Journal of Food Protection, Vol. 68, No. 3, 2005, Pages 636–648

Review

Bacillus cereus Food Poisoning and Its Toxins

JEAN L. SCHOENI† AND AMY C. LEE WONG*

Department of Food Microbiology and Toxicology, Food Research Institute, University of Wisconsin, 1925 Willow Drive, Madison, Wisconsin 53706, USA

MS 04-376: Received 26 August 2004/Accepted 11 November 2004

ABSTRACT

The genus *Bacillus* includes members that demonstrate a wide range of diversity from physiology and ecological niche to DNA sequence and gene regulation. The species of most interest tend to be known for their pathogenicity and are closely linked genetically. *Bacillus anthracis* causes anthrax, and *Bacillus thuringiensis* is widely used for its insecticidal properties but has also been associated with foodborne disease. *Bacillus cereus* causes two types of food poisoning, the emetic and diarrheal syndromes, and a variety of local and systemic infections. Although in this review we provide information on the genus and a variety of species, the primary focus is on the *B. cereus* strains and toxins that are involved in foodborne illness. *B. cereus* produces a large number of potential virulence factors, but for the majority of these factors their roles in specific infections have not been established. To date, only cereulide and the tripartite hemolysin BL have been identified specifically as emetic and diarrheal toxins, respectively. Nonhemolytic enterotoxin, a homolog of hemolysin BL, also has been associated with the diarrheal syndrome. Recent findings regarding these and other putative enterotoxins are discussed.

The genus *Bacillus* includes gram-positive and gram-variable rod-shaped bacteria that sporulate under aerobic conditions. The vegetative cells are 0.5 by 1.2 to 2.5 by 10 μm and occur singly or in chains. Most are motile via peritrichous flagella and produce catalase *(40, 46, 82, 94, 149, 162).* Aerobic sporulation and production of catalase distinguish members of this genus from those of *Clostridium. Bacillus* possesses aerobic or facultatively anaerobic modes of energy metabolism *(40, 46, 82, 149, 162).* Members of the genus survive in a wide variety of environmental conditions because of the ability to form endospores, which are resistant to heat, dehydration, and other physical stresses *(40, 149).* Optimal growth temperatures range from 25 to 37°C, although some psychrotrophic strains can grow at temperatures as low as 3°C and some thermophilic strains can grow at 75°C. Species that grow at extremes of acidity and alkalinity, ranging from pH 2 to pH 10, have been identified *(46).* As a result of this physiological diversity, *Bacillus* is found in a wide variety of habitats, from soil to thermal springs *(162).*

Several *Bacillus* species are known for their pathogenicity. *Bacillus anthracis* causes anthrax in mammals. *Bacillus thuringiensis* is widely used for its insecticidal properties; however, it has also been associated with foodborne disease *(86)* and infections in humans *(42, 78). Bacillus cereus* causes two types of food poisoning (the emetic and diarrheal syndromes) and a variety of local and systemic infections such as endophthalmitis, endocarditis, meningitis, periodontitis, osteomyelitis, wound infections, and septicemia *(46).* The pathogenesis of *B. cereus* is still largely undefined. The organism produces a large number of potential virulence factors, including multiple hemolysins, phospholipases, and proteases *(14, 46).* However, the roles of these factors in specific infections have not been established. The emetic toxin has been identified as cereulide *(3, 4)* and the tripartite hemolysin BL (HBL) has been established as a diarrheal enterotoxin *(19).* A homolog of HBL, nonhemolytic enterotoxin (Nhe), also has been associated with the diarrheal syndrome *(61, 101).* Many other enterotoxin candidates have been reported; however, their biological activity and involvement in *B. cereus* food poisoning have not been defined. In this review, we provide a historical perspective and evaluate and summarize recent findings regarding the toxins involved in *B. cereus*–associated foodborne illness.

TAXONOMY

The guanine plus cytosine content in chromosomal DNA from *Bacillus* species was reported to range between 32 and 69% *(125, 127),* indicating substantial genetic diversity. The genus includes 51 recognized species *(162)* and is divided into three groups based on the morphology of the spore and sporangium *(58, 59, 148, 162).* The species of group 1 have ellipsoidal or cylindrical spores that are centrally or terminally located and do not distend the sporangium. Group 1 is further subdivided into subgroups 1A and 1B based on cell size and presence of poly-beta-hydroxybutyrate globules in the protoplasm. The *B. cereus* group, consisting of *B. cereus, Bacillus mycoides, B. thuringiensis,* and *B. anthracis,* belongs to subgroup 1A, mem-

* Author for correspondence. Tel: 608-263-1168; Fax: 608-263-1114; E-mail: acwong@wisc.edu.
† Present address: TRAC Microbiology, Inc., 124 Owen Road, Madison, WI 53716, USA.

bers of which have bacillary body widths of ≥1 μm and protoplasmic globules. Recently, two additional species, *Bacillus pseudomycoides (114)* and psychrotolerant *Bacillus weihenstephanensis (96)* were described and proposed for inclusion in the *B. cereus* group.

Results from DNA-DNA hybridization studies revealed a high degree of similarity among *B. cereus*, *B. thuringiensis*, *B. anthracis*, and *B. mycoides (89, 137)*, and their 16S and 23S rRNA sequences are almost identical *(9, 10)*. Some workers have suggested that *B. anthracis*, *B. cereus*, and *B. thuringiensis* should be considered one species based on the high degree of relatedness indicated by multilocus enzyme electrophoresis and sequence analysis of genes *(75, 76)*. Comparison of the recently published genome sequences of *B. anthracis* Ames *(130)*, *B. cereus* ATCC 14579 *(85)*, and *B. cereus* ATCC 10987 *(129)* revealed that *B. anthracis* Ames and *B. cereus* ATCC 10987 are 93.94% identical, and *B. cereus* ATCC 14579 and *B. cereus* ATCC 10987 are 90.94% identical. Antigenic cross-reactions are also common among these species. Cross-agglutination between the flagellar antigens of *B. cereus* and *B. thuringiensis* and between spore antigens of *B. thuringiensis*, *B. anthracis*, and *B. cereus* has been observed *(46, 95)*.

