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Title: Bacterial Pathogens and Associated Toxins Involved in Erythrophore Cell Aggregation

Abstract approved:

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Janine E. Trempy

Current detection methods for bacterial contamination rely on structure based detection of proteins and nucleic acids. While these methods are easy to use and reliable, they cannot evaluate the toxicity of a sample and the potential to cause disease. Previously, erythrophore cells derived from *Betta splendens* had been suggested as a method to detect toxic agents such as chemicals and toxins.

The work described here, investigated the potential of erythrophore cells as a detection method for the model food-associated pathogenic bacteria *Salmonella enteritidis*, *Clostridium perfringens*, *Clostridium botulinum* and *Bacillus cereus*. Erythrophore cell response to each bacterial pathogen was unique and could be distinguished from the bacterial growth medium. These results demonstrate that erythrophore cell response can be used to detect a set of pathogenic bacteria.

A second focus of this research effort was to test the hypothesis that erythrophore cells respond to pathogenic bacteria differently then nonpathogenic bacteria. To test this hypothesis, erythrophore cells were exposed to pathogenic and nonpathogenic *Bacillus* strains. Erythrophore cells failed to respond to *Bacillus subtilis* and a nonpathogenic *Bacillus anthracis* vaccine strain. Erythrophore cells responded to members of the *B. cereus* group including: *B. cereus* ATCC 10987, *B. cereus* UW85, *B. cereus* ATCC 14579, emetic *B. cereus* NCTC 11143, and *Bacillus thuringiensis subsp. kurstaki* BGSC 4D1.

A third focus of this research effort was to identify the bacterial virulence factor(s) responsible for inducing erythrophore cell response. Random chemical mutagenesis of *B. cereus* ATCC 49064 resulted in a single amino acid conversion of alanine to threonine at residue 289 of L_2 toxin component of HBL. This mutation resulted in a delayed erythrophore cell response which was complemented with the wildtype *hbl* gene implementing the HBL toxin complex as a factor capable of inducing erythrophore cell response.

In conclusion, this study demonstrates that erythrophore cells can be optically monitored to detect several different bacterial pathogens. From this work, the utility of erythrophore cell response can be expanded from function based detection to include studying toxin activity as well as how pathogenic bacteria interact with eukaryotic cells.

Bacterial Pathogens and Associated Toxins Involved in Erythrophore Cell Aggregation

by
Janine R. Hutchison

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APPROVED:

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Chair of the Department of Microbiology

Dean of the Graduate School

I understand that my dissertation will become part of the permanent collection of Oregon State University libraries. My signature below authorizes release of my dissertation to any reader upon request:

Janine R. Hutchison, Author

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Chapter 1

Introduction

Cell-based biosensors utilize eukaryotic cells to detect a chemical or biological toxicant. Great interest has been expressed to investigate the capability of cell-based biosensors to monitor and detect food and water contaminates. These detection methods show great promise because of their portability and ease of use. Chromatophore cells represent a novel cell-based biosensor dependent on function-based detection that can be easily optically monitored.

Chromatophore cells are pigment cells found in fish, amphibians, and cephalopods (48). Chromatophore cells are grouped based on their color. Erythrophores contain red pigment, xanthophores contain yellow pigment, melanophores contain black or brown pigment, and cyanophores contain blue pigment (54). Chromatophore cells change their appearance in response to various physiological stimulants. This response is observed when pigment organelles within the cell move towards the outer edges of the cell (dispersion) or towards the center of the cell (aggregation). In nature chromatophores function as a mechanism for camouflage but can also reflect the environmental conditions and physiological stress of an animal (48). This ability of chromatophore cells to physically change their appearance in response to environmental conditions makes them an excellent model to study organelle transport, and most recently the response as been exploited to assess toxic conditions (31, 33, 39, 72, 98, 103).

Erythrophore cells from *Betta splendens* have been used to evaluate the presence of toxic agents such as pesticides, chemicals, and purified bacterial toxins (33, 103). The application of erythrophore cells as a method to detect bacterial pathogens has not been well described.

In this study evidence is presented in support of the hypothesis that erythrophore cells response can be used to evaluate the presence of the pathogenic bacteria, *Salmonella enteritidis*, *Clostridium perfringens*, *Clostridium botulinum,* and *Bacillus cereus*. The primary focus of this work is to understand what it is about *B. cereus* that causes erythrophore cells to respond by rapidly aggregating their pigment organelles. These data provide evidence that erythrophore cell response has the potential to be used to evaluate the presence of pathogenic bacteria and their associated toxins. In addition, I propose that this response is a novel model that can be used to study toxin activity and host-bacteria interactions.

Chapter 2

General Literature Review

This chapter will introduce foodborne illness in the United States, detection methods for bacterial pathogens associated with food, and the current limitations of these methods. Detection of toxic agents utilizing *Betta splendens* erythrophore cells will be covered as well. *Salmonella enteritidis, Clostridium perfringens, Clostridium botulinum* and *Bacillus cereus* will be highlighted as four food-associated bacterial pathogens of interest. Lastly, this chapter will focus on *B. cereus* foodborne illness, taxonomy, and production of virulence factors.

Foodborne Illness

It is estimated that there approximately 76 million food- and water-borne illnesses per year, costing the U.S. economy over $20 billion annually (24, 100). While the majority of foodborne illnesses are mild and symptoms typically last two to three days, the Centers for Disease Control and Prevention (CDC) estimates that there are 325,000 hospitalizations and 5,000 deaths (100). The most severe cases tend to occur in the very old, the very young, immune compromised individuals, and in healthy people exposed to a high dose of an organism. Of additional concern is the potential for several bacterial pathogens including *Bacillus anthracis*, *C. botulinum*, and *Yersinia* spp, to be used as bioweapons (13).

The Foodborne Disease Active Surveillance Network (FoodNet) was established to improve accuracy of estimating the incidence of foodborne illness. This network is focused on nine bacterial agents associated with severe foodborne disease:

Campylobacter spp., Escherichia coli O157:H7*, Listeria monocytogenes, Salmonella spp., Shigella spp., Vibrio spp., Yersinia spp., Cryptosporidium,* and *Cyclospora*. Other common agents associated with food- and water-borne illness which are not monitored by FoodNet include *Clostridium spp., Bacillus cereus,* norovirus, and *Giardia lamblia*. FoodNet is part of a collaborative network which includes the CDC, Emerging Infections Program (EIP), United State Department of Agriculture (USDA), Food Safety and Inspection Services (FSIS) and the Food and Drug Administration (FDA) (70). As of 2007, 44.5 million people (15% of the US population) were involved with these epidemiological surveys and studies (70).

Food- and water-borne illness will continue to be a problem for the world because of importing and exporting food products, poor sanitation, improper food preparation/storage, emergence of new pathogens, the potential for intentional tampering, and contamination of food stocks. Great efforts are required to prevent and minimize the potential of food-and water-borne illness.

Detection Methods

A variety of methods are employed to identify the source as well as the causative agent of foodborne illness. Rapid and accurate detection and monitoring allows government officials to prioritize hazards and interventions. Traditional detection of bacterial pathogens relies on growth of the bacterium in liquid growth medium, plating of culture-enriched sample onto selective/differential medium, and confirmation of pure culture isolate using a series of morphological, biochemical, serological, and other tests (46). Problems that complicate detection of bacterial

contamination of foods using traditional techniques include: (1) methods are time and labor intensive; (2) non-uniform distribution of pathogens in food (challenges in collecting global sample, uniform sample, homogenous representative sample, etc.); (3) low level of target pathogen compared to indigenous microbiota (which can be found at levels as high as 10^8 CFU/g in raw products); (4) heterogeneity of food matrices and food components interfering with growth or detection of an organism; and (5) inability to recover injured target organisms using selective enrichment media (46). To circumvent and overcome these obstacles, the development of rapid and accurate detection methods has become essential for monitoring food and water safety. Immunological assays, molecular techniques, and toxicity assays have become the gold standard for risk assessment and detection of bacterial pathogens (46).

Enzyme linked-immunosorbent assay (ELISA)

The ELISA was developed in 1971 and is the most common immunologic-based detection method. This assay relies on the specific binding of a bacterial antigen to either a monoclonal or polyclonal antibody. Sandwich ELISA involves immobilization of the antibody onto a solid phase, typically a microtiter plate, binding of the antigen (target microbial cell or toxin), rinsing, followed by the addition of a second antibody conjugated with an enzyme. The secondary antibody allows detection and enumeration based on enzyme-mediated colorimetric reactions (46). Many ELISA kits are commercially available for the detection of bacteria or their associated toxins. Limitations of immunoassays include a need for sample enrichment, cross-reactivity,

and the inability to assess functional toxicity or the presence of novel bacteria and their associated antigens/toxins (46).

Polymerase chain reaction (PCR)

The development of nucleic acid-based detection has revolutionalized molecular biology research and has many applications including diagnosis of microbial infection and genetic disease, as well as detection of pathogens in food, fecal, and environmental samples (46). Detection is dependent on the presence of a conserved nucleic acid sequence and thus is very sensitive.

The gold standard of nucleic acid detection is PCR, which is a very rapid and accurate method for detection of nucleic acids from known sources, and is more sensitive and accurate than immunoassays. PCR is an *in vitro* method that utilizes a DNA polymerase enzyme and oligonucleotide primers to amplify a specific region of DNA. The DNA region that is amplified determines the specificity of the reaction. Common targets include the genes encoding ribosomal RNA, enzymes, outer membrane proteins, heat shock proteins, toxins, and other virulence factors. Many PCR-based kits for detecting specific foodborne pathogens are commercially available. Problems associated with PCR detection include: (1) inhibition of the enzymatic reaction by food matrix components; (2) the need to separate and concentrate bacteria; (3) purification of DNA prior to detection; (4) DNA contamination; and (5) high percentage of false positives. (46). Real-time PCR and real-time reverse transcriptase PCR (RT-PCR) have been used to overcome some of these problems.

Real-time PCR combines the steps of target amplification and detection by quantifying a fluorescent signal associated with the production of the PCR product. These fluorescent signals can be from a thermostable intercalating dye such as SYBR green, or a TaqMan assay® (46). TaqMan® detection relies on the hybridization of probes to target PCR products. An oligonucleotide probe is labeled with a reporter and a quencher dye. When the probe binds to the internal region of the PCR product it is cleaved by the polymerase exonuclease activity separating the reporter from the quencher dye; thereby producing fluorescence. The fluorescence increases in proportion to the quantity of PCR products generated (46).

Real-time reverse transcriptase PCR allows detection of a target RNA molecule (mRNA or total RNA) using the enzyme reverse transcriptase to reverse transcribe RNA into cDNA. Fluorescent dyes or probes are incorporated into the product as the cDNA is copied. RT-PCR is more sensitive than conventional PCR, and has a distinct advantage of reporting cell viability via detection of specific mRNAs since these molecules have a short half life and are only found in viable cells (46).

Molecular detection methods have broad applications for the food and medical industries. These methods have increased our ability to ascertain the genes, proteins, networks, and cellular mechanisms that allow for the persistence of bacteria in food and other environments (46). While molecular and immunological (DNA and antibody) detection technologies are very reliable and have high sensitivity pathogenic bacteria still escape detection. Detection methods based on protein or DNA structure specific to an organism often cannot distinguish closely related isolates or the potential

of these organisms to cause disease (99, 108, 118). Methods that assess toxicity can circumvent the limitations of structure-based detection methods.

Toxicity Assays

Traditional detection, molecular detection, and biochemical assays do not indicate if a sample is toxic or has the potential to cause disease. Toxicity assays are a means to assess biological activity. Traditionally, toxicity assays rely on animal models to evaluate toxic effects of a substrate. These assays include skin injections to evaluate altered vascular permeability, ileal loop injections to assess fluid accumulation, and feedings to evaluate lethality (131) An increased interest in avoiding the use of animal models has resulted in several assays utilizing biosensors to assess toxicity (11, 14).

Biosensors

Biosensors utilize biological components for the detection of biologically relevant samples. The biological component varies greatly between systems but usually exploits an enzyme, antibody, cell receptor, peptide, oligosaccharide, nucleic acid or even living cells (98). Detection using cell-based biosensors is typically fast and/or real-time; they are portable, and have the potential to detect multiple targets (50).

Eukaryotic-cell-based biosensors have been successfully utilized in pharmaceutical screening, environmental monitoring, clinical diagnosis, and homeland security (11). This class of biosensor allows for functional assessment of

pathogenicity, or toxicity of a sample. These assays can infer physiological interactions and provide clues to the action (or mode of action) of the toxicant (chemical/pathogenic bacterium/toxin) (11). Eukaryotic cell-based biosensors can be used to make direct measurement of physiologic function (and changes induced by toxins), providing detection of previously unknown agents, they can distinguish between viable and non-viable bacterium as well as toxic and non-toxic conditions (11, 46, 50, 125). Neurons, stem cells, immune cells including T and B cells as well as epithelial cells have been used to detect an array of toxic conditions ranging from toxic chemicals, bacterial toxins and biologically active pathogenic bacteria (11). The use of eukaryotic-cell-based biosensors is very appealing for detection of bacterial pathogens because of the potential for function-based assessment of biological activity rather then only structure-based detection (113).

Biosensors composed of primary or secondary cell types are being used to evaluate food-associated pathogenic bacteria, their associated toxins, and study host-bacteria interactions. Primary cells (collected from tissue) have been shown to be more sensitive and can mimic physiological responses to a greater degree than secondary cells (11). It has been hypothesized that the increased sensitivity is attributed to the presence of original cellular characteristics including expression of various surface molecules (11, 46). Isolation of primary cells can be labor intensive and cells can have a short life *in vitro* (46). In contrast, secondary cell lines or immortalized cells, can survive indefinitely as long as they are maintained in the proper environment. While they may be more cost-effective, many cell lines have genetic aberrations resulting in

loss of original traits such as tissue receptors that may be required to study bacterial adhesion, invasion, or toxin action (11, 125).

One means of classifying cell-based biosensors is by the type of secondary transduction used to monitor cellular response (50). This discussion will focus on optical biosensors which have the advantage of rapid sensing, where the speed of detection is most often limited by the physiological interactions (recognition event), rather than by the speed of optical response (46). Optical biosensors can be monitored in a variety of ways including changes in fluorescence, chemiluminescence, and optical density (11, 46, 50, 125).

Fluorescence-based biosensors

An advantage of fluorescence-based biosensors is the ability to monitor intracellular activity, but this requires the use of invasive dyes (50). Immune cells have been a popular cell type for biosensors because of the availability of several cell lines and well characterized methods for measuring cell response such as cytokine release. Recently, a rat basophilic leukemia (RBL) mast cell-based biosensor was described with applications for detecting bacterial pathogens, specifically *Escherichia coli* and *Listeria monocytogenes* (30). This detection method utilizes fluorescent dyes as indicators of antigen recognition by mast cells. While this assay was very sensitive, mast cells were only responsive to bacterial antigens after they were sensitized using chimeric proteins and thus would be unable to detect novel antigens (30).

Chemiluminescent-based biosensors

Chemiluminescent cell-based biosensors are genetically engineered to luminesce in the presence of a toxic compound. Rider *et al*. reported the production of a pathogen-specific B lymphocyte line called CANARY (cellular analysis and notification of antigen risks and yields) (114). CANARY cells express cytosolic aequorin, a calcium sensitive bioluminescent protein from the *Aequoria victoria* jelly fish (114). CANARY was found to be capable of detecting 50 colony forming units per gram (CFU/g) of *Yersinia pestis* in less then 3 minutes and 500 CFU/g *E. coli* O157:H7 within 5 minutes (114). CANARY is rapid, sensitive and very specific for the listed bacteria but the ability to detect toxins or other bacteria has not been determined.

Optical density-based biosensors

Chromatophore cells are specialized pigmented nerve cells that can change appearance when physiologically stimulated (31, 39, 72). Historically, chromatophore cells from fish and amphibians have served as a model for the study of organelle transport, but they are now being employed as optical cell-based biosensors for detecting toxic chemicals.

Poikilothermal vertebrates including fish, chameleons, cephalopods and other lower vertebrates have chromatophore cells. Within a single organism, different types of chromatophore cells can be present allowing environmental adaptation by varying chromatophore size, shape, and color (49). The ability to change color or pattern serves several biological functions including the ability to camouflage and

communicate among members of the same species during courtship and mating (12, 48, 110).

There are 6 types of chromatophores which are differentiated by color of the pigment organelles within the chromatophore: melanophores (brown or black), erythrophores (red), xanthophores (yellow), cyanophores (blue), leucophores (white to opaque), and iridophores (metallic or iridescent) (48, 49). Chromatophore cells exhibit various morphologies ranging from round or oval, dendritic shape resembling neurons or sheet-like cells resembling squamous epithelial cells (48). An important feature of cell-based biosensors populated with chromatophore cells is the ability to confer rapid changes in cell appearance through the movement of pigment organelles within the chromatophore cell (49). Chromatophore cell responses can be categorized using the speed of response, whether the response involves the cells becoming darker or lighter in color, and the degree of change in optical density, specifically the change in pigment area occupied within the cell (103).

Pigment organelle movement can be dispersive (pigment organelles move towards the outer edges of the cell) or aggregative (movement towards the center of the cell) (126) (Figure 1). Pigment organelles move along the microtubule network. This movement is controlled through signal transduction pathways and other regulatory mechanisms in response to triggering events at various cell-surface receptor sites, including G-protein coupled receptor-binding events (12, 31). *In vivo*, rapid pigment organelle movement has been associated with signals from the peripheral nervous system, whereas slower translocation of pigment is controlled by the endocrine system (126).

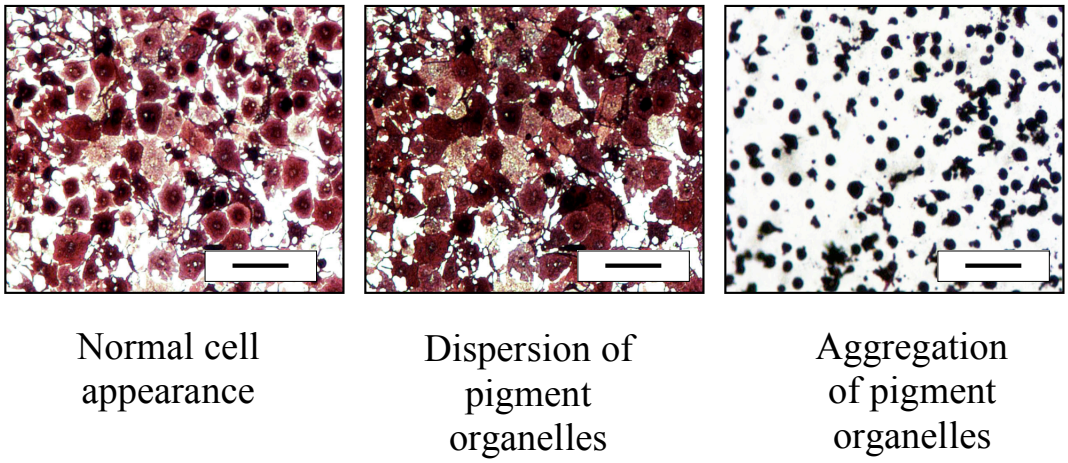


Figure 1. Erythrophore cells from *Betta splendens*. Left: pigment is evenly distributed through normal or untreated erythrophores. Center: dispersion of pigment organelles (movement towards the outer edges) causes erythrophores to appear larger. Right: aggregation of pigment organelles (movement towards the center of the cell) making the cell appear smaller. The size bar represents 100 μm.

Xenopus laevis (frog) melanophore cells have been described as cell-based biosensors of toxic chemicals. A *Xenopus* melanophore cell line has been developed, and currently this is the only example of a chromatophore cell-based biosensor that is populated by immortalized cells (95). Karlsson *et al*. transfected *Xenopus* melanophores with the human opiod receptor 3 making this cell line specific for detecting opiates (71). The authors suggest that a melanophore biosensor has the potential to be used in the analysis of body fluids such as blood plasma, urine, and saliva. Recently the *Xenopus* cell line was evaluated as a potential sensor for detecting toxins in water (68). Melanophore cells responded rapidly by dispersing pigment organelles following exposure to ammonia, arsenic, copper, mercury, pentachlorophenol and phenol. These chemicals were detected at military exposure guidelines (MEG) and human lethal concentrations (LEC). These results demonstrate

the potential for these cells to be used for the rapid assessment of chemical toxicity in drinking water (68).

Betta splendens, also known as Siamese fighting fish, are well known for their bright colors and the ability to respond to rival males by changing coloration and pigment intensity (Figure 2) (134). Mojovic *et al.* and Dierksen *et al.* reported that erythrophore cells respond by moving pigment organelles in a dispersive or aggregative direction in the presence of a variety of toxic samples including herbicides, fungicides, cell receptor agonists, and purified bacterial toxins (33, 103).

Figure 2. A red, male, *Betta splendens* also known as a Siamese fighting fish.

Henry Wallbrunn recognized that *B. splendens* pigment cells could be found with a varying cell morphology ranging from a dispersed pigmentation to clumping pigmentation resembling a small knot in the center of the chromatophore. These changes allow a rage of colors between fish as well as within a single fish (134). Frightened or sick fish can be pale in color as a result of pigment being aggregated

towards the center of the chromatophore cell, whereas fighting or courting fish are brilliantly colored with pigment dispersed throughout the chromatophore cell (54, 134).

Erythrophore cell response is easily detected by monitoring changes in optical density due to movement of pigment organelles within the erythrophore cell. The movement of the pigment organelles can be measured by recording the change in pigment area occupied by the erythrophore, allowing real-time monitoring (33, 103). Erythrophore cells are capable of distinguishing toxic samples from non-toxic samples without prior 'knowledge' of the agent, and categories of responses of the cells can be used to distinguish one toxic agent from another (103).

The potential of erythrophore cells as a cell-based detection method of bacterial pathogens has not been clearly defined. This work supports the hypothesis that erythrophore cell response, specifically the aggregative or dispersive movement of pigment organelles can be used to monitor for the presence of bacterial pathogens and their associated toxins. Additionally, this study contributes to a better understanding of bacterial interaction with erythrophore cells, as well as the potential for this response to be used to understand bacterial toxicity and bacterial physiology.

To test this hypothesis, *S. enteritidis, C. perfringens*, *C. botulinum,* and *B. cereus*, were chosen as model food-associated bacterial pathogens for this study. *Bacillus cereus* was selected as the model pathogen to identify bacterial factor(s) involved in eliciting an erythrophore cell response. Identifiying the factors involved will help to elucidate the mechanism behind erythrophore cell pigment movement in response to a toxic condition.

Salmonella enterica

The global incidence of foodborne outbreaks caused by *Salmonella* serovars has been on the rise around the world. It is believed that this increase is not associated with an improved surveillance program and medical response, but rather continuing pandemics (104). Recent massive recalls of produce and peanut butter contaminated products within the United States has increased public awareness and brought to light the need for accurate detection methods for this organism (1, 94, 104).

What makes *Salmonella* species such a growing concern? *Salmonella* species are facultative anaerobic gram-negative rod-shaped bacterium. They can grow at temperatures ranging from 2°C to 54°C; this ability to grow at lower temperatures increases the growth and survival of bacteria in refrigerated foods (104). Furthermore, *Salmonella* species have been observed to be incredibly resilient to pH changes, storage in high salt and anaerobic conditions (94). A major contribution of *Salmonella* persistence in the environment is though maintained reservoirs. These reservoirs include poultry, contaminated animal feed, and introduction of bacteria during harvest of fresh and dried produce (104). Of further concern is the marked increase of *Salmonella* isolates with multiple antibiotic resistance markers. This increase has been attributed to the use of antibiotics in agriculture (46).

Salmonella enterica subsp *enterica* includes 1,454 serotypes, and accounts for ~99% of the human cases of salmonellosis and can be associated with three distinct clinical syndromes: typhoid fever, bacteremia and enterocolitis (46, 81, 104). Typhoid fever is a systemic infection. Bacteremia is a systemic infection associated with highly invasive serotypes causing fever and septicemia (46). Enterocolitis is a localized

infection characterized by nausea, vomiting and diarrhea within 12 to 72 hours after infection (46). Treatment for *Salmonella* infections includes supportive therapy and in some cases treatment with antibiotics (104).

Virulence determinants of *Salmonella* are broadly defined by the scientific community (46, 104). Any factor that enables the bacterium to enter a host, avoid the host defenses, cause disease and be transmitted to the next host are considered virulence determinants. Genetic elements harboring *Salmonella*-specific virulence determinants include plasmids, bacteriophages, and pathogenicity islands (73).

