THE RELATIONSHIP AMONG CELL MEMBRANE FATTY ACIDS, EMETIC TOXIN PRODUCTION, AND CYTOTOXICITY IN *BACILLUS CEREUS*

By

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THE RELATIONSHIP AMONG CELL MEMBRANE

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AND CYTOTOXICITY IN *BACILLUS CEREUS*

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GLOSSARY

12 x shot gun status-is also known as genome shotgun sequencing. Long genomes cannot be sequenced in one piece; so the DNA is spliced into random fragments and the fragments are sequenced. This is done at 12x or greater coverage to ensure accuracy.

β-hemolysis- complete lysis of red blood cells in media; it is a clearing of the medium around the growth.

Ante-penultimate- third from last.

Cell pellet- is the solid material at the bottom of a tube after centrifugation of the bacterial culture.

Culture filtrate- cultured filtrate is the supernatant of a bacterial culture has been centrifuged. It is no longer media because it has been exposed to a bacterial culture for a period of time and can contain toxins, proteins, etc. that was not originally in the media.

Cytokines- are a group of proteins and peptides used for cell-to-cell signaling. They are often used in innate and adaptive immune responses. Two much cytokine production is known as a cytokine storm causing accumulation of too many immune cells and potential blockage of airways or blood.

Heat Stable toxin(s)- is/are the heat stable toxin(s) purified by autoclaving the cultured filtrate.

LD_{50}- a dose required to kill half of a tested population using a toxic substance or radiation.

Malonyl CoA- is a derivative of the coenzyme A and is important in fatty acid elongation.

Mesophilic- organisms that grow optimally at temperatures between 20°C and 45°C.

Monkey Emesis Assay- The feeding of emetic and non-emetic toxins to monkeys to the observe the effect(s) of different doses.

GLOSSARY

Oropharynx- oral part of the pharynx, from the soft palate of the mouth to the hyphoid bone.

Penultimate- next to last.

Psychrophilic- organisms that grow optimally at low temperatures, 15°C and below.

Rectal Tenesmus- a feeling of incomplete defecation, the inability or difficulty to empty bowel at defecation.

Sepsis- has two main definitions: (1) pathogenic organisms or toxins in the blood or tissues (2) a condition relating to poisoning from pathogenic organisms or toxins in the blood or tissues.

Vacuolation- the formation or multiplication of vacuoles, which are typically membrane bound components of the cytoplasm.

Variable Number Tandem Repeat- is a genetic tool using variable numbered (14 to 100 nucleotides) sequenced nucleotides that are in clusters that repeat in tandem from 4-40 times.

ABBREVIATIONS

ANOVA- analysis of variance

ATCC- American Type Culture Collection.

BHI- Brain Heart Infusion

CDC- Center for Disease Control

CF- Culture Filtrate

DMSO-Dimethyl Sulfoxide

DMEM- Dulbecco's Modified Eagles Minimal Essential Medium

FBDO- Food Born Disease Outbreak

GCMS- Gas Chromatography/ Mass Spectra

HuH-7- Hepatocellular Carcinoma Cell Line

LRN- Laboratory Response Network

MTT-3-[4,5-dimethylithiazol-2-yl]-2,5-dipheyl tetrazolium bromide

NIH- National Institutes of Health

OD- Optical Density

TSA II- Trypticase Soy Agar with 5% Sheep Blood

CHAPTER I

INTRODUCTION

The intentional attempt to spread anthrax through the U.S. Postal Service in 2001 (Inglesby et al., 2001) revealed a disturbing picture of a poorly structured and ill-equipped system to deal with consequences of such a bioterrorism event. The realization that neither the U.S. nor the rest of the world is prepared for the repercussions of bioterrorism has sparked research directed toward obtaining a better understanding of biological warfare agents.

Anthrax is caused by *Bacillus anthracis.* In 1877, Robert Koch used *B. anthracis* in his classic studies of microbial etiology. The studies later led to the investigation of tuberculosis and the formulate Koch Postulates regarding the germ theory of disease (Prescott, Harley, & Klein, 2002). *B. anthracis* is a Gram-positive, rod-shaped, spore-forming bacterium that measures approximately three to five micrometers (μm) in length (Todar, 2005). *B. anthracis* is a facultative anaerobe that produces ellipsoidal endospores which resist staining and are centrally located (Todar, 2005). *B. anthracis* is differentiated from similar species using growth on differential media, molecular genetics, and traditional biochemical analyses.

B. anthracis is found globally in a variety of soils and climates. However, the microorganism can be cultivated from soils where an epidemic of anthrax has occurred (Todar, 2005). Domestic and feral animals commonly ingest *B. anthracis* when consuming spore-containing soil. Animals develop anthrax either through the inhalation, gastrointestinal, or cutaneous routes of infection, typically resulting in death.

Human anthrax infections may be transmitted via the cutaneous, inhalation, and gastrointestinal routes of infection with an LD_{50} of approximately 8,000 to 40,000 spores ("*Bacillus anthracis",* 2005). Death is characteristically associated with a blood titer of 10^7 bacteria per milliliter of blood (Todar, 2005). The incubation period for *B. anthracis* endospores is from 1 to 43 days in humans; however, endospores were recovered up to 100 days after exposure in a non-human primate study ("AHFS DI Bioterrorism Research Manual", 2002). The mortality rates of human anthrax infections differ due to mode of infection and the administration of antibiotic treatment.

Cutaneous infections are the most common form of anthrax with an incidence rate of ninety-five percent in the United States ("Anthrax", 2005). A cutaneous infection is caused when *B. anthracis* spores enter the body through a lesion or abrasion of the epidermis often involving the head, the neck, and/or the upper extremities ("Cutaneous anthrax management algorithm", 2006). Once spores enter the break in the epidermal layer, the infection continues as follows: papule formation in 1 to 17 days; vesicle forms in 1 to 2 days post papule formation; and vesicle drying creates a black, necrotic escar surrounded by edema and purple secondary vesicles ("AHFS DI Bioterrorism Research Manual", 2002). The mortality rate for cutaneous anthrax infection is 20% without antibiotic treatment ("Anthrax", 2005).

Inhalation represents the second most common route by which anthrax infections are transmitted. Anthrax acquired in this manner was originally known as "woolsorter's disease" and was uncommon in the United States prior to 2001. The onset of inhalation anthrax varies, but incubation periods are approximately 10 days. *B. anthracis* spores are inhaled into the lungs where the alveolar macrophages ingest the spores and transport them to the mediastinal lymph nodes. (Murray et al, 2002) Germination of the vegetative forms occurs in the alveolar macrophages. The vegetative cells are transported to the mediastinal lymph nodes where they cause hemorrhagic mediasteinitis ("AHFS DI Bioterrorism Research Manual", 2002). Inhalation anthrax symptoms resemble influenza, but may be differentiated from influenza by a widening of the mediasteinum observed on chest x-ray. The inhalation infection is found to be more severe than anthrax due to other routes of infection because edema and inflammation can lead to life-threatening respiratory problems. Mortality rates in untreated inhalation anthrax human patients can exceed 80% ("Anthrax", 2005).

Gastrointestinal anthrax infection is the least common of all modes of infection and has not been reported in the United States ("AHFS DI Bioterrorism Research Manual", 2002). Gastrointestinal infection is caused by the ingestion of infected food, typically undercooked meat, from an infected animal. *B. anthracis* spores can cause ulcers in the mouth or esophagus, the terminal ileum, and cecum (Murray et al, 2002). Spores enter through a break in the mucosa thereby resulting in ulceration. Gastrointestinal infections cause acute edema and ulceration that, when left untreated, can quickly lead to sepsis. Mortality rates for untreated gastrointestinal anthrax infection vary from 25%-75% ("Anthrax", 2005).

The infectious nature of anthrax is due to two separate virulence determinates: (1) the capsule and (2) the three-part anthrax toxin. The capsule is composed of poly D-glutamate; an isomer of the L-glutamate amino acid typically found in bacterial species (Jones et al., 1985). The poly-D-glutamate capsule is non-toxic, but functions to elude phagocytic cells of the immune system (Todar, 2005). Capsule genes are encoded on the plasmid pX02 and can be transferred to non-capsulated bacteria via transduction (Todar, 2005). The second virulence determinate is the anthrax toxin that includes three factors. These are designated as edema factor (EF), protective antigen (PA), and lethal factor (LF) (Todar, 2005). The combination of EF and PA causes edema. The edema cripples the immune system through exhaustion and dilution of antibodies and natural killer cells. According to Todar (2005), the combination of LF and EF causes an increase in cAMP that reduces the phagocyte's ability to ingest. It also increases the amount of cytokine in macrophages and lymphocytes, thereby causing the accumulation of immune response cells in one area potentially blocking the circulatory system and airways. The LF factor is not a well-understood molecule, but the crystalline structure is similar to the family of enzymes known as mitogen-activated protein kinases. Mitogen-activated protein kinases aid in disrupting cellular signaling and communication (Todar, 2005). The combination of all three factors causes edema, necrosis, and finally death if not treated.

Due to the ability of *B. anthracis* to sporulate, spores can be carried through the air and water and survive on inanimate objects. The organism has been designated a Category A bioterrorism threat. According to the CDC (" Bioterrorism Agents/ Diseases", 2005) a threat must meet specific factors to be included as Category A bioterrorism threat, and thesis include:

- can be easily disseminated or transmitted from person to person
- result in high mortality rates, and have the potential for major public health impact
- might cause public panic and social disruption
- require special action for public health preparedness.

Category A agents pose a possible threat to the health and security of the nation, requiring the creation of a network of laboratories and regulations to perform research on *B. anthracis*. Presently labs are classified using two systems. The two systems are categorized based on the ability of the lab to safely work with hazardous biological and chemical agents. One system is known as the Laboratory Response Network (LRN). The LRN is a set of diagnostic laboratories used to identify biological and chemical agents. LRN labs are labeled 1, 2, 3 for chemical agents, but for biological agents the labs are labeled as sentential (identification), reference (investigation), and national laboratories (bioforensics, research, select agent activity) ("The Laboratory Response Network Partners in Preparedness", 2005). The system was initiated by the CDC for possible use in a bioterrorism situation for quick and effective identification. The second system categorizes labs according to the National Institutes of Health (NIH) Guidelines ("The need for biosafety laboratory facilities", 2006). The NIH Guidelines assign biosafety levels from one to four based on the threat of agents employed and the ability to work with agents safely. These labs are generally research facilities and hospitals. Biosaftey Level 4 would be utilized with extremely infectious biological agents.

B. anthracis can only be used in minimal quantities in laboratories lower than Reference/ Biosafety Level 3. The use of *B. anthracis* at lower levels is discouraged due to its infectious nature. Two avirulent strains are available: the Sterne strain lacks pX02 leaving the bacteria uncapsulated (Todar, 2005) and the Pasteur strain is void of pX01

and therefore does not produce the toxin (Read et al., 2003). Avirulent strains of *B. anthracis* are then available for laboratory use, but due to the missing genes are useless for toxin studies.

Bacillus cereus can be used as a surrogate for *B. anthracis*. *B. anthracis* and *B. cereus* are both part of the bacillus group named as the *B. cereus group* or *B. cereus sensu lato* (Turnbull, 1999). The *B. cereus* group bacteria are all Gram-positive, spore-forming, and rod-shaped bacteria. The differentiation between the "members" of the group is difficult because of genotypic and phenotypic similarities. Some studies suggest that *B. anthracis* and *B. cereus* should be considered one species because of the close similarity in genomic sequence (Helgason et al., 2000). *B. cereus* is a similar bacterium to *B. anthracis* in many aspects. Specifically, *B. cereus* and *B. anthracis* are exclusive research targets because both are pathogenic to humans. Similar DNA, toxins, fatty acids, and evolution enable the use of *B. cereus* as a surrogate for *B. anthracis* at a Biosafety level 2-laboratory setting.

Genetic similarities have been revealed between *B. cereus* and *B. anthracis.* The genome sequences of *B. cereus* and *B. anthracis* are completed or considered 12X shotgun status (Tourasse et al., 2006). Ribosomal sequences in the 16S rRNA were determined to be identical and only two differences were found in the 23S rRNA (Ash & Collins, 1992). Tandem repeats of a specific *B. cereus* strain (KCTC 1661) were found to be exact matches with *B. anthracis* strains when using variable number tandem repeat analysis. (Kim et al., 2002) The genetic relationships between *B. cereus* and *B. anthracis* are continually being researched due to newly found strains with more similarities.

