BACTERIAL UTILIZATION OF LIGHT:
RHODOPSINS AND CAROTENOIDS

by

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RHODOPSINS AND CAROTENOIDS

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ABSTRACT

Sunlight is the most abundant and sustainable resource of energy available. Organisms that can use sunlight for phototrophy fall into two categories: photoautotrophs and photoheterotrophs. Photoautotrophs are organisms that use light to carry out photosynthesis, utilizing carbon dioxide as their principal carbon source. Photoheterotrophs use light energy to break down organic carbon compounds from the environment to drive ATP synthesis. In particular, rhodopsin-based photoheterotrophic bacteria use a rhodopsin-retinal protein complex to capture light and potentially establish a transmembrane ion gradient, which could drive ATP synthesis. In addition, there are other ways for organisms to use sunlight. Some phototrophic organisms can utilize accessory molecules, such as carotenoids, as light-harvesting antennae for photosynthesis. Here, we discuss two ways bacteria can use light through rhodopsins and carotenoids.

Microbial rhodopsins are a family of transmembrane proteins, with photosensitive retinal cofactors, found in every domain of life. Rhodopsins respond to light by transporting ions across the cell membrane or by initiating a signaling cascade that leads to altered gene expression. Rhodopsins are abundant in nature, and recent estimates indicate that up to 70% of microbial cells in some aquatic environments possess rhodopsin genes, suggesting that more bacteria utilize sunlight than previously thought. However, these estimates are based on gene abundances, not direct observation. In order to determine the abundance of functional rhodopsins, visualization of these low-fluorescing proteins is essential. We recently developed a
method that uses total internal reflection fluorescence (TIRF) microscopy to identify rhodopsin-containing cells in environmental samples. Here, we use TIRF microscopy to quantify the total number of rhodopsin-containing cells in water samples collected along the Chesapeake Bay, demonstrating that rhodopsin production is correlated with daylight and salinity. Approximately up to 60 percent of cells produce functional rhodopsins; therefore, microbial capture and utilization of sunlight by rhodopsin-type photosystems is likely common throughout this estuary.

Carotenoids are pigmented, organic compounds that have been used in industry for years as food colorants, with up to 750 different carotenoids known today. The most abundant form of carotenoids are 40-carbon molecules (C40), but 50 carbon molecules (C50) exist as well. While most C40 carotenoid biosynthetic pathways have been well characterized, C50 pathways have not. Using genomic and phylogenetic analyses, we have identified two potential genes involved in the biosynthesis of a C50 carotenoid, bacterioruberin, in *Rhodoluna lacicola*. When the pathway is expressed in *Escherichia coli*, bacterioruberin is produced.

Overall, these insights into two different methods by which bacteria use sunlight can enhance our ability to produce alternative energy source technologies focused on biological organisms and their ability to generate power in the form of electron gradients.
Chapter 1

INTRODUCTION

1.1 Bacterial Utilization of Light

Sunlight is the most abundant and sustainable source of energy available. There are two main types of organisms that can use sunlight for phototrophy: photoautotrophs and photoheterotrophs. Photoautotrophs are organisms that carry out photosynthesis, utilizing carbon dioxide as their principal carbon source. Oxygenic photosynthesis, found in cyanobacteria and plants, uses chlorophyll a (Chl $a$) for light-energy capture for carbon fixation (Miyashita et al. 1997; Blankenship & Hartman 1998). However, of all the available energy in sunlight, only about 0.15% is converted by Chl $a$-dependent photosynthesis to chemical energy (Blankenship et al. 2011), and Chl $a$ is not the only means by which organisms are able to capture and utilize light. Anoxygenic photoautotrophic organisms, such as green sulfur bacteria, use bacteriochlorophyll for light-energy capture, but oxidize inorganic compounds, such as sulfur, rather than water (Bryant & Frigaard 2006). Photoheterotrophs are organisms that use light energy to break down organic carbon compounds from the environment to drive ATP synthesis. Rhodopsin-based phototrophic bacteria use a rhodopsin-retinal protein complex to capture light and establish a proton motive force (PMF), which could be used to drive ATP synthesis (Hartmann et al. 1980; Martinez et al. 2007).

There are other ways for organisms to use light aside from metabolic processes. Some organisms, such as bacteria and plants, are able to sense light and
move toward it. This is referred to as phototaxis, which is a way for organisms to actively move towards increasing light intensity to more effectively receive light for photosynthesis (Jékely et al. 2008). Photolyases are small, photoactivated enzymes capable of repairing DNA damaged by UV light and modulating circadian rhythms (Sancar 2003). In addition, phototrophic organisms can use accessory molecules to enhance light-capture for phototrophy. Carotenoids are pigmented, organic compounds that serve as light-harvesting antenna for photosynthesis and some retinal-based proton pumping rhodopsins (Imasheva et al. 2011; Heider et al. 2014).

Here, we explore two specific ways bacteria can utilize light by rhodopsins and carotenoids.

1.2 Rhodopsins

Rhodopsins are a family of light-sensing transmembrane proteins, consisting of seven alpha helices, with a retinal molecule covalently attached to the apoprotein via a protonated Schiff base formed with a lysine (Kandori 2015; Choi et al. 2014; Alexiev & Farrens 2014; Gerwert et al. 2014). They are found in every domain of life and are classified into two types: animal and microbial rhodopsins (Kandori 2015). Animal rhodopsins are specifically photosensory receptors, contain a 11-cis retinal cofactor (Figure 1.1), and are G-protein coupled receptors (Ernst et al. 2014). Microbial rhodopsins contain an all-trans retinal cofactor (Figure 1.1) and have a wide range of functions. Microbial rhodopsin functions include photosensory receptors, light activated transcriptional regulators, photoactivated enzymes, light-driven ion pumps, and light-gated ion channels (Kandori 2015) (Figure 1.2). Upon absorption of a photon, the retinal cofactor undergoes a conformational change, which initiates a cyclic reaction of the covalently bound retinal molecule isomerizing from an all-trans
to a 13-\textit{cis} orientation (Alexiev & Farrens 2014). This conformational change drives either the transport of an ion across the membrane or the transfer of information via protein-protein interactions and regulation of gene expression in response to light (Irieda et al. 2012; Keffer et al. 2015; Spudich et al. 2000).

\textbf{Figure 1.1} Retinal cofactors of microbial (left) and animal (right) rhodopsins produced from oxidative cleavage of beta-carotene (adapted from Kandori 2015).
Figure 1.2 Functions of microbial rhodopsins as pumps, channels, and light sensors. Arrows indicate the direction of transport or flow of signal. Green and blue arrows represent energy conversion and signal transduction, respectively (adapted from Kandori 2015).

A proton pumping rhodopsin, bacteriorhodopsin, was the first to be isolated and identified from *Halobacterium salinarum* in 1971, and has since become the most well-characterized rhodopsin type (Grote et al. 2014; Kandori 2015). Proton-pumping rhodopsins contribute to the proton motive force (PMF), and have been hypothesized to aid in cell motility or produce ATP. The *Anabaena* sensory rhodopsin (ASR), isolated in 2003 from freshwater cyanobacteria, is co-transcribed with a cytoplasmic protein called the ASR transducer (ASRT) (Jung et al. 2003; Irieda et al. 2012). ASR has become a well-studied sensory rhodopsin, and has been found to act as a transcriptional regulator (Irieda et al. 2012). Other sensory rhodopsin functions include signal transduction via phosphorylation cascades or soluble transducers that utilize protein-protein interactions (Spudich 2006).

