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Virulence of *Photorhabdus* spp.: Examining the Roles of Environment, Evolution,
and Genetics in Insect Mortality

Dana Blackburn

A dissertation submitted to the faculty of
Brigham Young University
in partial fulfillment of the requirements for the degree of

Doctor of Philosophy

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ABSTRACT

Virulence of *Photorhabdus* spp.: Examining the Roles of Environment, Evolution, and Genetics in Insect Mortality

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Entomopathogenic nematodes (EPNs) (genera *Heterorhabditis* and *Steinernema*) kill their invertebrate hosts with the aid of a mutualistic bacterium. The bacteria (*Xenorhabdus* spp. for steinernematids and *Photorhabdus* spp. for heterorhabditids) are primarily responsible for killing the host and providing the nematodes with nutrition and defense against secondary invaders. *Photorhabdus* is a Gram-negative bacterium in the Enterobacteriaceae family with high virulence towards their insect hosts. To achieve high mortality rates *Photorhabdus* produces a variety of virulence factors such as toxins, lipases, proteases, secretion systems, and fimbriae. EPNs are amenable to laboratory rearing and mass production for biocontrol applications against insects using *in vivo* or *in vitro* methods; however, *in vitro* liquid culture is considered to be the most efficient. In this method the symbiotic bacteria are cultured prior to the addition of their partner EPN. This can leave the bacteria susceptible to a number of problems such as genetic drift and inadvertent selection. Regardless of the culture method the symbiotic bacteria exhibit trait deterioration or changes due to laboratory rearing. This project had three primary aims: 1) investigate the role of nutrition in trait deterioration, 2) examine virulence evolution using a phylogenetic context, and 3) identify genes that are necessary for survival and virulence inside the insect host. Prior to studying these objectives we first determined the optimal conditions for growing and counting viable cells of *Photorhabdus*. We discovered that growth is enhanced by the addition of pyruvate to growth media. To determine the role of nutrition in trait deterioration we repeatedly sub-cultured *Photorhabdus* in three different media types. Throughout this study we found that, in contrast to previous studies, trait deterioration does not always happen and the environment influences trait deterioration. Furthermore, based on our phylogenetic studies we found that *Photorhabdus* spp. are evolving to an increase in insect virulence. Lastly, using Tn-seq we determined a list of 84 genes that are needed for efficient virulence inside the insect host and provide suggestions for ongoing research efforts.

Keywords: entomopathogenic nematodes, *Photorhabdus*, trait deterioration, nutrition, Tn-seq, evolution

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Introduction

Chemical pesticides have been commonly used since the mid-twentieth century and their application has increased ever since. An estimated 500 million kg are applied annually in the U.S. and about 3 billion kg are applied worldwide (1). High application rates have introduced new problems including secondary pest outbreaks, resistance, and hazards to the environment and human health (1). Due to these risks and strict regulations on chemical pesticides, more thoughtful pest control efforts are increasingly incorporating biological control (2, 3).

Diverse organisms have been investigated for use in biological control including insect parasitoids and predators and entomopathogens, which include bacteria, viruses, fungi, and nematodes. Many of these organisms are commercially produced and have widespread use. However, due to varying host-ranges, variable field efficacy, and practicality many biological control agents have had limited success and require further optimization. Despite the time and resources spent investigating biological control agents, only some have been used extensively (3-6).

Increasing the successful use of biological agents in classical, inundative or inoculative approaches requires efficient mass-production methods. However, isolating an organism and rearing it in the laboratory can lead to the deterioration of traits required for success in the field. Observed deterioration has been attributed to genetic factors such as drift, inbreeding, and inadvertent selection (7-11). However, these problems may also be driven, either alone, or in combination with non-genetic factors such as disease and nutrition (7).

While not much effort has gone into determining the effect nutrition has on trait deterioration, many studies have investigated the role of nutrition on the efficacy of various biological control agents. The first chapter of my dissertation aims to highlight the role nutrition

plays in the production and efficacy of entomopathogenic biocontrol agents (predators, parasitoids, bacteria, fungi, viruses, and nematodes); specifically, the effect of nutrition on important biocontrol traits such as environmental tolerance and survival, reproductive potential, longevity, and virulence.

Entomopathogenic nematodes (EPNs) kill insects with the aid of mutualistic bacteria. The most well-known EPN genera, *Steinernema* and *Heterorhabditis*, form symbiotic relationships with Gram-negative Enterobacteriaceae, *Xenorhabdus* and *Photorhabdus*, respectively. The life cycle is as follows: EPN infective juveniles (IJs), the only free-living stage, enter insect hosts through natural openings. Upon finding a suitable insect host the IJ enters through natural openings such as the mouth or anus, migrates to the bloodstream (hemolymph), and releases its symbiotic bacteria (12). The bacteria grow rapidly causing insect death through septicaemia. The nematode grows, develops and reproduces by feeding on the high-density of bacterial symbionts in the dead insect. The nematodes feed exclusively on the bacterial biomass within the insect and, after about 7-10 days, a new generation of IJs, each one colonized by the mutualistic bacteria, will emerge from the insect cadaver to search out new insect hosts (13-15).

Xenorhabdus nematophila exhibits virulence variability within a population, termed virulence modulation (vmo) (16). VMO has been invoked as an explanation for how individual colonies obtained from the same frozen stock kill their insect hosts at different rates. To understand the observed variation in virulence, Park et al. injected single colonies of *X. nematophila* into *Manduca sexta* larvae. Some colonies completely failed to kill their host while others had mortality rates of up to ninety percent (16).

While the vmo phenotype has been demonstrated in *Xenorhabdus* sp., it is still unknown if this occurs in *Photorhabdus* spp. Therefore, chapter two of my dissertation investigated the

vmo phenotype in *Photorhabdus* spp. Additionally, prior to investigating vmo we determined the optimal growth parameters of *Photorhabdus* spp. for verifying the number of viable cells injected into each insect.

EPNs are amenable to laboratory rearing and mass production using *in vivo* or *in vitro* methods (17, 18). Regardless of the culture method both the nematode and the symbiotic bacteria exhibit trait deterioration. While there have been investigations on trait deterioration in EPNs, most research has focused on the underlying causes in the nematode, which have suggested genetic sources for deterioration (10, 11). Furthermore, only one study has demonstrated trait deterioration in the bacterial symbionts without their nematode partner (19).

Photorhabdus spp. are primarily responsible for killing the host (20-22) and providing the nematodes with nutrition and defense against secondary invaders (23). For example, *Photorhabdus* spp. produce crystalline protein inclusion bodies that are crucial for supporting nematode growth (24, 25) and antimicrobial molecules that prevent other microbes from occupying the same insect (26, 27). Efficient reproduction and high virulence are also important *Photorhabdus* spp. traits needed for their use as effective biocontrol agents (21).

To our knowledge, there are no published results on the underlying causes of trait deterioration in *Photorhabdus* spp.; therefore, the purpose of chapter 3 was to understand the role of the environment in trait changes of *Photorhabdus* sp. observed *in vitro*. Using *Photorhabdus luminescens* subsp. *luminescens* isolated from *Heterorhabditis floridensis* K22 (Rhabditida: Heterorhabditidae) (28, 29) we monitored changes in important biocontrol traits before and after repeated sub-culturing in three different nutritional regimes. The traits we investigated were crystalline inclusion body production, reproductive potential, and virulence because these are

biocontrol traits specific to the bacterial symbiont and were previously shown to significantly change after repeated sub-culturing (19).

Photorhabdus spp. achieve high insect mortality rates using various virulence factors with high growth rates being tightly correlated with high virulence rates (30, 31). Genomic sequencing revealed that *Photorhabdus* contains more predicted toxin genes than any other sequenced bacterium, including the well described Tc and Mef toxins (32). Furthermore, *Photorhabdus* produces “*Photorhabdus* virulence cassettes” (PVCs) and a type III secretion system (TTSS) (12, 33). *E. coli* transformed with PVC-containing cosmids are toxic to wax worm moth larvae and cause destruction of phagocytes (34). The TTSS of *Photorhabdus* secretes effector proteins directly into host cells. One effector, LopT, is similar to the YopT effector of *Yersinia pestis* and prevents phagocytosis (35, 36). Additionally, some species and/or subspecies produce urease, DNase, and hemolysins.

Photorhabdus spp. stochastically produce primary form cells and small colony variant cells (37). Primary form cells are pathogenic while small colony variants are able to form a symbiotic relationship with the nematode (37, 38). Therefore, primary cells have been termed P form for pathogenic and small colony variants are called M form for mutualistic. M-form cells are smaller, less virulent, slower growing, less bioluminescent, and produce less secondary metabolites than their P-form counterparts (37).

Photorhabdus was initially classified as *Xenorhabdus luminescens*, within the genus *Xenorhabdus*, a group of bacterial endosymbionts of the Steinernematid family of EPNs. However, using phenotypic and molecular data, it was later placed in its own genus (39). Three species of *Photorhabdus* have been described: *P. asymbiotica*, *P. luminescens*, and *P. temperata*

based on a 16S rRNA phylogenetic analysis, phenotypic characterization, and DNA-DNA hybridization (40).

While there has been extensive work on understanding the mechanisms of virulence in *Photorhabdus* spp., the origin and maintenance of this virulence has not been explored in a phylogenetic context. To this end, chapter four examines how virulence has evolved in *Photorhabdus* using ancestral state reconstruction with LT_{50} values as a measure of virulence. Furthermore, we investigated correlations between patterns of virulence, growth rates and cell types.

Most studies that have identified specific genes involved in *Photorhabdus* virulence screened individual colonies from transposon mutant libraries (27, 31, 41). Additionally, genome-wide screens used cosmids expressed in *E. coli* to identify genes toxic to insects (42, 43). However, there have been no studies that have utilized high-throughput sequencing to examine *Photorhabdus* virulence. Transposon sequencing (Tn-seq) is a tool that combines transposon mutagenesis and high-throughput sequencing to quantitatively screen for single gene fitness (44).

In chapter five, I utilized Tn-seq to identify genes that are essential to *Photorhabdus* virulence and survival inside the insect host *Galleria mellonella*. We have identified 84 genes needed for survival inside the insect host with many genes showing consistent phenotypes with previous studies. Furthermore, we have discovered genes in *Photorhabdus* that are crucial for virulence in other bacterial species, but have not yet been characterized in *Photorhabdus*. We also discuss other important virulence genes that have not previously been well described and the potential for future work.

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Chapter 1

Biological control and nutrition: food for thought

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Abstract

Chemical pesticides are used frequently to combat arthropod pests that plague crops; however, these compounds come with potential risks to the environment and human health. Research efforts have focused on using natural agents as an alternative to these chemical insecticides. These biological control agents include a wide range of organisms including predators, parasitoids, and other entomopathogens (bacteria, fungi, nematodes, and viruses). Despite commercial availability of these biocontrol agents their widespread use is still limited due to biological and economic difficulties. Aside from conservation biocontrol, the success of introducing biological control agents into the field can be highly dependent on the ability to mass-produce these organisms. Efficient mass-production relies heavily on the environment in which the agent is grown. Nutrition can play a significant role in important biocontrol traits such as colonization and survival, tolerance to environmental stress, reproduction, and longevity. Therefore, to increase biocontrol potential nutritional aspects should be considered prior to commercial production. This review aims to explore the role nutrition plays in the production and efficacy of biocontrol agents by summarizing the effect nutrition has on important biocontrol traits, specifically traits in entomopathogenic organisms including predators, parasitoids, and microbial agents.

Keywords: Biological control, nutrition, entomopathogens, parasitoids, predators, microorganisms

Introduction

Chemical pesticides have been commonly used since the mid-twentieth century and their application has increased ever since. An estimated 500 million kg are applied annually in the U.S. and about 3 billion kg are applied worldwide (Pimentel, 2005). High application rates have introduced new problems including secondary pest outbreaks, resistance, and hazards to the environment and human health (Pimentel, 2005). Due to these risks and strict regulations on chemical pesticides, more thoughtful pest control efforts are increasingly incorporating biological control (Chandler et al., 2011; Kogan, 1998).

Diverse organisms have been investigated for use in biological control including insect parasitoids and predators and entomopathogens, which include bacteria, viruses, fungi, and nematodes. Many of these organisms are commercially produced and have widespread use. However, due to varying host-ranges, variable field efficacy, and practicality many biological control agents have had limited success and require further optimization. Despite the time and resources spent investigating biological control agents, only some have been used extensively (Chandler et al., 2011; Pedigo and Rice, 2009; Shapiro-Ilan et al., 2002; Vega and Kaya, 2012).

The infrequent use of biological control agents in general is due to economic as well as biological obstacles. Lidert suggested that the lack of biological pesticide products stems from insufficient understanding of market needs and strategy, cost efficiency, and shelf-life stability (Lidert, 2001). Additionally, the range (broad or narrow) of hosts affected by biological agents and the ability to be mass-produced influence the success of these products. Ultimately, for biological control methods to be more widely adopted their benefits must outweigh their costs.

Increasing the successful use of biological agents in classical, inundative or inoculative approaches requires efficient mass-production methods. However, isolating an organism and

rearing it in the laboratory can lead to the deterioration of traits required for success in the field. For example, numerous hymenopteran parasitoids used in biological control have been reported to decrease in host acceptance, fecundity, and longevity after long periods (generations) in the laboratory (Geden et al., 1992; Rojas et al., 1999; van Bergeijk et al., 1989). Furthermore, it has been shown that laboratory-reared entomopathogenic nematodes (EPNs) can lose their ability to find, infect, and kill their insect host, have decreased fecundity, and are less tolerant to environmental stress (Bilgrami et al., 2006). Sub-culturing has resulted in the reduced virulence in biological control agents such as viruses, bacteria, and entomopathogenic fungi (Dulmage and Rhodes, 1971; MacKinnon et al., 1974; Tanada and Kaya, 1993; Vandenberg and Cantone, 2004). Observed deterioration has been attributed to genetic factors such as drift, inbreeding, and inadvertent selection (Bai et al., 2005; Chaston et al., 2011; Hopper et al., 1993; Hoy, 1985; Roush, 1990). However, these problems may also be driven, either alone, or in combination with non-genetic factors such as disease and nutrition (Hopper et al., 1993).

While not much effort has gone into determining the effect nutrition has on trait deterioration, several studies have investigated the role of nutrition on the efficacy of various biological control agents. This review aims to highlight the role nutrition plays in the production and efficacy of entomopathogenic biocontrol agents (predators, parasitoids, bacteria, fungi, viruses, and nematodes); specifically, the effect of nutrition on important biocontrol traits such as environmental tolerance and survival, reproductive potential, longevity, and virulence. Our intent is to provide examples that demonstrate the importance of understanding nutritional aspects of producing biocontrol agents. Therefore, we have provided examples from a number of different biocontrol agents in each trait section rather than go in depth with each category of biocontrol agent.

Mass Production

An in-depth look at mass-production of biocontrol agents is outside the scope of this review; however, understanding the methods used to culture these organisms is important for determining how these methods affect the efficacy of these organisms. Production, formulation, and delivery have been reviewed extensively elsewhere (Ehlers, 2001; Fravel, 2005; Morales-Ramos et al., 2014; Vega and Kaya, 2012). Production of biocontrol agents can be achieved using *in vitro* or *in vivo* methods. For example, EPNs can be reared *in vivo* by inoculating insect hosts and harvesting the nematodes from host cadavers. Alternatively, these organisms can be reared on their symbiotic bacteria using *in vitro* solid or liquid cultures. Agents that are amenable to liquid culture such as EPNs, bacteria, and fungi typically begin in medium-sized flasks and are scaled-up to large fermentors (4000 L or more) (Ehlers, 2001; Fravel, 2005).

Large-scale production of predators and parasitoids can be significantly more complicated. Most are reared on artificial diets, which often requires supplementation with honey or sugar solutions (Thompson, 1999). Additionally, many parasitoids feed on host hemolymph and plant material. If artificial diets are unavailable or not possible, these insects must be reared on their natural host in addition to the host's natural plant food. Due to cost considerations, a factitious host is often used rather than the natural host; however, this tradeoff can have negative effects on fecundity, lifespan, and other traits (Bai et al., 1992; Bigler et al., 1987; Kazmer and Luck, 1995).

The major limiting factors in mass production of biological control agents are the costs associated with growth substrates, low reproductive rates, and/or limited economies of scale (Fravel, 2005). *In vivo* methods are often significantly more expensive than *in vitro* methods and are difficult to scale up. However, for most organisms, technological improvements continue to

make *in vivo* production more plausible (Gaugler et al., 2002). In each case methods must be carefully assessed and optimized individually before commercialization of a biocontrol agent is plausible.

Nutritional Effects on Biocontrol Traits

Although the field of molecular genetics has revolutionized our understanding of the relationship between genotype and phenotype, the role that the environment plays in gene expression and, ultimately, the phenotype of an organism, is frequently underappreciated. When an organism is isolated from its natural environment and reared in the laboratory, it is important to understand how laboratory conditions and/or nutrition can affect the organism's ability to control insect pests. The following sections will discuss how nutrition affects important biocontrol traits. There are many traits that make an organism suitable for mass production and application to combat agricultural pests and diseases. These traits include, but are not limited to survival and tolerance to environmental stress, reproductive potential, and infectivity or virulence.

Survival/Tolerance

Success of a biocontrol agent is dependent on numerous factors; however, the first step is the organism's ability to survive during storage and introduction into the field. The type of culture media used can increase the chances of survival. For example, the entomopathogenic fungus *Isaria fumosorosea* has higher survival rates after freeze-drying for distribution with increasing percentages of glucose in the medium (Cliquet and Jackson, 1999). High casamino acid concentration also increases *I. fumosorosea* survival rates following freeze-drying methods (Cliquet and Jackson, 2005). Furthermore, the use of galactose or sodium citrate as the carbon source improves desiccation-tolerance compared to glucose (Cliquet and Jackson, 1999).

Another fungal biocontrol agent *Beauveria bassiana* shows longer storage survival when grown in nitrogen-limited media (Lane and Trinci, 1991). Nutritional effects on storage survival are not exclusive to fungi. EPNs have a higher rate of survival when stored in high osmolarity media and low pH (Lunau et al., 1993; Strauch et al., 2000). Additionally, optimal formulation materials for commercial distribution are required. Depending on the species, EPNs may have a higher survival rate when they are stored in clay versus a sponge (Strauch et al., 2000).

In the field, the most common environmental stressors include changes in temperature, desiccation, humidity, and osmotic shock. Growth conditions can affect how an organism will respond to these changes when applied to agricultural systems after growth in the laboratory. For example, thermotolerance of *B. bassiana* is affected by the conditions in which it is cultured (Ying and Feng, 2006). Ideal conditions used 4% glucose or 1% starch as the carbon source with Mn^{2+} as the metal additive, whereas sucrose with Fe^{3+} or Cu^{2+} significantly decreases thermal resistance. Other studies suggest that using millet grain and corn oil increases thermotolerance (Kim et al., 2011; Kim et al., 2010).

Organisms that undergo various stressors accumulate inorganic ions and produce compatible solutes, which include small molecules such as amino acids, sugars, polyols, and betaines. These molecules are used by cells to stabilize proteins, likely by causing the proteins to remain in their native state instead of denaturing due to extreme conditions (Bolen and Baskakov, 2001; Roessler and Muller, 2001). The best studied of these molecules are trehalose, glutamate, and glycerol (Csonka and Hanson, 1991; da Costa et al., 1998; Miller and Wood, 1996; Potts, 1994; Welsh, 2000). These molecules accumulate in the cell to stabilize cellular processes during stressful growth conditions preventing mortality. Some, such as trehalose, are naturally produced in a wide array of organisms; however, others have only been reported in a few organisms.

Trehalose has been implicated in a wide array of organisms as a protectant from a number of extreme environmental conditions such as desiccation, temperature changes, and osmotic stress (Hallsworth and Magan, 1996; Perry et al., 2012; Ying and Feng, 2006). Liu et al. determined that as environmental conditions return to normal after thermal stress, so do trehalose levels, suggesting the importance of trehalose during stressful conditions (Liu et al., 2009). Trehalose production can be stimulated by stressing the organism during growth prior to field applications. Studies showed that during thermal stress trehalose levels increase in *B. bassiana* (Liu et al., 2009). Additionally, nutritional starvation can cause increased concentrations of trehalose (Thevelein and Hohmann, 1995). Other factors that affect trehalose levels include temperature, pH, and water availability (Hallsworth and Magan, 1996; Ying and Feng, 2006).

The composition of the growth medium used to culture microorganisms affects the types and amounts of compatible solutes produced. For example, adding certain carbohydrates to the medium can increase trehalose levels (Hallsworth and Magan, 1994). Additionally, methods discovered on non-biocontrol agents may provide useful information that can be applied to biocontrol agents. Pocard et al. showed that a variety of protectants accumulate in both *Pseudomonas mendocina* and *Pseudomonas pseudoalcaligenes*, according to the type of culture conditions in which they were grown (such as different ions or other compatible solutes) (Pocard et al., 1994). Furthermore, supplementing growth media with compatible solutes allows bacteria to uptake these molecules and utilize them as cross-protectants. D'souza-Ault et al. showed increased growth when glycine betaine is added to osmotically stressed cultures of *P. aeruginosa* (D'Souza-Ault et al., 1993).

To increase survival rates of predators released into crop systems or greenhouses a food source, either host eggs, a factitious host, or an artificial diet, are supplied at the same time as

release. The type of food source affects survival rates of the predator in this type of condition. The predator *Nesidiocoris tenuis* preys on insect pests such as whiteflies and mites. The number of host eggs supplied with the release of this predator affected the survival and establishment of the predator (Urbaneja-Bernat et al., 2015). Furthermore, sucrose alone was not enough to ensure survival of *N. tenuis* on plants, but in conjunction with small amounts of insect eggs it was effective.

