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REVIEW ARTICLE

Regulation of toxin production by Bacillus cereus and its food safety implications

Siele Ceuppens[1,3]*, Andreja Rajkovic[1], Marc Heyndrickx[2], Varvara Tsilia[1,2], Tom Van De Wiele[3], Nico Boon[3], and Mieke Uyttendaele[1]

[1]Ghent University, Faculty of Bioscience Engineering, Laboratory of Food Microbiology and Food Preservation (LFMFP), Ghent, Belgium, [2]Ministry of the Flemish Community, Institute for Agricultural and Fisheries Research (ILVO), Technology and Food Science Unit, Melle, Belgium, and [3]Ghent University, Faculty of Bioscience Engineering, Laboratory of Microbial Ecology and Technology (LabMET), Ghent, Belgium.

Abstract

Toxin expression is of utmost importance for the food-borne pathogen B. cereus, both in food poisoning and non-gastrointestinal host infections as well as in interbacterial competition. Therefore it is no surprise that the toxin gene expression is tightly regulated by various internal and environmental signals. An overview of the current knowledge regarding emetic and diarrheal toxin transcription and expression is presented in this review. The food safety aspects and management tools such as temperature control, food preservatives and modified atmosphere packaging are discussed specifically for B. cereus emetic and diarrheal toxin production.

Keywords: Bacillus cereus, toxin regulation, cereulide, enterotoxin, food safety management tools

1 Introduction

B. cereus is a Gram-positive spore forming bacterium that can cause systemic and local infections, including fulminant bacteremia, meningitis, brain abscesses, endophthalmitis, pneumonia, and gas gangrene-like cutaneous infections (Bottone, 2010) and two types of food-borne illness, i.e., the emetic and diarrheal syndrome (Stenfors Arnesen et al., 2008). Different toxins are implicated in both food-borne diseases, which result in different symptoms and incubation times. The emetic syndrome starts 1–5 h after the consumption of food contaminated with emetic *B. cereus* and/or preformed emetic toxin cereulide. The main symptoms are nausea and vomiting. The diarrheal syndrome is caused by enterotoxin production in the small intestine by vegetative *B. cereus* cells. As a consequence this type of disease starts later, 8 - 16 h after consumption of food contaminated with living *B. cereus* cells and/or spores. The most important symptoms are abdominal pain and diarrhea. In all types of *B. cereus* disease the virulence is closely linked with toxin production (Stenfors Arnesen et al., 2008; Bottone, 2010). Therefore this review addresses the regulation of toxin expression. Firstly the protein properties and clinical aspects of the toxins are briefly summarized. Secondly the regulation of toxin gene expression is discussed more in detail, in function of various bacterial and environmental parameters. Thirdly the potential exploitation of current knowledge about toxin expression is translated into recommendations or lessons to be learnt with regard to food safety management measures.

2 Cereulide, the emetic toxin

2.1 Protein properties of cereulide

Cereulide is the main virulence factor of emetic *B. cereus* strains, causing the emetic food poisoning syndrome. This toxin is a cyclic dodecadepsipeptide, namely [D-O-Leucine-D-Alanine-O-Valine-D-Valine]$_3$ (Agata et al.,

Address for correspondence: MiekeUyttendaele; Ghent University, Faculty of Bioscience Engineering, Laboratory for Food Microbiology and Food Preservation (LFMFP), Coupure Links 653, B-9000 Gent, Belgium; phone: +32 (0)9 264 61 78; fax: +32 (0)9 225 55 10; E-mail: Mieke.Uyttendaele@UGent.be; Webpage: www.foodmicrobiology.ugent.be

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1994). Cereulide shows structural and functional similarities to valinomycin, a potassium specific transporter that facilitates the movement of K^+ ions through lipid membranes following the electrochemical potential gradient. The emetic toxin is extremely stable. It can withstand a heat treatment of 2 h at 121°C at pH 7.0 (Rajkovic et al., 2008), frying, roasting, and microwave cooking (Agata et al., 2002), exposure to pH values from 2 to 11 and protease activity, for example digestion with pepsin and trypsin (Shinagawa et al., 1996). Accumulation of cereulide in food thus poses a health threat, as this toxin will not be inactivated during further food processing or preparation, nor during gastrointestinal passage.

2.2 Clinical aspects

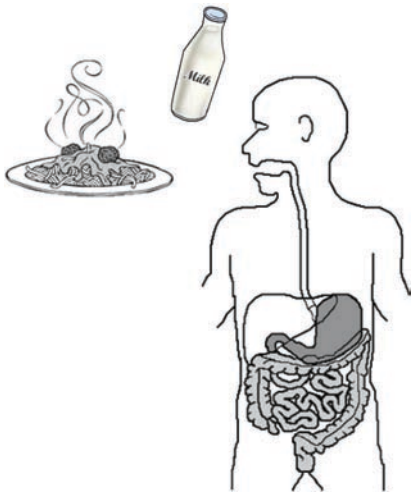
Cereulide causes emesis in Asian house shrew (*Suncus murinus*) at approximately 8 μg of cereulide per kg body weight (Agata et al., 1995b). After ingestion, cereulide reaches the stomach and duodenum and as currently understood it binds to serotonin 5-HT_3 receptors, which results in stimulation of the vagus afferent nerves and subsequently in vomiting. Cereulide also causes emesis in humans and the toxin can be lethal in the cases of brain edema and fulminate liver failure due to inhibition of mitochondrial fatty-acid oxidation (Mahler et al., 1997; Shiota et al., 2010). The mitochondrial and cellular damage is caused by the ionophoric properties of cereulide, noted as mitochondrial swelling and vacuolization of Hep-2 cells (Agata et al., 1994; Agata et al., 1995b). Furthermore cereulide has been shown to inhibit the natural killer cells (T cells) of the human immune system (Paananen et al., 2002). Patients suffering from cereulide food poisoning revealed cereulide to be present in gastric fluid (0.004 μg/mL), blood serum (0–0.004 μg/mL), urine (0–0.008 μg/mL) and stool (0.16–0.80 μg/g) (Shiota et al., 2010). Cereulide is probably excreted in urine in order to detoxify the body. Admission of intravenous fluids and blood-purification therapy can aid this process and thus the recovery of the patients. The food implicated in emetic food poisoning outbreaks show 0.01 to 1.28 μg of cereulide per gram food (Agata et al., 2002). The consumption of 100 g food by a person of 70 kg results in a dose of 0.02–1.83 μg cereulide per kg body weight, which is lower than for the Asian house shrew. Emetic *B. cereus* strains in the exponential growth phase produce 0.004 to 0.13 μg of cereulide per 10^6 cells (Jaaskelainen et al., 2004; Haggblom et al., 2002). Therefore *B. cereus* populations of 10^5 to 10^8 CFU/g food can produce sufficient amounts of emetic toxin to cause food-borne illness. This is not always the case, because various environmental factors influence cereulide production (Figure 1).

2.3 Cereulide genes

Cereulide is constructed by a non-ribosomal peptide synthase (NRPS), encoded in the cereulide synthase gene cluster (*cesHPTABCD* genes), located on pCERE01, a pXO1-like megaplasmid of 200–270 kb (Ehling-Schulz et al., 2006a; Horwood et al., 2004; Hoton et al., 2005). The *cesPTABCD* genes form an operon that is co-transcribed, while the putative hydrolase/acyltransferase *cesH* gene is under control of its own promoter (Dommel et al., 2010; Ehling-Schulz et al., 2006a). The large structural genes *cesA* (10 kb) and *cesB* (8 kb) are responsible for the activation and incorporation of two monomers in the peptide chain, while the *cesP* gene encodes a 28.9 kDa 4′-phosphopantetheinyl transferase for priming the NRPS, the *cesT* gene is coding for a thioesterases which removes misprimed monomers and finally a putative ABC transporter is encoded in the *cesC* and *cesD* genes (Ehling-Schulz et al., 2006a).

2.4 Prevalence and polymorphisms of cereulide genes

Cereulide producing *B. cereus* strains comprise a subgroup that carry the plasmid-encoded *ces* genes. The overall prevalence of emetic *B. cereus* group strains is low, approximately 1.5%, but it is much higher in non-random food and clinical samples, namely 32.8% (Hoton et al., 2009). Despite their relatively low prevalence, emetic



Factors involved in the toxin expression regulation of *Bacillus cereus*

- Temperature
- Atmospheric composition
- Oxidation-reduction potential
- pH
- Agitation
- Consistency
- Carbohydrate availabilityand relative ratios
- Cation concentrations and relative ratios

Figure 1. Overview of the food and gastrointestinal factors involved in the regulation of toxin expression by *Bacillus cereus*.

strains are widely distributed, as shown by isolates from various clinical, food and environmental samples. For example, 4.7% of the *B. cereus* isolates from ice cream were emetic strains, 1.6% in fish products, 11.0% in ready-to-eat foods, 3.9% in other food samples not implicated in food poisoning, 1.7% in soil, 1.5% in cow milk, 1.2% in cow bedding and 3.9% in farm rinsing water (Yang et al., 2005; Altayar & Sutherland, 2006; Svensson et al., 2006; Messelhausser et al., 2010; Rahmati & Labbe, 2008; Wijnands et al., 2006a). Emetic strains often display certain specific characteristics compared to other *B. cereus* strains, such as no or weak haemolysis, the inability to hydrolyze starch and salicin, an elevated lower temperature limit for growth of minimum 10°C and an increased heat resistance of their spores (Andersson et al., 2004; Shinagawa, 1993; Carlin et al., 2006). As a consequence emetic strains were initially considered to be a clonal *B. cereus* lineage based on phenotypic characteristics including serotype (Agata et al., 1996) and genetic similarities observed through multilocus sequence typing (MLST) (Priest et al., 2004). However, more genetic variation was found and a new independent emetic *B. cereus* lineage was discovered (Apetroaie et al., 2005; Vassileva et al., 2007). Food-borne isolates were often indistinguishable regardless of their origin, while environmental and intestinal isolates demonstrated more phenotypic variability. Today it seems that approximately 7.5% of the emetic *B. cereus* strains is capable of starch hydrolysis, salicin fermentation and enterotoxin Hbl production (Kim et al., 2010), although it was originally thought these strains never showed these features. In addition, the *nhe* genes have been detected in the majority of the emetic strains (Kim et al., 2010; Yang et al., 2005; Rahmati & Labbe, 2008). Therefore it seems plausible that emetic strains can also cause diarrhea through enterotoxin production. The minimal growth temperature of emetic strains was lowered by the discovery two emetic *B. weihenstephanensis* strains (Thorsen et al., 2006) and three emetic *B. cereus* strains which are able to grow at 8°C (Hoton et al., 2009). Furthermore, these strains possess other variants of the *ces* operon located on a larger unrelated plasmid or even their chromosome, showing more variability in the cereulide genes than previously thought and that cereulide (*cesB*) polymorphisms do exist.

The cereulide synthase genes *cesA* and *cesB* of emetic reference strain *B. cereus* AH187 (F4810/72) were used for a similarity search in the NCBI database of reference genomic sequences using megablast (Table 1). The search showed that the clinical emetic strain *B. cereus* H3081.97 possesses nearly identical (99%) cereulide synthase genes. A very small part of this large operon (3%) is conserved (92% identity) in some other strains, namely the environmental strains *B. cereus* BGSC 6E1, AH676, and *B. mycoides* Rock3-17 from soil isolates, the bio-insecticidal strain *B. thuringiensis* BMB171 and the clinical strain *B. cereus* 95/8201 from a endocarditis case. Further similarity searches using NCBI Blastx showed that these sequences correspond a variety of hypothetical proteins and proteins such as metallo-beta-lactamase/rhodanese-like domain proteins, FAD-dependent pyridine nucleotide-disulphide oxidoreductases, sensory box/GGDEP proteins, etc. (data not shown). In order to assess the extent of variation in the *cesB* polymorphisms, a more in-depth search was performed partially sequenced *cesB* gene from emetic *B. weihenstephanensis* MC67 (Thorsen et al., 2006). This confirms the existence of two emetic lineages with different *cesB* polymorphisms (86% identity), represented by emetic reference strain *B. cereus* AH187 (F4810/72) and emetic *B. weihenstephanensis* MC67 (Table 2). Furthermore also weaker homology (30–40% identity) was found with sequences encoding other NRPS genes, such as the zwittermicin A biosynthetic genes. Many *B. cereus* group strains isolated from soil produce the wide-range antibiotic zwittermycin A, which is regulated by specific nutrients and host exudates (Silo-Suh et al., 1998; Stabb et al., 1994; Milner et al., 1995). Moreover, zwittermycin A acts synergistically with the *B. thuringiensis* toxin and kanomycin, another antibiotic produced by *B. cereus* soil strains (Broderick et al., 2003; Silo-Suh et al., 1998). The huge differences that exist at first sight between the harmless, pathogenic and bio-control strains within the *B. cereus* group decrease with closer look which reveals that *B. cereus* isolates from soil can produce various antibiotics in the rhizosphere during the interbacterial competition and pathogenic isolates can produce multiple toxins in food and the host intestinal tract during infection. Furthermore, the translated DNA sequence of the cereulide synthase genes *cesA* and *cesB* were used to search for similar proteins with Expasy NCBI blastp against the UniProt Knowledgebase database for bacteria and archaea (Table 3). The results show that cereulide shares similarity with other non-ribosomal peptide synthases (NRPS) for the bacterial antibiotics gramicidin, bacitracin, mycosubtilin, surfactin, tyrocidine, plipastatin, and the siderophores enterobactin and phenyloxazoline. These (lipo-)peptides are important in the interbacterial competition and host interactions, suggesting a similar role for cereulide. In addition, cereulide synthase appears related to the D-alanine-poly(phosphoribitol) ligase subunit 1, which is responsible for the ATP-dependent biosynthesis of D-alanyl-lipoteichoic acid (LTA) and thus important for cell wall biogenesis in Gram-positive bacteria. This relation is probably based on the functional similarity, namely affinity for alanine.

