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Evidence that a partner-switching regulatory system modulates hormogonium motility in the filamentous cyanobacterium Nostoc punctiforme

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EVIDENCE THAT A PARTNER-SWITCHING REGULATORY SYSTEM MODULATES HORMOgonium MOTILITY IN THE FILAMENTOUS CYANOBACTERIUM NOSTOC PUNCTIFORME

By

Kelsey W. Riley

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EVIDENCE THAT A PARTNER-SWITCHING REGULATORY SYSTEM MODULATES HORMOGONIUM MOTILITY IN THE FILAMENTOUS CYANOBACTERIUM NOSTOC PUNCTIFORME

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EVIDENCE THAT A PARTNER-SWITCHING REGULATORY SYSTEM MODULATES HORMOGONIUM MOTILITY IN THE FILAMENTOUS CYANOBACTERIUM *NOSTOC PUNCTIFORME*

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by

Kelsey W. Riley
DEDICATION

This thesis is dedicated to my mother, Lynndee Riley, who raised two little girls as a single mom, worked full time, and made incredible personal sacrifices for us on a level that I’ll never truly comprehend, but for which I am eternally grateful. My mother is a strong, independent woman, and I learned early on that my mother was unafraid to take a stand, even when her opinions may have been unpopular. There were times growing up when her candidness and straightforwardness embarrassed me (like once when she walked right up to a boy in my class during the school Halloween parade and scolded him for making fun of my Halloween costume), but mostly I was in awe of her. She was beautiful and smart, both incredibly loving and a force to be reckoned with, and I admired her passion and tenacity. She was also a natural teacher, who always encouraged our learning and took us on educational trips to visit historic monuments and national parks. When I think back to myself as a little girl, I was full of confidence and curiosity, and I truly felt like I could do or be anything. That was because of my mother.

It is also because of my mother that I am here today, in this program, writing and defending this thesis. My mother has seen me at my highest highs, but she’s also seen me at my lowest lows. She has literally picked me back up when I couldn’t see any reason to move forward. Through some of my most difficult times, she has been the one person who was always there for me and saw me for who I really was: curious,
passionate, and in love with and in awe of the natural world. Even at times when I lost sight of my childhood confidence or questioned my self-worth, she maintained an unwavering belief in me. She’s the one who suggested that I go back to school and encouraged me to apply to the master’s program at the University of the Pacific. If it were not for her, I would never have moved back to Stockton, at the age of 32, to begin this program.

My years in this program have been a transformative experience. During this time, I have learned a tremendous amount about biology and research. Even more importantly, I have learned so much about myself. For the entire first year and a half of this program, I had total imposter syndrome. I felt like I wasn’t good enough to be here, like I didn’t know as much as everyone else, and like I didn’t quite fit in, as hard as I tried. I was significantly older than most of the other graduate students, and I’d already had a career spanning a decade and different life experiences. Age-wise, I felt closer to many of the faculty, but I wasn’t a faculty member, so I didn’t quite belong with them either. I recall that when I started this program, the thought of writing and defending my own research thesis was overwhelming and seemed unattainable, but I also had a deep level of appreciation for what it meant to be able to go back to school and to be a student again at this point in my life. I knew that this was an opportunity that I was incredibly grateful for, and I kept putting one foot in front of the other, trying to keep learning and growing in small ways every day, with the constant encouragement of my mom.

At some point along the way, I started to realize that I was actually understanding a lot of new things that I had never understood before, and that it was OK that I was different. I started to regain a small sense of confidence and I let myself be myself. I
found that I could connect with most everyone here, at some level, in my own way. The more I learned, the more my drive and passion to learn more was ignited and the less I questioned whether I belonged here, because I knew that I did. This was exactly where I belonged, and everything so far in my life had led me on a journey that brought me here. Luckily, my mom had been there by my side, to help buoy me once again through the challenges so that I could push through and experience the joy and sense of accomplishment that I feel today as I complete and defend this thesis.

So thank you, Mom. Thank you for always seeing in me the bold, confident, and yes, sometimes overly bossy little girl with a good heart who cares a bit too much about everything and everyone, and who loves to learn. Thank you for encouraging me to come home, take a leap of faith, and start something new at 32. As usual (95% of the time!), you were right.

I love you, more than you will ever know.
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It is not lost on me that the many opportunities and each of the successes I've had leading up to and throughout this master’s program are due in large part to a great number of “pivotal people” who have supported, taught, challenged, cajoled, encouraged, put up with, and inspired me along the way. To truly thank each one appropriately would take so much more than I can write here, but I will attempt to summarize my gratitude to some of those who have been so influential and helpful in this endeavor.

Having been a teacher myself, I’d first like to thank the two high school teachers I had who inspired me the most. My passion for studying biology was ignited by a most remarkable anatomy and physiology teacher, Mr. Manny Moreno, whose enthusiasm for science and whose enduring belief in me has been incredibly important in my life’s trajectory. I am also so grateful to my high school math teacher of three years, Mr. Glen Cornett, who taught me how to think critically and problem-solve, but who also went out of his way to make sure I knew that I mattered and that I was capable of great things.

I would like to express heartfelt gratitude to my dear friends, many of whom are my former teaching colleagues, and to my former students from the years that I taught middle and high school science. Knowing that I have their support and that they believe in me, and seeing their own perseverance through life’s challenges, has inspired me more times than I can count. I am also grateful to the friends that I’ve made here in the
graduate program for studying with me, hanging out with me, cheering me on, and generally commiserating with me through some of the struggles of graduate student life.

Thank you to my teammates in the Risser lab for tolerating my bossiness, humoring my silliness, and for engaging in lab chats with me when I needed human interaction and distractions from writing. Our undergraduate researchers, Thomas Harwood, Krithika Giresh, Daniel Nguyen, and Natalie Figueroa, are some of the very best and brightest, in addition to being exceptionally endearing. A special thank you goes to Adriana Pantoja for her assistance in optimizing protocols and helping me gather preliminary data for my thesis project during the summer after my first year, and for her ever-sweet and friendly demeanor. To my graduate student colleagues during my time in the lab, Behzad Khayatan and Alfonso Gonzalez, thank you so much for the assistance and support in research and with technology, and for always putting up with my jokes and quirky personality.

I’d like to express my sincere gratitude to the entire biology faculty at the University of the Pacific, for welcoming me into this program and showing me kindness and warmth from Day One. The quality of the faculty in this department is extraordinary, and I am so grateful to have had the opportunity to learn from and work alongside them for the past two and a half years. I’d like to especially thank Debbie Walker for all of her help when I taught Biology 61 and 51 labs, as well as the entire microbiology lab team that I’ve been privileged to work with (Drs. Geoff and Joan Lin-Cereghino, Dr. Doug Risser, Carrie Kozina, Behzad Khayatan, Sindhuja Krishnamurthy, and Henry Le). Their guidance, assistance, and collegiality made my teaching experience here truly wonderful. In addition, thank you to Dr. Lisa Wrischnik for lending me the use of her lab equipment
for my immunofluorescence imaging and bacterial two-hybrid assays, and for allowing me to sit in as an observer in her highly engaging and informative genetics and neurobiology lectures the past two semesters. To Dr. Mark Brunell, whose research lab is conveniently located next-door to the Risser lab, thank you for putting up with my antics and for being a source of kindness and steady encouragement, especially throughout the writing of my thesis.

A special thank you goes to Dr. Doug Weiser for serving on my thesis committee. Dr. Weiser was my professor for the molecular techniques course that I took my very first semester of grad school, back when I didn’t even remember how to set micropipettes correctly, let alone do any actual research techniques or read scientific journal articles. Even though I struggled that semester in his class, Dr. Weiser was always kind and helpful, and I hope that as a member of my committee, he can see the vast growth I’ve made over the past few years in this program and know that his support and patience with me that initial semester was instrumental to that growth. I also appreciate his willingness to help me with my research projects, whether it was lending me reagents or talking through my data and possible next steps.

I’d also like to express my sincere gratitude to Dr. Craig Vierra for serving on my thesis committee. As the department co-chair, Dr. Vierra was not only the very first person to welcome me to campus when I visited for my interview, but he was also the one who called to tell me that I was accepted into the program that exciting Tuesday in April 2015. Dr. V has been very encouraging and supportive of my entire experience here, and was gracious enough to allow me a third year in the program to continue to teach, learn and grow. Having had him as my professor for immunology and graduate
seminar courses, I can also attest to the fact that he is a great teacher, and I deeply thank him for all of the fascinating lectures and guidance he’s given me.

This brings me to a most crucial acknowledgment, that of my absolutely phenomenal research advisor, Dr. Doug Risser, without whom none of this thesis could have been possible. The best choice I made in graduate school was to ask Dr. Risser if I could join his lab, and the best thing that happened to me in graduate school was him agreeing to take me as a student. Dr. Risser is responsible for single-handedly teaching me the vast majority of what I’ve learned about research, and for guiding and supporting me in this thesis project every step of the way. He has walked me through every lab technique I’ve done for the first (and sometimes second, third, etc.) time I’ve done it, to ensure I learn the skills properly. He has been unbelievably patient with me, always willing to answer an absurd number of questions or to re-explain things to me multiple times when necessary. Dr. Risser revealed to me the complexity of my project and the skills and knowledge I needed to complete it little by little, as I was ready for each part, and in such a way that I never felt totally overwhelmed. He is one of the most hard-working, organized, and brilliant people I’ve ever met, and on top of being a distinguished and productive researcher, he is also an incredibly gifted teacher and engaging lecturer. No number of superlatives could ever fully capture the esteem and respect I have for Dr. Risser, and the gratitude I feel to have been his graduate student. It is difficult to imagine what my experience would have been like without him, and luckily for me, I’ll never have to.
Evidence that a Partner-Switching Regulatory System Modulates Hormogonium Motility in the Filamentous Cyanobacterium *Nostoc punctiforme*

