LEHRSTUHL FÜR MIKROBIELLE ÖKOLOGIE
DEPARTMENT FÜR GRUNDLAGEN DER BIOWISSENSCHAFTEN
TECHNISCHE UNIVERSITÄT MÜNCHEN

Development of genotypic and phenotypic methods for the identification and differentiation of hazardous *Bacillus cereus* group strains

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Vollständiger Abdruck der von der Fakultät Wissenschaftszentrum Weihenstephan für Ernährung, Landnutzung und Umwelt der Technischen Universität München zur Erlangung des akademischen Grades eines Doktors der Haushalts- und Ernährungswissenschaften

(Dr.oec.troph.)

genehmigten Dissertation.

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Prüfer der Dissertation:	1. Univ.-Prof. Dr.rer.nat.habil. Siegfried Scherer
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Die Dissertation wurde am 19.07.2007 bei der Technischen Universität München eingereicht und durch die Fakultät Wissenschaftszentrum Weihenstephan für Ernährung, Landnutzung und Umwelt am 27.09.2007 angenommen.

Nothing shocks me. I’m a scientist.

Harrison Ford as Indiana Jones

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SUMMARY

The *Bacillus cereus* group comprises the genetically closely related species *Bacillus anthracis*, *B. cereus*, *Bacillus thuringiensis*, *Bacillus weihenstephanensis* and *Bacillus mycoides*, which can cause quite different forms of disease. In this study, a rapid method for the phenotypic characterization and differentiation of this group of endospore forming organisms was developed: artificial neural network (ANN) assisted Fourier transform infrared (FTIR) spectroscopy. This metabolic fingerprinting method allows the rapid identification of *B. cereus* group members and was used for the population analysis of soil samples from different origins and of a rice dish associated with a food poisoning caused by emetic *B. cereus*. Determination of growth characteristics of 100 representative *B. cereus* strains from different origins highlighted the distinct phenotype of emetic *B. cereus* strain. In contrast to the high variance within diarrhoeal *B. cereus* strains, all emetic strains exhibited an elevated minimum and maximum growth limit. The distinct phenotype of emetic *B. cereus* strains was confirmed by FTIR spectroscopy and is accompanied by a rather monomorphic genotype as shown by MLST and toxin gene profiling.

Due to the increasing number of reports of food borne disease, especially of severe cases, fast detection methods are required for diagnostic purposes as well as for the prevention of food contamination and food borne outbreaks. Diagnosis of *B. cereus* linked to the emetic type of food poisoning was hampered by the lack of genetic information on the toxin genes. In this study, the gene cluster responsible for cereulide production was sequenced, and subsequently, the first molecular detection methods specific for emetic *B. cereus* strains were developed. These methods include a standard and a SYBR Green I PCR assay for high through-put applications, a SYBR Green I duplex assay for the one step differentiation between enterotoxigenic *S. aureus* and emetic *B. cereus* and a TaqMan assay including an internal amplification control for diagnostic purposes. The development of toxin gene profiling via a multiplex PCR assay for the detection of the enterotoxin genes and the cereulide synthetase genes enabled population studies on the distribution of these genes in different environments. All developed molecular methods have already been successfully applied in diagnostic laboratories and facilitate the rapid detection of potentially hazardous *B. cereus* strains or the causative agent of food poisonings. In general, the results of this study provide deeper insight into the genotypic and phenotypic characteristics of the *B. cereus* group of organisms, especially emetic *B. cereus*.

ZUSAMMENFASSUNG

Die *Bacillus cereus* Gruppe setzt sich aus den folgenden Spezies zusammen: *Bacillus anthracis*, *B. cereus*, *Bacillus thuringiensis*, *Bacillus weihenstephanensis* und *Bacillus mycoides*. Die Mitglieder dieser Gruppe endosporenbildender Mikroorganismen sind genetisch sehr nahe miteinander verwandt, können aber unterschiedliche Krankheitsformen auslösen. Im Laufe dieser Studie wurde eine phänotypische Methode basierend auf Fourier Transform Infrarot Spektroskopie (FTIR) und künstlichen neuronalen Netzen (KNN) zur Differenzierung der einzelnen Spezies der *B. cereus* Gruppe entwickelt. Diese Methode erlaubt die schnelle Identifizierung der Mitglieder der *B. cereus* Gruppe und kann für Populationsanalysen verwendet werden. In der vorliegenden Studie wurden verschiedene Bodenproben und eine Lebensmittelprobe aus einer emetischen Lebensmittelvergiftung in einem Kindergarten analysiert. Die Untersuchung der Wachstumscharakteristika von 100 repräsentativen *B. cereus* Isolaten unterschiedlichen Ursprungs, offenbarte eine enge Verwandtschaft emetischer Stämme. Alle emetischen Isolate wiesen eine einheitlich erhöhte minimale und maximale temperaturbedingte Wachstumsgrenze auf, wohingegen diarrhöe-auslösende Stämme hier einen sehr diversen Phänotyp besaßen. Der einheitliche Phänotyp emetischer *B. cereus* Isolate konnte mittels FTIR Spektroskopie bestätigt werden. Zusätzlich wiesen emetische Stämme einen einheitlichen Genotyp im Multilocus Sequence Typing (MLST) und im Toxingen-Profil auf.

Aufgrund der zunehmenden Fallzahlen von lebensmittelbedingten Krankheits-ausbrüchen, im Besonderen auch schwerwiegenden Fällen, werden schnelle und spezifische Nachweismethoden sowohl für diagnostische Zwecke, als auch zur Prävention von Lebensmittelkontaminationen und lebensmittelbedingten Ausbrüchen benötigt. Da der genetische Hintergrund für die Synthese des emetischen Toxins Cereulide bisher unbekannt war, konnten emetische *B. cereus* Stämme nur schwer diagnostiziert werden. Im Laufe dieser Arbeit wurde das entsprechende Gen-Cluster sequenziert und anschließend die ersten spezifischen, molekularen Nachweissysteme für emetische *B. cereus* entwickelt. Es handelt sich hierbei um eine Standard-PCR und eine SYBR Green I basierte Real-time PCR zur Analyse größerer Probenmengen, eine Duplex-SYBR Green I PCR zur schnellen Unterscheidung zwischen enterotoxigenen *S. aureus* und emetischen *B. cereus*, und eine TaqMan basierte Real-time PCR, die eine interne Amplificationskontrolle für diagnostische Analysen enthält. Ein neu entwickelter Multiplex-PCR Assay zur Bestimmung des Toxingen-Profils, der sowohl die Enterotoxingene als auch die Gene der Cereulid-Synthetase nachweisen kann, erlaubt es, die Verbreitung dieser Gene in Stämmen unterschiedlichen

Ursprungs zu untersuchen. Die beschriebenen molekularen Nachweismethoden werden bereits in diagnostischen Laboren eingesetzt und erleichtern sowohl den schnellen Nachweis von potentiell gefährlichen *B. cereus* Stämmen, als auch die Identifizierung lebensmittelvergiftungsauslösender Stämme. Die in vorliegender Arbeit erworbenen Erkenntnisse erlauben einen tiefen Einblick in die genotypischen und phänotypischen Merkmale der Mitglieder der *B. cereus* Gruppe, mit besonderem Augenmerk auf emetische *B. cereus* Stämme.

1 INTRODUCTION

1.1 The *Bacillus cereus* group

The *Bacillus cereus* group consists of the genetically closely related species *Bacillus anthracis*, *B. cereus*, *Bacillus thuringiensis*, *Bacillus weihenstephanensis* and *Bacillus mycoides* (Gordon et al. 1973; Priest et al. 1988; Turnbull and Kramer 1991; Lechner et al. 1998), which can produce different toxins and various extra cellular enzymes, several thereof are considered as potential virulence factors. *B. anthracis* is well known as the etiological agent of anthrax, and has gained notoriety as a bioterrorism weapon in 2001 as *B. anthracis* spores were placed inside common envelopes and sent to several locations in the United States. *B. cereus* is recognized as an opportunistic pathogen provoking two different types of food poisoning - emesis and diarrhoea - but also non-gastrointestinal infections like periodontitis, eye infections, septicaemia, or endocarditis (Helgason et al. 2000a; Kotiranta et al. 2000). Spore preparations of *B. thuringiensis* including its crystal toxin are commonly used as biopesticides (Schnepf et al. 1998). However, *B. thuringiensis* has also been described as the causative agent of human infections and the presence of enterotoxin genes has been reported frequently (Jackson et al. 1995; Damgaard et al. 1997; Hernandez et al. 1998; Gaviria Riviera et al. 2000; Jensen et al. 2002; Swiecicka et al. 2006). The two psychrotolerant members of the *B. cereus* group - *B. weihenstephanensis* and *B. mycoides* - are mainly food spoilage microorganisms, but they can also possess enterotoxin genes (Lechner et al. 1998; Prüß et al. 1999; Stenfors et al. 2002; Hendriksen et al. 2006).

Despite the variations in potential virulence, the differentiation between the *B. cereus* group members is not always an easy task. *B. anthracis* can be identified by lack of hemolysis, sensitivity to gamma phage and the presence of the two virulence plasmids pXO1 and pXO2 (Henderson et al. 1994; Turnbull 1999). In contrast, *B. thuringiensis* can only be differentiated from *B. cereus* by the presence of the *cry* genes in the former. These genes are located on a plasmid and are responsible for the formation of a crystalline insecticidal toxin (Kronstad et al. 1983; Frederiksen et al. 2006). The psychrotolerant members *B. weihenstephanensis* and *B. mycoides* can be identified by their ability to grow at low temperatures (below 7°C) and the presence of the cold shock protein A (*cspA*) gene (Lechner et al. 1998). *B. mycoides* is characterized by its rhizoid growth.

Several attempts have been made to differentiate the closely related *B. cereus* group members. Notably the differentiation between *B. cereus* and *B. thuringiensis* has challenged

the scientific community. Multilocus enzyme electrophoresis (MEE) and amplified fragment length polymorphism analysis (AFLP) revealed a rather monomorphic cluster for *B. anthracis* isolates, whereas *B. cereus* and *B. thuringiensis* isolates could not be separated from each other (Vilas-Boas et al. 2002; Hill et al. 2004).

1.2 Pathogenicity of *B. cereus*

The spectrum of potential *B. cereus* toxicity ranges from strains used as probiotics in animal feed to highly toxic strains already reported to be responsible for fatalities. *B. cereus* is capable of producing different non-specific virulence factors including cell surface proteins, cytotoxic components and degradative enzymes. Of these, proteases, phospholipases, haemolysins and enterotoxins contribute substancially to gastrointestinal and non-gastrointestinal diseases (Drobniewski 1993; Rowan et al. 2003; Gohar et al. 2005). Non-gastrointestinal manifestations of *B. cereus* infections include wound and eye infections, bacteremia, meningitis, endocarditis, and periodontitis (Beecher et al. 1995a; Miller et al. 1997; Helgason et al. 2000a; Haase et al. 2005; Pillai et al. 2006). The implicated individuals are often immunocompromised, preterm neonates, patients with indwelling catheters, prosthetic devices or traumatic injuries (Drobniewski 1993; Kotiranta et al. 2000; El Saleeby et al. 2004). The gastrointestinal manifestation of the disease caused by *B. cereus* is connected to two clinical pictures: diarrhoea and emesis. In general, both types of food poisoning are relatively mild and self-limiting, and symptoms usually disappear within 24 h. Nevertheless, during the last few years, severe forms of both types of disease have occasionally involved hospitalization or even deaths (Mahler et al. 1997; Lund et al. 2000; Dierick et al. 2005). Changing life styles with the trend to ready-to-eat and low-processed food and the increasing number of immunocomprimised individuals could increase the numbers of outbreaks in the near future.

The diarrhoeal type of *B. cereus* food poisoning is elicited by heat-labile enterotoxins: the two enterotoxin-complexes Nhe (non-hemolytic enterotoxin) and Hbl (hemolysin BL) and the single protein CytK (cytotoxin K) (Beecher et al. 1995b; Lund and Granum 1996; Lund et al. 2000). The toxicity of *entFM* is still in doubt and *bceT* was reappraised as a cloning artefact (Granum 2001; Hansen et al. 2003). Diarrhoea is caused by the ingestion of 10^5 to 10^7 enterotoxigenic *B. cereus* cells or spores and is mainly associated with proteinaceous foods like soups, meat products, vegetables and milk (Kramer and Gilbert 1989; Granum 2001). Symptoms are abdominal pain and diarrhoea, appear 8 to 16 hours after ingestion of contaminated food, and normally disappear within 12 to 24 hours. Nevertheless more severe cases requiring hospitalization have been described, and one case was even fatal (Lund et al.

2000). The diarrheal type of food poisoning corresponds to a classical infection, since the enterotoxins are inactivated by heat during processing and cleaved by trypsin in the stomach. Spores surviving the passage through the stomach germinate in the small intestine and produce enterotoxins "in place" (Granum and Lund 1997).

In contrast, the emetic syndrome is caused by an intoxication. The heat- and acid-stable emetic toxin cereulide is preformed in food and probably elicits emesis by stimulating the vagus afferent through binding to the 5-HT_3 receptor (Agata et al. 1995). In general, emetic food poisoning is mainly associated with farinaceous foods like rice, noodles, pasta and pastries (Ehling-Schulz et al. 2004a). The estimated infective dose derived from emetic food poisonings varies between 10^3 to 10^8 cfu/g food (Kramer and Gilbert 1989; Granum 2001). But as cereulide is extremely heat-stable and preformed in food, recovery of viable *B. cereus* from reheated foods associated with emetic food poisoning is not always possible (Drobniewski 1993). The emesis inducing dose of cereulide was reported to be about 10 µg/kg body weight in monkey feeding tests (Agata et al. 1994; Shinagawa et al. 1995) and seems to be similar for humans (Jääskeläinen et al. 2003). Cereulide is a cyclic depsipeptide chemically closely related to the potassium ionophore valinomycin with three repeats of the following amino acids: [D-*O*-Leu-D-Ala-L-*O*-Val-L-Val]$_3$ (Agata et al. 1994). Food poisoning caused by cereulide results in nausea and vomiting, 0.5 to 6 h after ingestion of contaminated food and normally lasts for up to 24 h. However, severe cases involving two children who died due to fulminant liver failure have been described (Mahler et al. 1997; Dierick et al. 2005).

1.3 Available methods for the detection of *B. cereus*

The true incidence of *B. cereus* food poisoning is unknown for a number of reasons, including misdiagnosis of the disease, which is symptomatically similar to other types of food poisoning. For example, the symptoms caused by diarrhoeal *B. cereus* resemble those caused by *Clostridium perfringens*, whereas symptoms caused by emetic *B. cereus* parallel those caused by *Staphylococcus aureus* (Ehling-Schulz et al. 2004a). In addition, *B. cereus* has often been regarded as a laboratory contaminant and has been excluded as a causative agent (Miller et al. 1997; Callegan et al. 1999).

Standard detection methods for *B. cereus* including enrichment, plating and further identification require at least three days (Rhodehamel and Harmon 1998; Malorny et al. 2003b). Isolation of *B. cereus* from foods, the environment, and as well from clinical settings is performed using conventional selective plating media. Polymyxin-egg yolk-mannitol-

bromothymol blue (PEMBA) or mannitol-egg yolk-polymyxin (MYP) agar are recommended by food authorities like the International Organization for Standardization (ISO), §64 Lebensmittel-, Bedarfsgegenstände- und Futtermittelgesetzbuch (LFGB) or the Food and Drug Administration (FDA) for the identification and enumeration of presumptive *B. cereus* colonies (Rhodehamel and Harmon 1998; Schulten et al. 2000; Anonymous 2004). The two recommended standard plating media have a slightly different formulation but are based on the same principles: (i) characteristic colony appearance, (ii) egg yolk hydrolysis resulting in a precipitation zone around suspect colonies, and (iii) the inability of *B. cereus* group strains to produce acids from mannitol (van Netten and Kramer 1992). However, some *B. cereus* strains are missing one or more of these key characteristics on the standard media and might therefore be misidentified or ignored (Szabo et al. 1984; Ehling-Schulz et al. 2004a). Recently, new chromogenic plating media were developed to overcome these limitations. These media contain synthetic chromogenic substrates that are cleaved by specific enzymatic activities of certain microorganisms, resulting in the formation of coloured colonies on media (Manafi 1996; Manafi 2000; Reissbrodt 2005). Chromogenic media for the *B. cereus* group have mainly been used for the identification of *B. anthracis* and/or its differentiation from *B. cereus* and *B. thuringiensis* (Reissbrodt 2004; Juergensmeyer et al. 2006; Tomaso et al. 2006).

Diarrhoeal *B. cereus* strains are comparatively well characterised and several PCR assays for the rapid detection of the different enterotoxin genes have been described (Prüß et al. 1999; Hansen and Hendriksen 2001; Guinebretiere et al. 2002). In addition, immunological assays are commercially available, e.g. the BCET-RPLA *B. cereus* Enterotoxin Test Kit (Oxoid, UK) for the detection of the L2 component of Hbl and the Tecra BDE kit (Tecra Diagnostics, Australia) for the detection of NheA, whereas such tools were missing for emetic *B. cereus* strains, at the beginning of this study. For the conclusive identification of the emetic toxin cereulide only laborious methods were available, e.g. high-performance liquid chromatography connected to ion trap mass spectrometry (HPLC-MS), a cytotoxicity assay using cell culture, or a bioassay working with boar sperms (Finlay et al. 1999; Häggblom et al. 2002; Andersson et al. 2004).

1.4 Aims of this work

Aim of this work was to perform an in-depth physiological and molecular characterization of the *B. cereus* group of organisms. In general, more information about the physiological and molecular characteristics are available for diarrhoeal *B. cereus* than for emetic strains. Therefore special emphasis was placed on the characterization of the latter. Diagnosis of emetic *B. cereus* was hampered by the lack of genetic information on the molecular basis of emetic toxin production and no suitable detection methods for routine diagnostics were available. Hence, a major goal of this work was the development of molecular detection systems for a fast and unequivocal identification of emetic strains, which can be implemented in food and clinical diagnostic laboratories.

2 MATERIAL AND METHODS

2.1 Bacterial strains

Different, partly overlapping strain sets of *Bacillus cereus* group isolates were compiled for the development and validation of the different methods presented in this work and are listed in the Appendix 7.1. Special care was taken, to include a representative number of *B. cereus* group strains isolated from various origins, like environments, food, food borne outbreaks and clinical settings in each strain set. In addition, a selection of type strains from other *Bacillus* species and isolates from non-*Bacillus* species were used to assess the specificity of the developed molecular methods (standard PCR, Real-time PCR and toxin gene profiling).

All *B. cereus* group strains were either characterized in previous studies (Prüß et al. 1999; Guinebretiere et al. 2002), or additional tests were accomplished to assure the correct classification of the strains. The following parameters were used for strain characterization: starch hydrolysis, hemolysis, presence of the emetic toxin (*ces*) genes in *B. cereus*, the crystal toxin (*cry*) genes and/or the crystal toxin in *B. thuringiensis* and the cold shock protein A (*cspA*) gene in psychrotolerant strains as described elsewhere (Bourque et al. 1993; Francis et al. 1998; Ehling-Schulz et al. 2005a; Hendriksen and Hansen 2006).

2.2 Cultivation methods

All *B. cereus* group strains were grown in plate count (PC) broth or on PC agar (5.0 g tryptone, 2.5 g yeast extract, 1.0 g D-glucose, 15 g agar per litre) at 30°C over night. *Bacillus* species and non-*Bacillus* species were grown in Luria-Bertani (LB) broth or on LB agar (10 g tryptone, 5 g NaCl, 5 g yeast extract, 15 g agar per litre) at 30°C or 37°C.

Isolates grown on agar slants were stored at 4°C for up to 4 weeks. For long-time storage, glycerine stocks of the isolates including 13 % glycerine were prepared from overnight cultures in PC or LB broth and were stored at -80°C. The main part of the isolates was already included in the WS (Weihenstephan) or WSBC (Weihenstephan *Bacillus* collection) culture collection. New isolates were freeze dried and either added to the WS or WSBC collection.

Standard reference culture methods

Cell counts in food samples were determined in accordance with standard reference culture methods recommended by food authorities (§64 Lebensmittel-, Bedarfsgegenstände- und Futtermittelgesetzbuch (LFGB), International Organization for Standardization (ISO), Food and Drug Administration (FDA)). In brief, 25 g food sample were homogenized in a Stomacher (Lab-Blender 400, Kleinfeld Labortechnik, Germany) with 225 ml BHIG broth (brain heart broth (Merck, Germany) supplemented with 0.1 % glucose) and were incubated at 37°C for 24 h without shaking. Serial dilutions of the enrichment were plated on LB, PEMBA (polymyxin-egg yolk-mannitol-bromothymol blue agar; Oxoid, Germany), MYP (mannitol-egg yolk-polymyxin agar, Oxoid) and Baird Parker agar (Oxoid) and incubated at 37°C as prescribed. In addition, two different chromogenic plating media specific for the detecion of *B. cereus* group strains were applied. The Chromogenic Bacillus Cereus agar (CBC; Oxoid) and both standard media (PEMBA and MYP) were prepared according to the manufacturer's instructions. The BCM *Bacillus cereus* group plating medium (Biosynth AG, Switzerland) - commercially available as Cereus Ident Agar (CEI; Heipha, Germany) - was provided by the Robert Koch-Institut (Wernigerode, Germany) as ready-to-use plates. Inoculated PEMBA and CBC plates were incubated for 24 h at 37°C, MYP, CEI and LB plates for 24 h at 30°C.

Natural and artificial contaminated food samples

Natural *B. cereus* contaminated food samples, like skimmed milk powder, rice, and two different spices, roughly ground dried ginger and mace, were used to challenge the four different selective plating media for *B. cereus* (PEMBA, MYP, CBC and CEI). In brief, a 25 g representative portion of each sample was homogenised with 225 ml BHIG broth. Serial dilutions were plated on the four media types and were incubated as described above. For spiking experiments, food samples were cooked and tested for the absence of any naturally occuring contamination with *B. cereus* and *S. aureus* by the standard reference culture methods described above. As no *B. cereus* and *S aureus* were detected, the food was used for artificial contamination. 25 g cooked rice or pasta was homogenized in 225 ml BHIG broth, and the mixture was inoculated with serial dilutions of the *B. cereus* reference strain for emetic toxin F4810/72 and/or the *Staphylococcus aureus* strain WS2608 overnight culture in the range of 10^0 to 10^3 cfu/g food. Enrichment was carried out at 37°C without shaking. Samples were taken after 0, 2, 4, and 6 h of enrichment for DNA isolation and for the determination of cell counts on LB, PEMBA or Baird Parker agar. 1 ml aliquots of enriched

samples were centrifuged (13,000 g for 4 min) and pellets were stored at -20°C until DNA isolation and PCR analysis.

Isolation of *B. cereus* group members from soil and a recent food poisoning

Soil samples were collected in spring 2006 on a field near Lausanne, Switzerland, and in the Golden Bay on Malta. In addition, a soil sample from a field in Freising-Weihenstephan (Germany) was added to the collection. 10 g of each soil sample were diluted 1:10 in ¼ Ringer solution (Merck, Germany). 10 ml of the mixture were incubated at 80°C for 10 min and serial tenfold dilutions were plated on PEMBA and PC agar. *B. cereus* group isolates were selected randomly and were subjected to further analysis. Isolates were named S1-110 for the sample from Switzerland, M1-110 for the sample from Malta, and R1-110 for the field sample from Freising-Weihenstephan. From the food sample (a rice dish) associated with a recent food poisoning in Southern Germany (for details see 3.6.2), 25 g of sample was homogenized in 225 ml BHIG broth and serial tenfold dilutions were plated as described for the soil samples. Isolates were named KG1-92. In addition, food remnants from the outbreak were tested for the presence of cereulide by the Hep-2 cytotoxicity assay (Finlay et al. 1999; Ehling-Schulz et al. 2005a).

2.3 DNA isolation methods

DNA used for standard PCR and slot-blot analysis was isolated from overnight cultures with the AquaPure genomic DNA isolation kit (Biorad, Germany) or the Puregene DNA isolation kit (Gentra, USA) according to the manufacturer's instructions. For inverse PCR, standard curves for Real-time PCR and Southern analysis, total chromosomal DNA was isolated by phenol-chloroform extraction (Sambrook et al. 1989). In brief, after lysis (0.5 % sodium dodecylsulfate (W/V), 0.1 mg/ml proteinase K, 37°C, 3 h), cell wall debris, denatured proteins and polysaccharides were complexed to hexadecyltrimethylammonium bromide and removed by phenol-chloroform extraction. DNA was precipitated with 2-propanol, washed with ethanol (70 %) and dissolved in water. RNA was digested with 0.1 mg/ml RNase A (37°C, 30 min). After phenol-chloroform extraction, DNA was precipitated with 3 M natrium acetate and 100 % ethanol, washed with 70 % ethanol and dissolved in water. For *Staphylococcus aureus*, the cell wall was digested with 23.5 U/ml lysostaphin (37°C, 1 h) prior to DNA isolation with an alkaline sodium dodecylsulfate (Carnio et al. 2001).

Total DNA from food samples was isolated using either the AquaPure genomic DNA isolation kit (Biorad, Germany) or the NucleoSpin food kit (Macherey-Nagel, Germany)

according to the manufacturer's instructions. In addition, DNA from food samples was extracted using a simple boiling method. In brief, cells from 1 ml enriched samples were harvested by centrifugation, cell pellets were resuspended in 300 µl sterile MilliQ, boiled for 10 min and then cooled on ice. After pelleting cell and food residues, a 5 µl aliquot of the supernatant was used as PCR template.

2.4 Molecular characterization

2.4.1 Standard PCR

Mixtures (50 µl) for standard PCR contained 0.2 mM of each dNTP, 1.5 mM $MgCl_2$, 1 µM of the forward and the reverse primer, 1 U of ThermoStart *Taq* DNA polymerase (ABgene, Epsom, UK), 5 µl 10 x polymerase buffer and 1 µl of template DNA. The standard PCR protocol started with a denaturation step for 15 min at 95°C, followed by 30 cycles of 30 s at 95°C, 30 s at the primer annealing temperature and an elongation step at 72°C. Elongation time was dependent on the length of the expected amplicon. The protocol ended with a final elongation step at 72°C for 2 min. All primers used in this study are listed in Appendix 7.2.

2.4.2 Inverse PCR and module jumping

Inverse PCR allows the amplification of unknown DNA sequences flanking a region with known sequence. 0.4 µg of total chromosomal DNA of the emetic reference strain F4810/72 were digested for 3 h with 10 U of the selected restriction enzyme (e.g. HpaI, Fermentas, Germany) in a total volume of 20 µl including the appropriate restriction buffer. The resulting DNA fragments were self ligated with T4-DNA-Ligase (Roche, Germany) in a total volume of 100 µl according to the manufacturer's instructions. Primers for inverse PCR pointed in opposite directions in the known sequence, whereupon one primer pointed to the selected restriction site and the other one in direction of the unknown sequence. 10 µl of the ligation was used for inverse PCR using the Expand High Fidelity PCR System (Roche, Germany) according to the manufacturer's instructions.

For module jumping, degenerated primers are designed, targeting e.g. highly conserved modules of non-ribosomal peptide synthetase (NRPS) genes. In combination with a specific primer located in the known sequence, entire modules can be amplified and sequenced. PCR mixture and conditions were applied as described for the standard PCR for

amplicons up to 3 kb. Longer templates were amplified with the Expand High Fidelity PCR System (Roche, Germany) as described for inverse PCR

2.4.3 Cloning of PCR amplicons, DNA sequencing and sequence analysis

Amplification products obtained from PCR performed with degenerated primers and amplicons obtained from module jumping were subcloned in the TOPO TA vector (Invitrogen, Germany) and sequenced (Sequiserve, Germany) using a DNA DyeDeoxy terminator cycle sequencing kit (Applied Biosystems). PCR products obtained by inverse PCR were either subcloned in TOPO TA or directly sequenced after purification with the QIAquick PCR purification kit (QIAGEN, Germany).

The fragments amplified by inverse PCR and module jumping were assembled with the software ContigExpress, a component of Vector NTI Suite 8.0 (InforMax, Inc.). The resulting sequence was searched against the sequenced genomes of *B. cereus* group members. Sequence similarity searches were performed using the Basic Local Alignment Search Tools BLASTX and BLASTP on the NCBI website (Altschul et al. 1990; Altschul et al. 1997).

2.4.4 Sequence typing

Selected isolates were subjected to multilocus sequence typing (MLST) analysis as described previously (Helgason et al. 2004). In brief, internal fragments of seven housekeeping genes (*adk*, *ccpA*, *ftsA*, *glpT*, *pyrE*, *recF*, *sucC*) were amplified and sequenced as described in 2.4.3. Concatenated sequences of all genes were prepared for each strain and were aligned together with published sequences with the software package Clustal_X (Thompson et al. 1997). Cluster analysis was performed using the TREECON software (Van de Peer and De Wachter 1997). Distances were calculated based on the Kimura's two parameter model (Kimura 1980) and tree topology was inferred by clustering and UPGMA.

