacillus* signal peptides (Simonen & Palva, 1993), and has not yet been shown to be secreted by *B. cereus*. Lund & Granum (1996) have characterized a non-haemolytic enterotoxin (NHE) complex from *B. cereus* isolated after a foodborne outbreak. The enterotoxin consisted of three proteins of 39, 45 and 105 kDa, and the 39 kDa protein showed 70% identity in a stretch of 23 aa to the L₁ component of HBL (Lund & Granum, 1996, 1997). In addition, several haemolysins have been identified in *B. cereus* strains (Baida & Kuzmin, 1995; Honda *et al.*, 1991). Thus the number and nature of enterotoxins and haemolysins in *B. cereus* is complex, and doubts have been raised whether the HBL complex expresses both haemolytic and enterotoxigenic activity.

To further investigate the role of HBL in the various aspects of *B. cereus* toxicity, we have constructed a gene inactivation mutant of the first gene in the *hbl* operon which in addition resulted in loss of transcription of the two downstream genes of the operon. The *B. cereus* type strain, ATCC 14579, was chosen for our studies, since this strain carries the *hbl* locus (Carlson *et al.*, 1996) and has been used previously in gene inactivation experiments (Lindbäck & Kolstø, 1997; Økstad *et al.*, 1997). The present study shows that disruption of the *hblC* gene causes a major reduction in the enterotoxigenic activity as well as in haemolytic activity against sheep erythrocytes, while no change was detected in haemolytic activity against human erythrocytes. The mutant described in this report will be employed in future work to further unravel the effects of HBL.

METHODS

Bacterial strains, plasmids and growth conditions. All bacteria were routinely grown in 100 ml LB broth (Sambrook *et al.*, 1989) in 1000 ml flasks, or on LB agar at 37 °C. The *B. cereus* strains ATCC 14579 (type strain) and ATCC 10987 were obtained from the American Type Culture Collection (Manassas, VA, USA). *B. cereus* F837/76 was obtained from the Public Health Laboratory Service (London, UK). *Escherichia coli* strains DH5 α and BK2118 (Evensen & Seeberg, 1982) were used as recombinant hosts. The DNA library was constructed in pUC19 and all subcloning was done in pUC18 or pUC19. Ampicillin was used at a concentration of 50 μ g ml⁻¹ and spectinomycin at a concentration of 100 μ g ml⁻¹, where appropriate. Bacterial culture supernatants used in immunoblotting and in the Vero cell toxicity test were obtained by centrifuging a culture of *B. cereus* grown to OD₅₅₀ 2.5 in Brain Heart Infusion broth (Difco) containing 0.5% glucose.

DNA manipulation and library preparation. Preparation, cloning and analysis of DNA were performed according to standard protocols (Sambrook *et al.*, 1989). *E. coli* BK2118 was used for electroporation according to Chuang *et al.* (1995). Genomic DNA from *B. cereus* was isolated from

200 ml cultures grown to mid-exponential phase. The bacteria were washed in TES (30 mM Tris, pH 8.0; 5 mM EDTA; 50 mM NaCl) and resuspended in 8 ml TES. One millilitre of lysozyme (5 mg ml⁻¹) and 0.4 ml RNase (2 mg ml⁻¹) were added and the suspension was incubated for 30 min at 37 °C. Triton X-100 (1.4 ml, 8%) and 0.6 ml proteinase K (5 mg ml⁻¹) were added and the bacteria were lysed for 1 h at 37 °C. PMSF (0.2 ml, 100 mM) was added before the DNA was purified by ultracentrifugation in a CsCl gradient (Sambrook *et al.*, 1989).

A genomic library was constructed by digesting the purified *B. cereus* ATCC 14579 DNA with *Bgl*II followed by ligation into *Bam*HI-digested pUC19. The library was screened using a probe covering the *hblC* and *hblD* genes from *B. cereus* F837/76. The probes were obtained as a gift from James D. Macmillan (Rutgers University, NJ, USA).

