AN ABSTRACT OF THE THESIS OF

Melissa J. Austin for the degree of Master of Science in Microbiology presented on November 18, 2005.
Title: Analysis of *Betta splendens* Erythrophore Responses to *Bacillus cereus* Cultured in Different Media.

Abstract approved:_____

Janine E. Trempy

Biological agents and their products commonly cause foodborne illnesses. In the United States it is estimated that there are over 76 million cases of foodborne illnesses each year, resulting in an economic loss of approximately $40 billion. These high figures demonstrate the need for bioassays that display a rapid and accurate response to biologically active agents such as the products produced by pathogenic bacteria. Current detection techniques consist of selective enrichment plating, which requires culturing time, or molecular probing techniques, that detect the presence of an agent, but not its actual biological toxicity. A rapid assay that detects biologically active agents would provide a superior method of pathogen detection and could act as a warning of environmental contamination. One pathogen of concern, *Bacillus cereus*, is ubiquitous in the environment and most notably known for causing food poisoning.

This research describes a chromatophore-based biosensor from *Betta splendens* (Siamese fighting fish), which can detect *B. cereus* at levels at or below the infectious dose needed to cause food poisoning. Chromatophores are pigmented cells found in cold-blooded animals that react physiologically to biologically active agents by altering their optical density. The red-pigmented chromatophores, or erythrophores, were used to determine the response limit to *B. cereus*, and the response patterns to *B. cereus* grown in different media. This

research found the lower limit of detection of *B. splendens* erythrophores for *B. cereus* cultured in Luria Bertani broth to be 10^4 viable *B. cereus* cells per milliliter. This sensitivity is equivalent to that expected of the sensitivity pertinent to food safety standards. Erythrophore responses to *B. cereus* cultured in Brain Heart Infusion broth supplemented with glucose support the hypothesis that culturing in this fashion results in maximally expressed enterotoxins. These results establish Brain Heart Infusion broth supplemented with glucose as the preferred medium for use in *B. cereus* gene expression analyses that assess enterotoxin regulation and function.

Analysis of *Betta splendens* Erythrophore Responses to *Bacillus cereus* Cultured in Different Media.

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APPROVED:

_____

Major Professor, representing Microbiology

_____

Chair of the Department of Microbiology

_____

Dean of the Graduate School

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

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Melissa J. Austin, Author

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INTRODUCTION AND LITERATURE REVIEW

Foodborne Illness

Foodborne illnesses result from the ingestion or consumption of contaminated beverages and food products. The contamination can be due to poisonous chemicals, but more commonly microbes and their toxins are the culprit. Over 200 foodborne illnesses have been characterized, of which most are caused by bacteria, viruses and parasites (4, 8).

In general, most foodborne illnesses go unreported; and of those reported, frequently the illness-causing agent is never identified. In the United States it is estimated that there are over 76 million cases of foodborne illness, 325,000 hospitalizations, and 5,200 deaths each year (4). The United States' estimated economic loss per year is between $20 billion and $40 billion. In actuality, the total disease burden for foodborne illnesses is not known nor is the total economic impact (75). As an example, the Centers for Disease Control and Prevention (CDC) estimates that for every case of salmonellosis diagnosed and reported, there are 38 undiagnosed or unreported cases (4).

In order to improve the accuracy of foodborne illness statistics, Foodborne Diseases Active Surveillance Network (FoodNet) was developed. FoodNet is part of the Emerging Infections Program (EIP) and is a collaborative project between ten EIP sites, the CDC, the United States Department of Agriculture's (USDA) Food Safety and Inspection Service (FSIS) and the Food and Drug Administration (FDA). Since its beginning in 1996, FoodNet has conducted surveys and performed epidemiological studies to provide national estimates and trends regarding foodborne illnesses. The goal of FoodNet and its affiliates is to identify, control and prevent risks associated with foodborne illnesses (5). This network focuses on the following nine severe foodborne disease-causing agents:

Campylobacter spp., *Escherichia coli* O157:H7, *Listeria monocytogenes*, *Salmonella spp*., *Shigella spp*., *Vibrio spp*., *Yersinia spp*., *Cryptosporidium*, and *Cyclospora*. Other common foodborne pathogens like *Bacillus cereus*, *Clostridium spp.,* noroviruses, and *Giardia lamblia* are not monitored at this time. As of 2004, 44.1 million people (15.2% U.S. population) were under the surveillance of FoodNet (7).

For additional safety in the food industry, the FDA created regulations termed Hazard Analysis and Critical Control Points (HACCP) to help eliminate hazards in the food industry that contribute to foodborne illnesses. HACCP safety regulations were applied to the seafood industry in 1995 and the juice industry in 2001 (3). In 1998 the USDA extended the regulations to meat and poultry processing, which later resulted in a decline of *E. coli* O157 infections (75). These regulations are continually extended to different food industries, with the future goal of all industries, small and large, in compliance.

Foodborne illnesses have been a problem in the past and will only continue to be a problem as long as poor sanitation, improper food preparation and improper storage practices continue. Health factors less in our control are the emergence of new pathogens and an increasing susceptible population of more than 30 million individuals (75).

Bacillus cereus Group

Of the organisms that cause foodborne illnesses, *Bacillus cereus* Group *spp*. are troublesome because they are ubiquitous in the environment and therefore their presence cannot be completely eliminated. However, their bacterial numbers can be kept low enough in food products to prevent illness.

Phylogenetically, the *Bacillus cereus* group consists of: *B. thuringiensis*, *B. anthracis*, *B. mycoides*, *B. pseudomycoides, B. weihenstephanensis,* and *B. cereus*. All *B. cereus* group species are Gram positive, spore-forming, aerobic bacilli that are ubiquitous in the environment. These organisms can be found naturally in

water, air, soil and soil-grown vegetables and grains (10). *B. anthracis* is the causative agent of anthrax. *B. anthracis* is the species of most concern because it causes an acute and often fatal disease and has the potential to be used in biowarfare. *B. thuringiensis* possesses insecticidal toxins and is commonly used as an insecticide (39). *B. mycoides* and *B. pseudomycoides* are nonpathogenic and are differentiated by their rhizoid colony morphology. *B. weihenstephanensis* is known most notably for its psychrotolerance (15). *B. cereus* is a human pathogen associated with a broad range of symptoms including dermonecrosis, necrotic enteritis, endophthalmitis (20), and food poisoning (66).

Overall, the species compromising the *B. cereus* group exhibit a high degree of genetic similarity. The differences arise when additional virulence factors are acquired from plasmids in the environment or through horizontal transfer (39). In general, the 16S rRNA (ribosomal ribonucleic acid) amongst the *B. cereus* group exhibits greater than 99% sequence similarity (13, 22, 39). Therefore, all species in the *B. cereus* lineage have the potential to become pathogenic and should be analyzed for in outbreak investigations (22).

Genetically, *B. thuringiensis, B. anthracis* and *B. cereus* are the same species. What distinguishes *B. thuringiensis* from the other *B. cereus* group *spp*. is the presence of larvicidal crystal protein toxins (Cry), which give it insecticidal properties (39, 46). Since the genes encoding the crystal proteins are found on plasmids, the loss of these plasmids result in a *Bacillus sp*. indistinguishable from *B. cereus*. *B. thuringiensis* is also potentially enterotoxic because its genome contains many genes for the same virulence factors that cause disease in *B. cereus* food poisoning infections (66).

B. anthracis contains virulence factors on its two plasmids (pXO1, pXO2), without which it becomes indistinguishable from *B. cereus*. The unique factors remaining are that most *B. anthracis* strains are penicillin-sensitive, and lack hemolytic and lecithinase activity; whereas *B. cereus* is penicillin-resistant and exhibits hemolytic and lecithinase activity (39). In addition, the 23S rRNA of *B.*

cereus and *B. anthracis* shares 99.9% sequence similarity and the 16S rRNA between all *B. cereus* group species is greater than 99% similar (14, 22).

B. cereus is an important bacterium in regards to food safety as it is the etiological agent for two types of food poisoning, the diarrheal and the emetic types (48). *B. cereus* emetic-type food poisoning is considered an intoxication because the disease is caused by toxins the bacilli produce while growing in food products. The diarrheal-type is considered an infection as it is caused by the cell growth and toxin secretion of the bacilli while germinating in the small intestine (35). Food is frequently contaminated with *B. cereus* because the bacilli are ubiquitous in the environment (78). *B. cereus* food poisoning is typically due to the improper refrigeration or inadequate reheating of foods that have been cooked. Common foods contaminated by the emetic-type food poisoning strains are rice, pasta, vegetables, milk and other dairy products (31). Common foods contaminated with the diarrheal-type strains are cooked meat and poultry, sauces, cooked vegetables, and puddings (54).

The actual number of cases of *B. cereus* food poisoning is unknown due to its symptoms being similar to other food poisoning illnesses (26). Since *B. cereus* is not a reportable disease nor is it under FoodNet's surveillance, the only reported cases are those involved in outbreaks (35). However, it is estimated that *B. cereus* causes 27,360 cases of illness per year in the U.S. (average of 1983-1992 outbreak data) (53).

Food products containing 10^4-10^5 colony forming units per gram (CFU/g) *B. cereus* are considered unsafe for human consumption (36, 76). More specifically, for the emetic-type food poisoning, food products contaminated with 1×10^3 to 5×10^{10} CFU/g have been implicated in food poisoning outbreaks. For the diarrheal-type food poisoning, food products contaminated with 5×10^5 to 9.5×10^8 CFU/g have been implicated in outbreaks (44). Induction of food poisoning is not based solely on colony forming units (cells or spores); rather the risk of illness is a result of toxin production (36).

Bacillus cereus Virulence

Virulence Profile of *Bacillus cereus*

B. cereus secretes over twenty exotoxins, which include several enterotoxins (58). The enterotoxin profile of *B. cereus* includes cereulide, non-hemolytic enterotoxin (NHE), hemolytic enterotoxin (HBL), hemolysin II (Hly II), cytotoxin K (CytK), and cereolysin AB, to name a few (51). There are many other toxins of which the function and mechanisms are unknown. These toxins include enterotoxin FM (*entFM*), and enterotoxin T (*bceT*) (12, 35).

Cereulide is the most dangerous enterotoxin of *B. cereus* and is responsible for emetic-type food poisoning (11, 36). Emetic-type food poisoning results in vomiting within one to five hours post-ingestion of contaminated foods (i.e. rice, milk, pasta). *B. cereus* emetic-type food poisoning is similar to *Staphylococcus aureus* food poisoning (42). Cereulide is heat stable (up to 100˚C), protease resistant, and alkali and acid stable. The dose for human illness is not known, but the average infectious dose needed for the emetic-type (10^9 CFU/mL) seems to be higher than for the diarrheal-type (10^7 CFU/mL) (42).

The biochemistry of cereulide synthesis is still being elucidated; although it is known that cereulide is synthesized via a nonribosomal pathway (i.e. enzymatically synthesized) by the peptide synthase *ces* gene (26). Cereulide [(D-*O*-Leu-D-Ala-L-*O*-Val-L-Val)$_3$] is a cyclic nonproteinaceous molecule structurally related to the potassium ionophore, valinomycin (35). Cereulide is classified as a mitochondriotoxin because is causes mitochondria to swell; most likely due to an uptake in potassium cations and loss of hydrogen cations (57).

In addition to the emetic-type food poisoning, *B. cereus* is responsible for a mild diarrheal-type food poisoning as well. *B. cereus* diarrheal-type food poisoning is likened to *Clostridium perfringens* food poisoning, although not as severe (1). *B. cereus* diarrheal-type food poisoning results in abdominal pain and

diarrhea eight to sixteen hours post-ingestion and lasting for six to twelve hours (42).

The two major enterotoxins responsible for *B. cereus* diarrheal-type food poisoning are non-hemolytic enterotoxin (NHE) and hemolysin BL (HBL). NHE and HBL are acid- and heat-labile, and sensitive to proteolytic enzymes (2). Both are three-component toxins, NHE containing NheA, NheB and NheC; and HBL containing L1 (lytic), L2, and B (binding) subunits. NHE displays enterotoxic and cytotoxic properties and is typically the sole enterotoxin produced by the diarrheal-type food poisoning *B. cereus* strains, while HBL is present in approximately half of the strains (37, 48). HBL is responsible for causing hemolytic and necrotic infections, and causes vascular permeability due to its pore forming ability in target cell membranes (17, 51).

Other toxins that play a role in *B. cereus* pathogenicity are hemolysin II, cytotoxin K, and cereolysin AB. Hemolysin II (Hly II) and cytotoxin K (CytK) are β-barrel pore-forming toxins (β-PFT). The cell permeation and lysis mechanism of β-PFTs consists of the toxins being secreted as water-soluble molecules, binding to the cell surface, and assembling to form transmembrane pores that disrupt the cellular osmolarity (58). CytK is a one-component toxin that is only released after cell lysis; and is similar to *Clostridium perfringens* β-toxin and *B. cereus* HlyII. CytK causes hemolytic, necrotic and cytotoxic effects although not as severe as that of *C. perfringens* (51). Cereolysin AB is a two-component cytolysin consisting of *cerA*, which encodes for phosphatidylinositol-specific phospholipase C (PI-PLC) and *cerB*, which encodes for sphingomyelinase (SPH) (31, 32). PI-PLC and SPH participate in the break down of membrane-bound lipids (45). In *B. cereus* it is thought that PI-PLC facilitates tissue destruction allowing the bacterium access to cellular nutrients (46). Cereolysin AB is also associated with endophthalmitis, which is a severe *B. cereus* infection of the eye (20).

Virulence Gene Expression

Lereclus, *et al*, found that many virulence genes of the *B. cereus* group *spp.* were regulated by a single pleiotropic regulator, PlcR (phospholipase C regulator) (46). In *B. cereus*, there are more than fifteen virulence genes regulated by PlcR scattered throughout the chromosome. Examples of regulated virulence factors include: proteases, cytotoxins, phospholipases C, and enterotoxins (HBL and NHE) (34, 60). Many of the PlcR regulated-genes encoding enterotoxins contain a secretory (*sec*) sequence, indicating that they are secreted from the cell (33).

Most molecular work regarding the regulation of *plcR* is performed with *B. thuringiensis* due to its heavy use in the agricultural industry. As mentioned earlier, *B. thuringiensis* differs from *B. cereus* in that it contains a plasmid encoding for crystal proteins (22, 39). More importantly, the similarity of the *plcR* gene between the two species is functionally equivalent, unlike the mutated and inactive *plcR* of *B. anthracis* (56). In *B. thuringiensis*, it has been discovered that *plcR* is both autoregulated and regulated by a response regulator, Spo0A (46). Spo0A is the master regulator of sporulation in *Bacillus spp*. Spo0A is activated through a phosphorelay, after which it binds to its '0A' box. Once bound to the '0A' box, sporulation specific genes are activated and vegetative phase genes are repressed (69).

