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ORIGINAL ARTICLE

Proteorhodopsin light-enhanced growth linked to vitamin-B_1 acquisition in marine Flavobacteria

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Proteorhodopsins (PR) are light-driven proton pumps widely distributed in bacterioplankton. Although they have been thoroughly studied for more than a decade, it is still unclear how the proton motive force (*pmf*) generated by PR is used in most organisms. Notably, very few PR-containing bacteria show growth enhancement in the light. It has been suggested that the presence of specific functions within a genome may define the different PR-driven light responses. Thus, comparing closely related organisms that respond differently to light is an ideal setup to identify the mechanisms involved in PR light-enhanced growth. Here, we analyzed the transcriptomes of three PR-harboring Flavobacteria strains of the genus *Dokdonia*: *Dokdonia donghaensis* DSW-1^T, *Dokdonia* MED134 and *Dokdonia* PRO95, grown in identical seawater medium in light and darkness. Although only DSW-1^T and MED134 showed light-enhanced growth, all strains expressed their PR genes at least 10 times more in the light compared with dark. According to their genomes, DSW-1^T and MED134 are vitamin-B_1 auxotrophs, and their vitamin-B_1 TonB-dependent transporters (TBDT), accounted for 10–18% of all *pmf*-dependent transcripts. In contrast, the expression of vitamin-B_1 TBDT was 10 times lower in the prototroph PRO95, whereas its vitamin-B_1 synthesis genes were among the highest expressed. Our data suggest that light-enhanced growth in DSW-1^T and MED134 derives from the use of PR-generated *pmf* to power the uptake of vitamin-B_1, essential for central carbon metabolism, including the TCA cycle. Other *pmf*-generating mechanisms available in darkness are probably insufficient to power transport of enough vitamin-B_1 to support maximum growth of these organisms.

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Introduction

The ocean's surface is the largest sun-lit environment on Earth. Most of the global biological activity such as photosynthesis and respiration takes place in the photic zone of the ocean, having a major impact on the overall carbon cycle. It has been recognized for many decades that marine organisms capture energy from sunlight via well-known chlorophyll-based photosynthesis and to a much lesser extent via bacteriochlorophyll-*a* (Shiba *et al.*, 1979). However, in recent years, it has been found that a third pigment type, proteorhodopsins (PR), may also be important. PRs are light-driven proton pumps that can be used for energy (ATP) generation (Béjà *et al.*, 2000; Martinez *et al.*, 2007). They are widely distributed in all environments where sunlight can reach, particularly in aquatic settings. The photic zone of the ocean contains the largest pool of PR, with an average of 75% of all bacterioplankton cells carrying these genes (Sabehi *et al.*, 2005; Rusch *et al.*, 2007). Since the discovery of PRs in the year 2000, a considerable amount of effort has been dedicated to discern their abundance, diversity and biochemical functioning (Béjà *et al.*, 2000; 2001; de la Torre *et al.*, 2003; Sabehi *et al.*, 2003; 2005; Martinez *et al.*, 2007). However, although they have been perceived as relatively simple photosystems, the consequences of their activity on cell physiology at both the individual and community level are still poorly understood.

Studies of PR gene expression in microbial communities *in situ* as well as in light-dark incubations have so far produced varied results, possibly indicative of multiple distinct utilization strategies at work (Fuhrman *et al.*, 2008). Physiological responses at the organism level such as light-enhanced growth,

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increased survival as well as substrate uptake, have been observed in phylogenetically diverse groups of PR-containing bacteria in culture. One example is *Candidatus* Pelagibacter ubique (*Ca.* P. ubique) HTCC1062, for which a greater survival capacity and ATP-dependent substrate uptake in the light have been reported (Steindler *et al.,* 2011). Also, two closely related marine *Vibrio* strains (AND4 and BAA-1116) have shown better survival to starvation or respiratory stress in the light (Gómez-Consarnau *et al.*, 2010; Wang *et al.*, 2012). However, a light-stimulated growth response could not be detected in other cultured bacteria like the gammaproteobacterium HTCC2207 in the SAR92 clade (Stingl *et al.*, 2007). To gain a complete understanding of the ecological impact of PR photoheterotrophy, the growth responses of PR-producing bacteria need to be resolved.

Flavobacteriia is the bacterioplankton class on which most physiological studies of PR have been conducted and most biological responses to light have been detected. For example, *Polaribacter* sp. MED152 has shown greater bicarbonate uptake in the light (González *et al.*, 2008) and the growth of *Psychroflexus torquis* ATCC 700755^T was improved under salinity stress upon illumination (Feng *et al.*, 2013). *Dokdonia* MED134 was the first bacterium shown to divide faster in the light compared to the dark under low organic carbon concentrations (Gómez-Consarnau *et al.*, 2007). Subsequent studies on the same strain have shown that growth stimulation by PR in the light is linked to changes in central metabolism (Palovaara *et al.*, 2014) and/or transcriptional changes associated with Na^+ pumping (Kimura *et al.*, 2011). Recently, another flavobacterium, *Nonlabens marinus* S1-08, containing three different rhodopsin types (H^+, Na^+ and Cl^- pumps) has been described. Similarly to MED134, this bacterium grows better in the light compared with dark in a low organic matter medium (Yoshizawa *et al.*, 2014). In contrast to these two *Dokdonia* (S1-08 and MED134), another strain of the same genus, PRO95, has not yet shown enhanced growth or increased PR gene expression in the light compared to the dark (Riedel *et al.*, 2010). PRO95 also has more than one rhodopsin type and, according to sequence similarities, one functions to pump H^+ and the other one to pump Na^+ ions (Yoshizawa *et al.*, 2014, Bertsova *et al.*, 2015). Together, the studies of PR in cultured bacteria reflect a diversity of physiological responses that still cannot be predicted solely based on whole-genome sequence information, and therefore a combination of different research approaches are required to elucidate the specific mechanisms of PR photoheterotrophy.

