Nachweis, Expression und Bedeutung der von *Bacillus cereus* produzierten Enterotoxin-Komplexe

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Abkürzungsverzeichnis

BcET	*Bacillus cereus* Enterotoxin
BCET-RPLA	Reverser Latexagglutinationstest
Cyt K	Cytotoxin K
EIA	Enzymimmunoassay
EntFM	Enterotoxin FM
HBL	Hämolysin BL
kDa	Kilodalton
mAK	Monoklonaler Antikörper
Nhe	Non-hemolytic enterotoxin
PCR	Polymerase-chain-reaction
RIL	Rabbit ileal loop
VPR	Vascular permeability reaction
ZT	Zellkulturtest

1 Einleitung

Im Zusammenhang mit *Bacillus cereus* sind eine Reihe von nicht gastrointestinalen Erkrankungen beim Menschen beschrieben worden. Einerseits konnten lokale Erkrankungen wie z.B. Wundinfektionen, Infektionen des Auges, Arthritiden, andererseits systemische Infektionen mit Septikämien, Endokarditiden, ZNS-Abszeß (DROBNIEWSKI, 1993) beobachtet werden. Bei Tieren wurde *B. cereus* als Ursache von akuten oder subklinischen Euterentzündungen beim Rind beschrieben (GEDEK, 1986). Als ubiquitär vorkommender gram-positiver Keim mit der Fähigkeit zur Sporenbildung ist *B. cereus* auch von großer lebensmittelhygienischer Bedeutung (KOTIRANTA et al., 2000). Er gehört zu den wichtigsten Verderbserregern von Milch und Milchprodukten (z.B. Süßgerinnung), kann aber auch regelmäßig aus verschiedensten anderen Lebensmitteln (z.B. Reis, Nudeln, Gemüse) isoliert werden. Neben seiner Rolle als Verderbserreger ist *B. cereus* auch bekannt als Ursache von Lebensmittelvergiftungen mit zwei unterschiedlichen Verlaufsformen (GRANUM, 2001; SCHOENI & WONG, 2005). Ursache dieser Erkrankungen sind bei der Diarrhö-Form unterschiedliche Protein(-komplexe) bzw. bei der emetischen Form ein zyklisches Peptid.

Erkrankungen mit dem Leitsymptom Durchfall sind neben dem bis jetzt bei nur einem Stamm nachgewiesenen Cytotoxin K auf die Bildung von zwei Enterotoxinkomplexen (Hämolysin BL und Non-hemolytic enterotoxin) zurückzuführen. Während die Enteropathogenität des HBL im Tierversuch bewiesen ist (THOMPSON et al., 1984; BEECHER et al., 1995), fehlen zu dem *in vitro* hoch potenten Nhe bislang aussagekräftige *in vivo* Studien. Verschiedene Untersuchungen in den letzten Jahren zeigten jedoch, dass dieser Komplex ebenfalls eine wichtige Rolle im Diarrhö-Krankheitsgeschehen spielt.

Derzeit ist es nicht möglich für Lebensmittelvergiftungen verantwortliche *B. cereus* Stämme von für den Menschen harmlosen Isolaten zu unterscheiden. Trotz des ubiquitären Vorkommens von *B. cereus* und den von bisherigen Studien zur Prävalenz der beiden Enterotoxin-Komplexe mit molekularbiologischen Verfahren ermittelten Daten (ca. 50% für HBL bzw. über 90% für Nhe, PRÜSS et al., 1999; HANSEN & HENDRIKSEN, 2001 und IN'T VELD et al., 2001) sind nur sehr wenige Ausbrüche von Lebensmittelvergiftungen dokumentiert. Aus dieser Diskrepanz zwischen häufigem Nachweis von toxinogenen Isolaten und tatsächlichen Erkrankungen ergibt sich die Frage nach den für die Virulenz eines

Stammes entscheidenden Faktoren. Eine mögliche Antwort gibt die quantitative Analyse der Toxinproduktion, die bisher nicht möglich war, da nur polyklonale Antisera zur Detektion von je einer Einzelkomponente der beiden Enterotoxin-Komplexe zur Verfügung stehen (BEECHER & WONG, 1994b; LUND & GRANUM, 1993). Um diese limitierte Nachweismöglichkeit zu erweitern, wurde im Rahmen dieser Arbeit die Entwicklung von monoklonalen Antikörpern (mAk) angestrebt, um zusammen mit den von DIETRICH et al. (1999) produzierten Antikörpern, den vollständigen und quantitativen Nachweis aller sechs Einzelkomponenten der beiden Toxine zu ermöglichen und somit einen Hinweis auf die pathogene Relevanz eines Isolates zu bekommen.

Anhand einer repräsentativen Studie an 100 ausgewählten *B. cereus* Stämmen, die sowohl geographisch als auch von ihrem Habitat ein breites Spektrum an Biodiversität repräsentieren (48 Isolate aus Lebensmittelvergiftungen bzw. 52 Isolate aus Umwelt und Lebensmitteln z.B. Fleisch, Milch, Gemüse, Babynahrung, Reis, Nudeln) sollte geklärt werden, ob i) Isolate aus Lebensmittelvergiftungsfällen sich in ihrer Toxinproduktivität von anderen Stämmen unterscheiden, ii) die Toxinkonzentration mit der toxischen Wirkung auf Zellen korreliert und iii) mit den daraus resultierenden Ergebnissen eine wissenschaftlich fundierte Aussage zum enteropathogenen Potential eines *B. cereus* Isolates getroffen werden kann.

Im Rahmen des Forschungsvorhabens: QLK-2001-00854 „Preventing *Bacillus cereus* foodborne poisoning in Europe – Detecting hazardous strains, tracing contamination routes and proposing criteria for foods“ wurden Teile dieser Arbeit durch die Europäische Komission gefördert.

2 Schrifttum

2.1 Allgemeines

Seit der ersten wissenschaftlich abgesicherten Beschreibung einer durch *B. cereus* verursachten Lebensmittelvergiftung (HAUGE, 1955), wurde die Rolle einer breiten Palette von Pathogenitätsfaktoren (z.B. Hämolysine, Sphingomyelinasen, Phospholipasen) diskutiert. In den 90er Jahren gelang eine genauere Charakterisierung von Enterotoxin-Komplexen, die ursächlich an der Entstehung von gastrointestinalen Erkrankungen beteiligt sind. So konnte von der Arbeitsgruppe um Ami WONG das Hämolysin BL (HBL) bzw. von der Arbeitsgruppe um Per Einar GRANUM das Non-hemolytic enterotoxin (Nhe) als Toxin identifiziert und charakterisiert werden. Eine Übersicht über die bisher als Diarrhö-Toxine identifizierten Proteine bzw. Proteinkomplexe gibt Tabelle 1. Andere als Enterotoxin beschriebene Proteine (BcET; AGATA et al., 1995; EntFM; ASANO et al., 1997) dürften nach dem derzeitigen Kenntnisstand keine Rolle spielen.

Tabelle 1: *Bacillus cereus* Diarrhö-Toxine

Referenz	Molekulargewicht (kDa)	Biol. Aktivität
HBL (L_1, L_2, B)		
THOMPSON et al. (1984)	38; 39,5; 43	RIL, VPR, ZT
BEECHER et al. (1995)	37,8; 38,5; 43,2	RIL, VPR, ZT
Nhe (A, B, C)		
LUND & GRANUM (1996)	39; 45; 105	ZT
GRANUM et al. (1999)	41; 39,8; 36,5	ZT
Cytotoxin K		
LUND et al. (2000)	34	ZT

2.2 Enterotoxine von *Bacillus cereus*

2.2.1 Hämolysin BL (HBL)

BEECHER & MACMILLAN beschrieben 1990 den hämolytisch wirksamen Enterotoxinkomplex (Hämolysin BL) bestehend aus drei Proteinen mit den Molekulargewichten 38.080 Da (Komponente B), 39.540 Da (L_1) und 44.800 Da (L_2; BEECHER & WONG, 2000). Nur nach Kombination aller drei Komponenten konnte im VPR- (vascular permeability reaction) und RIL-Test (rabbit ileal loop) eine biologische Aktivität nachgewiesen werden (BEECHER & WONG, 1994a; BEECHER et al., 1995). Die Gene der drei Komponenten (*hblC*, *hblD* und *hblA*) liegen auf einem Operon (Abb. 1) und werden in eine mRNA transkribiert (HEINRICHS et al., 1993; RYAN et al., 1997).

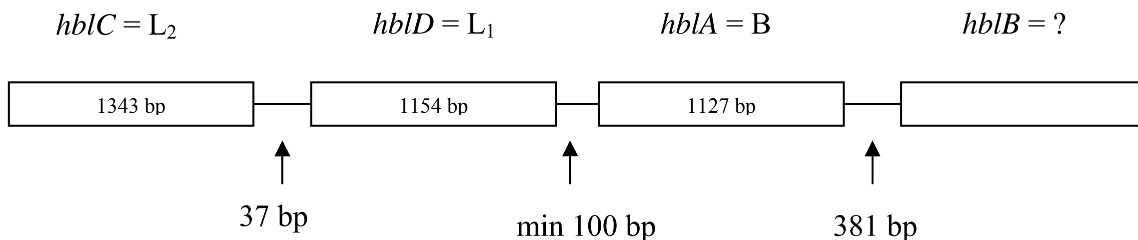


Abbildung 1: Aufbau des *hbl* Operons nach GRANUM (2001)

Die Funktion des von *hblB* codierten Proteins ist unbekannt und es konnte bis jetzt noch nicht isoliert werden, aber GRANUM et al. (1997) konnten in molekularbiologischen Untersuchungen zeigen, dass es zu 73 % identisch mit der B-Komponente ist. Die Expression wird, wie auch bei sehr vielen anderen Exoproteinen von *B. cereus* durch den ursprünglich für Phospholipase C (Plc) beschriebenen Regulator (PlcR) kontrolliert (AGAISSE et al., 1999). Studien von PRÜSS et al. (1999), HANSEN & HENDRIKSEN (2001) und IN'T VELD et al. (2001) zeigten, dass ca. 50-60 % aller untersuchten *B. cereus*-Isolate die Gene aller drei Komponenten besitzen. In Hinblick auf den Wirkungsmechanismus auf zellulärer Ebene wurden zwei Modelle vorgeschlagen: (1) die B- Komponente bindet sich an die Zelloberfläche und schleust die beiden L-Komponenten ein, die ihrerseits die metabolische Aktivität der Zelle stören (BEECHER & MACMILLAN, 1991) bzw. (2) alle Komponenten

von HBL binden sich unabhängig voneinander an die Zielzellen, wo sie sich dann zu einem sog. „membrane attack complex“ formieren (BEECHER & WONG, 1994a). Dadurch wird die Durchlässigkeit der Zellmembran erhöht, und aufgrund des kolloidosmotischen Druckes kommt es schließlich zum Austreten von Zellmaterial und zum Zelltod.

2.2.2 Non-hemolytic enterotoxin (Nhe)

Aus dem in eine Lebensmittelvergiftung involvierten Stamm NVH 0075-95 konnten LUND & GRANUM (1996) das Non-hemolytic enterotoxin (Nhe) isolieren. Ebenso wie Hämolysin BL besteht dieser Komplex aus drei Proteinen, die entsprechenden Molekulargewichte betragen 39.820 Da (NheB), 41.019 Da (NheA) bzw. 36.481 Da (NheC) (GRANUM et al., 1999). Wie auch bei HBL werden im Zellkulturtest alle drei Komponenten für die maximale zytotoxische Aktivität benötigt. Strukturelle Ähnlichkeiten bestehen einerseits zwischen der L_1-Komponente des HBL-Komplexes und der NheB Komponente, andererseits zwischen der B-Komponente des HBL-Komplexes und der NheC Komponente (LUND & GRANUM, 1997). Alle drei Gene der Komponenten liegen wie bei HBL auf einem Operon (Abb. 2; GRANUM et al.1999) und werden wie bei *hbl* durch PlcR kontrolliert.

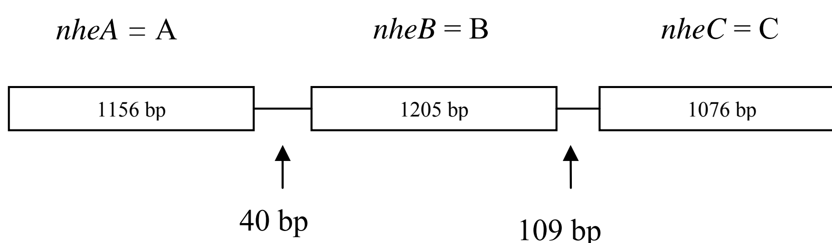


Abbildung 2: Aufbau des *nhe* Operons nach GRANUM (2001)

In verschiedenen Untersuchungen wurde eine Prävalenz dieser Gene von 90 % und mehr ermittelt (HANSEN & HENDRIKSEN, 2001; GUINEBRETIÈRE et al., 2002). Über den Wirkungsmechanismus des Toxins ist sehr wenig bekannt. LINDBÄCK et al. (2004) berichteten, dass bei einem Mengenverhältnis von 10:10:1 der Nhe-Komponenten A, B und C die höchste Zytotoxizität im Zellkulturtest zu beobachten ist. Unter Verwendung von Vero-Zellen konnte des Weiteren die B-Komponente des Nhe als Bindungskomponente identifiziert werden.

