Cereulide producing *Bacillus cereus* and amylosin producing *Bacillus subtilis* and *Bacillus mojavensis* : characterization of strains and toxigenicities

CAMELIA CONSTANTIN

Department of Applied Chemistry and Microbiology
Division Microbiology
Faculty of Agriculture and Forestry
University of Helsinki

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Supervisor: Prof. Mirja S. Salkinoja-Salonen
Department of Applied Chemistry and Microbiology
Faculty of Agriculture and Forestry
University of Helsinki
Helsinki, Finland

Reviewers: Doc. Dr. Pentti Kuusela
Department of Bacteriology and Immunology
Haartman Institute, University of Helsinki
Finland

Prof. Dr. Mieke Uyttendaele
Laboratory of Food Microbiology and Food Preservation
Department of Food Safety and Food Quality
University of Ghent
Belgium

Opponent: Prof. Christophe Nguyen-The
French National Institute for Agricultural Research (INRA), UMR 408,
Joint Research Unit for the Safety and Quality of Products of Plant Origin
University of Avignon et Pays de Vaucluse
Avignon
France

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Front cover: Monkey wondering with joy about the novel method to study cereulide, based on LC-MC, that eliminates the need of the monkey feeding test, previously used to assess and confirm the presence of cereulide.

To my beloved parents and to the most wonderful grandmother in the world

Content

List of original publications:

I. Max M. Häggblom, **Camelia Apetroaie**, Maria A. Andersson, and Mirja S. Salkinoja-Salonen. 2002. Quantitative analysis of cereulide, the emetic toxin of *Bacillus cereus*, produced under different conditions. Applied and Environmental Microbiology, 68, (5): 2479-2483.

II. Camelia Apetroaie, Maria A. Andersson, Cathrin Spröer, Irina Tsitko, Ranad Shaheen, Elina L. Jääskeläinen, Luc M. Wijnands, Ritva Heikkilä and Mirja S. Salkinoja-Salonen. 2005. Cereulide producing strains of *Bacillus cereus* show diversity. Archives of Microbiology, 184 (3): 141-151.

III. Camelia Apetroaie-Constantin, Ranad Shaheen, Lars Andrup, Lasse Smidt, Hannu Rita, and Mirja Salkinoja-Salonen. 2008 Environment driven cereulide production by emetic strains of *Bacillus cereus.* International Journal of Food Microbiology, 127: 60-67.

IV Camelia Apetroaie-Constantin, Raimo Mikkola, Maria A. Andersson, Vera Teplova, Irmgard Suominen, Tuula Johansson and Mirja Salkinoja-Salonen. 2008. Food and food poisoning strains from *Bacillus subtilis* group produce the heat stable toxin, amylosin. Journal of Applied Microbiology - accepted, in printing process.

The author's contribution

Paper I. Camelia Constantin was responsible for the experimental work except for the LC-MS analysis and wrote the article together with the other authors.

Paper II. Camelia Constantin wrote the article and planned and carried out the experimental work except for the 16S rRNA and *adk* gene sequencing, and analysis of a part of the LC-MS samples.

Paper III. Camelia Constantin wrote the article, planned and carried out the experimental work except for a part of the LC-MS analysis, plasmid profile and hybridization and statistical analysis.

Paper IV. Camelia Constantin wrote the article and is the corresponding author. She also planned and carried out the experimental work except for the purification and HPLC-MS analysis, part of the fatty acids analysis and Caco 2 cell assays.

Abbreviations

a_w	water activity
ATCC	American Type Culture Collection
ADP, ATP	adenosine 5'-diphosphate and 5'-triphosphate respectively
BHI	Brain heart infusion
Caco-2 cells	Colon adenocarcinoma
cfu	Colony forming unit
calcein AM	is a non-fluorescent, cell permeant compound that is hydrolyzed by intracellular esterases into the fluorescent anion calcein.
Da	dalton
DSMZ	Deutsche Sammlung von Mikroorganismen und Zellkulturen GmbH
EFSA	European Food Safety Authority
ESI	Electrospray ionization
EU	European Union
FTIR	Fourier transformed infrared spectroscopy
HBL	Haemolytic enterotoxin (*Bacillus cereus*)
HPLC	High-performance liquid chromatography
JC 1	5,5', 6,6'-tetrachloro-1,1', 3,3' -tetraethylbenzimidazolylcarbocyanine iodide
kb	kilobasepairs
LC-MS	Liquid chromatography - mass spectrometry
MLST	Multilocus sequence typing
m/z	Mass-to-charge ratio
MTT	3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide
NCBI	National Center for Biotechnology Information
NK cells	natural killer cells
PCR	polymerase chain reaction
PI	propidium iodide
RAPD	Random amplification of polymorphic DNA
RLM	rat liver mitochondria
TSA	Trypticase soy agar
TSB	Trypticase soy broth

Glossary

adduct	A new chemical species formed by direct combination of two separate molecular entities A and B into one molecule in such a way that there is change in connectivity, but no loss, of atoms within the moieties A and B.
auxotrophy	is the inability of an organism to synthesize a particular organic compound required for its growth. Auxotrophy is the opposite of prototrophy.
biotype	a group of micro-organisms sharing similar biochemical, physiological and morphological properties
hazard	anything that may cause injury or for the potential to cause injury.
fatty degeneration	The accumulation of fat globules within the cells of an organ, also called steatosis.
genotype	The genetic constitution (the genome) of a cell or an organism. The genotype is distinct from its expressed features, or phenotype.
infection	the detrimental colonization of a host organism by a foreign species.
mesophilic	thriving at moderate temperatures, between 20 and 45°C.
non-ribosomal peptide	is synthesized by peptide synthetases with no involvement of ribosomes.
outbreak	localized group of people or organisms infected with a disease.
pathogen	a biological agent that causes disease or illness to its host
pathotype	a pathogen distinguished from others of the species by its pathogenicity on a specific host(s).
pleiotropic regulator	a global regulator controlling the expression of several non-specific extracellular virulence factors
psychrotolerant	An organism able to grow at 7°C or below.
rhizosphere	area of soil immediately surrounding and influenced by plant roots
ropiness	having a gelatinous or slimy quality from bacterial or fungal contamination
risk	the probability that damage will occur as a result of a given hazard.
saprophyte	any organism that lives on dead organic matter
sensu stricto	strict meaning (Latin)
sensu lato	wide meaning (Latin)
serovar H	also called serotype H, it is a group of bacteria sharing the flagellin (H antigen) antigenicity.
surfactant	wetting agent
toxin	a poisonous substance produced by an organism
virulence	refers to the degree of pathogenicity of a given microbe

Abstract

B. cereus is one of the most frequent occurring bacteria in foods. It produces several heat-labile enterotoxins and one stable non-protein toxin, cereulide (emetic), which may be pre-formed in food. Cereulide is a heat stable peptide whose structure and mechanism of action were in the past decade elucidated. Until this work, the detection of cereulide was done by biological assays. With my mentors, I developed the first quantitative chemical assay for cereulide. The assay is based on liquid chromatography (HPLC) combined with ion trap mass spectrometry and the calibration is done with valinomycin and purified cereulide. To detect and quantitate valinomycin and cereulide, their $[NH_4^+]$ adducts, m/z 1128.9 and m/z 1171 respectively, were used. This was a breakthrough in the cereulide research and became a very powerful tool of investigation. This tool made it possible to prove for the first time that the toxin produced by *B. cereus* in heat-treated food caused human illness.

Until this thesis work (Paper II), cereulide producing *B. cereus* strains were believed to represent a homogenous group of clonal strains. The cereulide producing strains investigated in those studies originated mostly from food poisoning incidents. We used strains of many origins and analyzed them using a polyphasic approach. We found that the cereulide producing *B. cereus* strains are genetically and biologically more diverse than assumed in earlier studies. The strains diverge in the adenylate kinase (*adk*) gene (two sequence types), in ribopatterns obtained with *Eco*RI and *Pvu*II (three patterns), tyrosin decomposition, haemolysis and lecithine hydrolysis (two phenotypes). Our study was the first demonstration of diversity within the cereulide producing strains of *B. cereus*.

To manage the risk for cereulide production in food, understanding is needed on factors that may upregulate cereulide production in a given food matrix and the environmental factors affecting it. As a contribution towards this direction, we adjusted the growth environment and measured the cereulide production by strains selected for diversity. The temperature range where cereulide is produced was narrower than that for growth for most of the producer strains. Most cereulide was by most strains produced at room temperature (20 - 23ºC). Exceptions to this were two faecal isolates which produced the same amount of cereulide from 23 ºC up until 39ºC. We also found that at 37º C the choice of growth media for cereulide production differed from that at the room temperature. The food composition and temperature may thus be a key for understanding cereulide production in foods as well as in the gut. We investigated the contents of $[K^+]$, $[Na^+]$ and amino acids of six growth media. Statistical evaluation indicated a significant positive correlation between the ratio $[K^+]$:$[Na^+]$ and the production of cereulide, but only when the concentrations of glycine and $[Na^+]$ were constant. Of the amino acids only glycine correlated positively with high cereulide production. Glycine is used worldwide as food additive (E 640), flavor modifier, humectant, acidity regulator, and is permitted in the European Union countries, with no regulatory quantitative limitation, in most types of foods.

B. subtilis group members are endospore-forming bacteria ubiquitous in the environment, similar to *B. cereus* in this respect. *Bacillus* species other than *B. cereus* have only sporadically been identified as causative agents of food-borne illnesses. We found (Paper IV) that food-borne isolates of *B. subtilis* and *B. mojavensis* produced amylosin. It is possible that amylosin was the agent responsible for the food-borne illness, since no other toxic substance was found in the strains. This is the first report on amylosin production by strains isolated from food. We found that the temperature requirement for amylosin production was higher for the *B. subtilis* strain F 2564/96, a mesophilic producer, than for *B. mojavensis* strains eela 2293 and B 31, psychrotolerant producers. We also found that an atmosphere with low oxygen did not prevent the production of amylosin. Ready-to-eat foods packaged in micro-aerophilic atmosphere and/or stored at temperatures above 10 °C, may thus pose a risk when toxigenic strains of *B. subtilis* or *B. mojavensis* are present.

1. Heat-stable toxins produced by members of *B. cereus* and *B. subtilis* groups, a background

Whereas the diarrhoeal symptom induced by heat-labile toxins produced by *B. cereus* was documented since 1955 (Hauge, 1955), heat-stable toxin production by *B. cereus* group species was scientifically first reported only in 1976 (Melling *et al.*, 1976) and until recently limited to *B. cereus*. In the last years evidence appeared showing other species such as *B. licheniformis* (Salkinoja-Salonen *et al.* 1999; Taylor *et al.* 2005; Nieminen *et al.* 2007), *B. pumilus* (Suominen *et al.*, 2001; From *et al.* 2007), *B. amyloliquefaciens* (Mikkola *et al.*, 2004), *B. subtilis* and *B. mojavensis* (From *et al.* 2005), producing heat-stable substances toxic to mammalian cells. The heat-stable toxins may be pre-formed in food and are not inactivated by heating (Jay et al; 2005) and can induce illness that can vary in severity from mild to severe and even lethal (Mahler *et al.*, 1997; Salkinoja-Salonen *et al.* 1999; Dierick *et al.*, 2005). Yet, all the members of *B. subtilis* group are granted with Qualified Presumption of Safety status by the European Food Safety Authority (The EFSA Journal 2007, 587).

2. Review of literature

2.1. Genus *Bacillus*

The history of genus *Bacillus* starts in 1872, when Ferdinand Cohn recognized an aerobic Gram-positive organism, that forms a unique type of resting cell called endospore, and named it *Bacillus subtilis* (Harwood, 1989). This bacterium represented what was to become a large and diverse genus of bacteria named *Bacillus*, in the family *Bacillaceae*. The genus *Bacillus* is one of the most diverse groups of bacteria and comprise species widely distributed in the biota, soil, air and water (Harwood, 1989). The species important for human health are species that belong mostly to two groups, *B. cereus* and *B. subtilis*, both members of Bacillus RNA group 1 (Stackebrandt and Swiderski, 2002).

2.2. *B. cereus* group

B. cereus group, comprises *B. cereus* (*sensu stricto*), an opportunistic pathogen for human and some animals, *B. thuringiensis*, an insect pathogen, *B. anthracis* which causes anthrax in animals and human, *B. mycoides* and *B. pseudomycoides* (Nakamura, 1998), and the psychrotolerant *B. weihenstephanensis* (Lechner *et al.*, 1998). As a general trait, the species from *B. cereus* group share a G+C% of 35 % (Ravel and Fraser, 2005), hydrolyze lecithin but do not ferment mannitol (Parry *et al.*, 1983; Fritze, 2004). The DNA repeat element *bcr1* was found to be specific for the *B. cereus* group (Økstad *et al.*, 2004). Phenotypic traits used to

differentiate within *B. cereus* group are presented in Table 1. DNA based tools like Multilocus Sequence Typing Scheme (MLST) (Helgason *et al.*, 2004) or Randomly Amplified Polymorphic DNA (RAPD) analysis (Lechner *et al.*, 1998), have been found useful to distinguish the members of *B. cereus* group from each other but the golden standard for analyzing of phylogenetic relatedness remains still the DNA/DNA hybridization (Stackebrandt *et al.*, 2002).

Three species, *B. anthracis*, *B. cereus* and *B. thuringiensis*, also known as *B. cereus sensu lato*, were suggested on the basis of genetic evidence to be one species, with *B. cereus* as the ancestor (Helgason *et al.*, 2000; Daffonchio et. al., 2000, Bavykin *et al.*, 2004). The differences between these species are due to the pathogenic determinants located on plasmids (Helgason *et al.*, 2000). Indeed if *B. anthracis* or *B. thuringiensis* lose their plasmids they become indistinguishable from *B. cereus*. The small chromosomal differences found among *B. cereus* sensu lato cannot account for the diversity of hosts, the induced diseases or the acquisition of different ecological niches. Therefore, it was suggested that the genetic characteristics located on the plasmids together with genetic cross-talk between the plasmid and chromosomal genes may explain this diversity (Vilas-Bôas *et al.*, 2007). By acquiring a new metabolic capability for example by horizontal transfer, a population can get a new resource not used by parental population (Feldgarden *et al.*, 2003). It has been suggested that during bacterial evolution, the populations distinguished by horizontal transfer events are much more likely to co-exist over time (Feldgarden *et al.*, 2003). A case in which *B. cereus* acquired toxin genes from one of the *B. anthracis* plasmids and produced anthrax-like disease (Hoffmaster *et al.*, 2004) proves that plasmid exchanges between close species occur in nature. The cereulide production in strains of *B. cereus* is also plasmid encoded (Hoton *et al.*, 2005).

The members of the *B. cereus* group are wide spread in nature. Some researchers described *B. cereus* as a common soil saprophyte (Granum, 2002) whereas others suggest insect gut as its original habitat (Jensen *et al.*, 2003). A recent study showed that *B. cereus* is able to grow and have a life cycle in soil (Vilain *et al.*, 2006). However, in silico analysis of the metabolic potential of the core set of genes conserved between *B. cereus* and *B. anthracis* does not support the hypothesis of a soil bacterium as the common ancestor of these species (Ivanova *et al.*, 2003; Read *et al.*, 2003). For example *B. subtilis*, an usual inhabitant from soil, has 41 genes for the degradation of carbohydrate polymers which are plant-derived whereas *B. cereus* and *B. anthracis* possess only 14 and 15 genes, respectively, whose functions are limited to degrade glycogen, chitin and chitosan, that are important components in insect tissues (Ivanova *et al.*, 2003). Moreover, the abundance of proteolytic enzymes, peptide and amino-acid transporters and the variety of amino-acid degradation pathways indicates that proteins, peptides and amino acids may be the nutrient source of choice for *B. cereus* and *B. anthracis* (Ivanova *et al.*, 2003). Jensen at al. (2003) suggested that *B. cereus* sensu lato germinates and grows either in the rhizosphere or in an animal host resulting in either symbiotic or pathogenic interactions. The most common environmental niches of *B. cereus* group members are presented in Table 2. The ecology of these bacteria is still under debate.

Table 1. Phenotypic traits useful to differentiate within the members of *B. cereus* group

Species	**haemolysis**	**motility**	**parasporal inclusion**	**susceptibility to penicillin**	**colony morphology**	**growth temperature (°C)**	**lysis by gamma phage**
B. cereus sensu stricto	+	+	-	-	white	4-55	-
B. anthracis	-	-	-	+	white	15-40	+
B. thurigiensis	+	+	+	-	white/grey	10-45	-
B. mycoides	+	-	-	-[†]	rhizoid	10-40	-
*B. pseudomycoides**	+	-	-	+[†]	white/cream, rhizoidal	15-40	-
B. weihenstephanensis	+	+	-	-	white	7-38	-

*Distinguished from *B. mycoides* by differences in whole cell fatty acid composition 12:0 iso and 13:0 anteiso levels and from *B. cereus* by differences in 12:0 iso, 12:0, 15:0 iso and 16:0 (Nakamura, 1998).

[†] 4 of 5 strains of *B. mycoides* were resistant and 5 of 6 strains of *B. pseudomycoides* were susceptible - data from Luna *et al.*, 2007

Compiled from Granum, 2007 ; Gordon, 1973; Lechner et. al., 1998; Nakamura , 1998; von Stetten *et al.*, 1999; Roberts *et al.*, 1996b.

