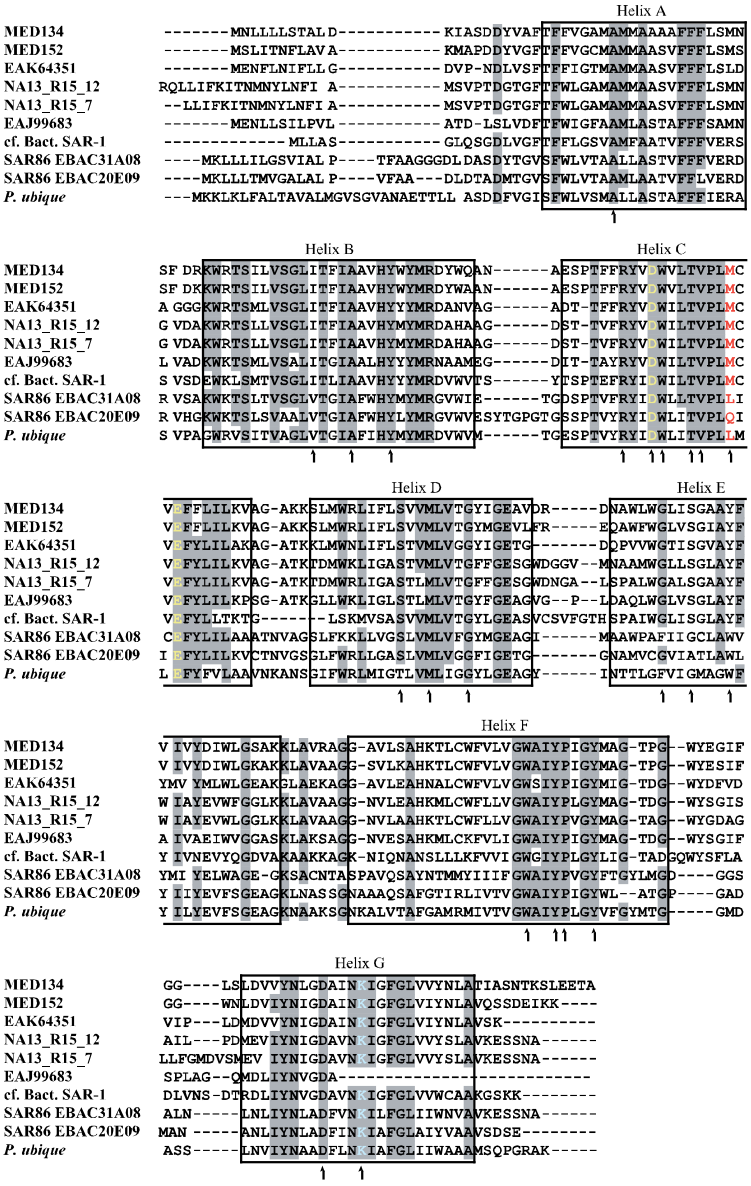
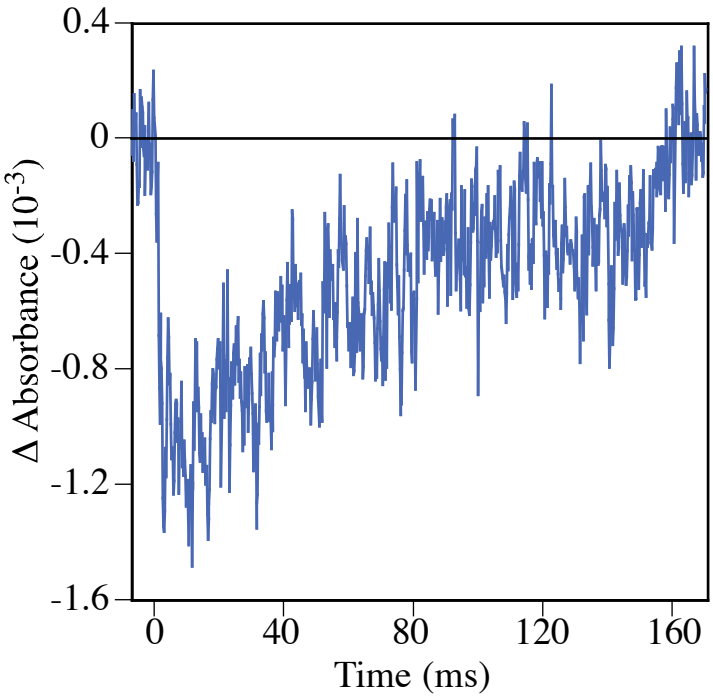
Light stimulates growth of proteorhodopsin-containing marine Flavobacteria

