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Toxigenic Bacilli Associated with Food Poisoning

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Abstract The genus *Bacillus* includes a variety of diverse bacterial species, which are widespread throughout the environment due to their ubiquitous nature. A well-known member of the genus, *Bacillus cereus*, is a food poisoning bacterium causing both emetic and diarrhoeal disease. Other *Bacillus* species, particularly *B. subtilis*, *B. licheniformis*, *B. pumilus*, and *B. thuringiensis*, have also recently been recognized as causative agents of food poisoning. However, reviews and research pertaining to bacilli have focused on *B. cereus*. Here, we review the literature regarding the potentially toxigenic *Bacillus* species and the toxins produced that are associated with food poisoning.

Keywords: *Bacillus*, food poisoning, emetic disease, diarrhoeal disease, toxigenic

Introduction

The genus *Bacillus* comprises a very large and diverse group whose members are widespread in nature. They are important in the food industry and also to consumers because of the association of some species with food poisoning and food spoilage. There are several reasons for the problems caused by *Bacillus* species in the food industry. First, they are difficult to eliminate from foodstuffs and food processing systems due to formation of spores which are ubiquitous and highly resistant to adverse conditions such as heat, dehydration, and other physical stresses (1-3). Second, the spores, as well as older cells, are hydrophobic and have adhesive characteristics, which facilitate their attachment to the surface of processing equipment (2,4-6). Third, both spores and vegetative cells appear to have become increasingly tolerant or resistant to environmental stressors, enabling them to survive under conditions or through treatments generally considered to stop growth or to inactivate all viable microorganisms (5).

The numbers of species in genus *Bacillus* fluctuated between the 1st edition and the 8th edition of Bergey's Manual of Determinative Bacteriology (7), due to heterogeneity within the genus. In the 8th edition (8), 22 species of *Bacillus*, including *Bacillus cereus*, were classified in Group I of the genus and were widely accepted as distinct species; whereas the 26 members of Group II received little attention (9). Of the Group I members, the *B. cereus* group and the *Bacillus subtilis* group have been widely recognised as causative agents of food-borne illness. The *B. cereus* group comprises 6 recognised species including *B. cereus*, *B. anthracis, B. thuringiensis, B. mycoides, B. pseudomycoides*, and *B. weihenstephanensis* (10). Studies of DNA-DNA hybridization and 16S rRNA sequences revealed *B. cereus, B. anthracis, B. mycoides*, and *B. thuringiensis*, referred to as the *B. cereus* group, are very closely related (11,12). In fact, extensive biochemical, physiological, and morphological studies of the genus failed to find characteristics that would consistently differentiate these 4 species (13); whereas members of the *B. subtilis* group, including *B. subtilis*, *B. amyloliquefaciens*, *B. licheniformis*, and *B. pumilus*, are accepted as distinct, easily differentiated species (14). However, recent ecological studies have also identified some very close relatives of *B. subtilis* which are *B. atrophaeus* (15), *B. mojavensis* (16), and *B. vallismortis* (17).

Most food poisoning incidents attributed to *Bacillus* species are associated with *B. cereus,* but other *Bacillus* species are increasingly recognized as food poisoning agents. Recent epidemiological evidence links *B. licheniformis*, *B. subtilis*, *B. pumilus*, and *B. thuringiensis* with incidents or outbreaks of food poisoning. The aim of this review is to systematically examine *Bacillus* species related to food poisoning, while providing background on the potentially toxigenic *Bacillus* species and a detailed review of their toxins.

Ecology of *Bacillus* Species

Bacillus species are found in a number of ecological niches, due to the resistance of their endospores to environmental stress, as well as their long-term survival under adverse conditions, but it is generally accepted that the primary habitat of endospore-forming bacilli is the soil (soil contains 10^5-10^6 spores of *B. cereus*/g).

