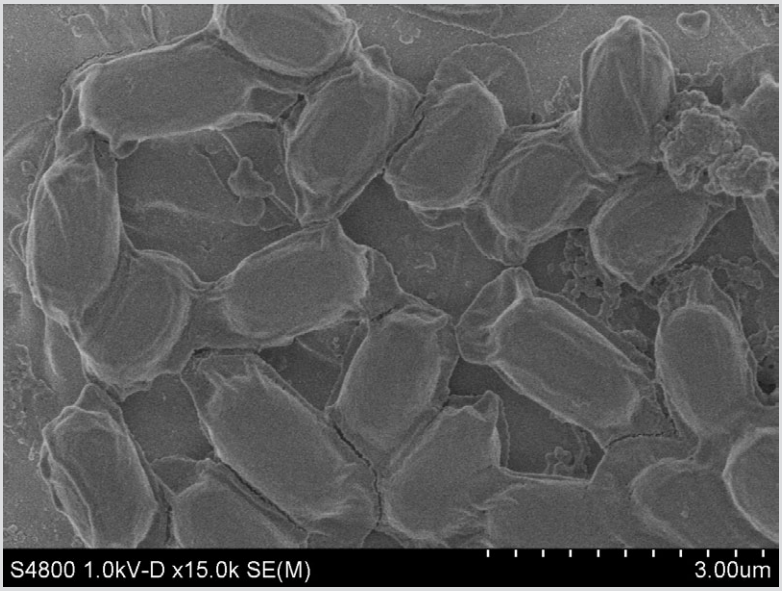

Bacillus cereus Spores and Cereulide in Food-Borne Illness

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Academic Dissertation in Microbiology

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The Cover image: Field emission scanning electron micrograph (FESEM) taken using the instrument Hitachi S-4800 (Tokyo, Japan) of spores of the *Bacillus cereus* strain UM 98 isolated from milk of dairy silo tank. For the microscopy, the spores were fixed with phosphate-buffered glutaraldehyde, dehydrated in an ethanol series and dried in hexamethyldisilazane. In the figure, the spores adhered to steel surface. The image was taken by Ranad Shaheen.

To my beloved Parents and Family

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I. Svensson, B., Monthan, A., **Shaheen, R**., Andersson M.A. & Salkinoja-Salonen, M.S. Christiansson A. 2006. Occurrence of emetic toxin producing *B. cereus* in the dairy production chain. International Dairy Journal 16,740-749.

II. **Shaheen, R**., Andersson, M.A., Apetroaie, C., Schulz, A., Ehling-Schulz, M., Ollilainen V-M., Salkinoja-Salonen M.S. 2006. Potential of selected infant food formulas for production of *B. cereus* emetic toxin, cereulide. International Journal of Food Microbiology 107, 287-294

III. **Shaheen, R**., Svensson, B., Andersson M.A., Christiansson, A., Salkinoja-Salonen, M.S. 2009. Persistence strategies of *Bacillus cereus* spores isolated from dairy silo tanks. Accepted for publication in Food Microbiology.

IV. Carlin, F., Fricker, M., Pielaat, A., Heisterkamp, S., **Shaheen, R**., Salkinoja-Salonen, M.S., Svensson, B., Nguyen-the, C., Ehling-Schulz M. 2006. Emetic toxin producing strains of *B. cereus* show distinct characteristics within the *B. cereus* group. International Journal of Food Microbiology 109, 132-138.

V. **Shaheen, R**., Andersson M.A., Pirhonen, T., Jääskeläinen, E.L., Svensson, B., Nguyen-the, C., Salkinoja-Salonen, M.S. 2009. Antagonistic activity and cereulide production of foodborne *B. cereus* may synergistically contribute to human morbidity. Submitted Aug 17,2009.

THE AUTHOR'S CONTRIBUTION:

Paper I
Ranad Shaheen identified and measured the emetic toxin produced by the strains and isolates of *B. cereus* in the dairy production chain and interpreted the results.

Paper II
Ranad Shaheen wrote the article, planned and carried out the experimental work.

Paper III
Ranad Shaheen designed the experiments, interpreted the results and wrote the paper. She was responsible for all experimental work except the phenotypic characterization of the *B. cereus* isolates, RAPD-PCR and the alkaline and acid resistance analysis of the spores.

Paper IV
This was a joint European project paper. Ranad Shaheen was responsible for the setup of the emetic toxin producing collection strains and for characterising the toxic properties of these strains. She executed all cereulide analyses in this multi-centre project.

Paper V
Ranad Shaheen designed the experiments, was responsible for the experimental work and executed most of it, interpreted the results and wrote the paper.

ABBREVIATIONS

aa	amino acid
ABC transporter	ATP binding cassette in a transporter
ADP, ATP	adenosine 5`-diphosphate and 5`-triphosphate, respectively
ATCC	American Type Culture Collection
a_w	water activity
AU	arbitrary unit
bp	base pair
Caco-2	colon adenocarcinoma cell line
CDSs	coding DNA sequences
CFU	colony forming unit
DNA	deoxyribonucleic acid
DPA	dipicolinic acid
EFSA	European Food Safety Authority
ESI	electron spray ionization
EU	European Union
HeLa	human cervical cancer cell line
HEp-2	human larynx carcinoma cell line
HepG2	human hepato carcinoma cell line
5-HT3	5-hydroxy tryptamine (serotonin) receptor 3
LC-MS	liquid chromatography – mass spectrometry
log K_{ow}	logarithm of the octanol / water partition coefficient
LRIL	ligated rabbit ileal loop
MTT	3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide
NL	The Netherlands
NRPS	non-ribosomal peptide synthesis
O-Ala	lactic acid
O-Leu	2-hydroxy isocaproic acid
O-Val	2-hydroxy isovaleric acid
Paju	human neural cell line
PBS	phosphate buffered saline
PCR	polymerase chain reaction
Tris	tris-(hydroxymethyl) aminomethane
TSB	tryptic soy broth
SASP	small acid-soluble proteins
UK	United Kingdom
USA	United States of America
VPR	vascular permeability reaction
$\Delta\Psi_m$	mitochondrial inner membrane transmembrane potential

DEFINITIONS

depsipeptide	a peptide in which one or more of the amide (-CONHR-) bonds are replaced by ester (COOR) bonds
dodeca	twelve, numerical prefixes derived from Greek
genotype	the genetic constitution of a cell or an organism. The genotype is distinct from its expressed features or phenotype
infection	the pathological state resulting from the invasion of the body by pathogenic microorganisms
isolate	a taxonomic unit, it means a pure culture that was isolated some time somewhere.
non-ribosomal peptide	is synthesized by peptide synthetases with no involvement of ribosome. It is made from amino acid precursors (AMP activated amino acid). The precursors and the conversion to hydroxy acid occur only after activation into AMP-amino acids, before or after their incorporation into the peptide.
phenotype	is any observable characteristic or trait of an organism.
species	a taxonomic unit, each species consists of one or more strains. The strains of any given species have been properly characterised and fulfil the definition of that specific species.
strain	a taxonomic unit defined as the descendants of a single isolate in pure culture and usually made up of a succession of cultures ultimately derived from an initial single colony.
toxin	biologically produced poisonous compound
toxic	a substance that damages one or more functions of an organism or of a living cell
toxinogenic	an organism that produces a toxin
virulence	refers to the degree of pathogenicity of a given microbe

ABSTRACT

B. cereus is a gram-positive bacterium that possesses two different forms of life: the large, rod-shaped cells (ca. 0.002 mm by 0.004 mm) that are able to propagate and the small (0.001 mm), oval shaped spores. The spores can survive in almost any environment for up to centuries without nourishment or water. They are insensitive towards most agents that normally kill bacteria: heating up to several hours at 90 ºC, radiation, disinfectants and extreme alkaline (≥ pH 13) and acid (≤ pH 1) environment. The spores are highly hydrophobic and therefore make them tend to stick to all kinds of surfaces, steel, plastics and live cells.

In favourable conditions the spores of *B. cereus* may germinate into vegetative cells capable of producing food poisoning toxins. The toxins can be heat-labile protein formed after ingestion of the contaminated food, inside the gastrointestinal tract (diarrhoeal toxins), or heat stable peptides formed in the food (emesis causing toxin, cereulide). Cereulide cannot be inactivated in foods by cooking or any other procedure applicable on food. Cereulide in consumed food causes serious illness in human, even fatalities.

In this thesis, *B. cereus* strains originating from different kinds of foods and environments and 8 different countries were inspected for their capability of forming cereulide. Of the 1041 isolates from soil, animal feed, water, air, used bedding, grass, dung and equipment only 1.2 % were capable of producing cereulide, whereas of the 144 isolates originating from foods 24 % were cereulide producers. Cereulide was detected by two methods: by its toxicity towards mammalian cells (sperm assay) and by its peculiar chemical structure using liquid-chromatograph-mass spectrometry equipment.

B. cereus is known as one of the most frequent bacteria occurring in food. Most foods contain more than one kind of *B. cereus*. When randomly selected 100 isolates of *B. cereus* from commercial infant foods (dry formulas) were tested, 11% of these produced cereulide. Considering a frequent content of 10^3 to 10^4 cfu (colony forming units) of *B. cereus* per gram of infant food formula (dry), it appears likely that most servings (200 ml, 30 g of the powder reconstituted with water) may contain cereulide producers. When a reconstituted infant formula was inoculated with $>10^5$ cfu of cereulide producing *B. cereus* per ml and left at room temperature, cereulide accumulated to food poisoning levels (> 0.1 mg of cereulide per serving) within 24 hours. Paradoxically, the amount of cereulide (per g of food) increased 10 to 50 fold when the food was diluted 4 - 15 fold with water. The amount of the produced cereulide strongly depended on the composition of the formula: most toxin was formed in formulas with cereals mixed with milk, and least toxin in formulas based on milk only.

In spite of the aggressive cleaning practices executed by the modern dairy industry, certain genotypes of *B. cereus* appear to colonise the silos tanks. In this thesis it was found that the *B. cereus* isolates from dairies possessed spores with properties that make them resistant towards cleaning: long survival in hot water with 1% sodium hydroxide (pH > 13), efficient adherence to steel and to many other surfaces, ability to germinate at + 8 ºC and to form biofilms in milk. Among the dairy silo isolates were cereulide producers, but these were low in frequency (1.1 %).

In this thesis it was shown that cereulide producing *B. cereus* was capable of inhibiting the growth of cereulide non-producing *B. cereus* occurring in the same food. This phenomenon, called antagonism, has long been known to exist between *B. cereus* and other microbial species, e.g. various species of *Bacillus,* gram-negative bacteria and plant pathogenic fungi. In this thesis intra-species antagonism of *B. cereus* was shown for the first time. This "brother-killing" did not depend on the cereulide molecule: also some of the cereulide non-producers were potent antagonists. Interestingly, the antagonistic clades were most frequently found in isolates from food implicated with human illness. The antagonistic property was therefore proposed in this thesis as a novel virulence factor that increases the human morbidity of the species *B. cereus*, in particular of the cereulide producers.

1. REVIEW OF THE LITERATURE

1.1. Taxonomic view on *B. cereus*

The genus *Bacillus* is a large and diverse group of bacteria belonging to the family *Bacillaceae,* Phylum Firmicutes. The species in this genus are aerobic or facultatively anaerobic, endospore forming, rod shaped gram positive bacteria widely distributed in nature, especially in the soil (Harwood, 1989). *B. cereus* group known as the causative agents of food-borne illness belongs to Group I of the genus *Bacillus* (Gibson and Gordon, 1974). Other species in the genus *Bacillus* including *B. subtilis* (Kramer and Gilbert, 1989; Shinagawa, 1990), *B. licheniformis* (Salkinoja-Salonen et al., 1999), *B. thuringiensis* (Jackson et al., 1995; Beattie and Williams, 1999) and *B. pumilus* (From et al., 2007) are increasingly recognized as food poisoning agents.

1.1.1. *B. cereus* group

The *B. cereus* group comprises six recognised species, *B. cereus*, *B. anthracis*, *B. thuringiensis, B. mycoides*, *B. pseudomycoides*, *B. weihenstephanensis* (Gordon et al., 1973; Lechner et al., 1998; Granum 2002; Jensen et al., 2003; Stenfors Arnesen et al., 2008). In general, species of the *B. cereus* group have a low G+C content of DNA (35%) (Ravel and Fraser, 2005), hydrolyze lecithin and do not ferment mannitol to acid (Parry et al., 1983; Fritze, 2004). *B. cereus*, *B. thuringiensis* and *B. anthracis* are members of a single species *B. cereus sensu lato.* They are genetically closely related based on genome sequence data both in the gene content and in synteny (Helgason et al., 2000; Rasko et al., 2004; Didelot et al., 2009) with 99% similarity of the 16S rRNA gene sequence (Helgason et al., 2000; Kolstø et al., 2002). These three species differ in virulence, which is encoded by genes located on plasmids recognized as mobile genetic elements (Van der Auwera et al., 2007). These include the *cry* gene encoding δ-endotoxins of *B. thuringiensis*, pXO1 plasmid carrying genes for the anthrax toxin complex and the pXO2 encoding the poly-γ-D-glutamic acid capsule of *B. anthracis* as well as the positive regulator of the virulence factor AtxA located on pXO1 (Stenfors Arnesen et al., 2008). The *B. cereus* emetic toxin genes (*ces*) are also present on a large plasmid (Hoton et al., 2005; Ehling-Schulz et al., 2006; Rasko et al., 2007).

B. anthracis causes the serious human disease anthrax (Mock & Fouet, 2001). The species *B. thuringiensis* is an insect pathogen which produces insecticidal δ-endotoxins during sporulation and is commercially used for crop protection (Aronson & Shai, 2001).