Amplification of *hblA* and *hblB* by PCR. Subregions of the *hblA* and *hblB* genes were amplified by PCR using the primers P3 (5'-GGC ACA TGC TGT TAC TTG GG-3') and P4 (5'-ACC CAA AAT AGC ACC CTT CA-3') for *hblA*, and P5 (5'-TGA ATC AGC TTC GTC CAT CA-3') and P6 (5'-AAT GAA AGC CAA TCA GCC C-3') for *hblB*. PCR was run as recommended by the suppliers for 30 cycles in a 50 μ l volume using an annealing temperature of 56 °C, 0.4 μ M of each primer, 100 ng of the cloned Bc300 fragment (Fig. 1) as DNA template and 1 U Dynazyme (Finnzymes Oy). The forward primers P3 and P5 are located at positions 13771 and 16086, respectively, and the reverse primers P4 and P6 are located at positions 14185 and 16275, respectively, in the EMBL sequence AJ237785.

RNA isolation and analysis. RNA was isolated from 10 ml samples as described by Igo & Losick (1986). Mapping of the 5' terminus of the *hbl* mRNA was performed on total RNA isolated from *B. cereus* ATCC 14579, using two different 5' end-labelled oligonucleotides, P1 (5'-TCC TTT CTG TCT GGT CAT ACC-3') and P2 (5'-TCA GCA AAC TCC TTA CTA GAC-3'), located 580 and 363 bp upstream of the putative translational start site of *hblC*, respectively. Annealing and extension were performed according to Luna *et al.* (1994) using 10 U AMV Reverse Transcriptase (Promega) in the extension reaction. Products were resolved in 6% polyacrylamide gels together with sequence reactions prepared according to the dideoxy chain-termination method using T7 DNA polymerase (Pharmacia). Northern blotting and hybridization were performed according to Narahara *et al.* (1992).

SDS-PAGE and immunoblotting. Supernatant (10 μ l) from cultures harvested in early stationary phase was applied on 10% SDS-PAGE gels. SDS-PAGE and immunoblotting were performed according to standard protocols (Harlow & Lane, 1988). Polyclonal antibodies directed against the L₁, L₂ and B components of HBL were obtained as a gift from Douglas J. Beecher (Rutgers University, NJ, USA) and used at a dilution of 1:5000. Anti-rabbit horseradish-peroxidase-conjugated antibodies from donkey (Amersham) were used as secondary antibodies (1:5000) and ECL Western blotting detection reagent (Amersham) was used to develop the immunoblot.

Vero cell toxicity and haemolytic activity assays. Vero cells were obtained from the Norwegian Radium Hospital and were grown in Dulbecco's MEM medium supplemented with 5% foetal calf serum. Cells were seeded into 24-well plates the day before testing. Bacterial culture supernatants (100 μ l) were added to triplicate wells containing a Vero cell monolayer. Cell cultures were incubated further at 37 °C under 5% CO₂ for up to 1 h. Before and immediately after the addition of bacterial culture supernatants, and at 10, 20 and 60 min post

addition, cell cultures were examined with a Nikon TMS microscope.

Blood agar plates were prepared with sheep, human, horse or chicken erythrocytes (10%), and bacteria or culture supernatant was spotted on the plates. The plates were incubated at 20, 30 or 37 °C overnight. After incubation the plates were checked for haemolytic zones.

RESULTS

Cloning of the *hbl* operon from *B. cereus* ATCC 14579

Screening of 20000 colonies from a genomic library with the *hblC/hblD* probe from *B. cereus* F837/76 resulted in the identification of two DNA fragments, Bc300 and Bc301, of 9.9 kb and 11.3 kb, respectively. Sequencing and analysis of the complete Bc300 and Bc301 clones is reported elsewhere (Økstad *et al.*, 1999). The DNA sequence of the *hbl* operon showed 97 % identity to the previously reported sequence from *B. cereus* F837/76 (Heinrichs *et al.*, 1993; Ryan *et al.*, 1997; Økstad *et al.*, 1999).

