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LETTERS

Light stimulates growth of proteorhodopsin-containing marine Flavobacteria

Laura Gómez-Consarnau[1], José M. González[2], Montserrat Coll-Lladó[3], Pontus Gourdon[4], Torbjörn Pascher[5], Richard Neutze[6], Carlos Pedrós-Alió[3] & Jarone Pinhassi[1]

Proteorhodopsins are bacterial light-dependent proton pumps. Their discovery within genomic material from uncultivated marine bacterioplankton caused considerable excitement because it indicated a potential phototrophic function within these organisms, which had previously been considered strictly chemotrophic[1]. Subsequent studies established that sequences encoding proteorhodopsin are broadly distributed throughout the world's oceans[2–5]. Nevertheless, the role of proteorhodopsins in native marine bacteria is still unknown[6]. Here we show, from an analysis of the complete genomes of three marine Flavobacteria, that cultivated bacteria in the phylum Bacteroidetes, one of the principal components of marine bacterioplankton, contain proteorhodopsin. Moreover, growth experiments in both natural and artificial seawater (low in labile organic matter, which is typical of the world's oceans) establish that exposure to light results in a marked increase in the cell yield of one such bacterium (*Dokdonia* sp. strain MED134) when compared with cells grown in darkness. Thus, our results show that the phototrophy conferred by proteorhodopsin can provide critical amounts of energy, not only for respiration and maintenance but also for active growth of marine bacterioplankton in their natural environment.

Rhodopsins are found in the domains Archaea, Bacteria and Eukarya. Rhodopsins in Archaea function as energy-transducing light-driven proton or chloride pumps, or as phototactic sensory proteins. In Eukarya, rhodopsins function primarily as sensory proteins and, for example, account for colour vision in the human retina. The recent discovery of rhodopsins in Bacteria (proteorhodopsin; PR) came after the sequence analysis of a cloned genome region from a marine bacterium of the uncultivated SAR86 clade[1]. Subsequent screening of DNA from different oceans revealed a very large diversity of PR in bacteria belonging to divergent clades of the Alphaproteobacteria and Gammaproteobacteria classes[3–8]. Studies on reconstituted PR overproduced in *Escherichia coli* have established that it functions as a light-driven proton pump with the potential to generate energy for cell growth or maintenance[1,6]. However, the physiological role and benefits of PR in native Bacteria in the marine environment have not been demonstrated.

Alphaproteobacteria and Gammaproteobacteria, together with members of the Bacteroidetes phylum, are the most abundant groups of heterotrophic bacteria in the sea[9–12]. In the present study we examined whole-genome sequences of three bacteria belonging to the class Flavobacteria, phylum Bacteroidetes (*Dokdonia* sp. strain MED134, *Polaribacter* sp. strain MED152 and *Leeuwenhoekiella blandensis* strain MED217^T). These bacteria were isolated from surface water from the Mediterranean Sea and were successfully cultured (Fig. 1).