In addition to the autoregulation and Spo0A regulation of *plcR*, other factors are indirectly required to activate its transcription. An oligopeptide permease (Opp) system composed of five genes (*oppA, B, C, D, F*) is also involved in the activation of *plcR* at the onset of stationary phase. Opp belongs to the family of ATP-binding cassette (ABC) transporters and is involved in the uptake of small peptides three to six amino acids in length (34). One important peptide, PapR (peptide activates PlcR), is encoded by *papR*; which is located downstream from *plcR*. PapR acts as an extracellular signaling peptide and quorum sensing effector. The PapR peptides are secreted by the cell and then taken up by the Opp system. Once back in the cell, PapR aids PlcR in binding to the

PlcR regulon. Once all of these steps have occurred, PlcR-regulated virulence genes are transcribed (66).

A summary of the interplay between PlcR and Spo0A is as follows (see figure 1), when conditions are favorable, ***B. cereus*** multiplies exponentially in what is known as the exponential growth phase. During this phase *spo0A*pv, a promoter of *spo0A*, and Opp, a regulator of *plcR*, are expressed at low levels (25, 27, 34). As the nutrient supply is exhausted, the cells enter the onset of stationary phase and *spo0A*pv and Opp are no longer utilized. During stationary phase, PapR, taken in by Opp, aids PlcR in binding to the PlcR regulon. This binding results in the transcription and expression of *plcR*-regulated genes. After stationary phase the onset of sporulation sets in. During sporulation, *spo0A*ps, another promoter of *spo0A*, is expressed and Spo0A, the regulator of sporulation, is phosphorylated. These events lead to the activation of sporulation-specific genes (25, 47). As more *spo0A* is expressed, less *plcR* is transcribed; therefore Spo0A acts as a repressor (negative regulator) of *plcR* transcription. Further, it has been shown by Gominet, *et al*, that *spo0A* knockout mutants result in increased *plcR* transcription (34).

Figure 1. A summary of the PlcR and Spo0A interplay. During exponential growth phase *spo0A*pv, a promoter of *spo0A*, and Opp, a regulator of *plcR*, are expressed at low levels. During stationary phase, PapR, taken in by Opp, aids PlcR in binding to the PlcR regulon. This binding results in the transcription and expression of *plcR*-regulated genes. During sporulation, *spo0A*ps, another promoter of *spo0A*, is expressed and Spo0A, the regulator of sporulation, is phosphorylated. These events lead to the activation of sporulation-specific genes. As more *spo0A* is expressed, less *plcR* is transcribed; therefore Spo0A acts as a repressor (negative regulator) of *plcR* transcription.

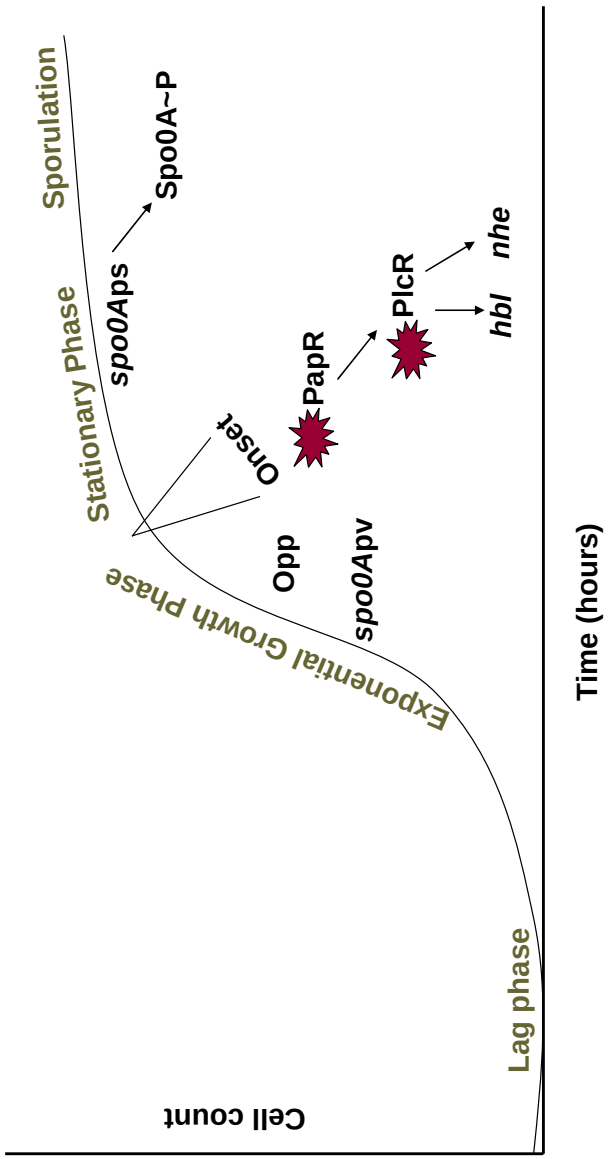

Cell count
Time (hours)
Lag phase
Exponential Growth Phase
Stationary Phase
Sporulation
spo0Apv
Opp
Onset
PapR
PlcR
hbl
nhe
spo0Aps
Spo0A~P

Figure 1

Figure 2. Spo0A and PlcR regulon binding. Spo0A binds to the two 0A boxes, which flank the PlcR regulon. During exponential growth phase PlcR is allowed to bind to the PlcR regulon with the aid of PapR. However, during sporulation, Spo0A binds to the 0A boxes, which hinders PlcR from binding to the PlcR regulon. Spo0A acts as a repressor (negative regulator) of *plcR* transcription.

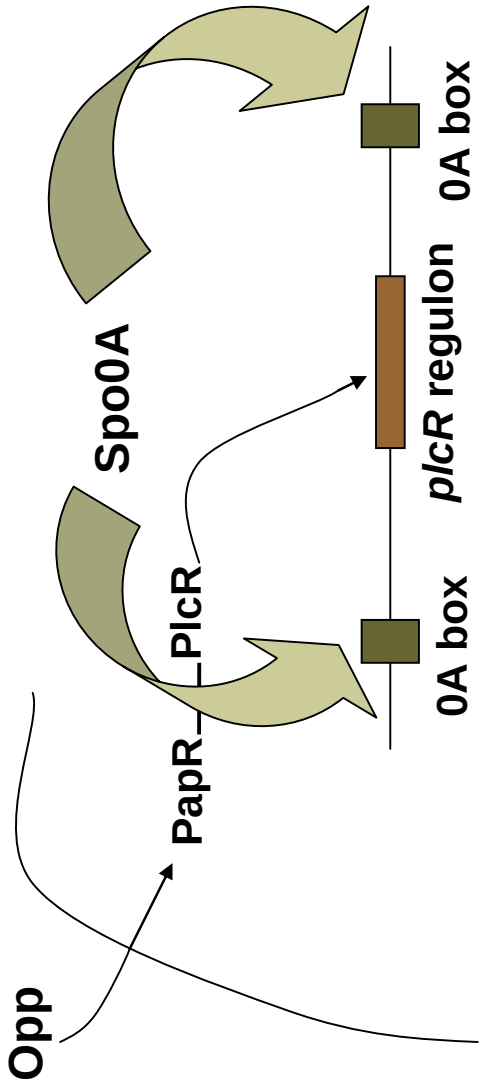


Figure 2

Media Effects on Gene Expression

Since sporulation is a response to starvation, experiments with the nutritional quality of media have been conducted. As it relates to sporulation, the presence of high levels of glucose in a medium represses the stationary phase promoter of *spo0A*, thereby repressing sporulation altogether (25). This type of repression is called carbon catabolite repression.

Carbon catabolite repression occurs when bacteria are exposed to a carbon source, typically glucose, which can be quickly metabolized. As a result the genes for metabolism of other carbon sources are reduced in expression (27). *B. subtilis* prefers glucose as its sole carbon energy source; therefore it is hypothesized that when *B. cereus* is exposed to glucose supplemented medium, cells will metabolize at a faster rate than the complex carbon sources. Rapidly metabolizing *B. cereus* would result in a higher expression of *plcR* and repression of sporulation (27). However, sporulation eventually occurs when all sources of carbon and nitrogen are exhausted.

The type of media *Bacillus spp*. are cultured in also has an effect on the timing of events in the growth curve. For example, when *B. thuringiensis* is cultured in rich medium, it enters the onset of stationary phase at a slower pace, which allows a higher level of *plcR* to be expressed. However, when *Bacillus spp.* are cultured in sporulation medium, the onset of stationary phase is entered sooner due to the rapid triggering of sporulation; and the window of *plcR* expression is extremely narrowed compared to other media. In this case the concentration of phosphorylated Spo0A increases sharply, and this increase is presumed to cause the repression of *plcR* (47).

In general, media containing glucose causes rapid metabolism in *Bacillus spp*., but a threshold is achieved when glucose is in excess. When an excessive amount of glucose is present in the medium or food product, inhibition of *B. cereus* growth and enterotoxin production occurs. Garcia-Arribas and Kramer found an increase in *B. cereus* toxin production in one liter of broth supplemented

with 10 grams glucose or less; but discovered a threshold at more than 50 grams of glucose per liter (g/L) broth (30). Sutherland and Limond discovered that *B. cereus* exhibited lowered toxin production in dairy desserts containing high amounts of sugar. High bacterial counts were calculated, and the conclusion was that media (or food products) containing less than 50 grams glucose per liter facilitated toxin production, while higher concentrations of glucose inhibited toxin production. Sutherland and Limond also determined that the maximum amount of glucose *B. cereus* can tolerate is 200 grams of glucose per liter of media or food product (70).

Detection Methods

In addition to traditional culturing techniques, current detection methods for *B. cereus* include toxicity assays, immunological assays and molecular techniques. Before these current methods were developed, selective enrichment plating was employed to test for the presence of a specific bacterium. However, culturing methods take many days and additional confirmatory testing is necessary. In addition, culturing methods do not give any information regarding the true toxicity of a sample containing bacteria (22).

Traditional Techniques

With *B. cereus* selective agar (BCSA), *B. cereus* can be identified by analyzing the colony color, morphology, and egg yolk hydrolysis properties. When grown on BCSA, *B. thuringiensis* is indistinguishable from *B. cereus*, so further analysis of cell and spore morphology is needed. On BCSA, *B. cereus* colonies appear blue and are surrounded with blue egg yolk precipitate (6).

Mannitol Egg Yolk Polymyxin Agar (MYP) is also used for the identification of *B. cereus*. Most strains of *B. cereus* are unable to utilize mannitol, but are able to digest egg yolk and produce phospholipase C. On MYP, *B. cereus* colonies appear bright pink with an area of egg yolk hydrolysis on MYP (6).

Toxicity Assays

Toxicity assays used to quantify the amount of *B. cereus* diarrheagenic enterotoxins in a sample include animal skin injections (rabbit and guinea pig), rabbit ileal loop injections, and animal feedings (monkeys, mice). The skin injections test for altered vascular permeability and the ligated rabbit ileal loop injections test for fluid accumulation that are a result of *B. cereus* enterotoxin production. The pathogenicity of *B. cereus* is tested by intravenously injecting

mice and by conducting monkey feedings to test for lethality (72). These techniques are used when it is not feasible to conduct whole animal assays (42).

Immunoassays

A commonly used immunoassay is the enzyme-linked immunosorbent assay (ELISA). The ELISA was developed in 1971, and displays specificity for antigens because of its utilization of monoclonal antibodies. The general methodology of the ELISA includes a bacterial antigen being captured by antibodies and detected through an enzyme-mediated color change (78). Presently there are two commercially available ELISA kits for the detection of *B. cereus* diarrheal enterotoxins (6, 71).

One *B. cereus* enterotoxin assay, the Reverse Passive Latex Agglutination assay (BCET-RPLA), is used to detect the nontoxic L_2 subunit of the 3 component diarrheal enterotoxin, HBL (51). Research has shown that this L_2 subunit does not correlate to overall toxicity of the food product (50). For this assay, latex is bound to a soluble antibody, which upon binding to the bacterial antigen causes a visible latex agglutination. This commercial kit can detect down to 2 nanograms per milliliter (ng/mL), but the manufacturer states that the sensitivity varies based on the dilution of the food product (6).

Another commercially available kit, the TECRA® Bacillus Diarrheal Enterotoxin Visual Immunoassay (BDEVIA) is an ELISA-based assay that detects the 45kDa protein of the 3 component NHE complex. BDEVIA can detect preformed toxin within 4 hours and enterotoxin production in less than 20 hours, down to a sensitivity of 1ng/mL (71). This assay is not correlated with enterotoxic activity, as strains with the same amount of the 45kDa protein had varying degrees

of enterotoxic activity. It has been shown that the 105kDa protein is responsible for the enterotoxic activity related to NHE (50).

Molecular Techniques

The polymerase chain reaction (PCR) is a more sensitive and accurate technique compared to the ELISA. PCR is similar to ELISA in that it can only detect the presence of an organism, not its toxicity. PCR can be used in sample analyses for the identification of bacteria. Typically the 16S rRNA is targeted, but in relation to the *B. cereus* group, the 16S rRNA exhibits greater than 99% sequence similarity (14, 22); therefore it is beneficial to target other genetic markers.

Different PCR methods exist to enhance the detection and identification of organisms in a sample. For example, reverse transcription PCR (RT-PCR) can detect 10^2 vegetative *B. cereus* cells per milliliter (21). Amplified fragment length polymorphisms (AFLP) is used in conjunction with PCR to distinguish between *B. thuringiensis* and *B. cereus* (67). PCR methods are useful because they allow for the detection and identification of *Bacillus spp.* in less than a day.

The obstacle of accurate detection of *B. cereus* is due to the illness being caused by toxins, for which culturing, immunoassays and molecular techniques can not accurately measure. The molecular techniques and immunoassays can detect the presence of a pathogenic organism before it becomes toxic, which could be beneficial for illness prevention. Molecular techniques and immunoassays are an improvement to the traditional techniques because they save time and materials, since the binding of specific targets is utilized instead of analyzing real tissue effects or waiting days for plate results.

Novel Methods

The current detection techniques mentioned above utilize enzymatic activity, antibody recognition, or nucleic acid binding and recognition. An even more technologically advanced tool, the biosensor, offers greater potential in the detection of toxins, pathogens, and environmental pollutants. Many prospective types are still in the research and development stage, but will be integrated into commercial applications in the near future. Currently, the most widespread commercially used biosensor is the blood glucose monitor (40).