Proton gradients and ATP generated by PR can potentially affect the global ocean carbon budgets in two ways: (i) they could decrease carbon respiration rates, if less dissolved organic carbon is respired and preferably used for anabolic processes, as shown for aerobic anoxygenic phototrophs (Holert *et al.*, 2011; Tomasch *et al.*, 2011), or (ii) they could enhance the uptake of solutes that could subsequently be respired via PR-powered ion gradients. Both of these mechanisms have been shown for *Ca.* P. ubique HTCC1062, a member of the abundant SAR11 clade. Respiration rates of SAR11 bacteria decreased under starvation in the light (Steindler *et al.*, 2011). However, when these bacteria were provided with taurine, ATP-dependent transport of the substrate was observed, suggesting that this resource became available for respiration under light conditions. Indeed, the intracellular transport of most substrates depends on either ATP (ABC transporters) or H^+ gradient pumps (TonB-dependent transporter (TBDT), H^+ antiporters) (Wong and Buckley, 1989; Bradbeer, 1993), suggesting that PR could have an important role in the transport efficiency of dissolved nutrients and growth factors such as B-vitamins.

The objectives of the study were to evaluate: (i) whether the observed specific PR-driven responses to light are due to the presence or absence of specific functions within a particular bacterial genome, as recently hypothesized (González *et al.*, 2011; Riedel *et al.*, 2013) and (ii) whether the energy generated by PR could be used for the uptake of exogenic organic molecules (for example, B-vitamins). To accomplish this, we compared three bacteria in the genus *Dokdonia* (MED134 from the Mediterranean Sea, PRO95 from the North Sea, and DSW-1^T from the East Sea of Korea), which contain PR genes and respond differently to light exposure. Nucleotide identity among 16S rRNA genes of the three marine *Dokdonia* was 99.5%. However, PRO95 belongs to a separate species, as the estimated DNA-DNA hybridization value was 25% with respect to DSW-1^T and 63% with MED134, lower than the discriminatory value of 70% for strains within the same species (Auch *et al.*, 2010). Our genome analysis revealed that although the three *Dokdonia* strains have a very similar gene content (Supplementary Table S1), only PRO95 has the *de novo* pathway to synthesize vitamin-B_1, an organic cofactor required for the primary metabolism of all living organisms (for example, TCA cycle of respiration) (Voet and Voet, 2004). Transcriptome comparisons showed that the expression of PR genes was higher in the light compared with dark in the three *Dokdonia*. However, the light-enhanced growth was only observed in DSW-1^T and MED134. Furthermore, the high expression of vitamin-B_1 TBDT in the two auxotrophic *Dokdonia* (DSW-1^T and MED134) and the potential powering of those transporters by PR functioning as a H^+ pump could explain the observed growth response to light. This inferred mechanism for light-enhanced growth has not previously been described.

Materials and methods

Genome sequencing of DSW-1^T

DSW-1^T was obtained from the culture collection DMSZ (DSM-17200). It was grown on ZoBell agar plates until colonies were identified and then

inoculated in 40 ml ZoBell liquid medium for 72 h at room temperature. Genomic DNA was extracted from 15 ml pellets using DNeasy kit (Qiagen, Hilden, Germany) and then sheared using Bioruptor NGS (Diagenode, Denville, NJ, USA). The genomic library was prepared using a liquid handling Apollo 324 robot (InteGenX, Pleasanton, CA, USA) and the KAPA HTP Library Preparation Kit for Illumina Platforms (Kapa Biosystems, Wilmington, MA, USA). Ligation products were purified and subjected to a final PCR amplification (10 cycles). The amplified library was assessed by Bioanalyzer 2100 (Agilent, Santa Clara, CA, USA) and real-time quantitative PCR (Kapa Biosystems) and subsequently sequenced on Illumina MiSeq for paired read of 250 bases (Illumina, San Diego, CA, USA). Sequence data were quality-trimmed to Phred 25 and read pairs merged using a Conveyor workflow (Linke *et al.*, 2011); non-overlapping read pairs were discarded. Owing to high coverage (~470 ×), random subsamples of the merged read pairs were individually assembled using the Roche GS *de Novo* Assembler and SPAdes version 3.0 software packages (Bankevich *et al.*, 2012). The assemblies were merged by re-sampling longer reads from the obtained contigs and performing a final assembly using the Roche GS *de Novo* Assembler (Roche Applied Science, Branford, CT, USA). The genome was finally assembled into a total of 20 contigs.

This Whole Genome Shotgun project has been deposited at DDBJ/EMBL/GenBank under the accession JSAQ00000000. The version described in this paper is version JSAQ01000000.

Growth conditions

Strains DSW-1^T, MED134 and PRO95 were grown in the same conditions in artificial seawater using sea salts (Sigma-Aldrich, St Louis, MO, USA) at 35 practical salinity units amended with 0.39 mM DOC from ZoBell medium, 2.1 µM NH_4Cl and 0.3 µM Na_2HPO_3 as in Gómez-Consarnau *et al.* (2007). No additional vitamins were added to the medium. The 1.9-l cultures were kept in 2-l polycarbonate bottles in a temperature-controlled room at 18 °C and exposed to light or darkness in duplicates. Light treatments were incubated under continuous light (130 µmol photons m^{-2} s^{-1}) using an artificial white light source. Dark bottles were covered with aluminum foil and a black plastic layer. Bacterial growth was monitored using cell counts every 24 h. Two milliliter samples were fixed with 10% formalin (4% formaldehyde), stained with acridine orange (Hobbie *et al.*, 1977), filtered through pre-blackened filters and counted with epifluorescence microscopy.