Insertional inactivation of *hblC*

The most downstream *Hind*III fragment of Bc301 (Fig. 1), with one *Hind*III site located in the pUC19 polylinker, was cloned into the *Hind*III site of the conjugative suicide vector pAT113 (Trieu-Cuot *et al.*, 1991) (Fig. 1). Subsequently a 1.2 kb *Eco*RI fragment covering the *hblD* gene (Fig. 1) was cloned into the *Eco*RI site and a spectinomycin-resistance cassette was cloned into the *Bam*HI site of the pAT113 construct. This new construct, pAT113-hbIs (Fig. 1), was used in transconjugal

Fig. 2. Southern blot analysis of chromosomal DNA digested with *Hind*III. Lanes: 1, BcΔ*hblC*; 2, single cross-over mutant used to create BcΔ*hblC*; 3, wild-type *B. cereus* ATCC 14579. The membrane was probed with both the 0.9 kb *Bgl*II-*Eco*RI fragment replaced by the spectinomycin-resistance cassette in BcΔ*hblC*, and the spectinomycin-resistance cassette of 1.4 kb.

transfer from *E. coli* JM83(pRK24) (Thomas & Smith, 1987) to *B. cereus* ATCC 14579, and chromosomal integration was verified by hybridization. Three separate

Fig. 1. Restriction map of a genomic region from *B. cereus* ATCC 14579 covering the *hbl* operon. The two *Bgl*II fragments, Bc300 and Bc301, are indicated. The transcriptional start site, location of primers (P1, P2, P3, P4, P5, P6) and the PlcR box are indicated by arrows. The construction of pAT113-hbIs used in conjugation experiments is shown.

Fig. 3. Northern blot analysis of total RNA isolated from different *B. cereus* strains. Lanes: 1, 0.24–9.5 kb RNA ladder from Gibco-BRL; 2, *B. cereus* ATCC 10987; 3, *B. cereus* F837/76; 4, *B. cereus* ATCC 14579; 5, *BcΔhblC*. The membrane was probed with a 0.9 kb *Bgl*II–*Eco*RI fragment covering the *hblC* gene (Fig. 1).

mating experiments between *B. cereus* ATCC 14579 and *E. coli* JM83(pRK24)(pAT113-hbIs) produced exclusively single cross-over transconjugants. One double cross-over transconjugant was identified after growing a single cross-over transconjugant at 45 °C for five passages of 24 h each. Hybridization of chromosomal DNA isolated from the double cross-over transconjugant, assigned *BcΔhblC*, showed that the 0.9 kb *Bgl*II–*Eco*RI fragment located within *hblC* (Fig. 1) had been replaced by the spectinomycin-resistance cassette of 1.4 kb (Fig. 2).

Expression analysis of the *hbl* locus

Northern blots of RNA isolated from different *B. cereus* strains probed with the 0.9 kb *Bgl*II–*Eco*RI fragment from *hblC* (Fig. 1) revealed a 5.5 kb transcript in *B. cereus* ATCC 14579 and *B. cereus* F837/76, while no transcript was detected in RNA from *B. cereus* ATCC 10987 and *BcΔhblC* (Fig. 3). Using a part of the *hblD* gene and a PCR-amplified subregion of *hblA* (primers P3 and P4; Fig. 1) as probes, hybridization was observed to the 5.5 kb transcript in wild-type *B. cereus* ATCC 14579 while no hybridization was detected in *BcΔhblC* (data not shown). PCR was used to amplify a 3' region of *hblB* that does not show sequence similarity to *hblA*, using

Fig. 4. Mapping of the 5' terminus of the *hbl* mRNA of *B. cereus* ATCC 14579 by primer extension. The transcriptional start site is indicated by an arrow. PE, primer extension.

oligonucleotide primers P5 and P6 (Fig. 1). When this *hblB*-specific probe was used in Northern hybridization, no transcript was detected in RNA isolated from wild-type or mutant cells during stationary phase (data not shown). These data show that the disruption of the *hblC* gene abolished transcription of the downstream genes, *hblD* and *hblA*, and indicated that *hblB* is not transcribed at a level detectable by Northern blot analysis, even in wild-type *B. cereus* ATCC 14579.