Given the diversity of rhodopsin function, it is not surprising that rhodopsins are widespread in illuminated environments such as marine, terrestrial and freshwater environments (Hahn et al. 2014; Lami et al. 2009; Campbell et al. 2008; Sharma et al. 2009). SAR11 Proteobacteria, containing proteorhodopsins, have been the main focus
of previous studies done in marine environments, while Actinobacteria, containing actinorhodopsins, have been the main focus of freshwater studies, because of their corresponding high abundances in each (Sharma et al. 2008; Campbell et al. 2008).

Previous studies using cultivation, metagenomic sequencing, targeted amplicon sequencing, and quantitative PCR (qPCR) have each been used to identify rhodopsin-containing microbes in these environments (Béjà et al. 2000; Rusch et al. 2007). Some estimates of microbial rhodopsin abundance based on metagenomic analysis suggest that from 48% to 70% of cells in some marine environments (Kirchman & Hanson 2013; Finkel et al. 2012) and up to 95% of microbes from freshwater and estuarine environments (Finkel et al. 2012) may host rhodopsins genes. However, these estimates cannot determine whether the rhodopsins identified are functionally expressed or not.

To quantify functional rhodopsin-containing cells in different environments, a method for direct observation is needed. The use of fluorescence spectroscopy for identifying structure and function of rhodopsins has been hampered because rhodopsins are weakly fluorescent due to their low quantum yield (Alexiev & Farrens 2014; Keffer et al. 2015). Therefore, standard fluorescence-based assays — such as flow cytometry and Förster resonance energy transfer (FRET) — are not useful techniques for observing functional rhodopsins (Alexiev & Farrens 2014).

We have recently reported a method that uses total internal reflection fluorescence (TIRF) microscopy to differentiate between rhodopsin-containing cells and other pigmented cells in environmental samples (Keffer et al. 2015). TIRF microscopy relies on the total internal reflection phenomenon that occurs when light encounters an interface between two substances with different refractive indexes (i.e.
the coverslip and liquid medium). The light hits the coverslip-liquid interface at an oblique angle that is internally reflected back into the objective. The reflection generates an evanescent field that only extends a few hundred nanometers past the coverslip (Axelrod 2001; Oheim & Schapper 2005). As a result, only cells within the evanescent field or at the coverslip-liquid interface will be illuminated while cells that are not within the evanescent field will not be illuminated, thereby reducing background fluorescence and scattering by the liquid media (Figure 1.3) (Axelrod 2001; Keffer et al. 2015; Prabhat & Erdogan 2014).
Figure 1.3 Diagram of total internal reflection fluorescence (TIRF) microscopy (by Dr. Jessica Keffer).
Abundance of rhodopsin genes has been quantified in marine, estuarine, and freshwater environments using gene-based methods. Functional rhodopsins in these environments have not yet been quantified, due to the lack of direct observational methods. Here, we use the Chesapeake Bay as our study site to quantify the total amount of functional rhodopsin-containing cells using TIRF microscopy. The Chesapeake Bay is an approximately 200 mile long estuary that lies inland of the Atlantic Ocean. With strong environmental gradients, it is an ideal system for quantifying the number of functional rhodopsins and determining the relationship between light utilization by rhodopsins and environmental parameters. Because it is an estuary, there is a salinity gradient along the bay’s length, from its headwaters at the Susquehanna River to the mouth, where the salinity is nearly identical to the ocean.

Previous research has shown a greater abundance of rhodopsin genes in marine environments than freshwater environments (Campbell et al. 2008; Lami et al. 2009; Sharma et al. 2009; Brindfalk et al. 2016); therefore, we hypothesize that the proportion of functional rhodopsin-containing cells will increase along the salinity gradient of the Chesapeake Bay due to the influx of rhodopsin-containing cells supplied by the Atlantic Ocean. In addition, the Chesapeake Bay is nutrient rich with carbon, nitrogen, and phosphorus inputs throughout (Murphy et al. 2011). If rhodopsin-containing organisms are strictly using rhodopsins to supplement cellular energy when nutrient sources are low (Brindfalk et al. 2016; McCarren & DeLong 2007; Kagawa 1978; Béjà et al. 2000), then few rhodopsins should be functional in the Chesapeake Bay. This study site fully allows us to test the hypothesis that light utilization by rhodopsins is related to salinity and nutrient availability.
1.3 Carotenoids

Carotenoids are highly conjugated isoprenoid compounds, and due to their extended polyene chain structure are pigmented compounds ranging from yellow to red (Krubasik, Kobayashi, et al. 2001; Krubasik, Takaichi, et al. 2001; Richter et al. 2015). Over 750 different carotenoids have been identified in nature, all of which have a linear, conjugated backbone structure (Britton et al. 2004; Richter et al. 2015; Takatani et al. 2015). Synthesized by plants, fungi, algae, and bacteria, carotenoids primarily occur as 40 carbon molecules (C40), although C30 and C50 carotenoids also occur. They are involved in photosynthesis, membrane stability, protection against ultraviolet radiation, and quenching of free radicals (Lazrak et al. 1988; Maresca et al. 2008; Maresca et al. 2009; Krubasik, Kobayashi, et al. 2001; Krubasik, Takaichi, et al. 2001; Shahmohammadi 1998; Yatsunami et al. 2014).

Photosynthetic organisms contain carotenoids to aid in light harvesting and to serve as photoprotectants by preventing the formation of reactive oxygen species (Imasheva et al. 2011; Heider et al. 2014). In addition, the conjugated double bond structure of carotenoids protects organisms against oxidative damage caused by free radicals (Richter et al. 2015; Shahmohammadi 1998). Carotenoids are often found in the membrane to make the membrane more rigid and impermeable to toxins or across membranes to aid in membrane stabilization. This can cause an increase in firmness of the membrane, potentially supporting resistance to toxic substances or stressors (Lazrak et al. 1988). In addition, carotenoids are thought to beneficially protect humans against diseases (Cooper et al. 1999). Since animals are not capable of synthesizing carotenoids, they require intake of carotenoids through their diets (Heider et al. 2014). Within the last two decades, carotenoids have come more and more into focus as a topic for research due to their potential health benefits in reducing the
incidence of free radical induced diseases such as some cardiovascular and neurological disorders (Sen & Chakraborty 2011). In 2010, the total commercial value of these isolated compounds was reported to be $1.2 billion (Heider et al. 2014; Richter et al. 2015). Carotenoids are mainly used as food and feed colorants (Gassel et al. 2013; Heider et al. 2014), but are also used in the cosmetic industry and to color beverages (Downham & Collins 2000; Heider et al. 2014). Aside from their role in industry as visually appealing pigments, industrial companies have been working to chemically synthesize carotenoids to use in animal feeds and vitamin supplements (Heider et al. 2014), but in order to chemically synthesize these natural products, we need to know their structures and biosynthesis pathways.

