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UNIVERSITY OF CALIFORNIA, SAN DIEGO

**Microscopic, Genetic, and Biochemical Characterization of  
Non-Flagellar Swimming Motility in Marine Cyanobacteria**

A dissertation submitted in partial satisfaction of the requirements for the degree

Doctor of Philosophy

in

Marine Biology

by

Jay William McCarren

Committee in charge:

Bianca Brahamsha, Chair  
Douglas Bartlett  
Brian Palenik  
Kit Pogliano  
Aristides Yayanos

2005

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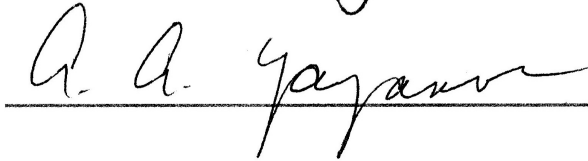
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Chair

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2005

## DEDICATION

To Alex

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## ABBREVIATIONS

ABC transporter	ATP binding cassette transporter
ATP	Adenosine triphosphate
BLOTTO	Bovine lacto-transfer technique optimizer
CM	Cytoplasmic membrane
CMi	Cytoplasmic membrane inner face
EDTA	Ethylenediaminetetraacetic acid
EL	External layer
FITC	Fluorescein isothiocyanate
FL	Fibrillar layer
HSP	High-speed pellet containing insoluble OM proteins
HSS	High-speed supernatant containing soluble OM proteins
IgG	Immunoglobulin G
MFP	Membrane fusion protein
MSCRAMMS	Microbial surface components recognizing adhesive matrix molecules
MWCO	Molecular weight cut-off
OM	Outer membrane
OMP	Outer membrane protein
ORF	Open reading frame
PAGE	Polyacrylamide gel electrophoresis
PAS stain	Periodic acid-Schiff stain



PBS	Phosphate buffered saline
PCR	Polymerase chain reaction
PPIase	Peptidyl-prolyl isomerase
Prot1E family	Protein-1 exporter family
RSCU	Relative synonymous codon usage
RTX	Repeats in toxin
S-layer	Surface layer
SAPS	Statistical analysis of protein sequences
SN	Natural seawater based medium
SOW	Synthetic ocean water
TOF	Time of flight
TEM	Transmission electron microscopy
WH8102	<i>Synechococcus</i> sp. strain WH8102

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The text of Chapter VI, in full, is being prepared for publication. The dissertation author was the primary author, and co-author B. Brahamsha directed and supervised the research, which forms the basis for this chapter.

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- McCarren, J., J. Heuser, R. Roth, N. Yamada, M. Martone, and B. Brahamsha. 2005. Inactivation of *swmA* results in the loss of an outer cell layer in a swimming *Synechococcus* strain. *J. Bacteriol.* 187: 224-230.
- Palenik, B. B. Brahamsha, F. W. Larimer, M. Land, L. Hauser, P. Chain, J. Lamerdin, W. Regala, E. E. Allen, J. McCarren, I. Paulsen, A. Dufresne, F. Partensky, E. A. Webb, and J. Waterbury. 2003. The genome of a motile marine *Synechococcus*. *Nature* 424:1037-1042.

## Conference Participation and Awards

- Wenner-Gren Foundations International Symposium “Marine cyanobacteria: evolution, function and genomes”. Poster titled: Identification and characterization of SwmB, an unusual protein that is required for non-flagellar swimming in marine *Synechococcus* (2005)
- ASM 104<sup>th</sup> General Meeting. Poster titled: Identification and characterization of SwmB, a 1.1 MDa protein that is required for non-flagellar swimming in marine *Synechococcus* (2004)
- ASLO Aquatic Sciences Meeting. Poster titled: Gene clusters required for swimming motility in marine *Synechococcus* (2003) *Winner of Student Poster Award*
- ASM 102<sup>nd</sup> General Meeting. Poster titled: Identifying components involved in marine *Synechococcus* swimming motility by transposon mutagenesis (2002)
- San Diego Microbiology Group Annual Symposia. Talk titled: Ultrastructural and genetic investigation of swimming motility in marine *Synechococcus* (2002)
- VIIth Cyanobacterial Workshop. Poster titled: SwmA forms an additional envelope layer in motile marine *Synechococcus* (2001)



**ABSTRACT OF THE DISSERTATION**

**Microscopic, Genetic, and Biochemical Characterization of  
Non-Flagellar Swimming Motility in Marine Cyanobacteria**

by

Jay William McCarren

Doctor of Philosophy in Marine Biology

University of California, San Diego, 2005

Bianca Brahamsha, Chair

The mechanism of motility in marine *Synechococcus*, which swim without any apparent extracellular appendages, remains a mystery 20 years after its discovery. A multifaceted investigation including direct microscopic visualization, genetic analyses, and biochemical approaches was carried out in order to better understand the physiology of this globally important primary producer. Ultrastructural analyses provided a detailed view of the cell envelope layers and aided in the identification of a structure important for motility. Electron microscope tomographic reconstructions

revealed the even distribution of SwmA, a protein required for motility, across the cell surface. Various cryo-fixation techniques were required for the preservation and visualization of a para-crystalline S-layer formed by this protein.

As complete genomic sequence information failed to identify genes involved in motility, a transposon mutagenesis technique was developed to identify components of the motility apparatus. Utilizing this genetic tool, 17 independent transposon insertions that abolish motility were localized to clusters in three separate chromosomal regions. Included within these clusters are several multicomponent transport systems, as well as a number of glycosyltransferases. One cluster is characterized by DNA with an exceptionally low % G+C content relative to the genome average. Additionally, inter-genome comparisons reveal the absence of this stretch of DNA in two non-motile strains of *Synechococcus*, suggesting acquisition of this genetic information by horizontal gene transfer. Contained within this region of low % G+C content is an extremely large gene called *swmB*, which is required for motility in these cells. The sequence of SwmB is highly repetitive, with 4 domains of tandem repeats comprising over 60% of the protein. Analyses confirm that this gene is indeed translated into a megadalton-size protein, which is localized on the cell surface. Cellular localization of the two motility proteins SwmA and SwmB revealed that all motility mutants in culture have a defect in the localization of either SwmA or SwmB and in some instances both of these proteins. Additionally, two outer membrane polypeptides of 70 kDa and 80 kDa are absent in some of these mutants, suggestive of a role in motility.

# **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 (~1 $\mu$ m) 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<sup>4</sup> cells·ml<sup>-1</sup>. 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,  $\beta$ -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  $15 \mu\text{m}\cdot\text{s}^{-1}$  on average, with speeds up to  $40 \mu\text{m}\cdot\text{s}^{-1}$  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: <sup>1)</sup> by generating

longitudinal or transverse surface waves and <sup>2)</sup> by bulk flow of the cell surface (11). The second proposal appears unlikely as experiments in which polystyrene beads (0.38  $\mu\text{m}$  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  $160 \mu\text{m}\cdot\text{s}^{-1}$  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 uncinatum* (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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## **CHAPTER II**

### **Ultrastructural analysis of SwmA, a protein required for non- flagellar swimming motility**

## **Abstract**

The mechanism of swimming motility in marine cyanobacteria remains poorly understood. Although the structural components used in swimming motility have not been detected, presumably motility structures do exist. Electron microscopic examination of motile cells, as well as comparisons between motile and non-motile strains, should begin to identify the structures used for locomotion. Three-dimensional tomographic electron micrographs verified the extracellular location of SwmA, a protein required for swimming motility in this bacterium. Comparative ultrastructural analysis of a motile strain and a non-motile mutant indicate possible differences in cell surface characteristics between these strains. These results provide a basis for further ultrastructural investigations and incentive for continued study presented in Chapter III.

## **Introduction**

Although diverse techniques have been employed in attempts to identify extracellular appendages on the surface of swimming *Synechococcus* cells (11, 12), to date no approach has identified such a structure, leaving the cell surface itself as the most likely thrust generating component. While the structures employed by *Synechococcus* to swim through their liquid environment remain mysterious, one protein that is required for their novel motility is known (1). SwmA is a glycosylated protein of approximately 130 kDa that is present in all motile strains screened and conspicuously absent in non-motile strains. Brief incubation of actively swimming

cells with proteinase K disrupts motility and whole cell extracts of treated cells show SwmA to be largely degraded, suggesting that this protein is accessible to the protease at or near the cell surface. Additionally, EDTA treatment, which strips the outer membrane off of whole cells (5), efficiently extracts SwmA from cells. Preliminary electron microscopic analysis of cells labeled with an antibody to SwmA indicated that this protein is associated with the outer membrane and that SwmA may be arranged on the cell surface with a periodic distribution (Brahamsha, unpublished). In order to better understand the localization and distribution of this motility protein on whole cells, additional experiments were undertaken. Ultrastructural analysis of cells labeled with an antibody raised against SwmA using intermediate voltage TEM combined with tomographic reconstruction was employed to investigate the three-dimensional distribution of SwmA on whole cells.

In addition to tomographic analysis of wild-type cells, a comparison of motile strain WH8102 and the non-motile, *swmA* mutant strain S1A1 was conducted. Strain S1A1, which has an insertional inactivation of the *swmA* gene, continues to produce all other cell surface proteins with the exception of SwmA itself (1). Thin sections of both strains were analyzed by TEM to detect potential structural differences between strains that could be correlated to swimming motility.

## **Materials and Methods**

**Bacterial strains and growth conditions.** *Synechococcus sp.* strains WH8102 (11) and its isogenic *swmA* mutant strain S1A1 (inactivated with the suicide plasmid pBB1000 as previously described (3)) were both grown in SN medium

(10) prepared with local seawater. 50-ml cultures were maintained in 125-ml flasks under constant illumination ( $10 \mu\text{E}\cdot\text{m}^{-2}\cdot\text{sec}^{-1}$ ) without shaking at  $25^\circ\text{C}$ . Cultures of S1A1 contained  $15 \mu\text{g}\cdot\text{ml}^{-1}$  kanamycin to select for and maintain the insertion inactivating *swmA*.

**Immunolabeling for tomographic analysis.** Cells were pre-fixed in 0.5% EM grade glutaraldehyde for 1 hour at room temperature and then pelleted by centrifugation at  $7500 \times g$  for 5 minutes. Following fixation, cells were washed three times for 5 minutes each in PBS. Cells were then incubated for 1 hour in 1:50 dilution of a rabbit polyclonal antibody to SwmA (Brahamsha, personal communication) in PBS + 1%  $\gamma$ -globulins, followed by another three washes in PBS. Secondary incubation with a 1:50 dilution of 10-nm gold conjugated goat anti-rabbit IgG (Ted Pella Inc., Redding, CA) in PBS was carried out for 1 hour at room temperature followed by 2 washes in PBS. Cells were post-fixed in 2% glutaraldehyde for 1 hour at  $4^\circ\text{C}$ . Cells were then enrobed in 1.5% low melting point agarose (BRL, Gaithersburg, MD), cut into 3mm cubes, and stained by soaking in a 2% solution of osmium tetroxide (w/v) for 1.5 hours, either alone or with a subsequent staining in 2% uranyl acetate for 30 minutes. Agar cubes were prepared as described by Rippka *et al.*(6) with some modifications. Briefly, the cubes were dehydrated in a graded series of ethanol and acetone washes. Following dehydration, the agar cubes were infiltrated with acetone-Durcupan (Electron Microscopy Sciences, Hatfield, PA) resin mixtures at room temperature (2:1 for 2 hours, 1:1 overnight, 1:2 for 2 hours) and then in pure

Durcupan EM resin (2×2hours). Finally the resin, containing the agar cubes, was polymerized in a vacuum oven overnight at 60°C.

**Chemical fixation for thin sectioning.** Cells were fixed directly in SN medium by the addition of 25% EM grade glutaraldehyde to a final concentration of 2% and incubated for 1 hour at 4°C. Cells were then enrobed in 1.5% low melting point agar and prepared exactly as described for immunolabeled cells.

**Electron microscopy and 3-D reconstruction.** Sectioning was performed on a Reichert-Jung ultramicrotome (Leica, Bannockburn, IL) using either a diamond blade or freshly prepared glass blades. Thick sections of approximately 500 nm were recorded using a JEOL 4000EX intermediate voltage electron microscope at an acceleration voltage of 400kV. For each tomogram produced, single-axis tilt series of images were recorded on film at angular increments of 2° from -60° to +60°. Images were digitized and manipulated as described by Perkins *et al.*(4). The computer software programs SUPRIM (8), FIDO (9), XVOXTRACE (S. Lamont, NCMIR), ANALYZE (7), and SYNUIVIEW (2) were used to generate and manipulate 3-D images. Thin sections (approximately 80 nm as determined by a silver refractive color to the sections) were visualized and recorded using a JEOL 100CX transmission electron microscope at an acceleration voltage of 80kV.

## Results

Intermediate voltage TEM allows for visualization of relatively thick sections (~500 nm), which contain intact, or nearly intact, whole cells. Thick sections containing anti-SwmA labeled cells demonstrate abundant labeling across the cell

(Fig. 1A). While allowing for visualization of intact cells, interpretation of where the labeling is positioned relative to the vertical axis of the image is difficult for these projections through the entire cell. Tomographic reconstruction of the same cell produces a three-dimensional image that more clearly displays the position of the gold labeling. The tomogram shows that the anti-SwmA labeling is associated with the cell surface (Fig. 1B). Labeling is located outside of the outer membrane (OM) yet still closely associated with the OM. Furthermore, labeling is evenly spread across the entire cell surface with no obvious pattern to its distribution apparent in the tomogram produced. Virtual ultra-thin sections produced by the 3-dimensional image processing software XVOXTRACE also demonstrate the close association of gold particles and the OM (Fig. 2). These virtual ultra-thin sections exhibit a diffusely staining layer of irregular thickness external to the OM. Labeling of SwmA was always closely associated with this extracellular material.

Thin sections of conventionally fixed cells also revealed diffusely stained material on the surface of cells. Both the motile wild-type strain WH8102 and *swmA* mutant strain S1A1 possess this cell-surface material (Fig. 3). Similar to the observations from tomographic reconstruction, this diffuse staining material is distributed around the entire cell surface of both strains in a layer of irregular thickness. Although wild-type cells appear to possess more of the extracellular material than do non-motile S1A1 cells, ultrastructural comparisons failed to detect any clear, unambiguous differences between strains. Neither thin sections nor tomographic reconstructions revealed any distinct structure present in one strain and absent in the other.

## Discussion

SwmA is associated with the cell envelope and is required for swimming motility. Understanding the location and arrangement of SwmA may provide clues as to the function of this protein. The immuno-labeling experiments presented here agree with prior results and confirm the extracellular location of SwmA. Three-dimensional tomographic reconstruction of a labeled *Synechococcus* cell provides a more detailed picture of the surface localization of SwmA yet fails to reveal a conspicuous pattern to its distribution. These results do however indicate the presence of some extracellular material associated with SwmA labeling.

Chemically fixed cells failed to show an unambiguous difference between the wild-type strain and a mutant lacking SwmA. Thin sections indicate that motile strain WH8102 may possess more extracellular material than the non-motile *swmA* mutant, but clear and reproducible differences were not observed. While these results did not provide conclusive results, they do suggest possible structural differences between strains, serve as a foundation for further studies, and provide a justification for continued ultrastructural characterization, which is presented in the following chapter.

The following chapter contains additional analyses of electron microscopic comparisons of wild-type strain WH8102 and *swmA* mutant strain S1A1. Those results show that the chemical fixation techniques that have been employed here are not sufficient for preserving the surface structures on these cells. Utilizing various quick-freeze techniques, the outer-most layer of WH8102 cells is observed to be a highly ordered S-layer, while S1A1 cells have some disordered fibrillar material



external to the OM. In retrospect, this largely explains the lack of a regular pattern to the anti-SwmA labeling in the tomogram as the S-layer was not preserved by glutaraldehyde fixation in these preparations. Perhaps with similar quick-freeze fixation techniques, the regular crystalline lattice structure of the S-layer would be observed in the distribution of the anti-SwmA gold label. Moreover, the fact that chemical fixation does not preserve all of the cell surface structures, likely accounts for the ambiguous differences in cell surface characteristics observed in comparisons of WH8102 and S1A1 strains.

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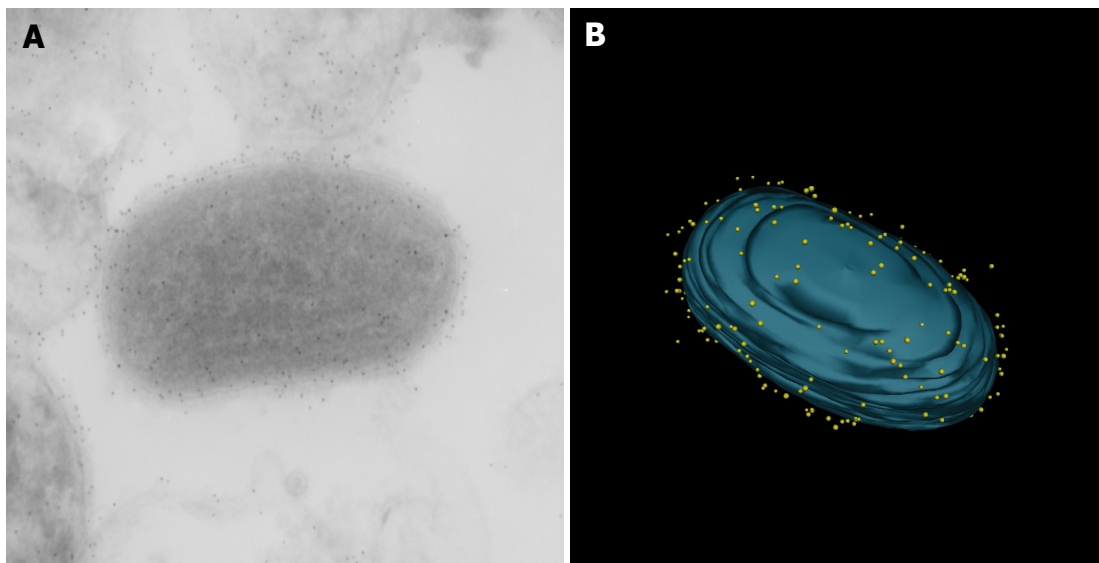


FIG. 1. Immunologically labeled *Synechococcus sp.* strain WH8102 cell. Thick section (~500nm) TEM (A) of a nearly intact cell labeled with an antibody raised against SwmA exhibits even gold-labeling across cell. Tomographic rendering of the same cell (B) illustrates the even distribution of extracellular localized gold-labeling (yellow spheres represent gold beads, blue surface represents the outer membrane).

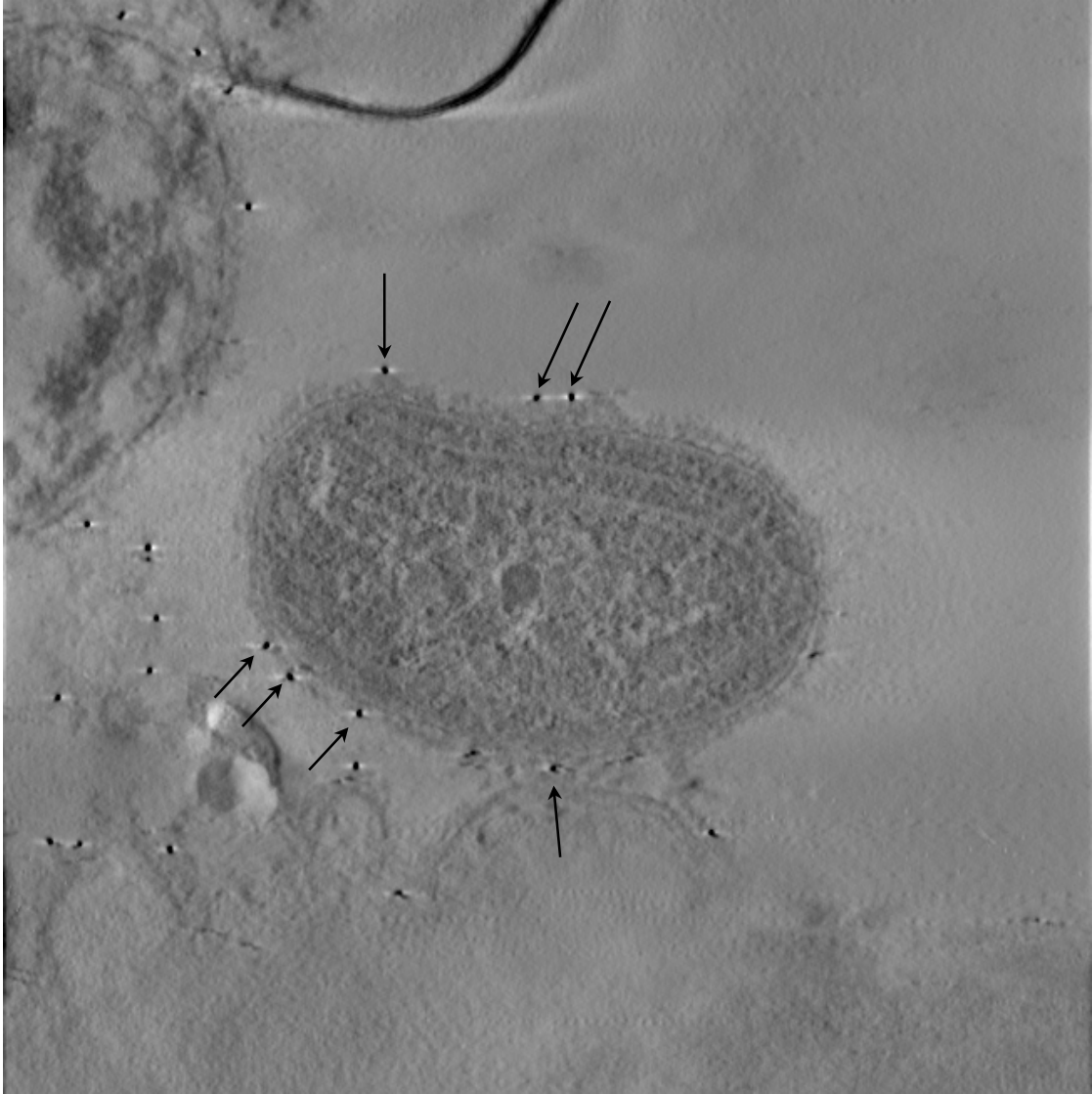


FIG. 2. Computer generated ultra-thin section from tomographic reconstruction of anti-SwmA labeled *Synechococcus* sp. strain WH8102 cells. Gold labeling (arrows) is intimately associated with extracellular material at the cell surface

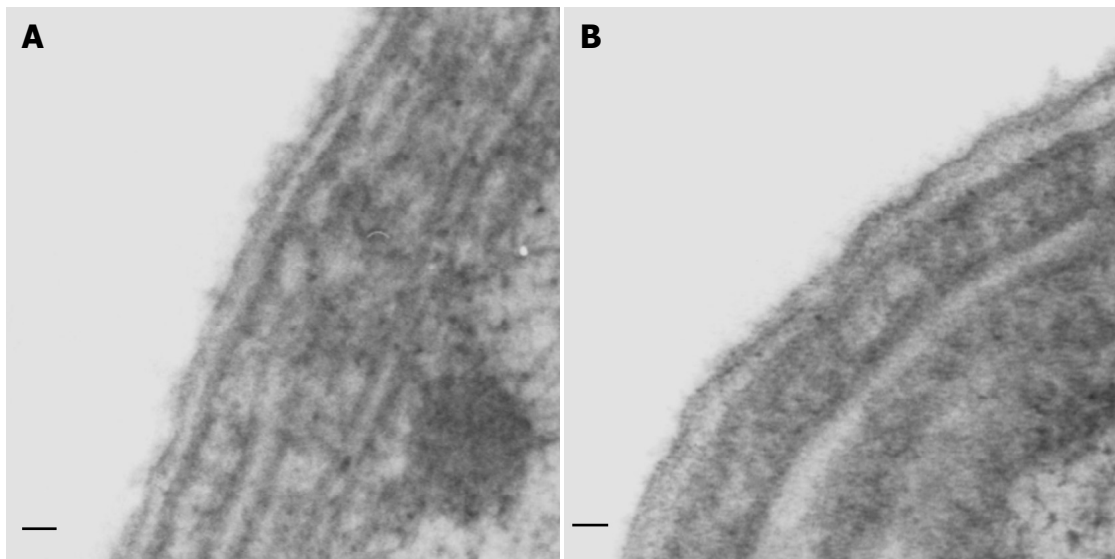


FIG. 3. Thin section transmission electron micrographs of *Synechococcus* sp. strains WH8102 (A) and S1A1 (B). Irregular layer of extracellular material is present outside of the outer membrane in both strains. Bar 50 nm .

## CHAPTER III

### **Inactivation of *swmA* results in the loss of an outer cell layer in a swimming *Synechococcus* strain**

## Inactivation of *swmA* Results in the Loss of an Outer Cell Layer in a Swimming *Synechococcus* Strain

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**The mechanism of nonflagellar swimming of marine unicellular cyanobacteria remains poorly understood. SwmA is an abundant cell surface-associated 130-kDa glycoprotein that is required for the generation of thrust in *Synechococcus* sp. strain WH8102. Ultrastructural comparisons of wild-type cells to a mutant strain in which the gene encoding SwmA has been insertionally inactivated reveal that the mutant lacks a layer external to the outer membrane. Cryofixation and freeze-substitution are required for the preservation of this external layer. Freeze fracturing and etching reveal that this additional layer is an S-layer. How the S-layer might function in motility remains elusive; however, this work describes an ultrastructural component required for this unique type of swimming. In addition, the work presented here describes the envelope structure of a model swimming cyanobacterium.**

The mechanism of swimming motility in marine *Synechococcus*, in the apparent absence of readily visible locomotor structures, remains a mystery 19 years after its discovery (33). The swimming behavior of marine *Synechococcus* resembles that of flagellated bacteria in several respects. Swimming *Synechococcus* strains are observed to rotate about their longitudinal axis as they translocate at speeds of up to 25  $\mu\text{m/s}$ . Moreover, cells that become fortuitously attached to a microscope slide or coverslip rotate about the point of attachment (34). Nevertheless, numerous attempts at visualizing an appendage or organelle, including transmission electron microscopy (TEM) of negatively stained cells, high-intensity dark-field microscopy, and shearing experiments, did not reveal any (33, 34).