The species *B. mycoides* and *B. pseudomycoides* are phenotypically distinguishable from the species *B. cereus sensu stricto* by their rhizoidal colony shape and whole cell fatty acid composition (Nakamura, 1998). *B. weihenstephanensis* is the psychrotolerant species within the *B. cereus* group, characterized by the ability to grow aerobically at 7 °C or lower in agitated liquid culture but not at 43 °C, possessing a signature sequences in the major cold shock gene *cspA* and in the 16 rDNA sequence (Lechner et al., 1998).

1.1.2. The species *B. cereus* (*sensu stricto*)

The species *B. cereus* is a gram positive rod-shaped bacterium generally 0.9 to 1.2 μm by 2 to 4 μm. The spore is

ellipsoidal, central or paracentral, rarely distending the sporangia (Gordon et al., 1973). *B. cereus* is widely distributed in the habitats like soil (Vilain et al., 2006, Jensen et al., 2003), water (Østensvik et al., 2004; Jensen et al., 2003), foods (Stenfors Arnesen et al., 2008) as well as the mammalian and insect guts (Jensen et al., 2003). *B. cereus* species grows from 4 to 55 °C (Roberts et al., 1996) at pH of 4.3 to 9.3 and requires a minimum water activity (a_w) of 0.93 (Forsythe, 2000). Three amino acids L-valine, L-leucine and L-threonine, are essential to *B. cereus* growth (Agata et al., 1999). Its growth is inhibited by lactoferrin at a concentration of 1000 µg/ml (Sato et al., 1999). *B. cereus* species have phenotypes differentiating it from the other members of the *B. cereus* groups: Cells of *B. cereus* (*sensu stricto*) are motile, do not have parasporal crystals, are resistant to penicillin and to gamma phage (Gordon, 1973; Granum, 2007; Apetroaie et al., 2005).

1.2. *B. cereus* spore

1.2.1. Ultrastructure of the *Bacillus* spore

All *Bacillus* species can form heat stable endospores (Harwood, 1989; Henriques and Moran, 2007). The bacterial endospore is a resting, dormant, tough, non reproductive structure and it is the most resistant living structure known (Atrih and Foster. 1999). Endospores formed by *bacillus* and related aerobic endospore-forming Firmicutes are a strategy to survive during unfavourable conditions.

The structures of the mature spore are: 1. The core which is the analog of the vegetative cell protoplast as it contains DNA, ribosomes, tRNA and a high concentration of dipicolinic acid (DPA) and of Ca^{+2} (Setlow, 2006). It contains only 25 – 50% of water, i.e. the content of free water is extremely low such that the macromolecular movement is greatly restricted (Cowan et al., 2003). 2. The germ cell wall is composed of

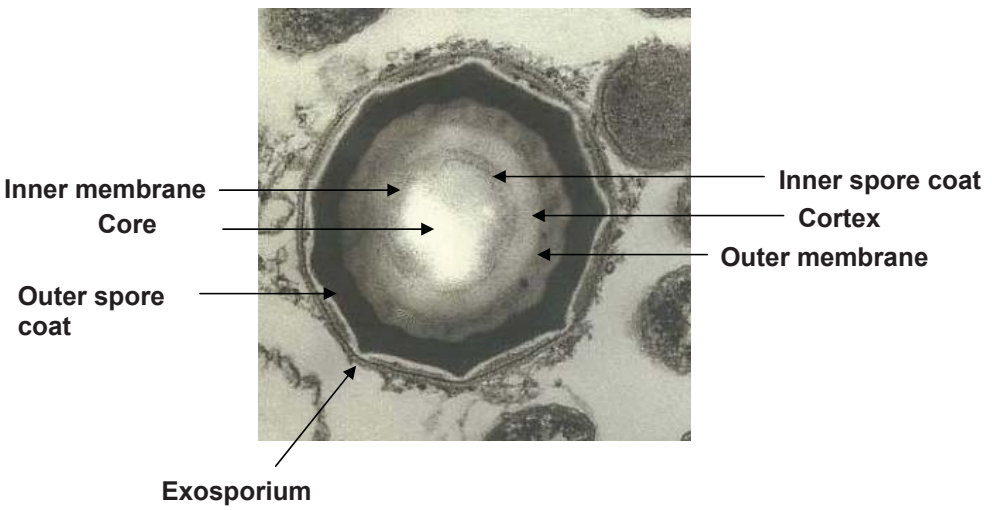

Figure 1. Transmission electron micrograph of a sporulating culture of the emetic *B. cereus* strain F4810/72. The culture was grown for 10 days on tryptic soy agar at 22 °C. Courtesy of Maria A. Andersson. The spore parts are named as described in F. Mayer (1999).

Table 1. Surface characteristics reported for *B. cereus* spores. The studied strains were the type strain ATCC 14579^T, one dairy isolate, 3 isolates from patients with diarrhoeal food poisoning and 2 strains isolated from the environment (Tauveron et al., 2006).

Properties	**Range**
Length of exosporium	2-3.2 μm
Number of appendages	1-23 per spore
Length of appendages	0.6-2 μm
Length of the hair like filaments (nap) of glycoprotein	27-35 nm

peptidoglycan identical to that of the vegetative cell (Setlow, 2006). 3. The cortex consists of a peptidoglycan which is different from the vegetative cell peptidoglycan (Atrih and Foster 1999). The cortex is essential for the formation of a dormant spore and for the reduction of its water content (Andersson, 1998). 4. The spore coat complex consisting of several layers of different proteins, mostly spore-specific and important for resistance towards chemicals and lytic enzymes (Setlow, 2006). 5. The exosporium which is a loose fitting balloon-like structure (Yan et al., 2007). The *B. cereus* exosporium contains more than 20 proteins (Steichen et al., 2003; Todd at al., 2003), amino and neutral polysaccharides, lipids and ash (Matz et al., 1970). Alanine racemase protein is a major component of the exosporium of *B. cereus* spores (Yan et al., 2007). It converts reversibly L-alanine to D-alanine (Steichen et al., 2003; Todd et al., 2003). The structures of the spores of the *B. cereus* emetic strain F4810/72 are shown in Figure 1.

The exosporium of the *Bacillus* spores is surrounded by a hair like external protein layer. It has been a suggested that these proteins are tightly absorbed on spore surface after the cell lyses or are included between the coat and the exosporium (Charlton et al., 1999; Todd et al., 2003). *B. cereus* spores are covered with appendages not present in many other *bacillus* species (Hachisuka et al., 1984). The appendages consist of protein as the main part together with a small amount of carbohydrate and lipid (Kozuka and Tochikubo 1985). There was a large difference in the protein profiles of the appendages of different strains of *B. cereus* (Stalheim and Granum, 2001) and a variation in the surface characteristics of *B. cereus* spores between strains (Andersson and Rönner, 1998; Husmark and Rönner, 1990; Hachisuka et al., 1984; Tauveron et al., 2006) (Table 1).

1.2.2. Properties of the *Bacillus* spores and those of *B. cereus* spores

Several properties reported for the spores of *B. cereus* make them a problem for the food industry. *B. cereus* spores are highly resistant to adverse conditions such as heat, dehydration, desiccation, radiation, disinfectants and cleaning agents (Table 2). The spores of *B. cereus* are hydrophobic and adhere to the processing equipment and subsequently form biofilm (Andersson et al., 1995, Peng et al., 2001). The resistance properties reported for the spores of *B. cereus* are also a problem for human health. *B. cereus* spores are highly resistant to acidity in a range of media simulating the conditions in the human stomach after food ingestion. The decrease in the spore counts was less than 1.5 log CFU ml $^{-1}$ after 6 h of incubation at pH 1 and 1.5 (Clavel et al., 2004).

The conditions prevailing during the sporulation: the temperature (Gonzales et al., 1999) and the composition of the sporulation medium (De Vries et al., 2005) affect the properties of the formed spores. The heat resistance of the *B. cereus* spores increases with the increase in sporulation temperature (Gonzalez et al., 1999). *B. cereus* spores showed higher survival at 90 °C when the spores were produced at 37 °C as compared to 15-20 °C (Gounina-Allouane et al., 2008).

Spores of *B. cereus* have extreme metabolic dormancy with respiratory activity of low as 10^{-4} of the maximum rate for vegetative cells metabolizing substrate (Andersson, 1998).

Several components are important for the resistance properties of the spores. Dipicolinic acid (pyridine-2, 6-dicarboxylic acid) is responsible for the reduction of the spore core water content during sporulation and for the UV photochemistry of the spore DNA. This molecule comprises ~5 to 20% of the dry weight of *Bacillus* spores. It is chelated with divalent cations, mainly Ca^{+2} (Setlow 2007). The small acid-soluble proteins (SASP) in the spore core play an important role of spore resistance. SASP proteins (α, β) represent 5-10 % of the total core protein which is sufficient to saturate and protect the spore DNA (Setlow and Setlow 1995, Setlow 2007) especially against UV radiation. SASP also play a role in the osmoresistance of spores (Ruzal et al., 1994; Tovar-Rojo et al., 2003).

Table 2. Resistance properties of *Bacillus* spores

Property	Due to
Resistance to heat	
wet heat	low water content of spores. mineral ions. stability of spore proteins. saturation of the spore DNA with α/β type SASP.
dry heat	saturation of the spore DNA with α/β type SASP.
Resistance to radiation	
γ-radiation	low core water content.
UV radiation	photochemistry of the DNA in spores, the spore photoproducts (SP) generated in the spores upon exposure to irradiation are less lethal than other photoproducts and are repaired in the first minute of spore outgrowth. low water content in the spore core. high level of DPA in the spore core. binding of α/β SASP to spore DNA.
Resistance to disinfectants (chlorine dioxide, hypochlorite, ozone, peroxynitrite, hydrogen peroxide)	spore coat proteins detoxifying the disinfecting chemicals (e.g CotA laccase protects against hydrogen peroxide) impermeability of the spore inner membrane saturation of DNA with α/β type SASP DNA repair mechanism.
Resistance to lysozyme	not yet known whether the outer coat functions are the main barrier against lysozyme or if the inner coat layer is essential for the resistance to lysozyme.

Compiled from Henriques and Moran. 2007; Setlow 2006

1.2.3. Sporulation and germination of *Bacillus* spores

Sporulation involves asymmetric cell division with a copy of the genome partitioned into each of the sister cells. The smaller cell develops into the mature endospore and the mother cell contributes to the differentiation process of the endospore and then autolyses releasing the mature spore into the environment (details of the sporulation process reviewed by Henriques and Moran 2007). It takes approximately 6 h for the process of spore formation of *B. cereus* to complete (Henriques and Moran 2007).

Germination is a non-log-linear event. Some spores form vegetative cells within 2 hours, others only after many hours or even days. For 12 *B. cereus* strains tested, 2 out of the 10 strains did not germinate and the maximum spore germination was obtained after 100 min with no additional germination was observed up to 160-200 min (Broussolle et al., 2008). Germination occurs without need for synthesising any new macromolecules and all the needs are present in the mature dormant spores (Moir 2006).

In the process of germination, substances acting as germinants permeate the outer coat and cortex layers of the spores and interact with receptors located in the inner spore membrane (Hudson et al., 2001; Paidhungat and Setlow 2001; Moir 2006). Then compounds such as monovalent cations (H^+, Na^+, K^+), divalent cations (Ca^{+2}, Mg^{+2}, Mn^{+2}) and DPA are released from the spore core (Moir, 2006). The germ cell wall becomes the bacterial cell wall when the spore germinates. The release of Ca-DPA triggers the hydrolysis of the spore´s peptidoglycan cortex by activating the cortex lytic enzymes (Moir, 2006). Hydrolysis of the peptidoglycan is required for germination and outgrowth of the spores (Atrih et al.,1998). The spore core rapidly takes up water so that the core water content rises to that in the protoplast of growing cells and the macromolecular motion and enzyme activity in the core are restored. Factors reported to affect the germination of *B. cereus* spores are compiled in Table 3.

In *B. cereus* L-alanine and the purine ribonucleoside inosine are effective germination-promoting compounds (Gounina-Allouance et al., 2008) and D- alanine is an effective inhibitor of L-alanine-induced germination. The most rapid germination of *B. cereus* spores was observed in a mixture of 0.1 mM L-alanine and 0.1 mM inosine. *B. cereus* spores failed to germinate in minimum salts medium with glucose plus yeast extract in 0.1 mM inosine (Warren and Gould 1968). Other germinants have also been identified, like L-phenylalanine, L-glutamine, a mixture of L-asparagine, glucose, fructose and K^+. Some mammalian cells like Caco-2 cells were reported to induce germination of enterotoxigenic *B. cereus* spores whereas HEp-2 cells did not trigger germination (Wijnands et al., 2007).

A number of germinantion receptors are present in the spores of *B. cereus*. *B. cereus* type strain ATCC14579 spore may contain seven functional receptors (Hornstra et al., 2006). *ger*P- encoded protein of *B. cereus* is believed to be important in establishing a coat that is permeable to germinants (for details see Moir, 2006). Variability of response to inosine or to L-alanine was observed between spores of *B. cereus*. Some strains can germinate at low germinant concentration (i.e. 0.05 mmol L^{-1}) (Broussolle et al., 2008).