3 Regulation of cereulide expression

3.1 Bacterial factors

3.1.1 Growth phase

Cereulide is produced by *B. cereus* vegetative cells, independent of sporulation (Haggblom et al., 2002). If conditions are suitable, *B. cereus* cells start cereulide expression during the exponential growth phase at cell densities of 10^5–10^6 CFU per g or mL and continue toxin production throughout the stationary growth phase (Rajkovic et al., 2006b; Agata et al.,

Table 1. Assessment of the variability in toxin DNA sequences of *Bacillus cereus* (by megablast in the NCBI database of reference genomic sequences on 19/10/2010).

Species	E value	Query coverage	Maximum identity
Cereulide synthase(*cesHPTABCD*; NC_010924 bp15094....38668)			
B. cereus	0.0	3– 100 %	92– 100%
B. thuringiensis	0.0	3%	92%
B. mycoides	2e-48	0%	88%
Clostridium cellulovorans	2e-13	0%	76%
Streptococcus bovis	0.002	0%	92%
Herpetosiphonaurantiacus	0.009	0%	90%
Nonhemolytic enterotoxin (*nheABC*operon; Y19005)			
B. cereus	0.0	98– 99%	92– 97 %
B. thuringiensis	0.0	99%	93– 97%
B. anthracis	0.0	99%	93– 94%
B. weihenstephanensis	0.0	99%	92%
B. mycoides	0.0	74– 99%	79– 91%
B. cereus subsp. cytotoxis	0.0	76%	81%
B. pseudomycoides	0.0	74%	79%
Hemolysin BL(*hblCDA*operon; AY822583)			
B. mycoides	0.0	89– 96 %	99– 100 %
B. cereus	0.0	82– 97 %	98– 100%
B. thuringiensis	0.0	88%	100%
B. weihenstephanensis	0.0	82%	100%
B. pseudomycoides	0.0	96%	96%
Cytotoxin K			
cytK-1 (AJ277962)			
B. cereus subsp.cytotoxis	0.0	99%	99%
B. thuringiensis	2e-86– 8e-90	80%	15%
B. cereus	2e-86– 8e-90	80%	15%
cytK-2 (AJ318875)			
B. cereus	e-86– 0.0	82– 99%	20– 84 %
B. thuringiensis	4e-175– 0.0	89– 98%	26– 85 %
B. anthracis	2e-178– 0.0	89– 90%	26%
B. weihenstephanensis	3e-117	87%	20%
B. mycoides	2e-93	83%	20%
Enterotoxin FM*(entFM*; AY789084)			
B. thuringiensis	0.0	94– 99%	100%
B. cereus	0.0	85– 99%	65– 100%
B. anthracis	0.0	94%	95– 100%
B. weihenstephanensis	0.0	90%	100%
B. mycoides	6e-63– 0.0	80– 90%	25– 100%
B. cereus subsp.cytotoxis	0.0	82%	100%
B. pseudomycoides	3e-61	80%	25%

2002). Cereulide expression is regulated by two transition state and sporulation regulators Spo0A and AbrB, independent of the pleiotropic transcription factor phospholipase C regulator (PlcR) which controls the majority of *B. cereus* enterotoxins (Lücking et al., 2009). AbrB directly suppresses the *ces* gene expression through binding to the *ces* promoter region. The *abrB* expression is maximal at the start of the exponential growth and declines until the mid-exponential phase. During the stationary phase Spo0A is expressed and this protein binds to AbrB, repressing it and allowing *ces* gene transcription. Transcription of the *ces* operon stops in the stationary phase and the emetic toxin production is then further regulated by other mechanisms linked to environmental and nutritional conditions. Although functional sporulation regulators are required for cereulide expression, there is no additional relation with the sporulation process itself (Lücking et al., 2009; Haggblom et al., 2002). Finally, it must be noted that the cereulide regulation in *B. cereus* by transcription factors Spo0A and AbrB is highly similar to the anthrax toxin regulation in *B. anthracis* (Saile & Koehler, 2002). In order to prevent food contamination with cereulide, outgrowth of emetic *B. cereus* beyond the early exponential phase should be avoided.

3.1.2 Strain specific variation

B. cereus strains that possess the *ces* genes express them upon favorable conditions, but the level of cereulide expression shows significant strain specific variation.

Table 2. Variability in cereulide synthase genes of the*Bacillus cereus* group members (assessed by a NCBI tblastn searchon 19/10/2010 against the nucleotide collection (nr/nt) of the*B. cereus* group (taxid:86661) with the translated translated partial *cesB* gene of emetic *Bacillus weihenstephanensis*MC67 (363 AA, DQ345790.1).

Species	DNA sequence	Query coverage	Maximum identity	E-value
B. weihenstephanensis	Cereulide peptide synthetase (*cesB* gene, partial cds)	100%	96%	0.0
B. thuringiensis	Cereulide peptide synthetase (*cesB* gene, partial cds)	100%	86%	0.0
B. cereus	Plasmid (pAH187_270, pH308197_258, pCER270, pBCE4810)	100%	86%	0.0
B. cereus	Cereulide peptide synthetase (*crs1* and *crs2* genes, complete cds)	100%	86%	0.0
B. cereus	Cereulide peptide synthetase (*cesB* gene, partial cds)	99– 100%	86– 87 %	5e-176– 0.0
B. thuringiensis	Complete genome	99%	34– 40%	7e54– 8e-73
B. weihenstephanensis	Plasmid (pBWB401)	99%	40%	4e-72
B. cereus	Complete genome	99%	34– 39 %	2e-51–7e-72
B. cereus	Zwittermicin A biosynthetic gene cluster (complete sequence)	100%	40%	9e-66
B. thuringiensis	Non-ribosomal peptide synthase gene (complete cds)	99%	36%	2e-65
B. weihenstephanensis	Complete genome	100%	36%	4e-61
B. thuringiensis	Plasmid (pBMB171)	99%	33%	1e-57
B. anthracis	Complete genome	99%	34%	5e-53
B. cereus	Cereulide peptide synthase-like gene (partial sequence)	23– 84%	37– 38%	2e-07–7e-45
B. thuringiensis	Non-ribosomal peptide synthetase gene (tzw1, complete cds)	53%	40%	6e-34
B. thuringiensis	Zwittermicin A biosynthesis gene cluster (partial sequence)	29%	42%	2e-19
B. weihenstephanensis	*yhfL* gene (partial cds)	40%	24– 25 %	2e-05–4e-05

Under identical cultivation conditions emetic strains show up to a 1000 fold difference in cereulide production, from 1 to 1200 μg per gram fresh weight biomass (Carlin et al., 2006; Rajkovic et al., 2006b). This strain variability can (partially) explain the differences in results obtained in conducted studies reviewed later on.

3.2 Environmental factors

3.2.1 Temperature

Cereulide production occurs in the temperature range of 8 to 40°C (Haggblom et al., 2002). The optimal temperature for emetic toxin production usually is between 20 and 30°C. The variability in optimal temperature is caused by the influence of other factors, mainly the bacterial growth rate and the food type (Agata et al., 2002; Rajkovic et al., 2006b). For a similar biomass of approximately 10^8 CFU/g at the end of the exponential growth phase a cereulide level of 20–30 μg/g is observed in the rice at 20, 30, and 35°C. Psychrotrophic emetic strains have been isolated (Thorsen et al., 2006) and sometimes significantly more emetic toxin is strains produced at lower temperatures (12–15 °C) than at 30°C if more incubation time is granted (Finlay et al., 2000). However, such strains and observation are more the exception than the rule. Keeping food at room temperature is thus considered to be most dangerous, while monitoring the integrity of the cold chain prevents bacterial outgrowth and emetic toxin production.

3.2.2 Atmospheric composition

Cereulide production is severely impaired by reduced atmospheric oxygen levels, suggesting that oxygen is an essential and stimulating factor in cereulide production (Jaaskelainen et al., 2004; Finlay et al., 2002; Rajkovic et al., 2006a). Under anaerobic conditions with less than 1–2% O_2, cereulide production does no longer take place (Rajkovic et al., 2006a; Jaaskelainen et al., 2004). *B. weihenstephanensis* strains, belonging to the *B. cereus* group, show similar sensitivity to modified atmosphere packaging (MAP) (Thorsen et al., 2009b). Oxygen restricted atmospheres (0–2% O_2 and 20% CO_2 in N_2) inhibit cereulide production: the lower the oxygen concentration and oxygen transfer rate of the packaging material, the lower the emetic toxin concentrations. Modified atmosphere packaging, i.e., replacing most of the head space atmosphere with nitrogen gas to eliminate atmospheric oxygen, can thus aid in the prevention of *B. cereus* emetic food poisoning. It must be noted that contradictory effects of oxygen on cereulide production have been described due to the variable influence of

Table 3. Proteins showing similaritiesto the *Bacillus cereus* toxins according to Expasy NCBI blastp, performed on 30/09/2010 for the amino acid queries against the UniProt Knowledgebase database Bacteria+Archaea with only Swiss-Prot curated sequences and fragment sequences excluded (except for CytK-1), results with an E-value ≤ 0.01 are shown.

Hit protein				
Cereulide synthase				
Query: CesA (translation NC_010924 25017..35141) 3374 AA	Gene	Species	Length (AA)	E-value
Linear gramicidin synthase (subunit A, B, C and D)	*lgrA, lgrB, lgrC, lgrD*	*Brevibacillusbrevis*	2273– 7756	0.0
Bacitracin synthase (1, 2 and 3)	*bacA, bacB, bacC*	*Bacillus licheniformis*	2607– 6359	0.0
Mycosubtilin synthase (subunit A, B and C)	*mycA, mycB, mycC*	*Bacillus subtilis*	2609– 5369	0.0
Surfactin synthase (subunit 1, 2 and 3)	*srfAA,srfA,srfAC*	*Bacillus subtilis*	1275– 3587	0.0–e-133
Tyrocidine synthase (1, 2 and 3)	*tycA, tycB,tycC*	*Brevibacillusparabrevis*	1088– 6486	0.0
	grsA	*Brevibacillusbrevis*	1098	0.0
Plipastatin synthase (subunit A, B, C, D and E)	*ppsA, ppsB, ppsC, pps, ppsE (pps5)*	*Bacillus subtilis*	1279– 2560	0.0–e-136
Gramicidin S (synthase 1 and 2)	*grsA, grsB*	*Aneurinibacillusmigulanus*	1098– 4451	0.0
	grsB	*Brevibacillusbrevis*	4450	0.0
Polyketide synthase (pksN, pksJ)	*pksN, pksJ*	*Bacillus subtilis*	2378– 5043	0.0– e-154
Dimodularnonribosomal peptide synthase	*dhbF*	*Bacillus subtilis*	3649	e-171
(ACV synthetase)	*pcbAB*	*Nocardialactamdurans*	3649	e-157
Enterobactin synthase (component F)	*entF*	*Escherichia coli*	1293– 1292	4e-96– 2e-95
	entF	*Shigellaflexneri*	1281	3e-93
Phenyloxazoline synthase	*mbtB*	*Mycobacterium tuberculosis*	1414	2e-73
	mbtB	*Mycobacterium bovis*	1414	2e-73
Protein AngR	*angR*	*Vibrio anguillarum*	1048	3e-69
High-molecular-weight protein 2 (HMWP2)	*irp2*	*Yersinia enterocolitica*	2035	3e-54
D-alanine-poly(phosphoribitol) ligase subunit 1	*dltA*	*Bacillus cereus*	504	2e-53– e-48
	dltA	*Bacillus anthracis*	503– 504	e-51
	dltA	*Bacillus thuringiensis*	504	e-51
	dltA	*Bacillus weihenstephanensis*	504	3e-50
	dltA	*Bacillus licheniformis*	502	3e-47
	dltA	*Lactococcuslactissubsp. lactis*	499	3e-46
	dltA	*Listeria monocytogenes*	510	3e-44– 3e-42
	dltA	*Bacillus amyloliquefaciens*	503	2e-43
	dltA	*Listeria innocua*	510	5e-43
	dltA	*Bacillus subtilis*	503	2e-42
	dltA	*Streptococcus pneumoniae*	516– 510	6e-42– 3e-41
	dltA	*Staphylococcus aureus*	485	6e-42– 2e-41
	dltA	*Staphylococcus haemolyticus*	485	7e-41
	dltA	*Listeria welshimeri*	510	7e-41
	dltA	*Staphylococcus epidermidis*	485	e-40– 9e-40
	dltA	*Streptococcus pyogenes*	512	3e-40– 4e-40
	dltA	*Lactobacillusplantarum*	508	8e-39
Hemolysin BL				
Hbl-B (AAA22522.1, 375 AA)				
Hemolysin BL-binding component precursor	*hblA*	*Bacillus cereus*	375	0.0
Uncharacterized 34.8 kDa protein	*hlyA 3′ region*	*Edwardsiellatarda*	311	e-163
Hbl-L1 (AAB52531.1, 384 AA)				

Table 3. continued on next page

Table 3. Continued.