Table 2. Environmental niches of *B. cereus* group

Species	**soil**[a]	**water**[b]	**food**[c]	**mammalian gut**[d]	**insect gut**[e]	**earth worm gut**[f]	**plant rhizosphere or endophyte**[g]
B. cereus sensu stricto	+	+	+	+[t]	+		+
B. anthracis	+				+		+
B. thurigiensis	+	+	+	+[t]		+	+
B. mycoides	+	+	+		+	+	+
B. pseudomycoides	+				+		+
B. weihenstephanensis	+		+		+		+

[a] Vilain *et al.*, 2006; Jensen *et al.*, 2003; von Stetten *et al.*, 1999; Thorsen *et al.*, 2006

[b] Østensvik et *et al.*, 2004; Jensen *et al.*, 2003

[c] Stenfors Arnesen *et al.*, 2008; Páčová *et al.*, 2003; Kajikazawa *et al.*, 2007; Frederiksen *et al.*, 2006; Hanson *et al.*, 2005

[d] Wilks *et al.*, 2007; Jensen *et al.*, 2003;

[e] Jensen *et al.*, 2003; Cook *et al.*, 2007

[f] Jensen *et al.*, 2003

[g] Jensen *et al.*, 2003; Okunishi *et al.*, 2005; Saile and Koehler, 2006; Jafra *et al.*, 2006;

[t] transient

2.2.1. *Bacillus cereus* sensu stricto

The species *B. cereus* is a large Gram positive rod-shaped bacterium of 0.5-2.5 x 1.2-10 µm in size often growing as chains. (Holt *et al.*, 1994). The cells are motile by peritrichous flagelli (Gordon *et al.*, 1973) and produces central or pericentral endospores. Usually after 2-3 days of growth on media the sporulation starts and motility is lost. The spores have no detectable metabolic activity and are resistant to heat, drying, toxic chemicals, UV and gamma radiations and other adverse conditions. The spores of *B. cereus* have a more hydrophobic surface than any other *Bacillus* sp spores and thus, adhere to steel or plastics and are difficult to remove from surfaces (Granum 2007).The spores of strains producing the emetic toxin cereulide, were reported to be about six-fold more heat resistant than the spores from the non-producing strains (Parry and Gilbert, 1980; Carlin *et al.*, 2006). Thus, due to their heat resistance and the ability to adhere, *B. cereus* spores, especially from the cereulide producing strains, are difficult to destroy.

B. cereus has an obligatory requirement for threonine, leucine and valine (Agata et. al., 1999) but auxotrophy for other amino acids has also been reported (Goldberg *et al.*, 1965). *B. cereus* is described as a predominantely mesophilic bacterium. Its growth range stretches from 4 to 55 °C (optimum 30-40 °C) (Roberts *et al.* 1996b). The pH range permitting growth is 4.3 - 9.3 and the minimum water activity (a_w) is 0.93 (Forsythe, 2000).

B. cereus has been implicated in a variety of non-gastrointestinal and gastrointestinal diseases (Drobniewski *et al.*, 1993). It is a recognized food pathogen that causes two different types of gastrointestinal diseases. In the diarrhoeal syndrome it is not clear whether diarrhoea is caused by the ingestion of pre-formed toxin or by toxin formation in the gut (Beecher, 2002). Mostly it has been suggested that diarrhoea is caused when an enterotoxin is formed in intestine (Kramer and Gilbert, 1989; Granum and Lund 1997). The emetic syndrome is caused by the toxin cereulide preformed in foods (Granum and Lund, 1997; Beecher, 2002).

2.2.2. *Bacillus cereus* in foods

B. cereus is found in many kinds of foods due to cooking survival of the spores or post-contamination of food. For example the mild heat treatment used for Refrigerated Processed Foods of Extended Durability (REPFEDs) like cooked chilled foods, may destroy the vegetative cells but not the spores. Twenty % of the cooked-chilled and pasteurized vegetable purees in France were reported to contain low levels of *B. cereus* (Choma *et al.*, 2000). Del Torre *et al.* (2001) found that 30 out of 110 of the investigated REPFED samples of Italian origin were contaminated with *B. cereus.* Integrating the data concerning the fatality rate, outbreaks per year, cases per year, relation to vegetable and vegetable-based foods, and growth temperatures, Carlin *et al.* (2000) quantified the health risk from spore forming

bacteria. Based on this, these authors classified psychrotrophic *B. cereus* as high risk in cooked chilled-vegetable foods. Recently, Guinebretière *et al.* (2008) found genetic evidence for a multiemergence of psychrotolerance in the *B. cereus* group. The risk from the psychrotrophic strains of *B. cereus* is under debate as the amounts of enterotoxin produced by psychrotrophic strains of *B. cereus* at 37°C have been reported to be low (Arnesen et al., 2007; Wijnands et al., 2006a).

Wijnands *et al.* (2006b) reported high numbers of *B. cereus* in milk and milk products, vegetable products, pastry and ready-to-eat foods in the Netherlands. In the same study it was found that within the ready-to-eat foods the numbers of *B. cereus* were high in the products containing rice and pasta. A survey of ready-to-eat moist foods from four cafeterias in Washington D.C. showed that after 20 h of simulated temperature abuse at 26°C, *B. cereus* spores were present in sufficiently high numbers to cause disease (10^5-10^7 cells for diarhhoeal and 10^5-10^8 cells g^{-1} according to Granum, 2007) in 88 % (7 from 8 samples) of the noodles (up to $6.7x10^6$ cfu/g), 92 % (11 from 12 samples) of rice (up to $1.9x10^6$ cfu/g), 100 % (10 from 10 samples) of the mashed potatoes (up to $1.4x10^6$ cfu/g), 100% (18 from 18 samples) of reconstituted non-fat milk (up to $33x10^6$ cfu/g), in 83 % (5 from 6 samples) of Lima beans, and in 75 % (3 from 4 samples) of turkey gravy (Harmon and Kautter, 1991). *B. cereus* spores are common in spices and herbs, detected in counts up to > 10^4 cfu/g (McKee, 1994), suggesting their presence in foods that contain these ingredients.
Due to the heat tolerance the spores are hard to destroy. In addition, heat stable toxins like cereulide will resist cooking and other heat treatments used in the food industry (Rajkovic *et al.*, 2008). The foods described in association with strains producing cereulide are listed in Table 3.

Table 3. Foods reported to contain cereulide producing *Bacillus cereus*.

Food	**Food poisoning associated**	**Country**	**Reference**
spaghetti with pesto	2 cases, 1 fatal	Switzerland	Mahler *et al.*, 1997
ready-to-eat foods	-	The Netherlands	Wijnands *et al.*, 2006b
pasta food	2 cases; 2 cases, 1 life threatening	Finland	Pirhonen *et al.*, 2005; Pósfay-Barbe, *et al.*, 2008
infant food formula	-	Finland	Shaheen *et al.*, 2006
rice dishes	+	Japan	Agata *et al.*, 1996; 2002
noodle	+	Japan	Agata *et al.*, 2002
fried and boiled vegetables	+	Japan	Nishikawa *et al.*, 1996
chocolate milk drink	+	Switzerland	Pósfay-Barbe, *et al.*, 2008
bean paste (Miso)	-	Japan	Mikami *et al.*, 1994
smoked salmon	-	Belgium	Rajkovic *et al.*, 2007
fruit yoghurt	-	Belgium	Rajkovic *et al.*, 2007
black olives in Greek manner	-	Belgium	Rajkovic *et al.*, 2007
pre-roasted turkey filet	-	Belgium	Rajkovic *et al.*, 2007
raw veal meat	-	Belgium	Rajkovic *et al.*, 2007
Camembert cheese	-	Belgium	Rajkovic *et al.*, 2007
pasta salad	5 cases, 1 fatal	Belgium	Dierick *et al.*, 2005
canned mushroom soup	-	Belgium	Rajkovic *et al.*, 2007
bacon	-	Belgium	Rajkovic *et al.*, 2007
Emmental cheese	-	Belgium	Rajkovic *et al.*, 2007
dried figs	2 cases	Norway	Hormazábal *et al.*, 2004
sweet red bean paste covered with sticky rice cake (An-Iri-Mochi)	346 cases	Japan	Okahisa *et al.*, 2008

- no association with food poisoning, + food poisoning associated but no case details were described

2.2.3. Phenotypic traits frequently useful for the characterization of *B. cereus*

Several biochemical properties like presence of exocellular lecithinase (phospholipase C), haemolysis, tyrosinase, caseinase, the starch hydrolysis and salicin decomposition were found useful for differentiation of *B. cereus* from other species of *Bacillus* (Claus and Berkeley, 1986) but also for the recognition of the specific group of cereulide producers in *B. cereus* (Shinagawa, 1993).

The lecithin hydrolysis test is based on the presence of phospholipase-C in the tested organism, capable to hydrolyze the lecithin of egg yolk in the growth substrate (Colmer, 1948). In mammalian cells lecithin (or phosphatidylcholine, with structure shown in Fig. 1) is a major component of cell membrane. In *B. cereus* phospholipase C is a virulence factor (Gilmore *et al.*, 1989), regulated by the transcriptional regulator PlcR (Phospholipase C Regulator). This pleiotropic regulator controls a large regulon of least 14 genes that encode degradative enzymes, cell surface proteins, and both haemolytic and non-haemolytic enterotoxins (Agaisse *et al.*, 1999), which represent most known virulence factors in *B. cereus* (Gohar *et al.*, 2008).

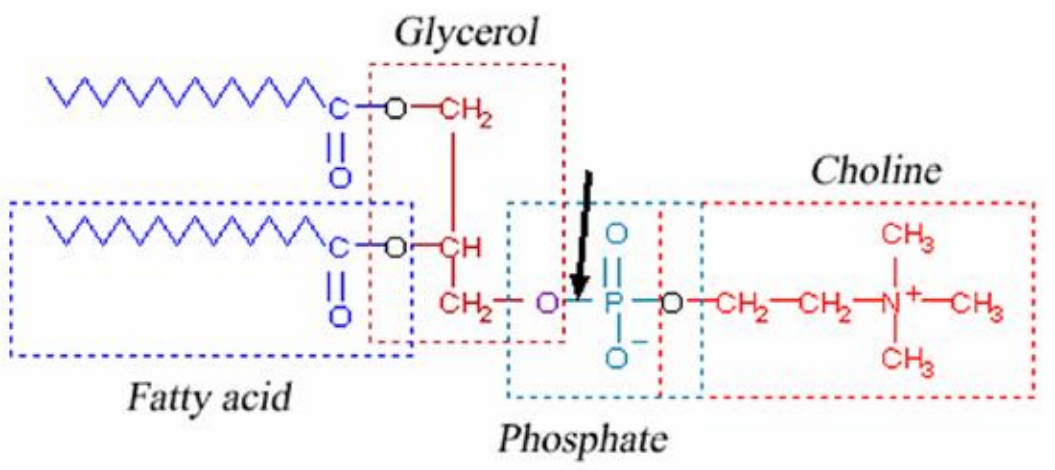


Fig 1. The lecithin structure and the phospholipase-C hydrolysis site, marked with an arrow.

Haemolysis in *B. cereus* is attributed to the presence of the cereolysin AB and hemolysin BL (HBL) (Beecher, 2002). HBL is a three-component toxin with components designated B, L_1, and L_2 which separately are non-toxic but combined exhibit a variety of toxic effects like haemolysis, cytotoxicity, vascular permeability, dermonecrosis, enterotoxicity and ocular toxicity (Beecher and Wong, 1997). Cereolysin AB possesses phospholipase C and sphingomyelinase activity. The genes involved in the production of cereolysin AB are the *plc* and *sph* genes producing phospholipase C and sphingomyelinase, respectively, and the expression of both genes is important for effective hemolytic activity (Gilmore *et al.*, 1989).

They are located in tandem on the *B. cereus* chromosome and are under the control of the global regulator PlcR. The emetic strains of *B. cereus* were found to share a lower haemolysis than the type strain and most non-emetic ones (Andersson *et al.*, 2004). The presence of haemolysis is also a major criterion to differentiate *B. cereus* from the closely related pathogen *B. anthracis* which lacks the haemolysis.

Tyrosin monooxygenase activity is best known by the formation of melanins. The dark pigments protect the bacterial cells and spores against UV radiation, oxidants, heat, enzymatic hydrolysis, antimicrobial compounds and phagocytosis, thus contributing to microbial pathogenesis (Claus and Decker, 2006). Tyrosinases are copper-containing enzymes which are ubiquitously distributed in prokaryotes and eukaryotes. Bacterial tyrosinases use molecular oxygen to catalyse two different enzymatic reactions (Claus and Decker, 2006): the hydroxylation of monophenols to o-diphenols (catechol) and the oxidation of o-diphenols to o-quinones, presented in Fig 2.

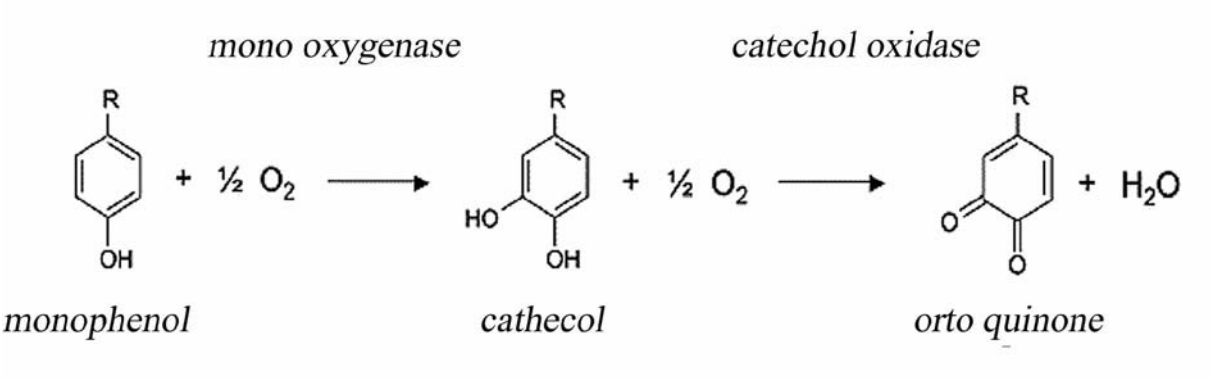


Fig 2. Enzymatic activities found in tyrosinases.

The reactive quinones polymerize non-enzymatically to macromolecular melanins. The tyrosinase reaction is used to differentiate *B. anthracis* (negative for this trait) from *B. cereus* and *B. thuringiensis* (positive) (Parry *et al.*, 1983).

Casein, a complex, globular, phosphoprotein is the main protein from milk (MacFaddin , 2000). The exocellular proteases cleave peptide bonds in the casein, producing peptides of various lengths (Rao *et al.*, 1998). Casein gives the opacity of the milk and this reaction causes the milk agar to clear around the growth area.
90 % or more of the *B. cereus* group strains hydrolyse casein (Claus and Berkeley, 1986). In milk, and other substrates containing casein or other proteins as the main source of carbon and nitrogen, extracellular proteolytic activity can be thus of competitive importance.

Ability to hydrolyze starch is a common property of *B. cereus* group species (Claus and Berkeley, 1986). Starch is the principal storage polysaccharide in plants, occurring in granular

form. The starch granules consist of two major molecular components, amylose (20-30%) and amylopectin (70-80%), both of which are polymers of α-D-glucose units (Fig 3).Amylose is essentially a linear polymer consisting of > 1000 glucose monomers linked by α-1,4 glucosidic bonds. Amylopectin has branched chains of about 20 glucose monomers linked by α-1,4 glucosidic bonds, that are connected by α-1,6 linkages (Warren, 1996). *Bacillus* is the most important amylolytic genus for industrial production of different starch degrading enzymes (Priest, 1977).

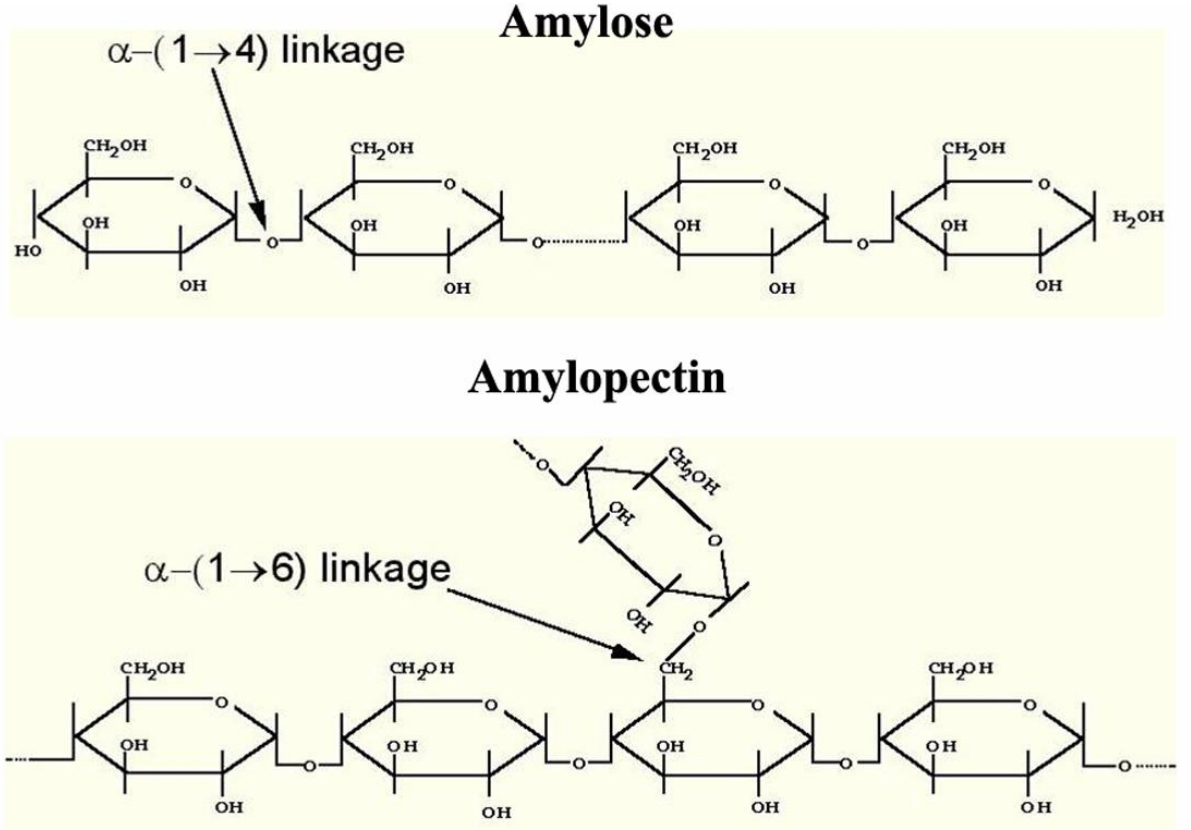


Fig 3. Structures of starch components, amylose and amylopectin

Salicin is a glycoside of o-hydroxybenzylalcohol, obtained from several species of *Salix* (willow) and *Populus* (poplar). Salicin (Fig 4) is hydrolyzed to glucose and saligenin (salicyl alcohol) by the enzyme β -glucosidase. Salicin hydrolysis is a biochemical trait used to differentiate *B. anthracis* (negative) from close relatives like *B. cereus* (positive, sometimes negative) (Parry *et al.*, 1983). The emetic strains of *B. cereus* were found to share the inability to ferment salicin (Shinagawa 1993; Ehling-Schulz, 2005a).

