REVIEW ARTICLE

From soil to gut: *Bacillus cereus* and its food poisoning toxins

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Abstract

Bacillus cereus is widespread in nature and frequently isolated from soil and growing plants, but it is also well adapted for growth in the intestinal tract of insects and mammals. From these habitats it is easily spread to foods, where it may cause an emetic or a diarrhoeal type of food-associated illness that is becoming increasingly important in the industrialized world. The emetic disease is a food intoxication caused by cereulide, a small ring-formed dodecadepsipeptide. Similar to the virulence determinants that distinguish *Bacillus thuringiensis* and *Bacillus anthracis* from *B. cereus*, the genetic determinants of cereulide are plasmid-borne. The diarrhoeal syndrome of *B. cereus* is an infection caused by vegetative cells, ingested as viable cells or spores, thought to produce protein enterotoxins in the small intestine. Three pore-forming cytotoxins have been associated with diarrhoeal disease: haemolysin BL (Hbl), nonhaemolytic enterotoxin (Nhe) and cytotoxin K. Hbl and Nhe are homologous three-component toxins, which appear to be related to the monooligomeric toxin cytolysin A found in *Escherichia coli*. This review will focus on the toxins associated with foodborne diseases frequently caused by *B. cereus*. The disease characteristics are described, and recent findings regarding the associated toxins are discussed, as well as the present knowledge on virulence regulation.

Introduction

Who is *Bacillus cereus*? It is a quiet soil dweller that thrives in a diversity of habitats or a part of the intestinal flora of different animals. It has the ability to withstand time and harsh environments because it can form endospores that are resistant to heat, dehydration and other physical stresses. When allowed access to mammalian tissues it is an opportunistic pathogen that may cause severe local or systemic infections such as endophthalmitis and septicaemia (reviewed in Drobniewski, 1993; Kotiranta *et al.*, 2000), and its close relative *Bacillus anthracis* is infamous for its potential to cause the severe disease anthrax (Mock & Fouet, 2001). *Bacillus cereus* is commonly present in food production environments by virtue of its highly adhesive endospores, spreading to all kinds of foods. It produces a range of virulence factors that may cause unpleasant disease in humans when present in food or the gastrointestinal tract and it is one of the major foodborne pathogenic bacteria, although in most cases disease is mild and of short duration. Interestingly, the spectrum of potential *B. cereus* toxicity ranges from strains used as probiotics for humans (Hong *et al.*, 2005) to highly toxic strains reported to be responsible for food-related fatalities (Mahler *et al.*, 1997; Lund *et al.*, 2000; Dierick *et al.*, 2005). The bacterium causes two types of gastrointestinal disease, the diarrhoeal and the emetic syndromes, which are caused by very different types of toxins. The emetic toxin, causing vomiting, has been characterized and is a small ring-formed peptide (Ehling-Schulz *et al.*, 2004b), while the diarrhoeal disease is caused by one or more protein enterotoxins, thought to elicit diarrhoea by disrupting the integrity of the plasma membrane of epithelial cells in the small intestine. The three toxins that have been implicated as aetiological agents of the diarrhoeal disease are the pore-forming cytotoxins haemolysin BL (Hbl), nonhaemolytic enterotoxin (Nhe) and cytotoxin K (CytK) (Beecher & MacMillan, 1991; Lund & Granum, 1996; Lund *et al.*, 2000). These cytotoxins are part of a virulence regulon that is activated by the transcriptional regulator PlcR (Lereclus *et al.*, 1996; Gohar *et al.*, 2002); however, it is becoming increasingly evident that other regulatory factors are involved, playing a role in determining the pathogenic potential of individual strains.

The organism: characteristics and identification

The '*B. cereus* group', also known as *B. cereus sensu lato*, is an informal but widely used term describing a genetically highly homogeneous subdivision of the genus *Bacillus*, comprising six recognized species: *B. cereus sensu stricto*, *B. anthracis*, *Bacillus thuringiensis*, *Bacillus mycoides*, *Bacillus pseudomycoides* and *Bacillus weihenstephanensis*. The type strain of *B. cereus sensu stricto* is American Type Culture Collection (ATCC) 14579, which was isolated from air in a cow shed more than one hundred years ago (Frankland & Frankland, 1887). *Bacillus thuringiensis* is distinguished from *B. cereus* by the production of insecticidal δ-endotoxins during sporulation, and is commercially used for biological control of insects in crop protection (Aronson & Shai, 2001). *Bacillus anthracis* causes the fatal animal and human disease anthrax, and has in recent years become known for its use as a biological weapon (Mock & Fouet, 2001; Jernigan *et al.*, 2002). The species *B. mycoides* and *B. pseudomycoides* are phenotypically differentiated from *B. cereus* by rhizoidal colony shape and fatty acid composition (Flugge, 1886; Nakamura, 1998). *Bacillus cereus* was originally described as a mesophilic organism, growing between 10 and 50 °C and with an optimum temperature of 35 and 40 °C (Johnson, 1984; Claus & Berkeley, 1986). During the last few decades, increasing numbers of psychrotolerant *B. cereus* strains were described, which led to the description of a new psychrotolerant species within the *B. cereus* group, named *B. weihenstephanensis*. This species is characterized by the ability to grow below 7 °C but not at 43 °C, and specific signature sequences in 16S rRNA and cold-shock protein genes (Lechner *et al.*, 1998). *Bacillus cereus sensu stricto* comprises all strains of the *B. cereus* group that do not belong to any of the other species due to the absence of distinctive traits.

The word bacillus means small rod, and cereus can be translated from Latin to mean wax-like. The name reflects the easily recognizable morphology of *B. cereus* when viewed in the microscope or on blood agar plates. *Bacillus cereus* is a large (1.0–1.2 μm by 3.0–5.0 μm) Gram-positive rod-shaped bacterium which grows on common agar media to large colonies (3–8 mm diameter) with a rather flat, greyish and 'ground-glass' appearance, often with irregular borders. On blood agar, the colonies are surrounded by zones of β-haemolysis (Kramer & Gilbert, 1989), the size of which is often large, but can vary depending on culturing conditions.

Most strains will form endospores within a few days on commonly used agar media. *Bacillus cereus* spores are ellipsoidal, centrally or paracentrally placed, and do not distend the cell (Gilbert & Kramer, 1986). Employing phase contrast microscopy or spore staining techniques, the placement and morphology of the spores are much used criteria to distinguish the species of the genus *Bacillus* (Fritze, 2002). Other commonly used features for identification are motility, haemolysis, carbohydrate fermentation (*B. cereus* does not ferment mannitol) and the very active lecithinase (phospholipase) production (Johnson, 1984). Various plating media are used for the isolation, detection and enumeration of *B. cereus* from foods, including MYP (mannitol-egg yolk-phenol red-polymyxin-agar) and PEMBA (polymyxin-pyruvate-egg yolk-mannitol-bromthymol blue-agar) (Holbrook & Anderson, 1980; Mossel *et al.*, 1967). In addition to selective compounds like polymyxin, these media utilize the bacterium's lecithinase production (egg-yolk reaction giving precipitate zones) and lack of mannitol fermentation. A thorough description of these media is found in Kramer & Gilbert (1989). More recently, chromogenic media have been developed for several food pathogens, including *B. cereus* (for instance Cereus–Ident-Agar from heipha Dr Müller GmbH, and chromogenic *B. cereus* Agar from Oxoid Ltd). These new media have been evaluated together with standard plating media by Fricker *et al.* (2008).

The dilemma in *B. cereus* group taxonomy

Although *B. anthracis, B. cereus* and *B. thuringiensis* are differentiated by phenotypic characteristics and pathological properties, genome sequencing data have shown that they are closely related, both in gene content and synteny (Helgason *et al.*, 2000; Rasko *et al.*, 2004), and their 16S rRNA gene sequences share greater than 99% similarity (Ash *et al.*, 1991). Phylogenetic studies based on chromosomal markers show that there is no taxonomic basis for *B. cereus* and *B. thuringiensis* having separate species status (Carlson *et al.*, 1994; Helgason *et al.*, 2004; Hill *et al.*, 2004; Ko *et al.*, 2004; Priest *et al.*, 2004; Guinebretière *et al.*, in press), while *B. anthracis* can basically be considered a clone of *B. cereus* (Keim *et al.*, 2000; Hill *et al.*, 2004; Muzzi *et al.*, 2007). The distinguishing features between the species are encoded by genes located on plasmids, which are well-recognized as highly mobile genetic elements, also within the species of the *B. cereus* group (Thomas *et al.*, 2000; Van der Auwera *et al.*, 2007). *Bacillus thuringiensis* is defined by the presence of plasmids carrying *cry* genes encoding δ-endotoxins, while *B. anthracis* carries two large plasmids encoding the two main virulence factors of this species; pXO1 encoding the anthrax toxin complex and pXO2 encoding the poly-γ-D-glutamic acid capsule, as well as the positive regulator of the virulence factors AtxA, located on pXO1 (Leppla, 2006). The importance of plasmids as virulence determinants within the *B. cereus* group is also demonstrated by the recent discovery that the genetic determinants of the *B. cereus* emetic toxin, the *ces* genes, are present on a large plasmid (Hoton *et al.*, 2005; Ehling-Schulz *et al.*, 2006a). This plasmid was observed to be almost exclusively present in a

single monomorphic cluster of *B. cereus sensu stricto* strains (Ehling-Schulz *et al.*, 2005a), although cereulide-producing strains have been described which differed from the highly homogeneous cluster in genotypic and phenotypic properties (Apetroaie *et al.*, 2005). Supporting this observation, a recent study employing multilocus sequence typing (MLST) identified cereulide-producing strains belonging to a phylogenetic cluster different from the main monomorphic emetic cluster (Vassileva *et al.*, 2007). Additionally, two *B. weihenstephanensis* isolates were demonstrated to produce cereulide and contain the *cesB* gene, even though detection of the plasmid was not reported (Thorsen *et al.*, 2006). The emetic type of *B. cereus* has been suggested to differ from nonemetic strains in properties such as starch hydrolysis, haemolysis, lecithinase reaction and temperature limits for growth (Andersson *et al.*, 2004; Ehling-Schulz *et al.*, 2004b; Carlin *et al.*, 2006).

The dilemma in definition of species within the *B. cereus* group is therefore that the principal virulence factors that distinguish *B. thuringiensis* and *B. anthracis* from *B. cereus* do not correlate with phylogeny studies based on chromosomal markers, as illustrated by the phylogenetic tree prepared using MLST shown in Fig. 1. An interesting exception is a newly discovered cluster comprising only three known strains, including the *B. cereus* strain NVH 391/98 responsible for three deaths due to diarrhoeal disease (Lund *et al.*, 2000). MLST analysis and genomic sequencing have indicated that this group is sufficiently far from the main *B. cereus* group cluster to warrant novel species status (Fagerlund *et al.*, 2007; Lapidus *et al.*, 2007; Fig. 1), and the name '*Bacillus cytotoxicus*' has informally been proposed for these strains (Lapidus *et al.*, 2007). These three strains can not be distinguished from the other *B. cereus* group strains based on virulence factors, but they are able to grow at temperatures 6–8 °C higher than the mesophilic *B. cereus* strains, making them thermotolerant representatives of the *B. cereus* group (Sorokin *et al.*, 2007; Guinebretière *et al.*, in press). In comparison, the species *B. weihenstephanensis* was described to distinguish psychrotolerant *B. cereus* strains from mesophilic strains. Several typing methods have suggested that *B. weihenstephanensis* strains group in a separate clade within the *B. cereus* group along with *B. mycoides* strains (Cherif *et al.*, 2003a, b; Priest *et al.*, 2004; Sorokin *et al.*, 2006; Guinebretière *et al.*, in press). However, psychrotolerant *B. cereus* group strains do not always conform to the *B. weihenstephanensis* species criteria (Stenfors & Granum, 2001; Stenfors Arnesen *et al.*, 2007), and a genetic group composed of psycrotolerant *B. cereus* and *B. thuringiensis* strains has been identified, which is phylogentically distant to the *B. weihenstephanensis* clade. Interestingly, the temperature tolerance limits for strains within the *B. cereus* group appear to correlate with different phylogenetic clusters (Guinebretière *et al.*, in press).