All carotenoids are produced from isopentenyl pyrophosphate (IPP) and its isomer, dimethylallyl pyrophosphate (DMPP). Two pathways of IPP synthesis exist: the mevalonic acid (MVA) pathway and the non-mevalonate (MEP) pathway. The MVA pathway is mainly found in eukaryotes, archaea, and a small number of bacteria. In most bacteria, IPP is synthesized from pyruvate and glyceraldehyde 3-phosphate through the MEP pathway (Lee & Schmidt-Dannert 2003; Heider et al. 2014). Through a series of condensation reactions, the C5 molecules of IPP and DMPP form geranyl pyrophosphate (GPP), farnesyl pyrophosphate (FPP), and geranylgeranyl pyrophosphate (GGPP). The first committed step in C40 carotenoid biosynthesis is the condensation of two GGPP molecules in a tail-to-tail configuration, which produces phytoene. The first carotenoid produced in some organisms is lycopene through the dehydrogenation reaction of phytoene (Figure 1.4) (Heider et al. 2014).
Figure 1.4 Diagram of the biosynthetic pathway of C40 carotenoid, lycopene. The selected carotenoids, their structure, names, and abbreviations are given.
C50 carotenoids are quite rare because they require an additional gene, which encodes a cyclase or an elongase, to catalyze the addition of two DMPP or IPP molecules to either end of the C40 precursor (Krubasik, Takaichi, et al. 2001). Only some species of Haloarchaea and Actinobacteria are known to synthesize C50 carotenoids (Dummer et al. 2011; Krubasik, Takaichi, et al. 2001; Krubasik, Kobayashi, et al. 2001; Lazrak et al. 1988; Shahmohammadi 1998; Yang et al. 2015).

Determining the biosynthetic pathway of C50 carotenoids could aid researchers in developing easy and cost-effective ways to synthesize pigments and use them as natural dyes in industry, rather than the synthetic dyes currently used. The biosynthetic pathways of one C50 carotenoid may vary between different bacterial species. Therefore, characterizing as many pathways as possible will benefit efforts to identify the most effective method of production. The biosynthetic pathways of C50 carotenoids are much less characterized than other carotenoid pathways. However, a recent study characterized the complete biosynthetic pathway of a C50 carotenoid, called bacterioruberin, in *Haloarcula japonica* (Yang et al. 2015).

Bacterioruberin is a red-pink pigmented, C50 carotenoid (Figure 1.5), and known to be produced in some halophilic archaea. This molecule requires an elongase enzyme to elongate its C40 precursor, lycopene, and it also requires dehydrogenase and hydratase enzymes (Yang et al. 2015). Bacterioruberin has specifically been identified to protect DNA against damages such as UV radiation, free radicals, and high intracellular salt concentrations (Shahmohammadi 1998). Bacterioruberin has also been shown to have a role in membrane stability, potentially protecting cells against toxins and osmotic stress (Lazrak et al. 1988).
Figure 1.5 Chemical structure of bacterioruberin.

The freshwater bacterium, *Rhodoluna lacicola*, was isolated from a lake in China called Lake Taihu. It forms small, circular, red-pigmented colonies and its red pigmentation is caused by carotenoid production. *R. lacicola’s* genome was fully sequenced in 2014 and is about 1.5 megabases in size, containing approximately 1,400 open reading frames (Hahn et al. 2014). Within those, *R. lacicola* encodes the genes to synthesize lycopene along with two other genes whose products are hypothesized to synthesize bacterioruberin.

1.4 Hypotheses and Experimental Aims

Previous studies to quantify the number of rhodopsin-containing cells in aquatic environments have used cultivation, metagenomic sequencing, and qPCR methods. These estimates have been strictly gene based analyses because there has not been an accurate method of direct observation to quantify functional rhodopsin-containing cells. Using TIRF microscopy will allow the identification of functional rhodopsin-containing cells in environmental samples. Previous research has shown a greater abundance of rhodopsin genes in marine environments than freshwater environments; therefore, we hypothesize that the proportion of functional rhodopsin-
containing cells will increase along the salinity gradient of the Chesapeake Bay due to the influx of rhodopsin-containing cells supplied by the Atlantic Ocean. In this study, we estimated rhodopsin gene abundance and functional rhodopsin-containing cells using qPCR and TIRF microscopy, respectively. In addition, we were able to examine how the abundance of rhodopsin-containing cells varies with environmental parameters.

Carotenoids are pigmented, organic compounds that have been used in industry for years as food and cosmetic colorants. Carotenoids are synthesized by every domain of life and close to 750 carotenoids have been isolated from nature. They most commonly occur as C40s molecules, but C50 carotenoids also exist. C40 carotenoid biosynthetic pathways have been elucidated for use in the chemical synthesis of natural dyes; however, the biosynthetic pathways of C50 carotenoids have remained elusive. Here, we have identified two potential genes involved in the biosynthetic pathway of bacterioruberin in the freshwater bacterium \textit{R. lacicola}. We hypothesize that these two genes encode an elongase and a dehydrogenase enzyme, respectively (Figure 1.6).
Figure 1.6 Hypothesized steps in the biosynthesis of bacterioruberin in *R. lacicola* starting from lycopene. Locus tags indicate the gene sequences that are predicted to encode carotenoid biosynthetic enzymes and the question mark indicates an unidentified carotenoid biosynthetic enzyme.
Chapter 2
MATERIAL AND METHODS

2.1 Rhodopsins

2.1.1 Sample Collection and Storage

A series of day, night, and depth samples were collected along the Chesapeake Bay on the R/V Hugh R. Sharp in April 2015 (Figure 2.1). Day and night samples consisted of surface samples between 1 to 3 meters deep, collected at 11:00 am and 11:00 pm, respectively. Depth samples were collected 3 to 9 meters below the surface at 11:00 am. Water quality data including temperature, salinity, dissolved oxygen, turbidity, and fluorescence were measured for each cast using a data sonde. Samples for TIRF microscopy were pre-filtered through 1 µm cellulose nitrate filters. Cells were fixed in 4% paraformaldehyde and stored at 4°C until analysis. Samples for DNA analysis were pre-filtered through 1 µm cellulose nitrate filters, collected on filters (0.2 µm) provided by the MoBIO PowerWater kit (MoBIO, catalog #14900, Carlsbad, CA), and stored at -20°C until analysis.
Figure 2.1 April research cruise track in the R/V Sharpe. Points indicate areas in which samples were obtained in sequential order starting at the Susquehanna River to the Atlantic Ocean.
2.1.2 DNA Extraction and qPCR

DNA extractions were performed using a MoBIO PowerWater kit following the manufacturer’s instructions. Preliminary PCR was performed on positive controls using LG1, SARPR, and 16S primer sets to optimize conditions (Table 2.1). Quantitative PCR was performed in triplicate with 5 µL of diluted DNA (Table 2.2) in a final volume of 20 µL using the Quanta Biosciences PerfeCTa SYBR Green FastMix for iQ (Quanta Biosciences, catalog #95071, Gaithersburg, MD). Degenerate primers were used to amplify proteorhodopsins and actinorhodopsins with qPCR conditions of 95°C 2.5 min; followed by 40 cycles of amplification at 95°C for 15 s, the indicated annealing temperature for 30 s, and 72°C for 30 s, with a final dissociation step. All primer concentrations were 0.25 µM. Average amplification efficiencies were as follows: 16S rRNA = 54%, LG1 = 39%, and SAR11 PR = 73%. We normalized rhodopsin gene copies to 1.9 copies of the 16S rRNA gene (Campbell et al. 2008).
Table 2.1  Primers and PCR conditions used in this study for examining 16S rRNA, actinorhodopsin, and SAR11 proteorhodopsin genes.

