Levels, Enterotoxigenicity, Growth and Physical Characteristics of B. Cereus From U.S Retail Rice

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LEVELS, ENTEROTOXIGENICITY, GROWTH AND PHYSICAL CHARACTERISTICS OF *Bacillus cereus* FROM U.S RETAIL RICE

A Thesis presented

by

CHANDRAKANT R. ANKOLEKAR

Submitted to the graduate school of the University of Massachusetts, Amherst in partial fulfillment of the requirements for the degree of

MASTER OF SCIENCE

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Department of Food Science
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Department of Food Science
I would like to thank God for giving me this opportunity to pursue my graduate studies. I would like to express my sincere appreciations for my advisor, Prof. Ronald Labbe for his support, guidance, and encouragement over the course of this work. I would like to thank him for his understanding and for his faith in me over the past couple of years. Special thanks to Prof. Kalidas Shetty for his support and encouragement. Together they have helped me to develop my skills not only as a student but also as an individual. I would also like to thank my committee members, Dr. Robert Levin and Dr. Lynne Melandsborough for being in my Committee and for their helpful ideas and guidance.

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ABSTRACT

LEVELS, ENTEROTOXIGENICITY, GROWTH AND PHYSICAL CHARACTERISTICS OF *B. CEREUS* FROM U.S RETAIL RICE

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*Bacillus cereus* is a ubiquitously found foodborne pathogen that is frequently associated with two types of illness: emesis and diarrhea. Two heat labile enterotoxins have been associated with the diarrheal syndrome whereas a heat stable acid stable peptide toxin has been associated with the emetic syndrome. In the U.S, *B. cereus* is responsible for 1-2% of the total outbreaks from bacteria. Although there are reports of isolation and characterization of this pathogen from various food stuffs all around the world, there are no reports on the levels, toxin producing ability, or growth characteristics from U.S retail rice. Considering that rice is grown mostly in developing countries and most of the rice in the U.S is imported, there is a high chance of the rice being contaminated with *B. cereus* spores. Therefore, the major objective of this thesis was to characterize *B. cereus* spores from U.S retail rice. The levels were determined and further the enterotoxigenic ability and the growth characteristics along with the physical characteristics of the isolates were studied.
Among the 178 samples analyzed, Spores of Bacillus species were found in 94 (52.8%) of the rice samples with an average concentration of 32.6 CFU/g (3.6-460 CFU/g). Eighty nine of the 94 isolates were tested positive for one of the two enterotoxins produced by B. cereus. none of the 94 isolates tested positive for the emetic gene.

All the isolates generally grew well in cooked rice. Levels of 10⁶/g were detected in cooked rice after 22h at 20⁰C and after 34h at 17⁰C whereas at 12⁰C the counts did not go above 10⁴/g even after 48h. A significant difference in the heat resistance of the emetic and the diarrheal strains was found. The emetic but not the diarrheal type grew well at inoculum levels of 10⁷/g and 10⁸/g level following cooking. So these results suggest although the diarrheal type are more predominant in U.S retail rice, the chances of foodborne illness arising from the diarrheal strains is low.

B. cereus, B. thuringiensis and B. mycoides were investigated for their physical characteristics. Appendages were not found on B. mycoides. By contrast, all the isolates had exosporia. The isolates were characterized to be moderately to highly hydrophobic and all the isolates had a net negative charge. Judging by their physical characteristics, it can be concluded that these spores may have a high affinity for adhering to inert surfaces.
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CHAPTER 1
INTRODUCTION

_Bacillus cereus_ is a gram-positive, endospore forming pathogenic bacterium that is ubiquitous in the environment and is frequently associated with emetic and diarrheal types of foodborne illness. The diarrheal type which resembles _Clostridium perfringens_ food poisoning is caused by an enterotoxin produced during vegetative growth of _B. cereus_ in the small intestine (Granum, 1994) whereas the emetic type which resembles _Staphylococcus aureus_ food poisoning is caused by a preformed toxin produced by growing cells in the food (Kramer and Gilbert, 1989). The emetic type is mostly associated with farinaceous foods whereas the diarrheal type is associated with meat, soups, sauces, milk, and vegetables (Kramer and Gilbert, 1989).

The thermo-labile diarrheal enterotoxin, which consists of 3 different protein components, is produced at an optimum temperature of 32-37°C during the late logarithmic phase (Drobneiwski, 1993). The illness, characterized by abdominal pain, diarrhea, and occasional nausea, usually subsides within 12-24 h and has an incubation period of 8-16 h. (Kramer and Gilbert, 1989). In contrast, the emetic toxin is an extremely heat stable (126°C for 90 min) peptide toxin formed during late exponential to stationary phase at optimal temperatures of 25-30°C (Drobneiwski, 1993). The emetic syndrome characterized by vomiting, nausea and in some cases diarrhea, has a rapid onset and usually lasts for about 6-24 h (Kramer and Gilbert, 1989).

_Bacillus_ is the dominant genus making up to 90% of the paddy soil bacteria and _B. cereus_ remains closely associated with the rice plant throughout its development (Kramer and Gilbert, 1989) and hence raw rice is likely to be contaminated with _B._
cereus. Spores of some B. cereus strains can survive the cooking procedure (Vijaylakshmi et al., 1981; Chung and Sun, 1986) and proliferate when cooked rice is stored at room temperature for a long time causing foodborne illness, resulting in the ingestion of large numbers of vegetative cells causing the emetic or diarrheal syndrome.

Although B. cereus spores are found on rice, even in refined polished form, with isolation rates ranging from 46-100% (Vijaylakshmi et al., 1981; Shinagawa et al., 1979; Blakey and Priest, 1980; Bryan et al., 1981; Gilbert and Parry, 1977), there are no reports investigating B cereus levels in U.S. retail rice. The illness caused by B. cereus is mild and usually does not last for more than 24 h (Kramer and Gilbert, 1989). However, three deaths caused because of a necrotic enterotoxin (Lund et al., 2000) produced by a severe diarrheal type of B cereus, along with deaths of two individuals caused by the intake of large amounts of the emetic toxin have been reported (Dierick., 2005; Mahler et al., 1997).

Considering that rice is the number one-consumed cereal in the world and that B. cereus has the potential to cause foodborne illness through rice, it is important to characterize the levels and growth of B cereus in cooked rice. There have been numerous studies reporting growth of emetic strains of B cereus in rice (Gilbert et al., 1974, Parry and Gilbert, 1980; Johnson et al., 1983) but no specific reports investigating the presence or growth of diarrheal type in rice or any other food.
CHAPTER 2
LITERATURE REVIEW

2.1. History

Hauge (1955), was the first to establish *B. cereus* as a food poisoning organism. Although there are reports in the European literature as early as the beginning of the 20th century about foodborne illness caused by *B. cereus* or *B. cereus*-like organism, there was no definite proof that *B. cereus* could cause food poisoning (Kramer and Gilbert, 1989).

Until 1970’s outbreaks caused by *B. cereus* were only characterized by watery diarrhea occurring 8-16h after ingestion of the contaminated food. However in 1971 a new form of *B. cereus* food poisoning characterized by nausea and vomiting was identified in the UK following outbreaks associated with the consumption of rice from Chinese restaurants and take-away outlets. In the UK, 192 such incidences involving more than 1000 cases were reported between a period of 1971 and 1984 (Kramer and Gilbert, 1989).

2.2. Classification

*B. cereus* is a facultative anaerobic, gram-positive, catalase-positive, endospore forming, motile organism that consists of six closely related species namely *Bacillus anthracis, Bacillus cereus, Bacillus mycoides, Bacillus pseudomycoides, Bacillus thuringiensis, and Bacillus weihenstephanensis*. Similar 16S and 23S rRNA sequences suggest that these species have diverged from a common evolutionary lineage (Ash and Collins, 1992; Ash et al., 1991). The spores of the six species do not distend the sporangia and produce cells which are large (>0.9µm) in size. Cells are typically 1-1.2µm
in diameter and 3-5µm in length (Claus and Berkeley, 1986, Lechner et al., 1998; Nakamura, 1998). The cells can grow anaerobically and sporulate freely on many media within 2-3 days under well aerated conditions. *B. thuringiensis* synthesizes a toxic crystalline inclusion that is highly toxic to specific insects. The cry genes of *B. thuringiensis* are located on the plasmid and may be lost during sub-culturing which makes them indistinguishable from *B. cereus* (Schraft and Griffiths, 2006). *B. weihenstephanensis* grows at <7°C but not at 43°C which forms the basis of its differentiation from *B. cereus* (Lechner et al., 1998). Also it can identified rapidly using rRNA or cold shock protein-targeted PCR (Lechner et al., 1998). *B. pseudomyoides* is differentiated from *B. mycoides* based on fatty acid composition and 16S RNA sequences but not by physiological or morphological characteristics (Nakamura, 1998).

2.3. *Bacillus cereus* food poisoning

*B. cereus* causes two types of illness caused by two different moieties; diarrheal toxin and the emetic toxin. The diarrheal syndrome is associated with a wide variety of foods and is characterized by an incubation period of 8-16h before the onset of watery diarrhea, abdominal pain and occasionally nausea and vomiting (Hauge, 1955). It is suggested that the diarrheal syndrome is caused by toxin produced by *B. cereus* in the intestine after ingesting large number of cells.

The emetic syndrome on the other hand is caused by consumption of preformed toxin mostly in farinaceous food items. The syndrome is characterized by an incubation period of 0.5-5h and is accompanied by symptoms such as vomiting, nausea and occasional diarrhea (Mortimer and McCann, 1974).
Usually it takes $10^5 - 10^7$ cells in total for the diarrheal syndrome and $10^5 - 10^8$ cells per gram of food to cause the emetic syndrome. Although foods with large \textit{B. cereus} populations ($10^7$/g) may not appear spoiled, the food may no longer be organoleptically acceptable (Granum, 1994). The total dose range of approximately $5 \times 10^4 - 10^{11}$ has been indicated in foods identified in outbreaks (Kramer and Gilbert, 1989; Granum, 1994; Granum and Baird-Parker, 2000; Hauge, 1955). The wide range of infective dosage partly may be attributed to the consumption of spores which can survive the acid barrier in the stomach and partly due to the ability of different strains to produce different amounts of the toxins (Granum, 1994).

