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I am submitting herewith a thesis written by Amy Tomaszewski entitled, "Expression of *luxI* in *Bacillus thuringiensis* for the Development of a Bacteriophage-based Bioreporter System for the Detection of Anthrax." I have examined the final electronic copy of this thesis for form and content and recommend that it be accepted in partial fulfillment of the requirements for the degree of Master of Science, with a major in Microbiology.

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Expression of *luxI* in *Bacillus thuringiensis* for the
Development of a Bacteriophage-based Bioreporter System
for the Detection of Anthrax

A Thesis
Presented for the
Master of Science
Degree
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Amy Tomaszewski
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Dedication

This thesis is dedicated in the memory of my father, who opened the door to the mysterious world of nature to me. His appreciation and fascination with the workings of the natural world fostered the same love of science in me.

Joseph Tomaszewski
1947-1998

Acknowledgments

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Abstract

In light of the recent anthrax attacks of 2001 in the United States, the need for a rapid, sensitive, and real-time surveillance system for detecting pathogenic bacteria has become readily apparent. A proof-of-principle study involving development of a bacteriophage-based bioreporter system for the detection of *Bacillus anthracis* spores has been initiated in the surrogate strain *Bacillus thuringiensis* 4Q7. The final goal of the project is to develop an engineered bacteriophage with the *luxI* gene from *Vibrio fischeri*, which upon infection of the target organism, would produce the signal molecule, *N*-(3-oxohexanoyl)-L-homoserine lactone (OHHL), capable of inducing bioluminescence in a neighboring OHHL bioluminescent bioreporter cell. Studies done in this project involve expression of a plasmid borne *luxI* gene in *B. thuringiensis*, quantification of OHHL produced, and examination of the effect of the OHHL degrading enzyme lactonase found in *B. thuringiensis*.

The *luxI* gene was expressed under a xylose-inducible promoter in *B. thuringiensis* 4Q7. This strain was capable of producing a detectable amount of OHHL within 3 hours post-induction as determined by bioluminescent assays with the OHHL bioreporter, RoLux. In addition, OHHL concentrations of approximately 500 nM were reached during late-log phase growth of the *luxI* engineered *B. thuringiensis* strain. Other aspects of the study included optimization of the detection of OHHL with the RoLux bioreporter. Finally, the *aiiA* gene, or lactonase, from *B. thuringiensis* 4Q7 was detected, cloned, and sequenced, and determined to be functional. Assays involving incubation of OHHL with growing cultures of *B. thuringiensis* provided evidence that the degradation

of OHHL by lactonase would not have an effect on detecting the molecule from the engineered strain at early log-phase of growth.

Recommendations for advancement of this project are made, along with future work in this area is provided.

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Chapter 1: Introduction

The deliberate use of biological agents as weapons to inflict harm and create terror in other human beings has been seen throughout history (Hawley, 2001 and Christopher, 1997). Although early attempts at biowarfare were done using crude methods, advances in the science of microbiology have allowed for the development of sophisticated weapons. *Bacillus anthracis* spores, the causative agent of anthrax, have been used on several occasions, including an attack by the Aum Shinrikyo terrorist group of Japan in 1993 (Miller, 2001), and most recently, in the United States following the terrorist attack of September 11, 2001 (CDC, 2001). The anthrax-laced letters of 2001 revealed how unprepared our system is regarding rapid detection of a bioterrorism agent. Some of the current detection methods include laboratory culture (Murray, 1999), various PCR techniques such as Quantitative PCR (Bader, 2001) and Multilocus Sequence Typing (Baillie, 2003), and MIDI Whole Cell Fatty Acid Analysis (Teska, 2001). Anthrax identification techniques have recently evolved for more rapid and reliable detection, but they do not allow for online surveillance of the organism.

One technology, originally employed for detection of food pathogens in 1987 by Ulitzur and Kuhn, is the use of recombinant phage-reporters (Ulitzer, 1987). This tool allows for rapid detection of bacterial host after infection with its corresponding bacteriophage. Because bacteriophage have high specificity of host for infection, they provide an accurate and reliable technique for pathogen identification. The recombinant

phage is created by inserting a reporter gene within the genome of the phage, then allowing it to infect the host followed by screening for the reporter gene product (Goodridge, 2002). Currently, there are phage-reporters for strains such as *E. coli* O157:H7 (Waddell, 2000), *Salmonella spp.* (Chen, 1996), *M. tuberculosis* (Jacobs, 1993), and *L. monocytogenes* (Loessner, 1996). These phage-reporters have been accurate at low cell numbers (10 cells/ml) and in a minimal incubation period (60 mins). The only disadvantage to this system is that it does not allow for online detection.

The development of bioluminescent bioreporter technology has opened the door for online monitoring of specific compounds found within the environment. Bioreporters for substances such as mercury (Selifonova, 1993), toluene (Applegate, 1998) and naphthalene (King, 1991) have been designed for use in conjunction with portable luminescence measuring devices (Ripp, 1999 and Ripp, 2000). Inherent in the design of bioluminescent bioreporters is the need for a specific compound to induce the reporter at concentrations at or above the detection limit. By genetically engineering a bacteriophage to produce a specific compound upon infection of an organism, development of an online monitoring system of bacterial pathogens may be accomplished. Bacteriophage offer several benefits for this type of system, including high specificity for the target organism, increased signal amplification, and ease of manipulation and transport.

In an effort to create an online monitoring system for the detection of *B. anthracis*, a proof-of-principle project was developed for *Bacillus thuringiensis*, a known surrogate for *B. anthracis* (Helgason, 2000), in which a bacteriophage specific for *B. thuringiensis* are engineered with the *luxI* gene from *Vibrio fischeri*. Upon infection, the

host will then produce a unique signal molecule, *N*-(3-oxohexanoyl)-L-homoserine lactone (OHHL), and induce the OHHL *lux*-based bioluminescent bioreporter, RoLux (*E. coli* STBL4 EZ:TN *luxRoCDABE*).

In this study, preliminary work on expression of the *luxI* gene and its product LuxI in the plasmidless host strain, *Bacillus thuringiensis* 4Q7, was performed. Specific aims included: 1) determining whether *B. thuringiensis* is capable of expressing the Gram-negative *luxI* gene from a vector, 2) if the LuxI protein is capable of functioning in *B. thuringiensis*, 3) if the necessary components for OHHL synthesis are present within the cell and finally, 4) quantification of the OHHL from *B. thuringiensis* with the OHHL bioluminescent bioreporter, RoLux.

One potential limitation to this system is the presence of the lactonase enzyme (AiiA) found within members of the *Bacillus cereus* group, including *B. thuringiensis* and *B. anthracis* (Dong, 2000 and Dong, 2002). The lactonase enzyme targets homoserine lactone molecules such as OHHL, rendering them inactive (Dong, 2001). An aspect of this study was to determine the presence of AiiA in *B. thuringiensis* 4Q7, what the activity of the enzyme was in different growth stages of the organism and what, if any, effect it had upon expression of OHHL from the host organism.

Chapter 2: Literature Review

Research Proposal

Although bioterrorism has been a reality of society for centuries, the development of increasingly dangerous and more highly purified pathogens has forced scientists to develop more sensitive, faster and accurate means of detecting these organisms before they have an opportunity to infect. In an effort to design a system for the detection of *B. anthracis* spores, a proof-of-principle study has been implemented to create a bacteriophage-based reporter system for the *B. anthracis* surrogate, *B. thuringiensis*. The proposed mechanism includes inserting the *luxI* gene from the bioluminescent marine organism, *V. fischeri*, into the genome of a bacteriophage that is specific for *B. thuringiensis*. This engineered bacteriophage will then infect the target organism, *B. thuringiensis*, and begin production of the signal molecule *N*-(3-oxohexanoyl)-L-homoserine lactone (OHHL). This freely diffusible signal molecule will leave the target organism, and enter a neighboring OHHL bioluminescent bioreporter cell. Once the OHHL in the confined area reaches a threshold concentration, induction of the bioluminescence genes will occur, producing a measurable bioluminescent signal (Figure 2-1).

Several questions arise in consideration of the feasibility of this project, including what is the necessity of this technology, why is *B. thuringiensis* a valid surrogate for *B. anthracis*, how do bacteriophage provide the required specificity for detecting a target organism, and how will the OHHL reporter respond to its inducer? In addition, there is

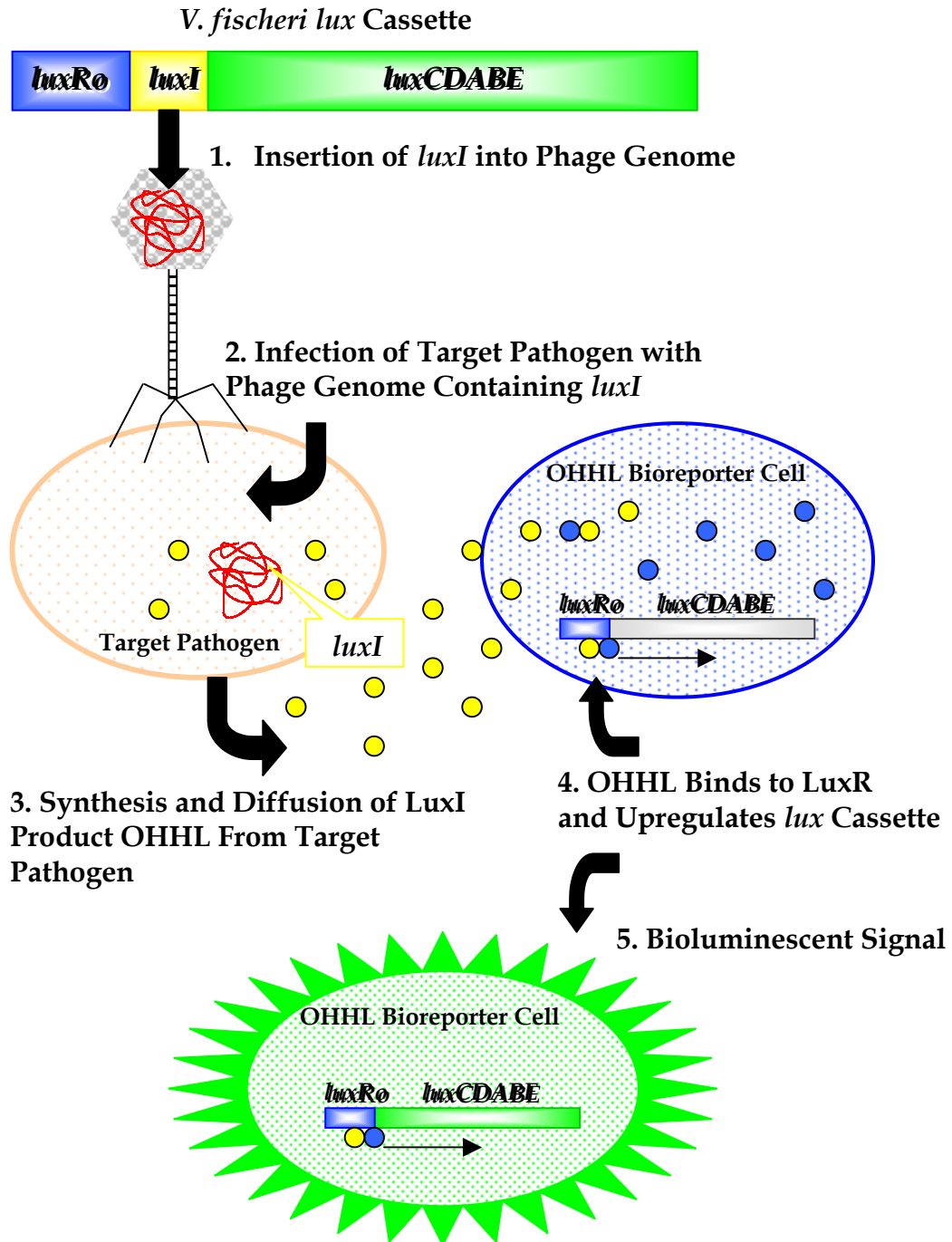


Figure 2-1. Bacteriophage-based Bioreporter System. Once the bacteriophage, which contains the *luxI*, infects the target organism, the signal molecule OHHL is diffused from the organism, binds to the LuxR protein, and induces bioluminescence in a neighboring OHHL bioreporter cell.

some question about the likelihood of a Gram-positive organism such as *B. thuringiensis* containing the proper components for OHHL assembly, since this naturally occurs in a Gram-negative bacterium. Finally, the question remains whether the enzyme lactonase will be capable of limiting the amount of OHHL produced by *B. thuringiensis*, and interfere with signal detection.

To address fundamental questions inherent in the design of this detection system, a comprehensive literature review of these subjects is described below. Topics include the use of *B. anthracis* as a bioweapon throughout history as a means for imparting the significance of this research, the relatedness of members of the *B. cereus* group for justification of using *B. thuringiensis* as a surrogate for *B. anthracis*, and a description of bacteriophage and their uses as proof of the efficiency, ease of manipulation, and inherent specificity. In addition, quorum-sensing is reviewed for understanding the mechanism of bioluminescence induction of the reporter, the appropriateness of choosing OHHL as a unique signal molecule from *B. thuringiensis*, and OHHL signal generation by the LuxI protein. A section on fatty-acid biosynthesis is included as well to provide evidence of its function in OHHL production and the homology of this pathway between Gram-negative and Gram-positive organisms. Finally, a description of the recently discovered enzyme lactonase is included, and its role in OHHL stability.

The History of Anthrax as a Bioweapon

Biological agents have been used throughout history as a means for infecting soldiers, civilians and even livestock. Reports of bioweapons date back as far as the 14th century where the Tatar forces succumbed to an epidemic of plague while attacking

Kaffa. In retaliation, the Tatar soldier catapulted the infected bodies of the deceased into Kaffa to initiate an epidemic in the city (Derbes, 1966). Hundreds of years later in the 18th century, Sir Jeffrey Amherst, commander of the British forces in North America, proposed a planned exposure of the Native Americans to smallpox. In 1763, Captain Ecuyer gave blankets and handkerchiefs from infected patients to the Native Americans, resulting in an epidemic of smallpox among Native Americans in the Ohio River Valley (Stearn, 1945).

Although these crude methods of biological warfare were effective, with the advancements made in the study of microbiology by Robert Koch, the ability to isolate and produce specific pathogens became available. Characteristics of *B. anthracis* spores lend themselves to being a useful tool for biowarfare, such as the ability to form spores which are heat, UV, and chemically resistant; high virulence and ability to cause acute illness in all mammals; and ease of manipulation and dissemination (U.S. Congress, Office of Technology Assessment, 1993). Research on the use of *B. anthracis* as a bioweapon began more than 80 years ago and *B. anthracis* spores have been developed in many biowarfare programs and by terrorist groups in that time (Christopher, 1997).

One of the first uses of *B. anthracis* as a bioweapon was seen during World War I by Germany. Substantial evidence showed that the Germans developed *B. anthracis* to infect livestock of neutral trading partners of the allied forces. One such case was the contamination of animal feed and infection of sheep from Romania for export to Russia. In addition, Argentinean livestock were also infected that were intended for Allied forces, resulting in the death of over 200 mules. Finally, attempts were made to infect feed and

livestock of U.S. horses, which were planned for export to Allied forces (Christopher, 1997).

Following World War I, the Japanese conducted biological weapons research while occupying Manchuria from 1932-1945. During that time, Unit 731, an extensive biowarfare research facility located near the town of Pingfan, conducted experiments on prisoners with numerous pathogens, including *B. anthracis*, resulting in the death of over 10,000 Chinese. In addition, weapons made by Unit 731 were used to attack 11 Chinese cities where water supplies and food items were contaminated with pure cultures of various agents including *B. anthracis* (Harris, 1994).

In addition to the Japanese developing biological agents post World War I, several other countries started research and developed their own biowarfare programs. One such country was the Soviet Union, whose biowarfare program, Biopreparat, developed numerous weaponized agents in massive quantities, although outlawed by the Geneva Protocol for the Prohibition of the Use in War of Asphyxiating, Poisonous or Other Gases, and of Bacteriological Methods of Warfare in 1925, and later by the Biological Weapons Convention (BWC) in 1972 (Harris, 1994; Frontline Interview). The Sverdlovsk facility had an accidental release of *B. anthracis* spores in 1979 when the air filters had not been activated. Over 95 cases of anthrax occurred, 64 resulting in death, in addition to the demise of numerous livestock in the area (Meselson, 1994).

The first reported case of a terrorist group using *B. anthracis* as a bioweapon occurred in 1995 in Japan. The religious cult, Aum Shinrikyo, allegedly released *B. anthracis* spores and botulinum toxin at least 8 times throughout Tokyo. Fortunately,

none of the attacks resulted in any casualties (Public Health Service Office of Emergency Preparedness, 1995).

Most recently, the U. S. suffered an attack with letters sent through the U. S. Postal Service laced with *B. anthracis* spores in September 2001. At least five letters were sent to Florida, New York City, and Washington, D.C from a location in Trenton, NJ. There were a total of 22 confirmed or suspected cases reported as a result of the mailing. Of the 22 cases, 11 people suffered from inhalation anthrax, with 5 deaths, and the other 11 contracted cutaneous anthrax (CDC, 2001). The organism was confirmed to be the *B. anthracis* Ames strain, although the specific source is still unknown (Inglesby, 2002).

Homology of the *Bacillus cereus* Group

The organisms *B. cereus*, *B. thuringiensis* and *B. anthracis* are closely related species making up what is known as the *B. cereus* group. *Bacillus anthracis* is a mammalian pathogen, while *B. thuringiensis* is an entomopathogen and *B. cereus* is a ubiquitous soil organism that may be an opportunistic pathogen for humans (Mignot, 2001). There are two phenotypes that may be used to distinguish between *B. anthracis* and *B. thuringiensis*. *Bacillus anthracis* is nonmotile, produces a capsule and is usually non-hemolytic. *Bacillus thuringiensis* produces intracellular toxins upon sporulation that have been widely used as insecticides (Kolstø, 2001). *Bacillus cereus* cannot be distinguished from *B. thuringiensis* before sporulation (Nakamura, 1994)

With regard to their genetic material, the genes encoding virulence factors for *B. anthracis* and *B. thuringiensis* are contained on plasmids. The only difference between

B. thuringiensis and *B. cereus* are the genes encoding for insecticidal toxins (Helgason, 2000). Upon examination of housekeeping genes within species of the *B. cereus* group, < 5% divergence in DNA homology was found between species (Helgason, 2000). However, analysis by amplified fragment length polymorphism (AFLP) of the members of the *B. cereus* group revealed that there are several unique locations in the *B. anthracis* genome which would allow for identification of this species when compared to *B. thuringiensis* and *B. cereus* (Radnedge, 2003).

Horizontal transfer between soil bacteria has been shown, specifically between species of the *B. cereus* group (Helgason, 1998). Genes responsible for virulence factors in *B. thuringiensis* were also found in *B. cereus* and *B. anthracis* (Guttmann, 2000). In addition, sequence from the pX01 plasmid from *B. anthracis*, which encodes the majority of the virulence factors, was also found in *B. cereus* and *B. thuringiensis* (Pannucci, 2002).

One significant difference in the genetic makeup of these organisms is the presence of a functional copy of the pleiotropic activator PlcR in *B. thuringiensis* and *B. cereus*, but not in *B. anthracis* (Agaisse, 1999). PlcR is responsible for secretion of non-specific toxins, such as hemolysins. The *plcR* gene has a nonsense mutation in *B. anthracis*, and this species uses AtxA as a transcriptional regulator for the virulence regulon. This may be how *B. anthracis* evolved with the transfer of genes for the virulence plasmid pAX01 (Mignot, 2001). It has been suggested that a *B. cereus* subgroup is the parent strain of *B. anthracis* (Pannucci, 2002), and that *B. cereus* and *B. thuringiensis* should be considered one species (Jackson, 1994).

Bacteriophage

Bacteriophage, or viruses that infect bacteria, were first discovered independently by Twort in 1915, and d'Herelle in 1917, as filterable, transmissible agents of bacterial lysis. Most Eubacteria, with the exception of *Neisseria meningitidis* and *N. gonorrhoeae*, have bacteriophage, which are specific for their host (Chanishvili, 2001). They are found in a variety of environments, including soil, water, sewage, and in general, most areas colonized by bacteria (Marks, 2000). The number of phage present in any given environment is relative to the concentration of bacteria, indicated by high numbers of phage in raw sewage and a lower numbers in unpolluted rivers. Bacteriophage diversity correlates with that of bacteria as well, with phage infecting at temperatures up to 95°C, and at a pH range from 1-11 (Sharp, 2001).

The mechanism of bacteriophage infection is well understood, and occurs in four stages. First, the bacteriophage attaches to its host via a receptor on the host cell surface for which the phage is specific. Next, the bacteriophage nucleic acid is injected into the cell, and then either begins replication (virulent phage), or inserts into the bacterial chromosome (temperate phage). Upon replication, formation of mature particles begins, followed by lysis of the host cells and release of daughter phage (Chanishvili, 2001).

The first stage in bacteriophage infection occurs when the phage is adsorbed to a receptor found on the bacterial surface. Almost every structure found on the cell membrane can act as a receptor, including flagella, pili, capsules, lipopolysaccharide, teichoic acid-peptidoglycan complexes, and surface proteins (for review, see Lindberg, 1973). Bacteriophage display high specificity toward the host organism, and will not infect other bacterial species that do not display the proper cell receptor. The specificity

of the adsorption step is determined by the presence of carbohydrates in the receptor. Although early work in the 1940's and 50's determined phage/host interactions and the specificity inherent in phage infection (for review, see Sharp, 2001), intensive study on Gram-negative and -positive cell envelopes and their appendages led to the elucidation of the structure of phage receptors (Lindberg, 1977).

The second step in bacteriophage infection involves the introduction of the phage genome into the bacterial host. This is accomplished in the majority of bacteriophage after binding to the host receptor through the tip of the tail attached to the capsid, which contains the genome. The nucleic acid of the bacteriophage is either injected into the cell, or taken up into the cell by the flagellae or pili, where, in the third step, it then utilizes host machinery to replicate the phage genome and to transcribe this to form new phage particles (Cann, 2001).

Phage that are lytic begin the process of replication immediately by releasing 50-200 new phage particles per infection (Marks, 2000). This is known as the “burst size”, and is preceded by the latent period when the bacteriophage are replicating. Other bacteriophage do not begin replication immediately, but instead, may insert their genome into the chromosome of the host. This is called lysogeny, where the viral genome persists, but gene expression is restricted. Some phage, such as the extensively studied phage λ , insert into a specific location in the chromosome, and remain stable until the phage is induced. Others, such as phage Mu, insert randomly into the chromosome. Once these lysogenic phage are induced by factors such as UV, stress, or chemical mutagens, the phage genome is released from the host chromosome, and begins the lytic cycle (Flint, 2000).

The inherent specificity of bacteriophage for a host has been utilized in numerous techniques. One of the earliest uses of phage was for treatment of bacterial infections, or phage therapy (for review, see Chanishvili, 2001 and Barrow, 1997). Although extensive study was done in the earlier part of the 20th century, interest waned in the United States with the advent of antibiotics. Both use and research with phage continued however in Russia. There, studies suggested that phage therapy would be highly effective for elimination and decrease of reoccurrence of infection as compared to antibiotics. Recent studies have shown promise for the use of phage in treating antibiotic resistant bacteria, and in alleviation of persistent infections caused by bacterial biofilms (Barrow, 2001; Levin, 1996; Tait, 2002; and Hanlon, 2001).

Phage specificity has also been harnessed in a technique known as bacteriophage typing, where bacterial species are identified by the susceptibility to infection by a panel of different phage. A set of bacteriophage was assembled for the epidemiological typing of *Staphylococcus aureus* in the early 1940's. In the following decade, The International Subcommittee on Phage Typing was formed to standardize the methodology (Marks, 2000). Phage typing allows for a simple, rapid, and inexpensive technique, and several bacterial groups now have phage typing systems, including *B. subtilis* and *B. thuringiensis* (Ackerman, 1995).

The ease of manipulation involved with bacteriophage has also led to the development of heterologous gene expression in phage. Phage display involves expression of recombinant proteins or peptides fused to the coat proteins for targeting of ligands for antigenic determination, production of single-chain antibodies, and construction of peptide libraries (Benhar, 2001 and Jestin, 2001). In addition, phage

display has also been used to introduce foreign DNA into mammalian cells in hopes of developing phage for gene therapy techniques (Kassner, 1999). Foreign gene expression has also been shown useful in bacteriophage as a means for detection of pathogenic bacteria (Marei, 2003), antibiotic sensitivity (Ulitzer, 1987) and as an alternative to the Most Probable Number (MPN) method for enumeration of bacteria (Turpin, 1993) with the use of reporter phage as discussed in detail in the next section.

There have been 33 bacteriophage species isolated specific for *Bacillus sp.* (Ackermann, 1994). The majority of these phage are tailed, and contain dsDNA. Little information exists on the molecular biology and relationships of phage for the *B. cereus* group. Attempts have been made to utilize the inherent specificity of bacteriophage to differentiate the members of the *B. cereus* group through phage-typing (Ackermann, 1995). Due to the high level of homology among the members of this group, several bacteriophage are characterized as “group specific,” infecting all members (Foldes, 1970). A number of studies in this area have been performed to isolate bacteriophage that would be type specific, allowing for identification of members among the group. In 1955, Brown and Cherry first characterized the well-known γ phage, specific for *B. anthracis* (Brown, 1955). Of over 250 *Bacillus sp.* tested, only the 41 strains of *B. anthracis* were susceptible to γ phage infection. Further characterization of the γ phage revealed that not all strains of *B. anthracis* are susceptible to γ phage infection, with 15% of *B. anthracis* strains not lysed (Buck, 1963). Later research elucidated the structure and protein composition of γ phage, and focused on determining the receptor site for attachment (Watanabe, 1975a; Watanabe, 1975b; and Watanabe, 1976). The γ phage assay is now defined as an appropriate method for detection of *B. anthracis* by the US

Centers for Disease Control and Prevention (CDC, Atlanta, GA). Recently, Schuch and colleagues (2002) harnessed the specificity of the lysin enzyme from γ phage, which is responsible for lysis of the host prior to release of daughter phage, as a means of detecting *B. cereus* RSVF1 (a closely related species) through bioluminescent assays.

Several other bacteriophage have also been found that are specific for *B. anthracis*. Bacteriophage AP 50 was isolated from soil and found to be highly specific for the *B. anthracis* Sterne strain (Nagy, 1974). Nagy and colleagues, (1982) later described the characterization of AP 50 and similar bacteriophage, which shared its unique characteristics. In addition, a temperate bacteriophage, Φ 20, has been isolated from *B. anthracis* and found to exist as a plasmoidial prophage (Inal, 1996).

The insecticidal toxins produced by *B. thuringiensis* make these bacteria of great interest to the industrial community. Generalized transducing phage have been isolated for genetic manipulation of *B. thuringiensis* for the purpose of creating engineered strains, optimized for its pathogenicity toward insects (Landen, 1981 and Reynolds, 1988). Bacteriophage lysin enzymes from *B. cereus* phage have also been characterized and found to be capable of lysing cell walls of closely related *Bacillus sp.* such as *B. thuringiensis* (Loessner, 1997).

Reporter Bacteriophage

Pathogenic bacterial contamination in food sources presents a dangerous and sometimes fatal issue for public safety (Goodridge, 2002). In addition, there is also a need for determining the antibiotic sensitivity of these bacterial strains for treatment of infection (Ulitzer, 1987 and Pearson, 1996). Current detection methods do not meet the

needs for the severity of the problems that arise when large foodborne or environmental disease outbreaks occur. For example, most diagnostics tests are too long, too costly, and too laborious and/or lack sensitivity (Goodridge, 2002). The development of better methodology for diagnostics is readily apparent.