Historically, the use of a canary in a cage was used to detect high levels of gas in mines. A new type of CANARY biosensor has been developed, the cellular analysis and notification of antigen risks and yields (CANARY) biosensor. This CANARY biosensor is based on engineering the membranes of B lymphocytes to display a pathogen-specific antibody and a bioluminescent protein. The CANARY biosensor is able to detect 50 colony forming units per gram (CFU/g) of *Yersinia pestis*, the causative agent of the plague, in less than 3 minutes; and 500 CFU/g *Escherichia coli* O157:H7 in less than 5 minutes. Another impressive finding is that this biosensor can detect 1000 CFU/g *Bacillus anthracis* spores from nasal swabs (63).

The researchers found that the B lymphocytes were easy to prepare and could be stored for 2 weeks, or even frozen, and still remain responsive. Since the antibody determines the sensitivity of the biosensor, the cells can be engineered to detect any pathogen of interest. B lymphocytes can detect one pathogen at a time, but perhaps it would be feasible to engineer a B lymphocyte capable of detecting multiple pathogens. CANARY can only detect the presence of a pathogen (bacteria and viruses), albeit at lower levels and in a more rapid manner than current molecular detection methods. It was not mentioned if CANARY can detect toxins or other pathogen by-products, but it assumed that this type of cellular engineering has not been conducted for this type of biosensor (63).

Molecular-based techniques using DNA recognition are popular in pathogen detection because of their sensitivity and accuracy. However, these techniques typically require hours for completion, so techniques with a more rapid response are needed. Currently in research, electrochemistry is being combined with molecular techniques to create an accurate and rapid biosensor. Researchers have created an electrochemical biosensor that can detect hemolysin BL (HBL) in enterotoxic strains of *B. cereus* and *B. thuringiensis.* This biosensor employs bead sandwich hybridization placed on an electric silicon chip, which results in an electrical current based on the amount of DNA molecules present in the sample. The researchers were able to engineer this biosensor so that it is less expensive than the typical optical devices, and small enough to be easily used in the field. The disadvantage of this electrochemical biosensor is that it was only capable of detecting to 10^7 cells/spores per 200 microliters of sample, and required a total of 4 hours to perform the analysis (29).

There are many types of biosensors in research and development designed for pathogen detection. However, the majority are not capable of detecting the actual toxicity of the sample. One type of biosensor, that utilizes a unique type of living cell, is the cytosensor utilizing chromatophores.

Chromatophores are found in cold-blooded animals such as fish, chameleons, cephalopods and other lower vertebrates. In the wild, chameleons utilize their chromatophores during mating rituals or for purposes of hiding and camouflaging to their surroundings. The color change is in response to the organism's physiological state (mood) and physical surroundings (temperature, light). The color change also plays a role in rivalry fighting, which is particularly seen in Siamese fighting fish (77).

Birds and mammals only harbor one type of chromatophore, the melanophore; whereas the lower vertebrates and nonvertebrates have 6 possible types of chromatophores. The six different types are melanophores (brown or black), xanthophores (ocher or yellow), erythrophores (red), leucophores (dull or whitish), iridophores (metallic or iridescent), and cyanophores (blue) (28). Each

type of chromatophore contains its specific type of pigment organelles called chromatosomes. For example, melanophores are composed of melanosomes and erythrophores are composed of erythrosomes. The chromatosomes move within the chromatophore cytoplasm, with the aid of pigment-motor proteins like dynein and kinesin, creating a change in optical density (16).

This change in optical density is initiated when a stimulant of hormonal or neural origin binds to a G-protein coupled receptor (GPCR) bound to the cellular outer membrane. Once a stimulant is bound, guanosine diphosphate (GDP) is phosphorylated, which in turn activates the α-subunit of a G-protein. The α-subunit interacts with the membrane bound enzyme adenylate cyclase until guanosine triphosphate (GTP) is hydrolyzed back to GDP. Adenylate cyclase converts adenosine triphosphate (ATP) to cyclic adenosine monophosphate (cAMP), which is a second messenger that binds to Kinase A. The binding of cAMP and Kinase A produces two free catalytic subunits that can phosphorylate substrates. In the case of a pigment-motor response, these substrates are the proteins kinesin and dynein (16, 28, 77).

To summarize the subcellular events of the chromatophore response, when inhibitory G-proteins (G_i) are activated by a GPCR, adenylate cyclase is inhibited, which causes a decrease in intracellular cAMP levels. This decrease results in an aggregated pigment-motor response in which dynein pulls the chromatosomes in towards the centrosome (center) of the chromatophore. Conversely, when stimulatory G-proteins (G_s) are activated by a GPCR, adenylate cyclase is stimulated, causing an increase in intracellular cAMP levels. Then, cAMP proceeds to bind to Kinase A, which creates catalytic subunits that go on to phosphorylate kinesin. This situation results in a dispersed pigment-motor response, in which kinesin moves the chromatosomes toward the periphery of the chromatophore (16, 28, 77).

Stimulants of dispersion are melanocyte-stimulating hormone (MSH) and adrenocorticotropic hormone (ACTH). MSH is produced in the pituitary gland and causes a dispersion of melanosomes within the melanophores. Melanin-

concentrating hormone (MCH) and melatonin (MT) have been shown to cause an aggregation in chromatophores. Epinephrine (adrenaline) and norepinepherine are recognized as reliable stimulants of aggregation, utilizing the β-adrenoreceptors (16).

Chromatophore-Based Cytosensors

Previous research has demonstrated that chromatophores provide a reliable and novel type of cytosensor. The chromatophores react physiologically, not solely structurally (i.e. receptor binding) due to molecular recognition; which allows for their responsiveness to biologically active compounds. Chromatophores can be employed to identify pharmaceuticals or to detect biohazardous materials. The application behind utilizing chromatophores is their property of altering their optical density. This alteration is monitored with computer optical imaging to create the chromatophore cytosensor.

Karlsson, *et al*, have transfected frog (*Xenopus laevis*) melanophores with the human opioid receptor 3 to use in the detection of opiates. Their research showed that both morphine and a synthetic opioid peptide stimulated aggregation in the melanophores. It was reported that this melanophore-based cytosensor could detect opioids in plasma at the range (1 μM) used for therapeutic use in humans. This research demonstrates that different types of chromatophore-based cytosensors can be produced to respond to specific agents. The researchers propose that their melanophore-based cytosensor has the potential to be used in the analysis of body fluids (blood plasma, urine, saliva) for the presence of controlled substances or in odor, pheromone and taste sensing (43).

Danosky and McFadden (23) analyzed the response of melanophores from Nile tilapia (*Tilapia nilotica*). Melanophores are the only type of pigment cell that Nile tilapia contains, and each fish only contains a small number. Norepinephrine and its α_2 adrenergic antagonist, yohimbine, were tested on the melanophores, and the researchers deduced that this type of cytosensor could be used to test for pharmacological drugs. The research utilized automated data collection and digital methods, which make the data analysis quick and simple. The difficulties with working with these cells are the temperature and media requirements for melanophore sustainability (23).

Numerous types of chromatophores are being employed in cytosensor applications. In addition to the *Xenopus* and *Tilapia* chromatophores, pigment cells from other organisms like the black tetra (*Gymnocorrymbus ternetzi*), Atlantic cod (*Gadus morhua*), peppered catfish (*Corydoras paleatus*) and European cuttlefish (*Sepia officinalis*) have been used in cell-based biosensors (49, 61).

Another organism well researched for its cytosensor applications, is *Betta splendens* (see figure 3). *B. splendens*, better known as Siamese fighting fish, are native to Southeast Asia. They live in freshwater at temperatures of 24-30ºC, are carnivorous, and as pets are typically fed brine shrimp. In the wild, they live in rice paddies and floodplains on a diet of zooplankton and insect larvae. Their natural coloration consists of dull green and brown, but being the popular aquarium fish they are, they have been bred for their brilliant color variation and fin styles (77).

B. splendens chromatophores have been used to analyze the response to neurotoxins and pathogenic bacteria. *B. splendens* chromatophores have shown to be responsive to virulence factors from pathogenic bacteria like *Salmonella enteritidis*, *Streptococcus pyogenes*, *Bordatella pertusis*, *Vibrio cholerae* and *V. parahemolyticus*, *Clostridium sp., Shigella sp*., and *Staphylococcus aureus. B. splendens* chromatophores have shown to be nonresponsive to nonpathogenic bacteria like *Lactococcus lactis* and *Bacillus subtilis* (24, 59). Using an organism's living cells is beneficial because the cells contain many biochemical targets that are shared with other living organisms'. Therefore, the chromatophores are sensitive to a broad range of neurological, chemical and biological agents (24).

The *B. splendens* chromatophore-based cytosensor does have its limitations, as the cells must be kept in cell culture medium and held at an appropriate temperature (25°C) (23). In order to analyze chromatophore responses, the cells must be combined with instrumentation that amplifies the signal and can optically detect and quantify the reaction change. The chromatophores do not require additional radioactive or fluorescent probes to aid in the reaction analyses. The benefits of high sensitivity, low cost, ease of preparation, and small cell size

far outweigh the limitations of cell culture medium and holding temperature (24, 59). Also, the *B. splendens* chromatophores are terminally differentiated, therefore they do not replicate, which allows for an increased lifespan of the cells (2 weeks to 1 month).

(A) 

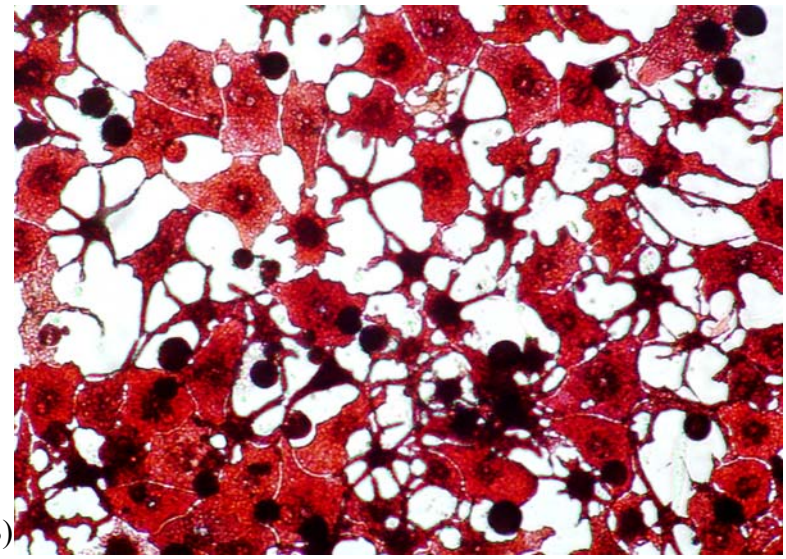
(B) 

Figure 3. (A) Red *Betta splendens* (Siamese Fighting Fish). (B) Cell preparation of *Betta splendens* erythrophores.

Experimental Approach

Oregon State University has been a pioneer in *Betta splendens* chromatophore research for the last 10 years. However, this subject area has lacked defined studies on specific pathogen interactions on the chromatophores. The goal of this research was to use defined media and experimental conditions to analyze the effects of *Bacillus cereus* on *Betta splendens* erythrophores.

MATERIALS AND METHODS

Bacterial Strain

The *Bacillus cereus* strain used in all experiments is catalogued as ATCC 49064. It is a diarrheal food-poisoning strain (4433) serotype 2 (73), isolated from meatloaf during an outbreak in Berkeley, California (55). Much is known about the genotype of *B. cereus* as its genome has been sequenced; and as a result many mutant strains exist. This strain of *B. cereus* contains all six genes for the HBL and NHE complexes.

Bacterial Propagation and Plate Counts

Initially, the *B. cereus* cells were streak plated from a –80ºC glycerol stock onto an agar plate of Brain Heart Infusion (BHIA, 52 grams of Bacto Brain Heart Infusion Agar, dehydrated (calf brains, beef heart, NaCl, protease peptone, disodium phosphate, dextrose), Difco #0418-17-7) into one liter of double distilled water, autoclaved at 121ºC for 20 minutes) or Luria-Bertani (LBA, 10 grams tryptone, 5 grams yeast extract, 5 grams NaCl, 15 grams agar into one liter of double distilled water, autoclaved at 121ºC for 20 minutes), depending on the type of media to be used in future experiments. The plate was incubated at 30ºC overnight, and subsequently streak plated under same medium and temperature conditions.

The next day, an isolated colony of *B. cereus* was inoculated into LB (10 grams tryptone, 5 grams yeast extract, 5 grams NaCl into one liter of double distilled water, autoclaved at 121ºC for 20 minutes), LB supplemented with glucose (LBG, LB containing 0.1% and 1% glucose, filter sterilized), BHI (37 grams of Bacto Brain Heart Infusion, dehydrated (Difco #0037-17-8) into one liter of double distilled water, autoclaved at 121ºC for 20 minutes), or BHI supplemented with glucose (BHIG, BHI containing 0.1% and 1% glucose, filter

sterilized) broth media according to the experiment. The inoculated culture was incubated overnight at 30ºC and 200 revolutions per minute (rpm). Typically, a second broth medium culture was needed so that the incubation time for experimental use could be exact (i.e. 7 hours). The new broth media was inoculated with a 1:100 (300µL in 30mL) dilution and incubated at 30ºC and 200 rpm.

Plate counts were conducted on every *B. cereus* broth medium culture used in the erythrophore response experiments. A dilution scheme was made with 0.1% peptone (Difco #0118-17-0) and dilutions, from 10^{-4} through 10^{-10}, were plated in triplicate. For consistency, the type of agar plates used corresponded with the type of broth the bacteria were previously cultured in (either BHIA or LBA). The plates were incubated at 30ºC for 12 hours and then counted.

Fish Preparation

Red *Betta splendens* were purchased from local pet stores and held for approximately one month to monitor and clear of any microbiological infections. The individual water containers were changed every week; the fish were dipped for 5 minutes in Dip-Away (Aquatronics) every other week to kill any existing microbial infection, and fed every other day. The fish were kept in individual containers containing 500 milliliters of double distilled water at 25ºC. Just prior to harvest, the fish were anesthetized and killed by immersing in ice water for at least 20 minutes.

The caudal and dorsal fins were snipped from the fish and washed twice in 10 milliliters of Phosphate Buffered Saline (modified PBS, 8.00 grams NaCl, 0.20 gram KCl, 1.44 grams Na_2HPO_4, 0.20 gram KH_2PO_4, and 1.00 gram of glucose in one liter of double distilled water, pH 7.3, filter-sterilized, supplemented with 10 milliliters of Antibiotic-Antimycotic (Gibco-BRL 15240-062)) for two minutes.

