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Phenotypic and Genomic Differences Between Wildtype and Mutant Synechocystis sp. Strain PCC 6803 and Positive Phototaxis

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Phenotypic and Genomic Differences Between Wildtype and Mutant *Synechocystis* sp. Strain PCC 6803 and Positive Phototaxis

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Abstract

Phenotypic and genomic differences between a wildtype non-motile strain and a motile mutant strain of *Synechocystis* sp. Strain PCC 6803 were determined by comparing the physical differences in their biofilm formation, their cell surface structures using scanning electron microscopy (SEM) and their genomes. The positive phototaxis of the motile mutant was also characterized using incandescent and fluorescent light sources and a variety of discrete wavelengths of light. It was found that the non-motile strain formed monolayer biofilms while the motile strain formed distinct pillars. SEM revealed that the non-motile cells had a more coarse surface than the motile cells. Four proteins that had different copy numbers between the two genomes were identified in genomic studies of the two species. Three of the proteins, related to twitching motility, exist in greater quantities in the motile strain than in the non-motile strain. Finally, it was determined that the motile mutant strain exhibits more phototaxis toward incandescent than toward fluorescent light. This might be due to the strain’s specific interest in the wavelength of 660nm. All of these results suggest that the mutant strain is distinctively different from the wildtype, both in its phenotypic as well as its genomic characteristics.
Acknowledgements

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Introduction

Biofilms:

Bacterial biofilms are aggregates of bacteria, consisting of one or more species, that are encased in a slimy matrix of polysaccharide, DNA and protein, and have the ability to adhere to various types of surfaces (Richards and Melander, 2009). Biofilms are found everywhere on the surface of the earth and can often be seen with the naked eye, from the bottom of lakes, to implanted medical devices and even to the film coating on teeth (Costerton et al., 1999).

Biofilms are of great interest to disease pathogenesis research because bacterial infections associated with biofilms are acknowledged to be more than a thousand times more resistant to antibiotics and environmental stress than free-floating, or planktonic, forms of bacteria (Parsek and Singh, 2003). The persistence of bacterial biofilms on implanted medical devices or damaged tissue may be attributed to the biofilm state of the microbes (Hall-Stoodley and Stoodley, 2005). Diseases such as the chronic lung infection cystic fibrosis and infections associated with the use of central venous catheters are all related to the fact that bacteria in biofilms are able to withstand antibiotics and evade natural host defenses (Costerton et al., 1999, Hall-Stoodley and Stoodley, 2005, Ramos et al., 2010).

Several hypotheses have been suggested to explain this phenomenon of biofilms and chronic persistent microbial infections. Antimicrobial treatment is able to destroy the individual planktonic cells that are free-floating and released by the biofilms, but is incapable of destroying the biofilm itself. This contributes to recurring disease symptoms after several cycles of antibiotics, so the most effective treatment is the surgical removal of the colonized surface of the biofilm (Stewart and Costerton, 2001).
Biofilms in nature are also resistant to a variety of environmental stressors such as nutrient deprivation, pH fluctuations, disinfectants and, of course, antibiotics (Jefferson, 2006). One defense mechanism employed by biofilms is the matrix’s ability to slow the diffusion and penetration of antibiotics due to the variety of polymeric substances that are found in the matrix (Stewart and Costerton, 2001).

Another hypothesis for antibiotic resistance is that some of the bacteria found in biofilms are in a semi-starved state due to nutrient limitation, so they exist in a slow-growing state, and cells in a non-active state are less susceptible to antibiotics (Stewart and Costerton, 2001). In biofilms, there exists a nutrient and waste gradient—with bacteria on the surface of the biofilm having more access to resources and producing more metabolic byproducts than those deep within the biofilm, which have less nutrients and experience greater concentrations of waste products (Costerton et al., 1999). Therefore, those interior bacteria enter a non-growing state. Since some antibiotics such as penicillin, which targets cell-wall synthesis, is only able to destroy actively growing bacteria, the bacteria in the non-growing state are protected as a result (Costerton et al., 1999).

A less supported hypothesis about the mechanism of antibiotic resistance states that there exists a population of bacteria within a biofilm that differentiates into a uniquely protected phenotype—similar to the concept of a spore (Costerton et al., 1999). Support for this theory came from studies of biofilm resistance in very thin biofilms with minimal matrix layer and metabolites (Costerton et al., 1999).
The life cycle of a biofilm can be broken down into five main stages—initial attachment, irreversible attachment, early development of biofilm, maturation of biofilm, and dispersion of single cells from the biofilm (Stoodley et al., 2002).

The initial attachment of cells is directed by environmental conditions that signal the transition to surface growth—these conditions vary among species, but generally include the nutritional content of the medium, temperature, pH, iron content and oxygen concentration (O'Toole et al., 2000). The initial stages of attachment are often unique and specific to the surface structure of a microbe. For example, in Pseudomonas aeruginosa, the most studied single species biofilm-forming gram-negative bacterium, it has been shown that flagella and type-IV pili play important roles in the adhesion of the bacteria to a variety of surfaces (O'Toole et al., 2000). Once there is surface contact, the bacterium rests on the surface and then uses twitching motility (a slow form of movement where bacteria extend and contract their type-IV pili) to move to form clusters with other twitching cells (O'Toole et al., 2000). This initial attachment may be transient and these microcolonies may disperse or move together across the surface.

Once the initial attachment has been made between bacteria and their surface, there is a change from a reversible to an irreversible attachment. This process has been described as going from a weak interaction of the cell with the surface to a permanent bond between them, usually with the aid of extracellular polymers (Stoodley et al., 2002). The physiological switch from flagellar movement to twitching motility, facilitated by type IV pili, is linked to irreversible attachment in some species of bacteria and is suspected to be related to the formation of microcolonies (Stoodley et al., 2002). Extracellular polysaccharide (EPS) is produced by
adherent cells to also help bind bacteria together and facilitate the formation of microcolonies and growth of the biofilm. EPS surrounding bacteria is unique to biofilms and is composed of a variety of biosynthetic polymers such as substituted and unsubstituted polysaccharides, proteins, nucleic acids and phospholipids (Stoodley et al., 2002).

After irreversible attachment has occurred between the bacteria and the surface, the biofilm begins to mature. In the maturation of the biofilm, more complex architecture is formed, including channels, pores, and a redistribution of bacteria away from the surface (Stoodley et al., 2002). The ability of the biofilm to grow and develop is dependent upon nutrient availability in its environment and the removal of waste. There is also an optimal hydrodynamic flow across the biofilm that does not erode the biofilm but rather supports its growth and development (Dunne, 2002). Other factors that may affect biofilm maturation include internal pH, oxygen perfusion, carbon source and osmolarity (Dunne, 2002).

Eventually, the biofilm reaches a critical mass and there exists an equilibrium in which the outermost layer of the biofilm begins to generate and release planktonic bacteria into the environment and they are able to colonize other surfaces and repeat the cycle (Dunne, 2002). It is hypothesized that the increase in the detachment of planktonic cells is correlated with starvation so that those planktonic cells can search for a more nutrient-rich environment (Stoodley et al., 2002).

Cyanobacterial biofilms are of great interest to researchers because ancient microbial carbonates serve as a geological record. For example, fossil stromatolites--layered sedimentoed structures formed through interactions between organisms such as bacteria and geochemical processes--serve as the oldest evidence of life on earth (Decho, 2000, Bosak et al., 2009).
Cyanobacteria are extremely important in stromatolite formation since they are the main species responsible for organic carbon production and nitrogen fixation; they also secrete most of the EPS which further stabilizes cells and sediment against resuspension (Decho, 2000).

Modern stromatolites are known as microbial mats—complex structures of large microorganism communities (mainly comprised of cyanobacteria) millimeters thick found in bodies of water (Riding, 2000). These mats are able to easily trap sediment, sand, and other grainy particles. Microbial mats have been closely examined and it was found that there is a distinct stratification, with aerobic phototrophic cyanobacteria found at the surface of the mat and chemoorganotrophs (which don't require oxygen or light) buried deeper within. These microbes are what determine the calcification and preservation of the microbial mats (Riding, 2000). These are some of the only cyanobacterial biofilms that have been investigated in a laboratory setting (Bosak et al., 2009).

Cyanobacterial biofilms are found not only in a variety of places in nature, but also around us. For example, biofilms are found on historical buildings as a part of the normal weathering process—usually containing cyanobacteria and algae (Crispim et al., 2003).

**Analysis of Biofilms:**

A popular method of bacterial biofilm analysis involves the use of confocal laser scanning microscopy (CLSM), due to its ability to capture the structure of biofilms non-invasively—without dehydrating, fixing or embedding the sample, procedures that are necessary in other microscopy techniques (Wood et al., 2000). The CLSM is a type of fluorescence microscope that has the ability to capture a three-dimensional specimen in many optical sections, and these optical sections can be generated into a 3D data set by a computer.
This ability to capture 3D images is extremely useful in analyzing the architecture of biofilms.

To analyze the three-dimensional biofilm images that are taken with the CLSM, the computer program COMSTAT is often used. COMSTAT is software with the ability to incorporate numerous features of the biofilm for the quantitative characterization of three-dimensional biofilm image stacks (Heydorn et al., 2000). The COMSTAT program has the ability to analyze three-dimensional biofilm images for a variety of characteristics including biovolume, area occupied by bacteria in each layer, the thickness distribution and mean thickness, the identification and area distribution of micro-colonies at the substratum, volumes of micro-colonies identified at the substratum, fractal dimension, roughness coefficient, distribution of diffusion distances—average and maximum diffusion distance, and surface to volume ratio (Heydorn et al., 2000).

Recent studies that observe the structure of biofilm growth in bacterial species using confocal microscopy and the COMSTAT program have generally seen two different types of biofilm structures in a developing biofilm. The first is what is called a “monolayer”, where the biofilm has essentially flat and uniform growth on a surface, whereas the other structure commonly seen is aggregates or clusters of cells, sometimes referred to as “mushrooms” or “pillars” due to their appearance (Shrout et al., 2006). One convincing hypothesis to explain these phenomena in *Pseudomonas aeruginosa* is the difference in surface motility employed by the bacteria—the monolayer biofilm would be caused by significant surface-associated motility, while the clusters would be formed as a result of minimal movement and clonal growth of
bacteria on the substrate (Shrout et al., 2006). Both mathematical simulation and time-lapse microscopy support this hypothesis.