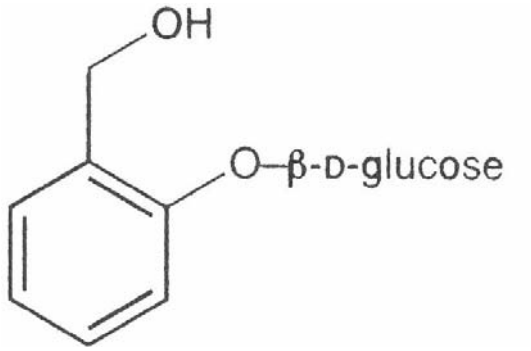


Fig 4. Chemical structure of salicin.

2.3. Cereulide producing *B. cereus*

The emetic type of *B. cereus* was first identified by Melling *et al.* (1976) who proposed that at least two enterotoxins are involved in *B. cereus* food poisoning: one responsible for the diarrhoeal illness and the other responsible for the emetic syndrome. In 1979, Turnbull *et al.* reported that the toxin inducing emesis in monkeys had appeared in rice slurry cultures at 6 h before the appearance of the spores. Fifteen years later, the structure of emetic toxin, cereulide, (Fig 5) was deciphered by Agata *et al.* (1994) and methods for detection were described since 1988 (Hughes *et al.*, 1988). In 2004 the gene responsible for cereulide synthesis was identified (Toh *et al.*, 2004; Horwood *et al.*, 2004), and in 2005 its location on a plasmid was revealed (Hoton *et al.*, 2005). A PCR method for detection of the cereulide synthetase gene is also available since 2005 (Ehling-Schulz *et al.*, 2005b). The complete genome of the emetic strain NC 7401 is in preparation and will bring more insight into the emetic *B. cereus*. (http://www.cb.k.u-tokyo.ac.jp/hattorilab/en/projects accessed on September 4th 2008).

2.3.1. Characterization of emetic strains

In 1979, Shinagawa *et al.* found that the *B. cereus* isolates from emetic syndrome outbreaks were negative for starch hydrolysis. Based on the inability to hydrolyze starch, prevalence of H1 serovar and lack of haemolytic enterotoxin production in the investigated emetic strains, Agata et. al. (1996), concluded that the emetic strains form a specific class of *B. cereus*. The properties reported specific for cereulide producing strains of *B. cereus* are listed in Table 4.

A specific ribopattern for the emetic strains was reported (Pirttijärvi *et al.*, 1999). Based on several genetic and biochemical properties (Table 4) it was suggested that the *B. cereus* emetic strains form a clonal complex that has recently emerged (Ehling-Schulz *et al.*, 2005a). The acquisition of the *ces* genes responsible for the cereulide production, located on a large

plasmid (Hoton *et al.*, 2005), may have been the result of a horizontal transfer. Recently the *B. cereus* strain Kinrooi 5975c carrying the c*es* bearing plasmid was shown capable of acting as either donor or recipient in biparental matings involving large plasmids (Van der Auwera, *et al.*, 2007).

The emetic toxin producing strains also present a distinct shift of growth limits towards higher temperatures combined with highly heat-resistant spores compared to non-emetic strains (Carlin *et al.*, 2006). Recently several exceptions have been reported. Thorsen *et al.* (2006) found from sandy soil two cereulide producing strains of *B. weihenstephanensis* that were positive for salicin fermentation and starch degradation and harbored Hbl enterotoxin complex genes *hblA* and *hblD*.

Table 4. Phenotypic and genotypic properties reported as specific for the cereulide producing strains of *B. cereus*.

	Properties	**Reference**
Phenotypic traits	weak haemolysis	Andersson *et al.* 2004
	inability to hydrolyse starch	Shinagawa *et al.*, 1979
	salicin decomposition negative	Shinagawa *et al.*, 1993
	predominance of H1 serovar	Agata *et al.*, 1996
	lack of the haemolytic enterotoxin production	Agata *et al.*, 1996
Genotypic traits	specific ribopattern	Pirttijärvi *et al.*, 1999
	specific RAPD profiles	Ehling-Schulz *et al.*, 2005a
	specific SDS-PAGE exoprotein profiles	Ehling-Schulz *et al.*, 2005a
	identical 16S rRNA gene sequences	Ehling-Schulz *et al.*, 2005a
	identical MLST sequence type	Ehling-Schulz *et al.*, 2005a
	identical FTIR spectra	Ehling-Schulz *et al.*, 2005a

2.3.2. Properties of the heat stable toxins of *B. cereus*

Cereulide was reported to be produced in strains of *B. cereus* (Agata *et al.*, 1994) and *B. weihenstephanensis* (Thorsen *et al.*, 2006) and it is a 1.2 kDa in size cyclic dodecadepsipeptide consisting of three repeating units of two amino acids, D-alanine and L-valine and two hydroxy fatty acids, D-O-leucine and L-O-valine (Agata *et al.*, 1994). The structure of cereulide is shown in Fig 5.

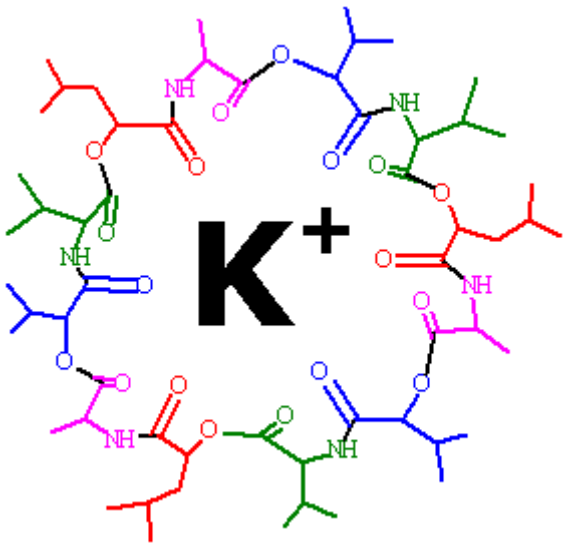


Fig 5. Cereulide, cyclo(*D*-Ala-*D*-*O*-Leu-*L*-Val-*L*-*O*-Val)$_3$, structure. Downloaded from: http://www.biocenter.helsinki.fi/groups/salkinoja/index.htm

The hydroxy fatty acids and the amino acids make the cereulide outer layer hydrophobic, with an octanol-water coeficient Log Kow 6.0 (Teplova *et al.*, 2006). The structure resembles that of valinomycin, a potassium ionophore produced by Streptomyces (Agata *et al.*, 1994). Like valinomycin, cereulide is a potassium ionophore, with affinity to potassium higher than that of valinomycin (Mikkola *et al.*, 1999; Teplova *et al.*, 2006). Cereulide is non-ribosomally produced by a peptide synthetase (Toh *et al.*, 2004; Horwood *et al.*, 2004), recently identified and described (Ehling-Schulz *et al.*, 2006).

The location of the cereulide gene was established on a megaplasmid of about 200-270 kb (Hoton *et al.*, 2005; Rasko *et al.* 2007) which has a high degree of similarity to the pXO1 plasmid from *B. anthracis* (Rasko *et al.*, 2007).
Since the first reports of the emetic type of food poisoning it was found that the involved toxin is heat-stable (Melling *et al.*, 1976). Cereulide is remarkably heat-stable, even at highly alkaline pH values in any tested temperature (up to 150°C) (Mikami *et al.*, 1994; Andersson *et al.*, 1998; Rajkovic *et al.*, 2008).

No heat treatment applicable in food industry will detoxify cereulide (Rajkovic *et al.*, 2008). It is colorless and odorless and thus not detectable by sensorial perception in foods. Cereulide does not lose toxicity upon exposure to pH from 2 to 11 and to proteolytic activity of pepsin and trypsin (Kramer and Gilbert, 1989; Mikami *et al.*, 1994; Shinagawa *et al.*, 1996). Cereulide is mitochondriotoxic (Hoornstra *et al.*, 2003; Mikkola *et al.*, 1999). Its actions on different biological targets are listed in Table 5.

Homocereulide is a 1166 Da depsipeptide with potent cytotoxic effect so far found only from a marine strain of *B. cereus,* SCRC. This toxin was never shown to act as the emetic toxin (Wang *et al.*, 1995). So far it has not been reported in foods.

Another heat-stable toxic substance produced by *B. cereus* (strain AH 682), is a non proteinaceous exotoxin, resistant to proteolysis, smaller than cereulide, of about < 1 kDa in size, lethal to *Anthonomus grandis* (cotton boll weevil) larvae. Its structure still needs to be deciphered (Perchat *et al.*, 2005).

Table 5. Heat stable toxins from *B. cereus* group

toxin	**Biological effects on:**	**Toxic threshold concentration**	**Detection and quantification method based on:**	**Reference**
cereulide (*B. cereus, B. weihenstephanensis*)	human HEp-2 cells	5-10 ng ml^{-1}	cell vacuolation test	Agata *et al.*, 1994; Kawamura -Sato *et al.*, 2005
		4 ng ml^{-1}		Andersson *et al.*, 2007
	human HepG2	2 ng ml^{-1}	inhibition of RNA-synthesis	Andersson *et al.*, 2007
	human HeLa cells	10-25 ng ml^{-1}	loss of mitochondrial transmembrane potential ($\Delta\Psi_m$) visualised by staining with JC-1	Jääskeläinen *et al.*, 2003b
	human Paju cells	2-4 ng ml^{-1} (slide culture) and 10 ng ml^{-1} (suspension culture)	loss of $\Delta\Psi_m$ visualized by staining with JC-1	Jääskeläinen *et al.*, 2003b
	human Calu-3 cells	2-4 ng ml^{-1} (slide culture) and 10 ng ml^{-1} (suspension culture)	loss of $\Delta\Psi_m$ visualized by staining with JC-1	Jääskeläinen *et al.*, 2003b
	human Caco-2 cells	2 ng ml^{-1} (slide culture) and 10-20 ng ml^{-1} (suspension culture)	loss of $\Delta\Psi_m$ visualized by staining with JC-1	Jääskeläinen *et al.*, 2003b
	human NK cells	20-30 ng ml^{-1}	loss of $\Delta\Psi_m$ visualized by staining with JC-1	Paananen *et al.*, 2002
	boar sperm cells	0.2-0.5 ng ml^{-1} sperm	sperm motility inhibition test and LC-MS	Andersson *et al.*, 1998; 2007
		2 ng ml^{-1} sperm		Jääskeläinen *et al.*, 2003b
		20 ng ml^{-1} sperm	CASA	Rajkovic *et al.*, 2006b
	Hepa-1 cells	0.9 ng ml^{-1}	measurement of cell protein content as the end point	Andersson *et al.*, 2007
	fetal porcine Langerhans islets	1 ng ml^{-1}	necrotic cell death visualized with calcein AM and PI	Virtanen *et al.*, 2008
	rat liver mitochondria (RLM)	50 ng ml^{-1}	measurement of the uncoupling effect on the respiratory activity	Kawamura -Sato *et al.*, 2005

			of RLM	
	Homo sapiens (Human)	≤ 8 μg kg^{-1} body weight	food analyzed by LC-MS	Jääskeläinen *et al.*, 2003b
	Macaca mulatta (Rhesus monkey)	10 μg kg^{-1} body weight	detection of emesis	Shinagawa *et al.*, 1995
	Suncus murinus (musk shrew)	8-10 μg kg^{-1} body weight	detection of emesis	Agata et. al., 1995
	MTT conversion	0.3 μg ml^{-1}	the yellow, soluble MTT is converted to purple insoluble formazan by metabolizing cells unaffected by emetic toxin.	Finlay *et al.*, 1999
homocereulide (*B. cereus*)	P388 cells Colon 26 cells	0.033 ng ml^{-1} 0.0082 ng ml^{-1}	N.S.	Wang *et al.*, 1995
non-proteinaceous insecticidal exotoxin (*B. cereus*)	larvae of *Anthonomus grandis* (cotton boll weevil)	N.S.	free ingestion method	Perchat *et al.* 2005

CASA - computer assisted sperm analysis

Cell lines: HEp-2 -human laryngeal carcinoma cells; HepG2 - human hepatocellular liver carcinoma cells; HeLa - human cervical cells; Paju - human neural cells; Calu-3 - human lung carcinoma cells; Caco-2 - human colon carcinoma cells; Hepa-1 - mouse hepatoma cells; P388 - murine leukemia cells; Colon 26 cells - mouse colon adenocarcinoma cells; **Primary cells**: NK - human natural killer cells; boar sperm cells; **Organotypic culture**: fetal porcine Langerhans islets; **Isolated cell organelles**: rat liver mitochondria;

JC-1 -5,5', 6,6'-tetrachloro-1,1', 3,3' -tetraethylbenzimidazolylcarbocyanine iodide; MTT - 3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide;

N.S. not specified

2.3.3. Methods for detection and quantification of cereulide

The methods used for the detection and quantification of cereulide are summarized in Table 5. The first successful demonstration that the vomiting and diarrhoeal symptoms were different entities came from the monkey-feeding test and ligated rabbit ileal loop (Turnbull *et al.*, 1979). By European legislation no whole animals are allowed for toxicity testing of other than drugs prior to their clinical testing. Cell toxicological methods are used instead (Registration, Evaluation, Authorization and Restriction of Chemicals, REACH) http://ec.europa.eu/environment/chemicals/reach/reach_intro.htm (accessed 5.09.2008).

In vitro methods have been available already from some time. An assay based on human larynx carcinoma cells (HEp-2 cells) was the first in vitro method to show the subcellular effects of cereulide, vacuolation of cells by swelling of the mitochondria (Hughes *et al.*, 1988). A modified version of the HEp-2 cell vacuolation assay uses 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide, a yellow, water-soluble tetrazolium salt which is converted to purple insoluble formazan by the metabolizing cells (Finlay *et al.*, 1999).
The boar spermatozoan test was developed in our laboratory (Andersson *et al.*, 1998). The boar sperm cells are excellent targets for hydrophobic toxins like cereulide. Since the mitochondria are the "engine" of the sperm cell, mitochondrial dysfunction will be reflected in impaired motility as observed by light microscopy. This test was later developed into a rapid bioassay (Andersson *et al.*, 2004). Objectivity of the read out was improved by semi quantitative recording by means of a computer assisted sperm analyzer (Rajkovic *et al.*, 2006b; 2007). Using the boar sperm cells, also other parameters such as the mitochondrial electric transmembrane potential ($\Delta\Psi_m$) can be visualized by staining with 5,5', 6,6'-tetrachloro-1,1', 3,3' -tetraethylbenzimidazolylcarbocyanine iodide (JC-1). This lipophilic dye shifts its fluorescence emission from orange-red to green if $\Delta\Psi_m$ is affected (Smiley *et al.*, 1991).

Due to the solubility of cereulide in organic solvents, it can be separated and purified by high-pressure liquid chromatography (HPLC). Cereulide can be then identified with mass spectrometry (MS) based on its molecular ions, ammonium [NH_4] *m/z* 1170.46, sodium [Na^+] *m/z* 1175.54 and potassium [K^+] *m/z* 1191.54 (Mikkola *et al.*, 1999; Andersson *et al.*, 1998).

Besides the methods based on identification of the toxin in biological or chemical assays, PCR methods were developed for detection of the cereulide synthase gene (*ces*) (Horwood *et al.*, 2004; Ehling Schulz *et al.* 2005b). *Ces* specific PCR can be used to screen for potential cereulide producing strains since *ces* genes are required for cereulide synthesis. However, the presence of the *ces* gene does not prove that cereulide will be produced, but its absence excludes the possibility. Other drawback of the PCR assay is the possibility of false negative results.

