Draft Screening Assessment for

Bacillus cereus **(ATCC 14579)**

Environment Canada

Health Canada

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SYNOPSIS

Pursuant to paragraph 74(*b*) of the *Canadian Environmental Protection Act, 1999* (CEPA 1999), the Ministers of Environment and of Health have conducted a screening assessment on a strain of *Bacillus cereus* (ATCC 14579). This strain was added to the Domestic Substances List (DSL) under subsection 105(1) of CEPA 1999 because it was manufactured in or imported into Canada between January 1, 1984 and December 31, 1986 and it entered or was released into the environment without being subject to conditions under CEPA 1999 or any other federal or provincial legislation.

B. cereus strains are generally considered ubiquitous, and have the ability to adapt to and thrive in many aquatic and terrestrial niches. *B. cereus* strains form endospores that permit survival under sub-optimal environmental conditions. They are resistant to a range of antibiotics and heavy metals. The ubiquity of *B. cereus* strains is partially explained by their minimal nutritional requirements and ability to grow over a wide range of temperatures and pH values. Various characteristics of *B. cereus* strains make them suitable for use as active ingredients in commercial and consumer products, including detergents, degreasers, additives for biodegradation and bioremediation, and in various industrial processes.

B. cereus ATCC 14579 is recognized as a Risk Group 2 animal pathogen by the Canadian Food Inspection Agency (Animal Pathogen Import Program). Generally, Risk Group 2 animal pathogens are any pathogens that can cause disease but under normal circumstances are unlikely to be a serious hazard to healthy organisms in the environment and from which effective treatment and preventive measures are available. For example, *B. cereus* can cause mastitis in cattle that is treatable with specific veterinary antibiotics. There are no other cases where *B. cereus* has been shown to cause adverse effects to organisms in the Canadian environment in the scientific literature. There are scientific reports of *B. cereus* ATCC 14579 causing a reduced rate of reproduction in springtail (an arthropod), and decreased shoot and root length in red fescue (a plant). However, these were under specific laboratory conditions, which are not a concern under the current known exposure scenarios.

B. cereus ATCC 14579 is also recognized as a Risk Group 2 human pathogen by the Public Health Agency of Canada. Information from the scientific literature indicates that *B. cereus* ATCC 14579 has pathogenic potential in both the otherwise-healthy general population and in susceptible groups (i.e., infants and the elderly, the immunocompromised and individuals with debilitating comorbidities). *B. cereus* is a gastrointestinal pathogen that can also cause other types of infection, including endophthalmitis and skin infections. As mentioned *B. cereus* is resistant to several clinical antibiotics, which could in some circumstances, compromise the effectiveness of treatment of *B. cereus* infections. *B. cereus* ATCC 14579 produces a wide variety of extracellular enzymes and toxins that are important factors for its pathogenicity in humans.

This assessment considers human and environmental exposure to *B. cereus* ATCC 14579 from its deliberate use in consumer or commercial products or in industrial processes in Canada. *B. cereus* ATCC 14579 was nominated to the DSL based on its use in consumer and commercial products. Potential uses of *B. cereus* ATCC 14579 reported in the public domain include bioremediation of aquatic systems, biodegradation of organic and inorganic waste, bioleaching of metals in mining and waste management, treatment of sewage sludge, and uses in the pulp and paper and textile industries.

The government launched a mandatory information-gathering survey (Notice) under section 71 of CEPA 1999 as published in the Canada Gazette Part I on October 3rd, 2009.There were no reports of import or manufacture of *B. cereus* ATCC 14579, except for limited quantities for academic research, teaching, and research and development activities. Therefore the likelihood of exposure to this living organism in Canada resulting from commercial and consumer activity is low. It is therefore proposed to conclude that *B. cereus* ATCC 14579 does not meet the criteria under paragraph 64(*a*) or (*b*) of CEPA 1999 as it is not entering the environment in a quantity or concentration or under conditions that have or may have an immediate or long-term harmful effect on the environment or its biological diversity or that constitute or may constitute a danger to the environment on which life depends. It is also proposed to conclude that *B. cereus* ATCC 14579 does not meet the criteria under paragraph 64(*c*) of CEPA 1999 as it is not entering the environment in a quantity or concentration or under conditions that constitute or may constitute a danger in Canada to human life or health.

Therefore, it is proposed that *B. cereus* ATCC 14579 does not meet any of the criteria as set out in section 64 of CEPA 1999.

However, given the potential pathogenicity and toxicity of this strain to healthy and susceptible humans and to some susceptible non-human species, there is concern that this strain could meet the criteria as set out in section 64 of the Act should consumer, commercial or industrial activities resume. Therefore, it is recommended that the above substance be subject to the Significant New Activity provisions specified under subsection 106(3) of the Act, to ensure that any manufacture or import for a new use undergoes ecological and human health assessments as specified in section 108 of the Act, prior to the organism being considered for re-introduction into Canada.

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INTRODUCTION

Pursuant to paragrph 74(b) of the *Canadian Environmental Protection Act, 1999* (CEPA 1999), the Ministers of Environment and of Health are required to conduct screening assessments of those living organisms listed on the Domestic Substances List (DSL) to determine whether they present or may present a risk to the environment or human health (according to criteria as set out in section 64 of CEPA 1999). This living organism was nominated and added to the DSL under Section 105 of CEPA 1999 because it was manufactured in or imported into Canada between January 1, 1984 and December 31, 1986 and it entered or was released into the environment without being subject to conditions under CEPA 1999 or any other federal or provincial legislation.

Screening assessments examine scientific information and develop conclusions by incorporating a weight-of-evidence approach and precaution. This screening assessment included consideration of hazard information obtained from the public domain as well as from unpublished research data and from internal and external experts. Exposure information was also obtained from the public domain as well as from a mandatory CEPA 1999 s. 71 Notice published in the Canada Gazette Part I on October 3, 2009. Further details on the risk assessment methodology used are available in the "Framework on the Science-Based Risk Assessment of Micro-organisms under the *Canadian Environmental Protection Act, 1999*", referred to in this document as the Risk Assessment Framework (which is available on the web at http://www.ec.gc.ca/subsnouvelles-newsubs/default.asp?lang=En&n=120842D5-1).

Data that is specific to DSL-listed *B. cereus* ATCC 14579 is identified as such. Where data concerning this particular strain were not available, surrogate information from literature searches of both *B. cereus* and the genus *Bacillus* was used. Surrogate organisms were identified to the taxonomic level provided by the source. Information identified as of September 2011 was considered for inclusion in this Report.

DISCLAIMER: A determination of whether one or more of the criteria of section 64 of CEPA 1999 are met is based upon an assessment of potential risks to the environment and/or to human health associated with exposure in the general environment. For humans, this includes, but is not limited to, exposure from air, water and the use of products containing the substance. A conclusion under CEPA 1999 may not be relevant to, nor does it preclude, an assessment against the criteria specified in the *Controlled Products Regulations*, which is part of the regulatory framework for the Workplace Hazardous Materials Information System (WHMIS) for products intended for workplace use. Individuals who handle *Bacillus cereus* ATCC 14579 in the workplace (i.e., laboratory and R&D facilities) should consult with their occupational health and safety representative about safe handling practices, applicable laws and requirements under WHMIS and the Laboratory Biosafety Guidelines.

1. HAZARD ASSESSMENT

A hazard assessment characterizes the micro-organism and identifies its potential adverse effects on the environment and/or human health and the extent and duration of those effects. Hazards may be posed by the micro-organism itself, its genetic material, toxins, metabolites or structural components.

1.1 Characterization

1.1.1 Taxonomic Identification and Strain History

The accurate taxonomic identification of a micro-organism is essential in distinguishing pathogenic from non-pathogenic species and strains. A polyphasic approach combining classical microbiological methods (such as culture-based methods) and molecular tools (such as genotyping and fatty acids analysis) is often required.

Bacillus cereus is a Gram-positive, facultatively anaerobic, spore-producing, motile, rod-shaped bacterium. *B. cereus* spores are ellipsoidal, subterminal and do not swell the sporangium. *B. cereus* cells tend to occur in chains and the stability of these chains determines the form of the colony, which may vary from strain to strain (Logan and De Vos 2009). Table 1 provides a comparison of colony morphologies of *B. cereus* from various sources.

B. cereus (*sensu stricto*) is a member of the *B. cereus* group, which consists of six very closely related species: *B. cereus, B. thuringiensis*, *B. anthracis*, *B. weihenstephanensis*, *B. pseudomycoides* and *B. mycoides*. Species differentiation within the *B. cereus* group is especially complex, and a polyphasic approach is required for clear identification.

B. cereus ATCC 14579 was first isolated from the air in a cow shed in the United Kingdom (Frankland and Frankland 1887). *B. cereus* ATCC 14579 is the type strain and has several accession numbers in other culture collections, including DSM 31 from Deutsche Sammlung von Mikroorganismen und Zellkulturen GmbH and NCCB 75008 from the Netherlands Culture Collection of Bacteria.

The phenotypic characteristics summarized in Table 2 provide an overview of the metabolic capabilities of *B. cereus* ATCC 14579 (for the complete list of DSL strains see http://www.ec.gc.ca/subsnouvelles-newsubs/default.asp?lang=En&n=C4E09AE7-1) compared to other members of the *B. cereus* group. Data generated by Health Canada[1], including growth in liquid media at different temperatures (Appendix 1A), growth on solid media and biochemical testing at 28ºC or 37ºC (Appendix 1B) and fatty acid methyl-ester (FAME) analysis (Appendix 1C) provided further confirmation of the identification. It should be noted that these techniques can not be used to differentiate the DSL-listed strain from other *B. cereus* strains. The discrepancies between data obtained by the nominator (submitted at the time of nomination to the DSL), Health Canada[1], ATCC, and Bergey's manual are within the range of acceptability for *B. cereus*, and may be due to variable culture conditions. The FAME analysis of *B. cereus* ATCC 14579 showed high similarity with *B. thuringiensis,* which is expected, given the genetic similarity among the *B. cereus* group members.

[1] Environmental Health Science and Research Bureau

Table 1: Colony morphology of *B. cereus* ATCC 14579 and *B. cereus sensu stricto*.

Organism	Source	Shape	Size (mm)	Margin	Elevation	Colour	Texture (surface)	Opacity	Pigment
ATCC 14579	Nominator[1]	irregular	N/A[2]	erose	flat	N/A	dull	opaque	N/A
ATCC 14579	Heath Canada[3]	circular irregular	5-8	undulate	flat	cream	moist	opaque	none
ATCC 14579	American Type Culture Collection[1]	irregular	N/A	erose	flat	N/A	dull	opaque	N/A
B. cereus sensu stricto	Bergey's manual[4]	circular to irregular	2-7	entire to undulate, cremate or fimbriate	N/A	whitish to cream	matte or granular (smooth and moist)	opaque	pinkish-brown, yellow diffusible or yellow-green fluorescent possible

1. appearance on nutrient agar at 30˚C
2. N/A not available.
3. appearance on TSB agar after 7 days of growth at room temperature
4. appearance on blood agar after 24-36 hours at 37˚C

Genotypic methods, such as full genomic sequencing (Ivanova *et al.* 2003), amplified fragment length polymorphism (AFLP) (Ticknor *et al.* 2001), rep-PCR fingerprinting (Cherif *et al.* 2003),16S rDNA and 23S rDNA analysis (Ash *et al.* 1991), multi-locus enzyme electrophoresis (MLEE)(Ash and Collins 1992;Helgason *et al.* 2000b), multi-locus sequence typing (MLST) (Helgason *et al.* 2004;Priest *et al.* 2004;Tourasse *et al.* 2006) and suppression subtractive hybridization (SSH) (Radnedge *et al.* 2003), have been extensively used to demonstrate phylogenetic relationships and to understand the few genomic variations among the *B. cereus* group species. The genetic relatedness between members of the *B. cereus* group is so close that from a strictly phylogenetic point of view they can be seen as a single species.

The *B. cereus* group members are usually divided into three main phylogenetic clades; Clade I comprises *B. anthracis* and some *B. cereus* and *B. thuringiensis*, mostly from clinical sources; Clade II contains *B. cereus* ATCC 14579 and several other *B. cereus* strains, but is mostly composed of *B. thuringiensis* strains, few from clinical sources; and Clade III contains the non-pathogenic *B. mycoides* and *B. weihenstephanensis* (Didelot *et al.* 2009;Helgason *et al.* 2000b;Kolsto *et al.* 2009;Priest *et al.* 2004;Vassileva *et al.* 2006)(see Appendix 2). Different lineages based on MLST have also emerged from Clades I and II. *B. cereus* ATCC 14579 belongs more specifically to the Tolworthi lineage (Barker *et al*. 2005; Priest *et al.* 2004;Vassileva *et al.* 2006).

16S rDNA sequence analyses of the DSL *B. cereus* strain, conducted by Health Canada[2], have shown 100% homology compared to *B. cereus* ATCC 14579 on the proprietary MicroSeq ® ID library and more than 99% homology compared to other members of the *B. cereus* group included on the database (*B. thuringiensis* ATCC 33679 and ATCC 10792, *B. anthracis* Ames and *B. mycoides* ATCC 6462). This confirmed that the 16S rDNA from the DSL-listed strain obtained from the ATCC matched the published 16S rDNA sequence from *B. cereus* ATCC 14579. The DSL-listed *B. cereus* 16S rDNA sequence also showed the same high similarity when compared to published *B. cereus* sequences in NCBI.

[2] Enviromental Health Science and Research Bureau

Central to the identification of the various members of the *B. cereus* group is an analysis of phenotypic characteristics and pathogenicity traits, and of the presence of extra-chromosomal elements, which reflect the species' virulence spectra. The extra-chromosomal elements that differ between members of the *B. cereus* group are presented in Appendix 3. The plasmids determining pathogenicity patterns in the *B. cereus* group include pXO1 and pXO2 of *B. anthracis,* which contain the anthrax pathogenicity island, pBtoxis of *B. thuringiensis,* coding for the insecticidal protein, and pCER270 of *B. cereus*, encoding an emetic toxin. Extrachromosomal elements can also differ between strains of the same species. While pXO1 has been found in some *B. cereus* strains, such as G9241, and others carry pCER270, these are not features of *B. cereus* ATCC 14579, which only contains one extrachromosomal element, pBClin15 (Ivanova *et al.* 2003). The plasmid pBClin15 does not contain genes associated with any known pathogenicity traits.

Table 2: Differentiation of *B. cereus* from closely related *B. cereus* group species

Characteristics	*B. cereus ATCC14579*[1]	*B. cereus*[2]	*B. cereus Emetic biovar*[2]	*B. anthracis*[2]	*B. thuringiensis*[2]	*B. weihenstephanensis*[2]	*B. pseudomycoides*[2]	*B. mycoides*[2]
Motility	+	+	+	-	+	+	-	-
Catalase	+	+	+	+	+	+	+	+
Oxidase	+	-	-	N/A	-	N/A	-	-
Egg-yolk reaction	N/A	+	+	+	+	+	+	+
Hydrolysis of Casein	+	+	+	+	+	+	+	+
Hydrolysis of Esculin	+	+	+	+	+	N/A	N/A	+
Hydrolysis of Gelatin	+	+	+	+	+	+	N/A	+
Acid from Glycogen	N/A	+	-	+	+	+	N/A	+
Acid from Starch	N/A	+	-	+	+	+	N/A	+
Degradation of Tyrosine	+	+	N/A	-	+	+	+	d
Utilization of Citrate	+	+	+	d	+	+	d	d
Utilization of Propionate	+	N/A	N/A	N/A	N/A	N/A	-	-
Parasporal Crystal	-	-	-	-	+	-	-	-
Reduction of Nitrate	+	d	+	+	+	d	+	d
Voges-Proskauer	+	+	+	+	+	+	+	+
Deamination of Phenylalanine	N/A	-	-	N/A	-	-	N/A	-

+ > 85%; - 0-15% positive; N/A indicates data not available; d, different strains give different reactions

1. Nominator data

2. based on information summarizing phenotype of several strains form various publications available in Bergey's manual (Logan and De Vos 2009)

1.1.2 Genetic Transfer

Horizontal gene transfer has been recognized as one of the major mechanisms driving the evolution of micro-organisms and it plays a key role in their ability to adapt to new environments through acquisition of traits. The *B. cereus* group is a highly dynamic population and genetic transfer between the group members is an important evolutionary mechanism. Various plasmids are found in different strains of *B. cereus* (Appendix 3) and some harbour genes linked to pathogenicity or environmental adaptation (Helgason *et al.* 2000a;Hoffmaster *et al.* 2004;Rasko *et al.* 2005;Rasko *et al.* 2007). Generally these plasmids are present in low copy number, and are not self transmissible, but they can be mobilized with the help of other plasmids carrying homologs of key components of a conjugative secretion system (Van der Auwera and Mahillon 2005). Transduction (phage-mediated horizontal gene transfer) is a potentially important mechanism of gene transfer in natural environments. Bacteriophage CP-51, a generalized transducing phage for *B. cereus*, *B. anthracis* and *B. thuringiensis*, mediates transduction of plasmid DNA (Ruhfel *et al.* 1984). The only plasmid found in *B. cereus* ATCC 14579 is linear plasmid pBClin15 (Ivanova, 2003), closely resembling the Bam35 phage, a common bacterial virus (Stromsten *et al.* 2003) but no transduction events have been associated with pBClin15 in the scientific literature.

