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 ultrastuctural 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with the outer membrane and that SwmA may be arranged on the cell surface with a periodic distribution (Brahamsha, unpublished).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 produces 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 were grown in SN medium

(10) prepared with local seawater. 50-ml cultures were maintained in 125-ml flasks under constant illumination (10 $\mu E \cdot m^{-2} \cdot sec^{-1}$) without shaking at 25°C. Cultures of S1A1 contained 15 $\mu g \cdot ml^{-1}$ 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 × *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% γ-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°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 SYNUVIEW (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 stains, 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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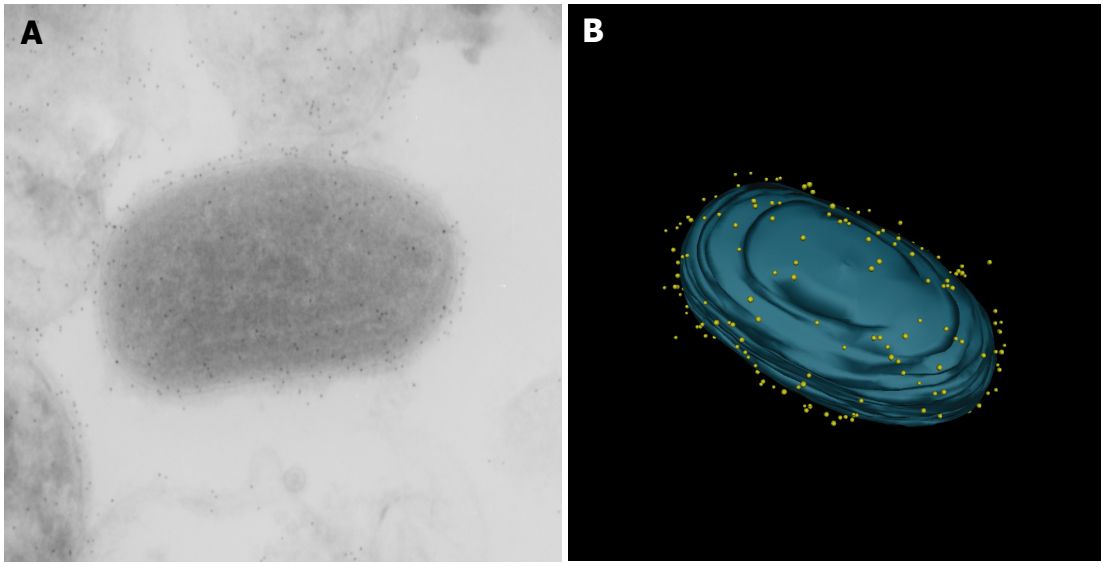

FIG. 1. Immunologically labeled *Synechococcus sp.* strain WH8102 cell. Thick section (~500nm) TEM (A) of a nearly intact cell labeled with an antibody raised against SwmA exhibits even gold-labeling across cell. Tomographic rendering of the same cell (B) illustrates the even distribution of extracellular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