Abstract

by Kelsey W. Riley

University of the Pacific
2018

Partner-switching regulatory systems (PSRSs) are utilized by many different bacteria to regulate a wide array of cellular responses, from stress response to expression of virulence factors. The filamentous cyanobacterium *Nostoc punctiforme* can transiently differentiate motile filaments, called hormogonia, in response to various changes in the environment. Hormogonia utilize a Type IV pilus (T4P) complex in conjunction with a secreted polysaccharide for gliding motility along solid surfaces. This study identified three genes, designated *hmpU*, *hmpW*, and *hmpV*, encoding the protein components of a PSRS involved in regulation of hormogonium motility in *N. punctiforme*. Although mutant strains with in-frame deletions in *hmpU*, *hmpW*, and *hmpV* differentiated morphologically distinct hormogonium-like filaments, further phenotypic analysis demonstrated significant distinctions among the strains. The ∆*hmpW* strain contained a higher percentage of motile filaments that moved faster than the wild-
type strain, while the $\Delta hmpU$ and $\Delta hmpV$ strains consisted of fewer motile filaments that moved at a slower rate compared to wild type. Immunoblotting and immunofluorescence of PilA, the major component of the pilus in the T4P system, showed that although all mutant strains appeared to express similar levels of PilA protein, the $\Delta hmpU$ and $\Delta hmpV$ strains displayed reduced extracellular PilA. Lectin blotting and staining with fluorescently-labeled UEA lectin demonstrated a decrease in extracellular hormogonium polysaccharide in the $\Delta hmpU$ and $\Delta hmpV$ strains, consistent with the current understanding that the polysaccharide is secreted via the T4P system. Epistasis analysis demonstrated that the $\Delta hmpW$, $\Delta hmpV$ double-deletion mutant strain displayed reduced spreading in plate motility assays, similar to the $\Delta hmpV$ single mutant. Together, these results support a model in which the HmpU phosphatase and HmpW serine kinase control the phosphorylation state of the HmpV protein, modulating its activity on a downstream target to ultimately promote activation of the T4P motor complex and enhance hormogonium motility.
TABLE OF CONTENTS

LIST OF TABLES ......................................................................................................................... 15

LIST OF FIGURES .......................................................................................................................... 16

CHAPTER

1. Introduction ................................................................................................................................. 18

   Significance of Cyanobacteria ................................................................................................. 18

   Four Developmental Alternatives of *Nostoc punctiforme* .............................................. 19

   Hormogonium Development and Motility ........................................................................... 23

   Partner-Switching Regulatory Systems ............................................................................... 29

   A PSRS Regulating Motility in *N. punctiforme* ............................................................. 34

2. Materials and Methods .............................................................................................................. 35

   Comparative Genomics Analysis ......................................................................................... 35

   Bacterial Two-Hybrid Analysis ............................................................................................ 35

   *Nostoc punctiforme* Culture Conditions .......................................................................... 37

   Plasmid and *N. punctiforme* Mutant Strain Construction ............................................. 37

   Hormogonium Induction ........................................................................................................ 40

   Light Microscopy ..................................................................................................................... 40

   Time Lapse Microscopy & Motility Analysis ....................................................................... 41
<table>
<thead>
<tr>
<th>Section</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>Immunoblot of PilA &amp; HmpD</td>
<td>41</td>
</tr>
<tr>
<td>Lectin Blot</td>
<td>42</td>
</tr>
<tr>
<td>Simultaneous PilA Immunofluorescence &amp; UEA-Fluorescein Staining</td>
<td>43</td>
</tr>
<tr>
<td>Fluorescence Microscopy</td>
<td>44</td>
</tr>
<tr>
<td>Plate Motility Assays</td>
<td>45</td>
</tr>
<tr>
<td>3. Results</td>
<td>49</td>
</tr>
<tr>
<td>Npun_F5169 is the Corresponding \textit{hmpV} for a PSRS in \textit{N. punctiforme}</td>
<td>49</td>
</tr>
<tr>
<td>Characterization of the Hmp PSRS Modulating Hormogonium Motility in \textit{N. punctiforme}</td>
<td>53</td>
</tr>
<tr>
<td>4. Discussion</td>
<td>65</td>
</tr>
<tr>
<td>REFERENCES</td>
<td>72</td>
</tr>
</tbody>
</table>
LIST OF TABLES

<table>
<thead>
<tr>
<th>Table</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>1. Strains and Plasmids Used in This Study</td>
<td>46</td>
</tr>
<tr>
<td>2. Oligonucleotides Used in This Study</td>
<td>47</td>
</tr>
</tbody>
</table>
### LIST OF FIGURES

<table>
<thead>
<tr>
<th>Figure</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>1. Phase contrast photomicrographs of <em>N. punctiforme</em>, illustrating its four developmental alternatives.</td>
<td>21</td>
</tr>
<tr>
<td>2. A model of the gene regulatory network promoting hormogonium development, depicting genes or gene clusters essential for functional hormogonia.</td>
<td>24</td>
</tr>
<tr>
<td>3. General type IV pilus (T4P) architecture, biogenesis and structure.</td>
<td>26</td>
</tr>
<tr>
<td>4. The T4P complex and gliding motility in filamentous cyanobacteria.</td>
<td>27</td>
</tr>
<tr>
<td>5. A model of how polar switching of HmpF regulates T4P activity and directional motility in <em>N. punctiforme</em>.</td>
<td>29</td>
</tr>
<tr>
<td>6. Conceptual model of the PSRS regulating σB in <em>B. subtilis</em>.</td>
<td>30</td>
</tr>
<tr>
<td>7. Models of PSRSs in <em>B. subtilis</em> and <em>V. fischeri</em>.</td>
<td>33</td>
</tr>
<tr>
<td>8. Schematic diagram depicting the genomic region harboring Npun_R5135 and Npun_R5134 in <em>N. punctiforme</em>.</td>
<td>50</td>
</tr>
<tr>
<td>9. Comparative genomic profiles of all RsbU, RsbW and RsbV homologs in the genome of <em>N. punctiforme</em>.</td>
<td>51</td>
</tr>
<tr>
<td>10. Bacterial two-hybrid analysis of protein-protein interactions between HmpW and each RsbV homolog in the <em>N. punctiforme</em> genome, as identified previously by the BLASTP search (NCBI).</td>
<td>52</td>
</tr>
</tbody>
</table>
11. Schematic diagram depicting the genomic region containing the open reading frame Npun_F5169 (the putative *hmpV*). .................................................................53

12. Light micrographs taken with a dissecting microscope of plate motility assays for wild-type and mutant strains, as indicated. ..............................................................55

13. Morphology of filaments at 0 h and 24 h post induction for hormogonia, by strain (as indicated). ........................................................................................................56

14. Quantitative analysis of motility from time-lapse microscopy for wild type (WT) and mutant strains Δ*hmpW*, Δ*hmpU*, and Δ*hmpV* at 24 h post induction for hormogonia. ................................................................................................................58

15. Immunoblot analysis of overall PilA and HmpD levels in wild type (WT), Δ*hmpW*, Δ*hmpU* and Δ*hmpV* strains. ........................................................................................................59

16. Immunofluorescence analysis of extracellular PilA for wild-type (WT) and mutant strains Δ*hmpW*, Δ*hmpU*, and Δ*hmpV* ..............................................................................61

17. Hormogonium polysaccharide (HPS) analysis for wild-type (WT) and mutant strains Δ*hmpW*, Δ*hmpU*, and Δ*hmpV*. ..................................................................................63

18. Light micrographs taken with a dissecting microscope of plate motility assays for wild type, single deletion mutant strains, and the Δ*hmpW*, Δ*hmpV* double-deletion mutant strain (as indicated). .................................................................64

19. A model of the partner-switching regulatory system in filamentous cyanobacteria comprised of HmpU, HmpW, and HmpV .....................................................................66
Chapter 1: Introduction

Significance of Cyanobacteria

In the vast and diverse microbial world, cyanobacteria stand out as prokaryotes of enormous significance. They are among Earth’s most ancient life forms, having first appeared as early as 3 to 3.5 billion years ago (Knoll 2015; Nisbet et al. 2007). As the first organisms on Earth to perform oxygenic photosynthesis and the only organism known to independently evolve the ability to do so, cyanobacteria were responsible for the rapid accumulation of atmospheric oxygen levels that occurred about 2.3 to 2.5 billion years ago, commonly referred to as the Great Oxidation Event, or GOE (Holland 2002; Lyons, Reinhard, Planavsky 2014). The biological ramifications of the GOE cannot be understated, as this significant change in the physical environment paved the way for the evolution of all oxygen-based life forms on Earth (Blaustein 2016). Additionally, multiple lines of evidence support the widely accepted theory that the chloroplasts performing photosynthesis inside plants today first originated through endosymbiosis of a proto-eukaryotic cell with cyanobacteria over a billion years ago (Gray 1999; McFadden 2014; Sagan 1967).

In addition to their extraordinary historical impact, cyanobacteria continue to play a major role in global carbon and nitrogen cycles today (Elbert et al. 2012). Photosynthesis by cyanobacteria converts solar energy into chemical energy through the
fixation of atmospheric carbon dioxide into organic molecules. Many cyanobacteria also possess the ability to fix nitrogen gas from the atmosphere into ammonia that can be used as a building block for biosynthesis of amino acids, nucleic acids, and other macromolecules (Meeks and Elhai 2002). Nitrogen-fixing cyanobacteria can form symbioses with plant and fungi partners to provide them with their nitrogen needs, and in return the cyanobacteria benefit by growing in cavities that protect them from competitors and predators (Meeks and Elhai 2002). The ongoing study of cyanobacteria continues to elucidate their myriad roles and significance, and could have potentially valuable biotechnology applications, including the bioengineering of cyanobacteria for production of biofuels or for use as biofertilizers to provide fixed nitrogen for economically important crops.

**Four Developmental Alternatives of Nostoc punctiforme**

Filamentous cyanobacteria such as *Nostoc punctiforme* are complex, multicellular organisms that can differentiate into multiple cell types, making them useful for the study of development and differentiation in prokaryotes (Meeks et al. 2002). *N. punctiforme* in particular serves as an excellent model organism for filamentous cyanobacteria, as it has a fully sequenced genome, is easily cultured in the lab, and can be readily genetically manipulated. *N. punctiforme* also has the capability to differentiate into the full range of all four distinct cell types, or developmental alternatives, that have been characterized in filamentous cyanobacteria (Meeks et al. 2002).