Vitamin-B_1 quantification in the culture medium

The concentration of vitamin-B_1 in the axenic growth medium was quantified using HPLC-MS (Sañudo-Wilhelmy *et al.*, 2012) after autoclaving (t_0) and after 5 days incubation in the light (t_5; sampling time for transcriptomic samples) in duplicate bottles, using triple injections per sample.

RNA sampling, processing and sequencing

After 5 days of growth, bacterial biomass was collected by filtering 1 l of culture onto 0.22-µm pore size, 47-mm diameter Durapore (Millipore, Billerica, MA, USA) filters using vacuum filtration. Immediately after filtration, filters were incubated for 1 min with 2 ml RNA-protect and stored dry at − 80 °C. RNA was extracted using the RNAeasy Mini kit (Qiagen), with two DNase digestions as in Riedel *et al.* (2013). The standard protocol for library construction began with 1 µg of total RNA. RNA was treated with the Epicentre Ribo-Zero ribodepletion kit for Gram-negative bacteria (Epicenter, Madison, WI, USA). Following ribodepletion, the entire sample was introduced into the Illumina TruSeq mRNA v2 protocol at the elute-prime-fragmentation step for cDNA generation using 12 PCR cycles. Libraries were visualized by Bioanalyzer (Agilent) and quantified for pooling and sequencing using Kapa Biosystems qPCR quantification kit.

For sequencing, libraries were diluted to 16 pM and then applied to a V3 flowcell using the Illumina cBot clonal amplification device. Libraries were sequenced on the Hi-Seq 2000 using HSCS v 1.5.15.1 obtaining more than 30 million reads per sample. The sequences were then quality-trimmed using FASTX-Toolkit (http://hannonlab.cshl.edu/fastx_toolkit), which discarded 2–3% of the reads. From these, 99% of the trimmed sequences mapped to the genome of the three strains using the program segemehl (Hoffmann *et al.*, 2009). Read abundance was extracted from resulting SAM mapping files with SAMtools version 0.1.19 (Li *et al.*, 2009) and coverage per base was calculated from BAM files. The read mapping accuracy and the levels of expression of the key genes used in this study (for example, PR, TBDT, vitamin-B_1 synthesis) as well as for other highly expressed genes (for example, ribosomal proteins) were visually inspected with the programs Rockhopper (Tjaden, 2015) and Integrative Genomics Viewer (Thorvaldsdottir *et al.*, 2013). In addition, plots based on aligned sequence reads per base were made with Python scripts for comparison. Read counts per gene were obtained from SAM alignment files with HTSeq software. To identify the differentially expressed genes, we used the DESeq R/Bioconductor package (Anders and Huber, 2010). The DESeq package normalizes for sequencing depth and it is based on a negative binomial distribution model of read counts (Robinson and Smyth, 2007; Anders and Huber, 2010).

The genomes of DSW-1^T and PRO95 were submitted to RAST for automatic annotation (Aziz *et al.*, 2008). The annotation of genes in DSW-1^T and PRO95 was then matched to the manual annotation of MED134 (González *et al.*, 2011) when the putative gene product was shared. That is, when reciprocal

Best Hits based on BLASTP against the peptides of MED134 had a minimum percent identity = 35, maximum evalue = 1 and a minimum percent coverage = 50. The genes of DWS-1^T and PRO95 that had no homologs in MED134 were annotated *de novo* as described in the study by González *et al.* (2011). The assignment of the individual peptides to functional categories as KEGG Orthology terms (KO; Kyoto Encyclopedia of Genes and Genomes (KEGG); Kanehisa *et al.*, 2007) was based on homology search by BLASTP against the KEGG database of peptides with a minimum percent identity of 35, minimum coverage of 60 and minimum bitscore of 50. Transcriptome sequences were uploaded to the European Nucleotide Archive under the Study accession PRJEB10816.

Results and Discussion

Genomic comparison among the three Dokdonia strains

We investigated the photo-physiology and gene expression of three closely related strains in the genus *Dokdonia*. Nucleotide identity between 16S rRNA genes of the three strains was 99.5%. On the basis of genome-to-genome distance calculator GGDC analysis (Auch *et al.*, 2010), PRO95 belong to separate species as the estimated DNA-DNA hybridization value (only 25% identity with DSW-1^T and 63% with MED134). This result was confirmed with an average nucleotide identity analysis (95.93%), which puts them on the lower limit range for strains within the same species (95–96%) (Supplementary Table S1) (Goris *et al.*, 2007). We refer to the strain designation hereafter because only the nomenclature of DSW-1^T has been formally described. The genomes of MED134 and PRO95 had been sequenced and described earlier (González *et al.*, 2011; Riedel *et al.*, 2013). In this study, we have sequenced the genome of the type strain, *D. donghaensis* DSW-1^T (Yoon *et al.*, 2005), allowing a broader comparison within this genus. The new genome is 3 223 976 nucleotides in length, similar to MED134 (3 302 550 nucleotides) and PRO95 (3 303 993 nucleotides). On the basis of the gene content of the three strains, the *Dokdonia* core genome contained 2389 protein-coding genes (77–82% of the genomes) (Figure 1, Supplementary Table S1). Although the overall genomic features among the three strains were very similar (Table 1), DSW-1^T and MED134 shared a larger number of protein-coding genes with each other (87.5% of DSW-1^T genome and 89.4% of MED134 genome) than with PRO95 (79%) (Figure 1, Supplementary Table S1), which is consistent with their phylogenetic proximity.