**Scanning Electron Microscopy:**

Scanning electron microscopes are a class of electron microscopes that visualize specimens by scanning the surface of the sample with a constant beam of electrons. The sample deflects the electrons and those deflected electrons are collected and converted into an image. Electron microscopes have the advantage of being able to magnify the sample up to a few hundred thousand times, which is extremely useful in looking at bacteria which are micrometers in size. However, some of their disadvantages include the fact that preparation of a specimen for electron microscopy essentially requires fixing the biological specimen in order to preserve its native structure; fixing is toxic and kills the specimen.

**Synechocystis sp. strain PCC 6803:**

Cyanobacteria are photoautotrophic microorganisms that are extremely similar to plants and algae in that they are also capable of oxygen-producing photosynthesis (Kaneko and Tabata, 1997). A well-accepted hypothesis is that current plant chloroplasts evolved from ancient cyanobacteria, which subsequently developed an endosymbiotic relationship with eukaryotic host cells (Nakamura et al., 1998). Due to the similarities in photosynthesis between these microorganism and algae and plants, cyanobacteria have been used as model organisms for the study of photosynthesis (Kaneko and Tabata, 1997).

*Synechocystis* sp. strain PCC 6803 is a species of unicellular cyanobacteria and it was the first photoautotrophic organism to have its entire genome sequenced (Kaneko and Tabata, 1997). The length of the genome was determined to be 3,573,470 bp in length, with the
accuracy of the sequence determined by analyzing both strands of the DNA (Kaneko et al., 1996).

There is some controversy about whether the wild type *Synechocystis* sp. strain PCC 6803 is non-motile or motile. However, the wild type strain of *Synechocystis* used in the present study is non-motile. This thesis also focused on the study and characterization of a mutant motile strain of PCC 6803. This motile strain was isolated from the wild type non-motile strain through a series of transfers in which the non-motile bacteria were grown on solid agar plates and exposed to unidirectional light (Mallama, 2010). The fastest moving cells were then transferred onto another plate and given unidirectional light again. This process was repeated until a stable motile mutant was obtained.

**Phototaxis:**

Many different types of photosynthetic bacteria, including cyanobacteria, are known to exhibit three different types of movement as a response to light: phototaxis, photokinesis and photophobic movement (Bhaya et al., 2001).

Phototaxis is the orientation and then subsequent movement either towards or away from the light source, known as positive or negative phototaxis, respectively (Bhaya et al., 2001). Photokinesis is a type of movement of the cell where speed is dependent upon the fluence rate of irradiation (radiation particles passing through a cross-sectional area). Finally, the photophobic response “consists of a reversal of movement, temporary stop or directional change upon a sudden change in the fluence rate” (Yoshihara et al., 2000).

The mechanism that enables cyanobacteria to exhibit these types of movements involves pili, which allow the gliding motion that occurs in *Spirulina*, *Oscillatoria* and *Anabena*.
(filamentous cyanobacteria), and the swimming motility characteristic of the marine unicellular cyanobacterium *Synechococcus* sp. WH8102 (Yoshimura et al., 2002).

*Synechocystis* sp. strain PCC 6803 has also been identified to exhibit both positive and negative phototaxis (Bhya et al., 2001). The motility that this strain exhibits is described as twitching motility, which has been traced to surface appendages known as Type IV pili (Tfp), also described as “thick pili”—they are shown to be flexible appendages with a diameter of 6nm and length of up to 2.5μm, and are mostly found at the poles of the cell (Bhya et al., 2002). Their functions were shown when an alternative sigma factor, SigF, was inactivated and resulted in the abolishment of cell-surface appendages and motility—the *sigF* gene is required for the biosynthesis of pili and the accumulation of *pilA1* mRNA, which encodes pilin, the main component of Type IV pili (Bhya et al., 2002). *pilA1* mutants are completely non-motile, whereas the other *pilA*-like genes that were inactivated (*pilA2-6*) had no effect on motility (Bhya, 2004).

Some *Synechocystis* sp. strain PCC 6803 mutants defective in the genes homologous to the *che* genes involved in flagellar switching for bacterial chemotaxis in other bacterial species were shown to lose their positive phototaxis ability (Yoshihara et al., 2000).

**Research Goals:**

The main focus of this thesis was to further elucidate the differences between the motile strain and the non-motile strain of *Synechocystis* sp. strain PCC 6803. More specifically, the physical differences between the two strains, analyzing both the architecture of their biofilms as well as their surface structure, the genetic differences between the strains, and
finally, the light signals involved in the positive phototaxis of the motile strain were investigated.

The differential biofilm growth of motile and non-motile *Synechocystis* involved growing the two types of mono-species biofilms in small “flow chambers” with a continuous flow of medium (Heydorn et al., 2000). These chambers were then analyzed using confocal microscopy and the three-dimensional images obtained were analyzed over time with COMSTAT, with collaborations at Olin College, to determine the statistical significance of differences in biofilm formation between the two species.

Physical differences between the wildtype and motile cyanobacteria were captured using scanning electron microscopy with collaborators at University of Toledo.

To characterize the genomic changes in the mutant motile strain, the DNA from both motile and non-motile *Synechocystis* was extracted and sequenced. The insertions and deletions of certain genes were identified and used to support the hypothesis that the motile strain is genetically different from the non-motile strain from which it was isolated.

Positive phototaxis was measured in the motile strain as a rate of the fastest growing cyanobacteria in response to two different light sources—fluorescent and incandescent illumination, as well as to light of specific wavelengths.
Materials and Methods

Cyanobacteria Strains:

*Synechocystis* sp. PCC 6803 was grown in liquid BG-11 medium or solid BG-11 agar plates under fluorescent light conditions. The standard BG-11 medium is shown in Appendix 1.

As seen below in Figure 1, the wildtype non-motile *Synechocystis* sp. strain PCC 6803 that was used is “ATCC 27184”; it is also glucose tolerant. This strain was derived from the Berkeley strain 6803, which was initially isolated from fresh water in Oakland, California in 1968.

**Figure 1. Strain history of *Synechocystis* sp. PCC 6803 (Ikeichi and Tabata, 2001).** This flow diagram depicts the genealogy of *Synechocystis* sp. PCC 6803 from its initial isolation to the many insertions and deletions that have occurred and their new strain names until 2001.
The motile mutant strain was derived from this non-motile of *Synechocystis* sp. PCC 6803 by Mallama (2010), using the set-up described in Figure 2 below. Only the mutant motile cyanobacterium responded to unidirectional fluorescent light and physically moved closer to that light. The bacteria that moved toward the light were then streaked on a fresh plate and put into a cardboard box to allow for unidirectional illumination again. This process was repeated again and again until a stable mutant strain of motile bacteria was isolated.

![Cardboard Box](image)

**Figure 2. Deriving the motile mutant from wildtype non-motile *Synechocystis* sp. PCC 6803 (Mallama, 2010).** The non-motile cyanobacteria were streaked onto a solid BG-11 plate and allowed to grow in unidirectional light. The light was ensured to be unidirectional by placing the agar plate inside a small cardboard box with fluorescent light shining on only one side and the other side closed.

**Analysis of Biofilms:**

The differences in biofilm formation of *Synechocystis* sp. strain PCC 6803 between the wildtype non-motile strain and the isolated motile strain were analyzed by growing biofilms in flow chambers and studying their structures using confocal microscopy.
Growing cyanobacteria in flow chambers

Wildtype non-motile and motile mutant strains of *Synechocystis* sp. strain PCC 6803 were grown in liquid BG-11 medium in 30°C on a shaker receiving fluorescent light until its growth had reached the log phase of its growth curve (an optical density of about 1.0), as determined by using the spectrophotometer at 750nm (Yoshihara et al., 2000).

The flow cell system is described below in Figure 3, with the fluorescent light source close to the flow channels. The whole system was autoclaved for sterilization, and then BG-11 medium was allowed to run through the system by turning on the peristaltic pump to 1rpm. The 205S peristaltic pump was obtained through the Watson Marlow Pumps group (http://www.watson-marlow.com/), and bubble traps were FC 34 Four-Channel Bubble Trap (http://www.biofilms.biz).

To grow the biofilm, the pump was stopped after medium had been completely pushed through the initial system. First, alcohol was used to clean the outside of the tubing between the bubble trap and the flow channels. Then, using a sterile needle (26G x 5/8 inch) attached to a 1mL syringe, about 300μL of one or the other type of bacteria at an OD<sub>750</sub> of 1.0 was carefully injected into the tubing a few centimeters in front of the chamber (ideally without introducing any air bubbles), and the puncture hole was then immediately sealed with silicone glue. The flow channel was then inverted (with the coverslip facing down) for about 2 hours to allow the bacteria to adhere to the coverslip. Then, the flow chamber was turned right side up and the peristaltic pump turned on to 1rpm—which pumped fluid at a rate of 2.4ml/hr. This whole set-up was done in room temperature and the flow chamber received 19 μmol/m<sup>2</sup>/s of fluorescent light in order for the cyanobacteria to grow.
Figure 3. Flow cell system setup (Nielsen et al., 2011). The basic flow cell system consists of medium pumped by a peristaltic pump through tubing which is freed of bubbles by using bubble traps. The medium is then pumped through the flow channels where the bacteria were inoculated, and the medium and bacterial waste flows out at the end of the system and is collected into a waste bottle. The flow channels were grown in front of a fluorescent light source and received a light intensity of 19μmol/m²/s. This whole setup was placed at room temperature.

