Tripartite haemolysin BL: isolation and characterization of two distinct homologous sets of components from a single *Bacillus cereus* isolate

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Haemolysin BL (HBL), a three-component enterotoxic/necrotizing/vascular permeability toxin, is a likely virulence factor of *Bacillus cereus* diarrhoeal food poisoning and necrotic infections. This paper describes the isolation of two distinct homologous sets of all three HBL components from a single *B. cereus* isolate, MGBC 145. The proteins of one set (designated HBL, consisting of B, L₁ and L₂), were about 87–100% identical in N-terminal amino acid sequences to their respective prototype components from strain F837/76, and the proteins of the homologous set (HBL_a, consisting of B_a, L_{1a} and L_{2a}) were all about 62–65% identical. Only the latter homologues differed immunochemically and physicochemically from the prototypes. HBL and HBL_a exhibited similar haemolytic and vascular permeability potencies, and the homologues could be interchanged freely. There were no notable differences in activity between the L component homologues. However, components B and B_a were significantly different. Both were secreted as monomers, but unlike B, B_a was isolated as a relatively inactive complex that could be reactivated with urea. When B_a was substituted for B in gel-diffusion assays the distinct discontinuous haemolysis pattern typical of the presence of B did not occur. In suspension assays, excess B inhibited the haemolysis of B-primed cells by L₁ (as previously described), but not that of B_a-primed cells. Excess B_a had the opposite effect and enhanced lysis of B_a-primed cells, but not that of B-primed cells. These differences reveal details about how the toxin components interact on target cell membranes. The authors' observations indicate that HBL represents a new family of multicomponent toxins that was generated by a process of gene and operon duplication that occurred either intracellularly or by horizontal transfer, and raise the possibility of the existence of other related toxins in the genetically diverse *B. cereus* taxonomic group.

Keywords: *Bacillus cereus*, hemolysin BL, enterotoxin, gene duplication, discontinuous hemolysis

INTRODUCTION

Bacillus cereus is a ubiquitous soil-borne bacterium that, under appropriate circumstances, causes diarrhoeal and emetic food poisoning and a variety of typically necrotic non-gastrointestinal infections (Drobniewski, 1993; Kramer & Gilbert, 1989; Turnbull, 1986). Among the many potential virulence factors of *B. cereus* is haemo-

lysin BL (HBL), a unique and potent three-component pore-forming toxin (Beecher & Macmillan, 1991; Beecher & Wong, 1994c; Beecher & Wong, 1997). The toxic activities so far identified for HBL include haemolysis, vascular permeability and necrosis in rabbit skin (Beecher & Macmillan, 1991; Beecher & Wong, 1994c), fluid accumulation in rabbit ileal loops (Beecher *et al.*, 1995b), toxicity to a number of transformed cell lines (Beecher, 1990), *in vitro* degradation of explanted rabbit retinal tissue, and *in vivo* ocular necrosis and inflammation in rabbits (Beecher *et al.*, 1995a). HBL is

Abbreviations: BCA, bichinchonic acid; HA, hydroxyapatite; HBL, haemolysin BL; TBS, Tris-buffered saline.

secreted by 45% of over 200 *B. cereus*, *B. thuringiensis* and *B. mycoides* isolates tested in our laboratory (Beecher & Wong, 1994b; Schoeni & Wong, 1999). A distinctive feature of HBL is an unusual discontinuous haemolysis pattern produced in blood agar (Beecher & Macmillan, 1991; Beecher & Wong, 1997). When HBL diffuses from a bacterial colony or a well in blood agar, lysis begins away from the well or colony, followed by slow lysis nearer the source.

In *B. cereus* strain F837/76, the three HBL components are encoded in an operon by tandemly arranged genes in the order *hblC*, *hblD* and *hblA*, which respectively encode components designated L_2 , L_1 and B (Heinrichs *et al.*, 1993; Ryan *et al.*, 1997). Immediately downstream from *hblA* is an open reading frame, *hblB*, which is about 85% identical to *hblA* in the first 158 predicted amino acids. The *hblB* gene product has not yet been isolated.

Many isolates in our *Bacillus* collection produce more than one antigen reactive to antibodies against individual HBL components (Schoeni & Wong, 1999). For example, on Western blots of denaturing electrophoresis gels *B. cereus* MGBC 145 produces two bands reactive with antibodies to HBL components B and L_1 , and one band reactive with antibodies to component L_2 . This strain is used in studies of experimental endophthalmitis in our laboratory and in others (Beecher *et al.*, 1995a; Callegan *et al.*, 1999a, b). Because the production of multiple toxin homologues might confound efforts to characterize the molecular basis of *B. cereus* virulence in intraocular infections, we decided to characterize the antigens responsible for producing multiple bands on Western blots. We isolated and characterized two distinct homologous variants of each HBL component from a single *B. cereus* strain, demonstrating that the variant antigens are distinct gene products. The activities of the variants were essentially the same as the prototype components, the homologues were interchangeable, and the requirement for three components to produce activity was maintained. A homologue of one component possessed several important differences in the details of its activity that elucidate some of its interactions with other components on erythrocyte membranes.