The rotational behavior of swimming and attached cells suggests that the cell surface or some component thereof rotates and hence may function in swimming. One component of the cell surface that is required for swimming has been identified. SwmA is a 130-kDa cell surface glycoprotein that is required for swimming (5). It is not an integral outer membrane (OM) protein but can be removed from cells by treatment with EDTA (5). Cells in which *swmA* has been insertionally inactivated, which hence do not express the SwmA protein, are nonmotile yet, when fortuitously attached to a microscope slide or coverslip, are still observed to rotate about their point of attachment. Thus, SwmA is somehow required for the generation of thrust but not torque (5). In order to understand how SwmA functions in the generation of thrust, we sought to determine its localization in cells of *Synechococcus*. Preliminary TEM experiments analyzing immunolabeled thin sections of motile *Synechococcus* cells indicated that SwmA is associated with the outer membrane (6; B. Brahamsha, unpublished

results). To understand better the localization of SwmA, further TEM analyses of thin sections as well as of whole cells were undertaken.

*Synechococcus* sp. strain S1A1, in which the *swmA* gene is insertionally inactivated, continues to express all of the other major and minor cell surface polypeptides found in the wild type and lacks only the SwmA protein (5). We compared the ultrastructure of strain S1A1 to that of the wild-type strain, WH8102. By the use of cryofixation combined with freeze-substitution, methods known for preserving delicate structures that may be destroyed by standard fixation procedures (12, 16), we show here that strain S1A1 lacks an outer cell layer. Furthermore, by use of the freeze-etching technique, we show that this additional layer is a surface layer (S-layer). The use of these techniques allowed us to detect an ultrastructural component important for this novel type of swimming motility.

### MATERIALS AND METHODS

**Bacterial strains and growth conditions.** Motile *Synechococcus* sp. strain WH8102 was used along with a nonmotile mutant strain, S1A1, in which the *swmA* gene had been insertionally inactivated. The inactivation was carried out as described in reference 5, using an internal fragment of the *swmA* gene (nucleotides 84507 to 84926 of the *Synechococcus* sp. strain WH8102 genome) (22) cloned into the EcoRI site of the suicide vector pMUT100. Both strains were grown in SN medium (32) prepared with local seawater as described previously (5). Cultures of S1A1 contained 15  $\mu\text{g}$  of kanamycin  $\text{ml}^{-1}$  to select for and maintain the inserted plasmid inactivating *swmA*. Fifty-milliliter cultures were maintained in 125-ml flasks under constant illumination (10 microeinsteins  $\cdot \text{m}^{-2} \cdot \text{s}^{-1}$ ) without shaking at 25°C.

**Chemical fixation.** Cells were fixed and prepared according to techniques described by Beveridge et al. (3) with some modifications. Cells were fixed in SN medium containing 2% (wt/vol) osmium tetroxide for 1.5 h at 4°C, rinsed three times with SN medium, and fixed with 2% glutaraldehyde (EM grade; Sigma) for 1.5 h at 4°C, followed by three rinses in phosphate-buffered saline. Following fixation, cells were enrobed in 1.5% low-melting-point agarose (FMC Bioproducts, Rockland, Maine) as described by Rippka et al. (25) with some modifications. The cell-containing agarose was cut into 1-mm cubes and washed with deionized water two times for 5 min each time. The cubes were stained with 1% (wt/vol) uranyl acetate for 30 min at room temperature and then rinsed with deionized water three times for 5 min each time. The cubes were then dehy-

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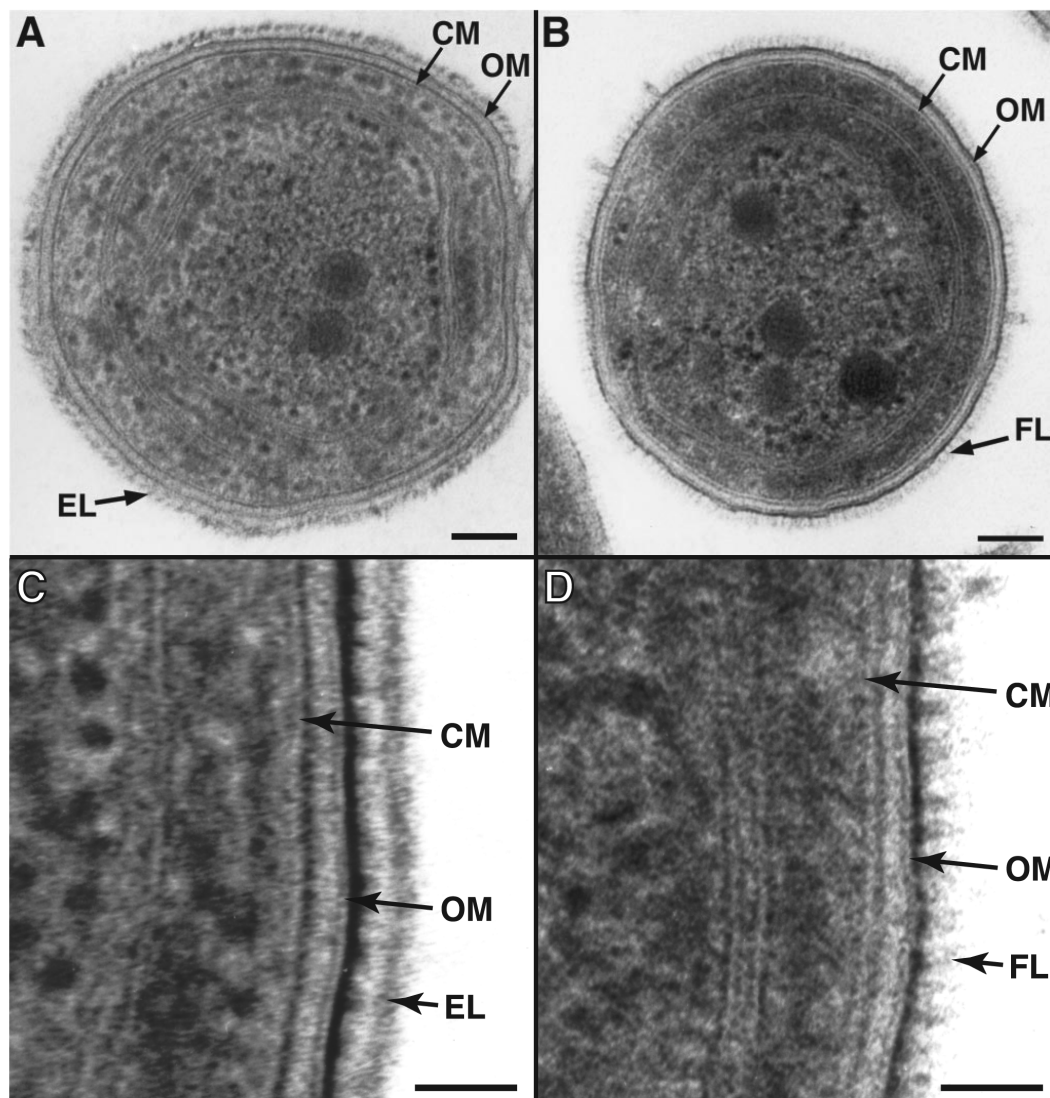


FIG. 1. Thin-section transmission electron micrographs of *Synechococcus* sp. strains WH8102 (A, C) and S1A1 (B, D) prepared by cryofixation and freeze-substitution. The external layer (EL) is observed to extend around the whole wild-type WH8102 cell and exhibits distinct periodicity (A). In strain S1A1 a fibrillar layer (FL), which also extends around the whole cell, is observed. Higher-magnification micrographs show detail of the cell envelope structures of wild-type (C) and S1A1 mutant (D) cells. CM, cytoplasmic membrane; OM, outer membrane. Bars, 100 (A, B) and 30 nm (C, D).

drated in a graded series of ethanol and acetone washes. Once dehydrated, the agarose cubes were embedded in Durcupan resin and polymerized in a vacuum oven at 60°C for a minimum of 24 h.

**Cryofixation and freeze-substitution.** In addition to conventional fixation, samples were prepared for TEM by cryofixation and freeze-substitution. Cells were centrifuged at  $7,000 \times g$  at room temperature for 10 min to form a loose pellet. A small amount of this pellet was then sandwiched between 300-mesh Gilder Fine Bar Beehive copper grids (Ted Pella, Inc.) and rapidly plunged into

liquid propane maintained at  $-188^{\circ}\text{C}$ . The grids, along with adhered cells, were incubated at  $-87^{\circ}\text{C}$  in anhydrous acetone containing 2% (wt/vol) osmium tetroxide for 48 h. The grids were then slowly ( $1^{\circ}\text{C min}^{-1}$ ) and incrementally (with a pause at  $-20^{\circ}\text{C}$  for 2 h and another at  $-4^{\circ}\text{C}$  for 1 h) brought up to room temperature. Grids were rinsed in anhydrous acetone and stained in anhydrous acetone containing 2% (wt/vol) uranyl acetate for 1 h at  $-4^{\circ}\text{C}$ . The grids were then dehydrated and embedded as described above. Thin sections ( $\sim 80$  nm) were cut on a Reichert-Jung Ultracut-E ultramicrotome and mounted on Form-



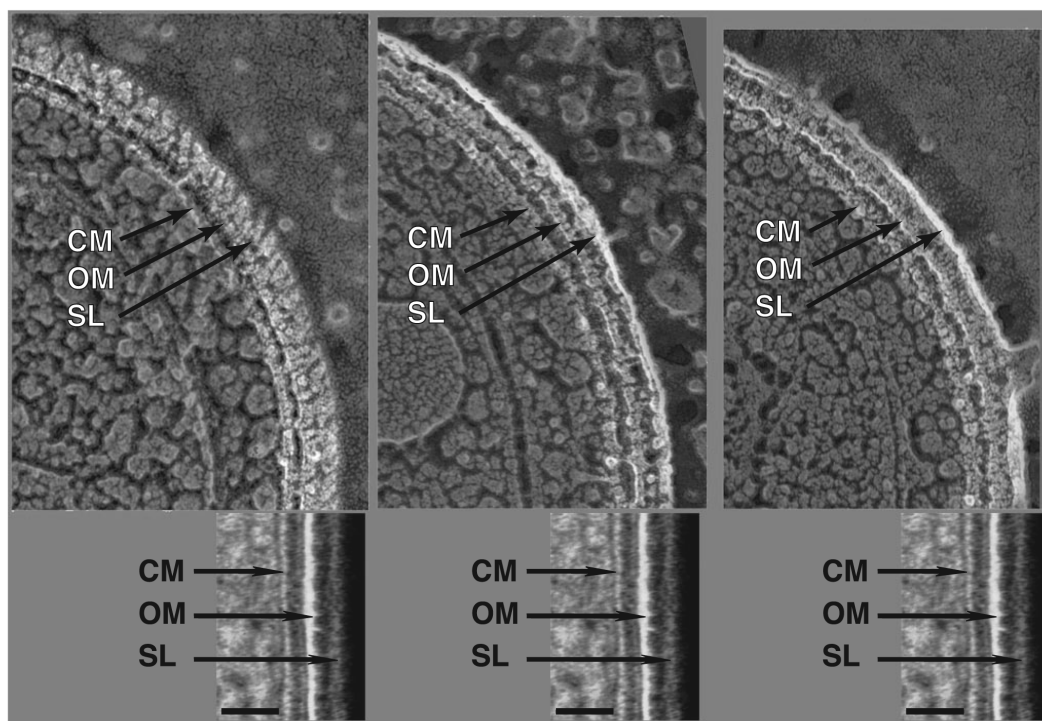


FIG. 2. Comparison of *Synechococcus* sp. strain WH8102 cells prepared by freeze fracturing and etching (upper panels) and cryofixation and freeze-substitution (lower panels). Both cryopreservation techniques yield similar cell envelope profiles. SL, S-layer. Bars, 30 nm.

var- and carbon-coated copper grids. Grids were poststained in 1% (wt/vol) uranyl acetate and then stained in Sato lead (27). Sections were then visualized and recorded using a JEOL 100CX transmission electron microscope at an acceleration voltage of 80 kV.

**Freeze fracturing and etching.** Samples were processed as previously described by Heuser and Salpeter (13–15). Briefly, cells were concentrated by centrifugation at  $7,000 \times g$  at room temperature for 10 min to form a loose pellet and then immediately quick-frozen by slamming onto a supercooled copper block. Samples were then fractured and freeze-etched to various degrees in a Balzer's vacuum evaporator at  $-100^\circ\text{C}$ . Replication with a 2-nm-thick layer of platinum, rotary-deposited from  $18^\circ$  above the horizontal, was achieved by standard evaporation techniques. Samples were backed with 4 to 6 nm of pure carbon, rotary-deposited from  $75^\circ$  above the horizontal. Replicas were visualized and recorded using a JEOL TEM microscope at an acceleration voltage of 100 kV.

## RESULTS

Thin sections of wild-type *Synechococcus* sp. strain WH8102 prepared by cryosubstitution revealed a multilayered gram-negative cell envelope profile characteristic of cyanobacteria. The cytoplasmic membrane (CM) is closely associated with a 6-nm-wide layer of darkly staining peptidoglycan (Fig. 1 A and C). This close association of peptidoglycan with the CM has been observed in other freeze-substituted preparations of cyanobacteria (16) and some other gram-negative bacteria (12). A double-tracked 9-nm-wide OM is then separated from the peptidoglycan and CM by a 9-nm-wide lightly stained periplasmic

space. The OM is asymmetrically stained, with the outer leaflet being more densely stained and thicker than the inner leaflet, as has been seen in various other cryopreserved bacteria (11, 12). Immediately external to the outer membrane there are a light-staining region 12 nm wide and then a dark-staining layer approximately 11 nm wide. In some cross sections, regions of this dark-staining external layer (EL) exhibit a distinct periodic arrangement (Fig. 1A). Cross sections show that this EL extends around the entire cell circumference. The location and periodic nature of this layer observed in thin sections suggest that this EL may be an S-layer. Such surface structures are better visualized by freeze fracturing and are described in more detail below.

In contrast, this EL is missing in the *swmA* mutant (Fig. 1B and D). Cryosubstituted thin sections of strain S1A1 exhibit the typical multilayered cell envelope observed in wild-type cells. Strain S1A1 cells also display staining of material external to the outer membrane, although its appearance is distinct from that of the EL of the wild type. These nonmotile cells possess a layer of diffuse-staining material with a periodic fibril-like appearance. This fibrillar layer (FL) appears as short, 18-nm-long fibrils of various thicknesses extending perpendicular from the external surface of the outer membrane (Fig. 1B and D). We have examined several hundred cells of both the wild type and the mutant, and the results shown here are

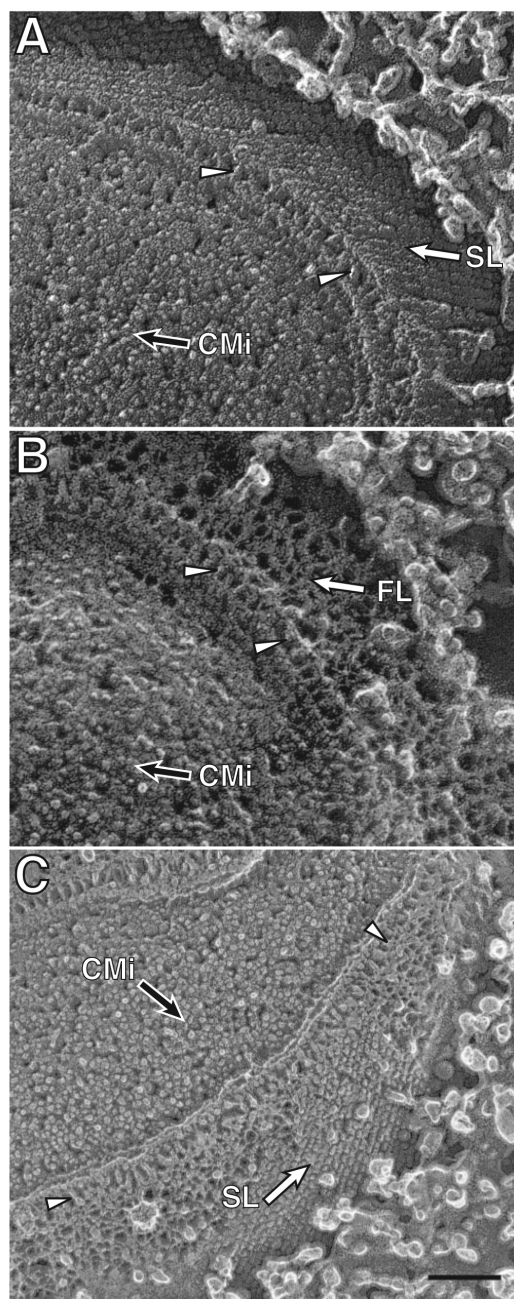


FIG. 4. Freeze fractured and etched preparations of *Synechococcus* sp. strain WH8102 (A, C) and S1A1 (B) cells. Etching has uncovered the outermost surfaces of the cells (open arrows), while partial cross-fractures reveal the cytoplasmic membrane inner leaflet face (CMi, filled arrows) and cell envelope layers. Fracturing has revealed fibril-

cell. These hemispheres have center-to-center spacing of 12 nm and yield an oblique  $110^\circ$  lattice. Cross-sectional fractures of wild-type cells reveal the S-layer as an 8-nm-thick outermost layer. The S-layer is separated from the outer membrane by a 14-nm space in which there are apparent columnar connections between the continuous S-layer and the outer membrane surface (Fig. 4A). Additionally, fractures removing the S-layer reveal a layer of fibrillar material beneath the S-layer in wild-type cells (Fig. 4C). These aerial views of fractured wild-type cells expose fibrillar material that is quite similar in appearance to the fibrillar material seen in surface views of the mutant (Fig. 3B and D and 4B). These observations are summarized in the model shown in Fig. 5.

The *swmA* mutant prepared by the quick-freezing and deep-etching method displays all of the same cell envelope structures as the wild type, with the exception of the missing S-layer. The outermost surface of S1A1 lacked regularity and the lattice-like appearance of the outer surface as seen in wild-type cells (Fig. 3B and D). Cross-sectional fractures reveal fibril-like structures arrayed perpendicular to the cell envelope (Fig. 4B). This FL extends 19 nm from the cell surface. Just as seen in thin sections, fractured cells of S1A1 lack the outermost S-layer. The outermost FL appears irregularly arranged in surface views (Fig. 3).

#### DISCUSSION

The mechanism of swimming motility in marine *Synechococcus* remains elusive due in large part to the lack of readily visible propulsive organelles. In order to address this question, we are making use of swimming motility mutants (5; J. McCarren and B. Brahamsha, unpublished data). The results presented here show, for the first time, an ultrastructural difference between a wild-type *Synechococcus* strain that is capable of swimming and a mutant that is not. Wild-type cells exhibit a cell envelope characterized by the presence of a crystalline S-layer, which is lacking in a mutant in which a single gene, *swmA*, has been insertionally inactivated. Polar effects of the insertion are unlikely since *swmA* does not appear to be part of an operon (5, 22), and 5' and 3' neighboring genes are encoded on the opposite strand.

Several observations suggest that the S-layer is composed of SwmA. First, *swmA* mutants lack the S-layer. Furthermore, as shown previously (5), a comparison of the outer membrane protein profiles of the wild type and the *swmA* mutant revealed that the only difference between these strains is the loss of SwmA. In addition, SwmA is one of the most abundant proteins in motile cells (5), which is a characteristic shared by other S-layer proteins (20) and is consistent with the hypothesis that this protein forms a layer covering the entire cell surface. Also, divalent cations, specifically  $\text{Ca}^{2+}$ , have been

like structures (arrowheads) immediately external to the outer membrane in both strains. Panel C shows a cell in which the S-layer (open arrow) has been fractured away, revealing the material immediately underneath. The fracture plane then crossed several cell envelope layers to reveal the cytoplasmic membrane inner leaflet face. SL, S-layer; CMi, cytoplasmic membrane inner leaflet. Bar, 100 nm (all panels).

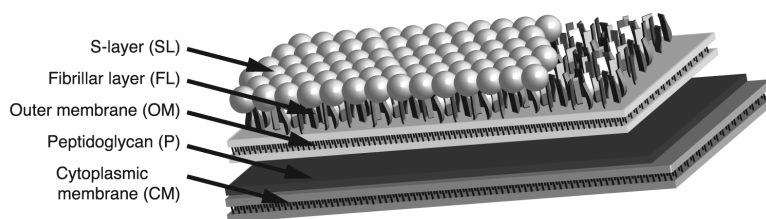


FIG. 5. Model of *Synechococcus* sp. strain WH8102 cell envelope structure.

shown to be important for the integrity, attachment, and re-crystallization of S-layers in various bacteria (9, 10, 21, 37), and treatment with divalent cation chelators causes some S-layers to dissociate from the cell surface. Treatment of *Synechococcus* sp. strain WH8102 with the chelator EDTA removes the outer membrane and solubilizes SwmA (5). Finally, while S-layer proteins are highly divergent, sharing essentially no homology in amino acid comparisons (9), SwmA, like other S-layer proteins, has numerous glycine- and aspartate-rich repeats that are thought to function in calcium ion bridging to the outer membrane (5).

S-layers are not uncommon in cyanobacteria (16, 17, 30). Additionally, fibrillar structures similar in size and arrangement to the ones we report here, spanning the region between the S-layer and outer membrane, have been observed in TEM analyses of both a coastal marine strain (23) and a freshwater strain of *Synechococcus* (28), neither of which exhibits swimming motility. Samuel et al. (26) have also used rapid-freezing and fracturing methods to examine cells of *Synechococcus* sp. strain WH8113, a motile strain closely related to strain WH8102 (31), and their results are similar to the ones reported here in several respects. Strain WH8113 was shown to have an S-layer arranged in a similar 110° rhomboid organization with 12-nm spacing separating individual units. In addition, however, these authors visualized short fibrils (5 nm wide and 150 nm long), or spicules, covering the surface of the cell. They proposed that these spicules, which were reported to span the entire cell envelope, may be able to transmit motion in the cytoplasmic or outer membrane to the surrounding medium. Wolgemuth et al. (36) further refined this model by postulating the presence of a mechanochemical filament located in the cytoplasmic membrane, which would be attached to these spicules and would cause them to move by bending. These are plausible models; however, it is clear from the results presented here that the presence of these spicules is not a characteristic of all swimming *Synechococcus* strains. Using methods like the ones used by Samuel et al. (26), we have never observed such spicules on the surface of WH8102 cells. The absence of spicules in the swimming strain WH8102 suggests that such structures are not required for motility in all swimming *Synechococcus* strains. Ehlers et al. (8) have proposed a model in which localized contractions and expansions of the OM generate surface waves of sufficient amplitude and frequency to propel *Synechococcus* at the observed speeds. This model does not require spicules and hence remains a possibility. The challenge to identify the mechanism of wave generation required by such a model remains.

How then does the S-layer of swimming *Synechococcus* strains function in motility? While S-layers are also ubiquitous in archaea and common in many bacteria (29), in only a few cases has the function of an S-layer been determined (4). S-layers are known to be sites of attachment for exoenzymes (7, 19). Perhaps in swimming *Synechococcus* strains, the S-layer serves as the site of attachment or the site for interaction of yet-to-be-discovered motility components. S-layers have also been shown to perform a shape-maintaining role in archaea (2). Conceivably, the S-layer may function to maintain cell shape or cell envelope integrity in the face of continuous disruption associated with the surface wave generation mechanism proposed by Ehlers et al. (8).

In several strains of *Oscillatoria*, a filamentous gliding cyanobacterium, an array of helically arranged fibers is present between the peptidoglycan and the outer membrane (1), and one model to explain the gliding motility of these organisms invokes the generation of surface waves produced by the contraction of such an array. While we have not observed such an array in *Synechococcus* sp. strain WH8102, there may be other ways of generating surface waves, such as localized swelling of the cell surface (18, 24). Could the S-layer be involved in such swelling? The source of energy for swimming of *Synechococcus* is the sodium motive force (35), suggesting that the “motor” responsible for converting chemical into mechanical energy is located in the cytoplasmic membrane. How is this energy transduced to the cell surface? While they are unable to generate thrust, and hence unable to translocate, *swmA* mutants can generate torque, since attached cells can still rotate about a point of attachment (5, 6). What is the mechanism of torque generation? How is it coupled to thrust? In order to answer these questions, it will be necessary to identify the other components of the motility machinery as well as to identify the proteins with which SwmA interacts.

The complete sequence of the genome of *Synechococcus* sp. strain WH8102 has recently been obtained (22). An examination of this genome provides further indication of the uniqueness of the mechanism of swimming of *Synechococcus* strains, as no homologs for proteins associated with other forms of prokaryotic motility were found (22). Recently, through transposon mutagenesis, three clusters of genes (*swmA* is in one of them) that are required for swimming were identified (22; McCarren and Brahamsha, unpublished). An analysis of these mutants, using both biochemical approaches and the ultrastructural methods described here, should provide further insights into what remains a fascinating mystery.

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The text of Chapter III, in full, is a reprint of the material as it appears in McCarren, J., J. Heuser, R. Roth, N. Yamada, M. Martone, and B. Brahamsha. 2005. Inactivation of *swmA* results in the loss of an outer cell layer in a swimming *Synechococcus* strain. *J. Bacteriol.* 187: 224-230. The dissertation author was the primary author, and co-author B. Brahamsha directed and supervised the research, which forms the basis for this chapter. Cryofixation, freeze-substitution and TEM work was performed at the National Center for Microscopy and Imaging Research, University of California, San Diego, by the dissertation author and N. Yamada under the direction of M. Martone. Freeze-fracturing and etching EM work was performed at Washington University, St. Louis, by R. Roth under the direction of J. Heuser.