The three *Dokdonia* genomes contain rhodopsin genes, which makes them photoheterotrophs. While MED134 and DSW-1^T have one copy of the rhodopsin gene, PRO95 contains two different rhodopsin genes: a H^+ rhodopsin (PR) and a Na^+ rhodopsin (NaR), based on sequence comparison (Yoshizawa *et al.*, 2014; Bertsova *et al.*, 2015). This

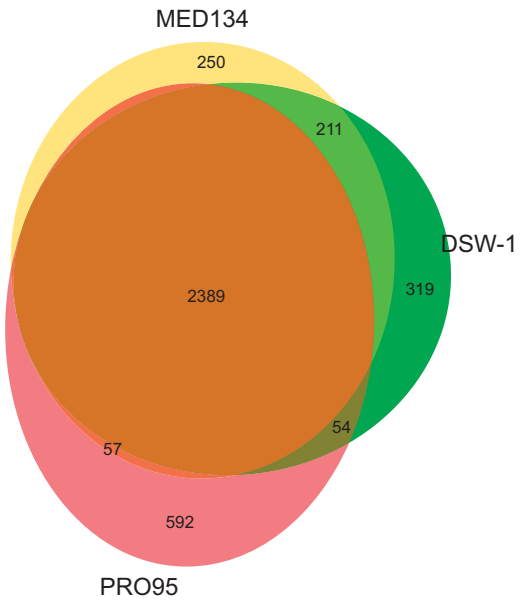

Figure 1 Venn diagram showing the genes shared by the *Dokdonia* strains DSW-1^T, MED134 and PRO95. Numbers indicate the genes that were unique for each organism as well as the ones shared by two or the three strains. The diagrams were made using the program eulerAPE (http://www.eulerdiagrams.org/eulerAPE/).

Table 1 General genomic characteristics of *Dokdonia* DSW-1^T, MED134 and PRO95

General genomic features	*DSW-1^T*	*MED134*	*PRO95*
Isolation	East sea, Korea, surface water	Mediterranean Sea, Spain, surface	North Sea, Germany, surface
Reference	Yoon *et al.* (2005)	Gómez-Consarnau *et al.* (2007)	Riedel *et al.* (2010)
No. of bp	3 223 976	3 302 550	3 303 993
GC content (%)	38.1	38.2	37.4
Predicted protein-coding genes	2973	2907	3092
tRNAs	40	43	36
Transporters	136	139	138
TBDT	26	27	31
Peptidases	98	101	100
Glycosyl hydrolases	17	15	13
Adhesion proteins	33	30	32

Abbreviation: bp, base pair.

would *a priori* suggest that PRO95 has a greater phototrophic capacity compared with the other two strains. However, previous light incubation experiments using this organism did not detect a light-enhanced growth response (Riedel *et al.*, 2010; 2013). The PR peptide of DSW-1^T differs from that of MED134 in only one amino acid while the identity with that of PRO95 is 74%. These differences in PR sequence might indicate that PRO95's PR is the result of lateral gene transfer, as the presence of genomic islands has frequently been found in Flavobacteria (González *et al.*, 2011; Fernández-Gómez *et al.*, 2013).

Another important feature that differentiates these organisms is that the PRO95 genome contains the *de novo* pathway to synthesize vitamin-B_1 (*thiE, thiF, thiD, thiH, thiG, thiC, thiS, thiL*) (Figure 2) (Jurgenson *et al.*, 2009), whereas MED134 and DSW-1^T do not. Figure 3 illustrates the presence/absence of the enzymes required at each step of the synthesis (or uptake) of vitamin-B_1 in the three *Dokdonia* strains. DSW-1^T and MED134 lack the entire set of genes for the synthesis of vitamin-B_1 except for *dxs*, which is also used in other metabolic pathways (Lois *et al.*, 1998). The three strains have a thiamin pyrophosphate riboswitch next to a TBDT receptor (MED134_10161 and homologs in the other two strains). This may indicate that the organisms could sense the intracellular presence of B_1-vitamin and repress the gene expression for the specific TBDT when the intracellular concentration of the vitamins is sufficiently high (Winkler and Breaker, 2005). PRO95 has an additional thiamin pyrophosphate riboswitch next to *thiC*, the first gene in an eight-gene cluster encoding thiamin synthesis genes (Figure 2), indicating that this organism could also accurately control the synthesis of this vitamin. Vitamin-B_1 is composed of two moieties: the HMP (4-amino-5-hydroxymethyl-2-methylpyrimidine) and THZ (4-methyl-5-(2-hydroxyethyl)-thiazole). In addition to the vitamin-B_1 uptake transporters, we identified the ABC transporter genes for the HMP moiety in MED134 (MED134_00580) and DSW-1^T but not for the transport of THZ (Figure 3).

Growth responses of Dokdonia in light and dark

To compare their growth response to light exposure, the three *Dokdonia* were grown in an artificial seawater medium. All strains grew in the dark and in the light, but with substantial differences in growth rates (Table 2, Supplementary Figure S1). Light-enhanced growth was higher in DSW-1^T (3.3-fold higher cell yields in the light compared to the dark) than in MED134 (2.1-fold higher yields

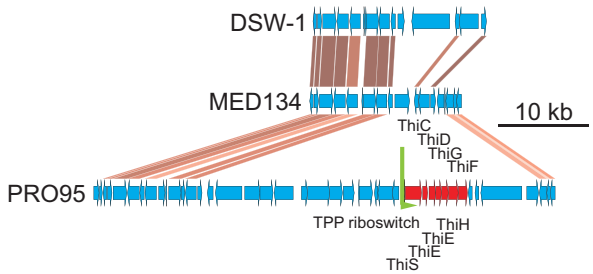

Figure 2 Genomic region where the vitamin-B_1 synthesis operon of PRO95 is located. Green arrow shows the thiamin pyrophosphate riboswitch and in red are the vitamin-B_1-synthesis genes. The genomic area around this operon is shared by the three *Dokdonia* strains although the synthesis genes are only present in PRO95.