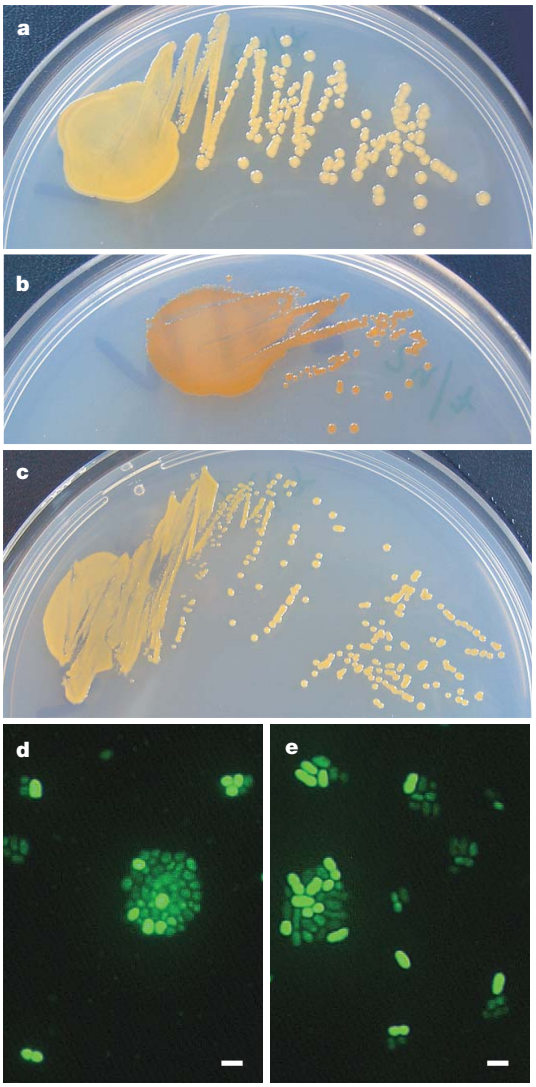

Figure 1 | Images of Flavobacteria isolates. a–c, Colony morphology of *Dokdonia* sp. MED134 (**a**), *Polaribacter* sp. MED152 (**b**) and *L. blandensis* MED217^T (**c**) growing on marine agar (Difco). **d**, **e**, Epifluorescence microscopy images of MED134 growing in the dark (**d**) and in the light (**e**), showing differences in cell morphology. Scale bars, 1 μm.

[1]Marine Microbiology, Department of Biology and Environmental Sciences, University of Kalmar, SE-39182 Kalmar, Sweden. [2]Department of Microbiology and Cell Biology, University of La Laguna, ES-38206 La Laguna, Tenerife, Spain. [3]Institut de Ciències del Mar, CMIMA-CSIC, ES-08003 Barcelona, Catalonia, Spain. [4]Department of Chemical and Biological Engineering, Chalmers University of Technology, SE-41296 Göteborg, Sweden. [5]Chemical Physics, Kemicentrum, Lund University, SE-22100 Lund, Sweden. [6]Department of Chemistry, Biochemistry and Biophysics, Gothenburg University, SE-40530 Göteborg, Sweden.

Genome analysis revealed the presence of genes encoding PR in MED134 and MED152 but not in MED217^{T}. Furthermore, these bacteria lacked genes for bacteriochlorophyll synthesis, reaction centres or light-harvesting complexes necessary to perform photosynthesis. This indicates that, if present, phototrophy in MED134 and MED152 must be due to the activity of PR.

Proteorhodopsin genes from MED134 and MED152 encoded peptides of 247 and 243 amino acid residues, respectively, sharing a sequence similarity of 83%. They are predicted to be heptahelical integral membrane proteins, as are PRs from other marine bacteria such as the SAR86 clade and '*Pelagibacter ubique*', the first cultivated member of the abundant SAR11 clade[13] (Supplementary Fig. S1). Phylogenetic analysis showed that PRs from MED134 and MED152 formed a distinct cluster with two unpublished PR orthologues from Flavobacteria isolates reported in GenBank and previously unclassified environmental sequences from the North Atlantic and the Sargasso Sea[5,8] (Fig. 2). The similarity between PR sequences within this putative Bacteroidetes cluster ranged from 57% to 85%, whereas the similarity to sequences outside this cluster ranged from 43% to 49%. Our results therefore substantiate a previous suggestion[5] that some PR sequences from uncultivated bacteria belong to the phylum Bacteroidetes.

Several key amino acid positions necessary for energy generation are conserved among rhodopsins functioning as proton or ion pumps (Supplementary Fig. S1). Lys 216, which binds retinal to helix G through a protonated Schiff base in bacteriorhodopsin, was conserved as Lys 230 in MED134 and MED152. Asp 85, the proton acceptor from the Schiff base, was conserved as Asp 97. In PR from marine bacteria Glu 108 replaces Asp 96, which facilitates Schiff-base reprotonation during the latter half of the bacteriorhodopsin photocycle[14,15]. Nevertheless, experiments with SAR86 and *P. ubique* PRs have demonstrated that they also function as proton pumps[1,6]. Thus, the essential mechanism of light-driven proton pumping by bacteriorhodopsin[16] seems also to apply for PR. Furthermore, laser flash (532 nm)-induced absorbance changes (probed at 500 nm) in membrane extracts from MED134 showed reaction times less than 50 ms, consistent with the presence of PR functioning as a proton pump (Supplementary Fig. S2).

