New Insights into Metabolic Properties of Marine Bacteria Encoding Proteorhodopsins

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Proteorhodopsin phototrophy was recently discovered in oceanic surface waters. In an effort to characterize uncultured proteorhodopsin-exploiting bacteria, large-insert bacterial artificial chromosome (BAC) libraries from the Mediterranean Sea and Red Sea were analyzed. Fifty-five BACs carried diverse proteorhodopsin genes, and we confirmed the function of five. We calculate that proteorhodopsin-exploiting bacteria account for 13% of microorganisms in the photic zone. We further show that some proteorhodopsin-containing bacteria possess a retinal biosynthetic pathway and a reverse sulfite reductase operon, employed by prokaryotes oxidizing sulfur compounds. Thus, these novel phototrophs are an unexpectedly large and metabolically diverse component of the marine microbial surface water.

Introduction

Proteorhodopsin (PR) proteins are bacterial retinal-binding membrane pigments that function as light-driven proton pumps in the marine ecosystem [1,2]. A gene encoding such a pigment was originally discovered on a large genome fragment [1] derived from an uncultured marine gammaproteobacterium of the SAR86 group [3,4]. Subsequently, many diverse PRs have been detected in marine plankton, via PCR-based gene surveys [5,6], environmental bacterial artificial chromosome (BAC) and fosmid libraries screening [7,8], or environmental shotgun libraries [9]. Recently, through comparative analyses of SAR86 rRNA-bearing genomic fragments, it was shown that diverse SAR86 members contain PR pigments belonging to different groups [7]. Furthermore, in another environmental genomics study, it was proposed that a Pacific PR is encoded by a planktonic alphaproteobacterium [8]. Although retrieval and comparative analyses of large genome fragments carrying PR genes is the most promising approach to phylogenetically assign and better understand uncultured PR-carrying organisms, the data accumulated to this day come from only five different PR genes contained within large insert BAC or fosmid clones: the original Pacific 31A08 clone [1], Antarctic ANT32C12 fosmid clone [8], Pacific Alphaproteobacteria-related clone HOT2C01 [8], Pacific clone HOT4E07, and eBAC20E09 clone from the Red Sea [7].

Results/Discussion

To better understand the extent of naturally occurring PR variability and physiological traits associated with PR-carrying organisms, we surveyed large insert BAC libraries (with inserts up to 170 Kb) from the photic zone of the Mediterranean Sea and Red Sea using Southern hybridization and newly designed general degenerated PR primers. The primers were designed based on alignments of PR sequences from the North Atlantic Ocean, the Mediterranean and Red Seas [5,6], the Pacific Ocean [7,8], and from the Sargasso Sea environmental shotgun project [9]. These primers amplified diverse PR sequences (red in Figure 1), which were not restricted to the three PR families we previously amplified using non-degenerate primers (orange in Figure 1). The diversity of PRs observed in the BAC library was comparable to recent findings from randomly sequenced small-insert shotgun libraries from the Sargasso Sea [9]. Fifty-five different BAC clones were found to contain PRs in the Mediterranean library, representing 0.52% of the total clones. Assuming (i) that an average marine bacterium had a genome size of 2.0 Mb, (ii) that the cloned DNA was recovered from exclusively prokaryotes, and (iii) that each PR-carrying microorganism carried only one PR gene copy on its genome, this PR abundance suggests that 13% of the bacteria in the photic zone of the Mediterranean Sea possess a PR gene (10,560 BAC clones × 80-Kb average insert size = 844.8 Mb; 844.8 Mb / 2.0 Mb = 422.2 genomes represented in the library; 55 PR genes in 422.2 genomes represent 13%). This estimation does not consider possible biases of environmental BAC libraries prepared in Escherichia coli against
Figure 1. Phylogenetic Tree of PR Proteins from the Mediterranean and Red Seas along with PR Homologs in GenBank

