CHAPTER I

Introduction

Introduction

From the very first discovery of microorganisms, their locomotion has been an important aspect of investigations into bacterial physiology. Even the realization that tiny "animalicules" were actually alive, is thanks to the fact that these organisms were moving about under the gaze of scientists using the very first microscopes (10). Accordingly, our knowledge of certain types of bacterial motility is quite advanced. Studies of the rotary motors of bacterial flagella are a good example of the advances in our understanding of the inner workings of complex yet miniscule molecular motors (5, 32). While some aspects of bacterial motility are understood down to the finest of details, many mechanisms of bacterial motility are just beginning to be elucidated. Among these is the remarkable motility exhibited by marine *Synechococcus*. These coccoid cells swim through their liquid environment in the complete absence of any visible extracellular appendages. While some characteristics of the motility exhibited by these cells are similar to other more well understood mechanisms of motility, most aspects of how these cells are able to move remain mysterious.

Marine *Synechococcus* occupy an important position at the base of the marine food web. These small (~1μm) unicellular phytoplankton are responsible for a major fraction of total oceanic primary productivity (44, 45). Marine *Synechococcus* are found in all of the world's oceans at abundances ranging from just a few to 10^4 cells·ml^{-1}. Together with the other numerically dominant marine phototroph *Prochlorococcus*, it is estimated that marine cyanobacteria may contribute as much as 20% of the total global primary production (21, 33). These marine cyanobacteria are adapted to living in the extremely low nutrient environment of the open ocean. While

motile *Prochlorococcus* strains have yet to be identified, numerous motile *Synechococcus* strains have been isolated. Motile strains represent a monophyletic clade of *Synechococcus* (41), all of which have been isolated from oligotrophic waters. *Synechococcus* are chemotactic towards the nitrogen containing compounds ammonia, nitrate, β-alanine, glycine, and urea at nanomolar concentrations expected to be environmentally relevant (48). Additionally, as many as a third of open ocean isolates are capable of swimming motility, while isolates from more nutrient replete coastal locations are non-motile. These observations indicate that the motility is an important adaptation to the oligotrophic open ocean, perhaps allowing these cells to seek out microscale patches of nutrients (48).

With the exception of the helical, wall-less *Spiroplasma,* which swim by means of conformational deformations of the cell's helical cytoskeletal filament (13, 51), *Synechococcus* are the only example of prokaryotic swimming in the absence of flagella. Flagella are common motility structures that are composed of two main parts: a cell wall anchored rotary motor to which is attached a semi-rigid helical filament. Either a proton or sodium ion gradient across the cytoplasmic membrane is used to power the motor which rotates the propeller-like flagella providing thrust. While a single polar flagellum is sufficient for motility, the number and localization of flagella can vary. For example, in the helically shaped spirochetes, the flagellum is internal, located in the periplasmic space between the cell wall and outer membrane (23), while *E. coli* and *Salmonella* possess multiple peritrichous flagella, which spin together as a cohesive bundle during a swimming "run" (22, 42).

Bacteria can sense stimuli such as nutrients, toxins, temperature, light, etc. and alter their motility to move towards a more favorable environment in a process called taxis (2). Directional control of motility is achieved by switching the speed and/or direction of rotation of the flagellum or flagella. In cells possessing multiple flagella, this switch results in an unbundling of the flagella and a random reorientation or "tumbling" of the cell. By controlling the frequency of runs versus tumbles (*e.g.* fewer tumbles as cells sense increasing concentrations of an attractant), cells are able to bias their movement resulting in a "random walk" towards a more favorable environment (4). In the marine environment, where bacteria frequently swim at higher speeds and exhibit abrupt reversals of direction (29-31), run-and-tumble behavior does not appear to be the norm. Modeling suggests the superiority of a "back-and-forth" strategy of chemotaxis in a high-shear environment such as the ocean (24).