Clostridium perfringens

Clostridium perfringens type A is the third most common cause of foodborne disease in the United States (104). Outbreaks are usually large and typically occur in settings where food is prepared in advance and held before consumption (104). The CDC estimates that 100% of recent outbreaks can be attributed to improper cooking, cooling, or holding of food items.

This bacterium is a gram-positive, non-motile, anaerobic, rod-shaped bacterium that can form heat tolerant endospores. Factors that contribute to *C. perfringens* virulence are rapid doubling time which allows for growth in food, the ability to form spores and the ability to produce enterotoxins. *Clostridium perfringens* can be isolated from soil, foods, dust, and the intestinal tract (46, 104). Virulence factors of *C. perfringens* include heat resistance and the ability to produce enterotoxins. The major toxins, alpha, beta, epsilon and iota toxins are used to classify *C. perfringens* isolates as types A through E. Additionally *C. perfringens* isolates

produce at least 15 different toxins (104). One of the most important of these toxins, and a major virulence factor, is the *C. perfringens* enterotoxin (CPE) (116). The CPE toxin is a 35 kDa protein that acts by altering membrane permeability within the intestine. CPE is produced during sporulation of the vegetative cell in the intestine and is released when the mother cell lyses following completion of sporulation (104).

Clostridium perfringens has been associated with human and animal disease ranging from cellulitis, septicemia, necrotic enteritis, and food poisoning (106). Food poisoning results from ingestion of contaminated meat products, typically poultry and beef. Symptoms include abdominal cramps and watery diarrhea, they occur 8 to 16 hours after ingestion of contaminated food. Symptoms typically resolve within 24 to 48 hours and supportive therapy can be helpful. The infectious dose for foodborne illness is $>10^6$ to 10^7 vegetative cells/gram of food (46).

Clostridium botulinum

Clostridium botulinum produces one of the most potent neurotoxins resulting in botulism, a severe neuroparalytic disease. Foodborne botulism is rare but results from intoxication of the pre-formed botulinum neurotoxin. Full recovery may take several weeks to several months; the fatality rate associated with foodborne botulism is ~10%. While the incidence of food botulism is relatively low compared to many other bacterial pathogens, illness can progress from mild to death within days (46, 104).

Clostridium botulinum is a gram-positive, anaerobic, rod-shaped bacterium capable of forming endospores. Factors that promote *C. botulinum* contamination of

food products include heat and pH resistance, and the ability to form radiation resistant spores (104). Foods associated with foodborne botulism include fish, meats, honey, and canned fruits and vegetables.

The ability to form spores and production of botulinum toxin are the main virulence factors for this bacterium. Botulinum toxin blocks the release of acetylcholine from synaptic vesicles at nerve terminals resulting in flaccid paralysis. The infectious dose is as little as 30 ng of toxin (104). Seven types of *C. botulinum* (A, B, C, D, E, F, G) based on the serological specificity of the neurotoxin produced have been identified. Human botulism is associated with types A, B, E, and occasionally type F (46, 104). Treatment involves removing or inactivating the botulinum toxin with antiserum in conjunction with supportive care.

Bacillus cereus group

Any gram-positive, spore-forming, aerobic, rod-shaped bacterium is classified as a *Bacillus* species (104). *Bacillus* species are ubiquitous and can be easily isolated from soil and plants. Analysis of 16S ribosomal RNA (rRNA) has produced evidence of ~16 different genera, suggesting that the *Bacillus* genus is far too diverse for a single genus (23, 63). The *B. cereus* group includes the closely related members *B. anthracis*, *B. cereus (sensus stricto), B. thuringiensis*, *B. mycoides, B. pseudomycoides*, and *B. weihenstephanensis*. All species within the *B. cereus* group have the potential to cause food poisoning, and these isolates cannot be distinguished using traditional detection and identification methods (46, 82). Vegetative cells are 0.5 by 1.2 μm to 2.5 by 10 μm and occur singly or in chains. They form endospores that

are heat- and dehydration-resistant, and resist other physical stresses. *Bacillus cereus* endospores are more hydrophobic than spores formed by other *Bacillus* spp. This hydrophobic property allows *B. cereus* spores to persist in the food production environment, and because of their ability to adhere to several types of surfaces, endospores are difficult to remove during equipment cleaning (104). *B. cereus* spores may also adhere to surfaces via a pili (104). Cells are motile via peritrichous flagella and produce catalase (117). Optimal growth temperature ranges from 25 to 37°C; psychotolerant strains can grow from 4 to 6°C (82).

Phylogenetic studies suggest *B. anthracis, B. cereus*, *B. mycoides*, *B. pseudomycoides*, *B. thuringiensis* and *B. weihenstephanensis* are closely related and should be placed within one species because of traditional classification they remain classified as different species (25, 26, 57, 63, 65, 66). While *B. cereus* group members are very similar at the genomic level, there is a high degree of diversity concerning virulence factors, pathogenesis, and the type of environmental niche each member prefers.

The phenotypic characteristics used to distinguish *B. cereus* group members include rhizoid growth on blood agar (*B. mycoides* and *B. pseudomycoides*) (44, 107). The most recent addition to the *B. cereus* group is the psychrotolerant species, *B. weihenstephanensis* which grows at low temperatures (82). *Bacillus anthracis* is the causative agent of anthrax in humans and animals, and has the potential to be used as bioweapon (62). *Bacillus anthracis* has the two large plasmids unique to this bacterium, pXO1 encoding the anthrax toxin complex, and pXO2 encoding the capsule (76). *Bacillus thuringiensis* harbors a plasmid that encodes the insecticidal

δ-endotoxins which are known to be toxic to insects (40). Any member of the *B. cereus* group that cannot be distinguished with the above characteristics is classified as *B. cereus sensu stricto* (*B. cereus*). The type strain *B. cereus* ATCC 14579 is the laboratory reference strain for this species and was isolated in 1887 by Frankland and Frankland from air in a cow shed (45). *Bacillus cereus* is recognized as a food poisoning organism and the virulence factors responsible for the diarrheal illness are generally chromosomally encoded. These genes are present in all species of the *B. cereus* group, thus it is not surprising that *B. thuringiensis* isolates have been implicated in cases of foodborne illness (104). *Bacillus cereus* group taxonomy will continue to be of interest to scientists and public health officials particularly in the case of severe anthrax-like illness caused by *B. cereus* isolates (66).

Bacillus cereus

Bacillus cereus is an opportunistic pathogen and has been linked to foodborne illness as well as numerous nongastrointestinal infections including, osteomyelitis, pulmonary and wound infections, bacteremia, and septicemia (76, 131). *Bacillus cereus* is the etiological agent of two forms of foodborne illness, the emetic and diarrheal form (104, 131-133). The two types of foodborne illness are characterized in Table 1.0.

Table 1.0. Characteristics of the diarrheal and emetic forms of *B. cereus* foodborne illness[a].

Characteristic	Diarrheal Form	Emetic Form
Dose causing illness	$10^5 – 10^7$ (total cells)	$10^5 – 10^8$ (cells/g)
Toxin production	In small intestine of host	Preformed in foods
Type of toxin	Protein: enterotoxin(s)	Cyclic peptide
Known toxins associated with illness	HBL, Nhe, CytK	Cereulide
Incubation period	8 to 16 hours	0.5 to 5 hours
Duration of illness	12 to 24 hours, occasionally days	6 to 24 hours
Symptoms	Abdominal pain, watery diarrhea, nausea	Nausea, vomiting, malaise
Foods most frequently implicated	Meat products, soups, vegetables, puddings, sauces, milk, milk products	Fried and cooked rice, pasta, pastries, noodles

[a]Modified from Montville, T. J., and K. R. Matthews, 2005. Food Microbiology an Introduction. ASM Press, Washington, D.C. p.216.

In general, *B. cereus* foodborne illness is mild, but severe cases are reported occasionally (36, 87). Treatment includes supportive therapies to relieve symptoms which usually have a short duration and resolve within ~ 24 hours. *Bacillus cereus* foodborne illness elicits symptoms similar to other foodborne pathogens. The presence of *B. cereus* in a food product (particularly milk) is not always a concern because the potential of an outbreak is directly related to a high number of bacterial cells. These three factors contribute to the low reported incidence of *B. cereus* foodborne illness (104). It is estimated that there are ~20,000 cases of illness per year associated with *B. cereus* food contamination and is therefore considered a minor foodborne pathogen in

the United States. However, *B. cereus* is considered an important foodborne pathogen in parts of Europe due to a higher incidence of *B. cereus* related illness (104). Due to different methods of surveillance of foodborne illness, it is not possible to compare the incidences of outbreaks in different countries.

Bacillus cereus toxins

Cereulide

Production of diarrheal enterotoxins is broadly distributed through members of the *B. cereus* group. Emetic toxin production is restricted to a specific group now known to harbor a large plasmid (61, 67). The emetic form of *B. cereus* foodborne illness was first identified in the early 1970's in the United Kingdom. The emetic toxin, cereulide, causes emesis (vomiting) one to five hours after ingestion and is associated with rice, milk and vegetables. Cereulide is a small, cyclic, heat and acid stable dodecadepsipeptide, has a molecular weight of 1.2 kDa and the structure: $(_{D}$-*O*-Leu-$_{D}$-Ala-$_{L}$-*O*-Val-$_{D}$-Val$)_3$ (4, 38, 128). Due to this protein being heat, and acid stable, it is often not destroyed during pasteurization or by gastric acid in the stomach (59). Cereulide is produced by a nonribosomal peptide synthetase (NRPS), encoded by the 24-kb cereulide synthetase (*ces*) gene cluster located on the large plasmid pCER270 (36, 38, 67).

Diarrheal Enterotoxins

Bacillus cereus enterotoxins are hypothesized to cause diarrheal symptoms by disrupting the integrity of the plasma membrane of intestinal epithelial cells.

Symptoms of the diarrheal foodborne disease are attributed to three enterotoxins: hemolysin BL (HBL), nonhemolytic enterotoxin (Nhe) and cytotoxin K (CytK) (15, 87, 88). Attributing disease symptoms to only these three toxins is controversial because *B. cereus* produces several secreted cytotoxins, hemolysin and degradative enzymes that could possibly contribute to diarrheal disease (3, 4, 7, 9, 10, 43, 55, 77, 78, 86, 88, 105, 117).

Hemolysin BL (HBL)

The tripartite toxin, HBL, was isolated by Beecher and Macmillian from *B. cereus* in 1990. The genes *hblA*, *hblD*, and *hblC* encode the B, L_1 and L_2 toxin components, respectively. These genes are arranged in an operon with the promoter upstream of *hblC* (18). All three toxin components are necessary for biological activity. Laboratory studies have concluded that HBL causes increased vascular permeability in rabbit skin, fluid accumulation in the rabbit ileal loop assay, necrosis of villi, submucosal edema, interstitial lymphocytic infiltration. HBL has also been shown to be toxic to a number of cell lines (55).

Hemolytic activity of HBL against sheep and calf erythrocytes is a feature of this toxin. HBL causes discontinuous hemolysis (ring shaped clearance) on blood agar where lysis begins away from the colony followed by slow lysis closer to the colony (55, 88). While the mechanism of toxicity is not known it is hypothesized that each component of HBL binds to the eukaryotic cell independently via an unknown receptor. Cell lysis is caused by the formation of a membrane attack complex resulting forming transmembrane pores (7). The stoichiomentry of the three components of the

HBL complex has not been determined but excess of any one component results in inhibition of cell lysis (7, 88).

Nonhemolytic enterotoxin (Nhe)

Nhe toxin is composed of the three proteins: NheA, NheB and NheC, encoded by the *nheABC* operon (87). Nhe was first characterized in 1995 from a large food poisoning outbreak in Norway caused by a *hbl*-negative *B. cereus* strain (87). Cytotoxic activity of Nhe towards epithelial cells occurs rapidly and it appears that there is disruption of the plasma membrane, followed by the formation of pores in lipid bilayers (7). Initial characterization of Nhe concluded that this toxin was non-hemolytic on bovine blood agar plates. Recently it was demonstrated that Nhe is cytotoxic to epithelial cells and has hemolytic activity towards erythrocytes from several mammalian species in suspension assays (41). The initial results and resulting nonhemolytic nomenclature of this toxin is likely due to the lower hemolytic activity of Nhe compared with that of HBL (58).

Attempts to delete the *nhe* operon in *B. cereus* and *B. thuringiensis* have been unsuccessful (42). *Bacillus cereus* mutant *nheBC* was created, and a strain with a frame shift mutation in the 5'-end of *nheC* has been identified (41). It has been observed that *B. anthracis* produces a low level of NheA, and the *nhe* operon has been found in every *B. cereus* group strain examined to date. This may suggest that *nheA* could be an essential gene in *B. cereus,* and that it may have a duel role with an additional function essential for cell viability in addition to its role as a secreted toxin component.

Cytotoxin K (CytK)

CytK, a 34kDa protein with dermonecrotic, cytotoxic and hemolytic activities, was characterized by Lund *et al.* in 2000 (77). CytK is a single component toxin, distinguishing it from HBL and Nhe. CytK is a member of the β-barrel pore forming toxin family which includes β-toxin from *C. perfringens*, and α-hemolysin of *Staphylococcus aureus* (9). β-barrel toxins are secreted as water-soluble monomers that associate into oligomeric 'prepores' at the target cell surface; a transmembrane pore is then formed at the cell membrane (5).

Two variants of CytK were identified in 1998; *cytK-1* and *cytK-2*. Isolates carrying *cytK-1* seems to be less prevalent then *cytK-2* (10). Outbreak associate with a *cytK-1* isolate tend to result in a more sever form of gastrointestinal disease compared to *cytK-2* isolates. The *cytK-1* variant had a five fold greater cytotoxic activity then a *cytK-2* variant against Caco-2 and Vero cells. (43). It is hypothesized that the difference in cytotoxicity between the two CytK variants could be the size of pores formed in an eukaryotic membrane as well as expression level of the toxin gene (78).

Regulation of virulence gene expression

Regulation of virulence factors in gram-positive bacteria is controlled by various types of regulatory processes including: (1) two-component systems; (2) alternative sigma factors; or (3) stand alone transcription regulators**.** These three processes can work independently or together to control the expression of virulence genes. The transcriptional regulator PlcR (Phospholipase C Regulator) is a 34kDa protein that controls the expression of 45 genes; many of which are known virulence

factors and include enterotoxins such as HBL, Nhe, CytK, hemolysins, Phospholipase C, and other proteases in members of the *B. cereus* group (52, 83, 84). PlcR was first characterized in *B. thuringiensis* and was found to control the expression of a phosphatidylinositol-specific Phospholipase C (*plcA*) gene (121). Many PlcR-regulated genes encode a secretion (*sec*) sequence, indicating that they can be secreted from the cell, and indeed it has been found that the majority of these proteins are extracellular, with a small subset bound to the cell wall (121).

plcR has been identified in all members of the *B. cereus* group, but the activity of this gene can vary between strains. The *plcR* gene of *B. anthracis* is nonfunctional due to a nonsense mutation that is conserved among all known *B. anthracis* isolates (32). PlcR is maximally expressed at the onset of stationary phase and plateaus two hours later (t_2). PlcR binds to a nucleotide sequence called the PlcR box, which is located upstream of the regulated genes and upstream of the -35 box of the sigma A promoter. The distance between the PlcR box and the -35 box varies between genes as well as strains (32). The PlcR box is a conserved palindromic sequence, wTATGnAwwwwTnCATAw (where w = a or t and n = any nucleotide) (32).

PlcR activation is dependent on PapR (peptide activating PlcR), which is encoded downstream of *plcR*. PapR is a short (44 amino acid) regulatory peptide that is translocated across the bacterial cell membrane and is then re-imported into the cytoplasm by ATP dependent Opp (oligo peptide permease, encoded by *OppA, B, C, D, E, F*) (52, 53). It is unclear if PapR is processed in the extracellular space or if it is cleaved by OPP, but the C-terminal pentapeptide LPFEF (PapR5) is the active intracellular form of PapR (7, 133). Once the concentration of PapR5 reaches the

appropriate level it binds to PlcR forming the active PlcR:PapR5 complex. It has been suggested that the active form of PlcR:PapR5 includes a PlcR dimer, but this form has never been successfully isolated (76, 133). Activated PlcR-PapR5 binds to PlcR Box recognition sequences located throughout the chromosome (76, 133). Declerck *et al.* proposed that initiation of PlcR-regulated transcription starts with circular polymerization of around DNA at the PlcR box. This binding may influence DNA topology, or displace other DNA-binding proteins to initiate gene expression (7).

Transcription of *plcR* and PlcR-regulated genes is activated at the end of exponential phase for cells grown in rich medium (examples: Luria Broth or Brain Heart Infusion). Transcription of *plcR* and PlcR-regulated genes is not activated in cells cultured in sporulation specific medium (84). This medium-dependent expression of *plcR* is controlled by the sporulation regulator Spo0A~P (84, 121). Spo0A~P is the master regulator of sporulation in *Bacillus* species and is activated through a phosphorelay pathway. This protein binds to two Spo0A boxes flanking the PlcR box upstream of *plcR* and represses *plcR* expression (84, 121).

What is the function of PlcR-regulated proteins and why are they expressed at the end of exponential phase? Degradative enzymes and proteases may be a bacterial mechanism to acquire additional nutrients from the environment. Enterotoxins and bacteriocins may provide cell protection, and two component sensors and chemotaxis proteins provide additional means for environmental sensing (104). In many cases pathogenic bacteria respond to their environment by producing toxins. The ability to produce toxins that result in human disease has become a very important issue regarding human health and the quality of life (104).

The ability of erythrophore cells to respond to functionally active bacterial pathogens and their associated toxins has not been well described. This work describes the response of erythrophore cells to the food-associated pathogenic bacteria *S. enteritidis*, *C. perfringens*, *C. botulinum* and *B. cereus*. An important question surrounding this research is: what is it about food-associated pathogenic bacteria that cause erythrophore cells to aggregate or disperse their pigment organelles? *Bacillus cereus* was selected as the initial model to address this question. Specifically, I investigated the hypothesis that a toxin or other virulence factor is responsible for the aggregative response induced by *B. cereus*.

Research Objectives

The research objectives of this study are three-fold: (1) evaluate erythrophore cell response to bacterial pathogens (*S. enteritidis, C. perfringens*, *C. botulinum* and *B. cereus*); (2) investigate erythrophore cell response to various members of the *B. cereus* group including *B. anthracis* and *B. thuringiensis*; and (3) identify the biological factor(s) of *B. cereus* that elicit the characteristic aggregative response from erythrophore cells.

Chapter 3

Materials and Methods

Erythrophore cell preparation

Erythrophore cells were isolated from the anal, caudal, and dorsal fins of male red *B. splendens* fish as described previously (103). Red *B. splendens* were purchased from local pet stores and maintained for at least one month prior to erythrophore cell harvesting to ensure that any parasitic or microbial infections cleared. Fish were maintained in individual quart aquariums at 28°C with 12 hours of light and 12 hours of dark exposure. Fish were fed every other day, water was changed weekly. Prior to erythrophore cell harvest, fish were anesthetized and killed by immersion in ice water for 20 minutes.

Fin tissue was removed from individual fish and was briefly washed in 10 ml of phosphate buffered saline [PBS, 128mM NaCl, 2.7mM KCl, 10mM Na_2HPO4, 1.46mM KH_2PO4, supplemented with 5.6mM glucose, 1:100 (w/v) antibiotic/antimycotic, pH 7.3 (Gibco #20012043)]. Fins were transferred to a 15 ml Falcon tube with 10 ml skinning solution [SK, 128mM NaCl, 2.7mM KCl, 10mM Na_2HPO4, 1.46mM KH_2PO4, 1mM EDTA, 5.6mM glucose, 1:100 (w/v) antibiotic/antimycotic, pH 7.3 (Gibco #20012043)], tissue was washed for 1 minute, and the skinning solution wash step was repeated for a total of ten washes using a rocking motion to mix the solution. Washing fin tissue in skinning solution removed scales and epithelial cells from the tissue surface.

To release erythrophore cells from the fin tissue, fins were digested with an enzyme mixture composed of 20-30 mg collagenase type 1 (Worthington #LS004196),

and 1-3 mg of hyaluronidase (Worthington #LS002594), dissolved in 7 ml of PBS [PBS, 128mM NaCl, 2.7mM KCl, 10mM Na2HPO4, 1.46mM KH2PO4, 5.6mM glucose, 1:100 (w/v) antibiotic/antimycotic, pH 7.3 (Gibco #20012043)] filter sterilized onto the fin tissue. Fins were mixed by rocking for 1 to 3 minutes, the enzyme solution was transferred to a new 15 ml Falcon tube and the erythrophore cells were pelleted in a swinging bucket centrifuge (IEC Centra CL3) at 600 rpm for 3 minutes. The enzyme mixture was transferred back to the fin tissue; typically the first pellet was discarded because it was composed of epithelial cells. The enzyme solution was mixed with the fin tissue for 5 – 10 minutes or until the tissue was completely digested. Erythrophore cells were spun out of the enzyme mixture by centrifugation as described above. In some cases it was necessary to collect several erythrophore cell pellets, these cell pellets were pooled and resuspended in 1 ml of Leibovitz L15 Medium (L15+) [L15+, Gibco #21083-027, 500 ml of dye-free L15 was supplemented with 10 ml 1M HEPES (Gibco #15630-080) buffer and 5 ml antibiotic/antimycotic, pH 7.3 (Gibco #15240-062)] to remove excess enzyme solution. Erythrophore cells were pelleted as described and then resuspended in a predetermined volume of L15+ medium to ensure the desired erythrophore cell density in the center of each well of a 24-well or 48-well microtiter plate.

Erythrophore cells were allowed to attach to the microtiter plate for 15minutes. L15 + medium was added in each well to a final volume of 1.5 ml (24-well microtiter plate) or 0.75 ml (48-well microtiter plate) and 5% fetal bovine serum (FBS, HyClone #SH30071-01) was added to each well 20 minutes after the addition of the L15+.

L15+ and FBS was changed every 7 days until cells were no longer viable and physiologically responsive.

This effort was performed under the requirements, regulations, and approved of Oregon State University's Institutional Animal Care and Use Committee, approval #2979 and #3513.

Erythrophore Cell Response Assay and Computer Analysis

Erythrophore cell response was monitored by observing and recording changes in the pigmented area of an erythrophore cell population exposed to bacterial-, chemical- and control-agents as previously reported (33, 103). Cell culture plates were mounted on the stage of an inverted microscope. A field of view containing approximately 100 erythrophore cells was monitored under a magnification of 100. This selected area was not moved throughout the course of the experiment. Prior to each experiment, physiological responsiveness was verified by treating erythrophore cells with a known dispersive agent, 400nM melanocyte stimulating hormone (MSH, Sigma M4135), and a known aggregative agent, 400nM clonidine (Sigma C7897) (33, 103). Bacterial suspension or physiological response testers (400 nM clonidine, Sigma C7897; 400 nM MSH, Sigma M4135, respectively) was added to each well to a final dilution of 1:10 in L15 medium. Digital images (JPEG format) at 300 x 300 resolution were taken using a SPOT Insight 320 color camera (Diagnostic Instruments, Inc., Sterling Heights, MI, USA) and SPOT software version 3.5.6.2 (Diagnostic Instruments, Inc), through a Leica (Leica, Inc., Wetzlar, Germany) DMIL inverted microscope (Bartels and Stout). Erythrophore response was recorded for 20 minutes;

images were captured at 0 min, 0.25 min, 0.5 min, 1 min, 2min, 4 min, 10 min, 20 min. For experiments lasting 6 hours, images were captured at the same time interval as the 20 minute experiments, then every 10 minutes up to 60 minutes. After 60 minutes, images were captured every 30 minutes until the experiment was completed. The images were opened in Image Pro Plus 4.1 (Media Cybernetics), exported and processed into Excel. The total area in pixels (digital area units) for each captured image was determined and the percentage pigmented area change (cell area change (%) = - ($A_0 - A_x$) / A_0 x 100, where A_0 is the initial area and A_x is the final area. Data was then represented graphically by plotting change in pigment area (%) over time (Figure 3). An aggregative response resulted in a negative percent area change and a dispersive response resulted in a positive percent area change. Experiments were repeated 2 or 3 times as indicated. Error bars represent the standard deviation of the mean.

