MOLECULAR PATHOGENESIS OF *BACILLUS* SPP., WITH EMPHASIS ON THE DAIRY INDUSTRY

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ABSTRACT

The bacterial species *Bacillus cereus* accounts for 1.4–12% of foodborne illness outbreaks worldwide, a statistic that is certainly an underestimate. This bacterial genus is capable of contaminating a wide range of food products, including rice, chicken, vegetables, spices, and dairy products. *B. cereus* endospores are partially resistant to pasteurization, dehydration, gamma radiation, and other physical stresses used in food processing, and their adhesive characteristics promote biofilm-forming capability on a variety of substrates in dairy operations. *B. cereus* and other closely-related species produce several types of exotoxins, including at least four hemolysins, three phospholipases, a heat/acid stable emetic toxin called cereulide, and three well-studied heat-labile enterotoxins that all cause gastroenteritis following ingestion. While a great deal of information on virulence gene presence and expression is known in *B. cereus*, very little has been done to explore the virulence potential of thermoduric spore-formers that may be found in ultrahigh temperature (UHT) pasteurized milk, and their ability to produce biofilms. Biofilm production is understood to be under similar regulation as toxins and other extracellular virulence determinants. This chapter describes the current status of knowledge with *Bacillus* spp. relevant to the dairy industry, virulence potential, and biofilm production from the perspective of food safety.

BACILLUS – GENERAL INFORMATION

Bacillus spp. bacteria show a wide range of characteristics that allow them to live in most natural environments (Griffiths, 2010). *Bacillus* comprise a large group of ubiquitous Gram-positive, rod-shaped, aerobic-to-facultatively anaerobic endospore-forming saprophytes (Weber & Rutala, 1988). Although the majority of *Bacillus* spp. are nonpathogenic, a few *(Bacillus cereus, Bacillus anthracis, and Bacillus thuringiensis)* opportunistically infect animal hosts (mammals and insects) (Vilian *et al.*, 2006). *Bacillus* microscopic morphology may be individual or as long chains in primary isolates from soil or water samples (Weber & Rutala, 1988). The size

of an individual rod can range from 0.5 x 1.2 um to 2.5 x 10 um. Spores produced by *Bacillus* spp. are resistant to heat (including to some extent, pasteurization conditions), cold, ionizing radiation, dehydration, and many disinfectants (Griffiths, 2010). The endospores are oval or cylinder shaped and are found centrally, sub-terminally or terminally. Over 30 species of *Bacillus* spp. are recognized, and are divided into two groups based mostly on the 16S rRNA/DNA sequences: the *Bacillus subtilis* group and the *Bacillus cereus* group. *Bacillus subtilis, Bacillus amyloliquefaciens, Bacillus licheniformis* and *Bacillus pumilus* are mesophilic, have ellipse shaped spores, and are the most common members of the *B. subtilis* group. *B cereus, B. anthracis, B. thuringiensis, Bacillus weihenstephanensis, and Bacillus mycoides* do not ferment mannitol, produce lecithinase, and comprise the *B. cereus* group.

The colony morphology of *Bacillus* spp. is diverse across species. *Bacillus* spp. grow on nutrient agar or peptone media and exhibit ideal growth at a pH 7; however some *Bacillus* spp. grow at a pH of 9, while other species can endure pH 2. *Bacillus* spp. grow best within a temperature range of 30°–45°C, but thermophilic variants grow optimally at 65°C. All *Bacillus* spp. metabolize organic substrates such as amino acids, organic acids and sugars by aerobic respiration, anaerobic respiration, or fermentation, depending on species and environment. The enzymatic processes and metabolic characteristics are typically the criteria for *Bacillus* species differentiation.

*Bacillaceae f*amily members demonstrate a wide range of characteristics, including the ability to produce a battery of enzymes, antibiotics, and other secondary metabolites (Schallmey *et al*., 2004). For example, *Bacillus* spp. have unique abilities to synthesize and/or secrete many substances which are beneficial and show great success in agriculture and industry. Many *Bacillus* species exhibit antibacterial and antifungal activity against phytopathogens through secretory products (Yu *et al*., 2002), a logical evolutionary strategy since *Bacillus* spp. are soilborne or are found in epiphytes (plant that grows non-parasitically on another plant) and/or endophytes (living within a plant host)(Fravel, 2005). Many antimicrobial compounds are well recognized in the biotechnology and biopharmaceutical industries for their surfactant properties are derived from *B. subtilis* (Jacques, 2007). A surfactant lowers surface tension between two liquids or a solid and a liquid (Singhal, 2007). Surfactants are used for foam creation and stabilization in food processing, household products (paint, detergent, fabric softener), solubilization of agrochemicals, oil recovery, crude oil drilling lubricants, and bioremediation of water insoluble pollutants.

Phenotypically, the genus is difficult to delineate into species, but using genotypic methods, determination of relatedness has been revisited in recent years (Sneath, 1986). The mole % G + C content of the DNA is a well-regarded metric by which organisms may be compared genetically. The *Bacillus* genus is diverse and has a G + C content from 33–69% (Winn *et al*., 2006). Sequencing of16S rRNA genes and DNA–DNA hybridization methods have been used to assign species names (Goto *et al*., 2000). 16S rDNA has a hypervariant region (HV region) on the 5' end. This HV region is highly specific to each *Bacillus* spp. and is a good genotyping target. Overall, much emphasis has been placed in recent years on defining criteria for species determination within the genus *Bacillus*, although no single accepted system or approach has been established yet.

