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Genetics and physiology of motility by Photorhabdus spp

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GENETICS AND PHYSIOLOGY OF MOTILITY BY *PHOTORHABDUS* SPP.

BY

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DEDICATION

This thesis is dedicated to my husband Josh Michaels,
and to my parents, George and Debra Day

Josh, I am indebted to you for your support, sacrifices, and encouragement to pursue all my personal and professional goals. Every day I am grateful that I have you to come home to. Your love, laughter, and advice are always there when I need it most.

Mom and Dad, since I was a child you encouraged me to pursue my education so that I could have a career I was passionate about. Mom, I especially want to thank you for instilling in me the belief and power that I am capable of anything. Dad, your example of exceptional work ethic has made me the hard worker that I am today.

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FOREWARD

Each chapter of this dissertation is written in the form of a manuscript. Consequently, each chapter is comprised of a separate abstract, introduction, materials and methods, results, and discussion section. A brief preface is included before each chapter to review the specific goals of each section. Chapter II is a reprint of a published paper reporting my early work on this project. A brief summary of the entire project is presented in Chapter VI. I recognize that further experimentation may be required before actual submission of the chapters for publication.

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ABSTRACT

GENETICS AND PHYSIOLOGY OF MOTILITY BY *PHOTORHABDUS* SPP.

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University of New Hampshire, May 2006

Photorhabdus is a bacterial symbiont of soil nematodes and a lethal pathogen of insects. Many pathogenic or symbiotic bacteria utilize various methods of motility to reach favorable conditions, colonize a host, or have motility genes that also regulate virulence expression. It is not known how motility is regulated, or how it may confer an advantage, in the complex life cycle of *Photorhabdus*.

We characterized motility in *Photorhabdus* and found that the bacterium was motile both by swimming (movement in liquid) and swarming (movement on surfaces) under appropriate conditions. Both types of motility utilized the same peritrichous flagella and shared genetic components. However, unlike swimming, swarming behavior was a social form of movement in which the cells coordinately formed intricate channels that covered a surface. The optimal conditions for motility were established including a Na+ or K+ requirement. Interestingly, microarray experiments imply that NaCl and KCl regulate motility post-transcriptionally and not at the gene expression level. This ionic salt post-transcriptional regulation of motility has not been observed in other

bacteria. We suggest that this form of regulation may be beneficial for an organism that must adapt quickly to changing environments.

To identify the genes involved in motility, *P. temperata* mutants with altered motility behavior were generated with random transposon mutagenesis. An *rssB* mutant that displayed a hyperswarming phenotype was isolated, suggesting that RssB acts as a negative regulator of swarming behavior. A *yidA* mutant, whose function remains unknown, had inhibited swimming behavior and dramatically attenuated virulence. A *plu3723* mutant (a *luxR* homolog) was isolated, that unlike the wild-type, was able to swim without NaCl or KCl. All together 86 motility mutants were isolated and physiologically characterized. Since many of the motility mutants had concomitant changes in expression of antibiotics, hemolysins, proteases, and insect virulence, expression of motility genes may be co-regulated with expression of virulence enzymes in *Photorhabdus*. The mutants isolated in this study will be useful long-term tools for additional experiments.

The ability of *Photorhabdus* to swarm could provide a rapid and coordinated colonization of either nematode or insect host, or in traveling from one host to another. The nematode environment is low in nutrients, ionic salts, and amino acids, while the insect hemocoel is high in these solutes. When the nematodes release their bacterial symbionts into the insect hemocoel, the bacteria are exposed to the ionic salts that would induce the flagella regulon. Ecologically, it would be beneficial for the bacteria to be motile upon entering the insect to rapidly colonize the hemocoel. If aspects of virulence expression are co-

regulated with motility genes as this research suggests, expression of virulence factors would also be induced upon exposure to the insect. The data presented in this study are the first steps for elucidating a model of motility in the life cycle of this insect pathogen and nematode symbiont.

CHAPTER I

LITERATURE REVIEW

General Aspects of the Bacteria-Entomopathogenic Nematode Association

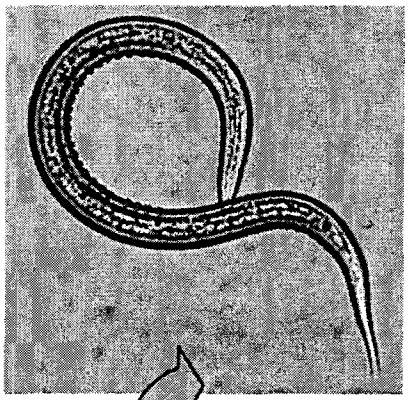
An interesting microbe-nematode interaction is found within the life cycle of two families of soil dwelling entomopathogenic nematodes, the *Steinernematidae* and the *Heterorhabditidae (*for a review see: Akhurst and Boemare, 1990; Boemare *et al.*, 1997; Burnell and Stock, 2000; Chattopadhyay *et al.*, 2004; Forst *et al.,* 1997; Forst and Nealson, 1996; Frackman and Nealson, 1990; Kim and Forst, 2005; Nealson *et al.*, 1990; Owuama, 2001; and Waterfield, *et al.*, 2004). These nematodes have evolved a mutualistic relationship with entomopathogenic proteobacteria *Xenorhabdus* and *Photorhabdus,* respectively. Although the genera *Photorhabdus* and *Xenorhabdus* are very similar, they differ in several important traits including nematode host specificity. Another major difference is that *Photorhabdus* is bioluminescent while *Xenorhabdus* is not.

The life cycle of *Photorhabdus* and its nematode partner is best described as a cyclic association that begins and ends with infective juvenile nematodes (Fig. 1). The bacteria are carried inside the gut of non-feeding third-instar infective stage nematodes (IJs). The IJ nematodes are relatively resistant to environmental stresses and provide protection to the bacteria. Since bacteria are

unable to penetrate an insect host by themselves, the IJ serves as a vector by transporting the bacteria into an insect host (Milstead, 1978).

Figure 1. *Photorhabdus/Heterorhabditis* Life Cycle

Photorhabdus spp. are present throughout the gut of infective juvenile (IJ) nematodes (*Heterorhabditis* spp.)



IJs infect an insect and release bacteria into hemocoel. Bacteria secrete toxins and virulence enzymes. One hermaphroditic nematode is sufficient for reproduction.

Insect dies within 24-48 hours. Bacteria and nematodes grow and multiply. IJ acquire new symbionts and emerge.

Nematode photo by Erick Janicki and Robert Mooney, Galleria mellonella larva photo by Brandye Michaels and Robert Mooney.

Taxonomy

The first bacterial isolates from *Heterorhabditidae* nematodes were characterized as bioluminescent, gram-negative, motile rods (Khan and Brooks, 1977 and Poinar *et al.*,1977). Initially, the bacterium was classified as *Xenorhabdus luminescens*, sharing a genus with non-bioluminescent X. *nematophilus* spp. (Thomas and Poinar, Jr., 1979). On the basis of phenotypic properties, nematode species specificity, and DNA relatedness of additional strains, the genus was later divided into *Xenorhabdus* for those bacteria found within *Steinernematidae* nematodes, and *Photorhabdus* for bacteria associated with *Heterorhabditidae* nematodes (Boemare *et al.,* 1993). Recently, the genus *Photorhabdus* has been sub-divided into three species (Fischer-Le Saux *et al.,* 1999 and Akhurst *et al.*, 2004): *Photorhabdus luminescens* including the subsp. *luminescens, akhurstii*, and *laumondii; Photorhabdus temperata* including the subsp. *temperata*; and finally the clinical isolates, *Photorhabdus asymbiotica* including the subsp. *asymbiotica* and *australis*. The clinical isolates are not associated with nematodes. These *Photorhabdus* species have been isolated from human skin infections in the United States (Farmer *et al.,* 1989) and Australia (Gerrard *et al.,* 2003 and Peel *et al.,* 1999). While the route of infection is unknown, the clinical isolates of *Photorhabdus* from these human infections have been cultured and characterized.

Life Cycle

As a symbiont, *Photorhabdus* is carried within the intestine of free-living dauer juvenile nematodes (Endo and Nickle, 1991). The nematodes actively seek

and infect a wide range of insect hosts by entering through natural openings or by burrowing directly through the insect cuticle. Upon exposure to insect hemolymph, the nematodes slowly regurgitate the bacterial symbionts through their mouth (Ciche and Ensign, 2003). Death of the insect, which occurs within 24 h of infection, is mainly due to the array of insect toxins produced by the bacteria (Khan and Brooks, 1976 and Poinar Jr., 1975). *Photorhabdus* also has a profound effect on the insect immune system. For example, *P. luminescens* strain W14 is known to secrete an anti-phagocytic factor, that even in cell-free supernatant, will inhibit phagocytosis by insect immune cells (Au *et al.*, 2004).

Photorhabdus also excretes a signal compound that induces "recovery" of the nematodes within the insect (Aumann and Ehlers, 2001). Recovery by entomopathogenic nematodes is the exit from the IJ stage allowing the next stage of development and reproduction. Generally, this transition is signaled by a food source, but recovery in *Heterorhabditis* nematodes is mediated by specific communication with their bacterial partners. When nutrients are low (approximately 2 weeks after infection) the bacteria switch to producing an antagonistic signal, which inhibits recovery and arrests the IJ stage of the nematodes (Aumann and Ehlers, 2001). This process allows the IJs and bacteria to reassociate and leave in search of a new insect host.

The nematode-bacterial interaction is very specific. Without the appropriate strain of bacterial symbiont, the nematodes will not grow and reproduce (Han *et al.,* 1991). The exact reason for this phenomenon is unknown. One hypothesis is that the bacteria play a direct or indirect role in providing

essential nutrients. *Photorhabdus* mutants defective in phosphopantetheinyl transferase activity fail to support nematode growth and reproduction (Ciche *et al.*, 2003). The gene may be involved in biosynthesis of an unknown metabolite required for symbiosis. Recent results (Watson *et al.,* 2005) suggest that iron may play a key role in the nutritional exchange between the bacteria and nematode. The bacteria probably produce siderophores to scavenge iron in the insect, while the nematodes feed on *Photorhabdus* to satisfy their own iron requirements. With many bacterial systems, the transport of these siderophores is coupled to the TonB complex, which serves as an energy-transducing system (for a review see: Andrews *et al.,* 2003; Braun, 2003; and Faraldo-Gomez and Sansom, 2003). *Photorhabdus* mutants defective in ExbD, a component of the TonB complex, grow poorly within the insect and are also unable to support nematode growth and development *in vitro* (Watson *et al.,* 2005).

Insect Virulence

These entomopathogenic bacteria are extremely virulent. Injection with as few as 1-15 bacterial cells will result in 100% insect mortality (Milstead, 1979). The main mechanism of insecticidal activity is toxin production. These bacteria produce several high molecular weight toxin complexes (Tcs) that are effective against many genera and orders of insects (for a review see ffrench-Constant and Bowen, 2000). The complexes are lethal when fed orally or directly injected into the hemocoel (Bowen and Ensign, 1998; and Bowen *et al.,* 1998). The bacteria also produce the *makes caterpillar floppy (mcf)* toxin, which triggers apoptosis of insect hemocytes and destruction of the insect epithelium (Daborn

et al., 2002; Waterfield *et al.*, 2003; and Dowling, *et al.*, 2004). A third class of toxins, called the "*Photorhabdus* insect-related" (Pir) proteins A and B, shows some similarity to a protein involved in beetle development and to Bt δ-endotoxins (Waterfield, *et al.*, 2005).

Secreted Enzymes

Photorhabdus produces a variety of extracellular enzymes to aid in the degradation of insect tissue. The proteases are primarily zinc metalloproteases of the RTX (Repeats-In-Toxin) family (Bowen *et al.*, 2000; Bowen *et al.*, 2003; Cabral *et al.*, 2004, Ong and Change, 1997; and Schmidt *et al.*, 1988). The proteases are not essential to insect pathogenicity. Strains or mutants lacking protease activity remain pathogenic towards insects (Marokhazi *et al.*, 2004) and purified proteases are not toxic when injected into the host (Bowen *et al.*, 2003). Instead, the proteases appear to play a major role in the bioconversion of insect tissue into accessible nutrients (Daborn *et al.*, 2001 and Bowen *et al.*, 2003) and in the inhibition of antibacterial factors secreted by the insect (Cabral *et al.*, 2004). Lipases (Wang and Dowds, 1993) and hemolysins (Brillard *et al.*, 2001 and Brillard *et al.*, 2002) are hypothesized to function in a similar biodegradation role.

Besides biodegradation enzymes, the bacteria also produce antibiotics to prevent scavengers (other nematodes, insects, bacteria, fungi, etc.) from invading the insect carcass (Akhurst, 1982 and Paul *et al.*, 1981). Several antimicrobial compounds have been identified, including a red pigment that is an anthraquinone derivative (Li *et al.*, 1995) and a hydroxystilbene with strong

antifungal activity (Richardson *et al.,* 1988). A novel catechol siderophore, "photobactin", was identified that is structurally related to the vibriobactins (Ciche *et al.,* 2003). Purified photobactin has detectible antibiotic activity against both gram-positive and gram-negative bacteria. A carbapenem-like antibiotic (a β-lactam broad-spectrum antibiotic) and the gene cluster involved in its biosynthesis (*cpmA* to *cpmH*) has been identified in *P. luminescens* strain TTO1 (Derzelle *et al.,* 2002). *P. luminescens* W14 produces bacteriocins (termed "lumicins") that have both DNase and RNase activity that kill competing *Photorhabdus* strains (Sharma *et al.*, 2002). To deter non-microbial pests, *Photorhabdus* produces an ant-deterrent factor (Zhou *et al.*, 2002) and nematicidal metabolites (Hu *et al.,*1995, 1996 and 1999). All of these compounds function to protect the nematode-bacterial partners within the insect carcass from scavengers.

To date, *Photorhabdus* is the only known terrestrial bioluminescent bacteria. *Photorhabdus* bioluminescence is catalyzed by a typical bacterial luciferase (requiring reduced flavin mononucleotide, aldehyde, and O_2) and is detectable within 20 h of insect infection (Poinar Jr. *et al.*, 1980). The ecological role of luminescence in the life cycle is currently unknown.

Phenotypic Phase Variation

Photorhabdus exists in two distinct phenotypic forms, termed primary and secondary-phase variants (Akhurst, 1980). Primary-phase variants are characterized as having higher levels of proteases, lipases, hemolysins, antibiotics, bioluminescence and pigmentation. These traits are absent or

diminished in the secondary-phase cells. The exact molecular mechanism and biological significance of phase variation in these species remains unknown, though nematodes grow preferentially with the primary-form cells (Akhurst, 1980; Boemare *et al.*, 1997). While equally pathogenic to insects, the secondary phase-variants are defective in supporting nematode reproduction, suggesting an alternative ecological role from the primary form (Ehlers *et al.,* 1990; Han and Ehlers, 2001; and Forst and Clarke, 2002). One hypothesis is that the secondary-phase cells are better adapted for survival as free-living organisms in nutrient deprived soil (Smigielski *et al.,* 1994). After starvation and subsequent addition of nutrients, secondary-phase cells recommence growth more quickly than primary-phase cells. The activity levels of major respiratory enzymes and the transmembrane proton motive force are both significantly higher in the secondary-phase cells, further supporting this hypothesis. Another possibility is that the secondary cells are simply laboratory artifacts. Aside from a few isolations from insect carcasses (Akhurst, 1980), phase II cells have only been isolated in the laboratory by prolonged culturing of the primary form under low osmolarity conditions (Krasomil-Osterfeld, 1995).

Primary-phase cells also differ from secondary-phase cells by harboring two types of cytoplasmic intracellular protein inclusions, encoded by the *cipA* and *cipB* genes (Bintrim and Ensign, 1998). These are hydrophobic proteins with no significant amino acid or gene sequence similarity to any other known protein. The protein inclusions are not used during long-term starvation of the cells, and the protein inclusions in non-viable *Photorhabdus* cells do not allow nematode

growth. Thus the inclusion proteins are not solely a requirement for bacterial or nematode nutrition (Bowen and Ensign, 2001). Since the Cip proteins are not toxic when injected into insect larva (Bowen and Ensign, 2001), they are not directly involved with insect pathogenicity. However, insertional inactivation of *cipA* or *cipB* in primary-phase cells produces mutants with altered phenotypic traits normally characteristic of the primary-phase variants (Bintrim and Ensign, 1998).

The molecular mechanism involved in phase variation is unknown. Genes involved in bioluminescence and lipase production have identical restriction patterns in the two phase variants (Frackman *et al.,* 1990; Wang and Dowds, 1991; and Wang and Dowds, 1993). The restriction analysis of entire chromosomal DNA also fails to show any differences between the two phase variants (Akhurst *et al.,* 1992). Both of these data suggest that DNA rearrangements or chromosomal differences between the phase variants is not responsible for phase variation. In addition, both primary and secondary-phase cells exhibit the same DNA, mRNA, and protein profiles for lipases, although only the primary-form has active lipase activity (Wang and Dowds, 1993). Therefore, lipase activity is not due to gene differences between the two phase variants, but to post-translational regulation.

To date, only two regulatory elements that are involved in phase variation have been identified: *ner* (O'Neill *et al.,* 2002) and *hexA* (Joyce and Clark, 2003). The *ner* gene product, when over-expressed, causes repression of primary-phase cell phenotypes. However, insertional activation of the gene in the

secondary variant does not cause reversion to the primary form, and the gene is expressed in both variants (O'Neill *et al.*, 2002). While not directly responsible for the secondary phenotype, one hypothesis is that the excess protein may affect a global regulator gene responsible for controlling a regulatory cascade. The *hexA* gene product, which has homology to *Erwinia carotovora hexA*, appears to repress all primary-phase traits while maintaining the secondary-phase (Joyce and Clarke, 2003). H*exA* knock-out mutants in secondary-phase cells express primary-phase phenotypic traits including normal symbiosis with nematodes, but have attenuated insect virulence (Joyce and Clarke, 2003). This suggests that the bacteria do not simultaneously express the genes required for both the pathogenic and symbiotic life styles. It is not known whether the *ner* and *hex* gene products affect gene regulation directly or indirectly through a cascade. The latter is more likely, given the large number of phenotypic difference between the two phase variants. The signals that induce phase variation also remains to be investigated.

Genomic Analysis

The first *Photorhabdus* genomic analysis was achieved by sample sequencing strain W14 to 0.5X coverage of the 5.5 Mb genome (ffrench-Constant *et al.,* 2000). Duchaud *et al.* (2003) sequenced the entire genome of *P. luminescens* TT01. Overall, the genome of *Photorhabdus* has been called "*Yersinia*-like" due to its large size and redundancy of toxins and pathogenicity islands. There are also a high number of mobile genetic elements, which may contribute to the array of toxins and overall genomic variability (for a review of the

Photorhabdus genome see ffrench-Constant *et al.*, 2003). While the sequence data is packed with information, genetic studies in *Photorhabdus* is in its infancy. Future work is required to further dissect the pathways of gene function and regulation in this complex organism.

Cell Surface Properties

Pathogenesis and/or colonization of a host by bacteria is usually through the action of cell structures such as fimbriae, pili, flagella, or adhesins. There have only been a few studies on the role of cell surface properties in the life cycle of *Photorhabdus*. Primary-phase cells of *Photorhabdus* strain K80 have capsular material (a glycocalyx) that is 2-3 times thicker than secondary phase (Brehelin *et al.*, 1993). Fimbriae are present when cells are grown on solid agar plates but absent when cells are grown in agitated liquid medium (Brehelin *et al.*, 1993). However, Meslet-Cladiere *et al.* (2004) found that mannose-resistant fimbriae, encoded by *mrfA*, were expressed in both phase variants of strain K122 in liquid culture. They concluded that *mrfA* expression levels were highest 20-25 hours post insect-infection, indicating that the fimbriae are not important for initial infection, but probably in the late stages of infection. A *pbgE1* mutant strain of TT01 defective in the production of O antigen for LPS is attenuated in insect virulence and is unable to colonize the gut of nematodes (Bennett and Clarke, 2005). This study supports that the bacterial surface may have an important role in symbiosis and pathogenicity. The genome sequence of *P. luminescens* TT01 predicts many genes potentially involved in the bacterial-host interaction

including several classes of adhesins, eleven clusters of fimbrial genes, *Yersinia*-like *inv* and *ail* paralogs, and type IV pili (Duchaud, *et al.*, 2003).

Bacterial Motility

While there are other forms of motility in bacteria, my research focuses on swimming and swarming motility. Swimming motility is characterized as movement through liquid, which may be mediated by polar or peritrichous flagella. Swarming motility is movement across solid surfaces. It is generally viewed as group movement in which rafts of cells continuously branch outward until the surface is fully colonized (for a review of swarming see Harshey, 2003). The same peritrichous flagella may be used for both swimming and swarming. Bacteria such as *Bacillus cereus* (Senesi *et al.*, 2002) up-regulate the quantity of flagella for swarming. The addition of more flagella may be complemented with the production of extracellular components such as polysaccharides (Gygi *et al.*, 1995) or surfactins (Ohgiwari *et al.*, 1992). These compounds make surfaces easier to swarm across by reducing surface tension. In the laboratory, the addition of the surfactant Tween 80 has been shown to enhance swarming behavior in several bacterial species (Niu *et al.*, 2005). In contrast to *Bacillus*, *Serratia marcescens* (Alberti and Harshey, 1990) and *Vibrio alginolyticus* (Ulitzur, 1975) switch from using polar flagella for swimming to peritrichous flagella for swarming. Bacteria that possess mixed flagellation may exhibit two separate flagellar systems for the two types of motility or have overlap of certain components.

Both types of motility are generally controlled by chemotaxis via a highly conserved two-component signal transduction system. Chemotactic bacteria detect attractants or repellants in the environment and respond by changing the direction and speed of flagella (for a review see Szurmant and Ordal, 2004). Chemotaxis confers a competitive advantage to bacteria since they are able to quickly escape detrimental conditions and reach a more favorable environment.

Motility or flagella can also be important in helping bacteria form biofilms or colonize hosts. Both *Pseudomonas aeruginosa* (O'Toole and Kolter, 1998) and *Escherichia coli* (Pratt and Kolter, 1998) utilize flagella in initial biofilm formation. In *Vibrio fischeri*, functional motility is required for the symbiotic bacteria to colonize the squid host (Graf *et al.*, 1994).

Flagellar genes are arranged in an ordered cascade in which the transcription of a gene at a higher level allows the expression of a gene further down the cascade (See Soutourina and Bertin, 2003 for a review). At the top of the hierarchy in enterobacteria is the *flhDC* master operon and the *fleQ* or *flrA* master genes in *Pseudomonas* and *Vibrio* spp. (Fig. 2). Expression of the *flhDC* operon is controlled by numerous environmental signals (pH, temperature, etc.) and/or global regulatory proteins (cAMP-CAP, H-NS, etc.), which vary depending on the organism (Fig 3). Genes early in the cascade may also control non-motility genes such as coordinated expression of virulence factors with flagella synthesis. Both *Proteus* (Allison *et al.*,1992) and *Bacillus* (Senesi *et al.*, 2002) co-express hemolysin and flagellar genes.

Figure 2. The *flhDC* master operon controlling flagella formation in *E. coli* is regulated by multiple signals and regulatory proteins. Source: Soutourina and Bertin. 2003. FEMS Micr. Rev. 27: 505-523.

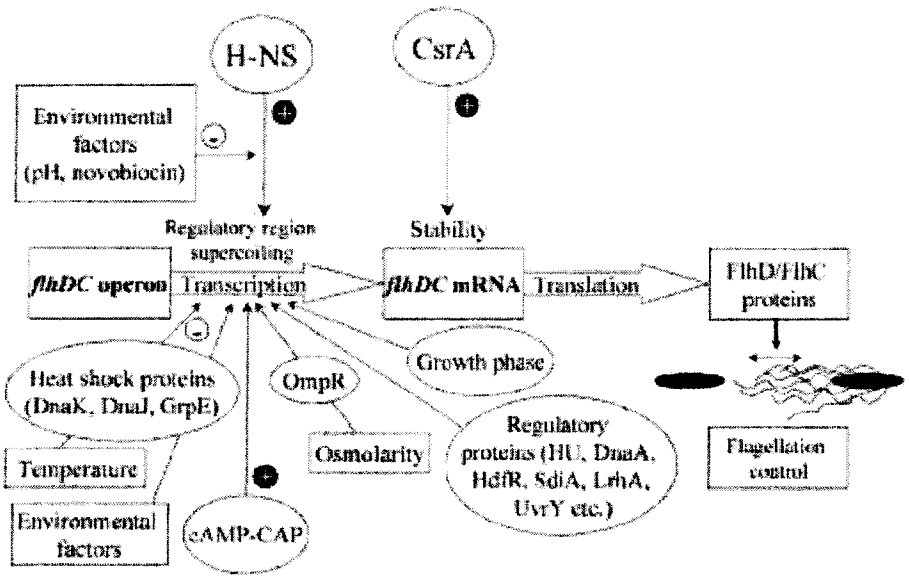
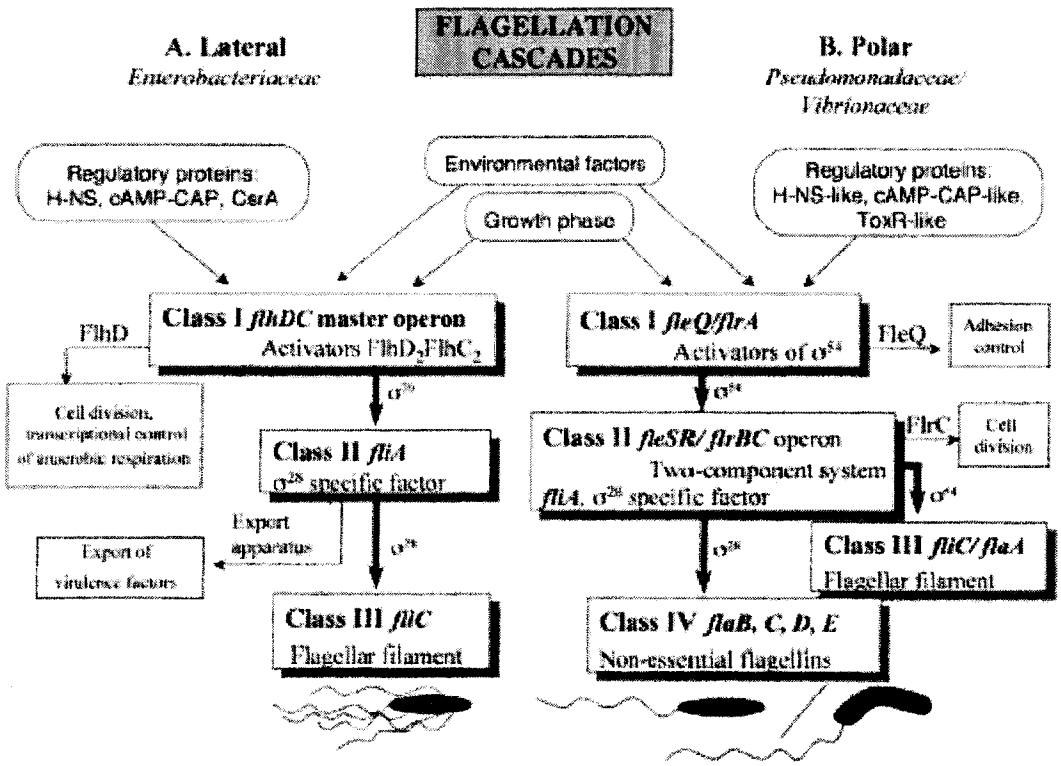


Figure 3. Overview of flagellation cascades in different bacteria. Expression of class I genes activate class II gene expression, whose product activates class III genes. Source: Soutourina and Bertin. 2003. FEMS Micr. Rev. 27: 505-523.