Mild preheating activates spores to germinate, in the presence of germination permissive environment. Optimal heating

Table 3. Factors promoting or inhibiting germination of *B. cereus* spores

Factor	Effects	Reference
Heat activation	Heat activation at 75 °C for 30 min is the optimum temperature which allows a rapid rise in germination in L-alanine + inosine or L-alanine +O-carbamyl-D-serine medium.	Warren and Gould 1968
pH of the medium for germination	The effect of pH on germination depends on the germinants: In inosine (1mmol L^{-1}) *B. cereus* spore germination decreased with a decreasing pH from 7.5 to 3.8. Some strains showed no germination at pH 6.8 and very low or no germination at pH 3.8 for all strains. In L- alanine (100 mmol L^{-1}) *B. cereus* spore germination decreased with a pH decreasing from 7.5 to 3.8.	Broussolle et al., 2008
NaCl	In L-alanine, most of the *B. cereus* spores germinate less efficiently with the increase of NaCl from 0.06 % to 5 %	Broussolle et al., 2008
Sporulation temperature	The spores of *B. cereus* produced at 15 °C and 20 °C showed a higher germination capacity in response to inosine at concentrations between 0.01 and 10 mmol $L^{-1}$and L-alanine at concentrations between 1 and 100 mmol L^{-1} than the spores produced at 37 °C.	Gounina-Allouane et al., 2008
Composition of the sporulation medium	Germination response enhanced by increasing the expression of the *B. cereus ger* operon when *B. cereus* spores were produced in nutrient rich medium (30 mM amino acid and 10 mM glucose) compared to a medium containing low amounts of nutrients (14 mM amino acid and no glucose).	Hornstra et al., 2006
Bacteriostatic agent	Bovicin HC5 is a small spore forming peptide produced by *Streptococcus bovis* HC5. In a concentration of 80 AU ml^{-1} bovicin HC5 reduced the outgrowth of *B. cereus* spores.	De Carvalho et al., 2007

AU: one arbitrary unit was defined as the reciprocal of the highest dilution that showed a zone of inhibition against *L. lactis* ATCC 19435 with at least 5 mm in diameter.

temperature depends on the sporulation temperature. *B. cereus* spores that were formed at 37 ºC require 80-90 ºC heat shock for activation, whereas those formed at room temperature, need only heat shock of 70-75 º C (Becker et al., 2005). Gamma-radiation, reducing agents such as thioglycolate or mercaptoethanol and oxidizing agents also may activate the spores (Andersson 1998).

1.3. Diversity of *B. cereus*

B. cereus is widespread in the environment and possesses a wide range of habitats. The diversity of *B. cereus* ranges from strains used as probiotics to strains that produce virulence factors causing serious disease. Most *B. cereus* strains produce one or several heat labile protein enterotoxins but some strains produce the

heat stable toxin, cereulide. Studies of the intraspecies diversity of *B. cereus* by genetic [M13-PCR, random amplification of polymorphic DNA (RAPD), multilocus sequence typing (MLST), ribotyping] and phenetic [Fourier transform infrared, (FTIR), protein profiling and biochemical typing assays] have shown that *B. cereus* strains other than the cereulide producing strains, have a high degree of heterogeneity (Ehling-Schulz et al., 2005a; Vassileva et al., 2007; Svensson et al., 2004; Andersson et al., 1999; Pirttijärvi et al., 1999; Guinebretière et al., 2008). *B. cereus* isolates from the dairy production chain, food, soil and other environments have a higher degree of heterogeneity as compared to clinical isolates (Helgason et al., 2000; Ehling-Schulz et al., 2005a).

Cereulide producing *B. cereus* was initially suggested to represent a specific class of *B. cereus* (Agata et al., 1996) because this class deviated in several traits from the cereulide non producers: weak hemolysis on sheep blood agar, negative for hydrolysis of starch and for fermentation of salicin, negative for tyrosine decomposition but positive for lecithinase (Agata et al., 1996, Shinagawa 1993, Pirttijärvi et al., 1999, Andersson et al., 2004) and possessing specific ribopatterns (Pirttijärvi et al., 1999). Several genotype properties grouped the cereulide producing isolates (Ehling-Schulz et al., 2005a; Priest et al., 2004, Ash and Collins, 1992; Vassileva et al., 2007). It was proposed that these belong to a virulent clone of *B. cereus* recently emerged within the *Bacillus* population (Ehling-Schulz et al., 2005a; Priest et al., 2004, Ash and Collins, 1992; Vassileva et al., 2007). However, phenotype and genotype diversity exist even within the cereulide producer of *B. cereus*. Apetroaie et al. (2005) found that some cereulide producing *B. cereus* were weakly haemolytic while others were nonhemolytic on sheep blood agar, positive or negative for tyrosine decomposition, positive or negative for lecithinase, exhibited different ribopatterns and showed variation in the housekeeping gene adk. Furthermore, a new phylogenetic cluster was found among cereulide producing strains of *B. cereus* using multilocus sequence typing (Vassileva et al., 2007; Hoton et al., 2009).

1.4. Interactions of *B. cereus* with living and nonliving environment

1.4.1. Adhesion of *B. cereus* to nonliving and living surfaces

B. cereus spores are the most adhesive and hydrophobic among *Bacillus* spp. spores (Rönner et al., 1990). Spores of the *B. cereus* type strain adhere nonspecifically to several types of industrially used materials (Hornstra et al., 2007). The adhesion of *B. cereus* spores to non living surface is due to three characteristics: The higher hydrophobicity of the spore surface; the low negative spore surface charge and the presence of appendages on the spore surface (Rönner et al., 1990; Tauveron et al., 2006).

B. cereus spores were found to adhere to human cells, shown for Caco-2 and HEp-2 (A. Andersson et al., 1998; Wijnands et al., 2007) indicating that ingested spores may adhere the intestinal epithelium (Granum, 2007). The spore coat associated proteins are involved in the adhesion of *B. cereus* spores to Caco-2 cells (Sánchez et al., 2009). The adhesion of enterotoxin producing vegetative *B. cereus* cells to Caco-2 cells may contribute to the severity and persistence of *B. cereus* causing food poisoning (A. Andersson et al., 1998).

1.4.2. Antagonistic property of *B. cereus* towards microorganisms

The antagonistic interaction between bacteria was described early in 1877 when Pasteur and Joubert noticed that some *E. coli* strains interfered with the growth of *B. anthracis* present in infected animals (cited by Oscáriz and Pisabarro 2001). *B. cereus* produces bacteriocins with antagonistic action towards other organisms. E.g. zwittermicin (ZmA) produced by *B. cereus* strain UW85 has a broad spectrum of activity towards plant pathogenic fungi and bacteria (Stabb et al., 1994; Handelsman et al., 1990; Kevany et al., 2009). Cerein 7A is a bacteriocin produced by *B. cereus* Bc7. It is 3949 Da in size, sensitive to proteolytic enzymes and active against gram positive but not gram negative bacteria (Oscáriz et al., 1999). It is produced at the end of the exponential growth phase but before sporulation and it is a membrane active compound which is highly hydrophobic (Oscáriz and Pisabarro 2000).

1.4.3. *B. cereus* as a probiotic

Probiotics are live organisms that, when administered in adequate amounts, confer a health benefit on the host (Quoted from: FAO/WHO 2002). They improve the efficiency of feed, protect against infectious disease (Zani et al., 1998), enhance the host immune responses or inhibit tumor growth in animal models (cited from De Los Santos et al., 2005).

Of the 305 species in the genus *Bacillus* (DSMZ web site) *B. cereus* belongs to the few that are used as probiotics. Probiotic *B. cereus* are used as animal feed supplements (Zani, et al., 1998; Alexopoulos et al., 2001; De Los Santos et al., 2005; Taras et al., 2005; Lodemann et al., 2008; Schierack et al., 2009), for aquaculture (Ravi et al., 2007) and for human (Duc et al., 2004, Sánchez et al., 2009) (Table 4).

The interest of using probiotics as animal feed supplements has increased in recent years due to the increased number of antibiotics banned from animal use as growth promoters in the European Union in 2006 (Council of the European Union, 2003). Spore forming bacteria are technically easy to use as probiotics for animal feed because the spores survive storage at ambient temperature as well as heating during the making of animal feeds. *B. cereus var. toyoi* is licensed by the European Food Safety Authority (EFSA) to be used as animal feed supplement.

B. cereus Bactinsubtil, *B. cereus* var. vietnami (Subtyl) and *B. cereus* (Biosubtyl ^{DL}) are three commercially available probiotic strains (Hoa et al., 2000) for human use. These strains were reported to have several advantages for use as a probiotic: they persist in the gastrointestinal tract and the spores are immunogenic (Duc et al., 2004). However, it was found that Biosubtyl ^{DL} and Bactinsubtil produce the HbL enterotoxin and the nonhaemolytic enterotoxin Nhe are produced by Biosubtyl ^{DL} and Subtyl indicating that they are not necessarily safe for human use (Duc et al., 2004). *B. cereus* IP5832 (Bactinsubtil) has been used as a human probiotic for many years but it is now banned in Europe. Nevertheless, it is still sold in other countries all over the world (Duc et al., 2004; Sánchez et al., 2009).

The uses of *B. cereus* as probiotics raise a big question of human safety. Reports of infection connected to the probiotic consumption include members of the genus *Bacillus* and production of enterotoxin has been reported. The single dose of spores probiotics is up to 10^9 spores/g or 10^9 spores/ml (cited from

Table 4. Reported uses of *B. cereus* as probiotic

Probiotics	Comments	Quoted by
For human use		
Bactisubtil from (*B. cereus* IP 5832, Marion Merrell S.A. Bourgoin-Jallieu, France). Biosubtyl [DL] (National Institute of Vaccines and Biological Substances, Da Lat, Vietnam). Subtyl (*B. cereus* var.vietnami) (Pharmaceutical Factory 24, Ho Chi Minh City, Vietnam).	Used for oral bacteriotherapy and bacterioprophylaxis of gastrointestinal disorders in humans.	Hoa et al., 2000; Duc et al., 2004
For veterinary use (animal feeds)		
B. cereus DQ915582	This strain was isolated from a marine sediment and was found to be effective in inhibiting the larval pathogens of shrimps.	Ravi et al., 2007
B. cereus Cen Biot	Reported to reduce the prevalence of diarrhoea and to improve feed conversion and weight gain in pigs.	Zani et al., 1998
B. cereus var. toyoi (Toyocerin®)	Licensed by the European Food Safety Authority (EFSA) to be used as a supplement to animal feed. Does not secrete toxin. Improved feed efficiency in broilers. Induces an increase of lactobacilli in the duodenum or in the caecum and reduction of *E. coli* or of *enterococci* in the intestine or faeces. Decreases the incidence of diarrhoea and the mortality in piglets.	Taras et al., 2005; De Los Santos et al., 2005; Lodemann et al., 2008
Paciflor (*B. cereus* CIP 5832)	Shown to be beneficial for the survival and growth of piglets. Withdrawn from production due to the presence of Nhe enterotoxin.	Alexopoulos et al., 2001, Duc et al., 2004

Duc et al., 2004). This is higher than the infective dose of the pathogenic *B. cereus* for diarrhea (10^5- 10^7 spores per consumption). There is at least one report on vegetative cells and spores of *Bacilli* detected in mesenteric lymph nodes and spleen of a mouse after giving *bacillus* spores as a probiotic (Spinosa et al., 2000). Furthermore, the species *B. cereus* is closely related to *B. anthracis* (Didelot et al., 2009). The emetic toxin producing strains of *B. cereus* and the *B. anthracis* have their pathogenic determinants located on plasmids. A case in which *B. cereus* acquired toxin genes from one of the anthracis plasmids (Hoffmaster et

al., 2004) proves that plasmid exchange between these close species and strains occurs in nature. Thus, using *B. cereus* as a probiotic can be a problematic and needs special attention. Lack of virulence traits in any specific strains of *B. cereus* should be checked carefully before any recommendation for the use as a probiotic.

1.5. Human virulence

1.5.1. *B. cereus* as a human pathogen

B. cereus belongs to the Hazard group 2 organisms as defined in the European legislation (European Commission, 1993). In 1950 *B. cereus* was first recognized to cause food borne illness. S. Hauge isolated in Norway in 1955 *B. cereus*, inoculated it into a sterile vanilla sauce to 10^6 cfu per ml and consumed it (Hauge, 1955). Severe abdominal pain, diarrhoea and rectal tenesmus followed 13 h after consuming of the contaminated sauce. This allowed Hauge to describe *B. cereus* as a causative agent of food poisoning (Hauge, 1955).

B. cereus is the aetiological agent of two major types of foodborne illness, the emetic syndrome and the diarrhoeal syndrome. The diarrhoeal syndrome involves diarrhea and abdominal pain initiating 8 h to 16 h after the ingestion of *B. cereus* in food, followed by toxin production in the small intestine (Kramer and Gilbert, 1989; Granum and Lund 1997). The emetic syndrome is characterized by nausea and vomiting 1 h to 5 h after the ingestion of the heat-stable emetic toxin cereulide, preformed in the food (Granum and Lund, 1997; Beecher, 2002). The dose of the emetic toxin, cereulide, causing acute serious illness in human was reported as ≤ 8 μg of cereulide per kg of body weight (Jääskeläinen et al., 2003b). The outcome of emetic food poisoning may be serious and fatal (Takabe and Oya, 1976, Mahler et al., 1997, Pirhonen et al., 2005, Dierick et al., 2005, Pósfay-Barbe et al., 2008) (Table 5). In addition to cereulide, the second toxin of *B. cereus* with known connection to fatal poisoning in human is cytotoxin K (Lund et al., 2000).

Health authorities have not declared *B. cereus* food poisoning as a reportable disease in any country and therefore its incidence is underreported. Food poisoning due to *B. cereus* may be misdiagnosed due to the similarity of symptoms with other types of food poisoning, for example *Staphylococcus aureus* intoxication and *Clostridium perfringens* (Shinagawa, 1990). Patients with diarrhoeal or emetic syndromes usually do not seek medical care due to the short duration of the symptoms (< 24 h) (Granum, 2007) which adds to underestimation of the reported rate of the illness caused by *B. cereus*. Furthermore *B. cereus* may have been involved in in the unclarified outbreaks involving heated food from which no viable bacteria could be isolated.

In Finland the causative agent of 19 outbreaks out of 55 in year 2005 remained unidentified (Niskanen et al., 2007). In most countries (for instance UK, NL and USA) > 90% of the registered outbreaks remain unclear, i.e. the causative agent is not identified (Pirhonen, 2009). In Finland this percentage is exceptionally low (40%, i.e. >50% of the registered outbreaks are clarified).