The sporulation stage III AB gene (*spoIIIAB*) was used as an additional genetic locus for sequence typing. The PCR protocol was adjusted to 59°C primer annealing and 1 min elongation time in order to amplify 577 bp of this gene with the primers Spo2F (5´-CGACG-AGGATAACCCAATTTGC-3´) and Spo2R (5´-CAGTGAGAGACCGAGGCAAC-3´) as described previously (Ehling-Schulz et al. 2005a). Sequence alignment and construction of phylogenetic trees were done as described for MLST

2.4.5 Amplification and sequencing of the *plcR* regulator gene

Nucleic acid sequence data for the *plcR* regulator gene was retrieved from sequences published in the GenBank database (*B. anthracis*: AF132086, AJ585425; *B. cereus*: AF132087, AF195601; *B. thuringiensis*: AJ582669, AJ582674, AJ582675, AJ583467). The primer combination PlcR_F1 + PlcR_R2 and PlcR_F2 + PlcR_R1 were used for the amplification of the *plcR* gene from selected strains. Primer sequences are listed in Table 1. DNA isolation and preparation of the PCR mastermix were performed as described in 2.4.1. The PCR protocol started with a denaturation step for 15 min at 95°C, 5 cycles of 30 sec at 95°C, 1 min at 48°C, 1 min at 72°C, followed by 25 cycles of 30 sec at 95°C, 1 min at 52°C, 1 min at 72°C and ended with a final elongation step at 72°C for 5 min. Sequencing of the PCR products, sequence alignment and cluster analysis were performed as described in 2.4.4.

Table 1: Oligonucleotides used for the amplification of the *plcR* gene.

Primer	Sequence (5´ to 3´)	Position in the reference sequence AJ582674
PlcR_for1	GCACGCAGAAAAATTAGGAAGTG	3 - 25
PlcR_for2	AGGGTGATGAGAGGATTAACAC	40 - 62
PlcR_rev1	ATGG(A/T)TTC(C/T)TAATATATC(A/G)AAAAAGAAG	822 - 792
PlcR_rev2	CTAATATATC(A/G)AAAAAGAAG(CT)(A/T)(A/T)GC	811 - 787

2.4.6 Standard PCR assay for detection of emetic *B. cereus*

Degenerate oligonucleotide primers targeting highly conserved motifs of known non-ribosomal peptide synthetases (NRPS, (Marahiel et al. 1997; Carnio et al. 2001)) were used to amplify and identify putative NRPS gene fragments in *B. cereus*. Based on the sequence information derived from an amplified DNA fragment, specific primers and probes for detection of emetic *B. cereus* strains were designed. The primers EM1F (5´- GACAAGAGA-AATTTCTACGAGCAAGTACAAT-3´) and EM1R (5´-GCAGCCTTCCAATTACTCCTT-CTGCCACAGT-3´) amplify a fragment of 635 bp from emetic *B. cereus* DNA. The standard PCR protocol (see 2.4.1) was adjusted to a primer annealing temperature of 60°C and an elongation time of 1 min.

The specificity of the assay was tested with the strain set listed in Appendix 7.1. The strain set comprised 162 *B. cereus* (including 52 emetic isolates), 35 *B. cereus* group, 8 *Bacillus* sp. and 41 non-*Bacillus* species isolates. Selected strains were amplified in parallel with general 16S rDNA primers (8-26/56: 5´-AGAGTTTGATCCTGGCTCA-3´; 1511-1493:

5′-CGGCTACCTTGTTACGAC-3′) derived from *E. coli* as a positive control (Stackebrandt and Liesack 1992).

2.4.7 SYBR green Real-time PCR for detection of emetic *B. cereus*

The emetic *B. cereus*-specific SYBR green I primers ces_SYBR_F (5′-CACG-CCGAAAGTGATTATACCAA-3′) and ces_SYBR_R (5′-CACGATAAAACCACTGAG-ATAGTG-3′) were derived from the cereulide synthetase (*ces*) gene sequence (accession no. DQ360825), thereby targeting a region of the *ces* genes shown to be highly specific for emetic *B. cereus* (Ehling-Schulz et al. 2006a). For the amplification of a *S. aureus*-specific genomic DNA fragment, primers targeting the *tuf* gene (Martineau et al. 2001), which encodes the elongation factur Tu, were included in the SYBR green I-based Real-time PCR assay (sa_SYBR_F: 5′-CGTGTTGAACGTGGTCAAATCA-3′ and sa_SYBR_R: 5′-CACCTTCG-TCTTTTGATAATACG-3′). More details on the primer are given in Table 2. The bacterial strain set used to assess the inclusivity and exclusivity of the Real-time PCR assay is overlapping with the strain set used for the standard PCR and is provided in Appendix 7.1.

A typical 25 µl Real-time PCR mixture consisted of 12.5 µl SYBR Premix Ex Taq (Takara Bio, Inc., Japan), 0.3 µM for each emetic *B. cereus* primer, 0.12 µM for each *S. aureus* primer, and template DNA. Usually 5 µl were applied from DNA isolated with the NucleoSpin food kit or from the simple boiling method, whereas only 1µl were applied from total chromosomal DNA isolated by phenol-chloroform extraction, DNA isolated with the AquaPure genomic DNA isolation kit or with the Puregene DNA isolation kit. No-template controls that contained 5 µl TE buffer instead of DNA were included in each run to detect any contamination. Typical cycling conditions using a SmartCycler II (Cepheid) were 95°C for 10 s, followed by 45 cycles at 95°C for 10 s and 60°C for 30 s. The ramp rate was set on maximum (10°C/sec) for the simplex detection of emetic *B. cereus* strains and altered to 3°C/sec for simplex and duplex detection involving the primers specific for *S. aureus*. In the subsequent melting curve analysis, the temperature was raised from 60°C to 95°C with a ramp rate of 0.2°C/sec. The specificity and robustness of the assay was tested in two independent labs on two different cycler systems (SmartCycler II and Stratagene MX3000P, Stratagene), revealing consistent results and similar detection limits.

Standard curves were generated using a tenfold dilution series of DNA ranging from 600 ng to 6 fg. C_T values were calculated with the standard algorithms provided with the two cycler systems. The PCR efficiency (E) was calculated according to Pfaffl (Pfaffl 2001) from

the standard curve with E = $10^{-1/slope}$-1. Additionally, an assay precision test according to Malorny et al. (Malorny et al. 2004) was performed for the SYBR Green I simplex assay.

Table 2: Oligonucleotide primers used for the different Real-time PCR assays.

Primer	Sequence (5´ to 3´)	Position	Sequence reference	Reference
ces_SYBR_F	CACGCCGAAAGTGATTATACCAA	8743 - 8765	DQ360825	Fricker et al. (2007)
ces_SYBR_R	CACGATAAAACCACTGAGATAGTG	8895 - 8918		
sa_SYBR_F	CGTGTTGAACGTGGTCAAATCA	389 - 410	AF298796	Derived from Martineau et al. (2001)
sa_SYBR_R	CACCTTCGTCTTTTGATAATACG	628 - 650		
ces_TaqMan_for	CGCCGAAAGTGATTATACCAA	8745 - 8765	DQ360825	Fricker et al. (2007)
ces_TaqMan_rev	TATGCCCCGTTCTCAAACTG	8828 - 8847		
ces_TaqMan_probe	FAM - GGGAAAATAACGAGAAATGCA - TAMRA	8798 - 8818		
IAC_for	GCAGCCACTGGTAACAGGAT	1216 - 1235	L09137	Fricker et al. (2007)
IAC_rev	GCAGAGCGCAGATACCAAAT	1314 - 1333		
IAC_probe	HEX - AGAGCGAGGTATGTAGGCGG - TAMRA	1240 - 1259		

2.4.8 TaqMan Real-time PCR including an internal amplification control

The emetic *B. cereus*-specific TaqMan primers and probe anneal in the same region of the *ces* gene cluster as the SYBR Green I primers. The commercially available plasmid pUC19 (Fermentas, Germany) was used as internal amplification control (IAC) without any modifications. IAC TaqMan primers and probe were placed in the pMB1 replicon *rep*. Optimal IAC concentration was assessed to be approximately 170 copies per PCR reaction. All TaqMan primers and probes were designed using Primer3 software available under www.frodo.wi.mit.edu/cgi-bin/primer3 and are specified in Table 2. The specificity of the sequences was tested by searches against NCBI´s nonredundant database using the BLASTN algorithm (Altschul et al. 1990). The emetic *B. cereus* target probe was labelled at the 5' end with the reporter dye 6-carboxyflourescein (FAM), and the IAC probe was labelled at the 5' end with 5-hexachloro-6-carboxyfluorescein (HEX). Both probes were labelled at the 3' end with tetramethyl-6-carboxyrhodamine (TAMRA). The TaqMan Real-time PCR system was challenged with the same strain set as described for the SYBR Green I assay.

A typical 25 µl PCR mixture contained 12.5 µl Brilliant QPCR Multiplex Mastermix (Stratagene, USA), 0.5 µM of each primer (for emetic *B. cereus* and IAC each), 0.2 µM

emetic *B. cereus* probe (ces_TaqMan_probe) and 0.2 µM IAC probe (IAC_probe), approximately 170 copies of plasmid DNA pUC19 (Fermentas, Germany) and 5 µl of the sample DNA. No-template controls that contained 5 µl TE buffer instead of DNA were included in each run to detect any contamination. Typical cycling conditions using a Stratagene MX3000P Real-time PCR system (Stratagene, USA) were 95°C for 10 min, followed by 40 cycles at 95°C for 15 sec and 55°C for 60 sec. The ramp rate of the Stratagene MX3000P Real-time PCR system has a maximum of 2.5°C/sec. Implementation of the assay on the SmartCycler II revealed consistent results and similar detection limits. Standard curves and efficiency were determined as described for the SYBR Green I assay.

2.4.9 Application of the novel Real-time assays in two recent food poisonings

DNA for Real-time PCR was isolated directly from the food remnants connected to two recent emetic outbreaks in Southern Germany using the NucleoSpin food kit and the simple boiling method as described in 2.3. Additional, cell counts were determined with the standard reference culture methods but without enrichment. Selected isolates were typed with toxin gene profiling as described below. Cereulide was extracted from the food remnants as follows: 5 g sample were homogenized in 5 ml sterile MilliQ and were autoclaved (20 min at 21°C). 10 µl of the supernatant were then tested in a HEp-2 cell culture assay as described previously (Ehling-Schulz et al. 2005b). The commerically available ELISA system VIDAS Staph. enterotoxin II (bioMérieux, France) was used to test the food remnants for *S. aureus* enterotoxins according to the manufacturer´s instructions.

2.4.10 Multiplex PCR Assay for toxin gene profiling of toxigenic *B. cereus*

Fragments of the enterotoxin genes *nhe*A, *nhe*B, *hbl*D and *hbl*A were amplified from diverse *B. cereus* strains and sequenced as described previously (Guinebretiere et al. 2002). Primers used for amplification and sequencing are provided in the Appendix 7.2. The resulting sequences and sequence data from *B. cereus* enterotoxin genes retrieved from databases were aligned using the software package Clustal W (Thompson et al. 1997). Positions and partial sequences were determined with reference to GenBank nucleic acid sequence data; accession no. AJ277962 for *cyt*K, AJ007794 for the *hbl* genes, and Y19005 for the *nhe* genes. 18-20 bp oligonucleotide primers were designed with the Primer Designer software (Becker et al. 1995). In a second step, sequence polymorphisms previously identified by sequence alignments, were taken into account by substituting the bases at variable

positions by inosine. For the detection of *B. cereus* strains possessing the emetic toxin gene cluster, previously published primers were added to the assay (Ehling-Schulz et al. 2005b). The resulting primers are presented in Table 3.

Table 3: Oligonucleotides used for toxin gene profiling (according to Ehling-Schulz et al. 2006b).

Primer[a]	Gene	Amplicon length (bp)	Sequence (5´to 3´)	Position[b]	Sequence reference strain (accession No.)
HD2F HA4R	hbl	1091	GTAAATTA**I**GATGA**I**CAATTTC AGAATAGGCATTCATAGATT	1188 2279	ATCC 14579^T (AJ007794)
NA2F NB1R	nhe	766	AAGC**I**GCTCTTCG**I**ATTC **ITI**GTTGAAATAAGCTGTGG	608 1374	NVH 1230-88 (Y19005)
CKF2 CKR5	cytK	421	ACAGATATCGG**I**CAAAATGC CAAGT**I**ACTTGACC**I**GTTGC	1859 2280	NVH 0391-98 (AJ277692)
CesF1 CesR2	ces	1271	GGTGACACATTATCATATAAGGTG GTAAGCGAACCTGTCTGTAACAACA	21816 23087	F4810/72 (DQ360825)

[a] Primers for the detection of the enterotoxin genes were designed during this study, whereas the primers for the detection of emetic toxin producing *B. cereus* strains (Ces-primers) were published by Ehling-Schulz et al. (2005b).
[b] Primer position relative to sequence reference.

PCR conditions were optimized for primer and $MgCl_2$ concentrations. The final mixture (50 µl) contained 0.2 mM of each dNTP, 3 mM $MgCl_2$, 0.2 µM of the oligonucleotide primers CesF1 and CesR2, 1 µM of HD2F and HA4R; 0.3 µM of NA2F and NB1R; 0.4 µM of CKF2 and CKR5; 1 U of ThermoStart *Taq* DNA polymerase (ABgene, Epsom, UK), 5 µl 10 x polymerase buffer and 1 µl template DNA. The PCR protocol was derived from the protocol for standard PCR (see 2.4.1) with 49°C as primer annealing temperature and 1 min elongation. The specificity of the multiplex PCR assay was evaluated with a divers panel of *B. cereus* strains with known toxin gene profiles (for details see Appendix 7.1). Additionally, strains from other *B. cereus* group members, other *Bacillus* species and non-*Bacillus* species were added to the test panel to assess the specificity of the assay (see Appendix 7.1).

2.4.11 Southern analysis

For Southern blot analysis, chromosomal DNA of selected strains was digested with *Hpa*I, Fermentas, Germany). The fragments were separated on a 1 % agarose gel and blotted onto Hybond-N+ nitrocellulose. Southern analysis (Southern 1975) was performed using a

260 bp digoxigenin-labelled probes (Roche, Germany) amplified with the primers HpaIF (5´-GCCAGAAGATGCAATGATTCCAGTATG-3´) and EM1F (see 2.4.6) located inside the amplicon specific for emetic *B. cereus* strains used for standard PCR. The probe was obtained from the emetic reference strain F4810/72. In order to ensure that the bacterial cell lysis protocol was efficient for all species tested, hybridizations with a digoxigenin-labelled probe targeting a conserved region of the 16S rDNA gene (Martineau et al. 1996) were performed.

2.5 Phenotypic characterization

2.5.1 Test of growth limits

Bacteria were grown for 18 h in 5 ml PC broth at 30°C and 170 rpm. Fresh PC broth (5 ml) was inoculated with 10 µl of the overnight culture (corresponding to approx. 10^5 cfu/ml), and incubated at different temperatures at 170 rpm. Lower growth limits were tested at 5°C, 7°C and 10°C, upper growth limits at 45°C and 48°C. In addition, strains that did not grow at 45°C were also tested at 42°C, and those that grew at 48°C were also tested at 52°C. The tubes were sealed with parafilm to avoid evaporation and concentration of the liquid medium at higher temperatures. Growth was determined via optical density at 585 nm with a portable WinLab photometer LF2400 (Windaus Labortechnik, Germany) after 1, 2, 5, 7, 9, and 12 days. Additionally, growth was measured with a Perkin-Elmer 550SE UV/VIS spectrophotometer at 600 nm (Perkin-Elmer, Germany) after 7 and 12 days. Growth was defined as an increase of OD_{600} by at least 0.1.

2.5.2 Influence of temperature and pH on growth

Growth curves were determined with a multiwell photometric plate reader Bioscreen C (Labsystems Corp., Helsinki, Finland). Overnight cultures of the test strains were grown in 5 ml PC broth, pH 7.0, at 30°C and 170 rpm. A 10 µl aliquot of each culture was diluted in 190 µl of fresh PC broth, and a 10 µl aliquot of this dilution was used to inoculate 240 µl of PC broth (corresponding to approx. 10^5 cfu/ml) with different pH values in multiwell plates of the Bioscreen C. PC broth was adjusted to pH 5 with 1 M piperazin solution (Aldrich, Germany), and PC broth with pH 7 or pH 8 was buffered with 1 M Tris-HCl solutions having the designated pH. The plates were incubated under continuous shaking in the plate reader at 24°C and at 37°C. OD_{600} was recorded every 60 min for 48 h with the

computer software Biolink (Transgalactic Ltd., Finland, version 5.30). Data were exported to Microsoft Excel for statistical analysis.

2.5.3 Gamma phage lysis assay and antibiotic resistance

Preparation of the *Bacillus anthracis* specific gamma phage solution was prepared as described elsewhere (Abshire et al. 2005). Strains were streaked on blood agar or CEI and 5 µl of the gamma phage solution were spotted after drying on the inoculated plate. Plates were incubated at 37°C and controlled for plaque formation after 9 and 22 h. Resistance to penicillin was tested with filter paper discs (5 mm in diameter), loaded with 10 µg of penicillin G (Sigma, Germany). Plates were flooded with an overnight culture of the strain and the filter paper discs loaded with the antibiotic were carefully placed on the inoculated agar surface. Inhibition zones were controlled after 8 and 24 h incubation at 37°C, except for the *B. weihenstephanensis* type strain that was incubated at 30°C.

2.5.4 Analysis of phosphatidylcholine-phospholipase C (PL-PLC) activity

Selected strains were tested for their PC-PLC activity. Therefore the chromogenic substrate 5-bromo-4-chloro-3-indoxyl-cholinphosphate (X-CP) was added to the components of the CEI formula. Respective plates were incubated at 30°C for 24h. Positive colonies are characterised by a homogeneous blue-turquoise colour.

2.5.5 Fourier transform infrared (FTIR) spectroscopy

The strains stored on plate count agar were streaked on tryptone soy agar (TSA) plates (Oxoid, Germany) and incubated at 25°C for 16 to 18 h. From this pre-culture the cells were grown as lawns on TSA plates at 25°C for 24 h ± 30 min. Samples were prepared as described previously (Oberreuter et al. 2002). In brief, one loop of cell material was suspended in 100 µl sterile deionised water. Isolates yielding a clumpy suspension were subjected to ultrasonication for 3 x 1 sec at 25 % power with a Bandelin Sonopuls HD2200 (Bandelin electronic, Berlin) in order to improve spectral quality. Spectra were recorded on an IFS 28/B spectrometer (Bruker Optics, Germany) and the quality of IR spectral data was evaluated using OPUS software (version 5.5; Bruker Optics). Additionally, first and second derivatives were calculated using the Savitzky-Golay algorithm with 9 smoothing points for further data processing. At least 10 independent measurements were prepared per strain to yield the number of spectra per strain required for the training of the artificial neural networks (ANN).

Spectral windows from 3030 to 2830, 1350 to 1200 and 900 to 700 cm^{-1} with weight factor 1 and repro-level 30 were used for univariate hierarchical cluster analysis (HCA). For the examination of the replicate measurements of each strain, the first derivative of the spectra with 9 smoothing points was used, and the HCA function of the OPUS software with the Ward's algorithm was applied. Ten replicate spectra of each strain with a heterogeneity level lower than 1.2 were chosen for further analysis. Additionally, one spectrum of each strain was randomly chosen to construct a HCA of all strains used in this study. Therefore the second derivative of the original spectra was calculated with 9 smoothing points and again the heterogeneity was determined with the Ward's algorithm.

2.5.6 Artificial neural networks (ANN)

A collection of 108 *B. cereus* group strains including 29 non-emetic *B. cereus*, 26 emetic *B. cereus*, 15 *B. weihenstephanensis*, 14 *B. mycoides*, and 18 *B. thuringiensis* isolates were used for the training and the validation of the developed ANN (details on strains are given in the Appendix 7.1). The software program NeuroDeveloper (Synthon, Germany) (Udelhoven et al. 2003) was used to perform feature selection and to establish a two-layer neural network. In a first step the 650 spectra of the reference strain set (for details see Appendix 7.1) were randomly distributed into a training set (5 spectra per strain), a pre-validation set (3 spectra per strain) and a test set (2 spectra per strain) for internal validation. Two spectral windows from 3100 to 2800 and 1800 to 700 cm^{-1} were pre-determined for the artificial neural network analysis. Again the second derivative of the original spectra with 9 smoothing points was selected and all spectra were vector normalized. The multivariate covar algorithm was applied to select the most discriminative wave numbers and their ranking for the calculation of the ANN (Udelhoven et al. 2003). The first level of the ANN had 100 input neurons (= wave numbers), one hidden layer, and three output units as depicted in Figure 14. The ANNs for the second level had two hidden layers and two output units. The developed ANN was then challenged with 43 strains of the *B. cereus* group in an external validation. Identification with the ANN results in three classifications: (i) correct identification, (ii) misidentification, if a spectra is assigned to a wrong *B. cereus* group member and (iii) non-identified, if the ANN was not able to assign a spectra to any of the *B. cereus* group members.

3 RESULTS

3.1 Genotyping

Multilocus sequence typing (MLST) was accomplished from selected *B. cereus* group strains using seven housekeeping genes: *adk* (adenylate cyclase), *ccpA* (catabolic control protein A), *ftsA* (cell division protein), *glpT* (glycerol-3-phosphate permease), *pyrE* (orotate phosphoribosyl transferase), *recF* (DNA replication and repair protein), and *sucC* (succinyl coenzyme A synthetase). Concatenated sequences of these strains and sequences from selected strains belonging to the *B. cereus* group of organisms retrieved from databases (http://mlstoslo.uio.no/index3.html, see also: Helgason et al. 2004) were aligned and used for cluster analysis. Three distinct clusters were observed: clinical *B. cereus* and *B. thuringiensis* cluster together with *B. anthracis* and the emetic type of *B. cereus* in cluster I. Cluster II represents mesophilic *B. thuringiensis* and non-emetic *B. cereus* isolates, whereas the psychrotolerant members of the *B. cereus* group – *B. weihenstephanensis* and *B. mycoides* – fall in cluster III (Figure 1A). Analysis of the sporulation stage III AB gene (*spoIIIAB*) as additional chromosomal genetic locus reveals the formation of the same three major clusters as for the MLST scheme (Figure 1B). With both sequence typing methods, all emetic *B. cereus* isolates except one (RIVM BC 67) form a monomorphic subcluster within cluster I. The latter strain is one of the two emetic *B. cereus* strains described so far, which possess the *cytK* gene, in addition to the emetic toxin gene clusterand the *nhe* gene cluster.

The gene encoding the pleiotropic regulator *plcR* from selected isolates with typical, and of all isolates with atypical growth on PEMBA, MYP and CEI was amplified and sequenced. The sequences were aligned and compared to *plcR* database sequences from strains belonging to the *B. cereus* group of organisms (Figure 2). The *plcR* gene of two strains (WSBC 10377 and SDA VI 273) could not be amplified. Both strains showed atypical growth on PEMBA, one strain also on CEI. All sequenced *plcR* genes, but one (F3351/87), amplified from *B. cereus* strains with atypical colony appearances cluster together with the *plcR* from *B. anthracis*. In contrast, no sequences from isolates with typical growth on the plating media cluster to *B. anthracis* (Figure 2).

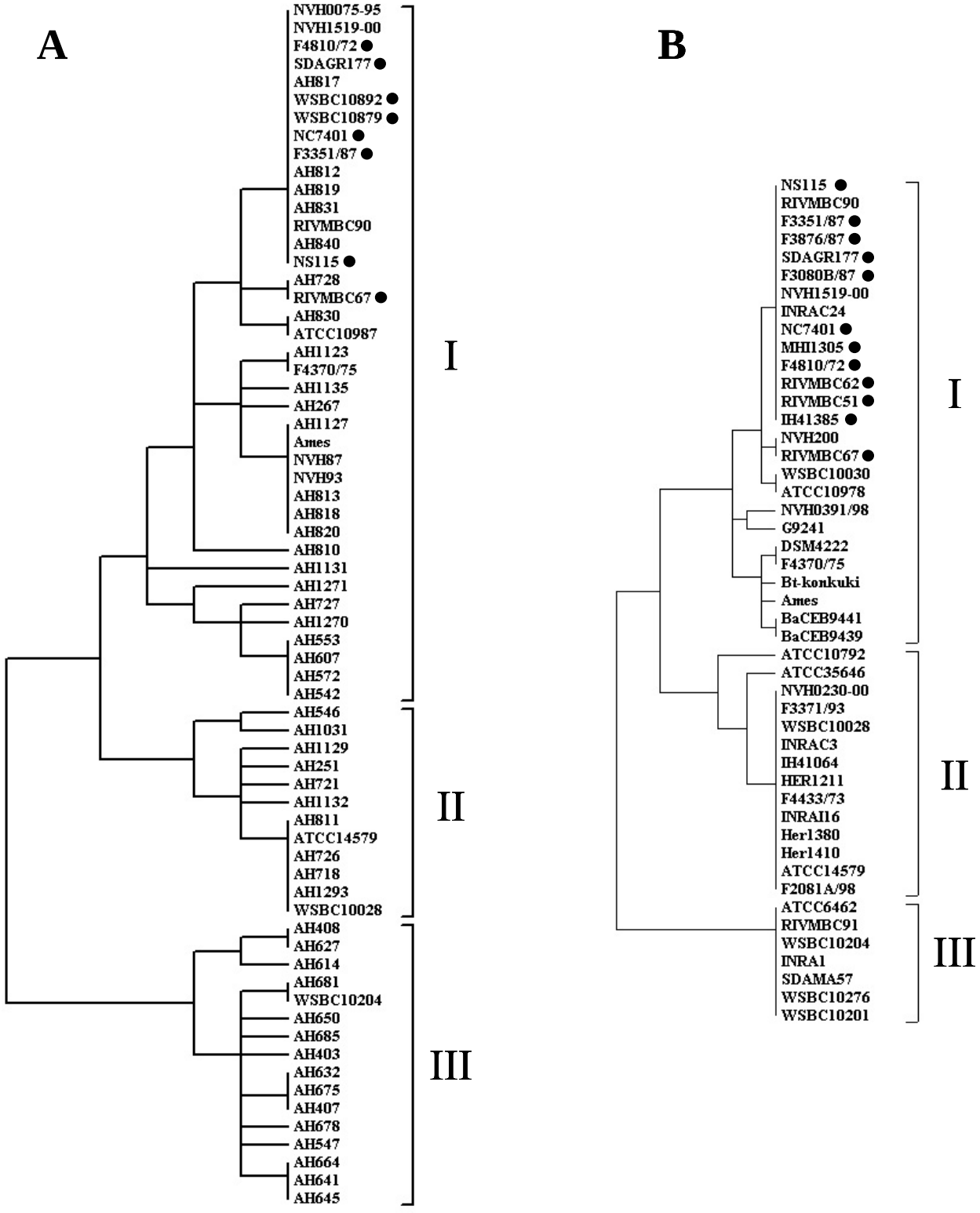

Figure 1: Comparison of MLST analysis and *spoIIIAB* as genetic marker gene for the genetic typing of *B. cereus* group members. Both methods revealed the formation of three distinct groups. Cluster I comprises the emetic type of *B. cereus* together with *B. anthracis* and *B. thuringiensis* isolates. Cluster II consists of mesophilic *B. cereus* and *B. thuringiensis* isolates, whereas the psychrotolerant members of the *B. cereus* group are found in Cluster III. A: MLST analysis of selected *B. cereus* group members with the MLST scheme according to Helgason et al, 2004. B: Cluster analysis of the *spoIIIAB* gene of selected *B. cereus* group members. Dots indicate emetic *B. cereus* strains.