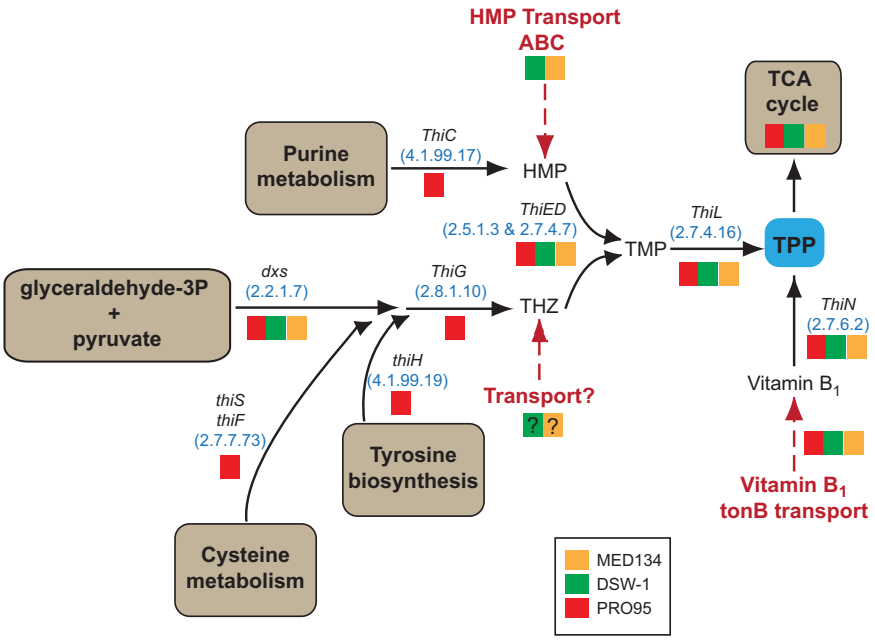

Figure 3 Diagram of the *de novo* synthesis pathway of vitamin-B_1. Green, orange and red boxes indicate the presence of the specific genes in DSW-1^T, MED134 and PRO95, respectively. The red dashed lines indicate transport functions. Question marks indicate that those genes have not been found but would be expected to be present in the genomes.

Table 2 Growth characteristics of DSW-1^T, MED134 and PRO95 in artificial seawater (C: 0.39 mM, N: 2.1 μM, P: 0.2 μM)

	DSW-1 light	*DSW-1 dark*	*MED134 light*	*MED134 dark*	*PRO95 light*	*PRO95 dark*
Bacterial yields after 120 h growth ($\times 10^6$)	0.90 (±0.14)	0.26 (±0.24)	5.76 (±0.68)	2.69 (±0.42)	7.76 (±0.51)	5.21 (±2.66)
Doubling time (days)	1.54	9.11	0.61	0.9	0.87	1.01

Doubling times were calculated at maximum growth rates. Transcriptome samples were taken after 120 h growth in seawater.

under illumination), while PRO95 showed no significant differences in growth between light and dark treatments. However, cell yields were significantly different; PRO95 reached the highest biomass of all (7.76×10^6 cells per ml), followed by the light treatments of MED134 (5.76×10^6 cells per ml) and DSW-1^T (0.9×10^6 cells per ml). These different growth responses observed in the *Dokdonia* strains were evident despite the high variability observed in the growth measurements of DSW-1^T and PRO95 (Table 2, Supplementary Figure S1). Light-mediated destruction of vitamin-B_1 was tested by measuring its concentration after 5 days in the light in sterile medium. We could not find evidence for a light effect on thiamin concentrations at around 100 pM.

Overall regulation of gene expression under light and dark conditions

We used a transcriptomic analysis to infer the mechanisms used by each *Dokdonia* strain to adjust its metabolism to light availability. To establish a threshold, we considered that differential gene expression was significant when the light vs dark transcript linear fold change was greater than 2 (higher in the light) or lower than 0.5 (higher in the dark) and with an adjusted *P*-value (*P*-adj) lower than 0.05. Consistent with the observed differences in cell yields (Table 2), the light-dark differential expression in these organisms was strikingly different despite their relatedness (Figures 4 and 5). DSW-1^T was the organism with the highest number of differentially expressed genes (617; 194 higher in the light and 423 higher in the dark), followed by MED134 (170; 144 in the higher light and 26 higher in the dark) and PRO95 (20; 16 higher in the light and 4 higher in the dark) (Figure 5).

Expression of rhodopsin under light-dark conditions

The expression of PR (and NaR of PRO95) was above the average gene expression for the three *Dokdonia* strains and significantly higher in the light (Figure 4). The only transcripts that were significantly higher in the light for the three strains were, in addition to the PR, a PAS domain sensor protein (MED134_10396 and homologs in DSW-1^T and PRO95) and a hypothetical protein (MED134_04254) (Supplementary Table S3A). PAS domains can respond to multiple stimuli like cellular energy levels, oxygen levels, redox potential and light (Taylor and Zhulin, 1999). Although this PAS domain sensor gene is not in the region nearby