Use of pulsed-field gel electrophoresis, multilocus enzyme electrophoresis, and multilocus sequence typing *(32, 74–76)* to compare multichromosomal factors revealed a high level of diversity among members of the *B. cereus* group, but isolates assigned to the same species using currently accepted criteria could not be differentiated with these methods. Efforts to differentiate species in this group by genome mapping were also unsuccessful. Although considerable variation was observed between isolates, the genomic maps of *B. thuringiensis* subsp. *canadiensis* HD224 and *B. cereus* ATCC 14579 did not suggest that they were different species *(34)*. Two recent studies using fluorescent amplified fragment length polymorphism (AFLP) analysis *(79, 158)* demonstrated a high degree of diversity among isolates of *B. cereus* and *B. thuringiensis*, with different isolates interspersed over all branches of the AFLP-based phylogenetic tree. In contrast, *B. anthracis* was very monomorphic.

The differentiation of *B. anthracis* is important because of its clinical significance. *B. anthracis* is the causative agent of anthrax, which is potentially fatal to animals and humans. Hemolysis, motility, penicillin resistance, tyrosine degradation, and phosphatase production have been used traditionally to distinguish *B. cereus* and *B. thuringiensis* from *B. anthracis*; *B. cereus* and *B. thuringiensis* are positive and *B. anthracis* is generally negative for all five characteristics *(40, 94, 162)*. However, Slamti et al. *(147)* reported recently that distinct mutations in the pleiotropic regulator PlcR caused some strains of *B. cereus* and *B. thuringiensis* to be nonhemolytic, and Klichko et al. *(90)* found that *B. anthracis* was hemolytic when grown anaerobically. Mignot et al. *(110)* observed that *B. anthracis* was not hemolytic against sheep erythrocytes but was weakly hemolytic against human erythrocytes. Key virulence genes, including those that encode for the anthrax toxin and capsule, are harbored by two virulence plasmids, pXO1 and pXO2, respectively, that are absent in *B. cereus* and *B. thuringiensis (46, 82, 123, 126)*. However, a recent report indicated that anthrax toxin genes were present in a *B. cereus* isolate from a patient with life-threatening pneumonia resembling inhalation anthrax *(80)*.

The presence of diamond-shaped parasporal crystals is critical in the differentiation of *B. thuringiensis* from *B. cereus*. *B. thuringiensis* produces a wide variety of crystal proteins known as delta endotoxins. These highly specific insecticidal toxins affect certain members of *Coleoptera* (beetles), *Diptera* (mosquitoes), and *Lepidoptera* (butterfly and moth larvae) *(81, 126)*. The delta endotoxins are plasmid encoded. Thus, mutants that have lost the plasmid cannot be differentiated easily from *B. cereus*.

ISOLATION AND IDENTIFICATION OF *B. CEREUS*

Methods for isolation and identification of *B. cereus* in foods and from clinical samples obtained from food poisoning cases have been described in detail by Bennett and Belay *(25)* and Kramer and Gilbert *(94)*. Two differential media in common use are mannitol–egg yolk–polymyxin agar and the same agar with pyruvate and bromothymol blue added. These formulations take advantage of the fact that unlike many *Bacillus* species, *B. cereus* does not ferment mannitol but does produce phosphatidylcholine-preferring phospholipase C (lecithinase). Polymyxin B is used as the primary selective agent for the *B. cereus* group.

Classical identification schemes for members of the *Bacillus* genus have been described extensively *(40, 115, 123, 162)*. Generally, *B. cereus* colonies on solid media are 5 to 6 mm in diameter and have a ground glass or matte appearance, with edges that range from circular and entire to irregular and fimbriate. Colonies often appear greenish on blood agar. The spores are ellipsoidal or cylindrical and do not swell the sporangia.

Confirmation of *B. cereus* involves a battery of biochemical tests. These tests can be performed using conventional methods *(25, 40, 71, 123, 162)* or using miniaturized commercial systems that combine biochemical profiles with information in large databases. Serotyping has been a useful tool in epidemiological studies. Forty-two serotypes have been identified based on serological classification of spore, somatic, and flagellar antigens. Twenty-three of these 42 serotypes are associated with *B. cereus*–related disease *(46)*. The flagellar (H) serotypes most commonly associated with diarrheal food poisoning are 1, 2, 6, 8, 10, 12, and 19. The H serotypes 1, 5, 8, 12, and 19 are commonly associated with the emetic food poisoning syndrome. Some *B. cereus* strains may cause both forms of food poisoning *(60, 94)*.

Phage typing as a means of *B. cereus* identification was explored because of the specificity of *Bacillus*-associated bacteriophages for their host strain *(154, 165)*. In a study by Väisänen et al. *(165)*, 21 different lysotypes were identified among the dairy isolates studied. Only 10% of the isolates could not be typed.

NONGASTROINTESTINAL ILLNESS

B. cereus has been associated with illness other than food poisoning, although these infections are not common.

The bacterium has been found contaminating postsurgical or traumatic wounds and burns and causes a variety of opportunistic infections, especially in immunocompromised patients, including bacteremia, septicemia, endocarditis, meningitis, pneumonia, pleuritis, osteomyelitis, and endophthalmitis *(46)*. These infections can be highly fulminant and sometimes fatal. *B. cereus* can also play a role in sepsis caused by use of contaminated needles by intravenous drug abusers or by following penetrating wounds *(14, 46, 160)*. In both instances, the outcome is usually poor.

Involvement of *B. cereus* in pneumonia is rare and is usually associated with other risk factors such as leukemia. However, two unusual cases were reported that involved two previously healthy middle-aged individuals who experienced fulminant bacteremia and pneumonia caused by *B. cereus*, with symptoms similar to those of *B. anthracis* pulmonary infection *(113)*. Both individuals experienced chills for three to five days, fever, cough, and bloody expectoration before hospitalization. Their conditions deteriorated in the hospital, and both patients died.