When environmental conditions are favorable and nutrients are unlimited, the vegetative cell cycle is maintained (Fig. 1A) (Meeks et al. 2002). Vegetative cells photosynthesize, grow, and divide by binary fission (Meeks and Elhai 2002). When
lacking a source of combined nitrogen, vegetative filaments can terminally differentiate into specialized nitrogen-fixing cells called heterocysts (Meeks and Elhai 2002). This differentiation takes place in a nonrandom spatial pattern, often occurring at the ends of filaments and evenly spaced out about every 8-15 cells along the filament (Meeks and Elhai 2002). The oxygen-sensitive enzyme nitrogenase is utilized by heterocysts to catalyze the conversion of atmospheric nitrogen into ammonia, so heterocysts provide a microoxic environment for nitrogenase by repressing the systems for photosynthesis and synthesizing a bilayered envelope of glycolipids and polysaccharides as a physical barrier around the cell (Meeks et al. 2002). Heterocysts can be visually distinguished from vegetative cells by their slightly larger size and rounder shape, thicker cell envelope, lack of green pigment, and by the accumulation of the nitrogen-storage polymer cyanophycin in granules at the cell poles (Fig. 1B) (Meeks et al. 2002; Muro-Pastor and Hess 2012).
Figure 1: Phase contrast photomicrographs of \textit{N. punctiforme}, illustrating its four developmental alternatives. \textbf{A.} \textit{NH}_4^+-grown filaments consisting of entirely undifferentiated vegetative cells. \textbf{B.} An \textit{N}_2-grown filament from a mid- to late-exponential phase culture showing three heterocysts (h) present in a nonrandom spacing pattern in the filaments. \textbf{C.} An early stationary phase \textit{N}_2-grown filament, showing akinete (a) differentiation starting near the midpoint of the interval between two heterocysts (h). The \textit{N}_2 filament is flanked by two \textit{NH}_4^+-grown filaments in this slide, made by immediately mixing differently grown cultures. \textbf{D.} Hormogonium filaments, showing the smaller size and different shape of the cells, relative to vegetative filaments. Bars = 10 µm (Meeks et al. 2002)
In response to light or nutrient deprivation, vegetative cells can differentiate into spore-like cells called akinetes (Meeks et al. 2002). Akinetes are significantly larger in size than vegetative cells, thicker walled, and usually contain visible granulation of glycogen and cyanophycin (Fig. 1C) (Adams and Carr 1981; Meeks et al. 2002). Like the endospores of gram-positive bacteria, akinetes are a more metabolically dormant cell type and are more resistant to extreme cold and desiccation than vegetative cells, so they can aid in overwintering and long-term survival (Meeks et al. 2002). However, akinetes are not resistant to high temperatures and typically maintain higher levels of metabolic activity than gram-positive endospores (Meeks et al. 2002). When environmental conditions become favorable again, akinetes can germinate to form vegetative filaments (Meeks et al. 2002).

In response to a variety of changes in environmental conditions, *N. punctiforme* and some other filamentous cyanobacteria can differentiate into a fourth developmental alternative, specialized filaments called hormogonia that are capable of gliding motility across surfaces (Meeks et al. 2002). Hormogonia enter a non-growth state and do not increase their biomass before dividing, resulting in visibly smaller cells of a distinct shape when compared to vegetative filaments (Fig. 1D) (Meeks et al. 2002). Hormogonium filaments also tend to be shorter in length than vegetative filaments, with terminal cells that often appear pointed or conical (Meeks et al. 2002). Hormogonium filaments lack heterocysts and do not possess the ability to fix nitrogen, although they do contain functional photosystems and continue to photosynthesize (Meeks and Elhai 2002). Hormogonium differentiation is essential for short-term dispersal of filaments and hormogonia serve as the infective unit to form symbioses with plant or fungi partners.
(Meeks and Elhai 2002). Hormogonia are a transient state; filaments typically stay motile for a period of 48 to 72 hours, after which they will begin to differentiate back to the vegetative state (Campbell and Meeks 1989).

**Hormogonium Development and Motility**

Detailed models of the mechanism by which hormogonia move and the molecular regulation of hormogonium differentiation are paramount to creating a deeper understanding of the developmental complexity of filamentous cyanobacteria, and could also play a key role in potential bioengineering applications. Although this represents an area of emerging and dynamic research and much remains to be studied, recent findings have begun to illuminate some of the key components of these models (Cho et al. 2017; Khayatan et al. 2017; Khayatan, Meeks, Risser 2015; Risser and Meeks 2013; Risser, Chew, Meeks 2014). A number of different environmental factors such as changes in light intensity, varying nutrient concentrations, and secretions from symbiotic plant partners have been shown to positively and negatively influence hormogonium development in *N. punctiforme* (Meeks and Elhai 2002), and the current working hypothesis is that these factors converge on a master regulator, from which two independent genetic pathways are initiated (Fig. 2) (Khayatan et al. 2017).
One of these pathways is dependent upon the *hmp* (hormogonium motility and polysaccharide) locus, which encodes five genes with homology to chemotaxis-like proteins (Risser, Chew, Meeks 2014). Four of these genes, *hmpB, C, D, and E*, have been shown to regulate hormogonium development and are essential for motility (Risser, Chew, Meeks 2014). A second locus, designated *hps* (hormogonium polysaccharide), is conserved exclusively in filamentous cyanobacteria and encodes a putative polysaccharide secretion system (Risser and Meeks 2013). The *hps* locus includes several genes for glycosyl transferases to synthesize a hormogonium polysaccharide (HPS) (Risser and Meeks 2013). Presumably, the master regulator activates expression of the *hmp* locus, which in turn activates expression of the *hps* genes that lead to synthesis of HPS (Khayatan et al. 2017; Risser, Chew, Meeks 2014).

**Figure 2:** A model of the gene regulatory network promoting hormogonium development, depicting genes or gene clusters essential for functional hormogonia. Arrows indicate positive interactions between genes or their mRNA or protein products, and lines with bars indicate negative interactions. Timing of events, based on gene expression, is depicted above. Based on the evidence presented here, regulation of *pilA* by *ogtA* is posttranscriptional, and it is currently unknown if *ogtA* regulates other components of the type 4 pilus (T4P) system in addition to *pilA*. (Khayatan et al. 2017)
The second independent pathway initiated from the master regulator involves expression of \( ogtA \), a gene encoding a putative O-linked \( \beta-N \)-acetylglucosamine transferase that is necessary for accumulation of PilA, the major pilin protein in the Type IV Pilus (T4P) complex that is required for motility in hormogonia (Khayatan et al. 2017). The exact manner in which \( ogtA \) acts in \( N. \) punctiforme is still undefined, but evidence points to posttranscriptional regulation of pilA (Khayatan et al. 2017). Together, pilA and a collection of other genes from the pil and hps loci are thought to produce components of the T4P system that drives gliding motility and the secretion of HPS in hormogonia (Khayatan, Meeks, Risser 2015; Risser and Meeks 2013).

Characterization of \( N. \) punctiforme mutant strains containing in-frame deletions of T4P-like genes has elucidated the roles of their protein products in the T4P complex (Khayatan, Meeks, Risser 2015). The T4P system in \( N. \) punctiforme appears to function in much the same way as the classic model of a T4P system (Fig. 3, Fig. 4A) (Hospenthal, Costa, Waksman 2017; Khayatan, Meeks, Risser 2015), constructed from studies of bacteria such as \( Myxococcus \) xanthus (McBride 2001). The pilus is composed primarily of PilA protein monomers, which are extended and retracted through the outer membrane pore-like secretin PilQ (Chang et al. 2016; Khayatan, Meeks, Risser 2015). The ATPases PilB and PilT1 power extension and retraction, respectively, via interaction with the inner membrane protein PilC, which is thought to transduce the force from PilB or PilT1 into rotational movement and result in the physical addition or removal of PilA monomers from the pilus (Chang et al. 2016; Khayatan, Meeks, Risser 2015). Together, PilM, N, O, and P form an alignment complex which aligns the PilQ pore in the outer membrane with the motor complex in the inner membrane consisting of PilB, T1, and C.
(Chang et al. 2016; Khayatan, Meeks, Risser 2015). The putative minor/pseudopilins HpsB, C, D, and H may also be incorporated into the structure of the pilus (Khayatan, Meeks, Risser 2015). HpsE, F and G are likely involved in the assembly of the HPS, which is thought to be secreted out of the cell via the PilQ pore through cycles of extension and retraction of the pilus (Khayatan, Meeks, Risser 2015).

Figure 3: General type IV pilus (T4P) architecture, biogenesis and structure. Components that constitute the T4P system are shown. Pilins are inserted into the inner membrane by the SecYEG translocon (not shown) and are then incorporated into the base of the growing pilus. The T4P machinery is composed of the outer membrane secretin subcomplex (PilQ, TsaP; the pilotin PilF is not included in this figure), the alignment subcomplex (PilM–PilN–PilO–PilP), and the inner membrane motor subcomplex (PilC–PilB–PilT). ATP hydrolysis by PilB and PilT provides energy for pilus elongation and retraction, which may involve the rotation of PilC. (Hospenthal, Costa, Waksman 2017)
The current evidence supports a model in *N. punctiforme* in which the pili are extended from the leading pole of each cell, attach to secreted HPS associated with the cell surface along the length of the filament and are subsequently retracted, thereby
pulling on the HPS and propelling the filament in the forward direction (Cho et al. 2017). This model is supported by immunofluorescence that shows the extension of PilA from the cell junctions in the direction of motility, opposite the location of the HPS trails (Fig. 4B-D) (Cho et al. 2017).

