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THE *PHOTORHABDUS TEMPERATA SSPAB* LOCUS IS REQUIRED FOR SYMBIONT TRANSMISSION IN *HETERORHABDITIS BACTERIOPHORA*

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By

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Abstract

THE *PHOTORHABDUS TEMPERATA SSPAB* LOCUS IS REQUIRED FOR SYMBIONT TRANSMISSION IN *HETERORHABDITIS BACTERIOPHORA*

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The entomopathogenic nematode, *Heterorhabditis bacteriophora*, and the Gram-negative, insect pathogen enteric bacterium, *Photorhabdus luminescens*, coexist in a mutualistic symbiotic relationship. As symbiont transmission is essential for the pair's insect parasitic lifestyle, the bacteria are transmitted from the maternal nematode intestine to offspring in a sophisticated pathway that involves multiple adhesion and invasion steps. To identify genes important in symbiont transmission, a *HimarGm* mariner transposon mutagenesis screen of ~8000 green fluorescent protein (GFP) labeled *P. luminescens* and *P. temperata* was conducted. A transmission defective mutant, TRN162, was identified that successfully completes the initial steps in the transmission cycle, but forms a spheroplast at 120 h post-recovery, resulting in a 0% transmission efficiency. TRN162 has a disrupted *sspA* gene, which is predicted to encode a homolog of the stringent starvation protein A. In *Escherichia coli,* SspA has been shown to be involved in survival under stressful conditions and during stationary phase, as well as being required for motility. *sspAB* is not essential for *P. temperata* virulence in insects and TRN162 survives growth under acidic conditions or in the presence of cationic microbial peptides, similar to its parent strain, NC1Tn7GFP. However, the mutant displays a growth defect, a hypermotile twitching motility phenotype and an increased sensitivity to growth in the presence of H_2O_2. The inability of TRN162 to fully complete the symbiont transmission cycle may be explained by its increased sensitivity to oxidative stress, an inability to evade a selective stress or a yet unidentified difference in gene expression as regulated by *sspAB*.

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Chapter 1: Introduction

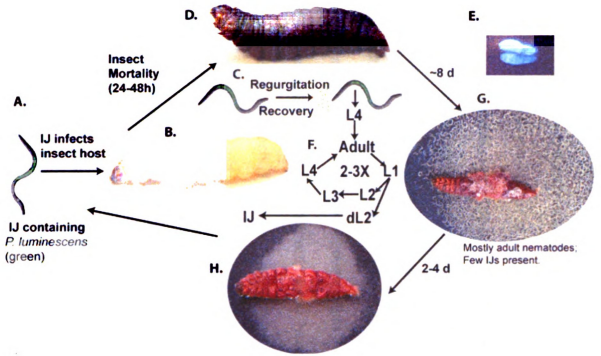
This introduction is divided into three parts. Part I describes the symbiotic nematode-bacteria relationship shared by *Heterorhabditis bacteriophora* and *Photorhabdus luminescens*, as well as the pathogenic relationship they share towards insect larvae. In Part II, the process of bacterial symbiont transmission in the nematode intestine is explained. Part III focuses on the role of SspA homologs in regulation of host-bacterial interactions, motility and stress response in several enteric and pathogenic microorganisms.

Introduction

Photorhabdus, Heterorhabditis bacteriophora and insect pathogenesis

According to Anton de Bary, symbiosis is "the living together of unlike organisms," (3). This pervasive biological phenomenon can comprise a wide spectrum of associations from the mutually beneficial to the acutely parasitic. Studying a genetically malleable symbiotic relationship provides an opportunity to not only learn more about genetic factors essential for mutualism, but also for comparison to pathogenic relationships, which results in a drastically different outcome for the host organism, disease. One such model system is that of the nematode, *Heterorhabditis bacteriophora*, and species of the Gram-negative bacteria, *Photorhabdus*, which colonize the worm's intestine (34). Although *Photorhabdus* and *H. bacteriophora* can be cultured separately in a laboratory setting, these two partners form an obligate mutualistic association in nature. Together they infect and kill the larval stage of a variety of insects, comprising three organisms of a tripartite relationship (31). While the nematode serves as *Photorhabdus'* vector between insect hosts, the bacteria are necessary for killing the insects and for nematode reproduction. This system has been used commercially for the biological control of insect pests of crops (14). The life cycle of the tripartite bacteria-nematode-insect relationship is initiated by the free-living, developmentally arrested infective juvenile (IJ) stage nematode, which

Figure 1-1: Life cycle of *H. bacteriophora* and *Photorhabdus luminescens* (adapted from Ciche). (A) Developmentally arrested IJ nematodes carry *P. luminescens* as intestinal symbionts and search for an insect host. (B) Upon encountering a host, IJs enter the insect hemocoel and (C) regurgitate *P. luminescens*, beginning the recovery process. (D) Toxins produced by *P. luminescens* kill the insect and secondary metabolites are produced, including antibiotics that inhibit other microbial competition. (E) Bacterial bioluminescence can be observed in insect cadavers. (F) The nematodes develop through 2-3 generations with 4 larval stages and 1 adult stage. (G) After initially laying eggs in the 1st generation, reproduction in the 2nd generation occurs via *endotokia matricida* where offspring develop into IJs (an alternative L3 stage) inside maternal nematodes. (H) The IJs reassociate with *P. luminescens* and disperse in search of a new insect host.



harbors bacterial symbionts within its intestine (Figure 1-1). The IJ actively seeks out its insect prey and enters the insect larva either through natural openings, such as the mouth or anus, or by using its buccal tooth to penetrate the exoskeleton (8, 9). Upon entering the insect hemocoel, a yet-unidentified cue causes the nematode to regurgitate its monoculture of intestinal symbionts (6). In this pathogenic phase, toxins produced by *Photorhabdus* kill the insect rapidly, within 24 to 48 hours. Germ free or axenic IJs are unable to kill insect larvae (20), while bacterial symbionts injected into insects are sufficient for insect death (31). The bacteria also produce compounds to discourage other saphrophytic organisms from feeding on the rich nutrients provided by the insect cadaver, such as antibiotics and nematicide (6, 25, 26, 35, 37). As the worm feeds on the bacteria and insect host, it develops through two to three generations within the insect cadaver in the saprophytic phase (20). Once all nutrients have been exhausted and the reproducing worms have reached a high density, the IJs reassociate with *Photorhabdus* and disperse in search of another insect larva to infect (20).

H. bacteriophora is a soil dwelling entomopathogenic nematode found in temperate climates (16) and has particular relevance in crop pest control. Species of *Heterorhabditis* nematodes belong to the family Rhabditidae and are phylogenetically related to *Caenorhabditis elegans* (29). Indeed, some features that make *C. elegans* a good model organism also apply to *Heterorhabditis*, such as its relatively small size and transparent body. For these reasons, *H.*

bacteriophora has been targeted for complete genome sequencing by the National Human Genome Research Institute (NHGRI).

Species of *Heterorhabditis* are heterogonic, that is, they are able to employ both hermaphroditic and gonochoristic (sexual) modes of reproduction. At low worm densities, when more nutrients are available, the nematodes develop gonochoristically by laying eggs outside of the female's body. These eggs will develop into either males, females or hermaphrodites, with no IJs being generated (10). However, as the number of nematodes grows and nutrients become sparse, both female and hermaphroditic nematodes began to produce eggs within their body cavities. Eggs produced exclusively by hermaphrodites will develop into IJs in a process that kills the nematode and is termed *endotokia matricida*. The IJs are developmentally arrested, non-feeding hermaphroditic nematodes that harbor intestinal symbionts of the genus *Photorhabdus* and disperse in search of new insects to infect (20).

Photorhabdus are Gram-negative, rod-shaped, bioluminescent microorganisms in the family Enterobacteriaceae. *Photorhabdus luminescens* subsp. lamondii TT01 and *P. temperata* strain NC1, are both able to colonize the intestinal tract of *H. bacteriophora* (7). The full genome of *P. luminescens* subsp. lamondii TT01 has been sequenced and is predicted to encode 4,839 protein-coding genes (13). The genome encodes many genes that presumably assist *Photorhabdus* in its roles of nematode mutualism and insect pathogenesis, including 11 fimbrial gene clusters and the largest number of predicted toxins in a bacterial genome sequenced to date. It is presumably these toxins that kill

insects infected by symbiont-harboring IJs. For example, there are multiple toxin-complex (*tc*) loci, encoding gene products that are predicted to have Tyr-Asp motifs (13). Proteins in this superfamily are predicted to be localized to bacterial surfaces and to bind carbohydrates (32), implicating them in assisting bacterial escape of the innate immune defenses of insect prey (13). Another class of toxin proteins, repeats-in-toxin (RTX), has been identified in *P. luminescens* ssp. TT01 (13). The organization of these genes is identical to that found in *Vibrio cholerae*, and is again predicted to play a large role in pathogenicity to insect prey (13). Not only does *Photorhabdus* encode toxins that contribute to insect pathogenicity, but also factors that aid in protection from other microorganisms that may want to benefit from the rich nutrients provided by insect cadavers, such as antibiotic synthesis genes (13). While the metabolism of *P. luminescens* is fairly similar to other enteric bacteria, the genome encodes many additional metabolic genes involved in degradation pathways not found in other enterics. These genes presumably aid in the bacteria's nematode mutualism and entomopathogenic lifestyles (13). In comparison to other sequenced genomes, the *P. luminescens* ssp. TT01 genome is similar to another insect and human pathogenic microorganism, *Yersinia pestis*, with 77% of their orthologous genes being syntenic (13, 33).

Photorhabdus ssp. exhibit the phenomena of phenotypic variation. Two wildtype phase variants of *Photorhabdus* have been identified, where the two extemes, primary and secondary phase, have been characterized. Primary variants are isolated from IJs and insects, while secondary variants arise after

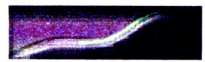
prolonged subculturing. Primaries are characterized by their ability to produce pigments, colonize IJs and biolumenesce (15, 17). Additionally, primaries produce crystalline inclusion proteins and display antibiotic, siderophore and hemolytic activities. In contrast, secondaries do not display any of the above phenotypes. Notably they are unable to colonize IJs, but are pathogenic to insects (19, 20).

Symbiont transmission in H. bacteriophora

Due to the essential function of *Photorhabdus* for the *H. bacteriophora's* insect parasitic lifestyle, it is not surprising that bacterial colonization of the nematode intestine and transmission to the next generation of nematodes involves many selective steps that are sure to involve regulation of gene expression from both partners. The symbiosis between *H. bacteriophora* and species of *Photorhabdus* is specific, with only *P. luminescens* and *P. temperata* being capable of completing the transmission cycle (16). This process begins with symbiont regurgitation by recovering IJs, where all intestinal symbionts are released (Figure 1-2). After regurgitation, the nematode feeds on *Photorhabdus*, and a subpopulation of bacteria then adhere to the posterior of the intestine and form a biofilm before adherent cells invade the rectal gland cells to gain access to the IJs developing in the pseudocoelom (10). These steps, occurring between 8 and 72 h post-recovery, likely require *Photorhabdus* to temporally regulate

Figure 1-2: *P. luminescens* transmission in *H. bacteriophora* (adapted from Ciche).

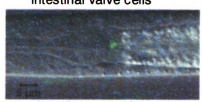
136 h, invasion of pharyngeal-intestinal valve cells



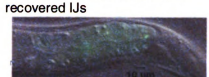
0 h, Infective juvenile

4 h

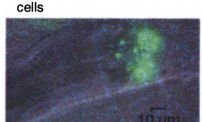
6 h, all bacteria released from recovered IJs



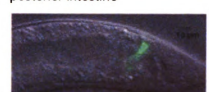
120 h, adherence of bacteria to pharyngeal-intestinal valve cells



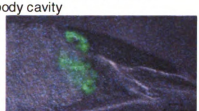
12 h, adherence of bacteria to posterior intestine



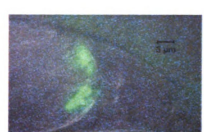
112 h, vacuoles lysed by pre-IJs developing in maternal body cavity



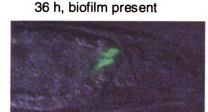
36 h, biofilm present



72 h, 12-30 vacuoles per rectal gland cells



48 h, all rectal gland cells are invaded



42 h, invasion of rectal gland cells begins

genes involved in host attachment and invasion processes. Only eggs that are retained within the body cavity of the nematode develop into IJs (10). As the pre-IJs feed on the maternal organs, the mother nematode is eventually killed in a process termed *endotokia matricida*. As the IJs develop, at approximately 112 h post-recovery, the maternal rectal gland cells are lysed, releasing the bacterial symbionts into the pseudocoelom. This allows the bacteria to be transmitted from the mother to the feeding IJs via a mechanism that is selective for true symbionts. In a clonal and presumably highly selective step, the bacteria then adhere to the pharyngeal intestinal valve cells (PIVCs) of the IJs. The bacteria ultimately invade these cells before exiting and fully colonizing the IJ intestine. Although this transmission process is symbiont specific, the molecular biology, specifically the gene regulation involved, is yet to be extensively studied and fully understood.

A forward genetics approach was applied to learn which bacterial genes play an essential role in the transmission of *Photorhabdus* species to *Heterorhabditis.* A *HimarGm* transposon mutagenesis screen composed of 28 independent mutagenesis experiments, was conducted using GFP-labeled *Photorhabdus* to identify mutants unable to complete the transmission cycle. The use of GFP-labeled *Photorhabdus* allowed for easy visualization of the presence or absence of symbiotic bacteria within the intestine of IJs (collected in the condensation on the lids of Petri dishes) under a fluorescent microscope. Broader implications of this study include gaining insight into generalized features of symbiotic relationships, in addition to uncovering genetic factors

important for host-bacterial interactions in general. Of the approximately 8,000 bacterial mutants produced, 30 were isolated that were defective in symbiont transmission to IJs. One of these, TRN162, was found to contain a mutation in *sspA*, which encodes a homolog of the stringent starvation protein A.

Roles of stringent starvation protein A

SspA is highly conserved throughout the Enterobacteriaceae family, where it has been implicated in the lytic development of the bacteriophage P1 in *Escherichia coli* (22) and in regulation of virulence factors in *Yersinia entercolitica* (1) (Table 1-1 and Figure 1-3). In addition, homologs have been identified in non-enteric bacteria, such as *Neisseria gonorrhoeae* (11) and *Francisella tularensis* (5, 18).