B. cereus is a common cause of eye infections, often causing irreversible tissue damage in a short time (within 24 h). It is one of the most common causes of posttraumatic endophthalmitis, where the organism is introduced into the eye by foreign bodies as a consequence of traumatic injury *(29, 43, 77, 164)*. It also causes metastatic endophthalmitis from hematogenous spread of the organism to the eye from other sites. Metastatic endophthalmitis is often highly fulminant and sometimes fatal. The rapid progression and fulminance of *B. cereus* endophthalmitis is attributed to multiple toxins elaborated by the organism, including the diarrheal enterotoxin HBL *(17, 18, 43)*.

FOODBORNE ILLNESS

***B. cereus* in foods.** *B. cereus* is a primary inhabitant of the rhizosphere and often is the most frequently isolated soil bacterium *(115, 148)*. Because of its abundance and the resistance of its spores, *B. cereus* contaminates nearly all agricultural products and plays a major role in the contamination and spoilage of food products *(94)*. It can be commonly isolated from a variety of food products, including spaghetti, other pasta, rice, dairy and dried milk products, spices, dried foodstuffs, meat, chicken, vegetables, fruits, grains, and seafood *(62, 88, 120, 132, 155, 165)*. *B. cereus* is a common spoilage organism in pasteurized milk and milk products *(107)*. The optimum heat-activation temperature for spore germination is 65 to 75°C. This range is similar to the temperature used in high-temperature short-time pasteurization of milk. Some *B. cereus* strains are psychrotrophic *(52, 107, 166)*. The combination of abundance, heat resistance, and psychrotrophy makes it difficult to control this organism in the food processing environment *(92)*. *B. cereus* can form biofilms on stainless steel *(5, 98, 124)* that are more resistant to sanitizers than are planktonic and attached single cells *(124)*. Significant increases in heat resistance were also observed in *B. cereus* spores attached to stainless steel surfaces *(144)*.

Epidemiology. The percentage of food poisoning outbreaks associated with *B. cereus* varies from country to country and is dependent on the reporting system. In The Netherlands, from 1991 to 1994 *B. cereus* was identified as the most common cause (19%) of food poisoning outbreaks *(145)*. In Taiwan, from 1986 to 1995 *B. cereus* outbreaks ranked third, behind those caused by *Vibrio parahaemolyticus* and *Staphylococcus aureus (121)*. Between 1973 and 1985, *B. cereus* caused 17.8% of the total bacterial food poisoning outbreaks in Finland, 11.5% in The Netherlands, 2.2% in Canada, 0.8% in Scotland, 0.7% in England and Wales, and 0.7% in Japan *(92)*. Mead et al. *(106)* estimated that more than 27,000 foodborne illnesses annually in the United States are caused by *B. cereus*, which from 1993 to 1997 ranked seventh among the etiological agents causing reported bacterial foodborne outbreaks (14 of 655; 0.5%) and sixth as the causative agent for reported cases (691 of 43,821; 0.8%) *(118)*.

Emetic food poisoning. The emetic syndrome was first identified in the United Kingdom in the early 1970s and was associated with the consumption of cooked (usually fried) rice from Chinese restaurants. This syndrome is characterized by nausea, vomiting, and abdominal cramping, which occur 1 to 5 h after ingestion of the contaminated food *(161)*. The symptoms are similar to those of *S. aureus* food poisoning, and the rapid onset indicates presence of preformed toxin. The illness is self-limiting, and recovery usually occurs within 6 to 24 h. On occasion, hospitalization is required because of excessive vomiting. Fatality is rare.

Kramer and Gilbert *(94)* evaluated the distribution of serotypes from 200 emetic outbreaks that occurred in 11 countries. The serotypes most frequently isolated were H1 (63.5%) and H8 (8%). H1 spores are more heat resistant than the other spore types and may more frequently survive cooking *(122, 161)*.

Foods implicated in emetic food poisonings include beef, poultry, vanilla sauce, pasteurized cream, milk pudding, pasta, and infant formulas *(54, 94)*. However, the majority of cases have been associated with cooked rice dishes. Various studies reported that 10 to 100% of raw and cooked rice samples were contaminated with *B. cereus (94)*. Contaminating organisms can multiply to large numbers after the rice is boiled, cooled, and held at room temperature. Temperatures achieved during reheating are usually not sufficient to inactivate the heat-stable emetic toxin *(49)*. Improper holding temperature of cooked foods is the most common factor associated with the emetic syndrome. An example of an emetic outbreak occurred in 1993 at two Virginia day care centers owned by the same provider. Rice was cooked and then cooled at room temperature before being refrigerated. The next morning, the rice was pan-fried with cooked chicken pieces, delivered to the day care centers, held without refrigeration, and served without reheating. *B. cereus* was isolated from leftover chicken fried rice and from the vomitus of one child *(7)*.

Emetic toxin. Melling et al. *(109)* first demonstrated that a toxin was associated with the emetic syndrome based

on rhesus monkey feeding studies using *B. cereus* strains isolated from emetic outbreaks. Further studies allowed Melling and Capel *(108)* to characterize the toxin as extracellular, heat stable, smaller than 10,000 daltons, and poorly antigenic.

The expense and impracticality of the monkey-feeding assay hampered research on the emetic toxin until Hughes et al. *(83)* noted that culture filtrates from emetic food poisoning samples produced vacuoles in HEp-2 cells. Using this detection assay, Agata et al. *(4)* isolated and identified the emetic toxin as cereulide, which is a dodecadepsipeptide produced primarily by *B. cereus* serotype H1 *(3)*. The structure, $(\text{D-}O\text{-Leu-D-Ala-L-}O\text{-Val-L-Val})_3$, is similar to that of the potassium ionophore valinomycin, consisting of a 36-member ring with alternating ester and amide bonds *(2, 84)*. The peptide is 1,165 Da with a predicted pI of 5.52. Cereulide is hydrophobic and not easily solubilized in aqueous solutions and may be delivered to its target cells bound to or dissolved in carriers found in food. Therefore, the cereulide activity of a sample may be underestimated if particulates are removed by filtration or centrifugation *(153, 163)*.