The creation of bacteriophage reporter technology by Ulitzer and Kuhn (1987) provided a fast, reliable and inexpensive method for bacterial detection and characterization. In this system, a reporter gene was introduced into the bacteriophage genome, which was then delivered to the target organism upon infection. The signal produced by the reporter was only seen if the target organism is present, as bacteriophage must have a specific host to replicate (Goodridge, 2002). The majority of bacteriophage reporter systems developed employ the luciferase gene from prokaryotic (*lux*) or eukaryotic (*luc*) systems (Loessner, 1996; Waddell, 2000; Chen, 1996; Turpin, 1993; Pearson, 1996; Maillard, 1996; Goodridge, 1999). There have been several different bacterial species for which this technology has been developed such as *E. coli* (Waddell, 2000), *Salmonella* spp. (Chen, 1996), *Listeria monocytogenes* (Loessner, 1997), and *Mycobacteria* (Pearson, 1996 and Sarkis, 1995)

The bacteriophage reporter system can be used in several ways. One such application is for detection of a bacterial species in a food sample. For example, the detection of viable *Listeria* cells was accomplished by Loessner (1996) who created a recombinant A511::*luxAB* bacteriophage. Bacterial counts of 5×10^2 to 10^3 cells was possible after 2 hours, with as few as 1 cell/gram of iceberg lettuce being detected after a 24-hour incubation period. Traditional testing methods for *Listeria* can take up to 6 days, making the bacteriophage system an attractive tool for assaying food sources for

contamination. Other bacteriophage reporters have also been developed for foodborne pathogens such as the *lux+* P22 for detection of *Salmonella*, (Chen, 1996) and ϕ V10::*luxA**luxB**camA*1-23 for detection of *E. coli* O157:H7 (Waddell, 2000).

The bacteriophage reporter system has also been useful in the detection of pathogenic bacteria in environmental samples. The standard technique for detecting *Salmonella* spp. in the environment, MPN, takes several days before a positive result can be confirmed. Turpin *et. al.* (1993) developed a bacteriophage reporter system for the detection of *Salmonella typhimurium* in environmental samples such as soils, water, lake water and sewage. Using the *lux+* P22 bacteriophage, they were able to detect *Salmonella* with 100% efficiency in only 24 hours as compared to the standard MPN, which takes 4 days.

Another aspect of the bacteriophage reporter system is its usefulness in determining antibiotic sensitivity of bacterial species. The system has been used to detect resistance and/or susceptibility of bacteria to various antibiotics by infecting the target strain with the recombinant bacteriophage, and monitoring the decrease in reporter signal after exposure to various drugs. Sarkis *et. al.* (1995) developed a *luc* recombinant L5 bacteriophage to detect *M. smegmatis* antibiotic sensitivity, and Pearson *et. al.* (1996) developed the D29 *lux* bacteriophage for antibiotic sensitivity for *M. tuberculosis* and *M. bovis*. This method allows for more rapid results as compared to several weeks required for traditional drug screening involving mycobacteria. In addition to mycobacteria, Ulitzer and Kuhn (1987) used the *lux* recombinant λ L28 with *E. coli* in urine samples for determining susceptibility to streptomycin.

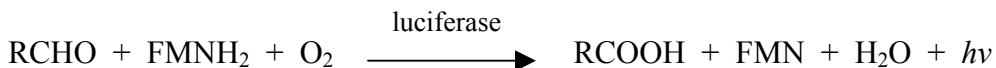
Quorum Sensing

For decades, the common thought in microbiology was that bacteria functioned as individuals within an environmental niche. It has recently become accepted that in contrast to the original thought, bacteria do indeed respond to environmental conditions by intercellular signaling to control expression of target genes. This mechanism of cell-to-cell signaling, or quorum sensing, enables a population of bacteria to function as a multicellular organism, and acquire the benefits from acting in a concerted manner. The first report of quorum sensing occurring in bacteria was 25 years ago in the marine organisms *Vibrio fischeri* and *Vibrio harveyi*, which use this system of communication to produce bioluminescence (Nealson, 1979). Identification of bacterial species utilizing quorum sensing has increased considerably over the past 10 years. Over 25 species of bacteria have been shown to utilize a quorum sensing system for controlling physiological activities such as bioluminescence, virulence, biofilm formation, plasmid transfer, symbiosis, competence and sporulation (for recent review, see Miller, 2001; Whitehead, 2001; Eberl, 1999; Schauder, 2001).

Intercellular signaling is accomplished by the production of autoinducers, which are excreted from the cell and allow bacteria to regulate their behavior according to their population density. As the number of organisms within a defined area increases, the concentration of the autoinducer also increases. Once a minimum threshold level of autoinducers is reached, alteration of gene expression occurs. Autoinducer molecules from bacteria can be placed into two separate categories: 1) short oligopeptides used by Gram-positive organisms (Kleerebezem, 1997), and 2) *N*-acyl-homoserine lactones, used by Gram-negative organisms (Eberl, 1999).

Gram-negative Quorum Sensing

Vibrio fischeri was one of the first quorum-sensing organisms characterized. The *lux* operon (*luxRoICDABE*), which is responsible for the production of bioluminescence, has been well characterized, and has served as the model for studying quorum-sensing genes in other Gram-negative bacteria. The *luxCDABE* genes encode for enzymes that are required for light production. Specifically, the *luxAB* genes encode for the luciferase enzyme (Engebrecht, 1983; Engebrecht, 1984), which oxidizes a reduced flavin and long-chain aldehyde, producing oxidized flavin and the corresponding long chain fatty acid. The *luxCDE* genes encode for a multienzyme fatty-acid reductase complex, which is responsible for synthesis of the aldehyde substrate utilized by the luciferase (Boylan, 1985). The reaction is as follows:



These genes are under the control of two regulatory proteins: 1) LuxI, which is the autoinducer synthase enzyme and directs the synthesis of *N*-(3-oxohexanoyl)-homoserine lactone (Engebrecht, 1983; Engebrecht, 1984; Eberhard, 1981) and 2) LuxR, the regulatory protein, which binds the autoinducer and is responsible for upregulating transcription of the *luxCDABE* (Stevens, 1994; Hanzelka, 1995; Stevens, 1999). The operon is arranged in two sections, with the *luxR* gene being transcribed in the opposite direction as the *luxICDABE* (Engebrecht, 1984). At low cell concentrations, the LuxI protein is transcribed at a basal level in conjunction with the synthesis of the autoinducer molecule (Eberhard, 1981). This autoinducer molecule is freely diffusible from the cell

and as such, accumulates within the surrounding area of the bacteria if in a confined area (Kaplan, 1985). The autoinducer molecule binds to the N-terminus of the LuxR protein (Hanzelka, 1995), which in conjunction with σ^{70} RNA polymerase then binds with the C-terminus to the *lux* box and the *luxI* promoter (Stevens, 1994 and Stevens, 1999), upregulating transcription of *luxICDABE* genes.

This system is positively autoregulated, allowing for a rapid increase in transcription through signal amplification, resulting in bioluminescence. Concentrations of autoinducer at 10 nM or 2 molecules per cell have been found sufficient to begin induction of the *luxICDABE* genes, with maximum induction reached at 200 nM (Kaplan, 1985). At the critical concentration of autoinducer, light output has been shown to increase by 1000-fold. With 10% or more of the cellular energy being utilized in a light reaction, strict regulation of the operon is necessary (Engebrecht, 1983). The specificity required for tight regulation of the system is derived from the synthesis of the autoinducer molecule, and binding of that molecule to the LuxR protein. Studies done with autoinducer analogs of OHHL have shown that although the *V. fischeri* LuxR protein is capable of binding structures similar to that of OHHL, none were as efficient at inducing bioluminescence (Schaefer, 1996 and Reverchon, 2002).

Many Gram-negative organisms that use quorum-sensing have been found to contain LuxR/I homologs in their genomes, and these proteins function in a similar fashion within those organisms. Each organism also makes its own unique homoserine lactone(s) and a corresponding LuxR protein that is specific for that molecule. One of the most well characterized quorum-sensing systems, aside from *V. fischeri*, is that of *Pseudomonas aeruginosa*, an opportunistic pathogen associated with infections in

immunocompromised patients, burn victims, neutropenic patients and individuals with cystic fibrosis (Johansen, 1996). *Pseudomonas aeruginosa* secretes several virulence factors including alkaline protease, toxin A, and elastases under the control of the LasR/LasI regulatory system (Passador, 1993). The LasI protein is an autoinducer synthase enzyme, similar to that of LuxI from *V. fischeri*, and directs synthesis of *N*-(3-oxododecanoyl) homoserine lactone (OdDHL [Pearson, 1994]). In addition to the LasR/LasI system, *P. aeruginosa* also contains RhlR/RhlI genes, which control expression of rhamnolipid biosynthesis by production of *N*-butyryl-homoserine lactone (BHL) through RhlI (Ochsner, 1995). Further studies have shown that the RhlR/RhlI system is dependent upon the LasR/LasI system, as transcriptional activation of the *rhlI* was dependent upon LasR/OdDHL (Pesci, 1997 and Latifi, 1996). *Pseudomonas aeruginosa* uses this hierarchical quorum-sensing cascade which combines the LasR/OdDHL, RhlR/BHL and the alternative sigma factor RpoS to integrate the regulation of virulence factors and secondary metabolites with survival in the stationary phase (Latifi, 1996).

AHL Structure and Synthesis

Engelbrecht and Silverman first elucidated the function of the LuxI protein through mutational assays of the *lux* genes from *V. fischeri*, and found that it was responsible for autoinducer synthesis (Engelbrecht *et. al.*, 1983 and 1984). The autoinducer signal molecules are *N*-acylhomoserine lactones (AHL), and although they are similar in structure, they all vary with respect to their acyl side chains. Of the current elucidated structures of autoinducers, all molecules have *N*-acyl side chains from 4-14

carbons and with either an oxo-, hydroxy-, or no substitution at the C3 position (Figure 2-2). In addition, they may contain a single carbon-carbon bond, or be fully saturated (Schaefer, 2000). The majority of known AHL's have an even number of carbons in the acyl side chain. Several different bacterial genera are known to produce the same AHL, and some individual bacteria may produce more than one type of AHL (Whitehead, 2001). In general, LuxI proteins are specific for the type of AHL they produce and the formation of the AHL is most favorable with the appropriate substrate, but it has been shown that several different shorter chain AHL can be made from the same LuxI protein (Schaefer, 1996 and Parsek, 1999). *N*-acylhomoserine lactones close in structure to that of the native AHL for a LuxR protein are capable of acting as antagonists, therefore, formation of alternative AHL may serve some ecological function (Schaefer, 1996).

There was some speculation that followed the discovery of the function of the LuxI protein as to whether the protein itself catalyzed the synthesis of the autoinducer, or rather directed other bacterial enzymes for autoinducer synthesis (Schaefer, 1996). In addition, the components required for autoinducer synthesis were also in debate. It was speculated after the *V. fischeri* autoinducer was first purified that the components came from two separate sources: 1) homoserine lactone from the amino acid metabolism pathway and 2) the acyl group from intermediates in the fatty acid metabolism pathway (Eberhard, 1981). Initial experiments for determining the substrates required for autoinducer synthesis involved crude extracts of *V. fischeri* with *S*-adenosylmethionine (SAM) and 3-oxohexanoyl coenzyme A, and showed that the autoinducer was produced

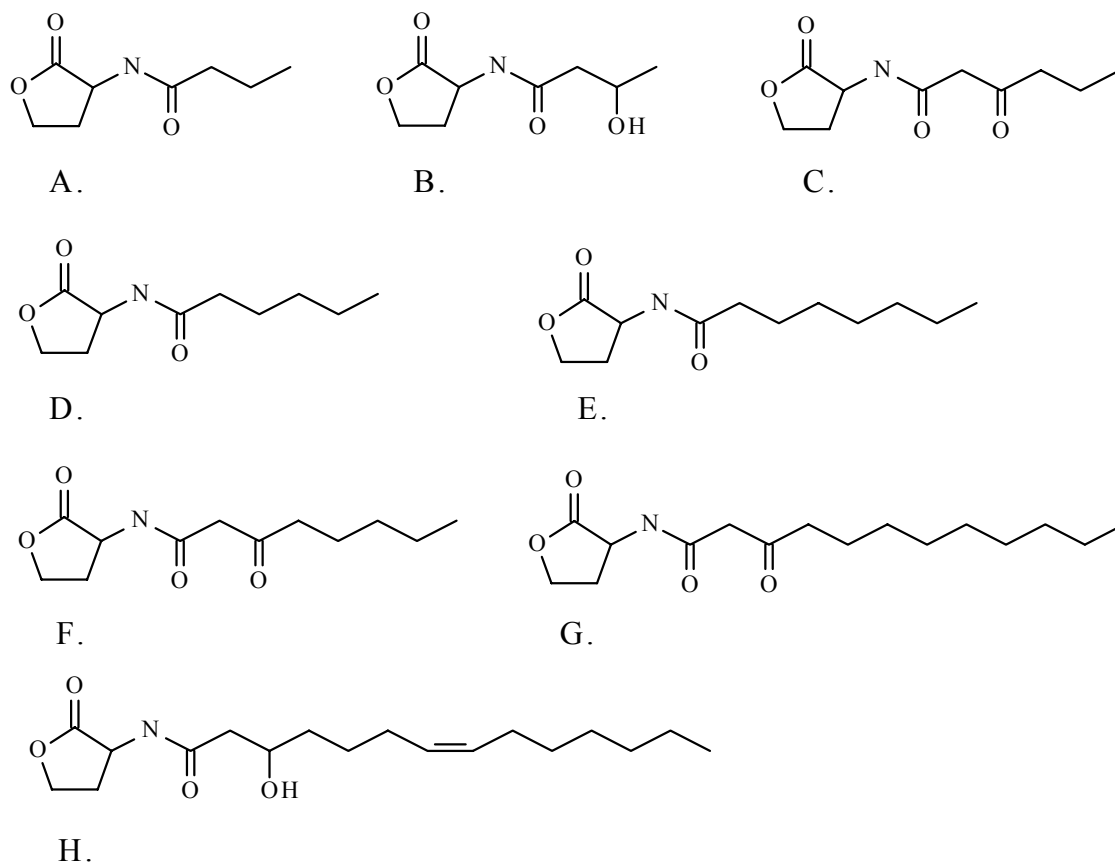


Figure 2-2. Known *N*-acyl-homoserine Lactone Signal Molecules. A. *N*-butanoyl-L-homoserine lactone (BHL); B. *N*-(3-hydroxybutanoyl)-L-homoserine lactone (HBHL); C. *N*-(3-oxohexanoyl)-L-homoserine lactone (OHHL); D. *N*-hexanoyl-L-homoserine lactone (HHL); E. *N*-octanoyl-L-homoserine lactone (OHL); F. *N*-(3-oxooctanoyl)-L-homoserine lactone; G. *N*-(3-oxododecanoyl)-L-homoserine lactone (OdOHL); H. *N*-(3-hydroxy-7-*cis*-tetradecenoyl)-L-homoserine lactone (HtdeDHL). Adapted from Whitehead, 2001.

(Eberhard, 1991). The question remained, however, if these substrates were further converted into other compounds by enzymes within the extract, or if SAM acted as a cofactor rather than a substrate. A study done by Henzelka and Greenberg (1996) showed that indeed it is SAM that is the amino acid substrate for the autoinducer molecule, and not homoserine or homoserine lactone.

Moré *et al.* (1996) showed that purified TraI protein from *Agrobacterium tumefaciens* was capable of synthesizing the autoinducer *N*-(3-oxo-octanoyl)-L-homoserine lactone from *S*-adenosylmethionine (SAM) and the 3-oxo-octanoyl-acyl carrier protein *in vitro*. Substitution with related compounds to SAM as well as 3-oxo-octanoyl-coenzyme A did not produce the *N*-(3-oxo-octanoyl)-L-homoserine lactone, providing evidence that SAM and an acyl-ACP is required for autoinducer synthesis. Schaefer *et al.* (1996) performed similar experiments with the purified LuxI protein from *V. fischeri*, with results consistent to that of the findings by Moré *et al.* (1996).

Interestingly, Scafer *et al.* (1996) found that the LuxI protein from *V. fischeri* had the highest production rate with the hexanoyl-ACP (967 pmol/min/mg protein) of *N*-(3-oxohexanoyl) homoserine lactone, which is its native autoinducer, as compared to synthesis rate with other acyl-ACP compounds which would result in synthesis of non-native autoinducers (butanoyl-ACP = 33 pmol/min/mg protein, octanoyl-ACP = 17 pmol/min/mg protein). These data show that the LuxI protein is specific for acyl-ACP in synthesis of autoinducer. In addition, when hexanoyl-CoA was supplied instead of the hexanoyl-ACP, the production rate of the autoinducer was <1 pmol/min/mg protein, giving evidence that it is fatty-acid biosynthesis components rather than fatty-acid metabolism products that are used in autoinducer synthesis. Further evidence for the

source of substrates in the autoinducer came from *in vivo* experiments using the TraI protein from *A. tumefaciens* by Val and colleagues (1998), where inhibitors of fatty acid synthesis blocked autoinducer synthesis, and, decreased levels of SAM by bacteriophage T3 *S*-adenosylmethionine hydrolase also caused a reduction in autoinducer synthesis. Following studies of LuxI and TraI, several reports were published on the function of the RhII autoinducer synthase from *P. aeruginosa*. Assays done on purified RhII proteins showed that the autoinducer synthase required SAM and butyryl-ACP as substrates for the formation of *N*-butyryl homoserine lactone, consistent to what had been found in LuxI and TraI (Hoang, 1999 and Parsek, 1999).

In terms of the actual structural characterization of LuxI and determination of how the enzyme catalyzes the reaction involving synthesis of AHL's, very little work has been published. Random mutations made in the LuxI protein of *V. fischeri* indicated two critical regions involved in the synthesis of AHL: 1) the N-terminus between residues 25-70 and 2) the C-terminus between residues 133-164 (Hanzelka, 1997). The N-terminal region contains residues that are highly conserved among LuxI homologs, and it was hypothesized that this area may be the active site of the protein (Hanzelka, 1997). The C-terminus showed less homology to other LuxI proteins, yet was important for activity of the enzyme. This area was hypothesized to be involved with binding of the appropriate acyl-ACP and delivery of the acyl group to the active site (Hanzelka, 1997). A more recent study where the EsaI protein from *P. stewartii* was examined through X-ray crystallography showed that the enzyme did in fact have the active site on the N-terminus, and in conjunction with several other residues, form the active site of the protein (Watson, 2002). In addition, they disproved the long-standing hypothesis that a

cysteine residue in the active site was responsible for forming a covalent thio-acyl-enzyme intermediate as this residue would not have access to the substrate in the described conformation. The C-terminal region of the enzyme was found to be responsible for specificity of the acyl-chain length, because when the binding pocket residues were modified, the enzyme efficiency was affected. Finally, the Thr140 residue showed preference for the 3-oxo-acyl-ACP, indicating that this residue might play a role in the specificity for the degree of oxidation at the AHL C3 position.

Watson and colleagues (2002) determined that the reaction mechanism for EsaI was in agreement with what had already been hypothesized as that of a bi-ter reaction, as shown in Figure 2-3. The AHL synthase binds the acyl-ACP first, followed by a conformational rearrangement of the N-terminal domain. SAM binds next, followed by a nucleophilic attack on the 1-carbonyl carbon of the acyl-chain by the amine of SAM. Next, lactonization occurs by a nucleophilic attack on the γ carbon of SAM by its own carboxylate oxygen to produce the homoserine lactone product. The byproduct of SAM lactonization results in release of 5'-methylthioadenosine (MTA), allowing the enzyme to return to its original state (Parsek, 1999 and Watson, 2002).

Fatty Acid Biosynthesis

The majority of fatty acid biosynthesis in prokaryotes occurs through a type II, or dissociated pathway, in which several enzymes interact in the formation of a multitude of fatty acid structures found in bacterial membranes (Rock, 1996). The genes responsible for fatty acid synthesis are conserved between prokaryotes and eukaryotes, although in

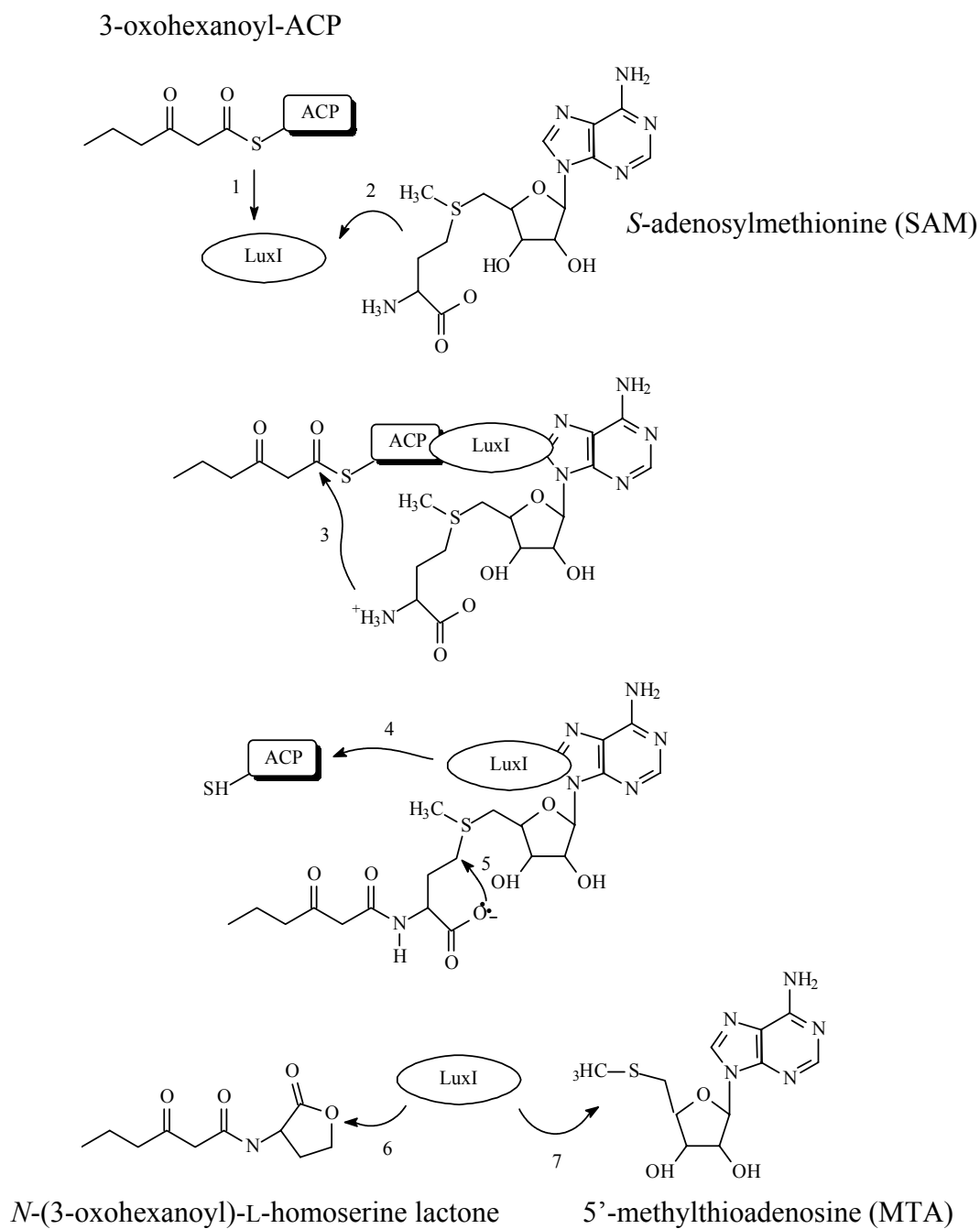


Figure 2-3. AHL Synthesis. 1) Acyl-ACP binds to LuxI, 2) followed by SAM; 3) acylation occurs through a nucleophilic attack by the amine of SAM, and 4) the holo-ACP is released; 5) an internal lactonization reaction in SAM by the nucleophilic attack of the carboxylate oxygen on the γ carbon, 6) followed by the release of the AHL, and 7) MTA from the protein.

eukaryotic systems, fatty acid synthesis occurs through a polyfunctional protein (type I fatty acid synthases), which catalyze each step with distinct domains contained in this very large protein. The fatty acid synthase system of *E. coli* serves as the archetype of the type II system for bacteria, where a number of even straight-chained saturated and unsaturated fatty acids are synthesized (for review see Rock, 1996 and Campbell, 2001). Unlike *E. coli*, *Bacillus sp.* have been shown to produce a variety of fatty acid structures containing odd- and even-carbon-numbered branched-chain fatty acids and glycolipids, in addition to utilizing an aerobic desaturation mechanism for unsaturated fatty acid biosynthesis (Mendoza, 1993).

Fatty-acid biosynthesis occurs in two stages: initiation and elongation. The first step is conversion of acetyl coenzyme A (acetyl-CoA) to malonyl-CoA by the enzyme acetyl-CoA carboxylase (Marini, 1995). Next, initiation is performed by β -ketoacyl-acyl carrier protein (ACP) synthase, the products of the *fabH* gene. In *B. subtilis*, this reaction can be carried out by two enzymes, FabH1 and FabH2, encoded by the genes *yjaX* and *yjaY*, respectively. These enzymes catalyze condensation of malonyl -ACP with various acyl-CoA substrates, and have a preference in *B. subtilis* for branched-chain acyl-CoA substrates over acetyl-CoA (Choi, 2000). The next phase in fatty acid synthesis is elongation, and begins with the condensation of malonyl-ACP with a growing acyl chain by the enzyme β -ketoacyl-ACP synthase II, or FabF, resulting in a β -ketoester. This is followed by a reduction reaction, which is catalyzed by the NADPH-dependent β -ketoacyl-ACP reductase, or FabG (Yamamoto, 2003). A water molecule is then removed by β -hydroxyacyl-ACP dehydrase (FabA or FabZ), encoded by *ywpB*, in *B. subtilis*. An NADH-dependent enoyl reductase I and III, or FabI and FabL, respectively,

catalyzes the final reaction. The FabL protein has recently been elucidated as part of the fatty acid synthesis pathway in *B. subtilis*, and although it shares similarity with FabI, its necessity to the pathway is still unknown (Marrakchi, 2002).

One unique characteristic of the fatty acid biosynthesis pathway type II is the use of ACP's (Prescott, 1972). All intermediates of the pathway are covalently bound to these enzymes through a 4'-phosphopantetheine moiety, which is added to ACP by a phosphodiester linkage at a modified Ser-36 residue (Carreras, 1997).

Phosphopantetheinylation is performed by the holo-ACP synthase enzyme, which receives the phosphopantetheine from coenzyme A (Parris, 2000). The gene encoding the ACP in *B. subtilis* is found in the cluster of lipid biosynthesis genes, similar to other prokaryotic organisms (Morbidoni, 1996).

Specificity for regulation of fatty acid chain length comes from the β -ketoacyl-acyl carrier protein synthase III, deemed FabH1 and FabH2 in *B. subtilis*. This enzyme is essential to the initiation of fatty acid synthesis, and dictates which fatty acid will be synthesized. Studies done on *B. subtilis* FabH1 and FabH2 have shown that this enzyme preferentially binds branched-chain acyl-CoA primers, unlike the FabH of *E. coli* that is most efficient with acetyl-CoA as a substrate (Choi, 2000). Several substrates were tested for their enzyme activity in a condensation reaction with malonyl-CoA, including butyryl-CoA, hexanoyl-CoA and octanoyl-CoA. Each substrate showed enzyme activity for both FabH1 and FabH2, unlike the *E. coli* enzyme, which produced an undetectable amount of product (Choi, 2000).