2.4. Isolation and Identification

Selective media is primarily used to isolate \textit{B. cereus} by direct plating. MYP (Mannitol-yolk-polymyxin) and PEMBA (Polymyxin-pyruvate-egg yolk-mannitol-bromothymol blue agar) are the most widely used selective media for isolating \textit{B. cereus}. Both the media are based on the diagnostic features of \textit{B. cereus} of lecithin hydrolysis and inability to ferment Mannitol. \textit{B. cereus} forms peacock blue and pink colonies on PEMBA and MYP respectively, surrounded by a halo of lecithin hydrolysis. Polymyxin acts as the selective agent by inhibiting the growth of competitive organisms (Holbrook and Anderson, 1980; van Netten and Kramer, 1992). A chromogenic agar BCM developed by Peng et al., (2001) relies on the phosphotidylinositol phospholipase C hydrolyase (PI-PLC) enzyme present in \textit{B. cereus} for detection.

Presumptive colonies are confirmed by biochemical tests described in the FDA Bacteriological Analytical Manual). These include anaerobic utilization of glucose,
Voges-Proskauer, L-tyrosine decomposition, nitrate reduction, and growth in 0.001% lysozyme. Enrichment if required can be carried out using brain heart infusion (BHI) or tryptic soy broth (TSB). Confirmed isolates can be maintained in sporulated state on NA at 4°C.

2.5. Reservoirs

*B. cereus* is ubiquitously distributed in nature. It is a very common contaminant isolated from foods especially of plant origin. It is frequently present in milk and milk products, rice, vegetables, spices, egg, ready to eat food stuffs etc (Kramer and Gilbert, 1989). Milk and rice perhaps are the two most commonly contaminated food items. *B. cereus* constitutes 90% of the paddy soil bacteria and contact of soil and grass with udders causes milk and milk products to be contaminated (Kramer and Gilbert, 1989). *B. cereus* spores can survive pasteurization of milk and cooking of rice to germinate and outgrow when they are stored at room temperature (Anderson et al., 1995). te Giffel, et al. (1995) reported that *B. cereus* is present in both raw and pasteurized milk with a prevalence of 2-37%. Also *B. cereus* enterotoxin was found in more than 1/3rd of the pasteurized milk samples analyzed by Odumeru et al. (1997). Not surprisingly the presence of *B. cereus* remains a significant sanitation issue in the dairy industry including its presumptive presence in biofilms. *B. cereus* spores are also a problem in dried products (Kramer and Gilbert, 1989). *B. cereus* levels can reach hazardous levels during the soaking dried legumes before cooking (Blakey and Priest, 1980).
2.6. Toxins

The two types of gastrointestinal diseases caused by *B. cereus* are derived from two significantly different types of toxins. The emetic syndrome is caused by a preformed emetic toxin whereas the diarrheal syndrome is caused by one or more diarrheal enterotoxin produced in the intestine (Granum, 1994; Kramer and Gilbert, 1989).

2.6.1 The emetic toxin

The emetic toxin named cereulide, is a small 1.2kDa dodecadepsipeptide consisting of a ring structure of three repeats of four amino acids (Agata et al., 1995). This toxin is closely related to valinomycin produced by *Streptomyces griseus* and is toxic to mitochondria by acting as a K\(^+\) ionophore. The emetic toxin is resistant to heat, pH, proteolysis and is not antigenic (Mikkola et al., 1999; Pitchayawasin et al., 2003). Highest amounts of toxin production have been reported before the beginning of the stationary phase and are not only dependent on the incubation temperature but also on other factors such as pH and aeration (Finlay et al., 2000). Glucose has been shown to stimulate cereulide production whereas repression of the toxin production has been reported by certain amino acids such as leucine, isoleucine and glutamic acid (Agata et al., 1999). Cereulide is synthesized by a Non-Ribosomal Peptide Synthetase (NRPS) (Horwood et al., 2004; Toh et al., 2004).

Commercial assay kits are not yet available for detection of cereulide due to its low antigenic properties (Melling and Capel, 1978). However, three methods of detection including a Boar sperm based bisoassay, LC-MS analysis and a cytotoxicity assay have been described. The boar sperm motility measures the loss of motility of the sperm cells
after exposure to the toxin (Andersson et al., 1998; Andersson et al., 2004). The cytotoxicity assay is based on the vacuole forming ability of cereulide in HEp-2 cells (Finlay et al., 1999). The downside of these assays is the lack of specificity of these assays and long precultivation times (Andersson et al., 1998). The LC-MS analysis is much more specific but involves laborious sample preparations, trained personnel and expensive equipment (Haggblom et al., 2002).

2.6.2 The diarrheal enterotoxin

Three different heat labile diarrheal enterotoxins are produced by *B. cereus* during vegetative growth in the small intestine. Two, NHE and HBL, are structurally related, three protein complexes and one, Cyt K, is a single protein (Heinrichs et al., 1993; Granum and Lund 1997; Lund et al., 2000). Two other proteins termed as enterotoxin T and FM have been reported but apparently they do not contribute to illness (Agata et al., 1995; Asano et al., 1997; Choma and Granum, 2002). The three component HBL consisting of three proteins: B, L₁, and L₂ was the first to be characterized whereas the three component NHE consisting of three proteins, A, B and C, is the most common enterotoxin produced by *B. cereus* (Beecher and Macmillan, 1991; Beecher and Wong, 1994). The multi-component enterotoxins require all three protein components for their maximal activities (Beecher and Macmillan, 1991; Lindback et al.; Granum, 2004). Although it has been reported that cytotoxicity is mainly due to *nhe*, it is not clear if the other two toxins contribute to diarrhea (Moravek et al., 2006).

Commercial assay kits are available for semi-quantitive detection of NHE and HBL enterotoxins. An Oxoid kit detects the presence of the L₂ component (HBL C), whereas a
Tecra visual immunoassay kit measures the A component of the NHE complex (Granum et al., 1993; Beecher and Wong, 1994; Granum and Lund, 1997).

2.7. Growth and survival conditions of *B. cereus* in foods

The temperature range for the growth of *B. cereus* has been reported to be between 4-50°C with the optimum being in the range of 30-40°C. Carlin et al. (2006), reported that none of the emetic strains were able to grow at a temperature below 7°C whereas 50 of 83 non-emetic strains were able to grow at a temperature of 4°C or 7°C. In contrast, all the emetic strains were able to grow at a temperature of 48°C whereas only 39% of the non-emetic strains tested were able to grow at that temperature.

The permissible range of pH for growth of *B. cereus* has been reported to be 4.9-9.3 (Kramer and Gilbert, 1989) with the optimum being 6.0-7.0. As with other microorganisms the minimum aw for growth is dependent on the type of humectant used. For example using glycerol, growth was possible at a water activity of 0.93 with the absence of NaCl (ICMSF, 1996). Bryan et al. (1981) reported samples of fried rice having an aw in the range of 0.912-0.961 and since fried rice has been shown to support the growth of *B. cereus*, 0.912 is reported as the minimum working limit (Kramer and Gilbert, 1989). Under optimal conditions a generation time of approximately 23 min was reported (ICMSF, 1996).

The reports on inhibitory effects of chemical preservatives are scanty. Sorbic acid at 0.26% and potassium sorbate at 0.39% were shown to be inhibitory in rice filling held at 23°C (Kramer and Gilbert, 1989). In beef gravy, Beuchat et al. (1997), reported 50μg/ml
and 5µg/ml nisin to be inhibitory to the growth and enterotoxin producing ability of *B. cereus* respectively, at 15°C.

### 2.8. Heat resistance

One of the primary factors of concern in the processing of safe foods with an extended shelf-life is the effective destruction of *B. cereus* and other spores, and one critical factor which has received significant attention in the food and pharmaceutical industry is spore heat resistance. In general spores have been shown to be more resistant to heat under reduced *a*w conditions (Molin and Snygg, 1967). Ababouch and Busta (1987), reported greater D-values for *B. cereus* spores in oil than in phosphate buffer (PB) solution. Molin and Snygg (1967), reported D$_{95}^{0}$C values of 13 min for spores suspended in PB solution and D$_{121}^{0}$C values of 17.5-30 min for the same spores in olive and soybean oils.

A wide range of D$_{95}^{0}$C values of 1.2-36.2 min in PB has been reported for *B. cereus* spores (Kramer and Gilbert, 1989). Parry and Gilbert (1980) observed greater heat resistance among 10 emetic strains as compared to 9 other non-emetic strains. Carlin et al (2006), demonstrated that the difference in the D$_{90}^{0}$C values of the emetic strains and other non-emetic strains was significant (P<0.05). This difference in heat resistance might be the reason why diarrheal syndrome is not associated with rice; if diarrheal strains possess their low heat resistance they would be easily destroyed when subjected to cooking. Valero et al., (2002), characterized *B. cereus* from fresh vegetables and refrigerated minimally processed foods and reported a D$_{90}^{0}$C value ranging from 1.4 to
21.2 min. Similarly, D_{90} values ranging from 2.23-23.36 min for B. cereus isolated from Spanish raw rice were reported (Sarrias et al., 2002).

2.9. Growth in foods

The list of foods serving as vehicles of B. cereus foodborne illness is huge and includes barbequed chicken, noodles, cakes, soups, steak, rice, egg, turkey, vanilla sauce, sprouts, desserts etc. It is literally impossible to identify raw food products not contaminated with B. cereus spores because of its ubiquitous nature (Schraft and Griffiths, 2006). The fact that many different kinds of foods have been incriminated in B. cereus foodborne illness indicates that it can grow and/or produce toxin in any kind of food under permissible growth conditions. Although, B. cereus is often associated with milk and rice it can easily spread to other foods, such as meat products through cross contamination (Kramer and Gilbert, 1989).