Polysaccharide Staining

Concanavalin Alexa Fluor® 488 Conjugate (Molecular Probes, Inc. Eugene, OR) was added to 0.5mL of a 0.1M sodium bicarbonate solution. This stock solution was diluted to make a 100μg/mL dye solution. The dye (300μL) was injected into a chamber of the growing biofilm and allowed to stain in the dark without medium passing through, for 20 minutes. Medium was then passed through the flow chambers for 10 minutes before the biofilm was observed with the Ar 488 laser and the He Ne 633. The He Ne 633 laser should excite chlorophyll a and the A 488 laser should excite any attached Con-A Fluor® 488.
Imaging

The biofilms grew 3 days before the first imaging. The flow cells were disconnected from the pump by clamping and removing the silicone tubing connecting the bubble traps to the tubing in the peristaltic pumps (and the tubing from the flow cell to the waste bottle was also clamped closed). Then the flow cells were easily placed onto the stage of the confocal microscope. Confocal data were taken by Professor Rebecca Christianson at Olin College; the specific protocol used is described in Appendix 2.

Data Analysis

The confocal microscopy image stacks were analyzed using the COMSTAT computer program by Professor Rebecca Christianson.

Scanning Electron Microscopy (SEM):

To determine any physical differences in the surface structure of the motile and non-motile strains of *Synechocystis*, these two strains were grown separately for three days on solid agar plates (0.8% BG-11 agar with 0.3% thiosulfate) until the plates were a vivid green color. They were then mailed to the Instrumentation Center of Natural Sciences and Mathematics Outreach S.C.O.P.E. Program at University of Toledo, Toledo, OH. Their preparation methods are described in Appendix 3. The SEM images were taken over the internet via a new program that allows remote users to control the SEM at Toledo.

Phototaxis:

Positive phototaxis towards light was observed in motile *Synechocystis* sp. strain PCC 6803 by growing the motile cyanobacteria on solid 0.8% BG-11 agar with 0.3% sodium thiosulfate.
thiosulfate, as is common in cyanobacterial phototaxis experiments (Yoshihara et al., 2000).

These plate conditions were also determined to be optimal based on preliminary trials comparing motility on different agar and sodium thiosulfate concentrations, as shown in Appendix 4. To determine the optimal environment for the fastest rate of movement, two different light conditions were utilized: incandescent and fluorescent. The wildtype and motile mutant *Synechocystis* sp. strain PCC 6803 strains had previously been grown only under fluorescent light.

*Preparing agar plates*

In order to conduct the motility assay, soft (0.8% BG-11) agar with 0.3% sodium thiosulfate (w/v) plates were prepared. This was achieved by mixing equal volumes of 2X concentrated BG-11 media dissolved with 0.6% (w/v) solid sodium thiosulfate (certified anhydrous, 500gm) with an equal volume of 1.6% (w/v) Bacto™ Agar (Becton Dickinson) dissolved in deionized water. Prior to combining the mixtures under a sterile hood, these solutions were autoclaved separately to ensure sterilization and cooled to 60°C in a water bath. After combining the two solutions, the mixture was poured into 100 X 15mm petri dishes. In order to allow enough transparency and illumination during image taking of the plates, the solution was poured to approximately half fill each plate.

The 1mM glucose plates in the wavelength-specific phototaxis experiment were prepared using the same method as described above, also with 0.8% BG-11 and 0.3% sodium thiosulfate. However, the glucose solution was not autoclaved with the medium and agar. Rather, the glucose was dissolved in deionized water and was then filter sterilized. The filter sterilized glucose was added to the medium after it had been cooled in the water bath.
Streaking

In order to observe and quantify phototaxis in motile *Synechocystis* sp. Strain PCC 6803, bacteria were streaked onto plates and placed in one of two different light sources. A line was marked across each plate at approximately 20mm from the edge (as seen below in Figure 4). About 1 ‘loop’ of bacteria was obtained from the tip of a fastest growing ‘finger’ from a previously streaked plate and bacteria were streaked onto the newly prepared plate. This process represented a ‘transfer’ and each transfer was labeled as a new generation. Plates were partially wrapped in aluminum foil with a small area left uncovered to allow unidirectional light through. Each plate was placed agar-side up with the streaked line perpendicular to the light source in a 30°C room (as shown in Figure 4). These plates were placed at a distance where they would receive 25 µmol/m²/s (measured using a Quantum Meter) in light intensity from the two difference sources of light: a 60 Watt soft white incandescent light, and cool white fluorescent light.

![Figure 4. Phototaxis set-up.](image)

The 0.8% BG-11 agar with 0.3% thiosulfate plate was streaked with a loop of bacteria about 20mm from the edge of the plate and about ¾ of the plate was covered with aluminum foil. The plate was placed at 30°C near the light source at a distance that received 25 µmol/m²/s of light intensity from either fluorescent light or incandescent light.
In the wavelength-specific phototaxis experiments, the streaked, partially aluminum foil covered agar plates, with and without added glucose, were placed in LED boxes or boxes attached to a Klett filter, with the latter described below in Figure 5. The 660nm (0.1 \(\mu\text{mol/m}^2/\text{s}\)) and 540nm (0.96 \(\mu\text{mol/m}^2/\text{s}\)) experiments were done using glass Klett filters, while the 470nm (1.32 \(\mu\text{mol/m}^2/\text{s}\)), 505nm (3.65 \(\mu\text{mol/m}^2/\text{s}\)), 610nm (5.96 \(\mu\text{mol/m}^2/\text{s}\)), 700nm (2.1 \(\mu\text{mol/m}^2/\text{s}\)), 720nm (3.6 \(\mu\text{mol/m}^2/\text{s}\)), 750nm (2.7 \(\mu\text{mol/m}^2/\text{s}\)), 880nm (0.41 \(\mu\text{mol/m}^2/\text{s}\)) and 1050nm (3.8 \(\mu\text{mol/m}^2/\text{s}\)) experiments were performed by placing the aluminum covered plates in cardboard boxes illuminated with LED lights of those specific wavelengths, courtesy of Professor Jean Huang of Olin College. Dark and fluorescent control plates were always incubated with experimental plates.

**Figure 5. Wavelength-specific phototaxis set-up.** The 0.8% BG-11 agar with 0.3% thiosulfate plates (with and without 1mM glucose) were streaked with a loop of bacteria about 20mm from the edge of each plate and about ¼ of the plate was covered with aluminum foil. The plates were then placed into boxes that held a glass Klett filter for the specific wavelengths.
660nm and 540nm or with a bank of LEDs. The boxes containing the two filters were placed extremely close to the either fluorescent light (540nm) or incandescent light (660nm).

Data collection

In order to determine the rate of movement in each light condition over a series of transfers (or generations), plates were analyzed every 24 hours for a visual indication of their cell mass (as indicated by how dark the green cyanobacteria appeared) and their average distance traveled was measured—every projection from the streaked line was measured and averaged with the other fingerlike projections on the same plate (each “arrow” in Figure 4 above would count as a data point). These plates were allowed to grow for up to 7 days before the fastest moving projection was transferred onto two fresh plates, which were again subject to the same two light sources, and their progress recorded. Rate of movement was plotted as a function of time and standard deviation was calculated.

For the wavelength-specific phototaxis experiment, the plates were grown for a week in room temperature before observations were made.

Genome Analysis:

To determine whether any genomic changes occurred between our wildtype and mutant strains of Synechocystis sp. Strain PCC 6803 and the sequences of published Synechocystis in NCBI (National Center for Biotechnology Information), DNA from the two strains was extracted, sequenced and analyzed.

Data collection:

Non-motile and motile Synechocystis sp. Strain PCC 6803 were grown on 0.8% BG-11 agar with 0.3% sodium thiosulfate (w/v) plates under cool white fluorescent light for a week
until the plates were visually a vivid green. The bacteria were scraped from their respective plates using sterile pipette tips and transferred to sterilized microcentrifuge tubes. The DNA extraction protocol that was followed was the “Powerlyzer UltraClean Microbial DNA Isolation Kit” from MoBio Laboratories (http://www.mobio.com/microbial-dna-isolation/powerlyzer-ultraclean-microbial-dna-isolation-kit.html), shown in Appendix 5. The cells were lysed with sodium dodecyl sulfate detergent and specialized beads, and all of the non-DNA material was precipitated. The DNA was then selectively bound to a silica membrane under high salt conditions, washed with ethanol to remove contaminants, and then finally, the DNA was released from the membrane with Tris buffer. This extracted DNA was then sent to be sequenced at the “MRDNA” laboratories (http://mrdnalab.com/sequencing-service.html).

Data analysis:

The sequenced DNAs were sent to us in fragments called contigs—both the annotated (where the DNA bases were translated into proteins) and unannotated files were received. Both types of files were analyzed using the computer software Geneious, where a published *Synechocystis* sp. Strain PCC 6803 genome (obtained from NCBI, ref: NC017277.1) was aligned to the sequenced genomes (both annotated and unannotated) separately.

It was decided that the annotated genome was easier to interpret; therefore the two annotated genomes were searched for genes that were known to be involved in cyanobacterial motility, mainly the pili and chemotaxis genes. The differential copies of the same genes were annotated for the two genomes and analyzed.
Results

Analysis of Biofilms:

To compare the biofilm formation of the wildtype non-motile Synechocystis sp. strain PCC 6803 with the mutant motile strain, the cyanobacteria were grown separately in liquid BG-11 medium as described in the Methods. Each strain was injected into one of three chambers in the flow cell system and allowed to grow under a continuous flow of BG-11 medium in the presence of 19 µmol/m²/s fluorescent light at 25°C. The flow cells were imaged using confocal microscopy, with a He Ne 633 laser to excite chlorophyll a and phycocyanin, and the early, middle and late stages of the non-motile and motile biofilm are depicted below in Figure 1. The images in Figure 1 are a compilation of “stacks” (referred to as a Z-stack) of images of a three-dimensional section of the flow chamber from the coverslip down. These Z-stacks are meant to provide not just a two-dimensional view of the surface of the cyanobacteria growing on top of the coverslip, but also the horizontal and vertical depth to which the biofilm grew, as indicated by the horizontal and vertical panels at the bottom and right of each image. Figure 2 was illustrated to provide a better understanding of how to interpret a Z-stack image: if the three-dimensional image of a heart was optically sectioned and reconstituted into a Z-stack image, then the vertical line through the stack provides the depth at that vertical section and is shown to the right of the image (the blue vertical line corresponds with the blue box to the right of the square image). The horizontal line (in green) provides the depth at the horizontal section and the image of that section is shown at the bottom of the image (in a green box).
Figure 1. Non-motile and motile *Synechocystis* sp. strain PCC 6803 biofilms in progressive stages of growth in 3-D image stacks (“Z-stacks”) taken by a Laser Scanning Confocal Microscope. Images A, B and C depict the early, middle and late stages of the biofilm growth of non-motile cyanobacteria. Images D, E and F show the same for motile cyanobacteria. White scale bars to the bottom right of each image represent a distance of 25μm. Incident laser was 633nm.