Two animal models, rhesus monkey (*Macaca mulatta*) and Asian musk shrew (*Suncus murinus*), have been used for cereulide assays. In one trial with three rhesus monkeys, Shinagawa et al. *(140)* found that 9 to 12 μg/kg induced emesis in 2 to 4 h in all three animals. The 50% emetic dose in the musk shrew was estimated to be 12.9 μg/kg by oral administration and 9.8 μg/kg by intraperitoneal injection *(4)*. These assays have received limited use because they can be difficult and expensive and because cell culture assays have been developed and improved. The HEp-2 vacuolation assay introduced by Hughes et al. *(83)* with colorimetric modifications *(50, 111, 153)* is commonly used to test for the emetic toxin. In this assay, the mitochondrial swelling caused by cereulide appears as cytoplasmic vacuoles in HEp-2 cells *(134)*. Paralysis of boar spermatozoa *(6)* and changes in oxidation rates in isolated rat liver mitochondria *(103, 112, 134)* have also been used as indicators of cereulide-induced toxicity. Measurement of oxygen consumption in these assays indicates that cereulide acts by uncoupling mitochondrial oxidative phosphorylation. Häggblom et al. *(68)* described a quantitative chemical assay based on high-performance liquid chromatography and ion trap mass spectrometry for cereulide. The assay has been calibrated to the boar sperm bioassay and can be used to detect concentrations ranging from 0.02 to 230 μg/ml. Recently, a PCR fragment of unknown function was identified as specific for emetic toxin–producing *B. cereus* strains and was used to develop a PCR assay for rapid detection *(47)*.

Cereulide was involved in a fatal case of *B. cereus* food poisoning where the patient died of fulminant liver failure *(103)*. High concentrations of the emetic toxin were found in the small intestine, liver, bile, and plasma of the patient and in the pan used to reheat food. Diffuse microvascular steatosis and midzonal necrosis were observed in the patient's liver and were attributed to inhibition of mitochondrial fatty acid metabolism by the toxin. When synthetic cereulide was injected intraperitoneally into mice, similar histopathological changes in the liver were observed *(168)*. Paananen et al. *(119)* reported that cereulide inhibited the activity of human natural killer cells, caused mitochondrial swelling, and eventually induced apoptosis, suggesting that this toxin might also have immunomodulating properties.

Diarrheal food poisoning. Hauge *(72)* provided the first and most comprehensive description of *B. cereus* diarrheal syndrome in the 1950s after investigating four Norwegian hospital outbreaks involving about 600 patients. High numbers of *B. cereus* were recovered from the vanilla sauce that was incriminated as the vehicle in the outbreaks. Hauge found that the cornstarch used to prepare the sauce contained about 10^4 *B. cereus* spores per g. To establish the toxicity of the suspected organism and vehicle of transmission he inoculated sterile vanilla sauce with the isolate (approximately 10^4 cells per ml) and incubated it for 24 h at room temperature. Hauge consumed 200 ml of vanilla sauce that contained 9.2×10^7 cells per ml, and within 13 h he experienced abdominal pain, diarrhea, and rectal tenesmus that lasted 8 h.

The onset time of the diarrheal syndrome generally ranges from 8 to 16 h, and the symptoms resolve in 12 to 14 h. Occasionally, nausea and vomiting can be experienced in addition to the symptoms described by Hauge *(72)*. This symptom profile is similar to that of food poisoning caused by *Clostridium perfringens*. Outbreaks with unusual onset and duration have also been reported. In an outbreak caused by consumption of barbecued pork that had not been refrigerated for the previous 18 h, 34% of the ill persons noted an onset time shorter or longer than the normal range of 6 to 24 h and 23% noted fever *(99)*. In a separate outbreak, Andersson et al. *(5)* noted prolonged diarrhea. Three of the 17 people affected were hospitalized, and one person was hospitalized for 5 weeks.

Meats, fish, vegetables, soups, sauces, and dairy products have been associated with the diarrheal syndrome, and a large number of cases are attributed to proteinaceous dishes. *B. cereus* concentrations are usually $>10^5$ cells per g of implicated food, suggesting that a high dose is required to cause illness *(94)*. Reports range from 200 to 10^9 cells per g of food, with calculated infective doses ranging from 5×10^4 to 10^{11} cells *(63, 72, 94)*.

History of diarrheal enterotoxin detection. Animal models that have been used in the detection of *B. cereus* diarrheal enterotoxin include the ileal loop, vascular permeability, and monkey feeding assays. At present, the rabbit ileal loop assay is considered the best in vivo model for assaying enterotoxigenic activity *(26)*. Mice *(141)* and rats *(159)* have also been used. However, the size of the ileum in these animals limits the number of samples that can be evaluated per animal. For assaying activity of the *B. cereus* enterotoxin, Spira and Goepfert *(150)* found that the age and weight of the rabbit are important; the rabbits should be $\leq$8 weeks old and weigh $<$1 kg. The volume of fluid accumulated (V) is expressed as a ratio of the length of each loop (L). Samples causing a V/L ratio of $\geq$0.5 in at least 50% of the test animals are considered positive *(157)*.

Glatz et al. *(55)* examined the correlation of the results from the vascular permeability assay with those of the rabbit ileal loop assay. In the vascular permeability assay, culture filtrates or protein samples (50 μl) are injected intradermally into the shaved backs of rabbits. Visualization of leakage of serum proteins due to increase in vascular permeability is aided with injection of Evan's blue dye into the ear vein. Necrosis surrounding the sample injection site can also be observed. Compared with the rabbit ileal loop, the vascular permeability assay is easier to perform and requires less protein. Occasionally, the results can be difficult to interpret. Glatz et al. *(55)* noted that the bluing response was transient; thus, a standard interval for reading the reactions had to be determined.