## **CHAPTER IV**

### **Transposon mutagenesis in a marine *Synechococcus* strain: Isolation of swimming motility mutants**

## Transposon Mutagenesis in a Marine *Synechococcus* Strain: Isolation of Swimming Motility Mutants

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**Certain marine unicellular cyanobacteria of the genus *Synechococcus* exhibit a unique type of swimming motility characterized by the absence of flagella or any other obvious organelles of motility. While the abundant cell surface-associated 130-kDa glycoprotein SwmA is known to be required for the generation of thrust, identification of other components of the motility apparatus has, until recently, been unsuccessful. Here we report on the development of a transposon mutagenesis system for use with marine *Synechococcus* sp. strain WH8102, a model organism for which the genome has been sequenced. Utilizing this mutagenesis technique, we have isolated 17 independent mutants impaired in swimming motility. These 17 transposon insertions are located in nine open reading frames, which cluster in three separate regions of the genome. Included within these clusters are several multicomponent transport systems as well as a number of glycosyltransferases.**

The unicellular marine cyanobacteria of the genus *Synechococcus* are ubiquitous in the world's oceans and comprise a major fraction of the photosynthetic marine picoplankton (29–31). As such, they are important contributors to global primary production and may generate as much as 30% of total oceanic primary production (29). A genetic manipulation system has been described previously (5, 6) which has allowed molecular investigation into the adaptations of this globally important organism. Here we describe an addition to the molecular tools available for use with marine *Synechococcus* spp. with the development of a transposon mutagenesis technique. We have applied this method of mutagenesis to investigate the unique swimming motility exhibited by marine *Synechococcus* spp.

Among the adaptations exhibited by marine *Synechococcus* spp. is the ability of certain strains to swim through their liquid environment without the use of flagella (33). Transmission electron microscopy techniques, such as negative staining and quick-freeze fixation, fracture, and etching as well as high-intensity dark-field microscopy, have been employed in attempts to visualize structures, without success (35). Additionally, shearing experiments and motility-dependent amplitude spectra confirm the lack of flagella or other extracellular appendages used for motility (35). Both jet propulsion and self-electrophoresis have been ruled out as possible mechanisms for motility (24). This leaves the cell surface itself as the only remaining structure with potential for generating a propulsive force. Ehlers et al. have proposed a model by which cells could move at the observed speeds by propagating longitudinal waves across their surfaces (9).

Nonflagellar motility is not limited to marine *Synechococcus* spp. A diverse array of bacteria (16), including other cyanobacteria (12), exhibit nonflagellar motility, but in virtually every

case this movement is not swimming but rather a movement associated with surfaces. Marine *Synechococcus* spp. are different in that in spite of their cells' clear lack of flagella, they swim through their liquid environment and do not move along surfaces. With the exception of the helical, wall-less bacteria of the genus *Spiroplasma*, which swim by means of conformational deformations of each cell's helical cytoskeletal filament (11, 36), *Synechococcus* spp. are the only bacteria known to swim without flagella. For a review of swimming motility in marine *Synechococcus* spp., see the work of Brahamsha (7).

While the basic tools required for genetic manipulations in *Synechococcus* spp. have been described previously (5, 6), a functional transposon mutagenesis system would be a powerful additional tool for molecular and genetic studies. The integration of a transposon into the host chromosome creates an insertional mutation, which can be easily cloned and sequenced to determine the site of insertion. We used the delivery vector pRL27 (15), which has a mini-Tn5 derivative engineered to enhance transposition frequency, for transposon mutagenesis in *Synechococcus* sp. strain WH8102, and our results have shown that this tool is useful in identifying new genetic loci involved in swimming motility. As this was the first use of transposon mutagenesis in a marine *Synechococcus* sp., preliminary experiments were conducted to characterize the use of this delivery vector and transposon. When combined with an appropriate screen or selection, the method described here will be applicable to the study of various aspects of *Synechococcus* physiology. We have developed a screen to identify mutants impaired in the ability to swim, and this screen in combination with transposon mutagenesis has identified several regions of the chromosome that contain genes necessary for nonflagellar swimming motility.

### MATERIALS AND METHODS

**Bacterial strains and growth conditions.** The bacterial strains and plasmids used in this study are listed in Table 1. *Escherichia coli* strains MC1061(pRK24, pRL528), DH5 $\alpha$ , BW20767 (15), and Transformax EC100D *pir*<sup>+</sup> (Epicentre, Madison, WI) were grown in Luria-Bertani medium (28). When appropriate,

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TABLE 1. Bacterial strains and plasmids used in this study

Strain or plasmid	Relevant characteristic(s) <sup>a</sup>	Source <sup>b</sup> or reference
<b>Strains</b>		
<i>Synechococcus</i> sp. strain WH8102	Motile strain, recipient in conjugations with pRL27 and pMUT100 constructions	J. Waterbury
<i>E. coli</i>		
MC1061	Host for pRK24, pRL528; used as a donor in pJM construction conjugations	8
DH5 $\alpha$	Recipient in transformations	BRL
BW20767	Used as donor for pRL27 conjugations	21
Transformax EC100D <i>pir</i> <sup>+</sup>	Expresses <i>pir</i> gene product for propagation of vectors containing the R6K $\gamma$ origin of replication	Epicentre
<b>Plasmids</b>		
pMUT100	Kan <sup>r</sup> , Tet <sup>r</sup> ; suicide vector	5
pRL27	Kan <sup>r</sup> ; mini-Tn5 plasmid ( <i>oriR6K</i> ) delivery vector	15
pRK24	Tc <sup>r</sup> , Amp <sup>r</sup> ; conjugal plasmid, RK2 derivative	5, 22
pRL528	Cm <sup>r</sup> ; helper plasmid, carries <i>mob</i>	5, 10
pCR2.1-TOPO	Kan <sup>r</sup> , Amp <sup>r</sup> ; PCR product cloning vector	Invitrogen
pJM37	pMUT100 containing SYNW0079 fragment of nt 78600 to 78939	This work
pBB1000	pMUT100 containing <i>swm4</i> fragment of nt 84507 to 84926	This work
pJM62	pMUT100 containing SYNW0087 fragment of nt 88793 to 89004	This work
pJM18	pMUT100 containing SYNW0088 fragment of nt 89414 to 89635	This work
pJM59	pMUT100 containing SYNW0192 fragment of nt 193443 to 193661	This work
pJM40	pMUT100 containing SYNW0193 fragment of nt 194020 to 194287	This work
pJM20	pMUT100 containing <i>swmB</i> fragment of nt 913153 to 913396	This work
pJM35	pMUT100 containing SYNW0957 fragment of nt 947811 to 948110	This work
pJM34	pMUT100 containing SYNW0960 fragment of nt 953811 to 954171	This work

<sup>a</sup> nt, nucleotide. Numbering according to reference 23.

<sup>b</sup> BRL, Bethesda Research Laboratories, Gaithersburg, MD; Invitrogen, Carlsbad, CA; Epicentre, Madison, WI.

ampicillin (100  $\mu$ g/ml), kanamycin (50  $\mu$ g/ml), and chloramphenicol (10  $\mu$ g/ml) were used for the selection and maintenance of plasmids in *E. coli*. Cyanobacterial strains were grown either in SN medium (32) made with seawater obtained from the Scripps Pier (Scripps Institution of Oceanography, La Jolla, CA) or in SN medium prepared with synthetic ocean water (25). Cyanobacterial cultures were incubated at 25°C with a constant illumination of 25 microeinsteins m<sup>-2</sup> s<sup>-1</sup> and were maintained as either 4-ml cultures in 17- by 100-mm polystyrene tubes (Becton Dickinson, Franklin Lakes, NJ) or as 50-ml cultures in 125-ml glass flasks without shaking. SN pour plates for obtaining isolated colonies were prepared as previously described (5), with the single modification of reducing the agar concentration to 0.2% (wt/vol), which aids in screening for nonmotile colonies. Kanamycin was added to a final concentration of 25  $\mu$ g/ml for pour plates and 20  $\mu$ g/ml for liquid cultures, where appropriate, to maintain selection for insertions.

**Transposon mutagenesis.** Biparental matings of *E. coli* and the *Synechococcus* sp. were carried out as previously described (5). *E. coli* strain BW20767, which harbors the plasmid delivery vector pRL27 (15), was used as the donor in conjugations with *Synechococcus* sp. strain WH8102. Following a 48-h incubation, cells were resuspended and plated in SN pour plates containing 0.2% agar and kanamycin (25  $\mu$ g/ml) to obtain isolated colonies. Following the appearance of colonies, which usually took from 2 to 3 weeks, the plates were screened visually for putative nonmotile mutants displaying a compact, dense colony morphology. These colonies were transferred into and grown in liquid SN medium and examined by phase-contrast microscopy to confirm the loss of swimming motility in liquid. The absence of the *E. coli* donor strain was verified as described previously (5).

**Plasmid rescue.** The plasmid pRL27 allows for one-step cloning of a transposon insertion and its flanking DNA (15). Chromosomal DNA was prepared from a 50-ml culture of a *Synechococcus* transconjugant as described previously (5). This purified DNA was digested with BamHI, which does not cut within the transposon sequence. The resulting fragments were ligated using T4 DNA ligase (Roche Applied Science, Indianapolis, IN) to generate a transposon junction plasmid consisting of the transposon and the flanking chromosomal DNA. The material from the ligation reaction was then electroporated into *E. coli* strain Transformax EC100D *pir*<sup>+</sup> (Epicentre, Madison, WI) following the manufacturer's recommendations. Following electroporation, the *E. coli* isolate was plated on Luria-Bertani plates containing kanamycin. Plasmid DNA was isolated from the transformants using a QIAprep spin miniprep kit (QIAGEN, Valencia, CA).

The site of insertion of the transposon was determined by sequencing, utilizing the outward-directed transposon-specific primer tpnRL 17-1 (5'-AACAGCC AGGGATGTAACG-3') (15). DNA sequencing was performed with Megabace reagents (Amersham, Piscataway, NJ) on a Megabace 500 sequencer.

**Directed mutagenesis.** Directed inactivations were accomplished by cloning a completely internal fragment of a gene into the suicide vector pMUT100 as previously described (5). Twenty-mer oligonucleotide primers (Integrated DNA Technologies, Inc., Coralville, IA) were used to amplify DNA fragments (details given in Table 1) for cloning into pMUT100. These constructions were introduced into *Synechococcus* sp. strain WH8102 by conjugation with *E. coli* followed by subsequent selection of exconjugants on solidified media containing kanamycin. Clonal isolates were grown in liquid medium to confirm the mutant phenotype of the original transposon mutant. Complete segregation of mutant chromosomes was confirmed by Southern blotting and by PCR. For the PCR test, chromosomal DNA from the cyanobacterial mutant strain was isolated using a DNeasy tissue kit (QIAGEN, Valencia, CA) with the following modification: prior to proteinase K treatment, cells were incubated in a solution containing 20 mM Tris-Cl (pH 8.0), 2 mM Na<sub>2</sub>EDTA, 1.2% Triton X-100, and 50 mg/ml lysozyme for 30 min at 37°C. This DNA was then used as a template for PCR analysis utilizing primers flanking the fragment used for inactivation. Primer pairs used in these tests were confirmed to amplify a fragment of the expected size from wild-type DNA. Failure to amplify a fragment of the wild-type size from a mutant strain's DNA confirms the absence of the intact gene among the clonal population of mutant cells. This same DNA sample was used as a template in another PCR, utilizing primers directed to another open reading frame (ORF) as a positive control to confirm that the DNA was of sufficient quality for PCR amplification.

**Genome information.** Complete genomic sequence information for *Synechococcus* sp. strain WH8102 is available at [http://genome.ornl.gov/microbial/syn\\_wh/](http://genome.ornl.gov/microbial/syn_wh/) (23).

## RESULTS

**Initial characterization.** After an initial conjugation of *Synechococcus* sp. strain WH8102 with *E. coli* strain BW20767 (containing the plasmid delivery vector pRL27), 11 kanamycin-resistant transconjugants were selected randomly without



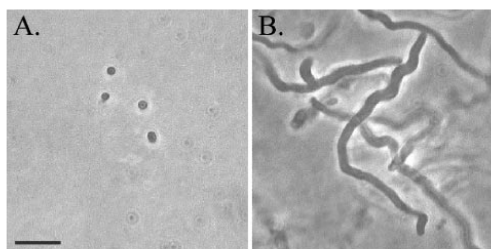


FIG. 1. Phase-contrast micrographs of wild-type WH8102 (A) and *minD* mutant (B) cells. Scale bar, 10  $\mu$ m (both panels).

application of a specific phenotypic screen. Following plasmid rescue, the site of transposon integration was determined for each strain by sequencing outwards from the transposon into flanking chromosomal DNA. The transposon insertion site was unique for each isolate. Genomic DNA isolated from these strains was also subjected to digestion with *Sma*I and to Southern analysis utilizing a probe designed against a portion of the kanamycin resistance gene present within the transposon. For each strain assayed, the probe hybridized with a single band consistent in size with that predicted by the genome sequence (data not shown). The majority of these strains did not have a phenotype that was obviously different from that of the wild-type strain, with a few exceptions. Two nonmotile strains were isolated and are described in greater detail below. The cells of another isolate, while still motile, were extremely elongated, ranging from 10 to 100 times the typical cell length (Fig. 1). In this strain, transposon insertion was into a putative septum site-determining gene, *minD*, which is present on the chromosome in the typical operon arrangement *minCDE*. The proteins of the *minCDE* system act in concert as a negative regulator of septation site formation in *E. coli* (26), and the phenotype of the *Synechococcus minD* mutant is consistent with a similar role for the Min proteins in *Synechococcus*. Directed mutagenesis at this locus confirmed the filamentous phenotype observed in the transposon mutant; nevertheless, these elongated cells retain the ability to swim.

**Nonmotile mutants.** To screen for mutants with impaired ability to swim, a screen was developed to distinguish between motile and nonmotile isolates based on colony morphology. Solid SN medium prepared as described previously (5), with the single modification of reducing the agar concentration to 0.2%, allowed for the identification of nonmotile isolates. Nonmotile cells produce small, dense colonies while motile cells produce larger, more diffuse colonies (Fig. 2). Nonmotile isolates were grown for multiple transfers in liquid medium to confirm total loss of swimming motility.

Utilizing this screen, 17 nonmotile transposon mutant strains were obtained (Table 2). These 17 independent transposon insertions are located in nine ORFs, which cluster into three separate regions of the genome (Fig. 3). Eight transposon insertions are located in a 12-kb region (cluster 1) that includes the previously identified motility gene *swmA* (5). Another five transposon insertions are tightly grouped into a 1.4-kb region that appears to be the beginning of an operon containing four genes (cluster 2). The last four insertions are spaced out over

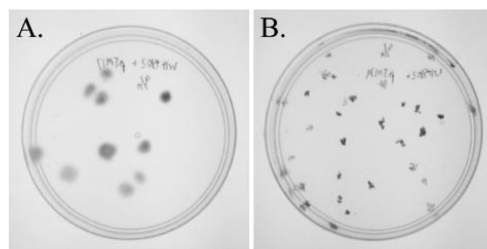


FIG. 2. Four plates of *Synechococcus* sp. strain WH8102 transconjugants for isolation and screening of mutants. Shown are both a motile transconjugant (A) and a nonmotile transconjugant (B).

a 41-kb region that contains, among other ORFs, one large ORF of 32.4 kb (cluster 3).

To confirm that the phenotype observed for these mutants was the result of transposon insertion, directed mutations were constructed for each ORF identified by transposon mutagenesis as required for swimming motility. These directed mutations confirmed the motility phenotype of the original mutations. None of the mutants described here exhibits the attached rotating behavior of the *swm4* mutant. Two ORFs (SYNW0957 and SYNW0960) interrupted by transposon insertion were resistant to inactivation by directed mutagenesis. Multiple attempts were made to create a directed mutation near the site of transposon insertion of these problematic ORFs, and in no instance were we able to obtain transconjugants. DNA from the original transposon mutant isolates (strains Tr A4-5 and Tr D2-3) was analyzed by PCR, utilizing primers flanking the site of transposon insertion. Positive PCR amplification of the wild-type-sized product from these isolates confirmed that the original transposon mutants were incomplete segregants which still possessed a nondisrupted intact gene.

On the basis of their proximity to one another, some genes in which insertions occurred are likely to be arranged in operons, and hence polar effects on downstream genes are possible.

TABLE 2. Transposon insertions yielding nonmotile mutants

Strain	ORF	Directed-mutant phenotype	Function
Tr C5-13	SYNW0079	Nonmotile	Conserved hypothetical
Tr B8-2	SYNW0085	Nonmotile	SwmA (cell surface S-layer protein [4])
Tr 3-4	SYNW0087	Nonmotile	Putative glycosyltransferase
Tr C10-3	SYNW0087		
Tr B7-3	SYNW0088		
Tr B5-2	SYNW0088	Nonmotile	Putative glycosyltransferase
Tr B1-4	SYNW0088		
Tr B3-10	SYNW0088		
Tr B8-5	SYNW0192	Nonmotile	Putative parvulin-like PPIase
Tr B6-4	SYNW0193	Nonmotile	Putative ABC transporter of the HlyB family
Tr A6-8	SYNW0193		
Tr A6-1	SYNW0193		
Tr B6-6	SYNW0193		
Tr A4-4	SYNW0953	Nonmotile	SwmB
Tr 15-2	SYNW0953		
Tr A4-5	SYNW0957	No transconjugants	Conserved hypothetical
Tr D2-3	SYNW0960	No transconjugants	Putative parvulin-like PPIase

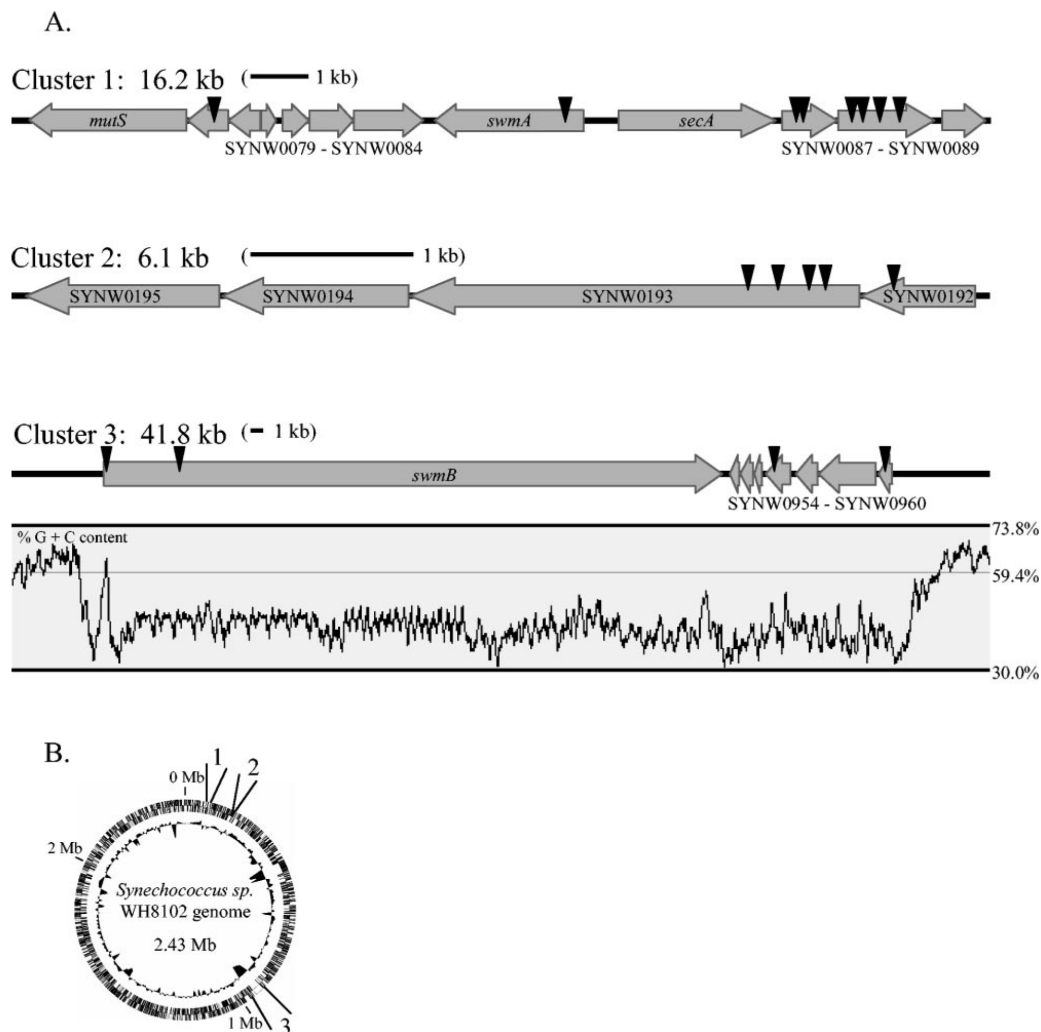


FIG. 3. Chromosomal regions containing genes involved in swimming motility as identified by transposon mutagenesis. (A) Gene arrangement and location of each transposon insertion (arrowheads) are shown. A 1-kb scale bar is included for each cluster. Percent G+C content (window = 200 bp) is included for the chromosomal region encompassing cluster 3. (B) Location of clusters 1 to 3 on a circular chromosome. The outer ring contains predicted ORFs for both strands, and the inner ring shows associated G+C content (deviation from average).

The start codon of SYNW0088 is 5 bp from the stop codon of SYNW0087. Likewise, in cluster 2, the stop codon of SYNW0192 overlaps the start codon of SYNW0193, and the stop and start codons of the next two downstream ORFs are 2 and 5 bp apart, respectively. On the other hand, neither *swmA* (SYNW0085) nor SYNW0953 is transcribed with other genes, while the intergenic distances between SYNW0079, SYNW0957, SYNW0960, and SYNW0088 and their downstream genes (159 bp, 59 bp, 118 bp, and 123 bp, respectively) indicate that they are unlikely to be cotranscribed. Determining which, if not all, of ORFs SYNW0087, SYNW0192,

SYNW0193, SYNW0194, and SYNW0195 are needed for motility will require complementation experiments. Unfortunately, these experiments are currently not possible with *Synechococcus* sp. strain WH8102, as kanamycin is the only selectable marker available. We are working to remedy this.

#### DISCUSSION

The transposon delivery vector pRL27 has been mobilized into a diverse array of bacterial species (15). As this was its first use in a marine *Synechococcus* sp., we report here on prelim-

inary characterization of the behavior of this transposon delivery vector in this host. Sequence analysis of the first 11 isolated mutant strains indicates that each transposon insertion is unique, and Southern analysis of DNA isolated from these same mutant strains shows that for each strain, transposon insertion occurred only once, indicating that insertion of multiple transposons is likely to be rare.

Motile cells grown embedded in solidified media produce large, diffuse colonies among which nonmotile isolates can be identified by their small, dense colony morphology. Using this simple visual screen, we have isolated 17 nonmotile transposon mutants and characterized the site of transposon insertion for each. All motility genes identified to date cluster into three discrete regions on the chromosome. Various transporters and transport-related genes are present within these clusters. Both cluster 2 and cluster 3 contain a set of three genes that appear to encode a multicomponent transport apparatus. Moreover, these three genes are arranged in the same order in both clusters. Each set contains a gene for an ABC transporter of the protein-1 exporter (HlyB) family (SYNW0193 and SYNW0959) (27), a gene for a membrane fusion protein (SYNW0194 and SYNW0958) which is an auxiliary component possibly spanning the periplasm to connect the ABC transporter with an outer membrane component, and a gene whose product has limited similarity to peptidyl-prolyl isomerases (PPIases) (SYNW0192 and SYNW0960). Such PPIases have been shown to exhibit chaperone-like activity involved in the maturation of outer membrane porins in *E. coli* (2). Additionally, the SYNW0957 product shows very weak similarity to the MotA/TolQ/ExxB proton channel family (PFAM accession no. PF01618), which is also involved in protein translocation across both membranes of the gram-negative bacterial cell wall. In addition to multicomponent transporters, the products of several ORFs in these clusters (SYNW0087, SYNW0088, SYNW0084, and SYNW0195) show similarity to glycosyltransferases. Interestingly, the only previously known component of the motility apparatus, SwmA, is glycosylated (4) and has been shown to form an S-layer on the surface of these cells outside of the outer membrane (20). Both of these characteristics of SwmA correspond well with the types of genes found in the motility clusters. The presence of multiple transporters and multiple glycosyltransferases suggests that other components of the motility apparatus may be located outside the outer membrane and may be posttranslationally modified by glycosylation.

One exceptional gene present in cluster 3 (SYNW0953), hereinafter called *swmB*, is more than 32 kb long and comprises more than 1% of the total genome of this organism. While the function of SwmB is still unclear, preliminary experiments have shown that this protein is associated with the outer membrane (J. McCarren and B. Brahamsha, unpublished). More than half of the sequence of *swmB* is composed of regions of large tandem repeats. Database searches against the SwmB sequence indicate limited similarity to bacterial RTX proteins (named for repeats in toxin, which are secreted toxins that possess tandem copies of a nine-amino-acid motif) (34) and that this similarity is restricted to the repetitive portions of SwmB. While SwmB shares similarity with RTX proteins, SwmB does not actually contain the RTX repeat. Cluster 3 is also exceptional in its percent G+C content compared to

that of the rest of the genome (Fig. 2). *Synechococcus* sp. strain WH8102 has a genome average G+C content of 59.4% (23) while the G+C content of cluster 3 is 42.2% and exhibits an abrupt departure from the genome average immediately before *swmB* and after SYNW0961. The dramatically different percent G+C contents suggest that these ORFs may have been acquired by a recent horizontal gene transfer event. While the ORFs in cluster 3 may be a recent acquisition, clusters 1 and 2 have percent G+C contents typical of *Synechococcus* sp. strain WH8102, suggesting that all of the components required for motility were not acquired in a single horizontal transfer event.

