


8-2013

Characterization of Phycoerythrin Physiology in Low-Light Adapted Prochlorococcus Ecotypes

Kathryn H. Roache-Johnson

Follow this and additional works at: <http://digitalcommons.library.umaine.edu/etd>

 Part of the [Environmental Microbiology and Microbial Ecology Commons](#), [Microbial Physiology Commons](#), and the [Organismal Biological Physiology Commons](#)

Recommended Citation

Roache-Johnson, Kathryn H., "Characterization of Phycoerythrin Physiology in Low-Light Adapted Prochlorococcus Ecotypes" (2013). *Electronic Theses and Dissertations*. 1964.
<http://digitalcommons.library.umaine.edu/etd/1964>

This Open-Access Thesis is brought to you for free and open access by DigitalCommons@UMaine. It has been accepted for inclusion in Electronic Theses and Dissertations by an authorized administrator of DigitalCommons@UMaine.

CHARACTERIZATION OF PHYCOERYTHRIN PHYSIOLOGY IN LOW-LIGHT ADAPTED

***PROCHLOROCOCCUS* ECOTYPES**

By

Kathryn H. Roache-Johnson

B.S. University of Delaware, 1997

M.S. University of Southern Maine, 2005

A THESIS

Submitted in Partial Fulfillment of the

Requirements for the Degree of

Master of Science

(in Microbiology)

The Graduate School

The University of Maine

August 2013

Advisory Committee:

Lisa R. Moore, Professor of Biological Sciences, University of Southern Maine,

Advisor

Mary Jane Perry, Professor of School of Marine Sciences

Mary Rumpho, Professor of Molecular and Biomedical Sciences

THESIS ACCEPTANCE STATEMENT

On behalf of the Graduate Committee for Kathryn H. Roache-Johnson I affirm that this manuscript is the final and accepted thesis. Signatures of all committee members are on file with the Graduate School at the University of Maine, 42 Stodder Hall, Orono, Maine.

5/29/13

Dr. Lisa R. Moore, Professor of Biology, University of Southern Maine Date

LIBRARY RIGHTS STATEMENT

In presenting this thesis in partial fulfillment of the requirements for an advanced degree at The University of Maine, I agree that the Library shall make it freely available for inspection. I further agree that permission for "fair use" copying of this thesis for scholarly purposes may be granted by the Librarian. It is understood that any copying or publication of the thesis for financial gain shall not be allowed without my written permission.

Signature _____

Date _____

CHARACTERIZATION OF PHYCOERYTHRIN EXPRESSION IN LOW-LIGHT ADAPTED

PROCHLOROCOCCUS ECOTYPES

By

Kathryn H. Roache-Johnson

Thesis Advisor: Dr. Lisa R. Moore

An Abstract of the Thesis Presented
in Partial Fulfillment of the Requirements for the
Degree of Master of Science
(in Microbiology)
August 2013

The marine cyanobacteria *Prochlorococcus* and *Synechococcus* are the most abundant phototrophs in the oceans. They cohabit the oligotrophic ocean and thus have coevolved together, yet they have distinctly different methods for harvesting light. *Synechococcus*, like other cyanobacteria, possess phycobilisomes with various combinations of phycobiliproteins to capture wavelengths of light not otherwise available to chlorophyll. *Prochlorococcus* lack phycobilisomes and use divinyl chlorophyll *b* (Chl *b*₂) as their primary accessory pigment to divinyl chlorophyll *a* (Chl *a*₂) to capture light energy. In addition to the divinyl chlorophylls, *Prochlorococcus* has genes associated with the phycobiliprotein phycoerythrin (PE), the role of which is still not fully understood, though it is unlikely to contribute significantly to photosynthesis. Past studies have focused on characterizing PE expression on a few isolates of *Prochlorococcus*, the Low Light adapted (LL) II/III ecotype SS120, High Light (HLI) ecotype

MED4 and HLII ecotype AS9601. These studies found that the LLII/III SS120 ecotype expressed both PE chromophores, phycoerythrobilin (PEB) and phycourobilin (PUB), whereas the HL ecotypes only expressed PEB. However, the LL ecotypes have more extensive phylogenetic diversity, so I sought to characterize the diversity in PE physiology for *Prochlorococcus* isolates from three LL ecotypes using a combination of flow cytometry, spectrofluorometry, and pigment measurements. I confirmed that the flow cytometric orange fluorescence (FL2) signal was due to PE in all LL *Prochlorococcus* strains examined and found differences in phycobilin composition among the LL *Prochlorococcus* strains. For instance, some LLIV ecotype strains did not have measurable PEB and most strains increased their PE expression per cell when grown at low light irradiances, suggesting PE is photoacclimating in LL *Prochlorococcus*. The physiological differences observed for LL *Prochlorococcus* strains and ecotypes highlight the necessity of examining more than one strain from an ecotype to make inferences about ecotypic physiology.

ACKNOWLEDGEMENTS

I would like to express my gratitude to my advisor Dr. Lisa R. Moore for the useful comments, remarks and engagement through the learning process of this master's thesis. She has been an excellent female role model, mentor, and friend. She provided me financial support through her NSF grant. I would also like to thank my other committee members Mary Jane Perry and Mary Rumpho. I would like to especially thank my husband, my children Hazel and Aran, and my extended family who helped me throughout this endeavor.

TABLE OF CONTENTS

ACKNOWLEDGEMENTS.....	iii
LIST OF TABLES.....	v
LIST OF FIGURES	vi
CHAPTER ONE: INTRODUCTION.....	1
CHAPTER TWO: METHODS.....	8
Culture conditions.....	8
Phylogenetic analysis.....	9
Flow cytometric parameters.....	10
Pigment analysis.....	10
<i>In vivo</i> fluorescence excitation and emission spectra.....	12
CHAPTER THREE: RESULTS.....	13
Phylogeny of new <i>Prochlorococcus</i> strains.....	13
Genetic differences.....	15
Flow cytometric fluorescence.....	17
<i>In vivo</i> fluorescence properties.....	21
Pigment content.....	24
Photoacclimation.....	26
PE emission with added glycerol.....	32
CHAPTER FOUR: DISCUSSION.....	34
REFERENCES.....	38
BIOGRAPHY OF THE AUTHOR.....	43

LIST OF TABLES

Table 1.	Summary of phycoerythrin related genes.....	16
Table2.	Phycoerythrin physiology in low light adapted <i>Prochlorococcus</i>	19

LIST OF FIGURES

Figure 1.	Neighbor-joining bootstrap consensus tree of 16-23S rDNA ITS sequences from <i>Prochlorococcus</i>	14
Figure 2.	Phycoerythrin flow cytometry and fluorescence emission spectra.....	18
Figure 3.	Fluorescence excitation spectra of LL <i>Prochlorococcus</i>	22
Figure 4.	Fluorescence emission spectra of LL <i>Prochlorococcus</i>	23
Figure 5.	Phycoerythrin physiological parameters compared to PE content.....	25
Figure 6.	Bar graphs of LL <i>Prochlorococcus</i> phycoerythrin physiological parameters.....	28
Figure 7.	Bar graphs of LL <i>Prochlorococcus</i> phycobilins.....	31
Figure 8.	Glycerol treated LL <i>Prochlorococcus</i> fluorescence emission spectra.....	33

CHAPTER ONE

INTRODUCTION

Approximately half of the net primary production (fixation of CO₂ into biomass) that occurs on earth is from phytoplankton in the oceans (Field et al. 1998). Within the phytoplankton community there resides a small, ubiquitous cyanobacterium, *Prochlorococcus* that is the dominant photosynthesizing microorganism in the oligotrophic oceans, reaching abundances as high as 10⁸ cells L⁻¹. This is one to two orders of magnitude greater than co-occurring *Synechococcus*, the next most abundant phototroph as well as the closest phylogenetic relative to *Prochlorococcus* within the cyanobacterial phylum (Partensky et al. 1999; Partensky and Garczarek 2010).

Prochlorococcus is closely related to the marine cluster A *Synechococcus* based upon their 16S-23S rDNA internal transcribed spacer sequences (>65%), and all *Prochlorococcus* ecotypes are 97% similar (Rocap et al. 2002). Despite the similarity in ribosomal DNA sequences, these genera have evolved different pigment compositions due to selection pressures found in their microenvironments, such as position in the oligotrophic water column or spatial ecological differences (i.e. coastal versus oligotrophic). *Prochlorococcus* occupies the oceans between the 40°S to 45°N latitudes (Johnson et al. 2006), extends vertically down to ~200m, and demonstrates resiliency after seasonally influenced mixing events which alter the local chemical environment (Partensky et al. 1999). Due to this wide distribution and adaptability, *Prochlorococcus* contributes a large portion of the net primary production in the tropical and subtropical oceans (Campbell et al. 1997; DuRand et al. 2001). Estimates of total global

picophytoplankton biomass is 0.53–1.32 Pg C with 17–39% *Prochlorococcus*, 12–15% *Synechococcus* and 49–69% picoeukaryotes (Buitenhuis et al. 2012).

Prochlorococcus is able to span vertically through four orders of magnitude in light intensity because of the existence of light adapted ecotypes that are able to thrive in a wide range of irradiances (Moore et al 1999). High-light (HL) ecotypes with low chlorophyll b_2/a_2 ratios, grow at light intensities found in the surface waters where low-light (LL) ecotypes are inhibited. The LL ecotypes with high chlorophyll b_2/a_2 ratios, are limited to irradiances deep in the euphotic zone (near the 1% light level). Culture independent quantitative polymerase chain reaction (qPCR) has revealed ecotypic distribution patterns for *Prochlorococcus*. These patterns consist of HLI and HLII with maximal abundance at the surface lit waters (<50m), LLII/III and LLIV most abundant in the dimmer, deeper portion of the water column (>100m), and LLI demonstrating maximal abundance between the HL and LL ecotypes (~50-100m) (Johnson et al. 2006; Zinser et al. 2006; Martiny et al. 2009; Malmstrom et al. 2010; Partensky and Garczarek 2010). While LLII and LLIII ecotypes have been separated, throughout this thesis these two ecotypes will be combined and referred to as LLII/III (sensu, Martiny et al. 2009). The HL and LL ecotypes are phylogenetically distinct (Rocap et al. 2002, 2003). In contrast to *Prochlorococcus*, which is confined to the oligotrophic ocean, marine *Synechococcus* has evolved greater genetic diversity such that it can occupy diverse horizontal gradients of nutrients and light quality (Scanlan et al. 2009).

Prochlorococcus and *Synechococcus* have overlapping niches in the ocean (Partensky et al. 1999) and share many physiological features; however, their light

harvesting apparatus is fundamentally different. Like most cyanobacteria and red algae, *Synechococcus* use phycobilisomes to harvest light not otherwise available to chlorophyll, and funnel this energy from the outer antenna down toward the photosystem reaction centers converting excitation energy to chemical energy (Sidler 1994; MacColl 1998; DeRuyter and Fromme 2008). The phycobilisome is an organized macromolecular light harvesting complex that is located in the stromal space between the thylakoid membranes. The phycobilisome is composed of an allophycocyanin core that is connected to the photosystems and is surrounded by six to eight rods composed of one or two types of phycobiliproteins. The phycobiliproteins absorb light between 450-660nm, with each protein having its own absorbance spectrum due to specific chromophore composition. Phycobiliproteins are water-soluble proteins that covalently bind chromophores (phycobilins) by thioester bonds to specific cysteinyl residues. Phycobilins are open chain tetrapyrroles. Different types of *Synechococcus* possess unique spectral properties that are responsible for the diversity of colors (blue-green to orange) (Six et al. 2007). Phycoerythrins (PE) are phycobiliproteins found on the most peripheral part of the antenna. Phycoerythrins bind two different chromophores, phycourobilin (PUB) and phycoerythrobilin (PEB). PUB is the main phycobilin for marine oceanic *Synechococcus* and is specifically adapted to absorb light in the blue portion of the visible spectrum that penetrates deepest in the oligotrophic waters.

In contrast to *Synechococcus*, *Prochlorococcus* has a drastically reduced cellular and genome size, resulting in a more efficient organism that thrives in the oligotrophic ocean. *Prochlorococcus* is able to take advantage of increased surface to volume ratio

for efficient uptake of nutrients and depleted resources available in the oligotrophic oceans (Partensky and Garczarek 2010). The reduced cellular size has resulted in a streamlined genome with only essential functions and genes remaining (Kettler et al. 2007; Scanlan et al. 2009). *Prochlorococcus* has simpler pigmentation than *Synechococcus*, made up of thylakoid membrane proteins binding divinyl chlorophyll a_2 and b_2 as their main antenna complex. While *Prochlorococcus* has lost the structure of the phycobilisome, some PE genes are still present. The genes related to PE biosynthesis and potential light harvesting function are remnants from ancestral *Synechococcus* (Scanlan et al. 2009).