2.2.3 Cytotoxin K (Cyt K)

Aus einem in eine tödlich verlaufende Lebensmittelvergiftung involvierten *B. cereus* Isolat konnten LUND et al. (2000) ein Einzelprotein mit einem Molekulargewicht von 33,8 kDa isolieren und sequenzieren. Dieses Isolat hatte nicht die Fähigkeit, HBL oder Nhe zu produzieren. Das zytotoxisch und hämolytisch wirkende Cyt K, das mit dem von BAIDA et al. (1999) beschriebenem *B. cereus* Hämolysin II eine Sequenzhomologie von 37 % aufweist, konnte allerdings bis jetzt nur bei einem einzigen Stamm nachgewiesen werden. Es ist stark zytotoxisch und wirkt hämolytisch auf Rinder- und Kaninchenblut. Die Aminosäurensequenz des Cyt K zeigte zudem Ähnlichkeiten mit von *Staphylococcus aureus* synthetisierten α- und γ- Hämolysinen und dem β-Toxin von *Clostridium perfringens*. In späteren Untersuchungen von STENFORS et al. (2001, 2002) wurde ein „Cyt K ähnliches" Toxin mittels PCR auch bei fünf anderen Stämmen nachgewiesen, die zusätzlich HBL und/oder Nhe produzierten.

2.3 Nachweisverfahren für *Bacillus cereus* Toxine

Nach der vollständigen Sequenzierung der Enterotoxin-Gene wurden in den letzten Jahren vermehrt molekularbiologische Verfahren, die meist auf der Polymerase-chain-reaction-Methode basieren, entwickelt (HEINRICHS et al., 1993; RYAN et al., 1997; GRANUM et al., 1999; LUND et al., 2000) und zum Nachweis des *nhe*- und *hbl*-Gens bei *B. cereus* Isolaten eingesetzt.

Früher wurde die Pathogenität von *B. cereus* Isolaten mittels verschiedener Tiermodelle, wie den Darmschlingen-Ligationstest (rabbit ileal loop = RIL; SPIRA & GOEPFERT, 1972), Mäuseletalitätstest (MLT; GLATZ & GOEPFERT, 1973), Gefäßpermeabilitäts-Reaktion (vascular permeability reaction = VPR; GLATZ et al.,1974) bzw. Affenfütterungstest (MELLING et al., 1976) überprüft. Diese *in vivo* Verfahren, die mittlerweile nur mehr zur Klärung spezifischer Fragestellungen eingesetzt werden, wurden danach durch *in vitro* Bioassays, insbesondere Zellkulturtests, abgelöst.

Unter Verwendung von Vero-Zellen (Affennierenzellen), die als besonders empfindlich gegenüber von *B. cereus* produzierten toxischen Substanzen gelten (WONG et al., 1988), entwickelten SEIDEL et al. (1996) einen sensitiven MTT-Zellkulturtest. Die Erstellung eines sensitiven und reproduzierbaren Zytotoxizitätstest zum Nachweis von *B. cereus* Diarrhö Toxin wurde durch Verwendung von CaCo-2-Zellen, sowie einer modifizierten simultanen Testdurchführung des MTT-Tests, ermöglicht (DIETRICH et al., 1997). Mit diesen biologischen Verfahren kann allerdings nur die Gesamttoxizität nachgewiesen werden, da sie nicht spezifisch für *B. cereus* Enterotoxine sind.

Zum spezifischen Nachweis von *B. cereus* Enterotoxin sind derzeit zwei immunchemische Nachweisverfahren, die auf polyklonalen Antikörpern basieren, kommerziell erhältlich. Im BCET-RPLA Toxin Assay (Oxoid), einem reversen Latexagglutinationstest, wird die L_2-Komponente des Hämolysin-BL Komplexes nachgewiesen. Mit dem Tecra-BDE, einem Sandwich-EIA, kann die 45 kDa Komponente (NheA) des Nhe Komplexes nachgewiesen werden. Beide Tests zeigen allerdings eingeschränkte Spezifität und weisen neben den Toxinkomponenten auch weitere Exoproteine von *B. cereus* nach (LUND & GRANUM, 1993; BEECHER & WONG, 1994b). Eine wesentliche Verbesserung der Spezifität immunchemischer Verfahren gelang DIETRICH et al. (1999) durch die Entwicklung monoklonaler Antikörper gegen alle drei Komponenten des Hämolysin-BL.

3 Publikationen

3.1 “Production and characterization of antibodies against each of the three subunits of the *Bacillus cereus* non-hemolytic enterotoxin complex“

Richard Dietrich, **Maximilian Moravek**, Christine Bürk, Per Einar Granum and Erwin Märtlbauer

APPLIED AND ENVIRONMENTAL MICROBIOLOGY 71, 8214–8220

ABSTRACT

The non-hemolytic enterotoxin (Nhe) is one of the two three-component enterotoxins which are responsible for diarrheal food-poisoning syndrome caused by *Bacillus cereus*. To facilitate the detection of this toxin, consisting of the subunits NheA, NheB and NheC, a complete set of high-affinity antibodies against each of the three components was established and characterized. A rabbit antiserum specific for the C terminal part (15 amino acids) of NheC was produced using a respective synthetic peptide coupled to a protein carrier for immunization. Using purified *B. cereus* exoprotein preparations as immunogens one monoclonal antibody against NheA and several antibodies against NheB were obtained. No cross-reactivity with other proteins produced by different strains of *B. cereus* was observed. Antibodies against the NheB component were able to neutralize the cytotoxic activity (up to 98 %) of Nhe. Based on indirect enzyme immunoassays the antibodies developed in this study were successfully used in the characterization of the enterotoxic activity of several *B. cereus* strains. For the first time it could be shown that strains carrying the *nhe* genes usually express the complete set of the three components including NheC. However, the amount of toxin produced varies considerably between the different strains.

INTRODUCTION

B. cereus is known to cause two different types of food poisoning (for reviews see references 9, 15, 26), which are characterized by either emesis or diarrhea. At present two different protein complexes, each consisting of three exoproteins, as well as a single protein (cytotoxin K; 19) are discussed as causative agents (9, 13). The enterotoxin described by Beecher and Wong (3, 5), consisting of the components B, L_1 and L_2, showed hemolytic activity and was therefore named hemolysin BL (HBL). HBL has been characterized intensively in view of the biological activity (5) as well as genetically (14, 25). The non-hemolytic enterotoxin (Nhe) described by Lund and Granum (17), contains the protein components NheA (41.0 kDa), NheB (39.8 kDa) and NheC (36.5 kDa). The genes encoding for the components of Nhe have been cloned and characterized, and it has been shown that they are transcribed as one operon (10, 16).

Specific monoclonal antibodies for immunochemical studies on the protein level are only available for HBL (6). Due to this limitation, the detection of the *B. cereus* enterotoxins is still not satisfactory, and a range of *in vivo* and *in vitro* tests are used to estimate the toxicity of *B. cereus* isolates, e.g., the mouse lethality test, the rabbit ileal loop test, the vascular permeability reaction, and cell culture assays (1, 3, 5, 27). These assays do, however, not allow differentiation between the specific activities of the individual toxins. On the other hand several studies published during the last years showed, that nearly all strains of *B. cereus* harbored *nhe*, whereas *hbl* genes were detected only in about 50 % of the tested isolates (7, 11, 12, 21, 22, 28). Although there is some evidence that both complexes are co-expressed frequently (1, 7, 11, 24), quantitative data on the amount of toxin produced by *B. cereus* isolates carrying both *hbl* and *nhe* genes are not available. To provide tools for such detailed studies and to improve the detection of Nhe we describe here the production of specific antibodies against NheA, NheB, and NheC.

MATERIAL AND METHODS

***B. cereus* strains, culture medium and culture conditions.** Enterotoxic strains of *B. cereus* used in this study were B-4ac (DSM 4384; DSM, Germany); NVH 0075/95 and 391/98 (*nhe* negative) (17, 19); a *B. subtilis* strain expressing recombinant NheB, and *E. coli* strains producing either NheA or NheC (16) were also used. All other *B. cereus* strains (prefix MHI) were isolated

from infant food or dried milk products (2). For cytotoxicity testing and indirect enzyme immunoassay (EIA) analyses cells were grown in CGY medium (3) supplemented with 1 % glucose for 6 h at 32 °C with shaking. To inhibit proteolytic cleavage of the toxins by metalloproteases EDTA (1 mM) was added at the time of harvesting. Cell-free supernatants obtained by centrifugation (10,000 x g at 4° C for 20 min) and filtration through 0.2 µm Millipore filters were used for purification of proteins and as coating antigens in the EIA. For production of recombinant NheB and NheC *B. subtilis* and *E. coli* strains were grown in BHI supplemented with 50 µg kanamycin per ml for 24 hours.

Production of monoclonal antibodies (MAb). Purified NheA was prepared according to Lund and Granum (17, 18) and used as immunogen. Additionally, an exoprotein preparation of strain B-4ac was produced as described by Dietrich et al. (6) and the fraction B2 obtained by gel filtration on Sephadex G-75sf was used for the immunization of mice.. Two groups of 12-week-old female mice (three BALB/c strain and a hybrid strain of BALB/c × [NZW × NZB] per group) were immunized by intraperitoneal injection of 30 µg of the respective protein preparation, dissolved in 0.01 M Tris-HCl buffer (pH 8.6), and emulsified in Freund's complete adjuvant (1:3). At day 84, the animals received a booster injection of the same amount of immunogen in incomplete Freund's adjuvant. Finally, at day 142, 3 days before cell-fusion, the animals got a final booster injection of 45 µg of antigen dissolved in Tris-HCl buffer. Cell-fusion experiments, establishment of hybridomas and antibody purification were done according to previously published protocols (6).

Production of NheC antisera. Due to the low immunogenicity of the recombinant protein, several attempts to produce monoclonal antibodies against NheC failed. Therefore, two rabbits were immunized with a synthetic peptide derived from the C terminal part of the NheC sequence [VKDYTEKLHEGVAK] and coupled to keyhole limpet hemocyanin (KLH), as protein carrier. The rabbits were immunized subcutaneously with 300 µg of the peptide protein conjugate emulsified in complete Freund's adjuvant and received booster injections at week 18 with 250 µg of the peptide protein conjugate in incomplete Freund's adjuvant. Blood samples were taken in 2-3 week intervals and the serum was obtained by centrifugation of the clotted blood.

Antibody screening by indirect enzyme immunoassays (EIA). To screen for antibody secreting hybridomas an indirect EIA system (6) was established by using recombinant NheA and NheB preparations as coating antigens. A similar approach was applied to determine the relative antibody titers of the rabbit antisera against the NheC-peptide. For this purpose plates were coated with a peptide-ovalbumin conjugate at a concentration of 0.25 µg/ml.

Cytotoxicity and neutralization assay. Cytotoxic activity of *B. cereus* culture supernatants was determined using Vero cells as previously described (6, 20). To check the neutralization capacity of the antibodies serial dilutions of *B. cereus* culture supernatants (0.1 ml) were placed into microtiter plates together with 10 µg of the purified MAb (1 mg/ml phosphate buffered saline, PBS). Cell suspensions (0.1 ml; 10^3 cells/well) were added immediately afterwards. For control, an identical preparation containing 10 µg of an unrelated MAb (Mab 1A6 against 3-acetyldeoxynivalenol, a *Fusarium* mycotoxin; unpublished results) was incubated in parallel on the same plate. After 24 h the mitochondrial activity of viable cells was determined by adding a tetrazolium salt (WST-1; Roche Diagnostics, Mannheim, Germany). The resulting dose-response curve was used to calculate the 50 % inhibitory value (expressed as the reciprocal dilution that resulted in 50 % loss of mitochondrial activity) by linear interpolation. Neutralization capacities of the antibodies tested were calculated by comparing the cytotoxicity titers seen in the samples with and without specific antibody.

SDS-PAGE. SDS-PAGE was carried out by using the PhastSystem (Amersham Biosciences, Freiburg, Germany) and precast minigels (PhastGel gradient 10 to 15). Separated proteins were stained with Coomassie brilliant blue.

Immunoblot. To further characterize the specificity of the monoclonal antibodies, culture supernatants containing recombinant Nhe components and exoprotein preparations from *B. cereus* were separated by SDS-PAGE and electrophoretically transferred to polyvinylidenfluoride membrane, Immobilon-PSQ (Millipore, Bedford, USA). Immunochemical staining was performed by blocking the membrane with Tris-buffered saline (50 mM of Tris-HCl, 150 mM of NaCl [pH 7.5]) containing sodium caseinate (10 g/liter) for 30 min and then incubating it with protein A-purified monoclonal antibodies (2 µg/ml) or rabbit antisera precipitated by ammonium sulfate. After a washing step, bound antibodies were detected with alkaline phosphatase-labeled

secondary antibodies (1:1,000) by using 4-nitroblue tetrazolium chloride-5-bromo-4-chloro-3-indolylphosphate (NBT-BCIP) as the chromogenic substrate according to the instructions of the manufacturer (Roche Diagnostics).

Immunoaffinity chromatography (IAC). Monoclonal antibody 1E11 (10 mg) was attached to 1g of CNBr-activated Sepharose 4b (Amersham Biosciences, Freiburg, Germany) according to the manufacturer's instructions. The purification procedure comprised the following steps: (a) storage buffer (PBS containing 0.1% sodium azide) was replaced with PBS; (b) sample (*B. cereus* supernatant diluted five times in PBS) was applied; (c) column was washed with PBS; (d) bound NheB was eluted with glycine/HCL buffer (pH 2.5); (e) column was washed with PBS and stored in storage buffer. During all steps, the flow rate was set to 1 ml/min.

