Metatranscriptomic and physiological analyses of proteorhodopsin-containing marine flavobacteria

by
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Abstract

Proteorhodopsin (PR) is a seven-helix integral membrane protein that uses retinal as a chromophore. PRs transport protons from the cytoplasmic (CP) to the extracellular (EC) side of the cell membrane utilizing the energy from light. Since PR was first discovered in marine Gammmaproteobacteria, similar types of rhodopsins have been found in all three domains of life (archaea, bacteria, and eukaryotes). Recent studies have suggested that some flavobacteria showed a light-dependent increase in cell yield and growth rate of cultures grown in low carbon media. Although their function as proton pumps with energy-yielding potential has been suggested in some strains, the photophysiological role of proteorhodopsins remains largely unexplored. This thesis describes the functional characterization of PR-containing flavobacteria previously identified from a (Gomez-Consarnau et al. 2007; Yoshizawa et al. 2012). We describe here experiments performed to help understand how PR-containing marine flavobacteria respond to varied DOC concentrations during light-dependent growth, using growth curve observations, inhibitor experiments and transcriptomic analyses.

The light-dependent growth effects demonstrated a dependence on carbon concentration, decreasing at increasing carbon concentration in all PR-harboring strains examined in this study. Interestingly however, the inverse results were observed at high carbon concentration (48.5 mM C) which resulted in higher cell yields when grown in the dark than in the light. Growth experiments using 2-(4-methylphenoxy)triethylamine (MPTA) as an inhibitor of β-carotene synthesis were performed for the representative isolates, Dokdonia sp. MED134 and Gilvibacter sp. SZ-19, at low and high concentrations of DOC. These experiments showed that inhibition of retinal biosynthesis abolished the light-stimulated growth response at low DOC concentrations. Transcriptomic experiments were designed to determine the effect of DOC concentration on gene expression of PR-
containing MED134 under light and darkness. The results show the both PR and retinal biosynthetic enzymes exhibit significant upregulation in the low carbon condition when they exposed to the light. Among protein-coding transcripts of high carbon concentration, beta-oxidation-associated proteins were expressed at significantly higher levels in the dark.

This work furthers our understanding of the details of light-enhanced growth rates and cell yields in diverse marine flavobacterial isolates, and demonstrate proteorhodopsin-associated light-dependent growth effects at various carbon concentrations in several different flavobacterial proteorhodopsin photosystems.

Thesis Supervisor: Edward F. DeLong
Title: Morton and Claire Goulder Professor of Civil and Environmental Engineering and Biological Engineering
Acknowledgements

This thesis would not have been possible without the guidance and help of several individuals who in one way or another contributed and extended their valuable assistance in the preparation and completion of this study.

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I am also thankful to have joined a group of caring, fun, and hardworking colleagues in the DeLong lab and in Parsons. I would like to especially thank Chon Martinez for her all the advice she gave me; my fellow graduate students, Mike Valliere, and Tsultrim Palden for their assistance in the metatranscriptomic data analysis and sharing many happy moments working on projects together; my lab mates Oscar Sosa, Jessica Bryant and post-doctoral fellow, Dr. Scott Gifford and Dr. Kristina M Fontanez, and John Eppley, for their insightful discussions and making my lab experience enjoyable.

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1. The important role of proteorhodopsin (PR) in marine ecosystem

1.1 Rhodopsins

Rhodopsins are light harvesting protein that contain retinal (Vitamin A aldehyde) as a chromophore and have seven transmembrane alpha helices (Spudich 2006). These light sensitive proteins absorb light and convert it into photonic energy to transport ions across the cell membrane. More specifically, upon light illumination, the photocycle begins when the retinal (Vitamin A aldehyde) in the protein undergoes photoisomerization causing conformational changes of the protein and the creation of the electrochemical gradient that facilitates ion transportation. Rhodopsins are basically classified into two protein families: type I and type II rhodopsins. The big difference between type I and type II rhodopsin is the photoisomerization mechanism of chromophore. In rhodopsin I, the photoisomerization causes retinal changes from all-trans to 13-cis whereas in rhodopsin II, changes occur from 11-cis to all trans retinal (Jung 2007). Type II rhodopsins function as photosensitive receptor proteins in animal eyes that are human rod and cone visual pigments. Type I rhodopsin is an archaeal-type rhodopsin and well studied in *Halobacterium salinarium*, halophilic archaea. The first discovered rhodopsins are bacteriorhodopsin (BR), halorhodopsin (HR), sensory rhodopsin I and II (SRI and SRII) in *H. salinarium* (Spudich et al. 2000). A number of other rhodopsins have been identified as playing a role in proton-motive force, although their mechanisms differ. Proteorhodopsins have been found in marine bacteria, archaea, and eukarya. Xanthorhodopsins (XRs) use two chromophores, the carotenoids salinixanthin and retinal, to broaden the spectral range for light harvesting (Balashov et al. 2005). Actinorhodopsins (ActRs) have been found in
abundance in freshwater, including actinobacteria from a hypersaline lagoon, an estuary, and a freshwater lake (Sharma et al. 2008). Bacteriorhodopsin (BR) and halorhodopsin (HR) are light-driven pumps that use light as a catalyst to translocate protons and chloride, respectively. Rhodopsins may be involved in regulating ionic content and the osmotic state (Mongodin et al. 2005). Sensory rhodopsins (SRs) function like photoreceptors rather than ion transporters, mediating phototaxis or signal transduction (Spudich et al. 2000).

Recently, type I rhodopsins have been found also in other lineages such as, Eubacteria, and unicellular Eukarya (Bieszke et al. 1999; Sineshchekov et al. 2002; Jung et al. 2003). That is the reason why type I rhodopsin is also called microbial rhodopsin.

Archaeal-type rhodopsins, type I rhodopsins, serve as a model for proteorhodopsin photosystems (PRPS) (Kawanabe et al. 2006). In reaction to a light signal, they establish a chemical gradient from light energy and change their conformation to facilitate the translocation of protons. In this study, I am focused on proteorhodopsin (PR), which belongs to the type I rhodopsin family.

1.2 Proteorhodopsins (PRs)

Proteorhodopsins (PRs), a member of the microbial rhodopsin superfamily of proteins, are retinal-binding transmembrane proteins that play a key role in light-activated proton efflux (Beja et al. 2000; Beja et al. 2001; Martinez et al. 2007).

Earlier studies showed microbial rhodopsins to be associated with Archaea but later studies found PRs in an uncultured marine gammaproteobacterial SAR86 group (Morris et al. 2002; Boichenko et al. 2006). PR-containing bacteria are found in a minimum of 13% of all microorganisms in the photic zone, the layer of the ocean that is exposed of sunlight.
Planktonic Bacteria, Archaea and Eukarya reside and compete for light in the photic zone of the ocean (McCarren and DeLong 2007). The PR-bearing marine microbes use light energy for the acquisition of adenosine triphosphate (ATP) by proteorhodopsin (Frigaard et al. 2006). The proton motive force (PMF) is generated by an electrochemical gradient that transports protons across an energy-transducing membrane. Through oxidative phosphorylation, bacteria use the PMF to synthesize ATP, drive chemiosmotic reactions, and power the rotary flagellar motor (Kashket 1985). The light energy absorbed by rhodopsins is used to translocated protons outside the cell, thereby generating the protonmotive force. Proteorhodopsin have been identified in the world’s oceans making this light-transducing protein a key source of solar energy absorption in photosystems.

Proteorhodopsins are composed of the opsins protein and the chromophore trans-retinal (Bielawski et al. 2004; Giovannoni et al. 2005). Retinal is formed through the conversion of the carotenoid β-carotene (Sabei et al. 2005; Walter et al. 2007). Many but not all marine bacteria contain the genes required for retinal biosynthesis, or genes that enable the conversion of the precursor beta carotene to retinal (Peck et al. 2001).