Therefore, raw foods of plant origin are a major source of *B. cereus* in food and the presence of *Bacillus* species in plant foods is the result of soil contamination rather than a specific association between the microorganism and plants (18). Cereal products and various types of peas and beans have been found to be frequently contaminated with *B. cereus* (19-21). Raw rice is a well known source of *B. cereus*, with most samples containing low levels of the bacterium (10^1-10^5 *B. cereus*/g) (22-24). Both raw and pasteurised milk is also often contaminated with *B. cereus*

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(25-27). Other *Bacillus* species, including *B. subtilis*, *B. licheniformis*, *B. pumilus, B. sphaericus, B. amyloliquefaciens*, and *B. circulans* have also been found in milk (28-30). Some bacilli survive ultra high temperature (UHT) treatment (31,32) as well as high temperature-short time (HTST) pasteurization (33).

The presence of *B. cereus* in processed foods stems from contamination from raw materials or is associated with the ability of spores to survive heat treatment processes. In the case of bread, though not a major source of *B. cereus*, spoilage may be due to *B. subtilis*, *B. licheniformis,* or other *Bacillus* species (21,34,35), and boiled rice or, more commonly, fried rice may contain high numbers of *B. cereus* (10^2-10^6 *B. cereus*/g) (36-38), although *B. pumilus* have also been found in cooked rice samples (39). Ninety-five % of *B. cereus* emetic syndrome outbreaks have been associated with the consumption of boiled or fried rice from Cantonese-style restaurants and thus food poisoning due to the emetic toxin has been termed the 'Chinese-restaurant syndrome' (40). *Kimbab*, a common ready-to-eat food in Korea that contains cooked rice as the main ingredient, is frequently associated with *B. cereus* contamination (41,42). Rosenquist *et al.* (43) reported that among 48,901 ready-to-eat food products at a Danish retail market, 98.7% contained *B. cereus*-like organisms at concentrations of $<10^3$ CFU/g, 0.7% of which were at 10^3-10^4 CFU/g, and 0.5% of which were at $>10^4$ CFU/g. Diary-based desserts such as custards, puddings, and mousses may contain *B. cereus* (44-46), though the relationship between the milk and the organism is not always certain. When 325 food samples were examined by Schlegelova *et al.* (47), 1/3 of the total samples and one half of the cream cheese and spreading butter samples were found to contain *B. cereus.*

Detection, Isolation, and Biochemical Properties of *Bacillus* Species

The only well-established detection methods for a *Bacillus* species of interest to the food microbiologist are for *B. cereus* (14). A number of selective-differential plating media for the detection and enumeration of *B. cereus* in foods have been developed, the major criteria for detection and identification of *B. cereus* on these plating media including haemolytic activity, lecithinase activity (egg yolk reaction), since most strains of the *B. cereus* group possess lecithinase activity, and/or inability to ferment mannitol. However, other *Bacillus* species including *B. thuringiensis*, *B. mycoides*, *B. licheniformis*, and *B. subtilis* were also shown to grow on *B. cereus* selective media (13,48), even though *B. subtilis* and *B. licheniformis* are able to ferment mannitol (13).

In mannitol-egg yolk-polymyxin (MYP) agar, a widely used medium, the single egg yolk diagnostic system of egg yolk agar (EYA) was expanded to a double diagnostic system by the addition of a mannitol/phenol red combination (49). However, due to the high peptone content (1%) of the medium, sporulation is often poor and re-streaking of suspect colonies may be necessary to confirm colony morphology and sporulation, thus increasing analysis time (14). Those limitations led to the development of polymyxin-egg yolk-mannitol-bromothymol blue agar (PEMBA) (50). PEMBA contains polymyxin B to inhibit growth of Gram-negative bacteria, mannitol/bromothymol blue and egg yolk to identify suspect colonies of *B. cereus*; and low levels of peptone to promote sporulation. Recently, chromogenic agar media have been developed to give more specificity by including chromogenic substrates, such as 5-bromo-4-chloro-3-indoxyl-myo-inositol-1-phosphate or 5-bromo-4-chloro-3-indoxyl-β-glucosidase, and greater selectivity by inclusion of ceftazidime (51), but these media, due to the cost of these substrates, are currently too expensive to use routinely. At present, none of the selective-differential media for *B. cereus* can differentiate between *B. cereus* and other members of the *B. cereus* group, such as *B. mycoides* and *B. thuringiensis* (52), though most isolates of *B. mycoides* are differentiated easily on agar media because they characteristically produce rhizoid colonies (13).