Insertion sequences (IS) are another type of mobile element that can be involved in horizontal gene transfer. IS elements are composed of inverted repeats flanking a transposase gene (De Palmenaer *et al.* 2004) and have been found in various members of the *B. cereus* group. IS*231* is one such element. Variants of IS*231* have been identified in the chromosomes and plasmids of *B. cereus* group members, including *B. cereus* ATCC 14579 (De Palmenaer *et al.* 2004). IS*231* has been implicated in the translocation of mobile insertion cassettes which may contain genes involved in antibiotic resistance or adaptation to the environment (Chen *et al.* 1999;De Palmenaer *et al.* 2004). The IS*231* variant identified in *B. cereus* ATCC 14579 is composed of two putative genes; one is 60% identical to a haloacid dehalogenase and the other is 55% identical to an acetyltransferase.

Group II introns were also identified in the genome of *B. cereus* group members (chromosome and plasmids), including one copy in *B. cereus* ATCC 14579. Even though these do not contain any pathogenicity genes, they are self-splicing, mobile retro-elements implicated in genetic transfer (Tourasse and Kolsto 2008). Other elements that can facilitate gene transfer may also be present in *B. cereus*. Økstad *et al* (2004) identified a DNA repeated element specific to the *B. cereus* group, *bcr1*. This element is present in *B. cereus* 14579 in 54 copies and possesses the characteristics of a mobile element. Therefore, *bcr1* could be implicated in horizontal gene transfer within the *B. cereus* group. Furthermore, the full genome analysis of the sequence of *B. cereus* ATCC 14579 (Ivanova *et al.* 2003) revealed the presence of 28 transposase genes, which could be involved in horizontal gene transfer (Kolsto *et al.* 2009).

Gene transfer is possible and could increase the hazard potential of *B. cereus*, as occurred when strain G9241 acquired the *B. anthracis* pXO1 plasmid carrying the anthrax pathogenicity island. However, the presence of vegetative cells seems to be essential for conjugation (Santos *et al*., 2010). Since *B. anthracis* exists in the natural environment mainly as dormant spores, and its vegetative cells survive poorly outside the host, the acquisition of *B. anthracis* plasmids by other members of the *B. cereus* group is extremely rare, and may be restricted to, or at least be more common in, areas where anthrax is endemic (Hoffmaster *et al*., 2006). Moreover, growth of *B. anthracis* outside a host usually leads to loss of

virulence (reviewed in Dragon and Rennie 1995). In a laboratory setting, the conjugal transfer of an insecticidal plasmid of *B. thuringiensis* to *B. anthracis* was observed at a ratio ranging from $6.9x10^{-4}$ to $1.9x10^{-7}$, but no naturally occurring insecticidal *B. anthracis* isolates have yet been reported (Yuan *et al.* 2010). Conjugation of *B. thuringiensis* plasmid pAW63 and pXO16 to one strain of *B. cereus* and between *B. thuringiensis* strains has been reported in food matrices under laboratory conditions (Van der Auwera *et al.* 2007). Conjugal transfer of plasmid pHT73- EM^R from *B. thuringiensis* var*. kurstaki* to *B. cereus* ATCC 14579 had frequencies of $1.1 \pm 0.90 \times 10^{-9}$ on nitrocellulose filter and was not detected on LB broth or on *Bombyx mori* larvae (Santos *et al*., 2010). While it is possible that *B. cereus* ATCC 14579 could acquire virulence plasmids from pathogenic relatives, the probability of such an occurrence is no higher than for other naturally occurring strains of *B. cereus*. The DSL strain does not contain plasmids bearing virulence factors, so it cannot be implicated in the conjugal transfer of virulence factors to other bacteria in the environment.

1.1.3 Pathogenic and Toxigenic Characteristics

The ability of *B. cereus* to produce infections in both human and non-human species is attributed to a wide array of mechanisms, including adherence, invasion, evasion of host defences and damage to host cells.

B. cereus can cause food poisoning and various opportunistic and nosocomial infections. It can cause two types of food poisoning, one resulting in vomiting through the action of the emetic toxin cereulide and the other resulting in diarrhea through the action of various enterotoxins (Granum 2001;Kotiranta *et al.* 2000;Stenfors Arnesen *et al*. 2008). Cereulide is a peptide toxin, that must be present in the food at the time of ingestion to cause vomiting (Agata *et al.* 2002). Live cells are not required to cause the emesis syndrome. For diarrheal syndrome, it is unclear if the enterotoxins are present in food or are produced in the small intestine by the live bacteria. However, enterotoxins are unstable at pH <4 and can be degraded by pepsin, trypsin and chymotrypsin (Granum 1994), so it is most likely that they are produced in the small intestine. Five toxins have been proposed as potential causes for the diarrheal syndrome: HBL, NHE, BceT, EntFM and CytK, but only three (HBL, NHE and CytK) have been related to food borne outbreaks (Agata *et al.* 1995a;Lund *et al.* 2000;Lund and Granum 1997; Stenfors Arnesen *et al.* 2008; Schoeni and Lee Wong, 2005).

B. cereus ATCC 14579 produces several different toxins including enterotoxins (hemolysin BL [HBL], nonhemolytic enterotoxin [Nhe], hemolysins (hemolysin II [HlyII] and III [HlyIII]) and phospholipase C (PLC) of which three variants are recognized: phosphotidylinositol hydrolase (PIH), phosphotidylcholine hydrolase (PC-PLC) and sphingomyelinase (SMase) (see Appendix 4) (Ivanova *et al.* 2003). Data generated by Health Canada by PCR-analysis confirmed the presence of *hBL*, *plC* and *hly-III* in the chromosome of *B. cereus* ATCC 14579 (Seligy *et al.* 2004). The emetic toxin-encoding gene is located on a plasmid, pCER270, which is not carried by the DSL-strain ATCC 14579, making it unlikely to produce cereulide (Haggblom *et al.* 2002). Phospholipase C and hemolysins produced by *B. cereus* are necrotic toxins that mimic the effects of some staphylococcal or clostridial toxins, resulting in invasive, non-gastrointestinal infections (Turnbull and Kramer 1983).

Adherence of enteropathogens to the intestinal epithelium is the essential first step required for colonization. Attachment of the bacterium is linked to the presence of fimbriae, which recognize a specific site on the enterocytes. A crystalline cell surface protein (S-layer) has also been implicated in attachment, but was not detected on the cell surface of *B. cereus* ATCC 14579 (Kotiranta *et al*. 1998). The enterotoxin components are either expressed on

the bacterial cell membrane or secreted into the intestinal lumen. There, the toxins cause diarrhea by perturbing the exchange of water and electrolytes (Belaiche 2000). It has been suggested that *B. cereus* HBL, Nhe and CytK enterotoxins form pores in the membrane of intestinal epithelial cells, which causes osmotic lysis (Beecher and Wong 1997;Hardy *et al.* 2001; Haug *et al.* 2010).

Other virulence factors specific to *B. cereus* include proteases, notably metalloproteases (Cadot et al., 2010; Guillemet et al. 2010), and other degradative enzymes play a role in the establishment and development of infection, and in circumventing the host immune system (Appendix 5). Some of these have been implicated in both human and non-human target infections (see Appendices 6A and 6B). The transcription factor PlcR is seen as a virulence factor as it is involved in the expression of most known virulence factors in *B. cereus*, including HBL, Nhe, CytK, PLCs and several proteases in the DSL strain and may be in part responsible for the variability of virulence amoung *B. cereurs* stains (Gohar *et al.* 2008). The ability of the *B. cereus* ATCC 14579 strain to grow at 37°C, as shown in Appendix 1A, is another concern from a human health standpoint.

Data generated by Health Canada[3] with *B. cereus* ATCC 14579 (cells and culture filtrates) showed cytotoxic activity towards a human colon cancer cell line and a mouse macrophage cell line at 37°C that is consistent with findings from other laboratories. Also, strain ATCC 14579 showed high cytotoxicity on Vero cells when grown at 37°C and 15°C in BHIG (L. P. Stenfors Arnesen, personal communication[4]). Linbäck *et al.* (1999) demonstrated the cytopathogenic effect of *B. cereus* ATCC 14579 (supernatant) on Vero cells and strong haemolytic activity against sheep erythtocytes, both at 37°C. Although cytotoxicity is evident in these studies, the results vary depending on the growth temperatures.

Due to the high genetic similarity among *B. cereus* group members, clinical isolates sharing the toxins known to be present in *B. cereus* ATCC 14579 are considered good surrogates for characterizing the potential human health hazard of *B. cereus* ATCC 14579, as long as it is recognized that *B. cereus* ATCC 14579 differs from the highly pathogenic strains of the *B. cereus* group in that it does not carry the virulence plasmids that are associated with the emetic syndrome or anthrax *(*Didelot *et al.* 2009*;* Helgason *et al.* 2000b; Kolsto *et al.* 2009; Rasko *et al.* 2005; Vassileva *et al.* 2006). *B. cereus* 14579 can also be distinguished from the highly pathogenic strains of the *B. cereus* group based on its genomic sequence and its phylogenetic position in Clade II of the *B. cereus* group. Clade II comprises the majority of *B. thuringiensis* isolates (Priest *et al.* 2004), which also include clinical isolates (Barker *et al*. 2005;Hoffmaster *et al*. 2008), whereas the highly pathogenic strains (*B. anthracis* and *B. cereus* emetic strains) are grouped in Clade I. Recently, Guinebretière *et al.* (2008) proposed a new classification of the *B. cereus* group based on AFLP. This new classification includes seven groups, each of which is associated with a particular temperature growth range and potential for pathogenicity. Under this scheme, *B. cereus* ATCC 14579 belongs to group IV, which includes those *B. cereus* and *B. thuringiensis* strains that grow at 37ºC and are implicated in food poisoning (Guinebretière *et al*. 2008; Guinebretière *et al.* 2008).

1.1.3.1 Effects on Human Health

Gastrointestinal illnesses are the most common infections associated with *B. cereus.* In healthy individuals the symptoms are generally mild, but complications can lead to more

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serious disease, or even death (Ginsburg, 2003; Girish *et al*. 2003; Lund *et al*. 2000; Shiota 2010; Dierick *et al*. 2005). *B. cereus* gastrointestinal outbreaks have been reported around the world (Appendix 7B). It is implicated in 1 to 33% of cases of food poisoning (Anonymous, 2005) with varying degrees of severity. The true number of cases is likely underestimated since most foodborne diseases are underreported. In Canada, *B. cereus*-related diseases are not notifiable and outbreaks are investigated at the local Health Unit level (J. Greig, personal communication). Only one foodborne outbreak has been reported for this species (J. Greig, personal communication[5], McIntyre *et al.* 2008). There have been no reported laboratory-acquired infections to date.

B. cereus also causes non-gastrointestinal illness (review in Bottone, 2010; Drowbnieski, 1993). Non-gastrointestinal *B. cereus* outbreaks (Appendix 7A) are less frequent, and most are identified as nosocomial in origin. Endophthalmitis is a severe infection caused by the introduction of bacteria into the eye following trauma or surgery. Case reports of *B. cereus* endophthalmitis or panophthalmitis have been reported in the literature (Al-Jamali *et al.* 2008;Altiparmak *et al.* 2007;Chan *et al.* 2003;Martinez *et al.* 2007;Tobita and Hayano 2007;Zheng *et al.* 2008). Among the organisms that infect the eye, *B. cereus* has one of the most rapidly evolving courses of infection (Brinton *et al.* 1984) and is one of the most destructive (Levin and D'Amico 1991;Parke 1986;Pflugfelder and Flynn 1992). An experiment conducted on rabbits by Callegan *et al.* 2003 showed reproducible endophthalmitis caused by *B. cereus* strain ATCC 14579. Despite aggressive drug and/or surgical intervention, *B. cereus* endophthalmitis typically results in migration of bacteria throughout the eye and a remarkably rapid progression to a severe intraocular inflammatory response, resulting in loss of functional vision within 24h to 48h (Davey and Tauber 1987;Vahey and Flynn 1991).

B. cereus can produce a variety of skin and soft tissue infections, including cellulitis (Dancer *et al.* 2002;Latsios *et al.* 2003) and necrotizing cellulitis (Darbar *et al.* 2005;Hutchens *et al*. 2010; Sada *et al.* 2009). Wound infections, mostly in otherwise healthy persons, have been reported following trauma, surgery and burns (Crane and Crane 2007;Dubouix *et al.* 2005;Moulder *et al.* 2008;Pillai *et al.* 2006;Ribeiro *et al.* 2010;Shimoni *et al.* 2008;Stansfield and Caudle 1997). Catheter use was often associated with *B. cereus* infection (Crane and Crane 2007;Flavelle *et al.* 2007;Koch and Arvand 2005;Monteverde *et al.* 2006;Ruiz *et al.* 2006;Srivaths *et al.* 2004).

B. cereus endocarditis is a rare condition that is associated with intravenous devices or recreational drug injections (Abusin *et al.* 2008). Morbidity and mortality associated with *B. cereus* endocarditis are high among patients with valvular heart disease (Cone *et al.* 2005;Steen *et al.* 1992).

Some cases of *B. cereus* meningoencephalitis (Evreux *et al.* 2007;Lebessi *et al.* 2009;Lequin *et al.* 2005;Manickam *et al.* 2008;Puvabanditsin *et al.* 2007) and bacteraemia (Girisch *et al.* 2003;Hilliard *et al.* 2003;John *et al.* 2007;Tuladhar *et al.* 2000;Van Der Zwet *et al.* 2000) have been reported in neonates. Neonatal meningoencephalitis caused by *B. cereus* is rare, but it carries a high mortality. Cases of infection are often associated with medical equipment or devices.

Some cases of *B. cereus* pneumonia have been reported. Pulmonary infections due to

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B. cereus are rare compared to those attributed to *B. anthracis,* but can be just as life threatening in immunocompromised persons. The majority of cases were in metalworkers and immunocompromised patients who have greater susceptibility to infection. Avashia *et al.* (2007) reported the cases of two healthy metalworkers who died from *B. cereus* pneumonia. Another case of death, in an area where anthrax occurs naturally in herbivores, was also reported in a metalworker (Hoffmaster *et al*. 2006). However, in all of those cases, plasmid pX01 (but not pX02) was found in all *B. cereus* samples and the route of exposure was suspected to be inhalation. Cases of *B. cereus* pneumonia in cancer patients were reported by Frankard *et al.* (2004), Fredrick *et al.* (2006), Katsuya *et al.* (2009), and Sotto *et al.* (1995). In most cases, the route of infection was unknown but linked to existing *B. cereus* infections in the patients. In all but one case, the infection resulted in death.

Two studies in BALB/c mice showed that inhalation of either *B. cereus* ATCC 14579 spores or vegetative cells had adverse effects. Salimatou *et al.* (2000) reported that ninety percent of mice died after 24h after nasal instillation of 10^7 spores, while all died after administration of 6 x 10^6 vegetative cells. The cause of death was not determined but did not seem to depend on the growth of bacteria in the mice. Flaws in the study make its results questionable. The experiment was done only once, and no results for control mice were presented. The instillation of a large dose volume could have been the cause death by asphyxiation and pulmonary hemorrhage. Tayabali *et al.* (2010) reported no toxicological effects in BALB/c mice exposed to 10^7 spores of *B. cereus* ATCC 14579 one week after endotracheal instillation. However, severe shock-like symptoms (lethargy, hunched appearance, ruffled fur, and respiratory distress) occurred 4 hours after exposure to 10^5 or 10^6 vegetative cells. An increase of inflammation cytokine levels was observed in the blood and lungs, but not in all mice, resulting in a high standard deviation. Post-testing revealed an intermediate cytokine response after exposure to 10^4 and no response to lower vegetative cell exposure (10^2 and 10^3) (A. Tayabali, personal communication). In comparison to the Salimatou study, the Tayabali study was better controlled and better standardized the production of spores and vegetative cells. Pre-study work on methodology was also done to limit the effect of the instillation procedure in the final results.

The range of reported non-gastrointestinal infections is wider in immunocompromised individuals. Necrotizing meningitis, subarachnoid hemorrhage and brain abscesses have been reported with systemic *B. cereus* infections in patients with leukemia (Gaur *et al.* 2001;Nishikawa *et al.* 2009). Other local and systemic *B. cereus* infections have also been reported in patients with compromised immunity (Akiyama *et al.* 1997;El Saleeby *et al.* 2004;Hernaiz *et al.* 2003; Hirabayashi *et al*. 2010;Kiyomizu *et al.* 2008;Kobayashi *et al.* 2005;Le Scanff *et al.* 2006;Musa *et al.* 1999;Nishikawa *et al.* 2009).

Clinical reports demonstrate that the severity of *B. cereus* infection significantly correlates with its ability to synthesize toxins (Beecher *et al.* 2000; Ghelardi *et al.* 2002) and depends on the immune competence of the host and the virulence of the microbe. As mentioned in section 1.1.3, genes encoding for hemolysin BL, nonhemolytic enterotoxin (Nhe), hemolysins (hemolysin II and III), and phospholipase C (phosphotidylinositol hydrolase, phosphotidylcholine hydrolase and sphingomyelinase) are present in *B. cereus* ATCC 14579, but no data is available on the levels of expression of toxins or virulence factors. Hemolysin II and metalloproteases InHA1 and NprA can also serve as indicators of pathogenicity (Cadot *et al.* 2010), however it is impossible to predict which *B. cereus* strains are able to cause gastrointestinal disease based solely on the presence of these virulence factors (Anonymous 2005) since not all strains containing these factors cause adverse effects.