Analyses of *B. cereus* culture supernatants. The HBL and Nhe titers of cell-free culture supernatants of *B. cereus* strains were determined using an indirect EIA as described recently (6) with slight modifications. Briefly, plates were coated with serial dilutions (in carbonate-bicarbonate buffer, 0.05 mol/liter, pH 9.6) of crude cell-free supernatants of the strains grown in CGY medium. After the blocking step with 3 % sodium caseinate/PBS for 45 minutes, 100 µl of purified monoclonal antibody were added to separate wells on the plates using a concentration of 2 µg ml^{-1} for 1 h. For the determination of NheC the rabbit antiserum was used in a dilution of 1:1,000. After a washing step, secondary antibodies (rabbit anti-mouse Ig, DakoCytomation, Hamburg, Germany, or goat anti-rabbit IgG, Sigma-Aldrich, Taufkirchen, Germany) labeled with horseradish peroxidase (1:3,000 in 1 % sodium caseinate/PBS) were added and incubated for 1 h at room temperature. Then the plate was washed again, and 100 µl/well of substrate/chromogen solution (1 mM 3,3',5,5'-tetramethylbenzidine and 3 mM H_2O_2 per liter potassium citrate buffer, pH 3.9) was added. After 20 min, the color development was stopped with 1 M H_2SO_4 (100 µl/well) and the absorbance was measured at 450 nm. Antigen titers were defined as the reciprocal of the highest dilution of crude supernatants that gave an absorbance value of 1.0 unit under these conditions.

Polymerase chain reaction. DNA from 100 µl aliquots of overnight cultures was extracted using the DNeasy Tissue Kit (Qiagen, Germany) according to the manufacturer's instructions. The strains were tested for the presence of all genes of the *nhe* operon (*nheA*, *nheB*, and *nheC*) and for

hblC of the *hbl*-operon. Primers and PCR conditions are summarized in Table 1. Primers for the detection of *nhe* genes were deduced from GenBank accession number Y19005 (10), and for *hblC* of GenBank accession number U63928 (25) by use of the program Primer3 (23). For the simultaneous detection of *nheB* and *nheC* a single primer pair was designed that spans the *nheB-nheC* intergenic region. Primers were produced in custom synthesis by MWG Biotech (Ebersberg, Germany). PCR (30 cycles) was performed in a total volume of 50 µl containing 25 pM of each primer, 200 µM of each desoxynucleoside triphosphate (peqlab, Erlangen, Germany), 1.5 mM $MgCl_2$ (*hblC* 2 mM), 1.5 U *Taq* polymerase (*hblC* 1.75 U; ABgene, Hamburg), 5 µl tenfold polymerase buffer, and 1 µl DNA preparation. The PCR products were visualized by ethidium bromide staining after electrophoresis on agarose gels (2 %).

TABLE 1. PCR primers and conditions used in this study

Primer pair	Nucleotide sequence	Target	Product size (bp)	PCR conditions	Reference
45c1	GAGGGGCAAACAGAAGTGAA	*nheA*	186	94 °C, 60 s	
45c2	TGCGAACTTTTGATGATTCG			52 °C, 60 s	(20)
				72 °C, 60 s	
nheBC1	ACATTGCGAAAGATAGCTGGA	*nheB/nheC*	300	94 °C, 60 s	
nheBC2	TGTTCTGCTGCAAAAGGATG			48 °C, 60 s	This study
				72 °C, 60 s	
L2aF	CGAAAATTAGGTGCGCAATC	*hblC*	411	94 °C, 60 s	
L2aR	TAATATGCCTTGCGCAGTTG			51 °C, 60 s	(20)
				72 °C, 60 s	

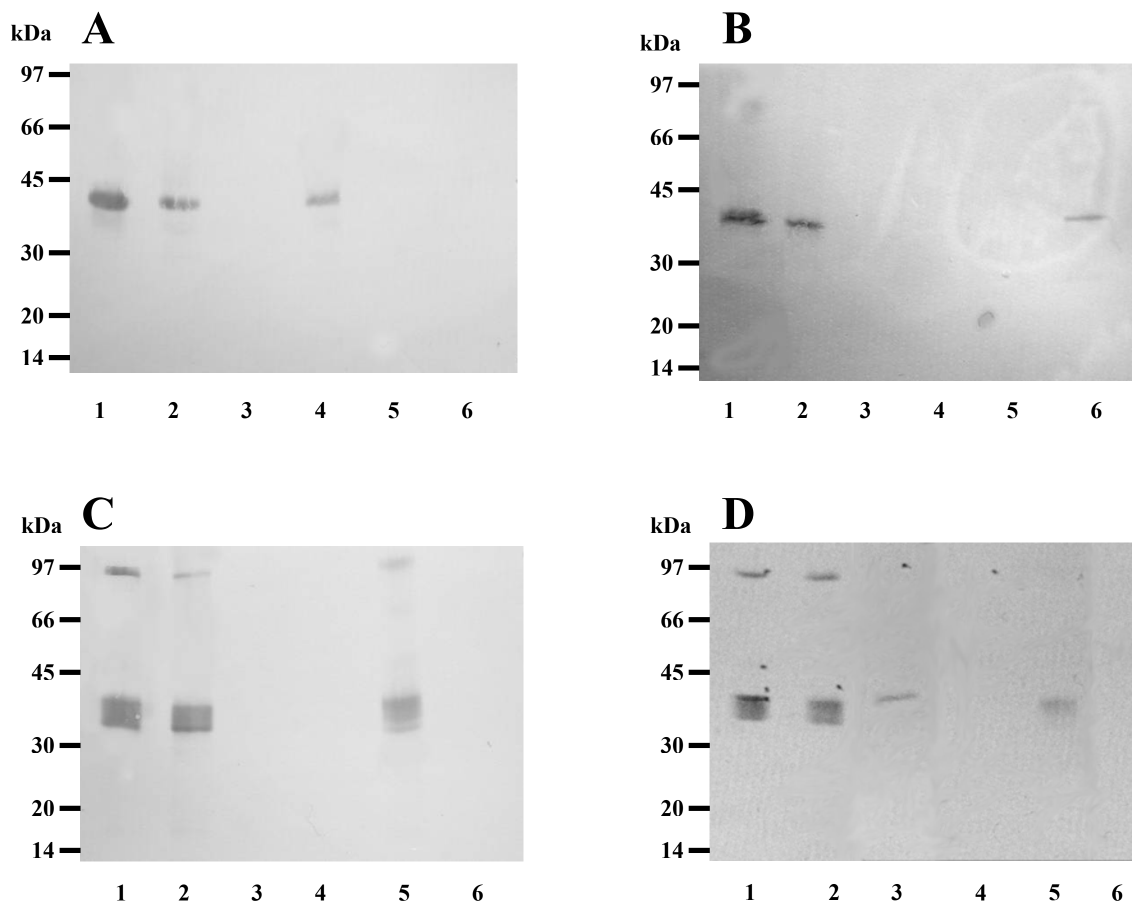
RESULTS

Antibody production. Culture supernatants of *B. cereus* strain B-4ac were purified by gel chromatography as described by Dietrich et al. (6) and one of the resulting exoprotein preparations, namely, fraction Sephadex G-75 B2, contained mainly proteins in the 35- to 42-kDa range. Using this fraction for the immunization of mice a broad range of monoclonal antibodies (MAb) were obtained and screened against the recombinant proteins NheA and NheB. A total of 25 hybridoma cell-lines secreting antibodies reactive with recombinant NheB were identified and these antibodies were further characterized by immunoblot. However, none of the antibodies obtained from this immunization showed reactivity with NheA and therefore mice were

immunized with a purified preparation of this component. A total of eight hybridoma cell lines, secreting specific antibodies against NheA, were obtained from two different fusions of mouse splenocytes with myeloma cells. However most of these antibodies were of the IgM class and only one high-affinity IgG-antibody (MAb 1A8) was identified. For the production of antibodies against NheC a peptide-protein conjugate was used. This was highly immunogenic when the respective antisera were tested in an indirect EIA using a peptide-ovalbumin conjugate as coating antigen. Detectable antibody titers were at > 1: 50,000 under the conditions described above.

Immunoblot analyses. Immunoblot analyses were done to further characterize the specificity of the produced antibodies. By using the supernatants of strains producing the recombinant proteins NheA, NheB or NheC, the results of the screening EIAs were confirmed. Monoclonal antibody 1A8 reacted with NheA, several antibodies with NheB and the polyclonal antisera with NheC (Fig.1, lanes 4-6). However, one of the rabbit antisera also showed a considerable reactivity with the recombinant NheB (results not shown) and was therefore not used for the further experiments. In parallel the antibodies were tested against crude culture supernatants of three *B. cereus* reference strains representing the different diarrheal toxin profiles commonly found in this food poisoning organism. In detail, strain B-4ac produces both Nhe and HBL (7, 20), strain NVH 0075/95 only Nhe (17, 18), and strain 391/98 lacks both the *hbl* and *nhe* genes (19). Analyzing culture supernatants of these strains by immunoblot the specificity of the monoclonal antibody 1A8 against NheA and the antiserum against NheC was demonstrated. Single bands were obtained for both strains producing Nhe (Fig. 1A and B, lane 1, 2) whereas strain 391/98 which has been found to be *nhe* negative by PCR (19) gave negative results (Fig. 1A and B, lane 3).

FIGURE 1. Immunoblot reactivity of the monoclonal antibodies (A) 1A8, anti-NheA; (C) 1E11, anti-NheB; (D) 3F1, anti-NheB, and (B) anti-NheC rabbit antiserum with exoprotein preparations from (lane 1) *B. cereus* strain B-4ac, (lane 2) *B. cereus* strain NVH 0075/95, (lane 3) *B. cereus* strain NVH 391/98, and recombinant (lane 4) NheA, (lane 5) NheB, and (lane 6) NheC.



However, testing the antibodies raised against NheB revealed, that most of the anti-NheB antibodies (20 out of 25) showed an additional reactivity with an unidentified exoprotein expressed by the Nhe-negative control strain. This reactivity is exemplified in Fig. 1D by the antibody 3F1. The remaining five antibodies (1E11, 1A5, 2C3, 2B11, 3G5) lacking this cross-reactivity showed a uniform reactivity pattern with crude culture supernatants of strains NVH 0075/95 and B-4ac which was characterized by a strong reactivity zone between approximately 33 kDa and 38 kDa and an additional band at approximately 97 kDa (Fig. 1C, lane 1, 2). Characteristics of the monoclonal antibodies are summarized in Table 2. To further verify the specificity of these antibodies culture supernatants of strain NVH 0075/95 were purified by an

immunoaffinity approach using the antibody 1E11 exhibiting the highest apparent affinity for NheB in the indirect EIA. The fractions obtained after the procedure described above were separated by SDS-PAGE (Fig. 2) and the N-terminal amino acid sequence of the protein band obtained for the eluate was determined by Edman degradation (TOPLAB Martinsried, Germany). The sequence found was VAKAYNDYEEYSL, a nicked form of NheB (17, 18).

Neutralizing properties of the antibodies. Besides the antiserum against NheC, the purified monoclonal antibodies against NheA and NheB were tested for neutralizing properties by using the Vero cell culture assay (Table 2). For this purpose 10 µg of each monoclonal antibody as well as the polyclonal antibodies against NheC were added to a serially diluted supernatant of strain NVH 0075/95 (producing only Nhe) and assayed in the cytotoxicity test. While the addition of the antibody 1A8 against NheA or of the NheC-antiserum showed only a minor effect on the apparent cytotoxic activity of the supernatants tested, all of the antibodies against NheB significantly reduced the cytotoxic effect of Nhe (Table 2). For supernatants of strain B-4ac containing both HBL and Nhe the residual cytotoxicity amounted to approximately 40 % of the untreated control (Table 3). By using previously described (6) monoclonal antibodies against the HBL components it could be verified that this residual cytotoxicity could be ascribed to HBL (details not shown).

TABLE 2. Characteristics of the monoclonal antibodies against Nhe of *B. cereus*

Monoclonal antibody	Subtype	Specificity	Neutralization of cytotoxic activity[a]
1A8	IgG1	NheA	15 %
1E11	IgG1	NheB	98 %
1A5	IgG1	NheB	87 %
2C3	IgG1	NheB	98 %
2B11	IgG2b	NheB	87 %
3G5	IgG2a	NheB	92 %

[a] Tested with culture supernatants of a strain producing only Nhe and not HBL (strain NVH 0075/95)

Characteristics of *B. cereus* strains. A total of 50 different strains of *B. cereus* including the reference strains were screened by PCR for *hblC, nheA* and *nheB/C*. It turned out that 49 of these strains harbored the *nhe* genes, 28 were positive for *hblC*, and strain NVH 391/98 was negative for both. To test the applicability of the antibodies for the characterization of cytotoxic activity of *B. cereus* strains and to avoid the influence of HBL on the cytotoxicity results, only the 20 food-

borne isolates which were positive for *nhe* and negative for *hbl*C were used for further analyses. Cell-free culture supernatants of these strains were tested in parallel for cytotoxicity and reactivity in the indirect EIA (Table 3). It could be demonstrated that all of these *nhe* positive strains produced the complete enterotoxin complex; all three Nhe components could be detected in the respective assays. Antigen titers, defined as the reciprocal dilution giving an absorbance value of 1.0 in the EIA, ranged from 180 to 6,000 for NheA and NheB. In contrast, measurable NheC titers were comparatively low, ranging from 5 to 180. The corresponding cytotoxicity values were at 135 to 2,500. Neutralization assays confirmed that most of the cytotoxicity measurable in the culture supernatants was due to the activity of Nhe (Table 3).