Figure 2. Z-stack Interpretation. If the left heart image was a 3-D sample, then the Z-stack representation of the heart will look like the diagram to the right, with the blue line giving the vertical depth of that section and the green line giving the horizontal depth of the section.
It can be seen that in the initial stages (2-4 days) of non-motile cell biofilm growth (Figure 1A), the bacteria, shown in red (because of red emission of fluorescence), have all attached to the surface of the coverslip and grew as a monolayer—the entire surface of the coverslip is covered with circular bacteria, with minimal depth in the biofilm, about 15µm deep. As the biofilm growth progressed (Figure 1B) to a depth of about 20 µm, the monolayer became thicker. And finally in Figure 1C, the biofilm was so thick that it was almost impenetrable by the confocal laser.

However, the motile cell biofilm growth, even in the initial stages of its development, had no monolayer of bacteria adhering to the surface of the coverslip, but rather many groups of smaller clusters were observed—seen in Figure 1D (40µm deep). As the biofilm growth progressed, the clusters expanded in surface area and depth, so much so that they were impenetrable by the 633nm laser (Figures 1E&F). Early stage was considered 2-4 days of growth, middle stage was 6-8 days and late stage was 10-14 days.

After analyzing the images of the middle and late stages of motile biofilms (Figure1E&F), the center of the large clusters appeared to have hollow pockets. To further investigate this phenomenon, a Z-stack image was taken of a late stage motile biofilm where different depths of the biofilm were clearly shown (Figure 3). In Figure 3B and 3C, where the images show the cross-section of the biofilm deeper into the cluster, there are pockets of darkness—which could indicate a hollow cavity or dead cyanobacteria.
**Figure 3.** Motile *Synechocystis* sp. strain PCC 6803 late stage biofilm at three different depths in Z-stacks. Images A, B and C show cross-sections of the biofilm right at the coverslip, partially down from the coverslip and at an even greater depth down from the coverslip, respectively. White scale bars to the bottom right of each image represent the distance of 25μm.

To further investigate the dark cavities seen in Figures 1 and 3, a fluorescent polysaccharide stain (Concanavalin A/Alexa Fluor 488® conjugate) was used and the Ar 480nm laser was used to excite the dye. The HeNe 633 nm laser was also used to show living cells with functional chlorophyll. The stain was used on three-week old biofilms; several of those images are shown below in Figure 4. Much green was seen inside the clusters shown in Figures 4A and B, which indicates that the cavities that were seen in Figures 1 and 2 contained mostly dead cyanobacteria and/or extracellular polysaccharide (EPS). The lectin (Concanavalin A) conjugated to the dye (Alexa Fluor® 488) selectively binds to α-mannopyranosyl and α-glucopyranosyl residues in EPS.
Figure 4. Polysaccharide stain of mature *Synechocystis* sp. strain PCC 6803 biofilm. Images A and B represent three-week old biofilms that were incubated with a polysaccharide dye for 20 min before imaging. The green indicates dead cells that wouldn’t fluoresce red and extracellular polysaccharide, whereas the orange represents the live cells. Scale bars on the bottom left of each image represent 25 μm. Both Ar480 and He 633 lasers were used.

To quantify the physical differences observed in biofilms such as those documented in Figure 1, the images taken using the confocal microscope were processed using the COMSTAT program by Professor Rebecca Christianson of Olin College. COMSTAT allowed statistical comparisons of two parameters of interest—the surface area covered by the two types of biofilms and the height differences between them. The two graphs are shown below in Figures 5 and 6.

The COMSTAT analysis of surface area in Figure 5A shows that the nonmotile wildtype biofilm surface area (WT as shown in the legend and colored as black circles) was on average greater than the surface area of the motile biofilms (MOT and shown as green circles). This is consistent with the observations in Figure 1 of the non-motile biofilm covering the entire
surface of the coverslip, while the motile biofilm had only small attachments to the surface due to its tendency to cluster and grow down from the coverslip.

The average heights of the two types of biofilms were also statistically compared, as shown in Figure 5B. Maximum heights of the biofilms analyzed (up to 70 μm for motile biofilms) were graphed in Figure 6, where the green and red circles represent data points collected from motile biofilms and black circles from non-motile biofilms. The results from Figure 5B and 6 also support the conclusions made from Figure 1. The motile biofilms were higher and deeper than the non-motile biofilms, as seen by the tall clusters or pillars formed by the motile biofilm and the flat non-motile biofilms.
Figure 5. COMSTAT analysis of non-motile and motile biofilms for surface area and average height. Image A shows the COMSTAT statistical analysis of the surface area covered by the motile and the non-motile biofilms. Image B statistically compares the average heights of the motile and non-motile biofilms. Analysis done by Professor Rebecca Christianson of Olin College. MOT is motile strain and WT is the nonmotile strain.
Figure 6. COMSTAT analysis of non-motile and motile biofilms for maximum height. This shows the COMSTAT statistical analysis of the maximum heights of the motile and the non-motile biofilms. Analysis done by Professor Rebecca Christianson of Olin College. MOT data from motile biofilms and WT is data from nonmotile biofilms.

Scanning Electron Microscopy (SEM):

To determine any phenotypic differences between the motile and non-motile strains of *Synechocystis* sp. strain PCC 6803, the two strains of bacteria were sent to the University of Toledo and were prepared for scanning electron microscopy. The images taken of the surfaces of the motile and non-motile cyanobacteria are compiled in Figure 7.
Figure 7. Scanning Electron Microscopy images comparing motile and non-motile *Synechocystis* sp. strain PCC 6803. SEM images were taken of the motile strain (A and C) and non-motile strain (B and D). Image A is the motile strain (X18,000). A higher magnification image of the motile strain is shown in C (X27,000). Image B depicts the surface structure of the non-motile strain (X16,000). A more highly-magnified image of B is shown in D (X25,000). White scale bars below each image represent the distance of 1μm. Arrows indicate dividing cells.

On the left column of Figure 7 (A and C) are representative images of the mutant motile strain. The surfaces of the cyanobacteria appear smooth with many hair-like structures -- termed pili -- projecting from their surface. The right column of Figure 7 (B and D) shows a cluster of the wild-type non-motile cyanobacteria at lower and higher magnifications.
Compared to the motile cells, the non-motile bacteria had a rougher surface—however, their pili structures were retained.

An interesting feature that was observed in the SEM images is that some of the cells are undergoing cellular division, as indicated by the arrows in Figure 12A, B and C.

Phototaxis:

Positive phototaxis is the ability of an organism to move toward light. Since the mutant strain of *Synechocystis* sp. strain PCC 6803 is motile, as opposed to the wildtype non-motile strain (both shown respectively on agar plates in Figure 8), positive phototaxis studies were pursued using the motile strain.

**Figure 8. Physical differences between motile and non-motile strains of *Synechocystis* sp. strain PCC 6803.** Image A depicts the movement of the motile strain towards the light source, with the direction of the incoming light source indicated by the blue arrow. Image B shows the non-motile strain. The motile plate contained BG-11 medium with 0.8% agar and 0.3% thiosulfate and the non-motile plate contained BG-11 medium with 1.5% agar.
To characterize the motility of the motile mutant, its positive phototactic response was measured. In a preliminary trial, motile cyanobacteria were grown 10 cm away from fluorescent and incandescent light sources on plates containing BG-11 medium, 0.8% agar and 0.3% thiosulfate with approximately 24 \( \mu \text{mol/m}^2/\text{s} \) of light intensity in a 30°C room. Their distance traveled was measured daily for up to eight days. Only the fastest growing projections were measured and graphed over seven days; the results are shown below in Figure 9. It is clear that the cells moved toward the light. Table 1 quantitatively describes the calculated rates of the motile cyanobacteria under the two different light conditions for the two experimental trials. A general trend that was seen in both trials was that the cyanobacteria moved faster toward incandescent light than to fluorescent light (Figure 9). From Table 1, it can be estimated that the cells moved about 66 µm/hr to 89 µm/hr faster toward incandescent light than to fluorescent light.
Figure 9. Graphs of preliminary experiments showing distance traveled over time for two transfers of motile *Synechocystis* sp. strain PCC 6803 under fluorescent or incandescent light. Graphs A and B depict two separate experiments where motile cyanobacteria were allowed to grow under either fluorescent or incandescent light. The distance traveled was measured in mm every 24 hours, and the average of the fastest moving projections was used as one data point. n=5 for the fastest moving projections in experiment A and n=15 for experiment B. The red squares indicate the distances traveled by motile cyanobacteria towards incandescent light while the blue diamonds represent the distances traveled by cells towards fluorescent light. The error bars are standard deviations.
Table 1. Rate of movement in initial phototaxis experiments. Rates of movement were calculated over a 4.5 day period for experiment A and over a 4 day period for experiment B for each experimental light condition.