Treatment of *B. cereus* human infections is hampered by resistance to antimicrobial drugs. Antibiotic sensitivity tests showed that the resistance of *B. cereus* to different antibiotics is widely variable between strains (Bernhard *et al.* 1978;Weber *et al.* 1988). Most strains of *B. cereus* produce ß-lactamase and are therefore considered to be resistant to ß-lactam antimicrobial agents (Coonrod *et al.* 1971). Most *B. cereus* strains have been found to be resistant to penicillin, semisynthetic penicillin, cephalosporin (Stretton and Bulman 1975;Weber *et al.* 1988), ampicillin, colistin, polymyxin, kanamycin, tetracycline, bacitracin and cephaloridine (Bernhard *et al.* 1978;Wong *et al.* 1988). Mols *et al*, 2007 reported that *B. cereus* ATCC 14579 is resistant to antibiotics targeting cell wall components such as cefazolin, ketoprofen and moxalactam. Even with appropriate antibiotic regimens, there are reports in the literature presenting refractory *B. cereus* infection leading to fatal outcomes (Musa, 1999; Tuladhar, 2000). Antibiotic susceptibility tests conducted by Health Canada on 10 classes of antibiotics have shown that *B. cereus* ATCC 14579 is highly resistant to amoxycillin, aztreonam and trimethoprim, that it had intermediate sensitivity to cephotaxim and nalidixic acid but that it is sensitive to doxycyline, erythromycin, gentamicin and vancomycin (Table 3).

Table 3: Minimal Inhibitory Concentration (MIC)[1] for *B. cereus* ATCC 14579.

Antibiotic	ATCC 14579
Amoxycillin	> 24 µg/mL
Aztreonam	> 24 µg/mL
Cephotaxime	> 12 µg/mL
Doxycycline	< 0.37 µg/mL
Erythromycin	< 0.37 µg/mL
Gentamicin	1.5 µg/mL
Nalidixic acid	6 µg/mL
Trimethoprim	> 24 µg/mL
Vancomycin	1.5 µg/mL

1. Work conducted using TSB-MTT liquid assay method (Seligy *et al.* 1997). The reported values are based on a minimum of three independent experiments. Values correspond to the minimal inhibitory concentration (µg/ml) for *B. cereus* ATCC 14579 (20, 000 CFU/well) grown in the presence of antibiotic for 72 hrs at 37°C.

1.1.3.2 Effects on the Environment

B. cereus, as a species, is recognized as a Risk Group 2 pathogen by the Canadian Food Inspection Agency (Animal Pathogen Import Program). Generally, a Risk Group 2 pathogen is any pathogen that can cause disease, but under normal circumstances is unlikely to be a serious hazard to organisms in the environment. Effective treatment and preventive measures are available, and the risk of spread is limited. However, *B. cereus* can have a range of effects on non-human species, depending on the host and method of exposure. Some examples include diarrhea (monkeys), mastitis (cattle), inflammation (rabbits) and death (range of organisms) (see Appendices 6A and 6B). With the exception of mastitis, all of the data are from experimental studies. In the studies cited below, those that utilized *B. cereus* ATCC 14579 are indicated by an asterix.

Four cases involved mastitis in cattle, which were lethal in some cases (Appendix 6B). However, it is known that with the appropriate treatment, animals can survive such infection (Schiefer *et al.*, 1976). With respect to invertebrates, two studies reported that *B. cereus* isolate WGPSB-2 has potential as a biocontrol agent against white grubs (Selvakumar *et al.*

2007;Sushil *et al.* 2008). A number of experimental studies (outside of what were considered natural settings) used *B. cereus* on a variety of target organisms. These included invertebrates (Lepidopteran*, Blattarian* and Coleopteran insects and crustaceans) and mammals (guinea pigs, rabbits, mice*, cattle, monkeys and cats). Some of the methods of exposure included free ingestion or gavage, injection (intravenous, intrahaemocoelic, intracoelomic, intradermal, intravitreal, intraperitoneal, subcutaneous), nasal instillation, or dermal exposure to cultures or cell-free supernatants.

The objectives of the studies varied and included some of the following: characterization of the role of specific genes in virulence, investigation of the opportunistic properties of *B. cereus*, the suitability of specific organisms as an oral infection model for entomobacterial pathogens, investigation of natural pathogens for different pests, pathogenicity testing to characterize cause of larval death, purification and identification of a soil bacterial exotoxin, and models for human *B. cereus* pathogenicity.

The results of the studies referred to above also varied, depending on the target organisms, the strains of *B. cereus* used and the method of exposure. In many of the studies on lepidopteran invertebrates, *B. thuringiensis* insecticidal crystal toxin (Cry1C) was co-administered with spores of *B. cereus* 14579. *B. cereus* spores contributed synergistically to mortality in these studies, and mortality in the absence of Cry1C was low. Nevertheless, a specific strain of *B. cereus* was identified as a lepidopteran pathogen by Koch's postulates in *Trichoplusia ni*, and *B. cereus* sphingomyelinase* was demonstrated to be toxic to silkworms and cockroaches. Elm bark beetle larvae suspended in *B. cereus* cultures showed 63.6% mortality. *B. cereus* was also pathogenic toward orally inoculated Southern pine beetle larvae and showed varying degrees of toxicity and mortality when freely ingested by Boll weevil and Black Bean aphids (but not by Egyptian cotton leafworm). Water fleas exposed to *B. cereus* cultures died within 8 to 16 days. Pathogenicity and toxicity testing on terrestrial organisms were also performed at Environment Canada laboratories. Results of chronic testing with *B. cereus* ATCC 14579 using the invertebrate species *Folsomia candida* (springtail) demonstrated no effect on adult mortality, but a depression in juvenile reproduction at 10^8 cfu/g soil (Princz, 2010).

In vertebrates, effects reported from various sources included necrotic inflammation at the site of subcutaneous injection, fluid accumulation in a rabbit ileal loop model, increased vascular permeability, presence of abscesses and/or nodules following intradermal injection, calcification and necrotic skin ulcers following intramuscular injection in rabbits, diarrhea following ingestion in monkeys, abortion in cattle and sheep injected intravenously with high doses, and mortality in mice. Specific details of these experiments are provided in Appendix 6A. Based on the available information, it is worth noting that the pathogenic effects noted in Appendix 6A are not expected to occur to biota in the environment given that the route of administration bypassed natural physical barriers to infection and/or the concentrations of bacteria used were higher than would be expected in the natural environment.

Pathogenicity and toxicity tests of *B. cereus* ATCC 14579 on plants were performed at Environment Canada laboratories. Plant testing using *Festuca rubra* (red fescue) demonstrated a significant decrease in shoot length (21% reduction relative to control response), root length (28% reduction) and root dry mass (42% reduction), but no effect on shoot dry mass in comparison to control growth in conducted tests (Princz, 2010). Despite the observed adverse effect on red fescue at the concentration tested, this strain is not suspected to be a frank plant pathogen nor is it expected to be used at this concentration in any industrial or consumer application to plants.

1.1.4 Other Ecological Characteristics

B. cereus has minimal nutritional requirements, grows over a range of temperatures and pH and has the ability to form spores; therefore, its vegetative cells have the capacity to colonize a variety of niches and its spores to persist indefinitely in many environments (Kotiranta *et al.* 2000). *B. cereus* forms endospores that permit survival under sub-optimal environmental conditions. These have a tough outer keratin-like layer which is heat-, chemical-, radiation-, disinfectant- and desiccation-resistant, often withstanding temperatures of 126°C for more than 90 minutes (Pillai *et al.* 2006). The spores are found in many types of soil and in sediments, dust and plant material, are described as having a ubiquitous presence in nature (Stenfors Arnesen et al, 2008) and may passively spread in the environment. The spores are not easily destroyed by means that eliminate vegetative cells and may germinate when in contact with organic matter, or once inside insect or animal hosts (Stenfors Arnesen *et al.* 2008).

Nevertheless, conditions required for growth and survival vary with the strain (Bassen *et al.* 1989; Gibriel and Abd-el Al 1973; Jaquette and Beuchat 1998; Rizk and Ebeid 1989; Rossland *et al.* 2003). The optimal growth temperature for most strains is between 30ºC and 37ºC, normally with no growth above 55ºC or below 5ºC. The optimal pH depends on the growth medium used (Andersson 1995), with no growth seen in media of pH lower than 4.3 or higher than 10.5.

Biofilm formation is in general associated with pathogenicity and increased resistance to antimicrobial agents. The species *B. cereus* is reported to have the ability to form biofilms; however, no biofilm formation was observed for *B. cereus* ATCC 14579 after incubation of an exponential phase culture at an OD (600 nm) of 0.01 into L-Broth medium in a 96-well polyvinylchloride microliter plate during 72 h at 30˚C, whereas biofilm formation was observed under the same conditions in *B. cereus* ATCC 10987 (Auger *et al.* 2006). In another study, *B. cereus* ATCC 14579 formed biofilms on Y1 medium after 24 h at 20°C and 30°C, but after 48h the biofilms dispersed (Wijman *et al*., 2007). The conclusion of this study was that bioflm production was found to be strongly dependent on incubation time, temperature, and medium, as well as the strain used.

It has been shown that *B. cereus* ATCC 14579 is able to produce a bacteriocin-like inhibitory substance (BLIS) that is highly active against closely related *Bacillus spp.* (Risoen *et al.* 2004). However, there are currently no published reports or research articles indicating that *B. cereus* ATCC 14579 is harmful to microbiota in the environment at the population level (for the purpose of this assessment, this indicates a significant number of organisms of the same species inhabiting a given area).

B. cereus is most likely involved in biogeochemical nutrient cycling, as it produces a wide range of extracellular enzymes and can grow on decaying organic matter (Borsodi *et al.* 2005).Therefore, it is capable of playing a role in the decomposition and recycling of soil organic matter, when it appears in a vegetative state, however its widespread occurrence in soils is notably as spores. *B. cereus* is also capable of reducing nitrate to nitrite and ammonium and can thereby play a role in the nitrogen cycle (Andersson 1995).

1.2 Hazard Severity

The **environmental hazard severity** for *B. cereus* ATCC 14579 is estimated to be **medium**[6]. Considerations that may result in a finding of medium hazard include that the micro-organism is known as an opportunistic pathogen, has some adverse but reversible effects, in the intermediate term, and effective treatments or mitigation measures are available. *B. cereus* is recognized as a Risk Group 2 animal pathogen by the Canadian Food Inspection Agency (Animal Pathogen Import Program). Such pathogens, under certain conditions can pre-dispose the host to infection, cause a range of symptoms that will debilitate the host and could kill it. In cases where it has been studied, such as in infections caused by *B. cereus* in an agricultural setting (mastitis in cattle), treatment with veterinary antibiotics allowed for survival of affected animals. Generally, in the absence of conditions that pre-dispose the host (which can include invasive routes of exposure), infection is unlikely to occur. This is consistent with the observation that there is no evidence in the scientific literature to suggest any adverse ecological effects at the population level.

The **human hazard severity** for *B. cereus* ATCC 14579 is estimated to be **medium**[6]. Considerations that resulted in a finding of medium hazard include: 1) cases of severe disease or fatality were limited to susceptible sub-populations (the immunocompromised) or were rare, localized and rapidly self-resolving in healthy humans; 2) there is little potential for transmission of infection to other humans; 3) effects in laboratory animal models of human infection were not lethal, and were limited to invasive exposure routes (i.e., endotracheal instillation) or were mild and rapidly self-resolving.

Information from the scientific literature indicates that this micro-organism has pathogenic potential in both otherwise healthy and immunocompromised humans. *B. cereus* is recognized by the Public Health Agency of Canada (PHAC) as a Risk Group 2 human pathogen. It produces a wide variety of extracellular enzymes and toxins that are important factors for its pathogenicity in susceptible and in healthy individuals. The vast majority of *B. cereus*-related diseases in healthy humans are mild, self-resolving and usually treatable. There are some reports of death related to *B. cereus* related infections in humans; however, the strains implicated in those cases contained important virulence plasmids that are not present in *B. cereus* ATCC 14579. No information was found to indicate that *B. cereus* ATCC 14579 has the ability to spread and acquire antibiotic resistance genes; however, the treatment of *B. cereus* ATCC 14579 infections could be hampered by its resistance to several antimicrobial drugs (refer to Table 3).

2. EXPOSURE ASSESSMENT

An exposure assessment identifies the mechanisms by which a micro-organism is introduced into a receiving environment (Section 2.1) and qualitatively and/or quantitatively estimates the magnitude, likelihood, frequency, duration, and/or extent of human and environmental exposure (Section 2.2). The exposure to the micro-organism itself, its genetic material, toxins, metabolites or structural components is assessed in this section.

2.1 Sources of Exposure

B. cereus is commonly found in the environment. It is widely distributed in nature and is able to survive and grow in a wide variety of environments, including soil, airborne dust, water, sediments, on plants and decaying matter (Logan and De Vos 2009; Stenfors Arnesen *et al.*,2008). Humans and non-human species are regularly exposed to *B. cereus*.

[6] see Appendix 8

Nevertheless, the purpose of this section is to characterize the exposure to the DSL-listed strain, *B. cereus* ATCC 14579, from its deliberate addition to consumer or commercial products or its use in industrial processes in Canada.

B. cereus as a species has properties that make it of commercial interest in a variety of industries. *B. cereus* ATCC 14579 was nominated to the DSL based on its past use in consumer and commercial products. A search of the public domain (internet, patent databases) suggests multiple potential uses, including food processing, pharmaceuticals, pulp and paper and textile processing, biochemical and enzyme production, bioremediation and biodegradation, bioleaching and biomining, and municipal and industrial wastewater treatment. For agricultural applications, some *B. cereus* strains have been used as livestock probiotics and as microbial pest control agents (Lodemann *et al.,* 2008).

In 2009, the government launched a mandatory information-gathering survey (Notice) under section 71 of CEPA 1999 as published in the Canada Gazette I on October 3rd, 2009 (hereafter "the Notice"). The Notice applied to any persons who, during the 2008 calendar year, manufactured or imported *B. cereus* ATCC 14579, whether alone, in a mixture, or in a product. Anyone meeting these reporting requirements was legally obligated to respond. Respondents were required to submit information on the industrial sector, uses and any trade names associated with products containing this strain, as well as the quantity and concentration of the strain imported or manufactured in the 2008 calendar year. No commercial or consumer activities using *B. cereus* ATCC 14579 were reported in response to the Notice. *B. cereus* ATCC 14579 was reported to be used in very small quantities for research and development (R&D), and teaching activities.

2.2 Exposure Characterization

The exposure characterization is based on activities reported in the Notice (R&D and teaching). As *B. cereus* ATCC 14579 is a Risk Group 2 human and animal pathogen, measures to reduce human and environmental exposure from its use in research and teaching laboratories are in place under the PHAC's *Laboratory Biosafety Guidelines* and the CFIA's *Containment Standards for Veterinary Facilities*. These include specific laboratory design, operational practices and physical requirements. For example, all material must be contained and is decontaminated prior to disposal or reuse in such a way as to prevent the release of an infectious agent, and equipment for emergency and decontamination response must be readily available and maintained for immediate and effective use. Arrangements for shipping of *B. cereus* ATCC 14579 must also meet requirements under Canada's *Transportation of Dangerous Goods Act and Regulations*. These measures are designed to prevent any human or environmental exposure to the organism in the laboratory setting. Human and environmental exposure to *B. cereus* ATCC 14579 through R&D and teaching uses reported under the Notice is therefore expected to be low.

2.2.1 Environment

The **environmental exposure** for *B. cereus* strain ATCC 14579 is estimated to be **low**[7] based on responses to the Notice, which indicate that this strain is no longer used in consumer or commercial products or for industrial processes in Canada.

[7] see Appendix 8

Nevertheless, environmental exposure scenarios, in the event that consumer, commercial or industrial activities with *B. cereus* ATCC 14579 resume, have been considered along with persistence and survival properties of this micro-organism.

The magnitude of non-human species exposure to this micro-organism will depend on the persistence and survival of *B. cereus* ATCC 14579 in the environment. Persistence test data were obtained by Environment Canada on *B. cereus* ATCC 14579 in agricultural soil. After inoculation of soil with live cells, samples from various time points were collected and the presence of *B. cereus* ATCC 14579 DNA was tested by specific AFLP PCR. DNA from this strain was detected for 127 days post-inoculation (Xiang *et al*., 2010). No testing for the presence of live cells was done. Another study (West *et al*., 1985) artificially inoculated natural (un-autoclaved) dry soil with 10^4 spores of *B. cereus* and the population level increased to 10^5 over the span of the experiment (64 days). This is consistent with the finding that spores can be maintained in the environment and are resistant to some of the factors that cause vegetative cell numbers to decline after artificial inoculation. Therefore, it is reasonable to believe that repeated releases of *B. cereus* spores into the natural environment could lead to increased numbers of spores being maintained in those environments.