In *E. coli*, it has been shown that SspA is a RNA polymerase associated protein (28) whose expression is upregulated during stationary phase and upon starvation for glucose, nitrogen, phosphate and amino acids (36). In addition, under nutrient limiting conditions, an *E. coli sspA* mutant had decreased survivability compared to the parental strain (36).

One of the first functional roles identified for SspA was as a transcriptional activator for the expression of late genes of the *E. coli* bacteriophage P1 (22). SspA, along with the late promoter activator (Lpa) protein, allows the phage P1 to enter the lytic cycle through the expression of late genes (22).

Table 1-1: Homologs of SspA

Protein Name	Organism	Function	Identity	Similarity	Reference
SspA	*Photorhabdus luminescens*	Essential for symbiont transmission, regulation of twitching motility and resistance to oxidative stress	NA*	NA	This study
SspA	*Yersinia enterocolitica*	Regulation of invasion and motility	85%	92%	(1,21)
SspA	*Yersinia pestis*	Confers acid resistance in *E. coli sspA* mutant and supports lytic development of phage P1	84%	92%	(21)
SspA	*Providencia stuartii*	Activation of genes involved in antiobiotic resistance	83%	92%	(12)
SspA	*Escherichia coli*	Resistance to starvation and acid stresses, lytic development of phage P1	78%	88%	(22,23)
SspA	*Vibrio cholerae*	Colonization of murine intestine, confers acid resistance in *E. coli sspA* mutant	72%	86%	(21,30)
SspA	*Pseudomonas aeruginosa*	Confers acid resistance in *E. coli sspA* mutant	55%	74%	(21)
RegF	*Neisseria gonorrhoeae*	Negative regulation of type IV pili protein	43%	66%	(11)
SspA	*Francisella tularensis subsp. Novicida*	Dimerizes with MglA	32%	54%	(3,5,18)
MglA	*Francisella tularensis subsp. novicida*	Growth and survival in macrophages, regulation of virulence gene expression and response to oxidative stresses	22%	54%	(3,5,18)

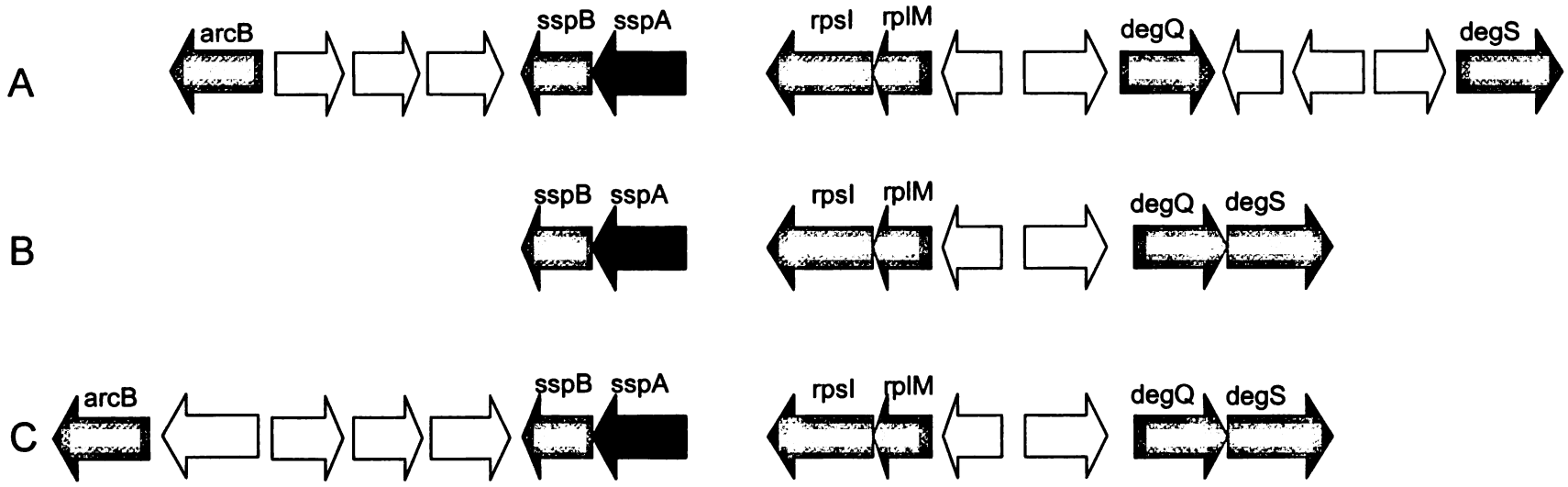
*NA, not applicable

In *E. coli*, it has also been observed that SspA negatively regulates H-NS post-transcriptionally (23). H-NS, the histone-like protein H1, functions as a negative regulator of gene expression (23, 27). In particular, it negatively regulates RpoS, the primary sigma factor responsible for gene expression under stressful conditions or during stationary phase (24). In *E. coli*, RpoS is required for stationary phase induced acid tolerance, while H-NS positively affects cell motility and flagella synthesis (4, 23).

Further insight into the mechanism of SspA comes from structural studies of its homologs. The crystal structure of the *Yersinia pestis* SspA homolog was determined to a resolution of 2 Å (21). Interestingly, SspA exhibits a glutathione S-transferase (GST) fold. However, neither *Y. pestis* nor *E. coli* SspA have GST activity and are unable to bind the glutathione substrate for GST. This is due to a substitution of the catalytically essential Cys residue with a Phe and a Tyr in *Y. pestis* and *E. coli*, respectively (21). This study determined that in *Y. pestis*, SspA functions as a dimer, with a surface exposed pocket present at the interface of the two subunits that is critical for the protein's function. This pocket is suspected to be the binding site for RNA polymerase and the high degree of conservation of SspA homologs indicates that these proteins share similar functions in their respective organisms.

It has further been established that SspA is an important regulator of virulence in some pathogenic bacteria. For instance, a SspA homolog has been studied in *Y. entercolitica*, a common enteric pathogen of humans (1). In this organism, SspA negatively regulates the expression of a flagellin protein, while

Figure 1-3: Synteny of *sspA* homologs in (A) *Photorhabdus luminescens* ssp. TT01, (B) *Escherichia coli* K12 and (C) *Yersinia enterocolitica* ssp. enterocolitica. Black arrows represent *sspA*, while gray arrows represent syntetic genes.



positively regulating the expression of invasin (*inv*) at the post-transcriptional level. Invasin is a primary factor involved in the bacteria's ability to invade the intestinal cells of its host organism and both *inv* and *sspA* are expressed at their highest levels in the transition between late exponential to stationary phase.

In the non-enteric pathogen *N. gonorrhoeae*, a SspA homolog named RegF, was identified as a regulator of *pilE* expression, the major subunit of the *N. gonorrhoeae* pili, which are essential for bacterial attachment to host cells (11). In this organism, pili serve as the bacteria's primary mode of attachment to cells of its human host (11). RegF and RegG share 42% and 44% identity with *E. coli* SspA and SspB, respectively. However, whereas *sspB* is immediately downstream of *sspA*, there are approximately 70 nucleotides between *regF* and *regG*. In this organism, a rho-independent terminator was identified downstream of *regF*, suggesting that in *N. gonorrhoeae*, these two genes are transcribed separately. A *regF* mutant displayed a slow growth phenotype and was hyperpiliated. As binding of the σ^{54}–associated RNA polymerase inhibits the σ^{70}–dependent expression of *pilE*, the proposed model for RegF upregulation of *pilE* involves the repression of transcription dependent on σ^{54}.

In the human urinary tract pathogen, *Providencia stuartii*, it was demonstrated that SspA is required for expression of *aarP*, a transcriptional activator of antibiotic resistance genes (12). This SspA-mediated expression also required a yet-unidentified extracellular signal. Although it is predicted that a nonfunctional SspA would decrease resistance to certain antibiotics in this microorganism, it was demonstrated that antibiotic resistance levels were

actually slightly higher. The authors suggest that in *P. stuartii*, SspA may play a role in drug efflux or alter the permeability of the cell (12).

In *F. tularensis*, an SspA homolog, MglA (macrophage growth locus A) has been recently identified (5, 18). MglA is essential for the microorganism to survive within macrophages of the host defense system and interacts with RNA polymerase in a SspA-dependent manner. A proteomic analysis of a *mglA* mutant indicated that MglA regulates several proteins important for the bacterium's stress response, including SspA, a known dimerization partner of MglA. It is possible that the MglA-SspA-RNA polymerase complex interacts with promoters of target genes directly. Alternatively, there could be a yet-unidentified *F. tularensis* protein that interacts with the MglA-SspA-RNA polymerase complex and makes contact with the promoter DNA, such as the phage P1-encoded Lpa (late promoter activator) that interacts with *E. coli* SspA to activate transcription (5, 22).

Although *sspA* has been shown to be a relevant regulator of processes linked to virulence and host-interaction processes in several microorganisms, much remains to be learned about the mechanism of *sspA*, especially its roles in symbiosis, stress response and virulence. The next chapter will characterize a new role for *sspA*, that of *Photorhabdus* transmission in *H. bacteriophora*.

References

1. **Badger, J. L., and V. L. Miller.** 1998. Expression of invasin and motility are coordinately regulated in *Yersinia enterocolitica*. J. Bacteriol. **180:** 793-800.

2. **Bary, H. A. d.** 1879. Die erscheinung der symbiose.

3. **Baron, G. S., and F. E. Nano.** 1998. MglA and MglB are required for the intramacrophage growth of *Francisella novicida*. Mol. Microbiol. **29:**247-259.

4. **Bertin, P., E. Terao, E. H. Lee, P. Lejeune, C. Colson, A. Danchin, and E. Collatz.** 1994. The H-NS protein is involved in the biogenesis of flagella in *Escherichia coli*. J. Bacteriol. **176:**5537-5540.

5. **Charity, J. C., M. M. Costante-Hamm, E. L. Balon, D. H. Boyd, E. J. Rubin, and S. L. Dove.** 2007. Twin RNA polymerase associated proteins control virulence gene expression in *Francisella tularensis*. PLoS Pathogens. **3:**770-779.

6. **Ciche, T.** 2007. The biology and genome of *Heterorhabditis bacteriophora*, *WormBook*, ed. The *C. elegans* Research Community, WormBook.

7. **Ciche, T. A., M. Blackburn, J. R. Carney, and J. C. Ensign.** 2003. Photobactin: a catechol siderophore produced by *Photorhabdus luminescens*, an entomopathogen mutually associated with *Heterorhabditis bacteriophora* NC1 nematodes. Appl. Environ. Microbiol. **69:**4706-4713.

8. **Ciche, T. A., C. Darby, R.-U. Ehlers, S. Forst, and H. Goodrich-Blair.** 2006. Dangerous liaisons: The symbiosis of entomopathogenic nematodes and bacteria. Biological Control **38:**22-46.

9. **Ciche, T. A., and J. C. Ensign.** 2003. For the insect pathogen *Photorhabdus luminescens*, which end of a nematode is out? Appl. Environ. Microbiol. **69:**1890-1897.

10. **Ciche, T. A., K.-s. Kim, B. Kaufmann-Daszczuk, K. C. Q. Nguyen, and D. H. Hall.** 2008. Cell invasion and matricide during *Photorhabdus luminescens* transmission by *Heterorhabditis bacteriophora* nematodes. Appl. Environ. Microbiol. doi:10.1128/02646-07.

11. **De Reuse, H., and M. K. Taha.** 1997. RegF, an SspA homologue, regulates the expression of the *Neisseria gonorrhoeae pilE* gene. Res. Microbiol. **148:**289-303.

12. **Ding, X., R. R. Baca-DeLancey, and P. N. Rather.** 2001. Role of SspA in the density-dependent expression of the transcriptional activator AarP in *Providencia stuartii*. FEMS Microbiol. Lett. **196:**25-29.

13. **Duchaud, E., C. Rusniok, L. Frangeul, C. Buchrieser, A. Givaudan, S. Taourit, S. Bocs, C. Boursaux-Eude, M. Chandler, J.-F. Charles, E. Dassa, R. Derose, S. Derzelle, G. Freyssinet, S. Gaudriault, C. Medigue, A. Lanois, K. Powell, P. Siguier, R. Vincent, V. Wingate, M. Zouine, P. Glaser, N. Boemare, A. Danchin, and F. Kunst.** 2003. The genome sequence of the entomopathogenic bacterium *Photorhabdus luminescens*. Nat. Biotech. **21:**1307-1313.

14. **Ehlers, R.-U.** 1996. Current and future use of nematodes in biocontrol: practice and commercial aspects with regard to regulatory policy issues. Biocontrol Sci. Tech. **6:**303-316.

15. **Ffrench-Constant, R., N. Waterfield, P. Daborn, S. Joyce, H. Bennett, C. Au, A. Dowling, S. Boundy, S. Reynolds, and D. Clarke.** 2003. *Photorhabdus*: towards a functional genomic analysis of a symbiont and pathogen. FEMS Microbiol. Rev. **26:**433-456.

16. **Fischer-Le Saux, M., V. Viallard, B. Brunel, P. Normand, and N. E. Boemare.** 1999. Polyphasic classification of the genus *Photorhabdus* and proposal of new taxa: P. *luminescens* subsp. *luminescens* subsp. nov., P. *luminescens* subsp. akhurstii subsp. nov., P. *luminescens* subsp. laumondii subsp. nov., P. temperata sp. nov., P. temperata subsp. temperata subsp. nov. and P. asymbiotica sp. nov. Int. J. Syst. Evol. Microbiol. **49:**1645-1656.

17. **Forst, S., B. Dowds, N. Boemare, and E. Stackebrandt.** 1997. *Xenorhabdus* and *Photorhabdus* ssp.: Bugs that kill bugs. Ann. Rev. Microbiol. **51:**47-72.

18. **Guina, T., D. Radulovic, A. J. Bahrami, D. L. Bolton, L. Rohmer, K. A. Jones-Isaac, J. Chen, L. A. Gallagher, B. Gallis, S. Ryu, G. K. Taylor, M. J. Brittnacher, C. Manoil, and D. R. Goodlett.** 2007. MglA regulates *Francisella tularensis* subsp. *novicida* response to starvation and oxidative stress. J. Bacteriol. **189:**6580-6586.