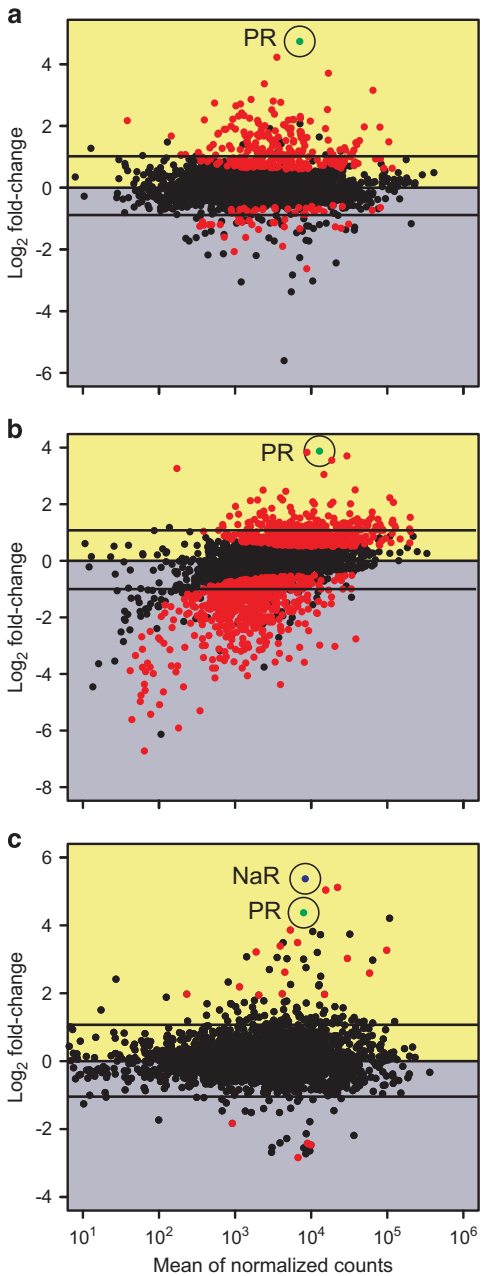

Figure 4 Normalized mean mRNA read counts of the *Dokdonia* transcriptomes. (**a**) MED134, (**b**) DSW-1^T and (**c**) PRO95. In red are the reads that are significantly higher in light or dark (*P*-adj < 0.05) regardless of the light/dark fold change value. Horizontal black lines denote the linear fold change levels of 2 and 0.5. Circled green and dark blue dots denote the PR and NaR transcript reads, respectively. Transcript sequence data were normalized by sequencing depth as described in the DESeq package (see Materials and methods).

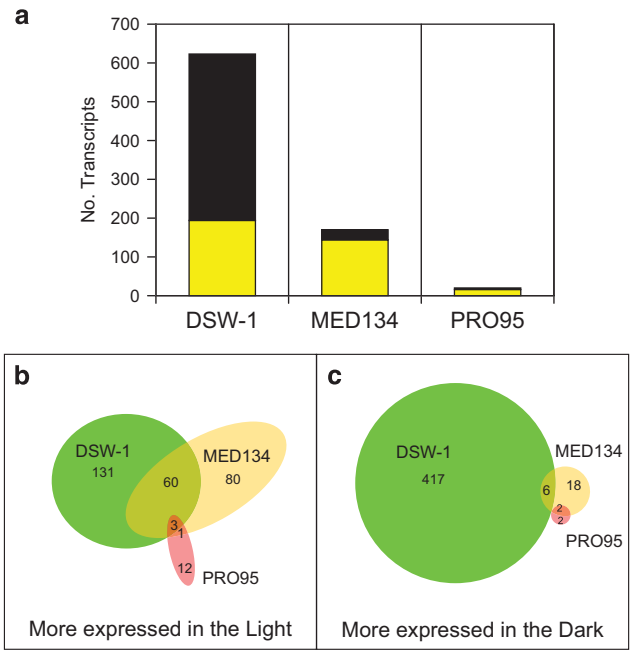

Figure 5 (**a**) Total transcripts that were significantly higher in light (in yellow) or dark (in black) in DSW-1^T, MED134 and PRO95. Significantly higher expression was considered when the linear fold change in light vs dark transcripts was larger than 2 or lower than 0.5 and the *P*-adjusted values were <0.05. Venn diagrams show the number of transcripts that were significantly higher in light (**b**) or dark (**c**) for each strain exclusively, as well as the number of transcripts shared by two or the three organisms in the two treatments. The Venn diagrams were made using the program eulerAPE (http://www.eulerdiagrams.org/eulerAPE/).

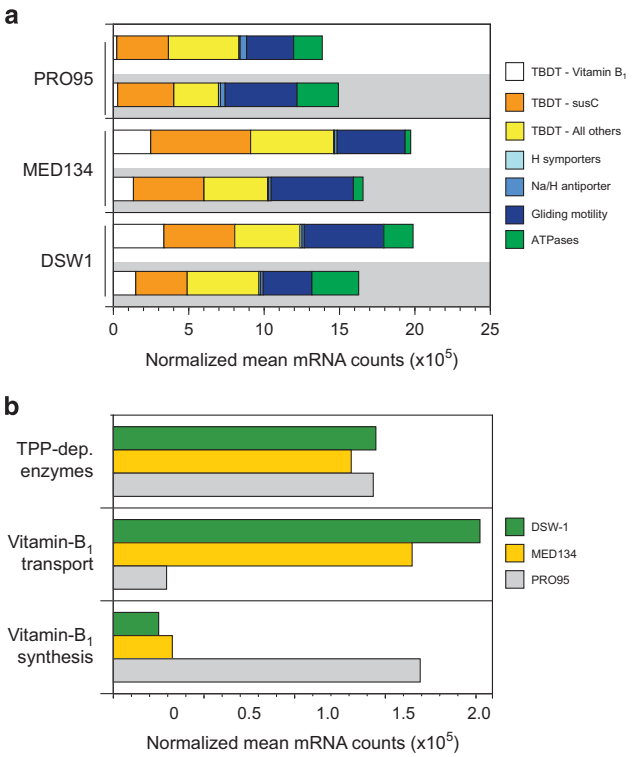

Figure 6 (**a**) Gene expression levels of *pmf*-dependent processes. Gray shading denotes the dark treatment transcriptome samples. (**b**) Average number of transcripts within light and dark treatments of vitamin-B_1 (thiamin pyrophosphate)-dependent enzymes as well as vitamin-B_1 transport and synthesis in DSW-1^T, MED134 and PRO95. mRNA sequence data were normalized by sequencing depth as described in the DESeq package (see Materials and methods).