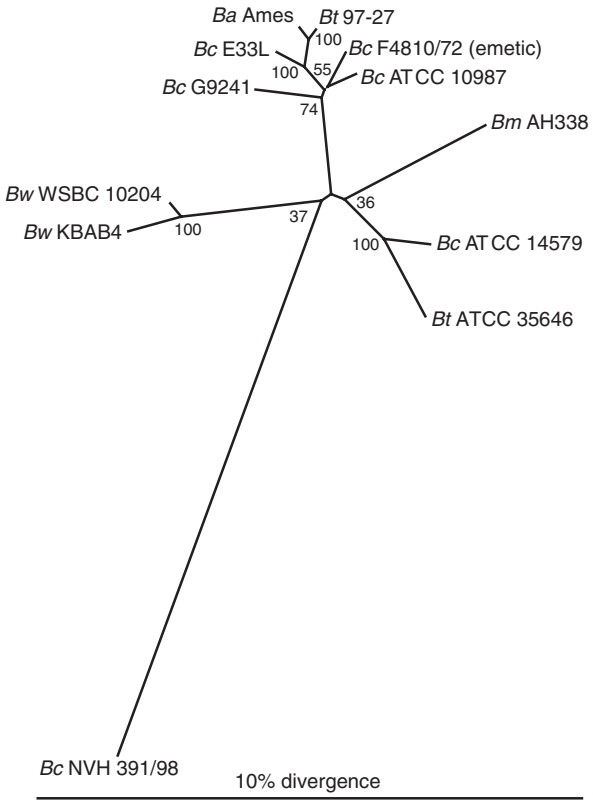

Fig. 1. Neighbor-joining phylogenetic tree prepared from the concatenated housekeeping gene sequences of different strains of the *Bacillus cereus* group. The tree was based on the MLST scheme described at the University of Oslo's *B. cereus* group MLST website (http://mlstoslo.uio.no). Genetic distances were estimated using the Kimura model and bootstrap confidence values were generated using 1000 permutations. Bootstrap values (in %) are shown next to the appropriate nodes. *Bc*, *B. cereus*; *Bt*, *Bacillus thuringiensis*; *Bw*, *Bacillus weihenstephanensis*; *Bm*, *Bacillus mycoides*.

The discussion of classification of *B. cereus* group strains is not only of academic and taxonomic interest, but also relates to issues concerning public health. For example, *B. cereus* isolates harbouring *B. anthracis* virulence factors have been detected in cases of severe anthrax-like illness (Hoffmaster *et al.*, 2004). Furthermore, while *B. cereus* is widely recognized as a food poisoning organism, *B. thuringiensis* is used as a biological insecticide for crop protection. However, because genes encoding the cytotoxins associated with diarrhoeal disease and other opportunistic *B. cereus* infections are generally chromosomally encoded, they are present in all species of the *B. cereus* group, although they are silent in *B. anthracis* (Mignot *et al.*, 2001). In particular, *B. thuringiensis* has a similar distribution and expression level of genes encoding extracellular virulence factors as *B. cereus* (Damgaard, 1995; Rivera *et al.*, 2000; Swiecicka *et al.*, 2006), and has caused human infections similar to those caused by *B. cereus* (Samples & Buettner, 1983; Jackson *et al.*, 1995;

Damgaard *et al.*, 1997; Hernandez *et al.*, 1998; Ghelardi *et al.*, 2007b). Food poisoning caused by *B. thuringiensis* is probably under-reported, as methods for identification of *B. cereus* group strains in food and clinical settings do not distinguish between *B. cereus* and *B. thuringiensis* (Granum, 2002). Therefore, unless otherwise stated, in the remainder of this review the discussion related to *B. cereus* also applies to *B. thuringiensis* and *B. weihenstephanensis* strains.

It has been proposed that *B. cereus*, *B. thuringiensis* and *B. anthracis* should be considered one species based on genetic evidence (Helgason *et al.*, 2000), but no consensus on this matter has been reached. This ambiguous taxonomic state of the *B. cereus* group illustrates the difficulties encountered with species definition within bacterial systematics, in particular in the genomic era. Traditional phylogenetic analysis of *B. cereus* group taxonomy is furthermore complicated by extensive horizontal gene transfer between strains (Cardazzo *et al.*, 2008). However, whereas genetically the *B. cereus* group could be considered one species, a good argument for retaining the current nomenclature is the principle that 'medical organisms with defined clinical symptoms may continue to bear names that may not necessarily agree with their genomic relatedness so as to avoid unnecessary confusion among microbiologists and nonmicrobiologists' (Stackebrandt *et al.*, 2002), according to rule 56a(5) in the *Bacteriological Code* (Lapage *et al.*, 1992).

Reservoirs and lifestyles

Bacillus cereus is described as being of ubiquitous presence in nature and can be found in many types of soils, sediments, dust and plants (Gilbert & Kramer, 1986; Kramer & Gilbert, 1989; von Stetten *et al.*, 1999; Kotiranta *et al.*, 2000; Schoeni & Wong, 2005). Spores may be passively spread and thus found also outside natural habitats. It is believed that *B. cereus sensu lato* exists in soil as spores, and germinates and grows when brought in contact with organic matter or an insect or animal host. Interest in the ecology of this bacterium spurred a study showing that *B. cereus* could germinate, grow and sporulate in soil, thus demonstrating a saprophytic life cycle (Vilain *et al.*, 2006). Furthermore, a multicellular phenotype with a filamentous mode of growth was observed and suggested to be a means of translocation through soil (Vilain *et al.*, 2006). A multicellular, filamentous mode of growth has also been observed in the gut of insects. The intestines of insects were suggested as a habitat for *B. cereus* when sporeforming bacteria, later identified as *B. cereus*, were isolated from guts of different soil-dwelling arthropod species, where the bacteria appear to exist in symbiosis with their invertebrate host (Margulis *et al.*, 1998). The role of the insect gut microbial communities as a natural niche for part of the *B. cereus* life cycle is further discussed by Jensen *et al.* (2003), and it is also suggested that the existence of different morphological modes used by *B. cereus*, such as the filamentous mode, may be adaptations to different life cycles like the 'normal' cycle of life as a symbiont or the more infrequent pathogenic life cycle with rapid growth.

Bacullus cereus has been reported to be present in stools of healthy humans at varying levels (Johnson, 1984; Kramer & Gilbert, 1989; Yea *et al.*, 1994; Jensen *et al.*, 2003). Its ubiquitous low level presence in environments, feed and foods would ensure *B. cereus* a transient presence in the mammalian gut (Kramer & Gilbert, 1989). However, genomic data from the *B. cereus* type strain ATCC 14579 and from *B. anthracis* suggested that their metabolic capacity is more adapted to the use of proteins as a nutrient source than carbohydrates, and furthermore that genes for establishment within a host were conserved (Ivanova *et al.*, 2003; Read *et al.*, 2003). Adding another nuance to the picture, a recent genomic and phenotypic comparison between *B. cereus* strains ATCC 14579 and ATCC 10987 revealed that ATCC 14579 actually has the capacity to metabolize a larger number of carbohydrates than what was initially believed based on genomic analysis alone (Mols *et al.*, 2007). These data suggest that in addition to a full life cycle in soil, where it is richly present, *B. cereus* is also adapted to a lifestyle in a host, as a pathogen or perhaps as a part of intestinal flora, as well as to growth in foods. The possible adaptation of *B. cereus* to the environment of the animal gut could be the basis of their proposed probiotic effect. Such use can not uncritically be considered safe for humans because all *B. cereus* strains are able to produce at least one of the toxins associated with diarrhoeal disease (Duc le *et al.*, 2004; Hong *et al.*, 2005). However, certain strains producing negligible amounts of toxin at 37 °C have been approved for probiotic use by the European Food Safety Authority (EFSA).

Being present in so many environments, it is expected that *B. cereus* should also be found in water; however, there are not many data on the presence of *B. cereus* in water sources, and standard methods for the detection from water are not available. Norwegian surface waters were investigated for presence of *B. cereus* spores, and cytotoxic strains were isolated from several rivers (Østensvik *et al.*, 2004). This suggests the possibility that the water supply may be a means by which *B. cereus* enters the food-processing chain.

Transfer from soil to food

Bacillus cereus can be isolated from a remarkable range of different foods and food ingredients, including rice, dairy products, spices, dried foods and vegetables (Kramer & Gilbert, 1989). Cross-contamination can distribute spores or cells to other foods, such as meat products (Johnson,

1984; Gilbert & Kramer, 1986; Granum, 2007). At harvest, *B. cereus* cells or spores may accompany plant material into food production areas and establish on food-processing equipment. *Bacillus cereus* is a common contaminant of milk (Johnson, 1984; Kramer & Gilbert, 1989; Andersson *et al.*, 1995; Te Giffel *et al.*, 1997; Lin *et al.*, 1998), and it can cause a defect known as sweet curdling in dairy products. Spores or cells of *B. cereus* can contaminate udders of cows during grazing (Andersson *et al.*, 1995), or enter the dairy farm through bedding material or feed (Kramer & Gilbert, 1989). In a recent study, high counts of *B. cereus* were found in the upper layers of dairy farm bedding (Magnusson *et al.*, 2007).

Bacillus cereus spores represent a huge advantage for the organism, allowing attachment, as well as survival of heat treatment or other procedures which remove species of vegetative bacteria which could otherwise outgrow *B. cereus*. Strain differences in spore characteristics, such as hydrophobicity, exosporium and appendages, have been shown to significantly affect the ability of the spore to adhere to surfaces such as food processing lines (Wiencek *et al.*, 1990; Tauveron *et al.*, 2006; Faille *et al.*, 2007). *Bacillus cereus* spores are not necessarily removed by regular cleaning of surfaces (Andersson *et al.*, 1995; Faille *et al.*, 2002). The ability of *B. cereus* to enter yet another lifestyle when forming biofilms (Wijman *et al.*, 2007) is most likely of importance for its persistence in food industry equipment, such as dairy pipelines. The biofilm protects spores and vegetative cells against inactivation by sanitizers (Ryu & Beuchat, 2005).

Modern large-scale food production technology, with extended use of refrigeration as a means of conservation, has created a cold niche well suited for bacteria that are not very competitive, but that can survive heat treatment and also grow at low temperatures. For instance, *B. weihenstephanensis* as well as *B. cereus* and other *Bacillus* species are frequently isolated from dairy products and environments which extensively use cooling as a means of controlling the growth of microorganisms (Wong *et al.*, 1988; Te Giffel *et al.*, 1997; Larsen & Jørgensen, 1999). In addition to dairy products, lightly heat-treated foods with extended refrigerated storage also represent a new and favourable environment for *B. cereus* group species.

Considering the ubiquitous presence of *B. cereus*, its resilient spores, and the nonfastidious nature of this microorganism, no type of food with pH > 4.8 (Gilbert & Kramer, 1986) can be excluded as a possible vehicle or as representing a risk of food spoilage or foodborne disease. Failure by consumers to follow basic food preparation rules, i.e. slow or inadequate cooling, storage at ambient temperature or prolonged heat-keeping at $< 60\,^{\circ}$C, may allow growth of *B. cereus* and is commonly part of the story in cases of foodborne disease.

Characteristics of foodborne disease

Two distinct foodborne disease types, emetic and diarrhoeal, are associated with *B. cereus*. Both are generally mild and self-limiting, although more serious and even lethal cases have occurred (Granum, 1994b; Mahler *et al.*, 1997; Lund *et al.*, 2000; Dierick *et al.*, 2005). *Bacillus cereus* was established as an organism of foodborne disease in the 1950s, with the first described outbreaks of the diarrhoeal type of disease in hospitals in Norway in 1947–1949 (Hauge, 1950, 1955). Earlier descriptions of disease which could probably be attributed to *B. cereus* lack the nomenclature and epidemiological framework that would allow this attribution, however there is little doubt that *B. cereus* has been implicated in foodborne disease historically (Kramer & Gilbert, 1989).

The emetic syndrome was first identified after several outbreaks caused by eating cooked rice in the United Kingdom in the early 1970s (Mortimer & McCann, 1974). This disease is an intoxication caused by the *B. cereus* emetic toxin, named cereulide, produced in foods before ingestion. The course of the disease is characteristic, with nausea and emesis occurring only a few hours after the meal. The incubation time was originally described as 1–5 h (Kramer & Gilbert, 1989), but more recently to be as short as 0.5 h, and up to 6 h (Ehling-Schulz *et al.*, 2004b; Table 1). The duration of the emetic disease is normally 6–24 h (Ehling-Schulz *et al.*, 2004b). The most important differential diagnosis is intoxication with *Staphylococcus aureus* enterotoxins, which causes similar symptoms; however, in this disease emesis is commonly accompied by diarrhoea (Seo & Bohach, 2007). Several severe and even lethal cases of emetic foodborne *B. cereus* disease have been reported (Mahler *et al.*, 1997; Jääskeläinen *et al.*, 2003; Ehling-Schulz *et al.*, 2004b; Dierick *et al.*, 2005; Fricker *et al.*, 2007).

The diarrhoeal syndrome is thought to be a toxicoinfection caused by vegetative cells, ingested as viable cells or spores, producing protein enterotoxins in the small intestine (Granum *et al.*, 1993; Andersson *et al.*, 1998a; Clavel *et al.*, 2004). It is easily confused with the foodborne disease caused by another sporeforming bacterium, *Clostridium perfringens* (Granum, 1990), and typically presents with abdominal pain, watery diarrhoea and occasionally nausea and emesis. The incubation time is over 6 h, normally in the range of 8–16 h, and on average 12 h, but in rare cases longer incubation times have been observed. The duration of the disease is normally 12–24 h but cases lasting several days have been reported (Kramer & Gilbert, 1989; Table 1).