The 15, 15' C=C bond is located at the central cleavage of β-carotene to form all-trans retinal. The conversion of the isoprenoid precursors into β-carotene is catalyzed by four crt genes: crtE, crtB, crtI, and crtY (Fig.1) (Martinez et al. 2007; McCarren and DeLong 2007). One molecule of β-carotene produces two retinal molecules. The rhodopsin chromophore retinal produces the oxidative cleavage of β-carotene.

This enzyme produced by cleaving the beta-carotene into two retinals is called 15, 15’-β-carotene-dioxgenase. Some proteobacteria can also synthesize β-carotene and convert it to retinal by using the enzyme encoded by the blh gene to produce functionally active PRs
Proteorhodopsin is all a photoactive retinylidene protein that functions as a heptahelical proton pump (de la Torre et al. 2003). It is simple heptahelical proton pumps containing a retinal chromophore covalently bound via a lysine Schiff’s base to helix G. A single molecule of all-trans retinal is binded by key residues lining the inner surface of the channel. Ion pumping is then induced by a corresponding change in protein conformation which causes ion transport accessory effector proteins to interact with the sensory rhodopsin family of proteins (Fig2) (Ottolenghi and Sheves 1989; Birge 1990).

Abundant evidence exists of PRs function as a transmembrane proton pump, including the light-mediated transport of protons in right-side-out PRs vesicles. Also, genetic community surveys suggested that proteorhodoopsin-harboring microorganisms are common in the different phylogenetic groups of the oceans by lateral gene transfer, indicating a fitness advantage (de la Torre et al. 2003; Frigaard et al. 2006; Martinez et al. 2007).
Fig. 1 Scheme of the carotenoid biosynthesis pathways from farnesyl pyrophosphate (FPP) to retinal. (FPP: farnesyl pyrophosphate, IPP: isopentenyl pyrophosphate (diphosphate), CrtE: GGPP synthase, GGPP: geranylgeranyl pyrophosphate, CrtB: phytoene synthase, CrtI: phytoene desaturase, CrtY: Lycopene cyclase, Bih: Bacteriorhodopsin-related-protein-like-homolog protein)
Fig. 2 Scheme of the all trans retinal change to 13-cis retinal.
1.3. Abundance and diversity of PR-containing marine bacteria

Oceanic picoplankton play a role in driving biogeochemical cycles. The following bacteria have been found to be the most prevalent in oceanic picoplankton: phyla Proteobacteria (63%), Bacteroidetes (13%), Cyanobacteria (7.9%), Firmicutes (7.5%), and Actinobacteria (4.6%) (Venter et al. 2004).

Following the discovery of PR in uncultured marine gamma proteobacterial SAR86 clade (Beja et al. 2001; Sabehi et al. 2004; Rusch et al. 2007), over 4,000 variants have been identified (Beja et al. 2001; Dioumaev et al. 2002; Spudich 2006) and several variants have been cloned into Escherichia coli. PR families have been found in Monterey Bay (Eastern Pacific Ocean), Hawaii Ocean Time (HOT, Central North Pacific Ocean), Palmer station (Beja et al. 2001), Mediterranean Sea, Red Sea (Sabehi et al. 2003), Sargasso Sea (Venter et al. 2004) and Pacific Ocean (Rusch et al. 2007). Most of the variants of PR fall under one of two groups depending on their photochemical properties: green-absorbing Proteorhodopsin (GPR) and blue-absorbing Proteorhodopsin (BPR) (Kralj et al. 2008). GPR has higher absorption efficiency. GPR absorbs light with a absorption maxima of $\lambda_{\text{max}}$ 525 nm (green) whereas BPR has a maxima of $\lambda_{\text{max}}$ 490nm (blue) (Man et al. 2003). Green-absorbing pigments predominately function in surface water while ‘blue-absorbing’ pigments function in deeper waters depending on light availability (Beja et al. 2001).

Recently, it has been suggested that there exist PR-like genes in CFB (Cytophya-Flavobacteria-Bacteroides) subdivision as well as Proteobacteria (Venter et al. 2004). In the Northwest Atlantic, among metagenome fragments, the Global Ocean Sampling (GOS) expedition found that the taxa of the PR gene was predominantly present in two assembled flavobacterial genomes (Rusch et al. 2007; Woyke et al. 2009). Flavobacteria,
which belong to the phylum Bacteroidetes previously called by the Cytophaga-Flavobacterium-Bacteroides (CFB), and Alpha- and Gammaproteobacteria are major carriers of photometabolic genes, including the microbial rhodopsin proteorhodopsin in marine environments (Giovannoni et al. 2005; Rusch et al. 2007). Flavobacteria play a role in maintaining the earth’s energy balance in the biogeochemical cycle of marine systems (Kirchman 2002; Abell and Bowman 2005; Alonso et al. 2007).

CHAPTER 2. THE INFLUENCE OF LIGHT AND CARBON ON PR-CONTAINING FLAVOBACTERIA

2.1 Introduction

Research in recent years has revealed new information about the role and diversity of PR in proteobacteria; however less attention has focused on the function and diversity of PR in Bacteroidetes (Zhao et al. 2009). Flavobacteria itself was found both free-living and attached to organic aggregates and are considered as major mineralizers of organic matter. (DeLong et al. 1993; Abell and Bowman 2005; Cottrell and Kirchman 2009; Zhao et al. 2009). Among them, PR in Flavobacteria (Dokdonia sp. MED134) was revealed to enhance the cell yields in the presence of light compared to in dark so that PR fulfils a phototrophic function in marine bacteria (Gomez-Consarnau et al. 2007). Also, in a PR-containing marine flavobacterial suspension, light-driven proton transport activity was sufficient for ATP generation was demonstrated (Yoshizawa et al. 2012). The effect of light on cell yields has been little studied in cultivated marine alpha proteobacterium, and primarily limited to the SAR11 strain HTCC1062 Pelagibacter ubique (Giovannoni et al. 2005) and gamma proteobacterium SAR92 strain HTCC2207. Therefore, it is expected
that flavobacteria will give us information about *in vivo* function of PR as well as the relationship between light and cell growth (Stingl *et al.* 2007).

This study will focus on the discovery of the effect of a light-enhanced growth yield in proteorhodopsin-containing flavobacterial strains. To see the physiological characteristics of PR-containing Flavobacteria, we performed growth experiment with various carbon concentrations in the light or in the darkness. Also, we explored the effect of retinal biosynthesis inhibitor, MPTA, on light-enhancement growth. To our knowledge, this is the first time a light-dependent growth yield effect under low and high carbon DOC concentrations has been demonstrated in native cells. The chapter will conclude with discussion of the significance of the findings and future directions.

2.2. Methods and Materials

**Bacterial strains and culture conditions**

Dr. Kazuhiro Kogure (The University of Tokyo, Japan) kindly provide us PR-containing marine Flavobacteria isolates from sea ice collected in Saroma-ko Lagoon and from the surface seawater collected at Sagami Bay Station P and western North Pacific Station S (Fig. 3.).