Hit protein				
Cereulide synthase				
Query: CesA (translation NC_010924 25017..35141) 3374 AA	Gene	Species	Length (AA)	E-value
Hbl-L2 (AAB52530.1, 447 AA)				
Hemolysin BL-binding component precursor	*hblA*	*Bacillus cereus*	375	2e-4
Nonhemolytic enterotoxin Nhe				
NheA (CAB53338.2, 386 AA)				
Hemolysin BL-binding component precursor	*hblA*	*Bacillus cereus*	375	3e-7
Cytadherence accessory protein 2	*hlp2 (hmw2)*	*Mycoplasma gallisepticum*	1931	8e-7
	hmw2	*Mycoplasma pneumoniae*	1818	6e-4
Uncharacterized protein		*Mycoplasma capricolum*	470	4e-6
Uncharacterized 34.8 kDa protein	*hlyA 3′ region*	*Edwardsiellatarda*	311	8e-5
DNA double-strand break repair rad50 ATPase	*rad50*	*Methanocaldococcusjannaschii*	1005	0.001
	rad50	*Pyrococcusfuriosus*	882	0.005
Methyl-accepting chemotaxis protein 2	*mcp2*	*Thermotogamaritima*	530	0.007
NheB (CAB53339.2, 402 AA)				
Hemolysin BL-binding component precursor	*hblA*	*Bacillus cereus*	375	3e-27
Uncharacterized 34.8 kDa protein	*hlyA 3′ region*	*Edwardsiellatarda*	311	2e-20
NheC (CAB53340.2, 359 AA)				
Hemolysin BL-binding component precursor	*hblA*	*Bacillus cereus*	375	4e-28
Uncharacterized 34.8 kDa protein	*hlyA 3′ region*	*Edwardsiellatarda*	311	8e-27
Extracellular matrix-binding protein	*ebh*	*Staphylococcus aureus*	9535–10746	2e-4– 0.002
	ebhB	*Staphylococcus aureus*	7031– 3890	2e-4– 0.019
	ebhA	*Staphylococcus aureus*	6713	2e-4
Cytotoxin K				
CytK-1 (CAC08440.1, 336 AA)				
Cytotoxin K		*Bacillus cereus*	336– 337	e-178– e-158
		Bacillus thuringiensis	368– 362	e-161– e-158
CytK-2 protein	*cytK-2*	*Bacillus cereus*	336	e-160
Hemolysin II		*Bacillus thuringiensis*	320	8e-52– 6e-50
		Bacillus cereus	412	2e-50– 8e-50
		Bacillus pseudomycoides	330	4e-43
		Bacillus anthracis	240	3e-36
Gamma-hemolysin component precursor	*hlgA, hlgB, hlgC*	*Staphylococcus aureus*	309– 325	2 e-19– 5e-32
Leukocidin-F subunit precursor	*lukF*	*Staphylococcus aureus*	323	2e-29
Alpha-hemolysin precursor	*hly (hla)*	*Staphylococcus aureus*	319	4e-29
Leukocidin-S subunit precursor	*lukS*	*Staphylococcus aureus*	315	3e-20
Uncharacterized leukocidin-like protein 1 and 2 precursor		*Staphylococcus aureus*	339–338	4e-22–2e-19
Enterotoxin FM				
EntFM (AAX14641.1, 426 AA)				
Endopeptidase precursor	*lytE (cwlF), lytF (yhdD,) cwlS (yojL,) yrvJ*	*Bacillus subtilis*	334– 518	4e-18–7e-28
Protein p60 precursor (Invasion-associated protein)	*iap*	*Listeria seeligeri*	523	2e-16
	iap	*Listeria welshimeri*	524	e-15
	iap	*Listeria grayi*	511	e-15
	iap	*Listeria innocua*	467	2e-15
	iap	*Listeria monocytogenes*	484	4e-15

Table 3. continued on next page

Table 3. Continued.

Hit protein				
Cereulide synthase				
Query: CesA (translation NC_010924 25017..35141) 3374 AA	Gene	Species	Length (AA)	E-value
Lipoprotein precursor	*spr*	*Shigellaflexneri*	188	e-9
	spr (yeiV), nlpC	*Escherichia coli*	154– 188	2e-9– e-9
	nlpC	*Haemophilusinfluenzae*	183	2e-9
Endopeptidase precursor	*cwlO*	*Bacillus licheniformis*	452	2e-8
	cwlO (yvcE, yzkA)	*Bacillus subtilis*	473	e-7
Protein P54 precursor		*Enterococcus faecium*	516	8e-8
Endopeptidase	*ykfC*	*Bacillus subtilis*	296	2e-5
Endopeptidase II		*Bacillus sphaericus*	271	5e-5
Beta-N-acetylglucosaminidase precursor	*lytD (cwlG)*	*Bacillus subtilis*	880	3e-4
Bacteriocin	*bcn*	*Clostridiumperfringens*	890	0.002
Cell wall-binding protein precursor	*ywsB*	*Bacillus subtilis*	178	0.004

agitation and thus aeration and oxygen concentration, further discussed in section 3.2.3.6 Agitation.

3.2.3 Nutrients and food properties

The food composition is of major influence on cereulide production. Non-acidic foods with high water and starch content will generally allow the highest accumulation of cereulide.

3.2.3.1 Carbon source (starch). Although emetic *B. cereus* strains generally are unable to hydrolyze starch, they produce high amounts of cereulide in many farinaceous foods, such as mashed potatoes, cooked rice, noodles, spaghetti, bread, and pastries (Rajkovic et al., 2006b; Agata et al., 2002; Jaaskelainen et al., 2003). However, this toxin can also be present in other types of food, such as vegetable burgers and milk (Rajkovic et al., 2005; Rajkovic et al., 2006b). Agata et al. (2002) found that cooked rice is most suitable for cereulide production by *B. cereus*, leading to 0.36 µg/g of emetic toxin after 12–16 h at 20°C. Other starchy food such as noodles, mashed potatoes and spaghetti followed with 0.08–0.16 µg/g cereulide, while bread and cake yielded only 0.02 µg/g. Finally, eggs, milk, fish, meat and their products sustain bacterial growth but without significant cereulide production (< 0.005 µg/g). In another study, the maximal cereulide content attained 4.080 µg/g in potato puree, followed by 3.30 µg/g in penne, 2.01 µg/g in rice and 1.14 µg/mL for milk after 48 h at 28°C (Rajkovic et al., 2006b). Furthermore pastries with meat are highly suitable for cereulide production, yielding 6.70 µg/g after 4 days at 22°C (Jaaskelainen et al., 2003). Pastries with rice contained up to 1.57 µg/g cereulide, even after baking at 250°C for 20 min. White and whole grain bread supported concentrations of 1.33 and 1.92 µg/g cereulide respectively, while rye bread only allowed a rather low level of cereulide production, namely 0.17 µg/g, despite its longer incubation time of 21 days at 22°C.

3.2.3.2 Amino acids. Early characterized emetic *B. cereus* strains were shown to require the amino acids L-leucine, L-valine, and L-threonine for growth (Agata et al., 1999). Supplementation of these three individual amino acids specifically stimulates the cereulide synthesis (Jaaskelainen et al., 2004). As leucine (E641) and valine also are frequently added to food as flavor enhancers, caution is needed in regard to emetic food poisoning by *B. cereus*.

3.2.3.3 pH Cereulide production is inhibited by low pH values. Acidification of potato salad, spaghetti and rice by addition of a dressing like mayonnaise, ketchup or vinegar decreased the bacterial growth in these foods and the cereulide concentration dropped below 0.01 µg/g (Agata et al., 2002). Moreover, béchamel sauce with pH 5.8 supported the growth of emetic *B. cereus* without cereulide production (Rajkovic et al., 2006b). Bakery products with water activity < 0.953 and pH values < 5.6 such as rye bread, muffins and jam rolls, were also not suitable for emetic toxin production during storage of 4 to 21 days at 22°C (Jaaskelainen et al., 2003). Acidification of food products is thus a possible strategy to prevent the risk of growth and cereulide production by *B. cereus*.

3.2.3.4 Cations The cation concentrations and their ratio seem to influence the *ces* gene expression (Apetroaie-Constantin et al., 2008). Cereulide production was stimulated by low concentrations of Na^+ and a high ratio $[K^+]:[Na^+]$ on the condition that Na^+ and glycine concentrations are constant. Modifying the salt content of food products or replacing NaCl by other salts are probably not relevant tools for influencing toxin production.

3.2.3.5 Food consistency. The physical properties of the food matrix, i.e. solid or liquid state, also influence toxin production. The onset of cereulide production is earlier on solid media than in the corresponding liquid broth, but the final cereulide concentration is lower (Jaaskelainen et al., 2004; Haggblom et al., 2002). Emetic toxin production on Tryptone Soya Agar (TSA) occurred during the exponential growth phase, while cereulide

production started during the stationary growth phase in Tryptone Soya Broth (TSB). However, the final cereulide concentration was lower on TSA than on TSB, up to 0.01 μg per 10^6 cells and 0.10 μg, respectively.

3.2.3.6 Agitation The shaking conditions are highly important factors for cereulide production in liquid media such as milk and soy milk. Unfortunately, contradictory results are found in literature regarding aeration and shaking: both stimulating and inhibiting effects are reported. The reason for these contrary findings is not known, but different toxin extraction protocols were performed. Agata et al. (2002) observed cereulide production in milk and soy milk, 0.640 and 0.320 μg/g respectively, during 24 h incubation at 30°C with shaking. When milk and soy milk were statically incubated no more cereulide was found. In accordance with these results Haggblom et al. (200) reported that growth of three emetic *B. cereus* strains was similar in liquid broth cultures with or without shaking, but cereulide production was not observed or drastically decreased during the static incubations (Haggblom et al., 2002). In contrast Rajkovic et al. (2006) found that aeration of cultures had a negative effect on cereulide production, e.g. cereulide was not detected anymore in shaken milk. Likewise, the cereulide content of dried infant food formulas was higher after static incubation than after incubation on a shaker at 60 rpm after 24 h storage at room temperature (Shaheen et al., 2006). It must be noted that all authors had used different detection methods and for a final understanding of these findings the use of a standard experimental setup and analytical technique such as liquid chromatography tandem mass spectrometry (LC/MS-MS) is suggested.

4 Enterotoxins

4.1 Protein properties

B. cereus expresses a variety of enterotoxic compounds, of which nonhemolytic enterotoxin (Nhe), hemolysin BL (Hbl), cytotoxin K (CytK), and hemolysin II (HlyII) are most important.

Nhe and Hbl are homologous three component toxins that display hemolytic and cytotoxic activity (Fagerlund et al., 2008; Lindbäck et al., 2004; Beecher et al., 1995). Nhe comprises the cytolytic protein NheA (41 kDa, 360 AA) and binding components NheB (40 kDa, 372 AA) and NheC (36 kDa, 329 AA) (Granum et al., 1999; Lindbäck et al., 2010). Maximal toxic effects are exerted by NheA, NheB and NheC in the ratio 10:10:1, because toxicity decreases when the relative NheC concentration in solution exceeds 10% (Lindbäck et al., 2004). This explains the lower *nheC* gene expression relative to that of *nheA* and *nheB*. Furthermore, also the order of administering the toxin components is important for toxicity towards Vero cells: maximal effect is obtained when NheC and NheB are added first followed by NheA (Lindbäck et al., 2010). Hbl is composed of the lytic components L2 alias HblC (46 kDa, 447 AA) and L1 alias HblD (38 kDa, 384 AA) and the binding protein B alias HblA (37 kDa, 375 AA) (Heinrichs et al., 1993; Ryan et al., 1997). Enterotoxin Hbl shows hemolytic, cytotoxic, dermonecrotic and vascular permeability activity and fluid accumulation in ligated rabbit ileal loops (Beecher et al., 1995). The optimal ratio of component L2, L1, and B for maximal toxicity is 1:1:1. The Hbl components independently bind to the cell membrane and after association and pore formation, osmotic cell lyses occurs (Beecher & Wong, 1997). The paradoxical zone phenomenon in blood agar is the result of haemolysis inhibition because B and L1 are mutually inhibitory above a threshold concentration and the binding of B is much slower than the binding of L1 and L2. All Nhe and Hbl components contain a predicted N-terminal signal peptide sequence for secretion of 26–32 AA (Nielsen et al., 1997). Moreover, the (main) source of cytotoxicity is the *B. cereus* culture supernatant and not the cell extract, indicating the actual secretion of the enterotoxins (Fermanian et al., 1996; Choma & Granum, 2002). Substantial protein sequence similarity is observed between components, i.e., 22–44% identity among the Nhe components and 18–25% identity among the Hbl proteins (Fagerlund et al., 2008). In addition, a high sequence identity was found between the Nhe and Hbl components with similar functions, namely NheA and Hbl-L2 (23%), NheB and Hbl-L1 (40 %) and NheC and Hbl-B (25 %). Moreover cross-reactivity of antibodies against Hbl-L1 with NheB suggests a common epitope (Dietrich et al., 1999; Dietrich et al., 2005). The Nhe and Hbl components also display structural similarities, e.g. NheA and Hbl-L2 have no predicted transmembrane helices, while NheB and Hbl-L1 have two predicted helices in the same position and NheC and Hbl-B have one (Granum et al., 1999). The highly similar Hbl and Nhe toxin components also show signification sequence identity (18–20%) with the pore-forming haemolysin cytolysin A (ClyA) from *Escherichia coli* and other Gram-negative enteric pathogens such as *Shigella flexneri* and *Salmonella enteric* (Fagerlund et al., 2008). Furthermore, all of these toxins also display structural similarity, because they all consist of four α-helices wrapped in a left-handed supercoil and a hydrophobic β-hairpin. Finally, functional similarities are also present in their hemolytic and cytotoxic mechanisms, as they all form pores in cell membranes which leads to osmotic cell lysis (Lindbäck et al., 2004; Beecher & Wong, 1997; Fagerlund et al., 2008; Beecher et al., 1995). These findings were confirmed by an Expasy NCBI blastp search for the amino acid sequences of the *B. cereus* enterotoxins (Table 3). Nhe and Hbl components also share significant homology with an uncharacterized 34.8 kDa protein *Edwardsiella tarda*. This Gram-negative fish pathogen possesses a β-hemolysin operon with three ORFs encoding three polypeptides of 15.9 kDa, 7.1 kDa and 34.8 kDa (Chen et al., 1996). In addition the Nhe components weakly resemble cytadherence proteins of *Mycoplasma* and extracellular matrix-binding proteins of *Staphylococcus*.

