Characterization of the *Bacillus cereus* Nhe enterotoxin

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The non-haemolytic enterotoxin (Nhe) is one of two three-component enterotoxins responsible for the diarrhoeal food-poisoning syndrome caused by *Bacillus cereus*. Nhe is composed of NheA, NheB and NheC. The three genes encoding the Nhe components constitute an operon, and the transcriptional start site is located 61 bp upstream of the *nheA* translational start site. The *nhe* genes were cloned separately, and expressed in either *Bacillus subtilis* or *Escherichia coli*. Separate expression showed that all three components were required for biological activity. In addition, NheA and NheB were purified from *B. cereus* culture supernatants. As NheC seems to be expressed in only small amounts by *B. cereus*, NheC was expressed and purified as a histidine-tagged fusion protein. The maximum cytotoxic activity was obtained when the molar ratio between NheA : NheB : His₆-NheC was 10 : 10 : 1, and it was shown that NheB was the binding component of the enterotoxin complex.

INTRODUCTION

Bacillus cereus is a Gram-positive, spore-forming, motile, aerobic rod, commonly found in soil, water and foods. The bacterium is an important food pathogen responsible for two different types of food poisoning: the emetic and the diarrhoeal types (Turnbull *et al.*, 1979; Granum, 2001). *B. cereus* has been isolated from a variety of foods, including rice, spices, meat, eggs and dairy products (Kramer & Gilbert, 1989), and also from drugs, including both topical and oral pharmaceutical products (Garcia-Arribas *et al.*, 1988).

Not all the factors that render *B. cereus* pathogenic to humans are precisely known, although several potential virulence factors have been characterized. All but one, the dodecadepsipeptide cereulide (Agata *et al.*, 1994), are proteins encoded by genes dispersed on the bacterial chromosome (Økstad *et al.*, 1999). The transcription of the genes encoding these potential virulence factors is controlled by the pleiotropic regulator PlcR (Agaisse *et al.*, 1999; Økstad *et al.*, 1999).

Two different three-component enterotoxins have been isolated from *B. cereus*. These are haemolysin BL (Hbl) (Beecher & MacMillan, 1991) and non-haemolytic enterotoxin (Nhe) (Lund & Granum, 1996), both of which are

positively regulated by PlcR (Gohar *et al.*, 2002). In addition to its enterotoxic activity, Hbl possesses haemolytic and dermonecrotic activities (Beecher & Wong, 1994). The genes encoding the B, L₁ and L₂ components of Hbl have been cloned and sequenced from *B. cereus* strain F837/76 (Heinrichs *et al.*, 1993; Ryan *et al.*, 1997). Beecher & Macmillan (1991) showed that all three components were required for complete lysis of sheep erythrocytes, and HblB was suggested to be the component that bound to the cell surface before the two L components could act to cause cell lysis. However, more recently, another model for the action of Hbl has been proposed, suggesting that the components of Hbl bind to target cells independently, and then constitute a membrane-attacking complex resulting in a colloid osmotic lysis mechanism (Beecher & Wong, 1997).

The three-component enterotoxin Nhe was first isolated from an Hbl-negative strain of *B. cereus*, which was involved in a large food-poisoning outbreak in Norway in 1995 (Lund & Granum, 1996). The proteins were different from the components of Hbl and had no detectable haemolytic activity. It was initially suggested that a collagenase of 105 kDa (Lund & Granum, 1999) was part of the Nhe complex (Lund & Granum, 1996, 1997), but this was later shown to be incorrect. Sequencing of the operon encoding NheA and NheB (Granum *et al.*, 1999) revealed a novel gene encoding a protein designated NheC, suggesting that this protein was part of the Nhe complex. NheC has previously not been detected in, or purified from, *B. cereus* culture supernatants.

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Abbreviations: Hbl, haemolysin BL; Nhe, non-haemolytic enterotoxin.

The present study shows specifically that all three components are necessary for cytotoxic activity, and that the molar ratio between the components is of importance.