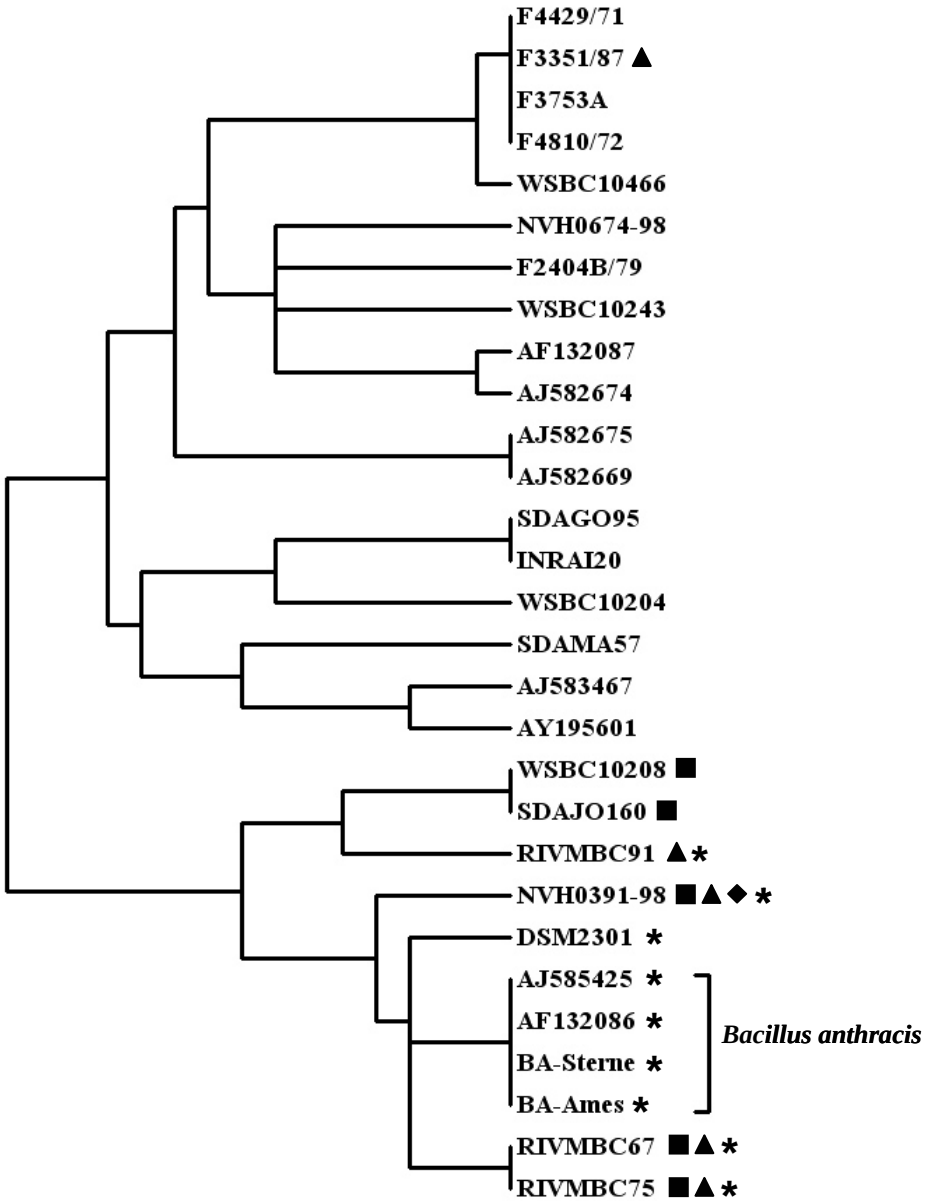

Figure 2: Genetic relationship based on the gene sequences encoding the pleiotropic regulator *plcR* of *B. cereus* group members. The tree was constructed with the TREECON software and shows the position of selected *B. cereus* isolates with typical and atypical growth appearance. Sequences designated AFxxxxxx, AJxxxxxx and AYxxxxxx refer to *plcR* sequences of *B. cereus* group strains retrieved from GenBank database. Symbols indicate atypical strains on the following media: squares on PEMBA, triangles on MYP, and diamonds on CEI. * indicates non-hemolytic isolates (for reference see Ehling-Schulz et al. 2005a).

3.2 Development of novel PCR assays

Starting from amplification products obtained with degenerated primers targeting A3 and A7 core motifs of non-ribosomal peptide synthetases (NRPS) (Ehling-Schulz et al. 2005b), the sequence of the *ces* gene cluster was determined by inverse PCR and module jumping. Subsequently, the resulting genetic information was used to develop standard and Real-time PCR assays.

3.2.1 Standard PCR assay for the detection of emetic *B. cereus*

Based on the amplification product obtained with degenerated primers targeting A3 and A7 core motifs of non-ribosomal peptide synthetases (NRPS), a PCR system specific for emetic *B. cereus* strains was developed. The 800 bp fragment exclusively amplified from emetic *B. cereus* strains was used for the design of the primer pair EM1F + EM1R. Sequence analysis of the respective amplicon showed only weak homology to AMP binding genes and acetyl-CoA synthetase and no significant homology to NRPS in a database search using BLAST. Comparison of the amplicons of ten emetic *B. cereus* isolates revealed identical sequences for all strains (data not shown).

The PCR systems was challenged with a strain set comprising emetic and non-emetic *B. cereus* isolates from different origins (clinical, food, food poisoning, and environment). Cross reactivity was evaluated with closely related *B. cereus* group strains and *Bacillus* species and non-*Bacillus* species. In total, 238 bacterial isolates were analyzed (see Appendix 7.1) and the assay turned out to be highly specific for emetic strains. Each of the 52 cereulide producing *B. cereus* isolates was detected while no non-cereulide producing isolate gave any signal. Selected isolates were amplified with universal primers targeting the 16S rDNA gene to confirm the absence of PCR inhibitory substances and to exclude false negative results (Figure 3).

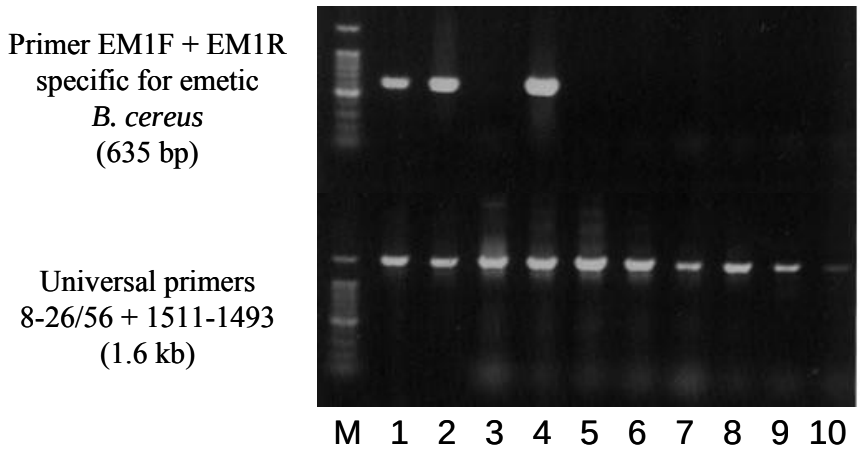

Figure 3: Test of specificity of the developed PCR assay for detection of emetic *B. cereus*. Upper part: amplicons with the primers EM1F + EM1R specific for emetic *B. cereus*; lower part: amplicons with universal primers targeting the 16S rDNA. M: marker (100-bp ladder, Promega); lane 1: reference strain for emetic toxin (F4810/72); lane 2 and 4: emetic *B. cereus* isolate from food (MHI 280, SDA GR 177); lane 3: non-emetic *B. cereus* isolate from food (INRA I20); lane 5: emetic-like *B. cereus* isolate from food (INRA C24); lane 6: *B. cereus* type strain (ATCC 14579); lane 7: *Bacillus anthracis* Sterne (CIP 7702); lane 8: *Bacillus brevis* (ATCC 9999); lane 9: *Staphylococcus aureus* (WS 2604); lane 10: *Salmonella enteritidis* (WS 2863).

In addition, the specificity of the derived amplicon was tested by Southern blot analysis. The digoxigenin labelled DNA probe amplified with the primers HpaIF and EM1R hybridized only to DNA from emetic *B. cereus* isolates, whereas no hybridization signals were observed from non-emetic *B. cereus* strains or DNAs from other bacterial species (Figure 4).

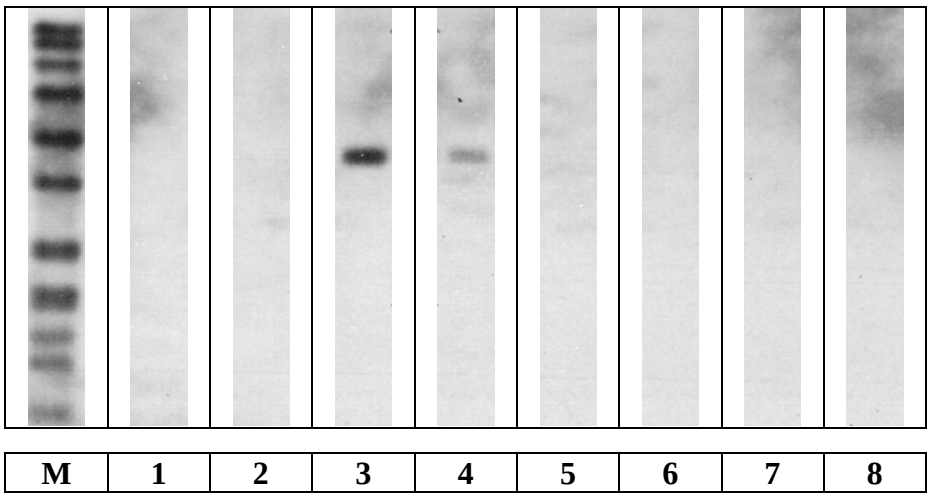

Figure 4: Southern blot analysis of selected strains (according to Ehling-Schulz et al. 2004b). The restricted genomic DNA was hybridized with a 260 bp probe targeting the identified emetic *B. cereus* specific DNA fragment. In total, 14 bacterial strains were tested: six *B. cereus* strains (four emetic, one emetic-like, two non-emetic strains), *B. weihenstephanensis*^{T} (WSBC 10204), *B. thuringiensis*^{T} (ATCC 10792), *B. subtilis*^{T} (ATCC 6051), *B. licheniformis* (WSBC 23001), *B. brevis* (ATCC 9999), *S. aureus* (WS 2604), and *Y. enterocolitica* (WS 2589). Results for the following strains are shown: lane 1, 2, 7, 8: non-emetic *B. cereus* group strains; lane 3 and 4: emetic *B. cereus* strains; lane 5: emetic-like *B. cereus* strain; lane 6: *B. brevis*; M: DNA molecular mass marker VII (dig labelled, Roche). Reprobing of the blot with the 16S rDNA universal probe confirmed the presence of DNA in all lanes.

3.2.2 Real-time PCR assays for the detection of emetic *B. cereus*

Two Real-time PCR assays were developed for the detection of emetic *B. cereus* strains using the two different chemistries available for Real-time detection of PCR products: fluorescent probes (e.g. TaqMan probes) that bind specifically to certain DNA sequences and fluorescent dyes (e.g. SYBR Green I) that intercalate unspecifically in any double-stranded DNA.

The primers and probe used in the TaqMan assay are located in the cesA1 domain of the ces gene cluster in a region with unknown function. For diagnostic purposes, an internal amplification control (IAC) was included in the assay. Inclusivity and exclusivity of the assay was assessed using a test panel of 100 strains, including 23 emetic strains from different origins, 16 non-emetic *B. cereus*, 16 *B. cereus* group, 9 *Bacillus* species and 36 non-*Bacillus*

species strains (see Appendix 7.1). Only the emetic *B. cereus* strains showed positive results, whereas all other strains tested negative. No cross reactions of the emetic *B. cereus*-specific or the IAC-specific primers and probes with non-emetic strains was observed, nevertheless the strain set included non-emetic *B. cereus* strains that carry potential *nrps* genes and known NRPS encoding bacteria like the gramicidin and tyrocidine producer *Bacillus brevis*. The detection limit of the developed TaqMan assay (including the IAC) was determined in two independent runs with tenfold serial dilutions from purified genomic DNA of the reference strain for emetic toxin F4810/72 and was 0.6 pg with an amplification efficiency of 108 %. In addition, serial dilutions of *B. cereus* DNA were used to determine the precision of the assays. The repeatability standard deviation s_r was calculated using the measured mean C_t values of 10 replicates from four consecutive runs. The assay precision test (Table 4) resulted in calculated s_r values between 1.5 to 2.2 %.

Table 4: Mean *CT* and s_r of tenfold serially diluted DNA from the reference strain for emetic toxin F4810/72 in the SYBR Green simplex PCR assay and in the TaqMan Real-time PCR assay in the presence of 170 IAC copy numbers (according to Fricker et al. 2007).

Amount of emetic *B. cereus* DNA/PCR	**Mean C_T and s_r using[a]:**		
	SYBR Green Simplex	***ces*-specific probe**	**IAC-specific probe**
600 ng	14.82 ± 0,24	18.98 ± 0.37	34.03 ± 2.76
60 ng	18.38 ± 0,27	22.60 ±0.40	31.14 ± 2.43
6 ng	20.72 ± 0.46	25.73 ± 0.54	30.49 ± 1.48
600 pg	23.61 ± 0,74	28.93 ± 0.59	30.80 ± 1.18
60 pg	29.17 ± 0.52	32.20 ± 0.49	30.87 ± 1.13
6 pg	32.64 ± 1.07	35.41 ± 0.63	31.07 ± 1.41
0.6 pg[b]	35.45 ± 0.54	37.69 ± 0.83	30.96 ± 1.26

[a] Results are shown as $C_T \pm s_r$. Values were calculated with the standard algorithms provided with the two cycler systems; s_r values were calculated from 10 replicates from four censecutive runs.
[b] 0.6 pg correspond to approximatly 100 genomic equivalents.

The primers used for the SYBR Green I based Real-time detection of emetic *B. cereus* were located in the same region of the *ces* gene cluster as the TaqMan primers and probe. The inclusivity and exclusivity of the SYBR Green I assay was determined with the same strain set as described above for the TaqMan assay. Again all emetic *B. cereus* strains could be detected and no cross reaction was observed for any non-emetic toxin producing strain included in the test panel. The detection limit of the SYBR Green I assays was determined to

be 60 fg (Figure 5) with a PCR amplification efficiency of 91.5 % (data not shown). The precision of the assay was determined as described for the TaqMan assay and resulted in calculated s_r values between 1.5 and 3.3 % (Table 4).

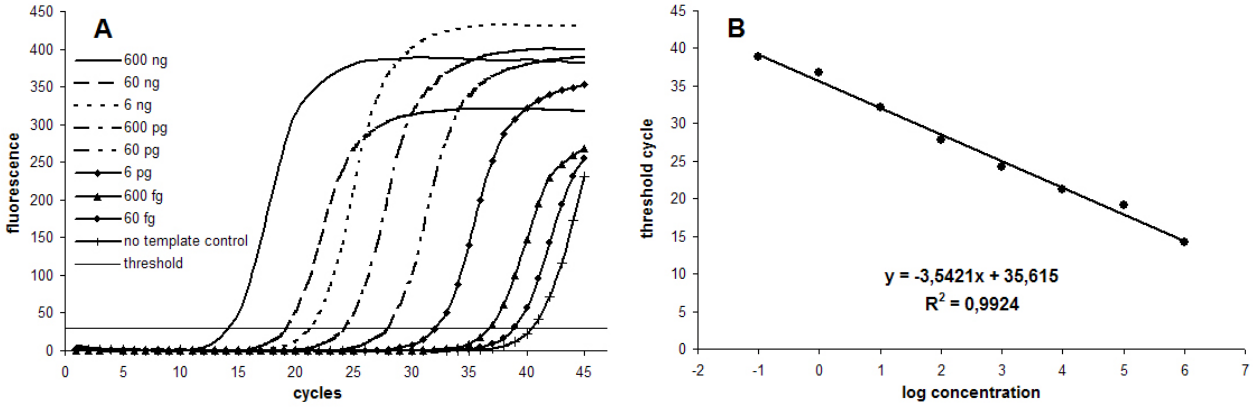

Figure 5: SYBR Green I detection of emetic *B. cereus*. (A) Primary curves of a dilution series from 600 ng to 60 fg derived from emetic *B. cereus* DNA with the primers of the developed SYBR Green I assay. The threshold was set to a fluorescence of 30. Melting curve analysis of the respective amplicons revealed 80°C as specific melting temperature, whereas the melting curve of the no template control shows the formation of primer dimers with a lower melting temperature (data not shown). (B) The threshold cycles (C_T) from (A) were used for the calculation of a standard curve for quantification purposes. The high correlation efficient (R^2) of 0.9924 confirms the accuracy of the developed assay.

For the one-step differentiation between emetic *B. cereus* and *Staphylococcus aureus*, the SYBR Green I simplex assay was extended with published primers specific for *S. aureus* (for details see 2.4.7). The two different primer pairs allowed the amplification of PCR products with distinct melting temperature values, resulting in the formation of two distinct peaks representing the two targets (Figure 6). The 176 bp - amplicon of emetic *B. cereus* (T_m 80.0°C; GC content of 35 %) could be clearly separated from the 262 bp - amplicon of *S. aureus* (T_m 83.5°C, GC content of 40 %). Melting points showed a higher degree of variation in the duplex Real-time PCR assay than in the simplex assay, but both amplicons were still clearly distinguishable from each other (Figure 6). The T_m in duplex PCR was 79.3 ± 0.2°C for the emetic *B. cereus* specific amplicon and 83.4 ± 0.2°C for the *S. aureus* specific amplicon.

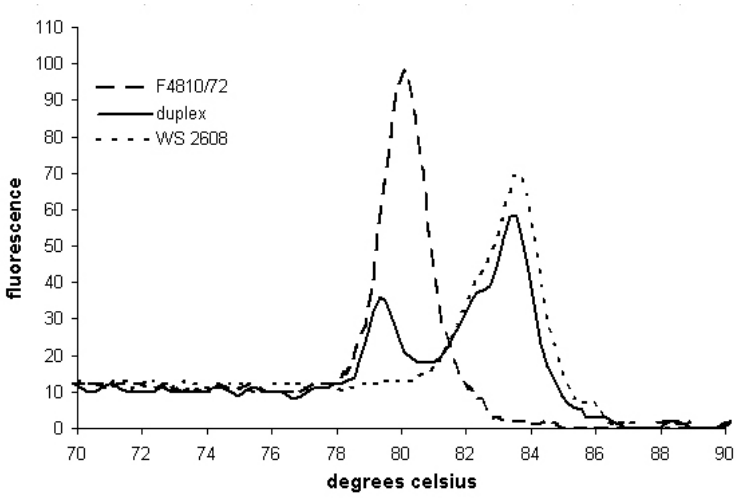

Figure 6: Melting curves of the SYBR Green I Real-time PCR products used for the detection of emetic *B. cereus* (F4810/72) and *Staphylococcus aureus* (WS2604). DNA was isolated with the NucleoSpin food kit from a duplex enrichment experiment with artificial inoculated rice after 6h. Cell counts are corresponding to 108 cfu/g F4810/72 and 107 cfu/g WS2608 (see also Table 6).

Detection limits in the SYBR Green reactions were 0.06 pg for emetic *B. cereus* and 5 pg for *S. aureus*. The robustness of the duplex Real-time PCR was tested with serial tenfold dilutions of DNA from one pathogen in the presence of constant amounts of DNA from the other pathogen. Results of two independent runs were comparable and are shown in Table 5. In brief, in the presence of 50 ng of *S. aureus* DNA 0.5 pg of emetic *B. cereus* DNA could be detected, while amounts as low as 5 pg of *S. aureus* DNA could be detected in presence of 50 ng of emetic *B. cereus* DNA.

Table 5: Robustness of the duplex SYBR Green I Real-time PCR for emetic *B. cereus* and *S. aureus* (according to Fricker et al. 2007).

Amount of DNA used as template		**Species detected**[a]	
Emetic *B. cereus*	***S. aureus***	**Emetic *B. cereus***	***S. aureus***
50 ng	0,5 pg	+	-
50 ng	50 pg	+	+
50 ng	50 ng	+	+
50 pg	50 ng	+	+
0.5 pg	50 ng	+	+

[a] +, species was detected; -, species was not detected.

The robustness of the Real-time assays was evaluated with artificially contaminated foods. Two commercially available kits and a simple boiling method were used for the DNA isolation. Different foods were tested for the absence of natural contamination with *B. cereus* and were then used for artificial contamination experiments. Cooked rice and pasta, which had been inoculated with different amounts of the emetic toxin reference strain F4810/72, were analyzed by Real-time PCR and in parallel by standard reference culture methods at different enrichment time points. The experiment was repeated three times independently and DNA was isolated from two of these experiments. Without enrichment, the detection limit of the TaqMan based assay including the IAC was 10^5 cfu/g (17 cfu per reaction) artificially contaminated rice for the boiling method and 10^3 cfu/g (2 cfu per reaction) for the kit based DNA isolation method. With SYBR Green I chemistry the detection limit was 10^3 cfu/g rice for the boiling method and 10^1 cfu/g with the kit based DNA isolation method. Considering the increase of cell numbers during enrichment (Table 6)**,** a reliable detection of 10^0 cfu of emetic *B. cereus* per g food was possible with the simple boiling method after 6 h enrichment for the TaqMan assay, and after 4 h enrichment for the SYBR Green I assay. Using the kit based DNA isolation methods the enrichment time could be reduced to 2 h for the SYBR Green I assay and to 4 h for the TaqMan assay. Spiking experiments performed with artificially contaminated pasta revealed similar detection limits as those depicted for the rice experiments (data not shown).

In addition, artificial inoculation experiments were conducted to determine the detection limit of the duplex Real-time PCR assay. Rice was inoculated with the emetic reference strain F4810/72 or the *S. aureus* strain WS2608 or both strains together. Cell counts are provided in Table 6**.** DNA from these enrichment experiments was isolated with the NucleoSpin food kit. After 6 h enrichment both pathogens could be detected simultaneously from the same artificially contaminated rice by the SYBR Green duplex assay (Figure 6).

Table 6: Cell counts of the enrichment experiment with the reference strain for emetic toxin *B. cereus* F4810/72 and *S. aureus* WS2608 in rice (according to Fricker et al. 2007).

Enrichment duration	Cell count at inoculation level (cfu/g) for:							
	Enrichment with fresh overnight culture of F4810/72				Duplex enrichment with fresh overnight culture of F4810/72 and WS2608			
	10^0	10^1	10^2	10^3	10^3 (only F4810/72)	10^3(only WS2608)	10^3 (F4810/72 and WS2608 together)	
					B. cereus	***S. aureus***	***B. cereus***	***S. aureus***
0 h	-[b]	2.0 x 10^1	3.3 x 10^2	1.8 x 10^3	2.6 x 10^3	7.5 x 10^3	2.5 x 10^3	7.9 x 10^3
2 h	6.0 x 10^1	1.3 x 10^2	*1.8 x 10^3*	*1.6 x 10^4*	*2.0 x 10^4*	2.1 x 10^4	*2.8 x 10^4*	2.2 x 10^4
4 h	*9.5 x 10^2*	*6.6 x 10^3*	***1.7 x 10^5***	***2.2 x 10^6***	*3.3 x 10^6*	*4.2 x 10^5*	*5.2 x 10^6*	4.1 x 10^5
6 h	***1.3 x 10^5***	***6.6 x 10^5***	***2.5 x 10^7***	***1.2 x 10^8***	*2.2 x 10^8*	*1.5 x 10^7*	*2.7 x 10^8*	*1.2 x 10^7*

[a] *Italic numbers* indicate a positive result in the SYBR Green assay, **bold numbers** indicate a positive result in the TaqMan assay. DNA was isolated with the simple boiling method for the simplex detection and with the NucleoSpin food kit for duplex detection.
[b] No colonies were detectable.

3.2.3 Toxin gene profiling

The primer pair CesF1 + CesR2 targeting the cereulide synthetase genes as described by Ehling-Schulz et al. (2005b) was combined with primers targeting the different enterotoxin genes to allow a toxin gene profiling of *B. cereus* strains. Taking into account sequence polymorphisms within the enterotoxin genes, the respective primers were designed after sequence alignment of *de novo* sequenced enterotoxin gene of 49 *B. cereus* strains together with enterotoxin sequences available from data base. Primers for the amplification of enterotoxin complex genes were located in different genes of the complexes, e.g. in *nhe*A and *nhe*B or *hbl*D and *hbl*A. The primers targeting the *cyt*K gene are able to amplify both variants of the gene (*cyt*K-1 and *cyt*K-2). Variable nucleotide positions were substituted by inosine. After optimization of primer and $MgCl_2$ concentration in the PCR master mix, the assay was validated with a panel of *B. cereus* strains with known toxin gene profiles. Figure 7 shows the toxin gene profiles of selected strains. The specificity of the assay was tested with other *Bacillus* sp. and non-*Bacillus* species, and no cross reactions occurred. Application of the assay on other members of the *B. cereus* group revealed, that the system is in principle suitable for the determination of toxin gene profiles of these strains (Table 7).

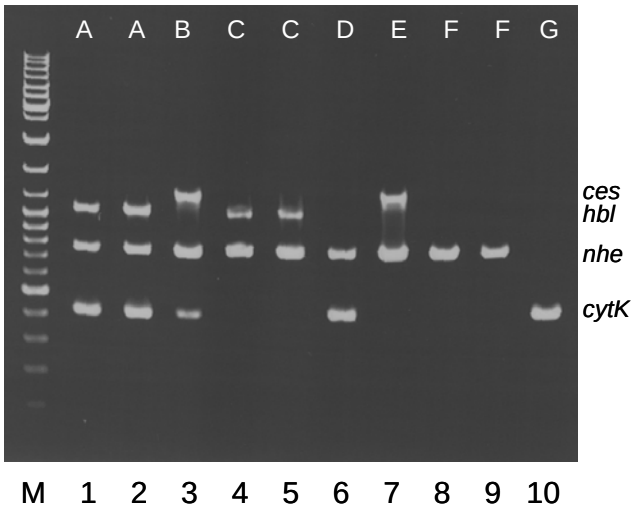

Figure 7: Toxin gene profiles of selected strains (according to Ehling-Schulz et al. 2006b). Possible amplicons sizes are: 1271 bp for the cereulide synthetase gene (*ces*), 1091 bp for the enterotoxin gene complexes of *hbl* and 766 bp for the complex of *nhe*, and 421 bp for cytotoxin K (*cytK*). Description of the toxin gene types (A-G) depicted in the upper part of the figure is given in Table 7. M: marker GeneRuler DNA ladder mix (Fermentas), lane 1: *B. thuringiensis* israelensis; lane 2: clinical *B. cereus* isolated from wound infection; lane 3: clinical *B. cereus* isolate (feces) connected to emetic food poisoning; lane 4: *B. cereus* isolate from cooked food; lane 5: *B. cereus* isolate from silo tank; lane 6: *B. cereus* isolate from milk powder; lane 7: emetic reference strain *B. cereus* F4810/72; lane 8: *B. anthracis* ATCC 6602; lane 9: *B. cereus* isolate from food remnants connected to diarrhoeal food poisoning; lane 10: original CytK strains *B. cereus* NVH 0391-98.

The specificity of the multiplex PCR assay was evaluated with a panel of *B. cereus* strains including 40 clinical isolates and isolates from food connected to food poisoning and 10 isolates from food and environment with known toxin gene profiles (see Annex 7.1). Results of the toxin gene profiling with the established multiplex PCR assay were in accordance to the known toxin gene profiles. The designed primers even allowed the amplification of enterotoxin genes from strains, which were previously only detected by Southern blot analysis. No cross reaction of non-*B. cereus* group strains with the primer system were observed.

Table 7: Toxin gene profiles of *Bacillus cereus* isolates obtained from clinical and food environments and from selected *B. cereus* group isolates (according to Ehling-Schulz et al. 2006b).

Source/species	Toxin profile							
	A (nhe$^+$, hbl$^+$, cytK$^+$)	**B** (nhe$^+$, cytK$^+$, ces$^+$)	**C** (nhe, hbl)	**D** (nhe$^+$, cytK$^+$)	**E** (nhe$^+$, ces$^+$)	**F** (nhe$^+$)	**G** (cytK$^+$)	No. of isolates
Test panel[a]								
B. cereus strains from food	7	-	1	-	1	1	-	10
B. cereus strains from food poisoning and clinical settings	9	2[b]	2	3	18[b]	5	1	40
B. anthracis	-	-	-	-	-	3	-	3
B. thuringiensis	1	-	2	-	-	-	-	3
B. mycoides	-	-	2	1	-	-	-	3
B. weihenstephanensis	-	-	3	-	-	-	-	3
Isolates from diagnostic labs and silo tank populations[c]								
Food isolates	10	-	9	9	12	17	-	56
Clinical isolates	1	-	2	3	4[d]	5	-	15
Silo tank isolates	12	-	34	3	1	30	-	80

[a] Compiled from a set of strains with known toxin profiles (Ehling-Schulz et al. 2005a).
[b] Emetic outbreaks.
[c] Most prevalent toxin profiles are printed in bold.
[d] Including three isolates from emetic food poisoning.

The evaluated multiplex PCR assay was applied for the typing of 71 clinical and food isolates provided by diagnostic laboratories (Technische Universität München, Freising; Landesanstalt für Verbraucherschutz, Halle, Technische Universität Dresden; Institut für Hygiene und Umwelt, Halle; private diagnostic labs) and of 80 *B. cereus* group strains collected during a population study from dairy silo tanks (Svensson et al. 2004). Results for

the determination and occurrence of toxin genes in *B. cereus* group strains from different origins are summarized in Table 7. Toxin profile C (nhe^+, hbl^+) was most prominent within the silo tank isolates, whereas it was rarely found within the clinical and food isolates. The latter ones were dominated by toxin type F (nhe^+).

3.3 Growth profiles of selected *B. cereus* isolates

3.3.1 Temperature correlated growth limits

Minimum and maximum growth limits were examined for a strain set comprising one hundred representative *B. cereus* strains including 15 environmental and 35 food isolates, 40 isolates from diarrhoeal and 10 isolates from emetic food poisonings. The detected combinations of minimum and maximum growth limits of the selected *B. cereus* isolates showed a correlation to the origin of the isolates as depicted in Figure 8 and 9.