While typically the method of choice, the direct plating method may not be sufficient to use for foods contaminated with low numbers of *B. cereus* or injured cells. Thus, enrichment is needed to isolate the organism. The enrichment method for *B. cereus* was developed with a selective medium containing polymyxin B, which suppresses Gram-negative organisms. However, this enrichment procedure is not specific for *B. cereus*; other *Bacillus* species as well as other Gram-positive bacteria can grow. Furthermore, it will not generate quantitative data because of the obvious variation in the population dynamics of enrichment. The limitations could be overcome with a centrifugation-plating method. The principle of this method is very simple. Microbial cells are readily sedimented by centrifugation at 3,000-10,000×g within minutes and centrifugation is a simple, efficient, and inexpensive physical method to concentrate and enrich microbial cells (53). Thus, centrifugation is a physical form of enrichment method that could assist in determining true population and diversity data. It can also overcome much of the uncertainty and unreliability associated with culture enrichment methods (54-57).

Isolates of bacilli can be identified by morphological studies based on Gram and spore staining, and biochemical tests. Table 1 shows some of the physical and biochemical characteristics of *Bacillus* species of public health concern. Miniaturised versions of traditional biochemical tests, embodied in API kits, Vitek cards, and Biolog plates, can make easier the performance of these laborious and time-consuming traditional biochemical tests. However, these commercial identification systems could not adequately differentiate between *B. cereus*, *B. cereus* var. *mycoides* and *B. thuringiensis* (58) and only allow the recognition of 30-34 species among the 123 valid species in *Bacillus* and the 7 new genera derived from it (59). Further, the reliability of these tests is, at times, questionable because ambiguous reactions may lead to misinterpretation. Therefore, these systems may not be sufficient to use alone for identification of bacteria.

Methods for Strain Typing of *Bacillus* Species

Monitoring foodborne pathogenic bacteria through typing at the subspecies (strain) level is an important part of investigative processes (60-62). Typing techniques that have good discrimination power are needed for epidemiological investigation of outbreaks of foodborne disease involving microbial pathogens, including *Bacillus* species, or tracking

Table 1. Identification of *Bacillus* species of public health significance[1)]

	B. cereus	*B. mycoides*	*B. thuringiensis*	*B. anthracis*	*B. subtilis*	*B. licheniformis*	*B. pumilus*
Cell diameter >1.0 μm	+	+	+	+	-	-	-
Anaerobic glucose fermentation	+	+	+	+	-	+	
Nitrate reduction	+	+	+	+	+	+	-
Voges-Proskauer	+	+	d	+	+	+	+
Tyrosine decomposition	+	d	d	d	-	-	-
Lysozyme sensitivity	+	+	+	+	d	d	d
Mannitol fermentation	-	-	-	-	+	+	+
Egg yolk reaction	+	d	d	+	-	-	-
Citrate utilization	+	d	+	d	+	+	+
Motility	+	-	+	-			
Rhizoid growth	-	+	-				
Haemolysis	+	+	+	-			
Toxin crystals	-	-	+	-			
Polymyxin resistance	+	+	+		+	+	+
Penicillin resistance	+	+	+	-	-	-	-

[1)]Modified from Jenson (13), and Jenson and Moir (14); +, 90% or more of strains are positive; -, 90% or more strains are negative; d, 11-89% of strains are positive.

of environmental sources of contamination for management of food safety and quality. The most common genotypic typing methods used in food microbiology include chromosomal DNA restriction analysis, plasmid typing, ribotyping, pulsed-field gel electrophoresis (PFGE), and polymerase chain reaction (PCR)-based methods such as randomly amplified polymorphic DNA (RAPD) analysis. Many of these molecular typing methods have been applied to isolates of *Bacillus* species, and those most commonly used are examined in this section.

