Signaling Mechanisms and Physical Structure of Biofilm Growth in Synechocystis sp. Strain PCC 6803

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Signaling Mechanisms and Physical Structure of Biofilm Growth in *Synechocystis* sp. Strain PCC 6803

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Submitted in Partial Fulfillment of the Prerequisite for Honors in Biological Chemistry

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# Table of Contents

Table of Contents  
Acknowledgements  
Abstract  
Introduction  
Materials and Methods  
Results  
Discussion  
References  

## Appendices

A - Biofilm growth in flow cells using *Synechocystis* sp. Strain PCC 6803
B - Biological sample preparation for scanning electron microscopy observation
C - Additional growth curve replicates of *C. violaceum* CV026 indicator
D - Additional confocal microscopy images of motile *Synechocystis* biofilms
E - Additional scanning electron microscopy images of *Synechocystis* cells
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Abstract

Bacterial cells use a molecular signaling mechanism known as quorum sensing in order to communicate with each other. Quorum sensing has been found in the past to play a central role in bacterial biofilm formation, and may be important for the cyanobacterium *Synechocystis* sp. Strain PCC 6803. Biofilms are cell aggregates which form on any available surface, including in nature and within the human body. In this investigation, wild type *Synechocystis* cells were observed to form a very uniform monolayer biofilm while a motile isolate of this species forms biofilm clusters which grow wider and deeper over time. In order to determine whether the communication system of this species involves acyl homoserine lactone (AHL) signaling molecules, the biosensor, *Chromobacterium violaceum* CV026, which produces the violacein pigment in the presence of AHLs, was used. The use of AHL standards demonstrated that there is a logarithmic correlation between the concentration of AHLs and the width of the detection, observed as purple pigmentation produced by the indicator species, and that this indicator species can recognize AHLs with chains of four to eight carbons. It was determined that the motile *Synechocystis* cells growing in liquid culture, a biofilm flow cell, or on solid medium do not appear to be producing an AHL, at least in a high enough concentration to be recognized by this indicator. AHL standards were found to be stable over time in pre-acidified effluent from the flow cells, pre-acidified BG-11 medium, and in pre-acidified growing *Synechocystis* cells. It was also observed that the *Synechocystis* cells respond positively to Octanoyl AHL and negatively to Hexanoyl AHL, but do not respond to 3-Oxodecanoyl AHL. Analysis of the *Synechocystis* genome identified possible quorum sensing and quorum quenching genes, which could account for the novel phenotype of the motile cells. In conclusion, this investigation determined that the motile *Synechocystis* cells form distinct cluster biofilms and that there is potential for a quorum sensing system, but AHLs were not yet identified as being produced by the *Synechocystis*. 
Introduction

Biofilms

Biofilm formation and significance

Biofilms are complex aggregates of bacterial cells attached to a surface and enclosed in a polymer matrix (Donlan and Costerton, 2002). They are ubiquitous in nature and it is thought that 99% of bacterial species regularly form biofilms (Costerton et al., 1987). The oldest fossilized biofilms actually date back 3.3-3.4 billion years ago and were found in hydrothermal environments (Westall et al., 2001). This indicates their evolutionary importance in bacterial survival. The formation of a biofilm is divided into specific stages (Figure 1). First, cells must irreversibly lose their motility and bind to a surface. The cells communicate their presence to the environment, which encourages more cells to bind. The biofilm grows as additional cells bind and the existing cells replicate. The cells also begin to secrete exopolysaccharides (EPS) to create the surrounding matrix. At this point, the biofilm is considered mature and complete. Eventually, the cells will stop producing the EPS matrix or the nutrient levels will decrease, some cells will begin to be released from the biofilm, and the entire film will detach (Prakash et al., 2003). The process is a cycle, as new biofilms form and detach regularly.

Figure 1 Schematic of biofilm formation (Costerton et al., 1999).
The main requirements for biofilm formation are the presence of a surface and appropriate nutrients (Donlan and Costerton, 2002). Almost any surface, whether rough or smooth and ranging from teeth to river stones, is usable (Prakash et al., 2003). Biofilms are actually stronger under high shear environments, such as in flowing water or in heart valves (Donlan and Costerton, 2002). The nutrients may be from trace organics on the surface or from shared resources and waste products from the other cells present (Prakash et al., 2003).

The EPS matrix produced by the cells is composed primarily of various polysaccharides (Prakash et al., 2003). However, *Vibrio cholera* biofilm matrix was found to include the proteins RbmA and Bap1, which recruit new cells and strengthen intercellular attachments respectively, while *Clostridium difficile* biofilms contain proteins, DNA and polysaccharides (Absalon et al., 2011; Dapa et al., 2013).

Biofilms form as a survival mechanism for cells. In terms of total biomass, variety of habitats and phenotypic adaptability in the environment, biofilms are the most successful form of life on the planet (Brown and Williams, 1985). Cells are more able to survive in hostile environments and can take advantage of neighboring cells, which are likely of a different species. They can share resources, or use the waste products of other cells. Many biofilms contain different zones of nutrient concentrations, including light, oxygen and signaling molecules, as well as canals through which nutrients and water can circulate (Prakash et al., 2003). Biofilms also allow for altered physiological traits such as slower growth rate and differential gene expression.

Study of biofilms is important in part because of their ubiquity in pathogenic infections of the human body. Biofilms are associated with many specific infectious diseases. The classic example is *Pseudomonas aeruginosa* infections in the lungs of cystic fibrosis patients (de Kievit
and Iglewski, 2000). Biofilms also readily form on medical devices such as urinary catheters and prosthetic heart valves, which provide a perfect surface for the infection (Donlan and Costerton, 2002). Often the only way to treat biofilm associated infections is to remove the prosthetic device, as such infections are particularly resistant to antimicrobial agents and thus very difficult to treat. Biofilms formed by \textit{C. difficile} in the human intestine, for example, have been shown to be more resistant than planktonic cells to the antibiotic vancomycin (Dapa et al., 2013). The thick EPS matrix of the cells may inhibit penetration of the antimicrobial agent, the decreased growth rate of biofilms may reduce the uptake of the agent, and reactive oxygen species present in the biofilm may degrade antibiotics (Donlan and Costerton, 2002; Prakash et al., 2003). In addition, biofilms provide the perfect environment for increased horizontal gene transfer, including the exchange of drug resistant plasmids (Prakash et al., 2003). The environment of the biofilm provides minimal shear and closer cell contact, which allows for enhanced conjugation (Prakash et al., 2003). In biofilms with more than one bacterial species, a diversity of genes can be exchanged as well. One study definitively showed the lateral gene transfer of Shiga toxin encoding genes to \textit{Escherichia coli} by transduction in a biofilm (Solheim et al., 2013).

Understanding the mechanisms by which biofilms form could be applied to the treatment of biofilm infections in the future.

\textit{Methods to visualize biofilms}

Our understanding of biofilm structure has been drastically improved through the use of different forms of microscopy. Initially, the main method of visualization was scanning electron microscopy, but the required dehydration of the sample resulted in distorted structures (Donlan and Costerton, 2002). Transmission electron microscopy allows for more in depth study of the extracellular matrix, with the use of specific polysaccharide stains (Donlan and Costerton, 2002).
More recently, confocal laser scanning microscopy has improved visualization of biofilms *in situ*. This method allows for study of the three dimensional structure of the biofilm in its natural form, and has been used very successfully for *V. cholera* biofilms (Berk et al., 2012). Although this method requires the use of fluorescent stains, it is particularly useful for cyanobacteria that are naturally fluorescent due to the energy transfer chain of the phycobiloproteins and chlorophyll in the cells (Stanier and Cohen-Bazire, 1977).

One method of visualizing biofilms is through the use of flow cells. A flow cell includes continuous media to be pumped through chambers attached to a glass coverslip, on which the biofilms form (Parsek and Greenberg, 1999). This method of observation of biofilms was found to be reproducible, and thus well controlled (Heydorn et al., 2000). One investigation of cyanobacterial biofilm growth in a flow cell showed an initial thin film on the slides, which spread across the entire surface after 30-33 days, and differences in growth and lipid production depending on the environmental conditions (Bruno et al., 2012).

*Cyanobacterial biofilms*

Cyanobacterial biofilms are very common in a variety of environments in nature. One of the classic examples of cyanobacterial biofilms is in stromatolites, which are layered sediment macrostructures formed through the interactions of bacteria and geochemical processes (Figure 2; Decho, 2000). Fossil stromatolites are the oldest known macroscopic evidence of life on earth and have been in existence for over 2.5 billion years (Decho, 2000). It is thought that cyanobacterial production of oxygen actually accounts for the contorted laminae and fossilized bubbles found on the surface of many stromatolites (Bosak et al., 2009). Stromatolites in the Bahamas include diverse communities of bacteria, which include cyanobacteria, whose biofilms secrete much of the EPS that stabilize the structure (Decho, 2000).
Cyanobacterial blooms in intertidal systems are also often considered biofilms. These blooms occur when an abundance of nutrients becomes present; the cells overproduce and the biofilm-like mats become very dense and are deposited (Decho, 2000). These blooms can be dangerous if the water source is used for swimming or drinking, meaning that the cells or their byproducts could come in contact with people or even be ingested. Cyanobacterial biofilms appear to be very stable, as evidenced by two examples found on granite in Antarctica; one biofilm was a layer of cell clusters while the other was a much more dense matrix of cells and polysaccharides (de Los Rios et al., 2007). Another mixed cyanobacterial biofilm in Antarctica was found to be a 5mm thick uniform mat with several distinct layers (Davey and Clarke, 1992). Cyanobacterial biofilms have also been detected on man-made structures. Mixed biofilms formed on ancient Mayan buildings, which are being degraded by cyanobacterial species including *Synechocystis*, on stone surfaces in Roman catacombs, and on granite monuments in Serbia, which contain five cyanobacteria taxa that are degrading the stone (Ortega-Morales et al., 2000; Albertano et al., 2000; Grbic et al., 2010).
One investigation of cyanobacterial biofilms in the laboratory looked at the effect of light and temperature on biofilm structure. A mixed biofilm of *Synechocystis* and *Chlorococcum* was shown to require moderate light amounts and to contain more capsular polysaccharide at 30°C than at 20°C (Di Pippo et al., 2012). Another method of observing cyanobacterial biofilms in the laboratory used alginate beads, in which the cells grew into dense clusters (Behrendt et al., 2012). A biofilm-forming mutant of *Synechococcus elongatus* was also isolated in the laboratory, and shown to adhere to glass during stationary phase (Schatz et al., 2012).

Cyanobacteria regularly secrete a variety of polysaccharides which likely serve foremost as a boundary from the environment and for biofilm formation, but also as protection against desiccation and antimicrobials (De Philippis and Vincenzini, 1998). Thus, it is likely that cyanobacteria have diverse EPS in their biofilm formations. In total, cyanobacterial species seem to produce ten different monosaccharides, but each species produces different ratios of specific molecules (De Philippis and Vincenzini, 1998). It was found in particular that *Synechocystis* sp. Strain PCC 6803 secretes the polysaccharides uronic acid, glucuronic acid, glucose and mannose (Mallama and Allen, 2010).

*Quorum sensing*

Quorum sensing is a general term for bacterial communication. The first quorum sensing system was discovered over 30 years ago in the two species *Vibrio fischeri* and *Vibrio harveyi*, both of which are marine luminescent species (Nealson and Hastings, 1979). The communication system works through the use of chemical signaling molecules that the bacterial cells produce and then detect in the environment. Once the concentration of the signaling molecules in the environment has reached a minimum threshold, a response such as a change in gene expression,
and thus behavior, occurs. Quorum sensing regulates physiological processes such as plasmid transfer, bioluminescence, antibiotic synthesis, virulence and biofilm formation (Fuqua et al., 2001). In nature, there are two known main quorum sensing systems, one for Gram-negative and one for Gram-positive bacterial species.

In Gram-negative species, such as cyanobacteria, communication works through acyl homoserine lactone (AHL) signaling molecules. In this system, synthase enzymes produce AHLs, which are freely diffusible across the cell membrane (Fuqua et al., 2001). The concentration of AHLs within the cell and in the environment will eventually reach equilibrium. AHLs are a class of molecules containing a five-member lactone ring attached to a chain varying in length from four to fourteen carbons; some chains have an additional carbonyl or hydroxyl substituent on the three carbon (Fuqua et al., 2001). N-hexanoyl-L-Homoserine lactone is one commonly found example of a quorum sensing AHL with a six carbon chain (Figure 3). Other typical AHLs include N-octanoyl-L-Homoserine lactone (eight carbon chain) and N-3-oxo-dodecanoyl-L-Homoserine lactone (twelve carbon chain with carbonyl on the three carbon). As the population density increases and the signal concentration increases, a threshold level is reached and a transcriptional change is activated in the cells (Figure 4; Fuqua et al., 2001). While the interactions between AHLs and the binding sites are not well understood, it appears that the lactone ring is most important for binding ability, regardless of the chain length and additional substituents (Fuqua et al., 2001). Over 70 Gram-negative bacterial species that produce AHLs have been identified, and the AHLs regulate a diverse range of processes (Fuqua et al., 2001).

![Figure 3 An Acyl Homoserine Lactone Molecule: N-Hexanoyl-L-Homoserine Lactone](image-url)
Gram-positive species, in contrast, use modified oligopeptides as signaling molecules to control transcriptional regulation (Waters and Bassler, 2005). These oligopeptide autoinducers consist of 5-17 amino acids, often with unusual side chain modifications (Federle and Bassler, 2003). The oligopeptides are not diffusible across the membrane, and thus require a membrane receptor to regulate DNA transcription (Waters and Bassler, 2005).

It appears as though quorum sensing is a more complicated as well as a more common system than at first thought. For example, it has been shown that the entire virulence regulon in *V. cholera*, containing 70 genes, is regulated by quorum sensing (Zhu et al., 2002). The universality of this system adds to the evidence that bacteria are more developed and evolved than typically believed, and do communicate in a community manner (Waters and Bassler, 2005). In addition, AHL-receptor interactions are very specific, and thus there is limited cross-talk in mixed populations of bacterial species (Federle and Bassler, 2003). Interspecies communication likely does occur, and appears to work through the autoinducer AI-2. Half of all sequenced bacterial genomes contain the *luxS* gene encoding the AI-2 synthase protein, and AI-2 can control gene expression in a variety of bacterial species (Waters and Bassler, 2005). For
example, *V. harveyi* uses two different signaling molecules, one of which may be for intraspecies communication and one for interspecies communication (Federle and Bassler, 2003).