B. cereus and *B. anthracis* exhibit similar toxin and virulence profiles. *B. cereus* is cited to cause an illness similar to inhalation anthrax (Hoffmaster et al., 2004). The *B. anthracis* toxin plasmid pX01 has been discovered in strains of *B. cereus.* The pX01 plasmid's backbone is similar to specific plasmid backgrounds found in isolated *B. cereus* strain ATCC 10987 (Rasko et al., 2004). *B. cereus* G9241 carries an almost complete pX01 plasmid known as pBCX01 (Hoffmaster et al., 2006). Also, the G9241 strain has the presence of a capsule. The capsule for *B. cereus* strain G9241 is not composed of poly D-glutamate as in *B. anthracis*, but is a unique characteristic relating virulence factors of the *B. cereus* and *B. anthracis* (Hoffmaster et al., 2006). Finally emetic toxin (cereulide) of *B. cereus* synthetase genes are located on a plasmid with a pX01 backbone (Ehling-Schulz et a.l, 2006).

PlcR transcriptional activator is an important component to the genetic expression and regulation of *B. cereus* toxins. The PlcR has been found as a nonsense mutation in *B. anthracis* (Gohar et al., 2002). Introduction of a functional PlcR activates *B. cereus* virulence factors in *B. anthracis* (Mignot et al., 2001). The ability to activate non-sense mutations in *B. anthracis* with a transcription factor from *B. cereus* further correlates the toxin profiles. According to Mendelson et al. (2004), non-hemolytic exotoxin genes have been found in *B. anthracis* with a 98% alignment. *B. anthracis* has been found to secrete a component of the non-hemolytic toxin (Mendelson et al., 2004) which may play a role in the diarrhea that can accompany gastrointestinal anthrax (Friedlander, 2000).

While clearly distinguishable from other *Bacillus* species, the nature and relative abundance of fatty acids produced by *B. anthracis* are found to be comparable to those of

B. cereus (Kaneda, 1967). Kaneda (1977) states that *B. cereus* and *B. anthracis* both produce anteiso/iso-C_{12} and anteiso/iso C_{13} fatty acids, which are not significantly produced by the majority of bacilli. Also, fatty acid profiles 17:1 ω10c and a17:1 are unique to *B. cereus* and *B. anthracis* (Whittaker, 2005). The similarities in fatty acid composition of *B. anthracis* and *B. cereus* is indicative of the close similarity in the biochemical activities involved in fatty acid metabolism (Kaneda, 1968).

Nonselective and selective media provide a method for discriminating *B. cereus* and *B. anthracis* phenotypically. Useful phenotypic tests include motility, hemolysis on sheep blood agar, gamma phage lysis, and D-glutamyl-polypeptide capsulation (Todar, 2005). *B. cereus* also produces β-lactamases and is penicillin, ampicillin, and cephalosporin resistant (Turnbull et al., 2004). Growth on differential media easily distinguishes the two bacteria apart; however, recently discovered strains of both species have given atypical results. Rare strains of *B. cereus* can be poorly hemolytic, minimally motile, may be lysed by the gamma phage, and are susceptible to penicillin (Turnbull, 1999). The atypical phenotypic results make *B. cereus* and *B. anthracis* difficult to distinguish with just the use of differential and biochemical tests.

The similarities between the two species make *B. cereus* an excellent surrogate for *B. anthracis.* As a spore-forming, Gram-positive bacterim *B. cereus* is a member of *B. cereus sensu lato* (Turnbull, 1999). *B. cereus* is 1μm to 3.0/5.0 μm in length, motile, rod-shaped, and able to survive at a pH rage of 4.3 or 9.3 (Burgess & Horwood, 2006). Most *B. cereus* strains are known to grow at moderate temperatures (mesophilic), yet strains have been found to thrive at low temperatures (psychrophilic) with tolerant growth temperature ranges from 8°C to 55°C (Burgess & Horwood, 2006). As a ubiquitous soil

bacterium *B. cereus* can be transferred to animals and plants in contact with *B. cereus* cultures in the soil (Helgason et al., 2000). *B. cereus* is a food borne human pathogen which causes diarrheal and emetic forms of illness.

The CDC has a Foodborne Disease Outbreak Surveillance System that monitors the occurrences and causes of foodborne-disease outbreaks (FBDOs) (Lynch et al, 2006). Recent national known outbreaks include *Escherichia coli* tainted spinach, *Salmonella* found in peanut butter, and Hepatitis A-contaminated green onions. Foodborne-disease epidemics are easily spread through the food industry and can be illustrated by the expansive infected area shown in **Figure 1** from the *Salmonella* peanut butter outbreak early in March of 2007 ("*Salmonellosis"*, 2007). It is possible for terrorists to use foodborne diseases, such as the emetic and diarrheal diseases caused by *B. cereus*, to cause extensive devastation. A pervasive food epidemic could have adverse effects on medical services, food supply, and economics of a billion dollar food service industry.

Figure 1: A map of the reported cases of *Salmonella* poisoning from peanut butter in March of 2007 ("*Salmonellosis"*, 2007).

The disease caused by *B. cereus* is mainly associated with food poisoning. *B. cereus* may be found in rice, spices, cereal, flour, vegetables, oils, meats, and milk

(Burgess & Horwood, 2006). *B. cereus* food poisoning is related to rice 95% of the time (Burgess & Horwood, 2006). The diarrheal disease is usually milder than the emetic infection. Diarrheal *B. cereus* infection symptoms range from abdominal cramps, watery stool, and rectal tenesmus ("Anthrax", 2005 ; Burgess & Horwood, 2006). Symptoms begin six to twelve hours after consumption of food and can last up to 24 hours ("Anthrax", 2005). The emetic infection is considered more acute due to the quick onset of symptoms. An emetic infection causes symptoms of nausea and vomiting with an onset 0.5 to 6 hours after the consumption of contaminated foods ("Anthrax", 2005) and symptoms can last up to 24 hours.

Different toxins produced by *B. cereus* cause the diarrheal and emetic infections. Toxins are substances that are capable of causing disease when introduced to animal or plant tissues ("Toxin", 2004). Bacterial toxins are typically proteins, peptides, or lipopolysaccharides that are divided into two groups, endotoxins and exotoxins. Endotoxins are produced predominately by Gram-negative bacteria only and are outer membrane structural components (lipopolysaccharides). Endotoxins are released through lysis of the bacterial cell. Exotoxins are protein/peptide toxins and released by the bacteria through cell membrane transport or cell lysis. Both Gram-negative and Gram-positive bacteria produce exotoxins. The toxins of *B. cereus* are exotoxins.

The diarrheal toxins of *B. cereus* have been well documented. The five most prominent toxins acknowledged to cause the diarrheal infection include hemolysin BL, non-hemolytic enterotoxin, cytotoxin K, enterotoxin FM, and enterotoxin T. Hemolysin BL and non-hemolytic enterotoxin are discussed in-depth below. Cytotoxin K was first identified by Lund, De Buyser and Granum (2000) and is thought to cause necrotic

enteritis. Enterotoxin FM and entertotoxin T are both single polypeptide proteins (Ghelardi, 2002). Little information is known about enterotoxin T and FM other than their involvement in the diarrheal infection.

Hemolysin BL (HBL) is suspected to be the major diarrheal toxin of *B. cereus* (Burgess & Horwood, 2006). HBL is a three-part enzymatic toxin that can only be activated when all three components are present (Lund & Granum, 1997). The three components consist of B, L_1, and L_2, which are not individually hemolytic. The concentration of L_1 and L_2 determines the lytic capabilities and concentration of the B component determines the diameter of the hemolysis ring (Burgess & Horwood, 2006). Approximately half of *B. cereus* strains produce diarrheal enterotoxin (Schoeni & Wong, 1999). HBL is easily viewed on a Trypticase soy agar plate with 5% blood agar (**Figure 2**). The toxin causes incomplete hemolysis ring and is considered a β-hemolysin.

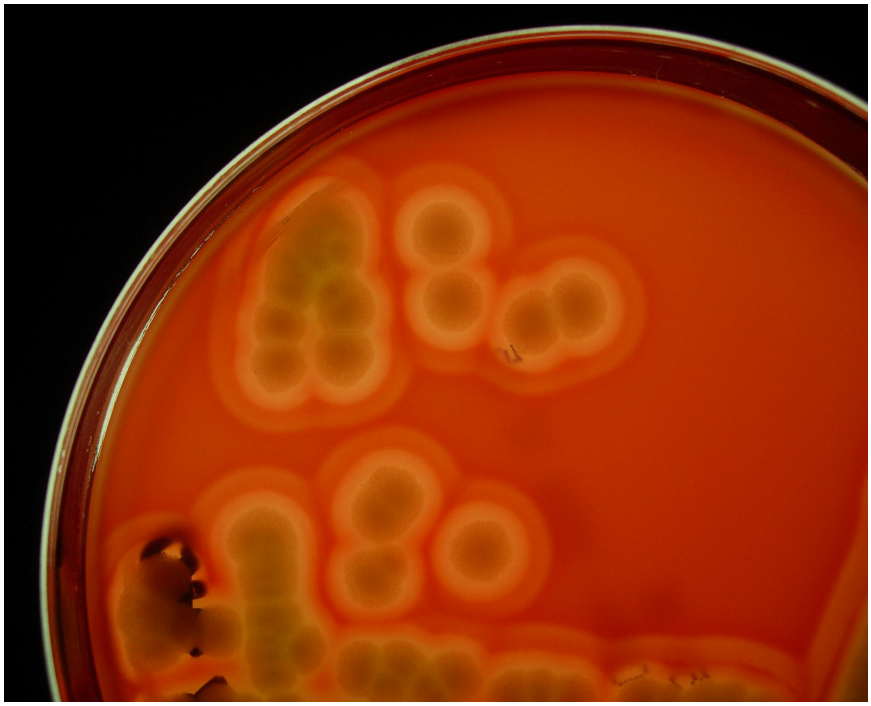

Figure 2: *Bacillus cereus* Arnold strain demonstrating β and incomplete hemolysis.

Another *B. cereus* diarrheal toxin is known as non-hemolytic enterotoxin (Nhe). This toxin is also a three-component toxin and was first isolated from a *B. cereus* food poisoning (Lund & Granum, 1999). The Nhe toxin is non-hemolytic but is shown to be highly cytotoxic to Vero cells (Lund & Granum, 1997). The Nhe components are Nhe A, Nhe B, and Nhe C (Lund & Granum, 1999) and not individually cytotoxic. The presence of the Nhe A component can be determined by the TECRA VIA BDE biochemical test (Beecher & Wong, 1994).

The *B. cereus* emetic toxin has been described as a single polypeptide known as cereulide (Agata et al., 1995). Cereulide is a small, heat and acid stable cyclic dodecadepsipeptide. Cereulide is closely chemically related to the potassium ionophore valinomycin with a chemical structure of [cyclic D-O-Leu-D-Ala-L-O-Val-L-Val]$_3$ and is shown in **Figure 3 (A) (B)** (Agata et al., 1995). Cereulide size is relatively small at 1.2 kDA (Carlin et al, 2006) and has been shown to be toxic to mitochondria (Mikkola et al, 1999)

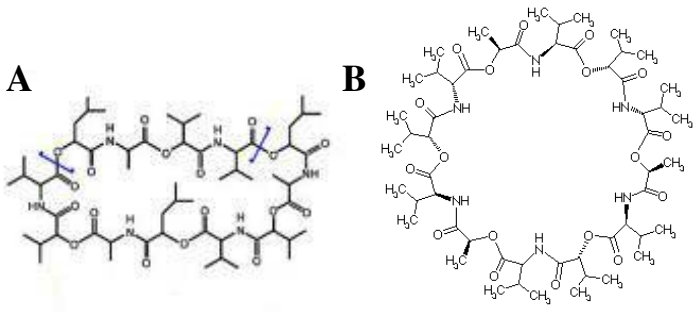

Figure 3: (A) Cereulide Chemical Structure (Ehling-Shulz et al.,2006) **(B) Valinomycin Chemical Structure** (Valinomycin, ND)

and human natural killer cells (Paananen et al., 2002),. Approximately 8µg of cereulide kg^{-1} dry weight of food is toxic to humans and can cause serious acute illness (Jääskeläinen et al., 2003). The presence of cereulide in cell cultures results in the vacuolation of mitochondria (Kawamura-Sato et al., 2005) and is highly toxic to hepatic

cells and a major health concern (Andersson et al., 2004). Cereulide may be detected by monkey emesis assay (Shinagawa et al., 1995), various cell culture toxicity assays (Agata et al., 1995), boar sperm assay (Andersson et al., 2004), and damage to rat liver mitochondria (Kawamura-Sato et al, 2005).