The fins were placed in a 15 milliliter Falcon® tube in which skinning solution (SK, 8.00 grams NaCl, 0.20 gram KCl, 1.44 grams Na_2HPO_4, 0.20 gram KH_2PO_4, 0.372 gram Na_2-EDTA, and 1.00 gram of glucose in one liter of double distilled water, pH 7.3, filter-sterilized, supplemented with 10 milliliters Antibiotic-Antimycotic) was added. The skinning solution was added in ten 10 milliliter washes of two minutes each. The purpose of using the skinning solution was to remove epithelial cells from the surface of the fins.

Erythrophore Digestion

The digest solution used to digest the dorsal and caudal fins consisted of 15 to 20 milligrams of collagenase (Worthington 4196) and 1 to 2 milligrams of hyaluronidase (Worthington 2594). These enzymes further digest the tissue matrix, releasing the erythrophores. The measured collagenase and hyaluronidase were added to 7 milliliters of PBS and filter-sterilized using a 0.22 micron Aerodisc® syringe filter and a 10 milliliter plastic syringe. The digest solution was added to the 15 milliliter Falcon® tube and gently mixed for 1 to 2 minutes, until the solution became turbid.

Erythrophore Plate Preparation and Maintenance

After digesting the fins, the harvested erythrophores were recovered by centrifugation for 3 minutes at 600 rpm. The supernatant (digest solution) was pipet back into the 15 milliliter Falcon® tube containing the digested fins for further enzymatic digestion. The erythrophore pellet was washed with 100 microliters of Leibovitz L15 Medium + (L15+, Gibco #21083-027, 500 milliliters of dye-free L15 was supplemented with 10 milliliters 1M HEPES (Gibco-BRL 15630-080) buffer and 5 milliliters Antibiotic-Antimycotic) and finally

resuspended in 50 to 100 microliters of L15+. On average, three digests were performed, but depending on cell yield, up to five were carried out. After a sufficient amount of cells were recovered and washed, they were plated, 5 microliters per well, into a 24-well cell culture plate. The cells were allowed to adhere to the plate for 30 to 60 minutes. After this time, 1.5 milliliters of Leibovitz L15 medium + (L15+) and 75 microliters (5%) of fetal bovine serum (FBS, HyClone #SH30071-01) were added to each well.

Between 24 and 48 hours after the erythrophores were plated, the existing L15+ and FBS media were removed and replaced with a fresh 1.5 milliliters of L15+ and 75 microliters of FBS. For erythrophore maintenance, the cell culture medium was changed weekly until the preparation was used or no longer viable.

Erythrophore Response and Computer Analysis

The erythrophore experiments were performed by running controls to test for reactivity, and then, upon appropriate responses, were tested with bacterial samples. The cell culture plates were mounted on the stage of an inverted microscope. A field of view containing approximately 20 to 50 erythrophores was monitored under a magnification of 200. This selected area was not moved throughout the course of the experiment.

A sample was added to a well by removing the 1.5 milliliters of cell culture medium and adding 1 milliliter of a control or experimental sample. After this addition, the experiment began and the first image (time zero) was captured. The erythrophore response was documented for 30 minutes and then the images were organized and processed.

The controls analyzed included: α-melanocyte stimulating hormone (MSH, at 400nM, Sigma M4135), clonidine hydrochloride (at 400nM, Sigma C7897), L15+ medium, and the experimental media (LB, BHI, etc.). MSH is the positive control for dispersion, due to its stimulation of kinesin to move the erythrosomes toward the periphery of the erythrophore. Clonidine, an adrenergic

neurotransmitter, is the positive control for aggregation, due to its stimulation of dynein to move the erythrosomes toward the center of the erythrophore (16, 28). The cell culture and bacterial media controls are the negative controls.

Dilutions were made of the *B. cereus* culture (0.5%-10%) in the total volume of 1 milliliter. To run the sample, the 1.5 milliliters of cell culture medium was removed from the well and a 1 milliliter *B. cereus* dilution was added, beginning the experimental analysis. The bacterial count (CFU/mL) of the sample was calculated after the results of the plated dilutions were known, 12 hours later.

Image Analysis

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 at a magnification of 200. Images were taken at times: zero, 5 seconds, 10 seconds, 15 seconds, 30 seconds, 45 seconds, 1 minute, 1.5 minutes, 2 minutes, 5 minutes, 7 minutes, 10 minutes, 15 minutes, 20 minutes, 25 minutes and 30 minutes. Upon analyzing the 30 minute response of bacterial samples, it became apparent that the bacteria were multiplying at 25 through 30 minutes; therefore the last two time points from all reactions were eliminated from the analyses.

The images were opened in Image Pro Plus 4.1 (Media Cybernetics), put in sequence from time zero to 20 minutes, and processed into Excel through software bundled with Image Pro Plus. The Image Pro Plus processing allows the user to process a sequence of images into an Excel workbook.

Excel Analysis

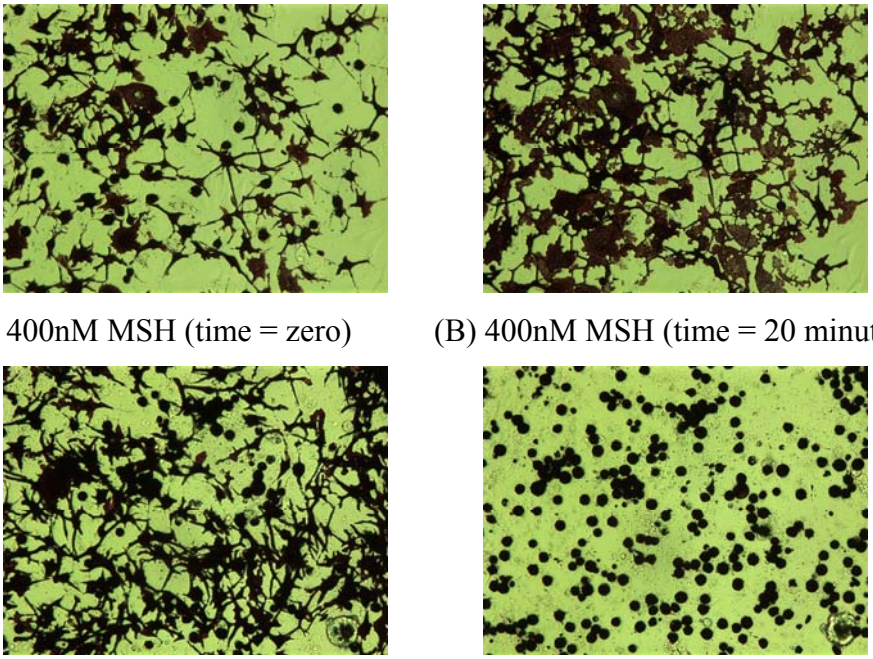
Excel computes the imaging data by calculating the percent area change using the equation: cell area change (%) = $-(A_0 - A_x) / A_0 \times 100$, where A_0 is the initial area and A_x is the final area. Where an aggregated response would result in a negative percent area change and a dispersed response would result in a positive percent area change. The data is plotted as time versus percent area change.

RESULTS

Introduction to Erythrophore Responses and Image Analysis

For all experiments, data was obtained using *Betta splendens* erythrophore cell preparations. The bacterium used in all experiments was *Bacillus cereus*, ATCC 49064. The bacterial culture was incubated at 30ºC at 200 rpm. Viable plate counts were carried out for every *B. cereus* broth culture used in an analysis, and these numerical figures are noted with their respective media type in the following graphs. Since the viable cell counts were not known until 12 hours after an experiment, obtaining identical cell counts from separate experiments was not always possible, so the closest cell counts (CFU/mL) are compared.

The percent area change was calculated using the equation: cell area change (%) = $- (A_0 - A_x) / A_0 \times 100$, where A_0 is the initial area and A_x is the final area. The images in figure 2 show the dispersed and the aggregated responses of *B. splendens* erythrophores. The dispersed response (figure 4B) was induced by the addition of 400 nanomolar (nM) α-melanocyte stimulating hormone (MSH) and the aggregated response (figure 4D) was induced by the addition of 400nM clonidine.



(A) 400nM MSH (time = zero) (B) 400nM MSH (time = 20 minutes)

(C) 400nM Clonidine (time = zero) (D) 400nM Clonidine (time = 20 minutes)

Figure 4. Erythrophore responses to α-melanocyte stimulating hormone and clonidine. (A) Erythrophore preparation in cell culture medium. (B) Erythrophore preparation 20 minutes after the addition of 400nM α-melanocyte stimulating hormone (MSH), the positive control for dispersion. (C) Erythrophore preparation in cell culture medium. (D) Erythrophore preparation 20 minutes after the addition of 400nM clonidine, the positive control for aggregation.

Erythrophore Responses to Standard Controls and Media Controls

The responses of erythrophores to standard controls were measured so as to assess the physiological state of responsiveness of the cells. Figure 5 illustrates the averaged responses of eight *B. splendens* erythrophore preparations to standard controls. The standard controls used were α-melanocyte stimulating hormone (MSH), clonidine, Leibovitz medium, and bacterial media (Luria Bertani and Brain Heart Infusion broth). As established by previous research, MSH is the positive control for dispersion, while clonidine is the positive control for aggregation (16, 28). Leibovitz medium is used as a control for disturbance, and the bacterial media controls are used as the background controls for experiments testing the erythrophore responses to the bacterium, *Bacillus cereus*.

The erythrophore response to MSH (at 400nM) resulted in the predicted dispersed response, with a change in area of 25.8% after 20 minutes. The erythrophore response to clonidine (at 400nM) resulted in the predicted aggregated response, with a total change in area of -57.4%. The erythrophore response to Leibovitz medium demonstrates that a very slight disturbance occurs in the erythrophores as the cell culture medium is removed and new medium is added; as the cells undergo a nominal area change of 0.42%. The erythrophore response to Luria Bertani broth (LB) resulted in a slight dispersion, ending at an area change of 3.43%. The erythrophore response to Brain Heart Infusion broth (BHI) remained reasonably steady, ending at an area change of 2.48%.

Figure 5. Erythrophore responses to standard controls and media controls. Erythrophore responses were documented by taking images throughout a 20 minute time period. The controls consisted of 400nM MSH, the positive control for dispersion, 400nM clonidine, the positive control for aggregation, Leibovitz medium to test the response to disturbance, Luria Bertani broth and Brain Heart Infusion broth to act as background responses for bacterial cultures.

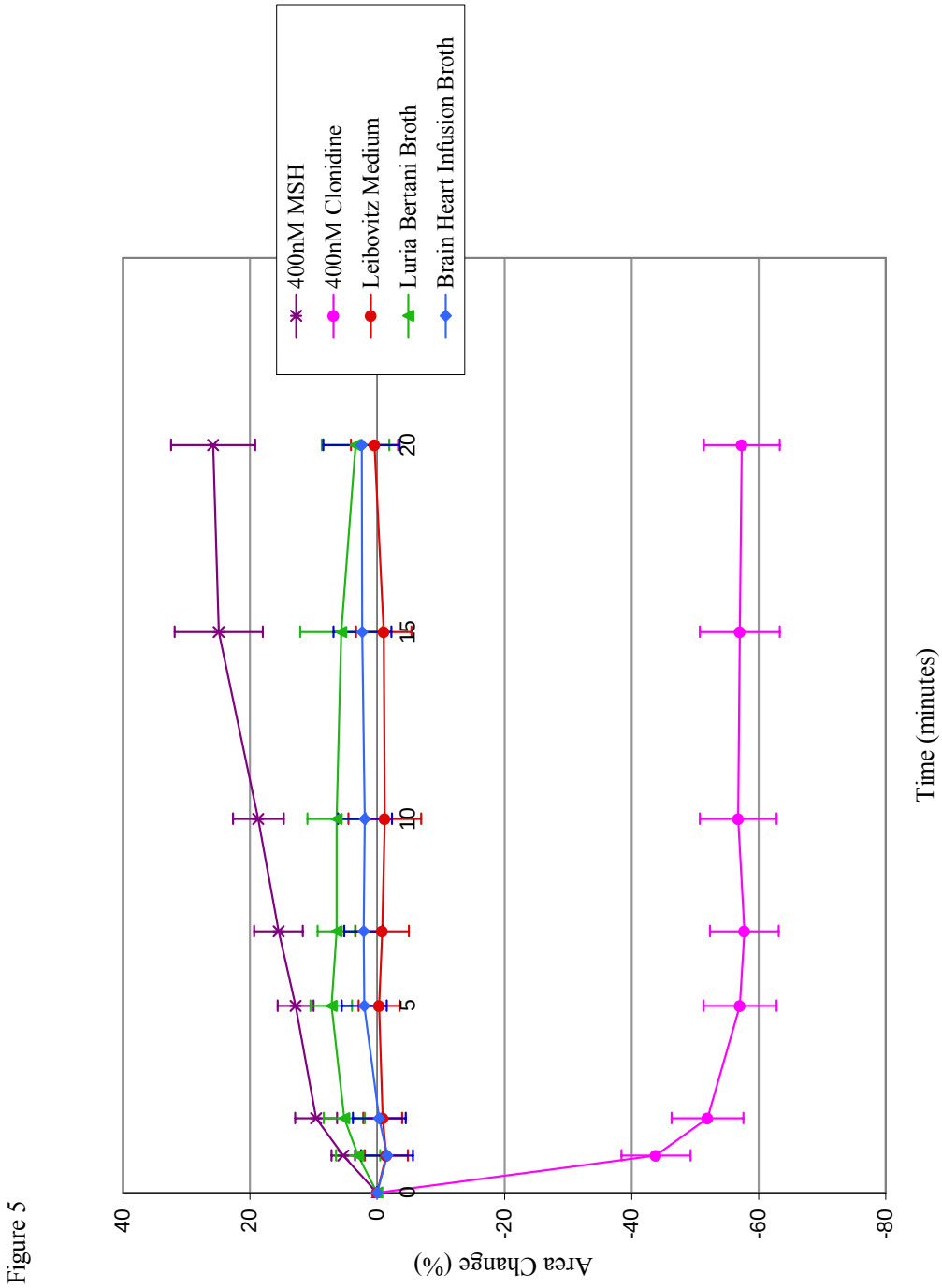

400nM MSH
400nM Clonidine
Leibovitz Medium
Luria Bertani Broth
Brain Heart Infusion Broth
Area Change (%)
40
20
0
-20
-40
-60
-80
0
5
10
15
20
Time (minutes)

Figure 5

Erythrophore Responses to *Bacillus cereus* Cultured in Brain Heart Infusion Broth

Erythrophore experiments were performed using *B. cereus* cultured in Brain Heart Infusion broth (BHI). BHI is considered a rich medium, in which *B. cereus* was calculated as having a doubling time of 24 minutes. BHI has been used as a culturing medium for pathogenic strains of *B. cereus* by previous researchers (9, 19).