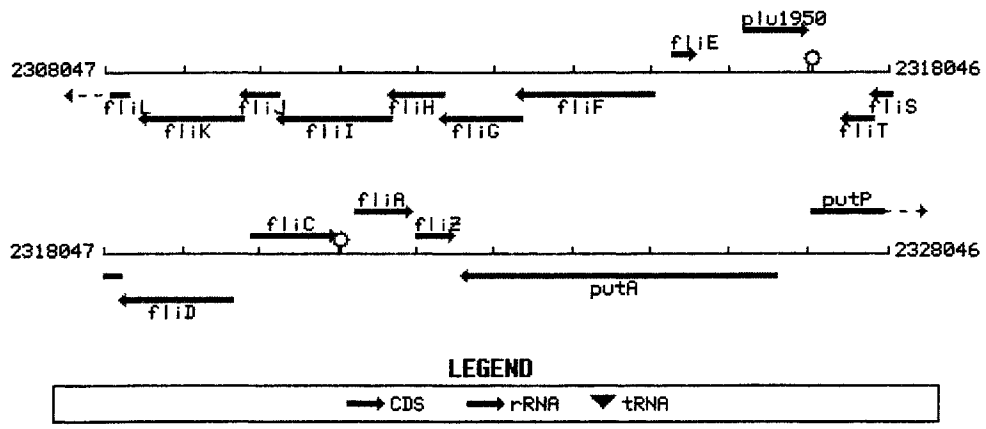


Motility in *Xenorhabdus* and *Photorhabdus*

In the closely related species *Xenorhabdus nematophilus*, primary-phase cells are motile by both swimming (0.35% agar) and swarming (0.8% agar), while the secondary cells are not (Givaudan, 1995). In *Xenorhabdus*, the *flhDC* operon controls flagellin expression for both swimming and swarming motility, as well as lipase and hemolysin activity (Givaudan and Lanois, 2000). OmpR (the response regulator of the OmpR-EnvZ two-component regulatory system in bacteria) is involved in regulation of *flhDC* expression (Kim *et al.*, 2003). A random sequencing read from *P. luminescens* strain W14 predicts *fli* and *flh*-like flagellar genes homologous with *Salmonella typhimurium*, *Escherichia coli*, and *Proteus mirabilis* (ffrench-Constant *et al.*, 2000). Flagella gene maps from the genome sequence of *P. luminescens* strain TT01 (Duchaud *et al.*, 2004) can be seen in Fig. 4.

Little is known about signal transduction and regulation of motility in *Photorhabdus*. PhoP-PhoQ, a two-component system associated with virulence in other bacterial pathogens, controls physiological adaptation to Mg^{2+} or Ca^{2+} availability (Garcia *et al.*, 1996 and 1997). Mutants with *phoP* deletions are more motile, more sensitive to antimicrobial peptides, and have reduced insect pathogenicity compared to the parental wild-type (Derzelle *et al.*, 2004). A novel two-component signal transduction system, AstR-AstS negatively regulates *flhDC*. Although not yet fully understood, AstR-AstS appears to be involved in the adaptation of cells to stationary phase and in regulation of phenotypic variation (Derzelle *et al.*, 2004).

Figure 4. Location of the *fli*, *flg*, and *flh* genes in the *P. luminescens* TT01 genomic map (**http://genolist.pasteur.fr/Photolist/**). Genes that are unnamed are designated with the prefix "plu" (Photorhabdus luminescens) and the gene number. *Fli* and *flg* genes include the structural and assembly proteins such as hook associated, filament, and flagellin proteins. *FlhD* and *flhC*, the regulatory genes, are found near the flagellar motor (*mot*) and chemotaxis (*che*) genes.



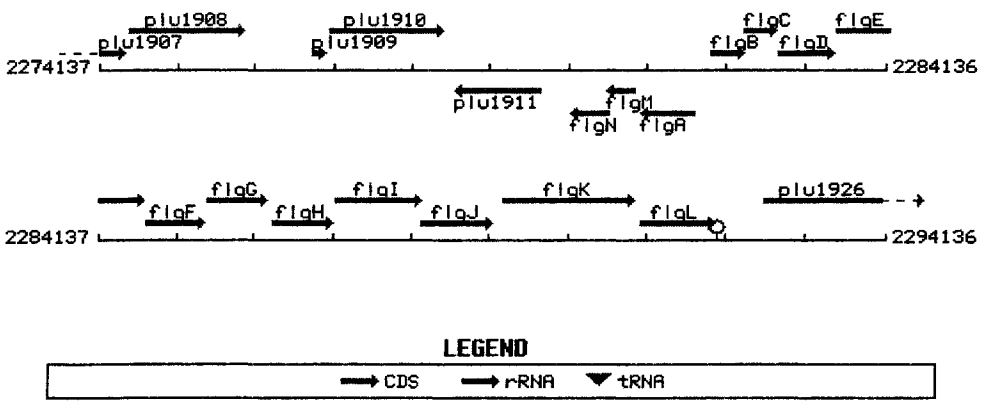
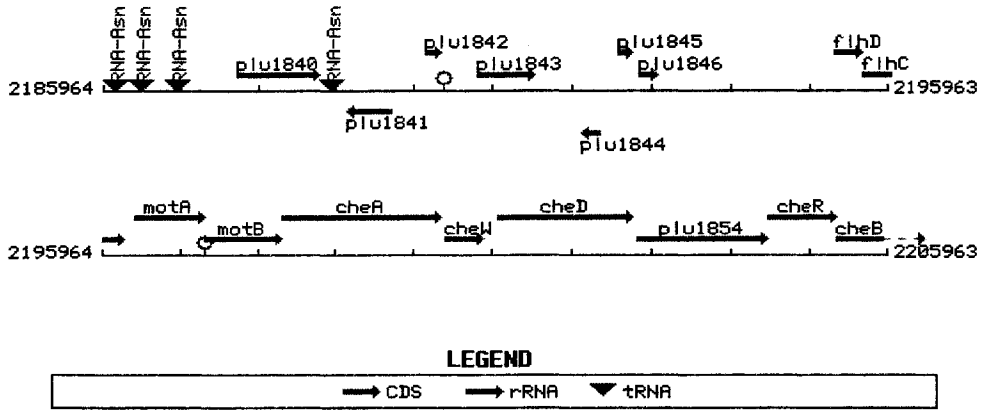


Figure 4 (cont.)



Research Approach

As discussed, motility can confer an advantage to symbiotic or pathogenic bacteria by helping them reach favorable conditions, avoid detrimental conditions, colonize a host, adhere to host tissue, and in biofilm formation. In pathogenic bacteria, regulation of flagellar genes may also be coordinated to expression of virulence factors. Since flagella are strong antigenic proteins, the regulation of flagella expression in either symbionts or pathogens may be important in evading host immune systems. Prior to the start of this project, genetic studies of *Photorhabdus* were limited, and motility studies were non-existent, therefore it was not known if any of these factors were important in this bacterium's role as an insect pathogen, nematode symbiont, or the switch between these two states.

To begin answering these questions, initial studies focused on characterizing motility in the wild-type (Chapter II and IV). I also utilized a genetic approach to identify key genes that may be controlling regulation of motility (Chapter III). One early important observation was that KCl or NaCl is essential for motility. A DNA microarray project stemmed from this observation to elucidate the regulation of all motility genes in the genome by these ionic salts (Chapter V). Overall, the goal of this research was to propose a model for the regulation and role of motility in the life cycle of *Photorhabdus.* As a result of these studies, a model is suggested in the Chapter VI Summary.

CHAPTER II

EFFECT OF GROWTH CONDITIONS ON SWIMMING MOTILITY BY *PHOTORHABDUS*

PREFACE

When I first began this project, there was little knowledge regarding *Photorhabdus* motility. Early publications described the isolations of these novel microbes and noted their motility, but there were no further studies. In addition, the genome of *Photorhabdus* had not yet been sequenced to elucidate the genetics of motility apparatus. In the absence of this knowledge, it seemed prudent to establish the environmental conditions associated with motility. As described in the Literature Review (Chapter I), bacterial movement is complex and has many forms. This study first centered on swimming motility and environmental conditions affecting swimming behavior.

The major goals of this section of the project were the following:

1. Characterize and compare motility in the primary and secondary phase-variants
2. Determine the effects of various growth conditions on motility
3. Establish conditions for optimal motility

The results of this section are presented in the form of a published manuscript [Archives of Microbiology (2003) 180: 17-24] with kind permission from Springer Science and Business Media.

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ORIGINAL PAPER

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Effect of growth conditions on the motility of *Photorhabdus temperata*

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Abstract *Photorhabdus temperata* is a bioluminescent bacterium that lives in mutualistic association with entomopathogenic nematodes of the genus *Heterorhabditis*. The bacterium exists in two morphologically distinguishable phases (primary and secondary). The swimming behavior of *P. temperata* was investigated. Both the primary and secondary variants were able to swim in liquid or semi-solid media under appropriate conditions. Variation in the oxygen levels had little affect on the chemotaxis and motility of the primary form, but greatly influenced the behavior of the secondary form. Under oxic conditions the secondary form was nonmotile, but motility was induced under anoxic conditions. Several phenotypic traits of the primary form were not expressed under anoxic conditions. The constituents of the growth media affected the motility of both variants. *P. temperata* required additional NaCl or KCl for optimum motility and chemotaxis. Optimal chemotactic behavior required the presence of bacto-peptone and yeast extract in the swim-migration medium. A mutant that was isolated from the secondary form was able to swim under oxic conditions and possessed an altered salt requirement for motility.

Keywords Chemotaxis · *Photorhabdus* · Signal transduction · Environmental signals · Nematode · Biocontrol agent · Anoxic conditions

Introduction

An interesting system of insect biological control is found during the life cycle of two families of entomopathogenic nematodes, the Steinernematidae and the Heterorhabditidae (for a review, see Forst et al. 1997; Forst and Nealson 1996; Owuama 2001). These nematodes have evolved a mutualistic relationship with entomopathogenic gram-negative γ-Proteobacteria (*Xenorhabdus* and *Photorhabdus*). The genera *Photorhabdus* and *Xenorhabdus* are similar in many characteristics, but they differ in several important traits. *Photorhabdus* is bioluminescent while *Xenorhabdus* is not. Another major difference between the genera is the specificity of their nematode host: Steinernematidae for *Xenorhabdus* and Heterorhabditidae for *Photorhabdus*.

The bacteria are carried inside the gut of the non-feeding third-instar infective stage nematode. These infective juvenile nematodes invade insects encountered in the soil. After the nematodes locate a susceptible insect host, they penetrate the haemocoel and release the facultative anaerobic bacteria into the hemolymph. The bacteria replicate and produce a variety of primary and secondary metabolites. The bacteria in conjunction with the nematode induce a lethal septicemia that kills the insect host within 48 h. These bacteria produce several antimicrobial compounds (Akhurst 1982; Richardson et al. 1988), hydrolytic enzymes (Boemare and Akhurst 1988; Bowen et al. 2000; Schmidt et al 1988; Wang and Dowds 1993), and insecticidal toxins (Bowen et al. 1998; Bowen and Ensign 1998; ffrench-Constant and Bowen 2000). Several of these metabolites have been purified and their mode of action is being investigated. The insecticidal toxin aids in the host killing process, while the antibiotics inhibit secondary invaders. Besides production of antibiotics and toxins, the bacteria also generate essential growth factors for the nematode. Thus, the bacteria create an optimum environment in the insect cadaver for nematode reproduction and development, and the eventual release of infective juveniles.

Both *Photorhabdus* and *Xenorhabdus* produce two phase variants, designated primary and secondary forms, which can be distinguished by biochemical tests and colony morphology (for review, see Akhurst 1980; Boemare et al. 1997; Forst et al. 1997; Forst and Nealson 1996). The primary form is often converted to the secondary form upon prolonged culturing and occasionally in vivo. Growth in

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low osmotic strength medium also triggers a primary to secondary phase-shift (Krasomil-Osterfeld 1995, 1997). Secondary forms are more stable and revert less frequently. The cells of the primary form produce increased amounts of antibiotics, pigments, and extracellular proteases and lipases, and in the case of *Photorhabdus* are more strongly bioluminescent than the secondary cells. Both phases are equally pathogenic when injected into the hemolymph of larvae. Within the nematode, bacteria are predominately maintained as the primary form, and nematodes grow preferentially in association with primary-form cells. Although both phase forms have been isolated from infective juveniles of their nematode partners (Ehlers et al. 1990), the numbers of infective juveniles released from their insect host are increased with the primary-phase form. *Xenorhabdus* and *Photorhabdus* show a seven-fold and three-fold increased production of infective juveniles with the primary form compared to the secondary form, respectively (Akhurst 1980). Phase variation occurs in a wide range of microbes (for a review, see Henderson et al 1999). One potential biological role for phase variation is to provide the bacterium with a strategy for adapting to more than one particular environment. The primary forms of *Photorhabdus* and *Xenorhabdus* are required for nematode development and reproduction (Forst et al. 1997; Forst and Nealson 1996; Han and Ehlers 2001). Little is known about the role of the secondary form in nature.

Our interest in *Photorhabdus* is an extension of our studies on signal transduction and bacterial diversity. Bacteria move in a coordinated manner toward attractants and away from repellents (Blair 1995). Givaudan et al. (1995) reported that the primary form of *Xenorhabdus nematophilus* is motile while the secondary form is nonmotile because it lacks flagella. In this communication, we report that both forms of *Photorhabdus* are motile under the appropriate environmental conditions.

Materials and methods

Strains

For this study, both primary- and secondary-phase variants of *Photorhabdus temperata* [formerly called *Photorhabdus luminescens* (Fischer-Le Saux et al. 1999) and *Xenorhabdus luminescens* (Boemare et al 1993)] NC19 (ATCC 29304) were used. For some experiments, primary- and secondary-phase variants of strains K122 (Griffin et al 1991) and NC1 (Bowen and Ensign 2001) were also used. Strains NC1 and NC19 are identical but were obtained from different sources (Ciche et al 2001). For each subculture, phase status was identified by pigmentation and by differential dye absorption (Boemare et al. 1997). The latter was determined by growing the strains on NBTA (nutrient agar supplemented with 25 mg bromothymol blue and 40 μg triphenyl 2,3,5 tetrazolium chloride per liter) or on MacConkey agar (bromothymol blue and neutral red absorption, respectively). Primary variant colonies are blue on NBTA and red on MacConkey agar, while secondary variant colonies are red on NBTA and off-white on MacConkey agar. On LB medium, primary-phase-variant colonies of strain NC19 were pigmented (yellowish-orange) while secondary-phase-variant colonies were off-white.

Growth conditions

Cells were grown and maintained at 28 °C in tryptone broth consisting of 1% bacto-tryptone (Difco Laboratories) and 0.5% NaCl unless otherwise noted. In addition to tryptone medium, five other growth media were used in this study: (1) LB medium consisting of 1% bacto-tryptone, 0.5% yeast extract (Difco Laboratories) and 0.5% NaCl; (2) peptone medium consisting of 1% Bacto-peptone and 0.5% NaCl; (3) LB-peptone medium consisting of 1% Bacto-peptone, 0.5% yeast extract, and 0.5% NaCl; (4) PP3 medium consisting of 1.0% proteose peptone no. 3 (Difco Laboratories), and 0.5% NaCl; (5) LB-PP3 medium consisting of 1.0% proteose peptone no. 3, 0.5% yeast extract, and 0.5% NaCl. Doubling times were determined by measuring optical density at 600 nm (OD_{600}) or turbidity with a Klett-Summerson colorimeter. Cultures were incubated with shaking at 28 °C.

Chemotaxis and motility assays

Swimming behavior was measured by the plate migration assay. Chemotactic ability of swimming cells was measured by the use of a swim-migration plate assay (Adler 1973). In this assay, bacteria migrate in response to a gradient of amino acids created by their metabolism. Swim plates containing 0.25% bactoagar (Difco Laboratories) and growth medium were inoculated in the center with a stab of approximately 10^6 cells and incubated at 22 °C unless otherwise noted. All six swim media were tested. Optimum conditions were obtained with swim medium consisting of 1.0% peptone, 1.0% yeast extract, 0.5% NaCl and 0.25% bactoagar.

Bacterial swimming behavior was observed by phase-contrast microscopy at a magnification of 400×. The cells in these behavioral assays were suspended in chemotaxis medium (10 mM K^+ phosphate, pH 7.0, 0.1 mM K^+ EDTA, and 1 mM L-methionine) or in filtered used growth medium at an optical density of 0.1 at 600 nm.

Anoxic conditions for swim plate assays

To achieve anoxic conditions, swim plates were incubated in an anaerobic hood at 22 °C under mixed gas atmosphere (85% N_2, 10% CO_2, and 5% H_2). The diameter of the chemotactic ring was measured at different time intervals for 24 or 48 h. When incubation at temperatures other than 22 °C was required, swim plates were incubated for 48 h in Brewer's jars that were rendered anoxic by the use of gas packets (BBL GasPak Anaerobic System).

Transmission electron microscopy

Bacterial suspensions were placed on Formvar-coated copper grids, negatively stained with 1% phosphotungstic acid, and viewed on a JEOL 100S transmission electron microscope.

Mutant isolation

Mutants with enhanced motility were isolated by selection. Cells from the outer ring of a tryptone swim plate were inoculated into a fresh swim plate and incubated at 28 °C for 24 h. Cells from the resulting outer chemotactic ring were streaked on tryptone agar plates and incubated overnight at 28 °C. Single colonies were inoculated into tryptone swim plates and incubated at 28 °C for 24 h. This selection procedure was repeated several times. Mutants DWA11 and DWB13 were isolated from the wild-type primary and secondary variants, respectively, and saved for further study.

Phenotypic characterization

Dye absorption with NBTA and MacConkey media was assayed as described above. In addition, EB agar (eosin Y and methylene blue

at 400 and 65 mg/l, respectively, in 2% PP3 agar) was used. Hemolytic activity was determined by observing a clearing surrounding the bacterial colonies cultured on blood agar. Lipase activity was tested on spirit blue agar containing 0.5% (v/v) Tween 20, Tween 40, Tween 60, or Tween 80. Catalase activity was determined by the addition of 10 µl of 30% H_2O_2 to isolated colonies on LB or PP3 agar. Protease activity was determined by the gelatin assay (Boemare et al. 1997). DNase activity was determined on DNase test agar containing methyl green. Antibiotic activity was evaluated by placing a 5-mm-diameter plug, taken 5 mm away from confluent growth of a 96-h culture of *P. luminescens* on PP3 agar, onto a plate of antibiotic medium 3 (Difco) that had been inoculated with *Micrococcus luteus* cells.

Biochemical traits were also measured by use of BIOLOG plates. Ten ml of peptone-yeast extract broth were inoculated with single colonies and the cultures were incubated overnight at 28 °C. Cells were harvested, washed twice with 0.85% KCl, and finally suspended in 0.85% KCl to an OD_{590} of 0.25. Washed cells (150 µl) were added to each well of a BIOLOG ECO plate. The plates were incubated at 28 °C and color formation was measured at 24 and 48 h by the use of an ELISA plate reader.

Protein profiles

Cells were grown in LB-peptone broth at 28 °C until the cultures reached an OD_{600} of about 1.0. The cells were harvested, resuspended in Laemmli sample buffer to an equivalent OD_{600}, and the samples were prepared by boiling in SDS sample buffer for 5 min. SDS-PAGE was carried out as described by Laemmli (1970). The gels were stained with Coomassie to visualize protein bands.

Results

Swimming ability and the effect of O_2

The plate migration assay was used to investigate the swimming behavior of *P. temperata* phase variants (Fig. 1). Since *Photorhabdus* is a facultative anaerobe, the effect of oxygen on chemotaxis and swimming movement was tested. The primary-phase cells formed spreading rings under oxic or anoxic conditions, indicating that the cells were motile and chemotactic (Fig. 1a, b). Secondary-phase cells formed spreading rings under anoxic conditions (Fig. 1d), but failed to spread when oxygen was present (Fig. 1c). Cells from the plate migration assays were also observed by phase-contrast microscopy. Primary-form cells taken from plates that had been incubated aerobically or anaerobically actively swam. Secondary-form cells taken from plates that had been incubated aerobically were nonmotile, whereas cells taken from plates that had been incubated anaerobically were actively motile and retained their motility when exposed to oxygen for short time periods. Several primary and secondary colonies from strains NC19, NC1, and K122 were tested and showed similar results (data not shown). Transmission electron microscopy revealed the presence of peritrichous flagella (Fig. 2). The secondary form only produced flagella under anoxic conditions (Fig. 2d), while the primary form produced flagella under both conditions. These results indicate that oxygen inhibits the motility of the secondary form by blocking flagella formation.

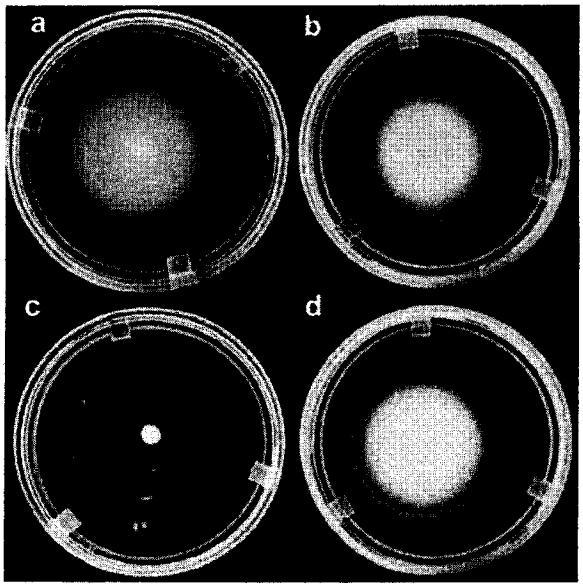

Fig. 1a–d Chemotactic properties of *Photorhabdus temperata* NC19 as determined by the swim-migration assay. Tryptone swim plates containing 0.25% bactoagar were incubated at room temperature (22 °C) for 48 h either under oxic or anoxic conditions (N_2: CO_2: H_2, 85:10:5 by volume). **a** Primary form under oxic conditions; **b** primary form under anoxic conditions; **c** secondary form under oxic conditions; **d** secondary form under anoxic conditions

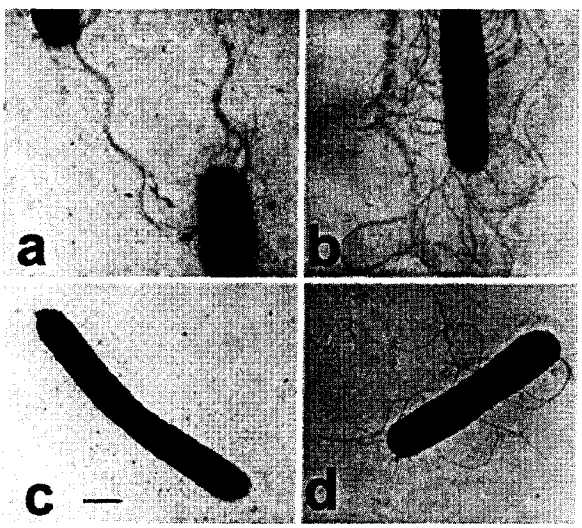

Fig. 2a–d Transmission electron microscopy of *P. temperata* NC19. Bacteria were gently placed onto Formvar-coated copper grids and negatively stained using 1% phosphotungstic acid. **a** Primary form under oxic conditions; **b** primary form under anoxic conditions; **c** secondary form under oxic conditions; **d** secondary form under anoxic conditions. *Bar* 1 µm

Table 1 Effect of media on the chemotactic properties of *Photorhabdus temperata* strain NC19. Swim migration was determined by the use of swim plates containing 0.25% bactoagar. Bacteria were grown overnight in tryptone broth and used as inocula for these experiments. The plates were incubated for 48 h at room temperature (22–24 °C). The diameters of the chemotactic rings were measured and are expressed in mm

Swim media	Swim ring diameter (mm)			
	Primary form		Secondary form	
	Oxic	Anoxic	Oxic	Anoxic
Tryptone	21.3±2.1	26.7±7.5	5.0±0.6	16.7±3.4
Tryptone+0.5% yeast extract	35.2±4.3	37.4±3.0	9.0±0.9	20.6±1.2
Tryptone+1.0% yeast extract	31.3±3.5	47.3±5.0	8.0±2.8	21.3±1.2
Peptone	52.9±3.3	64.4±2.7	6.2±0.6	30.0±1.7
Peptone+0.5% yeast extract	35.1±2.4	74.5±2.5	11.1±0.8	48.0±3.0
Peptone+1.0% yeast extract	37.0±1.9	54.0±4.9	8.7±5.3	44.0±1.6
PP3	27.3±2.3	18.0±1.7	4.0±1.0	10.7±2.5
PP3+0.5% yeast extract	35.7±1.5	32.0±3.0	10.7±2.3	33.0±3.0
PP3+1.0% yeast extract	43.0±7.5	35.0±1.7	7.0±1.4	27.0±3.0

Effect of growth media on chemotaxis

To better understand the factors influencing the motility and chemotactic behavior of *P. temperata*, the effect of growth conditions was investigated by the use of the swim-migration plate assay. The optimum temperature for swim ring formation was 28 °C for both forms (data not shown). Motility and chemotaxis were inhibited by elevated temperatures (35 °C and higher). Swim ring formation by the secondary form was only detected under anoxic conditions. Under oxic conditions, the secondary form was nonmotile at all temperatures tested.

Swim media composition greatly influenced ring formation (Table 1). Peptone resulted in larger rings than tryptone or proteose peptone. The addition of yeast extract to 0.5% stimulated swim ring formation by both forms under anoxic conditions. Elevated levels of yeast extract (1.0%) reduced the size of the primary form swim ring in peptone medium. The primary form was not influenced as strongly by yeast extract under oxic conditions, and swim ring formation was slightly inhibited by yeast extract addition to peptone media.

Effect of NaCl on motility and chemotaxis

Primary- and secondary-phase cells failed to form swim rings in LB-peptone swim agar unless additional salt was added. Addition of 86–176 mM NaCl allowed optimal swim ring formation. Phase-contrast microscopic observation confirmed that cells grown in LB-peptone without additional salt were nonmotile, whereas cells grown in the same medium with 86 mM NaCl exhibited rapid swimming motility. Transmission electron microscopy was used to demonstrate that cells grown in media without added salt lacked flagella, whereas cells grown in media with 86 mM NaCl exhibited peritrichous flagella. When peptone was replaced in the swim medium by tryptone or proteose peptone, the identical NaCl-requiring swim-migration patterns were observed for both forms (data not shown). These results suggest that medium composition did not influence the NaCl requirement for motility and chemotaxis.