Baculoviruses are not easily mass-produced and it is difficult to determine how nutrition affects biocontrol traits since they have a narrow host range resulting in a lack of growing options. However, the formulation for dispersal in the field has been studied. For example, optical brightener compounds that are often used in textile and detergent industries have been shown to protect baculoviruses from UV radiation (Dougherty et al., 1996; Shapiro, 1992). Not all optical brighteners are effective (Shapiro, 1992); therefore, optimizing the type and concentration of optical brighteners is necessary when formulating these viruses for biocontrol use. Furthermore, studies that have investigated other components, including lignin and corn flour, showed that formulations with pregelatinized corn flour and potassium lignate provided an increased protection against sunlight and rain (Tamez-Guerra et al., 2000).

Infectivity/Virulence

After application, an effective biological control agent must be able to infect and prevent the spread of its intended target, often through mortality. This is another biocontrol trait that is potentially affected by nutrition and should be taken into account when formulating methods for mass-production. As most parasitoid studies look at egg laying and host acceptance, far less work has been done on how host mortality is affected by nutritional rearing. However, Magro et al. tested the effects of various artificial diets on the ectoparasitoid *Bracon hebetor* and found

that of seven diets tested there was only one that affected host paralyzation rates (Magro and Parra, 2004). All other diets were equal to parasitoids raised on the natural host.

Entomopathogenic fungi have become increasingly more important components of integrated pest management programs. Studies have shown virulence in various fungi is often based on the environment in which they were reared. For example, Safavi et al. and Shah et al. tested various strains of *B. bassiana* and *M. anisopliae* for virulence after growth on different media types (Safavi et al., 2007; Shah et al., 2005). *M. anisopliae* and *B. bassiana* are most virulent when grown on osmotic stress medium (OSM) containing 8% glucose, 2% peptone, and 5.5% KCl (Shah et al., 2005). However, one strain of *B. bassiana* is more virulent when grown on nutrient-poor media with 2% peptone. This suggests that optimal media may be strain-specific. Furthermore, studies on *I. fumosorosea* showed that OSM produces the most virulent organisms in two strains, but not in a third (Ali et al., 2009).

When EPNs are isolated from their environment they are typically screened for virulence (usually host mortality). The effect nutrition has on nematode virulence appears to be species and even strain specific. Grewal et al. compared *in vitro* and *in vivo* methods for rearing *Steinernema carpocapsae* and *Steinernema scapterisci* and found no significant differences in infectivity or mortality between the methods for *S. carpocapsae* (Grewal et al., 1999). However, *S. scapterisci* grown *in vitro* caused higher mortality rates than when produced *in vivo*. On the other hand, *Steinernema riobrave* did not exhibit significantly different mortality rates when formulated from liquid mass production or *in vivo* production (Shapiro and McCoy, 2000). Other studies also showed that *S. carpocapsae* was equally virulent when raised by *in vivo*, *in vitro* solid, or *in vitro* liquid methods; however, *Heterorhabditis bacteriophora* was less virulent when grown using *in vitro* liquid methods compared to *in vivo* and *in vitro* solid methods (Gaugler and

Georgis, 1991). Therefore, effective EPNs require various types of growth conditions, which is species specific.

Another important consideration is how the diet of the host organism affects the virulence of the biocontrol agent. For example, different lipid- and protein-based supplements added to the host's diet resulted in significant differences in their susceptibility to *Heterorhabditis indica* (but not to *S. riobrave* (Shapiro-Ilan et al., 2008)). Additionally, host diet affects the virulence of EPNs against other hosts (Shapiro-Ilan et al., 2012). This suggests that in some cases the host's diet can be adjusted during the rearing phase to produce improved biocontrol agents.

Formulations for dispersal also affect the virulence of the organism. As discussed in the previous section understanding how nutrition directly affects virulence of baculoviruses is difficult. However, optical brighteners are not only important for environmental protection, but these compounds are also effective at enhancing viral activity when included in the final distribution formulation (Boughton et al., 2001; Dougherty et al., 1996; Lasa et al., 2007; Shapiro, 1992).

Reproductive Potential

An important aspect of producing and implementing a biological control agent is to have high yields at a low cost. Therefore, organisms with ideal life history traits such as early maturity, high fecundity, and long life spans are selected. Manipulating the media these organisms are grown on can alter the reproductive potential of the biological control agent. As reproduction is one of the easier traits to study, nutritional effects on fecundity have been explored for a wide variety of organisms.

Over the past half-century insect parasitoid and predator studies have emphasized growth on artificial diets. Rearing these insects on artificial media has made it possible to study

biological, behavioral, and physiological processes. Additionally, these techniques have made it possible to produce biological control agents. Therefore, the effect different nutrition regimens have on host acceptance and oviposition, or egg-laying, has been well-studied in regards to mass producing parasitoids. Most of these studies focus on finding diets that maximize reproductive yields.

Molecules such as amino acids, proteins, triglycerides, and inorganic salts are known to induce oviposition in insects (Kainoh and Brown, 1994; Nettles et al., 1983; Nettles et al., 1985; Rutledge, 1996). Various parasitoids oviposit at different rates when reared on the same artificial media. Additionally, some parasitoids have higher fecundity when reared on a factitious host rather than its natural host, suggesting that each biological control agent needs a specific diet for optimization (Dias et al., 2008). For some parasitoids specific molecules that induce oviposition have been identified, whereas for others the search is ongoing (Dias et al., 2010; Kainoh and Brown, 1994).

Sugar supplementation is important for rearing parasitoids and affects many different biocontrol aspects. Different sugars can increase progeny production. One example is that honeydew from aphid hosts causes an increase in progeny production when added to the normal food source (Hagley and Barber, 1992). Additionally, honey is a good source of sugar for parasitoid feeding as it increases progeny production in various parasitoids (Baggen and Gurr, 1998; Irvin and Hoddle, 2007; Schmale et al., 2001; Teraoka and Numata, 2000). The sugar concentration and feeding frequency can also cause an increase or decrease in fecundity (Heping et al., 2008). Furthermore, sugar concentration can expand the reproductive period of parasitoids (Heping et al., 2008).

Many of the concerns and considerations for rearing parasitoids are similar for rearing predator insects. There are various natural and artificial diets that affect reproductive potential in predators with important ingredients such as liver supplements, amino acids, and sugars (Thompson, 1999). Though, it is specific to the insect down to the species level. *Orius insidiosus*, a predatory thrips, grown on various diets including factitious host eggs, nymphs, adults, and pollen showed higher rates of fecundity when grown on factitious host eggs with no added supplements (Calixto et al., 2013). The predatory mite *Amblyseius swirskii* had shorter oviposition time and an increase in deposited eggs when grown on an artificial diet containing honey, sucrose, tryptone, yeast extract, egg yolk, and insect hemolymph (Nguyen et al., 2013). However, *N. tenuis*, grown on factitious hosts had a decrease in offspring compared to growth on the natural host (Mollá et al., 2013).

Fecundity and nutrition has also been studied extensively with EPNs. Traditionally, EPNs are grown on their symbiotic bacteria and lack sufficient growth without their symbionts. These nematodes are affected by the media composition they are grown in as well as the media in which their symbiotic bacteria are cultured. Adding to the complexity of rearing these organisms is the fact that the two may prefer different carbon sources. Gil et al. tested nematode yields using different carbon sources (Gil et al., 2002). Nematodes have highest yields when carbohydrate sources are used in combination with canola oil; however, their symbiotic bacteria prefer glucose as a carbon source. Therefore, efficient production of EPNs requires two different nutritional sources, an initial glucose source followed by oil supplements after the bacterial growth phase. Protein sources also play a role in EPN and bacterial yields. Media containing soybean flour is ideal for both bacterial and EPN reproductive potential compared to various other protein sources (Cho, 2011).

EPNs also require a lipid source in their growth medium because most lipids come from an external source rather than *de novo* synthesis (Fodor et al., 1994). Since the highest yields of EPNs are established *in vivo*, lipid sources in artificial media should be similar to insect lipids (Abu Hatab et al., 1998). In fact, it has been demonstrated that *in vitro* media with mono-unsaturated lipids similar to insect lipids, such as canola and olive oils, produce the highest yields of EPNs (Abu Hatab and Gaugler, 2001; Abu Hatab and Gaugler, 1999; Yoo et al., 2000). Furthermore, different lipid sources can influence bacterial yields, which also affects nematode production (Yoo et al., 2000). Optimizing the concentration of the medium (including carbohydrates, lipids, proteins, salts, and growth factors) also plays a role in bacterial and nematode yields (Yoo et al., 2001).

Nutrition has also been found to have an effect on the reproductive potential of entomopathogenic fungi. *I. fumosorosea* is a fungus that infects a number of insect species, including the important pest whitefly, *Bemisia argentifolii* (Zimmermann, 2008). This fungus has been investigated as a biocontrol agent, including how to grow it in the lab for the highest yields possible. Aspartate and glutamate as the nitrogen source produce the highest blastospore yields, and zinc is essential for high reproductive rates (Cliquet and Jackson, 1999). Additionally, casamino acids increase spore yields (Jackson et al., 2003). Higher concentrations of casamino acids result in higher spore yields regardless of glucose concentrations (Cliquet and Jackson, 2005).

Vega et al. conducted a study on varying media types and their effect on different entomopathogenic fungal species/strains (Vega et al., 2003). *B. bassiana* strains behave differently in varying environments; however, other genera seem to be more stable (Safavi et al., 2007; Vega et al., 2003). *I. fumosorosea* showed differences in spore production among six

media types as well as among different isolates (Ali et al., 2009). Two isolates reproduced best in nutrient-poor media whereas the third had the highest reproductive capacity when grown in a low C/N ratio. All isolates showed the least conidia yields in chitin peptone media. Overall trends of nutritional affects on fungi seem to be genus specific providing further evidence for individual organism optimization.

Growth and Size

Presumably, a biological control agent that has better growth rates and is larger in size is indicative of a healthier and more effective agent. Kouame et al. demonstrated that the quality of the host used to rear a parasitoid affects the quality of the wasp (Kouame and Mackauer, 1991). A larger host produces a larger and healthier parasitoid. However, the quality of the host is not necessarily a linear function of host size (Sequeira and Mackauer, 1992). Furthermore, EPNs show size variation in different media types, including *in vivo* and *in vitro* methods. Yang et al. showed that body length and width varied in different nutritional environments (Yang et al., 1997). *In vivo* environments provided the largest EPNs. Animal protein media and plant/animal protein mixture media produce the next largest EPNs whereas plant protein media resulted in the smallest EPNs, confirming that nutritional sources can affect nematode quality and/or size.

Optimal media for fungal growth are species, and sometimes even strain-specific. Most *B. bassiana* strains grow best in media with a C/N ratio of 10:1, 1% peptone, or chitin peptone (Safavi et al., 2007). However, *M. anisopliae* strains prefer media with a C/N ratio of 35:1 and *I. fumosorosea* exhibits higher growth rates on chitin peptone nutrient media (Ali et al., 2009; Safavi et al., 2007; Shah et al., 2005). Additionally, certain trace metals like zinc may be necessary for growth (Cliquet and Jackson, 1999).

Longevity

The longevity of an organism is an important life history and biocontrol trait. This aids in product shelf-life and productivity in the field. Often the influence nutrition has on longevity mirrors results seen with fecundity. For example, *Trichogramma ostriniae*, a parasitoid used to control the European corn borer, exhibit longer lifespans and higher reproductive potential during a natural host infection when reared on factitious hosts (*Sitotroga cerealella*, *Trichoplusia ni*, and *Ephestia kuhniella*) rather than their target host (*Ostrinia nubilalis*) (Hoffmann et al., 2001). Various sugars, such as sucrose, glucose, and fructose, are often beneficial to the longevity of parasitoids (Faria et al., 2008; Wäckers, 2001; Wyckhuys et al., 2008). Sometimes the lifespan is dependent on the concentration of sugars and the frequency of feeding (Heping et al., 2008). Additional supplements such as honey can also provide added longevity (Irvin and Hoddle, 2007; Irvin et al., 2007; Sime et al., 2006; Vattala et al., 2006; Wyckhuys et al., 2008).

Conclusions

While it is not yet a burgeoning discipline, studies on the effects of nutrition on biological control agents have clearly indicated that nutrition can have wide-reaching effects on the successful implementation of biological control agents (Bonaterra et al., 2007; Cabrefiga et al., 2011; Gil et al., 2002; Hoffmann et al., 2001; Teixido et al., 1998; Yoo et al., 2001). Nutritional differences among *in vitro* and *in vivo* methods can cause drastic changes in the efficiency of these agents (Abu Hatab and Gaugler, 1999; Blossey et al., 2000). Interestingly, different species that are mechanistically similar often respond to the same culture type in a variety of ways. The one commonality among the different biocontrol agents is their lack of similar nutritional or growth requirements. Even among strains of the same species efficiency varies in similar nutritional regimes. This suggests that production and formulation of each organism can benefit significantly from individual nutrition and culturing optimization.

Mass production of biocontrol agents includes various trade offs to consider. Some nutritional regimes increase some traits while limiting other traits. For example, predators grown on artificial media may have an increase in size, but have longer development times (Cohen and Smith, 1998). Producers must determine which traits are most important for the most efficient biocontrol agent to reduce arthropod pest numbers. Furthermore, considerations of economic trade offs are necessary to establish the best nutritional sources. With each biocontrol agent companies and growers must determine the best nutritional sources for effective biocontrol agents while keeping costs down to make production economical and competitive against chemical pesticides. For EPNs it may be the difference between using *in vivo* methods or *in vitro* (solid or liquid) cultures. However, for predators and parasitoids it means considering the best artificial diet versus a natural or factitious host.

Despite the many advances that have been made over the past century, much is still unknown about how specific methods for the production, formulation, and application affect biological control agents. Future research should focus on individual biocontrol agents or species to optimize nutritional sources that will increase production, increase traits that will improve the overall efficiency, decrease costs, and decrease any potential trait loss. Researchers and developers must ask important questions in regard to the rearing of biological control organisms, such as, “Should the organism be grown *in vivo* or *in vitro*? Are the desired qualities observed in a natural host or on a factitious host/artificial diet? Does this organism require additional supplements/molecules to produce ideal traits?” Answers to these and other pressing questions will go a long way towards an optimal implementation of biological control technologies.

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Chapter 2

Virulence Modulation in *Photorhabdus* spp.

Short title: Virulence Modulation

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Abstract

Photorhabdus is a genus of Gram-negative bacteria that forms a mutualistic relationship with nematodes (Heterorhabditidae) and is primarily responsible for insect mortality during nematode infection. The purpose of this study was to investigate virulence modulation (vmo) in *Photorhabdus* spp. where individual colonies exhibit different levels of virulence. Despite in-depth studies on culturing *Photorhabdus* spp. and its nematode partner for laboratory investigations or mass production, little is known about ideal growth conditions prior to virulence assays. Accordingly, eight *Photorhabdus* strains with representatives from each species were grown in four media types; Luria-bertani (LB) broth, LB + 0.1% pyruvate (LBP), tryptic soy broth + 0.5% yeast extract (TSY), and Grace's Insect Medium (GM). All strains grew best in either LBP or TSY broths. However, when strains were plated onto agar plates the only media on which all strains grew well were agar plates supplemented with pyruvate. To investigate vmo in this genus, individual colonies from three species were injected into *Galleria mellonella* larvae, and the LT_{50} was calculated for each strain. Vmo was exhibited in two out of the three tested species. Results of this study will aid in the design of *Photorhabdus* virulence assays.

Introduction

Entomopathogenic nematodes (EPNs) kill insects with the aid of mutualistic bacteria. The most well-known EPN genera, *Steinernema* and *Heterorhabditis*, form symbiotic relationships with Gram-negative Enterobacteriaceae, *Xenorhabdus* and *Photorhabdus*, respectively. The life cycle is as follows: EPN infective juveniles (IJs), the only free-living stage, enter insect hosts through natural openings. After entering the host's hemocoel, the nematodes release their symbiotic bacteria, which reproduce and cause host death through septicemia or toxemia. The nematodes molt and complete 1-3 generations within the host. After about 7-10 days IJs begin to emerge to search out new hosts [1, 2].

To effectively culture *Xenorhabdus* spp. *in vitro*, pyruvate is added to the media [3]; however, *Photorhabdus* spp. are easily reared in a variety of culture media [4-6]. Standard media include Luria-Bertani (LB) broth, nutrient broth, tryptic soy broth, and proteose peptone no. 3 (PP3) broth [7-10]. Additionally, other media have been described to produce high bacterial growth and to foster its relationship with the nematode [5, 6]. These media typically have additional salts, protein, and lipid sources. Furthermore, *Photorhabdus* spp. have viable but non-culturable cells (VBNC) [4]. The addition of pyruvate to solid media can increase the recovery of *Photorhabdus* spp. VBNC [4].

Both *Photorhabdus* spp. and *Xenorhabdus* spp. exhibit phenotypic variation with two phases, primary and secondary. Primary phase bacteria produce antibiotics, proteases, lipases, various enzymes, protein inclusion bodies, and in the case of *Photorhabdus* spp., bioluminescence. Secondary phase bacteria lack or are severely diminished in all of these characteristics. Additionally, the primary phase of *Photorhabdus* spp. have two colony types known as primary form colonies (Variance, primary phases; Vp) and small colony variants

(Variance, small colony; Vsm) [11]. Vsm colonies do not support nematode growth and are less virulent than the primary form.

Furthermore, *Xenorhabdus nematophila* exhibits virulence variability within a population, termed virulence modulation (vmo) [12]. VMO has been invoked as an explanation for how individual colonies obtained from the same frozen stock kill their insect hosts at different rates. To understand the observed variation in virulence, Park et al. injected single colonies of *X. nematophila* into *Manduca sexta* larvae. Some colonies completely failed to kill their host while others had mortality rates of up to ninety percent [12].

While the vmo phenotype has been demonstrated in *Xenorhabdus* sp., it is still unknown if this occurs in *Photorhabdus* spp. Additionally, while much work has been done to optimize growth media for the mass-production of *Photorhabdus* spp. and its nematode symbiont [5, 6, 13] optimal conditions for culturing *Photorhabdus* spp. to obtain accurate counts of viable cells prior to virulence assays have not been established.

Often single colonies are used to test virulence in *Photorhabdus* spp. (especially to avoid effects of Vsm) and *Xenorhabdus* spp.; however, the vmo phenotype complicates this process. The purpose of this study was to characterize vmo in *Photorhabdus* spp. in order to identify optimal, reliable methods for testing virulence. Preliminary studies revealed that variable colony-forming unit (CFU) counts complicate the interpretation of virulence assays, preventing accurate and comparable results (data not shown). Therefore, prior to investigating vmo we determined the optimal growth parameters of *Photorhabdus* spp. for verifying the number of viable cells injected into each insect. Results of this study will aid in the experimental design and analysis of future *Photorhabdus* spp. virulence studies.

Materials and Methods

Cultures and growth conditions

This study included nine strains of *Photorhabdus* with representatives from each of the three *Photorhabdus* species: *P. asymbiotica*, *P. luminescens*, and *P. temperata*. We used the following strains: *P. asymbiotica* subsp. *australis* Kingscliff, *P. luminescens* subsp. *laumondii* ARG and TT01, *P. luminescens* subsp. *luminescens* Hb and Hm, *P. luminescens* subsp. *akhurstii* W14, *P. temperata* subsp. *khanii* NC19, *P. temperata* Hepialius, and one unknown strain of *P. temperata* (H. Goodrich-Blair, personal communication).

To determine favorable growth conditions, we tested bacterial growth in liquid broth and on agar plates. We plotted growth curves for eight *Photorhabdus* spp. strains (all listed above except *P. temperata* subsp. *khanii* NC19) in four different liquid media types: Luria-Bertani (LB) (tryptone 10 g l⁻¹, yeast extract 5 g l⁻¹, 10 g l⁻¹), LB + 0.1% pyruvate (LBP), tryptic soy broth + yeast extract (TSY) (casein digest 17 g l⁻¹, soybean digest 3 g l⁻¹, dextrose 2.5 g l⁻¹, NaCl g l⁻¹, K₂HPO₄ 2.5 g l⁻¹, yeast extract 5 g l⁻¹), and Grace's medium, unsupplemented (GM) (Life Technologies, Carlsbad, CA, USA). To examine CFU growth, we grew three strains (*P. asymbiotica* subsp. *australis* Kingscliff, *P. luminescens* subsp. *laumondii* TT01, and *P. temperata* Hepialius) in LB, LBP, TSY, and TSY + 0.1% pyruvate (TSYP) broth and plated onto LB, LBP, TSY, and TSYP supplemented with 1.5% agar. For all other experiments we routinely grew strains in LBP.

Growth curves

Prior to calculating growth curves, we inoculated 10 mL of each media type in 18 x 150 mm glass tubes with a portion of a frozen stock from each strain and grew cultures for 12-16 hr at 30 C with shaking at 250 rpm in the dark until an OD₆₀₀=0.8 was reached. Then we inoculated 10 mL of fresh media with 100 µL of the overnight culture and grew cultures in the same manner.

We checked OD readings every four hours for forty-eight hours. We repeated this two more times for a total of three replicates for all strains and media types.

Growth on Solid Media

We inoculated 10 mL of each media type in 18 x 150 mm glass tubes with a portion of a frozen stock from each strain and grew cultures at 30 C with shaking at 250 rpm in the dark until an $OD_{600}=1.0$ was reached. Following growth, we took 500 μ L of culture, washed once in 1X phosphate buffered saline (PBS), and resuspended in 1X PBS. After a dilution series we plated 10 μ L of the 10^{-5} dilution onto agar. We counted colonies after 48 hr incubation at 30 C in the dark. We did this in triplicate.

Virulence Assays

To test for vmo, we injected single colonies from each strain into *Galleria mellonella* larvae. We picked four individual primary-form colonies from three strains (one representative from each species) and grew them in 10 mL of liquid LBP at 30 C in the dark with shaking at 250 rpm until they reached an $OD_{600}=0.8$. Then we froze these cultures at -70 C in 1/2X LB + 50% glycerol (v/v). We used these cultures (four per strain) and the original stock cultures for subsequent virulence assays.