The data illustrated in figure 6 is a compilation of two different erythrophore preparations using *B. cereus* cultured in Brain Heart Infusion broth (BHI) and incubated from 9.5 to 12 hours. The trend shown in figure 6 demonstrates how the kinetics of the erythrophore responses increase as the *B. cereus* viable cell counts increase. This is supported by the erythrophore response to 2.75 x 10^8 CFU/mL *B. cereus*, which resulted in the most rapid and aggregated response compared to the other reactions containing lesser viable cell counts. The erythrophore response to 8.23 x 10^6 CFU/mL *B. cereus* resulted in an initial change of -5.00% and maintained this degree of response +/- 1.00% through 15 minutes, after which it slightly declined, ending at a final area change of -12.6%. It is apparent that 8.23 x 10^6 CFU/mL *B. cereus* lies near the detection limit of the erythrophores as it treads near the erythrophore response to the Brain Heart Infusion (BHI) control. The erythrophore response to 4.12 x 10^7 CFU/mL *B. cereus* did not start to rapidly aggregation until 7 minutes, and ultimately reached an area change of –56.4%. The erythrophore response to 8.23 x 10^7 CFU/mL *B. cereus* began aggregating at 2 minutes and ended at a final area change of -45.5%. The erythrophore response to 1.38 x 10^8 CFU/mL *B. cereus* followed a similar reaction pattern as the response to 8.23 x 10^7 CFU/mL *B. cereus*. The final area change for the erythrophore response to 1.38 x 10^8 CFU/mL *B. cereus* ended 1.1% away from the response to 8.23 x 10^7 CFU/mL *B. cereus*, at -46.6%. The erythrophore response to 2.75 x 10^8 CFU/mL *B. cereus* deviated from the other

reactions after 2 minutes and caused the highest degree of aggregation, reaching a final area change of -61.6%.

Figure 6. Erythrophore responses to *B. cereus* cultured in Brain Heart Infusion broth. *B. cereus* was cultured in Brain Heart Infusion (BHI) broth for 9.5 to 12 hours at 30ºC and 200 revolutions per minute (rpm). Viable cell counts were obtained after overnight incubation and are listed in ascending order, with the respective erythrophore response induced. The purpose of showing the erythrophore response to the BHI broth control is to depict the background response of the medium alone.

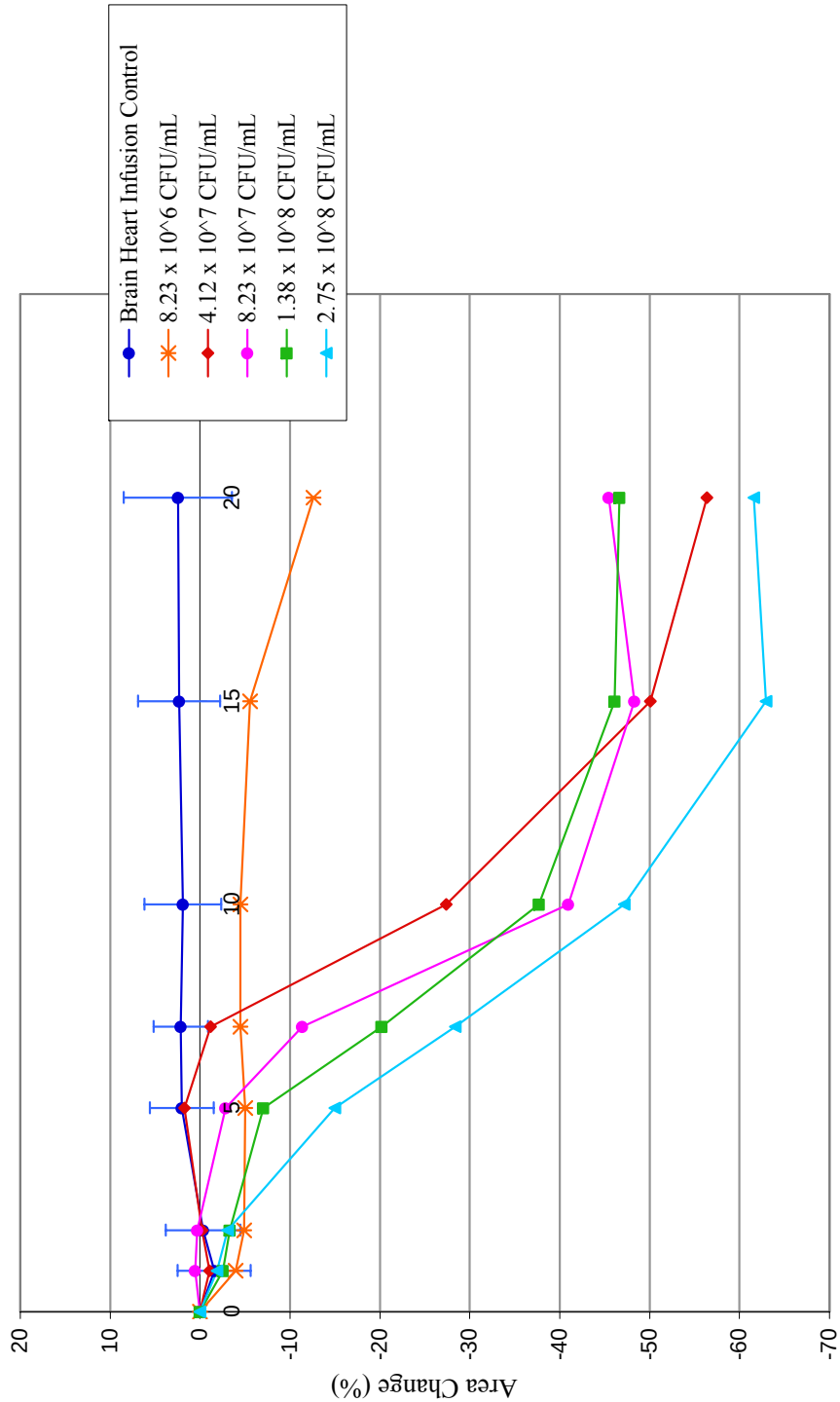

Brain Heart Infusion Control
8.23 x 10^6 CFU/mL
4.12 x 10^7 CFU/mL
8.23 x 10^7 CFU/mL
1.38 x 10^8 CFU/mL
2.75 x 10^8 CFU/mL
Area Change (%)
20
10
0
-10
-20
-30
-40
-50
-60
-70
0
5
10
15
20
Time (minutes)

Figure 6

Erythrophore Responses to *Bacillus cereus* Cultured in Luria Bertani Broth

Erythrophore experiments were performed using *B. cereus* cultured in Luria Bertani broth (LB). LB is considered a rich medium, in which *B. cereus* was calculated as having a doubling time of 27 minutes. Previous researchers have used LB as a culturing medium for *B. cereus,* as well as *Bacillus thuringiensis* used in molecular research (37, 46, 47, 64, 66).

Erythrophore responses to a range of concentrations of *B. cereus* cultured in Luria Bertani broth (LB) were compared, and the results are illustrated in figures 7 and 8. *B. cereus* was cultured for 6 to 12 hours to obtain a full range of cell counts (10^3 –10^{10} CFU/mL). Each erythrophore response is labeled with the calculated *B. cereus* viable cell count (CFU/mL).

Shown in figure 7 are the responses of erythrophores to increasing amounts of viable *B. cereus* cultured in LB. The erythrophores reacted to 7.30 x 10^3 CFU/mL *B. cereus* in a similar manner to the Luria Bertani broth (LB) control; therefore, it is apparent that 7.30 x 10^3 CFU/mL falls under the detection limit of the erythrophores. The erythrophore response to 3.80 x 10^4 CFU/mL *B. cereus* resulted in a dispersion greater than the LB control at 5 minutes, followed by a rapid aggregation consisting of 74.8% total area change through 15 minutes. The response to 3.80 x 10^4 CFU/mL steadies off and reaches a final area change of –62.7%. The erythrophore response to 3.80 x 10^4 CFU/mL *B. cereus* also induces the highest amount of aggregation than all of the other responses, even those of higher cell count; achieving an area change of –63.9% (see 15 minute point).

The erythrophore response to 7.60 x 10^4 CFU/mL *B. cereus* deviated from the response to the LB control after 5 minutes, and ended at an area change of –53.3%. The erythrophore response to 5.00 x 10^5 CFU/mL *B. cereus* and 1.00 x 10^6 CFU/mL *B. cereus* induced similar erythrophore responses, by both aggregating within the first 10 minutes, and then steadying off for the remaining 10 minutes. The erythrophore response to 5.00 x 10^5 CFU/mL *B. cereus* ended at –47.4%, whereas the response to 1.00 x 10^6 CFU/mL *B. cereus* ended at –52.7%.

Figure 7. Erythrophore responses to *B. cereus* cultured in Luria Bertani broth (10^3 through 10^6 CFU/mL). *B. cereus* was cultured in LB broth for 6 to 9 hours at 30ºC and 200rpm. Viable cell counts were obtained after overnight incubation and are listed in ascending order, with the respective erythrophore response induced. The erythrophore response to the LB broth control represents the background response to the medium alone.

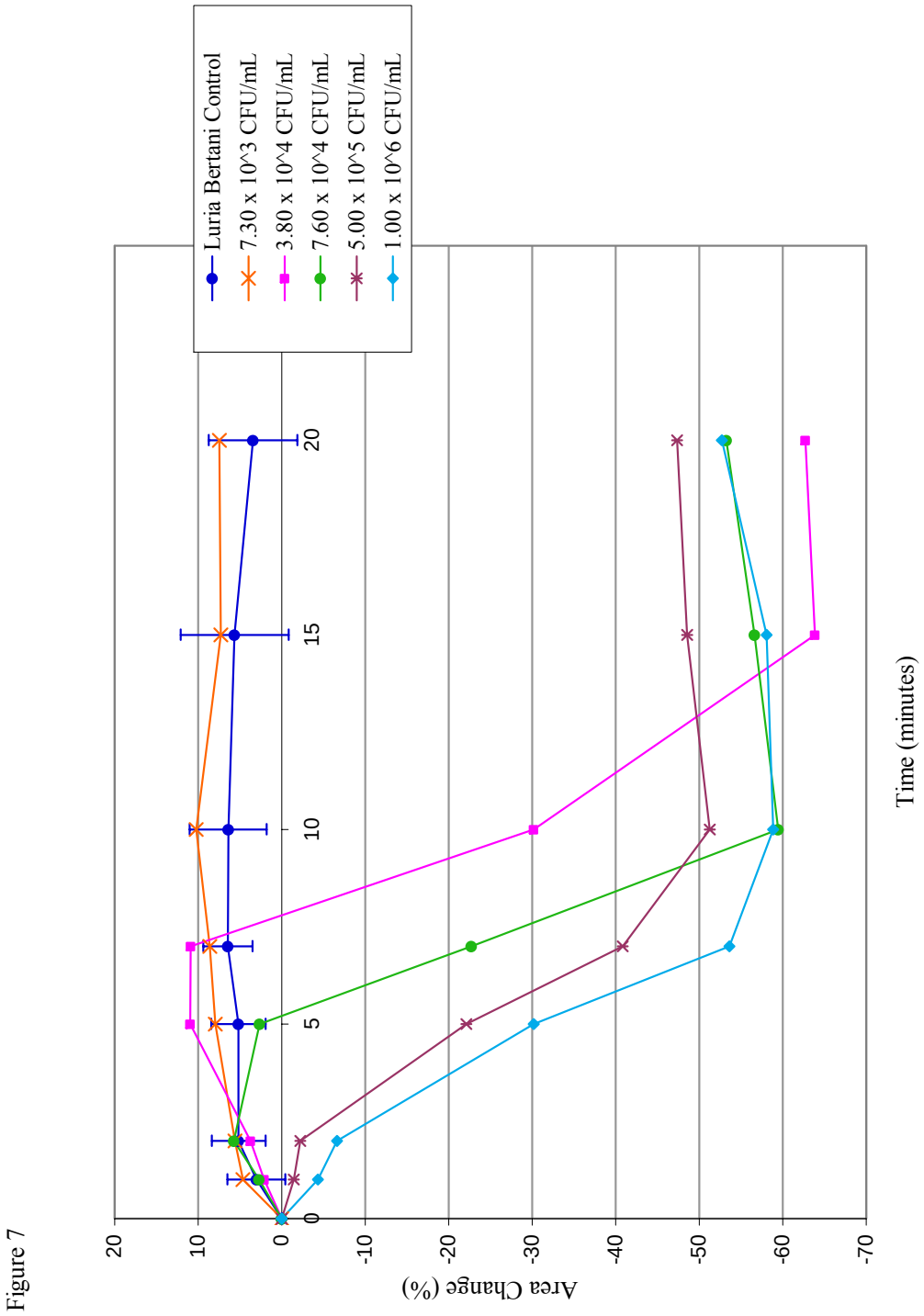

Luria Bertani Control
7.30 x 10^3 CFU/mL
3.80 x 10^4 CFU/mL
7.60 x 10^4 CFU/mL
5.00 x 10^5 CFU/mL
1.00 x 10^6 CFU/mL
20
10
0
-10
-20
-30
-40
-50
-60
-70
0
5
10
15
20
Area Change (%)
Time (minutes)

Figure 7

Figure 8. Erythrophore responses to *B. cereus* cultured in Luria Bertani broth (10^7 through 10^9 CFU/mL). *B. cereus* was cultured in LB broth for 9 to 12 hours at 30ºC and 200rpm. Viable cell counts were obtained after overnight incubation and are listed in ascending order, with the respective erythrophore response induced. The erythrophore response to the LB broth control represents the background response to the medium alone.