Supplementary Information

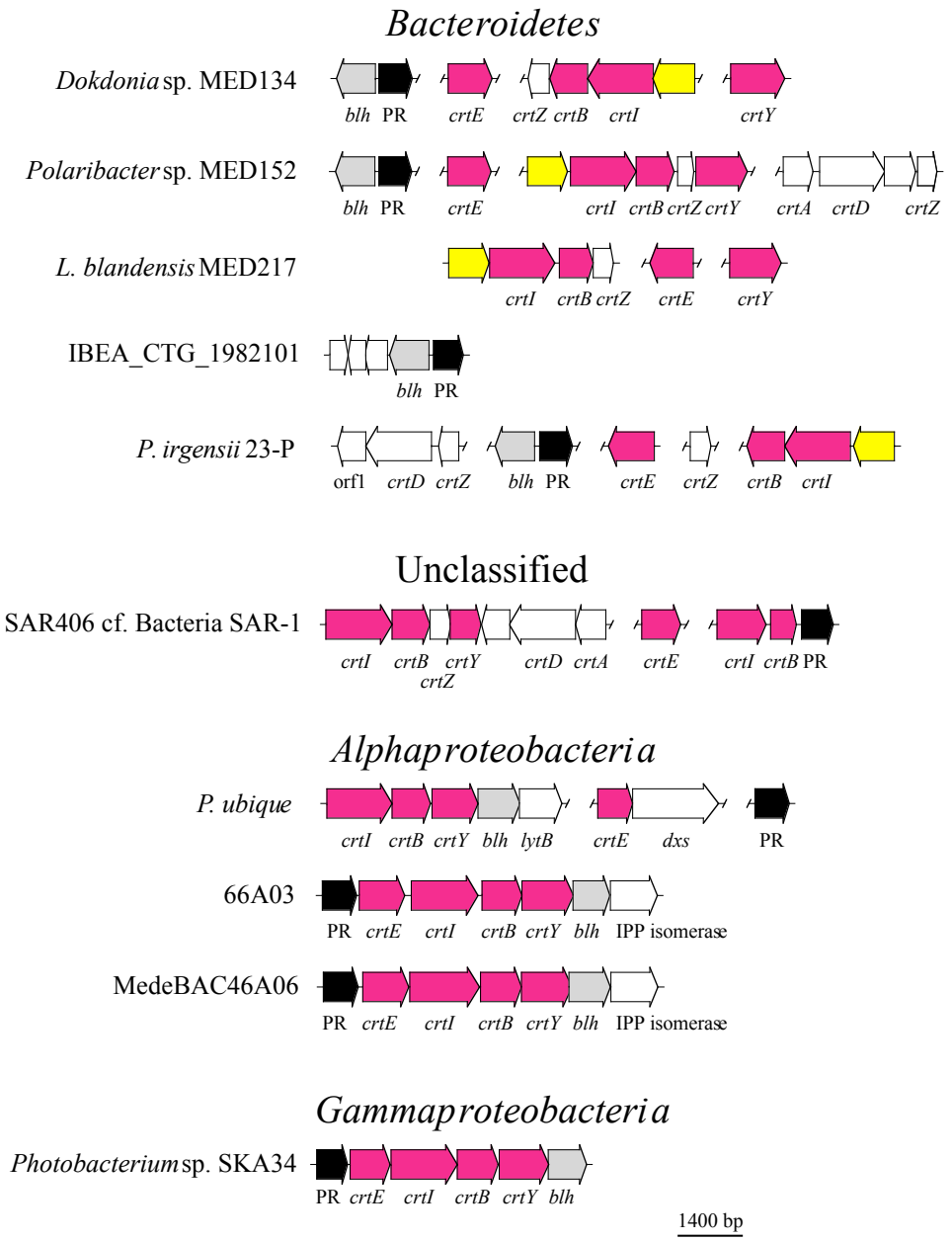
Supplementary figures.