In pasteurized milk B cereus has a generation time of 17 h at 60°C and may produce enterotoxin during extended storage at slightly unfavorable temperatures (te Giffel, 1995). In another study, a rapid growth of B. cereus in cream at 23°C was reported with sufficient population levels for toxin production within half a day (Feijoo, 1997). Gilbert et al. (1974) did a study in which they inoculated cooked rice with B. cereus spores and checked the growth at various temperatures ranging from 4-50°C. The growth was fastest at 37°C with log_{10} counts reaching 7.3-7.9 after 18h of storage starting with an initial log_{10} count of 1-1.6. It was also demonstrated in the study that B. cereus spores survived the frying procedure and those that survived were capable of germination and outgrowth. Parry and Gilbert (1980) did a similar study and determined that there was no significant
difference in the growth rates of emetic and non-emetic strains in cooked rice at 22\(^0\)C after 40h of storage. A comparison of growth of emetic and diarrheal strains in broth and rice was done by Johnson et al. (1983) at various temperatures ranging from 5-55\(^0\)C. The authors demonstrated that the growth rate was similar for both the strains in broth medium and rice although a higher maximum population was observed in rice due to a delay of the onset of stationary phase. A similar study by Carlin et al. (2006) indicated that there was no difference between the emetic and the diarrheal strains in the growth kinetics in mPlate Count Broth at 24\(^0\)C and 37\(^0\)C observed at a pH of 5.0, 7.0 and 8.0. Chung and Sun (1986) studied the effect of cooking on the survival of \textit{B. cereus} spores. They reported a 17.5 min cooking period caused a 100-fold reduction in the number of spores whereas a cooking time of 11.5 min reduced the \textit{B. cereus} spore number by about 10-fold.
CHAPTER 3
OBJECTIVES

The objectives of this thesis were to:

3.1. Determine levels and characteristics of *B. cereus* in rice (pg 13-36)

3.2. Determine the ability of spores of enterotoxigenic *B. cereus* to grow in rice (pg 37-54)

3.3. Determine the physical characteristics of *B. cereus* spores isolated from rice (pg 55-69)
CHAPTER 4
PREVALENCE OF TOXIGENIC BACILLUS CEREUS AND BACILLUS THURINGIENSIS SPORES IN U.S RETAIL RICE

4.1 Abstract

*Bacillus cereus* is a gram-positive, endospore forming pathogenic bacterium that is ubiquitous in the environment and is frequently associated with emetic and diarrheal types of foodborne illness. In this study, 178 samples of raw rice from retail food stores were analyzed for the presence of *B. cereus* spores. Spores of *Bacillus* species were found in 94 (52.8%) of the rice samples with an average concentration of 32.6 CFU/g (3.6-460 CFU/g). Eighty three of the 94 isolates were identified as *B. cereus* and 11 were identified as *B. thuringiensis*. Using PCR the isolates were checked for the presence of the cereulide synthetase gene (*ces*), the *hblA* and *hblD* genes of the hemolysin BL (HBL) complex and the *nheA* and *nheB* genes of the nonhemolytic (NHE) enterotoxin complex. The *ces* gene was not identified in any of the isolates. By contrast 47 (56.6%) *B. cereus* isolates possessed the *hblA* and *hblD* genes and 74 (89.1%) isolates possessed the *nheA* and *nheB* genes. As determined by commercial assay kits, forty four (53.0%) of the 83 *B. cereus* isolates produced both NHE and HBL enterotoxins whereas 78 (93.9%) were positive for either one or the other. Protein toxin crystals were detected visually in the 11 *B. thuringiensis* isolates. PCR analysis revealed 10 (90.9%) of those 11 isolates carried the *cry* gene. All the *B. thuringiensis* isolates were positive for NHE and HBL enterotoxins. Our results suggest that foodborne illness in the U.S. due to *B. cereus* with rice as the vehicle would be most likely associated with the diarrheal-type syndrome.
4.2. Introduction

The *Bacillus cereus* group consists of six different closely related species: *Bacillus anthracis, Bacillus cereus, Bacillus mycoides, Bacillus pseudomycoides, Bacillus thuringiensis, and Bacillus weihenstephanensis* of which *B. cereus* has been most commonly responsible for foodborne illness. *B. cereus* is a facultative anaerobic, spore-forming, motile microorganism that has been identified as a causative agent of two types of gastrointestinal diseases: emesis and diarrhea. The diarrheal type, characterized by abdominal pain and diarrhea, resembling *Clostridium perfringens* food poisoning, is caused by one or more heat labile enterotoxins whereas the emetic type, characterized by an acute attack of nausea and vomiting, resembling *Staphylococcus aureus* food poisoning, is caused by a heat stable peptide toxin (Gilbert, 1979; Agata et al., 1995).

From 1973-1987 *B. cereus* was responsible for 58 food-borne outbreaks in the U.S. accounting for approximately 3% of the total outbreaks from bacteria. Chinese food was the identified vehicle for 24 of those 58 outbreaks (Bean and Griffin, 1990). In the year 1997 alone an estimated 27,360 cases of illness arising from *B. cereus* occurred. All of them were food-borne (Mead et al., 1999). Nevertheless the incidence of confirmed outbreaks of foodborne illness due to *B. cereus* is widely underreported in the U.S. compared to Europe and Japan. For example its incidence is not tracked by the U.S. FoodNet program (CDC, 2008).

*B. cereus* is found in both vegetative cell and endospore form (Rusul and Yaacob, 1995). Farinaceous foods are the most common vehicles of the emetic type whereas the diarrheal type is associated with meat, soups, sauces, vegetables and milk products (Kramer and Gilbert 1989). *B. cereus* has been isolated from a wide variety of foods
including desert mixes (Warburton et al.; Peterkin et al., 1987), infant foods (Becker et al., 1994), milk products (Ahmed et al., 1983; Davies and Wilkinson; 1973; Wong et al., 1988; Reyes et al., 2006), spices (Konuma et al., 1988; Choo et al., 2007), ready to serve foods (Harmon and Kautter, 1991), seafood (Rahmati and Labbe, 2008; Wijnands et al., 2006), fresh vegetables (Valero et al., 2002), rice (Sarrias et al., 2002), and meat products (Konuma et al., 1988; Smith et al., 2004).

*B. thuringiensis* is distinguished from *B. cereus* by the presence of a crystalline inclusion during sporulation. One enterotoxin-producing strain of *B. thuringiensis* has been implicated in gastroenteritis (Jackson et al., 1995)

Cereulide, the emetic toxin produced by *B. cereus* is a heat stable (90 min at 121°C), pH stable (2-11), 1.2 kDa peptide, produced enzymatically by a non-ribosomal peptide synthetase (NRPS) complex transcribed by NRPS genes (Horwood et al., 2004; Toh et al., 2004). Three different enterotoxins are produced by *B. cereus*, two of which (NHE and hemolysis BL [HBL]) are composed of three different protein complexes and the third one (cytK) is a single protein (Heinrichs et al., 1993; Granum and Lund 1997; Lund et al., 2000). All three protein components namely B, L1, L2 and A, B and C are required for the maximal biological activity of HBL and NHE respectively (Beecher and Macmillan, 1991; Lindback et al.; Granum, 2004). Moravec et al. (2006) recently demonstrated that cytotoxicity is mostly due to NHE.

Rice exemplifies a classic way in which *B. cereus* poisoning can occur. During growth, harvesting, milling, and other agricultural operations rice can become contaminated with *B. cereus* spores which inhabit a wide variety of environments including soil, dust, sediment, and water (Goepfert et al., 1972; Norris et al., 1981;
Johnson, 1984). These spores survive normal cooking temperatures (Vijaylakshmi et al., 1981) and proliferate when the cooked rice is stored at room temperatures for long times; such temperature-abused rice can cause foodborne illness either by intoxication (emetic type) or infection (diarrheal).

Despite its common dietary role rice has rarely been investigated from a microbiological point of view. Here we sought to determine the levels of spores and toxigenicity of \textit{B. cereus} isolates in U.S. retail rice.

\section*{4.3. Materials and methods}

\subsection*{4.3.1 Control strains}

\textit{B. cereus} ATCC 14579, (HBL complex), 1230/88 (NHE complex), and F4810/72 (ces) strains were kindly provided by A. Wong, Univ. of Wisconsin. F4810/72 was originally obtained from an outbreak with cooked rice as the vehicle (Turnbull et al., 1979). Additional emetic stains were also provided by L. McIntyre, British Columbia (BC Center for Disease Control).

\subsection*{4.3.2. Enumeration and Isolation}

Isolates in this study were obtained from a total of 178 brown, white, wild type, black, and rice mixtures from small grocery stores, large supermarkets and ethnic food stores in western Massachusetts obtained from September 2006 to July 2007. For enumeration of spores, the most probable number (MPN) method was used. Because of perforation of various types of stomacher bags, rice samples were blended manually. Ten g samples were placed in 90 ml sterile water and then shaken by hand, vigorously for 2-3
min; after settling of rice, 10 ml of liquid were heated at 75°C for 15 min. After heat selection and cooling, two subsequent serial 1:10 dilutions were made. One ml from each of these 3 dilutions was inoculated into 3 tubes of 9 ml Trypticase soy broth. The tubes were incubated for 24-48 h at 32°C. Following incubation the contents of the tubes were streaked on plates of MYP agar base (Unipath-Oxoid, Columbia MD) with egg yolk. The plates were incubated for 18-24 h and typical pink colonies surrounded by a zone of lecithin hydrolysis were selected and transferred onto nutrient agar (NA) slants. After overnight incubation at 32°C isolates were maintained at 4°C in the spore state on NA slants. Confirmatory tests were performed as described in the FDA Bacteriological Analytical Manual. These included: motility, anaerobic utilization of glucose, nitrate reduction, Voges-Proskauer reaction, tyrosine decomposition, absence (in the case of B. cereus) or presence (in the case of B. thuringiensis) of parasporal crystal inclusions, hemolysis of sheep blood agar, and lysozyme resistance.

4.3.3. Starch hydrolysis

Plates of nutrient agar containing 0.25% soluble starch were inoculated with each isolate, incubated for 48 h at 32°C and flooded with Lugol’s iodine. Isolates in which the zone surrounding the colonies was colorless were considered positive for starch hydrolysis.

4.3.4. Toxin assays

Confirmed B. cereus and B. thuringiensis isolates were tested for NHE and HBL enterotoxins using the Tecra VIA visual immunoassay (VIA) (Tecra Diagnostics,
Roseville, Australia) and the Oxoid reversed passive latex agglutination (RPLA) (Unipath-Oxoid, Columbia, MD) test kits respectively. VIA kit measures the NheA component of the NHE complex whereas the RPLA measures the L2 component of the HBL complex (Granum et al., 1993; Beecher and Wong, 1994; Granum and Lund, 1997; Lund and Granum, 1997). The tests were carried out according to the directions supplied by the manufacturers.