Several other virulence factors have been found in *B. cereus* contributing to the virulence of this species: phospholipase-C which gives the capability to hydrolyse lecithin, the major component of the mammalian cell membrane (Gilmore et al., 1989), cereolysin AB and haemolysin BL which contribute to the haemolytic activity of *B. cereus* (Beecher, 2002) and

tyrosin monooxygenase responsible for the formation of melanins protecting the cells and spores of *B. cereus* from various environmental factors (Claus and Decker, 2006). Beecher et al. 1995 reported that hemolysin BL produced by *B. cereus* contributes to the virulence for *B. cereus* endophthalmitis.

B. cereus has been implicated in a variety of local and systemic infections in immunocompetent and immunocompromised hosts where *B. cereus* was the only microrganisms identified: pneumonia, endocarditis, meningitis, periodontitis, osteomyelitis, wound infection, necrotizing fascilitis and myonecrosis, septicemia and infection of the central nervous system of term and preterm neonates (Hilliard et al., 2003; Miller, 1997; Kotiranta et al., 2000; Gaur et al., 2001; Schoeni and Wong 2005; Hoffmaster, et al., 2004; Sada et al., 2009; Nishikawa et al., 2009; Lebessi et al., 2009). *B. cereus* strain G9241 was isolated from the sputum and the blood of a patient with life-threatening pneumonia. These strains contained a homologue of the *B. anthracis* pXO1-encoded PA gene, pagA, but not the pXO2-encoded poly-D-glutamic acid capsule biosynthetic genes (Hoffmaster, et al., 2004).

1.5.2. Cereulide, the emetic toxin of *B. cereus*

1.5.2.1. Structure and properties of cereulide

Cereulide is a small cyclic nonribosomally synthesized dodecadepsipeptide, 1152 Da in size (Fig 2). It consists of three repeating units of two amino acid residues and two hydroxy acids (D-O-Leu, D-Ala, L-O-Val and L-Val)$_3$ (Agata et al. 1994; Isobe et al., 1995). Cereulide has been reported produced also by isolates of *B. weihenstephanensis* (Thorsen et al., 2006; Hoton et al., 2009).

The chemical structure of cereulide resembles that of valinomycin, [-D-O-Val-D-Val-L-O-Ala-L-Val-]$_3$ produced by *Streptomyces fulvissimus, Streptomyces tsusimaensis* and *Streptomyces griseus*

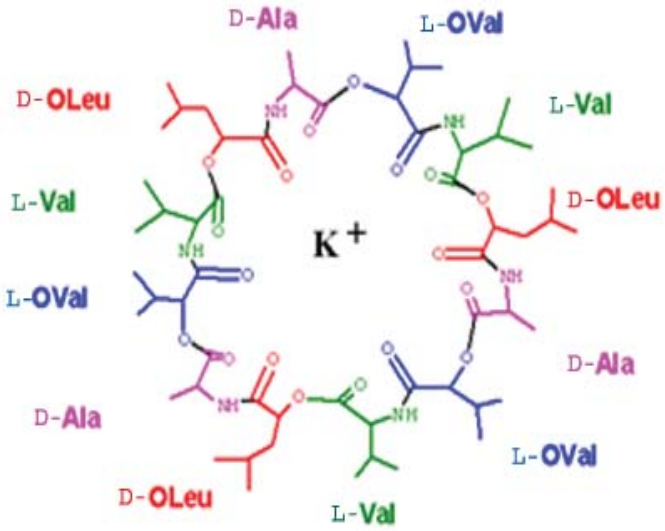

Figure 2. Structure of cereulide. Courtesy of prof. M.S. Salkinoja-Salonen and Raimo Mikkola

Table 5. Cases of *B. cereus* reported with details of emetic or emetic like food poisoning caused by this species

Country	Food involved	Description of the case	Reference
Japan	Noodle	• An outbreak with 50 affected. An eleven years old boy died with mycocardial degeneration and fatty liver. • Symptoms started 1-6 h after ingestion. • Acute gastroenteritis. • *B. cereus* was isolated from the intestine, peritoneal exudate, the cooked food and a sheet with the patient's vomit.	Takabe et al., 1976
Switzerland	reheated spaghetti and pesto	• Acute gastroenteritis to father and 17 year old son. The son died after developing a fulminant liver failure. • The symptoms started 30 min after ingestion. • Emetic *B. cereus* was isolated from the food remnants, patient ileum and colon. • Emetic toxin was detected in the remaining food, bile, plasma and intestinal content of the patient by HEp-2 cell vacuolation, HepG2 and rat liver mitochondrial assays.	Mahler et al., 1997
Norway	dried figs	• Two cases of food borne illness. The clinical symptoms and microbiological examination linked the cases with emetic toxin produced by *B. cereus*.	Hormazábal et al., 2004
Finland	reheated dish of pasta with minced meat	• Two adults with severe food poisoning involved both emesis and diarrhoea. • Emetic *B. cereus* was isolated from remnants of the food. • Toxicity of the remaining food was detected by the sperm bioassay. • Cereulide was measured in the remaining food by the LC-MS method.	Pirhonen et al., 2005; Jääskeläinen et al., 2003b
Belgium	Pasta salad	• Five children of a family, fatal for the 7 years old girl who died due to severe metabolic acidosis and liver failure. • The symptoms (vomiting) started 6 h after ingestion. • *B. cereus* was detected in the gut and the spleen of the patient.	Dierick et al., 2005
Germany	reheated rice dish	• 17 children in a day care centre. • *B. cereus* possessing the *ces* gene was detected in the remaining food using real time PCR assay with primers derived from the cereulide synthetase (*ces*) gene sequence. • Toxicity was detected by HEp-2 cytotoxicity assay in the remaining food.	Fricker et al., 2007
Germany	Cauliflower	• Single case. • *B. cereus* possessing cereulide synthesis genes was detected in the remaining food using real time PCR assay using primers derived from cereulide synthetase (*ces*) gene sequence. • Toxicity was detected by HEp-2 cytotoxicity assay in the remaining food.	Fricker et al., 2007
Japan	sweet red bean paste covered with sticky rice cake	• 346 persons suffered from food poisoning caused by cereulide.	Okahisa et al., 2008
Geneva	reheated pasta with sauce	• A 9 years old girl developed fulminant hepatitis, renal and pancreatic insufficiency and prolonged seizures. • The symptoms appeared 2 h after ingestion. • *B. cereus* possessing cereulide synthesis genes was detected in the remaining food using PCR with primers specific for the *ces* gene. • No toxicity assay was carried out.	Pósfay-Barbe et al., 2008

(Agata et al., 1994, Andersson et al., 1998b; Mikkola et al., 1999, Makarasen et al, 2009). Cereulide is a potassium ion selective ionophore, a highly lipophilic molecule (log K_{ow} ~6.0, Teplova et al., 2006) and therefore likely to be efficiently absorbed from the gut into the blood stream (Paananen et al., 2002). Cereulide does not haemolyse rabbit or sheep erythrocytes and has no phospholipase C or any other enzymic activity (Shinagawa at al., 1995). Cereulide is highly heat stable (121 °C for 90 min), stable upon exposure to pH from 2 to 11 and to the proteolytic activity of pepsin and trypsin (Table 6) therefore it may be found in food even when no live *B. cereus* is found due to heating of food (Pirhonen et al., 2005; Jääskeläinen et al., 2003b).

Cereulide begins to accumulate in the *B. cereus* cultures in the stationary phase of the growth independent of sporulation (Häggblom et al., 2002). No cereulide was produced by *B. cereus* in TSB at 10 ± 1 ºC (Häggblom et al., 2002) or by plate grown cells at ≤ 8 ºC (Thorsen et al., 2009).

1.5.2.2. Toxic mechanisms and detection of cereulide

In the 1970's, *B. cereus* was recognized as the causative agent of a specific type of *B.* cereus foodpoisoning which is the emetic syndrome. Rhesus monkey was used as the experimental animal to demonstrate that the heat stable toxin produced by *B. cereus* isolates from emetic outbreaks was associated with the emetic syndrome (Melling et al., 1976). The monkey feeding test and ligated rabbit ileal loop (LRIL) methods were used with success to show that the factors responsible for the vomiting and the diarrhoeal illnesses were distinct. The LRIL and the vascular permeability reaction (VPR) methods were found to be of value for studying the diarrhoeal toxin but not the vomiting factor (Turnbull et al., 1979). Research on the emetic toxin was hampered by the fact that rodents are insensitive to the orally given toxin (Yokoyama et al., 1999). Therefore primates were needed for each test until Hughes et al. (1988) developed an in vitro assay based on the vacuolisation of the human larynx carcinoma cells (HEp-2 cells). These authors tested samples connected to food poisoning and cultured isolates connected to food poisoning in rice. They noticed that the extracts obtained from some isolates caused vacuoles in the HEp-2 cells. Sakurai et al. 1994 observed that the vacuoles formed in the HEp-2 cells were swollen mitochondria.

Agata et al. (1994) extracted and purified the factor which causes the vacuolation in HEp-2 cells from the culture supernatant of *B. cereus* strain NC7401 connected to a case of emetic syndrome food poisoning and named the toxin cereulide. Shinagawa et al., 1995 and Agata et al. (1995) found that the factor causing vacuolisation of HEp-2 cells also caused vomiting when fed to rhesus monkey (*Macaca mulatta*) and the house musk shrew (*Suncus murinus*) and concluded that this was the emetic toxin. Cereulide was chemically synthesized by Isobe et al., 1995 and shown to possess the same emetic and pathogenic activities. Andersson et al. (1998a) developed a bioassay based on loss of the motility of boar spermatozoa upon 24 h exposure to the toxin. Finlay et al. (1999) developed the metabolic staining assay MTT using as an indicator 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide, a water soluble yellow tetrazolium salt. This salt was converted to an insoluble purple formazan (MTT) in HEp-2 cells but not in cells exposed to the emetic toxin. This allowed detecting the cytotoxicity of cereulide towards the HEp-2 cells.

Table 6. Selected biochemical properties of the *B. cereus* emetic toxin (cereulide)

Properties	Condition	Reference
pH stability	pH 2 for 2 h at room temperature	Mikami et al., 1994; Melling and Capel 1978
	pH 11 for 2 h at room temperature	Shinagawa et al., 1995
	pH 12 for 2 h at room temperature	Mikami et al., 1994; Melling and Capel 1978
	pH 12 for 4 h at room temperature	Shinagawa et al., 1995
		Sakurai et al., 1994
Heat stability	stable at 121 ºC for 20 min	Mikami et al., 1994; Shinagawa et al., 1995
	stable at 126 ºC for 90 min	Melling and Capel 1978
	stable at 4 ºC for 2 months	Melling and Capel 1978
	stable at -80 °C for 24 h	Sakurai et al., 1994
Protease stability	resistant to trypsin (2 mg / ml) for 2 h at 37 °C	Mikami et al., 1994, Melling and Capel 1978
	resistant to pepsin at 500 µg / ml	Shinagawa et al., 1995
	resistant to 2% pronase (w/v), pH 7.4 in PBS at 37 °C for 24 h	Sakurai et al., 1994
	resistant to 2% trypsin (w/v), pH 8 in 0.2 M Tris-HCl buffer at 37 °C for 24 h	Sakurai et al., 1994
	resistant to proteinase K (100 µg ml^{-1}, pH 7 at 37 °C for 2 h	Andersson et al., 1998a
Inactivated at pH 9.5 by	80 min at 121 °C or 60 min at 150 °C	Rajkovic et al., 2008
Hydrophobicity	highly hydrophobic (log K_{ow} ~6.0)	Teplova et al., 2006
Solubility in		
Water	No	Agata et al., 1994
Methanol	Yes	Andersson et al., 1998a
Ethanol	Yes	This thesis (paper II)
Pentane	Yes	Häggblom et al., 2002

A quantitative chemical assay was introduced by Häggblom et al. (2002) based on liquid chromatography followed by ion trap mass spectrometry (HPLC-MS). This method allows measuring the exact contents of the molecule cereulide in the *B. cereus* biomass as well as in food or samples from environmental origins (Table 7).

A rapid sperm bioassay was developed in 2004. It allows detecting the toxicity of *B. cereus* bacterial extract in a short period of time (Andersson et al., 2004). Table 8 shows the advantages of using the rapid sperm bioassay over bioassay methods previously developed for detecting the toxicity of cereulide.

1.5.2.3. Toxicity of cereulide in whole animals

In order to determine the dose of cereulide which may cause illness, Rhesus monkey and house musk shrew have been used as the animal models. Cereulide caused emesis in the house musk shrew following an oral dose of 12.9 µg per kg body weight and 9.8 µg per kg by intraperitoneal injection. In the rhesus monkey the dose causing vomiting was ≥ 70 µg per animal (approximately 10 µg per kg body weight) (Agata et al., 1995; Shinagawa et al., 1995). Using the house musk shrews for animal feeding tests, it was found that cereulide causes emesis through the serotonin receptor 5-HT 3 which stimulates the vagus afferent nerve that enervates the stomach (Agata

Table 7. Performance of the published in vitro assays for the emetic toxin of *B. cereus* (cereulide)

Assay	Toxicity end point	Toxicity target and the outcome of the assay	Reference
Human cell lines and primary cells as the toxicity targets			
HEp-2 cells	Formation of vacuoles in the mitochondria.	Detects mitochondrial toxicity. Generates the toxicity titre of a given extract.	Mikami et al., 1994
HEp-2 cells	Loss of the mitochondrial ability to convert the yellow tetrazolium salt into an insoluble purple formazan.	Detects a functional electron transfer chain (cytochrome c-a/a3).	Finlay et al., 1999
HeLa, Paju, Calu-3, Caco-2	Loss of the mitochondrial ability to emit yellow orange fluorescene using JC-1 staining indicating loss of transmembrane potential.	Detect mitochondrial toxicity.	Jääskeläinen et al., 2003b
Animal primary cells, cell lines and organelles as the toxicity targets			
Boar spermatozoa	Loss of motility.	Detects mitochondrial toxin. Screen for cereulide producing *B. cereus* isolates. Generates the toxicity titre.	Andersson et al., 2004
Mouse Hepa-1 cells	Measurement of the increase of cell protein content	Growth of the target cells.	Andersson et al., 2007
Rat liver mitochondria (RLM)	Rapid oxygen reduction in the mitochondria.	Detects toxins uncoupling the mitochondrial respiration.	Kawamura-Sato et al., 2005
Chemical assay			
LC-MS	Quantification of the mass ions specific for cereulide.	Specifically detects cereulide in bacteria or in food. Generates quantitative result.	Jääskeläinen et al., 2003a

Table 8. Essential features of three bioassays used for detecting the toxicity caused by cereulide.