Mapping of the 5' terminus of the *hbl* mRNA in *B. cereus* ATCC 14579 by primer extension analysis using oligonucleotide P2 (Fig. 1) revealed a product of 246 bp, thereby identifying a transcriptional start site at an adenine (Fig. 4) positioned 608 bp upstream of the putative translational start site. A putative –10 promoter sequence, TTTAAT, with T in position –10, was identified upstream of the putative transcriptional start site. Primer extension analysis using oligonucleotide P1 (Fig. 1) revealed no alternative transcriptional start site upstream of the one described above. The transcriptional start site is located 271 bp downstream of the putative binding site of the transcriptional regulator PlcR (Agaisse *et al.*, 1999).

By using polyclonal antibodies directed against components B, L₁ and L₂, respectively, in immunoblotting, no trace of the L₂ component, the product of the first gene of the operon (*hblC*), was found in the culture

Fig. 5. Western blotting of *B. cereus* ATCC 14579 and *BcΔhblC* culture supernatant probed with antisera to the individual HBL components. Lanes: 1, 3 and 5, *B. cereus* ATCC 14579 culture supernatant; 2, 4 and 6, *BcΔhblC* culture supernatant. Lanes 1 and 2 are probed with antiserum against L_2 , lanes 3 and 4 are probed with antiserum against L_1 and lanes 5 and 6 are probed with antisera against the B component of HBL. The size marker used was a prestained broad-range marker from Bio-Rad.

supernatant of *BcΔhblC* (Fig. 5). Faint bands observed when anti-B or anti- L_1 sera were used may be due to non-specific binding of the polyclonal anti-B and anti- L_1 antibodies.

Enterotoxin and haemolytic activity

Vero cell monolayers were examined microscopically after addition of culture supernatants from *B. cereus* ATCC 14579, *B. cereus* ATCC 10987, *B. cereus* F837/76

and *BcΔhblC*. A rapid cytopathogenic effect was observed within a few minutes for *B. cereus* ATCC 14579, *B. cereus* ATCC 10987 and *B. cereus* F837/76, while a reduced effect was observed when the *BcΔhblC* supernatant was added to the cells (Fig. 6). As a control we also included a mutant strain inactivated by the spectinomycin-resistance cassette in the *bctL* gene encoding a putative transporter protein (Økstad *et al.*, 1997). The effect of the *BcΔbctL* disruption mutant was similar to the effect of wild-type *B. cereus* ATCC 14579.

Wild-type *B. cereus* ATCC 14579 and *BcΔhblC* were grown on agar plates containing sheep, chicken, human or horse erythrocytes (10%). There was a pronounced reduction in the haemolytic activity of the mutant strain against sheep erythrocytes (Fig. 7), while no difference was seen against erythrocytes from human (Fig. 7) and other sources (data not shown). Interestingly, the strong haemolytic activity against sheep erythrocytes was observed when the cells were grown at 37 °C, but not at 30 °C, although a 'paradoxical haemolytic zone' described for HBL (Beecher & Wong, 1994b) was sometimes observed also at 30 °C (Fig. 7).

To analyse whether *B. cereus* ATCC 14579 harboured the gene(s) for the non-haemolytic enterotoxin NHE (Lund & Granum, 1996), a probe covering the promoter region and the 5' part of the first gene in the *nhe* operon (Agaisse *et al.*, 1999) was used to hybridize a Southern blot of digested genomic DNA. *nhe* was present in both wild-type and mutant *B. cereus* ATCC 14579, and also in strain *B. cereus* ATCC 10987. The expression of the gene was analysed by Northern blot hybridization, and while the gene was highly transcribed in ATCC 10987, it was only very weakly transcribed in both wild-type *B. cereus* ATCC 14579 and *BcΔhblC* (data not shown).