<table>
<thead>
<tr>
<th>Primer name</th>
<th>Sequence (5’ to 3’)</th>
<th>Gene</th>
<th>Product size (bp)</th>
<th>References and annealing temperatures</th>
</tr>
</thead>
<tbody>
<tr>
<td>LG1_F1</td>
<td>TAYMGNTAYGTNGAYTGG</td>
<td>Luna cluster actR and Gloeobacter violaceus PCC7421</td>
<td>300</td>
<td>Sharma et al. 2009 $T_a=46.6 , ^{\circ}C$</td>
</tr>
<tr>
<td>LG1_F2</td>
<td>MGNTAYATHGAYTGGYT</td>
<td>SARPR_125F</td>
<td>200</td>
<td>Lami et al. 2009 $T_a=54 , ^{\circ}C$</td>
</tr>
<tr>
<td>LG1_R*</td>
<td>ATNGGRTANACNCCCCA</td>
<td>SARPR_203R</td>
<td>Proteorhodopsin</td>
<td></td>
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<tr>
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<td>THGGWGGATAYTTAGGWAAGC</td>
<td>SAR11</td>
<td>200</td>
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<tr>
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<td>ACCTACTGTAACRATCATTCTYA</td>
<td>Proteorhodopsin</td>
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<td></td>
</tr>
<tr>
<td>BACT1369F</td>
<td>CGGTGAATACGTTTCYGG</td>
<td>16S rRNA</td>
<td>300 -350</td>
<td>Suzuki et al. 2000 $T_a=58 , ^{\circ}C$</td>
</tr>
<tr>
<td>PROK1541R</td>
<td>AAGGAGGTGATCCRGCCGCA</td>
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<tr>
<td>Sample</td>
<td>Sample #</td>
<td>DNA concentration (ng/µL)</td>
<td></td>
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<tr>
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<tr>
<td>4.11.15 surf 12pm_1</td>
<td>D1</td>
<td>3.2</td>
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<td>D3</td>
<td>3.2</td>
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<td>4.0</td>
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<td>3.6</td>
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<td>D4.2</td>
<td>3.2</td>
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<tr>
<td>4.14.15 D2_1</td>
<td>D4.3</td>
<td>6.1</td>
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<td>4.14.15 23pm_1</td>
<td>N4</td>
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<td></td>
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</tr>
<tr>
<td>4.15.15 11am_1</td>
<td>D5.1</td>
<td>7.3</td>
<td></td>
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<tr>
<td>4.15.15 D6_1</td>
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<td></td>
</tr>
<tr>
<td>4.15.14 D3_1</td>
<td>D5.3</td>
<td>5.0</td>
<td></td>
<td></td>
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<tr>
<td>4.15.15 23pm_1</td>
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<td>5.7</td>
<td></td>
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</tr>
<tr>
<td>4.16.15 11am_1</td>
<td>D6.1</td>
<td>4.7</td>
<td></td>
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</tr>
<tr>
<td>4.16.15 D1_1</td>
<td>D6.2</td>
<td>4.0</td>
<td></td>
<td></td>
</tr>
<tr>
<td>4.16.15 23pm_1</td>
<td>N6</td>
<td>5.7</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>
2.1.3 Sample Prep for TIRF Microscopy

Fisher brand coverslips (22 x 22 #1.5) were cleaned with 3 washes of DI water, followed by sonication for 15 minutes in DI water (2X). Coverslips were placed in 0.1 M HCl for 1 hour with shaking, washed with DI water (3X), and placed in 95% ethanol for 1 hour with shaking. The coverslips were then rinsed with DI water (2X) and stored in 95% ethanol until use. The washed coverslips were removed from the ethanol, air-dried, and sterilized with exposure to UV light for 15 minutes. The coverslips were dipped in 0.5% (wt/vol) gelatin (Sigma, catalog G6144) with 0.01% chromium ammonium sulfate, and air-dried overnight upright, at an angle (Keffer et al. 2015; Maresca et al. 2016). Thirty milliliters of pre-filtered and fixed Chesapeake Bay water samples were concentrated to approximately 3 mL on a 25 mm 0.2 µm white Isopore polycarbonate filter (EMD Millipore) and stained with NucBlue Fixed Cell ReadyProbes Reagent (Life Technologies, catalog #R37606) for 10 minutes. The remaining 3 mL was filtered onto the polycarbonate filter. The filter was then transferred onto a gelatin-coated coverslip. After 10 minutes, the filter was removed and discarded and the coverslips were sealed with nail polish to a glass slide containing 10 µL DI water. Each sample was prepared and analyzed in triplicate.

2.1.4 Microscopy

The light of each laser was expanded to approximately 1 in. diameter and focused onto the back aperture of the objective using a 500 mm achromatic doublet lens (Maresca et al. 2016; Keffer et al. 2015). A drop of immersion oil was added to the objective lens and each slide was mounted on the microscope stage with the coverslip facing down. The 405 nm laser was used to illuminate and focus each sample because all samples were stained with NucBlue Fixed Cell ReadyProbes.
Reagent. Once each sample was in view and focused, the slide was moved to a new field of view to start image collection. Each image was set to be collected in a sequential stack. Each field of view was illuminated with 641, 561, 488, and 405 nm lasers in order of decreasing wavelength with 49 sequential images collected for each excitation wavelength (Maresca et al. 2016).

2.1.5 Image Processing and Statistical Analysis

Images were processed using ImageJ version 1.47 (National Institutes of Health). Forty-nine sequential frames were averaged via intensity to reduce random background noise. The minimum fluorescence level was normalized for all images acquired with the same laser using control images to maximize visibility of cells. Cells were counted and recorded for all wavelengths. Regression analyses were performed and coefficient of determination ($R^2$) values were determined between the percentage of cells containing rhodopsins and salinity concentrations, Chl $a$ concentrations, temperature, nitrate concentrations, silicate concentrations, bacterial production, ammonium concentrations, and phosphate concentrations.

2.2 Carotenoids

2.2.1 Strains and Growth Conditions

*Rhodoluna lacicola* was grown at room temperature in 0.3% nutrient broth-soytone-yeast extract (NSY) medium (Hahn et al. 2004) for 14 days for optimal growth. *Escherichia coli* strain T7 Express (genotype fhuA2 lacZ, T7 gene1 [I;on] ompT gal sulA11 R(mcr-73::miniTn10—Tet$^6$)2 [dcm] R(gzb-210::Tn10—Tet$^5$) endA1 $\Delta$(mcrC-mrr)114;IS10; New England Biolabs, catalog #C2566I) containing plasmid pLY02 (Figure 2.2A), which encodes the genes that synthesize lycopene (Keffer et al.
2015) and pBR (Figure 2.2B), which encodes the elongase and dehydrogenase genes predicted to synthesize bacterioruberin from lycopene, were grown overnight at 37°C in M9CA minimal medium (AMRESCO; 2 g L\(^{-1}\) casamino acids, 6 g L\(^{-1}\) Na\(_2\)HPO\(_4\), 3 g L\(^{-1}\) KH\(_2\)PO\(_4\), 0.5 g L\(^{-1}\) NaCl, 1 g L\(^{-1}\) NH\(_4\)Cl, final pH 7.2, following sterilization, supplemented with 2 mL 1 M MgSO\(_4\), 10 mL 20% glucose, and 0.1 mL 1 M CaCl\(_2\)) supplemented with 30 µg mL\(^{-1}\) kanamycin and 50 µg mL\(^{-1}\) ampicillin.