19. **Han, R., and R.-U. Ehlers.** 2001. Effect of *Photorhabdus luminescens* phase variants on the *in vivo* and *in vitro* development and reproduction of

the entomopathogenic nematodes *Heterorhabditis bacteriophora* and *Steinernema carpocapsae*. FEMS Microbiol. Ecol. **35:**239-247.

20. **Han, R., and R. U. Ehlers.** 2000. Pathogenicity, development, and reproduction of *Heterorhabditis bacteriophora* and *Steinernema carpocapsae* under axenic *in vivo* conditions. J. Invertebr. Pathol. **75:**55-58.

21. **Hansen, A.-M., Y. Gu, M. Li, M. Andrykovitch, D. S. Waugh, D. J. Jin, and X. Ji.** 2005. Structural basis for the function of stringent starvation protein A as a transcription factor. J. Biol. Chem. **280:**17380-17391.

22. **Hansen, A.-M., H. Lehnherr, X. Wang, V. Mobley, and D. J. Jin.** 2003. *Escherichia coli* SspA is a transcription activator for bacteriophage P1 late genes. Mol. Microbiol. **48:**1621-1631.

23. **Hansen, A. M., Y. Qiu, N. Yeh, F. R. Blattner, T. Durfee, and D. J. Jin.** 2005. SspA is required for acid resistance in stationary phase by downregulation of H-NS in *Escherichia coli*. Mol. Microbiol. **56:**719-734.

24. **Hengge-Aronis, R.** 2002. Signal transduction and regulatory mechanisms involved in control of the {sigma}S (RpoS) subunit of RNA polymerase. Micriobiol. Mol. Biol. Rev. **66:**373-395.

25. **Hu, K., J. Li, and J. M. Webster.** 1999. Nematicidal metabolites produced by *Photorhabdus luminescens* (Enterobacteriaceae), bacterial symbiont of entomopathogenic nematodes. Nematology **1:**457-469.

26. **Hu, K., and J. M. Webster.** 2000. Antibiotic production in relation to bacterial growth and nematode development in *Photorhabdus--Heterorhabditis* infected *Galleria mellonella* larvae. FEMS Microbiol. Lett. **189:**219-223.

27. **Hulton, C. S. J., A. Seirafi, J. C. D. Hinton, J. M. Sidebotham, L. Waddell, G. D. Pavitt, T. Owen-Hughes, A. Spassky, H. Buc, and C. F. Higgins.** 1990. Histone-like protein H1 (H-NS), DNA supercoiling, and gene expression in bacteria. Cell **63:**631-642.

28. **Ishihama, A., and T. Saitoh.** 1979. Subunits of RNA polymerase in function and structure. IX. Regulation of RNA polymerase activity by stringent starvation protein (SSP). J. Mol. Biol. **129:**517-530.

29. **Kiontke, K., and D. H. A. Fitch.** 2005. The phylogenetic relationships of *Caenorhabditis* and other rhabditids, *WormBook*, ed. The *C. elegans* Research Community, WormBook.

30. **Merrell, D. S., D. L. Hava, and A. Camilli.** 2002. Identification of novel factors involved in colonization and acid tolerance of *Vibrio cholerae*. Mol. Microbiol. **43:**1471-1491.

31. **Milstead, J. E.** 1979. *Heterorhabditis bacteriophora* as a vector for introducing its associated bacterium into the hemocoel of *Galleria mellonella* larvae. J. Invertebr. Path. **33:**324-327.

32. **Minet, A. D., and R. Chiquet-Ehrismann.** 2000. Phylogenetic analysis of teneurin genes and comparison to the rearrangement hot spot elements of *E. coli*. Gene **257:**87-97.

33. **Parkhill, J., B. W. Wren, N. R. Thomson, R. W. Titball, M. T. Holden, M. B. Prentice, M. Sebaihia, K. D. James, C. Churcher, K. L. Mungall, S. Baker, D. Basham, S. D. Bentley, K. Brooks, A. M. Cerdeno-Tarraga, T. Chillingworth, A. Cronin, R. M. Davies, P. Davis, G. Dougan, T. Feltwell, N. Hamlin, S. Holroyd, K. Jagels, A. V. Karlyshev, S. Leather, S. Moule, P. C. Oyston, M. Quail, K. Rutherford, M. Simmonds, J. Skelton, K. Stevens, S. Whitehead, and B. G. Barrell.** 2001. Genome sequence of *Yersinia pestis*, the causative agent of plague. Nature **413:**523-527.

34. **Poinar, G. O.** 1975. Description and biology of a new insect parasitic rhabditoid, *Heterorhabditis Bacteriophora* N. Gen., N. Sp. (Rhabditida; Heterorhabditidae N. Fam.). Nematologica **21:**463-470.

35. **Richardson, W. H., T. M. Schmidt, and K. H. Nealson.** 1988. Identification of an anthraquinone pigment and a hydroxystilbene antibiotic from *Xenorhabdus luminescens*. Appl. Environ. Microbiol. **54:**1602-1605.

36. **Williams, M. D., T. X. Ouyang, and M. C. Flickinger.** 1994. Starvation-induced expression of SspA and SspB: the effects of a null mutation in *sspA* on *Escherichia coli* protein synthesis and survival during growth and prolonged starvation. Mol. Microbiol. **11:**1029-1043.

37. **Zhou, X., H. K. Kaya, K. Heungens, and H. Goodrich-Blair.** 2002. Response of ants to a deterrent factor(s) produced by the symbiotic bacteria of entomopathogenic nematodes. Appl. Environ. Microbiol. **68:**6202-6209.

Chapter 2: THE *PHOTORHABDUS TEMPERATA SSPAB* LOCUS IS REQUIRED FOR SYMBIONT TRANSMISSION IN *HETERORHABDITIS BACTERIOPHORA*

This chapter is formatted to be published in Journal of Bacteriology. It is reported here that a *Photorhabdus temperata sspA* mutant is essential for symbiont transmission in *Heterorhabditis bacteriophora* nematodes. The process of symbiont transmission is sophisticated, involving extra- and intracellular infection of maternal and IJ offspring intestines. The *sspA* mutant, TRN162, infects the maternal intestine normally, but fails to survive after adhesion to the IJ intestine. The addition of *sspAB* into the mutant chromosome restored the ability to complete symbiont transmission. In addition, the mutant has a growth defect, a hypermotile twitching motility and an increased sensitivity to H_2O_2, possibly providing insight into the role of *sspA* in *Photorhabdus* transmission.

Title. The *Photorhabdus temperata sspAB* locus is required for symbiont transmission in *Heterorhabditis bacteriophora*

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Abstract

The entomopathogenic nematode, *Heterorhabditis bacteriophora*, and the Gram-negative, insect pathogenic enteric bacterium, *Photorhabdus luminescens*, coexist in a mutualistic symbiotic relationship. Symbionts are maternally transmitted by a sophisticated pathway involving multiple adhesion and invasion steps. A transmission defective mutant, TRN162, was identified that successfully completes the initial steps in the transmission cycle, but forms a spheroplast at 120 h post-recovery, resulting in a 0% transmission efficiency. TRN162 contains a transposon insertion in *sspA*, which is predicted to encode a homolog of the stringent starvation protein A. *sspAB* is not essential for *P. temperata* virulence in insects, growth under acidic conditions nor in the presence of cationic microbial peptides. However, the mutant displays a slight growth defect, a hypermotile twitching motility phenotype and an increased sensitivity to growth in the presence of H_2O_2. The inability of TRN162 to fully complete the symbiont transmission cycle may be explained by its increased sensitivity to oxidative stress or to a yet unidentified difference in gene expression as regulated by *sspAB*.

Introduction

Species of the Gram-negative bacteria, *Photorhabdus*, colonize the intestine of the entomopathogenic nematode *Heterorhabditis bacteriophora* in an obligate mutualistic manner. The bacteria are required for insect killing and

serve as a substrate for nematode growth (22). The free-living, developmentally arrested infective juvenile (IJ) nematode, harboring bacterial symbionts within its intestine, seeks out its larval insect prey (23). Upon entering the insect, the nematode regurgitates its monoculture of *Photorhabdus,* which in turn produce toxins that kill the insect within 24 to 48 h. The bacteria also produce compounds to discourage other saphrophytic organisms from feeding on the nutrients and byproducts provided by the insect cadaver (7). As the worm feeds on the bacteria, it develops through two to three generations within the insect cadaver. Once all nutrients have been exhausted and the worm density peaks, the IJs reassociate with *Photorhabdus* and disperse to infect another insect larva (15).

Since *H. bacteriophora* and *Photorhabdus* require one another for insect virulence, colonization of the nematode intestine and transmission to the next generation of nematodes involves many selective steps that are sure to involve gene regulation in both partners (9). This process begins with symbiont regurgitation by recovering IJs, where all intestinal symbionts are expelled. After the nematode begins to feed on *Photorhabdus*, a few bacteria adhere to the posterior of the intestine and form a biofilm before adherent cells invade the rectal gland cells to gain access to the IJs developing in the maternal pseudocoelom (9). The bacteria then adhere to the pharyngeal intestinal valve cells (PIVCs) of the IJs and ultimately invade these cells before exiting and continuing on to fully colonize the IJ intestine (9). Although the stages of the transmission process are likely highly selective, the mechanisms by which symbionts negotiate this selective gauntlet are unknown.

The stringent starvation protein A (SspA), is a RNA polymerase associated protein that is highly conserved in many microorganisms (19). It has particular relevance in regulation of host-bacterial interactions and mediation of stress resistances. It regulates the resistance to acidic stresses in *Escherichia coli* (18), *Yersinia pestis, Vibrio cholerae* and *Pseudomonas aeruginosa* (16), while it plays a role in oxidative stress resistance in *Francisella novicida* (14). In *Providencia stuartii*, it has been shown to activate a regulator of antibiotic resistance genes (11). Additionally, in both *Y. enterocolitica* and *E. coli*, SspA has demonstrated regulation of motility (1, 18). In addition, *regF*, another homolog of *sspA*, negatively controls type IV piliation in *Neisseria gonorrhoeae* (10).

In *E. coli*, the expression of SspA has been shown to increase during stationary phase and upon glucose, nitrogen, phosphate and/or amino acid starvation (26). Moreover, when starved for arginine, an *E. coli sspA* mutant had decreased survivability compared to the parental strains (26). Additionally, SspA has been identified as a transcriptional activator for the expression of late genes of the *E. coli* bacteriophage P1 (17). SspA, along with the late promoter activator (Lpa) protein, is required for the P1 phage to enter the lytic cycle through the expression of late genes (17).

Studying a genetically malleable symbiotic relationship, such as that of *H. bacteriophora* and *Photorhabdus*, provides an opportunity to better understand gene regulation during the establishment of host-bacterial interactions. In this study, a *P. temperata sspA* mutant, with likely polar effects on the downstream

sspB, was identified as having an essential role in symbiont transmission in *H. bacteriophora* IJs. This mutant is defective only in a late stage of the transmission process. Here, we have characterized a mutant that forms a spheroplast near the PIVCs of developing IJs at 120 h post-recovery and *in vitro* studies were undertaken to explain a possible stress induced by the nematode at this time. Moreover, the *sspAB* mutant shows a decreased resistance to growth under oxidative conditions as well as an increase in twitching motility.

Materials and Methods

Plasmids, bacterial strains and culture media

All *Photorhabdus* strains were cultured in either PP3S (2% [w/v] Protease Peptone No. 3, Bectin Dickinson, Cockeysville, MD and 0.5% NaCl, Sigma-Aldrich, Saint Louis, MO), Grace's Insect Media (Gibco, Invitrogen, Carlsbad, CA) or nutrient broth (0.8%, Bectin Dickinson) at 28°C with agar (1.5% w/v), tetracycline (10 µg/ml), gentamicin (0.75 µg/ml), cornoil (1.2%, Mazola) or

Table 2-1: Strains and plasmids used in this study

Strain or Plasmid	Characteristic(s)	Source
Nematode strains		
Heterorhabditis bacteriophora TT01 – M31e	Inbred (self-fertilized 13 times)	(8)
Bacterial strains		
Photorhabdus luminescens		
subsp. *laumondii* TT01	Wild type (primary phase)	Nematode Host
subsp. *laumondii* TT01Tn7GFP	Labeled with Tn7-GFP	(9)
Photorhabdus temperata		
NC1	Wild type (primary phase)	ATCC29304
NC1Tn7GFP	Labeled with Tn7-GFP	(9)
TRN162	Transmission defective *P. temperata* NC1Tn7GFP, *sspA* disrupted	This study
TRN162Tn7*sspAB*	TRN162 complemented with pTeT^R-*sspAB*	This study
TRN16	Transmission defective *P. temperata* NC1Tn7GFP	
Escherichia coli		
DH5α	Cloning strain	
TransforMax EC100D *pir116*	Electrocompetent *E. coli*	EPICENTRE Biotechnologies
BW29427	*dap* auxotroph, *tra*	(K.A. Datsenko and B. L. Wanner)
BW29427 + pURE10	Mini-*HimarGm*	D. Lies and D. Newman
Micrococcus leutus		
Plasmid vectors		
pCRII	Cloning vector	Invitrogen
pURE10	Mini-*HimarGm* transposon	D. Lies and D. Newman
pUC18R6KT mini-Tn7T	Mini-Tn7, Tet^R,	(6)
pTet^R-*sspAB*	pUC18R6KT derivative, complementation vector	This study

Table 2-1 continued

pUX-BF13	Transposase complex, encoding TnsABC+E	(6)
pCM639	Template for Tet^R amplification	
Primers		
MarOUT	5' caagcttgtcatcgtcatcc 3'	This study
GmOUT	5' cggtaaattgtcacaacgcc 3'	This study
sspAB for	5' gtcgtcgtccacagttctcc 3'	This study
sspAB rev	5' tggctgatatctgcacaacc 3'	This study
Tet^R for	5' gatc**ctgcag**gcggccgcccacccggtggcgccaactattgcgataaca 3'	This study
Tet^R rev	5' **ctgcag**gtcatccaccggatcaattc 3'	This study

cholesterol (chol, 10 mg/ml, Sigma Aldrich) added when appropriate. *E. coli* was cultured in either SOC (super optimal broth (SOB) with 20 mM glucose) or Luria Broth (LB) (3) modified to contain 5 g/L NaCl with agar (1.5% w/v), ampicillin (100 µg/ml), tetracycline (10 µg/ml), or diaminopimelic acid (DAP, 300 µg/ml) added when appropriate. Ringer's solution (100 mM NaCl, 1.8 mM KCl, 2 mM $CaCl_2$, 1 mM $MgCl_2$, 5 mM HEPES, pH 6.9) and saline solution (0.85% NaCl) were used to wash and store *H. bacteriophora* axenic worm stock. Strains and plasmids used in this study are listed in Table 2-1.