Cytotoxin K (CytK, 34 kDa, 305 AA including a 31 AA signal peptide) and hemolysin II (HlyII, 46 kDA, 412 AA including a 32 AA signal peptide) are both single component β-channel forming toxins (Baida et al., 1999; Lund et al., 2000). HlyII shows no homology with other *Bacillus* spp. hemolysins, but the protein displays substantial sequence homology with CytK (37% identity) and with some β-channel forming toxins of *Staphylococcus aureus*, namely the α-toxin (31% identity), the F component of leukocidin R (29% identity) and the B component of γ-hemolysin, (29% identity) (Baida et al., 1999). CytK is also related to leucocidin LukF-PV (32%) and α-hemolysin (30%) of *S. aureus* and the β-toxin (26%) of *Clostridium perfringens* (Lund et al., 2000). Similar results are obtained in Table 3. Just like other β-channel forming toxins, CytK is cytotoxic due to pore-formation in the cell membranes, but the pore is non-selective with a slightly smaller (7 Å) diameter and the affinity for certain cell types also differs (Hardy et al., 2001). Two variants of the CytK toxin were found in *B. cereus* strains (Fagerlund et al., 2004). The firstly isolated CytK1 toxin variant is at least five times more toxic to human intestinal cell lines than the CytK2 variants. The CytK2 amino acid sequences share 97–99% identity with each other, in contrast to only 89% with that of CytK1. The differences between both toxin variants are of course more pronounced on the DNA level (Table 1). Furthermore, the regulatory DNA sequences also show some interesting differences: the CytK2 promoters contain a PlcR box that fully complies with the consensus sequence, while the CytK1 promoter contains a mismatch at the eleventh position (Fagerlund et al., 2004).

4.2 Enterotoxin genes

Bacteria in the *B. cereus* group carry the emetic, insecticidal and anthrax toxin genetic determinants on megaplasmids, but the enterotoxins are chromosomally located. Both the nonhemolytic enterotoxin (Nhe) and the hemolysin BL (Hbl) are encoded in an operon of three genes that are co-transcribed. The Nhe toxin is composed of the protein components NheA, NheB, and NheC encoded in the genes *nheA, nheB* and *nheC* respectively (Granum et al., 1999). The *nheC* expression is decreased relative to that of *nheA* and *nheB* due to a 13 bp inverted repeat between the *nheB* and *nheC* sequence. The whole *nhe* operon is transcribed but the expression of the *nheC* gene is lower due to translational repression (Lindbäck et al., 2004). As a result relatively low amounts of NheC are found in the culture supernatant in comparison with NheA and NheB (Dietrich et al., 2005). The *hbl* operon consists of the *hblC, hblD, hblA,* and *hblB* genes, encoding the toxin components L2, L1, and B respectively, while the *hblB* gene is not transcribed, so it is probably a pseudogene (Ryan et al., 1997; Okstad et al., 1999; Lindbäck et al., 1999). Moreover, an *hblCDA* operon was found, indicating that the *hblB* gene is not essential or functional (Lapidus et al., 2008; Stenfors Arnesen et al., 2008). According to the current hypothesis the *hbl* and *nhe* operons were formed by gene duplication of an ancestor gene and spread by horizontal gene transfer. The striking similarities between the Hbl and Nhe components suggest a common origin (Fagerlund et al., 2008). Horizontal gene transfer is suspected after the discovery of an inverted repeat with 78 % DNA sequence identity to a transposase-encoding region in *B. thuringiensis* plasmids downstream of *hblB* (Okstad et al., 1999) and the 400 kb plasmid with Nhe-like toxin sequences of *B. weihenstephansis* KBAB4 (Lapidus et al., 2008). In contrast to the three-component toxins Nhe and Hbl which are encoded in an operon, the single component toxins cytotoxin K (CytK) and hemolysin II (HlyII) are encoded in single chromosomal genes, the *cytK* and *hlyII* gene respectively (Baida et al., 1999; Lund et al., 2000). Finally, the enterotoxin FM (*entFM*) gene and the enterotoxin T (*BceT*) gene were cloned from *B. cereus* and proposed as an enterotoxin based on their structure and *in vitro* activity (Agata et al., 1995a; Asano et al., 1997). Although EntFM shows no statistically significant similarities to other known enterotoxins, it contributes significantly to the cytotoxic and hemolytic activity of *B. cereus* (Asano et al., 1997; Luxananil et al., 2003). In contrast, enterotoxin T showed no cytotoxicity and thereafter its activity and identity as enterotoxin was questioned (Choma & Granum, 2002). As currently understood, the *bceT* gene is a fusion product of genomic *B. cereus* DNA sequences and a gene homologous to ORF 101 in the *B. anthracis* virulence plasmid pOX1 (Hansen et al., 2003). The Expasy NCBI blastp search was also performed with the amino acid sequences of the hypothetical *B. cereus* enterotoxins EntFM and BceT (Table 3). Protein EntFM shares some similarities with a variety of cell wall catabolic enzymes such as endopeptidases CwlS, YojL, LytE, and LytF, which hydrolyze gamma-D-glutamyl bonds during cell separation, and the invasion-associated protein p60 of *Listeria* spp.

4.3 Prevalence and polymorphisms of the enterotoxin genes

In contrast to the cereulide genes, the enterotoxin genes show a higher degree of polymorphism and a higher prevalence among *B. cereus* strains (Guinebretiere et al., 2002; Schoeni & Wong, 1999; Ehling-Schulz et al., 2006b). The most common enterotoxin genes are *nhe* and *entFM*, as these genes are found in nearly all (84– 100 %) *B. cereus* strains, followed by the *cytK* gene (37–89%), the *hbl* genes (29–92%), *bceT* gene (12–71%) and the *hlyII* gene (19–56%) (Guinebretiere et al., 2002; Aragon-Alegro et al., 2008; Cadot et al., 2010; Wijnands et al., 2006a; Shadrin et al., 2007; Yang et al., 2005; Ngamwongsatit et al., 2008; Ouoba et al., 2008; Rahmati & Labbe, 2008; Bonerba et al., 2010).

It must be emphasized that this toxin gene prevalence is not only valid for *B. cereus s. s.* strains. For example, similar enterotoxin gene prevalences were obtained in a study specifically for B. thuringiensis strains (Swiecicka et al., 2006). The large variation in toxin gene prevalence can be partially attributed to the strain source. In general, clinical isolates possess multiple toxin genes and display

higher expression of these genes than food and environmental isolates. For example, 63% of the clinical *B. cereus* strains isolated from diarrheal food poisoning contained all *nhe, hbl* and *cytK* genes, compared with only 33% of the food isolates (Guinebretiere et al., 2002). Furthermore, there was more variation in enterotoxin gene sequences and their expression among *B. cereus* food isolates than among clinical strains (Yang et al., 2005; Guinebretiere et al., 2002).

Nhe and Hbl polymorphisms were revealed by Southern blotting of the enterotoxin sequences while PCR detection failed in 44% of the cases for *nhe* and 19% for *hbl* (Guinebretiere et al., 2002). Although older reports suggested that certain strains possessed only partial enterotoxin operons, it has been established that this misconceptions was due to primer bias for certain toxin gene polymorphisms (Beattie & Williams, 1999). After optimization and further development of primers, it seems today that the enterotoxin genes from the same operon always occur simultaneously (Ngamwongsatit et al., 2008; Guinebretière et al., 2010). Cytotoxin K polymorphism was discovered with the isolation of the highly toxic CytK1 from the clinical strain *B. cereus* NVH 391/98, while less hazardous strains usually possess the CytK2 toxin variant (Lund et al., 2000; Fagerlund et al., 2004). Further investigation revealed higher prevalence and sequence variability of CytK2 (Shadrin et al., 2007; Bonerba et al., 2010). Besides CytK1, *B. cereus* NVH 391/98 strain also contains a rare polymorphism of the *nhe* operon that was not detected before, because it has only 70–80% DNA sequence identity with other *nhe* operons (Fagerlund et al., 2007). This supports the most recent studies in their finding that all *B. cereus* strains possess the *nhe* operon, although they sometimes show considerable *nhe* polymorphism (Guinebretiere et al., 2002; Guinebretière et al., 2010; Ngamwongsatit et al., 2008). The enterotoxin gene sequences were used in a similarity search with megablast in the NCBI database of reference genomic sequences (Table 1). The results show that the enterotoxin genes are exclusively found in *B. cereus* group strains. High similarities are found among the *hbl* genes (> 96%) and the *nhe* genes (> 92% similarity), except for *B. cereus* subsp. *cytotoxicus* NVH 391-98 and some *B. mycoides* and *B. pseudomycoides* strains (> 78%). The *cytK-1* gene of *B. cereus* subsp. *cytotoxicus* NVH 391-98 is a rather rare variant of the Cytotoxin K, which is only 15% similar to the more abundant *cytK-2* sequences with 20–85% similarity. *B. cereus* group strains possess highly similar (> 94% similarity) *entFM* sequences, except for *B. cereus* Rock3-44 (65%) and *B. mycoides* and *B. pseudomycoides* (25%). In conclusion, the level of cytotoxicity is determined by the expression levels of the enterotoxin genes rather than the presence and polymorphisms of those genes. For example, *B. cereus* NVH 883/00 possesses the notorious CytK1 toxin variant but this strain is not cytotoxic (Fagerlund et al., 2007). As a consequence, the assessment of the genetic potential of *B. cereus* isolated from food does not suffice to evaluate the risk without confirmation of actual toxin production and preferably its quantification (Moravek et al., 2006).

4.4 Clinical aspects

The *in vivo* activity of the different enterotoxins and their (relative) importance is difficult to show, if not impossible, because diarrheal food poisoning strains rarely express only one enterotoxin. For example, the clinical strain *B. cereus* NVH 391/98 was thought to produce only enterotoxin CytK1, but another *nhe* polymorphism was discovered in its genome (Fagerlund et al., 2007), so it is not clear whether it produces another Nhe toxin variant or not. Moreover the toxicity of the enterotoxins is determined by their concentration and thus their gene expression levels. Nevertheless, Nhe is the most common and most important virulence factor of *B. cereus*. Cytotoxicity of the culture supernatant correlates well with Nhe production and not with the Hbl production (Moravek et al., 2006). Moreover monoclonal antibodies against NheB dramatically reduced cytotoxicity of Nhe producing *B. cereus* strains with > 95% (Dietrich et al., 2005). Therefore the *in vitro* cytotoxicity does not seem to be influenced by other virulence factors such as proteases, hemolysins and phospholipases. Monoclonal antibodies against Hbl showed that all Hbl producing strains are highly cytotoxic, but this toxicity was not fully attributable to enterotoxin Hbl (Dietrich et al., 1999). For example, the cytotoxicity of *B. cereus* DSM 4383, a strain that expresses both *hbl* and *nhe* operons, could be attributed to Nhe for 60% and to Hbl for 40% (Dietrich et al., 2005). The relative importance of the enterotoxins is probably strain dependent. For example, deletion of the *hbl* operon or the *cytK* gene in *B. thuringiensis* strain 407 Cry- did not affect its cytotoxicity (Ramarao & Lereclus, 2006), while inactivation of the *hbl* operon in *B. cereus* ATCC 14579 reduced the cytotoxic and hemolytic activity (Lindbäck et al., 1999). After elimination of Hbl expression the *B. thuringiensis* strain still produced Nhe and CytK, while *B. cereus* ATCC 14579 could only rely on its low Nhe expression. These studies emphasize that the importance of the enterotoxins is determined by their expression levels and combinations, which is strain dependent. The role of hemolysin II in virulence and food poisoning is not clear. The *hlyII* gene is reported to be associated with pathogenic strains responsible for food poisoning or infections (Cadot et al., 2010). However, an earlier study did not find a correlation of *hlyII* prevalence and polymorphism with pathogenicity (Shadrin et al., 2007). Although enterotoxin FM contributes to the cytotoxic and hemolytic activity of *B. cereus*, the EntFM protein itself is not cytotoxic (Asano et al., 1997; Luxananil et al., 2003; Tran et al., 2010). Moreover, EntFM is related to cell wall peptidases (Cwps) and is involved in motility, biofilm formation, adhesion to epithelial cells and insect virulence (Tran et al., 2010). HlyII and EntFM are probably of minor importance as toxins in human food poisoning. However, diarrhea is probably caused by the combined and possibly synergistic action of multiple toxins and degradative enzymes,

