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Paenibacillus larvae spore germination and American Foulbrood disease development in honey bee larvae

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PAENIBACILLUS LARVAE SPORE GERMINATION AND AMERICAN FOULBROOD DISEASE DEVELOPMENT IN HONEY BEE LARVAE

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Abstract

Paenibacillus larvae spore germination and American Foulbrood disease development in honey bee larvae

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Honey bee (*Apis mellifera*) pollination plays an important role in meeting our agricultural needs, yet we are incapable of completely preventing a decline in bee health, partially due to bacterial diseases. American Foulbrood (AFB), a bacterial disease of honey bee larvae, is particularly troublesome because the infectious agent is the bacterial spore of *Paenibacillus larvae*. Bacterial spores are resistant to extreme temperatures, unaffected by antibiotics, withstand exposure to disinfectants, and can remain dormant for years until they can revert back to vegetative cells.

Our research investigated *P. larvae* spore germination at the functional and pathogen-host level. We have found that *P. larvae* spores exit dormancy after exposure to an L-tyrosine plus uric acid solution. Germinated *P. larvae* spores were susceptible to killing with heat that would normally not affect dormant spores. These data suggest how triggering spore germination could help in decontamination of *P. larvae* spores in bee hives. We identified indole and phenol as inhibitors of *P. larvae* spore germination *in vitro*. Additional compound screens identified other indole analogs that inhibited *P. larvae* spore germination. These compound screens probed the

binding pocket(s) of the *P. larvae* germination receptor machinery. We identified compounds that interacted with the germination receptor(s) and blocked L-tyrosine plus uric acid spore germination. We also tested the effect of germination inhibitors on honey bee larvae. Larvae fed germination inhibitors had similar survival to the control groups. We found that prophylactic treatment with germination inhibitors prevented AFB disease in laboratory reared larvae. Lastly, we measured mRNA levels for the putative GerKA and PrkC germination receptors found in *P. larvae* spore. Two GerKA germination receptor mRNAs were found to be upregulated during sporulation. Our findings are relevant to beekeeping industry and a wide scientific audience because the spore germination is the first step in establishment of several diseases.

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Chapter 1: Introduction

1.1 Overview

Honey bees (*Apis mellifera*) play an important part in meeting our agricultural needs (Morse, R.A. & Calderone, N.W 2000). In the United States, the added value of agricultural productivity due to bee pollination in 2000 was approximately 15 billion dollars (Morse, R.A. & Calderone, N.W 2000). Honey bees support pollination of estimated 90-130 commercial crops that are important to our diet (Perez, Pollack 2003). Without bee pollination, crops planted in remote or desolate habitats would not be productive. Furthermore, as the number of farms/acreages devoted to farming decrease there is a higher density of plants per acre that require pollination. Thus, our modern planting and management schemes require that millions of bee colonies are transported by road to pollinate crops. However, we are unable to completely prevent a decline in bee health. This decline is partially due to bacterial diseases (Matheson 1993, Morse, R.A. & Calderone, N.W 2000, Genersch 2010a).

American foulbrood (AFB) is a widespread bacterial disease of honey bees that kills developing larvae (White 1906). *Paenibacillus larvae* spores are the infectious agent for AFB, but it is the vegetative cells that cause disease (Tarr 1938). In 2005, a survey of pollinating bee colonies indicated 4% of colonies had significant AFB load (Eischen, Graham 2005). Once a beekeeping operation is contaminated, the *P. larvae* spores are not easily removed (Shimanuki 1983). Although autoclaving and high concentrations of chemical disinfectants effectively kill spores, these treatments are not viable for the bee keeping industry (Dobbelaere, De Graaf et al. 2001). Autoclaves are expensive to purchase, install, and maintain thus reducing their use by beekeepers. Additionally, chemical disinfectants kill *P. larvae* spores when highly concentrated solutions are used. Traditionally, terramycin has been used for treatment and prevention of AFB,

however, the spore stage of *P. larvae* is not affected by antibiotic treatment and use of antibiotics leads to resistant strains (Alippi 1999, Alippi, López et al. 2007). Presently, burning of infected colonies and beekeeping equipment is the only accepted practice for controlling the spread of AFB (Shimanuki 1983, Genersch 2010a).

Because our long term goal is controlling spore germination in order to prevent AFB disease in bee larvae, characterization of the germination pathways that *P. larvae* spores used to exit dormancy is necessary. In nature, bacterial spores exit dormancy in response to an influx of nutrients, via Ger receptors, and/or muropeptides, via PrkC receptors (Peter 2003, Shah, Laaberki et al. 2008). Both Ger and PrkC spore germination pathways were previously characterized in the commensal bacterium, *Bacillus subtilis*.

This dissertation examined the role of Ger receptors in *in vitro* and *in vivo* spore germination and the molecular mechanisms allowing *P. larvae* spores to exit dormancy and cause AFB in honey bee larvae. We used molecular probes, bioinformatic tools, gene expression analyses, and genetic approaches to advance our understanding of the host-pathogen relationships causing AFB disease. Our research determined the importance of the two ubiquitous and widely studied germination pathways in AFB disease establishment. We determined the triggers and inhibitors of *P. larvae* spore germination that will be developed into an AFB disease treatment. Our findings are relevant to the beekeeping industry, but also to a wide scientific audience because spore germination is the first step in the establishment of several diseases (e.g.., anthrax, botulism, and *Clostridium difficile* infection) (Alvarez, Abel-Santos 2007, Dodatko, Akoachere et al. 2010, Howerton, Ramirez et al. 2011, Howerton, Patra et al. 2013b, Howerton, Patra et al. 2013a). This research yields new insights into factors shaping

the evolution of pathogen selectivity for a host; an evolutionary pressure that drives the germination receptor diversity seen in pathogenic spore formers.

1.2 Honey bee biology

The life cycle of a honey bee is composed of four stages (egg, larva, pupa, and adult) (Winston 1987). Queen bees lay eggs, and larva take approximately three days to hatch (eclose) after which they continue to develop through pupation into adulthood. Queen bees can lay unfertilized eggs that become drones/male bees and fertilized eggs that become worker/female bees. A larva takes 24 days to become a drone and 21 days to become a worker. The larval stage of workers is five days long and characterized by eating and the growth of a larva. When a larva reaches its full growth, it undergoes pupation and transforms into an adult after 13 days. Drone bees leave the colony to mate with virgin queens during growing seasons. On the other hand, worker bees that emerge from the cells begin working within the bee hive immediately. They are responsible for ensuring the health of the queen, drones, and developing bees by performing a series of tasks (Winston 1987).

The set of tasks/behaviors a worker bee performs is largely dependent on the age of the bee (Winston 1987). During the first 2-3 weeks of life, honey bees spend time cleaning comb and nursing developing larvae. As honey bees mature, they perform additional tasks including building comb and hive ventilation. Prior to becoming foragers, honey bees serve as guards at the entrance of the hive. The foraging bees are the oldest bees in the hive. They collect nectar, pollen, and water for the colony. They fly several hours and up to 2 miles to gather resources for the colony (Winston 1987). During flight, bees have the highest mass-specific metabolic rate measured in muscles (Suarez, Lighton et al. 1996). In the end, honey bees die while performing this metabolically expensive foraging behavior.

An essential need of the honey bee colony is to replenish and expand worker bee numbers for two reasons (Winston 1987). Firstly, the life expectancy of a honey bee is approximately 4 to 8 weeks, means bees are constantly dying. Secondly, extra worker bees are needed to utilize resources available during the peak flowering season. There can be between 40,000 to 60,000 honey bees reared within a single colony. Queens may lay up to 2,000 eggs per day to maintain the growth and health of the colony. Replenishment and expansion of the workers ensures that essential tasks continue to be performed (Winston 1987).

1.3 American Foulbrood (AFB) disease

P. larvae is the causative agent of American Foulbrood (AFB) disease. AFB disrupts the life cycle of the honey bees by killing the animals at the larval stage (White 1906). Honey bee larvae are infected by consuming larval diet that is contaminated with *P. larvae* spores (White 1906, Tarr 1938, Crailsheim, Riessberger-Galle 2001, Genersch 2010a). Although *P. larvae* spores can be found in every part of a contaminated bee hive, the spores are only known to germinate inside honey bee larvae (Shimanuki 1983, Piccini, D'Alessandro et al. 2002, Bakonyi, Derakhshifar et al. 2003).

American foulbrood disease occurs as newly germinated *P. larvae* cells proliferate in first or second instar larvae (Yue, Nordhoff et al. 2008). Once germinated, *P. larvae* cells are able to proliferate in the incoming larval diet and larval hemolymph (Djukic, Brzuszkiewicz et al. 2014). Furthermore, *P. larvae* produces toxins and cytolysins similar to other pathogenic bacteria which aid in killing the host (Fünfhaus, Poppinga et al. 2013, Djukic, Brzuszkiewicz et al. 2014). An important step for AFB disease development is breaching of the midgut by *P. larvae* cells. Proteases, collagenases, chitinase, and toxins are believed to allow *P. larvae* to breach the midgut via degradation of connective tissue. In fact, *in vitro* and larval exposure assays have

shown that *P. larvae* strains lacking toxins have reduced virulence. Extreme bacteremia causes the death of larvae several days after *P. larvae* spore infection (Davidson 1973, Genersch, Ashiralieva et al. 2005, Genersch, Forsgren et al. 2006). After the nutrients in honey bee larvae are depleted, *P. larvae* cells sporulate forming billions of spores that are distributed in the colony allowing the disease to continue.

The symptoms of AFB disease can be detected by inspection of honey bee colonies (White 1906, Shimanuki 1983, Shimanuki, Knox 2000). In a healthy colony, the cells within a comb have a solid and compact section/brood patch for bees at various developmental stages (egg, larvae, pupa, and emerging adults). By comparison, as AFB progresses the brood patch takes on an irregular appearance due to the presence of dead larvae or pupae in cells. The AFB diseased combs are drier, darker, and have a slight foul odor (White 1906, Shimanuki 1983, Shimanuki, Knox 2000).

As AFB disease progresses larval appearance changes from a pearly white to brown to almost black color. The deceased larva forms a sticky-ropy mess that can be stretched longer than 2.5 cm. Over time the dead larva dries out, toughens and becomes a scale that sticks to the side of cell walls. The caps of dead brood can puncture or remain intact thus preventing bees from cleaning cells containing dying or dead larvae (White 1906, Shimanuki 1983, Shimanuki, Knox 2000).

The majority of bee larvae in the brood chamber die from AFB after the cells are capped (White 1906, Shimanuki 1983, Shimanuki, Knox 2000). However, a few larvae have been shown to die of AFB disease prior to being capped (White 1906, Shimanuki 1983, Shimanuki, Knox 2000). Strains of *P. larvae* subspecies *pulvifaciens* were suspected to cause this rapid form of

AFB disease. This rapidly killing form of AFB disease is seldom reported because of the low incidence. These dead larvae are quickly removed by adult bees. The resulting powdery scale disease is characterized by a scale that crumbles when handled. (White 1906, Shimanuki 1983, Shimanuki, Knox 2000).

P. larvae spores are distributed within and between colonies by the transfer of food mouth-to-mouth (known as trophallaxis), swarming, robbing, and beekeeping practices (Shimanuki 1983, Fries, Camazine 2001, Fries, Lindström et al. 2006). Honey bees regularly exchange food and glandular secretions allowing spores to be distributed within bee hives. During the spring and summer when colonies are active and growing, honey bee colonies may divide into "swarms" forming two or more new colonies. Spores travel within the bees in the swarm to the new colonies. Weakened colonies have their food stores robbed by stronger neighbors. If a colony is debilitated by AFB, the robbing behavior allows spores to move between colonies and infect a new beehive (Fries, Lindström et al. 2006, Fries, Camazine 2001). Beekeepers actively move bees, honey, pollen, and brood amongst colonies and move colonies around the country. Beekeepers import and export honey bees and bee products worldwide (Shimanuki 1983, Alippi 1999, Bakonyi, Derakhshifar et al. 2003). *P. larvae* spores can contaminate any of these materials facilitating dispersal of AFB infection.

Honey is a major reservoir of *P. larvae* spores (Shimanuki 1983, Alippi 1999, Fries, Camazine 2001). One study found *Paenibacillus larvae* spores in one quarter of the raw honey samples tested. The presence of spores in honey is troubling because honey containing frames of honey comb and extracted honey are routinely shared by beekeepers (Sturtevant 1932, Lindström, Korpela et al. 2008). Furthermore, beekeepers move resources between hives to ensure health of their colonies. During the winter months, honey bee colonies can be fed honey

as resources within the colony are exhausted. The levels of spores in adults fed contaminated honey have been shown to peak during the winter. Feeding colonies contaminated honey produces clinically diseased larvae at similar levels as colonies with dead larvae in combs (Sturtevant 1932, Lindström, Korpela et al. 2008). These studies indicate that regardless of how *P. larvae* spores enter the colony, the disease causing agent persists within the colony.

1.4 Current AFB disease management

AFB disease management relies on antibiotics, breeding, natural products, and destruction of infected hives (Genersch 2010a). These management strategies do not eliminate AFB disease because the infectious agent is the resistant spore stage of *Paenibacillus larvae*. Decontamination of bacterial spores from hives and beekeeping equipment is difficult because spores are resistant to high temperatures, desiccation, UV irradiation, and harsh chemicals (Dobbelaere, De Graaf et al. 2001, Setlow, Loshon et al. 2002, Setlow 2006, Forsgren, Stevanovic et al. 2008). Moreover *P. larvae* spores can remain dormant in honey, pollen, wax, adult bees, and on hive surfaces (Shimanuki 1983, Shimanuki, Knox 2000, Alippi 1999) for years.

Antibiotics are used to prevent and control American Foulbrood disease (Alippi 1999, Shimanuki, Knox 2000). Antibiotics prevent AFB disease when they are incorporated into the larval diet produced by the adult bees where they kill newly germinated *P. larvae* cells (Peng, Mussen et al. 1992, Alippi 1999). Furthermore, antibiotics can control AFB disease by preventing additional *P. larvae* spore formation and thus reducing the number of larvae killed. Antibiotics such as terramycin/oxytetracycline, tylosin, and sodium sulfathiazole have been used in many countries (Alippi 1999). However, the use of antibiotics for AFB treatment has been

banned in most of Europe due to residue contamination, antibiotic resistance in bacteria, and inability to remove the infectious *P. larvae* spore (Alippi 1999, Genersch 2010a). As honey bees share food provided by beekeepers the antibiotic residues are spread throughout the colony. Overuse of antibiotics has led to resistant *P. larvae* strains (Alippi, López et al. 2007, Genersch 2010a). Moreover, the infectious spores are not affected by antibiotics and can remain dormant long after the antibiotic treatment (Peng, Mussen et al. 1992, Alippi 1999, Shimanuki, Knox 2000, Lodesani, Costa 2005, Alippi, López et al. 2007).

An alternative AFB disease treatment is to breed honey bees to have increased brood hygienic behavior (Spivak, Reuter 2001, Spivak, Downey 1998). Hygienic strains of honey bees detect and remove diseased brood from the colony more readily. Removal of diseased brood is thought to help control spread of AFB and other diseases within the colony. Colonies are selected based on their ability to remove freeze-killed brood. The daughters and sons of hygienic queens are mated to obtain new generations of hygienic bees. Hygienic bee colonies are susceptible to AFB disease although at a lower level than non-hygienic colonies. Although hygienic bees offer some protection from AFB, the disease persists because not all diseased larvae are removed and spores reservoirs remain (Spivak, Downey 1998, Spivak, Reuter 2001).

The effectiveness of bee venom, probiotics, essential oils, propolis, and plant extracts in the field of AFB disease control has been evaluated (Yoshiyama, Wu et al. , Antúnez, Harriet et al. 2008, Gende, Maggi et al. 2009, Fuselli, S., García de la Rosa, B., Eguaras, M., Fritz,R. 2010, Forsgren, Olofsson et al. 2010, Fernández, Porrini et al. 2014). *In vitro* assays have shown that these natural treatments have activity against *P. larvae* cells. Furthermore, toxicity assays have shown that these therapeutic agents can be incorporated in larval diet without changing larval development. Finally, exposure assays investigate the effects of therapeutic agents on AFB

disease development (Yoshiyama, Wu et al. , Antúnez, Harriet et al. 2008, Gende, Maggi et al. 2009, Fuselli, S., García de la Rosa, B., Eguaras, M., Fritz,R. 2010, Forsgren, Olofsson et al. 2010, Fernández, Porrini et al. 2014). The majority of these therapeutic agents can significantly lower AFB disease development. Unfortunately, these natural treatments target the active infection similarly to antibiotics but not the root of the disease which is the spore of *P. larvae*.

Our inability to control AFB reduces the number of hives available for pollination, reduces beekeeper profits, and threatens our food supply. A survey of bee hives pollinating California almond crops indicated 4% of colonies had significant AFB load (Eischen, Graham 2005). Another set of surveys in New South Wales, Australia, have shown 50% of beekeepers have a current or past history of AFB in their bee hives. Beekeeper profits decrease because AFB disease management requires purchasing disease treatments, higher labor costs, and cost of replacing bee hives (Rhodes, McCorkell 2007). For the past decade, the cost of pollinator services has increased due to a decline in bee health (Sumner, Boriss 2006). As a result, food prices increase and our food supply is threatened due to AFB disease.

Once a colony develops AFB disease, it needs to be destroyed to completely eradicate the infectious spores (Shimanuki 1983, Alippi 1999, Shimanuki, Knox 2000, Genersch 2010a). This represents a great cost to a beekeeper. Normally a hive costs approximately $552 (e.g. Dandant beginners hive $335 package of bees 3 pounds $163, 1 queen bee $54). When a hive is contaminated, all of the honey combs, bees, and hive equipment are burned in a large pit fire. What remains after the fire is buried to prevent the spread of the spores (Shimanuki 1983, Alippi 1999).

Establishment of alternative effective AFB disease treatments is needed. We propose to develop AFB treatments based on controlling *P. larvae* spore germination. Germinated spores are susceptible to treatments that prevent the AFB unlike dormant spores. Inhibiting spore germination would prevent cells from causing AFB disease. Thus, we needed to understand *P. larvae* spore germination to develop AFB treatments.

1.5 Bacterial spore biology

Studies of *P. larvae* spores are rare in part because AFB disease research focuses on bee biology (Genersch 2010a). As a result we have a limited knowledge of *P. larvae* spore characteristics. We know through microscopy studies that *P. larvae* produces spores in stages that are similar to other spore-formers (Bakhiet, Stahly 1985b). Furthermore, *P. larvae* spores are ellipsoidal and measure about 0.6 µm wide by 1.4 µm long (Alippi 1999). The spores are resistant to desiccation, high temperatures, UV light, and disinfectants. Our lack of knowledge of *P. larvae* spore biology prevents us from targeting the AFB disease vector.

Insights into *P. larvae* biology can be made by utilizing knowledge and tools developed for the study of other spore-forming organisms. Indeed studies have shown the importance of cell differentiation, sporulation, spore dormancy, spore resistance, and spore germination in the life cycle of bacteria. If we control any of these spore characteristics then we can alter the life cycle of the associated bacteria. Thus, one of the goals of this dissertation was to study the biology of *P. larvae* spores.

Sporulation, the differentiation of bacteria to dormant spores, occurs as a response to nutrient deprivation (Errington 2003, Errington 1993). Although sporulation has been studied in many bacteria, the best understood model system for sporulation is *B. subtilis*. At the end of exponential growth, *B. subtilis* cells use resources accumulated to either divide or form spores.

Because sporulation occurs in conjoined cells, the execution of this process takes place in several phases. One cell provides resources for spore formation and is ultimately broken down, while the other cell is packed into a resistant coat, which is endowed with germination machinery. Finally, the cell enters dormancy (Errington 1993, Errington 2003).

The physiological and biochemical properties of the spore are formed during a period of eight hours in *B. subtilis* (Errington 1993, Errington 2003). To produce viable spores during this process, the transcriptional activation and deactivation of several gene sets is required. There are transcriptional regulatory proteins in *B. subtilis* that regulate transcription of sporulation-specific genes. Initiation of sporulation depends on activation of the master regulator/protein Spo0A via phosphorylation by kinases. Phosphorylated Spo0A induces transcription of two sigma factors, SigA (σ^A) and SigH (σ^H), which promote RNA polymerase transcription. Genes induced by the activity of SigA and SigH allow for septum formation between the mother cell and forespore compartments. After septum formation, SigE (σ^E) and SigF (σ^F) activity allows for differential gene expression in the mother and fore-spore compartments. SigE allows for early mother cell genes to be activated, while SigF allows for the RNA polymerase to transcribe sporulation genes within the fore-spore compartment. Together the activity of SigE and SigF allows for engulfment of the fore-spore by the mother cell. After spore engulfment, the SigG and SigK factors are activated within the pre-spore and late mother cell. SigG induces transcription of genes involved in chromosome structure and germination machinery. SigK directs the synthesis of protective cortex and coat spore layers. The appearance of specialized transcription/sigma factors allows RNA polymerase to transcribe sporulation related promoters (Errington 1993, Errington 2003).

Bacterial spores are extremely resilient forms of bacteria (Setlow, Loshon et al. 2002, Setlow 2006). Treatment of *B. subtilis* spores with 1M HCl (pH 0) or NaOH (pH 14) solutions

for 40 minutes is not sufficient to kill them. Wet or dry heat at temperatures up to 120°C kills growing cells but not dormant spores. Bacterial spores are resistant to solutions we normally use to decontaminate surfaces (formaldehyde, chlorine, hydrogen peroxide, detergents, ethanol). UV resistance allows spores to remain on surfaces without incurring any DNA damage (Setlow, Loshon et al. 2002, Setlow 2006).

Bacterial spores are believed to be the longest lived cellular structures known (Gould 2006). There are two reports of revival and identification of spores from different environments. A bacterial spore was isolated from the abdominal contents of bees preserved in amber for 25-40 million years (Cano, Borucki 1995). The isolation of a 250 million year old spore-forming bacterium from salt crystals has also been reported (Vreeland, Rosenzweig et al. 2000). Sample quality and contamination concerns have made these studies less accepted. Nonetheless, multiple studies have shown spores have the capability of surviving for several years. In dry larval scales, *P. larvae* spores have been shown to be viable for 35 years or more (Alippi 1999).

Initiation of spore germination is determined by detection of cues from an environment that provides substances required for growth (Setlow 2003). Three spore germination pathways are well characterized in *Clostridium* and *Bacillus* species (Paredes-Sabja, Setlow et al. 2011). The first pathway, called the Ger receptor pathway, is triggered by detection of nutrients in the environment. Sugars, nucleosides, purines, amino acids, and organic salts promote spore germination via Ger receptors (Setlow 2003, Moir 2006). The Ger receptor pathway is thought to be the primary trigger of spore germination in nature (Paredes-Sabja, Setlow et al. 2011). The second pathway, called the PrkC receptor pathway, is triggered by the detection of muropeptides released from closely related growing bacteria (Shah, Laaberki et al. 2008). This pathway is analogous to bacterial quorum sensing in that it depends on a signal produced by a cell

population. Spores will only germinate if they detect a muropeptide signal released by cells. Even though the PrkC pathway has only been studied in *B. subtilis* and *B. anthracis, prkC* genes are found in over 75 bacterial species (Paredes-Sabja, Setlow et al. 2011). The third pathway, the non-nutrient germination pathway, is triggered by high concentrations of calcium-dipicolinic acid (Ca-DPA), surfactants, lysozyme, and high pressure. For example, 60 mM Ca-DPA is required to trigger *B. subtilis* spore germination. In biological systems DPA is only found in the spore of spore-forming microorganisms (Snyder, Thornton et al. 1998). Thus the rarity of the non-nutrient pathway triggers suggests that it may not play a role in nature (Paredes-Sabja, Setlow et al. 2011). The number of germination pathways available to a spore-forming organisms has profound effects on the ability of bacterial spores to exit dormancy.

Several studies have shown that the Ger and PrkC spore germination pathways may interact (Caipo, Duffy et al. 2002, Llaudes, Zhao et al. 2001, Paredes-Sabja, Torres 2010, Zhao, Montville et al. 2006, Zhao, Montville et al. 2000, Zhang, Garner et al. 2011, Webb, Stringer et al. 2012). The environments that spores occupy potentially contain mixtures of nutrient and muropeptide signals. The integration of two environmental signals compared to one environmental signal will allow spores to germinate more rapidly. Synergism between the germination pathways could explain the differences in ability of spores to germinate *in vitro* and *in vivo* (Caipo, Duffy et al. 2002, Llaudes, Zhao et al. 2001, Paredes-Sabja, Torres 2010, Zhao, Montville et al. 2006, Zhao, Montville et al. 2000, Zhang, Garner et al. 2011, Webb, Stringer et al. 2012).

Bacterial spore germination can be measured by following optical density over time (Powell 1950, Powell, Strange 1953, Vary, Halvorson 1965). The decrease in optical density is proportional to spore germination (Powell 1950). Spore germination curves have a sigmoidal

shaped with three separate stages: (i) lag phase, (ii) linear phase, and (iii) plateau phase. During the lag phase the spore binds the germinant and commits to germinate. In the linear phase, spore germination results in a significant decrease in turbidity over time. Finally, the germination curves plateau indicating no more spores will germinate. Germination curves can be further analyzed to characterize binding of germinant by spores.

1.6 Rearing of honey bee larvae

The life cycle of the honey bee is divided into four distinct stages (egg, larva, pupa, and adult) (Winston 1987). A queen lays eggs that will take approximately three days to eclose/hatch in the hexagonal wax cells. The subsequent honey bee larva lasts up to seven days and is characterized by distinct stages (instars). Nurse bees provide each larval instar specific food sources. During the first three instars which last 24 hours each, worker larvae are fed royal jelly. Larval instars 4 and 5 are fed a mixture of honey and pollen called bee bread. After seven days, larvae reach their full growth and enter the pupal stage. During the pupal stage the honey bee undergoes metamorphosis into an adult (Winston 1987).

The five developmental stages/instars of honey bee larvae have different susceptibility to AFB disease (Crailsheim, Riessberger-Galle 2001, Crailsheim, Brodschneider et al. 2013). First and second instar larvae are most susceptible to AFB infection, while older honey bee larvae are considered resistant to AFB disease. Thus, it is important to know the age of honey bee larvae used in experiments.

As in the colony, honey bee larvae can be reared in the laboratory to obtain adults (Peng, Mussen et al. 1992, Wolfgang 1998, Brodsgaard, Ritter et al. 1998, Genersch, Ashiralieva et al. 2005, Huang 2009, Crailsheim, Brodschneider et al. 2013). There are five crucial points for rearing larvae including caging of the queen, grafting, larval diet, incubation conditions, and

assessment of survival (Crailsheim, Brodschneider et al. 2013). Several protocols were adapted to rear honey bee larvae in our laboratory.

To start larval rearing and ensure a synchronous aged population the first step is to trap queens on empty cells and monitor oviposition/egg laying. Cages built with plastic queen excluders, a selective barrier that allows worker bees but not queens or drones to move through it, can be used to keep the queen on empty brood comb. The caged queen is placed within the brood chamber at the center of the hive. The brood chamber is inhabited by nurse bees and developing larvae (Huang 2009). Nurse bees can move through the queen excluders to care for the queen and comb, but due to its larger size the queen cannot leave the cage. After eggs have been laid, it can take between 66 and 93 hours for larvae to hatch. At this point the queen should be released from the cage to prevent the colony from developing problems (Crailsheim, Brodschneider et al. 2013).

Grafting, transferring of recently hatched honey bee larvae, allows movement of larvae from the hive to a semi-sterile environment (Crailsheim, Brodschneider et al. 2013). Larvae are scooped from combs to wells in plastic plates containing warm larval diet. Grafting tools can be made from paint brushes, spatulas, wires, bamboo, or flexible plastics. The grafting tool should be free of rough edges that could injure the larvae. During the grafting process, the orientation of larvae on the diet must be maintained to ensure that the breathing structures/spiracles are not blocked. If a larva is flipped over during the grafting process, it will drown in the liquid food. Additionally to ensure larval survival, grafting should not take more than 30 seconds per larva. If larvae are to be grafted from multiple combs, it is recommended that the combs are stored at 35° C and more than 60% relative humidity (Crailsheim, Brodschneider et al. 2013).

In the laboratory, larvae are fed artificial worker jelly composed of royal jelly, yeast extract, glucose, and fructose suspended in water (Rembold, Lackner 1981, Peng, Mussen et al. 1992). This artificial worker jelly (AWJ) replaces the worker jelly produced by the hypopharyngeal and mandibular glands of nurse bees. Prior to artificial worker jelly, reared honey bee larvae were fed primarily royal jelly which resulted in a development of queens or queen-worker hybrids rather than worker bees. The development of artificial worker jelly by Rembold and Peng (1981 and 1992) was crucial to AFB research because this disease affects primarily worker larvae. Thus, we can now control the composition and amounts of artificial worker jelly in every larval stage (Crailsheim, Brodschneider et al. 2013).

Test substances can be fed to honey bee larvae by supplementing the larval diet (Crailsheim, Brodschneider et al. 2013). It is recommended that compounds be dissolved in water or solvents like acetone and mixed with the larval diet. As a general rule the solvent used should not exceed 10% of the final larval diet volume. Pathogens can also be administered within larval diet to determine killing rates. Test substances can be administered once (acute exposure) or multiple (chronic exposure) times. It is recommended that preliminary dosage experiments be performed to determine how to apply test substances (Crailsheim, Brodschneider et al. 2013).

Fluctuations in temperature and relative humidity decrease honey bee larvae survival. For proper development, the larvae must be incubated at 37°C and 95% relative humidity (Crailsheim, Brodschneider et al. 2013). Maintaining a constant temperature is achieved by using laboratory incubators, while relative humidity can be obtained by placing saturated salt or glycerol solutions inside the incubator. Temperature and humidity conditions should be verified using data loggers (Crailsheim, Brodschneider et al. 2013).

Larval survival can be determined with a stereo microscope (Crailsheim, Brodschneider et al. 2013). The primary sign of larval death is when respiration via the spiracles stops. Larvae breathe through spiracles, a series of external openings, which allow air to enter and leave the larval respiratory system. The second sign of larval death is color change. Healthy larvae are a pearly white color but change to dark brown (AFB disease), yellow (EFB disease), or watery (sacbrood) in appearance with disease. The color changes are the result of pathogen activity within the larvae (Shimanuki 1983). Another sign is that healthy honey bee larvae exhibit movement and are flexible. Existing protocols which rely on common laboratory equipment can be used to rear larvae in the laboratory with at least 80% survival and similar developmental trajectories to the hive (Peng, Mussen et al. 1992, Huang 2009, Crailsheim, Brodschneider et al. 2013).

1.7 Research aims

American foulbrood disease (AFB) research has primarily focused on understanding the immune response of honey bee (*Apis mellifera*) larva and identifying growth inhibitors of *Paenibacillus larvae,* the causative agent of the disease (Genersch 2010b, Genersch 2010a, Genersch, Evans et al. 2010). As a result, AFB disease management relies on antibiotics, breeding, and natural products (Antúnez, Anido et al. 2012). However, there has been limited success using these treatments because of antibiotic resistance in bacteria and limited efficacy of natural alternatives. Therefore, new disease management tools for *P. larvae* are needed (Genersch 2010a).