Axenic nematode stock

50 µl of overnight cultures of TRN16 (completely defective in symbiont transmission) grown in PP3S was spread onto one half of a split well Petri dish of nutrient agar (NA) + cornoil. After 48 h of incubation at 28°C, 10 µl of *H. bacteriophora* M31e was added and plates were incubated at 28°C for an additional 11 d. Emerging IJs were collected in Ringer's solution on the empty half of the plate. Nematodes were harvested by centrifugation at 1,200 rpm for 1 min, surface sterilized in 1% commercial bleach for 5 min and washed three times with Ringer's before being stored in 10 ml of Ringer's solution. To ensure that the IJs were axenic, 50 µl of nematode stock was homogenized using a motorized tissue grinder (Kontes Glass Co., Vineland, NJ), plated on PP3S and incubated at 28°C for 48 h. Antibiotics were added to the nematode stock at the following concentrations: 100 µg/ml streptomycin, 100 µg/ml ampicillin, 30 µg/ml kanamycin and 10 µg/ml gentamicin.

Transposon mutagenesis and mutant screening

Overnight cultures of *P. temperata* strain NC1Tn7GFP and BW29427 carrying pURE10 were grown in PP3S and LB+DAP+Gm, respectively. 10 ml of fresh media was inoculated with 30 µL of overnight cultures and grown to an OD_{600} of 0.6. Cells were then pelleted by centrifugation, washed three times with LB+DAP and resuspended in a final volume of 500 µl LB+DAP. The two strains were then combined, centrifuged, resuspended in 50 µl LB+DAP and finally plated on LB+DAP. After 8 h of incubation at 28°C, cells were then washed off the plate using LB, centrifuged, washed three times with LB and resuspended in 1.5 ml LB before plating 100 µl on PP3S+Gm. Isolated colonies were patched onto PP3S+Gm and incubated at 28°C for 48 h. Each colony was then cultured in 250 µl PP3S+Gm for an additional 48 h at 28°C. 50 µl of the liquid culture was spread on NA+cornoil+Gm plates and incubated for an additional 48 h at 28°C, after which time 10 µl of axenic nematode stock (M31e grown on TRN16) that had been washed three times with saline solution was added. Plates were incubated at 28°C for 10-12 d until IJs formed and were isolated in the condensation that formed on the lids of the Petri dishes. Mutants were screened and identified by visualizing the lack of GFP-labeled symbionts in the intestine of *H. bacteriophora* IJs under a fluorescent stereomicroscope (Leica MZ16F, Leica Microsystems, Wetzler, Germany). Transmission efficiency was determined by scoring for the presence of bacteria in the IJ intestine, where a single GFP-labeled cell could be detected and was calculated by dividing the total number of

IJs observed (minimum 5,000 for mutants with 0% transmission efficiencies) by the number of colonized IJs.

Characterization of transmission processes

To characterize the stages in the transmission process of NC1Tn7GFP and TRN162, 10 µl of axenic nematode stock that had been washed three times in saline solution was placed on lawns of the strain to be tested grown on NA+chol plates and incubated between 8 h and 132 h at 28°C. Worms were then transferred from the GFP-labeled bacteria (NC1Tn7GFP or TRN162), rinsed in sterile Ringer's solution and transferred to lawns of unlabeled *P. temperata* strain NC1 for 4 h at 28°C to clear the intestine of transient labeled bacteria before being imaged, with images taken at 36, 48, 72, 120 and 132 h post-recovery. Nematodes were immobilized using 10 – 20 µm sodium azide and imaged on 1% agar pads using a fluorescent compound microscope (Leica DM5000).

Transposon retrieval and sequencing

Genomic DNA was purified from TRN162 grown in 3 ml of Grace's Insect Media overnight at 28°C using the DNeasy Tissue Kit (Qiagen, Valencia, CA). Direct retrieval of the transposon is possible because the *HimarGm* transposon has an R6Kγ origin of replication and can therefore be replicated in any strain expressing the π protein. Between 5 and 20 µg of genomic DNA was digested with *SphI* (New England Biolabs, Ipswitch, MA), diluted to 250 µl and then ligated with T4 DNA ligase (New England Biolabs). Circularized DNA fragments were

precipitated with isopropanol, washed with 70% ethanol and resuspended in 10 µl of double distilled (dd) H_2O before electroporating 1 µl of DNA into 10-15 µl TransforMax EC100D *pir-116* electrocompetent *E. coli* cells (EPICENTRE Biotechnologies, Madison, WI) using a Gene Pulser Xcell Electroporation System (BioRad, Hercules, CA). Transformed cells were recovered in SOC, harvested and plated on LB+Gm and incubated overnight at 37°C. Isolated colonies were then grown overnight in 3 ml LB+Gm and plasmid purified (Qiagen QIAprep Spin Miniprep Kit). The presence of the *HimarGm* was verified by plasmid digestion with *SacI* (New England Biolabs) releasing a 1 kb fragment. Digestion by *SphI* was used to determine size and digestion pattern of flanking DNA. Digestion products were analyzed by agarose gel electrophoresis. Plasmids verified to have a 1 kb fragment were submitted for sequencing to the Michigan State University Research Technology Support Facility with the *HimarGm* specific primers MarOUT and GmOUT. Analysis of sequencing data was performed using coliBLAST (http://xbase.bham.ac.uk/colibase/blast.pl) to determine what homolog of *P. luminescens* subsp. lamondii TT01 genes was disrupted by transposon insertion in *P. temperata*.

Complementation

The tetracycline resistance (Tet^R) gene was PCR amplified from pCM639 using primers Tet^R for and Tet^R rev with *PstI* restriction enzymes sites designed on the 5' ends (Table 2-1, *PstI* sequences in bold). The PCR product was ligated into pCRII (Invitrogen) and transformed into chemically competent *E. coli* DH5α.

Plasmids purified from the resulting colonies were digested with *PstI* (New England Biolabs) and cloned into the *NsiI* site of pUC18R6KT. Genomic DNA was purified from *P. luminescens* subsp. lamondii TT01 using the DNeasy Tissue Kit. Wildtype *sspAB* was then PCR amplified from TT01 genomic DNA using the primers *sspAB* for and *sspAB* rev. The PCR product was ligated into pCRII and transformed into chemically competent *E. coli* DH5α. Plasmids purified from the resulting isolated colonies were then digested with *XhoI* and *KpnI* cloned into pUC18-Tet^R at the same restriction sites. The resulting complementation vector, $pTet^R$-sspAB, was transformed into BW29427 and mobilized into TRN162 via a triparental mating as follows: Overnight cultures of TRN162 or BW29427 carrying either pTetR-sspAB or pUX-BF13 were grown in PP3S and LB+DAP respectively. Cells were pelleted by centrifugation, washed three times with LB+DAP before being combined and plated onto LB+DAP. After an 8 h incubation at 28°C, cells were washed off the plate with LB, centrifuged and washed three times with LB. They were then plated on PP3S+Tet and incubated at 28°C. Isolated colonies were verified by patching onto PP3S+Tet before being tested in the worm.

Phenotypic variation characterizations

Phase variation of *Photorhabdus* strains were characterized as described previously (4). Media used in dye absorption assays included nutrient agar supplemented with 2,3,5 – triphenyltetrazolium and bromthymol blue at 40 μg/μl and 25 μg/μl respectively, Congo Red agar (nutrient agar with 0.01% [wt/vol]

Congo Red) and eosin y-methylene blue agar (PP3S plus eosin y and methylene blue at 400 µg/µl and 65 µg/µl respectively). Bioluminescence was scored by observing 48 h mutant and parental strain colonies in the dark. Hemolytic and siderophore activities were determined by observing *Photorhabdus* growth on blood agar (Cole-Palmer) or chrome azurol S (CAS) agar (24), respectively. To assay for the production of extracellular antibiotics, *Photorhabdus* was spot inoculated onto PP3S agar and grown for 48 h at 28°C. Then 0.5 ml of an overnight culture of *M. luteus* was mixed with 20 ml of soft LB agar (0.75%) at 42°C and overlayed on top of the *Photorhabdus* patches. Antibiotic production was determined by the presence of zones of growth inhibition of *M. luteus.* The support of nematode growth and reproduction was monitored by growing M31e *Heterorhabditis bacteriophora* raised on TRN16 on lawns of TRN162.

Photorhabdus growth

Overnight cultures of *Photorhabdus* strains were inoculated in quintuple to an OD_{600} of 0.05 in 150 µL of Grace's Insect Media in a standard clear 96-well microtiter dish. Growth was carried out at 28°C with 30 sec of shaking every five min. Absorbance measurements were made at five min intervals for a total of 20 h in a SpectraMax M5 plate reader (Molecular Devices, Sunnyvale, CA).

Insect virulence

Manduca sexta eggs were obtained from North Carolina State University Insectary (Raleigh, NC) and raised on Gypsy Moth Wheat Germ Diet premixed

with agar (MP Biomedicals Inc., Solon, OH) at 25°C with a 14 h light/ 10 h dark cycle. 3 µl of a 24 h *Photorhabdus* culture was inoculated into 3 ml of fresh PP3S and grown for an additional 24 h at 28°C prior to injections. Fourth or fifth instar larvae of *M. sexta* were injected behind the 1st proleg with 10 µL of serial dilutions of liquid cultures of *Photorhabdus*. Insect mortality was monitored in 24 h intervals for 72 h.

Acid tolerance assay

Cultures of NC1Tn7GFP and TRN162 were grown overnight in PP3S. 200 µl of PP3S, pH adjusted with HCl and supplemented with 50 mM MES, was inoculated to an OD_{600} of 0.05 in a 96-well microtiter dish. Cultures were grown in a shaking 28°C incubator overnight and OD_{600} measurements were then taken in a SpectraMax5 plate reader. Relative OD_{600} values were calculated by dividing the OD_{600} value at a particular pH by the OD_{600} value at pH 7.

Motility assay

5 µl of *Photorhabdus* strains to be tested (OD_{600} adjusted to 2.0) were inoculated onto PP3S onto either swarming motility agar (0.35% agar) or twitching motility agar (0.75% agar). Plates were incubated at 28°C and diameter measurements were made under a fluorescent stereomicroscope at 20 h and 37 h.

Oxidative stress assays

Cultures of NC1Tn7GFP and TRN162 were grown overnight in PP3S. 3 ml of PP3S + H_2O_2 was inoculated to an OD_{600} of 0.05 and grown at 28°C for 16-18 h. Serial dilutions were plated out on PP3S to determine colony forming units per ml (CFU/ml). Methyl viologen dichloride hydrate (paraquat) was purchased from Sigma-Aldrich. 200 µl of PP3S was supplemented with paraquat to various concentrations in a 96-well microtiter dish and inoculated with NC1Tn7GFP and TRN162 to an OD_{600} of 0.05. The cultures were grown in a shaking 28°C incubator for 16-18 h and growth was measured by determining the OD_{600}. Relative OD_{600} values were calculated by dividing the OD_{600} value in the presence of a particular paraquat concentration by the OD_{600} value in the absence of paraquat.

Antimicrobial peptide assays

Protamine sulfate, lactoferrin and polymyxin B were purchased from Sigma-Aldrich (St. Louis, MO). For growth in the presence of antimicrobial peptides, overnight cultures of *Photorhabdus* strains to be tested were inoculated to an OD_{600} of 0.05 in 200 µL of PP3S plus appropriate antimicrobial peptide in a 96-well microtiter plate. The samples were incubated at 28°C for 16-18 h with constant shaking. OD_{600} measurements were made in a SpectraMax M5 plate reader. Each concentration was inoculated in triplicate and repeated a minimum of three times.

Results

sspA is disrupted in the transmission defective mutant TRN162.

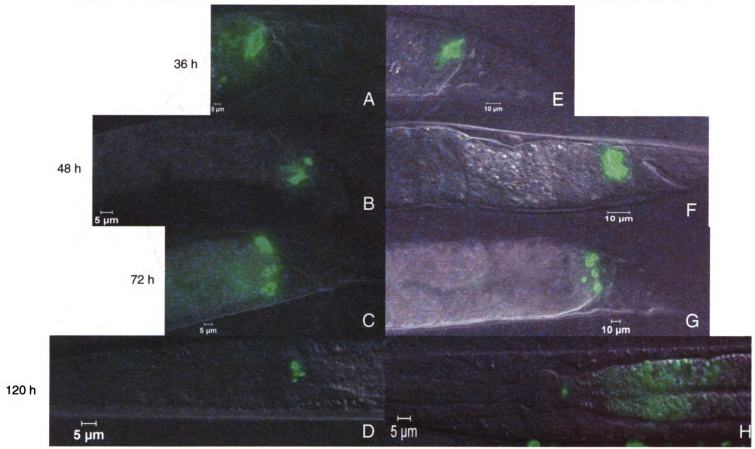
A transposon mutagenesis screen of green fluorescent protein (GFP) labeled *P. luminescens* subsp. laumondii TT01 and *P. temperata* strain NC1 was conducted with a *HimarGm* transposon to identify genes essential for bacterial colonization of the *H. bacteriophora* intestine. The screen produced approximately 30 transmission (TRN) defective mutants with various genes being disrupted. A *P. temperata* mutant, TRN162, was isolated that is defective in transmission. Retrieval by marker rescue and sequencing of the DNA flanking the *HimarGm* of mutant TRN162 revealed that the transposon was inserted near nucleotide 330 of *plu4013*, a gene predicted to encode a homolog of the stringent starvation protein A (SspA). *sspA* is 642 bp long and is predicted to encode a protein of 213 amino acids in length. 3 bp downstream of *sspA* is the predicted homolog of the stringent starvation protein B *(sspB)*, which is 516 bp long, encoding a 171 amino acid protein.