To obtain 50 CFUs/10 μ L for virulence assays, we had to first determine the number of CFUs in 500 μ L of culture from each bacterial population. We grew cultures from the frozen stocks to an $OD_{600}=1.0$ overnight as described above. We washed each 500- μ L culture once in 1x PBS and resuspended it in 1X PBS followed by a 10^{-5} dilution in 1X PBS. Then we plated on LB supplemented with 0.1% pyruvate. Following CFU counts we repeated these steps; however, we adjusted the 500- μ L-culture volume taken from the overnight culture to a volume that would ensure a final concentration of 50 CFUs/10 μ L at a 10^{-5} dilution factor. For the injection assays

we grew cultures in the same manner and used the adjusted culture volume previously determined for each population.

To examine vmo, we determined the LT_{50} values for each single colony culture and stock cultures discussed above by injecting *Galleria mellonella* with approximately 50 CFUs. We used fifth instar larvae that weighed between 0.19 and 0.30 g to determine the LT_{50} of each bacterial colony or stock culture. To minimize movement during the injections, we kept larvae on ice prior to injections. Following the bacterial preparations as described above, we injected 10 μ L into the hindmost left proleg using a 27-gauge needle. 1X phosphate buffered saline (PBS) served as our negative control (NaCl 8 g l^{-1} , KCl 0.2 g l^{-1} , Na₂HPO₄ 1.44 g l^{-1} , KH₂PO₄ 0.24 g l^{-1} , pH=7.4). Insects were stored in 94 x 16 mm petri dishes in the dark. Every ninety minutes we noted insect mortality until all larvae were dead (~40-48 hr) and determined LT_{50} values with a logistic regression. We assessed larval mortality based on the lack of movement upon contact with forceps and the “floppy” phenotype [14] caused by *Photorhabdus* spp. For statistical purposes, we did this in triplicate for each frozen stock.

Statistical Analysis

We used two statistical software packages in this study. To perform logistic regressions we used GraphPad Prism6 (Graphpad Software, La Jolla, CA, USA). To identify significant differences among growth conditions and among colony virulence, we used ANOVA, Student’s t-test, and the Tukey-Kramer test (JMP11; SAS, Cary, NC, USA).

Results and Discussion

Growth Conditions

Based on liquid culture, LBP and TSY provided the best environment for high growth rates (Fig. 1). LBP or TSY promoted the fastest growth in all eight strains tested. However, there

was some variation with LB and GM. In seven of the eight strains tested growth in LB was initially slow, but after 8-12 hr growth rates surpassed GM cultures and were often similar to LBP or TSY (ARG, Hepialius, Hm, Kingscliff, TT01). The *P. luminescens* Hb strain barely grew in LB (data not shown). Consequently, when testing a wide range of strains, our results suggest that LBP or TSY liquid media should be used instead of LB or GM.

Fig. 1. Growth curves of *Photorhabdus* spp. in liquid media. The strains *P. asymbiotica* subsp. *australis* Kingscliff (A), *P. luminescens* subsp. *laumondii* TT01 (B), and *P. temperata* Hepialius (C) are representatives of the eight strains of *Photorhabdus* that were grown in four media types.

GM cultures generally grew the slowest, implying that GM is the least suitable medium for growth. GM is commonly used to culture insect cells, providing an *in vitro* environment that is presumably similar to the insect haemocoel. Thus, our findings suggest that *Photorhabdus* spp. require nutrients for fast growth that are not in GM, highlighting nutritional differences between GM and the haemocoel. This result also hints at an explanation for the observed slower doubling times in the haemocoel [15] and GM versus the more rapid rates observed in LBP and TSY.

The three strains (*P. asymbiotica* subsp. *australis* Kingscliff, *P. luminescens* subsp. *laumondii* TT01, and *P. temperata* Hepialius) we tested on agar all grew on LBP and TSYP agar, but not all of the strains grew on the other media types (Fig. 2). LBP and TSYP agar plates had the highest number of CFUs for all liquid cultures despite the same amount being plated on all agar types. Additionally, TT01 growth was only detected on media supplemented with pyruvate. The other strains grew on all types, with the highest CFU numbers on LBP and TSYP agar.

Fig. 2. Growth of *Photorhabdus* spp. on solid media. Three strains were grown in LB, LBP, TSY, or TSYP and then plated onto LB, LBP, TSY, and TSYP agar. The three strains are:

P. asymbiotica subsp. *australis* Kingscliff (A), *P. luminescens* subsp. *laumondii* TT01 (B), and *P. temperata* Hepialius (C). Differing letters denote significant differences at $P < 0.05$.

Because our injection assays required only 50 CFUs/10 μ L, our experimental design was set up to count small numbers of CFUs. Lawns of bacteria can be obtained on all plates.

However, it is unlikely that this represents an accurate depiction of the number of viable cells in a culture rather just a selection of cells that can grow in a particular environment. For many strains growth on any media type would be appropriate; however, since one strain grew only on agar plates supplemented with pyruvate at this dilution factor we recommend using media containing pyruvate to obtain accurate CFU counts of *Photorhabdus* spp.

Virulence Modulation

Two of the three strains tested, *P. asymbiotica* subsp. *australis* Kingscliff and *P. luminescens* subsp. *khanii* NC19, exhibited the vmo phenotype (Fig. 3). In the Kingscliff strain ($F=17.0255$, $DF=4, 10$, $P=0.0002$) two colonies were significantly more virulent than the others, but the greatest variation occurred in the NC19 strain ($F=9.1620$, $DF=4, 10$, $P=0.0022$). TT01 did not display any detectable variation ($F=2.1373$, $DF=4, 10$, $P=0.1505$) among its individual colonies or with the stock culture. The Kingscliff strain had individual colonies with LT_{50} values that were significantly different from the stock culture. Additionally, the average LT_{50} of all the individual CFUs together was the same as the stock culture for all strains (Kingscliff $t=-0.99456$, $DF=13$, $P=0.8309$; NC19 $t=0.065435$, $DF=13$, $P=0.4744$; TT01 $t=1.330391$, $DF=13$, $P=0.1031$).

Fig. 3. Virulence modulation in *Photorhabdus*. Stock cultures and four individual colonies were injected into *G. mellonella* and LT_{50} values were calculated with three replicates. This was done with three strains: *P. asymbiotica* subsp. *australis* Kingscliff (A), *P. luminescens*

subsp. *laumondii* TT01 (B), and *P. temperata* susp. *khanii* NC19 (C). Differing letters denote significant differences at $P < 0.05$.

Photorhabdus spp. produce Vsm colonies known to be less virulent than Vp colonies [11]. To avoid the potential effects of Vsm colonies for future studies, individual Vp colonies can be used. However, due to vmo, our recommendation is to use multiple Vp colonies for virulence assays, though this would depend on the intended purpose of the virulence assay. Ideally, hundreds of individual colonies should be tested to determine virulence of a strain and its subpopulation of individual cells. However, this is likely to be impractical. Therefore, based on our results that the average of the individual colony LT_{50} values is equal to the stock culture virulence, a minimum of five colonies should be adequate.

Concluding Remarks

A uniform method for virulence assays in *Photorhabdus* spp. is currently lacking. There are numerous variables to take into consideration when designing these types of experiments. In this study we demonstrate that media supplemented with pyruvate provides a suitable environment for high growth rates and accurately counting viable cells. Furthermore, we show that one parent CFU is insufficient for capturing variation in virulence. Variation of growth or colony counts in different media types and variation of colony types can complicate the design, implementation, and analysis of *Photorhabdus* spp. bioassays, particularly those that involve virulence. Our work highlights the importance of growth media consideration and colony selection prior to bioassays. We conclude that the methods described in this study will yield the most accurate results for comparing virulence levels among *Photorhabdus* species and strains.

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Figures

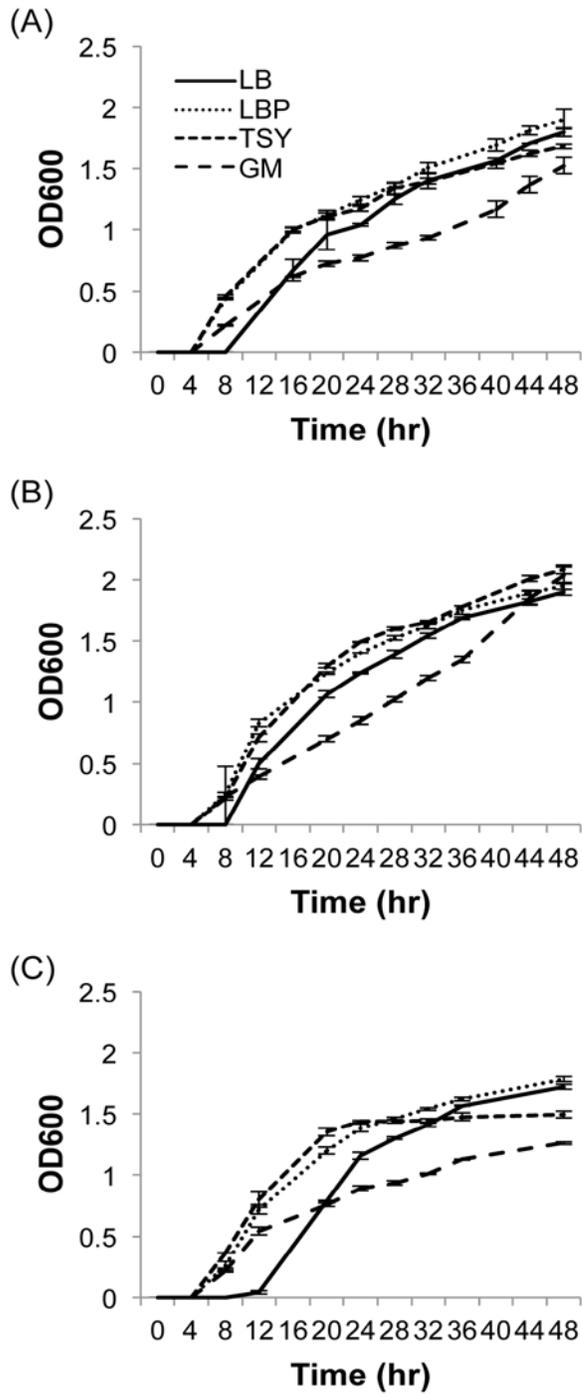


Figure 1. Growth curves of *Photorhabdus* spp. in liquid media.

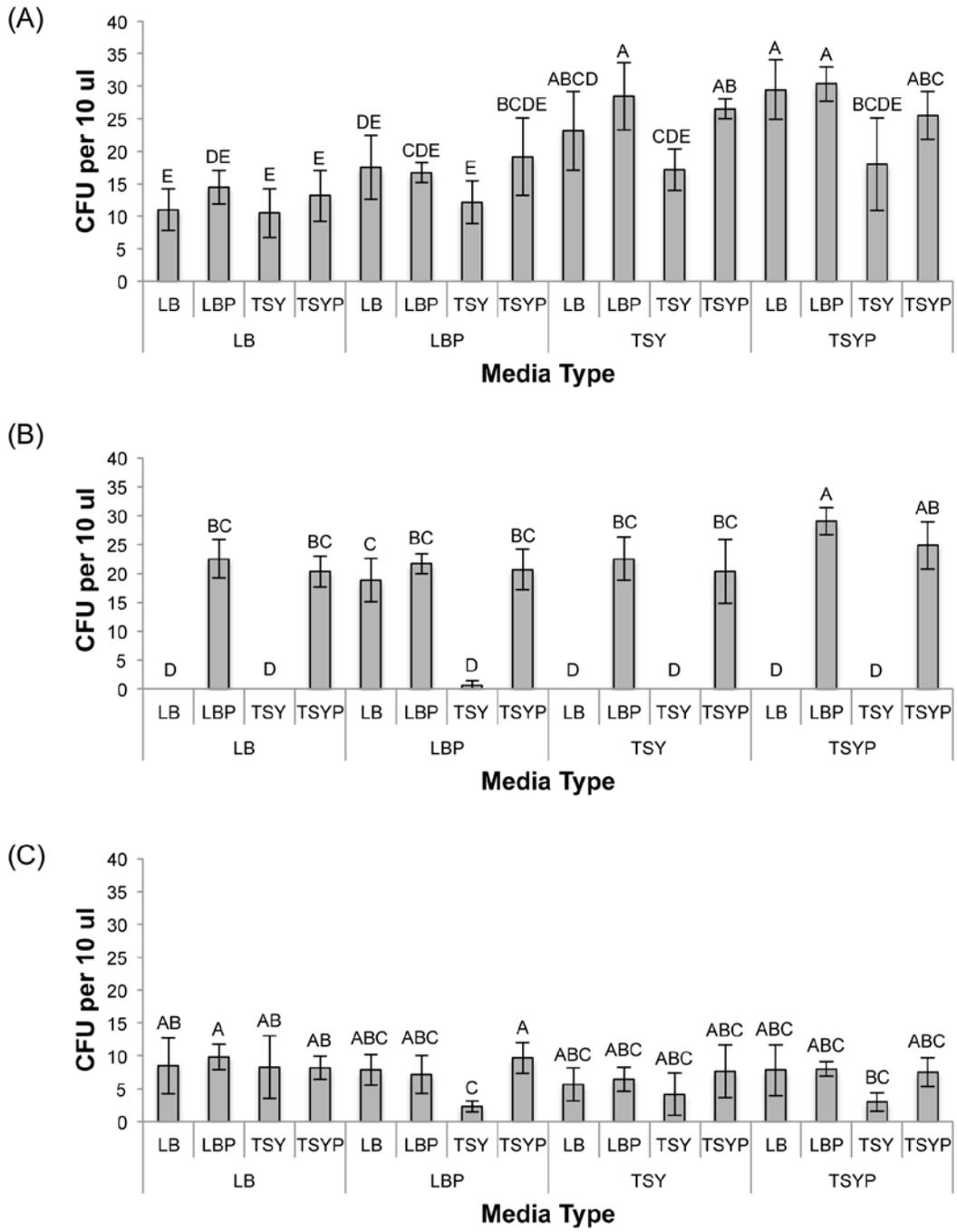


Figure 2. Growth of *Photorhabdus* spp. on solid media.

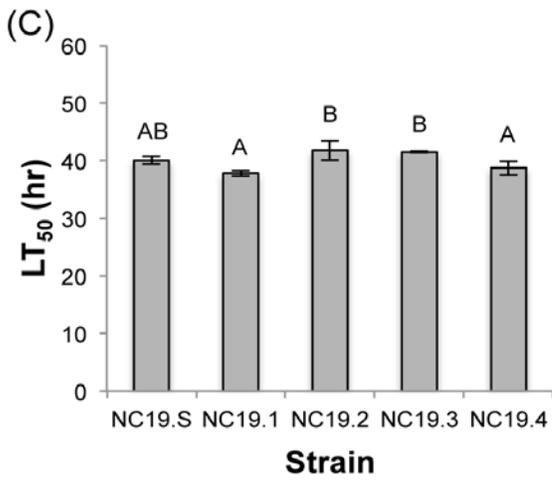
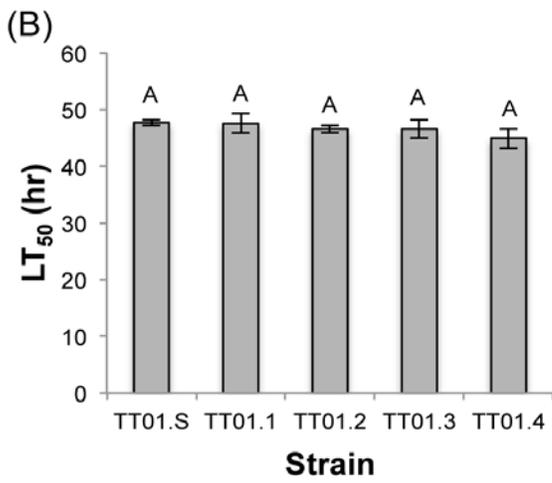
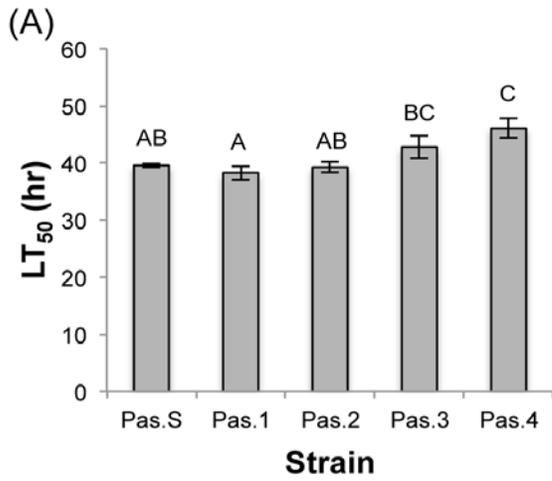


Figure 3. Virulence modulation in *Photorhabdus*.

Chapter 3

Environmental drivers of trait changes in *Photorhabdus luminescens*

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Abstract

Biological control agents have become increasingly important in integrated pest management programs. However, certain traits of these agents that are needed for efficient biocontrol often decrease or are lost during *in vitro* rearing. Entomopathogenic nematodes (EPNs) often exhibit trait deterioration when reared under laboratory conditions. EPN trait deterioration has been attributed (at least in part) to genetic causes; however, the underlying causes of trait deterioration in its bacterial endosymbiont have not been explored. In this study the EPN symbiont *Photorhabdus luminescens* was monitored for the deterioration of three traits; inclusion body production, reproductive potential, and virulence, in three different nutritional environments; lipid liquid medium (LLM), nutrient broth (NB), and tryptic soy broth+yeast extract (TSY). Significant trait deterioration did not occur for any of the traits in any environment. There was an increase in inclusion body production in TSY. Additionally, there was variation in growth within NB and TSY sub-cultured population lines and one TSY sub-population line was less virulent than the other two. However, returning bacteria to LLM restored all traits to wild-type levels. We infer the observed trait deterioration in *Photorhabdus* was minimal and appeared to be driven by environmental conditions as opposed to stable genetic changes. Our data suggest that variation among traits of *in vitro* cultures of *Photorhabdus* is more likely due to environmental variation than inadvertent laboratory selection or other genetic processes.

Keywords: *Photorhabdus luminescens*, entomopathogenic nematodes, nutrition, trait deterioration

Introduction

Chemical pesticides are commonly used against agricultural insect pests; however, in recent decades pest control efforts have refocused on finding new methods (Chandler et al., 2011). Chemical pesticides are effective but can have negative consequences on the environment and human health, as well as promote secondary pest outbreaks and the evolution of resistance (Coppel and Mertins, 1977; Pimentel et al., 1992). To combat these harmful effects individuals and agencies have implemented integrated pest management (IPM) programs. IPM programs utilize biological agents for insect control including bacteria, fungi, viruses, nematodes, and entomophagous insects (Chandler et al., 2011).

When a biocontrol agent is isolated from the environment and repeatedly cultured for experimental or commercial purposes essential traits or phenotypes, particularly virulence, host-finding abilities, and environmental tolerance (Kaya and Gaugler, 1993; Shapiro-Ilan et al., 2003), can be lost. These trait losses, or deterioration, are due to genetic processes such as drift, inbreeding, or inadvertent selection (Bai et al., 2005; Hopper et al., 1993; Hoy, 1985; Roush, 1990). However, nutrition plays a significant role in the efficiency of a mass-produced biocontrol agent (Cabrefiga et al., 2011; Shapiro and McCoy, 2000; Shapiro-Ilan et al., 2008). Therefore, changes in biocontrol traits may also arise from non-genetic factors such as poor nutrition and disease (Hopper et al., 1993).

Entomopathogenic nematodes (EPNs; genera *Heterorhabditis* and *Steinernema*) are important biocontrol agents that kill their invertebrate hosts with the aid of a mutualistic bacterium (Gaugler, 2002). The bacteria (*Xenorhabdus* spp. for steinernematids and *Photorhabdus* spp. for heterorhabditids) are primarily responsible for killing the host (Bilgrami et al., 2006; Gerritsen and Smits, 1993; Han and Ehlers, 2000) and providing the nematodes with

nutrition and defense against secondary invaders (Poinar, 1990). For example, *Photorhabdus* spp. produce crystalline protein inclusion bodies that are crucial for supporting nematode growth (Bintrim and Ensign, 1998; Bowen and Ensign, 2001) and antimicrobial molecules that prevent other microbes from occupying the same insect (Eleftherianos et al., 2007; Williams et al., 2005). Efficient reproduction and high virulence are also important *Photorhabdus* spp. traits needed for their use as effective biocontrol agents (Han and Ehlers, 2000).

EPNs are amenable to laboratory rearing and mass production using *in vivo* or *in vitro* methods (Ehlers and Shapiro-Ilan, 2005; Shapiro-Ilan and Gaugler, 2002). Regardless of the culture method both the nematode and the symbiotic bacteria exhibit trait deterioration. While there have been investigations on trait deterioration in EPNs, most research has focused on the underlying causes in the nematode, which have suggested genetic sources for deterioration (Bai et al., 2005; Chaston et al., 2011). Furthermore, only one study has demonstrated trait deterioration in the bacterial symbionts without their nematode partner (Wang et al., 2007). This study examined inclusion body production and size, reproductive potential, phase switching, and virulence in two strains (Hb-NJx and Hb-GA) of *P. luminescens* (unknown subspecies) before and after repeated sub-culturing in tryptic soy broth. *P. luminescens* exhibited trait deterioration in all traits except reproductive potential. Both strains demonstrated an increase in reproductive potential and one of the two strains also increased in virulence. To our knowledge, there are no published results on the underlying causes of trait deterioration in *Photorhabdus* spp.; therefore, the purpose of this study was to understand the role of the environment in trait changes of *Photorhabdus* sp. observed *in vitro*. We hypothesized that environment affects trait deterioration of *Photorhabdus* spp. Therefore, different nutritional sources would result in varying levels of deterioration. Using *Photorhabdus luminescens* subsp. *luminescens* isolated from

Heterorhabditis floridensis K22 (Rhabditida: Heterorhabditidae) (Nguyen et al., 2006; Shapiro-Ilan et al., 2014) we monitored changes in important biocontrol traits before and after repeated sub-culturing in three different nutritional regimes. The traits we investigated were crystalline inclusion body production, reproductive potential, and virulence because these are biocontrol traits specific to the bacterial symbiont and were previously shown to significantly change after repeated sub-culturing (Wang et al., 2007). Our results show that trait changes were not as drastic as previously described (Bilgrami et al., 2006; Wang et al., 2007); however, there was some nutritional effect on exhibited trait changes.