While swimming motility in marine *Synechococcus* spp. is distinct from the surface-associated gliding motility observed in other bacteria, it does share characteristics with the gliding motility observed in the *Cytophaga-Flavobacteria-Bacteroides* group, which occurs without slime extrusion or apparent appendages (17). Following chance attachment, both types of cells are observed to rotate about their point of attachment at a rate of 1 to 2 rotations per s (14, 35). Additionally, several parallels can be drawn between the motility genes of *Flavobacterium johnsoniae* identified in reports from the laboratory of McBride and coworkers (1, 13, 18, 19) and the types of genes identified in the data presented here. The first is the importance of transporters to both mechanisms of motility. Two sets of putative multicomponent ABC transporters have been identified in our current results. Similarly, several genes homologous to multicomponent ABC transporters (*gldA*, *gldF*, and *gldG*) and other transport-related genes (*secDF*) are all required for *F. johnsoniae* motility. A second similarity shared by both mechanisms of motility is that they employ PPIase-like proteins. While the PPIase-like ORF products required for *Synechococcus* and *F. johnsoniae* motility are from separate and evolutionarily unrelated families, the implication of functionally related proteins is of interest. Lastly, some genes with limited similarity to *swmB* have been implicated in *Cytophaga-Flavobacteria-Bacteroides*-type gliding motility. Recently, both a gene encoding an RTX autotransporter protein (T. Braun, S. Nelson, M. Uppal, and M. McBride, Abstr. Gen. Meet. Am. Soc. Microbiol. 2004, abstr. 104, 2004) and an exceptionally large and repetitive ORF (S. Nelson, personal communication) have been found to be involved in *F. johnsoniae* gliding motility. Also worthy of note in light of the low G+C content of cluster 3 genes, bacteria of the family *Flavobacteriaceae* have a characteristically low G+C content, ranging from 28 to 44% (3). How surface-associated gliding motility and nonflagellar swimming motility are related is difficult to envision, but the identification of similar types of genes involved in both phenomena suggests some possible relationship.

How the products of these recently identified genes function has yet to be determined. Exporting components of the motility apparatus to the cell surface appears to be important. In addition to allowing for the correct localization of the motility apparatus, perhaps these transporters play a more active role in generating swimming motility. Furthermore, a number of glycosyltransferases appear to be implicated. Whether these function to glycosylate protein components of the motility apparatus or whether they are involved in some other aspect of polysaccharide biosynthesis important for motility is unclear. Although how *Synechococcus* spp. are able to swim is still not

understood, these findings have begun to identify the genes required for swimming motility.

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The text of Chapter IV, in full, is a reprint of the material as it appears in McCarren, J., and B. Brahamsha. 2005. Transposon mutagenesis in a marine *Synechococcus*: isolation of swimming motility mutants. *J. Bacteriol.* 187:4457-4462. The dissertation author was the primary author, and co-author B. Brahamsha directed and supervised the research, which forms the basis for this chapter.

## CHAPTER V

**SwmB: a highly repetitive 1.12 MDa protein that is required for non-flagellar swimming motility in *Synechococcus***

## Abstract

An exceptionally large ORF (>33 kb) was identified in sequencing the genome of the marine unicellular cyanobacterium *Synechococcus sp.* strain WH8102. Both random and directed mutagenesis demonstrate that this gene, called *swmB*, is required for the unique non-flagellar swimming motility exhibited by these cells. The sequence of *swmB* is highly repetitive, with 4 domains of tandem repeats comprising over 60% of the protein. The genomic region encoding *swmB* and several other motility genes, including its putative cognate transporter, has an exceptionally low % G+C content relative to the genome average. This portion of the chromosome is not present in two sequenced non-motile strains suggesting acquisition of these genes by horizontal gene transfer. Gel electrophoresis confirms that the translated protein is approximately 1 megadalton in size. SwmB co-purifies with the outer membrane fraction and is also found in the culture medium. Inactivation of this gene does not appear to disrupt the proper positioning of at least one other known component of the motility apparatus, SwmA, although mutants do appear to be impaired in the generation of both torque and thrust.

## Introduction

While the mechanism of non-flagellar swimming motility in *Synechococcus* is still unexplained, at least one structure and several genes required for this process have been determined (18, 19). One protein required for motility in these cells, SwmA, is found in abundance in motile strains (4). SwmA is a glycosylated protein that contains repeated  $\text{Ca}^{2+}$  binding motifs. Inactivation of *swmA* results in complete loss

of motility, but cells can still generate torque as observed in fortuitously attached mutant cells that retain the ability to spin like wild-type cells. Microscopic analysis of wild-type and *swmA* mutant cells revealed that motile strains possess a paracrystalline S-layer that is absent in the *swmA* mutant, suggesting that SwmA is the S-layer protein (19).

While complete genomic sequence information did not identify other components of the motility apparatus (21), development of a transposon mutagenesis technique allowed for the identification of three chromosomal regions involved in non-flagellar swimming motility (18). One of these regions is particularly interesting, as it possesses a dramatically reduced % G+C content suggesting that this genetic material has been acquired through horizontal gene-transfer. Moreover, included in this chromosomal region is an exceptionally large and repetitive open reading frame (ORF). Two non-motile strains were obtained from independent transposon insertions within this ORF. These two transposon insertions occurred at 12 bp and 5237 bp downstream of the predicted start codon respectively, with both insertions completely eliminating motility. A directed inactivation of *swmB* was constructed by insertional mutagenesis, confirming the non-motile phenotype. This ORF has been named *swmB* (for swimming motility). No additional ORFs are found downstream of *swmB* on this coding strand and thus the loss of motility can be ascribed to inactivation of this gene and not to a polar effect of insertion. Repeated attempts were made to locate fortuitously attached cells exhibiting the attached spinning behavior of *swmA* mutants, but this behavior has yet to be observed in *swmB* mutant cells. Presented here is further characterization of this unusual bacterial protein.



## Materials and Methods

**Bacterial strains and growth conditions.** *Synechococcus sp.* strains WH8102 (30) and its isogenic *swmB* mutant strain Swm-2 (inactivated with suicide plasmid pJM20 as previously described (18)) were grown in SN medium (29) prepared with either local seawater from the Scripps Pier (Scripps Institution of Oceanography, La Jolla, CA) or synthetic ocean water (SOW) prepared as according to Price *et. al* (23) except components were not treated with chelex. Cultures were maintained as either 50-ml cultures in 125-ml glass Ehrlenmeyer flasks, or 1-L cultures in 2.8 L Fernbach flasks grown without shaking. Cultures were incubated at 25°C with constant illumination of 25  $\mu\text{E}\cdot\text{m}^{-2}\cdot\text{sec}^{-1}$ . Kanamycin was added to a final concentration of 20  $\mu\text{g}\cdot\text{ml}^{-1}$  for Swm-2, to select for and maintain mutational insertion.

**Sequence analysis.** The complete genomic sequence of *Synechococcus sp.* strain WH8102 and annotation was recently reported (21) and is available at the Joint Genome Institute website (<http://spider.jgi-psf.org>). BLAST-P analysis (1) was conducted using the non-redundant database at NCBI (<http://www.ncbi.nlm.nih.gov/BLAST/>). Repeats were identified using the MEME/MAST motif discovery and search tools available through the San Diego Supercomputer Center (<http://www.meme.sdsc.edu>). Additionally, motif searches and transmembrane predictions were conducted using proteomic and sequence analysis tools including ScanProsite, Motif Scan, FingerPRINTSscan, and HMMTOP, which are all available through the ExPASy website (<http://us.expasy.org>). Additionally secondary structure predictions were performed using the Robetta server (16). Due to

the large size of the *swmB* sequence, the ORF was subdivided into subregions according to repeat domains (see Fig. 1) and subjected to the above bioinformatic searches as the full-length coding sequence, as individual domains, and as individual repeats.

**Protein purification.** Outer membrane (OM) proteins were isolated from WH8102 and Swm-2 strains as described by Brahamsha (4) with some modifications. Briefly, exponentially growing cultures were centrifuged, washed once with 30 ml sterile SN medium, and resuspended at  $\sim 125\times$  concentration in ice-cold stripping buffer (50 mM Tris HCl + 10 mM Na<sub>2</sub>EDTA + 15% sucrose, pH 8.0) to strip off the outer membranes. After a 30 minute incubation on ice, cells were removed by centrifugation for 10 minutes at  $6277 \times g$ . The resulting material was subjected to a high speed spin of  $100,466 \times g$  for 90 minutes at 4°C to pellet the membrane fraction (HSP) and yield a supernatant containing soluble proteins not associated with the pelleted membranes (HSS). Proteins were routinely concentrated and subjected to buffer exchange using Amicon ultra 30,000 or 100,000 molecular weight cut-off (MWCO) centrifugal filters (Millipore, Bedford, MA) as directed by the manufacturer. Following removal of cells from media by centrifugation, proteins from spent media were recovered and concentrated using an ultra-filtration cell (Amicon) with 30,000 MWCO filters and further concentrated with 30,000 MWCO centrifugal filters. Gel electrophoresis was conducted using Nu-PAGE Novex Tris-Acetate 3-8% and Novex Tricine 10-20% gradient gels as recommended by the manufacturer (Invitrogen, Carlsbad, CA). Silver staining (Bio-Rad, Hercules, CA) and SYPRO Ruby staining (Sigma, St. Louis, MO) of gels was conducted as recommended by manufacturers.

Gels recorded on a ChemiImager 5500 (Alpha Innotech, San Diego, CA) and densitometry of SYPRO stained gels performed with AlphaEase FC version 3.2.2 software (Alpha Innotech).

Antibodies to SwmB were raised against full-length SwmB obtained from spent media and gel purified on 3-8% gradient gels. SwmB is several times larger than the next largest protein present in *Synechococcus* sp. WH8102 and is thus well separated from nearby bands on these gels. Multiple lanes were loaded identically and one lane cut off and stained to locate the position of the SwmB band on this gel. The equivalent portion of the remaining unstained gel containing SwmB, was excised for use as antigen in rabbit polyclonal antibody production by Strategic Biosolutions (Newark, DE). Raw sera were partially purified to remove antibodies that cross-react with other *Synechococcus* proteins utilizing French-pressed cell lysates from Swm-2 cells to adsorb non-specific antibodies. Briefly, approximately  $5 \times 10^9$  cells were collected by centrifugation for 10 minutes at  $7500 \times g$  and resuspended in 3 ml lysis buffer containing 3 $\times$  PBS (PBS: 9.56 mM  $\text{Na}_2\text{HPO}_4$ , 145 mM NaCl, pH 7.5), 4 $\times$  Complete Protease Inhibitor Cocktail (Roche, Indianapolis, IN). Cells were lysed with 4 passes through an Aminco french press mini-cell (Thermo Spectronic, Rochester, NY) at a pressure of 20,000 PSI. To this cell lysate solution 1 ml raw serum was added and incubated overnight at 4°C. Following incubation, debris and adsorbed antibodies were spun out of solution by centrifugation for 10 minutes at  $15000 \times g$ . SwmA was gel-purified by separating 800  $\mu\text{g}$  of protein from an OM soluble fraction preparation on a 7.5% Tris-glycine SDS gel followed by staining with copper using a Bio-Rad copper-staining kit (Bio-Rad Laboratories, Richmond, CA). The band

containing SwmA was excised from the gel and shipped to HRP, Inc (now Covance Research Products, Denver, PA) where it was used to prepare rabbit polyclonal antiserum.

For Western analysis, proteins were transferred to Invitrolon PVDF (Invitrogen) membranes in NuPAGE transfer buffer (Invitrogen) + 10% MeOH with 110V constant current for 2 hours. Following overnight blocking at 4°C in BLOTTO (15), membranes were incubated for 1.5 hours at room temperature with primary antibodies diluted in BLOTTO (1:500,000 and 1:50,000 for anti-SwmA and anti-SwmB respectively). Membranes were then washed 4 × 15 minutes in PBS + 0.05% Tween 20 (Fisher Scientific, Fair Lawn, New Jersey). Following washes, membranes were incubated 1.5 hours at room temperature with a peroxidase-conjugated anti-rabbit IgG (Sigma, St. Louis, MO) diluted 1:40000 in BLOTTO. Secondary antibody incubation was followed with another 4 × 15 minute washes in PBS + 0.05% Tween 20 and detected with Super Signal West Dura (Pierce, Rockford, IL) as recommended by the manufacturer. Periodic acid-Schiff staining was performed as described (26) to detect glycosylation.

Gel filtration and anion exchange chromatography were performed using an ÄKTA FPLC system with Superose 6 and HiTrap Q XL pre-packed columns, respectively (Amersham, Piscataway, NJ). Gel filtration was performed with 150 mM NaCl in 50 mM Tris-HCl pH 8.0. For anion exchange, a linear gradient of 0 to 1.5 M NaCl in 20 mM Tris-HCl pH 8.0 was used.

Sucrose density gradient centrifugation was performed with a Beckman L8-70M centrifuge and a SW-41 rotor. Stepwise gradients from 18% - 40% sucrose (w/v)

in 4% increments were centrifuged at  $288,000 \times g$  for 23 hours at  $4^{\circ}\text{C}$ .

Ultracentrifugation was carried out with a Beckman TL-100 ultracentrifuge and a TLA 100.1 rotor at  $436,000 \times g$  for 20 minutes at  $4^{\circ}\text{C}$ .

**Electron microscopy.** Partially purified SwmB was applied to freshly glow-discharged carbon-formvar coated grids (Ted Pella Inc., Redding CA) and incubated for 10 minutes at room temperature to allow for adsorption to carbon film. Grids were then washed twice on drops of milli-Q (Millipore, Billerica, MA) water, and negatively stained for 20 seconds on a drop of 2% uranyl acetate (Polysciences, Inc., Warrington, PA). Similarly, partially purified SwmB was applied to glow-discharged grids for immuno-gold labeling. Following adsorption, grids were floated on drops of blocking solution (PBS + 1% BSA + 5% Normal Goat Serum (Sigma)) for 1.5 hours at room temperature. Grids were transferred directly to drops of primary antibody diluted 1:1000 in blocking solution and incubated 1.5 hours at room temperature. Grids were washed  $3 \times 8$  minutes on drops of PBS before incubating 1.5 hours on drops of 10 nm-gold-conjugated anti-rabbit IgG (Ted Pella Inc.) diluted 1:100 in blocking solution. Grids were washed again  $3 \times 8$  minutes on drops of PBS before negatively staining as described. Samples were visualized and recorded at an acceleration voltage of 80kV on a JEOL 1200EX transmission electron microscope (TEM).

**Immuno-localization.** For gold labeling, cells were fixed directly in SN medium for 30min with EM grade glutaraldehyde (Sigma) at a final concentration of 0.5%. Following fixation cells were centrifuged 5 minutes at  $6500 \times g$  to collect cells, washed once for 5 minutes with PBS, then incubated for 15 minutes in blocking

solution of PBS + 1%  $\gamma$ -globulins (Sigma). Cells were then incubated for 2 hours in 1:50 dilution of a primary antibody in blocking solution, followed by two washes with blocking solution. Secondary incubation with a 1:100 dilution of 10-nm gold conjugated goat anti-rabbit IgG (Ted Pella Inc., Redding, CA) in blocking solution was carried out for 1 hour at room temperature followed by 1 washes in blocking solution and one wash in PBS. Cells were post-fixed in 0.5% glutaraldehyde for 1 hour at 4°C. After fixation cells were applied to glow-discharged carbon-formvar coated grids (Ted Pella Inc., Redding CA), allowed to adsorb for 5 minutes and then briefly stained by floating grid for 10 seconds on a drop of 2% (w/v) uranyl acetate and visualized by TEM as described above.

For fluorophore labeling whole cells were fixed directly in SN medium for 30 minutes with EM grade glutaraldehyde (Sigma) at a final concentration of 0.5%. After 30 minutes of fixation cells were applied to poly-L-lysine (Sigma) coated coverslips and incubated for another 30 minutes to adhere cells to coverslip for antibody incubations and washes. Coverslips were washed 3 $\times$  with PBS followed by blocking for 1 hour at room temperature with PBS + 1%  $\gamma$ -globulins (Sigma) + 1% normal goat serum (Sigma). Coverslips were incubated overnight at 4°C with primary antibodies diluted 1:25 in blocking solution. Following primary antibody incubation coverslips were washed 9 $\times$  with PBS and 1 $\times$  with blocking solution. Coverslips were then incubated with AlexaFluor 488-conjugated anti-rabbit IgG (Molecular Probes, Carlsbad, CA) diluted 1:50 in blocking solution for 2.5 hours at room temperature. Following another 8 washes with PBS, samples were equilibrated for 5 minutes in

Slow-Fade light equilibration buffer (Molecular Probes). Following equilibration, 10  $\mu$ l Slow-Fade Light (Molecular Probes) was applied to each coverslip prior to mounting. Paired images were collected on an Applied Precision Optical sectioning microscope (Issaquah, WA) equipped with a rhodamine filter set (Ex: 555/28 Em: 617/73) to detect fluorescence from chlorophyll and a FITC filter set (Ex: 490/20 Em: 528/38) to detect that from Alexa-488. Images were processed with softWoRx v3.3.6 software.

**Mass spectroscopy.** Analysis was conducted at the University of California San Diego, Chemistry & Biochemistry Mass Spectrometry Facility. For gel-purified bands, these bands were cut from a protein gel, reduced, alkylated, and extracted for subsequent protein digestion with trypsin (12, 25, 27). Additionally, preparations of partially purified SwmB were digested in solution for subsequent analysis. The resulting fragments were analyzed on an Applied Biosystems (Foster City, CA) QSTAR hybrid quadrupole-TOF mass spectrometer utilizing a Proxeon Biosystems (Denmark) nanospray source. Peptide masses and partial sequence information were matched against those predicted from genomic sequence information.

## Results

**Sequence analysis.** *swmB* is 32.38 kb in length and encodes a predicted protein of 10,791 amino acids with a molecular mass of 1.126 MDa and a pI of 3.98. This ORF is by far, the largest in the genome. *swmB* is almost 5 times larger than the next largest ORFs (conserved hypotheticals SYNW0985 and SYNW2303, both with similarity to RTX proteins, see discussion below) and comprises 1.33% of the entire

genome. In addition to its large size, SwmB is exceptional due to its highly repetitive primary structure. Greater than 60% of the predicted amino acid sequence is comprised of nearly identical repeats that are tandemly arrayed. Four repeat domains, each consisting of distinct repeats, are present (Figs.1 and 2). Repeat domain A consists of 28 highly conserved tandem repeats of 117 residues (type A repeats). Domain A repeats can be subdivided into three distinct types of nearly perfect repeats. A<sub>I</sub> and A<sub>II</sub> share 96.6% sequence identity and these repeats share 71.4% and 70.6% identity with type A<sub>III</sub> respectively. The three subtype repeats within domain A are then built into larger blocks arranged in consecutive order (A<sub>I</sub> - A<sub>II</sub> - A<sub>III</sub>) which itself is repeated multiple times (Fig. 1). The 14<sup>th</sup> repeat at the middle of this tandem array and the 28<sup>th</sup> repeat at the end, while still clearly related to the A repeat consensus, are less well conserved. Following domain A there is a short 225-residue region followed by a second array of 19 highly conserved tandem repeats of 127 residues each. Domain B repeats are nearly 100% identical with the exception of the first and last repeats, which have 55% and 66% identity with the consensus repeat respectively (Fig. 2). While domain A and domain B repeats do not share clear sequence homology, compositional analysis shows that these domains share similarly skewed amino acid usage (Table 1). These regions are especially rich in asparagine and threonine (highest 99% quantile in the Swiss-Prot database as analyzed by SAPS (6)) while deficient in methionine, arginine, and proline (lowest 5% - 1% quantile).

Additional repeats are present towards the C-terminus: domain C consists of 5 repeats of approximately 225 amino acids, and domain D contains 4 repeats of approximately 52 amino acids. The repeats within these domains are less well



conserved and do not exhibit the near identical nature seen in domains A and B, but are similar in that the first and last repeats of each tandem array are more degenerate. Additionally, these repeats show the same distinctive bias in amino acid composition similar to domains A and B.

In addition to its large size and repetitive sequence, *swmB* has a strikingly different % G+C content as compared to the rest of the *Synechococcus sp.* strain WH8102 genome (18). The genome average content of guanine and cytosine is 59.41%, while the sequence of *swmB* is only 42.91% G+C. In addition to this strikingly different % G+C content, *swmB* codon usage shows significant variation from the rest of the genome. A comparison of relative synonymous codon usage (RSCU) highlights differences between codon usage for this single gene as compared to the whole genome (Table 2). A Chi-square test of percent usage of each codon shows statistically significantly different ( $p < .001$ ) codon usage for all amino acids except for glutamate, cysteine and lysine. The low % G+C region that contains *swmB* also encompasses 10 other ORFs, two of which have been identified as motility genes by transposon mutagenesis (18). Comparison of this chromosomal region with the homologous regions in two non-motile *Synechococcus* strains, for which complete genomes are available, shows extensive conservation of both gene content and synteny in the sequence immediately flanking the low % G+C region (Fig. 3).

Due to the extreme length of this protein, similarity searches were conducted using the entire sequence of *swmB* as well as each region and each repeat separately. BLAST-P analysis (1) of SwmB was conducted and the predominance of hits come from genome sequencing projects with most of these annotated as hypothetical or

conserved hypothetical proteins. A few trends are observed however. Hits predominantly align to either domain B or to domain C with some aligning to the non-repetitive portion that precedes domain C as well. Several of these BLAST hits have reported similarity to the RTX (Repeats in ToXin) group of exotoxins, which are secreted, calcium-binding proteins that all share a common nonapeptide repeat (31). The sequence of SwmB however does not contain this RTX repeat.

While BLAST results yield no clear homologs to SwmB, examples of other bacterial proteins with some similarities to SwmB have been identified. A group of cell surface proteins involved in *Staphylococcus aureus* host-cell adhesion called MSCRAMMs (for Microbial Surface Components Recognizing Adhesive Matrix Molecules) are similarly large and repetitive (10). One member of this group is Ebh, a megadalton-sized protein from various *Staphylococcus aureus* strains which contains  $44 \times 126$ -residue tandem repeats and is responsible for cellular adhesion (7). LapA is a 900 kDa protein from *Pseudomonas fluorescens* that contains two regions of tandem repeats ( $9 \times 100$ -residues and  $29 \times 220$ -residues) and is required for surface attachment and biofilm formation (13). Lastly, rOmpA is a 190 kDa protein from *Rickettsia rickettsii* containing  $13 \times 72$ -residue tandem repeats (2) which are required for cell adhesion (17). These proteins are similar to one another in their tandemly repetitive primary structure, large size, extracellular localization, and function. Additionally, these proteins have atypical amino acid usage within their repeated regions that is similar to that of SwmB (Table 1). Whether the function of SwmB is similar to that of these proteins remains to be determined but these similarities have helped to direct efforts at cellular localization of SwmB.

Transmembrane prediction algorithms did not recognize any potential transmembrane helices and motif searches failed to identify any known prokaryotic motifs within the predicted amino acid sequence of *swmB*. SwmB also does not contain any apparent secretion signal sequences. Secondary structural predictions using the Robetta server (16), performed independently on each domain and repeat indicate that SwmB should fold into a predominantly  $\beta$ -sheet conformation.

**Protein identification and purification.** SDS-PAGE analysis of whole cells, soluble OM fractions, and proteins concentrated from spent media of motile strain WH8102 all show the presence of a high molecular weight band (Fig. 4A). While high molecular mass proteins do not penetrate far into the gel, using rabbit muscle proteins as a relative molecular mass standard, SwmB is observed to be over 1 MDa, as predicted by genomic sequence data. Mass spectrometry analysis of this band excised from a gel identified four unique peptides present within the SwmB sequence (residues 3698-3707, 6720-6728, 8379-8389, and 8602-8612) confirming that this band is SwmB. Periodic acid-Schiff staining did not detect glycosylation of SwmB (results not shown). Insertional inactivation yields cells that do not produce any detectable SwmB, as observed both on gels and by western analysis (Fig. 4B). Swm-2 cells do still produce SwmA at wild-type levels and with wild-type localization (Fig. 4B).

SwmB was partially purified both from spent medium and from whole cells. For the former, spent medium was concentrated approximately 75 fold with an ultrafiltration cell using a 30,000 MWCO filter followed by a further 12 fold concentration with a 30,000 MWCO centrifugal filter. This material was then purified

by sucrose density centrifugation to yield nearly pure SwmB (96.3% of total protein by densitometry) (Fig. 5A). Three contaminating bands with apparent molecular weights of 100 kDa, 80 kDa, and 58 kDa respectively (determined on Nu PAGE 3-8% tris acetate gels) are present in low amounts as detected by SYPRO staining. Similarly, SwmB was purified from whole cells by stripping the outer membrane (OM) with a modified EDTA treatment (24). The material released from cells by EDTA treatment is then subjected to high-speed centrifugation to pellet outer membranes (HSP) and yield a supernatant (HSS) fraction containing SwmB. This HSS was first purified by sucrose density gradient centrifugation followed by a variety of secondary purification steps based on size (gel filtration chromatography), density (ultracentrifugation), charge (anion exchange chromatography and differential ammonium sulfate precipitation). The same three bands were present as minor contaminants (Fig. 5B) in all of these preparations. Mass spectrometry analysis has identified the two largest of these bands as SYNW1565 (a conserved hypothetical protein) and SYNW0406 respectively, of which the latter is a major component the outer membrane insoluble fraction (4). Mass spectrometry analysis of the third contaminating band inconclusively identified this protein as the  $\beta$ -chain subunit of phycoerythrin: a structural component of phycobilisomes. Moreover another mass spectrometry analysis of the total contents in solution identified several phycobilisome components contaminating the sample.