Other molecular techniques are also used to identify bacterial species including *Bacillus* spp. The following molecular techniques are used to confirm identify of *Bacillus* and other bacterial species: (1) polymerase chain reaction (PCR)(Adzitey *et al*., 2013); (2) pulsed field gel electrophoresis (PFGE); (3) random amplified polymorphism deoxyribonucleic acid (RAPD); and (4) matrix assisted laser desorption/ionization time of flight (MALDI-TOF)(Murray, 2012). PCR is a DNA replication process that amplifies small portions of DNA (amplicons) exponentially with the help of oligonucleotide primers and DNA polymerase. PCR has many different variations, including real time PCR (qPCR). qPCR is a powerful approach wherein template bacterial DNA is amplified and quantified at the same time using a standard curve-based comparison of type strain standards. PFGE is an agarose gel electrophoresis method that separates large pieces of genomic DNA. This separation of DNA is done by applying an electrical current that periodically changes between three different directions, providing a means to accurately resolve small differences in genomic sequences for bacterial community analyses. RAPD is a PCR based method that uses arbitrary primers to randomly amplify segments of target DNA, essentially acting as a DNA fingerprinting system for bacterial species. MALDI-TOF is a simple and rapid technique. Bacterial colonies are removed from the plate, mixed with a UV absorbing matrix (saturated solution of α-cyano-4- hydroxy-cinnamic acid in 50% acetonitrile and 2.5% trifluoroacetic acid) and dried on a target plate. The target plates are exposed to laser pulses that develop an energy transfer from the matrix to the nonvolatile analyte molecules. The analyte is removed in the form of gas. The molecules are enhanced in a flight tube to the mass spectrometer. MALDI-TOF is accurate, rapid, and after initial purchase, inexpensive. These characteristics perhaps explain why MALDI-TOF is being used in hospitals for quick identification of bacterial infections.

BACILLUS IN CLINICAL SETTINGS

Through biofilm production, *B. cereus* has been implicated in contaminating intravenous catheters (Hernaiz *et al*., 2003) resulting in *B. cereus*-mediated sepsis (Kuroki *et al*., 2009; Ozkocaman *et al*., 2006). The formation of biofilms also allows the release of planktonic bacteria that produce additional biofilms increasing the severity of the infection (Costerton *et al*., 1999).

In addition to catheter contamination, *B. cereus* and its endospores have been shown to contaminate air filtration and ventilation equipment (Bryce *et al*., 1993), fiber optic bronchoscopy equipment (Goldstein & Abrutyn, 1985; Richardson *et al*., 1986), linens (Barrie *et al*., 1994), gloves (York, 1990), specimen collection tubes and balloons used in manual ventilation (VanDerZwet *et al*., 2000), alcohol-based hand wash solutions (Hsueh *et al*., 1999), plaster-impregnated gauze (Rutala *et al*., 1986), and many antiseptics such as chlorhexidine and povodone iodine (Dubuoix *et al*., 2005). The most common types of infections *B. cereus* causes, other than foodborne illness, include fulminant bacteremia, central nervous system (CNS) involvement (meningitis and brain abscesses), pneumonia, gas gangrene-like cutaneous infections and endophthalmitis.

B. CEREUS-MEDIATED ENDOPHTHALMITIS

B. cereus is not only capable of causing food-associated toxicoinfections, but can cause endophthalmitis as well (Davey & Tauber, 1987; Hermandy *et al.*, 1990; Ullman *et al.*, 1987). *B. cereus* is not the only pathogen capable of causing endophthalmitis, but is considered one of the most aggressive pathogens causing this condition. Because there is a limited immune response when a pathogen enters the eye, a wide spectrum of pathogens can enter and elicit a wide array of effects. Symptoms can range from a relatively painless anterior chamber inflammation (Aaberg *et al.*, 1998), to an explosive ocular and periorbital infection caused by *B. cereus* (Schemmer & Drebe, 1987). Specific toxin production by a particular microorganism is theorized to account for the difference in symptoms. *B. cereus* induced endophthalmitis is characterized by a corneal ring abscess followed by increased pain, chemosis, proptosis, retinal hemorrhage, and perivasculitis (Callegan *et al.*, 1999). Fever, leukocytosis, and general malaise often appear as the systemic manifestations of this condition (Martinez *et al.*, 2007).

B. cereus induced endophthalmitis can be divided into two categories: exogenous and endogenous. An exogenous source is due to blunt trauma that penetrates the eye, which may occur due to occupation (for example, metal workers), in an agricultural setting (David *et al.*, 1994) or infection resulting from unsterile instruments during cataract surgery. In one example in Rome, an ophthalmologist had four of his cataract patients lose vision in their treated eye one day after their cataract surgery (Simini, 1998). *B. cereus* is ranked second behind *Staphylococcus aureus* which is responsible for about 70% of post-cataract surgery endophthalmitis (Han *et al.*, 1996). The three main risk factors surgeons need to be aware of to reduce posttraumatic endophthalmitis are the presence of an intraocular foreign body, delay in closure of the globe, and the location/extent of the laceration of the globe.

Endogenous sources represent about 2–8% of all endophthalmitis cases (Romero *et al.*, 1999) and are due to bacteria entering the posterior segment of the eye. The most common pathogen to enter the posterior segment of the eye is *Candida albicans* but other common pathogens include S. *aureus, B. cereus, Escherichia coli, Neisseria meningitides* and *Klebsiella spp.* *B. cereus* can accomplish this route of entry through blood transfusion, contaminated needles/illicit drug injection paraphernalia (Grossniklaus *et al.*, 1985), or by iatrogenic administration of medications such as B vitamins or insulin (Motoi *et al.*, 1997).

Moyer *et al* (2009) demonstrated that *B. cereus* is capable of disrupting tight junctions between endothelial cells and the basement membrane of retinal capillaries and retinal pericytes as early as 4h post-infection. Such changes are hypothesized to be responsible for causing the loss of retinal structure and function (Kopel et al., 2008; Moyer *et al.*, 2009). The exact toxins from *B. cereus* responsible for causing this breakdown of the blood retinal barrier are unknown but are theorized to consist of the following molecules that may be working individually or in concert to achieve this effect: the Hbl enterotoxin, the Nhe enterotoxin, a crude exotoxin (CET) derived from cell-free *B. cereus* culture filtrates, phosphatidylcholine-preferring phospholipase C (PC-PLC), collagenase, cereolysin O (Shany *et al.*, 1974), or cereolysin AB (Scott *et al.*, 1996). However, only the Hbl enterotoxin protein has been identified for its role in endophthalmitis (Callegan *et al.*, 1999a).