The tree was divided into what we propose are distinct subfamilies of sequences, based on bootstrap values significance. The tree was constructed as follows: (i) All homologs of PR proteins were identified in GenBank including predicted proteins from the Sargasso Sea assemblies using BLASTp [36] searches with representatives of previously identified PR-like protein families as query sequences. (ii) All sequences greater than 300 nucleotides in length were aligned to each other using CLUSTALx [37], and a neighbor-joining phylogenetic tree was inferred using the neighbor programs of PAUP* [38]. Bootstrap resampling (1,000 pseudoreplications) of neighboring- and maximum parsimony trees were performed in all analyses to provide confidence estimation for the inferred topologies. Bootstraps values greater than 50% are indicated above the branches (neighbor-joining/maximum parsimony). The scale bar represents the number of substitutions per site. The sequences are colored according to the type of sample in which they were found: blue, cultured species; orange, sequences from uncultured organisms obtained using PCR-based methods; and red, BAC-derived sequences from uncultured species in the Mediterranean Sea and Red Sea (this study) or from previously reported Pacific, Antarctic, and Red Sea [1,7,8] BAC/fosmids. Black squares mark sequenced BACs in this study; red squares label BACs sequenced in previous reports. α, Alphaproteobacteria; γ, Gammaproteobacteria. Red circles mark the two abundant PR groups discussed in the manuscript.

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foreign DNA [10]. Interestingly, 50% of these PR-containing BAC clones fall into two distinct groups (red circles in Figure 1), which might represent the most abundant PR-containing bacteria in Mediterranean surface waters.

BAC clones representing each PR family (black squares in Figure 1) were partially or completely sequenced and annotated (11 clones in total). Two and seven out of these 11 BAC clones are suggested to be coming from prokaryotes related to the Gamma- and Alphaproteobacteria, respectively, based on top BLAST hits criteria (see Tables S1–S6 and previously published information [8]. Based on homology searches, we were able to assign BAC clone MED49C08 from one of the gammaproteobacterial groups to the SAR86 clade; thus, 14 other BAC clones with almost identical PR genes (Figure 1) were also considered as members of this group (assuming no lateral gene transfer in the case of PR). Three of the retrieved BAC clones (MED86H08, MEDPR45, and MED42A11) are predicted to be from the SAR11 group because they carry PR genes with high sequence homology to a PR recently identified by proteome analysis of a cultured alphaproteobacterium (SAR11) [11], and data from other genes on the BACs support alphaproteobacterial affiliations. The high abundance of genome fragments from SAR86 and Alphaproteobacteria found here is consistent with previous reports, which determined members of the SAR86 clade to account for up to 8% of the active bacteria in the photic zone of a coastal North Sea sample [3] while SAR11 members were found to represent as much as 50% of the total marine surface water microbial community [12]. Based on 16S rRNA surveys, both the SAR86 and SAR11 clades harbor very diverse populations [13]. This “microdiversity” is also reflected on the PR level (Figure 1). All PR representatives (Alphaproteobacteria MED18B02, MED46A06, MED66A3; Gammaproteobacteria MED49C08; and unassigned group MED13K09 and MED82F10) checked using the E. coli heterologous expression system showed light-driven proton pumping activity as well as fast photocycles typical of retinylidene transporters [14] (Figure 2). The photochemical reaction cycles observed are among the most rapid seen for proton-pumping rhodopsins. Of interest is that the pigments exhibiting blue absorption spectra (MED18B02, MED49C08, and MED13K09) have fast photocycles indicative of efficient proton pumps operating in a high solar radiation environment as found in surface water (12-m depth) from which the BAC library was prepared. In contrast, the only previously characterized blue absorbing PR, HOT75 [15], has an order-of-magnitude slower photocycle. This was previously attributed to its retrieval from 75-m depth, where solar flux intensities are greatly reduced [15]. Taken together, these data imply that the widespread marine SAR86 and SAR11 groups, as well as other bacterial groups, are using light-driven PR-based phototrophy as a way to harvest additional energy in oligotrophic marine environments.