the PR gene, its presence and high expression in the light observed in the three strains suggest that this is an important part of the light-dependent regulation. Notably, PR genes were also the transcripts with highest fold change in the light compared with the dark, being 14 in DSW-1^T and 26.6 in MED134. In PRO95, two adhesion proteins (that closest match to MED134_13296 and MED134_13296, 34× and 32×) showed the highest fold change followed by the PR (fold change of 20) (Figure 4, Supplementary Table S3). Thus, PRO95 had a higher expression of the rhodopsin genes in the light although it grew equally well in light or darkness, indicating that the differential expression of PR genes was not directly related to the light-enhanced growth and that other elements of their metabolism need to be considered.

Proton motive force (pmf)-dependent processes

Out of all the processes that require a *pmf* for their activity (that is, membrane transport and motility) (Saier, 2000; Jarrel and McBride, 2008), the highest numbers of transcript reads in the three strains were for TBDT genes followed by gliding motility (Figure 6a). The TBDT family contains integral membrane proteins that use proton gradients, like the ones produced by PR, to transport molecules of different molecular weight across the outer bacterial membrane (reviewed by Tang *et al*., 2012). Their high expression supports previous results on the lifestyle of Flavobacteria as degraders of high molecular weight compounds (Cottrell and Kirchman, 2000; Cottrell *et al*., 2005; Teeling *et al*., 2012). Most of the known TBDT have not been characterized yet, so the specific substrate they transport is currently unknown. However, analysis of operons suggests that they might be involved in the uptake of polysaccharides, peptides, heme groups and/or vitamins. In our study, we observed two *susC* TBDT of particularly high expression in MED134 and DSW-1^T (MED134_12381 and MED134_05219) (Supplementary Table S2), which are possibly involved in the uptake of high molecular weight compounds such as proteins or complex carbohydrates (González *et al*., 2011; Tang *et al*., 2012).

Vitamin-B_1 metabolism and PR phototrophy

PRO95 was the only *Dokdonia* strain among the three investigated here that contained the *de novo* pathway for vitamin-B_1 synthesis (Figure 3). We detected the expression of at least one gene in the synthesis pathway of vitamin-B_1, *thiC*, among the most highly expressed genes of PRO95 (Supplementary Table S2). Furthermore, the vitamin-B_1-TBDT (MED134_10161 and homolog in DSW-1^T) was the third most highly expressed TBDT gene in the vitamin-B_1 auxotrophs MED134 and DSW-1^T (Figure 6b, Supplementary

Table S2), suggesting that vitamin-B_1 was essential for them all. Indeed, the vitamin-B_1 TBDT transcripts were about 10 times higher in the vitamin-B_1 auxotrophs (DSW-1^T and MED134) compared with the synthesizer PRO95 (Figure 6b). Vitamin-B_1 transporter genes accounted for as much as 18% of all *pmf*-dependent processes. Our results are consistent with the transcriptome of MED134 reported by Kimura *et al.* (2011), where we found (reanalyzing their Supplementary Materials) that vitamin-B_1 TBDT were also among the most highly expressed genes.

Although the vitamin-B_1 requirements for heterotrophic bacteria have not been established, we suggest that the observed high expression of thiamin pyrophosphate riboswitch-regulated genes could be related to low vitamin-B_1 intracellular levels and availability. This is because the riboswitches should repress gene expression when the vitamin concentration meets their intracellular requirements (Winkler and Breaker, 2005). We did not observe any significant changes in vitamin-B_1 concentrations in the growth medium under our experimental conditions ($t_0 = 99.3 \pm 17.5$ pM vs $t_5 = 81.4 \pm 11.2$ pM; Mann–Whitney test, $P = 0.33$). This suggests that the gene expression patterns observed in the *Dokdonia* strains were not due to a light-mediated destruction of the vitamin. The light stability of vitamin-B_1 is consistent with the use of an artificial white light source in our experiments, as vitamin-B_1 light degradation has been observed only under x-rays, gamma rays and UV irradiation (Gubler, 1984). Vitamin-B_1 concentrations in the growth medium were within the threshold levels considered limiting for some phytoplankton species (Carlucci and Silbernagel, 1966; Tang *et al.*, 2010; Paerl *et al.,* 2015), suggesting that bacteria could have been vitamin-B_1-deficient during our experiments.

Moreover, when our transcriptomics samples were grouped by growth phase (regardless of light or dark treatment and isolate) only six gene transcripts were higher than the average in the exponential phase (>10%) but lower in the stationary phase (<10%) (Supplementary Table S5). One of those genes encoded a ThiJ/PfpI family protein (MED134_04464), involved in vitamin-B_1 metabolism, most likely in the transformation of thiamin into thiamin's active form thiamin pyrophosphate (*thiN*), suggesting that vitamin-B_1 is highly required during active growth. Of all the vitamin-B_1-dependent genes of *Dokdonia*, the most highly expressed were involved in the TCA cycle (2-oxoglutarate dehydrogenase E1 component, *sucA*, and pyruvate dehydrogenase E2 component; MED134_07711 and MED134_12071), one of the main metabolic steps in the oxidation of carbohydrates, fats and proteins into CO_2, fueling the production of ATP (Supplementary Table S4).