Restriction fragment length polymorphism (RFLP) and pulsed-field gel electrophoresis (PFGE) RFLP and PFGE are the typing techniques that involve visualising fingerprints of the complete genome. Since restriction endonuclease enzymes are very specific, complete digestion of a given DNA generates a reproducible pattern of DNA fragments and variations in the pattern of fragments is called RFLP (60). PFGE, which is similar in principle to RFLP, was developed for the separation of high molecular weight DNA fragments generated using rare-cutting restriction enzymes (63). These methods have been applied to the differentiation of species of bacilli such as *B. sphaericus* (64), and closely related *Bacillus* species such as those within the *B. cereus* group (65,66) and to assess genetic variability of bacilli isolated from wheat fields (67), and *B. anthracis* and related species (68). More practically, these methods have also been used for epidemiological purposes, such as the investigation of a pseudo-outbreak of *B. cereus* in a pediatric unit (69), or typing of *Bacillus* species isolated from food (70).

Ribotyping Ribotyping involves restriction enzyme digestion of chromosomal DNA, electrophoretic separation of fragments, transfer of those fragments from a gel onto a nitrocellulose or nylon membrane (71) and hybridization with labelled rRNA genes or a rDNA probe (72,73). Ribotyping can be considered as an interesting approach to taxonomic and epidemiologic studies of *Bacillus* species because *Bacillus sensu lato* members contain 9-12 rRNA operons (63). This method has been applied to the investigation of strains of *B. licheniformis* from food poisoning (74), and discrimination of *B. weihenstephanensis* from raw cow's milk (75) and aerobic mesophilic bacilli isolated from board and paper products (76). It has also been used to trace different pathotypes of *B. thuringiensis* (77) and analyse 2 closely related *Bacillus* species, *B. cerues* and *B. thuringiensis* (78). Several studies (79-81) have compared or used ribotyping with other DNA fingerprinting methods, and one study (79) revealed that RAPD-PCR is slightly more discriminatory than automated ribotyping for *B. cereus* isolates.

Randomly amplified polymorphic DNA (RAPD) RAPD is a very simple and quick method in which arbitrary oligonucleotides are used as primers in PCR. Even though there are many advantages to RAPD typing in terms of discriminatory power, ease of interpretation and performance (82,83), there is major concern with the reproducibility of this method. The method has been commercialised by Amersham-Pharmacia-Biotech (Sweden). The RAPD PCR method has been applied to the discrimination and characterisation of *B. sphaericus* (84), *B. thuringiensis* (85,86) and thermophilic members of the *Bacillus* genus (87-89). It was even applied to discriminate *B. anthracis* from closely related *Bacillus* species such as *B. cereus*, *B. thuringiensis*, and *B. mycoides* (90) and identify rope spoilage strains of *B. subtilis* and *B. licheniformis* (91).

Repetitive extragenic palindromic (Rep)-PCR REP-PCR is based on the observation that repetitive elements are randomly distributed in genomes of bacteria and relies on a primer derived from such repetitive sequences identified in many bacteria (92). Rep-PCR has been developed for enterobacterial repetitive intergenic consensus (ERIC) and REP sequences (53). The use of consensus primers may reduce stringency of PCR and this may negatively affect reproducibility of the method (63). This method has been

used to examine genetic diversity in *B. cereus* (93), *B. megaterium* (94), *B. sphaericus* (95), and bacilli including *B. cereus*, *B. licheniformis*, *B. subtilis*, and *B. pumilus* (96). It has been applied to the differentiation of *B. sporothermodurans* from other *Bacillus* species in milk (97).