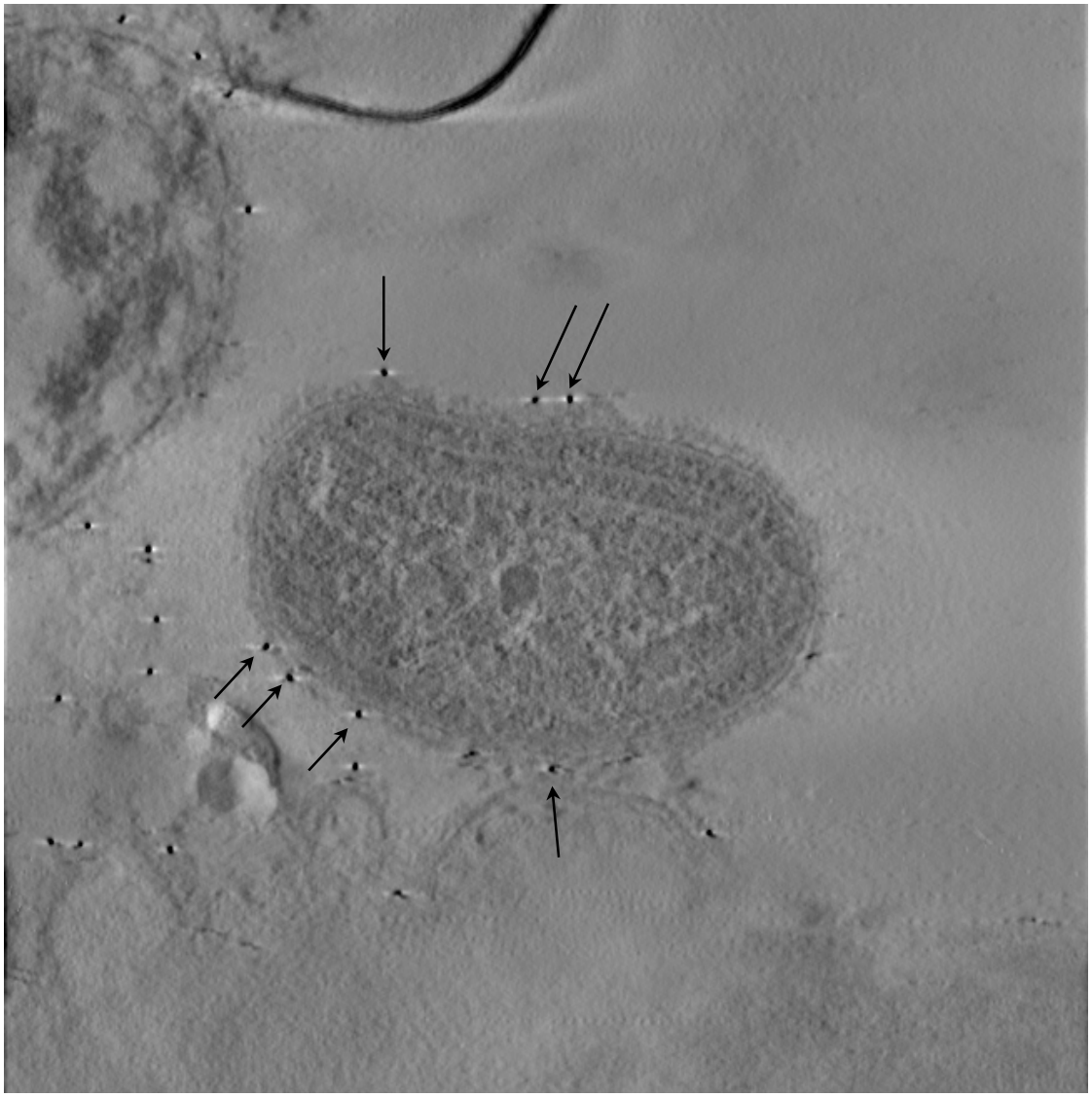

FIG. 2. Computer generated ultra-thin section from tomographic reconstruction of anti-SwmA labeled *Synechococcus* sp. strain WH8102 cells. Gold labeling (arrows) is intimately associated with extracellular material at the cell surface

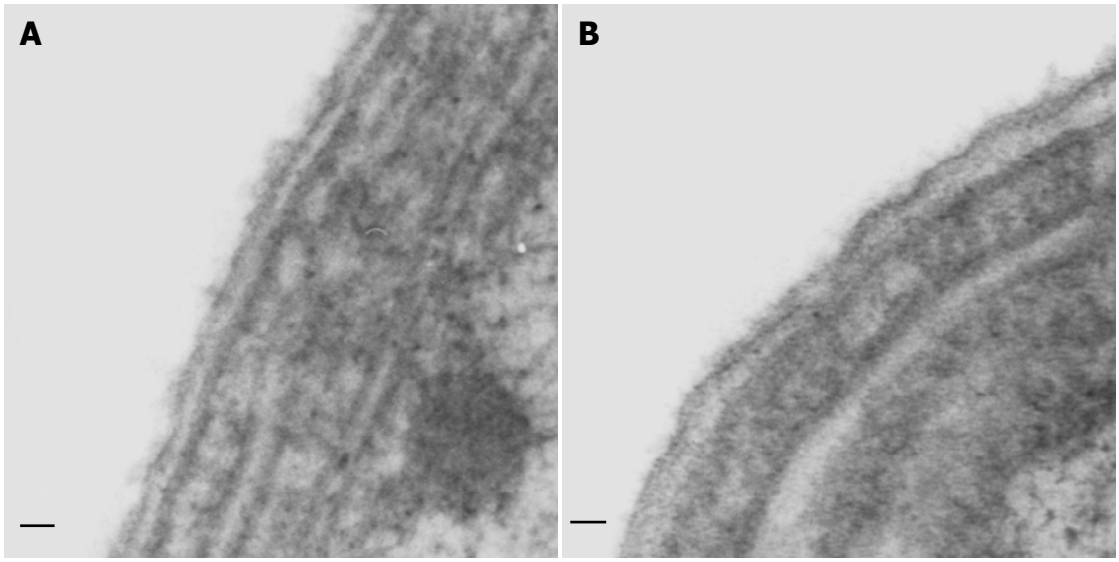

FIG. 3. Thin section transmission electron micrographs of *Synechococcus* sp. strains WH8102 (A) and S1A1 (B). Irregular layer of extracellular material is present outside of the outer membrane in both strains. Bar 50 nm .