In addition to swimming in liquid media, flagella are also utilized by bacteria for movement in viscous environments, as well as through thin films of liquid along surfaces in a process called swarming. Transition to swarming motility can be triggered by extracellular chemical cues and through physical contact with a surface (12). Cells elaborate many lateral flagella becoming hyper-flagellated, which allows for movement along surfaces. Distinct from this surface-associated flagellar motility, certain bacteria are able to move along surfaces in the absence of flagella. These behaviors are called either twitching or gliding. Undoubtedly, multiple mechanisms are employed for such surface motility, and in some cases it is clear that a single species is capable of employing multiple mechanisms of motility (27, 39). Originally classified as distinct types of motility (14), it is now clear that twitching motility is

equivalent to some forms of gliding motility due to the common use of pili for both. Thus the descriptive term of retractile motility may be more accurate (26) for this type of surface motility. As the name suggests, this pilus-dependent motility occurs through the extension, adhesion (to the substrate or another cell), and retraction of pili. Less well understood are various forms of gliding motility that do not involve detectable external cellular appendages such as pili. A variety of mechanisms have been proposed to explain the observed locomotion including directional extrusion of slime in cyanobacteria (16) and *Myxococcus* (50), directional propagation of waves along the surface of *Myxococcus* cells (25), "conveyor belt"-like coordinated export and import of extracellular polymers (28) or "tank tread"-like motion of outer membrane components (27) in *Flavobacteria*, inchworm-like extension and retraction of filaments in the anterior "head" of *Mycoplasma* cells (51), and even a type of walking on oar-like projections in *Mycoplasma* (38, 43). Again, there are certainly different mechanisms of motility being employed in these diverse bacteria, and no single explanation will describe the motility for all these bacteria.

There are no cyanobacteria that use flagella for motility, thus the discovery of strains of the unicellular cyanobacterium *Synechococcus* that were capable of swimming was quite unexpected (46). Cyanobacteria have been shown to employ some of the surface associated motility mechanisms described above. Several filamentous *Oscillatoriaceae* leave behind slime trails and tubes (17). These cyanobacteria possess pores at the junction between individual cells in the filament that are proposed to be sites of directional slime extrusion (18) and bear a striking resemblance to the slime nozzles found in *Myxobacteria* (50). The unicellular

Synechocystis sp. require functional type IV pili for movement (6, 7, 52). The motility of marine *Synechococcus* is distinct though, as these cells clearly lack flagella or any other detectable appendages, yet cells are swimming through a liquid environment and not moving along surfaces.

This unique type of prokaryotic motility was broadly characterized by Waterbury and Willey (46-49) and the extent of this field prior to the work presented in this dissertation was reviewed by Brahamsha (9). A summary of these publications and other relevant works is included here to serve as a foundation for the material to be presented in following chapters.

Marine *Synechococcus* swim at 15 $\mu m \cdot s^{-1}$ on average, with speeds up to 40 $\mu m \cdot s^{-1}$ observed (47). Generally, only a portion (50-80%) of cells in a culture are actively motile (47). Cells rotate about the axis of their direction of swimming as they translate (much like a corkscrew) (47). Additionally, following the chance attachment of cells to a microscope slide, cells will spin about the point of attachment (with equal numbers of cells spinning clockwise as counter-clockwise) (47). This behavior is reminiscent of flagellated cells, which produce rotational torque through the rotation of flagellar motors and similarly will spin if attached to surfaces. Another similarity to flagellar swimming is found in the relationship between medium viscosity and swimming speed. For both *Synechococcus* and flagellated cells, increasing viscosity decreases swimming speed, ultimately immobilizing cells (47). The swimming behavior of *Synechococcus* appears random, with cells swimming in irregular loopy paths. Cells do not tumble or reverse swimming direction, and only very rarely are non-motile cells observed to start moving (47). Similarly, fortuitously attached cells

have not been observed to reverse the direction of spinning. Blind well experiments have shown, however, that *Synechococcus* are chemotactic to a variety of nitrogen containing compounds (48). Thus, the behavior of these cells must not truly be random, yet how they direct their movement has yet to be realized.

Many different approaches have been taken to identify cellular structures involved in non-flagellar swimming motility. Many groups have employed a variety of TEM techniques, and in no case has an unambiguous motility structure been identified. Willey attempted thin sectioning with various stains, negative staining, as well as freeze fracture and etching. Samuel *et al.* also performed thin sectioning and freeze fracture and etching (37). Their results largely agree with the results presented in Chapter III, however their observation of "spicules" extending from the cell surface has now been attributed to an artifact of preparation (J. Heuser, personal communication). Our own results utilizing cryo-fixation and freeze-substitution, as well as freeze fracture and etching (presented in Chapter III), have begun to identify structures important for motility, but again, no structures extending from the cell surface were ever observed. Both high-intensity dark field microscopy and motility-dependent amplitude spectra also failed to detect extracellular appendages. Lastly, extended exposure to mechanical shearing in a blender (up to 15 minutes), which would have easily sheared flagella (10 – 15 seconds is sufficient to eliminate flagellar motility), failed to inhibit swimming in *Synechococcus* (46).