Quorum sensing regulates a variety of phenotypes, such as the bioluminescence in *V. fischeri* and the production of virulence factors in *P. aeruginosa* (de Kievit and Iglewski, 2000). There is strong evidence that biofilm formation occurs often as a result of quorum sensing signaling systems, including with AHL molecules. Quorum sensing was specifically implicated in the formation of *P. aeruginosa* biofilms, which were dependent on the presence of 3-Oxododecanoyl AHL for traditional biofilms (de Kievit and Iglewski, 2000; Davies et al., 1998). AHL molecules specifically were detected in living biofilms growing on submerged aquatic stones (McLean et al., 1997). In *Pseudomonas putida*, it was shown that AHL producing wild type cells formed homogenous unstructured biofilms, while non-AHL producing mutant cells formed structured microcolony biofilms, indicating the effect of AHLs on biofilm phenotype (Steidle et al., 2002). Similarly, it was found that *Bacillus cepacia* is dependent on AHLs for biofilm formation and that the addition of artificial Octanoyl AHL to AHL-production mutants can alone rescue the biofilm phenotype (Huber et al., 2001). Thus, it is very possible that cyanobacteria could use a similar signaling mechanism in their biofilm formation.

**Genetic systems of quorum sensing**

The quorum sensing system in Gram-negative species relies on two proteins, LuxI and LuxR. LuxI produces the AHL molecule while LuxR is the AHL receptor and DNA binding transcriptional activator (Waters and Bassler, 2005). The LuxR receptor is only activated once the concentration of AHLs reaches a critical threshold, which is determined by an appropriate cell density (Waters and Bassler, 2005). It is not energetically efficient for the cell population to undertake a change in phenotype unless there is a critical concentration of cells to make it
worthwhile. For example, bioluminescence is not favorable for the cells unless the density of cells is enough for the light to be visible. Normally, the LuxR proteins and their AHL signals are very species specific. However, there are other similar signaling systems used for interspecies communication (Waters and Bassler, 2005). Surprisingly, there is little general homology in LuxI/LuxR proteins among Gram-negative quorum sensing bacterial species (Steindler and Venturi, 2007).

**Quorum sensing in Cyanobacteria**

AHL signaling has only been studied in a limited manner in cyanobacterial species. The only published analysis of a specific AHL molecule in a single cyanobacterial species was the discovery of the use of N-octanoyl-L-Homoserine lactone by the cyanobacterium *Gloeothecce PCC 6909* (Sharif et al., 2008). One other study claims the production of AHLs by two cyanobacterial species (Braun and Bachofen, 2004). This investigation studied two lake systems dominated respectively by the cyanobacterial species *Planktothrix rubescens* and *Microcystis wesenbergii*, but did not isolate the species. Peptide signaling has also been implicated in *Anabaena*, the filamentous nitrogen-fixing cyanobacterium, where it inhibits heterocyst development (Winans and Bassler, 2002).

**Methods to detect acyl homoserine lactone molecules**

The detection of AHL molecules in the laboratory can be approached by several means. One method involves the use of biosensors, which do not produce any AHLs but do contain the LuxR protein that can respond to the presence of AHLs (Schaefer et al., 2000). Each biosensor recognizes a limited range of chain lengths and substitutions on AHLs (Steindler and Venturi, 2007). The stimulation of the LuxR receptor results in a visual phenotype, which indicates the presence of AHLs. For example, the *Photorhabdus luminescens* sensor results in
bioluminescence, the *Agrobacterium tumefaciens* uses the $\beta$-galactosidase reporter system, and some biosensors use the green fluorescent protein system (Steindler and Venturi, 2007). The biosensor can be inoculated in agar and put adjacent to the sample to be tested, whether it is a liquid sample or a thin layer chromatography plate used to separate the sample molecules (Schaefer et al., 2000; Shaw et al., 1997). One method of concentrating the AHLs in a sample is to extract with a solvent such as ethyl acetate, because these biosensors have limits of minimum detection (Schaefer et al., 2000). Use of the biosensors can also be used to quantify the concentration of AHLs present, with the use of a standard curve comparing response to AHL concentration (Schaefer et al., 2000). Unfortunately, biosensors can result in many false negatives because of low concentration or diverse affinities of biosensors to specific AHLs.

One common biosensor species is *Chromobacterium violaceum*. The wild type bacterial species produces the water soluble violacein pigment in response to AHLs it produces. For use as an indicator, a mutant defective in production of AHLs was created through mini-Tn5 transposon mutagenesis of the wild type *C. violaceum* ATCC 31532, with insertion in a putative repressor locus and a *luxI* homologue (*cvil*) (Winson et al., 1994). However, the pigment production by *C. violaceum* in the presence of AHLs is not affected. While the wild type *C. violaceum* naturally produces an AHL with a carbon chain of six, it is capable of recognizing a range of AHLs with a chain length of four to eight carbons, although at different sensitivities (McCleand et al., 1997).

In addition to biosensors, other methods of recognizing the presence of AHL molecules include chromatographic methods. Mass spectrometry can be used to discover the structure of AHLs present in a sample. Tandem MS, high resolution fast atom bombardment MS, electrospray ionization tandem MS and gas chromatography MS have been used to investigate the molecular mass and structure of AHLs (McCleand et al., 1997; Schaefer et al., 2000; Teplitski
et al., 2003). High performance liquid chromatography can also be used to both purify and confirm the identity of AHLs (McClean et al., 1997; Schaefer et al., 2000). Proton NMR can be used to differentiate the structure of some AHLs, depending on the length of the carbon chain and the presence of substituents which will shift the proton peaks (Schaefer et al., 2000). In addition, the synthesis of AHLs by bacterial species can be monitored with radiotracer techniques involving radioactive carbons (Schaefer et al., 2002).

**Acyl homoserine lactone stability**

Detection of AHL molecules by a biosensor is dependent on closure of the lactone ring. However, AHLs can regularly undergo lactonolysis, which is a natural mechanism by which the ring opens at the ester bond to create a COO⁻ and OH group. This is more likely to occur under basic conditions, because the molecules are much more stable at reasonably acidic pH, but are most stable when dry at -20°C (Schaefer et al., 2000). With increasing pH, the ring undergoes more lactonolysis and less is recognized by the biosensors (Yates et al., 2002). It was confirmed that lactonolysis occurs at basic pH, but the ring opening is reversed with acidification and the AHL can again be recognized by the biosensor (Yates et al., 2002). Thus the molecules can be recognized by a biosensor even after the ring is opened if they are put into an acidic solution.

**Quorum quenching genetic systems**

There are also natural quorum quenching systems, which specifically degrade AHLs so they are no longer functional or recognizable. The two main classes of quenching enzymes are acylase enzymes, which cut the amide bond of the AHL and split the molecule into two compounds, and lactonase enzymes, which cleave the ester bond and thus open the ring.
The acylase enzymes cleave the fatty acid side chain of AHLs and thus detach the intact lactone ring from the acyl side chain, which also deactivates the signal (Federle and Bassler, 2003). An example of this enzyme has been found in *P. aeruginosa*, where it cleaves side chains ranging from seven to fourteen carbons, including those with substituents on the three carbon (Huang et al., 2006). A novel acylase has been identified in *Anabaena* sp. PCC 7120, which indicates the possibility of quorum quenching activity in cyanobacteria (Romero et al., 2008). This enzyme has been found to have broad acyl-chain length specificity, and could either represent an internal response to the cyanobacterium’s own quorum sensing or a means of interfering with signaling of other cyanobacterial species within complex microbial communities. Another example is an acylase from *Ralstonia* (Lin et al., 2003). A BLAST search of the acylase protein in *Ralstonia* detected 94 sequences showing completely conserved domains from different organisms, including Cyanobacteria, although most organisms were gamma-Proteobacteria (Kalia et al., 2011).

Lactonases, which cause ring opening, completely inactivate the AHL signals (Federle and Bassler, 2003). *Bacillus* species produce AiiA, which is a lactonase protein (Dong et al., 2002). A BLAST search of the lactonase protein of *Bacillus* identified 110 completely conserved domains in a variety of species, although the results were dominated by Firmicutes and alpha-Proteobacteria (Kalia et al., 2011). Cyanobacteria were not found to have any lactonase enzyme sequences.

The use of quorum quenching in nature is debated. Quorum quenching may be used as a competitive device to disrupt the growth and perhaps biofilm formation of nearby species in nature (Kalia et al., 2011). This system gives one bacterial species an advantage over another species which relies on quorum sensing, particularly in an environment with limited resources
for which the species may be in competition (Waters and Bassler, 2005). Other species use AHLs as energy sources in metabolic processes (Kalia et al., 2011). A soil isolate of *Pseudomonas*, as well as *P. aeruginosa* PAO1, were found to preferentially utilize long chain AHLs such as 3-Oxododecanoyl AHL as a sole energy source (Huang et al., 2003). Increasing our understanding of quorum quenching systems could help in the development of therapies to disrupt quorum sensing regulated functions, which include bacterial pathogenesis, horizontal gene transfer and biofilm formation (Federle and Bassler, 2003).

There is evidence of another quorum sensing inhibition system in the cyanobacterial species *Lyngbya majuscula*. This species was found to naturally produce and secrete the antibiotic malyngolide, which disrupted the quorum sensing system of *C. violaceum* CV017 by inhibiting transcription of the LasR protein and thus secretion of violacein pigment (Dobretsov et al., 2010). The authors report that malyngolide did not act as an AHL mimic and did not degrade AHLs, so this system is different from the use of enzymes to degrade the AHL molecules.

**Synechocystis sp. Strain PCC 6803**

In this study, the cyanobacterial species *Synechocystis* sp. Strain PCC 6803 was investigated. Cyanobacteria are Gram-negative prokaryotic and photosynthetic cells, with a photosynthetic apparatus similar to eukaryotic chloroplasts and containing both chlorophyll a and phycobiliproteins (Stanier and Cohen-Bazire, 1977). Cyanobacteria first appeared in the fossil record 2.7 billion years ago, and may have been the earliest evolved organisms on earth (Stanier and Cohen-Bazire, 1977). This timing correlates with the development of the oxygenic atmosphere (Stanier and Cohen-Bazire, 1977). Cyanobacteria are actually believed to be the origin of the eukaryotic chloroplast; cyanobacterial cells may have been endocytosed by other...
cells and formed a permanent symbiotic relationship (Stanier and Cohen-Bazire, 1977). This particular cyanobacterial strain is an important laboratory model system, in part because it is very tolerant to diverse environments. In 1996, it was the first photosynthetic autotroph to have its genome sequenced, which enables comparison with other organisms and allows for increased molecular biology studies.

**Research Goals**

The work reported in this thesis was planned to investigate the biofilm formation and communication system of *Synechocystis* sp. Strain PCC 6803. Biofilm structure and formation of the *Synechocystis* cells was observed. To study the communication system of the cells, the indicator bacterium *C. violaceum* CV026 was characterized for its ability to detect AHLs. Production of AHLs by the *Synechocystis* cells was also investigated. In addition, AHL stability and the response of the *Synechocystis* cells to AHL standards were studied. Finally, genetic analysis of *Synechocystis* was undertaken.
Materials & Methods

Bacterial strains and culture methods

*Synechocystis* sp. Strain PCC 6803 (Table 1) cyanobacterial cells were cultured at 30°C under fluorescent light ranging in intensity from 4-20μmol/m²/s, in liquid or on solid BG-11 medium (17.6mM NaNO₃, 0.22mM K₂HPO₄, 0.03mM MgSO₄, 0.2mM CaCl₂, 0.03mM C₆H₈O₇/Citric Acid, 0.02mM Ammonium Ferric Citrate, 0.002mM Na₂EDTA, 0.18mM Na₂CO₃, Trace Metals (9μM MnCl₂, 0.77 μM ZnSO₄, 1.6 μM Na₂MoO₄, 0.3 μM CuSO₄, 0.17 μM Co(NO₃)₂), 0.3% sodium thiosulfate, 0.8% Bacto agar for solid). The wild type *Synechocystis* cells are non-motile (Ikeuchi and Tabata, 2001). In addition, a super-motile *Synechocystis* strain isolated previously was studied in this current investigation (Mallama and Allen, 2010). Frozen stocks of *Synechocystis* were made with 1 part concentrated liquid culture and 1 part 25% glycerol, and frozen at -80°C.

*Chromobacterium violaceum* CV026 was created through mini-Tn5 transposon mutagenesis of the wild type *C. violaceum* ATCC 31532, with insertion in a putative repressor locus and a luxI homologue (*cvil*) (Winson et al., 1994). *C. violaceum* cells were cultured at 30°C in liquid or at 29°C on solid Luria-Bertoni (LB) medium (10% Bacto-Tryptone, 5% Yeast extract, 10% NaCl, 1.5% agar for solid, pH 7.5) (McClean et al., 1997). Frozen stocks of *C. violaceum* were made with 1 part overnight liquid culture and 1 part 25% glycerol, and frozen at -80°C. To begin new culture from frozen stock, a loopful of frozen stock was steriley transferred to LB solid medium.
<table>
<thead>
<tr>
<th>Strain</th>
<th>Source</th>
<th>Notes</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>Synechocystis</em> sp. Strain PCC 6803</td>
<td>American Type Culture Collection</td>
<td>photosynthetic, nonmotile (Ikeuchi and Tabata, 2001)</td>
</tr>
<tr>
<td><em>Synechocystis</em> sp. Strain PCC 6803 motile isolate</td>
<td></td>
<td>photosynthetic, motile (Mallama and Allen, 2010)</td>
</tr>
<tr>
<td><em>Chromobacterium violaceum</em> ATCC 31532</td>
<td>Jean Huang, Olin College</td>
<td>Produces and recognizes acyl homoserine lactones</td>
</tr>
<tr>
<td><em>C. violaceum</em> CV026</td>
<td>Jean Huang, Olin College</td>
<td>Recognizes acyl homoserine lactones</td>
</tr>
</tbody>
</table>

**Growth curves of bacterial cells**

To observe the growth of the bacterial cells, an overnight culture of *C. violaceum* CV026 was subcultured in triplicate and a dense culture of *Synechocystis* cells was subcultured once. The cells were grown shaking at 30°C and the optical density of each culture was measured in regular intervals using a Klett colorimeter with a 660nm filter. The *C. violaceum* CV026 was measured approximately every 15 minutes for six hours, and the *Synechocystis* cells were measured once daily for several weeks. The log of measured optical density (Klett units with 660nm filter) was plotted against time and doubling time was calculated as \((\text{time})/((\log N_t - \log N_0)/\log 2)\).

**Biofilm flow cell set up**

*Synechocystis* biofilms were grown in a flow cell set-up (Figure 5) at room temperature with 19 rE/m²/s fluorescent light at the center of the flow cell. First, the flow cell was prepared by attaching a cover glass to the flow cell frame using silicone glue to enclose the channels. After the glue dried, the flow cell system was set up. Silicone tubing was inserted into a glass jar containing sterile BG-11 medium and then attached to bubble chambers (FC 34 Four-Channel Bubble Trap) and the flow cell channel. Finally, the tubing led into the effluent jar, which
contained acetic acid. The amount of acetic acid added was estimated based on how much effluent would be collected so that the final concentration would be about 1%. The entire set up was autoclaved, with aluminum foil wrapped around the bubble chambers and flow cell channel. Before addition of cells, BG-11 medium was allowed to run through the set up to eliminate bubbles and to check for leaks. To inoculate the flow cell channels, 300μL of *Synechocystis* cells at an OD$_{750}$ of 1.0 was injected with a sterile syringe. The channel was allowed to sit upright for two hours to allow attachment of the cells to the glass cover. Finally, BG-11 medium was pumped through the tubing by the peristaltic pump at a rate of 1rpm (2.4mL/hour). The effluent pumped out of the chambers was collected for further analysis. Visualization of the biofilms was done with confocal microscopy. Two separate trials of the motile biofilm growth were each observed for eleven days. (See Appendix A for detailed instructions).