Bacillus Species Associated with Food Poisoning

Most *Bacillus* food poisoning incidents are attributed to *B. cereus*. This bacterium is known to cause 2 different types of food poisoning, the diarrhoeal and the emetic syndromes. The first and better understood is the diarrhoeal syndrome, which results in diarrhoea and abdominal pain 8-16 hr after ingestion of contaminated foods, while the other is the emetic syndrome, which is characterised by nausea and vomiting within 1-5 hr of ingestion (98). The infective dose of diarrhoeal *B. cereus* in food poisoning ranges from 10^4 to 10^{11} CFU/g (99), while the level of *B. cereus* in emetic food poisoning has ranged from 10^3 to 10^{10} CFU/g (100). Although most illnesses caused by *B. cereus* are considered self-limiting, fatality as a result of emetic-type *B. cereus* food poisoning (101) and severe forms of the diarrhoeal syndrome have been reported, including a necrotic enteritis causing 3 deaths (102). The risk of *B. cereus* food poisoning was also highlighted in Australia in 2002, when 272 people were hospitalised due to emetic illness caused by *B. cereus* (103). The outbreak was associated with a meal consisting of rice, lamb, and potatoes and *B. cereus* was isolated from the rice sample.

Among the non-*B. cereus* related reports, *B. subtilis* is most frequently associated with food poisoning. Several episodes of food poisoning by *B. subtilis* in the UK (100), Japan (104), Canada (105), Australia and New Zealand (106) have been described. Generally, numbers greater than 10^5 CFU/g of *B. subtilis* are required to cause illness (100,107). Foods most commonly associated with the bacterium include meat dishes, pastry products, and meat or seafood with rice dishes. However, some incidences involved some of the more unusual implicated foods including infant cereals, and dried formulas (108), custard powder, mayonnaise and synthetic fruit drink (106). In most cases, the predominant symptom of *B. subtilis* food poisoning is vomiting and the illness has a short onset incubation period (14), similar to that of *B. cereus* emetic syndrome. Recent studies (109,110) revealed *B. subtilis* is capable of growing within the gastrointestinal (GI) tracts of animals. Therefore, foods contaminated with low levels of *B. subtilis* may not be safe for consumption.

Food poisoning due to *B. licheniformis* is most often associated with cooked meats and vegetables. The incubation period is about 2-14 hr and the predominant symptom is diarrhoea, with vomiting in about half the cases (100,107, 111). These characteristics are similar to those of *B. cereus* diarrhoeal syndrome. However, according to Salkinoja-Salonen *et al.* (74), a partially-purified toxin of *B. licheniformis*, isolated from foods (raw milk and industrially produced baby food) involved in a fatal case of food poisoning, showed physicochemical properties similar to the emetic toxin of *B. cereus* which was identified as lichenysin A recently (112).

Although some *B. pumilus* isolates were toxic in mice (113) and mammalian cells (114), only a few cases of food poisoning associated with *B. pumilus* have been reported (39,100,107). Incidences involved vomiting followed by abdominal pain and in some cases diarrhoea shortly after ingestion of food contaminated with large numbers of *B. pumilus* (10^5-10^7 CFU/g). Implicated foods include meat dishes, scotch eggs, cheese sandwiches, and canned tomato juice (100,107). However, in a recent report of food poisoning related to *B. pumilus*, the suspected source of outbreak was cooked and reheated rice (39). This incident, as well as a *B. cereus* emetic food poisoning in Australia, highlights the importance of proper food preparation, in particular, maintaining food at proper temperature. Storage at a growth-permissive temperature had allowed for growth and emetic toxin production. Upon reheating, while the bacterium may have been eliminated, the toxin remained active. In food service settings it is common to cook large quantities of rice which may then be left at ambient temperature prior to reheating and service (14).