TRN162 has a 0% transmission efficiency, where zero cells were observed in 5,000 IJs examined by fluorescent stereomicroscopy. Specifically, it is able to complete the initial stages in *Photorhabdus* transmission in *H. bacteriophora*, but is unable to fully colonize the resulting infective juvenile (Figure 2-1). The mutant is able to successfully form a biofilm near the rectal gland cells at 36 h post-recovery, indistinguishable from NC1Tn7GFP. At 48 h it can be observed that the rectal gland cells at the posterior of the intestine in the

maternal nematode are invaded, again analogously to the parental strain. By 72 h, both TRN162 and NC1Tn7GFP have entered the vacuoles of the rectal gland cells. However, at 120 h, a weakly-fluorescent spheroplast is seen near the PIVCs of the IJ raised on TRN162. This is in direct contrast to NC1Tn7GFP, which adheres to and ultimately invades the PIVCs. The spheroplast is indicative of a dying bacterium that has lost its structural integrity. The mutant is subsequently unable to completely colonize the intestine of the IJ and therefore fails to complete the transmission cycle.

Upon prolonged culturing of TRN162, the transmission defective phenotype changes in that the mutant is no longer able to complete even the initial steps of the transmission cycle. Therefore, samples of TRN162 were not cultured any longer than ten days to ensure that only the variant able to successfully adhere to and invade the rectal gland cells before forming a spheroplast at 120h was used for all experiments.

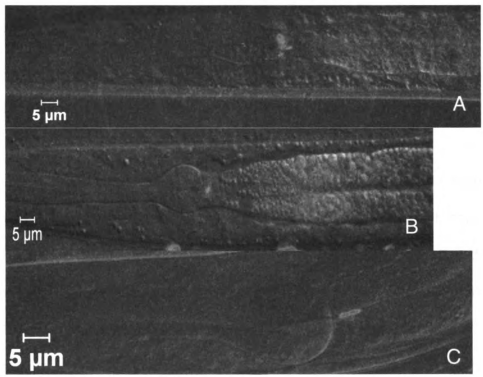
Figure 2-1: Transmission defective phenotype of TRN162. NC1Tn7GFP is shown in A-D. TRN162 is shown in E-H.



The disruption of sspAB is responsible for the transmission defective phenotype of TRN162.

Several unsuccessful attempts were made to complement the transmission defective phenotype of TRN162 by expressing wildtype *sspAB* from a plasmid construct. As the defective stage of symbiont transmission occurs at 120 h in this particular mutant, it is difficult to maintain antibiotic selection of a complementation plasmid in the intestine of the nematode for this length of time. Therefore, wildtype genes were stably inserted into the genome in order to

Figure 2-2: Complementation of TRN162. All pictures are 120h post-recovery.
(A) NC1Tn7GFP (B) TRN162 (C) TRN162Tn7*sspAB*



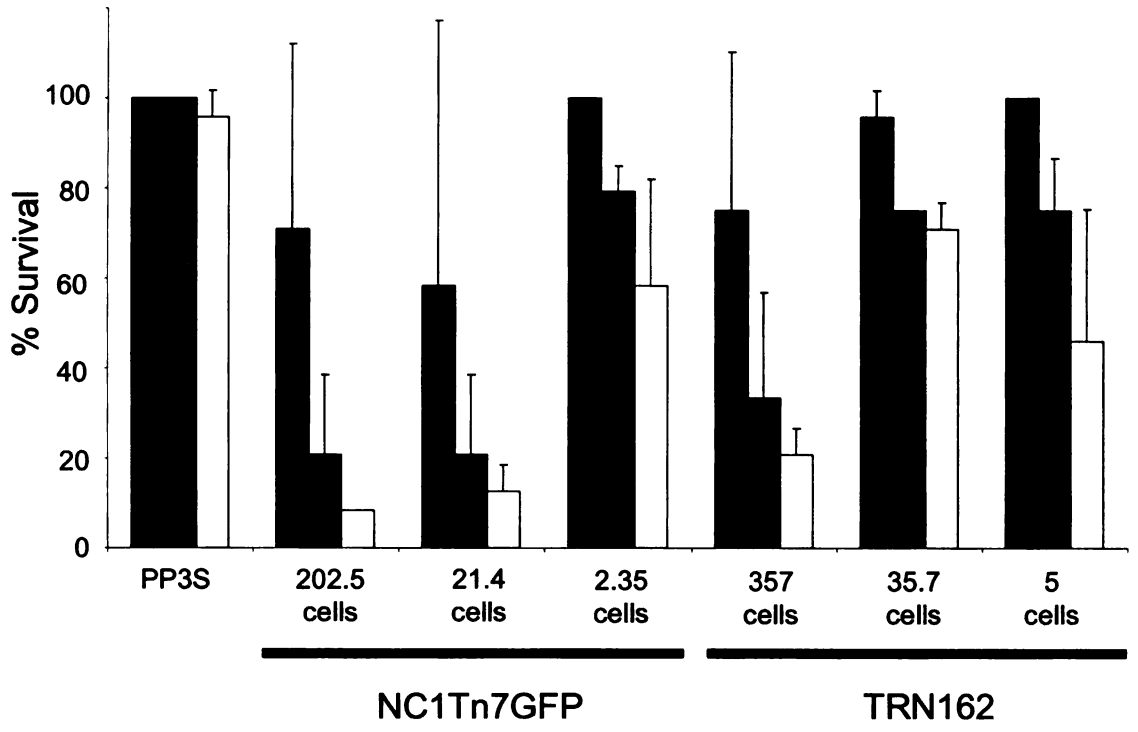
complement TRN162. This was accomplished by using a mini-Tn7-Tet^R transposon.

To restore the transmission defective phenotype, wildtype *sspAB* genes were mated into TRN162, resulting in strain TRN162Tn7*sspAB*. The resulting complemented mutant displays a full restoration of the transmission cycle (Figure 2-2). TRN162Tn7*sspAB* is able to successfully survive adherence to and invasion of the PIVCs after 120 h like NC1Tn7GFP, culminating in the colonization of IJs. The transmission efficiency of TRN162Tn7*sspAB* is 26.0% ± 5.3%. Although this transmission efficiency is not as high as NC1Tn7GFP, it still represents a significant increase over the 0% transmission efficiency of TRN162. These data support previous results indicating the defective transmission phenotype of TRN162 is due to the disruption of the *sspAB* operon.

sspAB is not essential for insect virulence.

Some factors required for symbiont transmission may also be required for insect pathogenesis. Additionally, there are known functions of SspA involvement in virulence. To determine if TRN162 has an altered pathogenicity to insect larvae, serial dilutions of NC1Tn7GFP and TRN162 were injected into 4th or 5th instar larvae of *M. sexta.* Virulence was assayed every 24 h for 72 h. TRN162 is able to cause significant death within 72 h after injections, similarly to NC1Tn7GFP (Figure 2-3). These results suggest that *sspAB* is not essential for insect virulence.

Figure 2-3: *P. temperata* virulence to *M. sexta*. Insect mortality was monitored after 24 h (black bars), 48 h (gray bars) and 72 h (white bars).



TRN162 has a growth defect compared to the parental strain NC1Tn7GFP.

It was possible that TRN162's inability to complete the symbiont transmission process was due to a growth defect where the mutant would be unable to completely colonize the IJ intestine after adhering to the PIVCs within the time assayed. The growth of TRN162 and its parental strain was assessed by measuring the OD_{600} in 5 min intervals for a period of 20 h. Both *Photorhabdus* strains reached similar final OD_{600}. From the portion of the growth curve corresponding to exponential phase, the growth rate (μ) and doubling time (T_2) was calculated (Table 2-3). With a growth rate of 0.210 ± 0.017 h^{-1}, TRN162

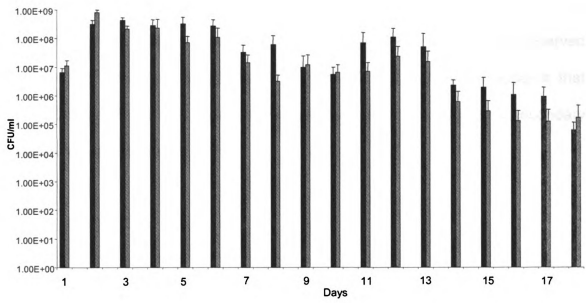
exhibits a growth defect compared to the 0.273 ± 0.013 h^{-1} growth rate of NC1Tn7GFP.

As it has been reported that *sspA* mutants in *E. coli* and the *mlgA* mutant of *F. novicida* have defects in stationary phase survival, the ability of TRN162 to survive prolonged culturing was evaluated in comparison to NC1Tn7GFP. After 18 d of prolonged stationary phase culturing in liquid media, no significant difference was observed between NC1Tn7GFP and TRN162 (Figure 2-4).

Table 2-2: Growth Rates of NC1Tn7GFP and TRN162

	Growth Rate	**Doubling Time**
NC1Tn7GFP	0.273 ± 0.013 h^{-1}	2.54 ± 0.12 h
TRN162	0.210 ± 0.017 h^{-1}	3.32 ± 0.28 h

Figure 2-4: Stationary Phase Survival of NC1Tn7GFP (black) and TRN162 (gray)



TRN162 displays characteristics of primary phase variants.

Photorhabdus species exhibit phase variation phenotypes. As only primary phase variants are transmitted by IJs, it was essential to determine the phase variation state of TRN162. This mutant displays primary phase characteristics, corresponding to those of NC1Tn7GFP (Table 2-3). *P. temperata* strain NC1 cells range in size between 3 and 6 μm (12). The cell length of TRN162 is comparable, with an average of 3.41 ± 0.91 μm. TRN162 absorbs pigments from its media supplemented with the dyes neutral red, eosin y- methylene blue, bromthymol blue or Congo red, which is a classic characteristic of primary phase variants. Furthermore, it displayed hemolytic activity, as well as siderophore and antibiotic activity, as do primary cells. Colonies of TRN162 are convex in shape, mucoid, and pigmented with an orange color. This pigmentation is in contrast to the yellow pigmentation of NC1Tn7GFP. Individual cells produced cellular inclusion proteins, as seen by light microscopy. No obvious difference in nematode growth was observed between IJs grown on NC1Tn7GFP or TRN162. These results suggest that TRN162's inability to complete the transmission cycle is not due to a secondary phase variant phenotype.

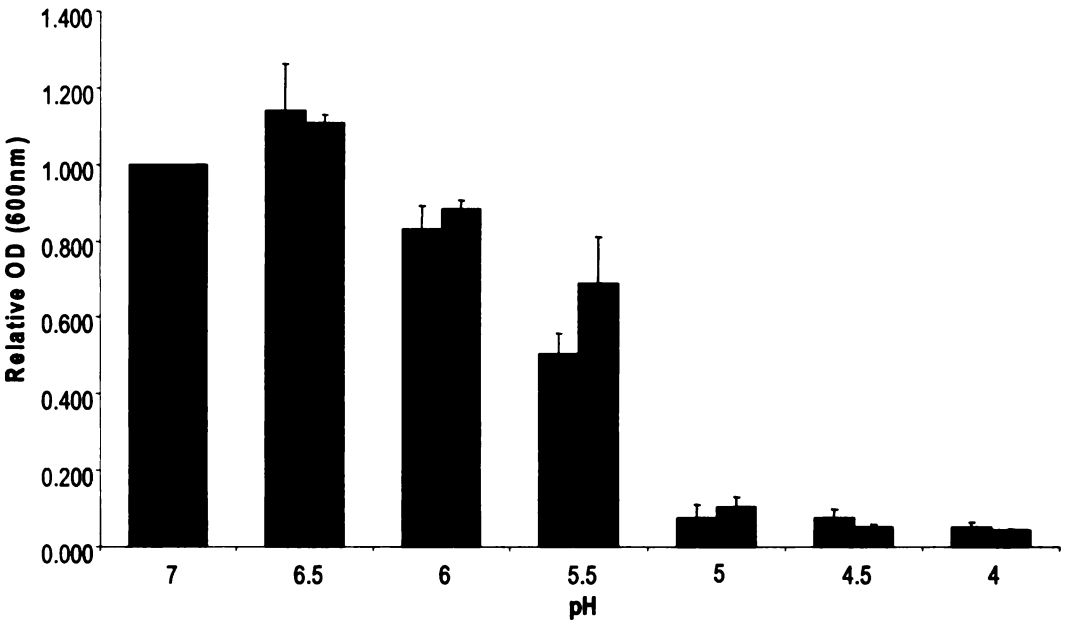
Table 2-3: Phase variation characterization of NC1 primary, NC1 secondary, NC1Tn7GFP and TRN162

Phenotype Assayed	NC1 Primary	NC1 Secondary	NC1Tn7GFP	TRN162
Dye Absorption				
Neutral red	+	–	+	+
Eosin Y-methylene blue	+	–	+	+
Bromthymol blue	+	–	+	+
Congo red	+	–	+	+
Bioluminescence	+	–	+	+
Extracellular Products				
Hemolytic activity	+	–	+	+
Siderophore activity	+	–	+	+
Antibiotic activity	+	–	+	+
Cellular Inclusion Proteins	+	–	+	+
Colony morphology	Convex, mucoid	Flat, nonmucoid	Convex, Mucoid	Convex, mucoid
Pigmentation	Yellow	White	Yellow	Orange
Support of nematode growth and reproduction	+	–	+	+

TRN162 does not display a significant difference in the bacteria's ability to grow under acidic conditions.

An *E. coli sspA* mutant displayed an increased sensitivity to growth under acidic conditions due to negative regulation of genes conferring acid resistance (18). Likewise, the nematode may produce some sort of stress, such as lowering the pH, to clear its intestine of nonsymbiotic bacteria at 120 h in the transmission cycle. Both TRN162 and NC1Tn7GFP grow optimally at a pH of 6.5, which is close to the unaltered pH of PP3S. Growth at pH 5.5 and above is similar between NC1Tn7GFP and TRN162, with growth being inhibited by 49.4 ± 5.1% and 31.2 ± 12.4% at pH 5.5, respectively. For both strains, growth is severely inhibited at or below pH 5. At this pH, NC1Tn7GFP displayed a 92.3 ± 3.3% growth inhibition and TRN162's growth was inhibited by 89.7 ± 2.6%.