Materials and Methods

Cultures and Growth conditions

In this study, we used *P. luminescens* subsp. *luminescens* previously isolated from fresh cultures of *H. floridensis* K22 (Shapiro-Ilan et al., 2014). We recovered *P. luminescens* subsp. *luminescens* K22 by spreading the hemocoel from infected insects onto lipid agar (nutrient broth, 5 g l⁻¹ yeast extract, 2 g l⁻¹ MgCl₂, 0.004% corn oil, and 0.007% karo syrup). Using *Photorhabdus* spp. from recently isolated EPNs is crucial since any established lab strain has a high likelihood of already being appreciably deteriorated. The bacteria can exist in two phases (primary and secondary), but the primary form produces antibiotics, proteases, crystalline inclusion protein bodies, and is preferable for nematode growth (Akhurst, 1980). Growth on NBTA (nutrient agar, 25 mg l⁻¹ of bromothymol blue, 40 mg l⁻¹ of triphenyl-2,3,5-tetrazolium chloride) (Akhurst, 1980) and lipid agar plates confirmed primary phase bacteria based on color (green/blue on NBTA and red/orange on lipid agar). We used lipid agar since NBTA alone can produce inconclusive or unreliable results (Boemare and Akhurst, 1988).

Our sub-culturing methods and bioassays were done in the same manner as the study done by Wang et al. with minor changes (Wang et al., 2007). Below we describe our methods in detail. Additionally, Table 1 compares the similarities and difference between our methods and two important EPN trait studies (Bilgrami et al., 2006; Wang et al., 2007)

To determine the effect of nutrition on trait deterioration, we used three media types: liquid lipid medium (LLM) as previously described with nutrient broth (EMD, Gibbstown, NJ) instead of soy flour (Yoo et al., 2000), nutrient broth (NB) (EMD, Gibbstown, NJ), and tryptic soy broth (BD, Sparks, MD) with 0.5% yeast extract (TSY) (BD, Sparks, MD) (Table 2). We used concentrations recommended by the manufacturer for commercially available media. NB and TSY are commonly used in routine lab culture of *Photorhabdus* spp. while LLM formulations are generally optimized for mass-production of EPNs (Ehlers, 2001; Yoo et al., 2000). We created base populations, or original populations, by inoculating 50 mL of each media type in a 250 mL flask with 50 colony-forming units (CFUs) to avoid potential founder effects. From each base population, we cultured three parallel sub-population lines, or experimental lines, in each media type for twenty cycles. Having three experimental lines allowed us to investigate any potential stochastic effects of the sub-culturing process. Previous research sub-cultured bacteria for twenty-five cycles; however, most trait deterioration was observed as early as ten cycles and always by twenty cycles (Wang et al., 2007). We stored a portion of each base population and all sub-cultured populations from every fifth cycle at -70°C in 1/2X LB+ 50% glycerol.

Each cycle consisted of 50 CFUs inoculated into 50 mL of liquid culture in a 250 mL flask followed by shaking at 250 rpm for 48 hr at 30°C. After growth in liquid culture, we plated all populations onto MacConkey agar. Due to the unreliability of NBTA in preliminary studies,

we plated cultures on five MacConkey agar plates between each cycle to select primary phase bacteria for each subsequent cycle (Boemare and Akhurst, 1988). Furthermore, MacConkey agar prevents growth of many bacterial types providing extra precautions against contamination. After 36 hr we initiated the next culture cycle. All incubation steps were done in the dark.

Prior to assessment of each beneficial trait (see below), the base population and sub-population lines for each medium were thawed and cultured in parallel through one cycle so that age of the culture and other variables would not be a factor. We included three replicates of each base population and sub-population line in each assay. We assessed inclusion body prevalence every fifth cycle whereas we assessed reproduction and virulence after 20 cycles.

Inclusion Body Prevalence

To determine the effect of nutrition on the number of inclusion bodies, we tested base populations and sub-cultured populations for the prevalence of inclusion bodies. Accordingly, we placed cultured cells on a glass slide with a 1:50 dilution and visualized the bacteria and their inclusion bodies using phase-contrast microscopy (Bowen and Ensign, 2001; Wang et al., 2007). We counted the number of total cells and the number of cells containing inclusion bodies in three different fields of view at 1,000x magnification.

Reproductive Potential

To understand the impact of nutrition on the deterioration of reproductive potential, we plotted growth curves using OD₆₀₀ values for base populations and all sub-population lines. Briefly, we added approximately 10⁶ CFUs from overnight cultures of base and sub-cultured populations to 50 mL of their respective medium in 250 mL flasks followed by shaking at 250 rpm in the dark at 30°C. We sampled all populations every four hours for 48 hrs to obtain OD₆₀₀ readings.

Prior to growth curves, we determined the number of CFUs per μL for each base and sub-culture population at $\text{OD}_{600}=1.0$ to ensure we were inoculating 10^6 CFUs. We inoculated a portion of each population's frozen stock into 10 mL of the appropriate media type in 18x150 mm glass tubes. Following inoculation, we grew these cultures overnight (12-16 hr) at 30°C with shaking at 250 rpm in the dark until an $\text{OD}_{600}=1.0$ was reached. We then performed serial dilutions to verify the number of CFUs per μL .

Virulence Assays

To obtain 50 CFUs/10 μL for virulence assays, we had to first determine the number of CFUs in 500 μL of culture from each bacterial population. We grew cultures from the frozen stocks to an $\text{OD}_{600}=1.0$ overnight as described above. We washed each 500 μL culture once in 1x PBS and resuspended it in 1X PBS followed by a 10^{-5} dilution in 1X PBS. Then we plated on LB supplemented with 0.1% pyruvate. Following CFU counts we repeated these steps; however, we adjusted the 500 μL culture volume taken from the overnight culture to a volume that would ensure a final concentration of 50 CFUs/10 μL at a 10^{-5} dilution factor. For example, instead of 500 μL of the LLM base population we took 1050 μL and resuspended it in 500 μL 1x PBS. For the injection assays we grew cultures in the same manner and used the adjusted culture volume previously determined for each population.

To examine the effect of nutrition on virulence, we found LT_{50} (median lethal time) values for base and deteriorated populations by injecting *Galleria mellonella* with approximately 50 CFUs. We used fifth instar larvae that weighed between 0.19 and 0.30 g. We injected three sets of ten insects per population for each replicate. To prevent movement during injections, we kept larvae on ice prior to injections. Following bacterial preparations as described above, we injected 10 μL into the hindmost left proleg using a 27-gauge needle. Additionally, we plated 10

μL onto LB with 0.1% pyruvate to ensure 50 CFUs were injected. After injections, we kept insects in 94x16 mm petri dishes in the dark at room temperature. Every ninety minutes we noted insect mortality until all larvae were dead (~40-48 hr) and determined LT_{50} values with a logistic regression. We assessed larval mortality based on the lack of movement upon contact with forceps and the “floppy” phenotype (Daborn et al., 2002) caused by *Photorhabdus* spp.

Mechanisms of trait changes

Sub-populations that exhibited changes in a particular medium were subsequently grown in a superior medium (one that did not induce change) to see if the trait loss was recovered. We performed all assays in the same manner as described above for each trait.

Statistical Analysis

To perform logistic regressions for LT_{50} values, we used GraphPad Prism6 (La Jolla, CA, USA). Within each medium, we used JMP11 (SAS, Cary, NC, USA) to conduct ANOVA and Tukey-Kramer tests to identify significant differences among the base populations and each sub-population line; these tests were applied for inclusion body and reproductive capacity parameters. We determined confidence intervals to detect significant differences in virulence using JMP11.

Results

Inclusion Body Prevalence

The nutritional environment affected the number of cells containing inclusion bodies. *P. luminescens* subsp. *luminescens* grown in NB had the highest percentage of cells with inclusion bodies whereas TSY cultures had the least in the base population ($F=64.4350$, $DF=2, 6$, $P=0.0001$) and the sub-population lines after the 20th cycle ($F=7.1495$, $DF=8, 18$, $P=0.0003$) (Fig. 1). In LLM ($F=1.2982$, $DF=9, 20$, $P=0.2978$) and NB ($F=2.0380$, $DF=9, 20$, $P=0.0887$) the percentage of cells containing inclusion bodies did not change over the sub-culturing process.

However, after sub-culturing in TSY (F=11.9896, DF=9, 20, P>F 0.0001) the percentage of cells containing inclusion bodies increased over time in two population lines (Fig. 1).

TSY base and 20th cycle sub-population lines grown once in LLM were no longer significantly different from one another (F=0.1396, DF=3,8, P=0.9335) (Fig. 2). Additionally, these cultures were no longer significantly different than LLM (F=1.1439, DF=13, 28, P=0.3669) and NB (F=1.5281, DF=13, 28, P=0.1684) cultures.

Reproductive Potential

Growth rates of *P. luminescens* subsp. *luminescens* were different in each media type at the 48hr time point (F=96.1652, DF=2,6, P<0.0001) (Fig. 3). *P. luminescens* subsp. *luminescens* grew the fastest in LLM and the slowest in NB. There were no significant differences between the base population and the sub-population lines grown in LLM (F=2.8645, DF=3, 8, P=0.1040). However, there were some differences between the base population and the sub-population lines grown in NB (F=6.9475, DF=3, 8, P=0.0128) and TSY (F=4.6670, DF=3, 7, P=0.0428). NB sub-population lines 1 and 3 actually increased in growth compared to the base culture. Furthermore, TSY sub-population line 2 grew slower than line 3.

NB and TSY base populations and sub-population lines grown in LLM no longer showed any significant difference in growth rates (NBLLM F=3.7015, DF=3, 8, P=0.0616; TSYLLM F=2.3560, DF=3, 8, P=0.1479). Additionally, NBLLM and TSYLLM base cultures were not significantly different from the LLM base culture (F=0.4964, DF=2, 6, P=0.6317).

Virulence

Nutrition did not have an overall effect on virulence changes during repeated sub-culturing in LLM and NB media. There were no significant changes in virulence between the base populations and their respective sub-cultured populations, as a group or individual sub-

population lines, in any of the nutritional environments (Tables 3-6). However, there were significant differences among the individual 20th cycle TSY-grown sub-population lines. Significant differences found among the individual TSY sub-population lines were not significant when subsequently grown in LLM prior to virulence assays.

Discussion

Contrary to previous studies (Bilgrami et al., 2006; Wang et al., 2007), our study on the effects of *in vitro* serial culture on *P. luminescens* subsp. *luminescens* did not reveal evidence of trait deterioration. We found no significant decrease between the base population and the sub-population lines in the percentage of inclusion bodies, reproductive potential, or virulence. There were some instances in the sub-population lines where traits exhibited variation or actually became better (as exhibited by increased growth or higher production of inclusion bodies); however, there was no clear sign of trait deterioration of *P. luminescens* subsp. *luminescens* in any of the three nutritional environments.

There are a number of possible reasons that our results differ from previous research. Some strains may be more resistant to trait loss than others, trait deterioration could be driven by environmental differences, or twenty sub-culture cycles may not have been an adequate amount of time to evolve observable trait changes. We suggest that while given enough generations one could expect evolutionary changes in just about any trait measured, over the 20 cycles for which we observed trait changes, these changes were most likely due to a combination of strain and environmental (nutritional) differences. This study used only one strain of *P. luminescens* subsp. *luminescens*. Other studies that showed trait deterioration used different strains (Bilgrami et al., 2006; Wang et al., 2007).

The one medium, TSY, where a change was found in virulence among the sub-populations lines is the same medium previously used to show trait deterioration (Wang et al., 2007). This may suggest that trait deterioration is dependent on the nutritional resources available for bacterial growth. However, even in TSY our strain of *P. luminescens* subsp. *luminescens* did not display trait deterioration from the base population to the deteriorated populations as previously shown (Wang et al., 2007). One potential reason for this is the difference in the host organism we used. We used *G. mellonella* whereas the Wang et al. study (Wang et al., 2007) used *T. molitor*. *G. mellonella* is not a natural host and is highly susceptible to *Photorhabdus* spp. However, other studies have demonstrated trait deterioration of the nematode-bacterium complex using *G. mellonella* (Bilgrami et al., 2006). Additionally, some of our unpublished work has shown similar virulence patterns in *G. mellonella* and *T. molitor*. Therefore, *G. mellonella* is an acceptable host for testing virulence and is likely not the reason for the differences between our study and previous studies.

Repeated sub-culturing of *P. luminescens* subsp. *luminescens* produced a variety of trait-specific results. Inclusion body production, which is essential for nematode growth, increased in two sub-population lines after repeated sub-culturing in the TSY medium. Two sub-population lines grown in NB also increased in growth rates after repeated sub-culturing. Furthermore, the three sub-population lines grown in TSY media displayed differing levels of reproductive potential and virulence. One line was significantly slower growing and less virulent than the other two lines suggesting stochastic effects associated with the study system; traits may not always deteriorate or respond the same way every time. Similarly, *in vitro* studies of trait deterioration may not be descriptive of what happens *in vivo*. While our study had multiple experimental lines to study random changes and providing repetition, it would be appealing for

future work to repeat this study. However, based on our observations of stochastic changes from population to population, we suspect that if sub-culturing were to be replicated results would differ from our study.

The only other study that investigated trait deterioration in *Photorhabdus* sp. showed that virulence does not always decrease over time (Wang et al., 2007). One *Photorhabdus* sp. strain decreased in virulence and one strain increased in virulence. In this same study, *Xenorhabdus nematophila*, the symbiont of *S. carpocapsae*, showed an increase in virulence. Thus, most evidence suggests that trait deterioration is not an inevitable outcome of lab culturing.

Previous studies showed that trait changes in EPNs can be attributed to genetic changes (Chaston et al., 2011). However, no one has demonstrated that *Photorhabdus* spp. trait changes are genetic or environmentally induced. If trait changes were observed over time, we determined if alterations were tied to the nutritional regimes or potentially genetically based by subsequently growing populations in LLM since there were no trait changes observed in populations sub-cultured in LLM. If the trait levels were recovered then an environmental basis was indicated. Our test to determine the underlying causes of virulence differences indicates environmental drivers of variation rather than stable genetic changes. If TSY cultures displayed stable genetic changes, we would have expected the same results when grown in LLM. However, virulence levels were restored to normal levels after overnight growth in LLM. We saw the same outcome with inclusion body production and reproductive potential. Therefore, the trait changes we observed were likely due to the nutritional environment in which they were grown and not genetic modifications.

The canola:olive oil combination in the LLM formulation we used in this study was shown to promote high nematode and bacterial yields compared to other lipid sources (Yoo et al.,

2000). Research on lipid media formulation has focused on stable yield production (Yoo et al., 2001; Yoo et al., 2000); however, there has not been any research on how lipid media affects the stability of other traits in the nematode or the bacterium over a prolonged period of time. Due to proprietary information it is unknown what each industrial company uses to produce their EPNs; though, lipid sources are needed in any formulation. Our study reinforces the use of lipid media in mass-production of EPNs as it prevented trait deterioration in *P. luminescens* subsp. *luminescens* and restored any trait changes displayed in other media to wild-type levels.

Previous research has determined optimal protein, carbon, and lipid sources for increasing yields of EPNs and their symbiotic bacteria (Abu Hatab and Gaugler, 2001; Cho, 2011; Gil et al., 2002; Yoo et al., 2001; Yoo et al., 2000). However, the components of various media have not previously been compared to trait changes in *Photorhabdus* spp. In our study the two media types in which *P. luminescens* subsp. *luminescens* displayed stable traits were NB and LLM. The base protein sources in our LLM formulation are the same as NB. NB has peptone and meat extract to provide essential amino acids, vitamins, energy sources, and growth factors. However, TSY is composed of casein and soybean digests for the main source of protein. Therefore, we suggest that the protein source may play an important role in trait stability. However, additional ingredients are necessary for high yields.

Concluding Remarks

EPNs and their symbiotic bacteria are valuable biocontrol agents that have shown much potential; however, widespread adoption has been slow, in part due to difficulties during mass-production, including trait deterioration. We have shown that environmental conditions affect essential phenotypes in *P. luminescens* subsp. *luminescens*. Therefore, using proper growth media should be taken into consideration for mass-producing EPNs to prevent trait loss.

Additionally, this study used only one strain of many *P. luminescens* strains; therefore, our results also highlight the need each newly isolated strain to be tested for optimal growth conditions. The environment affects biocontrol traits in a wide variety of organisms (Blossey and Hunt, 1999; Cabrefiga et al., 2011; Dias et al., 2008). Developing favorable growth conditions may aid in the development of superior biocontrol agents/strains, help prevent trait deterioration, and increase the effectiveness of biocontrol agents in IPM programs. Furthermore, Our findings emphasize the importance of exploring gene by environment interactions when assessing biocontrol-associated traits, especially as these traits are developed for applications in pest management programs, and the necessity of future work on environmental effects on trait deterioration.

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Figure Legends

Figure 1. Inclusion body production by *Photorhabdus luminescens*. Growth in (A) lipid liquid medium (LLM), (B) nutrient broth (NB), and (C) tryptic soy broth + 0.5% yeast extract (TSY) media. The percentages of cells with inclusion bodies were calculated using phase-contrast microscopy for base populations and after sub-culture cycles 10, 15, and 20. ANOVA and Tukey-Kramer tests were done within each nutritional type. Differing letters denote significant differences at $P < 0.05$.

Figure 2. Inclusion body production by *Photorhabdus luminescens*. After 20 cycles in TSY the base and sub-cultured populations were grown once in LLM (TSYLLM) and compared to LLM and NB base populations. The percentages of cells with inclusion bodies were calculated using phase-contrast microscopy. Differing letters denote significant differences at $P < 0.05$.

Figure 3. Reproductive potential of *Photorhabdus*. Growth in (A) lipid liquid medium (LLM), (B) nutrient broth (NB), and (C) tryptic soy broth + 0.5% yeast extract (TSY) media plus (D) NB cultures grown in LLM, and (E) TSY cultures grown in LLM. To examine growth rates, OD_{600} values were obtained every four hours for forty-eight hours and plotted to attain bacterial growth curves.

Tables

Table 1. Comparison of methods in trait deterioration studies.

	Blackburn et al.	Wang et al. (2007)	Bilgrami et al. (2006)
Organism	<i>P. luminescens</i> susp. <i>luminescens</i> (strain K22)	<i>P. luminescens</i> (strain Hb-NJx and Hb-GA)	<i>H. bacteriophora</i> - <i>P. luminescens</i> complex (strains Hb-NJx and Hb-GA)
Sub-culture method			
Culture Medium	<i>in vitro</i> 50 mL liquid culture	<i>in vitro</i> 50 mL liquid culture	<i>in vivo</i> using <i>G. mellonella</i>
Broth	LLM, NB, TSY	TSY	N/A
Inoculum	50 CFU	20-30 CFU	N/A
Phase Selection	Primary phase selection	Primary phase selection	N/A
Culture Time	48 hr	48 hr	2-3 EPN generations
Bioassays			
Cell and Inclusion Body Size	N/A	1:50 dilution	N/A
		1000x with 3 fields of view	
		Unknown method of microscopy	
Inclusion body production	1:50 dilution	1:50 dilution	N/A
	1000x with 3 fields of view	1000x with 3 fields of view	
	Phase-contrast microscopy	Unknown method of microscopy	
Cell Type (phase switching)	N/A	Repeated sub-culture without primary phase selection	N/A
Reproductive Potential	10 ⁶ inoculum in 50 mL	10 ⁹ inoculum in 50 mL	Investigated reproductive potential of nematode
	OD ₆₀₀ values	OD ₆₀₀ values	
	Every 4 hrs for 48 hrs	Every 4 hrs for 48 hrs	
Virulence	<i>G. mellonella</i>	<i>T. molitor</i>	<i>G. mellonella</i>
	LT ₅₀	LD ₅₀	% mortality at 72 hrs
	Number of cells injected based on CFUs	Number of cells injected based on counting total cells with a Petroff-Hausser chamber	

Table 2. Nutritional content of each media type.

	Energy Source	Protein Source (amino acids, vitamins, growth factors)	% Protein Source	Lipid Source	% Lipid	Salts	% Salt
NB	Beef extract	Peptone	0.5	-	0	-	0
		Beef extract	0.3				
LLM	Canola oil	Peptone	0.5	Canola oil	1.25	NaCl	0.4
	Beef extract	Beef extract	0.3	Olive oil	1.25	MgSO ₄	0.05
		Yeast extract	0.5	Cholesterol	0.02	CaCl ₂	0.03
		Lactalbumin hydrolyzate	1.0			KCl	0.03
		Liver extract	0.01				
TSY	Dextrose	Casein digest	1.7	-	0	NaCl	0.5
		Yeast extract	0.5			K ₂ HPO ₄	0.25
		Soybean digest	0.3				

Table 3. Virulence of liquid lipid medium (LLM)-grown base (B) and sub-cultured population lines after cycle 20 (lines 1, 2, and 3).

Media Type	LT₅₀	95% Confidence Intervals
LLMB ^a	37.7958	34.997-40.595
LLM-1 ^a	36.7958	33.997-39.595
LLM-2 ^a	38.0792	35.280-40.878
LLM-3 ^a	36.8708	34.072-39.670

*letters denote significant differences

Table 4. Virulence of nutrient broth (NB)-grown base (B) and sub-cultured population lines after cycle 20 (lines 1, 2, and 3).