A phototrophic role for PR implies that its absorption maximum would be tuned to optimize the overlap with environmental light[2,7]. Thus, PRs in near-surface seawater typically have leucine at amino

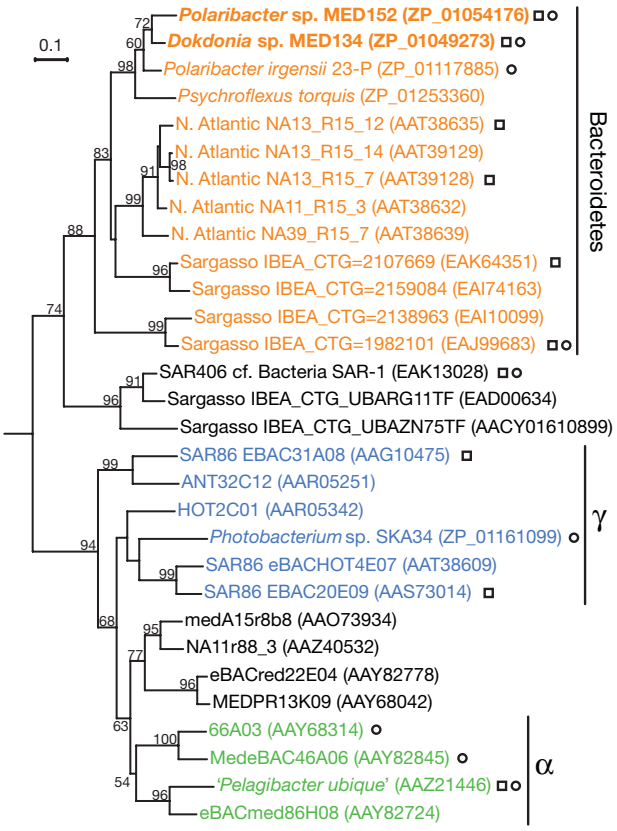

Figure 2 | Phylogenetic tree of PR amino acid sequences. The position of MED134 and MED152 (bold type) in relation to selected sequences of tentative Bacteroidetes PRs from the Sargasso Sea and the North Atlantic as well as Alphaproteobacteria (α) and Gammaproteobacteria (γ). Squares and circles denote sequences aligned in Supplementary Fig. S1 and included in the gene arrangement in Supplementary Fig. S3, respectively. The numbers at nodes are bootstrap values more than 50% after 100 replicates. The scale bar represents substitutions per site.