Since an analysis of chemotactic behavior by use of swim ring formation is influenced by growth rate, the effect of salts on growth rates was determined. The growth rate of both phase variants was not adversely affected by the absence of additional NaCl. Cells grown in medium lacking additional NaCl grew slower, with a 1.2-fold increase in the doubling time, suggesting that the NaCl requirement for motility was not directly related to a growth rate effect.

The salt requirement for motility and chemotaxis was not specific for Na^+ (Fig. 3). At 75 mM salt concentrations, NaCl, KCl, and $MgCl_2$ restored swimming motility under anoxic conditions by the primary form to an equiva-

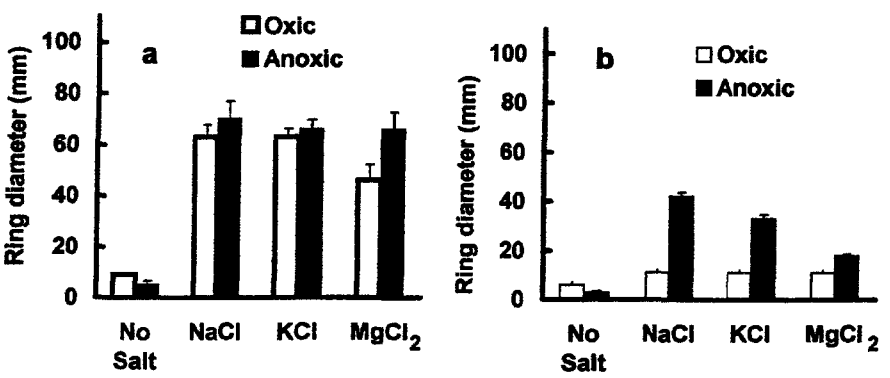

Fig. 3a, b Effect of salts on chemotactic properties of *P. temperata* NC19 as determined by the swim-migration assay. LB-peptone swim plates containing no salts or 75 mM NaCl, KCl, or $MgCl_2$ were incubated at 28 °C for 48 h. Results are shown for **a** primary-form cells and **b** secondary-form cells under oxic and anoxic conditions. Values are the average of 6–12 measurements

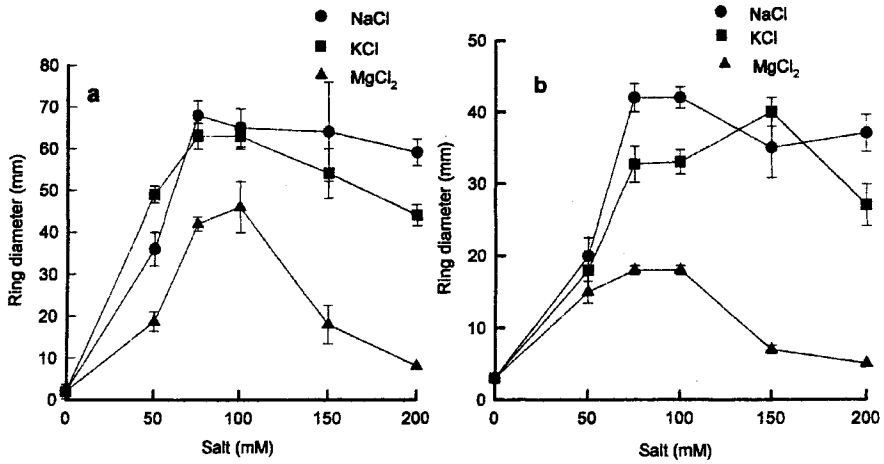

Fig. 4a, b The effect of different salt concentrations on swim ring formation by *P. temperata* NC19. LB-peptone swim plates containing different concentrations of NaCl, KCl, or $MgCl_2$ were incubated at 28 °C for 48 h. Results are shown for **a** primary-form cells under oxic conditions and **b** secondary-form cells under anoxic conditions. Values are the average of 3–6 measurements

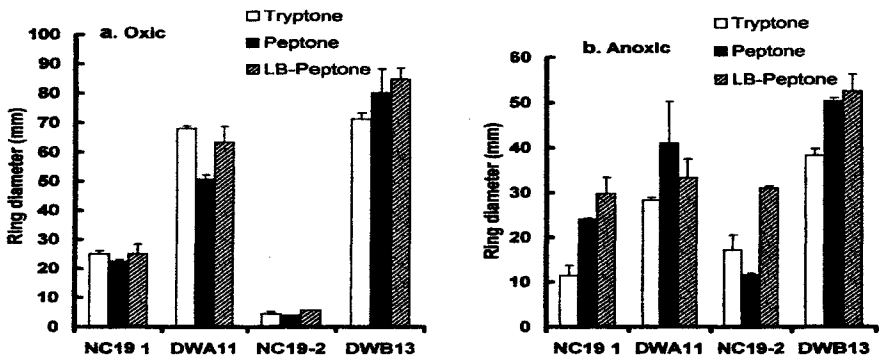

Fig. 5a, b Swim ring formation by mutants of *P. temperata* strain NC19 compared to their parental wild-types. Swim plates containing peptone, tryptone and LB-peptone media were incubated at 28 °C for 24 h. Results are shown for **a** oxic conditions and **b** anoxic conditions. Values are the average of 6–12 measurements

lent level (Fig. 3a). Under oxic conditions, there was a slight decrease in swim ring formation with $MgCl_2$ compared to the other two salts. Secondary-form cells showed the following order of preference: NaCl>KCl>$MgCl_2$ (Fig. 3b). Several primary and secondary colonies from strains NC19, NC1, and K122 were tested and showed similar results (data not shown).

A range of salt concentrations was tested and the results are shown in Fig. 4. Without added salt, swim rings were not observed. With the primary form, the addition of NaCl or KCl resulted in swim ring formation (Fig. 4a). $MgCl_2$ was not as effective as NaCl or KCl, and inhibited motility at elevated levels (>100 mM). $CaCl_2$ was unable to replace NaCl (data not shown). Similar results were observed for cells under anoxic conditions. With the secondary form, addition of NaCl resulted in the largest swim rings, followed by KCl and $MgCl_2$ (Fig. 4b). Elevated levels of $MgCl_2$ inhibited swimming motility. Secondary-form cells were nonmotile under oxic conditions.

Isolation of motility mutants and their properties

Two mutants with enhanced motility were isolated by selection as described in Materials and methods. Strain DWA11, a motility-enhanced mutant, was derived from the primary form. Strain DWB13 was derived from the secondary form and was selected for motility under aerobic conditions. Both mutants were stable and were highly motile under oxic and anoxic conditions as compared to their parental strains (Fig. 5). In contrast to its parental wild-type, oxygen did not inhibit motility by strain DWB13.

Mutants DWB13 and DWA11 did not have the same salt requirement as their parental wild-types (Fig. 6) and formed swim rings in the absence of added NaCl (ring diameters of 36.7±1.5 mm/day and 29.4±4.2 mm/day, respectively). Swim ring formation was greatest with the addition of KCl to the swim medium. Elevated levels of $MgCl_2$ inhibited swim ring formation. Motility was also inhibited by $CaCl_2$ (data not shown). Both mutant strains showed the same salt requirement patterns under anoxic conditions as was observed with oxic conditions (data not shown).

Several physiological properties of the behavioral mutants and their parental strains were investigated (Table 2). The phenotypic traits of primary-form cells under anoxic conditions differed from those exhibited under oxic conditions. Anoxic conditions down-regulated several phenotypic traits of the primary form, including hemolysin, protease, and lipase activities. Antibiotic production and catalase activity by primary-form cells were not inhibited by

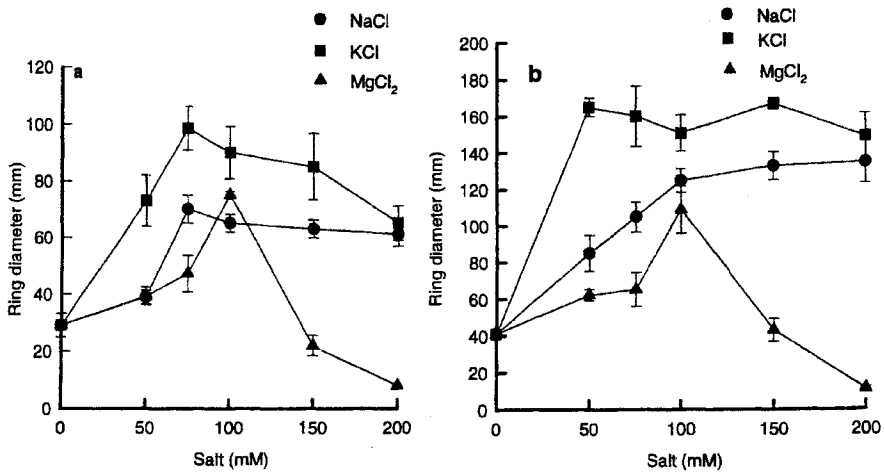

Fig. 6a, b Effect of salts concentrations on swim ring formation by the mutant strains of *P. temperata* as determined by the swim-migration assay. LB-peptone swim plates containing different concentrations of NaCl, KCl, or $MgCl_2$ were incubated at 28 °C for 48 h under oxic conditions. Results are shown for **a** DWA11 and **b** DWB13. Values are the average of 3–6 measurements

Table 2 Phenotypes of *P. temperata* mutants and their parental wild-types. Results for oxic and anoxic conditions are presented; values in parenthesis are for anoxic conditions. *1* Primary form, *2* secondary form. *w* Weakly positive. DNase and protease activities were determined by measuring the size of the halo (mm) surrounding the bacterial colony 24 h after inoculation. Strongly positive (++) >2 mm halo, positive (+) 1–2 mm halo, weakly positive (w) <1 mm halo, and negative (–) no halo. Antibiotic production was determined by measuring the size of the halo (mm) surrounding the bacterial colony 1 day after inoculation of the tester bacterium (*Micrococcus luteus*)

	NC19 1	NC19 DWA11	NC19 2	NC19 DWB13	NC1 1	NC1 2 Yellow	NC1 2 White	K122 1	K122 2
Dye absorption									
EMB	+ (–)	+ (–)	– (–)	– (–)	+ (–)	– (–)	– (–)	+ (–)	– (–)
MacConkey	+ (–)	+ (–)	– (–)	– (–)	+ (–)	– (–)	– (–)	+ (–)	– (–)
Extracellular products									
Lipase	+ (–)	+ (–)	– (–)	– (–)	+ (–)	– (–)	– (–)	+ (–)	– (–)
Hemolytic	+ (–)	+ (–)	– (–)	– (–)	+ (–)	w (–)	– (–)	+ (–)	– (–)
Protease	+ (–)	+ (–)	w (–)	w (–)	+ (–)	– (–)	– (–)	++ (–)	– (–)
DNase	+ (–)	+ (–)	+ (–)	+ (–)	+ (–)	+ (–)	– (–)	+ (–)	– (–)
Antibiotics	4 (3)	2 (3)	– (2)	– (1)	2 (3)	– (3)	– (1)	8 (4)	– (2)
Pigmentation	+ (+)	+ (–)	– (–)	– (–)	+ (+)	+ (+)	– (–)	+ (+)	– (–)
Catalase	+ (+)	+ (+)	w (w)	w (w)	+ (+)	w (w)	w (w)	+ (+)	w (w)

anoxic conditions. Secondary-form cells maintained their phenotypic traits under oxic or anoxic conditions except antibiotic production. Under anoxic conditions, secondary-form cells produced antibiotics, which they did not make under oxic conditions. However, the amount of antibiotics produced by secondary-form cells was less than that produced by the primary-form cells under anoxic conditions. The primary mutant DWA11 and the secondary mutant DWB13 maintained the physiological properties of their respective forms, including dye absorption, pigmentation, and extracellular enzyme activity. These results indicate that these are true "mutants" rather than phase variants.

Biolog ECO plates were used to identify substrate utilization patterns. The primary form was capable of using six out of the 33 substrates tested (L-serine, L-threonine, pyruvic acid methyl ester, *N*-acetyl-D-glucosamine, L-asparagine and D,L-α-glycerol phosphate) while the primary mutant DWA11 oxidized only four of these six substrates. The secondary form was able to use 11 substrates (the same six as the primary form plus glycogen, ketobutryric acid, D-malic acid, Tween 40 and Tween 80), while the secondary mutant DWB13 used these 11 substrates plus hydroxybutyric acid and 4-hydroxy benzoic acid. The protein profiles of the mutants under oxic and anoxic conditions were determined by SDS-PAGE analysis and were similar to the profiles of their corresponding parental wild-type (data not shown). These results suggest that the motility mutants maintained their respective phase forms.

Discussion

The optimum conditions for motility and chemotaxis for *P. temperata* were determined in this study. Oxygen inhibited the motility of the secondary form, but had little effect on the motility of the primary form. In contrast, only the primary form of *Xenorhabdus* was capable of swimming motility (Givaudan et al 1995). To our knowl-

edge, the effect of oxygen on the motility of *X. nematophilus* has not been studied previously. Both forms of *P. temperata* also required additional salt for optimal motility. The opposite effect was observed for *X. nematophilus* (Volgyi et al 1998); the addition of >35 mM NaCl to the medium inhibited motility.

One hypothesis to explain the oxygen effect is that the secondary form is more adapted to survival in anoxic environments. The increased motility under anoxic conditions could aid secondary-form cells in migrating toward nutrients or potential terminal electron acceptors. Rosner et al. (1997) investigated the metabolism of *P. luminescens* and found no apparent differences in the fermentation metabolism between the two phase variants, but they did not explore anaerobic respiration. The secondary form grows faster and has a higher cell yield than the primary form (Beakley and Nealson 1988; Rosner et al. 1997). Smigielski et al. (1994) postulated that secondary-phase cells are better adapted to the low-nutrient conditions found in the soil, while primary-form cells are better adapted to conditions in the insect and nematode. Although there have been no reports of the isolation of *Photorhabus* from uninoculated soil, the bacteria are able to survive and grow in soil (Bleakley and Chen 1999).

The mechanism of phase variation in *Photorhabdus* is unknown (for a review, see Forst and Nealson 1996; Forst et al. 1997). An analysis of the genome structure indicates that a major DNA rearrangement or instability is not responsible for phase variation (Akhurst et al. 1992). Other studies have ruled out the loss of a plasmid or phage as the mechanism (Leclerc and Boemare 1991). One current hypothesis is that a global regulatory system controls the phenotypic traits associated with phase variation. However, the presence of intermediate forms of phase variants (Akhurst 1980; Gerritsen et al. 1992; Hu and Webster 1998) suggests that regulation is probably more complex than a simple master-switch controlling many factors. Anoxic conditions down-regulated several primary-phase traits including DNase, protease, and hemolysin activities, but antibiotic production was not oxygen-regulated with primary-phase cells (Table 2). With secondary-phase cells, anoxic conditions initiated antibiotic production and motility, but did not stimulate expression of any other primary-phase traits. Our results support the more complex model rather than the simple master-switch hypothesis. Many other environmental factors may influence this control mechanism.

Flagellum formation by *Escherichia coli* is a response to environmental stress that appears to act at the level of *flhDC* expression and is affected by catabolite repression, temperature, and other factors linked to the cell cycle (Blair 1995). Our results suggest that anoxic conditions globally affect gene expression in *P. temperata*. Oxygen inhibited flagellum production and antibiotic production by secondary-phase cells (Fig. 2), while the absence of oxygen down-regulated many primary-phase traits (Table 2). These effects of oxygen on *P. temperata* imply a potential role for an FNR-like regulator and/or an ArcA/ArcB sensor-regulator system. The generation of a secondary motility mutant (DWB13) supports this hypothesis. This mutant was highly motile under oxic conditions and did not require added salt for expression of motility. These results suggest a mutation in a regulatory element. Current studies are directed toward understanding how oxygen levels regulate gene expression in *P. temperata*.

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CHAPTER III

ISOLATION AND CHARACTERIZATION OF MOTILITY MUTANTS

PREFACE

In Chapter II, I characterized swimming motility by various *Photorhabdus* strains. Conditions for optimal swimming motility and chemotaxis were defined. This work was essential for establishing phenotypic criteria to be used for my next objective: creating mutants altered in motility. By using a genetic approach, I would be able to identify the genes responsible for the motility phenotypes. These mutants could be tested for any other corresponding changes in physiology, including the expression of virulence enzymes. These results would elucidate whether other genes were under the same regulation as motility genes. Lastly, the mutants would be used for *in vivo* experiments to determine their effects on insect virulence.

The specific goals in this section of the project were the following:

1. Use transposon mutagenesis to generate and isolate motility mutants
2. Characterize mutants physiologically to determine if mutations also have an effect on virulence
3. Characterize mutants at the genetic level to determine the genes and pathways involved in regulation of motility

Isolation and Characterization of *Photorhabdus temperata* Motility Mutants

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ABSTRACT

Photorhabdus **is a lethal insect pathogen symbiotically associated with** ***Heterorhabditidae*** **nematodes. The bacteria are motile by both swimming and swarming, but the role of motility and its regulation in this complex life cycle is not understood. To identify motility genes,** ***P. temperata*** **mutants with altered motility were isolated from a bank of 10,000 transposon mutants. These mutants classified into five phenotypic groups: (1) non-motile; (2) hyperswimmers; (3) hyperswarmers; (4) mutants able to swim without NaCl; and (5) mutants with reduced motility. Several of the motility mutants were altered in the production of antibiotics, pigment, hemolysins, proteases, and/or DNases, suggesting that expression of motility genes may be coupled to some virulence enzyme genes. A** ***plu3723*** **(*luxR* transcriptional regulator homologue) mutant was isolated that is able to swim without NaCl. Directed by NaCl concentrations,** ***plu3723*** **may regulate the** ***Photorhabdus*** **flagellation cascade. An** ***rssB*** **(response regulator) mutant displayed a hyperswarming phenotype, suggesting RssB acts as a negative regulator of swarming behavior. A** ***yidA*** **mutant, whose function remains unknown, displayed reduced swimming behavior and dramatically attenuated virulence. The data presented in this study are the first clues for elucidating a model of motility in the life cycle of this insect pathogen and nematode symbiont.**

INTRODUCTION

Photorhabdus is a bacterial symbiont of soil dwelling entomopathogenic nematodes in the *Heterorhabditidae* family (for reviews see: Boemare *et al.*, 1997; Burnell and Stock, 2000; Forst *et al.*, 1997; Forst and Nealson, 1996). As a symbiont, these bioluminescent bacteria live in the intestine of free-living infective dauer juvenile (IJ or DJ) nematodes (Ciche and Ensign, 2003; Endo and Nickle, 1990; Milstead, 1978). The nematodes actively seek and infect a wide range of insect hosts by entering through natural openings or by burrowing directly through the insect cuticle (Poinar, 1975). Once inside the insect, the nematodes regurgitate the bacteria into the hemolymph (Ciche and Ensign, 2003). The bacteria release highly virulent toxins (Bowen, 1998; Bowen and Ensign, 1998; ffrench-Constant and Bowen, 2000), which results in insect death in less than 48 h. As the bacteria enter the stationary phase of their growth cycle, they secrete extracellular enzymes such as proteases (Schmidt *et al.*, 1988), lipases (Wang and Dowds, 1993), and hemolysins (Brillard *et al.*, 2001). These enzymes aid in breaking down insect tissue, thereby providing nutrients for both the bacteria and nematodes. In addition, the bacteria release antibiotics to prevent secondary invaders and putrefaction of the insect carcass (Akhurst, 1982; Richardson *et al.*, 1988). After several days of feeding, the nematodes and bacteria reassociate and leave in search of a new insect host.

Photorhabdus spp. exist in two distinct phenotypic forms, termed primary and secondary-phase variants (Akhurst, 1980). Primary-phase variants are easily

characterized by having higher production of extracellular enzymes and bioluminescence. The two variants also differ in colony and cell morphology, as well as in metabolism (Rosner *et al.*, 1996 and Smigielski *et al.,* 1994). The primary-phase cells are the variants that are naturally isolated from the infective-stage nematodes (for reviews see: Akhurst, 1980; Boemare *et al.*, 1997). The exact molecular mechanism and biological significance of phase variation in these species remains unknown. The secondary phase-variants are defective in supporting nematode reproduction suggesting an alternative ecological role from the primary form (Ehlers *et al.*,1990 and Han and Ehlers, 2001).

In the closely related species *Xenorhabdus nematophilus*, primary-phase cells are motile by swimming (movement through liquid or semi-solid media), while the secondary cells are not (Givaudan, 1995). We have shown that for *Photorhabdus,* both primary and secondary-phase cells swim by means of peritrichous flagella (Chapter 2; Hodgson *et al.*, 2003). Although oxygen levels do not affect the motility of the primary phase-variant, secondary-phase cells are motile only under anoxic conditions. These observations support the hypothesis that each phase variant may be adapted to a specific environment.

Motility confers an advantage to bacteria by allowing them to reach favorable conditions, avoid detrimental environments, and to compete with other microorganisms. Motility in pathogenic bacteria often contributes to the successful invasion of a host or adhesion to host cells. The expression of virulence products may also be coupled to flagellar synthesis (for reviews on motility see: Daniels *et al.*, 2004; Soutourina and Bertin, 2003; and Szurmant and

Ordal, 2004). We are interested in understanding the role of motility in the complex life cycle of *Photorhabdus*. The purpose of this study was to use transposon mutagenesis to generate mutants altered in swimming and swarming motility. We also screened for mutants able to swim without NaCl to understand why salt is required for motiltity, an important observation described previously (Chapter 2; Hodgson *et al.*, 2003).

MATERIALS AND METHODS

Bacterial strains and growth conditions. For this study, primary-phase variants of *Photorhabdus temperata* [formerly *Xenorhabdus luminescens* (Boemare *et al*, 1993; Fischer-Le Saux *et al*, 1999)] NC19 (ATCC 29304) were used. Cells were grown and maintained in LB medium as described previously (Chapter 2; Hodgson *et al.*, 2003).

Preparation of competent cells for electroporation. *Escherichia coli* and *Photorhabdus* cells were made competent using a modification of the procedure recommended by Bio-Rad Laboratories (Richmond, CA). All materials and reagents were chilled on ice before use. Primary-phase cells were grown to exponential phase (A_{600nm} = 0.50) in 50 ml of LB medium and chilled on ice for 15 min. Cells were harvested by centrifugation at 1300 X g for 10 min at 4°C. The harvested cells were washed 3X by resuspending in 10 ml 1mM HEPES (pH 7.0) and washed once with 5 ml 10% glycerol. After centrifugation, the pellet was resuspended in 500 ul 10% glycerol and frozen at -80°C in 200 ul samples.

Transformation Conditions. Transformation of *P. temperata* and *Escherichia coli* DH5α was done by electroporation using a BioRad gene pulser according to

the conditions suggested by the supplier (BioRad Laboratories; Hercules, CA). Competent cells (200 ul) that were prepared fresh as described above or thawed cells from -80°C storage were added to a chilled electroporation cuvette (0.2 cm gap, BTX Inc.; San Diego, CA) along with 10 ul of plasmid vector (approximately 10 ng). The plasmid was introduced into the cells by electroporation with a single pulse at a capacity of 25 uF and a field intensity of 2.5 kV at 600Ω. Immediately after the electrical pulse, the cells were diluted with 1 ml of SOC medium (2% w/v Bacto tryptone, 0.5% w/v Bacto yeast extract, 10 mM NaCl, 2.5 mM KCl, 10 mM $MgCl_2$, 10 mM $MgSO_4$, and 20 mM glucose) and incubated for at least 1 h at 28°C or 37°C, for *Photorhabdus* and *E. coli*, respectively. These cells were plated onto LB medium containing the appropriate antibiotic to select for successful transformants, which were suspended in 30% glycerol and stored at -80°C until needed.

Transposon mutagenesis. *P. temperata* cells were mutagenized by mini-Tn5 according to the method of Ciche *et al.* (2001). The mini-Tn5 delivery vector, pUB394 (Fig 1) was electroporated into competent primary-phase cells as described above. The streptomycin resistance trait encoded on the 9.4 kb plasmid allowed for easy selection of possible transformants. The plasmid also contains a *sacB* gene, conferring sucrose toxicity and a kanamycin resistance gene located within the mini-Tn5. The *sacB* gene allows selection against cells containing pUB394. Thus, cells containing a genomic mini-Tn5 insertion are selected on medium containing both sucrose and kanamycin.

Photorhabdus cells containing pUB394 were streaked onto LB plates containing 25 ug/ml streptomycin. Isolated colonies were used to inoculate LB broth containing 25 ug/ml kanamycin. Dilutions of the overnight culture (10^{-1} and 10^{-2}) were plated onto LB plates containing 7.5% sucrose and 25 ug/ml kanamycin. These potential transposants were isolated and individually inoculated into separate wells of 96-well microtiter plates containing LB medium supplemented with kanamycin. After the plates were incubated overnight at 28°C, glycerol was added to a final dilution of 30% and the plates were stored at –80°C. A total library of 10,176 transposants were generated and stored for further use.

Screening of transposants for changes in motility. The plate migration assay (Adler, 1973) was used to screen for mutants altered in motility behavior. In this assay, bacteria migrate in response to a gradient of amino acids created by their metabolism, forming a "swim migration ring" after inoculation into the center of the plate. Swim medium consisted of 0.5% yeast extract (Difco Laboratories), 1% Bacto-peptone (Difco), 0.5% NaCl and 0.25% (w/v) agar (Difco). For swarming or surface movement, PP3 swarm medium (2% Proteose Peptone 3 medium, 0.5% NaCl and 1% Bacto agar) was used. Swim or swarm media were poured into oversized petri dishes (150x15 mm; Fisher Scientific, Canada) or into 96-well plates. Using a 96-well replicator, the -80°C stored transposants were transferred into new 96-well microtiter plates containing fresh LB medium and incubated overnight at 28°C. The freshly grown overnight culture was replica plated directly into swim or on top of the swarm media. The motility plates were incubated at

28°C up to 72 h, and monitored every 6-8 h for mutants altered in motility behavior as compared to the parental wild-type. Thus, the library was rapidly screened and putative mutants were selected. All preliminary mutants were re-tested on individual motility plates using an inoculum of 2 ul from overnight broth cultures. Confirmed mutants were suspended in 30% glycerol and stored at -80°C for further study.

Physiological characterization. Phenotypic properties of the mutants were determined by the use of standard plate assays for virulence factors and dye binding as described previously (Chapter 2; Hodgson *et al*, 2003). Motility was determined by either the swim or swarm migration assay as described above.

***In vivo* pathogenesis assay**. Insect pathogenesis was tested in the greater wax moth larvae, *Galleria mellonella* (GrubCo. Inc., Hamilton, OH) according to the method of Clark and Dowds (1995). All larva were injected with approximately 280 cells (in 10 ul LB) from an overnight LB culture. At least 20 larvae were injected per mutant. The health of the larvae was monitored every 8 h at which time the number of dead larvae were counted. The LT_{50} values for each bacterial mutant strain were calculated and defined as the time taken for 50% of the larvae to die. The positive control was the parental wild-type. Negative controls included injection with LB and injection with *E. coli*. Insect death never occurred with negative controls as long as larvae were used within 2 ½ weeks of receipt.