B. cereus is a persistent micro-organism that is frequently isolated from natural environments. However, studies in the scientific literature that contain data on population levels of *B. cereus* in the natural environment are very limited. One study (Tucker and McHugh, 1991) showed that, in various soils containing varying floral populations, the concentration of *B. cereus* reached 6 x 10^4 CFU/g of soil. As well, no relevant reports concerning environmental persistence of toxins produced by *B. cereus* have been found. While large inputs of *B. cereus* ATCC 14579 into the environment could result in concentrations greater than background levels of *B. cereus*, high numbers of vegetative cells are unlikely to be maintained in water and in soil due to competition (Leung *et al.* 1995) and microbiostasis (van Veen *et al.* 1997), which is an inhibitory effect of soil, resulting in the rapid decline of populations of introduced bacteria. Nevertheless, *B. cereus* spores are likely to persist and accumulate in the environment, as indicated by the information presented above. No reports documenting elimination of *B. cereus* spores following environmental contamination were found in the literature.

The potential exposure scenarios are based on former and probable future uses as described in Section 2.1 *Sources of Exposure*. Former and potential uses are likely to introduce *B. cereus* ATCC 14579 to both aquatic and terrestrial ecosystems. For example, use of *B. cereus* ATCC 14579 in wastewater treatment or its discharge in wastes from industrial applications, such as pulp and paper processing, textile manufacturing and biochemical production, could introduce *B. cereus* ATCC 14579 into aquatic ecosystems. Similarly, its use in bioremediation and biodegradation as well as in livestock probiotics and pest control agents could introduce *B. cereus* ATCC 14579 into terrestrial ecosystems.

In the event that consumer, commercial or industrial activities resume the environmental exposure to *B. cereus* strain ATCC 14579 could change based on the exposure scenarios described above.

2.2.2 Human

The **human exposure** to *B. cereus* ATCC 14579 is estimated to be **low**[8] based on responses to the Notice, which indicate that this strain is no longer used in consumer or commercial products or for industrial processes in Canada.

Nevertheless, human exposure scenarios in the event that consumer, commercial or industrial activities with *B. cereus* ATCC 14579 resume have been considered. These are based on former and probable future uses as described in Section 2.1 *Sources of Exposure*. Workplace exposure to *B. cereus* ATCC 14579 is not considered in this assessment[9].

Human exposure would be expected during the direct use and application of consumer or commercial products containing *B. cereus* ATCC14579. Skin and eye contact, inadvertent ingestion and inhalation of aerosolized droplets or particles are likely routes of direct user and bystander exposure. The use of such products in food preparation areas could result in the contamination of surfaces and foods at the time of product application. Subsequent lapses in proper food handling practices could allow *B. cereus* ATCC 14579 to proliferate in foods, possibly resulting in the ingestion of large numbers of cells.

Human exposure may also be temporally distant from the time of application. Subsequent to application, *B. cereus* ATCC 14579 is expected to remain viable and establish communities where organic matter accumulates (for example: countertops, drains, sinks, grease traps and kitchen garbage disposals). From such reservoirs, aerosols containing *B. cereus* ATCC 14579 could inoculate surfaces and foods. As above, subsequent lapses in proper food handling practices could allow the organism to proliferate in foods and result in the ingestion of large numbers of cells and lead to adverse effects.

Certain uses may introduce *B. cereus* ATCC 14579 into bodies of water, as described in section 2.2.1. Nevertheless, human exposure to the strain through the environment is expected to be low. Moreover, drinking water treatment processes are expected to effectively eliminate these micro-organisms and so limit their ingestion through drinking water.

In the event that consumer, commercial or industrial activities resume, the **human exposure** to *B. cereus* strain ATCC 14579 could change based on the exposure scenarios described above.

3. RISK CHARACTERISATION

Based on the current level of exposure inferred from responses to the Notice and notwithstanding the potential hazards to human health or to the Canadian environment

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[8] see Appendix 8

[9] DISCLAIMER: A determination of whether one or more of the criteria of section 64 of CEPA 1999 are met is based upon an assessment of potential risks to the environment and/or to human health associated with exposure in the general environment. For humans, this includes, but is not limited to, exposure from air, water and the use of products containing the substance. A conclusion under CEPA 1999 may not be relevant to, nor does it preclude, an assessment against the criteria specified in the Controlled Products Regulations, which is part of the regulatory framework for the Workplace Hazardous Materials Information System (WHMIS) for products intended for workplace use. Individuals who handle *B.cereus* ATCC14579 in the workplace should consult with their occupational health and safety representative about safe handling practices, applicable laws and requirements under WHMIS and the Laboratory Biosafety Guidelines.

known to be associated with this organism, the risk is estimated to be **low**[10] to both the environment and human health from the DSL-listed strain *B. cereus* ATCC 14579.

Nevertheless, resumption of the import, manufacture or use of *B. cereus* ATCC 14579 could result in an increased level of human and environmental exposure, as described in Section 2.2, which would increase the estimation of risk. Therefore, with respect to future importation and manufacturing activities, and taking into account the known and potential uses of *B. cereus* ATCC 14579 in various industries, the exposure to the environment could change.

Non-human species are expected to be exposed to *B. cereus* ATCC 14579 primarily through water and soil. Specifically, terrestrial and aquatic species can come into contact with this organism mainly from its release from industrial or manufacturing activities. Uses involving introduction into terrestrial environments could become problematic, as it has been shown that high (10^7-10^8 CFU per gram of dry soil) concentrations of *B. cereus* ATCC 14579 can cause adverse effects in springtail and red fescue and there is a lack of information on the potential adverse effects of *B. cereus* on aquatic species.

In the event that consumer, commercial or industrial activities resume and result in increased environmental exposure to *B. cereus* strain ATCC 14579, the associated risk of adverse effects to the environment could increase. Therefore, it is recommended that any new activities with this organism be assessed to ensure that any new uses do not present additional new risks.

The risk to human health will depend on the route of exposure. Of all routes identified, exposure through ingestion is of primary concern since *B. cereus* is primarily a gastrointestinal pathogen. *B. cereus* ATCC 14579 is known to produce important pathogenic factors (e.g., extracellular enzymes and toxins) implicated in gastrointestinal disease. The infectious dose of *B. cereus* is reported to range from 10^2 to 10^8 cfu per gram of food or water and it is generally believed that any food containing concentrations of *B. cereus* exceeding 10^3 to 10^5 cells or spores per gram is not safe for consumption (Anonymous, 2005; Haggblom *et al.* 2002; Stenfors Arnesen *et al.* 2008). The use of products containing *B.cereus* ATCC 14579 in food preparation areas could result in the inoculation of foods and subsequent lapses in proper food handling practices could allow bacteria to proliferate. Cycles of reheating and inadequate refrigeration are particularly problematic for spore-forming bacteria like *B. cereus*, because spores are not inactivated during heating, and vegetative cells can germinate, multiply and re-sporulate between heating cycles. In this way, the number of viable cells in food increases in exponential fashion, eventually reaching a level that can lead to human gastrointestinal infection.

Skin and eye contact have been identified as potential routes of exposure, but these are less likely to result in adverse health effects. Wound infections have been documented for *B. cereus* in otherwise-healthy individuals; however, these are rare and there is no indication that *B. cereus* ATCC 14579 could penetrate intact skin to cause dermal infection. Since skin is a natural barrier to microbial invasion of the human body, infections are likely to occur only if the skin was damaged through abrasions or burns (Dubouix et al. 2005). Similarly, although *B. cereus* is highly virulent in the eye, infection is likely only in cases of prior injury to the eye.

[10] See Appendix 8

Inhalation of *B. cereus* ATCC 14579 cells or spores aerosolized through mechanical or air disturbances, either during or subsequent to product application, could lead to pulmonary exposure to spores or vegetative cells, but the number of inhaled spores or cells is unlikely to reach an infectious dose in healthy individuals. One survey conducted in the USA reported that a variety of *B. cereus* subgroup species are commonly present in urban aerosols across all seasons in 11 major American cities, but the reported incidence of respiratory infection due to *B. cereus* is extremely low in the USA. Exposure through inhalation is therefore not of concern (Merrill *et al*. 2006).

In the event that consumer, commercial or industrial activities resume and result in increased human exposure to *B. cereus* strain ATCC 14579, the associated risk of adverse health effects in humans could increase. Therefore it is recommended that any new activities with this organism be assessed to ensure that they do not present additional risks.

CONCLUSION

Based on responses to the Notice, it is concluded that *B. cereus* 14579 is not entering the environment in a quantity or concentration or under conditions that have or may have an immediate or long-term harmful effect in the environment or its biological diversity; constitute or may constitute a danger to the environment on which life depends; or constitute or may constitute a danger in Canada to human life or health. **Therefore, it is proposed that this substance does <u>not</u> meet the criteria as set out in section 64 of the CEPA 1999**.

Nevertheless, given the hazardous properties of *B. cereus* ATCC14579, reintroduction into Canada through import, manufacture or use could lead to this substance meeting the criteria set out in section 64 of the Act. Therefore, it is recommended that *B. cereus* ATCC 14579 be subject to the Significant New Activity (SNAc) provisions specified under subsection 106(3) of the Act, to ensure that any new activity involving this organism is notified and undergoes appropriate environmental and human health risk assessments as specified in section 108 of the Act, prior to the organism being re-introduced into Canada. Under CEPA 1999, the effect of the SNAc will be to channel any new activity not covered by Acts listed in Schedule 4 (*Pest Control Products Act*, *Heatlh of Animals Act*, *Feeds Act*, and the *Fertilizers Act*) into CEPA 1999 risk assessment so that a determination of risk can be made for new activities not addressed in this report.

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APPENDIX 1A: Growth of *Bacillus cereus* ATCC 14579 in liquid media at 28°C, 32°C, 37°C and 42 °C *

Growth of *B. cereus* ATCC 14579 in broth culture, as measured by increase in absorbance at 500nm, in four different growth media and over a range of temperatures. The concentration of bacteria at time 0 was 1×10^6 CFU/well. Measurements were taken every 15 minutes over a 24 hour period with a multi-well spectrophotometer at a wavelength of 500 nm.

Medium	Temperature (◦C)			
	28	32	37	42
Trypticase Soy Broth	+	+	+	+
Sheep Plasma	-	-	(+)	~
Fetal Bovine Serum	+	+	+	-
Dulbecco's Modified Eagles Medium (DMEM) (mammalian cell culture)	(+)	~	-	-

– no growth, + growth, ~ low level growth, (+) delayed growth (after 15h)

* Data generated by Health Canada's Environmental Health Science and Research Bureau

APPENDIX 1B: Characteristics of *Bacillus cereus* ATCC 14579-- Growth on Solid Media*

Medium		Temperature (°C)	
		28	37
Nutrient		+	+
TSB [1]		+	+
Citrate [2]		-	-
Lysine Iron [3]		+	+
Maconkey Agar [4]		-	-
Mannitol [5]		-	-
MYP supplements [6]		+	+
Starch [7]	Growth	N/A	+
	Hydrolysis	N/A	□
Triple Sugar Iron - w phenol red [8]		+	-
Urea [9]		+	+
Catalase activity [10]	TSB	-	+
	Sheep blood	+	+
Hemolysis [11]		+	+

□ Clearing zone extends beyond colony
N/A Data not available
(+) Positive for growth or test
(-) Negative for growth or test
(1) General purpose medium
(2) Citrate utilization test, ability to use citrate as the sole carbon source.
(3) Simultaneous detection of lysine decarboxylase and formation of hydrogen sulfide in the identification of Enterobacteriaceae, in particular *Salmonella* and *Arizona* according to Edwards and Fife (1961).
(4) Detection of coliform organisms in milk and water; tests for ability of organism to ferment lactose
(5) Isolation and differentiation of *Staphylococci*
(6) *B. cereus* selective agar
(7) Differential medium that tests the ability of an organism to produce extracellular enzymes that hydrolyze starch
(8) Gram-negative enteric bacilli based on glucose, lactose, and sucrose fermentation and hydrogen sulfide production
(9) Screening of enteric pathogens from stool specimens - Urea metabolism
(10) Catalase enzyme assay measures by enzymatic detoxification of hydrogen peroxide.
(11) Hemolysis of sheep, bovine, pig, goat, human and rabbit blood. Bacteria (5000 CFU, 20 µl) were spotted onto the blood-agar and incubated at 28°C or 37°C for 24h or 48h

* Data generated by Health Canada's Environmental Health Science and Research Bureau

APPENDIX 1C: Characteristics of *Bacillus cereus* ATCC 14579 – Fatty Acid Methyl Ester (FAME) Analysis*

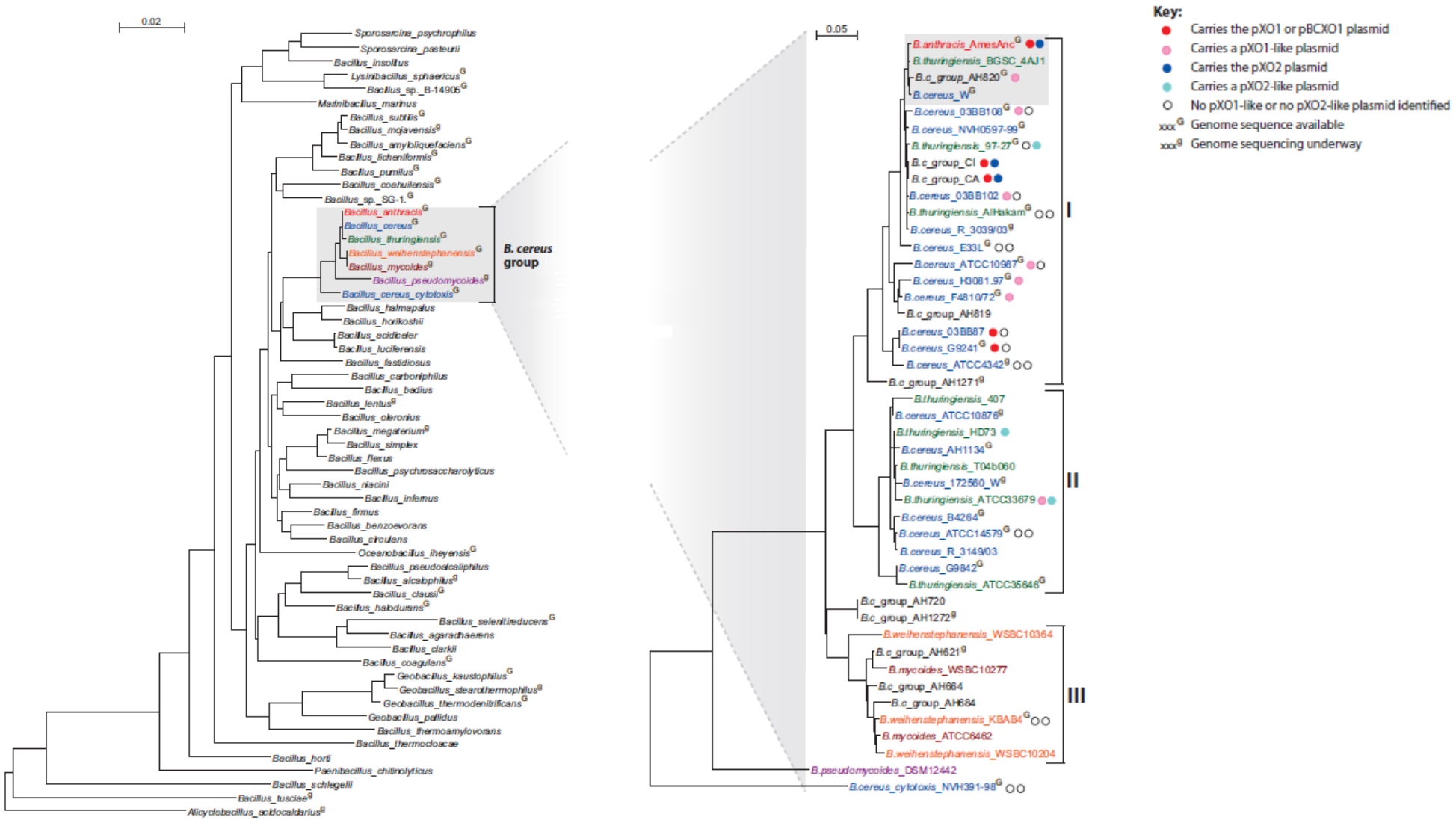
Data presented show the best match between the sample and different MIDI≅ databases (clinical and environmental), along with the number of matches (fraction of total number of tests) and the fatty acid profile similarity index (in parentheses; average of all matches).

Test Strain	Environmental Database		Clinical Database	
B. cereus ATCC 14579	*B. cereus* group A	39/46 (0.889)	*B. thuringiensis* group B (0.751)	24/35 (0.751)
	B. megaterium subgroup A	1/46 (0.045)	*B. cereus* group A	8/35 (0.751)
	No Match	6/46	No match	3/35

* Data generated by Health Canada's Environmental Health Science and Research Bureau

≅ MIDI is a commercial identification system that is based on the gas chromatographic analysis of cellular fatty acid methyl esters.

APPENDIX 2: Relationships within the *Bacillus cereus* group[11].



[11] Taken from Figure 1 with slight modifications, from Kolsto et al., 2009.