The bacterial strains used are listed in Table 1. The Flavobacteria strains were routinely grown in Marine Agar 2216 (Difco Laboratories, Detroit, MI, USA) at 22 ºC for 48 h.
Fig. 3. Geological maps showing location of the sampling sites of strains tested in this study.
Table 1. PR-containing Flavobacterial strains used in this work

<table>
<thead>
<tr>
<th>Organism</th>
<th>GenBank Accession Number</th>
<th>Reference or Source</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>16S rRNA</td>
<td>PR</td>
</tr>
<tr>
<td><strong>Dokdonia sp. Strain</strong></td>
<td>DQ481462</td>
<td></td>
</tr>
<tr>
<td>MED134</td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>Winogradskyella sp. PC-19</strong></td>
<td>AB557530</td>
<td>AB557551</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>Winogradskyella sp. PG-2</strong></td>
<td>AB557522</td>
<td>AB557552</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>Gilvibacter sp. SZ-19</strong></td>
<td>AB557542</td>
<td>AB557573</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>Persicivirga sp. S1-08</strong></td>
<td>AB602426</td>
<td>Not yet</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>Tenacibaculum sp. SZ-18</strong></td>
<td>AB557536</td>
<td>AB557572</td>
</tr>
</tbody>
</table>
Fig. 4. Phylogenetic tree based on PR amino acid sequences including strains that we studied in this paper. Figure adapted from Yoshizawa et al., 2007. Red boxes indicate strains examined in this study. Strains are color coded to indicate site of isolation (Blue, Saromako-Lagoon; Orange, western North Pacific, Green, Sagami Bay). Bootstrap values are shown at each node. Bar, 0.1 substitutions per nucleotide position.
Preparation of starter cultures for growth experiments

Starter cultures for the growth experiment at various carbon concentrations were grown in acid-washed borosilicate glass culture bottles (1 L, VWR), with 500 mL of artificial seawater (ASW, 15 practical salinity units, Sigma CAS No: 7647-14-5) supplemented with a full strength media (FSM, 1.1 mM C; Table 2). The ASW was filtered through 0.2 mm-pore-size bottle-top vacuum filter system (Nalgene, NY, USA) and autoclaved. A colony grown on marine agar plate streaked from frozen stock was inoculated in ASW amended with 1.1 mM C FSM media. The starter culture was covered with aluminum foil (Reynolds) and grown in the dark at 21°C - 22°C until near the middle of the exponential phase, which took from 3 to 5 days, depending on the strain, the conditions of growth, and the initial cell density. Cell density of the starter culture was determined during late-exponential phase by the direct cell counting method. Then, the starter culture was stored in a 4°C refrigerator and used to inoculate further growth experiments. And then all cultures were inoculated with a known number of cells (1000 cells/ml).

Culture conditions

Experiments to determine the effect of DOC concentration on light-dependent physiological effect of the PR-containing Flavobacterial strains were inoculated from a stationary phase of starter culture grown on 1.1 mM DOC in a dark condition. Inoculation was taken from this stationary phase culture stored in 4 °C. The culture density was followed (cells/mL) up to the time of inoculation and was used to determine the dilution of the starter culture into the DOC concentration experiment cultures. And then all cultures were inoculated with a known number of cells (1000 cells/ml). A common culture was
inoculated for all experiment conditions in ASW amended with FSM. Thorough mixing was provided at each step through rigorous vortexing.

Cultures of PR-containing flavobacterial strains were grown in artificial seawater (ASW, 15 practical salinity units, Sigma CAS No: 7647-14-5) supplemented with a dissolved organic carbon (DOC) at the indicated concentration (Table 2; full strength FSM contains 1.1 mM DOC). The ASW was filter-sterilized through 0.2 mm-pore-size bottle-top vacuum filter system (Nalgene, Rochester, NY, USA) and autoclaved. Then, 250 ml aliquots of ASW (containing a background concentration of 0.05 mM C of DOC) were partially supplemented with full strength medium (FSM). Concentrations of DOC in the ASW media tested were: 0.05 mM, 0.14 mM, 0.39 mM, 1.1 mM, and 48.5 mM. To avoid inorganic nitrogen and phosphate limitation, the media were also amended with 225 μm NH₄Cl (Sigma CAS No: 12125-02-9) and 44.7 μm Na₂HPO₄·12H₂O (Sigma CAS No: 10039-32-4). Cultures were grown in 50 mL glass tubes. Tubes for the dark condition were wrapped with aluminum foil and tubes for the light condition were left unwrapped. Cultures were incubated in a walk-in light room incubator. The temperature of the incubator is maintained at 21 °C – 22 °C.

To avoid DOC contamination in the growth experiment, it was extremely important to clean all glassware used in the analysis of growth characteristics very carefully. All glassware was acid-washed with 10% HCl (Macron Chemicals CAS No: 7647-01-0) with an overnight contact time and then thoroughly rinsed with distilled water. Acid-washed glassware was autoclaved before use.
Table 2. Composition of agar media used for cultivation studies.

<table>
<thead>
<tr>
<th></th>
<th>Marine Agar 2216 (g/L)</th>
<th>Full Strength Medium (g/L)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Peptone</td>
<td>5</td>
<td>5</td>
</tr>
<tr>
<td>Yeast extract</td>
<td>1</td>
<td>1</td>
</tr>
<tr>
<td>Ferric citrate</td>
<td>0.1</td>
<td>Artificial sea water (ASW), 1000 ml</td>
</tr>
<tr>
<td>Sodium chloride</td>
<td>19.45</td>
<td></td>
</tr>
<tr>
<td>Magnesium chloride</td>
<td>5.9</td>
<td></td>
</tr>
<tr>
<td>Magnesium sulfate</td>
<td>3.24</td>
<td></td>
</tr>
<tr>
<td>Calcium chloride</td>
<td>1.8</td>
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</tr>
<tr>
<td>Potassium chloride</td>
<td>0.55</td>
<td></td>
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<tr>
<td>Sodium bicarbonate</td>
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<tr>
<td>Potassium bromide</td>
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<tr>
<td>Strontium chloride</td>
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<td></td>
</tr>
<tr>
<td>Boric acid</td>
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</tr>
<tr>
<td>Sodium silicate</td>
<td>4 mg</td>
<td></td>
</tr>
<tr>
<td>Sodium Fluoride</td>
<td>2.4 mg</td>
<td></td>
</tr>
<tr>
<td>Ammonium nitrate</td>
<td>1.6 mg</td>
<td></td>
</tr>
<tr>
<td>Disodium phosphate</td>
<td>8 mg</td>
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<tr>
<td>Agar</td>
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<td></td>
</tr>
<tr>
<td>DDW, ml</td>
<td>1000</td>
<td></td>
</tr>
</tbody>
</table>
**Direct microscopic counting methods**

Total cell numbers were determined by direct microscopic count according to the method described previously (Kimura et al., 2011). The appropriate volume of culture was diluted using ASW and stained with 100 μL of 100X SYBR Green (Molecular Probes/Invitrogen) for 15 minutes under darkness. Then, the solution was vacuum filtered with a 25 mm diameter, 15-mL funnel filter apparatus (VWR) onto a black 0.22 μm isopore membrane (Millipore, #GTBP02500). Microscope slides were made using a glass slide (Gold Seal Microslides #3010) with immersion oil (Zeiss Immerisol 518) and coverslip (Thermoscientific mSeries Lifterlip 22x251-2-4816). Finally, cellular growth was determined using an epifluorescence microscope (Zeiss Axioskop 2 microscope) with a 100 X magnification objective (Zeiss Plan-NEOFLUAR). Bacterial cell densities with the proper dilution were counted at least 10 separate fields of view across the filter. Total cell densities of samples were calculated using the following equation (from Wetzel and Likens, 1991).

\[
\text{Bacteria mL}^{-1} = (\text{membrane conversion factor} \times \text{ND})
\]

\[
\text{Membrane conversion factor} = \frac{\text{Filtration area}}{\text{area of micrometer field}}
\]

\[
\text{N} = \frac{\text{Total number of bacteria counted}}{\text{number of micrometer fields counted}}
\]

\[
\text{D} = \frac{\text{Dilution factor; volume of sample stained}}{\text{total volume of sample available}}
\]
Culture experiments with specific inhibitors

Kimura et al. (2011) proposed that retinal-bound PR has an important role in the light-stimulated growth by culture experiment with MPTA inhibitor. MPTA act as a inhibitor of lycopene cyclization in retinal biosynthesis pathway (Cunningham et al., 1994; Armstrong, 1999).