Figure 2-5: Growth of NC1Tn7GFP (black) and TRN162 (gray) under acidic conditions.



Motility of TRN162

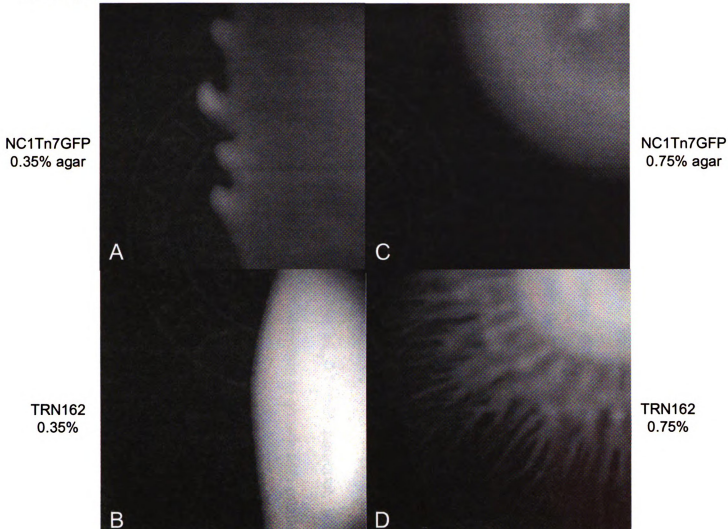
In addition to an increased sensitivity to growth under acidic conditions, the *E. coli sspA* mutant presents a hypermotile phenotype when grown on 0.3% semi-solid agar media (18). A similar phenotype has also been observed in the *Y. enterocolitica sspA* mutant (1). To determine if TRN162 displayed a motility phenotype different than NC1Tn7GFP, both strains (adjusted to OD_{600} = 0.2) were inoculated onto PP3S semi-solid agar plates (0.35% agar for swarming motility and 0.75% agar for twitching motility) and the diameter of the motility ring was measured after 20 h and 37 h. On 0.35% swarming motility agar, there was no significant difference in swarming ring diameter between the strains (Table 2-4). At 37 h, the swarming ring diameter of NC1Tn7GFP was 26.1 ± 3.8 mm and

the swarming ring diameter of TRN162 was 22.7 ± 7.5 mm. Interestingly, the boundary of the TRN162 swarming ring was smooth in comparison to the scalloped edges of the NC1Tn7GFP swarming ring (Figure 2-5). In comparison to NC1Tn7GFP, the *sspA* mutant twitched more than four times farther on 0.75% agar media (Table 2-4) and exhibited elaborate spiked outgrowth along the twitching ring boundary (Figure 2-5, C and D).

Table 2-4: Motility of NC1Tn7GFP and TRN162

	Swarming – 0.35% agar		Twitching – 0.75% agar	
Time	NC1Tn7GFP	TRN162	NC1Tn7GFP	TRN162
20 h	9.5 ± 1.3 mm	8.7 ± 1.7 mm	6.0 ± 0.3 mm	9.0 ± 1.7 mm
37 h	26.1 ± 3.8 mm	22.7 ± 7.5 mm	8.4 ± 0.6 mm	34.9 ± 3.1 mm

Figure 2-6: Motility of NC1Tn7GFP and TRN162. Swarming motility of (A) NC1TN7GFP and (B) TRN162. Twitching motility of (C) NC1Tn7GFP and (D) TRN162



TRN162 shows an increased sensitivity to H_2O_2

There is evidence that the *F. novicida sspA* homolog, *mglA*, has decreased resistance to oxidative stresses (14). To determine if *sspA* plays a role in *Photorhabdus'* response to oxidative stress, both TRN162 and NC1Tn7GFP were grown in media treated with up to 100 mM H_2O_2 or 10 mM paraquat, a compound that reacts with O_2 to produce superoxide and H_2O_2. While NC1Tn7GFP grew to a comparable CFU/ml in the presence of 100 mM H_2O_2 and the PP3S negative control, TRN162 displayed a two-order of magnitude decrease in CFU/ml between 0 mM H_2O_2 and 100 mM H_2O_2 (Figure 2-6).

In media inoculated with a dose as low as 0.1 mM paraquat, both NC1Tn7GFP and TRN162 showed a significant reduction in growth with relative OD_{600} values of 0.174 ± 0.122 and 0.405 ± 0.243, respectively. At the larger dose concentration of 10 mM paraquat, there was no significant difference between the two strains' ability to grow, with relative OD_{600} values of 0.110 ± 0.072 and 0.094 ± 0.030 for NC1Tn7GFP and TRN162, respectively.

Figure 2-7: Growth under oxidative stress. NC1Tn7GFP (black), TRN162 (gray) (A) Growth in the presence of H_2O_2. (B) Growth in the presence of paraquat.

(A)

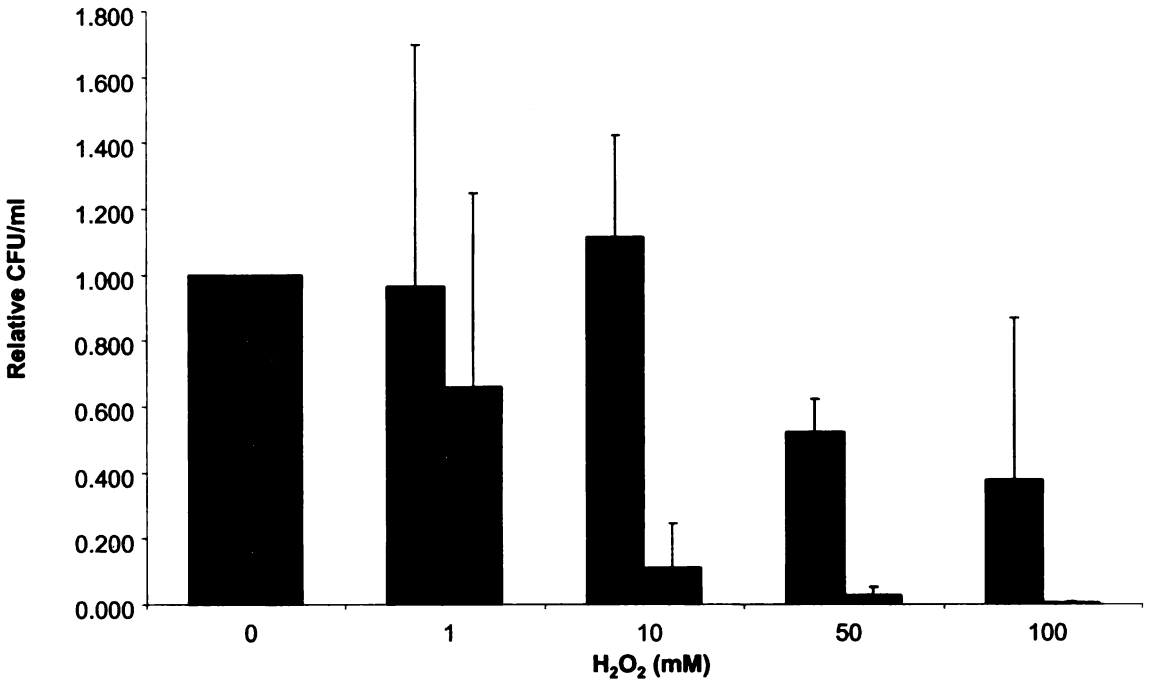
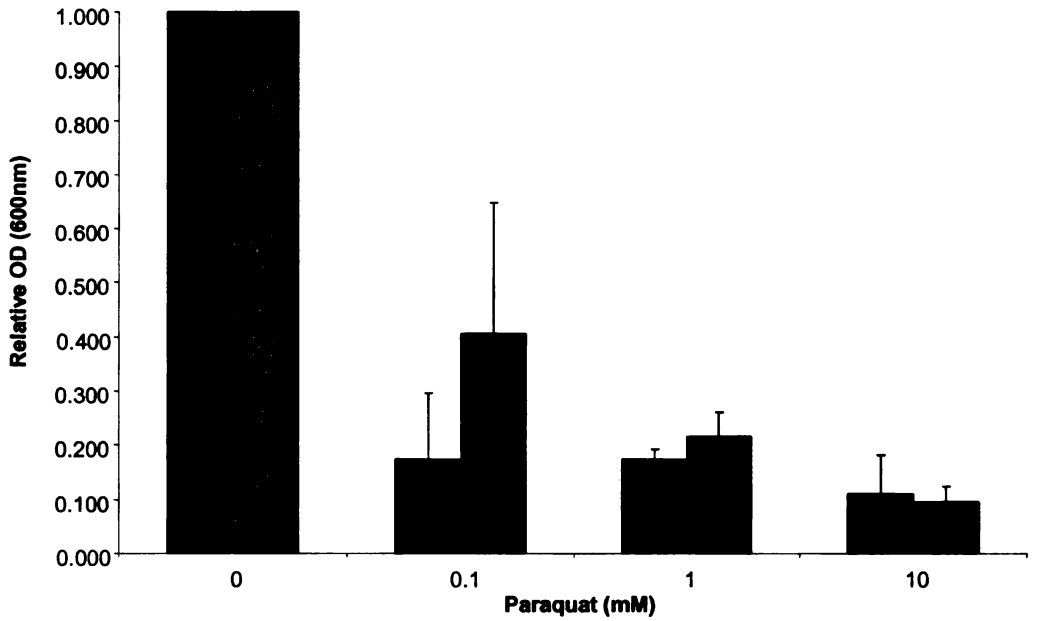


Figure 2-7 continued
(B)



sspAB is not essential for Photorhabdus ability to grow in presence of cationic antimicrobial peptides.

One mechanism shown to be employed by the innate immune systems of organisms commonly encountering microorganisms is the production of antimicrobial peptides (APs) (5). APs commonly are cationic in nature, and thus have a negative impact on the negatively charged bacterial capsule that leads to membrane permeabilization and eventually cell death. It is possible that *H. bacteriophora* produces one or more APs to clear its intestine of non-symbiotic bacteria, imposing a selection process to ensure that only true symbionts are transmitted to IJs.

To determine if the outer membrane or bacterial capsule of the *sspAB* mutant was defective, TRN162 and NC1Tn7GFP were grown in the presence of

the APs polymyxin B and lactoferrin (Figure 2-8). In the presence of 75 μg/ml polymyxin B, the growth of both strains was inhibited approximately 50%. When polymyxin B was added to the growth media at 300 μg/ml, growth was inhibited by 53.2 ± 9.3% in NC1Tn7GFP and 50.2 ± 8.5% in TRN162. In comparison, the highest concentration of lactoferrin tested in this study (450 μg/ml), produced no more than a 4.6% inhibition of growth for both strains.

Figure 2-8: Growth in the presence of antimicrobial peptides. NC1Tn7GFP (black), TRN162 (gray). (A) Polymyxin B, (B) Lactoferrin

(A)

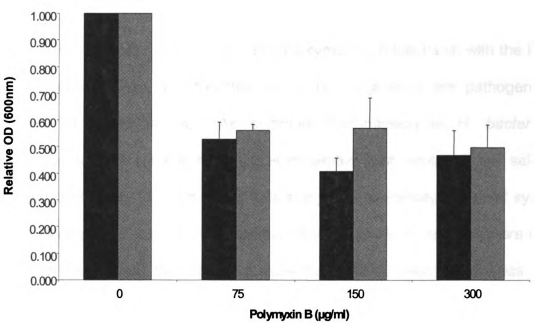
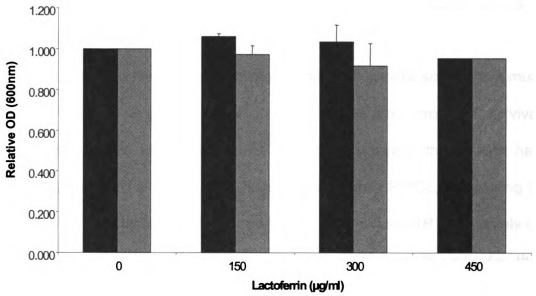


Figure 2-8 continued
(B)



Discussion

Photorhabdus exists in an obligate symbiotic relationship with the IJ stage of *H. bacteriophora* and together, these two organisms are pathogenic to a variety of insect larvas. As symbiont transmission in *H. bacteriophora* nematodes is essential in nature, it is imperative that symbionts are selectively transmitted to the IJ. The aim of this study was to identify essential symbiosis factors employed during *Photorhabdus* transmission in *H. bacteriophora* in order to gain insight into the molecular cues behind this selective process and, in particular, the gene regulation involved in the establishment of host-bacterial interactions. To this end, a transposon mutagenesis screen was conducted to isolate bacterial mutants unable to complete the transmission cycle, resulting in axenic nematode IJs. One mutant, TRN162, was determined to carry a

transposon disruption in the gene encoding a homolog of the stringent starvation protein A (*sspA*). It is also probable that there are polar effects on the downstream *sspB*.

Unlike all other mutants isolated during this genetic screen, this mutant is defective at a very late stage in the transmission process; survival after adherence to the IJ intestine. At 120 h post-recovery, this mutant has been observed to form a spheroplast near the nematode PIVCs, suggesting that the death of the bacterium might be due to an inability of *sspAB* to positively regulate stress response gene(s) required for evasion of a stress. In particular, this study aimed to demonstrate that the transmission defect of TRN162 is due to the disruption of *sspAB* and to determine the underlying cause(s) of why TRN162 is unable to successfully complete the transmission cycle in *H. bacteriophora* nematodes.

Our research has shown that the *sspAB* genes are essential for bacterial colonization of the nematode intestine and that TRN162 displays a slight growth defect compared to the parental strain NC1Tn7GFP. In the absence of competition from the parental strain, it is unlikely that this growth defect explains the mutant's inability to complete the transmission cycle. In addition, TRN162 displays characteristics that identify it as primary phase variant and not a secondary phase variant that may have arisen spontaneously upon prolonged culturing in the laboratory.