In the monkey feeding assays, 1.5- to 3.0-kg rhesus monkeys are fed toxin preparations, culture supernatants, or contaminated food extracts. They are then observed for 6 h for signs of diarrhea. However, this assay was invalidated when Thompson et al. *(157)* found that the combination of disodium phosphate from the growth medium and sodium bicarbonate in the neutralizing buffer administered prior to the culture supernatant could cause diarrhea in these animals.

Cytotoxicity in cell culture assays has been used as an indication of the presence of *B. cereus* diarrheal toxin in crude samples such as food extracts or culture supernatants *(11, 30, 31, 38, 51, 66, 167).* However, the validity of these assays must be confirmed. Although the enterotoxin is considered cytotoxic, a variety of enzymes and toxins produced by *B. cereus* can act on cell membranes and therefore also produce a cytotoxic effect. As yet, no cell culture system specific for *B. cereus* diarrheal toxin has been established.

Two commercial kits to detect *B. cereus* enterotoxin were independently developed. Oxoid distributes a *B. cereus* enterotoxin reverse passive latex agglutination kit (BCET-RPLA kit, Denka Seiken Ltd., Tokyo, Japan). BCET-RPLA is a semiquantitative assay. Beecher and Wong *(20)* found that this kit allows detection of the L_2 component of the tripartite toxin HBL. The second kit, *Bacillus* Diarrhoeal Enterotoxin Visual Immunoassay (Tecra Bioenterprises, Pty., Ltd., Roseville, Australia), allows detection of toxin using a double-sandwich enzyme immunoassay. The antigen detected by this kit is a 45-kDa protein *(20)*, which was later identified as NheA from the Nhe complex *(65).* Both kits have been used extensively *(38, 44, 62, 116, 151, 152)*, and kit results have been compared with each other and with results of cytotoxicity tests in tissue culture *(30, 31, 132).* Some of the discrepancies that arose in these studies can be explained by the fact that each kit detects a different enterotoxin component. Because the Oxoid kit detects L_2 from HBL and the Tecra kit detects NheA from Nhe, the kits may be useful as indicators of which samples contain a specific toxin complex, but they cannot be used to confirm the presence of biologically active toxin because both complexes require all three components for biological activity.

Identification of the diarrheal enterotoxin. The identification of *B. cereus* diarrheal enterotoxin(s) has long been a controversial topic. The earliest study demonstrating the toxigenicity of *B. cereus* was published by Chu *(39)*, who found that cell-free filtrates injected intravenously killed mice. This effect was thought to be caused by a single protein, the lethal toxin, that also possessed hemolytic and lecithinase activity. This work was performed using crude culture filtrates, and the claim that one protein produced multiple biological activities was never substantiated.

Using gel filtration chromatography, Johnson and Bonventre *(87)* separated the lethal, phospholipolytic (lecithinase), and hemolytic activities. There was considerable overlap in the activity of the fractions, which led the authors to suggest that more than one protein was responsible for the three activities. Ezepchuk and Fluer *(48)* purified a nonhemolytic, nonphospholipolytic protein (55 to 65 kDa) that they claimed was the *B. cereus* lethal toxin. This assertion contradicted that of Bernheimer and Grushoff *(27)*, who reported that purified cereolysin, a hemolysin produced by *B. cereus*, was lethal to mice.

In a study by Turnbull et al. *(163)*, two chromatographic fractions individually exhibited lethal activity. One fraction caused mouse death, was hemolytic, and contained cereolysin. The other fraction was enterotoxigenic, as determined by rabbit ileal loop assays, but the protein(s) responsible for this activity was not determined. Bernheimer and Grushoff *(27)* found that the toxin was produced during the transition from vegetative growth to sporulation and that biological activities included mouse death, vascular permeability in rabbits, fluid accumulation in rabbit ileal loops, necrosis, and cytotoxicity *(161, 163).*

In the late 1970s and early 1980s, researchers began to elucidate the identity of the enterotoxin. An unstable protein (approximately 50 kDa) with a pI of 4.85 was isolated originally *(93).* The protein was inactivated in 30 min at 56°C, induced fluid accumulation in rabbit ileal loops, increased vascular permeability in rabbit skin, and was lethal to mice. Later, it was found that the enterotoxin dissociated into two moieties with pI values of 5.1 and 5.6, suggesting a bicomponent toxin.

Thompson et al. *(157)* reported that diarrheal activity was caused by a complex of two or three proteins. A combination of chromatographic procedures yielded two fractions eluted from a hydroxylapatite column that contained three proteins of approximately 43, 39.5, and 38 kDa with pI values of about 5.3. Individually, the fractions were not biologically active; however, when combined they had all activities associated with *B. cereus* enterotoxin, including fluid accumulation in rabbit ileal loops, vascular permeability, hemolysis, cytotoxicity, and mouse lethality. Bitsaev and Ezepchuk *(28)* supported the idea of the diarrheal toxin being composed of two or more proteins. They had isolated a tricomponent complex, diarrheagenic-lethal toxin. The complex consisted of proteins A, B, and C with molecular masses of 37.5, 42, and 36 kDa, respectively. The combination of A and B produced fluid accumulation in rabbit ileal loops and edema in mouse paws, and B and C together were lethal to mice. The B protein bound a cellular receptor, which then allowed the A or C component to bind and produce biological activity.

Granum and Nissen *(64)* reported the purification of three proteins (34, 40, and 48 kDa) that were reactive to enterotoxin-specific antibodies (provided by J. M. Kramer, Public Heath Laboratory Service, London, UK). The 34-kDa protein, whose N-terminal sequence was nearly identical to that of *B. cereus* sphingomyelinase, was hemolytic, and the 40-kDa was cytotoxic to Vero cells. However, the proteins were only partially purified, and therefore the function of the individual proteins could not be defined.