Molecular biological techniques. Plasmid preparations were performed with a QIAprep Spin Mini Prep Kit according to the manufacturer's instructions (Qiagen Sciences; Valencia, CA). Restriction enzymes were used according to the

manufacturer's instructions (New England Biolabs; Beverly, MA). When required, DNA fragments were extracted from agarose gels using a QIAquick Gel Extraction Kit (Qiagen Sciences; Valencia, CA). Genomic DNA (gDNA) was extracted and purified by the cetyltrimethylammonium bromide (CTAB) method (Murray and Thompson, 1980).

Southern hybridization. Southern hybridization experiments were performed under high stringency conditions using a modification of Southern (1975) with a DIG High Prime DNA Labeling and Detection Starter Kit II (Roche; Mannheim, Germany). Purified DNA (10 ug) was digested with the restriction enzyme *NsiI* (the mini-Tn5 contains no *NsiI* sites). The fragmented DNA was separated on a 1% agarose gel in 1X TAE buffer (40 mM Tris-acetate and 1 mM EDTA). The DNA was transferred to a positively charged nylon membrane (Roche Diagnostics, Germany) by electroblotting at a constant voltage of 10V for 30 min. The membrane was washed, fixed, hybridized (at 42°C), and detected according to the DIG High Prime kit instructions.

The original vector pUB394 was used as the template for probe labeling of the mini-Tn5 kanamycin resistance gene sequence. The sequence was amplified with PCR using primers (Integrated DNA Technologies; Coralville, IA) to the kanamycin resistance gene with a Hot Start Taq Master Mix Kit (Qiagen Sciences, CA) and the primer set: Kan Forward primer 5'-GTAAACTGGATGGCTTTCTTGCCG-3' and Kan Reverse primer 5'-ATATCACGGGTAGCCAACGCTATG-3'. The probe for the mini-Tn5 insertion

was random prime labeled with digoxigenin-11-dUTP (DIG) according to the DIG-High Prime Kit instructions.

Retrieval of mutant DNA flanking the mini-Tn5. Mutant gDNA flanking the mini-Tn5 insertion was retrieved by rescue cloning as previously described (Ciche *et al.* 2001). gDNA was digested with *NsiI* and self-ligated at a concentration of 0.5 ng/ul. The ligated DNA (2-50 ng/20 ul) was concentrated and transformed by electroporation into 200 ul of competent *E. coli* DH5α cells (as described above). Transformants containing the mini-Tn5 on plasmids were selected by kanamycin resistance. These plasmids containing the mini-Tn5, flanking DNA, and the origin of replication (P15A), were isolated with a Qiagen kit as described above. Plasmids were either directly sequenced or amplified further with arbitrary primed PCR as described below.

Arbitrary primed PCR. When direct sequencing of the rescue plasmid failed, a modification of the arbitrary-primed PCR protocol was used to amplify the DNA regions flanking the mini-Tn5 insertion (Caetano-Annoles,1993; O'toole *et al.,* 1999). This PCR method uses primers specific to the ends of the transposon and primers of random sequence. Under low stringency temperatures, these arbitrary primers anneal to chromosomal DNA sequences in close proximity to the transposon insert. A nested PCR approach was used with two rounds of amplification. The second round of amplification enriched for the DNA-flanking the transposon insertion site. Both purified gDNA and rescued plasmids were used as DNA templates.

The first round of PCR used a primer specific to one end of the transposon, either M13 F –47 (5'-CGCCAGGGTTTTCCCAGTCACGAC-3') or M13 R –60 (5'-TCACACAGGAAACAGCTATGAC-3') and the arbitrary primer ARB1 (5'-GGC CAC GCG TCG ACT AGT AC $(N)^{10}$ - 3') . Thermocycler conditions were as follows: 5 min at 95°C, (30 s at 94°C, 30 s at 30°C, 1 min at 72°C) X 6, (30 s at 94°C, 30 s at 45°C, 1 min at 72°C) X 30, 5 min at 72°C , and 4°C forever.

First round PCR product was purified with a Qiagen PCR Purification Kit, resuspended in 30 ul H_2O, and 2 ul was used as template for the second (nested) PCR round. The second round PCR consisted of a primer specific to the end of the transposon (nested from the first round primers), M13F -20 (5'-GTAAAACGACGGCCAGTG-3') or M13R –27 (5'-GGAAACAGCTATGACCATG-3'), and the arbitrary primer ARB2 (5'-GGC CAC GCG TCG ACT AGT AC-3'). The following thermocycler conditions were used for the 2^{nd} round (nested): (30 s at 94°C, 30 s at 45°C, 1 min at 72°C) X 30, 5 min at 72°C , and 4°C forever. Amplified product was electrophoresed in a 1% agarose (w/v) gel with TAE buffer. Bands differing from the wild-type bands were excised as described above and saved for sequencing. Alternatively, second round product was purified and directly sequenced.

Gene sequencing. The sequence of DNA flanking the miniTn5 was obtained by using M13 F (-20 or -47) or M13 R (-27 or -60) primers for rescue cloned product and M13F (-20) or M13R (-27) for arbitrary PCR product. Dye terminator cycle sequencing using a DYEnamic ET Terminator Cycle Sequencing Kit (Amersham

Biosciences) was performed using the conditions recommended by the manufacturer. Product was analyzed on an ABI377 automated sequencer at the University of New Hampshire Hubbard Center for Genome Studies. The DNA sequences were compared to database sequences using a BLAST program in the NCBI database (http://www.ncbi.nlm.nih.gov/BLAST/) and/or the *Photorhabdus luminescens* TT01 genome database available at http://genolist.pasteur.fr/PhotoList/.

RESULTS

Transposon mutagenesis of *P. temperata*. A genetic approach was initiated to understand the role of motility in the life cycle of *Photorhabdus*. The plasmid, pUB394, was used to deliver a mini-Tn5 into *P. temperata* NC19 to construct a mutant library. To help reduce sibling mutations, the mutant bank was generated from 5 different batches of transpositions and about 500 to 2800 mutants were isolated in each batch. The resulting library consisted of 10,176 transposon mutants. The frequency of transposition defined as the ratio of cells resistant to kanamycin and sucrose to total cells present was 5.4×10^{-8}. Southern analysis of 7 randomly chosen mutants confirmed single, random mutations of the mini-Tn5 (Fig 2).

Screening and isolation of mutants. The transposon mutants were screened for mutants defective in motility by the use of the swim- or swarm-migration plate assays. A total of 86 mutants (Table 1) altered in motility were identified as altered in motility and were classified into 5 different phenotypic groups. First, 23 non-motile mutants were identified on swim medium by the absence of ring

formation (Fig 3A). These mutants formed a compact colony on this medium and did not form swim rings even after 72 h incubations.

A second class of mutants was identified that exhibited aberrant motility. These mutants were motile, but had defective or aberrant ring formation (Fig 2B). Thus, the swim ring was smaller and/or oddly-shaped. There were 12 mutants classified as this phenotype.

A third class of mutants were identified as hyperswimmers (Fig 3C). These 12 mutants formed swim rings noticeably earlier than the parental wild-type (Fig 2C).

P. temperata requires additional NaCl or KCl for optimal swimming motility (Chapter 2; Hodgson *et al.,* 2003). In the absence of this additional salt, the cells are non-motile because they do not produce flagella. A fourth class of mutants were isolated on swim media lacking additional salt (Fig 3D). Six mutants were isolated that were able to form swim rings in this swim medium.

The last motility mutant group consisted of 33 mutants with altered swarming behavior. In another study (Chapter 3) *Photorhabdus* cells swarmed optimally on complex media with 0.45% agar; swarming was reduced at higher concentrations of agar (above 1%). When the mutant library was screened for motility on swarm medium containing 1% agar, 33 mutants were identified as hyperswarmers. These mutants swarmed faster and formed swarm rings larger than the parental wild-types. All of the hyperswarmers formed unique patterns on these swarm plates (Fig 3E).

Physiological properties of the motility mutants. All five classes of mutants were retested for motility under three different conditions (Table 2). As expected, all of the non-motile mutants were non-motile in both swim media and on swarm media. Aberrant mutants showed reduced swim ring formation with NaCl and were non-motile without NaCl. The only exception was mutant P31, B12 which formed a small swim ring without NaCl. All of these mutants were also swarming defective. Hyperswimming mutants showed increased swim ring and four also had increased swarm ring formation (P2, H12; P10,D9; P7, E1; and P75, A4). However, all the hyperswimming mutants did not form swim rings in medium without NaCl. Hyperswarmer mutants displayed increased swarm ring formation that was 4 to 10-fold greater than the parental wild-type. These mutants also exhibited wild-type levels or greater of swim ring formation in the presence of additional NaCl. In the absence of NaCl, these mutants could be subdivided into 2 mutant groups: motile or non-motile. The mutants able to swim without NaCl swam as well as the wild-type with NaCl, with two mutants that were also hyperswarmers (P1,4 and P4,9).

Several phenotypic properties of the motility mutants and their parental strains were investigated (Table 3). In general, no overall patterns were observed for each mutant classification. Approximately 25 of the 86 motility mutants were altered in their production of extracellular enzymes. Four mutants (P35, B12, P61, A3, P5, 11 and P13,7) had profiles similar to wild-type secondary-phase cells showing altered pigment production and reduced levels of extracellular products. The down-regulation of numerous phenotypes suggests mutations in

global regulatory elements. Alternatively, these mutants could have converted to secondary-phase cells.

Antibiotic production was the most altered phenotype of the motility mutants, with 22 mutants being up-regulated or down-regulated. Three mutants (non-motile P72,C6; hyperswarmers, P11,A6 and P83,B10) had almost 2-fold increases in the zone of inhibition compared to the wild-type. Protease activity was also up-regulated or down-regulated in 5 mutants, while DNAse activity was only altered in one mutant (P39, E6) with a 2-fold increase in activity compared to the wild-type. Several mutants had reduced hemolysin production; none had increased hemolysin activity. Current studies are directed toward developing more quantitative methods of measuring enzyme expression.

Several mutants have reduced pathogenicity. We were interested in determining if motility plays a role in insect pathogenesis. Changes in virulence of the motility mutants were tested *in vivo* by injecting *Galleria mellonella* larvae with bacteria. Fig 4 demonstrates that four of the motility mutants had dramatically reduced virulence. The wild-type primary-phase cells will kill all of the larva injected by 48 h (LT_{100}) and require 35 h for an LT_{50}. While all of the mutants tested were lethal, these four mutants showed delayed responses. Two mutants (non-motile P88, D7 and aberrant mutant P13,7) were 16-18 h slower than the wild-type, while the hyperswarming mutants (P82, B9 and P12, A1) were 15 h slower.

Genetic analysis. The mini-Tn5 insertion sites of several mutants were identified (Table 5). Not surprisingly, two non-motile mutants (P4,27 and P59,G8) had

insertions in the structural genes *flgE* and *flgK*, whose products are part of the flagellar hook. Two mutants of the "swim without NaCl" class were identified. Mutant P6,22 had an insertion into a gene that had 90% identity to *P. luminescens plu3723*. This gene is a *luxR* homologue, a transcriptional regulator. This result suggests that this gene may be influenced by NaCl and regulating the flagellar gene cascade in *P. temperata*. The other mutant, P4,9, had an insertion in a gene that has 94% identity with plu3421, which has an unknown function. This gene does show similarity to lambda tail fiber assembly protein G.

Three mutations in the hyperswarming group were identified. One mutant, P82,69, had 80% identity to *plu1133*, a gene with unknown function. The other two hyperswarmers had increased antibiotic production (Table 3) and had interesting insertions. The site in mutant P57, G9 showed 80% identity with *plu3263*, known to be involved in antibiotic production, while P11, A6 showed 87% identity with *E. coli hnr* (*rssB* or *sprE*), a two-component regulator. The aberrant motility mutant had an insertion in a gene that has 91% identity with *E. coli yidA*.

DISCUSSION

Five phenotypic groups of motility mutants were isolated: non-motile, hyperswimming, hyperswarming, mutants capable of swimming without NaCl, and aberrant swimmers. We selected several mutants from each phenotypic group for gene sequencing of the mini-Tn5 insertion site. Two of the non-motile mutants (P4,27 and P59,G8) were identified as *flgE* and *flgK* mutants, respectively. Both of these gene products are structural components of flagella.

The most important observation regarding these mutants, is that though both were isolated as "non-swimmers", they were subsequently shown to be non-swarming. This indicates that, at least structurally, swimming and swarming motility may share the same components. Other evidence to support this idea, is that hyperswimmers were generally hyperswarmers and vice versa. We investigate swarming behavior further in a separate chapter (Chapter 4; Michaels and Tisa, to be submitted).

Motility genes high in the flagellation cascade have been shown to control virulence genes. For example, both *Proteus* (Allison *et al.*,1992) and *Bacillus* (Senesi *et al.*, 2002) co-express hemolysin and flagellar genes. To determine if motility is coupled to virulence expression, all motility mutants were tested for concurrent changes in the production of their virulence enzymes. While the profile of these traits varied from mutant to mutant, it is an important observation that nearly 1/3 had changes in expression of at least one virulence factor. Hemolysin, protease, DNase, and antibiotic production were all altered in at least one mutant, with antibiotic production appearing to be the most affected. One hyperswarming mutant (P57,G9) with increased antibiotic production was identified as a *plu3263* mutation. Though the identity of this gene product is currently unknown, one known function associated with *plu3263* is antibiotic synthesis. A second hyperswarming mutant, P11,A6, also displayed increased antibiotic production. P11,A6 possessed an *hnr* deletion, a two-component response regulator, which according to the EcoCyc *E.coli* database (Keseler *et al.*, 2005), is synonymous with *sprE, ychL*, and *rssB*. Interestingly, in *Serratia*

marcescens, a deletion in *rssB* caused the same hyperswarming phenotype (Wei *et al.*, 2005) we observed in *Photorhabdus.*

The motility mutants were tested for changes in insect virulence by *in vivo* caterpillar experiments. Four motility mutants, including P82,B9 (hyperswarmer); P12,A1 (hyperswarmer); P88,D7 (non-motile); and P13,7 (aberrant motility) had reduced pathogenicity. A non-motile mutant (P88, D7) had the most exaggerated delay in lethal time, with an LT_{50} 18 hours slower than the parental wild-type. P13,7; P82, B9; and P12, A1 all had LT_{50} values approximately 15 h slower. The gene deletion in P13,7 was identified as *yidA*, whose gene product has an unknown function. YidA may be controlling an important global regulatory element in *Photorhabdus*, since P13,7 not only possessed inhibited motility, but also attenuated insect virulence, and a reduction in all virulence enzymes typical of the parental wild-type 1° phase variant. Most likely motility itself does not play a direct role in virulence, since, for example, all the non-motile mutants were still able to kill the insects. More likely, after molecular investigations of these four mutants (with attenuated virulence), we will find that motility gene expression is coupled to some toxin or virulence genes. Screening for mutants with increased virulence will prove difficult since it has been reported that as few as 5 bacterial cells are lethal to the insect. A more useful approach to investigate the role of motility in the life cycle will be to use infection studies, mimicking their natural life cycle. Axenic nematodes will be inoculated with motility mutants and allowed to infect an insect. In this manner, the motility mutants will be tested for their ability

to travel between the nematode and insect, and for their ability to colonize each host.

Further analysis of the motility mutant genes revealed that a mutant capable of swimming without NaCl (P6,22) was a *plu3723* mutant. This gene is homologous to a DNA-dependent transcriptional regulator, *luxR.* The function of LuxR regulators is very diverse and differs immensely from organism to organism. Our *luxR (plu3723)* mutant is able to swim without NaCl unlike the wild-type. This suggests that in *Photorhabdus*, LuxR directly or indirectly senses NaCl levels, and activates the flagellation cascade when NaCl is present. Alternatively, Plu3723 may be negatively regulating motility when NaCl is not present. We further investigate the effect of NaCl on gene regulation in a separate chapter (Chapter 5; Michaels and Tisa, to be submitted).

Flagellation cascades are quite complex; the regulation and organization vary from one bacterium species to another. From genomic work (Duchaud *et al.,* 2003 and ffrench-Constant *et al.,* 2000), we now know that *Photorhabdus* motility apparatus share the most homology with the *Enterobacteriaceae* cascade. This cascade consists of three classes of motility genes with the master operon at the top of the cascade is *flhDC.* This operon, depending on the organism, can be regulated by pH, temperature, osmolarity, and various other environmental factors either directly, or indirectly through other regulatory proteins. (For a review of flagellation cascades see Soutourina and Bertin, 2003). To determine how NaCl is affecting the motility cascade in *Photorhabdus*, further work will focus on understanding how *rssB (hnr)* is regulating swarming behavior and the

importance of the genes *yidA* and *plu3263*. Here we have successfully generated and characterized a diverse group of motility mutants for further study in understanding the ecological role of motility in the complex life cycle of a bacterium that is both an insect pathogen and nematode symbiont.

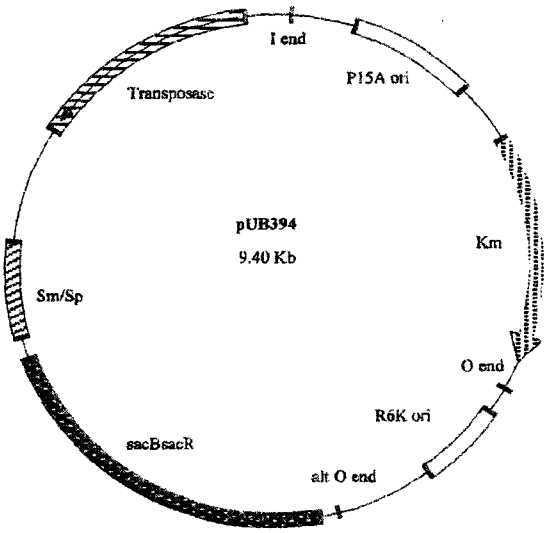


Figure 1. Genetic map of the mini-Tn5 delivery vector pUB394. This figure is courtesy of Ciche, *et al.,* (2001) J. Bact. 183:3120.

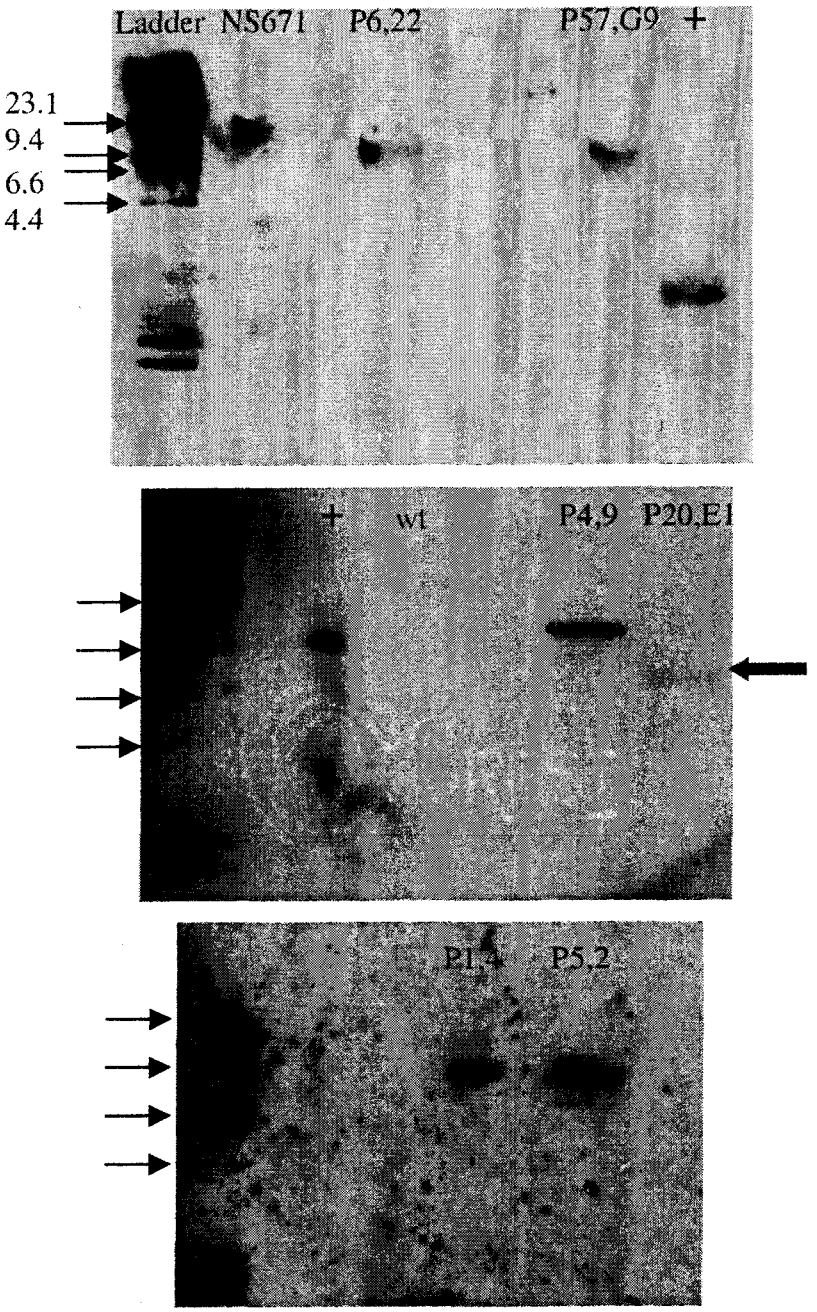


Figure 2. Southern blot of mutant gDNA hybridized to a DIG-labeled mini-Tn5 probe confirmed insertion of the mini-Tn5 into mutant gDNA. DNA (10 ug) was NsiI digested, separated on a 1% w/v agarose gel in TAE buffer, and electrophoresed to a nylon membrane at 10V for 30 min. Positive control was the original mini-Tn5 delivery vector. Negative control was parental wild-type gDNA.

Table 1. A total of 86 mutants altered in motility were isolated from the transposant library and categorized into 5 different phenotypic groups.

Mutant Phenotype (quantity):	Mutant ID:			
Non-swimmer (23)	P4, 27	P12,18	P39, C1	P26, E6
	P41, G10	P5, G7	P20, F12	P84, H2
	P7, F6	P39, E6	P76, H11	P4, C8
	P86, G6	P26, E8	P87, G4	P59, G8
	P84, B1	P90, G1	P3, C8	P30, B7
	P88, D7	P72, C6	P59, D11	
Swim w/o NaCl (6)	P1, 4	P4, 9	P6, 22	P30, H5
	P56, C3	P80, G4		
Hyperswimmer (12)	P5, 2	P62, H6	P7, E1	P35, B12
	P2, H12	P10, D9	P8, D3	P32, G7
	P93, A4	P75, A4	P35, C12	P83, A2
Inhibited swimmer (12)	P5, 11	P13, 7	P31, B7	P64, D5
	P43, B3	P47, F1	P58, C8	P28, A10
	P84, E4	P88, D8	P31, B12	P64, C3
Hyperswarmer (33)	P14, G2	P7, A8	P80, G7	P63, H9
	P8, C2	P90,G8	P5, A7	P61, A3
	P12, A1	P82, A5	P7, A11	P57, G9
	P11, A2	P82, G9	P83, B10	P64, B10
	P2, A9	P81, H7	P81, G7	P78, C2
	P82, B9	P81, G6	P83, A9	P83, H5
	P81, H8	P81, H5	P8, H6	P20, D5
	P11, A6	P38, B11	P46, G10	P50, A6
	P38, F2			

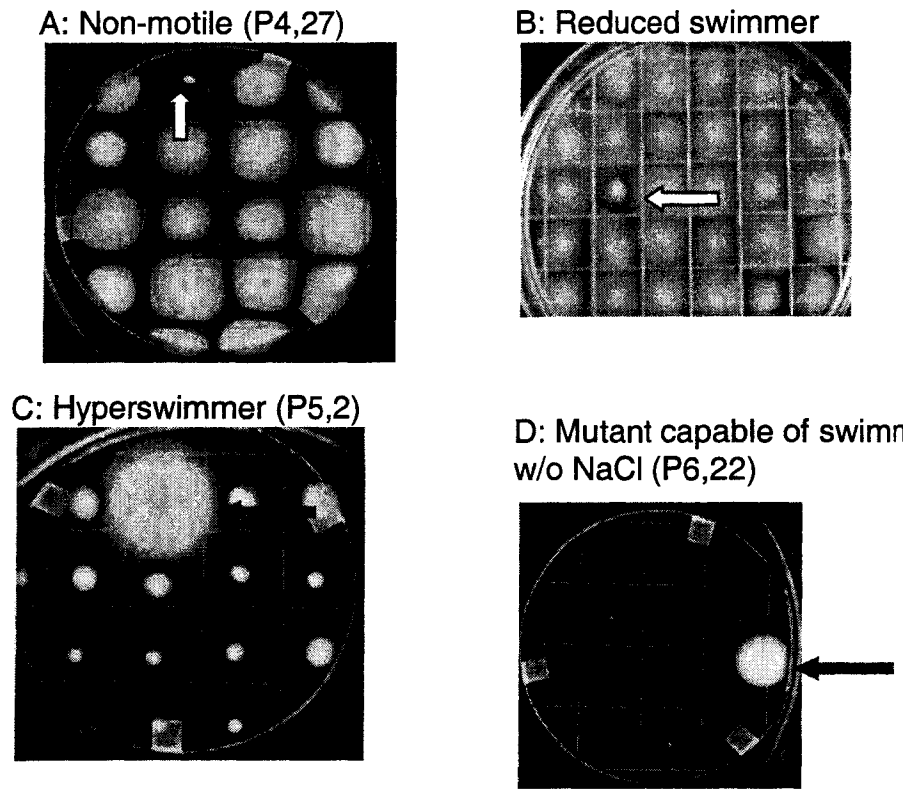


Figure 3. Pictures of typical isolated motility mutants. Cells from an overnight broth culture (2 ul) were used as an inoculum. Constituents of swim (0.25% w/v agar) and swarm medium (1.25% w/v agar) is described in Materials and Methods. All motility plates were incubated at 28°C for 24 h. Oversized swim plates used for screening mutants (containing multiple inocula) have arrows indicating a non-motile mutant (3A), a mutant with inhibited motility (3B), a hyperswimming mutant (3C) and a mutant capable of swimming without NaCl (3D; this swim plate contained no NaCl. Fig 3E demonstrates the unique patterns of four different hyperswarming mutants on individually inoculated swarm plates.

Table 2. Screening of all mutants for their ability to swim (in 0.25% w/v agar swim media) with NaCl and without NaCl, and to swarm on 1.25% (w/v) agar swarm plates. Growth conditions and media constituents are described in Materials and Methods. From overnight cultures, 2 ul was used for inoculation of motility plates, which were incubated at 28°C. Measurements (mm) of swim or swarm ring diameters were taken at 48 h. The maximum measurement is 100 mm, limited by the width of the petri dish. The parental wild-type is shown as a reference. All non-swimmers and inhibited swimmers were unable to swarm. Many of the hyperswarmers were robust swimmers with or without NaCl.