4.3.5. PCR

*B. cereus* and *B. thuringiensis* isolates were checked for the presence of the *ces* gene, two genes (*hblA* and *hblD*) of the HBL complex and two genes (*nheA* and *nheB*) of the NHE complex. For certain isolates the presence of *nheA* alone was also determined. Amplification of the rDNA 16S/23S spacer region of selected isolates was carried out to confirm the absence of PCR inhibiting compounds (Jensen et al., 1993). To avoid false negatives due to lysed or sporulating cells (Rahmati and Labbe, 2008), vegetative cells were prepared by inoculating nutrient agar slants and incubating for 4 h at 32°C and then overnight at 20°C to ensure that the cultures were in vegetative cell stage. Two mm loopfuls of the culture were suspended in 30 µl of double distilled water in microcentrifuge tubes. These tubes were then held in a boiling water bath for 20 min for DNA extraction, cooled and centrifuged (3-5 s at 13,500 x g). All PCR reactions were performed using Maxime™ PCR premix tubes (Boca Scientific, Boca Raton FL) using previously described conditions (Rahmati and Labbe, 2008) except that for *hblA* and *hblD*, the annealing temperature was reduced to 51°C. The primers used in this study are listed in Table 1.
Table 1 Oligonucleotide primers used in this study

<table>
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<th>Primera</th>
<th>Gene</th>
<th>Amplified Fragment</th>
<th>Sequences 5’→3’</th>
<th>Ref</th>
</tr>
</thead>
</table>
| rDNA SPACER G1 | 16S/23S rDNA spacer | 480-490 430-440 250-260 | GAA GTC GTA ACA AGG  
CAA GGC ATC CAC CGT | Jensen et al., 1993 |
| rDNA SPACER L1 | | | | |
| CES F1 | | | | |
| CES R2 | ces | 1271 | GGT GAC ACA TTA  
TCA TAT AAG GTG  
GTA AGC GAA CCT  
GTC TGT AAC AACA | Ehling-Schulz et al., 2006 |
| NA2 F | nheA and nheB | 766 | AAG CIG CTC TTC  
GIA TTT ITI GTT GAA ATA  
AGC TGT GG | Ehling-Schulz et al., 2006 |
| NB1 R | | | | |
| HD2 F | hblD and hblA | 1091 | GTA AAT TAI GAT  
GAI CAA TTT C  
AGA ATA GGC ATT  
CAT AGA TT | Ehling-Schulz et al., 2006 |
| HA4 R | | | | |
| NHEA F | nheA | 755 | GTT AGG ATC ACA  
ATC ACC GC  
ACG AAT GTA ATT  
TGA GTC GC | Guinebret-iere et al., 2002 |
| NHEA R | | | | |
| K3-CRYSTAL | | | | |
| K5-CRYSTAL | cry | 1635 | GCT GTG ACA CGA  
AGG ATA TAG  
CCA C  
AGG ACC AGG ATT  
TAC AGG AGG | Kuo et al., 2007, |

aF, Forward primer; R, Reverse primer  
rDNA spacer, spacer region between the 16S and the 23S rDNA  
CES, Cereulide synthetase  
NA2, non-hemolytic enterotoxin A component; NB1, non-hemolytic enterotoxin B component  
HD2, hemolytic enterotoxin D component; HA4, hemolytic enterotoxin A component  
NHEA, non-hemolytic enterotoxin A component  
K3 CRYSTAL, Forward primer for the *B. thuringiensis* crystal gene;  
K5 CRYSTAL, Reverse primer for the *B. thuringiensis* crystal gene
4.3.6. Low Temperature growth studies

The ability of *B. cereus* isolates to grow at 7°C and 12°C was determined. Five ml of Trypticase soy broth (TSB, Difco) was inoculated with a loopful of *B. cereus* isolates and incubated overnight at 32°C. Five ml of TSB was inoculated with 100 µl of this overnight-grown culture and held in a circulating low temperature water bath (Isotemp 1016S, Fisher Scientific, Pittsburgh, PA) at 12°C. Tubes were examined each day for visible growth for 14 days. Those isolates which grew at 12°C were checked for their ability to grow at 7°C using a similar protocol.

4.3.7. Antibiotic resistance

Isolates were grown to early exponential phase (A$_{600}$ = 0.2-0.3) in nutrient broth then spread plated (0.1 ml) on Mueller-Hinton agar. Antimicrobial sensitivity discs (Sensi Discs, BBL, Becton Dickinson) were placed on each plate. Two discs were used for each of the 10 antibiotics. The discs contained 10 µg of ampicillin, 30 µg of ceftriaxone, 30 µg chloramphenicol, 2 µg of clindamycin, 15 µg erythromycin, 30 µg nalidixic acid, 300 µg streptomycin, 30 µg tetracycline, 5 µg trimethoprim or 30 µg of vancomycin. Plates were incubated at 32°C for 24 h and the diameter of the zone of resistance around each disc was measured with reference to interpretive standards (Jorgensen and Turnidge, 2007).
4.4 Results and discussion

4.4.1. Levels of B. cereus and B. thuringiensis

Eighty three (46.6%) of the 178 rice samples were positive for B. cereus (Table 2). Bipyramid-shaped inclusions were detected visually in an additional 11 (6.1%) isolates confirming them as B. thuringiensis (Table 3). The levels ranged from 3.6 to 460 CFU/g for B. cereus and 3.6 to 23 CFU/g for B. thuringiensis. The ratio of B. cereus to B. thuringiensis isolates was similar to the ratio of these two Bacillus species in pasteurized milk (Zhow et al., 2008). The predominant (240 CFU/gm) aerobic spore-former in one rice sample was B. mycoides (not shown).

One isolate, #5, produced a red pigment, a characteristic of certain environmental strains of B. cereus (Canale-Parola, 1963).

4.4.2. Starch hydrolysis

The inability to hydrolyze starch has been reported to be indicative of the emetic subtype (Agata et al., 1996; Ehling-Schulz et al., 2005; Tuija et al., 1999; Valero et al., 2002) and indeed here 12 B. cereus strains previously implicated in emetic-type outbreaks were starch-hydrolysis negative (not shown). However 17 (18.0%) of the 94 Bacillus isolates here were negative for starch hydrolysis yet were ces-negative. Our results here and previously (Rahmati et al., 2008) indicate that this phenotype alone does not correlate with the ces-positive genotype (see also below).
Table 2. Characteristics of B. cereus isolates

<table>
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<th>MPN/g</th>
<th>Starch Hydrolysis</th>
<th>70°C</th>
<th>12°C</th>
<th>Tecra Test Kit Index&lt;sup&gt;a&lt;/sup&gt;</th>
<th>NHE complex</th>
<th>Oxoid Test kit Titer&lt;sup&gt;b&lt;/sup&gt;</th>
<th>HBL Complex</th>
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\(^a\) Isolate with an index of \(<3\) were considered negative

\(^b\) Isolates with a titer of \(<1:2\) were considered negative

\(^c\) ND, Not determined

\(^d\) NHE positive control strain

\(^e\) HBL positive control strain

\(^f\) Underlined isolates were obtained from white rice; sample numbers not underlined represent brown, black, or rice mixtures
Table 3. Characteristics of *B. thuringiensis* isolates

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*isolates with an index less than 3 were considered negative*

*isolates with a titer less than 1:2 were considered negative*

*underlined isolate numbers were obtained from white rice; isolate numbers not underlined were obtained from brown, black or rice mixtures*
4.4.3. Enterotoxin production

A number of *B. cereus* and *B. thuringiensis* isolates, 70 (84.3 %) and 11 (100 %) respectively, were positive for NHE enterotoxin using the Tecra VIA immunoassay kit (Tables 2 and 3). This level of toxin-positive *B. cereus* isolates is in accordance with the results of others (Hansen and Hendriksen, 2000; Guinebretiere et al., 2002; Dietrich et al., 2005; Yang et al., 2005; Moravek et al., 2006; Rahmati and Labbe, 2008). Similarly 51 (61.4%) and 11 (100 %) of *B. cereus* and *B. thuringiensis* isolates respectively tested positive for HBL using the Oxoid RPLA assay. In total 55 (58.5%) of the 94 isolates produced both the NHE and HBL whereas 89 (94.6%) were positive for either one of the two. We recently reported that 94% of *B. cereus* isolates from seafood also possessed either NHE or, less commonly, HBL (Rahmati and Labbe, 2008).

4.4.4. PCR

4.4.4.1 PCR for *B. cereus*

None of the *Bacillus* isolates possessed *ces* (Fig 1, lane 9). The absence of PCR-inhibiting compounds was confirmed by amplification of the rDNA 16S/23S spacer region (Fig 1, lane 9). To confirm further our PCR protocol we determined the presence of *ces* in 12 *B. cereus* isolates (in addition to the emetic control strain) from previous emetic-type outbreaks and which previously had been shown to possess *ces* (McIntyre, 2007). All produced a PCR reaction product corresponding to *ces* (not shown).

PCR for hemolysin BL components (*hblA* and *hblD* together) and NHE components (*nheA* and *nheB* together) was done using primers (Table 1) in which bases were substituted with inosine at known variable regions to account for the high degree of
polymorphism reported among the enterotoxin genes (Mantynen and Lindstorm, 1998; PrÜß et al., 1999; Hansen et al., 2000; Guinebertiere et al., 2002). For NHE the forward primer is located in the nheA gene whereas the reverse primer is located in the nheB gene and similarly, for HBL, the forward primer and the reverse primers are located in hblA and hblD respectively. This allows simultaneous detection of nheA and nheB genes of the NHE complex and hblD and hblA genes of the hemolysin BL complex (Ehling-Schulz et al., 2006).

The presence of the two genes (nheA and nheB) of the NHE complex was detected in 74 (89.1%) of the 83 B. cereus isolates (Table 2, Fig 1). Of the 83, 70 (84.3%) were positive for NHE as determined by the Tecra VIA assay yet seven of the NHE-positive isolates were negative for the composite primer pair for nheA/nheB. Therefore the PCR was repeated using the primer pair specific for nheA (which codes for the NHE toxin component in the Tecra assay). In this case five (#58, #139, #144, #154, #162) of the seven isolates gave a positive signal (Table 2; Fig. 1, lane 6). Three of the 83 B. cereus isolates produced NHE but were negative for nheA using either primer pair. We (Rahmati and Labbe, 2008) and others (Hansen and Hendriksen, 2001) have previously encountered B. cereus isolates producing NHE in the absence of nheA. These results highlight the polymorphism associated with nheA. Interestingly five isolates (#58, #139, #144, #154, #162) which were PCR-positive for nheA produced no PCR reaction product using NA2/NB1 primer pair. This is likely due to the absence of nheB which, together with nheA, the composite primer pair is designed to detect.