Several interesting operons providing new insights into the metabolisms of PR-encoding microorganisms were linked to PR genes or found on PR-containing BACs. On clone MED13K09, an entire dsr operon containing the genes for both subunits of a reverse siroheme sulfite reductase (dsrAB), typically used by chemotrophic or anaerobic phototrophic bacteria for exploiting reduced sulfur compounds as electron donor [16,17], was found. The reverse sulfite reductase encoded on this BAC clone forms a highly supported monophyletic cluster with nine reverse sulfite reductases for which genes (or gene fragments) were retrieved from the Sargasso Sea shotgun library [9] and with the respective enzyme of the anaerobic phototrophic purple sulfur bacterium Allochromatium vinosum [18] (Figure 3A), a member of the Gammaproteobacteria. This grouping is further supported by a highly conserved gene order of other dsr genes on the genome fragments (Figure 3B). Furthermore, some but not all phylogenetic analyses of three ribosomal proteins encoded on the genome fragment from BAC clone MED13K09 also suggest that the organism is a deep-branching gammaproteobacterium (Figure S1).

Since we could deduce that BAC MED13K09 is not a chimera (Figure S2), the close relationship of the reverse sulfite reductase from the PR-carrying MED13K09 clone with the enzyme of the gammaproteobacterium A. vinosum might suggest the existence of a novel anoxygenic phototroph exploiting light for energy generation not only by its bacteriochlorophyll-containing photosystem but also by PR. Alternatively, these genes might originate from a novel chemotrophic oxidizer of reduced sulfur compounds. In this context, it is interesting to note that some anoxygenic phototrophs [19] closely related to A. vinosum as well as thioacidi [20], which both possess dsrAB genes [17] (Figure 3), are capable of gaining energy from aerobic oxidation of dimethyl sulfide to sulfate. In contrast to reduced inorganic sulfur compounds, dimethyl sulfide is present in the analyzed oxygenated marine surface waters [21], and PR- and DsrAB-exploiting marine bacteria might thus be involved in degradation of this compound, which plays the key role in the transport of sulfur from oceanic to terrestrial systems [22] and as a precursor for cloud condensation nuclei [23]. Together with the recent finding that SAR11 bacteria consume significant amounts of dimethylsulfinopropionate [24], an osmoprotectant produced by marine algae and plant halophytes that is degraded by marine bacteria to DMS [25], our results suggest that bacteria exploiting PR phototrophy might be of importance for sulfur cycling in the marine photic zone.
Another interesting genomic feature linked to PR genes was a carotenoid biosynthesis gene cluster found on clones MED66A03, MED13K09, RED17H08, and MED82F10 (Figure 4 and Tables S1–S4). The arrangement of the respective genes was similar, containing the gene order \textit{crtIBY} in all BACs. These genes are predicted to encode for phytoene desaturase, phytoene synthase, and lycopene cyclase, respectively, which catalyze the formation of \(\beta\)-carotene from geranylgeranyl pyrophosphate through phytoene and lycopene intermediates [26]. In addition, the first gene in the carotenoid biosynthesis pathway coding for geranylgeranyl diphosphate synthase (\textit{crtE}) was found in the operon of MED66A03, RED17H08. MED13K09 carries the \textit{crtE} gene outside the operon approximately 25 kilobases downstream. This suggests that bacteria carrying these operons can synthesize \(\beta\)-carotene. Interestingly, the first reported bacterial gene coding for a homolog of the bacteriorhodopsin-related-protein-like homolog protein (Blh) from the archaeon \textit{Halobacterium} sp. NRC-1 was found in the operons of MED66A03, RED17H08, and MED13K09, leading to the operon arrangement of \textit{crtEIBY}, \textit{blh} on MED66A03, RED17H08 and \textit{crtIBY}, \textit{blh} on MED13K09. Bacteriorhodopsin-related protein was recently implicated in retinal biosynthesis [27] and was suggested to be the protein converting \(\beta\)-carotene to retinal, similar to the activity of 15,15'-\(\beta\)-carotene dioxygenase from \textit{Drosophila melanogaster} [28]. Although highly speculative, as the identity between the archaeal Blh and the bacterial proteins is only 20%, this may imply that bacteria possessing PR apoproteins also carry the ability to synthesize the retinal chromophore and to potentially form functional PR holoproteins. Indeed, expression of the Blh homolog in \(\beta\)-carotene-producing \textit{E. coli} cells resulted in the loss of the yellow color of these cells (Figure 4). When checked via HPLC, a clear all-\textit{trans} retinal signal was seen only in cells expressing the Blh gene. Moreover, co-expression of the bacterial Blh homolog on a \(\beta\)-carotene-producing and PR-expressing \textit{E. coli} background produced red-colored cells, indicating that the \(\beta\)-carotene is cleaved by the Blh homolog to retinal, which enters the membrane to form an active PR. The \(\beta\)-carotene cleaving enzyme Blh is the first one of its kind found in bacteria. The recently reported retinal biosynthetic enzyme from \textit{Synechocystis} PCC 6803 [29] cleaves apo-carotenoids only (i.e., single-ringed carotenes), while the bacterial Blh cleaves...
β-carotene. In addition, a predicted gene encoding for isopentenyl diphosphate isomerase was found in the carotenoid biosynthetic operons containing the blh gene. This protein was shown to enhance isoprenoid biosynthesis when expressed in E. coli cells [30].