Infective doses

For both types of *B. cereus* foodborne disease, a relatively high number of cells has generally been found in foods implicated in disease. For the diarrhoeal type, 10^5–10^8 cells

Table 1. Characteristics of the two types of *Bacillus cereus* foodborne disease. Adapted from Granum (2007)

Characteristics	Diarrhoeal disease	Emetic disease
Type of toxin	Protein; enterotoxin(s): Hbl, Nhe, CytK implicated	Cyclic peptide; emetic toxin (cereulide)
Location of toxin production	In the small intestine of the host	Preformed in foods
Infective dose	10^5–10^8 cfu (total) The total number required is lower for spores compared to vegetative cells*	10^5–10^8 cells g^{-1} is often found in implicated foods, but live cells are not required for intoxication Cereulide: 8–10 μg kg^{-1} body weight (animal models)†
Incubation time	8–16 h (occasionally > 24 h)	0.5–6 h
Duration of illness	12–24 h (occasionally several days)	6–24 h
Symptoms	Abdominal pain, watery diarrhoea and occasionally nausea Lethality has occurred‡	Nausea, vomiting and malaise. A few lethal cases (possibly due to liver damage)§
Foods most frequently implicated	Proteinaceous foods; meat products, soups, vegetables, puddings, sauces, milk and milk products	Starch-rich foods; Fried and cooked rice, pasta, pastry and noodles

*Clavel *et al*. (2004).
†Agata *et al*. (1994, 1995); Shinagawa *et al*. (1995).
‡Lund *et al*. (2000).
§Mahler *et al*. (1997); Dierick *et al*. (2005).

or spores have been indicated as the infective dose, although lower as well as much higher counts have been found in implicated foods. However, doses as low as 10^3 *B. cereus* CFU g^{-1} of food have been found in foods causing disease (Gilbert & Kramer, 1986). The lowest count of *B. cereus* found in a confirmed foodborne outbreak in Norway was 200 CFU g^{-1} food (Granum, 1994a), but further investigations showed that the actual number was closer to 10^4 CFU g^{-1} food, and that the underestimate was due to the bacilli being present as aggregated spores (T. Stalheim and P. E. Granum, unpublished data). Lower numbers of spores compared with vegetative cells can probably cause diarrhoeal disease, as the spores are better equipped to survive the passage through the gastric acid (Clavel *et al*., 2004).

The number of *B. cereus* cells required to produce sufficient emetic toxin to cause disease has not been determined, but in foods incriminated in cases of emetic disease, levels of 10^3–10^{10} CFU g^{-1} food have been found, in most cases at least 10^5 CFU g^{-1} food (Gilbert & Kramer, 1986). In animal experiments, a minimal emesis-causing dose of cereulide of 8–10 μg kg^{-1} body weight was reported (Agata *et al*., 1994, 1995; Shinagawa *et al*., 1995). This was supported by investigations on the cereulide content of a food dish that caused a serious outbreak of emetic disease, suggesting a dose of $\leq$ 8 μg kg^{-1} body weight (Jääskeläinen *et al*., 2003).

No specific population groups are described as being of special risk for *B. cereus* foodborne disease. However, individuals with lowered stomach acidity, for example elderly people or people suffering from achlorydria, may be more susceptible to *B. cereus* diarrhoeal disease, because a larger number of cells are expected to survive gastric transit (Clavel *et al*., 2004).

Outbreaks of *B. cereus* foodborne disease

Bacillus cereus is an important cause of foodborne disease worldwide (Clavel *et al*., 2007; Granum, 2007), although it is probably highly under-reported in official lists of foodborne disease causes. In the European Union, *Bacillus* species (including non-*cereus*) were reported to be responsible for 1.4% of foodborne outbreaks in 2005 (Anonymous, 2006). In the years 1992–2006, 45 outbreaks of gastroenteritis attributed to *Bacillus* spp. in England and Wales were reported to the Health Protection Agency Centre for Infections (www.hpa.org.uk/infections/topics_az/bacillus/fp/fpdata.htm). Between 1993 and 1998 in the Netherlands, *B. cereus* accounted for 12% of foodborne disease outbreaks where a causative agent was identified (Schmidt, 2001). Several factors contribute to the number of foodborne *B. cereus* disease being largely under-reported. It is a consequence of the generally short and mild course of disease, which does not motivate the patient to seek medical attention. Furthermore, when diagnosed, the disease is not reportable. In addition, cases and/or outbreaks may not always be attributed to *B. cereus*, because the symptoms of the emetic disease are not easily distinguished from those caused by *S. aureus* intoxication, and the *B. cereus* diarrhoeal disease shows the same symptoms as *C. perfringens* type A food poisoning. The number of cases of *B. cereus* foodborne disease is reportedly increasing in industrialized countries (Gilbert & Kramer, 1986; Kotiranta *et al*., 2000). However, as the surveillance systems for foodborne disease differ between countries, it is difficult to compare data and obtain true incidence estimates. Examples of cases and outbreaks are well described in several publications (see e.g. Johnson, 1984; Gilbert &

Kramer, 1986; Kramer & Gilbert, 1989; Kotiranta *et al.*, 2000; Granum, 2007).

Many kinds of food have been associated with *B. cereus* foodborne disease, including spices, meats, poultry, sprouts, rice and pasta (Johnson, 1984; Gilbert & Kramer, 1986; Kramer & Gilbert, 1989; Kotiranta *et al.*, 2000). Different types of food are more commonly associated with either of the two types of foodborne disease: the emetic type of disease has often been connected with consumption of fried and cooked rice (Gilbert & Kramer, 1986), pasta, pastry and noodles (Schoeni & Wong, 2005; Granum, 2007). The diarrhoeal type is commonly associated with proteinaceous foods, sauces and vegetables (Kramer & Gilbert, 1989), meat products, soups, puddings and milk products (Gilbert & Kramer, 1986; Kotiranta *et al.*, 2000; Granum, 2007). Paradoxically, the emetic type of *B. cereus* strains are thus more often found in starch-rich foods, although their metabolic capacities are not necessarily well adapted to the nutrient composition of these foods, as strains of this type are generally not able to hydrolyse starch (Ehling-Schulz *et al.*, 2004b). Perhaps a lack of readily available nutrients is a factor which can trigger the production of virulence factors?

A somewhat different distribution between countries is observed for the emetic and diarrhoeal diseases, which could partly be a reflection of the association of the two types of disease with different food vehicles: in Japan and the UK, the emetic disease dominates (Gilbert & Kramer, 1986; Shinagawa *et al.*, 1995), while in Northern Europe and North America, the diarrhoeal disease seems more prevalent (Kotiranta *et al.*, 2000). At least part of the difference in disease pattern is probably due to different eating habits, but it is difficult to document whether the distribution is truly different and not a result of reporting differences.

Cereulide, the emetic toxin

The rapid onset of the emetic disease caused by *B. cereus*, generally from 0.5 to 6 h after consumption of the meal, indicates that this is an intoxication by toxin preformed in the food. Cereulide, the emetic toxin, is a cyclic dodecadepsipeptide with molecular mass 1.2 kDa and the structure: $[\text{D-}O\text{-Leu-D-Ala-D-}O\text{-Val-D-Val}]_3$ (Agata *et al.*, 1994; Ehling-Schulz *et al.*, 2004b). Cereulide is produced by a nonribosomal peptide synthetase, encoded by the 24-kb cereulide synthetase (*ces*) gene cluster (Ehling-Schulz *et al.*, 2005b), which is located on a megaplasmid related to pXO1 (Ehling-Schulz *et al.*, 2006a). The plasmid was originally named pBCE4810, but has also been referred to as pCER270 (Rasko *et al.*, 2007). Because cereulide is resistant towards acid conditions, proteolysis and heat, it will not be destroyed by gastric acid, the proteolytic enzymes of the intestinal tract or by reheating foods that have been stored at room temperature after a first heating (Johnson, 1984; Agata *et al.*, 1994; Shinagawa *et al.*, 1996).

The mechanism by which cereulide causes emesis in humans has not been definitely determined, although animal feeding experiments have shown a receptor-mediated mechanism. Following release from the stomach into the duodenum, cereulide binds to the 5-HT_3 receptor, and stimulation of the vagus afferent causes vomiting in *Suncus murinus*, an animal model (Agata *et al.*, 1995). Several biological effects of cereulide have been described. The toxin acts as a cation ionopore, like valinomycin, and is therefore able to inhibit mitochondrial activity by inhibition of fatty acid oxidation (Mikkola *et al.*, 1999). This effect of cereulide was the reason for the liver failure in two lethal cases of emetic foodpoisoning where a 17-year-old Swiss boy and a 7-year-old Belgian girl died (Mahler *et al.*, 1997; Dierick *et al.*, 2005). In an experiment where mice were injected intraperitoneally with high doses of synthetic cereulide, massive degeneration of hepatocytes occurred. The serum values of hepatic enzymes were highest on days 2–3 after the inoculation of cereulide, and rapidly decreased thereafter. General recovery from the pathological changes, and regeneration of hepatocytes, were observed after 4 weeks (Yokoyama *et al.*, 1999). Cereulide has also been shown to cause cellular damage (Shinagawa *et al.*, 1996) and inhibit human natural killer cells of the immune system (Paananen *et al.*, 2002).

Cereulide production commences at the end of logarithmic phase during vegetative growth of *B. cereus*, with the highest level of production at early stationary phase of growth, and the production is not associated with sporulation. Cereulide synthesis takes place at temperatures ranging from *c.* 12 to 37 °C, although maximal production of emetic toxin appears to occur between 12 and 22 °C (Finlay *et al.*, 2000; Häggblom *et al.*, 2002). However, two isolates belonging to the psychrotolerant species *B. weihenstephanensis* were recently shown to produce cereulide at 8 °C (Thorsen *et al.*, 2006).

Different foods have varying ability to sustain cereulide production. In infant formulas, levels from 0.02 to 2 μg cereulide mL^{-1} food were reached after 24 h incubation at room temperature. Cereulide production was influenced by the composition of the formula, with a combination of dairy and cereal ingredients giving higher levels of cereulide production than rice and nondairy ingredients (Shaheen *et al.*, 2006). In another study, cereulide production was quantified in various types of food. In egg and meat products as well as in liquid foods such as milk and soy milk, only low cereulide levels were detected. In contrast, boiled rice and farinaceous foods could sustain production of high levels of cereulide (Agata *et al.*, 2002). In a study by Rajkovic *et al.* (2006), two *B. cereus* strains were used to inoculate potato puré, pasta and boiled rice. At static

incubation at 28 °C, lower cereulide levels were detected in boiled rice compared with the other two foods, while the counts of *B. cereus* were equally high (10^8 CFU g^{-1}) in all three foods.

Differences in levels of cereulide production between strains have also been observed (Häggblom *et al.*, 2002; Rajkovic *et al.*, 2006) and are possibly due to differences in regulation, because the *ces* genes themselves show only a low level of heterogeneity (Ehling-Schulz *et al.*, 2005b). Furthermore, environmental factors such as oxygen, pH, temperature and the presence of specific amino acids have been described to influence the production of cereulide (Agata *et al.*, 1999; Finlay *et al.*, 2000; Ehling-Schulz *et al.*, 2004b; Jääskeläinen *et al.*, 2004), and it has been shown that stationary incubation of food supports a higher level of cereulide production compared with aerated incubation (Rajkovic *et al.*, 2006; Shaheen *et al.*, 2006). However, the mechanisms regulating cereulide synthesis are still largely unknown.

Detection of cereulide

A range of different methods have been employed for cereulide detection. In early days, monkey feeding tests were used due to lack of other suitable detection methods (Melling *et al.*, 1976). Different variants of cell culture assays using HEp-2 cells take advantage of the ability of cereulide to cause vacuolization of this cell line (Hughes *et al.*, 1988; Sakurai *et al.*, 1994; Finlay *et al.*, 1999). A boar sperm biological assay was developed based on inhibition of boar sperm motility due to the mitochondria-damaging activity of cereulide (Andersson *et al.*, 1998b, 2004; Hoornstra *et al.*, 2003). Rat liver mitochondria were utilized to establish a quantitative detection method based on the ability of cereulide to uncouple mitochondrial respiratory activity (Kawamura-Sato *et al.*, 2005). Neither of the abovementioned tests specifically detects cereulide, and currently this can only be done conclusively by rather laborious and costly HPLC-MS analysis (Häggblom *et al.*, 2002). Nonetheless, in contrast to the use of live animals, or the somewhat labour-demanding cell culture assays, the boar sperm bioassay is easily performed and is well suited for screening *B. cereus* isolates (Andersson *et al.*, 2004; unpublished data from the Norwegian national reference laboratory). Furthermore, it was shown to correlate well with HPLC-MS analysis within a range of cereulide concentrations (Häggblom *et al.*, 2002) and with LC-MS analysis (Shaheen *et al.*, 2006).