including collagenases, phospholipases C, hemolysin III and cereolysin AB (Alouf & Popoff, 2006). For example, phospholipases C are reported to enhance the activity of enterotoxin Hbl (Beecher & Wong, 2000). Both phospholipases and enterotoxins cause cell lysis, but phospholipases enzymatically degrade the cell membrane, whereas hemolysins destroy it by pore formations (Alouf & Popoff, 2006). *B. cereus* can produce three phospholipase C enzymes, namely phosphatydylinositol phospholipase (PI-PLC), phosphatydylcholine phospholipase (PC-PLC alias lecithinase) and sphingomyelinase (SM-PLC), encoded in the *plcA*, *cerA* (alias *plcB*), and *cerB* (alias *smase*) genes respectively (Otnaess et al., 1977; Ikezawa et al., 1976; Ikezawa et al., 1978; Gohar et al., 2008). PC-PLC and SM-PLC combine to form the hemolysin cereolysin AB (CerAB) (Gilmore et al., 1989). The corresponding genes also form the *cerAB* operon but they are not co-transcribed. PC-PLC is responsible for the characteristic precipitation zone around typical *B. cereus* colonies on selective agar plates due to the degradation of lecithin (alias phosphatidylcholine) in egg yolk. SM-PLC is required for haemolysis of sheep erythrocytes. The concerted action of SM-PLC and PC-PLC is needed to lyse human erythrocytes due to tenfold higher concentration of the phosphatidylcholine in the human blood cell membrane (Gilmore et al., 1989). Furthermore, B. cereus group strains possess cholesterol-dependent cytolysins (CDC), i.e. cereolysin O (Clo) in B. cereus, thuringiolysin O (Tlo) in B. thuringiensis and anthrolysin O (Alo) of B. anthracis (Alouf & Popoff, 2006). CDC are found in wide variety of Gram-positive bacteria, e.g., streptolysin O (Slo) of Streptococcus pyogenes, perfringolysin (Pfo) of Clostridium perfringens, listeriolysin O (Llo) of Listeria monocytogenes (Shannon et al., 2003). These proteins display a high degree of homology and many have been shown to play an important role in virulence, for example the escape of the bacterium from the host macrophage or phagolysosome (O'Brien & Melville, 2004). At a molecular level, CDC associate with cholesterol and sometimes also with a specific receptor, followed by insertion, oligomerisation and β-barrel pore formation in the cell membrane, leading to cytolytic and hemolytic activity (Shannon et al., 2003).

Preformed enterotoxins in food are probably not relevant in the diarrheal syndrome, as enterotoxins are sensitive to cooking and gastrointestinal passage. Enterotoxic activity is eliminated by heating (20 min at 55°C), acid (pH 3.1 for 20 min at 37°C) and protease activity of pepsin, trypsin and chemotrypsin (1–2 mg/mL for 1–24 h) (Granum et al., 1993; Turnbull et al., 1979). However, it must be noted that inactivation experiments with toxins are often conducted in laboratory media, while the immunological thermostability of enterotoxin Nhe was shown to increase fourfold in milk compared with brain heart infusion (BHI) broth (Baker & Griffiths, 1995). Therefore the stability of the enterotoxins should also be investigated in food matrices to investigate whether biological activity can be retained during gastrointestinal passage due to physical protection by certain food components against gastric acid and proteolytic enzymes.

Besides food-borne illness, *B. cereus* can also cause non-gastrointestinal and gas gangrene-like infections, e.g., infection of the intraocular eye cavity (endophthalmitis), the blood (bacteremia, endocarditis), the respiratory tract (pneumonia) and the central nervous system (meningitis, encephalitis) (Bottone, 2010). *B. cereus* infections are rare but very severe, opposed to the very frequent but rarely lethal gastrointestinal illnesses. In both cases enterotoxins play an important role (Callegan et al., 2005).

5 Regulation of enterotoxin gene expression

5.1 Bacterial factors

5.1.1 Growth phase: phospholipase C regulator (PlcR)

Cell density is assessed by *B. cereus* through the phospholipase C regulator (PlcR) quorum sensing system (Declerck et al., 2007). At high population densities during the late exponential and stationary growth phase, the sensor protein PapR reaches the necessary threshold concentration to activate the effector protein PlcR. Activated PlcR-PapR complexes stimulate expression of the *plcR* and *papR* genes itself (autoinduction), while also inducing many virulence factor genes encoding the enterotoxins Nhe, Hbl, and Cytk, hemolysin III, phospholipases PC-PLC, and PI-PLC, with the exception of hemolysin II (HlyII) that is separately regulated by the adjacently encoded HlyIIR (Han et al., 2006; Gohar et al., 2008; Agaisse et al., 1999; Budarina et al., 2004). PlcR activates gene transcription by binding to a highly conserved palindromic DNA sequence TATGnAnnnnTnCAT(A) called the PlcR box located in various positions in the promoter region of the regulated gene (Agaisse et al., 1999; Slamti & Lereclus, 2002). PlcR expression is autoinduced just before the stationary growth phase, it sharply increases at the onset of the stationary phase, levels off two hours later and is inhibited during sporulation by the transition state regulator Spo0A (Gominet et al., 2001; Declerck et al., 2007; Lereclus et al., 2000; Slamti & Lereclus, 2002; Lereclus et al., 2000). In addition to the enterotoxin genes, PlcR upregulates the transcription of various other genes encoding other regulatory proteins and transcriptional regulators but also degradative and metabolic enzymes, motility and chemotaxis proteins, antibacterial peptides and a variety of transporters including antibiotic efflux proteins (Gohar et al., 2008; Agaisse et al., 1999; Ivanova et al., 2003). The regulated genes are spread throughout the chromosome and do not form a pathogenicity island, although they often are organized in pairs, in opposite orientation from a central PlcR box (Agaisse et al., 1999). Ninety percent of the PlcR regulated genes code for extracellular proteins that are important for sensing the environment, protecting the own bacterial cell and supplying food (Gohar et al., 2008). Furthermore, a functional *plcR* gene and thus expression of PlcR controlled genes is indispensible for pathogenicity of *B. cereus* in mice and

B. thuringiensis in insects (Salamitou et al., 2000). This suggests that the PlcR regulon is important to ensure survival and food supply of *B. cereus* in contact with bacterial competitors and eukaryotic host cells.

Maximal cytotoxicity of *B. cereus* culture supernatant coincides with maximal expression of PlcR controlled virulence factors (Gilois et al., 2007). However, contradictory results are found in scientific literature regarding the timing and kinetics of enterotoxin expression. Transcription of the *nhe, hbl,* and *plcR* gene starts during the exponential growth phase, but some studies report maximal toxin production during the early stationary growth phase (Gilois et al., 2007; Wijnands et al., 2006b; van der Voort & Abee, 2009), while others found maximal toxicity during the exponential growth phase (Duport et al., 2004; Fermanian et al., 1996; Zigha et al., 2006). Gene transcription of *cytK* starts at the onset of the stationary growth phase and peaks two hours later (Brillard & Lereclus, 2004). In conclusion, PlcR and high bacterial numbers are definitely key activators of enterotoxin production, but the enterotoxin gene expression varies significantly according to the specific bacterial growth rate and the presence and concentrations of oxygen and specific nutrients due to the complex regulation with influences from various bacterial and environmental signals.

5.1.2 Growth rate

The maximal enterotoxin production per unit of biomass coincides with the maximal specific growth rate under aerobiosis (Duport et al., 2004). In contrast, under anaerobiosis slower growth rates of 0.1 and 0.2 h^{-1} resulted in respectively 3 and 7 fold higher enterotoxin Hbl concentrations compared to higher growth rates between 0.3 and 0.9 h^{-1}. In agreement, the growth rate of 0.2 h^{-1} yielded maximal concentrations of both enterotoxin Nhe and Hbl at pH 7.2 and anaerobic conditions (Ouhib et al., 2006). Another study however reported that high growth rates (0.4 h^{-1}) under anaerobiosis at pH 7.0 resulted in maximal Nhe production while maximal Hbl production occurred at low growth rates (0.1 and 0.2 h^{-1}) (Thomassin et al., 2006). In conclusion, the bacterial growth rate regulates Hbl and Nhe production, but the pH and the oxygen availability also influence this regulation, and in a different way for both toxins. This again demonstrates the complex and differentiated regulation of enterotoxin expression in *B. cereus*.

5.1.3 Motility

The diarrheal toxins and degrading enzymes of *B. cereus* contain a signal peptide and are secreted during vegetative growth (Fermanian et al., 1996; Gilois et al., 2007). Interestingly, enterotoxin secretion and virulence of *B. cereus* are closely associated with flagella and swarming. According to the current hypothesis, the flagellar export system and the type III virulence secretion pathway have a common evolutionary origin and share overlapping functions (Hueck, 1998). Gram negative pathogenic bacteria use type III secretion systems to deliver effector proteins with cytotoxic or immune repressing effects directly into the host cell cytosol. Assemblage of the basal body flagellum shares genetic homology with the type III secretion apparatus, indicating a common evolutionary origin. Furthermore, virulence and motility are intimately linked in many pathogenic bacteria, for example swarming motility is associated with substantially increased secretion of virulence factors and host infection processes by *Proteus mirabilis, Vibrio cholerae,* and *Clostridium septicum* (Macfarlane et al., 2001; Gygi et al., 1995; Richardson, 1991). The flagella assembly, swarming ability and hemolysin production in *P. mirabilis* relies on the function of the *flhA* gene, encoding a soluble component of the flagellar export apparatus (Gygi et al., 1995; Macnab, 2003). Similarly, the *flhA* gene is essential for flagellin and enterotoxin Hbl export in *B. thuringiensis*, conferring swimming and swarming motility, hemolytic activity, adhesion and virulence (Heierson et al., 1986; Zhang et al., 1993; Ghelardi et al., 2002; Ramarao & Lereclus, 2006). The flagella export apparatus is also essential for enterotoxin Hbl export and haemolysis in *B. cereus s. s*. (Ghelardi et al., 2007). Hyperflagellated swarming cells are associated with quantitatively higher secretions of Hbl and clinical *B. cereus* strains more often display swimming and swarming motility, chemotaxis and haemolysis than food and environmental isolates (Ghelardi et al., 2007). Moreover, the non-motile mutant of *B. thuringiensis* BT407 Cry- showed significantly slower progression of the endophthalmitis in comparison with the wild-type (Callegan et al., 2005). In addition to the flagellum *flhA* gene, the *fliY* gene, encoding a flagellar switch protein that controls the direction of flagellum rotation and chemotaxis, is indispensible for swarming differentiation, chemotaxis and Hbl-L2 secretion by *B. cereus* (Bischoff & Ordal, 1992; Celandroni et al., 2000; Senesi et al., 2002). Enterotoxins Nhe, Hbl, and CytK possess a Sec-type signal peptide and are secreted through the Sec translocation pathway, independently of the flagellar export apparatus (Fagerlund et al., 2010). In conclusion, current research suggest that *B. cereus* virulence and motility are linked on a regulatory level.

5.1.4 Strain specific variation

The level of enterotoxin production is subject to substantial strain specific variability, noted as variable Nhe and Hbl antigen titers in the culture supernatant for different strains cultivated under identical conditions (Moravek et al., 2006; Dietrich et al., 2005). *B. cereus* strains isolated from food generally produce lower amounts of toxins, in comparison with clinical isolates (Guinebretiere et al., 2002; Moravek et al., 2006). Differences in virulence are hypothesized to exist predominately due to the presence of plasmids with specialized genes and alterations in gene expression regulation rather than mere presence or absence of chromosomal genes (Rasko et al., 2005a). For example, there was significant variability in the cytotoxicity of *B. cereus* strains carrying the CytK1 toxin variant,

which could be attributed to different toxin expression levels (Fagerlund et al., 2007). The *B. cereus* chromosome shows a high level of similarity with only subtle differences in gene content and encoded proteins (Rasko et al., 2005a). Often evidence is found of insertion and deletion events by mobile genetic elements (e.g. phages, transposons, IS elements) that promote gene exchange between plasmids and between plasmids and the chromosome (Rasko et al., 2005b). Moreover, horizontal toxin gene transfer could be spread virulence between *B. cereus* strains. For example, cry gene exchange occurred between *Bacillus cereus* group members in dead insect larvae through conjugational plasmid transfer (Yuan et al., 2007). Food matrices were also shown to hold significant potential for bacterial mating and plasmid transfer (Modrie et al., 2010; Van der Auwera et al., 2007). *B. cereus* species and strains can sometimes be distinguished by metabolic traits that could reflect niche specific adaptations, for example *B. cereus* ATCC 10987, isolated from spoiled cheese, possesses genes for the utilization of tagatose, a milk carbohydrate (Rasko et al., 2004). Similarly, virulence, toxin expression levels and host-specificity could be considered as niche specific adaptations as well.