Recently, McIntyre *et al.* (115) demonstrated *B. cereus* isolated from 39 outbreaks were characterized and reclassified as *B. cereus*, *B. thuringiensis, B. mycoides*, and mixed strains of *Bacillus*. As the *B. cereus* group is very closely related, some laboratories may fail to differentiate all species using routine methods. Thus, the incidence of food poisoning from *B. thuringiensis* and *B. mycoides* may be under reported. *B. thuringiensis*, which is used as a bioinsecticide, can reportedly produce enterotoxins (116-118) and cause food poisoning (119). Thus, extensive use of this organism as an insecticide could potentially cause serious problems in the food industry, as raw materials may carry a high load of potentially toxigenic organisms. It needs to be ensured that *B. thuringiensis* in use as a bioinsecticide should not carry enterotoxin genes and in turn, is unable to produce food-poisoning toxins. The Health and Consumer Protection Directorate-General of the European Commission has already accepted that only non-toxin producing *Bacillus* species should be allowed to be utilised in animal nutrition (120) and such regulatory concern should be expressed in other countries not yet aware of the issue. Reliable tests are needed to enable the toxigenicity of strains to be determined.

Toxins of *Bacillus* Species

***B. cereus* diarrhoeal toxin** Although the number of enterotoxins and their properties have been the subject of debate for some time (121,122) and some of them are still considered as only candidate enterotoxins (106), 5 different enterotoxins have been identified and characterised to date. There are 2 distinct enterotoxin complexes: 3-component (B, L_1, and L_2) haemolysin (Hbl) and non-haemolysin 3-component (*nhe*A, *nhe*B, and *nhe*C) enterotoxin (Nhe). In addition, there are 3 single enterotoxic proteins: enterotoxin T (BceT), enterotoxin FM, and cytotoxin K (CytK). These enterotoxins are heat-labile, being inactivated by heating to 56ºC for 5-30 min, stable between pH values of 4 and 11 (123), and sensitive to proteolytic enzymes (14). The mode of action of the toxin(s) has been suggested as reversing the absorption of fluid, Na^+ and Cl^-, in the large intestine, and causing malabsorption of glucose and amino acids, necrosis, and mucosal damage (124).

Traditionally, detection of enterotoxin has relied upon *in vivo* testing procedures such as mouse lethality, rabbit ileal loop, and the vascular permeability reaction in rabbit skin (125-127). There has also been the use of tissue culture systems such as the McCoy, HeLa, CHO, Vero, and HEL cell assay, whereby the enterotoxin produces a characteristic progressive destruction of cell monolayers (126,128,129). More recently detection of these toxins has been based on immunological techniques and 2 kits are available commercially. The diarrhoeal type *B. cereus* enterotoxin test kit (RPLA kit; Oxoid) is specific to the HblC (L_2) component while the *Bacillus* diarrhoeal enterotoxin visual immunoassay (BDE kit; Tecra) detects mainly the NheA (45-kDa) protein (99). Molecular techniques for detecting potentially enterotoxigenic organisms are also available as the genes encoding enterotoxin T, cytotoxin K, and the subunits of the complexes of haemolysin (Hbl) and the non-haemolytic enterotoxin (Nhe) have been identified.

***B. cereus* emetic toxin** The *B. cereus* emetic toxin was identified as a cyclic dodecadepsipeptide and named cereulide by Agata *et al.* (130). Generally, cereulide is more resistant to adverse conditions than the enterotoxins. Cereulide is an extremely stable molecule, able to withstand heating at 121ºC for 15-90 min (100), treatment with acid (pH 2 with HCl for 30 min), alkali (pH 12 with NaOH for 30 min), and the action of proteinase (131).