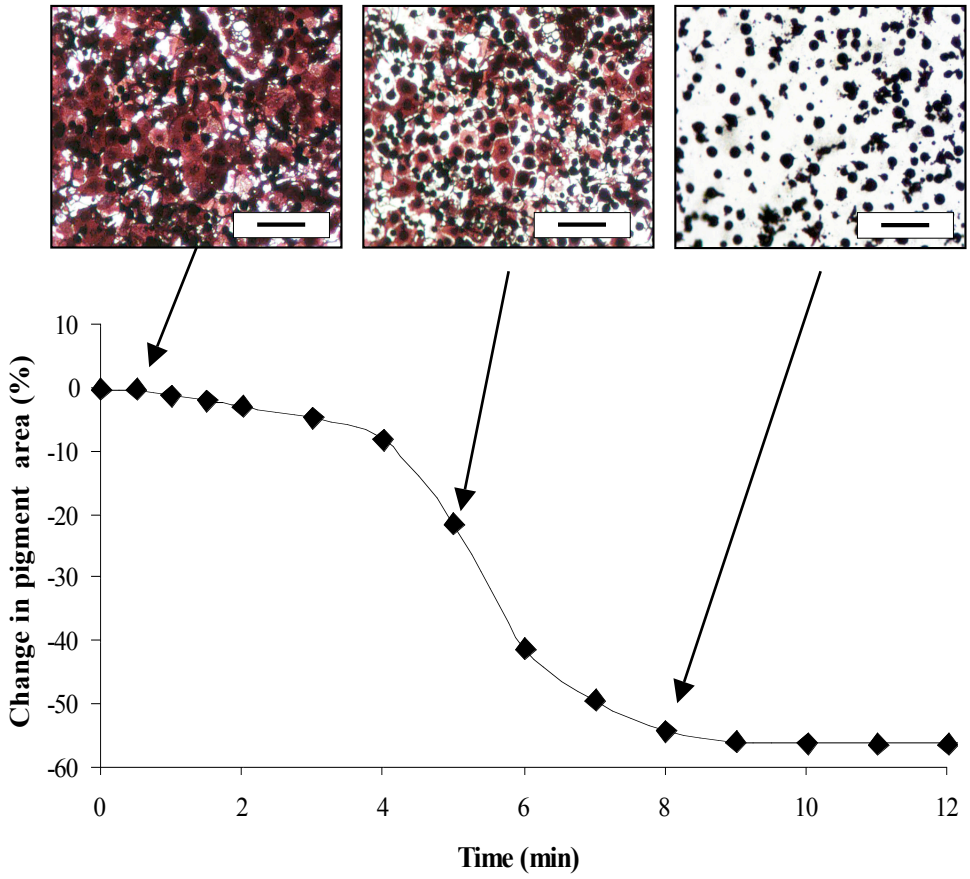


Figure 3. Pictorial and graphical display of erythrophore cell response. After the addition of a toxic compound, pigment within the erythrophore cell begins to migrate towards the center of the cell resulting in a negative change in pigment cell area. A positive change in pigment area is indicative of pigment dispersion (not shown). The size bar represents 100 μm.

Isolation and complementation of Bacillus cereus mutants

Bacillus cereus mutant clones and complemented clones were screened using erythrophore cells. The goal of the mutant screen was to isolate mutants that caused a delayed or no aggregation of pigment organelles compared to the wildtype *B. cereus* response; the complementation screen was used to determine if the wildtype phenotype could be recovered. Clones were stored at -80°C and were brought up on LB with or without antibiotic at 37°C overnight. Single colonies were used to

inoculate 5 ml of BHI with our without antibiotic depending on the origin of the clone. Cultures were incubated overnight at 37°C with aeration. Viable and responsive erythrophore cells were then exposed to 75 µl or 150 µl (volume was dependent on erythrophore cells being cultured in 48 well plates or 24 well plates). For initial mutant screening erythrophore cell response was monitored under an inverted microscope, for the final screen clones were subject to the above erythrophore cell response assay followed by computer analysis as described above.

Bacterial strains and plasmid extractions

All bacterial strains and plasmids used in this work and their sources are listed in Table 2. Plasmid extractions from *E. coli* were done using Qiagen QIAprep Spin Miniprep Kit (Cat. No. 27106). Plasmid extractions from *B. cereus* followed a modified User-developed Qiagen mini-prep protocol utilizing Qiagen QIAprep Spin Miniprep Kit (Cat. No. 27106). Bacterial cultures were grown in Brain Heart Infusion (BHI) medium overnight at 37°C with shaking at 200 rpm. Subcultures were inoculated with 1:100 dilution of overnight culture into fresh BHI and incubated 3 hours (OD_{600} 0.3 - 0.6) at 37°C with shaking at 200 rpm. Ten milliliters of culture was harvested by centrifugation at 3000 x g for 15 minutes at 4°C. The bacterial pellet was resuspended in 250 µl of Buffer P1 supplemented with 10 mg/ml lysozyme. Bacterial cells were incubated in this solution for 1 hour at 37°C. Standard Qiagen mini plasmid preparation protocol was followed after this step. Plasmids were eluted off of the Qiagen column with elution buffer (EB) and were stored at -20°C. DNA

concentrations were determined by A_{260} (NanoDrop ND-1000 UV-Vis Spectrophotometer; ThermoFisher Scientific, Wilmington, DE, USA).

Table 2. Bacterial strains and plasmids used in this study.

Strain or plasmid	Relative Characteristics	Source
E. coli JM110	F′ traD36 lacIqΔ(lacZ)M15 proA+B+/rpsL (Strr) thr leu thi lacY galK galT ara fhuA dam dcm glnV44 Δ(lac-proAB)	Dr. Martin Shuster, Oregon State University
E. coli GM2929	F– ara-14 leuB6 thi-1 fhuA3 lacY1 tsx-78 galK2 galT22 glnV44 hisG4 rpsL136 (Strr) xyl-5 mtl-1 dam13::Tn9 (Camr) dcm-6 mcrB1 hsdR2 (rK–mK+) mcrA recF143	E. coli Genetic Stock Center, Yale Univsity
B. anthracis 34F2 (Sterne)	Deficient in virulence plasmid pXO2	Dr. Bruce Geller, Oregon State University
B. cereus ATCC 10987	Dairy isolate, Xylose-positive variant	Laboratory stock
B. cereus ATCC 14579	Laboratory reference strain	Laboratory stock
B. cereus ATCC 14579 Δ*plcR*	Derivative of *B. cereus* ATCC 14579, deficient in global virulence gene regulator PlcR	Dr. Didier Lereclus, Institute of Pastuer
B. cereus ATCC 49064 [NCTC 11145 (F4433/73)	Gastroenteritis outbreak isolated, produces diarrheogenic enterotoxin	ATCC
B. cereus NCTC 11143 (F4810/72)	Emetic strain	ATCC
B. cereus UW85 (ATCC 53522)	Produces zwittermicin A, biological control of Phytophthora megasperma	Dr. Jo Handelsman, University of Wisconsin
B. subtilis 1A1	Laboratory reference strain	ATCC
B. thuringiensis subsp. kurstaki BGSC 4D1 (HD1)	Serotype 3a3b, *cry* +	ATCC
C. botulinum NCTC 7272	Produces type A toxin	PHLS Central Public Health Laboratory
C. botulinum NCTC 7273	Produces type B toxin	PHLS Central Public Health Laboratory

C. perfringens MRS101	Derivative of *C. perfringens* SM101, isogenic *cpe* knock-out	Dr. Mahfuzur R. Sarker, Oregon State University
C. perfringens SM101	Derivative of food poisoning type A isolate, NCTC8798, carries chromosomal *cpe*	Dr. Mahfuzur R. Sarker, Oregon State University
S. enteritidis ATCC 4931	Isolated from human gastroenteritis outbreak	Laboratory stock
pHT304	shuttle vector E.coli and Bacillus (low copy number (4 +-1)	Dr. Didier Lereclus, Institute of Pastuer
pHT370	shuttle vecotor E.coli and Bacillus (high copy number (70 +-20)	Dr. Didier Lereclus, Institute of Pastuer
pHT304_*hblC*	A ~ 1.5 kb PCR fragment which contains *hblC* was cloned into pHT304	This study

Culture conditions and media

Escherichia coli and *S. enteritidis* stock cultures were maintained in Luria-Bertani (LB) medium [10 g Tryptone (Difco), 5 g Sodium chloride (Difco), 5 g Yeast extract (Difco)] supplemented with 20% glycerol. *Bacillus* stock cultures were maintained in Brain Heart Infusion (BHI) medium (Difco) supplemented with 20% glycerol. Stocks were maintenance at -80°C. Frozen stocks were streaked onto LB agar and incubated at 37°C overnight and isolated colonies were used in experiments. *Escherichia coli* and *S. enteritidis* ATCC 4931 were cultured in LB medium with aeration overnight at 37ºC and 30°C respectively. All *Bacillus* species were cultured in BHI medium at 37ºC overnight with aeration. For experiments requiring heat inactivation of *Bacillus* cultures, 1 ml of overnight culture was transferred to an microcentrifuge tube and was heated for 15 minutes at 95°C, culture was allowed to equilibrate to room temperature prior to all experiments. *Bacillus* supernatants were collected from 1 ml of overnight culture. Samples were spun for 10 minutes at 13,000 x *g* in a microcentrifuge.

When required media was supplemented with the appropriate antibiotics (SIGMA) for *E. coli*: ampicillin (Amp) at 100 mg ml^{-1}, kanamcyin (Kan) 50 mg ml^{-1}, erythromycin (Erm) 125 mg ml^{-1}. For *Bacillus*: kanamcyin (Kan) 50 mg ml^{-1} and erythromycin (Erm) 5 mg ml^{-1}.

Clostridium perfringens and *C. botulinum* stock cultures were maintained at -20°C in Duncan Strong (DS) sporulation medium and cooked meat medium (Difco) respectively (97). Frozen stocks were used as starter cultures in both cases. *Clostridium perfringens* SM101 and *C. perfringens* MRS101 were generously provided by Dr. Mahfuzur R. Sarker (116, 136). Vegetative cultures were prepared by inoculated 0.1 ml aliquot of overnight culture into 10 ml fluid thioglycollate (FTG) (Difco). This culture was incubated for 14 h at 37°C Sporulating cultures of *C. perfringens* were prepared by inoculating 0.1 ml aliquot of a frozen stock into 6 ml of FTG which was then heat shocked for 20 minutes at 70°C. The heat-shocked culture was then incubated for 14 h at 37°C, and 0.4 ml of this starter culture was transferred to a second 6 ml FTG before this culture was incubated for 9 h at 37°C (75). An aliquot (0.4 ml) of this FTG culture was then added to 20 ml of (DS) sporulation medium and incubated for 8 h at 37°C (97). The presence of spores was verified using microscopy. *Clostridium botulinum* NCTC 7272 and *C. botulinum* NCTC 7273 were obtained from the National Collection of Type Cultures, PHLS Central Public Health Laboratory. A 0.1 ml aliquot of a cooked meat medium stock of *C. botulinum* NCTC 7272 and NCTC 7273 was transferred to 1.0 ml of brain heart infusion (BHI) medium. The starter culture was then incubated for 24 h at 37°C. A 0.1 ml aliquot of the starter culture was transferred to a second 1 ml BHI tube before the

culture was incubated for 24 h at 37°C. All *C. botulinum* culturing was done in an anaerobic chamber using AnaeroGen gas packs (Fisher). Bacterial filtrate was collected from 0.5 ml *C. botulinum* culture filtered through a 0.2 μM filter.

All Biosafety Level 2 (BSL2) bacterial agents were cultured in an approved BSL2 facility and performed under the requirements and regulations of and approved by Oregon State University's Institutional Biosafety Committee.

Bacterial growth curve and detection limit

Fresh *B. cereus* colonies were picked from LB agar plates and inoculated into 10 ml BHI. Cultures were incubated overnight at 37°C with 200 revolutions per minute. For bacterial growth curves, a secondary culture was started from the overnight starter culture with a 1:1000 dilution into 30 ml of BHI in a side arm flask. Cultures were grown at 37°C with shaking. At each hour optical density was read using Spectronic 20D+ spectrophotometer. A 500 μl sample was removed from the side arm flask to enumerate the bacteria and perform the erythrophore cell response assay. Bacteria were serially diluted in 0.1% peptone (Difco #0188-17-0) and enumerated on LB plates at 37°C using a modified drop plate method (3 x 20 μl) (28).

Bacillus cereus ATCC 49064 were cultured BHI medium or LB medium as indicated at 37ºC overnight with shaking at 200 rpm for detection limit experiments. Viable and responsive erythrophore cells were exposed to 75μl of overnight culture or a dilution of the overnight culture in 0.1% peptone After each experiment bacteria were enumerated as described above.

Preparation of genomic DNA

Genomic DNA was extracted from *Bacillus* cultures using Promega Wizard Genomic DNA Purification Kit (Cat #A1120) modified protocol. Briefly, cultures were grown in BHI medium overnight at 37°C with shaking at 200 rpm. Subcultures were inoculated with 1:100 dilution of overnight culture into fresh BHI and incubated 3 hours (OD_{600} 0.3 - 0.6) at 37°C with shaking at 200 rpm. Two ml of culture was spun down for 5 minutes in a microcentrifuge tube and the supernatant was discarded. Cells were resuspended in 480 µl of 50 mM EDTA, and 50 µl of 100mg/ml lysozyme (in 25mM Tris pH 8) was added. Cells were incubated for 1 hour at 37°C. Cells were then spun down for 2 minutes and the supernatant was discarded. Six-hundred µl of nuclei lysis solution was added and the cells were resuspended and incubated for 5 minutes at 80°C followed by cooling for 5-10 minutes to room temperature. To the cell lystates, 3 µl RNAse (10 mg/ml) (Fermentas) was added and lysates were incubated for 30 minutes at 37°C. After RNAse treatment, 200µl protein precipitation solution was added and cell lystates were vortexed for 15 seconds followed by incubation on ice for 5 minutes. Cell debris was removed by centrifugation for 3 minutes. To the supernatant, 600µl of cold 95% ethanol was added and incubated on ice for 30 to 60 minutes. Genomic DNA was pelleted by centrifugation for 4 minutes and ethanol was removed. DNA pellet was washed with 1 ml cold 70% ethanol and then spun for 2 minutes. Ethanol was decanted, and DNA pellet was air dried for 10 minutes at 37°C until all traces of ethanol had evaporated. DNA was resuspended in 100µl TE (10mM Tris, pH 7.5, 1mM EDTA). To get DNA into solution samples were heated at 65°C for 1hr. DNA was stored at 4°C.

Polymerase chain reaction

PCR utilized ~ 100 ng of template DNA, 0.8 μM of each primer (Table 3) and Platinum® PCR SuperMix (Invitrogen # 11306016) to a total volume of 50 μl. For cloning and sequencing Platinum® PCR SuperMix High Fidelity (Invitrogen #12532016) was used. The reaction was placed in a Perkin Elmer Gene Amp PCR system (Model 2400) for an initial period of 5 minutes at 95°C, then 30 cycles, each consisting of 30 seconds at 95°C, 30 seconds at the primer annealing temperature, 1 - 4 minute at 72°C, followed by a final extension period for 7 minutes at 72°C. Extension time was dependent on product size; in general 1 minute was used for every 1 kilobase of product. PCR products were stored at 4°. To verify the presence of the amplified product an aliquot was analyzed by agarose (1 – 2%) gel electrophoresis in TAE buffer. DNA was visualized by staining with ethidium bromide. PCR products were purified with QIAquick PCR Purification Kit (Qiagen #28104), or were excised from an agarose gel and purified using Qiagen Gel Extraction Kit (Qiagen #28704).

Table 3. Primers used in this study. All primers were purchased through IDT.

Primer Name	Primer Sequence (5' to 3')	Common Name	Annealing Temp (°C)	Fragment size (bp)	Reference
Bacillus 16sF 8-26	AGAGTTTGATCCTGGCTCA	*Bacillus* specific 16s	50	1500	(37)
Bacillus 16sR 1511–1493	CGGCTACCTTGTTACGAC	*Bacillus* specific 16s	50	1500	(37)
ba1F	TGCAACTGTATTAGCACAAGCT	*groEL*	55	533	(27)
ba1R	TACCACGAAGTTTGTTCACTACT	*groEL*	55	533	(27)
Adapt1	P-GATCGGAAGAGCTCGTATGCC GTCTTCTGCTTG	Varies: Illumina reaction	25	Varies	(29)
Adapt2	ACACTCTTTCCCTACACGACGCTC TTCCGATCT	Varies: Illumina reaction	25	Varies	(29)
PCR primer 1.1	AATGATACGGCGACCACCGACACT CTTTCCCTACAGGACGCTCTTCCGATCT	Varies: Illumina reaction	65	Varies	(29)
PCR primer 2.1	CAAGCAGAAGACGGCATACGAGCT CTTCCGATCT	Varies: Illumina reaction	65	Varies	(29)
M13F	GTAAAACGACGGCCAG	Varies: insert	55	Varies	Invitrogen
M13R	CAGGAAACAGCTATGAC	Varies: insert	55	Varies	Invitrogen
CesF1	GGTGACACATTATCATATAAGGTG	Cereulide	50	2200	(38)
CesR1	GTTTTCTGGTAACAGCGTTTCTAC	Cereulide	50	2200	(38)
CesR2	GTAAGCGAACCTGTCTGTAACAACA	Cereulide	50	1400	(38)

capBCA17	GAAATAGTTATTGCGATTGG	Capsule	55	873	(111)
capBCA20	GGTGCTACTGCTTCTGTACG	Capsule	55	873	(111)
cya65	CAGCATGCGTTTTCTTTAGC	Edema factor	55	929	(111)
cya65	CCCTTAGTTGAATCCGGTTT	Edema factor	55	929	(111)
lef59	GGATATGAACCCGTACTTGTAA	Lethal factor	55	993	(111)
lef60	TAAATCCGCACCTAGGGTTGC	Lethal factor	55	993	(111)
pag67	CAGAATCAAGTTCCCAGGGG	Protective antigen	55	747	(111)
pag68	TCGGATAAGCTGCCACAAGG	Protective antigen	55	747	(111)
BCE_5183 F1	ATGCGGCAGAAGCGATAGAA	Bacillolysin	40	2008	This study
BCE_5183 F2	GAGTATTGGGCGGTAAACAA	Bacillolysin	-	Sequencing	This study
BCE_5183 R1	AATGTCGTATGGAATCCGCCCT	Bacillolysin	40	2008	This study
BCE_5183 R2	ATGTATGGATACCTTGTCCTCG	Bacillolysin	-	Sequencing	This study
BCE_3869Fwd	CGTTGAGGAAAAATTAGCAACG	CodY	50	1000	This study
BCE_3869Rev	GAAATGCAATTCATGGCTTTTC	CodY	50	1000	This study
BCE_1209Fwd	AATTATGCATAACTATCCCTTCCG	CytotoxinK	40	1290	This study
BCE_1290Rev	ACAAAG AACAGGGAATTAGGCAC	CytotoxinK	40	1290	This study
BC0813 F1	GAAGCGCTATCAGGATATTTATGG	Enterotoxin	45	1500	This study
BC0813 F2	GCTAAAGCAGTGGAAGCAAC	Enterotoxin	-		This study
BC0813 R1	GTGGCTTCGAGACAATATTGC	Enterotoxin	45	1500	This study
BC0813 R2	CTTCAGCATCTGCCTGAC	Enterotoxin	-		This study

BC1953 F1	GGAACTGGATGAATATTTTACTTT	Enterotoxin	40	1544	This study
BC1953 F2	CACTGGTGCTTTAAAAGTACG	Enterotoxin	-	Sequencing	This study
BC1953 R1	CGTCCTAACTATTTCCATGAC	Enterotoxin	40	1544	This study
BC1953 R2	TAACCTGTGCGGCCGTTAT	Enterotoxin	-	Sequencing	This study
BC5239 F1	GTGTGTTTTCCCCAAATCCG	Enterotoxin	50	1000	This study
BC5239 R1	AGCCAGCTCTCAATAAAGAGA	Enterotoxin	50	1000	This study
BCE_5353F1	CAGATTCCCGCATAAGTCTTGAC	Enterotoxin	48	2000	This study
BCE_5353F2	GGTTGGGTGAAAATCAACC	Enterotoxin	-	Sequencing	This study
BCE_5353R1	GTGTATGAAATAGGGAAATAGATAC	Enterotoxin	48	2000	This study
BCE_5353R2	CCAGCTACCTTCAGAGCCAA	Enterotoxin	-	Sequencing	This study
BC_3102Fwd	CAACTGCTAATGTAGTTTCACCAG	HBL-B	45	700	This study
BC_3102Rev	CTCCCACTATGATACCGCC	HBL-B	45	700	This study
BC_3103F1	GATACAACTAACGAGCAGTCTC	HBL-L1	50	1400	This study
BC_3103F2	GCTACAAGACTTCAAAGGG	HBL-L1	-	Sequencing	This study
BC_3103R1	ACAGCGAGTAATTTGTAAGGG	HBL-L1	50	1400	This study
BC_3103R2	CCTTTCCCATCTGGACCA	HBL-L1	-	Sequencing	This study
BC_3104F1	CCAGTTTACAAAGAGAGCGGTCGT	HBL-L2	50	1400	This study
BC_3104F2	GCTCAAGCAGGGGTGGCAGCGT	HBL-L2	-	Sequencing	This study
BC_3104R1	GCTAAAGTTAGTACTTTGAATGG	HBL-L2	50	1400	This study
BC_3104R2	GTTCTGTCGCTTCTTGCGCCGC	HBL-L2	-	Sequencing	This study

hblC_HindIII	CCCAAGCTTGGGTAAGTAAAATCCTTACATTC	*hblC* insert	55	1508	This study
hblC_BamHI	CGCGGATCCGCGCCCGAAAAACGGAATATTA	*hblC* insert	55	1508	This study
BCE_4248Fwd	GAAATCGGTTTAACGACAGATGC	hemolysin A	50	1050	This study
BCE_4248Rev	GCACCTCACACTATCTCTTACC	hemolysin A	50	1050	This study
BCE_3795Fwd	GTGCTAATATACTTACATTGA	Hydrolase	50	984	This study
BCE_3795Rev	GTTTTTAAAGACCACTATATCACATG	Hydrolase	50	984	This study
BCE_0616 F1	GGATTCGGATTACTTTCGGGAG	Microbial Collagenase	40	1566/3151	This study
BCE_0616 F2	TAAATGAAAGAAGCTTCCAGGAC	Microbial Collagenase		Sequencing	This study
BCE_0616 F3	GAGACTGTACTGGGCTGCAAA	Microbial Collagenase	40	1758	This study
BCE_0616 F4	CTGAAACGTTGCCGATGAAAG	Microbial Collagenase		Sequencing	This study
BCE_0616 R1	TATTGTGACAGGCTCCTCC	Microbial Collagenase	40	1758/3151	This study
BCE_0616 R2	GTAACAGTTTTGTAGCCACTC C	Microbial Collagenase		Sequencing	This study
BCE_0616 R3	CCACCGTTGTTCGTTTCATATCC	Microbial Collagenase	40	1566	This study
BCE_0616 R4	CTGTTTCAACATCGCTTGACGC	Microbial Collagenase		Sequencing	This study
BCE_3539 F1	GCATGGTATGGATACATGTGTG	Microbial Collagenase	40	1586	This study
BCE_3539 F2	CCCGAATTTCAAACTCGGTACG	Microbial Collagenase	-	Sequencing	This study
BCE_3539 F3	CCGTCAATTATACGGATATGAAACG	Microbial Collagenase	40	1619	This study
BCE_3539 F4	GTGCGAAAGAATGGACAGGC	Microbial Collagenase	-	Sequencing	This study
BCE_3539 R1	CTTTCCCTTGGTAGTACCTATC	Microbial Collagenase	40	1619	This study
BCE_3539 R2	GTCCTGCTGCATTCACACGATAG	Microbial Collagenase	-	Sequencing	This study

BCE_3539 R3	CTGGCGTACGTTCATAAGTA AG	Microbial Collagenase	40	1586	This study
BCE_3539 R4	CGTTTCCACATCACTCGAAGC	Microbial Collagenase	-	Sequencing	This study
nheafmlu	CGACGCGTATAGTTGCTAATACATAGGA	*NheA*	49	1200	(85)
nhearmlu	TCACGCGTCTTCTCTCCCTATATCGCTA	*NheA*	49	1200	(85)
nheBF	AAGGAGGAAGCAGGTATGACAAAAAAAC	*NheB*	49	1200	(85)
nheBR	GACACGCACGAGGTTTATGCTTTTTTCG	*NheB*	49	1200	(85)
nhecf	ATGCAGAAACGATTTTATAAA	*NheC*	50	1000	(85)
nhecr	TTACTTCGCCACACCTTCAT	*NheC*	50	1000	(85)
BCE_5480Fwd	GAGGTGAATGCCTAGGGAAG	papR	40	500	This study
BCE_5480Rev	GTAGAAACGGAAGAAGAATGGG	papR	40	500	This study
BCE_0744Fwd	CGTGAAAGGTGGGATATTCTAG	Phospholipase C	48	1000	This study
BCE_0744Rev	ACTCCTAAACCAATCCCAAAGC	Phospholipase C	48	1000	This study
BCE_3793F1	AACGAAAGGAACTTCAGGTGAAG	1-phosphatidylinositol phosphodiesterase	40	1343	This study
BCE_3793F2	GAGTATGAGGATATGAAAGGAGCA	1-phosphatidylinositol phosphodiesterase	-	Sequencing	This study
BCE_3793R1	GCTTTTTCCCCAGGATTATTTATGCC	1-phosphatidylinositol phosphodiesterase	40	1343	This study
BCE_3793R2	CCCACGAGCATCTCCCAATTTT	1-phosphatidylinositol phosphodiesterase	-	Sequencing	This study

BCE_5481Fwd	GTTGAGCCTTAGCCCCAGTTACC	PlcR	40	1640	This study
BCE_5481Rev	CGCAGAAGGTGTAGAAACCGAAG	PlcR	40	1640	This study
BCE_0745F1	GCGAAACAAGATTACTCTGG	Sphingomyelinase	40	1381	This study
BCE_0745F2	GCAGGCTGAAGATAGTATGTG	Sphingomyelinase		Sequencing	This study
BCE_0745R1	CCACACTTTTTCTCTCCTCTTC	Sphingomyelinase	40	1381	This study
BCE_0745R2	GAACGTACTCGTCATTTGG	Sphingomyelinase		Sequencing	This study
BCE_3314F1	AGGTGGAGTAATACCTTTAGTCC	Thiol cytolysin	40	1700	This study
BCE_3314F2	GTGATGGTGGCGGCATATAAG	Thiol cytolysin	40	1700	This study
BCE_3314R1	CAGCTCTATAAGGCTATGTTGG	Thiol cytolysin	40	1700	This study
BCE_3314R2	CTCCTTTACGAGTTAGCTCATC	Thiol cytolysin	40	1700	This study

Capillary sequencing and sequence analysis

All sequencing was performed using an ABI 3730 capillary sequence machine by the Center for Genome Research and Biocomputing (CGRB) Core Laboratories at Oregon State University. Primer and template concentrations were optimized to CGRB specification. DNA sequences were aligned and analyzed using BioEdit sequence alignment editor version 7.0.9.