Media Type	LT₅₀	95% Confidence Intervals
NBB ^a	35.3083	32.434-38.183
NB-1 ^a	32.2875	29.413-35.162
NB-2 ^a	33.425	30.55-36.3
NB-3 ^a	32.4125	29.538-35.287

*letters denote significant differences

Table 5. Virulence of tryptic soy broth+yeast (TSY)-grown base (B) and sub-cultured population lines after cycle 20 (lines 1, 2, and 3).

Media Type	LT₅₀	95% Confidence Intervals
TSYB ^{ab}	35.1750	33.820-36.530
TSY-1 ^a	37.0417	35.687-38.397
TSY-2 ^b	33.1042	31.749-34.459
TSY-3 ^b	33.8583	32.503-35.213

*letters denote significant differences

Table 6. Virulence of tryptic soy broth+yeast (TSY)-grown base (B) and sub-cultured population lines after cycle 20 (lines 1, 2, and 3) when grown once in LLM.

Media Type	LT₅₀	95% Confidence Intervals
TSYLLMB ^a	37.3278	35.801-38.854
TSYLLM-1 ^a	38.0778	36.551-39.604
TSYLLM-2 ^a	37.7278	36.201-39.254
TSYLLM-3 ^a	37.7889	36.262-39.316

*letters denote significant differences.

Figures

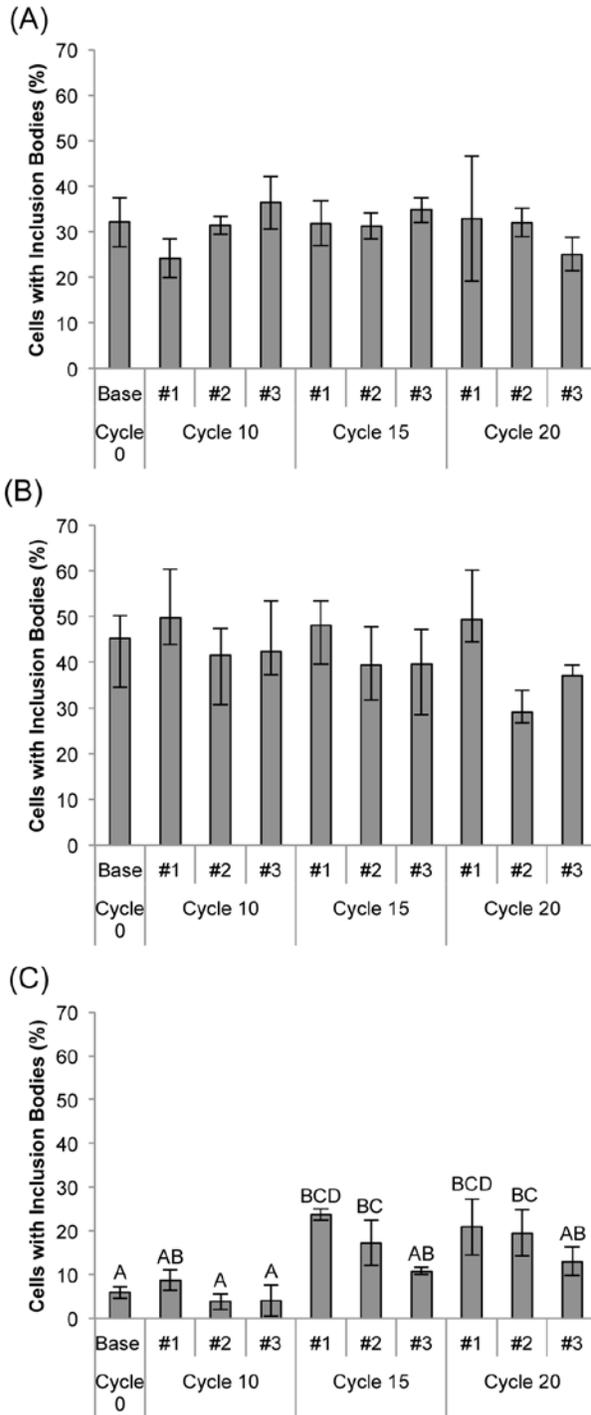


Figure 1. Inclusion body production by *Photothabdus luminescens*.

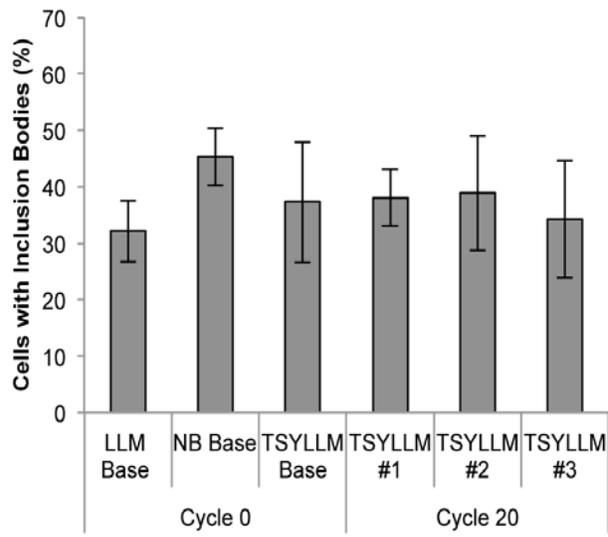


Figure 2. Inclusion body production by *Photorhabdus luminescens*.

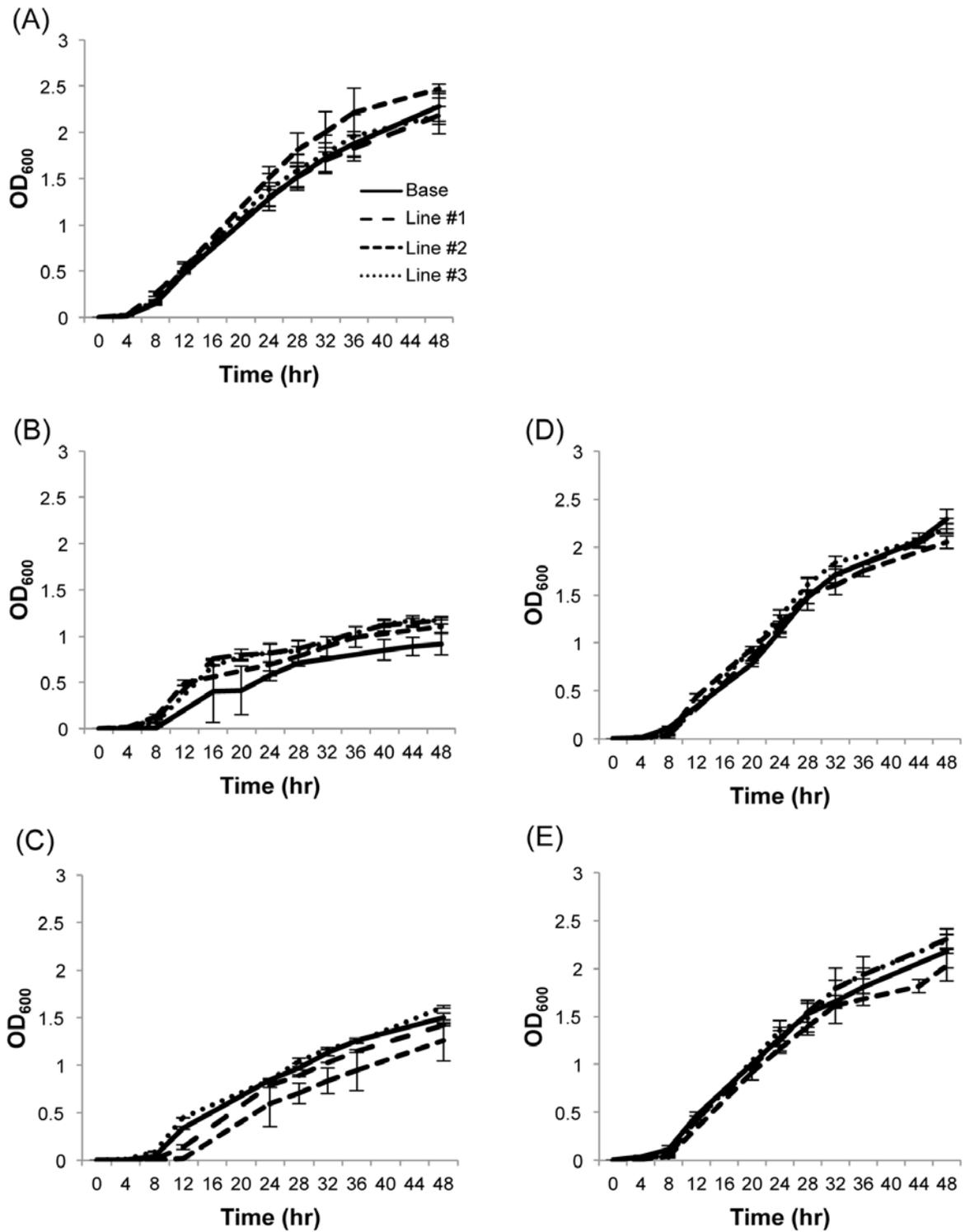


Figure 3. Reproductive potential of *Photorhabdus*.

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Chapter 4

Evolution of virulence in *Photorhabdus* spp., entomopathogenic nematode symbionts

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Abstract

Photorhabdus is a genus of Gram-negative bacteria belonging to the Enterobacteriaceae family. In addition to forming a mutualistic relationship with the Heterorhabditidae family of nematodes, these bacteria are the causal agent of insect mortality during nematode infection, and are commonly used as biological control agents against pest insects in managed ecosystems. There are three described species of *Photorhabdus*; *P. luminescens* and *P. temperata*, which are strictly entomopathogens, and *P. asymbiotica*, which has been isolated from wound infections in humans. While there has been extensive research on its virulence mechanisms, the evolution of virulence in *Photorhabdus* has not previously been investigated within a phylogenetic context. To investigate how virulence has evolved in this genus, we first reconstructed the phylogenetic relationships among 18 strains representing each of the main taxonomic lineages in the genus. Bacterial cells were injected into *Galleria mellonella* and *Tenebrio molitor* larvae, and the LT_{50} was calculated for each strain. These values were mapped onto the phylogeny using ancestral reconstruction methods. With few exceptions, we found that the general trend of *Photorhabdus* evolution is one of increasing virulence. We also explored the relationship between virulence and *Photorhabdus* cell types and growth rates. Although we found weak or no correlation between cell type and virulence, there was moderate to strong correlation between virulence and growth rates. A better understanding of the origin and maintenance of virulence in this bacterium will aid in unraveling the mechanisms of the *Heterorhabditis-Photorhabdus* complex, resulting in the selection of more effective nematode-bacterium complexes for biological control.

Keywords: *Photorhabdus*, entomopathogenic nematode, phylogeny, virulence, ancestral reconstruction

Introduction

Entomopathogenic nematodes (EPNs) utilize a bacterial endosymbiont to kill a wide range of insect hosts. The EPN genus *Heterorhabditis* forms a mutualistic relationship with species in the genus *Photorhabdus*. *Photorhabdus* cells are carried as symbionts in the gut of the infective juvenile (IJ) stage of the *Heterorhabditis* nematode [9]. The IJ is a non-feeding stage and the only free-living stage in the *Heterorhabditis* life cycle. Upon finding a suitable insect host the IJ enters through natural openings such as the mouth or anus, migrates to the bloodstream (hemolymph), and releases its symbiotic bacteria [23]. *Photorhabdus* grows rapidly causing insect death through septicaemia. The nematode grows, develops and reproduces by feeding on the high-density of bacterial symbionts in the dead insect. The nematodes feed exclusively on the bacterial biomass within the insect and, after about 7-10 days, a new generation of IJs, each one colonized by the mutualistic bacteria, will emerge from the insect cadaver to search out new insect hosts [9, 10, 39].

Photorhabdus spp. produce a wide array of virulence factors resulting in insect mortality within 24-48 hrs post-infection. Genomic sequencing revealed that *Photorhabdus* contains more predicted toxin genes than any other sequenced bacterium, including the well described Tc and Mcf toxins [16]. Furthermore, *Photorhabdus* produces “*Photorhabdus* virulence cassettes” (PVCs) and a type III secretion system (TTSS) [20, 23]. *E. coli* transformed with PVC-containing cosmids are toxic to wax worm moth larvae and cause destruction of phagocytes [43]. The TTSS of *Photorhabdus* secretes effector proteins directly into host cells. One effector, LopT, is similar to the YopT effector of *Yersinia pestis* and prevents phagocytosis [6, 7]. Additionally, some species and/or subspecies produce urease, DNase, and hemolysins.

Photorhabdus spp. stochastically produce primary form cells and small colony variant cells [35]. Primary form cells are pathogenic while small colony variants are able to form a symbiotic relationship with the nematode [26, 35]. Therefore, primary cells have been termed P form for pathogenic and small colony variants are called M form for mutualistic. Inside the maternal nematode, P-form cells switch to M form using a single promoter to initiate the symbiotic relationship with the IJ nematode. Inside the IJ, M-form cells use the same promoter to switch back to the P form prior to being released into the insect host. M-form cells are smaller, less virulent, slower growing, less bioluminescent, and produce less secondary metabolites than their P-form counterparts [35].

Photorhabdus was initially classified as *Xenorhabdus luminescens*, within the genus *Xenorhabdus*, a group of bacterial endosymbionts of the Steinernematid family of EPNs. However, using phenotypic and molecular data, it was later placed in its own genus [5]. Three species of *Photorhabdus* have been described: *P. asymbiotica*, *P. luminescens*, and *P. temperata* based on a 16S rRNA phylogenetic analysis, phenotypic characterization, and DNA-DNA hybridization [21].

While there has been extensive work on understanding the mechanisms of virulence in *Photorhabdus* spp., the origin and maintenance of this virulence has not been explored in a phylogenetic context. The purpose of this study was to determine how virulence has evolved in *Photorhabdus* using ancestral state reconstruction with LT_{50} values as a measure of virulence. Furthermore, we investigated correlations between patterns of virulence, growth rates and cell types.

Materials and Methods

Bacterial Strains and Culture Conditions

We obtained eighteen strains with representatives from all three *Photorhabdus* species and one *Xenorhabdus nematophila* strain for outgroup character state polarization (Table 1). Of the eighteen strains two are *P. asymbiotica*, nine are *P. luminescens*, and seven are *P. temperata*. We routinely grew strains in LB supplemented with 0.1% pyruvate (LBP) at 30°C with shaking in the dark.

To avoid high variation in the number of M-form colonies, we picked five P-form colonies from each strain, grew them in a mixed culture to an OD₆₀₀=0.8 into 10 mL of the appropriate media type in 18 x 150 mm glass tubes. Following inoculation, we grew these cultures overnight (12-16 h) at 30°C with shaking at 250 rpm in the dark until an OD₆₀₀=1.0 was reached, and froze them in ½X LB+50% glycerol. These stocks were used for subsequent virulence assays.

PCR

We amplified three genetic markers from all nineteen strains – the 16S rRNA gene, gyrase B gene (*gyrB*), and glutamine synthetase gene (*glnA*) [30] – using polymerase chain reaction on a DNA Engine DYAD thermal cycler (MJ Research). The standard reaction mixture included 1X Go Taq buffer, 1.25mM MgCl₂, 0.2 mM dNTPs, 0.5 uM of each primer, and 1 unit of Go Taq polymerase (Promega, Madison, WI). To obtain template DNA for PCR reactions, we placed microcentrifuge tubes containing 100 µL of bacterial cultures in boiling water for 5 min.

To amplify 16S and *gyrB* genes, we used the following parameters: an initial denaturation at 95°C for 10 min, 40 cycles of 95°C for 15 sec, 51°C for 1 min, and 72°C for 2 min, followed by a final elongation at 72°C for 7 min. Primers for 16S are forward primer 5'-GAAGAGTTTGATCATGGCTC-3' and reverse primer 5'-AAGGAGGTGATCCAGCCGCA-3'. We used universal Enterobacteriaceae *gyrB* primers with the forward primer 5'-

TAARTTYGAYGAYAACTCYTAYAAAGT-3' and the reverse primer 5'-
CMCCYTCCACCARGTAMAGTTC-3' [15].

We used the following parameters to amplify the *glnA* gene: an initial denaturation at 95°C for 6 min, 35 cycles of 94°C for 1 min, 54°C for 1 min, and 72°C for 2 min, followed by a final elongation at 72°C for 10 min. The primers used to amplify the *glnA* gene are forward primer 5'-CCGAGTATGTCCGTTGAACATG-3' and reverse primer 5'-
CGGAACCATTATCACCAACC-3'.

Sequencing and Sequence Editing

Prior to cycle sequencing we cleaned PCR reactions using 2 µL of ExoSAP-IT (Affymetrix, Santa Clara, CA) per 5 µL of PCR product. We performed cycle sequencing using DNA Engine Dyad (MJ Research) followed by a sephadex cleanup and capillary electrophoresis on an Applied Biosystems 2720xl DNA analyzer (Life Technologies, Carlsbad, CA). Reaction mixtures for cycle sequencing included 1X sequencing buffer, 0.5 µL BigDye Terminator v3.1 Cycle Sequencing RR mix, 0.32 uM primer, and 2 µL cleaned PCR product. We used Geneious 6.1.8 (<http://www.geneious.com>) [27] to analyze and edit DNA sequences. Sequences generated in this study have been submitted to GenBank; 16S (accession numbers KT899928-KT899945), *gyrB* (accession numbers KT899909-KT899927), and *glnA* (accession numbers KT899890-KT899908).

Alignment

To align all sequence data for each marker, we used Muscle under default parameters [17]. We visually inspected the alignments using MacClade 4.08 [28]. Following the individual alignments, we used MacClade 4.08 to concatenate all alignments into one dataset [28].

Phylogenetic Analyses

We performed parsimony analyses in TNT [22] using the new technology search with ratcheting, drift, and tree fusing set at 10. We calculated bootstrap values in TNT [22] with 1000 bootstrap replicates.

Additionally, we estimated phylogenetic relationships using a model-based maximum likelihood analyses in RAxML HPC v7.5.4 [36] via the command line, with an initial search of 200 replicates for the best tree, partitioned by gene. Due to computational limitations in RAxML we applied the most complex model of molecular evolution selected using the Akaike Information Criterion (AIC) implemented in jModeltest v2.1.3 [14] to each gene (Table 1). We calculated nodal support with 1000 bootstrap pseudoreplicates via the rapid-hill climbing algorithm [36].

To understand virulence evolution in *Photorhabdus* spp., we estimated ancestral reconstructions of the continuous trait LT_{50} on the RAxML best tree. The tree was transformed into an ultrametric tree using the penalized likelihood smoothing algorithm [34] implemented in the function `chronopl` in the R package ‘ape’ [31] with the lambda set to 0.1. Ancestral character state mapping was accomplished by estimating the states at the internal nodes using a ML function, `fastANC` in the R package `phytools` [32] with the interpolation of the states along each edge using equation (2) from Felsenstein [19]. The reconstructions were then plotted using the `contMap` [33] function in R package `phytools` [32].

Virulence Assays

To obtain 50 CFUs/10 μ L for virulence assays, we had to first determine the number of CFUs in 500 μ L of culture from each bacterial population. We grew cultures from the frozen stocks to an $OD_{600}=1.0$ overnight as described above. We washed each 500- μ L culture once in 1x PBS and resuspended it in 1X PBS followed by a 10^{-5} dilution in 1X PBS. Then we plated on

LBP. Following CFU counts we repeated these steps; however, we adjusted the 500- μ L culture volume taken from the overnight culture to a volume that would ensure a final concentration of 50 CFUs/10 μ L at a 10^{-5} dilution factor. For the injection assays we grew cultures in the same manner and used the adjusted culture volume previously determined for each population.

We found LT_{50} (median lethal time) values by injecting *Galleria mellonella* or *Tenebrio molitor* with approximately 50 CFUs. To determine the LT_{50} of each bacterial strain, we used larvae that weighed between 0.19 and 0.30 g. We kept larvae on ice prior to injections to prevent movement during injections. Following bacterial preparations as described above, we injected 10 μ L into the hindmost left proleg (*G. mellonella*) or between the sixth and seventh dorsal sclerites (*T. molitor*) using a 27-gauge needle. After injections, we kept insects in 94 x 16 mm petri dishes in the dark at room temperature. Every ninety minutes we noted insect mortality until all larvae were dead (~40-48 hr) and determined LT_{50} values with a logistic regression. We assessed larval mortality based on the lack of movement upon contact with forceps and the “floppy” phenotype [13] caused by *Photorhabdus* spp. For one logistic regression we used three replicates of ten insects and repeated this process twice.

M-Form Cells

Individual P-form colonies contain M-form cells. To determine the correlation of M-form cells with overall virulence, we calculated the percentage of M-form cells found in P-form colonies for 11 strains (at least one from each clade). After streaking for pure culture, we resuspended a single P-form colony from each strain in 1 mL of 1X PBS followed by serial dilutions to a 10^{-4} dilution and plated 10 μ L onto LB or LBP agar. Following 48 h of growth at 30°C in the dark, we counted the number of M-form colonies based on size and the total number of colonies to calculate the percentage of M cells in one P-form colony from each strain.

Growth Curves

To investigate the correlation between growth rates and virulence, we compared OD₆₀₀ readings to LT₅₀ values. Prior to calculating growth curves, we inoculated 10 mL of LBP and LB in 18 x 150 mm glass tubes with a portion of a frozen stock from each strain and grew cultures for 12-16 h at 30°C with shaking at 250 rpm in the dark until an OD₆₀₀=0.8 was reached. Then we inoculated 10 mL of fresh media with 100 µL of the overnight culture and grew cultures in the same manner. We checked OD readings at 8 h and 12 h time points for eight strains (at least one from each clade). We repeated this two more times for a total of three replicates for eight strains.

Statistics

To examine correlation between virulence and either M-form cells or growth rates we determined the correlation coefficient using JMP 12 (SAS, Cary, NC, USA).