Filamentous cyanobacteria display positive phototaxis (Campbell et al. 2015), but the T4P motor systems remain static in rings localized to both cell poles near septal junctions (Khayatan, Meeks, Risser 2015; Risser, Chew, Meeks 2014), so some mechanism must exist for coordinating the activation of the motor complex at the leading poles to facilitate the directional motility of filaments. A recent study provided key insights into the mechanism by which coordinated extension and retraction of the pili from the leading cell pole are thought to be achieved, via dynamic polar switching of the newly characterized protein HmpF (Cho et al. 2017). HmpF directly activates the T4P system at the leading pole, possibly through interaction with the extension ATPase PilB (Fig. 5) (Cho et al. 2017). In response to changes in light intensity, HmpF can relocate to the other side of the cell and activate the T4P complex at the opposite pole, resulting in a reversal in the direction of the filament’s movement (Cho et al. 2017). The coordinated polar switching of HmpF is hypothesized to be regulated by the chemotaxis-like proteins in the Hmp and Ptx systems to achieve phototaxis (Cho et al. 2017).
Partner-Switching Regulatory Systems

In a number of different bacteria, partner-switching regulatory systems (PSRSs) have been shown to control a variety of biological processes in response to environmental stimuli. The canonical example of the bacterial PSRS is the Regulator of SigmaB (Rsb) system involved in modulating the general stress response in the gram-positive bacterium *Bacillus subtilis* (Fig. 6) (Igoshin et al. 2007; Yang et al. 1996). In this system, an RsbU or RsbP protein phosphatase is activated in response to perceived environmental or energy stress, respectively (Igoshin et al. 2007). The active phosphatase can dephosphorylate a protein called RsbV, sometimes referred to as the “anti-anti-sigma factor” or “anti-sigma antagonist.” In its unphosphorylated form, RsbV is active and can bind and sequester the “anti-sigma factor” RsbW, to prevent it from binding and inhibiting the sigma factor $\sigma^B$ (Yang et al. 1996). This allows the free $\sigma^B$ to interact with RNA polymerase to form the holoenzyme complex, which can initiate transcription of

**Figure 5:** A model of how polar switching of HmpF regulates T4P activity and directional motility in *N. punctiforme*. Depicted are the regulatory interactions between HPS (yellow) and the T4P (blue), Hmp (grey) and Ptx (white) systems. (Cho et al. 2017)
specific target genes to respond to the stress. In this system, RsbW is often referred to as a “switch-protein,” as it can bind to either of two alternative partners, σ^B or RsbV (Yang et al. 1996). RsbW also has serine kinase capability and can phosphorylate RsbV, causing dissociation of the RsbW-RsbV interaction and freeing RsbW to re-bind and sequester the sigma factor (Yang et al. 1996). A similar PSRS also functions in *B. subtilis* to regulate availability of the sporulation-specific sigma factor σ^F (Igoshin et al. 2007), and partner-switching modules have been implicated in the regulation of virulence systems in gram-positive bacteria ranging from *Staphylococcus aureus* (Jonsson et al. 2004; Ziebandt et al. 2001) to *Mycobacterium tuberculosis* (Beaucher et al. 2002; Parida et al. 2005).

![Conceptual model of the PSRS regulating σ^B in *B. subtilis*.](image)

**Figure 6:** Conceptual model of the PSRS regulating σ^B in *B. subtilis*. Level of free σ^B is determined by the level of unphosphorylated RsbV anti-anti-sigma (V). Activity of either the RsbU environmental phosphatase or the RsbP energy phosphatase increases when a stress is perceived, turning the system on. W = the anti-sigma/serine kinase switch protein, RsbW. (Igoshin et al. 2007)
Although first characterized in gram-positive bacteria, there are also examples of gram-negative bacteria that have been shown to utilize PSRSs comprised of Rsb-like proteins. In the gram-negative respiratory pathogen *Bordetella*, a PSRS regulates the Type III Secretion System used to successfully colonize and infect a host’s trachea (Kozak et al. 2005). The aquatic proteobacterium *Shewanella oneidensis* can respond to environmental stressors and survive in harsh and diverse environments, both aerobic and anaerobic, by regulating the general stress response sigma factor $\sigma^B$ using a PSRS with homology to the Rsb system (Bouillet et al. 2016). In the parasitic bacterium *Chlamydia trachomatis*, the causative agent of the sexually transmitted infection chlamydia in humans, an Rsb-like PSRS has been identified that regulates availability of the primary sigma factor $\sigma^{66}$ and allows *C. trachomatis* to alter its growth rate in response to conditions inside the host cell and persist, surviving the host’s immune response (Thompson et al. 2015).

In some gram-negative bacteria, the Rsb-like PSRS may be working through a different mechanism than the direct regulation of a sigma factor by RsbW. The bioluminescent marine bacterium *Vibrio fischeri* uses a PSRS to regulate biofilm production in order to successfully colonize the light organ of its symbiotic partner, the Hawaiian bobtail squid *Euprymna scolopes* (Morris and Visick 2013). The two major protein components of this regulatory system, SypE and SypA, contain domains that are structurally homologous to Rsb proteins, although there are some key distinctions between this system and the Rsb system from *B. subtilis* (Fig. 7A-B) (Morris and Visick 2013). In addition to a conserved response regulator receiver (REC) domain, SypE
contains both an RsbU-like PP2C phosphatase and an RsbW-like serine kinase domain, and thus can function to either dephosphorylate or phosphorylate the RsbV-like protein SypA, presumably in response to an input received by phosphoryl transfer to, or removal from, the SypE REC domain (Morris and Visick 2013). The evidence supports a model in which the unphosphorylated form of the RsbV-like protein SypA is active and can work as the output of the regulatory system to influence an as-of-yet undefined target to promote biofilm formation and host squid colonization (Morris and Visick 2013).
Figure 7: Models of PSRSs in *B. subtilis* and *V. fischeri*.  
**A.** Model of the *B. subtilis* partner-switching system that regulates the activity of σ^B^ in the general stress response pathway. Shown are the relevant domains for partner-switching regulation. **B.** Model of *V. fischeri* biofilm regulation by SypE and SypA. SypE contains a central Response Regulator REC domain flanked by an N-terminal RsbW-like serine kinase domain and a C-terminal PP2C-like serine phosphatase domain. SypA contains a single STAS-domain conserved in anti-sigma factor antagonists, like RsbV. Under conditions in which SypE is unphosphorylated, its N-terminal kinase domain is active, resulting in the phosphorylation of SypA (on conserved serine residue S56) and inhibition of biofilm formation. When phosphorylated (presumably on conserved residue D192), SypE functions as a phosphatase. This results in the dephosphorylation of SypA, activating SypA to promote biofilm formation. A D192A mutation ‘locks’ SypE into a constitutive kinase, resulting in SypA phosphorylation and the inhibition of biofilms and colonization. Protein phosphatase 2C (PP2C) domain (black box); Serine/threonine kinase (RsbW) domain (white box); anti-sigma factor antagonist (STAS) domain (grey box); response regulator receiver (REC) domain (light grey box). (Morris and Visick 2013)
Recent research also provides support for a similar PSRS model in the purple bacterium *Rhodobacter capsulatus*, whereby the RsbV homolog is thought to work as the output of the system to directly interact with a target and promote the expression of gene transfer agents that can mediate the transfer of alleles to other nearby strains of *R. capsulatus* (Mercer and Lang 2014). Together, these examples from *V. fischeri* and *R. capsulatus* support a role for RsbV as the effector in some partner-switching systems and represent an alternative to the classically described Rsb PSRS model from *B. subtilis*. As of yet, there is no evidence to suggest that a sigma factor is being directly regulated by the RsbV homologs in either of these PSRSs.

**A PSRS Regulating Motility in *N. punctiforme***

This study identified and characterized the components of what is thought to be a complete Rsb-like PSRS in the gram-negative filamentous cyanobacterium *Nostoc punctiforme*. A transposon mutagenesis screen to identify genes involved in hormogonium development and motility recovered an insertion in the gene Npun_R5135, which encodes a homolog of the RsbU phosphatase in *N. punctiforme*. Immediately adjacent to Npun_R5135 and in the same orientation in the genome lies the gene Npun_R5134, with homology to *rsbW*. This led to the hypothesis that a PSRS was functioning to modulate hormogonium development and/or motility in *N. punctiforme*. A comparative genomics approach followed by bacterial two-hybrid analysis were used to identify the corresponding Rsb-V like component of the system, and a variety of experimental techniques were employed to characterize the roles of each of the components in the PSRS and develop a model for how the system may be working to regulate hormogonium motility in *N. punctiforme*. 
Chapter 2: Materials and Methods

Comparative Genomics Analysis

Comparative genomics analysis was performed using the IMG Genome Gene Best Homologs tool (Markowitz et al. 2012) set to the lowest percent identity cutoff (20+) with N. punctiforme as a reference genome to query each of 124 different cyanobacterial genomes with complete, partial, or draft sequences available in the database (Shih et al. 2013), or vice versa. This data was then used to assemble a database containing the percent identity for the corresponding reciprocal best hit homolog for each N. punctiforme predicted protein in each of the cyanobacterial genomes. Select genes from this dataset were then used to generate heat maps using Genesis (Sturn, Quackenbush, Trajanoski 2002).

Bacterial Two-Hybrid Analysis

**Plasmid construction for bacterial two-hybrid analysis.** To construct plasmids encoding proteins of interest fused to either the T18 or T25 fragment of Bordetella pertussis adenylate cyclase for bacterial adenylate cyclase two-hybrid (BACTH) analysis, the coding region of each gene was amplified via PCR and cloned into either pUT18c or pKT25 as XbaI-KpnI fragments using restriction sites introduced on the primers. For each RsbV-like coding gene, site directed mutagenesis using overlap extension PCR was
used to replace the codon for the putative phosphoserine with a codon for alanine, which has been previously shown to stabilize RsbW-RsbV interactions (Yang et al. 1996).

**Preparing cultures for bacterial two-hybrid analysis.** BTH101 (adenylate cyclase-deficient) *E. coli* strains transformed with appropriate plasmids were streaked onto Lysogeny Broth (LB) agar plates containing 100 µg/ml Ampicillin and 50 µg/ml Kanamycin, and incubated at 30°C for 48 h. For each strain, 1 ml of LB containing 100 µg/ml Ampicillin, 50 µg/ml Kanamycin and 0.5 mM Isopropyl β-D-1-thiogalactopyranoside (IPTG) was inoculated with several colonies from the plate, and cultures were then incubated overnight at 30°C with shaking at 230 rpm.