First, to confirm the effect of MPTA inhibitor on PR-containing flavobacterial strains, we cultured strains on Marine Agar 2216 amended with MPTA at a final concentration of 300 mM. The agar plates were incubated at room temperature for 3 days. When colonies were clearly visible, we determined the effect of MPTA against flavobacterial strains on the inhibition of β-carotene synthesis as indicated by colony pigmentation. Next, to see the PR-induced light enhancement in growth, Dokdonia sp. MED134 and Gilvibacter sp. SZ-19 were grown in ASW slightly enriched with FSM (0.14mM C and 48.5mM C) and amended with MPTA. MPTA was dissolved in methanol and added to ASW at a final concentration of 100 mM. The same volume of methanol was added to negative control cultures without MPTA. These cultures were incubated at 21-22 °C under continuous white light (approximately 150 mmol of photons m⁻² s⁻¹) or in the darkness in both carbon concentrations. The culture experiments with MPTA were performed in triplicate. Bacterial cell density was measured every day by the direct counting method with epifluorescence microscope described above.
Fig. 5. Specific inhibitor of Lycopene cyclization, 2-(4-Methylphenoxy)Triethylamine (MPTA) (a) Chemical formula of MPTA. (b) The bioregulatory action of MPTA in β-carotene synthesis.
2.3 Results and Discussions

**Responses of various carbon concentrations on light-dependent growth of PR-containing marine flavobacterial strains**

Several research studies have been shown that the light-stimulated ATP production or growth in PR-containing bacteria could be detected under carbon limiting conditions (Gomez-Consarnau et al., 2007; Martinez et al., 2007; Gomez-Consarnau et al., 2010; Steindler et al., 2011). In this study, we used marine flavobacterial strains to measure the sensitivity to light of the PR light-transducing genes as DOC concentration was varied in a culture media. To determine potential interference with very low levels of DOC caused by endogenous carbon or carryover from the starter culture, the effect of the DOC concentration was determined using a stationary phase culture grown on 1.1 mM glucose in the dark. Then, the cells were washed twice using ASW. Cell density was directly counted under the epifluorescence microscopy with SYBR Green (Applied Biosystems) staining.

Before the beginning of the growth experiment with various carbon concentrations, we confirmed the light-enhanced growth at low carbon concentration (0.14 mM C) on *Dokdonia* sp. MED134 and *Persicivirga* sp. S1-08 (Fig. 6). In both strains, cell densities were increased significantly, which were consistent with those seen in before. This shows that further growth experiments with different carbon concentrations are needed to investigate the PR-containing marine flavobacterial strains.

Flavobacteria strains (*Dokdonia* sp. MED134, *Winogradskyella* sp. PC-19, *Winogradskyella* sp. PG-2, *Gilvibacter* sp. SZ-19, *Persicivirga* sp. S1-08, *Tenacibaculum* sp. SZ-18; Yoshizawa et al, 2012) were grown under light and dark conditions while the
concentrations of DOC were varied. Our results showed that as the concentration of DOC was increased there was a diminishing advantage of growth in the light over the dark (Fig. 7-11). This result is consistent with those of other studies analyzing the effect of different carbon concentrations on light-induced increases in the growth yield in PR-containing flavobacterium. Figure 7-11 shows characteristic growth curves for flavobacterial strains grown on ASW media enriched with varied concentrations of carbon.

The measure of carbon concentrations showed that smaller amounts of carbon resulted in a different growth yields between cultures grown in light and dark conditions. Specifically, DOC concentrations below 1.1 mM showed a significant difference in the maximum growth yield with increased yields in cultures grown in the light compared to those in the dark (Fig. 7-11). In contrast, the difference between the light and dark cultures was not significant at a DOC levels of 1.1 mM. The cell yield ratio between light and dark showed a decreasing trend with increasing DOC concentration at DOC concentrations greater than 1.1mM (Figure 7-11). Therefore, it can be concluded that proteorhodopsin has a favorable impact on yields at low carbon concentrations. However, interestingly, the inverse was true at high carbon concentrations (48.5mM C) in the dark, which resulted in higher yields when grown in the dark than in the light. We could not fully explain the reason at this time, but it has been suggested that PMF generated respiration could interact with the PR.

In some microbial strains containing light transport-inducing rhodopsins, biomass yields increased and rates of aerobic respiration decreased when the bacteria were grown in light conditions (Gomez-Consarnau et al. 2007; Lami et al. 2009; Gomez-Consarnau et al. 2010; Kimura et al. 2011). Several theories have been presented to explain the mechanism, largely unknown, that could interfere with the ability of PRs to maintain
cellular proton mobility force even during respiratory distress. One theory states that "backpressure" from the PMF directly interacts between the respiratory proteins and proteorhodopsin, reducing its effect on proton pumping. As a result, PR proton pumping is replaced by respiration. With the electrochemical potential decreased, the PRs are unable to translocate protons and electrons across the cell membrane to a terminal electron acceptor during cellular respiration.

Another possibility would be regulation of metabolic pathways, such as through a decrease in available ATP, which results in fewer electron donors of the respiratory chain being formed. These include NADH and the FADH$_2$ of the tricarboxylic acid (TCA) cycle. Anabolic pathways competing for metabolites could result in a decrease in respiratory flux. The high respiration rate may be caused by the excess nutrients which may, in turn, interfere with the function of proteorhodopsin.

Studies on marine flavobacterial strains containing proteorhodopsin have similarly found that the light-stimulated growth effect in PR-harboring flavobacteria only occurs at low carbon substrate concentrations. However, the mechanism by which an increase in carbon decreases the light effect was not elucidated in this study. To get a better understanding, we performed transcriptomic analysis from cultures of *Dokdonia* sp. MED134 at various carbon concentration and different incubation time.
Fig. 6. Growth curves of *Dokdonia* sp. MED134 (a) and *Persicivirga* sp. S1-08 (b) incubated in the light (○) or in the dark (●). Strains were grown in ASW enriched to 0.14m C. Error bars indicate standard deviation for triplicate cultures.
Fig.7. Growth curves of *Winogradskyella* sp. PC-19 incubated in the light (○) or in dark (●). *Winogradskyella* sp. PC-19 was grown in ASW enriched to 0.14 mM C (a), in ASW enriched to 0.39 mM C (b), in ASW enriched to 1.1 mM C (c), and in ASW enriched to 48.5 mM C (d).
Fig. 8. Growth curves of *Winogradskyella* sp. PG-2 incubated in the light (○) or in dark (●). *Winogradskyella* sp. PG-2 was grown in ASW enriched to 0.14 mM C (a), in ASW enriched to 0.39 mM C (b), in ASW enriched to 1.1 mM C (c), and in ASW enriched to 48.5 mM C (d).
Fig. 9. Growth curves of *Gilvibacter* sp. SZ-19 incubated in the light (○) or in dark (●). *Gilvibacter* sp. SZ-19 was grown in ASW enriched to 0.14 mM C (a), in ASW enriched to 0.39 mM C (b), in ASW enriched to 1.1 mM C (c), and in ASW enriched to 48.5 mM C (d).
Fig. 10. Growth curves of *Tenacibaculum* sp. SZ-18 incubated in the light (○) or in dark (●). *Tenacibaculum* sp. SZ-18 was grown in ASW enriched to 0.14 mM C (a), in ASW enriched to 0.39 mM C (b), in ASW enriched to 1.1 mM C (c), and ), in ASW enriched to 48.5 mM C (d).
Fig. 11. Growth curves of *Dokdonia* sp. MED134 incubated in the light (○) or in dark (●). *Dokdonia* sp. MED134 was grown in unenriched ASW (0.05 mM C) (a), in ASW enriched to 0.14 mM C (b), in ASW enriched to 1.1 mM C (c), and in ASW enriched to 48.5 mM C (d). Error bars denote standard deviation for the triplicates.
Table 3. Maximum cell density (10^6 cells ml⁻¹) of PR-containing marine flavobacterial strains