![Figure 5 Biofilm flow cell set up](image)

**Figure 5 Biofilm flow cell set up** (1) Medium container. (2) Sterilizable silicon tubing. (3) Peristaltic pump. (4) Bubble chamber. (5) Flow cell channels sealed with cover slip: location of biofilm growth. (6) Effluent container. (7) Fluorescent light source for cyanobacteria.

**Confocal imaging of Synechocystis**

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 512 x 512 pixel resolution with a typical scale of 0.239μm/pixel in-plane and a resolution-optimized spacing in the Z direction of 0.125μm/slice. Fluorescence of healthy *Synechocystis* was excited with a 633nm Helium-Neon laser. Emission was detected typically from around 643nm up to around 765nm. 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 30-50μm without adopting some form of linear compensation, either through increasing the laser intensity or increasing the photomultiplier tube gain. (Modified from protocol written by Dr. Rebecca Christianson, Olin College).

*Scanning electron microscopy imaging of Synechocystis*

Sample preparation for scanning electron microscopy (SEM) was performed by the Kirschbaum laboratory at the University of Toledo in Ohio from plates sent to them. Briefly, samples were fixed in a glutaraldehyde and phosphate buffer saline solution. Samples were dehydrated using an ascending series of ethyl alcohol/deionized water solutions, and were then chemically dried. Finally, the dried samples were sputtered coated with gold for the SEM observation. (See Appendix B for detailed instructions).

*Use of high performance liquid chromatography to test for presence of AHLs*

Reverse phase high performance liquid chromatography (RP-HPLC) was analyzed for its ability to recognize AHL standards. Samples were run on a Waters 1525 Binary HPLC Pump with a Waters 2487 Dual λ Absorbance Detector. The column used was 15cm carbon-18 with
5μm beads. The gradient used was 10 mins 100% A, 70 mins 100% A to 100% B, 80 mins isocratic 100% B, 81 mins 100% B to 100% A, 100 mins isocratic 100% A. Buffers were A: 2.5% acetonitrile, 1% acetic acid in water; B: 95% acetonitrile, 1% acetic acid in water. Injection volumes used were 5μL and 50μL, flow rate was 1mL/min and detection was observed at 210nm. The standards Hexanoyl and Octanoyl AHL (5mM and 20mM) were dissolved in HPLC grade water with 1% acetic acid and filtered, before injection.

*Use of Chromobacterium violaceum CV026 indicator to test for the presence of AHLs*

Molten semi-solid Luria-Bertoni (LB) agar (0.3% w/v) was seeded with 0.5% of an overnight culture of *C. violaceum* CV026, and 10 mL was poured immediately over the surface of LB plates (1.5% agar) prewarmed to 30°C, prepared in 100mm diameter circular Petri dishes. When the overlaid agar solidified, wells were punched into the agar using the wide end of a sterile Pasteur pipette (diameter 6mm). Wells were filled with 20μL of the sample to be tested. Petri dishes were incubated in their upright position overnight at 29°C, and then examined for purple pigmentation of the bacterial growth around the well to indicate presence of AHLs.

*Environmental effects on C. violaceum CV026 growth*

The effect of temperature and acetic acid concentration on the growth of *C. violaceum* CV026 was tested. Samples of acetic acid ranging from 1%-100% concentration in ethyl acetate were added to wells as described above, and observed for effect on the growth of *C. violaceum* CV026. Growth of *C. violaceum* CV026 and its ability to recognize 5mM Hexanoyl AHL standard using the well assay was also compared at temperatures of 22°C, 29°C, 30°C and 37°C.
Effectiveness of *C. violaceum* CV026 indicator in recognizing acyl homoserine lactones from wild type *C. violaceum* in a T-test

The *C. violaceum* wild type and *C. violaceum* CV026 were transferred from separate agar plates and streaked on fresh LB agar perpendicular to each other (T-test) (Steindler and Venturi, 2007). The ability of the *C. violaceum* CV026 indicator to recognize the AHLs produced by the wild type was observed by the presence of purple pigmentation of the indicator. *C. violaceum* CV026 is stable in liquid medium for two-three days and on agar for six-seven days before it loses the ability to produce purple pigmentation in response to AHLs, at which point a new stock must be used.

Versatility of AHL recognition by *C. violaceum* CV026

To test for dependence of *C. violaceum* CV026 recognition of AHLs on AHL concentration, the indicator was seeded into LB agar and plated as described above. Wells were filled with 20μL of a range of concentrations (0.01nmoles to 400nmoles) of the Hexanoyl AHL standard (Cayman Chemical) dissolved in acidified ethyl acetate with 1% acetic acid. Plates were incubated and examined for purple pigmentation as described above. The width of purple pigmentation of the indicator was compared to the concentration of standard in each sample.

Ability of the indicator to recognize different AHLs was tested with the well assay using a variety of AHL standards. The standards tested were N-octanoyl-L-Homoserine lactone, N-decanoyl-L-Homoserine lactone, N-3-oxo-dodecanoyl-L-Homoserine lactone, N-butyryl-L-Homoserine lactone, and N-(β-ketocaproyl)-L-Homoserine lactone.
**Tests for the presence of AHLs in bacterial samples**

**AHLs in bacterial cultures and biofilm flow cell effluent**

To test for AHLs in bacterial cultures, a dense culture of the cells to be extracted was grown in the appropriate medium. *C. violaceum* wild type was grown overnight to an OD$_{690}$ of about 4. *Synechocystis* was grown to an OD of about 290, as measured by the Klett colorimeter with a 660nm filter. The culture was centrifuged (5 minutes at 4000rpm in a HERMLE Z232 MKII centrifuge for 20-35mL samples, or 20 minutes at 8000rpm in a Sorvall RC-5B Superspeed Centrifuge with GSA rotor for 100-200mL samples) to sediment out cells.

The next step was the extraction, which was performed either on one of the culture supernatants or the effluent from the biofilm flow cell set-up. The *C. violaceum* wild type culture tested was 25mL and the *Synechocystis* culture was 250mL. Effluent samples collected at four different time points throughout two flow cell trials were tested (Days 4, 7, 11 and 12 from first trial and Days 3, 6, 8 and 10 from second trial). First 50mL and then 200mL of each effluent sample were extracted with acidified ethyl acetate. Finally, 500mL of the Day 11 effluent sample was extracted and tested. Using the ethyl acetate layer, the sample was evaporated at room temperature, or *in vacu* for volumes greater than 200mL to concentrate AHLs. The extract was then dried with anhydrous MgSO$_4$. The sample was redissolved in acidified ethyl acetate and then tested with the *C. violaceum* CV026 indicator for the presence of AHLs as described above.

**Presence of AHLs on solid medium growth of motile Synechocystis cells**

As described above, *C. violaceum* CV026 indicator was seeded into molten semi-solid LB agar (0.3% w/v). 10mL was poured immediately over the surface of dense growth of motile *Synechocystis* cells on solid BG-11 agar, grown as described above for four weeks. The overlay of indicator cells was observed for any purple pigmentation.
Stability of AHLs

Effect of biofilm flow cell effluent on AHL stability

To test the effect of the effluent on AHL standards, 10mM Hexanoyl AHL was added to a 100mL sample of the Day 7 effluent sample from the biofilm flow cells. Half of the effluent sample was extracted with acidified ethyl acetate immediately, and tested as described above with the well assay. The other half of the effluent with the added standard was left at room temperature for 3.5 weeks and then extracted with acidified ethyl acetate and tested with the indicator with the well assay.

Effect of BG-11 medium and Synechocystis liquid cultures on AHL stability

The AHL standards Hexanoyl and Octanoyl were added to samples of pure acidified ethyl acetate, pre-acidified BG-11 medium (1% acetic acid) and a pre-acidified dense motile Synechocystis culture (1% acetic acid). 5μM of each AHL standard was added to 30mL of each sample, which were left to incubate at room temperature for four days. After four days, the sample of cells was centrifuged to pellet the cells. The BG-11 medium and the supernatant were extracted with acidified ethyl acetate. The presence of AHLs in each sample was tested for using the C. violaceum CV026 indicator.

The AHL standard Octanoyl was again added in a concentration of 5μM to 30mL samples of pure acidified acetate, plain BG-11 medium and a dense motile Synechocystis culture. At Day 0, Day 3 and Day 6, a 5mL aliquot of each sample was extracted with acidified ethyl acetate and tested for the presence of the AHL standard with the C. violaceum CV026 indicator.
Effect of AHLs on Synechocystis cells

Effect of AHL standards on Synechocystis liquid cultures

The AHL standards Octanoyl and Hexanoyl were tested for their effects on motile Synechocystis cells growing in liquid cultures (2mL). The final concentrations of AHL standards used were 1mM and 0.01mM. The AHL standards in acidified ethyl acetate were added first, and the ethyl acetate was allowed to evaporate before addition of cells. Synechocystis cells were diluted an OD$_{750}$ of 0.5, and were then added to the dried AHL standards. The cultures with AHL standards were compared to cultures with only evaporated unaltered acidified ethyl acetate and unaltered BG-11 medium. One set was put on a shaker at 100rpm and one was left still, both at 30°C. The growth pattern of the cells in all the samples was observed until the cells died.

Effect of AHL standards on Synechocystis growth on solid medium

To test for the response of motile and nonmotile Synechocystis cells to AHL standards on solid medium, cells were grown next to AHL standards. AHL standards (1μL of 5mM solutions) and pure acidified ethyl acetate (1μL) were each spotted on one half of a plate, 1cm from two parallel streaks of cyanobacterial cells. The cells were then grown with only one surface of the plate facing uniform fluorescent light of intensity of about 8μmol/m$^2$/s, and the rest was covered by aluminum foil. Response of the cells to the standards was compared to response to a control using only acidified ethyl acetate. The standards tested were Hexanoyl AHL, Octanoyl AHL, and 3-Oxododecanoyl AHL.
**Genomic analyses**

**DNA extraction and sequencing**

To extract genomic DNA from wild type and motile *Synechocystis* cells, the MoBio Labs PowerLyzer Ultra Clean Microbial DNA Isolation Kit was used. Briefly, cells were lysed with sodium dodecyl sulfate detergent and specialized beads. The released DNA was separated from the cell contents, isolated and bound to a silica spin filter with a high concentration salt solution, and washed with an ethanol wash solution. Finally, the DNA was eluted by a Tris buffer pH 8 (http://www.mobio.com/microbial-dna-isolation/powerlyzer-ultraclean-microbial-dna-isolation-kit.html). Cells for extraction were taken from dense solid medium growth. The DNA concentration from the motile cells was 28.1 ng/μL and the concentration from the nonmotile cells was 31.5 ng/μL. DNA sequencing was performed at MRDNA (http://mrdnalab.com/sequencing-service.html). Sequence analysis was performed using Geneious 6.1 software (http://www.geneious.com_web/geneious/home). The sequenced motile and nonmotile *Synechocystis* genomes were aligned using the Geneious software. Pilus and chemotaxis related genes were searched for in each genome, and the number of fragments corresponding to each gene was noted. Adjacent fragments were assumed to be part of the same gene.

**Bioinformatics of AHL genes**

Sequences of quorum sensing and quenching genes from a variety of species were obtained from NCBI (www.ncbi.nlm.nih.gov). Homologous sequences in *Synechocystis* were searched for using the BLAST tool (www.ncbi.nlm.nih.gov/BLAST). The percent identity, gaps, query coverage and the E value were noted for each homology identified.
Results

Growth of bacterial cells

In order to understand the growth of Chromobacterium violaceum, its growth in liquid medium was observed over time. A growth curve was created for the C. violaceum CV026 indicator cells by measurement of the optical density of cells growing at 30°C over time, using a Klett colorimeter with a 660nm filter (Figure 6). The doubling time was determined to be 60.9 minutes, based on an average of three cultures of cells (62.6, 63.4, and 56.8 minutes) remaining in exponential phase for three hours. (See Appendix C for additional growth curves).

Figure 6 Growth curve of C. violaceum CV026 indicator Example of growth curve from one culture growth. A dense culture of cells was diluted in triplicate, and the optical density of each was measured at regular intervals using a Klett colorimeter with a 660nm filter. The curve shows the logarithm of the optical density over time. The doubling time was calculated as (time)/((logN_t – logN_0)/log2). An exponential curve was fit to the data. The line indicates time points in exponential phase, to which the curve was fit.

The growth of the motile Synechocystis cells in liquid medium was also observed. A growth curve was created for the cells as described for the C. violaceum CV026 cells. The
doubling time was determined to be 6.15 days, based on an average of two cultures of cells (3.8 and 8.5 days) growing in exponential phase at 30°C under light intensity of about 4μmol/m²/s (Figure 7). (See Appendix C for additional growth curve).

**Figure 7 Growth curve of *Synechocystis*** Growth curve of cyanobacterial cells. A fresh subculture of cells was made, and the optical density was measured at regular intervals using a Klett colorimeter with a 660nm filter. The curve shows the logarithm of the optical density over time. The doubling time was calculated as (time)/(logNₜ – logN₀)/log2. An exponential curve was fit to the data. The line indicates time points in exponential phase, to which the curve was fit.

**Imaging of Synechocystis**

*Biofilm growth*

In order to observe the biofilm growth of the motile *Synechocystis*, cells were grown in the flow cell chambers, which provide a surface for their formation of biofilms. The cells were observed periodically using confocal microscopy. One example of the growth of the motile cells is shown in Figure 8 over time. The cells began by forming small clustered pillars, which were about 20μm deep (Fig 8a). These pillars grew into wider and deeper clusters, which became about 45μm deep after a few more days (Fig 8b). The clusters continued to grow, and
specifically spread across the surface, with some clusters even joining together (Fig 8c). The dark areas in the center of the clusters at the later stages appeared because the density of the cells inhibited the light penetration. Each cluster was considered an individual biofilm. Figure 9 shows a lower magnification, and thus wider representation, of the biofilm growth on the surface. Similarly, the growth started as small clusters (Fig 9a) which spread across the surface and actually joined together (Fig 9b). The pattern was consistent across this view of the surface. Two trials of the motile cells biofilm growth was observed over eleven days, resulting in some variation in shape of the clusters and the exact dimensions. The overall pattern was consistent in the two trials. (See Appendix D for additional images).

**Figure 8 Progression of biofilm formation of motile Synechocystis** (a) early pillar formation, 20μm deep at Day 3 (b) later stage clusters, 45μm deep at Day 6 (c) Late stage spreading of clusters at Day 8. Images were taken with confocal microscopy using the HeNe 633nm laser. The center of each image shows the surface of the coverslip of the flow cell, while the far right and bottom panels show the three dimensional view of the clusters. Marker indicates 25μm.
Figure 9 Surface representation of motile *Synechocystis* biofilm growth across flow cell

Tile and scan image (a) small clusters on surface at Day 6 (b) Later stage spreading of clusters across surface at Day 8. Images were taken with confocal microscopy using the HeNe 633nm laser. Marker indicates 100μm.