Multiple sequence alignments were performed using the ClustalW application from CGRB in-house-bioinformatics, Oregon State University, (http://bioinfo.cgrb.oregonstate.edu/ClustalW_bas.html) (127). Additional protein and nucleic acid sequences were retrieved from NCBI data base as indicated (http://www.ncbi.nlm.nih.gov/).

Bacterial Transformation

Transformation of *E. coli* cells was accomplished using a chemical competency protocol. Overnight *E. coli* cultures were diluted 1:100 in LB medium, incubated at 37°C with aeration until cultures reached mid-log phase. For the transformation, 2 ml of culture was spun down in a microcentrifuge tube in a microcentrifuge at maximum speed for 30 seconds. The supernatant was discarded, and the bacterial cells were resuspended with the residual culture medium. To this pellet, 200 µl of TSS (10g PEG 3350 or 8000, 0.5g MgSO4 7H2O, 88ml LB, 5ml DMSO, autoclaved and stored at 4°C) was added. One-hundred µl of this solution was used for each transformation. Cells were transformed with 1-5 µl of plasmid DNA or ligation mixture; cells were mixed by gentle flicking and incubated on ice for 20

minutes. The cells were heat shocked at 42°C for 1.5 minutes and allowed to recover on ice for 3 minutes. After recovery 900 µl of fresh LB was added to the transformation mixture. Cells were incubated for 1 hour at 37°C with gentle shaking for phenotypic expression prior to plating on selective media.

Bacillus cereus electroporation was attempted following several published protocols including Turgeon et al., Belliveau et al., Silo-Suh et al., and Dunn et al., (20, 35, 120, 130). Successful electroporation was accomplished by following a protocol described by Jaques Mahillon and Didier Lereclus (93). Overnight *B. cereus* was cultured in BHI broth at 37°C on a rotary shaker at 200 rpm for aeration. The next day 400 ml of fresh LB broth in a 2,000 ml flask was inoculated with 1 ml of overnight culture. Bacteria were incubated at 37°C on a rotary shaker at 200 rpm for 3 hours (or when culture was in approximately mid-exponential phase, OD_{600} 0.5 to 0.8). Cells were pelleted using a Beckman J2-21 centrifuge at 5,000 x g at room temperature; the pellet was resuspended in 400 ml of sterile demineralized water. Cells were pelleted three more times and resuspended in 400 ml and 200 ml of sterile demineralized water. The last pellet was resuspended in 10 ml of fresh 40 % (w/v) PEG_{6000} (polyethylene glycol molecular weight 6,000) prior to the final centrifugation step. Supernatant was removed and the pellet was weighed. The final pellet was resuspended in V ml of 40 % PEG_{6000} (where V = 1.5 x pellet wet weight in grams). Samples were aliquoted in 300 µl aliquots. These aliquots were used immediately or frozen in an ethanol-ice bath and stored at -80°C up to 3 months. Electrocompetent cells were mixed with 2 to 10 µl of non-methylated DNA (up to 10 µg), transferred to a room temperature 4 mm cuvette and a single electric pulse was delivered by a Bio-

Rad Gene pulser electroporation unit with the following settings: capacitance: 25 μF, resistance: 1000 Ω and voltage: 2.5 kV. The time constant was recorded and ranged from 7.0 to 9.5 ms. Immediately after electroporation 2.9 ml fresh LB culture medium was added to cells. Transformed bacteria were incubated for 90 minutes at 30°C or 37°C with gentle shaking. The entire pool of transformants were selected on LB plates as 100, 200 or 300 μl aliquots with the appropriate antibiotics at 30°C or 37°C. Bacterial clones were transferred to fresh antibiotic plates to avoid background as soon as they appeared (particularly with kanamycin). For each transformation a negative control of cells with no plasmid was conducted in parallel. These cells were plated on selective media to determine the frequency of spontaneous mutations.

Presence of plasmids in *B. cereus* was confirmed by (1) mini-plasmid preps followed by restriction digests; or (2) mini-plasmid preps followed by PCR analysis and sequencing. Electroporation efficiency of *B. cereus* ranged from $10^1 – 10^3$ CFU/ μg for *B. cereus* using the method reported by Mahillon (93). It should be noted that plasmids in addition to transposable elements were very unstable in *B. cereus* and it was pertinent to work from fresh glycerol stocks and verify the presence of plasmid or transposon prior to all experiments.

Plasmid Constructs

Ligation Reactions

Restriction enzymes, T4 DNA ligase and CIP were purchased from New England Biolabs (Beverly, MA) unless otherwise noted. Purified plasmids and inserts were digested with the appropriate restriction enzyme as recommended by the

manufacturer. Cut vector was then treated with alkaline phosphatase (CIP) to remove the 5' phosphate group form DNA as recommended by the manufacturer. Cut vector and inserts were cleaned up using QIAquick PCR Purification Kit (Qiagen #28104) as needed. Ligations were done using T4 DNA (NEB) as recommended by the manufacturer; ligations were allowed to proceed overnight at 16°C.

Construction of genomic library

A genomic library was constructed following the protocol reported by Silhavey *et al.* (119). Total genomic DNA was isolated from *B. cereus* ATCC 49064 as described above. Ten-micrograms of genomic DNA was partially digested with *Sau3A*1 (USB, Cleveland, Ohio) for 20 minutes at 37°C. DNA fragments ranging from 1- to 6-kb were purified from a 1% agarose gel after electrophoresis for 90 minutes at 100V using a QIAGEN Gel Extraction Kit (Qiagen #28704). The 1- to 6-kb fragments were ligated ino the shuttle vector pHT304 (6) digested with *Bam*HI as described above. Ligation mixture was then transformed into *E. coli* JM110 using the protocol described above. *E. coli* cells were plated on LB agar medium containing ampicillin (100 mg/ml), isopropyl-b-D-thiogalactopyranoside (IPTG) (10 μg/ml), and the chromogenic substrate 5-bromo-4-chloro-3-indolyl-b-D-galactopyranoside (X-Gal) (80 μg/ml). The plates were incubated overnight at 30°C. White and light blue Amp resistant colonies were gridded onto LB ampicillin agar plates and were incubated overnight at 30°C. Colonies were then pooled in LB liquid medium. Plasmid DNA was extracted from the pooled *E. coli* JM110 cells using Qiagen QIAprep Spin Miniprep Kit (Cat. No. 27106). Plasmid DNA was then electroporated

into *B. cereus* mutants, or ligation reactions were transformed directly into *B. cereus* mutants.

Construction of *hblC* complementation vector

PCR was used to amplify the *hblC* gene from *B. cereus* ATCC 49064 using primers described in Table 3. Restriction enzymes were designed as part of the primers to allow directional cloning into the shuttle vector pHT304. pHT304 and the PCR product were cut with HindIII and BamHI, and ligated together as described above. The ligation mixture was then transformed into *E. coli* JM110 using the described protocol, the cells were plated on LB agar medium containing erythromycin, IPTG, and X-Gal. The plates were incubated overnight at 30°C. White colonies were then selected to check for plasmid and insert. Clones were inoculated in 5 ml LB erythromycin medium and incubated overnight at 37°C. Plasmid DNA was extracted from *E. coli* cells as described, and insert orientation was verified by using sequencing primers M13F and M13R (Table 3).

N-methyl-N'-nitro-N-nitrosoguanidine (NTG) Mutagenesis

The carcinogen NTG is known to exert its mutagenic and lethal effects by methylating DNA**.** NTG was used to mutagenize *B. cereus* ATCC 49064, *B. cereus* ATCC 14579 and *B. cereus* UW85 following the protocol reported by Silhavey *et al*. (119). This approach was taken due to the difficulties of transforming *B. cereus*. Efforts were taken to investigate minute changes in gene expression rather then the gross changes typically observed with transposon mutagenesis. Fresh cultures were

started in 5 ml of LB medium and were incubated at 37°C overnight. The day of mutagenesis, 0.1 ml of overnight culture was inoculated in 5 ml of LB medium and incubated at 37°C until the cells reached late-log phase (OD_{600} ~1). To a centrifuge tube, 50 µl of fresh NTG (2.5 mg ml^{-1}, 5 mg ml^{-1}, 8 mg ml^{-1}, 10 mg ml^{-1}, 15 mg ml^{-1} or 30 mg ml^{-1} in 95% ethanol) and 2 ml of *B. cereus* cells were added and mixed well. Cells were incubated for 10 minutes at 37°C and then pelleted in a Beckman J2-21 centrifuge at 1500 x g for 10 minutes, supernatant was discarded and cells were resuspended in 2 ml of LB medium. Cells were pelleted by centrifugation an additional time and resuspended to a final volume of 5 ml of LB medium and were grown overnight at 37°C to allow mutant phenotype to be expressed. Glycerol stocks were made of each mutagenized strain and stored as a glycerol stock at -80°C. Aliquots were removed from glycerol stocks as needed and were grown on LB medium at 37°C until colonies appeared (overnight) Silhavy. Single colonies were picked and inoculated into 5 ml of BHI medium and grown overnight at 37°C, 75 µl of each sample was used in erythrophore cell response experiments. NTG concentration was varied for several pools to optimize mutagenesis to 1 mutation/1 bacteria.

Typing B. cereus ATCC 49064

In order to type *B. cereus* ATCC 49064 to better understand the genotype; two typing methods were employed: (1) 16s sequencing; and (2) PCR/ PCR-restriction fragment length polymorphism (RFLP). *Bacillus* specific primers were used to PCR amplify and sequence a fragment of the 16s gene (Table 3).

Chang et al. reported a method to differentiate *B. cereus* group members using PCR and PCR- restriction fragment length polymorphism (RFLP). *groEL* was amplified using PCR from *B. cereus* ATCC 49064, *B. cereus* ATCC 14579, *B. cereus* ATCC 10987, and *B. anthracis* 34F2 using primers ba1F and ba1R under reported conditions (27). PCR products were either (i) sequenced at CGRB facilities using PCR primers as sequencing primers, or (ii) RFLP analysis was conducted. For RFLP analysis 10 µl of PCR product was digested with the restriction enzyme, *pst*1 for 2 hours at 37°C. Samples were heat inactivated at 80°C for 10 minutes, and 10µl of each sample along with 5µl of NEB quick load 100bp DNA ladder was loaded on a 2% Low melt agarose gel (USB) in 1X TAE. For each RFLP analysis, a control sample without enzyme treatment was included.

Whole genome DNA sequencing using Illumina 1G sequencing technology

Genomic DNA was isolated from *B. cereus* ATCC 49064 WT and *B. cereus* ATCC 49064 JH1 as described. Samples were prepared for Sequencing following Illumina's guidelines using a kit described by Cronn & Liston Labs (29).

Fragmentation of Genomic DNA

Five µg of genomic DNA was sheared into fragments less than 800 bp using a nebulizer. The nebulizer (Invitrogen # K7025-05) was assembled according to manufacture instructions. Genomic DNA was added to the nebulizer in a total volume of 50 µl of TE buffer (USB #75893). To this, 700 µl of nebulization buffer (TE (pH 7.6) containing 25 % glycerol (80 µl 1M Tris pH 8.0, 16 µl 0.5mM EDTA, 2 ml 100%

glycerol, 5904 µl double distilled water). The nebulizer and DNA stored on ice and was connected to a compressed air source via PVC tubing (1/4 inch ID, 3/8 inch OD). Compressed air was delivered to the nebulizer at 32-35 psi for 6 minutes. The nebulizer was then placed in a centrifuge and spun at 450 x g for 2 minutes to collect the droplets, and 400 – 600 µl of solution was recovered. The sample was purified using a QIAquick PCR Purification Kit (Qiagen #28104) and DNA was eluted in 30 µl of EB.

End Repair

Overhangs as a result of fragmentation are converted to blunt ends. The following reaction mixture was set up: DNA sample (30 µl) , nuclease free water (45 µl) (USB), T4 DNA ligase buffer with 10mM ATP (10 µl) (NEB #B0202S), dNTP mix (4 µl) (Promega #U1240, 100mM of four dNTP's, 40 µmol each), T4 DNA polymerase (5 µl) (NEB #M0203S, 3 U/µl), Klenow DNA polymerase (1 µl) (NEB #M0210S, 5 U/µl), T4 PNK (5 µl) (NEB #M0201S, 500 units). This mixture was incubated in a thermal cycler for 30 minutes at 20°C. The solution was purified with QIAquick PCR Purification Kit (Qiagen #28104) and DNA was eluted in 32 µl of EB.

Addition of 'A' to the 3' End of the DNA Fragments

The following reaction mixture was set up to add an 'A' nucleotide to the blunt 3' end of the DNA fragments: DNA sample (32 µl), Klenow buffer (5 µl) (NEB #M0212S), dATP (10 µl) (Promega #U1240, 1mM), Klenow exo (3' to 5' exo minus) (3 µl) (NEB #M0212S, 5U/µl). This mixture was incubated for 30 minutes at 37°C.

The sample was purified and concentrated using QIAquick MinElute PCR Purification Kit (Qiagen #28004) and DNA was eluted in 10 µl of EB.

Ligation of Adapters to DNA Fragments

DNA adapters were ligated to the ends of DNA fragments to prepare them for hybridization to a flow cell. The reaction mixture was set up as follows: DNA sample (10 µl), DNA ligase buffer (25 µl) (NEB #M2200S), DNA ligase (5 µl) (NEB #M2200S, 1U/µl), 15 mM adapter oligo mix (10 µl) (Primers Adapt1 and Adapt2 Table 3). The DNA mixture was cleaned up and concentrated with QIAquick PCR Purification Kit (Qiagen #28104) and eluted with 30 µl of EB.

Purification of Ligation Products

Ligation products were purified to eliminate unligated adapters. The DNA mixture was loaded onto a 2% agarose gel (BIO-RAD, part # 161-3106). The gel was run at 80 V for 90 minutes and then stained with ethidium bromide. From the gel, the 150-200 bp range product was excised and purified using QIAGEN Gel Extraction Kit (Qiagen #28704). The DNA was eluted off of the column with 30 µl EB and concentrated using QIAquick MinElute PCR Purification Kit (Qiagen #28004) DNA with a final EB elution volume of 15 µl.

Enrich the Adapter-Modified DNA Fragments by PCR

PCR was used to enrich those DNA fragments which had the adapter molecules on both ends and amplified the amount of DNA in the library. The

following PCR reaction was set up: DNA (1 µl), Phusion DNA polymerase (Finnzymes Oy) (25 µl) (NEB #F-548S), PCR primer 1.1 (1 µl) (PCR primer 2.1 (1 µl) (34 µM), nuclease free water (22 µl) (USB). PCR reaction was used with the following PCR protocol: 1) 30 seconds at 98°C; 2) 18 cycles of: 10 seconds at 98°C, 30 seconds at 65°C, 30 seconds at 72°C; 3) 5 minutes at 72°C; 4) hold at 4°C. The PCR product was purified with QIAquick PCR Purification Kit (Qiagen #28104) eluting with 50 µl of EB.

Final Product Sequencing

The final DNA product from *B. cereus* ATCC 49064 and *B. cereus* ATCC 49064 JH1 was submitted as a 30 ul sample in10 nM sample buffer (EB supplemented with 0.1% Tween-20). DNA sequencing using Illumina 1G sequencing technology was performed by the Center for Genome Research and Biocomputing (CGRB) Core Laboratories at Oregon State University.

Data analysis whole genome assembly

Initial data analysis was processed by the CGRB Biocomputing Facility using the Solexa software pipeline (Illumina) and ELAND (Efficient Large-Scale Alignment of Nucleotide Databases) http://www.gersteinlab.org/proj/chip-seq-simu/eland.html. Raw data was processed into short reads, and the initial whole genome assembly was attempted. The genome reference file for the whole genome assembly was *B. cereus* ATCC 10987 which showed the highest similarity using RFLP analysis to *B. cereus* ATCC 49064. Secondary data analysis was conducted by Larry J. Wilhelm at Oregon

State University. Data from *B. cereus* ATCC 49064 and *B. cereus* ATCC 49064 JH1 was pooled. The raw data was processed to produce a file of reads matching the quality scores. The reads were then trimmed to 32 nucleotides. To understand the global alignment of *B. cereus* ATCC 49064, the reads were then aligned to the published reference genomes: *B. cereus* ATCC 14579 (genome: NC_004722 plasmid: NC_004721, *B. cereus* ATCC 10987 (genome: NC_003909, plasmid: NC_005707), and *B. cereus* E33L (also known as Zk) (genome: NC_006274, plasmids: NC_007103, NC_007104, NC_007105, NC_007106, and NC_007107). The reference genome, *B. cereus* ATCC 10987, was prepped as to remove IUPAC symbols. The U-category reads were aligned and assembled onto the genome using Mosaik (http://bioinformatics.bc.edu/marthlab/Mosaik). Three mismatches were allowed for a 90% identity over the read length. In addition, the reads were aligned and assembled with all *B. cereus* genes using Moasaik. Accession (number), Locus, Start-stop, Gene Name, Gene description, and Length (of gene) for the reference genes was recorded in Microsoft Excel; the comparison of the Solexa runs to each reference gene was reported as: Reads, Average Coverage, Peak Coverage, Zero Coverage, Weak Reference Base, Ambiguous, and SNP's. SNP's (single-nucleotide polymorphism) were called with rules developed in the Denver Lab (Oregon State University, Corvallis, Oregon) for calling *Caenorhabditis elegans* SNP's. In addition, the ratio of Length to Zero Coverage was calculated.

Data analysis gene assembly

Using the data obtained from whole genome sequencing, genes were given a ranking based on % Degree of Similarity = ((Length-Zero coverage)/Length)*100. The % Degree of Similarity helped establish the probability of a gene being present in *B. cereus* ATCC 49064, and the degree of relatedness to the reference gene. Initially, putative virulence factors, and PlcR-regulated genes were investigated. % Degree of Similarity was compared for genes present in the reference strains (*B. cereus* ATCC 14579, *B. cereus* ATCC 10987, and *B. cereus* E33L) the higher value gene was used as the reference gene for primer design. If these values were equivalent, then the reference with the higher average coverage was used. Primers used in this study are listed in table 3. In all cases, only the references sequence from *B. cereus* ATCC 14579 and *B. cereus* ATCC 10987 were used because *B. cereus* E33L was not available for laboratory analysis and PCR optimization. When available, previously reported primers were used rather then *de novo* primers (Table 3). PCR conditions were optimized for each set of primers by amplifying the target genes from genomic DNA of *B. cereus* ATCC 49064 and the corresponding reference strain. Optimized conditions were used for High Fidelity PCR reactions with genomic template from *B. cereus* ATCC 49064 and *B. cereus* ATCC 49064 JH1. PCR products were purified, sequenced, aligned, and analyzed as descried above. Analysis was done on DNA sequence and the translated protein sequence.

Statistical analysis

The statistical analysis was carried out with S Plus statistical software (Insightful Technologies, Seattle WA). All bacterial challenges were completed in triplicate or duplicate using three or two different erythrophore cell preparations as indicated. Reported values are the average of the trials. Final time points were analyzed using a two-sample *t* test, all reported *p*-values were declared significant at $p < 0.05$.

Chapter 4

Results

Erythrophore Cell Response to Salmonella enteritidis

An important parameter of each experiment was the effect uninoculated bacterial culture medium had on the erythrophore cells. Thus, for each experiment the corresponding uninoculated bacterial culture medium was included to establish if the observed response was bacterial- or bacterial culture medium-specific. *Salmonella enteritidis* ATCC 4931 was selected as a model gram-negative, pathogenic bacterium. Erythrophore cells responded to the addition of the media control (LB medium) by slow dispersion of pigment organelles whereas, *S. enteritidis* ATCC 4931 grown in LB caused slow aggregation initiating at ten minutes and continuing until pigment area change was ~ -40% (Figure 4). The difference between *S. enteritidis* and the media control (LB) was statistically significant ($p < 0.001$) at 20 minutes.

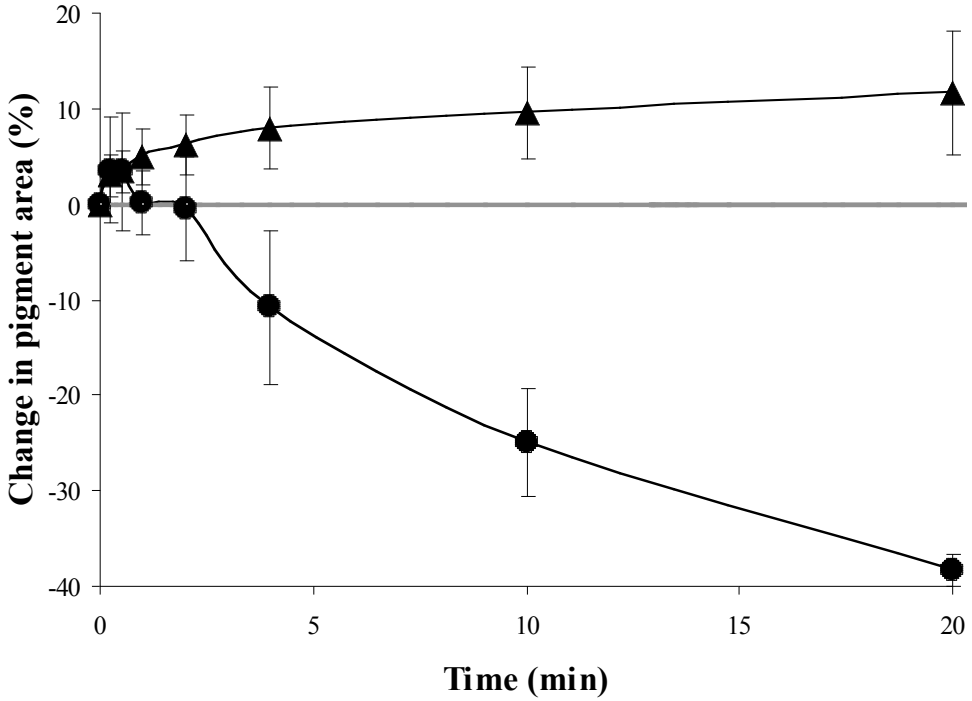


Figure 4. *Betta splendens* erythrophore cell response to *S. enteritidis.* Erythrophore cells were exposed to an overnight culture of *S. enteritidis* ATCC 4931 cultured in LB medium at 30°C. Uninoculated LB medium caused pigment organelle dispersion while the bacterial culture caused slow aggregation. ▲ - LB medium, ● - *S. enteritidis* ATCC 4931. A negative change in pigment area is indicative of pigment aggregation whereas a positive change in pigment area represents pigment dispersion. Data represents the average of three trials.