Insect virulence assays demonstrated that *sspAB* is not essential for insect virulence as TRN162 is still able to cause significant death within 72 h of

injection into *M. sexta*. However, comparing the calculated lethal dose or time required to kill 50% of the insects, LD_{50} and LT_{50} respectively, for NC1Tn7GFP and TRN162 would answer whether or not *sspAB* plays a role in pathogenicity. As it has been shown that some factors implicated in symbiont transmission are also involved in insect virulence (2, 13, 20, 25), it is interesting that there is no essential function for *sspAB* in insect pathogenicity. These results suggest that *sspAB* is a novel symbiosis factor in *Photorhabdus*. Under competitive conditions, where both NC1Tn7GFP and TRN162 were present, it may be that the *sspAB* mutant would have a competitive disadvantage in insect pathogenicity.

The weakly fluorescent spheroplast observed near the PIVCs of IJs raised on TRN162 at 120 h is indicative of a bacterium that has lost its structural integrity and is dying. We propose that the nematode produces a stress to clear its intestinal lumen of non-symbiotic bacteria and moreover, that TRN162's inability to complete the transmission process results from a defect in surviving or evading this particular stress. There was no significant difference in either of the strains' ability to grow in acidic media, suggesting that in *Photorhabdus*, *sspAB* does not play a role in the regulation of acid resistance genes as it does in *E. coli*. In addition, it is unlikely that the inability of TRN162 to survive a potential reduction in intestinal pH is the cause of the transmission defective phenotype. Furthermore, the ability of these strains to grow in the presence of APs was evaluated. Again, no significant difference in growth in the presence of the APs tested was found between TRN162 and NC1Tn7GFP, indicating that either

sspAB does not play a role in resistance to this particular type of stress or that the transmission defect is specific to a *Heterorhabditis* AP not tested in this study.

Conversely, when grown in the presence of H_2O_2, TRN162 displayed a two-order of magnitude decrease in survival compared to NC1Tn7GFP at 100 mM H_2O_2. Although there was no significant difference in the strains' ability to grow in the presence of another oxidative stressor, paraquat, this study has provided evidence that suggests an oxidative stress may be introduced into the nematode intestine at 120 h post-recovery and that resistance to this stress is mediated through *sspAB*. While it would be expected that TRN162 would respond similarly when exposed to paraquat and H_2O_2, the method of paraquat exposure in this study likely contributed to the observed phenotype. Had the strains been cultured in a larger volume with greater aeration during growth, TRN162 may have displayed an increased sensitivity to paraquat.

Lastly, TRN162 displayed a hypermotile twitching motility when grown on 0.75% agar plates, where it migrated over four times farther from the point of inoculation than NC1Tn7GFP. As twitching motility is mediated by type IV pili (21), it may be that this mutant is hyperpiliated. NC1Tn7GFP and TRN162 showed similar swarming motility. Therefore, while it is unlikely that *sspAB* plays a role in swarming motility, it is clear that it functions to negatively regulate twitching motility. These motility phenotypes may have particular significance in symbiont transmission. It may be that swarming or twitching motility is essential for the bacteria to invade the PIVCs in order to escape an intestinal-clearing

stress produced by developing nematodes to ensure only true symbionts are transmitted to IJs.

Much remains to be learned about the *sspAB* operon in *Photorhabdus* and in particular, its role in symbiont transmission to *H. bacteriophora* IJs. Analysis of genes differentially regulated by the *sspAB* mutant vs. the parental strain by DNA microarray experiments would offer great insight into the role *sspAB* plays in *Photorhabdus*. The upstream signals of *sspAB* have yet to be identified in this model organism, as well as genes functioning downstream. At this time, it is unknown if the phenotypes shown in this study are mediated solely through *sspA* or rather through a combined effect of the non-functional *sspAB* operon. A prudent line of investigation would be to construct a targeted in-frame gene knockout of *sspA* and *sspB* individually to determine the answer to this question. Additionally, a closer examination of the surface of TRN162 cells, perhaps by electron microscopy, could answer questions pertaining to the piliation of the mutant as compared to the parental strain. This study has provided the first evidence that *sspAB* plays a role in symbiosis.

References

1. **Badger, J. L., and V. L. Miller.** 1998. Expression of invasin and motility are coordinately regulated in *Yersinia enterocolitica*. J. Bacteriol. **180:**793-800.

2. **Bennett, H. P., and D. J. Clarke.** 2005. The *pbgPE* operon in *Photorhabdus luminescens* is required for pathogenicity and symbiosis. J. Bacteriol. **187:**77-84.

3. **Bertani, G.** 1951. Studies on lysogenesis I.: The mode of phage liberation by lysogenic *Escherichia coli*. J. Bacteriol. **62:**293-300.

4. **Bintrim, S. B., and J. C. Ensign.** 1998. Insertional inactivation of genes encoding the crystalline inclusion proteins of *Photorhabdus luminescens* results in mutants with pleiotropic phenotypes. J. Bacteriol. **180:**1261-1269.

5. **Campos, M. A., M. A. Vargas, V. Regueiro, C. M. Llompart, S. Alberti, and J. A. Bengoechea.** 2004. Capsule polysaccharide mediates bacterial resistance to antimicrobial peptides. Infect. Immun. **72:**7107-7114.

6. **Choi, K. H., J. B. Gaynor, K. G. White, C. Lopez, C. M. Bosio, R. R. Karkhoff-Schweizer, and H. P. Schweizer.** 2005. A Tn7-based broad-range bacterial cloning and expression system. Nat Methods **2:**443-448.

7. **Ciche, T.** 2007. The biology and genome of *Heterorhabditis bacteriophora*, *WormBook*, ed. The *C. elegans* Research Community, WormBook.

8. **Ciche, T., and P. Sternberg.** 2007. Postembryonic RNAi in *Heterorhabditis bacteriophora*: a nematode insect parasite and host for insect pathogenic symbionts. BMC Dev. Biol. **7:**101-112.

9. **Ciche, T. A., K.-s. Kim, B. Kaufmann-Daszczuk, K. C. Q. Nguyen, and D. H. Hall.** 2008. Cell invasion and matricide during *Photorhabdus luminescens* transmission by *Heterorhabditis bacteriophora* nematodes. Appl. Environ. Microbiol. doi:10.1128/02646-07.

10. **De Reuse, H., and M. K. Taha.** 1997. RegF, an SspA homologue, regulates the expression of the *Neisseria gonorrhoeae pilE* gene. Res. Microbiol. **148:**289-303.

11. **Ding, X., R. R. Baca-DeLancey, and P. N. Rather.** 2001. Role of SspA in the density-dependent expression of the transcriptional activator AarP in *Providencia stuartii.* FEMS Microbiol. Lett. **196:**25-29.

12. **Fischer-Le Saux, M., V. Viallard, B. Brunel, P. Normand, and N. E. Boemare.** 1999. Polyphasic classification of the genus Photorhabdus and proposal of new taxa: P. luminescens subsp. luminescens subsp. nov., P. luminescens subsp. akhurstii subsp. nov., P. luminescens subsp. laumondii subsp. nov., P. temperata sp. nov., P. temperata subsp. temperata subsp. nov. and P. asymbiotica sp. nov. Int. J. Syst. Evol. Microbiol. **49:**1645-1656.

13. **Goodrich-Blair, H., and D. J. Clarke.** 2007. Mutualism and pathogenesis in *Xenorhabdus* and *Photorhabdus*: two roads to the same destination. Mol. Microbiol. **64:**260-268.

14. **Guina, T., D. Radulovic, A. J. Bahrami, D. L. Bolton, L. Rohmer, K. A. Jones-Isaac, J. Chen, L. A. Gallagher, B. Gallis, S. Ryu, G. K. Taylor, M. J. Brittnacher, C. Manoil, and D. R. Goodlett.** 2007. MglA regulates *Francisella tularensis* subsp. *novicida* response to starvation and oxidative stress. J Bacteriol. **189:**6580-6586.

15. **Han, R., and R. U. Ehlers.** 2000. Pathogenicity, development, and reproduction of *Heterorhabditis bacteriophora* and *Steinernema carpocapsae* under axenic *in vivo* conditions. J. Invertebr. Pathol. **75:**55-58.

16. **Hansen, A.-M., Y. Gu, M. Li, M. Andrykovitch, D. S. Waugh, D. J. Jin, and X. Ji.** 2005. Structural basis for the function of stringent starvation protein A as a transcription factor. J. Biol. Chem. **280:**17380-17391.

17. **Hansen, A.-M., H. Lehnherr, X. Wang, V. Mobley, and D. J. Jin.** 2003. *Escherichia coli* SspA is a transcription activator for bacteriophage P1 late genes. Mol. Microbiol. **48:**1621-1631.

18. **Hansen, A. M., Y. Qiu, N. Yeh, F. R. Blattner, T. Durfee, and D. J. Jin.** 2005. SspA is required for acid resistance in stationary phase by downregulation of H-NS in *Escherichia coli.* Mol. Microbiol. **56:**719-734.

19. **Ishihama, A., and T. Saitoh.** 1979. Subunits of RNA polymerase in function and structure. IX. Regulation of RNA polymerase activity by stringent starvation protein (SSP). J. Mol. Biol. **129:**517-530.

20. **Joyce, S. A., R. J. Watson, and D. J. Clarke.** 2006. The regulation of pathogenicity and mutualism in *Photorhabdus.* Curr. Opin. Microbiol. **9:**127-132.

21. **Mattick, J. S.** 2002. Type IV pili and twitching motility. Annu. Rev. Microbiol. **56:**289-314.

22. **Milstead, J. E.** 1979. *Heterorhabditis bacteriophora* as a vector for introducing its associated bacterium into the hemocoel of *Galleria mellonella* larvae. J. Invertebr. Pathol. **33:**324-327.

23. **Poinar, G. O.** 1975. Description and biology of a new insect parasitic rhabditoid, *Heterorhabditis Bacteriophora* N. Gen., N. Sp. (Rhabditida; Heterorhabditidae N. Fam.). Nematologica **21:**463-470.

24. **Schwyn, B., and J. B. Neilands.** 1987. Universal chemical assay for the detection and determination of siderophores. Anal. Biochem. **160:**47-56.

25. **Watson, R. J., S. A. Joyce, G. V. Spencer, and D. J. Clarke.** 2005. The *exbD* gene of *Photorhabdus* temperata is required for full virulence in insects and symbiosis with the nematode *Heterorhabditis*. Mol. Microbiol. **56:**763-773.

26. **Williams, M. D., T. X. Ouyang, and M. C. Flickinger.** 1994. Starvation-induced expression of SspA and SspB: the effects of a null mutation in sspA on *Escherichia coli* protein synthesis and survival during growth and prolonged starvation. Mol. Microbiol. **11:**1029-1043.

Chapter 3: Summary and Future Directions

This chapter is divided into two parts. Part I is a summary of the work presented in this study. Part II focuses on future research to be done on this project.

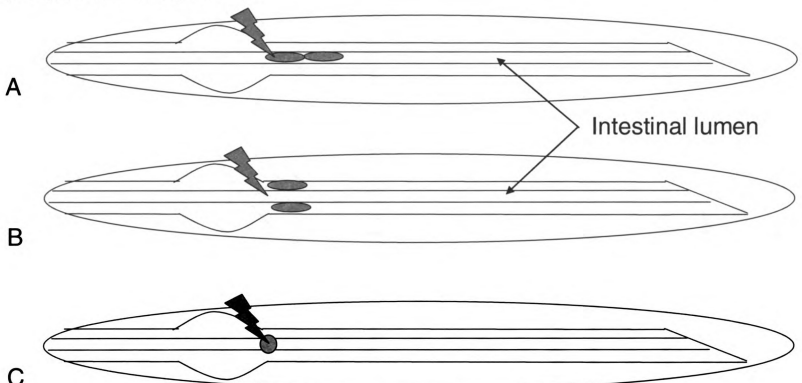
Summary

Research presented here describes a *Photorhabdus temperata* ssp. NC1 transmission defective mutant, TRN162, which is unable to complete the colonization process in *Heterorhabditis bacteriophora* nematodes. The disrupted gene in TRN162 is *sspA*, encoding a putative homolog of the stringent starvation proteins A. There is also a predicted polar effect on the downstream *sspB*. In addition, this study provides some insight into what kind of stress may be employed by the nematode to ensure that only true bacterial symbionts complete the transmission process. An oxidative stress, such as H_2O_2, seems to be the most likely to be produced by the nematode at 120 h post-recovery as this was the only type of stress studied here that showed a difference between NC1Tn7GFP and TRN162.

Moreover, the results of this study suggest that the *sspAB* regulon may function differently in *Photorhabdus* than it does in other microorganisms. For example, TRN162 does not show an increased sensitivity to acidic stresses, while the *E. coli sspA* homolog displayed just the opposite phenotype (2). Also, in *Photorhabdus*, *sspAB* seems to negatively regulate twitching motility. In both *E. coli* and *Y. enterocolitica*, *sspA* negatively regulates swarming motility (1, 2).

A proposed model for the role of *sspAB* in *Photorhabdus* transmission in *H. bacteriophora* is outlined here (Figure 3-1). As the nematode develops through its life cycle, its intestinal symbionts go through an elaborate selective process that ensures that only true symbionts are transmitted to the next

Figure 3-1: A proposed model for symbiont transmission in *H. bacteriophora* at 120 h post-recovery as mediated through *sspAB*. (A) A stress produced by the nematode to clear its intestinal lumen of non-symbiotic bacteria is survived only by true symbionts with a functional *sspAB* (B) Evasion of the selective stress by exiting the intestinal lumen and invading the PIVCs is only accomplished by true symbionts with a functional *sspAB* (C) Failure to either resist or escape the selective pressure, through a nonfunctional *sspAB*, results in formation of a weakly fluorescent spheroplast and ultimately cell death.



generation of IJs. At 120 h post-recovery, the developing IJ produces a stress that only true symbionts will be able to survive. Survival of this stress is mediated through *sspAB*. Data presented in this study suggest that an oxidative stress, such as H_2O_2, may be produced at this time. Alternatively, it may be that only true symbionts are able to invade the PIVCs, thus escaping the selective stress produced in the nematode intestine at 120 h. This process may be mediated through *sspAB* and its effects on motility. Failure to either resist or escape this selective pressure results in the formation of a spheroplast and ultimately, death of the bacterium.