The genes encoding the biosynthetic apparatus for production of cereulide appear to be restricted to emetic toxin-producing strains (Ehling-Schulz *et al.*, 2005b, 2006a), rendering PCR techniques highly relevant for identifying potentially harmful strains. The first PCR assay for detection of emetic strains was published in 2004 (Ehling-Schulz *et al.*, 2004a), and after identification of the nonribosomal peptide synthetase responsible for cereulide synthesis, a PCR assay specific for the *ces* genes was developed (Ehling-Schulz *et al.*, 2005b). More recently, a real-time PCR method for use in food, targeting the *ces* genes, was developed (Fricker *et al.*, 2007).

Cytotoxins associated with *B. cereus* foodborne disease

The diarrhoeal disease was early on attributed to an enterotoxin because culture filtrates of *B. cereus* caused fluid accumulation in rabbit ileal loops (Spira & Goepfert, 1972; Glatz *et al.*, 1974). This assay has traditionally been considered a decisive test of enterotoxic activity (Bergdoll, 1988). Because the toxins are presumed to elicit diarrhoea by disrupting the integrity of the plasma membrane of epithelial cells in the small intestine, cell culture assays measuring the cytotoxic activity of cell-free culture supernatants is now more commonly used to detect the presence of *B. cereus* diarrhoeal toxins, and these give a good indication of the cytotoxic potential of *B. cereus* strains. However, as *B. cereus* produces a large number of secreted cytotoxins and enzymes that may contribute to diarrhoeal disease, the identity of the enterotoxin(s) is still a controversial topic. The three cytotoxins Hbl, Nhe and CytK are currently considered the aetiological agents of *B. cereus* diarrhoeal foodborne disease (Beecher & MacMillan, 1991; Lund & Granum, 1996; Lund *et al.*, 2000). Hbl and Nhe are related three-component toxins, while the single-component CytK belongs to the family of β-barrel pore-forming toxins. In addition, several other protein cytotoxins, haemolysins and degradative enzymes have been described that may potentially contribute to the pathogenicity of *B. cereus* diarrhoeal disease. These include cereolysin O (Kreft *et al.*, 1983), haemolysin II (Baida *et al.*, 1999), haemolysin III (Baida & Kuzmin, 1995), InhA2 (Fedhila *et al.*, 2003) and three phospholipases C (Kuppe *et al.*, 1989).

Before the discovery of Nhe and CytK, Hbl was suggested to be the primary virulence factor in diarrhoea caused by *B. cereus*. However, food poisoning outbreaks have been caused by strains lacking Hbl (Granum *et al.*, 1996), for example the *hbl*- and *cytK*-negative strain *B. cereus* NVH 0075/95, in which Nhe was first identified (Lund & Granum, 1996), and *B. cereus* NVH 391/98, from which CytK was originally isolated (Lund *et al.*, 2000). Strain NVH 391/98 belongs to a phylogenetic group distantly related to the main cluster of *B. cereus* group strains, and was initially thought to be negative for both *hbl* and *nhe*. CytK was therefore acknowledged to be the enterotoxin responsible for the outbreak of foodborne disease caused by this strain, which presented as necrotic enteritis (Lund *et al.*, 2000; Dietrich *et al.*, 2005). However, Nhe was later detected in this strain (Fagerlund

et al., 2007; Lapidus *et al.*, 2007), and may therefore have contributed to its pathogenicity. Genes encoding Nhe are now thought to be present in all known *B. cereus* group strains. In contrast, *hbl* and *cytK* are present in less than 50% of randomly sampled strains (Ehling-Schulz *et al.*, 2005a, 2006b; Moravek *et al.*, 2006), although higher frequencies of *cytK* and *hbl* are observed in studies of clinical and food-associated isolates (Guinebretière *et al.*, 2002; Swiecicka *et al.*, 2006).

Several lines of evidence implicate Nhe as the most dominant diarrhoeal toxin, for example the strong correlation of cytotoxicity with the concentration of Nhe in culture supernatants in 100 *B. cereus* strains (Moravek *et al.*, 2006) and neutralization of cytotoxicity using an Nhe-specific antibody in 20 of 20 strains tested (Dietrich *et al.*, 2005). Furthermore, the cytotoxic activity of supernatant from the *hbl*- and *cytK*-negative *B. cereus* NVH 0075/95 was abolished by an *nhe* mutation (Fagerlund *et al.*, 2008), while no change in cytotoxicity was observed upon deletion of *hbl* or *cytK* in *B. thuringiensis* 407 Cry^- (Ramarao & Lereclus, 2006). However, because the disruption of the *hbl* operon in the laboratory strain *B. cereus* ATCC 14579 caused a major reduction in cytotoxic activity towards Vero cells (Lindbäck *et al.*, 1999), the most important toxin may vary between strains. Most likely, multiple toxins may act together to cause gastroenteritis (Callegan *et al.*, 2003; Fedhila *et al.*, 2003). Possibly, toxins may also act synergistically in the gastrointestinal tract, similar to that observed with erythrocytes where phospholipases C have been shown to enhance the lytic activity of Hbl (Beecher & Wong, 2000a).

Bacillus cereus tripartite cytotoxin family

Hbl and Nhe are both three-component toxin complexes, which are restricted to the *B. cereus* group (From *et al.*, 2005). Hbl consists of the three proteins L_2, L_1 and B (Beecher & MacMillan, 1991), encoded by the genes *hblC*, *hblD* and *hblA*, respectively, and are cotranscribed from one operon (Heinrichs *et al.*, 1993; Ryan *et al.*, 1997; Lindbäck *et al.*, 1999). Nhe is composed of the proteins NheA, NheB and NheC, encoded by the *nheABC* operon (Granum *et al.*, 1999). The proteins of Nhe and Hbl show homology, both between the three components of each complex and between the proteins of Nhe and Hbl, with amino acid identities ranging from 18% to 44%. The proteins show no significant sequence similarity towards any other known proteins. The observed similarities between the six *nhe* and *hbl* genes suggest that they have originated from a common gene, and that Hbl and Nhe constitute a family of tripartite toxins. Of all six Nhe and Hbl proteins, NheB and NheC show the highest sequence identity (44%), and of the three Hbl proteins both NheB and NheC are most similar to component L_1 (Fig. 2). This indicates that the latest gene duplication event in the *nhe*/*hbl* gene family was the generation of *nheB* and *nheC*. However, when it comes to similarities between hydrophobic regions of the proteins, NheA and Hbl L_2 contain no hydrophobic segments, NheB and Hbl L_1 each contain a hydrophobic segment of 54 and 60 amino acids, respectively, while NheC and Hbl B each contain one shorter stretch of 25 and 17 hydrophobic amino acids (Table 2). These hydrophobic regions are located in corresponding positions in the proteins, and were previously predicted to be one or two transmembrane helices (Granum *et al.*, 1999; Schoeni & Wong, 2005). Despite the similarities between the two toxins, co-operation between the components of Hbl and Nhe appears to be limited (Lund & Granum, 1997).

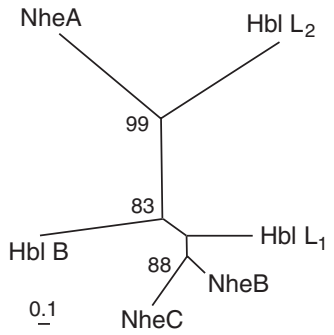

Fig. 2. Neighbour-joining tree showing the molecular relatedness of the Hbl and Nhe proteins from *Bacillus cereus* ATCC 14579. The tree was generated as described in Fig. 1 but using the proteins sequences of Hbl and Nhe from *B. cereus* ATCC 14579. Scale bar indicates 10% divergence.

For both Nhe and Hbl, all three components are necessary for maximal biological activity (Beecher & MacMillan, 1991; Beecher *et al.*, 1995b; Lindbäck *et al.*, 2004). Nevertheless, using proteins purified from culture supernatants, 10–15% haemolysis has been observed for Hbl in the absence of either L_1 or L_2 (Beecher & MacMillan, 1991; Beecher & Wong, 1994c), and limited toxic activity has been observed for NheA and NheB in the absence of NheC (Lindbäck *et al.*, 2004). However, because the limited lysis in the blood agar diffusion assay observed using only B and L_1 was abolished when recombinant B component was used (Heinrichs *et al.*, 1993), and recombinant clones containing NheA and NheB alone were not cytotoxic (Lindbäck *et al.*, 2004), it is likely that these observations were due to the presence of minute amounts of copurified Hbl L_1, L_2 or NheC, respectively. However, it has not been established whether limited lytic activity can occur for example at elevated concentrations of two of the three components of each toxin complex.

Biological activity and mechanism of Hbl

Hbl was originally purified from *B. cereus* strain F837/76, isolated from a postoperative wound (Turnbull *et al.*, 1979). It was initially thought to be a binary toxin composed of B,

the 'binding' component, and L, the 'lytic' component (Beecher & MacMillan, 1990), but upon further examination, the three components L_2, L_1 and B were identified (Beecher & MacMillan, 1991). Molecular properties of the Hbl proteins are summarized in Table 3. Hbl has been determined to cause fluid accumulation in rabbit ileal loops (Beecher *et al.*, 1995b), show dermonecrotic activity, vascular permeability (Beecher & Wong, 1994b), cytotoxic activity towards Vero cells and retinal tissue (Beecher *et al.*, 1995a; Lund & Granum, 1997), and haemolytic activity towards erythrocytes from several species (Beecher & MacMillan, 1990; Beecher & Wong, 2000a). A model for the action of Hbl has been proposed from studies of Hbl activity towards erythrocytes, including osmotic protection experiments, suggesting that the three components independently bind to erythrocytes, and then assemble into a membrane-attacking complex which lyses erythrocytes by a colloid osmotic lysis mechanism by forming a transmembrane pore (Beecher & Wong, 1997).

The stoichiometry of the three components of the Hbl complex forming the proposed transmembrane pore has not been determined, although a positive response in the rabbit ileal loop assay was obtained when the three Hbl components were injected in equimolar amounts (Beecher *et al.*, 1995b). However, maximal haemolytic activity appeared to occur also when the concentration of either Hbl L_1 or L_2 was lower than the concentration of Hbl B (Beecher & Wong, 1994c). Hbl produces a distinct ring-formed (discontinuous) haemolysis pattern when it diffuses from a well in blood agar, where haemolysis begins in a ring away from the well containing Hbl (Beecher & MacMillan, 1991; Beecher & Wong, 1994b). This pattern was suggested to be the result of a mutually inhibitory effect of B and L_1 and the slow reaction between the B component and the erythrocyte membrane, which was the rate-limiting step of haemolysis. Thus, in blood agar initiation of lysis begins at a distance away from the source of Hbl, where the priming reaction by B can occur before the B and L_1 components accumulate by diffusion to inhibitory concentrations (Beecher & Wong, 1997). Indeed, when the concentration of L_1 was reduced compared with that of Hbl B and L_2, lysis occurred more quickly near the well containing the Hbl components (Beecher & Wong, 2000b). The molecular basis for the inhibition of Hbl by excess Hbl L_1 and B remains elusive, but could indicate that optimal activity is obtained when one or two components is present in reduced amounts relative to the other components, although inhibition of

Table 2. Selected characteristics of the Hbl and Nhe toxin components

NheA	Detected by the TECRA-BDE kit from Tecra
	Does not contain hydrophobic segments
NheB	The only Nhe component that bound directly to the Vero cell surface
	Contains a hydrophobic segment of total length 54 amino acids (aa 234–287): AIIIGSSVATALGPIAIIGGAVVIATGAGTPLGVALIAGGAAAVGGG TAGIVLA*
NheC	Excess concentration inhibits cytotoxicity
	Produced in lower amounts than NheA and NheB
	Contains a hydrophobic segment 25 amino acids in length (aa 227–251), which contains a pair of cysteine residues: MVIAGGVLCVALITCLAGGPMIAVA*
Hbl	Detected by the BCET-RPLA kit from Oxoid
L_2	Does not contain hydrophobic segments
Hbl	Excess concentration inhibits haemolysis in blood agar
L_1	Possibly produced in lower amounts than L_2 and B under certain conditions
	Contains a hydrophobic segment of total length 60 amino acids (aa 234–293): VLAWSIGGGLGAAILVIAAIGGAVVIVVTGG TATPAVVGGLSALGAAGIGLGTAAGVTAS*
Hbl B	Excess concentration inhibits haemolysis in blood agar
	Reaction with erythrocytes is the rate-limiting step of haemolysis by Hbl
	Contains a hydrophobic segment 17 amino acids in length (aa 237–253): GAILGLPIIGGIIVGVA*
	The crystal structure has been determined (Protein Data Bank entry 2nrj)

*Hydrophobic sequences are from *B. cereus* ATCC 14579.