APPENDIX 3: List of some *Bacillus cereus* group mobile genetic elements and associated traits

Type	Name	*Bc*[1]	*Ba*[2]	*Bt*[3]	Associated traits	References
plasmid	pAW63[4]			subsp. *kurstaki*	• no known homology to *cry* and *cyt,* • contains mobile elements and putative proteins	(Schnepf *et al.* 1998;Van der Auwera and Mahillon 2005)
	pBc10987[5]	10987			• Tn*554*, AbrB (regulator hom.) • Bc1A (spore coat determinate)	(Rasko *et al.* 2004)
	pBC218	G9241			• polysaccharide capsule	(Hoffmaster *et al.* 2004)
	pBClin15[6],	14579			• prophage feature, Similar to Bam35	(Stromsten *et al.* 2003;Verheust *et al.* 2005)
	pBClin29	G9241			• prophage feature	(Hoffmaster *et al.* 2004)
	pBCOX1[7]	G9241			• lethal toxin complex *pagA, lef, cya*	(Hoffmaster *et al.* 2004)
	pBT9727[8]			97-27[9]	• no known homology to *cry* and *cyt,* • contains mobile elements and putative proteins	(Rasko *et al.* 2005)
	pBToxis			x	• insecticidal protein toxin (*cry, cyt*)	(Berry *et al.* 2002)
	pCER270	x AH1134 AH187			• emetic toxin (cereulide)	(Ehling-Schulz *et al.* 2006;El Emmawie *et al.* 2008;Rasko *et al.* 2007)
	pE33L[10] (series)	E33L[11]			• possesses a number of transposable genes and mobile elements	(Rasko *et al.* 2005)
	pPER272	AH820 AH818			• associated with periodontal isolates	(Rasko *et al.* 2007)
	pXO1		x		• lethal toxin complex, *pag*, *lef* and *cya* genes	(Okinaka *et al.* 1999)
	pXO2		x		• D-glutamic acid caspsule, • operon *cap* BCADE	(Drysdale *et al.* 2005)
	pXO16			subsp *israelensis*	• Aggregation phenotype	(Jensen et al. 1995)
	pCI-XO1[12]	CI			• lethal toxin complex, *pag*, *lef* and *cya* genes	(Klee *et al*. 2010)
	pCI-XO2[13]	CI			• D-glutamic acid caspsule, • operon *cap* BCADE	Klee *et al*. 2010)

Type	Name	*Bc*[1]	*Ba*[2]	*Bt*[3]	Associated traits	References
	pCI-14	CI			• Unknown function • Cryptic plasmid	Klee *et al*. 2010)
Phage	Bam35			*x*		(Ackermann *et al.* 1978)
	CP-51	x				(Ruhfel *et al.* 1984)
	GIL01			x		(Verheust *et al.* 2005)
Transposon	Tn5084	RC607 VKM684	x	x	• Resistance to mercury	(Huang *et al.* 1999;Narita *et al.* 2004)
Other						
DNA repeated element	*bcr1*	x (incl. 145790)	x	x	• exhibits characteristics of a mobile element	(Okstad *et al.* 2004)
Insertion Sequence	*IS*231	X (incl. 14579)	x	x	• transposase	(De Palmenaer *et al.* 2004)
Group I intron	*recA*	x (incl. 10987 E33L)	x	x	• Ribozyme (catalytic RNA)	(Tourasse *et al.* 2006)
	nrdE	x (incl. E33L G9241 10987	x	x	• Ribozyme (catalytic RNA)	(Tourasse *et al.* 2006)
Group I IStron	Bc*IS*t*1*	10987 E33L G9241 (not 14579)	x	x	• Self-splicing group I introns associated with IS element	(Tourasse *et al.* 2006)
Group II intron	*B.c.*I1	10987 14579)			•	(Tourasse *et al.* 2006)

[1] *Bacillus cereus* listed strains known to carry the extrachromosomal genetic element (x indicates multiple strains)
[2] *Bacillus anthracis* listed strains known to carry the extrachromosomal genetic element (x indicates multiple strains)
[3] *Bacillus thuringiensis* listed strains known to carry the extrachromosomal genetic element (x indicates multiple strains)
[4] Shares conserved backbone with *B. anthracis* pX02
[5] Shares conserved backbone with *B. anthracis* pX01
[6] Linear plasmid
[7] Shares 99.6% genetic identity with pX01
[8] Shares conserved backbone with pX02
[9] *B. thuringiensis* subsp. *konkukian* 97-27 isolated from a case of severe human necrosis
[10] Similar to pXO2 and pBC218
[11] Isolate from a dead zebra suspected of having died of anthrax, (phylogenitycally close to *B. anthracis*)
[12] Shares 99% genetic identity with pX01
[13] Shares 100% genetic identity with pX02

APPENDIX 4: Chromosomal genes coding for toxins in *Bacillus cereus* ATCC 14579[12] as analysed by PCR

CDSs in *B. cereus*	Function
BC3103, BC3102, BC3102	Hemolytic enterotoxin BL
BC1809, BC1810, BC0560	Non-hemolytic enterotoxin Nhe
BC2081	Enterotoxin T, BceT
BC1953	Enterotoxin FM1
BC1110	Cytotoxin K
BC3761	Phosphatidylinositol-specific phospholipase C
BC0670	Phosphatidylcholine-specific phospholipase C
BC0671	Sphingomyelinase
BC5101	Cereolysin O
BC3523	Hemolysin II
BC2196	Hemolysin III

[12] Adapted from Ivanova et al. 2003.

APPENDIX 5: List of toxins produced by *Bacillus cereus*

Toxins	Description	References
Cereulide	**Structural Characteristics** • Lipophilic peptide, heat-stable, emetic toxin • Cyclic dodecadepsipeptide resembling valinomycin (-OLeu-Ala-OVal-Val-)$_3$ • K+-specific ionophore similar to valinomycin **Toxic Dose and Effects** • Amount of cereulide found in food samples implicated in emetic food poisoning cases ranges from 0.01 to 1.28 µg/g of food • Toxic dose in human is 8 µg kg^{-1} body weight (human emesis-causing dose) in Rhesus monkey 10 µg/kg and in *Suncus murinus* is 8 µg/kg • The ED50 in *Suncus murinus* is 12.9 µg kg^{-1} by oral administration • Cytotoxic and mitochondriotoxic to primary cells and cell lines of human and other mammalian origins • In an assay for detection of cereulide production in *B. cereus* strains, boar sperm exposed *in vitro* to 2 µg/L of cereulide showed observable mitochondrial damage • Toxic towards porcine fetal Langerhans islets and beta cells • Inhibits hepatic mitochondrial fatty-acid oxidation which can cause liver failure • Inhibit natural killer cells at concentration 20-30 µg/L	(Agata *et al.* 1994;Agata *et al.* 1995b;Agata *et al.* 2002;Haggblom *et al.* 2002;Jaaskelainen *et al.* 2003;Mahler *et al.* 1997;Mikkola *et al.* 1999;Paananen *et al.* 2002;Shinagawa *et al.* 1995;Virtanen *et al.* 2008)
Cytotoxin K (CytK)	**Structural Characteristics** • Two variants of the protein: CytK-1 and CytK-2 • Sequence comparisons suggest that the protein may belong to the family of β-barrel pore-forming toxins **Toxic Dose and Effects** • CytK-1 and CytK-2 are able to form pores in lipid bilayers but the distribution of channel conductance is lower in CytK-2 • CytK-1 is associated with more severe forms of gastrointestinal disease • Highly cytotoxic, necrotic & haemolytic effects produced by CytK-1 or CytK-2 • This is the cytotoxin that may cause necrotic enteritis • Preliminary tests in guinea pigs using intracutaneous injections suggest that CytK is	(Brillard and Lereclus 2004;Fagerlund *et al.* 2004;Guinebretiere *et al.* 2006;Hardy *et al.* 2001;Lund *et al.* 2000)

Toxins	Description	References
	dermonecrotic • CytK-1 is highly toxic toward human intestinal Caco2 cells and Vero cells compare to CytK-2 • Cyt-K-2 proteins are toxic to Caco-2 and bovine erythrocytes but not to the same extent as the original CytK-1 • No information available on the effective dose of the toxin	
Hemolysin BL (HBL)	• The major virulence factor associated with diarrheal syndrome. HBL responsible for the major enterotoxigenic activity and the main cytopathogenic activity of *B. cereus* ATCC 14579 **Structural Characteristics** • Three-component (B, L_1,L_2) pore-forming toxin. **Toxic Dose and Effects** • Enterotoxin responsible for the diarrheal food poisoning syndrome • Toxic activities when three HBL components are combined: hemolysis, cytotoxicity, vascular permeability, dermonecrosis, enterotoxicity and ocular toxicity • Lysis caused by formation of a membrane attack complex on the cell surface • Enterotoxigenic; damages membranes of a variety of different cell types • Exhibits Vero cell, Chinese hamster ovary (CHO) cell and retinal cell cytoxicity and is lethal to mice upon injection • Causes necrosis of intestinal tissue, fluid accumulation in a ligated mouse ileal loop, and vascular permeability and necrosis in rabbit skin • HBL toxin does not contribute significantly to *B. cereus* haemolytic activity against human erythrocytes; HBL is most active against sheep and calf erythrocytes • Necrotic to rabbit retinal tissue with maximal activity in dose between 50 to 150 µg/L • Induces apoptosis in macrophages	(Agata *et al.* 1995a;Beecher and Macmillan 1991;Beecher *et al.* 2000;Beecher *et al.* 2000;Beecher *et al.* 1995b;Beecher and Wong 1994a;Beecher and Wong 1994b;Beecher and Wong 1994c;Beecher and Wong 1997;Beecher and Wong 2000;Lindback *et al.* 1999; Tran *et al.* 2010a)
Non Hemolytic enterotoxin (Nhe)	• Enterotoxin with no detectable haemolytic effects **Structural Characteristics** • Three-component complex (Nhe A, Nhe B and Nhe C). A binding factor (Nhe B), a complex formation factor (Nhe C) and a lysis factor (Nhe A).	(Fagerlund *et al.* 2008;Granum *et al.* 1999; Haug *et al*. 2010; Lindback *et al.* 2004; Linback *et al.* 2010; Lund and Granum

Toxins	Description	References
	• Nhe is fundamentally a two-component toxin (NheA and NheB) but a third component (NheC) is necessary for the full cytotoxicity in some cells • Optimal cytotoxic effect with ratio NheA:NheB:NheC of 10:10:1 . Concentration of NheC higher than 10% of that of NheA and NheB inhibited the toxic activity • Mechanism of cytotoxicity is osmotic lysis following pore formation in the plasma membrane **Toxic Dose and Effects** • Cytotoxic/enterotoxic properties	1996;Wijnands *et al.* 2001)
Enterotoxin T (BceT or bc-D-Ent)	• Unknown type of enterotoxic action • Proposed as an *B. cereus* enterotoxin but the proposition was disproved after the cloned *bceT* construct was suggested to be a cloning artifact	(Agata *et al.* 1995a;Choma and Granum 2002;Guinebretiere *et al.* 2006;Hansen *et al.* 2003;Lindbäck and Granum 2006)
Enterotoxin FM (entFM)	• Mechanism of action and role unknown • Increases vascular permeability in rabbit, and causes fluid accumulation in mouse ligated intestinal loops • Cytotoxic to Vero cells and lethal to mice • Sequence analysis revealed that EntFM is related to cell wall peptidases (CwpS) and has homology to *B. subtilis* cell wall hydrolase, suggesting that the protein might not be a toxin • However, EntFm might still have a role in *B. cereus* virulence	(Asano *et al.* 1997;Lindbäck and Granum 2006;Tran *et al.* 2010b; Shinagawa *et al.* 1991a;Shinagawa *et al.* 1991b)

Membrane-damaging virulence factors	Actions	References
Hemolysin II (HlyII)	• Hemolytic protein **Structural Characteristics** • Member of the β-barrel pore-forming toxin family • HlyII is a structural and functional homolg of staphylococcal α-hemolysin • Binds to surface of cells and assemble into oligomeric transmembrane pores leading to cell permeation and lysis **Toxic Dose and Effects** • Hemolysin II is able to lyse different kinds of eukaryotic cells. Hemolytic activity in rabbit erythrocytes have a HC_{50} value of 1.64 µg/L (HC_{50}:: Concentration of hemolysin to reach 50% of erythrocyte lyse) • Exhibit cytolytic activity on erythrocytes of human and rabbit. Bovine and mouse erythrocytes are least sensitive to HlyII	(Andreeva *et al.* 2006;Andreeva *et al.* 2007;Miles *et al.* 2002)
Hemolysin III (HLy-III)	• Hemolytic protein **Structural Characteristics** • Pore-forming hemolysis with functional diameter of pores about 3-3.5 nm • Three steps of hemolysis: i) the temperature-dependent binding of the Hly-III monomers to the erythrocyte membrane; ii) the temperature-dependent formation of a transmembrane pore by multiple molecules of the hemolysin; iii) temperature-independent erythrocyte lysis	(Baida and Kuzmin 1995;Baida and Kuzmin 1996)
Cereolysin O (CLO)	• Hemolytic protein **Structural Characteristics** • Pore-forming toxin from the cholesterol-binding cytolysin (CBC) family • Cross reacts with streptolysin-O **Toxic Dose and Effects** • Causes disorganization of the cytoplasmic membrane and intracellular organelles • Is thiol activated, heat labile and poorly susceptible to proteolysis • Pathogenic role in extraintestinal infection • CBCs are lethal to animals and highly lytic toward eukaryotic cells, including erythrocytes	(Alouf 2000;Granum 1994)
Phosphatidylinosol hydrolase (PIH)	**Structural Characteristics** • Phospholipase C that hydrolyzes	(Granum 1994) (Beecher and

Membrane-damaging virulence factors	Actions	References
	phosphatidylinositol (PI) and PI-glycan-containing membrane anchors, which are important structural components of one class of membrane proteins **Toxic Dose and Effects** • No hemolytic activity	Wong 2000)
Sphingomyelinase (SMase)	**Structural Characteristics** • Highly specific phospholypase C that hydrolyzes sphingomyelin (SM) to produce ceramide and phosphocholine **Toxic Dose and Effects** • SMase lysed ruminant erthrocytes (46-53% of SM) • Exhibits hemolytic action against mammalian erythrocytes and hemolyzes sheep erythrocytes with and without cold shock • Data for lysis is 222 HD^{50}/unit for Sheep erythrocytes and 27.8 HD^{50}/unit for human erythrocytes (HD^{50}: Higher enzyme dilution that cause 50% lysis of erythrocytes)	(Beecher and Wong 2000;Fujii *et al.* 2004;Ikezawa *et al.* 1980)
Phosphatidylcholine(PC) preferring phospholipase C (PC-PLC)	**Structural Characteristics** • Phospholipase C that hydrolyzes phosphatidylcholine, phosphatidylethanolamine and phosphatidycholine • The enzyme might be capable of binding to a membrane interface with little or no specific substrate present • Little published information regarding the binding of PL-PLC **Toxic Dose and Effects** • Cooperative action with SMase is needed to lyse swine and human erythrocytes (22-31% PC and 28-25% SM) • Inhibits HBL lysis of sheep erythrocytes and enhances the discontinuous hemolysis pattern • Second major contributor to retinal toxicity • PC-PLC is expressed by the great majority of isolates	(Beecher *et al.* 2000;Beecher and Wong 2000;Granum 1994)

Enzyme	Actions	References
ADP-ribosylating toxin (ADP-ribosyltransferase)	• Exoenzyme • Member of C3-like transferase which selectively ribosylates the small GTP-binding protein Rho • Produced by *Bacillus cereus* strain 2339, a clinical isolate	(Just *et al.* 1992)
Vip (vegetative insecticidal protein)	**Structural Characteristics** • Composed of VIP1, a cell-binding component, and VIP2, an ADP-ribosyltransferase that targets actin • Belongs to the family of binary bacterial toxins resembling mammalian clostridial toxins of the C2 and iota-like family **Toxic Dose and Effects** • VIP2 exerts its intracellular poisoning effect by modifying actin and preventing actin polymerization • Insect-killing properties on Northern and Western corn rootworms	(Barth *et al.* 2004;Jucovic *et al.* 2008)

APPENDIX 6A: Pathogenicity to invertebrates and vertebrates

Details of experiments mentioned in Section 1.1.3.2. The following three tables provide information specific to invertebrates and vertebrates, respectively.