Supplementary Figure 1. Proteorhodopsin amino acid sequence alignment. Multiple alignment of the predicted aminoacid sequences of PR in *Dokdonia* sp. MED134 and *Polaribacter* sp. MED152 compared to tentative *Bacteroidetes* orthologs and SAR86 and *P. ubique* sequences. Predicted transmembrane regions are marked by boxes with solid lines. Gray shading indicates conserved amino acid positions. Key amino acids for PR functionality are marked by colours: Asp97 and Glu108 in yellow, Met, Leu or Gln at position 105 in red, and Lys230 in light blue (SAR86 eBAC31A08 numbering). Amino acid positions predicted to be part of the retinal binding pocket are marked by arrows. Accession numbers and phylogenetic relationships of the sequences are presented in Fig. 2. Amino acid sequences were aligned using Clustal W. Predicted transmembrane regions were identified using TMpred, based on a statistical analysis of TMbase.



Supplementary Figure 2. Laser-flash induced absorbance changes in membrane preparation of *Dokdonia* sp. MED134. MED134 membrane absorption transient at 500 nm; the laser flash at time 0 was at 532 nm. The short reaction time, on the order of <50 ms, is consistent with the presence of PR functioning as proton pumps in the membranes. Laser flash-induced absorbance changes were measured with a laboratory-constructed flash-photolysis apparatus (Pascher 2001). The extracted cell membrane fractions were added to 1 cm cuvettes fitted with a stirrer. The sample was excited with a 10 mJ, 8 ns laser pulse at 532 nm. The acquisition cycle was: laser excitation (1 pulse), kinetic trace recording, stirring 10 s, resting 10 s, recording of reference kinetic trace (no laser), stirring 10 s and resting 10 s. This procedure was repeated 36 times for averaging.



Supplementary Figure 3. Arrangement of proteorhodopsin, ß-carotene and retinal synthesis genes. Position of proteorhodopsin (PR) genes and the putative *blh* homologs in Flavobacteria strains MED134 and MED152 compared to other marine bacteria. Genes marked in red denote *crtEBIY*, predicted to code for geranylgeranyl diphosphate synthase, phytoene synthase, phytoene desaturase and lycopene β-cyclase, respectively, needed to synthetize β-carotene from farnesyl diphosphate (FPP) and isopentenyl diphosphate (IPP). *crtZ* codes for a putative carotene hydroxylase probably involved in the synthesis of zeaxanthin. Furthermore, MED152 contains *crtD* (methoxyneurosporene dehydrogenase) and *crtA* (spheroidene monooxygenase) homologs. Strains MED134 and MED152 both contain a putative transcriptional regulator (MerR family, marked in yellow) in front of *crtI*. The genes *dxs*, IPP isomerase and *lytB* are involved in the synthesis of FPP and IPP precursors. Strain MED217^{T}, used as control in the present study, also contained the genes *crtEBIYZ*, but lacked PR and *blh* homologs. Interestingly, among the *Bacteroidetes*, the PR and *blh* genes are located next to each other but are transcribed divergently. Among other bacteria these genes are found separately and are transcribed in the same direction. Scale bar denotes 1400 base pairs.