Given its large size and repetitive primary sequence it was hoped that direct visualization of purified SwmB by electron microscopy might be possible. While a pure preparation of SwmB was not obtained, highly enriched samples were visualized

by TEM following heavy metal negative staining. Several distinct structures are visible in these preparations: hexameric rings with a diameter of 14 nm, thin filaments approximately 4 nm width (Fig. 6A, inset), and large bundles of filaments (Fig. 6). Individual filaments are highly variable in length with an average length of 623 nm for the 77 individual filaments measured. Several individual filaments of over 2000 nm were observed. These individual filaments appear quite flexible as many filaments are sharply bent and twisted yet still intact. The size of filament bundles was also highly variable with some greater than 200  $\mu\text{m}$  in length observed. Neither the ring-like or filamentous structures can be attributed to SwmB however as an identical preparation of material from Swm-2 cells also displays both of these structures. Anti-SwmB immunogold labeling of these preparations shows gold labeling is associated preferentially with these filament bundles however (Fig. 6B). Some labeling was observed unassociated with filaments as well, and this appears to be specific as compared to the pre-immune control (Fig. 6C).

While attempts to localize SwmB on whole cells using immunogold labeling and TEM visualization were unsuccessful, immunofluorescent labeling analyzed by deconvolution microscopy did reveal the subcellular localization of SwmB. SwmB is found on the cell surface with an irregular, punctate distribution (Figs. 7A and B). While SwmB has a punctate distribution, it is not localized exclusively to any one part of the cell and appears to be relatively evenly distributed across the entire cell surface. The sub-cellular distribution of SwmB revealed by immunofluorescence contrasts with that of SwmA, which appears as a bright homogenous layer surrounding the cell (Fig.

7E). Additionally, immunofluorescent localization reveals that Swm-2 cells possess SwmA with wild-type distribution (Fig. 7F).

## Discussion

Genome sequencing of *Synechococcus sp.* strain WH8102 was anticipated to provide insight to the novel motility exhibited by this bacterium (21). Complete genome sequence information failed to identify more than a few genes suspected to be involved in motility. The most promising candidates were several ORFs with homology to *pilT*, a motor protein involved in pilus retraction (20, 28). While WH8102 does not have the full complement of genes required for pilus formation and microscopic analyses have never observed pili in these cells, the presence of a motility motor protein was reason enough to generate inactivations of these genes but in no case was motility abolished (McCarren and Brahamsha, unpublished results). Consequently a method for random mutagenesis, utilizing a modified Tn5 transposon, was developed to identify genes involved in swimming motility (18). Among other motility genes identified, one extremely large and repetitive protein was discovered to be involved in swimming motility.

At the time of sequencing, this ORF was the largest prokaryotic gene identified to date that we could identify in the literature. Since that time, even larger prokaryotic ORFs have been sequenced but translated proteins corresponding to these ORFs have not been identified. For example, the incomplete genome sequences of *Magnetococcus* strain MC-1 ([www.jgi.doe.gov](http://www.jgi.doe.gov)) contains two extremely large ORFs of 15245 amino acids and 11699 amino acids respectively, but putative functions for

these ORFs have not been assigned nor have translated products for ORFs of this size been demonstrated. Additionally multiple strains of *Synechococcus* are currently being sequenced, one of which possesses an ORF nearly three times larger than SwmB (D. Scanlan, personal communication). Experimental work has shown several other bacterial proteins in the megadalton range that are produced and transported to the cell surface. All of the multiple strains of *Staphylococcus aureus* that have been sequenced to date (7) possess a gene, or genes, named *ebh* with predicted products ranging up to 1.13 MDa. *Ebh* is associated with the cell envelope and expression of a partial fragment of its gene shows host extracellular matrix binding activity (7). Similarly, multiple strains of *Pseudomonas fluorescens* have a gene encoding a large (approximately 900 kDa) cell-surface protein termed LapA that has been shown to be important for substrate attachment and biofilm formation (13).

SwmB is readily apparent in whole cell protein extracts as a large molecular weight band. Although accurate determination of molecular weight is difficult for large proteins that do not enter far into the gel, SwmB is clearly in the megadalton range. This finding suggests that transcription and translation of the entire reading frame likely occurs. Periodic acid-Schiff staining indicates this protein is not glycosylated as is the case for other *Synechococcus* cell surface proteins such as SwmA and a 70 kDa protein (4). Much like SwmA, SwmB copurifies with the outer membrane and does not appear to be an integral outer membrane protein as it purifies with the soluble fraction of outer membrane preparations. Furthermore, both SwmA and SwmB are found in abundance in spent medium. Their location on the cell surface and lack of membrane anchoring may make these proteins more susceptible to

being shed by living cells. These characteristics may provide a clue as to the function of these proteins in swimming motility. Perhaps for the surface wave generation model proposed by Ehlers et al. (9), proper functioning of the motility apparatus requires dynamic and more loosely attached cell envelope layers. It therefore would follow that SwmA and SwmB, as cell surface components of the motility apparatus, are easily shed and build up in the medium.

Inactivation of *swmB* results in a loss of motility but it does not affect the attachment of SwmA to the cell surface as determined by fractionation and immunolocalization experiments. Both WH8102 and Swm-2 strains contain SwmA in the outer membrane preparations (Fig. 4). Furthermore, spent medium from both wild-type and *swmB* mutant strains contain comparable amounts of SwmA (Fig. 4). Lastly, immunofluorescent localization of Swm-2 cells reveals identical distribution of SwmA as wild-type cells (Figs. 7E and F). Clearly SwmB is not just a structural protein involved in the attachment of SwmA to the cell surface. We have searched for fortuitously attached Swm-2 cells to see if these mutants retain the ability to produce torque as is seen in *swmA*<sup>-</sup> mutants. Such spinning cells have yet to be observed suggesting that torque production has been eliminated in this mutant. While this behavior is not uncommon in wild-type cells, such spinning cells are infrequently observed in *swmA*<sup>-</sup> cells. Thus we cannot rule out the possibility that *swmB*<sup>-</sup> cells still produce torque as we may have just not yet observed it.

Purification of SwmB by a variety of methods resulted in highly enriched preparations but several minor contaminants were never completely eliminated. One of these contaminants is a 70 kDa polypeptide that is particularly abundant in EDTA



stripped OM preparations (4), suggesting that following multiple purification steps, pieces of membrane still remain. Whether this is due to specific or non-specific interactions remains to be determined. Mass spectrometry also detected components of the light harvesting phycobilisome structures. Moreover, electron microscopic analysis of these samples revealed the presence of ring-like structures resembling phycobilisome discs in both size and shape (11). While the filamentous structures observed in these preparations are evidently not SwmB, there does appear to be an interaction between SwmB and these filaments as observed by immuno-gold labeling. Due to their extremely large size (several hundred times the size of an individual cell) it seems unlikely that the large bundles of filaments observed by TEM could correspond to actual structures found *in situ*. More likely these are accumulations of individual filaments that occur due to concentration effects. SwmB does associate with these filament bundles however, and it is tempting to speculate that the accumulation of individual filaments into large bundles may be mediated by SwmB.

While the function of SwmB remains uncertain, the origin of this gene poses interesting questions as well. The strikingly different % G+C content of this gene and flanking sequence, as compared to the genome, implies that this piece of DNA has been acquired by horizontal gene transfer. Even more convincing is a comparison of the homologous chromosomal region in two other sequenced marine *Synechococcus* strains: non-motile, oligotrophic strain CC9605 and non-motile, coastal strain CC9902 ([genome.jgi-psf.org/mic\\_home.html](http://genome.jgi-psf.org/mic_home.html)). Immediately outside of the low % G+C region encompassing *swmB* and several flanking genes, the gene content and synteny is highly conserved across all three genomes (Fig. 3). Other clusters of motility genes

present on separate regions of the chromosome do not exhibit the altered % G+C content of *swmB* (18). Apparently all the genes required for motility in marine *Synechococcus* were not gained by a single acquisition.

Sequence similarity provides few clues as to the function of SwmB. Of the few significant similarities found, many are to RTX proteins. RTX proteins, most specifically the HlyA hemolysin of *E. coli*, are the prototype substrate for type I secretion pathway across the gram-negative cell envelope (3). Type I secretion relies on a multi-component system comprised of an ABC transporter, a periplasm-spanning membrane fusion protein (MFP), and an outer membrane protein (OMP). While the substrates for type I secretion are quite varied (small peptides to proteins of varying molecular weights from 19 – 800 kDa,  $\beta$ -glucans, polysaccharides, and sialic acid (14)) several generalizations can be made. Type I secreted proteins are typically very acidic with a pI around 4, these substrates have very few or no cysteine residues, and many transported proteins that do not contain the actual RTX nonapeptide repeat still contain other types of repeats (8). All of these characteristics apply to SwmB.

Notably, an ABC transporter (SYNW0959) of the protein-1 exporter (Prot1E) family (<http://www.tcdb.org/tcdb>) and an MFP (SYNW0958) are present on the low % G+C region containing *swmB*. Transposon mutagenesis suggests the requirement of these genes for motility (18). Moreover Hinsa *et al.* have shown that the gene cluster adjacent to *lapA* (which encodes another extremely large cell surface protein) contains *lapEBC*, which encodes an OMP, a Prot1E family ABC transporter, and an MFP respectively. Their results show that this multi-component transporter is required for the correct localization of LapA on the cell surface. The sequence characteristics of

SwmB indicates it is exported by a type I secretion apparatus. The presence of both a Pro1E family ABC transporter and an MFP encoded on the same piece of DNA implies that SwmB and the ability to transport such a large protein were acquired together in a single step.

SwmA also contains multiple RTX repeats and may well be transported by a type I secretion mechanism as well. There is another set of genes encoding a second copy of a Pro1E family ABC transporter (SYNW0193) and an MFP (SYNW0194) that are also required for motility. Mutations in either SYNW0193 or SYNW0194 abolishes motility and these cells do not produce any SwmA (detailed in the following chapter). Lastly, there is one *tolC*-like OMP gene (SYNW2187) present in the *Synechococcus* strain WH8102 genome, completing the genetic complement required for type I secretion.

While the cell surface location of this exceptionally large protein has been determined and a likely mechanism for its transport is suggested, the function of SwmB, once it is in place, remains a mystery. Due to its highly repetitive primary structure, SwmB presumably has a repetitive tertiary structure. Whatever structure one domain assumes, each subsequent repeat should have a similar fold, resulting in a repetitive structure for the complete folded protein. Nevertheless, such a structure was not observed by TEM negative staining. Perhaps a repetitive structure, like that presumed for SwmB, is important for interaction with the highly repetitive S-layer formed by SwmA. If such an interaction occurs, conceivably conformational changes in SwmB could result in structural changes in the S-layer (*i.e.* the mechanical deformations (22) or regions of localized contractions (9) previously proposed). If

these structural changes were manifested and organized in a wave traveling the length of the cell it could result in the motility observed. While this model is largely hypothetical at this point it does provide queries for further research such as do SwmA and SwmB specifically interact, does SwmB undergo conformational changes, and under what conditions?

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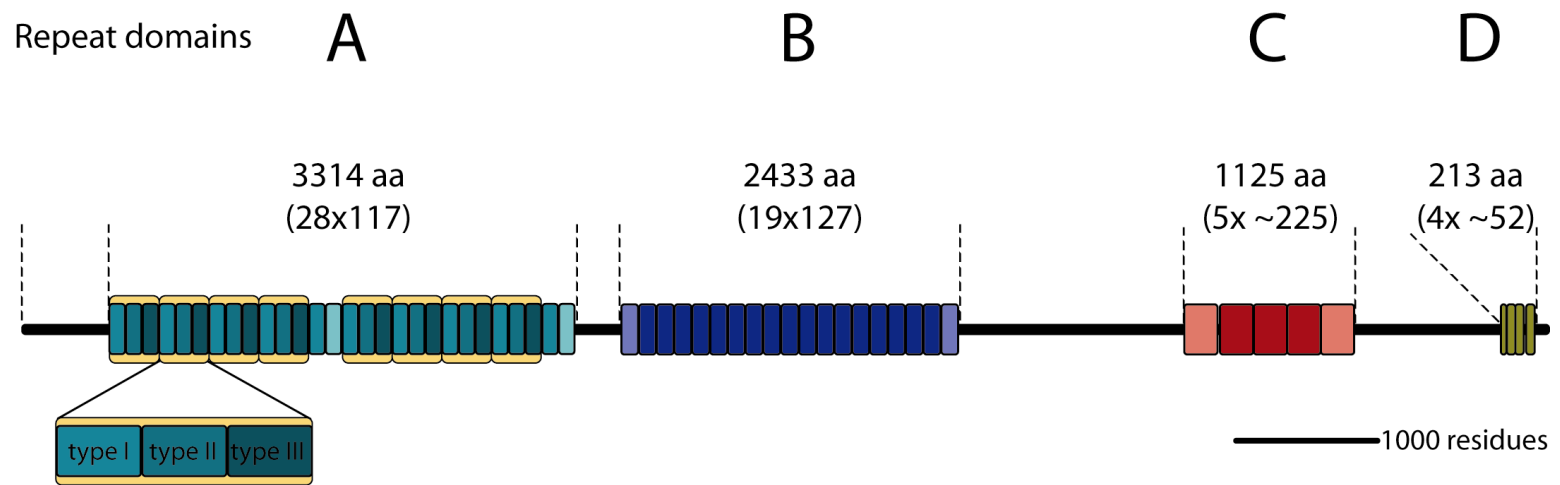


FIG. 1. Diagram of SwmB primary sequence divided according to repetitive domains A-D. Domain A contains three repeat types sharing over 70% identity that are arranged into a larger unit ( $A_I$ -  $A_{II}$ -  $A_{III}$ ) which is itself repeated. The central and C-terminal repeats in domain A as well as both terminal repeats in domains B and C are less well conserved than the central core repeats.



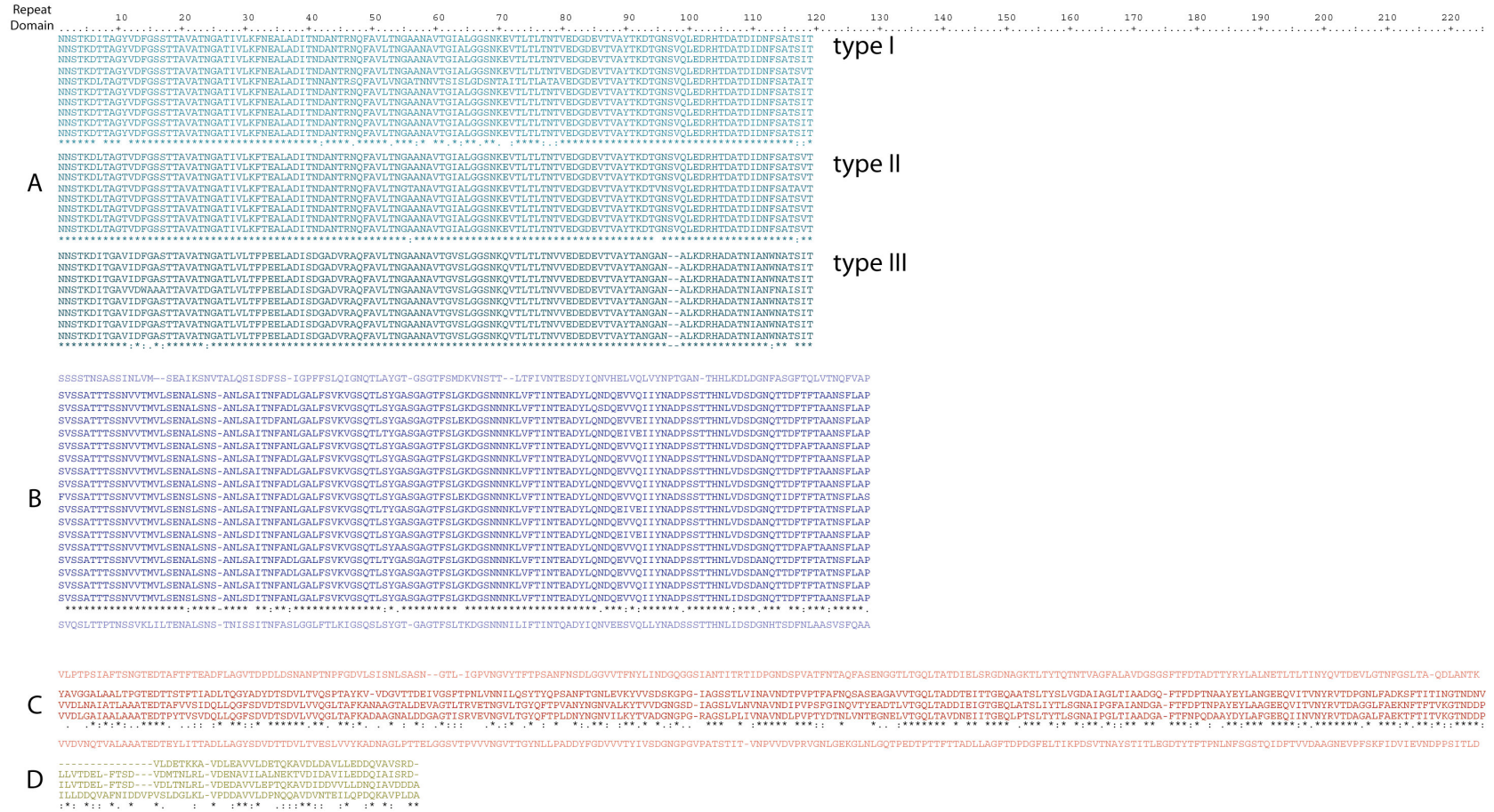


FIG. 2. Amino acid alignments of SwmB domains A-D color coded as in Fig 1. For each domain identical (\*), strongly similar (:), and weakly similar (.) residues are marked.

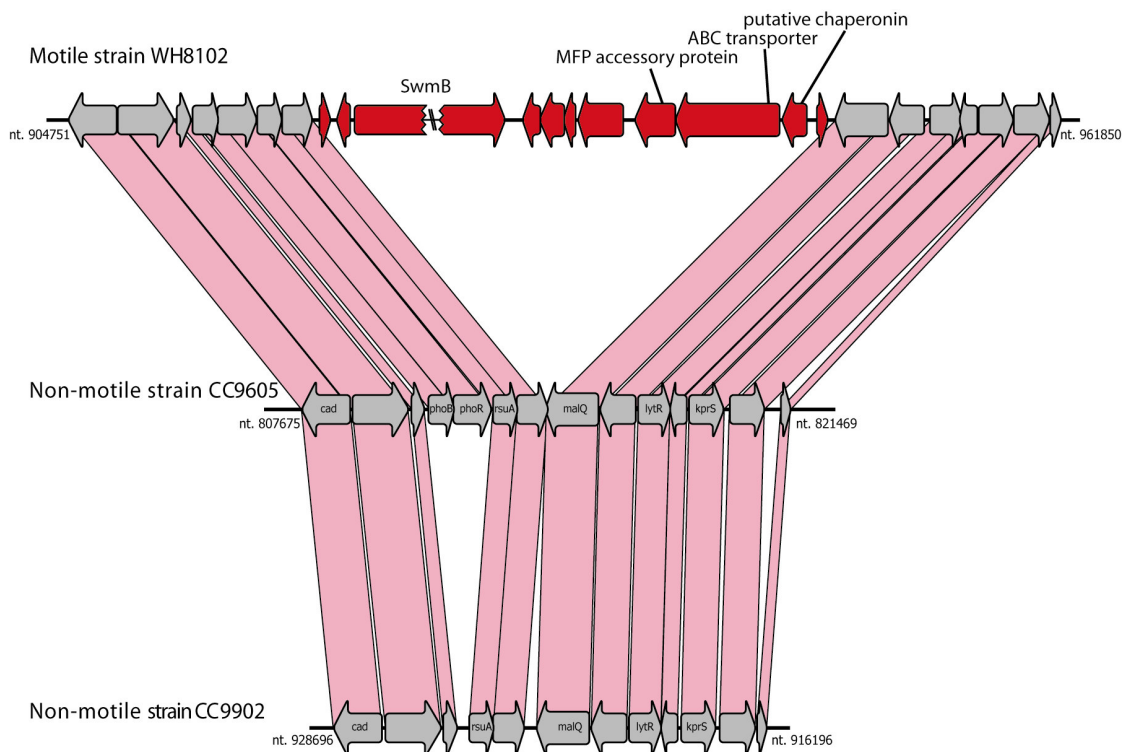


FIG. 3. Chromosomal region containing *SwmB* and flanking genes. Homologous regions from two non-motile strains CC9605 and CC9902 ([www.genome.jgi-psf.org/mic\\_home.html](http://www.genome.jgi-psf.org/mic_home.html)), show the absence of *SwmB* and other ORFs (in red) including a multicomponent transport apparatus implicated in motility. The twelve ORFs present only in *Synechococcus* sp. strain WH8102 are contained within a 41.8 kb region of DNA characterized by a % G+C content much lower than the genome average (18).

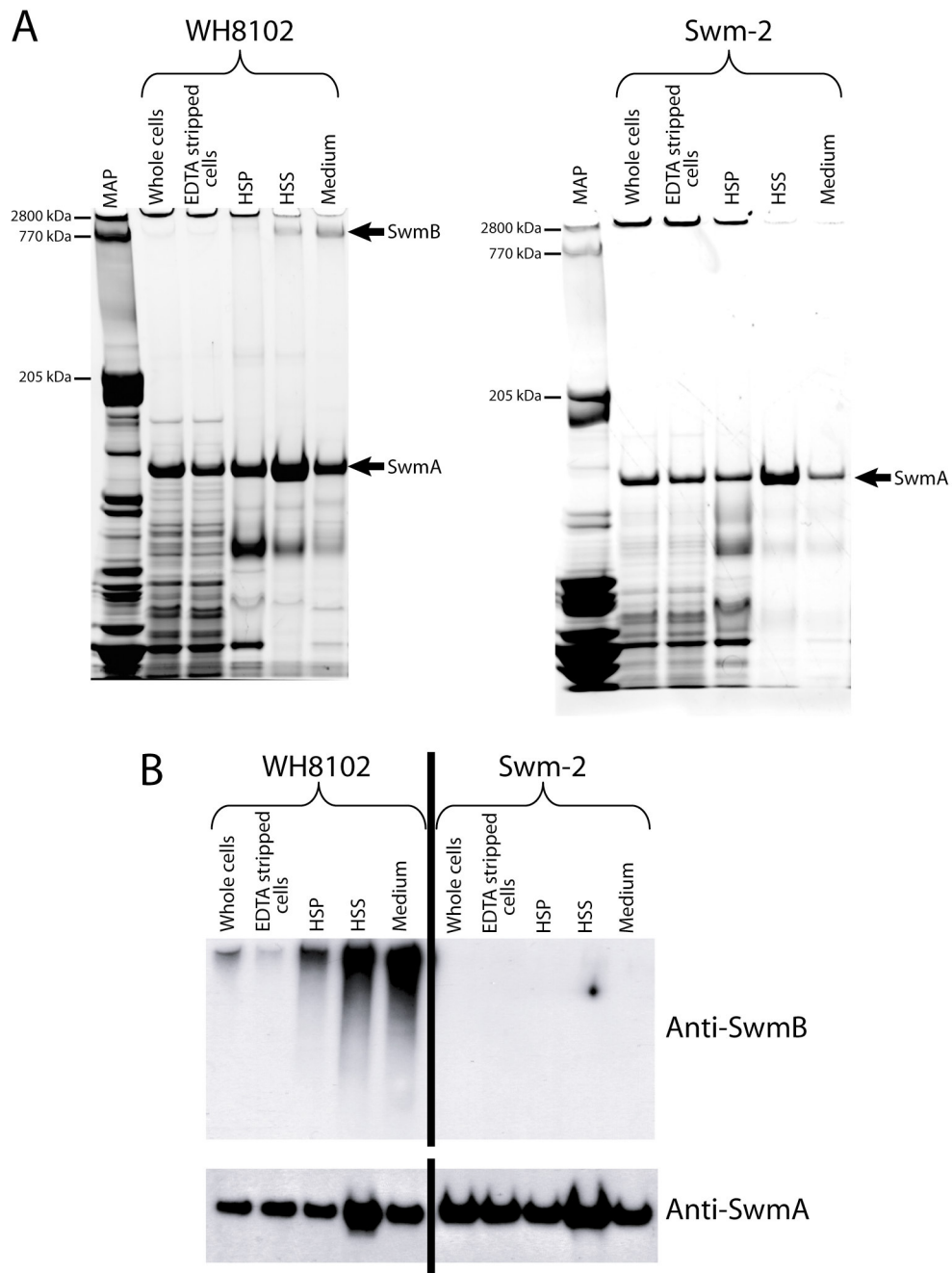


FIG. 4. (A) 3% - 8% SDS-PAGE analysis of motility proteins SwmB and SwmA. MAP, muscle acetone powder containing proteins titin (2800 kDa), nebulin (770 kDa) and myosin (205 kDa) used as molecular weight markers. (B) Western analysis of both motility proteins in cellular fractions from wild-type strain WH8102 and *swmB* mutant strain Swm-2.

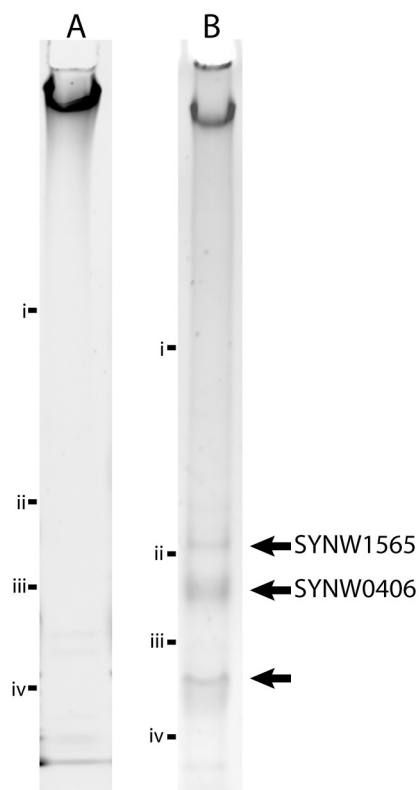


FIG. 5. NuPAGE 3-8% tris-acetate gels of SwmB from spent medium purified by sucrose gradient centrifugation (A). SwmB from HSS fractions purified by sucrose gradient centrifugation followed by ultracentrifugation (B). Identical MW markers used for both samples: 205 kDa (i), 97.4 kDa (ii), 66.2 kDa (iii), and 45 kDa (iv). Three contaminating bands marked with arrows.