	HEp-2 cell vacuolation assay (Hughes et al., 1988)	Boar sperm assay (Andersson et al., 1998a)	Rapid sperm microassay (Andersson et al., 2004)
Cultivation time for *B. cereus* (20-30 ºC)	1 d	10 d	1 d
Quantity of *B. cereus* biomass needed	50 ml of broth culture	500 mg wet wt of plate grown cells	5 to 10 mg wet wt of plate grown biomass
Time to prepare one extract	> 2 h	12 h	15 to 30 min
Solvent used to extract the *B. cereus* biomass	Water	methanol	methanol
Extraction protocol	Autoclaving the culture and centrifugation. The cell free supernatant obtained by filtration is used for the assay	Blend the biomass into 100 ml of methanol, soak overnight, evaporate with vacuum and redissolve the residue with a small volume of methanol	200 µl of methanol added, heated in water bath for 15 min at 100 °C
Exposure time of target cells	1 d	1 to 4 d	5 to 15 min
Time needed to prepare the exposed cells for microscopy	1 h	10 min	10 min
Threshold for detection of cereulide	5-10 ng per ml of the liquid culture[a]	1 ng per exposure	0.3 ng per exposure

[a] Quoted from Constantin. 2008

et al., 1995). Repeated feeding with an emetic toxin containing culture of the *B. cereus* strain F4810/72 at 16 occasions in rhesus monkeys showed no development of tolerance to this toxin indicating that the emetic toxin is poorly antigenic in contrast to the *staphylococcocal* enterotoxins known to produce resistance in the animals (Melling & Capel 1978). Recently Yabutani et al. (2009) tried to raise antibodies against cereulide but failed because of the non immunogenic chemical character of the cereulide molecule. The effects of cereulide on several biological targets are described in Table 9.

1.5.2.3. Biosynthesis of cereulide

Many microbial peptides are non-ribosomally synthesized by peptide synthetases. In the chemical structure of cereulide peptide bonds and ester bonds alternate. The presence of D- amino acids and a cyclic structure are often found in the products of nonribosomal peptide synthetases (NRPS). Cereulide is non-ribosomally produced by a peptide synthetase (Toh et al., 2004; Horwood et al., 2004; Ehling-Schulz et al., 2005b; Yabutani et al., 2009).

The genes (*ces*) responsible for the synthesis of cereulide were identified (Toh et al., 2004; Horwood et al., 2004) and found located on a large plasmid,

Table 9. Summary of the toxic activities of the emetic toxin, cereulide

Target organisms	Observed toxic effect	Reference
Whole organism		
Emesis in primates (rhesus monkey and human), *Suncus murinus* (house musk shrew)	Emesis.	Turnbull et al., 1979; Shinagawa et al., 1995; Agata et al., 1995, Jääskeläinen et al., 2003b
Primary cells		
Human natural killer cells (NK)	Inhibition of the cytotoxicity and production of cytokines by NK cells. Swelling of mitochondria and apoptosis.	Paananen et al., 2002
Boar sperm cells	Inhibition of motility, dissipation of the mitochondrial membrane potential.	Andersson et al., 1998a, 2007
Human cell lines		
HepG2 cells	Inhibition of RNA synthesis and cell proliferation.	Andersson et al., 2007
HeLa, Caco-2, Calu 3 and Paju cells	Dissipation of the mitochondrial $\Delta\Psi_m$	Jääskeläinen et al., 2003b
HEp2 cells	Inhibition of the growth and induction of acid production.	Mikami el al., 1994
Organelles		
Human hepatocytes	Swelling of the mitochondria.	Mahler et al., 1997
Mouse liver mitochondria	Acceleration of oxygen consumption, swelling of the mitochondria.	Sakurai et al., 1994, Yokoyama et al., 1999
Fetal porcine Langerhans islets	Necrotic cell death, inhibition of insulin secretion.	Virtanen et al., 2008

HEp-2: Human laryngeal carcinoma cells; HepG2: human hepatocellular carcinoma cells; HeLa: human cervical cancer cells; Paju: human neural cells; Calu-3: human lung carcinoma cells; Caco-2: human colon carcinoma cells

pCER270 (Hoton et al., 2005; Ehling-Schulz et al., 2006; Rasko et al., 2007). The plasmid pCER270 of the strain *B. cereus* F4810/72 (Rasko et al., 2007) and that from the strain NC7401 by Yabutani et al. (2009) have been fully sequenced. The plasmid pCER270 contains 270,082 nt with a GC content of 34% and with 71% of coding region. It has 250 genes, 235 genes code for proteins and 15 are pseudo genes. (http://www.ncbi.nlm.nih.gov/sites/entrez?Db=genome&Cmd=ShowDetailView&TermToSearch=22569; access date 07/08/2009). The enzymatic machinery for the biosynthesis of cereulide consists of the ces gene cluster of approximately 24 kb i.e. 10% of the pCER270 (Ehling-Schulz et al., 2006). Sequence analysis showed that the *ces* gene cluster contains 7 coding DNA sequences (CDSs): *cesH, cesP, cesT, cesA, cesB, cesC* and *cesD*. Details of the *ces* genes are explained in Table 10. The gene cluster for cereulide biosynthesis is shown in Figure 3. *CesH* is located at the 5' end of *cesP*. *CesT* is located downstream from *cesP* while *cesA* overlaps with *cesT*. *CesC* and *cesD* are located in the 3' part of the *ces* gene cluster. To date the *ces* genes are only found in strains producing the emetic toxin and a plasmid lacking the *ces* gene has been found in strains which

Table 10. Deduced functions of the proteins encoded by the ces operon of *B. cereus* strain F4810/72 by sequence homology

Product Name	**Start (nt)**	**End (nt)**	**Total Bp**	**Length aa**	**Mass (kDa)**	**Function**	**Comments**
CesH	37886	38668	782	260	31	Hydrolase/ Acyltransferase	58 % identities to putative hydrolases /acyltransferase from *B. cereus* group members.
CesP	36140	36895	755	251	28.9	Phosphopantheteinyl transferase	32–38% identity and approx. 60% similarity to the 4'-phosphopantetheinyl transferase from *B. brevis* and *B. subtilis* involved in the nonribosomal synthesis of gramicidin S and surfactin, respectively.
CesT	35158	35871	713	237	27.6	Type II thioesterase	33% identity and 56% similarity to BacT from *B. licheniformis*. 35% identity, 53% similarity to GrsT from *B. brevis*.
CesA	25017	35141	10124	3374	385.713	Cereulide synthesase A	Activate the precursors for D-O-Leu and D-Ala
CesB	16958	25003	8045	2681	304.305	Cereulide synthesase B	Activation of a precursor of L-O-Val, activation and incorporation of L-Val.
Ces C	15917	16813	896	298		ABC transporter ATP-binding protein	May be involved in the transport of cereulide or confer self resistance towards cereulide
Ces D	15094	15900	806	268	29.859	Putative permease (putative ABC transporter; permease	May be involved in the transport of cereulide or confer self resistance towards cereulide

Quoted from http://www.ncbi.nlm.nih.gov/sites/entrez?Db=genome&Cmd=ShowDetailView&TermToSearch=22569, http://www.uniprot.org/uniprot/?query=cereulide&sort=score, access date June/25, 2009, Rasko et al., 2007, Ehling-Schulz et al., 2006

do not produce the emetic toxin (Ehling-Schulz et al., 2006). So far no study has shown transfer of the plasmid carrying the *ces* gene between species. Thorsen et al. (2006) reported the presence of *B. weihenstephanensis* isolates producing cereulide identified using LC-MS. This made other authors to suggest that the plasmid could be subject to lateral transfer among the species of *B. cereus* group (Vassileva et al., 2007).

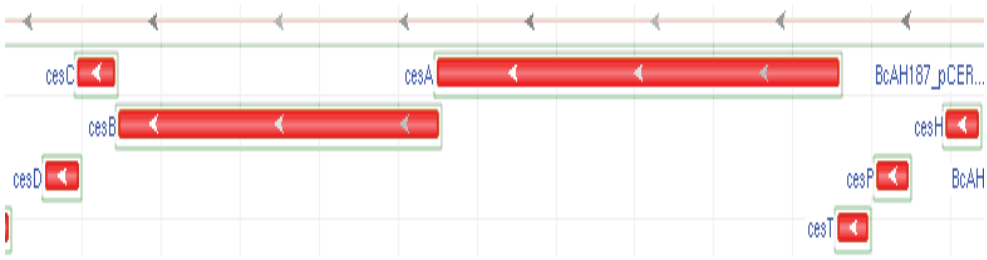

Figure 3. Gene cluster for the biosynthesis of cereulide in *B. cereus* AH187 (F4810/72) carrying the plasmid pCER270.
The figure obtained from http://www.ncbi.nlm.nih.gov/sites/entrez?Db=genome&Cmd=ShowDetailView&TermToSearch=22569, access date 25/06/2009

2. AIMS OF THE STUDY

The aim of this doctoral thesis was to understand the potential of cereulide producing *B. cereus* in causing food poisoning.

The specific aims were:

1. Assess the prevalence of cereulide producing *B. cereus* in different ecological niches (soil, water, foods, dairy farm, dairy industry)

2. Identify the conditions promoting cereulide production by *B. cereus* in food, using infant food formulas as an example.

3. Reveal the biological strategies of *B. cereus* spores enabling these to persist and propagate in food industry, using dairy silo tanks as an example.

4. Identify properties of *B. cereus* cereulide producing strains relevant for the risk of food poisoning.

3. MATERIALS AND METHODS

Table 11. Methods used in this thesis

Method	Described in	Reference
Extraction of cereulide from different materials		
Extraction of cereulide with methanol from plate grown bacteria for rapid detection	Paper I, II, III, IV, V	Andersson et al., 2004
Extraction of cereulide with pentane from food	Paper II	
Extraction of cereulide with ethanol from food	Paper II	
Biological assays for toxicity		
Inhibition of boar sperm motility	Paper I, II, III, IV, V	Andersson et al., 1998a, 2004
Chemical assay		
HPLC-MS for cereulide	Paper I, II, III, IV, V	Jääskeläinen et al., 2003a
Isolation method		
Isolation and enumeration of *B. cereus* in food	Paper II, V	ISO standard 7932, 2004
Methods for characterization of *B. cereus*		
Haemolysis *B. cereus* on blood agar	Paper V	Andersson et al., 2004
Starch hydrolysis	Paper V	Pirttijärvi et al., 1999
Ribopattern analysis	Paper II, III, V	Pirttijärvi et al., 1999
RAPD-PCR	Paper III	Nilsson et al., 1998
PCR detection of the cold shock gene in *B. cereus*	Paper III	Francis et al., 1998
Assay of antagonism	Paper V	
Methods for preparation and characterization of *B. cereus* spores		
Preparation of spores	Paper III	Magnusson et al., 2006
Adherence onto micro plates	Paper III	
Adherence onto stainless steel	Paper III	
Resistance of spores in hot alkaline or acid solutions	Paper III	
Scanning electron microscopy and methods for fluorescence detection		
Scanning fluorometry	Paper III	
Epifluorescence microscopy	Paper III	
Field emission scanning electron microscopy	Paper III	

4. RESULTS AND DISCUSSION

4.1. Strategies for isolating and identifying cereulide producers among strains and isolates of *B. cereus*

In this study, we identified the cereulide producers among the *B. cereus* strains and isolates of many origins and explored the occurrence of cereulide producing *B. cereus* in several ecological niches, the dairy production chain and in milk based infant food formulas (papers I, II, IV, V). I combined two independent methods to score cereulide production by the *B. cereus* isolates: the rapid sperm bioassay to detect the toxicity and to show that the toxin