<table>
<thead>
<tr>
<th>Experiment</th>
<th>Days</th>
<th>Light Condition</th>
<th>Rate of Movement</th>
</tr>
</thead>
<tbody>
<tr>
<td>A</td>
<td>4.5</td>
<td>Incandescent</td>
<td>309 ± 41 μm/hr</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Fluorescent</td>
<td>220 ± 27 μm/hr</td>
</tr>
<tr>
<td>B</td>
<td>4</td>
<td>Incandescent</td>
<td>290 ± 47 μm/hr</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Fluorescent</td>
<td>224 ± 39 μm/hr</td>
</tr>
</tbody>
</table>

To pursue the observations made with the initial phototaxis experiments in Figure 9 and Table 1, a more comprehensive experiment was set up. The bacteria streaked on agar plates were placed in front of fluorescent or incandescent light sources at a distance that they received equal intensities of light (25 μmol/m²/s). The average distance traveled was not only just an average of the fastest moving cells but was an average of all the projections. Data under each condition were graphed over a four day period to depict linear rates, and the data are shown below in Figure 10. Figure 11 shows how the motile bacteria grown under the two light sources physically differ.
Figure 10. Graphs of distance traveled over time for six consecutive transfers of motile *Synechocystis* sp. strain PCC 6803 under fluorescent and incandescent light. Graphs A, B, C, D, E and F depict the six consecutive transfers of the motile cyanobacteria compared under fluorescent and incandescent light conditions. Distance was measured in mm every 24 hours, and every projection of the cyanobacteria depicted a data point. The number of projections used for each data point in the graphs above are summarized in Appendix 7. All of the data points for each day were averaged and graphed. The blue diamonds indicate the distances traveled by motile cyanobacteria towards incandescent light while the red squares symbolize the distance traveled towards fluorescent light in the set-up described in Methods. Error bars indicate standard deviations of the daily averaged projections of cyanobacteria.
It can be concluded from Figure 10 that the rate of movement of motile cyanobacteria was significantly faster towards incandescent light than fluorescent light, which supports the preliminary phototaxis experiment, and this result was consistent during the six transfers that they were followed through. The average distances traveled by cells at day 4 were plotted in Figure 10 are summarized in Table 2—where all of the average rates for incandescent (I) light signaling are faster than the average rates for fluorescent (F) signaling.

Table 2. Average rates of phototactic movement of motile *Synechocystis* sp. strain PCC 6803 corresponding to Figure 10. The average rates of motile cyanobacteria were calculated over 4 days and recorded below for both light conditions: incandescent (I) and fluorescent (F).

<table>
<thead>
<tr>
<th>Generation</th>
<th>Days</th>
<th>Light Condition</th>
<th>Rate</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>4</td>
<td>I</td>
<td>334 ± 39 μm/hr</td>
</tr>
<tr>
<td></td>
<td></td>
<td>F</td>
<td>249 ± 23 μm/hr</td>
</tr>
<tr>
<td>2</td>
<td>4</td>
<td>I</td>
<td>285 ± 45 μm/hr</td>
</tr>
<tr>
<td></td>
<td></td>
<td>F</td>
<td>243 ± 26 μm/hr</td>
</tr>
<tr>
<td>3</td>
<td>4</td>
<td>I</td>
<td>265 ± 44 μm/hr</td>
</tr>
<tr>
<td></td>
<td></td>
<td>F</td>
<td>172 ± 58 μm/hr</td>
</tr>
<tr>
<td>4</td>
<td>4</td>
<td>I</td>
<td>353 ± 24 μm/hr</td>
</tr>
<tr>
<td></td>
<td></td>
<td>F</td>
<td>279 ± 28 μm/hr</td>
</tr>
<tr>
<td>5</td>
<td>4</td>
<td>I</td>
<td>275 ± 45 μm/hr</td>
</tr>
<tr>
<td></td>
<td></td>
<td>F</td>
<td>236 ± 37 μm/hr</td>
</tr>
<tr>
<td>6</td>
<td>4</td>
<td>I</td>
<td>322 ± 34 μm/hr</td>
</tr>
<tr>
<td></td>
<td></td>
<td>F</td>
<td>251 ± 44 μm/hr</td>
</tr>
</tbody>
</table>

To compare the phototaxis differences visually, the bacterial front in Figure 11B moved further away from the starting black line, where it was first streaked across, than the bacterial front in Figure 11A. It was observed that the bacterial growth was also more dense in the incandescent light—the bacteria in Figure 11B were a darker green than those in Figure 11A.
Figure 11. Comparison of motile *Synechocystis* sp. strain PCC 6803 grown under fluorescent light (A) and incandescent light (B) for 3 days. Image A depicts the movement of motile cyanobacteria toward fluorescent light and image B shows the movement toward incandescent light after three days of growth at 30° C. The bacteria were streaked on the black line and received unidirectional light of 25 µmol/m²/s, as indicated by the blue arrows.

The findings presented in Figure 11 and Table 2 are shown more clearly in Figure 12 below, which depicts the average rates of phototaxis of the cyanobacteria over the six consecutive transfers (or generations). The average rate was greater in response to incandescent light than to fluorescent light consistently over the six generations. However, no general trend was seen over the six generations—for example, the average rates of movement for the motile cells did not increase or decrease with every subsequent transfer (Figure 12).
Figure 12. Average rates of phototaxis in Table 2 plotted over six generations. The rates averaged over four days in Table 2 were graphed over the six consecutive transfers (generations) and the error bars indicate the calculated uncertainties. The blue diamonds represent movement toward the incandescent light source while the red squares indicate movement toward the fluorescent light.

Since motile cyanobacteria have a faster rate of movement and growth under incandescent light conditions, the differences between the two light sources were identified. Figure 13 shows a graph of wavelength versus intensity for both incandescent and fluorescent light sources. Fluorescent light has several high intensity peaks, the most prominent being 540nm and 610nm. However, the intensity of the incandescent light covers a broader range of intensities, peaking at around 660nm.

To investigate the wavelengths that motile *Synechocystis* sp. strain PCC 6803 use to grow and to move toward, a set of wavelength-specific phototaxis experiments were done. This
time, the agar plates used were 0.8% agar with BG-11 and 0.3% sodium thiosulfate with and without 1mM glucose. Glucose is a carbon and energy source for photoheterotrophic cyanobacteria (only a few cyanobacteria, such as *Synechocystis* sp. Strain 6803, are not only photoautotrophs), so by adding glucose to some of the plates, it might be easier to make a distinction between wavelengths that signal either movement (positive phototaxis) or growth (dark green cyanobacteria). Both plates with and without glucose were streaked with motile cyanobacteria and grown for 4-7 days at room temperature in the five wavelengths highlighted in Figure 13—470nm, 505nm, 540nm, 610nm, 660nm, 700nm, 720nm, 750nm, 880nm and 1050nm. Positive controls included plates with and without glucose grown in incandescent and fluorescent light at room temperature and at 30°C, and a negative control was made by covering up the streaked glucose and non-glucose plates entirely with aluminum foil and grown under room temperature fluorescent light.
Figure 13. Graph of intensity versus wavelength for fluorescent, incandescent and halogen lamps. The fluorescent lamp (in dark blue) has its greatest intensity at around 540nm and 610nm, while the incandescent lamp has its greatest intensity peak at around 660nm (all circled in blue), but the peak is very broad. Obtained from www1.union.edu.

The plates that were placed in the different control conditions as well as various wavelengths were imaged after 4-7 days, and the results are summarized in Table 3 below.

Table 3. Wavelength-specific phototaxis experiment results. A. Positive and negative control plates with and without glucose grown under different temperatures and both light sources. B and C. Plates with and without glucose were grown under single-wavelength conditions for 4-7 days before plates were photographed and described (* denotes 4 days of growth).
### A.

<table>
<thead>
<tr>
<th>Wavelength</th>
<th>Glucose added</th>
<th>No glucose added</th>
</tr>
</thead>
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<tr>
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<td><img src="image1.png" alt="Image 1" /></td>
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<tr>
<td></td>
<td>No Movement</td>
<td>No Movement</td>
</tr>
<tr>
<td></td>
<td>Light Green</td>
<td>Light Green</td>
</tr>
<tr>
<td>Fluorescent Light Control (RT)</td>
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<td><img src="image4.png" alt="Image 4" /></td>
</tr>
<tr>
<td></td>
<td>Very Minimal Movement</td>
<td>Movement</td>
</tr>
<tr>
<td></td>
<td>Very Light Green</td>
<td>Dark Green</td>
</tr>
<tr>
<td>Fluorescent Light Control (30°C)*</td>
<td><img src="image5.png" alt="Image 5" /></td>
<td><img src="image6.png" alt="Image 6" /></td>
</tr>
<tr>
<td></td>
<td>Movement</td>
<td>Movement</td>
</tr>
<tr>
<td></td>
<td>Light Green</td>
<td>Dark Green</td>
</tr>
<tr>
<td>Incandescent Light Control (RT)*</td>
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<td><img src="image8.png" alt="Image 8" /></td>
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<td>Movement</td>
</tr>
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<td>Green</td>
</tr>
<tr>
<td>Incandescent Light Control (30°C)*</td>
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<td><img src="image10.png" alt="Image 10" /></td>
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<tr>
<td></td>
<td>Movement</td>
<td>Movement</td>
</tr>
<tr>
<td></td>
<td>Green and Yellow</td>
<td>Green</td>
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</table>

*Note: Images and descriptions are placeholders for actual images and text.*
### B.

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<tr>
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<tr>
<td>540nm</td>
<td><img src="image3.png" alt="Image" /> • Minimal movement • Green</td>
<td><img src="image4.png" alt="Image" /> • Minimal movement • Green</td>
</tr>
<tr>
<td>610nm</td>
<td><img src="image5.png" alt="Image" /> • Movement • Light green</td>
<td><img src="image6.png" alt="Image" /> • Minimal movement • Dark green</td>
</tr>
<tr>
<td>660nm</td>
<td><img src="image7.png" alt="Image" /> • Minimal movement • Yellow</td>
<td><img src="image8.png" alt="Image" /> • Movement • Dark green</td>
</tr>
<tr>
<td>880nm</td>
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<td><img src="image10.png" alt="Image" /> • No movement • Light green</td>
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</tbody>
</table>
### C.