<table>
<thead>
<tr>
<th>Strain</th>
<th>ASW + 0.05mM C</th>
<th>ASW + 0.14mM C</th>
<th>ASW + 0.39mM C</th>
<th>ASW + 1.1mM C</th>
<th>ASW + 48.5mM C</th>
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<td></td>
<td>L</td>
<td>D</td>
<td>L</td>
<td>D</td>
<td>L</td>
</tr>
<tr>
<td>Dokdonia sp. MED134</td>
<td>0.23</td>
<td>0.06</td>
<td>9.32</td>
<td>6.28</td>
<td>-</td>
</tr>
<tr>
<td>Winogradskyella sp. PC-19</td>
<td>-</td>
<td>-</td>
<td>7.30</td>
<td>4.72</td>
<td>17.5</td>
</tr>
<tr>
<td>Winogradskyella sp. PG-2</td>
<td>-</td>
<td>-</td>
<td>10.1</td>
<td>7.44</td>
<td>18.2</td>
</tr>
<tr>
<td>Gilvibacter sp. SZ-19</td>
<td>0.13</td>
<td>0.05</td>
<td>9.27</td>
<td>6.31</td>
<td>-</td>
</tr>
<tr>
<td>Persicivirga sp. S1-08</td>
<td></td>
<td></td>
<td>3.26</td>
<td>2.46</td>
<td></td>
</tr>
<tr>
<td>Tenacibaculum sp. SZ-18</td>
<td>-</td>
<td>-</td>
<td>1.97</td>
<td>1.87</td>
<td>2.56</td>
</tr>
</tbody>
</table>
Table 4. Specific growth rate (day\(^{-1}\)) of PR-containing marine flavobacterial strains

<table>
<thead>
<tr>
<th>Strain</th>
<th>ASW + 0.05mM C</th>
<th>ASW + 0.14mM C</th>
<th>ASW + 0.39mM C</th>
<th>ASW + 1.1mM C</th>
<th>ASW + 48.5mM C</th>
</tr>
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<td>L</td>
<td>D</td>
<td>L</td>
<td>D</td>
<td>L</td>
</tr>
<tr>
<td><strong>Dokdonia sp. MED134</strong></td>
<td>1.53</td>
<td>0.62</td>
<td>3.20</td>
<td>2.96</td>
<td></td>
</tr>
<tr>
<td><strong>Winogradskyella sp. PC-19</strong></td>
<td></td>
<td></td>
<td>1.93</td>
<td>1.75</td>
<td>1.94</td>
</tr>
<tr>
<td><strong>Winogradskyella sp. PG-2</strong></td>
<td></td>
<td></td>
<td>0.74</td>
<td>0.73</td>
<td>0.81</td>
</tr>
<tr>
<td><strong>Gilvibacter sp. SZ-19</strong></td>
<td>0.76</td>
<td>0.49</td>
<td>1.44</td>
<td>1.31</td>
<td></td>
</tr>
<tr>
<td><strong>Persicivirga sp. S1-08</strong></td>
<td></td>
<td></td>
<td>1.55</td>
<td>1.47</td>
<td></td>
</tr>
<tr>
<td><strong>Tenacibaculum sp. SZ-18</strong></td>
<td></td>
<td></td>
<td>1.97</td>
<td>1.87</td>
<td>2.56</td>
</tr>
</tbody>
</table>
Fig. 12. Specific growth rate ($\mu$) determined by regression analysis based on the growth experiment of strain *Dokdonia* sp. MED134. (a) ASW + 0.05 mM C, (b) ASW + 0.14 mM C, (c) ASW + 1.1 mM C, (d) ASW + 48.5 mM C.
Specific growth rate of proteorhodopsin-containing marine flavobacterial strains

The maximum specific growth rates (μ) are one good way of expressing the relative ecological success of a species or strain in adapting to its natural environment or the experimental environment imposed upon it.

The maximum specific growth rates (the slope of a plot of the natural log of cell numbers and time yields the specific growth rate, μ) at various carbon concentrations were determined for PR-containing flavobacterial strains. The bacterium's growth was monitored during the exponential phase to measure the specific growth rate at each carbon concentration, as shown in Figure 12 and Table 4. The maximum cell density and specific growth rate reached are shown in Table 3 and 4, respectively.

The specific growth rates of the isolates under various growth conditions were summarized in graphs and compared to each other (Fig. 13 and 14). The μ values of the strains under light in ASW plus 0.14 mM C and LNHM plus 48.5 mM C ranged from 0.74 to 3.20 day⁻¹ and 1.04 to 4.99 day⁻¹, respectively (Table 2), and these values were lower than μ value of cultures in the dark condition at each strain.

All five strains in the Flavobacteria showed maximum μ values at low carbon concentrations (0.05 mM C and 0.14 mM C) in light-incubated cultures, while they showed maximum μ values at high carbon concentrations (Fig. 13 and 14). Interestingly, growth characteristics of the PR-containing flavobacterial strains were generally switched at media containing high carbon concentration. From this result, DOC concentration could be one of the criteria for differentiating light-dependent growth within the PR-harboring flavobacteria strains.
Fig. 13. Summary of specific growth rates (μ) of *Dokdonia* sp. MED134 (a) and *Winogradskyella* sp. PG-2 (b).
Fig. 14. Summary of specific growth rates ($\mu$) of *Winogradskyella* sp. PC-19 (a), *Gilvibacter* sp. SZ-19(b) and *Tenacibaculum* sp. SZ-18 (c).
Culture experiments with an inhibitor of retinal biosynthetic pathway, MPTA

To determine the effect of retinal biosynthesis inhibitor on light-dependent growth, we studied culture experiment with the MPTA in two different strains of PR-containing flavobacteria: *Dokdonia* sp. MED134 which was broadly studied and *Gilvibacter* sp. SZ-19.

In previous studies assessing the role of PR in light-induced growth in strain *Dokdonia* sp. MED134, it was observed that only light corresponding to the wavelengths absorbed by PR supported growth (Gomez-Consarnau *et al.* 2007; Kimura *et al.* 2011). Additionally, culture experiments on MPTA, an inhibitor of lycopene cyclization in the retinal biosynthetic pathway, were conducted to provide further evidence of the role of PR in stimulating growth in *Dokdonia* sp. MED134 (Kimura *et al.* 2011).

To assess the effectiveness of MPTA as an inhibitor of β-carotene synthesis (the direct precursor of retinal), MED134 was grown on Marine agar plates with and without MPTA. Bacterial cells accumulating β-carotene display yellow or orange colonies. In the presence of MPTA, MED134 produced light pink colonies, indicating the accumulation of lycopene (Fig. 15). In the absence of MPTA, yellow colonies were observed on agar plates (Fig. 16). Our results indicate that MPTA effectively prevented β-carotene generation, the precursor for retinal, in both *Dokdonia* sp. MED134 and *Gilvibacter* sp. SZ-19.
Fig. 15. Colony images of *Dokdonia* sp. MED134 in culture experiment with a MPTA.
(a, b) *Dokdonia* sp. MED134 was grown on Marine Agar (Difco 2216) plates with MPTA (a) and without MPTA (b). The strain was also grown on agar plate amended with methanol as negative control (c).
Fig. 16. Colony images of *Gilvibacter* sp. SZ-19 in culture experiment with a MPTA. (a, b) *Gilvibacter* sp. SZ-19 was grown on Marine Agar (Difco 2216) plates with MPTA (a) and without MPTA (b). The strain was also grown on agar plate amended with methanol as negative control (c).
Growth characteristics of representative flavobacterial strains on MPTA containing media

To determine the role of MPTA as an inhibitor of β-carotene, culture media at both low and high concentrations of DOC were used. Culture experiment with MPTA was performed in both *Dokdonia* sp. MED134 and *Gilvibacter* sp. SZ-19.