![Figure 2.2](image)

**Figure 2.2** Plasmids for the synthesis of lycopene (A) and the expression of predicted bacterioruberin biosynthesis enzymes (B).
2.2.2 Identification of Candidate Genes

Recently, the genome sequence of *R. lacicola* has been determined (Hahn et al. 2014). The genome of *R. lacicola* was searched for genes homologous to known genes in carotenoid biosynthesis (Keffer et al. 2015). Protein sequences for known carotenoid biosynthetic genes were gathered from NCBI and used as BLAST query searches against the *R. lacicola* genome. *Rhol_00000880*, *Rhol_00000870*, and *Rhol_0010900* were identified and predicted to be involved in carotenoid biosynthesis, based on gene annotations.

2.2.3 Cloning

The genes *Rhol_00000870* (AIC46917.1) and *Rhol_00000880* (AIC46918.1) were amplified from the genomic DNA of *R. lacicola* MWH-Ta8 using the primer pair F-Sall-BR and R-BR-BamHI (Table 2.3). The primers were designed to be complementary to the first MCS of pCOLA-Duet (Novagen, catalog #71406-3) and introduce a BamHI restriction site at the 5’ end of the PCR product and a Sall restriction site at the 3’ end. A 2.2 kb fragment encoding the two genes was amplified by PCR using Phusion DNA polymerase (ThermoScientific) under the following cycling conditions: 30 sec at 98°C, followed by 29 cycles of 5 sec at 98°C, 10 sec at 54°C, 38 sec at 72°C, and a final elongation step of 5 min at 72°C. The PCR product and pCOLA-Duet were digested to BamHI and Sall, and then ligated together with Electroligase (New England Biolabs, catalog #M0369S) to produce the plasmid pBR. The pBR plasmid was transformed into *E. coli* strain T7 Express (New England Biolabs, catalog #C2566I). Analysis using Sanger sequencing confirmed the identity of the cloned fragment.
Table 2.3  Primers used in this study to amplify both predicted carotenoid biosynthesis genes used for cloning.

<table>
<thead>
<tr>
<th>Primer name</th>
<th>Sequence (5′ to 3′)</th>
<th>Gene</th>
<th>Restriction Site</th>
</tr>
</thead>
<tbody>
<tr>
<td>F-Sall-BR</td>
<td>AGCGGTGACTTAGCTGCCGGTTTTAG</td>
<td>Predicted bacterioruberin elongase</td>
<td>Sall</td>
</tr>
<tr>
<td>R-BR-BamHI</td>
<td>CGGGATCCGTAAGTGCTAAGACGCCTAG</td>
<td>Predicted bacterioruberin dehydrogenase</td>
<td>BamHI</td>
</tr>
</tbody>
</table>

2.2.4 Pigment Analysis

For analysis of pigments produced by high-performance liquid chromatography (HPLC), *R. lacicola* cells were grown as previously stated and *E. coli* cells containing pLY02 and pBR were grown overnight at 37°C with shaking in minimal M9CA medium supplemented with 30 µg mL⁻¹ kanamycin, 50 µg mL⁻¹ ampicillin, 0.2% arabinose, and 1 mM isopropyl β-D-1-thiogalactopyranoside (IPTG). Cells were harvested by centrifugation and washed with TES buffer (200 mM Tris pH 8.0, 20 mM EDTA, 200 mM NaCl). Carotenoid pigments were extracted by sonication of cells resuspended in acetone-methanol (7:2 [vol/vol]). Cell debris was removed by centrifugation and organic extracts were dried with nitrogen gas. The dried pigments were resuspended in solvent B (see below) and filtered through 0.2 µm polytetrafluoroethylene syringe filters (Thermo Scientific) for HPLC analysis. The HPLC system was a Shimadzu Prominence system with a quaternary pump (LC-20AT), solvent degasser (DGU-20A5), and 996-element diode array detector (SPD-M20A) fitted with a Supelco Ascentis reverse-phase C18 column (100 x 3 mm, 3 µm)
beads; Sigma-Aldrich, catalog #581308-U). Solvent A was 
water/methanol/acetonitrile (62.5:21:16.5 by volume) and solvent B was 
methanol/ethyl acetate/acetonitrile (50:30:20 by volume). The gradient, at a constant 
temperature of 35°C, was as follows (min, %B): (0, 20), (5, 70), (12, 100), (25, 100).
Chapter 3

VISUALIZATION OF RHODOPSIN-CONTAINING CELLS IN THE CHESAPEAKE BAY

3.1 Estimates of Rhodopsin Gene Abundance in the Chesapeake Bay

We used qPCR to predict the abundance of microbes harboring rhodopsin genes of different types in the Chesapeake Bay. We observed SAR11-like and actinorhodopsin-like rhodopsin genes using two different sets of degenerate primers. The abundances of rhodopsin gene copies in surface water samples from the Chesapeake Bay were normalized to 1.9 copies of the bacterial 16S rRNA gene per genome.

Approximately 1 to 24% of cells were predicted to encode actinorhodopsin genes, and 1 to 115% were predicted to encode SAR11-type proteorhodopsin genes. Therefore, approximately 1 to 116% of all cells were predicted to encode rhodopsin genes.

Given these data, we predict the total percentage of rhodopsin-containing cells along the Chesapeake Bay to make up the majority of total cells. This wide range highlights the need for a method that relies on direct observation of functional rhodopsin-containing microbes.

3.2 Detection of Rhodopsin-Containing Cells in the Chesapeake Bay

Day, night (1-3 m) and depth (3-9 m) samples were collected from the Chesapeake Bay over the course of six days. Fixed Chesapeake Bay water samples
were imaged by TIRF microscopy. Lasers with excitation wavelengths of 405, 488, 561, and 641 nm were used to view each sample, in triplicate. The 405-nm laser enabled visualization of all DAPI stained cells. The 488-nm laser enabled visualization of pigment-expressing cells. The 561-nm laser selectively enabled visualization of retinal-rhodopsin-expressing cells, and the 641-nm laser enabled visualization of Chl a containing cells (Figure 3.1).
Figure 3.1  Example morning surface sample from the Chesapeake Bay imaged by TIRF microscopy indicating the presence of rhodopsin-containing cells (circle). 405 nm shows all cell stained with DAPI, 488 nm shows all carotenoid-containing cells, 561 nm shows all rhodopsin-containing cells, and 641 nm shows all Chl a containing cells.
In the day samples, 12 to 56% of microbial cells had functional rhodopsins in the Chesapeake Bay. The night samples showed 4% to 33% of cells produced rhodopsins. Similar to the day samples, 15 to 51% of microbial cells contained functional rhodopsins in depth samples. Overall, the rhodopsin-containing cells account for 4% to 56% of the total cells found in the Chesapeake Bay (Figure 3.2).