The expression of PE in *Prochlorococcus* has been observed through absorption and fluorescence properties on two HL and one LL cultured isolates (Hess et al. 1996; Steglich et al. 2003a, 2005). In LLII/III SS120, the absorption and fluorescence emission corresponding to PE was observed with PUB being the main chromophore. Additionally, low levels of orange fluorescence correlated with small amounts of flow cytometric excited PE has been reported from a natural population of *Prochlorococcus* found deeper in the water column (Hess et al. 1996). All LL *Prochlorococcus* strains have retained all the necessary genes for synthesizing one complete PE protein and the genes associated with the linker proteins that stabilize the phycobiliprotein structure and bind two chromophores (PUB and PEB) (Table 1). In contrast, HL *Prochlorococcus* strains have kept only a few PE related genes, including the genes for the biosynthesis of PEB and phycocyanobilin (PCB), a degenerated PE- β subunit (Ting et al. 2001), and a phycobilin lyase (*cpeS*) involved in the specific attachment of PEB to β -PE (Wiethaus et

al. 2010b). This β -PE has several mutations and has lost some of the cysteines needed for chromophore binding (Ting et al. 2001). The *cpeB* gene may not act as a structural gene, but instead may act as a green light sensing molecule (Steglich et al. 2005). It is thought that in HL *Prochlorococcus* the PE genes are in a process of being lost from the genome (Hess et al. 2001).

Expression of low levels of phycobilisome genes occur in both *Prochlorococcus* LLII/III SS120 and HLI MED4 (Hess et al. 1996; Steglich et al. 2005). All *Prochlorococcus* strains possess *cpeB*, the structural subunit for β -phycoerythrin, and *cpeB* has been used as a marker to identify HL and LL *Prochlorococcus* in natural populations from the Red Sea (Steglich et al. 2003b). Most LL *Prochlorococcus* have both α - and β -subunits for the heterodimer structure of phycoerythrin, the genes for the biosynthesis for phycocyanobilin and phycoerythronbilin, many lyase genes for chromophore attachment, and the gene *ppeC* for the attachment to the thylakoid membrane. Since PE genes form a phylogenetically distinct clade among *Prochlorococcus*, it has been suggested that they are driving forces in their evolution to maintain the sequences in many LL *Prochlorococcus* (Penno et al. 2000).

All LL *Prochlorococcus*, with the exception of MIT9303 and MIT9313, can synthesize *ppeC* linker polypeptide, which is thought to be involved in the transfer of excitation energy directly from PE to the photosystems (Hess et al. 1999). A disruption of this linkage can occur in the presence of glycerol (Wyman et al. 1985). None of the HL *Prochlorococcus* have the *ppeC* linker gene. LL *Prochlorococcus* possess a number of lyase genes (*mpeU*, *cpeZ*, *cpeY*, and *cpeT*) that are involved in chromophore attachment

(Wiethaus et al. 2010a). The genes code for enzymes (*ho1*, *pebA*, *pebB*, and *pcyA*) responsible for the biosynthesis of PEB and PCB are present in all *Prochlorococcus* strains. PEB and PCB originate from biliverdin IX α , an open-chain tetrapyrrole and product of the heme oxygenase reaction, followed by either the enzyme encoding *pcyA* which leads to PCB, or the two enzymes encoding for *pebA* and *pebB* that catalyze the four electron reduction of biliverdin IX α to PEB. Certain *Prochlorococcus* cyanophages carry a single gene (*pebS*) that encodes the enzyme phycoerythrobilin synthase (PebS) that replaces two enzymes 15,16-dihydrobiliverdin:ferredoxin oxidoreductase with gene *pebA* and PEB:ferredoxin oxidoreductase with gene *pebB*, making this two step process into a single reaction (Dammeyer et al. 2008). No PCB binding proteins have been identified in *Prochlorococcus* (Hess et al. 2001); however, absorption spectra have identified the presence of PEB in both HL and LL *Prochlorococcus* strains (Steglich et al. 2003a, 2005). *Prochlorococcus* also has the PUB chromophore, but no PUB biosynthesis enzyme has been identified, and it is thought that PUB is produced during the attachment process to the phycobiliprotein by an unidentified isomerase/lyase (Wiethaus et al. 2010a). Shukla et al. (2012) found that the gene *mpeZ* is involved in converting PEB to PUB in marine *Synechococcus*, but this gene has not been identified in *Prochlorococcus*.

The PE retained in *Prochlorococcus* is unique because in most cyanobacteria it is a peripheral pigment of the phycobilisome. Phycoerythrin is normally directly attached to phycocyanin, which is attached to an allophycocyanin core that is attached to the photosystem. However, in *Prochlorococcus*, neither allophycocyanin nor phycocyanin

expression have been documented. Therefore, the function of PE within the *Prochlorococcus* cell remains unsolved, with the exception of PE in HL *Prochlorococcus* possibly acting as a green light photoperceptor (Steglich et al. 2005). PE function in LL *Prochlorococcus* remains enigmatic.

In order to understand the potential role of PE and help characterize its physiological expression in other LL *Prochlorococcus*, this study measured *in vivo* excitation and emission spectra, PE content relative to chlorophyll content, and tracked changes in flow cytometric fluorescence under high and low growth irradiance in a variety of LL *Prochlorococcus* strains representing phylogenetically distinct ecotypes. Glycerol treatment was used to determine whether PE was attached to the thylakoid, indicating whether PE could serve a potential light harvesting function. I hypothesized that the weak orange fluorescence (FL2), measurable above the instrument noise of the flow cytometer, was due to PE fluorescence, and that the presence of *ppeC* indicates that PE transfers light energy to the photosystem. A broader goal of this study was to analyze whether PE may serve as a distinguishing characteristic for one or more LL ecotype sub-clades.

CHAPTER TWO

METHODS

Culture conditions

Several (LL) *Prochlorococcus* strains (LLI NATL1A, LLI NATL2A, LLII/III SS120, and LLIV MIT9313) that are representative of different LL ecotype subclades according to their 16-23S rDNA ITS were tested. HLI *Prochlorococcus* strain MED4 and *Synechococcus* WH8102 were used as a comparison. HLI MED4 should show no PE expression when excited at PUB maxima wavelength, and *Synechococcus* WH8102 should demonstrate enhanced PE expression compared to the LL *Prochlorococcus* isolates. New isolates LLI MIT0801 from Bermuda Atlantic Time-Series (BATS), and LLIV C3 from the South Atlantic (SA) were obtained by L. R. Moore using a high-throughput culturing technique (Connon and Giovannoni 2002). LLIV MIT0601 and LLIV MIT0603 from Hawaii-Ocean Time-series (HOT) were isolated with Pro99 media according to methods in (Moore et al. 2007) by L. Thompson, S. W. Chisholm and L. R. Moore. None of the strains tested were maintained as axenic; however, since photosynthetic pigments are being measured, any contaminating heterotrophic bacteria should not affect the results. Three replicate batch cultures were grown in acid-washed polycarbonate or glass containers containing 0.2 μ m filtered Woods Hole Oceanographic Institution-Environmental Systems Laboratory (WHOI-ESL) natural seawater based media Pro99 (Moore et al. 2007) at 24°C under L:D 14:10 using cool white fluorescent bulbs. Cultures were progressively acclimated to low growth irradiance (I_g)(10 μ mol Q $m^{-2} s^{-1}$) or high I_g (55-65 μ mol Q $m^{-2} s^{-1}$) light growth irradiances obtained using window

screening to obtain the appropriate light intensity. HL MED4 low Ig was grown at $20 \mu\text{E m}^{-2} \text{ s}^{-1}$ because of its inability to grow at a lower light irradiance, and high Ig LLIV C3 was grown at $36 \mu\text{mol E m}^{-2} \text{ s}^{-1}$ because it was photoinhibited at higher growth irradiances. The light levels chosen were from the initial portion of growth curves dependent on light irradiance prior to photoinhibition of cell growth. Acclimation was confirmed by obtaining successive, similar growth rates and consistent red fluorescence per cell as measured flow cytometrically. Irradiances were measured with a QSL-100 quantum scalar irradiance meter (Biospherical Instruments).

Phylogenetic analysis

DNA extraction and PCR amplification were performed with exponential phase cultures harvested by centrifugation ($14,000 \times g$ for 10 min), lysed using three rounds of a freeze/thaw (freeze at -70°C for 10 min) and thaw on (heat block at 70°C for 10 min), and extracted using a Qiagen DNeasy Kit (Cat#69506). The ITS/23S fragment was amplified using primers 16S-1247f and 23S-1608r (Rocap et al. 2002). For Sanger sequencing, PCR amplifications were performed at the Center for Genome Research & Biocomputing at Oregon State University. The ITS fragment was sequenced bi-directionally using primers 16S-1247f and primers internal to the PCR fragment: ITS-Alaf, ITS-Alar, and 23S-241r (Rocap et al. 2002). Sequences were edited and aligned manually with BioEdit (Hall 1999). Distance trees were inferred using the Neighbor-Joining method. The evolutionary distances were computed using the Maximum Composite Likelihood. There were a total of 316 positions in the final dataset. Phylogenetic analyses were conducted in MEGA4 (Tamura et al. 2007).

Flow cytometric parameters

Small aliquots (200 μ l) of cultures grown under low and high light irradiances were taken daily for flow cytometric measurements. Cell concentrations were determined using a BD FACS Calibur flow cytometer equipped with a 488nm laser and a high throughput sampler attachment. Flow cytometric detector parameters including red (>650 nm) and orange (564-606 nm) fluorescence were normalized with a standard 0.52 μ m bead (Polysciences) and reported for intercomparison between strains and light levels. Red fluorescence (FL3) corresponds to chlorophyll in the cell, orange fluorescence (FL2) corresponds to PE, and side-angle light scatter (SSC) is roughly correlated to cellular size. FL3, FL2, and SSC are established methods for enumerating and distinguishing different *Prochlorococcus*, *Synechococcus*, and picoeukaryotes in natural populations (Marie et al. 1999).

Pigment analysis

Cultures were harvested from late exponential growth phase by filtering a known volume of culture (typically 50 ml for chlorophyll and 100 ml for PE) onto 25-mm Whatman GF/F filters under low vacuum (~100 mm Hg), with approximately >99% cells retained (data not shown). Filters were immediately immersed in liquid nitrogen and then stored at -70°C until extraction (1-6 months). Cell concentration was determined flow cytometrically and used to calculate pigment content per cell.

Chlorophyll was measured on each *Prochlorococcus* culture (Knap et al. 1994). Filters were resuspended in extraction solvent (90% acetone) and sonicated with a small benchtop sonicator for 10 min under dim light. Samples were left in acetone overnight

at -20°C in the dark. The recovered extract was measured with a benchtop fluorometer (Turner Designs, TD700) equipped with a standard chlorophyll filter set. A known concentration of pure chlorophyll was determined spectrophotometrically (Shimadzu, BioSpec Mini) from Swiss chard that was extracted in 90% acetone and used for generating a standard curve within the range of the chlorophyll samples.

The PE content was determined for each *Prochlorococcus* culture on replicate filters to those used for chlorophyll measurements using a modified absorption method (Lawrenz et al. 2011). Our method used a fluorometric method to capture the small PE signal associated with *Prochlorococcus* cells. Filters were thawed, resuspended in 5 ml of 0.1M K-phosphate buffer (pH 7), and sonicated (Model #FB120, Fisherbrand) at 4°C, 25% max power for 45 sec. Samples underwent three rounds of sonication, freeze (-70°C) and thaw procedure, after which they were left to extract at 4°C for 24-48hrs. Samples were then vortexed and centrifuged (Beckman Model TJ-6, 5 min at 2,000RPM) to remove residual filter material and the supernatant was measured on a spectrofluorometer (FluoroMax2) with excitation set to 495nm and emission recorded at 573nm. PE was extracted from exponentially grown *Synechococcus* WH8102 and the concentration determined with a spectrophotometer (Cary-50Bio UV/VIS-spectrophotometer) using an extinction coefficient at 492nm of $2.78 \times 10^6 \text{ M}^{-1} \text{ cm}^{-1}$ (Ong et al. 1984). A serially diluted standard curve was generated and emission fluorescence was measured at 573nm (excitation set to 495nm) to estimate the PE concentration from fluorescence emission data collected from the *Prochlorococcus* samples.

***In vivo* fluorescence excitation and emission spectra**

In vivo fluorescence was measured on cells from the exponential phase using a Fluoromax-2 fluorescence spectrometer. Excitation for the emission spectra was set at 495nm (the maximum absorption band for PUB; Steglich et al. 2003a) and emission was measured from 515-700nm to detect both PE and chlorophyll. The emission spectra were normalized to the chlorophyll maximum (677nm). The excitation spectra were measured from 400-570nm while measuring emission at 590nm. The excitation spectra were normalized to the PUB maximum (500nm). The excitation and emission monochromator slits were set at 5-nm bandpass for both spectra. Fluorescence from Pro99 media was used as the blank and subtracted from the culture spectra.

To determine whether PE is attached to the photosystem in the thylakoid membrane, sub-samples of live *Prochlorococcus* cells were treated with glycerol (50% final concentration) and compared to subsamples without added glycerol, but with the same volume of media added. Glycerol has been used to interrupt the energy transfer between PE and the phycobilisome in *Synechococcus* and *Prochlorococcus* (Wyman 1992; Lokstein et al. 1999; Steglich et al. 2003a), resulting in an enhanced PE fluorescence signal when added to cells. Samples were excited at 495nm and emission spectra measured from 515-700nm. The fluorescence obtained from either (media or 50% (vol/vol) glycerol/media seawater) were subtracted from the spectra, and the emission spectra were normalized to number of cells.