Hbl enterotoxin has been shown to cause irreversible tissue damage to the photoreceptors of the retina in less than 12–24h causing blindness in the infected eye (Beecher *et al*., 1995; Davey & Tauber, 1987).

B. cereus is capable of disrupting the blood retinal barrier as early as 4h in retinal tissues, 6h post-infection in aqueous humor, and in all other ocular tissues 12h post-infection (Callegan *et al*., 1999b). *B. cereus* has been shown to be a more rapid and virulent endophthalmitis pathogen compared to S. *aureus* and *Enterococcus faecalis*. Additionally, *B. cereus* seems to exhibit an almost immediate inflammatory response despite low numbers of the organism present at the early stages of infection.

Limited research exists addressing the exact role the immune system plays in endophthalmitis, but the eye is known to be an immunoprivileged site as was first described by Medawar in 1948 (Cunha-Vaz, 1997). The eye restricts both the adaptive and innate immune systems in such a way to balance the challenge of pathogen infection against inflammation-induced vision loss (Streilien, 2003).

In most instances of *B. cereus* induced endophthalmitis, vision loss occurs regardless of the type of therapeutic or surgical intervention utilized because the severity of the disease has progressed to such a condition, that too many toxins have been released by *B. cereus* and many bacteria will have migrated in the eye out of the reach of antibiotics (Callegan *et al*., 2006). Thus within a 12–18h time frame, massive tissue destruction occurs to the retina and surrounding ocular tissues resulting in antibiotics no longer being maximally effective (Callegan *et al*., 2002). In addition, the inflammatory response inside the eye is so aggressive that even if the antibiotics control *B. cereus*, the inflammation produced causes damage to surrounding ocular structures thus making it difficult to manage ocular infections.

BACILLUS IN FOOD

Foodborne illness from a variety of microorganisms affects on average 76 million individuals in the U.S. each year resulting in some 5,000 deaths (Mead *et al*., 1999). Worldwide statistics on *Bacillus cereus* foodborne illness are underestimated due to a variety of factors, including emetic symptoms similar to *Staphylococcus aureus* intoxication and diarrheal symptoms similar to those elicited by *Clostridium perfringens* type A. Most affected individuals do not seek medical attention due to the short duration of signs and symptoms. *B. cereus* seems to account for between 1.4–12% of foodborne illness outbreaks worldwide (Stenfors *et al*., 2008).

Bacillus spp. are capable of contaminating a wide range of food products, including rice, chicken, vegetables, spices, and dairy products. Contamination in the dairy industry may occur when *B. cereus* spores come in contact with the udders of cows (Andersson et al. 1995), if the spores colonize feed or bedding, or if the spores survive pasteurization (Claus & Berkley, 1986; Sneath, 1986). This is a serious problem in the food industry because *B. cereus* endospores are in many instances partially resistant to the

heat of pasteurization, dehydration, gamma radiation, and other physical stresses. This resistance is due to the ultrastructure of the endospore of course, but also in part to the hydrophobic nature of the spores that allows them to adhere strongly to surfaces and develop biofilm-like properties (Mattson *et al.*, 2000; Ronner *et al.*, 1990). For example, an irradiation dose of 1.25–4 kGy needs to be administered to reduce spores by 90% (De Lara et al., 2002). Also, pasteurization may result in the activation and germination of spores (Hanson *et al.*, 2005). In addition, *B. cereus* endospores germinate in response to particular nutrients such as glycine or in response to physical stress such as temperature (spore germination can occur over 5–50°C in cooked rice) (Granum, 1994) and high pressures (i.e. 500 MPa). Thus foods need to be cooked at least at a temperature of 100°C (212°F) or above to kill most of the endospores (Griffiths & Shraft, 2002).

Thermoduric sporeformers have many important reasons to be the subject of great interest within the dairy industry (Burgess *et al.*, 2010). Thermophilic bacilli produce heat-resistant (80–100°C) and highly heat-resistant (>106°C) endospores in UHT treated products, which can lay dormant for years. Heat, chemicals, and pH levels can activate a spore for germination and outgrowth. This is particularly important in the dairy industry because heat is used as a preservation mechanism. *B. subtilis* has a low activation temperature of 65–70°C. Once the spores are activated, germination is elicited by nutrients that bind to germination receptors. A nutrient mixture of asparagine, glucose, fructose and K+ (AGFK) triggers *B. subtilis* spore germination (Setlow, 2003).

Many people consider *B. anthracis*, *B. thuringensis*, and *B. cereus* to be the same species (Helgason *et al.*, 2000). *B. anthracis* is found in the soil and infects primarily herbivorous animals, causing human disease (Winn *et al.*, 2006; Kolsto *et al.*, 2009). This disease may be contracted by local infections of skin lesions, through the gastrointestinal (GI) route, or by inhalation. Respiratory and GI-acquired routes are highly lethal forms of anthrax. *B. anthracis* virulence mechanisms easily allows for the spread of the bacteria to the lymph nodes. Once in the lymph nodes, the bacteria disseminate via the bloodstream and internal organs. *B. anthracis* spores are highly resistant to adverse environmental conditions and it is difficult to be certain that the organism has been fully eradicated from endemic areas (Winn *et al.*, 2006). The endospores are maintained in soil and stay dormant indefinitely. The virulence determinants produced by *B. anthracis* are composed of three proteins: a protective antigen (PA), an edema factor (EF), and the lethal factor (LF). Virulent strains are also typically capsule-producers. Toxin expression and production is enhanced by elevated CO2 and growth temperatures of 35–37° C. *B. anthracis* strains harbor two large plasmids, pXO1 and pXO2 (Kolsto *et al.*, 2009). These plasmids are needed for full virulence. pXO1 contains the coding for the PA (pag), EF (cya), and LF (lef). pXO2 contains a five-gene operon for the biosynthesis of a polyglutamate capsule. This capsule is important for the ability to escape the host immune system, by protecting the vegetative cells from phagocytosis.