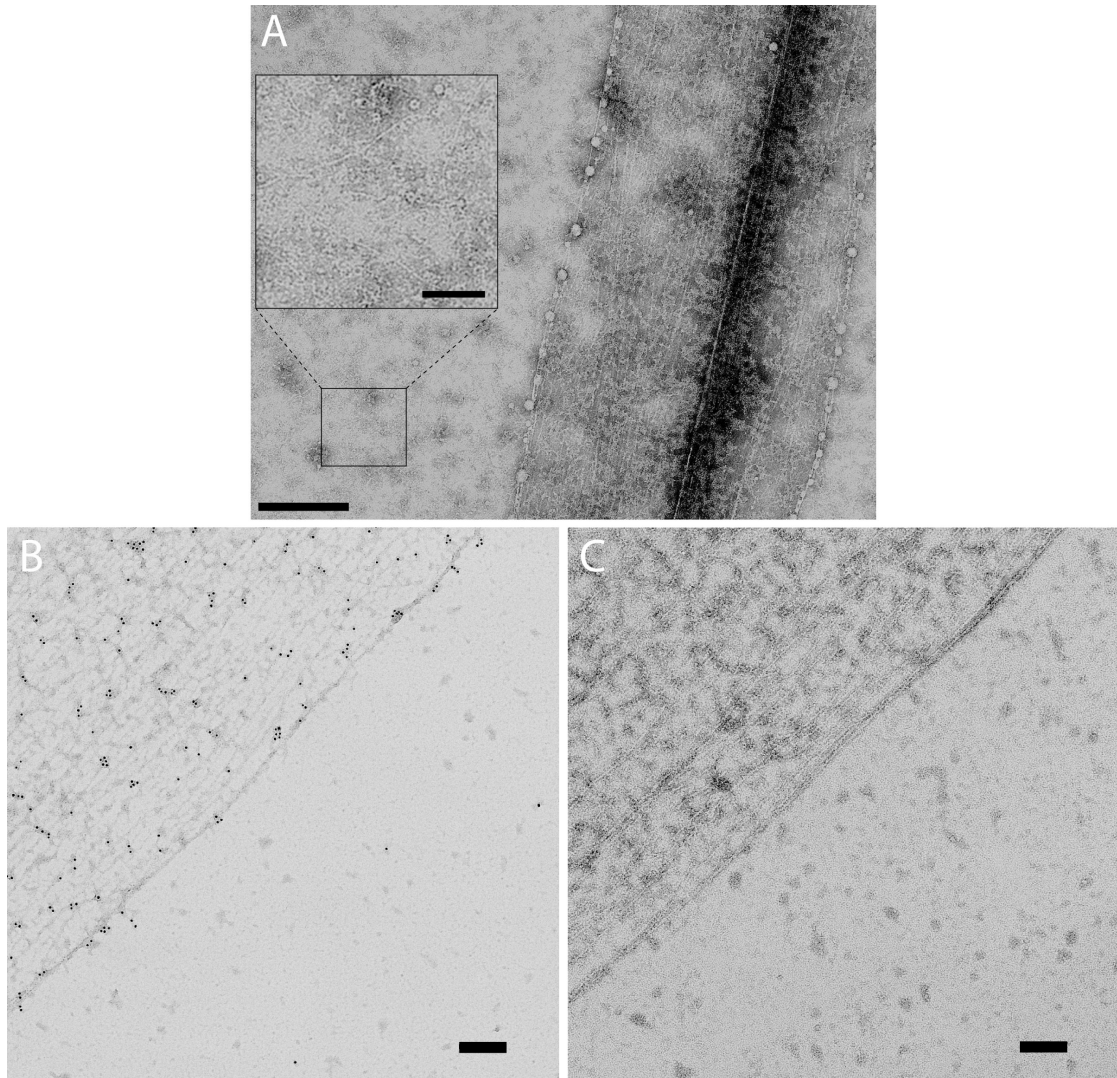


FIG. 6. Partially purified SwmB imaged by negative staining TEM (A, bar, 150 nm). Individual filaments and ring-like structures observed (inset, bar, 100 nm) as well as larger bundles of filaments. Anti-SwmB immunogold labeling of the same material shows gold labeling preferentially associated with filament bundles (B, bar, 200 nm) while the pre-immune control exhibits no labeling (C, bar, 200 nm).

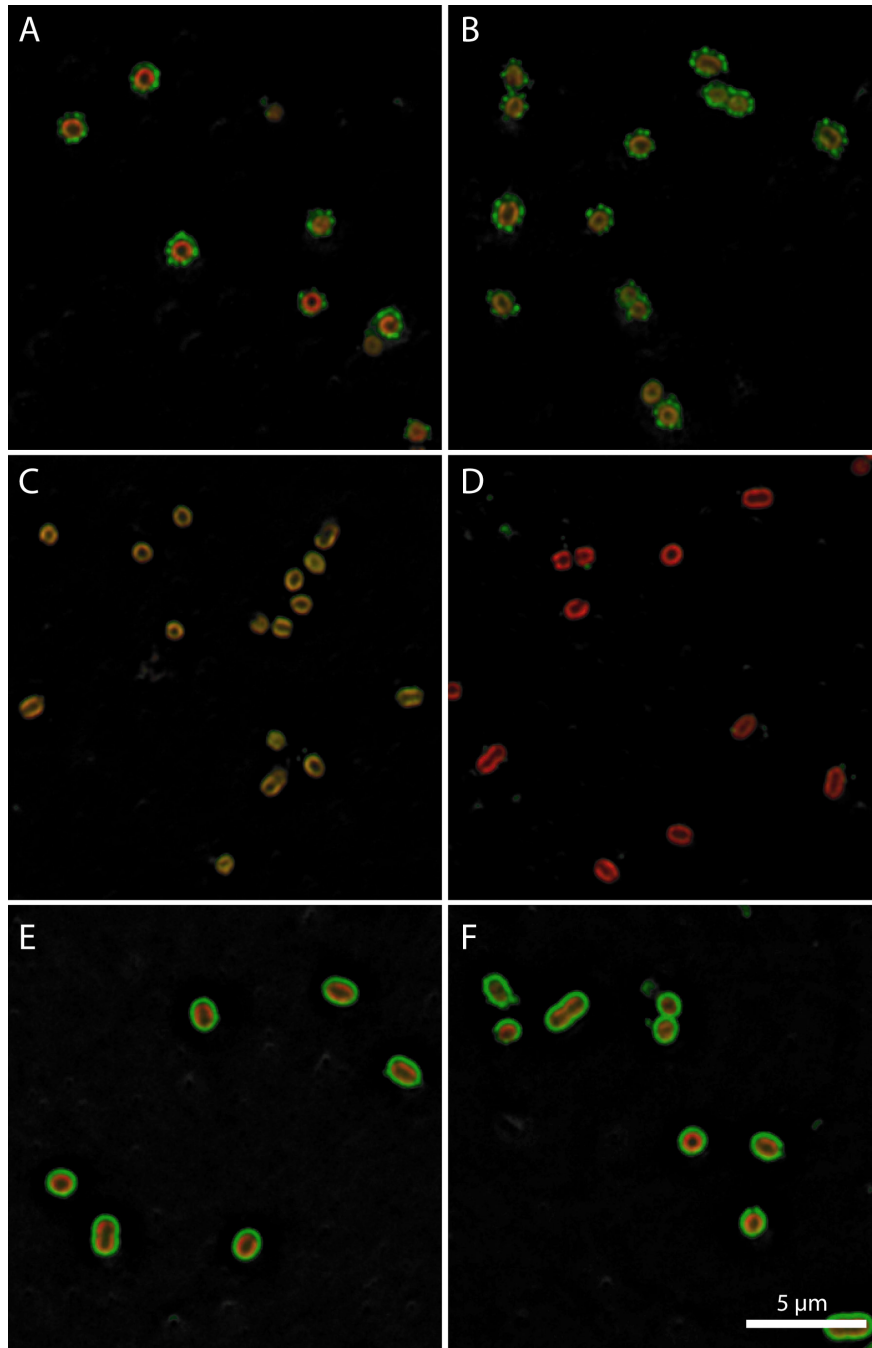


FIG 7. Immunofluorescent labeling of cell surface proteins SwmA and SwmB visualized by deconvolution microscopy. Red autofluorescence from chlorophyll shows the cell shape while immunolabeled proteins are displayed with green fluorescence. Wild-type cells labeled with anti-SwmB (A and B) reveal the punctate, cell-surface distribution of SwmB. Negative controls of wild-type cells labeled with a pre-immune antibody (C) and Swm-2 cells labeled with anti-SwmB do not possess this same distribution. SwmA is detected as a bright, homogenous layer on both wild-type (E) and Swm-2 (F) cells. Bar, 5  $\mu\text{m}$  (all panels).

Table 1. Amino acid usage analysis (6) for several large, repetitive, cell surface prokaryotic proteins

<b>Protein</b>	<b>Highest 99% quantile</b>	<b>Highest 95% quantile</b>	<b>Lowest 5% quantile</b>	<b>Lowest 1% quantile</b>
SwmB domain A	N, T	A, D	R	M, P
SwmB domain B	N, S, T		M, P	R
SwmB domain C	T	V, D	K	M, R, H
SwmB domain D	D, V	L	M, R, F	H, G, S
<i>S. aureus</i> Ebh <sup>a</sup> repeats	N	A, Q, T	P, R	F
<i>P. fluorescens</i> LapA domain 2 <sup>b</sup>	T	N, V	K, L	H, M, R
<i>P. fluorescens</i> LapA domain 3 <sup>b</sup>	T, V			M, R
<i>R. rickettsii</i> rOmpA <sup>c</sup> repeats	N, T, V	A, G	Q, F	E, H, M, P, R, Y
<b>Summary</b>		<b>N, T, V</b>		<b>M, R, P</b>

<sup>a</sup>*Staphylococcus aureus* strain COL Ebh (7)

<sup>b</sup>*Pseudomonas. fluorescens* strain WCS365 LapA (13)

<sup>c</sup>*Rickettsia. rickettsii* rOmpA (2)

Table 2. Comparison of Relative Synonymous Codon Usage (RSCU) for the entire *Synechococcus sp.* strain WH8102 genome versus SwmB.

Amino Acid	Codon	<u>Entire Genome</u>		<u>SwmB</u>	
		%		%	RSCU
Phe	UUU	33.62	0.67	73.67	1.47
	UUC	66.38	1.33	26.33	0.53
Leu	UUA	2.46	0.15	20.96	1.26
	UUG	15.07	0.90	18.50	1.11
	CUU	10.54	0.63	26.01	1.56
	CUC	19.59	1.18	9.30	0.56
	CUA	2.63	0.16	10.20	0.61
	CUG	49.71	2.98	15.02	0.90
Ile	AUU	25.59	0.77	59.32	1.78
	AUC	70.46	2.11	25.05	0.75
	AUA	3.95	0.12	15.63	0.47
Met	AUG	100.00	1.00	100.00	1.00
Val	GUU	18.12	0.72	50.49	2.02
	GUC	21.63	0.87	17.92	0.72
	GUA	4.12	0.16	21.82	0.87
	GUG	56.13	2.25	9.77	0.39
Ser	UCU	16.02	0.64	47.08	1.88
	UCC	39.89	1.60	14.48	0.58
	UCA	19.52	0.78	24.79	0.99
	UCG	24.57	0.98	13.65	0.55
Pro	CCU	16.62	0.66	46.43	1.86
	CCC	34.25	1.37	16.27	0.65
	CCA	16.87	0.67	28.57	1.14
	CCG	32.26	1.29	8.73	0.35
Thr	ACU	10.84	0.43	32.85	1.31
	ACC	51.87	2.07	18.91	0.76
	ACA	13.75	0.55	28.40	1.14
	ACG	23.54	0.94	19.84	0.79
Ala	GCU	19.02	0.76	37.67	1.51
	GCC	46.27	1.85	14.87	0.59
	GCA	13.51	0.54	32.41	1.30
	GCG	21.20	0.85	15.05	0.60
Tyr	UAU	36.21	0.72	72.91	1.46
	UAC	63.79	1.28	27.09	0.54
His	CAU	43.96	0.88	79.71	1.59
	CAC	56.04	1.12	20.29	0.41
Gln	CAA	25.68	0.51	46.01	0.92
	CAG	74.32	1.49	53.99	1.08
Asn	AAU	35.70	0.71	71.31	1.43
	AAC	64.30	1.29	28.69	0.57
Lys	AAA	35.27	0.71	27.14	0.54
	AAG	64.73	1.29	72.86	1.46
Asp	GAU	52.95	1.06	69.43	1.39
	GAC	47.05	0.94	30.57	0.61
Glu	GAA	42.26	0.85	48.66	0.97
	GAG	57.74	1.15	51.34	1.03
Cys	UGU	29.71	0.59	33.33	0.67
	UGC	70.29	1.41	66.67	1.33
Trp	UGG	100.00	1.00	100.00	1.00
Arg	CGU	20.39	0.82	46.30	1.85
	CGC	41.98	1.68	32.41	1.30
	CGA	11.33	0.45	18.52	0.74
	CGG	26.30	1.05	2.78	0.11
Ser	AGU	26.72	0.53	60.51	1.21
	AGC	73.28	1.47	39.49	0.79
Arg	AGA	41.13	0.82	72.34	1.45
	AGG	58.87	1.18	27.66	0.55
Gly	GGU	24.99	1.00	46.06	1.84
	GGC	42.07	1.68	21.12	0.84
	GGA	15.90	0.64	26.72	1.07
	GGG	17.04	0.68	6.11	0.24



The text of Chapter V, in full, is being prepared for publication. The dissertation author was the primary author, and co-author B. Brahamsha directed and supervised the research, which forms the basis for this chapter.

## CHAPTER VI

### Characterization of *Synechococcus* swimming motility mutants

## **Abstract**

Two components of the motility machinery involved in non-flagellar swimming in marine *Synechococcus*, SwmA and SwmB, have been localized to the cell surface. The nine motility mutants currently in culture, were characterized to determine the cellular localization of these two proteins as well as to identify other components of the outer membrane that may be involved in swimming motility. While SwmA and SwmB are not dependent on one another for proper localization on the cell surface, all motility mutants in culture have a defect in the localization of either SwmA or SwmB and in some instances both of these proteins. Additionally, two outer membrane polypeptides of 70 kDa and 80 kDa are absent in some of these mutants, suggesting a role in motility. This characterization implicates a multicomponent ABC transporter in the export of SwmA and reveals the importance of several glycosyltransferases for the proper localization of components of the motility apparatus.

## **Introduction**

The mechanism of non-flagellar swimming motility in marine *Synechococcus* remains mysterious following several advances anticipated to shed light on this problem. Genome sequencing failed to identify components of the motility apparatus (12). Not only were no motility genes identified, completion of the genome sequence exposed the absence of chemotaxis genes, making the novel motility of these cells even more intriguing. Development of a transposon mutagenesis technique for use in marine *Synechococcus* identified three chromosomal regions involved in swimming

motility (8). While this technique has begun to delineate the genes required for swimming motility, the genes identified in these regions do not point to an obvious mechanism for motility.

Due to the absence of extracellular appendages and having ruled out several other models, Ehlers *et al.* argue that the cell surface itself must be the thrust-generating structure in these cells (5). Two cell surface proteins involved in non-flagellar motility in *Synechococcus* have been identified. One cell-surface component of the motility apparatus is SwmA, a glycosylated protein that is required for motility (1). This protein forms a para-crystalline S-layer on the cell surface (9). Inactivation of *swmA* abolishes motility although cells retain the ability to generate torque.

Whether the S-layer plays a direct role in motility or a more indirect role, such as being required for the proper placement and functioning of other components of the motility apparatus, remains unclear. Another cell surface protein required for motility is SwmB. An extremely large protein, SwmB is found in the soluble fraction following removal of the outer membrane by EDTA treatment (1). How this megadalton sized protein functions in motility is still being investigated.

In addition to these two cell-surface proteins, transposon mutagenesis identified three chromosomal regions encoding genes required for motility (8). These genes include a number of multi-component ABC transporters, several putative glycosyltransferases, as well as conserved and hypothetical genes of unknown function (8). The requirement of particular transporters for swimming motility is not surprising as the few known components of the motility apparatus are located on or near the cell surface and these proteins must be exported from the cytoplasm to the

cell surface. Perhaps these transporters, which are non-essential for growth, are dedicated to exporting components of the motility apparatus. Similarly, the identification of glycosyltransferases required for motility was not unexpected. SwmA is a glycosylated protein (1) and glycosyltransferases are frequently involved in the biogenesis of other components of the cell envelope (3, 10). As the cell surface is of critical importance for non-flagellar swimming motility in *Synechococcus*, correct assembly of the cell envelope must be essential for the proper functioning of the motility apparatus.

For each motility gene identified by transposon mutagenesis, a directed mutation was constructed to confirm the non-motile phenotype of the original mutant (8). Several additional mutations were constructed to inactivate genes downstream of those directly affected by transposon insertion in an effort to rule out polar effects of insertion. In order to better understand swimming in *Synechococcus*, each motility mutant was then assayed for the production and cellular localization of the two known structural components of the motility apparatus: SwmA and SwmB. Outer membrane fractions of each strain were also analyzed to determine other differences in protein content between these mutants and wild-type swimming cells.

## **Materials and Methods**

**Bacterial strains and growth conditions.** The bacterial strains and plasmids used in this study are listed in Table 1. *E. coli* strains MC1061 (pRK24, pRL528) and DH5 $\alpha$  were grown in Luria-Bertani (LB) medium (14). Ampicillin (100  $\mu$ g/ml), kanamycin (50  $\mu$ g/ml), and chloramphenicol (10  $\mu$ g/ml) were used, when appropriate,

for the selection and maintenance of plasmids in *E.coli*. Cyanobacterial strains were grown in either SN medium (16) made with seawater obtained from the Scripps Pier (Scripps Institution of Oceanography, La Jolla, CA), or in SN medium prepared with synthetic ocean water (SOW) (13). Cyanobacterial cultures were incubated at 25°C with a constant illumination of  $25 \mu\text{E}\cdot\text{m}^{-2}\cdot\text{sec}^{-1}$  and were maintained as either 4-ml cultures in 17 mm  $\times$  100 mm polystyrene tubes (Becton Dickinson, Franklin Lakes, NJ) or as 50-ml cultures in 125-ml glass flasks without shaking. Kanamycin was added to a final concentration of 25  $\mu\text{g}/\text{ml}$  for pour plates and 20  $\mu\text{g}/\text{ml}$  for liquid cultures, where appropriate, to maintain selection for insertions.

**Directed Mutagenesis.** Directed inactivations were accomplished by cloning a completely internal fragment of a gene into the suicide vector pMUT100 as previously described (2). 20-mer oligonucleotide primers (Integrated DNA Technologies, Inc. Coralville, IA) were used to amplify DNA fragments (details given in Table 1) for cloning into pMUT100. These constructions were introduced into *Synechococcus sp.* WH8102 by conjugation with *E. coli* followed by subsequent selection of exconjugants on solidified medium containing kanamycin. Clonal isolates were grown in liquid medium to confirm the mutant phenotype of the original transposon mutant. Complete segregation of mutant chromosomes was confirmed by Southern blotting and by PCR. To confirm complete segregation of mutant chromosomes, DNA from each cyanobacterial mutant strain was isolated using the QIAGEN (Valencia, CA) DNeasy Tissue Kit with the following modification. Prior to proteinase K treatment, cells were incubated in 20 mM Tris-Cl (pH8.0), 2 mM  $\text{Na}_2\text{EDTA}$ , 1.2% Triton X-100, 50 mg/ml lysozyme for 30 minutes at 37°C. This

DNA was then used as a template for PCR analysis utilizing primers flanking the fragment used for inactivation. Primer pairs used in these tests were confirmed to amplify a fragment of the expected size from wild-type DNA. Failure to amplify a fragment of the wild-type size from a mutant strain's DNA confirms the absence of the intact gene among the clonal population of mutant cells. This same DNA sample was used as template in another PCR reaction utilizing primers directed to another ORF as a positive control to confirm that the DNA was of sufficient quality for PCR amplification.

**Cell fractionation.** Outer membrane (OM) proteins were isolated from all strains as described by Brahamsha (1). Briefly, exponentially growing cultures were centrifuged, washed once with 30 ml sterile SN medium, and resuspended at  $\sim 125\times$  concentration in ice-cold stripping buffer (50 mM Tris HCl + 50 mM Na<sub>2</sub>EDTA + 15% sucrose, pH 8.0) to strip off the outer membranes. After a 30 minute incubation on ice, cells were removed by centrifugation for 10 minutes at  $6277 \times g$ . The resulting outer membrane fraction was subjected to a high-speed spin of  $100,466 \times g$  for 90 minutes at 4°C to pellet the insoluble membrane fraction (high-speed pellet: HSP) and yield a supernatant containing the soluble OM proteins (high-speed supernatant: HSS). HSS proteins were concentrated using Amicon ultra 30,000 molecular weight cut-off (MWCO) centrifugal filters (Millipore, Bedford, MA) as directed by the manufacturer. Following removal of cells from spent media by centrifugation, proteins were recovered and concentrated using an ultra-filtration cell (Amicon) with 30,000 MWCO filters and further concentrated with 30,000 MWCO centrifugal filters. Cell density was determined prior to fractionating each sample using a Petroff-Hauser

counting chamber (Hauser Scientific Co., Horsham, PA). Gels were loaded normalizing cell number with a corresponding sample from wild-type strain WH8102 for comparison. Gel electrophoresis was conducted using Nu-PAGE Novex Tris-Acetate 3-8% and Novex Tricine 10-20% gradient gels as recommended by the manufacturer (Invitrogen, Carlsbad, CA). SYPRO Ruby staining (Sigma, St. Louis, MO) of gels was conducted as recommended by manufacturers.

For Western analysis, proteins were transferred to Invitrolon PVDF (Invitrogen) membranes in NuPAGE transfer buffer (Invitrogen) + 10% MeOH with 110V constant voltage for 2 hours at 4°C. Following overnight blocking at 4°C in BLOTTO (7), membranes were incubated for 1.5 hours at room temperature with primary antibodies diluted (production and purification described in Chapter V) in BLOTTO (1:500,000 and 1:50,000 for anti-SwmA and anti-SwmB respectively). Membranes were then washed 4 × 15 minutes in PBS + 0.05% Tween 20 (Fisher Scientific, Fair Lawn, New Jersey). Following washes, membranes were incubated 1.5 hours at room temperature with a peroxidase-conjugated anti-rabbit IgG (Sigma, St. Louis, MO) diluted 1:40000 in BLOTTO. Secondary antibody incubation was followed with another 4 × 15 minute washes in PBS + 0.05% Tween 20 and detected with Super Signal West Dura (Pierce, Rockford, IL) as recommended by the manufacturer. Densitometry analysis of these Westerns was performed using AlphaEase FC version 3.2.2 software (Alpha Innotech, San Leandro, CA). Bands from mutant strains were compared to the corresponding band in wild-type strain WH8102 to quantify protein abundance in relation to wild-type cells. Periodic acid-Schiff (PAS) staining was performed as described (15) to detect glycosylation.



## Results

**Mutagenesis.** Directed mutagenesis of each motility gene identified by transposon mutagenesis confirmed the non-motile phenotype of the transposon mutant (8). Due to their close downstream proximity, three additional genes (SYNW0089, SYNW0194, and SYNW0195) were inactivated by insertional mutagenesis (Fig. 1). Inactivation of SYNW0089 did not affect motility. Directed inactivation of both SYNW0194 and SYNW0195 resulted in a loss of motility.

**Cellular fractionation.** Whole cells, OM preparations separated into insoluble and soluble fractions by high-speed centrifugation (HSP and HSS, respectively), and spent medium were analyzed by gel electrophoresis and SYPRO staining as well as by western analysis using antibodies to both SwmA and SwmB for each mutant strain. Protein profiles of each fraction were analyzed by comparison to the corresponding wild-type fraction to detect differences in the protein content of each mutant strain (Figs. 2-10). Aside from changes in SwmA and SwmB, two major differences were detected. The abundant outer membrane protein of 70 kDa (1) appears to be absent in two mutant strains (SYNW0087<sup>-</sup> and SYNW0195<sup>-</sup>) (Figs. 4 and 9). Additionally, four mutant strains (S1A, SYNW0192<sup>-</sup>, SYNW0193<sup>-</sup>, and SYNW0194<sup>-</sup>) lack an 80 kDa protein typically present in the spent medium of wild-type cells (Figs. 3, and 6-8).

**SwmA Western analysis.** Wild-type cellular localization of SwmA was disrupted for every motility mutant except for the SwmB mutant strain Swm-2 (Figs 2-10). Inactivation of *swmB* had no apparent effect on SwmA localization. Excluding

Swm-2 cells, SwmA was not detected in either whole cells or the HSP for all motility mutants. SwmA was detected in the HSS of SYNW0195<sup>-</sup> cells at approximately 22% (by densitometry) of the level of wild-type (Fig. 9). While SwmA was, for the most part, not detected on cells or in cellular fractions for all of these mutants, several strains still produced SwmA (SYNW0079<sup>-</sup>, SYNW0087<sup>-</sup>, SYNW0088<sup>-</sup>, and SYNW0195<sup>-</sup>), which accumulated in the media in concentrations similar to wild-type (Figs. 2, 4, 5 and 9). Western analysis of these media fractions show a band of wild-type size but two smaller bands (approximately 108 kDa and 94 kDa) are also detected with the polyclonal anti-SwmA antibody (Figs. 2, 4, 5 and 9). These smaller bands are never seen in western analysis of the wild-type strain.