Fig. 6. Effect of supernatant from *B. cereus* ATCC 14579 wild-type and *BcΔhblC* on a Vero cell monolayer. (a) Total destruction of the monolayer 20 min after addition of a 10× dilution of *B. cereus* ATCC 14579 culture supernatant. (b) Confluent monolayer 20 min after addition of a 10× dilution of *BcΔhblC* culture supernatant. (c) Confluent monolayer with added LB medium.

Fig. 7. Haemolytic pattern shows reduction of haemolytic activity of BcΔhblC compared to wild-type *B. cereus* ATCC 14579 against sheep erythrocytes at 37 °C, but not against human erythrocytes. At 30 °C, the haemolytic activity of the wild-type strain was less profound.

DISCUSSION

HBL consists of three components which are all required to obtain full enterotoxigenic activity (Beecher & Macmillan, 1991). In this study we have inactivated the first gene of the *hbl* locus, *hblC*, encoding the L₂ component of HBL from *B. cereus* ATCC 14579. This strain was chosen since it was previously used for inactivation of other genes (Lindbäck & Kolstø, 1997; Økstad *et al.*, 1997). Other strains, like *B. cereus* F837/76 and *B. cereus* ATCC 10987, are not well suited for targeted gene disruption by the method used in this study. In addition, Southern blot analysis revealed no indication of more than one *hbl* locus in the ATCC 14579 strain.

Insertional inactivation of *hblC*, which is the first gene in the *hbl* operon, resulted in complete loss of transcription of the operon (Fig. 3). This effect rendered it difficult to correlate the phenotype with the inactivation of *hblC*. Immunoblot analysis (Fig. 5) showed that no L₂ component was synthesized in the mutant strain, while very weak bands were detected with antisera against L₁ and B components (Fig. 5). These weak bands were most likely due to cross-reaction with other unrelated proteins since no indication was found of separate transcription initiation from either of the two downstream genes of the operon by Northern blot analysis of the BcΔhblC strain (data not shown). However, it can not be excluded that small amounts of L₁ and B proteins are present due to a low level of independent transcription of the corresponding genes not detected by the Northern blot experiments. Beecher & Macmillan (1991) showed that L₁ + B did not exhibit detectable haemolytic activity. We therefore presume that if any traces of B and L₁ are

present in the disruption mutant they would not contribute to cytotoxic and haemolytic activity of the mutant strain.

The use of McCoy cells and Vero cells to assay *B. cereus* enterotoxin activity is well-established (Jackson, 1993; Agata *et al.*, 1995a; Lund & Granum, 1997). Other toxins like phospholipases and haemolysins other than HBL do not induce the characteristic destruction of the McCoy cell monolayer (Jackson, 1993). When testing the bacterial culture supernatants on Vero cell monolayers, we observed that inactivation of L₂ strongly reduced the rapid cytopathogenic activity observed in the wild-type strain of *B. cereus* ATCC 14579. However, we still observed a slower destruction of the cell monolayer when adding the culture supernatant from BcΔhblC.

The fact that BcΔhblC had a strongly reduced toxic effect towards Vero cells clearly indicated that HBL caused the main cytopathogenic activity of *B. cereus* ATCC 14579. The weak cytopathogenic effect observed from BcΔhblC was most likely due to small amounts of NHE, since low levels of *nhe* mRNA were observed in the wild-type and mutant strain of ATCC 14579. In accordance, strain ATCC 10987, which lacks the *hbl* operon, synthesized high levels of *nhe* mRNA and exhibited a strong cytopathogenic activity towards Vero cells. Both *hbl* and *nhe* are regulated by the PlcR regulator, present in *B. cereus* and *Bacillus thuringiensis* strains (Agaisse *et al.*, 1999). Sequencing of the *nhe* promoter region from both ATCC 14579 and ATCC 10987 strains and further analysis of the *nhe* and the *hbl* upstream regions may provide better understanding of the complexity of regulation of these operons.