<table>
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</tr>
</thead>
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<td><img src="image2" alt="Image" /></td>
</tr>
<tr>
<td></td>
<td>Movement</td>
<td>No Movement</td>
</tr>
<tr>
<td></td>
<td>Dark Green</td>
<td>Green</td>
</tr>
<tr>
<td></td>
<td>Dark Green</td>
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<tr>
<td>720nm</td>
<td><img src="image3" alt="Image" /></td>
<td><img src="image4" alt="Image" /></td>
</tr>
<tr>
<td></td>
<td>Movement</td>
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</tr>
<tr>
<td></td>
<td>Green</td>
<td>Green</td>
</tr>
<tr>
<td></td>
<td></td>
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<td><img src="image5" alt="Image" /></td>
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</tr>
<tr>
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<td>Movement</td>
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</tr>
<tr>
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<td>Green</td>
<td>Green</td>
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<tr>
<td></td>
<td></td>
<td></td>
</tr>
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<td>1050nm</td>
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<tr>
<td></td>
<td>No Movement</td>
<td>No Movement</td>
</tr>
<tr>
<td></td>
<td>Green</td>
<td>Green</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>470nm*</td>
<td><img src="image9" alt="Image" /></td>
<td><img src="image10" alt="Image" /></td>
</tr>
<tr>
<td></td>
<td>Minimal Movement</td>
<td>Very Minimal Movement</td>
</tr>
<tr>
<td></td>
<td>Green</td>
<td>Green</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>
The wavelength that the motile cells both grew and moved toward the best was 660nm without glucose—Table 3B shows that the bacteria moved toward the light source as indicated by the wave front being far away from the streak line and the growth was clearly evident by the dark green color of the plate. The cyanobacteria also moved toward 505nm, 610nm, 700nm, 720nm and 750nm light (Tables 3B and C), but only on plates without glucose. Tables 3B and C shows that the plates without glucose for two wavelengths had many finger-like projections toward the light source, with the exception being 720nm with a wave front. Based on these observations, it can be concluded that 505nm, 610nm, 700nm, 720nm and 750nm stimulate movement in Synechocystis sp. strain PCC 6803 while 660nm stimulates both movement and growth.

**Genome Analysis:**

To understand the genomic changes that may have enabled the mutant strain to become motile, DNA was extracted from the motile Synechocystis sp. strain PCC 6803 and was sequenced. Using the program Geneious, the sequenced fragments of the genome were aligned and compared to a published genome of Synechocystis sp. strain PCC 6803 from the National Center for Biotechnology Information (NCBI). The alignment is shown below in Figure 14. The yellow indicates matching between the sequenced fragments and the reference genome (shown in black), and the gaps between the yellow bands indicate the missing base pairs in the sequenced DNA, or no alignment between the contigs and the reference sequence.
Figure 14. Geneious alignment of fragmented motile *Synechocystis* sp. strain PCC 6803 genome to published NCBI genome (3.57Mb). The numbers on top act as markers for the numbers of base pairs there are in the entire genome. The yellow band in the center of the image represents the contigs that have been aligned to the *Synechocystis* sp. strain PCC 6803 NCBI Reference Sequence NC_017277.1 and the black band on the very bottom of the image is the reference sequence itself.

In order to ensure that the base pair gaps identified in Figure 14 were consistent with other published strains of *Synechocystis* sp. strain PCC 6803, Geneious was used to align the sequenced fragments from the motile strain to two other NCBI reference sequences. Figure 15 shows those results, with the blue squares highlighting the gaps that exist and match among all three genomes. The percentages in red to the left of each Geneious alignment indicate the percentage of base pairs that aligned between the motile mutant and each reference genome (97.4%, 99.4% and 99.4%, respectively).

Figure 15. Geneious alignment of fragmented motile *Synechocystis* sp. strain PCC 6803 genome against three different published NCBI genomes. The blue rectangles represent the gaps that are consistent when fragments are aligned across the three NCBI Reference Sequences for *Synechocystis* sp. strain PCC 6803 (NC_017277.1, NC_017052.1 and NC_017039.1 respectively).
However, the process of genome sequencing is prone to errors, so to ensure that the gaps identified are real genomic changes between the motile and non-motile strains, the DNAs of both the wild type non-motile and the mutant strains of bacteria were extracted. Using a nanodrop detector, it was determined that the concentrations of the extractions were 31.5ng/μL non-motile and 28.1ng/μL motile DNA. Both DNAs were sequenced, and their fragments were aligned to the same reference genome (NC_017277.1), as shown in Figure 16.

![Figure 16. Geneious alignment of fragmented motile and non-motile Synechocystis sp. strain PCC 6803 genome against NCBI Reference Sequence NC_017277.1. This is a side-by-side comparison of the two sequenced genomes to the same reference genome (black line), with the sequenced non-motile strain on top (yellow fragments) and the motile strain on the bottom (yellow fragments).]

To investigate the causes for the motility differences between the two genomes, a bioinformatics search was performed on the annotated proteins in the two sequences. The queries used in the bioinformatics search included “motility”, “pili” and “chemotaxis”. “SigF” was also searched since it has been implicated in motility in Synechocystis (Bhaya et al., 1999). These features were chosen due to previous studies that have linked pili and chemotaxis genes to cyanobacterial motility. The copy numbers of these genes were recorded for both strains of cells and are summarized in Table 4 below. Of all of the genes found related to the queries, only four proteins were found whose copy numbers differed between the mutant and wildtype
Synechocystis—Twitching motility protein PilT, Cyanobacterial SigF-related sigma factor CDS, Methyl accepting chemotaxis protein II and Chemotaxis protein (all highlighted in blue). It is interesting to note that (except for SigF) the copy numbers of these proteins are all greater in the motile mutant, and for the latter two proteins mentioned, no copies of those proteins were found in the wildtype genome.

Table 4. The motility and chemotaxis proteins found in the sequenced genomes of the motile and non-motile strains, along with their copy numbers. Motility and chemotaxis protein copies were searched for on the annotated genomes of the two strains of cyanobacteria using the program Geneious and the copy numbers were compiled below. The proteins that had a difference in copy numbers in the two strains are highlighted in blue.

<table>
<thead>
<tr>
<th>Category</th>
<th>Protein Name</th>
<th>Copies in Non-Motile</th>
<th>Copies in Motile</th>
</tr>
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<tr>
<td>Motility related Pili Proteins</td>
<td>Type IV pilin PilA</td>
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<tr>
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<td>Twitching motility protein PilT</td>
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<td>PilT protein domain protein</td>
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<td>Gliding motility protein Glf CDS</td>
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Discussion

The phenotypic and genomic differences between mutant motile and wildtype non-motile *Synechocystis* sp. strain PCC 6803 were characterized using confocal microscopy of biofilms growing in flow chambers, scanning electron microscopy and genome alignment and analysis. To further characterize the motile strain, its positive phototactic movement was studied.

Analysis of Biofilms:

Bacterial biofilms are how aggregates of bacteria exist in nature (Stewart and Costerton, 2001). To take advantage of their ability to adhere to numerous surfaces, biofilms of motile and non-motile cyanobacteria were grown on coverslips attached to a flow chamber setup. The biofilm imaging results shown in Figure 1 provided a clear contrast between the two types of biofilms formed in the two strains—with the motile strain forming clusters and pillars, while the non-motile strain formed uniform biofilm monolayers. Monolayers and pillars are commonly seen structures in bacterial biofilms (Shrout et al., 2006). Few published studies on cyanobacterial biofilms in the laboratory (Schatz et al., 2012) appear to have been done other than our study, so comparisons with other cyanobacterial biofilms cannot be made. Most of the studies done on cyanobacterial biofilms have been environmental studies, such as investigating stromatolites (Bosak et al., 2009).

However, the present results are different from those found in the studies done on the role of motility in biofilm formation of *Pseudomonas aeruginosa*, one of the most studied bacterial strains. It was found that conditions that favor surface motility of *P. aeruginosa* actually lead to the formation of uniformly flat biofilms (Parsek and Tolker-Nielsen, 2008), much
like the non-motile cyanobacterial biofilms observed in the present study. The *P. aeruginosa* biofilms characterized by clustered aggregates were found to mostly consist of non-motile bacteria (Parsek and Tolker-Nielsen, 2008).

A study performed by Klausen et. al (2003) compared the wildtype (motile) and motility mutants (inactivated pili genes, such as ΔpilA) of *P. aeruginosa* in a similar set-up as described here, using flow chambers, acquiring CLSM micrographs of the biofilm development and also using COMSTAT image analysis. They found that the motile wildtype cells formed a “flat carpet” similar to the monolayer that was seen in the present study with the non-motile cyanobacterial biofilms, and the non-motile *P. aeruginosa* formed a “hilly biofilm” with large dense clusters (Klausen et al., 2003) reminiscent of the motile cyanobacterial biofilms.

An explanation proposed for the phenomena of cluster biofilms, when compared to monolayer biofilms in *P. aeruginosa*, was that the monolayer biofilm is due to surface-associated motility of the motile strain, while the clusters in the non-motile biofilms are caused by the lack of movement and clonal growth on the substrate (Shrout et al., 2006). However, this interpretation seems to be the opposite for cyanobacterial biofilms. The biofilm images that were taken of the motile and non-motile *Synechocystis* seem to indicate that the non-motile cyanobacteria all settled on the surface of the coverslip and then grew in depth, while the motile strain had the ability to move and cluster together to form pillars that protrude from the coverslip where they first attached.

The conclusions drawn from Figures 3 and 4 in the Results indicate that there are hollow cavities containing dead cells and/or EPS within the clusters of very late stage biofilms. The
appearance of channels in biofilms is a common phenomenon in other bacterial biofilms and these structures are thought to provide the biofilm with a transport system—funneling water, nutrients and planktonic bacteria throughout the community (Richards and Melander, 2009).

To better understand the differences in initial attachment, future studies on imaging could be done on the flow cell a few hours after inoculation, and instead of imaging only the early, middle and late stages of biofilm formation, a daily snapshot could be taken to better understand the biofilm development. The EPS stain could also be employed during the initial, middle and late stages of biofilm growth as well to determine when the cavities within the clusters begin to form.