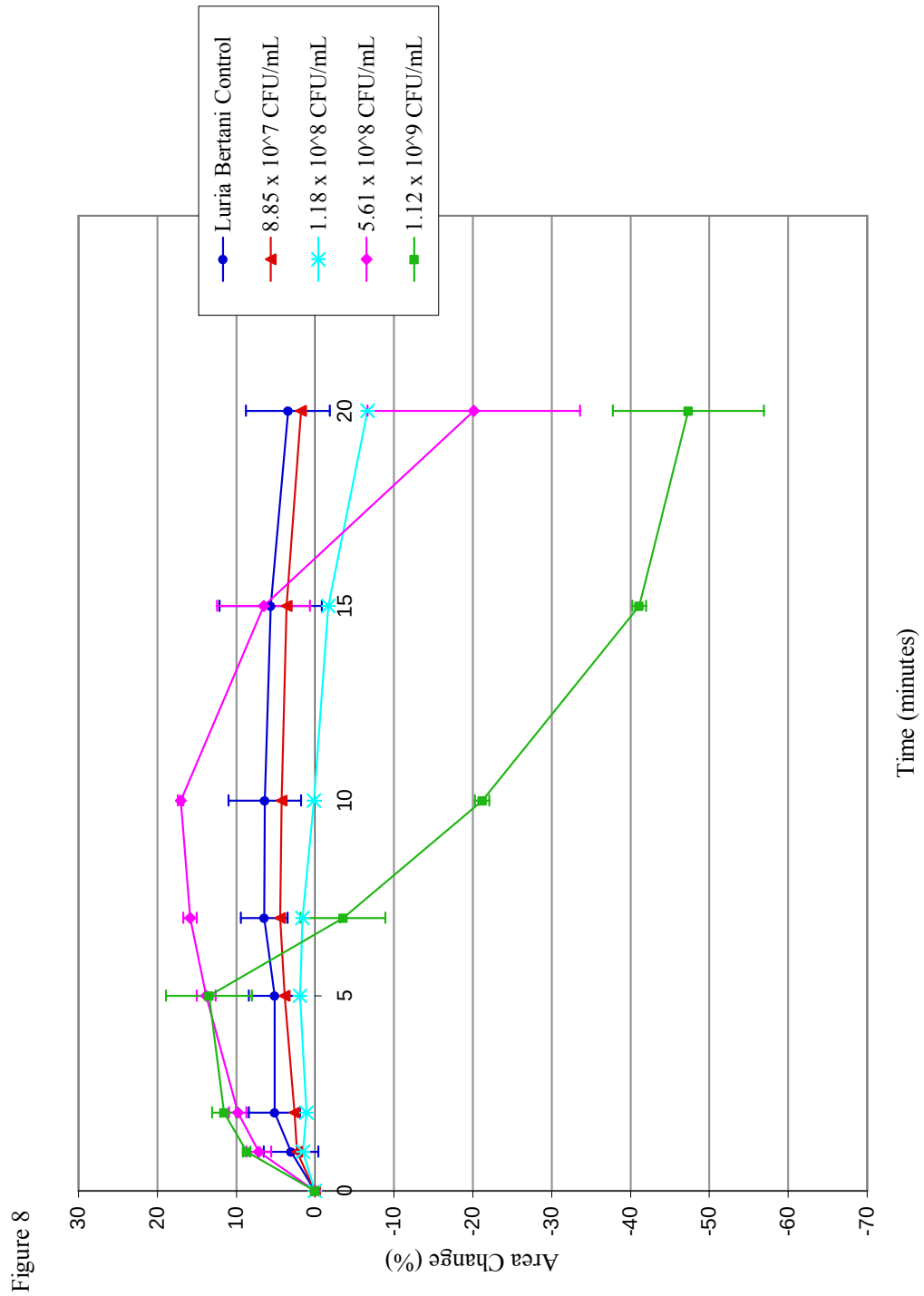

Luria Bertani Control
8.85 x 10^7 CFU/mL
1.18 x 10^8 CFU/mL
5.61 x 10^8 CFU/mL
1.12 x 10^9 CFU/mL
30
20
10
0
-10
-20
-30
-40
-50
-60
-70
0
5
10
15
20
Area Change (%)
Time (minutes)

Figure 8

As illustrated in figure 8, the erythrophore response to 8.85 x 10^7 CFU/mL *B. cereus* resulted in a similar response to the averaged erythrophore responses to the Luria Bertani broth (LB) control. The erythrophore response to 1.18 x 10^8 CFU/mL *B. cereus* did not elicit a strong aggregation and ended at -6.66%. The erythrophore response to 5.61 x 10^8 CFU/mL *B. cereus* resulted in a 17.0% dispersion at 10 minutes, followed by a slight aggregation, ending at a final area change of -20.1%. The erythrophore response to 1.12 x 10^9 CFU/mL *B. cereus* also resulted in an initial dispersion, of 13.5% at 5 minutes, followed by a steady aggregation, ending at a final area change of –47.3%.

Erythrophore Responses to *Bacillus cereus* Cultured in Brain Heart Infusion Broth Supplemented with Glucose

Experiments were performed using *B. cereus* cultured in Brain Heart Infusion broth supplemented with glucose in order to observe a stronger induction of erythrophore aggregation in response to the bacterium maximally expressing enterotoxins, as claimed by previous researchers (3, 30, 38, 41, 72, 74).

The BHI broth supplemented with glucose (BHIG) controls were reacted upon two different erythrophore preparations, and the BHI controls data was compiled from four different preparations. After the data was averaged and the standard deviations calculated (data not shown), no significant differences were found in the erythrophore responses to the BHI and BHIG controls. Since the erythrophore responses to these controls ended in only a slight dispersion, the experiments proceeded with analyzing the erythrophore responses to *B. cereus* cultured in BHI, BHI supplemented with 0.1% glucose, and BHI supplemented with 1% glucose broth media.

Figure 9. Erythrophore responses to *B. cereus* cultured in Brain Heart Infusion broth and Brain Heart Infusion broth supplemented with glucose. *B. cereus* was cultured in Brain Heart Infusion broth (BHI) supplemented with 0.1% or 1% glucose for 7 hours at 30ºC and 200rpm. *B. cereus* was cultured in BHI for 9.5 hours at 30ºC and 200rpm. Viable cell counts were obtained after overnight incubation and are listed in ascending order, with the respective erythrophore response induced. The erythrophore responses to the BHI broth control, BHI broth supplemented with 0.1% glucose control, and BHI broth supplemented with 1% glucose control represent the background responses to the media alone.

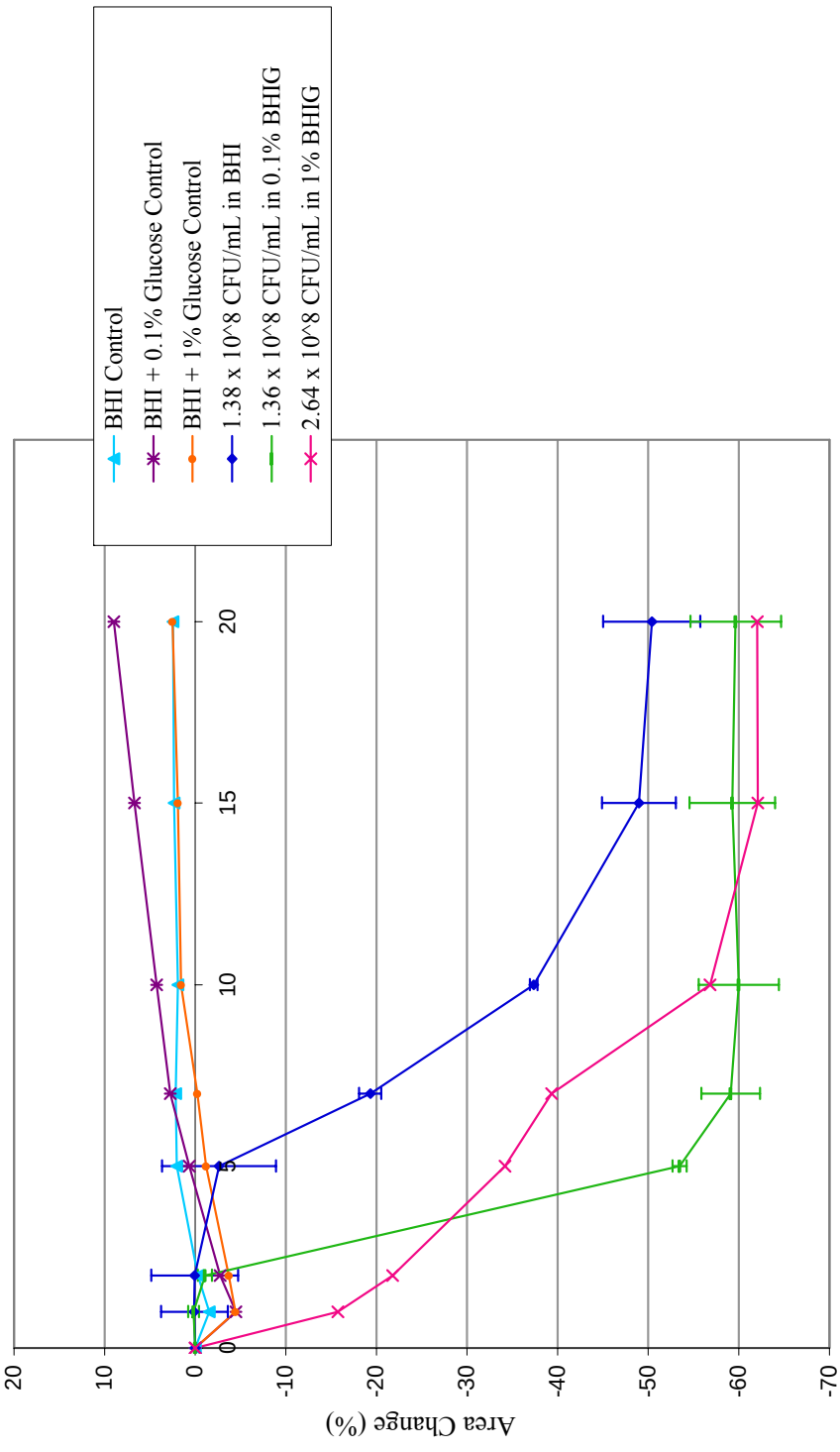

BHI Control
BHI + 0.1% Glucose Control
BHI + 1% Glucose Control
1.38 x 10^8 CFU/mL in BHI
1.36 x 10^8 CFU/mL in 0.1% BHIG
2.64 x 10^8 CFU/mL in 1% BHIG
20
10
0
-10
-20
-30
-40
-50
-60
-70
Area Change (%)
0
5
10
15
20
Time (minutes)

Figure 9

In figure 9, both of the erythrophore responses to 1.38 x 10^8 CFU/mL *B. cereus* cultured in BHI and 1.36 x 10^8 CFU/mL *B. cereus* cultured in BHI supplemented with 0.1% glucose represent the averages of duplicated responses, with error bars representing their standard deviations. These two separate erythrophore responses to these viable cell counts deviated from the erythrophore responses of their respective controls after 2 minutes, and then continued on causing significantly different aggregative responses. The erythrophore response to 1.36 x 10^8 CFU/mL *B. cereus* cultured in BHI with 0.1% glucose undergoes a rapid aggregation and ends at -59.7%; while the erythrophore response to 1.38 x 10^8 CFU/mL *B. cereus* cultured in BHI undergoes a gradual aggregation, ending at -50.4%. The erythrophore response to 2.64 x 10^8 CFU/mL *B. cereus* cultured in BHI with 1% glucose initially caused a sharp aggregation then steadily declined, reaching a final percent area change of –62.0%.

Erythrophore Responses to *B. cereus* Cultured in Luria-Bertani Broth Supplemented with Glucose

Experiments were performed using *B. cereus* cultured in Luria Bertani supplemented with glucose broth to observe whether the same glucose effect of increased enterotoxin production occurred in LB as seen in BHI supplemented with glucose.

The erythrophore responses to LB broth, and LB broth supplemented with glucose controls were reacted upon multiple erythrophore preparations. The data for the erythrophore responses to LB supplemented with 0.1% and 1% glucose were each averaged from two different erythrophore preparations, while the data for the LB control was performed on a total of six different preparations (see figure 10). After the data was averaged, the standard deviations were calculated (data not shown) and no significant differences were found amongst the erythrophore responses to LB broth or to LB broth supplemented with 0.1% and 1% glucose controls.

Since all of the controls performed satisfactorily upon the erythrophores, the experiments proceeded with analyzing the erythrophore responses to *B. cereus* cultured in LB, and LB supplemented with 0.1% and 1% glucose. For the erythrophore response to *B. cereus* cultured in LB for 7 hours, one reaction of 1.18 x 10^8 CFU/mL *B. cereus* is shown as a comparison to the cell counts of *B. cereus* cultured in LB supplemented with glucose. The erythrophore response to this cell count was chosen because it was the closest cell count obtained to the glucose supplemented cultures that resulted in a response that deviated from the response to the LB control.

In figure 10, the erythrophore responses to *B. cereus* cultured in LB with 0.1% and 1% glucose represent the average of duplicated experiments performed on two different erythrophore preparations. The erythrophore response to 1.18 x 10^8 CFU/mL *B. cereus* cultured in LB resulted in a gradual aggregation that ended

at -6.66% area change. The erythrophore response to 2.93 x 10^7 CFU/mL *B. cereus* in LB supplemented with 0.1% glucose caused the most rapid aggregation of all the LB samples, and ended at a final area change of -41.5%. The erythrophore response to 2.99 x 10^7 CFU/mL *B. cereus* in LB with 1% glucose deviated from the LB with 1% glucose control after 10 minutes. The response to 2.99 x 10^7 CFU/mL *B. cereus* in 1% LBG induced the weakest response and ended at a total area change of -23.7%. For the erythrophore responses to LB and LB supplemented with glucose, the *B. cereus* samples cultured in LB with 0.1% glucose induced the most aggregation, followed by a weaker response for *B. cereus* cultured in LB with 1% glucose.

Figure 10. Erythrophore responses to *B. cereus* cultured in Luria Bertani broth and Luria Bertani broth supplemented with glucose. *B. cereus* was cultured in LB broth supplemented with 0.1% or 1% glucose for 7 hours at 30ºC and 200rpm. *B. cereus* was cultured in LB for 9 hours at 30ºC and 200rpm. Viable cell counts were obtained after overnight incubation and are listed in ascending order, with the respective erythrophore response induced. The erythrophore responses to the LB broth control, LB broth supplemented with 0.1% glucose control, and LB broth supplemented with 1% glucose control represent the background responses to the media alone.