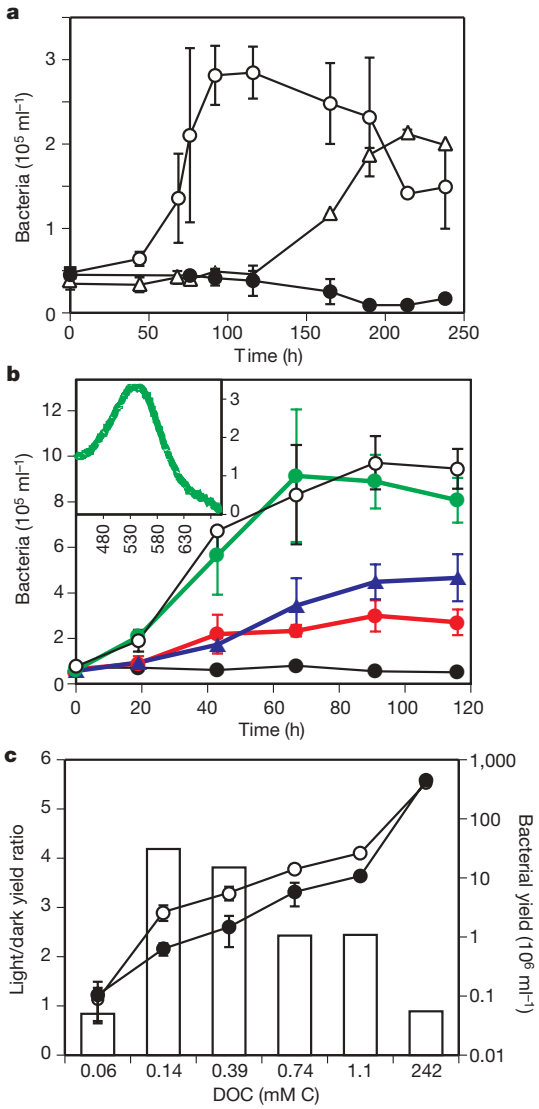

Figure 3 | Growth of MED134 in seawater cultures. **a**, Cultures grown in natural sterile filtered and autoclaved seawater, exposed to light (open circles) or in darkness (filled circles). After 120 h, duplicate cultures grown in darkness were exposed to light (open triangles). **b**, Cultures exposed to white light (open circles), blue light (blue triangles), green light (green circles) and red light (red filled circles) and cultures maintained in the dark (black filled circles). Inset: absorption spectrum of MED134 PR purified from *E. coli* membranes (for details see Supplementary methods); the *x* axis shows wavelength (nm) and the *y* axis absorbance (10^{-2}). **c**, Cell yields in artificial seawater, incubated in the light (open circles) and in the dark (filled circles), enriched with dissolved organic matter, in comparison with unenriched controls (0.06 mM C) and full-strength medium (242 mM C). Columns represent light/dark ratios of cell yield. Error bars denote s.d. for duplicate or triplicate cultures.

acid position 105 (eBAC31A08 numbering) and show absorption maxima near 530 nm (green light), whereas PRs from deeper water have glutamine at this position and absorb near 490 nm (blue light)[2,7,15]. Consistent with these findings is the observation that the near-surface Bacteroidetes PRs contained a hydrophobic side-chain Met 105 (similar to Leu; Supplementary Fig. S1) and showed an absorption maximum at 535 nm (Fig. 3b, inset).

Functional PR requires the covalent binding of retinal, which is synthesized from β-carotene. MED134 and MED152 contained the predicted genes *crtEBIY* (for gene arrangement see Supplementary Fig. S3) encoding enzymes needed to synthesize β-carotene from farnesyl diphosphate and isopentenyl diphosphate[17]. A gene encoding a candidate for Blh, an enzyme that converts β-carotene to retinal[8], was found next to the gene encoding PR in the Bacteroidetes, whereas in Proteobacteria the *blh* gene is next to putative genes in the pathway for retinal synthesis[6,8] (Supplementary Fig. S3).

Since its discovery in the sea by cloning environmental DNA, the lack of cultivated bacteria that have and express PR has hampered the determination of its physiological relevance to marine bacteria *in situ*. The gene encoding PR was recently found in cultivated *P. ubique*, and mass spectroscopy indicated that *Pelagibacter* PR is abundant in the surface ocean[6]. Laboratory experiments with *P. ubique*, however, showed no differences in cell yield when growing in light or darkness despite the fact that PR was present in cell membranes. The authors therefore speculated that the benefits of PR might be more pronounced when bacterial growth is limited by organic matter availability. We monitored the growth of MED134 in experiments with natural seawater exposed to light or darkness. Bacteria growing in moderate light reached a maximal abundance of 3×10^5 cells ml^{-1} after 100 h (Fig. 3a). In contrast, bacteria remained below 0.5×10^5 cells ml^{-1} in the dark. When dark cultures were exposed to light after 120 h, growth was initiated and bacteria reached 2×10^5 cells ml^{-1} within 100 h, whereas bacteria in the cultures remaining in darkness slowly decreased in numbers. This is the first confirmation *in vivo* that light has a definite positive impact on the growth of marine bacteria possessing the gene encoding PR.