Relatively few studies have been done on the fatty-acid biosynthesis components in *B. thuringiensis*. Nickerson and colleagues (1975a) have shown that certain nutritional

requirements are necessary for the proper germination of spores, such as glutamate, aspartate, citrate, thiosulfate, cystine, or ethylenediaminetetraacetic acid, which stimulate fatty acid synthesis. Upon germination, ribonucleic acid synthesis occurs first, followed by fatty acid synthesis (Nickerson, 1975b). Formation of fatty acids from acetate initiated a large transient increase in fatty acid synthesis, which coincided with the approach of cell division. The change from single to branched chained fatty acids occurred 30-minutes-post germination of spores (Nickerson, 1975c).

The connection of the fatty acid biosynthesis pathway products and autoinducer synthesis by RhII was verified in experiments by Hoang *et. al.* (1999). Production of BHL was demonstrated using the enoyl-acyl carrier protein reductase enzyme, FabI in conjunction with RhII, crotonyl-ACP, and SAM. In addition, FabI mutants of *P. aeruginosa* produced 50% less BHL than the wild type (Hoang, 1999). This report provides direct evidence that intermediates in the fatty acid biosynthesis pathway are involved in autoinducer synthesis. In a more recent study, Hoang and colleagues (2002) found that when the enzyme FabG, which is a NADPH-dependent β -ketoacyl-ACP reductase, was mutated, shorter-chain 3-oxo-AHL's were produced in an *in vitro* simulation of the fatty-acid biosynthesis system containing the LasI protein from *P. aeruginosa*. Because LasI is in direct competition with FabG for the 3-oxo-acyl-ACP precursors, this study indicates that modulation of the FabG activity of the fatty-acid biosynthetic pathway may affect acyl chain lengths of AHL's, in addition to showing that LasI is sufficient for synthesis of 3-oxo-AHL's.

Gram-positive Quorum Sensing

Gram-positive organisms also employ a quorum-sensing mechanism to respond to population density through regulation of gene expression. Unlike Gram-negative organisms, Gram-positive bacteria use secreted modified peptides as signal molecules instead of AHL's. To date, there have been no LuxR/I homologs found in Gram-positive organisms, and no species found that produce AHL's for regulation of gene transcription (for review see Miller, 2001 and Kleerebezem, 1997). The Gram-positive quorum-sensing system involves a two-component phosho-relay system for detection and response to autoinducers. The general mechanism involves the synthesis of a specific peptide, which is post-translationally processed and sometimes modified. A dedicated ATP-binding-cassette exporter then transports the peptide out of the cell. The peptide is recognized by a sensor component of a two-component signal transduction system. This system also contains a response-regulator, which uses phosphorylation as a mean of transferring information. Once this peptide binds, a series of phosphorylation events results in the activation of a cognate response regulator protein, which in turn allows it to bind to DNA and alter transcription of the target genes involved in the quorum-sensing system. Genes encoding the precursor of the peptide and the genes involved in the transport and regulatory system are transcriptionally linked, allowing for autoregulation of this process.

Similar to Gram-negative organisms, quorum-sensing mechanisms and genes are also conserved in Gram-positives. Several bacterial species have been well studied, allowing for elucidation of the genes and processes involved in the quorum-sensing mechanism. One example is that of genetic competence and sporulation in *B. subtilis*.

These organisms gain the ability to take up exogenous DNA and initiate sporulation at high cell densities under control of the Com system, where two separate signaling molecules are involved, ComX and the competence and sporulation factor (CSF). Both peptides act in density-dependent extracellular manner to induce competence, where CSF is transported back into the cell, and at low intracellular concentrations induces competence, and at high concentrations it also acts to inhibit competence and upregulate genes involved in sporulation (Lazazzera, 1999; Magnuson, 1994; Miller, 2001). These differences in timing allow for an increase in effectiveness of the regulation of signal transduction for the given environment, which is a common theme for Gram-positive quorum-sensing systems.

Lactonase

Many Gram-negative bacteria use *N*-acyl homoserine lactone signal molecules for controlling expression of virulence genes through quorum sensing. These pathogenic bacteria such as *P. aeruginosa* and *Erwinia carotovoria*, cause disease in humans and plants, respectively (de Kievit, 2000). In an effort to develop techniques to block or degrade these signal molecules, scientists have made a widespread search for bacterial species that can inhibit quorum-sensing in virulent bacterial strains. (Dong, 2000 and Reimmann, 2002)

The first reported bacterial species to contain an enzyme which degrades homoserine lactone was found to be a *Bacillus sp.*, subsequently named *Bacillus sp.* 240B1 (Dong, 2000). The gene responsible for the homoserine lactone elimination, *aiiA*, was cloned and sequenced, and determined to be 750 bp with the protein being 28 kDa

(Dong, 2000). Later studies showed that this enzyme, designated AHL lactonase, was found among numerous soil, plant and laboratory strains, with high sequence homology (91.2-94%) to the original isolate, *Bacillus sp.* 240B1 (Dong, 2002). Of the 800 environmental isolates, 20 showed through morphological and 16S ribosomal DNA sequence analysis, that the bacteria were *B. thuringiensis*. Other *Bacillus* species, including *B. cereus* and *B. mycoides*, which are considered members of the *B. cereus* group and are closely related to *B. thuringiensis*, also contained the *aiiA* gene. *Bacillus* species outside of the *B. cereus* group, such as *B. fusiformis* and *B. sphaericus*, did not have AHL lactonase activity (Dong, 2002). Laboratory isolates of *Bacillus thuringiensis* confirmed that the lactonase enzyme was found among all subspecies of this species, in addition to *B. anthracis*, also a member of the *B. cereus* group (Lee, 2002). No lactonase activity was detected with *B. subtilis*, once again confirming that the *aiiA* is found among members of the *B. cereus* group only (Reimann, 2002).

Study of the functional aspects of the lactonase enzyme are still in the early stages. The lactonase enzyme can degrade a variety of different homoserine lactone molecules, including OdDHL, BHL, OHHL, OOHl, OHL, and HHL (Dong, 2000; Lee, 2002; Dong, 2002; Reimann, 2002; Dong, 2001). One common element to the structure of these molecules is the lactone ring. Examination of digested homoserine lactone molecule by lactonase showed that the ester bond of the lactone ring had been hydrolyzed (Dong, 2001). Amino acid sequence analysis of the isolated lactonase genes have shown two conserved regions. These two regions correspond to a proposed zinc-binding motif in several other enzymes. Site directed mutagenesis of the highly conserved histidine and aspartate residues in these two regions led to reduced and/or

complete loss of activity, providing evidence that lactonase may be a metalloenzyme (Dong, 2000 and Dong, 2002).

Degradation rates of homoserine lactone by lactonase among species of the *Bacillus cereus* group have been found to be variable. Whole cell assays with environmental isolates of *Bacillus* spp. showed rates of 480-680 pmol/h/unit OD₆₀₀ (Dong, 2002). Whole cell assays with laboratory strains of *B. thuringiensis* subspecies showed that there was a wide range of degradation capabilities among subspecies, with *aizawai*, *galleriae*, *kurstaki*, *kyushuensis*, *ostrinae*, and *subtoxicus* being the strongest, and *alesti*, *darmstadiensis*, *israelensis*, *morrisoni*, and *thompsoni* being the weakest (Lee, 2002). Total protein extract and purified AiiA protein from the original isolate *Bacillus* spp. 240B1 were able to completely degrade 20 μ M OHHL, ODHL and OOHHL in 60 minutes (Dong, 2000). Expression of the lactonase enzyme in transgenic tobacco and potato plants showed rates of 1-6 pmol/hr/ μ g of enzyme (Dong, 2001). These data show that the enzyme is capable of functioning in whole cells and in heterologous environments.

The lactonase enzyme has proven useful in controlling pathogenic bacterial infections that are mediated by quorum sensing. The plant pathogen, *E. carotovora*, uses a quorum sensing mechanism for expression of virulence genes that produce exoenzymes, causing soft rot disease of various plants (Pirhonen, 1993). When the *aiiA* gene was expressed in transgenic plants and subjected to infection by *E. carotovora*, soft rot disease was significantly attenuated, and in some cases, abolished to the point where the plant's own defenses were able to take over (Dong, 2001). *Erwinia carotovora* expressing the lactonase gene product has also been shown to lose its virulence when

allowed to infect Chinese cabbage, cauliflower, and tobacco (Dong, 2000). The lactonase enzyme has also been useful in controlling virulence in human pathogens such as *P. aeruginosa*. This organism uses quorum sensing in virulence gene expression, and forms an antibiotic-resistant biofilm in the lungs of cystic fibrosis patients (Singh, 2000). When lactonase was expressed in *P. aeruginosa* PA01, production of several virulence factors was strongly reduced, including production of elastase, rhamnolipids, hydrogen cyanide, pyocyanin, and swarming motility (Reimann, 2002). Although biofilm formation was not prevented, this work has opened a new door for studying virulence gene expression in *P. aeruginosa* and may lead to other alternatives for eliminating the opportunistic pathogen by disrupting the quorum-sensing mechanism.

Research Goals

The scope of this research contained herein initiates the investigation of a bacteriophage-based bioreporter system for detection of *B. anthracis* in the surrogate strain *B. thuringiensis*. Components of the proposal examined in this study include expression of the *luxI* gene in *B. thuringiensis*, optimization of detection and quantification of the OHHL produced from *B. thuringiensis* by a bioluminescent OHHL bioreporter, and the effect of the enzyme lactonase encoded by *B. thuringiensis* which is responsible for OHHL degradation.

Chapter 3: Materials and Methods

Experimental Design

A flow diagram is depicted in Figure 3-1 of the experimental design and approach for this project.

Bacterial Strains, Plasmids, and Bacteriophage

All bacterial strains, plasmids, and bacteriophage used in this work are listed in Tables 3-1, 3-2, and 3-3, respectively.

Media

Escherichia coli and *Bacillus* strains were routinely cultivated in autoclaved sterilized Luria-Bertani (LB) broth (10.0 g tryptone, 5.0 g yeast extract, 10.0 g NaCl per liter, pH 7.0). Solutions and media were sterilized by autoclaving at 121°C and 15 psi for 20 minutes, unless otherwise noted. Filter-sterilized (0.2 µm) antibiotics were aseptically added to LB broth as necessary. Luria-Bertani agar plates were made by the addition of 15 g/L of agar prior to autoclave sterilization.

Agrobacterium strains were cultivated in *Agrobacterium Tumefaciens* (AT) media which is composed of 7.0 g K₂HPO₄, 3.7 g KH₂PO₄, 2g (NH₄)₂SO₄ in 1 L of deionized water with pH adjusted to 7.2. Glucose at a final concentration of 2 g/L was added after autoclaving along with 10 mL of 100x salt solution. The 100x salt solution contained 16

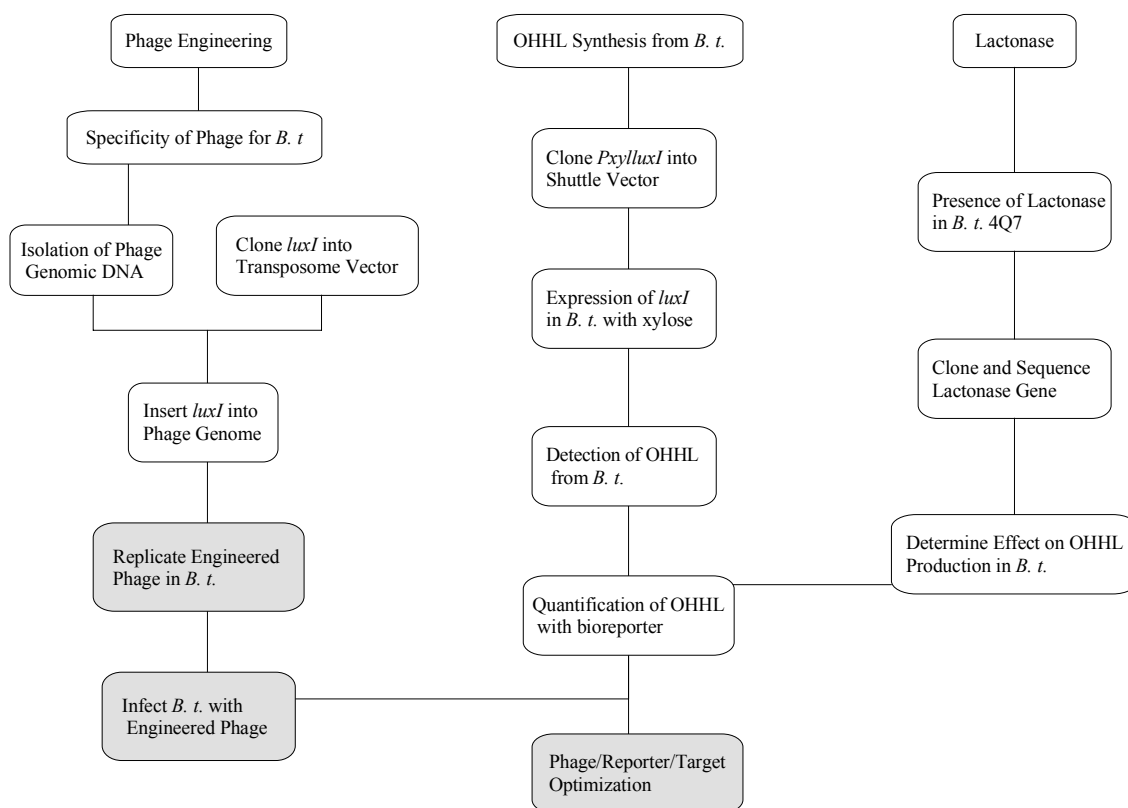


Figure 3-1. Experimental Design. Essential components pertaining to this project are shown in this diagram. The progression of work completed is indicated by white boxes, with aspects yet to be finished indicated by the gray boxes.

Table 3-1. Bacterial Strains

Bacterial Species	Strain	Description	Reference
<i>B. thuringiensis</i>	4Q7	Plasmidless mutant	BGSC, Clark, 1987 PhD Thesis (Ohio St. Univ.)
<i>V. fischeri</i>	ATCC 7744	Type strain	Hendrie, 1970
<i>E. coli</i>	RoLux	STBL4 (EZ::TN <i>luxRoCDABE</i>)	Bright, 2003
<i>E. coli</i>	DH5 α	F- ϕ 80 <i>lacZ</i> .M15 (<i>lacZYA-argF</i>)U169 <i>deoR recA1 endA1 hsdR17</i> (rk ⁻ , mk ⁺) <i>phoA supE44 thi-1 gyrA96 relA1</i> -	Invitrogen
<i>E. coli</i>	TOP10	F- <i>mcrA</i> (<i>mrr-hsdRMS-mcrBC</i>) ϕ 80 <i>lacZ</i> .M15 <i>.lacX74 deoR recA1 araD139 (ara-leu)7697 galU galK rpsL</i> (Str ^R) <i>endA1 nupG</i>	Invitrogen
<i>E. coli</i>	TOP10F'	F' <i>lacI</i> ^q , Tn10(Tet ^R) <i>mcrA</i> Δ (<i>mrr-hsdRMS-mcrBC</i>) ϕ 80 <i>lacZ</i> Δ M15 Δ <i>lacX74 deoR recA1 araD139 Δ(<i>ara-leu</i>)7697 <i>galU galK rpsL</i>(Str^R) <i>endA1 nupG</i></i>	Invitrogen
<i>A. tumefaciens</i>	A136	Ti plasmidless, <i>traR</i> overexpression plasmid (pCF218) and <i>traI-lacZ</i> (pCF372), Tet ^r , Sp ^r	Fuqua, 1996
<i>A. tumefaciens</i>	KYC6	<i>traM</i> null mutant with <i>traR</i> overexpression plasmid PCF218, Tet ^r , Km ^r	Fuqua, 1996
<i>E. coli</i>	BL21 (DE3) pLysS	F ⁻ <i>ompT hsdS_B</i> (<i>r_B⁻ m_B⁻</i>) <i>gal dcm rne131</i> (DE3) pLysS (Cam ^R)	Invitrogen

Table 3-2. Plasmids

Plasmid	Functional Usage	Relevant Characteristics	Reference
pHY300PLK	<i>E. coli</i> - <i>B. subtilis</i> shuttle vector	4870bp, Amp ^r (<i>E. coli</i>), Tet ^r (<i>E. coli</i> and <i>B. sp.</i>), MCS, ori- pAM α 1 and ori-177	Takara
pCR2.1 vector	TA cloning PCR products	Direct cloning of <i>Taq</i> polymerase-amplified PCR products	Invitrogen
pPFS2	Transposome vector for chromosomal insertion	3820bp, Ez::TN pMOD-2 Kan ^R , two transcriptional terminators from the <i>E. coli rrnB</i> operon	Bright,2002
pCR2.1-P1P2	Modified promoter <i>PIP2</i> for cloning	4095bp, Φ 105 holin gene promoter, P1P2, with <i>AvrII</i> restriction sites on 5' and 3' end	This work
pCR2.1-PLuxI	Source of <i>PIP1luxI</i>	4677bp, <i>luxI</i> gene from <i>V. fischeri</i> behind <i>PIP2</i> promoter in pCR2.1 vector	This work
pPLuxI	Chromosomal insertion of <i>PIP2luxI</i> into phage TP-15	4554bp, 743bp fragment of <i>PIP2luxI</i> in <i>EcoRI</i> site of pPFS2, Kan ^r , ME's	This work
pAX01	Source for <i>xylR</i> and <i>PxylA</i>	7781bp, Ectopic integration vector, Erm ^r , Bla ^r , <i>P_{xylA}</i> , <i>xylR</i> , <i>lacA</i> '-' <i>lacA</i> ,	BGSC, Härtl, 2001.
pCR2.1-BBLuxI	Modified <i>luxI</i> gene for cloning	4525bp, <i>luxI</i> gene from <i>V. fischeri</i> ATCC 7744 with <i>Bam</i> HI on 5' and <i>Bgl</i> II on 3'	This work
pHY-pXyl	Negative control shuttle vector	6383bp, 1519bp <i>Bam</i> HI- <i>Nru</i> I fragment containing the <i>xylR</i> and <i>PxylA</i> inserted in <i>Bam</i> HI- <i>Sma</i> I of pHY300PLK	This work
pHY-XylII	Xylose inducible shuttle vector for <i>luxI</i> expression in <i>B. thuringiensis</i> 4Q7	6975bp, 594bp <i>Bgl</i> II- <i>Bam</i> HI fragment containing <i>luxI</i> inserted upstream of 1519bp <i>Bam</i> HI- <i>Nru</i> I fragment containing the <i>xylR</i> and <i>PxylA</i> in pHY300PLK	This work

Table 3-2. cont.

Plasmid	Functional Usage	Relevant Characteristics	Reference
pCRT7/CT-TOPO	TA cloning of PCR products for expression	Direct cloning of <i>Taq</i> polymerase-amplified PCR products in an IPTG inducible expression vector with T7 promoter and 6xHis tag	Invitrogen
pCR T7/CT- <i>aiiA</i> #8	Expression of <i>aiiA</i> gene from <i>B. thuringiensis</i> 4Q7	3454bp, contains 752 bp PCR amplified fragment of <i>aiiA</i> gene from <i>B. thuringiensis</i> 4Q7	This work

Table 3-3. Bacteriophage

Bacteriophage	Description	Source
TP-15	<i>Siphoviridae</i> , temperate, tailed, double stranded DNA genome of 32.9 kb	BGSC; Reynolds, 1988

g $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$, 1 g $\text{CaCl}_2 \cdot 2\text{H}_2\text{O}$, 0.5 g $\text{FeSO}_4 \cdot 7\text{H}_2\text{O}$ and 0.2 g $\text{MnSO}_4 \cdot 7\text{H}_2\text{O}$ in 1 L of deionized water, autoclaved separately (Tempe, 1977).

Media used for the preparation of electrocompetent *B. thuringiensis* were made according to the manufacturer's protocol (Takara, Shiga, Japan): L-broth (tryptone 10 g, yeast extract 5g, and NaCl 5g in 1L of deionized water, pH 7.0-7.2), L-broth with 0.5 M sorbitol (28 mL of L-broth with 4 mL of 4 M sorbitol added post autoclave sterilization), Solution A (sorbitol 45.5 g, mannitol 45.5 g and 50 mL 10% glycerol in 450 mL deionized water, filter sterilized (0.2 μm)), and Solution B (tryptone 1 g, yeast extract 500 mg, NaCl 500 mg, sorbitol 9.1 g, and mannitol 6.9 g in 100 mL of deionized water, filter sterilized (0.2 μm)). Solution B was aliquoted into 10 mL fractions and stored at -20°C .

Bacteriophage propagation was performed using LB top agar or LB top agarose. The LB top agar contained LB broth with 4 g/L of agar, and the LB top agarose contained LB broth with 4 g/L of agarose.

Culture Conditions and Storage

All *Bacillus* strains were grown at 30°C in LB broth unless otherwise noted. *Agrobacterium* strains were also grown at 30°C in AT medium. *E. coli* strains were grown at 37°C in LB broth. Liquid cultures were grown shaking in 100 mL of media in a 250 mL Erlenmeyer flask at 200 rpm, unless otherwise noted.

Freezer stocks were prepared by adding 0.5 mL samples from an overnight culture with appropriate antibiotics to sterilized tubes containing 0.5 mL sterile 50% (v/v) glycerol. Tubes were maintained frozen at –80°C until ready for use.

Molecular Biology Techniques

All molecular biology techniques were performed as outlined in Molecular Cloning Manual (Sambrook , 1989) unless otherwise noted. All enzymatic reactions and routine purification of nucleic acids involving kits were performed as indicated in the manufacturer's instructions. All DNA modifying enzymes were obtained from Promega (Madison, WI) or New England Biolabs (Beverly, MA). All PCR reactions were conducted using Ready-To-Go PCR beads (Amersham Biosciences, Piscataway, NJ), unless otherwise specified, on a MJ Research PTC 200 Peltier thermal cycler (Waltham, MA). All primers were made by Sigma Genosys (The Woodlands, TX) and are listed in Table 3-4. Primers were diluted to a stock solution concentration of 500 µM in filter

Table 3-4. Primers

Primer Name	Sequence (5'-3')
P1P2 Forward	CCTAGGGGAAGGGAAACCTTTAAACAGTCTTAATCCCC CTTGATTTTATGTTCTCTGTAAACTGCGTCCGGTAAATC TCAGGATAG
P1P2 Reverse	CCTAGGTCCAATCCCTCCAATCATATTCTTTCTAAACTG CCCCGCACTCAAGCCGTTAACCGCCGATTGTCTATCCT GAGATTTACCGGA
P1P2 Forward Repair	CCTAGGGGAAGGGAAACCTT
AvrII LuxI Forward	CCTAGGATGACTATAATGATAAAAAAATCG
LuxI Reverse	TTAATTTAAGACTGCTTTTTTAAACTGTT CATTAATAGGC
BamHI LuxI Forward	GGATTCATGACTATAATGATAAAAAAATCGGATTTTT
BglII LuxI Reverse	AGATCTTTAATTTAAGACTGCTTTTTTAAACTGT TCATTAATAGGC
aiiA Forward	ATCGGATCCATGACAGTAAAGAAGCTTTATTTCG
aiiA Reverse	GTCGAATTCCTCAACAAGATACTCCTAATGATGT
aiiA Reverse #2	CTATATATACTCCGGAACA

sterilized HPLC-grade water with working stock diluted from this down to 5 μ M. All primers used in PCR reactions were at working stock concentration unless otherwise noted. All plasmid purification was done with either Qiagen Miniprep or Maxiprep kits (Qiagen, Valencia, CA) following the manufacturer protocol. All DNA quantification was done on a Quant 200 fluorometer (Hoeffer, San Francisco, CA).

DNA Sequencing

All DNA sequencing was performed by the Molecular Biology Resource Facility at The University of Tennessee, Knoxville, using an ABI Prism 3100 Genetic Analyzer (PE Applied Biosystems, Foster City, CA) with BigDye Terminator Cycle Sequencing v2.0 (PE Applied Biosystems).

Amplification of *luxI* Gene from *V. fischeri*

The *luxI* gene from *V. fischeri* ATCC 7744 was amplified using the AvrII LuxI Forward and LuxI reverse primer under the following conditions: 95°C for 5 min, followed by a cycle of 94°C for 30 sec, 55°C for 30 sec, 72°C for 1 min repeated 35 times, followed by an extension period of 72°C for 10 min. A 20 μ L aliquot of the PCR reaction was run on a 1% Tris Borate EDTA (TBE) gel to verify product size. After verification of correct amplicon size, the product was used in a TOPO TA cloning reaction. Colonies were screened for correct plasmid by purification with Qiagen Miniprep Kit, followed by restriction digest screening for insert and orientation with a double digest of AvrII and *Spe*I or *Xba*I. Clone #2 was picked for forward orientation and

sequenced. After the correct sequence was confirmed, the plasmid was named pCR 2.1 AvrII LuxI #2 and a large-scale plasmid prep was done using Qiagen Maxiprep kit.

To amplify the *luxI* gene with the appropriate restriction enzymes, another PCR reaction was done using primers BamHI LuxI Forward and BglII LuxI Reverse with the pCR 2.1 AvrII LuxI #2 plasmid as template. This reaction was done using the following components with enzyme, buffer and dNTP supplied by Takara: 1 µL pCR 2.1 AvrII LuxI #2 (100 ng), 1 µL BamHI LuxI Forward, 1 µL BglII LuxI Reverse, 5 µL 10x ExTaq Buffer, 4 µL dNTP's, 0.25 µL ExTaq, and 37.75 µL of sterile HPLC-grade water. The reaction was run under the same conditions as used for the initial *luxI* amplification. Amplicons were screened on a 1% TBE gel with ethidium bromide (EtBr) and product was cloned using TOPO TA cloning kit. Colonies were screening by preparing plasmid with Qiagen Miniprep kit, and resulting plasmid was digested with *Bam*HI and *Bg*II. Clone #10 showed insert and was sequenced. After confirming correct sequence, this plasmid was named pCR 2.1 BB LuxI and a large-scale plasmid prep was done using Qiagen Maxiprep kit.

Construction of Plasmid pHY-pXyl

The shuttle vector pHY300PLK was digested with *Sma*I at 37°C for 3 hr. Following this incubation, *Bam*HI was added and the reaction was incubated overnight. This restriction digest was then purified using the GeneClean Spin kit (Q·BIOgene, Carlsbad, CA) according to the manufacturer protocol and product quantified with final concentration at 327 ng/µL.

To isolate the *xylR* and *Pxyl* gene fragments, vector pAXO1 was digested with *NruI* at 37°C overnight. The following day, the digest was purified using GeneClean, and resuspended in 18 µL of sterile HPLC-grade water. To this, 0.5 µL of *BamHI* and 2 µL of Buffer E were added, and the plasmid DNA was allowed to digest for 3 hr at 37°C. The digest was then separated on a 1% TBE gel with no EtBr. To purify the band of interest (1520 bp), 10% of the digest was loaded in an adjacent lane to the remainder of the reaction on one side, and a DNA ladder on the other. The portion of the gel with the digest aliquot and the ladder were stained in an EtBr water bath. The stained portion of the gel was then visualized using UV light, and the 1520 bp band of interest containing the *xylR* and *Pxyl* fragment was marked by creating a hole in the gel with a transfer pipette. The stained portion of the gel was then aligned with the gel containing the remainder of the digest, and the band of interest was excised from the non-stained portion. This gel fragment was purified using GeneClean Spin kit (Q·BIOgene) and concentration was determined to be 23 ng/µL.

To ligate the *Pxyl/xylR* fragment into pHY300PLK, the *SmaI* digested pHY300PLK vector was diluted to 100 ng/µL, and the following reaction was assembled: 1 µL pHY300PLK vector, 4 µL *Pxyl/xylR* insert, 5 µL 2x Ligafast Buffer (Promega) and 1 µL T4 DNA ligase. This was incubated at room temperature for 20 min. Of this ligation mixture, 5 µL was transformed into 100 µL of chemically competent Library Efficiency *E. coli* DH5α (Invitrogen, Carlsbad, CA) according to the manufacturer protocol.