TABLE 3. Cytotoxic activity and reactivity in the indirect EIA and PCR of different strains of *B. cereus*

[a] Positive (+) and negative (–) results in the indirect EIA and PCR

[b] Indirect EIA were based on the antibody 1A8 against NheA, 1E11 against NheB, and the polyclonal antiserum against NheC

	Strains	indirect EIA[a,b]			PCR[a]			Cytotoxic activity[c]	
		1A8[d]	1E11[d]	NheC serum	*nhe A*	*nhe B/ nhe C*	*hbl C*		
Reference strains	*DSM 4384*	3	3	1	+	+	+	666	(60)
	NVH 0075/95	4	4	1	+	+	–	1,250	(>95)
	NVH 391/98	–	–	–	–	–	–	335	(<5)
Food-related strains	*MHI 1*	3	4	1	+	+	–	1,250	(93)
	MHI 13	3	3	1	+	+	–	862	(89)
	MHI 24	1	1	1	+	+	–	270	(>95)
	MHI 48	3	3	1	+	+	–	1,000	(>95)
	MHI 52	4	4	1	+	+	–	1,666	(94)
	MHI 61	1	1	1	+	+	–	135	(>95)
	MHI 64	2	2	1	+	+	–	357	(>95)
	MHI 69	2	2	1	+	+	–	526	(>95)
	MHI 97	4	4	1	+	+	–	769	(>95)
	MHI 109	3	3	1	+	+	–	555	(>95)
	MHI 124	3	2	1	+	+	–	322	(>95)
	MHI 126	2	2	1	+	+	–	277	(>95)
	MHI 144	2	2	1	+	+	–	400	(>95)
	MHI 183	2	2	1	+	+	–	152	(>95)
	MHI 195	2	1	1	+	+	–	217	(>95)
	MHI 1476	2	4	1	+	+	–	1,316	(>95)
	MHI 1493	3	4	1	+	+	–	2,500	(87)
	MHI 1496	3	4	1	+	+	–	1,818	(>95)
	MHI 1563	4	4	1	+	+	–	1,389	(>95)
	MHI 1662	2	3	1	+	+	–	690	(82)

[c] Values represent the reciprocal values of the titers obtained from the cytotoxicity test. Percentage of neutralization of the cytotoxicity after addition of antibody 1E11 is given in parentheses.

[d] Values represent relative reactivity of the culture supernatants tested: 1, antigen titer >10 – 500; 2, antigen titer 500 – 1,500; 3, antigen titer 1,500 – 2,500; 4, antigen titer > 2,500

DISCUSSION

During the last years it has become clear that, besides HBL, another enterotoxin complex, namely, Nhe, represents a major pathogenicity factor involved in the etiology of *B. cereus* diarrheal syndrome (17). Though multiple studies showed that nearly all *B. cereus* isolates possess the *nhe* genes (7, 11, 12, 22), only limited studies on the expression efficiency are available (8, 11). This is mainly caused by the lack of specific detection tools, such as well characterized antibodies.

The primary objective of the work was therefore the development of specific antibodies against each of the three Nhe components thus facilitating detection and quantification of this enterotoxin complex. Based on experiences made during the development of MAbs against HBL (6), initially impure preparations of exoproteins were used for immunization purposes. This approach worked well for the NheB but failed for the other two components insofar as no specific antibodies for these components were detectable in the respective antisera. Since the recombinant proteins were not available at this time, alternative immunogens were produced as described in the Materials and Methods section. As none of the used immunogens contained the complete tripartite Nhe, all of the applied preparations were well tolerated by the animals, adverse reactions were not observed.

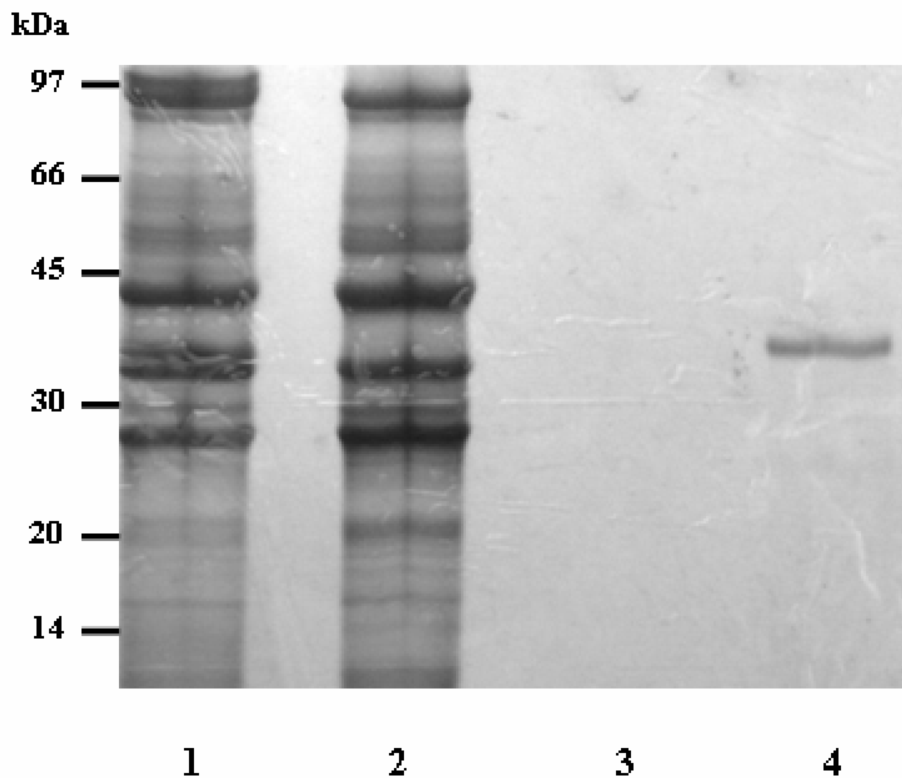
Owing to the formation of weakly immunogenic self-aggregates of the purified NheA only one MAb of the IgG subtype (1A8) was obtained against this component. This MAb, however, exhibited an extraordinary high affinity for the toxin enabling positive EIA results with culture supernatants of the reference strain diluted up to 1:10,000. Immunoblot analyses proved the desired specificity, with no cross-reactivity observed either within the Nhe complex or with other exoproteins produced by *B. cereus*, particularly not with the L2 component of HBL sharing sequence similarities with NheA (9, 10). For the detection of NheA a commercial test kit, *Bacillus* diarrheal enterotoxin visual immunoassay (BDE; Tecra) is available and frequently used

for the analysis of enterotoxin production of *Bacillus* species (7, 11, 24). However, the specificity of the employed polyclonal antibodies is unclear. Using immunoblot analyses Beecher & Wong (4) showed that besides NheA a broad range of other exoproteins with apparent molecular weights ranging from 41 to 114 kDa is recognized by the antibody-enzyme conjugate supplied with the EIA kit. Therefore, published results obtained with this test kit have to be interpreted carefully.

After a first screening a broad panel of MAbs reactive with recombinant NheB was obtained. However, detailed immunoblot analyses (Fig. 1D) revealed that most of these antibodies additionally reacted with an unidentified exoprotein expressed by the *B. cereus* strain 391/98 which lacks the *nhe* gene (19). In this context it is interesting to note that the previously described MAb 1C2 which detects the L1 component of HBL and exhibits cross-reactivity with NheB (6) showed exactly the same reactivity pattern. Therefore we assumed a common epitope exists in L1, NheB and the unknown protein. On the other hand, none of the MAbs against NheB showed reactivity towards the recombinant NheC as has been expected due to the substantial sequence homologies of these two proteins (10). To enable the specific detection of NheB, consequently further work concentrated on the remaining five MAbs which were characterized by a broad reactivity zone in the range from 36 to 39 kDa and an additional band at the top of the blot (Fig. 1C). A NheB aggregate with an apparent molecular weight of 105 kDa was also seen in a recent study (8) in which the extracellular proteome of *B. cereus* was first separated by 2D electrophoresis and then analyzed by MALDI-TOF mass spectrometry. It was speculated that this spot could be due to the formation of a stable complex between NheB and another protein (8). In our study this top protein was also found in the recombinant NheB preparations suggesting that only NheB contributes to the formation of this complex. So far, in all our experiments with Nhe positive *B. cereus* isolates this complex was commonly observed in the crude culture supernatants. However, despite the distinct reactivity of the MAb 1E11 with this complex, neither by SDS-PAGE (Fig. 2) nor by an immunoblot (data not shown) it was possible to detect the corresponding band after IAC. A possible explanation for this unexpected result could be that the complex is unstable under the acidic conditions used for the elution process.

After purifying culture supernatants from *B. cereus* strain NVH 0075/95 by IAC, a single band with an apparent M_r of 36,000 could be seen in the SDS-PAGE (Fig. 2) after staining the gel with Coomassie blue.

FIGURE 2. SDS-PAGE documentation of the NheB purification by immunoaffinity chromatography (IAC) using monoclonal antibody 1E11: (lane 1) crude culture supernatant of the *B. cereus* strain NVH 0075/95, (lane 2) flow through of the IAC column, (lane 3) rinse fraction of the IAC column, (lane 4) eluted NheB.



Use of the more sensitive immunoblot technique revealed that the preparation also contained some minor bands (results not shown). Subsequent analysis of the N-terminal amino acid sequence showed that the dominant protein represents a truncated form of the NheB enterotoxin proving the specificity of the MAb 1E11. A similar degradation product has been obtained by Lund & Granum (17, 18) after purifying NheB by chromatographic techniques. Obviously, the first 12 amino acids of the NheB protein are commonly degraded by simultaneously expressed proteases to a variable degree. This finding could also explain the observed broad reactivity zone of the MAbs (Fig. 1) which was probably caused by a mixture of differently truncated NheB derivates. Overall, the IAC purification yielded an average amount of approx. 300 µg per run

corresponding to a NheB concentration of at least 6 µg/ml of the crude supernatant, indicating that NheB belongs to the highly expressed exoproteins and this finding is consistent with data reported by Gohar et al (8). Taking into account this level of productivity and the antigen titers measured by indirect EIA it can be concluded that the detection limit of this assay is below 1 ng NheB/ml, a further indication for the high affinity of the produced MAbs.

All five MAbs against NheB possess distinct neutralizing properties. For instance, after the addition of MAb 1E11 negligible cytotoxic activity was observed for culture supernatants of strains producing only Nhe and not HBL, cytotoxicity titers decreased from > 1:1,000 to < 1:20. NheB represents the binding unit of the enterotoxin complex as shown recently by Lindbäck et al. (16). Consequently, the question arises if the neutralizing effect of the MAbs can be ascribed to an interaction with the receptor binding epitope of the toxin or inhibition of binding of the other components to NheB thus preventing the formation of an active toxin complex. Preliminary results (unpublished data) suggest that the latter case applies, but these findings need to be clarified in a more detailed study.

Recent findings (16) also proved the relevance of the NheC component for the formation of an active toxin complex despite the fact that several approaches failed to detect this component in culture supernatants of *B. cereus* (8, 10, 18). To produce polyclonal antibodies against this component, a peptide sequence corresponding to the C-terminal end of NheC was chosen for the immunization since the C-terminus of peptides coupled to a carrier protein represents usually an immunodominant epitope. As this peptide shows at the N-terminus a certain sequence homology with NheB, i.e. xKDYxEK, it was not surprising that only one of the two rabbits immunized produced antibodies specific for NheC (Fig. 1). By using the specific antiserum NheC could be detected for the first time in culture supernatants of *B. cereus* both under native (EIA) and denaturing (immunoblot) conditions. The EIA results also suggest that in untreated *B. cereus* supernatants the C-terminus of NheC represents a freely accessible epitope and is not assembled within the tertiary structure of the protein. However, during these experiments it also became obvious that, compared with the EIA systems for the detection of NheA and NheB, relatively low NheC antigen titers could be found in the cell-free supernatants. For instance, NheC titers in a culture supernatant of strain NVH 0075/95 were at 1:80, the corresponding NheA and NheB titers, however, were at approx. 1:2,500. Whether this finding is due to a considerably lower

affinity of the polyclonal antibodies against NheC or due to a low expression level of this component needs to be clarified. In a recent publication based on recombinant proteins Lindbäck et al. (16) report that maximum cytotoxic activity of the Nhe enterotoxin complex was obtained when the molar ratio for A:B:C was at 10:10:1 and it was postulated that, compared to NheA and B, *B. cereus* strains express only small amounts of NheC, possibly due to a predicted stem-loop structure between the *nheB* and *nheC* gene (10).

To demonstrate the applicability of the developed immunochemical methods and to get a first indication on the Nhe expression of *B. cereus*, 50 isolates were enriched under optimized conditions and the resulting supernatants were analyzed by EIA (presence/absence tests). Besides this the *nhe* and *hblC* genes were detected by PCR assays. By applying EIA and PCR methods consistent results were obtained. All isolates carrying the *hblC* gene expressed the L_2 component of HBL (details not shown) and all isolates carrying the *nhe* genes expressed the complete set of the three components. Thus, the PCR primers used in our study correctly identified all *nhe* positive isolates despite commonly observed *nhe* gene polymorphisms (11). These results indicate that the used primer sequences are located in rather conserved sections of the genes. The results obtained also suggest that the monoclonal antibodies against NheA (1A8) and NheB (1E11) react with a stable epitope, which is not affected by amino acid substitutions.

To avoid biased cytotoxicity results due to the simultaneous production of HBL, cell culture analyses concentrated on the 20 strains producing only Nhe (PCR negative for *hblC*). Comparison of cytotoxicity and Nhe antigen titers of the tested culture supernatants revealed a consistency between these two parameters. Simultaneously performed neutralization assays corroborated these findings. The addition of MAb 1E11 possessing neutralizing properties decreased the observable cytotoxic activity of most of the tested culture supernatants by more than 95 % (Table 3). Thus, it could be demonstrated that the cytotoxicity results are not biased by other virulence factors such as proteases, hemolysins and phospholipases (8), possibly produced by *B. cereus* under the enrichment conditions used for the preparation of the cell-free culture supernatants. With regard to the Nhe productivity marked differences between the particular isolates could be observed, for example NheB antigen titers ranged from 1:180 to 1:6,000. Similar results have been described for the productivity of HBL (6, 11, 21, 28).