METHODS

Bacterial isolates. *B. cereus* strain MGBC 145 was used as the source of HBL homologues. This strain was isolated from the infected eye of a patient with post-traumatic endophthalmitis (Beecher *et al.*, 1995a; Callegan *et al.*, 1999a, b). *B. cereus* strain F837/76, an isolate from an infected prostate, was used as the source of prototype HBL components in the present study. This isolate was originally chosen as the source of HBL because it is exceptionally toxigenic (Beecher & Macmillan, 1990).

Production of crude haemolysin BL. The *B. cereus* isolates were grown at 32 °C for 5 h in 3 litres of casein/glucose/yeast extract broth with shaking at 200 r.p.m. The culture supernatants were each concentrated by ammonium sulfate precipitation to 50 ml. The concentrates were used as sources of

crude HBL. The culture medium, growth conditions and concentration procedure are described in detail elsewhere (Beecher & Wong, 1994c).

Chromatography. Details (columns, flow rates, elution gradients, etc.) of purification steps involving anion-exchange (Whatman DE-52 and Pharmacia Mono-Q) and hydroxy-apatite (HA) chromatography were as previously described (Beecher & Wong, 1994c) with the following exceptions. The pH 5.9 Bistris anion-exchange equilibration buffer was 20 mM instead of 25 mM, and the HA was Macro-Prep ceramic HA (20 µm beads, Bio-Rad) instead of BioGel HTP. Salt concentrations of fractions were estimated assuming that the gradients were linear and began eluting one column volume after they were started. Column volumes were estimated empirically by changes in the apparent absorbance of eluate after abrupt changes in salt concentration.

Three additional chromatographic steps were used. First, rather than dialysis, buffer exchange of the concentrated culture supernatant was by gel-filtration chromatography on a 2.6 × 78 cm column of medium-grade Sephadex G-25 (Pharmacia) equilibrated with 20 mM Bistris/HCl, pH 5.9, and eluted at a flow rate of 7.5 ml min⁻¹. Second, for final isolation of the L_{1a} component (see below for nomenclature), fractions from the pH 5.9 Mono-Q column were dialysed against 25 mM Tris/HCl, pH 7.4, and applied to the Mono-Q column equilibrated with the same buffer. Proteins were eluted with a linear gradient of NaCl from 0 to 0.5 M over 50 ml at a flow rate of 1 ml min⁻¹. Third, for isolation of the L_{2a} the nonbound DE-52 fraction containing L_{2a} was applied to a 1 ml (0.5 × 5 cm) HA column (as compared to the 1.6 × 8 cm column usually used) equilibrated with 10 mM NaCl and eluted at 1 ml min⁻¹ with a linear gradient of sodium phosphate from 0 to 0.3 M over 30 ml and collected into 1 ml fractions.

Protein determinations. Concentrations of purified proteins were routinely determined by measuring A_{280} using previously estimated absorption coefficients (1 mg ml⁻¹) of 1.3, 1.8 and 0.8 for B, L_1 and L_2 (from strain F837/76) respectively. Initial experiments suggested that the absorption coefficients of B and B_a may differ significantly. Thus, when homologue doses were to be compared, concentrations were measured using a bicinchoninic acid assay (Pierce Chemical Co.) in which components from strain F837/76 were used as concentration standards for their respective homologues. The BCA assay measures primarily peptide bonds rather than aromatic amino acid residues, thus decreasing protein-to-protein variation.

Assay for HBL components and sphingomyelinase. HBL components and sphingomyelinase were identified in chromatography fractions immunochemically with dot blots. The different B and L_1 homologues were distinguished from one another by their different apparent molecular masses on Western blots. The L_2 homologues were identical in size, but could be distinguished by their widely different chromatographic behaviour. The antibodies and procedures used for the immunoassays were described previously (Beecher *et al.*, 1995a).

Electrophoresis and protein blotting. PAGE was performed using precast ReadyGels (Bio-Rad) composed of polyacrylamide with a Tris/glycine buffer system, using Mini Protean II dual-slab electrophoresis cells (Bio-Rad). SDS gels (Laemmli, 1970) were composed of 12% polyacrylamide, and native gels (Davis, 1964; Ornstein, 1964) were composed of 4–15% acrylamide gradients. The minigels were stained with Coomassie brilliant blue R-250 (0.1%, w/v) or silver (Nesterenko *et al.*, 1994), or were used for protein blotting.

The Pharmacia LMW Marker Kit was used for size standards (phosphorylase *b*, bovine serum albumin, ovalbumin, carbonic anhydrase and lactalbumin).

Proteins resolved in SDS and native PAGE gels were transferred to PVDF membranes (Millipore) in a Mini Trans-Blot electrophoretic transfer cell (Bio-Rad) at 100 V, constant voltage, for 1 h or 1.25 h, respectively. Isoelectric points were determined on a PhastSystem (Pharmacia) using Pharmacia IEF standards and Phastgel IEF gels with pH ranges of 3–9 and 4–6.5. All gels were run at 2000 V, 2 mA, 3.5 W for 75 V h prior to loading samples and at 200 V, 2 mA, 3.5 W for 15 V h during sample loading. Proteins were focused at 2000 V, 5 mA, 3.5 W for 510 V h (pH 3–9 gels) or 410 V h (pH 4–6.5 gels).