**Scanning electron microscopy of Synechocystis**

The motile and nonmotile *Synechocystis* cells exhibit very different motility phenotypes. In response to light, the motile cells move towards the light while the nonmotile cells do not move at all (Figure 10). In order to observe the motile and nonmotile *Synechocystis* cells in greater detail, scanning electron microscopy (SEM) was used to visualize the cells (Figure 11). The motile and nonmotile cells were observed under similar magnification, specifically for differences in morphology. Both cells exhibited projections off the surface, which form a matrix-like structure around the cells. However, the actual outer surface of the cells is strikingly different. The motile cell surface was much smoother than the surface of the nonmotile cells, which had a rough, striated pattern that is uniform across the surface. (See Appendix E for additional images).
**Figure 10 Comparison of motility of *Synechocystis* cells**

Motile and nonmotile cyanobacteria were streaked on solid BG-11 medium (1.5% agar). Cells were given uniform fluorescence light of intensity 20μmol/m²/s at 30°C, and observed for their response to the light.

**Figure 11 Scanning electron microscopy of *Synechocystis***

Samples of motile and nonmotile cyanobacterial cells were imaged under scanning electron microscopy. The phenotypes of the cells, particularly their outer surface, were compared. Marker indicates 1μm. Arrows indicate dividing cells. Circle indicates loss of part of the outer cell wall, likely as an artifact of preparation.

**Use of high performance liquid chromatography to test for presence of AHLs**

In order to investigate a possible method for the detection of AHLs, reverse phase high performance liquid chromatography (RP-HPLC) was used on the Hexanoyl and Octanoyl AHL
standards. A 5mM sample of both Hexanoyl and Octanoyl AHL, as well as a 20mM sample of Hexanoyl AHL, were tested with 5μL and 50μL injection volumes. The highest concentration tested was 50μL of 20mM Hexanoyl AHL (Figure 12). The amount of AHL injected in this sample was 1μmole. A small peak can be seen at 28.66mins. It is not known what the large negative peak represents.

Figure 12 RP-HPLC chromatogram of Hexanoyl AHL standard The Hexanoyl AHL standard (20mM) was subjected to RP-HPLC with a gradient of 10 mins 100% A, 70 mins 100% A to 100% B, 80 mins isocratic 100% B, 81 mins 100% B to 100% A, 100 mins isocratic 100% A. Buffers were A: 2.5% acetonitrile, 1% acetic acid in water; B: 95% acetonitrile, 1% acetic acid in water. The injection volume was 50μL, detection was measured at 210nm and flow rate was 1mL/min.

Use of C. violaceum CV026 indicator to test for the presence of AHLs

The C. violaceum CV026 indicator was tested for its ability to recognize the AHL molecules produced by the wild type C. violaceum. A T-test shows the indicator C. violaceum CV026 growing perpendicular to the wild type C. violaceum on solid LB medium (Figure 13). The wild type was dark purple, indicating that it was producing violacein pigment in response to the presence of AHL molecules it presumably produced and secreted. The indicator cannot produce its own AHLs, and thus only the cells closest to the wild type were producing the
violacein pigment. This indicates that these cells were responding to the AHLs produced by the wild type. The indicator cells appear only to be able to recognize these AHLs up to a certain distance on the solid medium.

![Image of T-test of wild type C. violaceum and C. violaceum CV026 indicator](image)

**Figure 13** T-test of wild type *C. violaceum* and *C. violaceum* CV026 indicator The wild type *C. violaceum* cells were grown perpendicular to the *C. violaceum* CV026 indicator (not producing AHLs) on solid LB medium. Growth is shown after two days at 30°C.

**Effect of environment on growth of *C. violaceum* CV026 indicator**

All of the solutions tested in this investigation were diluted in a solvent of ethyl acetate containing 1% acetic acid. Thus, it was ensured that this solvent would not affect the growth of the *C. violaceum* CV026 cells. The effects of ethyl acetate with increasing concentrations of acetic acid on the growth of the *C. violaceum* CV026 were tested. Cells were grown adjacent to samples of ethyl acetate acidified with a range of concentrations of acetic acid, using the well assay described in Materials and Methods (Figure 14). The area of cells directly around the wells was observed for lack of growth. Ethyl acetate with 1% acetic acid, which is used in this investigation, did not affect the growth of the cells at all. Growth was not affected at concentrations at least up to 10% acetic acid, and only slightly up to 25% acetic acid. Higher acetic acid concentrations killed the cells, indicated by clearing of turbidity around the wells.
Figure 14 Effect of ethyl acetate and acetic acid on growth of *C. violaceum* CV026 indicator

Using the well assay described in Materials and Methods, samples of ethyl acetate with increasing concentrations of acetic acid were tested with the *C. violaceum* CV026. Growth was observed as turbidity around the wells containing the samples. Growth is shown after two days at 30°C.

To ensure the optimal temperature for growth of *C. violaceum* CV026, cells were grown at four different temperatures with a sample of the Hexanoyl AHL standard (Figure 15). While the cells grew well at all temperatures, the best growth occurred at 29°C, as indicated by the evenness of the growth. The cells also were able to recognize the AHL standard at all temperatures. The darkest and most even pigmentation occurred at 29°C, with decreased recognition of AHL at both lower and higher temperatures. Thus, all further experiments were done with the indicator growing at 29°C.
Figure 15 Effect of temperature on growth of *C. violaceum* CV026 indicator The ability of the indicator bacterium to grow and recognize the presence of AHL standards was observed at a range of temperatures. Growth is shown after three days at the particular temperature indicated. The Hexanoyl AHL was used as a standard to observe the ability of the indicator to recognize AHLs and produce the purple pigment at different temperatures.

Quantitative recognition of AHLs by *C. violaceum* CV026 indicator

The sensitivity of the *C. violaceum* CV026 indicator in its recognition of AHL standards was tested. *C. violaceum* CV026 was tested for its ability to recognize the Hexanoyl AHL standard at a range of concentrations, using the well assay described above. The width of recognition, measured by the width of purple pigmentation, of the indicator was observed (Figure 16a). The minimum concentration of recognition of this AHL was 0.08nmoles. The darkness of the pigment was very faint at the lower concentrations, and increased up to 0.32nmoles. The pigment did not become more dense above this point. The radius of detection appeared to level off as the concentration of AHL reached above 50nmoles, as the width of purple pigment stopped increasing. The radius of the violacein pigment was quantitatively compared to the concentration of the AHL in each sample (Figure 16b). There is a strong logarithmic correlation between the width of purple pigment and the concentration of AHL, through 2.56nmoles, after which there was leveling off of recognition.
(a) Using the well assay described above, the indicator bacterium was used to test a range of concentrations of the Hexanoyl AHL standard. The width of recognition, measured as the width of purple pigmentation, was measured at each increasing concentration. (b) The width of purple pigmentation was graphically compared to the concentration of AHL. A logarithmic curve was fit to the data. Growth was observed after one day at 30°C.

**Figure 16 The quantitative recognition of Hexanoyl AHL by the* C. violaceum CV026* indicator** (a) Using the well assay described above, the indicator bacterium was used to test a range of concentrations of the Hexanoyl AHL standard. The width of recognition, measured as the width of purple pigmentation, was measured at each increasing concentration. (b) The width of purple pigmentation was graphically compared to the concentration of AHL. A logarithmic curve was fit to the data. Growth was observed after one day at 30°C.

**Versatility of C. violaceum CV026 indicator recognition of a variety of AHLs**

The versatility of the *C. violaceum CV026* indicator was tested based on its ability to recognize different AHL standards. The standards (N-butyryl-L-Homoserine lactone, N-(β-ketocaproyl)-L-Homoserine lactone, N-hexanoyl-L-Homoserine lactone, N-octanoyl-L-Homoserine lactone, N-decanoyl-L-Homoserine lactone and N-3-oxo-dodecanoyl-L-Homoserine lactone) were tested against the indicator strain using the well assay (Figure 17). These AHLs
have carbon chains ranging in length from four to twelve carbons. The β-Ketocaproyl and 3-Oxododecanoyl AHLs also have an additional carbonyl group substituent at the three carbon. The width of detection was determined to be a measure of the sensitivity of the indicator to the particular AHL. The indicator can recognize AHL chain lengths of four, six or eight carbons, even with the carbonyl substituent, but not AHL chain lengths of ten or twelve carbons.

Figure 17 The recognition of AHLs with varying length of carbons chains by the C. violaceum CV026 indicator Six different AHL molecules, differing in their carbon chain lengths and substituents, were tested with the indicator strain using the well assay. Growth was observed after one day at 30°C. The width of purple pigmentation was considered a measure of the sensitivity of the indicator to each AHL.

Tests for the presence of acyl homoserine lactones in bacterial samples

In order to test the ability of the C. violaceum CV026 indicator to recognize naturally produced AHLs, a liquid culture of wild type AHL-producing C. violaceum cells was tested. The supernatant (20-30mL) of this culture was extracted with ethyl acetate with 1% acetic acid to purify the AHLs, and the C. violaceum CV026 indicator was observed for purple pigmentation in response to the samples using the well assay (Figure 18). The indicator was able to recognize AHLs both in the untouched supernatant of the wild type culture and in the extracted supernatant. The presence of AHLs in the untouched supernatant indicates that the AHLs did not pellet after centrifugation. The increased width of purple pigmentation after extraction indicates a higher concentration of AHLs, and thus that the extraction procedure did concentrate the AHLs in the supernatant.
Figure 18 Purification of AHLs from wild type *C. violaceum* (a) supernatant and (b) purified acidified ethyl acetate extract of the supernatant of wild type *C. violaceum* were tested against the indicator bacterium for the presence of AHL molecules using the well assay. The density and width of purple color of the bioassay strain indicates the relative concentration of AHL molecules. All wells on each plate are identical in sample volume. Growth was observed after one day at 29°C.

*Presence of AHLs in growing Synechocystis supernatant extracts*

Motile *Synechocystis* cells were tested for production of AHLs when growing in liquid cultures. The supernatant of a dense culture was extracted and tested for the presence of AHLs by the *C. violaceum* CV026 indicator using the well assay (Figure 19). There was no purple pigmentation around the well containing the extract from the cyanobacterial culture, in comparison to the pigment around the well containing the standard. This indicates that the *Synechocystis* cells do not appear to be producing an AHL, at least in a high enough concentration that can be recognized by this indicator.
Presence of AHLs in liquid culture of *Synechocystis* cells

The supernatant (250mL) of a cyanobacterial liquid culture of optical density of 290, as measured by a Klett colorimeter with a 660nm filter, was extracted with acidified ethyl acetate and tested for the presence of AHLs by the *C. violaceum* CV026 indicator strain. The top well contains the extracted sample and the bottom well contains Hexanoyl AHL standard, to ensure the ability of the indicator to produce the violacein pigment. Growth was observed after one day at 29°C.

Presence of AHLs in growing *Synechocystis* biofilms

The presence of AHLs was also tested in *Synechocystis* biofilms grown in flow cells. While medium was pumped through the flow cells, effluent flowing out of the chambers was collected. This effluent should contain any molecules or substances secreted by the biofilm-forming *Synechocystis*, including any AHLs. Effluent samples collected at four different time points throughout two flow cell trials were tested (Days 4, 7, 11 and 12 from first trial and Days 3, 6, 8 and 10 from second trial). First 50mL and then 200mL of each effluent sample were extracted with acidified ethyl acetate to concentrate any AHLs, and were then tested with the *C. violaceum* CV026 indicator. Finally, 500mL of the effluent sample from Day 11 of biofilm growth was similarly extracted with acidified ethyl acetate and tested with the *C. violaceum* CV026 indicator strain (Figure 20). No pigmentation was observed around the well containing any of the samples, as compared to the well containing the AHL standard. This indicates that the
Synechocystis cells growing in biofilms do not appear to be producing an AHL, at least in a high enough concentration to be recognized by this indicator.

Figure 20 Presence of AHLs in biofilm growth of Synechocystis cells One effluent sample (500mL) from the Synechocystis biofilm growth in flow cells was extracted with acidified ethyl acetate and tested for presence of AHLs with the C. violaceum CV026 indicator strain. The top well has the extracted sample. The lack of purple growth indicates the lack of presence of AHLs. The bottom well contains the Hexanoyl AHL standard control, which was recognized by the indicator. Growth was observed after one day at 29°C.

Synechocystis was tested for production of AHLs on solid medium as well. The C. violaceum CV026 indicator was overlaid on dense growth of motile cyanobacterial cells and observed for purple pigmentation (Figure 21). The lack of pigmentation by the indicator cells indicates that the cyanobacterial cells do not appear to be producing an AHL while growing in solid medium, at least in a high enough concentration to be recognized by this indicator.
Figure 21 Presence of AHLs on solid medium growth of Synechocystis cells C. violaceum CV026 indicator was overlaid on dense growth of motile cyanobacterial cells to test for the presence of AHL molecules. The indicator cells were observed for purple pigmentation. The cyanobacteria cells were grown for four weeks at 30°C before the C. violaceum CV026 overlay. Growth of the C. violaceum CV026 was observed after three days at 29°C. Both cyanobacteria plates are identical and show no purple pigmentation.

Stability of AHLs

It is also possible that any AHLs present in the acidified effluent from the Synechocystis biofilm growth in flow cells were degraded, and thus not recognized by the C. violaceum CV026 indicator. To eliminate this possibility, the Hexanoyl AHL standard was added to the Day 7 effluent sample. Half of the sample was first extracted immediately and tested for AHLs; the remaining sample was left for 3.5 weeks, and then extracted again and tested for presence of AHLs (Figure 22). After the first extraction, the purple pigmentation of the C. violaceum CV026 indicator shows that the AHL was clearly present in the sample (Fig 22a). This indicates that the AHL was not degraded immediately. After the second extraction, the C. violaceum CV026 indicator clearly recognized the presence of AHLs in the sample (Fig 22b). This indicates that the AHL standard in the effluent was still not degraded over time. The width of recognition was measured in order to indicate the relative concentration of AHL standard present in the well. The radii of detection of the samples were compared to the radii of detection of AHL standards of known concentration, and shown to be of a concentration of 0.29nmoles (Fig 22c). The amount
of AHL standard added to the effluent sample was expected to result in 0.5nmoles in each well, after extraction. The radii of purple pigment from the effluent samples were less than expected if the extraction was ideal. It is important to note that the radius was the same for both extraction samples, indicating that the concentration of AHL standard did not change.

![Image](image1.png)

(a) Initial  (b) After 3.5 weeks

![Image](image2.png)

(c) 0.16nmoles  0.32nmoles  0.64nmoles

Figure 22 Stability of Hexanoyl AHL standard in effluent from biofilm flow cells

1000nmoles of Hexanoyl AHL standard was added to 100mL of acidified effluent from Day 7 of biofilm flow cell growth. (a) Half the sample was extracted immediately in order to purify the AHL standard. (b) The other half was extracted 3.5 weeks later. The *C. violaceum* CV026 indicator was used to test for the presence of AHLs in each extracted sample and the widths of recognition were compared to those of (c) known standard concentrations.