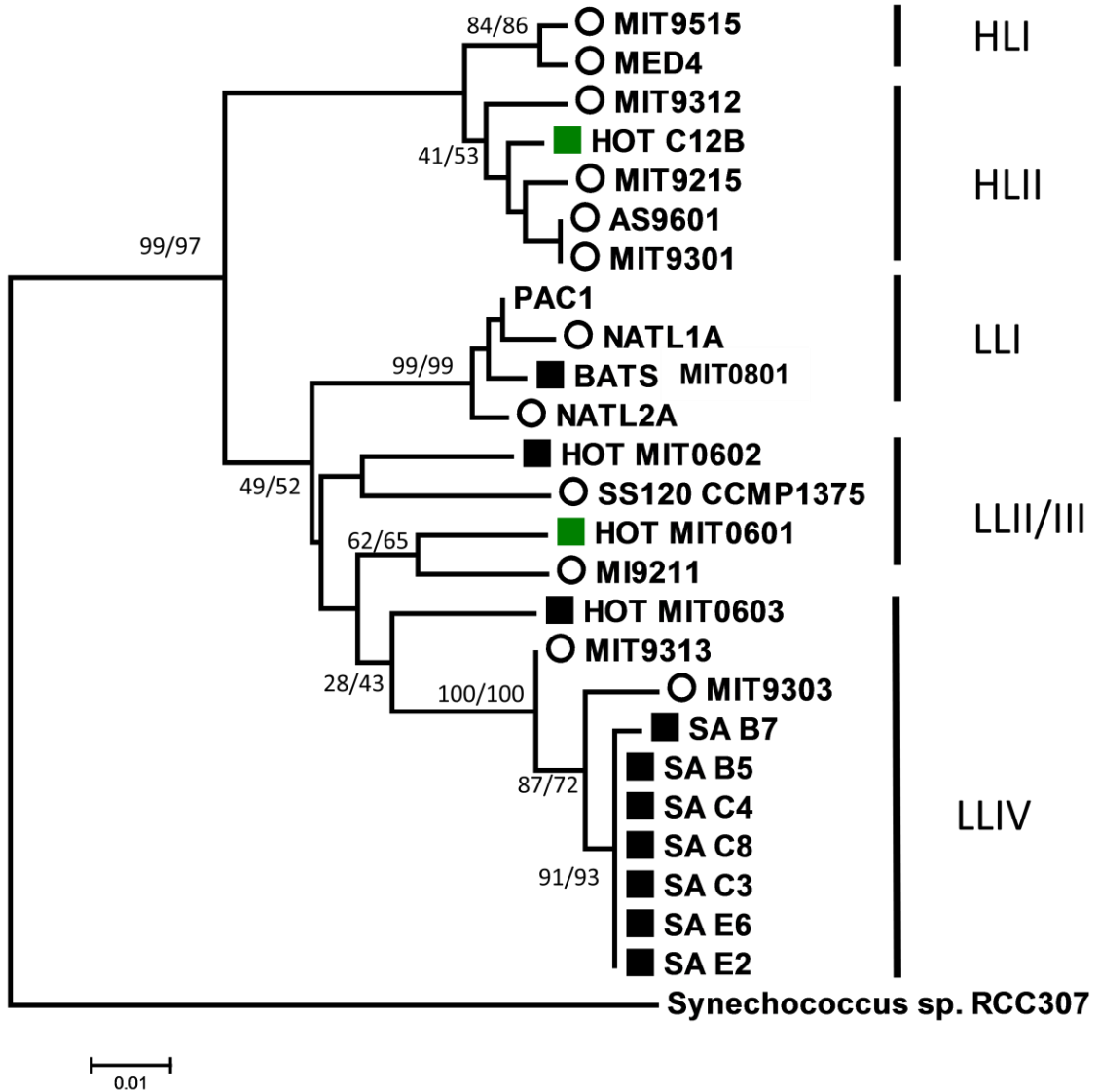
CHAPTER THREE

RESULTS

Phylogeny of new *Prochlorococcus* strains

The 16S and 23S ribosomal DNA internal transcribed spacer (ITS) sequence was used in this study to achieve finer resolution between closely related *Prochlorococcus* isolates (Rocap et al. 2002) to categorize the phylogeny of the new *Prochlorococcus* isolates based upon neighbor-joining bootstrap consensus tree (Figure 1). New strains BATS MIT0801 grouped with the LLI clade, HOT MIT0601 grouped with the LLII/III clade, and South Atlantic C3 grouped with the LLIV clade. HOT MIT0603 also grouped with LLIV clade although had a weak relationship according to neighbor-joining and minimum evolution with the LLIV clade (23%/48%). Analysis of the ITS region further separated the HL and LL ecotypes into subclades or ecotypes with possible physiological differences that represent oceanic niche differentiation, spatial, and temporal specialization (Rocap et al. 2003; Zinser et al. 2006; Martiny et al. 2009).

Figure 1. Neighbor-joining bootstrap consensus tree of 16-23S rDNA ITS sequences from *Prochlorococcus*. New isolate sequences were generated from PCR amplification of cultured isolates preceded by a square and each name is preceded by the location isolated (SA=South Atlantic, BATS=Bermuda Atlantic Times-Series, and HOT=Hawaii Ocean Time-series). All other sequences were obtained from BLAST NCBI. The green boxes indicate new isolates that possibly utilize nitrate. The bootstrap values at the nodes are based on neighbor-joining and minimum evolution algorithms by MEGA 4.0 phylogenetic software. Bootstrap values less than 50 are not shown. The tree is rooted with *Synechococcus sp.* RCC307.



Genetic differences

While *Prochlorococcus* has completely lost the distinctive cyanobacterial phycobilisomes, many of the PE-related genes are still present. A summary of PE-related genes among the LL *Prochlorococcus* strains used in this study indicate the presence of genes for structure (*cpeA* and *cpeB*), lyases used for chromophore attachment (*mpeU*, *cpeZ*, *cpeY*, *cpeT*, and *cpeS*), biosynthesis of phycobilins (*hol1*, *pebA*, *pebB*, and *pcyA*), and linker protein for the attachment of PE to the photosystems (*ppeC*) are presented and compared with published *Prochlorococcus* sequenced strains (Table 1) (Hess et al. 1996, 1999, 2001; Dammeyer et al. 2008; Wiethaus et al. 2010a; b). The new strains (LLI MIT0801, LLII/III MIT0601, and LLIV MIT0603 and LLIV C3) were sequenced and kindly made available to me (unpublished sequences by S. Biller, P. Berube, and S.W. Chisholm, MIT). Both newly sequenced strains LLI MIT0801 and LLII/III MIT0601 have all the necessary genes for a complete PE (including both α - and β -subunits), biosynthesis of PEB, and the γ linker (*ppeC*), the possible linker protein that attaches PE to the photosystem. Interestingly, LLIV MIT0603 also possesses these genes, while LLIV C3 is missing the γ linker gene, like LLIV MIT9313 and MIT9303. The protein sequence that is identified as a possible linker to the thylakoid, corresponding to the gene *ppeC*, was searched for amino acid similarity using the NCBI blastp tool to understand the results from the glycerol experiments (see PE with added glycerol section). Analysis of the *ppeC* corresponding amino acid sequence was compared to *Prochlorococcus* LLII/III SS120 and found to be less than 60% identical for all the other LL

ecotype strains (Table 1), indicating possible mutations at the sites that attach to the thylakoid membrane.

Table 1. Summary of phycoerythrin related genes. Summary of genes (presence or absence) related to phycobiliproteins and amino acid % similarity of the *ppeC* gene in SS120 for *Prochlorococcus* strains that have had their genomes sequenced and new isolates. Isolates in red are newly sequenced.

Gene	<i>cpeA</i> (NP874732)	<i>cpeB</i> (NP874731)	<i>xmpeU</i> , <i>cpeZ</i> , <i>cpeY</i> , <i>cpeT</i>	<i>cpeS</i> (NP874737)	<i>hol1</i> , <i>pebA</i> , <i>pebB</i> , <i>pcyA</i>	<i>ppeC</i>	% amino acid similarity of <i>ppeC</i> to SS120
Strain	α -subunit structural protein	β -subunit structural protein	PE linker (PUB or PEB)	PE linker PEB β -subunit	Biosynthesis phycobilins PEB or PCB	PBS linker PE to thylakoid	
Syn WH8102	X	x	X	X	X	X	43
LLI MIT0801	X	X	X	X	X	X	43
LLI (NATL1A, NATL2A)	X	X	X	X	X	X	42
LLII/III (SS120, MIT9211)	X	X	X	X	X	X	100
LLII/III MIT0601	X	X	X	X	X	X	52
LLIV MIT0603	X	X	X	X	X	X	58
LLIV (MIT9313, MIT9303)	X	X	X	X	X		n/a
LLIV C3	X	X	X	X	X		n/a
HLIMED4		X		X	X		n/a

Flow cytometric fluorescence

Along with detection of the typical chlorophyll (FL3) fluorescence emission, orange (FL2) fluorescence emission was observed for *Synechococcus* WH8102 (Figure 2A,B). A low but measurable amount was detected for *Prochlorococcus* LLII/III MIT0601 (Figure 2D,E), and none was detected out of the FCM noise for *Prochlorococcus* HLI MED4 strain (Figure 2G, H). All LL ecotypic strains examined showed measurable FL2, though the amount varied for different LL strains (Table 2). The corresponding fluorescence emission spectra verified that the FL2 emission signal was consistent with PE emission in LLII/III MIT0601 and the absence of FL2 emission signal in HLI MED4 (Figure 2F,I). The difference in FL2 emission level between *Synechococcus* WH8102 and LLII/III MIT0601 also was consistent with the higher level of PE emission observed for WH8102 (Figure 2C).

Figure 2. Phycoerythrin flow cytometry and fluorescence emission spectra. [A], [D], and [G] Red (FL3) and [B], [E] and [H] orange (FL2) flow cytometric analysis versus side-angle scatter of *Synechococcus* WH8102 (orange dots), *Prochlorococcus* LLII/III MIT0601 (green dots), and *Prochlorococcus* HLI MED4 (green dots). Black dots are instrument noise or dying cells. Fluorescence intensity of chlorophyll (>650 nm) and phycoerythrin (564-606 nm) versus side-angle scatter after excitation with 488 nm laser. The relative phycoerythrin normalized to a standard bead (0.52 μm Polysciences) ratio is inset within the plots. [C], [F], and [I] Relative fluorescence emission spectra of whole cells depicting the wavelengths where phycoerythrin emission is detected by the flow cytometer (orange block). Emission spectra were excited at 488nm and emission was detected between 520-700 nm.