N-terminal sequence analysis. Purified proteins were prepared for N-terminal sequence analysis as described by Matsudaira (1990). Electroblotted proteins were sent to the Protein/Nucleic Acid Shared Facility at the Cancer Center of the Medical College of Wisconsin for N-terminal sequence analysis by Edman degradation.

Haemolysis assays: gel diffusion. For all haemolysis assays (diffusion and turbidimetric), defibrinated sheep blood (Crane Laboratories) was washed in 50 mM Tris/HCl, 150 mM NaCl, pH 7.4 (TBS) prior to use. Gel-diffusion assays for haemolysin BL were as described previously (Beecher, 1990; Beecher & Wong, 1994c) except that 0.3 U ml⁻¹ of *B. cereus* phosphatidylcholine-preferring phospholipase C (Boehringer-Mannheim) was added to the gels. This enzyme enhances and preserves the discontinuous haemolysis pattern of HBL by an as-yet unknown mechanism (Beecher & Wong, 1996). All gel-diffusion assays were at 22–25 °C.

Haemolysis assays: turbidimetric. Haemolysis was also measured with turbidimetric assays (Beecher & Wong, 1997) modified to a microtitre plate format. Washed sheep erythrocytes were diluted into TBS such that 200 µl in a well of a 96-well microtitre plate had an OD₆₂₀ of 0.7. At this OD₆₂₀ there were approximately 6.1 × 10⁷ sheep erythrocytes ml⁻¹. Cell suspensions were warmed to the assay temperature prior to addition to the assay plate. Lysis was measured as the decrease in OD₆₂₀ over time using a temperature-controlled Spectra-Fluor spectrophotometer/microplate reader (Tecan). Haemolysis rates were calculated as the maximum rate of change during the assay period in OD₆₂₀ min⁻¹ × 1000 (ΔmOD₆₂₀ min⁻¹) using DeltaSoft 3, a microplate analysis software package for the Macintosh (Biometallics Inc.).

Lysis of naive erythrocytes. Assay samples consisted either of individual HBL components, combinations of components, or control samples. Samples were either first mixed and diluted, then transferred to empty microtitre wells for the assay, or diluted and then added separately to the empty assay wells. Premixed samples were added to assay wells in volumes from 5 to 20 µl (usually 10 µl), but always in the same volume for a given assay. Samples added separately were generally in 5 µl. Sample volumes were all made identical with TBS when necessary. Immediately prior to the start of the assay, 200 µl of naive erythrocytes (cells that have not been treated with any HBL component) warmed to the assay temperature were added to wells containing the samples and lysis was monitored as described above.

Priming of erythrocytes by B components. The priming activity of the HBL B component was described in detail by Beecher & Wong (1997). Erythrocytes treated with the B component do not lyse, but slowly become sensitized to the rapid lytic action of the combined L₁ and L₂ components. The

priming reaction nearly stops at or below 25 °C, whereas the L components rapidly (1–5 min) lyse primed cells, but not unprimed cells. Here, B or B_a was added to erythrocytes at 42 °C, then cooled to 25 °C for the lysis reaction. The extent of priming was quantitated by adding 5 nM L₁₊₂ to B-treated erythrocytes at 25 °C and measuring the percentage of cells that lysed in less than 5 min.

Quantitation of L component activity. To determine the activity of the L components the rate of the rapid lysis of B-primed erythrocytes was measured (Beecher & Wong, 1997). Sheep erythrocytes were treated with B homologues at 42 °C until 100% of the cells were primed. Primed cells were then added to wells containing the desired amount of L components and lysis was measured as described above and expressed as ΔmOD₆₂₀ min⁻¹.

Urea treatments. Some samples of component B_a were treated with 8 M urea to dissociate complexes and then tested for haemolysis and priming activity. When these samples were to be compared with non-urea-treated components, the same final concentration of urea was added to the erythrocytes separately from the components. During the priming reaction, if present, the urea concentration was 0.11 M. When 25 nM excess B or B_a was tested, if urea was present, the final concentration was 0.4 M. None of the urea concentrations used had an effect on the lysis rate of L₁₊₂ components or the priming rate of B. Urea at 4 M in wells of gel-diffusion assays had no effect on activity of binary combinations of components (i.e. there was no lysis), and had the effects on ternary combinations shown in Results.

Vascular permeability assay. Vascular permeability activity was assayed as described by Glatz *et al.* (1974). Briefly, 50 µl samples were injected intradermally in the shaved backs of rabbits, and after 3 h, Evans blue dye was injected into the marginal ear vein. Samples possessing vascular permeability activity produced areas of oedema and bluing in proportion to the concentration of the toxin. High toxin concentrations also produced areas of necrosis. Female New Zealand White rabbits of 3–4 kg (Covance Research Products) were used for all assays. All activities are reported as means of duplicate or triplicate samples.