Table 3. Molecular properties of Hbl proteins

	Signal peptide* (amino acids)			Mature protein (amino acids)			Predicted MW of mature protein (kDa)			MW from SDS-PAGE F837/76‡	Estimated pI†		
Hbl component	F837/76	ATCC 4579	KBAB4 (Hbl_a)	F837/76	ATCC 14579	KBAB4 (Hbl_a)	F837/76	ATCC 4579	KBAB4 (Hbl_a)		F837/76	ATCC 4579	KBAB4 (Hbl_a)
L_2	32	32	32	415	407	407	46.8	46.0	46.2	43.2	5.65	5.14	6.19
L_1	30	30	29	354	376	379	38.2	40.6	41.0	38.5	5.81	5.21	5.45
B	31	31	31	344	344	346	38.3	38.4	38.8	37.8	5.10	5.25	5.57

*Predicted using SignalP at www.cbs.dtu.dk/services/SignalP/

†Predicted from the mature sequence using www.expasy.ch/tools/pi_tool.html

‡Beecher & Wong (1994c).

The included *Bacillus cereus* group strains were selected to represent some of the diversity between Hbl proteins.

MW, molecular weight, SDS-PAGE, sodium dodecyl sulphate-polyacrylamide gel electrophoresis.

Hbl haemolysis was also seen in suspension assays when the concentration of all three components was increased at a constant ratio (Beecher & Wong, 1994c). Although all three Hbl components have readily been isolated from culture supernatants and often appear to be present in approximately equal amounts (Beecher & MacMillan, 1991; Beecher & Wong, 1994c; Dietrich *et al.*, 1999; Gohar *et al.*, 2005), Hbl component L_1 was not always detected on two-dimensional gels (Gohar *et al.*, 2002; Gilois *et al.*, 2007), indicating that, at least during certain phases of growth, it may be produced in lower amounts than L_2 and B.

Biological activity and mechanism of Nhe

Nhe was first characterized after a large food poisoning outbreak in Norway in 1995 caused by the *hbl*-negative *B. cereus* strain NVH 0075/95 (Granum *et al.*, 1995; Lund & Granum, 1996). Initially, Nhe was thought to be a cytotoxin complex composed of NheA, NheB and a 105-kDa protein (Lund & Granum, 1996, 1997), but the 105-kDa protein was later shown to be a collagenase (Lund & Granum, 1999), not part of the Nhe complex. Sequencing of the *nhe* operon identified the gene encoding NheC (Granum *et al.*, 1999), which was subsequently confirmed to be a component of Nhe (Lindbäck *et al.*, 2004). Molecular properties of the Nhe proteins are summarized in Table 4. Both NheA and NheB appear to be present in culture supernatants in two forms with slightly differing sizes, where the smallest form represents a further processed variant of the largest form. The smallest forms of NheA and NheB lack 11 and 12 N-terminal amino acids, respectively, in addition to the 26 and 30 residues of their signal peptides (Beecher & Wong, 1994a; Lund & Granum, 1996, 1997). Trypsin digestion of the largest form of NheA yielded a fragment with mobility identical to the smaller one (Lund & Granum, 1997). Both variants of NheA and NheB show similar biological activity (Lund & Granum, 1996, 1997).

The maximal cytotoxic activity towards Vero cells was obtained when the molar ratio between NheA, NheB and NheC was *c.* 10 : 10 : 1. Furthermore, addition of excess NheC inhibited the cytotoxic activity of Nhe against Vero cells, both in *B. cereus* culture supernatants and using purified proteins (Lindbäck *et al.*, 2004). Presumably, the initial lack of identification of NheC as part of the Nhe toxin was a result of NheC being produced by the bacterium in much lower concentration than NheA and NheB, in order to obtain a toxin complex with optimal ratio of components. This probably also explains why NheC was not detected among the secreted proteins of *B. cereus* by two-dimensional electrophoresis analysis (Gohar *et al.*, 2005), and why the NheC antigen titre in *B. cereus* culture supernatants were considerably lower than the titre of NheA and NheB, although the latter could be related to differing affinities of the antibodies used (Dietrich *et al.*, 2005; Moravek *et al.*, 2006). An inverted repeat located between *nheB* and *nheC* has been suggested to mediate translational repression of *nheC* resulting in lower expression of *nheC* compared with that of *nheA* and *nheB* (Granum *et al.*, 1999; Lindbäck *et al.*, 2004).

Recently, the nature of the cytotoxic activity of Nhe towards epithelial cells was further examined, showing rapid disruption of the plasma membrane following exposure to Nhe, and formation of pores in planar lipid bilayers (Fagerlund *et al.*, 2008). These results were consistent with the insertion of transmembrane pores rather than activation of endogenous channels. Osmotic protection experiments and measures of increases in cell size upon Nhe exposure further indicated that Nhe causes cell death through colloid osmotic lysis by forming transmembrane pores. Nhe was also shown to have haemolytic activity towards erythrocytes from several mammalian species in suspension assays (Fagerlund *et al.*, 2008). However, the relative levels of haemolytic activity of Nhe and Hbl have not been examined, and it is possible that Nhe was previously found to be

Table 4. Molecular properties of Nhe

	Signal peptide* (amino acids)			Mature protein (amino acids)			Predicted MW of mature protein (kDa)				Estimated pI†		
Nhe component	NVH 0075/95	KBAB4 (plasmid)	NVH 391/98	NVH 0075/95	KBAB4 (plasmid)	NVH 391/98	NVH 0075/95	KBAB4 (plasmid)	NVH 391/98	MW from SDS-PAGE	NVH 0075/95	KBAB4 (plasmid)	NVH 391/98
NheA	26	26	24	360/349‡	363	363	41.0/39.8‡	41.3	41.5	45§/40‡and 41¶	5.07/5.04‡	4.89	5.12
NheB	30	29	30	372/360‡	355	371	39.9/38.7‡	39.2	40.4	39§	5.61/5.44‡	5.04	6.04
NheC	30	30	30	329	355	323	36.5	39.8	36.3		5.18	5.11	6.03

*,†Predicted as described in Table 3.

‡Values are for the 'further processed' forms (Beecher & Wong, 1994a; Lund & Granum, 1996, 1997).

§*Bacillus cereus* NVH 0075/95 Lund & Granum (1996).

¶*Bacillus cereus* F837/76 Beecher & Wong (1994a).

The included *B. cereus* group strains were selected to represent some of the diversity between Nhe proteins.

MW, molecular weight; SDS-PAGE, sodium dodecyl sulphate-polyacrylamide gel electrophoresis.

nonhaemolytic on bovine blood agar plates (Lund & Granum, 1996) due to lower haemolytic activity compared with that of Hbl.

Several independent attempts to prepare deletion mutants of the complete *nhe* operon in *B. cereus* and *B. thuringiensis* strains have failed (M. Ehling-Schulz, pers. commun.; Ramarao & Lereclus, 2006; Fagerlund *et al.*, 2008), although a *B. cereus nheBC* mutant has been obtained (Fagerlund *et al.*, 2008) and a *B. cereus* strain with a frameshift mutation in the 5′-end of *nheC* has been identified (E. Märtlbauer, pers. commun.). In *B. anthracis*, which produces a low level of NheA protein despite a nonfunctional PlcR protein, an *nheA* insertional mutant has successfully been prepared; however, this mutant renders two truncated proteins, one of which lacks only the first 77 amino acids (Mendelson *et al.*, 2004). As the *nhe* operon is found in every *B. cereus* group strain examined to date, these observations may suggest the intriguing possibility that *nheA* could be an essential gene in *B. cereus*. As cytotoxic activity is not likely to be crucial for cell viability, it could be possible that NheA may have a dual role, with a function essential to cell viability in addition to its role as a secreted toxin component.

The Hbl/Nhe family and ClyA constitute a toxin superfamily

The Hbl and Nhe proteins do not show significant sequence homology towards any other known protein family. However, the crystal structure of Hbl component B determined by a structural genomics consortium (Protein Data Bank entry 2nrj; Fig. 3a) showed remarkable tertiary structure resemblance with the pore-forming toxin cytolysin A (ClyA) (Fig. 3b; Protein Data Bank entry 1qoy; Wallace *et al.*, 2000; Fagerlund *et al.*, 2008). ClyA, also known as HlyE or SheA, is a haemolytic and cytotoxic monooligomeric protein toxin of 34 kDa expressed during anaerobic growth in *Escherichia coli*, *Shigella flexneri* and *Salmonella enterica* serovars Typhi and Paratypi A (Oscarsson *et al.*, 1996, 2002; Ludwig *et al.*, 1999; Wallace *et al.*, 2000).

The crystal structures of ClyA and Hbl B consist of long, four/five α-helix bundles that wrap around each other in left-handed supercoils, and a unique subdomain containing a hydrophobic β-hairpin flanked by two short α-helices. The main structural difference, the orientation of the subdomain (Fig. 3c), may possibly represent two different conformational states that both molecules may adopt, with the subdomain and the main helix bundle being connected by a hinge region. This is supported by the observation that the two structures represent different crystallization states, as Hbl B was crystallized as a monomer while ClyA was a dimer in a head-to-tail conformation, where the subdomain containing the β-hairpin was buried against a second hydrophobic surface patch on the opposite end of the protein structure (Wallace *et al.*, 2000). Hinge movements within the subdomain containing the β-hairpin in ClyA were also suggested by results from electron microscopy showing that the oligomeric pores formed by ClyA were of significantly greater length than the water-soluble monomeric protein structure, indicating significant structural changes upon

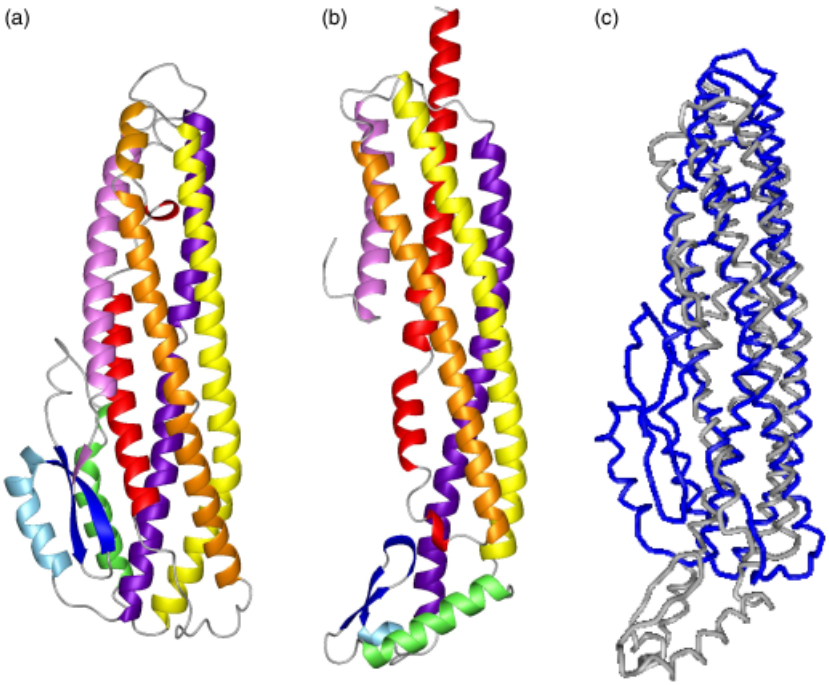

Fig. 3. Comparison of the structures of Hbl component B and ClyA determined by X-ray crystallography. (a) Hbl component B, (b) *E. coli* ClyA. Protein structures are shown in ribbon format, with the β-hairpins in blue. (c) Structural alignment visualized as a 3D superimposition of Hbl B (blue) and ClyA (grey), viewed as a Cα-trace. Figures from Fagerlund *et al.* (2008).

pore formation (Wallace *et al.*, 2000; Eifler *et al.*, 2006; Tzokov *et al.*, 2006).

NheB and NheC show sufficient sequence identity towards Hbl B for generation of 3D homology models based on the Hbl B structural template. Interestingly, as observed for Hbl B and ClyA, the hydrophobic segments of NheB and NheC correlate with the predicted β-hairpin in the homology models. Despite limited sequence identities, the strong structural and functional similarities suggested that the Hbl/Nhe family and the ClyA family of toxins constitute a new superfamily of toxins (Fagerlund *et al.*, 2008).

Membrane binding and pore formation

To date, no host cell receptor for Nhe or Hbl has been identified, and the nature of a putative receptor is an open question. For Hbl, all three components have been determined to bind individually to the erythrocyte surface in experiments where the addition of one component to erythrocytes, followed by washing and subsequent addition of the two remaining components, resulted in haemolysis regardless of which component was the initial component added (Beecher & Wong, 1997). For Nhe, NheB was the only component for which binding directly to the Vero cell surface could be demonstrated, and this association was inhibited by the presence of excess NheC (Lindbäck *et al.*, 2004). The ability of Nhe to rapidly form pores in synthetic lipid bilayer membranes using low toxin concentration shows that Nhe has innate pore-forming ability in phospholipid membranes (Fagerlund *et al.*, 2008), and it is thus possible that Nhe is not dependent on a protein or carbohydrate receptor for toxin binding and activity. For the structurally related ClyA, the hydrophobic β-hairpin was suggested to be the part of the toxin responsible for membrane interaction (Wallace *et al.*, 2000; Eifler *et al.*, 2006; Tzokov *et al.*, 2006). As the β-hairpins in the Hbl B crystal structure and in the NheB homology model also comprise hydrophobic residues, membrane binding by these proteins could occur by a similar mechanism, whereby reorientation of hinge regions in the subdomain correlates with association of the β-hairpin with the membrane. However, the hydrophobic sections of Hbl B and L_1 have also been suggested to serve as mediators of oligomerization (Schoeni & Wong, 2005).