The goal of our project was to control the root of the problem by identifying cues that trigger or inhibit *P. larvae* spore germination. Establishing the relationship between the host environment and its pathogen is an important aim because it reveals what is necessary for the

disease to develop (Genersch, Evans et al. 2010). In *Bacillus subtilis*, Ger and PrkC germination pathways permit bacterial spores to germinate in favorable environments. Ger receptors bind to specific nutrient signals, and PrkC receptors bind to specific muropeptide fragments released by growing bacteria (Powell 1950, Paidhungat, Setlow 2000, Peter 2003, Shah, Laaberki et al. 2008), however, a link between Ger and PrkC receptors and germination within a natural host remains to be studied. Our experiments sought to determine if Ger and PrkC germination pathways act independently or synergistically with each other. The 3 aims of this dissertation were:

Aim 1: To identify triggers/germinants and inhibitors of *P. larvae* spore germination *in vitro*. Our identification of compounds that trigger and inhibit *P. larvae* spore germination is described in chapters 2 & 4.

Aim 2: To test if germinants and inhibitors of spore germination prevent AFB disease in laboratory-reared larvae. We determined the ability of five compounds to prevent AFB disease in laboratory-reared larvae reported in chapter 4.

Aim 3: To identify *P. larvae* spore germination receptors. We used quantitative polymerase chain reactions to measure mRNA levels of germination receptors in vegetative cells and sporulating cultures. Our results are described in chapter 5.

Chapter 2: Requirements for *in vitro* germination of *Paenibacillus larvae* spores

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2.1 Abstract

Paenibacillus larvae is the causative agent of American foulbrood (AFB), a disease affecting honey bee larvae. First- and second instar larvae become infected when they ingest food contaminated with *P. larvae* spores. The spores then germinate into vegetative cells that proliferate in the midgut of the honey bee. Although AFB affects honey bees only in the larval stage, *P. larvae* spores can be distributed throughout the hive. Because spore germination is critical for AFB establishment, we analyzed the requirements for *P. larvae* spore germination in vitro. We found that *P. larvae* spores germinated only in response to L-tyrosine plus uric acid under physiologic pH and temperature conditions. This suggests that the simultaneous presence of these signals is necessary for spore germination *in vivo*. Furthermore, the germination profiles of environmentally derived spores were identical to those of spores from a biochemically typed strain. Because L-tyrosine and uric acid are the only required germinants *in vitro*, we screened amino acid and purine analogs for their ability to act as antagonists of *P. larvae* spore germination. Indole and phenol, the side chains of tyrosine and tryptophan, strongly inhibited *P. larvae* spore germination. Methylation of the N-1 (but not the C-3) position of indole eliminated

its ability to inhibit germination. Identification of the activators and inhibitors of *P. larvae* spore germination provides a basis for developing new tools to control AFB.

2.2 Introduction

American foulbrood (AFB) is a bacterial disease of honey bees that kills developing larvae (White 1906, Brodsgaard, Ritter et al. 1998). *Paenibacillus larvae* spores are the infectious agent for AFB, but it is the vegetative cells that cause disease (Tarr 1938, Genersch 2010a). In 2005, a survey of almond pollinating bee colonies indicated 4% of colonies had significant AFB load (Eischen, Graham 2005). Once a beekeeping operation is contaminated, the bacterial spores are not easily removed (Shimanuki 1983). Although autoclaving and high concentrations of chemical disinfectants effectively kill spores, these treatments are not viable for the bee keeping industry (Dobbelaere, De Graaf et al. 2001). Traditionally, terramycin and other antibiotics have been used for treatment and prevention of AFB. However, the spore stage of *P. larvae* is not affected by antibiotic treatment and use of antibiotics leads to resistant strains (Lodesani, Costa 2005, Alippi, López et al. 2007). Presently, burning of infected colonies and beekeeping equipment is the only accepted practice for eradicating AFB from bee hives (Shimanuki 1983, Genersch 2010a). Because destroying hives is economically unfeasible for beekeepers, developing a new AFB control strategy is essential.

AFB occurs when first or second instar larvae (within 48 hours after the egg hatches) ingest food contaminated with bacterial spores (Crailsheim, Riessberger-Galle 2001). Twelve hours after ingestion, *P. larvae* spores germinate and the new vegetative cells start proliferating inside the larval gut (Yue, Nordhoff et al. 2008). Several days post-infection, bacteremia causes the death of the honey bee larva (Davidson 1973, Genersch, Ashiralieva et al. 2005, Genersch, Forsgren et al. 2006). After nutrients are depleted, *P. larvae* cells stop dividing and sporulate. As

a result billions of spores are found in the remains of each bee larva (Sturtevant 1932, Lindström, Korpela et al. 2008). Within the colony, spores are transmitted by adult bees that eat larval remains (Fries, Camazine 2001, Gillard, Charriere et al. 2008). Additionally, robbing of honey from infected colonies by neighboring bees and contaminated beekeeping equipment help transmit *P. larvae* spores between colonies (Fries, Lindström et al. 2006).

Because *P. larvae* spore germination is the first step in infection, controlling spore germination could lead to new approaches to prevent AFB (Alvarez, Abel-Santos 2007). However, little is known about the environmental cues required to trigger *P. larvae* spore germination. In *Clostridia* and *Bacilli* species, spores require metabolites including sugars, nucleosides, amino acids, and/or inorganic salts to stimulate germination (Paredes-Sabja, Setlow et al. 2011). The complexity of germination signals varies and commonly requires several types of germinants (Ross, Abel-Santos 2010a). Several labs including our own have also identified molecules that can inhibit spore germination (Akoachere, Squires et al. 2007, Cortezzo, Setlow et al. 2004, Yasuda-Yasaki, Namiki-Kanie et al. 1978, Setlow 2003, Howerton, Ramirez et al. 2011, Alvarez, Abel-Santos 2007).

Not knowing the requirements for *P. larvae* spore germination has limited AFB disease detection (Rose 1969, Hornitzky, Nicholls 1993, Goodwin, McBrydie et al. 2013, Forsgren, Laugen 2014). Culture based detection methods are superior than PCR based assays in identifying diseased colonies (Forsgren, Laugen 2014). PCR based methods are inferior to culture based methods because they depend on DNA extraction from complex hive samples that contain PCR inhibitors. However, one drawback of culture dependent techniques is that it requires *P. larvae* spores to germinate in laboratory media. The highest spore germination reported is approximately 2% on J agar. Incorporating larval and adult bee extracts to medium

can improve *P. larvae* spore germination (Goodwin, McBrydie et al. 2013). The triggers of *P. larvae* spore germination can replace bee extracts to help detect AFB diseased colonies.

We tested the ability of metabolites to promote *P. larvae* spore germination. We found that *P. larvae* spores exclusively recognize L-tyrosine and uric acid as co-germinants. We determined the germination half maximal effective concentration (EC_{50}) of L-tyrosine and uric acid. Because L-tyrosine and uric acid are strong germinants in vitro, we screened chemical analogs for their ability to inhibit spore germination. Indole and phenol, the side chains of tryptophan and tyrosine, were thus identified as germination inhibitors. Methylation of the N-1 position of indole inactivates its inhibitory activity. In contrast, methylation of the C-3, C-5, or C-7 positions has no effect on indole`s inhibitory potential.

2.3 Materials and Methods

Materials: Chemicals were purchased from Sigma Aldrich Corporation (St. Louis, MO). Dehydrated culture media was purchased from BD Difco (Franklin Lakes, NJ).

Paenibacillus larvae subsp. *pulvifaciens* B-3685/ATCC 49843 strains were purchased from the American Tissue Culture Collection (ATCC). Environmental American Foulbrood scales (the remains of infected larvae collected from infected hives) were kindly donated by Dr. Jay D. Evans at the USDA Bee Research Facility in Beltsville, MD. The environmental strain was identified as a strain of *Paenibacillus larvae* subsp. *larvae* based on its phenotypic characteristics and 16S rRNA analysis (Piccini, D'Alessandro et al. 2002). *Paenibacillus larvae* subsp. *larvae* strain B-3650/LMG 16245 was purchased from the Belgian Coordinated Collections of Microorganisms (BCCM) culture collection.

Media preparation: Tryptic soy broth (TSB) consists of 15 g tryptone, 5 g soytone, 5 g sodium chloride per liter of medium (pH 7.3 ± 0.2). MYPGP broth consists of 10 g Mueller-

Hinton broth, 15 g yeast extract, 3 g K_2HPO_4, 2 g glucose, and 1 g sodium pyruvate per liter of medium (pH 7.2 ± 0.2). Our media was autoclaved at 121°C for a minimum sterilization time of 30 minutes. Glucose was filtered sterilized and added to media prior to use. For solid media (TSA or MYPGP agar) we used 20 g of agar per liter of medium.

UNLV *P. larvae* spore preparation: *P. larvae* cells from a glycerol stock were used to inoculate MYPGP agar plates. The plates were incubated for two days at 37 °C under aerobic conditions. Individual bacterial colonies were used to inoculate 5 ml of MYPGP broth. The liquid culture was incubated at 37 °C with shaking 200 rpm to obtain exponentially growing cultures. Each TSA plate was inoculated with 200 µl of exponentially growing cultures. *P. larvae* strains were grown on tryptic soy agar plates for 7 days in a 5% CO_2 incubator at 37 °C. The resulting bacterial lawns were collected by flooding with ice-cold deionized water. Spores were pelleted by centrifugation at 8000 rpm at 4 °C for 5 minutes. The supernatant was discarded and the pellet was resuspended in fresh deionized water. After three washing steps, spores were separated from vegetative and partially sporulated forms by centrifugation at 11500 rpm at 4 °C for 35 minutes through a 20%-50% HistoDenz gradient. The spore pellet was washed five times with water and stored at 4 °C (Akoachere, Squires et al. 2007). Spore preparations were more than 90% pure as determined by microscopic observation of Schaeffer-Fulton stained samples (Schaeffer, Fulton 1933).

Preparation of germinant solution: Sixteen complex media (MYPGP, TSB, BHI, Nutrient, LB, TMYGP, NZ amine, NZCYM, Lactobacillus, SOC, Bailey, Clostridium, Michael, Terrific, MD, and J broths) were prepared (Bailey, Lee 1962, Dingman, Stahly 1983, Zimbro 2009). A defined medium was prepared as previously described (Ramirez, Abel-Santos 2010). An artificial worker jelly (AWJ) medium was prepared based on the published composition of

worker jelly (Rembold, Dietz 1966). For AWJ, the following stock solutions were prepared: 100 mM inosine in 220 mM NaOH, 400 mM for each sugar (fructose, glucose, and arabinose) in water, 30 mM for each of the 20 proteinogenic L-amino acids in 0.36 N HCl, 100 mM uric acid in 220 mM NaOH and 0.2 mg/ml vitamins (thiamine, riboflavin, pyridoxine, β-alanine, para-aminobenzoic acid, nicotinic acid, pantothenic acid, biotin, folic acid, and inositol) in water. To prepare AWJ, inosine, uric acid, sugars, and amino acids were dissolved to 3 mM final concentration in 0.1 M sodium phosphate buffer (0.06 mM Na_2HPO_4 and 0.04 mM NaH_2PO_4) and adjusted to pH 7.0.This solution was supplemented with vitamins to 1 µg/ml final concentration.

Determination of germinants for *P. larvae* spores: The decrease in optical density (OD) is inversely proportional to spore germination (Powell 1950). Changes in light diffraction during spore germination were monitored at 580 nm (OD_{580}) on a Biomate 5 (ThermoElectron Corporation, Waltham, MA) or a Tecan Infinite m200 (Tecan group, Männedorf, Switzerland) spectrophotometer. Experiments were carried out in 96-well plates (200 µL/well). In preparation for germination assays, *P. larvae* spore suspensions were washed three times with water. Spores were then heat activated at 70 °C for 30 minutes in water. The heat-activated spores were allowed to reach room temperature and transferred to 0.1 M sodium phosphate buffer (pH 7.0) to an approximate OD_{580} of 0.70. Spores were monitored for auto-germination for 30 minutes. Germination experiments were carried out with spores that did not auto-germinate. Putative germinants were added individually or in combinations to a final concentration of 3 mM. Experiments were performed in triplicate with at least two different spore preparations. After germinant addition, OD_{580} of the spore suspension was measured every minute for up to two hours. Relative OD values were derived by dividing each OD_{580} reading by the initial OD_{580}.

Spore germination rates (v) were calculated from the initial linear decrease in relative OD (23). Germination rates were set to 100% for *P. larvae* spores that had the fastest germination rate in an assay. Germination rates for other conditions were divided by the maximum germination rate for that condition and are reported as percent germination (Akoachere, Squires et al. 2007). Standard deviations were calculated from at least six independent measurements and are typically below 10%. Spore germination was confirmed in selected samples by microscope observation of Schaeffer-Fulton stained aliquots (Schaeffer, Fulton 1933).

Effect of temperature and pH on *P. larvae* germination: For temperature experiments, *P. larvae* spores were germinated in 3 mM L-tyrosine and 3 mM uric acid. Germination rates were determined as above, except that the germination temperature was varied between 25 and 42°C. The germination rate was set to 100% for spores germinated at 42°C because at this temperature we measured the maximum germination. Germination rates for other conditions were divided by the maximum germination rate at 42°C and are reported as percent germination. Germination rate differences were analyzed using ANOVA followed by a Tukey-Kramer procedure (SigmaPlot v.9).

For pH experiments, *P. larvae* spores were re-suspended in 0.1 M sodium phosphate, potassium/sodium phosphate, or citrate phosphate buffer. The pH of the buffers was adjusted between 3.0 and 9.0. Spores were germinated in the presence of 3 mM L-tyrosine and 3 mM uric acid. Germination rates were determined as above. Germination rate was set to 100% for spores germinated at pH 7.0. Germination rates for other conditions were divided by the maximum germination rate at pH 7 and are reported as percent germination. As above, germination rate differences were analyzed using ANOVA followed by a Tukey-Kramer procedure (SigmaPlot v.9).

Activation of *P. larvae* spore germination by L-tyrosine and uric acid: *P. larvae* spore germination was tested with different combinations of L-tyrosine and uric acid. For L-tyrosine titrations, spores were exposed to varying concentrations of L-tyrosine and a constant 3 mM uric acid. For uric acid titrations, spores were exposed to varying concentrations of uric acid and a constant 3 mM L-tyrosine. Germination rates were determined as above. Germination rate was set to 100% for *P. larvae* spores germinated in the presence of 3 mM L-tyrosine/3 mM uric acid. Germination rates for other conditions were divided by the maximum germination rate obtained with 3 mM L-tyrosine/3 mM uric acid and are reported as percent germination. Percent germination was plotted against compound concentrations. The resulting sigmoidal curves were fitted using the four parameter logistic function of the SigmaPlot v.9 software to calculate EC_{50} values (for enhancers of spore germination). EC_{50} is defined as the concentration of a germinant required to increase the germination rate to 50% of the maximal value (Rodbard, Lenox et al. 1976, Sebaugh 2011).

The linear portion of germination curves can be used to calculate kinetic parameters. Germination rates can be calculated as the slope of linear portion of germination curves (optical density versus time). The rate of spore germination can be influenced by the concentration of germinants. A titration of the germinants can be performed to measure the half maximal effective concentration (EC_{50}). The EC_{50} value indicates at what germinant concentration spore germination will be induced. The rate of spore germination can be altered by the presence of inhibitors. Calculation of the half maximal inhibit concentration (IC_{50}) from germination rates allows us to compare the strength of inhibitors.

Agonists of *P. larvae* spore germination: To test for possible agonists of *P. larvae* spore germination, spores were individually supplemented with 3 mM of a purine analog and 3 mM L-

tyrosine. Separately, *P. larvae* spores were incubated with 3 mM of an amino acid analog and 3 mM uric acid. Spore germination was monitored as above. Germination rates for other conditions were divided by the maximum germination rate obtained with 3 mM L-tyrosine/3 mM uric acid and are reported as percent germination.

Antagonists of *P. larvae* spore germination: To test for possible antagonists of *P. larvae* spore germination, spores were individually supplemented with 3 mM of a purine analog or 3 mM of an amino acid analog. Spore suspensions were incubated for 15 min at room temperature while monitoring OD_{580}. If no germination was detected, L-tyrosine and uric acid were added to 3 mM final concentrations and germination monitored as above. Germination rates for other conditions were divided by the uninhibited maximum germination rate obtained with 3 mM L-tyrosine/3 mM uric acid and are reported as percent germination.

Inhibition of *P. larvae* spore germination by indole and phenol: *P. larvae* spores were individually incubated with varying concentrations of indole, phenol, 1-N-methylindole, 3-mehtylindole, 5-methylindole, or 7-methylindole. After 15 minute incubation, spores were treated with 3 mM L-tyrosine/3 mM uric acid. Germination rate was set to 100% for *P. larvae* spores germinated in the absence of inhibitor. Germination rates for other conditions were divided by the uninhibited maximum germination rate obtained with 3 mM L-tyrosine/3 mM uric acid and are reported as percent germination. Percent germination was plotted against inhibitor concentrations. The resulting sigmoidal curves were fitted using the four parameter logistic function in SigmaPlot v.9 to calculate IC_{50} values. IC_{50} is the concentration of a germination inhibitor required to reduce the germination rate to 50% of the maximal value (Akoachere, Squires et al. 2007, Rodbard, Lenox et al. 1976).

Heat resistance of germinated *P. larvae* spores: Loss of heat resistance occurs upon the germination of a spore suspension (Powell 1950). We determined the percentage of germinated spores by calculating heat resistant counts on MYPGP agar plates. Initially, spores were exposed to (1) sodium phosphate buffer, (2) MYPGPG broth, or (3) 3 mM L-tyrosine plus uric acid for 2 hours at 37 °C. The spores were heated at 68°C for 30 minutes, diluted in buffer, and plated onto MYPGP agar. Plates with 30-300 colonies per plate were used to calculate the heat resistant spore counts per MYPGP plate used. Heat resistant counts were performed in triplicate with two different spore preparations.

Enhancing spore germination on MYPGP plates: The highest spore germination on agar plates is approximately 2% (Goodwin, McBrydie et al. 2013). We measured the ability of L-tyrosine plus uric acid to enhance spore germination on MYPGP agar plates. *P. larvae* spore suspensions were washed 3 times with water. Spores were then heat activated at 70 °C for 30 minutes, after they reached room temperature spores were transferred to 0.1 M sodium phosphate buffer (pH 7.0) to an OD_{580} of 0.40. The spore stocks were diluted in buffer and plated onto MYPGP agar with or without 3 mM L-tyrosine plus uric acid. Plates with 30-300 colonies per plate were used to calculate the heat resistant spore counts per MYPGP plate used. Heat resistant counts were performed in triplicate with two different spore preparations.

2.4 Results

We were unable to detect significant *P. larvae* spore germination in any of the 16 different complex media tested even after 24 hour incubation. In comparison, spores of *Bacillus anthracis* and *Bacillus cereus* germinate within two hours in rich medium (Johnson, Nelson et al. 1983, Sanz, Teel et al. 2008). Similarly, *P. larvae* spores failed to germinate in defined medium

containing metabolites commonly used as germinants by *Bacillus* and *Clostridia* species (Figure 2.1).

Honey bee larvae are fed royal or worker jelly that can be contaminated with *P. larvae* spores (Winston 1987). *P. larvae* spores were resuspended in a chemically defined medium (AWJ) that differ from worker jelly only in its pH values. The optical density of *P. larvae* spores suspended in AWJ decreased, indicating that the spores were germinating (Figure 2.1). Spore germination was confirmed by Schaeffer-Fulton staining (Schaeffer, Fulton 1933).

To determine compounds necessary to trigger *P. larvae* spore germination, groups of compounds were systematically left out of the AWJ medium. *P. larvae* spores germinated well in the absence of sugars and vitamins, suggesting that germination onset required uric acid and proteinogenic amino acid(s). Testing of individual amino acids showed that only L-tyrosine was able to synergize with uric acid to produce a strong germination response in *P. larvae* spores (Figure 2.2). A mixture of uric acid and the remaining 19 proteinogenic amino acids induced negligible germination response (data not shown).

To determine the effects of temperature on *P. larvae* spore germination a range of 25-42 °C was tested (Figure 2.3). At temperatures below 30°C, germination of P. larvae spores was slow. The maximal germination rates were at temperatures above 35°C.

The ability of *P. larvae* spores to germinate was also tested at different pH values (Figure 2.4). In acidic or basic conditions spores failed to germinate. Germination was optimal near neutral pH.

Titration of L-tyrosine at a saturating uric acid concentration yielded an EC_{50} of 1.2 mM for L-tyrosine activation of *P. larvae* spore germination (Figure 2.5). Our dose response assays resulted in sigmoidal curves that passed the Durbin-Watson statistical test for autocorrelation.

Titration of uric acid at a saturating L-tyrosine concentration yielded an EC_{50} of 0.2 mM for uric acid activation of *P. larvae* spore germination (Figure 2.6). Our dose response assays resulted in sigmoidal curves that passed the Durbin-Watson statistical test for autocorrelation.

Uric acid is a degradation product of purine catabolism. Hence, we tested the ability of purine analogs to act as co-germinants of *P. larvae* spores. Since L-tyrosine is the only amino acid able to trigger *P. larvae* spore germination, we also tested its stereoisomer (D-tyrosine) and its side chain (phenol) as co-germinants with uric acid. None of the compounds tested was able to activate *P. larvae* spore germination (data not shown).

L-tyrosine and purine analogs were also tested for their ability to inhibit *P. larvae* germination. None of the purine analogs tested inhibited uric acid/L-tyrosine induced germination of *P. larvae* spores (data not shown). Similarly, D-tyrosine did not inhibit *P. larvae* spore germination.

Of the compounds tested, indole (the side chain of tryptophan) and phenol (the side chain of tyrosine) were able to inhibit *P. larvae* spore germination. Titrations of indole yielded an IC_{50} of 0.37 mM for indole (Figure 2.7) inhibition of *P. larvae* spore germination.

Titrations of phenol yielded an IC_{50} of 0.46 mM for phenol (Figure 2.8) inhibition of *P. larvae* spore germination.

3-methylindole, 5-methylindole and 7-methylindole retained inhibitory properties with an IC50 of 0.38, 0.37, and 0.28 mM, respectively (Table 2.1). In contrast, 1-N-methylindole did not activate nor inhibit *P. larvae* spore germination. All dose response assays resulted in sigmoidal curves that passed the Durbin-Watson statistical test for autocorrelation.

To test the generality of *P. larvae* spore response, we prepared spores from the type strain *P. larvae* subsp. *pulvifaciens* strain ATCC 49843 and from an environmental AFB sample. Based

on phenotypic characteristics, this environmental sample was identified as *P. larvae* subsp. *larvae* (13, 14). Spores of both *P. larvae* sub-species responded identically to L-tyrosine and uric acid. Their germination was similarly inhibited by indole and phenol (data not shown).

Spores exposed to buffer, a complex medium, and L-tyrosine plus uric acid were heat treated (Figure 2.9). Although vegetative cells die from heat treatment, dormant spores are resistant to heat treatment (Forsgren, Stevanovic et al. 2008). We observed that buffer and complex medium did not initiate germination because the spores were not susceptible to heat killing. However, upon germination *P. larvae* spore were susceptible to heat killing. Approximately, 3% of the *P. larvae* spores exposed to germinants were not susceptible to heat treatment.

AFB disease detection is performed by culture based methods that detect spores in bees and bee related products (Goodwin, McBrydie et al. 2013). Unfortunately, *P. larvae* spores do not germinate well on currently available media. Furthermore, the material inspected (bees or bee products) can alter germination rates. A paper recently found that adult bee and larval extracts improve *P. larvae* spore germination (Goodwin, McBrydie et al. 2013). The average number of colonies per plate was between 8-300 times greater than in conventional media. We incorporated L-tyrosine plus uric acid into medium to determine if we could enhance spore germination (Figure 2.10). Incorporating uric acid plus L-tyrosine enhance our ability to detect spores on laboratory media. For strain B-3650, we had approximately 3,000 times more colonies per plate when L-tyrosine plus uric acid were incorporated into medium. For strain B-3685, incorporation of germinants in medium enhanced spore germination approximately 60 times.

2.5 Discussion

Bacterial spore germination is a critical step for infection onset in numerous hosts (Guidi-Rontani, Weber-Levy et al. 1999, Guidi-Rontani, Pereira et al. 2002). The nature of the spore germination signals have been widely studied in *Bacilli* and *Clostridia* (Paredes-Sabja, Setlow et al. 2011, Howerton, Ramirez et al. 2011, Alvarez, Lee et al. 2010, Barlass, Houston et al. 2002, Broussolle, Gauillard et al. 2008, Dodatko, Akoachere et al. 2009, Dodatko, Akoachere et al. 2010, Huo, Yang et al. 2010, Smith, Sullivan 1989, Warren, Gould 1968), but the triggers for *P. larvae* spore germination have not been identified. In order to cultivate *P. larvae*, specialized media has been produced (White 1906, Bailey, Lee 1962, Nordstrom, Fries 1995, Alippi 1995, Hornitzky, Nicholls 1993). Under the best conditions fewer than 10 percent of *P. larvae* spores plated on complex laboratory media germinate (Shimanuki, Knox 2000). In this study, we show that *P. larvae* spores specifically recognize L-tyrosine and uric acid as germinants.

P. larvae spores germinate sluggishly at room temperature and thus could remain dormant on hive surfaces and beekeeping equipment for long periods. Honey bees are endothermic and use heat producing muscle contractions to actively keep the colony temperature in a narrow range around 35 °C (Winston 1987). To develop correctly in the laboratory, reared honey bee larvae are maintained at a constant temperature of 35-37 °C (Peng, Mussen et al. 1992), which is also optimal for *P. larvae* spore germination. This means that *P. larvae* spores depend on multiple signals including temperature to escape from dormancy.

Maximal germination of *P. larvae* spores was observed between pH 5-7, which matches the intestinal pH of both adult bees (pH 5.6-6.3) and bee larvae (pH 6.8) (Colibar, Popovici et al. 2010). Honey, nectar, pollen, and royal jelly are much more acidic (pH 3-4). We suggest that the acidity of these products will may prevent *P. larvae* spores from germinating prematurely outside the honey bee gut.

Although the concentration of L-tyrosine in the bee larvae gut is not known, honey bee larvae are fed either worker or royal jelly that contains free L-tyrosine at approximately 0.11 mM (Liming, Jinhui et al. 2009). Royal jelly also contains proteins that, upon digestion, could increase the concentration of L-tyrosine to 22 mM (Liming, Jinhui et al. 2009). Thus, the *P. larvae* spores are exposed to saturating concentrations of L-tyrosine within the bee larvae.

Once metabolized, proteinaceous materials are converted to uric acid which is excreted as a waste product (Yadav 2003). Uric acid in the midgut of honey bee larvae must be at saturating concentrations because when larvae first defecate uric acid precipitates as large crystals (Yadav 2003). In fact, the appearance of uric acid crystals has been used as a marker for pupation onset (Winston 1987) and insect infestation of stored grains (Jood, Kapoor 1993).

The *Bacilli* and *Clostridia* spore germination response results from complex interacting pathways (Foerster, Foster 1966, Peter 2003). Indeed, six different strategies have been described that integrate the multiple signals required for spore germination (Ross, Abel-Santos 2010a). We recently showed that active germination pathways in *Bacillus* can cooperate or interfere with each other (Luu, Akoachere et al. 2011). In contrast, the simplicity of signals required for *P. larvae* spore germination can be described as a single integrator logical gate (Ross, Abel-Santos 2010a). By narrowing the germination signals to two germinants, *P. larvae* spores further ensure that germination only occurs in the larval gut where amino acids and uric acid are abundant.

The midgut and the hindgut of honey bee larvae are disconnected until the final larval molt. Hence, in the AFB-susceptible young larvae, food and waste products will co-localize in the midgut (Winston 1987). This will ensure that *P. larvae* spores are simultaneously exposed to high concentrations of uric acid and L-tyrosine, allowing for germination and infection onset. In contrast, in adult honey bees amino acids are thought to be absorbed in the midgut and uric acid

will only be present in the hindgut (Yadav 2003, Crailsheim 1988). The spatial and temporal separation between food and waste products in adult honey bees will preclude *P. larvae* spores from detecting both germination signals, thus preventing germination (Winston 1987).

Spore formers can have strain specific differences in their germination response. For example, while adenosine is a germinant for *B. cereus* strain 3711, it inhibits inosine-mediated germination in *B. cereus* strain 569 (Hornstra, De Vries et al. 2006, Abel-Santos, Dodatko 2007). *P. larvae* spores from both subspecies show identical germination response. Our result is consistent with polyphasic taxonomic studies that have found few differences between *P. larvae* strains (Genersch, Forsgren et al. 2006).

The spore germination response has been studied in other *Paenibacillus* species. Spores of *P. polymyxa* germinate in response to fructose and alanine (Huo, Yang et al. 2010). The difference in germination response between *P. larvae* and *P. polymyxa* was expected since *P. polymyxa* spores are associated with plant roots, where the concentration of uric acid is diminishingly small. Other members of the *Paenibacillus* genus are insect pathogens that specifically target the larval stage. Indeed, *P. popilliae* spores are sold commercially to control Japanese beetle infestation (McSpadden Gardener 2004). It is tempting to speculate that uric acid could serve as a general germination signal for insect larvae pathogens. Indeed, while there are no other reports of bacterial spores that germinate in response to uric acid and L-tyrosine, *Bacillus fastidiosus* spore germination occurs on medium containing only uric acid (Salas, Ellar 1985), while *Clostridium cylindrosporum* spores germinate in medium containing bicarbonate, uric acid, and calcium (Smith, Sullivan 1989). Both of these bacteria are present in poultry litter and bird droppings where uric acid is abundant (Smith, Sullivan 1989, Bergey, Boone et al.

2009). The ability to recognize uric acid and a co-germinant could allow bacteria to select for specific hosts.

Although D-amino acids are strong spore germination inhibitors of *Bacillus* species (Romick, Tharrington 1997, Woese, Morowitz et al. 1958, O'Connor, Halvorson 1961, Fey, Gould et al. 1964, Hills 1949), D-tyrosine failed to inhibit *P. larvae* spore germination. Of the analogs tested only indole (the side chain of tryptophan) and phenol (the side chain of tyrosine) inhibited *P. larvae* spore germination.

Indole and phenol could inhibit *P. larvae* spore germination by non-specific binding to hydrophobic regions of the tyrosine or uric acid binding sites. Methylindole derivatives are more hydrophobic than indole, but none of these analogs show increased anti-germination activity. On the contrary, methylation at the N-1 position eliminates anti-germination activity, suggesting that the N-H group of indole forms an essential hydrogen bond with the tyrosine and/or uric acid binding sites.