2.3.4. Factors affecting the cereulide production

Szabo *et al.* (1991) found that the optimum temperature for production of the emetic toxin by *B. cereus* was between 20-30 °C. Agata *et al.* (2002) showed that *B. cereus* grew and produced the emetic toxin in cooked rice and other starchy foods stored at room temperature. Two emetic toxin producing strains of *B. weihenstephanensis* grew at temperatures as low as 8°C, but produced no cereulide at this temperature (Thorsen *et al.*, 2006). Finlay *et al.* (2000) reported that the optimal temperature for toxin production was 15°C. Rajkovic *et al.* (2006a) found high counts (ca 10^7 CFU/g) of *B. cereus* 5964a strain resulting in cereulide production in penne and potato puree at 12°C (after 4 and 5 d, respectively) and at 22°C (after 42 and 24 h respectively) but not in liquid medium (BHI) at 12°C at any tested pH (Rajkovic *et al.*, 2006b). Therefore, the medium composition may also be an important factor for the synthesis of cereulide.

Agata *et al.* (1999), studied the cereulide production using a defined medium with amino acids. They found that three amino acids were essential for the growth of *B. cereus* and also for cereulide production: L-valine, L-leucine and L-threonine. Jääskeläinen *et al.* (2004) found that adding L-leucine and L-valine (0.3 g $^{l-}$1) stimulated cereulide production 10 to 20 fold in R2A media and in rice water agar. Agata *et al.* (1999), reported for strain NC 7401, that commercial skim milk supported higher cereulide titres than brain heart infusion broth (BHI), trypticase soy broth (TSB), or nutrient broth. Skim milk was previously reported as a good medium for the production of the heat-stable toxin by *B. cereus* strain F 4810/72 whereas BHI and TSB supported four and 250 times respectively, less the production of the emetic toxin (Szabo *et al.*, 1991).

Szabo *et al.* (1991) found that milk and white rice were superior to all the other tested foods in their ability to support emetic toxin production. The toxicity titres were 1024 (milk) and 512 (white rice) compared to titres of 256 (converted rice), 128 (brown rice), egg (32) and pasta (16). These studies, executed before cereulide was found and identified, were based on toxicity titers of *B. cereus* cell extracts tested with HEp-2 cells. Agata *et al.* (2002) reported that boiled rice supported cereulide production (320 ng/g) better than bread and cake with only 20 ng/g and in egg or egg products with < 5-10 ng/g.

In the studies cited above, water extracts were used for the assessment of cereulide content. It is possible that, by using extraction protocols involving solvents in which cereulide will dissolve, higher values would have been obtained. Jääskeläinen *et al.* (2003a), found using methanol-pentane (1:1) at 100°C and 10,340 kPa with a robotized apparatus that 1.6 µg cereulide / g of bread was produced as measured by LC-MS. The *B. cereus* counts (ca 10^8 CFU) in the bread were similar to those in the study of Agata *et al.* (2002). Rajkovic *et al.*, (2006a) extracted with methanol potato puree, penne, rice and milk, that had been inoculated with the *B. cereus* strain 5964a and incubated for 48h at 28°C. They found by HPLC-MS that ca 4 µg g^{-1} cereulide was produced in potato puree. A high concentration of cereulide was also

found in penne (ca 3 μg g^{-1}) whereas in rice the strain produced only half the amount of cereulide produced in potato puree, and in milk ca 1 μg ml^{-1}.

Jääskeläinen *et al.* (2003a) investigated bakery products and found that most cereulide accumulated in those with the highest a_w and pH: the rice pastry with an a_w of 0.982 and a pH of 6.55 and the meat pastry filling with an a_w of 0.988 and a pH of 6.2. Rajkovic *et al.* (2006a) found that no detectable amount of cereulide was produced in the food with the lowest pH and a_w of all tested foods, grown at 12°C. These data suggest that a high a_w and pH might promote the cereulide production.

A further factor of importance for cereulide production is aeration and access to oxygen. Several authors (Hughes *et al.*, 1988, Szabo *et al.*, 1991, Agata *et al.*, 1996) reported that shaking promoted high production of cereulide, suggesting that oxygenation could be a stimulus for cereulide producing *B. cereus*. Finlay *et al.* (2002) reported that static skim milk cultures yielded only 10 % toxin compared to similar cultures that were shaken (200 rpm orbital shaking). Agata *et al.* (2002) also noticed that if the milk was shaken, the toxin production increased.
Rajkovic *et al.* (2006a) reported that the aeration of BHI cultures decreased cereulide production by more than 10 fold compared to the static conditions. Another study reported that no cereulide was detected in shaken (150 rpm orbital shaking) milk whereas 1,140 ng ml^{-1} accumulated in stationary incubated milk (Rajkovic *et al.*, 2006b). An increase by a factor of 10 fold of cereulide production was reported in potato slurry for the strain 5964a (Rajkovic *et al.*, 2000a) and up to 100 fold in infant foods for F 4810/72 (Shaheen *et al.*, 2006) when kept stationary compared to shaking. These data suggest that aeration by shaking might promote cereulide production in substrates of homogenous liquids, as seen in several studies but not all, whereas in slurry substrates, containing suspended particles, the toxin production is enhanced by static conditions.

Jääskeläinen *et al.* (2004) found that aeration was important for cereulide production and a nitrogen atmosphere (> 99.5 % N_2) suppressed cereulide production in beans by 90% and almost completely (≤ 0.05 μg g^{-1}) in rice. Rajkovic *et al.* (2006b) found that the cereulide producing strains NS 117 and 5964a, produced up to 1 μg cereulide mg^{-1} of biomass on TSA plates when the O_2 concentration was 4.5 vol % but none when the atmosphere contained less then 1.6 vol % O_2. However, cereulide was produced by *B. cereus* strains B 116, B 203 and F 4810/72 up to 400 μg g^{-1} in anoxic atmosphere when CO_2 (9-13 %) was present (Jääskeläinen, 2008). These results suggest that in fact not O_2 is the essential gas in the air that allow or promote the cereulide production but CO_2.

The presence of the resident background microbiota was also reported to influence cereulide production, by having an inhibitory effect (Rajkovic *et al.*, 2006a).

2.4. *Bacillus subtilis* group and its relevance to foods

2.4.1. The *B. subtilis* group

B. subtilis group comprises *B. subtilis ssp. subtilis, B. amyloliquefaciens, B. atrophaeus, B. licheniformis, B. mojavensis, B. pumilus, B. subtilis ssp. spizizenii*, *B. vallismortis* (Fritze, 2002) and *B. sonorensis* (Palmisano *et al.*, 2001). The *B. subtilis* group species share a G+C % of 43-44% (Ravel and Fraser, 2005). The traits used to differentiate the species within the *B. subtilis* group are presented in Table 6. The species *B. atrophaeus* is closely similar to *B. subtilis* but can be distinguished from it on the basis of DNA relatedness, multilocus enzyme electrophoresis analysis and pigment production (Nakamura, 1989). The genetic tools most reliably used for identification of *B. subtilis* group members are DNA-DNA reassociation, gyrase *gyr*A (Chun and Bae, 2000) and gyr B genes sequencing (Wang *et al.*, 2007).

Whole cell fatty acid composition has been reported as an additional trait useful for species identification in this group (Roberts *et al.*, 1994). *B. subtilis* was one of the early bacterial genetic models and significant amount of information is available on its genetics whereas little information is available of the other members of the *B. subtilis* group and this information is restricted to strains of economic importance. Bacteria from *B. subtilis* group are important sources of enzymes and antibiotics (Priest, 1989), starters in fermented foods (Harwood, 1989) and of probiotics (Sanders *et al.*, 2003). The pathogenic potential of the strains from *B. subtilis* group was investigated mostly in connection to their use as probiotics (Sanders *et al.*, 2003). There are sporadic reports on these species implicated in food poisoning incidents and infections as reviewed by Drobniewski, (1993).

Compared with *B. cereus* group, the ecology of the *B. subtilis* group is less arguable. Its members are considered as saprophytic inhabitants of soil and decomposing materials (Priest, 1993). *B. subtilis* has historically been called "Hay bacillus" or "Grass bacillus" (Lewis, 2001). In Japan, *B. subtilis* is called "Kosoukin" which means "bacteria present in dead grass" (Ochiai, 2007). Recently, it was discovered that *B. subtilis* and its close relatives can enter an intestinal life cycle and have adapted to carry out their entire existence within this environment (Tam *et al.*, 2006). Table 7 summarizes the environmental niches most frequently reported for one or more members of the *B. subtilis* group.

Table 6. Phenotypic traits for species description within *Bacillus subtilis* group

Species	**anaerobic growth**	**acid from lactose**	**growth temperature °C**	**utilization of propionate**	**nitrate reduced to nitrite**	**starch hydrolysis**	**growth in 5-10% NaCl**	**G+C content (mol %) of DNA**	**ribitol in the cell wall**
B. subtilis ssp. *subtilis*	-	-	15-55	-	+	+	+	43	-
B. amyloliquefaciens	-	+	15-50	-	+	+	+	44	ND
B. licheniformis	+	-	15-55	+	+	+	+	46	ND
B. mojavensis	+	-	10-50	-	+	+	+	43	ND
B. pumilus	-		10-50		-	-		42	ND
B. vallismortis	-	-	10-50	-	+	+	+	43	ND
B. atrophaeus	-	-	5-55	-	+	+	+	42	ND
B. subtilis ssp. spizizenii,	-	-	15-55	-	+	+	+	43	+
B. sonorensis	+	ND	15-55	+	+	+	-	46	ND

ND-not determined

Compiled from Claus and Berkeley, 1986; Roberts *et al.*, 1996a; Palmisano *et al.*, 2001; Nakamura, 1989; Nakamura *et al.*, 1999; Priest *et al.*, 1987.

Table 7. Environmental niches reported for the members of *B. subtilis* group

Species	**soil**[a]	**water**[b]	**food**[c]	**mammalian gut**[d]	**insect gut**[e]	**industrial fermentations**[f]	**in rhizosphere or as endophyte of plants**[g]
B. subtilis ssp. subtilis,	+	+	+	+	+	+	+
B. amyloliquefaciens,	+					+	+
B. atrophaeus	+						
B. licheniformis	+	+	+	+	+		+
B. mojavensis	+		+				+
B. pumilus	+	+	+				+
B. subtilis ssp. spizizenii	+						
B. vallismortis	+	+					+
B. sonorensis	+		+				

[a] Priest F. 1989; Nakamura *et al.*, 1999; Roberts *et al.*, 1996a; Nakamura 1989; Roberts *et al.*, 1994; Palmisano *et al.*, 2001

[b] Priest F. 1989; Østensvik *et al.*, 2004; Jeong *et al.*, 2007

[c] Priest F. 1989; From *et al.*, 2005; Sorokulova *et al.*, 2003

[d] Tam *et al.*, 2006; Leser et. al., 2007

[e]König H. 2006

[f] Ochiai *et al.*, 2007; Priest F. 1989

[g] Idriss *et al.*, 2002; Bacon and Hinton, 2001; Mano *et al.*, 2006; Park *et al.*, 2007

2.4.2. Members of *Bacillus subtilis* group in foods

Within *B. subtilis* group, the species that were frequently reported in foods are *B. subtilis* and *B. licheniformis*. Strains of these species are used to ferment vegetable foods such as natto in Japan, using *B. subtilis* (Yokotsuka and Sasaki, 1998) and several West African products using *B. subtilis* and *B. licheniformis* (Odunfa and Oyewole, 1998). The presence of endospores enable the species of the *B. subtilis* group to survive heat treatment during food preparation (Pendurkar and Kulkarni, 1989). The spores of these species are also contained in herbs, spices and seasonings and can contaminate the food (te Giffel *et al.*, 1996). High microbial loads, of 10^7 cfu/g were found in black pepper, ginger and Lebanon bologna spice mixture, the predominant species being *Bacillus subtilis* (Palumbo *et al.*, 1975). Table 8 lists the foods in which five of the *B. subtilis* group species described in Table 6 were investigated.

Table 8. Members of *B. subtilis* group reported in foods.

Species	**Food**	**food-borne illness associated**	**spoilage / toxins produced**	**Reference**
B. subtilis ssp. subtilis	fermented like natto	-	-	Priest F. 1989
	bread / crumpets	-	ropiness	Leuschner *et al.*, 1998; Kramer and Gilbert 1989
	spices	-	-	Palumbo *et al.*, 1975; te Giffel *et al.*, 1996
	fried marinated chicken	+	putative emetic toxin	From *et al.* 2005
	cocoa/chocolate	-	-	te Giffel *et al.*, 1996
	meat products	-	-	Kramer and Gilbert, 1989; te Giffel *et al.*, 1996
	meat/seafood curries with rice			Kramer and Gilbert, 1989
	pasta products	-	-	te Giffel *et al.*, 1996
	Chinese meals	-	-	te Giffel *et al.*, 1996
	custard powder	+	putative emetic toxin	Kramer and Gilbert, 1989
	mayonnaise	+	putative emetic toxin	Kramer and Gilbert, 1989
	canned bean salad	+	putative emetic toxin	Kramer and Gilbert, 1989
	synthetic fruit drink	+	putative emetic toxin	Kramer and Gilbert, 1989
B. licheniformis	bread	-	ropiness	Leuschner *et al.*, 1998
	infant food formula	+	lichenysin A	Salkinoja-Salonen *et al.*, 1999; Mikkola *et al.*, 2000
	Curried chicken and mayonnaise sandwich	+	heat stable toxin	Salkinoja-Salonen *et al.*, 1999;
	Curry rice	+	heat stable toxin	Salkinoja-Salonen *et al.*, 1999;
	Profiteroles	+	heat stable toxin	Salkinoja-Salonen *et al.*, 1999;

	raw milk (postmastitic or mastitic)		heat stable toxin	Salkinoja-Salonen *et al.*, 1999; Nieminen *et al.*, 2007
	cider	-	ropiness	Grande *et al.*, 2006
B. mojavensis	spices	-	surfactin	From *et al.* 2005; 2007
	figs	-	putative emetic toxin	From *et al.* 2005
B. pumilus	bread		ropiness	Leuschner *et al.*, 1998
	raw milk (mastitic)	-	heat stable toxin	Nieminen *et al.*, 2007
	rice dishes	+	heat-stable toxin/pumilacidin A	Suominen *et al.*, 2001; From *et al.*, 2007
	curry paste	+	heat-stable toxin	Suominen *et al.*, 2001
	chewing tobacco	-	virulence factor that evokes plasma exudation from the oral mucosa	Rubinstein and Pedersen, 2002
B. sonorensis	bread	-	ropiness	Sorokulova *et al.*, 2003

2.5. Heat stable toxins from *B. subtilis* group

Toxic strains from *B. subtilis* group were first reported from outbreaks in the United Kingdom (Kramer and Gilbert, 1989). Since then three toxins associated with human illness have been purified and characterized, all of them heat-stable: lichenysin (Salkinoja-Salonen *et al.*, 1999; Mikkola *et al.*, 2000; Nieminen *et al.*, 2007), amylosin (Mikkola *et al.*, 2004; 2007), pumilacidin (From *et al.* 2007b). Another heat-stable cytotoxic peptide was identified as surfactin (From *et al.* 2007a). *B. subtilis* group species are known to produce many nonribosomally synthesized peptides. *B. subtilis* and *B. amyloliquefaciens* use 4-7 % of their genomes to code for biosynthesis of bioactive compounds (Stein, 2005). Besides the three peptides described in association with human illness and surfactin, listed in Table 9, bacillomycins, mycobacilin, mycosubtilin, plipastatins, and rhizocticin A, are known to be produced by *B. subtilis*, bacitracin, produced by *B. subtilis* and *B. licheniformis*, fengycins and iturin A by *B. subtilis* and *B. amyloliquefaciens*, halobacillin, amoebicins, and fungicin M4 by *B. licheniformis*, reviewed by Mikkola, 2006. All of these are peptides, many of them known to be nonribosomally synthesized (Mikkola, 2006).

Lichenysin A was found in *B. licheniformis* connected to a fatal case (Salkinoja-Salonen *et al.* 1999). It was also found in strains isolated from vomit (Taylor *et al.* 2005) and from mastitic milk (Nieminen *et al.* 2007). Lichenysin A is a family of 992-1034 Da cyclic heptalipopeptides, known as biosurfactants and antibacterial agents produced by several *B. licheniformis* strains (Mikkola *et al.*, 2000). It is produced in aerobic as well as anaerobic conditions, it is resistant to heat (100°C, 20 min), pronase, acids and alkali (Yakimov *et al.*, 1995; Salkinoja-Salonen *et al.*, 1999). The reported biological effects of lichenysin A, on which detection and quantitation are based, are presented in Table 9. The *B. licheniformis* strains producing lichenysin A were beta-haemolytic, grew anaerobically and at 55°C but not at 10°C, were not distinguishable from the type strain of *B. licheniformis* DSM 13^T by a broad array of biochemical tests, and presented four different ribopatterns with *Pvu*II and six with *Eco*RI (Salkinoja-Salonen *et al.*, 1999).

Amylosin was reported in *B. amyloliquefaciens* isolates from indoor dust and building material from water damaged buildings where the occupants suffered respiratory health symptoms 2004). Amylosin is a 1,197 Da lipopeptide moderately lipophilic, heat-stable, forming cation-permeant channels to K^+, Na^+, and Ca^{2+} (Mikkola *et al.* 2004; 2007). It contains six different amino acids: leucine, proline, serine, aspartic acid, glutamic acid and tyrosine and a polyene structure (Mikkola *et al.*, 2007). The reported biological effects of amylosin are presented in Table 9.