Colonies were prepared using the Qiagen Miniprep kit, and plasmids were screened using *Bam*HI and *Eco*RI. Digested product was separated on a 1% TBE gel containing EtBr and visualized under UV light. Clone #7 showed the expected bands, was named pHY-pXyl, and was prepared using the Qiagen Maxiprep kit.

Construction of Plasmid pHY-XylII

A schematic of the construction of plasmid pHY-XylII is seen in Figure 3-2. The vector pHY-pXyl was digested with *Bam*HI and *Bgl*II at 37°C overnight. This reaction was then purified using the GeneClean Spin kit (Q·BIOgene). Vector concentration was confirmed at 430 ng/μL.

The pCR 2.1 BB LuxI plasmid was digested with *Bam*HI and *Bgl*II at 37°C for 1 hr. The digested product was separated on a non-EtBr gel and purified in the same manner as for the *Pxyl/xylR* insert as mentioned above. Concentration of the insert was determined to be 17 ng/μL.

The *luxI* gene was ligated into the pHY-pXyl vector in the following reaction: 0.5 μL of pHY-pXyl vector, 3.5 μL BB luxI insert, 5 μL 2x Ligasfast Buffer (Promega), and 1 μL T4 DNA ligase. This was incubated at room temperature for 10 min, and then transformed into chemically competent Library Efficiency *E. coli* DH5α (Invitrogen) according to the manufacturer protocol. Plasmids were prepared using the Qiagen Miniprep kit, and screened using *Bam*HI and *Bgl*II. Clone #4, 6 and 9 were sent for sequencing, and all clones contained the insert with correct sequence. Clone #4 was named pHY-XylII and prepared using Qiagen Maxiprep kit.

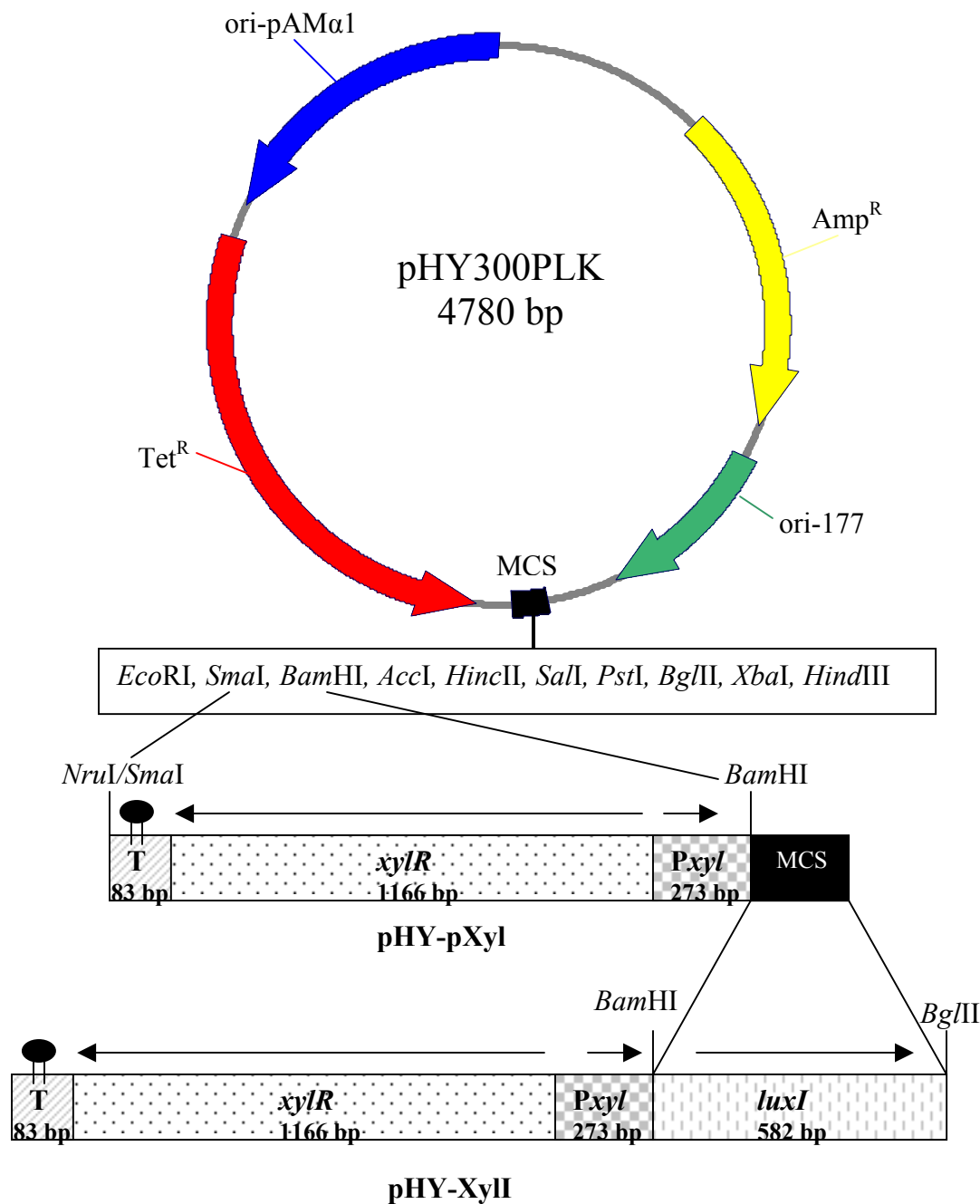


Figure 3-2. Construction of plasmid pHY-XylII. Vector pAXO1 was digested with *NruI* and *BamHI* excising the *Pxyl/xylR/T* fragment. This was cloned into the *SmaI* and *BamHI* sites of vector pHY300PLK, resulting in vector pHY-pXyl. pCR2.1-BBLuxI was digested with *BamHI* and *BglII*, excising the *luxI* gene, which was then cloned into vector pHY-pXyl, resulting in plasmid pHY-XylII. Amp^R = Ampicillin resistance, Tet^R = Tetracycline resistance, MCS = Multi-cloning site, ori-pAMα1 = Gram-positive origin of replication, ori-177 = *E. coli* origin of replication

***B. thuringiensis* 4Q7 Electrocompetent Cell Preparation**

Electrocompetent cells were made according to the manufacturer protocol for *B. subtilis* ISW 1214 (Takara). In brief, *B. thuringiensis* 4Q7 was streaked on an LB plate and incubated at 30°C overnight. A single colony was chosen to inoculate 100 mL LB broth which was grown at 30°C, 200 rpm overnight. Of this overnight culture, 2 mL were transferred to 28 mL of L-broth along with 4 mL of sterile 4 M sorbitol into a 250 mL baffled bottom Erlenmeyer flask. This culture was incubated at 37°C, 200 rpm for approximately 3 hours. An OD₆₆₀ measurement was made using a Beckman Coulter DU-640B spectrophotometer (Fullerton, CA) with OD₆₆₀ = 1.2427. The culture was then transferred to a 40 mL Oakridge tube and centrifuged at 6,500 rpm for 5 minutes at 4°C in a Sorvall SS-34 rotor with a Sorvall RC-5B High Speed Floor Model Refrigerated Centrifuge (Sorvall, Newton, CT). The supernatant was decanted and cells were washed with 10 mL of ice-cold Solution A. Cells were pelleted at 6500 rpm for 5 min at 4°C in a Sorvall SS-34 rotor. This was repeated three more times followed by final resuspension in 800 µL of Solution A. Cells were pipetted into 50 µL aliquots into ice-cold sterile eppendorf tubes, and stored at -80°C until use.

Electroporation of pHY-pXyl and pHY-XylII into *B. thuringiensis*

Electroporation conditions were followed as stated in manufacturer's protocol (Takara). In brief, electrocompetent *B. thuringiensis* 4Q7 were thawed on ice, and then diluted 1:1 with ice-cold Solution A. Approximately 100 ng of pHY-pXyl and 100 ng of pHY-XylII were added to separate tubes, and the mixtures were transferred to ice-cold 1

mm electroporation cuvettes (BTX, San Diego). The cuvettes were incubated on ice for 1 minute, then subjected to electroporation in the ECM 630 (BTX) under the following conditions: 2.0 kV, 200 Ω , and 25 μ F. Following electroporation, 1 mL of Solution B was added immediately to the cuvette, and the sample was transferred to a 10 mL culture tube (Fischer) and incubated at 37°C, 200 rpm for 3 hours. The cells were then spread-plated on LB Tet 30- μ g/mL with 200 μ L per plate over five plates and incubated at 37°C overnight.

Plasmid DNA was isolated from transformants using the Qiagen Miniprep kit following the protocol for *B. subtilis* with a few modifications. In brief, one colony was picked from each of the pHY-XylII and pHY-pXyl plates and grown up in 10 mL of LB broth with Tet 30- μ g/mL in a 40 mL Oakridge tube at 37°C, 200 rpm for 6 hr. Cells were centrifuged at 3000 x g for 15 min at 4°C in a Sorvall SS-34 rotor. The cells were resuspended by vortexing in P1 buffer with 5 mg/mL of lysozyme. This was incubated at 37°C for 20 min at 200 rpm. This was followed by a 5-min incubation and lysis of the cells with 250 μ L of buffer P2. Next, 350 μ L of ice-cold buffer N3 was added, and cellular debris was precipitated. The precipitate was centrifuged at 10,000 x g for 10 min at 4°C in a Sorvall SS-34 rotor. Supernatant containing plasmid DNA was applied to purification column supplied by the manufacturer, and centrifuged for 60 sec at 10,000 x g. The column was then washed with 500 μ L of buffer PB to remove nuclease activity and centrifuged at 10,000 x g for 60 seconds. Two additional wash steps were performed with 750 μ L of buffer PE under the same conditions. The column was then transferred to a sterile eppendorff tube, where 50 μ L of buffer EB was added to elute plasmid DNA.

This was incubated at room temperature for 60 sec, and centrifuged under the same conditions. Plasmids were then screened by restriction digest with *Bam*HI and *Bgl*II for *luxI* insert at 37°C for 1 hr, and bands were separated on a 1% TBE gel with EtBr and visualized under UV light.

RT-PCR of *B. thuringiensis* pHY-XylI

Bacillus thuringiensis pHY-XylI and *B. thuringiensis* pHY-pXyl were grown overnight in 10 mL LB Tet 30-μg/mL at 37°C, 200 rpm. Of this, 5 mL were transferred to 100 mL LB and grown at 37°C, 200 rpm for approximately 4 hours. Optical density was verified and found to be OD₆₀₀ = 0.7051 for pHY-XylI and OD₆₀₀ = 0.7976 for pHY-pXyl. Each culture was then transferred to a 250 mL sterile centrifuge bottle and pelleted at 6,000 rpm for 10 min at 4°C in a Sorvall GSA rotor. The supernatant was decanted, and the cells were washed with 25 mL of PBS prewarmed to 37°C, followed by centrifugation at 6,000 rpm for 10 min at 4°C. The supernatant was decanted, and both cultures were resuspended in 100 mL prewarmed PBS. The pHY-pXyl culture was then split into two separate 50 mL samples by transferring 50 mL into a sterile 250 mL centrifuge bottle. To one of these, 1 mL of 1 M xylose was added (20 mM final), serving as negative induced control, while the other acted as the uninduced negative control. For the pHY-XylI culture, 2 mL of 1 M xylose were added (20 mM final). This was denoted time 0, and 3 mL of the culture were removed for monitoring OD₆₀₀, and for RNA isolation. Cultures were placed at 37°C, and sampled every 30 min for 3 hr for the same parameters. For the positive control, *V. fischeri* was grown in LB broth at 30°C for 24

hours, after which a sample was taken to measure bioluminescence. Light output was measured on the Azur Environmental Deltatox PS1 (Strategic Diagnostics Inc., Newark, Delaware) in ATP mode and found to be ~ 7,000,000 CPS. RNA from this culture was prepared using the Qiagen RNeasy kit following the manufacturer's protocol for Gram-negative bacteria.

RNA Extraction

RNA extraction was done using Qiagen RNeasy Mini kit following the manufacturer protocol. In brief, 2 mL of cells were resuspended in 100 μ L of TE pH 8.0, 10-mg/mL lysozyme followed by a 20 min incubation at 37°C. Next, 350 μ L of buffer RLT 10 μ L/mL β -mercaptoethanol was added followed by brief vortexing to resuspend the cells. To this suspension, 250 μ L of 100% ethanol was added, and the entire solution was pipetted onto a purification column supplied by the manufacturer. The column was centrifuged at 8,000 x g for 15 sec, and transferred to a sterile eppendorf tube. The column was then washed two times with 500 μ L of buffer RPE under the same conditions. For the final elution, the column was transferred to a sterile eppendorf tube, and 50 μ L of RNase-free water was added. This was centrifuged at 8,000 x g for 60 sec. Samples were stored at -20°C until use.

Quantification of RNA

RNA was quantified using the Beckman Coulter DU-640B spectrophotometer by determining the absorbance at 260 nm and 280 nm of 2 μ L of sample in 998 μ L of DEPC

water (1:500 dilution). The following formula was used to calculate concentration (Doyle, 1996):

$$\frac{40 \times \text{Dilution Factor} \times A_{260}}{1000} = \mu\text{g}/\mu\text{L RNA}$$

RT-PCR Reaction

An aliquot from each RNA sample was adjusted to 1 µg of RNA/ 7µL of DEPC water. For the RT-PCR reaction, the Platinum Quantitative RT-PCR ThermoScript One-Step System (Invitrogen) was used. Each reaction contained 7 µL RNA template, 25 µL 2x ThermoScript Reaction Mix, 1 µL BamHI LuxI Forward (10 µM), 1 µL BglII LuxI Reverse (10 µM), 1 µL ThermoScript/PlatinumTaq Mix, and 15 µL DEPC water. The thermocycler was pre-warmed to 60°C and tubes were then placed inside. RT-PCR reaction conditions were as follows: 60°C for 30 min, 95°C for 5 min, 35 cycles of 95°C for 15 sec, 55°C for 30 sec and 72°C for 1 min with a final extension period of 72°C for 10 min.

Reactions were mixed well by vortexing, and 30 µL of each was loaded in a 1% TBE gel with EtBr and visualized under UV light.

Xylose Induction Assay of *B. thuringiensis* pHY-XylI for Qualitative Response

B. thuringiensis pHY-XylI and pHY-pXyl were grown overnight in 100 mL of LB Tet 30-µg/mL at 30°C, 200 rpm. Of these overnight cultures, 1 mL was used to inoculate 100 mL LB broth and cultures were grown at 30°C, 200 rpm to an OD₆₀₀ ≈ 0.1. To each culture, 20 mM xylose (final) was added, and samples were taken for measuring

OD₆₀₀ and analyzing supernatant for OHHL production. For the supernatant, 2 mL of culture were transferred to a sterile 2 mL Micro tube (Sarstedt, Newton, NC) and centrifuged at 14,000 rpm for 2 min. Supernatants were then filter sterilized (0.22 µm) into a sterile 2 mL Micro tube and stored at -20°C. Samples were taken every 30 min for 3 hr for each parameter.

RoLux Qualitative Reporter Assay

Escherichia coli RoLux was grown overnight in LB Kan 50-µg/mL at 37°C, 200 rpm. Of this overnight culture, 1 mL was used to inoculate 100 mL LB Kan 50-µg/mL and grown at 37°C, 200 rpm for approximately 5 hr. Optical density was found to be OD₆₀₀ = 0.4689. An aliquot of 30 mL of this culture was transferred to a sterile 40 mL Oakridge tube and cells were pelleted at 6,000 rpm for 10 minutes at 4°C in a Sorvall SS-34 rotor. The supernatant was decanted, and the cells were resuspended in 30 mL fresh LB broth. Supernatants from the induced time course assay of *B. thuringiensis* pHY-XylII and pHY-pXyl were mixed in a 1:1 ratio (100 µL of RoLux to 100 µL supernatants) with of RoLux culture for each time point in triplicate and also in a 3:1 ratio (150 µL RoLux to 50 µL supernatants) and assembled in a Costar black with clear bottom 96 well microtiter plate (Corning, Inc., Corning, NY). Controls for RoLux reporter included culture with 1 µM synthetic OHHL (Quorum Science, Coralville, IA) added in triplicate and another with no OHHL, also in triplicate for each volume used. Bioluminescence and absorbance at 490 nm was measured on a Wallac Victor2 1420 Multilabel Counter (Perkin Elmer Life Sciences, CT). The assay was run at 30°C and included 10 min of

shaking, bioluminescence count (CPS), absorbance (490 nm) followed by 5 min of idle time. This was repeated over a 15-hr period. Analysis of bioluminescent response was performed by dividing the CPS by the OD₄₉₀ of the RoLux during the assay to determine peak specific bioluminescence (SB). Earliest induction time was determined by comparing triplicate samples of the SB produced by each supernatant to that of triplicate samples of the background SB (time 0) by the Student's T-test program on SigmaPlot. The first point in the assay that had a $P < 0.05$ value was considered the earliest induction time. Peak SB was determined by finding the maximum average SB produced by each supernatant sample. The number of RoLux cells per well was calculated based on the RoLux growth curve experiments where colony-forming units per mL (CFU/mL) was determined. The log-phase portion of the CFU/mL was analyzed on a semi-log plot by determining the linear best-fit line. The equation of this line was determined to be $y = 2 \times 10^8 x - 5 \times 10^7$. This equation was used to determine the CFU/mL of reporter cells at the time of peak SB based on the OD₄₉₀ at that point. The number of cells per well was determined by calculating the number of cells per 100 μ L and 150 μ L for the 1:1 and 3:1 ratio experiments, respectively, based on CFU/mL.

RoLux reporter induction was also measured with the same supernatant and same parameters for reporter strain preparation on Wallac Microbeta 1450 Plus (Perkin Elmer Life Sciences). Reporter to supernatant ratios included 1:1 (100 μ L RoLux to 100 μ L of Supernatant) and 3:5 (75 μ L of RoLux to 125 μ L of Supernatant). The assay was assembled in a Costar black 96-well microtiter plate with all samples in triplicate. Bioluminescence was measured every 15 min for 9 hr at 30°C. The earliest induction time and peak CPS was determined in the same manner for these samples.

***B. thuringiensis* pHY-XylII Growth Curve and Xylose Induction Assay for**

Quantitative Response

Bacillus thuringiensis pHY-XylII and pHY-pXyl was grown overnight in LB broth at 30°C, 200 rpm. The overnight culture of pHY-XylII was used to inoculate 500 mL of LB broth with 20 mM xylose in triplicate, and the pHY-pXyl was used to inoculate one 500 mL flask of LB broth with 20 mM xylose. The time of inoculation was designated time 0. Optical density was measured at 546 nm, and an additional 5 mL was transferred to a 10 mL culture tube (Fischer), where it was centrifuged at 3,000 rpm in a GH-3.8 rotor in a Beckman Allegra 6 benchtop centrifuge for 15 min for each culture.

Supernatants were filter sterilized (0.22 µm) into a sterile 15 mL centrifuge tube (Fischer), and stored at -20°C. Supernatants were collected every 30 min for the first 3 hr, then every hour for an additional 4 hr. Optical density was measured every 30 min for the full 9 hr. At various points along the growth curve, 1 mL of each of the pHY-XylII culture was used for serial dilutions in 9 mL sterile PBS, with 100 µL spread plated on LB agar for four of the dilutions to determine colony forming units per mL of culture (CFU/mL). Plates were incubated at 30°C overnight. Colonies were counted after 16-hr incubation in the range of 30-300 per plate and growth rate was determined. Generation time (GT) of *B. thuringiensis* pHY-XylII was calculated using the following formula (Cappucino, 1996):

$$GT = \frac{t \log 2}{\log b - \log B}$$

where t is equal to the time, B is equal to the number of cells at a point during exponential growth phase, and b is equal to the number of cells at a second point during exponential growth phase.

RoLux Quantitative Bioreporter Assay

A fresh culture of *E. coli* RoLux was inoculated from an overnight culture, and grown at 37°C, 200 rpm to an $OD_{490} = 1.0908$. The culture was then pipetted into 200 μ L aliquots into sterile eppendorf tubes. These tubes were centrifuged at 14,000 rpm for 2 min and the supernatant was decanted. The RoLux cell pellets were then resuspended in 200 μ L of *B. thuringiensis* pHY-XylII supernatant from xylose induction time course and transferred to a Costar black with clear bottom 96 well microtiter plate (Corning). Two separate standard curves were also assembled using supernatant from the pHY-pXyl culture at log ($OD_{546} = 0.4252$) and stationary phase ($OD_{546} = 1.1252$). Synthetic OHHL (Quorum Science) was added to the standard curve wells in increasing concentrations: 0, 5, 10, 25, 50, 100, 200, 400 and 600 nM. Bioluminescence (CPS) and absorbance (490 nm) were monitored at 30°C on a Wallac Victor2 1420 Multilabel Counter (Perkin Elmer Life Sciences, CT). Measurements included 10 min of shaking, bioluminescence (CPS), absorbance (490 nm) and 5 min of idle time repeated over a 15-hr period.

All CPS values were converted to SB by dividing the CPS by the OD_{490} of RoLux during the assay. Standard curve values were plotted on a semi-log scale, and the best-fit line was determined for each with the equation: $y = [a \cdot x / (b + x)] + c \cdot x$. The equation for the log-phase standard curve was determined to be: $y = [11,0048.01 \cdot x / (156.85 + x)] + (-$

$30.72) \cdot x$ with $R^2 = 0.9975$, and for the stationary-phase standard curve to be: $y = [11,276.38 \cdot x / (133.58 + x)] + 11.22 \cdot x$ with $R^2 = 0.9986$. Both curves began to reach saturation after the 100 nM sample, therefore, values in this range were plotted on a linear scale, with a best fit and 95% confidence intervals determined for each. The linear equation for the log-phase standard curve was found to be: $y = 382.94x + 2912.20$ with an $R^2 = 0.9561$, and $y = 278.13x + 58.79$ with an $R^2 = 0.9776$ for the stationary phase standard curve.

To calculate the concentration of OHHL in each supernatant, peak SB values of each triplicate sample were used. Samples from time points 0-240 were calculated with the linear equation from the log-phase standard curve. Samples from time points 300-480 were calculated using the hyperbolic equation from the stationary-phase standard curve, as these SB values were not within the linear range. OHHL concentration was determined for each triplicate sample at each time point, and the average value including the standard deviation arising from the average was calculated.

RoLux Growth Curve

Escherichia coli RoLux was grown overnight in LB Kan 50- μ g/mL at 37°C, 200 rpm. Of this overnight culture, 2 mL were transferred to 100 mL LB Kan 50- μ g/mL in triplicate. Optical density was measured every 30 min (OD₄₉₀) until the cultures reached stationary phase. Serial dilutions of the three cultures were done in sterile PBS at various points along the growth curve. Dilutions were plated on LB agar and incubated at 37°C overnight. Colonies were counted 16-hr later in the 30-300 colonies per plate range, and CFU/mL were determined in the same manner as that of *B. thuringiensis* pHY-XylI.

***B. thuringiensis* 4Q7 Genomic Prep**

A large-scale preparation of genomic DNA from *B. thuringiensis* 4Q7 was done with a modified version of the protocol found in Current Protocols in Molecular Biology (Ellington, 1988). In brief, *B. thuringiensis* 4Q7 was grown overnight in 100 mL of LB broth at 30°C, 200 rpm. This culture was transferred to a 250 mL centrifuge bottle and centrifuged at 6,000 rpm for 15 min at 4°C in a Sorvall GSA rotor. The supernatant was decanted, and the cell pellet was resuspended in 9.5 mL of TE 2 mg/mL lysozyme. The resuspension was transferred to 40 mL Oakridge tube and incubated at 37°C, 200 rpm for 1 hr. Next, 500 µL of 10% SDS and 50 µL of 20 mg/mL Proteinase K was added and mixed gently. The cells were then incubated at 37°C for 30 min.

Following this incubation, 1.8 mL of 5 M NaCl and 1.5 mL of CTAB/NaCl solution were added, mixed gently and incubated at 65°C for 20 min. A chloroform extraction was then done by adding 13.3 mL of chloroform/IAA which was then mixed vigorously for 1 min. This was centrifuged for 15 min at 10,000 rpm in a Sorvall SS-34 rotor. The aqueous layer was removed and transferred to a sterile 40 mL Oakridge tube where the genomic DNA was precipitated by adding 6.6 mL of isopropanol and gently inverting the tube. The genomic DNA was removed from the tube with a sterile transfer loop and transferred into an Eppendorf tube containing 1 mL of 70% EtOH. The genomic DNA was then pelleted by centrifuging for 10 min at 14,000 rpm after which the EtOH was removed, and 1 mL TE buffer was added to resuspend the pellet. The resuspension was carried out at 4°C overnight. Once the pellet was resuspended, the

sample was transferred to 3 mL of TE pH 8.0 and aliquoted into four separate Eppendorf tubes. DNA concentration was determined to be 591 ng/μL.

Amplification of *aiiA* from *B. thuringiensis* 4Q7

The lactonase gene, *aiiA*, from *B. thuringiensis* 4Q7 was amplified using primers AiiA Forward and AiiA Reverse (Table 3-4) and genomic DNA as template (Dong, 2001). PCR product was visualized on a 1% TBE gel with EtBr and found to be approximately 900 bp, larger than the expected 750 bp. The amplicon was cloned with a TOPO TA Cloning kit (Invitrogen) and sequenced. After reviewing the sequence data, it was determined that product extended 150 bp past the stop codon of the gene. A new primer designated AiiA Reverse #2 was designed to amplify only the gene itself. The PCR reaction was repeated with AiiA Forward and AiiA Reverse #2 under the same conditions, and cloned into TOPO TA cloning expression vector pCR CT/T7-TOPO (Invitrogen), following the manufacturer protocol. Plasmid preps on colonies were done using the Qiagen Miniprep kit, and screened through restriction digest with *HindIII*. Clone #8 and 9 were sequenced, and clone #8 was designated pCR CT/T7 *aiiA* #8 and used for future experiments.

Expression of Lactonase (AiiA) from pCR CT/T7 *aiiA* #8

Expression of the AiiA protein was monitored following the manufacturer protocol for Pilot Expression experiment (Invitrogen). In brief, pCR CT/T7 *aiiA* #8 was transformed into expression cell line *E. coli* BL21 (DE3) pLysS following manufacturer protocol. Of this overnight transformation culture, 500 μL was used to inoculate 10 mL

LB Amp 50 µg/mL, Chlor 34 µg/mL. This culture was grown at 37°C, 200 rpm for 2 hr. The culture was then divided into two separate 5 mL cultures, one for the induction experiment, and one to serve as a negative control. To the induction culture, 1 mM IPTG was added, and a 500 µL sample was taken from each culture. Cells were pelleted at 14,000 rpm for 30 sec, the supernatant was decanted, and cell pellets stored at -20°C. Both cultures were incubated at 37°C, 200 rpm and samples were taken every hour for 4 hr.

SDS-PAGE Analysis of AiiA Expression

Cell pellets from expression experiment were resuspended in 80 µL of sample buffer, then boiled for 5 min in a heating block. Samples were centrifuged briefly at 14,000 rpm, and placed on ice. To each sample, 10 µL of loading dye was added, and 10 µL of each was loaded into a 12% SDS gel. Protein was separated at 150 V for approximately 1 hr in a Mini-PROTEAN II Cell apparatus (BioRad, Hercules, CA) after which the gel was stained with Gel Code Blue Stain Reagent (Pierce, Rockford, IL) as per the manufacturer protocol.