Experiments using light of different wavelengths showed that this enhanced growth was stimulated primarily by green rather than blue or red light (Fig. 3b). This correlated well with the measured absorption spectrum of MED134 PR (Fig. 3b, inset) and substantiates our hypothesis that the observed growth enhancement derives from the direct absorption of light by PR. Furthermore, analysis of the expression of the gene encoding PR with the use of reverse-transcriptase-mediated polymerase chain reaction for detecting the corresponding mRNA showed a significant upregulation of the PR mRNA in light in comparison with dark cultures (Fig. 4). These observations support the notion that PR fulfils a phototrophic function in marine bacteria.

We further investigated how different concentrations of dissolved organic matter (DOM, as peptone and yeast extract) affected the growth of MED134 (Fig. 3c). In cultures with artificial seawater without enrichment (0.06 mM C) little growth was observed in either light or darkness, whereas enrichment with DOM corresponding to 0.14 or 0.39 mM C (final concentration) resulted in bacterial cell yields about fourfold higher in light cultures. Greater enrichment (0.74 or 1.10 mM C) gave yields about 2.5-fold higher in the light cultures, whereas in full-strength medium (242 mM C) similar bacterial cell yields were observed in both light and dark conditions. In addition, cells grown in light-exposed cultures seemed larger than those in dark cultures (Fig. 1d, e). Thus, exposure to light confers a stronger selective advantage when MED134 grows at low or intermediate concentrations of labile organic matter, closer to those found in marine surface waters, than in richer media. It is noteworthy that *Dokdonia* sp. MED134 thrives in conditions under which resources are abundant, whereas *P. ubique*, in which PRs have been suggested to have a subtle function[6], grows only under oligotrophic conditions. Factors limiting its growth have yet to be identified[6]. These findings emphasize the need to clarify the intricate relationships between a PR-mediated light response, and the availability and composition of organic matter for typically oligotrophic bacteria, in comparison with bacteria adapted to eutrophic environments.

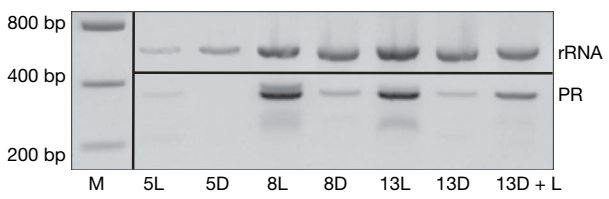

Figure 4 | RT–PCR analysis of PR expression in MED134 compared with 16S rRNA levels. PR was expressed preferentially in the light. Bacteria were grown in natural sterile filtered and autoclaved seawater exposed to light or in the dark. Numbers indicate days since inoculation; D, dark incubation; L, light incubation. 13D + L denotes a sample incubated for 10 days in the dark followed by 3 days in the light. M, DNA size marker; bp, base pairs.

Members of the Bacteroidetes phylum in general, and Flavobacteria in particular, are important in the degradation of organic matter during and after algal blooms in the sea[18–20]. Our findings indicate that phototrophy may allow these organisms to maintain net growth during periods when concentrations of organic matter are declining. If this were true for other PR-bearing bacterioplankton, PR-mediated photoheterotrophy would increase their growth efficiency. An interesting consequence is that, for a given amount of DOM, bacterial assemblages dominated by species expressing genes encoding PR would produce more biomass than assemblages of bacteria lacking PR, making it available to higher trophic levels in the marine microbial food web.