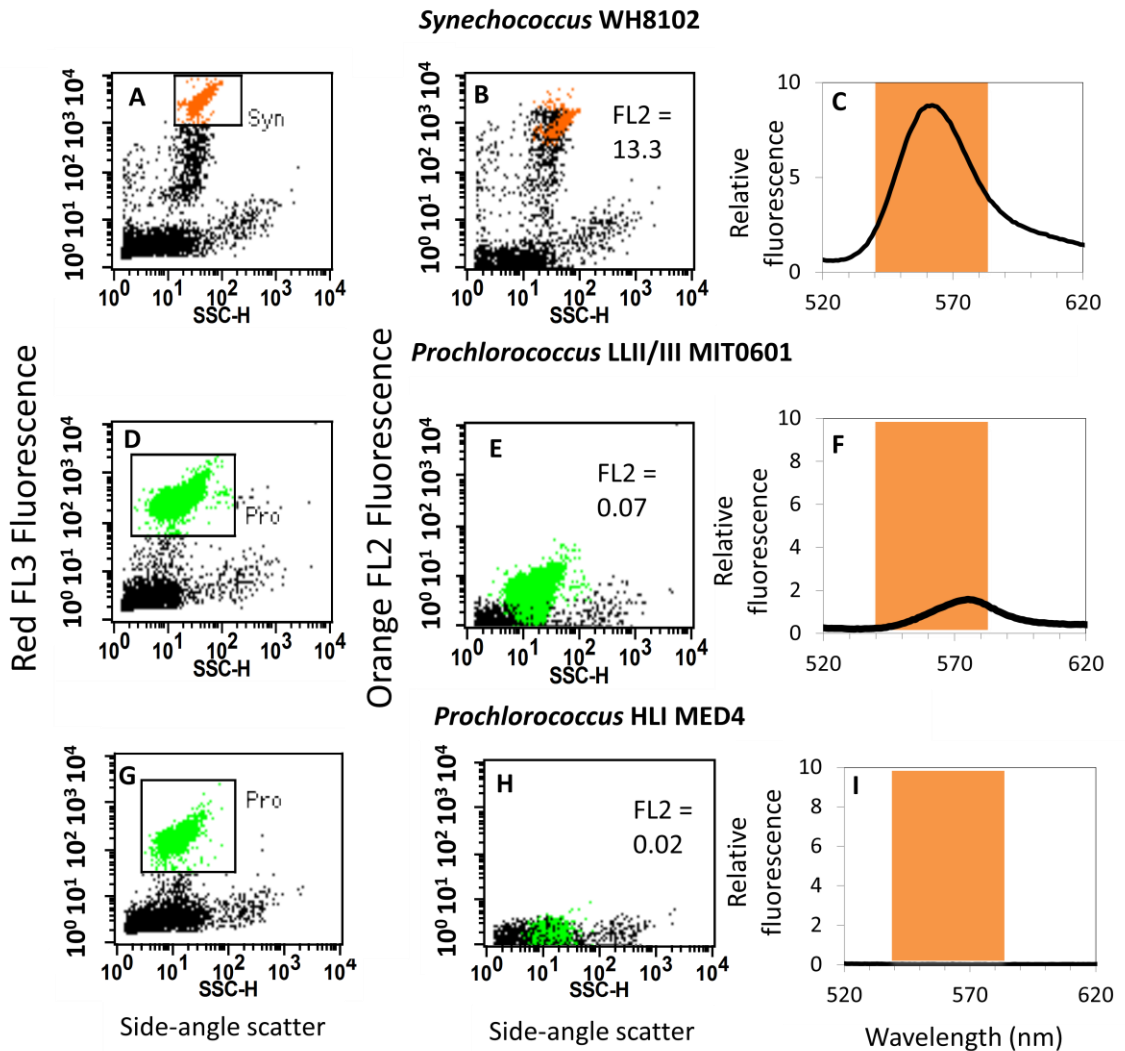


Table 2. Phycoerythrin physiology in low light adapted *Prochlorococcus*. Flow cytometric parameters (red and orange fluorescence normalized to 0.52 μm reference bead), fluorescence excitation maxima PUB (500 nm) and PEB (559 nm) normalized to cell concentration (emission set to 590nm), PUB:PEB ratios, fluorescence emission per cell (Ex set to 495 nm), and pigment content (phycoerythrin and chlorophyll) for various *Prochlorococcus* strains grown under high (55-65 $\mu\text{mol Q m}^{-2} \text{s}^{-1}$) and low (10 $\mu\text{mol Q m}^{-2} \text{s}^{-1}$) growth irradiances (n=3; n/a = no value available). For each parameter differences among the two conditions (Low and High I_g grown cultures) were tested using a student's t test, *p \leq 0.05, **p < 0.001.

		Flow cytometric parameters		Fluorescence Properties			Pigment analysis		
Strain	Growth irradiance	Relative Chl fluor cell ⁻¹	Relative PE fluor cell ⁻¹	PUB excitation Ex500/Em590 (cell ⁻¹)	PEB excitation Ex559/Em590 (cell ⁻¹)	Ratio of PUB to PEB	PE emission Ex495/Em573 (cell ⁻¹)	PE (fg cell ⁻¹)	Chl (fg cell ⁻¹)
HLI MED4	Low I	5.1** (0.9)	0.0204 ** (0.0003)	n/a	n/a	n/a	n/a	0.0011 (0.0004)	4.1** (0.6)
	High I	2.5** (0.2)	0.0190** (0.0002)	n/a	n/a	n/a	n/a	0.0018 (0.0002)	2.7** (0.1)
Syn WH8102	Low I	103 (11)	15 (1)	0.11 (0.06)	0.07 (0.03)	1.75 (0.02)	0.27 (0.14)	91 (9)	5.7 (0.7)
LLI ecotypes									
NATL1A	Low I	9.42** (0.3)	0.0502** (0.0005)	0.00163* (0.00001)	0.00089** (0.00002)	1.82* (0.04)	0.00177 (0.00005)	0.17** (0.02)	10.6** (0.4)
	High I	3.5** (0.8)	0.036** (0.003)	0.0011* (0.0001)	0.00061** (0.00006)	1.75* (0.04)	0.0013 (0.0003)	0.043** (0.004)	3** (1)
NATL2A	Low I	10* (1)	0.047* (0.003)	0.0007 (0.0006)	0.0004 (0.0004)	1.8 (0.1)	0.00095 (0.00084)	0.0130* (0.0001)	5.1** (0.7)
	High I	3.6* (0.2)	0.035* (0.001)	0.00032 (0.00001)	0.00019 (0.00001)	1.72 (0.07)	0.00042 (0.00002)	0.0096* (0.0002)	1.78** (0.09)
BATS MIT0801	Low I	30** (2)	0.076** (0.003)	0.00074** (0.00004)	0.00047** (0.00003)	1.57 (0.01)	0.00092** (0.00007)	0.051 (0.009)	9.4** (0.5)
	High I	6.59** (0.3)	0.041** (0.001)	0.00040** (0.00006)	0.00027** (0.00004)	1.5 (0.1)	0.0005** (0.0001)	0.034 (0.005)	3** (1)

Table 2. Continued

		Flow cytometric parameters		Fluorescence Properties			Pigment analysis		
Strains	Growth irradiance	Relative Chl fluor cell ⁻¹	Relative PE fluor cell ⁻¹	PUB excitation Ex500/Em590 (cell ⁻¹)	PEB excitation Ex559/Em590 (cell ⁻¹)	Ratio of PUB to PEB	PE emission Ex495/Em573 (cell ⁻¹)	PE (fg cell ⁻¹)	Chl (fg cell ⁻¹)
LLII/III ecotypes									
SS120	Low I	6.0** (0.3)	0.0305** (0.0005)	0.00077** (0.00002)	0.00048** (0.00002)	1.6* (0.03)	0.00098** (0.00004)	0.011 (0.008)	3.2** (0.2)
	High I	3.1** (0.5)	0.0256** (0.0008)	0.00039** (0.00002)	0.00025** (0.00002)	1.54* (0.04)	0.00054** (0.00003)	0.008 (0.003)	1.9** (0.1)
MIT0601	Low I	10.4** (0.1)	0.081** (0.001)	0.00082* (0.00002)	0.00045** (0.00001)	1.81 (0.01)	0.00135* (0.00004)	0.022 (0.003)	3.9** (0.1)
	High I	3.6** (0.3)	0.059** (0.004)	0.00067* (0.00004)	0.00036** (0.00002)	1.85 (0.03)	0.00119* (0.00006)	0.021 (0.003)	2.5** (0.3)
LLIV ecotypes									
MIT0603	Low I	10.15** (0.08)	0.035** (0.001)	0.00022* (0.00002)	0.00014** (0.00001)	1.61** (0.03)	0.00032** (0.00002)	0.004 (0.002)	2.2** (0.2)
	High I	2.8** (0.2)	0.0298** (0.0004)	0.00018* (0.00001)	0.000103** (0.000007)	1.73** (0.03)	0.00024** (0.00001)	0.0051 (0.0005)	0.81** (0.08)
MIT9313	Low I	28** (2)	0.063* (0.002)	0.00063 (0.00005)	n/a	n/a	0.00103 (0.00002)	0.05 (0.01)	7.0** (0.2)
	High I	14** (1)	0.055* (0.001)	0.00054 (0.00006)	n/a	n/a	0.0021 (0.0002)	0.016 (0.002)	2.1** (0.8)
SA C3	Low I	43.4** (0.5)	0.103 (0.003)	0.00091 (0.00003)	n/a	n/a	0.00160 (0.00009)	0.034 (0.004)	4.3 (0.5)
	High I	25** (2)	0.108 (0.003)	0.0011 (0.0002)	n/a	n/a	0.0020 (0.0003)	0.042 (0.007)	2.4 (0.9)

***In vivo* fluorescence properties**

To better characterize the flow cytometric FL2 measurements among the LL *Prochlorococcus* ecotypes, both *in vivo* fluorescence excitation spectra (Figure 3A-H) and emission spectra (Figure 4A-H) were measured for a variety of *Prochlorococcus* cultures from each LL ecotype: LLI ecotypes NATL1A, NATL2A, MIT0801, LLII/III ecotypes MIT0601, SS120, and LLIV ecotypes MIT0603, MIT9313, SA C3. The *in vivo* fluorescence excitation spectra for *Prochlorococcus* revealed PE excitation maxima at 500 and 559 nm, corresponding to the excitation wavelengths of the chromophores PUB and PEB, respectively, similar to that observed for LLII/III SS120 (Steglich 2003; Steglich et al. 2003a) and for *Synechococcus* WH8102, a high PUB containing strain (Six et al. 2004). Two exceptions were SA C3 and MIT9313 (both members of the LLIV ecotype subclade) (Figure 4E, H), which only exhibited PUB excitation at 500 nm. The physiological expression of PEB is consistent with the presence of the genes for the biosynthesis of PEB, which all LL *Prochlorococcus* have (Table 1). Under this study's experimental conditions no PEB chromophore was detected for HLMED4 (data not shown). Steglich et al. (2005) detected PEB in HLMED4 and AS9601 and found 100-fold less PE protein than SS120. For the strains that have PUB and PEB, the PUB:PEB excitation peak maxima ratio was similar for all strains, ranging from 1.6-1.9, and close to that observed for *Synechococcus* WH8102 (Table 2; Six et al. 2004).

Figure 3. Fluorescence excitation spectra of LL *Prochlorococcus*. [A]-[H] Fluorescence excitation spectra with emission set to 590 nm and excitation detected between 450-570 nm. Excitation spectra are normalized to PUB absorption maxima at 495 nm.

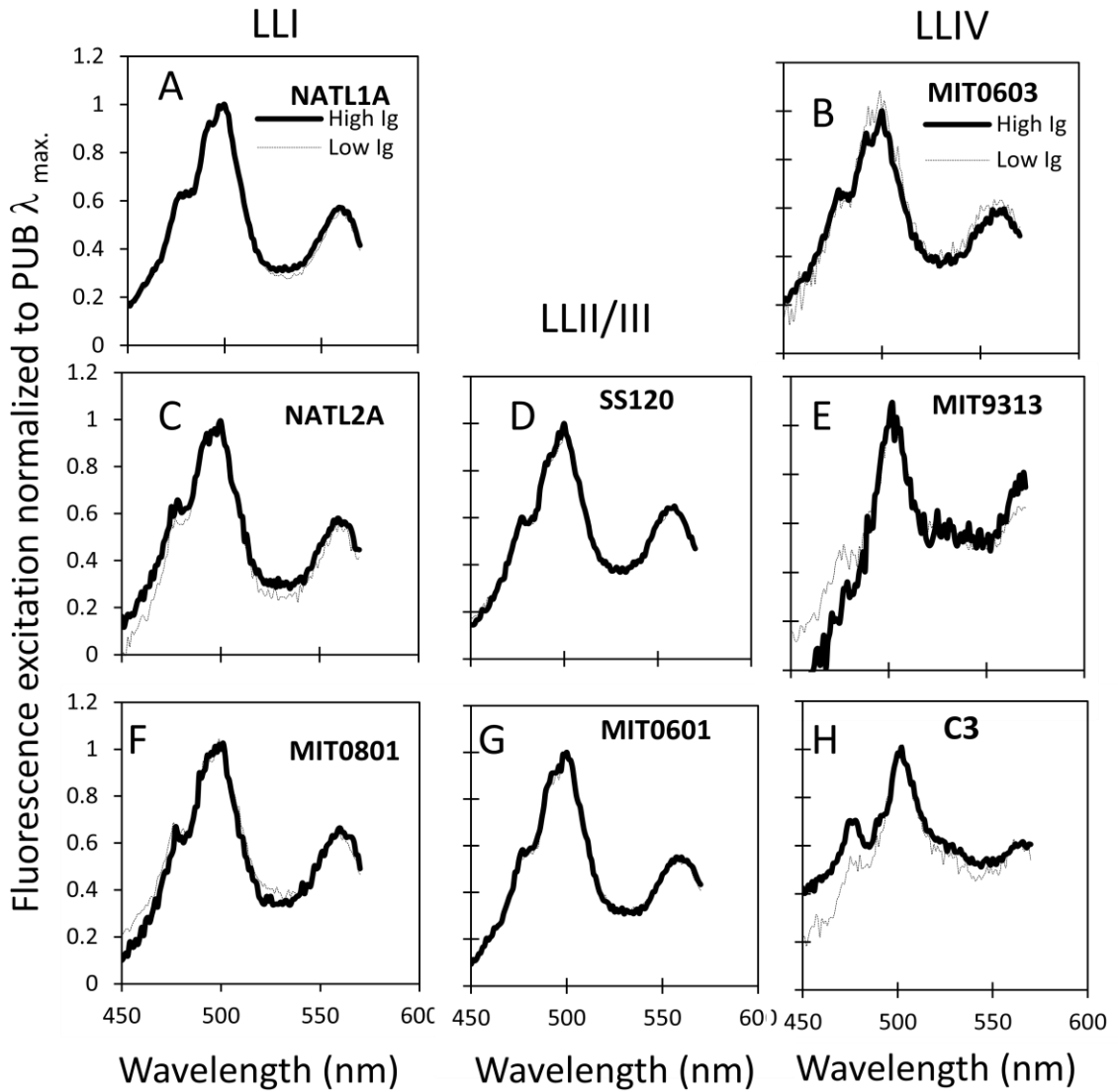
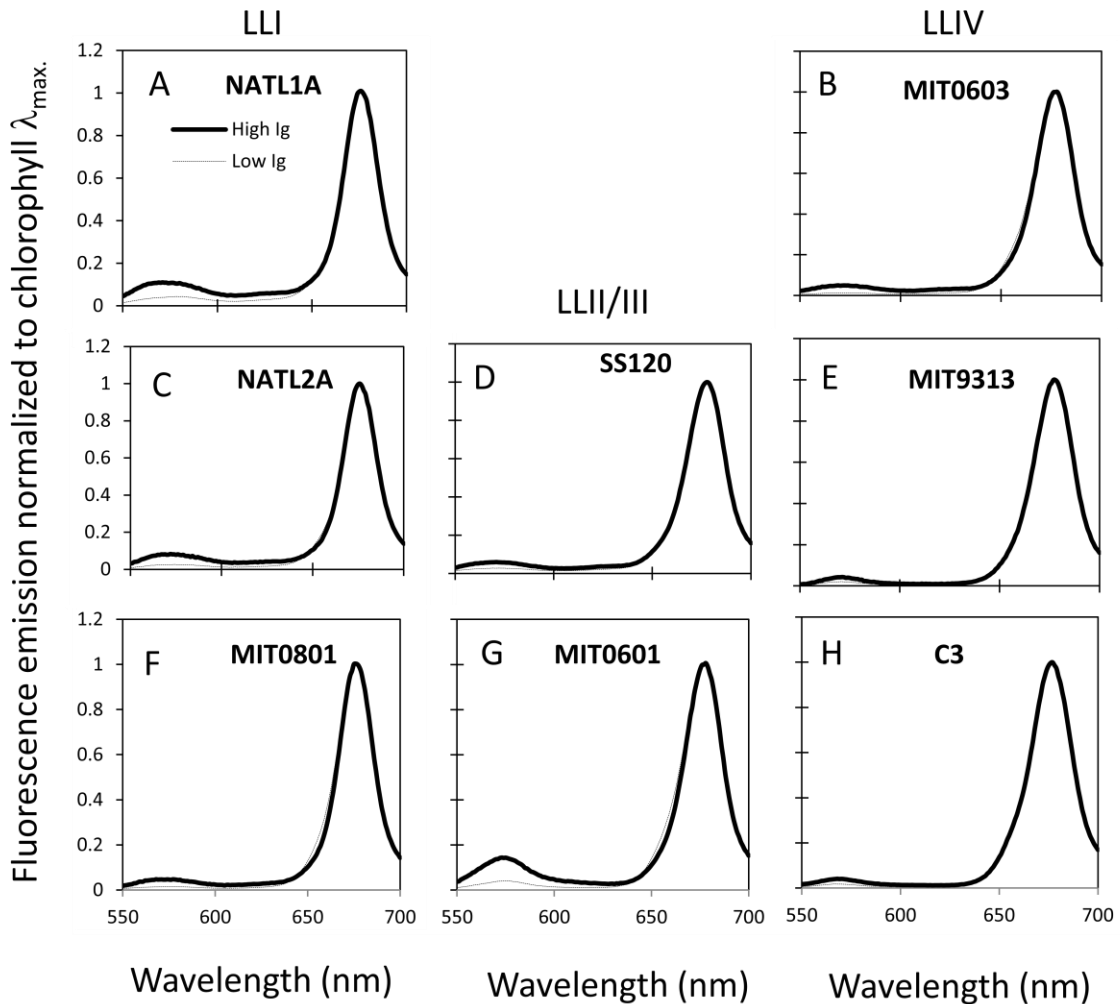