Erythrophore cell response to Clostridium perfringens

Clostridium perfringens produces several different toxins during vegetative growth whereas CPE (*C. perfringens* enterotoxin) is released with formed spores during sporulation. CPE is a major virulence factor of this bacterium and has been attributed to the symptoms associated with *C. perfringens* food-borne illness. Erythrophore cells responded by dispersing pigment when exposed to *C. perfringens* SM101 cultured in the vegetative medium, FTG. This was similar to the response observed when erythrophore cells were exposed to uninoculated FTG.

Clostridium perfringens SM101 was cultured in Duncan-Strong (DS) sporulation medium to allow production of the potent enterotoxin CPE (116, 136). *Clostridium perfringens* SM101 induced aggregation of erythrophore cell pigment within four minutes followed by slow dispersion for the remainder of the assay (Figure 5). This response was very different from the dispersive response of the media control (DS). The difference observed in erythrophore cell response induced by *C. perfringens* SM101 and was statistically significant compared to the media control at 20 minutes ($p < 0.004$). Additionally, erythrophore cells failed to respond to *C. perfringens* MRS101 a derivative of SM101 lacking *cpe* when cultured in vegetative medium (FTG) or sporulation medium (DS).

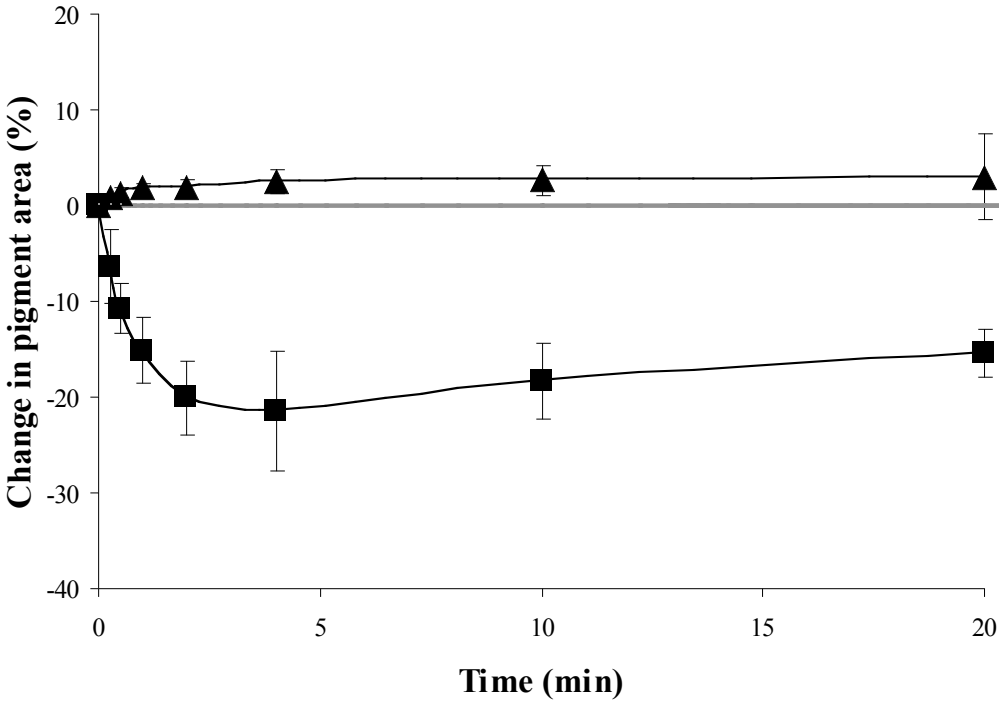


Figure 5. *Betta splendens* erythrophore cell response *C. perfringens*. *Clostridium perfringens* was cultured in Duncan-Strong sporulation medium to allow for production of CPE. Uninoculated sporulation medium caused slight dispersion of pigment within the chromatophores. *Clostridium perfringens* SM101 culture caused rapid aggregation which stabilized ~ 4 minutes after the addition of the culture. ▲- Duncan-Strong sporulation medium, ■ - *C. perfringens* SM101. Data represent the mean values of three trials.

Erythrophore cell response to Clostridium botulinum

Clostridium botulinum was chosen as a second representative of gram-positive, spore forming, anaerobic bacterium associated with food contamination. Besides being a food-associated bacterium, *C. botulinum* also has the potential to be used as a biological weapon. Erythrophore cells were challenged with *C. botulinum* NCTC 7272 (Type A) and *C. botulinum* NCTC 7273 (Type B) cultured in BHI. No change was observed in erythrophore cells in response to *C. botulinum* NCTC 7272 or NCTC 7273 within the first hour so monitoring time was extended. Two hours after exposure to pigment organelles began to slowly aggregate (Figure 6A) and aggregation of

pigment organelles was complete at approximately 5 hours. The erythrophore response to both *C. botulinum* cultures was statistically significant compared to the media control (BHI) (*C. botulinum* NCTC 7272 $p < 0.005$ and *C. botulinum* NCTC 7273 $p < 0.006$ respectively). Erythrophore cells responded in an identical manner to *C. botulinum* NCTC 7272 whole culture and culture filtrate. The observed morphological changes of the erythrophore cells were unique from changes induced by other bacterial pathogens. Extensive dendrite formation was observed in erythrophore cells exposed to *C. botulinum* (Figure 6B), while other bacterial pathogens induced pigment organelles to centrally localize within the erythrophore cell. The media control (BHI), did not induced pigment aggregation for the length of the experiment (Figure 6C). Additionally, the nonpathogen model organism, *B. subtilis*, did not induce pigment aggregation for the length of the experiment.

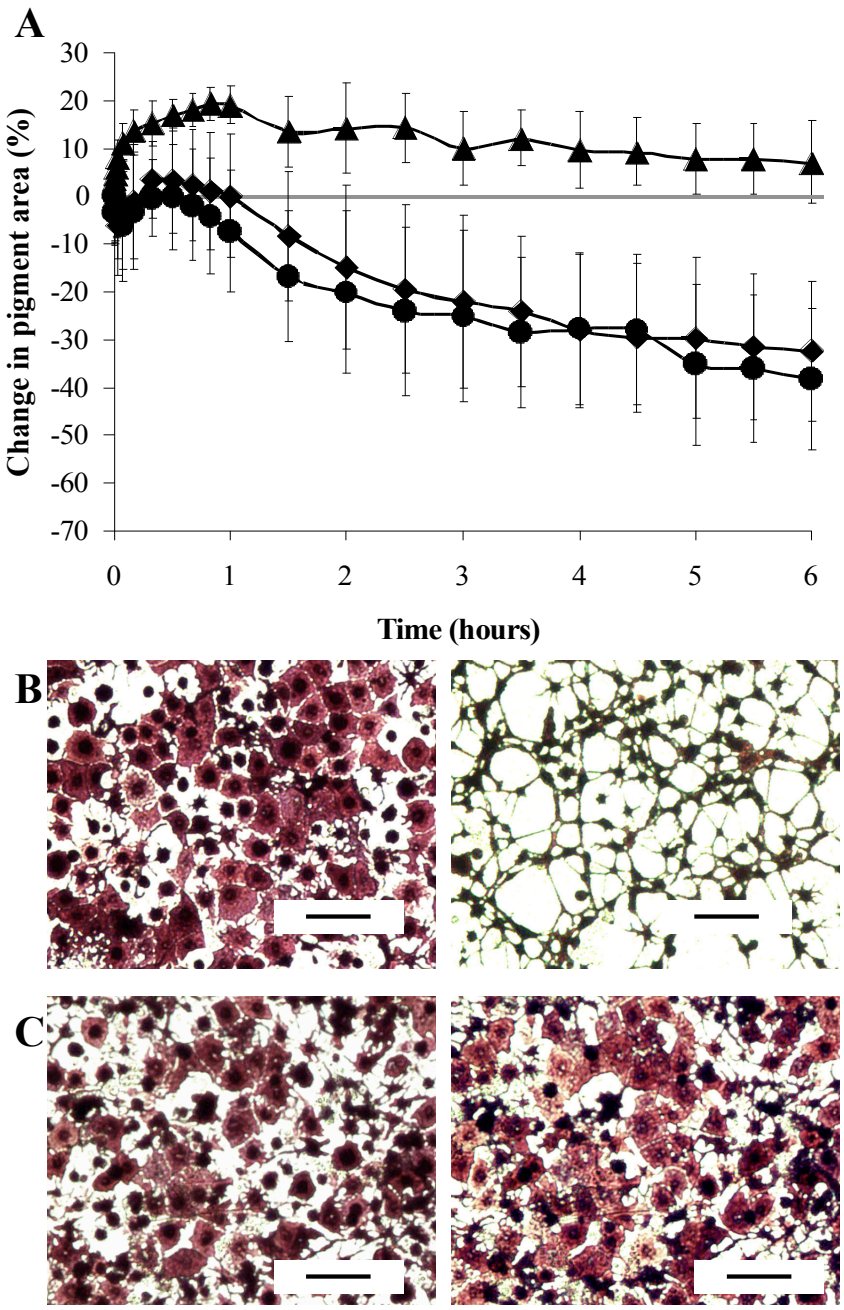


Figure 6. (A) *Betta splendens* erythrophore cell response to *C. botulinum.* Erythrophore cell response time recorded was extended to account for the slower response time. Both *C. botulinum* NCTC 7272 and *C. botulinum* NCTC 7273 caused slow pigment aggregation. ▲- BHI medium. ● - *C. botulinum* NCTC 7272. ♦- *C. botulinum* NCTC 7273. **(B)** and **(C)** = *B. splendens* erythrophore cells at 100X, the size bar represents 100 μm**;** Left, time = 0 hours, Right, time = 6 hours, exposure to: **(B)**. *C. botulinum* NCTC 7272 Erythrophores exhibited a dendritic morphology following exposure to *C. botulinum*. **(C)** BHI medium. The media control, BHI, caused erythrophore cells pigment to disperse. Data is the mean values of three trials.

Erythrophore cell response to Bacillus subtilis and members of the Bacillus cereus group

Erythrophore cells were exposed to a set of characterized *Bacillus* species cultured in BHI (Brain Heart Infusion) medium overnight: *B. subtilis* 1A1 (laboratory reference strain) (135), *B. cereus* ATCC 49064 (gastroenteritis outbreak isolate) (101), *B. thuringiensis* subsp. *kurstaki* BGSC 4D1 (34), and the nonpathogenic *Bacillus anthracis* 34F2 vaccine strain (Sterne). The media control (BHI) caused slight dispersion of pigment organelles within the erythrophore cell (Figure 7). The observed dispersion was slow, but stabilized 10 minutes after exposure. Erythrophore cell response to *B. subtilis* 1A1 culture was similar in strength and rate to the response elicited by the media control (BHI) (Figure 7), and this response was found to be statistically insignificant ($p = 0.562$). Addition of *B. cereus* ATCC 49064 induced rapid erythrophore pigment organelles aggregation which occurred within 3 minutes after the addition of the bacterial culture (Figure 7). By 5 minutes, erythrophore cells remained in the aggregated state indefinitely. Erythrophore cell response to *B. cereus* ATCC 49064 was found to be statistically significant compared to the media control ($p< 0.003$). *Bacillus thuringiensis* BGSC 4D1, an insect pathogen, also induced pigment organelle aggregation in erythrophore cells, but this rate was slower in comparison to the rate of aggregation induced by *B. cereus* (Figure 7). This response was statistically significant from the media control ($p< 0.008$). *Bacillus anthracis* 34F2 caused pigment organelles to aggregate slightly followed by pigment dispersion. This response found to be statistically insignificant compared to the control ($p = 0.327$).

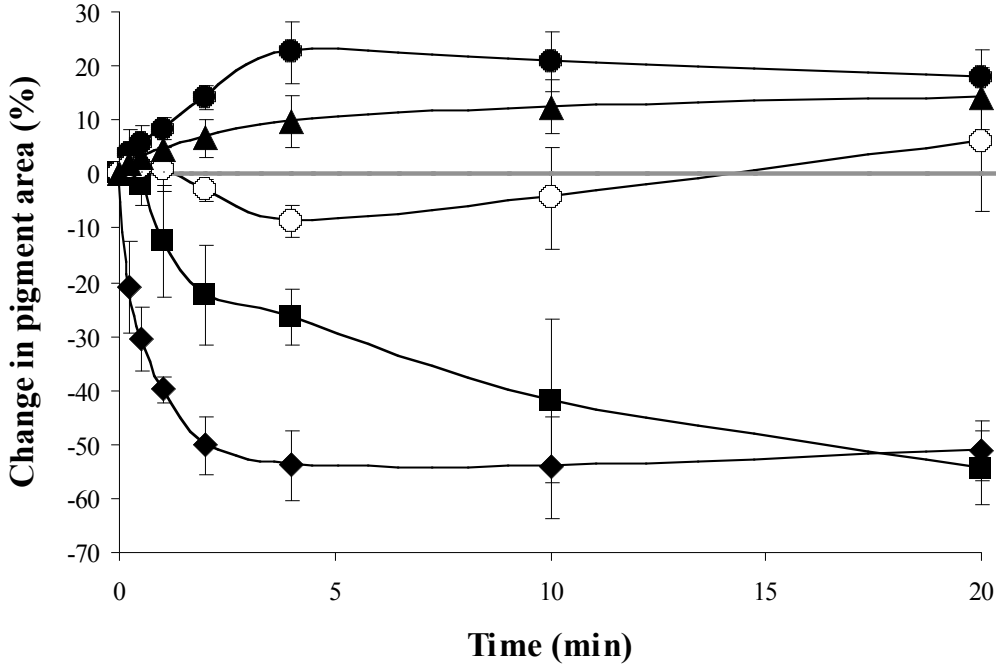


Figure 7. *Betta splendens* erythrophore cell response to *B. cereus* group members and *B. subtilis*. *Bacillus cereus* and *B. thuringiensis* both caused pigment aggregation within erythrophore cells, but the rate of aggregation was different between the two strains. The media control (BHI), *B. subtilis*, and to a slightly lesser extent *B. anthracis* caused pigment organelle dispersion, this change was found to be statistically insignificant. ▲- BHI medium. ● - *B. subtilis* 1A1. ○ - *B. anthracis* 34F2. ■ - *B. thuringiensis subsp. kurstaki* BGSC 4D1. ♦ - *B. cereus* ATCC 49064. Data represent the mean values of three trials.

Erythrophore cell response to Bacillus cereus isolates

The rapid rate of pigment aggregation induced by *B. cereus* and *B. thuringiensis* was very unusual from observed rates of other bacterial pathogens. Given the variation of toxin production by *B. cereus* isolates addition bacterial strains were investigated. Erythrophore cells were exposed to *B. cereus* UW85, (60), *B. cereus* ATCC 10987 (64, 123) and *B. cereus* ATCC 14579 (45). These strains were

chosen because they are widely available and *B. cereus* ATCC 10987 and *B. cereus* ATCC 14579 were recently sequenced (112). *Bacillus cereus* UW85 is a biological control agent for *Phytophthora megasperma* f. sp. *medicaginis,* which causes alfalfa seedling damping-off (60). *Bacillus cereus* ATCC 10987 was isolated from a study on cheese spoilage in Canada in 1930, and *B. cereus* ATCC 14579 is a laboratory reference strain. In each experiment, erythrophore cell response to *B. cereus* cultures was compared to the media control, BHI. Erythrophore cells were exposed to these three *B. cereus* isolates, and each one caused pigment organelles to rapidly aggregate 2 minutes after the addition of the bacterial culture (Figure 8). By 10 minutes, erythrophore cells remained in the aggregated state indefinitely for each isolate. Interestingly, the erythrophore cell response curves from these three isolates were indistinguishable from each other. The observed changes were statistically significant compared to the media control (*B. cereus* UW85 $p < 0.003$, *B. cereus* ATCC 10987 $p < 0.004$, and *B. cereus* ATCC 14579 $p < 0.002$, respectively).

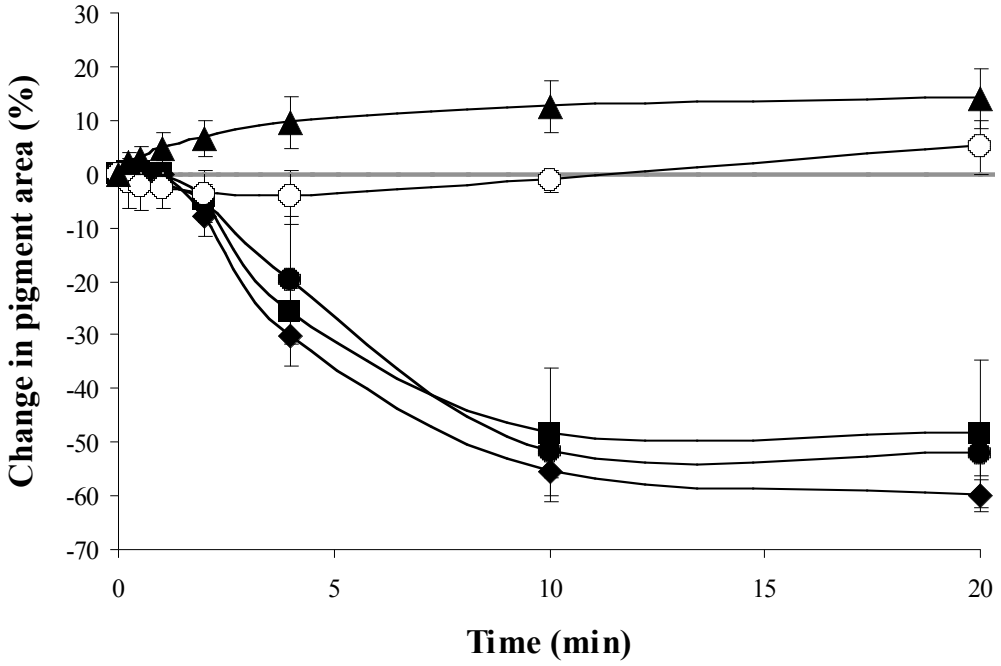


Figure 8. Erythrophore cell response to *B. cereus* isolates. *Bacillus cereus* UW85, *B. cereus* ATCC 10987, and *B. cereus* ATCC 14579 caused rapid pigment aggregation that was complete within 10 minutes, whereas *B. cereus* ATCC 14579 Δ *plcR* caused slight aggregation followed by pigment dispersion similar to the response caused by the media control. ▲- BHI medium. ■ - *B. cereus* UW85. ♦ - *B. cereus* ATCC 10987. ● - *B. cereus* ATCC 14579. ○ - *B. cereus* ATCC 14579 Δ *plcR*. Data represents the average of three individual experiments.

Bacillus cereus isolates produce several known toxins as well as other putative virulence factors. One hypothesis is that the rapid aggregation of pigment organelles within erythrophore cells could be caused by a virulence factor or toxin produced by *B. cereus*. To test this, erythrophore cells were exposed to *B. cereus* ATCC 14579 strain lacking the global gene regulator, *plcR* (115). PlcR controls the expression of numerous secreted proteins, toxins, known virulence factors and PlcR and PlcR-

regulated proteins are conserved among *B. cereus* group members. The *B. cereus* Δ*plcR* mutant strain induced a similar response (slightly dispersive rather than aggregative) in erythrophore cells as the media control, (p value = 0.075) (Figure 8), and this response was found to be statistically insignificant. These data suggest that a PlcR-regulated factor is involved in erythrophore cell response.

Erythrophore cell response to Bacillus species culture, heated culture, and culture supernatant

Twenty-two known enterotoxins and proteases are regulated by PlcR within *B. cereus* ATCC 14579 (52). These 22 proteins are secreted outside of the bacterial cell and into the extracellular environment. Additionally, PlcR regulates the expression of 18 proteins associated with the cell wall and 5 cytoplasmic proteins (52). To determine if the factor(s) responsible for erythrophore cell aggregation were (1) secreted; (2) associated with the cell membrane; or (3) cytoplasmic, the effect of bacterial supernatant was evaluated. Bacterial cells were removed from the bacterial culture medium by centrifugation and the supernatant was applied to erythrophore cells. The results of this experiment are summarized in Table 4. Supernatant from stationary phase cultures caused erythrophore cells to respond in a manner identical to that observed with stationary whole cultures. These results were observed for all *Bacillus* strains tested.

Several secreted PlcR-regulated proteins are heat labile. To determine the effect of heat, stationary phase cultures were held at 95°C for 15 minutes prior to erythrophore cell exposure. The results of these experiments are summarized in Table 4. Erythrophore cells exposed to heat treated *B. cereus* cultures failed to respond by

aggregation. A single exception was observed: *B. cereus* ATCC 49064 caused erythrophore cells to aggregate their pigment organelles in a manner similar to un-heated bacterial culture (Table 4).

Table 4. Erythrophore cells were exposed to whole bacterial culture, culture supernatant (cells were removed from culture by centrifugation), or heated culture (15 minutes at 95°C). Erythrophore cell response was scored: (+ +) very rapid aggregation (complete pigment aggregation within 5 minutes), (+) rapid aggregation (complete pigment aggregation within 10 minutes), or (-) dispersion (positive change in pigment cell area).

Bacterial Strain	Erythrophore cell reaction rate: whole culture	Erythrophore cell reaction rate: culture supernatant	Erythrophore cell reaction rate: heated culture
B. cereus ATCC 49064	+ +	+ +	+ +
B. cereus ATCC 14579	+	+	-
B. cereus ATCC 14579 ΔplcR	-	-	-
B. cereus ATCC 10987	+	+	-
B. cereus NCTC 11143	+ +	+ +	-
B. cereus UW85	+	+	-
B. thuringiensis subsp. kurstaki BGSC 4D1	+	+	-
B. subtilis 1A1	-	-	-
BHI	-	-	-

Detection Sensitivity

Erythrophore cells exposed to *B. cereus* ATCC 49064 cultures in lag phase or early exponentially phase did not induce pigment aggregation in erythrophore cells. Only *B. cereus* cultures that completed exponential growth and were entering stationary phase induced rapid pigment aggregation in erythrophore cells in a similar fashion as overnight cultures. Erythrophore cells respond to as little as 2 µl of stationary phase *B. cereus* ATCC 49064 culture, corresponding to ~ 2 µg of crude

toxin as quantified by Gray *et al*. The number of bacteria required to induce erythrophore cell response from an overnight stationary phase culture was found to be >6 x 10^6 CFU when cultured in BHI medium and >4 x10^6 CFU when cultured in LB medium.

Erythrophore cell response to Bacillus cereus emetic strain

Bacillus cereus produces heat labile and heat stable toxins. The heat labile enterotoxins have been associated with the diarrheal form of foodborne illness whereas the heat stable toxin, cereulide, is associated with the emetic form. Erythrophore cells exposed to *B. cereus* NCTC 11143 emetic strain, rapidly aggregated pigment towards the center of the cell (Table 4, Figure 9). Erythrophore cells did not aggregate pigment when were exposed to *B. cereus* NCTC 11143 culture heated for 15 minutes at 95°C (Figure 9). Rapid pigment aggregation was observed in erythrophore cells exposed to *B. cereus* ATCC 49064 whole culture, and heated culture (15 minutes at 95°C) (Figure 9). *Bacillus cereus* ATCC 49064 was isolated from a diarrheal outbreak and has not been reported to produce the known heat stable emetic toxin, cereulide. Using cereulide specific primers reported by Ehling-Schulz *et al*., PCR analysis confirmed the presence of the emetic toxin, cereulide in *B. cereus* NCTC 11143 but failed to detect the presence of cereulide in *B. cereus* ATCC 49064. The failure of heated *B. cereus* NCTC 11143 to elicit an aggregative response suggests that erythrophore cells are not sensitive to the heat stable emetic toxin, but rather the heat labile, secreted, PlcR-regulated factor(s). Heat treatment of *B. cereus* ATCC

49064 suggests the presence of an unknown heat stable factor that may be involved in the very rapid aggregation of pigment organelles.