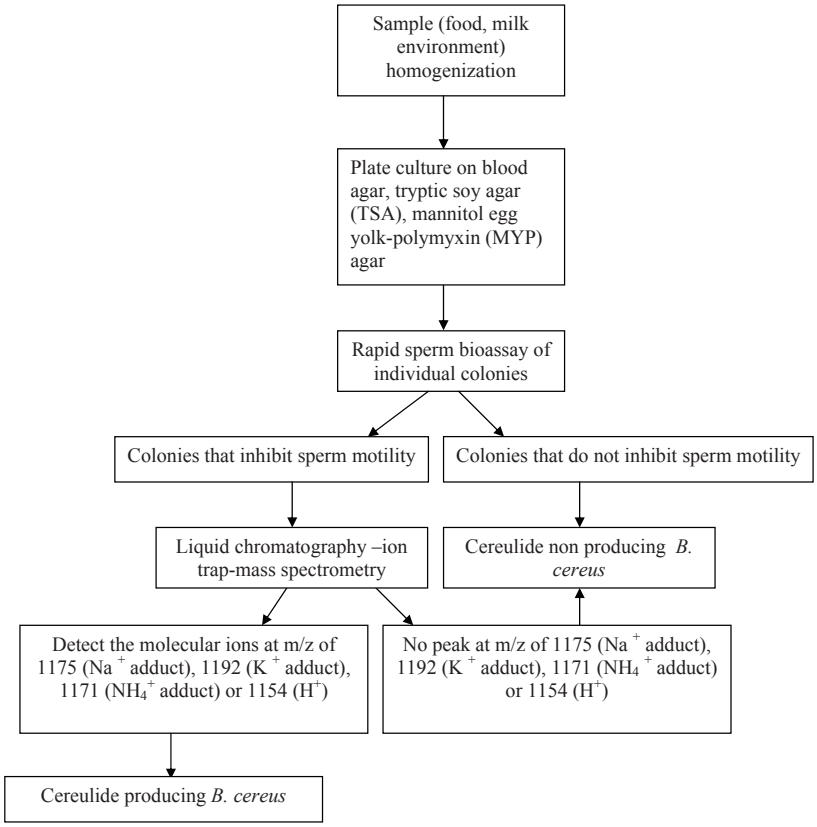

Figure 4. A flow chart for distinguishing the cereulide producing *B. cereus* isolates from the nonproducers in foods or in environmental samples. Plate grown biomass of *B. cereus* is suspended in methanol. Viable bacteria and spores are inactivated by heating this methanol suspension in a boiling water bath. This treatment also inactivates any protein toxins in the same extracts. Boar sperm cells are obtained from commercially sources. The sperm can be handled at ambient temperature with no need for special cabinets. The sperm cells are exposed to ≤ 5 vol % of the heated methanol extract or its methanol dilution (10x, 100x….) for 5-15 min at 21 °C. Cereulide inhibits the spermatozoa motility. Methanol, in the absence of cereulide, is tolerated by the sperm cells up to 5 vol % for 30 min. Outcome of the bioassay is a toxicity titre of the tested extract. The detection threshold is 0.9± 3 ng of cereulide mg^{-1} wet wt of the extracted material. Extracts that inhibit the motility of the boar sperm cells are fractionated by liquid chromatography and screened by mass spectrometry for the cereulide specific mass ions.

is in the active form, and the chemical assay (LC-MS) to specifically detect the cereulide molecules and to accurately determine the concentration of cereulide in the bacterial extracts (papers I, II, IV, V). Figure 4 describes the strategy used to isolate and identify cereulide producing *B. cereus*. The rationales behind the steps of the rapid sperm assay are explained in the legend.

4.2. Cereulide productivity of *B. cereus* isolates of geographically and temporally diverse origins

B. cereus isolates from food, environment and food borne illness representing 191 independent samples were collected from eight countries to serve as a European reference collection (EU project QLK1-CT-2001-00854). In my thesis I analysed the toxicities and cereulide contents of these isolates. The results were published in (Papers IV, V and Ehling-Schulz et al., 2005 a & b) and are compiled in Tables 12 & 13 in this thesis.

In Table 12 and 13, I divided the *B. cereus* strains and isolates into four categories based on the content of cereulide: high (>1 μg mg^{-1} of wet wt cells), medium (0.1-1 μg mg^{-1} of wet wt cells), low (0.0005-0.1 μg mg^{-1} of wet wt cells) producers and (Table 13) non producers (<0.0005 μg mg^{-1} of wet wt cells). These numbers refer to the cereulide content of *B. cereus* biomass grown for 24 h at 28 °C on tryptic soy agar. The results revealed differences of 100 fold and more between the content of cereulide in cells of different strains grown in the same laboratory, extracted and measured under identical conditions (Table 12).

Medium (0.1-1 μg mg^{-1}) and low (0.0005-0.1 μg mg^{-1}) production of cereulide was found with isolates from food connected and not connected to an emetic food poisoning. High amounts of cereulide (>1 μg mg^{-1}) were found in two isolates originating from dairy milk (A116, A16). Similarly by high amounts of cereulide has been reported of endophytes (NS 115, NS 117, NS 58, NS 88) of live spruce trees, one isolate (HS-10b) from brown paper and of one isolate (B308) from a risotto connected to food poisoning in Finland (Apetroaie-Constantin et al., 2008; Jääskeläinen 2008). Inspection of the origin of these strains (Table 12) revealed no obvious connection of cereulide productivity to the geographic, temporal or material origins of the strains. The differences were strain dependent.

B. cereus strains identified by me as cereulide producers were subsequently used in several other studies by other authors: Ehling-Schulz et al., 2005b; Apetroaie et al., 2005; Apetroaie-Constantin et al., 2008, Fricker el al., 2007; Dommel, 2008. These strains were used to identify the cereulide biosynthesis genes (Ehling-Schulz et al., 2005b). The mutant strains prepared by disruption of the peptide synthetase genes were confirmed by me using the LC-MS to be no longer cereulide producer. The thirty eight strains identified as cereulide producers by the sperm assay and the LC-MS assay all possessed the *ces* gene, detected by the PCR method or by plasmid profiling followed by southern hybridization blot (paper IV, Apetroaie et al., 2005, Apetroaie-Constantin et al., 2008, Ehling-Schulz et al., 2005a, Dommel, 2008).

The strain IH 41385 identified as low producer (0.5 -1 ng cereulide mg^{-1} bacterial biomass) and the emetic reference strain F4810/72 identified as

medium producer (240-600 ng mg $^{-1}$ bacterial biomass) (Paper IV) were also studied by Dommel, 2008. The author of that study found a lower level of mRNA transcripts *cesP*, *cesA* and *cesB* in the low producer strain IH 41385 as compared to the emetic reference strain F4810/72. The reasons for the vast differences in the expression of the toxin genes are yet unknown but unlikely to be related to promoter strength of the *ces* operon (Dommel, 2008).

In the very large number of *B. cereus* strains that I investigated (191), I found two *B. cereus* strains GR285 and NVH 506 that were toxic in the sperm bioassay but did not contain cereulide (Table 13). We conclude that both the sperm bioassay and the LC-MS should be used in order to confirm the identity of cereulide in the toxic *B. cereus*.

4.3. Occurrence of cereulide producing *B. cereus* in some environmental niches

This chapter deals with the occurrence and significance of cereulide producing *B. cereus* in certain ecological niches: the dairy production chain and in milk based infant food formulas.

In paper I we identified 34 (1.9 %) emetic toxin producers among the 1757 *B. cereus* isolates from the dairy farm: milk, soil, grass, dung, rinsing water, bedding of the cows and air (Table 4, paper I). Forty four emetic toxin producers (1.1 %) were found among the 3911 *B. cereus* isolates from milk sampled from eight Swedish dairy plants (Table 5, paper I). The results in Paper I show that dairy production chains are a potential but not a major source of emetic *B. cereus*.

The presence of *B. cereus* spores in farm environment is well known (Christiansson et al., 1999, Svensson et al., 2004; Vissers et al., 2007; Bartoszewicz et al., 2008; Banyko & Vyletova, 2009) but only 1-2% of all *B. cereus* isolates retrieved from farms and dairies were emetic toxin producer. Our data thus show that emetic toxin producing *B. cereus* were rare in these environments.

None of the *B. cereus* isolates from farms identified as emetic toxin producers came from soil (0/374) (Table 4 Paper I). I also investigated additional soil isolates (strains WSBC 10310, WSBC10441, INRA SZ) from Germany and France and found no cereulide producer (Table 14). These results may indicate that soil is not the main reservoir of emetic toxin producing *B. cereus*. Similar finding was reported by Altayar and Sutherland 2005 where none of the 101 isolates from soil and animal faeces were emetic toxin producer. Also a recent study by Hoton et al., 2009 reported only one emetic toxin producer out of 543 isolates from soil. However, a study in Japan which investigated the occurrence of emetic *B. cereus* in soil from rice field, 10 out of 20 isolates were emetic toxin producer (Ueda and Kuwabara, 1993). May be this explain why in Japan most cases of emetic *B. cereus* food poisonings are due to the ingestion of rice dishes.

We found cereulide producing *B. cereus* in dairies and milk (Paper I). This raises the question whether cereulide producers are also present in dairy milk derived foods like milk based infant food formulas. I investigated 100 isolates from infant formulas and found 11 cereulide producers. The *B. cereus* isolates from the infant foods produced similar amounts of cereulide as those isolated from emetic food poisonings. I found cereulide producing *B. cereus* among the strains isolated from commercial baby foods

in Germany (Table 2, paper II). These findings indicate potential for cereulide production in commercial infant foods.

Spores of cereulide producing *B. cereus* are more resistant to heat (paper IV) than those of *B. cereus* not producing cereulide. Evaporation exposes milk to heat. This may promote the survival of spores of emetic toxin producing *B. cereus* in dried milk products compared to those of non producers, explaining the high frequency of cereulide producing *B. cereus* in infant food formulas based on dried milk (paper II). Further studies are necessary along this line.

Another finding was that cereulide producers are more common in food than in other environments (Table 1, paper V). Thirty five (24%) out of the 144 isolates from food connected or not connected to food borne illness were cereulide producer (Table 1, paper V). In contrast to this, 1-2% of the environment isolates (Table 14) were cereulide toxin producer. This result shows that cereulide producing *B. cereus* is relatively common among food isolates.

Cereulide producing *B. cereus* share several deficiencies distinguishing them from the cereulide nonproducers: Weak or no haemolysis on blood agar, inability to hydrolyse starch or to decompose tyrosine (Table 12; Agata et al., 1996; Andersson et al., 2004; Apetroaie et al., 2005; Pirttijärvi et al., 1999; Shinagawa, 1993), inability to grow < 10 ºC (Table 5, Paper IV) and the very slow germination of spores (Table 2, Paper IV). These negative physiological traits may explain why cereulide producers usually are poor competitors in the environment.

When investigating a collection of 86 *B. cereus* isolates (one isolate per case) originating from the remnants of food connected to cases of food poisoning, we found that 25 (29%) were cereulide producer. This percentage indicates that emetic foodpoisoning is relatively common among the cases of foodpoisoning connected to *B. cereus*. According to the international standard for identifying the causative agents of foodpoisoning outbreaks, identification of *B. cereus* is based on one or two isolates per case. When the primary plate is blood agar, colonies exhibiting remarkable zone of haemolysis are recommended to be selected by the currently valid standard protocols of ISO (Standard 7932, 2004), NMKL (2003) and FDA (1998). All cereulide producing *B. cereus* isolates identified in our laboratory were weakly haemolytic (≤ 1mm) or non hemolytic (Table 12). Therefore the representation of cereulide producing strains among the *B. cereus* isolates from food poisoning outbreaks is likely underestimated in laboratories following the most widely used international standards protocols.

4.4. Potential for cereulide production in selected infant food formulas

I investigated 8 infant food formulas to determine their amenability to support cereulide production. I used the sperm assay to determine the toxicity and the LC-MS analysis for identifying and quantifying cereulide in the food. I found that 50 to 200 µg of cereulide accumulated within 24 h at 21-23 ºC in 100ml of the infant food formulas composed of both milk and cereals, when the food had been inoculated with > 10^5 cfu ml^{-1} of vegetative cells of *B. cereus* F4810/72. The amount of cereulide accumulated in the infant food would have caused serious illness if consumed. Jääskeläinen et al. (2003) reported that the food caused serious emetic food poisoning

Table 12. Cereulide production by the *B. cereus* strains and isolates analysed in this thesis. All strains were grown on tryptic soy agar plates for 1 d at 28 ºC. The plate grown biomass was analysed for cereulide by LC-MS and for toxicity by the sperm motility inhibition assay. -, trait is negative; +, trait is positive; w= weak with ≤1 mm zone of hemolysis; nd= not determined

Origin and source			The measured traits						
Strain	Country	Source	Toxicity sperm assay	Cereulide measured μg mg $^{-1}$ wet wt of bacteria	*Ces* gene	HBL	Haemo-lysis	Starch hydrolysis	Reference for the description of the strain
Food borne isolates connected to illness									
B315	Finland	Cake	+	0.0005-0.1	+	nd	-	-	Paper IV
B412	Finland	Cake	+	0.1-1	nd	nd	w	-	a
B308	Finland	Risotto	+	> 0.1	+	nd	w	-	a, b
B208	Finland	Cake	+	0.1-1	nd	nd	nd	nd	c
F3080B/87	UK	Rice	+	0.1-1	+	-	w	-	Paper IV
F3350/87	UK		+	0.0005-0.1	+	-	-	-	This thesis, d
F4108/89	UK		+	0.0005-0.1	+	-	w	-	This thesis, d
F3605/73	UK	Rice	+	0.0005-0.1	+	-	-	-	This thesis, d
F3942/87	UK	Rice	+	0.0005-0.1	+	-	w	-	This thesis, d
F3752A/86	UK	Rice	+	0.0005-0.1	+	-	w	-	This thesis, d
F6921/94	UK	Rice	+	0.1-1	+	-	w	-	This thesis, d
LMG17604	UK	Pancake	+	0.1-1	+	nd	-	nd	b
F3942/87	UK		+	0.0005-0.1	+	-	w	-	This thesis, d
F47/94	UK		+	0.1-1	+	-	w	-	This thesis, d
F3976	UK		+	0.1-1	nd	-	nd	nd	This thesis, d
F3876/87	UK		+	0.1-1	+	-	w	-	This thesis, d
F4426	UK		+	0.1-1	+	-	w	-	This thesis, d
F5881/94	UK	Fried rice	+	0.1-1	+	-	w	-	Paper II
F4552/75	UK	Vomit	+	0.0005-0.1	+	-	-	-	This thesis, d
F4810/72	UK	Vomit	+	0.1-1	+	-	w	-	Papers II, IV
RIVM BC 00051	NL	Rice	+	0.1-1	+	-	w	-	This thesis, d
RIVM BC 00052	NL		+	0.0005-0.1	+	-	w	-	This thesis, d
RIVM BC 00061	NL		+	0.0005-0.1	+	-	w	-	This thesis, d
RIVM BC 00062	NL	Vomit	+	0.0005-0.1	+	-	w	-	This thesis, d
MHI 1305	Germany	Rice	+	0.1-1	+	nd	w	-	Paper IV

Table 12 cont.