In the case of HBL 47 (56.6%) B. cereus isolates had both the hblD and hblA genes of the HBL complex (Fig. 1, lane 4). The genes of the HBL complex are generally
less common than that of the NHE complex (Al-Khatib et al., 2007; Moravek et al., 2006; Wong et al., 1998). In addition 51 (61.4%) of the *B. cereus* isolates were positive in the Oxoid test kit which measures the L$_2$ component of HBL (*hblC*).
Fig. 1. Agarose gel electrophoresis of PCR assay of *Bacillus cereus*. Lane 1 and 10, 1-kb ladder; lane 2, *B. cereus* ATCC 14579 using primer pair NA2 and NB1 (for *nheA* and *B*); lane 3, isolate #19 using primer pair NA2 and NB1; lane 4, *B. cereus* ATCC 14579 using primer pair HD2 and HA4 (for *hblD* and *hblA*); lane 5, isolate #8 using primer pair HD2 and HA4; lane 6, *B. cereus* 1230/88 using primer pair NHEA (for *nheA*), lane 7, isolate #58 using primer pair NHEA; lane 8, *B. cereus* F4810/72 using primer pair CES (for *ces*); lane 9, isolate #1 using primer pair G1 and L1 (for 16S-23S rDNA spacer region); lane 10, 1-kb ladder.
4.4.4.2. PCR for *B. thuringiensis*

In addition to visual determination of the presence of the crystal inclusion, 10 of the 11 *B. thuringiensis* isolates showed PCR reaction products using primers for the *cry* gene (Table 3; Fig. 2, lane 3). All the 11 isolates produced NHE toxin and were positive for *nheA* using either the composite or the individual primer pair for *nheA*. Nine of the 11 *B. thuringiensis* isolates possessed *hblA* and *hblD* (Fig 1, lane 7). Though the presence of *hblC* was not specifically determined, its gene product, L2, was detected in all 11 isolates using the Oxoid assay (Table 3). *B. thuringiensis* isolates have previously been shown to possess *nhe* and *hbl* and their associated toxins, NHE and hemolysin BL. (Garcia-Rivera et al., 2000; Hansen and Hendriksen, 2001; Kyei-Poku et al., 2007; Ngamwongsatit et al., 2008; Phelps and McKillip, 2002; Pruss et al., 1999). For insect control application of $10^6$ of *B. thuringiensis* or more per gram of leaf material is not unusual (Petersen et al., 1995). In fact NHE toxin has been found in commercial preparations of *B. thuringiensis*-based insecticides (Damgaard, 1995).
Fig. 2. Agarose gel electrophoresis of PCR assay of *B. thuringiensis*.

Lanes 1 and 10, 1-kb ladder; lane 2, *B. thuringiensis* using primer pair K3 and K5 (for *cry*); lane 3, isolate #136 using primer pair K3 and K5; lane 4, *B. cereus* ATCC 14579 using primer pair NA2 and NB1 (for *nheA* and *B*); lane 5, isolate #105 using primer pair NA2 and NB1; lane 6, *B. cereus* ATCC 14579 using primer pair HD2 and HA4 (for *hbl* D and *hbl*A); lane 7, isolate #105 using primer pair HD2 and HA4; lane 8, *B. cereus* 1230/88 using primer pair NHEA (for *nheA*), lane 9, isolate #136 using primer pair NHEA.
4.4.5. Low temperature studies

Sixty two (74.6%) of the *B. cereus* (Table 2) and seven (63.6%) of the *B. thuringiensis* isolates (Table 3) grew at 12°C while only one isolate (#14) was able to grow at 7°C. This isolate was positive in both toxin assays. Psychotrophic isolates are of importance since diarrheal enterotoxin has been shown to be produced at low temperatures (Dufrenne et al., 1995).

4.4.6. White vs. Brown rice

Forty three brown rice samples were analyzed of which 24 (55.1%) were positive for *B. cereus* with an average count of 52.9 CFU/g. For white rice, 89 samples were analyzed and 42 (47.2%) of those were positive for *B. cereus* with an average of 30.2 CFU/g. It is likely that the processing steps of milling and polishing reduce the counts in the final product (white rice). However the percent of white versus brown rice samples containing *B. cereus* was not statistically significant (P>0.05). All but one *B. thuringiensis* isolate were obtained from samples other than white rice. Of the eight wild rice samples analyzed four (50%) were positive for *Bacillus* spores with an average of 11.3 CFU/g (not shown). Thirty eight of the samples examined in this study were mixtures of different combinations of white, brown, black, wild rice with or without some kind of seasoning. Twenty four of these (63.1%) were positive for *B. cereus* with an average of 23.3 CFU/g. Spices in seasonings would also be a likely source of *Bacillus* spores.
4.4.7 Antibiotic resistance

The antibiotic resistance of 50 randomly selected enterotoxin-positive isolates was determined using a panel of antibiotics previously employed by our laboratory in a study of *B. cereus* from fish (Rahmati et al., 2008). Half or more of 50 isolates were resistant to three of the 10 antibiotics tested: ceftriaxone, streptomycin and tetracycline (Table 4). Resistance to ceftriaxone was also demonstrated in our earlier work. Resistance to tetracycline by foodborne *B. cereus* was observed previously (Rusul and Jaacob, 1995).
<table>
<thead>
<tr>
<th>Antimicrobial</th>
<th>Conc (µg/disc)</th>
<th>Resistant</th>
<th>Intermediate</th>
<th>Susceptible</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ampicillin</td>
<td>10</td>
<td>0</td>
<td>17 (34%)</td>
<td>33 (66%)</td>
</tr>
<tr>
<td>Ceftriaxone</td>
<td>30</td>
<td>50 (100%)</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Chloramphenicol</td>
<td>30</td>
<td>0</td>
<td>0</td>
<td>50 (100%)</td>
</tr>
<tr>
<td>Clindamycin</td>
<td>2</td>
<td>0</td>
<td>16 (32%)</td>
<td>34 (68%)</td>
</tr>
<tr>
<td>Erythromycin</td>
<td>15</td>
<td>9 (18%)</td>
<td>12 (24%)</td>
<td>29 (58%)</td>
</tr>
<tr>
<td>Nalidixic acid</td>
<td>30</td>
<td>19 (38%)</td>
<td>18 (36%)</td>
<td>13 (26%)</td>
</tr>
<tr>
<td>Streptomycin</td>
<td>10</td>
<td>50 (100%)</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Tetracycline</td>
<td>30</td>
<td>49 (98%)</td>
<td>1 (2%)</td>
<td>0</td>
</tr>
<tr>
<td>Trimethoprim</td>
<td>5</td>
<td>0</td>
<td>14 (28%)</td>
<td>36 (72%)</td>
</tr>
<tr>
<td>Vancomycin</td>
<td>30</td>
<td>22 (44%)</td>
<td>28 (56%)</td>
<td>0</td>
</tr>
</tbody>
</table>
B. cereus has been responsible for outbreaks in many countries including Japan, Sweden, Iceland, Canada and the U.S (Kramer et al., 1989; Schmidt, 2001). However there have been few reports on the prevalence and toxigenicity of B. cereus (or B. thuringiensis) in foods in U.S retail food in general or rice in particular. To our knowledge, this is the first attempt determine the potential toxigenic risks associated with B. cereus in U.S retail rice. Outbreaks with rice as the vehicle are typically associated with the emetic biotype; however there have been reports of outbreaks associated with cooked rice caused by the diarrheal subtype (Shinagawa et al., 1979). For this potential vehicle our results demonstrate that, if properly investigated, foodborne outbreaks due to B. cereus in the U.S. would most likely be associated with diarrheal-type cases. The potential risks of enterotoxins produced by B. thuringiensis have been noted (Jensen et al., 2002; Ngamwongsatit et al., 2008; Swiecicka et al., 2006) and their significance in foods remains to be determined.

Acknowledgements

We thank A. Wong for the control strains and L. McIntyre for the food-poisoning emetic strains.
CHAPTER 5
THE ABILITY OF SPORES OF ENTEROTOXIGENIC
B. CEREUS TO GROW IN RICE

5.1. Abstract

Bacillus cereus is a gram-positive, spore forming, facultative anaerobe that is responsible for two types of gastrointestinal diseases: emesis and diarrhea. In this study, the heat resistance of B. cereus spores at 95\(^0\)C, growth of spores in cooked rice in the range of 12-20\(^0\)C, effect of cooking on spore survival, and toxin production during growth were assessed. A significant difference in the heat resistance of spores of the emetic and the diarrheal type were found. The ability of an inoculum of a diarrheal- and emetic-toxin type spore mixture to germinate and grow in cooked rice was determined. Levels of 10^6/g were detected in cooked rice after 22h at 20\(^0\)C and after 34h at 17\(^0\)C whereas at 12\(^0\)C the counts did not go above 10^4/g even after 48h. The diarrheal strains did not grow well at inoculum levels of 10^2/g and 10^3/g level following cooking. In contrast, the emetic spores survived and outgrew at 10^3/g level but not at 10^2/g level. Our results suggest that the spore levels of the diarrheal strains found naturally in U.S retail rice are sufficiently inactivated during cooking that keeps the rice safe for consumption up to 48h at 20\(^0\)C at least with regard to the B. cereus diarrheal biotypes. No enterotoxin was detected in cooked rice when cell count reached 10^8/g after 48h. These results indicate that although diarrheal strains are predominant in U.S retail rice, the chances of foodborne illness arising from these strains are low because of their inability to survive cooking and outgrow, attributed to the low heat resistance of their spores.
5.2. Introduction

*Bacillus cereus* is a pathogenic bacterium that is frequently associated with emetic and diarrheal types of gastrointestinal diseases. The emetic syndrome is caused by ingesting heat stable, pH stable, peptide toxin preformed in the food whereas the diarrheal syndrome is caused by ingesting cells of *B. cereus* which cause illness by producing one or more enterotoxins in the intestine. Starchy foods are most commonly associated with the emetic biotype whereas the diarrheal subtype is associated with meat, soups, sauces, vegetables and milk products (Kramer and Gilbert, 1989). In countries where rice is the staple diet, the emetic type is more frequently reported, for example, in Japan whereas in the U.S and Europe the diarrheal type is more common (Granum, 2007).