METHODS

Bacterial strains, plasmids and growth conditions. *B. cereus* strain 0075-95, responsible for diarrhoeal-syndrome food poisoning in Norway in 1995, was used for enterotoxin production in this study (Lund & Granum, 1996). DNA isolated from strains 1230-88 and 0075-95 (Granum *et al.*, 1993) was used in primer extension experiments. The culture medium used for production of enterotoxins was a modification of CGY medium (Beecher & Wong, 1994) described by Lund & Granum (1997). The cells were grown at 32 °C for 5–7 h before harvesting by centrifugation (10 000 *g* at 4 °C for 20 min). *Escherichia coli* BL21(DE3)pLysS (Invitrogen) was used for recombinant expression of His₆-NheC. Recombinant NheA and NheC were expressed in *E. coli* XL10-Gold, while NheB was expressed in *Bacillus subtilis* JH642. *E. coli* and *B. subtilis* were grown in BHI medium (Difco).

Purification of NheA and NheB from *B. cereus* culture supernatants. The purification schemes for NheA and NheB were similar to those described in detail for purification of Hbl (Beecher & Wong, 1994) and Nhe (Lund & Granum, 1996, 1997). Proteins from 2 l culture supernatant were concentrated by precipitation with 70 % saturated (NH₄)₂SO₄, resuspended in 25 ml water, and dialysed against 25 mM Bistris/HCl pH 5.9. Chromatography on a DEAE-Sephacel column (Amersham Biosciences), with Bistris/HCl and a gradient of 0–0.5 M NaCl, was followed by chromatography on a HA column (Bio-Rad), using a sodium phosphate gradient of 0–0.25 M at pH 6.8 for elution. Fractions were analysed on SDS-PAGE, and combinations of fractions were tested for cytotoxic activity. Protein concentration was estimated by the difference in absorbance at 235 and 280 nm (Whitaker & Granum, 1980).

Expression of recombinant NheA (rNheA) and NheC (rNheC) in *E. coli*. The shuttle vector pMS10 was constructed by cloning the kanamycin-resistance gene from pUC4K (Amersham Biosciences) into the *SacI*–*NcoI* site of pGK12 (Kok *et al.*, 1984). The strong, constitutive promoter p32 from pMG36e (van de Guchte *et al.*, 1989) was cloned into the *KpnI*–*MluI* site of pMS10, and the construct was designated pMS20. Primers nhexfmlu and nhexrmlu (Table 1), with restriction enzyme recognition site sequences (*MluI*) at their ends, were used to PCR-amplify *nheA* and *nheC*. The PCR products were cloned into the *MluI* site of pMS20, and the constructs were named pMS21 (rNheA) and pMS23 (rNheC). *E. coli* XL10-Gold/pMS21/23 was grown for 6 h, then the cells were pelleted by centrifugation, resuspended in water and lysed by sonication. Cell debris was removed by centrifugation, and the supernatants were used in toxicity tests on Vero cells.

Expression of recombinant NheB (rNheB) in *B. subtilis*. Due to difficulties expressing NheB in *E. coli*, it was expressed in *B. subtilis*. *nheB* was cloned into the pDG148-Stu vector (Joseph *et al.*, 2001) obtained from the Bacillus Genetic Stock Center (BGSC accession no. EC145). *nheB* was PCR-amplified using primers nheBF and nheBR (Table 1). The primers had 5' overhangs to allow ligation-independent cloning. After amplification, the PCR products were treated with T4 DNA polymerase (Amersham Biosciences) in the presence of dATP (Amersham Biosciences). The pDG148-Stu vector was digested with *StuI*, and treated with T4 DNA polymerase in the presence of dTTP (Amersham Biosciences). Then, the *nheB* insert and the vector were mixed and transformed into *E. coli* TOP10F'. The plasmid, designated pDG148-nheB, was transformed into *B. subtilis* strain JH642 by electroporation (Stephenson & Jarrett, 1991). *B. subtilis* JH642/pDG148-nheB was grown for 6 h, and cells were removed by