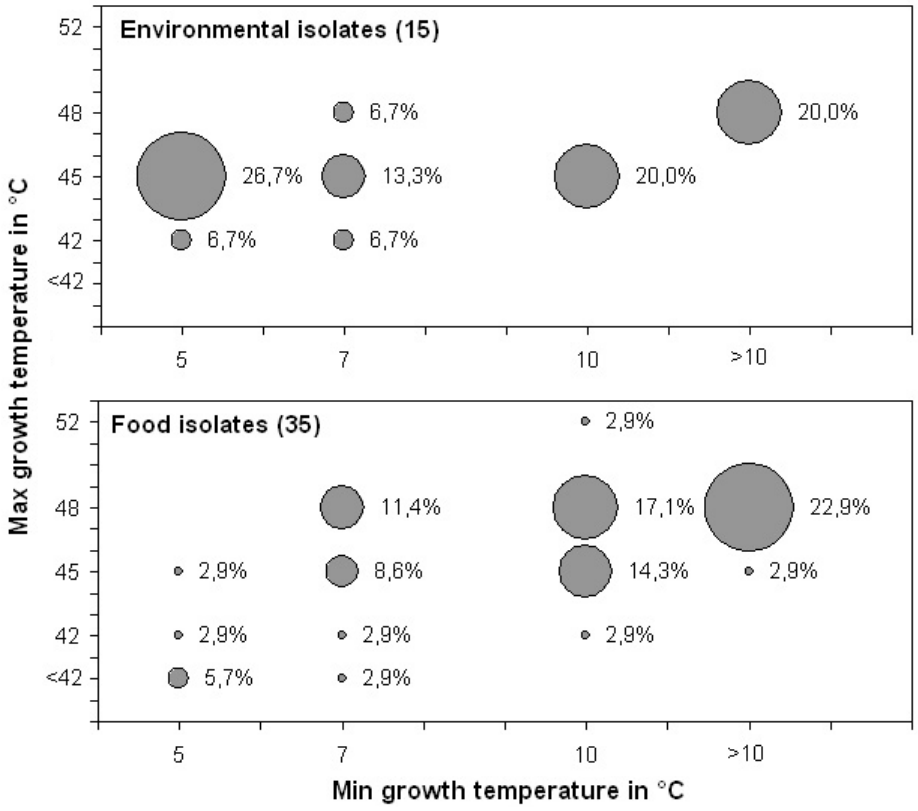

Figure 8: Comparison of growth profiles of *B. cereus* isolates from environment and food. Percentages given in the diagrams refer to the number of isolates written in brackets behind the origin.

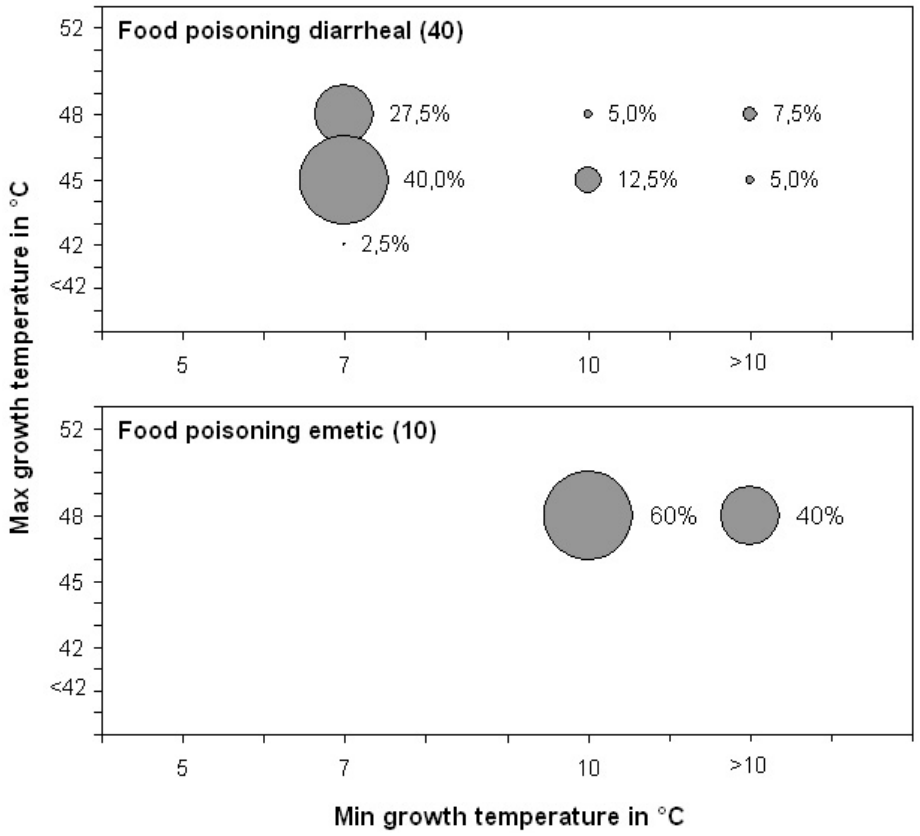

Figure 9: Comparison of growth profiles of *B. cereus* isolates from diarrhoeal and emetic food poisonings. Percentages given in the diagrams refer to the number of isolates written in brackets behind the origin.

In general, environmental and food isolates showed higher variations in the range of the determined growth limits than the isolates from food poisonings. Environmental isolates showed a tendency to grow at temperatures of 7°C or even below (60 % of the strains). Strains growing at 4°C were isolated either from food or environment while none of the food poisoning strains showed detectable growth at 4°C. The main part (70 %) of the isolates from diarrhoeal food poisonings had a minimum growth temperature of 7°C. All isolates from emetic food poisonings, and also the emetic isolates from environment (2) and from food (5) showed the same growth profile with a minimum growth temperature of at least 10°C and a maximum of 48°C. In contrast, only 26.7 % of the environmental isolates were able to grow at temperatures above 45°C, whereas 40 % of the isolates from diarrhoeal food poisoning, and 54.3 % of the food isolates showed visible growth at these temperatures. One food isolate even tolerated 52°C.

3.3.2 Influence of different temperatures and pH

Growth curves were recorded for all strains at 24°C and 37°C at different pH. Emetic toxin producing, diarrhoeal, food, and environmental strains showed nearly no significant difference in their estimated growth kinetic. Typical growth curves are depicted in Figure 10 for the strain NVH 1519-00 as representative isolate. In general, the differences between strains were more pronounced at pH 5.0 and pH 8.0 than at pH 7.0, anyhow strains isolated from food poisoning outbreaks tended to grow better than environmental and food isolates at pH 7.0. At low pH a better growth was observed for all strains at 24°C than at 37°C. At pH 5.0 only 30 % of the strains showed measurable growth at 37°C, while all strains grew under this condition at 24°C. The combination of 37°C and pH 8.0 inhibited the growth of 19 % of the strains but the strains with measurable growth had a shorter lag time compared to 24°C at the same pH.

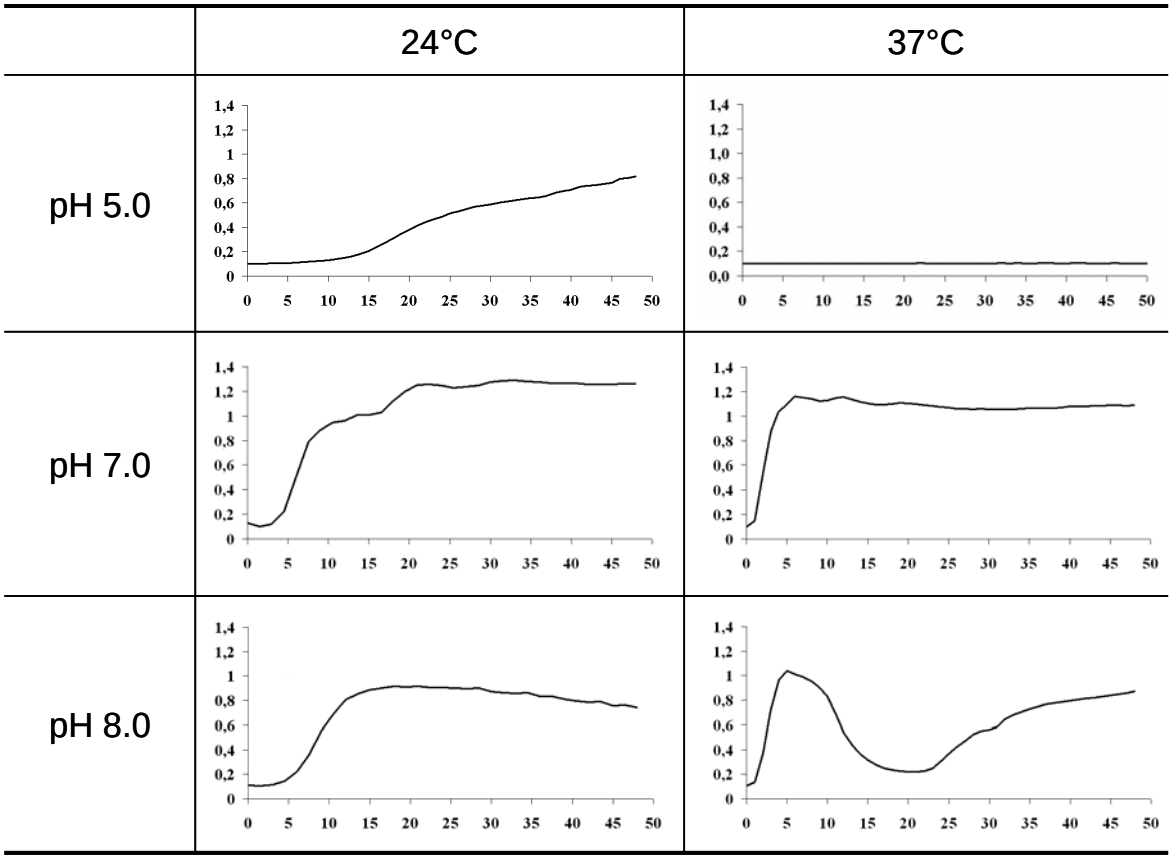

Figure 10: Representative growth curves at different temperatures and pH from strain NVH 1519-00. The optical density (y-axis) was measured at 600 nm for 48 h (x-axis).

3.4 Comparison of different selective plating media for the detection and identification of *B. cereus* group strains

3.4.1 Plating media assessment

A test panel, including a large number of *Bacillus cereus* strains (n=100) isolated from food, food borne outbreaks (40 isolates, each) and clinical origin (20 isolates) was compiled and used to assess the reliability and ease of use of two conventional ISO recommended, and two new chromogenic selective plating media for detection and enumeration of *B. cereus*. Results of this survey are summarized in Table 8 and 9, while detailed information on individual strains and the results are given in Appendix 7.1.

Typical colonies of *B. cereus* group strains on PEMBA and MYP were surrounded by a precipitation zone due to egg yolk hydrolysis and had a peacock-blue and pink colour, respectively. CBC contains 5-bromo-4-chloro-3-indolyl-β-glucopyranoside that is cleaved by β-D-glucosidase and results in white colonies with a blue-green centre. On CEI, 5-bromo-4-chloro-3-indoxyl myoinositol-1-phosphate is cleaved by phosphatidylinositol phospholipase C (PI-PLC) and gives a homogeneous blue-turquoise colour to *B. cereus* colonies. For several strains no unequivocal reactions could be observed on the different selective plating media (Figure 11A-D). Therefore a differentiation between typical and weak reaction was introduced. A typical reaction was defined as the reactions described above. Weak reaction on PEMBA and MYP was characterized by lack of the typical colour of the colonies or a precipitation zone only underneath and not around the colonies. On CBC, a weak reaction corresponded to a very light blue point in the middle of the colonies. Isolates with no precipitation zone and no colour formation on PEMBA and MYP, white colonies on CEI or either white or totally blue colonies on CBC were classified as isolates with atypical growth (e.g. Figure 11A-D).

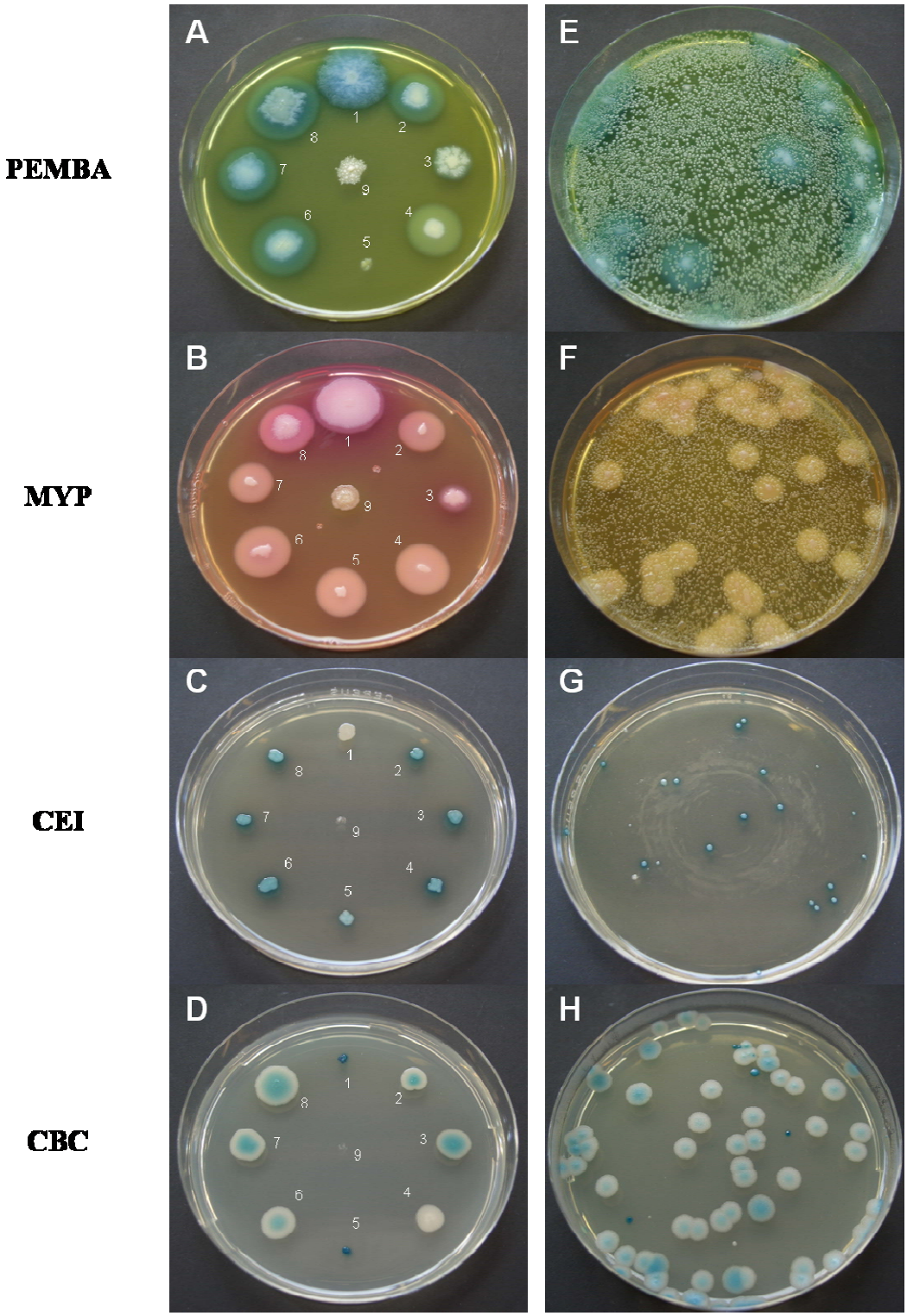

Figure 11: Comparison of the colony appearance on the four plating media for single strains and for complex food samples. **A-D:** Growth of selected *B. cereus* strains on the standard (PEMBA and MYP) and the chromogenic plating media (CEI and CBC). Identical numbers correspond to the same strains. 1: NVH 0391-98 (CytK reference strain), 2: NC 7401 (isolate from emetic outbreak), 3: RIVM BC 67 (human feces), 4: RIVM BC 90 (human feces), 5: WSBC 10204 (*B. weihenstephanensis*^{T}), 6: WSBC 10882 (isolates from emetic outbreak), 7: F4810/72 (reference strain for emetic toxin), 8: ATCC 13479 (*B. cereus*^{T}), 9: *B. licheniformis* (negative control, food isolate). **E-H:** plating of naturally contaminated roughly ground dried ginger on the different plating media.

The ratio of isolates with typical to weak reactions was 25 % to 68 % on PEMBA and 35 % to 60 % on MYP. The chromogenic media revealed more precise results. Only 8 isolates showed a weak reaction on CBC and none on CEI. Strains with atypical growth were found on all four plating media (Table 8).

Table 8: Comparison of the four different media for the detection of *B. cereus* strains.

	PEMBA	**MYP**	**CBC**	**CEI**
Typical reaction	25	35	65	97
Weak reaction[a]	68	60	8	-
Growth, but no typical reaction	7	5	27	3

[a] For PEMBA and MYP agar: no colour formation or precipitation zone only under the colonies; for CBC: white colonies with very light blue point in the middle.

In total, 29 isolates showed atypical growth on one or more plating media. 19 isolates thereof showed atypical growth only on CBC. Eight isolates grew atypically on two, one isolate on three (SDA VI273) and one even on all four plating media (NVH 0391-98, see also Figure 11A-D). The latter strain is a highly toxic isolate and was responsible for a fatal food poisoning in 2000 (see also 4.2). The identification with CBC failed for 27, with PEMBA for seven, with MYP for five and CEI for three isolates (Table 9).

Table 9: Origin of strains used in this study and distribution of strains with atypical growth reactions on the different selective plating media within the strain set.

Strain origin	**Number of strains**	**Number of strains with atypical growth reactions[a]**			
		PEMBA	**MYP**	**CBC**	**CEI**
Food	40	4	-	15	1
Food borne outbreaks	40	1	2	6	1
Clinical isolates	20	2	3	6	1
Total number of strains	100	7	5	27	3

[a] Explanations to atypical growth and media abbreviations are given in Material and Methods (2.2).

3.4.2 Gamma-phage sensitivity and antibiotic resistance of selected strains

Three *B. cereus* isolates (NVH 0391-98, SDA VI273 and RIVM BC 63) with a *Bacillus anthracis*-like phenotype on CEI (white colonies) were further tested for their gamma phage sensitivity and their antibiotic resistance. The three isolates could be differentiated from *B. anthracis* according to their resistance to the gamma phage (Table 10). Nevertheless, two of the isolates (NVH 0391-98 and SDA VI273) were sensitive against penicillin as is *B. anthracis*. Interestingly, the *B. weihenstephanensis* type strain (WSBC 10204) was gamma phage sensitive when incubated at 37°C, but lost this sensitivity when incubated at 30°C. Other *B. weihenstephanensis* strains (n=12) tested in this assay were not sensitive against the gamma phage when grown at 37°C (data not shown).

Table 10: Sensitivity to penicillin, gamma phage sensitivity, and colour formation on CEI of selected strains.

Strain	Sensitivity to penicillin	Gamma phage sensitivity	Colour formation on CEI
ATCC 14579^T	-	-	turquoise
ATCC 10987	-	-	turquoise
F4810/72	-	-	turquoise
WSBC 10204	-	+/-[a]	turquoise
NVH 0391-98	+	-	white
SDA VI273	+	-	white
RIVM BC 63	-	-	white
F4370-75	-	-	turquoise

[a] Variable results; sensitivity towards the gamma phage was lost when incubation temperature was lowered to 30°C.

3.4.3 Detection of *B. cereus* from complex food samples on selective plating media

The suitability of the four plating media for the detection of *B. cereus* from complex food samples was determined using roughly ground dried ginger and mace, skimmed milk powder and rice, all naturally contaminated with *B. cereus*. Colony appearance of isolates from the ginger sample plated on the four different media is depicted in Figure 11E-H, while images of other food samples plated on the different media are provided in Figure 12. The presence of *B. cereus* strains in the ginger sample is reflected by opaque precipitation zones on these media, which are visible more distinctly on MYP than on PEMBA agar. However, no characteristic pink colour formation could be observed on MYP because of the high acid formation of the background flora. In contrast, skimmed milk powder contained mainly

B. cereus isolates with typical colony morphology on the different plating media, whereas the predominant isolates of the mace sample were lecithinase negative on MYP (Figure 12). The rice sample included background flora on the standard plating media, but *B. cereus* isolates showed the characteristic growth reactions and could be identified unambiguously. In general, identification, isolation and enumeration of *B. cereus* from the tested food samples was easier on the chromogenic plating media than on the standard plating media since *B. cereus* was overgrown by background flora on the latter ones (Figure 11E-F). The chromogenic plating media contain additional inhibitory substances in their formula that inhibit potential background flora more precise than polymyxin B, which is included in PEMBA and MYP. Both chromogenic media contain trimethoprim, CEI additionally contains sulfamethoxazole and cycloheximide. The latter media are therefore more inhibitory for non-*B. cereus* species than the standard plating media PEMBA and MYP facilitating thereby cell count determination and isolation of *B. cereus* strains. Nevertheless, the diversity in the colony morphology of *B. cereus* on CBC, resulted not only in totally white or turquoise, but also in atypical white colonies with a blue-green centre (Figure 11H). Atypical colonies of the different food samples on all four plating media were subjected to toxin gene profiling as described in 2.4.10. All toxin gene profiles except B, E and F were observed.

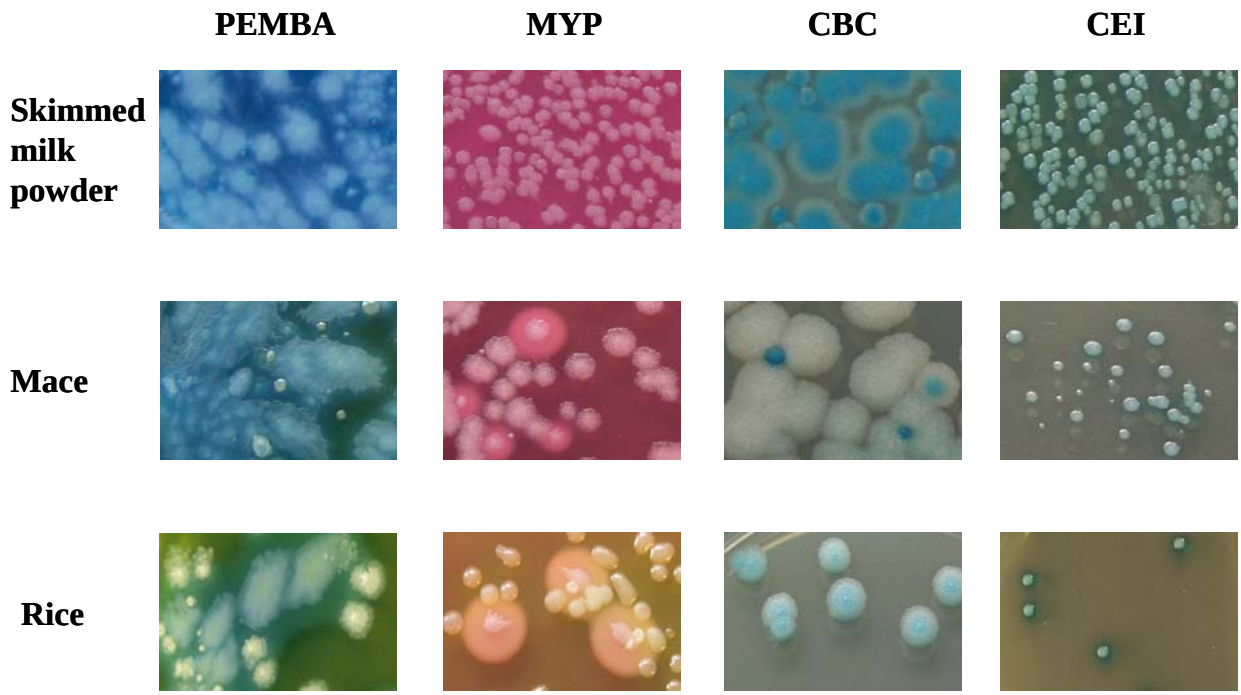

Figure 12: Plating of different food samples on selective plating media. *B. cereus* isolates from the skimmed milk powder and rice yielded typical growth reactions on all plating media, whereas in mace a high portion of the colonies showed atypical growth reactions.

3.5 ANN assisted Fourier transform infrared spectroscopy

Fourier transform infrared (FTIR) spectroscopy is a metabolic fingerprinting method, which is based on the absorbance of mid-infrared light by the molecules present in the cells. In this study, FTIR spectroscopy was combined with artificial neural networks (ANNs) to improve the resolution of this method. For the development of an ANN assisted FTIR spectroscopy method for the rapid discrimination between the *B. cereus* group members, ten independently measured FTIR spectra were recorded from each of 108 well characterized strains including 26 emetic and 29 non-emetic *B. cereus*, 18 *B. thuringiensis*, 15 *B. mycoides*, and 14 *B. weihenstephanensis* (for details see Annex 7.1).

3.5.1 Analysis of FTIR spectra

The quality of all measured FTIR spectra was verified by the quality test provided with the OPUS software. Only FTIR spectra passing this test were used for further analysis. In addition, all spectra of one strain derived from independent measurements were subjected to hierarchical cluster analysis (HCA) using the Ward's algorithm to audit the reproducibility of the measurements. Ten spectra per strain with a heterogeneity below 1.2 were selected for the training and validation of the artificial neural network (ANN). Figure 13 demonstrates the reproducibility of the independent measurements for individual strains. Spectra of the same isolate are arranged in discrete subcluster. One spectra per strain used for the training and evaluation of the ANN was randomly selected. HCA of these spectra revealed a similar structure of this phenotypic method as described for the genetic typing with MLST or the *spoIIIAB* gene (Figure 1). A distinct cluster I with emetic *B. cereus* isolates and III with the psychrotolerant members of the *B. cereus* group was also found with HCA, whereas the cluster II was divided in two separate mixed clusters with non-emetic *B. cereus* and *B. thuringiensis*. This structure is also reflected in Figure 13 although only a selection of *B. cereus* group strains is shown. Nevertheless, the resolution of HCA is limited to the formation of mixed subgroups.

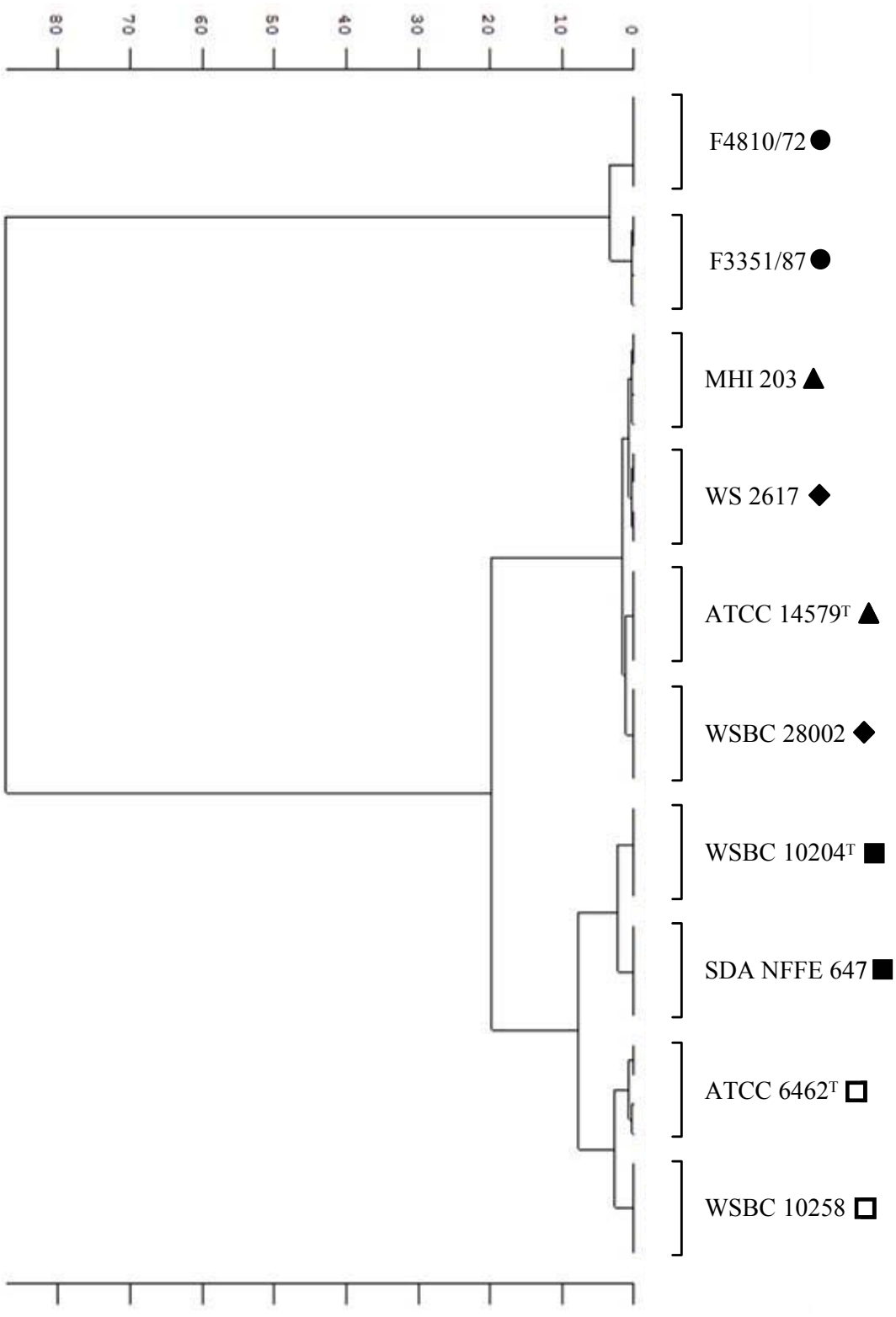

Figure 13: Hierarchical cluster analysis (HCA) of four independent replicate measurements of different *B. cereus* group strains using the Ward's algorithm and the first derivative of the original spectra. Spectra of the same strain are arranged in discrete subclusters, confirming the good repeatability of the measurements. Black dots: emetic *B. cereus*, black triangles: non-emetic *B. cereus*, black diamonds: *B. thuringiensis*, black squares: *B. weihenstephanensis*, white squares: *B. mycoides*.