Analysis of the upstream region of the *hbl* operon of *B. cereus* F837/76 revealed the presence of the highly conserved palindromic region postulated to be the specific recognition target for transcriptional activation by the pleiotropic regulator PlcR (Agaisse *et al.*, 1999). The transcriptional start site was located 271 bp downstream of the putative PlcR recognition target (Agaisse *et al.*, 1999). The transcriptional start site of the *B. cereus* ATCC 14579 *hbl* operon was identified by primer extension analysis at a location 608 bp upstream of the putative translational start site of *hblC* (Fig. 4). This differed from the one in F837/76 reported by Agaisse *et al.* (1999) by 2 bp, probably due to variation between strains.

The *hbl* transcript from the *B. cereus* ATCC 14579 wild-type strain was about 5.5 kb, similar to that which has been reported for *B. cereus* F837/76 (Heinrichs *et al.*, 1993; Ryan *et al.*, 1997). The expected size of a transcript including the three genes *hblC*, *hblD* and *hblA* would be about 4.4 kb. A transcript of 5.5 kb transcribed from the start site as determined by primer extension in this report will be terminated within the *hblB* gene. No evidence was found supporting the presence of a second transcriptional start site upstream of *hblC*. This indicates that a putative stem-loop structure found downstream of *hblA* (Økstad *et al.*, 1999) is not functional as a transcription terminator. Considering the primer extension analysis and the Northern blot analysis results, we conclude that transcription of the *hbl* operon most likely starts 608 bp upstream of the translational start site, 271 bp downstream of the PlcR regulatory sequence, and terminates within *hblB*. This conclusion is reinforced by the fact that no transcript was observed using a probe located at the 3' end of *hblB* in Northern blot analysis. Hybridization with this *hblB*-specific probe also indicated that *hblB* is not transcribed alone, although it cannot be excluded that at other time points or growth conditions, transcription of *hblB* may occur.

HBL is most active against sheep and calf erythrocytes (Beecher & Wong, 1994b). Accordingly, inactivation of the *hbl* locus reduced the haemolytic activity of BcΔ*hblC* compared to wild-type ATCC 14579. A smaller difference was seen at 30 °C, indicating a temperature dependence perhaps due to conformational changes of the HBL toxin. Since there was no reduction of the haemolysis when comparing mutant and wild-type ATCC 14579 strains on human blood agar plates, the HBL toxin does not contribute significantly to *B. cereus* haemolytic activity against human erythrocytes. The reason for the preferential lysis of sheep erythrocytes is not known, but the presence of a higher amount of sphingomyelin in the plasma membrane in the sheep erythrocytes could perhaps be of importance.

We conclude that the enterotoxigenic HBL is responsible for the major enterotoxigenic activity of *B. cereus* ATCC 14579. The haemolytic activity of *B. cereus* ATCC 14579 against sheep erythrocytes is partly caused by HBL, while HBL does not contribute significantly to the haemolytic activity against human, chicken or horse erythrocytes.

ACKNOWLEDGEMENTS

We gratefully acknowledge James D. Macmillan for providing us with the probes used for screening of the genomic library, and Douglas J. Beecher for providing the polyclonal antibodies directed against the L₁, L₂ and B components of HBL. The authors are thankful to Ewa Jaroszewicz for skilful technical assistance.