Nomenclature. Prototype HBL components isolated from strain F837/76 are referred to as B(F) L₁(F) and L₂(F) or else the strain from which they were purified is stated. Among the proteins isolated from strain MGBC 145, the homologues exhibiting the closest relationship to the prototypes from F837/76 (i.e. 87–100% identity in the N-terminal sequences) are designated B, L₁ and L₂. The more distantly related homologues (i.e. 62–65% identity in the N-terminal sequences) are designated with a subscript 'a', i.e. B_a, L_{1a} and L_{2a}. Component B_a was isolated as a complex. Under the assumption that the complex is a dimer, B_a in complex form is sometimes referred to as 2B_a and the urea-treated monomer as B_a. Combinations of the L components are similarly indicated by L₁₊₂(F), L₁₊₂ and L_{1a+2a}. Other combinations are specifically identified, e.g. B(F) + L₁ + L_{2a}. Combinations, such as HBL or L₁₊₂, were composed of equimolar concentrations of each component unless otherwise specified.

RESULTS

Isolation of HBL homologues

The following description applies to the isolation of components from *B. cereus* MGBC 145. For comparisons with the components from MGBC 145, the

Fig. 1. Chromatographic elution profiles of HBL and HBL_a components. (a) Elution profiles from HA columns. The top four profiles are from the 16 ml HA column and the bottom profile is from the 1 ml HA column (details in Methods). (b) Elution profiles from Mono-Q (anion-exchange) columns. All Mono-Q columns were run at pH 5.9 except for one depicted in the inset, which was run at pH 7.4. Proteins were eluted with linear salt gradients and flow rates as described in Methods. Labels adjacent to peaks identify the major proteins in those peaks. The L_{1a}/S peaks contained both L_{1a} and sphingomyelinase (S). Inset, separation of L_{1a} from sphingomyelinase on the Mono-Q column at pH 7.4. The L_{1a} and S peaks are labelled and eluted at 110 and 140 mM, respectively. The first peak contained no proteins larger than 10 kDa. The x- and y-axes do not apply to this profile, which is included only to illustrate the effectiveness of separation at pH 7.4.

prototype HBL components were also isolated from *B. cereus* F837/76 (data not shown).

DE-52 anion-exchange chromatography. The desalted fraction from the G-25 column was applied directly to the DE-52 column and eluted with a linear gradient of NaCl as described in Methods and by Beecher & Wong (1994c). The elution characteristics of the B, L₁ and L₂ components from MGBC 145 were essentially identical to those from F837/76, while elution of the 'a' components varied to different extents. The following list indicates what fractions were pooled to isolate each component and the calculated concentration range of NaCl comprised by the fractions. L_{2a}, nonbound fraction (isocratic elution); L_{1a} and L₂, fractions 23–26 (0.10–0.13 mM NaCl); L₁, (some L₂) fractions 27–32 (0.13–0.17 mM NaCl); B, fractions 33–38 (0.17–0.21 mM NaCl); B_a, fractions 39–46 (0.21–0.26 mM NaCl).

HA chromatography. Except for L_{2a}, the fraction pools listed above were each applied to the 1.6 × 8 cm HA column and eluted with identical gradients of phosphate buffer. L_{2a} was applied to a smaller HA column as described in Methods. Upon elution from HA, the proteins are nearly pure, so that comparison of their A₂₈₀ elution profiles becomes informative. Fig. 1(a) compares the elution profiles of each component from HA relative to the phosphate eluent concentration. Only

the elution profiles of L₂ and L_{2a} were exceptionally different from one another. At this point, L_{2a} was essentially pure, and no further separations were performed. Also of interest, L_{1a} and sphingomyelinase behaved identically on HA.

Mono-Q anion-exchange chromatography. Except for L_{2a}, the fractions containing the respective HBL component homologues were pooled and applied to a Mono-Q column. Fig. 1(b) compares the Mono-Q elution profiles for each component. All of the homologous component pairs behaved differently on this column (essentially reflecting their behaviour on DE-52). Note that both peaks that eluted from the column depicted in the top panel in Fig. 1(b) represent the B component. The later peak represents intact B, and the early peak represents B with its first two N-terminal residues (Ser-Glu) cleaved by endogenous protease (unpublished observations). Also note that, as on HA, L_{1a} and sphingomyelinase behaved identically on the Mono-Q column at pH 5.9, but could be easily separated at pH 7.4 (inset, Fig. 1b). Under these conditions, the L_{1a} peak occurred at 110 mM NaCl, and the sphingomyelinase peak was at 140 mM NaCl.