**MacConkey agar assay.** 2 µl were taken from each overnight culture and spotted onto MacConkey agar plates containing 100 µg/ml Ampicillin and 50 µg/ml Kanamycin. Plates were incubated at 30°C for 48 h.

**Quantitative beta-galactosidase assay.** Quantitative beta-galactosidase assays were performed as previously described (Zhang and Bremer 1995), with several modifications. 20 µl of each overnight culture were added to 980 µl of LB containing 100 µg/ml Ampicillin, 50 µg/ml Kanamycin and 0.5 mM IPTG, and incubated at 30°C with shaking at 230 rpm until an OD_{600} of 0.28-0.70 was reached. 20 µl of each sample were added to 80 µl of permeabilization solution (100 mM Na_{2}HPO_{4}, 20 mM KCl, 2 mM MgSO_{4}, 0.8 mg/ml hexadecyltrimethylammonium bromide, 0.4 mg/ml sodium deoxycholate, 5.4 µl/ml β-mercaptoethanol), and incubated for 30 min at 30°C. 600 µl of substrate solution (60 mM Na_{2}HPO_{4}, 40 mM NaH_{2}PO_{4}, 1 mg/ml ortho-Nitrophenyl-β-galactoside, 2.7 µl/ml β-mercaptoethanol, warmed to 30°C) were added to each sample to
initiate the reaction. After incubation for 40 min at 30°C, there was a detectable yellow color in the positive control sample and all reactions were terminated by addition of 700 µl of stop solution (1 M Na₂CO₃). Samples were centrifuged at maximum speed for 5 min to separate out particulate matter, and OD₄₂₀ was measured for the supernatant of each sample. The average Miller Units (Miller 1972) were calculated for each strain as a standardized measure of beta-galactosidase activity, from two technical replicates for each of three biological replicates.

**Nostoc punctiforme Culture Conditions**

*N. punctiforme* strain ATCC 29133 and mutant strains were cultured in Allen and Arnon medium diluted fourfold (AA/4) as described previously (Campbell et al. 2007) with 4 mM sucralose added to the medium to inhibit hormogonium differentiation (Splitt and Risser 2015). Cultures were grown at room temperature under continuous light with slow shaking at 120 rpm.

**Plasmid and N. punctiforme Mutant Strain Construction**

The strains and plasmids used in this study can be found in Table 1. The primers used for construction of plasmids are listed in Table 2. All constructs were sequenced to ensure sequence fidelity. For in-frame deletion of genes, approximately 900 bp of flanking DNA on either side of the gene and the first and last three or four codons of each gene were amplified via overlap extension PCR and were then cloned into pRL278 as BamHI-SacI fragments using restriction sites introduced on the primers.

To create *E. coli* strains harboring the deletion constructs, the appropriate plasmids were each transformed into UC585 competent cells as follows: 1 µl of plasmid
DNA was added to 16 µl of UC585 competent cells, mixed, and then incubated on ice for
15 min. This was followed by a 2 min heat shock at 42°C and then recovery on ice for 2
min. 800 µl of LB were added and samples were incubated at 37°C for 2 h with shaking
at 230 rpm. 10 µl of each sample were plated on LB agar containing 50 µg/ml
Kanamycin, 50 µg/ml Ampicillin, and 15 µg/ml Chloramphenicol, and plates were
incubated overnight at 37°C. A single colony from the plate was used to inoculate 2 ml
of Terrific Broth (TB) containing 50 µg/ml Kanamycin, 50 µg/ml Ampicillin, and 15
µg/ml Chloramphenicol, and liquid cultures were grown overnight at 37°C with shaking
at 230 rpm.

To create each of the _N. punctiforme_ deletion mutant strains ΔhmpW (UOP119),
ΔhmpU (UOP113), and ΔhmpV (UOP150), the corresponding plasmid was introduced by
conjugal transfer from _E. coli_ into the _N. punctiforme_ wild-type strain ATCC 29133. To
create the ΔhmpW, ΔhmpV (UOP151) double-deletion mutant strain for epistasis analysis,
the ΔhmpV plasmid (pKR101) was transferred by conjugation from _E. coli_ into the
ΔhmpW strain. Conjugations were performed as follows: 250 µl of the overnight _E. coli_
culture was used to inoculate 20 ml of LB containing 50 µg/ml Kanamycin, 50 µg/ml
Ampicillin, and 15 µg/ml Chloramphenicol, and tubes were incubated at 37°C with
shaking (230 rpm) for approximately 3 h, until an OD_{600} of 0.9-1.2 was reached.
Samples were then centrifuged for 10 min at 3,000 g, the supernatant was discarded, and
the cell pellet was washed once with 4 ml of fresh LB containing no antibiotics. The
concentrated _E. coli_ pellet was then mixed with the equivalent of 50 µg/ml chlorophyll _a_
(Chl _a_) of cell material from the appropriate _N. punctiforme_ strain. Here, and throughout
the study, the equivalent concentrations of Chl _a_ were established by methanol extraction.
as follows: 1 ml of the *N. punctiforme* culture was centrifuged at maximum speed for 1 min, and then 900 µl of the supernatant was discarded and replaced by 900 µl of methanol. Samples were resuspended in the methanol and incubated at room temperature in the dark for 5 min, followed by centrifugation at maximum speed for 1 minute, and then the absorbance of the supernatant was measured at 665 nm.

200 µl of the *E. coli / N. punctiforme* cell mixture were plated on AA/4 containing 0.5% LB, 5 mM 3-Morpholinopropane-1-sulfonic acid (MOPS), 2.5 mM NH$_4$Cl, and 10mM sucralose. Plates were incubated overnight at room temperature under continuous light. The next day, cells were transferred to AA/4 plates containing 5 mM MOPS and 2.5 mM NH$_4$Cl. After incubation for 3 days at room temperature under continuous light, each plate was overlayed with 5 ml of liquid AA/4 medium containing 5 mM MOPS, 2.5 mM NH$_4$Cl, and 50 µg/ml Neomycin. Plates were then incubated at room temperature under continuous light for several weeks for selection.

A Neomycin-resistant single recombinant colony was selected, inoculated into liquid AA/4 media containing 50 µg/ml Neomycin, 5 mM MOPS, and 2.5 mM NH$_4$Cl, and incubated under continuous light with shaking (120 rpm) for 2 weeks. The single-recombinant was then subcultured in liquid AA/4 + 5 mM MOPS + 2.5 mM NH$_4$Cl media containing 50 µg/ml Neomycin for 2 weeks, and then subcultured again in liquid AA/4 + 5 mM MOPS + 2.5 mM NH$_4$Cl media without antibiotic selection for 2 weeks to allow for the second recombination event. This was followed by plating on AA/4 with 5% sucrose for counter-selection, and the plates were grown at room temperature under continuous light until colonies formed. The mutants were distinguished from the wild type by performing a colony PCR in which half of a colony was mixed with 5 µl 2x
MangoMix™ (BIOLINE kit), 0.2 µl of each primer (NpR5134-5'-F, NpR5134-3'-R for ΔhmpW, or NpR5135-5'-F, NpR5135-3'-R for ΔhmpU, or NpF5169-5'-F, NpF5169-3'-R for ΔhmpV), and 4.6 µl of water. The colony PCR reactions were performed in an Applied Biosystem 2720 Thermal Cycler under the following conditions: 5 min at 94°C, followed by 35 cycles of 30 sec at 94°C, 30 sec at 55°C, and 4 min at 72°C, followed by 7 min at 72°C and a final hold at 4°C.

**Hormogonium Induction**

The cell material equivalent of 30 µg/ml of Chl a was harvested from cultures grown in sucralose-containing media and centrifuged at 2,000 g for 3 min. The supernatant was discarded, and the cell pellets were washed twice in fresh AA/4 media, and finally resuspended in a final volume of 2 ml of fresh AA/4 media in a 24-well plate. Plates were incubated for 24 h at room temperature under continuous light with shaking at 120 rpm.

**Light Microscopy**

Ten µl of each sample were spotted on a slide containing dehydrated 1% agarose pads and covered with a cover slip. Samples were imaged at 400x using a Leica DME microscope attached to a Leica DFC290 camera controlled by Micro-Manager imaging software (Edelstein et al. 2014), and images were analyzed using ImageJ (NIH). 50 filaments were chosen at random and the presence/absence of heterocysts and average cell length from 5 contiguous vegetative cells were determined for each filament, in order to calculate the percent of total filaments with heterocysts and average vegetative cell length for each strain at 0 h and 24 h post-induction. This process was repeated in triplicate.
Time Lapse Microscopy & Motility Analysis

2 µl of each strain at 24 h post-induction for hormogonia were spotted onto a plate containing AA/4 with 0.5% Noble agar, and covered with a cover slip. Samples were imaged at 50x using a Leica MZ APO dissecting microscope attached to a Leica DFC290 camera controlled by Micro-Manager imaging software (Edelstein et al. 2014). Images were taken every 15 sec for 20 min, and resulting time lapse videos were analyzed using the wrMTrck plugin (Nussbaum-Krammer et al. 2015) for ImageJ (NIH) to determine the percent of total filaments that were motile, and the average and maximum speeds for motile filaments in each strain. These assays were repeated in triplicate.