Invertebrates

Target	Conditions	Strains	Results	Reference
Lepidopteran insects				
Tobacco hornworm *Manduca sexta* 5th instar larvae Sex not specified **Purpose or context:** Insect infection model to characterize the role of the iron-responsive regulator *fur* gene in the virulence of *B. cereus*.	**Route of exposure**: • Injection of vegetative cells (compartment not specified. **Test conditions:** • Not specified. **Dose regimen**: • Single injection of known but unspecified doses • Single replicate of at least 20 larvae per dose group **Controls:** • Not specified. **Duration of study:** • 48 hours.	• 569 WT • 569 *Δfur* • Both grown in LB medium with antibiotics. • *fur* is a homologue of the *B. subtilis* fur gene identified in *B. cereus*.	**Observation intervals:** • Not specified. **Mortality observed:** • Median lethal dose (LD50) calculated by Probit analysis of mortality data (values in parentheses are 95% confidence limits). • Wild-type LD_{50} value = 1859 cfu (1142-2774) • Mutant LD_{50} value = 4932 cfu (3609-6912) **Conclusions:** • Reduced virulence for the *B. cereus* 569 *Δfur* mutant. • The *Δfur* mutant constitutively expresses siderophores and accumulates iron intracellularly to a level threefold greater than the WT.	(Harvie *et al.* 2005)

Target	Conditions	Strains	Results	Reference
Wax moth *Galleria mellonella* Last instar larvae Sex not specified **Purpose:** Investigation of the opportunistic properties of acrystalliferous *B. thuringiensis* (Bt) and *B. cereus* strain and the role of the *plcR* gene, a pleiotropic regulator of extracellular factors.	**Routes of exposure**: • Force-feeding co-ingestion. • Intrahaemocoelic injection. **Methods of administration, respectively:** • 0.5 X 25 mm needles and microinjector. • At the base of last proleg, using a microinjector with a 1 ml hypodermic syringe and 0.45 X 12 mm needles. **Dose regimen**: • 10 µl of spore suspension per larvae for both methods. • For the force-feedings, spores were in association with crystal toxins. **Repetitions and replications:** • No repetition on the same animal. • 30 larvae used for each dose and for each method. **Controls:** • Force-feedings with spores (10^6) or Cry1C toxins alone. **Duration of study:** • Casualties recorded over 7 days.	• ATCC 14579 • Spores were obtained by culturing cells in HCT medium at 30°C for 4 days, centrifuged and resuspended in 10 mL sterile distilled water. • Spore preparations were heated for 20 min at 80°C.	**Observation intervals:** • Organisms checked daily. • After injection, organisms were kept individually in boxes containing beeswax and pollen at 25°C. **Mortality observed:** • After 2 days. • Very low (>10%) with crystals or spores alone (controls). • ≈70% mortality caused by co-ingestion of 10^6 spores with a sublethal (1µg) quantity of Cry1C toxin. **Conclusions:** • Clear pattern of synergism between the spores of *B. cereus* and the toxin of *B. thuringiensis*.	(Salamitou *et al.* 2000)

Target	Conditions	Strains	Results	Reference
Wax moth *Galleria mellonella* 2^{nd} and 5^{th} instar larvae Reared on beeswax and pollen. **Purpose:** To evaluate whether *Galleria mellonella* can function as an oral infection model for human and entomo-bacterial pathogens.	**Route of exposure**: • Oral infection **Free ingestions:** • On 2^{nd} instar • Mixtures of 50% pollen with 50% water containing 10^8 spores/mL alone or along with 2 µg of Cry1C diluted in PBS at pH 7.4. • Both preparations (2 ml) were added to 5 cm diameter plastic Petri dishes and allowed to surface dry. • Larvae then placed in each dish and incubated at 37°C **Force feedings:** • On 5^{th} instar, weighing 200 mg and starved for 24 hours prior to the test. • Used a micro injector. • 5×10^5 - 1×10^6 spores or vegetative cells per larva, with (2 – 3 µg) and without Cry1C toxin. **Repetitions and replications:** • No repetition on the same animal. • 10 X 2^{nd} instar larvae in each dish for free ingestions. • 20 X 5^{th} instar larvae for force-feedings. • Experiments replicated 3 times. **Controls:** • Spores alone. • Cry1C toxin alone. **Duration of study:** • 72 hours.	**1 environmental isolate:** • ATCC 14579 **5 other diarrheal strains:** • D6 (F4370/ 75) • D23 (F284/78) • D17 (1651-00) • D19 (NvH391/ 98) • D24 (F352/90) **4 of them were tested in force feedings:** • D19 • D23 • D6 • ATCC 14579 **Spores:** • Produced in HCT medium, washed, resuspended in water, heat treated and enumerated. **Vegetative cells:** • Collected at exponential growth phase ($OD_{600nm} \approx 1$) in LB medium.	**Observation intervals:** • Mortality recorded daily. **Mortality observed for free ingestion:** • 2 ± 2% for ATCC 14579 spores alone. • 5 ± 5% for Cry1C toxin alone. • Ranging from 12 ± 7% (D24) to 57 ± 20% (D23) for co-ingestion of Cry1C toxin. **Mortality observed for force feeding:** • 0% (D19) to 8 ± 6% (D23) without toxin. • 10 ± 8% (D19) to 50 ± 13% (D23) in co-ingestion. **Conclusions:** • Important variation among strains was observed. • These results demonstrate synergy. • The low virulence of D19 (10%) was unexpected since it is known to be a highly virulent human pathogen. • Insect mortality values did not correlate with the pathogenic potential of the bacterial strains.	(Fedhila *et al.* 2010)

Target	Conditions	Strains	Results	Reference
Cabbage looper *Trichoplusia ni* 1 to 8-day old healthy larvae from a stock culture. **Purpose:** Pathogenicity test to characterize the non-viral cause of larvae death in a study on NPV.	**Route of exposure**: • Free ingestion of contaminated diet pathogenicity test. **Method of administration** • Suspension pipetted onto the surface of freshly prepared artificial diet in a 1-oz plastic cup. • One larvae at a time was transferred in the cup to feed. **Physical conditions:** • 25-28°C. • 75-85% relative humidity. **Dose regimen**: • 0.1 ml of suspension • bacteria, virus or combination used (3 test groups) • 2.5 or 5.0 × 10^7 cells/cup **Repetitions and replications:** • No repetition on the same larvae. • 50 larvae per dosage. • 2 experiments. **Controls:** • Sterile saline or water. **Duration of study:** • 12 days.	• No strain designation specified. • Consistent isolate from dead or moribund larvae. • Cells suspended in sterile saline.	• The cause of death and symptoms was identified as *B. cereus* on the basis of criteria of A. Krieg's key, 1970. **Parameter measured:** • Daily mortality counts. • Corrected according to Abbott's formula. **Range of effects:** • The highest level, 7.2 × 10^8 cells/cup, caused 100% mortality in 11 days. • 69 and 50% mortality occurred among larvae exposed to 3.6 and 1.8 × 10^8 cells/cup, respectively. • 70 to 100% died within 10 days. • Symptoms were identical to those observed in larvae from which original isolations were found: larvae ceased to feed, showed paralysis, darkening of integument and ultimately died. • 1-day-old larvae appeared more susceptible to the bacterium than 2 to 8-day-old larvae. • 1-day-old cultures of *B. cereus* caused greater and more rapid mortality than did 2, 3 or 20-day-old cultures. **Conclusions:** • Combinations of the two pathogens resulted in slightly higher mortality than either pathogen alone, but there were no synergistic effects. • Pathogenicity to *T. ni* was not associated with any demonstrable toxin.	(To *et al.* 1975)

Target	Conditions	Strains	Results	Reference
Silkworm 5th instar larvae Raised from fertilized eggs in the laboratory. Fed antibiotic-free food for 1 day. **Purpose:** Purification and identification of a soil bacteria exotoxin, sphingomyelinase C.	**Route of exposure**: • Injection into the hemolymph through the dorsal surface. **Method of administration** • 27-gauge needle. **Physical conditions:** • Not specified. **Dose regimen** • 3 test groups • 0.05 ml of an overnight culture or culture supernatant. • Two-fold dilutions of purified sphingomyelinase. **Repetitions and replications** • 2 silkworms for each dose of culture or culture supernatant. • 5 silkworms for each dose of the toxin. **Controls** • None specified. **Duration of study** • 24 hours.	• ATCC 14579 • 25 distinct colonies of which 16 killed silkworm • 9 undesignated strains of *Bacillus* sp isolated from soil. • Soil samples were spread onto brain-heart infusion agar plates and colonies isolated after overnight incubation at 30°C. • Cultures were centrifugated and filtrated through a 0.22-µm filter.	**Parameter measured:** • Number of silkworms alive after 24 hours. **Range of effects for soil isolates:** • Of 25 distinct isolates, 16 killed silkworms. • 5 out of 16 culture supernatants had a killing activity against silkworms. These 5 strains were identified as *Bacillus* species (16S rRNA sequences). • The toxin purified from isolate #11 was identified as sphingomyelinase C from *B. cereus*. **Range of effects for sphingomyelinase from *B. cereus* ATCC 14579:** • Killed silkworms with an LD_{50} of 0.7 µg.	(Usui *et al.* 2009)

Target	Conditions	Strains	Results	Reference
Blattarian insects				
German cockroaches *Blattela germanica* Adult males **Purpose:** Purification and characterization of insect toxicity of sphingomyelinase C produced by *B. cereus*.	**Route of exposure**: • Injection into the abdomen **Method of administration** • Not specified **Physical conditions:** • Insects reared at 26 and 60% relative humidity. **Dose regimen**: • 2 µl of cell-free supernatant or solution of protein sample. • Untreated, heat-shocked and proteinase K treated culture broths tested on cockroaches (3 groups). **Repetitions and replications:** • 5 cockroaches used for each dose. **Controls:** • Not specified. **Duration of study:** • 10 minutes.	• ATCC 14579 (99.9% rRNA sequence homology) • Isolated from the mandibles of last instars of antlions, *Myrmeleon bore.* • Produced insecticidal factors when cultured aerobically.	**Parameter measured:** • Symptoms observed 10 minutes after injection. • Minimum paralysis dose (MPD) at which at least four or five insects were paralysed. **Range of effects:** • Rapid paralysis after injection. • MPD of 262 ± 29 ng protein/insect. • The bacterium was able to grow even at 50°C. • The insecticidal activity was abolished by heating at 100°C and by proteinase K treatment. **Conclusions:** • Sphingomyelinase C produced by *B. cereus* is able to kill insects rapidly at low doses. • The insecticidal factors produced by *B. cereus* may aid the prey-capturing action of the antlions. • The insecticidal effect of sphingomyelinase C is due to its action on the nervous system.	(Nishiwaki *et al.* 2004)
Cockroaches *Leucophaea maderae*	• Intrahemocoelic challenge	• 4 strains comprising: • B1 • NCIB 3329	• B1 was the most pathogenic. • NCIB 3329 was the least pathogenic	(Rahmet-Alla and Rowley 1989)

Target	Conditions	Strains	Results	Reference
Coleopteran insects				
Elm bark beetles *Scolytus scolytus* 5th instar larvae Collected from infested elm logs. **Purpose:** Investigations for a biological control for the vector of Dutch elm disease, among *Bacillus* spp.	**Test conditions:** • Larvae suspended in a suspension of cells. • Time of suspension: 1 hour. • Afterwards, reared on an artificial medium at 27°C . • Dead larvae removed daily. **Dose regimen:** • 8×10^5 cells/ml **Controls:** • Larvae suspended in distilled water. **Duration of the study:** • 21 days.	• *B. cereus* 11796	**Mortality observed:** • Corrected for natural mortality: 63.6% of 40 larvae were killed. • Control gave 17.5% mortality (corrected to 0%) in 40 larvae.	(Jassim *et al.* 1990)
Southern pine beetle *Dendroctonus frontalis* larvae	• Oral inoculation	• No strain designation provided.	• Strains isolated from diseased beetle were pathogenic.	(Moore 1972)
Other insects				
Boll weevil *Anthonomus grandis,* Egyptian cotton leafworm *Spodoptera littoralis* Black bean aphid *Aphis fabae*.	• Free ingestion method of supernatant. • Colonies used.	• 575 strains used for *A. grandis*. • 270 strains used for *S. littoralis* and *A. fabae*.	• 4 of the 575 strains were toxic for *A. grandis* (85 to 100% mortality). • 5 of the 270 strains resulted in 41 to 97% mortality in *A. fabae*. • No effect on *S. littoralis*.	(Perchat *et al.* 2005)

Target	Conditions	Strains	Results	Reference
Crustacean				
Water flea *Daphnia magna* Newborns	• Culture serial dilutions (200 mL) added to 20 jars containing individual neonates (24-hours old) • Final concentration 10^4 – 10^6 CFU mL^{-1}. • Presence of death events checked each day. • Control of uninfected animals.	• BD170 EH2, an originally non hemolytic *Bacillus subtilis* expressing an introduced *B. cereus* hemolysin II gene, *hlyII*. • *B. cereus* VKM B-771.	o Animal death resulted within 8 to 16 days. o BD170 EH2 decreased fecundity.	(Sineva *et al.* 2009)

Vertebrates

Target	Conditions	Strain	Results	Reference
Guinea pigs				
Guinea pigs *Cavia porcellus*	**Route of exposure:** • Injection (compartment not specified). **Dose regimen:** • Not specified. • Surface culture at 24 h on an agar plate. **Control:** • 4.0 cc. glucose broth.	• ATCC 21 subcultured rapidly for a few generations. • N. R. Smith No. 156. • Both strains subcultured rapidly for a few generations.	• Guinea pigs killed only when strains were subcultured. • Glucose broth failed to kill the animals.	(Clark 1937)
Guinea pigs *Cavia porcellus*	**Route of exposure:** • Injection of culture filtrates (0.05 mL) intradermally in albino guinea pigs of either sex. **Control:** • Positive and negative	• B-4ac used for the dermal assay. • 24 other B. cereus strains tested • No designation provided.	• B-4ac and 21 strains gave necrotic reactions surrounded by inflammation at the site of injection.	(Glatz and Goepfert 1973)

Target	Conditions	Strain	Results	Reference
	controls included. • Animals were observed after 6 and 24 hours.			
Rabbits				
New Zealand white rabbits *Oryctolagus cuniculus* Ligated ileal loop	• Food poisoning experimental model. • 6 test loops per rabbit. • 1 negative and 1 positive control per rabbit	• 22 different strains designated	• Rapid accumulation of 3 to 20 mL of straw-colored, often bloody fluid. • Positive responses for 19 of the 22 strains. • Consistently positive responses for younger rabbits. • Most of the rabbits with at least one positive loop died within 10 hours following the surgery.	(Spira and Goepfert 1972)
New Zealand white rabbits *Oryctolagus cuniculus*	• 0.05 mL of cell-free culture filtrate injected intradermally in rabbits weighing 2 to 3 kg. • 3 hours after injection, Evans blue dye was injected into the ear vein.	• 11 strains of *B. cereus* • Only one of them had the designation *B. cereus* B-4ac, which was known to be positive in both the ileal loop and guinea pig dermal assays.	• Blueing area representing the increase in vascular permeability ranged from 4 to over 100 mm^2 for strain B-4ac. • 9 of the 10 other strains produced a positive vascular reaction.	(Glatz *et al.* 1974)
Dutch rabbits *Oryctolagus cuniculus* Males weighing 1.8 ± 0.2 kg.	• 0.1 or 0.3 mL injected intramuscularly into the flank. • 0.15 mL injected subcutaneously • Animals were killed before the infection proved fatal. • Vegetative cells and spore suspensions used. • Concentration ca. 10^2 cells/mL.	• SV1 lecithinase negative variant	• Presence of abscesses showing inflammatory response. • Presence of nodules under the skin with necrotic fibres and fibrosis around its periphery. • Calcification observed in 80% of the animals after 7 days.	(Stretton and Bulman 1975)
Rabbit	• Injected intradermally (0.1 mL) into rabbits weighing from 2.5 to 3 kg.	• 50 of 136 strains isolated from dairy products. • 102 positive strains	• All 102 strains caused vascular permeability in rabbit skin (intense blueing caused by the Evans blue dye.	(Christianss on *et al.* 1989)

Target	Conditions	Strain	Results	Reference
		for extracellular toxins.		
Rabbits *Oryctolagus cuniculus*	• 3 enterotoxins in concentrated cell-free culture filtrate. • Ligated rabbit ileal assay. • Preliminary results for 23 isolates. • 4 selected strains.	• Strain isolated from an incident which caused diarrhea in 6 of 10 monkeys • Strain isolated from raw rice which failed to produce symptoms in eight monkey feedings. • Strain isolated from a brain abscess (2141/74, serotype 11). • B-4ac	• Although just 11 of the strains were tested more than twice, only 2 of the 11 exhibited a >50% probability of being positive on repeated testing. • Fluid accumulation in rabbit ileal loop for the first two strains • Severe disruption of the mucosa in the ileal mucosa for the third strain.	(Turnbull 1976)
New Zealand adult white rabbits *Oryctolagus cuniculus*	• In vitro retinal toxicity assay:Measure of the cytolytic release of lactate dehydrogenase (LDH) from retinal buttons treated with *B. cereus* toxins (600 ng/mL HBL_{eq}*).* • In vivo sterile endophtalmitis model::Intravitreal injection of pure or crude exotoxin (0.1 to 1.15 mL). • Control samples and toxin-containing samples included.	• MGBC 145	• Retinal buttons treated with either CET or HBL became completely disaggregated into cells and cell debris and collapsed upon removal. • Within 4 hours, all eyes receiving ≥ 0.8 µg crude exotoxin exhibited marked exudate, conjunctival edema and hyperemia. • When receiving 1-4 µg, no or little red reflex, vitreal hemorrhage, hemorrhagic chemosis of the conjunctiva, and corneal haze. • Milder responses to low doses.	(Beecher *et al.* 1995a)
Rabbit *Oryctolagus cuniculus*	• Ileal loop fluid accumulation model. • Purified 3 components of HBL.	• F837/76	• Caused fluid accumulation and 3 components were required together to cause maximal activity.	(Beecher *et al.* 1995b)
New Zealand white	• Eyes injected intravitreally	• MGBC145	• Intraocular inflammation and reduction in retinal	(Callegan