In conclusion, this study describes the production and characterization of the first complete set of high-affinity antibodies against all three components of the Nhe enterotoxin produced by *B. cereus*. The antibodies enable the specific and sensitive detection of these compounds in culture supernatants and may be used together with the previously produced MAbs against the HBL enterotoxin for the quantitative evaluation of the toxin expression of *B. cereus* strains. Furthermore, particularly the neutralizing properties of the MAbs against NheB enable detailed studies on the mode of action of this toxin and provide a basis for further differentiated studies on *B. cereus* strains expressing simultaneously the HBL and the Nhe enterotoxin complex.

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3.2 “Determination of the toxic potential of *Bacillus cereus* isolates by quantitative enterotoxin analyses”

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SUMMARY

Hemolysin BL (HBL) and non-haemolytic enterotoxin (Nhe), each consisting of three components, represent the major enterotoxins produced by *Bacillus cereus*. To evaluate the expression of these toxins, a set of 100 *B. cereus* strains was examined. The strains, representing a broad spectrum of biodiversity, included clinical and food isolates connected to food-borne outbreaks, as well as isolates from diverse foodstuffs and the environment. Molecular biological characterization showed that 42 % of the strains harboured the genes for HBL and 99 % for Nhe. The production of all Nhe and HBL components were analyzed by using specific antibodies and, in culture supernatants, detectable levels of HBL and Nhe were found for 100 % of *hbl* positive and 96 % of *nhe* positive strains. The concentrations of the HBL-L_2 and NheB component ranged from 0.02 to 5.6 µg ml^{-1} and from 0.03 to 14.2 µg ml^{-1}, respectively. Comparison of the amount of NheB produced by food poisoning and food/environmental strains revealed that the median value for all food poisoning strains was significantly higher than for the food/environmental isolates. Further on, a clear correlation was found between the cytotoxic activities of *B. cereus* supernatants on Vero cells and the level of NheB. The data presented in this study provide evidence that the specific and quantitative determination of the enterotoxins is necessary to evaluate the toxic potential of *B. cereus*. In particular, the level of Nhe seems to explain most of the cytotoxic activity of *B. cereus* isolates and may indicate a highly diarrheic potential.

INTRODUCTION

B. cereus is able to produce different enterotoxins, which cause two types of food poisoning named after their main symptoms of emesis and diarrhoea [for reviews see 1,2]. The diarrhoeal type has been linked to a single protein [3] as well as two enterotoxin complexes as causative agents [4,5]. One of these, a non-haemolytic enterotoxin (Nhe) consisting of three components (NheA, NheB, NheC) was described by Lund and Granum [5]. The second complex (hemolysin BL, HBL) [4] contains the protein components B (37.5 kDa), L_1 (38.2 kDa), and L_2 (43.5 kDa). A number of studies has been published on the prevalence of the *nhe* and *hbl* genes [6-8] in *B. cereus* and other species of the *B. cereus* group [9-12]. Until now, immunochemical characterization of *B. cereus* strains regarding the HBL and Nhe complexes has been limited by the non-availability of specific antibodies. Most studies so far [6,7] have used commercial test kits which allow only qualitative estimation of the L_2 component of HBL [13,14] or NheA [14] and no detailed and quantitative characterization of the enterotoxic potential of *B. cereus* isolates. Production and characterization of monoclonal antibodies against HBL [16] and Nhe [17] enabled us to study the expression of the single components of both enterotoxin complexes. The results of this study provide a comprehensive set of molecular biological, immunochemical and cell cultural data on the enterotoxic profile of 100 *B. cereus* strains representing a broad spectrum of biodiversity. This is also the first report providing quantitative data on the amount of HBL-L_2 and NheB produced by *B. cereus in vitro*.

MATERIAL AND METHODS

***B. cereus* strains, culture medium and culture conditions.** The *B. cereus* strains used in this study were collected and characterized within the EU-research project "Preventing *Bacillus cereus* food borne poisoning in Europe" (QLK1-CT-2001-00854). The strain set includes clinical isolates and isolates from food remnants connected to food-borne outbreaks, as well as isolates from diverse food stuffs and the environment (Table 1). For cytotoxicity testing and EIA analyses, cells were grown under conditions optimized for toxin production [14, 15]. In detail strains were incubated in CGY medium supplemented with 1 % glucose for 6 h at 32 °C or overnight at 25 °C (psychrotolerant strains only; n = 4) with shaking. EDTA (1 mM) was added at the time of harvesting. Cell-free supernatants were obtained by centrifugation (10,000 x g at 4° C for 20 min) and filtration through 0.2 µm Millipore filters and were used for purification of proteins and as coating antigens in the indirect enzyme immunoassays (EIA).

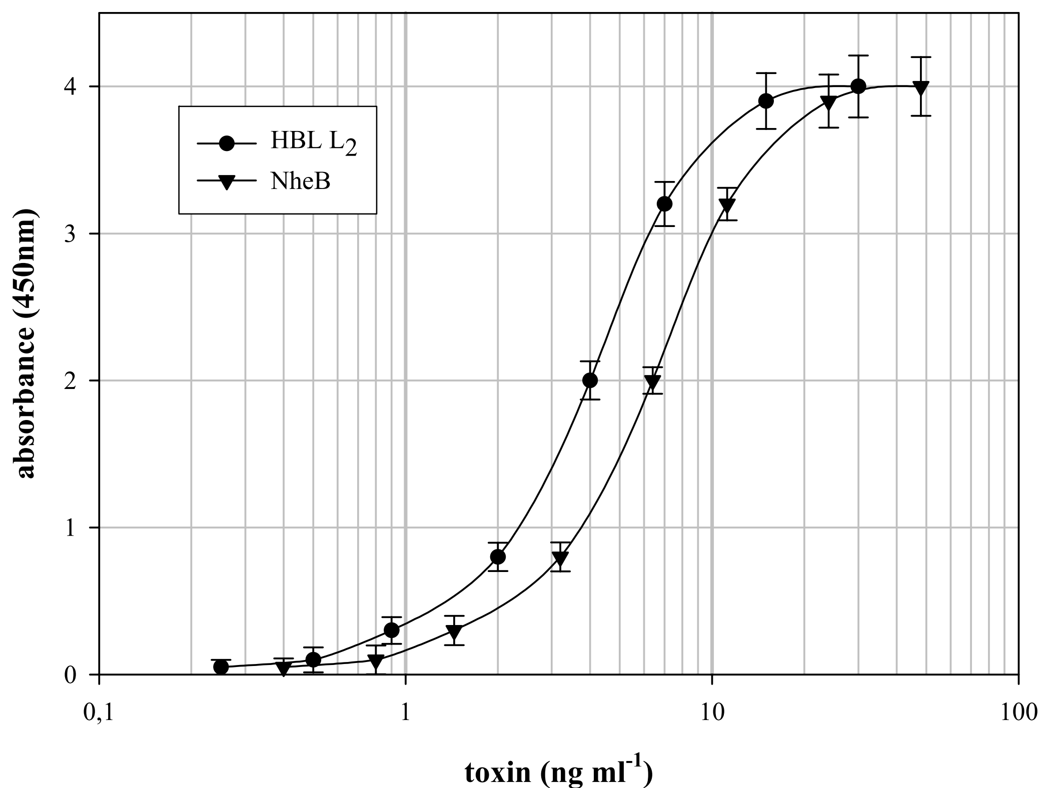
Toxins. Purified HBL-L_2 and NheB components which served as standards in the specific immunoassays were prepared by immunoaffinity chromatography as previously described [16,17]. Briefly, monoclonal antibodies were attached to CNBr-activated Sepharose 4b (Amersham Biosciences, Freiburg, Germany) according to the manufacturer's instructions. The purification procedure comprised the following steps: (i) storage buffer (phosphate-buffered saline, PBS, containing 0.1% sodium azide) was replaced with PBS; (ii) sample (*B. cereus* supernatant diluted five times in PBS) was applied; (iii) column was washed with PBS; (iv) bound NheB was eluted with glycine/HCL buffer (pH 2.5); (v) column was washed with PBS and stored in storage buffer. During all steps, the flow rate was set to 1 ml/min.

Indirect enzyme immunoassays (EIA). Using specific antibodies the absence or presence of HBL L_1, HBL B, NheA and NheC in cell-free culture supernatants of the *B. cereus* isolates was determined by indirect EIAs as described recently [16,17].

Sandwich enzyme immunoassay. To obtain quantitative data on the amount of toxin produced by *B. cereus* isolates, novel Sandwich enzyme immunoassays were established within this study based on previously described monoclonal antibodies. In detail, microtitre plates were coated with 10 µg ml^{-1} monoclonal antibody 1A12 (directed against HBL-L_2) or 5 µg ml^{-1} monoclonal antibody 2B11 (reactive with NheB) overnight at room temperature. Free protein binding sites of the plates were blocked with PBS (pH 7.3) containing sodium caseinate (30 g l^{-1}) for 45 min. Then the plates were washed with Tween 20 solution (0.25 ml l^{-1} of 0.15 mol l^{-1} of sodium

chloride solution) and made semidry. Subsequently, serial dilutions (in PBS containing 0.5% of Tween 20) of cell-free, crude culture supernatants of *B. cereus* strains were added to each well (100 µl/well) and incubated for 1 h. After a washing step, monoclonal antibody 8B12 (L_2) or monoclonal antibody 1E11 (NheB) labelled with horseradish peroxidase (1:1,000 or 1: 4,000 in PBS containing sodium caseinate [10 g l^{-1}]) was added and again incubated for 1 h at room temperature. Then the plate was washed, and 100 µl of substrate–chromogen solution (1 mmol of 3,3´,5,5´-tetramethylbenzidine, 3 mmol of H_2O_2 per litre of potassium citrate buffer [pH 3.9]) per well was added. After 20 min, the colour development was stopped with 1 mol of H_2SO_4 (100 µl/well), and the absorbance was measured at 450 nm. For quantification calibration curves using purified toxin components (L_2 and NheB) were prepared and toxin concentration was calculated by linear interpolation (Figure1).

Figure 1: Calibration curves for HBL-L_2 and NheB in the sandwich EIA. Error bars indicate the standard error of four determinations.



Cytotoxicity tests. Cytotoxicity of the *B. cereus* culture supernatants was determined using Vero cells as described recently [16]. Briefly, serial dilutions of the supernatants were placed into microtiter plates (0.1 ml per well) and Vero cell suspensions (0.1 ml; 10^4 cells/well) were added immediately afterwards. The growth medium and diluent consisted of Eagle minimum essential medium (Biochrom KG, Berlin, Germany) with Earle salts supplemented with 1 % foetal calf serum and 2 mmol l^{-1} glutamine. The test was incubated for 24 h at 37 °C in a 5 % CO_2 atmosphere and then the mitochondrial activity of viable cells was determined at 450 nm by using the tetrazolium salt WST-1. The resulting dose-response curve was used to calculate the 50 % inhibitory concentration (expressed as the reciprocal dilution that resulted in 50 % loss of mitochondrial activity) by linear interpolation.

PCR.

All strains were tested for the presence of the *hbl* and *nhe* enterotoxin genes by PCR experiments according to previously described methods [7, 18]. The absence of the tested gene in the PCR negative strains was confirmed by Southern blotting [7].

RESULTS AND DISCUSSION

As several recent studies [6,7,19] indicate that nearly all strains of *B. cereus* possess the genes of at least one of the diarrhoeal enterotoxins, the present study was initiated to provide data about the expression of the HBL and Nhe components under *in vitro* conditions. To cover the biodiversity of *B. cereus*, the strains used in this study were selected on differences in strain properties, geographical origin, and source of isolation. To address the pathogenicity of this species about half of the strains were selected from documented food poisoning outbreaks (Table 1).

First, the strains were analysed by PCR for the presence of the *hbl* and *nhe* genes. Since a high degree of sequence polymorphism was found for *hbl* in food associated strains of *B. cereus* [7], two different sets of primers were used for this purpose. In the first assay, performed according to Guinebretière *et al.* [7], 53 isolates out of the 100 strains tested reacted positive, 42 could be confirmed with a primer pair specific for *hblC* [18]. A similar percentage of PCR positive strains has been reported by Prüß *et al.* [9], whereas in other studies one or more *hbl* genes were detected in 59 to 73 % of the strains analyzed [6,19]. By using specific immunoassays, expression of the

HBL components could be verified for 38 out of the 42 *hbl*-positive strains enriched at 32 °C in CGY broth. After modifying the enrichment conditions, i.e. incubation at 25 °C overnight, HBL components were also detected in the supernatants of the remaining four strains. This unusual productivity behaviour was ascribed to the psychrotrophic growth characteristics of these strains (details not shown). Furthermore, 40 (95 %) out of the 42 *hbl*-positive strains produced complete HBL consisting of the three components; the two remaining *hbl* positive strains were negative for the B component but produced traces of the L_2 and L_1 component. By using the newly developed sandwich EIA (Fig. 1), enabling the quantification of the L_2 component, 0.02 to 5.6 µg ml^{-1} (Table 1) of this protein could be found in the culture supernatants of the analyzed strains. Since the genes encoding for the components of HBL are transcribed from the same operon in one mRNA [20] and maximum biological activity is obtained at an approximately equi-molar ratio of the single compounds [4,16] it may be assumed that HBL B and L_1 are produced to a similar level as the L_2 component. All 42 *hbl*-positive strains carried the *nhe* operon and produced detectable levels of Nhe (details see below).