Mutant:	Isolated Phenotype:	Swim +NaCl:	Swim -NaCl:	Swarm:
1°wt	Swimmer	35	3	10
P4, 27	Non-motile	3	4	10
P12, 18	Non-motile	3	3	10
P5, G7	Non-motile	3	3	10
P3, C8	Non-motile	3	3	11
P4, C8	Non-motile	3	3	11
P41, G10	Non-motile	3	3	8
P39, E6	Non-motile	3	3	11
P39, C1	Non-motile	4	3	10
P59, D11	Non-motile	3	7	8
P59, G8	Non-motile	3	5	9
P7, F6	Non-motile	3	3	10
P26, E8	Non-motile	3	3	8
P20, F12	Non-motile	3	3	9
P26, E6	Non-motile	4	4	7
P30, B7	Non-motile	4	4	9
P86, G6	Non-motile	5	6	9
P90, G1	Non-motile	4	6	8
P76, H11	Non-motile	4	7	11
P84, H2	Non-motile	4	3	10
P88, D7	Non-motile	4	2	11
P84, B1	Non-motile	6	5	10
P72,C6	Non-motile	4	4	8
P87, G4	Non-motile	4	4	8
P1, 4	Swim w/o NaCl	47	24	30
P4, 9	Swim w/o NaCl	34	24	47
P6, 22	Swim w/o NaCl	38	27	13
P30, H5	Swim w/o NaCl	27	27	11
P56, C3	Swim w/o NaCl	30	28	8
P80, G4	Swim w/o NaCl	37	45	15
P5, 2	Hyperswimmer	45	8	14
P2, H12	Hyperswimmer	47	5	50
P62, H6	Hyperswimmer	45	3	10

Table 2 (cont.)

Mutant:	Isolated Phenotype:	Swim +NaCl:	Swim -NaCl:	Swarm:
P10, D9	Hyperswimmer	45	5	38
P7, E1	Hyperswimmer	44	3	25
P8, D3	Hyperswimmer	42	3	12
P35, C12	Hyperswimmer	49	3	12
P35, B12	Hyperswimmer	47	3	10
P32, G7	Hyperswimmer	47	3	10
P83, A2	Hyperswimmer	42	4	10
P93, A4	Hyperswimmer	41	3	15
P75, A4	Hyperswimmer	52	7	32
P5, 11	Inhibited Swimmer	8	2	10
P13, 7	Inhibited Swimmer	11	2	7
P31, B7	Inhibited Swimmer	18	3	9
P31, B12	Inhibited Swimmer	11	10	12
P28, A10	Inhibited Swimmer	22	7	11
P43, B3	Inhibited Swimmer	5	3	8
P47, F1	Inhibited Swimmer	5	3	8
P58, C8	Inhibited Swimmer	5	5	10
P64, D5	Inhibited Swimmer	10	3	15
P64, C3	Inhibited Swimmer	18	7	12
P84, E4	Inhibited Swimmer	22	6	15
P88, D8	Inhibited Swimmer	12	4	8
P7, A11	Hyperswarmer	24	5	50
P14, G2	Hyperswarmer	24	20	63
P8, C2	Hyperswarmer	27	9	69
P12, A1	Hyperswarmer	30	12	35
P11, A2	Hyperswarmer	44	12	100
P2, A9	Hyperswarmer	24	20	40
P7, A8	Hyperswarmer	30	11	47
P5, A7	Hyperswarmer	36	6	57
P8, H6	Hyperswarmer	35	19	100
P11, A6	Hyperswarmer	40	30	100
P38, B11	Hyperswarmer	26	100	100
P46, G10	Hyperswarmer	40	100	100
P20, D5	Hyperswarmer	45	20	100
P50, A6	Hyperswarmer	30	7	100
P38, F2	Hyperswarmer	18	6	45
P63, H9	Hyperswarmer	29	100	57
P61, A3	Hyperswarmer	20	7	36
P57, G9	Hyperswarmer	29	7	100
P64, B10	Hyperswarmer	32	9	100
P78, C2	Hyperswarmer	49	50	100
P83, H5	Hyperswarmer	27	10	70
P83, A9	Hyperswarmer	50	12	63
P83, B10	Hyperswarmer	47	50	100

Table 2 (cont.)

Mutant:	Isolated Phenotype:	Swim +NaCl:	Swim -NaCl:	Swarm:
P81, G7	Hyperswarmer	30	10	70
P81, G6	Hyperswarmer	30	5	100
P81, H5	Hyperswarmer	50	50	30
P81, H8	Hyperswarmer	54	22	100
P82, B9	Hyperswarmer	45	100	100
P90,G8	Hyperswarmer	23	6	26
P82, A5	Hyperswarmer	27	3	100
P82, G9	Hyperswarmer	40	14	100
P81, H7	Hyperswarmer	33	8	68
P80, G7	Hyperswarmer	35	25	55

Table 3. Phenotypes of *P. temperata* mutants and their parental wild-types. Hemolysis, protease, and DNase activities were determined by measuring the size of the halo (mm) surrounding the bacterial colony 48 h after inoculation. Dye absorption on EMB and MacConkey plates were noted at 48 h. A positive result on EMB plates was indicated by metallic green colonies, and a negative result by dull purple colonies. A positive result on MacConkey plates was indicated by bright red colonies and a negative result by colorless or pink colonies. Antibiotic production was determined by measuring the zone of inhibition (mm) surrounding the bacterial colony after 24 h incubation with the tester bacterium *M. luteus*. All plates were incubated at 28°C. Growth conditions for cultures and constituents of media are described in Materials and Methods.

Strain:	Pigment on PP3:	Hemolysis:	Protease:	DNase:	EMB:	MacConkey:	Antibiotics:
1°wt	Dark	2	4	3	+	+	4
2°wt	Light	0	3	2	-	-	0
Non-motile:							
P4, 27	Dark	2	4	1.5	+	+	5
P12, 18	Dark	1	3	1.5	+	+	4
P5, G7	Dark	2	3	1.5	+	+	5
P3, C8	Dark	2	3	3	+	+	3
P4, C8	Dark	2	3	3	+	+	4
P41, G10	Dark	2	3	3	+	+	4
P39, E6	Dark	2	4	6	+	+	4
P39, C1	Dark	2	4	3	+	+	3
P59, D11	Dark	2	4	4	+	+	6
P59, G8	Dark	2	4	1.5	+	+	5
P7, F6	Dark	2	4	3	+	+	5
P26, E8	Dark	2	2.5	3	+	+	5
P20, F12	Dark	0	4	3	+	+	5
P26, E6	Dark	0	4	3	+	+	2
P30, B7	Dark	2	4	1.5	+	+	4
P86, G6	Dark	2	5	1.5	+	+	6
P90, G1	Dark	2	4	1.5	+	+	4

Table 3 (cont.)

Strain:	Pigment on PP3:	Hemolysis:	Protease:	DNase:	EMB:	MacConkey:	Antibiotics:
P76, H11	Dark	2	5	1.5	+	+	4
P84, H2	Dark	2	3	4	+	+	4
P88, D7	Dark	2	4	1.5	+	+	6
P84, B1	Dark	2	4	2	+	+	4
P72,C6	Dark	2	5	2	+	+	7
P87, G4	Dark	2	3	2	+	+	4
Swim w/o NaCl:							
P1, 4	Dark	2	4	3	+	+	4
P4, 9	Dark	2	4	3	+	+	5
P6, 22	Dark	1	3	1.5	+	+	3
P30, H5	Dark	1	4	3	+	+	5
P56, C3	Dark	1	4	3	+	+	4
P80, G4	Light	0	9	3	-	-	0
Hyperswimmer:							
P5, 2	Dark	2	3	3	+	+	5
P2, H12	Dark	0	2	2	-	-/+	3
P62, H6	Dark	2	4	3	+	+	4
P10, D9	Dark	1	4	3	+	+	4
P7, E1	Dark	1	4	3	+	+	4
P8, D3	Dark	2	5	3	+	+	5
P35, C12	Dark	2	5	3	+	+	4
P35, B12	Light	0	0	0	-/+	-	0
P32, G7	Dark	2	4	3	+	+	4
P83, A2	Dark	2	3	3	+	+	5
P93, A4	Dark	2	3	1.5	+	+	4
P75, A4	Dark	2	3	1.5	+	+	5

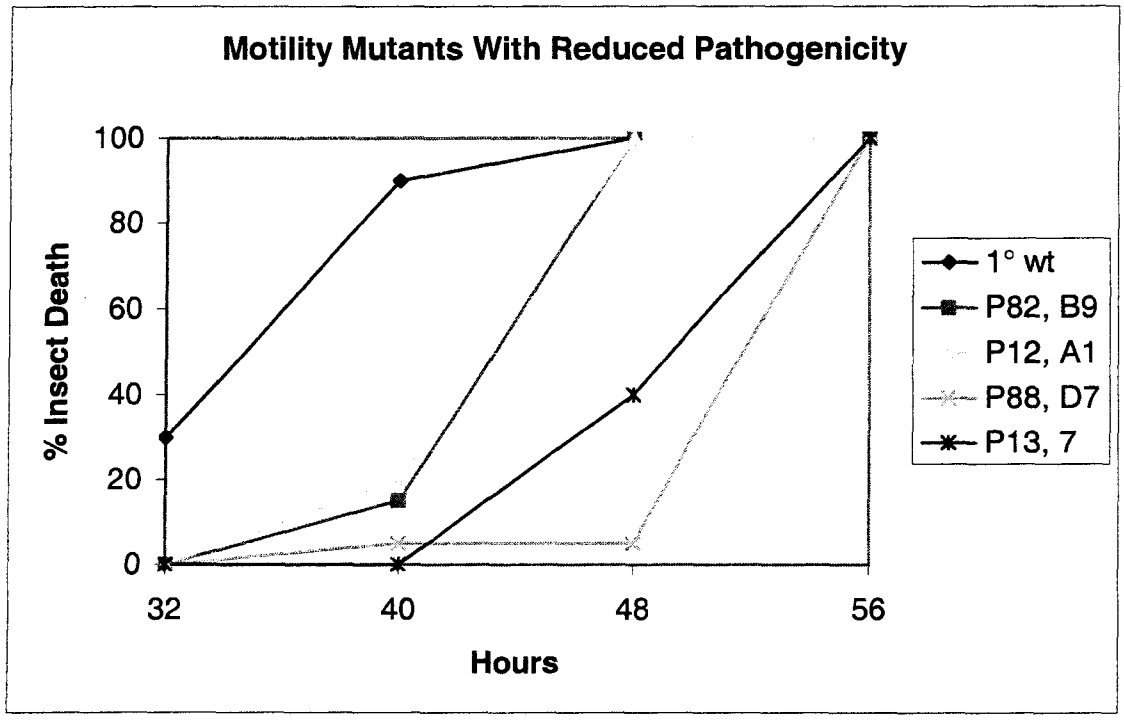
Table 3 (cont.)

Strain:	Pigment on PP3:	Hemolysis:	Protease:	DNase:	EMB:	MacConkey:	Antibiotics:
Reduced swimmer:							
P5, 11	Light	0	2	3	-/+	-	0
P13, 7	Light	0	0	4	-	-	0
P31, B7	Dark	2	4	3	+	+	5
P31, B12	Dark	2	3	3	+	+	4
P28, A10	Dark	2	5	3	+	+	1
P43, B3	Dark	2	5	3	+	+	4
P47, F1	Dark	2	4	3	+	+	4
P58, C8	Dark	2	4	3	+	+	4
P64, D5	Dark	2	4	3	-	-	2
P64, C3	Dark	2	4	3	-	-	2
P84, E4	Dark	2	4	1.5	+	+	1
P88, D8	Dark	2	3	4	-/+	-/+	1
Hyperswarmer:							
P7, A11	Dark	2	4	3	+	+	4
P14, G2	Dark	2	3	3	+	+	5
P8, C2	Dark	2	5	1.5	+	+	5
P12, A1	Dark	2	4	1.5	+	+	6
P11, A2	Dark	2	5	2	+	+	3
P2, A9	Dark	2	3	2	+	+	3
P7, A8	Dark	2	3	2	+	+	2
P5, A7	Light	2	4	2	+	+	3
P8, H6	Dark	2	5	1.5	+	+	5
P11, A6	Light	2	3	4	+	+	7
P38, B11	Dark	2	3	1.5	+	+	4
P46, G10	Dark	2	5	1.5	+	+	5
P20, D5	Dark	1	4	3	+	+	4

Table 3 (cont.)

Strain:	Pigment on PP3:	Hemolysis:	Protease:	DNase:	EMB:	MacConkey:	Antibiotics:
P50, A6	Dark	2	5	1.5	+	+	6
P38, F2	Dark	2	3	1.5	+	+	4
P63, H9	Dark	2	4	3	+	+	2
P61, A3	Light	1	2.5	2	-	-	0
P57, G9	Dark	2	4	1.5	+	+	6
P64, B10	Dark	2	5	1.5	+	+	6
P78, C2	Dark	2	5	2	+	+	4
P83, H5	Dark	2	4	4	+	+	4
P83, A9	Dark	2	3	1.5	+	+	5
P83, B10	Dark	2	5	1.5	+	+	7
P81, G7	Dark	2	3	1.5	+	+	0
P81, G6	Dark	2	4	1.5	+	+	2
P81, H5	Dark	2	5	1.5	+	+	4
P81, H8	Dark	2	3	1.5	+	+	3
P82, B9	Dark	2	4	1.5	+	+	4
P90,G8	Dark	2	4	1.5	+	+	4
P82, A5	Dark	2	2	1	+	+	4
P82, G9	Dark	2	4	1.5	+	+	5
P81, H7	Dark	2	5	1.5	+	+	4
P80, G7	Dark	2	5	1.5	+	+	5

Figure 4. *In vivo* pathogenicity assays, using *G. mellonella* larvae, revealed four motility mutants with reduced insect virulence. Larva (20) were injected per test mutant with approximately 280 bacterial cells, and repeated 3X. Larva were monitored for death every 8 h. LT_{50} is defined as the time (h) required for 50% of the injected larva to die, and LT_{50} for 100% larval death.



	Phenotype	LT_{50}	LT_{100}
1° wt	wild-type	35	48
P82, B9	hyperswarmer	43	48
P12, A1	hyperswarmer, increased antibiotics	43	48
P88, D7	Non-motile, increased antibiotics	52	56
P13, 7	inhibited motility, all 1° traits down-regulated	50	56

Table 4. Identification of mini-Tn5 insertion sites in motility mutants. M13F or R primers were used to identity the site of genomic insertion by mini-Tn5 in motility mutants. Identity of the genes were acquired by comparing the DNA sequences to database sequences using the BLAST program in the NCBI database (http://www.ncbi.nlm.nih.gov/BLAST/) and/or the *Photorhabdus luminescens* TT01 genome database available at http://genolist.pasteur.fr/PhotoList/.

Mutant:	Gene:	% Identity:	BLAST Score:	E-value:	Other altered phenotypic traits:
Non motile					
P4,27	*flgE,* flagellar hook protein	65/74 (87%)	75.8	5e-15	None
P59, G8	*flgK,* flagellar hook associated protein (HAP1)	78/90 (87%)	69.9	8e-9	None
Swim w/o NaCl					
P6,22	*plu3723, luxR* homologue	60/67 (90%)	71.9	1e-12	None
P4, 9	*plu3421*, Unknown; some similarity to lambda tail fiber assembly protein G	169/179 (94%)	280	4e-72	None
Hyperswarmer					
P11,A6	*E. coli hnr (rssB);* Two component response regulator	145/166 (87%)	159	5e-39	Increased antibiotic production
P57,G9	*plu3263,* Unknown; involved in antibiotic synthesis; transmembrane protein	246/306 (80%)	119	4e-27	Increased antibiotic production
P82, G9	*plu1133,* Unknown; weakly similar to prophage protein or transmembrane protein	84/105 (80%)	65.9	5e-8	None
Aberrant motility					
P13,7	*E.coli yidA,* Hydrolase/phosphatase, function unknown	162/178 (91%)	232	6e-58	Attenuated insect virulence and 1° traits

CHAPTER IV

PHYSIOLOGICAL CHARACTERIZATION OF SWARMING BEHAVIOR

PREFACE

In Chapter III, hyperswarming mutants were generated through transposon mutagenesis. While swimming behavior was investigated in Chapter II, swarming behavior by *Photorhabdus* had not been fully characterized. In order to understand the significance of the hyperswarming mutants, it was essential to characterize swarming behavior by the parental wild-type in more detail. The specific goals in this chapter were to:

1. Define swarming behavior in the parental wild-type
2. Compare swarming behavior to swimming behavior
3. Compare hyperswarming mutant characteristics to the parental wild-type

Physiological Characterization of Swarming Behavior by *Photorhabdus temperata*

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To Be Submitted

ABSTRACT

***Photorhabdus temperata*, an insect pathogen and nematode symbiont, is motile in liquid medium by swimming. We found that *P. temperata* was capable of surface movement termed swarming behavior. Several lines of evidence indicated that *P. temperata* uses the same flagella for both swimming and swarming motility. Both motility types required additional NaCl or KCl, had the presence of peritrichous flagella, and had the same flagellin as detected by immunoblotting experiments. Mutants defective in flagellar structural protein were non-motile for both motility types, indicating that the same flagellin protein is involved. Unlike swimming, we observed swarming behavior to be a social form of movement in which the cells coordinately formed intricate channels covering a surface. The constituents of the swarm media affected motility: swarming was optimal on low agar concentrations, and as agar concentrations increased, swarm ring diameters decreased. In Chapter III, several hyperswarming mutants were generated using random transposon mutagenesis that were also hyperswimmers. Additionally, all non-swimming mutants that were isolated were unable to swarm; further evidence that swarming and swimming flagella are the same.**

INTRODUCTION

Photorhabdus spp. are bacterial symbionts of soil dwelling entomopathogenic nematodes in the *Heterorhabditidae* family (Thomas and Poinar, 1979). These bioluminescent bacteria have a complex life cycle in which they play a dual role as nematode symbiont and insect pathogen. (For recent reviews see Kim and Forst, 2005; Owuama 2001; and Forst *et al.,* 1997.) The nematodes carry their symbiotic bacteria throughout their gut, and actively seek insect prey in the soil. The nematodes infect a wide range of insect hosts by entering through natural openings or by burying directly through the insect cuticle. The nematodes release the bacteria into the insect hemolymph, which release an array of extracellular virulence factors such as toxins (Bowen, 1998; Bowen and Ensign, 1998; ffrench-Constant and Bowen, 2000), proteases (Schmidt *et al.*, 1988), lipases (Wang and Dowds, 1993), and hemolysins (Brillard, *et al.* 2001). In addition, the bacteria release antibiotics to prevent secondary invaders and putrefaction of the insect carcass (Akhurst, 1982). After several days of feeding, the nematodes and bacteria reassociate and leave in search of a new insect host.

Due to the complex life cycle of *Photorhabdus*, motility may play an important role in the colonization of either its insect prey or nematode host, or in transportation of the bacteria from one host to another. There are several forms of bacterial motility, including swimming (movement through liquid) and swarming (movement on surfaces). We have previously investigated *Photorhabdus*

swimming behavior (Hodgson *et al.,* 2003; Chapter 2). These cells swim by means of peritrichous flagella and several environmental factors control this type of motility. Swarming is an important mode of group surface movement, which allows bacteria to quickly colonize an abiotic or biotic surface. (For a review see Harshey, 2003.) Some bacteria, such as *Escherichia coli* and *Salmonella typhimurium*, utilize the same flagellar systems for both swimming and swarming motility. Others, such as *Vibrio parahaemolyticus*, switch from polar flagella for swimming to peritrichous flagella for swarming. (For a review on regulation of motility see Soutourina and Bertin, 2003). Here we examine swarming behavior by *Photorhabdus* in detail for the first time.

MATERIALS AND METHODS

Strains and growth conditions. *Photorhabdus temperata* [formerly *Xenorhabdus luminescens* (Boemare *et al*, 1993; Fischer-Le Saux *et al*, 1999)] strain NC19 (ATCC 29304) was used. Cells were grown and maintained at 28°C in LB medium consisting of 1% Bacto-tryptone (Difco), 0.5% yeast extract (Difco) and 0.5% NaCl, or in Proteose Peptone 3 (PP3) medium consisting of 1% PP3 (Difco) and 0.5% NaCl. When appropriate, kanamycin was added to a final concentration of 25 ug/ml.

Growth Curves. Overnight broth (PP3) cultures were inoculated 1:50 into fresh PP3 medium warmed to 28°C. The cultures were incubated at 28°C and growth was monitored hourly with a Klett-Summerson Photoelectric Colorimeter (Klett Mfg. Inc., NY).

Transposon Mutagenesis. *P. temperata* cells were mutagenized by mini-Tn5 as described previously (Chapter 3; Michaels and Tisa, to be submitted).

Chemotaxis and motility assays. The swim migration assay was performed as described previously (Chapter 2; Hodgson *et al.*, 2003).

For the swarm migration assay, swarm plates (2% w/v PP3, 0.5% yeast extract, 0.5% NaCl, and 0.65% agar, unless otherwise noted) were inoculated by spotting 2 ul of an overnight culture (approximately 10^6 cells) onto the surface of the agar medium and incubated at 28° C for 24-48 h. Each measurement was performed in triplicate and each experiment was repeated at least twice.

Microscopy. For phase-contrast microscopy, cells were observed on slide cultures with an Olympus BH-2 phase contrast microscope (Olympus Microscopes, Japan) at 100X and 400X magnification. Slide cultures were prepared by placing 1 ml of swarm media on the surface of a glass microscope slide. After the medium solidified, 2 ul of an overnight culture was inoculated onto the agar surface and the slides were incubated at 28° overnight.

Transmission Electron Microscopy (TEM) was performed as described previously (Chapter 2; Hodgson *et al.*, 2003). Cells were scraped from the surface of a 24 h swarm plates, place on a Formvar-coated copper grid, and negatively stained with 1% phosphotungstic acid. Samples were viewed at 5000X to 16000X magnification on a Carl Zeiss/LEO922 Omega transmission electron microscope (Leo Electron Microscopy, Inc., NY).

Immunoblots. Flagellin protein from *P. temperata* NC19 primary-phase swimming cells was isolated and purified as described by Sprott *et al.* (1994).

The purified, filter sterilized flagellin protein was injected into Bal b/c mice. Injections of approximately 70-140 ng protein per mouse were made at three different intervals, 5 days apart. Polyclonal antiserum was collected and tested against whole motile cells, as well as purified flagellin, by the Enzyme-Linked Immunosorbent Assay (ELISA) (Engvall and Perlmann,1971).

For the immunoblotting experiments, cells were harvested directly from either swim or swarm migration plates. The cells were suspended in Laemmli sample buffer (6X buffer: 7 ml 4X TrisCl/SDS, pH 6.8; 3 ml glycerol; 1 g SDS; 0.93 g DTT; 1.2 mg bromophenol blue; dH_20 up to 10 ml) and solubilized by boiling for 10 min. The solubilized proteins were separated by SDS-PAGE (sodium lauryl sulfate polyacrylamide gel electrophoresis) as described by Laemmli (1970). Either an 8-16% Express pre-cast polyacrylamide mini gel (ENEMate, UT) or a 10% polyacrylamide Life Gel (Life Therapeutics, Australia) was used as a matrix and was electrophoresed at 70V. To visualize the proteins, the gel was stained with Coomassie brilliant blue. Gels for immunoblotting were performed by electrophoretically transferred to a nitrocellulose membrane (BioRad Trans Blot Nitrocellulose Transfer Medium; BioRad, CA) as described by Towbin *et al.* (1979). Proteins were transferred overnight using a Hoefer TE Series Transphor Electrophoresis Unit (Hoefer Scientific Instruments, CA) at 50 mAmp in transfer buffer (4.56 g ethanolamine, 43.2 glycine, 2400 ml dH_2O, 600 ml 20% methanol, pH 9.6). After the transfer was complete, the membrane was incubated in 5% blotto (5% w/v dry milk in PBS; 1X PBS: 80 g NaCl, 2.2 g KCl, 9.9 g Na_2HPO_4, 2 g K_2HPO_4, dH_2O to 1L, pH 7.4) for 1 h at 37°C. This treatment

blocked unoccupied sites in the nitrocellulose membrane. After blocking, the membrane was incubated with the primary antibody, mouse anti-flagella polyclonal serum (produced as described above) at a dilution of 1:4 in blotto for 1 h at 37°C. The membrane was washed 3X for 10 min each with fresh changes of blotto at 37°C. To detect the antigen-antibody complex, the membrane was then incubated for 1 h at 37°C with a secondary antibody, goat anti-mouse IgG conjugated with peroxidase (Pierce Biotechnology, IL) at a titer of 1:1000 or 1:500. After washing 3X for 10 min each with fresh blotto, detection buffer (10 mg 4-chloro-1-napthol, 3 ml methanol, 17 ml PBS) and 20 ul 30% H_2O_2 was used to visualize bands.

RESULTS

Swarming properties of the parental wild-type.

During our previous study on swimming behavior (Chapter 2; Hodgson *et al.,* 2003), we observed that *P. temperata* exhibited swarming movement on media containing 1% agar. The plate migration assay was used to test the effect of agar concentrations on cell swarming. Swarming was optimal on PP3 medium with low agar concentrations. As agar concentrations increased, the ability of the bacteria to swarm decreased, as indicated by smaller swarm rings (Fig 1A). Even at agar concentrations as low as 0.45% (w/v), cells were found on the surface of the plate rather than swimming within the medium. This was determined by gently scraping the cells from the surface of the plate and observing that no cells were embedded in the agar (Fig 1B). At higher concentrations of agar, the swarm ring edges were more lobed, while at lower concentrations they appeared more

filamentous. Swarm rings almost always possessed a "fried egg" appearance with a dark yellow center, tapering to paler pigment on the edges. The most dramatic change in swarming patterns was observed between 0.85 and 1.05% (Fig 1A).

Similar to swimming motility (Chapter 2; Hodgson *et al.,* 2003), swarming motility required additional NaCl (Fig 2A and 2B). A range of three different salt concentrations were tested (Fig 3). The salt requirement for swarming was not specific for Na+. Both NaCl and KCl allowed almost equivalent motility and swarm ring formation was optimal at 75-100 mM for both salts. $MgCl_2$ was not as effective as NaCl or KCl. All three salts inhibited motility at elevated levels (>100 mM). These characteristics were identical to those observed with swimming behavior (Chapter 2; Hodgson *et al.*, 2003), suggesting swimming and swarming operate by the same system.

Swarming and swimming cells use the same flagella.

TEM of swarming cells revealed the presence of peritrichous flagella, which appeared similar to those observed on swimming cells (Fig 4 and 5C). The number of flagella present were similar in both swimming and swarming cells. Since both swimming and swarming motility also required additional NaCl or KCl, we predicted that both motility systems used the same flagella. To test that hypotheisss, the prescne of the flagellin protein was detected by immunoblotting (Fig 6). Both swimming and swarmin cells produced flagellin protein. Furthermore, mutants defective in FlgE and FlgK hook protein are nonmotile for both types of motility (Chapter 3; Michaels and Tisa, to be submitted). These

data indicate that swarming and swimming motility share the same structural genes.

Swarming cells show coordinated movement.