Target	Conditions	Strain	Results	Reference
rabbits *Oryctolagus cuniculus* 2 to 3 kg	with viable *B. cereus* (log 2.06 CFU) or cell-free supernatant. • Surgical and absolute controls.		responses after 3 hours. • Retinal detachment and photoreceptor layer folding and disrupting observed after 9 hours. • At 18 hours, eyes demonstrated maximal inflammation, including in peri-ocular tissues. • Injection of culture supernatant produces similar results.	*et al.* 1999)
Mice				
Mice *Mus musculus* Albino Namru strain 6- to 9-week old	• Intraperitoneal (0.5 mL) and subcutaneous (0.25 mL) injections • 4 dilutions injected in each of 12 or more mice • Vegetative forms and spores tested.	• NRS 201 • NRS 232 • NRS 1256	• 10 to 100 times more spores were required to kill mice. • Death occurred upon intraperitoneal injection but not subcutaneous. • Subcutaneous injections resulted in an open necrotic lesion.	(Lamanna and Jones 1963)
Mice *Mus musculus*	• Subcutaneous or intraperitoneal injections (0.25 mL) of a suspension (approx. 500 million bacilli per mL) • Cultures were frequently transferred from one medium to another to increase virulence • Groups of 4 to 8 mice	• Activated strains • No strain designation provided.	• Acute lethal illness at high doses, almost all within 6 hours. • The severity of the disease was clearly dose-dependant. • The minimal dose causing 84 to 100% mortality was approx. 2.2 X 10^7 bacilli. • Low doses resulted in mild illness and sometimes by necrotic skin ulcers at the injection site.	(Burdon *et al.* 1967)
Mice *Mus musculus*	• 0.5 mL of culture filtrate injected into the caudal veins of 4 adult ICR mice. • 2 clinical isolates were used as positive controls and culture medium was used as negative control.	• 183 strains isolated from dairy products	• Of 11 isolates having strong hemolysin activity, 3 of them killed mice.	(Wong *et al.* 1988)
Mice *Mus musculus*	• Intravenous injection of 8 µg of purified hemolysin II	• FS-1	• Death within 2 minutes	(Shinagawa *et al.* 1991a)
Mice	• Vascular permeability test,	• 116 strains.	• Good correlation between production of necrosis in	(Turnbull *et*

Target	Conditions	Strain	Results	Reference
Mus musculus	intestinal necrosis reaction and mouse lethal test.	• About 13 of them designated.	the skin and intestinal tests and the fluid accumulation test.	*al.* 1979)
Mice *Mus musculus* BALB/c strain 5-week-old females Kept in a biosafety containment facility in groups of 5, with sterile water and food. **Purpose:** Investigation of the opportunistic properties of a *B. thuringiensis* mutant and *B. cereus,* and the role of the *plcR* gene.	**Route of exposure**: • Nasal instillation under slight ether anaesthesia. **Method of administration:** • The spore or bacterial suspension was carefully deposited at the corner of the nostril. The mouse inhaled the inoculum by breathing. **Dose regimen**: • 50 µl of the suspension (spores or vegetative cells). **Repetitions and replications:** • No repetition. • Groups of 5-10 mice used for each strain. **Controls:** • None specified. **Duration of study:** • Mortality observed after 24 hours.	**2 *B. cereus* strains tested:** • ATCC 14579 • ATCC 14579 Δ*plcR* • Vegetative cells were prepared from cultures in TSB at 37°C, recovered after culture for 18 h (late stationary phase) by centrifugation. • Spore suspensions were prepared from a 10 days old culture on agar medium. They were washed and resuspended in sterile water, incubated for 1 h at 65°C to kill the vegetative forms.	**Mortality observed:** • 10^8 spores per mouse resulted in 100% mortality for both strains. • 5 X 10^7 spores per mouse resulted in 90% and 22% mortality, respectively. • 10^7 spores per mouse resulted in 90% and 0% mortality, respectively. • 6 × 10^6 vegetative cells per mouse resulted in 100% and 0% mortality, respectively. **Conclusions:** • ATCC 14579 possesses additional factors, not regulated by PlcR, which may potentiate its opportunistic properties. • Rapid death of the host if large doses of vegetative or sporulated cells are used. • The cause of death is unlikely to be due to the growth of the bacteria.	(Salamitou *et al.* 2000)
Mice *Mus musculus* BALB/c strain	**Route of exposure**: • Endotrachea	• ATCC 14579	• Exposure to spores results in negligible effects • Exposure to vegetative cells • experiments terminated at 4h due to severity of symptoms; • elevated pyrogenic cytokines, • pulmonary granulocyte infiltration, • acute phase response markers.	(Tayabali et al. 2010)
Monkeys				
Monkeys *Macaca mulatta*	**Route of exposure**: • Force-feeding.	• B-4ac, isolated from a food poisoning	**Observation intervals:** • continuous	(Goepfert 1974)

Target	Conditions	Strain	Results	Reference
Rhesus strain **Purpose:** Determine the usefulness of Rhesus monkeys model for enteropathogenicity of *B. cereus*.	**Method of administration:** • Monkeys fasted 18 hours prior to feeding. • Given to monkeys by stomach tubes. **Dose regimen**: • 3 types of test material fed: whole cultures, sterile culture filtrates or purified precipitated toxin. • 40 ml by monkey. • Normal food available afterwards. **Repetitions and replications:** • 6 monkeys for each test material. • Fluid accumulation in rabbit ileal loops and skin capillary permeability tests also performed. **Control:** • Sterile BHIG as negative. **Duration of study:** • till symptoms were observed (at least 210 min)	outbreak. • 6 other strains with no specified designation, isolated from the rice-associated outbreaks	**Symptoms observed:** • Diarrhea elicited by the three test materials 35-150 minutes after administration. • Lasted 60-210 minutes. • Control was negative. • Considerable variation in sensitivity among test monkeys. • Approx. 50% of the monkeys showed positive responses. • Vomiting never observed. • 4 of the 6 undesignated strains were positive diarrheal but negative vomiting. • When grown on rice, B-4ac induced diarrhea in 3 of 6 monkeys but not vomiting. **Conclusions:** • Direct correlation between ability to cause fluid accumulation in rabbit ileal loops, alteration of skin capillary permeability and ability to induce diarrhea in monkeys. • Rhesus monkeys are a suitable model. • Diarrhea is due to synthesis and excretion of a toxin by logarithmically growing cells.	
Monkeys *Macaca mulatta* Sex not specified Young Rhesus strain of approximately 3 kg. **Purpose:** Attempt to confirm that	**Route of exposure**: • Force-feeding. **Method of administration:** • Contaminated rice was homogenized and concentrated. • Organisms were also grown in liquid broth. • Under anaesthesia, the test material was administered	**3 strains of *B. cereus*:** • 4810/73 (formerly strain 88) isolated from vomitus, associated in illness. • 4433/73 isolated from meat loaf, implicated in food outbreak. • 2532B/74 isolated	**Parameter measured:** • Emetic activity: vomiting within 5 hours. • Diarrhea: presence of watery or loose stools within 24 hours. • Faecal examination. • Ligated ileal assays **Range of effects for emetic activity:** • Feeding of uninoculated media had no adverse effect. • Only cultures grown on rice could cause vomiting.	(Melling *et al.* 1976)

Target	Conditions	Strain	Results	Reference
food-associated outbreaks were caused by *B. cereus* and to determine the involvement of a new enterotoxigenic material.	*per os via* a lubricated feeding tube. **Dose regimen**: • 30 ml of an 8-hour culture • In food, about 10^{10} viable organisms. • In broth, about 10^{11} organisms. • 100 to 150 ml of the material. • Also, ileal fluid accumulation tested with 12-15 fold concentrated filtrates. **Repetitions and replications:** • Between 4 and 24 monkeys for each combination of strain and culture medium. **Control:** • Uninoculated medium **Duration of study:** • 24 hours.	from rice.	• 10 of 24 monkeys showed positive vomiting for strain4810/73. **Range of effects for diarrheic activity:** • Feeding of uninoculated media had no adverse effect. • Largely confined to strain 4433/73. • Chiefly, diarrhea in 6 of 10 monkeys, for rice. • Present in both rice and broth cultures. • Faecal examination. • Bacteriological picture accurately reflected the quantities in the material fed. **Ligated ileal assays** • Of 13, 15 and 12 tests respectively for each strain, 2, 12 and 9 were loop positives. **Conclusions:** • A clear distinction is made between the strains causing vomiting and diarrheal. • The difference between the activities of the 2 first strains is reinforced by the rabbit loop test. • The range of foods involved in diarrheal outbreaks was wide whereas vomiting outbreaks have been restricted to the consumption of rice.	
Other studies involving more than one organism				
Monkeys *Macaca mulatta* Rhesus strain 6-8 kg Mice *Mus musculus* ICR, strain not specified. Sex not specified. Weighing 20-24 g. **Purpose:** Study the correlation	**Tests conducted**: • Mouse lethality. • Emetic activity to monkeys. **Route of exposure**: • Intravenous injection. • Intragastric administration. **Method of administration:** • Details not specified for mice. • Oral administration by catheter for monkeys.	• *B. cereus* No. 35, produces enterotoxin, but no vacuole factor. • *B. cereus* No. 55, isolated from the outbreak. Produces vacuolation factor but no enterotoxin.	**Mouse lethal activity:** • Was not observed for 100-500 units for both substances. • Was found for more than 1000 units of toxin. **Monkey emetic activity:** • No activity was observed for controls. • For cereulide at 14 000 units, all 3 monkeys showed emesis within 2-4 hours. • For partially purified factor at 30 000 units, 1 of 2 monkeys showed emesis after 6 hours. • For partially purified factor at 36 000 units, the 2	(Shinagawa *et al.* 1995)

Target	Conditions	Strain	Results	Reference
between emetic toxin and vacuolation factor HEp-2 produced by *B. cereus* isolated from an outbreak of vomiting-type food poisoning.	**Dose regimen:** • 0.2 ml of the test sample. • 20 ml of the test sample. **Substances tested:** • Purified cereulide. • Partially purified vacuolation factor. **Test conditions:** • Foods and water were provided prior to administration of test sample to monkeys. **Replication:** • Not specified. **Control:** • Tested on 2 monkeys each. • Supernatant of *B. cereus* No. 35 as negative control. • Physiological saline. **Duration of study:** • Mice observed for 60 minutes. • Monkeys observed for 24 hours.		monkeys showed emesis after 2 and 4 hours. **Significance:** • Demonstrates that the HEp-2 vacuolation factor is an emetic toxin like cereulide. • These toxins can produce emesis in monkeys	
Sheep and cattle Young females	**Route of exposure**: • Intravenous injection. **Number and condition of animals:** • 5 ewes, pregnant 90 to 110 days. • 6 heifers, pregnant 7 months. • All animals housed in an isolation building for 10 days prior to inoculation. **Dose regimen for ewes:**	• No strain designation provided. • Isolated from an aborted bovine fetus.	**Symptoms observed for ewes:** • 4 aborted dead lambs between 3 to 8 days postinoculation. • One died after having fever, tachycardia, tachypnea and central nervous system disorders. **Symptoms observed for heifers:** • Groups 1 and 2 aborted dead calves between 7 to 12 days postinoculation. • Group 3 had normal calves at term. **Examination of lambs and calves:** • Varying degrees of autolytic change. • Blood-tinged ascites, hydrothorax, hydropericardium	(Wohlgemuth *et al.* 1972b)

Target	Conditions	Strain	Results	Reference
	• 5.1 X 10^5 organisms. **Dose regimen for ewes:** • 3 groups (2 animals each): • Group 1: 8 X 10^6 organisms. • Group 2: 8 X 10^5 organisms. • Group 3: 8 X 10^3 organisms. **Replications:** • Duplicate sections of tissues were stained.		and subcutaneous edema. • The foetal membranes were hyperemic and edematous. **Bacteriological findings:** • *B. cereus* isolated in pure cultures from tissues of the dead ewe, lambs and calves. **Conclusion:** • Necrotic placentitis was consistent in all the abortions, indicating that the placenta is the principal site of infection.	
Rabbits and mice	• Purified enterotoxin.	• FM-1	• Vascular permeability in rabbits. • Lethal to mice. • Caused fluid accumulation in mouse ligated intestinal loop.	(Shinagawa *et al.* 1991b)
Mice and cats	• Intravenous injection of purified enterotoxin.	• *B. cereus* 96.	• Minimum lethal dose of 300 µg per mouse. • 70 to 80 µg per kg caused vomiting in cats.	(Gorina *et al.* 1975)

APPENDIX 6B: Pathogenicity of *Bacillus cereus* to invertebrates and vertebrates in natural settings.

Cases where *B.cereus* was isolated from animals showing disease symptoms in a natural setting.

Organism	Conditions	Strain	Results	Reference
Lepidopteran insects				
Pectinophora gossypiella larvae	**Conditions of the animals:** • Throughout 2 resting seasons, the rate of sick larvae carrying dermal brown lesions were 4.1 and 1.7%. • The rates of dead larvae carrying dermal brown lesions were 2 and 0.4%. **Number of animals studied:** • For each year: • 50/ 28 in July • 347/ 51 in August • 1612/ 458 in September **Control:** • None specified. **Duration of study:** • 2 years	• No strain designation given.	**Symptoms observed:** • When these larvae were kept in the laboratory, many of them died within 8-45 days. • Death rates from December to April were 45, 54, 20, 8 and 0% in the first season. • In the second season, rates were 56, 20, 20, 20 and 0%. **Bacteriological observation:** • *B. thuringiensis* var. *finitimus* and *B. cereus* were isolated from these larvae, but not from the healthy larvae or dead larvae not presenting the lesions. **Conclusion:** • Decreasing virulence with the advance of the resting period may indicate that the larvae catching the disease late may be or may become more resistant to its effect.	(Abul Nasr *et al.* 1978)
Coleopteran insects				
White grubs *Anomala dimidiata*	• Isolates from an atrophied pupa.	• WGPSB-2 (MTCC 7182)	• The strain was able to infect and cause 92 and 67% mortality in second instar larvae of *Anomala dimidiata* and *Holotrichia seticolis*, respectively.	(Selvakumar *et al.* 2007)
White grubs *Anomala dimidiata* and *Holotrichia seticollis*	• Up to one-fifth of the population was found to exhibit symptoms of bacterial infection.	• WGPSB-2	• Of 27 bacterial isolates tested against *A. dimidiata*, the most highly toxic strain was identified as *B. cereus.*	(Sushil *et al.* 2008)
Mammals				
Dairy cattle *Bos taurus* **Purpose:**	**Route of exposure**: • Injection into quarters. **Dose regimen**:	• None specified. • *B. cereus* identified according to cases	**Symptoms observed:** • Some of the affected cows developed acute mastitis within 24 hours, most of them shortly after calving.	(Schiefer *et al.* 1976)

Organism	Conditions	Strain	Results	Reference
Describe the pathology of bovine *B. cereus* mastitis after intramammary treatment with antibiotic preparations.	• Contaminated commercial antibiotic product. **Number and condition of the animals:** • 8 dairy herds. • Total 80 cows affected. **Study conducted:** • Two whole carcasses which died of acute mastitis were examined. • Selection of tissues was made on the carcasses and also on 9 cows: mammary tissue, supramammary lymph nodes, liver, spleen and kidney. **Controls:** • None specified. **Duration of study:** • Approximately one year (1974)	previously described (Perrin *et al*. 1976)	**Gross examination:** • Watery blood that had failed to clot. • Marked subcutaneous edema over the udder. • Numerous dark red, well demarcated areas were scattered throughout the affected quarters. • Markedly enlarged supramammary lymph nodes. • Moderately edematous and emphysematous lungs. • Twice the normal size, dark red and turgid spleens **Histological findings:** • Mammary glands: interstitial septa were found to be edematous, acute thrombosis of veins and lymph vessels was noted. • Erythrocytes found in the interstitial tissue. • Gram-stained sections revealed Gram-positive organisms in the necrotic alveoli only. • Acute lymphadenitis in sections of supramammary lymph nodes with focal areas of necrosis and large numbers of inflammatory cells. • Liver showed presence of centrolobular hypoxic necrosis. • Renal tissue revealed hemoglobinemic casts in the tubules. • Hyaline thrombi were evident in capillaries of glomerular tufts and in the corticomedullary junction. • Lungs revealed thickened alveolar septa due to edema. Alveolar capillaries were engorged with blood and many had hyaline thrombi.	
Cattle Various sexes and ages.	**Case 1**: • Male bovine fetus, 8 months in gestation. • Second abortion in an 8-month period in a herd of 80 Brown Swiss cows. **Case 2:** • Male bovine fetus, 8	• No strain designation provided.	• 3 case reports of abortions. • Necropsy, microbiologic and histopathologic examinations conducted for each fetus and fetal membranes when available. **Necropsy findings:** • Atelectatic, firm and dark red lungs. • Fibrinous pleuritis, pericarditis and peritonitis. • Yellow liver, twice the normal size.	(Wohlgemuth *et al.* 1972a)