**Scanning Electron Microscopy (SEM):**

SEM images were taken of the motile and non-motile *Synechocystis* sp. strain PCC 6803 to visualize any phenotypic differences between the two types of outer surfaces, as shown in Figure 12. There was a discernible physical difference between the two strains—the motile strain seems to be much smoother than the non-motile strain, which has a very coarse surface. However, both strains were similar in the number of pili projecting from their surfaces. This was unexpected since experiments have shown that when a gene for an alternative sigma factor, *sigF*, was disrupted, the transcription of two *pilA* genes (which help encode Type IV pili) was affected and the number of pili on the surface of cyanobacteria was drastically reduced and the cells’ motility was abolished (Bhya et al., 1999). Since the wildtype cyanobacteria were non-motile, it was hypothesized that there might be fewer pili on their surface compared to the motile mutant that might have many more surface appendages.
As seen in Figure 7, both strains appear to be covered with comparable numbers of pili. The only discernible phenotypic difference lies in the roughness of the surface of the two strains. One hypothesis is that the smoothness of the cell surface of the motile strain, when compared to the non-motile strain, gives cells an aerodynamic advantage that enables them to move more easily.

To further explore the surface structures of the motile and non-motile cyanobacteria, transmission electron microscopy (TEM) images could be acquired of sectioned cyanobacteria and negatively stained cyanobacteria. This technique would allow even higher magnification so that a more detailed and magnified image of surface structure and pili could be obtained. From our SEM images, it is difficult to distinguish what types of pili the surface appendages are—they could be thick pili (important for motility), thin pili (not involved in motility), or a combination of both (Bhaya et al., 2002). Perhaps in TEM images, there might be more thick pili observed on the motile cell surface than on the non-motile cyanobacteria surface, which would support the hypothesis that the wildtype *Synechocystis* gained thick pili when it became motile.

**Phototaxis:**

Positive phototaxis, or the movement towards light, was observed in the motile *Synechocystis* sp. strain PCC 6803. Cyanobacteria are photoautotrophs and their growth rates can be reduced at low light intensities (Bhaya, 2004). However, extremely intensive light can also cause photodamage, so some species of cyanobacteria can physically orient themselves towards or away from light, termed positive or negative phototaxis, respectively (Bhaya, 2004). There are many types of motility that have been characterized in cyanobacteria, the most well-known are gliding, swimming and twitching motility (Sarma, 2013). *Synechocystis* sp. strain PCC
6803 has been shown to move via twitching motility, with the aid of surface structures termed pili (Bhya et al., 2000). Pili can be subdivided into two types—thin and thick (Bhya, 2004). Thin pili are more rigid and have an average diameter of 3-4 nm and about a length of 1 μm, whereas thick pili, which are more flexible, have an average diameter of 6-8 nm and about 4-5 μm long (Bhya, 2004). It has been shown that the thick pili, also termed Type IV pili, are linked to the surface-dependent twitching motility (Bhya et al., 2000). Type IV pili are primarily composed of the protein pilin or PilA, and their biogenesis involves several other pili genes such as pilD, pilT and pilC (Bhya et al., 2000).

Chemotaxis is the physical response of bacteria towards nutrient gradients and has been best characterized in *Escherichia coli* (Partridge and Harshey, 2013). The bacterial chemotaxis response has been divided into three steps: the first step is signal perception by the chemoreceptors located on the membranes of the bacteria, the second is signal transduction of the signal from the receptors to the motor of the flagella, and the third is the “adaptation of the signal to desensitize the initial signal output” (Sarma, 2013). Many genes in its signal transduction pathway, such as the *che* genes, have been linked to phototaxis, (Yoshihara et al., 2000).

It was determined in the present experiments that there was a greater phototactic response to incandescent light than to fluorescent light at the same light intensity of 25 μmol/m²/s (Figures 9-12, and Tables 1 and 2). This was unexpected due to the fact that *Synechocystis* sp. strain PCC 6803, and cyanobacteria in general, are typically cultured in the presence of fluorescent light. However, one study did cite the use of incandescent light where a
light intensity at 70 μmol/m²/s was used to grow the *Synechocystis*, and greater intensities, in
the range of 250 μmol/m²/s to 2000 μmol/m²/s, were used to induce photodamage
(Allakhverdiev and Murata, 2004).

In the present results, the average speeds of movement of the motile cells were
between 150 μm/hr and 350 μm/hr (Tables 1-2). This seems to be incredibly fast considering
that the diameter of the cells is only around 1 μm. *P. aeruginosa* also exhibits twitching
motility; a study measured its rate of movement to be approximately 0.6mm/hr, which
translates to 600 μm/hr (Semmler et al., 1999). This rate is of the same magnitude as the rate
of the motile cyanobacteria in this study.

Due to the different intensity spectra of incandescent and fluorescent light as
highlighted in Figure 13, it was suspected that the motile cyanobacteria might respond
phototactically to specific wavelengths. A wavelength-specific phototaxis experiment was
conducted to isolate the wavelengths that cyanobacteria respond to for both movement and
growth. Growth and movement were separated using both glucose and non-glucose plates,
with the theory that for plates with glucose, there is less incentive for the cyanobacteria to
move toward light to use photosynthesis to produce their own carbon and energy sources, so
their movement indicates a greater response to light signaling. The glucose would provide
carbon and energy for growth.

The results in Table 3 from the different wavelengths used suggest 505nm, 610nm,
700nm, 720nm and 750nm wavelengths of light signal movement, whereas 660nm is optimal
for both movement and growth. The glucose-grown cells in 505nm, 610nm, 700nm, 720nm and
750nm all moved towards the light source, as evident by their finger-like projections and wave fronts pointing towards the light source (Tables 3B and C in results). If they moved towards the light even though they have their nutrition source in the medium, then it suggests that they move purely in response to those two wavelengths. The cells on the plate in the 660nm wavelength light without glucose both moved toward the light and had very dark growth, which means that that wavelength was conducive to both phototaxis and growth.

Comparing the absorbance spectrum of *Synechocystis* sp. strain PCC 6803 to the wavelengths used in the experiment, it makes sense that 660nm wavelength light might be the optimal wavelength for growth and movement because there is a very broad absorption maximum around 660nm (Figure 1 below). This wavelength is where both phycocyanin and chlorophyll a absorb (610nm, 700nm and 720nm also correspond to these photopigments and exhibited movement). The phototaxis data showing *Synechocystis* sp. Strain 6803 moving faster and growing better under incandescent light conditions can also be explained since 660nm is where intensity peaks in the incandescent light spectrum (Figure 1). The minimal growth and movement of cells at 540nm, 880nm and 1050nm wavelength is also supported by the figure below because no photosynthetic pigments can absorb at 880nm. That wavelength of 505nm which supports phototaxis corresponds to another broad peak of photopigments, which could explain the fingerlike projections seen in Table 3 of Results, however, it was unexpected that 470nm which also corresponded to the peak of those pigments induced no movement. The observation that a wavelength of 540nm has minimal effects on movement and growth can be interpreted by the fact that the corresponding wavelength in the absorption spectrum is where photopigment absorption is at a minimum (Figure 1 below).
Future follow-up experiments would include testing more wavelengths to find more wavelengths that induce either movement or growth. Also, the experiments described in Table 3 did not use a consistent light intensity and varied between 4 and 7 days of growth, so in
future experiments, the light intensities of all of the wavelengths and temperatures should be better controlled and the growing time will also be consistent.

**Genome Analysis:**

In order to determine any changes in the genome that could have occurred when the wildtype non-motile *Synechocystis* sp. strain PCC 6803 became motile, the DNA of the motile strain was extracted and sequenced. When this sequenced DNA was compared to published wildtype strains’ DNA, there were very obvious “gaps” or deletions that were seen, visualized in Figures 14-16. To ensure that those deletions were not just due to unsequenced DNA fragments caused by errors in sequencing technology (as well as to be sure that the comparisons were between the actual two strains studied in this thesis), DNA was extracted from motile and wildtype cyanobacteria and sequenced at the same time.

The genome fragments of the sequenced motile and non-motile strains were both aligned to the same reference NCBI genome of *Synechocystis* sp. strain PCC 6803, and the genes known to be involved in motility were characterized by searching for their protein products in the annotated genomes of the two strains. The proteins that were focused on were the pili proteins, more specifically, Type IV pili proteins (such as PilA, PilD and pilT) as well as chemotaxis proteins—including CheY, CheA and CheD (Yoshihara et al., 2000, Bhaya et al., 2002). These pili genes had previously been implicated in the motility of *Synechocystis* and the chemotaxis genes are homologous to the che genes involved in flagellar switching for bacterial chemotaxis in other species of bacteria (Yoshihara et al., 2000, Bhaya, 2004).

There were four proteins found in the search that had different numbers of copies in the motile and non-motile strains. These are highlighted in blue in Table 4—two of the genes
were related to the function and formation of Type IV pili, while the other two were chemotaxis genes. Twitching motility protein PilT is a highly conserved motor protein that is required for the retraction of Type IV pili (Bhaya, 2004), and there were two copies of this protein in the motile strain and only one in the non-motile strain. Studies with pilT mutants have shown that the mutants are non-motile and strongly hyperpiliated, both of which support the function of PilT in Type IV pili retraction mechanisms (Bhaya, 2004). SigF is an alternative sigma factor which is known to have an effect on the transcription of pilA genes-- sigF mutants reduced PilA levels, and PilA makes up a large structural component of Type IV pili; when PilA is reduced, phototactic movement is eliminated (Bhaya et al., 1999).

The two chemotaxis-related proteins had one copy in the motile strain and none in the wildtype. Methyl accepting chemotaxis protein II (MCP) is a protein that plays an important role in the chemotaxis signal perception cascade—MCPs are stimulated by specific ligands and pass information to downstream signaling proteins in the cytoplasm. The signaling proteins in turn signal the bacteria (in this case Escherichia coli) to either “tumble” or “swim” in response to gradients of nutrients (Derr et al., 2006). Chemotaxis protein, also known as CheY, is more downstream than the MCP in the cascade. It transmits sensory signals from the chemoreceptors to the flagellar motors in E. coli (Paul et al., 2010).