Though swimming and swarming motility may share some motility apparatus, direct observations of swarm plates with a phase contrast microscope revealed that, unlike swimming, swarming showed coordinated group movement (Fig 5). At low magnification (100X, Fig 5A), the swarming cells formed streaming appendages that migrated forward at the tips. The migrating tips eventually connected with another appendage. At higher magnification (400X, Fig 5B; 5000X, Fig 5C), these appendages were shown to consist of swarming cells grouped into rafts, aligned along their long axises. Some cells appeared to be elongated compared to the parental wild-type. The cells moved only forward and backward, coordinately forming channels that continuously connected until the surface of the plate was covered. Swarming cells may be chemotactic, though further investigations are required. When a gradient swarm plate was inoculated with bacteria, they preferentially swarmed towards the 2% peptone half and away from the half containing no PP3 (Fig 7).

Physiological properties of hyperswarming mutants.

As part of a larger study to generate a library of motility mutants (Chapter 3; Michaels and Tisa, to be submitted), several hyperswarming mutants were isolated that swarmed at an accelerated rate compared to the parental wild-type. Many of these mutants formed unique swarming patterns (Fig 8). Since wild-type swarming behavior was inhibited at high agar concentrations, hyperswarming

mutants were isolated on 1.25% agar swarm plates (Chapter 3; Michaels and Tisa, to be submitted). The hyperswarming mutants were further characterized in this study. When motility was tested on swarm plates containing 0.65% agar, the mutants covered the surface of the plate in less than 36 h, while the parental wild-type took 48 h (Fig 9). The most rapid change in swarm ring diameter occurred between 24 and 36 h. The ability of these mutants to swarm faster was not due to an increased growth rate (Fig 10).

The effect of agar concentrations on swarming by the hyperswarming mutants was tested. Many of the mutants required different amounts of agar (and thus surface tension) in the swarm medium to induce swarming. For example, P83, B10 began swarming at 0.65% (w/v) agar and swarmed up to 1.25%, while P12, A1 would only swarm at 0.85% and 1.05% (Fig 11). Since these phenotypes are not identical, many of the hyperswarmers probably contain different mutations.

DISCUSSION

Swimming motility is generally characterized as an individual endeavor of a bacterial cell through liquid or semi-solid media. Swarming is viewed as a social or group movement of cells over solid surfaces. Some bacteria, including *Vibrio parahaemolyticus*, possess two different flagella for these two types of movement, switching from polar flagella for swimming to peritrichous flagella for swarming motility (McCarter, 1995). Organisms that display this mixed sort of flagellation may harbor one regulatory cascade, or two distinct systems. Most enterobacteriaceae, *Escherichia coli* included, possess the *flhDC* cascade, and

express peritrichous flagella for both forms of motility. (For a review of flagellar regulation see Soutourina and Bertin, 2003.) To date, genomic studies have confirmed that *Photorhabdus* possesses the *flhDC* cascade, and their motility genes are most homologous to *E. coli* and *S. typhimurium* genes (ffrench-Constant *et al.*, 2000 and Duchaud *et al.*, 2003). This study was executed to discern what flagellar systems exist for swimming and swarming motility in *Photorhabdus temperata.*

We have provided evidence that *P. temperata,* like *E. coli,* uses the same flagella for both types of motility. Microscopy revealed that numerous peritrichous flagella were present for both swimming and swarming motility. NaCl or KCl was required for both swimming and swarming indicating that the two types of motility are under the same regulation. Immunoblots confirmed that the same flagellin protein is involved in both types of motility. Previously, we demonstrated that non-swimming mutants are unable to swarm, and hyperswarming mutants are also hyperswimming (Chapter 3; Michaels and Tisa, to be submitted). These data collectively support the idea that there is overlap between swimming and swarming regulation.

While the two forms of motility may share some components, *Photorhabdus* swarming behavior was distinctly different compared to swimming. We observed that swarming behavior was a form of coordinated group movement. Cells were aligned in rafts along their long axis, with individual cells moving only forward and backward. The movement appeared to be highly coordinated since the rafts of cells moved only in one direction. Intricate channels

were formed by a group effort until all the channels eventually joined, covering the surface of the swarm plates. Quorum sensing regulation of swarming in a diverse range of bacteria has been observed (for a review see Daniels *et al.*, 2004). This idea should be investigated to elucidate whether quorum sensing coordinates this social movement in *Photorhabdus.*

In many bacteria, swarming cells differ from swimming cells by being hyperflagellated and elongated. Typically this change in cell morphology is more striking in bacteria that swarm at high agar concentrations of 1-2%. We report that swarming by *Photorhabdus* is optimal on lower concentrations of agar. This is most similar to *E. coli* and *S. typhimurium*, which cannot swarm above 1% (for a review of swarming see Harshey, 2003). We did not observe any radical cell differentiation in *Photorhabdus.* Some cells appeared elongated, but this idea deserves further experimentation. In addition, EM should be repeated to discern if there are any differences in either the quantity or length of flagella in swarming vs. swimming cells.

The hyperswarming mutants in this study were isolated for their ability to swarm over 1.25% w/v agar plates, which the parental wild-type is unable to do. At 0.65% w/v agar, a concentration at which the parental wild-type can swarm efficiently, the hyperswarmers were able to cover the swarm plates approximately 12 h sooner. Thus they are not only able to swarm on more solid surfaces, but they can swarm faster. Growth curves proved that the faster motility of the mutants was not due to a faster growth rate. Previously, we identified two genes responsible for hyperswarming phenotypes (Chapter 3; Michaels and Tisa,

to be submitted). One of the mutants, P11, A6 had an *hnr* (or *rssB*) deletion. This gene is known to control swarming behavior in *Serratia marcescens* (Wei *et al.*, 2005). The other hyperswarming (P57,G9), possessed a *plu3263* deletion, whose function is unknown, but is hypothesized to be involved with antibiotic production. Interestingly, this mutant (as well as P11, A6) may have up-regulated antibiotic production (Chapter 3; Michaels and Tisa, to be submitted), but future experimentation is required. Current studies are also directed toward understanding the NaCl requirement for motility and the significance of NaCl in the life cycle.

The ability of *Photorhabdus* to swarm could provide a rapid and coordinated colonization of either nematode host or insect body, or in traveling from one host to another. In the future we will use a genetic approach to dissect the components required for swarming and swimming behavior, and utilize *in vivo* experiments to discern if motility is important for symbiosis or pathogenicity.

Figure 1. Effect of agar concentrations on swarming by *P. temperata* NC19.

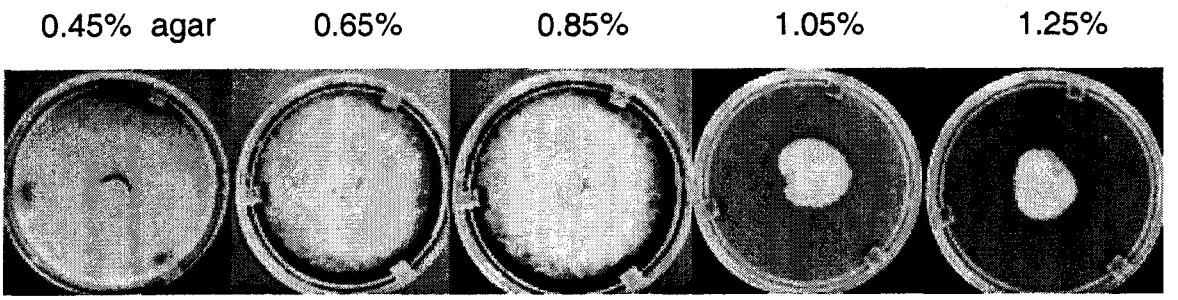


Figure 1A. Swarm ring diameters decreased as agar concentrations increased. *P. temperata* NC19 cells were inoculated (2 ul of overnight culture) onto the surface of PP3 swarm plates containing varying agar concentrations (w/v) and incubated at 28°C for 48 h.

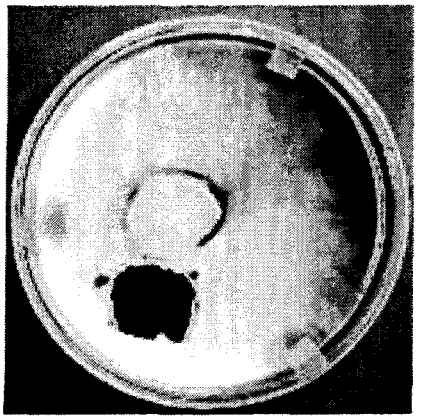


Figure 1B. Even at an agar concentration of 0.45%, the swarming cells were found on the surface of the plate rather than being embedded in the agar. This was demonstrated by gently scraping the cells from the surface of the plate, and observing that no additional cells were left.

Figure 2. Effect of NaCl on swarming by *P. temperata* NC19

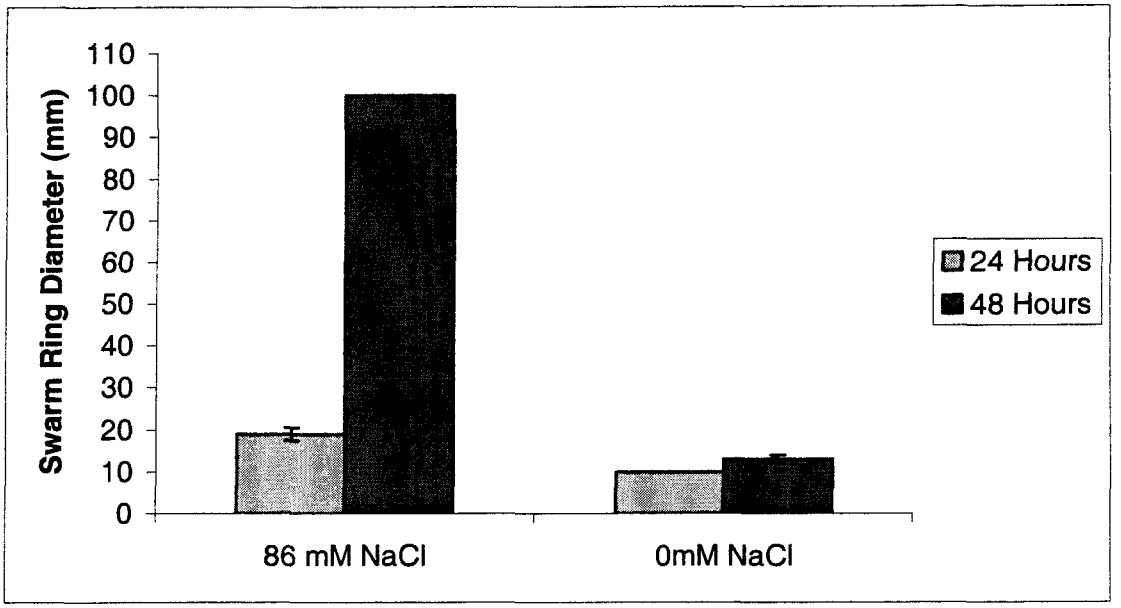


Figure 2A. *P. temperata* swarming required the presence of NaCl in PP3 swarm medium. Swarming was determined by the swarm-migration plate assay as described in Material and Methods. Plates were incubated at 28°C. Values are the average of 5 measurements. The maximum measurement was 100 mm (the width of the petri dish).

Figure 2B. *Photorhabdus* was unable to swarm without at least 50 mM NaCl. *P. temperata* NC19 cells were inoculated (2 ul of overnight culture) onto the surface of PP3 swarm plates (constituents described in Materials and Methods) without additional NaCl.

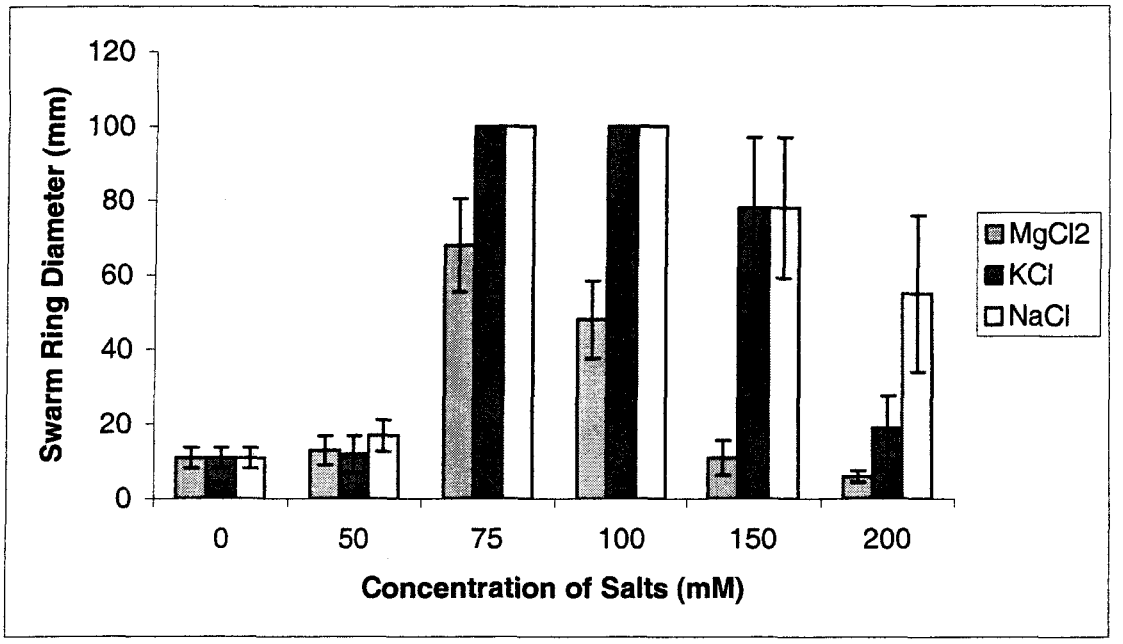


Figure 3. The effect of salt concentrations on swarm ring formation by *P. temperata* NC19. PP3 swarm plates (0.65% w/v agar) containing different concentrations of NaCl, KCl, and $MgCl_2$ were incubated at 28°C for 48 h. Values are the average of 3 measurements. The maximum value is 100 mm (the width of the petri dish).

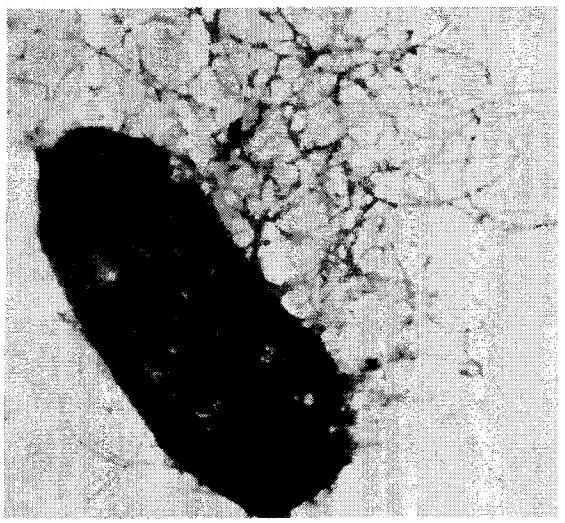


Figure 4. Transmission electron microcopy of an individual swarm cell (25,000X) stained with 1% phosphotungstic acid.

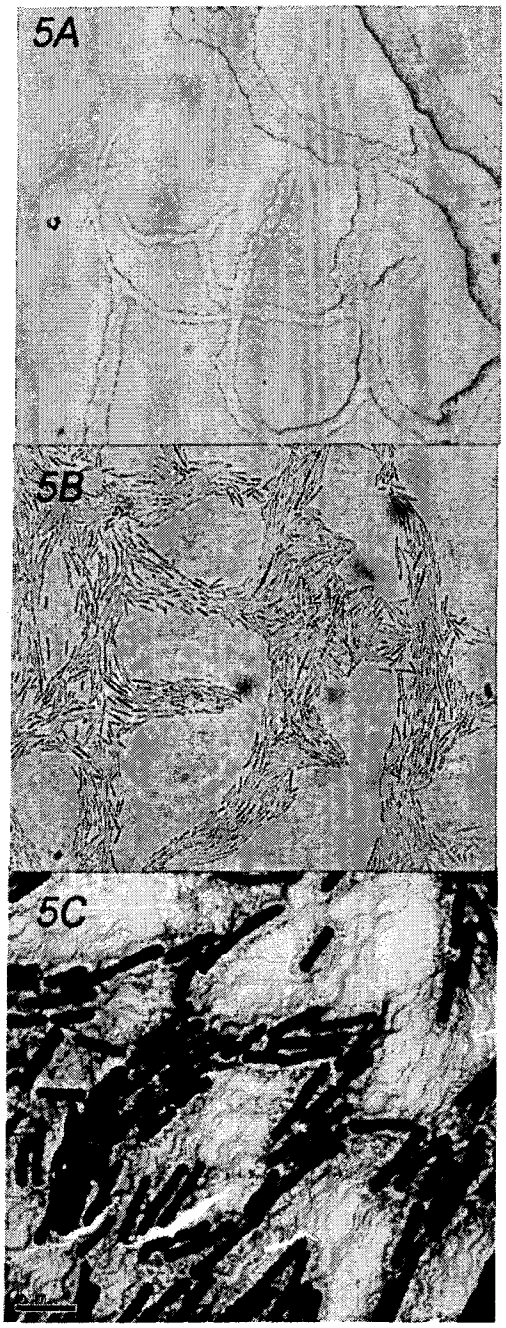


Figure 5. Microscopy of swarming cells. Swarming cells showed coordinated movement. (A) Phase contrast microscopy at 100X magnification of a slide culture of *P. temperata* NC19. (B) Phase contrast microscopy at 400X. (C) TEM at 5000X of cells scraped from swarm plate. Bar represents 5 um.

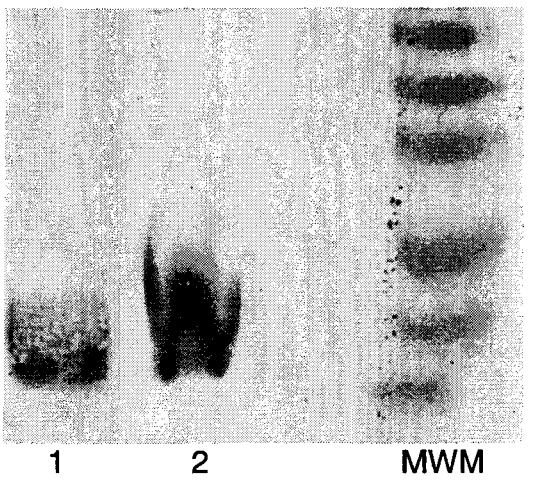
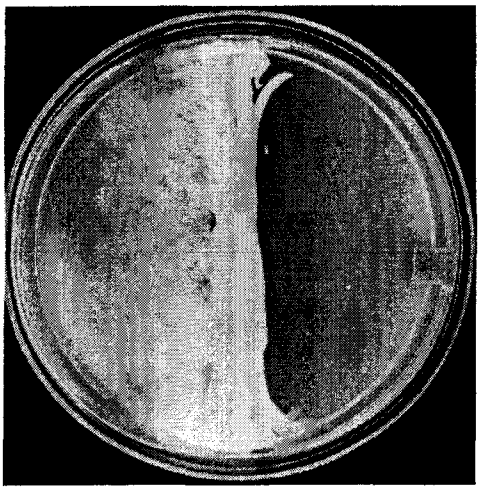


Figure 6. Immunodetection of flagellin protein. Cells were harvested from swim and swarm migration plates. The solubilized proteins were separated with SDS-PAGE and detected by immunoblotting as described in Materials and Methods. Both swimming (Lane 1) and swarming (Lane 2) cells produced a positive result against the anti-swimming flagellin indicating that they are the same protein. MWM bands from top to bottom are: 120, 84, 60, 39.2, 28, and 18.3 kDa).



2% PP3 No PP3

Figure 7. Swarming cells of *P. temperata* were chemotactic. Swarm plate (0.65% w/v agar, 0.5% yeast extract, and 0.5% NaCl) containing 2% PP3 on the left half, and no PP3 side on the right half (sides were poured separately and allowed to cool in between). *P. temperata* cells from an overnight culture were streaked in a single line down the center of the plate and incubated at 28°C for 48 h.

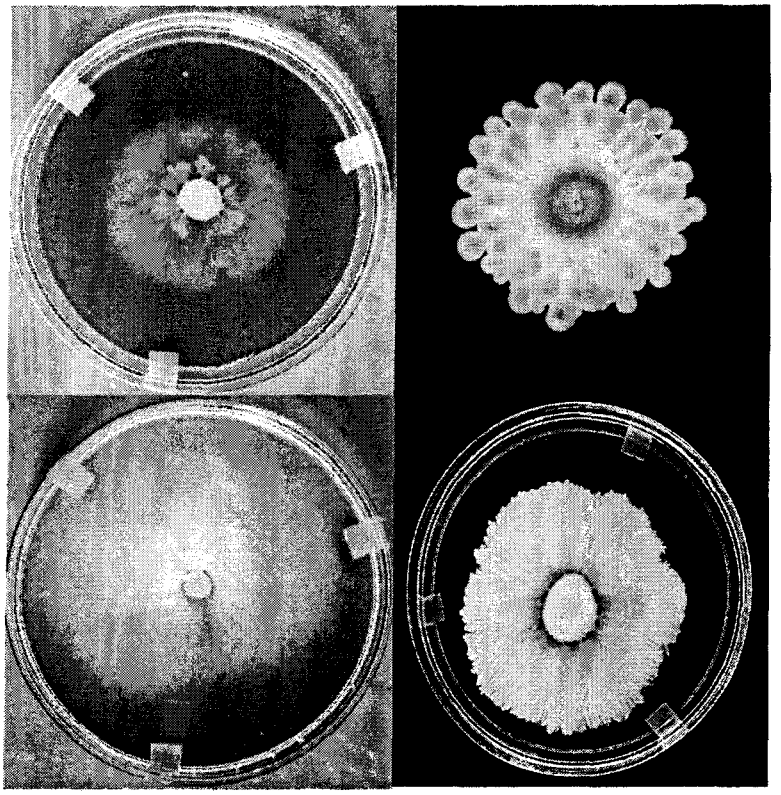


Figure 8. Hyperswarming mutants formed unique swarming patterns. Cells from an overnight broth culture (2 ul) were inoculated onto the surface of PP3 swarm plates (1.25% w/v agar) and incubated at 28°C for 24 h.

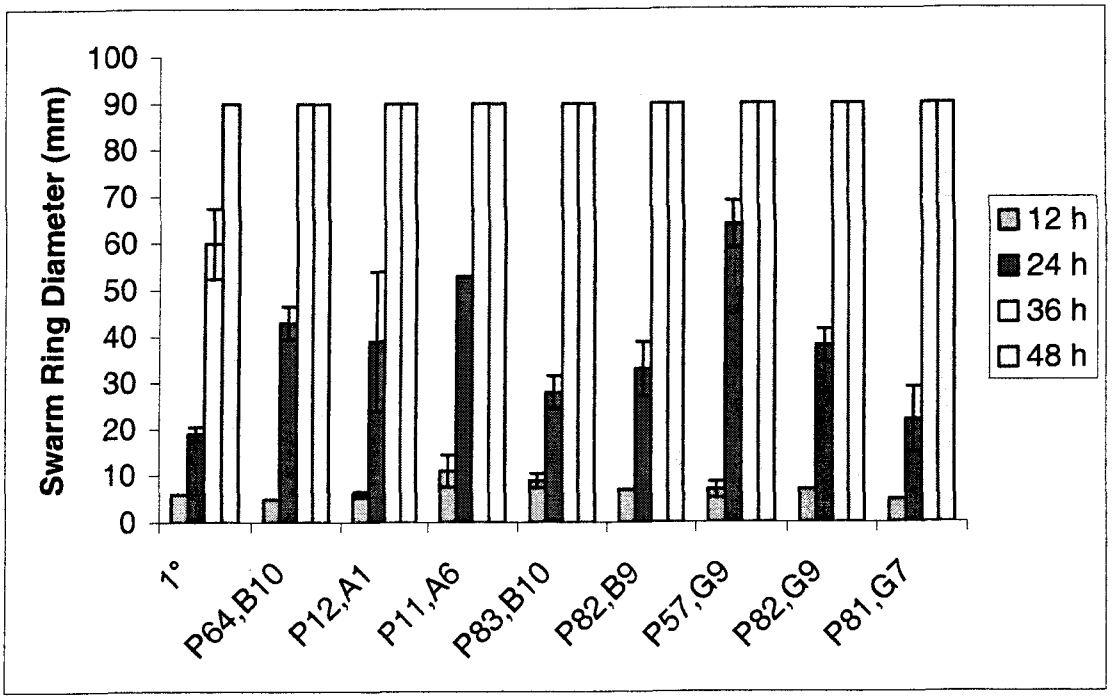


Figure 9. Swarm rates of hyperswarming mutants compared to the wild-type. Hyperswarming mutants reached maximum coverage of the swarm plate (100 mm) approximately 12 h sooner than the parental wild-type. For this experiment, PP3 swarm plates (0.65% agar w/v) were inoculated with 2 ul of cells from an overnight culture and incubated at 28°C. Values are the average of 4 plate measurements. The maximum measurement was 100 mm (the width of the petri dish).

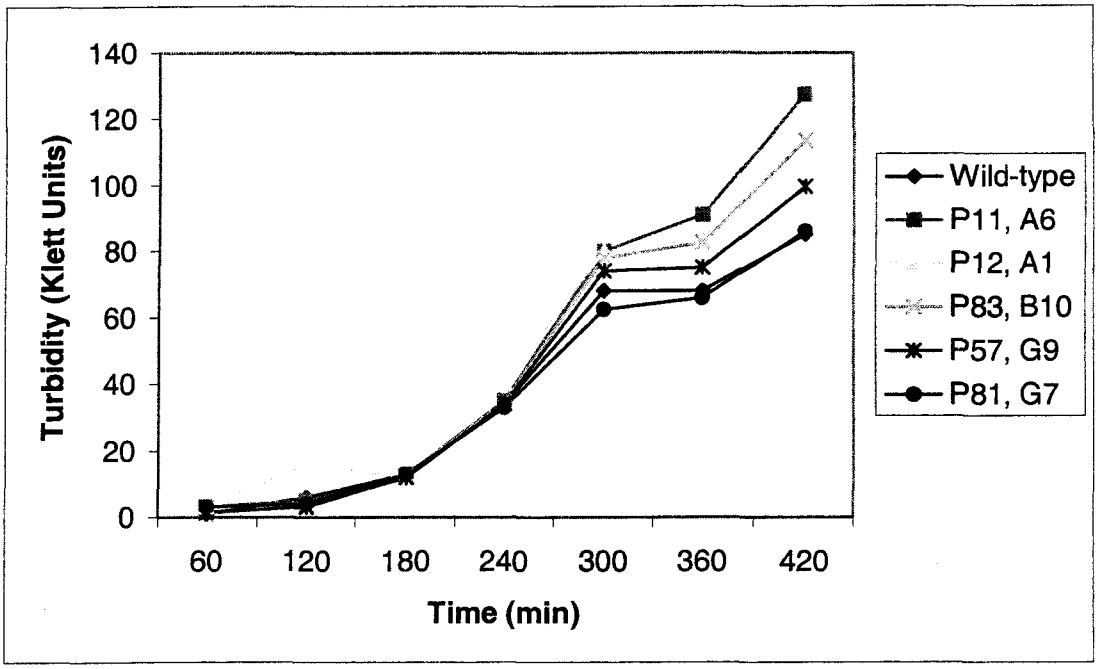


Figure 10. Hyperswarming mutants had the same growth rate as the parental wild-type. Growth curve was determined as described in Materials and Methods.