Each of the two different enterotoxins, NHE and HBL, produced by *B. cereus* are composed of three different protein complexes namely A, B, C and B, L₁ and L₂ (Heinrichs et al., 1993; Granum and Lund 1997; Lund et al., 2000). Commercial kits are available to detect enterotoxin (NHEA or L₂) production by *B. cereus*. The Tecra VIA visual immunoassay kit (Tecra Diagnostics, Roseville, Australia) measures the *nheA* component of the NHE enterotoxin whereas Oxoid reversed passive agglutination (RPLA) (Unipath-Oxoid, Columbia, MD) measures the L₂ component of the HBL enterotoxin.

Although farinaceous foods are almost exclusively associated with emetic outbreaks there has been at least one report of an outbreak from cooked rice caused by the diarrheal toxin type (Shinagawa et al., 1979). Recently we reported that diarrheal strains are predominant in U.S retail rice and are present at levels ranging from 3.6-460
CFU/g (Ankolekar et al., 2008). Considering that rice is a widely consumed cereal and *B. cereus* is so closely associated with rice, the objectives of this work were to a) determine the spore heat resistance of selected *B. cereus* strains, isolated from rice, in aqueous suspension, b) characterize the spore germination and growth of diarrheal strains of *B. cereus* in cooked rice at different temperatures, c) determine survival and outgrowth of emetic and diarrheal strains of *B. cereus* spores during cooking, and d) determine the production of NHE enterotoxin during growth in cooked rice.

### 5.3 Materials and Methods

#### 5.3.1. Bacterial strains

The emetic strains used in this study were a) F4810/72 originally obtained from an outbreak associated with cooked rice (Turnbull et al., 1979), b) strains #2, #4, #6, and #8 from L. McIntyre, British Columbia (BC Center for Disease Control) and c) emetic strain (#10) isolated from raw fish in a study previously done in our lab (Rahmati and Labbe, 2008).

The diarrheal strains (# 85, #102, #113, #115, #133, #145) used in this study were isolated from U.S retail rice (Ankolekar et al., 2008) and were selected on the basis of i) ability to grow at 12°C, ii) the presence of the *nhe* or *hbl* genes along with the ability to produce the corresponding gene product (enterotoxins) at elevated titers. All the isolates were maintained in the spore state on nutrient agar (NA) slants at 4°C.
5.3.2. Preparation of spore suspensions

Tryptic Soy Broth tubes were inoculated with a loop full of the stock culture and incubated overnight at 32°C to obtain vegetative cells. 100µl of this overnight culture was surface spread onto a sporulation medium containing nutrient agar with added salts of MnSO₄ and CaCl₂ at 0.001% level each. The plates were incubated at 37°C for four days. Spores were harvested with cold, sterile sodium-potassium phosphate buffer (0.05M, pH 7) using disposable sterile plastic spreaders. Vegetative cells, cell debris and other impurities were removed by employing low speed centrifugation (5000g for 10 min), resuspending in phosphate buffer and repeating the process a sufficient number of times (at least thrice or more) to obtain suspensions with >95% refractile spores. The spores were stored in the same buffer at 4°C.

5.3.3 Rice

White, organic rice (Nature’s Promise brand) from a local supermarket (Stop and Shop) was used in this study. Raw rice was cooked and stored for 2 days at 20°C and checked for growth of any microorganism. None was observed indicating that any growth in cooked rice in subsequent studies was due to external addition of *B. cereus*.

5.3.4. Determination of heat resistance of spores

The method followed here was as described by Gilbert *et al.*, (1974). The stock spore suspensions were diluted appropriately to an absorbance of 0.1-0.2 at 600nm in sodium-potassium phosphate buffer, pH 7.0. A diarrheal spore mix based on equal number of spores from three different strains with the highest D₀₅ was prepared and
stored in sodium-potassium phosphate buffer at 4°C. Into 2ml freeze drying ampoules, 0.2ml samples were distributed and the ampoules were then flame-sealed. Ampoules containing spores were heated in a thermostatically controlled water bath at 95°C by complete immersion.

Ampoules were removed from the water bath at specific time intervals, immediately cooled in an ice-water mixture, opened, the contents serially diluted with 0.1% peptone, and plated in duplicate. Nutrient Agar plates supplemented with 1% glucose were used for enumeration. Two ampoules were used for each strain tested and the plating was done in triplicates. On a semi-log scale, the survivor curves were plotted and the negative reciprocal of the slopes was used to compute the D95 values. Colonies of *B. cereus* are typically visible within 24h on NA at 37°C. To assess spore injury preliminary experiments were done which included prolonged incubation (2-3 additional days) of plates.

5.3.5. Growth of *B. cereus* diarrheal strains in cooked rice

In order to characterize the germination and growth from spores of diarrheal strains in cooked rice, 10g of cooked rice were distributed in 25 ml beakers and inoculated with 200-300µl of 1.25 x 10^4 spores/ml spore mix to give a initial concentration of approximately 10^2 spores/g raw rice. The spores were not heat-shocked since little effect on subsequent vegetative cell growth in boiled rice was observed by this treatment (Gilbert and Parry, 1977). Inoculated rice samples were then incubated at 12°C, 17°C, and 20°C representing the different temperature-abused conditions. The beakers were taken at appropriate time intervals (at least 7), serially diluted and plated onto NA
plates to obtain cell numbers. Growth curves were plotted from the resulting data. Generation times were determined using the slope of the regression line of the exponential phase of growth.

5.3.6. Spore survival and outgrowth following cooking of rice

Eighty ml of water was taken in a rice cooker. An appropriate number of the diarrheal-type spore mixture representing $10^2$ and $10^3$ spores/g raw rice was added to the water. This level is approximately twice the average of and is equal to the maximum counts of *B. cereus* (diarrheal toxin type) spores detected in U.S retail raw rice (Ankolekar et al., 2009). The spores were well distributed by shaking the vessel. Forty g of raw rice were added and heated until completion as determined by the unit’s cooking cycle. Rice was allowed to cool to room temperature and 10g samples were distributed into 25ml beakers and incubated at 20°C. At times zero h, 24 h and 48 h cell numbers were determined using a 1:5 dilution with 0.1% peptone (Difco, Becton Dickinson, Sparks, MD), followed by enumeration by MPN as previously described (Ankolekar et al., 2009). For comparison, an emetic spore mixture was prepared and a similar protocol was followed.

5.3.7. Enterotoxin production:

NHE enterotoxin was assayed using an enterotoxin visual immunoassay kit (Tecra Bioenterprises, Pty, Roseville, Australia). Ten g rice samples were inoculated with 200µl of $10^4$ spore/ml spore mix. The cell numbers were enumerated after 48 h using a 1:5 dilution with peptone (0.1%). Ten ml of the dilution were distributed into 1.5 ml
Eppendorf centrifuge tubes (1 ml each) and concentrated 10 times using a Speed Vac (specifications) to obtain a final volume of 0.1ml in each tube. The immunoassay was carried out according to the manufacturer's instruction using the collective volume of 1 ml from all the tubes. This represented an undiluted sample of the rice.

5.4. Results and Discussion

5.4.1. Heat resistance of spores

A wide range of $D_{95}^0$ values of 1.2-36.2 min in phosphate buffer (PB) has been reported for $B. cereus$ spores (Kramer and Gilbert, 1989). $D_{95}$ values of spores of the emetic and the diarrheal strains are shown in Table 5. A representative inactivation plot is shown in Fig. 3). The range of $D_{95}$ values for six diarrheal strains tested were in the range of 1.78-3.44 min with an average 2.79 min. In contrast, the $D_{95}$ values for the six emetic strains ranged from 12.72-27.93 min with an average of 19.33 min. This difference in heat resistance was found to be statistically significant (P<0.05). Similar differences between emetic and diarrheal strains results have been shown in another study (Carlin et al., 2006). Parry and Gilbert (1980) demonstrated that the average $D_{95}$ values of 14 emetic strains were significantly higher than 13 non-emetic strains. The $D_{95}$ values of spores of eight strains (heated in distilled water) of $B. cereus$ isolated from Spanish raw rice ranged from 0.69 to 5.17 (Sarrias et al., 2002). These strains produced one of the two enterotoxins but the emetic toxin potential was not determined.
Table 5. Heat resistance of *B. cereus* spores

<table>
<thead>
<tr>
<th>Strain</th>
<th>Toxin type</th>
<th>$D_{05}$ (min)</th>
</tr>
</thead>
<tbody>
<tr>
<td>102</td>
<td>Diarrheal</td>
<td>2.87</td>
</tr>
<tr>
<td>85</td>
<td>Diarrheal</td>
<td>1.98</td>
</tr>
<tr>
<td>113</td>
<td>Diarrheal</td>
<td>3.23</td>
</tr>
<tr>
<td>133</td>
<td>Diarrheal</td>
<td>2.24</td>
</tr>
<tr>
<td>36</td>
<td>Diarrheal</td>
<td>3.03</td>
</tr>
<tr>
<td>75</td>
<td>Diarrheal</td>
<td>3.44</td>
</tr>
<tr>
<td>F4810/72</td>
<td>Emetic</td>
<td>13.79</td>
</tr>
<tr>
<td>#10</td>
<td>Emetic</td>
<td>15.74</td>
</tr>
<tr>
<td>2</td>
<td>Emetic</td>
<td>27.93</td>
</tr>
<tr>
<td>4</td>
<td>Emetic</td>
<td>12.72</td>
</tr>
<tr>
<td>6</td>
<td>Emetic</td>
<td>24.51</td>
</tr>
<tr>
<td>8</td>
<td>Emetic</td>
<td>21.3</td>
</tr>
</tbody>
</table>
Fig 3 Inactivation of spores of representative emetic and diarrheal *B. cereus* strains.

Spores were heated at 95°C in 0.05 M, pH 7.0 phosphate buffer.
5.4.2. Growth in cooked rice:

Based on the $D_{95}$ values, a diarrheal spore mixture and an emetic spore mixture consisting of thereof the most heat resistant strains were made. The diarrheal spore mix consisted of equal numbers of the isolates #36, #75 and #113 whereas the emetic spore mix consisted of equal numbers of isolates #2, #6, and #8.