Indole is the last intermediate in the biosynthesis of tryptophan in plants and bacteria (Radwanski, Last 1995). Indole also acts as intracellular and extracellular signal for virulence, biofilm formation, acid resistance, drug resistance, and sporulation in bacteria (Hirakawa, Kodama et al. 2009, Lee, Lee 2010, Lee, Jayaraman et al. 2007, Nikaido, Yamaguchi et al. 2008, Kim, Lee et al. 2011). Phenol, on the other hand, is toxic in its free form but is found as a functional group in many plant secondary metabolites (Karakaya, El et al. 2001). Since honey bees gather pollen and nectar that are very rich in phenolic compounds, it is possible that collected polyphenols and indole compounds could protect the honey bee larvae from infection.

We have begun to study the use of spore germinants to reduce *P. larvae* resistant properties. Others have already demonstrated that germinated spores are susceptible to heat,

radiation, ethanol, and desiccation (Nerandzic, Donskey 2010). Indeed, we have shown that approximately 98% of spores exposed to germinants are killed by heat treatment. Furthermore, we have shown that inclusion of germinants on solid media enhances spore germination. Addition of germinants to media will enhance the limit of detection for *P. larvae* spores. Early detection of *P. larvae* spores will allow beekeepers to start preventative AFB treatments and quarantine diseased colonies.

In conclusion, we have found activators and inhibitors of *P. larvae* spore germination. We also presented evidence to suggest that *P. larvae* spores have evolved to germinate only in the gut of the larvae and to remain dormant in the food, exposed environments, and the adult bee.

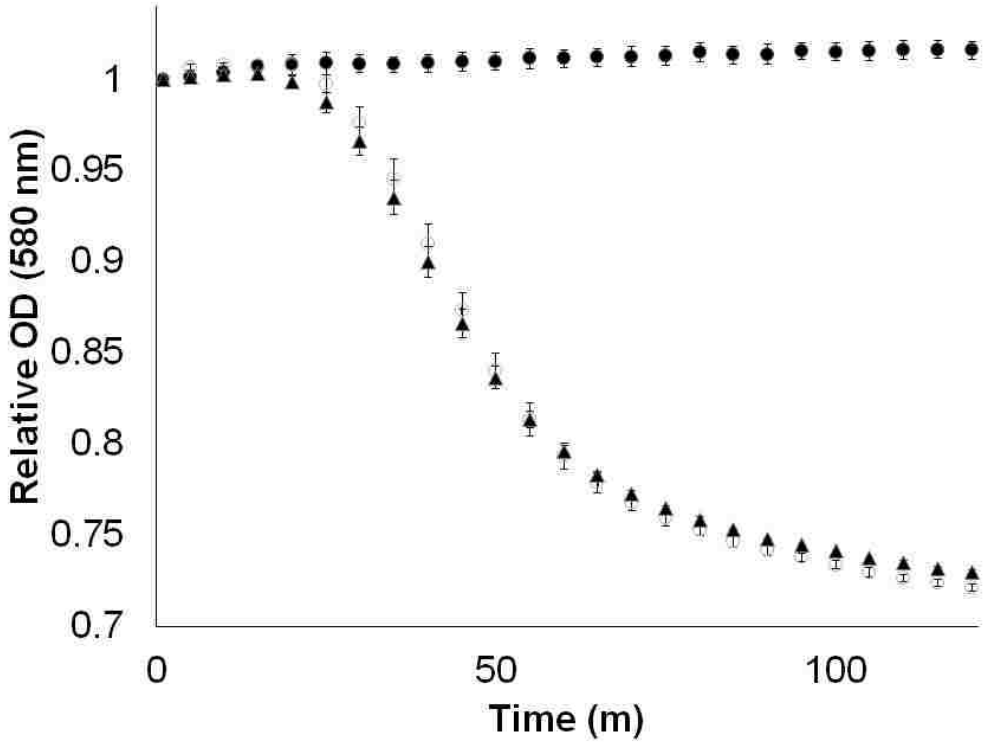


Figure 2.1: *P. larvae* spores germinate in artificial worker jelly. *P. larvae* strain B-3685 spores were suspended in defined medium (●), artificial worker jelly (○), or uric acid plus L-tyrosine (▲). Data are shown for every 5 min for clarity. Spore germination was followed by decreases of the relative OD over time. Each error bar represents a standard deviation obtained from at least six independent measurements.

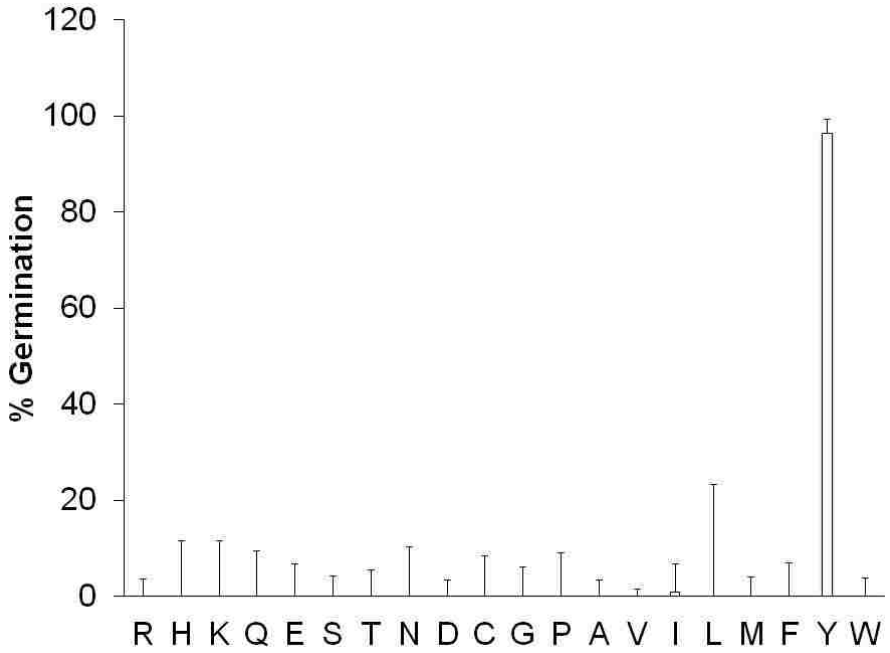


Figure 2.2: *P. larvae* spore germination is triggered by uric acid plus L-tyrosine. *P. larvae* strain B-3685 spores were suspended in the uric acid plus an L-amino acid. L-tyrosine plus uric acid was the only germinant combination that resulted in significant spore germination. Each error bar represents a standard deviation obtained from at least six independent measurements.

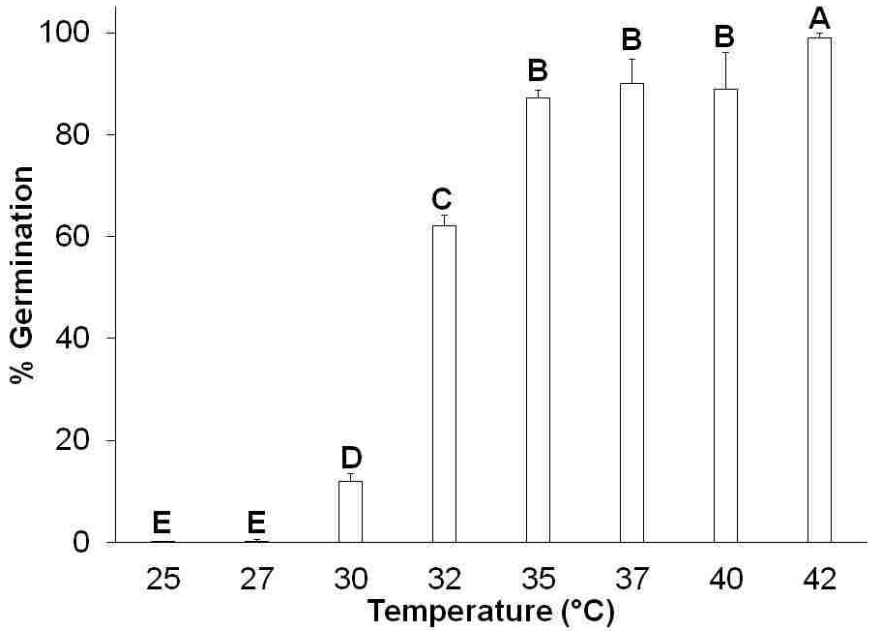


Figure 2.3: Effects of temperature on *P. larvae* spore germination. *P. larvae* spores were suspended in 3 mM L-tyrosine–3 mM uric acid and exposed individually to temperatures between 25 and 42°C. The maximum germination rate was set to 100% for spores germinated at 42°C. Percent germination for other conditions was calculated relative to 42°C. Each error bar represents a standard deviation obtained from at least six independent measurements. Columns that are labeled with different letters are statistically different ($P > 0.05$).

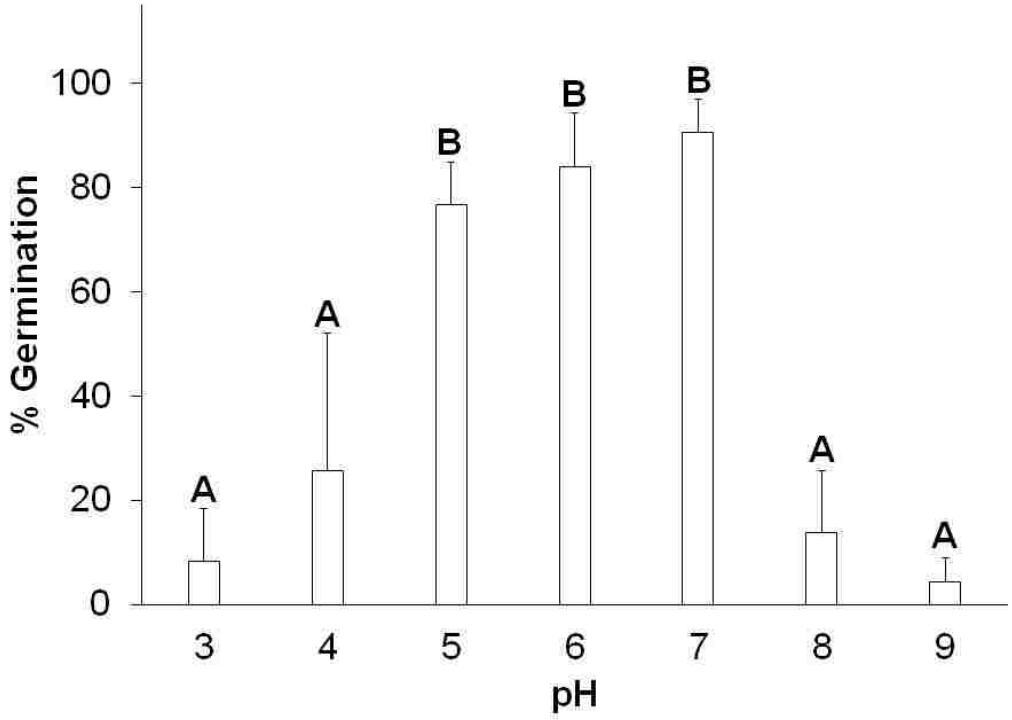


Figure 2.4: Effects of pH on *P. larvae* spore germination. *P. larvae* spores were suspended in 3 mM L-tyrosine–3 mM uric acid and were exposed individually to pH values between 3 and 9. The maximum germination rate was set to 100% for spores germinated at pH 7. Percent germination for other conditions was calculated relative to pH 7. Each error bar represents a standard deviation obtained from at least six independent measurements. Columns that are labeled with different letters are statistically different ($P > 0.05$).

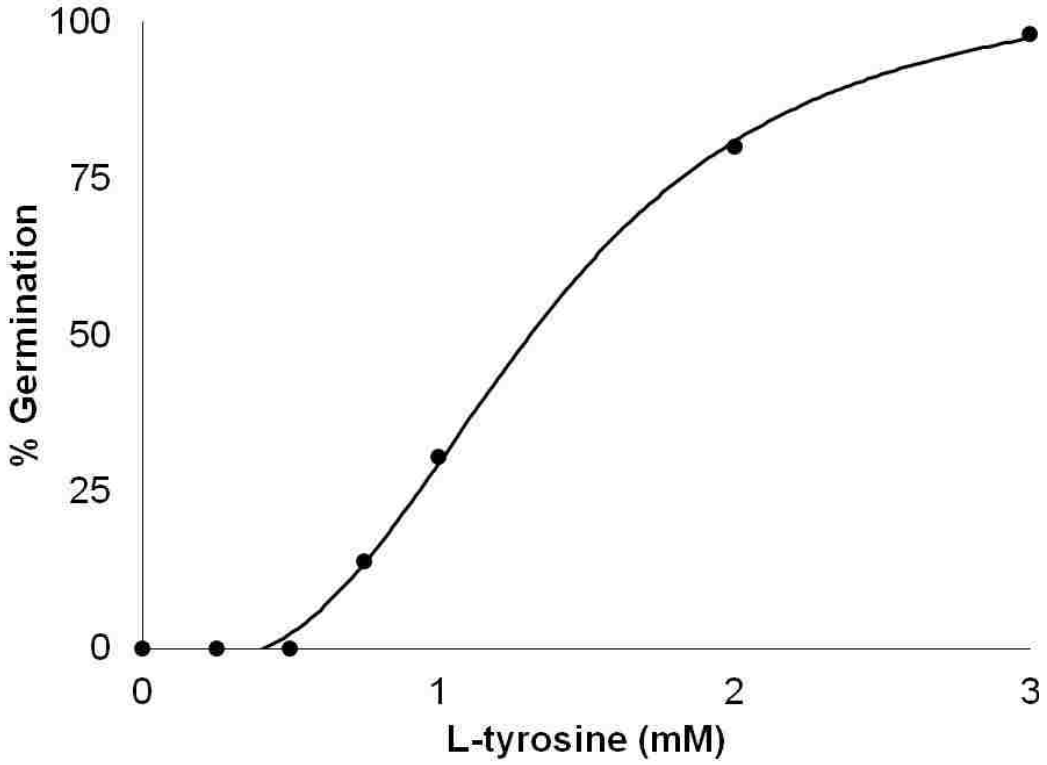


Figure 2.5 Calculation of 50% effective concentration for L-tyrosine. Dose-response curve of *P. larvae* spores germinated at a saturating concentration of uric acid and various concentrations of L-tyrosine. The EC_{50} for L-tyrosine was determined based on these data.

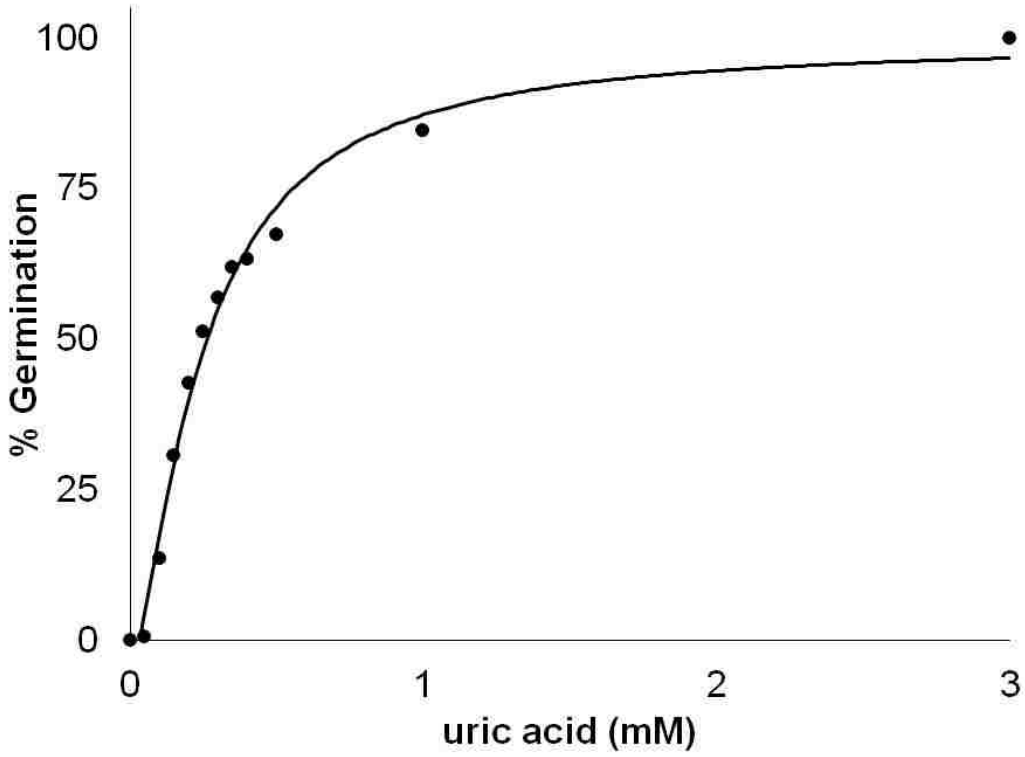


Figure 2.6 Calculation of 50% effective concentration for uric acid. Dose-response curve of *P. larvae* spores germinated at a saturating concentration of L-tyrosine and various concentrations of uric acid. The EC_{50} for uric acid was determined based on these data.

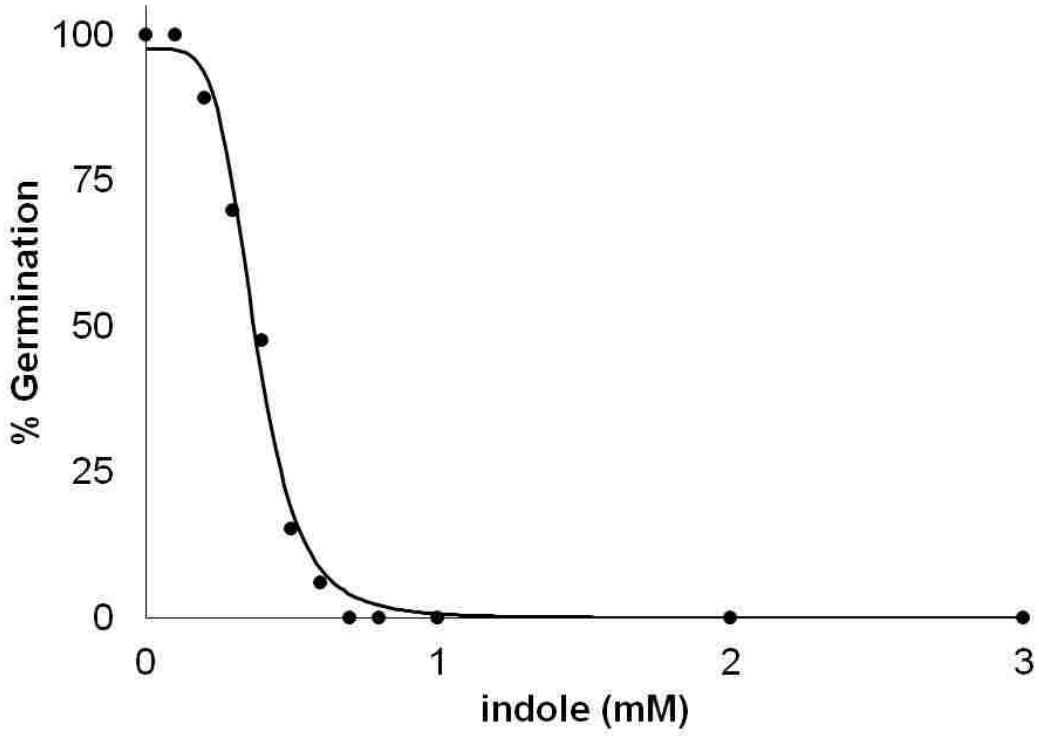


Figure 2.7: Calculation of 50% inhibitory concentration for indole. Dose-response curve of *P. larvae* spores germinated with L-tyrosine–uric acid in the presence of various concentrations of indole. The IC_{50} for indole was determined based on these data.

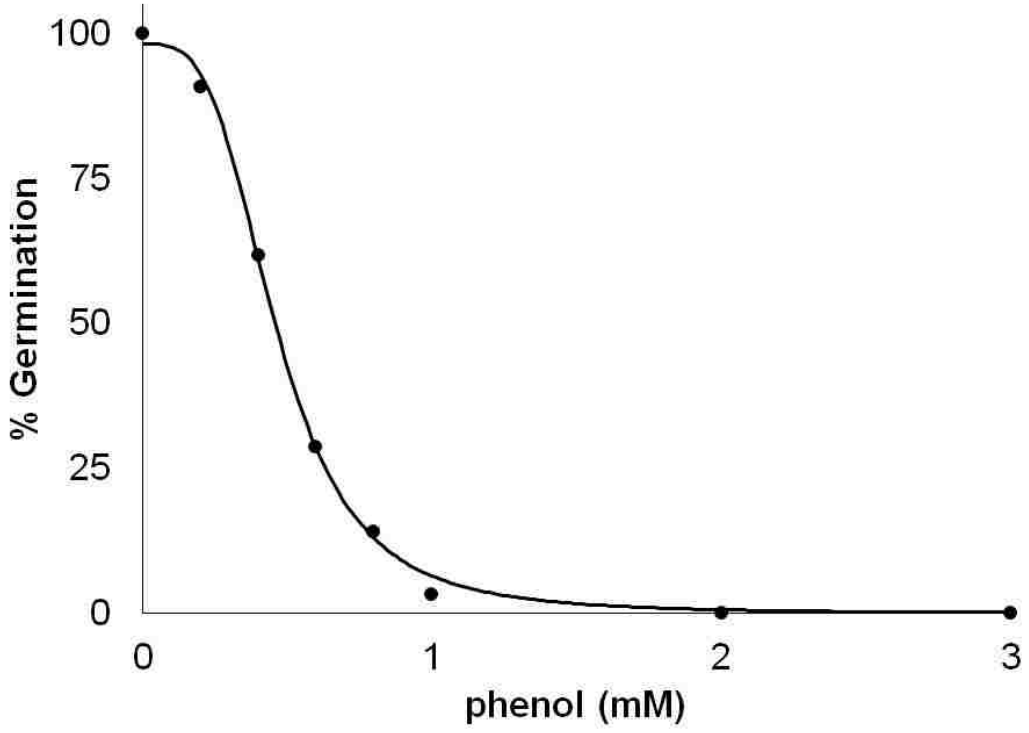


Figure 2.8: Calculation of 50% inhibitory concentration for phenol. Dose-response curve of *P. larvae* spores germinated with L-tyrosine–uric acid in the presence of various concentrations of phenol. The IC_{50} for phenol was determined based on these data.

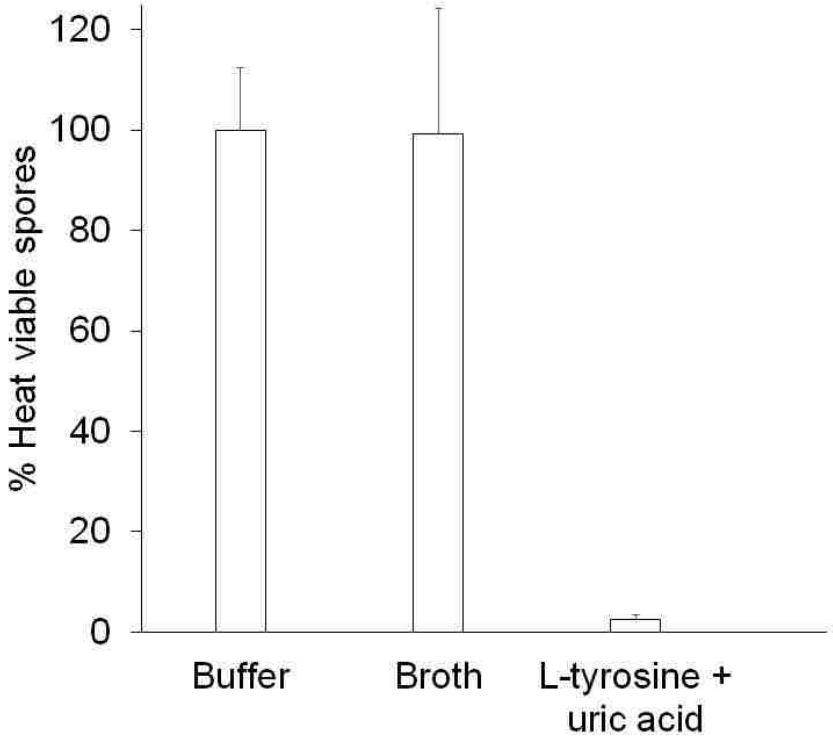


Figure 2.9: Measure of heat resistance in germinated spores. *P. larvae* strain B-3685 spores were suspended in buffer, broth, or uric acid plus L-tyrosine. After incubation *P. larvae* spores were heat treated to measure the average number of heat resistant spores. Maximum heat viability was set to 100% for spores in sodium phosphate buffer. Error bars are standard deviations from at least 6 independent measurements.

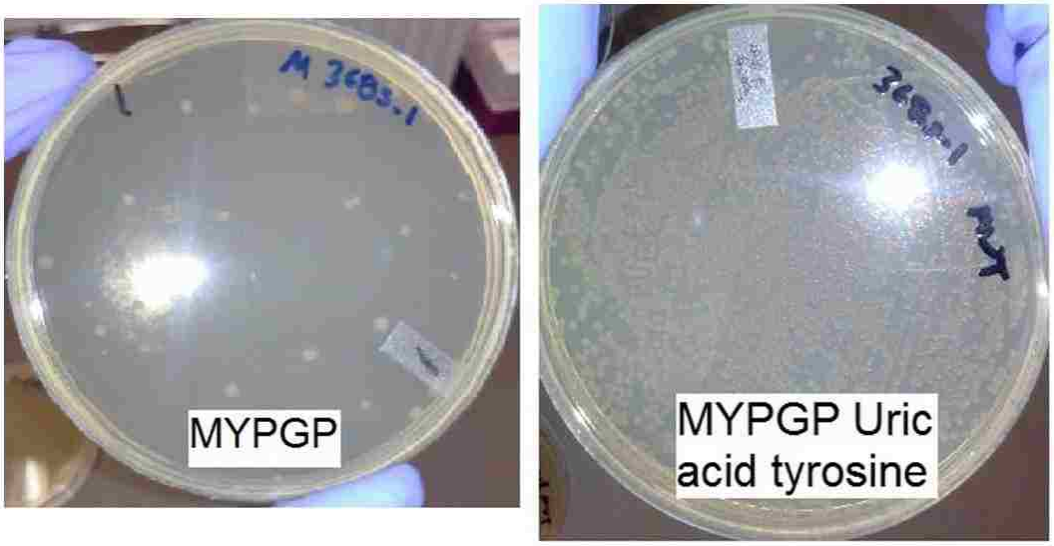
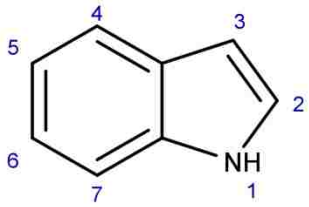


Figure 2.10: L-tyrosine + uric acid enhance germination: Spores of *P.larvae* strain B-3685 were incubated on MYPGP plates with or without 3 mM uric acid plus L-tyrosine. The number of germinated spores was assessed by plating a stepwise dilutions of spores in water.

Table 2.1: Effects of indole methylation on *P. larvae* spore germination. *P. larvae* spores were incubated with various concentrations of indole analogs for 15 min prior to addition of 3 mM L-tyrosine–3 mM uric acid. IC_{50} was calculated by plotting percent germination versus indole analog concentration. Standard deviations are shown in parentheses. NA, no activity under the conditions tested.



Indole Analog	**IC_{50} (mM)**
Indole	0.37 ± .02
phenol	0.46 ± .02
1-*N*-methylindole	0.38 ± .01
5-methylindole	0.37 ± .02
7-methylindole	0.28 ± .01

Chapter 3: Comparison of *in vitro* methods for the production of *Paenibacillus larvae* endospores

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3.1 Abstract

Paenibacillus larvae endospores are the infectious particles of the honey bee brood disease, American Foulbrood. We demonstrate that our previously published protocol (Alvarado, Phui et al. 2013) consistently yields higher numbers and purer preparations of *P. larvae* endospores, than previously described protocols, regardless of strain tested (B-3650, B-3554 or B-3685).

3.2 Article

The Gram-positive, spore-forming bacterium *Paenibacillus larvae* is the causal agent of American Foulbrood disease (White 1907). Honey bee larvae become infected when contaminated brood food containing infectious spores is fed to larvae by the "nurse" bees, which, like all adult honey bees, are resistant to AFB (William T. 1972). The spores of *P. larvae* are

highly resilient and can remain viable for at least 35 years (Haseman 1961). Eradication of *P. larvae* spores from infected hives is commonly achieved by burning the entire hive and all associated equipment, but this highly destructive solution increases the economic losses associated with this disease (Matheson 1993, Calderone 2001). Targeted biological approaches that eradicate spores from equipment have significant potential, but the development of such strategies requires large scale and pure preparations of *P. larvae* endospores.

While three procedures for *in vitro* sporulation of *P. larvae* have been commonly used (Dingman 1983, Dingman, Stahly 1983, Genersch, Ashiralieva et al. 2005) and recently described in (de Graaf, Alippi et al. 2013), these methods do not yield large enough numbers of spores of sufficient purity for studies that seek to identify chemical inhibitors and/or germinants of *P. larvae* spores (Powell 1950, Akoachere, Squires et al. 2007, Alvarez, Abel-Santos 2007, Ramirez, Abel-Santos 2010, Howerton, Ramirez et al. 2011). Consequently, a new method was developed ["UNLV"; (Alvarado, Phui et al. 2013)]. Here, we present a comparison of the UNLV *in vitro* sporulation protocol (Alvarado, Phui et al. 2013) with a protocol (described in (de Graaf, Alippi et al. 2013)) that has traditionally yielded the highest number of spores (Dingman, The Connecticut Agricultural Experiment Station; personal communication). Notably, in both protocols spores are prepared from solid growth medium.

To account for strain differences, three different *P. larvae* strains were used in this comparative study (Table 1). In the first protocol (de Graaf, Alippi et al. 2013), spores were harvested from MYPGP agar inoculated with *P. larvae*. Since "sporulation efficiency can decline when high numbers of colonies are present on a plate" (de Graaf, Alippi et al. 2013), cultures were serially diluted using MYPGP broth to obtain both low (50-200) and high (1,000-5,000) CFU counts per MYPGP plate prior to spore preparation. This allowed a thorough comparison of

this protocol (de Graaf, Alippi et al. 2013) with the other (Alvarado, Phui et al. 2013). After 7 days of incubation, spores were removed from MYPGP plates by gently scraping and washing the agar surface with sterile water three times. The spore suspension was concentrated via centrifugation at 12000 rpm/19800 g at 4 °C for 15 minutes, the supernatant was discarded and the remaining pellet was resuspended in sterile ice-cold water. Additional centrifugation and pellet resuspension steps were repeated four times to clean spores. Spore stocks produced in this manner were stored in sterile distilled water at 4°C.