Table 1. Primers

Primer	Sequence (5'–3')
P5452	TCGTAGATACTGCTGTAACCA
P0045	GTTAGGATCACAATCACC GC
P1141	CGACTTCTGCTTGTGCTCCTG
P1143	GAGTAATGATATGGTCATTGC
P4088	CCAATGGTACACAATGGGATC
P7376	CCTTCTGGTCCTAATGAGTA
P517	CGGTTTCATCTGTTGCGACAGC
nhecf	ATGCAGAAACGATTTTATAAA
nhecr	TTACTTCGCCACACCTTCAT
nheafmlu	CGACGCGTATAGTTGCTAATACATAGG- AGGAG
nhearmlu	TCACGCGTCTTCTCTCCCTATATCGCTA
nheBF	AAGGAGGAAGCAGGTATGACAAAAAAA- CCTTATAAAAGTAATGGCTC
nheBR	GACACGCACGAGGTTTATGCTTTTTTC- GTATCTACTACTTTAATATC
nhecfmlu	TTACGCGTCTTATAAAGAGAATAGGTG
nhecrmlu	TAACGCGTATTGCAAGTTCAACCCCTTAC

centrifugation. The supernatant was concentrated tenfold by ammonium sulphate precipitation, dialysed against 20 mM Tris/HCl pH 7.6, and used in toxicity tests on Vero cells.

Expression and purification of His₆-NheC. Since free-existing NheC has not been detected in culture supernatants from *B. cereus*, NheC was expressed as a histidine-tagged fusion protein, His₆-NheC, to facilitate the purification of the predicted protein. The primers nhecf and nhecr (Table 1) were used to PCR-amplify *nheC*, which was subsequently cloned into the pCR T7/NT-TOPO vector (Invitrogen). His₆-NheC was purified using Ni-NTA agarose (Qiagen) under denaturing conditions (8 M urea), according to the procedure provided by the supplier. The urea was removed by stepwise dialysis against 3 M urea/20 mM Tris/HCl pH 7.6, 1 M urea/20 mM Tris/HCl pH 7.6, 0.1 M urea/20 mM Tris/HCl pH 7.6, and then 20 mM Tris/HCl pH 7.6.

SDS-PAGE, native PAGE and immunoblotting. PAGE and Western immunoblotting were carried out using a Bio-Rad Mini-Protein II Dual Slab Cell, according to standard protocols (Harlow & Lane, 1988). The gels were silver stained according to Blum *et al.* (1987). The molecular mass of the proteins was estimated using Bio-Rad SDS-PAGE standards, and prestained kaleidoscopic standard (Bio-Rad) for Western blots. Monoclonal antibodies against NheA and NheB were obtained as a gift from Dr Erwin Märklbauer (Ludwig-Maximilians-Universität, Munich, Germany). His₆-NheC was detected on Western blots using monoclonal antibodies against the histidine tag (Invitrogen) in a dilution of 1:5000. Biotin-conjugated anti-mouse antibodies from goat (Amersham Biosciences) were used as secondary antibodies (1:10 000). A complex of streptavidin (Bio-Rad) and biotinylated alkaline phosphatase (Bio-Rad) was used at a dilution of 1:3000, prior to development with nitro blue tetrazolium/5-bromo-4-chloro-3-indolyl phosphate (Bio-Rad). Native PAGE was performed according to Yoshida *et al.* (2002), using a 10 % polyacrylamide separation gel without SDS in 187.5 mM Tris/HCl (pH 8.9). The gel was run for 4 h at 60 V in 82.6 mM Tris/HCl (pH 9.4) and 33 mM glycine.

Cytotoxicity test on Vero cells. Cytotoxicity was tested using a Vero cell assay according to Sandvig & Olsnes (1982). The assay

monitors inhibition of protein synthesis in Vero cells after addition of toxic proteins. Vero cells were grown in minimal essential medium, MEM (Gibco), with 10 % fetal calf serum added.

RNA isolation and analysis. RNA was isolated from 10 ml samples as described by Igo & Losick (1986). Mapping of the 5' terminus of the *nhe* mRNA was performed on total RNA isolated from *B. cereus* 0075-95 and 1230-88, using a 5' end-labelled oligonucleotide, P5452 (Table 1), located 28 bp downstream of the translational start site of *nheA*. Annealing and extension were performed according to Luna *et al.* (1994), using 10 U cloned AMV Reverse Transcriptase (Invitrogen) in the extension reaction. Products were resolved in 6 % polyacrylamide gels together with sequence reactions prepared according to the dideoxy chain-termination method using the Thermo Sequenase Radiolabelled Terminator Cycle Sequencing Kit (USB) according to protocol.