The germination and growth of a spore mixture of diarrheal strains of *B. cereus* at different temperatures is shown in Table 6. Three different temperatures of $12^9C$, $17^9C$ and $20^9C$ were used to characterize the growth of the diarrheal toxin type. At $20^9C$ the counts reached $10^6/g$ within 24h of storage with a starting inoculum of $1.0 \times 10^2/g$. At $17^9C$, the counts reached $10^6/g$ within 36h of storage with a starting inoculum of $2.5 \times 10^2/g$ (Fig 4). There was slight growth at $12^9C$ but counts did not go beyond $10^4/g$ even after 48h of storage. This indicates that, with regard to growth of diarrheal strains, numbers of *B. cereus* would remain below hazardous levels even after two days in cooked rice held at $12^9C$ (at initial spore levels found in raw rice) instead of storing it at refrigerated temperatures, thereby reducing the damage to quality done by refrigeration.

Maximum counts of $10^8/g$ were obtained at both $20^9C$ and $17^9C$. At $20^9C$ and $17^9C$, the mean generation time observed were 93 min and 121 min respectively. Similar generation times in rice at $20^9C$ and $17^9C$ has been reported (Johnson et al., 1983). A similar growth rate at $22^9C$ in boiled rice was observed by Parry and Gilbert, (1980), starting with a slightly higher inoculum level of $10^3/g$. 

46
Table 6. Growth (log$_{10}$) per g rice of spores of diarrheal-type *B. cereus* spore mixture in cooked rice. Initial inoculum level: 2-3 x 10$^2$/g

<table>
<thead>
<tr>
<th>Time (hr.)</th>
<th>Temperature of incubation</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>20$^0$C</td>
</tr>
<tr>
<td>0</td>
<td>2.03</td>
</tr>
<tr>
<td>4</td>
<td>2.24</td>
</tr>
<tr>
<td>10</td>
<td>3.13</td>
</tr>
<tr>
<td>14</td>
<td>4.44</td>
</tr>
<tr>
<td>18</td>
<td>5.34</td>
</tr>
<tr>
<td>22</td>
<td>6.54</td>
</tr>
<tr>
<td>28</td>
<td>7.1</td>
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<td>34</td>
<td>7.68</td>
</tr>
<tr>
<td>39</td>
<td>8.14</td>
</tr>
<tr>
<td>41</td>
<td>N.D</td>
</tr>
<tr>
<td>44</td>
<td>8.33</td>
</tr>
<tr>
<td>48</td>
<td>8.43</td>
</tr>
</tbody>
</table>

N.D, Not determined
Fig 4. Growth from diarrheal-type *B. cereus* spores.

A three strain mixture (1-2.5x10^2 spores) was added to cooked rice an incubated at the indicated temperature.
5.4.3. Spore survival and outgrowth during cooking of rice:

A graph of temperature vs. time (Fig5) was plotted for the rice cooker to comprehend the conditions the spores were subjected to. The results of spore survival and growth of diarrheal versus emetic strains of *B. cereus* following the cooking of rice reflected the $D_{95}$ values of the spores of each toxin type. The diarrheal strains did not grow well at $10^2/g$ and $10^3/g$ levels. In contrast, the emetic spores survived and outgrew at $10^3/g$ level but not at $10^2/g$ level (Table 7). Gilbert et al (1974), studied survival and outgrowth with two emetic strains of *B. cereus* with $D_{95}$ values of 21 and 5 min and observed good growth of these strains following cooking within 24h. Chung and Sun (1986), reported a two log$_{10}$ decrease in the number of spores with a 17.5 min cooking period and a single log$_{10}$ decrease with a 11.5 min cooking period. However, no data on outgrowth, following cooking, were reported.
Figure 5 Temperature vs. Time plot for the rice cooker
Table 7. Survival and growth (MPN/g) at 20° C of *B. cereus* spores following cooking in rice

A mixture of emetic- or diarrheal-type spore strains was added at levels of $10^2$ or $10^3$/g to rice and heated in a rice cooker.

<table>
<thead>
<tr>
<th>Time (hr)</th>
<th>Diarrheal</th>
<th>Emetic</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>$10^2$/g</td>
<td>$10^3$/g</td>
</tr>
<tr>
<td>0</td>
<td>&lt;18</td>
<td>15</td>
</tr>
<tr>
<td>24</td>
<td>30.5</td>
<td>37</td>
</tr>
<tr>
<td>48</td>
<td>55</td>
<td>180</td>
</tr>
</tbody>
</table>
5.4.4. Enterotoxin production

NHE enterotoxin production was not detected even at stationary phase levels of cells, i.e., when cell counts reached $10^9/g$ (48h at 20°C). Finlay et al. (2000) studied emetic toxin production in skim milk medium and observed that greater quantities of the toxin were produced at 12°C and 15°C than any other temperatures tested and also the lowest viable count capable of producing detectable amount of emetic toxin was at 12°C. Lee et al., (2006) studied HBL enterotoxin production in oriental-style rice cakes, and observed that toxin was detected in white rice flour cakes only on day 7. These reports, along with our results, suggest that toxin production by *B. cereus* in food is not only dependent on the cell numbers but likely on other factors such as pH, $a_w$, temperature, nature of the food etc. Current thinking is that diarrheal toxins are formed after ingestion of large numbers of vegetative cells which produce one or more enterotoxins in the intestine as opposed to the emetic toxin which is preformed in food (Granum, 2007). It is important to characterize toxin production so that measures applied to control diarrheal enterotoxins in food could also be applied to controlling emetic toxin in food.

In summary, although diarrheal strains of *B. cereus* are predominant in U.S retail rice, at levels found naturally in raw rice, the chances of a diarrheal outbreak associated with cooked rice is low because the cooking process sufficiently inactivates the spores and heat injury likely renders survivors incapable of outgrowth. Even though emetic strains are associated with raw rice to a lesser extent than diarrheal strains (reference), they have the potential to cause illness owing to the extremely heat resistant nature of their spores and ability to grow at high temperatures. Growth at high temperatures may
especially be a problem in Chinese restaurants where large amounts of rice are prepared well before consumption and which require several hours to cool down. Our data suggests that the chances of diarrheal-type foodborne illness in the U.S from cooked rice is low but, because \textit{B. cereus} is so commonly isolated from foods, cross contamination is always possible and one must be careful about exposing food to the temperature range of 4-40\textdegree C for more than 4 hrs.
CHAPTER 6
PHYSICAL CHARACTERISTICS OF B. CEREUS SPORES ISOLATED FROM RICE

6.1. Abstract

Five strains of *B. cereus* representing three species, all isolated from U.S retail rice, were studied with respect to their physical characteristics; specifically their morphology, surface hydrophobicity and $\zeta$-potentials were studied. All the strains tested had an exosporium. By contrast all except *B. mycoides* had appendages. The length of the appendages ranged from 0.45-3.8$\mu$m. The isolates tested were classified as being moderately to highly hydrophobic. All the isolates tested had a net negative charge. The long length of the appendages, high hydrophobicity and a net negative charge indicated that these isolates could have high potential to adhere to inert surfaces.

6.2. Introduction

*Bacillus cereus* is a world-wide food borne pathogen causing diarrheal or emetic type illnesses. The presumptive toxins have been identified in each case (Gilbert, 1979; Agata et al., 1995; Granum and Baird-Parker, 2000).

Two heat-labile enterotoxins, hemolysin BL (HBL) and a non-hemolytic enterotoxin (NHE) are associated with the former and a heat and acid stable cyclic dodecadepsipeptide (Cereulide) with the latter. A wide variety of foods have been associated with the diarrheal syndrome whereas farinaceous foods are typically associated with the emetic illness.
Best known for the insecticidal activity of its parasporal crystals, *Bacillus thuringiensis*, has been associated with gastroenteritis and isolated in rare cases from outbreaks of food borne illness (Jackson et al., 1995). Isolates of *B. thuringiensis* have been shown to posses all NHE and HBL component genes (Ankolekar et al., 2009; Kyei-Poku et al., 2007) and produce one or both of the enterotoxins, HBL and NHE (Ankolekar et al., 2009; Hansen and Hendriksen, 2001; Phelps and Mckillip, 2002; Pruss et al., 1999) including commercial based insecticides (Damgaard, 1995).

Spores of certain *Bacillus* species possess appendage like structures (Abram, 1966; Hachisuka and Kuno, 1976; Hodgkiss, 1971). In the case of *B. cereus* the appendages, the hydrophobicity of spore surfaces, and the spore exosporium may be responsible for the ability of *B. cereus* spores to adhere to inert surfaces (Faille et al., 2007; Husmark and Ronner, 1992; Tauveron et al., 2006). However in the case of appendages, Stalheim and Granum (2001) reported only minor difference in the ability to adhere to stainless steel between spores with and without appendages. Controversy remains over the generally-accepted fact that appendages may initiate contact with surfaces by penetrating potential barriers which might otherwise prevent the spores from adhering (van Loosdrecht et al., 1989). The length of the appendages and the size of the exosporium were reported to influence the adhering ability of *B. cereus*. In general, a longer length of the appendages and smaller size exosporium resulted in greater number of spores adhering to inert surfaces (Faille et al., 2007; Tauveron et al., 2006). The presence or absence of appendages and exosporia has also been proposed as a supplementary diagnostic test for classification of the *Bacillus* species (Hachisuka et al., 1984).
Rice has been a vehicle of foodborne illness due to emetic strains of *B. cereus* (Finlay et al., 2002; Kramer and Gilbert, 1989). We recently reported that diarrheal type was the more common toxigenic type in U.S. retail rice. Spores of *B. thuringiensis* and *Bacillus mycoides* were also isolated. Here we present some of the physical properties of spores of the *B. cereus* group isolated from rice.

6.3. Materials and Methods

6.3.1 Spore production

All the strains were isolated from U.S retail rice (Ankolekar et al., 2009). Two diarrheal *B. cereus* strains (#85, #133), two *B. thuringiensis* strains (#105, #129) and one *B. mycoides* strain (#157) were used in this study. The strains were selected on the basis of the presence of *nhe* or *hbl* gene along with the ability to produce the corresponding gene product at elevated titers. As a comparative reference, *B. subtilis* (ATCC 6633) was used. All the isolates were maintained in the spore state on nutrient agar (NA) slants at 4°C.