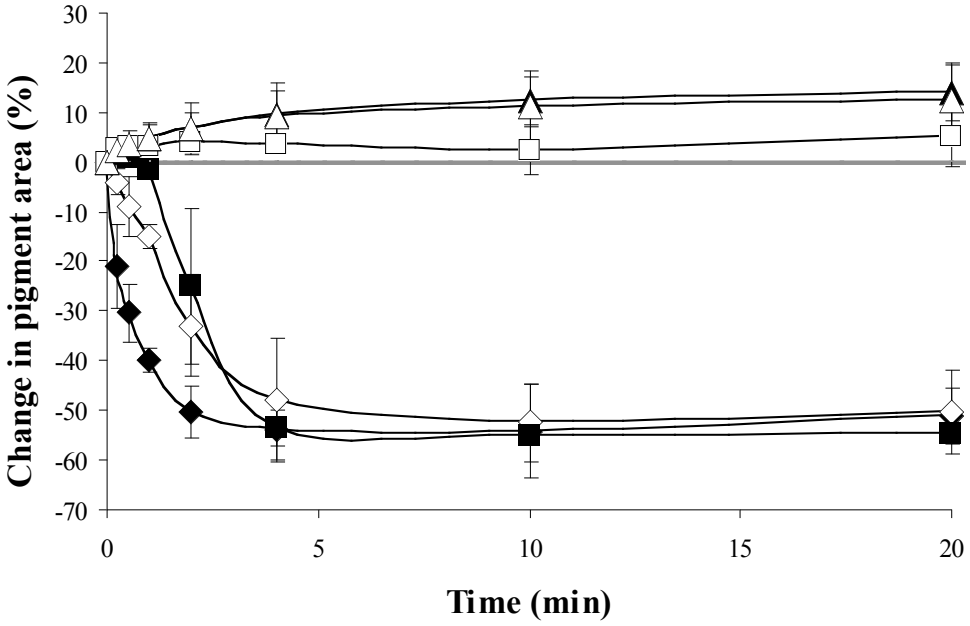


Figure 9. Erythrophore cell response to *B. cereus* ATCC 49064 and *B. cereus* NCTC 11143 cultures with and without heat treatment. Heat treatment of *B. cereus* ATCC 49064 culture at 95°C for 15 minutes caused a slight delay of pigment aggregation compared to the unheated culture. Heat treatment of *B. cereus* NCTC 11143 cultures resulted in total loss of aggregation whereas the untreated culture caused pigment aggregation. ▲- BHI medium. Δ - BHI medium heated. ♦- *B. cereus* ATCC 49064. ◊ - *B. cereus* ATCC 49064 heated culture. ■ - *B. cereus* NCTC 11143, □ - *B. cereus* NCTC 11143 heated culture. Data represent the mean values of three trials.

Identification of bacterial factor(s) responsible for erythrophore cell aggregation

What is it about *B. cereus* that causes erythrophore cells to respond by rapidly aggregating their pigment organelles? Erythrophore cell response to *B. cereus* ATCC Δ *plcR* mutant strain causes minimal pigment organelle aggregation suggesting that

one or more PlcR-regulated factors may be responsible for pigment aggregation. One hypothesis is that one or more PlcR-regulated toxin(s) is involved in pigment aggregation. To test this hypothesis mutants were isolated that induced a delayed aggregative erythrophore cell response compared to the wild-type bacterium. Gene knockouts were unsuccessful in *B. cereus* ATCC 49064 using reported protocols and vector systems. Transposon mutagenesis of *B. cereus* ATCC 49064 and *B. cereus* ATCC 10987 was conducted utilizing the following transposon vectors: pMarA, pMarB, pIC333, and EzTn10 transposome (74, 80, 124). All transposon vectors failed to generate a stable insertional event, and thus we were unable to isolate a transposant that caused a delayed erythrophore cell response compared to the response induced by wildtype bacterium. Chemical mutagenesis of *B. cereus* ATCC 49064, *B. cereus* ATCC 14579, and *B. cereus* UW85 was used as an alternative approach to investigate the factor(s) involved in erythrophore cell response. Putative NTG mutants, their backgrounds, and concentration of NTG used are listed in Table 5. *Bacillus cereus* ATCC 49064 JH1 was selected for further characterization. *Bacillus cereus* ATCC 49064 JH1 will be referred to as JH1 for the remainder of this work.

Table 5. Putative *B. cereus* clones identified that cause a delayed erythrophore cell response.

Background	Clone ID	Concentration of NTG
B. cereus ATCC 49064	JH1 thru JH7, JH22, JH23	5 mg ml^{-1}
B. cereus ATCC 49064	JH12 thru JH 21	15 mg ml^{-1}
B. cereus ATCC 49064	JH8 thru JH11	30 mg ml^{-1}
B. cereus ATCC 14579	JH24, JH25, JM1	8 mg ml^{-1}
B. cereus ATCC 14579	JM19 thru JM21	15 mg ml^{-1}
B. cereus UW85	JM2 thru JM15	2.5 mg ml^{-1}
B. cereus UW85	JM17, JM18, JM22 thru JH25	10 mg ml^{-1}

Erythrophore cell response to B. cereus ATCC 49064 *and JH1*

Erythrophore cell response to wildtype *B. cereus* ATCC 49064 was very rapid as shown previously (Figure 10). JH1 caused a delayed erythrophore cell response compared to wildtype, and pigment organelle aggregation did not initiate until 4 minutes after the addition of the bacterial culture. The aggregation rate was slower then the wildtype and was complete at 10 minutes (Figure 10). Bacterial growth curves and viable cell counts of *B. cereus* ATCC 49064 and JH1 failed to identify a growth deficiency between the wildtype and the mutant clone, suggesting the mutant phenotype is not due to growth variations. The failure to abolish aggregation suggests that multiple factors are involved in inducing erythrophore cell aggregation.

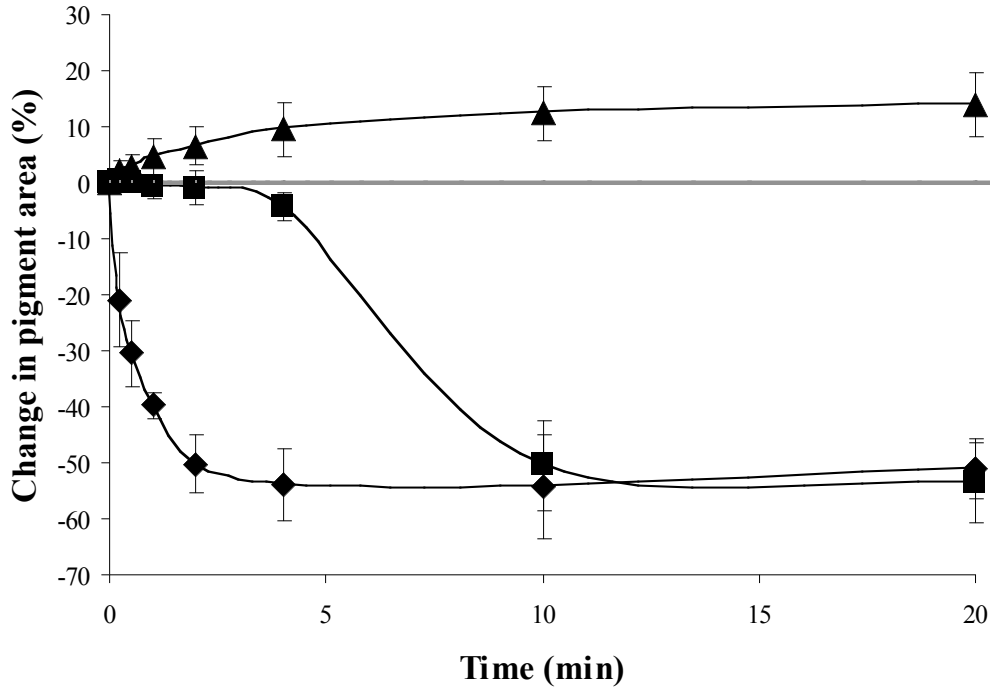


Figure 10. *Betta splendens* erythrophore cell response to *B. cereus* ATCC 49064 and JH1 cultures. *Bacillus cereus* ATCC 49064 caused very rapid pigment aggregation. JH1 culture did not cause pigment aggregation until 4 minutes after the addition of the bacterial culture, this aggregation was slow and was complete by 10 minutes. ▲- BHI medium. ♦ - *B. cereus* ATCC 49064. ■ - JH1. Data represent the mean values of three trials.

Genomic library complementation of B. cereus ATCC 49064 JH1

A genomic library of *B. cereus* ATCC 49064 was constructed by cloning DNA fragments into the *B. cereus* low copy number shuttle vector, pHT304. This library was electroporated into JH1. Very few transformants were recovered that contained the shuttle vector. Putative transformants were screened using erythrophore cell response assay but this method failed to identify a vector/insert that complemented the mutant phenotype. Thus, alternative approaches became necessary to identify the genetic variation between *B. cereus* ATCC 49064 and JH1.

Typing B. cereus ATCC 49064

To date, the *B. cereus* ATCC 49064 genome has not been sequenced so Illumina 1G sequencing technology was employed for whole genome analysis. Initial data analysis requires a reference genome for comparison and preliminary genome assembly. At the time of this study, *B. cereus* ATCC 14579, *B. cereus* ATCC 10987 and *B. cereus* E33L (Zk) were the only *B. cereus* isolates sequenced. To deduce the appropriate reference genome we used 16s rRNA sequencing, *groEL* RFLP and *groEL* sequence analysis. *Bacillus cereus* E33L was not included in these analyses because the strain was not available for laboratory evaluation.

16s rRNA sequencing

One hundred percent sequence similarity was observed between *B. cereus* ATCC 49064, *B. cereus* ATCC 14579, and *B. cereus* ATCC 10987 using *Bacillus* specific primers (37). These results are in agreement with previously published data

concluding that the 16s rRNA gene is too highly conserved among *B. cereus* group members to be used as a genetic differentiating factor.

groEL RFLP analysis

PCR was used to amplify *groEL,* a highly conserved housekeeping protein, from *B. cereus* ATCC 49064, *B. cereus* ATCC 14579, and *B. cereus* ATCC 10987. RFLP analysis with the restriction enzyme *PstI* was performed on each *groEL* PCR product. While the sample size for this analysis was small, the purpose was to identify the most appropriate reference genome for initial Illumina analysis and subsequent gene targeted sequencing. The RFLP pattern for *B. cereus* ATCC 49064 was similar to *B. cereus* ATCC 10987. Whereas the pattern observed for *B. cereus* ATCC 14579 was unique compared to the other two isolates (Figure 11).

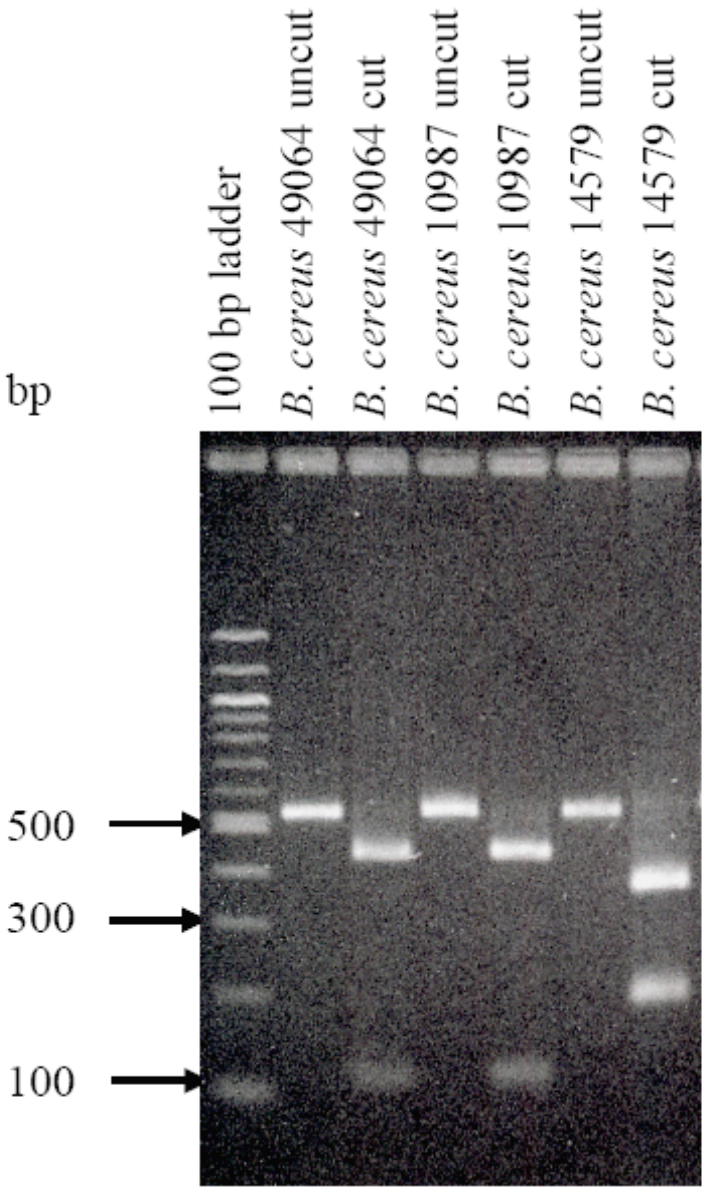


Figure 11. RFLP of *groEL* PCR product. Uncut PCR product from all three isolates was observed to be ~ 550 bp. *PstI* restriction digest of *B. cereus* ATCC 49064 and *B. cereus* ATCC 10987 resulted in two bands running at ~ 450 and ~ 100 bp. Two bands at ~ 300 and ~ 200 bp was observed for *B. cereus* ATCC 14579 digest.

groEL gene sequences

A 533 bp PCR product was obtained for *groEL* from *B. cereus* ATCC 49064, *B. cereus* ATCC 14579, and *B. cereus* ATCC 10987. Nucleotide sequence similarity was observed to be 99.6% different between *B. cereus* ATCC 49064 and *B. cereus* ATCC 10987, and 97.6% similar between *B. cereus* ATCC 49064 and *B. cereus* ATCC 14579 (97.6%). This sequence data and *groEL* RFLP assay suggest that *B. cereus* ATCC 10987 is the most appropriate reference genome for initial Illumina sequence analysis.

Whole genome assembly using Illumina

We generated 6,799,404 random shotgun reads from *B. cereus* ATCC 49064 and 7,412,957 reads from JH1 using Illumina sequencing. Initial data analysis was processed by CGRB Biocomputing Facility using ELAND and the reference genome *B. cereus* ATCC 109897. The results are summarized in Table 6.

Table 6. Output from Illumina. The differences in outputs between *B. cereus* ATCC 49064 and JH1 could be due to several factors inkling adaptor ligation efficiency and cluster formation efficiency.

Output	*B. cereus* ATCC 49064	JH1
Clusters per Tile	22,968	23,144
Total Reads	6,799,404	7,412,957
Perfect Hit	246,152	1,751,373
1 Miss Match	176,609	558,523
2 Miss Match	110,226	269,710
Single Hit to genome	518,328	2,560,745
Multi Hit to genome	14,659	18,861
No Hit / Too many MM	6,266,417	4,833,351

Gene specific alignment using Illumina output

Assembly of the entire genome of *B. cereus* ATCC 49064 was not accomplished. We feel this is due to two factors, (1) not enough coverage with the output generated, and/or, (2) technology and reference genome coverage limitations prevented a *de-novo* whole genome assembly. To circumvent this limitation the Illumina data from *B. cereus* ATCC 49064 and JH1 was pooled in order to conduct gene specific alignments. Three data bases were created using the reference genes from *B. cereus* ATCC 14579, *B. cereus* ATCC 10987 and *B. cereus* E33L (Zk). The U-category reads were aligned and assembled using Moasaik. For the 32 nucleotide reads, 3 mismatches were allowed for a 90% identity over the read. For each gene in the database the following output was generated: Reads (aligned to gene), Average Coverage (over length of gene), Peak Coverage (to the gene), Zero Coverage (nucleotides with no coverage within the gene), Weak Reference Base (IUPAC nucleotides in the reference gene file), Ambiguous (reference base call were cryptic), and SNP's (single-nucleotide polymorphism along read length to the gene). The following output was included in the final analysis: Reads, Average Coverage, Zero Coverage, and SNP's. The goal of the final analysis was to assess the degree of similarity between the three reference genomes and *B. cereus* ATCC 49064 as to determine if a given gene was present, and which reference genome was most appropriate for primer design. To assess the degree of similarity the following ratio was calculated and represented as a percentage:

$$[(\text{Length} - \text{Zero Coverage})/\text{Length}]*100 = \text{Degree of Similarity (\%)}$$

A small subset of these data are represented in Table 7. Genes of interest were selected initially if they were known to be regulated by PlcR and those genes identified as putative virulence factors within the literature (Table 7). The reference gene sequence with the highest degree of similarity was used for primer design

Table 7. Results of sequence data compared to published reference genomes. Gray shading indicates reference gene sequence used for primer design; genes in white were not evaluated. Primers for NheA, NheB, and NheC were those reported by Lindbäck *et al.*

Accession	Locus[a]	Gene Description	Length	Average Coverage	Zero Coverage	Snps	Degree of similarity	PCR results for *B. cereus* ATCC 49064[c]	Amino acid changes in mutant compared to WT[c]
NC_003909	BCE_5183	Bacillolysin	1776	12.81	91	51	94.88%	+	No
NC_004722	BC_5036	Bacillolysin	1650	9.89	146	53	91.15%		
NC_003909	BCE_3869	CodY	780	10.78	22	5	97.18%	+	No
NC_004722	BC_3826	CodY	780	8.38	49	28	93.72%		
NC_003909	BCE_1209	Cytotoxin K	1011	12.78	6	10	99.41%	+	No
NC_004722	BC_1110	Cytotoxin K	1011	10.34	49	37	95.15%		
NC_003909	BCE_5353	Enterotoxin	1749	17.43	5	23	99.71%	+	No
NC_004722	BC_1953	Enterotoxin	1294	7.53	72	20	94.44%	+	No
NC_004722	BC_5239	Enterotoxin	893	14.03	55	27	93.84%	+	No
NC_004722	BC_0813	Enterotoxin	1296	8.87	160	33	87.65%	-	ND
NC_004722	BC_2952	Enterotoxin	1490	1.35	947	11	36.44%		
NC_004722	BC_3104	*hblC*	na	na	na	na	na	+	Yes: Alanine289 to Threonine 289
NC_004722	BC_3102	*hblA*	na	na	na	na	na	+	No
NC_004722	BC_3103	*hblD*	na	na	na	na	na	+	No
NC_003909	BCE_4248	Hemolysin A	840	11.38	8	15	99.05%	+	No
NC_004722	BC_4175	Putative Hemolysin A	840	8.18	57	32	93.21%		
NC_003909	BCE_3795	Hydrolase	798	7.92	18	12	97.74%	+	No
NC_003909	BCE_2449	Hydrolase	729	5.45	70	13	90.40%		
NC_004722	BC_1170	Hydrolase	735	8.33	81	16	88.98%		
NC_004722	BC_3414	Hydrolase	828	5.34	119	25	85.63%		
NC_003909	BCE_3539	Microbial collagenase	2916	6.37	274	52	90.60%	-	ND
NC_003909	BCE_0616	Microbial collagenase	2898	10.41	342	77	88.20%	-	ND
NC_004722	BC_0556	Microbial collagenase	2871	7.24	674	64	76.52%		

NC_004722	BC_3529	Microbial collagenase	2895	3.15	1008	73	65.18%		
NC_004722	BC_3161	Microbial collagenase	2883	0.19	2718	3	5.72%		
NC_003909	BCE_1968	NheA	1161	5.7	99	22	91.47%	+	No
NC_003909	BCE_1969	NheB	1209	6.19	92	19	92.39%	+	No
NC_003909	BCE_1970	NheC	1080	5.7	139	25	87.13%	+	No
NC_004722	BC_1810	NheB	1209	5.28	97	26	91.98%		
NC_004722	BC_1809	NheA	1161	5.15	196	17	83.12%		
NC_004722	BC_1811	NheC	1080	4.26	236	18	78.15%		
NC_003909	BCE_5480	PapR	147	8.05	33	6	77.55%	+	No
NC_004722	BC_5349	PapR	147	3.57	59	2	59.86%		
NC_003909	BCE_0744	Phospholipase C	852	15.01	32	10	96.24%	+	No
NC_004722	BC_0670	Phospholipase C	852	11.39	81	37	90.49%		
NC_003909	BCE_3793	1-phosphatidylinositol phosphodiesterase	990	5.75	95	25	90.40%	-	ND
NC_004722	BC_3761	1-phosphatidylinositol phosphodiesterase	991	5.02	135	27	86.38%	NT	NT
NC_003909	BCE_5481	PlcR	859	3.28	516	10	39.93%	+	No
NC_004722	BC_5350	PlcR	858	2.87	546	10	36.36%		
NC_003909	BCE_0745	Sphingomyelinase	1137	8.7	158	32	86.10%	+	No
NC_004722	BC_0671	Sphingomyelinase	1004	3.75	399	27	60.26%		
NC_003909	BCE_3314	Thiol-activated cytolysin	1539	6.11	80	23	94.80%	+	No
NC_004722	BC_5101	Thiol-activated cytolysin	1540	4.48	254	36	83.51%	NT	NT

Note: NT, not tested; NA, not available due to lack of data, primers were designed from the reference genome *B. cereus* ATCC 14579.
[a] Locus ID, BCE_xxxx *B. cereus* ATCC 10987 reference; BC_xxxx *B. cereus* ATCC 14579 reference
[b] +, PCR product of the expected size was seen; -, no PCR product was formed
[c] Yes, change in amino acid residue of JH1 compared to *B. cereus* ATCC 49064; No, no amino acid changes were observed; ND, not determined because of PCR failure

Genes of interest

Twenty-three genes were selected as initial genes of interest. These genes were selected based on (1) degree of similarity (%); (2) published results suggesting involvement with virulence; and (3) PlcR-regulation. A PCR product could not be generated for BC_0813 (enterotoxin), BCE_3539 (microbial collagenase), BCE_0616 (microbial collagenase), and BCE_3793 (1-phosphatidylinositol phosphodiesterase). PCR amplification was successful for 19 of these genes using genomic DNA from *B. cereus* ATCC 49064 and JH1. PCR products were sequenced using PCR primers as sequencing primers. Nucleotide sequences were assembled into full length gene sequences, and then translated. Amino acid sequences from *B. cereus* ATCC 49064 and JH1 were compared using BioEdit or ClustalW. The gene targets, PCR results, and amino acid changes between *B. cereus* ATCC 49064 and JH1 are listed in Table 7 Additionally, the following *B. anthracis* virulence genes were screened for using PCR: protective antigen (*pag*), edema factor (*cya*), lethal factor (*lef*) and capsule *(capABC*). PCR failed to detect the presence of all 4 genes in *B. cereus* ATCC 49064.

The only gene identified with an amino acid substitution was in the protein L_2, one of the three proteins composing the HBL toxin. A guanine to adenine conversion was observed at nucleotide position 865 that caused an alanine to threonine residue change at position 289 (Figure 12). This suggests that the L_2 protein is at least partially involved in erythrophore cell aggregation caused by *B. cereus* ATCC 49064.

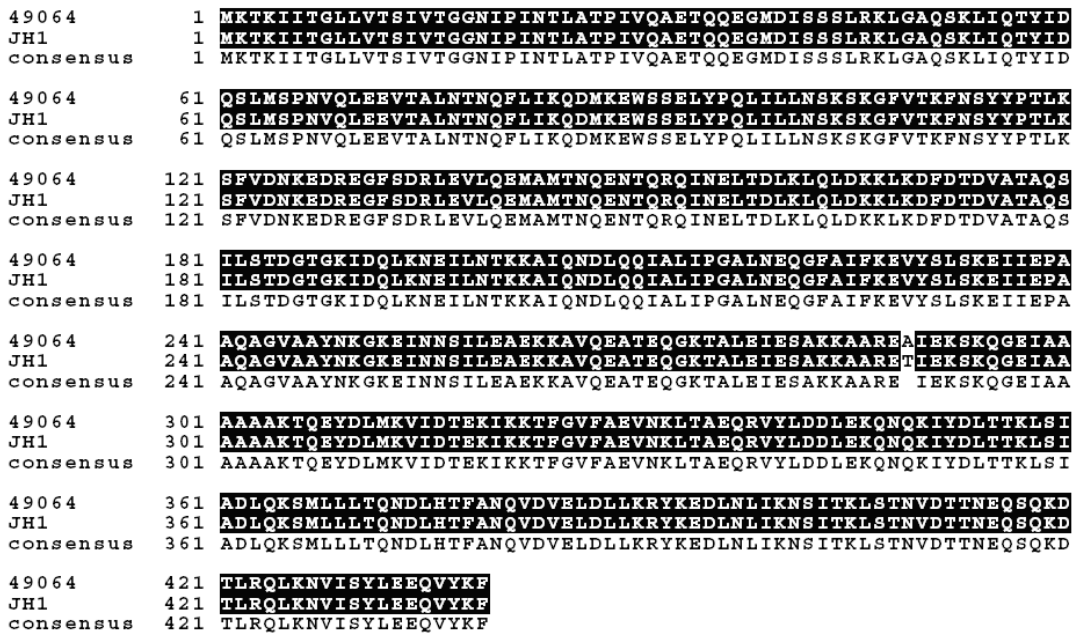


Figure 12. Comparison of amino acid sequence of L_2 from *B. cereus* ATCC 49064 and JH1. All residues were identical except at position #289 with an alanine to threonine conversion. Globally conserved residues are shown in black, normal residues in blue, similar residues in grey, and identical residues in red. Amino acid alignment was done using ClustalW program.