Reverse transcriptase PCR was performed using 1 µg total RNA treated with 2 U RNase-Free DNase (Promega) at 37 °C for 30 min. The extension reactions were carried out using 10 U cloned AMV Reverse Transcriptase (Invitrogen) in the supplied reaction buffer with 0.5 mM dNTPs (Finnzymes, Finland), all in a total volume of 20 µl at 45 °C for 1 h. The reverse primers used in the extension reactions (Table 1) were used in a final concentration of 1 pmol µl⁻¹. A 1 µl volume of the extension reaction was used as a template in standard PCR reactions.

PCR amplification. All PCRs were carried out in an MJ Research Minicycler PTC-150 with heated lid, or an Eppendorf Mastercycler gradient. DyNAzyme II DNA polymerase (supplied with 10 × buffer) and dNTP Mix from Finnzymes were used as instructed by the manufacturer. The primers used in the reactions are listed in Table 1. Depending on the primers, the standard programme was as follows: 95 °C for 1 min, 30 cycles of 95 °C for 1 min, 48–52 °C for 1 min, and 72 °C for 1 min, then a final extension step of 72 °C for 7 min.

Binding of Nhe components to Vero cells. Purified Nhe proteins (10 nM) were added to wells containing Vero cell monolayers, and incubated for 10 and 20 min at 37 °C. The monolayers were washed five times with MEM, and then suspended in 50 µl SDS-PAGE sample buffer. A 20 µl volume of each sample was applied to 12 % SDS-PAGE, and transferred to nitrocellulose membranes (Bio-Rad) by Western immunoblotting.

RESULTS

Expression analysis of the *nhe* operon

Northern blotting of RNA isolated from *B. cereus* strain 1230-88 revealed a 3.7 kb transcript (data not shown), indicating that the genes encoding the three Nhe proteins were transcribed as an operon. Mapping of the 5' terminus of the *nhe* mRNA by primer extension analysis in *B. cereus* strains 1230-88 and 0075-95 revealed products of 111 and 115 bp respectively (Fig. 1a), thereby identifying a transcriptional start site at a T (Fig. 1b) positioned 62 and 66 bp upstream of the translational start site in the two strains (Granum *et al.*, 1999). A putative -10 promoter sequence TCTTAA, with T in position -10, was identified upstream of the transcriptional start site in strain 1230-88, while a putative -10 promoter sequence in strain 0075-95, TCTCAA, revealed T in position -6.

RT-PCR

RT-PCR was performed to confirm that the operon was as predicted, and to investigate the role of the predicted stem-loop structure between *nheB* and *nheC* (Granum *et al.*, 1999). RNA isolated from toxin-producing *B. cereus* 0075-95 cultures in early stationary phase was treated with DNase prior to the extension reaction with specific primers and reverse transcriptase. Two different reverse primers, p7376 and p1141, were used for extension with reverse transcriptase, (Fig. 2a). RT-PCR products of the correct size (1300 bp) were obtained when primers p0045 and p7376 (Fig. 2b) were used in the PCR following the extension reaction using primer p7376. This PCR product covered the intergenic region between *nheA* and *nheB* (Fig. 2a). When using two internal primers of *nheC* (p1141 and p1143) in the PCR following the extension reaction with primer p1141, the resulting product of 150 bp indicated that intact *nhe* mRNA was present in the extension mixture. Attempts to amplify PCR products covering the intergenic region between *nheB* and *nheC* did not succeed (Fig. 2b), even though several primer pairs were tested under different conditions. Primer pairs p4088–p1141 and p517–p1141 failed to amplify PCR products of the predicted size from cDNA, while a product of the correct size was obtained from traces of DNA in the reaction when the RNA was not treated with DNase. A weak band of the correct size was observed in lane 5c in Fig. 2(b), but the major product was much smaller.