**SwmB Western analysis.** In contrast to SwmA, SwmB was detected among total cellular proteins in all strains (Figs. 2-10). Three strains (SYNW0079<sup>-</sup>, SYNW0088<sup>-</sup>, and SYNW0193<sup>-</sup>) lack SwmB in the HSP fraction and although it could be detected in the HSS fraction it was only at a fraction of the wild-type level (29%, 26%, and 23% respectively, by densitometry) (Figs. 2, 5 and 7). In all strains however SwmB is present in spent media in amounts similar to wild-type. Much like what was observed in anti-SwmA Westerns, the spent medium of one strain (SYNW0087<sup>-</sup>) contains full-length SwmB as well as three smaller bands (approximately 98 kDa, 64 kDa, and 57 kDa) that are detected using the polyclonal antibody to SwmB (Fig. 4). Again, these smaller bands that react with the SwmB antibody are never seen in the wild-type strain. A summary of all cellular fractionation results is presented in Table 2.

**PAS staining.** For each strain that still produces SwmA, samples of spent medium concentrate were assayed for SwmA glycosylation by PAS. All strains revealed a band of 130 kDa, corresponding to SwmA, that tested positive for glycosylation by this test.

## Discussion

SwmA and SwmB represent the only two characterized proteins required for non-flagellar swimming in marine *Synechococcus*. Presumably the production and proper localization of each of these components of the motility apparatus is crucial for locomotion. Inactivation of *swmB* has no apparent effect on SwmA production and localization on the cell surface. The reverse is also true. Mutation of *swmA* results in cells that still produce SwmB at wild-type levels and with wild-type localization. There does not appear to be any interdependence of one protein for the other in regards to the production and surface localization of these two proteins.

The same is not true for a number of the other mutants screened. Every mutant, other than *swmB* mutant strain Swm-2, had some defect in SwmA physiology. Three mutants (four including the *swmA* mutant strain S1A) produce no detectable SwmA at all. The corresponding genes inactivated in these mutants are all sequentially encoded in cluster two (Fig. 1) and are predicted to encode the pieces of a multi-component ATP-binding cassette (ABC) transporter. None of these mutations affect SwmB transport across the cell envelope however. While mutation to SYNW0193 results in SwmB being found primarily in spent media, mutations to SYNW0192 and SYNW0194 do not affect SwmB localization. Clearly SwmB can be

exported and attached on the cell surface in the absence of this transporter. Perhaps SYNW0192, SYNW0193, and SYNW0194 comprise a transporter dedicated to exporting SwmA. The observation that no SwmA is found in the medium supports this assertion. It appears that without this transporter, SwmA is not produced at all. How *swmA* transcription and/or translation is controlled remains to be determined. The four strains that lack SwmA all appear to lack an 80 kDa protein that is typically present in the spent media. Discussed in greater detail below, this polypeptide is unlikely to be merely a breakdown product of SwmA as Western analysis of media from wild-type cells does not detect any polypeptides other than intact SwmA. Perhaps this is a component of the motility apparatus that is dependent on the presence of SwmA for production.

Four mutations result in SwmA being found almost exclusively in the media. While SwmA transport out of the cytoplasm is not a problem for these motility mutants, correct localization is. Three of the four predicted gene products are glycosyltransferases, and the fourth is a conserved hypothetical. It is tempting to speculate that these proteins function in the modification of SwmA, which is known to be glycosylated (1). Although the predicted glycosyltransferases can be assigned to glycosyltransferase protein families based on sequence homology (3) (<http://afmb.cnrs-mrs.fr/CAZY/>), these families are general classifications including many enzymes involved in cell envelope biogenesis. Thus determining whether any would act directly on SwmA is not possible based solely on sequence information. Moreover, SwmA from these strains has the same electrophoretic mobility as wild-type and is positive for glycosylation by PAS staining. Thus it seems that the products

encoded by these motility genes do not act to directly modify SwmA, or do not act independently. They are clearly important for motility however, suggesting that certain components of the cell envelope must be modified for proper interaction with SwmA.

Intriguingly, Western analysis of spent medium proteins from these same mutants show wild-type size SwmA as well as two bands of lesser molecular weight. These smaller molecular weight polypeptides (approximately 108 kDa and 94 kDa) are unlikely to be merely non-specific breakdown products as these bands are never seen in the wild-type. Perhaps SwmA is specifically degraded when not properly attached and these bands represent breakdown products. Another possibility is that these two polypeptides are SwmA in partially modified form. The predicted molecular weight of SwmA is 83.6 kDa although the mature post-translationally modified protein has an apparent molecular weight of 130 kDa (1). Both of the additional bands detected by Western analysis are larger than 83.6 kDa leaving open the possibility that they represent the full intact peptide sequence of SwmA that has not been modified to the full extent that wild-type SwmA is.

While all of the motility mutants obtained produce SwmB (with the exception of *swmB* mutant strain Swm-2), three of the mutants have aberrant SwmB localization. In each of these three strains SwmB is detectable in whole cells as well as in the HSS but at dramatically reduced levels compared to wild-type cells. SwmB accumulates in the spent medium at concentrations similar to wild-type. The disrupted genes resulting in this phenotype are present in chromosomal regions 1 and 2 (Fig 1.) and encode an MFP component of an ABC transporter, a predicted glycosyltransferase and

a conserved hypothetical. How the products of these genes with disparate functions result in the same abnormal localization of SwmB is not clear. These mutations seem similar in some respects to those previously described in that SwmB is being produced and traverses the cell envelope but appears to have a defect in proper attachment at the cell surface.

Western analysis of spent medium from SYNW0087<sup>-</sup> cells reveals three polypeptides, in addition to full length SwmB, reacting with antibodies to SwmB. As these bands are never observed in the wild-type strain they are unlikely to be non-specific breakdown products of SwmB. SwmB appears to have normal localization in this strain however and an explanation for the additional anti-SwmB reactive bands is not obvious.

One other major component of the outer membrane fraction appears greatly reduced or absent in two strains. The glycosylated 70 kDa protein (1) found in abundance in the HSP and spent media fractions of wild-type cultures, appears absent in strains SYNW0087<sup>-</sup> and SYNW0195<sup>-</sup>. Perhaps the 70 kDa protein is either not present at all in these cellular fractions or is not glycosylated as it is in wild-type cells, resulting in a shift of electrophoretic mobility. ORFs SYNW0087 and SYNW0195 are both predicted to be glycosyltransferases. Again, it is tempting to speculate that these proteins function in the modification of the 70 kDa glycoprotein. Alternatively, the 70 kDa protein may require other components of the cell to be properly modified before it can be properly localized itself.

Analysis of the present collection of motility mutants reveals a variety of differences in the protein content of outer membrane and spent media fractions. Two

general trends regarding the two known motility components have been observed. One class of mutations affects proper localization of a given motility protein while a second class of mutation affects production of the protein. Another trend observed is the presence of multiple forms of these motility proteins in some mutants as detected by western analysis. It is not clear whether this is due to the same process affecting both SwmA and SwmB (*e.g.* specific degradation of components of the motility apparatus when it is improperly constructed), or if it is simply a similar result caused by disparate processes. Three basic SwmA phenotypes are observed: 1) wild-type abundance and distribution of SwmA, 2) SwmA present but only in the media, and 3) total lack of SwmA. Likewise the same three classes can be applied to mutations affecting SwmB. Additionally mutations affecting the 70 kDa OM protein are observed as well. Unfortunately no trends are observed in the combinations of these phenotypes. Mutations affecting one of these three proteins does not necessarily have an effect on another. The presence or location of one of the proteins cannot predict that of another. Perhaps with the isolation of additional motility mutants, commonalities in protein complements of mutant strains will become apparent. For the time being, several proteins of interest, specifically the 70 and 80 kDa proteins, appear to have a role in swimming motility and warrant further investigation.

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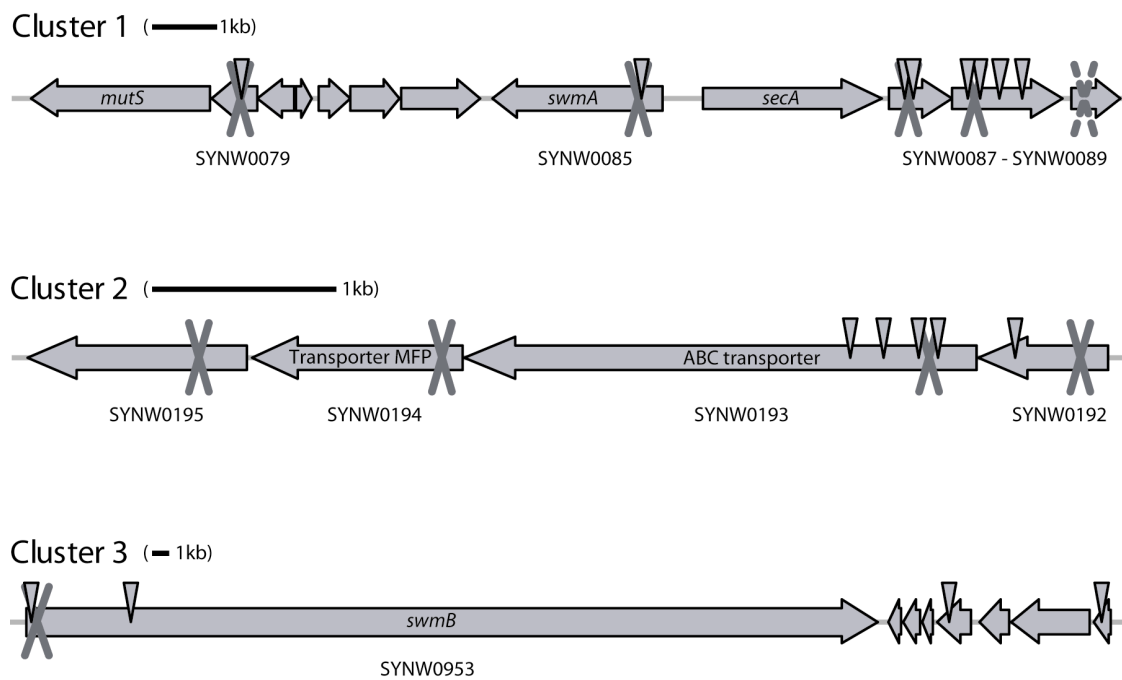


FIG. 1. Chromosomal regions containing clusters of motility genes (8). Arrowheads represent transposon insertions disrupting motility; X, directed inactivation disrupting motility; dashed X, directed inactivation with no effect on motility.

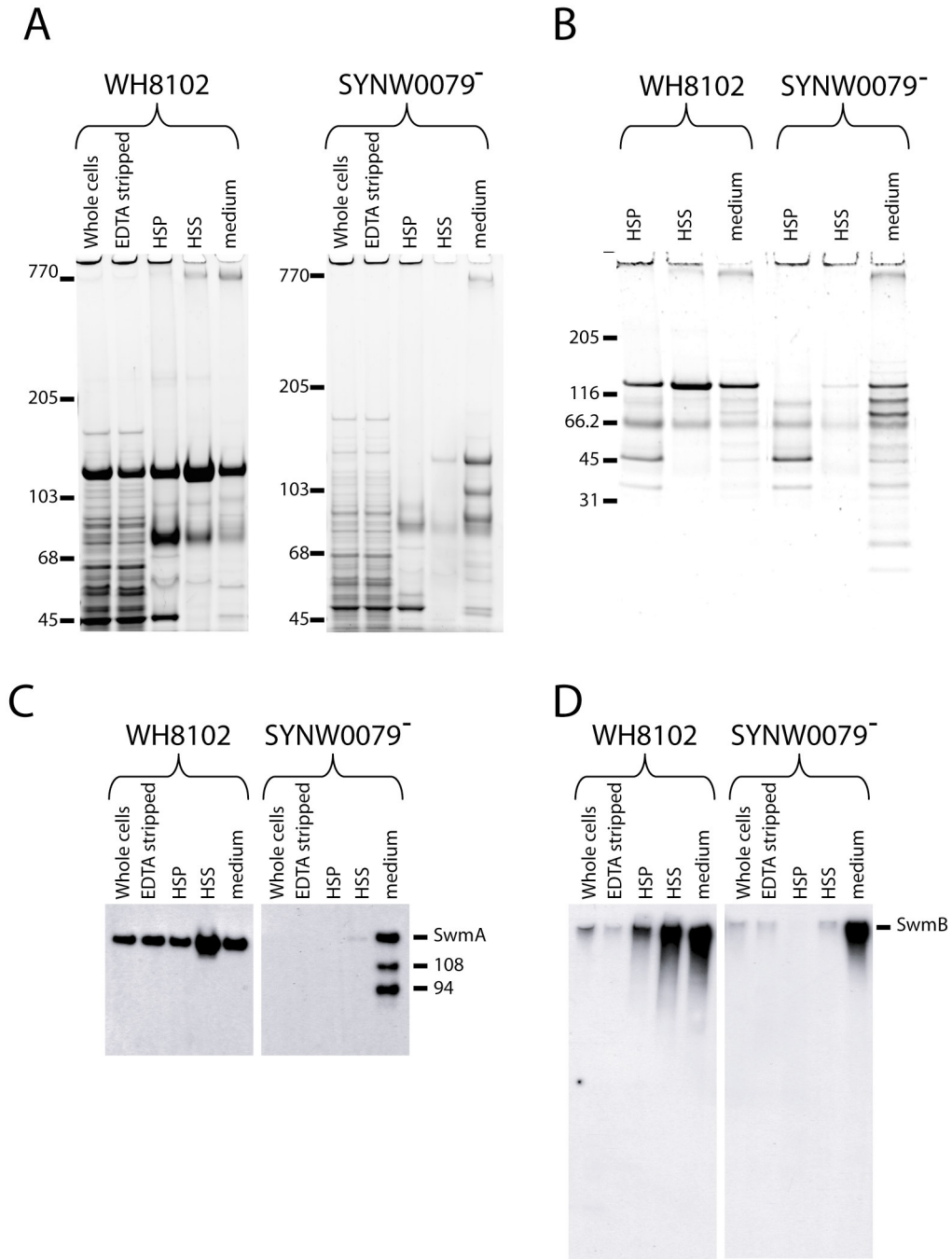


FIG. 2. SYPRO stained 3-8% tris-acetate gels (A), SYPRO stained 10-20% tricine gels (B), anti-SwmA Westerns (C), and anti-SwmB Westerns (D) comparing cellular fractions from wild-type strain WH8102 and SYNW0079<sup>-</sup> mutant strains. Cells were treated with EDTA to strip outer membranes (EDTA stripped). This material is then subjected to high-speed centrifugation to yield a pellet (HSP) containing the outer membrane and a supernatant (HSS) containing soluble proteins. Spent medium included for comparison as well.

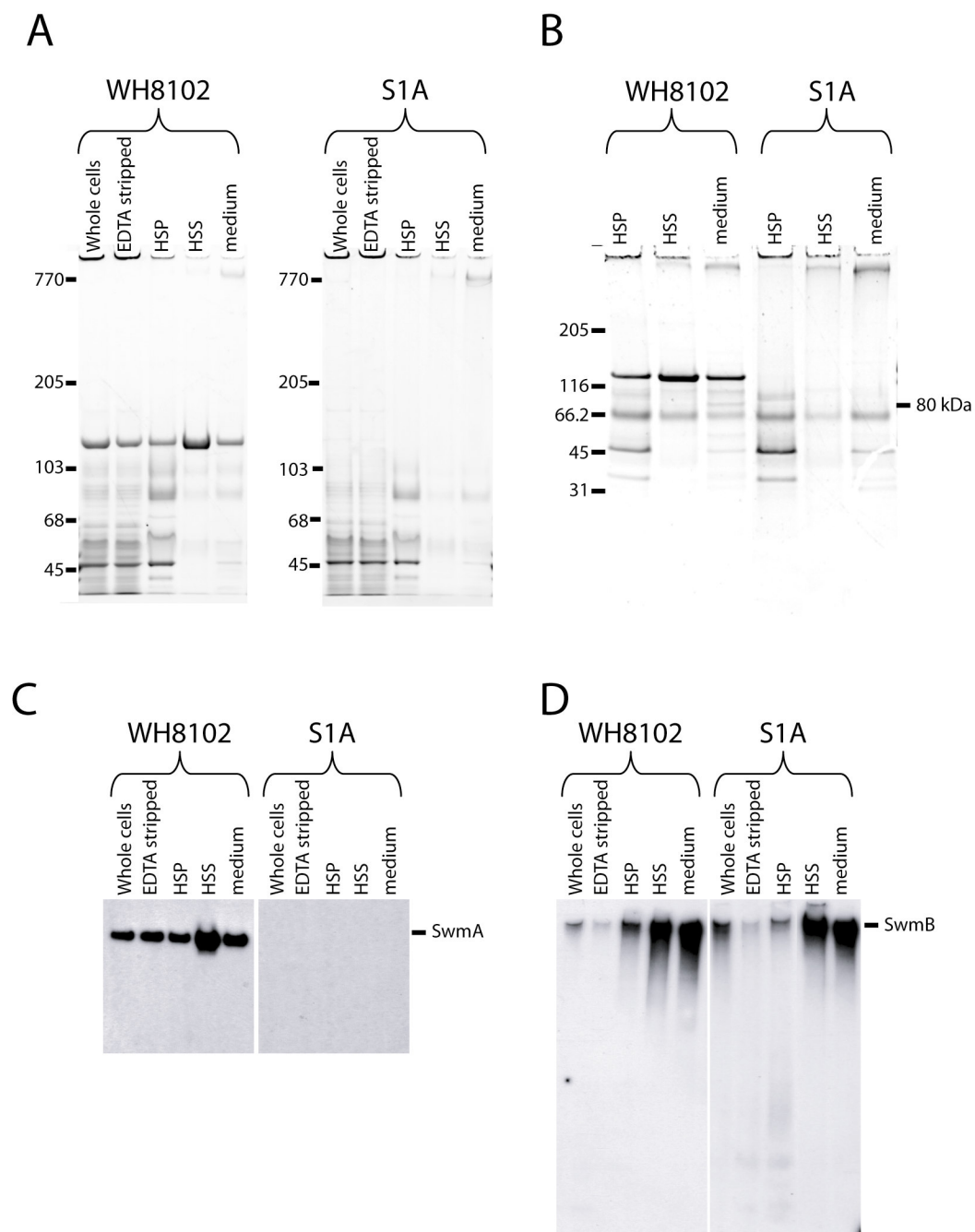


FIG. 3. SYPRO stained 3-8% tris-acetate gels (A), SYPRO stained 10-20% tricine gels (B), anti-SwmA Westerns (C), and anti-SwmB Westerns (D) comparing cellular fractions from wild-type strain WH8102 and S1A mutant strains.

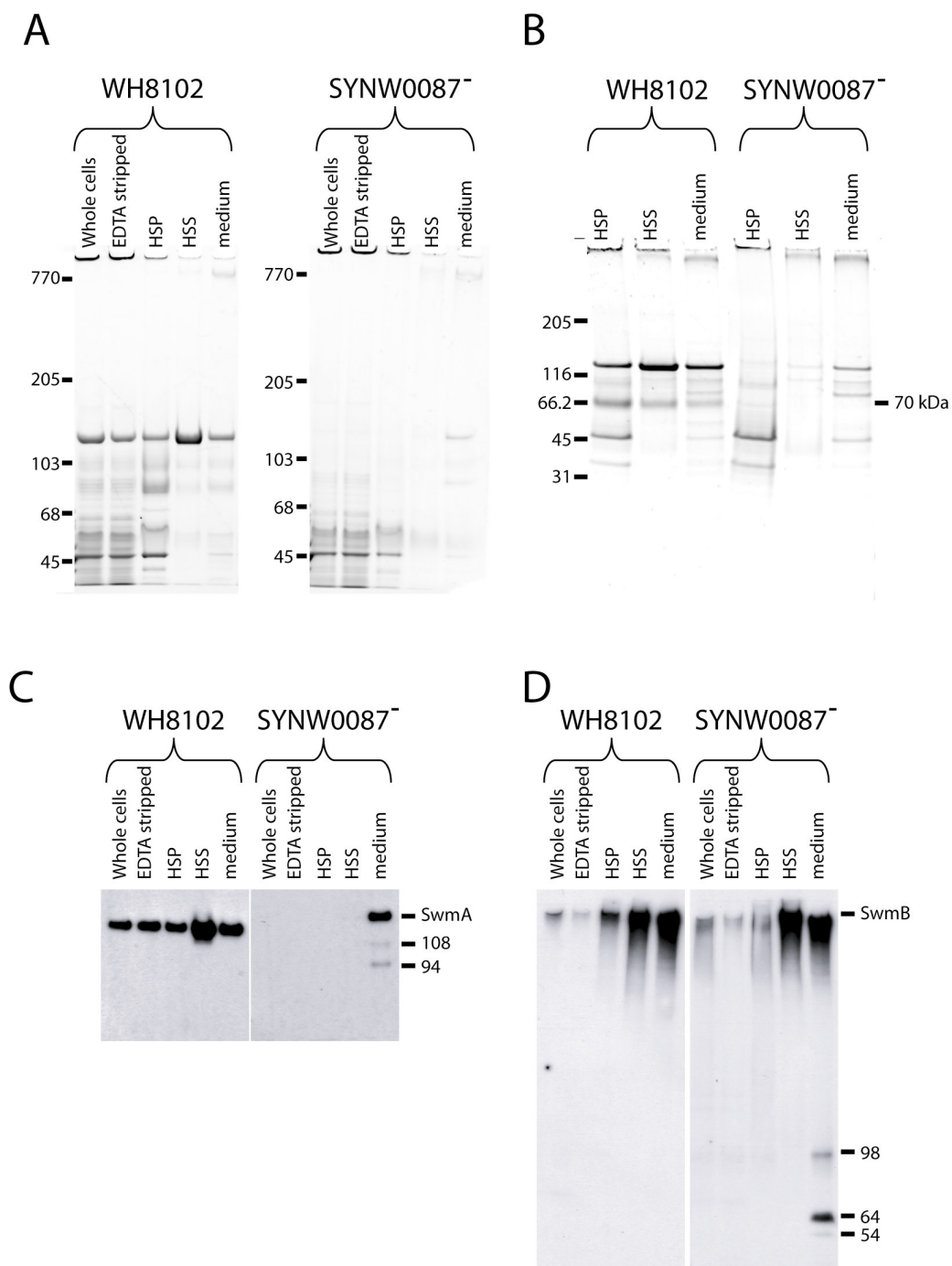


FIG. 4. SYPRO stained 3-8% tris-acetate gels (A), SYPRO stained 10-20% tricine gels (B), anti-SwmA Westerns (C), and anti-SwmB Westerns (D) comparing cellular fractions from wild-type strain WH8102 and SYNW0087<sup>-</sup> mutant strains.

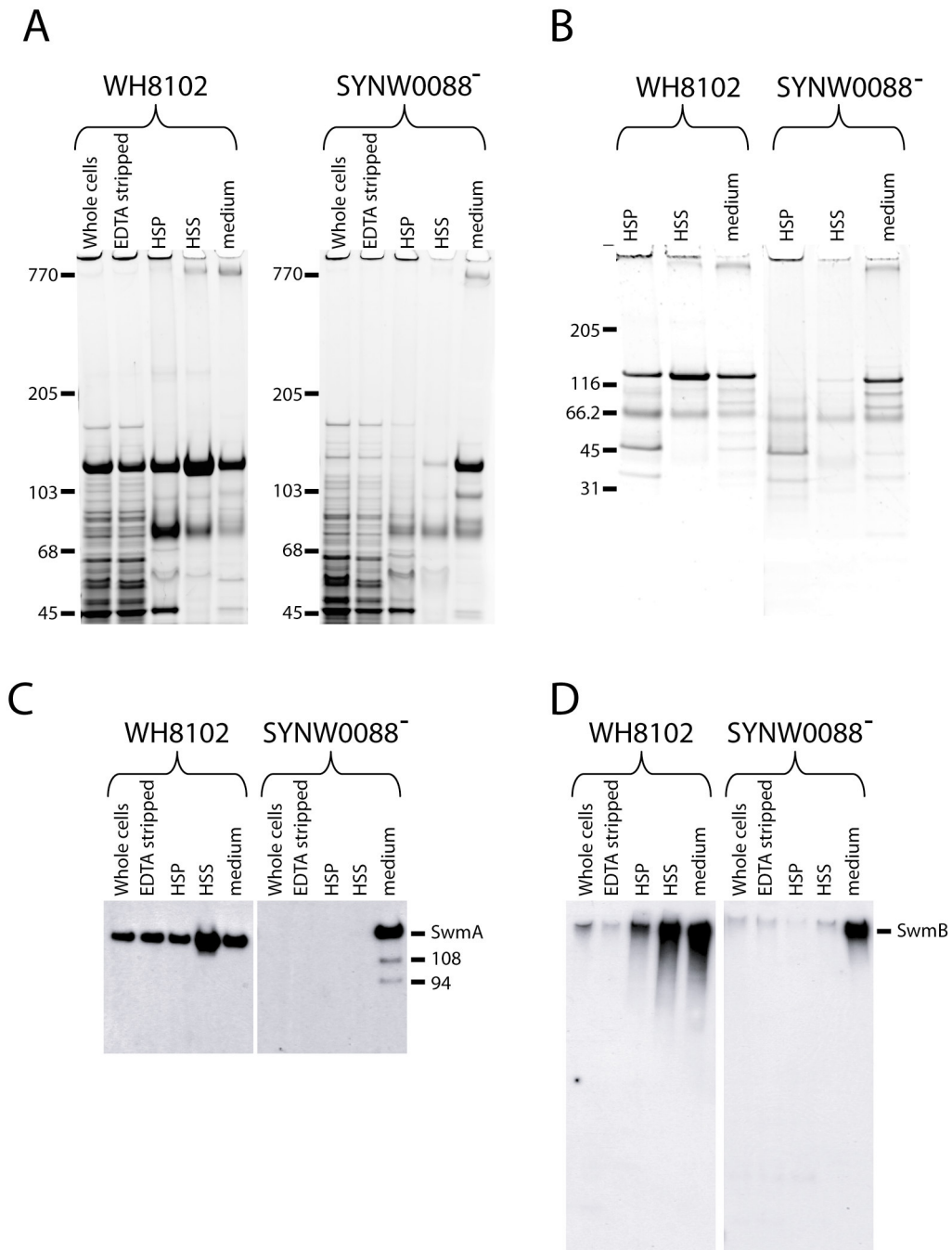


FIG. 5. SYPRO stained 3-8% tris-acetate gels (A), SYPRO stained 10-20% tricine gels (B), anti-SwmA Westerns (C), and anti-SwmB Westerns (D) comparing cellular fractions from wild-type strain WH8102 and SYNW0088<sup>-</sup> mutant strains.