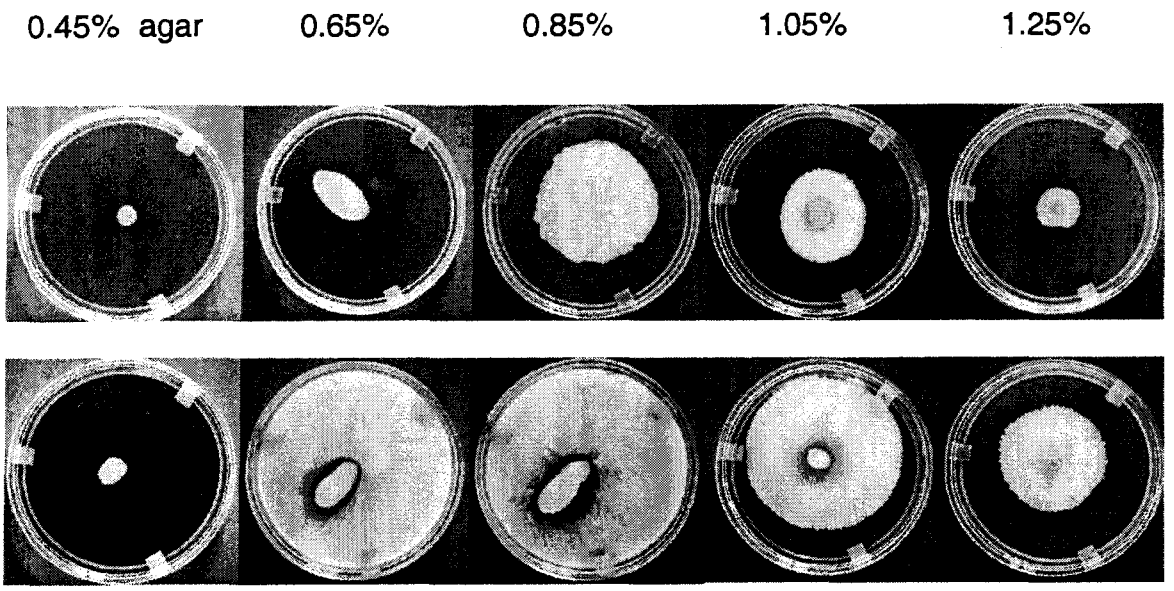


Figure 11. The effect of agar concentrations on swarming by hyperswarming mutants. Many of the mutants required different amounts of agar (and thus surface tension) in the swarm medium to induce swarming. Two examples are shown here. Mutant P12, A1 (top row) swarmed robustly only at 0.85% agar (w/v) and 1.05%. However, mutant P83, B10 (bottom row) began swarming at 0.65% (w/v) agar and swarmed up to 1.25%.

CHAPTER V

EFFECT OF NACL AND KCL ON GLOBAL GENE EXPRESSION AND THE MOTLITY REGULON

PREFACE

One of the many important observations made thus far is that NaCl or KCl is essential for both swimming and swarming motility by *Photorhabdus* (Chapters II and IV). It is not clear how these ionic salts regulate motility, nor their physiological relevance. The purpose of this section was to use global gene expression studies to elucidate potential mechanisms of regulation.

NaCl and KCl Regulate Motility at the Post-Transcriptional Level in *Photorhabdus* spp.

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ABSTRACT

Photorhabdus **spp. are bacterial insect pathogens symbiotically associated with nematodes of the** ***Heterorhabditidae*** **family. These bacteria are motile by swimming and swarming, but require the presence of NaCl or KCl for both types of motility. We used microarray analysis to examine the effect of these ionic salts on global gene regulation, and particularly on the motility regulon. Of the 4683 represented genes included on the array, 93 were significantly up-regulated in the presence of NaCl. These genes were categorized into four physiological functional groups: metabolism, macromolecule synthesis or transport, environmental stress responses, and unknown function. Of these 93, roughly half (43) were also significantly up-regulated in the presence of KCl. Surprisingly, both the +NaCl/No salt and +KCl/No salt gene expression profiles for motility genes were unchanged, suggesting that NaCl and KCl do not regulate motility at the gene expression level. RT-PCR confirmed the microarray data. Immunoblotting experiments indicated that flagellin protein was not produced in the absence of NaCl. These data indicate that ionic salts must regulate motility genes post-transcriptionally in** ***Photorhabdus spp.***

INTRODUCTION

Entomopathogenic nematodes of the *Heterorhabditidae* family are insecticidal due to the presence of their symbiotic bacteria, *Photorhabdus spp.*, which are carried throughout the nematode gut. (For reviews see: Burnell and Stock, 2000; Forst *et al.*, 1997; and Forst and Nealson, 1996). Once inside an insect, the nematodes release the bacteria, which in turn release an array of extracellular virulence enzymes and insect toxins. After several days of feeding, the nematodes and bacteria reassociate and leave in search of a new insect host. Although the exact reason is unknown, *Photorhabdus* is essential for normal nematode development.

As *Photorhabdus* switches roles from beneficial symbiont to lethal pathogen, the bacteria are exposed to several different environments: nematode gut, insect hemocoel, and possibly soil. This lifestyle subjects the bacteria to changes in osmolarity, nutrient levels, and host defense mechanisms. These bacteria must possess the ability to respond to these changes within their complex life cycle. We hypothesize that the motility of *Photorhabdus* may aid the bacteria in traveling from host to host and/or colonization, and is most likely highly regulated and coordinated. *Photorhabdus spp.* are motile by both swimming and swarming motility Interestingly, the bacteria are completely non-motile without the presence of NaCl or KCl (Hodgson et al., 2003; Chapter 2; and Michaels and Tisa, to be submitted; Chapter 4). To understand this ionic salt

requirement for bacterial motility, we have taken a genomic approach utilizing DNA microarray technology.

MATERIALS AND METHODS

Strains. *Photorhabdus luminescens* subsp. *laumondii* strain *TT01* (CIP 105565) was utilized in the microarray experiments. For all other experiments, *Photorhabdus temperata* strain *NC19* (ATCC 29304) was used in addition to strain TT01.

Growth and maintenance of cultures. Bacterial cells were grown and maintained at as described previously (Hodgson *et al.*, 2003; Chapter 2).

Growth conditions for DNA microarrays. *P. luminescens TT01* was streaked on to an LB plate from a -80°C glycerol-preserved stock. After a 48 h incubation, a single colony was used to inoculate LB medium (1% w/v tryptone and 0.5% yeast extract) with or without 86 mM (0.5% w/v) NaCl and the cultures were incubated overnight at 28°C. The overnight cultures were inoculated (1:50) into their respective fresh LB medium and incubated at 28°C until the cultures reached exponential phase (approximately 5-6 h and an OD_{600} of 0.5). The cells were harvested for RNA isolation as described below. The RNA preparations from 3 different cultures were pooled for each sample for +/- NaCl. Each microarray assayed used a pool RNA preparation from different cultures.

RNA isolation. Prior to cell harvesting, Qiagen RNA Protect Reagent (Qiagen Sciences, CA) was added to the 4.5 ml exponential phase cultures. After mixing, the samples were incubated at room temperature for 5 min. Cells were harvested

by centrifugation at 10,000 X g for 10 min. To lyse the cells, pellets were resuspended in 100 ul of lysis solution [1 mg/ml lysozyme in 0.1% DEPC (diethyl pyrocarbonate) treated TE Buffer (1 mM EDTA, 10 mM Tris)] and incubated at room temperature for 5 min. From this step, the Qiagen RNAeasy Mini Kit protocol was followed (Qiagen Sciences, CA) with the exception of the final elution step: 50 ul of RNase-free water was pipetted directly onto column and allowed to sit for 10 min. before eluting RNA. RNA was stored at -80°C.

DNase treatment and purification of RNA. RNA samples were treated with RNase-free DNase to remove residual DNA. DNase reactions were as follows: 50 ul RNA (from above isolation), 10 ul 10X DNase I buffer (Ambion, Inc.,TX) 5U DNase (2U/ul; Ambion, Inc.,TX), 5 ul RNase Out ribonuclease inhibitor (40U/ul; Invitrogen, CA) and 32.5 ul RNase free water (total reaction of 100ul). Samples were incubated at 37°C for 30 min. To inactivate DNase activity, 1 ul RNase-free 0.5M EDTA pH 8.0 (Ambion, Inc., TX) was added to reaction and incubated at 75°C for 10 min.

Following DNase inactivation, RNA was extracted with 1 ml water-saturated nuclease-free phenol (pH < 7.0) using gentle inversion of tube to mix, and harvested by centrifugation at 21,200 x g. Aqueous phase containing RNA was transferred to a new tube and extracted with 1 ml 50:50 v/v phenol:chloroform. After the tube was inverted to mix, the mixture was partitioned by centrifugation at 21,200 x g for 3 min. The aqueous RNA phase was transferred to a new tube and extracted with 1 ml chloroform and mixed by gentle inversion. The aqueous RNA was partitioned by centrifugation at 21,200 x g for 3

min. This chloroform extraction step was repeated. To precipitate and concentrate the RNA, 0.1 volume of DEPC-treated 3 M sodium acetate (pH 5.2) and 2.5 volume of -20°C 100% ethanol were added to the collected supernatant. The mixture was incubated at -80°C overnight and RNA was harvested by centrifugation at 21,200 x g for 25 min. at 4°C. After the supernatant was discarded, 1 ml DEPC treated 80% ethanol was gently added to the pellet and inverted to mix. After the mixture was centrifuged at 21,200 x g for 5 min at 4°C, the supernatant was discarded. The RNA pellet was air dried and resuspended in 30 ul RNase-free water.

Determination of RNA yield and quality. RNA was quantified by measuring the absorbance at 260 nm using a Beckman DU 640 spectrophotometer (Beckman Instruments, Inc., MA). The quality of RNA was tested by separating 5 ug RNA on a 2% (w/v) agarose gel in MOPS buffer (20 mM MOPS, 5 mM NaAcetate, 1 mM EDTA, pH 7.0) containing 0.66 M formaldehyde. Gels were run in 1X MOPS buffer at 70V and stained in ethidium bromide to visualize strong 5S, 16S, and 23S rRNA bands.

PCR was used to determine if RNA was contaminated with DNA. The Hot Start Taq Master Mix Kit (Qiagen Sciences, CA) was used according to the manufacturer's instructions to amplify, if present, 16s rDNA, with the primers listed in Table 1. PCR product was separated on a 1% (w/v) agarose gel in TAE buffer (40 mM Tris-acetate and 1 mM EDTA) run at 70V. The absence of PCR amplicon confirmed the absence of DNA. Control samples included parental wild-type gDNA and a sample with no DNA or RNA.

cDNA synthesis. cDNA synthesis from purified mRNA was performed according to the method provided by NimbleGen Systems Inc. (WI). Frozen RNA samples were thawed and RNase Out (3 ul; 120U) was added to 10 ug RNA to protect against degradation. If necessary, the RNA suspension was dried down in a speed-vac, or RNase-free water was added, to adjust the total volume of RNA to 8 ul before adding RNase Out (RNA + 3 ul RNase Out + water = 12 ul).

To begin cDNA synthesis, 1 ul of random hexamer primers (1.0 ug/ul; Invitrogen, CA) were added to the mixture. The samples were incubated in a thermocycler: 10 min at 70°C, 2 min at 60°C, 2 min at 50°C, 2 min at 40°C, 2 min at 30°C and 10 min at 25°C. When the thermocycler reached 25°C, 42 ul of master mix [3 ul 10 mM dNTPs, 12 ul 5X First Stand Buffer (Invitrogen, CA), 12 ul 0.1 DTT (Invitrogen, CA) and 15 ul DEPC-treated water] was immediately added to the sample. The samples were incubated for several more cycles: 10 min at 25°C, 3 min at 29°C, 3 min at 32°C, 2 min at 37°C, and 55 min at 42°C. When thermocycler reached 42°C, 6 ul Superscript II Reverse Transcriptase (Invitrogen, CA) was immediately added to the reaction mixture, which was then incubated for 55 min at 42°C, followed by 15 min at 72°C, and finally 4°C indefinitely.

RNase treatment and purification of cDNA. The cDNA was RNase treated to remove any residual RNA. The RNase reaction mixture contained 60 ul of the whole cDNA reaction, 1 ul RNase H (2U) (Invitrogen, CA) and 39 ul nuclease free water (for a total reaction volume of 100ul) and was incubated at 37°C for 20 min.

The RNase-treated cDNA was phenol extracted, ethanol precipitated, and quantified according to the NimbleGen procedure. The cDNA was extracted with 100 ul 25:24:1 nuclease-free phenol:chloroform:isoamyl alcohol (saturated with 10 mM Tris-HCl, pH 8.0, 1 mM EDTA) followed by centrifugation at 12,000 x g for 2 min. The aqueous layer containing cDNA was transferred to a clean tube and 1 ul of Pellet Paint NP Co-Precipitant (Novagen/EMD BioSciences, Germany) was added to visualize precipitated cDNA. The cDNA was precipitated by the addition of 0.1 volume 7.5 M ammonium acetate and 2.5 volumes of -20°C absolute ethanol and harvested by centrifugation at 12,000 x g for 20 min. After discarding the supernatant, the cDNA pellet was washed twice with 0.5 ml 80% ethanol (pre-chilled to -20°C) and pelleted by centrifugation at 12,000 x g for 5 min. The cDNA pellet was allowed to air-dry and resuspended in 30 ul of nuclease-free water.

cDNA fragmentation and biotin labeling. A modified version of the NimbleGen procedure was utilized to fragment cDNA into a range of 50-200 bases. The reaction mixture contained 30 ul cDNA, 4 ul 10X DNase buffer, 4 ul nuclease free water, and 2 ul DNase I (0.1 U/ul; diluted fresh in 1X DNase buffer). After the reaction was incubated at 37°C for 13 min, an additional 2 ul DNase I (0.1 U/ul) was added and incubated at 37°C for an additional 4 min. DNase activity was inactivated by incubating at 99°C for 10 min and immediate storage on ice.

The fragmented cDNA was biotin end-labeled by incubating the cDNA with 20 ul 5X Terminal Deoxynucleotidyl Transferase (TDT) Buffer (Promega, WI), 2.5

ul 1 mM Biotin-N6-ddATP (Enzo Life Sciences, NY), 1.7 ul 30U/ul TDT (Promega, WI) and 35.8 ul nuclease-free water at 37°C for 2 h.

Biotin-labeled cDNA was concentrated using Microcon YM-10 Centrifugal Filter Devices (Millipore, MA). The columns were washed with 100 ul nuclease-free water by centrifugation at 14,000 x g. The labeled cDNA was added to the column and centrifuged at 14,000 x g until the volume in the column was reduced to approximately 20 ul. An additional 100 ul nuclease-free water was added on top of the column, and the column was centrifuged until the remaining volume reached 20 ul. This step was repeated. The remaining 20 ul sample was recovered by inverting the spin-filter into a fresh tube and centrifuging at 1000 x g for approximately 6 min or until all liquid was transferred. The concentrated biotin-labeled cDNA was stored at -20°C until ready for shipment to Nimblegen.

Hybridization to microarray chips. The biotin-labeled samples (12 ug in 12 ul) were submitted to NimbleGen for hybridization. A total of 6 samples were submitted: 3 were from cells grown with additional NaCl, and 3 from cells grown with no salt (as described above in "Growth conditions for DNA microarray"). One "no salt" sample did not pass quality control specifications; therefore those results were not included in this study. NimbleGen's DNA microarray chips constructed for *P. temperata TT01* consisted of 4683 genes and 20 probe pairs per gene in duplicate (termed "Block 1" and "Block 2"). Each probe pair consisted of a perfect match (PM) oligonucleotide and a mismatch (MM) oligonucleotide. Gene intensity differences were normalized at NimbleGen by calculating PM minus MM (http://www.nimblegen.com) and subtracting local background signal.

Microarray data analysis. The experimental reproducibility (r^2 values) between blocks within the same array ranged from 0.93 to 0.97 (an example is shown in Fig 1). In this study, the normalized data per condition (with and without NaCl) for all arrays were averaged. The log2 of the +NaCl/ - NaCl ratio was calculated. A two-fold increase or decrease was considered differential gene expression. Thus, the log2 ratio of +NaCl/-NaCl greater or equal to 1.0, and less than or equal to –1.0, are an indication of these increases or decreases, respectively.

Reverse Transcriptase PCR. To confirm the microarray results, mRNA levels were measured by the use of Reverse Transcriptase (RT)-PCR. A Titan One Tube RT-PCR Kit (Roche Molecular Biochemicals, Germany) was utilized for these experiments. RNA (500 ng) isolated from + and – NaCl conditions (as described above) was converted to cDNA, and motility genes of interest were amplified with the primers described in Table 1, according to the manufacturer's instructions. The gene *plu1575* was used as a control, since it was one of the most differentially expressed genes in the microarray data. PCR products (5 ul) were run on a 1% (w/v) agarose gel in TAE buffer (40 mM Tris-acetate, 1 mM EDTA) at 70V and visualized with ethidium bromide.

Immunoblots. For the immunoblotting experiments, strains NC19 and TT01 were harvested directly from swim migration plates or from exponentially grown cultures (to mimic microarray conditions). Overnight cultures grown in LB (with and without NaCl) were used to inoculate swim plates with and without NaCl respectively (1% peptone, 0.5% yeast extract, 0.25% agar and 0.5% NaCl if noted) as described previously (Chapter 2, Hodgson *et al.*, 2003). The swim

plates were incubated at 28°C for 48 h. Cells were directly pipetted from plates and suspended in 4X Laemmli sample buffer as described previously (Chapter 4; Michaels and Tisa, to be submitted). In addition, both strains were grown to exponential phase, with and without additional NaCl (as described above in "Growth Conditions for DNA Microarray"). These cells were also suspended in 4X Laemmli sample buffer. The same immunoblotting procedure was utilized as described previously (Chapter 4; Michaels and Tisa, *to be submitted*) where whole cells were immunoblotted against polyclonal anti-flagella to determine if flagella were produced under +/- NaCl conditions.

RESULTS

DNA microarray. Gene expression levels were compared between cells grown with and without NaCl. Fig 2 shows a scatter plot comparing the mRNA levels of cells grown in the presence or absence of 86 mm NaCl. The $\log_2$ of the +NaCl/ -NaCl ratio was calculated for each gene as described in Materials and Methods. A gene was considered differentially expressed if the $\log_2$ ratio was greater or equal to 1.0, or less than or equal to – 1.0, representing a two-fold difference in gene expression between the two conditions. Of the 4683 genes represented on the array, 93 had values that were greater than 1.0 (in the range of 2.7-1.0) indicating that these genes were up-regulated at least 2-fold under +NaCl conditions (Table 2). Approximately half of these genes were also up-regulated at least 2-fold under +KCl conditions. No genes had expression levels of –1.0 or less in the presence of the salt. Thus, no genes showed a 2-fold or more up-regulation of genes when cells were grown without NaCl (many genes had

values near –0.9 to –0.8; data not shown). The genes with $\log_2$ ratio values of 1.0 or greater fit into four physiological functional groups: metabolism, macromolecule synthesis/transport, environmental stress, and unknown function (Table 2). The majority were ribosomal genes in the growth related category.

The array data was screened for all genes involved in motility (Table 3). All of these genes had $\log_2$ ratio values in the range of 0.36 to –0.45. None of these genes were considered differentially expressed since their $\log_2$ values do not fall anywhere near the cutoffs of 1.0 to –1.0. Thus, there is no difference in expression of any motility genes between +NaCl or +KCl and no salt.

RT-PCR confirmed the microarray results. RT-PCR and gel electrophoresis were used to visualize any relevant differences in mRNA levels for selected motility genes under +/- NaCl conditions (*flhC, flhD*, and *fliA)*. The gene *plu1575* was chosen as a control because it was one of the most differentially expressed genes in the microarray experiment (with a value of 2.10). The RT-PCR experiment confirmed the microarray data for the motility genes. No change in gene expression was observed for these 3 genes. However, the control *plu1575* did not show any difference in mRNA levels for +/- NaCl. This deserves further investigation since the array data contradicts this result. Quantitative RT-PCR will be utilized to repeat the experiment since RT-PCR and gel electrophoresis may not be sensitive enough to detect 2-fold differences in gene expression.

Immunoblotting evidence suggests that flagella production was blocked at the post-transcriptional level. Cells isolated from swim plates without NaCl do not possess flagella (Chapter 2; Hodgson et al., 2003). However, there was no

change in mRNA levels for the motility genes in cells grown +/- NaCl or +/-KCl, To test if the gene products were regulated post-transcriptionally, we analyzed these cells immunologically with anti-flagellin anti-sera (Fig 4). Cells grown with NaCl showed a positive cross-reaction indicating flagella were produced. Cells grown without NaCl did not cross-react, indicating that there is no translational product or protein. These data infer that flagella biosynthesis may be regulated at the post-transcriptional level.

DISCUSSION

In the absence of additional NaCl or KCl, *Photorhabdus* is non-motile and does not produce flagella (Chapter 2; Hodgson *et al.,* 2003 and Chapter 4; Michaels and Tisa, to be submitted). Gene expression profiles of *Photorhabdus* cells grown with/without NaCl and with/without KCl showed that many potentially interesting genes were regulated (porin and toxin genes, for example), but NaCl and KCl did not regulate motility genes at the transcriptional level. There were no motility genes that were up-regulated in the presence of these ionic salts. Immunoblotting data indicated that flagellin was not produced in the absence of NaCl. Collectively, these data suggest that motility was regulated by NaCl at the post-transcriptional level: mRNA was transcribed for motility genes but not translated. It is not clear how this regulation occurs.

In post-transcriptional regulation, mRNA is degraded before translation may begin. The decay of mRNA is commonly controlled by ribosomes or by small un-translated RNAs (sRNAs) (for a review see: Deana and Belasco, 2005). Use of post-transcriptional regulation has the advantage that transcripts are rid from

the cell, and energy is instead used to translate newly needed transcripts. Interestingly, *Photorhabus* regulates the expression of several phenotypes both post-translationally and post-transcriptionally: in production of lipases (Wang and Dowds, 1993), proteases (Wee *et al.,* 2000) and bioluminescence (Wang and Dowds, 1991). In the closely related bacterium *Xenorhabdus*, which has a similar life cycle to *Photorhabdus,* there are two phase-variants in which one is motile and one is not. It was noted, though not discussed in detail, that the amount of mRNA for *flhDC* was identical for the two variants, indicating that in this instance as well, motility may be regulated post-transcriptionally (Givaudan and Lanois, 2000). In a life cycle where this bacterium must survive within two different hosts, it may be advantageous to use post-transcriptional control to adapt quickly.

We are less certain as to why NaCl is important for motility in *Photorhabdus*. It should be noted that this requirement is not NaCl specific; KCl is able to support motility as well, but $MgCl_2$ cannot (Chapter 2; Hodgson *et al.*, 2003 and Chapter 4; Michaels and Tisa, to be submitted). These data indicate that the requirement is not for Cl-, but may be an ionic requirement since both the monovalent ions K+ and Na+ can support motility equally well. Recently, *Bacillus subtilis* was shown to have a potassium ion-dependent form of spreading motility (Kinsinger *et al.,* 2005). It was suggested that the cells require potassium for normal growth, surfactin production, and/or pH control, all of which are necessary for this organism to colonize surfaces with spreading motility. Our future studies will be directed toward understanding why ion concentrations and motility may be important in the life cycle of *Photorhabdus*.

Table 1. Primers utilized in this study as described in Materials and Methods.

Gene:	Primer Set:	Reference:
plu1575, *cipA*-like	F-5'-ACCTGAACAGACTTCTCCAAACCC-3'	This study[1]
	R-5'-TAACCACCAGGAGAAACCGCTGAT-3'	This study[1]
flhC (*plu1848)*, flagellum biosynthesis transcriptional activator	F-5'-ACACAATTGAGCAGAGGGCGACTA-3'	This study[1]
	R-5'-GTTGCAACATGCCACTGTCCACAA-3'	This study[1]
flhD (*plu1847)*, flagellar transcriptional activator	F-5'-GAGTACGGTTGAATTGCTCAAACA-3'	This study[1]
	R-5'-ACTCGAGATTCCCTAGTAAGCTGC-3'	This study[1]
fliA (*plu1955)*, sigma 28; RNA polymerase sigma factor for flagellar operon	F-5'-AGCCAGTGAGCAGGAAGTTGCTAA-3'	This study[1]
	R-5'-TTAATCGCCTGGCTGTGTAGCTGA-3'	This study[1]
Universal16s rRNA 27F	F-5'-AGAGTTTGATCTTGGCTCAG-3'	Lane, 1991
Universal 16s rRNA 1492R	R-5'-GGTTACCTTGTTACGACTT-3'	Lane, 1991

[1] Primers were designed through Integrated DNA Technologies (www.idtdna.com) using gene sequences acquired from the *Photorhabdus luminescens TT01* genome available at http://genolist.pasteur.fr/PhotoList/.

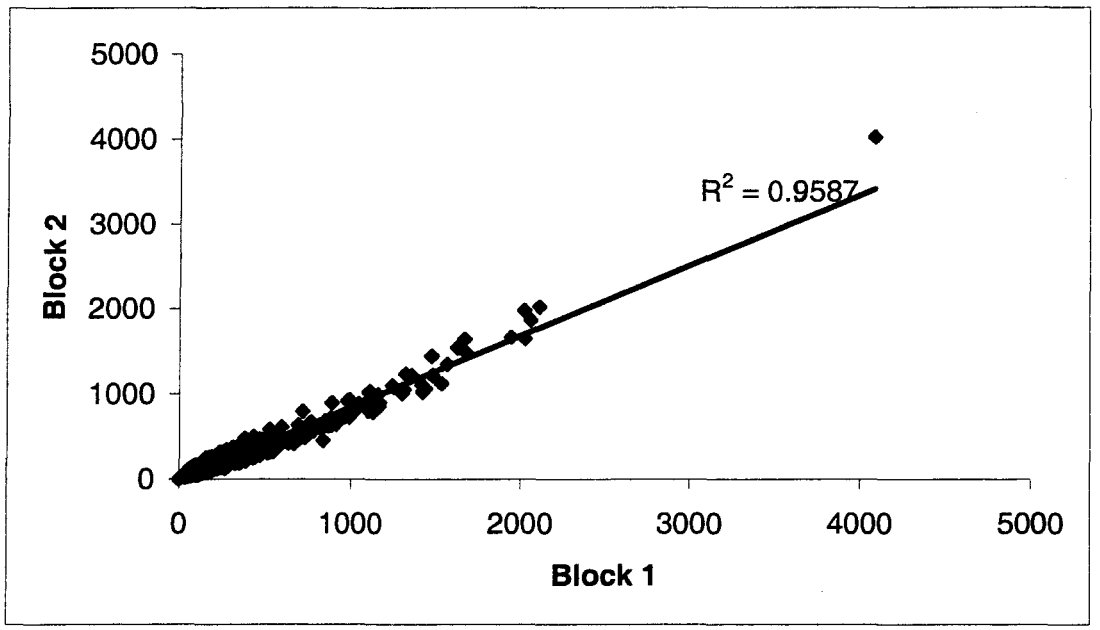


Fig 1. Scatter plot of normalized fluorescence signal for No Salt condition (array 3) comparing Block 1 to Block 2. The linearity of the plot indicates consistency of gene expression data between the two blocks.