It is likely that the loss of two important chemotaxis proteins in the wildtype and the presence of only one copy of pilT in the wildtype strain of Synechocystis would contribute to its non-motile phenotype. But the presence of SigF in the non-motile strain, and its absence in the motile strain, is unexpected because a deletion of SigF would imply the loss of phototaxis. This
gene discrepancy could be due to a sequencing error. The suppositions made from the variable copy numbers of the other genes aren’t conclusive because it is possible that during the DNA sequencing process, those genes in the wildtype cyanobacteria were not sequenced as well. Further experiments should be done, such as knocking out these genes in the motile mutant to see if the strain becomes non-motile or re-sequencing the genomes.

Another important consideration when comparing the genomes of bacteria in general is that bacteria have the ability to exchange genetic information via conjugation, transformation or transduction. Studies have shown that the rate of conjugation for bacteria within biofilms occurs at rates of up to 1000-fold higher than in planktonic cells (Richards and Melander, 2009). This transfer of genes also complicates genomic analyses of bacteria.

Further studies in the future could include re-extracting the DNAs of both strains and having it sequenced again, and having the same bioinformatics search performed and analyzed.

Conclusion:

Based on the physical and genomic differences found in these current studies between the wildtype Synechocystis sp. strain PCC 6803 and the motile mutant derived from this strain, it can be concluded that the two strains are both phenotypically and genotypically different. These two strains form significantly different biofilms, have different cell surfaces and vary in the copy numbers of specific proteins important for motility. This new motile strain also responds to light signaling like other motile cyanobacteria strains, and the optimal wavelength that it responds to was tentatively found to be 660 nm.
References


Appendix

1. Preparing standard BG-11 medium
The standard BG-11 liquid medium concentration included 1.5 g/L NaNO3, 0.078 g/L K2HPO4, 0.02 g/L Na2CO3, 0.058 g/L Na2SiO3•9 H2O, 0.075 g/L MgSO4•7 H2O, 0.035 g/L CaCl2•2 H2O, 1.0 mg/L diNa EDTA, 6.0 mg/L Fe citrate-citric acid, and 1.0 mL trace elements as described: Trace elements: 2.86 mg/L H3BO3, 1.8 mg/L MnCl2•4 H2O, 0.22 mg/L ZnSO4•7 H2O, 0.39 mg/L NaMoO4•2 H2O, 0.08 mg/L CuSO4•5 H2O, and 0.05 mg/L Co(NO3)•6 H2O.

2. Confocal Microscopy of Cyanobacterial Biofilms
Confocal data were taken using a Leica SP5 RS AOBS laser-scanning confocal microscope. The line scan operates at a fixed rate of 8000 lines/second unidirectionally, 16000 bidirectionally. The objective was a 63x, 1.3NA glycerine immersion objective. Digital data was collected at 512x512 pixel resolution with a typical scale of 0.239 microns/pixel in-plane and a resolution-optimized spacing in the z direction of 0.125 microns/slice. Fluorescence of healthy cyanobacteria was excited with a 633nm Helium-Neon laser. Emission was detected typically from around 643nm up to around 765. When dead or unhealthy, the spectrum of the cyanobacteria changed, reducing the emission in the red, and increasing the response of the cells to a 488nm excitation with emission spectra in the 550nm range. The number of images in a stack depended on the height of the biofilm under study, but strong absorption of the excitation light limited our ability to consistently image the biofilm bulk deeper than 30-50 microns without adopting some form of linear compensation, either through increasing the laser intensity or increasing the photomultiplier tube gain (Rebecca Christianson).

Fixation:

0.5 mL of 2.5% glutaraldehyde in sodium cacodylate buffer solution was added to a mixture of bacteria in Phosphate Buffer Saline (PBS) solution in a 1 mL plastic centrifuge tube. The sample tubes were stored in a refrigerator overnight. The mixture then was washed twice with PBS solution.

Dehydration:

The samples were dehydrated in the ascending series of ethyl alcohol (ETOH)/deionized water mixture at the ETOH concentration of 25%, 50%, 75%, and 100%. They were chemically dried with 50% ETOH/Hexamethyl disilazane (HMDS) and 100% HMDS.

In each step of the washing and dehydrating, the sample suspension was centrifuged in the Fisher Scientific Micro Centrifuge model 59A at speed #7 for 5 min. The solvent was discarded. The dehydrating mixture was added to the pellet in the tube. The mixture was mixed in the Scientific Products Deluxe mixer model S8220. The process was repeated to the 100% HMDS.
The suspension of a sample in HMDS was dropped directly on to an aluminum stub, air dried, and sputtered coated with gold for the SEM observation (S.C.O.P.E. Program at University of Toledo).

4. Determining the best agar concentrations for phototaxis experiments.

![Incandescent light source](image)

1.5%agar+1mM thiosulfate (19 days)  
0.8%agar+0.3% thiosulfate (19 days)

A comparison of phototaxis between BG-11 medium containing 1.5% agar with 1mM thiosulfate with 0.8% agar containing 0.3% thiosulfate grown in front of incandescent light. There was a greater movement observed in the 0.8% agar containing 0.3% thiosulfate.

5. Experienced User Protocol for DNA isolation (“Powerlyzer UltraClean Microbial DNA Isolation Kit” from MoBio Laboratories)

Please wear certified RNase-Free gloves at all times.

1. Properly identify each Glass MicroBead Tube on both the cap and on the side; See “Important Notes For Use” for more information.
2. Add 1.8 ml of microbial (bacteria, yeast) culture to a 2 ml Collection Tube (provided) and centrifuge at 10,000 x g for 30 seconds at room temperature. Decant the supernatant and spin the tubes at 10,000 x g for 30 seconds at room temperature and completely remove the media supernatant with a pipette tip.

Note: Based on the type of microbial culture, it may be necessary to centrifuge longer than 30 seconds.
3. Resuspend the cell pellet in 300 µl of MicroBead Solution and gently vortex to mix. Transfer resuspended cells to a PowerLyzer™ Glass MicroBead Tube, 0.1 mm.
4. Check Solution MD1. If Solution MD1 is precipitated, heat the solution at 60°C until the precipitate has dissolved. Add 50 µl of Solution MD1 to the Glass MicroBead Tube.
Optional: To increase yields, to minimize DNA shearing, or for difficult cells, see Alternative Lysis Methods in the “Hints & Troubleshooting Guide” section before continuing.

5. Homogenization options:

A. PowerLyzer™ 24 homogenizer: Place the PowerLyzer™ Glass MicroBead Tubes onto the Tube Holder for the PowerLyzer™ 24. The Glass MicroBead Tubes must be balanced (evenly spaced) on the Tube Holder. Homogenize for 5 minutes at 2000 RPM. **Note:** Depending on your sample less time at a higher speed may be used.

B. Vortex: Secure PowerLyzer™ Glass MicroBead Tubes horizontally using the MO BIO Vortex Adapter tube holder for the vortex (MO BIO Catalog# 13000-V1) or secure tubes horizontally on a flat-bed vortex pad with tape. Vortex at maximum speed for 10 minutes. (See “Hints & Troubleshooting Guide” for less DNA shearing).

6. Make sure the Glass MicroBead Tubes rotate freely in the centrifuge without rubbing. Centrifuge the tubes at 10,000 x g for 30 seconds at room temperature. **CAUTION:** Be sure not to exceed 10,000 x g or tubes may break.

7. Transfer the supernatant to a clean 2 ml Collection Tube (provided).

**Note:** Expect 300 to 350 μl of supernatant.

8. Add 100 μl of Solution MD2, to the supernatant. Vortex for 5 seconds. Then incubate at 4°C for 5 minutes.

9. Centrifuge the Tubes at room temperature for 1 minute at 10,000 x g.

10. Avoiding the pellet, transfer the entire volume of supernatant to a clean 2 ml Collection Tube (provided). Expect approximately 450 μl in volume.

**Note:** A small carryover of glass beads is possible. This will not affect the results.

11. Shake to mix Solution MD3 before use. Add 900 μl of Solution MD3 to the supernatant and vortex for 5 seconds.

12. Load about 700 μl into the Spin Filter and centrifuge at 10,000 x g for 30 seconds at room temperature. Discard the flow through, add the remaining supernatant to the Spin Filter, and centrifuge at 10,000 x g for 30 seconds at room temperature.

**Note:** A total of 2 to 3 loads for each sample processed are required. Discard all flow through liquid.

**High Throughput Option:** Step 12 can become tedious when many samples need to be processed. For this reason, MO BIO has developed a vacuum protocol. It does require the purchase of our aluminum Spin Filter Adapters (catalog # 11992-10) which will allow you to fit our flat bottom spin filters on to any vacuum manifold with Luer lock fittings. Please read **Vacuum Protocol using the PowerVac™ Manifold** on page 13.

13. Add 300 μl of Solution MD4 and centrifuge at room temperature for 30 seconds at 10,000 x g.

14. Discard the flow through.

15. Centrifuge at room temperature for 1 minute at 10,000 x g.

16. Being careful not to splash liquid on the spin filter basket, place Spin Filter in a new 2 ml Collection Tube (provided).

17. Add 50 μl of Solution MD5 to the center of the white filter membrane.

18. Centrifuge at room temperature for 30 seconds at 10,000 x g.
19. Discard **Spin Filter column**. The DNA in the tube is now ready for any downstream application.
No further steps are required.
We recommend storing DNA frozen (-20°C). **Solution MD5** contains no EDTA.

6. Representative confocal Z-stack images of motile and non-motile *Synechocystis* sp. strain PCC6803 biofilms.

Image A depicts the middle stage of a non-motile biofilm and B represents the late stage of a non-motile biofilm. The white scale bars represent 25μm.

Images A, B and C depict the initial, middle and late stages of the motile biofilm development. The white scale bars represent 25μm.
7. Table of the number of projections measured for each data point depicted in Figure 10 of Results.

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