Supplementary methods

Annotation and phylogenetic analysis. Open reading frames were predicted and autoannotated using GenDB (Meyer et al. 2003). In addition, all relevant genes discussed here were manually annotated. For the phylogenetic analysis of PR, amino acid sequences were aligned using Clustal W, and a tree was constructed based on a Kimura's distance matrix and the Neighbour-Joining method using the PHYLIP package (Version 3.2) (Felsenstein 1989). For the phylogenetic tree, the *Halobacterium salinarum* S9 bacteriorhodopsin was used as outgroup (not shown in Fig. 2).

Seawater culture experiments. For the experiment with natural seawater, water collected in the North Sea was mixed with an equal volume of aged seawater from the North Atlantic. For each culture, 1.5 litres of seawater, used as growth medium, was filter-sterilized through a 0.2 µm-pore-size filter (Sterivex; Millipore) and autoclaved. For the DOM gradient experiment, an artificial seawater medium (Sea Salts; Sigma: salinity 35 psu) was filter-sterilized through 0.2 µm-pore-size filters (Sterivex; Millipore) and autoclaved. Cultures of 520 ml each were enriched with 0, 0.23, 0.74, 1.48 and 2.25 ml of full strength medium (5 g peptone [Bacto Peptone; BD] and 1 g yeast extract [Bacto Yeast Extract; Difco] in 1 l artificial seawater). For the coloured light experiment, cultures were grown in artificial seawater at a DOC concentration of 0.14 mM C. Blue (400-500 nm), green (490-570 nm) and red (600-800 nm) light were obtained using coloured filters (HQ Power).

Cultures were maintained in polycarbonate bottles (Nalgene). The natural seawater cultures received final concentrations of 2.1 µM NH_4Cl and 0.3 µM Na_2HPO_4 to avoid inorganic nutrient limitation. In the DOM gradient experiment, N and P were further supplied by the added medium (36-285 µM N and 0.6-3.4 µM P). Cultures were inoculated with bacteria previously grown in seawater. Light cultures were incubated under an artificial light source (180 µmol photons m^{-2} s^{-1}) at 20°C. All material in contact with the samples was rinsed with 1 M hydrochloric acid and extensively washed with MilliQ-water prior to use.

Dissolved organic carbon (DOC) was measured with a high temperature carbon analyzer (Shimadzu TOC 5000) on samples filtered through 0.2 µm-pore-size polycarbonate filters (Supor-200; Gelman Sciences) and acidified with 16 mM HCl (final concentration).

For bacterial counts, samples were fixed with 0.2 µm-pore-size filtered formaldehyde (4%, final concentration), stained with SYBR Gold (1:100 dilution, Molecular Probes), filtered onto black 0.2 µm-pore-size polycarbonate filters (Poretics, Osmonics Inc.) and counted by epifluorescence microscopy within 48 hours.

Cloning, production and determination of the absorption spectrum of proteorhodopsin from *Dokdonia* sp. MED134. The PR gene was cloned from MED134 cells using PCR, incorporating a C-terminal 6xHis-tag. The amplified fragment was inserted into the pBAD-TOPO vector (Invitrogen) and subsequently transformed into the *E. coli* outer membrane protease-deficient strain UT5600. PR was over-produced in cells grown at 37°C and induced at an optical density (600 nm) of 2 with 1.25 g l^{-1} L(+)-arabinose and 1.5 mg l^{-1} all trans-retinal for 16 hours. The cells were disrupted with a French Press (Thermo Spectronic) at 1,500 psi, additional all trans-retinal was added (1 mg retinal per gram of cells), and the membrane fractions were collected by centrifugation at 150,000 g for 90 min. Membranes were then solubilised in n-octyl-β-d-glucopyranoside (Anatrace). Extracted histidine-tagged PR was purified with affinity column chromatography using Chelating Sepharose Fast Flow

(GE Healthcare) immobilized with Ni^{2+}. The column with bound PR was washed using 35 mM imidazole and PR was subsequently eluted using 150 mM imidazole. Subsequent ion exchange chromatography resulted in a peak containing PR from which the absorption spectrum could be determined after the fractions had been concentrated in Vivaspin concentration tubes (Sartorius) to 100 µl. The absorption spectrum was measured in a Shimadzu UV-1601 spectrophotometer, using the same buffer as in the last purification step as a baseline.