3.5.2 Architecture and validation of the artificial neural networks (ANN)

The architecture of the first level net was designed according to the results of the MLST and HCA analysis described in 3.1 and 3.5.1. In the first level, the spectra of an unknown *B. cereus* group isolate is either assigned to (i) the emetic type of *B. cereus* (including emetic and emetic-like isolates), (ii) non-emetic *B. cereus* and *B. thuringiensis* or (iii) *B. weihenstephanensis* and *B. mycoides* (Figure 14). In the second level the two latter clusters are further differentiated with species-specific subnets that allow the final classification to non-emetic *B. cereus*, *B. thuringiensis*, *B. weihenstephanensis* or *B. mycoides*.

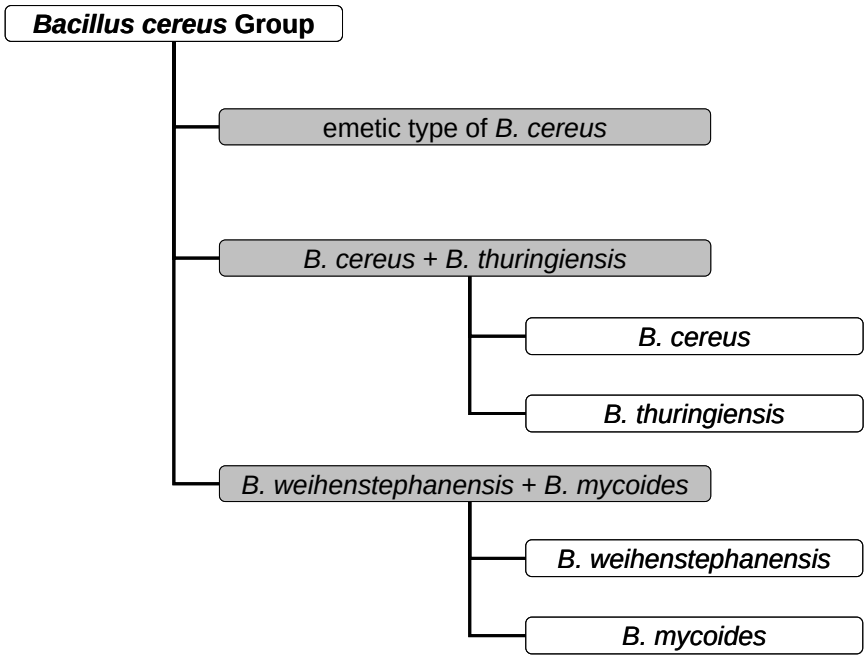

Figure 14: Architecture of the ANN trained with FTIR spectra of the *B. cereus* group members. The first level of the ANN divides the *B. cereus* group in the three subgroups shaded in grey. According to the output of the first level net, species-specific subnets are activated for the differentiation between *B. cereus* / *B. thuringiensis* and *B. weihenstephanensis* / *B. mycoides*.

The first and second level nets of the ANN were trained with 65 strains of the *B. cereus* group listed in Appendix 7.1. These strains could be unequivocal assigned to specific *B. cereus* group members. The internal validation of the ANN with unknown spectra of strains that were used for the training to the ANN yielded 96.9 % correct identification (Table 11). Only one spectra of non-emetic *B. cereus*, one of *B. mycoides* and two of *B. thuringiensis* isolates were misidentified, whereas all spectra of *B. weihenstephanensis* and emetic *B. cereus* were identified correctly.

Table 11: Internal validation of the ANN for the identification of *B. cereus* group isolates.

Species	No. of strains in training	No. of spectra	Misidentified spectra	Correct identified spectra in %
Emetic type of *B. cereus*	12	24	0	100
Non-emetic *B. cereus*	23	46	1	97.8
B. thuringiensis	12	22	2	91.7
B. weihenstephanensis	10	20	0	100
B. mycoides	8	16	1	93.8
In total	65	130	4	96.9

The external validation with 43 strains that were unknown to the ANN achieved an overall correct identification of 95.6 % (Table 12). The ANN was able to identify all spectra of emetic *B. cereus* strains (100 %) correctly, 96.0 % of the *B. weihenstephanensis*, 95.0 % of the *B. thuringiensis*, 93.6 % of the non-emetic *B. cereus* and 90.0 % of the *B. mycoides* spectra.

Table 12: External validation of the ANN for the identification of *B. cereus* group isolates**.**

Species	No. of spectra	Misidentified spectra	Non-identified spectra	Correct identified spectra in %
Emetic type of *B. cereus*	140	0	0	100
Non-emetic *B. cereus*	110	7	0	93.6
B. thuringiensis	60	3	0	95.0
B. weihenstephanensis	50	1	1	96.0
B. mycoides	70	7	0	90.0
In total	430	18	1	95.6

3.5.3 Wave numbers selected for the differentiation between the *B. cereus* group members

The selected wave numbers and their importance for the differentiation between the *B. cereus* group members varies between the first level net and the two different second level nets. Analysis of the 30 most discriminative wave numbers demonstrates the significance of

the carbohydrate (1200 - 900 cm^{-1}) and fingerprint region (900 - 600 cm^{-1}) for the first level net and the second level net for *B. weihenstephanensis* and *B. mycoides* (Table 13).

Table 13: Assignment to biological molecules of the 30 most discriminative wave numbers selected for the ANN assisted FTIR spectroscopy method.

Range (cm^{-1}) and assignment[a]	**Quantity of the 30 most discriminative wave numbers selected per range (total quantity of wave numbers selected per range)**		
	First level net	**Second level net: Non-emetic *B. cereus* and *B. thuringiensis***	**Second level net: B. weihenstephanensis and B. mycoides**
3100 – 2800 Fatty acids	2 (22)	6 (27)	4 (23)
1690 – 1620 Amide I	3 (7)	2 (4)	2 (4)
1570 – 1515 Amide II	0 (2)	3 (4)	0 (1)
1305 – 1200 Amide III	2 (4)	2 (6)	1 (10)
1200 – 900 Carbohydrates	7 (24)	3 (18)	13 (25)
900-600 Fingerprint region	8 (17)	10 (14)	7 (22)
Not assigned[b]	8 (24)	4 (15)	3 (15)
In total	30 (100)	30 (88[c])	30 (100)

[a] According to Naumann et al. (1991) and Maquelin et al. (2002).
[b] Wave numbers outside the defined ranges.
[c] The ANN found only 88 discriminative wave numbers for this net.

The fatty acid region (3100 - 2800 cm^{-1}) in combination with the fingerprint region is more important for the differentiation between non-emetic *B. cereus* and *B. thuringiensis*. Between 63 to 70 % of the selected wave numbers per net can be assigned to the three aforementioned regions. Two wave numbers were selected for the training of all three nets. One can be assigned to the fatty acid region (3018.4 cm^{-1}) and has a weighting below 0.22 in all nets. In contrast, the second wave number is outside the defined ranges (1799.5 cm^{-1}) and has a weighting of 0.54 for the first level net, 0.88 for the second level net for non-emetic *B. cereus* and *B. thuringiensis*, but 0.1 for the second level net for *B. weihenstephanensis* and *B. mycoides*. Eight selected wave numbers were identical for the first level net and the second level for non-emetic *B. cereus* and *B. thuringiensis*, and seven for the first level net and the second level net for *B. weihenstephanensis* and *B. mycoides* (data not shown).

3.5.4 Application of ANN assisted FTIR spectroscopy in population analysis in soil samples and a rice sample from a food poisoning outbreak

The analyzed soil samples were collected near Lausanne (Switzerland), Golden Bay (Malta) and Freising-Weihenstephan (Germany) in 2006. The rice dish was sampled in 2006 in a day care centre in Southern Germany, where 17 children started vomiting after consumption of the reheated meal. FTIR spectra of all isolates were first analysed by HCA (Figure 15) and in a second step with the novel ANN (Table 14).

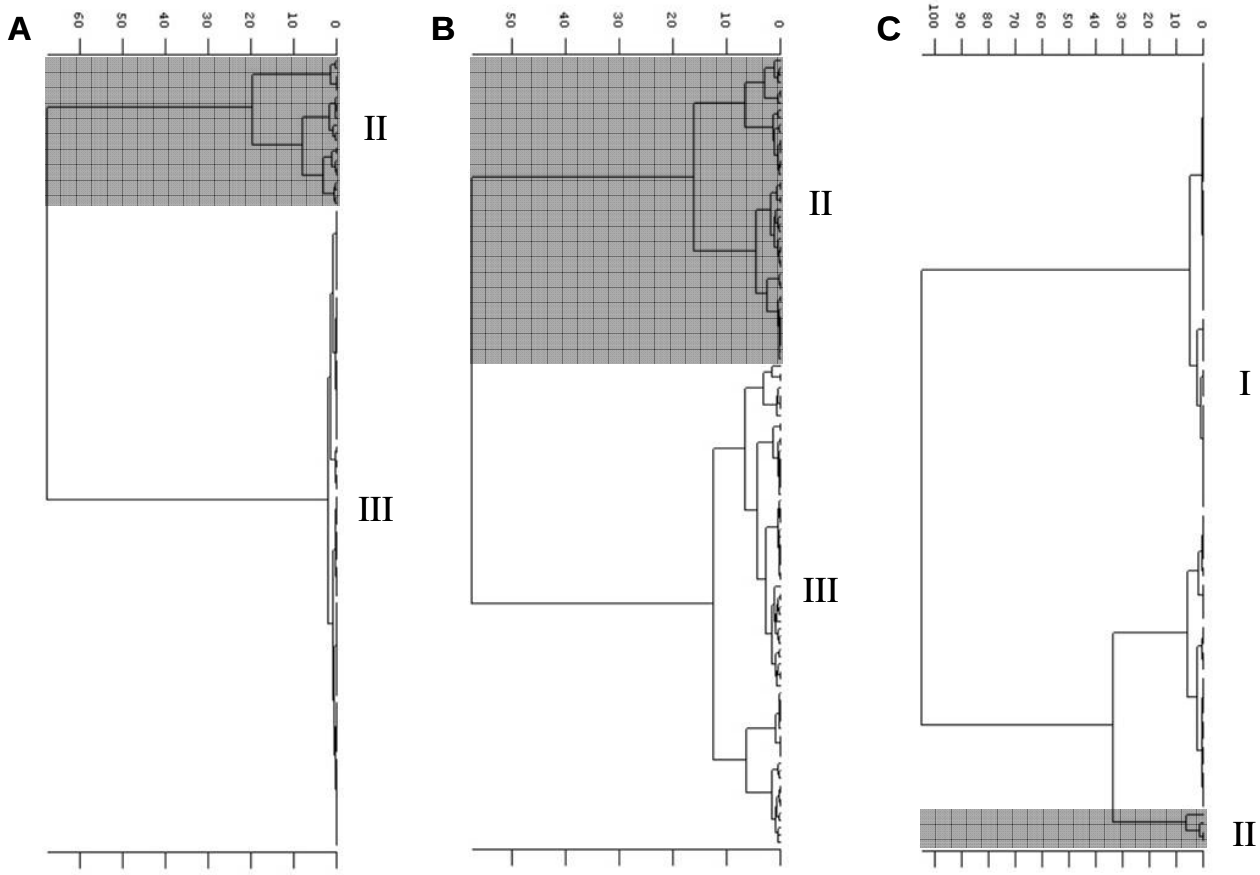

Figure 15: HCA of the FTIR spectra of isolates from different soil samples (**A:** Golden Bay, Malta; **B:** field near Lausanne, Switzerland) and a rice dish associated to an emetic food poisoning (**C**). Distances are expressed as heterogeneity. ANN assisted FTIR spectroscopy revealed mainly mesophilic *B. cereus* isolates in the grey shaded cluster II of all three samples. Cluster III of the soil samples (**A**-**B**) mainly consisted of psychrotolerant *B. cereus* group members, whereas the isolates of cluster I from the rice dish (**C**) were all identified as emetic type of *B. cereus*. (Note: no isolates belonging to cluster I are present in the soil samples, while none of the rice isolates belonged to cluster III)

The total heterogeneity varied between 55 and 105 within the different samples. Two main clusters could be observed in each HCA, examples are given in Figure 15A-C. Identification of the isolates with the ANN revealed the formation of clusters mainly consisting of isolates identified as mesophilic and non-emetic *B. cereus* for all samples that

are named cluster II in Figure 15. Cluster III of the soil samples (Figure 15A-B) included mainly psychrotolerant members of the *B. cereus* group, namely *B. weihenstephanensis* and *B. mycoides*, whereas all isolates in cluster I of the rice dish sample (Figure 15C) were identified as emetic type of *B. cereus*. The main population components varied within the different samples. The main part of the Malta isolates (79 out of 110 isolates) was classified as *B. mycoides*, whereas the majority of the isolates from the other soil samples were identified as *B. cereus* and *B. mycoides* for the sample from Switzerland, and *B. cereus* and *B. weihenstephanensis* for the sample from Germany (Table 14). In contrast, 88 out of 92 isolates from the rice dish connected to a food borne outbreak clustered to the emetic type of *B. cereus*. Thereof 11 randomly selected isolates were analysed by PCR for the presence of the *ces* gene cluster, and 4 isolates could be confirmed as emetic toxin producing *B. cereus*.

Table 14: Classification of soil sample isolates from different origin and from a food poisoning case by ANN assisted FTIR spectroscopy.

Species	Golden Bay, Malta	Field near Lausanne, Switzerland	Field near Weihenstephan, Germany	Rice dish from an emetic food poisoning
Emetic type of *B. cereus*	-	1	1	**88**
Non-emetic *B. cereus*	22	33	36	3
B. thuringiensis	7	3	6	1
B. weihenstephanensis	-	25	**41**	-
B. mycoides	**79**	**41**	26	-
Failed identification	2[a]	7[b]	-	-
Total no. of isolates	110	110	110	92

[a] Further characterization revealed that one isolate was *B. weihenstephanensis*, the other one *B. thuringiensis*.
[b] Further characterization revealed that one isolate was *B. weihenstephanensis*, the others non-emetic *B. cereus*.

The 12 isolates with failed identification were further characterized. One isolate from Malta and one from Switzerland possessed the *cspA* gene and no rhizoid growth and can be counted to *B. weihenstephanensis*. The second isolate from Malta with failed identification showed the formation of the crystal toxin typical for *B. thuringiensis*. One of the remaining isolates with failed identification from Switzerland formed white colonies on CEI agar and was suspected to be *B. anthracis*, but specific PCRs targeting the *pagA* and *lef* gene tested negative. The respective isolate had the toxin gene profile A (nhe^+, hbl^+, $cytK^+$). This isolate and the others with failed identification therefore can be counted to non-emetic *B. cereus* as they neither showed the formation of the crystal toxin typical for *B. thuringiensis* nor

possessed the *cspA* gene (*B. weihenstephanensis*), the *ces* genes (emetic *B. cereus*), or the *pagA* or *lef* genes specific for *B. anthracis*.

3.6 Application of the developed methods for the characterization of *B. cereus* strains isolated from foods and in recent food poisonings

3.6.1 Characterization of *B. cereus* isolates from naturally contaminated cream

Cream samples naturally contaminated with high amount of *B. cereus* (about 10^4 cfu/ml) and possessing a bitter taste were analyzed for their risk potential for the consumer. Sample material consisted of PEMBA plates with dilutions derived from two different cream samples, obviously containing *B. cereus* (Figure 16), and six cream samples. Sample 1- 3 were unopened, whereas sample 4-6 were already opened. According to a sensoric examination of sample 4-6 by the manufacturer, sample 4 had a normal taste, whereas sample 5 and 6 tasted bitter.

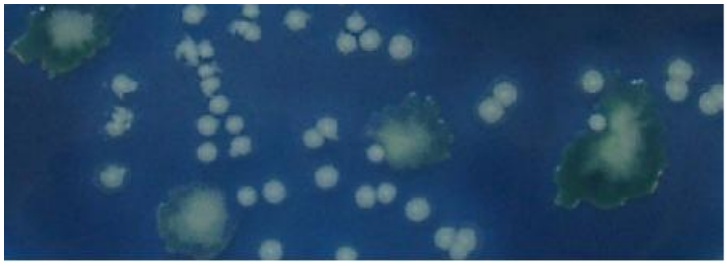

Figure 16: *B. cereus* isolates on the PEMBA plates provided by the manufacturer of the contaminated cream. Two different colony morphologies are present in the sample. Both show typical growth characteristics of *B. cereus* on PEMBA with blue colour formation and precipitation zones around the colonies.

The *B. cereus* isolates on the PEMBA plates provided by the manufacturer of the cream exhibited two different colony morphologies (Figure 16). PCR analysis of four randomly selected small and four large colonies revealed the absence of the *cspA* gene indicating that no *B. weihenstephanensis* were present. All isolates with large colonies possessed the toxin gene profile A (nhe^+, hbl^+, $cytK^+$), isolates with small colonies the profile F (nhe^+). None of the isolates was positive for the emetic toxin gene cluster *ces*. In addition, all isolates were typed by FTIR (see below).

B. cereus cell counts of the cream samples were about 10^6 cfu/ml for the already opened samples (4 - 6) and less for the unopened samples (see Table 15). In sample 1 no *B. cereus* colonies were detected on PEMBA and PC agar. In addition, total DNA was

isolated from the cream samples and subjected to toxin gene profiling. Both unopened samples naturally contaminated with *B. cereus* showed the toxin gene profile G, whereas the opened sample with normal taste (sample 4) had profile A, the samples with bitter taste (samples 5 and 6) had profile D (Table 15).

Table 15: Results for the analysis of the cream samples naturally contaminated with *B. cereus*.

Cream sample	Taste	cfu *B. cereus* / ml cream	Toxin gene profile[a]
1	n. d.[b]	-[c]	-[c]
2	n. d.	9×10^4	G ($cytK^+$)
3	n. d.	8×10^3	G ($cytK^+$)
4	normal	2×10^6	A (nhe^+, hbl^+, $cytK^+$)
5	bitter	6×10^6	D (nhe^+, $cytK^+$)
6	bitter	5×10^6	D (nhe^+, $cytK^+$)

[a] Toxin gene profiles were determined from whole cream samples.
[b] Not determined.
[c] No *B. cereus* were detected.

The same two colony morphologies as shown in Figure 16 were found on the PEMBA plates inoculated with serial dilutions of cream sample 2 - 6. Four colonies per cream sample (including both colony morphologies) were subjected to FTIR spectroscopy. Figure 17 shows the HCA of all typed isolates. Isolates derived from the PEMBA plates provided by the manufacturer and isolates from the cream samples 2, 3, 5 and 6 are forming mixed subclusters according to their colony morphology. Nevertheless, the isolates from sample 4 form a distinct third cluster. ANN assisted FTIR spectroscopy identified all isolates forming big colonies and the isolates from cream sample 4 as *B. cereus*. Seven of the isolates of the cluster with small colonies were identified as *B. cereus*, eight as emetic type of *B. cereus*. Thereof four isolates had the toxin gene profile F (nhe^+).

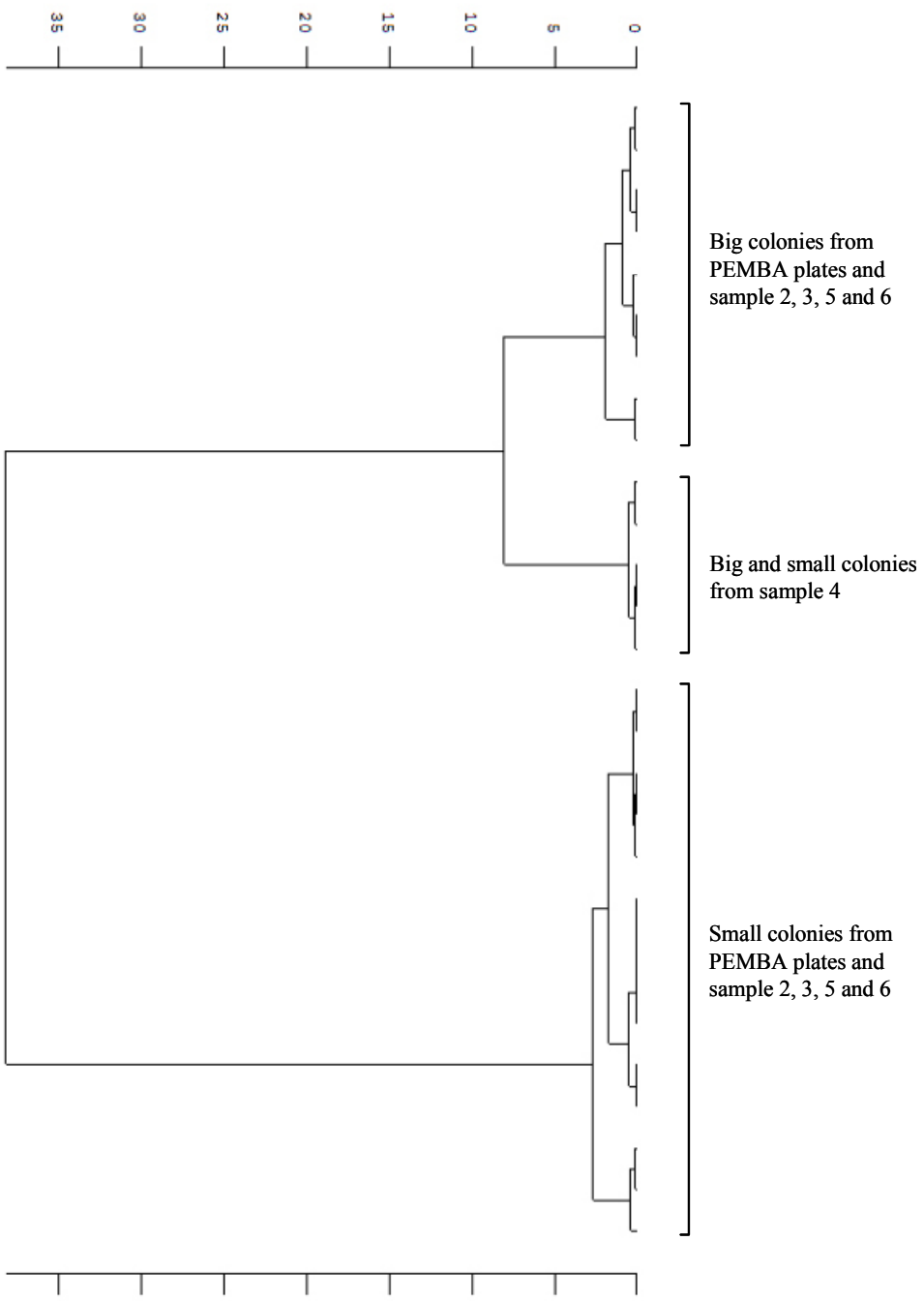

Figure 17: HCA of isolates from cream sample 2 - 6. Except for the isolates from sample 4, all other isolates with similar colony morphology from PEMBA plates provided by the manufacturer and from cream are located in the same cluster.

3.6.2 Identification of the etiological agent in recent food borne outbreaks

In June 2006, 17 children (aged 3 - 5) visiting a day care center became sick after eating a rice dish with vegetables. 1 h after the meal the children began vomiting, collapsed and were hospitalized. Food remnants contained 10^4 cfu *B. cereus*/g rice dish. Emetic *B. cereus* was detected after DNA isolation directly from the rice dish by TaqMan Real-time PCR, while *S. aureus* enterotoxin as a causative agent was excluded using the commercial

ELISA system VIDAS Staph. enterotoxin II (bioMérieux, France). These results were confirmed by the duplex SYBR Green I Real-time PCR assay developed in this study (see Figure 18). A representative number of *B. cereus* strains isolated from the implicated food were subjected to ANN assisted FTIR spectroscopy analysis. Results are summarized in chapter 3.5.4.

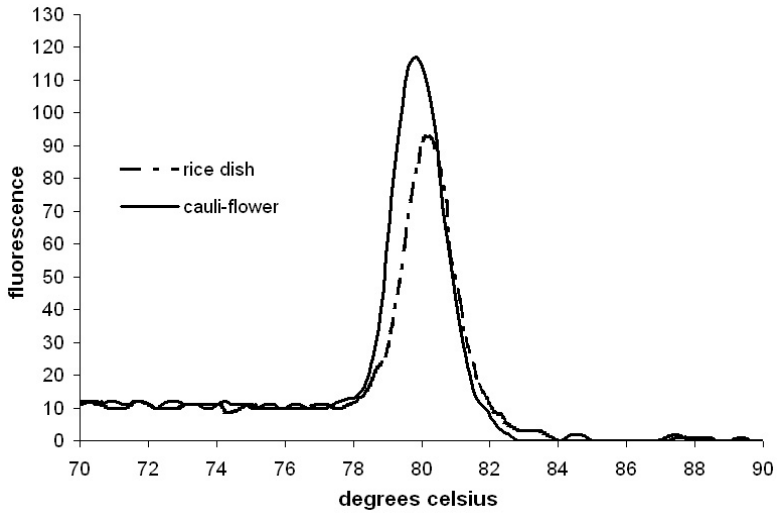

Figure 18: SYBR Green duplex Real-time PCR with DNA isolated from food samples connected to emetic food poisonings.

The second emetic food poisoning involved one student who consumed cooked cauliflower stored at room temperature for 1.5 days. The student began vomiting 75 min after consumption of about 20 g of the reheated food. DNA was isolated directly from the food remnants and used as template in the TaqMan and the duplex SYBR Green I assay. Both assays were positive for emetic *B. cereus* but the duplex assay was negative for *S. aureus* (Figure 18). Plating of serial dilutions of the original sample confirmed the absence of *S. aureus* and the presence of 10^9 cfu *B. cereus*/g food. The mainpart of the isolates could be identified as emetic toxin producing *B. cereus* as they possessed the gene toxin profile E (nhe^+, ces^+). About 10^7 cfu/g showed a different colony morphology and had the toxin type C (hbl^+, nhe^+). In both food poisoning cases the presence of the *B. cereus* emetic toxin cereulide in the food remnants was confirmed by the HEp-2 cytotoxicity assay.

4 DISCUSSION

4.1 Genotypic and phenotypic characterization of *B. cereus* group isolates

4.1.1 Sequence typing confirms the monomorphic structure of emetic *B. cereus* strains

There are quite a few studies trying to differentiate the closely related members of the *B. cereus* group. Differentiation between their members, namely *B. anthracis*, *B. cereus*, *B. thuringiensis*, *B. weihenstephanensis* and *B. mycoides*, is not possible with 16S rDNA sequence-based analysis (Ash et al. 1991; Yamada et al. 1999). Other typing methods like multilocus enzyme electrophoresis (MEE) or amplified fragment length polymorphism (AFLP) are able to differentiate *B. anthracis* from other *B. cereus* group members, but fail to differentiate the remaining group members from each other (Helgason et al. 2000b; Hill et al. 2004). Nevertheless, it has been shown by MEE and AFLP analysis that pathogenic strains are more closely related to each other and to *B. anthracis* than environmental ones (Helgason et al. 2000b; Ticknor et al. 2001; Hill et al. 2004). Multilocus sequence typing (MLST) as proposed by Helgason et al. (2004) showed a higher phylogenetic resolution than the typing methods applied before. Sequencing of the seven housekeeping genes proposed in the first MLST scheme (Helgason et al. 2004) from emetic and emetic-like *B. cereus* isolates revealed the location of these strains near *B. anthracis* together with clinical *B. cereus* and *B. thuringiensis* isolates. Emetic-like *B. cereus* isolates share the same phenotypic characteristics as emetic *B. cereus* isolates except they are not able to neither produce the emetic toxin cereulide nor possess the *ces* gene cluster necessary for the production of cereulide (Ehling-Schulz et al. 2005a). In this work, emetic and emetic-like *B. cereus* isolates are merged under the term "emetic type of *B. cereus*". The isolates of the emetic type of *B. cereus* showed a rather monomorphic structure with an identical sequence type in the MLST. Only one emetic *B. cereus* strain (RIVM BC 67) owned a different sequence type, but was still located very close to the cluster of the emetic type of *B. cereus*. In addition, strain RIVM BC 67 is one of the rare strains of the emetic type of *B. cereus* possessing the gene for *cytK*. In total, only four isolates of the emetic type were positive for the gene encoding *cytK*. These isolates include two out of eight emetic-like isolates (NVH 200, F4429/71) and two out of 37 emetic *B. cereus* isolates (RIVM BC 67, RIVM BC 75). The latter emetic strains were isolated from human feces associated to the same outbreak and might represent clones of the same isolate. In general, toxin gene profiling confirmed the low heterogeneity within the

emetic type of *B. cereus* and revealed that all strains were positive for the enterotoxin complex *nhe* and negative for *hbl*. Emetic toxin producing isolates additionally possessed the genes necessary for cereulide synthesis.