First, strains were grown in ASW enriched to 0.14 mM C and amended with MPTA for low carbon concentration media. When *Dokdonia* sp. MED134 and *Gilvibacter* sp. SZ-19 were incubated in ASW without MPTA, bacteria grew well to $1.14 \times 10^6$ cells/ml and $1.17 \times 10^6$ cells/ml in the presence of light, respectively (Fig. 17a and Fig. 18a). However, cultures incubated in ASW with MPTA in the light showed significantly lower yields than those in ASW without MPTA. In culture experiments in the dark with or without MPTA, cell density of both strains similar to the results with MPTA-treated condition in the light (Fig. 17a and Fig. 18a).

Second, *Dokdonia* sp. MED134 and *Gilvibacter* sp. SZ-19 were grown on the media of ASW enriched to 48.5 mM C and amended with MPTA for high carbon concentration (Fig. 17b and 18b). Under culture experiment with MPTA at high carbon concentration conditions, whether present of light of not, *Dokdonia* sp. MED134 were reached up to $5.15 \times 10^7$ cells/ml and $5.77 \times 10^7$ cells/ml in the light or in the darkness, respectively, which was at least two times higher than the maximum cell density in the MPTA-treated cultures. Also, the similar results were shown in the *Gilvibacter* sp. SZ-19.

Although the results of MPTA growth experiment at high carbon concentration are hard to explain clearly at this point, we can still suggest that PR bound to retinal has a
important role in the light-stimulated growth of the marine flavobacterial strain containing proteorhodopsin.
Fig. 17. Growth curves of *Dokdonia* sp. MED134 in culture experiments with a MPTA inhibitor. 
(a, b) *Dokdonia* sp. MED134 was grown in ASW enriched to 0.14 mM C with and without MPTA (a), in ASW enriched to 48.5 mM C with and without MPTA (b). The cultures in enriched ASW amended with MPTA exposed to light (○) and in the dark (△), and in the enriched ASW without MPTA in culture exposed to light (●) and in the dark (▽). The epifluorescence microscopy was used to observe and count bacterial cells.
Fig. 18. Growth curves of *Gilvibacter* sp. SZ-19 in culture experiments with a MPTA inhibitor. (a, b) *Gilvibacter* sp. SZ-19 was grown in ASW enriched to 0.14 mM C with and without MPTA (a), in ASW enriched to 48.5 mM C with and without MPTA (b). The cultures in enriched ASW amended with MPTA exposed to light (○) and in the dark (△), and in the enriched ASW without MPTA in culture exposed to light (●) and in the dark (▼). The epifluorescence microscopy was used to observe and count bacterial cells.
CHAPTER 3. TRANSCRIPTOMIC ANALYSIS OF LOW AND HIGH CARBON CONCENTRATIONS ON LIGHT-DEPENDENT GROWTH OF *Dokdonia* sp.

MED134

3.1 Introduction

A branch of biology 'genomics' was coined by Thomas Roderick as a name for the new journal *Genomics* in 1986, and refers to a new scientific discipline of mapping, sequencing, and analyzing genomes (McKusick 1997). And, at the beginning of this century, genomics greatly changed the face of biology with the completion of large-scale genome projects. Not only has the scale of molecular biology changed, but also its scope. Genomics is now undergoing a transition or expansion from the mapping and sequencing of genomes to an emphasis on genome function (Debouck and Metcalf 2000; DeLong et al. 2006; Rusch et al. 2007; Rodrigue et al. 2009). To reflect this shift, genome analysis may now be divided into "structural genomics" and "functional genomics". Structural genomics represents an initial phase of genome analysis such as linkage analysis, molecular cytogenetics, physical mapping, expressed sequence tag (EST) sequencing, genome sequencing and genome organization (McKusick 1997). And functional genomics refers to the development and application of global (genome-wide or system-wide) experimental approaches to assess gene function by making use of the information and reagents provide by structural genomics (Hieter and Boguski 1997; Tringe and Rubin 2005). It is characterized by high throughput or large-scale experimental methodologies combined with statistical and computational analysis (bioinformatics) of the results (Fig. 19)
Functional genomics studies for the definition of the functions of large sets of genes that are involved in or regulate processes in plant growth and development are called plant functional genomics.

In functional genomics, "transcriptomics", the study of gene expressions at transcription level, is one of the flourishing areas that will have a great impact in understanding the functional role of genes (Eymann et al. 2002; Ernst et al. 2005; DeLong et al. 2006; Toledo-Arana et al. 2009). Since mRNA is the initial and intermediate product of gene expression, most cases studies at the transcriptional level may reflect the function of the respective genes. To analysis transcriptomes in large-scale, several genomic technologies were developed. They include: PCR-based assays, serial analysis of gene expression (SAGE) (Velculescu et al. 1995), rapid analysis of gene expression (RAGE), expressed sequence tags (ESTs) (Poretsky et al. 2009), macroarrays (Steward et al. 2004) and microarray (biochip) technology (Lindell et al. 2007; Zinser et al. 2009). Among these technologies microarray technology was considered as the powerful tool for transcriptomes analysis. The DNA microarray is invented for studying gene expression patterns simultaneously on a global scale. Large numbers of different gene fragments are immobilized in an ordered array on a solid support; it is available for the interrogation of transcriptomes. To do this, an entire population of mRNAs from a tissue can be simultaneously hybridized, in a single experiment, with tens of thousands of known DNA probes that are immobilized at precisely known positions on the array. After the hybridization, the expression levels of each gene can be determined with a high-resolution laser scanner, and computer programs can group genes that are up- or down-regulated in response to the
interrogated stress. Therefore, it is possible to detect the differences in relative abundance of several mRNAs between two sets of conditions run in parallel.

Despite these advantages (Zhou and Thompson 2002), a non-targeted, sequence approach is increasingly used to profile community transcripts due to the technological limitations of microarrays. While microarrays employ complex quantification algorithms, increasingly more complex gene expression solutions are required. Factors that can affect probe specificity include outputs of signal intensity instead of nucleotide sequence identities. Furthermore, microarrays depend on massively parallel nucleic acid hybridization and therefore have a risk of cross-hybridization of highly related sequences. The scope and size of studies enabled by non-targeted, sequence is evident in a 2005 study that generated and sequenced a cDNA clone library of ~400 clones by random priming of microbial community RNA collected from a hypersaline lake (Poretsky et al. 2005).

Sequence-based profiling of community transcriptomes on a large scale has benefited from technological advances. Next-generation sequencing techniques can perform millions of sequence reads in a run. These include pyrosequencing (Margulies et al. 2005), Illumina technology (formerly Solexa sequencing), and Ion Torrent technology (Ion Torrent Systems, Inc., Guilford, CT). Pyrosequencing, in particular, has been increasingly used in microbial ecology and its use has been peer reviewed many times since 2007. First used in soil analysis (Leininger et al. 2006), it is conducive to a number of open ocean microbial assemblage experiment procedures, including measuring size-fractionation and collecting bacterioplankton biomass.

A major advantage of next-generation sequencing applying metatranscriptomic analysis is the lack of a need for cloning in the process of processing the extracted RNA.
(rRNA subtraction, amplification and so forth) and converting it into cDNA (Fig. 20). In terms of analysis, metatranscriptomic technology provides insight into the regulation and expression patterns of the biological processes of genes at the molecular level (Frias Lopez et al., 2008). Accordingly, it is being more widely applied to understand the unknown and increasingly complex biology of microbes under different and untested environmental conditions. Methodological and technological approaches and protocols continue to evolve.
Fig. 19 Scheme showing how the integration of results from different technological levels of functional genomics leads to construction of a virtual microorganism.
Fig. 20. Scheme of sample processing pipeline for the metatranscriptomic analysis; adapted from Stewart et al (2010).
3.2. Materials and methods

**Cultivation for transcriptomic analyses**

Our experiment was designed to determine the effect of DOC concentration on gene expression of PR-containing *Dokdonia* sp. MED134 under light and dark growth conditions. For transcriptomic analyses, MED134 was grown in ASW amended with FSM to yield DOC concentrations of low (0.14 mM C) and high (48.5 mM C), respectively.