Shinagawa *(139)* and Shinagawa et al. *(141, 142)* described a 45-kDa protein with a pI of 5.5 isolated from *B. cereus* strain FM-1. The protein induced vascular permeability, mouse lethality, and fluid accumulation and was cytotoxic but nonhemolytic. However, the reported minimum amount of protein required to elicit these activities was high, raising concerns about protein purity. The amounts were 50 and 200 ng for rabbit and mouse vascular permeability, respectively, 12 μg for mouse lethality, and 30 to 50 μg or 500 μg/mg for fluid accumulation in mice and rabbits, respectively *(142)*.

Asano et al. *(8)* cloned and sequenced a gene (*entFM*) from *B. cereus* strain FM1, *B. thuringiensis* subsp. *sotto*, and *B. thuringiensis* subsp. *israelensis*, which they identified as the gene for the 45-kDa protein described by Shinagawa et al. *(143)*. However, as pointed out by Beecher *(14)*, the predicted pI (9.6) of the entFM protein is quite different than that for the 45-kDa protein of Shinagawa et al. *(143)*; in addition, it is similar to a phosphatase-associated protein with cell-wall hydrolase activity from *Bacillus subtilis* described by Margot et al. *(105)*.

The *bceT* gene was identified in a clone from a genomic library of *B. cereus* strain B-4ac *(4)*. The bceT protein has a predicted size of 41 kDa. Lysates from *Escherichia coli* containing the *bceT* gene were cytotoxic to Vero cells, caused fluid accumulation in the mouse ileal loop assay, and increased vascular permeability in rabbit skin. However, the protein has not yet been demonstrated to be secreted by *B. cereus*. Hansen et al. *(69)* reappraised the *bceT* sequence and suggested that the cloned *bceT* gene did not exist as a single gene in *B. cereus* B-4ac but as four independent DNA fragments that were joined during ligation. One of the fragments had 93% homology to an open reading frame (ORF 101) in the pathogenicity island of the virulence plasmid pXO1 in *B. anthracis*. Hansen et al. suggested that the enterotoxic activity observed by Agata et al. *(4)* was due to either the fusion gene or to the fragment with homology to *B. anthracis* ORF 101 in pX01.

Lund et al. *(100)* isolated a 34-kDa cytotoxin (CytK) from a *B. cereus* strain associated with a food poisoning outbreak that resulted in three deaths in France. CytK is identical to hemolysin IV, which was identified independently by Beecher et al. *(17)* and shown to be toxic to retinal tissue in vitro and hemolytic. The deduced amino acid sequence of CytK has about 30% identity to proteins belonging to the family of β-barrel channel–forming toxins *(100)*. Hardy et al. *(70)* found that CytK could form pores in planar lipid bilayers and was cytotoxic to human colon cancer Caco-2 cells. These authors suggested that CytK possessed potential enterotoxic activity and could be a cause of necrotic enteritis in humans and animals *(70, 100)*.

HBL. Beecher and Macmillan *(15, 16)* isolated HBL, a tripartite toxin produced by *B. cereus*, from strain F837/76. HBL is the only factor that has been highly purified *(15, 16, 21)* and established to be a diarrheal toxin by the ligated rabbit ileal loop assay *(19)*. In addition to demonstrating enterotoxigenic activity, Beecher et al. *(19)* found that HBL is identical to the toxin of Thompson et al. *(157)* by performing Western blots and immunodiffusion assays with antiserum to HBL components and antiserum that Thompson et al. *(157)* produced to their enterotoxin.

The three components of HBL, B, L_1, and L_2, have been purified from *B. cereus* strain F837/76 and have molecular masses of 37.8, 38.5, and 43.2 kDa, respectively, and pI values of approximately 5.3 *(21)*. Nucleotide and deduced amino acid sequences have been reported for all components in strain F837/76 (GenBank accession nos. L20441 and U63928) *(73, 133)* and ATCC 14579 (GenBank accession no. AJ237785) *(117)*. The genes *hblC* (L_2), *hblD* (L_1), and *hblA* (B) are arranged in tandem in an operon *(117, 133)* with the promoter located upstream of *hblC*. Alignment of the deduced amino acid sequences of the three proteins revealed significant similarities *(13, 14, 23)*. The proteins are 20 to 24% identical to each other. Structural analysis of the HBL proteins indicates that all three components consist almost entirely of alpha-helix. Components B and L_1 contain predicted transmembrane segments of 17 and 60 amino acid residues, respectively, in the same position, whereas L_2 does not contain predicted transmembrane segments. These observed similarities suggest that the HBL components resulted from the duplication of a common gene *(13, 14)*.

The DNA sequences of the *hbl* genes of strains F837/76 and ATCC 14579 are 97% identical *(73, 117)*. The major difference is at the C-terminal of L_1; 22 additional amino acids are present in ATCC 14579 that are absent in F837/76. Immediately downstream of *hblA* is *hblB*, which has a predicted amino acid sequence that is about 69% identical to that of the mature B component. There are 91 amino acids at the C-terminal of the protein encoded by *hblB* that are absent from the B component encoded by *hblA*. This difference could be the result of a C-terminal fusion with an open reading frame during the duplication process *(117)*. The protein encoded by *hblB* has not been isolated, but the presence of a signal peptide is predicted from the nucleotide sequence, suggesting that the protein could be exported from the cell.

All three components in HBL are required for biological activity. HBL produces a unique discontinuous hemolysis pattern on blood agar *(15, 21, 22)*. Hemolysis begins several millimeters from the edge of a colony or a well containing HBL, forming a ring-shaped clearing zone (discontinuous). With time, the zone moves inward toward the source. Hemolytic potency varies depending on the species of mammalian blood tested, with guinea pig > swine > calf > sheep > goat > rabbit > human > horse. Beecher and Wong *(22)* found that the discontinuous hemolysis phe-

nomenon is mediated by the B and L_1 components. Sheep erythrocytes do not lyse when incubated with the B component alone. Rather, the erythrocytes become sensitized or primed and are rapidly lysed with the addition of L_1 and L_2. Excess concentrations of B, however, inhibit the activity of L_1 on the lysis of B-primed erythrocytes, and excess L_1 inhibits the priming activity of B. The L_2 component is required for lysis but does not interfere with the action of B or L_1. Therefore, hemolysis of erythrocytes in the blood agar plate assay occurs at the point in the diffusion gradient (away from the well) where appropriate concentrations of both B and L_1 exist.