The manner in with *B. cereus* regulates cell membrane transport and regulation of toxins production is important for a better understanding of the two types of infections. The *B. cereus* toxins are exotoxins and therefore must be released through (1) cell lysis or (2) transmembrane regulation. There is no evidence of cell lysis of *B. cereus* cells noted in previous studies on the bacterium or toxins. Without literature regarding the use of cell lysis of *B. cereus* to release toxins, it can be postulated that the toxins are released through the cell membrane transport. Cell membrane fatty acids and emetic toxin production could be correlated through the investigation of cell membrane composition.

The cytoplasmic membrane is a crucial part in the regulation of solute entrance and exit of the cell. Cell membranes are made up of phospholipids and proteins (Campbell & Reece, 2001). Phospholipids are the major component of all biological membranes and fatty acids occur in nearly all-living organisms as the predominate components of membrane phospholipids (Kaneda, 1977). Fatty acids are carboxylic acids with long aphilic tails that can be saturated or unsaturated. Saturated fatty acids are categorized into straight-chain, branched-chain, and cyclohexyl fatty acids. Only monounsaturated fatty acids are found in bacteria. As a major part of the cytoplasmic membrane, the fatty acid composition is crucial to understanding cell envelope transport.

Branched-chain fatty acids are common constituents of the lipids contained in bacteria (Christie, 2006). *B. cereus* is made up of 80% branched-chain fatty acids

(Kaneda, 1991). Branched–chain fatty acids are divided into iso, anteiso, and ω-alicyclic methyl branched. The naming of iso or anteiso is dependent on the position of the methyl group branching. If the methyl is on the penultimate carbon (one from the terminal carbon) then the fatty acid is iso branched (Christie, 2006). However if the fatty acid is on the ante-penultimate carbon (two from the terminal carbon) it is said to be anteiso branched (Christie, 2006). ω-Alicyclic fatty acids are major contributors to membrane composition in specific bacterial species and are present as minor contributors in most other bacteria (Kaneda, 1991). These specific branched chain fatty acids are synthesized in bacteria using specific primers and malonyl CoA (enzyme for fatty acid chain elongation).

Branched-chain fatty acids are synthesized using specific primers that are unique from the synthesis of other fatty acids. The synthesis of branched-chain acids is important to help understand the mechanisms involved in regulating the cell membrane composition of *B. cereus*. De novo synthesis of branched-chain fatty acids is done through the use of two different primers (1) Short chain carboxylic acids (2) α-keto acids (Kaneda, 1991). Ruminal and mutant *Bacillus subtilis* bacteria typically use short chain carboxylic acids as primers for branched-chain fatty acid synthesis (Kaneda, 1991). Short chain carboxylic acid primers are not commonly used in branched-chain fatty acid synthesis and must be supplemented by the addition of specific substrates into the media. The second forms of primers used for branched-chain fatty acid synthesis are α-keto acids. The α-keto acid primers derive from isoleucine, leucine, and valine (Kaneda, 1977). Branched-chain fatty acid synthesis with the use of α-keto acids as primers is the main synthesis pathway used by bacteria (Kaneda, 1991).

The fatty acid composition of the bacterial cell envelope may be changed by variable factors. Fatty acids ratios change when different stressors are introduced to the environment. The stressors include desiccation, reduced temperature, and the presence of soluble rice starch (Haque & Russell, 2004). The fluidity or rigidity of the cell membrane is also changed with different proportion of fatty acids. It has been proposed that branched-chain fatty acids function in the membrane may be used as an alternative to double bonds to increase the fluidity because the oxidative nature of double bonds (Christie, 2006). Guffanti et al. (1987) stated that changes in membrane fatty acids can affect the overall change in the energy synthesis, specifically ATPase synthesis. Branched-chain fatty acids must be present in minimal levels, stated to at 28%, for growth for *Bacillus* species (Kaneda, 1991). Phospholipids have been found to play a role in the transport of branched-chain amino acids in *Pseudomonas aeruginosa* and *Streptococcus cremoris* (Kaneda, 1991).

Toxins and branched-chain fatty acids play an important role in the composition and virulence of *B. cereus*. The relationship between the two has yet to be investigated through scientific study. The purpose of this study is to determine whether the fatty acid profiles and cytotoxicity assay results differ among *B. cereus* strains. It is hypothesized that a relationship among cell membrane fatty acids, emetic toxin production, and cytotoxicity does exist.

CHAPTER II

MATERIAL AND METHODS

Selection and Maintenance of *B. cereus* strains

Four strains of *B. cereus* that differed phenotypically with regard to emetic toxin production were selected for the present study (**Table 1**). *B. cereus* strains F4810/72 and NC7401 are positive for emetic toxin production (Turnbull, 1999; Andersson et a.l, 1998). *B. cereus* Arnold strain was obtained from a soil sample found adjacent to Oklahoma I-35 (Miller, 2005) and identified by Oklahoma State University-College of Health Sciences Forensic Science Laboratory for Forensic Pathology and Infections Disease. The *B. cereus* strain Arnold ability to produce emetic toxin is unknown, but

Table 1: Bacillus Cereus Strains.

Strain	Emetic Toxin Production	Isolated From	Reference
F4810/72	Positive	Vomitus	Norio Agata, Nagoya City Public Health Institue, Nagoya, Japan
NC7401	Positive	Feces	Norio Agata, Nagoya City Public Health Institue, Nagoya, Japan
Arnold	Unknown	Soil (Oklahoma I-35)	Dena Miller, 2005
ATCC 14579	Negative	Unidentified	American Type Culture Collection, Manassas, Virginia

Red indicates emetic strain
Blue indicates non-emetic strain

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suspected to be non-emetic. The *B. cereus* strain ATCC 14579 (American Type Culture Collection, Manassas, VA) is negative for the emetic toxin (Andersson et al, 1998).

The *B. cereus* strains were routinely maintained on Trypticase soy agar with 5% sheep blood and kept at 0-8°C. Deep–stabs were made of each strain and kept on 2% agar at room temperature

Analyses of *B. cereus* Growth

Trypticase soy agar plates with 5% sheep blood (TSA II) were streak inoculated and incubated at 30±1˚C overnight. Confluent growth from each *B. cereus* strain TSA II culture were used to inoculate to 10 ml of sterile brain heart infusion (BHI) and incubated for 24 hours at 30±1˚C. 50ml of sterile BHI broth was added to a pre-warmed 250 ml Nephelo culture flask (side arm). Overnight cultures were added drop wise to the BHI broth until an optical density (OD) of 0.020 ($OD_{620\ nm}$) was obtained. 50 mililiters of the inoculated media were used to perform analyses of growth. The samples aerated at 30 ±1˚C with rapid shaking (180 rpm) (Lab-line table top Orbit Environ-Shaker). Optical densities were recorded at 30-minute intervals until stationary phase was attained and maintained for 24 hrs. Experiments were performed in triplicate from independent cultures.

Culture Filtrate and Cell Sample Preparation

Early and late stationary phase optical densities were determined from analyses of *B. cereus* growth and subsequent OD points were used for sample harvesting. The cultures were removed from the incubator at OD 1.6-1.7 (early stationary phase) and 2.0

(late stationary phase). Cultures were centrifuged at 10,000 x g for 10 minutes at 4°C. 10 ml of culture filtrate (CF) were enriched for cereulide through the partial purification of cereulide. Partial purification of cereulide follows the method used by Finlay, Logan and Sutherland (1999) modified by the use of BHI. Samples were heat-sterilized for 15 minutes at 121°C, sterile filtered using a 0.22 μm filter, frozen in liquid nitrogen, and stored at -80°C.

The cell pellet, solid centrifuged material, from the centrifuged sample was used to analyze fatty acids by gas chromatography/ mass spectral scans. The cell pellet was washed with DiH_20 three times and placed in sterile low temperature freezer vials. The samples were frozen in liquid nitrogen and stored at -80°C.

HuH-7 Cell Line Maintenance

The cell line of choice was the human hepatoma cell line HuH-7 due to availability and sensitivity of hepatic cells to cereulide. HuH-7 was obtained from Dr. Rashmi Kaul at Oklahoma State University-CHS. The cell line was originally derived by Nakabayashi et al. (1982) from a hepatocellular carcinoma that was found to replicate continuously.

The HuH-7 cells were grown in 75-cm^2 flasks (Falcon) at 37°C in 5% CO_2 with a humidified atmosphere. HuH-7 cells were cultivated in Dulbecco's modified medium (DMEM) containing high glucose, 2mM L-glutamine, 1mM sodium pyruvate (Invitrogen, Carlsbad, CA), supplemented with 10% heat-inactivated fetal bovine serum (30 minutes, 56° C, Atlanta Biologicals, Lawrenceville, GA), and 1% penicillin/

streptomycin/glutamine. The cell line was grown to approximately 60% confluence and harvested with Trypsin-EDTA (0.05% trypsin, 0.53 mM EDTA, GiBCO, Carlsbad, CA).

In Vitro MTT Cell Toxicity and Mitochondrial Permeability Assays

The *in vitro* 3-[4,5-dimethylithiazol-2-yl]-2,5-dipheyl tetrazolium bromide (MTT) cell toxicity and mitochondrial permeability assays employed were both obtained from Sigma-Aldrich in St. Louis, Missouri. Cells for both assays were trypsinized and suspended in complete DMEM at 10^6/ml, and 125 µl were added to 88 of the wells of a 96-well microtiter plate. Black plates were used for the mitochondrial permeability assay. The cultures were grown overnight for confluencey and adherence.

Toxicity analyses included valinomycin (positive control), BHI (negative interference control), and CF samples. Valinomycin was originally solubilized in DMSO and then diluted in complete DMEM. Valinomycin was serial diluted with a starting concentration of 400ng/ml. Both BHI and CF samples were serial diluted in DMEM. Over night cell culture media was removed by aspiration. 100 µl of toxicity samples were added according to **Figure 4**. Once the toxicity samples were added to the wells, an additional 100 µl of complete DMEM was added to the well and incubated for 48 hours. At the end of the time period the *in vitro* MTT cell toxicity and the mitochondrial permeability assays were performed according to the protocols stated by the manufacturer.

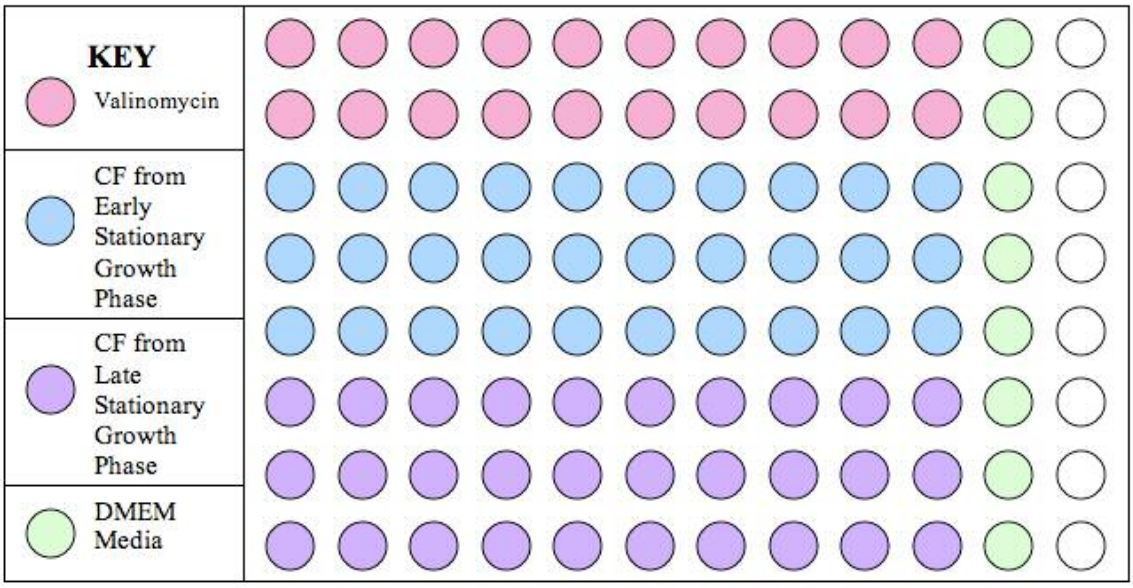


Figure 4: The contents of the 96-well microtiter plate in both the *in vitro* MTT cell toxicity and the mitochondrial permeability assays.