Although both Hbl and Nhe appear to be pore-forming toxins, it is not known how the three components in each complex interact or to what extent they oligomerize in the process of forming a transmembrane pore. Because NheB appears to be the sole component of Nhe that binds to the cell membrane (Lindbäck *et al.*, 2004), it is also possible that Nhe, instead of acting as a classical heterooligomeric pore-forming toxin, may act in a manner reminiscent of the A-B type toxins, in which the catalytic and receptor binding functions reside on separate polypeptides or protein domains. The size of the functional pores formed by Hbl has been estimated by osmotic protection experiments using carbohydrates of increasing size to have an approximate diameter of $\leq$ 1.2 nm (Beecher & Wong, 1997), while lipid bilayer experiments and osmotic protection assays have indicated that Nhe and ClyA forms pores of comparable size (Fagerlund *et al.*, 2008), which in the case of ClyA were estimated to be 2.5–3.0 nm in diameter (Ludwig *et al.*, 1995; Ludwig *et al.*, 1999; Oscarsson *et al.*, 1999). The pores formed by Nhe and ClyA were also similar in that they were moderately cation-selective in lipid bilayers (Ludwig *et al.*, 1999; Fagerlund *et al.*, 2008). Given that Hbl and Nhe each require three separate proteins for maximum cytotoxic action it is difficult to predict the pore structures formed by Hbl and Nhe based on those formed by the homooligomeric ClyA. However, assuming that Hbl and Nhe, like ClyA, do not encounter major changes in secondary structure upon pore-formation, it is likely that putative oligomeric pores will be built mainly from α-helices, because β-barrels formed from the β-hairpins would be too short to span the predicted thickness of the target membrane (Eifler *et al.*, 2006).

Heterogeneity and genetic organization of *hbl* and *nhe*

It has been reported that Hbl proteins from different strains show a high degree of hetereogeneity. Two homologous sets of all three Hbl components, with distinct physical properties, were isolated from *B. cereus* strain MGBC145 (Beecher & Wong, 2000b), indicating that two distinct homologues of *hbl* genes may exist in a single strain. Western blot analysis of several strains has furthermore identified proteins of several sizes for each of the three Hbl proteins, and in one strain three Hbl B protein bands were detected (Schoeni & Wong, 1999). Although it is possible that some of the detected Hbl proteins represent forms processed after secretion, similar to those observed for NheA and NheB (Beecher & Wong, 1994a; Lund & Granum, 1996, 1997), the current availability of genomic sequences from several *B. cereus* group strains has made it possible to establish that two different types of *hbl* operons indeed exist.

In what appears to be the most common variant of the *hbl* operon, the ORF *hblB* is located immediately downstream of the *hblCDA* genes. The *hblB* gene was originally identified when the *hblA* gene was sequenced in *B. cereus* F837/76 (Heinrichs *et al.*, 1993), but is probably a pseudogene, as it is not transcribed at a detectable level and the *hblCDA* mRNA transcript appears to terminate within *hblB* (Agaisse *et al.*, 1999; Lindbäck *et al.*, 1999). Based on sequence analysis, *hblB* appears to have been generated by duplication of the first 1092 bp of the 1128 bp long *hblA* gene and a fusion with

an ORF in the 3′ end (Økstad *et al.*, 1999). The *hblCDAB* operon is highly conserved, with DNA sequence identities of 97–99% between strains. Nevertheless, frameshift mutations in the *hblC* and *hblD* genes in strain F837/76 have resulted in the C-terminal ends of the L_2 and L_1 proteins being eight amino acids longer and 23 amino acids shorter, respectively, in this strain compared with in the strains for which genomic sequences are currently available (Table 2).

Sequence similarity searches revealed a second variant of the *hbl* operon in *B. cereus* 03BB108 (GenBank entry ABDM00000000) and in *B. weihenstephanensis* KBAB4 (GenBank entry CP000903; Lapidus *et al.*, 2007). These two *hbl* operons consist of only three genes, organized as *hblCDA* operons lacking the *hblB* pseudogene. *Bacillus cereus* 03BB108 also harbours the *hblCDAB* operon and hence contains two *hbl* operons. Interestingly, the determined N-terminal sequences of the two sets of Hbl proteins isolated from *B. cereus* MGBC145 (Beecher & Wong, 2000b) indicated that one set of Hbl proteins originated from an *hblCDAB* operon, while the second set, denoted Hbl_a and consisting of proteins L_{2a}, L_{1a} and B_a, appeared to be more similar to the Hbl proteins encoded by the three-gene *hbl* operons from strains 03BB108 and KBAB4, which correspondingly are referred to as hbl_a. The hbl_a genes show 75–82% identity towards the corresponding genes of the *hblCDAB* operons. There is a greater divergence between the sequences of the two identified hbl_a operons, showing only 86% sequence identity, than between the more homogeneous *hblCDAB* operons which show 97–99% sequence identity between strains. Differences in molecular properties between Hbl proteins from *B. cereus* strains F837/76 and ATCC 14579 and the Hbl_a proteins from *B. weihenstephanensis* KBAB4 are listed in Table 2.

The *hblCDAB* operon is chromosomally encoded and the genomic location is conserved between strains. It is part of a 17.7-kb 11-gene insertion flanked on one side by a degenerate IS*Rso11* transposase fragment, and on both sides by a direct repeat covering the 3′ end of an *uvrC*-like gene, suggesting that these *hbl* genes have been acquired as a mobile genetic element (Han *et al.*, 2006). Other genes in this inserted cluster include *gerIABC* encoding spore germination proteins, and *trrA* encoding a transcriptional regulator. In contrast, the genomic location of the hbl_a operons differed between strains 03BB108 and KBAB4. The KBAB4 hbl_a operon is chromosomally encoded, and flanked upstream by a β-lactamase gene and downstream by a gene encoding a predicted 99-kDa S-layer domain protein. In contrast, the 03BB108 hbl_a operon is located in a region containing genes with similarities towards genes present on the pXO1 plasmid, potentially indicating that it could be plasmid-borne. However, as the 03BB108 genome sequence is currently not assembled it is not known whether this hbl_a operon is chromosomally encoded or located on a plasmid.

Heterogeneity for Nhe proteins has been reported for the *nhe* operons present in the group of strains represented by *B. cereus* NVH 391/98 (Fagerlund *et al.*, 2007; Lapidus *et al.*, 2007). The *nhe* operons in these strains show only about 77% identity towards the *nhe* operons in the main *B. cereus* group cluster, in correspondence with the remote phylogenetic relationship that these strains show towards other *B. cereus* group strains (Fagerlund *et al.*, 2007; Fig. 1). In contrast, the identities between *nhe* operons from other strains are on average about 90%, but approach 100% between strains belonging to the *B. anthracis* and emetic *B. cereus* clonal clusters. The greatest differences between *nhe* operons from different strains reside in the intergenic regions, in particular in the region between *nheB* and *nheC*, which also varies in length between strains. All currently available genome sequences of *B. cereus* group strains contain a single *nhe* operon, except *B. weihenstephanensis* KBAB4, which contains two distinct *nhe* operons. One of these operons is similar to the *nhe* operons found in the other *B. cereus* group strains, and likewise chromosomally encoded. The second operon, however, is located on a 400-kb megaplasmid named pBWB401 (GenBank entry CP000904; Lapidus *et al.*, 2007). The sequence identity of this operon towards the other known *B. cereus* group operons is only around 58%. The Nhe proteins encoded by the plasmid-borne *nhe* operon are thus more divergent from the known chromosomally encoded Nhe proteins than the Hbl and Hbl_a groups of proteins are divergent from each other, as illustrated in the tree representation in Fig. 4. Differences in molecular properties between the Nhe proteins from *B. cereus* strains NVH 0075/95, NVH 391/98, and those encoded by the *B. weihenstephanensis* KBAB4 megaplasmid pBWB401 are listed in Table 3.

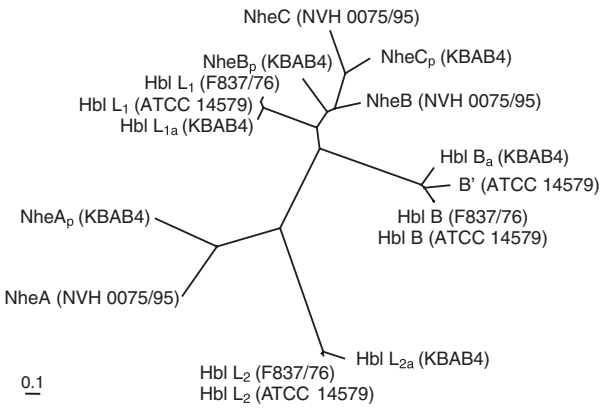

Fig. 4. Neighbor-joining tree showing the molecular relatedness of different variants of Hbl and Nhe proteins from selected *Bacillus cereus sensu lato* strains. The tree was generated as described in Figs 1 and 2. B′ indicates the deduced protein sequence of *hblB*. $NheA_p$, $NheB_p$ and $NheC_p$ denotes the Nhe proteins encoded on the KBAB4 megaplasmid pBWB401. Scale bar indicates 10% divergence.

For Hbl, functional differences have been determined between the two Hbl toxins isolated from *B. cereus* MGBC145, because the Hbl_a variant did not produce the ring-formed haemolysis pattern in blood agar diffusion assays characteristic for Hbl, although both toxins showed haemolytic activity (Beecher & Wong, 2000b). The biological activity of the Nhe toxins encoded by the recently described *nhe* operons in strain NVH 391/98 and on the KBAB4 megaplasmid remains to be determined, as these proteins have not been isolated and characterized. Interestingly, the only *B. weihenstephanensis* strain sequenced to date, KBAB4, contains both variants of the *nhe* operon and the rare *hbl* variant, hbl_a. This may reflect the higher level of genetic exchange that is observed among *B. weihenstephanensis* strains compared with other species of the *B. cereus* group (Sorokin *et al*., 2006). In any case, it is clear that a greater diversity than what was initially apparent exists within the family of *B. cereus* tripartite cytotoxins. Furthermore, the implications for genetic transfer suggested by the observation that the *hblCDAB* operon may have been acquired as a transposable element and the presence of a new variant of the *nhe* operon on a plasmid are intriguing.

The *B. cereus* β-barrel pore-forming toxins

Bacillus cereus produces two single-component protein toxins that are members of the family of β-barrel pore-forming toxins, namely CytK and HlyII (Baida *et al*., 1999; Lund *et al*., 2000). This toxin family includes β-toxin of *C. perfringens* (Steinthorsdottir *et al*., 2000) and α-haemolysin of *S. aureus* (Gouaux, 1998), the best characterized member. These toxins are secreted as water-soluble monomers that associate into oligomeric prepores at the target cell surface, which subsequently insert their pore-forming regions into the cell membrane forming a transmembrane pore (Bhakdi & Tranum-Jensen, 1991). The crystal structure of *S. aureus* α-haemolysin shows a mushroom-shaped heptamer of about 10 nm in height and diameter, with an amphiphatic β-barrel membrane-spanning pore ranging in size from 1.4 to 4.6 nm (Song *et al*., 1996).

CytK is a 34-kDa protein with dermonecrotic, cytotoxic and haemolytic activities, and shows similar cytotoxic potency towards cell cultures as Hbl and Nhe (Lund *et al*., 2000). It is identical to the toxin referred to as haemolysin IV (HlyIV), partially characterized by Beecher *et al*. (2000). CytK was originally isolated from *B. cereus* strain NVH 391/98, which was responsible for a severe foodborne outbreak of diarrhoeal disease in a French nursing home in 1998, in which several people developed bloody diarrhoea and three elderly people died (Lund *et al*., 2000). Based on the necrotic activity of CytK and the apparent lack of both Nhe and Hbl in the outbreak-associated strain, CytK was implicated as the toxin responsible for the severe symptoms and uncharacteristic bloody diarrhoea presenting in this outbreak (Lund *et al*., 2000; Dietrich *et al*., 2005). However, because genes encoding *nhe* were later identified in this strain (Fagerlund *et al*., 2007; Lapidus *et al*., 2007), contribution by Nhe to the pathogenicity of *B. cereus* NVH 391/98 cannot be excluded.