RNA extraction and purification. Cells were harvested from seawater cultures by pipetting 10 ml samples into 15-ml tubes on ice. Samples were centrifuged and the pellets were stored with 0.5 ml RNAlater® (Ambion Inc., Austin, TX) at –80 ºC. For RNA extraction, samples were thawed on ice. The RNAlater was discarded and the pellet was washed in PBS 1X. A total of 500 µl of lysis/binding solution provided by the RNAqueous®-4PCR kit (Ambion, Inc.) were added to the cells. The samples were transferred to 2 ml screw-cap microcentrifuge tubes containing 1.2 g of 100-µm-diameter zirconia-silica beads (BioSpec Products, Inc.). Samples were mechanically disrupted in a Mini-beadbeater-8™ cell disrupter (BioSpec Products Inc., Bartlesville, OK). After disruption, samples were incubated on ice for 5 min and the beads were allowed to settle out of the lysis mixture. The lysate was clarified by centrifugation and the aqueous phase was transferred to a new tube. An equal volume of 64% ethanol was added to the lysate and samples were purified according to the RNAqueous®-4PCR Kit. The isolated total RNA was treated with DNase I - RNase-free (Ambion Inc.). RNA preparations were checked for DNA contamination with PCR using primers 358F and 907RM for the 16S rRNA gene. Total RNA was quantified by spectrophotometry at 260 nm.

Reverse transcriptase-PCR. A proteorhodopsin primer set was designed to amplify a 349-bp fragment of the *Dokdonia* sp. MED134 PR gene. The sequence of forward primer 238F was 5'-GCAAGCAAATGCTGAGTCAC-3' and the sequence of reverse primer 548R was 5'-AATTGCCCATCCCACAAGTA-3'. This primer set was designed to amplify a 349-bp fragment of the MED134 proteorhodopsin gene. Total RNA was used to reverse-transcribe proteorhodopsin mRNA into cDNA using primer 548R and the two-step protocol of the RETROscript® Kit (Ambion Inc.). To denature any secondary structures, aliquots of each of the RNA extracts and primer 548R were heated for 3 min at 75 ºC and immediately placed on ice before they were mixed with the final reaction solution (20 µl). 2 µl of the RT reaction product were used in a subsequent PCR. The PCR amplification conditions were as follows: 94 ºC for 2 min, and then 40 cycles of 94 ºC for 30 s, 55 ºC for 30 s, and 72 ºC for 40 s and finally 1 cycle of 72 ºC for 5 min. As positive RNA controls, 16S ribosomal RNA was reverse transcribed and thereafter amplified with bacterial 16S rRNA gene-specific primers 358F (5'-CCTACGGGAGGCAGCAG-3') and 907RM (5'-CCGTCAATTCA/CTTTGAGTTT-3'). The same amplification protocol as for the PR reaction was used, except that the number of cycles was reduced to 25. PCR products were analyzed by agarose gel electrophoresis (Fig. 4).

Supplementary notes.

Felsenstein, J. PHYLIP-Phylogeny Inference Package. Cladistics 5, 164-166 (1989).

Meyer, F. et al. GenDB – an open source genome annotation system for prokaryote genomes. Nucl. Acids Res. 31, 2187-2195 (2003).

Pascher, T. Temperature and driving force dependence of the folding rate of reduced horse heart cytochrome c. Biochemistry 40, 5812-5820 (2001).