By taking advantage of large insert environmental BAC libraries and heterologous expression assays, we were able to show that PR-carrying bacteria are an important component of the microbial communities in the photic zone of the Mediterranean Sea and Red Sea, and that several phylogenetically diverse PR genes encode functional light-driven proton pumps. Furthermore, we revealed previously unrecognized links between PR genes and different and partly unexpected metabolic traits and thus gained novel insights into the biology of some uncultured PR-carrying bacteria. Some of these PR-carrying bacteria are apparently energy scavengers, ideally adapted to oligotrophic marine surface waters by exploiting not only light but possibly also some reduced organic sulfur compounds for energy generation.

Materials and Methods

BAC library construction. BAC libraries were constructed from plankton samples collected in the Red Sea or from 12-m water collected on a transect from Haifa to Cyprus (33°25′N, 35°56′E to 32°54′N, 34°44′E). Construction was carried out as described [10] with minor modifications (for more details, see http://www.tigr.org/tdb/MBMO/MBMO.shtml). The approximately 800 l of pre-filtered waters (Whatman GF/A filter) (Middlesex, United Kingdom) expression vector and transformed into E. coli XLI-Blue (Bethesda, United States) and pBAD expression system [1], and their light-driven proton-pumping activity was measured as previously described [32].

β-carotene dioxygenase activity. XLI-Blue E. coli cells transformed with pBCAR [33] with the creT, creB, creR, and creY genes for β-carotene biosynthesis from Eureinia herbicola, pGB-Inpi carrying ip (IP isomer-atom to DMAPP) from Haematococcus pluvialis [34] and plasmid pBAD-blh carrying the blh gene under the arabinose promoter were grown overnight at 37 °C in the dark to early stationary phase. Bacteria were harvested from samples of 10 ml of culture for carotenoids and retinoids analysis at time 0, 1, 2, and 6 h after addition of 0.1% (v/v) L-arabinose. Uninduced cells were harvested at 6 h of growth. Cells were resuspended in 200 μl of 6M formaldehyde and incubated for 2 min at 37 °C. Two ml of dichloromethane were added, and carotenoids and retinoids were extracted twice with 4 ml of hexane. The solvent was dried under a stream of nitrogen and the carotenoids dissolved in 75 μl hexane:ethanol 99.5:0.5 to be injected to the HPLC. Carotenoids and retinoids were separated by HPLC using a Waters (Milford, Massachusetts, United States) system and a Spherisorb ODS2 C18 (5 μm, 4.6 × 250 mm) reversed-phase column. Samples of 25 μl were injected to a Waters 600 pump. A gradient of acetonitrile-water (9:1) containing 0.1% (w/v) ammonium acetate (A) and ethylamine (B), at a constant flow rate of 1.6 ml/min was used as follows: 100% A during the first 15 min; 100% to 80% A during 8 min; 80% to 65% A during 4 min, followed by 65% to 45% A during 14 min and a final segment at 100% B. Light absorption peaks were detected in the range of 200–600 nm using a Waters 996 photodiode array detector. Carotenoids and retinoids were identified by their absorption spectra and characteristic retention time. Pure all-trans-retinal and pure β-carotene were used as standards.