In contrast, the UNLV method utilized Tryptic Soy Agar (TSA) plates (supplied by Becton, Dickinson and Company) inoculated with 200 µl of an exponentially growing culture (about 1.2 x 108 colony forming units, CFUs) that were incubated for 7 days in a 5% CO2 incubator at 37°C to produce lawns of *P. larvae* (Alvarado, Phui et al. 2013). The resulting spore-containing bacterial lawns were collected by flooding plates with ice-cold deionized water. Spores were pelleted by centrifugation at 8000 rpm/8820 g at 4 °C for 5 minutes and resuspended in fresh deionized water. After three washing steps, the spores were separated from their vegetative and partially sporulated forms by centrifugation at 11500 rpm/18200 g at 4 °C for 35 minutes through a 20-50% HistoDenz™ (Sigma Aldrich) gradient. Spore pellets were washed five times with sterile distilled water and stored at 4°C.

To measure the concentration of viable spores in each spore stock, counts of heat resistant colony forming units were calculated from MYPGP agar plates (Dingman 1983). Briefly, spore stocks were normalized to an OD_{580} of 0.2, heated at 68°C for 15 minutes, diluted in water and plated onto MYPGP agar. Plates bearing about 30-300 colonies per plate were used to calculate the number of heat-resistant spores in each stock (Dingman 1983). Assays were performed in triplicate and average CFUs, standard deviations and statistically significant

differences ($p < 0.05$) for all pairs of mean heat resistant spore counts per stock were determined using a Student's one-tailed t test assuming equal variances.

As expected, spore yields varied from strain to strain regardless of the protocol was used. Consistent with previous findings, yields from B-3554 and B-3650 were higher when spores were harvested from plates with lower CFU density than higher; a trend not seen for B-3685. Importantly, regardless of the strain tested or whether the spores were isolated from either low or high density plates, the UNLV protocol (Alvarado, Phui et al. 2013) yielded significantly higher numbers of spores than the established MYPGP plate method (de Graaf, Alippi et al. 2013) (more than 385-fold higher for strain B-3554, 2.6-fold higher for strain B-3650 and 142-fold higher for strain B-3685; see Figure 3.1A), allowing us to conclude that the UNLV protocol routinely yields higher numbers of viable spores than the de Graaf protocol, regardless of the *P. larvae* strain used.

The purity of spore stocks was next determined by microscopic analysis. Briefly, 10 µl of each spore stock was smeared onto a glass slide, air dried, and observed after Schaeffer-Fulton staining (Schaeffer, Fulton 1933). At least 10 images of each spore stock were randomly acquired and three random images were analyzed using image processing software (ImageJ, NIH). The purity of spore preparations viewed in these images was expressed as: (number of spores/number of spores and vegetative cells) X 100. Statistically significant differences ($p < 0.05$) for all pairs of mean spore preparations or percent purity were determined using a Student's two-tailed t test assuming equal variances.

This analysis revealed that spore stocks prepared from strains B-3554 and B-3685 using the de Graaf protocols consisted of less than 20% spores (Figure 3.1B). Furthermore, spores prepared using this protocol, were frequently contaminated with cell debris, although the amount

was difficult to quantify by microscopy. For example, even though spore stocks generated from strain B-3650 were found to be 100% pure (did not contain any vegetative cells) cell debris could still be observed by microscopy (Figure 3.2A). These results indicate that four washes used in the previously reported protocols (Dingman, Stahly 1983, de Graaf, Alippi et al. 2013) were not sufficient to remove cell debris from the spore stocks. While it is possible to remove vegetative cells from spore stocks by heating, this approach is not recommended because muropeptides released from vegetative cells have been shown to trigger spore germination (Shah, Laaberki et al. 2008). In the UNLV protocol, this problem was solved by using a HistoDenz™ density gradient. This approach separated the spores from vegetative cells and cell debris and yielded virtually pure preparations of spores, regardless of the strain tested (Figure 3.2B).

In sum, this comparative analysis demonstrates that the UNLV *in vitro* sporulation method is more effective at generating higher numbers of spores with greater purity than the established *in vitro* sporulation protocol (Dingman, Stahly 1983, de Graaf, Alippi et al. 2013). The use of a commercially available rich medium, TSA, facilitated spore production and streamlined media production compared to other protocols (Dingman 1983, Dingman, Stahly 1983, Genersch, Ashiralieva et al. 2005, de Graaf, Alippi et al. 2013) and the use of a centrifugation density gradient ensured that a pure stock was obtained. Although, we acknowledge that similar results might be obtained if the numbers of plates and tubes were increased while following older protocols (Dingman 1983, Dingman, Stahly 1983, Genersch, Ashiralieva et al. 2005), a differential centrifugation step would still be required to increase the purity of the spores generated. This comparative analysis highlights the strengths and weaknesses of the currently published protocols for the *in vitro* preparation of *P. larvae* spores from solid medium (Alvarado, Phui et al. 2013, de Graaf, Alippi et al. 2013), and as such will

help those requiring large scale and pure preparations of *P. larvae* endospores and possibly other bacterial endospores.

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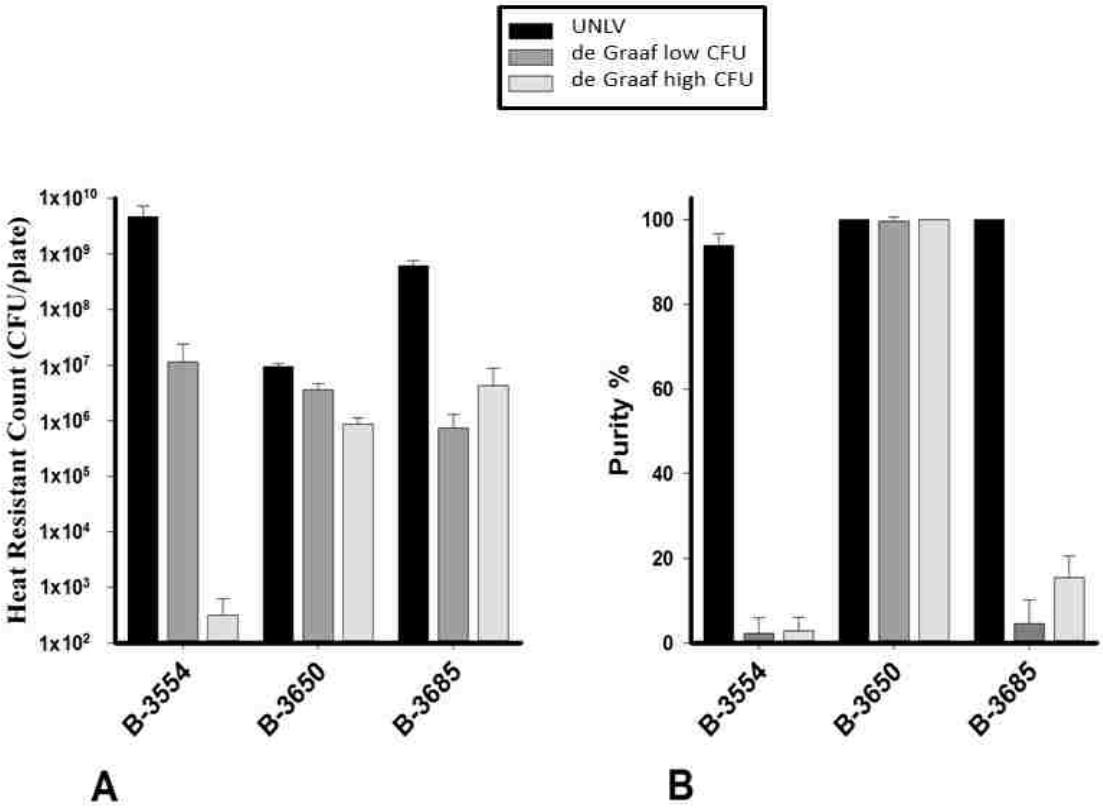


Figure 3.1 - Effect of sporulation protocol on *P. larvae* strains spore production (A) Average number of heat resistant spores for *P. larvae* strains spore stocks produced by two methods. NOTE: log scale. The error bars represent standard deviations from the mean obtained from at least three independent measures of CFU ml^{-1}. (B) *P. larvae* spore stock smears were analyzed via microscopy after Schaeffer-Fulton staining (Schaeffer, Fulton 1933). The error bars represent standard deviation obtained from the mean based on at least three independent measures of purity. In all cases, the number of spores collected using the UNLV protocol was significantly higher ($p < 0.05$) than those collected using the de Graaf protocol. Furthermore, the purity of the resulting spore samples was significantly higher when the UNLV protocol was used ($p < 0.05$) compared to the de Graaf protocol, with one notable exception; spores harvested from strain B-3650 using the UNLV protocol were quantified to be equivalently pure as spores harvested using the de Graaf protocol.

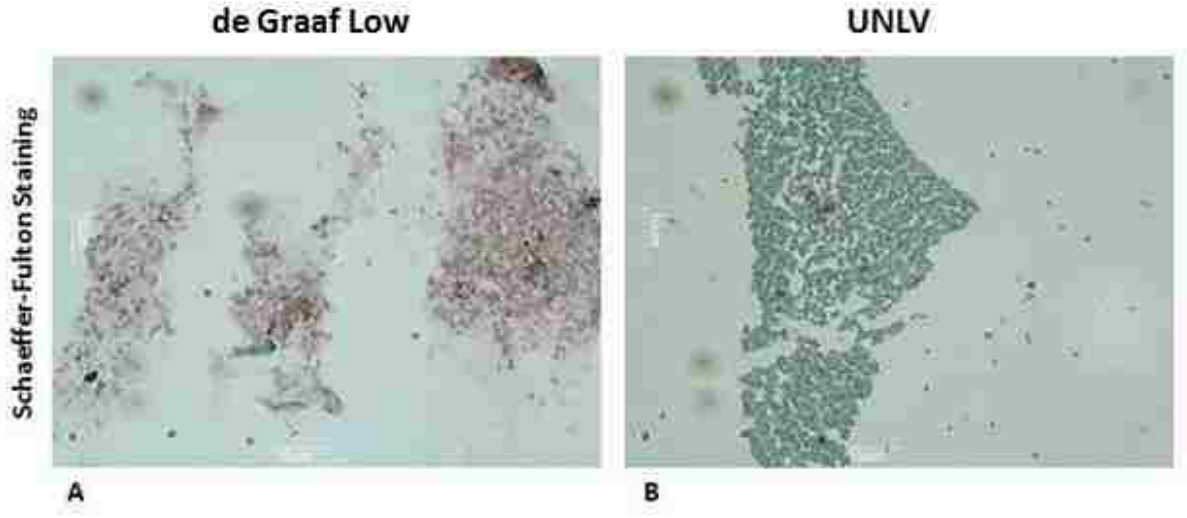


Figure 3.2 - Purity of spore stocks determined by microscopic analysis (A) Representative image of spore stocks stained using the Schaeffer-Fulton method prepared for strain B-3650 (A) using the de Graaf protocol or (B) the UNLV protocol. Cell debris retained safranin-O dye while spores retained malachite green dye. The scale bars indicates 10 μm.

TABLE 3.1: Strains of *P. larvae* used.

Species	ERIC classification[a]	Strain number[b]	Isolation
P. larvae	I	NRRL B-3650	Isolated from diseased honey bee, RE Gordon
P. larvae	I	NRRL B-3554	Isolated from diseased honey bee, NRRL
P. larvae	IV	NRRL B-3685/ATCC-49843	Powdery scale of honey bee larvae, RE Gordon

[a]Evaluated at UNLV by Enterobacterial repetitive intergenic consensus sequence-based PCR.

[b]Strain numbers sourced from the American Tissue Culture Collection (www.atcc.org) are prefixed with ATCC, those from the Agriculture Research Services (ARS) Culture Collection Database Server (nrrl.ncaur.usda.gov; formerly known as the Northern Regional Research Laboratories, NRRL) are prefixed with NRRL.

Chapter 4: The inhibitory effect of indole analogs against *Paenibacillus larvae*, the causal agent of American Foulbrood disease

4.1 Abstract

The antagonistic effects of indole and phenol structural analogs on the honey bee pathogen *Paenibacillus larvae* were evaluated. Indole structural analogs strongly inhibited L-tyrosine plus uric acid mediated *P. larvae* spore germination and the inhibitory effect was observed in a medium capable of supporting growth. These results suggests that indole analogs could prevent *P. larvae* spore germination and cellular growth *in vitro*. Addition of indole analogs to larval diet significantly decreased AFB disease in laboratory-reared larvae. The identification of inhibitors of *P. larvae* spore germination provides a basis for new tools to control AFB disease.

4.2 Introduction

Honey bees are vital pollinators of agricultural and horticultural crops (Matheson 1993, Morse, R.A. & Calderone, N.W 2000). However, recent losses of managed honey bee colonies have compromised pollination of crops (Neumann, Carreck 2010). The decline in honey bees is the result of many maladies including virus, parasites, pesticides, and bacterial diseases. American Foulbrood (AFB) disease is a lethal disease for honey bee larvae and ultimately colonies (White 1920, Genersch 2010a).

American Foulbrood (AFB) disease occurs in first or second instar larvae as newly germinated *Paenibacillus larvae* cells proliferate (Yue, Nordhoff et al. 2008). Extreme bacteremia causes the death of larvae several days after *P. larvae* spore infection (Yue, Nordhoff et al. 2008). As nutrients in honey bee larvae are depleted, *P. larvae* cells sporulate forming billions of spores. The infectious spores can be transmitted within the colony or amongst

colonies by bees and beekeeping practices (Lindström, Korpela et al. 2008, Sturtevant 1932). Because the infectious agent is the spore stage of *Paenibacillus larvae* and spores are hard to eradicate, AFB disease persists everywhere honey bees are found (Tarr 1938, Shimanuki 1983, Genersch 2010a).

Eradication of AFB is difficult because the bacterium produces spores are resistant to high temperatures, desiccation, UV irradiation, and harsh chemicals (Dobbelaere, De Graaf et al. 2001). Moreover *P. larvae* spores can remain dormant in honey, pollen, wax, adult bees, and on hive surfaces (Adjlane, Haddad et al. 2014, Lindström, Korpela et al. 2008, Fries, Lindström et al. 2006). *P. larvae* spores are only known to germinate, exit dormancy, in the honey bee larvae. Terramycin and other antibiotics have been used to prevent AFB disease. However, overuse of antibiotics has led to resistant *P. larvae* strains. Furthermore, spores are not affected by antibiotics and can remain dormant long after the antibiotic treatment (Alippi, López et al. 2007, Peng, Mussen et al. 1992, Lodesani, Costa 2005).

Because *P. larvae* spore germination is the first step of infection, preventing spore germination is a promising avenue to prevent AFB (Alvarez, Abel-Santos 2007). Inhibition of *Clostridium difficile* spore germination has been shown to be an effective means to prevent infection in rodent models (Howerton, Ramirez et al. 2011, Howerton, Patra et al. 2013b, Howerton, Patra et al. 2013a). Previous research in our laboratory identified agonists (uric acid and L-tyrosine) and inhibitors (indole or phenol) of *P. larvae* spore germination (Alvarado, Phui et al. 2013). Because spores need to germinate to cause the disease, preventing spore germination could potentially protect honey bees from AFB disease.

The aim of this work was to assess the efficiency of germination inhibition as a treatment strategy for AFB. First a series of indole and phenol analogs were tested as antagonists of uric

acid and L-tyrosine mediated germination of *P. larvae* spores. The half maximal inhibitory concentration (IC_{50}) of the best inhibitory indole analogs was calculated. The effect of indole analogs on cellular outgrowth from spores and growth of *P. larvae in vitro* and the toxicity of indole inhibitors on honey bee larval development was tested. Finally, indole inhibitors were tested for their ability to protect honey bee larvae from AFB disease. 5-chloroindole was identified as strong inhibitor of spore germination, cell growth, and AFB disease development.

4.3 Materials and Methods

Materials. Chemicals were purchased from the Sigma-Aldrich Corporation (St. Louis, MO) and VWR International (Radnor, PA). The dehydrated culture medium components including yeast extract, Mueller-Hinton broth, tryptic soy agar were purchased from BD Difco (Franklin Lakes, NJ) and Amresco (Solon, OH). *Paenibacillus larvae* ERIC IV strain B-3685/ATCC 49843 was obtained from the American Tissue Culture Collection (ATCC). Lyophilized royal jelly from GloryBee Foods (Eugene, OR) was stored at -20°C until used for diet preparation.

***P. larvae* spore preparation.** *P. larvae* strains were grown on BD tryptic soy agar plates for 7 days under 5% CO_2 at 37°C. The resulting bacterial lawns were collected by flooding with ice-cold deionized water and scrapped from plates using a cell spreader. Spores were pelleted by centrifugation and resuspended in fresh deionized water. After three washing steps, the spores were separated from their vegetative and partially sporulated forms by centrifugation through a 20%-50% HistoDenz density gradient. The spore pellet was washed five times with water and stored at 4°C. Spore preparations were 90% pure as determined by microscopic observation of Schaeffer-Fulton-stained samples as reported previously (Schaeffer, Fulton 1933, Alvarado, Phui et al. 2013).

Testing for antagonists of *P. larvae* spore germination. To test for possible antagonists of *P. larvae* spore germination, spores were resuspended in 0.1 M sodium phosphate buffer (pH 7) to an average OD_{580} of 1.0 and individually supplemented with varying concentrations of individual indole or phenol analogs dissolved in dimethyl sulfoxide (DMSO) as a vehicle. Spore suspensions were incubated for 15 min at room temperature while monitoring the OD_{580}. If no germination was detected, L-tyrosine and uric acid were added to final concentrations of 3 mM, and germination was monitored for 120 minutes. Germination rates for all conditions were divided by the uninhibited maximum germination rate obtained by treating spores with 3mM L-tyrosine plus 3mM uric acid and are reported as percent germination. Percent germination was plotted against the log of inhibitor concentrations. The resulting sigmoidal curves were fitted using the four-parameter logistic function in SigmaPlot v.9 to calculate the half maximal germination inhibitory concentrations (IC_{50}s).

Artificial worker jelly preparation and larvae feeding regime. Artificial worker jelly was prepared using different concentrations of lyophilized royal jelly, D-glucose, D-fructose, yeast extract, and autoclaved double distilled water (Table 4.1) (Crailsheim, Brodschneider et al. 2013). All ingredients except lyophilized royal jelly were dissolved in autoclaved double distilled water. Lyophilized royal jelly was then added to this sugar-yeast extract solution and mixed thoroughly with a vortex mixer. Artificial worker jelly was prepared an hour prior to use for larval rearing. Larvae were fed daily in different amounts according to Table 4.1 (Crailsheim, Brodschneider et al. 2013).

Acquisition of first instar larvae. First instar honey bee larvae were collected from two colonies headed by naturally mated queens (Honey Bee Genetics, Vacaville CA). Queens were confined in a queen excluder cage on empty frames (Crailsheim, Brodschneider et al. 2013). This

allows for the approximate time and place for the queen to lay the eggs to be controlled. After 24 hours of being caged the queen was released and the eggs were incubated in the hive until first instar larvae could be collected. A group of similarly aged first instar larvae were transferred on to artificial worker jelly with a Chinese grafting tool (Amazon, Seattle WA).

Incubation conditions. Plates containing grafted honey bee larvae were placed in a 304 cm by 228 cm by 101 cm (l x w x h) plastic container fitted with a tight lid. To maintain 95% relative humidity, 500 ml of 20% glycerol was poured inside the container that was placed inside an incubator at 35° C (Segur 1953, Crailsheim, Brodschneider et al. 2013). Plates containing larvae were place on top of a plastic island above the glycerol. The desired relative humidity and temperature was verified with an iButton data logger (Maxim Integrated, San Jose CA). The plastic containers were decontaminated after bees pupated with dishwashing detergent followed by 70% ethanol. Plastic containers were irradiated with an ultraviolet lamp overnight.

Toxicity assay. To determine if spore germination inhibitors would harm larvae, toxicity assays were performed. Each indole analog was dissolved in warm autoclaved water to provide a 0.5 mM stock solution. The concentrations of indole analogs used in the toxicity assay were determined based on preliminary experiments with indole. D-glucose, D-fructose, and yeast extract were dissolved in the indole analog stock solution (Table 4.1). Lyophilized royal jelly was then added to this indole-sugar-yeast extract solution and mixed thoroughly via vortex mixer. Artificial diet was prepared an hour prior to use for larval rearing. Chronic feeding of indole analogs was performed to assess toxicity on first instar honey bee larvae (Figure 4.1). Larval survival was determined every 24 hours under a stereo microscope. Larvae that stopped breathing, showed a color change, or failed to pupate after 7 days were considered dead (Evans

2004, Genersch, Ashiralieva et al. 2005). Each experiment used three replicate trials with 30 larvae, for a total of 90 larvae per treatment (Crailsheim, Brodschneider et al. 2013).

Larval exposure assays. To determine if spore germination inhibitors would protect larvae, *P. larvae* spore exposure assays were performed. Each exposure assay consisted of: (1). negative control group fed normal diet, (2). positive control group fed one dose of spores, and (3). experimental groups fed one dose of spores and a diet including 0.5 mM of each individual indole analog.

Initially, first instar larvae were brought into the laboratory and fed larval diet for a 24 hour period (Figure 4.2). The experimental groups were fed diet including 0.5 mM of each individual indole analog. The negative and positive control groups were initially provided normal diet.

During the next 24 hours, second instar larvae in the positive control and experimental groups were fed diets containing a defined number of *P. larvae* spores prepared as described previously (Alvarado, Phui et al. 2013). A stock spore suspension with an optical density of 1.0 at 580 nm was prepared (4.4×10^8 spores per ml based on a microscopic count with a counting chamber). The stock spore suspension was diluted 1:100 times, and then 100 µl of the dilution was added to 5 ml larval diet. The larval diet containing *P. larvae* spores was mixed and then dispensed to 6 well plates (2.5 ml/well). Assuming that honey bee larvae consume 10 µl of larval diet during the 24 hours spore exposure period, then each larvae consumes approximately 880 spores (Crailsheim, Brodschneider et al. 2013). Larvae in the negative control group continued to be fed normal diet.

On the third day, all honey bee larvae were removed from larval diets, cleaned using Kim wipes, and then moved to 48 well plates (one larva per well containing 250 µl larval diets without spores). Larval survival was determined daily by observing signs of respiration until the onset of pupation (approximately 5-7 days). Infected honey bee larvae developed a red color as *P. larvae* (a red pigmented bacterium) proliferated in the gut. Later on, decomposing larvae became characteristic brown to black color and soft appearance (Shimanuki 1983, Crailsheim, Riessberger-Galle 2001, Yue, Nordhoff et al. 2008, Crailsheim, Brodschneider et al. 2013). Each experiment used three replicate trials with 30 larvae, for a total of 90 larvae per treatment (Crailsheim, Brodschneider et al. 2013).

Survival analysis. The larval survival data was analyzed using Kaplan-Meier Survival plot and the LogRank statistic in SigmaPlot. The LogRank test determines if there are significant differences between survival curves. The Holm-Sidak statistic method was used to identify pairs of survival curves that are significantly different (significance level = 0.05).

5-chloroindole larval toxicity and exposure assays. Toxicity and exposure assays were performed as described above with different concentrations of 5-chloroindole. For larval toxicity assays, 0.5, 0.75, and 1.0 mM final concentration of 5-chloroindole was used to prepare larval diets. Larval exposure assays were conducted using a range of concentrations of 0.125-1.0 mM 5-chloroindole to prepare larval diet.

Testing for 5-chloroindole antibiotic activity against *P. larvae* cells and spore outgrowth. Overnight *P. larvae* cultures were diluted to an OD_{580} of 0.19. 5-chloroindole dissolved in DMSO was added to cells to a final concentration of 0.0, 0.5, 1.0, 1.5, or 2.0 mM in a 200 µl volume. Cultures were incubated at 37°C with shaking at 335 rpm every 15 minutes for

a 24 hour period. Optical density was measured every 15 minutes in a Tecan Infinite m200 (Tecan group, Männedorf, Switzerland) spectrophotometer. Experiments were performed in triplicate with at least two different cultures in 96 well plates (200 µL/well). Relative OD values were derived by dividing each OD_{580} reading by its initial OD_{580} reading.

The conversion of *P. larvae* spores to cells and subsequent growth was monitored over a 24 hour period. *P. larvae* spores were diluted to an OD_{580} of 0.15. 5-chloroindole dissolved in DMSO was added to spores to a final concentration of 0.0, 0.5, 1.0, 1.5, or 2.0 mM. MYPGP broth was used to support the growth of *P. larvae* cells (40 µl). Cultures were incubated at 37°C with shaking at 335 rpm every 15 minutes for a 24 hour period. Optical density was measured every 15 minutes in a Tecan Infinite m200 (Tecan group, Männedorf, Switzerland) spectrophotometer. Experiments were performed in triplicate with at least two different cultures in 96 well plates (200 µL/well). Relative OD values were derived by dividing each OD_{580} reading by its initial OD_{580} reading.

4.4 Results

It was previously demonstrated that indole (IC_{50} 0.4 mM) and phenol (IC_{50} 0.5 mM) inhibited *P. larvae* spore germination triggered by uric acid plus L-tyrosine (Alvarado, Phui et al. 2013). To identify stronger inhibitors, the effect of 29 indole and phenol structural analogues on *P. larvae* spore germination *in vitro* was tested. The compounds were tested at a concentration of 0.4 mM to identify germination inhibitors that are stronger than indole. We expected this compound screen to provide information regarding the functional groups that enhance inhibitor binding to germination receptor.

Inhibition of *P. larvae* spore germination by indole and phenol analogs

Indole consists of a six carbon benzene ring fused to a heterocyclic five-member ring similar to uric acid an agonist of *P. larvae* spore germination. To identify indole analogs that inhibited *P. larvae* germination, spores were treated individual indole analogs and subsequently with uric acid plus L-tyrosine (Figure 4.3). The indole analogs tested differed from the original molecule by a couple atom or functional modifications.

Substitution of the nitrogen in position 1 of indole with a sulfur group to form thionaphthene (compound 1) failed to significantly inhibit *P. larvae* spore germination. Addition of nitrogen to position 2 (compound 2), 2 + 3 (compound 3), 3 (compound 4), or 7 (compound 5) of indole also did not significantly reduce spore germination. Addition of a sulfur group to position 3 of indole did not significantly prevent *P. larvae* spore germination (compound 6). It was observed that position 1 of indole could only support nitrogen (compound 7, 13, and 19) and not any other atoms. In general, alterations to the atoms on the benzene or heterocyclic five-member ring reduced the effectiveness of indole as an inhibitor of *P. larvae* spore germination (compound 8, 9, 10, 11, 12, and 20).

It was previously shown that methylindole derivatives did not have improved anti-germination activity of indole. In this work, we observed that the addition of a bulky group to indole as seen in 3-ethanamineindole did not enhance indole inhibition. Addition of electron donating groups (amino and hydroxyl) reduced the inhibitory effect indole has on *P. larvae* spore germination (compounds 24 and 26).

P. larvae spore germination was significantly reduced by indole analogs with electron withdrawing groups (compounds 21-23, 25, 27, 28). Electron withdrawing groups (chloro, bromo, cyano, fluoro, and nitro) draw electrons from the benzene ring. Titrations of these indole analogues at saturating uric acid plus L-tyrosine concentrations were performed to obtain IC_{50}

values (Table 4.2). The maximum effect on *P. larvae* spore germination was observed on indole with halide groups. With 5-chloroindole and 6-chloroindole having the lowest IC_{50} measured with *P. larvae* spores (Figure 4.4). As expected the dose response assays resulted in sigmoidal curves, which passed the Durbin-Watson statistical test for autocorrelation.

None of the phenol analogs we tested inhibited *P. larvae* spore germination more than phenol (compounds 14, 15, 16, 17, and 18). In fact, any modification or addition to the benzene ring reduced *P. larvae* spore germination inhibition of phenol.

Indole analogs do not alter honey bee larvae development

Several standard methods were followed for rearing honey bee larvae in the laboratory (Peng, Mussen et al. 1992, Huang 2009, Crailsheim, Brodschneider et al. 2013). Larval survivorship was measured until the onset of pupation, i.e., after defecation was observed. In our hands, larvae fed worker jelly had mortality below 8%. The typical mortality for *in vitro* rearing reported by others is between 10-20% (Peng, Mussen et al. 1992, Huang 2009).

To test for toxicity, honey bee larvae were fed artificial worker jelly (WJ) containing 0.5 mM of each indole analog (Figure 4.5) through pupation. Larval survival was monitored every 24 hours. Under our experimental conditions the larval phase lasted 7 days. By 7 days, honey bee larvae defecated uric acid crystals and stringy material. Any larvae that had not defecated by 7 days were designated as dead. The only significant difference in survival curves was between larvae fed different 6-chloroindole (91%) and those fed the control diet (97%).

Indole analogs protect honey bee larvae from AFB disease.

Second instar larvae were fed approximately 880 spores during a 24 hour period. Honey bee larvae started dying two days after exposure to *P. larvae* spores (Figure 4.6). Honey bee larval survival was significantly higher for larvae fed spores (22%) than for larvae fed indole analogs plus spores (36-75%). The highest survival percentages were observed in larvae fed with

spores plus either 5-bromoindole (75%) or 5-chloroindole (65%). However, honey bee larvae fed indole analogs plus had a significantly lower larval survival for larvae fed control diet (97%).

5-chloroindole does not alter honey bee larvae development

Among the indole analogues tested, we focused on 5-chloroindole because it had the lowest IC_{50} value, is inexpensive, and highly soluble in water. To determine the highest concentration of 5-chloroindole that larvae could tolerate, larvae were fed artificial worker jelly (WJ) containing 0.5, 0.75, 1.0, or 1.5 mM of 5-chloroindole (Figure 4.7) through pupation. There were no significant differences in survival between larvae fed 0.5-1.0 mM doses of 5-chloroindole and those fed the control diet. However, at 1.5 mM 5-chloroindole 100% mortality was observed 24 hours after treatment (n=30).

5-chloroindole protects honey bee larvae from AFB disease.

Honey bee larval survival was significantly higher for larvae fed 5-chloroindole (27-55%) plus spores than for larvae fed spores alone (8%). The highest survival percentages were observed in larvae fed with 0.5 (51%) and 0.75 (55%) mM 5-chloroindole. However, honey bee larvae fed 5-chloroindole had a significantly lower larval survival than the larvae fed control diet (99%).

Antimicrobial activity of 5-chloroindole on *P. larvae* cells and spores

At 6, 12, 18, and 24 hours there were no significant differences in growth of *P. larvae* cells supplemented with the two controls, MYPGP or MYPGP plus dimethyl sulfoxide as a vehicle (DMSO). In the presence of 0.5 mM 5-chloroindole *P. larvae* cellular growth did not differ from MYPGP controls. However, *P. larvae* cellular growth decreased significantly when higher concentrations of 5-chloroindole (1.0, 1.5, 2.0 mM) were used. 5-chloroindole had an antagonistic effect of on *P. larvae* cells growing in MYPGP broth over a 24 hour period (Figure 4.8). Hence, we conclude that 5-chloroindole prevented *P. larvae* cellular growth *in vitro*.

P. larvae spores converted to vegetative cells over a 24 hour period in a medium supporting growth (Figure 4.10). When *P. larvae* spores were exposed to 5-chloroindole over a 24 hour period in complex medium no growth was obtained). After 18 hours, spores in MYPGP and MYPGP plus DMSO had significantly higher growth than other conditions. During the 24 hour period, spores in any of the 5-chloroindole (0.5, 1.0, 1.5, 2.0 mM) concentrations tested failed to germinate and produce detectable cellular growth.