For bulk production of spores, tryptic soy broth was inoculated with a loop full of the stock spore and incubated overnight at 32°C. One hundred μl of this overnight culture of vegetative cells were surface-spread onto a sporulation medium. The sporulation medium consisted of nutrient agar with 0.001% CaCl₂ and MnSO₄ each as sporulation aides. The spores were harvested with cold, sterile, sodium-potassium phosphate buffer (molarity, pH 7.0), after incubating the plates for four days at 37°C. Low speed centrifugation was used (5000g, 10min) to remove cell debris, vegetative cells and other impurities. This process was repeated a sufficient number of times (at least three) to
obtain suspensions with >95% spores when observed under a phase-contrast microscope. The spores were stored in sodium-potassium phosphate buffer at 4°C.

6.3.2. Separation of inclusion bodies from spores for *B. thuringiensis*

The spores of *B. thuringiensis* were separated from the inclusion bodies (IB) by centrifuging in linear gradients of sucrose. Linear gradients of 0.6-1.8 g/ml sucrose in two 30ml centrifuge tubes were prepared with a 100ml density gradient apparatus for this purpose. The stock spore suspension in sodium-potassium-phosphate buffer was diluted to an absorbance of 0.4 at 600nm. Two-three ml of the diluted suspension was layered on top of the gradient. The gradients were centrifuged for 2.5 hr in a swinging bucket rotor at 450 x g at 10°C. A visible white layer of spores were collected from the bottom with a Pasteur pipette. The layer was washed with 0.85% saline (at least twice) and stored in the same at 4°C.

6.3.3. Spore morphology by TEM

After adsorption of samples onto carbon coated colloidal filmed grids for 4-10min, the excess was drained to filter paper and negative stains were made by immediately touching the grid to a drop of 2% aqueous uranyl acetate, staining 30s, draining the stain to filter paper and air drying. Shadowed preparations were adsorbed the same way, excess sample drained and air dried. The grids for shadowing were placed in a Balzers BA080T evaporator and evacuated to 1x10-4pa and shadowed at 25 degrees elevation with 2nm (measured at normal incidence) electron-beam generated platinum-
carbon. All preparations were observed in a JEOL 100s transmission electron microscope at an accelerating voltage of 80 kV.

6.3.4. Hydrophobicity of \textit{B. cereus} spores

Spore hydrophobicity was measured using the bacterial adhesion to hydrocarbon (BATH) assay (Rosenberg et al., 1980). In this method, spore suspensions in aqueous media were mixed with a hydrocarbon and the hydrophobicity was determined by the decrease in the absorbance of the aqueous media before and after mixing. The extent of decrease in the absorbance is directly related to the hydrophobic nature of the spores. Usually, 3 mls of aqueous spore suspension (1-2.5 x \(10^7\) spores/ml) in saline (0.145M) and 1 ml of hexadecane were layered in a test tube and vortexed vigorously (60s). The mixture was then allowed to stand for 30 min to allow phase separation. Absorbance of the aqueous phase before and after mixing was determined at 600nm. The mean and the standard deviations were calculated from a minimum of seven measurements.

6.3.5. \(\zeta\)-potential measurements

\(\zeta\)-potentials of the spores were measured in a Malvern Zetasizer (Specifications) using the Smoluchowski equation. The spores were suspended in saline (0.145M) at a pH of 6-7. The \(\zeta\) potentials were determined from a mean value of five measurements.
6.4. Results and Discussion

6.4.1. Spore morphology

The TEM images of spores of two diarrheal *B. cereus* strains, two *B. thuringiensis* strains and one *B. mycoides* strain were analyzed. Appendages were observed on *B. cereus* (Fig 6,7) and *B. thuringiensis* (Fig 8,9,10) but not *B. mycoides* (Fig 11). By contrast, exosporia were seen in all the isolates examined (Table 8). Similar observations have been reported by others (Tauveron et al., 2006; Hachisuka et al., 1984; Husmark and Ronner, 1992; Mizuki at al., 1998). In previous studies, contradictory results have been reported regarding the presence of appendages on *B. mycoides* (Husmark and Ronner, 1992). This could be attributed to the fact that the appendages of some species are more fragile and could be easily lost during the preparation procedures (Husmark and Ronner; 1992; Tauveron et al., 2006). Here where the appendages and the exosporia were present, they were very clear and easy to observe.

The number of appendages varied among strains. *B. cereus* #85 had many appendages (4-9) whereas *B. cereus* #133 had only a few (1-4). On the other hand, *B. thuringiensis* #105 and #129 had higher (14-16) and (12-18) respectively] numbers per spore. The appendage length for *B. cereus* varied from 0.45-3.8µm. In all cases, most of the appendages on a spore were seen protruding from the ends and were whip-like in shape.
Table 8. The presence of appendages and exosporia in *Bacillus* species isolated from rice

<table>
<thead>
<tr>
<th>Isolates</th>
<th>Figure</th>
<th>Appendages</th>
<th>Exosporium</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>B. cereus</em> #85</td>
<td>1</td>
<td>Yes</td>
<td>Yes</td>
</tr>
<tr>
<td><em>B. cereus</em> #133</td>
<td>2</td>
<td>Yes</td>
<td>Yes</td>
</tr>
<tr>
<td><em>B. thuringiensis</em> #105</td>
<td>3</td>
<td>Yes</td>
<td>Yes</td>
</tr>
<tr>
<td><em>B. thuringiensis</em> #129</td>
<td>4,5</td>
<td>Yes</td>
<td>Yes</td>
</tr>
<tr>
<td><em>B. mycoides</em> #157</td>
<td>6</td>
<td>No</td>
<td>Yes</td>
</tr>
</tbody>
</table>
Figure 6 Negative stain of *B. cereus* #85 showing appendages and exosporium

Figure 7 Negative stain of *B. cereus* #133 showing appendages and exosporium
Figure 8 Negative stain of a cluster of *B. thuringiensis* #105 spores with appendages and exosporium

Figure 9 Shadowed image of *B. thuringiensis* #129 showing appendages, exosporium and inclusion
Figure 10 Negative stain of *B. thuringiensis* #129 showing tail fibers.

Figure 11 Negative stain of *B. mycoides* #157 showing exosporium without appendages.
6.4.2. Hydrophobicity

The relative hydrophobicity of the five strains representing three species was measured. Large differences was not observed between the species tested (Table 9). The hydrophobicity values for the isolates tested were observed to be in a considerably narrow range of 42.4 to 55.6%. Similar values for spores of the *B. cereus* group have been reported by others (Husmark and Ronner, 1992; Ronner et al., 1990; Doyle et al., 1984). From the values obtained here these isolates can be classified to be moderately to highly hydrophobic. Exosporia have been shown to be responsible for surface hydrophobicity of spores (Ronner et al., 1990, Husmark and Ronner, 1992). So the results reported here support this fact considering that an exosporium was seen in images of all the species tested.

Doyle et al., (1984) mentioned the hydrophobicity values for *B. thuringiensis* but no separation of the inclusions from the suspension was reported. The values reported here are assuming that the presence of the inclusions do not alter the hydrophobicity values significantly.

There are several methods reported in the literature to measure surface hydrophobicity of spores with each method having its own advantages and disadvantages (Dillion et al., 1986; Van der Mei et al., 1987; Mozes and Rouxhet, 1987). The BATH assay is generally regarded as the simplest and the least time consuming method of all with results being comparable to those obtained by other methods (Husmark and Ronner, 1992).
6.4.3. Charge

The electrophoretic mobility of spores was determined by the $\zeta$-potential (Table 9). The highest negative charge of -12.8 (#129) and -12.2 (#105) was observed amongst \textit{B. thuringiensis} followed by -10.9 (#133) and -10.6 (#85) amongst the two strains of \textit{B. cereus}. \textit{B. mycoides} had a slightly net lower negative charge (-8.2). Although it has been reported that lower charge indicates a higher adhering ability of spores (Husmark and Ronner, 1992; Gilbert et al., 1991) some authors suggest that the net charge matters only when the hydrophobicity of the spores is low (van Loosdrecht et al., 1987). Complete separation of the inclusions in \textit{B. thuringiensis} isolates was not possible. After separation, the suspensions had <20% inclusions (relative to spores) as observed by phase contrast microscopy. However, no significant difference in the net charge was observed between \textit{B. thuringiensis} isolates with and without the inclusions separated.
<table>
<thead>
<tr>
<th>Isolates</th>
<th>Relative hydrophobicity (% ± SD)</th>
<th>Zeta potential (mV ± SD)</th>
</tr>
</thead>
<tbody>
<tr>
<td>B. cereus #85</td>
<td>44.4 ± 4.9</td>
<td>-10.6 ± 1.04</td>
</tr>
<tr>
<td>B. cereus #133</td>
<td>51.9 ± 4.4</td>
<td>-10.9 ± 0.63</td>
</tr>
<tr>
<td>B. thuringiensis #105</td>
<td>55.6 ± 3.0</td>
<td>-12.2 ± 3.23</td>
</tr>
<tr>
<td>B. thuringiensis #129</td>
<td>42.4 ± 4.2</td>
<td>-12.8 ± 2.55</td>
</tr>
<tr>
<td>B. mycoides #157</td>
<td>51.5 ± 3.8</td>
<td>-8.18 ± 0.94</td>
</tr>
</tbody>
</table>

Table 9 The relative hydrophobicity and the ζ-potential of *Bacillus* isolated from rice
Here the spore morphology, hydrophobic characteristics and the net charge of two *B. cereus*, two *B. thuringiensis* and one *B. mycoides* isolated from U.S retail rice were investigated. Although there are studies on differences in the physiological characteristics of *Bacillus* spores relating to the species, strains, growth conditions and survival, primarily because of the changes to the exosporium, there are no reports on such examination relating to the origin of these species.

All the isolates observed here had exosporia surrounding the spores. Appendages were seen around all isolates except *B. mycoides*. The number and the length of appendages varied among the strains. The spores had a net negative charge on all the strains tested. Long appendages, high hydrophobicity and net negative charge observed in the strains here suggest that they have a high potential to adhere to inert surfaces. The results obtained here conform well to the previous work on these species and indicate that the physiological characteristics of *Bacillus* spores are independent of the source although more data testing spores from different sources is required to confirm this hypothesis. It is clear that the role of surface charge, exosporia, and hydrophobicity in spore adhesion, whether in natural conditions or on processing equipment (as, e.g., in biofilms), remains to be clarified.
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