B. thuringensis classification has been accomplished by H serotyping, which utilizes bacterial flagellar antigens (Sneath, 1986). This species has unique insecticidal properties demonstrating activity against several insect orders, as well as nematodes, mites and protozoa. *B. thuringensis* produces protoxins during sporulation (Aronson *et al.*, 1986). These toxins are either parasporal inclusions or found on the spore surface. *B.*

thuringensis produces parasporal crystals during sporulation, which are inclusions of insecticidal toxins. The midgut of the larvae have proteases that convert protoxins to toxins, activating the toxin to bind to receptors on columnar midgut cells. This binding event results in pore formation of the midgut epithelium, and susceptible insects die from this extensive damage and pH changes as midgut contents mix with the hemocoel cavity. Three common subspecies variants have been recognized and well characterized over the last 40 years: (1) *B. thuringiensis subsp. kurstaki*; (2) *B. thuringiensis subsp. israelensis*; and (3) *B. thuringiensis subsp. japanensis*. Each produces crystalline endotoxin specific for a unique order of insect for selectively toxic biological control. Interestingly, each is also extremely genetically similar to the type strain pathogen in this family, *B. cereus*.

B. cereus and other *Bacillus* spp. are a major cause of foodborne illness globally and a major cause of endophthalmitis (Weber & Rutala, 1988; Stenfors *et al*., 2008; Moyer *et al*., 2008). *B. cereus* has an optimum growth temperature of 30°–40°C, although psychotrophic members can grow in temperatures as low as 4°C. *B. cereus* can grow in a pH of 5.0–8.8 with optimal pH of 6.0–7.0. Food poisoning due to *B. cereus* is underreported because it is short-term and self-limiting. In 2005, *Bacillus* spp. were responsible for 1.4% of foodborne illness in Europe. In the Netherlands 12% of foodborne illness was caused by *B. cereus* between 1993–1998. In 2006, an average of 63,400 (0.4%) people were domestically affected with *B. cereus food* poisoning (Scallan *et al*., 2011). Reports of *B. cereus* induced food poisoning has increased in industrialized countries, however reporting and testing is variable. In the US passive surveillance is usually performed due to low hospitalization of *B. cereus* food poisoning.

Foods frequently contaminated by *B. cereus* include milk, dairy products, dry foods, rice, egg products and legumes. Two types of food-related illnesses are caused by *B. cereus*: (1) Type 1: short-incubation "emetic" and (2) Type 2: long incubation "diarrheal." Type 1 has an incubation time of 2 hours and lasts approximately 9 hours. Type 2 has an incubation time of 9 hours and lasts 24 hours. Type 1 is mostly associated with contaminated rice and type 2 is associated to contaminated meat or vegetables.

The main virulence factor for type 1 food poisoning caused by *B. cereus* is cereulide (Cueppens et al., 2011). Cereulide is a small molecular weight heat stable exotoxin that can withstand treatment at 121°C for 2 hours at a pH of 7.0. This stability means the toxin can withstand frying, roasting, and microwave exposure, eliciting a foodborne emetic intoxication in susceptible individuals. The main causes of type 2 foodborne illness are hemolysin B (Hbl) and non-hemolytic enterotoxin (Nhe), both comprised of three components encoded by separate operons (Fig. 1) – typical AB toxin architecture. Hbl is made of the cytolytic subunits HblC and HblD, and the protein B binding domains. The Hbl operon also has a fourth member, the *hblB* gene. However, *hblB* is not transcribed and is likely a pseudogene. Nhe is made of the cytolytic protein NheA, and the protein B binding sections NheB and NheC. In recent research, 7.5% of reported emetic symptoms have been linked to Hbl and Nhe. These toxins are a product of aerobic, spore forming *B. cereus*.

Aerobic spore formers in food are ubiquitous. This ubiquity makes it impossible to prevent aerobic spore formers from being present in many fresh foods. Spore counts in raw milk vary throughout the year, but are highest in winter when dairy cows are primarily indoors. Pasteurization is effective

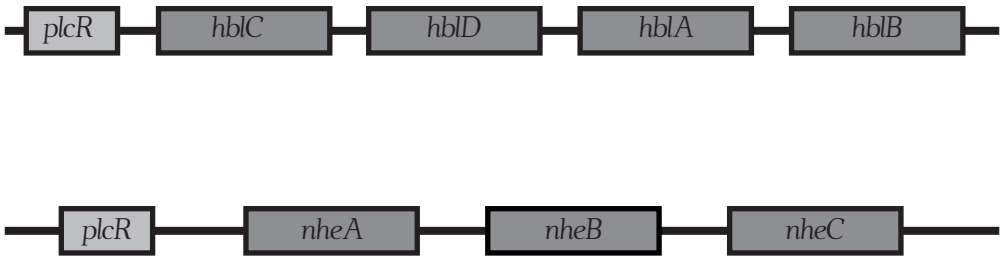

Figure 1. *Bacillus* spp. HBL and NHE operons. The *plcR* region is the regulator gene as described in the text [24]."

in inactivating vegetative cells in raw milk, but fails to kill many spores. The spores have no competition from vegetative cells, so they proliferate rapidly if the product is mishandled or improperly stored. The sporulated *Bacillus*, upon germination, can adhere to pipelines and equipment, causing biofilm formation. These spores and vegetative cells in equipment and raw milk may be tolerant to sterilization. Biofilm extrapolymeric substances (EPS) offer a significant survival strategy to established populations of bacteria. These counteractive techniques include ultra-high temperature (UHT) processing, previously known to inactivate all living material, however spores are now known to survive UHT-processing.