Pumilacidin was found from *B. pumilus* strains connected to a serious food poisoning (From *et al.* 2007a). It is a non-ribosomally produced 1035-1077 Da cyclic acylheptapeptide containing seven components: pumilacidin A, B, C, D, E, F and G (Naruse *et al.*, 1990)

Pumacilidin is lipophilic and soluble in organic solvents and thus easily absorbed through biological membranes. The reported biological effects of pumilacidin are presented in Table 9.

Strains of *B. mojavensis* and *B. subtilis* producing surfactin have been isolated from food (From *et al.* 2005). Surfactin is a family of 993-1035 Da, heat-stable cyclic lipoheptapeptides, non-ribosomally produced (Peypoux *et al.*, 1999). It is the most powerful biosurfactant known, exerting detergent-like action on biological membranes, shown to lyse erythrocytes and to have antiviral and antibiotic activity (Stein, 2005). Toxic effects of surfactin are listed in Table 9. Surfactin production is necessary for the swarming activity of *B. subtilis* and it is involved in biofilm formation (Stein, 2005). Surfactin shares with the other non-ribososmal peptides several properties such as heat-stability, pH and proteolytic degradation resistance, toxicity to Vero cells and inhibition of sperm cell motility.

Temperature has been shown to influence the production of pumilacidin in *B. pumilus* (From *et al.*, 2007b). To our knowledge, other factors affecting the heat-stable toxins production in *B. subtilis* group have not been described.

Table 9. Heat stable toxins reported from *B. subtilis* group

Heat stable toxin	**Species**	**Toxicity**		**Detection and quantification method based on:**	**Reference**
		target cell / organelle	**concentration**		
lichenysin A	*B. licheniformis*	boar sperm	4-8 μg ml^{-1}	sperm motility inhibition test, loss of plasma membrane integrity visualized by staining with Calcein AM and propidium iodide, depletion of cellular ATP, acrosome swelling.	Mikkola *et al.*, 2000
amylosin	*B. amyloliquefaciens*	boar sperm	0.2-0.3 μg ml^{-1}	sperm motility inhibition test, loss of $\Delta\Psi_m$ visualized by staining with JC-1	Mikkola *et al.*, 2004
		feline		depletion of cellular ATP and NADH	
		foetal lung	0.2-0.3 μg ml^{-1}	loss of $\Delta\Psi_m$ visualized by staining with JC-1	
		Paju	1-2 μg ml^{-1}	loss of $\Delta\Psi_m$ visualized by staining with JC-1	
		RLM	200 ng ml^{-1} in K^+ medium or > 250 ng ml^{-1} in Na^+ medium	uncoupling of oxidative phosphorylation of RLM, oxidation of PN, loss of $\Delta\Psi_m$ visualized by staining with JC-1 and suppression of ATP synthesis.	Mikkola *et al.*, 2007
pumilacidin	*B. pumilus*	boar sperm	8 μg for pumilacidin A 13 μg for pumilacidin B 14 μg for pumilacidin G > 15 μg for pumilacidin C and D	sperm motility inhibition test-inactivation of oxidative phosphorylation in mitochondria due to destruction of cell membrane	From *et al.*, 2007
		Vero	> 20 μg for pumilacidin E 30 μg for pumilacidin A and B > 30 μg for pumilacidin C and D	reduction in protein synthesis by more than 30 % in Vero cells assay	

surfactin	*B. subtilis*	boar sperm	25 μg ml^{-1}	sperm motility inhibition test (1d)	Hoornstra et al., 2003
			6.25 μg ml^{-1}	loss of $\Delta\Psi_m$ visualized by staining with JC-1	
		Vero	N.G.	reduction in protein synthesis by more than 30 % in Vero cells assay	From *et al.*, 2005
	B. mojavensis	boar sperm	3-4 μg	sperm motility inhibition test	
		Vero	6-27 μg	reduction in protein synthesis by more than 30 % in Vero cells assay	From *et al.*, 2007

Paju - human neural cells, Pumilacidin A, B, C, D, E, and G concentrations - 1 mg ml^{-1} methanol; RLM - rat liver mitochondria; N.G. - not given

2.6. Properties important for the growth of *B. cereus* and *B. subtilis* group strains in foods

The factors reported to contribute to growth of bacteria in food, with application to *Bacillus cereus* and *B. subtilis* group members are enumerated in Table 10.

2.6.1. Mechanisms for resistance to physical stressors

The spores help *Bacillus* sp. to survive the processes involved in food technology. The spores have no metabolic activity but ensures the survival during environmental stresses like heat, desiccation, radiation, toxic chemicals. Living *Bacillus* spores as old as 250 million years have been reported (Vreeland *et al.*, 2000). Parry and Gilbert (1980) showed that the spores of 14 *B. cereus* strains isolated from a vomiting type syndrome were about 8 times more heat resistant at 95°C (range 9.5-36.2 min, mean 24.8 min) than those of 13 strains isolated from rice (range 1.5-6 min, mean 3.3 min). Carlin et al, (2006) found that the spores from 17 cereulide producing strains survived four to five log better heating at 90°C for 120 min than the spores of 81 cereulide non producers.
In the presence of water or milk, *B. subtilis* spores were found more resistant to heat (100°C) than the spores of *B. cereus*, whereas in rice subjected to boiling *B. cereus* spore counts were about 3 times higher than those of *B. subtilis* (Pedurkar *et al.*, 1989). The same authors found that frying of rice at 180-190°C for 5-7 min killed the spores of both species.

Spores of *B. subtilis* PS832 subjected to Martian atmospheric pressure (7-18 mbar) and gas composition (100 % CO_2) for up to 19 days had a similar survival rate as the spores exposed to the conditions on the Earth (Nicholson and Schuerger, 2005). Another exceptional property of the spores is the resistance to radiation. *B. subtilis* spores strain WN648 in liquid suspension exposed to simulated Mars solar radiation for 42 min retained the potential to germinate (Tauscher *et al.*, 2006). Resistance of the spores to desiccation may explain why *Bacillus* species are the most frequent contaminants in the dried herbs (McKee, 1995) and in dried infant foods (Le Duc *et al.*, 2005; Shaheen *et al.*, 2006).

Once the spore germinates the survival depends on environment (Table 10). An interesting feature reported for the emetic strain SA-50 was that its vegetative cells localized inside the raw kernels of the rice whereas the diarrhoeal-type of *B. cereus* strains grew on the surface of the kernels (Nishimura *et al.*, 2002). The ability to penetrate inside the rice kernel may enhance survival during cooking and possibly explains the selectivity for emetic strains in outbreaks where rice dishes were involved. In a study investigating the time and temperature exposures during the cooking of rice in Cantonese-style restaurants, it was found that the highest temperature reached during cooking ranged from 93 to 99°C for 8 to 30 min (Bryan *et*

al., 1981). Of the 110 emetic outbreaks reported in UK between 1971-1978, rice was implicated in 108 (cited by Drobniewski, 1993).

Table 10. Intrinsic and extrinsic properties of *B. cereus* and *B. subtlis* group important for growth in foods

Bacterial biochemical properties	
starch degrading activity	Ability to hydrolyze starch is a common property of *B. cereus* group species[1] and *Bacillus* is the most important amylolytic genus for industrial production of different starch degrading enzymes[2]. Most emetic strains are negative for starch hydrolysis although they are most frequently reported in starchy foods.
protease activity	important for making amino acids available from protein substrates
lipolytic activity	important for making fatty acids available from lipid substrates
salt tolerance	allows survival in salt stress environment. Important also for the cross protection between stresses. For example in one study on *B. cereus*, salt protected against hydrogen peroxide, which protected against ethanol, which protected against heat shock.[3]
Intrinsic parameters of the food [4]	
pH	*B. cereus* and *B. subtilis* group grow at a pH 5-9.5. The presence of salt may widen the pH range. Too low or too high pH alter the functioning of enzymes and the transport of nutrients into the cell.
moisture content	the water activity (a_w) of most fresh foods is above 0.99 and *B. subtilis* for example can grow at minimum 0.95. The minimum a_w reported for *B. cereus* growth is 0.91 to 0.96 in fried rice[5]. Temperature and nutrients might permit growth at lower values of a_w: the range of a_w over which growth occurs is greatest at the optimum temperature for growth and the presence of nutrients increases the range of a_w over which the organisms can grow. A low a_w increases length of the lag phase of growth and decreases the growth rate.
oxidation-reduction potential Eh	generally the members of the genus *Bacillus* prefer a positive Eh values (oxidized) for growth.
nutrient content	includes the presence of water, source of energy (such as sugars and amino acids), source of nitrogen (like amino acids), vitamins and related growth factors and minerals.
antimicrobial constituents and food preservatives	Includes essential oils in plants (like eugenol in cloves, allicin in garlic, thymol in sage and oregano, etc), lysozyme in eggs and milk, lactoferrin in milk, etc. Food preservatives affecting spore formers include nisin, benzoate, sorbate.

biological structure	the natural covering of some foods provides protection against the entry of spoilage organisms (i.e. testa of seeds, shells of eggs, of nuts, the skin, etc). Presence of organic matter may protect bacteria from the action of disinfectants.
Extrinsic parameters[4]	
temperature of storage	Generally, a temperature lower than 4-7°C or higher than 55°C would impede the growth of any member from *B. cereus* or *B. subtilis* group. (Table 1 and Table 6).
relative humidity of environment	When the a_w of a food is for example 0.6, it is important to store it under conditions that prevent the food absorbing moisture.
presence and concentration of gases	CO_2 is the most important atmospheric gas that is used to control microorganisms in foods. *Bacillus* sp. are among the most sensitive microorganisms to CO_2 relative to modified atmosphere packaging where the CO_2 content is 20-30 % CO_2. However for the cereulide producing strains of *B. cereus* the presence of CO_2 in nitrogen atmosphere was permissive for toxin production[6].
exposure to radiation	microwaves, UV, X or gamma radiation ("cold sterilization")
exposure to high hydrostatic pressure	A $\log_{10}$ 3.5-5.0 cfu/ml reduction from initial of 10^6 of spores of *B. subtilis* in milk was effected by a combined treatment of ≤ 1.0 % sucrose laurate and 392 MPa for 10 min at 45°C. In hyperbaric conditions, cell morphology is altered and ribosomes are destroyed. Between 450 and 800 MPa are needed to destroy spore formers but some spores require > 1000 MPa.
presence and activity of other organisms	some other microorganisms from food produce substances that are inhibitory /lethal or simply inhibit the growth of *Bacillus* by competition for the same resources and a higher growth rate.

[1]Claus and Berkeley, 1986
[2]Priest, 1977
[3] Browne and Dowds, 2001
[4]adapted from Jay *et al.*, 2005
[5]Bryan *et al.*, 1981
[6]Jääskeläinen, 2008

2.7. Food-borne illness in connection with emetic *B. cereus, B. subtilis* and *B. mojavensis*

The symptoms of the *B. cereus* emetic illness mimic those from *Staphylococcus aureus* food poisoning and are described in Table 11. Generally the illness is mild and short-lasting, but fatal cases have been reported involving young persons and children (Takabe and Oya, 1976; Mahler *et al.*, 1997; Dierick *et al.*, 2005;). In two cases fulminant liver failure was reported as the cause of death. Although the report of Mahler *et al.* was the first to show linkage between *B. cereus* and fatal liver failure, this may not be new disease (Schafer and Sorell, 1997).

Takabe and Oya, (1976) reported an outbreak of food poisoning in Nagoya, that affected 50 out of 51 persons following ingestion of cooked Chinese noodle. An 11 year old boy began to complain of nausea, sever abdominal pain and diarrhoea about 1 h after meal. The symptoms persisted during the night and in the morning he died. Subsequently, the autopsy showed fatty degeneration of the heart, liver and kidneys and the bacteriological examination of the peritoneal exudate and intestinal content detected *Bacillus cereus* but no other food poisoning bacteria. In the Udorn province of Thailand, a rice-growing region, many children died due to encephalopathy and fatty degeneration of the viscera (cited by Schafer and Sorell, 1997). When the food is unrefrigerated during the storage for several days high amounts of cereulide may accumulate (Jääskeläinen *et al.*, 2003a; Rajkovic *et al.*, 2006a). In two of the reported fatal cases the food was improperly stored for 4 days.

Yokoyama *et al.* (1999) showed that pure cereulide (20 μg cereulide) injected intraperitoneally in mice produced fatty degeneration in liver similar to a human case. However with lower doses of cereulide (10 μg) the regeneration of the liver was observed within 4 weeks (Yokoyama *et al.*, 1999). Recently a life-threatening case of acute liver failure with renal and pancreatic insufficiency, shock and mild encephalopathy due to cereulide producing *B. cereus* in a pasta dish was reported (Pósfay-Barbe *et al.*, 2008). In that case it was observed that in toxin-related *Bacillus* disease, peak transaminases was reached in about 48 h. Protecting the patient liver for this time rescued the liver which regained its structure and function (Pósfay-Barbe *et al.*, 2008).

In *Suncus murinus* (house musk shrew) abdominal vagotomy abolished the vomiting (Agata *et al.,* 1995). The ventral vagus nerves are attached to the oesophagus just below the diaphragm and the 5-HT_3 (serotonin) receptors are present on presynaptic nerve terminals. The abolition of emesis by vagotomy suggests that the cereulide vomiting in *Suncus murinus* was mediated through the 5-HT_3 receptor via the vagus afferent (Agata *et al.*, 1995).

By inhibiting cytotoxic activities and cytokine production of human killer cells cereulide may act as an immunosuppressant (Paananen *et al.*, 2002).

Table 11. Food-borne illness in connection with heat-stable toxins from *B. cereus, B. subtilis* or *B. mojavensis*.

bacteria	**toxin**	**symptoms**	**Reference**
Bacilllus cereus	cereulide	-0.5-5 h after the ingestion of contaminated food -usually nausea and vomiting lasting for 6-24 h -in the fatal cases involving a 17 years old, after 2 d the symptoms included listlessness, somnolence, icteric presentation and pain in the upper right quadrant of the abdomen -in the fatal case involving a 7 years old, after 6 hours she started vomiting and complaint of respiratory distress and before death (occurred at 13 h after the meal) she had severe pulmonary haemorrhage and severe muscle cramps. -no fever	Mahler *et al.*, 1997; Dierick *et al.*, 2005
Bacillus subtilis	putative emetic toxin	-at 1- 6 to 14 h after ingestion of contaminated food -nausea, vomiting -severe stomach pain -headaches -flushing/sweating -later diarrhoea can follow	Kramer and Gilbert, 1989 From *et al.*, 2005
Bacillus mojavensis	surfactin	not known (no reported involvement in food poisoning)	From *et al.*, 2007a

Data on illness due to heat-stable toxins produced by *Bacillus subtilis* is scanty (one reference in Table 11). No illness due to heat-stable toxins produced by *B. mojavensis* has been described to our knowledge.

3. Aims of study

The aim in this doctoral thesis was primarily to characterise the emetic strains of *Bacillus cereus* and their toxin production. To achieve this aim we developed an assay to detect and to quantify cereulide. The secondary aim was to reveal whether other food-borne species of *Bacillus* produced heat-stable toxins. The target was to offer reliable tools for revealing the properties of *Bacillus* strains in order to understand their potential as food poisoning agents.

Specific aims were to:

1. Design a chemical assay for accurate identification and quantification of cereulide.

2. Characterize by biochemical and molecular methods the cereulide producing strains of *B. cereus.*

3. Reveal the relation between the medium, temperature for growth and production of cereulide by *B. cereus.*

4. Find species of *Bacillus* outside the *B. cereus* group producing heat-stable toxins and determine their properties relevant for risk of food poisoning.

4. Materials and methods

The methods used for this thesis are listed in Table 12.