Complementation of hblC

PCR was used to amplify *hblC* from *B. cereus* ATCC 49064. This PCR product was cloned into the low copy number shuttle vector pHT304 and then transformed into JH1. Transformation was verified by recovering the appropriate plasmid from the bacterium and with DNA sequencing. Erythrophore cells were exposed to JH1:pHT304 and JH1:pHT304_*hblC* in addition to experimental controls. Erythrophore cells responded by rapidly aggregating their pigment organelles in the presence of JH1:pHT304_*hblC*. The aggregation rate was similar to *B. cereus* ATCC 49064 (Figure 13). Pigment aggregation induced by JH1:pHT304 was similar to the response induced by JH1 culture (Figure 13). These data suggest that one possible candidate involved in erythrophore cell aggregation is L_2 protein of the HBL toxin

complex. The mutant described here did not abolish the aggregative response, suggesting that other factors could be involved in *B. cereus* induced erythrophore cell pigment aggregation.

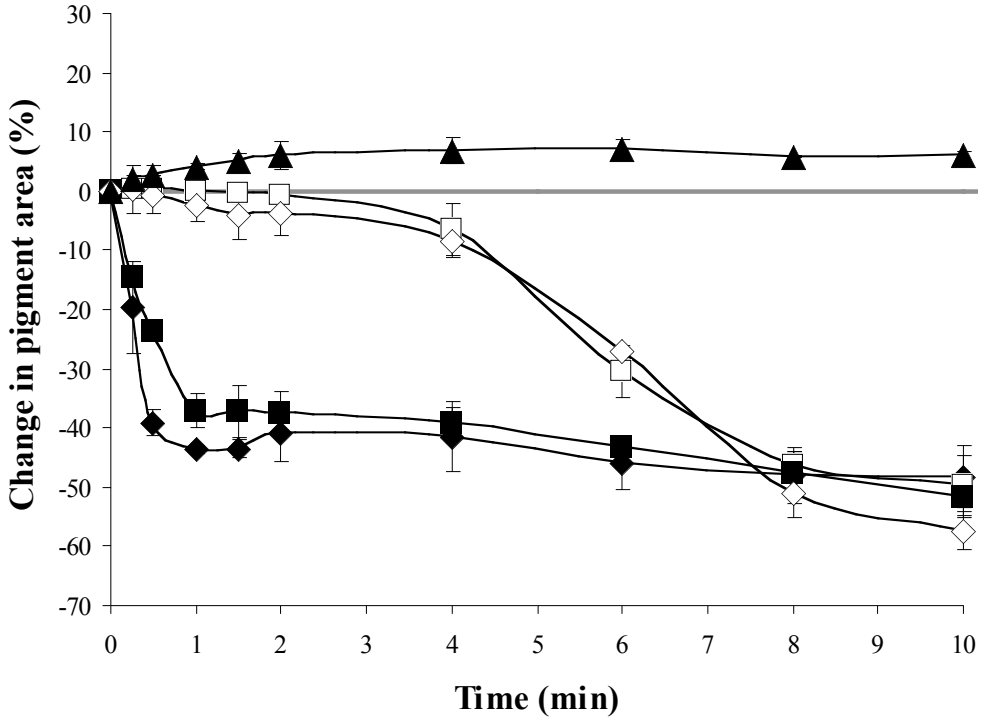
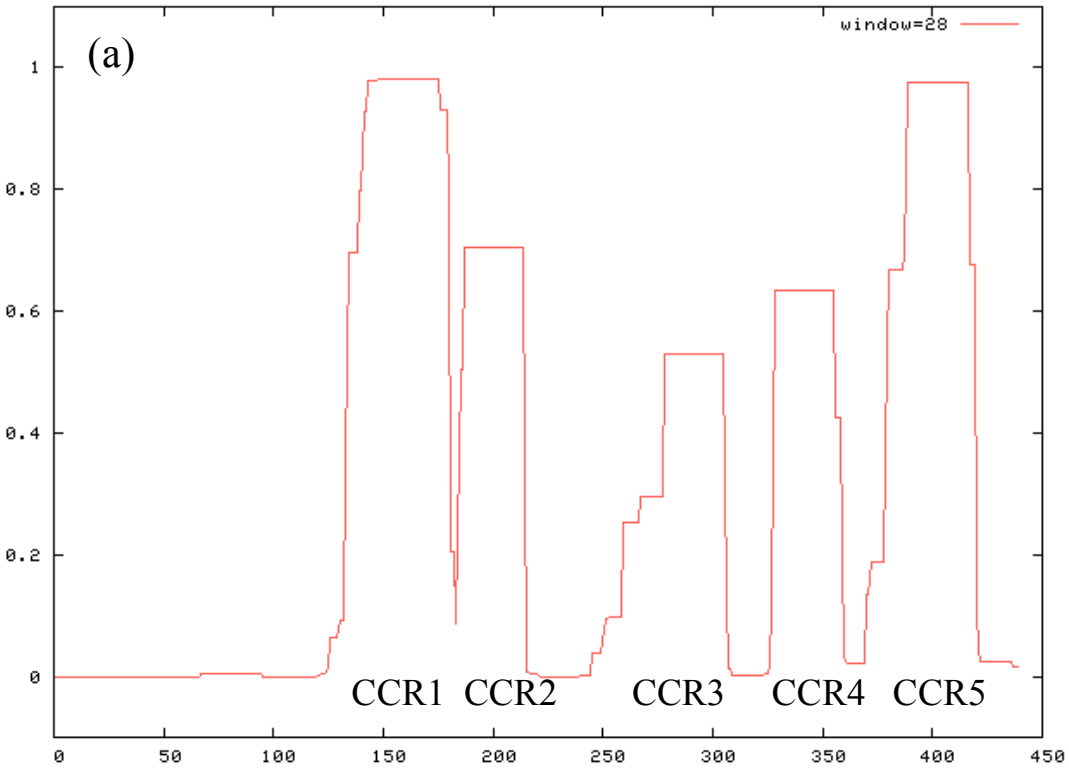


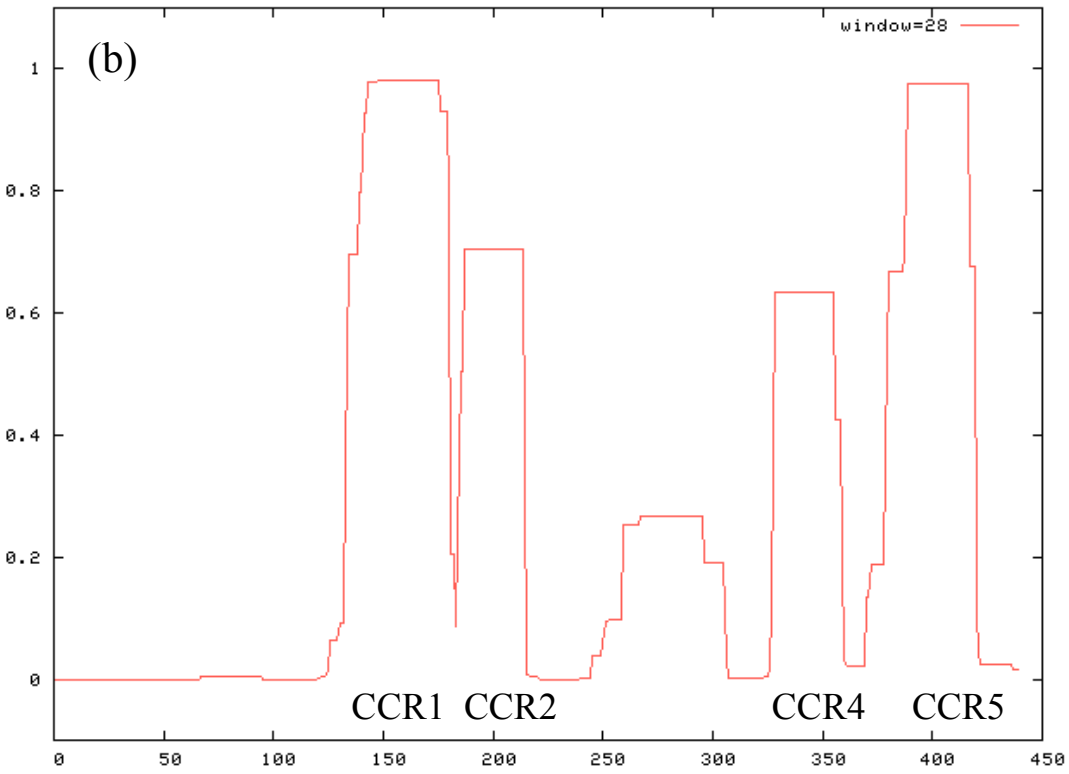
Figure 13. Complementation assay. Erythrophore cells were exposed JH1 with plasmid and insert and JH1 with empty plasmid cultured in BHI medium supplemented with erythromycin in addition to the wildtype and mutant strains. The complemented strain with insert elicited the wildtype response curve from erythrophore cells and the completed strain with empty vector caused a similar response as the mutant. ▲- BHI Erm5 medium. ♦- *B. cereus* ATCC 49064 culture. ◊ - JH1. ■ – JH1:pHT304_*hblC*, □ - JH1:pHT304 culture. Data represents average of two experiments.

Coiled Coil Prediction L_2 component of HBL of B. cereus ATCC 49064 and JH1

Coiled coils motif is a structural element that mediates protein-protein interactions (89, 91). Coiled coils are helical bundles of 2 to 5 helices that have a distinctive packing of amino acid side chains with a heptad repeat in the amino acid sequence (90). The COILS prediction software was used to predict coiled coils in the wild-type (*B. cereus* ATCC 49064) and mutant (JH1) L_2 protein sequence. The results for window 28 are presented in Figure 14. Residues with a probability >50 % in one or more reference windows of 28, 21, or 14 residues are designated as part of a coiled coil region (CCR). L_2 is predicted to have five CCRs which are located between residues 134 and 180 (CCR1), residues 185 and 214 (CCR2), residues 278 and 305 (CCR3), residues 328 and 355 (CCR4), and 379 and 419 (CCR5). Four CCRs are predicted in the mutant protein (CCR1, CCR2, CCR4, and CCR5).

Figure 14. Prediction of coiled coil regions (CCR's) of L_2 using COILS (http://www.ch.embnet.org/software/COILS_form.html). Shown here is window 28 from the COILS output. The probability of residues being in a CCR is on the y-axis and residue number along the x-axis. Window 28 is shown because it was the only window with a significant change in the number of predicted coiled coils. The COILS program predicted 5 CCR's in the wild-type L_2 (a) and 4 CCR's in the mutant (b). Residues with a probability of >50% were designated part of CCRs. The location of CCR1, 2, 3, 4, and 5 as predicted from the wild-type sequence is detailed in the text. Each set of peaks is labeled with the corresponding CCR designation.


(a)
window=28
1
0.8
0.6
0.4
0.2
0
CCR1
CCR2
CCR3
CCR4
CCR5
0
50
100
150
200
250
300
350
400
450


(b)
window=28
1
0.8
0.6
0.4
0.2
0
CCR1
CCR2
CCR4
CCR5
0
50
100
150
200
250
300
350
400
450

Chapter 5

Discussion

Erythrophore cell response to food-associated bacterial pathogens

The *Betta splendens* erythrophore cell response described here represents a unique class of optical cell-based biosensors with potential to improve current detection protocols by reporting on sample toxicity rather then structure presence or absence in a sample. Erythrophore cells respond to toxic agents, including pathogenic bacteria, by moving pigment organelles within the cell. Pigment movement can be dispersive or aggregative. Erythrophore cells respond by aggregating pigment organelles in the presence of *Bacillus cereus, Salmonella enteritidis, Clostridium perfringens* and *Clostridium botulinum*. This response varied with each pathogen ranging from rapid aggregation, to a slow aggregation with some cells remaining unchanged over time. By understanding the erythrophore cell response physiology to chemical toxicants as well as bacterial pathogens, additional applications for these cells can be defined.

The controls for this study included uninoculated bacterial culture medium (media control) and a nonpathogen, *Bacillus subtilis*. Erythrophore cells responded in a similar manner to both controls by slowly dispersing pigment organelles. This dispersion stabilized over time. Bacterial pathogens induced a varying degree of aggregation from erythrophore cells that can be easily distinguished from the uninoculated bacterial culture media. The difference in erythrophore cell response to the pathogenic bacteria compared to the controls, suggest that an aggregative response

is not media-dependent rather the response is bacterial-dependent. Additional experiments evaluating other model nonpathogens, as well as nonpathogenic variants of known pathogens would support this hypothesis.

Error bars on graphs are correlated to erythrophore cell population density and degree of fully dispersed cells. This variation was overcome by selecting similar erythrophore cell populations, but even if variation was large, the overall trend of the response can be easily distinguished from the media control and even the response induced by other bacterial cultures. Three replicates were conducted for each experiment, and each replicate utilized different erythrophore cell populations to eliminate the possibility of the observed response being an anomaly.

When erythrophore cells were exposed to *S. enteritidis* ATCC 4931, pigment aggregation was slow. Virulence factors of *S. enteritidis* are broadly defined but a main attribute of this bacterium is the ability to invade host epithelial cells. Identifying mutant bacterial gene products will help define the mechanism(s) by which this bacterial pathogen induces a change in pigment organelle location within erythrophore cells.

Clostridium perfringens SM101 cultures induced slow aggregation of erythrophore cells. The aggregative response induced by *C. perfringens* culture was only observed when bacteria were cultured in DS sporulation medium and a dispersive response was observed when bacteria were cultured in FTG vegetative medium. A major virulence factor of *C. perfringens* is production of CPE (*C. perfringens* enterotoxin) (46). CPE is produced during sporulation in the intestine and is released when the mother cell lyses following completion of sporulation (104). Erythrophore

cell response to *C. perfringens* MRS101, a derivative of *C. perfringens* SM101 lacking the potent enterotoxin CPE, failed to elicit an aggregative response from erythrophore cells. The lack of erythrophore cell response to a *cpe* knockout and the observation that only bacterial cultures grown in DS sporulation medium elicit a response, suggests that CPE may be involved in the aggregative erythrophore cell response. CPE toxin induces intestinal damage within 15 to 30 minutes by altering membrane permeability and disrupting ion transport. The aggregation response observed when erythrophore cells were exposed to *C. perfringens* SM101 occurred immediately after the addition of the culture and was complete within four minutes. If CPE is involved in erythrophore cell response, one would hypothesize that the effect observed on erythrophore cells may be similar to the effect on intestinal cells and would follow a similar mechanism of action. CPE binds to intestinal receptors belonging to the claudin family of proteins, followed by small complex formation, exposing CPE to the membrane surface. Subsequently, intermediate and large complex formation occurs, and finally CPE affects the plasma membrane permeability by inducing lesions in the membrane (96). The claudin family of proteins have been found to be important in the formation of tight junctions in epithelial and endothelial cells (129). It has been proposed that members of the claudin family confer permeability properties to epithelial cells (129). Erythrophore cells are isolated from the epithelial tissue, specifically fin and tail tissue of *B. splendens,* and while the movement of pigment organelles has been attributed to G-protein interactions, binding of claudin receptors could change the membrane permeability and induce pigment movement as well. Future studies testing this hypothesis include additional

experiments involving erythrophore cell exposure to *C. perfringens* MRS101 *cpe* - complemented *cpe* + strain, purified CPE toxin, and testing with known claudin binding proteins. These experiments would not only help to answer the question of if CPE is responsible for erythrophore aggregation, but would also contribute to the understandings of erythrophore cell physiology and pigment organelle movement.

Clostridium botulinum is the causative agent of botulism associated with the ingestion of the botulinum toxin, is a potential bio-weapon, and has recently become a very popular cosmetic treatment for smoothing moderate to severe frown lines (104). Botulinum toxin, one of the most toxic proteins known, prevents the release of the neurotransmitter acetylcholine, causing flaccid paralysis. Erythrophore cells exposed to *C. botulinum* NCTC 7272 (type A) or *C. botulinum* NCTC 7273 (Type B) failed to respond until one hour after the addition of the bacterial culture. Erythrophore cells morphology became very dendritic during this reaction. *Clostridium botulinum* NCTC 7272 culture filtrate induced an identical erythrophore cell response as the whole culture. This suggests that the factor responsible for erythrophore cell aggregation is secreted. Erythrophore cells exposed to the nonpathogen model *B. subtilis* failed to elicit an aggregative response. The erythrophore cell response observed was specifically associated with *C. botulinum* cultures not just a long exposure time to a bacterial culture. Current literature describing detailed culture conditions and optimal conditions for the production of botulinum toxin could not be found. This may reflect the current governmental restrictions because of the select agent status of *C. botulinum,* or patent and trade secrets for the production of BoTox. In 1965 Bonventre *et al.* conducted a quantitative study concerning the effects of pH and

temperature incubation on the growth, autolysis and toxin production of *C. botulinum* Type A and B. The pH of the culture medium did not affect synthesis of botulinum toxin, but the toxicity of culture filtrates (as observed using a maximum lethal dose in a whole animal model) was dependent on a narrow pH and corresponded to the optimum condition for autolytic enzymes to function. Culture growth was maximal at 24 hours with maximum toxin synthesis (correlated by lethal dose) varying between 24 hours and 48 hours (22). The current standard method for determining toxicity of *C. botulinum* cultures and botulinum toxin utilizes the mouse model. This was not conducted in parallel with erythrophore cell response assays, but a hypothesis can be formed using the data presented in this study and the data from Bonventre *et al.* A similar erythrophore cell response was observed for whole culture or *C. botulinum* filtrates at 24 hours and 72 hours. This correlates well with reported toxin synthesis time line (maximal between 24 and 48 hours). While erythrophore cell response cannot be directly attributed to botulinum toxin, the hypothesis for this interaction is that erythrophore cells respond to a secreted, stable, *C. botulinum* product, which may include botulinum toxin or autolytic enzymes of the cell.

The observation of a slow response time and unique erythrophore cell morphology to *C. botulinum* suggests that bacterial pathogens are interacting through different mechanisms and differences in the kinetics of erythrophore cell response may provide another means to measure and characterize different bacterial species.

Erythrophore cell response to members of the Bacillus cereus group

The *Bacillus cereus* group, also known as *B. cereus sensu lato,* comprises six recognized species: *B. cereus sensu stricto* (*B. cereus*)*, B. anthracis, B. thuringiensis, B. mycoides, B. pseudomycoides,* and *B. weihenstephanesis* (112). Within this group, *B. cereus*, *B. thuringiensis,* and *B. anthracis* have been given the most attention due to their economic and social importance. *Bacillus thuringiensis* has been traditionally used as a biocontrol agent for insects in crop production. It is distinguished from *B. cereus* by the production of insecticidal δ-endotoxins. *Bacillus anthracis* is the causative agent of anthrax which can be fatal to animals and humans. Most recently, the bacterium has been adapted as a biological weapon. *Bacillus cereus* is an opportunistic pathogen that is most often associated with gastrointestinal disease, but can also cause several non-gastrointestinal diseases in susceptible populations. Members of the *B. cereus* group have come under scrutiny because of reports of *B. thuringiensis* isolates associated with gastrointestinal illness and *B. cereus* isolates associated with an anthrax like illness (66, 104). The traditional means of differentiating these three species is based on plasmid populations and pathological properties. As more genomic information has become available it is now recognized that these species are closely related in both gene content and synteny.

Bacillus cereus is an opportunistic pathogen and has been linked to gastrointestinal infections and numerous nongastrointestinal infections including, osteomyelitis, pulmonary and wound infections, bacteremia, and septicemia (76, 132). *Bacillus cereus* is the causative agent of two forms of foodborne illness, emetic and diarrheal. The emetic syndrome is associated with intoxication of the heat stable

emetic toxin cereulide. The diarrheal syndrome is associated with toxicoinfection and vegetative cells producing heat labile enterotoxin(s) in the intestine. Erythrophore cells exposed to a gastrointestinal outbreak strain, *B. cereus* ATCC 49064, responded by rapidly aggregating their pigment organelles to the center of the cell. This aggregation rate was considerably faster then the rate observed from the other bacteria evaluated. Thus, *B. cereus* was selected for further evaluation.

A set of well known and genetically characterized *B. cereus* group members were selected to determine if erythrophore cell response was correlated with the ability to cause disease, or attributed to a conserved genetic factor of this species. Erythrophore cells exposed to *B. cereus* ATCC 10987 (dairy isolate), *B. cereus* UW85 (biocontrol agent), *B. cereus* ATCC 14579 (laboratory reference strain), *B. thuringiensis subsp. kurstaki* BGSC 4D1 (biocontrol agent), and *B. cereus* NCTC 11143 (emetic strain) aggregated their pigment organelles. This response was delayed compared to the response induced by *B. cereus* ATCC 49064. *Bacillus cereus* NCTC 11143 produces cereulide, and to deduce the potential involvement of this heat stable toxin, cultures were heat treated to destroy heat labile enterotoxin(s). Erythrophore cells failed to respond to the heat treated *B. cereus* NCTC 11143 culture. This result suggests that erythrophore cells are not sensitive to the heat stabile emetic toxin cereulide, rather are responding to heat labile factor(s) which could include one of many virulence factors, such as enterotoxins. Interestingly, a heat stable factor other then cereulide was identified in the diarrhoeagenic *B. cereus* ATCC 49064 culture. This heat stable factor was not present in any other *Bacillus* strain tested, but seems to contribute to the faster erythrophore cell response induced by *B. cereus* ATCC 49064.

This study was unable to determine the identity of this heat stable factor. Current experiments are being conducted to identify this novel heat stable factor. My hypothesis is that this factor could be an uncharacterized signaling molecule, a known protein with altered heat stability, or a heat stabile bacteriocin.

The similar erythrophore cell response curves induced by *B. cereus* ATCC 10987, *B. cereus* UW85, *B. cereus* ATCC 14579, and *B. thuringiensis subsp. kurstaki* BGSC 4D1 suggest that erythrophore cell response may have great potential for use in the food industry to detect *B. cereus* group members as well as other food-associated pathogenic bacteria. Important parameters of this detection system are the detection limit and the ability to access risk. *Bacillus cereus* ATCC 49064 was isolated from meatloaf during an outbreak of gastroenteritis and was chosen as the model organism for evaluating the detection limit of erythrophore cells (133). As stationary phase bacterial cultures were diluted, the rate of erythrophore cell aggregation slowed, and the final change in pigment area decreased. This was observed for bacteria cultured in LB or BHI media. The number of bacteria required to cause erythrophore cell aggregation was >6 x 10^6 CFU when cultured in BHI medium and >4 x10^6 CFU when cultured in LB medium. Previously, it was suggested that the number of *B. cereus* ATCC 49064 cells required to elicit erythrophore response was 10^6 CFU when cultured in BHI and 10^4 CFU when cultured in LB medium (8). Review of the work conducted by Austin *et al.* suggest that the different results could be attributed to inconsistencies of culture age, lack of replicate experiments and varying culture techniques. The experimental procedures described in this dissertation were designed to take into account the above inconsistencies. Another contributing factor to the

differences reported in detection limit particularly with cells cultured in LB medium include how the secondary culture was started. The previous study reported that the secondary culture was started using a 1:100 dilution of an overnight culture. Erythrophore cells respond to as little as two µl of stationary phase *B. cereus* ATCC 49064 culture, corresponding to ~ two µg of crude toxin. It is possible that this dilution resulted in enough carry over from the starter culture to skew results. Indeed it was observed that when a 1:1000 dilution was used to inoculate the secondary culture the detection limit was higher, but was also very reproducible. Both studies enumerated the number of bacterial cells using traditional plating techniques. The number of bacterial cells added in each experiment was not known until the next day. Use of an automated cell counter would provide a definitive answer to the detection limit.