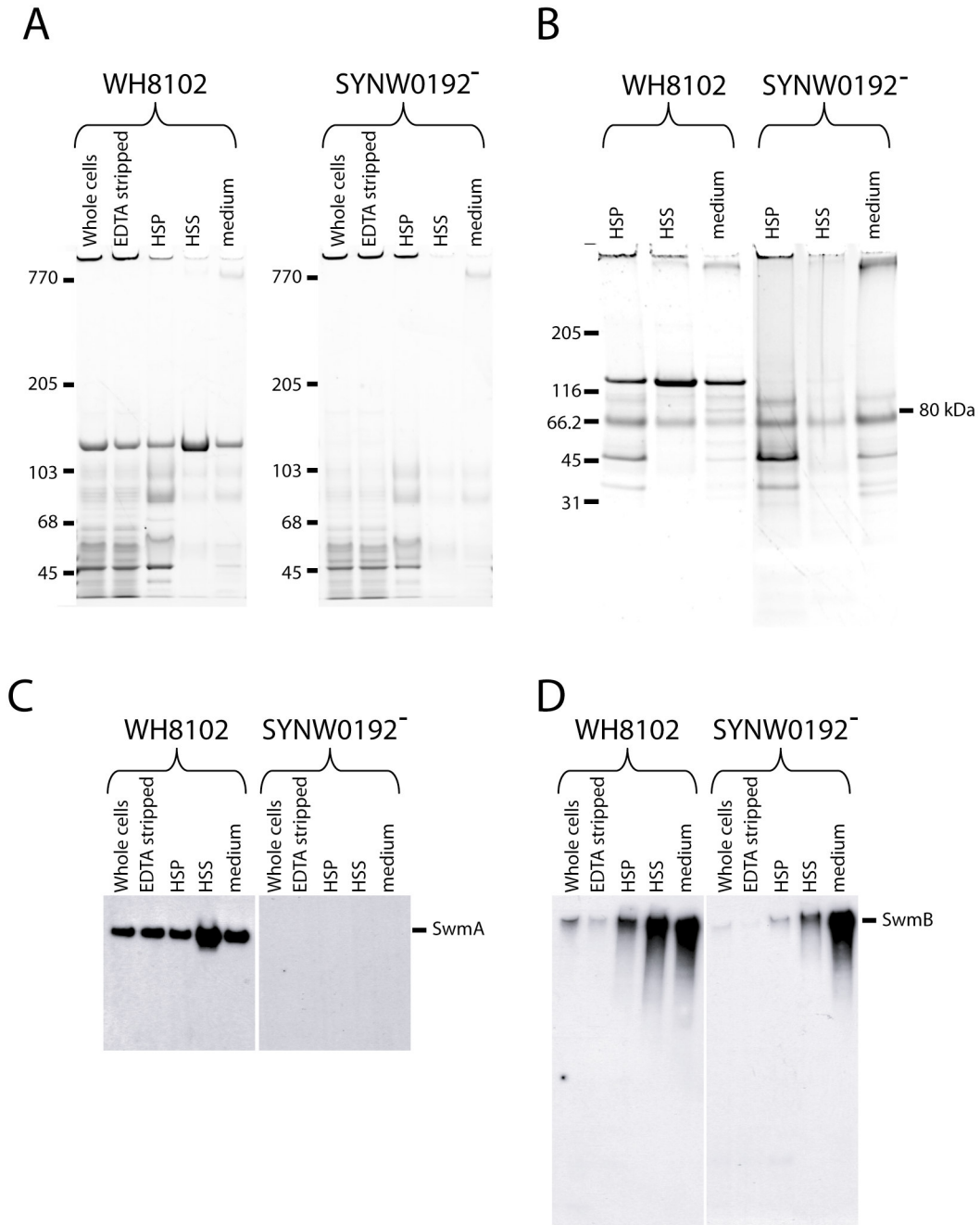


FIG. 6. SYPRO stained 3-8% tris-acetate gels (A), SYPRO stained 10-20% tricine gels (B), anti-SwmA Westerns (C), and anti-SwmB Westerns (D) comparing cellular fractions from wild-type strain WH8102 and SYNW0192<sup>-</sup> mutant strains.

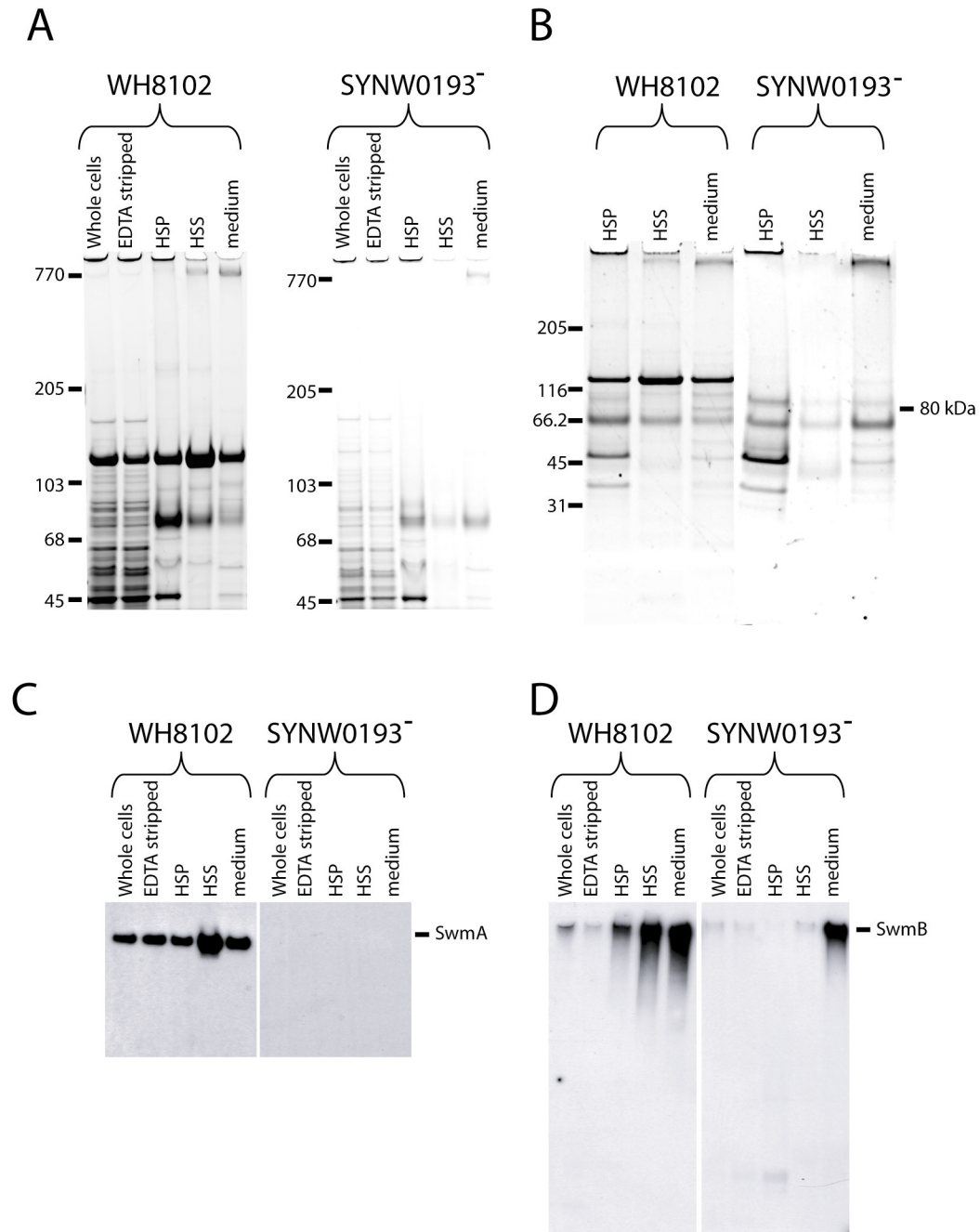


FIG. 7. SYPRO stained 3-8% tris-acetate gels (A), SYPRO stained 10-20% tricine gels (B), anti-SwmA Westerns (C), and anti-SwmB Westerns (D) comparing cellular fractions from wild-type strain WH8102 and SYNW0193<sup>-</sup> mutant strains.



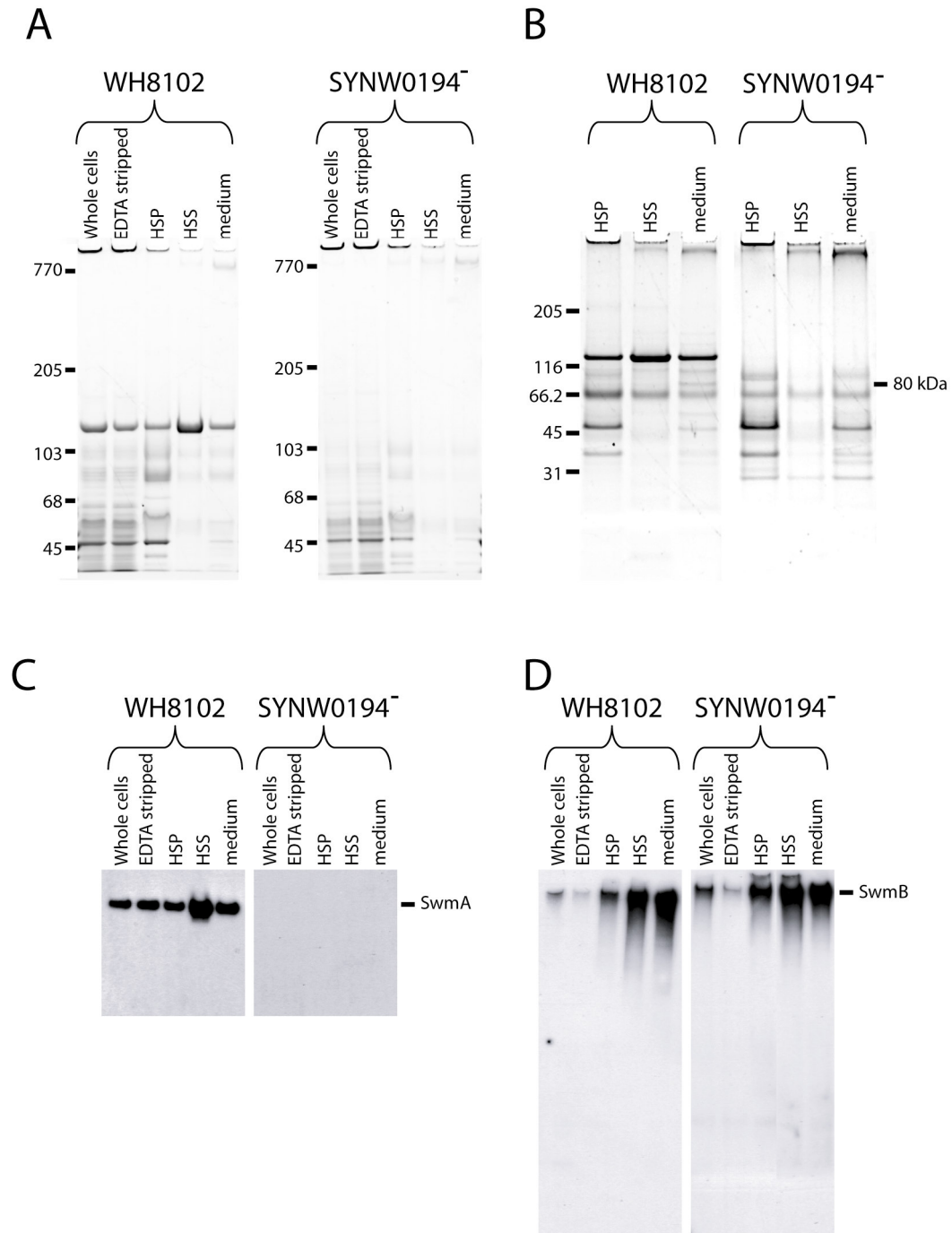


FIG. 8. SYPRO stained 3-8% tris-acetate gels (A), SYPRO stained 10-20% tricine gels (B), anti-SwmA Westerns (C), and anti-SwmB Westerns (D) comparing cellular fractions from wild-type strain WH8102 and SYNW0194<sup>-</sup> mutant strains.

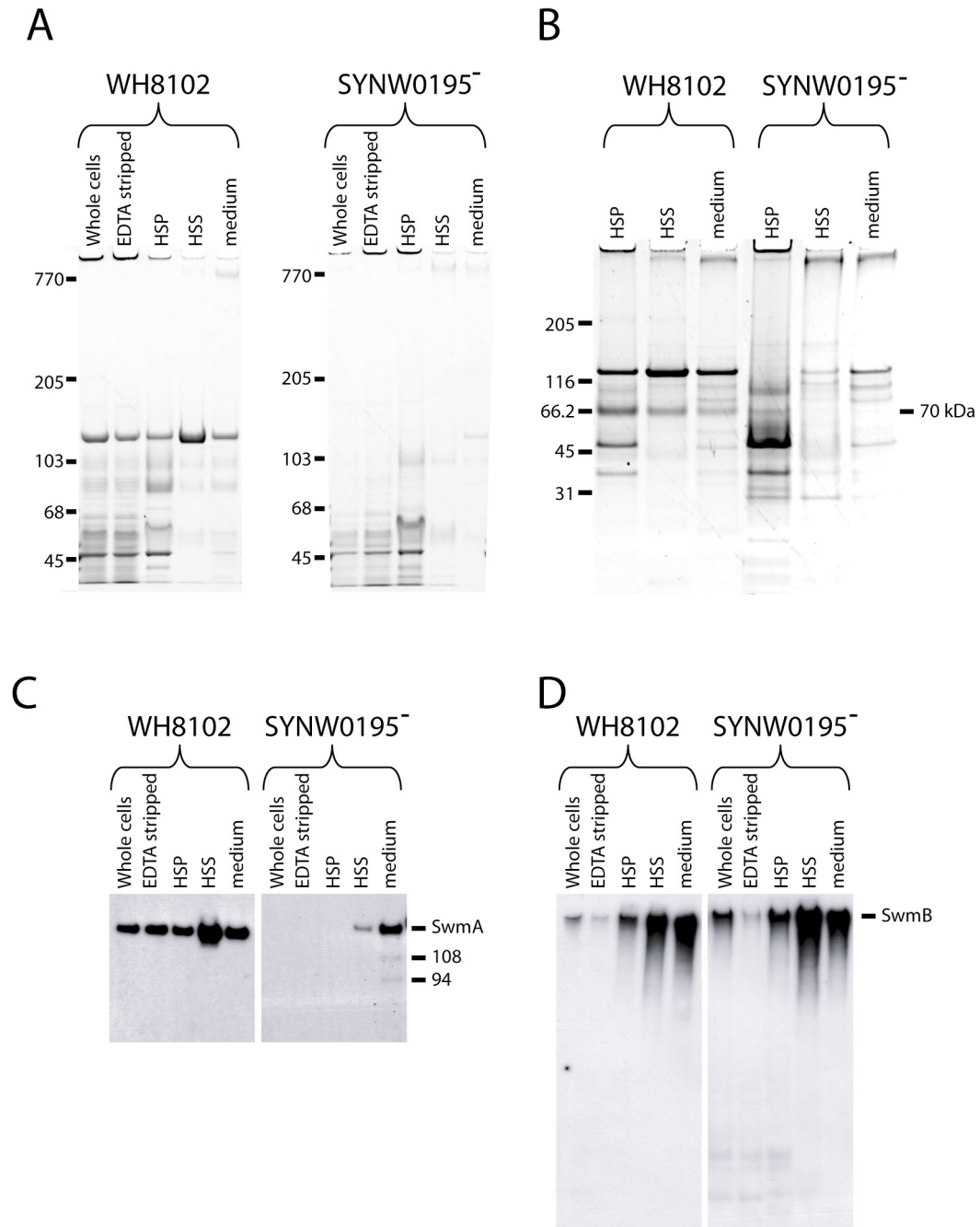


FIG. 9. SYPRO stained 3-8% tris-acetate gels (A), SYPRO stained 10-20% tricine gels (B), anti-SwmA Westerns (C), and anti-SwmB Westerns (D) comparing cellular fractions from wild-type strain WH8102 and SYNW0195<sup>-</sup> mutant strains.

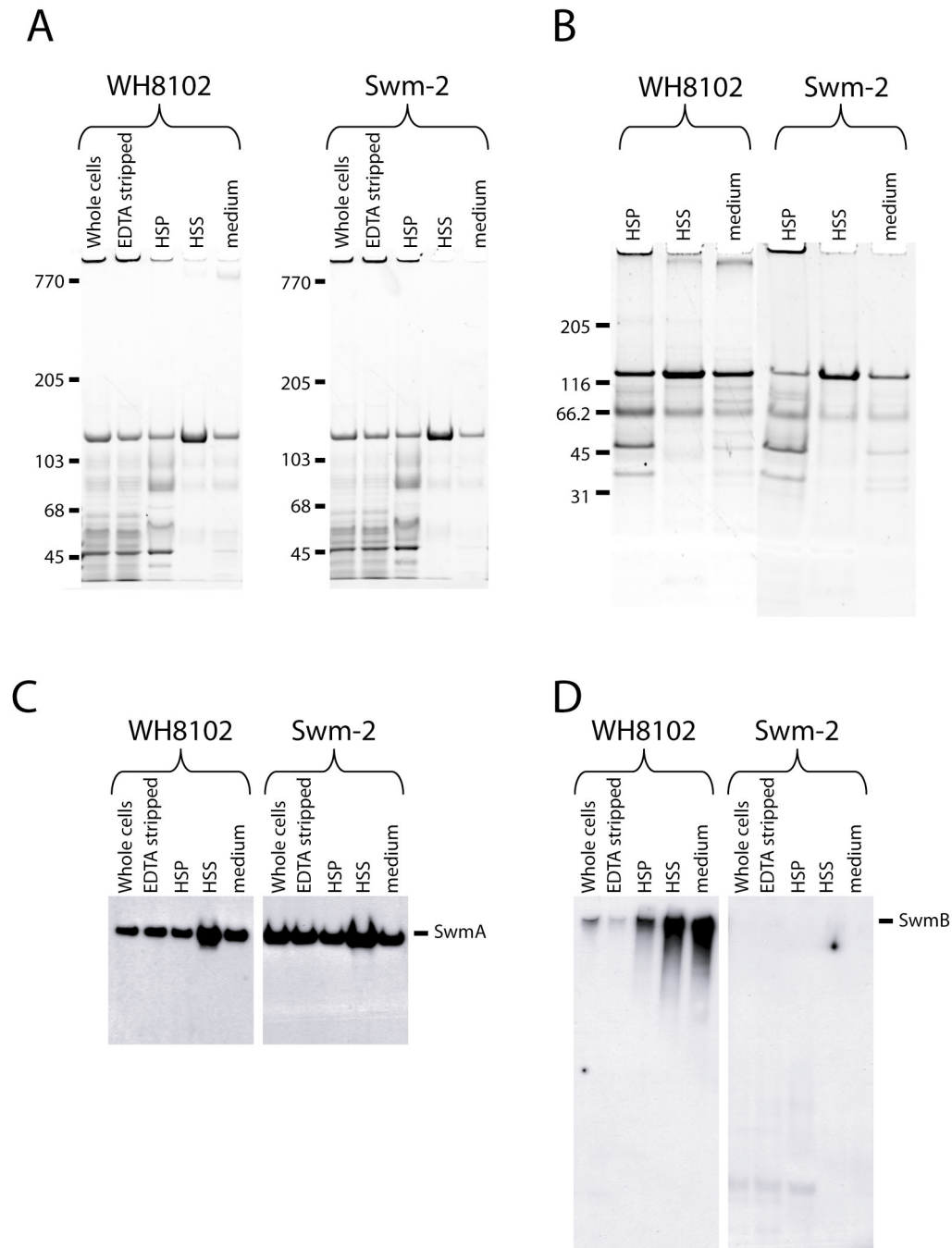


FIG. 10. SYPRO stained 3-8% tris-acetate gels (A), SYPRO stained 10-20% tricine gels (B), anti-SwmA Westerns (C), and anti-SwmB Westerns (D) comparing cellular fractions from wild-type strain WH8102 and Swm-2 mutant strains.

TABLE 1. Bacterial strains and plasmids used in this study

Strain or Plasmid	Relevant Characteristics <sup>a</sup>	Source or reference <sup>b</sup>
<b>Strains</b>		
<i>Synechococcus sp.</i>		
WH8102	Motile strain, recipient in conjugations with pMUT100 constructions	J. Waterbury
S1A	<i>swmA</i> mutant, inactivated with pBB1000	(8)
Swm-2	<i>swmB</i> mutant, inactivated with pJM20	(8)
SYNW0079 <sup>c</sup>	SYNW0079 (conserved hypothetical) inactivated with pJM37	(8)
SYNW0087 <sup>c</sup>	SYNW0087 (putative glycosyltransferase) inactivated with pJM62	(8)
SYNW0088 <sup>c</sup>	SYNW0088 (putative glycosyltransferase) inactivated with pJM18	(8)
SYNW0192 <sup>c</sup>	SYNW0192 (putative PPIase) inactivated with pJM59	(8)
SYNW0193 <sup>c</sup>	SYNW0193 (ABC transporter) inactivated with pJM40	(8)
SYNW0194 <sup>c</sup>	SYNW0194 (MFP transport accessory) inactivated with pJM60	(8)
SYNW0195 <sup>c</sup>	SYNW0195 (putative glycosyltransferase) inactivated with pJM61	(8)
<i>E. coli</i>		
MC1061	Host for pRK24, pRL528; used as a donor in pJM construction conjugations	(4)
DH5 $\alpha$	Recipient in transformations	BRL
<b>Plasmids</b>		
pRK24	Tc <sup>r</sup> , Amp <sup>r</sup> ; conjugal plasmid, RK2 derivative	(2, 11)
pRL528	Cm <sup>r</sup> ; helper plasmid, encodes <i>mob</i>	(2, 6)
pCR2.1-TOPO	Kan <sup>r</sup> , Amp <sup>r</sup> ; PCR product cloning vector	Invitrogen
pJM37	pMUT100 containing SYNW0079 fragment 78600..78939	(8)
pBB1000	pMUT100 containing <i>swmA</i> fragment 84507..84926	(8)

TABLE 1 (continued). Bacterial strains and plasmids used in this study

pJM62	pMUT100 containing SYNW0087 fragment 88793..89004	(8)
pJM18	pMUT100 containing SYNW0088 fragment 89414..89635	(8)
pJM19	pMUT100 containing SYNW0089 fragment 91118..91367	This work
pJM59	pMUT100 containing SYNW0192 fragment 193443..193661	(8)
pJM40	pMUT100 containing SYNW0193 fragment 194020..194287	(8)
pJM60	pMUT100 containing SYNW0194 fragment 197115..197342	This work
pJM61	pMUT100 containing SYNW0195 fragment 198491..198714	This work
pJM20	pMUT100 containing <i>swmB</i> fragment 913153..913396	(8)

<sup>a</sup> nt, nucleotide. Numbering according to (12).

<sup>b</sup> BRL, Bethesda Research Laboratories, Gaithersburg, MD; Invitrogen, Carlsbad, CA

Table 2. Protein content of cellular fractions for all motility mutants.

Strain	Putative function of inactivated gene	SwmA				SwmB				70 kDa	80 kDa
		WC	HSP	HSS	Med	WC	HSP	HSS	Med	All	Med
WH8102		X	X	X	X	X	X	X	X	X	X
SYNW0079 <sup>-</sup>	conserved hypothetical				X <sup>+</sup>	X		29%	X	X	X
S1A	cell surface motility component					X	X	X	X	X	
SYNW0087 <sup>-</sup>	glycosyltransferase				X <sup>+</sup>	X	X	X	X <sup>+</sup>		X
SYNW0088 <sup>-</sup>	glycosyltransferase				X <sup>+</sup>	X		26%	X	X	X
SYNW0192 <sup>-</sup>	chaperonin					X	X	X	X	X	
SYNW0193 <sup>-</sup>	ABC transporter					X		23%	X	X	
SYNW0194 <sup>-</sup>	MFP					X	X	X	X	X	
SYNW0195 <sup>-</sup>	glycosyltransferase			22%	X <sup>+</sup>	X	X	X	X		X
Swm-2	cell surface motility component	X	X	X	X					X	X

SwmA and SwmB specifically assayed for by Western analysis. 70 kDa and 80kDa proteins visualized by SYPRO staining. WC, whole cells; HSP, EDTA stripped high-speed pellet; HSS, EDTA stripped high-speed supernatant; Med, spent media concentrate; X, present at wild-type levels; %, present but at a fraction of the level present in wild-type cells (by densitometry); X<sup>+</sup>, present at wild-type size along with other smaller bands detected by western analysis.

The text of Chapter VI, in full, is being prepared for publication. The dissertation author was the primary author, and co-author B. Brahamsha directed and supervised the research, which forms the basis for this chapter.