Method	Description	Reference
Extracting methods of cereulide and amylosin		
methanol extraction of cereulide from bacterial cultures	Papers I and IV	Andersson *et al.*, 1998
Pentane extraction of cereulide from bacterial liquid cultures	Paper I	
Extraction of cereulide from bacterial cultures for rapid detection	Papers II, III and IV	Andersson *et al.*, 2004
Biological assays for toxicity		
Boar sperm motility inhibition	Paper I	Andersson *et al.*, 1998
Boar sperm motility inhibition for rapid detection	Papers II, III, and IV	Andersson *et al.*, 2004
JC-1, Calcein AM and PI staining for detection of electric transmembrane potentials and cell membrane integrity	Paper IV	Hoornstra *et al.*, 2003
Caco-2 cells (human colon carcinoma) assay with cereulide	Paper IV	Jääskeläinen *et al.* 2003b
Chemical methods		
HPLC-MS for cereulide	Paper I	
HPLC-ESI-IT-MS for amylosin	Paper IV	Mikkola *et al.*, 2004
HPLC-MS using for adducts for cereulide	Papers II, III, and IV	Jääskeläinen *et al.* 2003a
Gas chromatography of methyl esters of whole cell fatty acids	Paper IV	Pirttijärvi *et al.*, 1996
Methods for identification and characterization of *Bacillus* strains		
16S rRNA gene sequencing	Paper II and IV	Rainey *et al.*, 1996
adk gene sequencing	Paper II	Helgason *et al.*, 2004
PCR for the detection of cereulide producing strains of *B. cereus*	Paper II	Ehling-Schulz *et al.*, 2004)
Light Cycler Real time PCR for the detection of *rpoB* gene and of differential genetic markers for *B. anthracis* using RealArt™ *B. anthracis* LC PCR kit	Paper II	Qi *et al.*, 2001; Artus-Biotech, Hamburg, Germany
Automated riboprinting	Paper II, III and IV	Pirttijärvi *et al.*, 1999
plasmid profiling	Paper III	Jensen *et al.*, 1995
Southern hybridization	Paper III	Sambrook *et al.*, 1989
Methods for physiological characterization of *Bacillus* strains		
haemolysis	Papers II, III and IV	

lecithin hydrolysis, tyrosine decomposition,	Papers II and III	
salicin fermentation, gelatine liquefaction, starch hydrolysis	Paper II	
caseinase activity	Paper III	
Strain motility	Paper II	
determining the growth range temperature	Papers II, III, IV	
antibiotics susceptibility	Papers II and III	
sensitivity to Gamma phage	Paper II	Turnbull *et al.*, 1998
Microscopy methods		
Light microscopy	Paper I, II, III and IV	
Fluorescence microscopy	Paper IV	
Isolation methods		
Isolation of emetic strain UB 1020 from faeces	Paper II	

5. Results and discussion

5.1. The first chemical assay for cereulide detection and quantification

In this study we investigated the cereulide content of *Bacillus cereus* cultures grown under various conditions (Paper I and III). The bioassays used for measuring the heat-stable toxin cereulide give toxicity titres but not accurate concentrations (Mikami *et al.*, 1994; Andersson *et al.*, 1998; Finlay *et al.*, 1999; Andersson *et al.*, 2004). Due to solubility of cereulide in organic solvents, it can be separated by high-pressure liquid chromatography (HPLC). Identification of cereulide can be done by mass spectrometry based on specific mass ions (Mikkola *et al.*, 1999; Andersson *et al.*, 1998). We developed a chemical assay based on separation by HPLC, on-line connected with ion trap mass spectrometry for ion detection. With a C8 column and a solvent made up to 95 % acetonitrile, 4.9 % water, and 0.1 % trifluoroacetic acid, the closely similar depsipeptides, valinomycin and cereulide, were well separated, with retention times of 5.32 and 5.78 min respectively. The ion (*m/z*, 1,171.1; NH_4^+ adduct) was chosen for identification based on its specificity for cereulide deduced from its described structure (Agata *et al.*, 1994; Isobe *et al.*, 1995). The total mass ion current from 500 to 1,300 *m/z* were scanned and integrated after smoothing. The assay was calibrated with purified cereulide and with commercially obtained valinomycin, a similar depsipeptide with *m/z*, 1,128.9 (NH_4^+ adduct). The sample injection volume was 1.0 µl. The detection limit of this assay for cereulide was of 10 pg per injection.

Extracts from biomass grown on solid medium were prepared as described by Andersson *et al.* (1998). For liquid cultures the following protocol was used:

- liquid culture (10 ml TSB) was extracted twice, each time with an equal volume of pentane for 1 h with mild agitation (25 rpm) in vertical motion,
- after shaking the tubes were frozen,
- the organic phase layer was separated from the aqueous phase layer in a smaller test tube (if the separation is not clear the tubes can be sonicated),
- The combined pentane phases were evaporated to dryness under a stream of nitrogen.
- The residue was dissolved in 1 ml of methanol.

The efficiency of extraction for spiked valinomycin was > 80 %. This extraction protocol was subsequently used in other researches (Shaheen *et al.*, 2006; Jääskeläinen et.al., 2004; Nakano *et al.*, 2004). The extracts used for the chemical assays were also analysed by the sperm motility inhibition assay (Andersson *et al.*, 1998). The findings in Paper I (Fig 2) show that the results obtained for *B. cereus* extracts with the boar sperm motility inhibition assay matched well with those obtained with the chemical assay. The chemical assay was more accurate (standard deviation, ± 10 % within a sample) than the bioassay (50 %, based on twofold dilution step).

The assay we describe in Paper I is the first direct chemical assay applicable for detection and quantitation of cereulide. This method was subsequently used for cereulide detection and

quantification in several other studies (Rajkovic *et al.*, 2006b, 2007; Thorsen *et al.*, 2006; Ehling-Schulz *et al.*, 2005; Pirhonen *et al.*, 2005; Toh *et al.*, 2004; Jääskeläinen *et al.*, 2003b). Paper I showed the validity of valinomycin as a calibration standard for cereulide and made this assay easier to use, since valinomycin is commercially available. Our assay stays also at the base of an improved LC-MS assay using four molecular adducts for cereulide [H^+], [NH_4^+], [Na^+], and [K^+] (Jääskeläinen *et al.*, 2003a). Using this chemical assay the dose of cereulide causing illness in humans (8 μg kg^{-1} body weight) was determined for the first time (Jääskeläinen *et al.*, 2003b).

Using the method described in Paper I, we analysed the effect of growth and incubation conditions on cereulide production. We found that *B. cereus* strain NC 7401 started to produce cereulide in the stationary phase in trypticase soy broth where this strain did not sporulate within 120 h (Fig. 4 in Paper I). This suggests that cereulide production was independent from sporulation. This finding together with the cyclic structure and the presence of D-amino acids in cereulide guided Toh *et al.*, 2004 to the suggest that cereulide synthesis could be by a nonribosomal biosynthetic mechanism pathway. Using PCR with primers targeted to recognise a fragment of the nonribosomal peptide synthetase and our chemical assay they provided the first evidence for the synthetic mechanism of cereulide.

In Paper I (Fig. 3) we showed that three strains, F 5881, F 4810/72 and NC 7401 accumulated more cereulide (80 to 166 μg g^{-1}) at room temperature (21°C) than at 11 and 40°C where cereulide production was low (0.5 - 2.8 μg g^{-1} and < 0.2 - 0.9 μg g^{-1} respectively).

Another finding was that the trypticase soy broth cultures of NC 7401 when shaken (150 rpm) for 70 h at 21°C, produced about 100 times more cereulide than stationary even though the cultures were equally turbid (Table 2 in Paper I). Several authors investigated the emetic toxin production in shaken cultures and found it in high amounts (Table 13). In these studies the used media as well as the extraction and toxin assessment protocols differed from the ones used in our experiments (Table 13). However, the finding that the shaking promoted the toxin production was similar to ours. Szabo et al., 1991 tested the cereulide production in TSB and found no production after 18 h of incubation. This is not surprising since we found that in TSB it took at least 30 h for the strain NC 7401 to produce cereulide (Fig. 4 in Paper I). In milk, however, 18 - 24 h were enough for the production of cereulide (Table 13). There are other studies (Shaheen *et al.*, 2006; Rajkovic *et al.*, 2006a) where shaking inhibited the cereulide production instead of increasing it. In these cases the growth substrates were slurries rather than liquids (cereal containing infant food, penne, potato and rice slurries) (Table 13). Shaheen *et al.* (2006), used our extraction protocol and assessed the toxicity in the same way as we did except that we used TSB cultures and they used reconstituted infant food. TSB is a homogenous liquid with only 3 % solids. The infant food formulas were 15 % solids and the slurries 10 % solids (Table 13). Thus, it is possible that the amount of suspended solids and the consistencies may have influenced the different outcomes of theses studies.

Table 13. Comparison of different studies approaches to evaluate the emetic toxin production in shaken or static conditions.

Reference	**Media and growth conditions**	**Extraction protocol of the extracts used in the assay**	**emetic toxin assay**	**emetic toxin production**
Paper I	TSB -24 h at 21°C with (150 rpm) or without shaking -70 h at 21°C with (150 rpm) or without shaking	extraction with pentane, the solvent phase was evaporated to dryness and the residue dissolved in methanol.	boar sperm motility assay	-24 h cultures: 1.07-1.36 μg ml^{-1} shaken < 0.02 μg ml^{-1} static -70 h cultures: 21.98 μg ml^{-1} shaken 0.16 μg ml^{-1} static
Szabo *et al.*, 1991	BHI or BHIG, skim milk, TSB, Evans medium, foods - 18 h at 27°C, with shaking (200 rpm)	the cultures/foods were centrifuged (2000 × g for 10 min), supernatants filtered (through 0.8 μm filters) and filtrates heated at 100°C for 10 min	Hep-2 cells	-toxin titer 256 in milk, white rice, and BHI -toxin titre 4 in TSB
Agata *et al.*, 1996	10 % skim milk - 20 h at 30°C with shaking (200 rpm)	the cultures were centrifuged (2000 × g for 10 min) at RT, and supernatants autoclaved (121°C for 10 min).	Hep-2 cells	> 5 μg ml^{-1}
Agata *et al.*, 2002	milk - 24 h at 30°C with or without shaking	cultures were centrifuged (5500 × g for 15 min), supernatant collected and autoclaved.	Hep-2 cells	0.64 μg g^{-1}- shaken < 0.005 μg g^{-1} static
Finlay *et al.*, 2002	skim milk medium - 24 h at 30°C with (200 rpm) or without shaking	cultures were centrifuged (4500 × g for 40 min at 4°C), supernatants collected and autoclaved	MTT metabolic staining assay	-reciprocal toxin titer 3191-shaken -reciprocal toxin titer 358-static
Shaheen *et al.*, 2006	reconstituted dairy based infant food (15 %) - 24 h at 28°C with (60 rpm) or without shaking food	the foods were extracted with pentane, the solvent phase was evaporated to dryness and the residue dissolved in methanol.	boar sperm motility assay	0.2 μg ml^{-1} - shaken 0.2 μg ml^{-1} - static

Shaheen *et al.*, 2006	reconstituted cereal-dairy based infant food (15 %) - 24 h at 28°C with (60 rpm) or without shaking	the foods were extracted with pentane, the solvent phase was evaporated to dryness and the residue dissolved in methanol.	boar sperm motility assay	0.05 μg ml^{-1} - shaken 3 μg ml^{-1} - static
Rajkovic *et al.*, 2006a	penne, potato puree, rice, 10 % slurries and milk -48 h at 28°C with (150 rpm) or without shaking	the diluted foods were extracted with methanol at 100°C for 20 min, the methanol extract evaporated and the dry residue was dissolved in dimethyl sulfoxide	boar sperm motility assay using a computer aided semen analyzer.	penne: 0.2 μg ml^{-1} shaken 3 μg ml^{-1} static potato: 0.2 μg ml^{-1} shaken 4 μg ml^{-1} static rice: 0.7 μg ml^{-1} shaken 2 μg ml^{-1} static milk: < 0.01 μg ml^{-1} shaken 1 μg ml^{-1} static

Abreviations: BHIG - brain heart infusion with 0.1 % (w/v) glucose; RT - room temperature; MTT -3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide

5.2. A physiological and genetic investigation on the diversity of cereulide producing strains

I characterized 13 cereulide producing strains of different origins that were low or high producers of cereulide, ranging from 20 ng mg^{-1} wet weight biomass to 1,800 ng mg^{-1} wet weight biomass (Table 2 in Paper II) when measured from 24 h cultures grown on TSA. Differences around 100 fold or more in the capacity to produce cereulide when cultivated and measured under identical conditions, were found also by other authors (Andersson *et al.*, 2004, Carlin *et al.*, 2006) and made us to raise the question whether there are more differences between the strains.

5.2.1. Physiological properties found to characterise the cereulide producing strains

Out of 10 tested physiological properties, three properties namely haemolysis, tyrosine decomposition and lecithin hydrolysis, divided the emetic strains in two phenotypes, as shown in Table 14 and Fig 6.

We found three *B. cereus* cereulide producing strains, of faecal origin, that were completely non-haemolytic on sheep-blood agar after 24 h at 37°C, resembling in this *B. anthracis*. The cereulide producing strains have been reported with a narrow area of haemolysis (about 1 mm) compared with the non-producers and the type strain (Andersson *et al.*, 2004 and Paper II). Haemolysis in *B. cereus* is attributed to the presence of cereolysin AB and hemolysin BL (HBL). The genes involved in the production of cereolysin AB are the *plc* and *sph* genes producing phospholipase C and sphingomyelinase, (Gilmore *et al.*, 1989). These genes are under the control of a global regulator PlcR (Agaisse *et al.*, 1999). The non-haemolytic strains were also negative for lecithinase, which is a phospholipase C controlled by PlcR. In *B. anthracis,* the pleiotropic regulator PlcR, regulating activities of phospholipase C and haemolysins, is inactive due to the production of a truncated polypeptide (Agaisse *et al.*, 1999). It is possible that the cereulide producing non-haemolytic strains also have a mutation in the *Plc*R gene. This novel type of cereulide producing *B. cereus* is not likely to be recognised as *B. cereus,* since it is lacking these key properties of *B. cereus*, haemolysis and lecithinase.

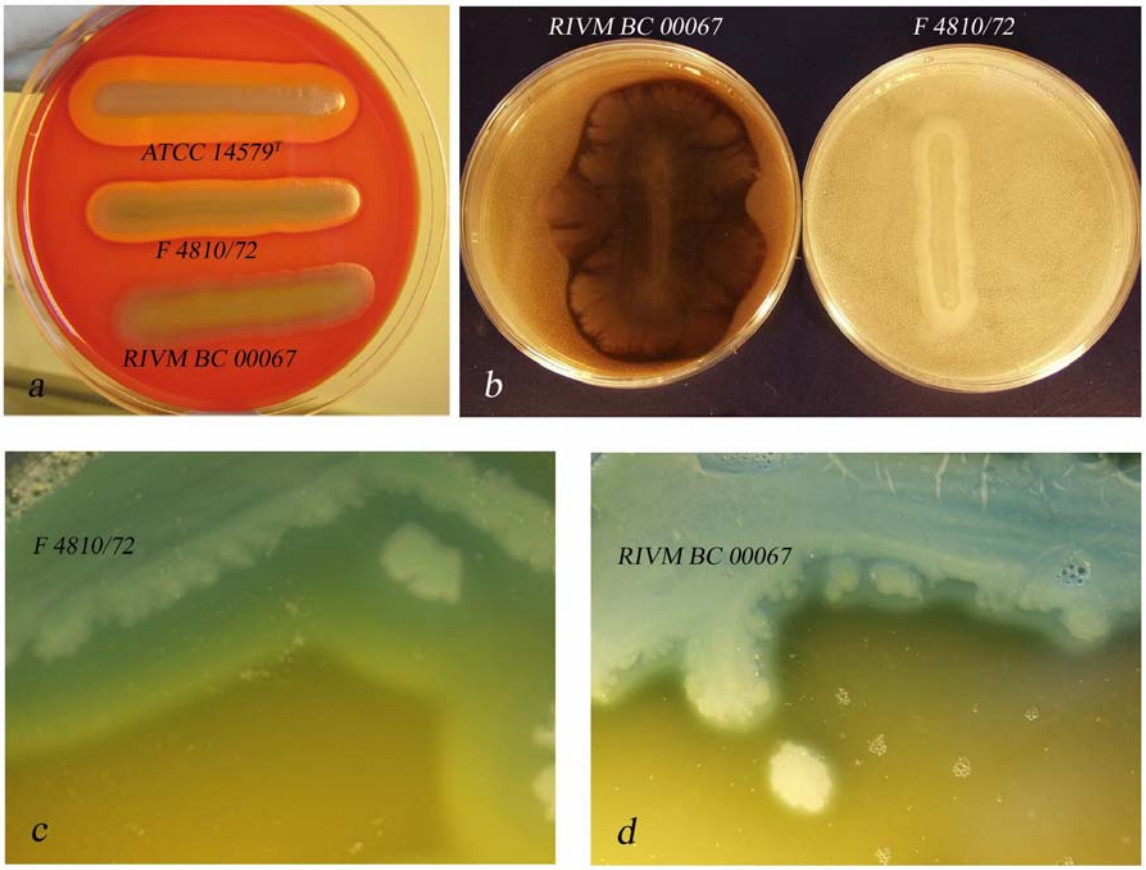


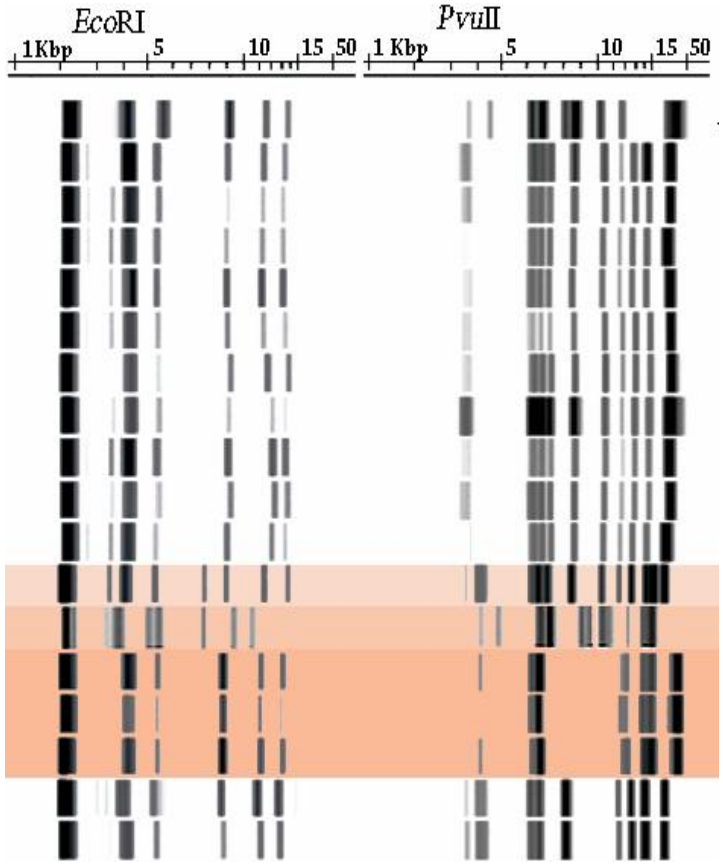
Fig 6. Physiological traits in which the cereulide producing strains of *Bacillus cereus* were found to differ. Panel a, haemolysis on sheep blood agar plates after 24 h at 37°C; panel b, positive (left) and negative (right) tyrosine decomposition; panel c and d, positive and negative lecithine hydrolysis respectively, on polymyxin pyruvate egg yolk mannitol bromothymol blue agar (PEMBA).