UHT-processing is achieved by treating fluid milk at 135–150°C for 1–8 seconds. The milk flows continuously during this process and is packaged into pre-sterilized containers (aseptic packaging). The UHT process is designed to kill almost all organisms including spores. The concern is that some spores still survive and there is no competition for these spores, giving them an ideal environment to proliferate. The growing concern for psychrotolerant spore formers is that they show potential to induce foodborne illness and produce spoilage defects caused by enzymatic activity. These concerns are due to a combination of the following reasons: (1) longer refrigeration storage pre-pasteurization; (2) higher temperatures used for pasteurization; (3) prolonged shelf life; and (4) pasteurization activates the germination of spores. A combination of these "advantages" are beneficial for *B. cereus* endospores to form from vegetative cells or vegetative cells to form endospores.

Production length of milk treatment has been reduced to 6–8 hours to help reduce thermophile growth (Burgess *et al*., 2010). Once a production cycle is complete, a cleaning-in-place (CIP) method is performed on the equipment. CIP consists of the following steps: (1) a warm water rinse; (2) a 1.5% caustic wash at 75°C for 30 minutes; (3) a water rinse; (4) a 0.5% nitric acid wash at 70°C for 20 minutes; and (5) a second water rinse. These steps have helped with growth within the equipment, but not within the milk itself. Table 1 indicates the time and temperature requirements laid out by the Food and Drug administration for pasteurization regimes, including UHT pasteurization.

BACILLUS SPP. BIOFILMS

Adherence of microbial biofilms to dairy production surfaces makes sanitization more difficult, and increases cost via labor and chemical usage along with lost production time. FDA involvement and subsequent product recalls can also occur causing further financial problems for dairies. Araújo et al. (2009) have proposed a basic mechanism for biofilm adhesion based on six general stages. First, the biofilm surface must be primed for adhesion with the existence of food deposits. The biofilm-producing microorganism must then come into contact with the primed surface. Positive and negative biochemical forces including van der Waals forces and other electrostatic forces then allow the biofilm to make a non-permanent attachment to the surface when microorganism are between 20 and 50 nm away. Irreversible adhesion results within 1.5 nm when extracellular polysaccharide production, ionic bonds, and hydrophobic forces occur. The fourth stage is described by the multiplication of bacterial cells and an increase in secreted polysaccharides and the fifth stage involves strong metabolism in the biofilm. Lastly, microorganisms begin to be released from the biofilm during the sixth stage, shedding bacteria to generate new biofilms elsewhere.

Several authors have identified a variety of mesophilic *Bacillus* subspecies capable of surviving ultra-high temperature pasteurization via endospore formation (Araújo *et al.* 2009; Lindsay *et al.* 2002; Scheldeman et al. 2006; Sutyak *et al.* 2008). Using bacterial cultures sampled from dairies, 16s rRNA, and PCR amplification some of the most prevalent and potentially problematic species, in regards to biofilm production, have been characterized. These species include *B. cereus*, *B. amyloliquefaciens*, and several others.

The level of virulence activity in *B. cereus* cells is due to a number of different environmental factors, including temperature, pH, oxygen tension, glucose concentrations, and specific antimicrobial chemical compounds (Glatz and Goepfeort, 1976; Sutherland and Limond, 1993). Biofilm production is understood to be under similar regulation as toxins and other extracellular virulence determinants, which suggests that subinhibitory stress may have great influence on overall potential for *Bacillus spp.* to become problematic in dairy microbiology settings.

QUORUM SENSING

Quorum sensing is a regulatory system where the bacterium (*B. cereus*) recognizes an extracellular signal caused by an autoinducer (AI) to sense the density of *B. cereus* in the immediate environment. Quorum sensing is used to govern cell density, and the corresponding regulation of relevant gene expression that would enhance survival during the log-to-stationary phase transition in dense cultures, or in a natural environment such as soil, food, or within a host [28]. Quorum sensing mechanisms control many processes in the bacterial cell, including sporulation, biofilm production, and virulence factor secretion [27]. Quorum sensing involves direct or indirect activation of a related receptor protein by the AI (Graumann, 2012). This activation results in up- or down-regulation of specific genes. All quorum sensing routines are dependent on three principles: (1) the bacterial species produces AIs; (2) AIs are detected by membrane or cytoplasm receptors; and (3) AIs produce a positive feedback loop (Rutherford *et al.*, 2012).

Gram-positive bacteria use small, post-translationally modified peptides as same species AIs, called Autoinducing Peptides (AIPs) (Graumann, 2012). AIPs are expressed as large, precursor peptides and processed into smaller, cyclic, thiolactone-containing peptides that are transported across the membrane. This transportation can happen in two ways: (1) two-component signaling (Fig. 2) or (2) AIP-binding transcription factor signaling (Fig. 3). In the two-component signaling method, once the AIPs are transported outside of the cell they are too hydrophilic to cross the membrane without help. The AIPs remain in the extracellular matrix. The bacteria sense the AIP as it binds to the receptor protein (histidine kinase) located in the neighboring bacterial cell surface. This binding induces phosphorylation of the kinase. The phosphoryl group is then transferred to an aspartate residue of the response regulator. Then this binds to the promoter region of target genes, which activates or represses transcription.