Three bioassays have been developed for detecting cereulide. The HEp-2 vacuolation (132) and boar sperm motility inhibition (133) assays rely on microscopic examination to assess the proliferation of HEp-2 cells (a human laryngeal-carcinoma cell line) and loss of motility of boar sperm through blockage of oxidative phosphorylation in mitochondria by cereulide. The 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyl tetrazolium bromide (MTT) assay (134), currently the most widely used bioassay, uses tetrazolium salt, MTT as a material for assessing the toxicity of cereulide in cells. Molecular techniques for detecting potentially emetic toxin-producing organisms are also available now because 2 recent studies (135,136) reported. They hypothesized that the emetic toxin, cereulide is synthesised enzymatically by a non-ribosomal peptide synthetase (NRPS) and a PCR based screening assay using degenerate primers was targeted to known NRPS sequences. Recently, it was shown that cereulide production is plasmid-mediated (137).

Toxins of other *Bacillus* species The toxins produced by *Bacillus* species other than *B. cereus* have chemical and biological properties similar to those of *B. cereus* toxins. A few of the toxic materials produced by non-*B. cereus* species have been purified and identified, but, until recently, there was little information regarding the association between these toxins and outbreaks of foodborne illness. This was due, at least in part, to the lack of reliable detection methods for these toxins. The techniques presently used to detect toxins produced by other *Bacillus* species are the same as those used to detect *B. cereus* toxins. These include immunological assays, the boar spermatozoa motility test, and cell cytotoxicity assays.

The non-*B. cereus* including *B. mycoides*, *B. thuringiensis*, *B. subtilis*, *B. lentus*, *B. circulans*, *B. lichenifomis,* and *B. pumilus* shown to produce molecules detected by immunological assays (BDE and RPLA) for the diarrhoeal toxin of *B. cereus* (138,139). *Bacillus* isolates, including *B. licheniformis, B. subtilis, B. circulans, B. thuringiensis,* and *B. pumilus*, showed cytoxicity in Chinese hamster ovary (CHO) cells, Hep-2 and Caco-2 epithelial cells, suggesting these species produce enterotoxins (138,140). However, the involved toxins were not purified from these isolates and thus may not be identical to the enterotoxins of *B. cereus*; although the BDE and RPLA kits used are known to detect Hbl and Nhe enterotoxins of *B. cereus*.

Recently, it was reported that 8 strains, assigned to the 4 different species *B. subtilis*, *B. mojavensis*, *B. pumilus*, and *B. fusiformis*, produced cytotoxic or putative emetic toxin when they were analysed by a vero cell cytotoxicity assay and the boar spermatozoa motility assay (141). Taylor *et al.* (142) also demonstrated that strains of *B. cereus*, *B. firmus*, *B. megaterium*, *B. simplex,* and *B. licheniformis* produced heat-stable toxin. These studies indicate potentially toxigenic *Bacillus* species, particularly in the *B. subtilis* group, produce a heat-stable toxin that is very similar to the *B. cereus* emetic toxin, cereulide. Recently, responsible virulence factors related to the *B. subtilis* group were shown to be surfactin and several peptidic variants of surfactin. Surfactin is a bacterial cyclic lipopeptide produced by some *B. subtilis* isolates, and functions as a potent biosurfactant (143). It is structurally characterized by the presence of a heptapeptide with a β-hydroxy fatty acid tail. Peptidic variants of surfactin, named lichenysin and pumilacidin, were isolated from *B. licheniformis* (112) and *B. pumilus* (39), respectively. Both of these surfactin variants have been linked to food poisoning incidents. These surfactin analogues were resistant to heat, pH alterations, and proteolytic enzymes and proved toxic in boar sperm cells through pore formation and destruction of the cell with subsequent loss of mitochondrial activity (114,144). *B. licheniformis* strains are also known to produce peptides called amoebicins that have exhibited cytotoxicity in murine cells (145,146). Recently, *B. mojavensis* was also shown to produce heat stable toxic components identical in molecular mass and amino acid composition to surfactin (147). Boar sperm-toxic strains of *B. amyloliquefaciens* were isolated from a moisture-damaged building, and the toxins were identified as surfactin and amylopsin, a novel potassium channel-forming peptide (148,149).