Gas Chromatography/ Mass Spectral Fatty Acid Scans

The Gas Chromatography/ Mass Spectral experimental for fatty acids design was performed according to Supelco, Inc Bulletin 767A (1977). Fatty acid methyl esters (FAME) standard were obtained from Matrace Company (Pleasant Gap, PA). The GCMS was an Aligent 6890 Series ALS GC coupled to a Aligent 5973 MSD with a Restek RXI-5m3, 30m x 0.25mm x 0.25 film thickness, column. Cell pellets from previous section were used.

All GCMS samples were analyzed using the following programmed method. The initial ramp temperature was 150°C for 4 minutes. This was an increased to a final temperature of 250°C at a rate of 6°C/min. Column head pressure of helium carrier gas was set at 13.27 psi. Helium carrier was ultra high purity (99.99%). The split ratio was

changed from 10:1 to 5:1 with a 1 μl sample size. The mass spectrum range was 35.0 to 550.0 amu for the entire scan due to 3-minute solvent delay.

Data Collection and Statistics

The optical densities recordings were plotted in a semi-logarithmic curve to determine the stationary growth optical density points. The samples were graphed using SigmaPlot® Version 10 (2006) ©.

Mitochondrial permeability assay results did not warrant statistical analysis. The *in vitro* MTT assay results were graphed using OD points at 570 nm and –log toxin dilutions. The points were graphed using Graph Pad Prism 4©. An ANOVA with Tukey's multiple comparison tests were used to determine significant differences among *B. cereus* strains toxicity at early and late stationary growth phases. Student T-Tests were performed for each individual strains OD points from early to late stationary growth phases. Concentrations of the amount of heat stable toxin(s) of *B. cereus* strains were determined by comparing them to the standard curve generated from valinomycin. The concentrations were tested for statistical significance among and within individual strains. Difference in toxicity from early and late stationary phases were determined using Tukey's multiple comparison test applied to an one-way ANOVA and Student T-tests.

GCMS spectra results were analyzed for the presence of fatty acids in the different strains. The area percent and most likely fatty acid from individual peaks generated by ChemStation software (Allegen, 2003) © were normalized by the equation in **Figure 5.** All graphs and analysis were performed using Graph Pad Prism 4 ®. All

normalized fatty acid data were statistically analyzed using an ANOVA with Tukey's multiple comparison tests to determine the differences among the strains. Individual strain differences were determined with a Student T-test. Analyses of fatty acid with respect to branche were performed and statistically analyzed using Tukey's multiple comparison tests as applied to an ANOVA. All data were placed in tables and graphed.

Figure 5: Normalization of original area percent from GCMS library search report of fatty acid extraction.

$$\frac{\text{Individual Area Percent for Identified Fatty Acid}}{\text{Total Area Percent for All Identified Fatty Acids}} \times 100 = \text{Normalized Area Percent}$$

CHAPTER III

RESULTS

Analyses of *B. cereus* Growth

The results for the analyses of *B. cereus* growth are demonstrated in **Figure 6**. Under the experimental conditions early stationary phase began at 1.6-1.7 OD_{620nm}, approximately at 4-5 hours from the initial inoculation. Late stationary phase began at OD_{620nm} of 2.0; 9-10 hours from inoculation. The OD's for early and late stationary phases were used for all sampling for GCMS and cell toxicity assays

Figure 6: Growth Curves for *Bacillus cereus* Strains with Differing Virulence Factors.

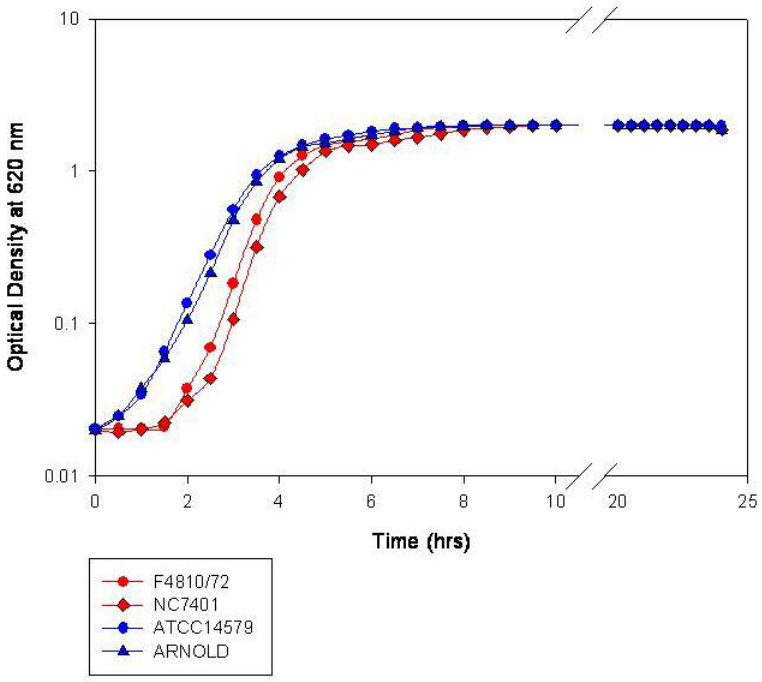


Table 2: *B. cereus* Regressions and Slopes from Exponential Growth Phase

Strain	Regression	Slope
F4810/72	0.8653	0.1605 ± 0.02394
NC7401	0.7482	0.08400 ± 0.01842
Arnold	0.8376	0.1074 ± 0.01788
ATCC 14579	0.7978	0.1048 ± 0.01995

Red indicates emetic strain. Blue indicates non-emetic strain.

Regression and slopes for all *B. cereus* strains were determined for the exponential growth phase. The values were determined to not be significantly different.

In vitro MTT Assay

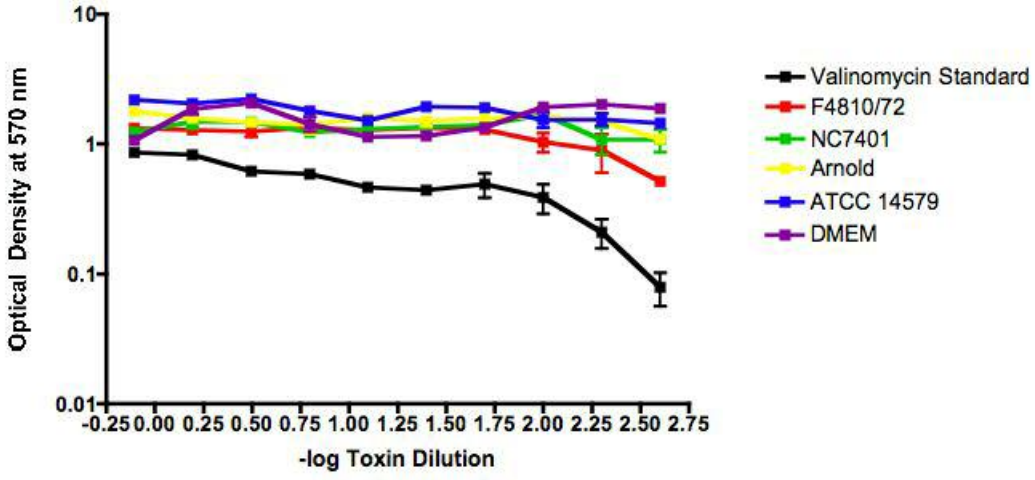
The *in vitro* MTT assay results for all strains at both early and late stationary growth phases are shown in **Figure 7** and **Figure 8**. Valinomycin was used as the positive control standard for cytotoxicity with concentrations ranging from 400 ng/ml to 0.75 ng/ml. The LD_{50} for valinomycin in HuH-7 cell cultures was which is approximately 50 ng/ml at OD_{570nm} at 0.4815.

Normal HuH-7 growth was at approximately OD 0.90 because this was the lower limit to DMEM only controls. The mean OD for DMEM was 1.53 and the upper limit being at OD 2.288. Any OD above 0.90 was determined to be normal HuH-7 growth and below was determined to be due to cytotoxicity.

The relative toxicity of CF from the various *B. cereus* strains in early stationary phase is shown in **Figure 7**. When these results were compared each strain to both valinomycin and DMEM it is apparent that all *B. cereus* strains stayed above the OD 0.90

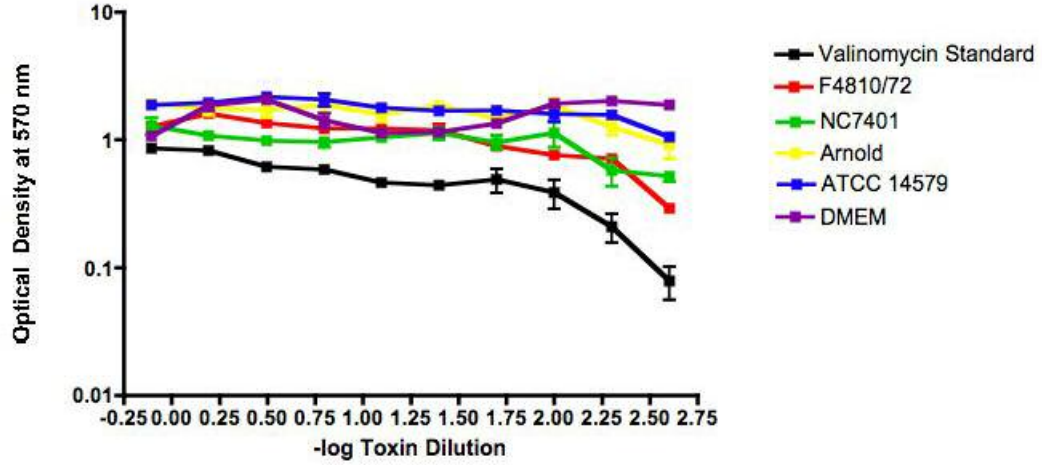
point until the last dilution (CF dilution). Both NC7401 (OD 0.811) and F4810/72 (OD 0.269) were under the limits of normal HuH-7 growth in early stationary phase.

Figure 7: *B. cereus* Strains *in vitro* MTT Assay in Early Stationary Growth Phase.



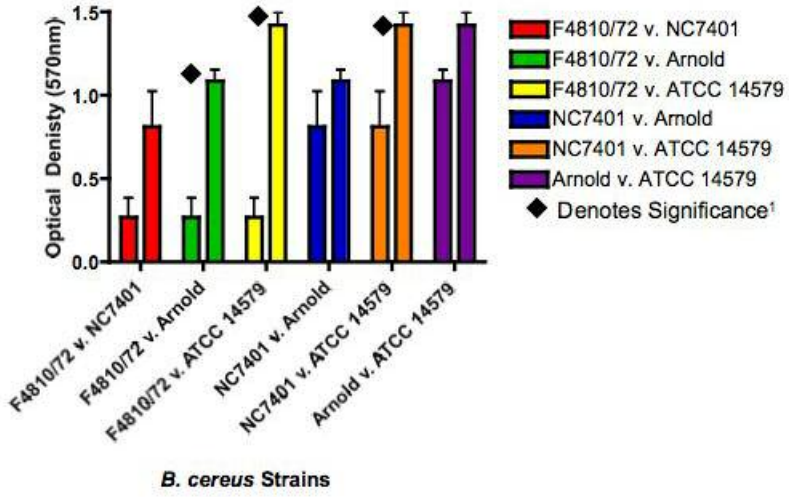
The relative toxicity of CF from the various *B. cereus* strains in late stationary phase are shown in **Figure 8**. *B. cereus* strains Arnold and ATCC 14579 optical density points stay above the normal HuH-7 cell growth lower limits. F4810/72 last three optical density points 0.2097, 0.713, and 0.76 are below the normal growth limitations and show effects of cytotoxicity. NC7401 last two optical density points, OD 0.379 and OD 0.786, are under normal HuH-7 cell growth and exhibit effects of cytotoxicity .

Figure 8: *B. cereus* Strains *in vitro* MTT Assay in Late Stationary Growth Phase.