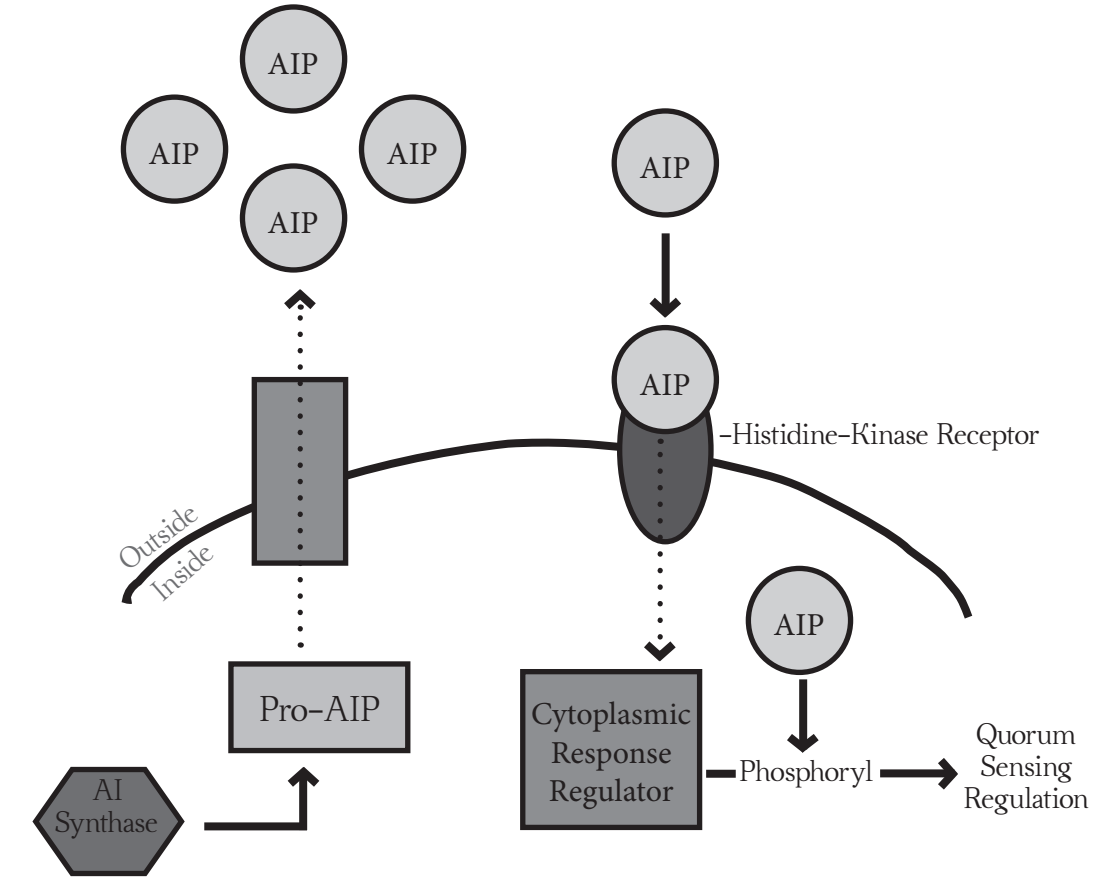

Figure 2. Two-component quorum sensing of Gram-positive bacteria. AI synthase is used to process and transport the pro-AIP out of the cell. Once the concentration of AIP outside the cell is high, AIP binds to histidine kinase receptors. This binding activates the kinase activity of the receptor, inducing autophosphorylation. The phosphoryl group binds to the response regulator and activates transcription of the quorum sensing system genes [27].

QUORUM SENSING & *BACILLUS* SPP. PATHOGENESIS

Quorum sensing in *B. cereus* is dependent on a protein PlcR. PlcR is a pleiotropic regulator of most virulence factors specific to the B. cereus group (Nhe and Hbl) (Rutherford *et al.*, 2012). The activity of PlcR depends on binding to the AIP that is produced from the PapR protein. PapR is a small signaling peptide that acts as a quorum sensing effector (Slamti & Lereclus, 2005) (Fig. 3). PapR is 48 amino acids long and is encoded by an open reading frame located downstream from plcR. PapR is secreted from the cell forming a PapR pro-AIP. PapR pro-AIP is processed by neutral protease B (NprB) to form the active AIP. The AIP is transported back into the cell by an oligopeptide permease system (Opp). AIP then binds to the transcription factor PlcR, activating the protein. This PlcR-AIP complex regulates the production of virulence factors and a positive feedback loop for *papR*. It has been shown that PlcR expression is positively regulated by CodY expression (Frenzel *et al.*, 2012).

CodY is a global transcriptional regulator that facilitates advantageous changes in response to variations of available nutrients in Gram-positive bacteria (Sonenshein, 2005). CodY is a GTP and isoleucine binding protein that also initiates endosporulation. The binding of GTP and isoleucine act as co-repressors of the transcription of many genes.