Immunoblot of PilA & HmpD

Protein extraction. Cell pellets with the equivalent of 22.5 µg/ml Chl a taken from strains at 24 h post-induction for hormogonia were stored at -20°C. For protein extraction, pellets were thawed on ice, resuspended in 30 µl lysozyme solution (20 mg/ml lysozyme, 10 mM Tris, 25 mM ethylenediaminetetraacetic acid [EDTA], pH 8.0), and then incubated on ice for 1 hour. 60 µl of 20% sodium dodecyl sulfate (SDS) was mixed with each sample, and then tubes were incubated at 100°C for 10 min and gently mixed again. Samples were centrifuged at maximum speed for 1 min, the supernatant of each sample was transferred to a new tube, 800 µl of acetone was added to each tube, and tubes were vortexed. Tubes were then incubated on ice for 10 min, followed by centrifugation at maximum speed for 5 min. Supernatants from each sample were discarded, the samples were air-dried, and then 50 µl of 8% SDS was mixed with each sample and tubes were incubated at 50°C until resolubilized.
**Immunoblot Analysis.** Standard SDS-PAGE and previously described immunoblot procedures were followed (Risser, Chew, Meeks 2014; Wall, Wu, Kaiser 1998) using primary anti-PilA, anti-HmpD, and anti-RbCL rabbit polyclonal antisera in a 1:10,000 dilution, and horseradish peroxidase (HRP)-conjugated goat anti-rabbit secondary antibodies (Chemicon) in a 1:20,000 dilution. Enhanced chemiluminescent (ECL) detection of immunoblots was performed by addition of Clarity™ Western ECL blotting substrate (Bio-Rad Laboratories) and imaging in a Bio-Rad Molecular Imager ChemiDoc™ XRS+. Quantitative analysis of immunoblots was performed using ImageJ (NIH) to create a plot profile of the pixel intensities for each band, and then the area under the curve for each plot profile was calculated and normalized to the wild type average value. Immunoblot analysis was repeated in biological triplicate.

**Lectin Blot**

Supernatants taken from strains at 24 h post-induction for hormogonia were stored at 4°C. One hundred microliters100 µl of each supernatant sample were vacuum blotted onto a wet nitrocellulose membrane and the membrane was dried at 55°C for 5 min. The membrane was blocked for 30 min at room temperature with gentle shaking at 50 rpm in Carbo-Free™ Blocking Solution (Vector Laboratories), and then incubated for 1 h at room temperature with gentle shaking (50 rpm) in a Tris-buffered saline + 0.1% Tween20 (TBST) wash solution containing 0.5 µg/ml of biotinylated-*Ulex europaeus* agglutinin (UEA) lectin. The membrane was then washed 3 times in TBST, followed by incubation for 1 h at room temperature with gentle shaking (50 rpm) in VECTASTAIN® ABC reagent (Vector Laboratories). The membrane was again washed 3 times in TBST, followed by one wash in Tris-buffered saline (TBS). ECL detection of lectin blots was
performed by addition of Clarity™ Western ECL blotting substrate (Bio-Rad Laboratories) and imaging in a Bio-Rad Molecular Imager ChemiDocTM XRS+. Quantitative analysis of lectin blots was performed using ImageJ (NIH) to create a plot profile of the pixel intensities for each spot, and then the area under the curve for each plot profile was calculated and normalized to the wild type average value. The procedure was repeated for each of 3 biological replicates.

**Simultaneous PilA Immunofluorescence & UEA-Fluorescein Staining**

100 µl were taken from each strain at 0 h and 24 h post-induction for hormogonia and centrifuged at 2,000 g for 2 min, supernatant was discarded, pellet was resuspended in 100 µl of 4% paraformaldehyde, and samples were stored at room temperature in the dark for 1 to several days. To prepare for staining, stored samples were centrifuged at maximum speed for 1 min, and supernatant was discarded to remove the paraformaldehyde. Samples were washed twice with 500 µl of PBS (137 mM NaCl, 2.7 mM KCl, 10 mM Na₂HPO₄, 1.76 mM KH₂PO₄, pH 7.4) and resuspended in 50 µl of GTE solution (50 mM glucose, 25 mM Tris-Cl, pH 8.0, 10 mM EDTA). 20 µl of each sample was spotted on a poly-L-lysine coated slide (Sigma-Aldrich Co.) and air-dried. Slides were incubated for 5 min in -20°C methanol, followed by a 30 sec incubation in -20°C acetone, and allowed to air dry completely. Slides were incubated at room temperature for 30 min in a blocking solution of 2% bovine serum albumin (BSA) in PBS, followed by incubation at room temperature for 1 h in blocking solution containing a 1:250 dilution of anti-PilA rabbit polyclonal antiserum. Slides were then washed 3 times with PBS, followed by incubation for 1 h at room temperature in blocking solution containing a 1:500 dilution of CF405m-labeled goat anti-rabbit secondary antibodies (Biotium, Inc.)
and a 1:200 dilution of UEA-fluorescein (Vector Laboratories). Slides were washed again 3 times with PBS, allowed to air dry completely, and then 10 µl of EverBrite™ Hardset mounting medium (Biotium, Inc.) was added to fix each sample and samples were covered with a cover slip. Slides were incubated in the dark overnight for the mounting medium to set, and then stored at 4°C until visualization with fluorescence microscopy.

**Fluorescence Microscopy**

Images of cellular autofluorescence, PilA immunofluorescence (IF), and UEA-fluorescein labeled hormogonium polysaccharide (HPS) were obtained with a Leica DMIRE2 inverted fluorescence microscope using MetaMorph software (Molecular Devices) and a Yokogawa CSU-X1 spinning disc confocal scanner unit with a QuantEM: 5125C camera. Excitation and emission were as follows: 491 nm excitation and 525 (±25) nm emission for UEA-fluorescein, 405 nm excitation and 525 (±25) nm emission for fluorescently-labeled PilA, and 561 nm excitation and 605 (±25.5) nm emission for cellular autofluorescence. Images were analyzed using ImageJ (NIH). Quantitative analysis of PilA IF was performed as follows: The average of the total pixel intensity values for PilA IF images of samples at 0 h (uninduced for hormogonia) was subtracted from each of the total pixel intensity values of PilA IF images of samples at 24 h post-induction for hormogonia, and then a ratio was taken of each resulting value to the corresponding pixel intensity of the cellular autofluorescence for the image. The mean ratio value was calculated from 3 images of each of 3 biological replicates for each strain, and then normalized to the average wild type value. Similarly, for UEA-fluorescein quantitative analysis, the average of the total pixel intensity values for UEA-fluorescein
images of samples at 0 h (uninduced for hormogonia) was subtracted from each of the total pixel intensity values of UEA-fluorescein images of samples at 24 h post-induction for hormogonia, and then a ratio was taken of each resulting value to the corresponding pixel intensity of the cellular autofluorescence for the image. The mean ratio value was calculated from 3 images of each of 3 biological replicates for each strain, and then normalized to the average wild type value.

**Plate Motility Assays**

All plate motility assays were performed in triplicate. Strains were streaked onto plates containing AA/4 supplemented with 10 mM sucralose, and then individual colonies were picked from the plates and patched onto AA/4 plates containing 0.5% NA. Plates were incubated at room temperature under continuous light for 48 h, and then colonies were imaged using a Leica MZ APO dissecting microscope equipped with a Leica DFC290 camera controlled by Micro-Manager imaging software (Edelstein et al. 2014).
Table 1. Strains and Plasmids Used in This Study

<table>
<thead>
<tr>
<th>Strains</th>
<th>Relevant Characteristic(s)</th>
<th>Source</th>
</tr>
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<tbody>
<tr>
<td>Nostoc punctiforme strains</td>
<td></td>
<td></td>
</tr>
<tr>
<td>ATCC 29133</td>
<td>Wild type</td>
<td>ATCC</td>
</tr>
<tr>
<td>UCD153</td>
<td>Laboratory derivative of <em>N. punctiforme</em> ATCC 29133 with reduced motility</td>
<td>(Buikema and Haselkorn 2001)</td>
</tr>
<tr>
<td>UOP113</td>
<td>ΔhmpU (Npun_R5135) *</td>
<td>This study</td>
</tr>
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<td>UOP119</td>
<td>ΔhmpW (Npun_R5134)</td>
<td>This study</td>
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<tr>
<td>UOP150</td>
<td>ΔhmpV (Npun_F5169)</td>
<td>This study</td>
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<td>TNM219</td>
<td>UCD153 with a Tn5-1063 insertion after nucleotide 546 of hmpU (Npun_R5135)</td>
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† numbers in brackets correspond to primers used to construct plasmid. Detailed information on primers can be found in Table 2.
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Chapter 3: Results

Npun_F5169 is the Corresponding hmpV for a PSRS in *N. punctiforme*

Previously, a transposon mutagenesis study to find genes essential for hormogonium motility and/or development in *N. punctiforme* isolated a mutant strain (TNM219) with a transposon insertion in the gene Npun_R5135 (Fig. 8), which encodes a homolog of RsbU, a phosphatase that participates in the canonical Rsb PSRS of *B. subtilis* (Yang et al. 1996). Immediately downstream of Npun_R5135 and in the same orientation is Npun_R5134, which encodes a homolog of RsbW, the anti-sigma factor for the *B. subtilis* Rsb system (Yang et al. 1996). Given their proximity in the *N. punctiforme* genome, it was hypothesized that Npun_R5135 and Npun_R5134 could be co-transcribed, and that their protein products could be working together as Rsb-like components of a PSRS modulating hormogonium development and/or motility. Based on the results presented in this study (see below), the genes that encode the proteins in this putative PSRS in *N. punctiforme* were given the designation *hmp*, which has been used previously to denote genes involved in hormogonium motility and polysaccharide secretion (Risser and Meeks 2013). The gene Npun_R5135 was designated *hmpU*, and the gene Npun_R5134 was designated *hmpW*, following the letter designations in the *B. subtilis* Rsb system. Based on the Rsb system, it was hypothesized that there must also exist a gene *hmpV*, which encodes an RsbV homolog in *N. punctiforme*. 


Comparative genomics analysis identifies a putative HmpV for the Hmp PSRS. To identify a potential HmpV for the PSRS, a comparative genomics analysis was performed. BLASTP searches (NCBI) identified 4 RsbU homologs, 4 RsbW homologs, and 5 RsbV homologs in the predicted *N. punctiforme* proteome. A genome comparison across 124 cyanobacteria species revealed that HmpU and HmpW are exclusively conserved in nearly all filamentous cyanobacteria, with only the most distantly related filamentous species lacking these proteins (Fig. 9). Of the five RsbV homologs in *N. punctiforme*, only that encoded by Npun_F5169 had a similar conservation pattern to HmpU and HmpW, indicating that Npun_F5169 could encode the corresponding HmpV for the PSRS.