Qualitative Lactonase Assay using *B. thuringiensis* 4Q7

Detection of the presence of the lactonase enzyme was performed as described previously (Dong, 2000). In brief, 20 µL of overnight culture of *B. thuringiensis* 4Q7 was mixed with 20 µL of fresh LB broth with synthetic OHHL (Quorum Science) at a final concentration of 40 µM. This was incubated at 28°C, 200 rpm for 4 hr. The

samples were then exposed to UV for 1 hr to destroy viable cells, followed by pelleting at 14,000 rpm for 1 min and supernatant being filter sterilized (0.22 μ m) into a sterile Eppendorf tube. The *B. thuringiensis* 4Q7 treated OHHL was then spotted on an AT medium with X-gal plate at point 0, with LacZ AHL reporter strain *A. tumefaciens* strain A136 spotted at 0.5 cm intervals vertically over 5 cm from point 0. This was incubated overnight at 30°C. Diffusion of OHHL over the plate was observed by induction of the *lacZ* gene resulting in blue color of *A. tumefaciens* A136 colonies. Controls were also done under the same conditions with positive control omitting the *B. thuringiensis* 4Q7 culture and negative control omitting the OHHL.

Bioluminescent Lactonase Assay for *B. thuringiensis* pHY-XylI

Bacillus thuringiensis pHY-XylI was grown overnight in LB at 30°C, 200 rpm. Of this overnight culture, 3 mL were transferred to 100 mL of LB in triplicate. Cultures were grown at 30°C, 200 rpm. At each hour along the growth curve, OD₅₄₆ was monitored, and one mL of the three experimental cultures was transferred to an eppendorf tube with 500 nM synthetic OHHL (final [Quorum Science]). All tubes were placed at 30°C for 60 min. These tubes were centrifuged at 14,000 rpm for 60 sec. Supernatant from these samples was then passed through a 0.22 μ m filter and stored at -20°C. A 5 mL aliquot of supernatant from one of the cultures was collected at various points along the growth curve and prepared in the same manner as the experimental samples.

RoLux Quantitative Lactonase Reporter Assay

Escherichia coli RoLux was grown overnight in LB Kan 50 µg/mL at 37°C, 200 rpm. Of this overnight culture, 200 µL was centrifuged at 14,000 rpm for 2 min and the supernatant was decanted. Aliquots (200 µL) of *B. thuringiensis* pHY-XylII treated OHHL supernatants from the lactonase assay were used to resuspend the RoLux reporter cells. Two separate standard curves were assembled with supernatant from *B. thuringiensis* pHY-XylII in log-phase ($OD_{546} = 0.3977$) and stationary phase ($OD_{546} = 1.2995$) under the same conditions with 200 µL of supernatant and synthetic OHHL(Quorum Science) added at 0, 25, 75, 125, 250, and 500 nM. All samples were arranged in a Costar black with clear bottom 96-well microtiter plate (Corning, Inc.) and bioluminescence output was measured on the Wallac Victor2 1420 Multilabel Counter (Perkin Elmer Life Sciences, CT). Measurements were as follows: 30°C, 10 min shaking, bioluminescence (CPS), absorbance (490 nm) and 10 min idle, repeated over 15 hr.

All bioluminescence values were converted to specific bioluminescence by dividing the CPS by the OD_{490} of the RoLux during the assay. Peak SB values for each standard curve were determined and plotted on a linear scale. The linear best-fit was determined along with the 95% confidence intervals. The linear equation for the log-phase standard curve was found to be $y = 52.72x + 14573.26$ with a $R^2 = 0.9102$, and for the stationary-phase standard curve, the linear equation was $y = 7.72x + 1431.42$ with a $R^2 = 0.8429$. The OHHL concentration in each sample was determined by taking the average of the SB produced and calculating OHHL with the linear equation from each of

the standard curves. For samples 0-240-min, the log-phase standard curve equation was used. The stationary-phase standard curve was used for the 480-min sample. Sample 300-420-min did not have supernatant similar enough to that used in the standard curves, therefore, the values calculated for the OHHL concentration were inappropriate.

Contruction of Plasmid pCR2.1-PLuxI

The holin gene promoter from *B. subtilis* phage Φ 105 (Leung, 1995) was amplified using primers P1P2 Forward and P1P2 Reverse at a concentration of 50 pmoles/ μ L in the following reaction: 2.5 μ L Pfx buffer, 2.5 μ L Enhancer, 2.5 μ L 10mM dNTP (Perkin Elmer), 0.5 MgCl, 0.5 μ L Pfx, 0.5 μ L P1P2 Forward, 0.5 μ L P1P2 Reverse, and 15.5 μ L of sterile water. All amplification components were purchased from Invitrogen unless otherwise stated. The reaction was carried out under the following conditions: 94°C for 5 min, followed by 40 cycles of 94°C for 15 sec, 58°C for 15 sec, and 68°C for 15 sec with a final incubation at 68°C for 10 min. The PCR product was screened on a 2% TBE gel with EtBr, and resulting bands excised and gel purified with GeneClean. To allow for TA cloning, the purified product was added in the following mixture: 15 μ L PCR product, 2 μ L 10 mM dATP (Perkin Elmer), 2 μ L ExTaq 10X Buffer (Takara), and 0.5 μ L ExTaq (Takara). This solution was incubated at 72°C for 10 min, and used in a TA cloning reaction. White transformed colonies were subjected to mini-plasmid preps, and plasmids were screened through restriction digest with *AvrII*. Clone #5 contained insert and had functional restriction sites. This plasmid was sequenced, and a large-scale plasmid prep was done with final concentration at 600 ng/ μ L.

Vector pCR2.1-AvrII LuxI was digested with *AvrII* (NEB) and Multi-core buffer (Promega) overnight at 37°C. The digest was then purified with GeneClean and resuspended in 30 µL. The vector was dephosphorylated with 0.5 unit of Shrimp Alkaline Phosphatase (SAP [USB, Cleveland, OH]) and 3.4 µL of 10X SAP buffer (USB). This reaction was incubated at 37°C for 90 min, and then heat inactivated by incubation at 65°C for 20 min. Plasmid concentration was verified and determined to be 101 ng/µL.

The insert plasmid, pCR2.1-P1P2 was digested with *AvrII* (NEB) and Multi-core Buffer (Promega) overnight at 37°C. The digest was then separated on a 1.5% TBE gel with no EtBr, followed by removal of the band of interest as described above, and gel purification with GeneClean. Fragment concentration was determined to be 22 ng/µL.

The ligation reaction contained 1 µL of P1P2 insert, 2.15 µL of *AvrII* LuxI vector, 5 µL of 2X Ligafast buffer (Promega), 1 µL T4 DNA ligase, and 0.85 µL of sterile water. This was incubated at room temperature for 5 min, and transformed into Library Efficiency chemically competent DH5α (Invitrogen) according the manufacturer's protocol. Plasmid from transformed colonies was prepared, and screened through restriction digest with *AvrII* (NEB) and *SpeI* (Promega). Clone #6 contained the promoter, and was sequenced. After conformation that the P1P2 promoter was in the correct orientation, the plasmid was named pCR2.1-PLuxI, and a large-scale plasmid prep was done (900 ng/µL).

Construction of Plasmid pPLuxI

A schematic of the construction of plasmid pPLuxI is seen in Figure 3-3. The vector plasmid pFSD2 was digested with *Eco*RI in Buffer H at 37°C overnight. The digest was then purified using GeneClean, resuspended in 15 µL sterile water and dephosphorylated with 0.5 unit of SAP (USB) and 1.5 µL of 10X SAP Buffer (USB) at 37°C for 90 min. The SAP was heat-inactivated at 65°C for 20 min, and vector concentration quantified at 189 ng/µL.

Insert DNA was obtained from vector pCR2.1-PLuxI by digesting with *Eco*RI in buffer H (Promega) at 37°C overnight. The digest was then separated on a 1.5% TBE gel with no EtBr, and the band of interest containing the *PIP2luxI* fragment was purified as described above. The fragment was gel purified using GeneClean, resuspended in 15 µL, and the concentration found to be 60 ng/µL.

To ligate the *PIP2luxI* fragment into the pFSD2 transposome vector, the following reaction was assembled: 1 µL vector, 2.5 µL insert, 5 µL 2X Ligafast buffer, 1 µL T4 DNA ligase (Promega), and 0.5 µL sterile water. This was incubated at room temperature for 15 minutes, followed by transformation into Library Efficiency *E. coli* DH5α chemically competent cells according to the manufacturer's protocol.

Transformed colonies were screened with Colony Screen Fast (Epicentre) according to the manufacturer's protocol, followed by mini-plasmid preps of plasmids that were considered possible successful ligations. These plasmids were screened using PCR in the following reaction: 1 µL P1P2 Forward Repair, 1 µL LuxI Reverse, 0.5 µL of mini-prep plasmid, 22.5 µL of sterile water, and a Ready-To-Go PCR bead.

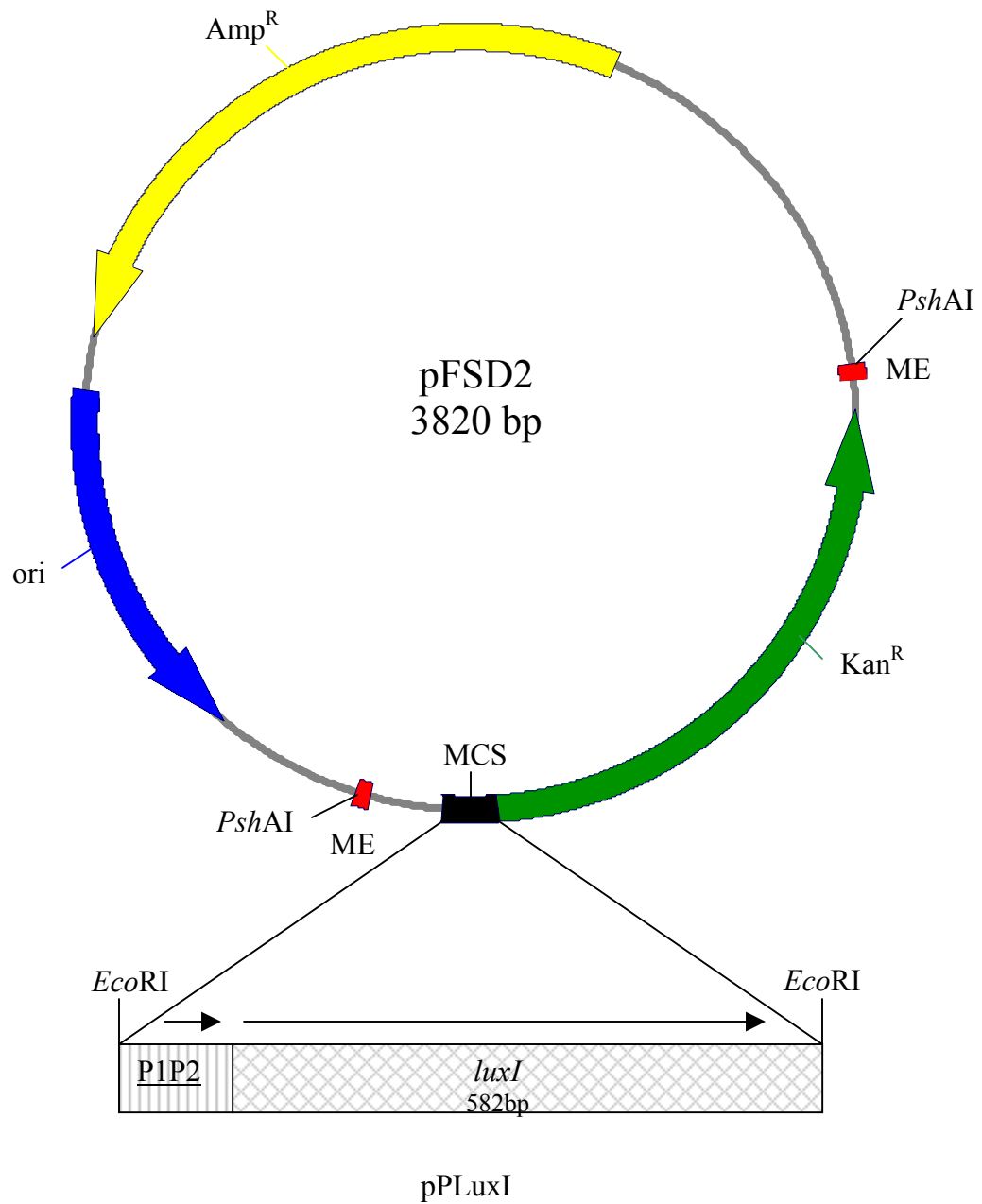


Figure 3-3. Construction of plasmid pPLuxI. Vector pCR2.1-P1P2LuxI was digested with *EcoRI* and cloned into the *EcoRI* site of pFSD2. ME = Mosaic end, Kan^R = Kanamycin resistance, Amp^R = Ampicillin resistance, ori = *E. coli* origin of replication

The reaction was subjected to the following conditions: 94°C for 5 min, followed by 30 cycles of 94°C for 30 sec, 55°C for 30 sec, and 72°C for 1 min with a final extension time of 10 min. PCR product was separated on a 1% TBE gel with EtBr and visualized under UV light. All clones tested had the expected band of 746 bp. Clone #14 was chosen for further screening, and a large-scale plasmid prep was done, with concentration determined to be 507 ng/μL. This plasmid was then digested with *EcoRI* overnight at 37°C, and separated on a 0.9% TBE gel with EtBr. The bands were visualized under UV light, and confirmed to contain the *PIP2luxI* insert. The plasmid was sequenced and then named pPLuxI.

TP-15 Bacteriophage Propagation

Bacillus thuringiensis 4Q7 was grown overnight in LB broth at 30°C, 200 rpm. Of this overnight culture, 2 mL were pipetted into a sterile 10 mL glass culture tube, along with 0.500 mL of bacteriophage suspension and 4 mL of molten LB top agar. This was poured onto a prewarmed LB agar plate and incubated at 30°C overnight. The following day, the top agar was removed and the plate was washed with 3 mL sterile PBS. This mixture was placed into a 50 mL tube, and 4 drops of chloroform added. The tube was then vortexed vigorously for 1 minute, and centrifuged at 3,000 rpm for 10 min. The supernatant containing bacteriophage was then passed through 0.45 μm filter and stored at 4°C until further use.

To determine pfu/mL of TP-15, the stock solution of phage was serially diluted by adding 10 μL of stock solution of phage to 90 μL of PBS. These dilutions were then

added to 2 mL of overnight culture of *B. thuringiensis* 4Q7 and 3 mL of molten LB top agar. The mixture was poured onto prewarmed LB agar plates, and incubated at 30°C overnight. Plaques were counted the following day and pfu/mL was determined.

TP-15 Bacteriophage Genomic Prep

Bacteriophage genomic preparation was done according to the protocol outlined in Current Protocols in Molecular Biology with slight modifications as stated (Besmond, 1988). In brief, *B. thuringiensis* 4Q7 was grown overnight in 50 mL LB broth at 30°C, 200 rpm. Approximately 4.5×10^8 TP-15 phage were added to this culture, and incubated at 37°C for 24 hr at 200 rpm. After the infection period, 4 drops of chloroform were added to destroy any bacterial cells that had not lysed, and the mixture was then centrifuged at 10,000 rpm, 4°C for 10 min. The supernatant was decanted into a sterile 40 mL Oakridge tube, where 15 µL of the Qiagen DNase-RNase solution was added (90 µg DNase and 300 µg RNase), and incubated at 37°C, 200 rpm for 1 hr. The solution was then ultracentrifuged at 25,000 rpm in a SW28 rotor for 2 hr at 4°C. The supernatant was decanted and the pellet containing phage was resuspended in 200 µL of 0.5 M Tris-HCl, pH 8.0 in an eppendorff tube. To this, 200 µL of buffered phenol (pH 8.0) was added, and the tube was vortexed at max speed for 20 min. After vortexing, the tube was centrifuged at 12,000 rpm in a bench top centrifuge for 2 min. The aqueous layer was removed and transferred to a clean Eppendorff tube. The phenol extraction was repeated in the same manner a second time. Next, 200 µL of chloroform was added and vigorously mixed for 1 min to remove any residual phenol. The solution was then

centrifuged for 1 min at 12,000 rpm, and the aqueous layer was transferred to a clean tube. TP-15 genomic DNA was then precipitated with 20 μ L of 3 M Sodium Acetate (pH 5.5) and 400 μ L of 100% ethanol. Precipitated DNA was pelleted at 12,000 rpm for 10 min. The supernatant was decanted, and 1 mL of 70% ethanol was added to remove any residual salts. The DNA was again pelleted at 12,000 rpm for 5 min, the supernatant decanted, and allowed to air-dry overnight. The following day, the pellet was resuspended in 100 μ L of TE buffer, with concentration determined to be 408 ng/ μ L. Verification of the phage genomic prep was done by digesting the DNA with *Eco*RI and separating resulting bands on a 0.4% TBE gel at 50V for 10 hr.

Insertion of *P1P2luxI* Into TP-15 Phage Genome

Plasmid pPLuxI was digested with *Psh*AI in buffer #4 (NEB) overnight at 25°C to excise the transposome fragment. The digest was separated on 1% TBE gel with no EtBr, and the band of interest was excised as described above. The transposome fragment was gel purified, resuspended in 15 μ L of sterile HPLC-grade water, and quantified at 227 ng/ μ L.

For the *in vitro* insertion of *P1P2luxI* into the TP-15 genome, the following reaction was assembled according to the manufacturer's protocol: 1 μ L EZ::TN 10X Reaction Buffer (Epicentre), 1 μ L (200 ng) of TP-15 genomic DNA, 1 μ L (9 ng) of P1P2luxI insert, 1 μ L EZ::TN Transposase (Epicentre), 6 μ L sterile water. This mixture was incubated at 37°C for 2 hr. To stop the reaction, 1 μ L of EZ::TN 10X Stop Solution (Epicentre) was added, and the mixture was heated at 65°C for 10 minutes.

To propagate the TP-15 phage, 0.5 μ L, 1 μ L, and 2 μ L of the *in vitro* insertion reaction was electroporated into electrocompetent *B. thuringiensis* 4Q7 cells as described above. The negative control reaction contained no DNA. After the 3 hr recovery period, the entire 1 mL of cells was mixed with 3 mL of LB top agar warmed to 45°C, and poured onto warmed LB agar plates. These were incubated at 30°C overnight, followed by monitoring of plaque formation the next day.

Chapter 4: Results

Detection of *luxI* Transcript from *B. thuringiensis* pHY-XylII

Figure 4-1 shows the gel separation of RT-PCR product from xylose-induced cultures of *B. thuringiensis* pHY-XylII and pHY-pXyl in a time course assay. RT-PCR reaction products were first seen in the pHY-XylII total RNA at the 0 time point. Amplicon size corresponded to that of the *luxI* gene, 582 bp. Transcript was present in all samples, with a faint band in the 0 time point, and a greater amount of product seen in all other samples up to 3 hr. There was no band seen in the negative control where no template was added, and an equal size band seen in the positive control with *Vibrio fischeri* total RNA added (Figure 4-1B).

The negative control reactions containing total RNA from pHY-pXyl, which does not contain the *luxI* gene, showed bands of approximately 600 bp in all samples (Figure 4-1A). The bands were of increasing concentration up to 150 min, with a fainter band in the 180 min post induction, but appear to be less product than seen in the pHY-XylII reactions.

Bioluminescent Detection of OHHL from Xylose Induced *B. thuringiensis* pHY-XylII

A RoLux OHHL bioreporter assay was performed with filter sterilized supernatants from *B. thuringiensis* pHY-XylII and pHY-pXyl xylose induced cultures in a time course assay, and were analyzed for bioluminescent output with varying ratios of RoLux culture and supernatant on two separate instruments. Data for experiments done

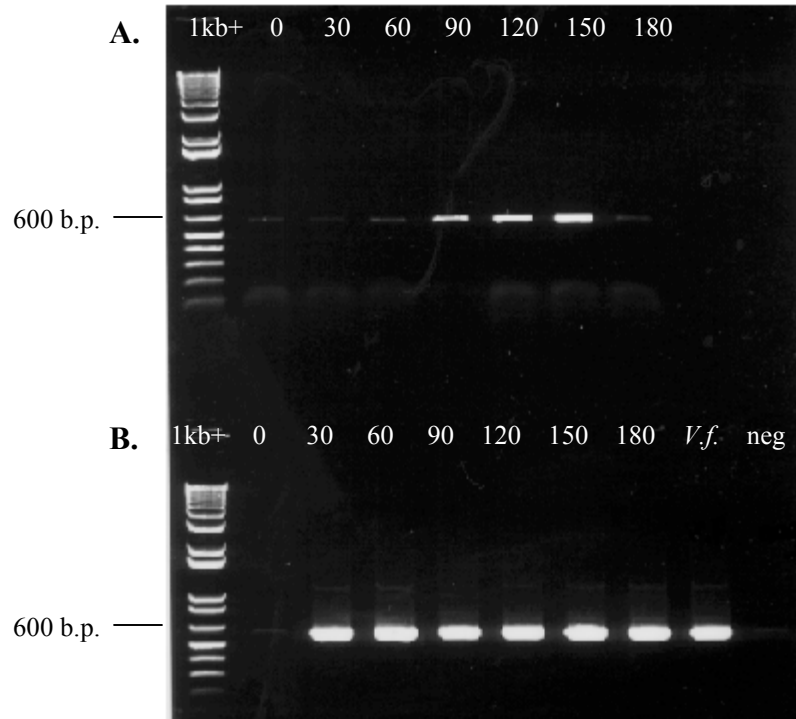


Figure 4-1. Time Course of *luxI* Transcript Presence in *B. thuringiensis* pHY-pXyl and pHY-XylII Xylose Induced Cultures. (A) RT-PCR reaction products of RNA from the negative control culture, pHY-pXyl and (B) from pHY-XylII containing the *luxI* gene, at 30-minute intervals after induction with 20 mM xylose. *V. fischeri* (*V.f.*) RNA, and negative control with no template (neg) reactions are also shown. Sampling times are indicated above the corresponding wells.

on the Wallac Victor2 1420 Multilabel Counter (Perkin Elmer Life Sciences, CT) are reported as specific bioluminescence (CPS/OD₄₉₀) as this instrument has the capability to measure absorbance. Data for experiments done on the Wallac Microbeta 1450 Plus (Perkin Elmer Life Sciences, CT) are reported as bioluminescent counts per second (CPS) only. Analysis of peak bioluminescence for each sample was measured as this is an indicator of OHHL concentration, in addition to the earliest time where bioluminescence values were significantly higher than that of the background (time 0).

Wallac Victor2 1420 Multilabel Counter Experiments

Figures 4-2A shows the specific bioluminescence (SB) of the RoLux reporter in a 1:1 (100 μ L: 100 μ L) ratio of reporter to supernatants from *B. thuringiensis* pHY-XylII induced culture at increasing time after induction with xylose. Peak SB of the reporter was obtained for all samples near 5.5 hr. A significant increase in the peak SB above background (time 0) at the 95% confidence level ($P=0.00403$) was detectable at 30 minutes post induction for the 5.5 hr point, where the SB then approximately doubled in a linear response at each 30 min interval up to 150 min where RoLux then reached saturation with the 180 min supernatant. The 180 min post induction culture supernatant produced the greatest amount of light, with peak SB at $19,957 \pm 2,696$. Supernatant from the 150 min induced culture produced similar results, reaching peak SB of $19,640 \pm 3,033$. There was no induction seen with any of the supernatant used from the *B. thuringiensis* pHY-pXyl negative control (Figure 4-2B).

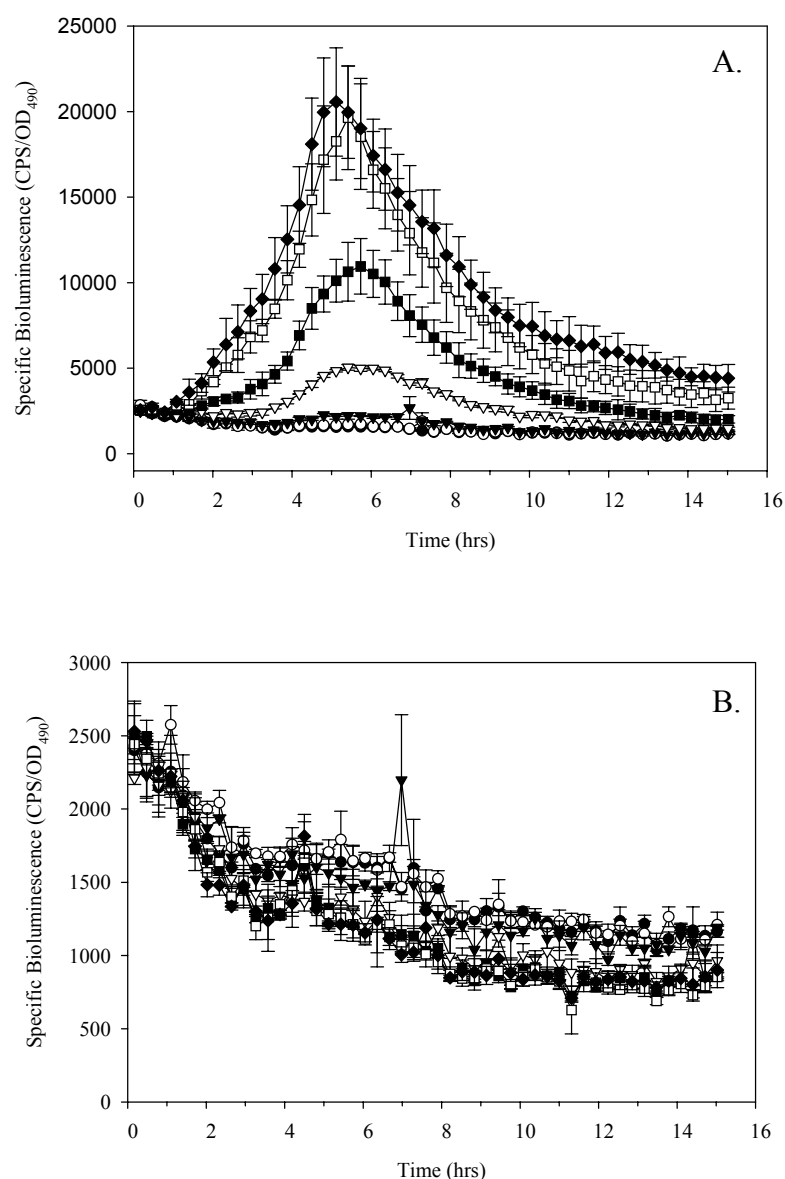


Figure 4-2. Detection of OHHL From *B. thuringiensis* Culture Supernatant at 100 μ L: 100 μ L Reporter to Supernatant Ratio on the Wallac Victor2 1420 Multilabel Counter. Supernatants were collected at various time points from xylose-induced cultures of *B. thuringiensis* pHY-XylI and pHY-pXyl. Bioluminescent response with 100 μ L of the OHHL reporter RoLux to 100 μ L of these supernatants was monitored over time. (A) Bioluminescent response of RoLux to *B. thuringiensis* pHY-XylI supernatant and (B) to *B. thuringiensis* pHY-pXyl supernatant with sampling times of supernatant as indicated: (●) 0, (○) 30, (▼) 60, (▽) 90, (■) 120, (□) 150, (◆) 180. SB values are the average of triplicate samples

In Figure 4-3A, RoLux specific bioluminescence values are shown for the 3:1 ratio (150 μ L: 50 μ L) RoLux reporter to *B. thuringiensis* pHY-XylII xylose induced culture supernatant assay. In this experiment, peak SB was also reached near 5.5 hr, but values were much lower as compared to the 1:1 ratio assay. The peak SB for time points 0-90 showed no significant difference. A significant increase at the 95% confidence level ($P = 0.0090$) of peak SB above background was first seen in the 120 min post induction sample. Peak SB approximately doubled as in a linear response for each of the next two samples, 150 and 180 min. The 180 min post induction supernatant produced the greatest SB at $12,029 \pm 672$. The negative control reporter assay using *B. thuringiensis* pHY-pXyl showed no induction for all time points (Figure 4-3B).