Pathogenicity is not univocally associated with certain species within the *B. cereus* group, noted by the fact that nearly all species comprise both harmless and pathogenic strains. As currently understood, pathogenic strains do not possess specific genes or polymorphisms, but they display higher enterotoxin expression levels and thus higher cytotoxicity (Rasko et al., 2005a; Guinebretiere et al., 2002). The *B. cereus* group comprises the following 6 species: *B. cereus s.s., B. thuringiensis, B. anthracis, B. weihenstephanensis, B. mycoides,* and *B. pseuodmycoide*s, which can be divided into 7 phylogenetic groups (Table 4) (Guinebretière et al., 2010). *B. pseuodmycoide*s strains are found in group I, *B. mycoides* and *B. weihenstephanensis* constitute group VI, *B. anthracis* strains make up subgroup III-4, *B. cereus s.s.* and *B. thuringiensis* strains are spread over group II, III, IV, and V and finally group VII is represented by *B. cereus* subsp. *cytotoxicus*, a specific *B. cereus s.s*. lineage known for its specific polymorphisms in enterotoxins Nhe and CytK. Each phylogenetic group is associated with a probable cytotoxicity level and thus a certain risk in respect with food-borne disease. *B. cereus* group strains can be assigned to a phylogenetic group based on their *panC* sequence, which subsequently gives a risk indication. It must be noted that this is only an indication, as significant heterogeneity still exist in the cytotoxicity within the groups. For example, the most toxic group III comprises subgroups of strains representing both the highest (III-1 and III-2) as the lowest risk (III-3). Non-toxic strains are found in groups with high cytotoxicity and vice versa. For example *B. cereus* NVH 883/00 with the notorious CytK1 from group VII (Fagerlund et al., 2007) and the emetic *B. weihenstephanensis* strains in the low-risk group VI (Thorsen et al., 2006; Hoton et al., 2009). The distribution of emetic strains is limited to groups III-2 and III-3, but it must be noted that the screening was done with primer pairs that do not take *cesB* polymorphism into account. Therefore it is not unlikely that other cereulide synthase polymorphisms will be discovered in other phylogenetic groups with other primers, similar to Nhe polymorphisms.

5.2 Environmental factors

5.2.1 Temperature

The optimal temperature for diarrhoea1 toxin production is approximately 30 °C, for both mesophilic as psychrotrophic *B. cereus* strains (Rowan & Anderson, 1998; Fermanian et al., 1997; Mahakarnchanakul & Beuchat, 1999). The minimum temperature for enterotoxin production is as low as 6°C for some psychrotrophic strains in reconstituted milk-based infant formulae (MIF) (Rowan & Anderson, 1998). Higher incubation temperatures generally yield higher enterotoxin concentrations,

Table 4. Phylogenetic groups and species of the *Bacillus cereus* group and their cytotoxic and growth characteristics (adapted from Guinebretière et al., 2010).

Phylogeneticgroup	Cytotoxicity	Species	Growthtemperature range (°C)
I-1	notdetermined	B. pseudomycoides	10 - 43
I-2	notdetermined		
II	high	B. cereus, B. thuringiensis	7 - 40
III-1	high	B. cereus, B. thuringiensis, emetic B. cereus(III-2 and III-3), B. anthracis(III-4)	15 - 45
III-2	low		
III-3	high		
III-4	high		
IV-1	moderate	B. cereus, B. thuringiensis	10 - 45
IV-2	high		
IV-3	moderate		
V	moderate	B. cereus, B. thuringiensis	8 - 40
VI-1	low	B. weihenstephanensis, B. mycoides	5 - 37
VI-2	low		
VII	high	B. cereus subsp. cytotoxicus	20 - 50

both for psychrotrophic as mesophilic strains (Rowan & Anderson, 1998; Park et al., 2008; Baron et al., 2007). However, the temperature dependence of toxin production is not universal, as some strains show similar toxin production at high (32°C) and low (10°C) temperatures for cultures in BHI with similar biomass (Fermanian et al., 1997). Moreover, psychrotophic strains produced similar levels of enterotoxins in BHI, milk, minced meat, lasagna, and a rice meal after 24 days at 7°C than after 2 days at 17°C (Van Netten et al., 1990).

5.2.2 Atmospheric composition and the oxidation-reduction potential (ORP)

Anaerobic atmospheres result in slower bacterial growth but increased enterotoxin production, because the *nhe* and *hbl* gene expression is down-regulated by atmospheric oxygen (Duport et al., 2004; van der Voort & Abee, 2009; Duport et al., 2006). Maximal enterotoxin production takes place during the exponential phase under anaerobic conditions, but in comparison with aerobiosis it is reported as unaltered (Zigha et al., 2006; Fermanian et al., 1996), delayed (Duport et al., 2004) or accelerated (van der Voort & Abee, 2009; Gilois et al., 2007). Furthermore, the absence of oxygen stimulated the expression of the *cerAB* genes (encoding cereolysin AB) and the *picA* gene (phosphatidylinositol phospholipase C), while no or minor changes in *cytK* gene expression were observed (van der Voort & Abee, 2009). An apparent contradiction is found in literature regarding the effect of anaerobiosis on *plcR* expression: both no effect (van der Voort & Abee, 2009) and stimulation (Duport et al., 2006) were reported. This may be due to method of mRNA quantification, because the former study compared mRNA levels between the aerobic and anaerobic culture, while the latter study compared mRNA levels with that of the 16S rRNA gene of the same culture.

Under anaerobiosis the expression of the *plcR, nhe* and *hbl* genes is additionally up-regulated by low oxidation-reduction potentials (ORP) (Duport et al., 2006). Lowering the ORP resulted in higher and prolonged *nhe* and *hbl* gene expression (Zigha et al., 2006). Furthermore, under low OPR conditions both the intra- as extracellular protein production increased from 29 to 41%, with a more pronounced increase for Hbl (8 fold) than Nhe (2.5 fold). Regulation of enterotoxin expression in function of the redox potential is mediated by the fumarate and nitrate reduction regulator (Fnr) and the two-components system resDE. Fnr is essential for fermentative growth of *B. cereus* and for enterotoxin production during both aerobic and anaerobic growth (Zigha et al., 2007). The redox state of the cell and the environment determines the oligomerization state of Fnr through reversible formation of disulfide bonds (Esbelin et al., 2008). Monomeric Fnr binds to the promotor region and stimulates gene expression of the enterotoxin gene operons *hbl* and *nhe*, its regulatory genes *resDE* and *plcR* and the *fnr* gene itself (Esbelin et al., 2008). The two-component system (TCS) ResDE is an anaerobic redox regulator that controls fermentative genes and enterotoxin genes to allow anaerobic growth and adaptation to low ORP conditions (Duport et al., 2006). Low ORP causes autophosphorylation of histidine sensor kinase ResE located in the cell membrane, which in turn leads to phosphorylation of the response regulator resD in the cytoplasm. The activated phosphorylated ResD up-regulates the transcription of fermentation genes, enterotoxin *hbl* and *nhe* genes and regulatory genes *fnr,* resDE, and *plcR* (Esbelin et al., 2009). Without the two-component system ResDE, *nhe,* and *hbl* gene expression is not completely eliminated but up-regulation beyond the aerobic level by anaerobic and low ORP conditions is no longer possible (Duport et al., 2006). The most recent research hypothesizes that ResD-Fnr-complexes stimulate *nhe* and *hbl* gene expression when ResD is phosphorylated and acts as co-activator of Fnr, while non-phosphorylated ResD is an Fnr anti-activator that inhibits gene transcription (Esbelin et al., 2009). Low ORP conditions result in high concentrations of phosphorylated ResD and could confer high enterotoxin expression through high levels of phosphorylated ResD-Fnr-complexes. In any case, Fnr and ResDE play a major role in the regulation of enterotoxin production under anaerobic conditions, with particular importance of ResDE under highly reducing conditions.

5.2.3 Two-component systems (TCS) for unknown environmental factors

The *B. cereus* genome contains many TCS: approximately 50–58 sensor proteins, i.e., histidine kinases (HK), and 48–52 response proteins, i.e., response regulators (RR) (de Been et al., 2006). Their biological role and target were predicted based on classification of the DNA-binding output domain of the RRs. *B. cereus* TCSs included ABC transporters conferring resistance against antimicrobial compounds and proteins involved in nutrient uptake and temperature dependent control of membrane fluidity (de Been et al., 2008). A specific promoter region often contains multiple inaction sites for different RRs with varying affinity, offering a possible explanation for the complex regulation of the certain genes by a variety of environmental signals, as it is also observed for the enterotoxin genes. The role of TCS system ResDE in enterotoxin up-regulation under to low oxidation-reduction potentials has been elucidated (Duport et al., 2006; Esbelin et al., 2009) and is discussed above. The *yvfRSTU* and *ywjDE* loci were discovered in the vicinity of the plcR gene (Okstad et al., 1999). The TCS yvfTU stimulates the *plcR* gene expression during the stationary growth phase, but the corresponding environmental signal is currently unknown (Brillard et al., 2008). Mutation of *yvfTU* slightly decreased the virulence of *B. cereus* in an insect model and slightly modified the expression of 24% of PlcR regulated genes. Expression of the *papR, plcR, nprB, cerAB,* and *nhe* genes was up-regulated by a functional YvfTU system, while the *hbl* expression was down-regulated. The partial and differential influence of yvfTU on the PlcR regulon suggests a mode of action more than simple

up-regulation of the *plcR* gene expression alone. The TCS YvrGH was suggested to regulate *cytK* transcription in addition to PlcR, but no studies have been conducted to elucidate its function and putative role in toxin regulation (Lund et al., 2000; Fagerlund et al., 2007).

5.2.4 Nutrients and food properties

5.2.4.1 Carbon source Glucose is required for enterotoxin production in low to moderate concentrations, the optimum is about 10 g/L (Spira & Silverman, 1979). No enterotoxin production was observed in the presence of 0.1 g/L glucose while growth was only slightly impaired (< 0.5 log CFU/mL) (Rowan & Anderson, 1997). Interestingly, high glucose concentrations (> 50 g/L) completely inhibit toxin production without affecting the growth of *B. cereus* (Sutherland & Limond, 1993). The repressing effect of high sugar levels is specific for glucose, because the toxin production was in accordance with growth in Nutrient Broth (containing 50 g/L glucose) supplemented with high concentrations of lactose (up to 150 g/L) and fructose (up to 300 g/L and possibly higher). This phenomenon could be used to prevent toxin production by addition of glucose in food products (Sutherland, 1993). Increased glucose concentrations also reduced the *hbl* gene expression under anaerobiosis (Duport et al., 2004). Repression of the *nhe* and *hbl* genes by high glucose levels is concerted through the catabolite control protein (CcpA) regulation system (van der Voort et al., 2008). Carbon catabolite repression (CCR) ensures the use of the preferential (rapidly metabolizable) carbon source from the available ones in order to optimize the energy yield. In Gram-positive bacteria with low G+C content, such as *B. cereus*, the repressor CcpA binds with the co-repressor P-Ser-HPr and the resulting CcpA-P-Ser-HPr complex binds to the Catabolite Responsive Element (*cre*) sequence of genes, which leads to their repression. *Cre* sequences were found before and in the beginning of the *nheA* gene and between the *hblC* and *hblD* genes (van der Voort et al., 2008). The effect of high glucose concentration on enterotoxin CytK production has not yet been studied, but CytK will probably be unaffected by catabolite repression, because the *cytK* gene lacks a *cre* site and *cytK* gene expression was unaffected in the *ccpA* mutant (van der Voort et al., 2008).

Bacteria take up and concomitantly phosphorylate numerous carbohydrates through phosphoenolpyru vate:carbohydrate phosphotransferase systems (PTS) (Deutscher et al., 2006). Phosphate groups are transferred from phosphoenolpyruvate (PEP) to the carbohydrates via two "general" cytoplasmic components EI and HPr and the carbohydrate specific component EII in the cell membrane. These PTS components cycle between their phosphorylated and non-phosphorylated state during the carbohydrate uptake and phosphorylation. HPr becomes phosphorylated by PEP at His-15, resulting in P-His-HPr, but also by Hpr kinase/phosphatase (HprK/P) and ATP at Ser-46, resulting in P-Ser-HPr. Bacterial growth on rapidly metabolizable carbohydrates such as glucose results in relatively low levels of phosphorylated PTS proteins and elevated fructose-bisphosphate (FBP) levels inside the cells, which stimulates the kinase activity of HprK/P and the subsequent formation of P-Ser-HPr. CcpA is a LacI/GalR-type repressor and a pleiotropic regulator of many genes involved in carbohydrate and protein metabolism and even the enterotoxin operons *nhe* and *hbl* (van der Voort et al., 2008). The glucose metabolism is regulated by CcpA through the activation of genes encoding glycolytic enzymes and the repression of genes encoding citric acid cycle enzymes. Repression by CcpA also occurs for enterotoxin genes and genes involved protein and amino acid metabolism. In conclusion, *B. cereus* prefers glycolysis but has plentiful alternative energy generation pathways, including protein metabolism. In environments where easily metabolizable nutrients like glucose are scarce such as the host's intestines, the expression of enterotoxins could be of particular use to aid in the degradation of host tissue to set free proteins as alternative energy and nutrient sources. This hypothesis is supported by the fact that many other bacterial pathogens also regulate toxin gene expression through CCR, such as the Cry4A toxin in *B. thuringiensis*, the enterotoxin Cpe and collagenase (kappa toxin) in *Clostridium perfringens* and the alfa-hemolysin and toxic shock syndrome toxin 1 (TSST-1) in *Staphylococcus aureus* (Varga et al., 2004; Khan & Banerjee-Bhatnagar, 2002; Seidl et al., 2008). The CcpA regulation of virulence factors by *S. aureus* occurs both dependently and independently of glucose (Seidl et al., 2009). Whether this is also the case for *B. cereus* is currently not known.