**Figure 8:** Schematic diagram depicting the genomic region harboring Npun_R5135 and Npun_R5134 in *N. punctiforme*. The gene Npun_R5135 encodes a protein product with an N-terminal regulatory receiver (REC) domain and a GAF domain, as well as a region encoding a C-terminal PP2C type (RsbU-like) protein phosphatase domain. Adjacent to Npun_R5135, the gene Npun_R5134 encodes a protein product with a single RsbW-like domain, indicating possible serine kinase and anti-sigma activity. The red triangle indicates the location of the transposon insertion in the GAF domain of Npun_R5135.
Bacterial two-hybrid analysis demonstrates protein-protein interaction between HmpV and HmpW. To further support the hypothesis that Npun_F5169 encodes the HmpV for the putative Hmp PSRS, a bacterial two-hybrid analysis was performed to test for protein-protein interactions between HmpW and each of the five RsbV homologs present in *N. punctiforme*. A previous study demonstrated that alteration of the putative phosphoserine on RsbV enhances the RsbW-RsbV protein-protein interaction (Yang et al. 1996). Therefore, the codon for the putative phosphoserine on each of the *rsbV* homologs was mutated to code for an alanine. Qualitative analysis by plating on MacConkey agar (Fig. 10A) was consistent with quantitative results from the beta-galactosidase assay (Fig. 10B), demonstrating that of the five RsbV homologs tested...
for interaction with HmpW, the only strong interaction occurred between HmpW and the putative HmpV protein encoded by Npun_F5169 (at a level comparable to that of the positive control). The data provides further evidence that Npun_F5169 encodes the corresponding HmpV in the PSRS.

**Figure 10:** Bacterial two-hybrid analysis of protein-protein interactions between HmpW and each RsbV homolog in the *N. punctiforme* genome, as identified previously by the BLASTP search (NCBI). **A.** Pictures of colonies grown on MacConkey agar. **B.** Quantification of Miller Units from beta-galactosidase assay. Error bars = +/- 1 SD
The gene *hmpV* (Npun_F5169) encodes a single domain protein, and the genomic context of *hmpV* indicates that it is monocistronic (Fig. 11). Following identification of *hmpV* as the cognate *rsbV* ortholog for the PSRS, an ongoing transposon mutagenesis study isolated a mutant strain with a transposon insertion in *hmpV*, providing additional evidence that this gene is important in hormogonium development and/or motility.

**Figure 11:** Schematic diagram depicting the genomic region containing the open reading frame Npun_F5169 (the putative *hmpV*). Red triangle indicates location of the transposon insertion.

**Characterization of the Hmp PSRS Modulating Hormogonium Motility in *N. punctiforme***

Mutant strains with in-frame deletions in Δ*hmpW*, Δ*hmpU*, and Δ*hmpV* were created for each of the components identified in the putative Hmp PSRS, and the strains
were characterized through a series of experimental methods to determine the role of each protein in the system and to further investigate how the system affects hormogonium development and/or motility.

**Plate motility assay reveals difference in colony spreading among deletion mutant strains.** Plate motility assays were performed on the wild-type strain and each mutant strain Δ*hmpW*, Δ*hmpU*, and Δ*hmpV* to determine the effect of each gene deletion on colony spreading. Previous research has shown that sucralose inhibits hormogonium differentiation (Splitt and Risser 2015), so colonies were patched onto media lacking sucralose to induce hormogonia development, and plates were then imaged under a dissecting microscope after 48 h (Fig. 12). When compared to the wild type, the Δ*hmpW* strain showed such an increase in colony spreading that very little of the center of the colony remained after 48 h and the dispersed filaments had spread so far that they were very diffuse and difficult to see in the image. The Δ*hmpU* and Δ*hmpV* strains both showed an obvious decrease in colony spreading compared to the wild type after 48 h, indicated by the presence of a dark, dense central colony in both images. The results are consistent with a model in which the PSRS is modulating some aspect of hormogonium development and/or motility.
Similar development of morphologically distinct hormogonia in all 3 deletion mutant strains after induction. Light microscopy images taken at 0 h and 24 h post induction for hormogonia demonstrate similar morphology among the wild type and all three deletion mutant strains ΔhmpW, ΔhmpU, and ΔhmpV (Fig. 13A). At 0 h (uninduced), the wild type and mutant strains all contain filaments with similarly sized vegetative cells and the presence of evenly spaced N₂-fixing heterocysts. Images taken 24 h post induction for hormogonia showed that all three deletion strains have similar morphological features as the wild type strain, including a smaller average cell length compared to 0 h and the absence of heterocysts from the filaments, known to be characteristics of hormogonia (Meeks et al. 2002). Quantitative analysis confirmed these observations (Fig. 13B).
Figure 13: Morphology of filaments at 0 h and 24 h post induction for hormogonia, by strain (as indicated). **A.** Light microscopy images at 0 h (uninduced) and 24 h (induced). Bar= 10 μm. **B.** Quantitative analysis of light microscopy images at 0 h (blue) and 24 h post induction for hormogonia (orange), showing the average cell length and the percent of total filaments with heterocysts present. Error bars = +/- 1 SD.
Difference in motility of deletion mutant strains after induction for hormogonia. Time-lapse microscopy was used to characterize the motility of wild-type and mutant strains Δ*hmpW*, Δ*hmpU*, and Δ*hmpV* at 24 h after induction for hormogonia, and analysis revealed a drastic reduction in motility for both the Δ*hmpU* and Δ*hmpV* strains compared to the wild type (Fig. 14). The percent of total filaments that were motile in the Δ*hmpU* and Δ*hmpV* strains was significantly reduced compared to the wild type, as were the average and maximum filament speeds in these mutant strains. In contrast, the percent of total filaments that were motile in the Δ*hmpW* strain was comparable to that of the wild type, but the Δ*hmpW* strain had a significantly faster average and maximum filament speed. Notably, despite a significant increase in the rate of motility, the variation between filaments in the Δ*hmpW* strain was lower than the wild-type strain, indicating that the motility of filaments in the Δ*hmpW* population was both faster and more homogenous.
Similar overall levels of PilA and HmpD protein expressed in all 3 deletion mutant strains after induction for hormogonia. As described previously in the introduction, PilA and HmpD are proteins that are specifically expressed in hormogonia and are part of independent genetic pathways (Khayatan et al. 2017), so they can be assessed to further characterize the role of the PSRS in hormogonium development and/or motility. To determine whether there is a significant difference in levels of PilA and HmpD proteins produced among the mutant strains that could account for the differences in motility observed through time-lapse microscopy, immunoblotting for PilA and HmpD was performed on the wild-type and mutant strains ΔhmpW, ΔhmpU, and ΔhmpV. Although there was some variation between immunoblots from the biological replicates, quantitative analysis demonstrated no major difference in levels of PilA or
HmpD among strains that could account for the differences observed in motility (Fig. 15A+B).

**Figure 15:** Immunoblot analysis of overall PilA and HmpD levels in wild type (WT), ΔhmpW, ΔhmpU and ΔhmpV strains.  

A. Image of immunoblot for PilA and HmpD from one biological replicate. Large subunit of RuBisCO (rbcL) was simultaneously detected as a loading control.  

B. Quantification of PilA and HmpD proteins from immunoblot images.  Values expressed as a percentage of the average PilA or HmpD for wild type hormogonia at 24 h post induction.  

Error bars = +/-1 SD
**Difference in levels of extracellular PilA among the 3 deletion mutant strains after induction for hormogonia.** Although no significant difference in the level of overall PilA protein was observed between the wild-type and mutant strains from the immunoblot analysis, the amount of PilA that is actually exported out of the cells through the T4P system could differ if the Hmp PSRS is regulating some component(s) of the T4P machinery. PilA immunofluorescence and visualization with fluorescence microscopy showed a significant reduction in levels of extracellular PilA in the ΔhmpU and ΔhmpV strains compared to the wild type strain (Fig. 16A), which was confirmed through quantitative analysis (Fig. 16B). The data implies that some aspect of the T4P motor complex could be affected in the ΔhmpU and ΔhmpV mutant strains that results in less PilA protein being exported out of the cells.
Difference in levels of cell-associated and soluble HPS among the 3 deletion mutant strains after induction for hormogonia. Previous research has shown that the extension and retraction of the pilus in the T4P system is essential for the secretion of the HPS (Khayatan, Meeks, Risser 2015; Risser and Meeks 2013). Because the results of the PilA immunofluorescence appeared to suggest that activity of the T4P motor could be
negatively affected in the ΔhmpU and ΔhmpV strains, HPS levels were assessed to determine if the amount of HPS secreted by each mutant strain differed significantly from the wild-type strain. Lectin blotting demonstrated reduced levels of soluble HPS in the ΔhmpU and ΔhmpV strains when compared to wild type (Fig. 17A). Levels of cell-associated HPS were also reduced compared to wild type when assessed by UEA-fluorescein staining and visualized by confocal microscopy (Fig. 17B). Quantitative analysis of both soluble and cell-associated HPS confirmed a reduction in the ΔhmpU and ΔhmpV strains when compared to wild type (Fig 17C), concomitant with the results of the PilA immunofluorescence, which further suggested that this PSRS could be influencing the T4P machinery.
Figure 17: Hormogonium polysaccharide (HPS) analysis for wild-type (WT) and mutant strains ΔhmpW, ΔhmpU, and ΔhmpV. A. Lectin blot image depicting levels of soluble HPS. Trials represent 3 biological replicates. B. Merged images from fluorescence micrographs depicting UEA-fluorescein staining of cell-associated HPS (green) and cellular autofluorescence (red) at 24 h post induction for hormogonia. Bar= 50 µm. C. Quantification of soluble HPS from lectin blot (blue) and cell-associated HPS from fluorescence micrographs (orange). Values expressed as a percentage of the soluble or cell-associated HPS for wild type hormogonia at 24 h post induction. Error bars = +/-1 SD

Epistasis analysis of double mutant strain ΔhmpW, ΔhmpV supports a model in which HmpV is the output of the PSRS. In the classic Rsb PSRS model described in B. subtilis, RsbW is the downstream effector and the system works to regulate a sigma factor (Yang et al. 1996). However, recent studies have shown that in some partner-switching systems, it is the RsbV-like protein that works as the output of the system to modulate a target (Mercer and Lang 2014; Morris and Visick 2013). To test whether HmpW or HmpV is the downstream effector of the putative Hmp PSRS in *N.*
punctiforme, an epistasis analysis of a ΔhmpW, ΔhmpV double-deletion mutant strain was conducted. If HmpW is the downstream effector, then the double mutant should have a phenotype indistinguishable from the ΔhmpW single mutant. In contrast, if HmpV is the downstream effector, then the double mutant should have a phenotype indistinguishable from the ΔhmpV single mutant. Plate motility assays with the double deletion strain (Fig. 18) showed an obvious reduction in colony spreading when compared to the wild type, similar to the phenotype of the single mutant strain ΔhmpV. This data supports a model in which HmpV is the output of the PSRS.