Cluster I of the MLST analysis includes two different monomorphic subclusters: *B. anthracis* and the emetic type of *B. cereus*. *B. anthracis* is already known for its lack of diversity (Henderson et al. 1994; Keim et al. 1997). The monomorphic structure within emetic *B. cereus* strains was confirmed by RAPD, but also with phenotypic methods like protein profiling and Fourier transform infrared spectroscopy (Ehling-Schulz et al. 2005a). However, recent studies have observed limited diversity within emetic *B. cereus* isolates (Apetroaie et al. 2005; Vassileva et al. 2007). Vassileva et al. (2007) observed a second cluster of cereulide-producing strains within cluster I. Nevertheless, strains belonging to this new subcluster seem to be rare. One might speculate that cereulide-producing strains either are progressively diversifying (Vassileva et al. 2007) or that these strains acquired the cereulide synthetase gene cluster that is located on a mega-plasmid via horizontal plasmid transfer as has been shown recently by an isolate of *B. weihenstephanensis* (Thorsen et al. 2006).

In contrast to the monomorphic emetic type of *B. cereus*, all non-emetic *B. cereus, B. thuringiensis* and psychrotolerant members of the *B. cereus* group displayed a high degree of diversity. MLST failes to differentiate between the different species, but two separate clusters can be observed for the mesophilic and psychrotolerant strains. Cluster II comprises the non-emetic *B. cereus* and *B. thuringiensis* and cluster III the psychrotolerant members *B. weihenstephanensis* and *B. mycoides*. Analysis of the single chromosomal genetic locus of the *spoIIIAB* gene revealed similar cluster formation as described above for MLST (Figure 1) and represents therefore a cost efficient alternative compared to the extensive sequencing effort necessary for MLST.

4.1.2 Emetic *B. cereus* strains show distinct growth characteristics

Bacillus cereus has been described to multiply and grow at temperatures between 10°C and 50°C, with the optimum between 28-35°C, and pH values of 4.9 to 9.3 (Kramer and Gilbert 1989). Nevertheless, also more extreme temperature values are tolerated, e.g. 4°C or 55°C (International Commission on Microbiological Specifications for Foods (ICMSF) 1996). The determination of minimum and maximum growth limits of a strain set comprising 100 representative *B. cereus* isolates confirmed the monomorphic characteristics of emetic toxin producing strains. All emetic isolates from food poisonings, food and environment were unable to grow below 10°C and had a maximum growth limit of 48°C. The emetic toxin

producing *B. cereus* isolates showed also additional distinct phenotypic characteristics like a higher heat resistance of spores and lower germination rates at 7°C compared to spores of the remaining strains of the set (Carlin et al. 2006). In contrast to the elevated minimum growth limit of the emetic isolates, 60.1 % of the non-emetic isolates from environment and 37.3 % from foods grew at 7°C or even below. Generally, food and environmental isolates showed a broader diversity concerning their growth limits. None of the isolates connected to food poisonings was able to grow at 4°C. The only strains growing at this temperature were isolated from environment and food. These results are a hint that strains able to grow at 4°C might have less potential to cause food poisonings, which would have important implication for the risk assessment of *B. cereus* food poisoning.

Temperature and pH had a significant influence on the shape of the growth curves for all strains. Nevertheless, no relationship between curve shape and origin of strains could be established. The additional stress of an acid or alkaline pH seems to have a higher impact on growth at 37°C than at 24°C, as all strains showed growth for all tested pH conditions at 24°C, whereas inhibition of growth was observed at 37°C and pH 8.0 for 19 % of the strains, and in combination with pH 5.0 for 70 % of the strains. The unusual profile of the growth curve at 37°C and pH 8.0 was observed for 78 % of the isolates under this condition. Several authors have observed a sudden decrease of the OD in growth curves and explained this by the production of spores (Marahiel et al. 1979; Hornstra et al. 2005). The subsequent increase of the OD would then correspond to the germination of the spores. In general, better growth was observed for all strains with the combination pH 5.0 and 24°C than 37°C, and pH 8.0 and 37°C than 24°C. These two conditions can be linked to different scenarios where *B. cereus* can grow. As the main part of foods has a rather acidic pH, the combination pH 5.0 and 24°C can be linked to growth of *B. cereus* in food stored at room temperature. The combination of pH 8.0 and 37°C corresponds to the more alkaline conditions during outgrowth of *B. cereus* spores in the intestine, where enterotoxin production takes place and causes diarrhoea (Granum 2001). These results present a linkage between spoilage of improper stored food or food poisoning caused by *B. cereus* and conditions that enhance the growth of this pathogen.

4.1.3 Metabolic fingerprinting of *B. cereus* group organisms by Fourier transform infrared spectroscopy

The discrimination between the members of the *B. cereus* group is mainly based on phenotypical characteristics as for example the formation of crystalline inclusion bodies for *B. thuringiensis*, the ability to grow at low temperature for *B. weihenstephanensis* or the

rhizoid colony morphology of *B. mycoides*. Comparison of the chromosomal DNA of sequenced *B. cereus* group members has shown a very high overall similarity, and according to their close genetic relationship it was proposed to regard them as one species (Carlson et al. 1994; Helgason et al. 2000b; Rasko et al. 2005). However, this concept would cause major problems concerning the classification of the virulence of strains (Priest et al. 2004). Therefore, the differentiation between the closely related *B. cereus* group members is still of major interest. In this study, the phenotypic method Fourier transform infrared (FTIR) spectroscopy was applied in combination with hierarchical cluster analysis (HCA) and artificial neural networks (ANN) to establish a classification system for the *B. cereus* group members *B. cereus*, *B. thuringiensis*, *B. weihenstephanensis* and *B. mycoides* with special emphasis on emetic *B. cereus* strains. It was not possible to record spectra of *B. anthracis* because of the need for category 3 containment facilities.

As described in chapter 4.1.1 and 4.1.2, emetic *B. cereus* isolates show low phenetic and genetic variability when compared with other *B. cereus* isolates (Ehling-Schulz et al. 2005a). The close relationship within emetic *B. cereus* isolates is confirmed in cluster I of the MLST analysis of *B. cereus* group members in Figure 1A, this cluster is also reflected with HCA of FTIR spectra (data not shown). Non-emetic *B. cereus* and *B. thuringiensis* isolates were mainly distributed in cluster II with MLST analysis. The formation of mixed clusters of the two strains has also been demonstrated with other genotypic methods like MEE and AFLP, often the isolates of the two species were scattered over the whole phylogenetic tree or were separated in different mixed clusters (Vilas-Boas et al. 2002; Helgason et al. 2004; Hill et al. 2004). Cluster III represents the psychrotolerant members of the *B. cereus* group namely *B. weihenstephanensis* and *B. mycoides*. These two species can only be separated from each other according to the rhizoid growth of *B. mycoides* and the differentiation in two species has been questioned recently (Tourasse et al. 2006). The formation of a psychrotolerant cluster as described here has also been reported with MLST using different house keeping genes and AFLP (Keim et al. 1997; Priest et al. 2004; Sorokin et al. 2006).

The three main clusters observed with phenotypic as well as with genotypic methods were selected as basis for the application of the supervised learning algorithm of the ANN. The implementation of ANNs in combination with FTIR spectroscopy has been shown to allow a more accurate differentiation of closely related strains than for example HCA (Rebuffo et al. 2006). ANNs have the advantage that the most discriminative wave numbers between species or isolates are weighted according to their importance for the differentiation (Schmitt and Udelhoven 2001), whereas only windows between wave numbers can be

selected for HCA. The trained two-level ANN reached an overall correct identification rate of 96.6 % in the internal and 95.6 % in the external validation. This result demonstrates the high performance of ANN assisted FTIR spectroscopy for the differentiation of the closely related *B. cereus* group members (Table 11 and 12). It has already been shown that the interspecies differentiation was improved for *Listeria* species when more strains were included in the ANN reference database because of a better coverage of intraspecies variability (Rebuffo et al. 2006). Therefore, the number of *B. mycoides* and *B. thuringiensis* strains should be increased in the training of the ANN because of the reduced correct identification rate in the internal validation of these species with 93.8 % and 91.7 %, respectively.

The 100 % correct identification rate for emetic *B. cereus* strains in the internal as well as the external validation suggests that the intraspecies variability for this subgroup of *B. cereus* is already covered with the present selection of isolates. This is in accordance with the low phenetic and genetic variability described for these strains (Ehling-Schulz et al. 2005a). However, the current ANN is not capable to differentiate between emetic and emetic-like *B. cereus* strains; both are classified as emetic type of *B. cereus*. Emetic-like strains share the same phenotypic characteristics as emetic *B. cereus* strains but are not producing detectable amounts of the emetic toxin cereulide nor possess the *ces* gene cluster necessary for the production of the emetic toxin (Ehling-Schulz et al. 2005a). Since suitable PCR based detection methods for emetic toxin producing *B. cereus* isolates have been developed within this study, a rapid differentiation between emetic and emetic-like *B. cereus* isolates is now possible.

By the selection of the covar algorithm during the training of the ANNs, the most discriminative wave numbers for the differentiation between the *B. cereus* group members are selected and ranked automatically. The main part of the discriminative wave numbers can be assigned to the fatty acids, carbohydrates and fingerprint region, whereas the amide regions are of minor importance. Others have described a characteristic absorbance peak between 1740 and 1738 cm^{-1} for the members of the *B. cereus* group (Lin et al. 2004), but this region seems not to be important for the differentiation between the species as no wave numbers in between were selected for the training of the ANNs.

The established ANN assisted FTIR spectroscopy method was successfully applied to analyze soil samples from different origins and a food sample from a recent outbreak caused by emetic *B. cereus*. Classification results in Table 14 highlight the population differences in such kind of samples. Non-emetic *B. cereus* and the psychrotolerant members of the *B. cereus* group were predominant in the different soil samples, whereas they were rarely or not at all

found in the sample from the food borne outbreak. Only few of the soil isolates were classified as *B. thuringiensis* (3 to 7 per sample) in this study. Hendriksen et al. (2006) examined Danish sandy loam and made similar observations. This type of soil was free of *B. thuringiensis* and *B. anthracis*, contained mainly *B. weihenstephanensis* and *B. mycoides* (94 %), and only 6 % of the population represented *B. cereus* isolates. von Stetten et al. (1999) described the annual average temperature as a possible explanation for the differences in populations of soil samples concerning mesophilic and psychrotolerant *B. cereus* group members. The low number of soil isolates classified to the emetic type of *B. cereus* is in accordance with Altayar and Sutherland (2005). They found *B. cereus* in 61 of 80 soil samples. None of 209 presumptive *B. cereus* isolated from soil and feces were positive for emetic toxin production.

In contrast, none of the isolates from an emetic food borne outbreak connected to rice were classified to the psychrotolerant members of the *B. cereus* group. The majority of these isolates were classified to the emetic type of *B. cereus*. Thereof, 11 randomly selected isolates were analyzed via PCR for the presence of the *ces* gene cluster necessary for emetic toxin production, but only four isolates tested positive. The seven isolates negative in the PCR assay belong to the group of the emetic-like *B. cereus*. This result implicates that although the rice dish was the causative agent of an emetic food poisoning involving 17 children, the majority of the isolates are probably emetic-like *B. cereu*s, that are not able to produce the emetic toxin cereulide. This strengthens the demand to analyze more than one colony from food samples implicated in food poisonings as also others have observed mixed populations of emetic and non-emetic *B. cereus* isolates in such samples (Pirhonen et al. 2005).

In summary, an ANN assisted FTIR spectroscopy method was established for the rapid and cost efficient differentiation between the closely related *B. cereus* group members. The established ANN has already an overall correct identification rate of 95.6 %. Still, an increased number of strains used for the training might provide an even superior coverage of the intra- and interspecific diversity of the *B. cereus* group members and decreases the number of isolates with failed identification. The established method was successfully applied for the analysis of populations of *B. cereus* group members in different soils and a food sample implicated in a recent food poisoning and demonstrated high differences in the population structures of these samples. The developed method is a promising tool to elucidate the composition of *B. cereus* group populations in different kinds of samples, e.g. soil or food habitats.

4.2 Evaluation of selective plating media

Currently, two selective plating media, PEMBA and MYP, are recommended by food authorities as standards for the detection of *B. cereus*. One main identification feature of these media is the lecithinase activity, resulting in opaque precipitation zones around suspect colonies. Strains that lack this characteristic have already been described (Szabo et al. 1984; Ehling-Schulz et al. 2004a). However, not only a lack of lecithinase activity but also the colour of the colony and its morphology may result in misidentification of specimens (Figure 11). In our experiments, 72 % did not show the expected blue colour on PEMBA, and 29 % showed unusual colony morphologies on at least one of the tested standard plating media. *B. cereus* group organisms, except for *B. anthracis*, in general show a strong expression of degrading enzymes such as proteinases, which results in an increase of pH and the appearance of the typical peacock blue colonies on PEMBA (Figure 11A). Since many of these degrading enzymes are regulated by the pleiotropic regulator PlcR (Gohar et al. 2005), atypical colony appearance on PEMBA and MYP might be explained by mutations in the *plcR* gene. Slamti et al. (2004) showed that distinct mutations in the *plcR* gene could significantly affect the PlcR activity.

The two chromogenic plating media tested in this study are based on the activity of two different hydrolyzing enzymes specific for *B. cereus*, phosphatidylinositol phospholipase C (PI-PLC) for CEI, and β-D-glucosidase for CBC. The expression of β-D-glucosidase of *B. cereus* strains grown on CBC turned out to be quite variable. The majority of strains showed white colonies with a blue centre (65 %), but some showed totally turquoise pinpoint colonies (16 %), and 11 % of the strains did not yield a colour formation at all and remained white (Figure 11D). This chromogenic plating medium therefore does not seem to be an appropriate alternative to the conventional standard plating media. In contrast, the identification of the tested *B. cereus* strains with PEMBA, MYP or CEI was superior, less than 10 % of the tested strains showed atypical growth reactions. The best overall performance was obtained with the chromogenic plating media CEI. The advantage of CEI, in comparison to the standard plating media, is the unambiguous results (Table 8). On PEMBA and MYP more than 50 % of the tested strains showed only weak reactions (for definition see material and methods section), which makes identification difficult and requires highly trained and experienced lab staff and/or further conformation tests. These results suggest that the use of direct plating on ISO recommended standard plating media may result in failure to detect a significant portion of *B. cereus* specimens. In particular this was seen if specimens from complex matrices like food and clinical environments, with high background flora, were

analysed (Figure 11E-F and 12). The routine use of the standard plating media might therefore contribute to an underestimation of the occurrence of *B. cereus* in foods or clinical samples, and might be a reason for significant underestimation of the actual proportion of acute food borne gastrointestinal diseases attributable to *B. cereus*. Analysis of four naturally contaminated food samples showed that enumeration and isolation of colonies was much easier on the chromogenic plating media, in particular on CEI, than on the standard plating media (Figure 11E-F and 12). These results are in accordance with previous studies showing that new chromogenic media are more selective and reduce the competing background flora present on the agar when complex samples are examined (Manafi 1996; Juergensmeyer et al. 2006). In summary, the best results were obtained with the chromogenic plating medium CEI, but there are still few unusual strains that would be misidentified even on the latter. Since these unusual strains include highly toxic strains like the original cytK strain NVH 0391-98 (Figure 11C), which was responsible for a fatal outbreak in France (Lund et al. 2000), improved detection methods are urgently needed.

Due to the growing interest in *B. anthracis* identification several authors have tested the suitability of chromogenic plating media for the detection of *B. anthracis*. E.g., two studies tested the suitability of the CEI agar for *B. anthracis* diagnostics (Reissbrodt et al. 2004; Tomaso et al. 2006). Usually, *B. anthracis* grows on CEI as white colonies, while all other *B. cereus* group members are supposed to form blue colonies (Figure 11C). However, Tomaso *et al.* found a notable portion (30 %) of *B. cereus* and *B. weihenstephanensis* strains that showed a *B. anthracis*-like morphology on CEI and were misidentified as *B. anthracis*. *B. weihenstephanensis* is a psychrotolerant member of the *B. cereus* group with a lowered temperature for optimum of growth compared to the mesophilic members of the *B. cereus* group (Lechner et al. 1998). In preliminary tests we have found that by lowering the incubation temperature to 30°C, instead of the recommended 36 ± 1°C for CEI, the portion of misidentified psychrotolerant *B. cereus* group organisms could be substantially reduced (below 5 % in this study). The sensitivity of psychrotolerant strains to higher temperature was also reflected by the *B. weihenstephanensis* type strain in the gamma phage assay. This strain was sensitive against the gamma phage at 37°C but not at 30°C. The question if gamma phage sensitivity of the *B. weihenstephanensis* type strain at 37°C is the result of heat stress of the psychrotolerant strain or has other reasons requires further analysis. Nevertheless, at 30°C there are still some *B. cereus* isolates which show a *B. anthracis*-like phenotype on CEI. Additionally to an inactive PI-PLC resulting in white colonies on CEI, those strains also had an inactive PC-PLC (phosphatidylcholine-phospholipase C) and were not able to cleave the

artificial chromogenic substrate X-CP (data not shown). Recently, a new selective chromogenic plating medium to distinguish *B. anthracis* from *B. cereus* has been described (Juergensmeyer et al. 2006). However, since this agar is based on the reduced PC-PLC activity of *B. anthracis*, the aforementioned PC-PLC negative *B. cereus* isolates might be misidentified as *B. anthracis* on this plating media too. An unequivocal identification of isolates showing a *B. anthracis*-like phenotype was only possible with the gamma phage test (Table 10) and molecular tests.

Expression of PI-PLC as well as PC-PLC is dependent on the pleiotropic regulator PlcR. Disruptions or mutations of the *plcR* gene greatly reduce the production of the aforementioned phospholipases and a nonsense mutation in the *plcR* gene in *B. anthracis* has been reported to be responsible for the non-hemolytic phenotype of *B. anthracis* (Agaisse et al. 1999; Slamti et al. 2004). A cluster analysis of the *plcR* sequences from selected *B. cereus* group strains showed that the *plcR* genes of isolates with atypical growth on PEMBA, MYP and CEI fall in the same branch as *B. anthracis* (Figure 2). It is therefore tempting to speculate that the atypical growth reactions observed on these plating media are attributable to variations in the *plcR* gene sequence. This hypothesis is supported by a recent study by (Slamti et al. 2004) providing evidence that distinct variations in the *plcR* gene significantly influence the expression of a variety of unspecific virulence factors, including phospholipases. The current concept of selective plating media, utilisation of PlcR regulated enzyme activities for differentiation purposes, should therefore be reconsidered.

In conclusion, this survey showed that chromogenic media can improve and facilitate *B. cereus* diagnostics since (i) the background flora is effectively suppressed and (ii) these media rely on a single enzymatic reaction resulting in less ambiguous results. But, this study also highlighted that the selection of the synthetic substrate is a crucial step (Table 8). Since there are still *B. cereus* strains that could not be identified with these new chromogenic plating media, including a very toxic isolate, alternative systems for the detection of *B. cereus* have to be considered. Research should be directed towards culture independent methods like quantitative Real-time PCR systems, as have already been described for the detection of other food pathogens (e.g. (Berrada et al. 2006; Hein et al. 2006)).

4.3 Development of genotypic methods for the detection of emetic *B. cereus* isolates

4.3.1 PCR assays for the detection of emetic *B. cereus* isolates

Diagnosis of *B. cereus* linked to the emetic type of food poisoning was hampered by the lack of genetic information on the toxin genes. Based on the fact, that the emetic toxin cereulide has a cyclic dodecadepsipeptide structure (Agata et al. 1994) and is closely related to the potassium ionophore valinomycin, a similar mode of synthesis as described for valinomycin via a non-ribosomal peptide synthetase (NRPS) was assumed for cereulide (Perkins et al. 1990; Stachelhaus and Marahiel 1995). In a PCR screening assay searching for NRPS homologue sequences, a specific DNA fragment for emetic toxin producing *B. cereus* strains was identified. Subsequently a standard PCR assay with the primers EM1F and EM1R targeting a region with unknown function (Ehling-Schulz et al. 2004b) was developed, and represents the first molecular assay for the rapid detection of emetic *B. cereus* isolates. The specificity of the assay was confirmed analyzing 238 bacterial isolates, including 162 *B. cereus* isolates (thereof 52 were emetic) and by Southern blot analysis. Finally, after sequencing the complete *ces* gene cluster by inverse PCR and module jumping, the location of the primers EM1F and EM1R could be demonstrated in a region of unknown function between the A8 and A9 core motifs of c*esB1* domain (Ehling-Schulz et al. 2006a).

In addition, the sequence information on the *ces* gene cluster was used for the design of primers and probes suitable for the Real-time detection of emetic *B. cereus* isolates. In principle, two different chemistries are available for the Real-time detection of PCR products: fluorescent probes that bind specifically to certain DNA sequences and fluorescent dyes that intercalate in any double-stranded DNA. Within this study, a diagnostic 5' nuclease (TaqMan) Real-time PCR for diagnostic purposes, as well as a SYBR Green I based system representing an interesting economic alternative for high throughput screening purposes, were developed. Both methods allow a more rapid identification of emetic *B. cereus* isolates. As the use of internal amplification controls (IAC) in diagnostic PCR is becoming mandatory (Hoorfar et al. 2003; Malorny et al. 2003a), an IAC was included in the TaqMan assay. Such an internal control indicates the presence of PCR amplification inhibitors or malfunction of the thermal cycler (Al-Soud and Radström 1998; Schoder et al. 2003). The primers of both Real-time assay anneal within an unusual insertion in the *cesA1* domain of the *ces* gene operon which encodes a novel type of α-ketoreductase that perform chiral reduction of α-ketoacyl-S-carrier

proteins as opposed to the typical ketoreductases found in polyketide synthetases (PKS) (Magarvey et al. 2006) This insertion has been shown to be highly specific for emetic *B. cereus* by hybridization studies and database analysis (Ehling-Schulz et al. 2006a).

Both assays were challenged with a strain set (100 isolates) comprising emetic and non-emetic *B. cereus*, *B. cereus* group isolates and other bacterial species. No false negative, false positive or cross reactions were observed with the SYBR Green I or TaqMan primers and the *ces*- or IAC- specific probes. The detection limit for the TaqMan assay (including the IAC) was 0.6 pg and 0.06 pg for the SYBR Green I assay (Fricker et al. 2007). Recently, it has been shown that the cereulide synthetase gene cluster is located on a 208 kb mega plasmid that has high homology to the plasmids pXO1 of *B. anthracis* and pBc10987 of *B. cereus* ATCC 10987 (Ehling-Schulz et al. 2006a; Rasko et al. 2007). According to Rasko et al. (Rasko et al. 2004; 2005) these are low copy plasmids with between 1 - 3 copies per cell. Assuming similar numbers for the pBCE4810 plasmid, the detection limit of the Real-time PCR assay (for pure cultures) would correspond to approximately 10 genomic equivalents for the SYBR Green I simplex PCR assay and 100 genomic equivalents for the TaqMan assay (deduced from the genome size of *B. cereus* ATCC 10987 and calculated with the formula proposed by (Rodríguez-Lázaro et al. 2005)). The repeatability standard deviation s_r of both assays was calculated according to (Malorny et al. 2004) and were quite similar for the TaqMan (1.5 to 2.2 %) and the SYBR Green I simplex assay (1.5 to 3.3 %), thus indicating the high precision of the assays.

Since the emetic syndrome caused by *B. cereus* can not be differentiated symptomatically from intoxications with *S. aureus* (Ehling-Schulz et al. 2004a), the SYBR Green I PCR assay was extended to a duplex assay for the one step differentiation between emetic *B. cereus* and *S. aureus*. Both pathogens are broadly distributed microorganisms that are often transferred to foodstuff. Typical cell counts in food samples connected to food poisoning are 10^5 - 10^8 cfu *B. cereus*/g (Granum 2001) or 10^6 - 10^8 cfu *S. aureus*/g (Johnson et al. 1990), sometimes less. Microbial norms in most countries tolerate low numbers of these pathogens, but threshold values are often dependent on the foodstuff in question, its further preparation (re-heating or ready-to eat) and the potential consumer (young, old, immunocompromised people). E.g. threshold values for *B. cereus* in Germany vary from 10^2 cfu/g in baby food to 10^4 cfu/g in spices, or for *S. aureus* in France from 0 cfu/g in semi-canned food to 10^3 cfu/g in some raw milk cheeses (Le Loir et al. 2003; Becker 2005). Of food poisoning cases in which *S. aureus* had been suspected to be the causative agent, in only 2 % *S. aureus* was detected (Dietrich Mäde, Halle, Germany, personal communication).

However, *B. cereus* was the main cause of food borne disease in mass-catered food prepared by and served to German Armed Forces from 1985 to 2000 and the most common pathogen isolated from food borne illness in 1990 in Norway (Aas et al. 1992; Kleer et al. 2001). It is therefore tempting to speculate that a significant portion of those cases in which the causative agent remained unknown were actually elicited by emetic *B. cereus*.

SYBR Green I based duplex PCR has been shown to be suitable for the one-step detection and differentiation of food and plant pathogens (Aarts et al. 2001; Jothikumar et al. 2003; Brandfass and Karlovsky 2006). Product accumulation during PCR is monitored by the dsDNA-specific cyanine dye SYBR Green I, followed by a melting curve analyses of the amplicons. Different PCR products can be distinguished by their respective melting temperature (T_m) which is dependent on the GC content, length and sequence characteristics (Ririe et al. 1997). The specific amplicons for emetic *B. cereus* and *S. aureus* could be clearly separated and allowed the detection of 0.5 pg emetic *B. cereus* DNA and 5 pg *S. aureus* DNA, when DNA both pathogens where present in the same PCR reaction (Fricker et al. 2007).

The detection limit of the SYBR Green simplex, duplex and TaqMan assay from artificially contaminated food were dependent on the applied DNA isolation method. Various methods have been described in the literature for DNA extraction from foods for Real-time PCR, since it is highly sensitive towards inhibitory substances originating from the food matrix (McKillip and Drake 2004). Often these methods include a complexing step (e.g. cetyltrimethylammonium bromide), magnetic beads or commercially available kits (Romero and Lopez-Goni 1999; Rudi et al. 2002; Nakano et al. 2004) but also simple methods, like boiling, have been reported to be suitable (De Medici et al. 2003). Verification of the diagnostic accuracy of the Real-time assays showed that they are suitable for the detection of 10^0 cfu/g emetic *B. cereus* in foods after a short enrichment time (4 to 6 hours) while higher cell numbers (10^1 - 10^3 cfu/g) can be detected directly from food samples without the need for further enrichment steps. Since the typical cell counts reported in foods incriminated in emetic outbreaks have been reported to be about 10^5 - 10^8 cfu *B. cereus*/g (Granum 2001), samples from emetic food poisonings can be processed and analyzed within 1.5 to 4 hours, depending on the DNA isolation method applied. In general, kit-based DNA isolation methods have a better recovery rate, but are more time-consuming and more expensive than the simple boiling method applied in this study. A longer enrichment in combination with the simple boiling method can accelerate the results of the Real-time PCR and lower the costs. In food samples connected to *S. aureus* intoxications, cell counts between 10^5 - 10^6 cfu/g have been reported (Bennett 2005). The SYBR Green duplex assay allowed the detection of 10^3 cfu *S. aureus*/g

food after 4 h enrichment and 6 h enrichment when emetic *B. cereus* were present in the same enrichment, respectively. However, due to the heat stability of the emesis causing toxins from both pathogens the molecular analysis by Real-time PCR should be supplemented by toxin analysis methods, especially in case of re-heated foods.

In summary, the introduced TaqMan and SYBR Green I Real-time PCR assay allow the fast and conclusive identification of emetic *B. cereus*. The TaqMan assay includes an IAC to avoid false-negative results and therefore allows its implementation in routine food diagnostic laboratories. The SYBR Green I based assay represents an economically interesting alternative for the analysis of a large number of samples (e.g. in epidemiological studies) and can be extended to a duplex Real-time PCR for the one-step differentiation of emetic *B. cereus* and *S. aureus*. The novel Real-time PCR assays were shown to be fast, sensitive and reliable diagnostic tools, which complement the existing toxin analysis methods.

4.4 Application of the developed methods

B. cereus is increasingly recognized as the causative agent of gastrointestinal and non-gastrointestinal diseases in humans and animals. Hence, there is an increasing awareness of the importance to detect possible contaminations with the facultative pathogen and to determine the risk potential of the respective isolates. At the beginning of this study, no fast and simple methods for the detection of emetic *B. cereus* strains or the emetic toxin cereulide were available. Therefore, emetic food poisonings were mainly associated with entero-toxigenic *Staphylococcus aureus*, a pathogen provoking the same symptoms as reported for emetic *B. cereus*. But in the majority of the cases (49 of 50), *S. aureus* was not detected and the causative agent of the emetic food poisoning remained unknown (Dietrich Mäde, Halle, Germany, personal communication). The developed molecular detection methods include a standard, a SYBR Green I and a TaqMan PCR assay for the rapid detection of emetic *B. cereus* strains. All three assays have already been applied in different diagnostic laboratories for the routinuous examination of food samples and have helped to elucidate the causative agent of recent and former food poisonings (Messelhäußer et al. 2007). In addition, the developed duplex SYBR Green I assay for the one step differentiation between emetic *B. cereus* and *S. aureus* provides the possibility to increase the detection and identification rate of the causative agents in emetic food poisonings. Therefore appropriate tools are now available to investigate the true incidence of food poisonings caused by emetic *B. cereus*. In addition, the developed toxin gene profiling has been successfully applied to examine the distribution of the enterotoxin genes and the *ces* gene cluster within clinical, food,

environmental and dairy isolates (Ehling-Schulz et al. 2006b). Apart from population studies, this method can be used to analyze the potential hazard of *B. cereus* strains isolated from foods for the consumer.