For the detection of the *nhe* operon, gene primers, amplifying successively *nheA, B* and *C,* were used and like in other recent PCR studies [6-8] with the exception of strain 391/98 (producing cytotoxin K) all strains (99) were positive in this assay. However, by applying specific immunoassays it could be shown that only 93 strains were capable to produce NheA, NheB and NheC simultaneously. Mutations in the PlcR regulon described by Gohar *et al.* [21] and Slamti *et al.* [22] may explain that two strains produced only two components and five isolates were negative for all three compounds, including strain 391/98, which has an *nhe* operon quite different from other strains (Fagerlund and Granum, unpublished result). Overall, the levels of NheB found in the culture supernatants ranged from 0.03 to 14.2 µg ml^{-1} (Table 1), thus covering a broader and higher range of toxin concentrations than HBL L_2. Moreover, nearly all strains expressing both enterotoxin complexes simultaneously produced higher amounts of Nhe. As also shown in the study of Dietrich *et al.* [17] compared to NheA and B the EIA results for the NheC component were very low, which could be explained by a predicted stem-loop structure (in mRNA) between the *nheB* and *nheC* gene [23]. Also the recent report of Lindbäck *et al.* [24] showing maximum cytotoxic activity of the Nhe enterotoxin complex at a molar ratio of NheA, B and C of 10:10:1, would suggest that NheC is probably produced at low levels.

Table 1: Characteristics of *B. cereus* isolates collected in Belgium, Denmark, Finland, France, Germany, India, Japan, Norway, Sweden, Thailand, United Kingdom and USA from 1972 to 2002.

Origin	No. of strains	Clinical symptoms	PCR		Immunoassay								Cytotoxicity[f]
					HBL				Nhe				
			hbl[c]	*nhe*[c]	B pos.[c]	L_1 pos.[c]	L_2 pos.[c]	L_2 range ($\mu g\ ml^{-1}$)[e]	A pos.[c]	C pos.[c]	B pos.[c]	NheB range ($\mu g\ ml^{-1}$)[e]	
Meat	5[a]	-	1	5	1	1	1	0.03	5	5	5	3.08 - 11.05	129 - 752
	11[b]	diarrhoea, emesis	5	11	5	5	5	0.50 - 5.10	11	11	11	0.16 - 13.73	369 - 1,818
Milk	10	-	5	10	5	5	5	0.02 - 5.56	9	10	10	0.11 - 10.61	< 10 - 1,316
	3	diarrhoea	1	3	1	1	1	0.90	3	3	3	0.48 - 11.54	238 - 3,030
Vegetable	8	-	4	8	4	4	4	0.04 - 1.73	8	8	8	2.55 - 10.34	212 - 2,857
	8	diarrhoea	5	7	5	5	5	0.08 - 4.45	7	7	7	2.44 - 14.18	226 - 2,500
Baby food	7[d]	-	1	7	1	1	1	1.84	7	7	7	1.09 - 10.39	37 - 1,724
Pasta & rice	1	-	0	1	0	0	0	0	1	1	1	12.07	690
	9	diarrhoea, emesis	6	9	5	6	6	0.05 - 3.25	8	8	8	0.03 - 13.65	< 10 - 1,429
Environment	13[d]	-	7	13	6	7	7	0.03 - 1.96	13	13	12	0.63 - 10.87	< 10 - 1,786
Clinical	2	-	0	2	0	0	0	0	1	1	1	13.59	< 10 - 855
	11	diarrhoea, emesis	2	11	2	2	2	2.03 - 2.78	11	11	11	0.11 - 11.74	<10 - 1,695
Other[g]	6	-	4	6	4	4	4	0.11 - 1.99	5	5	5	5.45 - 10.96	42 - 1,124
	6	diarrhoea, emesis	1	6	1	1	1	1.05	5	5	5	0.03 - 9.71	< 10 - 1,587
Total	100		42	99	40	42	42	0.02 - 5.56	94	95	94	0.03 - 14.18	< 10 - 3,030

[a] Random sampled food/environment isolates.
[b] Isolates connected to food poisoning.
[c] No. of strains positive in the respective test.
[d] No isolate connected to food poisoning available.
[e] Range of toxin concentration, zero indicates negative EIA result.
[f] Reciprocal cytotoxicity titre.
[g] Including tobacco, manure, spices and starch.

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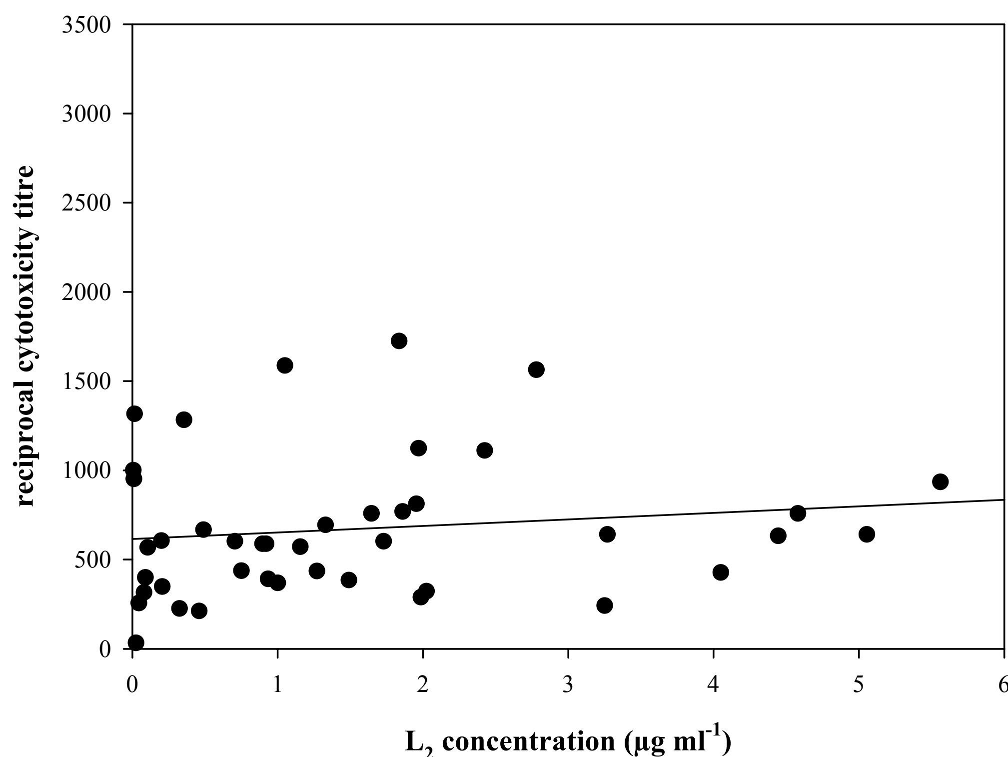
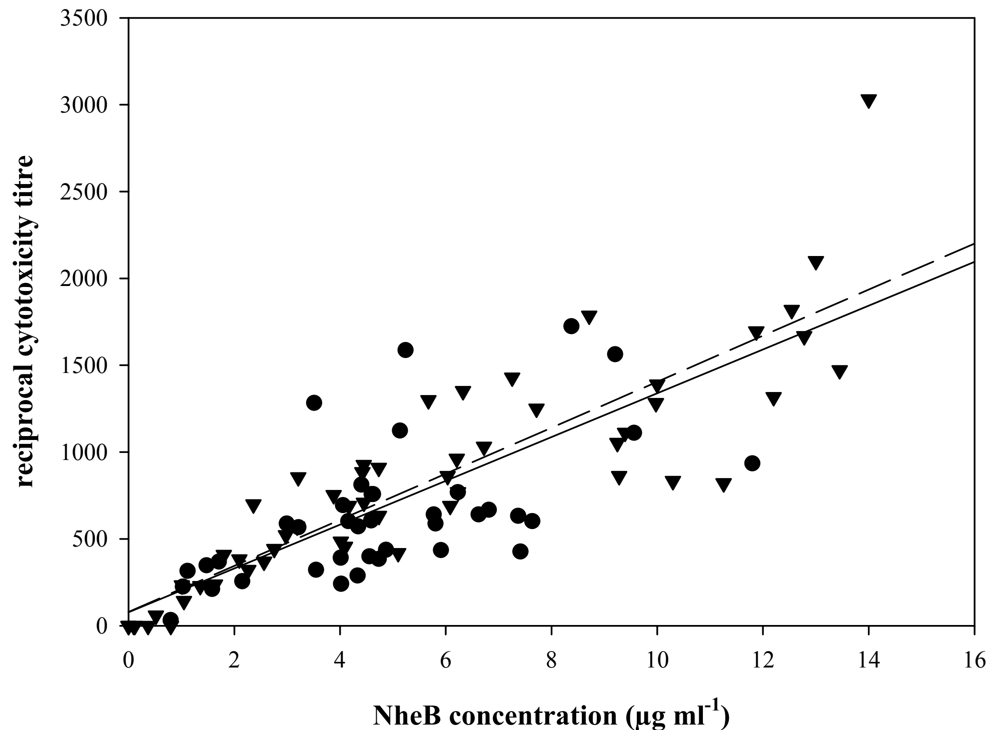
The cytotoxicity titres of the strains producing all three Nhe components varied from 37 to 3,030. As expected, negative results were obtained for the two strains producing only two components of Nhe and lacking the *hbl* gene. Cytotoxicity of HBL positive strains ranged from 30 to 1,700 but could not be attributed to HBL alone since all strains positive for HBL also produced Nhe. Therefore, comparison of the amount of L_2 detectable in the culture supernatants with the cytotoxicity titres resulted in a very poor correlation (Fig. 2). On the other hand, a good correlation between the concentration of NheB and the toxic activity of culture filtrates of *B. cereus* on Vero cells was obtained (Fig. 2). This interesting finding clearly indicates that cytotoxicity on Vero cells is dominated by Nhe rather than HBL and shows that *B. cereus* strains producing two enterotoxin complexes are not more cytotoxic than sole Nhe producers. The reasons for this striking result need to be clarified, particularly with regard to earlier studies which suggest comparable toxic activities of the both enterotoxins, HBL and Nhe [25].

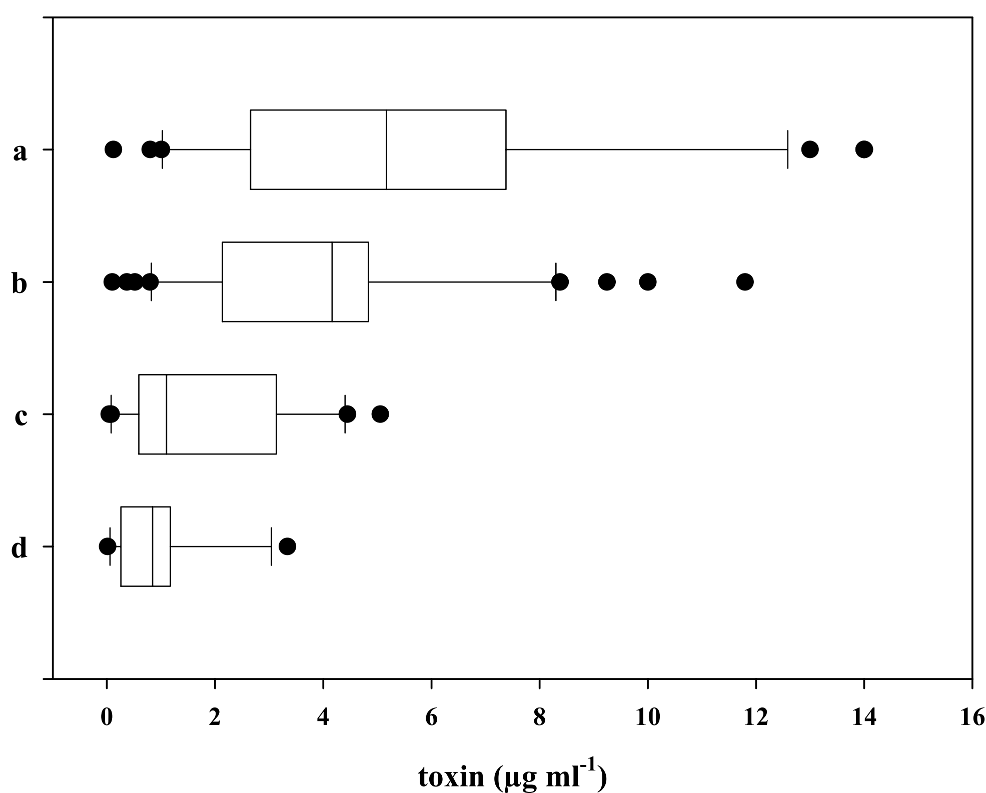
Figure 2: Correlation between toxicity to Vero-cells and expression of L_2 (●, n = 42; regression line ─,y = 614.81 + 0.04x; r = 0.02) or NheB of sole Nhe producers (▼, n = 57; regression line - -, y = 74.76 + 0.13x, r = 0.78) and all Nhe producing isolates (▼and ■, n = 99; regression line ─, y = 70.44 + 0.13x; r = 0.71).