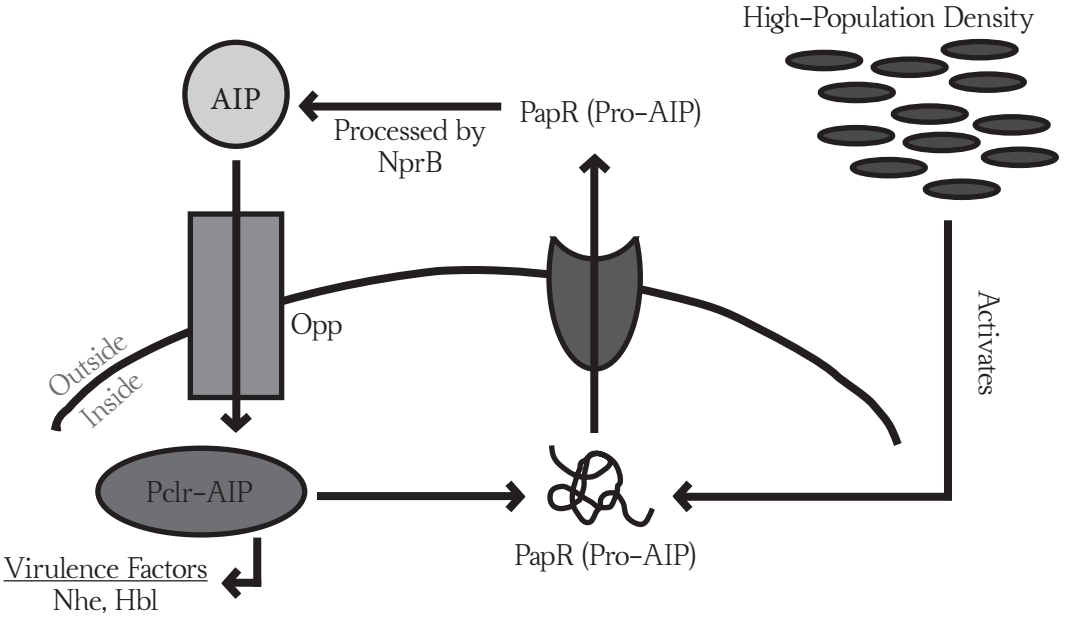

Figure 3. AIP binding PlcR (transcription factor) quorum sensing signaling in *B. cereus*. A high population density outside the cell activates PapR (pro-AIP) and then the PapR (pro-AIP) is secreted outside of the cell. PapR (pro-AIP) is processed by the protease NprB to become a heptapeptide AIP. AIP is transported back into the cell using an Opp. Once AIP is inside the cell, it binds and activates PlcR. This PlcR-AIP complex regulates virulence factors and also produces a positive feedback loop for PapR secretion [27].

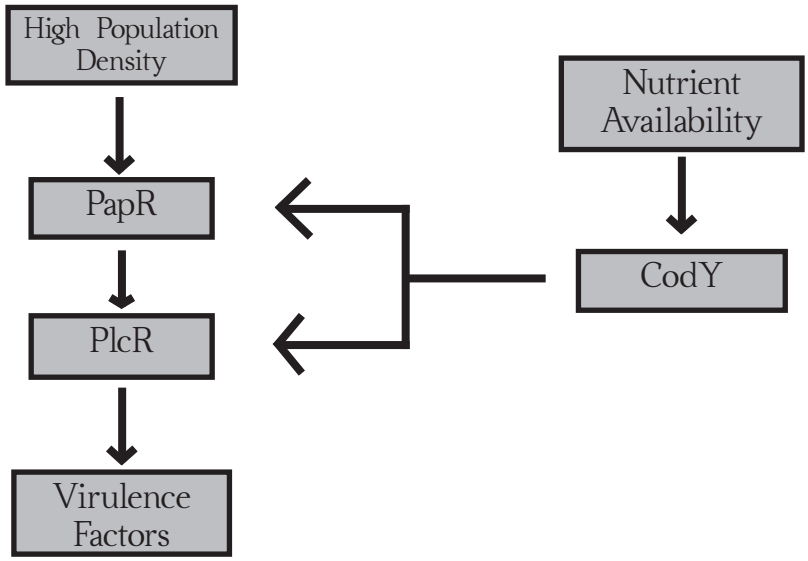

Figure 4. CodY regulates PapR and PlcR expression in *B. cereus*. Nutrient availability for the cell regulates the expression of CodY [27, 30].

Endosporulation happens when there is a response to bacterial starvation by limited levels of carbon, nitrogen or phosphorous. Endosporulation is a defense mechanism of Gram-positive bacteria, like a turtle hiding in its shell, to protect its genome. Nucleotide synthesis is dependent on carbon, nitrogen, and phosphorus. This spore keeps the genome dormant until the environment is favorable enough to replicate. CodY is a transcriptional activator of the plcR gene [30] (Fig. 4). In a Δ*codY B. cereus* strain, PlcR expression was strong in exponential, late exponential, and stationary phases of growth. In contrast, a wildtype *B. cereus* strain showed expression of PlcR in only the stationary phase of growth. CodY was first found in *B. subtilis* to control expression of more than 100 stationary phase genes. Thus, it is generally accepted that (like *plcR*), *codY* is widely conserved among Bacillaceae family members.

B. amyloliquefaciens belongs to the *B. subtilis* group (Priest *et al.*, 1987). Members of this group exhibit similar behaviors physiologically, although *B. amyloliquefaciens* is not a subspecies of *B. subtilis* due to the difference in □-amylase production. *B. amyloliquefaciens* has been found to share less than 5% homology at the DNA level with *B. subtilis*. *B. subtilis* has been shown to express CodY (Serror & Sonenshein, 2002; Ratnayake-Lecamwasam *et al.*, 2001). Phelps and McKillip (2002), using DNA PCR, found that hblC, hblD, hblA, nheA, and nheB genes or gene homologues were present in a different strain of *B. amyloliquefaciens* obtained from a Louisiana creamery, although expression of these genes was not measured. Thus, the potential for this species to harbor and express these or other virulence factors (via global effectors CodY and/or PlcR) is a realistic possibility, despite this species being placed (at least currently) in the *B. subtilis* group rather than the *B. cereus* group.

SUMMATION & FUTURE WORK

The debate over proper identification and understanding of *Bacillus* virulence has been ongoing for over 50 years (Rasko et al., 2005). Recent public awareness of potential bioterrorism using the anthrax toxin produced by *B. anthracis* has lead government agencies to fund multiple studies aimed at rapidly differentiating *B. anthracis* from other closely related *Bacillus* species such as *B. cereus* and *B. thuringiensis*, since *B. anthracis* produces the anthrax toxin encoded by two plasmid-based operons, pXO1 and pXO2. The anthrax toxin primarily kills herbivore mammals, but can also kill humans. Not to be underestimated, *B. cereus* can cause severe food poisoning through its production of emetic and diarrheal toxins. While heavily used as an insecticidal agent in crops with its Cry crystalline toxins, *B. thuringiensis* has also recently been demonstrated to cause food poisoning symptoms in humans similar to *B. cereus*. Ironically, species like *Bacillus coagulans*, which has been found to harbor the *nheA* gene, are readily used as probiotics in human health.