Alternatively, for the co-expression with the PR, Blh homolog from MED06A03 was amplified using the BLH66A3fwd 5'-ACGATGGCCGCTTGATGTTAATTGATTGGTG-3' and BLH66A3rev 5'-ATTTTTGATTTAATCTGGAAGATGTGGTGC-3' primers, cloned into the pBAD-TOPO (Invitrogen, Carlsbad, California, United States) expression vector and transformed into β-carotene accumulating E. coli cells carrying plasmid pACCAR16 with the creT, creB, creR, and creY genes for β-carotene biosynthesis from E. herbicola [20]. For the transformation, the cells carrying the plasmid carrying the co-expression with the PR gene, the plasmid carrying the 31A08 PR gene under the control of the lacUV-protomer [K.-H. Jung, V. D. Trivedi, E. N. Spudich, J. L. Spudich, unpublished data]. β-carotene-accumulating E. coli cells were grown with L-arabinose and IPTG to induce the blh and PR genes, respectively.

Supporting Information

Figure S1. Phylogenetic Analysis of Ribosomal Proteins L21, L27, and S20 from BAC Clone MED13K09. Ribosomal protein L31, which is also present on BAC clone MED13K09, was excluded from the analysis because lateral gene transfer of this protein has been reported [35]. The dataset consisted of 51 reference organisms ranging from domain Bacteria to archaea for which whole genome sequences have been reported. A concatenated dataset and a 30% amino acid sequence conservation filter (234 alignment positions) was used for phylogeny inference. Polytopic nodes connect branches for which a relative order could not be determined unambiguously, while using distance matrix, maximum parsimony, and maximum-likelihood methods. In contrast to the consensus tree, trees inferred from distance matrix (DM) and maximum-likeliness (ML) methods do support a clustering of MED13K09 proteins with the Gamma-βetaproteobacteria (see insets).

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A

Sulfate/Sulfite Reducers

Genus Thermodesulfobacterium
Desulfococcus beani
Desulfitobacca acetoxidans
Genus Archaeoglobus
Genus Thermodesulfovibrio

Firmicutes and Desulfobacterium anilini
Desulfobulboseae
Desulfobacteriae

Thermodesulfobium naranganse

10%

Chlorobium tepidum
Allochromatium vinosum
Theobacillus denitrificans
Magnetospinum magnetotacticum

Oxidizers of Reduced S-compounds

B

MED13K09 (95 Kb)

IBEA_CTG_2027414 (12 Kb)

IBEA_CTG_1982486 (3 Kb)

A. vinosum (14 Kb)

C. tepidum (2155 Kb)

A. fulgidus (2178 Kb)

1Kb
**Figure 3.** Dsr Operons in PR-Carrying BAC and Sargasso Sea Scaffolds

(A) Phylogenetic tree showing the affiliation of DsrAB from MED13K09. Alignment regions of insertions and deletions were omitted in DsrAB amino acid sequence analyses. Polytopic nodes connect branches for which a relative order could not be determined unambiguously by using distance-matrix (FITC with the Dayhoff PAM matrix, global rearrangements, and randomized input order of species), maximum-parsimony, and maximum-likelihood (with JTT-f as the amino acid replacement model) methods. Maximum-parsimony bootstrap values (%) are indicated at each node (1,000 re-samplings).

(B) Organization of the dsr operons on MED13K09, Sargasso Sea shotgun clones IBEA_CTG_2027414 and IBEA_CTG_1982486, and in *A. vinosum*, *Chlorobium tepidum* TLS, and the sulfate-reducer *Archaeoglobus fulgidus*. Asterisk indicates an authentic frame shift in the second copy of dsrB in the genome of *C. tepidum.*

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**Figure 4.** Retinal Biosynthesis Pathways in PR-Carrying BACs

(A) Schematic comparison of different carotenoid biosynthesis gene clusters linked to PR genes. ORF marked in gray represent predicted carotenoid biosynthesis genes while PR is marked in black.