Origin and source			The measured traits						
Strain	Country	Source	Toxicity sperm assay	Cereulide measured μg mg $^{-1}$ wet wt of bacteria	*Ces* gene	HBL	Haemo-lysis	Starch hydrolysis	Reference for the description of the strain
Specimens implicated with human illness									
LKT 1/1	Finland	Indoor wall of a building	+	0.1-1	+	nd	w	-	a, b
7/PK4	Finland	Indoor wall of a building	+	0.0005-0.1	nd	nd	nd	nd	a
IH41385	Finland	Dialysis liquid from an artificial kidney	+	0.0005-0.1	+	-	w	-	Paper IV
F3876/87	UK	Person with illness	+	0.1-1	+	-	w	-	This thesis, d
F3351/87	UK	Faeces	+	0.0005-0.1	+	nd	w	-	Paper IV
NC7401	Japan	Person with illness	+	0.1-1	+	-	w	-	Paper IV
RIVM BC 00075	NL	Faeces	+	0.1-1	+	-	-	-	a, b
RIVM BC 00067	NL	Faeces	+	0.0005-0.1	+	-	-	-	Paper IV
RIVM BC 00068	NL	Faeces	+	0.0005-0.1	+	nd	-	-	a, b
RIVM BC 124	NL	Faeces	+	0.0005-0.1	+	-	w	-	Paper IV
Foods not connected to illness									
CIF1	Finland	Infant food	+	0.0005-0.1	+	-	w	-	Papers II, IV, V
CIF3	Finland	Infant food	+	0.0005-0.1	nd	nd	w	-	Paper IV
MIF1	Finland	Infant food	+	0.0005-0.1	nd	N	nd	nd	Paper II
B116	Finland	Meat pastry	+	0.1-1	nd	nd	nd	nd	e
B203	Finland	Rice mush	+	0.1-1	nd	nd	w	-	a, e
JP31	Finland	Meat pastry filling	+	ND	nd	nd	nd	nd	This thesis, d
RIVM BC 00379	NL	Chicken	+	0.0005-0.1	+	-	w	-	Paper IV
A116	Sweden	Milk from dairy farm	+	>1	nd	nd	w	-	Papers I, V
A16	Sweden	Milk from dairy farm	+	>1	nd	nd	w	-	Papers I, V

Table 12 cont.

Origin and source			The measured traits						
Strain	Country	Source	Toxicity sperm assay	Cereulide measured μg mg $^{-1}$ wet wt of bacteria	*Ces* gene	HBL	Haemo-lysis	Starch hydrolysis	Reference for the description of the strain
Foods not connected to illness									
mjA1	Sweden	Milk from dairy farm	+	0.1-1	nd	nd	w	-	Papers I, III, V
Jo 331	Sweden	Milk from dairy plant	+	0.1-1	+	nd	w	-	Paper I
GR314	Sweden	Milk from dairy plant	+	0.1-1	nd	nd	w	-	Papers I, V
GR516	Sweden	Milk from dairy plant	+	0.1-1	nd	nd	w	-	Papers I, V
LU1	Sweden	Dairy plant	+	0.1-1	nd	nd	w	-	Papers I, V
LU37	Sweden	Dairy plant	+	0.1-1	nd	nd	w	-	Papers I, V
GR177	Sweden	Milk	+	0.0005-0.1	+	-	w	-	Papers III, IV
GR651	Sweden	Milk	+	ND	nd	nd	nd	nd	Paper III
MHI 87	Germany	Baby food	+	0.0005-0.1	nd	nd	w	-	Papers II, IV
Environments not connected to illness									
NS 115	Finland	Endophyte from a live spruce tree	+	>1	+	nd	-	-	Paper IV
NS 58	Finland	Endophyte from a live spruce tree	+	>1	+	nd	w	-	b
NS 117	Finland	Endophyte from a live spruce tree	+	>1	nd	nd	nd	nd	c
NS 88	Finland	Endophyte from a live spruce tree	+	>1	nd	nd	nd	nd	c
3/pk1	Finland		+	0.1-1	+	nd	nd	nd	Paper IV
HS-10b	Finland	Brown paper	+	>1	nd	nd	nd	nd	d
Strö 10	Sweden	Sawdust bedding	+	0.1-1	nd	nd	w	-	Papers I, V
V1	Sweden	Rinsing water from farm	+	0.1-1	nd	nd	w	-	Papers I, V

[a]Apetroaie et al., 2005, [b] Apetroaie-Constantin et al., 2008, [c] Jääskeläinen 2008, [d]Ehling-Schulz et al., 2005, [e]Jääskeläinen et al., 2003.

of two adult persons contained 100-200 µg of cereulide per100 g.

I found that the infant food formula composed of dried milk as well as cereals accumulated 60 times more cereulide when the reconstituted formula was incubated in stationary position as compared to shaking (60 rpm) (Figure 2, paper II). I concluded from these data that the non shaking condition promoted the production of cereulide at least in these foods. It is possible that non shaking acts as a stressor on the emetic *B. cereus* causing it to produce more cereulide. Similar finding was reported by Rajkovic et al (2006) who showed that more cereulide accumulated in slurries of penne, potato, rice and as well as in whole consumer milk when incubated in static position as compared to shaking.

Although cereulide production is growth phase dependent when tested in laboratory media (Häggblom et al., 2002), I nevertheless found that growth of cereulide producing *B. cereus* to 8 log cfu /ml (Figure 1, Paper II) did not necessarily lead to accumulation of cereulide. Cereulide was detected in the cereal plus milk based formula 24 h after the food was inoculated with $\geq 10^5$ cfu per ml whereas in the milk based infant formula accumulation of cereulide was detected after 24 h only when inoculated with 100 times more of *B. cereus,* $\geq 10^7$ cfu per ml (Figure 2, paper II). Both infant foods supported the growth of *B. cereus* F4810/72 similarly, i.e. to $> 10^8$ cfu per ml. These findings show that the factors affecting the accumulation of cereulide in foods differ from those affecting the growth of the producer strain in the food.

I also found that 1000 times cereulide accumulated in the dairy and cereal based formula than in the formula based on dairy only. Both formulas were prepared by reconstituting dry powder with water, inoculated, treated and analysed similarly (Figure 2, paper II). The inoculated strain *B. cereus* F4810/72 grew in both foods to similar densities during 24 h (Figure 2 paper II). The result shows that these two infant food formulas differed in supporting cereulide production and environmental factor other than the temperature, time, inoculum and incubation conditions affected the production of cereulide.

I also investigated cereulide production in three dairy based formulas amended with cereal or rice, three dairy only formulas and one rice formula with no dairy component. The highest concentration of cereulide (200 to 300 µg /100 ml) was found in the formulas with dairy plus cereal and those with dairy plus rice (Figure 4, paper II). The lowest concentration of cereulide was measured from the formula based on dairy only. Therefore, cereulide accumulated to highest density in the infant food formulas containing farinaceous ingredients like grain and rice when inoculated with the strain F4810/72. Emetic outbreaks have frequently been associated with farinaceous rather than proteinaceous foods. It is interesting to note that most emetic producing strains of *B. cereus* including the F4810/72 do not hydrolyse starch (Table 12; Agata et al., 1996, Apetroaie et al., 2005; Shinagawa 1993) indicating that a nutritional stress factor may have upregulated the cereulide production. However, the substrate preferences of different cereulide producer strains of *B. cereus* may differ from one another, as was shown by Apetroaie-Constantin et al. (2008).

I studied the influence of diluting formulas with water on the accumulation of cereulide by *B. cereus* in cereal and milk based infant formulas. I found that

Table 13.The strains and isolates of *B. cereus* investigated in this thesis and found not to produce cereulide. All strains were analysed with LC-MS and were found to contain less than the detection limit of cereulide (<0.0005 µg mg^{-1} wet wt)

Strains and isolates and their sources	Country	Sperm toxicity assay	Description of the strain
Food connected to illness			
B106 (potato flour), B154 (cake)	Finland	-	Paper IV
98HMPL63 (cooked salsify)	France	-	Paper I
NVH 200 (meat dish with rice), NVH 141/1-01 (vegetarian pasta), NVH 0075-95, NVH 0154-01 (figs), NVH 0165-99 (dear steak), NVH 0226-00 (turkey), NVH 0230-00 (oriental stew), NVH 0309-98, NVH 0391-98 (vegetable puree), NVH 0500-00 (potatoes in cream sauce), NVH 0597-99 (mixed spices), NVH 0674-98 (scrambled eggs), NVH 0784-00 (ground beef), NVH 0861-00 (ice cream), NVH 1104-98 (fish soup), NVH 1105-98 (topping on steak), NVH 1230-88, NVH 1230-98 (oriental stew), NVH 1518-99 (soft ice cream), NVH 1519-00 (stew with deer meat), NVH 1651-00 (caramel pudding)	Norway	-	Paper IV
F352/90(chow mein), F528/94 (chow mein and rice), F2081A/98 (cooked chicken), F2085/98 (cooked rice), F3371/93(chop suey), F3453/94 (rice), F4430/73 (pea soup), F4433/73 (meat loaf), FH3502/72 (DSM 2301)	UK	-	Paper IV
F352/90, F284/78, F0210/76, F1942/85, F2081B/98 (cooked chicken), F2404B/79, F2769/77 (lobster plate), F284/78 (pork pie), F3003/73, F4094/73, F4096/73, F4346/75, F4370/75, F4431/73, F4432/73, F4370/75, F4429/71, F4430/73 (pea soup), F4432/73, F4433/73, F4815/94, DSM4282, LMG17605, LMG17615 (pork pie)	UK	-	This thesis, a
MHI 203 (pasta)	Germany	-	Paper IV
Specimens implicated with human illness			
F837/76 (DSM 4222) (postoperative infection), DSM 8438	UK	-	Paper IV
RIVM BC0063 (faeces), RIVMBC 0070, RIVM BC0090 (faeces), RIVM BC 91 (faeces from a control person), RIVM BC 122 (faeces)	NL	-	This thesis, a
IH41064 (faeces) , B217(faeces)	Finland	-	Paper IV
Food not connected to illness			
CIF13 (infant food) (dry formula)	Finland	-	Paper IV
B102 (meat pie)	Finland	-	Paper IV
INRA 1(zucchini puree), INRA A3 (starch), INRA I20 (cooked leek), INRA I21 (cooked carrot), INRA C24 (pasteurized vegetables), INR C3 (pasteurized vegetables), INRA I16 (pasteurized vegetables), INRA C57 (potato puree), INRA PA (milk protein)	France	-	Paper IV
INRA C1 (pasteurized zucchini), INR A32 (zucchini purée)	France	-	This thesis , a
KA96 (raw milk), MA57 (raw milk)	Sweden	-	Paper IV
Go95 (milk), JO 160 (milk, VI273 (milk), UM37 (milk), MA60 (milk), JO164 (milk), JO164 (milk),	Sweden	-	This thesis ,a
GR 285 (milk)	Sweden	+	Paper IV

Table 13 cont.

Strains and isolates and their sources	Country	Sperm toxicity assay	Description of the strain
Food not connected to illness			
WSBC 10206, WSBC10210, WSBC 2454 (pasteurized milk)	Germany	-	This thesis
WSBC 1020, WSBC 10208, WSBC 10211, WSBC 10030, WSBC 100035, WSBC 100042, WSBC 10204 (pasteurized milk),	Germany	-	This thesis, Paper IV
WSBC 10286 (cream), WSBC 10377 (raw milk), WSBC 10395 (raw milk), WSBC 10466 (red rice), WSBC 10483 (tobacco)	Germany	-	Paper IV
MHI 124 (baby food), MHI 13 (baby food), MHI 32 (baby food) (dry formulas)	Germany	-	Paper IV
NVH 445 (meat), NVH 449 (spices),	Norway	-	Paper IV
NVH 506 (spices)	Norway	+	This thesis
RIVM BC 485 (chicken ragout), RIVM BC 934 (lettuce), RIVM BC 938 (lettuce), RIVM BC 964 (kebab)	NL	-	Paper IV
Environments not connected to illness			
WSBC10310 (soil), WSBC 10441 (soil)	Germany	-	Paper IV
INRA SZ (soil)	France	-	Paper IV
NVH 460 (equipment), NVH 512 (equipment), NVH 655 (river water)	Norway	-	This thesis , a
IR177 (manure), IR183 (manure), IR72(soil), NFFE640 (air), NFFE 647 (water), NFFE664 (water hose of a dairy farm).	Sweden	-	Paper IV
TSP9 (paper board)	Finland	-	Paper IV
NS 113, NS 61 (endophytes from live spruce trees)	Finland	-	b, c, d

[a]Ehling-Schulz et al., 2005, [b]Hallaksela et al., 1991, [c]Pirttijärvi et al., 1999; [d]Hoornstra et al., 2006

Table 14. Screening for cereulide producers among environmental isolates of *B. cereus* not connected to illness (one isolate per sampled item). Compilation of strains and isolates described in this thesis and elsewhere.