![Image](image3.png)

Figure 22 Stability of Hexanoyl AHL standard in effluent from biofilm flow cells

In order to test the stability of AHLs in BG-11 medium and in medium of growing cells, both the AHL standards Hexanoyl and Octanoyl were added to acidified ethyl acetate, acidified BG-11 medium and acidified growing motile *Synechocystis* cells, and after four days at room temperature, each sample was extracted and tested for the presence of AHLs (Figure 23). The AHLs are very stable in the acidified ethyl acetate, so no degradation was expected. For both the
Hexanoyl and Octanoyl AHL samples, the motile cells and the BG-11 medium show a very similar width of purple pigmentation, which was only slightly smaller than that of the ethyl acetate standard. This indicates that there was no significant degradation of the AHLs in acidified BG-11 medium or growing motile *Synechocystis* cells.

**Figure 23 Stability of Hexanoyl and Octanoyl AHL standards in acidified BG-11 medium and *Synechocystis* cells** 5μM of AHL standard was added to 30mL of pre-acidified ethyl acetate, acidified BG-11 medium and acidified growing motile *Synechocystis* cells. Solutions were left shaking at room temperature, and then extracted after four days to test for the presence of AHLs with the well assay described above.

In order to test the stability of AHLs in greater detail, AHL Octanoyl standards were tested without pre-acidification. The AHL Octanoyl standard was added to solutions of acidified ethyl acetate, unacidified BG-11 medium and unacidified motile *Synechocystis* cells. Periodically, samples of each solution were extracted and tested for the presence of AHLs (Figure 24). Initially, all three samples contained undegraded AHLs. At Day 3 and Day 6, though, only the acidified ethyl acetate control sample still contained AHLs, as indicated by the lack of purple pigment around the well samples of BG-11 medium and cell culture extracts.
Figure 24 Stability of Octanoyl AHL standard in medium and in *Synechocystis* culture

5μM Octanoyl AHL was added to 30mL of pure acidified ethyl acetate, pure BG-11 medium and growing motile *Synechocystis* cells. Samples of each were extracted regularly and tested for the presence of AHLs with the well assay described above.

**Response of *Synechocystis* to AHL standards**

In order to investigate if the cyanobacterial cells can sense the presence of AHLs, their response to AHL standards was tested. The AHL standards, Hexanoyl and Octanoyl, were added in two different concentrations to growing cyanobacteria cultures. The growth of the cells was compared both in still and shaking cultures (Figure 25). All still cells (even the control) died after two days, and thus the standards did not have any effect on their growth. The cultures with standards did not differ at all from the cultures with acidified ethyl acetate alone or with medium alone. For the shaking cells, clumping occurred in all cultures before three days. Even the cultures without standard and without ethyl acetate clumped. It is interesting to note that most cultures had a ring of dense growth (biofilm) at the top of the liquid culture, but at least at first, the cultures with standards had the darkest ring of growth. The most growth appeared in the 0.01mM Hexanoyl AHL and 1mM Octanoyl AHL culture. Thus, there does not appear to be a pattern in the effect of the addition of the two standards.
Figure 25 Effect of AHL standards on liquid cultures Cyanobacterial cells were grown in small volumes and left (a) still and (b) shaking at 30°C. From left to right the tubes contain only cells (nothing added), 1mM Hexanoyl AHL, 0.01mM Hexanoyl AHL, 1mM Octanoyl AHL, 0.01mM Octanoyl AHL, and acidified ethyl acetate, all in BG-11 medium.

In addition to the response of cyanobacterial cells to standards in liquid culture, their response to standards while growing on solid medium (0.8% BG-11 agar with 0.3% sodium thiosulfate) was also observed. The AHL standards, Hexanoyl, Octanoyl and 3-Oxododecanoyl, were all spotted next to parallel streaks of motile Synechocystis cells (Figure 26). The response of the cyanobacterial cells to the standards was compared to a blank plate (streaked cells but no standards) and to pure ethyl acetate with 1% acetic acid on the plate. First, the AHL standards were each tested on separate plates (Fig 26a). The cells appeared to move toward the Octanoyl AHL standard but away from the Hexanoyl AHL standard, and did not respond at all to the 3-Oxododecanoyl AHL. Next, the Hexanoyl and Octanoyl AHL standards were tested on the same plate in order to eliminate variation in experimental conditions (Fig 26b). The movement of the cells was much less uniform. The cells were not uniformly moving towards or away from either AHL standard or the acidified ethyl acetate. On the left experimental plate, they barely moved at
all, and did not seem to have a preference for any of the samples. On the right plate, the cells appeared to move toward the Octanoyl AHL, as observed before. However, they also moved toward the acidified ethyl acetate. On the part of the plate containing the Hexanoyl AHL, they moved both toward and away from the standard.

(b) **Figure 26 Response of motile *Synechocystis* to AHL standards on solid BG-11 medium (0.8% agar with 0.3% sodium thiosulfate)** Cyanobacterial cells were grown in two parallel lines. (a) One AHL standard was dotted on one half of the plate and pure acidified ethyl acetate was dotted on the other half down the center between the two streaks of cells. (b) Both Hexanoyl and Octanoyl AHL, as well as pure acidified ethyl acetate, were dotted down the center between the two streaks of cells. The cells were observed for their response to the ethyl acetate and the standards. Growth was compared on a plate without any test substance (blank). Cells were given completely uniform light (intensity 8μmol/m²/s). Growth shown after four weeks at 30°C.

The effect of the addition of standards to the nonmotile cyanobacterial cells was also tested. Similarly, the AHL standards, Hexanoyl and Octanoyl, were spotted next to parallel streaks of nonmotile *Synechocystis* cells (Figure 27). The response of the cells to the presence of the AHLs was observed over time. These cyanobacterial cells did not respond at all to either the acidified ethyl acetate or the AHLs. They did not move at all, and their growth was not affected.
Nonmotile cyanobacterial cells were grown in two parallel lines in uniform light (intensity 8μmol/m²/s). The AHL standards were dotted on one half, while acidified ethyl acetate was dotted on the other half between the two parallel streaks. Cells were observed for their response to the AHLs and the ethyl acetate. Growth is shown after two weeks at 30°C.

**Figure 27 Response of nonmotile *Synechocystis* to AHL standards on solid medium growth**

*Genetics of quorum sensing and quorum quenching*

*Genetic quorum sensing system*

The presence of potential quorum sensing genetic systems in *Synechocystis* was also investigated. The LuxR and LasR protein sequences in several other Gram-negative bacterial species were found using NCBI and were used to search for potential homologues in the *Synechocystis* genome (Table 2). Several different candidate homologues were identified. In particular, the NarL subfamily is known to include transcriptional regulation proteins. The region of homology of *V. fischeri* LuxR to *Synechocystis* proteins is specifically residues 157-230. In the LuxR protein of *V. fischeri*, residues 184-230 are particularly similar to a class of transcriptional regulators involved in two component regulatory systems, and are known to be involved in DNA sequence recognition and DNA binding. As a comparison, the known LuxR systems of three other bacterial species were also compared (Table 3). The LuxR systems of *V. fischeri* and *V. cholera* are very similar. However, the LuxR systems of *V. fischeri* and *A. tumefaciens* have a similar identity value to the examples in *Synechocystis*, but higher query coverage and lower E value than the examples in *Synechocystis*. 
Table 2 Investigation of candidate quorum sensing system proteins in *Synechocystis*

<table>
<thead>
<tr>
<th>Species</th>
<th>Protein</th>
<th><em>Synechocystis</em> sp. Strain PCC 6803 homologue</th>
<th>Identity (%)</th>
<th>Gaps (%)</th>
<th>Query Coverage (%)</th>
<th>E Value</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>Vibrio fischeri</em> ES114</td>
<td>LuxR</td>
<td>sll0782 (transcriptional regulator)</td>
<td>38</td>
<td>8</td>
<td>32</td>
<td>5x10^{-8}</td>
</tr>
<tr>
<td><em>Yersinia pestis</em> CO92</td>
<td>LuxR</td>
<td>slr1909 (rre9; two component response regulator (NarL subfamily))</td>
<td>33</td>
<td>0</td>
<td>32</td>
<td>4x10^{-8}</td>
</tr>
<tr>
<td><em>Vibrio cholera</em></td>
<td>LuxR</td>
<td>sll0098 (hypothetical protein)</td>
<td>31</td>
<td>13</td>
<td>28</td>
<td>0.44</td>
</tr>
<tr>
<td><em>Agrobacterium tumefaciens</em></td>
<td>LuxR</td>
<td>slr0697 (hypothetical protein in glutathione metabolism)</td>
<td>31</td>
<td>20</td>
<td>26</td>
<td>0.24</td>
</tr>
<tr>
<td><em>Escherichia coli</em> KO11FL</td>
<td>LuxR</td>
<td>sll1708 (rre17; two component response regulator (NarL subfamily))</td>
<td>25</td>
<td>13</td>
<td>56</td>
<td>0.005</td>
</tr>
<tr>
<td><em>Pseudomonas aeruginosa</em></td>
<td>LasR</td>
<td>sll1592 (rre19; two-component response regulator (NarL subfamily))</td>
<td>37</td>
<td>0</td>
<td>26</td>
<td>2x10^{-3}</td>
</tr>
</tbody>
</table>

Table 3 Comparison of known quorum sensing system proteins

<table>
<thead>
<tr>
<th>Species</th>
<th>Protein</th>
<th>Identity (%)</th>
<th>Gaps (%)</th>
<th>Query Coverage (%)</th>
<th>E Value</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>V. fischeri</em> to <em>V. cholera</em></td>
<td>LuxR</td>
<td>71</td>
<td>0</td>
<td>92</td>
<td>2x10^{-114}</td>
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<tr>
<td><em>V. fischeri</em> to <em>A. tumefaciens</em></td>
<td>LuxR</td>
<td>24</td>
<td>3</td>
<td>95</td>
<td>2x10^{-13}</td>
</tr>
</tbody>
</table>

*Genetic quorum quenching system*

The possibility of potential quorum quenching systems being present was also investigated in the *Synechocystis* genome. Both acylase and lactonase protein sequences from several bacterial species were used to search for possible homologues in *Synechocystis* (Table 4).
Consistently, the protein 7-beta-(4-carbaxybutanamido) cephalosporanic acid acylase was identified with somewhat low identity, reasonably high query coverage and low E values. Note that *Anabaena* sp. PCC 7120 is a filamentous nitrogen-fixing cyanobacterium.
Table 4 Investigation of candidate quorum quenching system proteins in *Synechocystis*

<table>
<thead>
<tr>
<th>Species</th>
<th>Protein</th>
<th><em>Synechocystis</em> sp. Strain PCC 6803 homologue</th>
<th>Identity (%)</th>
<th>Gaps (%)</th>
<th>Query Coverage (%)</th>
<th>E Value</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>Pseudomonas aeruginosa</em></td>
<td>PvdQ</td>
<td>7-beta-(4-carboxybutanamido) cephalosporanic acid acylase</td>
<td>30</td>
<td>13</td>
<td>96</td>
<td>$5 \times 10^{-18}$</td>
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<tr>
<td><em>Pseudomonas aeruginosa</em></td>
<td>QuiP</td>
<td>7-beta-(4-carboxybutanamido) cephalosporanic acid acylase</td>
<td>22</td>
<td>20</td>
<td>83</td>
<td>$6 \times 10^{-13}$</td>
</tr>
<tr>
<td><em>Actinoplanes utahensis</em></td>
<td>AAC</td>
<td>7-beta-(4-carboxybutanamido) cephalosporanic acid acylase</td>
<td>24</td>
<td>16</td>
<td>90</td>
<td>$2 \times 10^{-28}$</td>
</tr>
<tr>
<td><em>Anabaena</em> sp. PCC 7120</td>
<td>AiiC</td>
<td>7-beta-(4-carboxybutanamido) cephalosporanic acid acylase</td>
<td>31</td>
<td>0</td>
<td>60</td>
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<tr>
<td><em>Ralstonia</em> XJ12B</td>
<td>AiiD</td>
<td>7-beta-(4-carboxybutanamido) cephalosporanic acid acylase</td>
<td>24</td>
<td>14</td>
<td>91</td>
<td>$7 \times 10^{-39}$</td>
</tr>
<tr>
<td><em>Bacillus thuringiensis</em></td>
<td>AiiA</td>
<td>hypothetical protein slr1259</td>
<td>22</td>
<td>18</td>
<td>51</td>
<td>0.041</td>
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</table>

Alignment of nonmotile and motile *Synechocystis* genomes

In order to investigate potential differences between the nonmotile and motile *Synechocystis* genomes, their DNA was extracted and the full genomes were sequenced and analyzed using Geneious software. The two genomes were aligned and compared for major sequence differences (Table 5). Several pilus related and chemotaxis genes were analyzed in each sequence. The number of fragments in each genome was noted. Adjacent fragments were assumed to be part of the same gene. One gene of note is that of twitching motility protein PilT, which occurs once in the nonmotile genome but twice in the motile genome. There is an additional fragment of this gene present in the motile genome at a distance from the original
gene. One copy of the PilT appears at approximately 2.20 megabases in both the motile and nomotile genome. The additional fragment in the motile genome appears at approximately 2.05 megabases. Also, two chemotaxis proteins, methyl accepting chemotaxis protein II and chemotaxis protein were only present in the motile genome. The asterisk (*) indicates genes which show differences between the nonmotile and motile genomes.