None of the cereulide producing strains I investigated hydrolyzed starch or lecithin. These are traits reported to be negative for emetic strains of *B. cereus* by Agata *et al.* (1996), Shinagawa (1993) and Pirttijärvi *et al.* (1999). Thorne *et al.* (2006) described two soil isolates of *B. weihenstephanensis*, a psychrophilic species of *B. cereus* sensu lato (Lechner *et al.*, 1998), that produced cereulide and hydrolysed both starch and salicin. Recently, Colavita *et al.* (2007) claimed to have found a cereulide positive strain of *B. cereus* that hydrolysed starch. However their data were limited to a positive PCR amplification of a 635 bp fragment with primers EM1F and EM1R (Ehling-Schulz *et al.*, 2004). This fragment of unknown function is not a part of the cereulide synthase gene (Ehling-Schulz *et al.*, 2006), although it was initially thought to have some connection with it. Colavita *et al.* (2007) did not show cereulide nor toxicity in their strain. Cereulide producing *B. cereus* that would hydrolyse starch have yet to be found.

Our data show that despite shared properties a phenotypic diversity within cereulide producers certainly exist.

Table 14. Phenotypic (marked in light and dark grey) and genotypic (in pink shades and emboldened) groups found within 14 cereulide producing strains of *B. cereus* of several different origins. The two strains of *B. anthracis* and the type strain of *B. cereus* are given as reference.

B. cereus **and *B.anthracis*** **(*B.a.*) strains**	**Origin**	**Biochemical traits***			**Genetic traits**	
		Tyr.	**haem. (mm)**	**Lec.**	ribopatterns	***adk*** **gene ST**
ATCC 14579^T	type strain	+	4	+		1
B 308	food poisoning, Fin	-	1	+		6
B 203	food, Fin	-	1	+		6
B 412	food poisoning, Fin	-	1	+		6
F 5881/94	food poisoning, UK	-	1	+		6
F 4810/72	food poisoning, UK	-	1	+		6
NC 7401	food poisoning, Japan	-	1	+		6
LKT 1/1	building material, Fin	-	1	+		6
7pk4	indoor wall, Fin	-	1	+		6
UB 1020	human faeces, Fin	-	1	+		6
NS 58	live spruce tree, Fin	-	1	+		**4**
LMG 17604	food poisoning, Belgium	-	1	+		N.D.
RIVM BC 00067	human faeces, NL	+	**0**	-		6
RIVM BC 00068	human faeces, NL	+	**0**	-		6
RIVM BC 00075	human faeces, NL	+	**0**	-		6
B. a. NC 08234-02		-	0	-		7
B.a. CIP 7702		-	0	N.D.		7



***** Tyr. -tyrosine decomposition; Haem. - haemolysis; Lec.- lecithin hydrolysis

ST - sequence type; N.D. - not determined

5.2.2. Genetic traits found to vary among the cereulide producing strains

To see if the cereulide producing strains presented any genetic diversity we sequenced two housekeeping genes (16S rRNA and *adk* genes) and analysed their ribopatterns. We found that the sequence type in *adk* gene for the environmental strain NS 58 differed from that in the other cereulide producing strains in the study (Table 14). The sequencing of housekeeping genes which are genes constitutively expressed was found useful in several phylogenetic studies on *B. cereus* group (Priest *et al.*, 2004; Helgason *et al.*, 2004; Ehling-Schulz *et al.*, 2005a; Vassileva *et al.*, 2007; Olsen *et al.*, 2007; Cardazzo *et al.*, 2008). *Adk* gene encodes for adenylate kinase, which is a phosphotransferase enzyme that catalyzes the interconversion of the adenine nucleotides, and plays an important role in cellular energy homeostasis (2 ADP $\Leftrightarrow$ ATP + AMP). In a recent phylogenetic study using 295 *Bacillus cereus* group members, *adk* gene sequence comparison was found as a tool suitable for screening the genetic variation, and also labour and time saving (Olsen *et al.*, 2007).
We found in Paper II three different ribopatterns among the cereulide producing strains. In Paper III we detected a fourth new ribopattern, for the strain LMG 17604 (Table 14). A further *B. cereus* ribopattern differing from those shown in this study was recently reported by Shaheen *et al.,* (2006).

The cereulide producing strains investigated in this study were found negative for tyrosine decomposition and two strains also for haemolysis. These traits resemble *B. anthracis*. Using the Sacchi *et al.* (2002) typing scheme for 16S rRNA sequence, we found that the 13 cereulide producers in this study belonged to the Sacchi type 7, together with *B. anthracis* strain NC 08234-02 (non-virulent). Due to these findings we investigated the 13 *B. cereus* for the presence of genetic markers of *B. anthracis* (*rpo*B gene, *lef* and *cap*A genes), and found none of these in the cereulide producing strains of *B. cereus*.

The data in Paper II represented the first report on diversity, even limited, for cereulide producing strains. My unexpected findings motivated Vassileva *et al.* (2007) to explore the diversity of cereulide producing strains and to find a new phylogenetic cluster. These authors hypothesized that cereulide-producing strains are progressively diversifying. It is possible that the observed diversification resulted from horizontal gene transfer. The *ces* genes responsible for the cereulide production were found on a large plasmid pCERE01 (Hoton *et al.*, 2005). In Paper III (Fig. 2) we also found that all the cereulide producing strains possessed a similarly sized plasmid (about 200 kb) carrying the *ces* gene. Recently the *B. cereus* strain Kinrooi 5975c, carrying the c*es* bearing plasmid, was shown capable of acting as either donor or recipient in heterologous biparental matings involving large plasmids (up to $\sim$ 400 kb) (Van der Auwera, *et al.*, 2007).

Before my work, the cereulide producing strains of *B. cereus* were known only as a specific subset (Agata *et al.*, 1995, 1996) that differed from the most non-producers by inability to hydrolyse starch and to ferment salicin (Agata *et al.* 1996), low haemolysis (Andersson *et al.*,

2004), and possessing a specific ribopattern (Pirttijärvi *et al.*, 1999). Several genotypic properties also grouped the cereulide producing strains (Priest et. al., 2004) and the available information on cereulide diversity made Ehling-Schulz *et al.* (2005a) to conclude that the cereulide producing strains represented a virulent clone of *B. cereus*. Our findings brought a new light on the cereulide producing strains, showing that diversity of phenotypes and genotypes does exist within this subset of *B. cereus*.

5.3. Influence of environment on the cereulide production

Interactions between the growth environment of *B. cereus* and cereulide production were investigated in Papers I and III. In Paper III we used strains of a wide spectrum of origins, including a set of strains from Paper II showing diversity. Other studies indicated that the amount of emetic toxin in different growth conditions may be very different even when cell density in the cultures was similar, indicating that the toxin production also depended on environmental factors and the strain properties (Szabo *et al.*, 1991; Jääskeläinen *et al.*, 2004; Shaheen *et al.*, 2006; Rajkovic *et al.*, 2006a).

We found that on TSA but not on oatmeal agar, an increase of temperature downregulated the production of cereulide in five of the seven investigated strains (Fig. 1 in Paper III). Two strains (sampled from stools of a patient during and after the acute phase of food poisoning) were little if at all affected in their cereulide production by temperature. Maybe this feature reflects adaptation of the strains to the constant temperature of the gut. All the cereulide producing strains of different origins possessed a similarly sized plasmid (about 200 kb) as the plasmid pCERE01 (Hoton *et al.,* 2005), carrying the *ces* gene (Fig. 2 in Paper III). The differences in temperature response of cereulide production could result from the cross talk of the *ces* genes, present on the plasmid pCERE01 and the different chromosomal or plasmidic backgrounds (Table 15). Such cross talk maybe explains also the different response of the strains towards growth substrate (Paper II and III).

In Paper III (Fig. 3) we found for the strain LMG 17604 a novel ribopattern, different from the ones reported previously (Pirttijärvi *et al.*, 1999; Fig 1 in Paper II). This strain had also a different biotype (Tables 14 and 15). It had a weak caseinase (3 mm) compared to the other cereulide producers (10 mm) even though it grew well and produced cereulide on skim milk agar similarly to the highly caseinolytic F 4810/72. This suggests that the cereulide production was not dependent on protease (caseinase) activity.

Table 15. Cereulide production in different environments and strains properties. The seven cereulide producing strains possessed pCERE01 plasmid, had various ribopatterns (marked in colours), plasmid profiles and biotypes.

Strain	Additional plasmids *	Biotype**	Media supporting high cereulide production (ng cereulide mg^{-1} wet weight)	Influence of temperature increase (from 23 to 39°C) on cereulide production
NS 58	2	1	160-920 on TSA and 150-530 on blood agar	downregulated on TSA, but not on oatmeal agar
F 4810/72	2	1		
NC 7401	1	1		
UB 1020	0	1		
LMG 17604	2	2	200-520 on TSA, 120-330 on blood agar and 110-490 on oatmeal agar	downregulated on TSA, but not on oatmeal agar
RIVM BC00067	0	3	blood agar (170-380)	unaffected
RIVM BC00075	0	3		

***** The numbers indicate the number of other plasmids detected in the strain besides the pCERE01 (Paper III). The number of additional plasmids in F 4810/72 is inferred from Hoton *et al.*, 2005.

****** Biotypes:

1. negative tyrosinase, low haemolysis (1 mm), positive lecithinase and caseinase (10 mm), sensitive to 10 of the tested antimicrobials but resistant to penicillin, oxacillin and bacitracin.
2. negative tyrosinase, low haemolysis (c.a. 1 mm), positive lecithinase but weak caseinase (3 mm), sensitive to the 13 tested antimicrobials including penicillin, oxacillin and bacitracin.
3. positive tyrosinase and caseinase (10 mm), no haemolysis, negative lecithinase, sensitive to 10 of the tested antimicrobials and intermediate sensitive to bacitracin but resistant to penicillin, oxacillin.

When we found that the type of media influenced the cereulide production and knowing that cereulide is a powerful potassium ionophore (Teplova *et al.*, 2006), we decided to investigate the possible correlation with the amino acid and alkali metal composition of the media (Paper III). We found three parameters, $[Na^+]$, $[K^+]$:$[Na^+]$ and glycine, significantly associated with cereulide production. An increasing of $[K^+]$:$[Na^+]$ ratio of the growth media, correlated significantly positively with high cereulide production but only when supported by constant concentrations of glycine and of $[Na^+]$. Similarly, rising concentration of glycine in the medium would promote cereulide production provided that $[Na^+]$ and $[K^+]$:$[Na^+]$ ratio remained unchanged. On the other hand increases in $[Na^+]$ was correlated with a low cereulide production, when $[K^+]$:$[Na^+]$ ratio and glycine remained constant. Since an increase of $[Na^+]$ would not decrease the cereulide production unless the $[K^+]$:$[Na^+]$ ratio and the glycine content are kept constant, the mechanism is unlikely a simple salt stress.

In the present study, of the total peptide bonded and free amino acids, glycine was the only amino acid that correlated with high cereulide. Jääskeläinen et al. (2004) reported stimulatory effects of L-valine and L-leucine supplementation on cereulide production by *B. cereus* strains originating from food and food poisoning incidents. These amino acids increased 4 to 10 fold the cereulide production but only when added in free form. When added as peptone there was no effect (Jääskeläinen *et al.*, 2004). The biological significance of glycine with regard to cereulide production is unknown and the present state of research is too immature for speculations in this direction.

When we designed the experimental setup with the temperature range in which the seven strains in Paper III were investigated for cereulide production, we were particularly interested to include the 37°C. We had three strains originated from patients faeces and we wanted to know if this temperature such as it is in the gut would allow the cereulide production. We found that 37°C was not a limiting factor for cereulide production with up to 250 ng mg^{-1} wet weight (Table 2 in Paper III). Oxygen concentration in human gut varies from about 22 to 25 % of that in the air (Wilson, 2005), which means around 4.6-5.2 % oxygen in the gas present in the gut. At more than 4.5% O_2, high amounts (c.a 1225 ng mg^{-1}) of cereulide were reported but no toxin was found in nitrogen atmospheres containing 1.6 % and 0.7 % of oxygen (Rajkovic *et al.*, 2006b). Jääskeläinen (2008) found that an N_2 atmosphere in the absence of CO_2 did not allow the cereulide production but in the presence of CO_2 cereulide was produced also in anaerobiosis. In the colon CO_2 is one of the principal gases produced (Suarez *et al.*, 1997). These data suggest that indeed cereulide production in the gut would be possible.

5.4. Amylosin production by *B. subtilis* and *B. mojavensis*

5.4.1. Screening of the strains for toxicity, and amylosin detection

B. subtilis F 2564/96 was isolated from an incident of food poisoning involving chicken korma from an Indian take-away. The symptoms, 8 h after consumption, consisted of abdominal pain, vomiting and diarrhoea. The bacterial count in the food was 9.5×10^6 cfu g^{-1} (determined at Health Protection Agency, Centre for Infections London, UK). When grown on laboratory media this strain produced a methanol soluble substance that inhibited the spermatozoa motility after 30 min of exposure to 8 µg dry weight ml^{-1} extended semen (Table 2 in Paper III). The toxin depolarized the mitochondria, $\Delta\Psi_m$, after 1 d of exposure to 0.8 µg dry weight ml^{-1} extended semen, Fig 7. Plasma membrane damage was noticed only after 3 d of exposure and with a higher dose (6.3 µg dry weight ml^{-1})(Table 2 in Paper III).

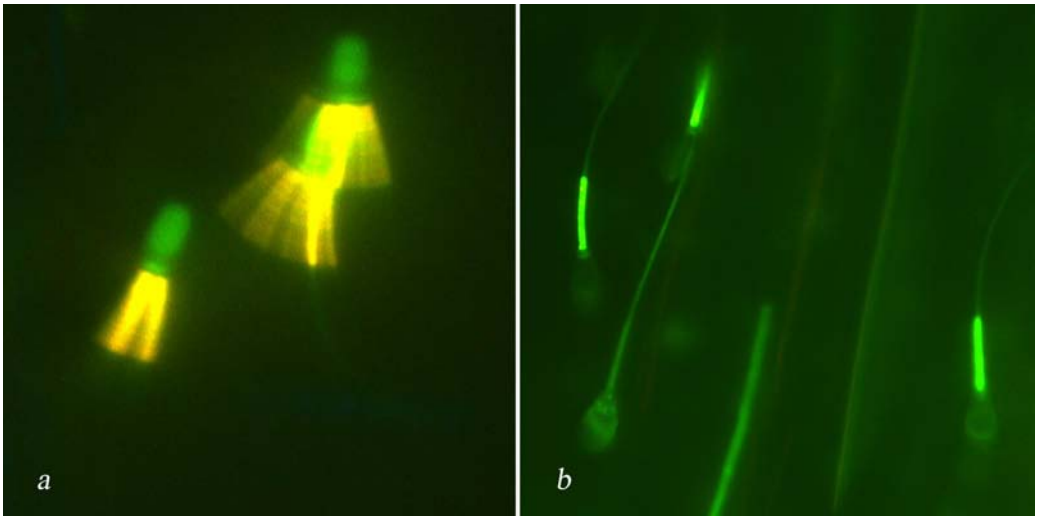


Fig. 7. Effects of the methanol extracted toxin from the strain *B. subtilis* F 2564/96 on boar sperm visualised by JC-1 staining. Panel a shows the sperm cells exposed for 1 d to vehicle only (methanol). Panel b shows sperm cells after 1 d of exposure to the extract from *B. subtilis* F 2564/96 (0.8 µg dry weight ml^{-1}). Image taken by Camelia Constantin and Maria Andersson.

The extract from *B. subtilis* F 2564/96 was tested on human colon adenocarcinoma cell line (Caco-2). Already after 3 h of exposure to 25 µg dry weight ml^{-1}, the mitochondria became depolarised. Concentrations of $\geq$ 50 µg (dry weight) ml^{-1} induced plasma membrane damage, visualised in Fig. 8 by staining with Calcein AM and propidium iodide (PI).

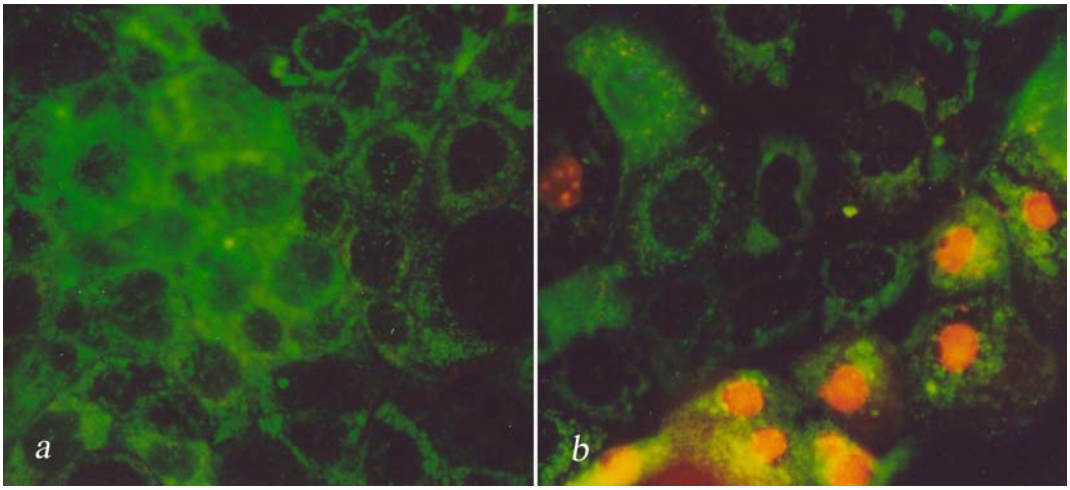


Fig.8. The effects of the methanol soluble substances from *B. subtilis* F 2564/96 on the membrane integrity of Caco-2 cells stained with Calcein AM and PI. Panel a shows the cells exposed for 5 h to the vehicle only (methanol). Panel b shows the cells after 5 h of exposure to 50 µg (dry weight) ml^{-1} of the extract from the strain F 2564/96. Image taken by Camelia Constantin and Vera Teplova.