When evaluating *B. cereus,* it is important to not only evaluate viable cell numbers, but also evaluate toxicity potential (the risk of illness as a result of toxin production). *Bacillus cereus* foodborne illness is directly associated with production of toxins, while quantification of bacteria or presence of DNA does not always suggest the potential to cause disease. Toxin assays using mammalian cell culture assays have been developed to assess biological activity of *Bacillus* spp. (14, 21, 56, 69). The majority of these cell-based toxicity assays relay on metabolic activity of cultured cell lines. These cells lines include CHO, Vero and HEp-2 cells. These assays can be time consuming ranging from 24 to 48 hours. Recently, Gray *et al.* described an assay utilizing Ped-29 cells (56). Cytotoxicity results were determined using an alkaline phosphatase release assay, and test results were complete within one hour. The assays

utilizing mammalian cell culture described above rely on *Bacillus* culture supernatant rather than whole culture. Erythrophore cell response represents a novel *Bacillus* cytotoxicity assay that is rapid with results within 10 minutes. The sample requirements are not limited to supernatant, and the detection limit is within the infectious dose of 10^5 – 10^7 total cells. Preliminary studies evaluating crude toxin preparations suggest that the detection limit for erythrophore cells is similar to that of the Ped-29 cytotoxicity assay reported by Gray *et al*. Additional studies need to be conducted to understand the applications of erythrophore cells as a *Bacillus* cytotoxicity assay. It would also be extremely valuable to conduct a more extensive evaluation of *Bacillus* spp. isolates to determine applications and limitations of this model.

Erythrophore cell detection relies on a primary cell-culture which includes the collection of pigment cells from commercially available *B. splendens*. This introduces a high degree of variability in (i) the genetic background of each fish; (ii) the health and age of the fish; (iii) the types of erythrophores present; and (iv) variations between erythrophore cell populations when cultured *in vitro*. The most significant of these variables is isolating consistent erythrophore cell populations. Some erythrophore cell populations move easily from the animal model to *in vitro* cell culture. The variation between successful and unsuccessful erythrophore cell isolation has is not understood. The development of an erythrophore cell line will address many of these issues, and will also help to standardize erythrophore cell response.

Factors involved in erythrophore cell response

The last objective of this study was to determine what factor(s) are involved in eliciting an erythrophore cell response. The results of this study demonstrate that erythrophore cells isolated from *B. splendens* respond to several members of the *B. cereus* group, thus providing evidence that this would be a strong model organism. Erythrophore cells respond to *B. cereus* culture supernatant in an identical manner as to whole bacterial culture, suggesting that erythrophore cells are interacting with a factor secreted outside of the bacterial cell. It was also observed that culture age was important to induce rapid erythrophore cell response. Overnight *B. cereus* ATCC 49064 cultures induced a similar aggregation rate as sample taken during late stationary phase. It was observed that cultures leaving exponential phase induced erythrophore cell aggregation, but this was at a slower rate when compared to the response induced by overnight cultures.

The nonpathogenic, vaccine strain, *Bacillus anthracis* 34F2 ($pXO1^+$ $pXO2^-$), and *B. cereus* ATCC 14579 Δ*plcR* mutant failed to elicit a response from erythrophore cells. Both strains fail to express a functional PlcR. Within *B. anthracis*, the *plcR* gene contains a nonsense mutation resulting in the expression of a non-functional PlcR (2, 102). *Bacillus cereus* ATCC 14579 Δ*plcR* has an insertion which inactivates the *plcR* gene (115). The transcriptional regulator PlcR (Phospholipase C Regulator) controls the expression of many secreted virulence factors in *B. cereus* group members, and is required for virulence in *B. thuringiensis* and *B. cereus* (115). Initial identification and characterization of PlcR was done by Lereclus and coworkers by analyzing the expression of the *plcA* gene, encoding phosphatidylinositol-specific phospholipase C

(PIPLC). From this analysis they determined that transcription starts at the onset of stationary phase and requires a trans activator encoded by the *plcR* gene (84). *plcR* transcription is autoinduced and is repressed by the sporulation factor Spo0A (83, 84). PlcR also requires the short peptide PapR. PapR is exported out of the cell, processed into the active peptide and then imported through the oligopeptide permease system OppABCDF (53, 121). Transcription of *plcR* starts before the onset of stationary phase, and reaches a plateau two hours later (83). Samples taken from an actively growing culture of *B. cereus* ATCC 49064 failed to induce erythrophore cell aggregation until the culture reached an OD_{600} >1. The aggregation rate at this time was slow, taking up to 10 minutes to complete whereas cultures taken just after the onset of stationary phase and throughout stationary phase induced rapid aggregation of pigment organelles. The slower aggregation rate did not appear to be bacterial cell number dependent.

plcR is not expressed in *B. cereus* or *B. thuringiensis* cells growing in sporulation specific medium, but is expressed when cells are growing in a rich medium such as LB (83). Cultures grown in Nutrient Broth, while not the same as sporulation medium, failed to cause the rapid and complete aggregation of erythrophore cells cultured in a rich medium. This convincing evidence suggests that erythrophore cells are responding to a PlcR-regulated factor, produced by *B. cereus*, and that this response is temporal as well as medium-dependent.

PlcR regulates the transcription of 45 known genes, many of which are secreted into the extracellular medium. PlcR-regulated genes include phospholipases, proteases, toxins, bacteriocins, and transporters (52). Hemolysin BL (HBL),

nonhemolytic enterotoxin (Nhe) and cytotoxin K (CytK) have been implicated as the virulence factors involved in the diarrheal form of *B. cereus* foodborne illness (15, 87, 88). The failure of *B. cereus* ATCC 14579 Δ*plcR* and *B. anthracis plcR* - to induce erythrophore cell aggregation led to the hypothesis that a PlcR-regulated virulence toxin(s) is involved in erythrophore cell response. To test this hypothesis, *B. cereus* ATCC 49064 mutants were isolated that induced a delayed aggregation rate compared to the wild-type.

Genomic analysis followed by complementation of the mutant JH1 identified a single nucleotide substitution in *hblC* at position 864 resulting in a guanine to adenine conversion. This nucleotide change resulted in an alanine to threonine conversion at residue 289 in the protein L_2 of the HBL toxin complex. HBL is hemolytic, cytotoxic, dermonecrotic, alters vascular permeability, and has been implicated as a factor contributing to *B. cereus* diarrheal illness (15-17). HBL is composed of B, L_1 and L_2 toxin components encoded by the genes *hblA*, *hblD*, and *hblC* respectively. The mechanism of action for HBL has not been determined, but it is hypothesized that red blood cell lysis initiates with independent binding of the three toxin components to the membrane, assembly into a membrane attack complex, and formation of a transmembrane pore (38).

Maximal hemolytic activity appears to occur when the concentration of either L_1 or L_2 is lower then the concentration of B (19). Gilios *et al.* reported that L_2 and B appeared in the supernatant at the same time and their concentrations peak two hours later. However, after this time point the concentration of B decreased while the concentration of L_2 did not decrease for the remainder of the study (51). The half-life

for L_2 was reported as 7.3 hours and B was 5.05 hours. No data was presented on the concentration or half-life of L_1. Future studies evaluating the half-life and protein concentrations of B, L_1 and L_2 toxin components in the JH1 mutant could provide information on the function of L_2. The results presented in this dissertation suggest that the HBL toxin component is involved in erythrophore cell response, and that a misformed complex fails to induce erythrophore cell pigment aggregation. This observation suggests that erythrophore cell response may have more applications then initially proposed, and shows potential as a tool to understand the mode of action for the HBL toxin complex.

L_2 wild-type protein sequence was queried against the NCBI-nr database (as of May 3, 2009) using TBLASTN at the National Center for Biotechnology Information (NCBI) website http://www.ncbi.nlm.nih.gov/blast. Results of BLAST confirmed that HBL is a unique protein specific to the *B. cereus* group of organisms. The top 17 hits had complete coverage across the protein sequence. The first 16 hits had an E value of 0.0, and 17^{th} hit E value was 2e-135. All subsequent hits were partial matches to the N-terminal region of the protein. The top 17 hits were used for a ClutalW alignment; the results of this alignment suggest that amino acid position 289 is conserved within the sequences analyzed and that this residue and protein region could be important for protein function.

The B and L_1 components of HBL have predicted transmembrane sequences, but no structural predictions have been made for the L_2 component (55). The COILS prediction software was used to predict coiled coils in the wild-type (*B. cereus* ATCC 49064) and mutant (JH1) L_2 protein sequence. Wild-type L_2 is predicted to have five

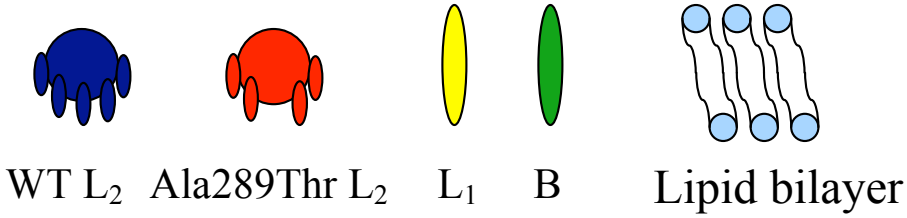
CCRs. Four CCRs are predicted in the mutant protein. From this, I hypothesize that a coiled coil region of L_2 is involved in initiating erythrophore cell aggregation. The coiled coils observed could be involved in facilitating protein-protein interactions and perhaps involved in receptor binding/recognition and/or stabilizing the transmembrane structure formed by B and L_1 components.

As discussed previously, the host receptor(s) and the mechanism of toxicity of HBL is not well known. HBL and Nhe show a high degree of sequence homology to one another, but not to any other protein family. The crystal structure of the B component of HBL was recently characterized, and while at the sequence level there was little homology towards any other known protein family, at the tertiary level the B component resembles the pore-forming toxin cytolysin A (ClyA) (92). This observation implies that HBL, Nhe, and ClyA are members of a new superfamily of toxins (41). ClyA also known as HlyE or SheA, is a hemolytic and cytotoxic monooligomeric protein toxin expressed by *Escherichia coli, Shigella flexneri and Salmonella enterica* serovars Typhi and Paratypi A (7). The mechanism of ClyA toxicity is thought to occur by osmotic lysis through the formation of transmembrane homo-oligomeric pores (41). This is similar to the proposed model of red blood cell lysis mediated by HBL. Red blood cell lysis initiates with independent binding of each of the HBL components to the cell membrane. Cell lysis is caused by the formation of a membrane attack complex and the formation of transmembrane pores as illustrated in Figure 15B. The role of L_1 and L_2 toxin components are not known. The observation that there are several coiled coil regions in the amino acid sequence of the L_2 toxin component, suggests that this protein could be involved in protein-protein

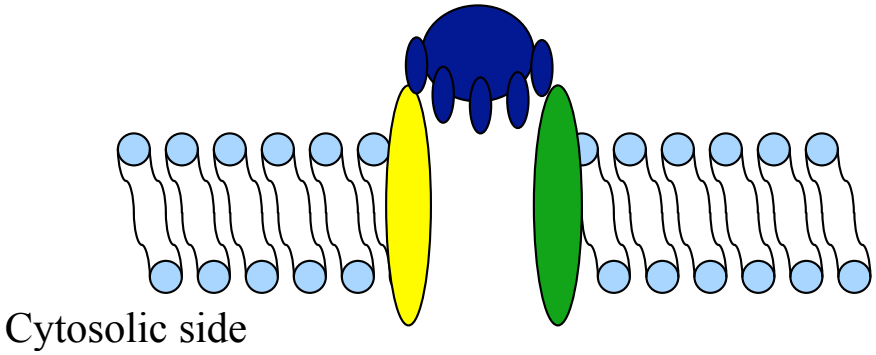
interactions. I propose that the L_2 toxin component is involved in receptor binding or stabilization of the membrane attack complex, and residue 289 is important for this function (Figure 15C). It is unclear how HBL toxin components are interacting with erythrophore cells, but the rapid pigment aggregation suggests that this could be a receptor binding event rather then formation of a membrane attack complex.

Figure 15. Model for the mechanism of HBL cell lysis and erythrophore cell aggregation. **(A)** Diagram key illustrating the toxin components L_2, L_1, and B and the lipid bilayer. Wild-type L_2 (WT L_2) is illustrated with 5 prongs representing the 5 coiled coil regions. The mutant L_2 protein (Ala289Thr L_2) is illustrated with 4 prongs representing the 4 coiled coil regions as predicted by COILS. L_1 and B components have predicted transmembrane segments involved in the pore structure. **(B)** L_1 and B components bind to an unknown receptor and form a transmembrane structure that is stabilized by the WT L_2 protein allowing pore formation in red blood cells and rapid erythrophore cell aggregation. **(C)** L_1 and B components form the transmembrane structure but Ala289Thr L_2 toxin component cannot stabilize the complex resulting in delayed erythrophore cell aggregation and potentially slower pore formation. It is unknown if the erythrophore response is due to pore formation or some other interaction with the HBL toxin components. Illustrations are not drawn to scale; stoichiometry is unknown so only one of each of the toxin components was used.

A

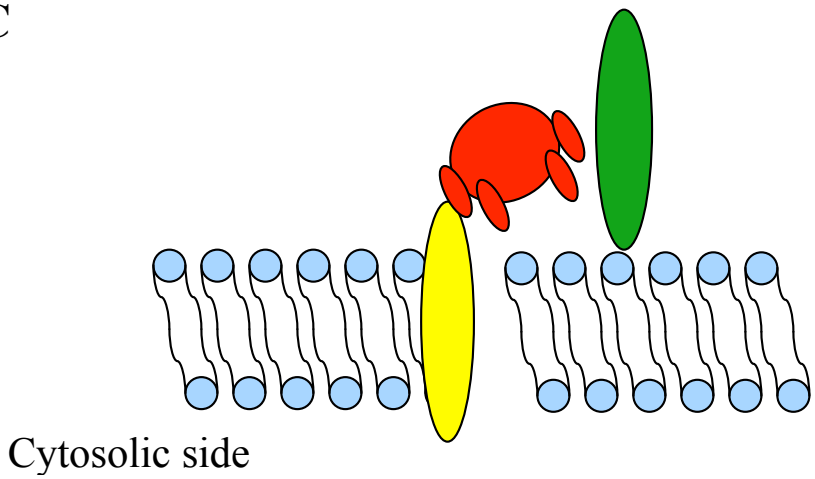


B



Pore formation

C



Delayed pore formation

The single amino acid change in L_2 did not completely abolish the ability for this bacterium to cause pigment aggregation in erythrophore cells. Thus, additional PlcR-regulated factors must be involved in this response. Toxin producing ability varies among members of the *B. cereus* group (47, 79, 105). Moravek *et al*. analyzed 100 *B. cereus* strains. Molecular characterization demonstrated that 42% of the strains harbored the genes for HBL and 99% for Nhe. The production of Nhe and HBL components was found to range from 0.02 to 5.6 μg mL^{-1} for HBL-L_2 and from 0.03 to 14.2 μg mL^{-1} for NheB (105). Of the strains tested in this dissertation, *B. cereus* ATCC 49064 and *B. cereus* ATCC 14579 have genes encoding HBL and Nhe toxins, but these two strains induced different rates of aggregation from erythrophore cells. Of particular interest is the observation that JH1 induces a very similar response from erythrophore cells as *B. cereus* ATCC 10987 which lacks genes encoding HBL. This observation provides additional evidence that HBL is not the only factor involved in erythrophore cell aggregation, and that variation in toxin production maybe important. It has been demonstrated that the cytotoxicity of *B. cereus* varies greatly, this has been attributed to mutations in the *plcR* gene (122). While it is unclear why the majority of *B. cereus* isolates tested here induce a similar erythrophore cell aggregation, one could hypothesize that this is correlated to the high degree of genetic relatedness, and those that deviated from the group could fall into the class of isolates with an altered *plcR* sequence.

Future Directions

Betta splendens erythrophore cells used in this study are a primary tissue culture line. Working with these cells requires isolation of erythrophore cells prior to each experiment. Reports suggest that primary cell lines are more sensitive than immortalized cell lines, but the development of an immortalized erythrophore cell line may overcome potential limitations because it would decrease preparation time and remove fish-to-fish variation (11). Erythrophore cells detect several different types of functionally active bacteria. In this regard, erythrophore cell-based detection is not limited to a specific group of bacterial pathogens and may represent a universal detection system. Future studies should address field applications for erythrophore cells-based detection. Specifically, which pathogenic bacteria can be monitored, the appropriate sample compositions, and methods for rapid field analysis. There are several reports utilizing chromatophore cells to detect chemical and biological toxicants, standardization of analysis, particularly how images are analyzed will help to understand the full potential of these cells. Additionally, continued identification of the bacterial factors involved in erythrophore cell response will increase our understanding of how these factors interact with erythrophore cells providing a model to study toxin activity and bacterial-host interactions.

Chapter 6

Conclusions

In the current study, the following research questions were asked: do erythrophore cells respond to bacterial pathogens, specifically *Salmonella enteritidis, Clostridium perfringens*, *Clostridium botulinum,* and *Bacillus cereus*? If so, is this response distinguishable from the media control? Can erythrophore cells respond to various members of the *B. cereus* group? What is it about the bacterial pathogen *B. cereus* that elicits a rapid aggregative response from erythrophore cells?

To address the first two questions, erythrophore cells were exposed to selected food-associated bacteria *S. enteritis, C. perfringens, C. botulinum,* and *B. cereus*. The observed response was statistically different from the respective media control using an end point paired T-test. Erythrophore cell response to *S. enteritidis* ATCC 4931 and *C. perfringens* SM101 caused slow aggregation over 20 minutes that stabilized before pigment became concentrated at the center of the erythrophore cell. *Bacillus cereus* ATCC 49064, the causative agent of a gastroenteritis outbreak, caused a rapid and complete aggregation of aggregated pigment organelles; aggregation was complete within 3 minutes and remained unchanged for the duration of the experiment. When erythrophore cells were exposed to *C. botulinum* NCTC 7272 and *C. botulinum* NCTC 7273 pigment aggregation initiated 1 hour after the addition of the bacterial culture and was complete within 4 hours, this was observed for both isolates. Interestingly, erythrophore cells exposed to *C. botulinum* had a dendritic morphology that was distinct from the morphology after exposure to the other bacterial pathogens. The

observation of specific erythrophore cell morphology in response to specific bacterial pathogens, as well as differences in the kinetics of erythrophore cell response, suggests that bacterial pathogens may interact with erythrophore cells through different mechanisms.

The graphical and statistical evidence presented here suggests that erythrophore cells are responding to a bacterial-dependent rather then bacterial culture-dependent factor. With these results an intriguing question then arises, do erythrophore cells respond to nonpathogenic bacteria in a similar manner as bacterial pathogens? Erythrophore cells are unable to distinguish the nonpathogenic bacterial strain, *Bacillus subtilis* 1A1 from the media control, BHI medium. This evidence supports the conclusion that erythrophore cells can respond to select food-associated pathogenic bacteria in a manner that can be easily distinguished from a nonpathogen or to the media control.

The rapid erythrophore cell response induced by *B. cereus* ATCC 49064 was very interesting because this response was unique compared to the kinetics of the other bacterial agents tested. *Bacillus cereus* group members including *Bacillus thuringiensis*, *Bacillus anthracis*, and additional *B. cereus* isolates were investigated to determine if this response was unique to outbreak strain or conserved among members of this group. Erythrophore cells failed to respond to *B. anthracis* 34F2 but responded by aggregating pigment organelles when exposed to *B. cereus* ATCC 10987, *B. cereus* UW85, *B. cereus* ATCC 14579, *B. thuringiensis subsp. kurstaki* BGSC 4D1, and *B. cereus* NCTC 11143. *Bacillus cereus* NCTC 11143 produces the heat stable toxin cereulide responsible for the emetic form of *B. cereus* food-poisoning. Erythrophore

cells failed to respond when exposed to heat treated *B. cereus* NCTC 11143 culture suggesting that the observed aggregation response was associated with a heat labile factor produced by members of the *B. cereus* group. These data suggest that there seems to be a conserved factor found in *B. cereus* and *B. thuringiensis* strains that is not produced by *B. anthracis*.

What is it about a bacterial pathogen, such as *B. cereus*, that causes erythrophore cells to respond by rapidly relocating pigment organelles to the center of the erythrophore cell? I hypothesized that a toxin or virulence factor is involved in erythrophore cell response. The failure of erythrophore cells to respond to the nonpathogenic *B. cereus* Δ*plcR* mutant strain and *B. anthracis*, which lacks a functional *plcR* gene, provides insight to this intriguing question. PlcR, a pleiotropic transcriptional regulator, controls the expression of numerous extracellular compounds, including several virulence factors and toxins (2, 115, 121). The data presented here infers that one or more PlcR-regulated gene products are required to induce erythrophore cell aggregation. Two additional observations support the involvement of PlcR-regulated virulence factors in erythrophore cell response. First, PlcR is maximally expressed at the end of vegetative phase and is maximal 2 h after the onset of stationary phase (2, 83, 84, 115). Only cultures of *B. cereus* that completed exponential growth and were in stationary phase induced erythrophore cell aggregation. Secondly, most of the PlcR regulated gene products have a Sec type signal peptide, imparting the ability to export PlcR-regulated virulence factors to the outer surface of *B. cereus* (109). Supernatant of spent culture medium that had

stationary phase *B. cereus* cells removed induced erythrophore cells to aggregate in a manner identical to whole culture.

Hemolysin BL (HBL) is a PlcR-regulated, membrane-lytic toxin produced by *B. cereus* group members. HBL is composed of a binding component, B, and two lytic components, L_1 and L_2 (17). This work describes the isolation of a mutation in the *hblC* component of HBL. This mutation results in a single amino acid substitution, Ala289 to threonine in the lytic component L_2. The bacterial mutant was no longer able to induce rapid pigment aggregation from an erythrophore cell population. Complementation of this gene returned the wild-type aggregation rate. The amino acid substitution observed in L_2 did not cause complete loss of aggregation of erythrophore cell pigment, suggesting that this change may have only diminished HBL toxin activity, or that an additional PlcR-regulated factor is involved in erythrophore cell response. While HBL is a major toxin of *B. cereus* group members, the mechanism of toxin activity is not well understood. With a better understanding of the mode of action it will become clear how HBL is involved in pigment aggregation of erythrophore cells. The observations presented here support the hypothesis that stationary phase *B. cereus* secretes certain PlcR-regulated virulence factors, such as toxins, that are responsible for inducing pigment aggregation in erythrophore cells.

In conclusion, results from this study have contributed to the understanding of erythrophore cell response to the food-associated pathogens *S. enteritis, C. perfringens, C. botulinum,* and *B. cereus*. Erythrophore cell response could not distinguish the nonpathogen model, *B. subtilis*, from the media control suggesting that factors involved in pathogenicity induce erythrophore cell change. The work described

here highlights the potential for erythrophore cell response to be utilized as a novel method to detect food-associated pathogenic bacteria and distinguish them from nonpathogenic bacteria. Analysis of members of the *B. cereus* group demonstrated that erythrophore cells respond to a secreted, PlcR-regulated, heat labile factor produced at the end of exponential growth phase. The protein L_2, belonging to the HBL toxin complex was found to be involved with inducing erythrophore cell aggregation, but it is unclear if additional PlcR-regulated gene products are also involved. The discovery that a single amino acid substation in L2 of *B. cereus* ATCC 49064 results in a delayed erythrophore cell response provides a new direction to investigate toxin activity and bacteria-host interactions. Conducting future studies to identify additional *B. cereus* factor(s) involved in erythrophore cell response, as well as analysis of additional bacterial pathogens, will contribute to our understanding the biology of these cells and how we may exploit this response as a detection method and as a model to study toxicity as it relates to bacteria-host interactions.

Chapter 7

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APPENDIX

Biographical Information

Janine Roberta Hutchison was born April 20, 1981 in Riverton, Wyoming to Robert H. and Daphne Y. Hutchison. She was raised in Dubois, Wyoming. After graduation from high school, she moved to Riverton, Wyoming where she earned an A.S. in Biological resource management at Central Wyoming College in May 2001. She was employed at ACE hardware while attending Central Wyoming College. She then transferred to Idaho State University in Pocatello, Idaho where she earned a B.S. in biochemistry and a B.S. in microbiology in May 2004. She worked at ACE hardware and outdoor sports in Pocatello as a hardware department manager and a general manager. During the summer of 2004 she was an Idaho BRIN fellow at Idaho State University in Pocatello, Idaho. Fall term, 2004, she began graduate training in microbiology in the laboratory of Dr. Janine E. Trempy at Oregon State University in Corvallis, Oregon. She was the recipient of a Department of Homeland Security pre-doctoral fellowship from Fall 2005 through Summer 2008.

Publications

Hutchison, J. R., Dukovcic, S.R., Dierksen, K.P., Carlyle, C.A., Caldwell, B. A., and Trempy, J.E. 2008. Erythrophore cell response to food-associated pathogenic bacteria: Implications for detection. *Journal of Microbial Biotechnology*. **1:**425-431.

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Abstracts

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