Phototrophism has now been found in all main microbial groups in the sea. These include eukaryotic algae and cyanobacteria performing chlorophyll *a*-driven photosynthesis, Proteobacteria using bacteriochlorophylls for generating energy[21–23], and Proteobacteria[1,3]—and now also Bacteroidetes—using PR. The phylogenetic diversification and wide geographic distribution of genes for PR in the world's oceans indicate that evolution has favoured organisms with the potential to complement their chemotrophic life style by phototrophy[3,24–26]. Surface and near-surface marine microorganisms are bathed in light and it is therefore not surprising that diverse strategies have evolved to exploit such an abundant energy source.

METHODS

Isolation of Flavobacteria strains. Bacteria were isolated from Northwest Mediterranean Sea surface water (0.5 m depth) collected 1 km off the Catalan coast at the Blanes Bay Microbial Observatory (41° 40′ N, 2° 48′ E, Spain). Strains MED134 and MED152 were isolated from ZoBell agar plates inoculated on 20 March 2001, and strain MED217^T on 23 May 2001.

Sequencing, annotation and phylogenetic analysis. Whole-genome sequencing was performed by the J. Craig Venter Institute through the Gordon and Betty Moore Foundation initiative in Marine Microbiology (https://research.venterinstitute.org/moore/). PR sequences were aligned by using ClustalW, and a Neigbour-Joining phylogenetic tree was constructed (see Supplementary Methods).

Seawater culture experiments. For the natural seawater experiment, water from the North Sea was mixed with aged seawater from the North Atlantic. For the DOM gradient experiment, artificial seawater (35 practical salinity units, prepared from Sea Salts; Sigma) was enriched with peptone and yeast extract. All cultures were enriched with N and P to avoid inorganic nutrient limitation. Cultures were incubated under continuous light (180 μmol photons m^{-2} s^{-1}) or in the dark at 20 °C. Dissolved organic carbon was measured with a high-temperature carbon analyser. Bacteria were counted by epifluorescence microscopy. For details see Supplementary Methods.

Expression analysis. RNA were extracted and purified from cell pellets with RNAqueous-4PCR Kit (Ambion Inc.). mRNA was reverse transcribed with the two-step protocol of RETROscript Kit (Ambion Inc.) and a MED134 PR-specific primer set. For details see Supplementary Methods.