Swimming motility in marine *Synechococcus* relies upon a sodium motive force (49). Although the most well studied flagellar motility mechanisms utilize a proton motive force, the motility of various alkaliphilic *Bacillus (15)* and marine

bacteria also utilize a sodium motive force to power motility (3, 29). *Synechococcus* cells continue to swim in the presence of the oxygenic photosynthesis inhibitor DCMU, indicating that respiration alone can power motility. Conversely, cells also continue to swim in the presence of cyanide demonstrating that photosynthesis alone can also power motility (35). Pitta *et al*. have also shown a calcium requirement for swimming motility (35). Interpretation of these results are complicated by the fact that removal of calcium by treatment with EDTA removes the outer membranes of these cells (36). Calcium is clearly important for motility, however, as careful resuspension of cells in calcium-free medium results in a loss of motility that can be restored by the addition of millimolar levels of calcium. The authors suggest the involvement of a calcium potential for motility.

These observations then provide a framework and starting point for more detailed investigation into the mechanism by which *Synechococcus* cells produce thrust. Several models have been proposed to explain the mechanism of swimming motility in these cells. Both jet propulsion and self-electrophoresis have been proposed as possible mechanisms (34). Jet propulsion is implausible due to the small size of these cells and the correspondingly low Reynolds number interaction they have with their environment in which viscous forces dominate. Cells would have to eject a volume comparable to its own contents to move a single cell length. Experimentally, self-electrophoresis has been ruled out, as *Synechococcus* cells do not migrate in an applied electric field due largely to the high ionic strength of seawater (34). This leaves the cell surface itself as the remaining thrust generating structure. Two mechanisms by which these cells might swim were proposed: [1)] by generating

longitudinal or transverse surface waves and [2)] by bulk flow of the cell surface (11). The second proposal appears unlikely as experiments in which polystyrene beads (0.38 μm diameter) were added to swimming cells revealed that the occasional bead stuck to a cell would remain fixed as the cell rotated about the axis of translation. Additionally, incompressible cell surface streaming has been mathematically ruled out (40). Ehlers *et al.* point out that longitudinal compression waves would be sufficient to propel microorganisms and that such waves would not generate any cell shape change. Further calculations estimate that a combination of transverse and longitudinal waves 20 nm in amplitude, 200 nm wide traveling at 160 $\mu m \cdot s^{-1}$ could produce the swimming speeds observed in marine *Synechococcus*. Such waves are small enough to be consistent with the lack of detectable shape change of cells during swimming.

Paying particular attention to the cell surface, as it appears to be of special importance for non-flagellar swimming, one cell surface component has been identified that is required for swimming motility. SwmA is a glycosylated 130 kDa protein that is associated with the outer membrane of the cell (8). The amino acid sequence of *swmA* contains two different types of calcium-binding domains: an EF-hand loop domain and twelve RTX repeats. Database searches using the BLASTP algorithm (1) primarily yield matches to other proteins containing the same RTX repeat. One of these matches is to oscillin, a cell protein that forms a parallel helical surface array arranged in the same orientation as the direction of rotation that accompanies gliding in the filamentous cyanobacterium *Phormidium unicinatum* (19). Based on this directional correlation and the observation that non-motile cells do not

produce oscillin, these authors suggest a role for oscillin in gliding motility. A direct role for oscillin in gliding motility has yet to be proven and the sequence similarity to SwmA is limited to the repeats present in these two proteins. Whether SwmA is evolutionarily or functionally related to oscillin is uncertain but the possibility does raise the question as to how swimming motility could share a common mechanism with gliding motility.

Insertional mutagenesis of *swmA* results in a complete loss of swimming motility yet fortuitously attached mutant cells still rotate about their point of attachment (8). SwmA is clearly required for motility and may be involved in the conversion of torque into thrust. How these cells produce torque and the role of SwmA in the generation of thrust remains a mystery. There are undoubtedly more components of the motility apparatus that have yet to be discovered. Looking to the better-understood types of prokaryotic motility, both flagellar motility and pili mediated retractile motility require a complement of approximately 40 genes for the proper biogenesis and function of their respective components (20, 26).

This dissertation is organized into three broad areas encompassing my research into the swimming motility exhibited by marine *Synechococcus*:

1. TEM ultrastructural investigations (chapters II and III)
2. Development of genetic tools to identify motility genes (chapters IV)
3. Biochemical characterization of one protein component of the motility apparatus and characterization of several motility mutants (chapters V and VI)

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