Results

Phylogeny Reconstruction

The topologies of the maximum parsimony and likelihood trees are identical and the three *Photorhabdus* species formed monophyletic clades (Fig. 1). The *P. asymbiotica* clade is supported with a parsimony bootstrap value of 76 and likelihood bootstrap of 90. *P. luminescens* is strongly supported with bootstrap values of 100 and 96 for parsimony and likelihood, respectively. The *P. temperata* clade is also strongly supported with a parsimony bootstrap value of 100 and likelihood bootstrap value of 95. Though weakly supported (parsimony bootstrap=55, likelihood bootstrap=78), *P. asymbiotica* is the sister taxon to *P. luminescens* with *P. temperata* being the sister taxon to *P. asymbiotica* + *P. luminescens*.

Ancestral State Reconstruction

LT₅₀ values showed that *G. mellonella* is more susceptible to *Photorhabdus* sp. than *T. molitor* (Table 2). In all strains, *Photorhabdus* spp. had higher LT₅₀ values for *T. molitor*. Therefore, *Photorhabdus* spp. are demonstrably less virulent towards *T. molitor*.

With few exceptions *Photorhabdus* spp. are evolving towards a more virulent state (Fig. 2). Using *G. mellonella* as a host showed that high virulence is evolving within the *P. asymbiotica* and *P. temperata* clades. However, in the *P. luminescens* clade virulence did not appear to show a clear evolutionary trend. While the more derived strains (W14, IND, ARG, Pa) were more virulent than the ancestral state, there was a decrease in virulence followed by a subsequent increase in virulence. We observed the same overall trend in *T. molitor* and *G. mellonella* with two exceptions. In *T. molitor* the *P. asymbiotica* Kingscliff and *P. temperata* Hepialius strains both display a trend towards decreased virulence.

Virulence vs. M-form cell production

There were varying percentages of M-form cells found in the tested *Photorhabdus* strains (Table 1). On LB agar, six of the eleven strains tested had higher than 10% M-form cells and all had more than 5% M-form cells in their respective P-form colonies. However, on LBP agar only three strains had more than 5% M-form cells in a P-form colony; TT01, K122, and Hm. TT01 and K122 had more than 20% M-form cells.

When compared to their respective LT₅₀ values there was no strong evidence for correlation between M cell production and virulence (Table 2). There was no correlation between M cell production and virulence in *G. mellonella*. Though there is a negative correlation between M cell production and virulence, in *T. molitor* it is a weak correlation.

Virulence vs. Reproductive Potential

We compared LT₅₀ values in *G. mellonella* and *T. molitor* with OD₆₀₀ readings at 8 h and 12 h for eight strains (Table 2). At 8 h there is a moderate negative correlation between growth rates and virulence in both *G. mellonella* and *T. molitor*. After 12 h of growth there is still only a moderate negative correlation between growth rates and virulence in *G. mellonella*. However, there is a strong negative correlation between growth rates and virulence in *T. molitor*.

Discussion

Overall, *Photorhabdus* seems to be evolving increased virulence. In *G. mellonella* we found that *P. asymbiotica* is more virulent than its reconstructed ancestral state. However, when using *T. molitor* as its host, one strain of *P. asymbiotica* has evolved an increase in virulence while the other has evolved avirulence. While this points to the possibility of that these strains are evolving host-specific virulence, validation of this trend requires additional, replicated assays from multiple strains and a broader diversity of insect hosts. In the *P. temperata* clade the most recently diverged strains have evolved high virulence in both *G. mellonella* and *T. molitor*. Therefore, with the exception of the Hepialius strain, virulence in *P. temperata* appears to be evolving towards increased insect mortality rates.

Additionally, in the *P. luminescens* clade the trend towards or away from virulence is identical in both insect hosts. Of the three main lineages in this clade, the closely related strains TT01, Pl, and Hb, reverted to decreased avirulence relative to their reconstructed ancestral state. While this seems incongruous, an alternative explanation to natural selection could be artificial selection imposed due to the length of time these strains have been in the laboratory and cultured in the absence of their nematode symbiont and insect host. Hb and TT01 are two of the oldest isolated strains in the genus. In fact, Hb was the first *Photorhabdus* sp. strain to be isolated and characterized [38]. Studies have shown that traits such as virulence can be lost after repeated

sub-culturing and this can also happen when stored in the freezer for long periods of time [2, 4, 29, 40, 41].

We observed varying levels of M-form cell production in different strains. Due to the avirulence of the M-form cells we hypothesized that the reason for the observed patterns in virulence evolution may be due to selection favoring colonies that produce fewer M-form cells. However, there was no correlation between M-form cell formation and virulence. Our interpretation of this lack of correlation is that since *Photorhabdus* spp. can have LD₅₀ values as low as 5 cells [3], the number of P-form cells must be sufficient to compensate for any variation in the number of M-cells produced.

Because faster growing *Photorhabdus* spp. have higher mortality rates [11, 12, 42], we expected to find that growth rate plays an important role in virulence evolution. Based on the results of our OD₆₀₀ readings for 8 sister strain pairs from each of the major lineages, after 8 h of growth we found a moderate negative correlation between reproductive potential and virulence in both host insect species. Thus, strains that have evolved increased growth rates have a subsequent decrease in LT₅₀, or an increase in virulence. It is important to note, however, that after 12 h of growth in *T. molitor* there was a strong negative correlation between reproductive potential and virulence there is only a moderate negative correlation between reproductive potential and virulence in *G. mellonella*, suggesting that growth rate alone is insufficient to explain the increased virulence in these strains and that virulence is more likely a much more complex and nuanced trait.

There are several reasons why it would be advantageous for entomopathogenic nematodes to associate with bacteria that grow and kill faster. First, faster growing bacterial endosymbionts kill the insect host faster than slow ones, allowing the nematodes with a more

rapidly growing endosymbiont to reproduce sooner than nematodes with a slow growing endosymbiont. Additionally, since the bacteria are the primary food source for the nematode, endosymbionts with high growth rates would provide more food, earlier, for their nematode host than slow growing bacteria. As resources in the host insect diminish, more bacteria in the insect cadaver could result in an increased chance of successful re-association with their nematode symbiont. Ultimately, the best explanation for strong selection for rapid bacterial growth is that it results in a more efficient, competitive harvesting of resources from the insect host.

Another explanation for the observed trend of evolving increasingly higher virulence is that this trait is an important part of maintaining successful pathogenesis across a broad host range [8]. High virulence hinders insects from vectoring nematodes to places of similar insect species such as host nests. Therefore, because highly virulent EPN/B can have dramatic effects on the population density of local hosts [37], IJs emerging from an extinguished cadaver are increasingly likely to encounter a different host species than the one from which they most recently emerged. A broad host range increases the need for high virulence, and vice versa, creating a positive feedback loop that is reinforced by the requirement for a highly virulent pathogen to have a broad host range [1, 18, 24, 25].

Interestingly, the virulence ancestral reconstructions were almost identical between *G. mellonella* and *T. molitor*. *G. mellonella* infest beehives and EPNs are found in the soil; therefore, *G. mellonella* is not a natural host of EPNs or *Photorhabdus* spp. Though *G. mellonella* has been used extensively to test virulence in *Photorhabdus*, it has been suggested that because it is not a natural host, it is unsuitable for this purpose [41]. Although *T. molitor* is also not a natural host, its many coleopteran relatives are, suggesting it much more closely resembles a natural host for EPNs. Therefore, the similarities in virulence patterns between *G. mellonella* and *T. molitor*

suggest that *G. mellonella* may be a suitable model for testing virulence in *Photorhabdus* spp. after all.

Concluding remarks: EPNs are important biocontrol agents that are used frequently in integrated pest management programs. However, EPNs have not gained widespread use partially due to trait loss such as virulence during production. Insights into the origin and maintenance of virulence among *Photorhabdus* spp. may aid in choosing strains for commercial applications. For example, depending on the application, choosing a *P. temperata* strain over a *P. luminescens* strain may be beneficial, since it has clearly evolved very high virulence. Additionally, understanding the reasons for high virulence, such as M-form vs. P-form cells and the factors responsible for growth rates can also help in choosing particular strains and improving production methods. Taking these factors into consideration may help increase the use of EPNs in crop production, resulting in decreased use of harmful pesticides.

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Figure Captions

Fig. 1. Phylogenetic Reconstructions. Maximum parsimony (A) and maximum likelihood (B) trees for all *Photorhabdus* spp. strains included in this study. Nodal support as inferred from bootstrap replicates are indicated below branches.

Fig 2. Ancestral Reconstruction of Virulence. LT_{50} values from injecting *G. mellonella* (A) and *T. molitor* (B) with *Photorhabdus* spp. mapped onto the maximum likelihood tree.

Tables

Table 1. The best-fit models of molecular evolution for each gene.

Gene	AIC	BIC	Applied
16S rRNA	HKY+I+G	HKY+I	GTR+G
<i>glnA</i>	SYM+G	SYM+G	GTR+G
<i>gyrB</i>	GTR+G	K80+G	GTR+G

Table 2. Virulence, M-form cells, and reproductive potential of *Photorhabdus* strains.

Strain	LT ₅₀ (hr) <i>G. mellonella</i>	LT ₅₀ (hr) <i>T. molitor</i>	% M-Form Cells (LB)	% M-Form Cells (LBP)	OD ₆₀₀ (8 hr)	OD ₆₀₀ (12 hr)
ARG	34.15±1.41	42.19±1.02	15.93±19.36	3.16±1.43	0.45±0.02	0.88±0.003
Hb	54.24±3.06	88.89±5.97	5.25±4.10	3.16±1.59	0.16±0.02	0.51±0.03
Hepialius	34.78±1.58	61.37±5.90	11.24±2.33	0.12±0.35	0.26±0.04	0.72±0.04
Hm	39.76±0.45	50.43±3.13	36.99±30.46	3.16±1.59	0.15±0.01	0.74±0.06
HP88	34.86±2.66	45.98±4.32	20.36±7.32	1.17±1.10	NA	NA
IND	33.72±1.30	40.83±1.64	7.28±5.40	3.10±2.69	NA	NA
K22	35.98±0.22	43.0±1.51	NA	NA	NA	NA
K122	35.97±0.60	44.41±1.12	77.12±14.74	20.92±5.81	NA	NA
Kesha	34.65±0.64	44.57±2.70	NA	NA	NA	NA
Kingscliff	33.16±2.43	51.38±5.64	5.055±3.36	NA	0.43±0.002	0.714±0.01
NC1	34.29±0.92	45.04±2.52	NA	NA	NA	NA
NC19	34.26±1.19	44.09±1.73	7.95±5.85	0.76±1.03	NA	NA
Pa	33.37±1.67	42.56±1.29	NA	NA	NA	NA
Pl	47.41±5.55	63.32±6.05	NA	NA	NA	NA
Ps	34.6±2.56	41.71±1.49	NA	NA	NA	NA
Pt	36.37±1.93	45.37±3.77	NA	NA	0.26±0.02	0.64±0.03
TT01	39.01±3.65	45.56±3.10	64.81±14.4	28.06±10.08	0.25±0.02	0.83±0.03
W14	37.53±1.09	49.11±2.21	7.002±3.54	3.37±0.93	0.489±0.07	0.84±0.06
<i>X. nematophila</i>	42.17±1.14	65.99±8.45	NA	NA	NA	NA

Table 3. Virulence correlation coefficients.

Insect Host	M-Form Cells (LB)	M-Form Cells (LBP)	OD₆₀₀ (8 hr)	OD₆₀₀ (12 hr)
<i>G. mellonella</i>	-0.024*	0.0622*	-0.6007*	-0.6718*
<i>T. molitor</i>	-0.2954*	-0.2193*	-0.5012*	-0.7899*

*p<0.0001 for all correlation coefficients.

Figures

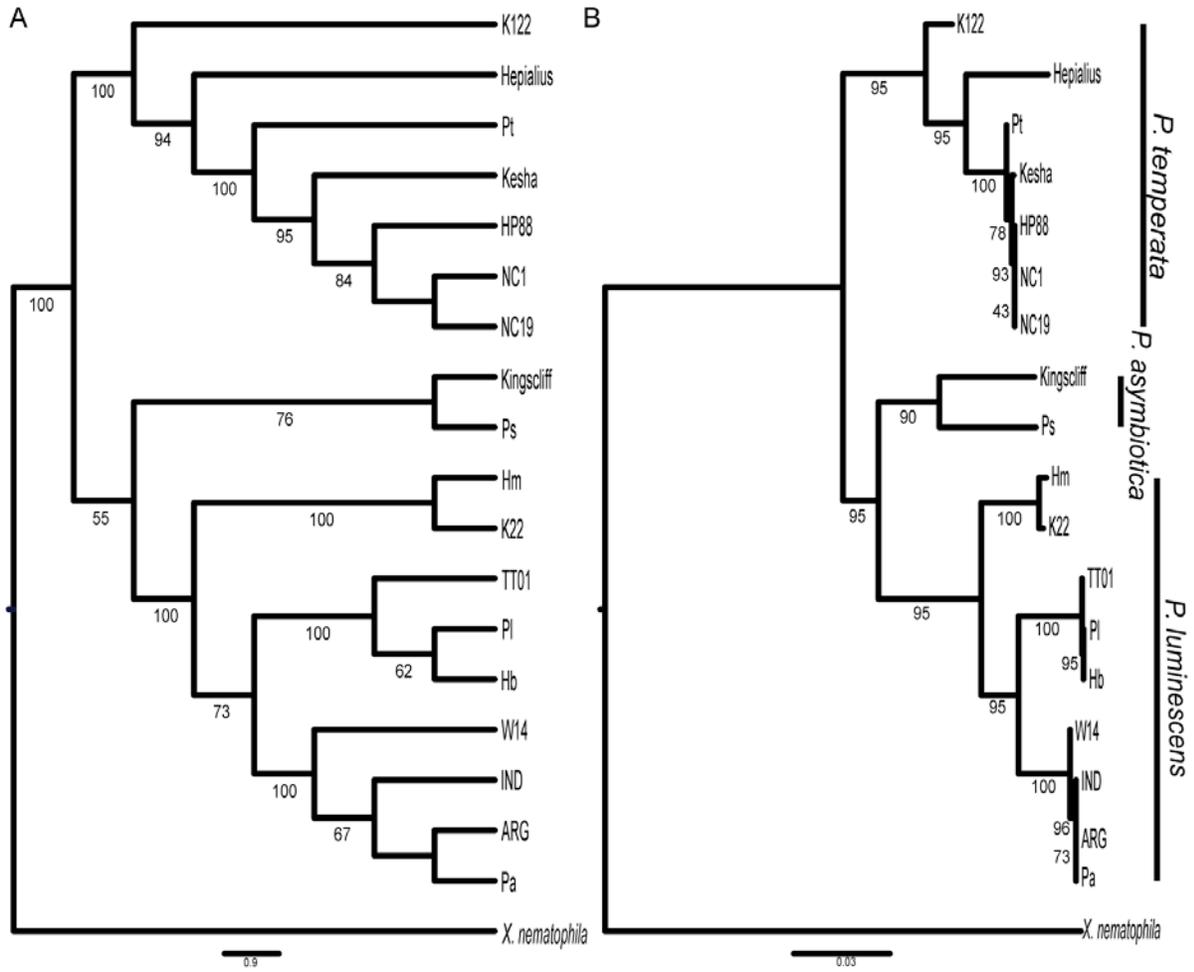


Figure 1. Phylogenetic Reconstructions.

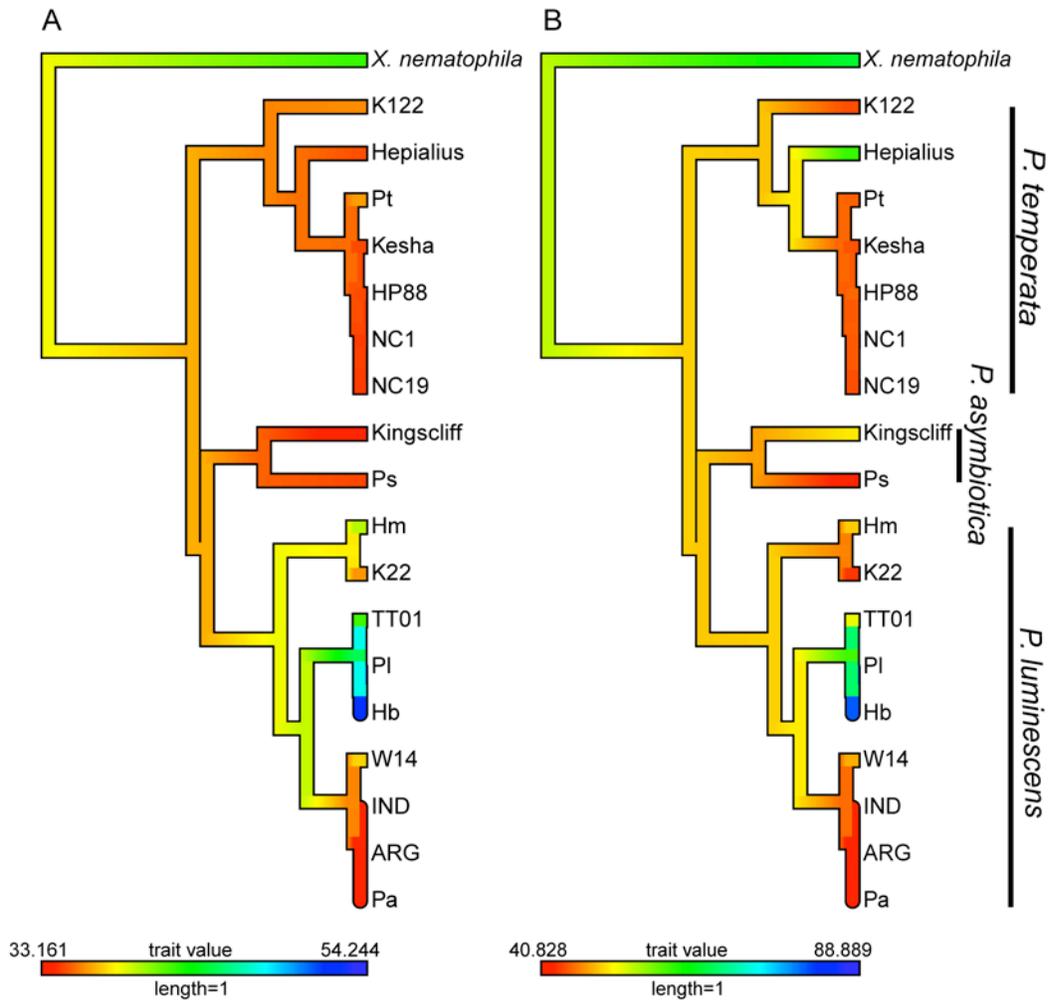


Figure 2. Ancestral Reconstruction of Virulence.

Chapter 5

Identification of critical virulence genes of *Photorhabdus* using Tn-seq

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Abstract

Photorhabdus is a genus of Gram-negative bacteria belonging to the Enterobacteriaceae family. *Photorhabdus* forms a symbiotic relationship with the Heterorhabditidae family of entomopathogenic nematodes (EPNs) and together this complex is pathogenic to a wide variety of insect hosts. *Photorhabdus* plays a number of roles in its mutualistic relationship with *Heterorhabditis*. In addition to forming a specific, symbiotic relationship with the nematode, *Photorhabdus* is primarily responsible for insect mortality. *Photorhabdus* spp. achieve high insect mortality rates using various virulence factors including toxins, proteases, lipases, and a number of secretion systems. Most studies that have identified genes involved in *Photorhabdus* virulence screened transposon mutant libraries a single colony at a time. Transposon sequencing (Tn-seq) is a tool that combines transposon mutagenesis and high-throughput sequencing to quantitatively screen for single gene fitness [1].

However, there have been no studies that have utilized Tn-seq to examine *Photorhabdus* virulence. In this study we employed Tn-seq to identify genes that are essential to *Photorhabdus* virulence and survival inside the insect host *Galleria mellonella*. We have identified 84 genes needed for survival inside the insect host with many genes showing consistent phenotypes with previous studies. We discuss a number of these genes in detail and the potential for future studies of these genes.

Keywords: *Photorhabdus*, entomopathogenic nematode, Tn-seq, virulence

Introduction

Entomopathogenic nematodes (EPNs) are soil-dwelling organisms that utilize a bacterial symbiont to kill a wide variety of insect hosts. The two main genera (*Steinernema* and *Heterorhabditis*) exhibit similar life cycles. An infective juvenile (IJ), similar to the *Caenorhabditis elegans* dauer juvenile and the only free-living stage, enters an insect host via natural openings. Their symbiotic bacteria are released and send molecular cues to the nematode to exit the IJ stage and develop into adults [2, 3]. After 2-3 generations when nutrients inside the insect cadaver become limited a new generation of IJs, each colonized with their symbiotic bacteria, exit in search of a new insect host [4-6].

Photorhabdus is a genus of Gram-negative bacteria belonging to the Enterobacteriaceae family. *Photorhabdus* forms a symbiotic relationship with the Heterorhabditidae family of EPNs by lining the gut of each nematode [4]. *Photorhabdus* plays a number of roles in its mutualistic relationship with EPNs. In addition to forming a specific, symbiotic relationship with the nematode, *Photorhabdus* is primarily responsible for insect mortality [7] and is the primary food source for the nematodes providing nutrients for growth and development [8]. Each role has been extensively studied; however, there is still a great deal that is poorly understood.

Photorhabdus spp. achieve high insect mortality rates using various virulence factors with high growth rates being tightly correlated with high virulence rates [9, 10]. Additionally, *Photorhabdus* contains more predicted toxin genes than any other sequenced bacterium [11]. The best-described toxins are the Mcf and Tc toxins. The Mcf (makes caterpillars floppy) toxins are a group of large toxins that cause apoptotic cell death in the insect midgut and hemocytes resulting in the “floppy” phenotype where insects lose body turgor [12]. Tc toxins were originally discovered in *Photorhabdus* [13, 14], but have since been discovered in a variety of other

bacteria such as *Xenorhabdus*, *Serratia*, *Bacillus*, *Burkholderia*, and *Yersinia* [15]. In *Photorhabdus*, there are at least four Tc toxin complexes (A, B, C, and D) with multiple open reading frames associated with each one (ex: TcaA, TcaB, TcaC, etc.) [14]. The A, B, and C subunits of each complex are needed for full activity [16, 17]; though, subunits from different complexes can be functionally mixed and matched [16, 18]. The number of Tc toxins and *tc*-like homologs being discovered continues to grow with many genomes containing multiple Tc complexes [19].