Physicochemical comparison of components

Fig. 2 shows the purified HBL homologues from strains F837/76 and MGBC 145 on SDS-PAGE. Table 1

Fig. 2. SDS-PAGE gel of purified HBL and HBL_a components. Lanes 3–5, 7–9 and 11–13 contain 1 µg of each purified component as labelled at the top of the gel. Components with a dot above them were isolated from *B. cereus* F837/76. All other components were from *B. cereus* MGBC 145. Lanes 1, 2, 6, 10, 14 and 15 contained low-molecular-mass protein standards (s). The gel was run and stained as described in Methods.

compares their N-terminal sequences and their estimated molecular masses and isoelectric points. Also shown for comparison is the predicted N-terminal sequence from the *hblB* open reading frame immediately downstream from the B gene (*hblA*).

Between aligned residues, B, L₁ and L₂ were 92, 100 and 87 % identical to their respective prototype components

from F837/76. B_a, L_{1a} and L_{2a} were respectively 60, 65 and 63 % identical to B, L₁ and L₂. Interestingly, L_{2a} was more similar to L₂(F) over 36 residues (67 % identical) than to L₂ (63 % identical). B_a was 67 % identical to the *hblB* predicted amino acid sequence from F837/76. It is doubtful that these identities reflect the overall identities between the respective sequences; for example, B(F) and the predicted *hblB* amino acid sequence are approximately 85 % identical over 158 residues, but only 60 % identical over 43 aligned N-terminal residues.

Isolation of B_a as a complex

We recently found, in experiments unrelated to this study, that treatment of the B component from strain F837/76 at 65 °C converts it to stable but relatively inactive complexes, primarily dimers (analysis to be presented elsewhere). The complexes can be identified as slowly migrating bands on native PAGE gels and can be dissociated to the monomer with 8 M urea, which also restores activity. The native PAGE gel in Fig. 3(a) shows that, in our preparation, B_a was a complex that can be dissociated with urea. Unlike B, which forms mixtures of increasingly larger complexes over time, B_a was present primarily as a single complexed species that migrated at the same rate as the initial complex formed by B. The other proteins purified in this study were all monomers, because their migration distances on native PAGE were not affected by 6–8 M urea (not shown).

Fresh culture supernatants were run on a native PAGE gel and analysed by Western blotting to determine whether B_a is secreted as a dimer. Fig. 3(b) compares

Table 1. N-terminal sequence alignments and characteristics of HBL and HBL_a components

Component	Strain	N-terminal sequence*	Source of sequence data†	M _r ‡	pI§
B	F837/76	--SEIEQTNNGDTALSANEAKMKETLQKAGLFAKSMNAYSMLIK	Pred. ^a	38080	5.3 , 5.4
<i>hblB</i>	F837/76	--I..Q...TENRS....EQ..KA..d...V.a..E...L..h	Pred. ^a	—	—
B	MGBC 145	--.....R.....N.....X.....	Exp.	38080	5.3
B _a	MGBC 145	SE.k....STE.IS..V.sE...KA..d..V.....D...L..N	Exp.	42500	6.0
L ₁	F837/76	--QETTAQEQQVGNYALGPEGLKKALAETGSHILVMDLYAKT	Pred. ^b	39540	5.3
L ₁	MGBC 145	--.....X.....	Exp.	39540	5.2, 5.3
L _{1a}	MGBC 145	QA..q...K..IE.....D...l..TGS.I..md.Y..T	Exp.	40930	5.5
L ₂	F837/76	ETQQENMDISSLRKLGAQSKLIQTYIDQSLMSPNV	Pred. ^b	44800	5.3
L ₂	MGBC 145	Q.....H...V.....N¶	Exp.	45450	5.2
L _{2a}	MGBC 145	..K.D.I....A...I..H...t..FV.GA.A....	Exp.	45900	6.4

* The prototype sequence is at the top of each group. For compared sequences, identities are depicted by dots; letters indicate differing residues. Differences requiring two base substitutions from the known nucleotide sequence are indicated by lower-case letters. Gaps are indicated by dashes. Residues marked by X could not be determined chemically.

† Pred., predicted from nucleotide sequences [a, Heinrichs *et al.* (1993); b, Ryan *et al.* (1997)]; Exp., experimental data of present study.

‡ M_r, molecular size determined from SDS-PAGE gel in Fig. 2.

§ pI, isoelectric point determined by PhastGel electrophoresis in this study; bold indicates the major band.

|| Protein product not yet isolated and characterized. Nucleotide sequence in Heinrichs *et al.* (1993).

¶ First residue interpreted once as Glu, and once as Gln with a weaker Glu signal.

Fig. 3. Production of B_a as a monomer and isolation as a dimer. Lanes 1–5, native PAGE of components B and B_a in complex and monomer forms. Each lane was loaded with 0.25 μ g protein and the gel was stained with silver. Lane 1 (B65), component B treated at 65 °C, 10 min, to form complexes. Lane 2 (B), untreated component B. Lane 3 (B_a), untreated component B_a . Lane 4, blank. Lane 5 (B_a U), component B_a added to gel in the presence of 6 M urea. Note: 8 M urea eliminates upper band (not shown). Lanes 6 and 7, Western blot probed with polyclonal antiserum to B. Lane 6 (F), 20 μ l culture supernatant from *B. cereus* F837/76 (HBL prototype strain). Lane 7 (M), 20 μ l culture supernatant from *B. cereus* MGBC 145 (HBL $_a$ -producing strain).

supernatants from strains MGBC 145 and F837/76 (which produces only B). No complex was detected in either supernatant. Both supernatants did exhibit one slow-moving band, which did not correspond to the complexes. The identities of these bands are unknown.