In addition to its hemolytic activity, HBL is dermonecrotic, increases vascular permeability in rabbit skin *(21)*, and is cytotoxic to Chinese hamster ovary cells *(12)* and retinal tissue both in vitro and in vivo *(18)*. It causes fluid accumulation in the rabbit ileal loop assay, and necrosis of villi, submucosal edema, interstitial lymphocytic infiltration, and variable amounts of blood were also observed in loops that were positive for fluid accumulation *(19)*. The potency in the rabbit ileal loop assay (5 μg per component) is in a range similar to that of cholera toxin (≤1 μg), and the tissue necrosis is similar to that observed by Turnbull et al. *(163)* when working with two chromatographic fractions that exhibited lethal activity.

HBL forms pores in eukaryotic cell membranes, with each of the components binding the membrane independently and reversibly *(22)*. One hypothesis was that once bound, the components oligomerize and form transmembrane pores consisting of at least one of each component. The transmembrane segments in B and L_1 may serve as mediators of oligomerization *(14, 23)*. Membrane receptors have not been identified.

Heterogeneity of HBL. A high degree of molecular heterogeneity exists in HBL from different strains. In a study of 127 *B. cereus* isolates by Western blot analysis, four sizes of B (38, 42, 44, and 46 kDa), two L_1 (38 and 41 kDa), and three L_2 (43, 45, and 49 kDa) were identified *(136)*. Individual strains produced various combinations of single or multiple bands of each component. In addition, some strains produced only one or two of the three HBL components. A total of 13 different band patterns were observed with various forms of B, L_1, and L_2.

Beecher and Wong *(24)* reported the isolation of two distinct sets of HBL proteins from a single *B. cereus* strain, MGCB 145. One set of proteins, 87 to 100% identical in their respective N-terminal amino acid sequences to those of the prototype *B. cereus* strain F837/76, was designated HBL. The second set, designated HBL_a, was 62 to 65% identical to the prototype and consisted of B_a, L_{1a}, and L_{2a}. These antigens reacted to polyclonal antibodies raised against HBL purified from strain F837/76. The authors suggested that high sequence similarity between the two sets of homologs indicates that genes may have been either duplicated or transferred horizontally.

HBL and HBL_a from strain MGBC145 induced hemolysis in erythrocyte suspensions and increased vascular permeability *(24)*. As with HBL, all three HBL_a components were required for activity, and in most activity assays the components of HBL and HBL_a were interchangeable. No notable differences in activity between individual L_1 and L_{1a} or L_2 and L_{2a} components were observed, but B and B_a were significantly different in the hemolysis assay. On blood agar plates, B_a with L_{1a} and L_{2a} or with L_1 and L_2 produced a continuous rather than the discontinuous pattern typical of HBL. In the erythrocyte suspension assay, excess B inhibited the activity of L_1 in lysing erythrocytes that had been primed by exposure to B but not those primed by exposure to B_a. Excess B_a enhanced lysis of B_a-primed cells. The exact mechanism is unknown, but B and B_a must interact differently on the target cell membrane.

Two sets of HBL proteins were also isolated from a second *B. cereus* strain, S1C *(135)*. The N-terminal amino acid sequences of the first set of B, L_1, and L_2 had 90 to 100% identity with their respective HBL counterparts from strain F837/76, whereas the second set shared 50 to 71% identity. The second set of proteins had 45 to 86% identity with HBL_a from strain MGBC 145.

Nhe. After screening over 300 *B. cereus* strains using PCR, Western blot, and cell cytotoxicity tests, Granum and coworkers *(61, 101)* concluded that there was at least one enterotoxin complex in addition to HBL involved in *B. cereus* enterotoxigenicity. *B. cereus* strain 0075-95, responsible for a Norwegian diarrheal syndrome food poisoning outbreak, was used to produce and characterize Nhe *(101)*. Three proteins (39, 45, and 105 kDa) reacted to polyclonal antiserum that was reported to detect *B. cereus* enterotoxin. The 39- and 45-kDa proteins were isolated. Some similarity was observed between the N-terminal amino acid sequence determined for the 39-kDa protein and that of the HBL L_1 component. The 45-kDa protein, NheA, was the same as the main antigen in the *Bacillus* Diarrhoeal Enterotoxin Visual Immunoassay kit from Tecra, previously reported by Beecher and Wong *(20)*. The 105-kDa protein was identified as a protease that is not part of the Nhe complex *(102)* but is similar to ColH from *Clostridium histolyticum* and to the collagenase encoded by *colA* from *C. perfringens*, possessing both gelatinolytic and collagenolytic activity.

Granum et al. *(65)* cloned and sequenced *nhe* from *B. cereus* strain 1230-88 (EMBL accession no. Y19005). The *nhe* operon contains three open reading frames that correspond to the genes *nheA*, *nheB*, and *nheC*. The deduced sizes of the encoded proteins are 41 kDa (NheA), 39.8 kDa (NheB), and 36.5 kDa (NheC), with predicted pI values of 5.13, 5.61, and 5.28, respectively. NheA and NheB have been isolated, but NheC has not. The functions of these proteins have not been determined.

The Nhe proteins demonstrate homology with each other and with HBL components. NheA is 19% identical to NheB and NheC, and NheB is 44% identical to NheC. The identities observed between NheA and L_2, NheB and L_1, and NheC and B are 24, 37, and 25%, respectively *(14, 65)*. Similarities were also observed in predicted transmembrane helices for the proteins. Like L_1 and B, NheB and NheC have two and one predicted transmembrane helices, respectively, located in the same position, whereas NheA,

like L_2, has none. These similarities suggest that HBL and Nhe are homologs in a family of tripartite toxins consisting of HBL, HBL_a, and Nhe *(14)*.