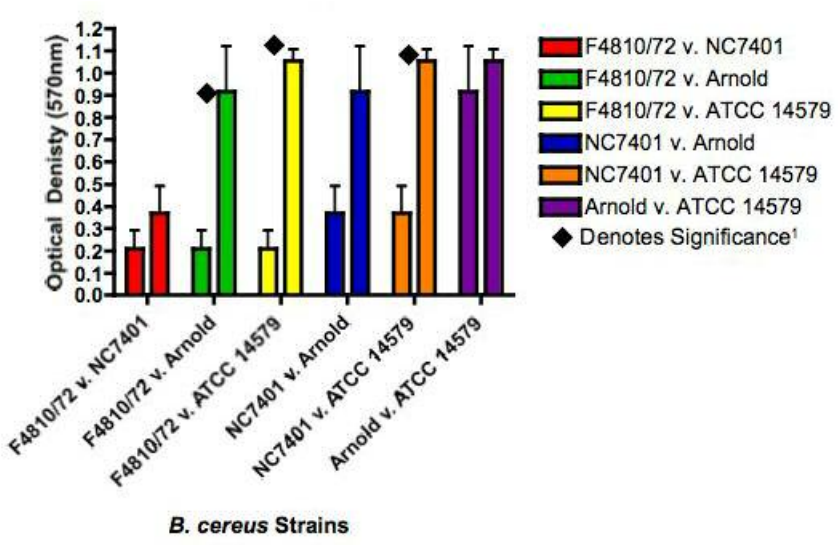
Statistical analyses compared the relative toxicity of CF in early and late stationary phases. Both early and late stationary phases produced the same statistical results at a 95% confidence interval. In each comparison, F4810/72 differed from strains Arnold and ATCC 14579. NC7401 also differed from ATCC 14579, but not from Arnold. The results are summarized in **Figure 9** and **Figure 10**.

Figure 9: A Comparison of *B. cereus* Strains Culture Filtrate *in vitro* MTT Assay in Early Stationary Growth Phase.



[1] Significance is based on Tukey's multiple comparison test applied to an ANOVA,; n = 3

Figure 10: A Comparison of *B. cereus* Strains Culture Filtrate *in vitro* MTT Assay in Late Stationary Growth Phase.



[1] Significance is based on Tukey's multiple comparison test applied to an ANOVA,; n = 3

When the CF's were examined individually, statistical analyses (Student T-test) revealed that only ATCC 14579 differed from early to late stationary growth phase, when comparing OD's. The plots are shown in **Figures 11-14**.

Figure 11: *In vitro* MTT Assays of *B. cereus* Strain F4810/72 in Early and Late Stationary Growth Phases.

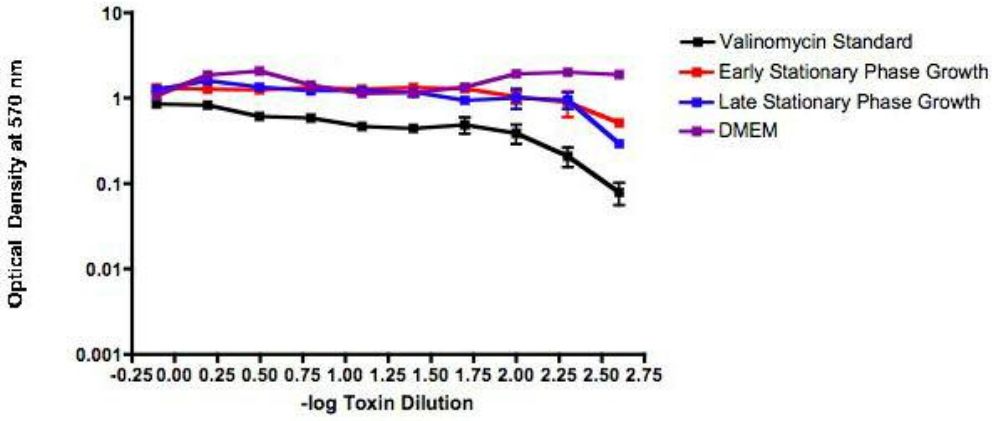


Figure 12: *In vitro* MTT Assays of *B. cereus* Strains NC7401 in Early and Late Stationary Growth Phases

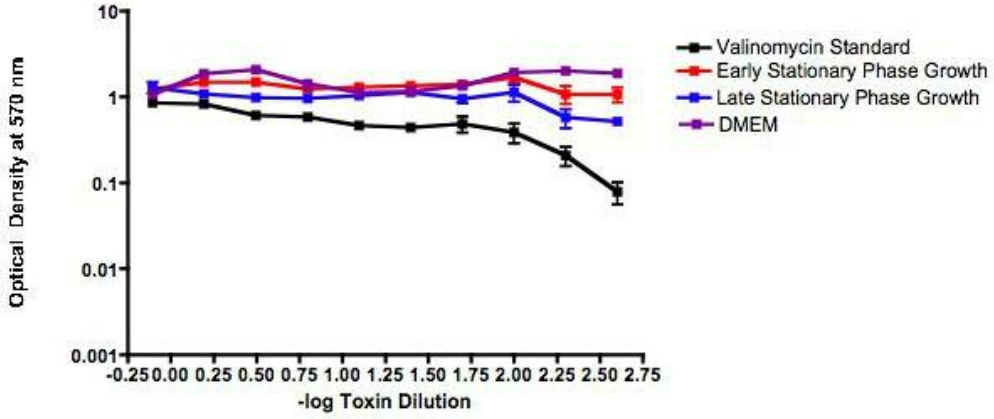


Figure 13: *In vitro* MTT Assays of *B. cereus* Strain Arnold in Early and Late Stationary Growth Phases

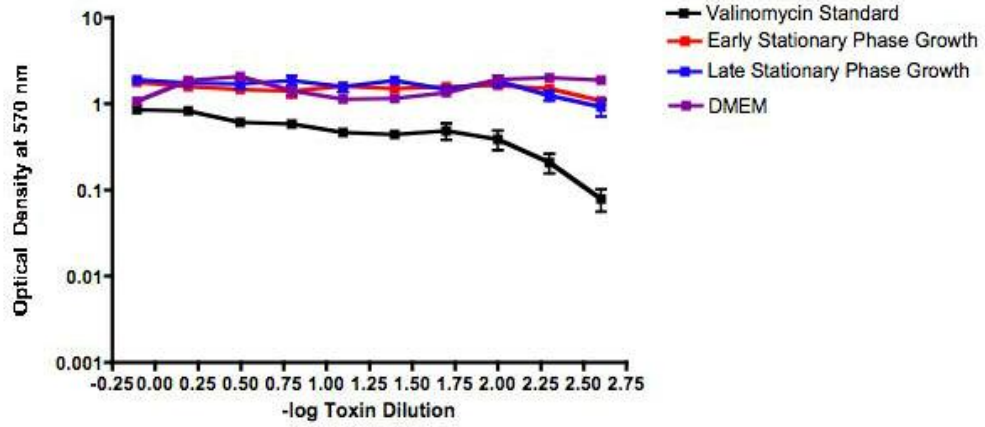
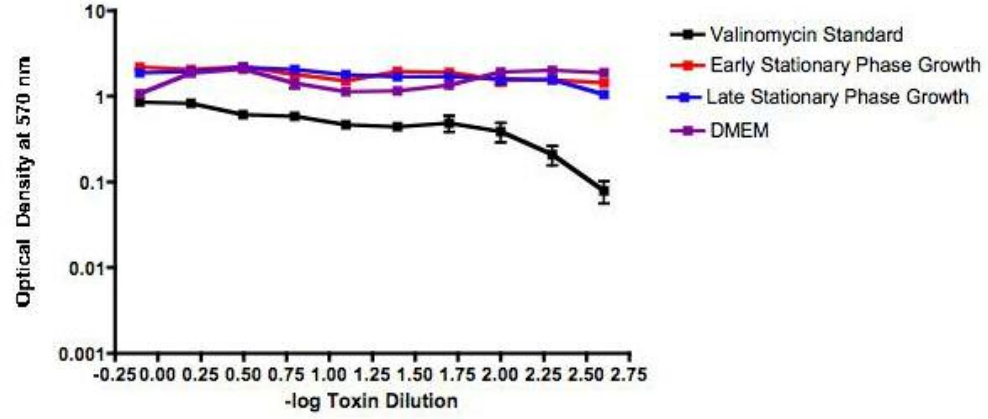


Figure 14: *In vitro* MTT Assays of *B. cereus* Strain ATCC 14579 in Early and Late Stationary Growth Phases



CF optical densities from all *B. cereus* strains were compared to the standard curve for valinomycin concentration (**Table 3**). The DMEM negative controls exhibited background cytotoxicity concentrations (0.70425) and were subtracted from all *B. cereus* values of heat stable toxin(s). Both non-emetic and emetic strains showed an increase in cytotoxicity in early stationary growth phase. However, no significant differences were found when using Tukey's multiple comparison assays applied to an one-way ANOVA. Differences were found in the late stationary phase growth with F4810/72 when compared to the other stains (**Table 4**).

Table 3: *B. cereus in vitro* MTT Assay Heat-Stable Toxin(s) Concentration Results from *in vitro* MTT Assay in Early and Late Stationary Growth Phases. Heat stable concentrations were derived from heat-purified CF for cereulide, the first dilution point in the assays.

Strain	Emetic Toxin Production	Heat Stable Toxin Concentration (ng/ml)	
		Early Stationary Phase Growth	Late Stationary Phase Growth
F4810/72	Positive	3.98	20.56
NC7401	Positive	3.02	9.12
Arnold	Negative	2.92	5.6
ATCC 14579	Negative	1.31	4.65

Red indicates emetic strain. Blue indicates non-emetic strain.

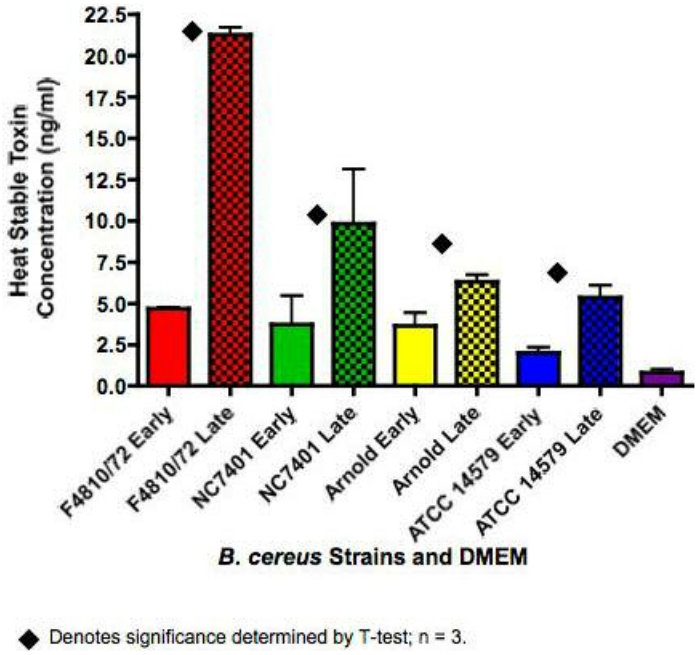
Table 4: Statistical Analyses of *B. cereus* Heat-Stable Toxin(s) Concentration Results using *in vitro* MTT Assay in Early and Late Stationary Growth Phases.

Stationary Phase Growth	F4810/72 vs NC7401	F4810/72 vs Arnold	F4810/72 vs ATCC 14579	NC7401 vs Arnold	NC7401 vs ATCC 14579	Arnold vs ATCC 14579
	P-values at 95% Confidence Interval[1]					
Early	>0.05	>0.05	>0.05	>0.05	>0.05	>0.05
Late	<0.01	<0.01	<0.001	>0.05	>0.05	>0.05

[1]P-values were determined by a Tukey multiple comparison method, which was applied to an ANOVA; n= 3. Red indicates emetic strain. Blue indicates non-emetic strain. ☐ = Signficant.

Significant changes in concentrations of heat stable toxin from early to late stationary phase growth were analyzed by using Student T-tests and are shown in all strains (**Figure 15**). The p-values, based on a 95% confidence interval, <0.001 for F4810/72; 0.0485 for NC7401; 0.0395 for Arnold; and 0.0149 for ATCC 14579. The p-values were based on a 95% confidence interval.