Haemolysis by HBL and HBL $_a$ components: similarities

Activity of component combinations. In turbidimetric haemolysis assays, sheep erythrocytes were treated with all possible binary and ternary combinations of the prototype-like and 'a' components from MGBC 145. None of the binary combinations was lytic. Among the 20 possible three-component combinations, all eight of those possessing B, L_1 and L_2 homologues were lytic. None of the 12 combinations that possessed redundant homologues (e.g. B + L_1 + L_{1a} , B + B_a + L_1 , etc.) was lytic. Thus, the three-component requirement for HBL activity was maintained, and homologous components could be interchanged freely.

Priming activity of B and B_a . During the priming reaction, which occurs independently of the L components, naive erythrocytes gradually become sensitive to rapid lytic action by the L components (Beecher & Wong, 1997). This slow priming occurred here for both B and B_a from MGBC 145. B at 5 nM completely primed erythrocytes at 40 °C in 1 h, whereas 10 nM B_a monomer (urea-treated) required 1.25 h to prime all of the cells. Untreated B_a complex also primed erythrocytes. How-

ever, its activity has not yet been characterized thoroughly because we had only limited quantities, and the monomer appears to be the natural form (Fig. 3b).

Rapid lytic activities of all L components. We tested all combinations of L and L_a components for their abilities to lyse B- and B_a -primed cells. For all combinations possessing L_1 and L_2 homologues (e.g. L_{1+2} , L_{1a+2} , L_{1a+2a} , etc.) lysis by 0.5–10 nM of the L components was rapid and complete (data not shown). L_{1a} appeared to be about half as potent as L_1 . However, minor concentration inaccuracies cannot yet be ruled out. There were no apparent differences in activities between L_2 and L_{2a} .

Haemolysis by HBL and HBL $_a$ components: differences

Haemolysis in blood agar. When B was present the discontinuous haemolysis pattern typical of HBL occurred for all L component combinations (i.e. L_{1+2} , L_{1+2a} , L_{1a+2} and L_{1a+2a}). This suggests that the L_1 and L_{1a} components interact in similar ways with the B component to produce the pattern (L_2 neither inhibits B or L_1 , nor is it inhibited by them and thus does not contribute to the discontinuous pattern: Beecher & Wong, 1997). However, when B_a replaced B, the discontinuous pattern did not occur for any L combination, i.e. lysis was continuous from the well edge to the edge of the lysis zone.

Fig. 4 shows the effect of varying the concentration of L_1 on the haemolysis pattern in the presence of B or B_a (L_{1a} produced the same effects in blood agar; not shown). The discontinuous pattern occurred at all L_1 concentrations in the presence of B, but lysis occurred more quickly near the well at lower L_1 concentrations, which appear nearly continuous in the figure. A discontinuous pattern was not evident at any time around the wells containing B_a .

Fig. 4 also indicates that 8 M urea significantly enhanced the activity of B_a , but quantitation of the enhancement was not possible because of the inhibitory effects of excess L_1 . The relative potencies of B and B_a in blood agar were estimated by placing serial dilutions of each in wells surrounding a central well containing 100 ng L_{1+2} . In this method B primes cells surrounding the well without the inhibitory effect of excess L_1 (Beecher & Wong, 1997). The lowest amounts of B, B_a and B_a + 8 M urea exhibiting lytic activity were 0.3, 4 and 1.25 ng per well respectively.

Inhibition of L_1 components by B and B_a . Just as excess L_1 inhibits the priming reaction of B to produce discontinuous haemolysis on blood agar, it also inhibits priming in erythrocyte suspensions (Beecher & Wong, 1997). Likewise, the addition of excess B to previously primed erythrocytes inhibits the lysis rate of L_1 (Beecher & Wong, 1997). We examined the abilities of excess B and B_a components to inhibit the lysis of B- and B_a -primed cells by L_1 (preliminary experiments indicated that there were no discernible differences between L_1

Fig. 4. Effect of urea and L_1 concentration on the haemolysis pattern in sheep blood produced by B and B_a . Wells in rows received 100 ng of either B or B_a in the presence or absence of 8 M urea as indicated on the left. Wells in columns received L_1 at the concentrations indicated at the bottom. All wells contained 100 ng L_2 . All four horizontal strips were on the same blood agar gel. The image was recorded after 6 h at 25 °C.