Origin of isolates		Number of *B. cereus* isolates		Method of emetic toxin detection	Origins of the strains described in
Item	Country	Tested isolates	Emetic		
This thesis					
Soil	Germany, France	3	0	Boar sperm assay	Tables 12 & 13
Equipment	Norway	2	0	Boar sperm assay	Tables 12 & 13
River water	Norway	1	0	Boar sperm assay	Tables 12 & 13
Soil	Sweden (dairy farm)	374	0	Boar sperm assay	Paper I
Feed	Sweden (dairy farm)	43	0	Boar sperm assay	Paper I
Grass	Sweden (dairy farm)	19	0	Boar sperm assay	Paper I
Dung	Sweden (dairy farm)	44	0	Boar sperm assay	Paper I
Rinsing water in the farm	Sweden (dairy farm)	339	4	Boar sperm assay	Paper I
Used bedding	Sweden (dairy farm)	204	8	Boar sperm assay	Paper I
Air	Sweden (dairy farm)	12	0	Boar sperm assay	Paper I
Endophytes from live spruce trees	Finland	6	4	Boar sperm assay, LC-MS	Andersson et al., 1998a; Hoornstra et al., 2006; Tables 12 &13
Other studies					
Environment (stream water, soil, lake water)	Japan	14	11	HEp2 vacuole cell assay	Vassileva et al. 2007
Soil and animal faeces	UK	101	0	MTT assay	Altayar and Sutherland 2005
Soil	Belgium	543	1	Boar sperm assay, LC-MS	Hoton et al., 2009
Arthropods (insects and isopods)	Belgium	58	0	Boar sperm assay, LC-MS	Hoton et al., 2009
Mammals	Belgium	109	18	Boar sperm assay, LC-MS	Hoton et al., 2009
Commercial wastewater powders	Belgium	57	0	Boar sperm assay, LC-MS	Hoton et al., 2009
Soil	Japan (rice field)	20	10	HEp2 vacuole cell assay	Ueda and Kuwabara, 1993
Air	Japan (rice mills)	29	12	HEp2 vacuole cell assay	Ueda and Kuwabara, 1993

more cereulide accumulated per gram of food (dry wt) when the food was more diluted: Diluting the formula with water from 15 g dry formula /100 ml which is the recommended density, to 6, 3 or 1 g/100 ml increased cereulide accumulation by factors 10 to 50 (Figure 3, Paper II). Lücking et al. (2009) recently showed that cereulide production is regulated by *SpoOA-AbrB* regulon but independent from the later steps of the sporulation process and that *AbrB* may act as a repressor of cereulide production. Diluting the food causes a nutritional stress and this may upregulate the *SpoOA* gene. The *SpoOA* product may repress the transcription factor *AbrB*, as speculated by Lücking et al. (2009), releasing the repressor of the cereulide synthetase operon, thus explaining why more cereulide was produced.

4.5. Strategies of *B. cereus* spores for persistence in the dairy process environment

This part of my study was initiated by the observation of Svensson et al. (2004) that certain recurrent genotypes (RAPD-PCR) of *B. cereus* were found in the silo milk of more than one dairy plant in different parts of Sweden. I collaborated with the group of Svensson et al. (2004, 2006) to reveal the survival strategies of *B. cereus* spores to explain how these *B. cereus* genotypes managed to colonise the dairy silo tanks.

I took a detailed look on the spores of 23 isolates of *B. cereus* selected to represent those RAPD-PCR patterns that were frequent in the dairy silo tanks. This study included: 1. assessment of survival of the spores in liquids used at the dairy industry for cleaning-in-place, 1% sodium hydroxide (pH 13.1, +75 ºC) and 0.9% nitric acid (pH 0.8, +65 °C); 2. adhesivity of the spores from water or from milk to various material surfaces at 4 °C, and 3. spore germination and biofilm formation in milk environment.

I identified four strategies explaining how the spores may have survived in the dairy silo environment. One strategy was the extreme resistance of spore suspension to hot 1% NaOH shown as an extremely low log 15 min kill of ≤ 1.5. This strategy was strengthened by an ability to adhere to stainless steel at 4 °C and to germinate and grow to biofilm in full milk at 21 °C. The second strategy was shown by isolates that adhered as spores to steel from cold water. These spores may have survived by adhering to steel as they did not germinate or grow to biofilm in full milk and also were not particularly resistant (log 15 min kill >3) towards hot alkaline washing. The third strategy was exhibited by isolates with spores that germinated extremely slowly even though the conditions were permissive for growth (+30 ºC). These spores also expressed an extended viability when exposed to heating at +90 ºC. The fourth group of survivors consisted of psychrophiles that possessed the cold shock gene and were able to grow at 8 ºC.

I found that the spores of *B. cereus* RAPD-PCR group 1 (Svensson et al., 2004), representing the group most prevalent in the dairy silo tanks, possessed the survival strategy number 1. Three isolates from this group had spores that survived in hot alkali with a high $D_{initial}$ (> 40 min) and a low 15 min kill value (log of < 0.3). Such extreme tolerance to alkali may be the highest reported so far for *B. cereus*. The previous published record was by Langsrud et al. (2000) who described a strain of *B. cereus* of which the spores suspended in 1% NaOH at 80 ºC for 20 min lost 3 logs of viability.

The spores of cereulide producing strains (n=17) of *B. cereus* exhibited higher $D_{90°C}$ values ($P < 0.001$) and survival rates after 120 min of heating at 90 °C ($P < 0.001$) compared to those of cereulide non producing strains (n=83) (Table 14, Figure 2, Paper IV). The 17 emetic *B. cereus* strains also possessed a strategy of germinating extremely slowly (≤ 1 log in 7 d) in rich medium (at +7 °C or at + 30 °C (Figure 1, Paper IV). Postponed germination is likely to promote chances for survival in dairy industry where most milk is heat treated resulting to inactivation of the spores that germinated. An example of this strategy is one silo tank in a Swedish dairy plant from which 307 *B. cereus* isolates were collected during two weeks in the autumn and two weeks in the winter. Among these isolates, we found 40 (13%) emetic toxin producers (Table 5, Paper I). The high heat resistance combined with a delayed germination of the spores may explain why these cereulide producing *B. cereus* had been successful in surviving in the silo tank environments.

4.6. Are the cereulide producing *B. cereus* successful in competition with the non producing *B. cereus* in food?

I investigated if antagonism exists between isolates of cereulide producing and non producing *B. cereus* (*sensu lato*). I found that cereulide producing *B. cereus* antagonized the cereulide non producing *B. cereus* strains and isolates when inoculated on the same plate (Figure 5). In paper V, I showed that this novel property was common but not obligatory connected to cereulide producing *B. cereus*: 35 (83%) of the 42 cereulide producing strains inhibited the growth of *B. cereus* ATCC 4342 and C477 and also of *B. mycoides* ATCC 6462^T. Cereulide thus was not required for such inhibition.

All strains studied were members of the species of *B. cereus sensu lato* Among the genus *Bacillus*, the species *B. cereus* is defined as a species that does not produce any acid or gas from sugars (e.g. glucose or starch) (Slepecky and Hemphill 2006). This means that the possibility that the antagonism substance would be an organic acid, is very unlikely. I tested the heat stability of the antagonism substance and found that the effective agent was heat sensitive. This means that it is not possible to rule out that the antagonistic agent was an enzyme or some other protein (e.g. bacteriocin), but it rules out (once more) that the agent was cereulide, which is heat stable. The nature of the antagonistic substance was not investigated further within this thesis.

Antagonism was twice more common (83 %) among cereulide producers isolated from foods than among cereulide nonproducers isolated from foods (36%). Of the 44 cereulide producing isolates or strains originating from foods implicated with food poisoning, 93 % were antagonistic and only 37 % of the nonproducers (Table 1, paper V). This indicates that the antagonistic property may contribute to the morbidity of the cereulide producing *B. cereus* more than to that of the nonproducing representative.

I also found that cereulide producing isolates from a food connected to an emetic food poisoning inhibited the growth of cereulide non producers isolated from the same food. The capability to antagonise nonproducers may explain why cereulide producers *B. cereus* succeeded to conquer the food in the case of the food poisoning (Pirhonen et al., 2005). A similar change of *B. cereus* population

Figure 5. Intraspecies antagonistic activity of *B. cereus*. For testing the sensitivity, the target strain of *B. cereus* was plated as a lawn and the potential antagonists were spotted on the lawn. In this figure the lawn of *B. cereus* ML60 was spotted with antagonistic strains F4810/72, B208 and ML30 and the nonantagonistic strain RIVMBC0067. The clear zone around the spotted F4810/72, B208 and ML30 show that these were antagonistic towards ML60.

was observed at the end of shelf life of bakery products (Table 7, paper V). I found that cereulide producing *B. cereus* is relatively common (29%) among food isolates connected to food borne illness (one isolates per item of food per case, Table 1, paper V). The antagonistic property of cereulide producing *B. cereus* may contribute to the success of emetic *B. cereus* in some foods and when time and ambient conditions are permissive, leading to food borne illness.

It important to note that the emetic food poisoning cases described in the literature were most of the time associated with reheated cooked food (Mahler et al., 1997; Pirhonen et al., 2005; Fricker et al., 2007; Pósfay-Barbe et al., 2008). Reheating kills vegetative *B. cereus* but may activate the spores to germinate (See 1.2.3) The emetic *B. cereus* spores are more heat resistant compared with the non emetic *B. cereus* spores (paper IV). Each time the food reheated, there will be selection in favour of the emetic *B. cereus* to grow and produce cereulide. The germinated cereulide producer cells may die during subsequent reheating, but the cereulide is stable and remains. The slow germination strategy (paper IV) of emetic *B. cereus* ensures that sufficient spores remain to survive beyond all heating steps. This sequence of events increase the potential of that food for emetic *B. cereus* food poisoning as the time passes on.

5. Summary and Conclusions

The work presented in my thesis started at a time when little was known about the prevalence of the cereulide producing *B. cereus* in food and non-food environments. The assays for toxicity testing available at that time were not rapid and did not allow screening large numbers of isolates. The contribution of factors other than the toxins to the morbidity of emetic *B. cereus* was not known. The major outcomes of my work are the following:

1. We screened a large number (191) of *B. cereus* isolates from food, outbreaks of food borne illness and environments sampled from several countries within an EU project. The isolates identified as *B. cereus* were investigated for cereulide production. I used the rapid sperm bioassay to test the strains for toxicity and used the LC-MS technique to identify the toxin as cereulide and to quantify its amount in the bacteria. I found more than 100 fold differences in the amount of cereulide produced by the strains. The difference in cereulide production was shown to be strain dependent. We conclude that for the purpose of assessing food safety it is not sufficient to draw conclusions based on the cfu numbers of *B. cereus* or the presence of the *ces* gene but it is necessary to know the toxicity and to measure cereulide in the food.

2. We searched for cereulide producing *B. cereus* in the dairy production chain and found that cereulide producers do occur in the farm environment and the dairy production chain but at low frequency, 1.9% of the total numbers of *B. cereus* isolates from the farms and 1.1% from the dairy plants when one isolate per sample item was analysed. We conclude that the dairy production chain is a potential but a minor source of cereulide producing *B. cereus*.

3. We found that cereulide producing *B. cereus* is more common in food than other environments. Among the 144 food borne *B. cereus* isolates, we found 35 (24%) cereulide producers, in foods connected (29%) or not connected (17%) to illness. Only 12 (1.8%) out of 1041 *B. cereus* isolates collected from environments other than food were identified as cereulide producers.

4. We searched for cereulide producers among *B. cereus* isolates randomly collected from commercial formulas of dry infant foods. Out of 100 isolates from the cereal and dairy based infant foods, 11 were cereulide producers (11%). This is more than the frequency of cereulide producers among dairy process isolates (1.1%) indicating that cereulide producing *B. cereus* may be more common in dried milk based products than in liquid milk.

5. I studied the susceptibility of different infant food formulas to accumulate cereulide when cereulide producers were present. I found that up to 0.1 to 0.4 milligrams of cereulide accumulated in 200 ml of presterilised food within 24 h after the food was contaminated with $\geq 10^5$ cfu of cereulide producing *B. cereus* ml^{-1} and left at room temperature (21- 23 °C). This amount of cereulide would cause serious food poisoning even for an adult consumer. The cereal and dairy based infant food formulas accumulated 1000 times more cereulide than in the formulas based on dairy only although both types of

reconstituted foods supported the growth of the inoculant *B. cereus* similarly. We conclude that the composition of the food had a major impact on cereulide production when a producer strain was present.
Interestingly, diluting the infant food formula with water from the recommended density of 15 g dry weight /100 ml to 1g / 100 ml increased the accumulation of cereulide ca. 50 fold. We suggest that the cereulide production in *B. cereus* is enhanced by nutrient stress. Improper cleaning of infant food bottles may thus contribute to the risk of emetic food poisoning.

6. We investigated the spore survival of *B. cereus* in dairy industry environment. We identified 8 RAPD-PCR groups of *B. cereus* that colonised more than one dairy. When 23 representatives of these groups were investigated, we identified four strategies to explain their survival of their spores in dairy silos. First, high survival (log 15 min kill ≤ 1.5) in the hot alkaline (pH >13) wash liquid, used at the dairies for cleaning-in-place. Second, efficient adherence of the spores to stainless steel from cold water. Third, a cereulide producing group with spores characterized by slow germination in rich medium and well preserved viability when exposed to heating at 90 ºC. Fourth, spores capable of germinating at 8 ºC and possessing the psychrotolerance gene, *cspA*. There were indications that spores highly resistant to hot 1% sodium hydroxide may be effectively inactivated by hot 0.9% nitric acid. Eight out of the 14 dairy silo tank isolates possessing hot alkali resistant spores were capable of germinating and forming biofilm in whole milk, not previously reported for *B. cereus*.

7. We detected that there are *B. cereus* strains that kill other *B. cereus* strains. This phenomenon, intraspecies antagonism or cannibalism, is for the first time described in this thesis. We measured the antagonistic action of strains of many origins and found that antagonism was almost universal (93%) among cereulide producing *B. cereus* isolates from food connected to human illness. We propose that the antagonistic potency may increase the morbidity of cereulide producing *B. cereus* towards the human consumer.

8. We identified the antagonistic activity as a trait that may give advantage for cereulide producing *B. cereus* to gain prevalence in a food that undergoes several heatings and the logistic chain from manufacturer to consumer becomes long. The cereulide producers may outcompete other biotypes of *B. cereus* from a given food commodity by the antagonistic activity and the superior heat survival of spores of the cereulide producers over that of non producer spores.

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