Figure 15: Comparison of *B. cereus* Heat-Stable Toxin(s) Concentrations using *in vitro* MTT Assay in Early to Late Stationary Phase Growth



Mitochondrial Permeability Assay

The results from the mitochondrial permeability assay showed no differences from the valinomycin control, CF dilutions, and DMEM dilutions (data not shown). The samples fluoresced from 1.54 to 2.0 ratio of JC-1 aggregates to JC-1 monomers. JC-1 aggregates are healthy cells, while JC-1 monomers are apoptotic cells.

Gas Chromatography/ Mass Spectra Fatty Acid Scans

Identified fatty acid names and abbreviations are shown in **Table 5**. An example of electron ion mass spectra fatty acid extraction of F4810/72 late stationary phase sample is shown in **Figure 16**. Area percentages from the library search report were normalized using the equation in **Figure 5**. Normalized average area percentages are shown in **Table 6** and **Table 7**.

Figure 16: Electron mass spectra of fatty acid extraction from *B. cereus* F4810/72 in late stationary phase.

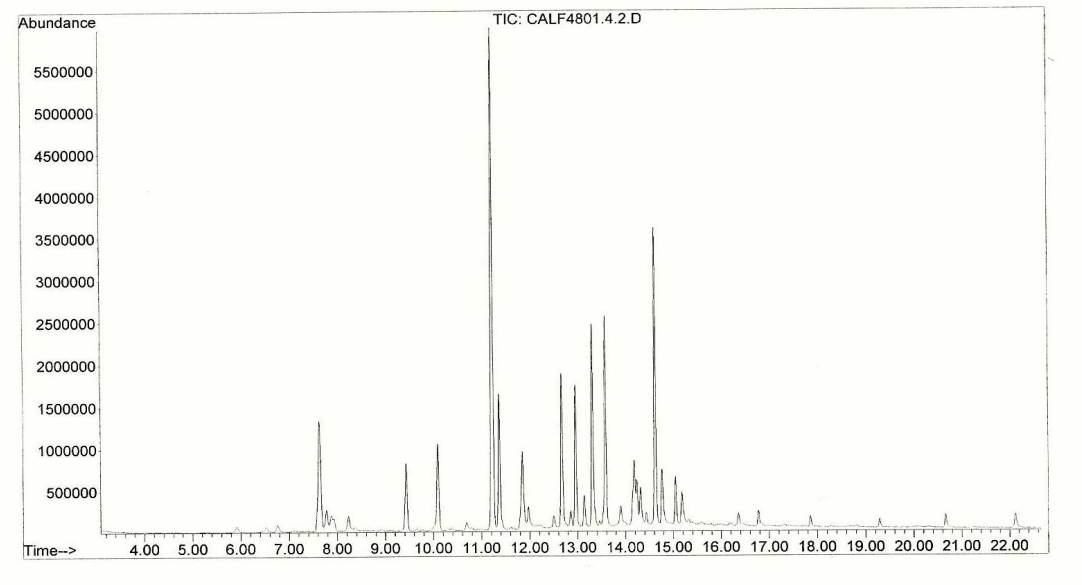


Table 5: Identified Fatty Acids from four *B. cereus* strains.

Fatty Acid	
Abbreviation	**Chemical Name**
i[1]C13:0	11 - Methyldodecanoic
a[2]C13:0	10 - Methyldodecanoic
iC14:0	12 - Methyltridecanoic
C14:0	Tetradecanoic (Myristic)
iC15:0	13 - Methyltetradecanoic
aC15:0	12 - Methyltetradecanoic
iC16:0	14 - Methylpentadecanoic
C16:1	*cis* - 9 - Hexadecanoic
C16:0	Hexadecanoic (Palmitic)
aC17:1	*cis* - 11 - 15 - Methylhexadecanoic
iC17:0	15 - Methylhexadecanoic
aC17:0	14 - Methylhexadecanoic

[1] i = Iso. [2] a = Anteiso.

Table 6: Fatty Acid Composition for *B. cereus* strains in Early Stationary Growth Phase.

Fatty Acid	Strains of *B. cereus* Area Percents[1] (%)			
	F4810/72	NC7401	Arnold	ATCC 14579
i[2]C13:0	8.36 ± 0.12	6.88 ± 0.40	7.17 ± 0.75	6.76 ± 0.61
a[3]C13:0	1.97 ± 0.13	2.24 ± 0.20	3.75 ± 0.12	2.64 ± 0.36
iC14:0	3.41 ± 0.02	4.43 ± 0.41	5.36 ± 0.78	4.67 ± 0.01
C14:0	5.71 ± 0.23	3.8 ± 0.29	6.35 ± 1.24	4.8 ± 0.44
iC15:0	25.69 ± 0.90	23.62 ± 0.55	20.38 ± 1.7	23.09 ± 1.33
aC15:0	10.37 ± 0.77	13.09 ± 1.1	13.72 ± 1.86	12.87 ± 1.28
iC16:0	4.46 ± 0.21	7.2 ± 1.25	7.27 ± 0.25	6.8 ± 0.34
C16:1	16.21 ± 0.76	12.56 ± 2.38	12.23 ± 5.25	14.38 ± 2.44
C16:0	7.86 ± 0.41	6.55 ± 0.91	8.69 ± 0.60	5.97 ± 1.22
aC17:1	2.69 ± 0.52	3.15 ± 1.24	1.68 ± 1.45	3.96 ± 0.26
iC17:0	9.77 ± 0.79	10.76 ± 0.73	8.12 ± 0.31	9.7 ± 0.65
aC17:0	3.48 ± 0.27	5.71 ± 1.04	5.3 ± 0.16	4.89 ± 0.32

[1] All values are means ± standard deviation of three determinations of independent experiments. [2] i = Iso.
[3] a = Anteiso. Red indicates emetic strain. Blue indicates non-emetic strain.

Table 7: Fatty Acid Composition of *B. cereus* strains in Late Stationary Growth Phase.

Fatty Acid	Strains of *B. cereus* Area Percents[1] (%)			
	F4810/72	NC7401	Arnold	ATCC 14579
i[2]C13:0	7.88 ± 0.08	8.59 ± 0.84	7.99 ± 2.08	9.09 ± 1.95
a[3]C13:0	1.05 ± 0.18	1.37 ± 1.3	2.67 ± 1.6	2.28 ± 0.44
iC14:0	3.8 ± 0.3	5.27 ± 0.19	5.88 ± 2.4	5.15 ± 0.84
C14:0	5.66 ± 0.47	5.23 ± 0.76	4.97 ± 1.22	5.71 ± 1.52
iC15:0	27.42 ± 0.94	27.06 ± 0.98	23.29 ± 0.51	24.08 ± 1.71
aC15:0	7.37 ± 0.32	11.54 ± 2.31	11.31 ± 3.9	9.74 ± 0.43
iC16:0	6.28 ± 0.92	6.75 ± 1.83	6.13 ± 1.12	5.67 ± 0.68
C16:1	11.62 ± 2.62	10.8 ± 2.0	15.54 ± 2.96	15.3 ± 2.15
C16:0	10.3 ± 0.30	7.3 ± 0.61	6.77 ± 2.93	8.74 ± 2.64
aC17:1	2.23 ± 0.18	1.47 ± 1.27	3.69 ± 1.72	3.16 ± 1.25
iC17:0	13.21 ± 1.19	10.6 ± 2.64	9.07 ± 3.63	8.3 ± 1.56
aC17:0	3.18 ± 0.25	4.04 ± 0.4	2.69 ± 0.47	2.52 ± 0.27

[1] All values are means ± standard deviation of three determinations of independent experiments. [2] i = Iso.
[3] a = Anteiso. Red indicates emetic strain. Blue indicates non-emetic strain.

The statistical analyses of early stationary growth phase are shown in **Table 8**. F4810/72 has the most significant differences when compared to the other strains. NC7401 is not notably different than ATCC 14579 (p-values > 0.05). F4810/72 and Arnold have the greatest differences, with five fatty acids being significantly different. At iC 13:0 all strains are differnt to Arnold. Throughout the strain comparisons between iC13:0 and iC15:0 tended to differ more than between aC15:0 and aC17:0. When compared to late stationary growth phases (**Table 9**), early stationary phase growth overall has more deviation in fatty acid composition among the strains.

Table 8: Statistical Analyses of Fatty Acid Composition of *B. cereus* Strains in Early Stationary Growth Phase.

Fatty Acid	F4810/72 vs NC7401	F4810/72 vs Arnold	F4810/72 vs ATCC 14579	NC7401 vs Arnold	NC7401 vs ATCC 14579	Arnold vs ATCC 14579
	P-values at 95% Confidence Interval[1]					
i[2]C13:0	<.05	>.05	<.05	>.05	>.05	>.05
a[3]C13:0	>.05	<.001	<.05	<.001	>.05	<.01
iC14:0	>.05	<.01	<.05	>.05	>.05	>.05
C14:0	<.05	>.05	>.05	<.01	>.05	>.05
iC15:0	>.05	<.01	>.05	<.05	>.05	>.05
aC15:0	>.05	>.05	>.05	>.05	>.05	>.05
iC16:0	<.01	<.01	<.05	>.05	>.05	>.05
C16:1	>.05	>.05	>.05	>.05	>.05	>.05
C16:0	>.05	>.05	>.05	>.05	>.05	<.05
aC17:1	>.05	>.05	>.05	>.05	>.05	>.05
iC17:0	>.05	>.05	>.05	<.01	>.05	>.05
aC17:0	<.01	<.05	>.05	>.05	>.05	>.05

[1] P-values were determined by a Tukey multiple comparison method, which was applied to an ANOVA; n= 3. Red indicates emetic strain. Blue indicates non-emetic strain. [2] i = Iso. [3] a = Anteiso. ☐ = Signficant.

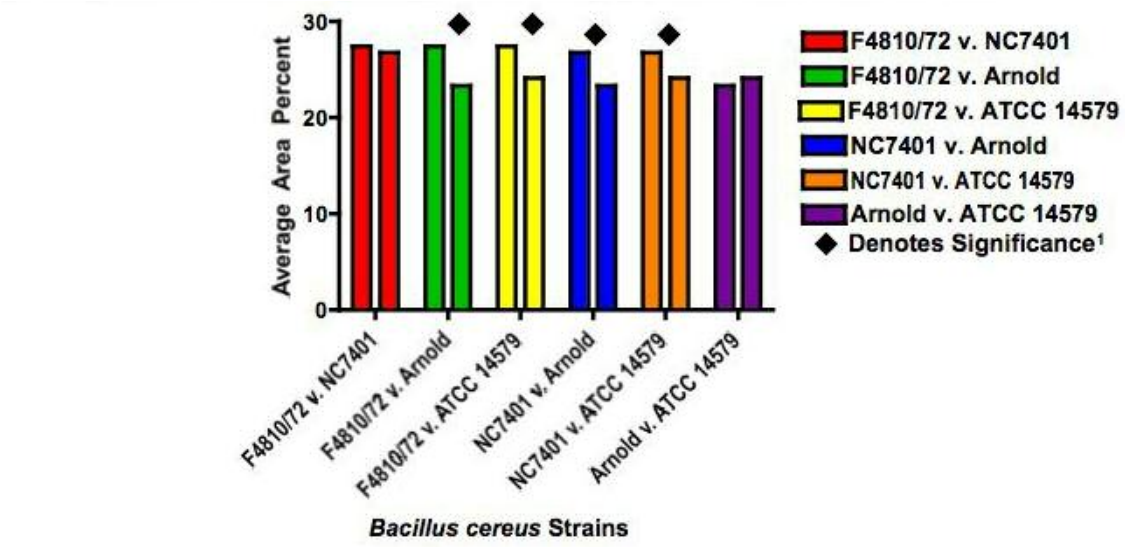
When late stationary phase fatty acid profiles were compared (**Table 9**) the strains did not generally differ. At iC15:0, there was a significant difference between the fatty acid content of the emetic strains and Arnold/ ATCC 14579 (**Figure 17**). At aC17:0, NC7401 was significantly different from both Arnold and ATCC 14579.

Table 9: Statistical Analyses of Fatty Acid Composition of *B. cereus* Strains in Late Stationary Growth Phase.