Recombinant expression of the Nhe components

As the combination of NheA and NheB, purified from culture supernatants, sometimes gave a small but significant inhibition of protein synthesis in Vero cells, we decided to test this combination using recombinant Nhe components. The *nhe* genes were individually cloned and expressed in either *E. coli* or *B. subtilis*. Expression of rNheA, rNheB and His₆-NheC was verified by Western immunoblotting (Fig. 3a). We previously reported (Lund & Granum, 1997) that NheB exists in an intact form and a shorter form lacking 21 amino-terminal amino acids. Both forms were observed on the Western blot of the SDS-PAGE (Fig. 3a) and the native PAGE (Fig. 6). No cytotoxic activity was observed when combining recombinantly expressed rNheA and rNheB in the Vero cell assay. After addition of small amounts of His₆-NheC or rNheC, 100 % inhibition of protein synthesis in the Vero cells was obtained. Combinations of either rNheA/His₆-NheC or of rNheB/His₆-NheC did not show any cytotoxic effect towards Vero cells.

Cytotoxic activity of Nhe purified from *B. cereus* culture supernatant

NheA and NheB were purified from *B. cereus* 0075-95 early-stationary-phase culture supernatants by ion-exchange chromatography. The purified proteins are shown in Fig. 3b. Each Nhe component, tested either individually

(b)

PlcR recognition site 50

1230-88 CGATGTGGAAATGAGGGTCGATAGAAAATTATGCAATGTTATTCATAC
0075-95 CAATGTGGGAAATAAGATGAAATAGAAAATTATGCAATGTTATTCATAC

100

1230-88 TAAGCACATATGAAAAGTTTATAAAAAATAAGTAAGTACTGTAGCAATAA
0075-95 TAACCACATATGAAAAGTTTATAACGATAAGTAAGTACTGTAGCAATAA

-35 150

1230-88 CAGTTGGGAGAGATGGCTCGTACGTCAATCACGGTGAGTACGTCTCTAT
0075-95 AATTGGGAGAGATGGTTCGTGTGTAATCGCGGTGAGAACGTTCTCTAT

-10 +1 200

1230-88 TTTAACCATTCTTAAATTATATGCAAGAAAATAGAATAATAAAATGCGATA
0075-95 TTTAACCATTCTCAAACTATGTGCAAGAAAATAGAATAATAAAATGCGATA

+1

RBS 250

1230-88 GTAATAGTTGCTAATACATAGGAGGAGTTAAAGTGAAAAAGACTTTAATT
0075-95 GTAATAGTTACTAATACATAGGAGGAGTTAAAGTGAAAAAGACTTTAATT

Fig. 1. (a) Mapping of the 5' terminus of the *nhe* RNA of *B. cereus* strains 1230-88 and 0075-95. The transcriptional start sites are indicated by arrows. The -10 promoter sequences (TATA box) are boxed. (b) Alignment of the *nhe* promoter regions of *B. cereus* strains 1230-88 and 0075-95. The PlcR recognition site, the -10 region, and the transcriptional start sites (+1) are indicated.

or in a pair, gave no inhibition of protein synthesis in the Vero cell assay.

To elucidate the composition of the Nhe complex, different concentrations of the Nhe proteins were tested in the Vero cell assay. Representative results from inhibition of protein synthesis in Vero cells when altering the concentration of the individual Nhe components are presented in Table 2. The concentration of NheA was held constant at 1 nM, as this was the lowest concentration resulting in significant inhibition. The components were added one by one in the order NheA, NheB and His₆-NheC. One nanomolar NheA, 1 nM NheB and 0.1 nM His₆-NheC were the lowest concentrations of each Nhe component necessary for maximal inhibition in the linear part of the toxicity curve (linear between 30 and 70 % inhibition).

Both rNheA and rNheB were able to substitute for their equivalents purified from *B. cereus* culture supernatant in the Vero cell assays. Replacing His₆-NheC with rNheC did not change the cytotoxic activity of the Nhe complex,

indicating that the histidine tag did not interfere with the biological activity of NheC. Incubation of the three Nhe components (ratio 10:10:1) at 37 or 22 °C for 15 min prior to their addition to the Vero cells resulted in cytotoxic activity in the same range as when adding the three components one at a time.