4.5 Discussion

Molecular analogs had been previously used to understand how germination receptors interact with germinants (Abel-Santos, Dodatko 2007, Dodatko, Akoachere et al. 2010, Howerton, Ramirez et al. 2011). The strongest inhibitors of *P. larvae* spore germination were indole analogs with electron withdrawing groups. Electron withdrawing groups may have improved receptor interaction by displaying a negatively charged atom similar to uric acid. Uric acid has hydroxyl groups at positions 2, 5, and 7 that provide a negative charge. To inhibit *P. larvae* spore germination further, indole analogs with multiple electron withdrawing atoms at positions 2, 5, and 7 should be tested.

The majority of the compounds tested did not significantly reduce *P. larvae* spore germination *in vitro*. Changing the nitrogen or carbon atoms in indole rings with sulfur or nitrogen groups reduced the inhibitory effect of the molecule as a whole. Indeed, 13 of the 33 compounds tested differed from indole only by two atoms but were unable to inhibit *P. larvae* spore germination. These results suggest that the carbon and nitrogen groups of indole are essential for binding the putative germinant binding site.

When indole analogs are incorporated in worker jelly they are not toxic to honey bee larvae at a 0.5 mM final concentration. Furthermore, all of the indole analogs tested protected

honey bee larvae from AFB disease development. Thus, as previous studies suggest, indole and indole analogs can serve as protective compounds against infectious organisms (Ueno, Kihara et al. 2005, Kim, Lee et al. 2011). Unfortunately, none of the indole analogs tested completely prevented AFB in bee larvae. However, amongst the compounds identified in this compound screen 5-chloroindole had a strong effect against *P. larvae*.

The antagonistic activity of 5-chloroindole against *P. larvae* was evaluated with additional toxicity and exposure assays. 5-chloroindole can be incorporated into worker jelly daily at a 0.5, 0.75, and 1.0 mM concentrations without altering larval development even at the highest concentration. We expected 5-chloroindole not to be toxic to honey bee larvae because of the low concentrations tested.

The effect of indole analogues against the vegetative and spore stages of *P. larvae in vitro* was evaluated. Exogenous 5-chloroindole delayed *P. larvae* cellular growth during a 24 hour period at a 1.0, 1.5, or 2.0 mM concentration. Addition of 5-chloroindole to *P. larvae* spores in complex medium prevented spore outgrowth over a 24 hour period. A complex medium was used instead of uric acid plus L-tyrosine to provide germinating cells nutrients to grow. Thus, complex medium served as a surrogate to the nutritious environment in the larval gut. The inhibitory effect observed *in vitro* could serve to protect honey bee larvae from AFB disease development.

Developing a suitable inhibitor delivery system for honey bee colonies is the final step towards a potential disease treatment. Ideally the treatment substances would be ingested, be effective at low concentrations, and degrade prior to contaminating honey stores. Honey bees can be fed sugar water mixtures, sugar powder, or patties infused with treatments (Elzen, Westervelt et al. 2002, Yoder, Jajack et al. 2014). In the colony, both nectar and pollen are transferred from

foraging bees to bees performing nurse duties (Crailsheim, Schneider et al. 1992, Grüter, Farina 2007). Once inside the nurse bees the compounds can spread similarly as *P. larvae* spores spread to honey bee larvae (Gillard, Charriere et al. 2008, Lindström, Korpela et al. 2008). The compounds identified need to work at low concentrations so that the AFB treatment remains cost effective. The half-life of the compounds needs to be limited to weeks so that no residue remains in wax, honey, or pollen (Frison, Breitkreitz et al. 1999, Bogdanov 2006, Lopez, Pettis et al. 2008). Ultimately, in order to prevent AFB disease, compounds with lower toxicity and increased inhibitory effect on spore germination need to be synthesized.

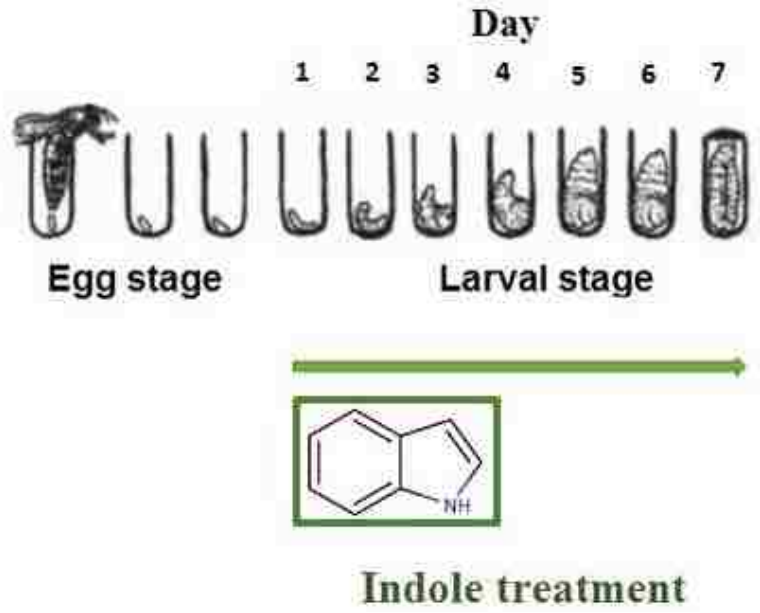


Figure 4.1: Chronic feeding of indole analogs to honey bee larvae. Indole analogs were fed to honey bee larvae until the onset of pupation. The larval diet was prepared daily in order to ensure indole analogs remained in solution.

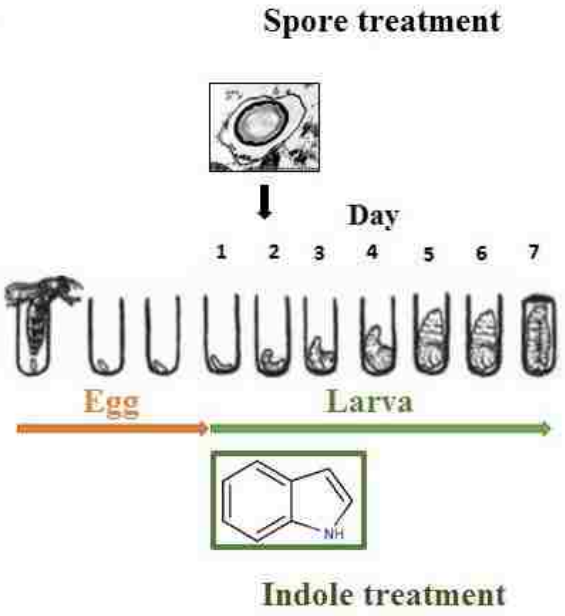


Figure 4.2: Honey bee larvae exposure assay. Second instar larvae were exposed to *P. larvae* spores during a 24 hour period. AFB disease development was monitored via microscopy. Larvae that developed AFB disease were consumed by *P. larvae* cells.

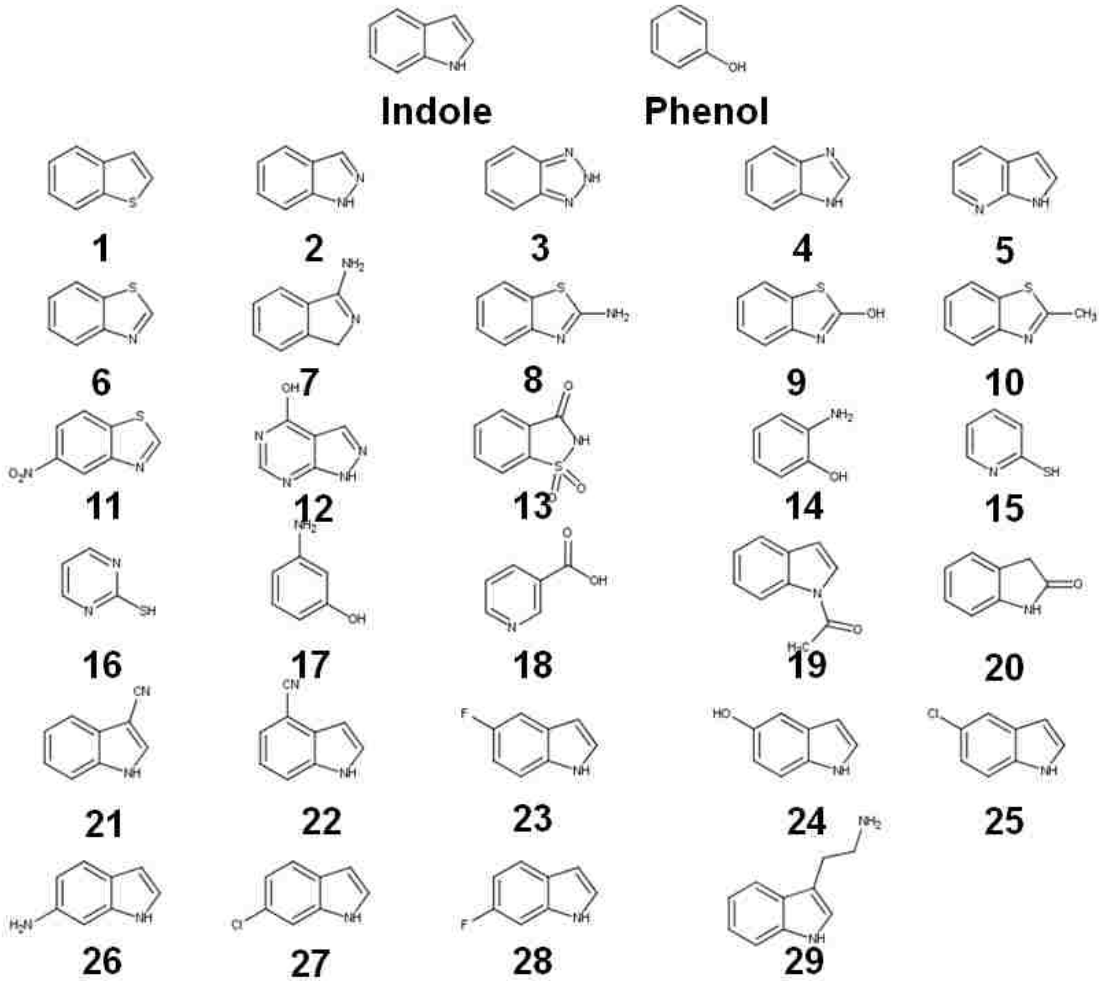


Figure 4.3: Compounds tested as antagonists of *P. larvae* spore germination. 1. Thionaphthene; 2. Indazole; 3. 1H-Benzotriazole; 4. Benzimidazole; 5. 7-Azaindole; 6. Benzothiazole; 7. 1H-Isoindol-3amine; 8. 2-aminobenzothiazole; 9. 2-Hydroxybenzothiazole; 10. 2-Methylbenzothiazole; 11. 6-nitrobenzothiazole; 12. Allopurinol; 13. Saccharin; 14. 2-Aminophenol; 15. 2-Mercaptopyridine; 16. 2-Mercaptopyrimidine; 17. 3-Aminophenol; 18. Nicotinic acid; 19. 1-Acetylindole; 20. 2-oxoindole; 21. 3-Cyanoindole; 22. 4-Cyanoindole; 23. 5-Fluoroindole; 24. 5-hydroxyindole; 25. 5-Chloroindole; 26. 6-Aminoindole; 27. 6-Chloroindole; 28. 6-Fluoroindole; 29. 3-Ethanamineindole.

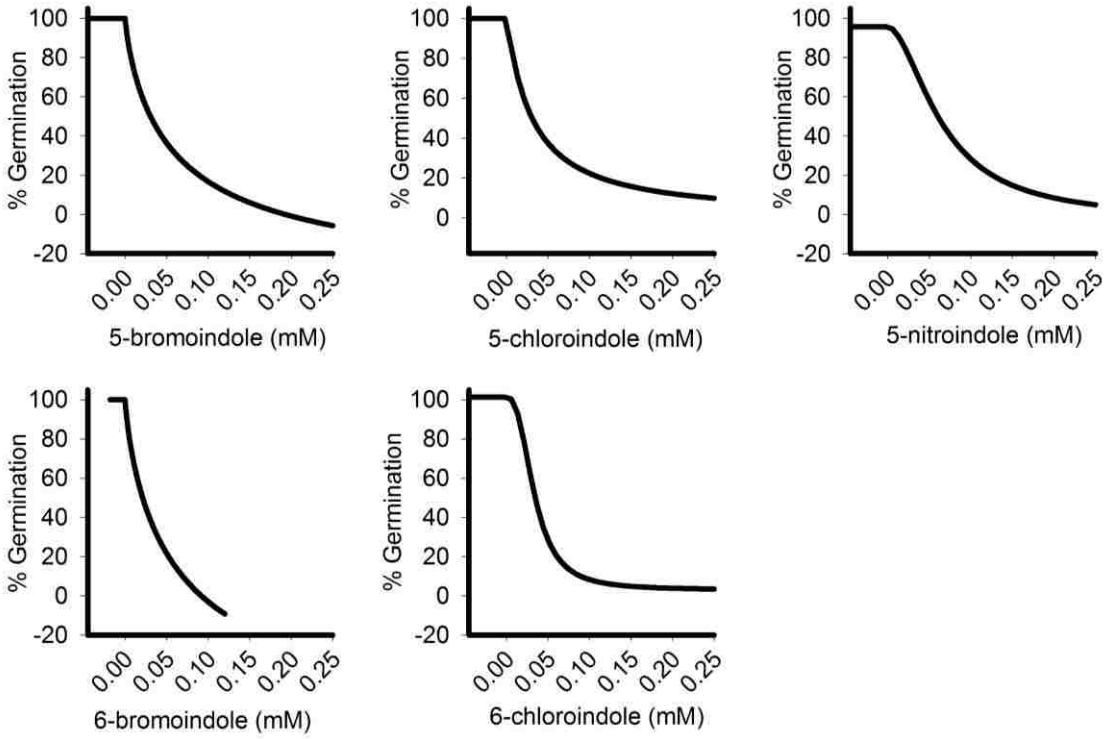


Figure 4.4: Calculation of 50% inhibitory concentration. Dose-response curve of *P. larvae* spores germinated with L-tyrosine–uric acid in the presence of various concentrations of indole analogs. The IC_{50} for indole analogs was determined based on these data.

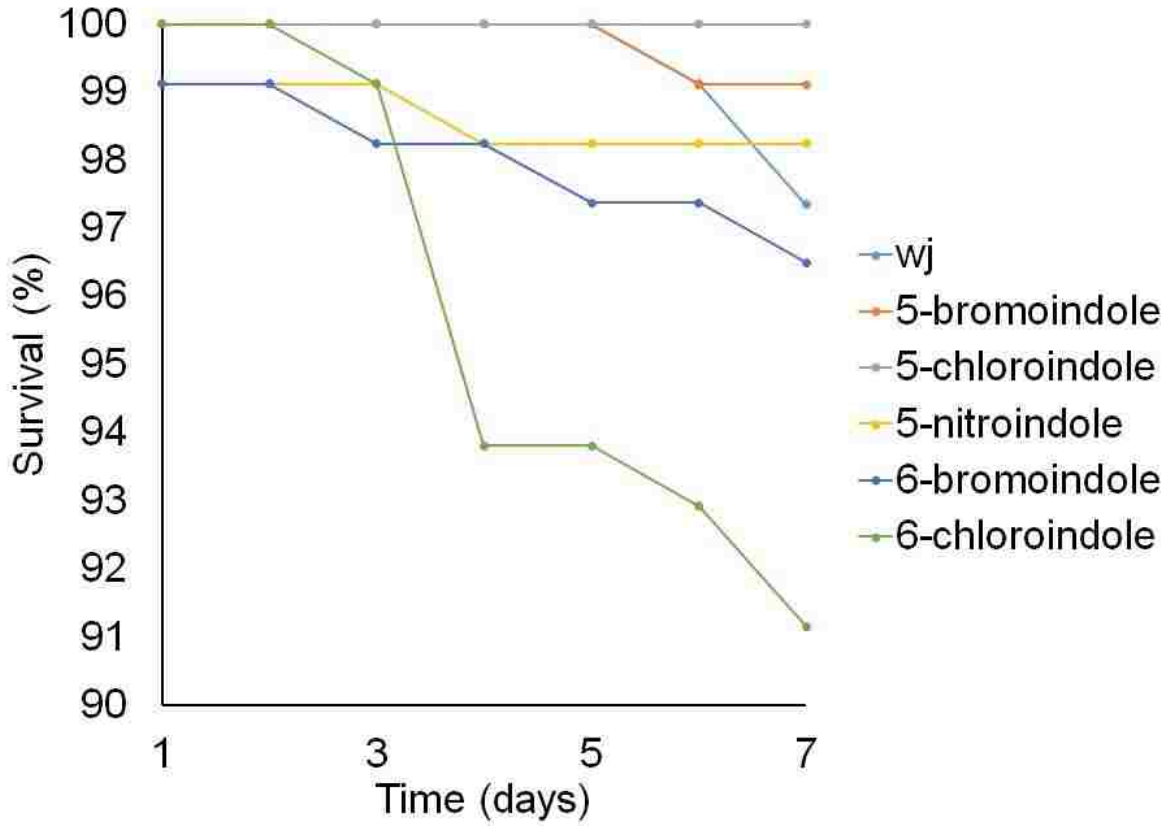


Figure 4.5: Effects of indole analogs on larval survival. Survival curves of honey bee larvae fed larval diet supplemented with indole analogs did not differ from control diet (wj). Differences between survival curves were determined using Kaplan-Meier analysis, Log-Rank test, and the Holm-Sidak method with significance level = 0.05.

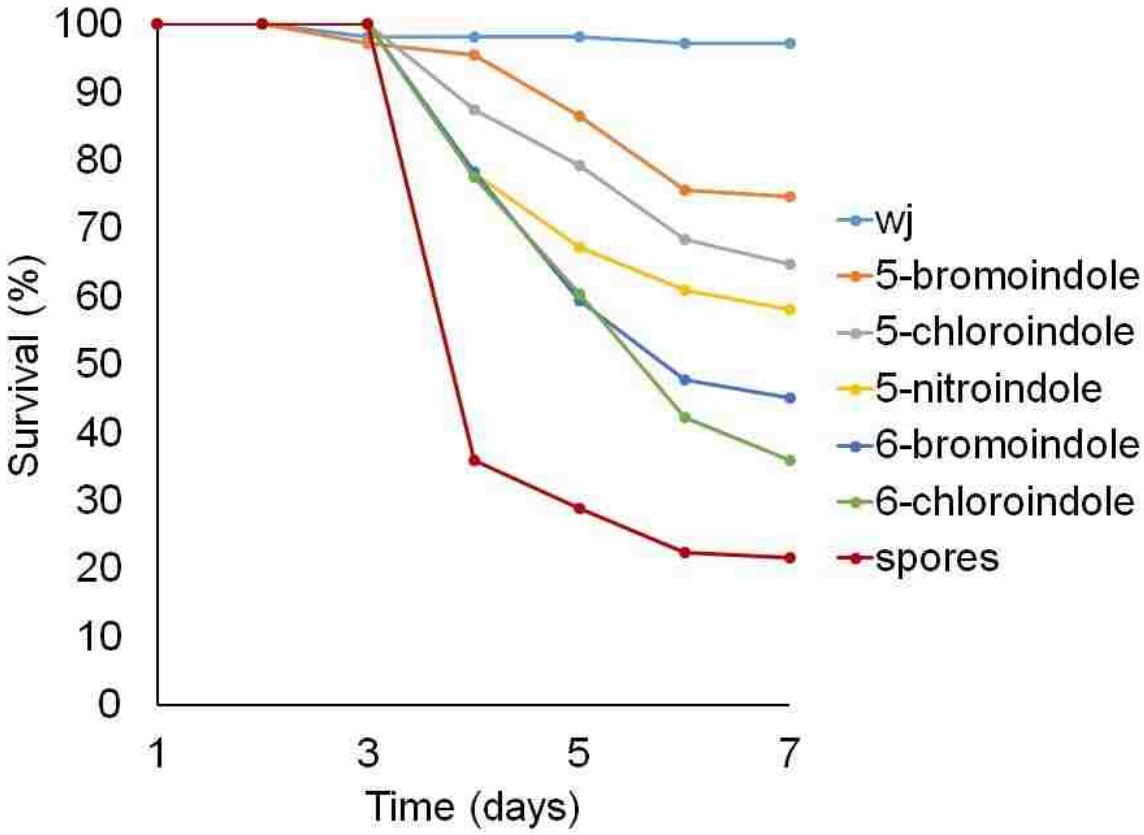


Figure 4.6: Effects of indole analogs on AFB disease in larvae. Survival curves of honey bee larvae fed larval diet (wj) supplemented with different indole analogs and *P. larvae* spores. Differences survival curves were determined to be significant using the Holm-Sidak method with significance level = 0.05.

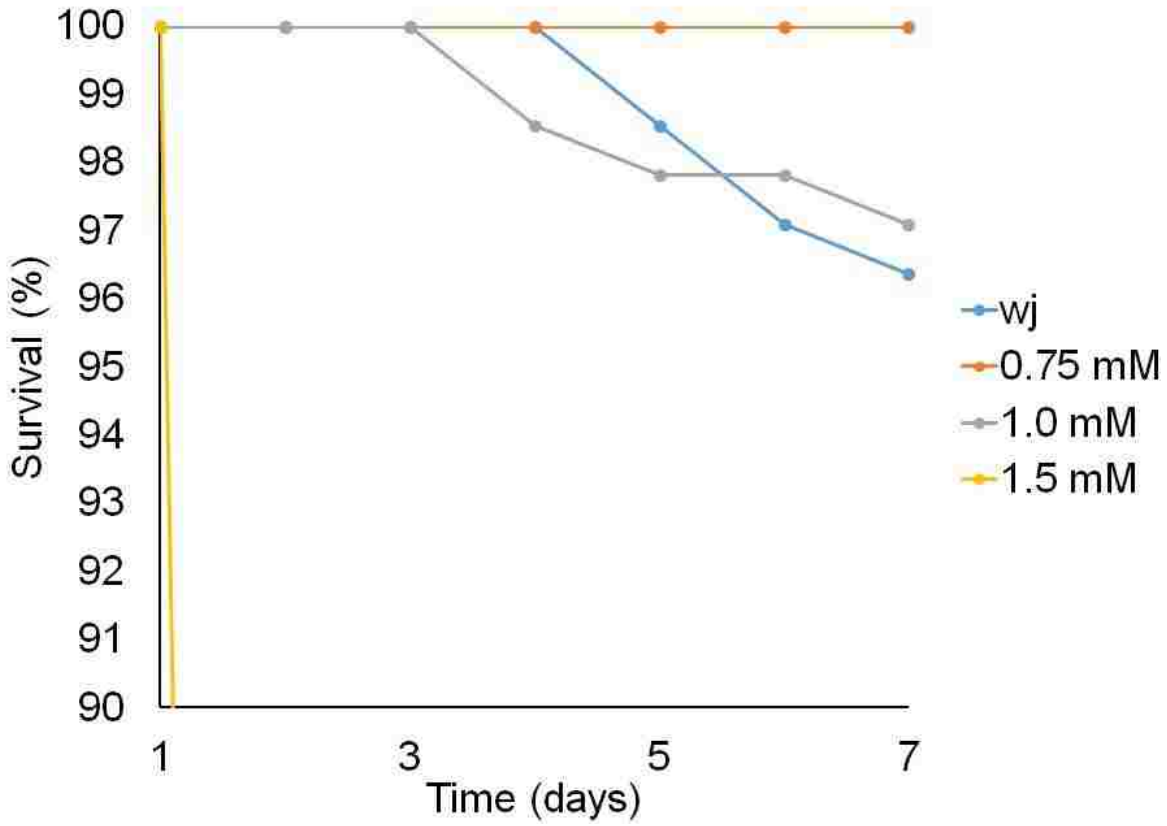


Figure 4.7: Maximum 5-chloroindole dosage larvae tolerate. Survival curves of honey bee larvae fed larval diet supplemented with different concentrations of 5-chloroindole did not differ from control diet (wj). Differences between survival curves were determined using Kaplan-Meier analysis, Log-Rank test, and the Holm-Sidak method with significance level = 0.05.

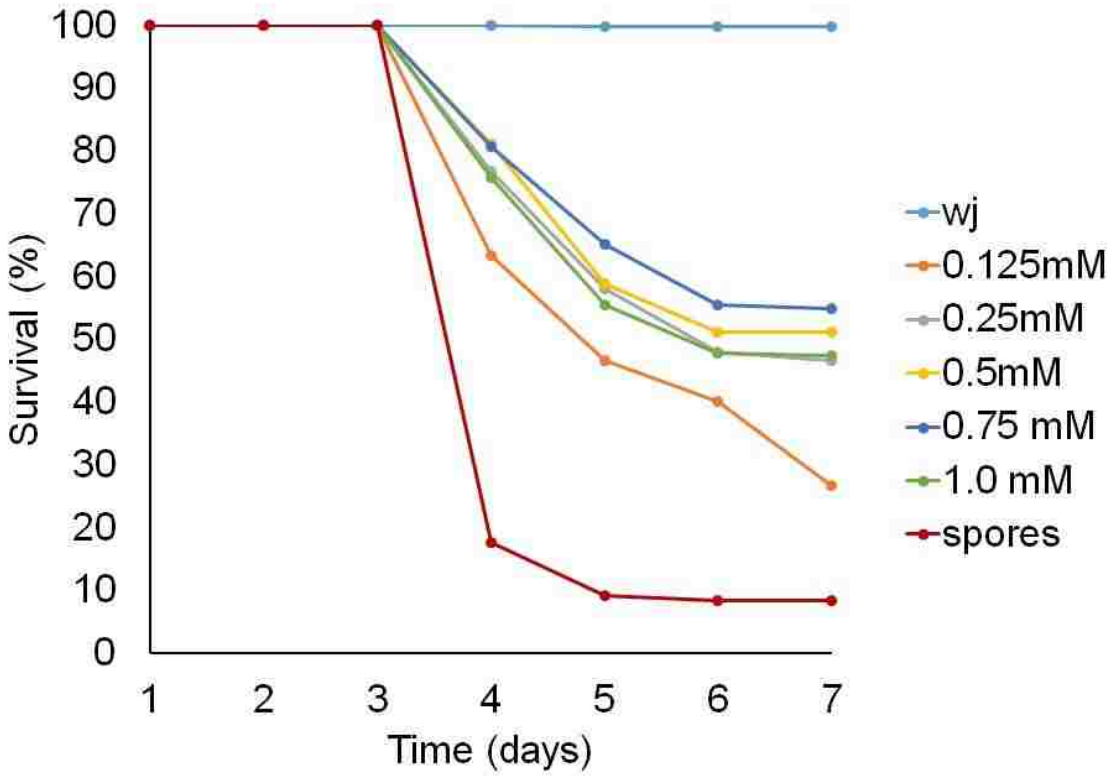


Figure 4.8: 5-chloroindole increases larval survival after *P. larvae* spore exposure. Survival curves of honey bee larvae fed larval diet (wj) supplemented with different concentrations of 5-Chloroindole and *P. larvae* spores. Differences between all 5-chloroindole concentrations and the spore treatments survival curves were determined to be significant using the Holm-Sidak method with significance level = 0.05.

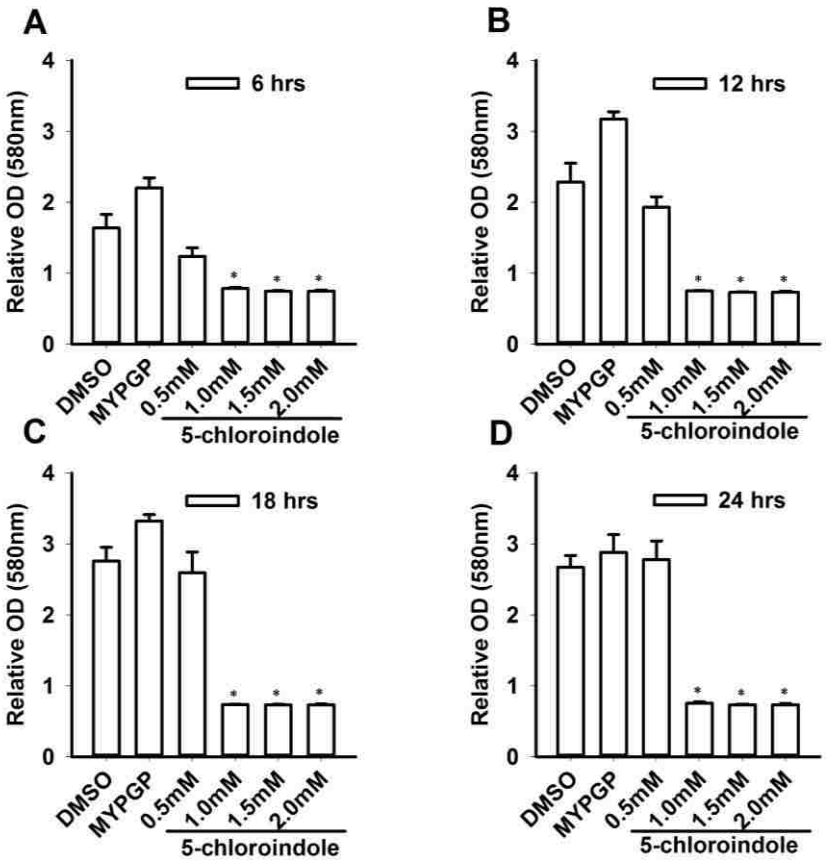


Fig. 4.9: Inhibition activity of 5-chloroindole on *P. larvae* cells. *P. larvae* cells were grown in medium containing different concentrations of 5-chloroindole. Cellular growth was monitored over a 24 hour period (A-D). Asterisks denote significant differences from the growth of medium control (Analysis of Variance, Log-rank test, $p<0.05$). Relative OD_{580} values are obtained by dividing each data point by the initial optical density (OD_{580} approximately = 0.19 at 0 hour).