(B) HPLC separation of the retinoids formed in the β-carotene producing *E. coli* and expressing the Blh protein. Left panel, extract from non-induced cells; right panel, after 60 min of induction (L-arabinose). Insights, absorption spectra of peaks 1 (β-carotene) and 2 (all-trans retinal).

(C) Color shift due to the cleavage of β-carotene to retinal in *E. coli* cells. Color shift from orange (β-carotene; non induced) to almost white (retinal; L-arabinose induced cells) in β-carotene producing and accumulating *E. coli* cells caused by expression of the blh gene and, the same β-carotene producing cells co-expressing the blh and a PR gene; color shift to red (L-arabinose and IPTG induced cells).

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**Figure S2.** BAC Clone MED13K09 Is Not a Chimera

Schematic illustration showing that BAC clone MED13K09 is not a chimera and that the *dsr* genes identified are linked to the *pr* gene on the genome of the respective unknown marine bacterium. In addition to BAC clone MED13K09, a partially overlapping BAC clone (MED47G02) was detected by BAC end sequencing. This clone does not carry *dsr* (>100% identity on DNA level) as demonstrated by PCR amplification and sequencing. Specific primer sets were designed and used to amplify overlapping +4-kilobase PCR fragments (shown in red) using DNA isolated directly from the environment as a template, which demonstrate that the sequence region of MED13K09 identical to the *dsr* gene is actually connected to the *pr* gene. *dsrA*, *dsrE*, *pr*, and BAC MED47G02 end positions relative to MED13K09 are marked. In addition, a shotgun sequence scaffold from Sargasso Sea carrying *dsr* genes and a PR has been deposited by Venter et al.[9], providing independent evidence for co-occurrence of these genes on bacterial genomes. 

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**Table S1.** List of Genes on BAC Clone MED13K09

This clone contains four genes encoding ribosomal proteins (S20, L27, L21, L31). Based on these proteins, a phylogenetic analysis was performed (see Figure S1). Of the 100 ORFs annotated, 54%, 12%, and 34% were provisionally assigned based on the top BLAST hit to the *Gammaproteobacteria, Alphaproteobacteria,* and other prokaryotes, respectively.

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**Table S2.** List of Genes on BAC Clone MED66A03

Of the 40 ORFs annotated, 15%, 50%, and 35% were provisionally assigned based on the top BLAST hit to the *Alphaproteobacteria, Gammaproteobacteria,* and other prokaryotes, respectively.

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**Table S3.** List of Genes on BAC Clone RED17H08

Of the 38 ORFs annotated, 16%, 42%, and 42% were provisionally assigned based on the top BLAST hit to the *Gammaproteobacteria, Alphaproteobacteria,* and other prokaryotes, respectively.

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**Table S4.** List of Genes on BAC Clone MED82F10

Of the 18 ORFs annotated, 28%, 22%, and 50% were provisionally assigned based on the top BLAST hit to the *Gammaproteobacteria, Alphaproteobacteria,* and other prokaryotes, respectively.

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**Table S5.** List of Genes on BAC Clone MED49C08

*ORF* has highest homology to a protein from the SAR86-related environmental BAC clone EBAC31A08 [1]. Of the 67 ORFs annotated, 60%, 25%, and 15% were provisionally assigned based on the top BLAST hit to the *Gammaproteobacteria, Alphaproteobacteria,* and other prokaryotes, respectively.

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**Table S6.** List of Genes on BAC Clone MED35C06

*ORF* has highest homology to a protein from the SAR86-related environmental BAC clone EBAC31A08 [1]. Of the 39 ORFs annotated, 77%, 13%, and 10% were provisionally assigned based on the top BLAST hit to the *Gammaproteobacteria, Alphaproteobacteria,* and other prokaryotes, respectively.

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**Competing interests**

The authors have declared that no competing interests exist.

**Author contributions.** GS, AL, JLS, and OB conceived and designed the experiments, GS, AL, RP, TI, and JH performed the experiments, GS, AL, RP, JLS, TI, JH, MW, and OB analyzed the data, GS, RP, JLS, and JH contributed reagents/materials/analysis tools, AL, JLS, JH, MW, and OB wrote the paper.