Figure 4. Fluorescence emission spectra of LL *Prochlorococcus*. [A]-[H] Fluorescence emission spectra with excitation set to 495 nm and emission was detected between 550-700 nm. Emission spectra are normalized to chlorophyll maxima.



To relate the flow cytometric FL2 signal to PE content in whole cells, *in vivo* fluorescence emission spectra were also obtained. The excitation wavelength was set at the PUB maxima of 495 nm and showed two main peaks for all LL *Prochlorococcus* strains, consistent with that observed previously for *Prochlorococcus* LLII/III strain SS120 (Steglich et al. 2003; Lokstein et al. 1999). The first peak occurred at 573 nm corresponding to PE emission, and the other peak at 677 nm corresponded to

chlorophyll *a* emission, the terminal energy acceptor. Interestingly, none of the *Prochlorococcus* strains tested exhibited a phycocyanin peak, as found at 650 nm in the emission spectra of *Synechococcus* WH8102 when excited at 495 nm (Six et al. 2004), despite the fact that all *Prochlorococcus* genomes have the necessary genes for phycocyanobilin biosynthesis. A plot of FL2 emission versus PE emission at 573 nm (Figure 5A) showed a linear correlation ($R^2=0.42$, p value <0.05), providing additional evidence that flow cytometric orange fluorescence is a direct measure of *in vivo* PE expression.

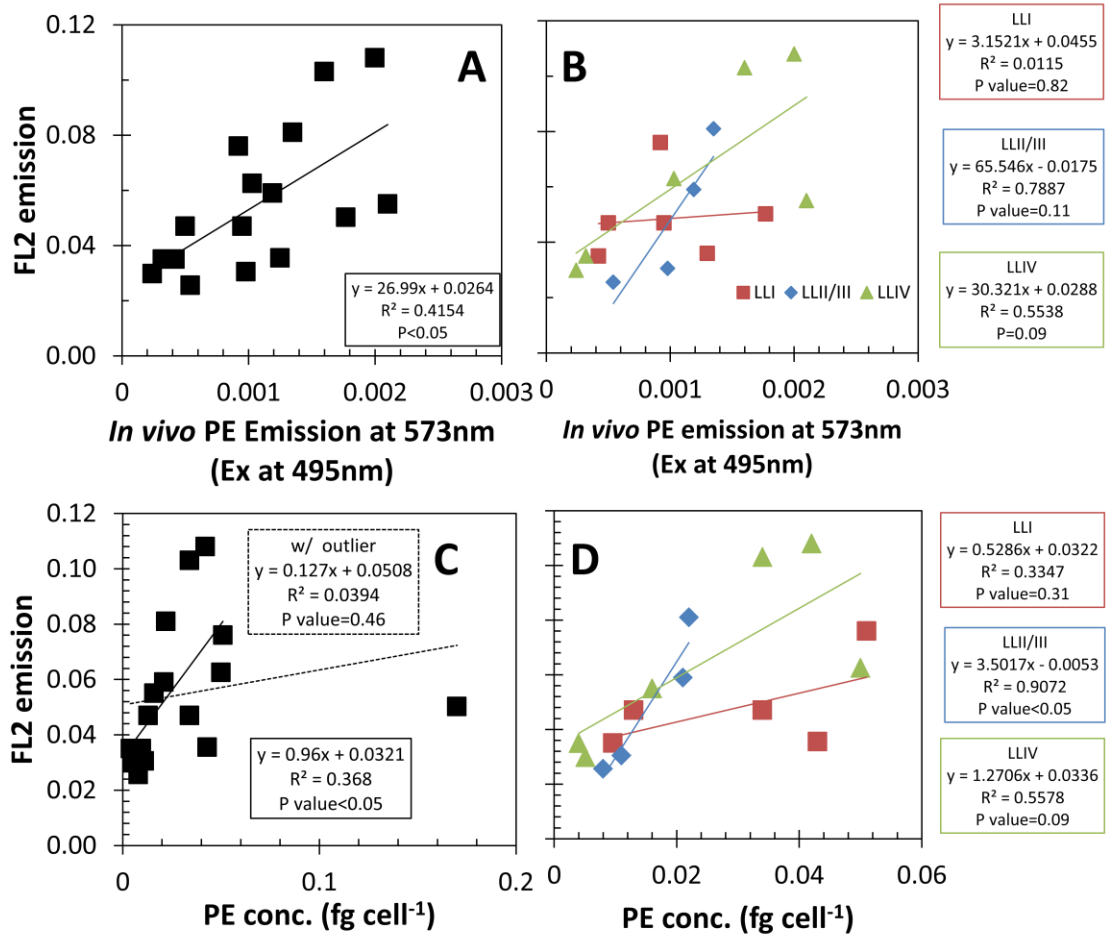
Pigment content

The amount of PE measured per cell in all LL *Prochlorococcus* was small and significantly less (3-5 orders of magnitude) than in *Synechococcus* (Six et al. 2004) (Table 2). Surprisingly, a plot of flow cytometric FL2 versus PE content per cell (Figure 5C) did not show a correlation ($R^2=0.04$, p value=0.46) as expected from the correlation observed with FL2 and PE peak emission. The correlation improved ($R^2=0.4$, p value <0.05) when one outlier, that may have had double the volume filtered, was removed. Although this may be a true outlier due to sampling error, no documentation can verify this, so the outlier has been included in the dataset. When the same variables (FL2 vs. PE Emission; Figure 5A) and (FL2 vs. PE content; Figure 5C) are plotted with each ecotype indicated by a different symbol, different slopes suggest ecotypic differences (Figure 5B; LLI slope=3.2, $R^2=0.01$, p value=0.82; LLII/III slope=65.5, $R^2=0.79$, p value=0.11; LLIV slope=30.3, $R^2=0.55$, p value=0.09) and FL2 vs. PE content (Figure 5D;

LLI slope=0.5, $R^2=0.3$, p value=0.31; LLII/III slope=3.5, $R^2=0.9$, p value<0.05; LLIV

slope=1.3, $R^2=0.6$, p value=0.09) despite the high p values.

Figure 5. Phycoerythrin physiological parameters compared to PE content. [A] Comparison of flow cytometric orange fluorescence (FL2) versus *in vivo* relative fluorescence emission values corresponding to PE (573 nm, Excitation set to 495nm). [B] same as [A] with each ecotype depicted a different symbol. [C] Comparison of FL2 emission versus phycoerythrin concentration (fg cell^{-1}). [D] same as [C] with each ecotype depicted as a different symbol.



Photoacclimation

In order to understand the possible function of PE in LL *Prochlorococcus*, the response of PE related measurements were observed under two different growth irradiances. *Prochlorococcus* cultures grown under low and high light irradiances varied both their relative FL3 and FL2 (Table 2, Figure 6A). As expected FL3 showed an increase as growth irradiance was decreased, consistent with photoacclimation of chlorophyll content observed previously (Moore and Chisholm 1999). FL2 increased for most LL *Prochlorococcus* strains as light irradiance decreased, with the exception of LLIV C3, suggesting that most of the LL *Prochlorococcus* strains are photoacclimating to changes in growth irradiance. As expected there was no significant difference in FL2 signal between low and high light grown HLI MED4. Thus, flow cytometric FL2 signal is correlated to changes in growth irradiance in all LL *Prochlorococcus*, with the exception of the newly isolated LLIV C3 strain.

The photoacclimation observed for FL2 was reflected in the *in vivo* PE emission per cell at 573nm (Figure 6B) and the PE content per cell (Figure 6C) as growth irradiance increased, for some of the LL *Prochlorococcus* strains tested. This is in contrast to the findings of Hess et al. (1999) for LLII/III SS120, which did not change its PE protein (α -PE and β -PE) when grown between 8-38 $\mu\text{E m}^{-2} \text{s}^{-1}$. It should be noted, however, that Hess et al (1999) examined changes in protein expression as measured with a western blot, and the difference in growth irradiance was not as great as used in this study. For those strains that changed PE emission per cell, this could indicate that the numbers of PE proteins are decreasing as growth irradiance increased. Two LLIV

strains, MIT9313 and C3, did not significantly alter the PE emission per cell (Figure 6B), possibly because PE functions differently considering that these two strains also do not have the gene *ppeC*, which encodes the γ linker protein connecting PE to the thylakoid.

Phycoerythrin emits maximally between 566-572nm in *Synechococcus* WH8102 as growth irradiance is increased from 15-650 $\mu\text{E m}^{-2} \text{s}^{-1}$ (Six et al. 2004), whereas *in vivo* PE emission did not vary its maximum wavelength with changes in growth irradiance (Figure 4A-H). For PE content per cell, only two LLI strains (NATL1A and NATL2A) showed statistically significant increases with decreased growth irradiance (Figure 6C). The lack of statistical significance may be due to the high variation of the PE measurements. Despite this, most LL *Prochlorococcus* strains showed increasing average PE content per cell with decreasing growth irradiance, consistent with what has been observed for *Synechococcus* WH8102 (Six et al. 2004). When *in vivo* PE emission maxima (at 573 nm) was normalized to chlorophyll emission maxima (at 677 nm), the PE to chlorophyll (573nm:677nm) peak ratio decreased with decreased growth irradiance for all LL *Prochlorococcus* (Figure 6D), indicating that PE emission increased to a lesser extent (or not at all) relative to chlorophyll emission. This is in contrast to *Synechococcus* WH8102 that increased its PE emission greater than its chlorophyll emission with changes in growth irradiance (Six et al. 2004), though WH8102 showed a greater change in PE content than chlorophyll.

Figure 6. Bar graphs of LL *Prochlorococcus* phycoerythrin physiological parameters. [A] Bar graph of relative FL2 cell⁻¹. [B] Bar graph of PE emission per cell. [C] Bar graph of PE content (fg cell⁻¹). [D] Bar graph of fluorescence emission at chlorophyll to phycoerythrin (573:677). *Prochlorococcus* strains grown under high irradiance (High I_g; 55-65 μmol Q m⁻² s⁻¹) and low irradiance (Low I_g; 10 μmol Q m⁻² s⁻¹). Error bars are standard deviation. Two tail t test, *p value < 0.05, **p < 0.01.