Control induction assays included the RoLux reporter with fresh medium as a negative, and with 1 μ M synthetic OHHL in fresh media as a positive for both the 1:1 and 3:1 ratios as seen in Figure 4-4. For both ratios, negative controls did not induce bioluminescence. In the positive control with 1 μ M OHHL in fresh medium at a 1:1 ratio, the maximum SB was reached after 4.25 hr at $76,740 \pm 13,088$. In the 3:1 positive control, maximum SB was reached after 4.75 hr at $11,3707 \pm 1,172$.

A summary of all induction assays done on the Wallac Victor2 1420 Multilabel Counter are shown in Table 4-1 and 4-2. Analysis between the two ratio assays shows that in the 1:1 experiment, significant increase in peak SB at the 95% confidence interval ($P < 0.05$) occurred in supernatant 30 min post induction, while in the 3:1 experiment, significant increase in peak SB occurred 90 min later with the 120 min post induction supernatant. In addition, the peak SB with the 180 min post induction supernatant for the 1:1 ratio was $39.7\% \pm 14.2$ higher than the 3:1 ratio.

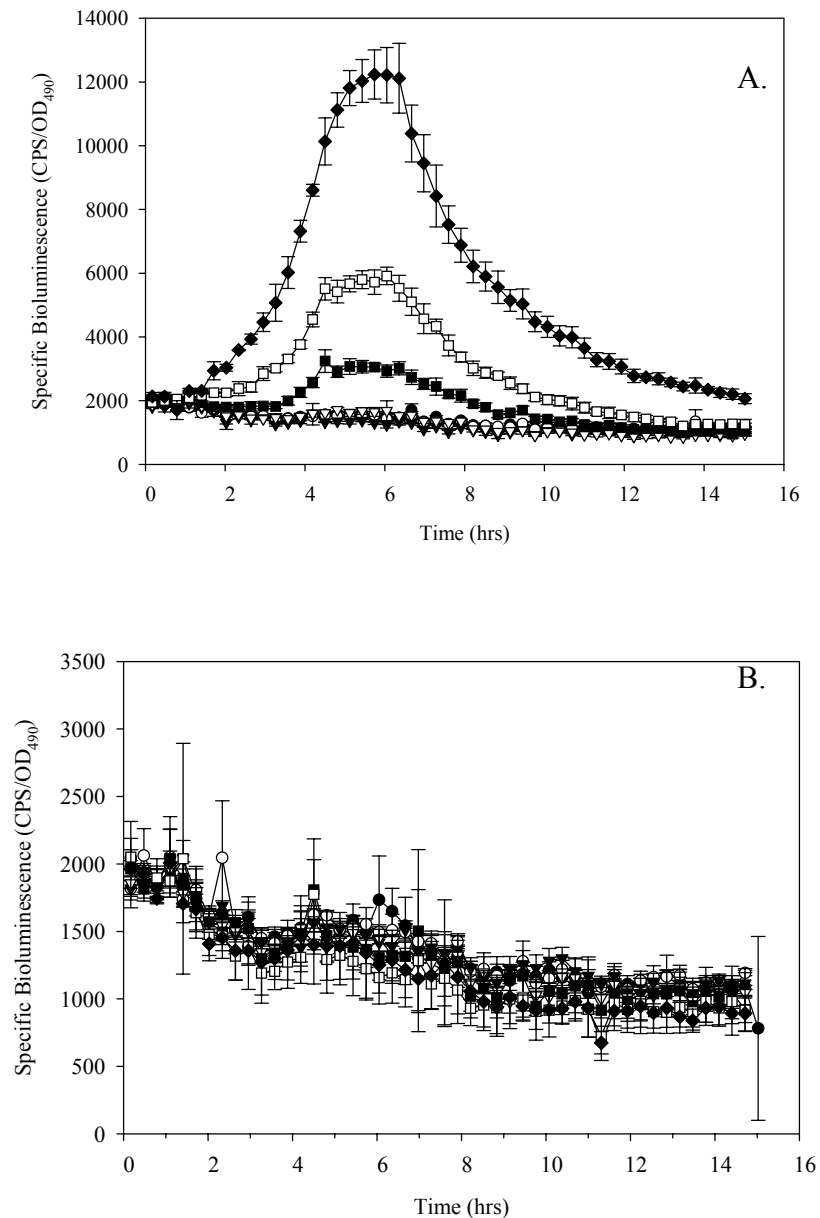


Figure 4-3. Detection of OHHL From *B. thuringiensis* Culture Supernatants at 150 µL: 50 µL Reporter to Supernatant Ratio on the Wallac Victor2 1420 Multilabel Counter. Supernatants were collected at various time points from xylose-induced cultures of *B. thuringiensis* pHY-XylII and pHY-pXyl. Bioluminescent response with 150 µL of the OHHL reporter RoLux to 50 µL of these supernatants was monitored over time. (A) Bioluminescent response of RoLux to *B. thuringiensis* pHY-XylII supernatant and (B) to *B. thuringiensis* pHY-pXyl supernatant with sampling times of supernatant as indicated: (●) 0, (○) 30, (▼) 60, (▽) 90, (■) 120, (□) 150, (◆) 180. SB values are the average of triplicate samples

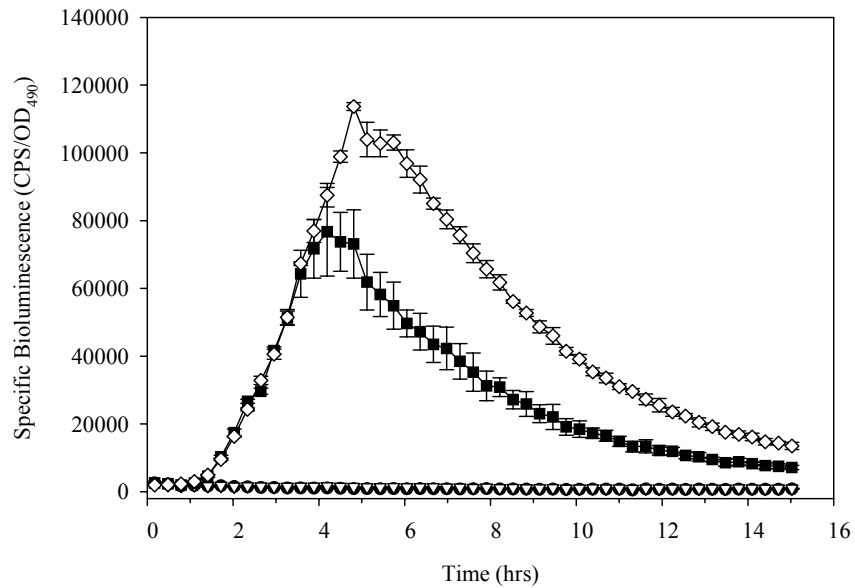


Figure 4-4. Bioluminescent Reponse of RoLux to a Known Quantity of OHHL on the Wallac Victor2 1420 Multilabel Counter. RoLux bioluminescent bioreporter was monitored for bioluminescent production with different ratios of reporter culture to fresh medium, or fresh medium with 1 μ M OHHL to confirm reporter induction in the presence of OHHL. (◇) 150 μ L RoLux with 50 μ L 1 μ M OHHL in fresh medium (3:1), (■) 100 μ L RoLux with 100 μ L 1 μ M OHHL in fresh medium (1:1), (▽) 150 μ L RoLux with 50 μ L fresh medium (3:1), (●) 100 μ L RoLux with 100 μ L fresh medium (1:1). SB values are the average of triplicate samples

Table 4-1. Summary of RoLux Induction with *B. thuringiensis* pHY-XylII Supernatant Experiments on the Wallac Victor2 1420 Multilabel Counter

Time Post Induction (mins)	Peak SB (CPS/OD ₄₉₀) of RoLux with 1:1 ratio	Average Number of Reporter Cells (CFU x 10 ⁵ /100 µL)	Time of Earliest Detection (mins)	Peak SB (CPS/OD ₄₉₀) of RoLux with 3:1 ratio	Average number of Reporter Cells (CFU x 10 ⁵ /150 µL)	Time of Earliest Detection (mins)
0	1585 ± 47	27.9 ± 2.1	N/A	1611 ± 50	46.0 ± 0.71	N/A
30	1706 ± 35	26.1 ± 2.7	270	1523 ± 66	48.5 ± 0.93	N/D
60	2210 ± 191	27.6 ± 1.6	177	1373 ± 35	73.8 ± 3.5	N/D
90	5048 ± 170	28.8 ± 1.3	84	1674 ± 103	82.1 ± 6.3	N/D
120	10638 ± 1717	33.9 ± 8.8	65	3065 ± 197	87.8 ± 8.1	214
150	19640 ± 3033	34.5 ± 9.0	65	5797 ± 282	93.6 ± 6.7	47
180	19957 ± 2696	40.5 ± 17.4	65	12029 ± 672	82.3 ± 2.5	105

The time post-induction of *B. thuringiensis* pHY-XylII cultures with xylose, peak specific bioluminescence (SB) of RoLux obtained with corresponding supernatant, and the number of RoLux cells per well is indicated. The earliest significant induction above the background (time 0) at the 95% confidence interval ($p < 0.05$) for both ratio experiments is also shown. N/A = Not applicable, N/D = Not detectable.

Table 4-2. Summary of RoLux Induction Controls on the Wallac Victor2 1420 Multilabel Counter

Ratio of RoLux to Treatment	Treatment	Peak Specific Bioluminescence (CPS/OD ₄₉₀)	Time of Earliest Detection (mins)
1:1	Fresh Media	1071 ± 66	N/A
1:1	1 µM OHHL	76,740 ± 13,088	105
3:1	Fresh Media	1431 ± 95	N/A
3:1	1 µM OHHL	113,707 ± 1172	47

RoLux peak SB is indicated during negative (fresh media) and positive (1µM with fresh media) controls for both ratio experiments. The earliest time of detectable SB above background (fresh media) at the 95% confidence interval ($P < 0.05$) is indicated. N/A = Not applicable.

For the 1:1 ratio experiment, earliest detection of significant induction at the 95% confidence interval ($P < 0.05$) for RoLux as compared to the background SB (time 0) was seen in the 30 min post induction supernatant at 270 min, with the fastest response time seen in the 180 min supernatant after 65 min. The earliest detection of SB in RoLux for the 3:1 experiment was seen with the 120 min supernatant after 214 min, with the fastest seen in the 150 min supernatant at 47 min. Response time in the 3:1 ratio 180 min supernatant did not follow the same trend as seen in the 1:1 ratio experiment, with earliest detection seen later than the 150 min supernatant.

The number of reporter cells in the 1:1 ratio experiment remained consistent from the time 0 supernatant up to the 90 min supernatant ($2.8 \times 10^6 \pm 2.1 \times 10^5$ to $2.9 \times 10^6 \pm 1.3 \times 10^5$, respectively), where it then increased slightly up to the 180 min supernatant ($4.1 \times 10^6 \pm 1.7 \times 10^6$). In contrast, reporter cell number increased greatly in the 3:1 experiment, from the initial cell number in with the time 0 supernatant at $4.6 \times 10^6 \pm 7.1 \times 10^4$ CFU up to $8.2 \times 10^6 \pm 2.5 \times 10^5$ CFU in the 180 min supernatant.

In contrast to the experimental assay, greater SB was seen with the 3:1 ratio in the control assay. In the positive control assays, the RoLux reporter produced $32.5 \% \pm 10.7$ less SB at maximum induction in the 1:1 ratio than the 3:1 ratio. In addition, response time was faster for the 3:1 ratio at 47 min as compared to 105 min for the 1:1 ratio control.

Wallac Microbeta 1450 Plus Experiments

Figure 4-5 shows the RoLux bioluminescent output as counts per second (CPS) in a 1:1 (100 μ L: 100 μ L) ratio with *B. thuringiensis* pHY-XylII xylose induced culture supernatant. CPS maximum values for each sample were reached at approximately 6 hr. A significant increase ($P < 0.05$) in CPS was seen in the 30 min post induction supernatant above background (time 0). Peak CPS for the later samples increased in sigmoidal fashion.

The greatest induction of RoLux was measured for the 180 min post induction supernatant with peak bioluminescence at $514,680 \pm 3,349$ CPS, followed closely by the 150 min post induction supernatant at $430,057 \pm 18,174$ CPS. Figure 4-6 shows the RoLux bioluminescent output (CPS) in a 3:5 ratio with *B. thuringiensis* pHY-XylII supernatant. Results from this experiment were similar to that seen in the 1:1 ratio assay, with maximum bioluminescence reached at 6 hr. Peak CPS values for supernatants also increased in a sigmoidal fashion in this assay, with the greatest induction of the RoLux reporter was attained with the 180 min post induction supernatant, with bioluminescence at $647,383 \pm 7,889$ CPS.

A summary of the results from both assays done on the Wallac Microbeta 1450 Plus is shown in Table 4-3. A higher ratio of RoLux to supernatant (1:1) produced less bioluminescence ($20.5\% \pm 0.5$) than the lower ratio (3:5). In the 1:1 ratio experiment, the earliest detection of bioluminescence over the average of the background (time 0) was 117 min with the 30 min supernatant, and fastest at 11 min with the 180 min supernatant. In the 3:1 experiment, response time was the same as seen in the 1:1 ratio assay. Overall, bioluminescent output was greater for the 1:1 ratio assay, yet response time was the same.

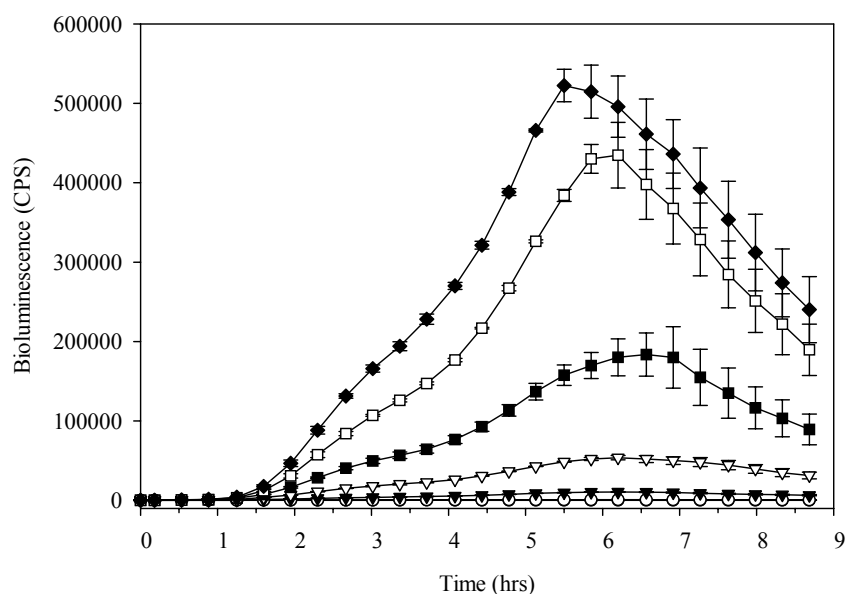


Figure 4-5. Detection of OHHL From *B. thuringiensis* Culture Supernatants at 100 μ L: 100 μ L Reporter to Supernatant Ratio on the Wallac Microbeta 1450 Plus. Supernatants were collected at various time points from xylose-induced cultures of *B. thuringiensis* pHY-XylII. Bioluminescent response with 100 μ L of the OHHL reporter RoLux to 100 μ L of these supernatants was monitored over time. (A) Bioluminescent response of RoLux to *B. thuringiensis* pHY-XylII supernatant with sampling times of supernatant as indicated: (●) 0, (○) 30, (▼) 60, (▽) 90, (■) 120, (□) 150, (◆) 180. CPS values are the average of triplicate samples

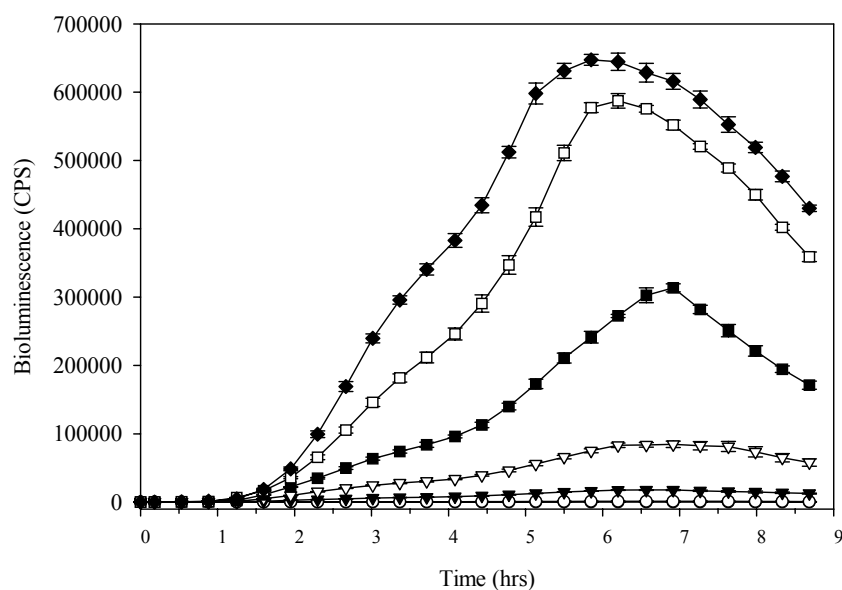


Figure 4-6. Detection of OHHL From *B. thuringiensis* Culture Supernatants at 75 μ L: 125 μ L Reporter to Supernatant Ratio on the Wallac Microbeta 1450 Plus. Supernatants were collected at various time points from xylose-induced cultures of *B. thuringiensis* pHY-XylI. Bioluminescent response with 25 μ L of the OHHL reporter RoLux to 175 μ L of these supernatants was monitored over time. (A) Bioluminescent response of RoLux to *B. thuringiensis* pHY-XylI supernatant with sampling times of supernatant as indicated: (●) 0, (○) 30, (▼) 60, (▽) 90, (■) 120, (□) 150, (◆) 180. CPS values are the average of triplicate samples

Table 4-3. Summary of RoLux Induction with *B. thuringiensis* pHY-XylII Supernatant on the Wallac Microbeta 1450 Plus

Time Post Induction (min)	Peak Bioluminescence (CPS) of RoLux with 1:1 ratio	Time of Earliest Induction Detectable (min)	Peak Bioluminescence (CPS) of RoLux with 3:5 ratio	Time of Earliest Induction Detectable (min)
0	280 ± 17	N/A	280 ± 26	N/A
30	857 ± 23	117	1,183 ± 30	117
60	10,473 ± 124	75	16,353 ± 335	75
90	52,107 ± 1,948	32	75,207 ± 2,964	32
120	169,803 ± 16,486	32	241,407 ± 8,575	32
150	430,057 ± 18,174	32	577,463 ± 7,164	32
180	514,680 ± 3,349	11	647,383 ± 7,889	11

Sampling time of *B. thuringiensis* pHY-XylII culture is indicated as Time Post-Induction, with the peak CPS of RoLux reached after 6 hr with the corresponding supernatant. Earliest detection times were calculated with 95% confidence ($P < 0.05$) as compared to the background (time 0).

RoLux Growth Curve

The growth curve of RoLux in LB Kan 50 µg/mL at 37°C can be seen in Figure 4-7. A semi-log plot of the CFU/mL determined during the growth curve experiment is in Figure 4-8. The generation time for RoLux under these conditions was found to be 109.04 ± 23.79 min by direct calculation.

***B. thuringiensis* pHY-XylII Growth Curve**

The growth curve for *B. thuringiensis* pHY-XylII in LB broth 20 mM xylose at 30°C is in Figure 4-9. A semi-log plot of the CFU/mL determined during the growth curve experiment is in Figure 4-10. Generation time was calculated by direct method, and found to be 63.95 ± 5.18 minutes.

Quantification of OHHL Produced by *B. thuringiensis* pHY-XylII Induced Cultures

OHHL production from *B. thuringiensis* pHY-XylII was determined through a time course assay where supernatant from the induced culture was taken at various points after induction, and specific bioluminescence production from OHHL reporter strain RoLux with these supernatants was monitored. Figure 4-11 shows the induction of RoLux with supernatants from the time course assay with specific bioluminescence measured on the Wallac Victor2 1420 Multilabel Counter (Perkin Elmer Life Sciences). Figure 4-12 shows the peak specific bioluminescence obtained by RoLux with its corresponding supernatant.

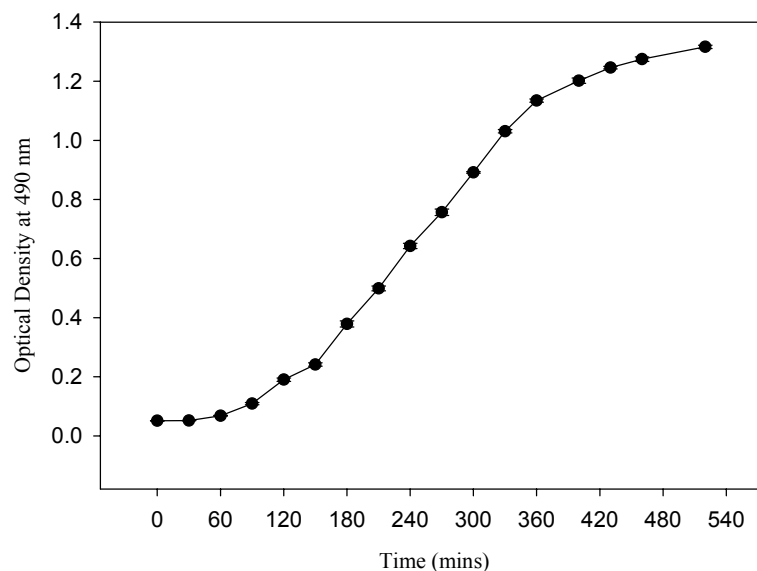


Figure 4-7. RoLux Growth Curve. The optical density (OD) at 490 nm of a RoLux culture was monitored over 9 hr. OD values indicated are the average of triplicate samples.

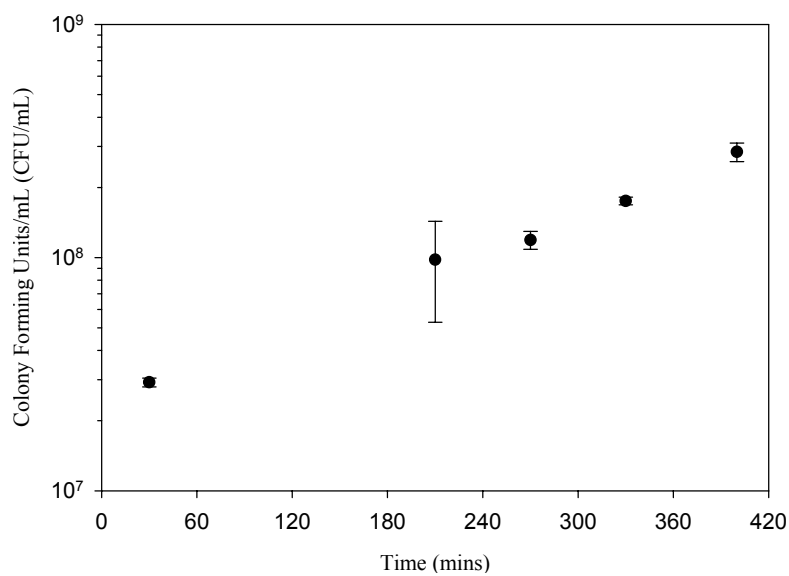


Figure 4-8. RoLux Viable Cell Count During Growth. The RoLux CFU/mL was calculated during growth curve at indicated sampling times. CFU/mL values represent the average of triplicate samples.

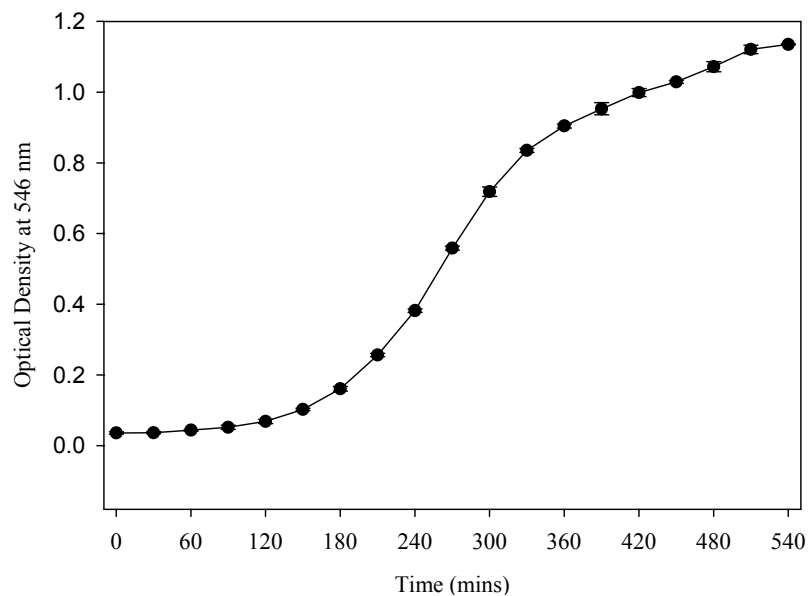


Figure 4-9. *B. thuringiensis* pHY-XylI Growth Curve. The optical density (OD) at 546 nm of a *B. thuringiensis* pHY-XylI culture was monitored over 9 hr. OD values are the average of triplicate samples.

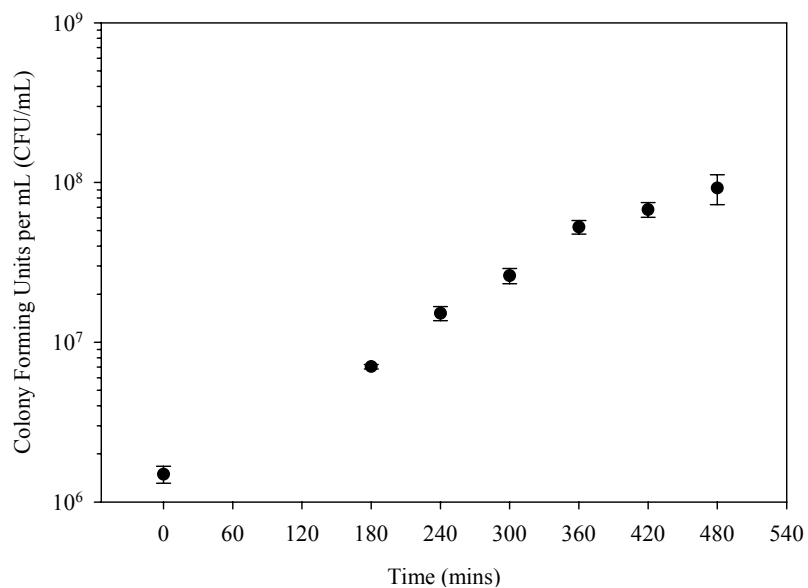


Figure 4-10. *B. thuringiensis* pHY-XylI Viable Cell Count During Growth. The *B. thuringiensis* pHY-XylI CFU/mL were calculated during the growth curve at indicated sampling times. CFU/mL values represent the average of triplicate samples.