To get further insight into the respective role of the two complexes in enterotoxicity we compared the amount of NheB produced by food poisoning and food/environmental strains. Overall, the median value (5.10 µg ml^{-1}, Fig. 3a) of all food poisoning strains was approximately 1 µg ml^{-1} higher than that of the food/environmental isolates (4.10 µg ml^{-1}, Fig. 3b). Less pronounced results, 1.10 µg ml^{-1} vs. 0.85 µg ml^{-1} (Fig. 3c, d), were obtained for the HBL-L_2 production of *hbl* positive strains. Altogether the results demonstrate that food poisoning strains tend to produce higher amounts of enterotoxins, particularly Nhe. While interpreting these data it must be kept in mind that "food/environmental strains" probably also include strains which could cause food poisoning. On the other hand the "food poisoning strains" also could include isolates which lost some of their toxic potential and it cannot fully ruled out, that some strains may have been wrongly associated with illness. We also know far too little about expression of the toxins when *B. cereus* is growing in the gut. With the protein amounts found in this study for NheB and for HBL L_2, both enterotoxin compounds are expressed at a high level, a finding which particularly for NheB is consistent with data reported by Gohar *et al.* [21].

Figure 3: Levels of HBL L_2 and NheB compared in different subsets of strains. The boundary of the box closest to zero indicates the 25th percentile, the line within the box marks the median, and the boundary of the box farthest from zero indicates the 75th percentile. Whiskers right and left the box indicate the 90th and 10th percentiles. In addition, outlying results are shown as dots. – (a) indicates the amount of NheB produced by the *B. cereus* isolates related to food poisoning, (b) the NheB production of the *B. cereus* strains isolated from food or environment, (c, d) the L_2 production of the isolates producing HBL related to food poisoning (c) or isolates from food or environment (d).



On the other hand the amount of enterotoxins produced by individual *B. cereus* strains showed an extremely broad variation. Consequently, the question arises if the detection of enterotoxin genes in *B. cereus* by PCR or an absence/presence test for toxin production is an appropriate method to estimate the virulence of *B. cereus* isolates. The data obtained in this study rather show that the quantitative determination of the enterotoxins allow a better assessment of the toxic potential. Particularly, the newly developed sandwich assays (Fig. 1) for L_2 and NheB represent valuable tools enabling accurate measurements of the toxin productivity of *B. cereus* isolates.

In conclusion, this is the first report on qualitative and quantitative aspects of enterotoxin production by *B. cereus* strains. The most striking result was that the toxic activity of strains producing both enterotoxin complexes, HBL and Nhe, was not significantly different from that of sole Nhe producers. The cytotoxicity of the *B. cereus* strains was dominated by the amount of secreted Nhe. Even though cytotoxicity on Vero cells only partly reflects the mechanisms involved in the aetiology of diarrhoea, the results obtained suggest that Nhe is the most important toxin in food poisoning. To verify this statement further research on the pathogenic role of the two enterotoxin complexes produced by *B. cereus* is required.

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4 DISKUSSION

In der Lebensmittelmikrobiologie werden zum Schutz des Verbrauchers vor Lebensmittelvergiftungen entweder wie bei den Salmonellen sämtliche Vertreter eines Genus als pathogen eingestuft oder es wird wie bei *Escherichia coli* die Fähigkeit einzelner Isolate zur Bildung bestimmter Virulenzfaktoren (z.B. Bildung von Shigatoxinen bei STEC) als Pathogenitätskriterium verwendet. Bei *B. cereus* sind Stämme mit emetischem Toxinbildungsvermögen als zweifelsfrei pathogen anzusehen, der Anteil der emetischen Isolate in Europa beträgt allerdings nur ca. 2 % (SCHULZ, 2004). Bei allen anderen *B. cereus* Isolaten stellt sich aber auch noch 50 Jahre nach der Erstbeschreibung durch HAUGE (1955) die Frage, welche der vielen von diesem Keim produzierten Virulenzfaktoren (Hämolysine, Phospholipasen, Proteasen, Zytotoxine) die Enteropathogenität eines Isolates bestimmen.

Prinzipiell gibt es bei *B. cereus* eine Diskrepanz zwischen dem ubiquitären Vorkommen dieses Keims in Lebensmitteln und der geringen Anzahl an bekannt gewordenen Ausbrüchen. Dies ist zum einen sicherlich auf Probleme mit der Datenerhebung zurückzuführen – durch *B. cereus* verursachte Erkrankungen sind nach Infektionsschutzgesetz (IfSG) nicht meldepflichtig bzw. sind oft auch durch einen milden und kurzen Krankheitsverlauf charakterisiert – zum anderen wäre es aber auch möglich, dass die Prävalenz von hoch pathogenen Stämmen in Lebensmitteln relativ gering ist. Bei den Enterotoxinen zeigte sich in den letzten Jahren, dass neben dem HBL-Komplex, dessen Enteropathogenität im Tierversuch verifiziert wurde (THOMPSON et al., 1984; BEECHER et al., 1995) auch das Non-hemolytic Enterotoxin eine wichtige Rolle als Pathogenitätsfaktor im Krankheitsbild der Diarrhö-Form spielt (LUND & GRANUM, 1996). Ein weiteres Enterotoxin, Cytotoxin K, wurde bisher nur bei einem einzigen *B. cereus* Stamm (LUND et al., 2000) beschrieben und dürfte somit von geringer epidemiologischer Bedeutung als Ursache von Lebensmittelvergiftungen sein. In dieser Studie sollte nun der Versuch gemacht werden, durch qualitative und quantitative Analyse der beiden beschriebenen Enterotoxin-Komplexe (HBL und Nhe) zu einer wissenschaftlich fundierten Aussage zum enterotoxischen Potential von *B. cereus* Isolaten zu kommen.

Um einerseits hoch virulente Stämme in der Lebensmittelkette identifizieren zu können bzw. andererseits die Quantifizierung der bekannten Enterotoxin-Komplexe HBL und Nhe zu ermöglichen, war es zuerst notwendig, neue Antikörper zu entwickeln. Die bisherigen

Nachweisverfahren basieren auf der Verwendung von polyklonalen Antiseren und weisen jeweils nur eine Komponente der beiden Enterotoxin-Komplexe nach (GRANUM et al., 1993; BEECHER & WONG, 1994). Die maximale biologische Aktivität der Enterotoxine wird allerdings nur bei der Kombination jeweils aller drei Komponenten erreicht (BEECHER et al., 1995; LINDBÄCK et al., 2004). Dies unterstreicht die Wichtigkeit des Nachweises jeweils aller drei Komponenten und verdeutlicht, dass der alleinige Nachweis einer Komponente völlig unzureichend für eine Beurteilung des enteropathogenen Potentials von *B. cereus* Isolaten ist.

In einem ersten Schritt wurden Antikörper gegen Nhe produziert, wobei es erstmals gelang trotz der Strukturähnlichkeiten zwischen den Nhe-Komponenten bzw. zum HBL-Toxinkomplex einen kompletten Satz an hochspezifischen Antikörpern gegen alle drei Nhe-Einzelkomponenten herzustellen. So reagierte der mAk gegen NheA, der sich auch durch eine sehr hohe Affinität auszeichnete, nicht mit den anderen Komponenten der beiden Toxin-Komplexe, insbesondere auch nicht mit dem strukturähnlichen HBL-L_2. Dieser mAk wurde auch zur Etablierung eines neuen Nachweisverfahrens, nämlich einem Kolonieimmunoblot, verwendet, das zur einfachen Charakterisierung der Enterotoxinprofile von *B. cereus* Stämmen eingesetzt werden kann (MORAVEK et al., 2004). Insgesamt wird durch diesen mAk die Analyse von NheA deutlich verbessert, da die in dem kommerziell erhältlichen Testkit (BDE; Tecra) implementierten polyklonalen Antikörper auch mit einer ganzen Reihe weiterer *B. cereus* Exoproteine reagieren (BEECHER & WONG, 1994b).

Des Weiteren konnte durch die Entwicklung von polyklonalen Antikörpern gegen NheC erstmals gezeigt werden, dass diese Komponente tatsächlich von *B. cereus* Stämmen exprimiert wird. Die Existenz dieser Komponente war zwar aufgrund genetischer und toxinologischer Untersuchungen postuliert worden, keiner der verschiedenen Arbeitsgruppen war es aber bislang gelungen, NheC aus Kulturüberständen von *B. cereus* zu isolieren (GOHAR et al., 2002; LINDBÄCK et al., 2004).

Zum Nachweis von NheB wurde eine breite Palette an spezifischen Antikörpern hergestellt. Keiner dieser Antikörper zeigte eine Kreuzreaktion mit NheC, was aufgrund der großen Ähnlichkeit der beiden Aminosäuresequenzen (GRANUM et al., 1999) zu Beginn der Arbeiten als potentielles Problem angesehen worden war. Jedoch trat bei einer ganzen Reihe von Antikörpern eine unerwartete Reaktivität mit Kulturüberständen des als *nhe*-negativ

eingestuften Cyt K Referenzstammes auf. Neue Untersuchungen zeigten, dass entgegen früherer Schlussfolgerungen auch dieser Stamm ein wenn auch stark verändertes Nhe-Gen besitzt (GRANUM, pers. Mitteilung). Bei der weiteren Charakterisierung der Antikörper mittels Immunoblot wurde beobachtet, dass bei allen untersuchten *B. cereus* Stämmen statt der erwarteten einzelnen Bande eine breite Reaktivitätszone im Bereich von 36 – 39 kDa auftrat. Nach Aufreinigung des Kulturüberstandes mittels Immunaffinitätschromatographie und nachfolgender Sequenzierung des eluierten Proteins wurde ein um zwölf Aminosäuren reduziertes NheB Protein erhalten. Offensichtlich wird bei *B. cereus* das ursprüngliche NheB durch gleichzeitig produzierte Exoproteasen N-terminal angedaut. Ähnliches wurde auch von LUND & GRANUM (1996 und 1997) berichtet. Hinweise darauf, dass es sich dabei um eine mögliche Aktivierungsreaktion des Proteins, ähnlich der bei *Bacillus anthracis* beschriebenen proteolytischen Spaltung des protektiven Antigens (PA) handelt (COLLIER & YOUNG, 2003), konnten jedoch nicht gefunden werden. Vielmehr beeinflusst nach WEGSCHEIDER (2004) die Substitution des N-terminal angedauten NheB's durch von *Escherichia coli* produziertem, rekombinantem NheB mit intaktem N-Terminus nicht die zytotoxische Aktivität des Toxinkomplexes. Neuere Untersuchungen bei denen die neutralisierenden Eigenschaften der Antikörper im Vordergrund standen, zeigten auch, dass vielmehr der C-Terminus des NheB einen für die Bildung eines aktiven Toxinkomplexes eminent wichtigen Proteinbereich darstellt (Ergebnisse nicht dargestellt).

Zusammen mit den von DIETRICH et al. (1999) beschriebenen mAk gegen die HBL-Komponenten standen somit Antikörper gegen alle sechs Einzelkomponenten der zwei Enterotoxinkomplexe zur Verfügung. Im Rahmen eines Projektes der Europäischen Union wurde unter Verwendung dieser Immunreagenzien die Expression von HBL und Nhe bei 100 ausgewählten *B. cereus* Stämmen detailliert analysiert. Unter diesen 100 Stämmen befanden sich einerseits Isolate, die in Lebensmittelvergiftungen (n = 48) involviert waren, andererseits auch 52 Umwelt- (z.B. Wasser, Erde, Baum) und Lebensmittelisolate (z.B. Fleisch, Milch, Gemüse, Babynahrung, Reis, Nudeln) von *B. cereus* aus der ganzen Welt. Die ausgewählten Isolate wurden mit immunchemischen und molekularbiologischen (Polymerase-Chain-Reaction, PCR) Methoden untersucht, was eine vollständige Charakterisierung der vorliegenden Enterotoxin-Gene und der daraus resultierenden Proteinexpression ermöglichte.

Bisherige molekularbiologische meist auf PCR-Verfahren basierende Untersuchungen zeigten, dass fast alle *B. cereus*-Stämme zumindest ein Enterotoxin-Gen besitzen (HANSEN

& HENDRIKSEN, 2001; IN'T VELD et al., 2001), wobei für das HBL-Gen von GUINEBRETIERE et al. (2002) Polymorphismen beschrieben wurden. In eigenen Untersuchungen konnte mittels PCR bei 42 % aller Isolate *hbl* nachgewiesen werden. Eine ähnliche Prävalenz wurde auch von PRÜSS et al. (1999) ermittelt, während in anderen Studien (HANSEN & HENDRIKSEN, 2001; IN'T VELD et al., 2001) bei der Detektion von einem oder mehreren HBL-Genen 59 – 73 % der untersuchten Stämme positiv waren.

Unter optimalen Bedingungen für die Toxinproduktion (GLATZ & GOEPFERT, 1976; BEECHER & WONG, 1994) konnten bei 38 Isolaten eine oder mehrere HBL-Komponenten mit den jeweiligen spezifischen Antikörpern nachgewiesen werden. Bei den restlichen vier Isolaten wurden aufgrund derer psychrotrophen Wachstumseigenschaften die Anzuchtbedingungen geändert (25 °C, über Nacht). Mit dieser Methode konnte auch bei diesen Isolaten HBL-Komponenten nachgewiesen werden. Von den 42 *hbl*-positiven Stämmen produzierten 40 (95 %) alle drei Toxinkomponenten des HBL- Komplexes; bei zwei Isolaten konnten Spuren von L_1 und L_2, aber kein B nachgewiesen werden. Möglicherweise wird die B-Komponente auch exprimiert, konnte aber aufgrund der Nachweisgrenze durch den EIA nicht mehr detektiert werden.

Von den 100 ausgewählten Stämmen waren alle mit Ausnahme des Stammes 391/98 (produziert CytK) *nhe*-positiv. Ähnlich hohe Prävalenzen von *nhe* wurden auch von anderen Arbeitsgruppen berichtet (HANSEN & HENDRIKSEN, 2001; GUINEBRETIÈRE et al., 2002). Aber nur 93 der 99 *nhe*-positiven *B. cereus* Isolate waren in der Lage, alle drei Nhe-Komponenten zu exprimieren. Dabei produzierten zwei Isolate je zwei der drei Komponenten und vier Stämme waren zu keiner Expression fähig. Möglicherweise ist diese unvollständige bzw. nicht vorhandene Expression der Nhe-Gene auf Mutationen im PlcR wie von SLAMTI et al. (2004) und GOHAR et al. (2002) beschrieben, zurückzuführen.