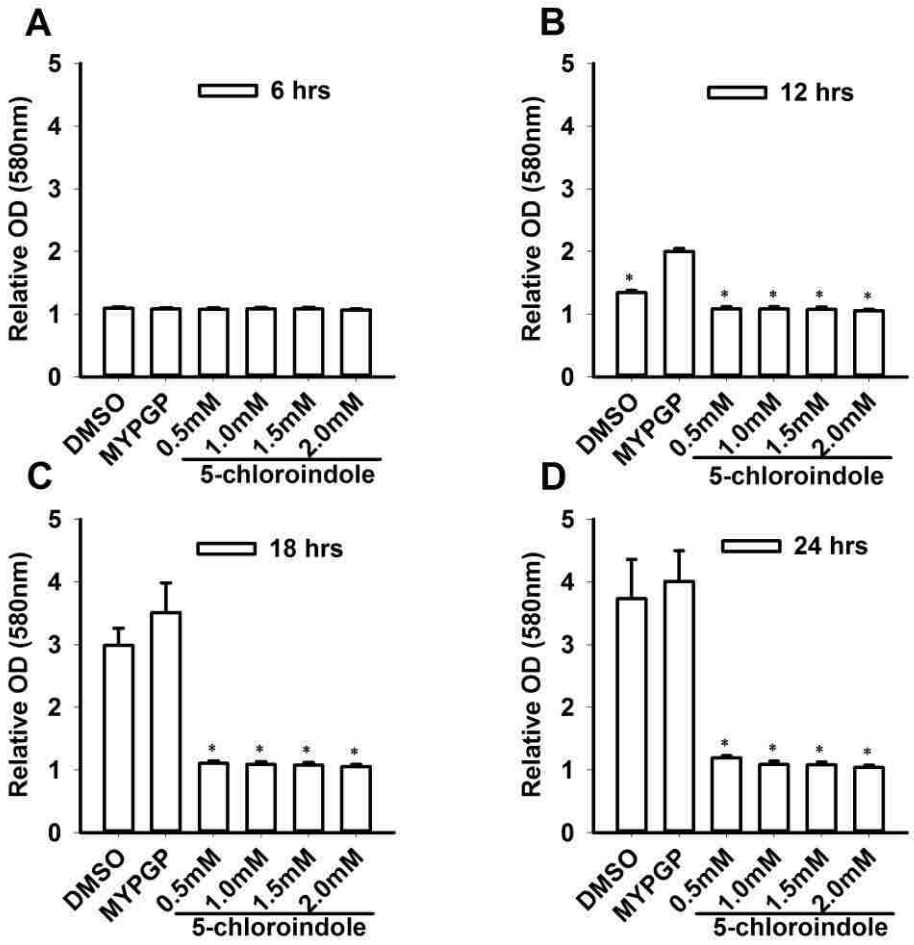
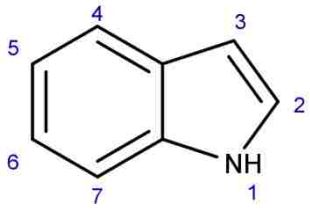


Figure 4.10: Inhibition activity of 5-chloroindole on *P. larvae* spores. *P. larvae* spores were grown in medium containing different concentrations of 5-chloroindole. Growth was monitored over a 24 hour period (A-D). Asterisks denote significant differences from the growth of the medium control (Analysis of Variance, Log-rank test, $p<0.05$). Relative OD_{580} values were obtained by dividing each data point by the initial optical density (OD_{580} approximately = 0.15 at 0 hour).

Table 4.1. Daily amounts and composition of artificial diet provided to larvae							
Day	Amount per well (µl)	Culture plates used (wells/ plate)	Number of larvae (larvae/ well)	% D-glucose (w/v)	% D-fructose (w/v)	% yeast extract (w/v)	% royal jelly (w/v)
1	2500	6	40	5.1	5.1	0.9	50
2	2500	6	40	5.1	5.1	0.9	50
3	250	48	1	6.4	6.4	1.3	50
4	30	48	1	7.7	7.7	1.7	50
5	40	48	1	7.7	7.7	1.7	50
6	50	48	1	7.7	7.7	1.7	50
7	60	48	1	7.7	7.7	1.7	50

Table 4.2: The effects of indole analogues on *P. larvae* spore germination. *P. larvae* spores were incubated with various concentrations of indole analogs for 15 minutes prior to addition of 3 mM L-tyrosine and 3 mM uric acid. IC_{50} was calculated by plotting percent germination versus indole analog concentration. Error represents standard error from at least six independent measurements.



Indole Analog	**IC_{50} (mM)**
Indole	0.37 ± .02
4-cyanoindole	0.20 ± .02
5-bromindole	0.06 ± .07
5-chloroindole	0.03 ± .01
5-fluoroindole	0.12 ± .02
5-hydroxyindole	0.55 ± .17
5-nitroindole	0.07 ± .01
6-bromoindole	0.06 ± .06
6-chloroindole	0.02 ± .00
6-cyanoindole	0.11 ± .01
6-fluoroindole	0.12 ± .01

Chapter 5: Expression of *Paenibacillus larvae* spore germination receptors

5.1 Abstract

Spores are dormant forms of bacteria resistant to extreme heat, desiccation and chemicals that are produced during the process of sporulation as a response to nutrient deprivation. During sporulation, germination receptors are incorporated within the inner membrane to allow spores to germinate and become active cells again under favorable environmental conditions. The genome of *P. larvae* strain B-3650 contains five loci with gene sequences that are similar to known *B. subtilis ger* and *prkC* germination receptors. The mRNA level of germination receptors in exponentially growing and sporulating cultures was measured. Germination receptor mRNAs for *gerKA 3* and *gerKA 4* were more abundant in sporulating than in growing cultures. Furthermore, no germination receptor mRNAs were detected in exponentially growing cultures. These results suggest that *P. larvae* spores exit dormancy due to detection of environmental cues via germination receptors. The identification of *P. larvae* spore germination receptors provides a basis for understanding how AFB disease begins in honey bee larvae.

5.2 Introduction

Sporulation, the differentiation of bacterial cells into dormant spores, occurs as a response to nutrient deprivation (Errington 1993, Errington 2003). Although sporulation has been studied in many species, the best understood model system for sporulation is *B. subtilis*. At the end of exponential growth of vegetative cells, the cells use accumulated resources to divide or form spores. Because sporulation occurs in conjoined cells, the execution of this process takes place in several phases. One cell provides resources for spore formation and is ultimately broken down, while the other cell is packed into a resistant coat, which is endowed with germination machinery. Finally, the cell enters dormancy. The physiological and biochemical properties of

the spore form during a period of several hours. To produce viable spores during this process, the transcriptional activation and deactivation of several gene sets is required. There are four sigma factors/proteins (σ^e, σ^f, σ^g, and σ^k) in *B. subtilis* that regulate transcription of sporulation specific genes. The expression of *ger* germination receptors and several other genes is regulated by the specialized sigma factor G (SigG/σ^g) protein, which is only active during sporulation. Localization of the germination receptors occurs during spore cortex formation (Errington 1993, Errington 2003).

The process of germination allows for spores to convert to metabolically active cells (Moir, Corfe et al. 2002, Peter 2003, Moir 2006). Germination receptors respond to environmental cues, germinants, and initiate the conversion to metabolically active cells. *Ger* germination receptors genes are normally encoded in transcriptional units (operons) consisting of three genes/subunits designated A, B, and C. Although isolated monocistronic units are also found. For example, the first germination receptor described GerA is composed of GerAA, GerAB, and GerAC subunits. All three subunits are necessary for germination receptor formation. Fourteen phylogenetically distinct germination receptors have been described including GerA, GerB, GerG, GerH, GerI, GerQ, GerK, GerL, GerM, GerR, GerS, GerU, GerX, GerY (Ross, Abel-Santos 2010a, Ross, Abel-Santos 2010b).

The PrkC receptor germination pathway, is triggered by the detection of muropeptides released from closely related growing bacteria (Shah, Laaberki et al. 2008). This pathway is analogous to bacterial quorum sensing in that it occurs in response to population density. Spores will only germinate if they detect a muropeptide signal released by cells. Even though the PrkC pathway has only been studied in *B. subtilis* and *B. anthracis*, PrkC proteins are found in over 75

bacterial species (Paredes-Sabja, Setlow et al. 2011). When chemical cues from the environment, i.e. germinants, bind to germination receptors it triggers spore germination.

Spore germination, the conversion of dormant spores to actively dividing cells, results in a loss of spore specific characteristics. Initially, germinated spores lose their resistance to wet and dry heat that would not kill dormant spores. As germination continues the spore's inner membrane becomes permeable to ions. Monovalent ions including H^+, Na^+, and K^+ are released from the spore as the membrane permeability increases. Then the calcium-dipicolinic acid (Ca-DPA) complex exits the spore core. Ca-DPA release activates hydrolysis of peptidoglycan in the spore cortex. Together movement of ions and hydrolysis of peptidoglycan allow for spore hydration (Peter 2003, Moir 2006, Paredes-Sabja, Setlow et al. 2011). In nature, the germination process is triggered by binding of nutrients to germination receptors.

The *ger* and *prkC* receptor sequences in the *P. larvae* genome are assigned a name and function based on homology to *B. subtilis* proteins (Chan, Cornman et al. 2011). In *B. subtilis* germination proteins were identified by studying germination mutants (Sammons, Moir et al. 1981). For example, mutations in any of the three genes in the *gerA* operon prevented *B. subtilis* spores from germinating in response to a known germinant, L-alanine. However, experimental tools to generate *P. larvae* gene mutants are not readily available.

There are very few molecular tools available to study the germination receptors of *P. larvae*. The effect of overexpression of germination receptors on germination rates of *P. larvae* spores has not been studied because of a lack of expression plasmids. In *B. subtilis*, it has been shown that overexpression of germination receptors increases germination rates (Cabrera-Martinez, Tovar-Rojo et al. 2003). Furthermore, only recently efficient introduction of plasmids into *P. larvae* cells has been improved through a new electroporation method (Murray, Aronstein

2008). The electroporation method replaced conjugation and polyethylene glycol based protoplast transformation methods that had low reported efficiencies (Bakhiet, Stahly 1985a). In 2012, the TargeTron mutagenesis system was used to create the first *P. larvae* gene mutants (Zarschler, Janesch et al. 2009). This system has allowed researchers to study virulence genes in *P. larvae*. Unfortunately, none of the published works have the experimental criteria to satisfy all of Koch's molecular postulates (Falkow 1988, Garcia-Gonzalez, Müller et al. 2014a, Garcia-Gonzalez, Müller et al. 2014b, Hertlein, Müller et al. 2014, Fünfhaus, Poppinga et al. 2013). There is currently no molecular tool to show reversion or replacement of mutated genes in *P. larvae*.

P. larvae sporulation is not as well characterized as it is in *B. subtilis*. However, the genome of *P. larvae* contains four putative *ger* and one *prkC* gene sequences that are homologous to *ger* genes in *B. subtilis (Chan, Cornman et al. 2011)*. Because *B. subtilis* receptor production is regulated by the sigma factor (σ^g), we expect *P. larvae* sporulation to work in a similar fashion. If this is the case, *P. larvae* likely produces Ger and/or PrkC receptor proteins that allow spores to germinate. By measuring the mRNA levels of *ger* and *prkC* genes, we can infer which receptors are expressed during sporulation.

In this work, methods necessary to study sporulation in *P. larvae* grown on MYPGP agar plates were developed. Germination receptor mRNAs were measured in growing and sporulating cultures via quantitative polymerase chain reaction (qPCR). The relative levels of two germination receptor mRNAs were higher in sporulating than in growing cultures. The expression of *ger* genes coincided with the expression of sporulation regulator *sigG* gene expression as expected.

5.3 Materials and Methods

Materials. Chemicals were purchased from the Sigma-Aldrich Corporation (St. Louis, MO) and VWR International (Radnor, PA). The dehydrated culture media were purchased from BD Difco (Franklin Lakes, NJ). *Paenibacillus larvae* ERIC group I strain B-3650/LMG 16245 was purchased from the Belgian Coordinated Collections of Microorganisms (BCCM).

Experimental design. *P. larvae* protein sequences were analyzed via structure prediction software to identify key germination receptor structural features. Three replicate *P. larvae* cultures were used to inoculate MYPGP plates (4 plates per culture). The inoculated plates were incubated and their growth was monitored over a 96 hour period. RNA was isolated from three *P. larvae* plates after 24 hours of growth. Similarly, RNA was isolated from three sporulating *P. larvae* plates after 48, 72, and 96 hours of growth. Thus, qPCR could be used to compare mRNA levels between growing (24 hour) and sporulating (48, 72, and 96 hour) time points. The relative expression of five putative germination receptor genes (*gerKA 1*, *gerKA 2*, *gerKA 3*, *gerKA 4*, and *prkC*), a sporulation specific gene (*sigG*), and a housekeeping gene (*rpoB*) was measured. The qPCR workflow followed standard methods including: DNase I digest of gDNA, cDNA synthesis, primer design, primer PCR test, qPCR, and data analysis.

Identification of *P. larvae* B-3650 germination receptors. *P. larvae* strain B-3650 was used because its genome had been sequenced previously (Chan, Cornman et al. 2011). The gene coding sequences (CDS) for this *P. larvae* strain have been deposited on Pathosystems Resource Integration Center (PATRIC) website (Wattam, Abraham et al. 2014). The *P. larvae* feature table was searched to identify CDS related to sporulation, germination, and housekeeping genes (Table 5.1). The sporulation specific sigma factor G (*sigG*) gene was identified using a BLAST search of *P. larvae* DNA sequences (Chan, Cornman et al. 2011).

Published BLAST searches of genomic DNA sequences of *P. larvae* resulted in the identification of four distinct Ger germination receptor sequences (Chan, Cornman et al. 2011). There are two genome sites that encode putative canonical tricistronic Ger operon (containing A, B, and C-subunits). There is also one orphan germination receptor A-subunit and a truncated orphan A-subunit in the *P. larvae* genome. Finally, a eukaryotic-like serine/threonine kinase (*prkC*) germination receptor was also identified using this published BLAST-based approach (Figure 5.1) (Chan, Cornman et al. 2011). The PrkC protein has a conserved kinase and penicillin-binding protein and serine/threonine kinase associated (PASTA) domain similar to the protein identified in *B. subtilis* (Shah, Laaberki et al. 2008).

A BLAST search identified an RNA polymerase beta subunit (*rpoB*) gene within the *P. larvae* genome. In *B. subtilis* and *C. difficile*, the *rpoB* gene is considered a housekeeping gene whose expression should not change during sporulation (Fimlaid, Bond et al. 2013). Thus *P. larvae* sporulation and germination gene expression was normalized relative to *rpoB*.

Amino acid sequences were pasted into OCTOPUS a program that predicts membrane protein topology and signal peptides (Viklund, Elofsson 2008). Furthermore, the amino acid sequences were uploaded to RaptorX a protein structure prediction server (Källberg, Wang et al. 2012).

Media preparation: TMYGP consists of 15 g yeast extract, 4 g glucose, 1 g sodium pyruvate, 7.1 g Tris-maleate per liter of medium (pH 7.0 ± 0.2). MYPGP broth consists of 10 g Mueller-Hinton broth, 15 g yeast extract, 3 g K_2HPO_4, 2 g glucose, and 1 g sodium pyruvate per liter of medium (pH 7.2 ± 0.2). Media were autoclaved at 121°C and 15 PSI for a minimum

sterilization time of 30 minutes. Glucose was filtered sterilized and added to media prior to use. For solid media (MYPGP agar) 20 g of agar were used per liter of medium.

***P. larvae* strain B-3650 sporulation in TMYGP broth.** *P. larvae* B-3650 was grown aerobically on MYPGP agar plates for 2 days at 37°C. A single colony from the MYPGP plate was used to inoculate 8 ml of MYPGP broth and it was incubated overnight at 37°C and 195 rpm. TMYGP broth medium was inoculated with a 10% inoculum of the overnight culture. The turbidity of the cultures was monitored by optical density readings at 580 nm. Spore formation in cultures was monitored using the Schaeffer-Fulton staining method (Schaeffer, Fulton 1933).

***P. larvae* strain B-3650 sporulation on MYPGP agar plates.** *P. larvae* B-3650 was grown aerobically on MYPGP agar plates for 2 days at 37°C. A single colony from the MYPGP plate was used to inoculate 2 ml MYPGP broth, then incubated over 4.5 hours at 37°C and 195 rpm. MYPGP agar plates were spread plated with 100 µl of a culture with an approximate optical density A_{580} of 0.01. The inoculated plates were incubated for 5 days at 37°C in an incubator at 5% CO_2. Spore formation in cultures was monitored using the Schaeffer-Fulton staining.

***P. larvae* RNA extraction with lysozyme/SDS/Trizol Reagent.** Total RNA was isolated from three plate cultures at 24, 48, 72, and 96 hours after plating. A three step protocol was used to obtain total RNA from *P. larvae* grown on MYPGP agar plates (Ambion & Invitrogen protocols). Initially, sporulating bacterial lawns were collected from MYPGP plates by flooding with 5 ml of a solution of 10 mM Tris HCl and 0.1 mM EDTA in RNase free water (Ambion, Austin TX). Sporulating cells were pelleted by centrifugation at 3082 g for 10 minutes at 4°C. The supernatant was discarded and the pellet was resuspended in 1 ml of a lysis solution containing 10 mM Tris HCl, 0.1 mM EDTA, 10 mg/ml lysozyme, and 0.1% SDS in RNase free

water (Ambion, Austin TX). Cells were incubated with the lysozyme-SDS solution at room temperature for 5 minutes.

Next, a 5 minute homogenization step using a pulsing vortex bead mixer (set to the maximum speed of 3200 rpm) was performed at room temperature in the presence of 200 mg 100 µm zirconia beads and 1 ml Trizol reagent (Invitrogen, Carlsbad CA). The sample was incubated for 5 minutes at room temperature and 0.2 ml chloroform was added and mixed by inversion for 15 seconds. The sample was incubated for 3 minutes at room temperature and then centrifugation at 12,000 g for 10 minutes at 4°C. The centrifugation step allows for separation of the organic and aqueous phases. The aqueous phase was moved to a new tube and two additional chloroform extraction steps were performed. Afterwards, RNA was precipitated from the aqueous phase with one volume of isopropanol at room temperature. The RNA was pelleted by centrifugation at 12,000 g for 10 minutes and 4°C. One 70% ethanol wash step was performed to remove contaminants. RNA was resupended in RNA free water and stored at -82°C until use.

DNase I treatment to remove genomic DNA contamination from RNA. The extracted RNA was treated with a Turbo DNA-free kit (Ambion, Austin TX). To each RNA sample, 0.1 volume of Turbo DNase I buffer and 1-4 µl of DNase I were added. Samples were incubated at 37°C for 30 minutes to remove contaminating DNA. After sample incubation 0.1-0.4 volumes of the Ambion inactivation reagent were used to remove divalent cations and DNase I enzyme. Centrifugation at 10,000 g for 1.5 minutes was performed to separate RNA from Turbo DNase I components. The supernatant containing RNA was transferred to a new tube and RNA was precipitated with 2.5 volumes of reagent ethanol and 0.1 volumes of 3 M sodium acetate (pH 5.2). The pellet was washed with 70% ethanol to remove salts and contaminants. RNA was resuspended in RNase free water and stored at -82°C prior to use.

RNA quantification and quality control. The RNA concentration was determined using either a NanoView (GE, Piscataway NJ) or the NanoDrop 1000 (Nanodrop, Wilmington DE) spectrophotometer. The spectrophotometer provided RNA concentrations (ng/µl), A_{260}/A_{280}, and A_{260}/A_{230} ratios. Quality RNA has an A_{260}/A_{280} ratio of approximately 2.0 and an A_{260}/A_{230} ratio ranging from 2.0-2.2 (Thermo Scientific, Wilmington DE).

Minus reverse transcriptase (RT) PCR reactions were performed with 2.5 ng of DNase I treated RNA following the same qPCR reaction setup. A minus RT PCR reaction consisted of 5 µl of RNA template at a final concentration of 0.5 ng/µl, 0.4 µM primer pairs, 12.5 µl EconoTaq DNA polymerase mix (Lucigen, Middleton WI), and water to a final volume of 25 µl. A 4 µl aliquot from the Minus RT PCR reaction was visualized using a 1% agarose gel stained with ethidium bromide (0.5 µg/ml). RNA contaminated with genomic DNA would yield a visible PCR product on an agarose gel. Only RNA free from genomic DNA was used for complementary DNA (cDNA) synthesis.

cDNA synthesis from DNase I treated RNA. The qScript cDNA synthesis kit (Quanta Biosciences, Gaithersburg MD) was used for mRNA quantification using quantitative PCR. The cDNA synthesis kit contains random primers that allow for first-strand synthesis from a broad range of RNA templates. A maximum of one microgram of RNA was used in a reverse transcription reaction to produce first strand cDNA. The cDNA synthesis step was assumed to have 100% efficiency. Thus one microgram of RNA was assumed have converted to one microgram of cDNA.

qPCR primer design. PrimerQuest, a program from Integrated DNA Technologies (IDT, Coralville IA), was used to design primers for qPCR. The sequence for the four *gerA*-subunits, *prkC*, *sigG*, and *rpoB* genes were provided to the program. The amplicon size was

limited to below 200 bases as suggested by PerfeCta SYBR Green SuperMix instructions. The primer sequences were checked for specificity using NCBI's Primer-Blast program. Primer-Blast determines if primer pairs will amplify more than one sequence in an organism's genome. Primer specificity was also measured by conducting PCR with each primer pair using genomic DNA as a target. Amplified samples were detected on an agarose gel and visualized with ethidium bromide. The primers were approximately 20 bases long and had a melting temperature of 57-58°C (Table 5.2).

qPCR reaction setup. cDNA, genomic DNA, and water were used as templates for qPCR reactions (Figure 5.2). To measure mRNA abundance, 25 ng of cDNA was used as template for qPCR reactions. For positive controls, 0.125 ng of genomic DNA was used as template because this resulted in reliable amplification of genes of interest. For negative controls, nuclease free water was used as template. A qPCR reaction consisted of 5 µl of template, 0.4 µM of primer pairs, 12.5 µl PerfeCta SYBR Green SuperMix (Quanta Biosciences, Gaithersburg MD), and water to a final volume of 25 µl. mRNA abundance was measured during each time point in triplicate with at least three different RNA isolations.

Bio-Rad iQ iCycler qPCR program. The Bio-Rad iQ iCycler qPCR program was setup using the PerfeCta SYBR Green SuperMix guidelines (Table 5.3). An initial heat activation step is required to denature the antibodies that maintain the AccuStart Taq DNA polymerase inactive (cycle 1). Next, 40 cycles of a standard three step (denaturation, annealing, and extension) PCR protocol were used to amplify cDNA (cycle 2). Fluorescence was measured during every extension step of PCR.

A melt curve analysis was performed to measure the number of DNA fragments produced during PCR amplification (cycle 5). The temperature changed from 55° to 94°C by

0.5°C increments while relative fluorescence was measured. Initially, at 55° C all of the PCR products were bound by fluorescent dye (SYBR green) and maximum fluorescence is measured. As the temperature increased, the fluorescence detected decreased proportionally. After the melt curve analysis was completed the IQ iCycler qPCR program provides a delta fluorescence/delta temperature versus temperature graph. If the qPCR primers are specific, then a single peak is observed in the melt curve graph.

***P. larvae* qPCR gene analysis.** For each gene of interest, mRNA abundance in 12 RNA samples was measured via qPCR in triplicate. The presence of four *ger* and one *prkC* mRNA transcripts was analyzed. To confirm that the isolated RNA belonged to sporulating cultures the mRNA levels for sigma factor G (σ^g) were measured. The sigma factor G (σ^g) gene encodes for a protein that promotes transcription of sporulation specific genes. *SigG* gene should be transcribed at approximately the same time as *ger* and *prkC* genes. The *rpoB* gene that encodes the β subunit of bacterial RNA polymerase was used to normalize the gene expression data generated.

The qPCR results were analyzed via the delta-delta C_t method that assumes a real-time PCR reaction efficiency of 100% (Schmittgen, Livak 2008). Initially, the delta C_t for the experimental group is calculated (C_t target gene - C_t reference gene). Next, the delta C_t for the control group is calculated (C_t target gene - C_t reference gene). Finally, the fold change is calculated by comparing the delta C_t for the experimental and control groups ($2^{-(\text{Experimental delta Ct-Control delta Ct})}$). A one way ANOVA was used to detect statistical differences between experimental groups.

5.4 Results

Analysis of genes encoding germination receptors in *P. larvae*

Four sequences in the genome of *P. larvae* are related to the *gerKA* gene of the *B. subtilis* (Chan, Cornman et al. 2011). The GerKA subunit in *B. subtilis* and three of the GerKA *P. larvae* are predicted to have five or six membrane spanning domains along with N- and C-terminal hydrophilic domains (Ross, Abel-Santos 2010a). To predict protein structure and function of *P. larvae* GerKA subunits the amino acid sequences were analyzed using protein prediction websites.

Initially, Octopus, a program that predicts membrane topology and signal peptides was used (Käll, Krogh et al. 2004, Viklund, Elofsson 2008). According to Octopus both *P. larvae* and *B. subtilis* germination receptors contain multiple transmembrane-helices. The GerKA subunit of *B. subtilis* and three of the *P. larvae* GerKA subunits are predicted to contain four transmembrane-helices and one predicted to contain five transmembrane-helices (Figure 5.3). All of the transmembrane-helices are located near the C-terminus of the protein. Additionally, Octopus predicted that the N- and C-terminal domains of the GerKA subunits analyzed can be found outside the membrane. In contrast to the other GerKA subunits analyzed, *P. larvae* GerKA 3 appeared to be a truncated protein with a smaller soluble domain than other proteins analyzed. Together these Octopus results suggest *P. larvae* GerKA 1, 2, and 4 are intact proteins.

The expected similarity between GerKA in *B. subtilis* and *P. larvae* proteins was also confirmed with RaptorX (Figure 5.4). The predicted 3D structures provided by RaptorX, indicate that all *P. larvae* GerKA subunits share a common shape with GerKA from *B. subtilis*. As Octopus indicated, all of the GerKA proteins have helices near the C-terminus that are buried in a membrane. However, RaptorX predicts that *P. larvae* and *B. subtilis* germination receptors have five helices instead of four as Octopus predicted. Moreover, at the C- and N- terminus GerKA proteins form structures that are exposed to the environment. These soluble domains are

absent in the truncated GerKA 3. Because *P. larvae* GerKA proteins contain similar domains as the *B. subtilis* GerKA protein they are likely to play a role in spore germination.

P. larvae contains a gene that is similar by BLAST to a eukaryotic-like serine/threonine kinase gene (*prkC*) that codes for a protein that signals spores to exit dormancy in response to peptidoglycan fragments in *B. subtilis*. The *P. larvae* PrkC protein is predicted to contain an N-terminal kinase domain, a membrane spanning domain, and three penicillin-binding protein and serine/threonine kinase associated (PASTA) domain. RaptorX and Octopus protein were used to analyze the *B. subtilis* and *P. larvae* PrkC amino acid sequences (Figure 5.5). Both PrkC protein sequences consist of two domains that are related to the serine/threonine kinase PrkC from *Staphylococcus aureus*. Octopus detected a membrane spanning domain characteristic of PrkC proteins studied. The cytoplasmic (N-terminus) and extracellular (C-terminus) domains of PrkC proteins were also detected correctly with Octopus. The results from RaptorX and Octopus protein prediction programs indicate that the PrkC protein is the *P. larvae* genome is likely functional.

qPCR primer specificity

PCR amplification with the primers designed (Table 5.1) and genomic DNA template was performed. All of the designed primer sets produced one product as seen on an agarose gel (Figure 5.6). The PCR products were approximately 200 bp long as expected. A melt curve analysis was also performed to check PCR specificity. The melt curve for all of the genes analyzed was a single sharp peak indicating a single PCR product (Figure 5.7). No primer dimers were detected in the sample lacking template.

***P. larvae* growth and sporulation in TMYGP broth**

To assess the expression of *P. larvae* strain B-3650 germination receptors, sporulation studies were first performed with TMYGP liquid cultures. *P. larvae* strain B-3650 had been previously shown that sporulate in TMYGP broth at a level of up to 1000 times more ($5x10^8$ spores per milliliter) spores relative to previously used liquid media (Dingman 1983, Dingman, Stahly 1983). This corresponds to approximately 68% of the bacterial population being capable of sporulation.

In our hands, less than 1% of cells were able to sporulate in TMYGP medium (determined by microscopic analysis of bacterial smears stained using Schaeffer Fulton method). One explanation for the sporulation efficiency differences could be medium composition. TMYGP is composed of three compounds (sodium pyruvate, glucose, tris-maleate) and yeast extract. The compounds purchased (sodium pyruvate, glucose, tris-maleate) had a percent purity above 99% based on manufacturer analyses indicating purity was not an issue. As previously observed in the Abel-Santos laboratory, the choice in yeast extract could alter sporulation in different species (data not shown). Four different brands of yeast extract were tested as supplements to TMYGP medium. However, changing yeast extract did not increase sporulation percentages in TMYGP medium.

***P. larvae* growth and sporulation of on MYPGP agar plates**

P. larvae strain B-3650 grew and sporulated well on MYPGP agar plates when incubated in a 5% CO_2 atmosphere. MYPGP plates were utilized instead of Tryptic Soy agar (Chapter 3) because *P. larvae* sporulation had been previously characterized using this medium (Dingman 1983). *P. larvae* made confluent lawns on MYPGP agar. The Schaeffer-Fulton endospore staining method allowed us to follow the sporulation process by microscopy (Figure 5.8). At 24 hours, only vegetative bacterial cells could be detected on MYPGP agar plates. At 48 hours,

sporulating cells could be detected along with vegetative cells. At 72 hours, the majority of the cells were sporulating. At 96 hours, the majority of the cell mass on plates were fully formed spores and very few cells.

RNA extraction from *P. larvae* grown on MYPGP agar plates

The RNA extracted using the lysozyme, SDS, zirconium beads, and Trizol protocol was of high quality. The average 260/280 ratio was 1.98 indicating RNA samples were pure nucleic acids. The average 260/230 ratio was 1.57 which was lower than expected indicating RNA samples contained some chaotropic salts, organic molecules, or proteins. However, the 260/230 ratio was high enough to conduct downstream analyses according to personal communication with UNLV genomics core. RNA samples were treated with DNase I following the rigorous treatment instructions. After DNase I digestion an RNA precipitation and an ethanol wash step was performed to remove PCR contaminants. For cDNA synthesis 500 ng and 1000 ng of RNA were then utilized.

Quality control of RNA and cDNA from *P. larvae* grown on MYPGP agar plates

A quality control test with RNA and cDNA from *P. larvae* prior to conducting qPCR assays was performed. Using 2.5 ng of cDNA and RNA as a template PCR was performed with *rpoB* primers. PCR products were produced from cDNA template from the 24, 48, and 72 hour time points (Figure 5.9). However, PCR products were also detected in the RNA samples from the 48 hour time point indicating genomic DNA contamination (these RNA samples were not used). To remove genomic DNA contamination from RNA samples a rigorous DNase I treatment was performed. Eight units of the DNase I were used, capable of degrading 8 μg of DNA in 10 minutes, to remove gDNA contamination from a new RNA aliquot. A second quality control test

with RNA and cDNA was performed after DNase I treatment. In the end, all of the RNA used was not contaminated by genomic DNA and was used to make cDNA.

qPCR assays using cDNA from *P. larvae* grown on MYPGP agar plates

qPCR assays were performed a with cDNA made from our cultures at 24, 48, 72, and 96 hour time points. The PCR amplification vs cycle number graph and melt curve indicated our qPCR assays were successful. Amplification was seen amongst most technical replicates and in all positive controls. Furthermore, the melt curves indicate the amplification of a single product within each PCR reaction. qPCR data was analyzed using the delta delta Ct method to measure the target gene expression patterns (Schmittgen, Livak 2008). The 24 hour time point was used as the control group because only bacterial cells were observed during microscopic analysis of cultures. The 48, 72, and 96 hour time points were treated as the experimental groups.