REFERENCES

- Agaisse, H., Gominet, M., Økstad, O. A., Kolstø, A.-B. & Lereclus, D. (1999). PlcR is a pleiotropic regulator of extracellular virulence factor gene expression in *Bacillus thuringiensis*. *Mol Microbiol* **32**, 1043–1053.
- Agata, N., Mori, M., Ohta, M., Suwan, S., Ohtani, I. & Isobe, M. (1994). A novel dodecadepsipeptide, cereulide, isolated from *Bacillus cereus* causes vacuole formation in HEp-2 cells. *FEMS Microbiol Lett* **121**, 31–34.
- Agata, N., Ohta, M., Arakawa, Y. & Mori, M. (1995a). The *bceT* gene of *Bacillus cereus* encodes an enterotoxigenic protein. *Microbiology* **141**, 983–988.
- Agata, N., Ohta, M., Mori, M. & Isobe, M. (1995b). A novel dodecadepsipeptide, cereulide, is an emetic toxin of *Bacillus cereus*. *FEMS Microbiol Lett* **129**, 17–20.
- Baida, G. E. & Kuzmin, N. P. (1995). Cloning and primary structure of a new hemolysin gene from *Bacillus cereus*. *Biochim Biophys Acta* **1264**, 151–154.
- Beecher, D. J. & Macmillan, J. D. (1991). Characterization of the components of hemolysin BL from *Bacillus cereus*. *Infect Immun* **59**, 1778–1784.
- Beecher, D. J. & Wong, A. C. L. (1994a). Improved purification and characterization of hemolysin BL: a hemolytic dermonecrotic vascular permeability factor from *Bacillus cereus*. *Infect Immun* **62**, 980–986.
- Beecher, D. J. & Wong, A. C. L. (1994b). Identification of hemolysin BL-producing *Bacillus cereus* isolates by a discontinuous hemolytic pattern in blood agar. *Appl Environ Microbiol* **60**, 1646–1651.
- Beecher, D. J., Schoeni, J. L. & Wong, A. C. L. (1995). Enterotoxin activity of hemolysin BL from *Bacillus cereus*. *Infect Immun* **63**, 4423–4428.
- Carlson, C. R., Johansen, T. & Kolstø, A.-B. (1996). The chromosome map of *Bacillus thuringiensis* subsp. *canadensis* HD224 is highly similar to that of the *Bacillus cereus* type strain ATCC 14579. *FEMS Microbiol Lett* **141**, 163–167.
- Chuang, S.-E., Chen, A.-L. & Chao, C.-C. (1995). Growth of *E. coli* at low temperature dramatically increases the transformation frequency by electroporation. *Nucleic Acids Res* **23**, 1641.
- Evensen, G. & Seeberg, E. (1982). Adaptation to alkylation resistance involves the induction of a DNA glycosylase. *Nature* **296**, 773–775.
- Garcia-Arribas, M. L., Plaza, C. J., de la Rosa, M. C. & Mosso, M. A. (1988). Characterization of *Bacillus cereus* strains isolated from drugs and evaluation of their toxins. *J Appl Bacteriol* **64**, 251–264.
- Harlow, E. & Lane, D. (1988). *Antibodies: a Laboratory Manual*, 1st edn. Cold Spring Harbor, NY: Cold Spring Harbor Laboratory.
- Heinrichs, J. H., Beecher, D. J., Macmillan, J. D. & Zilinskas, B. A. (1993). Molecular cloning and characterization of the *hblA* gene encoding the B component of hemolysin BL from *Bacillus cereus*. *J Bacteriol* **175**, 6760–6766.