Binding of NheB to Vero cells

To examine if any of the Nhe components were able to bind to the Vero cells, the individual Nhe components were incubated with Vero cells. Following incubation, the cells were washed five times, suspended in sample buffer, and applied to SDS-PAGE. Western immunoblots of the Vero cells incubated with NheA, NheB or His₆-NheC, individually, showed that NheB was the binding component of the Nhe complex (Fig. 4). After both 10 and 25 min, NheB was the only single component attached to the Vero cell monolayer. When NheB was incubated with an equal amount of His₆-NheC prior to addition to the Vero cells, no NheB was detected attached to the Vero cells, indicating

Fig. 2. (a) Schematic representation of the *nhe* operon and the primers used in RT-PCR. (b) A 1% agarose gel of RT-PCR products. Lanes: 1, GeneRuler 100 bp ladder Plus; a, RT-reaction with DNase, without reverse transcriptase; b, RT-reaction without DNase, with reverse transcriptase; c, with DNase and reverse transcriptase; 2, primer pair 0045-7376; 3, primer pair 1143-1141; 4, primer pair 517-1141; 5, primer pair 4088-1141.

that His₆-NheC prevents NheB from binding to the Vero cell surface.

Fig. 5 shows that the percentage inhibition of protein synthesis in Vero cells was gradually reduced upon the addition of mixtures of the Nhe proteins with increasing concentrations of His₆-NheC. To show that this reduction in cytotoxic activity was due to a lack of binding of free

Table 2. Percentage inhibition of protein synthesis in Vero cells when varying the relative amounts of the three Nhe proteins added

The concentration of NheA was held constant at 1 nM, as this was the lowest concentration resulting in significant inhibition. The concentrations of the Nhe components are presented as relative values to indicate the relative amounts of the Nhe components.

NheA	NheB	His ₆ -NheC	Inhibition (%)
10	10	1	69
10	10	0.8	64
10	10	0.4	36
10	13	1	71
10	13	0.8	67
10	13	0.4	31
10	7	1	62
10	7	0.8	59
10	7	0.4	35

NheB to the Vero cell surface, the Vero cells were incubated with NheB prior to addition of the Nhe complex. This restored the cytotoxic activity of the complex, even though the concentrations of His₆-NheC in the added mixtures were equal to the inhibiting concentration of His₆-NheC. Increasing the amount of His₆-NheC resulted in further reduction of cytotoxic activity. These results indicated that unbound NheB was necessary for cytotoxic activity, and that an excess of His₆-NheC prevented NheB from binding to the Vero cells. Incubation of Vero cells with NheA prior to addition of Nhe complex did not alter the inhibiting effect of increasing the His₆-NheC concentration, indicating that NheA is not important for the initial binding of the components.

To investigate the inhibitory effect of His₆-NheC in culture supernatant, the amount of concentrated supernatant from *B. cereus* 0075-95 resulting in 50% inhibition of protein synthesis in Vero cells was determined. Increasing concentrations of purified His₆-NheC were added to this amount of concentrated supernatant. The samples were incubated at room temperature for 30 min, and then mixtures with and without His₆-NheC were subjected to the Vero cell cytotoxicity assay. The addition of sufficient amounts of His₆-NheC abolished the cytotoxic activity of the concentrated supernatant.

Formation of complex between NheA/His₆-NheC and NheB/His₆-NheC

To investigate the association between the individual Nhe components, different combinations of the Nhe proteins were mixed in equal concentrations (1:1:1), incubated for 15 min at room temperature, subjected to native PAGE, blotted onto nitrocellulose membranes, and probed with Nhe or His antibodies. The Western immunoblot of the native gel showed that the individual Nhe bands disappeared when combining NheA/His₆-NheC, NheB/His₆-NheC and

Fig. 3. (a) Western blot of the recombinant Nhe proteins. (b) Silver-stained SDS-PAGE of purified NheA and NheB from *B. cereus* culture supernatant.

Fig. 4. Western immunoblotting of Vero cells incubated with the three Nhe proteins probed with monoclonal antibodies against NheA, NheB and His₆. For the positive controls (A+, B+ and C+), SDS-PAGE sample buffer was added directly to the culture medium containing Nhe proteins that overlaid the Vero cells.