Photorhabdus also produces “*Photorhabdus* virulence cassettes” (PVCs) that are prophage-like loci that contain type VI secretion system-type genes and toxin effector proteins. *E. coli* transformed with PVC-containing cosmids are destroyed by wax worm moth phagocytes [20]. Additionally, *Photorhabdus* contains a type III secretion system (TTSS) [21, 22] that secretes effector proteins directly into host cells such as LopT, which is similar to the YopT effector of *Yersinia pestis* and prevents phagocytosis [23, 24]. Other molecules and proteins that have been shown to be important in *Photorhabdus* virulence include molecules such as regulators, secondary metabolites, and stress response proteins [25-27].

Most studies that have identified specific genes involved in *Photorhabdus* virulence screened individual colonies from transposon mutant libraries [25, 28, 10]. Additionally, genome-wide screens used cosmids expressed in *E. coli* to identify genes toxic to insects [29, 30]. However, there have been no studies that have utilized high-throughput sequencing to examine *Photorhabdus* virulence. Transposon sequencing (Tn-seq) is a tool that combines transposon mutagenesis and high-throughput sequencing to quantitatively screen for single gene fitness [1]. Tn-seq was initially designed to understand genetic interactions in *Streptococcus pneumoniae*, but has since been widely used to discover essential genes in various traits, such as virulence,

antimicrobial targets, biofilm production, cell wall biogenesis, and growth in diverse microorganisms [31-35].

In this study we utilized Tn-seq to identify genes that are essential to *Photorhabdus* virulence and survival inside the insect host *Galleria mellonella*. We have identified 84 genes needed for survival inside the insect host with many genes showing consistent phenotypes with previous studies. Furthermore, we have discovered genes in *Photorhabdus* that are crucial for virulence in other bacterial species, but have not yet been characterized in *Photorhabdus*. We also discuss other important virulence genes that have not previously been well described and the potential for future work.

Material and Methods

Strains and Culture Conditions

To discover strains useful for transposon mutagenesis we used eighteen strains with representatives from all three *Photorhabdus* species. Of the eighteen strains two are *P. asymbiotica*, nine are *P. luminescens*, and seven are *P. temperata*. We used the *P. luminescens* subsp. *akhurstii* IND strain to identify genes crucial for virulence. For all cultures we routinely grew strains on LB agar supplemented with 0.1% pyruvate (LBP) at 30°C in the dark while liquid cultures were also grown with shaking at 250 rpm.

Additionally, we used *E. coli* c812 and c814, which are both MFDpir strains. However, c814 also contains pJG714, a 4,500bp plasmid containing the Tn5-110 transposon. We grew *E. coli* strains in LB media supplemented with diaminopimelic acid (DAP) (12.5 mg/mL) at a 1:250 ratio. We added kanamycin (30 mg/mL) (Kn30) at a 1:1000 ratio to all *P. luminescens* and *E. coli* cultures containing the plasmid and/or the transposon.

Phylogenetic Reconstruction

We used three genetic markers to reconstruct the phylogeny of the 18 *Photorhabdus* strains tested for transposon mutagenesis efficiency that we previously sequenced (Blackburn et al. 2015, submitted): the 16S rRNA gene, gyrase B gene (*gyrB*), and glutamine synthetase gene (*glnA*) [36]. GenBank accession numbers are KT899928-KT899945 for the 16S rRNA gene, KT899909-KT899927 for *gyrB*, and KT899890-KT899908 for *glnA*. To align all sequence data for each marker, we used Muscle under default parameters [37]. Following the individual alignments, we used MacClade 4.08 to concatenate all alignments into one dataset [38]. We performed parsimony analyses in TNT [39] using the new technology search with ratcheting, drift, and tree fusing set at 10. We calculated bootstrap values in TNT [39] with 1000 bootstrap replicates.

Transposon Mutagenesis

We inoculated 30 mL of LB+1 mM MgCl₂ (MgLB) in a 125-mL Erlenmeyer flask with overnight cultures of c814 and each target *Photorhabdus* strain to an OD₆₀₀=0.1. We used c812 as a negative control for any strain that was positive for transposon mutagenesis and was treated in the same manner. When cultures reached an OD₆₀₀=0.6 we centrifuged and washed 1 mL of each culture 2X with fresh MgLB+DAP. We then resuspended cultures in 100 µL of MgLB+DAP and combined the donor and recipient strains for a 1:1 ratio. We spotted cultures onto LBP+DAP agar plates and incubated at 30°C in the dark for 5 h. Following incubation, we scraped conjugation reactions off plates with 650 µL LBP and spread 100-µL aliquots onto LBP+Kn30 agar plates. We incubated plates at 30°C in the dark for 48 h. After incubation colonies were counted to determine the efficiency of transposon mutagenesis per strain.

To obtain the final transposon mutant library for virulence screening, *P. luminescens* subsp. *akhurstii* IND and c814 were grown as described above, but in 40 mL MgLB. After

centrifuging and washing the entire culture, we resuspended each culture in 1 mL and combined together. Then we aliquoted 200 μ L onto 10 LBP+DAP agar plates and incubated for 4 h. After incubation, we scraped off each conjugation reaction using 2 mL of LB+15% glycerol, combined cultures, and froze them at -70°C . To determine the number of mutants, we diluted a portion of the conjugation reaction and plated onto LBP+Kn30. After a 48-h incubation, we counted CFUs. We then plated the frozen stock onto 20 plates to obtain approximately 2,000 CFUs per plate. Following incubation, we added 2 mL of LB+15% glycerol to each plate and all colonies were scraped off and combined in a 50 mL conical vial. We vortexed the culture, aliquoted 100 μ L into 300 microcentrifuge tubes, and froze them at -70°C .

Virulence Assays

To prepare for insect injections, we inoculated 3 mL of LBP+Kn30 broth with an individual aliquot of the transposon mutant library and grew cultures for 2 h prior to injections. Following growth, we centrifuged 100 μ L of culture, washed once, and resuspended in 100 μ L of 1X PBS. After a 1:10 dilution, we injected 15 μ L (~2,000 cells) into the hindmost left proleg of a single *Galleria mellonella* larva using a 27-gauge needle. We injected two biological replicates of 1,000 larvae to reach 50X coverage of the mutant library in each replicated. Following injections, we incubated larvae in 94 x 16 mm petri dishes at room temperature in the dark. After 24 h and every hour thereafter, we placed dead larvae in 50-mL conical vials and froze at -70°C . We assessed larval mortality based on the lack of movement upon contact with forceps and the “floppy” phenotype [12] caused by *Photorhabdus* spp.

Tn-seq Library Preparation

To prepare bacteria for DNA extraction, we added each biological replicate (1,000 insects each) to a Waring blender with 300 mL of LB+20% glycerol. After blending for 5 m on the

lowest setting, we aliquoted 8 mL into 20-15mL conical vials and stored aliquots at -70°C. Prior to DNA extraction, we inoculated a single aliquot from each replicate in 16 mL of LBP+Kn30 in a 125-mL Erlenmeyer flask and grew cultures for 24 h. Additionally, we inoculated two flasks of 24 mL of LBP+Kn30 with 300 uL each of the original transposon mutant library and grown for 24 h in the same manner. Therefore, we did DNA extractions on two input (prior to insect injections) replicates and two output (passed through insects) replicates.

We collected 1.7 mL of each culture and extracted genomic DNA using the PowerLyzer UltraClean Microbial DNA Isolation Kit (MoBio, Carlsbad, CA) according to manufacturer instructions with the following modifications. Following vortexing and incubation at 4°C, centrifugations were done for 3 m instead of 30 s. After discarding the MD4 solution flow through, we washed filters once with 500 µL of PE buffer (Qiagen, Valencia, CA) followed by a 1-m centrifugation after discarding PE buffer flow through. Then we transferred filters to a new 2 mL tube and applied 55 µL of warm Tris-2.5mM. After a 2-m incubation, we centrifuged tubes for 1 m.

Following DNA extraction, we fragmented the genomic DNA with fragmentase. We added 2 µL of fragmentase (vortexed first) (NEB, Ipswich, MA) to 16 µL of genomic DNA + 2 µL of 10X fragmentase v.2 buffer. Next, we incubated samples at 37°C in a heating block for 13 m. To stop the reactions, we added 10 µL of 0.25 M EDTA. We cleaned samples using the QIAprep Spin Miniprep Kit (Qiagen, Valencia, CA). We added 300 µL of PB buffer to each sample and applied samples to a filter column, centrifuged for 30 s, and discarded flow through. Then we added 650 µL of PE buffer, centrifuged for 30 sec, discarded flow through, and centrifuged for an extra minute. We placed filter columns in new 1.7-mL tubes and incubated

with 50- μ L warm Tris-2.5 mM followed by centrifugation for 1 m. We did this cleanup between each step.

After fragmentation, we added C-tails to the fragmented DNA using terminal deoxynucleotidyl transferase (TdT). To 30 μ L of fragmented DNA, we added 4 μ L of 10X TdT buffer, 4 μ L of 2.5 mM CoCl_2 , 2.1 μ L of 9.5 mM dCTP/0.5 mM ddCTP mix, and 0.6 μ L of TdT enzyme (NEB, Ipswich, MA). We incubated reactions for 30 m at 37°C.

We amplified DNA using two rounds of polymerase chain reaction on a DNA Engine DYAD thermal cycler (MJ Research). The standard reaction mixture for the first round included 8 μ L of 5X Q5 buffer, 1.2 μ L 10 mM dNTPs, 2.5 μ L of primer 1TN (10 μ M), 5 μ L of primer 1OLIGOG (10 μ M), 0.5 μ L of Q5 polymerase (NEB, Valencia, CA), and 5 μ L of template DNA (cleaned up TdT reaction). The standard reaction mixture for the second-round PCR included 8 μ L of 5X Q5 buffer, 1.2 μ L dNTPs (10 mM), 3.0 μ L of primer 2TNX (10 μ M), 3.0 μ L of primer 2BARX (10 μ M), 0.5 μ L of Q5 polymerase (NEB), and 2.5 μ L of template DNA (cleaned up first-round PCR product).

Both PCR reactions used the following parameters: an initial denaturation at 96°C for 1 min, 25 cycles of 96°C for 20 sec, 60°C for 30 sec, and 72°C for 20 sec, followed by a final elongation at 72°C for 1 min. Primers for the first round PCR are 1TN 5'-

CTGACCCGGTCGAC-3' and primer 1OLIGOG 5'-CAGACGTGTGCTCTTCCGATCG

GGGGGGGGGG-3'. Primers used for the second round PCR are 2TNA 5'-AATGATAC

GGCGACCACCGAGATCTACTCTTTCCCTACACGACGCTCTTCCGATCTTCGAGAT

GTGTATAAGAGACAG-3', 2TNB 5'-AATGATACGGCGACCACCGAGATCTACTCT

TTCCCTACACGACGCTCTTCCGATCTATCGAGATGTGTATAAGAGACAG-3', 2TNC 5'-

AATGATACGGCGACCACCGAGATCTACTCTTTCCCTACACGACGCTCTTCCG

ATCTGATCGAGATGTGTATAAGAGACAG-3', 2BAR1 5'-CAAGCAGAAGACGGCATA
CG AGATCGTGATGTGACTGGAGTTCAGACGTGTGCTCTTCCGATC-3', 2BAR2 5'-
CAAGCAGAAGACGGCATAACGAGATACATCGGTGACTGGAGTTCAGACGTGTGCTCT
TCCGATC-3', 2BAR3 5'-CAAGCAGAAGACGGCATAACGAGATGCCTAAGTGACTGG
AGTTCAGACGTGTGCTCTTCCGATC-3', 2BAR4 5'-CAAGCAGAAGACGGCATAACGA
GATATTGGCGTGACTGGAGTTCAGACGTGTGCTCTTCCGATC-3'. We used the
following combination of primers for each sample: sample input 1 (I1) 2TNA/2BAR1, input 2
(I2) 2TNB/2BAR4, output 3 (O3) 2TNC/2BAR3, and output 4 (O4) 2TNA:2TNB:2TNC
(1:1:1)/2BAR2.

Illumina sequencing and Assembly

We isolated genomic DNA from the IND strain in the same manner as described above. We submitted samples to the Brigham Young University DNA Sequencing Center for library preparation and sequencing. They performed library preparation according to Illumina TruSeq® DNA PCR-Free Library Prep protocols (Illumina, San Diego, CA). To quantify the library, they used KAPA Library Quantification Kits (Kapa Biosystems, Wilmington, MA) for qPCR. Then they loaded 18pM of the library onto the Illumina HiSeq 2500 flow cell (Illumina, San Diego, CA) for a 125 bp paired-end run with 3,818,052 read pairs.

The sequencing center cleaned Tn-seq libraries using a SPRI bead cleanup according to TruSeq® DNA PCR-Free Library Prep (Illumina, San Diego, CA) guidelines with fragments less than 150 bp removed. Following quantification described above, they loaded 10pM onto the Illumina HiSeq 2500 for a 50 bp single end run with 8,240,00-20,140,000 single-end reads per sample.

We assembled the IND genome using Velvet 1.2.10. We determined an initial kmer

length of 111 based on Velvet Advisor (http://dna.med.monash.edu.au/~torsten/velvet_advisor/) estimations. To determine the optimal kmer length, we assembled contigs with a range of 87-121 with increments of 2. Based on the resulting contig numbers, N50 scores, and maximum contig lengths for each kmer, we determined the optimal kmer length. For the final assembly we used a kmer length of 105 and discarded anything with 14X coverage or less. We annotated the final assembly using the Rapid Annotation using Subsystem Technology (RAST) server.

We assembled the sequence reads from the input and output samples to our IND reference genome using Bowtie2. We used the following parameters: mismatches=3, genetrim=0, readlength=25, and minimum hop count=2.

Gene Selection Criteria

We identified important virulence genes using two main criteria: insect fitness and hop sites. To determine insect fitness, we calculated the output to input ratio ($O1+O2/I1+I2$). Furthermore, we discarded any gene with no representations in any one of the samples. We considered any gene with an insect fitness below 0.2 when knocked down with at least 2 hop sites across all samples to be necessary for virulence or growth in the insect host. However, we considered genes with an insect fitness of 5 or higher when knocked down to decrease virulence or survival in the insect host. We compared our genome to the *P. luminescens* subsp. *akhurstii* TT01 and *X. nematophila* ATCC 19061. We discarded any gene that only appeared in the IND genome.

Statistics

We used JMP12 (SAS, Cary, NC, USA) to conduct ANOVA and Tukey-Kramer tests to identify significant differences among the strains used for transposon mutagenesis.

Results

Efficiency of Transposon Mutagenesis

There was a significant difference (DF=17,36, F=22.5928, $p < .0001$) in the efficiency of transposon mutagenesis among all of the tested strains with the majority of the strains not being suitable for transposon mutagenesis (Fig 1). Three strains showed potential for efficient transposon mutagenesis (ARG, IND, Pa) and two strains had low efficiencies, but were able to uptake the transposon (Kingscliff, Ps). All other strains either had little to no mutant colonies rendering them unusable for our purposes. ARG, IND, and Pa strains form a monophyletic clade. Kingscliff and Ps also formed a monophyletic clade.

IND Genome

Based on our *de novo* genome assembly the IND genome is 5,362,084 nucleotides with 4,898 predicted genes. Our assembly had 265 contigs with a N50 of 59,763.

Genes Involved in Insect Pathogenesis

We identified 33 genes that result in low fitness when they are knocked out (Table 1). Of these, we determined 8 to be of interest based on their function. These are primarily functions needed for virulence in other bacterial species, but have never been characterized in *Photorhabdus* spp. or were unexpected results such as toxins. One is a potential inclusion body, 1 is a potential toxin, 3 are involved in type VI secretion systems, 2 are antitoxins, and 1 is a flagellin protein. We also assessed genes based on fitness level and the presence of multiple gene copies. There were 6 genes that fit these criteria. Two are potential toxin genes, 1 is an oxidoreductase, 1 is an ATPase, 1 is a hypothetical protein with an immune protein domain, and 1 is a protein with no known or predicted function. There are two genes of interest that overlap based on function and copy number: the potential inclusion body and the potential toxin.

There were 54 genes we identified that increased bacterial fitness when they were

knocked out (Table 2). We determined 21 were of interest based on function in the following categories: flagellar production (6), secretion systems (2), fimbrial assembly (3), regulators (7), chemotaxis (1), toxins (1), and proteases (1). Based on the number of copies in the genome there were 5 genes of interest: an aldolase, a toxin, a fimbrial protein, and 2 proteins of unknown function. Again, two genes were found to be of interest based on both function and genome copy, a toxin and a fimbrial protein.

We verified our results by comparing our study to other studies that have investigated single-gene mutants in *Photothabdus* (Table 3). All of the genes we compared were consistent with previous studies. We also examined as many flagellar genes as we could find in the genome (Table 4). We found 42 genes with a wide range of fitness among these genes. Some gene knockouts resulted in a decrease in virulence and some had an increase in virulence, while others had no effect. We found that 11 of these genes were not in the input samples. Furthermore, we found 4 more of these genes that were in the input samples, but not in the output samples.

We narrowed our list of potential genes of interest using the presence of multiple copies or similar genes in the genome (Table 5). When we did BLASTp searches and percent identities among the sets of genes we found most are not true gene copies, but are similar proteins such as different Tc toxins or multiple fimbrial assemblage proteins. We took a closer look at 5 sets of genes.

We found the following similarities based on a percent identity matrix. The two Photopexin B genes were 76% similar to each other, but only 60-65% similar to Photopexin B. Another set of genes that we identified were a number of Tc toxins. They are not located near each other and there were no two genes more than 60% similar with most being about 20% similar to each other. However, gene 1998 is 92% similar to TcdB2 in the TT01 genome and

gene 2950 is 95% similar to the TcaC protein in TT01. The fimbrial usher proteins were only 20-40% similar to each other. Though, gene 3333 is 90% similar to the MadH usher protein. The genes containing PixA are not highly similar to the PixA protein (20-30%) and only two of the genes in this set are more than 45% similar to each other. The set of hypothetical proteins are all 70-85% similar to each other.

Discussion

Prior to selecting genes for further investigation we determined the reliability of this study by comparing our results to previous genetic studies on *Photorhabdus* virulence. With a small subset of genes we determined that our study is consistent with previous findings suggesting that we can rely on these results for future work. For example, the HcaR regulatory protein controls expression of various toxins and oxidative stress proteins [26]. When the *hcaR* gene is knocked out virulence is decreased. In our study, the *hcaR* mutant was decreased in fitness by 63%. Furthermore, knocking out the ExbD protein, a protein involved in iron uptake, also results in a decrease in virulence [10]. Our data had a high number of *exbD* mutants in the input samples, but none in output samples for a fitness of 0. On the other hand, some genes that have been shown to play roles in nematode colonization or development, but do not affect virulence such as *uvrY*, *hdfR*, *flgG*, *motA*, and *sctC* showed no reduction in virulence in this study [40-43]. None of these genes met our conservative criteria for determining essential genes involved in virulence; however, these results do coincide with predictions based on previous studies. Therefore, the findings we present are reliable and Tn-seq is a valid method for unraveling virulence mechanisms in *Photorhabdus*.

Flagella play important roles in virulence for a wide range of bacterial pathogens [44]. We noticed that a number of genes involved in flagellar biosynthesis met all of our essential-

gene criteria. After compiling a list of genes involved in flagellar biosynthesis and their relative fitness levels in the insect it became clear that flagella are important to *Photorhabdus* infections. Little is understood about flagella in *Photorhabdus*. However, it was shown that knocking out FlgG (distal rod of the flagellum) or MotAB (motor proteins that rotate the flagellum) resulted in a lack of flagella/motility, but had no effect on virulence [42]. Though, it is possible that other proteins are still secreted without the flagellum. In *Xenorhabdus* it was shown that pyocin products are secreted via the flagellar apparatus [45]. It has been predicted that bacteriocins in *Photorhabdus* located near the flagellar genes would be secreted the same way [45, 46]. Perhaps with most of the secretion system intact *Photorhabdus* is still able to secrete bacteriocins and virulence factors.

Additionally, our results are consistent with what has been studied in *Xenorhabdus*. For example, FlhC was shown to be a transcriptional activator of flagellar genes as well as lipolytic and hemolysin activity [47]. In our study the *flhC* mutant showed a decrease in virulence. Two other regulators (FliAZ) involved in flagellar, lipase, and hemolysin production also resulted in decreased virulence in our study, which also happens in *Xenorhabdus* [48, 49]. Not only did we find flagellar regulatory genes involved in virulence, but we also found that structural proteins such as flagellin, FliC, resulted in less virulence when they were knocked out. Furthermore, some genes when knocked out resulted in higher virulence such as the hook protein, FlgE. Most work investigating the effect of flagella on virulence in *Xenorhabdus* has been done on regulatory genes rather than structural proteins [50, 47, 49, 48]. However, based on the number of genes that displayed variations in virulence in our study it is clear that flagellar regulatory and structural proteins are playing a key role in *Photorhabdus* virulence and persistence inside the insect host. Future investigations are needed to understand this vital system.

Furthermore, we identified genes of interest based on our initial criteria, function, and the presence of multiple copies in the genome. Most of these are not true gene copies, but are similar proteins such as different Tc toxins or multiple fimbrial assemblage proteins. We limited our investigation to five sets of genes. One set of genes has Photopexin B domains. Photopexin B is a protein predicted to be used in host cell attachment or binding iron-containing molecules in the insect host [51]. Though they were not highly similar to the Photopexin B protein found in *P. luminescens* W14, the two proteins were similar to each other and are located closely to each other on the genome. These may be gene duplications since Photopexin B is suspected to be a duplicate of Photopexin A. Interestingly, one gene increased bacterial fitness when knocked out and the other decreased in fitness. Therefore, if these are duplicates they are both functional, but may have evolved separate functions.