Organism	Conditions	Strain	Results	Reference
	months in gestation. • Second abortion in an 8-month period in a herd of 150 Holstein-Friesian cows. **Case3:** • Female bovine fetus, 7 months in gestation. • The only abortion in a 1-year period in a herd of 21 Holstein-Friesian cows.		• Enlarged and congested lymph nodes. **Microbiological findings:** • *B. cereus* was the only microorganism isolated from gastric contents and tissues. **Histopathologic findings:** • Vasculitis, edema, inflammation and necrosis in the intercotyledonary space. • Hyperplasia in spleen. • Congested liver.	
Dairy cattle *Bos taurus* Adult females	• Quarters inoculated with *B. cereus*.	• No strain designation provided.	• Acute mastitis developed, followed by atrophy and cessation of milk secretion.	(Horvath *et al.* 1986)
Dairy cattle *Bos taurus* Adult females **Purpose:** Accidental occurrence of *B. cereus* mastitis in several herds involved in efficacy trials of a proposed "dry-cow" therapy product.	**Route of exposure**: • Injection into quarters. **Dose regimen**: • Experimental product containing 500 mg of cloxacillin in peanut oil and 3% monostearate base. **Number and condition of the animals:** • 5 herds of 120, 70, 1 600, 1 500 and 1 500 milking cows, respectively. • Deliberate injection in 151 non-lactating cows. • Inadvertent injection in 33 lactating cows. **Study conducted:** • Sample of the foremilk from all 4 quarters was taken immediately before the last milking of the lactation period.	• No strain designation provided. • Isolated from the experimental product and from the quarters.	**Symptoms observed:** • Gangrenous mastitis developed in 5 cows at calving. • Clinical mastitis developed in 15 other infected quarters, chiefly at calving or during lactation. • Only 26 of 184 cows and 37 of 735 quarters exposed were infected. **Culture study:** • Agreement between the double samples was excellent. • *B. cereus* was recovered in 9.9% of the treated quarters, in 3.6% of quarters having another infection at the time of exposure, and in 15.5% of cultures negative at the time of exposure, when later recultured. • Most of the isolations were made 33 to 56 days after exposure and from quarters with no clinical evidence of mastitis. **Conclusions:** • The numbers of organisms in infected quarters vary widely, often being low. • It is postulated that the organism is chiefly in spore form and less responsive to simple cultural or	(Jasper *et al.* 1972)

Organism	Conditions	Strain	Results	Reference
	• Quarters were treated soon after completion of this milking. • Teats were dipped in an iodophor teat-dip after treatment and adverse reactions were checked by owners. • Re-sampling and culture of the samples were made. **Controls:** • None specified. **Replications:** • Double sample (2 independent samples collected aseptically with cleansing and drying of the teat prior to collection) • Single postreatment samples taken. **Duration of study:** • Approximately 3 months.		treatment procedures. • The number of organisms in each product tube was low and not all tubes were contaminated.	
Dairy cattle *Bos taurus* Adult females	• 11 cows with acute mastitis between 1963 and 1973.	• None specified.	• *B. cereus* was isolated from 1 cow.	(Inui *et al.* 1979)
Holstein dairy cattle *Bos taurus* Adult females **Purpose:** Antibiotic therapy using cloxacillin as part of a herd health program.	**Physical conditions:** • Well managed cows with no serious mastitis problems. • Antibiotic program initiated in 67 cows. o Infusions of the antibiotic during the dry period or the lactating period, or	• No strain designation given. • Isolate from the milk of infected cows. **Preparation of the bacterin:** • Incubation of the isolate in brain-heart infusion broth. Sediment	**Symptoms observed for infusions:** • Acute severe mastitis occurred in 62 of the 67 cows infused with cloxacillin. • During the dry period: o Of 25 that were infused 11 developed severe mastitis (average 24 days later, range 2 to 94 days). o Post mortem examination of one of the cows revealed scarlet-colored mammary glands surrounded by	(Perrin *et al.* 1976)

Organism	Conditions	Strain	Results	Reference
	both. • Vaccination of 41 cows, before or after the antibiotic treatment. Subcutaneous injection of 10 ml of the bacterin. **Number of animals studied:** • 129 out of a 140 cow herd. **Control:** • None specified. **Duration of study:** • 3 months.	resuspended in 0.85% saline with 0.25% formaldehyde and tested for sterility. Finally diluted to MacFarland No. 3.	gelatinous material and filled with serosanguineous fluid. Mammary lymph nodes were wet in appearance and surrounded by gelatinous material. • During lactation: o All of 33 cows infused developed mastitis 1 to 30 days later (the majority within 1 to 3 days). o Observation of one of the cows the day after parturition revealed a very hard hind quarter that contained only serous, red fluid. The cow refused to eat and her rectal temperature was 39.5°C, feces slightly diarrheic. In the succeeding days the mammary gland became cold, black and started to slough. • During both dry and lactating periods: o All 4 cows infused developed mastitis. • 5 cows infused with cloxacillin did not develop mastitis. **Symptoms observed when given the bacterin:** • Of 21 non-vaccinated cows, 6 died suddenly and 15 survived. • All of 41 vaccinated cows developed less severe but recurrent mastitis and showed poor milk production. **Conclusions:** • The disease usually occurs as the result of injection of *B. cereus* into the teat cistern when treating mastitis of other causes. Contaminated antibiotics, teat tubes, syringes and dilators have been described as the source of infection. • Both gangrenous inflammation and acute mastitis with systemic involvement have been reported. • The secretion was serous and frequently contained erythrocytes, fibrin and leukocytes.	

Organism	Conditions	Strain	Results	Reference
			• Very low numbers of *B. cereus* can produce profound pathogenic effects.	
Dairy cattle *Bos taurus* Goat *Capra hircus* Adult females **Purpose:** Report of bovine mastitis apparently caused by *B. cereus*.	**Physical conditions:** • Trimmed tissues from one affected animal were fixed for sectioning. • Toxins tests with the rabbit skin vascular permeability and necrosis reaction. **Number of animals studied:** • 28 cows • 1 goat • Distributed on 4 farms. **Control:** • None specified. **Duration of study:** • Not specified.	• No strain designation given. • Identified as *B. cereus* by colony morphology.	**Symptoms observed:** • 5 rapidly fatal. • Others ranging from gangrenous to mild. **Farm 1:** • 3 cases of very acute mastitis in one week. • First cow died within 24 hours. • No response to antibiotic therapy. • Milk "port-wine" in color. • Second animal had subnormal temperature and a swollen and cold udder. Both milk and urine were port-wine; animal died within 24 hours. • Examination of viscera revealed deep red kidney and udder, blood in the pelvis, congested liver and large white clots and blood stained fluid in the teat cistern. • These latter two were in late lactation. • The third cow was newly calved and developed mastitis 2 days later. • She had pale brown milk and recovered from antibiotic therapy. **Farm 2:** • Symptoms were mild. • Response to therapy was poor. **Farm 3:** • One cow recumbent after milk fever suddenly developed peracute mastitis and died. Port-wine milk. • Second case occurred in newly-calved, in the same calving box. *B. cereus* was recovered from the udder. **Farm 4:** • One cow died of acute mastitis the morning following a cut in the teat. **Bacteriological examination of faeces and brewer's**	(Jones and Turnbull 1981)

Organism	Conditions	Strain	Results	Reference
			grains: • Organisms present in faeces of affected and non-affected cows at levels of 10^5-10^6 per g. • 10^2-10^3 cells of *B. cereus* per g recovered from well preserved brewer's grains and 10^4-10^5 when spoilage had occurred. • 7.5 X 10^5 and 4 X 10^8 in grains obtained from the same supplier. • *B. cereus* has been isolated on 17 other occasions in pure culture from mastitic bovine milk. **Histopathological examination:** • Lesions, interstitial septa oedematous and containing erythrocytes. • Thrombi in veins. • Necrosis of alveolar cells. **Permeability test:** • Only one of 19 mastitic and environmental isolates showed strong toxic activity. **Conclusions:** • Possibility of brewer's grain being the source of infection. • The organism is more likely to establish itself when there is no pre-existing infection in the udder.	
Dairy cattle *Bos taurus* Adult females	• Bovine mastitis	• 1820/77 • 1419/77 • 1414/77 • 1589/77 • 624/76	• 1820/77: Death • 1419/77, 1414/77 and 1589/77: 2 deaths. • 624/76: not available.	(Turnbull *et al.* 1979)

APPENDIX 7A: Selected non gastrointestinal outbreaks caused by *Bacillus cereus* and reported in the literature.

Year	Place	Type of infection
2004	Georgia (US), University Military Program	94/660 cadets with non puritic, impetigo-like lesions on their scalps caused by *Bacillus cereus*. Infections are linked to the following potential factors: haircut, poor hygiene, sunscreen, exposure to soil and water (CDC 2005)
1998	Amsterdam (Netherlands) Neonatal Intensive Care Unit	Three neonates developed a series of invasive blood infections with *B. cereus* between January and August 1998. One died and the two recovered. Thirty-five neonates were found to be colonized with *B. cereus*. The source of infection was contaminated balloons used for manual ventilation.(Van Der Zwet *et al.* 2000)

APPENDIX 7B: Reported *Bacillus cereus* Food-Related Outbreak[13]

Vehicle	Country	Year	Cases	Story
Rice	Australia	2002	37	
Potato	Australia	2004	6	National franchised fast food restaurant - potato & gravy.
Chicken	Australia	2006	14	cooked chicken
Sauce	Australia	2007	3	81-year-old male died 12 hours after consuming asparagus cream sauce
Pasta salad	Belgium	2003	5	Severe illness & death of 1 child. The temperature of the fridge where the pasta salad was stored was 14°C.
Pasta	Belgium	2004	50	
Rice	Belgium	2005	6	
Milk products	Belgium	2006	70	
Potato salad	Canada	1999	25	Meal prepared by a restaurateur inexperienced in catering services & temperature control
Chicken	Denmark	2005	4	
Pizza	Denmark	2005	16	
Sauce	Finland	2004	5	Confirmed in left-overs; inadequate cooling and reheating and improper storage; mushroom sauce
Cake	Finland	2004	10	Confirmed in left-overs; layer cake
Eggs	Finland	2005	2	Listed as egg-butter
Pork, mixed dishs	Finland	2005	20	Ham casserole
Fruit	Finland	2005	15	Berries imported from Poland
Macaroni and Cheese	Finland	2005	18	
Soup	Finland	2005	9	Meat soup
Spices	France	2007	146	School / kindergarten - herbs and

[13] Information courtesy of Judy Greig, food Safety Microbiologist/Epidemiologist, Laboratory for Foodborne Zoonoses, Public Health Agency of Canada

Vehicle	Country	Year	Cases	Story
				spices source
Rice	India	2006	140	
Milk, pasteurized	Japan	2000	3	Murayama milk recalled 4 tons of dairy products because investigators found *B. cereus* in bottles of milk.
Bean jam	Japan	2001	335	Kindergarten – *B. cereus* in rice cakes & bean jam inside - bean jam kept longer than usual at room temperature.
Milk products	Jordan	2007	51	Distributed under the government's School Nutrition Programme.
Chicken	Norway	2004	19	Confirmed in left-overs
Chili	Norway	2005	6	Workplace canteen
Stew	Norway	2005	22	
Rice	Norway	2005	3	
Pizza	Norway	2005	3	
Infant Cereal	United Kingdom	2005	2	
Rice	United States	1995	21	
Marinara sauce	United States	1996	22	
Stuffing	United States	1997	400	
Chicken, BBQ	United States	1997	3	
Seafood	United States	1997	2	Seafood corn chowder
Rice, fried	United States	1997	4	*These 2 are separate outbreaks*
Rice, fried	United States	1997	4	*These 2 are separate outbreaks*
Rice, fried	United States	1997	19	
Pork, BBQ	United States	1997	33	
Shrimp	United States	1998	118	
Rice, fried	United States	1998	6	
Meat	United States	1998	19	Turkey, roast beef
Rice, fried	United States	1998	7	
Sandwich, submarine	United States	1998	25	
Meat	United States	1998	19	
Rice, fried	United States	1998	11	
Rice, fried	United States	1998	4	
Coleslaw	United States	1999	8	
Rice, fried	United States	1999	4	
Potato, mashed, with gravy	United States	1999	4	
Rice	United States	1999	32	
Rice	United States	1999	4	
Sandwich, beef	United States	1999	2	
Rice Milk	United States	2000	2	Rice Dream Original Enriched beverage.
Rice, fried	United States	2000	18	
Rice	United States	2000	15	
Rice, fried	United States	2000	10	
Salmon	United States	2000	3	
Taco	United States	2000	4	
Salad	United States	2000	3	
Dips	United States	2001	10	Buttermilk peppercorns dip

Vehicle	Country	Year	Cases	Story
Rice, fried	United States	2001	5	Fried rice, ethnic style
Rice, fried	United States	2001	17	
Salad	United States	2001	3	Vegetable-based salad, lettuce-based salad
Chicken	United States	2002	11	
Chicken	United States	2002	3	
Rice, fried	United States	2002	8	
Rice, egg-fried	United States	2002	2	
Pizza	United States	2002	6	Meat pizza
Chicken, fried	United States	2002	4	
Chicken, mixed dish	United States	2002	8	
Potato, fried	United States	2003	42	
Chicken, mixed dish	United States	2003	8	
Chinese food	United States	2004	3	Chicken chow mein
Chicken	United States	2004	11	
Pizza	United States	2004	4	Cheese, meat and vegetable pizza
Chicken, mixed dish	United States	2004	2	Chicken and pasta
Rice, fried	United States	2004	26	
Chinese food	United States	2004	2	
Taco	United States	2005	27	Taco meat
Sauce	United States	2005	4	Tzatziki sauce
Grains	United States	2006	2	
Pasta	United States	2006	2	Lo mein
Pancakes	United States	2006	2	
Rice, fried	United States	2006	5	Pork fried rice
Pork	United States	2006	20	Roasted
Chicken, baked	United States	2006	5	
Beef	United States	2006	3	Prime rib steak
Rice	United States	2006	4	Spanish rice
Rice, fried	United States	2007	16	Vegetable fried rice
Rice, fried	United States	2007	3	

APPENDIX 8: Considerations for Levels of Hazard Severity, Exposure and Risk as per Health Canada and Environment Canada's "Framework for Science-Based Risk Assessment of Micro-organisms regulated under the *Canadian Environmental Protection Act,* 1999".

Considerations for hazard severity (environment)

Hazard	Considerations
High	Considerations that may result in a finding of high hazard include a micro-organism that: • Is known as a frank pathogen; • Has irreversible adverse effects (e.g., loss of biodiversity, loss of habitat, serious disease); • Has significant uncertainty in the identification, characterization or possible effects..
Medium	Considerations that may result in a finding of medium hazard include a micro-organism that: • Is known as an opportunistic non-human pathogen or for which there is some evidence in the literature of pathogenicity/toxicity; • Has some adverse but reversible or self-resolving effects.
Low	Considerations that may result in a finding of low hazard include a micro-organism that: • Is not known to be a non-human pathogen; • Is well characterized and identified with no adverse ecological effects known; • May have theoretical negative impacts for a short period but no predicted long term effect for microbial, plant and/or animal populations or ecosystems; • Has a history of safe use over several years.

Considerations for hazard severity (human health)

Hazard	Considerations
High	Considerations that may result in a finding of high hazard include:: • Disease in healthy humans is severe, of longer duration and/or sequelae may result; • Disease in susceptible humans may be lethal; • Potential for horizontal transmission/community-acquired infection; • Lethal or severe effects in laboratory mammals at maximum hazard/challenge dose trigger multiple-dose testing.
Medium	Considerations that may result in a finding of medium hazard include: • Case reports of human disease in the scientific literature are limited to susceptible populations or are rare, localized and rapidly self-resolving in healthy humans; • Low potential for horizontal transmission; • Effects at maximum hazard/challenge dose in laboratory mammals are not lethal, and are limited to invasive exposure routes (i.e., intraperitoneal, intravenous, intratracheal) or are mild and rapidly self-resolving.
Low	Considerations that may result in a finding of low hazard include: • No case reports of human disease in the scientific literature, or case reports associated with predisposing factors are rare and without potential for secondary transmission and any effects are mostly mild, asymptomatic, or benign. • No adverse effects seen at maximum challenge dose in laboratory mammals by any route of exposure.

Considerations for level of exposure (environment and human health)

Exposure	Considerations
High	Considerations that may result in a finding of high exposure include a micro-organism for which: • The release quantity, duration and/or frequency are high. • The organism is likely to survive, persist, disperse proliferate and become established in the environment. • Dispersal or transport to other environmental compartments is likely. • The nature of release makes it likely that susceptible living organisms or ecosystems will be exposed and/or that releases will extend beyond a region or single ecosystem. • In relation to exposed organisms, routes of exposure are permissive of toxic or pathogenic effects in susceptible organisms.
Medium	Considerations that may result in a finding of medium exposure include a micro-organism for which: • It is released into the environment, but quantity, duration and/or frequency of release is moderate. • It may persist in the environment, but in low numbers. • The potential for dispersal/transport is limited. • The nature of release is such that some susceptible living organisms may be exposed. • In relation to exposed organisms, routes of exposure are not expected to favour toxic or pathogenic effects.
Low	Considerations that may result in a finding of low exposure include a micro-organism for which: • It is no longer in use. • It is used in containment (no intentional release). • The nature of release and/or the biology of the micro-organism are expected to contain the micro-organism such that susceptible populations or ecosystems are not exposed. • Low quantity, duration and frequency of release of micro-organisms that are not expected to survive, persist, disperse or proliferate in the environment where released.

Considerations for level of risk

Risk	Considerations
High	A determination of high risk implies that severe, enduring or widespread adverse effects are probable for exposure scenarios predicted from known, foreseeable or intended uses. A conclusion of CEPA-toxic would result and control measures or risk management would be recommended.
Medium	A determination of medium risk implies that adverse effects predicted for probable exposure scenarios may be moderate and self-resolving. The conclusion (CEPA toxic or not) is chosen based on the particulars of the case. If the conclusion is not CEPA-toxic, for intended (proposed) use(s) or exposure scenario(s) but, under another significant new activity, may become toxic, application of the **Significant New Activity provision** may be recommended to allow for the assessment of new uses/activities.
Low	A determination of low risk implies that any adverse effects predicted for probable exposure scenarios are rare, or mild and self-resolving. The conclusion would be not CEPA toxic, and SNAc provisions may or may not be applied.