Table 2. Effect of excess B, B_a or $2B_a$ on erythrocyte lysis by components L_{1+2}

Components*		Lysis rate ($\Delta\text{mOD}_{620} \text{ min}^{-1}$)†		
Cells‡	Excess§	Control	Sample	%I
B'	B	354 ± 1.6	111 ± 7.7	69
B'	B_a	354 ± 1.6	352 ± 0.2	1
B'	$2B_a$	443 ± 1.1	454 ± 6.2	-3
B'_a	B	276 ± 3.0	290 ± 2.1	-4
B'_a	B_a	276 ± 3.0	446 ± 1.4	-48
B'_a	$2B_a$	214 ± 4.6	206 ± 3.6	2

* Component designations: B, component B; B_a , B_a monomer, i.e. treated with 8 M urea; $2B_a$, B_a dimer (no urea).

† Lysis of primed cells was produced by 5 nM L_{1+2} . Samples were in duplicate; the values shown are means $\pm$ range. Control, rates of haemolysis of cells treated with the respective component combinations in the absence of excess B, B_a or $2B_a$. Sample, lysis rates in the presence of excess B, B_a or $2B_a$. %I, percentage inhibition of lysis in the presence of excess B, B_a or $2B_a$ versus control; negative numbers indicate enhanced lysis.

‡ Naive erythrocytes were primed at 40 °C either with 5 nM B for 1 h (B') or with 10 nM B_a for 1.25 h (B'_a).

§ After cells were primed, 25 nM B, B_a or $2B_a$ was added in excess of that used to prime the cells.

and L_{1a} components; therefore only results using L_1 are shown). The results are shown in Table 2.

Sheep erythrocytes were primed at 42 °C with 5 nM B or 10 nM B_a . The primed erythrocytes were lysed with 5 nM L_{1+2} in the presence or absence of 25 nM B or B_a . The B_a was added either in 8 M urea to test the monomer, or only in buffer to test the complex ($2B_a$). Final urea concentrations in samples and controls are described in Methods.

There were striking differences between the effects of excess B and excess B_a on the lysis rate produced by L_1 . As expected, excess B inhibited the lysis of B-primed cells. However, excess B_a actually enhanced haemolysis

of B_a -primed cells. Neither excess B nor excess B_a significantly affected the lysis rate of cells primed by the other protein. Complexed B_a ($2B_a$ in Table 2) did not significantly affect lysis of cells primed by either B or B_a .

Most proteins unfold in 8 M urea, and it was possible that the enhanced activity of urea-treated B_a was due to the unfolding and refolding of B_a rather than dissolution of the complex *per se*. When B, which was already in monomer form, was first dissolved in 8 M urea, its inhibition of lysis differed by less than 10 % compared to untreated B. This suggests that urea had little effect on the monomer.

Vascular permeability activity of HBL and HBL_a

The ability of the HBL and HBL_a components to cause vascular permeability and necrosis in rabbit skin was tested using combinations that appeared likely to be active based on their haemolytic characteristics. Samples containing 0.5–1.5 μg of single components and binary combinations produced no reaction. There were no clear differences between samples containing 0.5 μg of each component of HBL from F837/76, or HBL and HBL_a from MGB C 145, which produced respectively 20 ± 1.6 , 21 ± 1.2 and 20 ± 2.0 mm of bluing and 3.7 ± 2.5 , 4.4 ± 3.7 and 4.0 ± 1.2 mm of necrosis (means $\pm$ SD, $n = 3$). Similar reactions were obtained when B_a was mixed with L_{1+2} and when B(F) was mixed with L_{1a+2a} . When B and B_a concentrations were varied with constant L_{1+2} , necrosis occurred with 0.5 μg of B and B_a , but not with 0.25 and 0.125 μg . The only difference appeared in the bluing response between 0.125 μg of B and 0.125 μg of B_a , which produced 23 ± 0.5 and 9 ± 2.6 mm zones respectively.

B_a was not treated with urea prior to vascular permeability testing to avoid any complications that high concentrations of urea might produce. This component will be further characterized when conditions are developed to produce significant concentrations of B_a monomer in physiological salt concentrations.

DISCUSSION

We have noted that numerous *B. cereus* strains produce multiple bands on Western blots probed with antibodies specific for components of HBL. The presence of multiple toxin homologues might complicate attempts to understand virulence mechanisms. Therefore, we tried to determine whether the different bands seen on Western blots represent distinct gene products. Using antibodies to track the chromatographic behaviour of the different forms of the HBL components, we isolated two distinct homologues of each component from the medically important *B. cereus* isolate MGBC 145. Both sets of homologues, HBL and HBL_a, were haemolytic, dermonecrotic, and caused vascular permeability in rabbit skin with similar potencies. Overall, there were no discernible differences in activity between the L homologues, but there were significant differences in activity between B and B_a.

The homologous components differed from one another in size, net charge and chromatographic behaviour. L₁ and L_{1a} behaved most similarly, and L_{1a} coeluted with sphingomyelinase from HA and from anion-exchangers at pH 5.9. However, it could be separated from sphingomyelinase at pH 7.4. L₂ and L_{2a} were essentially identical in size, but because they differed in pI by a full pH unit, they were readily separated by ion-exchange chromatography, and the exceptionally late elution of L_{2a} from HA made it easy to isolate in as few as two chromatographic steps.