B. subtilis strain F 2564/96 thus produced substances toxic to boar sperm cells and to human Caco-2 cells. Based on the effects observed with boar sperm cells we noticed that the toxicity pattern resembled that induced by similarly prepared extracts of *B. amyloliquefaciens* 19b, an amylosin producer isolated from a mouldy building (Mikkola *et al.*, 2004). Fractionation by HPLC and subsequent ES-IT-MS analysis of the toxic peak showed that the substance from *B. subtilis* strain F 2564/96 indeed was amylosin (Fig. 2a. in Paper IV).

These exciting results were a motivation to screen for toxicity a collection of 93 food-borne isolates of *Bacillus* species in our laboratory. These isolates (Table 1 in Paper IV), other than *B. cereus*, were associated (75 isolates) or not associated (18 isolates) with food-borne illness. Results of this work are presented in Paper IV. Out of this collection we found four toxic isolates (EELA 2290, EELA 2291, EELA 2292, EELA 2294), identified as *B. subtilis* and one isolate EELA 2293 identified as *B. mojavensis* (Paper IV). These strains were isolated from a pumpkin curry dish associated with a food-borne illness in which six persons were affected by unexpected abdominal pain, at least one having also fever about 39°C. The extracts prepared from the *B. subtilis* strains EELA 2290, EELA 2291, EELA 2292, EELA 2294 inhibited the boar sperm cells motility after 30 min exposure concentrations ranging from 15 to 31 µg dry weight ml^{-1} (Table 2 in Paper IV). The extract from *B. mojavensis* EELA 2293 induced the loss of sperm motility at 4-8 µg dry weight ml^{-1} after 30 min of exposure and loss of $\Delta\Psi_m$ after 1 d at 0.2 µg dry weight ml^{-1} (Table 2 in Paper IV). Thus, the six strains, one *B. subtilis* from UK, four *B. subtilis* from Finland and one *B. mojavensis* from Finland, showed similar cellular and subcellular toxicity with boar sperm cells. Only the

concentration of the toxic end points varied from strain to strain (Table 2 in Paper IV) indicating that the strains may differ in their quantities of toxin produced.

The extracts with observed toxicity were fractionated by HPLC and the fractions tested for toxicity by the boar sperm motility inhibition assay. The fractions found toxic in this assay, were analysed by ESI-IT-MS and found to contain amylosin (Paper IV). Other fractions contained also surfactin but these were not toxic in the sperm motility inhibition assay. No further toxic substances besides amylosin were found in these strains. Thus, the toxic compound synthesised in the five strains of *B. subtilis* and one *B. mojavensis* strain involved in food-borne illness was most likely amylosin.

B. mojavensis B 31 was reported by From *et al.* (2007a) to contain surfactin. We analysed also this strain and confirmed the finding of From *et al.* (2007a) but we also showed that the boar sperm toxicity of this strain was unlikely to have been caused by surfactin as assumed by From *et al.* (2007a). We found that this strain also produced amylosin (Fig. 2b in Paper IV) which is about 200 times more toxic than surfactin (Mikkola, 2006). From *et al.* (2007a) evidence was indirect, based on the observation that the toxicity was abolished when a gene essential for surfactin synthesis (*sfp*) was deleted. However they did not take into account that the *sfp* gene required for surfactin production might also be needed in the synthesis of other non-ribosomal peptides. It could also be possible that the deleted area affected amylosin synthase gene.

5.4.2. Amylosin production in different temperatures and atmospheres

Once we found that strains of *B. subtilis* and *B. mojavensis* isolated from foods connected to food-borne illness produced amylosin we went on to search for the conditions with relevance to the storage of food promoting or restricting amylosin production. I investigated amylosin production at temperatures from 11 to 56°C and atmospheres with different contents of O_2 and CO_2. The results are described in Table 4 and Fig. 4 in Paper IV. We found that on TSA plates, the *B. mojavensis* strains EELA 2293 and B 31 produced most amylosin (800-900 ng mg^{-1} wet weight bacteria) at 11-21°C, whereas the *B. subtilis* strain F 2564/96 produced most amylosin (700 ng mg^{-1} wet weight bacteria) at 21-37°C. No amylosin (< 20 ng mg^{-1} wet weight bacteria) was detected at ≥ 46°C. Thus, amylosin production was not as thermotolerant as growth: all three strains grew up to 54-56°C (Fig 4 in Paper IV).

From applying different atmospheres for growth we learned that *B. subtilis* strains F 2564/96 and *B. mojavensis* strain EELA 2293 produced amylosin in air and in 8 - 9 % O_2 with 7 - 8 % CO_2 but little or no amylosin was produced under anoxic conditions (< 1 % O_2 with 9 - 13 % CO_2). Interestingly, *B. mojavensis* strain B 31 produced amylosin in all these atmospheres including the anoxic (Table 4 in Paper IV). Tam et al. (2006) showed that orally

administered *B. subtilis* spores germinate, proliferate and then resporulate within the gut of a mouse model. We found that 37°C was supportive for amylosin production of *B. subtilis* strain F 2564/96 and, although in little amount, amylosin was produced in anoxic conditions (Table 4, Paper IV). Thus, cannot be excluded the possibility that some strains of *B. subtilis* might produce amylosin in the gut.

Our data show that amylosin producing strains of *B. subtilis* or *B. mojavensis* may pose a risk for foods stored unrefrigerated and ingested spores may be a toxin risk in the gut.

6. Conclusions

The work presented in my thesis started in a time when little was known about the physiology of cereulide producing strains of *B. cereus* and nothing was known about their genetic determinants concerning cereulide synthesis. The assays available to detect and quantify the production of cereulide were measurements of toxicity titers using cell lines or sperm cells as target. The production of heat-stable toxins by *Bacillus* species other than *B. cereus* was even less known. The major outcomes of my work are the following:

1. We designed the first chemical assay for the detection and quantification of cereulide. The assay is based on liquid chromatographic separation followed by ion trap mass spectrometric detection and identification. The molecular adduct $[NH_4^+]$ *m/z* 1171 was selected for cereulide detection. For quantitation, valinomycin or cereulide molecular adducts $[NH_4^+]$ *m/z* 1129 and *m/z* 1171 respectively, were used as calibration standards. Bioassay for toxicity was performed using the sperm motility inhibition test with the same extract used for the chemical assay. The results of chemical assay matched with those of the bioassay and verified that cereulide was responsible for toxicity in the *B. cereus* extracts.

2. Using the newly developed chemical assay we showed that cereulide production by *B. cereus* grown in trypticase soy broth started in the beginning of stationary phase of growth. *B. cereus* does not sporulate in this medium and thus cereulide was produced independent from sporulation.

3. We found two different phenotypes and four different genotypes of cereulide producing *B. cereus*. All these different cereulide producers carried the cereulide synthetase *ces* gene on a similar large sized (c.a. 200 kb) plasmid pCERO1. I conclude that the phenotypic differences reflected different chromosomal backgrounds of the cereulide producing strains and were not likely encoded by the acquired plasmid carrying the cereulide synthetase *ces* gene. My results showed for the first time that within the cereulide producing strains there is diversity even if limited.

4. Seven *B. cereus* strains representing different phenotypes, genotypes and origins of isolates, were studied for temperature response of cereulide production. Cereulide content of *B. cereus* biomass grown on trypticase soy agar was highest at 21-23°C and low at 11 °C and from non-detected to low at 40°C. An increase of temperature from 23 to 39°C downregulated the cereulide production in five strains. In two strains of *B. cereus* that originated from the gut of food poisoning patients, the cereulide content was similar, independent of the temperature (from 23 to 39°C). On oatmeal agar, however, the cereulide production was independent on the temperature for all the strains. These results show that the temperature dependence of cereulide production is coupled to the composition of the growth medium.

5. To find out how cereulide production was affected by the growth substrate we searched for correlations between the amino acids composition of the growth medium, its content of sodium and potassium ions and cereulide production. We found that the substrate contents of glycine, $[Na^+]$, and the $[K^+]$:$[Na^+]$ ratio, were significantly associated with cereulide production. An increase of $[K^+]$:$[Na^+]$, promoted cereulide production only when $[Na^+]$ and the glycine content remained constant. Increased concentrations of glycine would upregulate cereulide production, only when the other two parameters remained unchanged. $[Na^+]$ would decrease cereulide production provided that the $[K^+]$:$[Na^+]$ and glycine remained constant. I conclude from the results that the composition of the substrate had a major impact on cereulide synthesis and that the increase of production in response to increased $[K^+]$:$[Na^+]$ is not a simple salt stress.

6. Using the newly developed chemical assay it was possible to assess the amounts of cereulide produced in response to access to oxygen. We found that the cereulide production in trypticase soy broth cultures was promoted by shaking compared with the static conditions. These results are in agreement with the findings of some previous studies who found that aeration by shaking was beneficial for the emetic toxin production in substrates of homogenous liquids like culture media. In slurry substrates like diluted foods other studies reported an inhibitory effect of shaking on cereulide production. We conclude that that the amount of suspended solids and the consistencies may have influenced the different effects of shaking on the cereulide production.

7. The novel chemical assay developed in this thesis validated valinomycin as a surrogate standard for cereulide to calibrate the assay. This is useful for laboratories because purified cereulide is presently not commercially available.

8. From 75 isolates of *Bacillus species*, other than *B. cereus*, associated to incidents of food-borne illness we found five isolates of *B. subtilis* and one of *B. mojavensis* that induced toxicity in cell toxicological assays. No toxin producing isolates were found in the 18 strains not associated with food borne illness. The substance produced by the six toxic isolates was identified as amylosin. It may have been the agent responsible for the food-borne illness, since no other toxic substance was found from the strains. My results show that amylosin is a toxin produced by more than one *Bacillus* species in addition to the earlier known *B. amyloliquefaciens.*

9. We found that *B. mojavensis* produced most amylosin at 11-21°C whereas *B. subtilis* produced the best at 21-37°C. No amylosin was produced at ≥ 46°C despite the growth of the strains up to 54-56°C. The temperature requirement for amylosin production for both species was less thermotolerant than that for growth. Amylosin was produced by both species in air and under reduced O_2 with increased CO_2 but *B. mojavensis* produced amylosin also in absence of O_2. Carbon dioxide enriched or anoxic atmosphere thus does not prevent amylosin

production in food stored unrefrigerated (≥ 10°C). This implies a risk for food safety when amylosin producing strains of *B. subtilis* or *B. mojavensis* are present

7. Tiivistelmä

Bacillus cereus on elintarvikkeiden yleisimpiä bakteereja. Se tuottaa useita erilaisia enterotoksiineja, joista yksi on kuumennusta kestävä peptidi (oksetus- eli emeettinen toksiini, kereulidi), muut toksiinit kuumennusherkkiä. Oksetustoksiinin määritys elintarvikkeissa perustui myrkkyvaikutusten toteamiseen, kunnes viime vuosikymmenen kuluessa tämän toksiinin rakenne selvitettiin. Ensimmäinen, toksiinin rakenteeseen perustuva kereulidin kvantitointimenetelmä on minun kehittämäni, yhdessä opettajieni kanssa. Menetelmä perustuu korkean erotuskyvyn nestekromatografiaan (HPLC) yhdistettynä joniloukku massaspektrometriaan. Kalibrointiin käytin valinomysiiniä (kereulidin sukulaismyrkky) ja tuottajabakteerista eristettyä kereulidia. Kvantitointi perustuu kummankin yhdisteen [NH_4^+] -adduktien massajoneihin, jotka ovat edelliselle m/z 1128.9 ja jälkimmäiselle m/z 1171. Tämä luotettava työmenetelmä mahdollisti läpimurron kansainvälisessä kereuliditutkimuksessa. Sen avulla voitiin ensimmäistä kertaa osoittaa että *B. cereus*'ksen tuottama toksiini, eikä välttämättä itse bakteeri, oli syyllinen elintarvikkeen aiheuttamaan ruokamyrkytykseen.

Ennen väitöstutkimustani uskottiin yleisesti, että kereulidia tuottavat *B. cereus* kannat ovat toistensa klooneja, eli homogeeninen ryhmä samankaltaisin ominaisuuksin. Tämä käsitys perustui siihen, että oli tutkittu vain ruokamyrkytystapauksista löydettyjä bakteerikantoja. Tutkin työssäni kereulidia tuottavia kantoja monista eri lähteistä ja vertasin niitä toisiinsa polyfaasisilla menetelmillä, eli yhdistämällä biokemian, fysiologian ja genetiikan keinoja. Tulokset osoittivat että kereulidin tuottajat eroavat toisistaan sekä perintötekijöiltään että biologisilta ominaisuuksiltaan. Kereulidin tuottajilta löytyi kaksi erilaista adenylaattikinaasin geeniä (*adk*). Niiden ribosomaalista RNAta tuottavat geenit olivat sijoittuneet erilaisiin geeniympäristöihin eri kannoissa, mikä näkyi kolmena erilaisena ribopattern'ina, kun genomia pilkottiin restriktioentsyymeillä *Eco*RI ja *Pvu*II. Eri kannat poikkesivat toisistaan myös kyvyissään hajottaa tyrosiinia, veren punasoluja (hemolyysi) ja lesitiiniä (kaksi biotyyppiä kussakin). Tutkimukseni oli ensimmäinen osoitus heterogeenisyydestä kereulidia tuottavien *B. cereus* bakteerien ryhmässä.

Jotta kereulidimyrkyn muodostumisen riskejä pystyttäisiin arvioimaan ja hallitsemaan, on välttämätöntä tuntea ne tekijät, jotka lisäävät tai vähentävät sen tuottoa elintarvikkeessa. Valitsin tähän tutkimukseeni useita ominaisuuksiltaan erilaisia kereulidin tuottajakantoja, ja tutkin niiden toksiinituottoa erilaisissa vakioympäristöissä. Tulokset osoittivat, että ympäristön lämpötila rajoitti tiukemmin kereulidin tuottoa kuin tuottajakantojen kasvua. Useimmat kannat tuottivat kereulidia eniten kun lämpötila oli 20 - 23ºC. Sensijaan ihmisen ulosteista eristettyjen kantojen toksiinintuottotahti oli vakio lämpötila-alueella 23 ºC - 39ºC. Havaitsimme myös, että 37º C lämmössä tuotetun kereulidin määrä riippui joillakin mutta ei kaikilla kannoilla ravinnnon laadusta, vaikka ravinnosta riippuvuutta ei havaittu kun samoja kantoja tutkittiin huoneenlämmössä. Kereulidin tuoton riippuvuus ympäristön ravinteista ja lämpötilasta lienee ratkaisevaa sille, tuottaako kyseinen kanta kereulidia jossakin tietyssä ruuassa tai, kun ruoka on syöty, suolistossa. Valitsimme lähempään tutkimukseen kuusi

kasvualustaa, jotka suosivat tai hidastivat kereulidin tuottoa. Koostumusanalyysien tilastollinen tarkastelu paljasti merkitsevän positiivisen korrelaation kereulidin tuoton ja kasvualustan [K^+]:[Na^+] suhteen välillä, mutta vain jos eräät toiset muuttujat, glysiinin ja natriumin ([Na^+]) pitoisuudet vakioitiin. Aminohapoista vain glysiinin pitoisuus edisti korkeaa kereulidin tuottoa. Glysiini on elintarvikelisäaine (E 640), käyttö hyväksytty Euroopan Unionissa elintarvikkeiden aromivahventeena, kosteuden ja happamuuden tasaajana. Sen käytölle ole säädetty määrällisiä rajoituksia.

Bacillus subtilis ryhmä kuuluu lämpökestoisia itiöitä tuottaviin ympäristöbakteereihin kuten *B. cereus*. Eri *Bacillus* lajeja on kirjallisuudessa silloin tällöin epäilty ruokamyrkytysten aiheuttajiksi. Väitöstyössäni tutkin ruokamyrkytyksiä aiheuttaneiksi epäillyistä elintarvikkeista eristettyjä *B. subtilis* ja *B. mojavensis* kantoja. Tunnistin osan niistä toksiinin tuottajiksi. On mahdollista, että sairastumisen aiheutti amylosiini, koska amylosiinin tuottajien lisäksi ei löytynyt muita toksiinin tuottajia. Nämä tutkimustulokset olivat ensimmäinen kerta kun amylosiinia tuottavia bakteereja löydettiin ruuasta. Lämpötilavasteita tutkiessani havaitsin, että amylosiinin tuotto mesofiilisellä kannalla *B. subtilis* F 2564/96 edellytti korkeampaa lämpötilaa kuin psykrofiileillä *B. mojavensis* kannoilla eela 2293 and B 31. Havaitsin myös, että näiden bakteerien amylosiinin tuotto ei edellyttänyt hapen läsnäoloa. Muunnettuun ilmakehään pakatuissa valmisruuissa amylosiinin muodostus voi olla mahdollista, jos niissä on toksigeenisiä *B. subtilis* tai *B. mojavensis* kantoja ja jos lämpötila ylittää 10°C.

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