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1. Béjà, O. *et al.* Bacterial rhodopsin: evidence for a new type of phototrophy in the sea. *Science* **289,** 1902–1906 (2000).
2. Béjà, O., Spudich, E. N., Spudich, J. L., Leclerc, M. & DeLong, E. F. Proteorhodopsin phototrophy in the ocean. *Nature* **411,** 786–789 (2001).
3. de la Torre, J. R. *et al.* Proteorhodopsin genes are distributed among divergent marine bacterial taxa. *Proc. Natl Acad. Sci. USA* **100,** 12830–12835 (2003).
4. Sabehi, G. *et al.* Novel proteorhodopsin variants from the Mediterranean and Red Seas. *Environ. Microbiol.* **5,** 842–849 (2003).
5. Venter, J. C. *et al.* Environmental genome shotgun sequencing of the Sargasso Sea. *Science* **304,** 66–74 (2004).
6. Giovannoni, S. J. *et al.* Proteorhodopsin in the ubiquitous marine bacterium SAR11. *Nature* **438,** 82–85 (2005).
7. Man, D. *et al.* Diversification and spectral tuning in marine proteorhodopsins. *EMBO J.* **22,** 1725–1731 (2003).
8. Sabehi, G. *et al.* New insights into metabolic properties of marine bacteria encoding proteorhodopsins. *PLoS Biol.* **3,** e273 (2005).
9. Giovannoni, S. & Rappé, M. in *Microbial Ecology of the Oceans* (ed. Kirchman, D.) 47–84 (Wiley-Liss, New York, 2000).
10. Glöckner, F. O., Fuchs, B. M. & Amann, R. Bacterioplankton composition of lakes and oceans: a first comparison based on fluorescence in situ hybridization. *Appl. Environ. Microbiol.* **65,** 3721–3726 (1999).
11. Hagström, Å. *et al.* Use of 16S ribosomal DNA for delineation of marine bacterioplankton species. *Appl. Environ. Microbiol.* **68,** 3628–3633 (2002).
12. Kirchman, D. L. The ecology of Cytophaga–Flavobacteria in aquatic environments. *FEMS Microbiol. Ecol.* **39,** 91–100 (2002).
13. Rappé, M. S., Connon, S. A., Vergin, K. L. & Giovannoni, S. J. Cultivation of the ubiquitous SAR11 marine bacterioplankton clade. *Nature* **418,** 630–633 (2002).
14. Dioumaev, A. K. *et al.* Proton transfers in the photochemical reaction cycle of proteorhodopsin. *Biochemistry* **41,** 5348–5358 (2002).
15. Wang, W.-W., Sineshchekov, O. A., Spudich, E. N. & Spudich, J. L. Spectroscopic and photochemical characterization of a deep ocean proteorhodopsin. *J. Biol. Chem.* **278,** 33985–33991 (2003).
16. Neutze, R. *et al.* Bacteriorhodopsin: a high-resolution structural view of vectorial proton transport. *Biochim. Biophys. Acta* **1565,** 144–167 (2002).
17. Teramoto, M., Takaichi, S., Inomata, Y., Ikenaga, H. & Misawa, N. Structural and functional analysis of a lycopene L-monocyclase gene isolated from a unique marine bacterium that produces myxol. *FEBS Lett.* **545,** 120–126 (2003).
18. Abell, G. C. J. & Bowman, J. P. Ecological and biogeographic relationships of class Flavobacteria in the Southern Ocean. *FEMS Microbiol. Ecol.* **51,** 265–277 (2005).
19. Pinhassi, J. *et al.* Changes in bacterioplankton composition under different phytoplankton regimens. *Appl. Environ. Microbiol.* **70,** 6753–6766 (2004).
20. Riemann, L., Steward, G. F. & Azam, F. Dynamics of bacterial community composition and activity during a mesocosm diatom bloom. *Appl. Environ. Microbiol.* **66,** 578–587 (2000).
21. Shiba, T., Simidu, U. & Taga, N. Distribution of aerobic bacteria which contain bacteriochlorophyll *a*. *Appl. Environ. Microbiol.* **38,** 43–48 (1979).
22. Kolber, Z. S., Van Dover, C. L., Niederman, R. A. & Falkowski, P. G. Bacterial photosynthesis in surface waters of the open ocean. *Nature* **407,** 177–179 (2000).
23. Béjà, O. *et al.* Unsuspected diversity among marine aerobic anoxygenic phototrophs. *Nature* **415,** 630–633 (2002).
24. Bielawski, J. P., Dunn, K. A., Sabehi, G. & Béjà, O. Darwinian adaptation of proteorhodopsin to different light intensities in the marine environment. *Proc. Natl Acad. Sci. USA* **101,** 14824–14829 (2004).
25. Frigaard, N.-U., Martinez, A., Mincer, T. J. & DeLong, E. F. Proteorhodopsin lateral gene transfer between marine planktonic Bacteria and Archaea. *Nature* **439,** 847–850 (2006).
26. Sabehi, G., Béjà, O., Suzuki, M. T., Preston, C. M. & DeLong, E. F. Different SAR86 subgroups harbour divergent proteorhodopsins. *Environ. Microbiol.* **6,** 903–910 (2004).

Supplementary Information is linked to the online version of the paper at www.nature.com/nature.

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Author Information The genomes of strains MED134, MED152 and MED217 are deposited in GenBank under accession numbers AAMZ00000000, AANA00000000 and AANC00000000, and their 16S rRNA gene sequences under accession numbers DQ481462, DQ481463 and DQ294290, respectively. The amino acid sequences of MED134 and MED152 PR are deposited in GenBank under accession numbers ZP_01049273 and ZP_01054176. Reprints and permissions information is available at www.nature.com/reprints. The authors declare no competing financial interests. Correspondence and requests for materials should be addressed to J.P. (jarone.pinhassi@hik.se).