**Table 5 Analysis of genomes of nonmotile and motile *Synechocystis***

<table>
<thead>
<tr>
<th>Gene</th>
<th>Nonmotile fragments/gene repeats</th>
<th>Motile fragments/gene repeats</th>
<th>Notes</th>
</tr>
</thead>
<tbody>
<tr>
<td>Type IV pilin PilA</td>
<td>1/1</td>
<td>1/1</td>
<td></td>
</tr>
<tr>
<td>Pili retraction protein PilT</td>
<td>1/1</td>
<td>1/1</td>
<td></td>
</tr>
<tr>
<td>Type IV pilus biogenesis protein PilQ</td>
<td>2/1</td>
<td>1/1</td>
<td></td>
</tr>
<tr>
<td>Type IV pilus biogenesis protein PilO</td>
<td>1/1</td>
<td>1/1</td>
<td></td>
</tr>
<tr>
<td>Type IV pilus biogenesis protein PilN</td>
<td>2/1</td>
<td>2/1</td>
<td></td>
</tr>
<tr>
<td>Type IV fimbrial assembly protein PilC</td>
<td>1/1</td>
<td>1/1</td>
<td></td>
</tr>
<tr>
<td>*Twitching motility protein PilT</td>
<td>2/1</td>
<td>3/2</td>
<td>Additional piece in motile</td>
</tr>
<tr>
<td>Type IV fimbrial assembly ATPase PilB</td>
<td>3/2</td>
<td>4/2</td>
<td></td>
</tr>
<tr>
<td>Gliding motility protein GldF</td>
<td>2/1</td>
<td>1/1</td>
<td></td>
</tr>
<tr>
<td>CheY subfamily protein</td>
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<td></td>
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<td>Methyl accepting chemotaxis protein</td>
<td>5/1</td>
<td>5/1</td>
<td></td>
</tr>
<tr>
<td>Chemotaxis protein CheA</td>
<td>2/1</td>
<td>1/1</td>
<td></td>
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<tr>
<td>tsr or; CheD</td>
<td>4/1</td>
<td>2/1</td>
<td></td>
</tr>
<tr>
<td>Signal transduction histidine kinase CheA</td>
<td>11/2</td>
<td>7/2</td>
<td></td>
</tr>
<tr>
<td>*Methyl accepting chemotaxis protein II</td>
<td>0/0</td>
<td>2/1</td>
<td>Only present in motile</td>
</tr>
<tr>
<td>*Chemotaxis protein</td>
<td>0/0</td>
<td>1/1</td>
<td>Only present in motile</td>
</tr>
</tbody>
</table>
Discussion

Growth of bacterial cells

The growth of both the *Chromobacterium violaceum* CV026 indicator strain and *Synechocystis* sp. Strain PCC 6803 was investigated. The doubling time of *C. violaceum* CV026 was determined to be 60.9 minutes, based on an average of three cell cultures (Fig 6). This doubling time, while slower than the model growth of *Escherichia coli*, is relatively fast. This growth allows the indicator to be diluted and seeded into agar for the well assay, after one night of growth. Similarly, McClean et al. (1997) notes that the indicator could be seeded into agar after one night at growth. Indicator growth after being seeded into the agar was dense enough after one night for observation of purple pigment and the presence of AHLs. The growth of this indicator was more than sufficient for the analyses performed here.

The doubling time of *Synechocystis* was determined to be 6.14 days, based on an average of two cell cultures (Fig 7). This growth rate initially appears slow in comparison to other growth analyses of *Synechocystis*. Previously, it has been found that a mutant lacking the apcE gene coding for the anchor protein linking the phycobilisome to the thylakoid membrane grew with a slow doubling time of 5-6 days under photoautotrophic conditions, which was much slower than the wild type (Shen et al., 1993). In comparison, in this current investigation, the wild type growth under very similar light intensity (about 5μmol/m²/s) matched that of the apcE mutant. Another investigation determined a doubling time of the wild type *Synechocystis* under photoautotrophic conditions to be only 11-12 hours, although this was under about 10 times higher light intensity (Haag et al., 1993). The slow growth of the *Synechocystis* in this current investigation may be due to somewhat low light intensity. However, the growth was still sufficiently dense for all experiments carried out.
**Imaging of Synechocystis**

**Biofilm growth**

In order to observe the growth of motile *Synechocystis* cells in biofilm structures, cells were grown in flow cell chambers and visualized with confocal microscopy. The biofilms began as small pillars, which grew into wider and deeper clusters. Over time (observed for up to 11 days), the clusters continued to become more dense and spread across the surface (Fig 8-9). These individual cluster formations are very typical of the classical biofilm structure (Costerton et al., 1999). It is interesting that in all the replicates of the biofilm growth, the clusters began to spread across the flow cell surface and joined together to create very large clusters. The lower magnification view of a wider surface indicates that the pattern was not localized to one area of the flow cell (Fig 9). The phenotype of the cells was very uniform across all of the samples tested and within each sample. The confocal microscopy allowed for a three dimensional view of the biofilm growth, so that the width as well as depth of the clusters could be observed. Many of the clusters were so dense that they became impenetrable by the laser, and thus appeared dark in the images. The darkness does not indicate lack of cells, but rather dense growth.

The growth of these biofilms is in direct contrast to other previously studied cyanobacterial biofilms. Natural cyanobacterial biofilms are usually in the form of blooms or biofilm-like mats, either in intertidal systems or on buildings (Decho, 2000; Ortega-Morales et al., 2000; Albertano et al., 2000; Grbic et al., 2010). In a laboratory study of three environmental cyanobacterial isolates, all three grew in mats which homogenously covered the entire surface, even when grown in a flow-lane incubator prototype designed for biofilm growth (Bruno et al., 2012). Thus, it is particularly interesting that these motile *Synechocystis* cells display a very unique biofilm phenotype.
This phenotype leads to many other questions. It is not clear why these photosynthetic cells are forming such biofilms. Forming these dense clusters would block the inner cells from light, which would appear to be disadvantageous for the growth of the cells. It is not clear why the cells form these structures, despite this drawback. Perhaps this phenotype is an evolutionary vestige which previously promoted survival. There may also be another unknown advantage to this type of biofilm formation, despite its apparent growth disadvantage. In Antarctic environments, it has been hypothesized that the formation of mat-like biofilms covered by a layer of inorganic material actually protects the cyanobacterium from desiccation and high irradiance in the extreme environment (Davey and Clarke, 1992). This could in part explain the origin of this phenotype, because the *Synechocystis* likely were exposed to extreme environments in the past.

In addition, it is not yet clear how the cells determine the presence of other like cells with which they would like to associate. Likely, the cells have some sort of communication system that the cells use to notify each other that they are present and should form a biofilm. This communication system must somehow induce the phenotypic changes required for aggregation and biofilm formation.

*Scanning electron microscopy of Synechocystis*

In the laboratory, the nonmotile and motile *Synechocystis* cells show very different phenotypes in response to light. The motile cells actually migrate towards the light while the nonmotile cells are completely immobile (Fig 10). In order to try to investigate the differences between these two strains in greater detail, scanning electron microscopy (SEM) was used to image the surface structure of both types of cells (Fig 11). Both cell types seem to be excreting some sort of matrix around all of the cells, including long filaments which actually connect the
cells. These structures are likely pili that have been described as the motility structures of some bacteria (Bhaya et al., 2000). However, the surface of the two types of cells is visibly different. The motile cells have a very smooth surface, with slight ridges. The nonmotile cells have a much rougher surface, with clear indentations across the whole surface. Perhaps the smooth surface of the motile cells allows them to glide along the solid medium surface, and thus allows their motility. The nonmotile cells may adhere better to the solid medium, because of their increased surface area. There is no clear indication of completely new structures, though, which could enhance the motility of the motile cells. The motile cells are also able to form distinctive biofilm-cluster formations. Perhaps their smooth surfaces allow them to aggregate more closely and to attach together in the biofilms.

The surface of the motile *Synechocystis* cells appears to be more similar to that of other *Synechocystis* in previous imaging studies. The surface of *Synechocystis* BAS0670 and BAS0672 cells was shown to be very smooth, perhaps even smoother than the motile cells observed in this investigation, although the exact surface morphology was less clear because of the lower magnification (Ozturk et al., 2009). These two *Synechocystis* strains also show limited matrix-like structures around the cells, some of which connect the cells (Ozturk et al., 2009). Specifically, the surface of cells of *Synechocystis* sp. PCC 6803 were observed with Cryo-SEM, and showed somewhat smooth surfaces, very similar to the motile cells observed in this current investigation (van de Meene et al., 2006). In addition, the matrix-like structures around these cells are even more pronounced and much thicker than in the samples in this current investigation (van de Meene et al., 2006). Thus, the presence of the matrix structure of the *Synechocystis* used here matches previous imaging. However, the differences in surface morphology between the motile and nonmotile cells imaged here is unexpected. The previously
Imaged cells exhibit a very smooth surface, which is more similar to the motile *Synechocystis* cells isolated in the current investigation, which are hypothesized to be a mutant. Perhaps the differences are an artifact of the growth environment, preparation method, or imaging parameters.

**Use of high performance liquid chromatography to test for presence of AHLs**

Reverse phase high performance liquid chromatography (RP-HPLC) was studied for its ability to recognize the presence of AHLs (Schaefer et al., 2000; Teplitski et al., 2003). At first, samples of 5mM Octanoyl and Hexanoyl AHL standards were tested with a 5μL injection volume. These chromatograms did not show any peak. Next, a 20mM Hexanoyl AHL standard sample with a 5μL injection was tested, but no clear peak was visible. Finally, a 20mM Hexanoyl AHL standard sample with a 50μL injection volume was tested (Fig 12). On this chromatogram, a very tiny peak was observed at just over 20 minutes. It is not clear what the very large negative peak at the end of the gradient is; it may be a result of aggregation or nonspecific binding to the column. The amount of AHL present in this sample is 1μmole, which is a high concentration of AHL. Thus, it was determined that this method was not sensitive enough for the detection of AHLs in future samples.

**Use of *C. violaceum* CV026 indicator to test for the presence of AHLs**

**Effect of environment on growth of *C. violaceum* CV026 indicator**

In order to ensure optimal growth of the *C. violaceum* CV026 indicator, the effect of the growth conditions was investigated. In the well assay being used in this investigation, the solvent of all samples was ethyl acetate with 1% acetic acid. Ethyl acetate plus acetic acid ranging in
concentration from 0% to 100% was tested with the *C. violaceum* CV026 indicator with the well assay described above (Fig 14). No effect on growth was seen with the pure ethyl acetate or with up to 25% acetic acid. Not surprisingly, the very high concentrations of acetic acid interrupt the growth of the cells, as indicated by a ring of non-growth around the well. However, in this investigation, only 1% acetic acid was ever used and this concentration did not have any effect on growth. Thus it was determined that these growth conditions were acceptable for *C. violaceum* CV026.

It was hypothesized that the acidity could be detrimental to the growth of the cells, but the effect of acidity is very variable across different bacterial species and across a range of concentrations of acid. Acetic acid in vinegar in particular was shown to decrease growth of cells of *Salmonella aertrycke* by 98% and *Staphylococcus aureus* by 99% at the low pH of 3.3 and acetic acid concentration of 0.15% (Levine and Fellers, 1940). Similarly, the lag phase for growth of *Saccharomyces cerevisiae* was lengthened and growth was significantly inhibited at concentrations of 0.6% acetic acid and 2.5% lactic acid (Narendranath et al., 2001). In contrast, though, acetic acid was actually found to be a carbon source for bacteria in drinking water pipes, and to increase the overall bacterial biomass (Lu and Chu, 2005). The variation in concentrations of different types of acid and different bacterial species shows that acidity has a variety of effects on growth. Thus, it was important to test the effect of acetic acid specifically on *C. violaceum* CV026 indicator growth.

Both ethyl acetate and acetic acid are very volatile compounds. They evaporate readily at room temperature, and even more quickly at elevated temperatures. Thus it is possible that the solvent in these samples evaporated quickly, leaving only the AHL solid, and thus it would have
less of an effect on growth of the cells. The evaporation of the solution may help explain the lack of effect on the growth of the cells.

In addition, the optimal temperature for growth of *C. violaceum* CV026 was determined. The optimal growth temperature was determined to be 29°C (Fig 15). Growth was also very good at 30°C, but growth decreased at lower and higher temperatures. While the *C. violaceum* CV026 was able to grow at all temperatures tested, it recognized the AHL standards with different efficiencies. The most even and darkest purple pigmentation was observed from the cells growing at 29°C, while the purple pigmentation was much splotchier at the other temperatures. This supports the use of 29°C as the optimal temperature for this investigation. The traditional temperature for growth of this indicator is 30°C (McClean et al., 1997), which was found to work well; however 29°C appears to be even better.

**Quantitative recognition of AHLs by *C. violaceum* CV026 indicator**

In order to investigate the ability of the indicator to recognize the Hexanoyl AHL standard at a range of concentrations, the relationship between the radius of purple pigmentation and AHL standard concentration was determined (Fig 16). There appears to be a strong logarithmic correlation between the radius of detection and concentration of AHLs. To ensure that logarithmic fit was the best for the data, a quadratic and linear fit were both attempted but resulted in a much lower $R^2$ value (0.7128 and 0.5545 respectively). Thus, this method of AHL detection is at least partially quantitative. Similarly, calibration curves of violacein pigment production and β-galactosidase activity in response to different concentrations of synthetic AHLs have been used as a method of quantifying changes in AHL concentration over time (Delalande et al., 2005). For the *A. tumefaciens* indicator, quantification of AHLs was determined through a standard curve of AHL concentration to the distance of blue colonies from the AHL origin,
which was determined to have an exponential relationship (Dong et al., 2000). This is in contrast to the logarithmic relationship identified here, but this assay used a different indicator and detection method.

Based on observing the purple pigmentation, it appeared that the width of detection leveled off at a concentration above 50nmoles (2.5mM, in the 20μL sample). There appears to be a limit to diffusion of the AHLs through the agar, because the molecules do not seem to spread beyond about 3cm from the well. The *C. violaceum* CV026 indicator has a limit of detection of 0.08nmoles (2μM, in the 20μL sample). If the AHLs were able to diffuse throughout the agar, the width of purple pigment would increase until the AHLs diluted enough. Thus, the width of purple pigment would continually increase with increasing AHL concentration up to the edge of the plate. However, the leveling off of the diameter of purple pigment indicates that the AHLs are not diffusing all the way to the edge of the plate through the agar, and there must be some limit to their movement.

Another study determined that the minimum quantity for detection of Hexanoyl AHL by the *C. violaceum* CV026 indicator was 0.03nmoles (McClean et al., 1997), which is close to the limit detected here. The response to AHL standards of the indicator *C. violaceum* CV0blu(pSB403) was found to have a minimum detection at 1nM and a saturated response at 20nM (Blosser and Gray, 2000). *E. coli* biosensors based on the LuxR and LasR genetic systems recognized AHLs with a minimum sensitivity of 10nM 3-Oxohexnaoyl and 3-Oxododecanoyl AHL (Lindsay and Ahmer, 2005).

It is not clear from the present investigation how quantitative this assay is for measuring the concentration of AHLs present in a sample. There is a tight logarithmic correlation, which at least hints at a quantitative method. However, in order to more reliably quantify the amount of
AHL present, it may be useful to extract the violacein pigment from the C. violaceum CV026 indicator growth, and to measure its absorbance (Blosser and Gray, 2000). It was found previously that the amount of violacein produced by the C. violaceum CV0blu(pSB403) indicator reached a maximum value, as if the response became saturated at higher AHL concentrations (Blosser and Gray, 2000). These results confirm that in the present investigation, the relationship of AHL concentration to width of recognition is quantitative and reliable.

Versatility of C. violaceum CV026 indicator recognition of a variety of AHLs

For this investigation, it was important that the indicator used could recognize different AHL molecules. Thus, the C. violaceum CV026 indicator was tested for its ability to recognize a range of AHL standards (Fig 17). This indicator successfully recognized the standards N-butyryl-L-Homoserine lactone, N-(β-ketocaproyl)-L-Homoserine lactone, N-hexanoyl-L-Homoserine lactone, and N-octanoyl-L-Homoserine lactone, but not N-decanoyl-L-Homoserine lactone and N-3-oxo-dodecanoyl-L-Homoserine lactone. Thus, it is able to detect AHLs with chain lengths of four, six and eight carbons with or without a carbonyl substituent on the three carbon. These results match exactly with the previously determined versatility of this indicator. C. violaceum CV026 was previously found to recognize chains of four, six and eight carbons all with carbonyl substituents on the three carbon (McClean et al., 1997). Similarly, this indicator was not able to detect AHLs of ten, twelve or fourteen carbons with or without substituents on the three carbon (McClean et al., 1997).