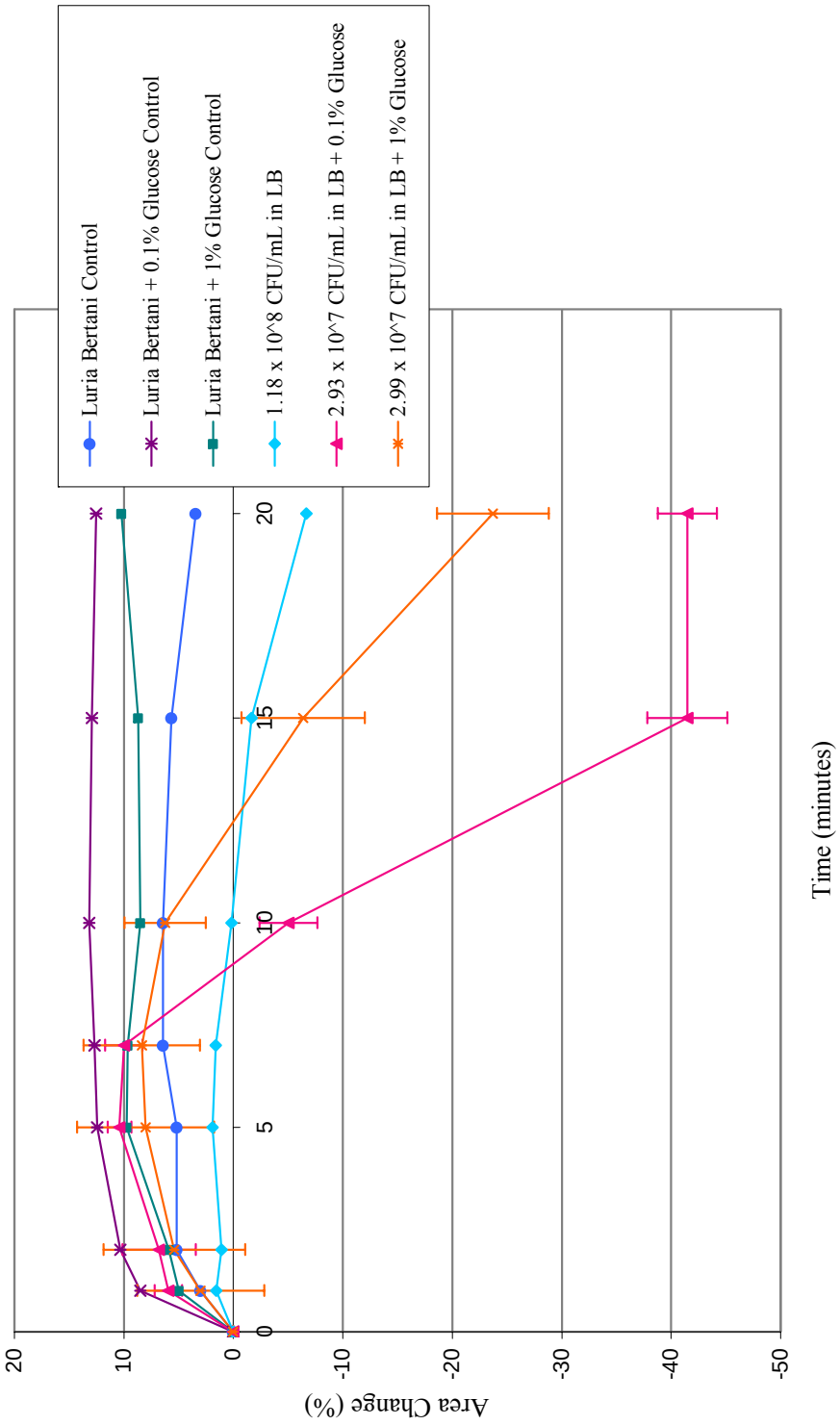

Luria Bertani Control
Luria Bertani + 0.1% Glucose Control
Luria Bertani + 1% Glucose Control
1.18 x 10^8 CFU/mL in LB
2.93 x 10^7 CFU/mL in LB + 0.1% Glucose
2.99 x 10^7 CFU/mL in LB + 1% Glucose
Area Change (%)
20
10
0
-10
-20
-30
-40
-50
0
5
10
15
20
Time (minutes)

Figure 10

Erythrophore Responses to *Bacillus cereus* Cultured in Luria Bertani Broth Versus Brain Heart Infusion Broth

Figure 11 illustrates how *B. cereus* cultured in Brain Heart Infusion (BHI) induced a stronger erythrophore response than *B. cereus*, of similar cell counts, cultured in Luria Bertani (LB). The responses to *B. cereus* cultured in BHI induced rapid erythrophore aggregation after 5 minutes, whereas the *B. cereus* cultured in LB induced a weak erythrophore response at 10^8 CFU/mL, and induced no significant response at 10^7 CFU/mL *B. cereus.*

Figure 11. Erythrophore responses to *B. cereus* cultured in Luria Bertani broth and Brain Heart Infusion broth. *B. cereus* was cultured in LB and BHI broth for 9 to 12 hours at 30ºC and 200rpm. The 8.23 x 10^7 CFU/mL *B. cereus* in BHI and 8.85 x 10^7 CFU/mL *B. cereus* in LB are used in a comparison of the erythrophore responses to *B. cereus* cultured in two different media. The 1.38 x 10^8 CFU/mL *B. cereus* BHI and 1.18 x 10^8 CFU/mL *B. cereus* in LB are also used in the comparison.

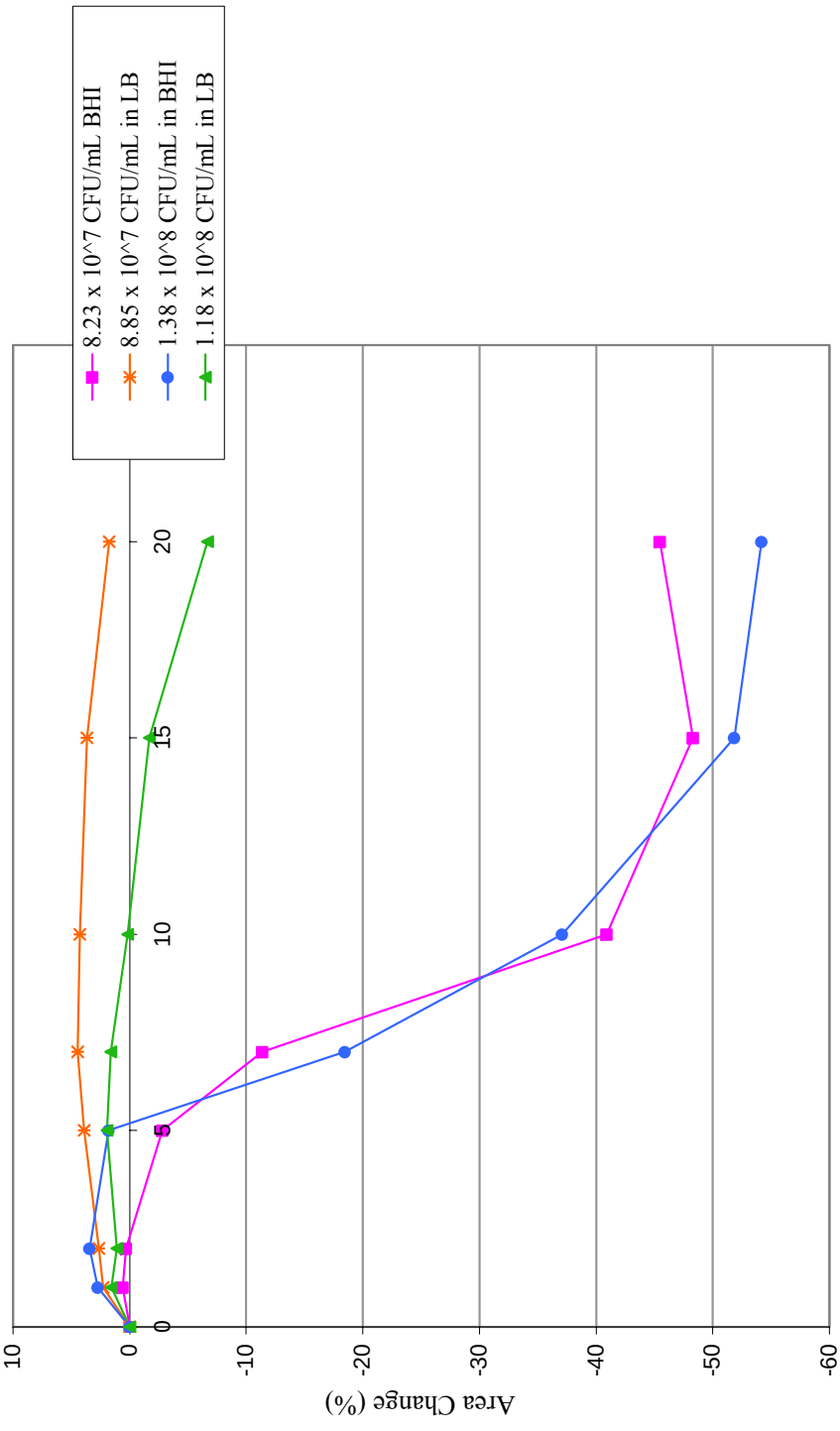

8.23 x 10^7 CFU/mL BHI
8.85 x 10^7 CFU/mL in LB
1.38 x 10^8 CFU/mL in BHI
1.18 x 10^8 CFU/mL in LB
10
0
-10
-20
-30
-40
-50
-60
Area Change (%)
0
5
10
15
20
Time (minutes)

Figure 11

Erythrophore Responses to *Bacillus cereus* Cultured in Luria Bertani Broth Supplemented with Glucose Versus Brain Heart Infusion Broth Supplemented with Glucose

The erythrophore responses to LB and BHI supplemented with 0.1% and 1% glucose are illustrated in figure 12. The 5.85 x 10^7 CFU/mL *B. cereus* in LB with 0.1% glucose and 6.80 x 10^7 CFU/mL *B. cereus* in BHI with 0.1% glucose make for a good comparison because the viable cell counts are similar. The erythrophore responses to each of these cultures are also similar, with the two responses never separating more than 8.00% throughout the 20 minute response. The 5.85 x 10^7 CFU/mL *B. cereus* in LB with 0.1% glucose ended at -47.6%, and the 6.80 x 10^7 CFU/mL *B. cereus* in BHI with 0.1% glucose ended at -52.1%.

Viable cell counts of *B. cereus* cultured in LB and BHI supplemented with 1% glucose are not identical, however, are the closest available. The 2.99 x 10^7 CFU/mL *B. cereus* in LB with 1% glucose induces dispersion in the erythrophores for 10 minutes, then induces a slight aggregation, ending at a final area change of -23.7%. The 1.32 x 10^8 CFU/mL *B. cereus* in BHI with 1% glucose induces a very rapid aggregative response in the erythrophores in the first 2 minutes, then slightly decreases in pace, ending at a final area change of -56.6%.

Figure 12. Erythrophore responses to *B. cereus* cultured in Luria Bertani broth supplemented with glucose and Brain Heart Infusion broth supplemented with glucose. *B. cereus* was cultured in LB and BHI broth supplemented with 0.1% or 1% glucose for 7 hours at 30ºC and 200rpm. The 5.85 x 10^7 CFU/mL *B. cereus* in LB supplemented with 0.1% glucose and 6.80 x 10^7 CFU/mL *B. cereus* in BHI supplemented with 0.1% glucose are used in the comparison of the erythrophore responses to *B. cereus* cultured in media supplemented with 0.1% glucose. The 2.99 x 10^7 CFU/mL *B. cereus* in LB supplemented with 1% glucose and 1.32 x 10^8 CFU/mL *B. cereus* in BHI supplemented with 1% glucose are used in the comparison of the erythrophore responses to *B. cereus* cultured in media supplemented with 1% glucose.

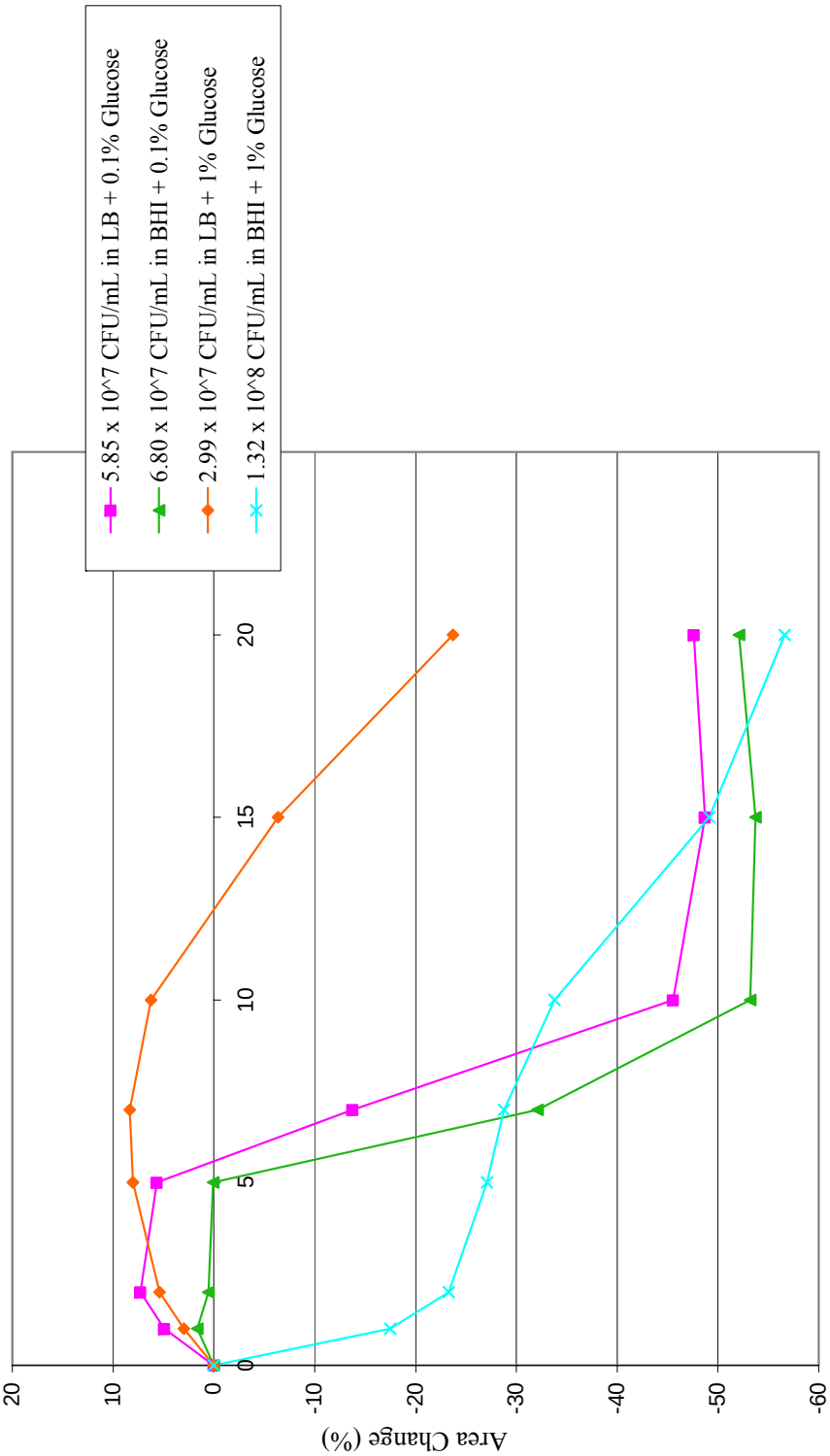

5.85 x 10^7 CFU/mL in LB + 0.1% Glucose
6.80 x 10^7 CFU/mL in BHI + 0.1% Glucose
2.99 x 10^7 CFU/mL in LB + 1% Glucose
1.32 x 10^8 CFU/mL in BHI + 1% Glucose
Area Change (%)
20
10
0
-10
-20
-30
-40
-50
-60
0
5
10
15
20
Time (minutes)

Figure 12

DISCUSSION

Bacillus cereus Food Poisoning and Detection Techniques

Foodborne illnesses are the result of chemical, parasitic and biological contamination of food products, and are most commonly caused by microbiological agents. In the United States it is estimated that there are over 76 million foodborne illness cases each year, resulting in an approximate economic loss of $40 billion (4, 75).