B_a presented a peculiar case. It was isolated as a complex, which may explain its incongruous chromatographic elution. Its apparent pI was 6.0 (Table 1), and one would expect that it would elute earlier from anion-exchange columns than B (pI 5.3). Instead, it eluted later, at 1.3 times the elution volume of B. The heat-generated complexes of B (upper bands, Fig. 3a, lane B65) behave in the same way (data to be published elsewhere). It appears, however, that B_a is naturally produced as a monomer, which is how it appeared in fresh *B. cereus* culture supernatant (Fig. 3b). We are investigating the factors that result in complex formation during purification.

All of the 'a' components were 62–65% identical to their respective homologues over 30–43 residues. Similar identity (60%) in strain F837/76 between the N-termini of B and the predicted *hblB* sequence rises to approximately 85% over the first 158 residues (Heinrichs *et al.*, 1993), suggesting that the HBL_a components may prove to be more closely related to HBL components than indicated by the N-termini. The high sequence similarity among the 'a' homologues suggests that the three genes may have been either simultaneously duplicated or simultaneously transferred horizontally, and this would have been facilitated by their tandem arrangement in an operon (Ryan *et al.*, 1997). We have also determined that the four *hbl* genes in strain F837/76 all arose via duplication of a single gene (Beecher, 1997). Note that HBL has been mapped to a portion of the *B. cereus* chromosome that exhibits exceptional variability com-

pared with other regions, and that this variable region is sometimes located on large extrachromosomal DNA fragments that appear to be stable, but might also prove to be large mobile plasmids (Carlson *et al.*, 1996a, b; Carlson & Kolstø, 1994). This raises fundamental questions about the extent and role of gene duplication and horizontal transfer in the evolution of *B. cereus*.

In addition to HBL and HBL_a, we have found (data not shown) that strain MGBC 145 expresses another homologous three-component toxin designated nonhaemolytic enterotoxin (NHE) by Lund & Granum (1996). Data from several studies suggest that, among strains that express either HBL or NHE, 80% of them express both (Buchanan & Schultz, 1994; Day *et al.*, 1994; Rusul & Yaacob, 1995). These data are based on detection of the L₂ protein of HBL, or the NHEA protein, by two commercial kits (Beecher & Wong, 1994a).

Our observations highlight practical problems both for the diagnosis of toxigenic strains and for the identification of *B. cereus* virulence factors, particularly since there may be additional unidentified HBL homologues. Since the discontinuous haemolysis pattern typical of HBL appears to be produced only in the presence of the prototype B component, discontinuous lysis around colonies or culture supernatants (Beecher & Wong, 1994b) now appears to be of limited diagnostic value. For maximum value, other methods should detect all relevant toxin components or component combinations. However, not all of these components and combinations are completely defined. In attempts to identify virulence factors, possible contributions from multiple, potentially interacting, multicomponent toxins must be kept in mind.

Although numerous physical phenomena have been documented for HBL, little is known about how the components interact at the molecular level beyond the fact that they bind to cells independently and must physically interact to form pores. It is also clear that there is a change in the physical state of B between its initial association with the membrane (B^m) and its primed state (B'). This change may involve conformational changes, oligomerization, membrane insertion, or all three. The experiment represented in Table 2 provides some insight into such additional interactions as discussed below.

We previously found that the lysis of B-primed erythrocytes by L₁₊₂ was inhibited by excess B added simultaneously with the L components, and that it is L₁ that is inhibited, not L₂ (Beecher & Wong, 1997). Table 2 shows that excess B and B_a have different effects, and that the specific effect depends on which was used to prime the cells. A significant effect occurred only when the protein added in excess was the same as that used to prime cells. This indicates that the excess B^m must physically interact with B' (likewise for excess B_a^m and B_a'), and that B and B_a are sufficiently unrelated that they do not interact with one another.

The excess B may inhibit lysis simply by obstructing L₁ from contact with B', or by producing inefficient lytic

complexes composed of B^m, B' and L₁. Enhancement of lysis by B_a was unexpected, and the mechanism by which this occurs is unclear. It might occur either if B_a^m enhances the incorporation of L₁ into the lesion, or if B_a^m-B_a'-L₁ complexes are more efficient than B_a'-L₁ complexes.

There is a structural basis for suggesting that L₁ binds more efficiently to B_a^m-B_a' complexes. B and L₁ from strain F837/76 appear to be distant homologues, and the most significant structural difference between them is that B is missing 40 residues of a 60-residue apparent transmembrane segment present in L₁ (Beecher, 1997). The observed size difference of approximately 4.4 kDa between B and B_a (Table 1) equates with a difference of approximately 40 residues. In essence, B_a may act as a hybrid between B and L₁ by virtue of retaining the full transmembrane segment that is characteristic of L₁.

While our observations emphasize practical difficulties for diagnosis and identification of virulence factors, they also provide promising opportunities for structure-function analysis of the HBL family of toxins. The structural and functional differences between B and B_a are significant and may provide insight into the mechanism of HBL toxicity as well as an explanation for the unique haemolytic phenomena of this toxin.

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