To examine the gene expression of *Dokdonia* sp. MED134 at low carbon concentration under both light and darkness, MED134 was grown in 4L of ASW enriched to 0.14 mM C at 22 °C in the darkness for 2 days. At this time, 500 mL of culture was filtered on to a pore-size 0.22 mm Durapore membrane filter (25mm diameter, Millipore), yielding the T0 sample. The remaining culture was split in two 1 L flasks that were incubated again at 22 °C under the continuous white light (approximately 150 mmol of photons m\(^{-2}\) s\(^{-1}\)) or in darkness. After 2 more days, the cultures were taken from two time points, mid to late exponential phase and stationary phase and filtered onto Durapore membrane filters (Millipore), yielding samples L1 (exponential phase and light conditions) and L2 (stationary phase and light conditions) and D1 (exponential phase and dark conditions) and D2 (stationary phase and dark conditions), respectively. Sampling time points were determined by pilot experiment. The same procedures were performed in cultures of high carbon concentration (48.5 mM C) condition. All filtered samples were immediately placed into screw-cap tubes containing 1 mL of RNALater (Ambion, Austin, TX, USA) and stored at -80 °C until RNA extraction. All treatments were performed in triplicate.
Total RNA extraction

Total RNA was extracted from the filter samples using a modification of the mirVana miRNA isolation kit (Ambion) as described previously (Shi et al., 2009; McCarren et al., 2010). Briefly, Sterivex filter cartridges containing samples were taken from the -80°C freezer, thawed on ice and the RNAlater was carefully removed by pushing through with a syringe. Then, the filters were immersed with Lysis/Binding (Ambion) and mixed to lyse attached cells. The yellowish color on the filter due to cells disappeared during vortexing suggesting cells were lysed. Total RNA was extracted from the lysate according to the manufacturer’s protocol. Remaining genomic DNA was removed using a TURBO DNA-free kit (Ambion). Finally, extracted total RNAs (pellets) from the filters were purified, concentrated, and resuspended in 50 μl DEPC treated RNase-free water by using the MinElute PCR Purification Kit (Qiagen) and stored at -80°C.

ribosomal RNA (rRNA) subtraction

rRNA frequently represents 53% - over 90% of the total RNA (Frias-Lopez et al., 2008; Stewart FJ et al., 2010). Thus, rRNA needs to be removed so that the majority of sequencing effort goes to mRNA.

To remove bacterial 16S and 23S rRNA molecules from total RNA samples, we carried out the subtractive hybridization using sample specific biotinylated rRNA probes as previously described by Stewart et al (Stewart et al., 2010). Ribonucleotide probes were generated from the total DNA extracted from the Dokdonia sp. MED134. Templates for probe were first prepared by PCR using strain-specific primers flanking
nearly the full length of the bacterial 16S gene and 23S rRNA gene and Herculase II Fusion DNA Polymerase (Stratagene, La Jolla, CA, USA), with reverse primers modified to contain the T7 RNA polymerase promoter sequence. Biotin-labeled antisense rRNA probes were generated by in vitro transcription (IVT) using T7 promoter-containing 16S and 23S amplicons as templates based on MEGAscript High Yield Transcription Kit (Ambion, Austin, TX, USA). Biotinylated rRNA probes were hybridized to rRNA in the total RNA sample. The labeled double-strand rRNA was then removed via hybridization to streptavidin-coated magnetic beads (New England Biolabs, Ipswich, MA, USA), followed by magnetic separation. After subtracted RNA purification, purified RNA was confirmed the removal of bacterial rRNAs using a 2100 Bioanalyzer (Agilent, Santa Clara, CA, USA).

RNA amplification, complementary DNA (cDNA) synthesis and Illumina sequencing

The rRNA-subtracted RNA was amplified using the ScriptSeq™ v2 RNA-Seq Library Preparation kit (Epicentre, Madison, WI). cDNA was synthesized according to the manufacturer’s protocol.

In brief, the rRNA-depleted sample is fragmented using the RNA Fragmentation Solution. The fragmented RNA was converted to double-stranded cDNA via random-sequence primers containing a tagging sequence at their 5’ ends. The 5’-tagged cDNA is then tagged at its 3’ end by the terminal-tagging reaction to make di-tagged at both its 5’- and 3’-end, single-stranded cDNA. Following purification, the di-tagged cDNA is amplified by PCR, which completes the addition of the Illumina adaptor sequences, amplifies the library for subsequent cluster generation and adds an optional Illumina
Index. The amplified RNA-seq library is purified using AMPure kit (Beckman Coulter Genomics, Danvers, MA, USA) and is ready for cluster generation and sequencing. The resulting cDNA libraries were then pyrosequenced on the MiSeq platform (Illumina).
3.3 Results and Discussions

**Cultivation for transcriptomic analyses**

Before the beginning of the transcriptomic analysis with various carbon concentrations, we confirmed the light-enhanced growth on *Dokdonia* sp. MED134 at both low (0.14 mM C) and high (48.5 mM C) carbon concentration. Sampling time points were also determined by this pilot experiment.

Cell densities were increased significantly in light condition at low DOC concentration whereas MED134 grew well slightly in darkness at high DOC concentration, which were consistent with those seen in before. This shows that further transcriptomic analysis with different carbon concentrations are needed to investigate the PR-containing marine flavobacterial strains. Twelve samples were collected at TO, T1 and T2 of low DOC condition and T0, T1 and T2 of high DOC condition under both light and darkness, respectively (Fig. 21).
Fig. 21 Determining optimal sampling time points for transcriptomic experiments, based on growth curves of *Dokdonia* sp. MED134. Growth curves of *Dokdonia* sp. MED134 incubated in the light (○) or in the dark (●). MED134 was grown in ASW enriched to 0.14 mM C (a), and in ASW enriched to 48.5 mM C. Error bars indicate standard deviation for triplicate cultures. Red arrows represent the sampling time points for each condition.
Transcriptomic analyses

Illumina sequencing of *Dokdonia* sp. MED134 transcriptomes (samples T0, T1 triplicates in light, T1 triplicates in dark at low carbon concentrations) yielded 3.16E4 to 6.72E5 reads per sample (Table 5). Poor quality reads were removed by demultiplexing, dereplication, removal of short reads and reads with low fastq quality scores. Quality control was performed using pipelines for CLC Genomics workbench. Table 5 reflects that the number of reads that survive quality-control step in read processing via our scripts across samples.

Transcriptomic analyses revealed that several genes showed significant upregulation in the presence of light at T1 and T2 low carbon condition (Table 6). In particular, two predicted Fe assimilation proteins (MED134_06839, MED134_06834, MED134_22457) were significantly upregulated in the light at T1 low carbon condition (Table 6). In T2 low carbon cultures, genes of a proteorhodopsin (MED134_07119), retinal biosynthetic enzymes (MED134_13081), putative adhesion protein (MED134_03969) are exhibited significant upregulation in the light (Table 7). Further, bacterial cryptochrome gene (MED134_10201) also exhibited significant upregulation in the light at T2 low carbon cultures. These results suggested the importance of the proteorhodopsin for the light-stimulated growth in the light.