Diese qualitativen Analyseergebnisse zeigen, dass bei nahezu jedem *B. cereus* Isolat mindestens ein Enterotoxin-Gen nachgewiesen werden kann. Auch die Expression von zumindest einer Komponente der Enterotoxin-Komplexe kann bei 94 % der untersuchten Isolate beobachtet werden. Zusammen mit den Daten aus der Zellkultur verdeutlicht dies, dass fast jedes *B. cereus* Isolat in der Lage ist, einen aktiven Toxinkomplex zu produzieren. Daraus jedoch Rückschlüsse auf die Virulenz der Stämme zu ziehen, ist nicht möglich, da die bloße Anwesenheit der Gene von HBL und/oder Nhe bzw. deren Expression keinen Beweis für die

Enteropathogenität darstellt. Bestes Beispiel dafür ist das kommerziell erhältliche Probiotikum Bactisubtil®. Die in diesem Präparat enthaltenen *B. cereus* Sporen (HOA et al., 2000) sind in der Lage, beide Enterotoxin-Komplexe (HBL und Nhe) zu produzieren (unveröffentlichte Ergebnisse). Vergiftungsfälle in Verbindung mit diesem Präparat sind jedoch nicht beschrieben. Dies unterstreicht nochmals die Bedeutung einer quantitativen Toxinanalyse bei *B. cereus*.

Im Rahmen der vorliegenden Arbeit wurden dafür zunächst nicht-kompetitive indirekte EIA's etabliert, mit denen die Konzentration der einzelnen Toxinkomponenten in Kulturüberständen von *B. cereus* semiquantitativ bestimmt werden konnte. Die so erhaltenen Daten gaben erste Hinweise auf eine stark differierende Produktivität bei den jeweiligen *B. cererus* Stämmen. Bei NheC konnte die von LINDBÄCK et al. (2004) postulierte geringe Expression dieses Proteins im Vergleich zu den beiden anderen Nhe-Komponenten bestätigt werden. Im Überstand des Nhe-Referenzstammes (NVH 0075/95) lagen die NheA und NheB-Titer bei ca. 1:2500, für NheC konnten hingegen nur Titer von 1:80 nachgewiesen werden.

Unter Verwendung von immunaffinitätschromatographisch gereinigten Toxinkomponenten als Referenzstandards und von Sandwich-EIA's als Analyseverfahren, konnte schließlich erstmals der quantitative Nachweis der HBL-L_2-, sowie der NheB-Komponente realisiert werden. Dabei wurde in den Kulturüberständen HBL-positiver Stämme zwischen 0,02 – 5,6 µg/ml L_2-Protein detektiert. Da die Gene für die HBL- Komponenten auf einem Operon liegen (RYAN et al., 1997) und maximale biologische Aktivitäten nur erreicht werden wenn alle drei Proteine in äquimolaren Konzentrationen vorliegen (BEECHER et al., 1995; DIETRICH et al., 1999), kann vermutet werden, dass auch L_1 und B in ähnlichen Mengen wie L_2 produziert werden. Die in den Kulturüberständen gefundenen Mengen an NheB lagen im Bereich von 0,02 bis 14,2 µg/ml, bei fast allen Stämmen lagen die Werte höher als diejenigen für HBL-L_2.

Wenn man nun die detektierten Toxinmengen mit der Herkunft der Stämme vergleicht, kann man feststellen, dass die Medianwerte der in Verbindung mit Lebensmittelvergiftungen isolierten *B. cereus* Stämmen bei NheB um 1 µg/ml und bei L_2 um 0,25 µg/ml höher liegen als bei Umwelt- und Lebensmittelisolaten. Berücksichtigt man, dass bei den Isolaten aus der Umwelt bzw. Lebensmitteln wahrscheinlich auch potenziell pathogene Stämme inkludiert sind bzw. dass unter den Isolaten aus Lebensmittelvergiftungen möglicherweise auch falsch

eingeordnete Stämme sein könnten, dürfte der tatsächliche quantitative Unterschied in der Expression der Enterotoxinkomplexe für beide Komponenten noch größer sein.

Ähnliche Unterschiede konnten auch für die zytotoxische Aktivität der beiden Keimgruppen festgestellt werden. Prinzipiell konnte dabei nur in *B. cereus* Kulturüberständen, die eines der bisher beschriebenen Enterotoxine wie HBL, Nhe oder Cyt K enthielten, ein zytotoxischer Effekt nachgewiesen werden. Bei reinen Nhe-Produzenten konnte über die vollständige Neutralisation der toxischen Aktivität von Kulturüberständen mittels der mAk's gegen NheB die Hypothese untermauert werden, dass andere in der Literatur erwähnte und als Enterotoxin titulierte Exoproteine wie BcET, EntFM oder Cyt K-like Proteine kein ausgeprägtes toxisches Potenzial haben. Zudem konnte dadurch gezeigt werden, dass andere von *B. cereus* produzierte Pathogenitätsfaktoren wie Proteasen, Hämolysine oder Phospholipasen (GOHAR et al., 2002) nicht zytotoxisch sind.

Die quantitativen Daten zur Toxinproduktion erlauben auch eine bislang nicht mögliche Einschätzung des toxischen Potenzials der beiden Enterotoxin-Komplexe. Die vergleichende Analyse der Zytotoxizität von Kulturüberständen und der exprimierten Toxinmenge zeigte, dass im Gegensatz zu L_2 bei NheB eine eindeutige Korrelation der beiden Parameter festgestellt werden konnte. Bei Isolaten, die sowohl HBL als auch Nhe produzierten, konnten keine höheren Titer im Zellkulturtest festgestellt werden als bei Isolaten, die nur Nhe in gleicher Menge produzierten. Daraus ergibt sich, dass die von einigen Forschergruppen vor allem wegen der hohen Sequenzhomologien der Komponenten der beiden Enterotoxinkomplexe postulierten möglichen antagonistischen oder synergistischen Effekte offenbar nicht vorhanden sind. Des Weiteren kann davon ausgegangen werden, dass im Zellkulturtest die Bedeutung des HBL für die Zytotoxizität, im Gegensatz zu den Schlussfolgerungen der Arbeit von LINDBÄCK et al. (1999), sehr gering ist. Dies ist umso mehr überraschend, als in früheren Studien eine gleichwertige und vergleichbare Zytotoxizität der beiden Enterotoxinkomplexe HBL und Nhe beschrieben wurde (GRANUM et al., 1997). Obwohl die bei Vero-Zellen gemessene Zytotoxizität sicher nur teilweise die Mechanismen bei der Entstehung einer Diarrhö durch *B. cereus* reflektiert, weisen die Daten dieser Studie darauf hin, dass Nhe möglicherweise die weit bedeutendere Rolle in diesem Krankheitsgeschehen spielt.

Für eine umfassende Beurteilung der Pathogenität eines *B. cereus* Isolates sind sicherlich noch weitere Faktoren mit einzubeziehen. So berichtet BORGE et al. (2001) von besonders kurzen Generationszeiten bei zwei in Lebensmittelvergiftungen involvierten *B. cereus* Isolaten. Aus anderen Arbeiten ergeben sich Hinweise darauf, dass die Säurestabilität der *B. cereus* Sporen bei der Magenpassage bzw. deren Adhärenz-Eigenschaften wichtige Virulenzfaktoren darstellen könnten. Sporen von *B. cereus* besitzen durch ihre oberflächlichen Bestandteile und Strukturen stark hydrophobe Eigenschaften (RÖNNER et al., 1990). Diese verbessern nach ANDERSSON et al. (1998) möglicherweise die Anhaftung der Sporen an die Epithelzellen des Dünndarmes und erhöhen somit ihre Virulenz.

Zusammenfassend konnte in dieser Arbeit erstmals gezeigt werden, dass in Lebensmittelvergiftungen involvierte *B. cereus* Stämme tendenziell eine höhere Toxinproduktivität aufweisen als andere Lebensmittel- und Umweltisolate. Für die Lebensmittelüberwachung ergibt sich daraus die Konsequenz, dass zukünftig bei der Untersuchung von Verdachtsproben neben der *B. cereus* Keimzahl auch die Toxinproduktivität des entsprechenden Isolates für die Beurteilung stärker berücksichtigt werden sollte. Mit den entwickelten Verfahren können zudem in lebensmittelherstellenden Betrieben Eintragsquellen pathogener *B. cereus* zuverlässig identifiziert und durch gezielte Maßnahmen beseitigt werden. Psychrotrophe *B. cereus* Stämme, die häufig als Verderbserreger in gekühlten Lebensmitteln wie z.B. pasteurisierter Milch zu finden sind, spielen hierbei jedoch keine Rolle, da sie offensichtlich nicht in der Lage sind, bei der normalen Kerntemperatur des menschlichen Körpers von 37 °C Enterotoxine zu produzieren.

5 ZUSAMMENFASSUNG

Die vorliegende Arbeit beschäftigt sich mit der Produktion von Antikörpern gegen die drei Einzelkomponenten des Non-hemolytic-enterotoxins (Nhe) von *Bacillus cereus* und deren Einsatz zur qualitativen und quantitativen Analyse der Enterotoxinprofile von 100 *B. cereus* Stämmen.

Es wurde ein kompletter Satz an hoch-affinen Antikörpern gegen jede Einzelkomponente von Nhe (NheA, NheB und NheC) produziert und charakterisiert. Mit Hilfe von synthetisch hergestellten Peptiden, die den C-Terminus des NheC repräsentierten, konnte ein spezifisches Kaninchenantiserum zum Nachweis dieser Toxinkomponente hergestellt werden. Unter Verwendung von gereinigten *B. cereus* Exoproteinen als Immunogen, konnte ein monoklonaler Antikörper (mAk) gegen NheA und mehrere mAk gegen NheB gewonnen werden. Es wurden keine Kreuzreaktionen mit anderen Proteinen verschiedenster *B. cereus* Stämme beobachtet. Alle Antikörper gegen NheB zeigten im Zellkulturtest neutralisierende Eigenschaften (bis zu 98 %).

Zur Evaluierung der Expression von HBL und Nhe wurden 100 *B. cereus* Stämme untersucht. Mit molekularbiologischen Methoden konnte festgestellt werden, dass 42 % der Stämme Gene für HBL und 99 % für Nhe besitzen. Spuren der Einzelkomponenten von HBL und Nhe konnten mit Hilfe der spezifischen Antikörper bei 100 % für *hbl*-positiven und 96 % der *nhe*-positiven Isolate nachgewiesen werden. Eine Quantifizierung der Expression aller Stämme ergab für HBL-L_2 Mengen von 0,02 to 5,6 µg/ml bzw. für NheB 0,03 to 14,2 µg/ml. Wenn man nun die detektierten Toxinmengen mit der Herkunft der Stämme vergleicht, kann man feststellen, dass die Medianwerte der in Verbindung mit Lebensmittelvergiftungen isolierten *B. cereus* Stämmen bei NheB und HBL-L_2 höher lagen als bei Umwelt- und Lebensmittelisolaten.

Mit dieser Arbeit konnte gezeigt werden, dass eine Quantifizierung der Enterotoxin-Komplexe zur Einschätzung des enterotoxischen Potentials eines *B. cereus* Isolates zwingend notwendig ist. Des Weiteren weisen die Daten dieser Studie darauf hin, dass Nhe möglicherweise eine weit bedeutendere Rolle im Krankheitsgeschehen der Diarrhö-Form spielt als bisher angenommen.

SUMMARY

Detection, expression and significance of the enterotoxin-complexes produced by *Bacillus cereus*

This work deals with the production of antibodies against each component of the Non-hemolytic enterotoxin (Nhe) of *Bacillus cereus* and their use for qualitative and quantitative analyses of the enterotoxin profiles of 100 *B. cereus* strains.

To facilitate the detection of this toxin, consisting of the subunits NheA, NheB, and NheC, a complete set of high-affinity antibodies against each of the three components was established and characterized. A rabbit antiserum specific for the C-terminal part of NheC was produced using a respective synthetic peptide coupled to a protein carrier for immunization. Using purified *B. cereus* exoprotein preparations as immunogens, one monoclonal antibody against NheA and several antibodies against NheB were obtained. No cross-reactivity with other proteins produced by different strains of *B. cereus* was observed. Antibodies against the NheB component were able to neutralize the cytotoxic activity (up to 98 %) of Nhe.

To evaluate the expression of HBL and Nhe, a set of 100 *B. cereus* strains was examined. Molecular biological characterization showed that 42 % of the strains harboured the genes for HBL and 99 % for Nhe. The production of all HBL and Nhe components was analyzed using specific antibodies and, in culture supernatants, detectable levels of HBL and Nhe were found for 100 % of *hbl*-positive and 96 % of *nhe*-positive strains. The concentrations of the HBL–L_2 and NheB component ranged from 0.02 to 5.6 µg/ml and from 0.03 to 14.2 µg/ml, respectively. Comparison of the amount of NheB and HBL-L_2 produced by food poisoning and food/environmental strains revealed that the median values for all food poisoning strains were higher than for the food/environmental isolates.

The data presented in this work provide evidence that specific and quantitative determination of the enterotoxins is necessary to evaluate the enterotoxic potential of *B. cereus*. In particular, the level of Nhe seems to explain most of the cytotoxic activity of *B. cereus* isolates and may indicate an higher diarrheic potential than anticipated.

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