NheA/NheB/His₆-NheC (Fig. 6), indicating the formation of complexes that were too large to enter the gel. Combining NheA and NheB did not result in the disappearance of the NheA band, indicating no (or weak) complex formation between these two proteins. The result shows that both NheA and NheB form complexes with His₆-NheC, without the presence of the third component. His₆-NheC appeared at the upper part of the native gel. Combining His₆-NheC with NheA or NheB weakened the His₆-NheC band, while combining all three components resulted in complete disappearance of the His₆-NheC band (Fig. 6).

DISCUSSION

Substantial progress has been made in recent years in the elucidation of toxin production in *B. cereus* (Beecher & MacMillan, 1991; Agata *et al.*, 1994; Beecher *et al.*, 1995a, b; Lund & Granum, 1996, 1997; Lund *et al.*, 2000). Hbl was the first three-component toxin isolated from *B. cereus*. Hbl differs from Nhe in several ways: Hbl has haemolytic,

dermonecrotic and enterotoxic activity (Beecher & MacMillan, 1991; Beecher & Wong, 1994; Beecher *et al.*, 1995b), while to date, Nhe has revealed exclusively cytotoxic/enterotoxic properties. Expression of both Hbl and Nhe is positively regulated by the pleiotropic regulator PlcR (Agaisse *et al.*, 1999; Gohar *et al.*, 2002). While the *hbl* operon is only present in about 50 % of *B. cereus* isolates, Nhe is produced by almost 100 % of the strains, and is the sole cytotoxin/enterotoxin produced by many food-poisoning strains (Granum, 2001).

The transcriptional start site of the *nhe* operon in two *B. cereus* strains was identified at positions 62 and 66 bp upstream of the translational start site (Fig. 1). Variations in the location of transcriptional start sites between virulence genes of different *B. cereus* strains have been reported previously (Agaisse *et al.*, 1999; Lindbäck *et al.*, 1999). The putative -10 promoter sequence for both of the *B. cereus* strains examined in this study showed 50 %

Fig. 5. Binding of NheB to Vero cells prior to addition of the Nhe complex restored the cytotoxic activity that was inhibited by the addition of excess His₆-NheC. White bars, Nhe complex added to untreated Vero cells; black bars, Vero cells preincubated with 10 nM NheB before addition of the Nhe complex. The results are representative.

Fig. 6. Western blot of native 10% polyacrylamide gel showing combinations of Nhe components.

homology to the classical Pribnow box (TATAAT). In addition, the -35 region was conserved to 50/33 % homology with the classical -35 sequence (TTGACA). The -10 sequence of *B. cereus* 0075-95 was located 3 bp closer to the transcriptional start site than that of strain 1230-88, but this difference is most probably of no significance, as both strains are high producers of Nhe.

Since attempts to purify NheC from *B. cereus* culture supernatant have not succeeded, NheC was expressed in *E. coli* as a histidine-tagged fusion protein. The addition of His₆-NheC to NheA and NheB was essential to obtain cytotoxic activity of the Nhe complex in the Vero cell assay. The maximum cytotoxic activity of the Nhe complex was obtained when the NheA:NheB:NheC ratio was 10:10:1, as determined by the Vero cell assay (Table 2). The accuracy of the Vero cell assay and protein concentration measurements is not precise enough to give a definite ratio between the three components, so a ratio down to 8:8:1 might be possible. The important observation is that a concentration of NheC higher than about 10 % of that of NheA and NheB inhibited the toxic activity. As the correlation between percentage inhibition of protein synthesis in Vero cells and concentration of toxin is linear in the range from about 30 to 70 %, the minimum cytotoxic concentrations presented in Table 2 were kept within this range.

Native PAGE showed that both NheA and NheB formed complexes with His₆-NheC, which were probably too large to enter the gel (Fig. 6). Combining rNheA and rNheB in the Vero cell assay did not result in any inhibition of protein synthesis, strongly indicating that NheC is needed for toxic activity of the Nhe complex. The presence of the histidine tag did not seem to affect the properties of the NheC protein, as rNheC could replace the His₆-NheC in the Vero cell assay, with identical results. In some experiments, combination of NheA and NheB from different batches of purified proteins resulted in up to 30 % inhibition of protein synthesis in Vero cells, without the addition of His₆-NheC/rNheC. This was probably due to minor contamination of NheC, as NheC can be associated with both NheA and NheB.