![Image of light micrographs](Figure 18: Light micrographs taken with a dissecting microscope of plate motility assays for wild type, single deletion mutant strains, and the ΔhmpW, ΔhmpV double-deletion mutant strain (as indicated). Bar = 5 mm.)
Chapter 4: Discussion

The evidence presented here supports a model (Fig. 19) in which HmpU, HmpW, and HmpV comprise a discrete PSRS involved in the regulation of hormogonium motility in *Nostoc punctiforme*. Supporting evidence for this model include the genomic proximity of *hmpU* and *hmpW*, the co-occurrence of HmpU, HmpW and HmpV orthologs in nearly all filamentous cyanobacteria, the specificity of HmpW for protein-protein interaction with HmpV, but not other RsbV homologs, and the antagonistic effects of HmpW versus HmpU and HmpV on hormogonium motility and extracellular levels of PilA and HPS.

As discussed previously, in canonical PSRSs the RsbW-like protein is the downstream effector and regulates a sigma factor (Igoshin et al. 2007), while in alternative PSRSs, the RsbV-like protein is the output of the system, working through an unknown mechanism (Mercer and Lang 2014; Morris and Visick 2013). Epistasis analysis between *hmpV* and *hmpW* indicates that *hmpV* is epistatic. This indicates that HmpU/V/W form an alternative PSRS, which appears to be functioning in a similar manner to those of other gram-negative bacteria such as *R. capsulatus* and *V. fischeri* (Mercer and Lang 2014; Morris and Visick 2013), in which the phosphorylation state of HmpV determines its ability to interact with a downstream target. In this model, the HmpW serine kinase binds to and phosphorylates HmpV, causing dissociation of the
HmpW-HmpV complex and inactivating HmpV. The HmpU phosphatase can dephosphorylate HmpV, activating it to influence some unknown target to promote PilA and HPS secretion through the T4P system and ultimately enhance hormogonium motility.

**Figure 19:** A model of the partner-switching regulatory system in filamentous cyanobacteria comprised of HmpU, HmpW, and HmpV. Unphosphorylated HmpV works as the effector of the system through a currently-unknown mechanism to promote PilA and HPS secretion through the T4P, ultimately enhancing motility.
The \( \Delta \text{hmpV} \) strain produces wild-type cellular levels of PilA but the secretion of PilA and HPS is significantly impaired and there is a drastic reduction in both filament speed and percent of motile filaments. This specific phenotype leads us to hypothesize that the Hmp PSRS could be working to regulate the T4P machinery directly or indirectly, since the T4P system is responsible for secretion of PilA and HPS and necessary for gliding motility (Khayatan, Meeks, Risser 2015; Risser and Meeks 2013; Risser, Chew, Meeks 2014). To determine if HmpV is interacting directly with some component of the T4P motor complex to activate the motor, fluorescence microscopy with GFP tagged-HmpV could be used to visualize localization of HmpV to the septal junctions, where the T4P motors are located (Risser, Chew, Meeks 2014). If HmpV does localize to the same region of the cell as the T4P motor, subsequent protein-protein interaction studies, such as bacterial adenylate cyclase two-hybrid (BACTH) assays, could be done to look for interactions between HmpV and T4P motor complex components or associated proteins. HmpF and PilB are cytoplasmic proteins required for the accumulation of extracellular PilA (Cho et al. 2017; Khayatan, Meeks, Risser 2015), making them intriguing candidates for direct interaction with HmpV.

If HmpV is working to activate the T4P motor and ultimately enhance motility through indirect means, additional experiments may be needed to determine the immediate downstream target of HmpV. A co-immunoprecipitation followed by SDS/PAGE and mass spectrometry could be used to identify possible interacting protein partners for HmpV. A BACTH assay could also be utilized to screen a library of proteins for interactions with HmpV.
Alternatively, the PSRS could be working to activate the expression of genes encoding the T4P motor to promote PilA/HPS secretion and hormogonium motility. To test this hypothesis, qPCR could be used to compare expression of T4P-system encoding genes in the wild-type and ΔhmpV strains. A reduction in the transcription of T4P-encoding genes would be consistent with a model where HmpV affects motility by modulating gene expression.

It is also currently unknown what the input signal, or signals, are for the PSRS that modulate the activity of the HmpU phosphatase. Presumably, the system is sensing and responding to some stimulus, and this input is received through the REC (receiver) domain and/or GAF (cGMP-specific phosphodiesterases, adenylyl cyclases and FhLA) domain at the N-terminus of HmpU, resulting in modulation of the C-terminal PP2C phosphatase domain and therefore regulating the level of phospho-HmpV (Fig. 19).

REC domains are common modules found in bacterial response regulators as part of two-component signaling systems. In the simplest model of these systems, a sensor histidine kinase perceives some input through its sensory domain and autophosphorylates a histidine residue, followed by phosphoryl transfer to an aspartate on the REC domain of a response regulator protein (Lohrmann and Harter 2002). This changes the conformation of the REC domain, which induces a conformational change in a nearby output domain on the response regulator (typically a DNA binding domain or enzymatic domain) (Lohrmann and Harter 2002). In the case of HmpU, the REC domain could be serving as the target for phosphoryl transfer by a histidine kinase and this could modulate the activity of the PP2C phosphatase domain. Currently, the only histidine kinase known to influence motility in *N. punctiforme* is HmpE of the Hmp chemotaxis system (Risser,
Chew, Meeks 2014). It is conceivable that this or some other histidine kinase is directly targeting the REC domain of HmpU. Future studies could include phosphotransfer assays with purified HmpU and HmpE or other candidate histidine kinases to determine if they indeed target the REC domain of HmpU. Targeted deletion of the REC domain, followed by characterization of the mutant strain, could also serve as a next step to determine whether the REC domain is essential to the normal functioning of HmpU as a phosphatase.

GAF domains are known to act as sensory domains and bind to small molecules like cyclic nucleotides or chromophores, with the latter typically being involved in sensing light (Martinez, Beavo, Hol 2002; Rumyantsev et al. 2015). Therefore, it is possible that the GAF domain of HmpU modulates the activity of the adjacent PP2C phosphatase domain in response to changes in light, or levels of nucleotide-based second messengers. One way to test this could be to use time lapse microscopy to compare the rates of motility in response to different light qualities and intensities, or the presence of different nucleotide-based second messengers in the wild-type strain and the ΔhmpW mutant strain, where HmpV is locked in the active (unphosphorylated) state. If the wild type shows variation in motility in response to various light regimens or second messengers, but the ΔhmpW strain does not, this could indicate that light may be serving as an input for the PSRS. Another possible future study could include creating a targeted deletion of just the GAF domain of hmpU and characterizing the phenotype to determine if the GAF domain is essential for the normal functioning of the HmpU phosphatase.

It is notable that the Hmp PSRS described here appears to be almost exclusively conserved in filamentous cyanobacteria, indicating a specific selective advantage for this
system in filamentous versus unicellular strains. As mentioned in the introduction, it is widely accepted that the photosynthetic activity of Earth’s ancient cyanobacteria is what led to the rapid accumulation of atmospheric oxygen known as the GOE around 2.5 billion years ago (Holland 2002; Lyons, Reinhard, Planavsky 2014), and recent studies indicate that the GOE may have coincided with the evolution of filamentous cyanobacteria (Schirrmeister et al. 2013; Schirrmeister, Gugger, Donoghue 2015). If this is the case, multicellularity may have provided selective advantages during the earth’s early history that allowed for rapid expansion and diversification of cyanobacteria and led to the resulting rapid increase in atmospheric oxygen levels (Schirrmeister, Gugger, Donoghue 2015). Filamentous species could coordinate movement up and down in stromatolites to access the light necessary for photosynthesis while avoiding lethal overexposure to UV radiation, and could colonize new habitats more rapidly with better surface attachment to microbial mats (Schirrmeister, Gugger, Donoghue 2015; Stal 1995). Based on its gene conservation pattern, it is conceivable that the Hmp PSRS arose early in filamentous cyanobacteria and allowed for enhanced motility and ability to modulate the coordinated movement of the filaments in response to the environment. The evolution of the Hmp PSRS may therefore have played an important role historically with regard to the GOE, which paved the way for the evolution of oxygen-based life forms on Earth (Lyons, Reinhard, Planavsky 2014).

Furthering our understanding of hormogonium development and motility may have significant applications for potential biotechnological advances in the future. The production of biofuels is becoming an increasingly important alternative to the use of fossil fuels, but one major disadvantage to producing biofuels from biomass such as corn
is the need for vast amounts of cropland, which may present a diversion of land resources from food production (Tenenbaum 2008). Cyanobacteria and algae have already successfully been bioengineered to produce biofuels such as ethanol and isoprene (Luan and Lu 2018). When filamentous cyanobacteria such as *Nostoc* exist in the hormogonium state, they continue to photosynthesize but they do not grow and accumulate biomass (Meeks and Elhai 2002). The potential therefore exists to engineer a biological system in *Nostoc* that maintains filaments in the hormogonium state and has the ability to directly convert the products of photosynthesis to biofuels rather than movement, without the accumulation of biomass. Another potential biotechnology application involves the use of diazotrophic cyanobacteria such as *Nostoc* as biofertilizers for plants. The development of motile hormogonia is necessary for establishing symbioses with plant partners (Risser, Chew, Meeks 2014), so further understanding of hormogonium development and motility could lead to the genetic engineering of *Nostoc* to establish more efficient nitrogen-fixing symbioses and limit the need for addition of nitrogen-based chemical fertilizers to plants.
REFERENCES