Also, MED134 has a gene associated with light sensing contains the BLUF (blue light sensing using FAD) domain, which specifically responds to blue light (Gomelsky and Klug, 2002). The BLUF domain is also found in the genome of Polaribacter sp. MED152 (Gonzalez et al., 2008). In addition to light sensors, ABC transporter (MED134_03404) and molecular chaperone DnaK (MED134_02409), which might have important roles for response regulation, were significantly upregulated in the light.
Transcriptomic survey for High carbon conditions

In transcriptomic experiments of high carbon concentration condition, upregulated genes were similar to the results with low carbon condition at T2 in the light T1 (Table 8). At T1 in high carbon condition, genes of a proteorhodopsin (MED134_07119), ABC transporter (MED134_03404), bacterial cryptochrome gene (MED134_10201), retinal biosynthetic enzymes (MED134_13081), putative adhesion protein (MED134_03969) are exhibited significant upregulation in the light (Table 8). However, the pattern of upregulated genes in T2 high carbon condition was changed compared to the results of low carbon condition. The lesser upregulation of a proteorhodopsin (MED134_07119) was observed at T2 higher carbon concentration. Also, β-oxidation pathways involved in ATP/energy production are up-regulated in the high carbon concentration at T2 culture with the former being statistically significant (Table 9). In particular, genes encoding electron transfer flavoprotein alpha-subunit (MED134_12996), electron transfer flavoprotein beta-subunit (MED134_00130) and Enoyl-CoA hydratase (MED134_06229) exhibited significant upregulation in the dark.

Fatty acid β-oxidation is a multi step process by which fatty acids are broken down by various tissues to produce energy. The long chain acyl-CoA enters the fatty acid β-oxidation pathway, which results in the production of one acetyl-CoA from each cycle of fatty acid β-oxidation. This acetyl-CoA then enters the mitochondrial tricarboxylic acid (TCA) cycle. The NADH and FADH₂ produced by both fatty acid β-oxidation and the TCA cycle are used by the electron transport chain to produce ATP. The proton gradient built up in electron transport chain then allows for ATP synthesis. These results suggested the potential importance of the β-oxidation for growth of PR-containing MED134 in the high carbon concentration condition.
Table 5. Number of reads remaining after quality control step in the pipeline for quality control.

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<td>39134</td>
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<td>15280</td>
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<td>6</td>
<td>3</td>
<td>6</td>
<td>7</td>
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<td>24187</td>
<td>24187</td>
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<td>36447</td>
<td>24691</td>
<td>10279</td>
<td>363742</td>
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<th>log2FoldChange</th>
<th>pval</th>
<th>pvalFDRadj</th>
<th>Genbank annotation</th>
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<td></td>
<td></td>
<td></td>
<td></td>
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<td>MED134_06839</td>
<td>107 27</td>
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<td>0.017</td>
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<td>MED134_06834</td>
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<td>1.95E-09</td>
<td>5.55E-06</td>
<td>TonB-dependent outer membrane receptor</td>
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<td>MED134_22457</td>
<td>11827 3598</td>
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<tr>
<td>MED134_01225</td>
<td>109 360</td>
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<td>1.7</td>
<td>5.00E-06</td>
<td>0.005</td>
<td>Secretion protein HlyD</td>
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</table>

Red character indicates significant upregulation in the light. Black character indicates significant upregulation in the darkness.
Table 7. Read number and significant upregulated genes in the light in low carbon condition at T2.

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<th>Locus tag</th>
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<td>MED134_07119</td>
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<td>0.1</td>
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<td>3.49E-05</td>
<td>Bacteriorhodopsin</td>
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<tr>
<td>MED134_07089</td>
<td>25/2</td>
<td>0.1</td>
<td>-3.7</td>
<td>4.94E-09</td>
<td>2.80E-06</td>
<td>Putative uncharacterized protein</td>
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<td>MED134_10201</td>
<td>41/4</td>
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<td>-3.4</td>
<td>1.29E-10</td>
<td>1.82E-07</td>
<td>Probable bacterial cryptochrome</td>
</tr>
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<td></td>
<td></td>
<td></td>
<td>Transcriptional regulator, MerR family protein</td>
</tr>
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<td>69/11</td>
<td>0.2</td>
<td>-2.7</td>
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<td>2.80E-06</td>
<td>Putative uncharacterized protein</td>
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<tr>
<td>MED134_14276</td>
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<td>-2.6</td>
<td>1.43E-05</td>
<td>0.005</td>
<td>Putative uncharacterized protein</td>
</tr>
<tr>
<td>MED134_04999</td>
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<td>0.2</td>
<td>-2.4</td>
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<td>MED134_01275</td>
<td>456/102</td>
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<tr>
<td>MED134_13076</td>
<td>33/9</td>
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<td>Phytoene dehydrogenase</td>
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<tr>
<td>MED134_05424</td>
<td>66/27</td>
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<td>-1.3</td>
<td>0.001</td>
<td>0.140</td>
<td>ThiJ/PfpI family protein</td>
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<tr>
<td>MED134_03409</td>
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<td>-1.3</td>
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<td>0.004</td>
<td>(dnaK) Molecular chaperone DnaK</td>
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Table 8. Read number and significant upregulated genes in the light in high carbon condition at T1.

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<td>MED134_07119</td>
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<td>1.11E-04</td>
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Red character indicates significant upregulation in the light. Black character indicates significant upregulation in the darkness.
Fig. 22. Plot of normalized mean versus log$_2$ fold change for low carbon T1 light sample versus low carbon T1 dark sample (a) and for low carbon T2 light sample versus low carbon T2 dark sample (b).
Fig. 23. Plot of normalized mean versus log₂ fold change for high carbon T1 light sample versus high carbon T1 dark sample (a) and for high carbon T2 light sample versus high carbon T2 dark sample (b).
Fig. 24. Plot of normalized mean versus log₂ fold change for T1 light low carbon versus T1 light high carbon (a) and for low carbon T1 dark sample versus high carbon T1 dark sample (b). Red dots indicate differentially expressed genes.
Fig. 25. Plot of normalized mean versus log₂ fold change for T2 light low carbon versus T2 light high carbon (a) and for low carbon T2 dark sample versus high carbon T2 dark sample (b). Red dots indicate differentially expressed genes.
Conclusions

The light-enhanced growth rates and cell yields in marine flavobacterial strains demonstrate the proteorhodopsin-associated light-dependent growth effect at various carbon concentrations for the first time in a native cell in a proteorhodopsin photosystem (PRPS). In this study, we found that increasing concentrations of DOC in the culture media decreased the effect of the PR-stimulated growth. Also, interestingly, we found that cell densities of dark-incubated cultures with PR-containing flavobacterial strains were higher than light-incubated cultures at high carbon concentration. Together, the results show strong evidence that PR is beneficial in carbon and respiration limited conditions. The results also show that this effect is seen in a variety of different PR-containing flavobacteria, suggesting that this phenomena is widespread and not just limited to a few strains.

To verify that proteorhodopsins are responsible for the observed effect, we conducted a growth experiment in which we inhibited the synthesis of proteorhodopsin with an inhibitor of MPTA. Cultures incubated in ASW with MPTA in the light showed significantly lower yields than those in ASW without MPTA. In culture experiments in the dark with or without MPTA, cell density of both strains similar to the results with MPTA-treated condition in the light. This result demonstrated the critical role of PR in light-enhanced growth.

Additionally, to better characterize the photophysiology of PR-containing marine flavobacteria, metatranscriptomics studies were performed to see the gene expression patterns in strain MED134 in this study. Metatranscriptomic analysis will lead to a better understanding of functional capacity under different carbon conditions different staged of growth. We still need to work more on metatranscriptomics analyses, but this study has addressed several questions including: “What are the active genes at
different DOC conditions in light?”, “When are they active?”, and “How do flavobacteria to different DOC conditions in the dark compared to light” which provides new insight into the photophysiological characteristics of PR-containing marine flavobacteria. In the future, it will be useful to examine the gene expression patterns associated with the higher growth rates and cell yields in flavobacterium strain MED134 in both low-carbon and high-carbon media in even greater detail.


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