A recent study also used *Dokdonia* MED134 grown in yeast extract and peptone medium (YEP), and discussed the reactions of the TCA cycle that may be involved in the light-enhanced growth (Palovaara *et al.*, 2014). They quantified the expression of a small set of genes (11), including only one vitamin- B_1-dependent enzyme, 2-oxoglutarate dehydrogenase. In both, their and our study, this enzyme was not differentially expressed in the light or in the dark when growing in YEP medium. As this enzyme is central for cellular growth, and vitamin-B_1 is its coenzyme, we hypothesize that vitamin-B_1 auxotrophic *Dokdonia* did not grow well in the dark owing to vitamin deprivation.

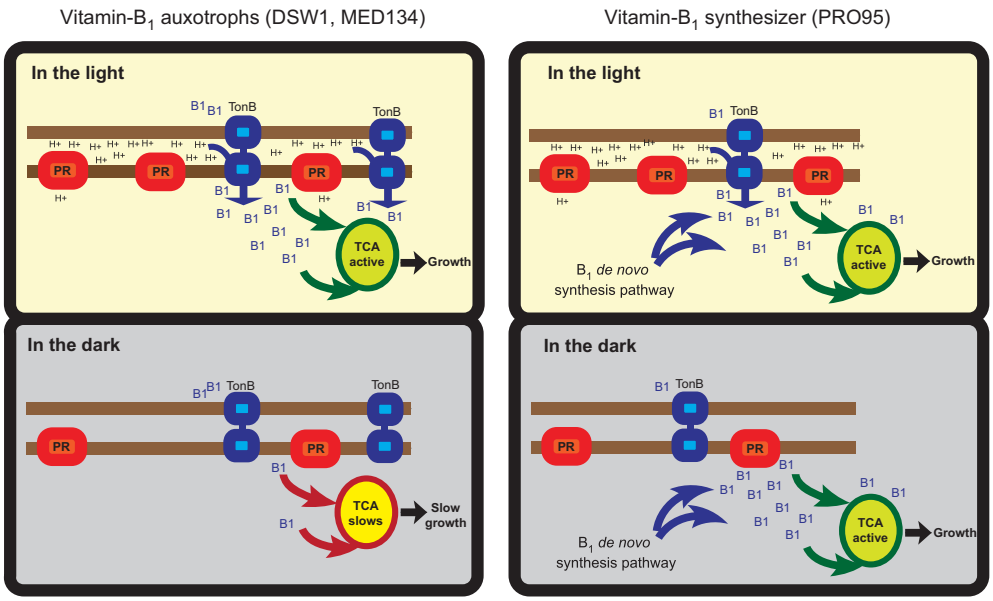

Figure 7 Hypothetical scenarios of vitamin-B_1 uptake and utilization in the context of PR phototrophy.

Figure 7 illustrates the proposed scenarios where the vitamin, being either transported into the cell (DSW-1^T and MED134) or synthesized *de novo* (PRO95), can have an impact on growth of *Dokdonia*. For the vitamin-B_1 auxotrophs DSW-1^T and MED134, vitamin-B_1 transport could be substantially enhanced by the *pmf* generated by PR in the light. In the dark, even though PR and vitamin-B_1 -TBDT might be present and abundant, the lack of PR *pmf* would lead to insufficient intracellular vitamin-B_1 concentrations, and growth rates would be reduced to suboptimal operation of the TCA cycle and other vitamin-B_1-dependent pathways. In contrast, the vitamin-B_1 synthesizer (PRO95) would not depend on environmental concentrations or the transport of vitamin-B_1, allowing the organism to continue maximum growth even in the dark (Figure 7).

Conclusions

The aim of this study was to try to understand the mechanisms by which *Dokdonia* (and perhaps other bacteria) that contain PR photosystems grow better in the light while others do not. In *Dokdonia*, vitamin-B_1 metabolism and the use of specific vitamin-B_1 TBDT seem to be relevant for the light-dependent growth response. In contrast to other substrates that can be utilized for growth, B-vitamins have extremely precise functions that cannot be replaced by any other molecule (Voet and Voet, 2004; Sañudo-Wilhelmy *et al*., 2014), thus leading to growth limitation when absent. It has recently been shown that large areas of the ocean are vitamin-depleted, suggesting that vitamin limitation is commonly found *in situ* (Sañudo-Wilhelmy *et al*., 2012). Moreover, all genome-sequenced members of the abundant marine SAR11 clade can synthesize only the THZ moiety of the vitamin-B_1, and their growth can be significantly limited by the absence of the HMP moiety or thiamin itself (Carini *et al*., 2014). These results suggest that the PR light-mediated uptake of vitamin-B_1 and its auxotrophy could be important in vitamin-B_1-limited environments. Having the *de novo* pathway for vitamin-B_1 synthesis, other microbes (for example, *Dokdonia* PRO95) would be unaffected by fluctuations in ambient vitamin-B_1 availability, growing equally well in the light or in the dark. However, the auxotrophy and limitation by other vitamins or any other irreplaceable coenzymes would potentially lead to the situation observed in the strains *Dokdonia* DSW-1^T and MED134 with respect to vitamin-B_1.

Finally, we hypothesize that the light-enhanced growth seen in some *Dokdonia* strains is not caused through reduction of respiration under illumination, but to the possibility to sustain aerobic respiration (via the TCA cycle) when vitamin-B_1 limitation is alleviated through PR activity. Future studies will need to address the effects of this hypothetical PR light-enhanced respiration in *Dokdonia* and other marine PR photoheterotrophs to further evaluate its impact on the carbon cycle.

Conflict of Interest

The authors declare no conflict of interest.

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Author contributions

LG-C and IW-D conceived and designed the experiments; LG-C and TR performed the experiments; LG-C and JMG analyzed the data; LG-C, JMG, IW-D, JAF and SJ contributed reagents/materials/analysis tools; LG-C, JMG, TR, SAS-W, IW-D and JAF contributed to the writing the manuscript.

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