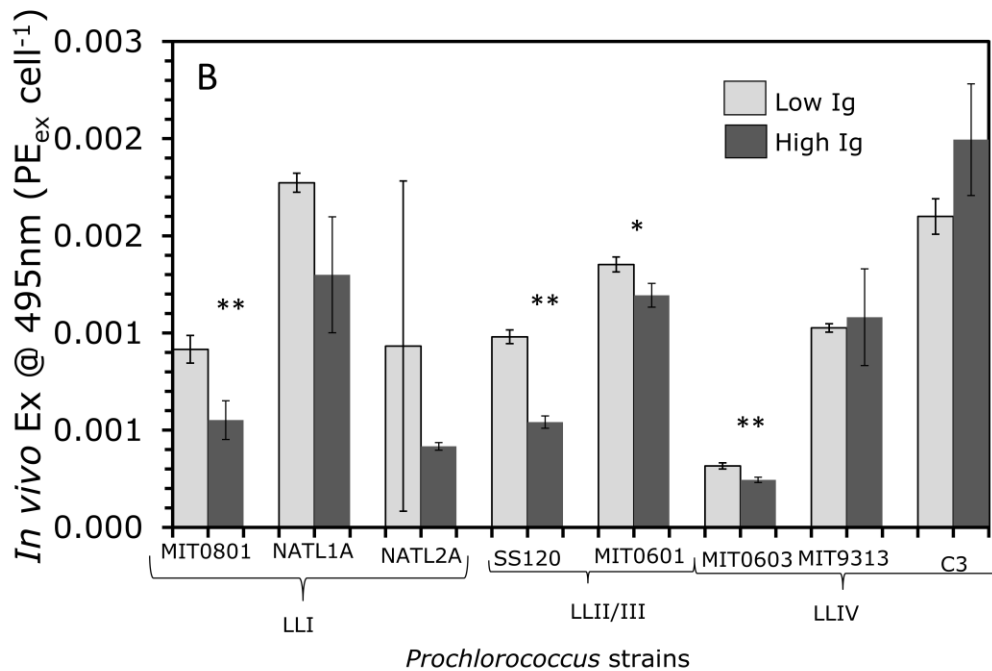
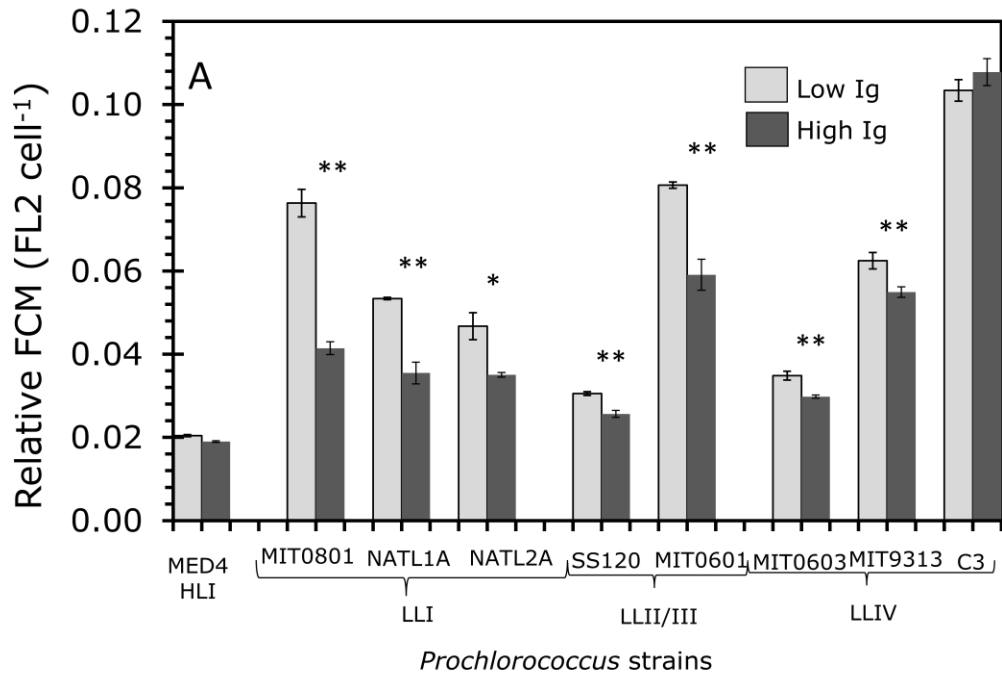
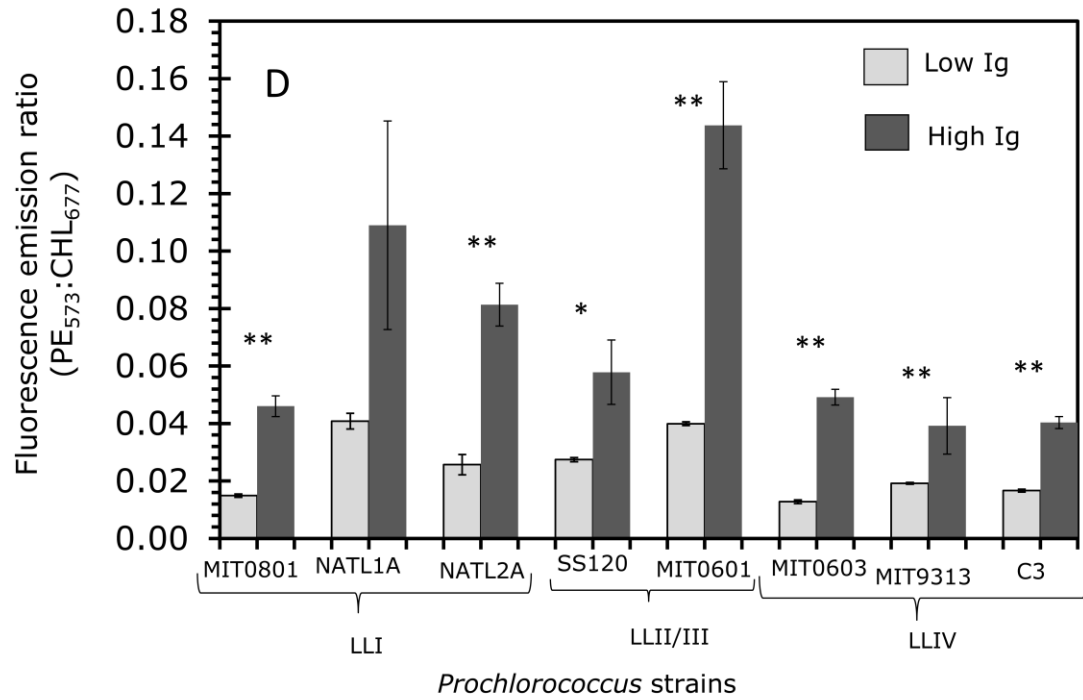
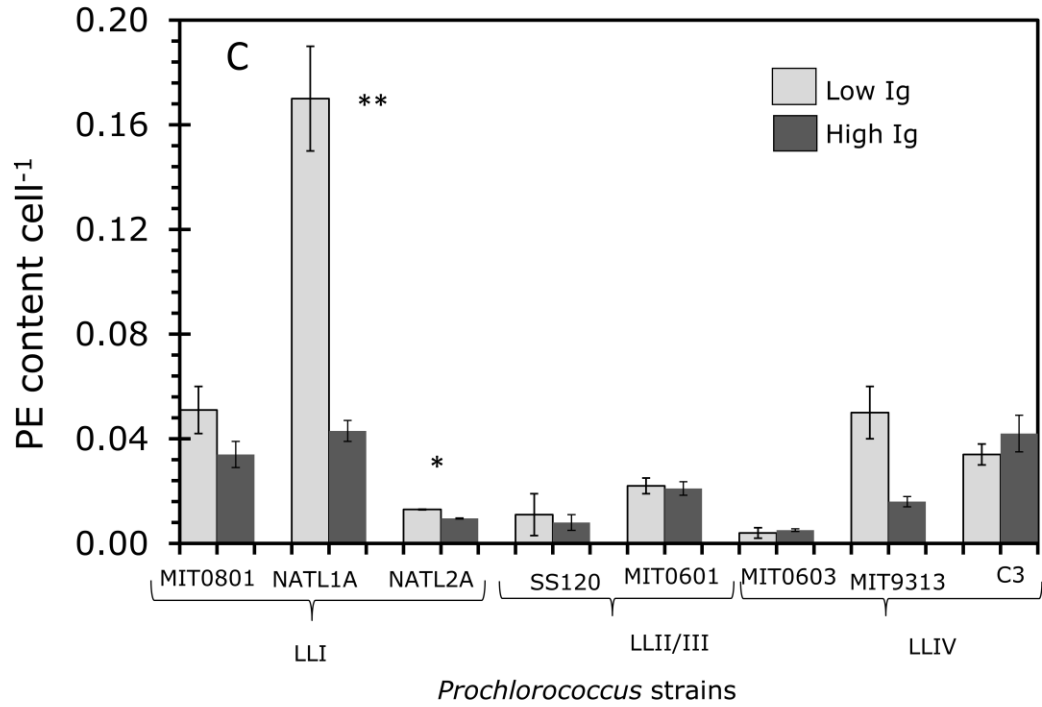


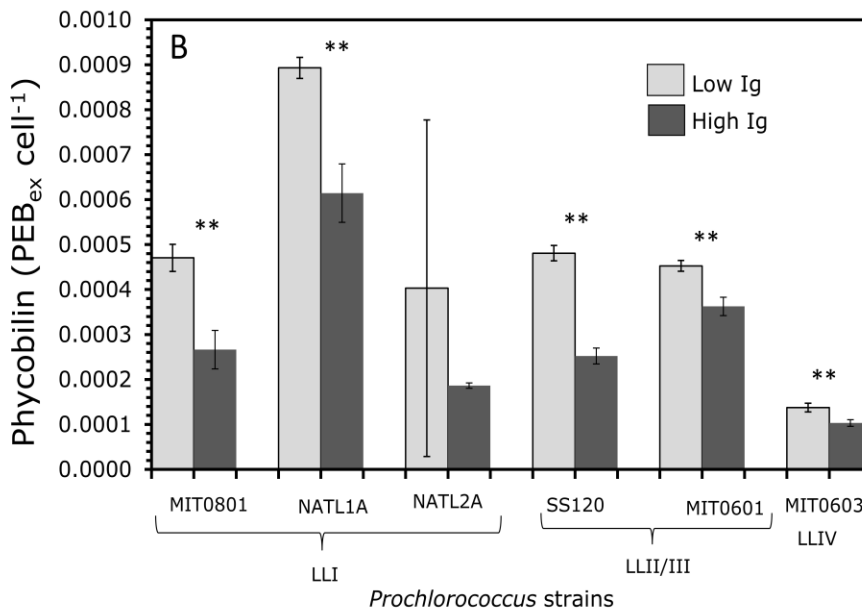
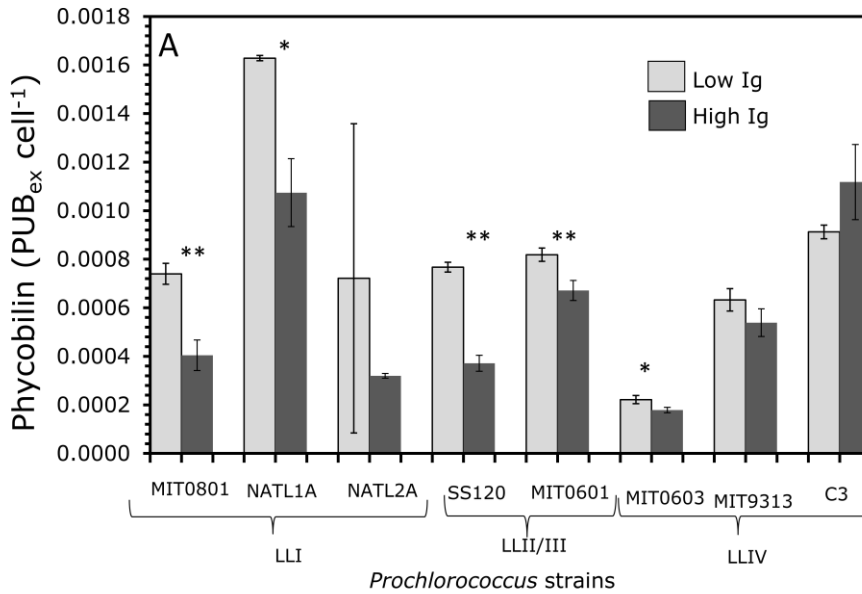
Figure 6. Continued



In *Synechococcus* WH8102 PEB and PUB absorbance indexes (related to quantity per cell) increased with decreased growth irradiance indicating a reduction of phycobilisomes per cell and changes within the PBS structure (Six et al. 2004). Although PEB and PUB concentrations were not measured directly, it is likely that each of these chromophores are increasing with decreasing growth irradiance, as indicated by the change in PUB and PEB excitation spectra (Figure 7A&B). For all LL *Prochlorococcus*, the PUB and PEB excitation peaks (with the exception of LLIV MIT9313 and LLIV C3 that do not have PEB) increased with decreased growth irradiance (Table 2, Figure 7A&B), perhaps indicating a slight change in PE structure as growth irradiance changed or a reduction in the number of PE molecules in the thylakoid. Neither LLIV MIT9313 nor LLIV C3 showed changes in PUB per cell with changes in growth irradiance, whereas LLIV MIT0603 did alter its PUB per cell with growth irradiance. The ratio of PUB:PEB excitation peak maxima for all LL *Prochlorococcus* was 1.69 ± 0.13 and did not vary within a strain under different growth irradiances for most of the LL *Prochlorococcus* (Table 2). The PUB:PEB ratio in *Synechococcus* WH8102 also did not change with growth irradiance (1.78 ± 0.11) (Six et al. 2004). Two exceptions were LLII/III SS120 that increased with decreased growth irradiance and LLIV MIT0603 that decreased its PUB:PEB ratio with decreased growth irradiance, indicating possible changes in the phycobilin makeup as growth irradiance changed. Chromatic adaptation has been observed in *Synechococcus*, where the PUB:PEB ratio varies depending upon the light quality of the growth conditions (i.e. greater PUB in blue light grown cultures) (Palenik 2001; Shukla et al. 2012). This was beyond the scope of this study, but would be an

interesting trait to test with *Prochlorococcus* strains that appear to vary their PUB and PEB phycobilin ratios.