Results from cell-binding experiments followed by Western immunoblotting showed that NheB was the only individual component that bound to the Vero cells. Neither NheA nor His₆-NheC was observed attached to the Vero cells. In the Vero cell assay, the addition of His₆-NheC in concentrations equal to NheA and NheB resulted in complete loss of cytotoxic activity of the Nhe complex. When Vero cells were incubated with a high enough concentration of NheB prior to addition of NheA:NheB:NheC in the ratio 1:1:1, the cytotoxic activity was restored (Fig. 5). This was, however, not observed when the Vero cells were incubated with NheA prior to addition of the Nhe complex. The inhibitory effect of increasing the NheC concentration was probably due to complex formation between NheB and NheC, which prevented the binding of NheB to the membrane. Free His₆-NheC bound to NheB, and NheB in

complex with His₆-NheC was not able to bind to the surface of the Vero cells. Pre-incubation of the components of the Nhe complex prior to addition to the Vero cell assay was not necessary in order to obtain maximum cytotoxic activity. Furthermore, differences in the temperature (22 and 37 °C) used in the pre-incubation of the Nhe components prior to addition to Vero cells did not influence the level of inhibition obtained.

RT-PCR products obtained using internal primers in *nheC*, in addition to a forward primer in *nheA* and a reverse primer in *nheB* (Fig. 2a), showed that intact *nhe* mRNA was present in the reaction mixture (Fig. 2b). RT-PCR products covering the intergenic region between *nheA* and *nheB* indicate no, or easily dissolved, secondary structure in the mRNA between these two genes. All attempts to amplify RT-PCR products of the correct size containing the section between *nheB* and *nheC* failed. This indicated that the predicted structural conformation between the two genes (Granum *et al.*, 1999) prevented amplification of complete cDNA during the reverse transcriptase reaction. The efficiency of translational initiation and regulation is strongly correlated with the structure of the ribosome-binding site (RBS) (Schlax & Worhunsky, 2003). The primary interaction between mRNA and the 30S subunit rRNA requires local single-stranded mRNA (Draper, 1987, 1996; Gualerzi & Pon, 1990; Draper *et al.*, 1998). Secondary structure in the RBS can lower translational efficiency through a competitive mechanism, where *cis*-mRNA sequences compete with the 30S subunit for binding to the RBS (de Smit & van Duin, 1994; de Smit, 1998). We suggest that the low level of expression of NheC is caused by translational repression due to the structure of the RBS or the sequence surrounding the RBS. Alternatively, the 5' end of the mRNA could be more susceptible to degradation, as the predicted secondary structure between *nheB* and *nheC* might protect against degradation. A combination of both possibilities cannot be excluded. The formation of an active enterotoxin complex requires the concentration of NheC to be lower than that of NheA and NheB. This observation is supported by the results obtained by Gohar *et al.* (2002), where NheA and NheB, but not NheC, were identified on a two-dimensional SDS-PAGE, and we suggest that NheC was not identified because of its low concentration.

According to Granum *et al.* (1999), two transmembrane regions in NheB were predicted from the nucleotide sequence, while the predictions for NheA and NheC were no and one transmembrane region, respectively. By comparison with Hbl, where L₁ has two transmembrane regions, while B, the binding component, and L₂ have one and none, respectively, we expected NheC to be the binding component. Final binding studies of the Hbl components to Vero cells (or other epithelial cells) have not yet been carried out.

The composition of the enterotoxin described in this report is unique. The concentration of each Nhe component is of

importance for the function of the enterotoxin, and NheA and NheB together have negligible activity. An increase in the concentration of NheC results in a decrease in toxic activity, possibly due to the binding of NheC to NheB inhibiting the binding of NheB to the receptors. The function of NheC is not yet elucidated, but it seems likely that it functions as some kind of 'catalyst', either by bringing NheA and NheB together (after binding of NheB to the target cells), or by enhancing conformational changes. The structure of the Hbl complex has not been resolved, although a 1:1:1 ratio has been postulated, so it would be of great interest to further study the difference between the two three-component enterotoxins produced by *B. cereus*.

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