Sigma G is a protein responsible for the expression of many spore specific genes including germination receptors. The expression of *sigG* was greater in experimental groups (48, 72, and 96 hours) than in the control group at 24 hours of growth (Figure 5.10). In fact, *sigG* expression was approximately 16-44 times greater in experimental cultures than in control cultures. *SigG* gene expression data correlates with the appearance of sporulating cells at 48, 72, and 96 hours but not in the 24 hour control culture as observed via microscopy.

The relative abundance of germination receptor mRNAs was higher in experimental cultures than in control cultures (Figure 5.11). For *P. larvae gerKA 1* and *gerKA 2* receptors, the average fold change measured was approximately 1 indicating the mRNA levels did not change amongst cultures. The levels of *gerKA 3* and *gerKA 4* mRNA were 5-37 times higher in sporulating cultures than in control cultures. Thus, *ger* gene expression correlated with higher *sigG* mRNA expression in *P. larvae*.

The PrkC germination receptor allows *B. subtilis* spores to exit dormancy in response to muropeptide. The levels of PrkC germination receptor in growing and sporulating *P. larvae* cultures was examined. At the time points analyzed the expression of *prkC* was approximately equal to 1 indicating no transcription increase during sporulation (Figure 5.12).

Discussion

Transitioning from cells to spores for *P. larvae* strain B-3650 did not occur readily in TMYGP broth as previously described (Dingman, Stahly 1983, Dingman 1983). This could be due to "domesticating" the organism to a laboratory setting. Indeed, another *P. larvae* strain obtained from a microbial stock center lost the ability to form spores after more than 30 years in the laboratory (Dingman 1983, Dingman, Stahly 1983). Nonetheless, the reason for the difference in sporulation efficiency observed for *P. larvae* strain B-3650 is not clear.

The inability of *P. larvae* to form spores in liquid cultures limited this study. Comparison of this work to any previous sporulation studies that utilized liquid cultures was thus difficult (Dingman, Stahly 1983, Errington 1993, Igarashi, Setlow 2006). Additionally, the onset and abundance of sporulation gene mRNAs as previously determined using synchronized liquid cultures.

Sporulation of *P. larvae* on MYPGP agar plates was used because it proceeds as expected and with large number of spores produced. As expected, *sigG* was transcribed during sporulation. The levels of *sigG* in *P. larvae* were high as those seen in *B. cereus* (140 fold) (de Vries, Hornstra et al. 2004). Additional time points need to be made to monitor induction and loss of *sigG* mRNAs. The expression of *sigG* indicates the induction of germination receptor gene expression (Errington 1993, Errington 2003).

The levels of the five *P. larvae* germination receptors ranged between 1-37 fold in experimental versus control growing cultures. *GerKA 3* expression was significantly higher in all experimental cultures than in the controls. *GerKA 4* expression was significantly higher in experimental cultures (48 and 96 hours) than in controls. Transcription levels of *P. larvae* germination receptors are consistent with those seen in other studies (Igarashi, Setlow 2006, Madslien, Granum et al. 2014). The number of time points used here are not sufficient to detect changes in germination receptor mRNAs observed by other researchers (Igarashi, Setlow 2006). In *B. subtilis* the relative levels of germination receptor mRNAs fluctuate during the sporulation process. The initial peak in the levels of *B. subtilis ger* mRNAs have been shown to occur 3.5 hours after the onset and prior to the end of sporulation based on dipicolinic acid (DPA) concentration. Thus, additional RNA extractions are required to determine *P. larvae* receptor mRNA levels during sporulation. The concentration of dipicolinic acid (DPA) will be measured to identify the start and end of sporulation.

In conclusion, the relative abundance of germination receptor mRNAs in *P. larvae* was measured via qPCR. Gene expression studies were conducted on RNA isolated from *P. larvae* grown on MYPGP plates. The expression of *P. larvae* germination receptors follows the developmental model first described in *B. subtilis* but our work is complicated by non-synchronous growth of *P. larvae* on solid medium. Future work will focus on meeting MIQE guidelines for publication and determining the role of the highly expressed *gerKA 4* gene in *P. larvae* spore germination (Huggett, Foy et al. 2013).

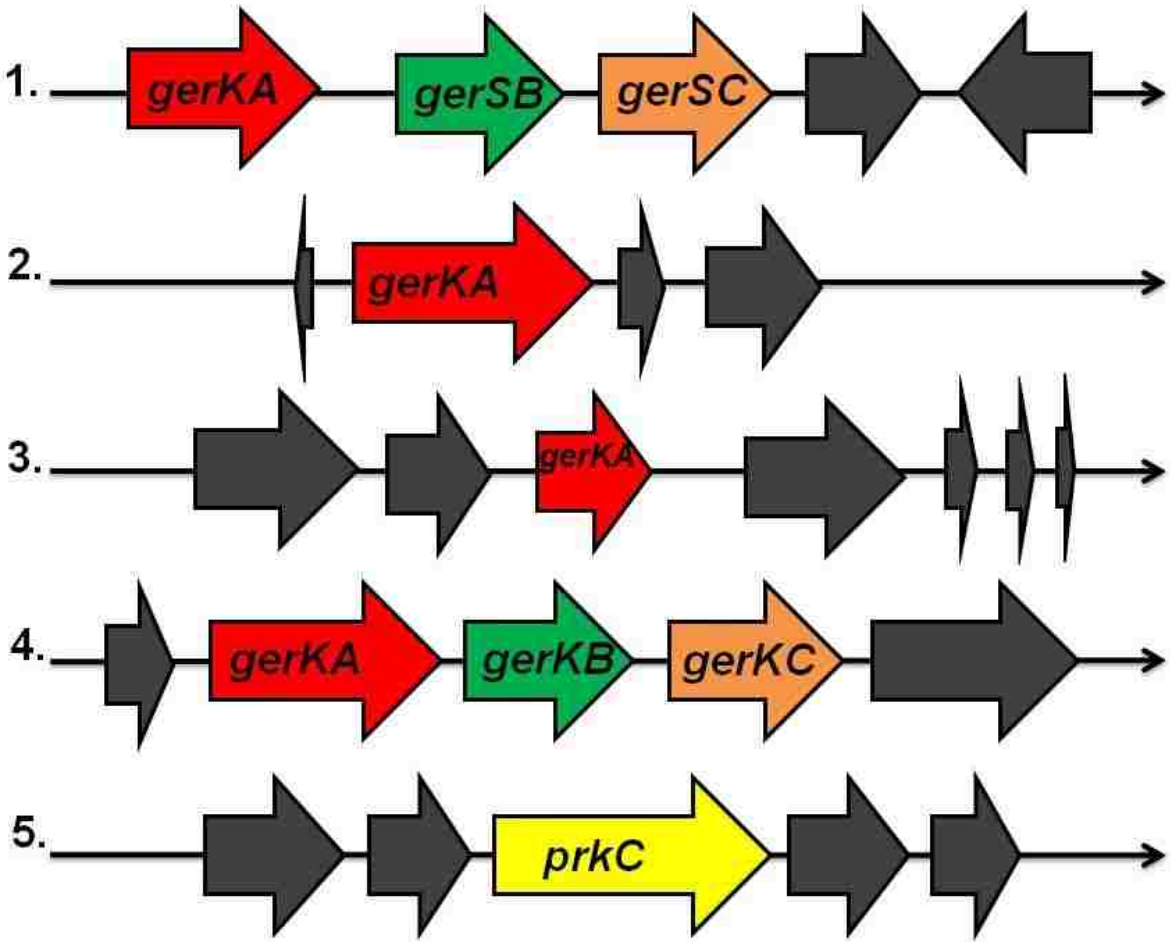


Figure 5.1: Genomic organization of *P. larvae* germination receptors. Related germination receptor subunits are shown in the same color. Proteins with non-germination related functional roles are shown in gray color.

	1	2	3	4	5	6	7	8	9	10	11	12
A	cDNA 1 *gerKA 1*			cDNA 2 *gerKA 1*			cDNA 3 *gerKA 1*				*gerKA 1*	*gerKA 2*
B	cDNA 1 *gerKA 2*			cDNA 2 *gerKA 2*			cDNA 3 *gerKA 2*				*gerKA 3*	*gerKA 4*
C	cDNA 1 *gerKA 3*			cDNA 2 *gerKA 3*			cDNA 3 *gerKA 3*				*prkC*	*sigG*
D	cDNA 1 *gerKA 4*			cDNA 2 *gerKA 4*			cDNA 3 *gerKA 4*					*rpoB*
E	cDNA 1 *prkC*			cDNA 2 *prkC*			cDNA 3 *prkC*					
F	cDNA 1 *sigG*			cDNA 2 *sigG*			cDNA 3 *sigG*				*gerKA 1*	
G	cDNA 1 *rpoB*			cDNA 2 *rpoB*			cDNA 3 *rpoB*			*gerKA 2*	*gerKA 3*	*gerKA 4*
H										*prkC*	*sigG*	*rpoB*

			cDNA template
			gDNA template
			water template

Figure 5.2: qPCR assay plate setup. qPCR was used to measure mRNA abundance in growing and sporulating cultures (wells marked in gray). Each assay performed included positive and negative controls for the seven mRNA targets. Genomic DNA was use as a template for our positive qPCR control (wells marked in green). Similarly, water was used as a template for our negative qPCR control (wells marked red). Note that one qPCR plate was used to measure mRNA abundance for one culture.

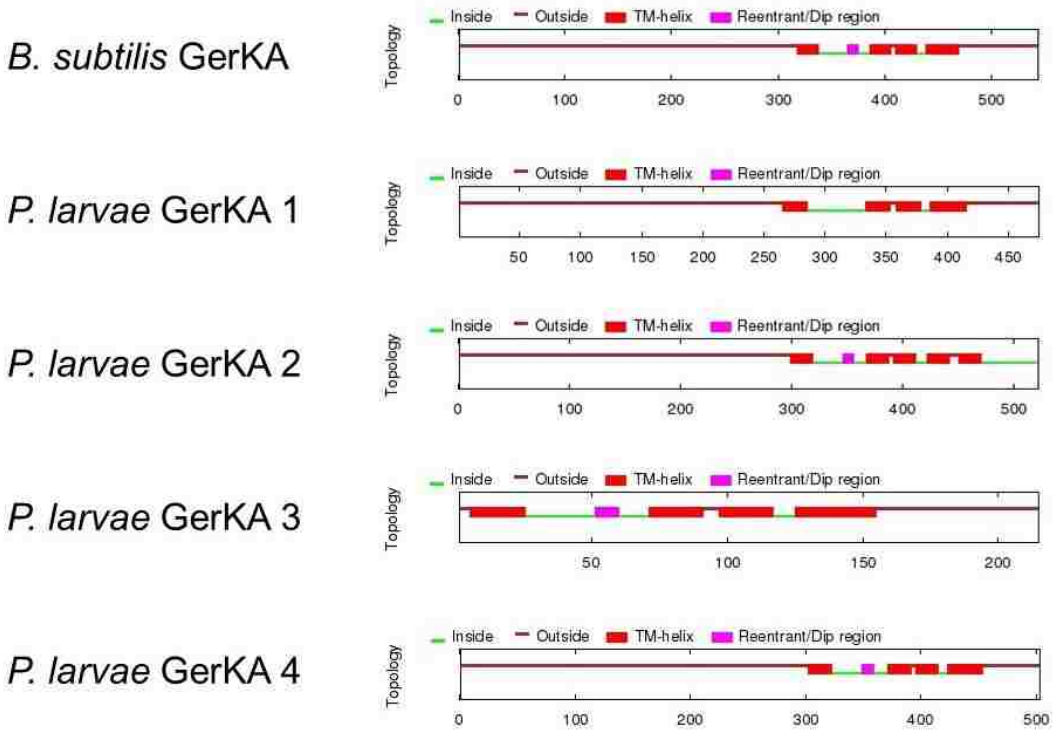


Figure 5.3: Topology prediction for germination receptors. Results for *B. subtilis* and *P. larvae* germination receptor A-subunit indicate topology similarities. The transmembrane domains are shown in red and soluble domains are shown in brown and green.

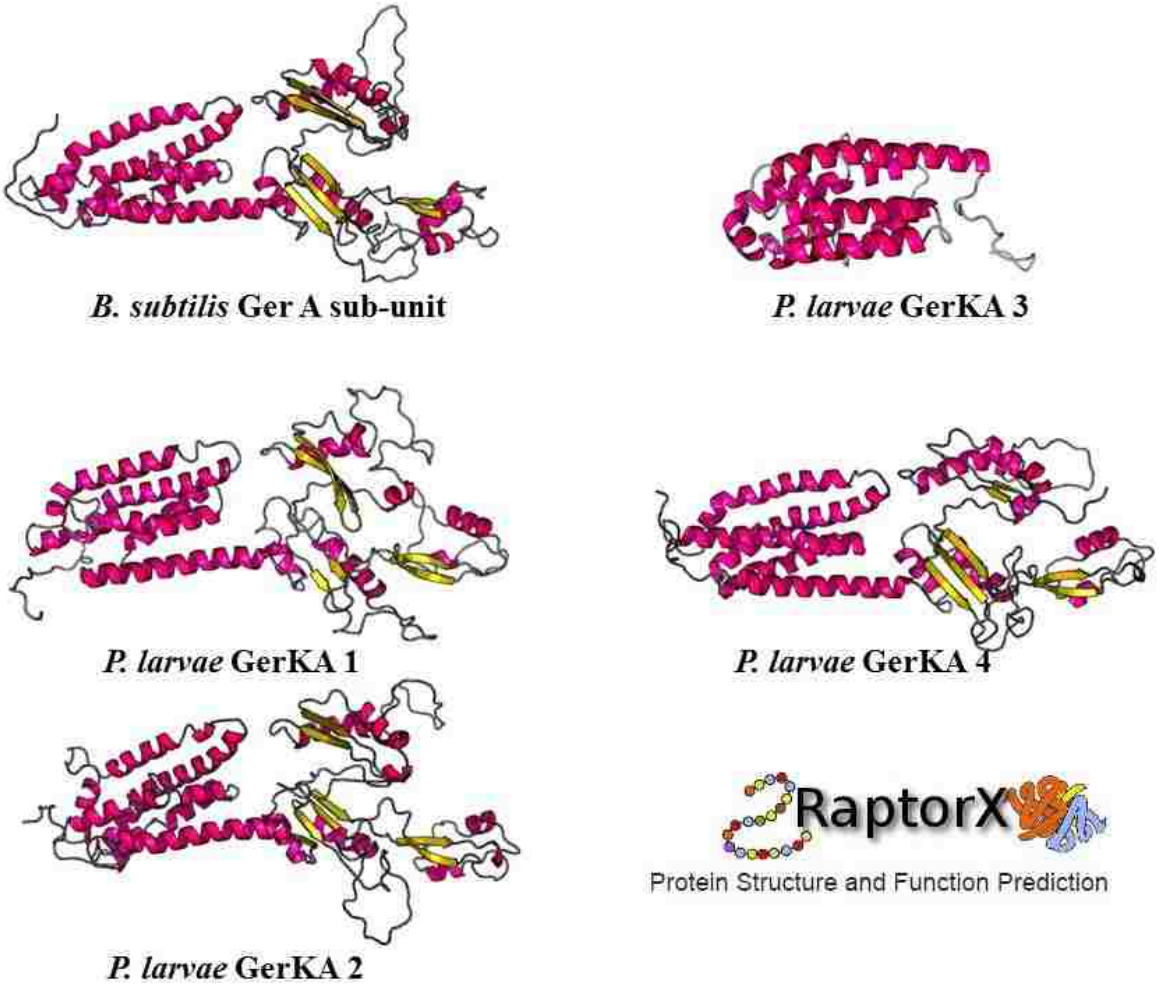


Figure 5.4: Predicted structure of germination receptor subunit A. Protein prediction software indicates the genome of *P. larvae* contain sequences for germination receptors. There are no apparent differences in protein structure topology between canonical *B. subtilis* and putative *P. larvae* protein structures.

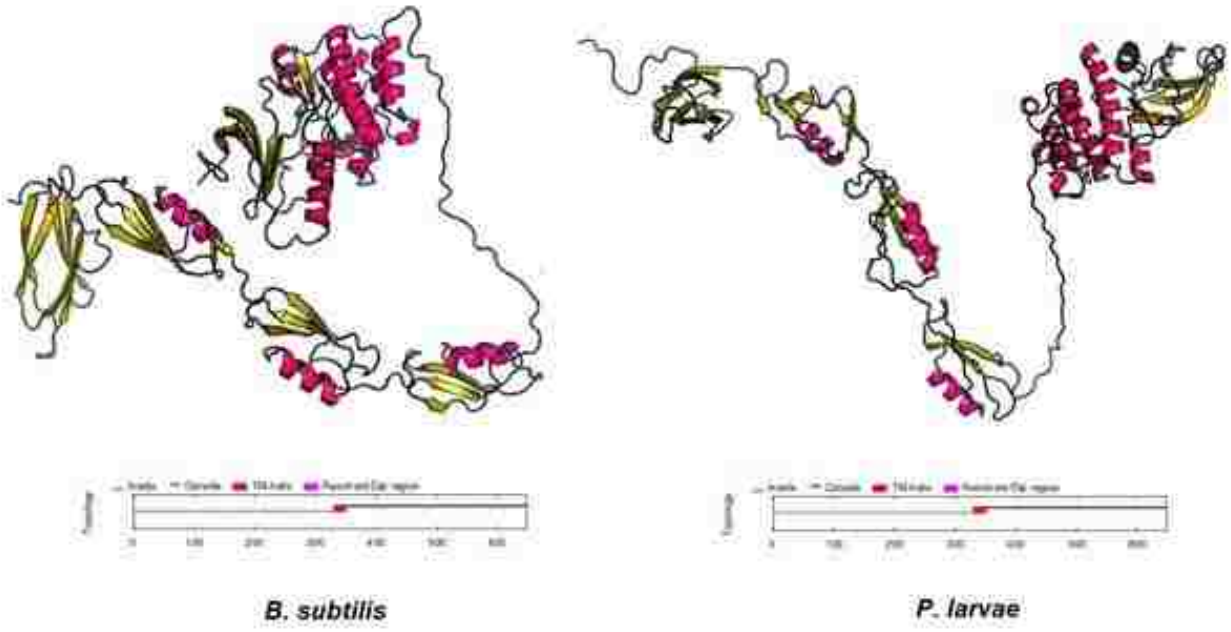


Figure 5.5: Predicted *P. larvae* prkC receptor. The PrkC protein in *B. subtilis* and *P. larvae* are predicted to have similar domains. Peptidoglycan fragments are thought to bind to PrkC proteins found in *B. subtilis* spores membrane via PASTA domains and trigger spore germination via the cytoplasmic kinase domain.

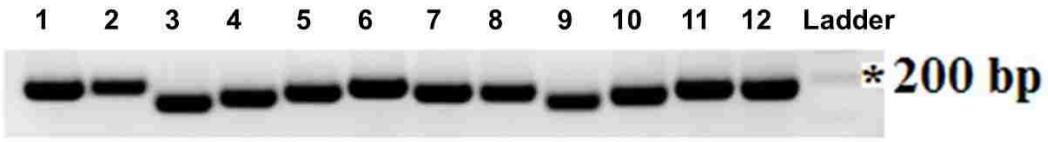
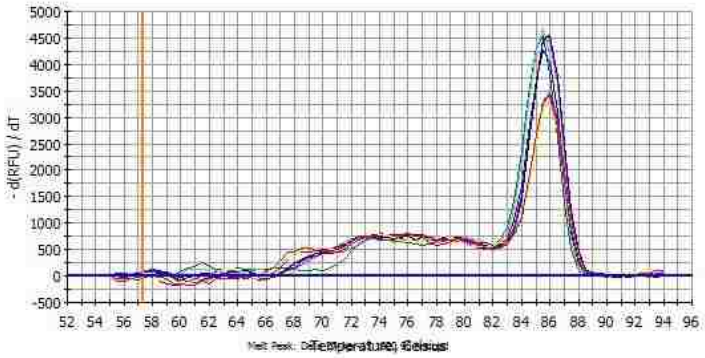
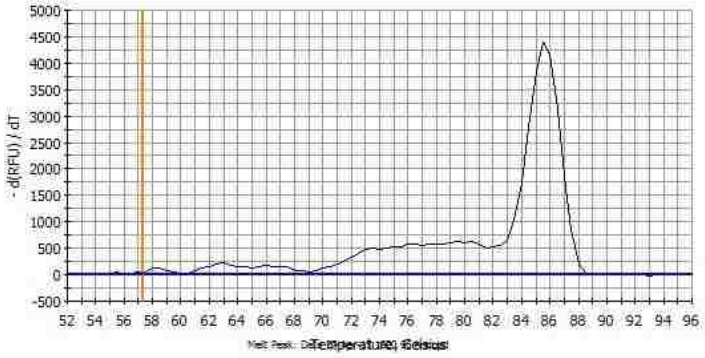


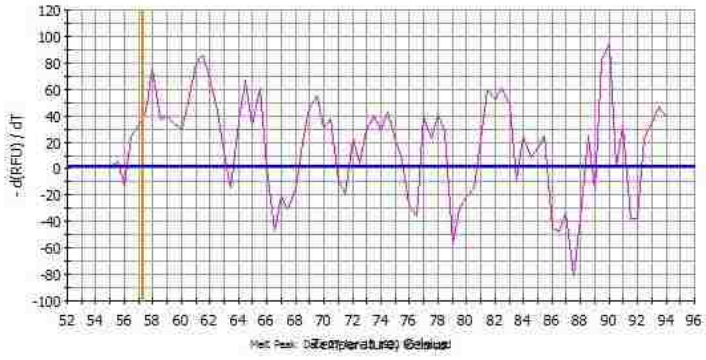
Figure 5.6: Specificity of qPCR primers. Agarose gel (1%) analysis of PCR products from qPCR primer and genomic DNA template. Lane 1: *rpoB*; Lane 3: *prkC*; Lane 6: *sigG*; Lane 9: *gerKA 1*; Lane 10: *gerKA 2*; Lane 11: *gerKA 3*; and Lane 12: *gerKA 4*.



***rpoB* cDNA template**



***rpoB* genomic DNA template**



***rpoB* lacking template**

Figure 5.7: Specificity of qPCR primers. After qPCR reactions we performed a melt curve analysis. A single peak in a melt curve analysis indicates a single PCR product. No primer dimers were detected in the positive and negative controls.

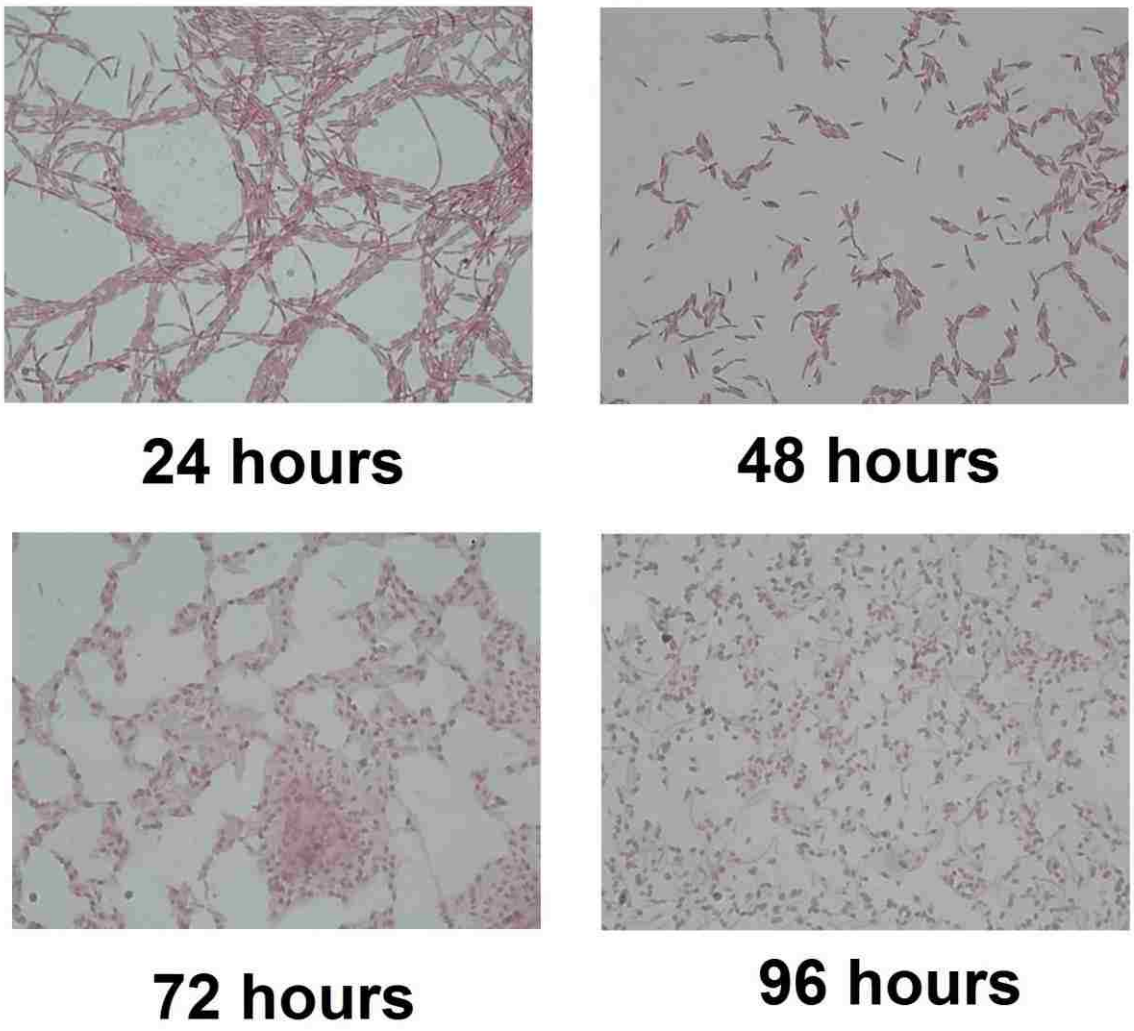


Figure 5.8: *P. larvae* sporulation in MYPGP broth. The growth of *P. larvae* in three cultures was followed during a 96 hour period via microscopy. At 24 hours, the cultures consisted of long growing bacilli chains. As time progressed the number of sporulating cells and spores increased. Cells are stained red and spores are stained green. *P. larvae* spores are approximately half the size of vegetative cells.

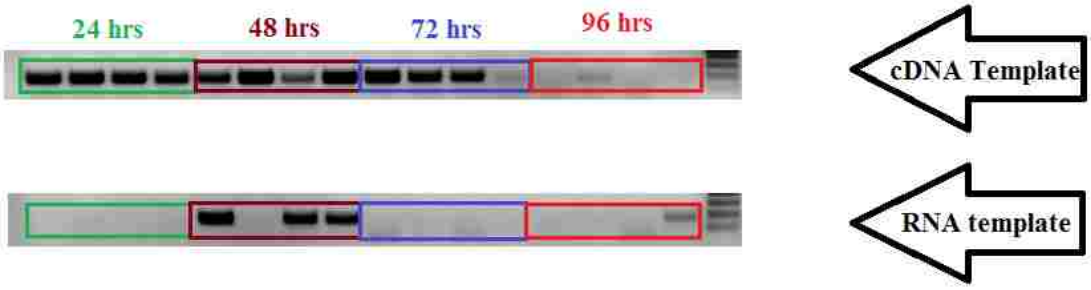


Figure 5.9: PCR products detected from cDNA and RNA templates. After 40 PCR cycles, amplification products were detected using cDNA as a template. Genomic DNA contamination in 48 hour RNA samples was very noticeable. As a result only cDNA from the 24 and 72 hour time point was used for qPCR assays.

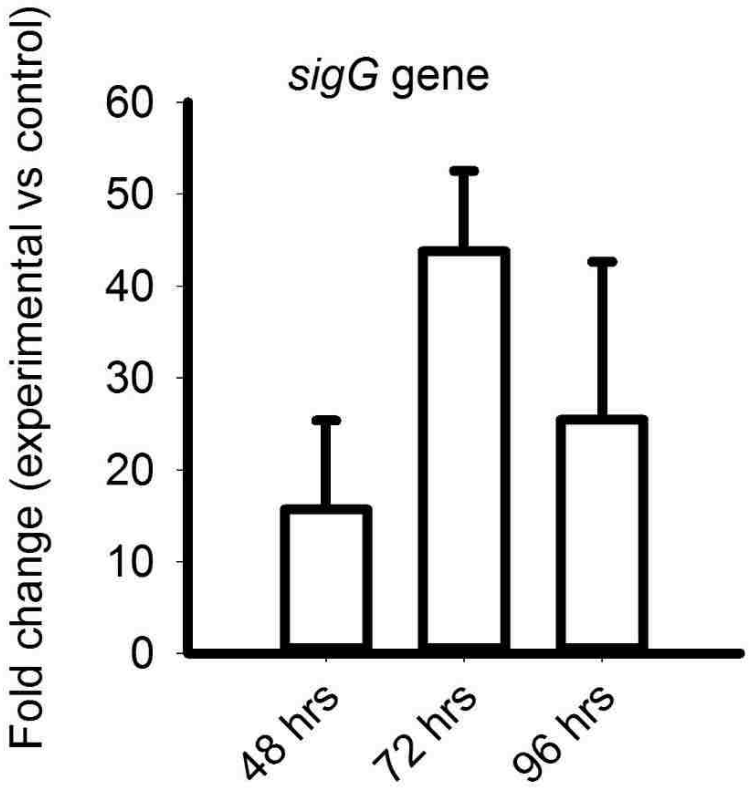


Figure 5.10: Relative abundance of *P. larvae sigG* sporulation gene. RNA was extracted from *P. larvae* cultures and mRNA levels quantified relative to *rpoB. SigG* expression was relatively higher in sporulating than in growing cultures. Each error bar represents a standard deviation obtained from at least nine independent measurements. There are no significant differences in *sigG* expression between sporulating cultures ($p < 0.05$). Each error bar represents a standard deviation obtained from at least nine independent measurements.