Fatty Acid	F4810/72 vs NC7401	F4810/72 vs Arnold	F4810/72 vs ATCC 14579	NC7401 vs Arnold	NC7401 vs ATCC 14579	Arnold vs ATCC 14579
	P-values at 95% Confidence Interval[1]					
i[2]C13:0	>.05	>.05	>.05	>.05	>.05	>.05
a[3]C13:0	>.05	>.05	>.05	>.05	>.05	>.05
iC14:0	>.05	>.05	>.05	>.05	>.05	>.05
C14:0	>.05	>.05	>.05	>.05	>.05	>.05
iC15:0	>.05	<.01	<.05	<.05	<.05	>.05
aC15:0	>.05	>.05	>.05	>.05	>.05	>.05
iC16:0	>.05	>.05	>.05	>.05	>.05	>.05
C16:1	>.05	>.05	>.05	>.05	>.05	>.05
C16:0	>.05	>.05	>.05	>.05	>.05	>.05
aC17:1	>.05	>.05	>.05	>.05	>.05	>.05
iC17:0	>.05	>.05	>.05	>.05	>.05	>.05
aC17:0	>.05	>.05	>.05	<.01	<.01	>.05

[1]P-values were determined by a Tukey multiple comparison method, which was applied to an ANOVA; n= 3. Red indicates emetic strain. Blue indicates non-emetic strain. [2] i = Iso. [3] a = Anteiso. ☐ = Signficant.

Figure 17: A Comparison of *B. cereus* Strains Average Area Percent at iC15:0 in Late Stationary Growth Phase.



[1] Significance is based on Tukey's multiple comparison test as applied to an ANOVA: n = 3

Differences between fatty acid composition in early and late stationary phase fatty acid profiles of individual *B. cereus* strains were statistically analyzed using a Student T-test and shown in **Table 10**. In F4810/72 five long chain and one short chain fatty acid changed from early to late stationary phase. With the exception of aC13:0, NC7401 short chain fatty acids (iC13:0- iC15:0) show difference between early to late stationary growth. At fatty acid aC17:0, both Arnold and ATCC 14579 showed changes between early and late phase growths. Non-emetic strains showed changes at C15:0: iso for Arnold and anteiso for ATCC 14579.

Table 10: Statistical Comparison of Fatty Acid Composition from Early and Late Stationary Growth Phases of Individual *B. cereus* Strains.

Fatty Acid	F4810/72	NC7401	Arnold	ATCC 14579
	P-values at 95% Confidence Interval[1]			
i[2]C13:0	0.0041	0.0336	0.5535	0.1222
a[3]C13:0	0.5389	0.3171	0.3077	0.7716
iC14:0	0.1617	0.0314	0.7405	0.3736
C14:0	0.8765	0.033	0.2423	0.3754
iC15:0	0.0858	0.0061	0.0472	0.4751
aC15:0	0.0032	0.351	0.3973	0.016
iC16:0	0.0292	0.7392	0.1609	0.0612
C16:1	0.0433	0.3843	0.3956	0.6511
C16:0	0.0012	0.3061	0.3293	0.1759
aC17:1	0.2186	0.1766	0.194	0.339
iC17:0	0.0141	0.9275	0.6672	0.2225
aC17:0	0.2347	0.0602	0.0008	0.0006

[1] P-values were determined by T-Test statistical analysis; n = 3.[2] i = Iso. [3] a = Anteiso. Red indicates emetic strain. Blue indicates non-emetic strain. ☐ =Signficance for T-Test.

Other authors (Lawrence et al., 1991) have stated that a method of differentiating between *B. cereus* and *B. anthracis* involves the ratio of iso to anteiso fatty acid composition. Early stationary phase ratios of odd numbered iso and anteiso fatty acids were compared. As noted in **Table 11**, F4810/72 is significantly different than Arnold at each iso/anteiso ratio statistical comparison, while NC7401 and ATCC 14579 are different in two comparisons.

Table 11: Statistical Analyses of Odd Numbered Iso and Anteiso Fatty Acid Ratios of *B. cereus* Strains in Early Stationary Growth Phases

Fatty Acid	F4810/72 vs NC7401	F4810/72 vs Arnold	F4810/72 vs ATCC 14579	NC7401 vs Arnold	NC7401 vs ATCC 14579	Arnold vs ATCC 14579
	P-values at 95% Confidence Interval[1]					
i[2]C13:0/ a[3]C13:0	<0.01	<0.001	<0.001	<0.01	>0.05	>0.05
iC15:0/ aC15:0	<0.01	<0.001	<0.01	>0.05	>0.05	>0.05
iC17:0/ aC17:0	>0.05	<0.01	>0.05	>0.05	>0.05	>0.05

[1]P-values were determined by a Tukey multiple comparison method, which was applied to an ANOVA; n= 3. Red indicates emetic strain. Blue indicates non-emetic strain. [2] i = Iso. [3] a = Anteiso. ☐ = Signficant.

Late stationary growth phase odd numbered iso and anteiso fatty acid composition rations are shown in **Table 12**. Only F4801/72 differs from all other strains at iC13:0/aC13:0 and iC15:0/aC15:0. All other strains show know difference from each other at any iso/anteiso comparisons.

Table 12: Statistical Analyses of Odd Numbered Iso and Anteiso Fatty Acid Composition of *B. cereus* Strains in Late Stationary Growth Phases

Fatty Acid	F4810/72 vs NC7401	F4810/72 vs Arnold	F4810/72 vs ATCC 14579	NC7401 vs Arnold	NC7401 vs ATCC 14579	Arnold vs ATCC 14579
	P-values at 95% Confidence Interval[1]					
i^2C13:0/ a^3C13:0	<0.05	<0.01	<0.01	>0.05	>0.05	>0.05
iC15:0/ aC15:0	<0.05	<0.05	<0.05	>0.05	>0.05	>0.05
iC17:0/ aC17:0	>0.05	>0.05	>0.05	>0.05	>0.05	>0.05

[1]P-values were determined by a Tukey multiple comparison method, which was applied to an ANOVA; n= 3. Red indicates emetic strain. Blue indicates non-emetic strain. [2] i = Iso. [3] a = Anteiso. ☐ = Signficant.

The average total area percentages for even iso, odd iso, and odd anteiso fatty acids are shown in **Table 13** and **Table 14**. The results showed differences in early stationary growth phase between F4810/72 and the other strains for even iso and odd anteiso fatty acids. Even iso fatty acids for F4810/72 and NC7401 are the only strains to increase from early to late stationary growth phase. F4810/72 is the only strain to increase in odd numbered anteiso fatty acids from early to late stationary phase.

Table 13: *B. cereus* Average Iso and Anteiso Branched-Chain Fatty Acid Area Percent in Early Stationary Growth Phase.

Strain	Fatty Acid Percents		
	Even Iso	Odd Iso	Odd Anteiso
F4810/72	7.87	43.82	15.82
NC7401	11.63	41.26	21.04
Arnold	12.63	35.67	22.77
ATCC 14579	11.47	39.55	20.14

Red indicates emetic strain. Blue indicates non-emetic strain.

Table 14: *B. cereus* Average Iso and Anteiso Branched-Chain Fatty Acid Area Percent in Late Stationary Growth Phase.

Strain	Fatty Acid Percents		
	Even Iso	Odd Iso	Odd Anteiso
F4810/72	10.08	48.51	11.6
NC7401	12.02	46.25	16.95
Arnold	12.01	40.35	16.67
ATCC 14579	10.82	41.47	14.54

Red indicates emetic strain. Blue indicates non-emetic strain.

CHAPTER IV

DISCUSSION

The purpose of the present study was to determine whether a correlation exists among cell membrane fatty acid composition, emetic toxin production, and cytotoxicity in *B. cereus*. Analyses of *B. cereus* growth, *in vitro* MTT assays, mitochondrial permeability assays, and GCMS were employed.

Analyses of *B. cereus* Growth

The *B. cereus* strains exhibited similar patterns of growth with minimal differences, regarding growth kinetics. Emetic strains remained in lag phase for a longer period of time than did non-emetic strains. The reason for an extended lag phase and subsequent separation in exponential growth phase could be due to undefined physiological differences between the strains. Apetroaie et al. (2005) found that cereulide producers, including F4810/72 and NC7401, present atypical physiological test results when compared to the non-cereulide producers.

Growth rates are important to the present study, as they can also be indicative of physiological differences between the strains**.** Growth rates for all *B. cereus* strains employed exhibited no significant changes as assessed on the basis of exponential growth

phase slopes. Also, the r-squared values of all strains were similar. R-squared values are used to report "goodness of fit" or how well the points make a straight-line.

There is evidence to support harvesting the samples at later times, including 18 hours (Finland, Logan, & Sutherland, 2005), 30 hours (Kawamura-Sato, 2005), and 48 hours (Toh et al, 2004). The papers suggest longer incubation time ensure cereulide production; however, longer incubation could induce sporulation. Sporulation was not an objective for this study, so the time period and designation of OD points for early (1.6-1.65 OD) and late (2.0) stationary phases were used to ensure that sporulation of the cultures had not yet occurred.

In vitro MTT Assays

Valinomycin, the standard ionophore for assessing cereulide cytotoxicity and concentration, was determined to have a LD_{50} of approximately 50 ng/ml for the HuH-7 cell line, a human hepatocellular carcinoma. This value is was high when compared to that reported using a boar sperm assay by Haggblom et al. (2002), but this could be due the ability of liver cells to filter toxins. The LD_{50} for the *B. cereus* strains examined here could not be determined because the heat-stable toxin(s) from CF failed to reach lower limits of the assay determined by valinomycin control (.047 OD). CF samples were considered to contain heat stable toxin(s) rather than cereulide because no definitive identification of cereulide was possible.

The final CF dilution point of the *B. cereus* strains from early stationary growth phase produced cytotoxic effects on HuH-7 cells. F4810/72 exhibited a larger concentration of heat-stable toxin(s) when compared to the other *B. cereus* strains. The

increase in heat-stable toxin(s) concentration may be explained due to the fact F4810/72 has been reported by others to produce cereulide (Andersson et al., 1998). NC7401 and Arnold exhibited comparable heat-stable toxin(s) concentrations in early stationary phase. Strain NC7401 has been shown to produce less heat-stable toxin when compared to F4810/72 (Andersson et al., 1998). The cytotoxicity results for ATCC 14579 show minimal levels of heat stable toxin(s) at 1.31 ng/ml. ATCC is known to be negative for cereulide production (Andersson et al, 1998).

Late stationary growth phase heat-stable toxin(s) production is significantly different among the *B. cereus* strains. F4810/72, with a concentration of 20.56 ng/ml of heat-stable toxin(s), was significantly different than NC7401, Arnold, and ATCC 14579 with p-values ranging from <0.01 to <0.001. F4810/72 and NC7401 are known cereulide producers (Andersson et al., 1998) and would then be expected to produce greater concentrations of heat stable toxin(s) than the non-emetic strains. Arnold and ATCC 14579 have produced 5.6 and 4.65 ng/ml of heat-stable toxin(s), respectfully. These concentrations were not significantly different and may be typical of non-emetic *B. cereus* stains. A clear distinction between emetic and non-emetic strains of *B. cereus* in late stationary growth phase heat-stable toxin(s) concentrations was demonstrated to exist.

The OD and heat-stable toxin(s) concentrations of *B. cereus* individual strains were statistically compared in the transition of early to late stationary growth phases. Only ATCC 14579 demonstrated significant OD decreases from early to late stationary growth phase, however, decreases in all *B. cereus* strains were observed from early to late stationary phase. The *in vitro* MTT assay revealed significant increase in heat-stable

toxin(s) production for all strains. OD and heat-stable toxin(s) concentrations have an inverse relationship. The decrease in OD exhibited by all strains correlates with an increase in heat stable toxin(s) concentration from early to late stationary phase growth.

Heat-stable toxin(s) production in early stationary phase was less for all strains than the concentrations produced in late stationary phase. The production of *B. cereus* heat-stable toxin cereulide has been shown to begin in late exponential to early stationary phases (Drobniewski, 1993). With the production of cereulide commencing before early stationary phase detection of the toxin may not occur until late stationary phase due to an insufficient amount of toxin to cause observable cytotoxic effects. The increase of heat-stable toxin(s) concentration from early to late stationary phase in all strains correlates with the previously shown production of heat-stable toxin cereulide. The elevation of heat-stable toxin(s) concentrations for F4810/72 and NC7401 in late stationary phase could have been due to the production of heat-stable toxin cereulide. However, the importance of the non-emetic strains exhibiting cytotoxicity with known negative cereulide production could be indicative of other heat-stable toxin(s) and cereulide could not be distinguished from other toxins.