Bacillus cereus is a Gram positive, spore-forming, aerobic bacilli that can be found in the water, air, soil and soil-grown vegetables and grains (10). *B. cereus* is an important bacterium in regards to food safety as it is the etiological agent for two types of food poisoning, a diarrheal-type and an emetic-type (48). *B. cereus* food poisoning is typically due to the improper refrigeration or inadequate reheating of foods that have been cooked. The actual number of cases of *B. cereus* food poisoning is unknown due to its symptoms being similar to other food poisoning illnesses and because it only causes a mild illness (26, 35). Data from previous *B. cereus* food poisoning outbreaks have been compiled and from this data it has been elucidated that food products containing 10^5 to 10^8 colony forming units of diarrheal-type *B. cereus* per gram can cause food poisoning (44).

Current detection methods for detecting the presence of *B. cereus* include traditional plating techniques, molecular techniques, and immunoassays. The traditional techniques require culturing time, and the molecular and immunoassays detect based on the structure of the agent, and cannot report on its pathogenicity. The molecular and immunoassay techniques are sensitive, as the molecular techniques can detect 10^2 CFU/mL of *B. cereus* and immunoassays can detect down to 1 to 2 nanograms per milliliter of *B. cereus* enterotoxic proteins (6, 21).

A unique type of biosensor, based on the red-pigmented chromatophores, or erythrophores, of *Betta splendens* (Siamese fighting fish) can respond to

biologically active agents, and distinguish the pathogenic from the nonpathogenic. This erythrophore-based biosensor utilizes the change in optical density caused by pigmented cell movements, which results in a visual reaction that can be monitored.

An Erythrophore-Based Cytosensor for *Bacillus cereus* Detection

The objective of this research was to determine the response limit of *Betta splendens* erythrophores to viable cells of *Bacillus cereus*. In addition, erythrophore responses to *B. cereus* cultured in different media were analyzed.

The responses of the erythrophores to *B. cereus* cultured in Brain Heart Infusion broth and Luria Bertani broth were analyzed in order to find the medium that resulted in the highest degree of response. Upon this discovery, the specified culturing methods could be used in future research analyzing *B. cereus* gene expression and strain pathogenicity.

Brain Heart Infusion broth (BHI) has been used in previous research as a culturing medium for *B. cereus* enterotoxin production (9, 19). Luria Bertani broth (LB) has been used in previous research as a culturing medium for *B. cereus* and *Bacillus thuringiensis* (37, 46, 47, 64, 66). BHI supplemented with 0.1% glucose is commonly used to maximally produce *B. cereus* enterotoxins (18, 30, 38, 41, 72, 74). BHI supplemented with 1% glucose has also been used to enhance enterotoxin production (52). LB supplemented with 0.1% and 1% glucose was analyzed to test whether enterotoxin production could also be maximized in this media with the addition of glucose.

Bacillus cereus Growth Curve and Viable Cell Counts

It is general microbiological knowledge that as a bacterial culture multiplies, it passes through lag phase, exponential growth phase, stationary phase, death phase, and for some bacteria, sporulation. The growth curves for *B. cereus* cultured in LB, LB supplemented with glucose, BHI, and BHI supplemented with glucose were carried out and observed to be very similar. Lag phase was found to occur from initial inoculation through approximately 1 hour; then the cultures entered exponential growth phase, which continued through approximately 4 hours. The onset of stationary phase occurred from approximately 4 hours to 7 hours, and then the cultures entered stationary phase.

B. cereus entered the onset of sporulation only after all of the nutrients were exhausted. Sporulation occurred at different times depending on the nutritional quality of the medium. The timing of sporulation in *B. cereus* was monitored with spore stains and wet mounts. For *B. cereus* cultured in LB, the onset of sporulation occurred by 27 hours. When cultured in LB supplemented with glucose, BHI, and BHI supplemented with glucose media, sporulation occurred some time between 27 hours and 4 days after initial inoculation.

For the erythrophore experiments described herein, *B. cereus* was cultured in BHI for 9.5 hours and 12 hours to obtain a range of cell counts (10^6 through 10^8 CFU/mL) that resulted in a high degree of response in the erythrophores; and to provide comparable viable cell counts to the BHI supplemented with glucose cell counts. In addition, *B. cereus* was cultured in LB and incubated for 6 hours through 12 hours to obtain a range of cell counts (10^3 through 10^{10} CFU/mL). The BHI supplemented with glucose, as well as the LB supplemented with glucose, cultures were incubated under the same conditions as was performed by previous researchers desiring maximal expression of enterotoxins, which was 7 hours at 30ºC and 200 rpm (18, 30, 38, 41, 72, 74).

Temporal *plcR* Gene Expression

A summary of the key players in *Bacillus thuringiensis* (in the *Bacillus cereus* group) and *Bacillus subtilis* toxin production begins in exponential growth phase as PapR, an extracellular signaling peptide, is accumulated within the bacterial cell by an oligopeptide permease (Opp) system (34). At the onset of stationary phase, PapR activates the key regulator of virulence factors, *plcR,* by increasing the affinity of PlcR to its DNA targets (66). PlcR regulates proteases, cytotoxins, phospholipases C, and enterotoxins produced by *B. cereus* (see figure 1).

Lereclus, *et al* (46), showed that when *B. thuringiensis* and *B. subtilis* are cultured in LB medium, *plcR* transcription begins one half hour before the onset of stationary phase and continues to be expressed for 1 hour into this phase. The transcription of *plcA*, which encodes for a phospholipase C, begins at the onset of stationary phase, and continues to be expressed for 4 hours. So, as time progresses into stationary phase, the level of *plcR* quickly decreases and the level of *plcA* gradually dissipates. The level of intracellular PlcR is dependant on the level of PapR taken into the cell by oligopeptide permease (Opp) during exponential phase (46).

Hypotheses for changes in this temporal gene regulation would include the effect of culturing *B. cereus* in different media. It would be hypothesized that higher than normal levels of *plcA* could be present in a bacterial culture grown in high glucose-containing medium if a superabundance of *plcR* was transcribed due to the higher influx and production of PapR. This could allow for *plcR* and *plcA* to be expressed for a prolonged period of time, possibly exceeding the time points observed by Lereclus (46).

To observe the erythrophore responses to the hypothesized temporal gene expression proposed by Lereclus (46), experiments were performed using *B. cereus* cultured in Brain Heart Infusion broth, Luria Bertani broth, Brain Heart

Infusion broth supplemented with two concentrations of glucose (0.1% and 1%), and Luria Bertani broth supplemented with two concentrations of glucose (0.1% and 1%).

Analysis of the Erythrophore Responses to *Bacillus cereus* Cultured in Brain Heart Infusion Broth

When *B. cereus* was cultured in Brain Heart Infusion broth (BHI) (figure 6) the erythrophore response to 10^6 colony forming units per milliliter (CFU/mL) *B. cereus* registered just below the erythrophore response to the BHI control; therefore 10^6 CFU/mL is the lowest detectable viable cell count of *B. cereus* cultured in BHI to induce an aggregated erythrophore response. Overall, viable cell counts of 10^6 CFU/mL through 10^8 CFU/mL *B. cereus* cultured in BHI elicited an aggregative response. The observed trend in erythrophore response to *B. cereus* cultured in BHI is, the higher the cell count, the stronger the aggregative response. This trend is most likely the result of higher bacterial cell counts producing a higher amount of enterotoxins, which in the erythrophores results in increasing degrees of aggregation.

Analysis of the Erythrophore Responses to *Bacillus cereus* Cultured in Luria Bertani Broth

In analyzing the results of *B. cereus* grown in LB (figure 7, 8), it becomes evident that 10^3 CFU/mL *B. cereus* contained too few cells to induce a significant response in the erythrophores; whereas 10^4 through 10^6 CFU/mL *B. cereus* induced rapid aggregative responses in the erythrophores. It is unknown why samples containing 10^7 CFU/mL *B. cereus* cultured in LB did not register a significant response in the erythrophores, while viable cell counts of 10^8 through 10^{10} CFU/mL *B. cereus* proceeded to induce responses consisting of an initial dispersion, followed by gradual aggregation.

What is possibly being observed with this range of cell counts is the response to *plcR*-regulated enterotoxins (i.e. HBL, NHE) in the first hour of the onset of stationary phase (corresponding to 10^4 to 10^5 CFU/mL) and the continuation of the activation of *plcA* through the first 4 hours (corresponding to 10^4 to 10^8 CFU/mL). As time passes and the levels of *plcR* and *plcA* dissipate, the toxicity of the sample undergoes a substantial decrease in virulence, or the levels of dispersion-inducing and aggregation-inducing toxins are equalized (corresponding to 10^7 CFU/mL). Then, what remains to be discovered, is the virulence factor(s) expressed in stationary or late stationary phase that elicit a slight dispersion (~20%) followed by aggregation (corresponding to 10^8 to 10^{10} CFU/mL).

Perhaps at the time (9 to 12 hours) corresponding to 10^8 through 10^{10} CFU/mL, what is seen is a response to a second phase (or newly produced) set of toxins, or virulence factors that were earlier masked by the aggregative response of the diarrheagenic enterotoxins; or this could be a transition to the next phase, sporulation.

Analysis of the Erythrophore Responses to *Bacillus cereus* Cultured in Brain Heart Infusion Broth Supplemented with Glucose

The observed trend in erythrophore response to *B. cereus* cultured in BHI and BHI supplemented with glucose (BHIG) is *B. cereus* cultured in BHI supplemented with 0.1% glucose and 1% glucose elicited a stronger aggregative response in the erythrophores than *B. cereus* cultured in BHI without glucose (figure 9). *B. cereus* cultured in BHI supplemented with 1% glucose elicited the strongest initial aggregation within the first 2 minutes, and *B. cereus* cultured in BHI supplemented with 0.1% glucose resulted in the most aggregative erythrophore response overall. When *B. cereus* is cultured in BHI, an aggregative erythrophore response is observed, but to a lesser degree than that of *B. cereus* cultured in BHI supplemented with 0.1% and 1% glucose. In summary, the research presented here demonstrates that when *B. cereus* is cultured in BHI supplemented with glucose, an increased aggregative response is observed in the erythrophores.

Due to an increased amount of glucose in the media, exponential growth phase is extended until all of the glucose is metabolized. When *B. cereus* is cultured in media supplemented with glucose, *plcR* is expressed at higher levels than in media without glucose; therefore more *plcR*-regulated enterotoxins (i.e. HBL, NHE) would be present in the culture (47). This is a possible explanation for the trend of higher glucose concentrations in BHI eliciting a stronger aggregative response in the erythrophores.

Analysis of the Erythrophore Responses to *Bacillus cereus* Cultured in Luria Bertani Broth Supplemented with Glucose

For the experiments involving *B. cereus* cultured in LB supplemented with glucose, *B. cereus* was cultured under the same time (7 hours) and temperature (30°C) conditions as in the BHI supplemented with glucose experiments. The observed trend in erythrophore response to *B. cereus* cultured in LB supplemented with glucose, as seen in figure 10, is when *B. cereus* is cultured in LB with a small amount of glucose (0.1%), enterotoxin production is enhanced, but when *B. cereus* is cultured in LB containing an excess of glucose (1%), enterotoxin production is hampered, although the level is still higher than that of *B. cereus* cultured in LB without glucose. Explanations for this trend could be based on the gene expression of *plcR* and *plcR*-regulated virulence factors, in that added glucose causes rapid metabolism and enterotoxin production in the culture, but too much glucose causes inhibition of certain *B. cereus* virulence-related genes.

The metabolism of *B. cereus* does not seem to be repressed by the higher amount of glucose, as the *B. cereus* cultured in LB supplemented with 1% glucose attained a similar cell count as the *B. cereus* cultured in LB supplemented with 0.1% glucose (see figure 10). The glucose effect on *B. cereus* cultured in LB appears to influence the pathogenicity of the bacterium, but not the growth.

Concluding Remarks

In conclusion, this research demonstrates that *Betta splendens* erythrophores can detect a diarrheal strain of *Bacillus cereus* (ATCC 49064) at or below 10^5 CFU/gram when grown in Luria Bertani medium, which induces a response in the erythrophores at 10^4 CFU/mL. This data supports the use of an erythrophore-based biosensor for food safety analyses related to *B. cereus* food poisoning.

This research also supports the current use of Brain Heart Infusion supplemented with 0.1% glucose medium to induce *B. cereus* to maximally express enterotoxins; establishing this medium as a preferred for use in research regarding *B. cereus* virulence factor gene expression.

The erythrophore-based cytosensor has the potential to be used in temporal gene expression analyses of virulence factors. As this research shows, the toxins secreted by *B. cereus* cause the erythrophores to respond in different ways, depending on the medium and the temporal stage of the culture. Varying responses are observed in *B. cereus* cultured in LB, where responsiveness is not the result of increasing cell counts, but of temporal toxin production. Conducting future studies in order to identify the virulence factors causing the dispersive and aggregative erythrophore responses would be of great interest. Also of interest would be further research on the limit of detection if *B. cereus* in media supplemented with glucose; and additional erythrophore response analyses to *B. cereus* during different growth stages (i.e. exponential, sporulation), to other strains of *B. cereus*, and to *B. cereus* cultured in different media (i.e. sporulation media).

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APPENDIX

Biographical information

Melissa Austin was born May 23, 1981 and raised in Salem, Oregon. After graduation from Salem Academy High School in Salem, Oregon, she began her undergraduate studies at George Fox University in Newberg, Oregon. She subsequently transferred to Western Oregon University in Monmouth, Oregon, where she earned a Bachelor's of Science in Biology with a minor in Forensic Science. Fall term, 2003, she began graduate school in the Department of Microbiology at Oregon State University in Corvallis, Oregon. She was offered a teaching assistantship for her first year, while she rotated through two laboratories. She began with a rotation in the laboratory of Dr. Mafuz Sarker, working on the insertion of green fluorescent protein into *Clostridium perfringens*. The following term, she rotated into the laboratory of Dr. Claudia Hase, working with chemotaxis in *Vibrio cholerae*. In the summer of 2004, she began research in the laboratory of Dr. Janine Trempy. During the fall of 2004 she was a private microbiology consultant for Hach Homeland Security Technologies in Grants Pass, Oregon. She was the recipient of the Oregon Sports Lottery Scholarship for research for the academic year 2004-2005.

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