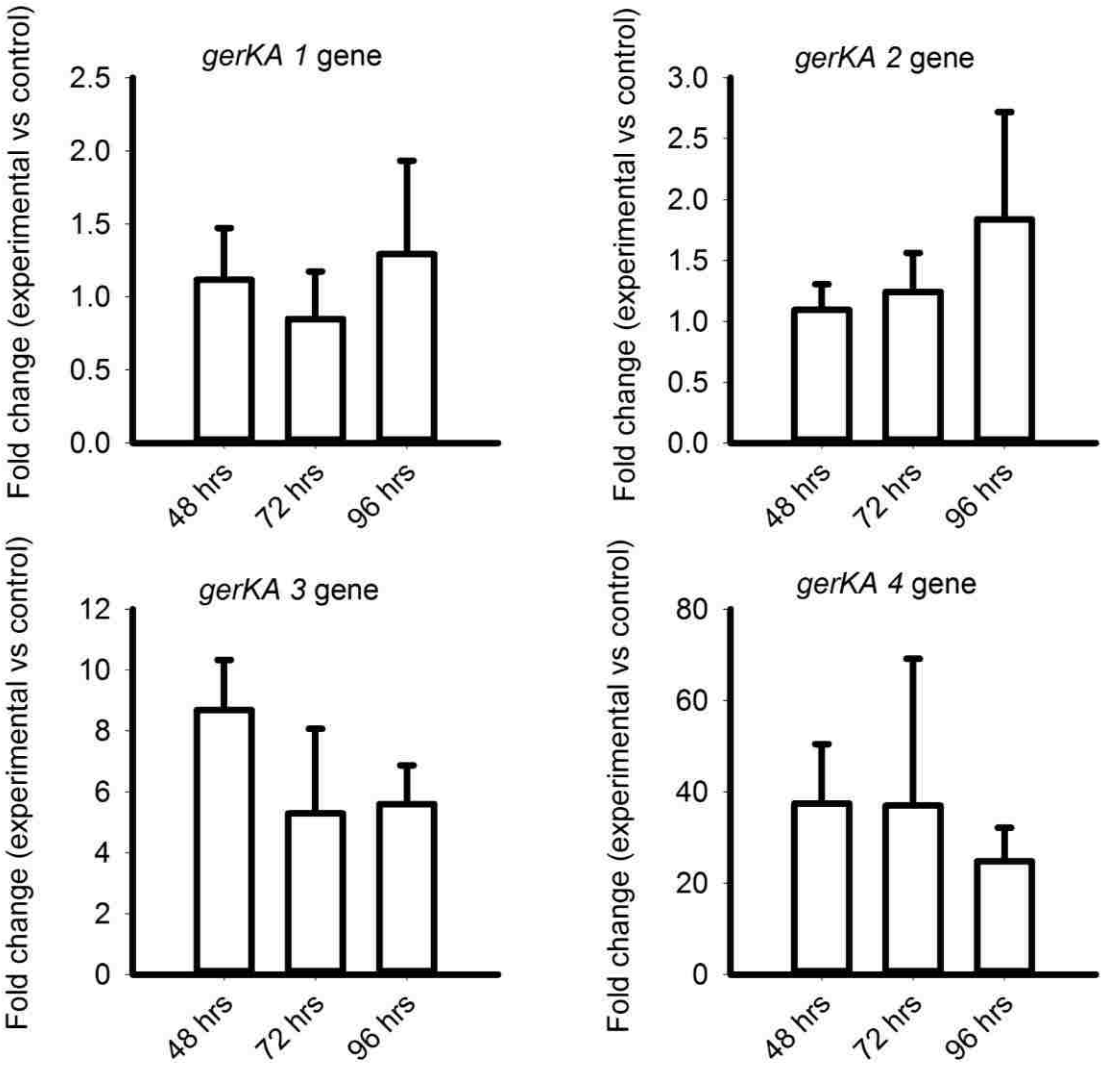


Figure 5.11: Relative abundance of *P. larvae gerKA* germination genes. We compared the relative abundance of *gerKA* mRNAs in growing and sporulating cultures. *GerKA* expression was relatively higher in sporulating than in growing cultures. Each error bar represents a standard deviation obtained from at least nine independent measurements. There are no significant differences in *gerKA* expression between sporulating cultures ($p < 0.05$). Note: the scales are not the same for each graph. Each error bar represents a standard deviation obtained from at least nine independent measurements.

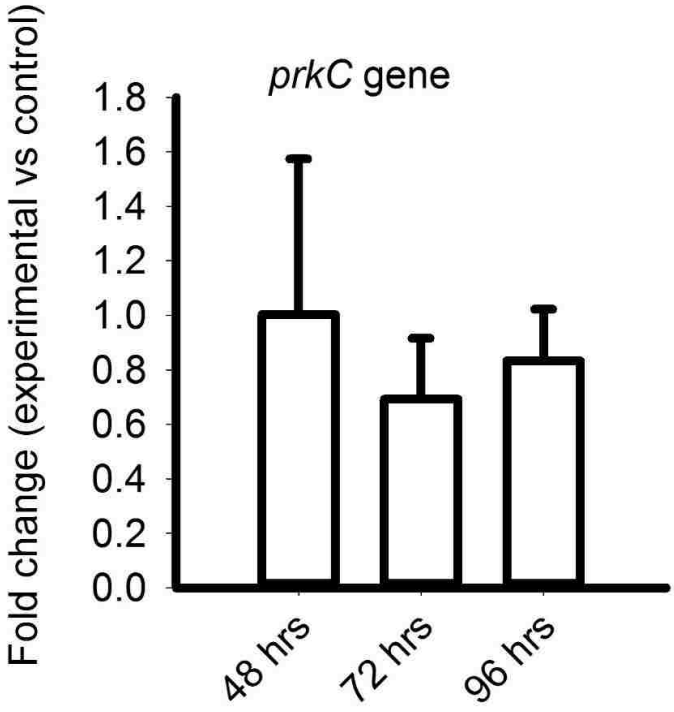


Figure 5.12: Relative abundance of *P. larvae prkC* germination gene. The relative abundance of mRNA was compared in growing and sporulating cultures. The expression of *prkC* was similar in sporulating and growing cultures. Each error bar represents a standard deviation obtained from at least nine independent measurements. There are no significant differences in *prkC* expression between sporulating cultures ($p < 0.05$). Each error bar represents a standard deviation obtained from at least nine independent measurements.

Table 5.1: *P. larvae* genes of interest: Potential germination receptors, a sporulation specific gene, and a housekeeping genes of interest.

Designation (Protein)	Product	Length	AA Length	Strand	Protein ID
GerKA 1	GerKA	1428	475	+	ZP_08055564.1
	GerSB	1101	366	+	ZP_08055565.1
	GerSC	1131	376	+	ZP_08055566.1
GerKA 2	GerKA	1569	522	+	ZP_08057011.1
GerKA 3	GerKA	648	215	-	ZP_08056748.1
GerKA 4	GerKA	1512	503	+	ZP_08057959.1
	GerKB	1098	365	+	ZP_08057960.1
	GerKC	1140	379	+	ZP_08057961.1
PrkC	Serine/threonine protein kinase PrkC	2070	689	-	ZP_08058023.1
SigG	RNA polymerase sporulation specific sigma factor	783	260	-	ZP_08057830.1
RpoB	DNA-directed RNA polymerase beta subunit	3537	1178	-	ZP_08055137.1

Table 5.2: Primers designed for qPCR assay. Primer pairs produced one PCR product from *P. larvae* genomic DNA.

Primer	Bases	Sequence	Melting temperature °C
Forward gerKA 1	20	TAC AGC ATA CGC TGG CTT GT	57
Reverse gerKA 1	20	TCC CTG GAT TCC GCT ATG GA	58
Forward gerKA 2	20	TCC AGA GAA CGA GTC CCC TT	58
Reverse gerKA 2	20	GTT GCT GAC TAG TCC GGC TT	57
Forward gerKA 3	20	GTA TCT ACC GCA GCT GTC CC	57
Reverse gerKA 3	20	CCG TCA GAG CTT CTG GTC AA	57
Forward gerKA 4	20	GGC CCA TTT GAT GGA GGG AA	58
Reverse gerKA 4	20	AAA AAG CGG GAA GCG TTG TC	57
Forward prkC	20	ACC GTT ATG GCA GCA AGT GA	57
Reverse prkC	20	CGG CCA CAA GAA CCA AAA GG	57
Forward rpoB	20	AAA TCC ATG CCC GTT CCA CT	57
Reverse rpoB	20	AAG TCT TCA CAC GAC CGA CC	57
Forward sigG	20	CTC GCT TCG GGA TAT TGC CT	57
Reverse sigG	20	CGT AAA TCG GAT CTC CCC CG	57

Table 5.3: qPCR assay program. Summary of qPCR conditions used in this study.

Cycle	Step	Temperature	Duration (mm:ss)	Comment
Cycle 1:	(1X)			
	Step 1:	95.0ºC	for 02:00	
Cycle 2:	(40X)			
	Step 1:	95.0ºC	for 00:15	
	Step 2:	58.0ºC	for 00:30	
	Step 3:	72.0ºC	for 00:30	Data collection enabled.
Cycle 3:	(1X)			
	Step 1:	95.0ºC	for 01:00	
Cycle 4:	(1X)			
	Step 1:	55.0ºC	for 01:00	
Cycle 5:	(80X)			
	Step 1:	55.0ºC	for 00:10	Melt curve data collection and analysis enabled.
Increase setpoint temperature after cycle 2 by 0.5ºC				
Cycle 6:	(1X)			
	Step 1:	4.0ºC	HOLD	

Chapter 6: Conclusions and Future Directions

6.1 Conclusions

Although bacteria can inhabit several environments, most bacteria have a preferred environmental niche. The obligate pathogen, *P. larvae*, is only known flourish inside honey bee larvae. For *P. larvae* the conditions required for growth, sporulation, and germination have been poorly recreated in the laboratory. Dr. Douglas Dingman had previously shown that oxygen toxicity and possibly CO_2 removal could alter *P. larvae's* life cycle. We observed that aeration was crucial for adequate sporulation of *P. larvae* in the laboratory. In Chapter 2, we observed that microaerophilic conditions enhanced *P. larvae* sporulation in the laboratory.

Environmental specificity is provided by germination receptors found in the inner spore membrane. In Chapter 3, we provide evidence supporting L-tyrosine plus uric acid as triggers of *P. larvae* spore germination. Exposure to one germinant molecule (L-tyrosine or uric acid) is not sufficient to trigger spore germination. As far as we know, in the colony L-tyrosine and uric acid are only found in the honey bee larvae. Thus, germination receptors allow for *P. larvae* to survive in an environment that is conducive for growth and only germinate when the target environment is available.

Studies have shown that structural analogs of germinants can inhibit spore germination. Inhibitors of spore germination interact with germination receptors displacing the germinants. Studies have shown that spore germination is the most critical step in the life cycle of spore forming bacteria. Understanding *P. larvae* spore germination is important to prevent disease development in honey bee larvae. In Chapter 4, our data indicate that *P. larvae* germination inhibitors can be used to reduce AFB disease development. We used *in vitro* assays to measure the ability of 30 molecules to prevent *P. larvae* spore germination. Five inhibitors of

germination were used in larval exposure assays. The presence of *P. larvae* germination inhibitors decreased AFB disease development in bee larvae. If we can synthesize stronger, yet non-toxic germination inhibitors, then we can protect honey bee larvae from AFB disease.

Germination receptors allow spores to monitor and respond to their environments. Gene expression, protein quantification, and mutagenesis studies have shown the importance of germination receptors in spores exiting dormancy. It is believed that there are less than a dozen germination receptors in spores. Indeed the level of gene expression observed in sporulating cultures for germination receptor genes is insignificant. Consequently, mutation of any of the germination receptor subunits abolishes spores response to environmental cues. In Chapter 5 & 6, we provide evidence for the role of five genes in *P. larvae* spore germination. Using qPCR we measured the relative abundance of the five germination related genes in growing and sporulating cultures. We found that the abundance of two germination receptor sequences was higher in sporulating cultures than in growing cultures. Furthermore, the expression of two germination sequences coincided with expression of a sporulation specific gene. In parallel, we attempted to disrupt germination receptor sequences and measure the effect on *P. larvae* spore germination. A clone containing a mutation in a germination receptor gene sequence was unable to be isolated. Additionally, *P. larvae* transformed with a plasmid that contained a germination receptor sequence failed to germinate faster. Our preliminary experiments will be validated in our future research.

6.2 Significance

Spore germination is a critical step in establishment of many diseases including anthrax, antibiotic associated colitis, gas gangrene, tetanus, botulism, and American Foulbrood disease. Controlling spore germination could provide a means of preventing disease establishment.

Triggering spore germination would induce the loss of spore-specific properties that permit the organisms to survive in harsh environments, while inhibition of spore germination would prevent the disease causing organism from exiting dormancy. Characterization of germination pathways is crucial for this disease management strategy to work. Identification of germinants is difficult because spore forming organisms inhabit nearly every environment. Thus the environmental signals that spores detect are unique and more complex than previously thought.

Through *in vitro* and *in vivo* assays we learned about *P. larvae* spore germination and its influence on AFB disease in honey bee larvae. In the presence of L-tyrosine plus uric acid *P. larvae* spores exit dormancy and become as susceptible as vegetative cells. Additionally, we have shown that inhibition of *P. larvae* spore germination alleviates AFB in laboratory-reared larvae. Stronger inhibitors of *P. larvae* spore germination need to be discovered to block the appearance of disease causing cells in honey bee larvae. Controlling spore germination will allow for the life cycle of *P. larvae* to be altered. Future studies will be conducted to measure the susceptibility of cells from germinated spores to alcohol based products, soaps, ultraviolet radiation, and environmental insults.

6.3 Future studies

The sigmoidal shape of *P. larvae* spore germination with uric acid plus L-tyrosine indicates cooperative binding to germinants (Chapter 2). The extent of cooperativity can be measured by double reciprocal plots (Lineweaver-Burke) or Hill plot of germination data (Neet 2009). However, the inconsistency of uric acid plus L-tyrosine solubility must be resolved by identifying other solvents to obtain consistent germination data in order to measure cooperativity. Alternatively, we can measure the order of germinant binding via dilution experiments (Luu, Akoachere et al. 2011). It is possible that *P. larvae* binds to germinants in a

random or sequential order. Determining kinetic parameters is important because it allows us to understand how spores respond to germination cues in their environment.

We have the opportunity to establish a connection between spore germination biology and disease development in the natural host. Development of a *P. larvae* germination receptor mutant will allow us to determine the role of germination pathways in disease development. It is possible that the interaction between the three germination pathways (*ger*, *prkC*, and non-nutrient) is necessary for AFB disease development in honey bees. Isolation of *ger* or *prkC* gene receptor mutant is necessary to determine germination pathway interactions. If *P. larvae* depends on one germination pathway then that pathway will become the focus future projects.

Additionally, creation of *P. larvae* germination receptor mutants can aid in the identification of indole target. If germination receptor mutants fail to grow in the presence of indoles, then Ger receptors are not the only site of inhibition. It is possible that *P. larvae* DNA replication and/or RNA transcription are also inhibited by indole and indole analogs. Indole analogs have been shown to interfere with dNTP binding during DNA replication (Coggins, Maddukuri et al. 2013). Furthermore, indole analogs inhibit nucleotide synthesis by *B. subtilis* and *E. coli* RNA polymerase (Doan, Rickards et al. 2000, Doan, Stewart et al. 2001). Future studies are required to identify the mechanism(s) by which indole alters *P. larvae* spore germination and subsequent cellular growth.

In vitro assays which exposed *P. larvae* to indole analogs in liquid and solid media showed great promise. However, when the same concentrations of germination inhibitors were used in larval exposure assays they failed to prevent AFB. It is possible that the germination inhibitors fall out of solution or are inactivated in the larval diet. Our fellow UNLV researchers, Dr. Helen Wing and Mrs. Jasmin Khilnani, found that the method used to measure antimicrobial

activity altered the effectiveness of compounds (manuscript in preparation). Thus, we need to determine and improve the solubility of the compounds used against *P. larvae* by synthesizing compounds with hydrophilic groups.

The genetic tools that we are developing for *P. larvae* can be used by other researchers. A simple and cost effective means to perform gene inactivation in *P. larvae* would help the field. In *Bacillus subtilis*, mutagenesis via homologous recombination has been an effective means to study protein function since 1978. Development of genetic tools in *P. larvae* will allow us to utilize characteristics of the organism. For example, *P. larvae* is unique in that it produces antimicrobial compounds that eradicate competing organisms within honey bee larvae (Garcia-Gonzalez, Müller et al. 2014b).

Our larval exposure assays do not fully represent the natural environment found in honey bee larvae. Because honey bees feed larvae through transfer of food by mouth the microbial community in our laboratory-reared larvae is incomplete. Field studies will allow us to test if germination inhibitors plus beneficial bacteria would prevent AFB disease development. It is also possible that the antimicrobial activity of our indole analogs might alter the larval microbiome. Future studies should examine the larval microbiome to understand the effect of germination inhibitors on the larval gut microbial community.

Future studies must also measure the fate of germination inhibitors within the honey bee colony. For example, indole residues in bee wax can be detected using phase extractions and gas chromatography-mass spectroscopy (GC-MS). There are established methods for extracting residues in wax. Furthermore, the solvents used in testing wax for residues are compatible with a method for determination of indole. Initially, wax would be solubilized in hexane and centrifuged. The supernatant fraction containing indole would then be collected and

concentrated. A hexane plus aqueous acetonitrile mixture would then be added to the residue. A separation funnel would be used to collect the acetonitrile phase containing indole. GC-MS would then be used to detect traces of indole in honey bee wax. Determining the persistence of indole in honey bee wax is important because honey is deposited in wax cells.

Previous studies indicate that germinated spores should have a survival rate similar to vegetative cells. Thus, for germinated *P. larvae* spores it would be important to know their susceptibility to environmental stress, disinfectants, and viability over time. We have already shown that germinated *P. larvae* spores are no longer heat resistant. Future experiments will need to measure the ability of germinating spores to survive pH, desiccation, and UV irradiation. In addition we can measure the susceptibility of germinated spores to alcohol, detergents, and other disinfectants. Lastly, it would be good to know the viability of germinated spores overtime. We expect that germinated *P. larvae* will lose the ability to survive in the environment with increasing time as observed when other types of spores germinated.

Appendix A

Alvarado, Israel, and Ernesto Abel-Santos. How enteric pathogens know they hit the sweet spot. Future microbiology 9, no. 1 (2014): 13-16.

This appendix is a primary paper evaluation by Dr. Ernesto Abel-Santos and myself of work conducted by: Ng, Katharine M., Jessica A. Ferreyra, Steven K. Higginbottom, Jonathan B. Lynch, Purna C. Kashyap, Smita Gopinath, Natasha Naidu et al. Microbiota-liberated host sugars facilitate post-antibiotic expansion of enteric pathogens. Nature 502, no. 7469 (2013): 96-99.

My contribution to the paper evaluation was conducted under the direction of Dr. Ernesto Abel-Santos.

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This appendix was published in the journal of Future Microbiology.

How enteric pathogens know they hit the sweet spot

Summary

The human gut microbiota is a complex system of commensal microorganisms required for normal host physiology. Disruption of this protective barrier by antibiotics creates opportunities for enteric pathogens to establish infections. Although the correlation between the use of antibiotic and enteric infections have been known for some time, the specific signals that allow enteric pathogens to recognize a susceptible host have not been determined. In a recent article, Ng et al. showed that the expansion of both *Salmonella typhimurium* and *Clostridium difficile* infections is enhanced by the availability of host-specific sugars liberated from the intestinal mucus by commensal bacteria. These results show how antibiotic-removal of specific species from the gut microbiome allows a normal symbiotic function to be hijacked by pathogenic species.

Keywords

Microbiota, fucose, sialic acid, *Salmonella typhimurium*, *Clostridium difficile*, *Bacteroides thetatiotaomicron*

Summary of methods and results

In order to study the effect of an antibiotic-depleted microbiota on intestinal infections, Ng et al. firstly developed a murine model that was monocolonized with *Bacteroides thetatiotaomicron* (Bt), a major component of the human GI microbiota.

Ng et al. used *S. typhimurium* as a model gram-negative enteric infection. In Bt-monoassociated mice, *S. typhimurium* showed upregulation of genes needed to metabolize fucose and sialic acid. The presence of Bt-released monosaccharaides, however, did not provide

a growth advantage to *S. typhimurium* in the murine gut. This surprising result that was attributed to residual sialic acid shed normally in the intestinal tract.

To further determine the effect of mucus-derived monosaccharides in the infection process, Ng et al. created a *S. typhimurium* mutant that was unable to metabolize fucose and sialic acid. This mutant was able to colonize Bt-monoassociated mice but was outcompeted by wild-type *S. typhimurium*.

C. difficile has the genetic machinery necessary to catabolize sialic acid but not fucose. Similar to *S. typhimurium*, *C. difficile* upregulated genes necessary for sialic acid catabolism in the presence of Bt. Even more, Bt provided a distinct advantage for *C. difficile* colonization of the mouse intestines.

Ng et al. showed that the levels of sialic acid and fucose are low in conventional mice but increase rapidly after antibiotic treatment. In conventional, antibiotic treated animals, wild type *S. typhimurium* is able to outcompete mutants that cannot degrade sialic acid. Similar results were observed for *C. difficile*.

To further address the importance of Bt-mediated sialic acid release in enteric infections, Ng et al. created a Bt mutant that lacks sialidase activity. In the presence of this mutant, the upregulation of sialic acid degradation genes is lost in both *S. typhimurium* and *C. difficile*. The colonization advantage provided to *C. difficile* by wild-type Bt was also lost in mice monocolonized with the Bt sialidase mutant but was rescued by feeding mice free sialic acid.

Discussion

The mammalian gastrointestinal tract contains a complex and dense microbial ecosystem [1, 2]. This gut microbiota is important for the normal physiological functions of the host including digestion, vitamin production, and immune system development [3-6].

The gut microbiota is separated from intestinal epithelial cells by a thin protective barrier arranged in two layers. The innermost layer is the glycoalyx, a structure formed by glycan attached to the epithelial cells. The most external layer is formed by secreted mucus, mostly composed of host glycosylated mucins [7]. Some commensal bacteria can degrade mucus-derived glycans and the resulting saccharides can be used by specialized microbial enteric species as food source [8].

Members of the gut microbiota also synergize with host defenses to prevent colonization of the gut by pathogenic invaders [9]. The use of antibiotics weakens the defensive barrier. Disruption of the gut microbiome creates niches that pathogens can exploit [10, 11]. Two well-known opportunistic pathogens of the microbiome-depleted gut are *S. typhimurium* (a gram-negative facultative anaerobe) and *C. difficile* (a gram-positive, spore-forming, strict anaerobe).

The genus *Salmonella* is one of the most common causes of food-borne illness in developed countries and diarrheal diseases in developing countries [12]. In the United States there are approximately 40,000 cases of salmonellosis reported each year. Although treatable, the mortality rate of salmonellosis can reach 24% for children living in developing countries.

C. difficile is uniquely associated with antibiotic exposure since, unlike other enteric pathogens, antibiotic treatment is almost a prerequisite for disease. Hence, prior to the discovery of antibiotics, *C. difficile* infections were rare. *C. difficile* infection (CDI) is the major identifiable cause of antibiotic-associated diarrhea. In the U.S., there are over 500,000 cases of CDI annually, with mortality rate of up to 2.5% and costs up to $3.2 billion [13].

Bacteroides thetatiotaomicron (Bt) plays a major role in the normal GI microbiota. Bt can hydrolyze non-digestible glycans but does not metabolize the released monosaccharaides [15]. Indeed, almost no host-derived monosaccharides were found in the feces of conventional mice. In contrast, Ng et al. found that high levels of mucus-derived sialic acid in the feces of Bt-monoassociated mice. These results reinforce the notion that monosaccharides released from the mucus by Bt provide a carbon source for more-antibiotic sensitive microbial species [8, 16]. *S. typhimurium* and *C. difficile* showed increased transcription of genes needed to metabolize host-derived sugars in BT-monocolonized mice. The alterations of gene expression reveal adaptations of these pathogens to recognize the lower complexity environment found after antibiotic-induced microbiota disruption.

Mucus-derived monosaccharide catabolism positively influenced *C. difficile* expansion in a susceptible host. These results show that Bt must provide *C. difficile* with assets not available through the normal diet or metabolism of mice. In contrast to *C. difficile*, excess host-derived monosaccharaides did no help the expansion of *S. typhimurium* in the host. These results suggest that while sialic acid is required for *C. difficile* expansion, other cues must be used by *S. typhimurium*. This is further supported since the colonization disadvantage of a *S. typhimurium* mutant unable to use host monosaccharaides disappeared in germ-free mice. Nevertheless, competition experiments showed that the utilization of fucose and sialic acid confer an evolutionary advantage to wild-type *S. typhimurium*. Taken together, these results suggest that the availability of host monosaccharaides is important in the dissemination of enteric infections of both gram-positive and gram-negative pathogens.

The GI tract is a complex environment that contains nutrients and bacteria [17]. Commensal bacteria assist the host by processing ingested food. Absorption of foodstuff in the intestine

creates a low nutrient environment [18]. The resulting competition for scarce resources could create a hostile environment for invading organisms that do not have the adapted metabolic capabilities of the natural microbiota. Thinning of the dense commensal microbial community by antibiotics increases the nutrient rich environment that is likely to have a role in pathogen expansion [19].

When microorganisms are exposed to a mixture of carbon sources there is a ranking in how the nutrients are utilized [20-24]. Not surprisingly, similar regulatory mechanisms are used by pathogenic organisms to control various steps of their infection process [25]. Pairing activation of metabolic processes with virulence factors is a common theme in bacterial pathogens. As an example, bacteria modify the transcription of carbohydrate utilization and virulence factor genes in response to environmental conditions in a susceptible host [26]. The efficient metabolism of a carbon source might serve as signal for proper fitness. Alternatively, the presence of a specific carbon source might indicate to bacterial cells the presence of a susceptible host and that specific virulence genes should be turned on or switched off. The availability of mucus-derived monosaccharides in antibiotic-treated guts, produce a rich environment that can be exploited by enteric pathogens for continuous proliferation

Future perspectives

The results obtained by Ng et al. suggest that there are universal molecular beacons that signal a disrupted gut microbiota. Enteric pathogens can take advantage of these signals to identify susceptible hosts. Once we understand how these beacons are detected, we could use it to our advantage. Targeting the fucose and/or sialic acid degradation pathways of enteric pathogens could contain infections. Alternatively, replenishing the intestines with sialic acid and/or fucose

degrading commensal microbes will deny pathogens the necessary resources to expand the infectious process.

The results of Ng et al are tantalizing and lead to a number of intriguing questions. Determining whether increased fucose and/or sialic acid concentrations are sensed by other intestinal pathogens (including eukaryotic parasites) could lead to a general mechanism for the recognition of antibiotic-depleted microbiome. Even more, it would be interesting to determine whether commensal produced, host-derived metabolites are signals of stressed in other commonly colonized tissues (e.g. mouth, vaginal tract, skin). This could lead to a general understanding on the role of commensal species in the promotion of infections. Finally, a link could be made between host metabolite utilization and the virulence of a pathogen. Answer to these questions could improve our understanding how pathogens recognize a susceptible host. In turn, these general mechanisms could be used as new anti-infective targets.

Executive summary

Objectives

To determine adaptations enteric pathogens use to expand in the gastrointestinal tract.

To confirm carbohydrate concentrations increases in infected mice and associate them with enteric pathogen virulence.

Methods

Germ free mice and Bt-monoassociated mice were infected with enteric pathogens to identify virulence genes.

Enteric pathogens unable to upregulate virulence genes were analyzed by in in vitro and in vivo studies.

Nutrient levels during pathogen infection in the mouse intestinal tract were measured in germ free mice and Bt-monoassociated mice.

Results

S. typhimurium and *C. difficile* both upregulate carbohydrate metabolism genes during their expansion in the mouse gastrointestinal tract.

Levels of free carbohydrates increase after antibiotic-induced disruption of microbiota.

Enteric pathogens unable to utilize free carbohydrates have a competitive disadvantage when competing with wild-type pathogens in Bt-monoassociated mice.

Enteric pathogens expansion is promoted by free carbohydrates and inhibited by competition with bacteria that catabolize free carbohydrates.

Conclusion

Infecting enteric pathogens encounter a gut environment that is low in nutrients.

The ability of enteric pathogens to expand within the host gut depends on the detection and utilization of carbohydrates.

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Education

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- B.S.- Biology. California State University San Marcos, 2006.
- A.A.- University studies- MiraCosta College, 2003.

Awards and Honors

- UNLV GPSA travel grant
- UNLV Hermsen Fellowship
- UNLV Strategic Plan Graduate Research Assistant Award
- CSUSM RISE scholarship from the Office of Biomedical Research and Training
- CSUSM MARC scholarship from the Office of Biomedical Research and Training

Publications

- Alvarado, Israel et al. Requirements for *in vitro* germination of *Paenibacillus larvae* spores. Journal of Bacteriology 195.5 (2013): 1005-1011.
- Alvarado, Israel and Ernesto Abel-Santos. How enteric pathogens know they hit the sweet spot. Future Microbiology 9, no. 1 (2014): 13-16.
- In preparation: Alvarado, Israel et al. Methods for developing treatment strategies against the honeybee pathogen *Paenibacillus larvae*, the causal agent of American Foulbrood disease. BMC Microbiology.
- Technical report phase 1: Fernando Flores-Mendes, Israel Alvarado, and Ernesto Abel-Santos. Requirements for Germination of *Bacillus* species Spores *In Vitro*. Novozymes
- Technical report phase 2: Fernando Flores-Mendes, Israel Alvarado, and Ernesto Abel-Santos. Requirements for Germination of *Bacillus* species Spores *In Vitro*. Novozymes

Presentations and Outreach

- Presented a poster at the 55th Annual Wind River Conference which focuses on prokaryotic and lower eukaryotic research. The conference provided exposure to bacterial pathogenesis experts in the field.
- ASM Student Chapter Outreach:
  - Oral presentation on the structure of eukaryotic and prokaryotic cells, Natural History Museum, North Las Vegas, NV, March 24 2013.
  - Oral presentation on the structure of eukaryotic and prokaryotic cells, Natural History Museum, North Las Vegas, NV, April 7 2013.
  - Oral presentation for high school students on the value of scientific research, Northwest Technical and Career Academy, North Las Vegas, NV, December 6 2013.
- 3rd Grade classroom oral presentation on the importance of honey bees and science, Tartan Elementary School, North Las Vegas, NV, 2012

- 4th Grade classroom oral presentation on the importance of honey bees and science, Tartan Elementary School, North Las Vegas, NV, 2012
- 5th Grade classroom oral presentation on the importance of honey bees and science, Green Valley Christian Academy, Henderson, NV, 2012
- Oral Presentation for adults focusing on the importance of honey bees and science. Whole Foods, Las Vegas, NV, 2012
- Oral Presentation for adults focusing on the importance of honey bees and science. Whole Foods, Henderson, NV, 2012
- Introductory beekeeping class assistance and honey bee research presentation. UNLV extension Orchard, North Las Vegas, NV, 2011
- Introductory beekeeping class assistance and honey bee research presentation. UNLV extension Orchard, North Las Vegas, NV, 2012
- 2nd Grade classroom oral presentation on the importance of honey bees and science, International Christian Academy, Las Vegas, NV, 2012
- 5th Grade classroom oral presentation on the importance of honey bees and science, Green Valley Christian Academy, Henderson, NV, 2014
- 1st Grade classroom oral presentation on the importance of honey bees and science, Doral Academy of Nevada- Cactus Campus, Las Vegas, NV, 2015
- Springs Preserve ScoutQuest Insect Study presentation on the importance of insects, honey bees, and science, Springs Preserve, Las Vegas, NV, 2015
- Outreach event for the Clark High School Science Club. Helped host 15 students to show the importance of education, science, and the role of UNLV researchers, UNLV, Las Vegas, NV, 2015.