The phenotypic method Fourier transform infrared spectroscopy (FTIR) was utilized in combination with hierarchical cluster analysis (HCA) and artificial neural networks (ANN). In combination with HCA, a comparison of infrared spectra is possible and the degree of heterogeneity between the spectra provides an insight into the relationship and similarity of the respective isolates. This application has already been used for contamination route analysis by determining whether different isolates are identical and subsequently by identifying the source of contamination. Another application is the population analysis of various samples as shown by the analysis of soil samples from different origins and of a food sample connected to an emetic food poisonings. The developed ANN assisted FTIR spectroscopy method provides the possibility for a rapid identification and differentiation between *B. cereus* group strains and was applied for the samples described above and for the routinous identification of unknown *B. cereus* group isolates.

4.5 General conclusions and perspectives

During this study, the first molecular assays for the fast and conclusive detection of emetic *B. cereus* strains were developed: (i) a standard PCR assay and a SYBR Green I based Real-time PCR assay for high through-put applications, (ii) a duplex SYBR Green I assay for the one step differentiation of enterotoxigenic *S. aureus* and emetic *B. cereus*, and (iii) a TaqMan based Real-time PCR assay including an internal amplification control (IAC) for diagnostic purposes. The TaqMan assay has already been implemented in routine food diagnostic laboratories and has been proposed as a §64 LFGB method for the standardized detection of emetic *B. cereus* (Busch 2007). In addition, the developed Real-time PCR assays allow the quantification of emetic *B. cereus*.

The comparison of new chromogenic plating media (CBC and CEI) with standard selective plating media (PEMBA and MYP) used for the detection and enumeration of *B. cereus* revealed the superior performance of the former. However, there are still some *B. cereus* strains that could not even be detected with this new type of chromogenic plating media and sometimes growth reactions were ambiguous. The development of alternative methods for the conclusive identification of *B. cereus* therefore has to be considered, e.g. culture independent methods like the Real-time PCR systems introduced in this work.

Physiological and phenetic analysis of *B. cereus* group strains revealed a distinct phenotype for emetic *B. cereus* strains with an elevated minimum and maximum growth limit, and a rather monomorphic phenotype and genotype, as shown by FTIR spectroscopy, MLST, and toxin gene profiling, whereas diarrhoeal *B. cereus* strains show high variance in these parameters. In summary, the developed metabolic fingerprinting method using ANN assisted FTIR spectroscopy can be combined with genotypic fingerprinting methods like MLST, AFLP or the introduced toxin gene profiling method to gain a deeper insight into the population structure and pathogenicity of the *B. cereus* group of organisms.

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7 APPENDIX

7.1 Bacterial strains

The bacterial strains used for the development and evaluation of the applied methods are listed in the following tables. Table 16 comprises *Bacillus* sp. and non-*Bacillus* strains, whereas Table 17 comprises all *Bacillus cereus* group strains. The first column of each table indicates the affiliation of the respective strain to the different strain sets compiled during this study with the following encoding:

a: strain set used for the standard PCR assay

b: strain set used for the Real-time PCR assays

c: strain set used for the toxin gene profiling

c1: test panel used for the toxin gene profiling

c2: strains set used for the application of toxin gene profiling

d: strain set used for the determination of growth limits

e: strain set used for the evaluation of different selective plating media

f1: strain set used for the training of ANN assisted FTIR spectroscopy

f2: strain set used for the external validation of ANN assisted FTIR spectroscopy

Table 16: *Bacillus* sp. and non-*Bacillus* strains used during this study.

strain set[a]	species	code	additional codes	additional information	origin
a, b, c	*Bacillus amyloliquefaciens*	WSBC 27001			pasteurized milk
a, b, c	*Bacillus brevis*	ATCC 8185		produces tyrocidine	
a, b, c	*Bacillus brevis*^{T}	ATCC 8246	WS 1536		
a, b, c	*Bacillus brevis*	ATCC 9999		produces gramicidin	
a, b, c	*Bacillus licheniformis*^{T}	ATCC 14580	WS 1528		
a, b, c	*Bacillus licheniformis*	WSBC 23001			pasteurized milk
a, b, c	*Bacillus licheniformis*	WSBC 23007			pasteurized milk
b	*Bacillus megaterium*^{T}	ATCC 14581			
a, b, c	*Bacillus subtilis*^{T}	ATCC 6051	WS 1525		
a, b, c	*Campylobacter jejuni*	DSM 4688	WS 3510		animal
a, b, c	*Campylobacter pylori*	DSM 10242			clinical
a, b, c	*Campylobacter pylori*	DSM 9691			clinical
a, b, c	*Clostridium perfringens*	ATCC 3626	WS 3004		
a, b, c	*Clostridium perfringens*	ATCC 3628	WS 3005	toxin type B	
a, b, c	*Clostridium perfringens*	DSM 798	WS 2953	toxin type C	
a, b, c	*Escherichia coli*	WS 2577		H1196/13	clinical
a, b	*Escherichia coli*	WS 2578		H1303/92	
a, b, c	*Escherichia coli*	WS 2579		H19 026	clinical
a, b, c	*Escherichia coli*	WS 2580		C1403-83 **O157**	clinical

strain set[a]	species	code	additional codes	additional information	origin
a	*Listeria monocytogenes*	WSLC 10209	WS 2923		
a, b	*Listeria monocytogenes*	WSLC 1132		serovar 1/2b	Silberdistel
a, b	*Listeria monocytogenes*	WSLC 1211		serovar 3a	Brie
a, c	*Listeria monocytogenes*	WSLC 1363	WS 3549	serovar 4b	
a, b, c	*Listeria monocytogenes*	WSLC 1364	WS 3550	serovar 4b	food
a, b, c	*Listeria monocytogenes*	WSLC 1526		serovar VI/VII+	
a, b, c	*S.choleraesuis* subsp. *choleraesuis*	DSM 554	WS 3468		
a, b, c	*Salmonella dublin*	WS 2692			
a, b	*Salmonella typhimurium*	WS 2709			
a, b, c	*S. cholerae-suis/typhi-suis*	WS 2743			
a	*S. enterica* group I	WS 2863			
a	*Salmonella enterocolitica* Serovar *typhimurium* LT2				
a, b, c	*Staphylococcus aureus*	WS 2604		toxin type SEA	
a, b	*Staphylococcus aureus*	WS 2605		toxin type SEA	
a, b	*Staphylococcus aureus*	WS 2606		toxin type SEB	
a, b	*Staphylococcus aureus*	WS 2607	ATCC 14458	toxin type SEB	stool, child
a, b	*Staphylococcus aureus*	WS 2608		toxin type SEC	
a, b, c	*Staphylococcus aureus*	WS 2609	ATCC 19095	toxin type SEC	leg abscess
a, b	*Staphylococcus aureus*	WS 2610		toxin type SED	
a, b, c	*Staphylococcus aureus*	WS 2611		toxin type SED	
a, b	*Staphylococcus aureus*	WS 2612	ATCC 27664	toxin type SEE	chicken
a, b	*Staphylococcus aureus*	WS 2613		toxin type SEE	
b	*Staphylococcus aureus*	WS 4387			clinical
b	*Staphylococcus aureus*	WS 4388			clinical
b	*Staphylococcus aureus*	WS 4389			clinical
b	*Staphylococcus aureus*	WS 4390			clinical
a	*Staphylococcus equorum*	WS 2733			
a, b, c	*Yersinia enterocolitica*	WS 2589			
a, b	*Yersinia enterocolitica*	WS 2594			
a, b	*Yersinia enterocolitica*	WS 2596			
a	*Yersinia enterocolitica*	WS 2599			
a	*Yersinia enterocolitica*	WS 2602			
a, c	*Yersinia enterocolitica*	WS 3371			
a, b	*Yersinia enterocolitica*	WS 3372			
a, c	*Yersinia enterocolitica*	WS 8081			

[a] Definitions on the different strain sets are given in 7.1

Table 17: *Bacillus cereus* group strains used during this study.

affiliation of strains to the different strain sets[a]	Informations on strains					Determination of growth limits		Toxin gene profiling					Results for selective plating media[d]			
	species[b]	code	additional codes	additional information	strain origin	minimum growth temperature	maximum growth temperature	Ces	HBL	NHE	CytK	toxin type[c]	PEMBA	MYP	CBC	CEI
a, c	*Ba*	ATCC 6602	CEB 9533	pXO1-/pXO2+				-	-	+	-	F				
a, b	*Ba*	CEB 9534		pX01-/pX02+	Davis historic											
a, b, c	*Ba*	CEB 9606		pX01+/pX02-	Cepanco historic			-	-	+	-	F				
a, b	*Ba*	CIP 7700	CEB 9531	pXO1-/pXO2-	Sterne											
a	*Ba*	CIP 7702	CEB 9439	Sterne (pXO1+/pXO2-)												
a, b, c	*Ba*	CIP A2	CEB 9441	pX01+/pX02-	human			-	-	+	-	F				
a	*Ba*	NC 08234-02														
c2	*Bc*	13982-3 (M13)		food	fresh milk			-	-	+	-	F				
c2	*Bc*	14177-3 (M14)		food	fresh milk			-	+	+	-	C				
c2	*Bc*	15472-2 (M15)		food	fresh milk			-	+	+	-	C				
a, c1, d	*Bc*	98HMPL63	WSBC 10560	food borne outbreak	cooked salsify	7°C	45°C	-	+	+	+	A				
f1	*Bc*	ATCC 10876	WS 2173													
a, b, e, f2	*Bc*	ATCC 10987	WSBC 10865	food isolate	dairy isolate								2	2	3	1
f2	*Bc*	ATCC 12826	WS 2452													
a, b, f2	*Bc*^{T}	ATCC 14579 (DSM 31)	WSBC 10528	environment	soil											
a, b, f1	*Bc*	ATCC 27877	WS 2454													
f1	*Bc*	ATCC 7064	WS 2630													
a	*Bc*	F0210/76	WSBC 10579	food poisoning	indonesian rice dish											
a	*Bc*	F1942/85	WSBC 10580	food borne outbreak												
a, c1, d	*Bc*	F2081A/98	WSBC 10581	food borne outbreak	Cooked chicken	7°C	48°C	-	+	+	+	A				
a, c1, d	*Bc*	F2085/98	WSBC 10583	food borne outbreak	Cooked rice	10°C	48°C	-	-	+	+	D				
a, c1	*Bc*	F2141/74 (DSM 4282)	WSBC 10567	food borne outbreak	diarrheal			-	+	+	+	A				

affiliation of strains to the different strain sets[a]	Informations on strains					Determination of growth limits		Toxin gene profiling					Results for selective plating media[d]			
	species[b]	code	additional codes	additional information	strain origin	minimum growth temperature	maximum growth temperature	Ces	HBL	NHE	CytK	toxin type[c]	PEMBA	MYP	CBC	CEI
a, b, e	*Bc*	F2404B/79	WSBC 10584	food borne outbreak	food poisoning								2	2	3*	1
a, e	*Bc*	F2769/77	WSBC 10585	food borne outbreak	lobster pate								2	1	3*	1
a	*Bc*	F284/78	WSBC 10586	food poisoning	pork pie											
a	*Bc*	F2875/77	WSBC 10587	food poisoning	rice											
a, c1	*Bc*	F289/78 (LMG 17615)	WSBC 10622	food borne outbreak				-	+	+	+	A				
a, c1, e	*Bc*	F3003/73	WSBC 10588	food borne outbreak				-	-	+	-	F	2	2	1	1
a, c1, d	*Bc*	F3371/93	WSBC 10590	food borne outbreak	chop suey	7°C	45°C	-	+	+	+	A				
a, d	*Bc*	F3453/94	WSBC 10591	food borne outbreak	rice dish	10°C	45°C									
a	*Bc*	F3465/73	WSBC 10592	food borne outbreak												
a, c1, d	*Bc*	F352/90	WSBC 10593	food poisoning	Chow Mein	10°C	45°C	-	+	+	+	A				
e	*Bc*	F3753A/86	WSBC 10541	food borne outbreak	cooked rice								1	3	3	1
a	*Bc*	F4094/73	WSBC 10597	food borne outbreak												
a	*Bc*	F4096/73	WSBC 10598	food borne outbreak												
a, e	*Bc*	F4370/75	WSBC 10602	food borne outbreak	barbecued chicken								2	2	1	1
a, c1, e	*Bc*	F4429/71	WSBC 10604	food borne outbreak	vanilla pudding			-	-	+	+	D	2	2	1	1
a, c1, d, e	*Bc*	F4430/73	WSBC 10605	food borne outbreak	Pea soup	7°C	45°C	-	+	+	+	A	1	1	1	1
a, c1, d, e	*Bc*	F4433/73	WSBC 10608	food borne outbreak	meat loaf	7°C	45°C	-	+	+	+	A	1	1	1	1
a, c1, d	*Bc*	F528/94	WSBC 10613	food borne outbreak	beef&chow mein&rice	7°C	45°C	-	+	+	-	C				
a, b, c1, d, e, f1	*Bc*	F837/76 (DSM 4222)	WSBC 10566	diarrheal - HBL-Referenz	postoperational infection	10°C	48°C	-	+	+	-	C	2	2	1	1
a, c1, d, e	*Bc*	FH3502/72 (DSM 2301)	WSBC 10565	food borne outbreak		7°C	48°C	-	-	+	-	F	2	1	1	1
f1	*Bc*	HER 1399	WS 2622													
c2	*Bc*	Hi1206 PC3		food	baby food			-	+	+	+	A				
c2	*Bc*	Hi1206 PC47		food	baby food			-	+	+	+	A				
a, d, e	*Bc*	IH41064	WSBC 10618	clinical isolate	feces	7°C	45°C						1	1	1	1

affiliation of strains to the different strain sets[a]	Informations on strains					Determination of growth limits		Toxin gene profiling					Results for selective plating media[d]			
	species[b]	code	additional codes	additional information	strain origin	minimum growth temperature	maximum growth temperature	Ces	HBL	NHE	CytK	toxin type[c]	PEMBA	MYP	CBC	CEI
a, c1	*Bc*	INRA 32	WSBC 10551	food + environment	zucchini puree			-	+	+	+	A				
a, c1, d	*Bc*	INRA A3	WSBC 10561	food + environment	Starch	>10°C	45°C	-	+	+	+	A				
a, b,c2, e	*Bc*	INRA C1	WSBC 10562	food isolate	pasteurized zucchini			-	+	+	+	A	2	2	1	1
a, c2, d, e	*Bc*	INRA C24	WSBC 10564	food isolate	pasteurized carrot	>10°C	48°C	-	-	+	-	F	1	2	3	1
a, c1, d	*Bc*	INRA C3	WSBC 10563	food + environment	Pasteurized carrot	10°C	45°C	-	+	+	+	A				
a, d	*Bc*	INRA C57	WSBC 10718	food	Potato purée	10°C	<45°C									
a, c1, e	*Bc*	INRA I16	WSBC 10615	food + environment	pasteurized potatoe			-	+	+	+	A	2	1	1	1
a, c1, d	*Bc*	INRA I21	WSBC 10617	food + environment	carrot	7°C	45°C	-	+	+	-	C				
a, c2, d	*Bc*	INRA PA	WSBC 10730	food isolate	milk proteins	>10°C	48°C	-	-	+	+	D				
a, c2, d	*Bc*	INRA SZ	WSBC 10635	environment	soil	7°C	45°C	-	+	+	+	A				
a, b, d, f2	*Bc*	MHI 124	WSBC 10709	food	baby food	10°C	48°C									
a, b, d	*Bc*	MHI 13	WSBC 10705	food	baby food	10°C	45°C									
a, d, f1	*Bc*	MHI 203	WSBC 10702	food	pasta	7°C	45°C									
a, e	*Bc*	MHI 294	WSBC 10534	food									1	2	1	1
a, d	*Bc*	MHI 32	WSBC 10708	food	baby food	7°C	45°C									
a, c1, d	*Bc*	NRS 404 (DSM 8438)	WSBC 10568	clinical isolate		7°C	45°C	-	-	+	+	D				
a, b, c1, e	*Bc*	NVH 0075-95	WSBC 10552	nhe reference strain	stew with vegetables	>10°C	48°C	-	-	+	-	F	1	2	1	1
a, b, d, e, f2	*Bc*	NVH 0154-01	WSBC 10779	food borne outbreak	figs	10°C	45°C						2	1	3	1
a, d	*Bc*	NVH 0165-99	WSBC 10764	food borne outbreak	Deer-steak	7°C	48°C									
a, d	*Bc*	NVH 0226-00	WSBC 10772	food borne outbreak	Turkey	7°C	45°C									
a, c1, d	*Bc*	NVH 0230-00	WSBC 10553	food borne outbreak	mushroom stew	7°C	48°C	-	+	+	+	A				
a, d	*Bc*	NVH 0309-98	WSBC 10760	food borne outbreak		10°C	45°C									
a, b, c1, d, e, f1	*Bc*	NVH 0391-98	WSBC 10559	cytK reference strain	vegetable pure	>10°C	45°C	-	-	-	+	G	3	3	3*	3
a, d	*Bc*	NVH 0500-00	WSBC 10554	food borne outbreak	Potatoes in cream sauce	7°C	48°C									
a, d, e	*Bc*	NVH 0597-99	WSBC 10766	food borne outbreak	mixed spices	7°C	48°C						2	1	1	1

affiliation of strains to the different strain sets[a]	Informations on strains					Determination of growth limits		Toxin gene profiling					Results for selective plating media[d]			
	species[b]	code	additional codes	additional information	strain origin	minimum growth temperature	maximum growth temperature	Ces	HBL	NHE	CytK	toxin type[c]	PEMBA	MYP	CBC	CEI
a, b, d, e	*Bc*	NVH 0674-98	WSBC 10761	food borne outbreak	scrammbled eggs	7°C	45°C						2	1	3*	1
a, d, f2	*Bc*	NVH 0784-00	WSBC 10774	food borne outbreak	Ground beef	7°C	45°C									
a, d, e	*Bc*	NVH 0861-00	WSBC 10555	food borne outbreak	soft ice	7°C	45°C						2	2	1	1
a, d, e, f1	*Bc*	NVH 1104-98	WSBC 10762	food borne outbreak	Fish-soup	7°C	42°C						1	1	1	1
a, d	*Bc*	NVH 1105-98	WSBC 10763	food borne outbreak	Topping on steak	7°C	45°C									
a, d, e, f1	*Bc*	NVH 1230-88	WSBC 10556	food borne outbreak	oriental stew	7°C	45°C						2	1	1	1
a, d	*Bc*	NVH 141/1-01	WSBC 10778	food borne outbreak	Vegetarian pasta	7°C	45°C									
a, d	*Bc*	NVH 1518-99	WSBC 10770	food borne outbreak	Soft ice (ice-cream)	>10°C	45°C									
a, c1, d, e	*Bc*	NVH 1519-00	WSBC 10557	food borne outbreak	stew with deermeet	>10°C	48°C	-	-	+	-	F	2	2	1	1
a, d, e, f1	*Bc*	NVH 1651-00	WSBC 10558	food borne outbreak	caramel pudding	7°C	45°C						2	1	1	1
a, c2, d, e	*Bc*	NVH 200	WSBC 10624	food borne outbreak	meat dish with rice	7°C	48°C	-	-	+	+	D	1	1	1	1
a, d	*Bc*	NVH 445	WSBC 10797	food	Meat	10°C	48°C									
a, d, f1	*Bc*	NVH 449	WSBC 10788	food	Spices	10°C	45°C									
a, d	*Bc*	NVH 460	WSBC 10801	environment	Equipment	7°C	48°C									
a, d, f2	*Bc*	NVH 506	WSBC 10791	food	Spices	10°C	52°C									
a, d	*Bc*	NVH 512	WSBC 10805	environment	Equipment	7°C	45°C									
a, d, f1	*Bc*	NVH 655	WSBC 10811	environment	River water	7°C	42°C									
c2	*Bc*	R 1	1a	food	milk powder			-	+	+	+	A				
c2	*Bc*	R 10	FA041208 23 oben	food	ice cream			-	-	+	-	F				
c2	*Bc*	R 2	1b	food	milk powder			-	+	+	+	A				
c2	*Bc*	R 28	6a	food	instant dry product			-	-	+	-	F				
c2	*Bc*	R 3	2a	food	milk powder			-	+	+	-	C				
c2	*Bc*	R 4	2b	food	milk powder			-	+	+	-	C				
c2	*Bc*	R 41	mil0425-1-1	food	milk powder			-	-	+	-	F				
c2	*Bc*	R 5	HWW 194 A/2	food	past. milk powder			-	+	+	+	A				

affiliation of strains to the different strain sets[a]	Informations on strains					Determination of growth limits		Toxin gene profiling					Results for selective plating media[d]			
	species[b]	code	additional codes	additional information	strain origin	minimum growth temperature	maximum growth temperature	Ces	HBL	NHE	CytK	toxin type[c]	PEMBA	MYP	CBC	CEI
c2	*Bc*	R 56	G292	food	food			-	+	+	+	A				
c2	*Bc*	R 6	HWW 293 A/2	food	past. milk			-	-	+	-	F				
c2	*Bc*	R 7	HWW 32/2	food	milk powder			-	-	+	-	F				
c2	*Bc*	R 74	ze0614d 187-1	food	almond paste			-	-	+	+	D				
c2	*Bc*	R 85	ATY0708 3-1	food	nuts from cereals			-	+	+	-	C				
a, d, e	*Bc*	RIVM BC 122	WSBC 10820	clinical isolate	patients feces	10°C	45°C						2	1	1	1
a, d, f2	*Bc*	RIVM BC 485	WSBC 10845	food	chicken ragout	7°C	45°C									
a, b, c2, d, e	*Bc*	RIVM BC 63	WSBC 10629	clinical isolate	human feces	7°C	48°C	-	-	+	-	F	2	2	3*	3
a, c2	*Bc*	RIVM BC 70	WSBC 10631	clinical isolate	human feces			-	-	+	-	F				
e	*Bc*	RIVM BC 893	WSBC 10831	food isolate	mixed vegetables								2	1	1	1
a, c1, d, e	*Bc*	RIVM BC 90	WSBC 10633	clinical isolate	human feces	>10°C	48°C	-	-	+	-	F	2	2	3	1
a, c2, e	*Bc*	RIVM BC 91	WSBC 10634	clinical isolate	human feces			-	+	+	-	C	2	3	3	1
a, d, f1	*Bc*	RIVM BC 934	WSBC 10834	food	lettuce	>10°C	48°C									
a, d, f1	*Bc*	RIVM BC 938	WSBC 10836	food	lamb's lettuce	7°C	48°C									
a, d	*Bc*	RIVM BC 964	WSBC 10850	food	kebab	7°C	42°C									
a, d, f1	*Bc*	SDA 1R 177	WSBC 10648	environment	manure	4°C	45°C									
a, d, f1	*Bc*	SDA 1R 183	WSBC 10649	environment	manure	10°C	45°C									
a	*Bc*	SDA 1R 41	WSBC 10640	environment	soil											
a	*Bc*	SDA 1R 49	WSBC 10641	environment	soil											
a, d	*Bc*	SDA 1R 72	WSBC 10643	environment	soil	4°C	45°C									
a, e	*Bc*	SDA GO 95	WSBC 10569	food isolate	vegetables								2	2	3*	1
f1	*Bc*	SDA GR 281	WSBC 10694		raw milk											
a, d	*Bc*	SDA GR 285	WSBC 10871	raw milk in silo tank	food	7°C	48°C									
a, b, e	*Bc*	SDA JO 160	WSBC 10570	food isolate	vegetables								3	2	3*	1
a, c1	*Bc*	SDA JO 164	WSBC 10577	food + environment	raw milk			-	+	+	+	A				

affiliation of strains to the different strain sets[a]	Informations on strains					Determination of growth limits		Toxin gene profiling					Results for selective plating media[d]			
	species[b]	code	additional codes	additional information	strain origin	minimum growth temperature	maximum growth temperature	Ces	HBL	NHE	CytK	toxin type[c]	PEMBA	MYP	CBC	CEI
a, c1, d	*Bc*	SDA KA 96	WSBC 10574	food	raw milk	10°C	45°C	-	+	+	+	A				
a, e, d, f1	*Bc*	SDA MA 57	WSBC 10571	food isolate	raw milk	7°C	48°C						2	2	1	1
a, c2	*Bc*	SDA MA 60	WSBC 10575	food isolate	raw milk			-	+	+	+	A				
a, d	*Bc*	SDA NFFE 640	WSBC 10685	environment	air	4°C	45°C									
a, d, f2	*Bc*	SDA NFFE 664	WSBC 10688	environment	water hose	4°C	45°C									
a, e	*Bc*	SDA VI 273	WSBC 10572	food isolate	vegetables								3	2	3*	3
a, d	*Bc*	UHDAM 1IFI (13)	WSBC 10747	infant food	food	10°C	48°C									
a, c1, d	*Bc*	UHDAM B102	WSBC 10737	food isolate	meat pie	10°C	48°C	-	-	+	-	F				
a, d	*Bc*	UHDAM B106	WSBC 10738	food borne outbreak	'potatoe flour	7°C	48°C									
a, d, f2	*Bc*	UHDAM B154	WSBC 10736	food borne outbreak	cake	7°C	48°C									
a, d	*Bc*	UHDAM B217	WSBC 10735	food borne outbreak	human feces	7°C	48°C									
e	*Bc*	UHDAM ML102	WSBC 10739	food borne outbreak	pasta								2	1	1	1
a, d, f2	*Bc*	UHDAM TSP9	WSBC 10854	environment	Paper board	10°C	45°C									
a, c1, e	*Bc*	WSBC 10028		food isolate	pasteurized milk			-	+	+	+	A	1	1	1	1
a, d	*Bc*	WSBC 10030		food isolate	pasteurized milk	10°C	48°C									
a, c1, e	*Bc*	WSBC 10035		food isolate	pasteurized milk			-	-	+	-	F	2	2	1	1
e	*Bc*	WSBC 10243		food isolate	water filter								2	2	3*	1
a, d, f1	*Bc*	WSBC 10286		food isolate	cream	>10°C	48°C									
a, d	*Bc*	WSBC 10310		environment	soil	>10°C	48°C									
a, d	*Bc*	WSBC 10395		food isolate	raw milk	7°C	n.g. at 42°C									
e	*Bc*	WSBC 10421		food isolate	lemon gras								2	1	1	1
a, b, d, f1	*Bc*	WSBC 10441		environment	soil	10°C	45°C									
a, d, e, f2	*Bc*	WSBC 10466		food isolate	red rice	7°C	48°C						2	1	3*	1
a, d, e, f1	*Bc*	WSBC 10483		food isolate	tobacco	10°C	45°C						2	1	1	1

affiliation of strains to the different strain sets[a]	Informations on strains					Determination of growth limits		Toxin gene profiling					Results for selective plating media[d]			
	species[b]	code	additional codes	additional information	strain origin	minimum growth temperature	maximum growth temperature	Ces	HBL	NHE	CytK	toxin type[c]	PEMBA	MYP	CBC	CEI
c2	*Bc*	WSBC 10883		clinical isolate	wound infection			-	-	+	-	F				
c2	*Bc*	WSBC 10884		clinical isolate	wound infection			-	-	+	+	D				
c2, e, f2	*Bc*	WSBC 10885		clinical isolate	wound swab			-	-	+	+	D	2	2	3	1
c2, e	*Bc*	WSBC 10886		clinical isolate	wound swab			-	+	+	+	A	2	1	1	1
c2	*Bc*	WSBC 10887		clinical isolate	wound infection			-	+	+	-	C				
c2	*Bc*	WSBC 10890		clinical isolate	human feces			-	-	+	+	D				
c2, e	*Bc*	WSBC 10891		clinical isolate	human feces			-	-	+	-	F	2	1	1	1
c2, e	*Bc*	WSBC 10892		clinical isolate	human blood			-	-	+	-	F	2	2	1	1
c2	*Bc*	WSBC 10893		food isolate	butter cream			-	-	+	-	F				
c2, e	*Bc*	WSBC 10894		food isolate	buttercream			-	-	+	-	F	2	2	1	1
c2	*Bc*	WSBC 10896		food isolate	butter cream			-	-	+	-	F				
c2	*Bc*	WSBC 10901		food isolate	dried apricots			-	-	+	-	F				
c2, e	*Bc*	WSBC 10902		food isolate	dried apricots			-	-	+	-	F	2	2	1	1
c2, e, f1	*Bc*	WSBC 10903		food isolate	cake								2	2	1	1
c2, e	*Bc*	WSBC 10904		food isolate	chocolate milk rice			-	+	+	+	A	2	1	3*	1
c2	*Bc*	WSBC 10916		food	baby food			-	-	+	-	F				
c2	*Bc*	WSBC 10917		food	baby food			-	-	+	+	D				
c2	*Bc*	WSBC 10918		food	baby food			-	-	+	+	D				
c2	*Bc*	WSBC 10919		food	baby food			-	+	+	-	C				
c2	*Bc*	WSBC 10920		food	milk powder			-	-	+	-	F				
c2	*Bc*	WSBC 10921		food	milk powder			-	-	+	+	D				
c2	*Bc*	WSBC 10922		food	milk powder			-	-	+	+	D				
c2	*Bc*	WSBC 10923		food	milk powder			-	-	+	+	D				
c2	*Bc*	WSBC 10924		food	milk powder			-	-	+	+	D				
b, c2	*Bc*	WSBC 10926		food poisoning	cauliflower			-	+	+	-	C				