The indicator detects these AHLs with different amounts of sensitivity. All of the AHL standards used in this investigation were tested with the same concentration, but the width of purple pigmentation varied. Based on the width and darkness of purple pigment around the samples, the C. violaceum CV026 indicator clearly recognizes the six carbon chain best,
followed by the four carbon chain and finally the eight carbon chain. McClean et al. (1997) similarly found that the indicator was most sensitive to the Hexanoyl AHL and was less sensitive to AHLs with a carbonyl substituent on the three carbon, but in contrast, found that the indicator recognized the Octanoyl AHL at a lower concentration than the Butanoyl AHL standards. It is not surprising that the *C. violaceum* CV026 indicator recognizes the Hexanoyl AHL the best, because this is the AHL produced by the wild type *C. violaceum*. Similarly, it has been found that *E. coli* biosensors recognize the cognate molecule of the wild type most effectively, followed by the closest analogue, and all in a quantitative manner (Winson et al., 1998). All of these data support the idea that both the length and composition of the carbon chain is important in allowing the indicator species to recognize the AHLs.

The AHLs recognized by this indicator are short and middle range AHLs, so the indicator is useful for testing for the presence of these AHLs in biological samples. It would not be effective for detecting long chain AHLs. In a comparison of four major biosensors, *C. violaceum* CV026 was determined to be very sensitive to all short and medium-chain length AHLs, although not as sensitive as the *A. tumefaciens* reporter (Cha et al., 1998). Most bacterial biosensors are similarly limited in their sensitivity to different AHLs (Steindler and Venturi, 2007).

**Tests for the presence of acyl homoserine lactones in bacterial samples**

The use of the *C. violaceum* CV026 indicator to test for naturally produced AHLs was investigated first with the wild type *C. violaceum* cells. The untouched supernatant and the extracted supernatant of a 25mL culture of the wild type cells were tested for the presence of AHLs with the indicator strain (Fig 18). Both samples caused purple pigmentation, indicating the
presence of AHLs. The purple pigmentation in the supernatant sample indicates that the AHLs stayed in the supernatant during centrifugation and therefore were secreted by the cells and not attached to them. The increase in width of purple pigmentation after extraction shows that the extraction purified and concentrated the AHLs present. It was estimated, based on the width of purple pigmentation, that the supernatant samples contained 0.10nmoles of AHL in each well and the extracted samples contained 0.20nmoles of AHL in each well. The liquid culture of the *C. violaceum* wild type is purple naturally because the cells are able to both produce and recognize the AHLs. Thus, it was known that the tested culture contained AHLs. Given the dense purple color of the wild type culture, it was interesting that the samples contained such a small concentration of AHLs.

The extraction procedure is a common procedure used to concentrate AHLs, because they are often present in such a small concentration (Schaefer et al., 2000; Shaw et al., 1997). Our results from the extraction procedure support the use of extraction. The extraction with ethyl acetate was determined to be more effective than extraction with C18 cartridges, but even ethyl acetate extraction recovers longer chain AHLs much more effectively than short chain AHLs like Butanoyl AHL and more hydrophilic, oxygen-modified AHLs (Teplitski et al., 2003). Thus the extraction procedure is variable depending on the samples used.

*Presence of AHLs in growing Synechocystis cells*

After the investigation of the wild type *C. violaceum*, the secretion of AHLs by *Synechocystis* cells was tested. *Synechocystis* cells growing in liquid medium, in the biofilm flow cells and on solid medium were all investigated using the *C. violaceum* CV026 indicator for the presence of AHLs. A dense liquid culture of 250mL *Synechocystis* was centrifuged and the supernatant was extracted as described above (Fig 19). The effluent (500mL) from a biofilm
flow-cell was also extracted with acidified ethyl acetate, and tested with the indicator strain (Fig 20). Finally, the indicator strain was overlaid on solid medium growing *Synechocystis* cells (Fig 21). The *C. violaceum* CV026 indicator did not detect AHLs in any of these three samples. This analysis indicates that the motile *Synechocystis* cells are not secreting an AHL that can be recognized by this indicator in a high enough concentration. In addition to simple lack of production of AHLs, there are several other possible explanations for this lack of recognition, which will be discussed below.

**Stability of AHLs**

The biosensor is only able to recognize AHLs if the structure is untouched, specifically if the ring is closed and the chain is intact. Thus, it is possible that the *Synechocystis* are producing AHLs which are being degraded and, therefore, are not recognized by the *C. violaceum* CV026 indicator.

First the stability of the AHLs in the effluent from the biofilm flow cells was tested. The Hexanoyl AHL standard was added to an effluent sample. Half the sample was extracted immediately and half was extracted after 3.5 weeks, and the presence of AHLs was tested in both (Fig 22). It was found that the width of purple pigment of the *C. violaceum* CV026 indicator did not change over time, indicating that the concentration of AHLs did not change over time and thus the AHLs did not degrade in the supernatant in 3.5 weeks time.

Next, the stability of the Hexanoyl and Octanoyl AHL standards in BG-11 medium and in growing *Synechocystis* cells was investigated. The AHL standards were added to pre-acidified ethyl acetate, BG-11 medium and growing cyanobacterial cells. After four days, the samples were extracted and tested for the presence of AHLs (Fig 23). Based on the amount of Hexanoyl
AHL added, it was expected that there would be 0.1nmole in each well, which would correspond to about a 0.3cm width of purple pigment. The width around the ethyl acetate sample is actually slightly wider, but this could be due to evaporation of the ethyl acetate and thus concentration of the AHL. For the Hexanoyl AHL samples, the width of purple pigmentation around the culture and the medium were both about 0.3cm, and were slightly smaller than the ethyl acetate sample. It is important to note that the width of purple pigment around the BG-11 and Synechocystis cells wells is identical. Thus it appears that the AHLs did not degrade at all over time, and particularly that the cells did not interfere with the AHL structure. The same pattern is apparent for the Octanoyl AHL. The purple pigmentation for the Octanoyl AHL-containing wells was less dark and less wide than that for the Hexanoyl AHL-containing wells because the C. violaceum CV026 indicator is less sensitive to Octanoyl AHL than to Hexanoyl AHL.

The same assay was repeated without pre-acidification of the BG-11 medium and Synechocystis cells. Octanoyl AHL was added to acidified ethyl acetate, unacidified BG-11 medium and unacidified motile Synechocystis cells. The samples were extracted periodically and tested for the presence of AHLs (Fig 24). Initially, all three samples contained some AHLs, although in different amounts. Because this first extraction was done immediately after addition of AHLs, the different widths of purple pigmentation are likely not due to different amounts of AHLs. At Day 3 though, only the ethyl acetate control still contained AHLs and none seemed to be left in the BG-11 medium and Synechocystis culture. These results indicate the importance of pre-acidifying the samples to ensure stability of AHL. Even though acid was added later during the extraction procedure, it likely did not allow rescue of the AHL rings.

One factor affecting AHL stability is pH. An acidic environment is preferable, because the lactone ring will open under basic pH. AHLs were not detectable in Yersinia
*pseudotuberculosis* as the pH increased from 7.1 to 8.5, but AHLs could be recovered if the pH was lowered to 3.0 or if the pH of the culture was initially adjusted to 7.0 (Yates et al., 2002). The rate of ring opening and the pH range at which ring opening begins very much depends on the chain composition of the AHL (Yates et al., 2002). Specifically, the rate of ring opening decreases with increased length of the AHL carbon chain, while the addition of a carbonyl group on the three carbon increases the rate of opening (Yates et al., 2002). For 3-Oxohexanoyl AHL, the half-life was determined to be 1 day at pH 7.0 and 2-3 hours at pH 8.0 (personal communication in Schaefer et al., 2000). The half life of Hexanoyl AHL was found to range from 4 hours to 21 days depending on the conditions, and to increase at higher temperature and lower pH. Specifically, at a pH of 6.5 and 28°C, the half life was four days (Delalande et al., 2005). In comparison, the half life of 3-Oxooctanoyl AHL was estimated to be 7 hours at pH 8.0 and 30°C (Delalande et al., 2005). The lactonolysis process is reversible in acidic conditions, although the exact mechanism for ring closure is not simply the reverse of the ring opening (Schaefer et al., 2000; Yates et al., 2002).

Specifically, in a *Y. pseudotuberculosis* culture, it was found that the AHLs degraded as the culture grew because the pH naturally increased from 7.0 to 8.5 (Yates et al., 2002). Buffering a culture at a lower pH, for example 6.8, seems to increase stability of the AHLs (Byers et al., 2002). The initial addition of acid to the effluent of the biofilm flow-cell set up in this investigation would ensure that any AHLs were almost immediately put into acidic conditions, and would have little time to stay in the slightly basic BG-11 medium in the flow cells. Thus, any AHLs produced by the *Synechocystis* cells in the biofilm flow cells were likely not degraded, eliminating this possibility for why AHLs were not seen. However, the
*Synechocystis* liquid culture which was tested was not buffered and was likely at a slightly basic pH. This could explain why no AHLs were detected in that sample.

The process of natural degradation is relevant in soil samples. It has been shown that Hexanoyl and Oxohexanoyl AHLs were decomposed in soil samples at a maximum rate of 13.4 nmoles per hour even under protein synthesis inhibition conditions, indicating that the degradation was likely not enzymatic (Wang and Leadbetter, 2005). Again, the exact rate of degradation and optimal temperature and pH varied depending on the structure of the AHL (Wang and Leadbetter, 2005). This process of signal decay over time may serve different purposes. The decay could ensure that the AHLs in the environment are accurately representative of the cells still present (Wang and Leadbetter, 2005). However, it could also be a competitive mechanism used to hinder growth of other species or to insulate one population from molecules produced by other cells, which could be beneficial or harmful (Wang and Leadbetter, 2005).

**Response of *Synechocystis* to AHL standards**

Although AHLs were not identified as being naturally produced by the *Synechocystis* cells, it was also investigated whether the cells can respond to the artificial addition of AHL standards. First, the Hexanoyl and Octanoyl AHL standards were added to liquid cultures of motile *Synechocystis* cells (Fig 25). There did not appear to be a pattern in terms of the effect of the AHL standards on the growth of the cells. In many of the samples, the cells did appear to clump and even form dense rings of biofilm on the glass. However, this occurred even in the control tubes without any additions and did not seem to be related to the concentration or identity of the AHL present.
AHLs were also artificially put adjacent to growth of motile and nonmotile *Synechocystis* cells growing on solid medium. It appears as if the motile cells migrate towards Octanoyl AHL but away from Hexanoyl AHL, although the pattern was not always uniform (Fig 26). When the Hexanoyl and Octanoyl AHLs were tested on different plates, the phenotype was very obvious and uniform. The cells on both parallel streaks moved either all away or all towards the standards. However, when the two standards were tested on the same plate, the pattern was less uniform. Some cells moved towards the Octanoyl AHL, but some did not move at all. Also, many cells actually moved towards the Hexanoyl AHL and towards the acidified ethyl acetate solvent. This second replicate, with two standards on one plate, was meant to eliminate differences in the medium, light intensity and location, which may account for the different results. It is not yet clear how the cells recognize and respond to these AHL standards.

The nonmotile strain was also tested for its ability to recognize and respond to AHL standards (Fig 27). The nonmotile cells did not have any change in phenotype due to the presence of AHLs. Their growth pattern did not change and they did not move. This indicates that the AHLs do not cause a visible change in phenotype in the nonmotile cells.

In other investigations, the artificial addition of AHL standards was found to induce a variety of phenotypes. The addition of AHLs to the cyanobacterium *Anabaena* caused inhibition of cell growth by inhibiting heterocyst formation and thus nitrogen fixation (Romero et al., 2011). Specifically, 3-Oxododecanoyl AHL was especially toxic to this cyanobacterium because of its inhibition of nitrogen metabolism. The addition of synthetic AHLs to a non-AHL producing mutant of *Pseudomonas putida* actually changed the biofilm structure to more homogenous and unstructured biofilms, in contrast to the microcolonies formed without the presence of AHLs (Steidle et al., 2002).
The addition of synthetic AHLs to human oral squamous carcinoma cells was actually found to have an effect on proliferation and apoptosis (Chai et al., 2012). Differently modified Dodecanoyl AHLs were found to suppress growth of two different cell lines and to induce apoptosis in those cell lines (Chai et al., 2012). Dodecanoyl AHL was also found to stimulate immunoresponse (specifically the production of cytokines) in mouse and human monocytic cells, but shorter chain AHLs were not found to have any phenotypic effect (Gomi et al., 2006). This indicates that the AHL chain is important for inducing phenotypic change.

A more clinically relevant examination was done on the effect of the 3-Oxododecanoyl AHL produced by P. aeruginosa on lung fibroblasts and epithelial cells (Smith et al., 2001). This AHL was found to stimulate interleukin production in human fibroblasts and epithelial cells in vitro, which supports the idea that the lung damage caused by P. aeruginosa infections is caused by excessive neutrophil response stimulated by 3-Oxododecanoyl AHL at a minimum concentration of 25μM (Smith et al., 2001). 3-Oxododecanoyl AHL was also able to induce apoptosis in bone-marrow derived macrophages and neutrophils (Tateda et al., 2003). These investigations of the effect of different AHLs on different cell types allow an increased understanding of the broad implications of AHL signaling, and can contribute to knowledge specifically on the pathogenesis of bacterial infections.

**Genetics of quorum sensing and quorum quenching**

*Genetic quorum sensing system*

In order to identify candidate quorum sensing proteins in *Synechocystis*, known quorum sensing protein sequences were used to search for homologous sequences in the *Synechocystis* genome (Table 2). Each of the known sequences from other bacterial species matched to a
unique protein in the *Synechocystis* genome. The identity was relatively consistent, ranging only from 25-38%. This identity appears to be rather low. In a comparison of two known quorum sensing proteins from *V. fischeri* and *A. tumefaciens*, though, the identity is only 24% (Table 3). However, the query coverage for this alignment is 95%, which is much higher than the query coverage of the *Synechocystis* searches. The E values for the *Synechocystis* searches range significantly from $4 \times 10^{-8}$ up to 0.44, meaning that these homologies vary in reliability. The E value for the alignment of *V. fischeri* and *A. tumefaciens* is very low, at $2 \times 10^{-13}$. Thus, this investigation identified several possible candidates for a quorum sensing system in *Synechocystis*, albeit with low query coverage and middle-range E values.