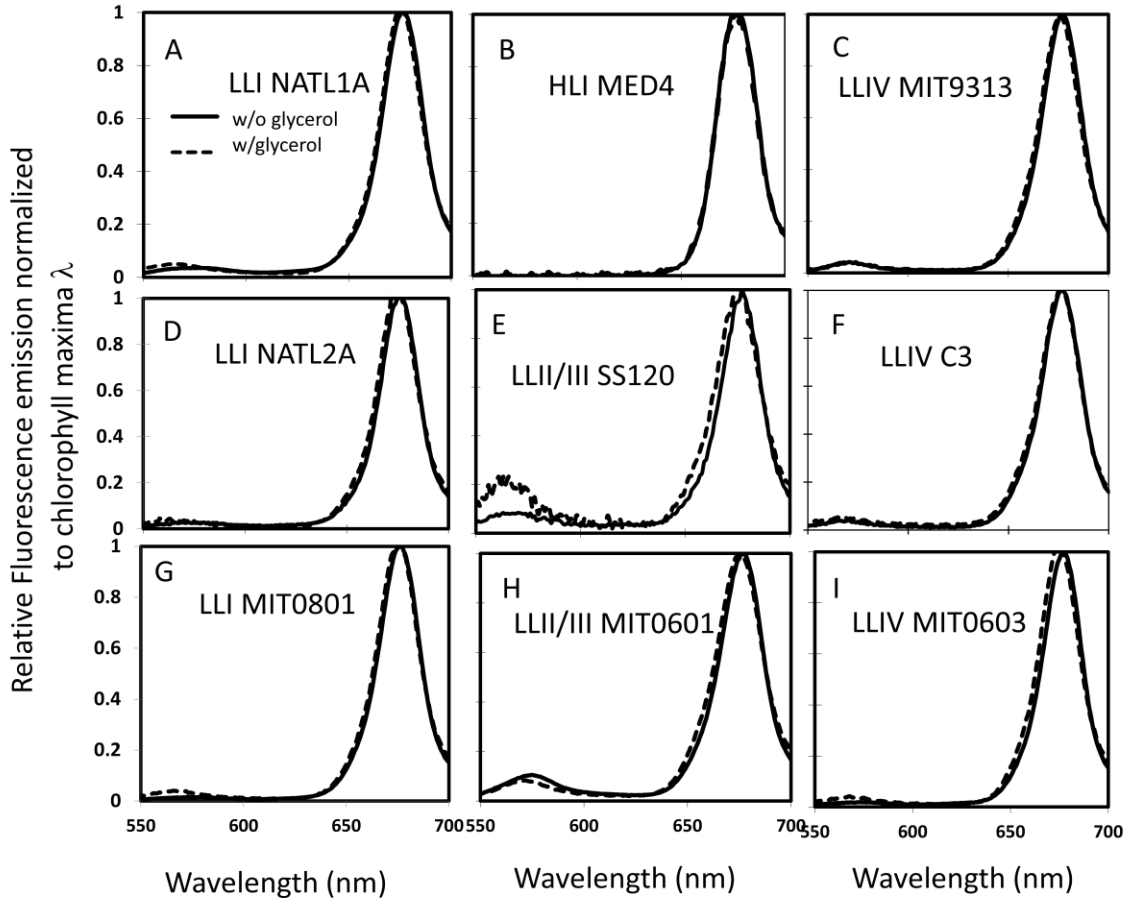
Figure 7. Bar graphs of LL *Prochlorococcus* phycobilins. [A] Bar graphs of fluorescence excitation of PUB cell⁻¹. [B] Bar graphs of fluorescence excitation of PEB cell⁻¹. *Prochlorococcus* strains grown under high irradiance (High I_g; 55-65 μmol Q m⁻² s⁻¹) and low irradiance (Low I_g; 10 μmol Q m⁻² s⁻¹). Error bars are standard deviation. Two tail t test, *p value < 0.05, **p < 0.01.



PE emission with added glycerol

The small but measurable change in PE emission as a function of growth irradiance would imply that PE is contributing to photosynthesis. In order to further test this, the *in vivo* fluorescence emission of PE with and without 50% glycerol was measured, as done previously with *Synechococcus* and *Prochlorococcus* LLII/III SS120 (Wyman 1992; Lokstein et al. 1999; Steglich 2003). The addition of glycerol uncouples PE from the thylakoid, thereby enhancing the PE emission signal with a concurrent decrease in chlorophyll emission. Slightly enhanced PE emission relative to chlorophyll emission was observed in the presence of glycerol with only LLI MIT0801, LLII/III SS120, and LLIV MIT0603 (Figure 8A-I), indicating that glycerol decoupled PE from the photosystem in those strains. This would also be consistent if the γ linker protein connects PE to the thylakoid membrane. HLIMED4, LLIV SA C3 and MIT9313 do not have the *ppeC* gene and were not expected to exhibit enhanced PE emission in the presence of glycerol. However, no enhancement due to glycerol was observed for LLI NATL1A, NATL2A, MIT0801, or LLII/III MIT0601 despite the presence of *ppeC*, implying that *ppeC* is not expressed or is regulated differently in these strains. Thus, in these LL *Prochlorococcus* strains it appears that the PE autofluorescence observed is not contributing to photosynthesis.

Figure 8. Glycerol treated LL *Prochlorococcus* fluorescence emission spectra. [A]-[I] Fluorescence emission spectra of *Prochlorococcus* strains with excitation at 495 nm (PUB absorption maximum) with 50% glycerol (dashed line) and without glycerol (solid line). Spectra were normalized to DV-Chl *a* maxima.



CHAPTER FOUR

DISCUSSION

This comparative physiological assessment of PE in *Prochlorococcus* strains from different LL ecotypes is consistent with the hypothesis that the flow cytometric FL2 is a measure of PE in the cell. Thus, FL2 can now be used in combination with FL3 and SSC to better distinguish HL from LL *Prochlorococcus* in natural populations. However, it must be kept in mind that the ability to distinguish between these two populations using FL2 also is dependent upon the type of flow cytometer and the particular emission filters used to detect FL2 emission. In addition, *in vivo* PE emission and PE content were well correlated with the FL2 signal for LLII/III and LLIV strains, though with different linear relationships. This linear relationship may be useful for quantifying PE content in cultured cells, assuming their ecotypic designation is known phylogenetically. Oddly, there was no relationship of FL2 vs. PE content for LLI strains, though the error on some of the measurements of these parameters was quite high and there was a spurious PE content outlier, which points to the need to repeat some of the measurements and/or examine more LLI strains.

The measurement of *in vivo* excitation spectra allowed the determination of relative phycobilin chromophore expression. In contrast to HL *Prochlorococcus* that only exhibits the PEB chromophore, all LL *Prochlorococcus* strains examined, except for LLIV MIT9313 and LLIV C3, have both PUB and PEB chromophores, similar to that observed for marine *Synechococcus* WH8102. As mentioned, two of the three LLIV strains only exhibited PUB expression, even though all *Prochlorococcus* have all the necessary genes

for PEB biosynthesis (Table 1). However, these two strains lack the *ppeC* gene, whose protein product is thought to link PE to the thylakoids (Hess et al. 1999), which might have some connection to the absence of PEB emission. Unlike the other LLIV strains tested, LLIV MIT0603 exhibited both PUB and PEB expression. This, along with the weak phylogenetic analysis relationship of the ITS region with the other LLIV ecotypes, indicates that MIT0603 may represent another new LL clade, or a subclade within the LLIV ecotype.

It is tempting to hypothesize that the presence of PUB, a blue light absorbing pigment, would give LL *Prochlorococcus* an ecological edge in the deeper portions of the blue lit waters (Hess et al. 1996; Steglich et al. 2003a). While the divinyl derivatives of chlorophylls present in *Prochlorococcus* absorb maximally at wavelengths that penetrate into the euphotic zone (Moore et al. 1995), PUB also absorbs in the blue (maximum absorbance at 495 nm). Together, Chl *b*₂ and PUB pigments could increase the overall absorbance of light energy in the deeper euphotic zone where LL *Prochlorococcus* predominate. In fact, the specific PE chromophore content of the HL and LL ecotypes is consistent with the depth distributions of ecotypes and water quality. At the surface of the oligotrophic oceans where the wavelengths of light cover a broader range of the visible spectrum (Morel et al. 2007), HL *Prochlorococcus* ecotypes that contain only the PEB chromophore dominate. As one goes down in the water column towards the middle of the euphotic zone, the predominant *Prochlorococcus* ecotypes are the LLI and LLII/III ecotypes (Zinser et al. 2006; Malmstrom et al. 2010) that contain both PUB and PEB for absorbing the blues and greens that penetrate to these depths. In the deepest

euphotic zone where blue wavelengths of light penetrate, the LLIV ecotype with only PUB, are most prevalent. This scenario first hypothesized for HL MED4 as a green light sensing chromophore (Steglich et al. 2005) is similar to how marine bacteria with photorhodopsins adjust their absorption properties through the water column, shifting from green- to blue-light absorbing at surface down to deeper euphotic zone water layers (Beja et al. 2001).

The actual functional role of PE in *Prochlorococcus* is still somewhat of a mystery, in large part because the concentrations of PE are so low compared to levels in marine *Synechococcus*. As Steglich et al. (2005) hypothesized specifically for HL MED4, *cpeB*-PE may provide a green-light sensing role rather than a structural function, since no phycobilisome is present. This also might be the case for most of the LL strains of *Prochlorococcus* because they adjust PE content in response to growth irradiance, but do not appear to participate in light harvesting. Light sensing in cyanobacteria functions as photoreception and signal transduction (Montgomery 2007). While photoacclimation might indicate that the PE chromophores serve a role in light harvesting, as was hypothesized for LLII/III SS120 (Lokstein et al. 1999; Steglich et al. 2003a), my results from the glycerol experiment indicate that most of the LL *Prochlorococcus* strains, with the exception of LLII/III SS120 and possibly LLI MIT0801, do not use PE for light harvesting. The presence of *ppeC* did not indicate that PE would transfer energy to the photosystem, as predicted due to the putative linker function of *ppeC*. The per cent amino acid sequence similarity of the *ppeC* gene in all of the LL *Prochlorococcus* showed very low similarity to SS120 (less than 60% for all strains that had *ppeC*), pointing to

possible mutations in the *ppeC* gene preventing its expression. A comparison of the *ppeC* nucleotide sequences is underway to verify this. Both LLIV C3 and LLIV MIT9313, which lack *ppeC*, showed no change in PE emission or PUB per cell with changes in growth irradiance. Thus, not only is the amount of PE found in *Prochlorococcus* so small that it is unlikely to play a role in light harvesting, as proposed by Steglich et al. (2003a), it also appears that the potential for light harvesting is present only in a select few strains.

One of the broader goals for this study was the correlation between ecophysiological characteristics with ecotype phylogenetic groupings. While shot-gun sequencing has become commonplace, gene presence does not necessarily indicate expression. The need to characterize multiple representative cultured isolates from a phylogenetic clade persists. PE is but one physiological trait that helps characterize a *Prochlorococcus* strain. Lastly, sea-going flow cytometry can now be used to assess PE *in situ* to separate not only different types of *Synechococcus* (Olson et al. 1988), but also to discriminate between HL and LL *Prochlorococcus* populations, further enhancing our understanding of this ubiquitous picophytoplankton in the world's oceans.