Future Directions

While some fundamental characterizations of TRN162 were made in this study, many questions remain unanswered. Are the phenotypes described in this study mediated solely through *sspA* or *sspB*, or are both genes involved? What are the genes regulated by *sspAB* in *Photorhabdus* and how do these genes function in the transmission process? How do *H. bacteriophora* nematodes ensure only true symbionts complete the transmission process?

It is unknown if the phenotypes shown in this study are mediated solely through *sspA* or rather through a combined effect of the non-functional *sspAB* operon. It would be prudent to construct targeted in-frame gene knockouts of *sspA* and *sspB* individually, so that there are no polar effects on *sspB*. By then

examining the oxidative stress resistance and twitching motility of these strains, the answer to this question could be determined.

Additionally, a DNA microarray study would be useful to identify genes differentially regulated between TRN162 and NC1Tn7GFP. Similar previous approaches in an *E. coli sspA* mutant led researchers to the acid sensitive *sspA* phenotype (2). In particular, using a microarray approach with the *Photorhabdus-H. bacteriophora* model system may provide greater insight into the stress imposed upon intestinal inhabitants near the PIVCs of pre-IJs. Reverse transcription PCR (RT-PCR) experiments would confirm the suggested *sspAB* regulation of genes involved in the resistance to oxidative stresses or the biosynthesis of type IV pili.

In an effort to discover how *H. bacteriophora* controls the transmission of symbionts to developing IJs, growth in the presence of varying stresses was monitored in this study. However, an alternative approach to this would be to monitor the response of TRN162 to these stresses over a shorter amount of time. In this way, one would be monitoring survival as opposed to growth and there may be a more distinct phenotype in the presence of these stresses, which could offer greater insights into the particular stress that is hypothesized to be introduced in the nematode intestine. For example, much of the H_2O_2 may be degraded by bacteria not initially killed in a shorter exposure. When the H_2O_2 has reached a lower, more tolerable level for these bacteria, they may resume growth, resulting in a reduced CFU/ml as seen in this study.

It was shown here that TRN162 has an increased twitching motility phenotype compared to the parental strain. As twitching motility is mediated by type IV pili, electron microscopy of individual cells of TRN162 or fluorescent microscopy using antibodies specific for type IV pili would confirm if this strain is hyperpiliated. Additionally, targeted deletions of type IV pili biosynthetic genes would confirm that *Photorhabdus* symbionts need to have functional twitching motility in order to invade the PIVCs of developing IJs.

References

1. **Badger, J. L., and V. L. Miller.** 1998. Expression of invasin and motility are coordinately regulated in *Yersinia enterocolitica*. J. Bacteriol. **180:**793-800.

2. **Hansen, A. M., Y. Qiu, N. Yeh, F. R. Blattner, T. Durfee, and D. J. Jin.** 2005. SspA is required for acid resistance in stationary phase by downregulation of H-NS in *Escherichia coli*. Mol. Microbiol. **56:**719-734.

Appendices

RT-PCR of H-NS and RpoS

As it has been shown in an *E. coli sspA* mutant, there are altered levels of the H-NS and RpoS proteins (3), RT-PCR was performed on *hns* and *rpoS* to determine if these genes were being expressed in TRN162. RNA was isolated using Trizol (Invitrogen) from TRN162 and NC1Tn7GFP grown in Grace's Insect Media at 28°C for 24 h, 12 h and 4 h. RNA was DNaseI (Invitrogen) treated and converted to cDNA using Thermoscript reverse transcriptase (Invitrogen) at 53°C using the primers below. cDNA was then used as a template for amplification PCR.

hns RT for: 5' cgtactttacgagcccaagc 3'

hns RT rev: 5' gcacgacgttttgctttacc 3'

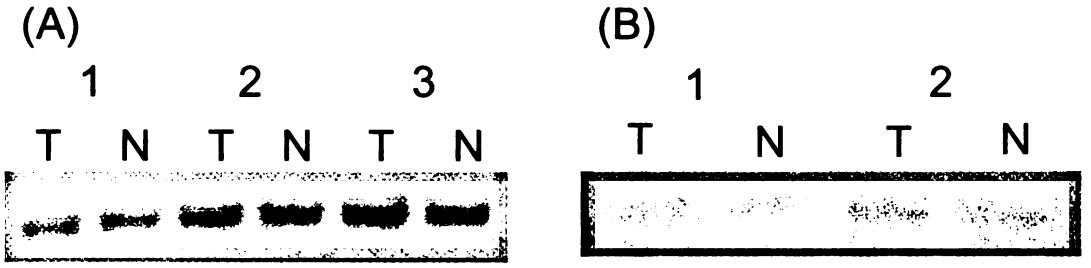
rpoS RT for: 5' ttcggttgatacccccatta 3'

rpoS RT rev: 5' ttccaaaagaccaaaacgac 3'

In both NC1Tn7GFP and TRN162, *hns* is expressed at all time points tested here (Figure 3-2). In the *rpoS* samples, the gene is being expressed by both strains at 12 h and 24 h of growth. However, at 4 h growth, neither strain showed strong expression of *rpoS*. This could be due to an un-optimized annealing temperature for these primers, as amplification was not as robust as in the *hns* samples. Alternatively, the absence of *rpoS* mRNA at 4 h could be due to the early growth stages of these cultures, as the expression of *rpoS* is increased under stressful or starvation conditions (2). While this experiment examined the presence or absence of *hns* and *rpoS* mRNA, quantitative RT-PCR

could be used to determine if there are altered mRNA levels corresponding to these genes in TRN162 compared to the parental strain.

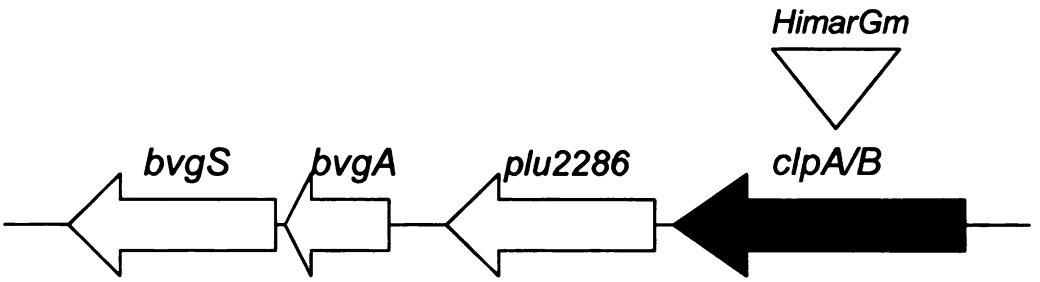
Figure A-1: RT-PCR of *hns* and *rpoS*. (A) *hns*. 1, 24h; 2, 12h; 3, 4h. (B) *rpoS*. 1, 24h; 2, 12h. T, TRN162. N, NC1Tn7GFP.



A ClpA/B type chaperone is disrupted in TRN17-96

Another NC1Tn7GFP-derived mutant identified in the *HimarGm* transposon mutagenesis screen was TRN17-96. This mutant is defective at the initial stage of biofilm formation in the *H. bacteriophora* intestine and the gene disrupted is *plu2287*, which is predicted to encode a homolog of a ClpA/B-like chaperone protein (Figure 3-1). Located just downstream at *plu2285* and *plu2284* are genes encoding putative homologs of the *Bordetella* virulence gene (*Bvg*) two component response regulator (*BvgA*) and sensor kinase (*BvgS*), respectively. However, primer extension analysis has shown that in *Photorhabdus*, these genes are not co-transcribed with *plu2287*, but in fact have their own promoter (1).

Figure A-2: Gene organization surrounding transposon insertion in TRN17-96. *clpA/B* is disrupted by the *HimarGm* transposon.



Growth rate of TRN17-96

As with any mutant being studied, it is prudent to determine the growth rate and doubling time. Growth rate (μ) and doubling time (T_2) was measured as described in Chapter 2 (Table 3-1).

Table A-1: Growth rate of NC1Tn7GFP and TRN17-96

	Growth Rate	**Doubling Time**
NC1Tn7GFP	0.273 ± 0.013 h^{-1}	2.54 ± 0.12 h
TRN17-96	0.248 ± 0.017 h^{-1}	2.80 ± 0.19 h

While the growth rate of TRN17-96 (0.248 ± 0.017 h^{-1}) is slower than NC1Tn7GFP (0.273 ± 0.013 h^{-1}), it is not likely that this causes the defective transmission phenotype. Just as with TRN162, examining this mutant at later times post-recovery does not resolve the mutant's inability to complete the transmission process, although in a competitive situation, TRN17-96 may be at a disadvantage compared to NC1Tn7GFP.

Phase variation of TRN17-96

As secondary phase variants cannot colonize the nematode intestine, several phase characteristics of TRN17-96 were determined as described previously. This mutant displays mostly primary phase characteristics comparable to NC1Tn7GFP (Table 3-2). It has a yellow pigmentation, produces crystalline inclusion proteins and is positive for siderophore activity. TRN17-96 is able to absorb pigments from its media supplemented with neutral red, bromthymol blue or Congo red. However, when grown in the presence of eosin y-methylene blue, TRN17-96 did not produce the metallic green sheen observed when NC1Tn7GFP was grown on the same media, but rather exhibited purple pigmented growth.

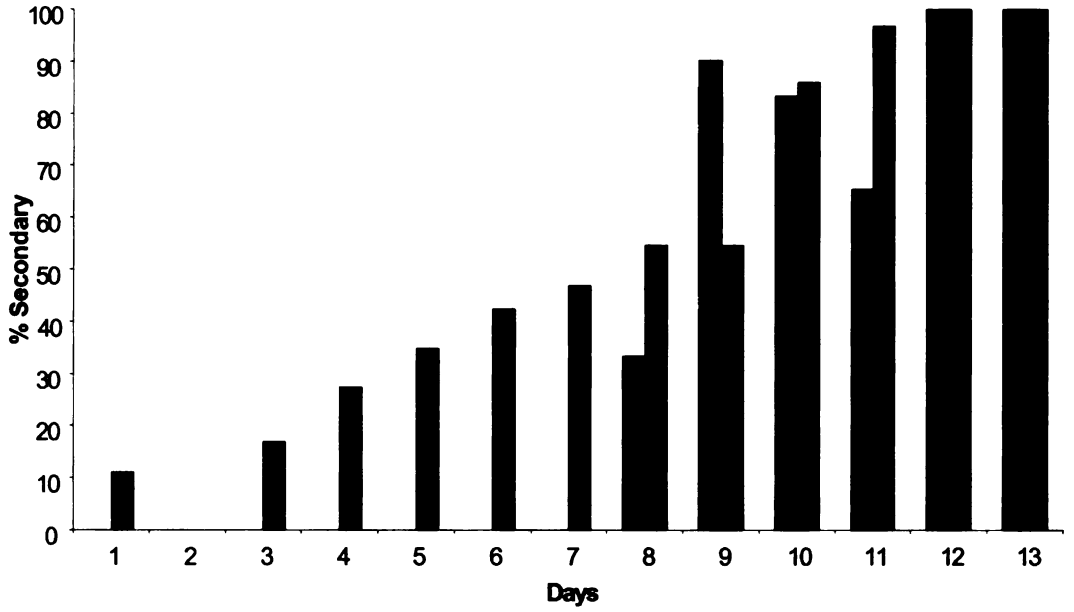
Although the majority of phase variation characteristics studied in TRN17-96 suggest that it is a primary phase variant, the rate at which it switches to a secondary phase variant is much more rapid than NC1Tn7GFP (Figure 3-2). Interestingly, a mutation in the *Photorhabdus bvgA* homolog has been shown to increase the rate at which the strain transitions from primary to secondary phase variants (1). To monitor this phase switching, NC1Tn7GFP and the mutant were inoculated to the same OD_{600} (0.2) in 100 ml of PP3S and grown at 28°C. At 24 h intervals, 10-fold serial dilutions were made and plated on PP3S. Colonies were scored for phase variation on the basis of their translucence observed under a dissecting microscope. The production of crystalline inclusion proteins, a hallmark of primary phase variants, makes colonies opaque under this microscope. Even after only 24 h of growth, more than 10% of the colonies of

TRN17-96 exhibited translucence. Between 7 to 8 d of growth, approximately 50% of the colonies of TRN17-96 were scored as secondaries. In comparison, NC1Tn7GFP did not exhibit any translucence until 8 d of growth. However, there is doubt that this increased rate of switching to a secondary phase variant is the cause of the transmission defect in TRN17-96 because even at 72 h of growth, more than 80% of the cells can be classified as primaries.

Table A-2: Phase variation characterization of NC1 primary, NC1 secondary, NC1Tn7GFP and TRN17-96.

Phenotype Assayed	NC1 Primary	NC1 Secondary	NC1Tn7GFP	TRN17-96
Dye Absorption				
Neutral red	+	–	+	+
Eosin Y-methylene blue	+	–	+	–
Bromthymol blue	+	–	+	+
Congo red	+	–	+	+
Bioluminescence	+	–	+	+
Extracellular Products				
Siderophore activity	+	–	+	+
Cellular Inclusion Proteins	+	–	+	+
Colony morphology	Convex, mucoid	Flat, nonmucoid	Convex, mucoid	Convex, mucoid
Pigmentation	Yellow	White	Yellow	Yellow

Figure A-3: Rate of phase variation switching of NC1Tn7GFP (black) and TRN17-96 (gray)



References

1. **Derzelle, S., S. Ngo, E. Turlin, E. Duchaud, A. Namane, F. Kunst, A. Danchin, P. Bertin, and J. F. Charles.** 2004. AstR-AstS, a new two-component signal transduction system, mediates swarming, adaptation to stationary phase and phenotypic variation in *Photorhabdus luminescens*. Microbiol. **150:**897-910.

2. **Gentry, D. R., V. J. Hernandez, L. H. Nguyen, D. B. Jensen, and M. Cashel.** 1993. Synthesis of the stationary-phase sigma factor sigma s is positively regulated by ppGpp. J. Bacteriol. **175:**7982-7989.

3. **Hansen, A. M., Y. Qiu, N. Yeh, F. R. Blattner, T. Durfee, and D. J. Jin.** 2005. SspA is required for acid resistance in stationary phase by downregulation of H-NS in *Escherichia coli*. Mol. Microbiol. **56:**719-734.