The *in vitro* MTT assay determined that the cytotoxicity and heat-stable toxin(s) production of *B. cereus* emetic and non-emetic strains differ. In this study F4810/72 and NC7401 were confirmed to produce increased heat-stable toxin(s) concentrations and cytotoxicity; Arnold and ATCC 14579 were found to have decreased heat-stable toxin(s) concentration and cytotoxicity. Arnold's emetic toxin production was previously undocumented, but determined to be non-emetic due to the heat-stable toxin(s) concentration in late stationary growth phase being comparable to that of ATCC 14579.

Mitochondrial Permeability Assay

Triplicate results of the mitochondrial permeability assay were precise, yet proved to be inconclusive. The ratio of JC-1 aggregates and monomers remained within the range of 1.54 to 2.0 fluorescence. The lack of difference between the valinomycin standard, DMEM, and CF dilutions from all *B. cereus* strains could be due to assay insensitivity, use of fluorescent spectrometry rather than microscopy, or human error.

Gas Chromatography/ Mass Spectra Fatty Acid Scans

The fatty acid content of *B. cereus* has been previously been employed as a means for differentiating species in the *B. cereus* group (Whittaker et al., 2005). The fatty acid composition of different toxin-producing *B. cereus* strains has not yet been examined. Gas chromatography/mass spectral scans were employed in the present study to determine if differences in fatty acid composition exist among *B. cereus* emetic and non-emetic strains.

The fatty acids identified from the four *B. cereus* strains in this study correlated with those previously reported by Kaneda (1991), Whittaker et al (2005), and Lawrence et al., (1991). The *B. cereus* strains in the present study displayed strain-specific differences in fatty acid area content in each growth phase and individual strain fatty acid differences occurred with regard to changes between the growth phases. Changes in fatty acid composition can occur in response to temperature changes, pH, stress, transportation, energy synthesis, and primer availability (Haque & Russell, 2004; Kaneda, 1991; Guffanti et al., 1987; Beck et al., 2004). In order to eliminate

physiological variables the *B. cereus* strains in this study were cultivated under standard conditions.

Analyses of fatty acid composition of *B. cereus* strains in early stationary phase growth revealed four general trends: (1) the majority of significant differences involved *B. cereus* F4810/72 strain, especially as it differed from non-emetic strains; (2) a preponderance of fatty acid differences were between iC13:0 and iC15:0 which distinguished F4810/72 and NC7401 from non-emetic strains (3) NC7401 was not significantly different than ATCC 14579, and (4) Arnold differs from F4810/72 and NC7401 at iC15:0, which is a major contributor to *B. cereus* fatty acid composition (Lawrence et al., 1991). Generally in early stationary phase growth, a separation of emetic and non-emetic strains was exhibited, particularly involving strain F4810/72.

Late stationary growth phase fatty acid composition did not generally differ among *B. cereus* strains as much as that seen in early stationary growth phase. There were significant differences between the emetic and non-emetic strains in iC15:0, which is known to be the largest branched-chain fatty acid produced by *B. cereus* (Lawrence et al., 1991). F4810/72 and NC7401 exhibited greater amounts of iC15:0 than either Arnold or ATCC 14579. Elevated iC15:0 could then be a distinguisher of emetic versus non-emetic strains of *B. cereus* in late stationary phase growth. Also, fatty acid aC17:0 statistically differed from NC7401 than the non-emetic strains, which could help discriminate this strain.

B. cereus strain-specific fatty acid content differed significantly in early and late stationary growth phases. F4810/72 exhibited significant changes in longer chain fatty acids, while NC7401 demonstrates the opposite; changes in shorter chain fatty acids.

Overall, the fatty acid content of emetic strains was more likely to exhibit changes during the transition from early to late stationary growth phase than in non-emetic strains. The differences between early and late stationary growth phases exhibited by the emetic strains could be representative of the differences in physiology or fatty acid synthases.

Odd numbered iso/anteiso branched chain fatty acids were used to distinguish *B. cereus* from *B. anthracis* by Lawrence et al. (1991). In the present study, the ratios of iso/anteiso branched-chain fatty acids were also shown to be distinguishing factors among the four strains. For example, F4810/72 could be discriminated from all other strains by virtue of iso/anteiso fatty acid content at iC13:0/aC13:0 and iC15:0/aC15:0 in early and late stationary growth phases. F4810/72 was distinguished from NC7401, Arnold, and ATCC 14579 with regard to iso/anteiso fatty acid content in both growth phases. Lawrence et al. (1991) stated that the differences in iso/anteiso fatty acid content ratios could be indicative of different fatty acid synthase activities.

Branched-chain fatty acids are generally synthesized from specific α-keto acid primers, specifically even numbered iso (valine primer), odd numbered iso (leucine primer), and odd numbered anteiso (isoleucine primer) fatty acids (Beck et al., 2004). Exogenous supplies of α-keto acids have been shown to be assimilated into the membrane in the form of branched-chain fatty, therefore increasing branched-chain fatty acid composition (Beck et al., 2004; Zhu et al., 2005). Changes in area percent of α-keto acid primer branched-chain fatty acid derivatives provide evidence of the emetic strain F4810/72 being different from non-emetic strains. F4810/72 and NC7401 exhibited increases in even iso fatty acids as the early to late stationary growth phase transition occurred, thereby indicating of increased valine specificity or availability. F4810/72 is

the only strain when underwent an increase in odd numbered iso fatty acids when early and late stationary phase were compared, thus an increase in leucine availability could be suspected. All strains decrease in odd anteiso fatty acid composition potentially thereby demonstrating a decrease in isoleucine abundance in the culture or the strains ability to synthesize this α-keto acid. The comparison of branched-chain fatty acid α-keto acid derivatives may further lead to a relationship between membrane composition and the emetic toxin, cereulide, produced by *B. cereus* due to the α-keto acid monomer composition of cereulide.

The physiological ramifications of fatty acid content changes in bacteria have been well cited throughout the literature. Generally, changes in fatty acid content occur in response to temperature and pH changes causing physiological changes thereby maintaining optimal membrane fluidity and enhance survival of the bacteria in the new environment. A study on a protonophore-resistant mutant of *Bacillus subtilis* revealed the change in fatty acid content to be associated with protonophore-resistance (Krulwich et al., 1987). The *B. subtilus* fatty acid content has been noted to decrease monounsaturated C16 (C16:1) by 50% and increase in the iso/anteiso C15:0 ratio (Krulwich et al., 1987). The *B. cereus* cereulide-producing strains are known to exhibit self-resistance towards cereulide (Ehling-Schulz et al., 2006). F4810/72 and NC7401 exhibit the same deduction in C16:1 and increase in iso/anteiso C15:0 ratio; where as, the non-emetic show an increase in C16:1 fatty acid and iso/anteiso C15:0 ratio. This clear difference between emetic and non-emetic strains may then confer self-resistance to cereulide-producing strains of *B. cereus.*

Distinguishing emetic and non-emetic strains through fatty acid composition was possible, especially when discriminating the emetic strains and Arnold. F4810/72 fatty acid content was distinguished from non-emetic strains. F4810/72 was primarily different than non-emetic strains in early stationary phase growth and iso/anteiso ratios. NC7401 has been shown to be similar both to emetic and non-emetic strains, but could generally be differentiated from Arnold. Arnold and ATCC 14579 possess minimal significant differences in all fatty acid content comparisons were considered alike. The most distinguishing factor was that F4810/72 and NC7401 were distinguished from non-emetic strains at iC15:0 in early and late stationary phases of growth.

CHAPTER V

CONCLUSION

The results of this study led to several conclusions. First, analyses of *B. cereus* growth results revealed similar growth patterns among the *B. cereus* strains. The ability to study different strains in the same growth phase was a crucial step in the comparison of the *B. cereus* strains. Subtle differences of the four *B. cereus* strains at lag and exponential phases of growth were detectable, but the slopes revealed no statistical differences between the strains, therefore growth rates are generally similar among the strains under study.

Second, the results from the *in vitro* MTT assay provides a clear distinction between emetic and non-emetic strains heat-stable toxin(s) production and cytotoxic affects on HuH-7 cells line (hepatocellular carcinoma). The production of heat stable toxin was elevated in known emetic strains F4810/72 and NC7401. The non-emetic strains (Arnold and ATCC 14579) were found to cause decreased amounts of cytotoxicity when compared to known emetic strains. The amplified heat-stable toxin(s) production exhibited by emetic stains was determined to be due to the emetic toxin, cereulide.

Third, variations exist among the *B. cereus* strains when fatty acid composition was compared. It was determined that in early stationary growth phase, the emetic strains are different than the non-emetic strains in the analyses performed. While late

stationary growth phases provided minimal discrepancies in all fatty acid analyses, clear differences of emetic and non-emetic strains at iC15:0 could be differentiated. Another distinguishing factor was the iso/anteiso fatty acid ratios of *B. cereus* strain F4810/72 when compared to *B. cereus* strains NC4701, Arnold, and ATCC 14579. Individual strain analyses from early to late stationary growth phases produced noteworthy changes in fatty acid content. Overall F4810/72 can be distinguished from the other strains utilized in this study through cell membrane fatty acid content.

The potential for correlation among fatty acid composition, emetic toxin production, and cytotoxicity of *B. cereus* does exist, but cannot be completely defined in this study. However the present study demonstrates reproducible results that encourage further study on this topic. Investigations that involve changing the fatty acid content of the *B. cereus* strains and performing the same experimental design will provide greater insight into the possible correlations.

Microbial forensic research will prove to be crucial to the better understanding of bioterrorism agents. The information presented in this study could be used as a basis for future research on the biochemical physiology of *B. cereus* and the pathology of the diseases. The use of *B. cereus* as a bioterrorism agent is possible; however, more life threatening agents, such as *B. anthracis,* would be more likely employed in a terrorism event. Ultimately, future research in the possible relationship between the fatty acids and toxin production of *B. anthracis* would be the main target of the knowledge gained from this study.

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VITA

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Master of Science

Thesis: THE RELATIONSHIP AMONG CELL MEMBRANE FATTY ACIDS, EMETIC TOXIN PRODUCTION, AND CYTOTOXICITY IN *BACILLUS CEREUS*

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Title of Study: THE RELATIONSHIP AMONG CELL MEMBRANE FATTY ACIDS, EMETIC TOXIN PRODUCTION, AND CYTOTOXICITY IN *BACILLUS CEREUS*

Pages in Study: 57 Candidate for the Degree of Master of Science

Major Field: Forensic Science

Scope and Method of Study: The purpose of this study was to determine if there is a correlation among cell membrane fatty acids, emetic toxin production, and cytotoxicity in *Bacillus cereus*. *B. cereus* was used as a surrogate for *Bacillus anthracis*, the causative agent for anthrax. Four *B. cereus* strains were selected (F4810/72, NC7401, Arnold and ATCC 14579) because the strains phenotypic differences with regards to emetic toxin production. Analyses of *B. cereus* growth were performed using optical density readings. Samples were harvested at early and late stationary growth phase and centrifuged. The culture filtrate was partially purified for the emetic toxin cereulide. The cell pellet was washed with deionized H_20 and kept for gas chromatography/mass spectral scans (GCMS). *In vitro* MTT and mitochondria permeability assays were employed to determine emetic toxin concentration and cytotoxicity. GCMS scans were performed on the cell pellet using a programmed method to determine differences in fatty acid composition.

Findings and Conclusions: Statistical analyses of the slopes of *B. cereus* strains in exponential phase showed no differences between the four strains. The *in vitro* MTT assay results revealed differences in heat-stable toxin(s) concentrations distinguishing emetic from non-emetic *B. cereus* strains. Heat-stable toxin(s) concentration increased from early to late stationary phase for each *B. cereus* strain. Elevated concentrations of heat-stable toxin(s) for emetic strains when compared to non-emetic strains were determined to be due to cereulide production. GCMS scans of fatty acid composition of the four *B. cereus* strains showed differences, specifically distinguishing the fatty acid content of *B. cereus* stain F4810/72 from non-emetic strains. The emetic strains were differentiated from non-emetic strains at iC15:0 fatty acid in early and late stationary phase growth. The fatty acid content of emetic strains changed significantly from early to late stationary phase more than non-emetic strains of *B. cereus.* Odd numbered iso/anteiso ratios significantly distinguished F4810/72 from all other strains employed. Overall, a correlation among cell membrane fatty acids, emetic toxin production, and cytotoxicity of *B. cereus* is probable but cannot be definitively defined from the results of this study.

ADVISER'S APPROVAL: R. Thomas Glass, D.D.S., Ph.D.