Another set of genes that we identified was a set of various Tc toxins. They are not located near each other and there were no two genes highly similar to each other. Therefore, these are likely multiple different Tc toxins, which is consistent with previous findings that *Photorhabdus* and *Xenorhabdus* genomes contain multiple Tc toxins [19]. What is surprising is that deleting one of these toxins decreased virulence and another one increased in virulence. The other two stayed neutral, which is what we would have expected to happen with any toxin. With high numbers of toxins in the genome one missing toxin should not have an effect on virulence. Furthermore, the gene knock out resulting in decreased virulence is about half the size of the other Tc toxins, but contains the same protein domains.

There was a set of five potential usher proteins that span the outer membrane and are required for fimbrial assembly. Previous work showed that the Mad fimbriae are used for mutualistic association and cells expressing these fimbriae are less virulent [52, 53]. The likely

madH (usher protein) homolog in the IND strain doubled in fitness levels when it was knocked out. However, there was another usher protein that resulted in cells that were 8X more virulent. This is potentially another fimbria involved in colonizing the nematode. Furthermore, there are 11 sets of predicted fimbrial genes in *Photorhabdus* [11] with some likely used in pathogenesis rather than nematode colonization. Two of the predicted usher proteins we identified were 26-43% reduced in virulence. These fimbriae would be a promising place to start investigations on fimbriae involved in pathogenesis.

Perhaps the two most intriguing sets of genes we found, especially from an evolutionary perspective, are a set of 6 genes that all contain a PixA domain and a set of hypothetical proteins with no known function or protein domains. These two sets are particularly interesting because they are all approximately the same size and are all located in tandem on the genome. PixA is the inclusion body protein produced by *Xenorhabdus* species essential to nematode development, but are not involved in virulence [54]. The genes we identified containing the PixA domain are not highly similar to the PixA protein or to each other. Furthermore, the TT01 genome did not contain any of these proteins, but the *Xenorhabdus* genome contains the PixA protein and another non-similar protein with the same domain. Inclusion bodies are not implicated in virulence in either *Photorhabdus* or *Xenorhabdus* [54, 55]; however, five of the six genes in this cluster all had a decrease in virulence when knocked out and the other had an increase in virulence. Is this a new undiscovered inclusion body that plays a role in virulence like protein inclusion bodies in other insect pathogens or are these unique proteins with a domain that can serve multiple purposes? Maybe these do not form protein inclusion bodies, but use a similar domain for a different function. Future research should aim at unraveling the roles of these proteins as well as their evolutionary significance as a gene family and/or gene duplications.

Lastly, there is a set of hypothetical proteins with no known function and no known domains. These proteins are also clustered together on the genome, but they are highly similar to each other. Again, five of the six had some level of decrease in virulence when knocked out and the other one drastically increased in virulence. These are more likely to be gene duplications, which leads to several questions. What are the origins of these genes? Are these genes true duplications? Are they all functional? What are their functions? What selective pressures have increased or decreased their role in virulence? Which nucleotides or amino acids are under selective pressure? These and other questions make this gene set a good candidate for future research.

We have strongly demonstrated the use of Tn-seq for identifying genes that play a role in virulence in *Photorhabdus*. Furthermore, we have identified genes that play a role in virulence providing preliminary data for numerous future research projects. Tn-seq is a useful tool for studying various bacterial life history traits. We suggest that future research should use Tn-seq to understand the roles of *Photorhabdus* genes in nematode colonization and development. Furthermore, future research can use this tool for understanding *Photorhabdus* antimicrobial targets, interactions with the insect host, and simple growth mechanisms among other questions.

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Tables

Table 1. List of knocked-down genes resulting in low fitness.

Gene	Gene ID	BLAST	InsectFitness*
2325†	pind 29488.6.peg.2760	oxidoreductase	0.02978798
4547**	pind 29488.6.peg.546	Flagellin	0.047145488
1750	pind 29488.6.peg.2169	hypothetical protein	0.058229631
3607	pind 29488.6.peg.4313	hypothetical protein	0.07128752
751**	pind 29488.6.peg.732	putative antitoxin	0.07713311
3606	pind 29488.6.peg.4312	membrane protein	0.07960199
3076	pind 29488.6.peg.3641	metallo-beta-lactamase	0.079918033
582	pind 29488.6.peg.4882	4-alpha-glucanotransferase	0.095572117
2319	pind 29488.6.peg.2754	oxidoreductase	0.101156847
326	pind 29488.6.peg.3843	membrane protein	0.102242152
3924	pind 29488.6.peg.4740	murein transglycosylase	0.120377085
4109†	pind 29488.6.peg.67	putative immune protein (Imm32 domain)	0.12495911
2255**	pind 29488.6.peg.2690	Lysozyme (Type VI secretion domain)	0.127852454
4073	pind 29488.6.peg.31	aminotransferase	0.139067524
2265**	pind 29488.6.peg.2700	Hypothetical protein (Type VI secretion/ImcF domain)	0.152463768
2974	pind 29488.6.peg.3539	chorismate mutase	0.154666168
2295	pind 29488.6.peg.2730	aminopeptidase	0.155392069
4678	pind 29488.6.peg.857	rRNA methyltransferase	0.157223796
4512	pind 29488.6.peg.511	nudix hydrolase	0.157637872
559**†	pind 29488.6.peg.4802	Putative toxin (has SpvB domain)	0.160540263
2004	pind 29488.6.peg.2430	hypothetical protein	0.171244635
3613†	pind 29488.6.peg.4319	hypothetical protein	0.17323601
2329	pind 29488.6.peg.2764	protein of unknown function	0.173913043
3508**†	pind 29488.6.peg.4105	Hypothetical protein (PixA domain)	0.178461538
3386	pind 29488.6.peg.3983	glutamate synthase	0.180898876
2216**	pind 29488.6.peg.2649	Type VI secretion system protein	0.181980057
3421**	pind 29488.6.peg.4018	Antitoxin	0.186118541
667	pind 29488.6.peg.591	anhydro-N-acetylmuramic acid kinase	0.186756418
278	pind 29488.6.peg.3460	Phosphoethanolamine transferase	0.187516961
2575	pind 29488.6.peg.3033	ATPase AAA	0.188366798
3647	pind 29488.6.peg.4353	murein transglycosylase	0.189918534
4660	pind 29488.6.peg.839	ankyrin	0.198081023
1061†	pind 29488.6.peg.1165	ATPase AAA	0.20288296

*fitness of mutant cells

**genes of interest based on predicted function

†genes of interest based on multiple copies in the genome

Table 2. List of knocked-down genes resulting in high fitness.

Gene	Gene ID	Function	Insect Fitness*
1430**	pind 29488.6.peg.1712	transcriptional regulator	5.023738872
3415**	pind 29488.6.peg.4012	hypothetical protein (VirK domain)	5.034844668
2427**	pind 29488.6.peg.2879	LuxR family transcriptional regulator	5.079069767
3885	pind 29488.6.peg.4701	argininosuccinate synthase	5.090062112
3472	pind 29488.6.peg.4069	regulator	5.106463878
4535**	pind 29488.6.peg.534	flagellar biosynthesis chaperone (FliJ family)	5.181818182
3298	pind 29488.6.peg.3895	exoribonuclease II	5.248309179
1425	pind 29488.6.peg.1707	Homoserine dehydrogenase	5.258536585
1674	pind 29488.6.peg.2002	terminase, endonuclease subunit	5.404360056
3228	pind 29488.6.peg.3794	dicitrate transport ATP-binding protein FecE	5.524109015
59	pind 29488.6.peg.1428	dehydrogenase	5.52617801
3649	pind 29488.6.peg.4355	synthase, synthetase subunit / Phosphoribosylformylglycinamide synthase, glutamine amidotransferase subunit	5.595486111
1062†	pind 29488.6.peg.1166	hypothetical protein	5.622073579
539**	pind 29488.6.peg.4528	LysR family transcriptional regulator	5.720496894
1328	pind 29488.6.peg.1595	desulfurase	5.736613603
3674**	pind 29488.6.peg.4380	hypothetical protein (OmpA domain, possible type VI or flagellar role)	5.814661134
3227	pind 29488.6.peg.3793	dicitrate transport system permease protein FecD	5.921161826
2692	pind 29488.6.peg.3151	phosphoribosyltransferase	6.019512195
606	pind 29488.6.peg.104	hypothetical protein	6.067692308
123	pind 29488.6.peg.2024	predicted calcium/sodium:proton antiporter	6.24301676
3182**	pind 29488.6.peg.3748	DNA-binding response regulator	6.298076923
4779	pind 29488.6.peg.1501	hypothetical protein	6.36585366
3683**†	pind 29488.6.peg.4389	photopexin B	6.374531835
277	pind 29488.6.peg.3459	translation elongation factor	6.567226891
4715	pind 29488.6.peg.1051	phosphoribosylamine-glycine ligase	6.574132492
1198	pind 29488.6.peg.1314	PAS domain-containing sensor histidine kinase	6.872340426
3331**	pind 29488.6.peg.3928	fimbrial chaperone protein (PapD chaperone domain)	6.915829146
4340	pind 29488.6.peg.339	system, N-acetylmuramic acid-specific IIB component/ PTS system, N-acetylmuramic acid-specific IIC component	6.985232068
2479**	pind 29488.6.peg.2931	preprotein translocase J (EscJ)	7
4546**	pind 29488.6.peg.545	flagellar cap protein FliD	7.151898734
4206	pind 29488.6.peg.175	TonB receptor	7.19895288
3670	pind 29488.6.peg.4376	dehydrogenase	7.552697095
1982	pind 29488.6.peg.2408	permease	7.571428571
1312	pind 29488.6.peg.1579	4,6-dehydratase	7.620547945
1966**†	pind 29488.6.peg.2392	fimbrial assembly protein (PapC usher)	7.958083832

		domain)	
3528	pind 29488.6.peg.4234	hypothetical protein	8.079646018
4536**	pind 29488.6.peg.535	ATP synthase (FliI family)	9.239583333
1598**	pind 29488.6.peg.1918	intracellular growth attenuator protein IgaA (potential flagellar regulator)	9.377643505
2200	pind 29488.6.peg.2633	hypothetical protein	9.76
4619	pind 29488.6.peg.673	hypothetical protein	9.853174603
3686**	pind 29488.6.peg.4392	membrane protein (rhomboid family)	10.58585859
566**	pind 29488.6.peg.4809	flagellar hook protein FlgE	11.2231405
3525**	pind 29488.6.peg.4231	hypothetical protein, pilin (fimA domain)	11.76470588
823**	pind 29488.6.peg.893	chemotaxis protein	15.76699029
1311	pind 29488.6.peg.1578	thymidyltransferase	22.71779141
3856	pind 29488.6.peg.4672	Bcr/CflA family drug resistance efflux transporter	23.23333333
192†	pind 29488.6.peg.2093	phosphate aldolase	24.30927835
706	pind 29488.6.peg.687	dehydrogenase	40
1309	pind 29488.6.peg.1576	aminotransferase	55.3826087
1314	pind 29488.6.peg.1581	2-epimerase	57.1961326
3308	pind 29488.6.peg.3905	symport protein	61.94444444
260**	pind 29488.6.peg.3442	transcriptional regulator	68.28947368
1305	pind 29488.6.peg.1572	UDP-N-acetyl-D-mannosaminuronic acid transferase	90.2
3889**	pind 29488.6.peg.4705	transcriptional regulator	113.937576

*fitness of mutant cells

**genes of interest based on predicted function

†genes of interest based on multiple copies in the genome

Table 3. Insect fitness of published mutants in *Photorhabdus luminescens* TTO1.

Gene	Insect Fitness*	Virulence Phenotype	Reference
<i>hcaR</i>	0.369955157	-	Chalabaev et al. (2007)
<i>uvrY</i>	0.970424346	+	Krin et al. (2008)
<i>hdfR</i>	1.341071429	+	Easom and Clarke (2012)
<i>exbD</i>	0	-	Watson et al. (2005)
<i>flgG</i>	0.732057416	+	Easom and Clarke (2008)
<i>motA</i>	0.741935484	+	Easom and Clarke (2008)
<i>sctC</i>	2.75297619	+	Brugirard-Ricaud et al. (2005)

*fitness of mutant cells

Table 4. Insect fitness of flagellar genes.

Gene	Gene ID	Function	Insect Fitness*
4532	pind 29488.6.peg.531	FliL	0
4545	pind 29488.6.peg.544	FliS	0
560	pind 29488.6.peg.4803	FlgN	0
829	pind 29488.6.peg.899	FlhD	0
565	pind 29488.6.peg.4808	FlgD	0.021164021
4547**	pind 29488.6.peg.546	FliC	0.047145488
828	pind 29488.6.peg.898	FlhC	0.096153846
567	pind 29488.6.peg.4810	FlgF	0.102179837
4544	pind 29488.6.peg.543	FliT	0.217277487
572	pind 29488.6.peg.4815	FlgK	0.263697627
571	pind 29488.6.peg.4814	FlgJ	0.304832714
4528	pind 29488.6.peg.527	FliP	0.394915254
4166	pind 29488.6.peg.135	FlhA	0.402414487
573	pind 29488.6.peg.4816	FlgL	0.434447301
4548	pind 29488.6.peg.547	FliA	0.463312369
4549	pind 29488.6.peg.548	FliZ	0.549187808
3252	pind 29488.6.peg.3818	FliY	0.577194753
568	pind 29488.6.peg.4811	FlgG	0.732057416
827	pind 29488.6.peg.897	MotA	0.741935484
4534	pind 29488.6.peg.533	FliK	0.776035834
1168	pind 29488.6.peg.1284	FliY	1.3020265
4527	pind 29488.6.peg.526	FliQ	1.912087912
4526	pind 29488.6.peg.525	FliR	2.00656168
4530	pind 29488.6.peg.529	FliN	2.872093023
4539	pind 29488.6.peg.538	FliF	3.730769231
4541	pind 29488.6.peg.540	FliE	4.545454545
4535**	pind 29488.6.peg.534	FliJ	5.181818182
4546**	pind 29488.6.peg.545	FliD	7.151898734
4538	pind 29488.6.peg.537	FliG	7.714285714
4536**	pind 29488.6.peg.535	FliI	9.239583333
566**	pind 29488.6.peg.4809	FlgE	11.2231405
4529	pind 29488.6.peg.528	FliQ	N/A
4531	pind 29488.6.peg.530	FliM	N/A
4537	pind 29488.6.peg.536	FliH	N/A
561	pind 29488.6.peg.4804	FlgM	N/A
562	pind 29488.6.peg.4805	FlgA	N/A
563	pind 29488.6.peg.4806	FlgB	N/A
564	pind 29488.6.peg.4807	FlgC	N/A
569	pind 29488.6.peg.4812	FlgH	N/A
570	pind 29488.6.peg.4813	FlgI	N/A
826	pind 29488.6.peg.896	MotB	N/A
4165	pind 29488.6.peg.134	FlhB	N/A

*fitness of mutant cells

**meets all of the initial criteria

Table 5. Selected genes with genome duplicates.

Gene Cluster	Gene	Gene ID	Location	Length	Insect Fitness*
PixA	3507	pind 29488.6.peg.4104	3814859..3815455	597	0.459799581
	3508	pind 29488.6.peg.4105	3815495..3816076	582	0.178461538
	3509	pind 29488.6.peg.4106	3816714..3817310	597	0.794811321
	3510	pind 29488.6.peg.4107	3817350..3817931	582	0.577106742
	3511	pind 29488.6.peg.4108	3818447..3819055	609	1.410579345
Hypothetical Protein	3512	pind 29488.6.peg.4109	3819092..3819613	522	0.372577172
	3610	pind 29488.6.peg.4316	3933753..3935273	1521	0.855555556
	3611	pind 29488.6.peg.4317	3935384..3936904	1521	0.500897666
	3613	pind 29488.6.peg.4319	3937321..3938823	1503	0.17323601
	3614	pind 29488.6.peg.4320	3939205..3940710	1506	0.332355407
Tc Toxins	3615	pind 29488.6.peg.4321	3941166..3942671	1506	6.767241379
	1998	pind 29488.6.peg.2424	2181627..2186054	4428	1.194852941
	3995	pind 29488.6.peg.4833	4404511..4408941	4431	0.939046915
	2950	pind 29488.6.peg.3514	3192423..3196880	4458	2.915277778
Photopexin B	559	pind 29488.6.peg.4802	569855..572212	2358	0.160540263
	3682	pind 29488.6.peg.4388	4012088..4013092	1005	0.504809895
Usher Proteins	3683	pind 29488.6.peg.4389	4013509..4014534	1026	6.374531835
	1961	pind 29488.6.peg.2387	2126715..2129195	2481	0.578496042
	1966	pind 29488.6.peg.2392	2133805..2136447	2643	7.958083832
	2389	pind 29488.6.peg.2824	2603058..2605514	2457	0.836749634
	3488	pind 29488.6.peg.4085	3798523..3801039	2517	2.022964509
	3333	pind 29488.6.peg.3930	3623064..3625814	2751	2.013044685

*fitness of mutant cells

Figure Legends

Figure 1. The efficiency of transposon mutagenesis. Eighteen different strains of *Photorhabdus* spp were tested for their ability to be mutated using a transposon. The average number of mutant colonies per strain are shown from three different conjugation reactions. Differing letters denote significant differences at $P < 0.05$.

Figure 2. Phylogenetic location of efficient strains. Boxes show which strains were able to be mutated with a transposon. The dark gray box highlights strains that were highly efficient. The light gray box displays strains that were weakly efficient.

Figures

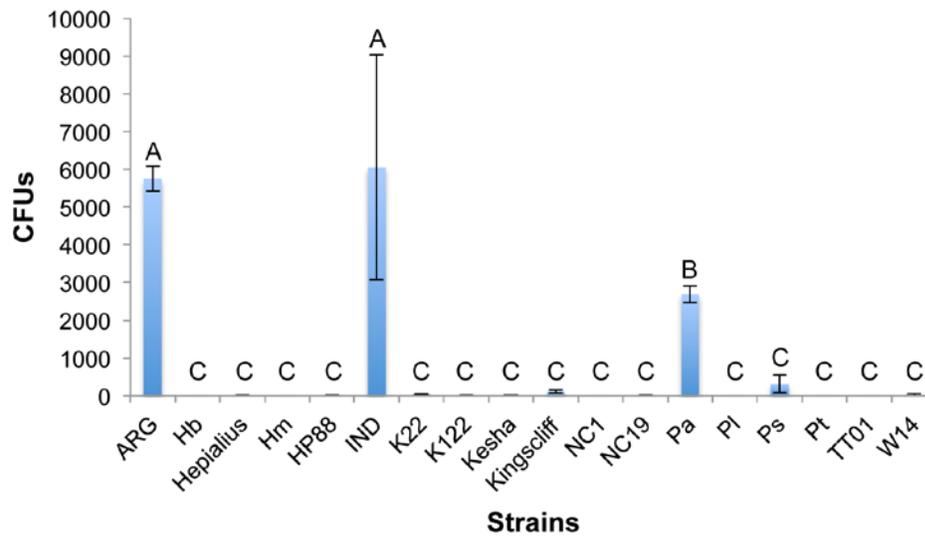


Figure 1. The efficiency of transposon mutagenesis.

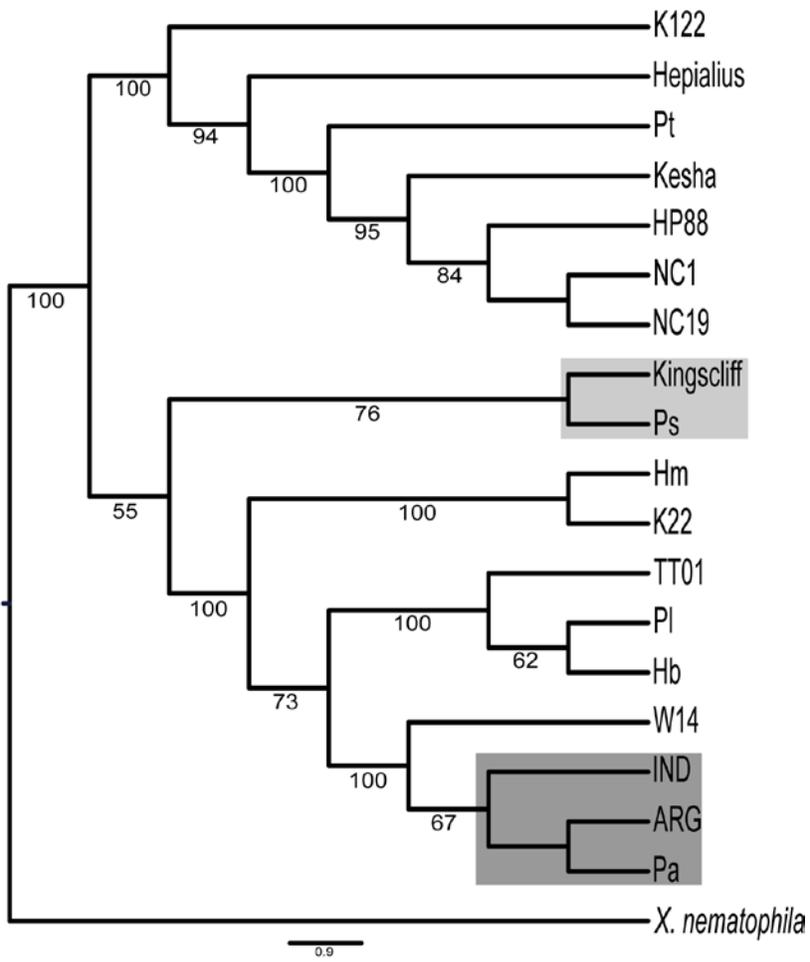


Figure 2. Phylogenetic location of efficient strains.