affiliation of strains to the different strain sets[a]	Informations on strains					Determination of growth limits		Toxin gene profiling					Results for selective plating media[d]			
	species[b]	code	additional codes	additional information	strain origin	minimum growth temperature	maximum growth temperature	Ces	HBL	NHE	CytK	toxin type[c]	PEMBA	MYP	CBC	CEI
c2	*Bc*	WSBC 10928		food	cocos milk			-	-	+	-	F				
a, c1, d, e	*Bc*, emetic	F3080B/87	WSBC 10542	food borne outbreak	chicken korma&rice			+	-	+	-	E	1	2	1	1
a, c1, e	*Bc*, emetic	F3350/87	WSBC 10589	food borne outbreak				+	-	+	-	E	2	2	2	1
a, c1, d, e, f1	*Bc*, emetic	F3351/87	WSBC 10544	clinical isolate	human feces	10°C	48°C	+	-	+	-	E	1	2	1	1
a, c1, e	*Bc*, emetic	F3605/73	WSBC 10594	food borne outbreak	boiled rice			+	-	+	-	E	2	2	2	1
a, c1, e	*Bc*, emetic	F3752A/86	WSBC 10595	food borne outbreak	cooked rice			+	-	+	-	E	2	2	2	1
a, c1, e, f2	*Bc*, emetic	F3876/87	WSBC 10546	clinical isolate	patient with pyrexia			+	-	+	-	E	2	2	1	1
a, c1, e	*Bc*, emetic	F3942/87	WSBC 10596	food borne outbreak	boiled rice			+	-	+	-	E	2	2	2	1
a, c1	*Bc*, emetic	F4108/89	WSBC 10599	food borne outbreak				+	-	+	-	E				
a, c1	*Bc*, emetic	F4426/94	WSBC 10603	food borne outbreak	boiled rice			+	-	+	-	E				
a, c1, e	*Bc*, emetic	F4552/75	WSBC 10609	clinical isolate	vomit			+	-	+	-	E	2	2	1	1
a, c1, b, e, f1	*Bc*, emetic	F47/94	WSBC 10610	food borne outbreak	indian restaurant chicken&rice			+	-	+	-	E	1	2	1	1
a, b, c1, d, e, f1	*Bc*, emetic	F4810/72 (SMR-178)	WSBC 10530	food borne outbreak	cooked rice	10°C	48°C	+	-	+	-	E	1	2	1	1
a, b, c1, e	*Bc*, emetic	F5881/94	WSBC 10548	food borne outbreak	fried rice			+	-	+	-	E	1	2	1	1
a, c1, e	*Bc*, emetic	F6921/94	WSBC 10614	food borne outbreak	rice			+	-	+	-	E	1	1	1	1
a, b, c1, d, e, f1	*Bc*, emetic	IH41385	WSBC 10619	clinical isolate	dialysis liquid	>10°C	48°C	+	-	+	-	E	1	2	1	1
a, e	*Bc*, emetic	INRA I9	WSBC 10729	food isolate	flour								1	1	1	1
a, b, c2, e	*Bc*, emetic	MHI 1305	WSBC 10536	food borne outbreak	indian rice dish	>10°C	48°C	+	-	+	-	E	2	2	1	1
a, e, f2	*Bc*, emetic	MHI 135	WSBC 10532	food	baby food								2	2	1	1
e, f2	*Bc*, emetic	MHI 1471	WSBC 10908	food borne outbreak	noodles/outbreak								2	2	1	1
c2, e, f2	*Bc*, emetic	MHI 1631	WSBC 10909	food borne outbreak	pudding/outbreak			+	-	+	-	E	1	2	1	1
e, f2	*Bc*, emetic	MHI 1745	WSBC 10910	food borne outbreak	fish panade								2	2	1	1
a, b, e	*Bc*, emetic	MHI 280	WSBC 10533	food									2	2	1	1
a, b, e	*Bc*, emetic	MHI 297	WSBC 10535	food									1	2	1	1

affiliation of strains to the different strain sets[a]	Informations on strains					Determination of growth limits		Toxin gene profiling					Results for selective plating media[d]			
	species[b]	code	additional codes	additional information	strain origin	minimum growth temperature	maximum growth temperature	Ces	HBL	NHE	CytK	toxin type[c]	PEMBA	MYP	CBC	CEI
a, d, e, f2	*Bc*, emetic	MHI 87	WSBC 10531	food isolate	baby food	>10°C	48°C						2	2	1	1
a, b, c1, d, e, f2	*Bc*, emetic	NC 7401	WSBC 10537	food borne outbreak	vomit (Chow mein)	>10°C	48°C	+	-	+	-	E	2	2	1	1
b	*Bc*, emetic	PA21		food												
b	*Bc*, emetic	PA24		food												
b	*Bc*, emetic	PA25		food												
b	*Bc*, emetic	PA29		food												
b	*Bc*, emetic	PA3		food												
b	*Bc*, emetic	PA35		food												
b	*Bc*, emetic	PA36		food												
a, c2, d, f2	*Bc*, emetic	RIVM BC 124	WSBC 10821	clinical isolate	human feces	10°C	48°C	+	-	+	-	E				
a	*Bc*, emetic	RIVM BC 143	WSBC 10825	clinical	control feces											
a, d, e, f2	*Bc*, emetic	RIVM BC 379	WSBC 10843	food	chicken	>10°C	48°C						2	2	1	1
a, c1, e, f1	*Bc*, emetic	RIVM BC 51	WSBC 10625	food borne outbreak	rice dish			+	-	+	-	E	2	2	1	1
a, c1	*Bc*, emetic	RIVM BC 52	WSBC 10626	food borne outbreak	rice dish			+	-	+	-	E				
a, b, e	*Bc*, emetic	RIVM BC 61	WSBC 10627	clinical isolate	vomit								1	2	3	1
a, c2, e	*Bc*, emetic	RIVM BC 62	WSBC 10628	clinical isolate	vomit			+	-	+	-	E	1	2	2	1
a, b, c1, d, e	*Bc*, emetic	RIVM BC 67	WSBC 10630	clinical isolate	human feces	10°C	48°C	+	-	+	+	B	3	3	1	1
a, c1, e	*Bc*, emetic	RIVM BC 75	WSBC 10632	clinical isolate	human feces			+	-	+	+	B	3	3	1	1
a, e, f1	*Bc*, emetic	SDA A 116	WSBC 10870	food isolate	commercial farm								2	1	1	1
a, b, c1, e, f2	*Bc*, emetic	SDA GR 177	WSBC 10576	food + environment	dairy	10°C	48°C	+	-	+	-	E	2	2	1	1
a	*Bc*, emetic	SDA JO 303	WSBC 10701		raw milk											
a	*Bc*, emetic	UHDAM 141/9	WSBC 10751	environment	dust											
a	*Bc*, emetic	UHDAM 143/pl	WSBC 10750	environment	dust											
a, d, e, f1	*Bc*, emetic	UHDAM 1IFI (1)	WSBC 10744	food isolate	infant food	>10°C	48°C						2	1	3	1
a, d, e, f1	*Bc*, emetic	UHDAM 1IFI (3)	WSBC 10746	food isolate	infant food	>10°C	48°C						2	1	2	1

affiliation of strains to the different strain sets[a]	Informations on strains					Determination of growth limits		Toxin gene profiling					Results for selective plating media[d]			
	species[b]	code	additional codes	additional information	strain origin	minimum growth temperature	maximum growth temperature	Ces	HBL	NHE	CytK	toxin type[c]	PEMBA	MYP	CBC	CEI
a	*Bc*, emetic	UHDAM 2/pkl	WSBC 10755	environment	filler material from hospital											
a, e, d, f2	*Bc*, emetic	UHDAM 3/pkl	WSBC 10756	clinical isolate	filler material from hospital	>10°C	48°C						1	1	1	1
a	*Bc*, emetic	UHDAM 7/pk4	WSBC 10758	environment	filler material from hospital											
a	*Bc*, emetic	UHDAM 8/pk4	WSBC 10757	environment	filler material from hospital											
a	*Bc*, emetic	UHDAM 9/pk4	WSBC 10759	environment	filler material from hospital											
a	*Bc*, emetic	UHDAM B208	WSBC 10741	food borne outbreak	creamy cake											
a	*Bc*, emetic	UHDAM B308	WSBC 10743	food borne outbreak	risotto											
a, e, d, f1	*Bc*, emetic	UHDAM B315	WSBC 10742	food isolate	cake	10°C	48°C						2	1	2	1
a	*Bc*, emetic	UHDAM LKT1/1	WSBC 10753	environment	filler material											
a, d, f1	*Bc*, emetic	UHDAM ML127	WSBC 10740	food borne outbreak	pasta	10°C	48°C									
a, b	*Bc*, emetic	UHDAM NS 58	WSBC 10539	environment	spruce											
a	*Bc*, emetic	UHDAM NS 88	WSBC 10540	food isolate	spruce											
a, d, e	*Bc*, emetic	UHDAM NS115	WSBC 10538	food isolate	spruce	>10°C	48°C						1	1	3	1
a, b	*Bc*, emetic	WSBC 10879		food	cooked rice											
c2, e, f2	*Bc*, emetic	WSBC 10881		food borne outbreak				+	-	+	-	E	2	2	1	1
b, c2, e	*Bc*, emetic	WSBC 10882		food borne outbreak	meat dish with rice			+	-	+	-	E	1	2	1	1
c2, e, f1	*Bc*, emetic	WSBC 10888		clinical isolate	human feces			+	-	+	-	E	2	2	2	1
c2, e, f1	*Bc*, emetic	WSBC 10895		food isolate	buttercream			+	-	+	-	E	2	2	1	1
c2	*Bc*, emetic	WSBC 10897		food isolate	butter cream			+	-	+	-	E				
c2, e, f2	*Bc*, emetic	WSBC 10898		food isolate	dried apricots			+	-	+	-	E	2	2	1	1
c2, e	*Bc*, emetic	WSBC 10899		food isolate	dried apricots			+	-	+	-	E	2	2	1	1
c2, e, f1	*Bc*, emetic	WSBC 10900		food isolate	dried apricots			+	-	+	-	E	2	2	1	1
b, c2	*Bc*, emetic	WSBC 10914		food	baby food			+	-	+	-	E				
c2	*Bc*, emetic	WSBC 10925		food	water bottle			+	-	+	-	E				
b, c2	*Bc*, emetic	WSBC 10927		food poisoning	cauliflower			+	-	+	-	E				

affiliation of strains to the different strain sets[a]	Informations on strains					Determination of growth limits		Toxin gene profiling					Results for selective plating media[d]			
	species[b]	code	additional codes	additional information	strain origin	minimum growth temperature	maximum growth temperature	Ces	HBL	NHE	CytK	toxin type[c]	PEMBA	MYP	CBC	CEI
b, c, f1	*Bm*^{T}	ATCC 6462	WS 2641					-	+	+	-	B				
f2	*Bm*	BmA2		environment	soil											
f2	*Bm*	BmA3		environment	soil											
f2	*Bm*	BmCM1		environment	soil											
f1	*Bm*	BmFL2		environment	soil											
f2	*Bm*	BmFL3		environment	soil											
f2	*Bm*	BmLC1		environment	soil											
f1	*Bm*	WSBC 10256		environment	soil											
f1	*Bm*	WSBC 10257		environment	soil											
f1	*Bm*	WSBC 10258		environment	soil											
f2	*Bm*	WSBC 10264		environment	soil											
a	*Bm*	WSBC 10276		food isolate	pasteurized milk											
f1	*Bm*	WSBC 10277		food isolate	pasteurized milk											
a	*Bm*	WSBC 10278		food isolate	pasteurized milk											
a, b	*Bm*	WSBC 10279		food isolate	pasteurized milk											
a, b, c	*Bm*	WSBC 10291		environment	soil from Thailand			-	+	+	-	C				
a	*Bm*	WSBC 10292		environment	soil from Thailand											
a, b, c, f1	*Bm*	WSBC 10293		environment	soil from Thailand			-	-	+	+	D				
f1	*Bm*	WSBC 10360		environment	soil											
f2	*Bm*	WSBC 10361		environment	soil											
a	*Bpm*^{T}	NRRL B-617	WS 3118	environment	soil											
a	*Bpm*	NRRL BD-10	WS 3120	environment	soil											
a	*Bpm*	NRRL NRS-322	WS 3119	environment	soil											
b, c, f1	*Bt*^{T}	ATCC 10792	WS 2734					-	+	+	-	C				
f1	*Bt*	ATCC 29730	WS 2623													

affiliation of strains to the different strain sets[a]	Informations on strains					Determination of growth limits		Toxin gene profiling					Results for selective plating media[d]			
	species[b]	code	additional codes	additional information	strain origin	minimum growth temperature	maximum growth temperature	Ces	HBL	NHE	CytK	toxin type[c]	PEMBA	MYP	CBC	CEI
a	*Bt*	HER 1211	WS 2614													
f1	*Bt*	HER 1231	WS 2616													
a, b, f2	*Bt*	HER 1232	WS 2617													
f1	*Bt*	HER 1236	WS 2620													
f1	*Bt*	HER 1357	WS 2621													
a, f1	*Bt*	HER 1380	WS 2624													
a, f2	*Bt*	HER 1387	WS 2625													
a, f1	*Bt*	HER 1404	WS 2626													
a, f2	*Bt*	HER 1410	WS 2627													
f2	*Bt*	HER 1418	WS 2629													
f2	*Bt*	WS 2632														
f1	*Bt*	WSBC 28001		environment	soil											
f1	*Bt*	WSBC 28002		environment	soil											
f2	*Bt*	WSBC 28020		environment	soil											
b, f1	*Bt*	WSBC 28022														
b, f1	*Bt*	WSBC 28023														
c, f1	*Bt*	WSBC 28024						-	+	+	+	A				
c	*Bt*	WSBC 28009		environment	soil			-	+	+	-	C				
a, b, c, d, e, f1	*Bw*^{T}	DSM 11821	WSBC 10204	food isolate	pasteurized milk	4°C	n.g. at 42°C	-	+	+	-	C	2	2	3*	1
a, c2, d	*Bw*	INRA 1	WSBC 10550	food isolate	zucchini puree	4°C	<45°C	-	+	+	-	C				
a, f1	*Bw*	INRA 5	WSBC 10710	food	zucchini puree											
a, b, c1, e, f1	*Bw*	INRA I20	WSBC 10616	food isolate	cooked leek	4°C	45°C	-	+	+	-	C	2	1	3*	1
a, f2	*Bw*	INRA I3	WSBC 10733	food isolate	cooked mussel											
a, b, d, f1	*Bw*	SDA NFFE 647	WSBC 10690	environment	water	4°C	42°C									

affiliation of strains to the different strain sets[a]	Informations on strains					Determination of growth limits		Toxin gene profiling					Results for selective plating media[d]			
	species[b]	code	additional codes	additional information	strain origin	minimum growth temperature	maximum growth temperature	Ces	HBL	NHE	CytK	toxin type[c]	PEMBA	MYP	CBC	CEI
f2	*Bw*	WSBC 10067		food isolate	pasteurized milk											
a, e, f1	*Bw*	WSBC 10201		food isolate	pasteurized milk								2	1	3	1
f1	*Bw*	WSBC 10202		food isolate	pasteurized milk											
a	*Bw*	WSBC 10206		food isolate	pasteurized milk											
a, e	*Bw*	WSBC 10207		food isolate	pasteurized milk								2	1	3*	1
a, e	*Bw*	WSBC 10208		food isolate	pasteurized milk								3	1	3*	1
a	*Bw*	WSBC 10210		food isolate	pasteurized milk											
a, c2	*Bw*	WSBC 10211		food isolate	pasteurized milk			-	+	+	-	C				
f1	*Bw*	WSBC 10295		environment	soil											
f2	*Bw*	WSBC 10363		environment	soil											
f1	*Bw*	WSBC 10365		environment	soil											
a, b, d, e, f1	*Bw*	WSBC 10377		food isolate	raw milk	4°C	n.g. at 42°C						3	2	3*	1
f1	*Bw*	WSBC 10378		food isolate	raw milk											
f2	*Bw*	WSBC 10379		food isolate	pasteurized milk											

[a] Definitions on the different strain sets are given in 7.1.
[b] Species are abbreviated as follows: *Ba*: *B. anthracis*; *Bc*: *B. cereus*; *Bc*, emetic: emetic *B. cereus*; *Bm*: *B. mycoides*; *Bt*: *B. thuringiensis*; *Bw*: *B. weihenstephanensis*; [T] type strain
[c] Toxin types are defined in Table 7: A: nhe^+, hbl^+, $cytK^+$; B: nhe^+cytK^+, ces^+; C: nhe^+, hbl^+; D: nhe^+, $cytK^+$; E: nhe^+, ces^+; F: nhe^+; G: $cytK^+$.
[d] Reactions on the different selective plating media are defined in 3.4.1: 1: typical reaction, 2: weak reaction, 3: atypical reaction, 3*: formation of total blue colonies on CBC agar.

7.2 Oligonucleotide primers and probes

Table 18: Oligonucleotide primers and probes used during this study.

Oligonucleotide name	Sequence (5´to 3´)	Target gene	Description	Reference
BcAPF1	GAGGAAATAATTATGACAGTT	*cspA*	detection of *B. weihenstephanensis*	Francis et al. 1998
BcAPR1	CTT(C/T)TTGGCCTTCTTCTAA	*cspA*	detection of *B. weihenstephanensis*	Francis et al. 1998
BcFF2	GAGATTTAAATGAGCTGTAA	*cspF*	detection of *B. weihenstephanensis*	Francis et al. 1998
RB-19	GGGACTGCAGGAGTGAT	CryIA(c)	detection of *B. thuringiensis*	Bourque et al.1993
SB-1	TGCATAGAGGCTTTAAT	CryIA(a)	detection of *B. thuringiensis*	Bourque et al.1993
U3-18c	AATTGCTTTCATAGGCT	CryIA(b)	detection of *B. thuringiensis*	Bourque et al.1993
U8-15c	CAGGATTCCATTCAAGG	CryIA(a)	detection of *B. thuringiensis*	Bourque et al.1993
SB-2	TCGGAAAATGTGCCCAT	CryIA(b)	detection of *B. thuringiensis*	Bourque et al.1993
8-26/56	AGAGTTTGATCCTGGCTCA	16S rDNA	PCR detection of genomic DNA	Stackebrandt and Liesack 1992
1511-1493	CGGCTACCTTGTTACGAC	16S rDNA	PCR detection of genomic DNA	Stackebrandt and Liesack 1992
16S rRNA	GGAGGAAGGTGGGGATGACG	16S rRNA	Southern detection of genomic DNA	Martineau et al. 1996
16S rRNA	ATGGTGTGACGGGCGGTGTG	16S rRNA	Southern detection of genomic DNA	Martineau et al. 1996
PlcR_for1	GCACGCAGAAAAATTAGGAAGTG	*plcR*	partial sequencing of plcR gene	This study
PlcR_for2	AGGGTGATGAGAGGATTAACAC	*plcR*	partial sequencing of plcR gene	This study
PlcR_rev1	ATGG(A/T)TTC(C/T)TAATATATC(A/G)AAAAAGAAG	*plcR*	partial sequencing of plcR gene	This study
PlcR_rev2	CTAATATATC(A/G)AAAAAGAAG(C/T)(A/T)(A/T)GC	*plcR*	partial sequencing of plcR gene	This study
EM1F	GACAAGAGAAATTTCTACGAGCAAGTACAAT	*ces*	PCR detection of emetic *B. cereus*	Ehling-Schulz et al. 2004b
EM1R	GCAGCCTTCCAATTACTCCTTCTGCCACAGT	*ces*	PCR and Southern detection of emetic *B. cereus*	Ehling-Schulz et al. 2004b
HpaIF	GCCAGAAGATGCAATGATTCCAGTATG	*ces*	Southern detection of emetic *B. cereus*	Ehling-Schulz et al. 2004b
ces_SYBR_F	CACGCCGAAAGTGATTATACCAA	*ces*	Real-time detection of emetic *B. cereus*	Fricker et al. 2007
ces_SYBR_R	CACGATAAAACCACTGAGATAGTG	*ces*	Real-time detection of emetic *B. cereus*	Fricker et al. 2007
sa_SYBR_F	CGTGTTGAACGTGGTCAAATCA	*tuf*	Real-time detection of *S. aureus*	derived from Martineau et al. 2001
sa_SYBR_R	CACCTTCGTCTTTTGATAATACG	*tuf*	Real-time detection of *S. aureus*	derived from Martineau et al. 2001
ces_TaqMan_for	CGCCGAAAGTGATTATACCAA	*ces*	Real-time detection of emetic *B. cereus*	Fricker et al. 2007
ces_TaqMan_rev	TATGCCCCGTTCTCAAACTG	*ces*	Real-time detection of emetic *B. cereus*	Fricker et al. 2007
ces_TaqMan_probe	FAM-GGGAAAATAACGAGAAATGCA-TAMRA	*ces*	Real-time detection of emetic *B. cereus*	Fricker et al. 2007

Oligonucleotide name	Sequence (5´to 3´)	Target gene	Description	Reference
IAC_for	GCAGCCACTGGTAACAGGAT	*rep*	Internal amplification control for Real-time PCR	Fricker et al. 2007
IAC_rev	GCAGAGCGCAGATACCAAAT	*rep*	Internal amplification control for Real-time PCR	Fricker et al. 2007
IAC_probe	HEX-AGAGCGAGGTATGTAGGCGG-TAMRA	*rep*	Internal amplification control for Real-time PCR	Fricker et al. 2007
HD F	ACCGGTAACACTATTCATGC	*hblD*	sequencing of *hblD*	Guinebretiere et al. 2002
HD R	GAGTCCATATGCTTAGATGC	*hblD*	sequencing of *hblD*	Guinebretiere et al. 2002
L1 A	AATCAAGAGCTGTCACGAAT	*hblD*	sequencing of *hblD*	Hansen and Hendriksen 2001
L1 B	CACCAATTGACCATGCTAAT	*hblD*	sequencing of *hblD*	Hansen and Hendriksen 2001
HD F3	ATT(A/G)GCTGAAACAGG(A/G)TC(C/T)C	*hblD*	sequencing of *hblD*	Ehling-Schulz et al. 2006b
HD R1	C(A/G)ATCCACCACC(A/G)ATTGACC	*hblD*	sequencing of *hblD*	Ehling-Schulz et al. 2006b
HA F	AAGCAATGGAATACAATGGG	*hblA*	sequencing of *hblA*	Guinebretiere et al. 2002
HA R	AGAATCTAAATCATGCCACTGC	*hblA*	sequencing of *hblA*	Guinebretiere et al. 2002
NA F	GTTAGGATCACAATCACCGC	*nheA*	sequencing of *nheA*	Guinebretiere et al. 2002
NA R	ACGAATGTAATTTGAGTCGC	*nheA*	sequencing of *nheA*	Guinebretiere et al. 2002
NA F2	GAATGT(A/G)CGAGA(A/G)TGGATTG	*nheA*	sequencing of *nheA*	Ehling-Schulz et al. 2006b
NA R2	GC(C/T)GCTTC(C/T)CTCGTTTG(A/G)CT	*nheA*	sequencing of *nheA*	Ehling-Schulz et al. 2006b
NB F	TTTAGTAGTGGATCTGTACGC	*nheB*	sequencing of *nheB*	Ehling-Schulz et al. 2006b
NB R	TTAATGTTCGTTAATCCTGC	*nheB*	sequencing of *nheB*	Ehling-Schulz et al. 2006b
HD2 F	GTAAATTA**I**GATGA**I**CAATTTC	*hbl*	toxin gene profiling (*hbl*)	Ehling-Schulz et al. 2006b
HA4 R	AGAATAGGCATTCATAGATT	*hbl*	toxin gene profiling (*hbl*)	Ehling-Schulz et al. 2006b
NA2 F	AGGC**I**GCTCTTCG**I**ATTC	*nhe*	toxin gene profiling (*nhe*)	Ehling-Schulz et al. 2006b
NB1 R	**ITI**GTTGAAATAAGCTGTGG	*nhe*	toxin gene profiling (*nhe*)	Ehling-Schulz et al. 2006b
CK F2	ACAGATATCGG**I**CAAAATGC	*cytK*	toxin gene profiling (*cytK*)	Ehling-Schulz et al. 2006b
CK R5	CAAGT**I**ACTTGACC**I**GTTGC	*cytK*	toxin gene profiling (*cytK*)	Ehling-Schulz et al. 2006b
CesF1	GGTGACACATTATCATATAAGGTG	*ces*	toxin gene profiling (*ces*)	Ehling-Schulz et al. 2005b
CesR2	GTAAGCGAACCTGTCTGTAACAACA	*ces*	toxin gene profiling (*ces*)	Ehling-Schulz et al. 2005b
Spo2F	CGACGAGGATAACCCAATTTGC	*spoIIIAB*	sequence typing	Ehling-Schulz et al. 2005a
Spo2R	CAGTGAGAGACCGAGGCAAC	*spoIIIAB*	sequence typing	Ehling-Schulz et al. 2005a
adk F	CAGCTATGAAGGCTGAAACTG	*adk*	sequence typing	Helgason et al. 2004
adk R	CTAAGCCTCCGATGAGAACA	*adk*	sequence typing	Helgason et al. 2004
ccpA F	GTTTAGGATACCGCCCAAATG	*ccpA*	sequence typing	Helgason et al. 2004
ccpA R	TGTAACTTCTTCGCGCTTCC	*ccpA*	sequence typing	Helgason et al. 2004
ftsA F	TCTTGACATCGGTACATCCA	*ftsA*	sequence typing	Helgason et al. 2004

Oligonucleotide name	Sequence (5´to 3´)	Target gene	Description	Reference
ftsA R	GCCTGTAATAAGTGTACCTTCCA	*ftsA*	sequence typing	Helgason et al. 2004
glpT F	TGCGGCTGGATGAGTGA	*glpT*	sequence typing	Helgason et al. 2004
glpT R	AAGTAAGAGCAAGGAAGA	*glpT*	sequence typing	Helgason et al. 2004
pyrE F	TCGCATCGCATTTATTAGAA	*pyrE*	sequence typing	Helgason et al. 2004
pyrE R	CCTGCTTCAAGCTCGTATG	*pyrE*	sequence typing	Helgason et al. 2004
recF F	GCGATGGCGAAATCTCATAG	*recF*	sequence typing	Helgason et al. 2004
recF R	CAAATCCATTGATTCTGATACATC	*recF*	sequence typing	Helgason et al. 2004
sucC F	GGCGGAACAGAAATTGAAGA	*sucC*	sequence typing	Helgason et al. 2004
sucC R	TCACACTTCATAATGCCACCA	*sucC*	sequence typing	Helgason et al. 2004

8 ACKNOWLEDGEMENT

I was fortunate to perform the work on my Ph.D. thesis at the "Abteilung Mikrobiologie, Zentralinstitut für Ernährungs- und Lebensmittelforschung (ZIEL), Weihenstephan, TU München" headed by Prof. Dr. Siegfried Scherer. I would like to acknowledge and thank Prof. Dr. Siegfried Scherer for the initiation of this work and the opportunity to work in such a pleasant atmosphere.

My cordial thanks to my supervisor Dr. Monika Ehling-Schulz for her scientific contribution, excellent support and encouragement throughout this work, that should not be taken for granted.

To all members of the –not always- "serious *B. cereus* group", present and former ones: I am very grateful for your assistance and the pleasant working atmosphere! Special thanks to my Ph.D. collegues Monica Dommel, Genia Lücking and Li Lin, and the technicians Romy Renner and Michaela Bauer for excellent technical assistance: you form the backbone of the lab! I am very thankful to Sebastian Sedlmeier, Christina Räuber and Eva-Maria Sedlmeier for their contribution to this work.

Sincere thanks are given to all my colleagues at the institute, especially to these with whom I shared office and labs. You all made me feel at home at work!

Special thanks is addressed to Monica Dommel for proof-reading all my manuscripts written in English. It must be really hard to be the only native speaker at the institute!

Furthermore I am very thankful to Cecilia Rebuffo for introducing me to the artificial neural networks and for her support and encouragement in this part of my work.

I am in debted to my collaborators Ute Messelhäußer and Ulrich Busch (LGL, Oberschleißheim), Petra Rieck and Martin Wagner (VUW, Wien), Marie-Helene Guinebretiere and Frederic Carlin (INRA, Avignon), Birgitta Svensson (SDA, Lund), and all the partners from the *Bacillus* EU project (QLK1-CT-2001-00854).

Finally, I would like to cordially thank my parents, grandparents, my sister Dodo and especially my better half Martin for their interest in my Ph.D. work, their constant, infinite encouragement and support throughout this time.

This work was partially supported by the European Comission, by the „Bayerisches Staatsministerium für Landwirtschaft und Forsten", and by the „Vereinigung zur Förderung der Milchwissenschaftlichen Forschung an der Technischen Universität München in Freising – Weihenstephan e.V.".

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