In accordance with the remote phylogenetic relationship of *B. cereus* NVH 391/98 towards the majority of other *B. cereus* group strains, the CytK protein from these two groups of strains show only 89% sequence identity (Fagerlund *et al*., 2004, 2007). Characterization of the two variants of CytK showed that the CytK protein from NVH 391/98 had five-fold greater cytotoxic activity towards Caco-2 and Vero cells than the most common CytK variant, represented by CytK from *B. cereus* NVH 1230/88, which was initially named CytK-2. The differences in cytotoxicity correlated with the most common CytK variant forming a greater number of small-sized pores in synthetic lipid bilayers (Fagerlund *et al*., 2004). The greater cytotoxic activity of the CytK protein from NVH 391/98 offered a plausible explanation for the severe food poisoning outbreak caused by this strain. However, while NVH 391/98 was shown to have an exceptionally high level of *cytK* expression (Brillard & Lereclus, 2004), *B. cereus* NVH 883/00 harbouring the same *cytK* variant as NVH 391/98 was noncytotoxic in a cell culture assay due to low amounts of toxins produced under the tested conditions (Fagerlund *et al*., 2007). This implies that the level of virulence gene expression is probably more important than the presence of specific gene variants for determining the level of cytotoxicity of a particular strain.

HlyII, the second β-barrel pore-forming toxin of *B. cereus*, is haemolytic and cytotoxic towards human cell lines (Andreeva *et al*., 2006), but has never been implicated as the toxin responsible for diarrhoea caused by *B. cereus*. It has been suggested that this may be due to a trypsin digestion site in the β-loop constituting the transmembrane domain of the toxin, resulting in inactivation by trypsin in the small intestine (Lund *et al*., 2000), as is observed for β-toxin of *C. perfringens* (Granum, 1990), but this remains to be tested experimentally. The toxin, with a deduced mass of 42.3 kDa, was originally characterized by Sinev *et al*. (1993) and Baida *et al*. (1999). Compared with other known members of the family of β-barrel pore-forming toxins family, HlyII has a 94-amino acid, C-terminal extension not required for pore formation or haemolytic activity (Baida *et al*., 1999; Miles *et al*., 2002). Interestingly, HlyII expression appears to be independent of PlcR, the central transcriptional regulator for virulence genes in *B. cereus*, which is required for transcription of *hbl*, *nhe* and *cytK*. This may suggest that HlyII may have a different role than Hbl, Nhe and CytK in *B. cereus*.

Both CytK and HlyII have been shown to form anion-selective channels with functional diameters of *c*. 7 Å in planar lipid bilayers (Hardy *et al*., 2001; Miles *et al*., 2002;

Andreeva *et al.*, 2007). Because they readily form pores in synthetic lipid bilayers, both toxins have innate pore-forming ability in phospholipid membranes. It is thus unlikely that protein or carbohydrate receptors are absolute requirements for binding and lysis by CytK and HlyII, and it has correspondingly been reported that HlyII has no specific receptor on erythrocytes (Andreeva *et al.*, 2006). However, for the related *S. aureus* α-haemolysin, phosphocholine appears to be the host cell binding receptor. A low affinity binding site for a phosphocholine head group allows concentration of α-haemolysin toxin monomers in microdomains enriched in cholesterol and sphingolipids (lipid rafts). This results in high local concentrations allowing toxin oligomerization and thus stable membrane-anchored binding to target host cells, giving the appearance that certain cell types have high-affinity toxin binding sites (Valeva *et al.*, 2006). β-toxin from *C. perfringens* has likewise been shown to concentrate in lipid rafts (Nagahama *et al.*, 2003). Based on the functional similarities within the family of β-barrel pore-forming toxins, it is tempting to speculate that membrane binding of CytK and HlyII may occur by a similar mechanism.

Secretion of cytotoxins from the bacterial cell

CytK and all six components of Hbl and Nhe all contain secretory signal peptides indicating that they are secreted by the general secretory (Sec) pathway, which is considered to be the main translocation system on which bacterial protein secretion relies (van Wely *et al.*, 2001). Nevertheless, it has been suggested that the three Hbl components are secreted from the bacterial cell using the flagellar export apparatus (Ghelardi *et al.*, 2002). This conclusion was based on the absence of all three Hbl components in culture supernatant from a nonflagellated *flhA* mutant. Observations of reduced Hbl protein levels in culture supernatants have recently also been observed in two additional nonflagellated strains (Ghelardi *et al.*, 2007a) and in an *flhF* mutant strain also showing reduced numbers of flagella and altered motility behaviour (Salvetti *et al.*, 2007). However, these studies did not address the question of whether the reduced levels of Hbl proteins in culture supernatant were mediated at the transcriptional, translational or post-translational level, or due to a secretion defect. Interestingly, a study by (Bouillaut *et al.*, 2005) demonstrated that the nonflagellated *flhA* mutant showed a 50% reduction in *hbl* transcription.

Recent evidence has, however, suggested that all three Hbl proteins, in addition to the Nhe components and CytK, are indeed secreted by the Sec pathway, as inhibition by azide of SecA, an essential component of the Sec translocase, resulted in reduced secretion and intracellular accumulation of the toxin components. In contrast, the nonflagellated *flhA* mutant showed reduced secretion of Hbl, Nhe and CytK, but the lack of intracellular accumulation of toxin proteins in this strain suggested that the absence of secreted toxin proteins was due to reduced toxin production and not a secretion defect (A. Fagerlund & P. E. Granum, unpublished results).

Detection of *B. cereus* cytotoxins

Owing to the widespread presence and hardy nature of *B. cereus*, it must be expected to be present in different foods and raw materials, and thus detection of the bacterium is not always the main issue for food safety purposes. Instead, ability to detect the possibly harmful strains, or their toxic products, is the highly desired goal. As cereulide and the three cytotoxins Hbl, Nhe and CytK are the main known virulence factors in *B. cereus* foodborne disease, focus has been on their detection.

Antibodies have been produced for the three-component toxins Nhe and Hbl (Dietrich *et al.*, 1999, 2005), and two antibody-based detection kits targeting these toxins are commercially available (Buchanan & Schultz, 1994; Day *et al.*, 1994). The BCET-RPLA kit (Oxoid Ltd., UK) is a semi-quantitative assay detecting, by reversed antibody agglutination, the L_2 component of Hbl in foods and in cultures of *B. cereus* (Beecher & Wong, 1994a). The sensitivity of the test is reported to be $2\ ng\ mL^{-1}$ test extract. The TECRA-BDE kit (Tecra International Pty Ltd., Australia) detects the NheA component of the Nhe toxin by an enzyme-linked immunosorbent assay (ELISA) sandwich test (Beecher & Wong, 1994a). The sensitivity reported by the manufacturer is $> 1\ ng\ mL^{-1}$ prepared sample, and the kit is intended for use on foods and environmental samples. Neither of the kits will confirm the presence of biologically active toxin, because only one of each of the three-component toxins is detected. For the third and more recently described toxin CytK, there is at present no commercially available detection kit.

For nonspecific detection and characterization of *B. cereus* enterotoxins, different laboratory animal and tissue culture assays have been employed. Among the tests involving live animals are the rabbit ileal loop (RIL) test, performed by injection of *B. cereus* cultures or extracts into ligated rabbit intestinal loops followed by observation of fluid accumulation, the guinea pig skin reaction, and the vascular permeability assay (Kramer & Gilbert, 1989). The use of tissue culture assays for detecting *B. cereus* enterotoxins has been shown to correlate well with results from traditional methods, and represent a convenient alternative for screening purposes (Gilbert & Kramer, 1984; Thompson *et al.*, 1984; Shinagawa *et al.*, 1991; Jackson, 1993; Fermanian *et al.*, 1996). The cell culture lines used include CHO cells (Buchanan & Schultz, 1994; Beattie & Williams, 1999; Hsieh

et al., 1999), McCoy cells (Jackson, 1993; Fletcher & Logan, 1999), Caco-2 cells (Hardy *et al*., 2001; Rowan *et al*., 2001) and Vero cells (Lund & Granum, 1996; Dietrich *et al*., 1999; Prüss *et al*., 1999; From *et al*., 2005).

For specific detection of the genes encoding the *B. cereus* toxins Hbl, Nhe and CytK, several PCR schemes, including multiplex PCR, have been developed (see for instance Mäntynen & Lindström, 1998; Hansen & Hendriksen, 2001; Guinebretière *et al*., 2002; Yang *et al*., 2005). Considering the wide distribution of cytotoxin genes among *B. cereus* strains (Rusul & Yaacob, 1995; Mäntynen & Lindström, 1998; Prüss *et al*., 1999; Rivera *et al*., 2000; Guinebretière *et al*., 2002; Ehling-Schulz *et al*., 2005a), the use of PCR techniques to identify diarrhoeal strains is of little use for practical food safety purposes, because detection of a toxin gene does not reveal the level of toxin production and thus can not predict the potential pathogenicity of a particular *B. cereus* strain.

Regulation of cytotoxin expression

In general, production of bacterial toxins is tightly regulated. Bacteria sense their environment and respond by producing virulence factors when they are needed, for example in the host environment or upon encountering nutrient limitation, and by turning off toxin production when it would be a selective disadvantage, as synthesis and transport of toxins requires a considerable amount of energy. Because virulence factors of bacteria are often co-ordinately regulated, toxin genes are frequently members of regulons that include additional genes encoding virulence determinants (Finlay & Falkow, 1997). The majority of the *B. cereus* protein cytotoxins are members of a regulon controlled by the transcriptional activator PlcR, but it is becoming increasingly clear that additional regulatory mechanisms must be involved.

The PlcR quorum sensing system

PlcR is the major virulence regulator of *B. cereus* (Lereclus *et al*., 1996). It is part of a 'quorum sensing' system that allows *B. cereus* to regulate virulence genes in a cell density-dependent manner, and it activates expression of a regulon comprising several extracellular virulence factors, including Hbl, Nhe, CytK, degradative enzymes (phospholipases, proteases) and surface proteins (Lereclus *et al*., 1996; Agaisse *et al*., 1999; Gohar *et al*., 2002). Activation of the 34-kDa PlcR protein is dependent on PapR, a 48 amino acid peptide encoded downstream of *plcR*, which is thought to be secreted by the Sec pathway and extracellularly processed. The PapR autoinducer peptide is then reimported via an oligopeptide permease (Opp), apparently as a heptamer (Agaisse *et al*., 1999; Gominet *et al*., 2001; Slamti & Lereclus, 2002; Declerck *et al*., 2007). When high bacterial densities are reached, the concentration of PapR inside the cells increases, and PapR then interacts with PlcR facilitating binding of PlcR to a conserved palindromic motif known as the PlcR box ($TATGNAN_4TNCATA$) upstream of target genes to activate their transcription (Agaisse *et al*., 1999; Slamti & Lereclus, 2002).

PlcR has been crystallized as an asymmetric dimer in complex with PapR, and contains an N-terminal helix-turn-helix DNA binding domain and a C-terminal regulatory domain composed of 11 helices with which PapR interacts. Structure modelling based on small angle X-ray scattering analysis has further suggested that binding of PapR triggers oligomerization of PlcR dimers into a supramolecular structure forming a right-handed spiral that may associate with DNA (Declerck *et al*., 2007). PlcR is phylogenetically related to all quorum sensors that bind directly to their autoinducer peptide inside the cell, which form a superfamily referred to as the RNPP family, restricted to the Gram-positive class *Firmibacteria* and the orders *Bacillales* and *Clostridiales* (Declerck *et al*., 2007).

As PlcR is positively autoregulated, activation of PlcR by PapR causes a positive feedback loop, presumably responsible for the sharp initiation of PlcR activation. The initiation of PlcR expression at the transition between exponential and stationary phase in liquid culture indicated that PlcR was also regulated by transition state regulators (Lereclus *et al*., 1996). The *plcR* promoter contains two Spo0A binding sites on either side of the PlcR box, and *plcR* transcription was strongly upregulated in a *spo0A* deletion mutant, but abolished in sporulation specific medium, indicating that PlcR was repressed by the transcriptional regulator Spo0A∼P (Lereclus *et al*., 2000), which is responsible for initiation of sporulation in the stationary phase of growth (Phillips & Strauch, 2002). Activation of PlcR thus most likely requires at least two conditions to be fulfilled: (1) that the cell density is high enough for quorum sensing to occur, and (2) that the nutritional state of the cell is such that Spo0A∼P is at a sufficiently low concentration to allow *plcR* transcription.

The significance of PlcR was demonstrated by showing that a deletion of *plcR* resulted in a 50% decrease in the amount of proteins secreted at the onset of stationary phase compared with the wild-type *B. cereus* strain, at which time the majority of secreted proteins were putative virulence factors (Gohar *et al*., 2002). Furthermore, PlcR was shown to directly influence *B. cereus* and *B. thuringiensis* pathogenicity as disruption of *plcR* caused a strong reduction in virulence against both insect larvae, mice and rabbit eyes (Salamitou *et al*., 2000; Callegan *et al*., 2003). However, although virulence was reduced it was not abolished, indicating that additional factors not regulated by PlcR contributed to virulence, and that activation of PlcR was not sufficient to account for the pathogenicity of *B. cereus*. In *B. anthracis*, the PlcR regulon is silent due to a nonsense

mutation in *plcR*, resulting in a truncated inactive PlcR protein (Agaisse *et al.*, 1999; Gohar *et al.*, 2005). The PlcR regulon may have been counterselected in *B. anthracis* due to incompatibility between the AtxA and PlcR regulons, as their simultaneous expression resulted in a sporulation defect (Mignot *et al.*, 2001). Interestingly, the upstream promoter regions of both the hbl_a operon and the plasmid-borne *nhe* operon of *B. weihenstephanensis* KBAB4 contain consensus PlcR-boxes, indicating that these operons are regulated by PlcR. A PlcR-box was, however, not identified upstream of hbl_a in *B. cereus* 03BB108.