References

- Beja, O., E. Spudich, J. Spudich, M. Leclerc, and E. F. DeLong. 2001. Proteorhodopsin phototrophy in the ocean. *Nature* **411**: 786–789.
- Buitenhuis, E., W. Li, D. Vaultot, M. W. Lomas, M. Landry, F. Partensky, D. M. Karl, O. Ulloa, L. Campbell, S. Jacquet, F. Lantoiné, F. Chavez, D. Macias, M. Gosselin, and G. McManus. 2012. Picoplankton biomass distribution in the global ocean. *Earth System Science Data* **4**: 37–46.
- Campbell, L., H. Liu, H. A. Nolla, and D. Vaultot. 1997. Annual variability of phytoplankton and bacteria in the subtropical North Pacific Ocean at Station ALOHA during the 1991-1994 ENSO event. *Deep-Sea Research I* **44**: 167–192.
- Connon, S., and S. Giovannoni. 2002. High-Throughput methods for culturing microorganisms in very-low-nutrient media yield diverse new marine isolates. *Applied and Environmental Microbiology* **68**: 3878–3885.
- Dammeyer, T., S. C. Bagby, M. B. Sullivan, and S. Chisholm. 2008. Efficient Phage-Mediated Pigment Biosynthesis in Oceanic Cyanobacteria. *Current Biology* **18**: 442–448.
- DeRuyter, Y. S., and P. Fromme. 2008. Molecular structure of the photosynthetic apparatus, p. 217–226. *In* *The Cyanobacteria Molecular Biology, Genomics and Evolution*. Caister Academic Press.
- DuRand, M. D., R. J. Olson, and S. W. Chisholm. 2001. Phytoplankton population dynamics at the Bermuda Atlantic Time-series in the Sargasso Sea. *Deep-Sea Research II* **48**: 1983–2003.
- Field, C. B., M. J. Behrenfeld, J. T. Randerson, and P. Falkowski. 1998. Primary Production of the Biosphere: Integrating Terrestrial and Oceanic Components. *Science* **281**: 237–240.
- Hall, T. 1999. BioEdit: a user-friendly biological sequence alignment editor and analysis program for Windows 95/98/NT. *Nucleic Acids Symposium* **41**: 95–98.
- Hess, W. R., F. Partensky, G. W. van der Staay, J. M. Garcia-Fernandez, T. Börner, and D. Vaultot. 1996. Coexistence of phycoerythrin and a chlorophyll a/b antenna in a marine prokaryote. *Proceedings of the National Academy of Sciences of the United States of America* **93**: 11126–11130.

- Hess, W. R., G. Rocap, C. S. Ting, F. Larimer, S. Stilwagen, J. Lamerdin, and S. W. Chisholm. 2001. The photosynthetic apparatus of *Prochlorococcus*: insights through comparative genomics. *Photosynthesis Research* **70**: 53–71.
- Hess, W. R., C. Steglich, C. Lichtle, and F. Partensky. 1999. Phycoerythrins of the oxyphotobacterium *Prochlorococcus marinus* are associated to the thylakoid membrane and are encoded by a single large gene cluster. *Plant Molecular Biology* **40**: 507–521.
- Johnson, Z. I., E. R. Zinser, A. Coe, N. P. McNulty, E. M. S. Woodward, and S. W. Chisholm. 2006. Niche Partitioning Among *Prochlorococcus* Ecotypes Along Ocean-Scale Environmental Gradients. *Science* **311**: 1737–1740.
- Kettler, G., A. C. Martiny, Haung, Zucker, and M. L. Coleman. 2007. Patterns and Implications of Gene Gain and Loss in the Evolution of *Prochlorococcus*. *PLoS Genetics* **3**: 2515–2528.
- Knap, A. H., A. Michaels, A. Close, H. Ducklow, and A. Dickson, eds. 1994. Measurement of Chlorophyll a and Phaeopigments by Fluorometric Analysis, p. 119–122. *In* Protocols for the Joint Global Ocean Flux Study (JGOFS) Core Measurements.
- Lawrenz, E., E. J. Fedewa, and T. L. Richardson. 2011. Extraction protocols for the quantification of phycobilins in aqueous phytoplankton extracts. *Journal of Applied Phycology* **23**: 865–871.
- Lokstein, H., C. Steglich, and W. R. Hess. 1999. Light-harvesting antenna function of phycoerythrin in *Prochlorococcus marinus*. *Biochimica et Biophysica Acta* **1410**: 97–98.
- MacColl, R. 1998. Cyanobacterial Phycobilisome. *Journal of Structural Biology* **124**: 311–334.
- Malmstrom, R. R., A. Coe, G. C. Kettler, A. C. Martiny, J. Frias-Lopez, E. R. Zinser, and S. W. Chisholm. 2010. Temporal dynamics of *Prochlorococcus* ecotypes in the Atlantic and Pacific oceans. *ISME J* 1–13.
- Marie, D., F. Partensky, D. Vaultot, and C. Brussaard. 1999. Enumeration of Phytoplankton, Bacteria and Viruses in marine samples, John Wiley & Sons. Inc.
- Martiny, A. C., A. P. K. Tai, D. Veneziano, F. Primeau, and S. W. Chisholm. 2009. Taxonomic resolution, ecotypes and the biogeography of *Prochlorococcus*. *Environmental Microbiology* **10**: 1–10.

- Montgomery, B. L. 2007. Sensing the light: photoreceptive systems and signal transduction in cyanobacteria. *Molecular Microbiology* **64**: 16–27.
- Moore, L., A. Coe, E. R. Zinser, M. A. Saito, M. B. Sullivan, D. Lindell, K. Frois-Moniz, J. Waterbury, and S. W. Chisholm. 2007. Culturing the marine cyanobacterium *Prochlorococcus*. *Limnology and Oceanography Methods* **5**: 353–362.
- Moore, L. R., and S. W. Chisholm. 1999. Photophysiology of the marine cyanobacterium *Prochlorococcus*: ecotypic differences among cultured isolates. *Limnology and Oceanography* **44**: 628–638.
- Moore, L. R., R. Goericke, and S. W. Chisholm. 1995. Comparative physiology of *Synechococcus* and *Prochlorococcus*: influence of light and temperature on growth, pigments, fluorescence and absorptive properties. *Marine Ecology Progress Series* **116**: 259–275.
- Morel, A., B. Gentili, H. Claustre, M. Babin, A. Bricaud, J. Ras, and F. Tieche. 2007. Optical properties of the “clearest” natural waters. *Limnology and Oceanography* **52**: 217–229.
- Olson, R., S. Chisholm, E. Zettler, and E. Armbrust. 1988. Analysis of *Synechococcus* pigment types in the sea using single and dual beam flow cytometry. *Deep-Sea Research* **35**: 425–440.
- Ong, L. J., A. N. Glazer, and J. B. Waterbury. 1984. An unusual phycoerythrin from a marine cyanobacterium. *Science* **224**: 80–83.
- Palenik, B. 2001. Chromatic adaptation in marine *Synechococcus* strains. *Applied and Environmental Microbiology* **67**: 991–994.
- Partensky, F., and L. Garczarek. 2010. Advantages and Limits of Minimalism. *Annual Review of Marine Science*. **2**: 305–331.
- Partensky, F., W. R. Hess, and D. Vaultot. 1999. *Prochlorococcus*, a Marine Photosynthetic Prokaryote of Global Significance. *Microbiol Mol Biol Rev* **63**: 106–127.
- Penno, S., L. Campbell, and W. R. Hess. 2000. Presence of phycoerythrin in two strains of *Prochlorococcus* (cyanobacteria) isolated from the subtropical North Pacific Ocean. *Journal of Phycology* **36**: 723–729.
- Rocap, G., D. L. Distel, J. B. Waterbury, and S. W. Chisholm. 2002. Resolution of *Prochlorococcus* and *Synechococcus* Ecotypes by Using 16S–23S Ribosomal DNA Internal Transcribed Spacer Sequences. *Applied and Environmental Microbiology* **68**: 1180–1191.

- Rocap, G., F. W. Larimer, J. Lamerdin, S. Malfatti, P. Chain, N. A. Ahlgran, A. Arellano, M. Coleman, L. Hauser, W. R. Hess, Z. I. Johnson, M. Land, D. Lindell, A. F. Post, W. Regala, M. Shah, S. L. Shaw, C. Steglich, M. B. Sullivan, C. S. Ting, A. Tolonen, E. A. Webb, E. R. Zinser, and S. W. Chisholm. 2003. Genome divergence in two *Prochlorococcus* reflects oceanic niche differentiation. *Nature* **424**: 1042–1047.
- Scanlan, D. J., M. Ostrowski, S. Mazard, A. Dufresne, L. Garczarek, W. R. Hess, A. F. Post, M. Hagemann, I. Paulsen, and F. Partensky. 2009. Ecological Genomics of Marine Picocyanobacteria. *Microbiol. Mol. Biol. Rev.* **73**: 249–299.
- Shukla, A., A. Biswas, N. Blot, F. Partensky, J. Katy, L. Hammad, L. Garczarek, A. Gutu, W. M. Schluchter, and D. M. Kehoe. 2012. Phycoerythrin-specific bilin lyase-isomerase controls blue-green chromatic acclimation in marine *Synechococcus*. *Proceedings from the National Academy of Sciences* **109**: 20136–20141.
- Sidler, W. 1994. Phycobilisome and phycobiliproteins structures, p. 139–216. *In* The Molecular Biology of Cyanobacteria. Kluwer Academic Publishers.
- Six, C., J. C. Thomas, B. Brahamsha, Y. Lemoine, and F. Partensky. 2004. Photophysiology of the marine cyanobacteria *Synechococcus* sp. WH8102, a new model organism. *Aquatic Microbial Ecology* **35**: 17–29.
- Six, C., J. C. Thomas, L. Garczarek, M. Ostrowski, A. Dufresne, N. Blot, D. J. Scanlan, and F. Partensky. 2007. Diversity and evolution of phycobilisomes in marine *Synechococcus* spp.: a comparative genomics study. *Genome Biology* **8**.
- Steglich, C. 2003. Biochemical, spectroscopic and molecular genetic characterisation of novel phycoerythrin species from *Prochlorococcus* sp.
- Steglich, C., N. Frankenberg-Dinkel, S. Penno, and W. R. Hess. 2005. A green light-absorbing phycoerythrin is present in the high-light adapted marine cyanobacterium *Prochlorococcus* sp. MED4. *Environmental Microbiology* **7**: 1611–1618.
- Steglich, C., C. W. Mullineaux, K. Teuchner, W. R. Hess, and H. Lokstein. 2003a. Photophysical properties of *Prochlorococcus* marinus SS120 divinyl chlorophylls and phycoerythrin in vitro and in vivo. *FEBS Letters* **553**: 79–84.
- Steglich, C., A. F. Post, and W. R. Hess. 2003b. Analysis of natural populations of *Prochlorococcus* spp. in the northern Red Sea using phycoerythrin gene sequences. *Environmental Microbiology* **5**: 681–690.

- Tamura, K., J. Dudley, M. Nei, and S. Kumar. 2007. MEGA4: Molecular Evolutionary Genetics Analysis (MEGA) software version 4.0. *Molecular Biology and Evolution* **24**: 1596–1599.
- Ting, C. S., G. Rocap, J. King, and S. W. Chisholm. 2001. Phycobiliprotein genes of the marine photosynthetic prokaryote *Prochlorococcus*: evidence for rapid evolution of genetic heterogeneity. *Microbiology* **147**: 3171–3182.
- Wiethaus, J., A. W. U. Busch, T. Dammeyer, and N. Frankenberg-Dinkel. 2010a. Phycobiliproteins in *Prochlorococcus marinus*: Biosynthesis of pigments and their assembly into proteins. *European Journal of Cell Biology* **89**: 1005–1010.
- Wiethaus, J., A. W. U. Busch, K. Kock, L. I. Liechert, C. Hermann, and N. Frankenberg-Dinkel. 2010b. CpeS is a lyase specific for attachment of 3Z-PEB to Cys82 of b-phycoerythrin from *Prochlorococcus marinus* MED4. *The Journal of Biological Chemistry* **285**: 37561–37569.
- Wyman, M. 1992. An in vivo method for the estimation of phycoerythrin concentration in marine cyanobacteria (*Synechococcus* spp.). *Limnology and Oceanography* **37**: 1300–1306.
- Wyman, M., R. Gregory, and N. Carr. 1985. Novel role for phycoerythrin in a marine cyanobacterium, *Synechococcus* strain DC2. *Science* **230**: 818–820.
- Zinser, E., A. Coe, Z. I. Johnson, A. Martiny, N. Fuller, D. Scanlan, and S. Chisholm. 2006. *Prochlorococcus* ecotype abundances in the North Atlantic Ocean as revealed by an improved Quantitative PCR method. *Applied and Environmental Microbiology* **72**: 723–732.

BIOGRAPHY OF THE AUTHOR

Kathryn Johnson was born in South Korea, raised in Falmouth, Massachusetts and currently lives in Long Island, ME, Casco Bay. She graduated from Falmouth High School in 1993. She attended the University of Delaware Honors Program and graduated in 1997 with a Bachelor's degree in Biochemistry and a minor in Biology. She attended the University of Southern Maine and graduated in 2005 with a Master of Science degree in Biology. She entered the BMB graduate program at The University of Maine in the fall of 2009. After receiving her degree, Kathryn will be teaching Microbiology at Southern Maine Community College. Kathryn is a candidate for the Master of Science degree in Microbiology from The University of Maine in August 2013.