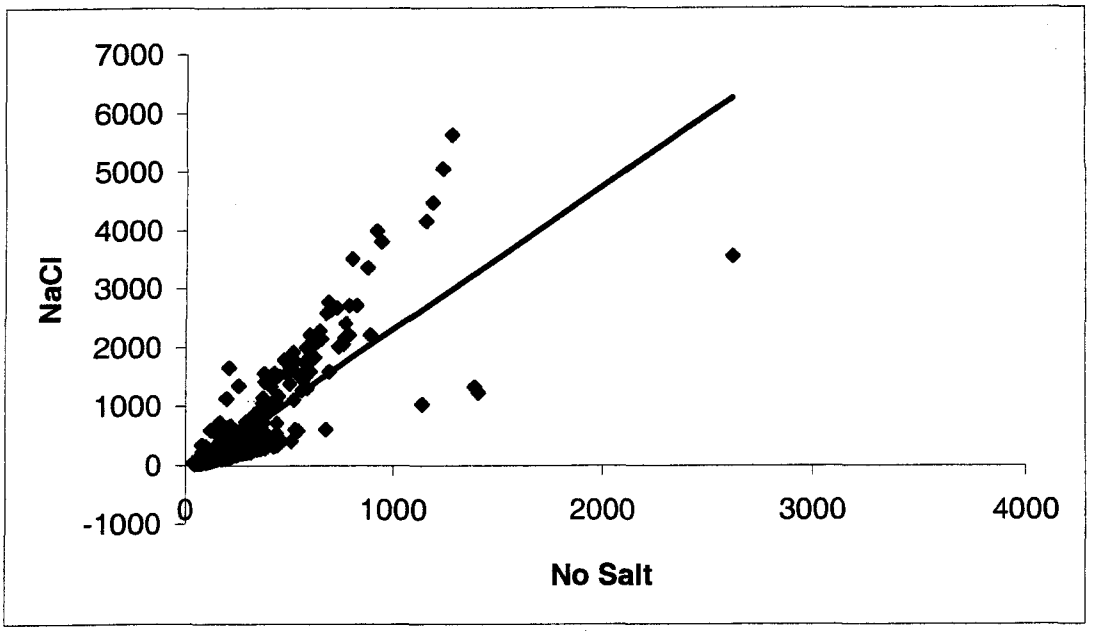


Fig 2. Scatter plot analysis of the array data. The normalized fluorescence signal for No Salt gene expression is plotted against signal for NaCl conditions. Genes that are differentially expressed appear off the linear regression line.

Table 2. Genes from microarray data that were up-regulated at least two-fold under NaCl conditions (compared to No NaCl conditions) categorized into physiological groups. The log2 of the NaCl/No NaCl ratio was calculated as described in Materials and Methods. A gene was considered differentially expressed if the log2 ratio of NaCl/No NaCl was greater/equal to 1.0, or less than/equal to −1.0, representing a two-fold difference in gene expression under the two conditions. Also listed are the log2 ratios of the same genes for KCl/No Salt and NaCl/KCl.

Locus:	Gene:	Function:	NaCl/No Salt:	KCl/No Salt:	NaCl/KCl:
Macromolecule Synthesis/Transport					
plu4703	*rpsD*	30S ribosomal protein S4	1.77399544	1.356222562	0.417772878
plu4720	*rpsC*	30S ribosomal protein S3	1.759637061	1.021351747	0.738285314
plu4711	*rplF*	50S ribosomal protein L6	1.739975384	1.045147697	0.694827687
plu0589	*rpsT*	ribosomal protein S20	1.737858061	1.368507944	0.369350117
plu4714	*rplE*	50S ribosomal protein L5	1.65610179	0.701988929	0.954112862
plu4707	*rplO*	50S ribosomal protein L15	1.638173039	0.967822119	0.67035092
plu0438	*rplL*	50S ribosomal protein L7/L12 (L8)	1.617126884	1.060820198	0.556306686
plu4702	*rpoA*	RNA polymerase alpha subunit	1.568776149	1.177749047	0.391027102
plu4719	*rplP*	50S ribosomal protein L16	1.567976746	0.965832589	0.602144157
plu4708	*rpmD*	50S ribosomal protein L30	1.565316615	0.824976173	0.740340442
plu4532	*secG*	Protein-export membrane protein SecG	1.558908697	1.108722811	0.450185886
plu4725	*rplD*	50S ribosomal protein L	1.550227126	1.037152994	0.513074132
plu4704	*rpsK*	30S ribosomal protein S11	1.549396236	1.046408461	0.502987775
plu4726	*rplC*	50S ribosomal protein	1.544169395	0.81457035	0.729599045
plu4524	*nlpL*	Lipoprotein Nlpl precursor	1.534898751	1.057451419	0.477447332
plu4712	*rpsH*	30S ribosomal protein S14	1.518071634	0.906019132	0.612052502
plu4721	*rplV*	50S ribosomal protein L22	1.516283643	0.762039921	0.754243722
plu4717	*rpsQ*	30S ribosomal protein S17	1.50138156	0.817697741	0.683683819
plu4701	*rplQ*	50S ribosomal protein L17	1.483239123	0.654629573	0.82860955
plu4709	*rpsE*	30S ribosomal protein S5	1.479125934	0.681002806	0.798123129
plu4705	*rpsM*	30S ribosomal protein S13	1.477940311	0.849207718	0.628732593

Table 2 (cont.)

Locus:	Gene:	Function:	NaCl/No Salt:	KCl/No Salt:	NaCl/KCl:
Macromolecule Synthesis/Transport (cont.)					
plu0430	*rpsG*	30S ribosomal subunit protein S7	1.449955925	0.627347166	0.822608759
plu4573	*rpsF*	30S ribosomal protein S6	1.440139482	1.043246056	0.396893425
plu4572	*priB*	primosomal replication protein N	1.438239418	1.123483358	0.31475606
plu4723	*rplB*	50S ribosomal protein L2	1.438153692	0.80049585	0.637657842
plu4864	*rpmB*	50S ribosomal protein L28	1.432523293	1.111638068	0.320885224
plu4724	*rplW*	50S ribosomal protein L23	1.42694209	0.880755804	0.546186287
plu4523	*deaD*	inducible ATP-independent RNA helicase	1.424970678	1.047188088	0.37778259
plu4863	*rpmG*	50S ribosomal protein L33	1.416667434	1.127034248	0.289633186
plu4727	*rpsJ*	30S ribosomal protein S10	1.411137669	0.607737352	0.803400317
plu4713	*rpsN*	30S ribosomal protein S14	1.406649604	0.725826253	0.680823351
plu1259	*trmD*	tRNA (guanine-N1-)-methyltransferase (M1G-methyltransferase)	1.403603497	1.036888177	0.36671532
plu4722	*rpsS*	30S ribosomal protein S19	1.399390171	0.768805335	0.630584836
plu4715	*rplX*	50S ribosomal protein L24	1.367969654	0.727762468	0.640207187
plu4541	*rpmA*	50S ribosomal protein L27	1.363897808	1.185929159	0.177968649
plu4718	*rpmC*	50S ribosomal protein L29	1.35848536	0.736678497	0.621806862
plu2869	*rplY*	ribosomal protein L25	1.357126742	1.068024935	0.289101807
plu4262	*N/A*	hypothetical protein; Similar to L-ornithine 5-monooxygenase (L-ornithine N5-oxygenase)	1.353971993	2.281719334	-0.92774734
plu4706	*secY*	Preprotein translocase SecY subunit	1.351484246	0.580196016	0.77128823
plu4710	*rplR*	50S ribosomal protein L18	1.331494621	0.594211758	0.737282863
plu0672	*rpsB*	30S ribosomal subunit protein S2	1.323228057	1.088674992	0.234553066
plu0431	*fusA*	translation elongation factor EF-G	1.323311116	0.49085883	0.832452286
plu0437	*rplJ*	50S ribosomal subunit protein L10	1.284027848	0.692332231	0.591695618
plu4716	*rplN*	50S ribosomal protein	1.26884405	0.559572721	0.709271329
plu0429	*rpsL*	ribosomal protein S12	1.217781674	0.601083927	0.616697747

Table 2 (cont.)

Locus:	Gene:	Function:	NaCl/No Salt:	KCl/No Salt:	NaCl/KCl:
Macromolecule Synthesis/Transport (cont.)					
plu4015	*rplM*	50S ribosomal protein L13	1.202468093	0.980315172	0.222152921
plu1258	*rimM*	16S rRNA processing protein	1.183662944	1.044595043	0.139067901
plu4529	*infB*	translation initiation factor IF-2	1.134815463	1.134815463	1.134815463
plu2668	*infC*	translation initiation factor IF-3	1.132063207	0.62965404	0.502409166
plu4135	*groES*	10 kDa chaperonin (Protein CPN10) (Protein GROES)	1.12852942	0.856133107	0.272396313
plu0432	*tufB*	translation elongation factor EF-Tu.B	1.120973602	0.421575805	0.699397798
plu2837	*rpmF*	50S ribosomal protein L32	1.116159256	0.827237089	0.288922167
plu4542	*rplU*	50S ribosomal protein L21	1.103952299	1.101560017	0.002392283
plu0435	*rplK*	ribosomal protein L11	1.091378647	0.851127346	0.240251301
plu0436	*rplA*	50S ribosomal subunit protein L1	1.078160374	0.678037849	0.400122525
plu4730	*tufA*	elongation factor Tu (EF-Tu)	1.076858094	1.076858094	1.076858094
plu1622	*rpsA*	30S ribosomal protein S1	1.075476885	0.636112516	0.439364369
plu4089	*fis*	DNA-binding protein (HIN recombinational binding protein)	1.07406453	0.961528342	0.112536188
plu4134	*groEL*	60 kDa chaperonin (protein Cpn60) (GroEL protein)	1.066280939	0.73192607	0.334354869
plu1257	*rpsP*	ribosomal protein S16	1.054244398	0.919278061	0.134966337
plu0673	*tsf*	elongation factor EF-Ts	1.043270559	0.675743958	0.367526601
plu2667	*rpmL*	50S ribosomal protein L35	1.037158108	0.692082258	0.34507585
plu0052	*asnA*	Aspartate--ammonia ligase (Asparagine synthetase A)	1.025408859	3.744432279	-2.71902342
plu4571	*rpsR*	30S ribosomal protein S18	1.008457996	0.761350284	0.247107712
plu3977	*rpsU*	30S ribosomal subunit protein S21	1.002309967	0.801938502	0.200371465

Table 2 (cont.)

Locus:	Gene:	Function:	NaCl/No Salt:	KCl/No Salt:	NaCl/KCl:
Metabolism					
plu3623	*aceE*	pyruvate dehydrogenase E1 component	1.618366431	0.925223507	0.693142924
plu4088	*yhdG*	Highly similar to putative dehydrogenase YhdG of Escherichia coli	1.219730662	0.880979996	0.338750666
plu1533	*phnA*	hypothetical protein; Similar to alkylphosphonate uptake protein	1.219044421	1.08156529	0.137479131
plu3624	*pdhR*	pyruvate dehydrogenase complex repressor	1.174341387	0.619728831	0.554612557
plu4530	*nusA*	N utilization substance protein A (nusA protein) (L factor)	1.031950302	0.353087005	0.678863297
plu3622	*aceF*	dihydrolipoamide acetyltransferase component of pyruvate dehydrogenase complex	1.026984503	0.713949302	0.313035201
plu2145	*ldhA*	D-lactate dehydrogenase (D-LDH)	1.0235785	0.366498254	0.657080246
Unknown Function					
plu4502	*ybiJ*	Similar to unknown protein YbiJ precursor of Escherichia coli	2.198055756	2.023897144	0.174158613
plu0015	N/A	Unknown, probable tail sheath structure	2.085906494	1.174831045	0.911075449
plu0014	N/A	Unknown, probable tail tube structure	1.809290961	0.772329696	1.036961265
plu0480	N/A	hypothetical protein; Highly similar to unknown protein of Photorhabdus	1.72541418	0.979988852	0.745425328
plu4261	N/A	hypothetical protein; Similar to unknown protein	1.567012902	2.272604775	-0.70559187
plu3988	*ybjX*	Similar to unknown protein YbjX of Escherichia coli	1.34317087	1.076387791	0.266783079
plu0503	*bax*	BAX protein	1.288015041	1.060161372	0.22785367
plu0022	N/A	Unknown, probable tail baseplate synthesis	1.199711648	0.453480096	0.746231553

Table 2 (cont.)

Locus:	Gene:	Function:	NaCl/No Salt:	KCl/No Salt:	NaCl/KCl:
Unknown Function (cont.)					
plu4531	N/A	hypothetical protein; Highly similar to unknown protein YhbC Escherichia coli	1.193511421	0.562429999	0.631081422
plu4501	*yhbP*	Similar to unknown protein YhbP of Escherichia coli	1.142893323	1.218240274	-0.07534695
plu0372	N/A	hypothetical protein; Highly similar to unknown protein	1.112537708	0.428847941	0.683689767
plu1973	N/A	hypothetical protein; Hypothetical gene	1.026921311	0.970394109	0.056527202
plu0010	N/A	Unknown, probable tail synthesis	1.002724419	0.535602918	0.467121501
Environmental Stress					
plu1752	N/A	Unknown, probable porin	2.772522749	2.772522749	2.772522749
plu1537	N/A	Unknown, hypothetical toxin	2.553171283	1.895875278	0.657296005
plu1575	N/A	hypothetical protein; Similar to crystalline inclusion protein type II (CipA-like protein)	2.195793809	1.337877125	0.857916685
plu3790	*cspL*	cold shock-like protein (CPS-I)	2.187765527	2.178882592	0.008882935
plu1842	N/A	Unknown, probable cold shock protein	1.664743782	1.14894561	0.515798172
plu2144	*hslJ*	Heat shock protein HslJ	1.239275155	0.474702252	0.764572903
plu2838	*yceD*	Highly similar to probable membrane protein of Escherichia coli	1.218224419	0.952120136	0.266104283
plu0579	*dnaK*	chaperone protein (heat shock protein 70)(HSP70)	1.015286994	0.769499465	0.245787529

Table 3. Expression levels of motility genes from microarray data. The log2 of the +NaCl/ - NaCl ratio was calculated as described in Materials and Methods. A gene was considered differentially expressed if the log2 ratio of +NaCl/-NaCl was greater/equal to 1.0, or less than/equal to –1.0, representing a two-fold difference in gene expression under the two conditions. Thus, all motility genes did not show any differential expression under these conditions.

Locus:	Gene:	Function:	NaCl/No Salt:	KCl/No Salt:	NaCl/KCl:
plu1956	*fliZ*	FliZ protein	0.364498835	0.270084063	0.094414771
plu1951	*fliT*	flagellar protein	0.338030839	0.078472822	0.259558017
plu1924	*flgK*	flagellar hook-associated protein 1 (HAP1)	0.184931536	0.104020357	0.080911179
plu1953	*fliD*	flagellar hook-associated protein 2 (HAP2)(Flagellar cap protein)	0.180074607	-0.004622225	0.184696832
plu1925	*flgL*	flagellar hook-associated protein 3 (HAP3)	0.122092319	0.113039227	0.009053093
plu1954	*fliC*	Flagellin	0.026378372	0.37728605	-0.350907678
plu1949	*fliE*	flagellar hook-basal body 11K protein	0.018738949	0.056667199	-0.03792825
plu1947	*fliG*	flagellar motor switch protein	-0.056727061	0.085337374	-0.142064435
plu1912	*flgN*	flagella synthesis protein	-0.063667932	0.054331891	-0.117999823
plu1937	*fliQ*	flagellar biosynthetic protein	-0.082512798	0.12644592	-0.208958719
plu1952	*fliS*	flagellar protein	-0.085323893	-0.000174878	-0.085149015
plu1944	*fliJ*	flagellar protein	-0.106187492	-0.106575809	0.000388317
plu1943	*fliK*	flagellar hook-length control protein	-0.10966323	-0.119869365	0.010206135
plu1916	*flgC*	flagellar basal-body rod protein FlgC	-0.111418911	0.08870891	-0.20012782
plu1921	*flgH*	flagellar L-ring protein precursor (Basal body L-ring protein)	-0.113049828	-0.05366919	-0.059380638
plu1915	*flgB*	flagellar basal-body rod protein (Putative proximal rod protein)	-0.125155986	-0.142586476	0.01743049
plu1918	*flgE*	flagellar hook protein	-0.131109692	-0.071781395	-0.059328297
plu1939	*fliO*	flagellar FliO protein	-0.17882449	-0.11855951	-0.06026498
plu1848	*flhC*	flagellum biosynthesis transcription activator	-0.18000012	-0.077638007	-0.102362113
plu1940	*fliN*	flagellar motor switch protein	-0.182940749	-0.18003821	-0.002902539
plu1914	*flgA*	flagella basal body P-ring formation protein precursor	-0.189674685	-0.096349036	-0.093325649

Locus:	Gene:	Function:	NaCl/No Salt:	KCl/No Salt:	NaCl/KCl:
plu1913	*flgM*	negative regulator of flagellin synthesis (Anti-sigma-28 factor)	-0.203339717	-0.248333129	0.044993412
plu1920	*flgG*	flagellar basal-body rod protein FlgG (Distal rod protein)	-0.226485627	-0.079188824	-0.147296803
plu1917	*flgD*	basal-body rod modification protein FlgD	-0.240977867	-0.220134494	-0.020843373
plu1936	*fliR*	flagellar biosynthetic protein	-0.244881785	-0.170662118	-0.074219667
plu1948	*fliF*	flagellar basal-body M-ring protein	-0.253826802	-0.238279488	-0.015547314
plu1938	*fliP*	flagellar biosynthetic protein FliP	-0.267205979	-0.221438925	-0.045767053
plu1896	*flhA*	flagellar biosynthesis protein	-0.268688366	-0.080478196	-0.18821017
plu1942	*fliL*	flagellar protein	-0.291749291	-0.239500897	-0.052248394
plu1895	*flhB*	flagellar biosynthetic protein	-0.330910981	-0.179626373	-0.151284608
plu1919	*flgF*	flagellar basal-body rod protein (Putative proximal rod protein)	-0.339604544	-0.09043374	-0.249170804
plu1941	*fliM*	flagellar motor switch protein	-0.367830399	-0.282182105	-0.085648294
plu1945	*fliI*	flagellum-specific ATP synthase	-0.404134714	-0.234776999	-0.169357714
plu1946	*fliH*	flagellar assembly protein	-0.446493344	-0.251438858	-0.195054486

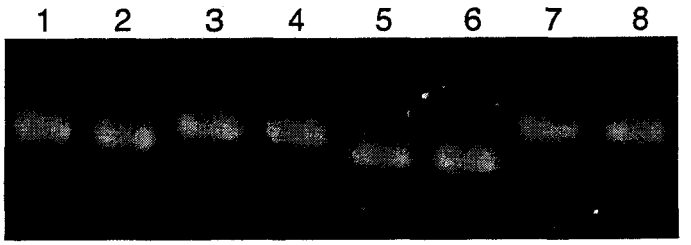


Fig 3. RT-PCR analysis of flagellar gene expression confirmed microarray results. mRNA was extracted from cells grown in LB with and without NaCl and analyzed by RT-PCR as described in Materials and Methods). Lane (1,2) *plu1575,* (3,4) *flhC,* (5,6) *flhD* , (7,8) *fliA*; (1,3,5,7) +NaCl, (2,4,6,8) no NaCl.

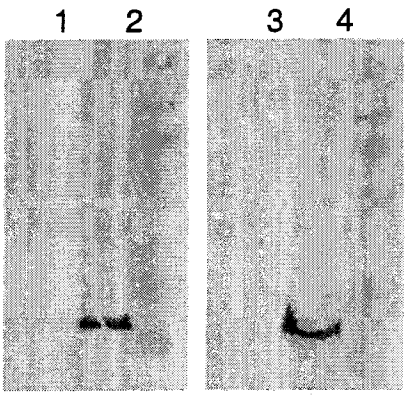


Fig 4. Immunodetection of flagella showing that cells without NaCl do not produce flagella. *P. temperata NC19* cells were harvested from swim plates and broth cultures without NaCl (Lanes 1 and 3) and with NaCl (Lanes 2 and 4).The solubilized proteins were separated by SDS-PAGE in a 10% polyacrylamide gel and detected by immunoblotting as described in Materials and Methods. Molecular weight marker is a Blue Ranger protein marker (Pierce Biotechnology, IL). Bottom marker is 18.3 kb, followed by 28 kb above, and 39.2 kb.

CHAPTER VI

SUMMARY

As *Photorhabdus* switches roles from beneficial symbiont to lethal pathogen, the bacteria are exposed to several different environments: nematode gut, insect hemocoel, and possibly soil. We hypothesized that the motility of *Photorhabdus* may aid the bacteria in traveling from host to host, in colonization, and/or in virulence. When this project was first started, there were no reported *Photorhabdus* motility studies. The genome of this bacterium had not yet been sequenced to facilitate the identification of motility apparatus. In this project, a physiological, genetic, and genomic approach was utilized to begin characterization of motility in *Photorhabdus*.

We first determined that *Photorhabdus* was motile both by swimming (movement in liquid) and swarming (movement on surfaces) under the appropriate conditions. Both swimming and swarming cells possessed the same peritrichous flagella and the same flagellin protein, indicating that these two forms of motility share some structural components. However, unlike swimming behavior, swarming was an elaborately coordinated group movement in which the cells migrated in an organized manner to colonize a surface. Clearly there are some aspects of swarming behavior that are regulated differently from swimming.

After characterizing motility in the wild-type, a genetic approach was used to elucidate the genes regulating these two forms of motility. *Photorhabdus temperata* mutants with altered motility behavior were generated with transposon mutagenesis. After screening a library of over 10,000 mutants, several of the mutant genes were identified with DNA sequencing. Non-motile mutants were isolated with deletions in *flgE* and *flgK*. These mutants were unable to swim or swarm, further supporting the idea that some of the same genes are involved in both swimming and swarming behavior. A *plu3723* mutant (*luxR* homolog), unlike the wild-type, was able to swim without NaCl. Directed by NaCl concentrations, the *plu3723* product may be a transcriptional regulator of the *Photorhabdus* flagellation cascade. An *rssB* mutant was also isolated that displayed a hyperswarming phenotype. This gene may act as a negative regulator of swarming behavior. A *yidA* mutant, whose function remains unknown, had reduced swimming behavior and dramatically attenuated virulence. This may be an important mutant in determining the link between expression of virulence and motility. Upon further phenotypic characterization, many of the motility mutants were found to have altered expression of virulence enzymes, or had overall reduced insect pathogenicity. The results of this study suggest that motility may directly play a role in pathogenicity or that some virulence factors may be co-regulated with expression of motility genes.

One of the most striking observations of swimming and swarming behavior was that NaCl or KCl must be present for flagella production. I used

global gene expression studies to investigate the potential regulation mechanism of these ionic salts on the motility regulon. Interestingly, microarray data suggested that motility genes are transcribed regardless if NaCl or KCl is present, but immunoblotting experiments did not detect flagellin in the absence of NaCl. Collectively, these data suggest that NaCl and KCl levels regulate motility genes at the post-transcriptional level and not at the gene expression level. This is the first report of post-transcriptional regulation of bacterial motility. Perhaps we will learn that this form of regulation confers an advantage in bacteria with complex life cycles that must rapidly adapt to changing environments.

The physiological and ecological relevance of these ionic salts remains to be elucidated. I propose a preliminary model for the correlation of flagella expression, motility, and ionic salt levels for *Photorhabdus* in Fig 1. It is known that the insect hemocoel is relatively high in nutrients, ionic salts, and solutes. In contrast, the symbiotic host of these bacteria, the infective juvenile nematode, does not feed, closes its mouth and anus, and waits in the soil for an insect prey. The nutrients and solutes are probably low within the nematode compared to the nutrient-rich insect. Since NaCl and KCl are required for expression of flagella, it is presumed that within the nematode, the bacteria would be non-motile. Ecologically this would make sense: it would be a waste of energy for the bacteria to produce flagella since they are unable to leave the nematode. However, when the nematodes release their bacterial symbionts into the insect hemocoel, the bacteria are exposed to the ionic salts that would induce the

flagella regulon. It would be beneficial for the bacteria to be motile upon entering the insect so that they could rapidly colonize the hemocoel. If aspects of virulence expression are co-regulated with motility genes as my research suggests, expression of virulence factors would also be induced upon exposure to the insect.

The physical consistency of an insect carcass is probably semi-solid, much like the conditions that were optimal for swarming motility. Thus, swarming may prove to be the ecologically important form of motility for *Photorhabdus*. In many gram-negative bacteria, quorum sensing is involved in regulation of swarming motility and colonization of hosts. Quorum sensing could be important for the bacteria to communicate and strategically colonize the insect cavity when bacterial numbers are optimal. This is an important aspect of motility that I did not focus on and should be further explored.

This was the first reported study on motility by *Photorhabdus*. As such, I have just begun to open the door towards understanding the importance of motility in the life cycle of this bacterium as both a pathogen and a symbiont. The mutants that were generated in this study will be important long-term tools for future experiments. While many important discoveries were made in this project, even more questions have been raised. Many different projects remain to be initiated, including:

- Quorum sensing and swarming motility
- Quantitative methods for accurately assessing hemolysin, antibiotic,

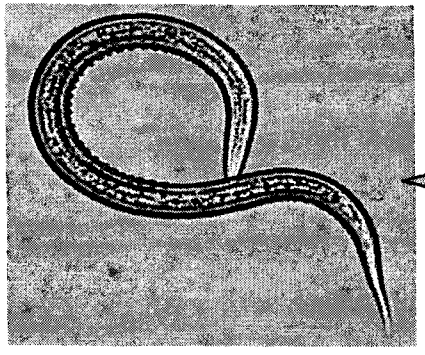
protease activity, etc. of mutants

- Targeted mutagenesis, causality, complementation, etc. to prove effect of mutations
- Quantifying number of flagella for swarming mutants, swarming vs. Swimming in the wild-type, etc.
- Quantitative RT-PCR for confirming microarray data
- Microarrays/quantitative RT-PCR with mRNA extracted at varying time points/points in growth curve
- Microarrays/quantitative RT-PCR for motility mutants
- *In vivo* nematode infection studies with motility mutants

It seems feasible that future work will unravel the questions this research has raised and begun to answer.

Figure 1. Model for Motility in the Life Cycle of *Photorhabdus*: Link among NaCl / Virulence / Motility

Nematode: Low osmolarity, salts, nutrients



NOT MOTILE?

- No expression of flagella or virulence factors?

Travel?
Colonization?

Insect: High osmolarity, salts, nutrients

MOTILE?

-Expression of flagella allowing subsequent colonization, production of enzymes and toxins

-Group swarming, quorum sensing?

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