Some other carbon sources stimulate enterotoxin production, for example maltodextrose (16 g/L) in reconstituted milk-based infant formulae (IMF) (Rowan & Anderson, 1997). Similarly the addition of soluble starch (50 g/L) to nutrient broth resulted in fourfold higher enterotoxin Nhe and Hbl concentrations while bacterial outgrowth was only slightly higher (0.4 log CFU/mL) (Sutherland & Limond, 1993). However, high starch is no guarantee for toxin production, as mashed potatoes did not support Hbl synthesis (Mahakarnchanakul & Beuchat, 1999). On the other hand, some carbon sources inhibit enterotoxin production. For example lactose as the sole carbon source up to 35 g/L only supports growth without toxin production (Rowan & Anderson, 1997). Also under anaerobiosis, the enterotoxin production levels are highly dependent on the available carbon source (Ouhib et al., 2006). Fructose (3.6 g/L) as a carbon source results in tenfold higher transcription of the *hbl, nhe* and *plcR* genes in comparison to glucose (3.6 g/L) and sucrose (6.8 g/L), but surprisingly the Hbl and Nhe production was highest during growth on respectively sucrose and a glucose-fructose mixture (Ouhib-Jacobs et al., 2009). The discrepancy between the gene transcription and translation level indicates that the carbon source and the bacterial carbon metabolism are involved in additional transcriptional or post-transcriptional regulation of enterotoxin expression, besides the direct control of toxin gene transcription.

5.2.4.2 pH Enterotoxin expression is clearly regulated by the pH, but this effect is connected with and modified by other parameters, especially growth rate and nutrient availability. As a result, different studies report different pH values that confer maximal toxin production: pH 7.0–8.0 in basal medium (Spira & Silverman, 1979), pH 8.0–9.0 in Nutrient broth (Sutherland & Limond, 1993), pH 8.0–8.5 in Brain Heart Infusion broth with 0.1% glucose (BHIG) (Christiansson et al., 1989), pH 7.0 in BHIG (Garcia-Arribas & Kramer, 1990), pH 7.0 in basal medium (Beattie & Williams, 2002), pH 9.0 for Hbl and pH 6.0 for Nhe in J broth (Thomassin et al., 2006). Adjustment of BHIG with neutral pH 7.0 to alkaline pH 9.0 and acidic pH 5.0 delayed and reduced the enterotoxin production (Garcia-Arribas & Kramer, 1990). Acidic foods are not associated with enterotoxin production, but the inhibitory effect of acidification on toxin production might be indirect due to growth inhibition of *B. cereus* cells rather than direct control of the enterotoxin gene expression (Sutherland & Limond, 1993; Sutherland, 1993). Moreover, significant toxin production can still occur at low pH values (5.0–6.0) despite the slightly reduced growth (Sutherland & Limond, 1993; Beattie & Williams, 2002; Thomassin et al., 2006; Garcia-Arribas & Kramer, 1990).

5.2.4.3 Iron The iron availability inside the host is usually very low due to binding to the specialized host proteins such as transferrin, ferritins, hemoglobins, and myoglobulins. As a consequence several pathogenic bacteria regulate virulence factor expression in response to iron availability, e.g., the cytolysin expression in *S. aureus* (Torres et al., 2010). In *B. cereus* the ferric uptake regulator (Fur) and a ferric dicitrate uptake channel (Fec) encoded in the *fec* operon are important for iron sensing and iron metabolism. Fur and Fec control the expression of genes encoding iron metabolism proteins, and also genes encoding hemolysin II and cell surface proteins involved in cellular adhesion and invasion, which are essential for full virulence (Harvie & Ellar, 2005; Harvie et al., 2005). Hemolysin II is the only virulence factor of *B. cereus* that is regulated by iron availability through Fur. Furthermore, the *hlyII* gene is also regulated by the repressor HlyR, which binds in the *hlyII* promoter region and inhibits *hlyII* transcription (Budarina et al., 2004). However the *hlyII* gene is not regulated by PlcR, which differs completely from the regulation of the other enterotoxins and virulence factors that are under the control of PlcR and not of Fur (Gohar et al., 2008).

5.2.4.4 Aeration Aeration of BHIG resulted in a clear increase of cytotoxicity (Christiansson et al., 1989). Moreover, toxin was only produced in aerated milk, whereas static and shaken incubation of sealed milk packages did not reveal any toxicity. The stimulatory effect of aeration on enterotoxin production seems in conflict with the stimulatory effect of anaerobiosis. It must be noted that aeration of the milk resulted in higher biomasses ($2*10^8$ CFU/mL) than shaken and static milk ($1*10^7$ CFU/mL– $4*10^7$ CFU/mL), which could partially explain the observed stimulation of enterotoxin production. Another possible explanation is that the aeration alters the interactions of the enterotoxins with milk proteins, which in turn influences the cytotoxicity detection.

5.2.5 Food additives and preservatives

B. cereus growth and enterotoxin production are inhibited by the addition of the peptide bacteriocin nisin to beef gravy (Beuchat et al., 1997). Concentrations in the range of 0.001–0.005 g/L prevented toxin production, while higher concentrations of nisin (0.005–0.050 g/L) also inhibited the growth. The cationic cyclic peptide bacteriocin AS-48 of *Enterococcus faecalis* (0.0075 g/L) completely inhibited the *B. cereus* growth and enterotoxin production in BHI, but lower concentrations (0.005 g/L) resulted in delayed but full outgrowth and delayed and decreased maximal enterotoxin production (Abriouel et al., 2002). Similarly, the essential oil carvacrol (0.06 g/L) reduced the maximal growth rate (from 0.48 h^{-1} to 0.24 h^{-1}) and inhibited Nhe production by *B. cereus* in BHI (Ultee & Smid, 2001). To obtain similar results in mushroom soup a 50-fold higher concentration of carvacrol (3 g/L) was required. These studies show that some food additives and preservatives have the potential to decrease enterotoxin production in food by affecting toxin production and/or growth of *B. cereus*. Depending on the stability and toxicity of the components and their sensorial properties, it might be worthwhile to look for food preservatives that continue to control the toxin gene expression in the consumer's intestines.

Food additives and preservatives that delay or prevent bacterial growth also inhibit toxin production indirectly, as high toxin concentrations are correlated with high bacterial numbers. Following measures can control *B. cereus* growth in food and thus toxin synthesis: sodium chloride (2–4%) (Mahakarnchanakul & Beuchat, 1999), sodium lactate (3%) and calcium lactate (1.5%) (Aran, 2001), sorbic acid (0.09%), citric acid (0.16%), and lactic acid (0.27%) (Del Torre et al., 2001; Van Netten et al., 1990) and a variety of essential plant oils (Oroojalian et al., 2010). Synergistic effects of multiple additives are often reported, as well as more pronounced effects at suboptimal conditions, e.g. lower pH and temperature.

6 Food safety implications

6.1 Cereulide

The very stable toxin cereulide is pre-formed in food by emetic strains growing in food. Thus, outgrowth of *B. cereus* in food beyond the early exponential phase holds the risk of emetic food poisoning syndrome for the consumer later on, even if the bacteria are inactivated during further food processing or preparations. To avoid emetic food poisoning caused by *B. cereus*, temperature control to prevent toxin production is essential. Food should be kept > 55 °C after preparation or cooled as quickly as possible through the temperature range 55–10°C and stored < 10°C or ideally < 4 °C (The EFSA journal, 2005). Following additional

measures are recommended to prevent cereulide contamination of food products: lowering the pH below 5.6, lowering the water activity below 0.953, modified atmosphere packaging with < 2% oxygen and elimination or substitution of the food additives leucine and valine. The majority of the cereulide producing strains is mesophilic, while both mesophilic and psychrotrophic strains produce enterotoxins (Carlin et al., 2006; Rowan & Anderson, 1998). Psychrotrophic strains can grow at refrigeration temperatures and could attain high contamination levels in refrigerated food products at the end of their shelf life. A general remark regarding the definition of psychrotrophic and mesophilic should be made, because this term is used differently by many authors. Psychrotrophic is defined as a strain which can grow at refrigeration temperatures, although its optimal growth temperature lies in the room temperature range (Mossel et al., 1995). It is usually defined as the ability to grow at ≤ 7°C (American Public Health Association, 1992), but this exact temperature limit may differ according to the author.

The conventional plating methods for detection of *B. cereus* by plating on Mannitol Egg Yolk Polymyxin (MYP) and Polymyxin Egg Yolk Mannitol Bromothymol Blue Agar (PEMBA) rely on haemolysis and lecithinase activity. As a consequence, the non-hemolytic and lecithinase-negative emetic strains are not detected by the standard plating methods. As an alternative, numerous real-time PCR assays have been developed for *B. cereus*, targeting the hemolysin gene (Wang et al., 1997), the phospholipase C gene (Martinez-Blanch et al., 2009), the 16S rRNA gene (Priha et al., 2004; Reekmans et al., 2008), the nonhemolytic enterotoxin genes (Yang et al., 2007), the hemolysin BL genes (Gore et al., 2003) and the emetic toxin genes (Fricker et al., 2007; Ehling-Schulz et al., 2004). Specific detection of emetic strains through PCR screening offers possibilities, but only if suitable general primers are developed which allow detection of all *ces* polymorphisms, as this is currently not the case (Hoton et al., 2009). Furthermore, screening for emetic *B. cereus* strains is often conducted only on isolates which do not show Hbl production, starch hydrolysis and salicin fermentation. All this points out that to date the detection of emetic *B. cereus* is biased, leading towards an underestimation of the prevalence of emetic *B. cereus*. So, the apparent distinct characteristics of emetic strains may reflect the lower strain variability due to the recent emergence of the emetic lineages from other *B. cereus* strains, but it might as well merely follow from biased screening.

6.2 Enterotoxins

Because the symptoms of *B. cereus* food-borne disease are usually mild, a bias in the food poisoning data is created due to underreporting of *B. cereus* food poisoning, and even more so for the small scale domestic cases. In these food poisoning cases an incriminating role of psychrotrophic B. cereus strains is important. Especially in situations where the average refrigeration temperature is just few degrees higher than 4°C psychrotrophic B. cereus is of a great problem.. In contrast the large scale outbreaks often involve catering and temperature abuse, often with mesophilic and emetic strains. This leads to an additional underestimation of psychrotrophic and diarrheal *B. cereus* strains as food-borne pathogens. On the other hand the growth and germination of psychrotrophic strains at body temperature and under intestinal conditions are impaired in comparison with mesophilic strains (Wijnands et al., 2007; Wijnands et al., 2006b).

Diarrheal *B. cereus* food poisoning has been associated with a wide diversity of food products including meat, fish, stew, poultry, cereals products, ready-to-eat-meals, milk, salads, and pasta. This again demonstrates the complex regulation of virulence and enterotoxin expression by various environmental signals and the opportunistic nature of *B. cereus* as a food-borne pathogen. Numerous studies attempted to unravel the regulatory effects of specific nutrients and environmental factors on *B. cereus* enterotoxin expression, but no general rules or correlations were discovered. Besides extensive strain variability there is also a variable response to environmental signals depending on their concentrations and the combination with various other parameters. Generally speaking, food products with a neutral to alkaline pH, an intermediate glucose concentration, a high starch content and a low oxidation-reduction potential pose a potential threat, as their nutrient composition stimulates enterotoxin expression in the food and possibly also in the small intestine. Enterotoxin production in food prior to consumption is generally assumed not to be of major concern, because enterotoxins are probably completely inactivated by cooking and gastrointestinal passage (Granum et al., 1993; Turnbull et al., 1979). Nevertheless, certain characteristics of the food commodity partially determine the conditions for *B. cereus* later on in the small intestine, such as the specific nutrients that are present in the gastrointestinal tract. In this way the food type can also influence the enterotoxin production *in vivo*.

Some psychrophic strains synthesize significant amounts of enterotoxins in food at refrigerator temperatures, so this could lead to accumulation of these bacteria and their toxins in Refrigerated Processed Foods of Extended Durability (REPFED). Moreover, if temperature abuse takes place during the extended shelf life, mesophilic strains may also multiply to higher numbers in REPFED foods. For example, cooked-chilled and pasteurized vegetable products such as broccoli, carrot and potato purée are regularly (approx. 25%) naturally contaminated with low numbers of *B. cereus* (< 10 CFU/g), of which > 97% could produce enterotoxins Nhe and/or Hbl (Choma et al., 2000). Although none of the strains grew at 4°C, mild (5°C) and moderate (10°C) temperature abuse resulted in outgrowth of 10% respectively 63% of the isolates. This risk of growth to higher levels in particular when cooked chilled meals are subjected to temperature abuse is further illustrated by the fact that only 4% of the 394 *B. cereus* isolates from ready-to-eat/cook foods was psychrotrophic, while 6.2%, 43.1%, and 90.0% could grow at

8°C, 9°C, and 10°C respectively (Samapundo et al., 2009). It must be noted that the minimal growth temperature may be higher in food products than in nutrient rich optimal laboratory media (Choma et al., 2000). However, *B. cereus* strains isolated from food and clinical samples display a cold adaptation response that could result in growth at a lower temperature by prolonged refrigerated incubation (Foegeding & Berry, 1997). Although preformed enterotoxins in food will probably not retain their toxicity during gastrointestinal passage (Granum et al., 1993; Turnbull et al., 1979), *B. cereus* multiplication is always hazardous in a food product, because it increases the consumption and gastric passage of viable and potential enterotoxic bacteria, leading to elevated numbers of surviving bacteria in the small intestine and thus the risk of diarrheal food poisoning. The minimal growth temperature is 5°C, so food should be kept below this temperature throughout the food chain. Moreover, refrigeration of food is often more effective than altering the food composition in the prevention of growth and enterotoxin production by *B. cereus* (Beattie & Williams, 2002).