**Figure 3.2** Range of rhodopsin-containing cells using TIRF microscopy indicating a higher percentage of cells containing rhodopsins in the day than at night.
3.3 Relationship of Rhodopsin Gene Abundance and Rhodopsin-Containing Cells to Environmental Factors

The relationship between percentage of rhodopsin-containing cells and environmental parameters was analyzed (Table 3.1). When using TIRF microscopy, the variation in rhodopsin-containing cell abundance appeared to be a result of varying salinities (Table 3.2). Abundance of total rhodopsin-containing cells demonstrated a linear correlation with salinity in day and night samples, and a linear correlation with ammonium concentrations in night samples alone. In both day and night samples, there were no observed relationships between rhodopsin-containing cells and temperature, bacterial production, phosphate, nitrate, silicate, and total Chl \( \alpha \) concentrations.

When using qPCR, the variation in actinorhodopsin gene abundance showed no correlation to any environmental parameters. The abundance of SAR11 proteorhodopsin genes demonstrated a linear correlation with salinity and bacterial production in the day samples, and linear correlation to phosphate, nitrate, ammonium, and silicate concentrations in night samples. The variation in the total abundance of rhodopsin genes, detected using qPCR, showed similar correlations to that of the SAR11 proteorhodopsin genes, due to their overall high abundances.
Table 3.1  Percentage of rhodopsin-containing cells in both day and night samples collected in the Chesapeake Bay with corresponding environmental data (n.d. = not determined) obtained per sample by Barbara Campbell (Clemson University).

<table>
<thead>
<tr>
<th>Date</th>
<th>Time</th>
<th>Percentage of rhodopsin-containing cells</th>
<th>Salinity (parts/thousand)</th>
<th>Phosphate (μmol/L)</th>
<th>Nitrate (μmol/L)</th>
<th>Ammonium (μmol/L)</th>
<th>Silicate (μmol/L)</th>
<th>DAPI count (cells/mL)</th>
<th>Bacterial production (ngC/L/h)</th>
<th>Chl a (μg/L)</th>
<th>Temperature (°C)</th>
</tr>
</thead>
<tbody>
<tr>
<td>4.11.15</td>
<td>Day</td>
<td>19%</td>
<td>0.07</td>
<td>0.202</td>
<td>61.9435</td>
<td>3.3205</td>
<td>60.933</td>
<td>3050000</td>
<td>40.6</td>
<td>2.1</td>
<td>8.04</td>
</tr>
<tr>
<td></td>
<td>Night</td>
<td>4%</td>
<td>0.07</td>
<td>0.208</td>
<td>63.1145</td>
<td>3.19</td>
<td>61.787</td>
<td>2520000</td>
<td>32.2</td>
<td>3.1</td>
<td>9.28</td>
</tr>
<tr>
<td>4.12.15</td>
<td>Day</td>
<td>19%</td>
<td>6.65</td>
<td>-0.0085</td>
<td>56.3955</td>
<td>2.488</td>
<td>35.106</td>
<td>2540000</td>
<td>42.4</td>
<td>15</td>
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<td>7.9</td>
<td>-0.022</td>
<td>55.0195</td>
<td>2.355</td>
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<td>4.5</td>
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<td>0.2495</td>
<td>2300000</td>
<td>19</td>
<td>4.9</td>
<td>9.17</td>
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<td>Night</td>
<td>11%</td>
<td>15.1</td>
<td>-0.1125</td>
<td>8.794</td>
<td>0.8915</td>
<td>0.1875</td>
<td>1590000</td>
<td>18.6</td>
<td>5.6</td>
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<td>1.801</td>
<td>1.5265</td>
<td>0.3315</td>
<td>2320000</td>
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<td>4.6</td>
<td>10.98</td>
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<td>Night</td>
<td>22%</td>
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<td>-0.1505</td>
<td>2.8615</td>
<td>1.5065</td>
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<td>-0.0976667</td>
<td>3.078333</td>
<td>2.185333</td>
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</tr>
<tr>
<td></td>
<td>Night</td>
<td>20%</td>
<td>24.09</td>
<td>-0.1137</td>
<td>0.4165</td>
<td>0.4645</td>
<td>0.4145</td>
<td>3240000</td>
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<td>2.4</td>
<td>11.1</td>
</tr>
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<td>4.16.15</td>
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<td>56%</td>
<td>31.56</td>
<td>-0.1015</td>
<td>0.7685</td>
<td>2.9145</td>
<td>1.576</td>
<td>640000</td>
<td>9.3</td>
<td>1.6</td>
<td>9.77</td>
</tr>
<tr>
<td></td>
<td>Night</td>
<td>33%</td>
<td>31.75</td>
<td>n.d.</td>
<td>n.d.</td>
<td>n.d.</td>
<td>n.d.</td>
<td>3180000</td>
<td>34.5</td>
<td>1.4</td>
<td>8.22</td>
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</table>
**Table 3.2** Coefficient of determination ($R^2$) values between relative abundance of rhodopsin-containing cells or rhodopsin gene and Chl $a$, nutrient concentrations, and temperature in day and night samples. *Correlation is significant ($p < 0.05$), **Correlation is significant ($p < 0.01$).

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Coefficient of determination ($R^2$) values</th>
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</thead>
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<td>Total Rhodopsins</td>
</tr>
<tr>
<td></td>
<td>TIRF</td>
</tr>
<tr>
<td>---------------------------</td>
<td>------</td>
</tr>
<tr>
<td>Salinity (parts per thousand)</td>
<td>Day</td>
</tr>
<tr>
<td></td>
<td>Night</td>
</tr>
<tr>
<td>Phosphate ($\mu$M)</td>
<td>Day</td>
</tr>
<tr>
<td></td>
<td>Night</td>
</tr>
<tr>
<td>Nitrate ($\mu$M)</td>
<td>Day</td>
</tr>
<tr>
<td></td>
<td>Night</td>
</tr>
<tr>
<td>Ammonium ($\mu$M)</td>
<td>Day</td>
</tr>
<tr>
<td></td>
<td>Night</td>
</tr>
<tr>
<td>Silicate ($\mu$M)</td>
<td>Day</td>
</tr>
<tr>
<td></td>
<td>Night</td>
</tr>
<tr>
<td>Temperature ($^\circ$C)</td>
<td>Day</td>
</tr>
<tr>
<td></td>
<td>Night</td>
</tr>
<tr>
<td>Bacterial Production (ng C h$^{-1}$ L$^{-1}$)</td>
<td>Day</td>
</tr>
<tr>
<td></td>
<td>Night</td>
</tr>
<tr>
<td>Total Chl $a$ (mg L$^{-1}$)</td>
<td>Day</td>
</tr>
<tr>
<td></td>
<td>Night</td>
</tr>
</tbody>
</table>
Chapter 4  
SYNTHESIS OF BACTERIORUBERIN BY LIGHT-RESPONSIVE ACTINOBACTERIA  

4.1 Identification of *R. lacicola* Carotenoid Synthesizing Genes  

To investigate the biosynthetic production of bacterioruberin, a search for homologs of known carotenoid biosynthetic genes in the *R. lacicola* genome was performed. All genes required to synthesize lycopene were identified, with the addition of three putative carotenoid synthesis genes: *Rhol_00000870*, *Rhol_00000880*, and *Rhol_00010900*. *Rhol_00000870* exhibited 23% identity and 37% similarity to the phytoene dehydrogenase (*crtI*) from *Rhodobacter capsulatus*, *Rhol_00000880* demonstrated 35% identity and 49% similarity, while *Rhol_00010900* showed 52% identity and 70% similarity to the lycopene elongase (*crtEb*) from *Clavibacter michiganensis*. In addition, genes that were identified to be required for the biosynthesis of bacterioruberin by Yang et al (2015) in *H. japonica* were also analyzed. *Rhol_00000870* displayed 28% identity and 43% similarity to *c0507* (Yang et al. 2015). *Rhol_00000880* exhibited 35% identity and 49% similarity, while *Rhol_00010900* showed 33% identity and 50% similarity to *c0506* (Yang et al. 2015). These homologous gene sequences were then used for phylogenetic analysis to further elucidate their predicted functions in bacterioruberin biosynthesis.