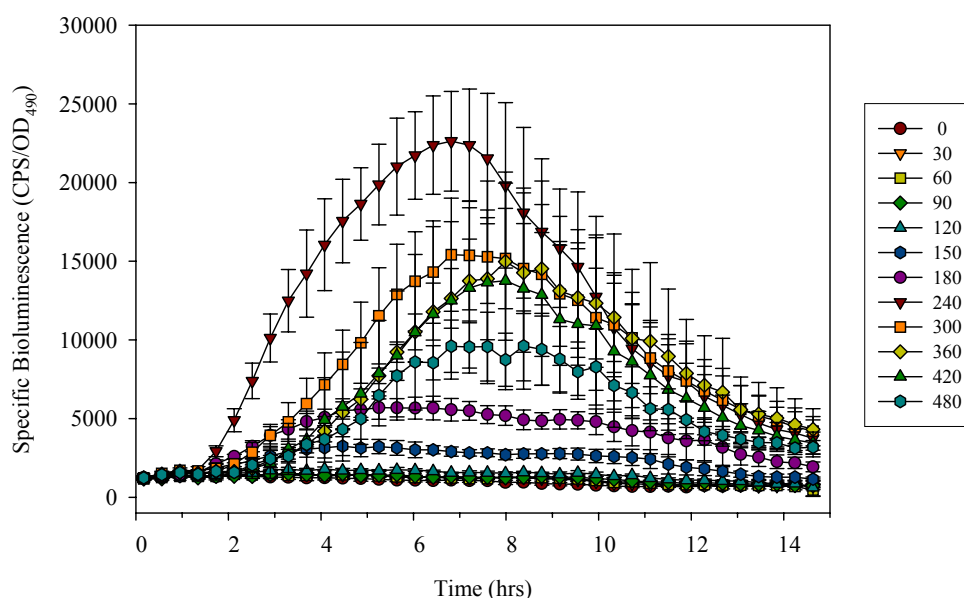


Figure 4-11. Detection of OHHL from Supernatants of *B. thuringiensis* pHY-XylII With Increasing Culture Age. Supernatants from a xylose-induced *B. thuringiensis* pHY-XylII culture were monitored for the presence of OHHL through bioluminescent bioreporter assay with RoLux. Values shown are the average of triplicate samples and sampling time (min) of the supernatants are indicated in the legend.

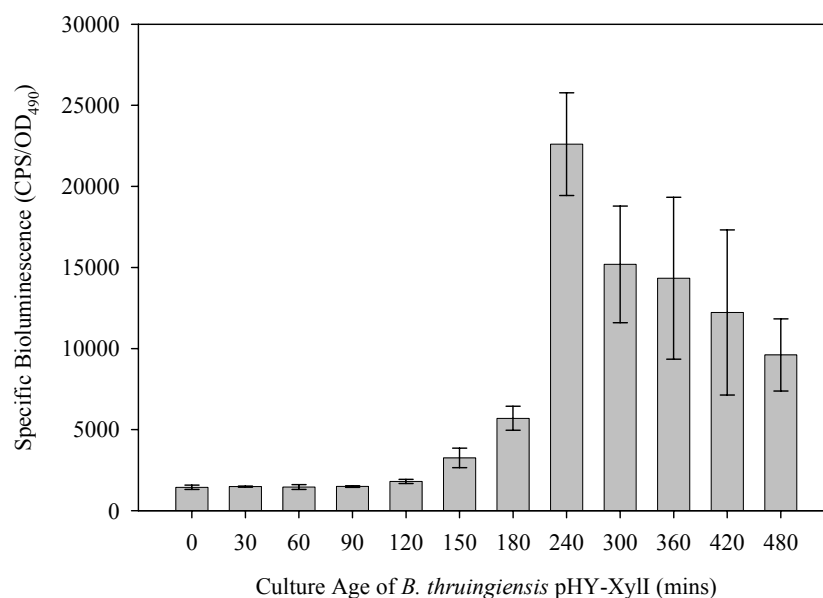


Figure 4-12. Peak Bioluminescence of RoLux Obtained with *B. thuringiensis* pHY-XylII Supernatants With Increasing Culture Age. The maximum specific bioluminescence of RoLux obtained with each supernatant is shown. Values are the average of triplicate samples.

To calculate the concentration of OHHL in each of the supernatants, two separate standard curves were obtained, using supernatant from the negative control culture, *B. thuringiensis* pHY-pXyl at log ($OD_{546} = 0.4252$) and stationary phase ($OD_{546} = 1.1252$) of the growth curve with known concentrations of synthetic OHHL added. Separate standard curves were used to simulate supernatant conditions at various stages of growth. Both the log and stationary standard curves began to reach saturation after 100 nM OHHL (Figures 4-13A and 14A, respectively). The linear response range of each of the curves was plotted, and the linear equation was used to calculate OHHL concentrations that fell into that range (Figures 4-13B and 4-14B).

Values for OHHL concentration during each stage of growth were based on the corresponding standard curve as indicated in Table 4-4. There was no detectable OHHL in supernatants from the 30-120 min post induction supernatants as the peak specific bioluminescence values for these samples were not significantly above background ($P < 0.05$). Bioluminescent values for the lag and log phase supernatant samples (0-240) fell into the linear range, excluding time point 150, where although there was detectable increase in bioluminescence, the OHHL concentration was below that of the lowest concentration in the standard curve (5 nM). Specific bioluminescence of the stationary phase supernatant samples (300-480) did not fall into the linear response range of the stationary phase standard curve, and were therefore estimated with the equation from the hyperbolic plot (Figure 4-14A). OHHL concentration increased from the 150 min supernatant to the 420 min sample, with the greatest concentration of OHHL in the 300 min sample. There was a sharp decrease in the OHHL concentration found in the 480 min supernatant. Although the stationary phase supernatants produced less

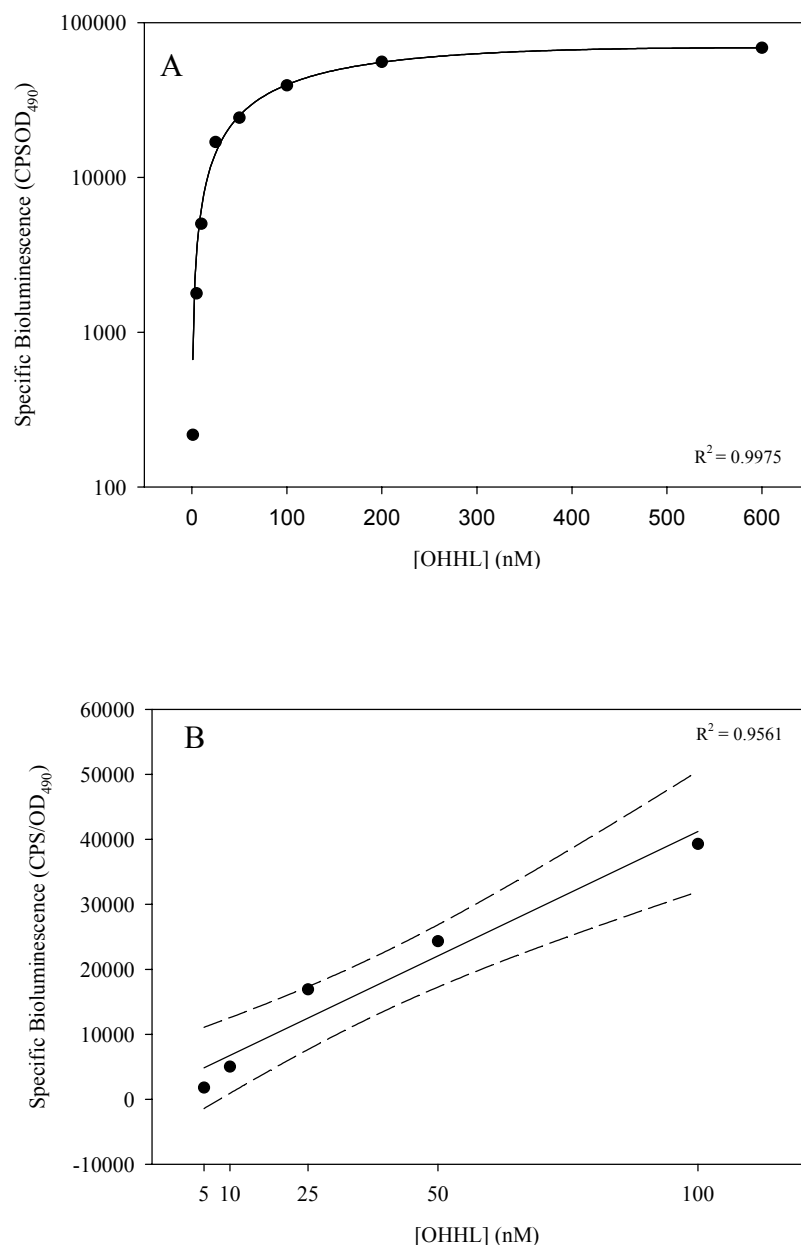


Figure 4-13. RoLux Induction With Synthetic OHHL added in *B. thuringiensis* Log-Phase Supernatant. **A.** Hyperbolic-fit plot of peak SB of RoLux with 0, 5, 10, 25, 50, 100, 200 and 600 nM OHHL added to OD₅₄₆ = 0.4252 supernatant from *B. thuringiensis* pHY-pXyl. **B.** Linear response range of hyperbolic plot. The linear best fit is indicated by the solid line, and the 95% confidence intervals shown with dashed line.

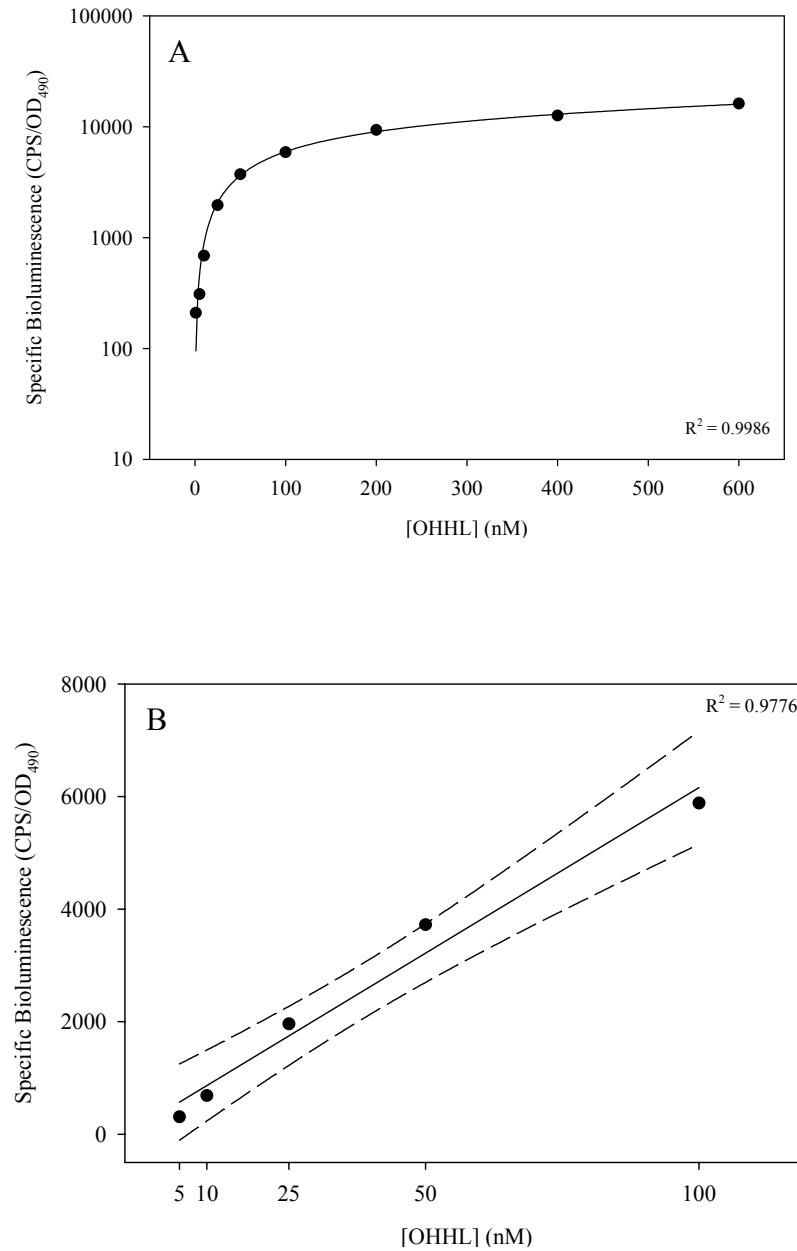


Figure 4-14. RoLux Induction With Synthetic OHHL added in *B. thuringiensis* pHY-pXyl Stationary-Phase Supernatant. **A.** Hyperbolic-fit plot of peak SB of RoLux with 0, 5, 10, 25, 50, 100, 200, 400 and 600 nM OHHL added to OD₅₄₆ = 1.1252 supernatant from *B. thuringiensis* pHY-pXyl. **B.** Linear response range of hyperbolic plot. The linear best fit is indicated by the solid line, and the 95% confidence intervals shown with dashed line.

Table 4-4. OHHL Concentrations from *B. thuringiensis* pHY-Xyll Supernatants

Sampling Time (mins)	OD ₅₄₆	Peak SB	Avg. Peak SB	[OHHL] nM	Standard Curve
0	0.0398 0.0339 0.0341	1,368 1,378 1,608	1,451 ± 136	0	Log
30	0.0401 0.0352 0.0348	1,527 1,474 1,456	1,486 ± 37	0	Log
60	0.0460 0.0426 0.0423	1,373 1,630 1,384	1,462 ± 145	0	Log
90	0.0587 0.0493 0.0479	1,505 1,442 1,535	1,494 ± 47	0	Log
120	0.0751 0.0681 0.0631	1,788 1,938 1,690	1,805 ± 125	0	Log
150	0.1064 0.1015 0.0997	2,709 3,899 3,141	3,249 ± 602	NQ	Log
180	0.1687 0.1574 0.1565	5,158 6,549 5,399	5,702 ± 743	7.29 ± 1.94	Log
240	0.3868 0.3828 0.3764	20,004 26,143 21,722	22,623 ± 3,167	51.47 ± 8.27	Log
300	0.7334 0.7073 0.7150	12,974 19,548 13,732	15,418 ± 3,597	483.11 ± 103.60	Stationary
360	0.9081 0.8978 0.9070	10,676 13,796 20,450	14,974 ± 4,992	442.45 ± 162.86	Stationary
420	1.0078 1.0025 0.9863	8,043 21,437 11,791	13,310 ± 5,093	366.02 ± 219.45	Stationary
480	1.0882 1.0654 1.0625	7,372 9,672 11,811	9,618 ± 2,220	234.98 ± 97.29	Stationary

Sampling time of the supernatant is indicated along with corresponding OD₅₄₆ of pHY-Xyll culture at sampling time. Peak specific bioluminescence produced by RoLux with pHY-Xyll supernatants was used to calculate OHHL concentration in each sample, based on a standard curve for the appropriate growth phase as indicated. NQ = Detectable but not quantifiable

specific bioluminescence than the log phase supernatants, the OHHL concentration was determined to be higher. This anomaly in the bioluminescent response of the reporter may be due to physiological effects, or degradation of the OHHL by the enzyme lactonase, which is found in *B. thuringiensis* 4Q7.

Qualitative Lactonase Assay using *B. thuringiensis* 4Q7

To determine the presence of lactonase in *B. thuringiensis* 4Q7, an overnight culture was incubated with 40 μ M OHHL for 4 hr at 28°C. Control assays included the same incubation conditions with *B. thuringiensis* and no OHHL and fresh medium with OHHL. Supernatant from these incubations were then spotted on an AT plates with X-gal (point 0), and 5 μ L of the AHL indicator strain *A. tumefaciens* 136A was spotted vertically at intervals of 1 cm from point 0. The control plate with 40 μ M OHHL induced the *A. tumefaciens* 136A LacZ reporter strain up to approximately 3 cm from point 0, showing that the OHHL was capable of diffusing across the plate (Figure 4-15A). The *B. thuringiensis* 4Q7 treated OHHL sample did not induce the reporter strain at any distance, indicating that there was no OHHL diffusion across the plate due to degradation (Figure 4-15B). Negative control sample containing only LB broth showed similar results (Figure 4-15C).

***B. thuringiensis* pHY-XylII Bioluminescent Lactonase Assay**

OHHL degradation by *B. thuringiensis* pHY-XylII was monitored with a RoLux bioluminescent assay. Synthetic OHHL at 500 nM final concentration was added to *B.*

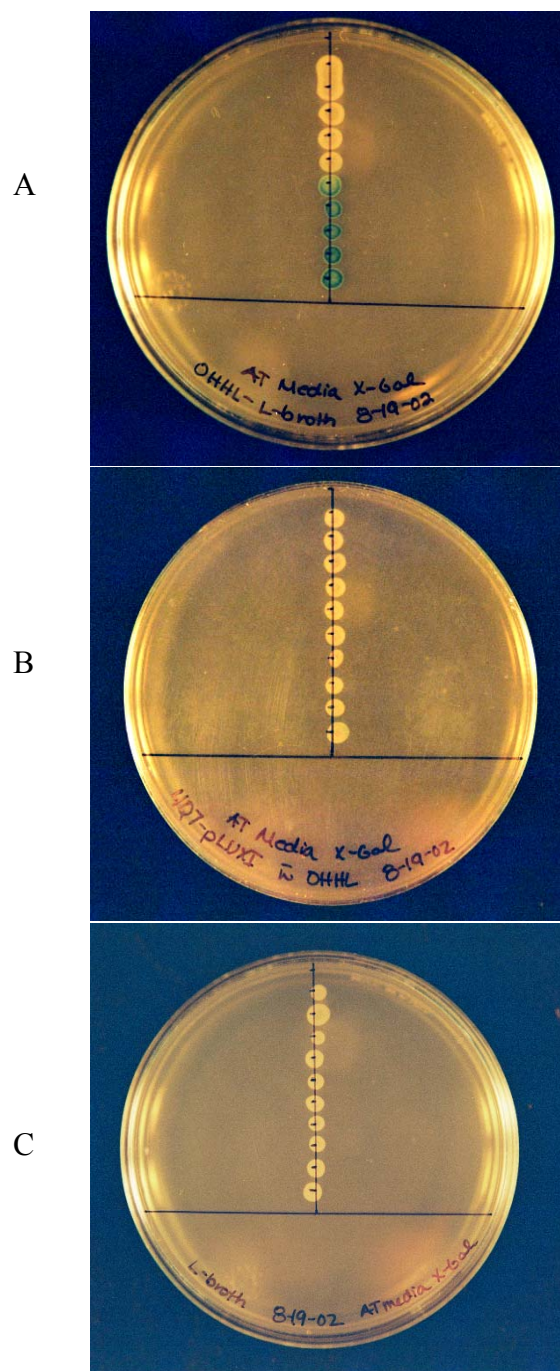


Figure 4-15. Degradation of OHHL by *B. thuringiensis* 4Q7 in Stationary Phase Culture. Synthetic OHHL was incubated with stationary phase *B. thuringiensis* 4Q7 culture and OHHL presence was monitored by diffusion across the plate inducing LacZ reporter strain *A. tumefaciens* 136A A. OHHL(40 μ M) added to LB broth as a positive control. B. OHHL (40 μ M) exposed to *B. thuringiensis* 4Q7 stationary phase culture. C. LB broth alone.

thuringiensis pHY-XylI culture at various points along the growth curve, and supernatants from this assay added to the OHHL reporter RoLux where specific bioluminescence was measured. Figure 4-16 shows the growth curve of *B. thuringiensis* pHY-XylI during the assay with OD₅₄₆ monitored over 8 hr. The generation time for this culture was determined to be approximately 60 min, calculated by the indirect method.

Figure 4-17 shows the peak specific bioluminescence of RoLux with *B. thuringiensis* pHY-XylI treated OHHL added. The average specific bioluminescence values show a gradual decline in the first five samples corresponding to the culture entering log phase. In the 300 min sample, or mid-log phase (OD₅₄₆ = 0.7535), there is a sharp decline in peak bioluminescence. The peak SB continues to decrease with increasing optical density of the culture.

Two separate standard curves for this assay were also performed using supernatant from *B. thuringiensis* pHY-XylI at the log (OD₅₄₆ = 0.3977) as seen in Figure 4-18, and stationary phase (OD₅₄₆ = 1.2995) as seen in Figure 4-19, with known quantities of synthetic OHHL added to determine how much OHHL was being lost by treatment with *B. thuringiensis* pHY-XylI culture. OHHL concentrations were calculated from the average peak SB produced with each supernatant with the linear equation of the standard curve corresponding to the growth phase of the culture at sampling time. Although the SB produced from supernatants at culture age 300, 360 and 420 min fell into both the log and stationary standard curve ranges, supernatant condition for these samples were not similar enough to those of the standard curves to represent the OHHL concentration found in these supernatants. Figure 4-20 shows a scatter plot of the average concentration of OHHL determined by the standard curves for the remaining

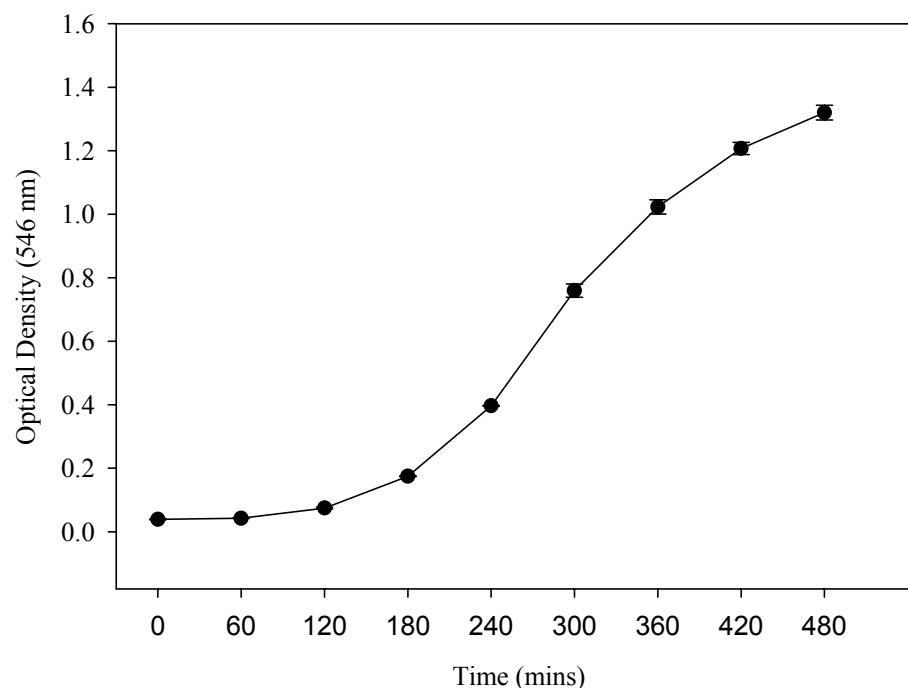


Figure 4-16. *B. thuringiensis* pHY-XylII Growth Curve for Bioluminescent Lactonase Assay. Optical density at 546 nm was monitored every hour for 8 hr. Values represent the average of triplicate samples.

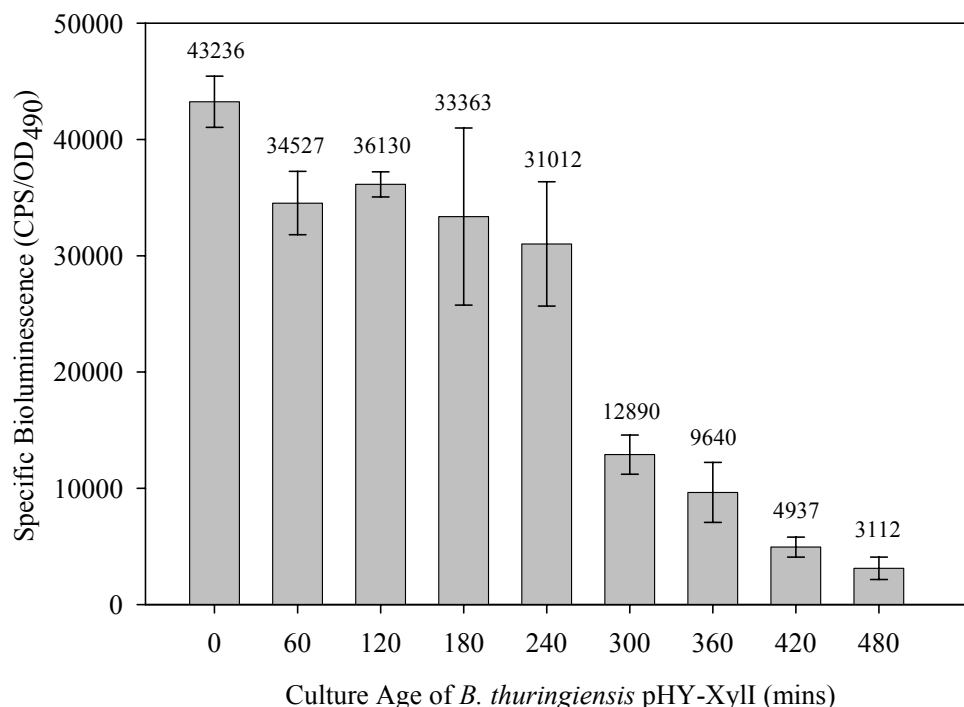


Figure 4-17. Influence of *B. thuringiensis* pHY-XylII Culture Age on the Stability of OHHL. Synthetic OHHL was incubated with *B. thuringiensis* pHY-XylII at increasing points during the growth phase and supernatants were analyzed for OHHL loss through a bioluminescence assay with OHHL bioreporter RoLux. The Peak specific bioluminescence reached with each supernatant is shown. Values are the average of triplicate samples.

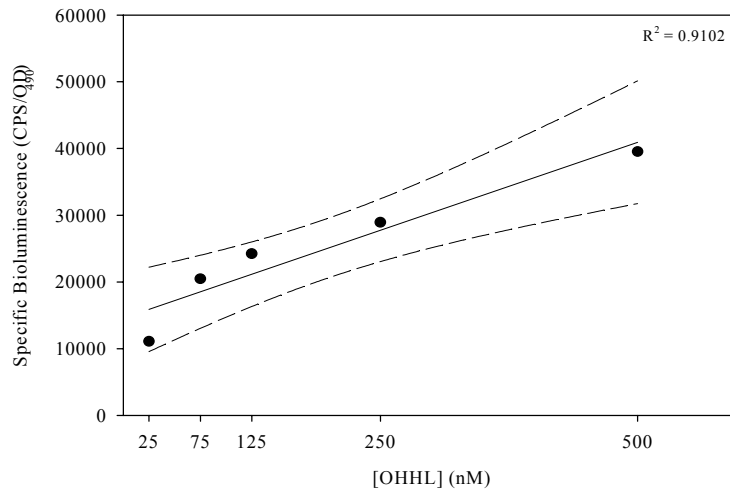


Figure 4-18. RoLux Induction With Synthetic OHHL added in *B. thuringiensis* pHY-XylI Log-Phase Supernatant. Scatter plot of peak SB of RoLux with 25, 75, 125, 250, and 500 nM OHHL added to OD₅₄₆ = 0.3977 uninduced supernatant from *B. thuringiensis* pHY-XylI. The solid line is the linear best fit and dashed lines indicate the 95% confidence intervals.

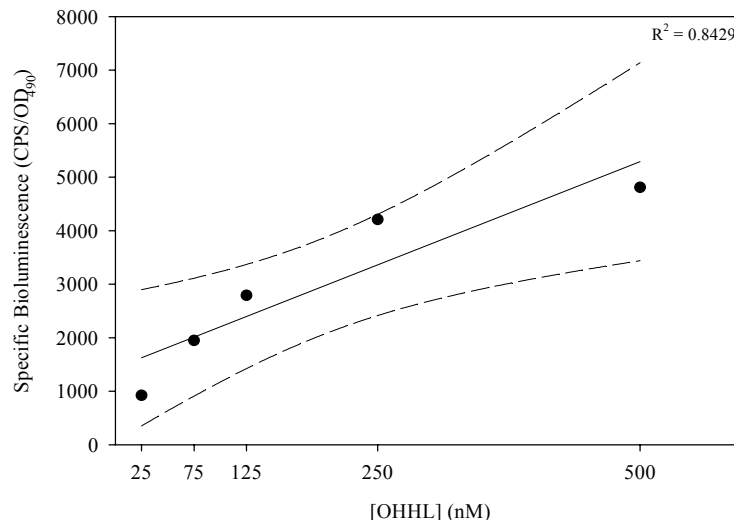


Figure 4-19. RoLux Induction With Synthetic OHHL added in *B. thuringiensis* pHY-XylI Stationary-Phase Supernatant. Scatter plot of peak SB of RoLux with 25, 75, 125, 250, and 500 nM OHHL added to OD₅₄₆ = 1.2995 uninduced supernatant from *B. thuringiensis* pHY-XylI. The solid line is the linear best fit and dashed lines indicate the 95% confidence intervals.