The specific regions of homology between the known LuxR proteins and the *Synechocystis* proteins were also studied. The region of the *V. fischeri* LuxR which is homologous to the *Synechocystis* proteins is specifically residues 157-230. In the LuxR protein of *V. fischeri*, residues 184-230 are known to be important for DNA sequence recognition and transcriptional regulation (Slock et al., 1990). This is evidence that the homologous *Synechocystis* protein may have transcriptional regulation functions. Half of the proteins identified are part of the NarL subfamily, which is known to include proteins involved in transcriptional response regulation. Similarly, LuxR is involved in targeting gene transcription (Waters and Bassler, 2005). Thus it is possible that these candidate *Synechocystis* proteins could be involved in functions related to quorum sensing and regulation. A LuxR type transcriptional regulator PedR (ssl0564) has been previously identified in *Synechocystis*; this protein works as a sensor linking the perception of reducing equivalents in the environment to transcriptional regulation of the electron transport chain (Nakamura and Hihara, 2006). Other than this example,
which is not specifically related to AHL quorum sensing, other homologues have not yet been identified in *Synechocystis*.

**Genetic quorum quenching system**

In addition, candidate quorum quenching proteins were searched for in *Synechocystis* (Table 4). Several different known acylases and one known lactonase from different species were used to search for possible quorum quenching proteins in *Synechocystis*. All of the acylase proteins tested matched with the 7-beta-(4-carboxybutanamido) cephalosporanic acid acylase in *Synechocystis* with an identity of 22-31%. For these alignments, all of the query coverages were over 80% except for one, and all of the E values were below $4 \times 10^{-10}$, indicating high confidence in this match.

The role of this acylase would presumably be to degrade AHLs present in the sample. Thus, it is possible that even if the *Synechocystis* cells were producing an AHL, the acylase may have degraded it before it could be recognized by the *C. violaceum* CV026 indicator even though it was present. However, it is not guaranteed that the acylase was even expressed regularly. Even if it was expressed, it likely did not degrade all of the AHLs present, especially given the number of replicates tested of different growth systems. Finally, it is also possible that the acylase exists only as a competitive mechanism, and may selectively degrade AHLs produced by other species and not the *Synechocystis*’ own AHLs. Thus the presence of this acylase does not guarantee degradation of the *Synechocystis* signaling molecules, but its function is not yet known.

A similar method to the one used here to identify potential quorum sensing proteins was used to identify potential hydrolases in *A. tumefaciens*. Using the known sequence of the *Bacillus* lactonase aiiA, a BLAST search was used to look for homologous proteins in *A. tumefaciens* (Carlier et al., 2003). Three putative hydrolases were identified, and two were discovered to have
AHL degradation activity (Carlier et al., 2003). Thus, this method is an effective means of identifying novel proteins.

Alignment of nonmotile and motile Synechocystis genomes

In order to further analyze the differences between the nonmotile and motile Synechocystis cells, DNA from both cell types was extracted and full genome sequencing was obtained. The two genomes were aligned using Geneious software. Particular pilus related and chemotaxis genes were searched for in each genome, and compared for the numbers of copies present (Table 5). All but three genes showed the exact same number of gene copies present in both the nonmotile and motile genomes. The twitching motility protein PilT has an extra copy present in the motile genome. One copy of this gene is present at approximately 2.20 megabases in both genomes, which is the expected location of the PilT1 gene (http://genome.microbedb.jp/cyanobase/Synechocystis/genes/slr0161). However, there is an additional fragment at about 2.05 megabases in the motile genome which is not present in the nonmotile genome. This fragment corresponds to the known location of the PilT2 gene (http://genome.microbedb.jp/cyanobase/Synechocystis/genes/sll1533). The PilT protein is known to be involved in pilus production and cell motility (Okamoto and Ohmori, 2002; Bhaya et al., 2000). Thus, it is possible that this gene is involved in the difference in the motility phenotype between the two strains in this current investigation.

In addition, two chemotaxis related proteins were found to be present in the motile genome but not in the nonmotile genome. The methyl accepting chemotaxis protein II was found at approximately 3.1 megabases in the motile genome. Homologues of this protein are present in other bacterial species, and it is known to be a membrane protein which is methylated to allow bacterial adaptation to environmental cues, which could include light (Yoshihara et al., 2000). It
was found that mutants of this protein did have decreased positive phototaxis (Yoshihara et al., 2000). The chemotaxis protein was identified at about 491 kilobases, but it is not clear which exact protein was identified. At this approximate location in the genome, there are proteins identified for a two-component response regulator, a CheW-like protein required for motility and a methyl-accepting chemotaxis protein. These three differences could help to explain the differences in phenotype between the motile and nonmotile strains. A recent study investigated horizontal gene transfer among cyanobacterial species. It was found that about 50% of gene families in cyanobacteria have a history of horizontal gene transfer either between cyanobacteria and other phyla or within cyanobacteria (Zhaxybayeva et al., 2013). This indicates that gene transfer has likely affected the evolutionarily history of cyanobacteria, and thus has increased the complexity of the genome. It is important to note that any genomic analyses are made more complicated by the transfer of genes across many different bacterial species.

Conclusions and Future Directions

The goals of this project were to investigate the physical structure and communication system of Synechocystis biofilms. Towards the first goal, confocal imaging was performed on motile Synechocystis biofilm structures. These cells form very distinct cluster structures, which grow taller and wider over time. In order to determine the communication system used by the cells in the process of biofilm formation, the C. violaceum CV026 indicator strain was characterized for its use in recognizing AHL signaling molecules. This indicator effectively and quantitatively recognizes a variety of AHLs. Thus, it is an effective method for detecting the presence of specific AHLs.
However, no AHLs were detected by this indicator in *Synechocystis* growing in liquid culture, in biofilm flow-cells or on solid medium. It was also determined that the lack of recognition of AHLs by the *C. violaceum* CV026 indicator was not due to degradation of the AHLs. It was shown that AHL standards are stable in pre-acidified effluent from the biofilm flow-cells, pre-acidified medium and pre-acidified *Synechocystis* cell culture. The effect of acidity could be analyzed further, in order to quantify the stability of AHLs in relation to pH.

This investigation showed that the *Synechocystis* cells are not producing one of the AHLs recognized by the *C. violaceum* CV026 indicator in a high enough concentration. This leads to several new questions, because the communication system used by the *Synechocystis* cells is still unknown. The cells could be using a different AHL molecule, which would not have been recognized by this indicator. It is also possible that the cells are using an entirely different communications system, which does not involve AHLs. For example, biofilm formation in another cyanobacterial species was actually found to be a result of suppression, as opposed to production of another signaling molecule. A mutant of *Synechococcus elongates* was found to gain a biofilm forming phenotype as a result of inactivation of the type II secretion/type IV pilus assembly systems, which in the wild type bacterial cells likely helps to secrete an inhibitory compound to the formation of biofilms; the authors hypothesize this system may have evolved to prevent aggregation and maximize light absorption for photosynthesis (Schatz et al., 2012).

Another common signaling molecule is cyclic di-GMP. This molecule is known to be involved in many bacterial signaling pathways, including those involved in biofilm formation (Romling et al., 2013). Cyclic di-GMP promotes biofilm formation through regulation of different aspects of biofilm formation, including the motility-sessility transition and extracellular polysaccharide
production (Romling et al., 2013). Perhaps the *Synechocystis* cells also use a completely novel method of communication.

*Synechocystis* cells appear to respond to the presence of AHL standards. Growing on solid medium, the cells respond positively to Octanoyl AHL, negatively to Hexanoyl AHL and do not respond to 3-Oxododecanoyl AHL. This response could be analyzed in greater detail by testing more AHL standards and different concentrations.

Finally, potential quorum sensing and quorum quenching system proteins were identified in the *Synechocystis* genome. There are several candidate quorum sensing proteins present. The exact function of these proteins could be elucidated in greater detail in order to determine if they are involved in AHL production or in another quorum sensing system. A similar approach was used for *A. tumefaciens*, in which three putative hydrolases were expressed in *E. coli* to determine their function (Carlier et al., 2003). In addition, an acylase protein was identified in *Synechocystis*. It is not yet clear what the target of this acylase is, and if it could have an impact on the quorum sensing system of *Synechocystis* in their biofilm formation.
References


Appendices

Appendix A – Biofilm growth in flow cells using *Synechocystis* sp. Strain PCC 6803
Modified from protocol outlined by Michelle Xu, Wellesley College

**PROTOCOL** for growing and analyzing *Synechocystis* sp. Strain PCC 6803 biofilms in flow cells
[v]: subject to variation depending on experiment

**Materials**

Inoculum of *Synechocystis* sp. Strain PCC 6803
70% and 96% (v/v) ethanol
BG-11 medium
3M Super Silicone Sealant Clear glue
Flow cell system (3 channels)
1mL sterile syringes
23-G syringe needles
Clamps
Fisher Scientific Microscope Cover Glass 12-544-E 24X50 #1.5
1000mL autoclavable glass bottles
Glass bottle lids with 4 holes of 5-mm diameter
Helix Mark (60-805-08 50ft) Standard Silicone tubing; ID 0.062”, OD 0.125”
  4” for air vent
  15” for media bottle to pump
  25” from bubble trap to flow cell
  18” for flow cell to waste bottle
Watson Marlow 205S Peristaltic Pump
Stovall Life Science Incorporated Bubble traps (Ref: ACCFL0002)
Watson Marlow Marprene Manifold Tubing (978.0102.000), 18 inch
Straight T-connectors, 1/16” + 1/16”: Cole Parmer Model 6365-90
Scalpel
Metal (Aluminum) foil
Micropipette tips
Confocal Laser Scanning Microscope (CLSM)

**Constructing the Flow Cell**

1. Apply beads of silicone glue along the surface of the flow cell frame with channels facing upright
2. Use a micropipette tip and spread the glue along the frames of the channel taking care not to get silicone in the channels
3. Set a cover glass on the silicone to seal the channels
4. Press the cover slip into place using the tip of the micropipette tip in a gentle tapping motion
5. Be cautious to not get fingerprints on the coverslip
6. Let constructed flow cell sit overnight to dry
Assembling Flow Cell System

1. Insert the short silicone tubing into a hole in the lid\(^1\)
2. Cover short tubing on top with metal foil
3. Insert the longer silicone tubing(s) into hole(s) in the lid\(^2\)
4. Cap the lid with tubing onto the medium bottle\(^3\)
5. Attach ends of the silicone tubing (that is now lying exterior to the medium bottle) to T-connectors
6. Connect the Marprene tubing(s) to the other side of the T-connector(s)
7. Run Marprene tubing through the peristaltic pump with the white holders inside the hook on the pump\(^4\)
8. Connect the end(s) of the Marprene tubing(s) to the channel(s) on the bubble trap
9. Connect another line of silicone tubing from the bubble trap to the flow cell channel inlet
10. Attach silicone tubing from the outlet(s) into the hole(s) of the waste bottle lid\(^5\)

Sterilizing Components of Flow Cell

1. Ensure the appropriate amount of medium is present in the bottle and that all connections are tight.
2. Cover the bubble chambers and flow cells with aluminum foil.
3. Autoclave entire system with a slow liquid cycle.
4. Run medium through set-up at 1rpm to ensure no leakage

Inoculating the Flow Cell

1. Stop the medium flow in flow system
2. Clamp silicone tubing near inlet of flow cell and after bubble trap\(^6\)
3. Sterilize tubing near inlet with 70% ethanol
4. Load a 1.0mL sterile syringe (23-G syringe needle) with inoculum of *Synechocystis* sp. Strain PCC 6803 at an optical density of 1.0\(\text{v}\) to 1mL
5. Tap syringe until bubbles are removed
6. Penetrate silicone tubing near inlet with syringe needle *at an angle* taking care not to penetrate the opposite end of tubing
7. Inject about 300μL inoculum slowly into the flow chamber
8. Reseal tubing by applying silicone glue above the needle during injection
9. Slowly remove needle taking care not to inject bubbles into the flow!
10. Allow flow cell to sit upright for 2 hours.
11. After 2 hours, remove clamps and turn the flow rate on to resume BG-11 flow

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\(^1\) Short tubing acts as an air vent. Length of tube is short enough to not reach into the liquid

\(^2\) Tubing allows medium flow. Long enough to almost reach the bottom of the medium bottle

\(^3\) DO NOT contaminate the tubing by making sure the lines do not touch the exterior of the bottle.

\(^4\) This prevents the Marprene tubing from being pulled into the pump when the flow is running.

\(^5\) Make sure the tubing end does not dangle in the waste

\(^6\) Clamping prevents backflow of inoculum
Running the Flow Cell System

1. Turn peristaltic pump on to a flow rate of 1rpm
2. Turn bubble trap valves open to allow medium to flow half way up the bubble trap, then turn valve to the close position
3. While running the system initially, check periodically for leakage.

Changing Bottles for extended experiments

1. Prepare fresh media bottle and sterilize by autoclaving
2. Turn off peristaltic pump
3. Turn lid until it unscrews from bottle
4. Gently pull the lid with hanging silicone tubing
5. Switch the old media bottle with a new one and gently lower the tubing(s)
6. Screw lid back onto new media bottle
7. Resume flow rate on peristaltic pump.

Imaging biofilms using Confocal Laser Scanning Microscopy (CLSM)

1. Clamp inlet and outlet of each channel tubing of flow cell
2. Turn off peristaltic pump
3. Mount flow cell onto the stage of the CLSM microscope
4. Hold flow cell in place with tape
5. Begin imaging from the center of chamber
6. HeNe 633nm laser was used to excite chlorophyll a in cyanobacteria

Disassembling Flow Cell

1. Disassemble flow cell
2. Soak pieces in bleach solution
3. Use rod to dislocate cells from walls of tubing

O.D. for injection
Desired absorbance at 750nm: 1.0 A

1. Dilute sample to a reading of less than 0.4 A750nm on spectrophotometer. Multiply by dilution factor to obtain actual A750.
2. X mL to make 1.0A

(Absorbance from Spectrophotometer)(X mL) = (1mL)(1.0A)
Plug in knowns to obtain how much of the sample needed to create the desired absorbance

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7 Do not allow the silicone tubing to touch any exterior surfaces
8 Do not allow the silicone tubing to touch any exterior surfaces
Diagram of Flow Cell Setup including 1x and 2x flow
Appendix B – Biological sample preparation for scanning electron microscopy observation

Instrumentation Center of Natural Sciences and Mathematics Outreach S.C.O.P.E. Program
University of Toledo, Toledo, OH

Samples:
Sample N and NM (Synechocystis sp. Strain PCC 6803) on culture plates grown for one week, were prepared in the same way within 2 days of their arrival.

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.
Appendix C – Additional growth curve replicates of *C. violaceum* CV026 indicator

\[ y = 1.7876e^{0.6665x} \]
\[ R^2 = 0.982 \]

\[ y = 1.2627e^{0.7473x} \]
\[ R^2 = 0.9912 \]
Additional growth curve replicate of *Synechocystis*

![Graph showing growth curve]

\[ y = 124.56e^{0.0035x} \]

\[ R^2 = 0.9791 \]
Appendix D – Additional confocal microscopy images of motile *Synechocystis* biofilms

Day 3

Day 4

Day 6
Appendix E – Additional scanning electron microscopy images of *Synechocystis* cells

Motile cells:

![Motile cells at 5,000X magnification](image1)

![Motile cells at 18,000X magnification](image2)

Nonmotile cells:

![Nonmotile cells at 7,500X magnification](image3)

![Nonmotile cells at 16,000X magnification](image4)

![Nonmotile cells at 27,000X magnification](image5)