- Honda, T., Shiba, A., Seo, S., Yamamoto, J. & Miwatani, T. (1991). Identity of hemolysins produced by *Bacillus thuringiensis* and *Bacillus cereus*. *FEMS Microbiol Lett* **63**, 205–209.
- Igo, M. M. & Losick, R. (1986). Regulation of a promoter that is utilized by minor forms of RNA polymerase holoenzyme in *Bacillus subtilis*. *J Mol Biol* **191**, 615–624.
- Jackson, S. G. (1993). Rapid screening test for enterotoxin-producing *Bacillus cereus*. *J Clin Microbiol* **31**, 972–974.
- Kramer, J. M. & Gilbert, R. J. (1989). *Bacillus cereus* and other *Bacillus* species. In *Foodborne Bacterial Pathogens*, pp. 21–70. Edited by M. P. Doyle. New York: Marcel Dekker.
- Lindbäck, T. & Kolstø, A.-B. (1997). A *Bacillus cereus* member of the SNF2 family. *Microbiology* **143**, 171–174.
- Luna, L., Johnsen, Ø., Skartlien, A. H., Pedeutour, F., Turc-Carel, C., Prydz, H. & Kolstø, A.-B. (1994). Molecular cloning of a putative novel human bZIP transcription factor on chromosome 17q22. *Genomics* **22**, 553–562.
- Lund, T. & Granum, P. E. (1996). Characterization of a non-haemolytic enterotoxin complex from *Bacillus cereus* isolated after a foodborne outbreak. *FEMS Microbiol Lett* **141**, 151–156.
- Lund, T. & Granum, P. E. (1997). Comparison of biological effect of the two different enterotoxin complexes isolated from three different strains of *Bacillus cereus*. *Microbiology* **143**, 3329–3336.
- Narahara, A., Naterstad, K., Kristensen, T., Lopez, R., Bork, P. & Kolstø, A.-B. (1992). Cloning of a gene from *Bacillus cereus* with homology to the *mreB* gene from *Escherichia coli*. *Gene* **122**, 181–185.
- O'Day, D. M., Smith, R. S., Gregg, C. R., Turnbull, P. C. B., Head, W. S., Ives, J. A. & Ho, P. C. (1981). The problem of *Bacillus* species infection with special emphasis on the virulence of *Bacillus cereus*. *Ophthalmology* **88**, 833–838.
- Økstad, O. A., Grønstad, A., Lindbäck, T. & Kolstø, A.-B. (1997). Insertional inactivation of a Tet(K)/Tet(L) like transporter does not eliminate tetracycline resistance in *Bacillus cereus*. *FEMS Microbiol Lett* **154**, 181–186.
- Økstad, O. A., Gominet, M., Purnelle, B., Rose, M., Lereclus, D. & Kolstø, A.-B. (1999). Sequence analysis of three *Bacillus cereus* loci carrying PlcR-regulated genes encoding degradative enzymes and enterotoxin. *Microbiology* **145**, 3129–3138.
- Pflugfelder, S. C. & Flynn, H. W., Jr (1992). Infectious endophthalmitis. *Infect Dis Clin North Am* **6**, 859–873.
- Ryan, P. A., Macmillan, J. D. & Zilinskas, B. A. (1997). Molecular cloning and characterization of the genes encoding the L₁ and L₂ component of hemolysin BL from *Bacillus cereus*. *J Bacteriol* **179**, 2551–2556.
- Sambrook, J., Fritsch, E. F. & Maniatis, T. (1989). *Molecular Cloning: a Laboratory Manual*, 2nd edn. Cold Spring Harbor, NY: Cold Spring Harbor Laboratory.
- Simonen, M. & Palva, I. (1993). Protein secretion in *Bacillus* species. *Microbiol Rev* **57**, 109–137.
- Thomas, C. M. & Smith, C. A. (1987). Incompatibility group P plasmids: genetics, evolution and use in genetic manipulation. *Annu Rev Microbiol* **41**, 77–101.
- Trieu-Cuot, P., Carlier, C., Poyart-Salmeron, C. & Courvalin, P. (1991). An integrative vector exploiting the transposition properties of Tn1545 for insertional mutagenesis and cloning of genes from Gram-positive bacteria. *Gene* **106**, 21–27.
- Turnbull, P. C. B., Kramer, J. M., Jørgensen, K., Gilbert, R. J. & Melling, J. (1979). Properties and production characteristics of vomiting, diarrheal, and necrotic toxins of *Bacillus cereus*. *Am J Clin Nutr* **32**, 219–228.

Received 26 May 1999; revised 27 July 1999; accepted 5 August 1999.