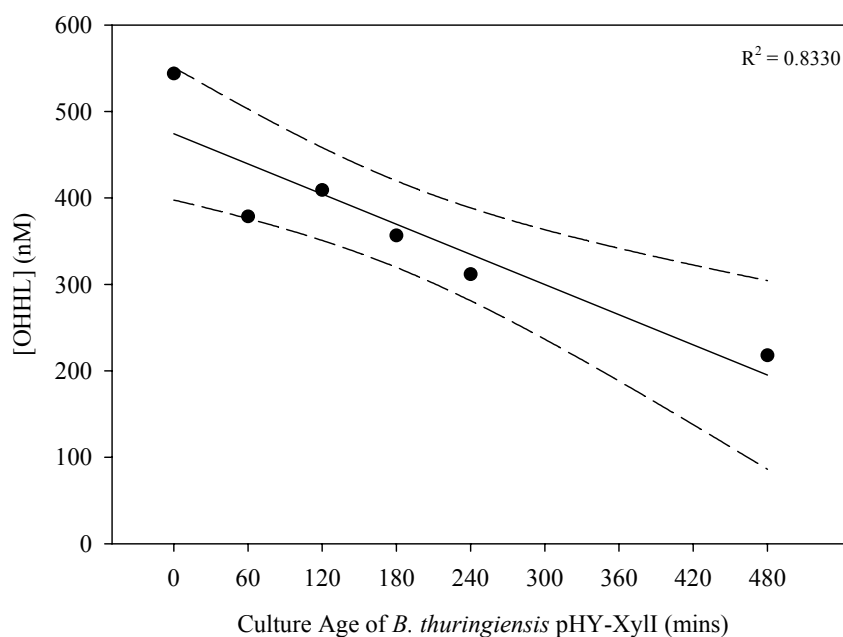


Figure 4-20. Decrease in OHHL Concentration After Treatment with Aging *B. thuringiensis* pHY-XylII Culture. The decrease in OHHL concentration after treatment with *B. thuringiensis* pHY-XylII culture of increasing age was determined by averaging triplicate samples of the specific bioluminescence produced by supernatants added to OHHL reporter RoLux and calculation the OHHL concentration as compared to standard curves. Samples for culture age 0, 60, 120, 180 and 240 min were based on the log-phase standard curve and sample from the 480 min culture was based on the stationary phase standard curve. The solid line is the linear best fit, and dashed lines indicate the 95% confidence interval.

supernatants. OHHL concentration decreased continuously from 500 nM in the 0 min sample down to 217 nM with the 480 min sample.

Expression of Lactonase (AiiA) from pCR CT/T7 *aiiA* #8

The *aiiA* gene from *B. thuringiensis* 4Q7 was amplified from genomic DNA using primers AiiA forward and AiiA reverse #2 and cloned into expression vector pCR CT/T7-TOPO (Invitrogen). The fragment was sequenced and found to have > 90% identity with several *B. thuringiensis aiiA* genes in the GenBank database (accession numbers AF478051.1, AF478057.1, AF478053.1, AF478048.1, AF478046.1, AF350931.1, and AF350927.1). In addition, the *B. thuringiensis* 4Q7 *aiiA* gene had 90% nucleotide identity with the *B. anthracis* Ames strain (AE017035.1).

As seen in Figure 4-21, expression of the AiiA protein in *E. coli* produced a band of increasing intensity at the expected size (28kDa). The greatest intensity of the suspected AiiA band was seen 4 hr post induction with IPTG. Cell extract from the uninduced culture after 4 hour incubation did not produce a similar band.

Analysis of TP-15 Genomic DNA

A genomic preparation of bacteriophage TP-15 was performed to isolate DNA for inserting the *luxI* gene. The genomic preparation of bacteriophage TP-15 was subjected to restriction digest using enzyme *EcoRI*, based on previous studies with this bacteriophage (Reynolds, 1988). The digest was electrophoresed overnight, and compared to several high molecular weight ladders as seen in Figure 4-22. Digestion of

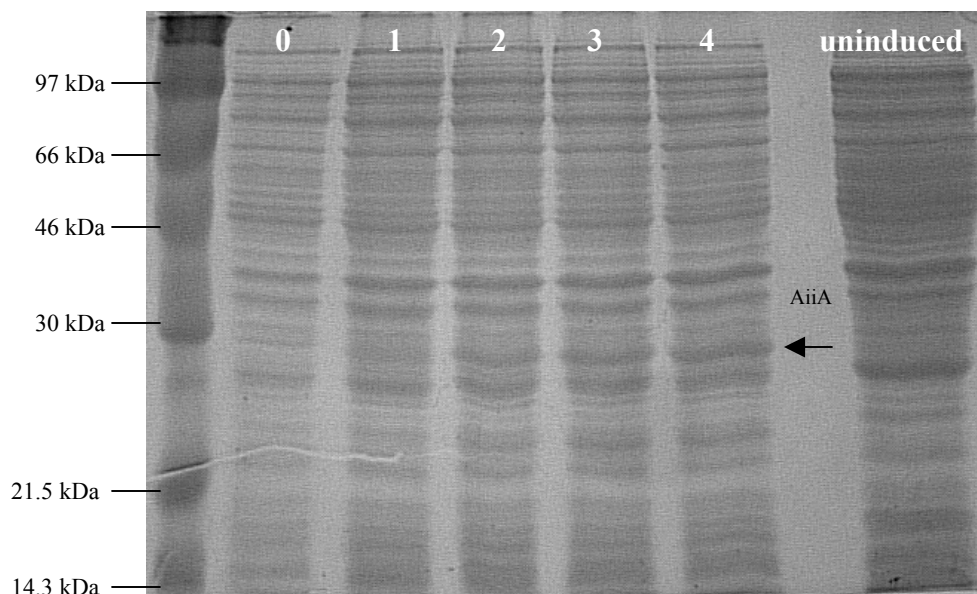


Figure 4-21. Expression of AiiA From *B. thuringiensis* 4Q7. The *aiiA* gene from *B. thuringiensis* 4Q7 was cloned into expression vector pCR T7/CT TOPO and expressed under an IPTG inducible T7 promoter at 1mM IPTG. Cells were harvested every hour, and total protein separated on a SDS-PAGE gel. The expected size of the AiiA protein is 28 kDa, and this band can be seen in increasing quantity over the time course after induction as indicated by the arrow. There was no band of equal proportion seen in the uninduced culture after 4 hr.

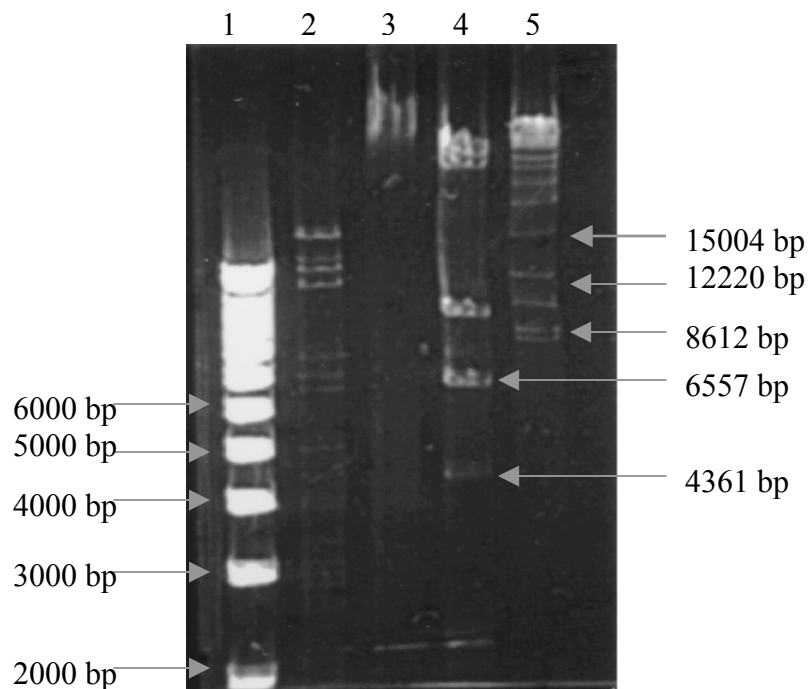


Figure 4-22. Restriction Analysis of Bacteriophage TP-15. Approximately 400 ng of TP-15 genomic DNA was digested with *Eco*RI for 1 hr at 37°C. The digest was then separated on a 0.4% TBE gel at 50V for 10 hrs. Lane 2 shows the digestion of TP-15 genomic DNA with numerous discrete bands ranging from 2 Kb to 15 Kb. 1 = 1Kb+ ladder (Invitrogen), 2 = *Eco*RI digest of TP-15 DNA, 3 = uncut TP15 DNA, 4 = λ DNA *Hind*III Fragments (Invitrogen), and 5 = High Molecular Weight Ladder (Invitrogen).

the TP-15 genome resulted in distinct bands, confirming that there was no carry over of genomic DNA from the *B. thuringiensis* 4Q7 propagation culture.

Engineering of Bacteriophage TP-15

An *in vitro* transposition reaction was performed to insert the *PIP2luxI* fragment into the genomic DNA of bacteriophage TP-15. This reaction mixture was then electroporated into *B. thuringiensis* 4Q7 cells and monitored for the formation of plaques. There were no plaques seen following incubation for any of the reaction conditions.

Chapter 5: Discussion and Conclusions

A principle goal of this research was to demonstrate that *B. thuringiensis* (as a surrogate for *B. anthracis*) was capable of expressing an introduced *luxI* gene and that the gene product, AHL synthase, would function to produce sufficient OHHL to induce a subsequent bioluminescent reporter strain. To accomplish these objectives, a *luxI* xylose-inducible expression plasmid, pHY-XylII, was constructed and introduced into *B. thuringiensis* 4Q7. The *luxI* gene was induced with xylose, and cultures were monitored for the presence of OHHL. Once confirmation of OHHL product was made, further experiments were performed to quantify the amount of OHHL being produced, in addition to optimizing conditions for detection of OHHL using the bioluminescent bioreporter *E. coli* RoLux.

Another aspect of this research was to determine what effect the enzyme lactonase, which is responsible for AHL degradation, would have on detection of OHHL from the *luxI*-engineered *B. thuringiensis* strain. The presence of a functional lactonase enzyme in *B. thuringiensis* 4Q7 was determined, in addition to cloning and sequence of the lactonase gene, *aiiA*. Bioluminescence assays were also performed using OHHL exposed to aging cultures of *B. thuringiensis* in order to monitor the amount of OHHL degradation over time.

To determine that *B. thuringiensis* pHY-XylII was capable of transcribing the *luxI* gene, RT-PCR was performed on total RNA from xylose induced cultures to amplify the *luxI* mRNA. The *luxI* transcript was detected in total RNA samples that were prepared immediately after the addition of xylose (time 0), indicating some background level of

transcription (Figure 4-1B). Previous work with the *P_{xyl}* promoter has shown that there is indeed some basal level of transcription in the absence of inducer (Kim, 1996).

At the second time point, 30 min post-induction, the quantity of cDNA detected following the RT-PCR reaction increased demonstrably (Figure 4-1B). This, too, is consistent with previous work, in which a 200-fold increase in expression was reported 30 min after induction (Kim, 1996). Subsequent time points up to 180 min resulted in similar levels of expression as detected by gel electrophoresis of amplified cDNA. This equivalence, however, may be artificial and the result of saturation of the PCR reaction and maximum consumption of the reagents (e.g. dNTP's).

A small quantity of *luxI* mRNA was also detected in the negative control culture, *B. thuringiensis* pHY-pXyl, which did not contain the *luxI* gene. Amplification products were less than those seen in the *B. thuringiensis* pHY-XylII culture, and did not exhibit the same response with respect to a substantial increase in product 30 min post induction. The amount of product did appear to increase from time 0 up to 150 min; yet, the 180 min post induction sample produced an equivalent amount of product as time 0 (Figure 4-1A). Genome sequence comparison of the *luxI* gene primers to *B. cereus* ATCC 14579 (GenBank Accession number NC_004722.1), a close relative of *B. thuringiensis*, revealed several locations of 100% match to 10 bp of the forward primer. There was one match to a small region of the reverse primer, but the location was greater than 582 bp from the match of the forward primer. No homologous sequence was found for the entire *luxI* gene. This analysis suggests that the template RNA for the RT-PCR reaction did not arise from genomic DNA transcription. Given the robust ability of PCR to exponentially amplify DNA from a minute quantity of initial template, it is likely that the faint bands

appearing in the negative control reaction are indicative of cross contamination from culture preparation. Subsequent attempts to test this hypothesis were hindered by difficulties in cell lysis and mRNA extraction from the Gram-positive organism, *B. thuringiensis*.

Bioluminescent assays, performed using *B. thuringiensis* pHY-XylII culture supernatants to induce an OHHL bioluminescent bioreporter (RoLux) were used to determine relative and, later in the study, actual concentration of OHHL being produced. Peak bioluminescence produced by RoLux with each sample of varying culture age was chosen as a marker for measurement of OHHL. It is expected that a direct relationship exists between the amount of an inducer in the supernatant to the level of reporter signal, up to the saturation point. Another possibility for determining the OHHL concentration in the *B. thuringiensis* pHY-XylII supernatant is to examine the initial velocity of bioluminescence induction by the reporter. Although both methods are valid for determining inducer concentration, only the peak bioluminescence values were examined to allow for consistency when comparing data sets.

Whenever possible, bioluminescence values (CPS) were converted to specific bioluminescence (CPS/OD₄₉₀) to take into account the change in reporter cell number. Normalization of the data in this manner provides a means of eliminating extremely high or low bioluminescence due to varying concentrations of reporter cells in the test wells. This factor is important when dealing with a reporter like the one used in this study, *E. coli*. This organism has a generation time of 109.04 ± 23.79 minutes, and cell numbers have the potential to vary greatly during the bioluminescence detection assay, which occurs over 8 hr or more. If the starting concentration of reporter cells is slightly unequal

in the initial stages of the bioluminescent assay, these differences can become substantially greater over a period of several hours if the cells are in exponential growth. In consideration of the fact that each reporter cell encodes a copy of the *lux* cassette, an equal concentration of OHHL will produce a greater amount of bioluminescence in a well that has a higher number of cells as compared to a well that has fewer reporter cells.

Initial experiments involving detection of OHHL produced by *B. thuringiensis* pHY-XylII and its negative control, *B. thuringiensis* pHY-pXyl, were preformed using two separate bioluminescence measuring instruments, and with varying volumes of reporter culture to *B. thuringiensis* supernatant. Experiments were preformed in this manner to determine optimal conditions for detecting OHHL from *B. thuringiensis* pHY-XylII, with respect to the number of reporter cells required for producing a significant amount of signal, and sensitivity of bioluminescence detection. The Wallac Victor2 1420 Multilabel Counter (WVMC) has the advantage of measuring reporter culture optical density during the assay, but provides less bioluminescence sensitivity. In contrast, the Wallac Microbeta 1450 Plus (WMP) is generally more sensitive in regards to bioluminescence detection, but does not allow for optical density measurement. Upon comparison of the peak bioluminescence obtained in the 100 μ L: 100 μ L ratio experiments performed on the two separate instruments, the WMP detected up to approximately 60 times more bioluminescence than the WVMC as seen in Table 5-1.

Peak bioluminescence values obtained were generally higher for the same components on the WMP, although, this instrument does not allow for aeration of the wells as does the Wallac Victor2 Multilabel Counter. Because oxygen can be a limiting

Table 5-1. Comparison of Bioluminescence Values for Plate Reader Instruments

Time Post Induction (mins)	WVMC (CPS)	WMP (CPS)	Fold Decrease/Increase of Bioluminescence
0	617	280	0.4
30	649	857	1.3
60	858	10473	12.2
90	1990	52107	26.2
120	4411	169803	38.5
150	8208	430057	52.4
180	8903	514680	57.8

Comparison of the average CPS values at peak bioluminescence obtained with 100 μ L: 100 μ L ratio of RoLux reporter culture to *B. thuringiensis* pHY-XylII supernatant on the Wallac Victor2 Multilabel Counter (WVMC) and the Wallac Microbeta 1450 Plus (WMP). The time of *B. thuringiensis* pHY-XylII culture post induction with xylose is indicated, as well as fold decrease/increase of bioluminescence obtained on the WMP as compared to the WVMC (WMP CPS/ WVMC CPS).

factor for the bioluminescence reaction, it is expected that the bioluminescent reporter will have decreased performance under conditions in the WMP assay. Despite this fact, this instrument still allowed for greater sensitivity than the WVMC.

Data from experiments performed on the WVMC indicated that a 1:1 volume of reporter cell culture to supernatant provided better sensitivity for detecting OHHL than a 3:1 ratio of reporter to supernatant (Table 4-1). Reporter cell numbers in the test wells for the 1:1 experiment averaged 3.2×10^6 cells/well at the time of peak specific bioluminescence, with a slight increase in the number of cells per well with supernatants from the increasing exposure time of the *B. thuringiensis* pHY-XylII culture to xylose. In the 3:1 ratio test wells, the number of reporter cells per well increased dramatically from incubation with supernatant at 30 min post induction ($4.8 \times 10^6 \pm 9.3 \times 10^5$ CFU) to 60 min post induction ($7.3 \times 10^6 \pm 3.5 \times 10^5$ CFU). The cell numbers continued to increase

from this point, up to $9.3 \times 10^6 \pm 6.7 \times 10^5$ CFU with the 150 min post induction supernatant, and decreased with the 180 min post induction supernatant to $8.2 \times 10^6 \pm 2.5 \times 10^5$ CFU. The lesser number of reporter cells used in the 1:1 ratio experiment allowed for earliest detection above background in the *B. thuringiensis* pHY-XylII 30 min post induction supernatant after 270 min. With the increased amount of reporter cells used in the 3:1 assay, the earliest detectable signal above background was not seen until the 180 min post induction supernatant after 214 min of bioluminescence detection.

One consideration in the analysis of these data is the amount of OHHL present in the supernatants. The 1:1 ratio experiment received double the concentration of OHHL as there was 100 μ L of supernatant added, compared to the 3:1 ratio experiment where there was only 50 μ L added. Although there was at least double the number of reporter cells found in the 3:1 ratio assay (see Table 5-1), this was not enough to compensate in bioluminescence for the decreased concentration of OHHL as compared to the 1:1 ratio assay. These aspects of the dynamics of bioluminescence produced per cell based on OHHL concentration should be considered for the detection of OHHL from a target organism in the bacteriophage-based bioreporter system.

There was a general trend in both ratio experiments of decreased amount of time for earliest detection of bioluminescence above background with increase amount of time for induction of the *B. thuringiensis* pHY-XylII culture with xylose (Table 4-1). One exception was found in the 3:1 ratio experiment, when the earliest time to detection was 47 min with the 150 min post induction supernatant from *B. thuringiensis* pHY-XylII. This was faster than the 180 min post induction supernatant, which was not detectable until 105 min. The number of reporter cells found in the test wells may explain this

anomaly in the time to detection trend. The 150 min post induction wells contained over 1×10^6 more reporter cells than that of the 180 min post induction wells ($9.3 \times 10^6 \pm 6.7 \times 10^5$ CFU as compared to $8.2 \times 10^6 \pm 2.5 \times 10^5$ CFU, respectively). This greater number of reporter cells may have produced an increased amount of bioluminescence due to the higher copy number of the *lux* cassette, allowing for detection at an earlier time, even though it is expected that a lower OHHL concentration in the 150 min post induction supernatant compared to the 180 min post induction supernatant.

Culture supernatants from the xylose-induced negative control *B. thuringiensis* pHY-pXyl did not induce the reporter at any point. These data validate the OHHL detection assays with *B. thuringiensis* pHY-XylII and prove that there was no other molecule being produced from the *B. thuringiensis* genomic DNA or plasmid DNA that was capable of inducing the RoLux reporter. In addition, this also provides evidence for culture contamination for the RT-PCR reactions where a similar band to that of the *luxI* product was seen in the negative control cultures, as previously discussed.

Experiments performed on the WMP showed similar results as those performed on the WVMC with respect to the amount of relative bioluminescence produced among samples. One significant difference between the two sets of experiments was the earliest detection times for each supernatant. In the WMP experiments, earliest detection times were much shorter than those found for the WVMC. For example, in the 1:1 ratio experiments, supernatants from the 180 min post induction were detectable in 11 min on the WMP, while this same sample was not detectable above background until 65 min with the WVMC.

Response times were identical for both reporter to supernatant ratios performed on the WMP (Table 4-3), indicating that although there was a higher concentration of OHHL in the 3:5 (75 μ L of reporter: 125 μ L supernatant) ratio experiments, the reporter functioned in a similar manner to that of the 1:1 (100 μ L reporter: 100 μ L supernatant) ratio experiment. The amount of reporter culture between the 1:1 and 3:5 ratio experiments were similar (100 μ L to 75 μ L, respectively), and earliest detection above background values were not affected by this decrease in the number of reporter cells in the assay.

Overall, these data indicate that a decreased amount of reporter cells with a higher concentration of OHHL allows for a greater bioluminescent response, and a faster time for induction above background without affecting the amount of signal obtained from the reporter. In addition, a more sensitive instrument would be required for detecting OHHL when analyzing supernatants from the target organism. These studies show the importance of optimizing the ratio of reporter culture to supernatant for detecting OHHL, and provide a base line for using this reporter in the bacteriophage-based bioreporter system.

OHHL quantification experiments were performed on an induced aging culture of *B. thuringiensis* pHY-XylI to quantify the amount of OHHL produced from this culture and at what point in the growth phase would a detectable quantity of OHHL be produced. OHHL production from this culture followed that of the growth curve, with increased amount of OHHL being seen in early log-phase and continuing to increase up to early stationary phase. The OHHL concentration then began to level off, and eventually decreased into stationary phase (Figure 4-12). These results were expected, as the sigma

factors controlling transcription of housekeeping genes involved in macromolecule synthesis are active during log phase, followed by a cascade of induction of alternative sigma factors, which alter the transcription specificity to genes involved in spore formation with the onset of stationary phase (Helmann, 2002). The first detectable amount of OHHL was seen at culture age 150 min, with the greatest amount found in the 300 min culture at 483.11 ± 103.60 nM.

An interesting phenomenon seen with the RoLux bioluminescent reporter assays was the decrease in the amount of bioluminescence produced when the reporter was resuspended in *B. thuringiensis* stationary-phase supernatant. Although there was a higher concentration of OHHL found in the stationary-phase supernatants of *B. thuringiensis* pHY-XylII, these supernatants produced approximately 25-50% less light when added to RoLux than the supernatants from log-phase which contained less OHHL. There are several possible explanations for this anomaly in the reporter's response to inducer. One possibility is that the stationary-phase supernatant contained less of the carbon source than the log-phase supernatants, and the energy required for the *lux* reaction was not available. As previously mentioned, the *lux* bioluminescence reaction requires 10% or more of the cellular energy being utilized in the light reaction (Engebrecht, 1983). Alternatively, secondary metabolites and/or extracellular virulence factors produced by *B. thuringiensis* entering stationary phase may have a deleterious affect on reporter physiology. *Bacillus sp.* have been shown to produce antibiotics, proteases, and other degradative enzymes during the onset of stationary phase (Bizani, 2002; Gupta, 2002; Price, 2002 and Agaisse, 1999).

Another interesting point of the quantification assay was the decrease in OHHL in *B. thuringiensis* pHY-XylII supernatants after the onset of stationary phase. It was expected that the OHHL concentration to be maintained at a constant level once the culture reached stationary phase as the cells began to initiate stationary-phase related functions, such as sporulation, or as the cells began to die off. This decrease in OHHL concentration may be attributed to two factors: 1) an increase of the pH of the *B. thuringiensis* supernatant and 2) degradation of OHHL by the enzyme lactonase.

OHHL has been found to be subject to lactonolysis, or the opening of the lactone ring, in basic pH conditions (Yates, 2002 and Schaefer, 2000). Alkaline pH conditions have been shown in *Bacillus sp.* cultures, and remains the most frequently used source in industry for alkaline proteases, which function optimally from pH 8-11 (Gupta, 2002). Schaefer *et. al.* (2000) reported that the stability of OHHL at pH 7 was approximately 1 day, while at pH 8, the stability was between 2-3 hr. An overnight culture of *B. thuringiensis* at $OD_{546} = 1.8443$ was found to have a pH of 8.13, although culture pH from log-phase was found to be 6.88 at an $OD_{546} = 0.9103$ (data not shown). The pH value in stationary-phase culture might degrade any OHHL produced by *B. thuringiensis* pHY-XylII if allowed to remain under those conditions for an extended period of time, such as the bioluminescent bioreporter assays with RoLux which are carried out over 8 hr or more.

In addition, OHHL degradation in stationary-phase culture may be due to lactonase activity. *Bacillus thuringiensis* 4Q7 was found to contain the sequence for lactonase, or *aiiA*, in its genomic DNA. This sequence matched with > 90% sequence identity to other *aiiA* genes from *Bacillus sp.* in Genbank, and the enzyme was found to

be functional in this strain. Data from the bioluminescent lactonase assay showed that there was an increased level of OHHL degradation with culture age (Figure 4-20). The decrease in OHHL concentration seen in log-phase cultures indicates activity of the lactonase enzyme, as the pH of the supernatant would still be near neutral at this point. The loss of OHHL seen in the stationary phase samples (OHHL final concentration 217 nM from 500 nM initial) may be a compound effect of lactonase and the change to alkaline conditions in the media.

Previous work with lactonase has shown that this enzyme is unlikely to be secreted from the bacterium as it does not contain a hydrophobic signal peptide at the N-terminus, and AHL-inactivation was not seen with culture supernatant alone (Dong, 2000). It is expected that the inactivation of exogenously added OHHL occurring through exposure to *B. thuringiensis* culture is therefore a result of diffusion of the molecule into the cell, where it was then degraded by lactonase. With regard to OHHL synthesis from the LuxI-producing *B. thuringiensis* pHY-XylII strain, it is possible that there may be some OHHL inactivation occurring in the cell before the signal molecule is diffused into the medium. In light of the data presented in Figure 4-20 from the bioluminescent lactonase assay, it appears that the amount of OHHL degradation in early growth phase will have only a minimal effect on OHHL detection.

Initial attempts at propagating a *luxI*-engineered bacteriophage were unsuccessful. One of the difficulties encountered in this process was electroporation of the bacteriophage DNA once the *in vitro* insertion of the *luxI* was complete. TP-15 genomic DNA is over 39 Kb, and electroporation of extremely large segments of DNA is inefficient. This method of creating the engineered bacteriophage requires some

optimization of the electroporation reaction. Future work in this area might include an alternative method for inserting the *luxI* gene into TP-15, and sequencing of the TP-15 genome to look for regions best suited for *luxI* expression.

Based on the analysis of data presented within this work, several conclusions can be drawn: 1) *B. thuringiensis* is capable of expressing the *luxI* gene from a plasmid, 2) the necessary components for the assembly of OHHL are available within the cell, 3) a detectable amount of OHHL is produced in under 3 hr from a growing culture, 4) up to 500 nM of OHHL may be produced during log-phase and, 5) the enzyme lactonase, while produced, will not have a significant effect on detection of OHHL during early log-phase. These findings will facilitate future work involving the development of a bacteriophage-based bioreporter system for detection of *B. thuringiensis*.

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