Bacillus spp. were originally differentiated into species at a time when biologists did not possess the molecular tools to delve deeper than biochemical tests and phenotypical observations. While this strategy worked well for other genera, 16S rRNA analysis of differences among *B. cereus*, *B. thuringiensis*, and *B. anthracis* have shown these species to have a nucleotide sequence difference of less than 1%. Recent advances in molecular biology have allowed scientists to scrutinize the genetic properties of these three "species". After exhaustive studies using DNA-DNA hybridization, 16S and 23S rRNA comparative analyses, multilocus sequence typing (MLST), fluorescent amplified fragment length polymorphism analysis, rep-PCR, and small nucleotide polymorphism (SNP) analyses, scientists have been unable to reliably differentiate these three *Bacillus* species.

While many methods have been pursued, most results have suggested that *B. cereus*, *B. thuringiensis*, and *B. anthracis* should be considered the same species due to highly conserved nucleoidal genetic sequences. Due to the easily identifiable symptoms of *B. anthracis* and *B. cereus*, there is recent concern among biologists that the "*B. anthracis*" species may in fact be an oversampled subset of *B. cereus*. Other scientists speculate that *B. anthracis* may have only recently evolved to the point to be considered distinct from *B. cereus*. Unfortunately, recent literature is contradictory when discussing how similar two separate *Bacillus* genomes need to be in order to be considered the same species. There are claims that *B. thuringiensis*, *B. cereus*, and *B. anthracis* should be considered one species on the basis of genetic evidence. Alternatively, other scientists claim that current taxonomy has not divided *Bacillus* strains enough, suggesting that more species or subspecies than currently listed in literature exist. No commonly accepted definition that separates these species on genetic evidence has been found.

There are three nhe genes that are encoded on the nheABC operon, and have been shown to remain conserved as a cluster during genetic recombination. It can reasonably be assumed that the presence of the most proximal subunit of nhe indicates the presence of the other two genes. In the literature, all genes encoding the Nhe and Hbl enterotoxins have been readily located downstream in both *B. cereus* and *B.*

thuringiensis (Phelps & McKillip, 2002).

The presence of the nheABC operon does not necessarily indicate a virulent strain, but has a very high likelihood of expressing these genes in a host environment or in food under permissive conditions. Thus, future work to determine the pathogenicity of nheA positive samples could include the use of a Tecra VIA to detect enterotoxin proteins. Without this step, the virulence of nheA positive samples cannot be definitively determined. A large degree of genetic variation exists in nhe sequences among Bacillus spp., giving rise to false negative results in PCR-based detection assays. Strains negative for nheA in real-time PCR have been found to produce the enterotoxin Nhe as determined using a Tecra VIA kit.

The nheABC operon is mobile among Bacillus spp. through horizontal gene transfer (HGT). Indeed, HGT has been observed among *Bacillus* spp. and can serve as a mechanism explaining the incidence of non-*B. cereus* samples positive for nheA. While no data has been found to suggest that this gene transfer mechanism uses an integron, the anthrax-like operon pXO16 found in *B. thuringiensis* is part of a conjugative plasmid. It is reasonable conjecture that other *Bacillus* species may also harbor conjugative plasmids that aid in HGT.

Within *Bacillus*, most virulence factors are encoded on plasmids (55), which have been demonstrated to readily transfer between differing species. Indeed, a recent study indicated that the virulence genes associated with *B. cereus* infection undergo frequent rearrangement both within the bacterial nucleoid and between species. Thus, a better method than traditional biochemical tests to detect pathogenic *Bacillus* strains is to screen for virulence operons present in plasmids or in nucleoidal DNA.

Bacillus genomes that have been sequenced display a high level of genetic synteny in their gene order. Two genes that encode for bacterial ribosomes, 16S and 23S rDNA, contain genetic sequences that are less than 1% different when compared between B. *cereus, B. thuringiensis*, and *B. anthracis* (12). A dissimilarity of 3% between 16S or 23S rDNA sequences is the minimal "cut off" between two strains to be considered as distinct species. Additionally, the *gyrB* gene sequence shared among these species is very homologous. Because these genes are shared among different species within the *Bacillus* genus, they cannot be used to differentiate species. However, 16S and 23S rRNA can be used to differentiate between different strains of *B. anthracis*.

Interestingly, there are a number of mechanisms that facilitate the movement of genes between different members of the *Bacillus* genus. One such mechanism is through the natural action of bacteriophage. After lysing its host cell, the bacteriophage will insert its genes into *Bacillus* genomes. While normally either lytic or lysogenic, it is possible for prophage to undergo random mutation, which renders it unable to enter the lysogenic cycle. In this way, genes from one species of bacteria can be transferred to *Bacillus spp.* As previously mentioned, *Bacillus* operons may be on conjugative plasmids. Additionally, *Bacillus* spp. are naturally competent, allowing these microbes to naturally take up random DNA in their vicinity.

The virulence genes for Nhe are present in more strains of Bacillus than is currently accepted within the scientific community. This research identified several "species" of *Bacillus* that were not previously known to harbor the Nhe enterotoxin operon. Given

that a debate is currently underway about the very identity of *B. cereus* and other strains, it is improper for food safety experts to screen food products only for *B. cereus*. Phenotypic-based classification techniques have failed to accurately differentiate *Bacillus* species. Additionally, no molecular-based approach can accurately differentiate *Bacillus*. The bottom line is the determination of species within *Bacillus* does not even matter when concerned with food safety. Molecular techniques should instead screen for virulence determinants in microbes instead of identifying said microbes. Since endospore formation enables *Bacillus spp.* to be ubiquitous in the environment and on food, all foods should be examined in this way. This is the only true way to determine whether food products are safe for human consumption.

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