Suboptimal growth conditions with nutrient and oxygen limitations generally serve as a stimulus for *B. cereus* enterotoxin production. For example, enterotoxin gene expression is stimulated by anaerobiosis and fructose, while the highest biomass is obtained by aerobic growth on glucose and even during fermentation glucose is the preferential carbon source of *B. cereus* (Ouhib-Jacobs et al., 2009). On the other hand, the toxin production is often impaired stronger and more easily by suboptimal growth conditions than bacterial outgrowth itself, as shown for temperature, carvacrol, nisin and bile salts (Park et al., 2008; Rowan & Anderson, 1998; Beuchat et al., 1997; Clavel et al., 2007; Ultee & Smid, 2001). Therefore modified atmosphere packaging (MAP) of food should be thoroughly investigated before application as food preservation technique. Anaerobiosis, limited oxygen availability and low oxidoreduction potentials stimulate enterotoxin and virulence factor expression by diarrheal *B. cereus* strains. This makes sense, as diarrhea occurs through enterotoxin production by *B. cereus* cells in the human intestine, which is a reducing environment where oxygen availability is limited. Thus MAP with no or decreased oxygen content is expected to stimulate enterotoxin production by *B. cereus* (Duport et al., 2004; van der Voort & Abee, 2009) and the virulence of bacteria that survive MAP and remain or multiply in the food product could be higher. Moreover, the atmospheric oxygen concentration influences the stress resistance of *B. cereus* (Mols et al., 2009). During microaerobic and anaerobic growth the bacteria show increased heat and acid resistance, so MAP packaging might also enhance the survival of this bacterium during later food processing and the gastrointestinal passage. Nevertheless, there is potential for MAP as a food preservation technique, as shown by a study that inactivated the present *B. cereus* bacteria in raw milk by flushing the headspace of milk vessels with pure nitrogen gas (Munsch-Alatossava et al., 2010). MAP and vacuum packaging of ready-to-eat potato based food products stored at 7°C with lowered atmospheric oxygen concentrations resulted in decreased growth rate and lower bacterial populations of diarrheal *B. weihenstephansis* (Samapundo et al., 2010). Enterotoxin Hbl production was decreased by vacuum packaging but it was unaffected by MAP packaging in the range of 1 to 21% O_2 in nitrogen atmospheres. MAP packaging with 40% CO_2 led to complete growth inhibition and thus also toxin production. Such application orientated experiments should be conducted in real MAP packaged food products under refrigeration conditions, because the diffusion and solution of gases differs according to the temperature and the exact food component in heterogeneous composite food products. More fundamental experiments with strict control of growth parameters are also necessary to further investigate the effect of MAP on *B. cereus* virulence and toxin production. The application of mild food processing and preservation techniques and its consequential sub-lethal stress signals may induce (cross) resistance or virulence (Rowan, 1999). Application of MAP and combinations of food preservative and additives (i.e., hurdle technology) that delay or inhibit growth provide a way to prolong the shelf life of food products, but the effect of these treatments on *B. cereus* growth capacity in the intestines, virulence and toxin production is currently not known and therefore potentially dangerous. The way virulence regulation and toxin production of *B. cereus* are affected by minimal food processing requires further research.

6.3 General food safety issues

It is clear that the mere presence of emetic and enterotoxin genes is only an indication of the pathogenic potential of *B. cereus* strains, but the actual risk for food poisoning is linked to the expression level of the toxin genes (Guinebretiere et al., 2002; Carlin et al., 2006). *B. cereus* is often present as a contaminating organism in food ingredients and finished products, although usually at low levels. Currently a few food safety questions still need to be answered. How can pathogenic *B. cereus* strains be identified, i.e., what are specific and discriminating characteristics of the food poisoning strains? Which enterotoxins are most important in *B. cereus* food poisoning and in which concentrations and combinations do they cause diarrhea? At the moment it is not clear which combination and/or polymorphisms of enterotoxin genes are associated with *B. cereus* strains responsible for diarrheal illness. Moreover no link has been found so far between virulence and polymorphisms in virulence factors and their regulators (Fagerlund et al., 2007). What is the infectious dose that will lead to food-borne illness? Food poisoning is not simply linked to the concentration of *B. cereus* in the ingested food, but it is highly strain dependent (emetic and/or diarrheal, psychrotrophic or mesophilic), the type of cells (vegetative cells or spores), the food product and the health status of the consumer. For example, healthy people did not suffer from food-borne illness after consumption of milk naturally contaminated with < 10^8 CFU/mL psychrotrophic *B. cereus* strains with

low enterotoxin expression levels (Langeveld et al., 1996), while formerly healthy people died of cereulide (Dierick et al., 2005; Mahler et al., 1997). Are there some food types, due to their composition, intrinsic factors or conditions of storage, more prone to cause food poisoning by stimulation of toxin production? The risk food products for *B. cereus* food poisoning are pasteurized (dairy foods or cooked chill foods) and dry foods (spices and to be reconstituted formulates), because these products commonly contain low numbers of *B. cereus* spores and vegetative cells either present from ingredients and during processing or due to post-contamination, which have the ability to germinate during storage and/or preparation. Emetic food poisoning cases usually involve starch-containing food products such as pasta, rice and mashed potatoes, while diarrheal food poisoning occurs with a wide variety of food commodities.

7 Future research

7.1 Cereulide

Currently the cereuilde regulation mechanisms are not fully understood, shown by seemingly contradictory results in literature and unexplained strain variability. Future research should address the influence of various stress conditions and the prior history of bacterial cells on the *ces* gene transcription and subsequent cereulide production. For example, the exact temperature and the duration of temperature abuse determines the effect on cereulide expression (Thorsen et al., 2009a). Studies that broaden the fundamental knowledge on cereulide expression and virulence regulation will allow improvement of the efficacy of food safety control measures. The cereulide promoter activity was studied by fusion of the *ces* promoter to the luciferase genes and subsequent bioluminescence measurement in a reporter strain (Dommel et al., 2010). Strong promoter activity and high cereulide concentrations were found in cooked rice, béarnaise sauce and liver sausage, while camembert cheese and quark dessert showed intermediate values and cream and a pastry snack did not contain any cereulide and displayed a low promoter activity. However, luciferase bioluminescence requires molecular oxygen for the synthesis of oxyluciferin and light. The use of flavin mononucleotide-based fluorescent proteins could enable promoter activity studies to assess virulence gene expression in both the presence and absence of oxygen (Drepper et al., 2007). Alternatively, cereulide gene transcription can be studied with the recently developed *ces* mRNA quantification method (Yasukawa et al., 2010), if it also proves applicable to food and intestinal samples, which can be a challenge (Ceuppens et al., 2010). Of course gene expression is also subject to posttranscriptional regulation, which should also be investigated with additional peptide detection methods, for example with highly sensitive LC/MS-MS methods (Delbrassinne, 2010).

7.2 Enterotoxins

It has become clear that enterotoxin expression by *B. cereus* is extensively and punctually regulated at transcriptional level (Figure 2). Strains harboring the same toxin gene variants still show substantial variability in enterotoxin transcription and expression, which cannot be attributed to

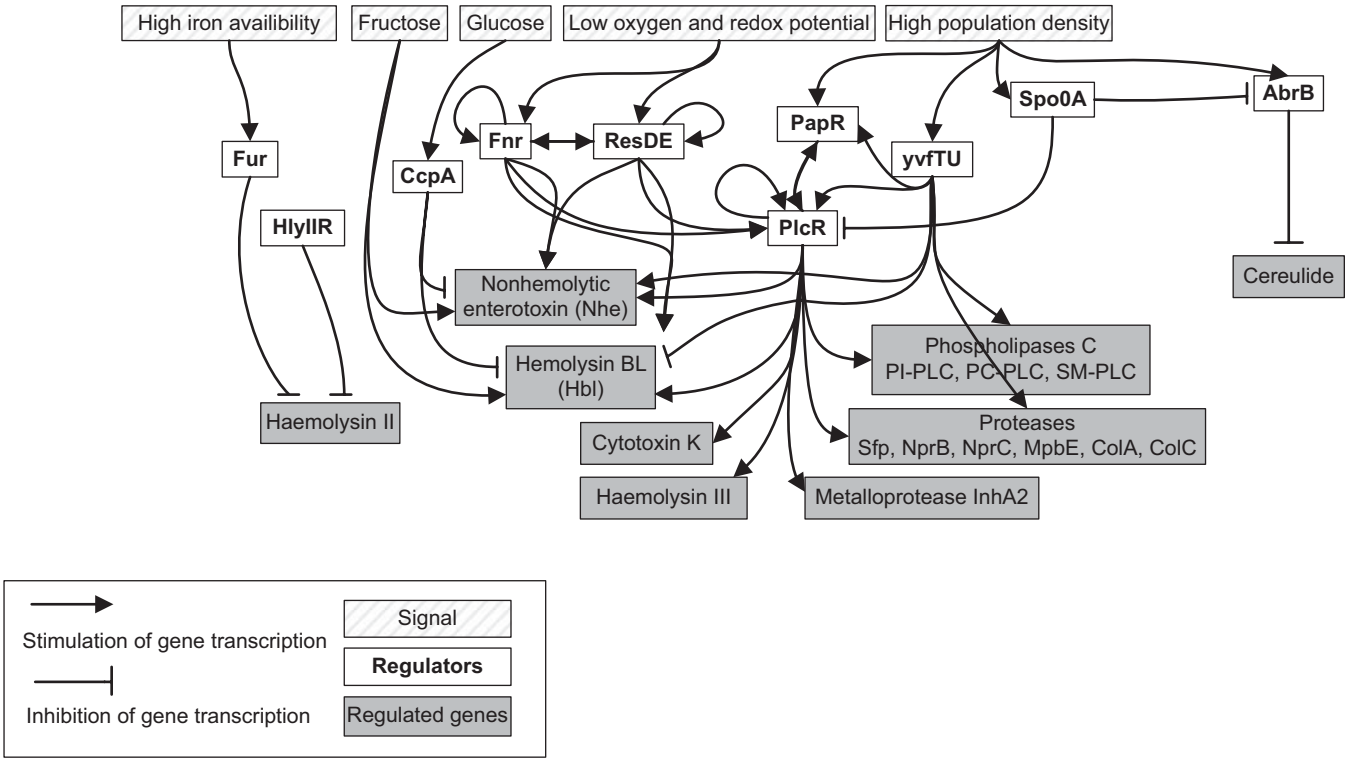


Figure 2. Regulation of toxin and virulence factor gene transcription in *Bacillus cereus*.

differences in the toxin promoter, PlcR–PapR, YvrGH, and YvfTU sequences (Fagerlund et al., 2007; Brillard & Lereclus, 2004). Moreover, the *hbl* and *nhe* operons have large promoter regions and the corresponding messenger RNA molecules contain long untranslated regions. This indicates that there are additional unidentified regulatory mechanisms for toxin expression in *B. cereus* yet to be discovered. Furthermore, discrepancies between gene expression and gene translation have been reported (Ouhib-Jacobs et al., 2009). Therefore it seems plausible that posttranscriptional regulation systems also exist. For example, the whole nhe operon is transcribed, but the expression of NheC is always approximately 10-fold lower than that of NheA and NheB due to translational repression (Lindbäck et al., 2004).

Future studies regarding enterotoxin production by *B. cereus* should be conducted by preference under conditions mimicking the human gastrointestinal environment, because the toxin production in food may not be relevant. One such study showed that in intestinal simulation medium the *hbl* gene expression and Hbl production by *B. cereus* is impaired more strongly than growth is reduced (Clavel et al., 2007). Intestinal stress conditions including bile salts inhibited toxin production and growth, but supplementation of certain food products mitigated this adverse effects. For example, toxin production never occurred in intestinal medium with skimmed milk, while in the case of pea soup enterotoxin Hbl was produced in intestinal medium with up to 0.9 g/L bile salts, with whole milk and chicken up to 0.6 g/L and with semi-skimmed milk up to 0.3 g/L. Experiments with similar approaches should elucidate the effect of certain food components on enterotoxin expression and thus the role of the food commodity in the diarrheal food poisoning syndrome. Expression studies should simultaneously investigate the effect of environmental parameters on the expression of the *hbl, nhe,* and *cytK* enterotoxin genes and differentiate between the effects on the different toxins, even though the final result could be a similar total cytotoxicity level.

8 Conclusions

Toxin expression is subject to complex regulation which is still far from completely elucidated. Besides the influence of numerous environmental parameters on toxin expression there is also huge strain dependent variability under identical circumstances. The difference between strains with high and low toxin expression remains obscure, despite numerous worthy efforts to comprehend the *B. cereus* genome and its variability. Future research should consist of experiments conducted in relevant food matrices or intestinal environments and take into account that toxin production is not always proportional to growth. Prevention of outgrowth and toxin production of *B. cereus* in food is the key to avoid both emetic and diarrheal food poisoning and maintain food safety standards. Refrigeration at < 4°C and limitation of residence at elevated temperatures (e.g., fast cooldown between 55°C and 10°C) are recommended as the most powerful food safety management tools (The EFSA journal, 2005). Moreover, acidification of food products and lowering the water activity can also be very useful to inhibit growth and associated toxin production. Modified atmosphere packaging with the exclusion of oxygen is effective to prevent cereulide production but there are indications that anaerobic conditions (as is also the case in the small intestine) stimulate enterotoxin production. Moreover the combination of MAP packaging and prolonged refrigerated storage of food products such as cooked chilled meals and dairy desserts, could enable growth and potentially enterotoxin production by psychrotrophic *B. cereus* strains or affect their virulence.

Declaration of interest

The authors report no conflict of interest. The authors alone are responsible for the content and writing of the article.

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