PCR-based methods to detect toxin-producing *B. cereus* genes have provided information about other *Bacillus* species, as well. *B subtilis* group members were not thought to possess genes coding for putative enterotoxins (150), but a recent study showed the possibility of enteropathogenicity in this group. Ouoba *et al.* (151) detected a weak presence of Hbl and CytK genes in isolates of *B. subtilis* and *B. licheniformis*, though results were inconsistent, especially for Hbl genes. The psychrotolerant *B. cereus* group member *B. weihenstephanensis* is considered to be potentially toxin producing; 2 *B. weihenstephanensis* strains have been found to produce cereulide (152). Cereulide production was previously thought to be restricted to *B. cereus*. Furthermore, 2 new diarrheic toxin operons were recently detected in this strain, although further phylogenetic and biochemical characterization is required (153). Kim and Batt (78) reported that 5 *B.*

thuringiensis strains examined by PCR were found to harbor 7 virulence-associated genes: cerAB, pi-plc, entFM, bceT, hblA, hblC, and hblD.

Limitations of current toxin detection methods and the future To date, several bioassays have been developed for detection of *B. cereus* toxins, but these currently practiced assays have several limitations. First, all bioassays are laborious and time consuming. Second, these assays depend on the use of cell cultures that are not convenient to use and they bring with them the problem of maintenance due to dealing with living cells. Most importantly, the methods are based on indirect biological activity of the toxin, and generally do not allow for providing accurate or precise analysis that will generate quantitative data.

Therefore, it is necessary to develop the appropriate detection methods that will generate quantitative data for analysis of *Bacillus* toxins and examine the relationship between cell growth and toxin production in food. While toxicologically relevant substances may be detected by screening procedures, such as immunoassays or receptor assays (at present, Tecra International is undertaking development of an immunoassay for detection of cereulide), there is a need to exploit analytical methods that generate real quantitative data such as chromatographic techniques. Recently, the quantitative analysis of cereulide, using high-performance liquid chromatography-ion trap mass spectrometry (HPLC-MS) was reported (154).

Molecular methods for detecting the toxin-producing genes of *B. cereus* are extremely useful in the specific isolation (such as detecting toxigenic strains of *Bacillus*) and characterization of microorganisms. Nevertheless, the results of PCR may not represent the real health threat because even though the gene is present, the toxin may not be produced or not at the level required for human intoxication. Furthermore, the existing primers may not be valuable for species other than *B. cereus*. Therefore, in order to prevent toxin-mediated food poisoning, quantitative determination of a population of cells and/or the toxin present is important. In addition, research to develop molecular methods for detecting other toxigenic *Bacillus* species is needed.

Conclusion

Since *Bacillus* species are widespread in nature, there are few foodstuffs that are free of these species. *B. cereus* is the most prevalent pathogenic *Bacillus* species found in food and other industrial processes. Strains of this bacterium cause food spoilage during production, storage, and distribution, and food poisoning by gastrointestinal intoxication. Several studies have suggested that diarrheal enterotoxins produced by non-*B. cereus Bacillus* species, including *B. thuringiensis*, *B. subtilis,* and *B. lichenifomis*, are similar to well-characterized *B. cereus* toxins. More recent studies have reported that members in the *B. subtilis* group also produced a putative emetic toxin that is extremely heat-stable. The toxic components were identified as cyclic lipopeptides, including surfactin, lichenysin, and pumilacidin. Many *Bacillus* species are important because of their ability to produce commercially important biomolecules such as pesticides, antibiotics, and enzymes and for their fermentation of food products. However, because they can potentially produce toxic substances, there should be careful monitoring of their environmental occurrence, particularly in foods. Therefore, intensive research is required to develop sensitive and specific methods to detect toxins and toxin-producing genes in all potentially toxigenic *Bacillus* species.

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