Location of *hbl* and *nhe*. Numerous studies on genome mapping of *B. cereus* and *B. thuringiensis* have revealed that the chromosome sizes vary greatly, ranging from 2.4 to 6.3 Mb, and extrachromosomal bands are frequently observed *(33, 37, 91)*. The genetic organization of one region of the chromosome appears to be constant, whereas that of the other is variable in terms of presence and location of genes. The constant region contains housekeeping and ribosomal genes *(33–36)*, whereas genes in the variable region are often plasmid encoded. One of the strains studied by Carlson and Kolstø *(37)*, F837/76, considered the prototype strain for production of HBL *(12, 16, 21)*, has the smallest chromosome (2.4 Mb) of the 10 strains analyzed; however, it carries a large amount of extrachromosomal DNA (2.6 Mb), which exists as large plasmids that are each reported to be greater than 40 kb and stably maintained. The small chromosome of F837/76 contained many of the genes in the constant part of the larger chromosomes observed in other strains, whereas genes in the variable region were absent or present in the extrachromosomal elements. The *hbl* operon is located in the variable region, and the *nhe* operon is in the constant portion of the *B. cereus* and *B. thuringiensis* chromosome *(34, 35)*.

Distribution of HBL and Nhe. Production of both HBL and Nhe is quite widespread; 34 to 84% of *B. cereus* isolates produce HBL *(20, 45, 67, 104, 128, 132)*. Some isolates secrete only one or two of the HBL components *(136, 156)*, whereas others possess the HBL genes but the proteins are not expressed *(128, 136, 156)*. The ability of *B. cereus* to produce Nhe is even more common, with estimates of its production in 92 to 100% of isolates *(31, 44, 132)*. HBL and Nhe production is also prevalent among *B. thuringiensis (41, 128, 131)*, and HBL can be produced by *B. mycoides (128)*. The *B. anthracis* genome contains the genes for Nhe but not those for HBL *(130)*. Whether Nhe is expressed is not known.

Regulation of *hbl* and *nhe*. PlcR is a 34-kDa protein that was first described in *B. thuringiensis* as a *trans* activator for the phosphatidylinositol-specific phospholipase C gene *plcA*. PlcR regulated its own transcription at the onset of stationary phase *(97)*. Agaisse et al. *(1)* provided evidence that PlcR is a pleitropic regulator that controls the expression of at least 15 genes, many of which encode virulence factors in *B. cereus* and *B. thuringiensis*. These genes encode for degradative enzymes, cell-surface proteins, and toxins including Nhe and HBL. A highly conserved palindromic region ($TATGNAN_4TNCATA$), known as the PlcR box, is the specific recognition target for PlcR activation and is located at various positions upstream of the transcription start site of the target gene *(1, 117)*. The genes regulated by PlcR are widely dispersed and do not constitute a pathogenicity island. Many of the proteins encoded by these genes are regulated by PlcR in *B. cereus*, as determined by two-dimensional gel electrophoresis *(56)*.

Agaisse et al. *(1)* found that *plcR* is present in *B. anthracis* and *B. cereus* but not *B. subtilis*. The polypeptide produced by the *B. cereus* gene is similar to that of *B. thuringiensis*, but the *B. anthracis* polypeptide is truncated because of a nonsense mutation in the *plcR* gene and presumably is not functional. The genome sequences of *B. cereus* strains 14579 *(85)* and 10987 *(129)* contain 55 and 57 putative PlcR-binding motifs, respectively, which potentially could regulate over 100 genes in each isolate. In *B. anthracis* Ames, there are 56 putative PlcR-binding motifs in the chromosome and two on the virulence plasmid pXO2 *(130)*.

Gominet et al. *(57)* found that the *B. thuringiensis* oligopeptide permease system was required for *plcR* expression, and Slamti and Lereclus *(146)* found that a small peptide, PapR, was secreted from the cell and reimported via the oligopeptide permease system. Once inside the cell, PapR is processed into a pentapeptide and activates the PlcR regulon by promoting binding of PlcR to its DNA target.

Results of two recent studies suggest that components of the flagellar apparatus may be involved in the secretion of virulence-associated proteins, including HBL. Senesi et al. *(138)* found that a *fliY* mutant of *B. cereus* lost the ability to swarm and secrete L_2 and that L_2 was only produced in the swarm-cell state. Ghelardi et al. *(53)* observed that *flhA* was required for export of flagellin, HBL, and phosphatidyl-preferring phospholipase C in *B. thuringiensis*.

SUMMARY

B. cereus is widespread in nature and frequently contaminates a wide variety of food products. The incidence of both the diarrheal and emetic syndromes caused by *B. cereus* probably has been underestimated because the illnesses are usually self-limiting with mild symptoms. Despite the recognition of *B. cereus* as a foodborne pathogen over 50 years ago, its virulence mechanisms are still not fully elucidated. Cereulide has been identified as the causative agent in the emetic syndrome, and HBL is associated with diarrheal food poisoning. Nhe, a homolog of HBL, probably possesses biological activities similar to those of HBL and could be a factor in the diarrheal syndrome; however, this hypothesis has not been tested. In addition to causing food poisoning, HBL can play a role in nongastrointestinal infections caused by *B. cereus*. The in vivo roles of many of the putative and potential virulence factors produced by *B. cereus*, such as hemolysins, phospholipases, and proteases, have not been defined. Based on their biological activities, these factors are likely to be involved in *B. cereus* infections and illnesses. Many of these factors are also expressed by *B. thuringiensis* and *B. anthracis*, which are close relatives of *B. cereus*. Continued research is needed to elucidate the virulence mechanisms of *B. cereus* and the regulation and significance of virulence factor expression in infections and in nature.

ACKNOWLEDGMENTS

This work was supported by Hatch funds (WIS04188) and by the College of Agricultural and Life Sciences of the University of Wisconsin–Madison.

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