Regulation of HlyII

HlyII is one of the few secreted virulence factors of *B. cereus* that does not appear to be regulated by PlcR (Budarina *et al.*, 2004; Gohar *et al.*, 2005). It has instead been shown to be negatively regulated by the transcriptional regulator HlyIIR, encoded immediately downstream of *hlyII* (Budarina *et al.*, 2004). HlyII expression is additionally predicted to be regulated by the ferric uptake regulator (Fur), as the *hlyII* promoter contains a Fur binding site overlapping the transcriptional start site (Harvie *et al.*, 2005). Fur regulates iron metabolism and represses genes involved in iron uptake when sufficient iron is present, but upon sensing iron limitation the repression of genes involved in iron uptake and transport is lifted, enabling the bacterium to obtain sufficient iron for growth. Deletion of *fur* in *B. cereus* resulted in reduced virulence in an insect infection model, demonstrating a link between virulence and iron metabolism (Harvie *et al.*, 2005). The observations that HlyII has neither been implicated as the enterotoxin responsible for an outbreak of foodborne disease, nor demonstrated to be important in nongastrointestinal infections, in addition to the predicted coregulation with iron metabolism genes, could suggest that the haemolytic action of HlyII is a mechanism by which the bacterium gains access to iron.

Other regulatory mechanisms

It has long been observed that the level of toxic activity produced by a *B. cereus* culture is dependent on environmental factors such as pH, temperature, glucose concentration and oxygen tension (Glatz & Goepfert, 1976; Sutherland & Limond, 1993). More recently, the regulation of Hbl and Nhe expression has been linked to the metabolic state of the cell, as *B. cereus* produced more Hbl during fermentative (anaerobic) growth than during respiratory (aerobic) growth (Duport *et al.*, 2004), and a low oxidoreduction potential (ORP) during anaerobiosis strongly favoured Hbl and Nhe production (Zigha *et al.*, 2006). These results seem to reconcile well with the anaerobic, highly reducing fermentative conditions present in the small intestine (Moriarty-Craige & Jones, 2004), where *B. cereus* must produce toxins in order to induce diarrhoeal disease (Granum *et al.*, 1993). The two-component system ResDE and the transcriptional regulator Fnr, whose primary roles are to modulate the metabolism of the cell in response to oxygen availability and redox conditions, also mediates the regulation of Hbl and Nhe expression in response to these factors (Duport *et al.*, 2006; Zigha *et al.*, 2007). Production of Hbl and Nhe was essentially abolished under all conditions tested in a *fnr* mutant and in a *resE* (sensor kinase) mutant with intact *resD* (response regulator) (Duport *et al.*, 2006; Zigha *et al.*, 2007). ResDE is also a positive regulator of the *B. anthracis* toxin complexes (Vetter & Schlievert, 2007), and interestingly, Fnr is a positive regulator of *E. coli* ClyA (Green & Baldwin, 1997), belonging to the same superfamily of toxins as Hbl and Nhe. The control of Hbl and Nhe production by ResDE and Fnr is not mediated through PlcR, and putative ResD binding sites have been identified in the promoter regions of *hbl* and *nhe* (Duport *et al.*, 2006).

Hbl and Nhe have also been suggested to be subject to catabolite repression, at least during anaerobiosis, because transcription of *hbl* was repressed by increasing concentrations of glucose (Duport *et al.*, 2004), and growth on sucrose gave higher levels of Hbl and Nhe production compared with growth on glucose (Ouhib *et al.*, 2006). Genes regulated by catabolite repression harbour catabolite responsive element(s) (*cre* sites), for which the consensus sequence in *Bacillus subtilis* has been determined to be TGWNANCGNT NWCA (Hueck & Hillen, 1995) or WWTGNAARCGNWW WCAWW (Miwa *et al.*, 2000). A search of the *hbl*, *nhe* and *cytK* regulatory regions for the presence of this sequence revealed two potential *cre* sites in the *nhe* regulatory region (Fig. 5). The concept of *B. cereus* toxin regulation by catabolite repression is perhaps not unexpected, as from a bacterial point of view, deploying virulence factors to liberate required nutrients does not appear necessary when easily metabolized carbohydrates are available.

Observations also point towards a regulatory link between expression of motility and virulence factor genes in *B. cereus*. For example, in a *plcR* mutant, flagellin expression and motility was reduced (Gohar *et al.*, 2002; Callegan *et al.*, 2003), and inactivation of *flhA*, encoding a component of the flagellar export apparatus, has been shown to affect flagellation, sporulation, secretion of Hbl, transcription of *hbl* and *plcA*, and production of Hbl, Nhe and CytK (Ghelardi *et al.*, 2002; Bouillaut *et al.*, 2005; A. Fagerlund & P. E. Granum, unpublished results). Also, Hbl production increases during swarming migration (Ghelardi *et al.*, 2007a), which is a differentiated state where elongated and hyperflagellate swarm cells collectively move across solid surfaces (Henrichsen, 1972; Harshey, 1994). However, the molecular mechanisms that putatively couple the expression of virulence factors to motility have not been elucidated, and it is not known whether motility plays a role in *B. cereus* infection.

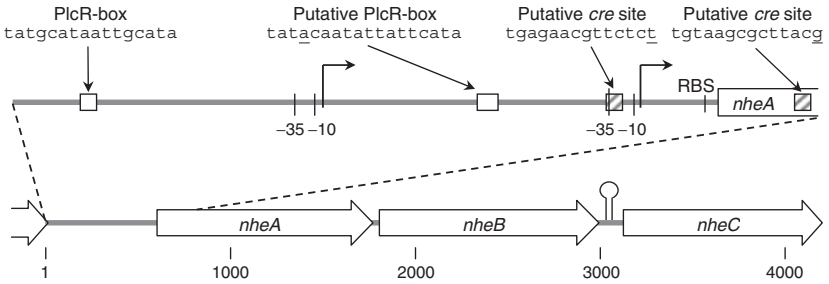

Fig. 5. The *nhe* operon with promoter and regulatory sites. The indicated regulatory sequences are from *Bacillus cereus* ATCC 14579. The consensus PlcR-box (Agaisse *et al*., 1999) and the putative PlcR-box with one mismatch in strain ATCC 14579 (underlined) and six bases between the palindromic flanks of the recognition sequence instead of four as in the established consensus (Granum *et al*., 1999), and the two predicted *cre* sites (with mismatches towards the *B. subtilis* consensus underlined) are shown as boxes. The inverted repeat between *nheB* and *nheC* (Granum *et al*., 1999) is indicated as a stem loop structure. The bent arrows indicate the positions of transcriptional start sites, preceded by putative −10 and −35 regions. The transcriptional start site closest to the *nheA* gene was identified using RNA isolated from strains NVH 0075/95 and NVH 1230/88 (Lindbäck *et al*., 2004), while the one further upstream was identified using a plasmid carrying the *nhe* promoter from *Bacillus thuringiensis* strain 407 (Agaisse *et al*., 1999). The scale in basepairs is shown in the lower part of the figure.

The complexity of regulation of toxin production by *B. cereus* is also illustrated by the apparent differential regulation of Hbl, Nhe and CytK synthesis, illustrated by the variation in time-course of production of these toxins (Gilois *et al*., 2007). The highest specific production of Nhe was determined to occur early during exponential growth, while Hbl was produced later, early in the stationary phase of growth (Zigha *et al*., 2006). Furthermore, the *nhe* operon appears to contain two transcriptional start sites (Agaisse *et al*., 1999; Lindbäck *et al*., 2004;Fig. 5), indicating the presence of two promoters which may potentially be differently regulated. Furthermore, it appears that the temperature of growth affects toxin production in *B. cereus* group species in a strain-dependent manner (Christiansson *et al*., 1989; Stenfors Arnesen *et al*., 2007), although the mechanism of such control is not known. However, the most notable observation regarding *B. cereus* virulence regulation is the substantial variation in the level of toxin production between individual strains. While certain *B. cereus* strains have been used as probiotics (Hong *et al*., 2005), others are the cause of lethal foodborne disease (Mahler *et al*., 1997; Lund *et al*., 2000; Dierick *et al*., 2005). Because the presence of cytotoxin genes or gene variants does not seem to be sufficient to explain the level of virulence of a particular strain with respect to the diarrhoeal type of disease, it is likely that the reason why only some strains of *B. cereus* appear to be pathogenic lies in strain-dependent differences in regulation of toxin expression. Virulence gene regulation therefore appears be essential for understanding *B. cereus* pathogenesis.

Concluding remarks

Bacillus cereus shows a wide range of variation in phenotypes and virulence types. The diverse nature of the bacterium is all the more fascinating when the strong elements of likeness within the *B. cereus* group members are considered. There is a huge contrast between the strongly clonal strains with plasmid-borne virulence factors, such as *B. anthracis* and the emetic type of *B. cereus*, and the more diverse strains of *B. cereus* and *B. thuringiensis*. As described in this review, the level of virulence between strains is highly variable, ranging from harmless to lethal strains. While the role of cereulide in causing the emetic syndrome of *B. cereus* is well established, the role of the protein cytotoxins as aetiological agents of diarrhoeal disease appears more complex. Strong evidence points towards the Hbl, Nhe and CytK cytotoxins being the main virulence factors in *B. cereus* foodborne diarrhoeal disease. However, final proof of their role has not been obtained, and the disease appears to be more complex than for example that observed for *C. perfringens* type A food poisoning, where a single enterotoxin (CPE) alone accounts for all symptoms. Currently, evidence points towards Nhe being the major cytotoxic membrane-damaging factor secreted by most *B. cereus* strains (Dietrich *et al*., 2005; Moravek *et al*., 2006). However, the difficulties in establishing a single factor as the aetiological agent of gastroenteritis due to *B. cereus* probably reflects that the basis for the disease is multifactorial, where a number of virulence factors may contribute to the overall cellular damage, possibly in a strain-dependent manner.

The most intriguing toxins of *B. cereus* are the related three-component pore-forming toxins Hbl and Nhe. These unique toxins appear to be distantly related to the homo-oligomeric toxin ClyA found in certain species of *Enterobacteriaceae*, a relationship that was discovered due to the structural similarities between the recently determined crystal structure of Hbl component B and ClyA (Fagerlund *et al*., 2008). This superfamily of toxins appears to represent the only known pore-forming toxins with mammalian targets

that are mainly α-helical in structure. However, while ClyA assembles into a transmembrane pore of identical subunits (Wallace *et al.*, 2000), Hbl and Nhe appear to have evolved into tripartite toxin complexes through gene duplication. Further structural and functional studies will hopefully reveal more about the role of the three proteins in each complex, including the molecular basis of the unusual inhibitory effect on cell lysis by excess concentration of individual toxin components. At least two genetically different operons encoding both Hbl and Nhe have been revealed through genomic sequencing of *B. cereus* group strains, suggesting that the family of *B. cereus* tripartite toxins may harbour much greater diversity than originally conceived. Further study remains in order to determine the significance of these newly discovered toxin complex variants in relation to foodborne disease.

Increasing evidence points towards gene regulation being the key to understanding the ecology and pathogenesis of *B. cereus*. Because *B. cereus* is not a strict pathogen, it may have developed its regulation of pathogenesis using 'established' regulatory systems. Apparently, several systems are involved in *B. cereus* virulence regulation, in a cross-talk between metabolism and toxin production. As an organism inhabiting a diversity of niches, *B. cereus* employs a complex network of gene regulation for optimal use of resources in all situations, a complexity which is also reflected in its regulation of foodborne virulence. Expression of *B. cereus* virulence factors implicated in foodborne disease is temporally controlled in response to cell density, environmental conditions, nutrient availability and the metabolic state of the cell, in addition to being co-ordinately regulated with motility genes. The level of toxin gene expression appears to play a major role in determining the pathogenic potential of a particular strain, while in comparison, the presence of specific toxin genes or gene variants appear to be of less importance. A better understanding of the multiple regulatory mechanisms involved in *B. cereus* toxin production will help to understand the adaptation of *B. cereus* to its pathogenic lifestyle, and may prove to be the key for identification of potentially harmful strains.

Like its relative *B. subtilis,* widely used as a model organism, *B. cereus* is a species receiving considerable attention where we can learn even more because it exists as both virulent and avirulent types. This may reflect the diverse nature of *B. cereus*, primarily existing as a soil saprophyte, with physiology well adapted for the intestinal tract, also acting as an opportunistic pathogen involved in local and systemic infections.

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