In the analysis of known desaturase (*crtD*) and *crtI* sequences, *Rhol_00000870* clustered with *crtI* sequences, while the annotated *crtI* sequence clustered with *crtD* sequences (Figure 4.1). *Rhol_00000880* clustered with known bacterioruberin
lycopene elongase sequences and *R. lacicola*’s other predicted lycopene elongase, *Rh* _ol_ _00010900_, clustered with lycopene elongases involved in decaprenoxanthin biosynthesis (Figure 4.2). Therefore, only *Rh* _ol_ _00000870_ and *Rh* _ol_ _00000880_ were predicted to be involved in the biosynthesis of bacterioruberin from lycopene in *R. lacicola*. 
Figure 4.1 Phylogenetic analysis of Rhol_00000870 to predict function in carotenoid biosynthesis of bacterioruberin. Yellow indicates R. lacicola protein, Rhol_00000870, red indicates c0507 (Yang et al. 2015), gray specifies a R. lacicola CrtI annotated protein that groups with CrtD proteins, and red asterisks indicate known bacterial species that produce bacterioruberin. Bootstrap values for nodes are indicated and determined from 1000 replicates.
Figure 4.2 Phylogenetic analysis of Rhol_00000880 to predict function in carotenoid biosynthesis of bacterioruberin. Yellow indicates the two R. lacicola predicted elongase enzymes, Rhol_00010900 (top) and Rhol_00000880 (bottom). Red indicates c0506 (LyeJ) (Yang et al. 2015). Red asterisks indicate known bacterial species that produce bacterioruberin, while orange asterisks indicate known bacterial species that produce decaprenoxanthin. Bootstrap values for nodes are indicated and determined from 1000 replicates.
4.2 Investigation of Predicted Carotenoid Genes in E. coli

Carotenoid pigments were extracted from R. lacicola and analyzed via HPLC. The carotenoids produced were compared to a bacterioruberin standard from Halobacterium salinarum MPK414 (Ronald Peck, Colby College). Four peaks were observed in the bacterioruberin standard and were identified as bacterioruberin (Figure 4.3A, peak 1), monoanhydrobacterioruberin (peak 2), bisanhydrobacterioruberin (peak 3), and isopentenyldehydrorhodopin (peak 4) (Yatsunami et al. 2014). Bacterioruberin was identified in R. lacicola’s elution profile (Figure 4.3B, peak 1*) based on retention time and absorption spectra, along with several unidentified intermediates.

To explore the involvement of the predicted genes in bacterioruberin biosynthesis, both genes were cloned, transformed, and expressed in E. coli, which already contained the pLY02 plasmid that synthesizes lycopene under the control of an arabinose promoter. Cells were grown in minimal media overnight, pelleted, and pigments were extracted for pigment analysis via HPLC. Both the cell suspension and pigment extract of E. coli/pLY02/pBR cells were red in color. Peak 1* of the elution profile was predicted to be bacterioruberin (Figure 4.3C) based on retention time and absorption spectra, but has not yet been confirmed through mass spectrometry.
Figure 4.3 HPLC elution profiles of pigments produced in *H. salinarum* MPK414 (A), *R. lacicola* (B) and *E. coli* pLY02/pBR (C), which synthesizes lycopene and bacterioruberin. Absorption spectra on the right correspond to the numbers peaks. (A) Bacterioruberin (peak 1), monoanhydrobacterioruberin (peak 2), bisanhydrobacterioruberin (peak 3), and isopentenyldehydrorhodopin (peak 4) produced by *H. salinarum* MPK414. (B) Peak 1# is bacterioruberin produced by *R. lacicola*. (C) Peak 1* is bacterioruberin produced by *E. coli* pLY02/pBR.
Chapter 5
DISCUSSION AND FUTURE DIRECTIONS

5.1 Rhodopsins

Several studies have examined the diversity of rhodopsin-encoding microbes, but have not been able to estimate the number of functional rhodopsin-containing microbes (Atamna-Ismaeel et al. 2008; Brindefalk et al. 2016; Campbell et al. 2008; Finkel et al. 2012; Lami et al. 2009; Sharma et al. 2009). Unlike other molecules, rhodopsins cannot be quantified using direct methods such as microscopy or flow cytometry (Alexiev & Farrens 2014). It was not until recently that a method for direct observation of these functional rhodopsin-containing microbes in environmental samples was developed (Keffer et al. 2015). Our study provides the first abundance estimates of functional rhodopsin-containing microbes in estuarial waters using TIRF microscopy and allows us to use these data to understand what might control rhodopsin distribution.

We have presented the first data using TIRF microscopy to identify and quantify functional rhodopsin-containing cells in the Chesapeake Bay. More functional rhodopsin-containing cells are present in the day compared to the night. Similar to previous research on rhodopsin gene abundance, we found that during the day, 12 to 56% of cells in the Chesapeake Bay contain functional rhodopsins. These cells are found along the length of the Chesapeake Bay indicating their presence in both fresh and saltwater environments. In addition, we have demonstrated that the distribution of functional rhodopsin-containing cells correlates with salinity, which has not been previously discovered before. We also observed a weak correlation with
ammonium concentrations; however, ammonium gradients in the Chesapeake Bay are known to fluctuate with season (Fisher et al. 1988). This suggests that salinity and season might be key factors in the determination of geographical distribution of functional rhodopsin-containing cells.

Our qPCR results of rhodopsin gene abundance have confirmed the presence of rhodopsin genes in the Chesapeake Bay, even though the estimates of abundance vary greatly from the TIRF analysis. We quantified actinorhodopsin and SAR11 proteorhodopsin genes and found a high abundance of SAR11 proteorhodopsin genes overall. Actinorhodopsin genes displayed an even distribution along the entire length of the Chesapeake Bay seen in day samples alone. This suggests constant low abundances in both fresh and saltwater environments. However, night samples demonstrated a higher rhodopsin-containing cell abundance in freshwater and lower abundance in marine environments, which is not surprising because Actinobacteria are known to mainly inhabit freshwater environments (Hahn et al. 2014; Sharma et al. 2009). These estimates may not provide a complete picture of all actinorhodopsin genes. Degenerate primers were used to cover a wide diversity of sequences and thus may not cover all sequences present in an environment. In one case, it was suggested that degenerate primers would only retrieve 1 to 25% of rhodopsin sequences sampled from surface waters (Campbell et al. 2008).

Day samples demonstrated a steady increase in the abundance of SAR11 proteorhodopsin genes in both day and night samples along the salinity gradient from the Susquehanna River to the Atlantic Ocean. This is also not surprising because SAR11 Proteobacteria are known to thrive in marine environments (Campbell et al. 2008; Lami et al. 2009; Brindefalk et al. 2016). The total number of rhodopsin genes
shows a similar trend to the SAR11 proteorhodopsin genes, mainly because of the large percentage of SAR11 proteorhodopsin genes obtained by using qPCR. This large range of SAR11 proteorhodopsin genes, from 0 to 115%, indicates the drawbacks of using qPCR for environmental analysis of genes. The large range may be due to the use of degenerate primers, which may lack the specificity to only amplify rhodopsin genes.

Actinorhodopsin genes were not correlated with any environmental gradients; however, SAR11 proteorhodopsin and total rhodopsin genes were correlated with salinity and bacterial production in day samples and phosphate, nitrate, ammonium, and silicate concentrations in night samples. Previous studies have seen no correlation of rhodopsin gene abundance with any environmental gradients (Campbell et al. 2008), with the recent exception of salinity (Brindefalk et al. 2016). These data observed could be the result of seasonal variation within the Chesapeake Bay, correlations between nutrients, or a correlation of those nutrients with salinity in the Chesapeake Bay (Morris et al. 1981; Fisher et al. 1988) and not necessarily correlation with the abundance of rhodopsin genes. In addition, the lack of specificity in using degenerate primers could contribute to the correlations seen only in the qPCR data.

While TIRF is a new method for direct observation of functional rhodopsin-containing cells in environmental samples, it lacks the ability to differentiate between different rhodopsin bacterial types. We propose that this TIRF microscopy method be used in conjunction with gene-based methods that identify specific types of rhodopsin genes for different bacterial phyla. To make TIRF microscopy a more efficient method for this process, an automated TIRF microscopy system for cell sorting of functional rhodopsin-containing organisms is under development in our laboratory.
The discovery in 2001 that a large percentage of microbes in marine environments were aerobic anoxygenic phototrophs (AAPs) revolutionized our understanding of the role of sunlight in the oxidation of organic matter (Kolber et al. 2000; Moran & Miller 2007). Some estimates indicate that rhodopsin-containing cells are more widespread than AAPs (Kirchman & Hanson 2013). This would suggest that even more bacteria in aquatic environments that are capable of this type of phototrophy and that there is a much higher abundance of these bacteria than previously thought (Bryant & Frigaard 2006; Finkel et al. 2012). Because rhodopsin-producing cells are photoheterotrophs, sunlight may play a large role in organic carbon consumption, and these data could have important implications in global carbon cycling.

There is a commonly held hypothesis that rhodopsins are used for the supplementation of energy in low-nutrient environment. Although the Chesapeake Bay is not a nutrient-limited system, a large percentage of functional rhodopsin-containing cells were observed. Therefore, this hypothesis may not hold true in all environments. We suggest that in the Chesapeake Bay, rhodopsins may play other physiological roles.

5.2 Carotenoids

Carotenoids are highly conjugated isoprenoid compounds that are synthesized by every domain of life (Krabasik, Kobayashi, et al. 2001; Richter et al. 2015). They are involved in photosynthesis, membrane stability, and protection against ultraviolet radiation (Lazrak et al. 1988; Maresca et al. 2009; Shahmohammadi 1998). There have been over 750 different carotenoids identified which primarily occur as C40s; however, C50 carotenoids, although rare, also occur. Carotenoids have been a main
focus of research due to their potential health benefits and their use in cosmetic and food industries (Heider et al. 2014). Industry has attempted to chemically synthesize carotenoids to use as food, feed, and cosmetic colorants, but in order to synthesize these products, an understanding of how they are made naturally is needed (Heider et al. 2014; Downham & Collins 2000; Gassel et al. 2013). The more abundant C40 carotenoids have well characterized biosynthetic pathways; however, the pathways of C50 carotenoids are largely unknown (Heider et al. 2014). This study identifies two potential genes involved in the biosynthesis of the rare C50 carotenoid, bacterioruberin, in *R. lacicola*.

Through genome and phylogenetic analysis, two genes were identified in the genome of *R. lacicola* to have predicted functions in the synthesis of bacterioruberin. These genes were cloned into *E. coli*/pLY02 to create *E. coli*/pLY02/pBR. When expressed and the pigments were analyzed using HPLC, the two genes were able to produce a peak in the elution profile resembling bacterioruberin, based on retention time and absorption spectra. However, it remains unconfirmed by mass spectrometry if this peak is in fact bacterioruberin.

*Rhol_00000870* and *Rhol_00000880* are related to known carotenoid biosynthesis genes and are predicted dehydrogenase and lycopene elongase enzymes, respectively. Yang et al (2015) identified three enzymes to be responsible for synthesizing bacterioruberin from lycopene in *H. japonica*: an elongase, dehydrogenase, and a hydratase. Although a hydratase related to the one found in *H. japonica* has not been identified in *R. lacicola*, another hydratase could be completing this task or, like in *H. japonica*, the elongase could have dual properties, elongase and hydratase functions, and not require an additional hydratase (Yang et al. 2015). It is
also possible that without the required hydratase, \textit{E. coli}/pLY02/pBR is not producing a bacterioruberin variant or intermediate. The predicted bacterioruberin peak is also relatively small; therefore, in future work, pigment production should be optimized.

The complete pathway of bacterioruberin in \textit{R. lacicola} remains unconfirmed. However, progress has been made to elucidate its biosynthetic pathway. When determining a pathway, each step must be made clear and all intermediates described. Currently, \textit{E. coli} is producing several intermediates and a small amount of our predicted bacterioruberin. Plasmids containing only the sequence of one predicted enzyme involved in the pathway should be expressed in \textit{E. coli}. This would allow us to more easily identify intermediates and potentially determine which enzyme acts in the process first to generate bacterioruberin. In addition, to enhance pigment production, growth optimization steps should be taken for any future work.

Carotenoids have antioxidant capacity linked to their conjugated double bond structure, and consumption of antioxidants can reduce incidence of free radical induced diseases such as some cardiovascular and neurological disorders (Sen & Chakraborty 2011). Bacterioruberin contains 13 conjugated double bonds and is known to be a more effective free radical scavenger than beta-carotene (Yatsunami et al. 2014). Determining the biosynthetic pathway of bacterioruberin would help researchers to more easily synthesize bacterioruberin for food and cosmetic industrial purposes and could potentially lead to more cost effective methods than currently used.

\textbf{5.3 Significance of Two Ways Bacteria Use Light}

Here, we characterize two ways in which bacteria can utilize light and the potential impacts on carbon cycling and industrial progress, respectively. The global
primary energy production is dominated by fossil fuels; unfortunately, their combustion products contribute to greenhouse effects. This, coupled with their diminishing and unreliable sources, is driving the innovation for alternative energy sources (Bradley et al. 2012). Photovoltaics is the conversion of light into electricity using semiconducting materials that can create an electrical current (Blankenship & Hartman 1998). By understanding how bacteria utilize light in their own environments, we could potentially harness their abilities to adapt photovoltaic systems into biological photovoltaic systems and employ biological organisms to generate power in the form of electron gradients.
REFERENCES


