Characterization of an unconventional rhodopsin from the freshwater Actinobacterium 

Rhodoluna lacicola

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Abstract

Rhodopsin-encoding microorganisms are common in many environments. However, knowing that rhodopsin genes are present provides little insight into how the host cells utilize light. The genome of the freshwater actinobacterium, *Rhodoluna lacicola*, encodes a rhodopsin of the uncharacterized actinorhodopsin family. We hypothesized that actinorhodopsin was a light-activated proton pump, and confirmed this by heterologously expressing *R. lacicola* actinorhodopsin in retinal-producing *Escherichia coli*. However, cultures of *R. lacicola* did not pump protons, even though actinorhodopsin mRNA and protein were both detected. Proton pumping in *R. lacicola* was induced by providing exogenous retinal, suggesting that the cells lacked the retinal cofactor. We used HPLC and oxidation of accessory pigments to confirm that *R. lacicola* does not synthesize retinal. These results suggest that in some organisms, the actinorhodopsin gene is constitutively expressed, but rhodopsin-based light capture may require cofactors obtained from the environment.

Importance

Up to 70% of microbial genomes in some environments are predicted to encode rhodopsins. Because most microbial rhodopsins are light-activated proton pumps, the prevalence of this gene suggests that in some environments, most microorganisms respond to or utilize light energy. Actinorhodopsins were discovered in an analysis of freshwater metagenomic data and subsequently identified in freshwater Actinobacterial cultures. We hypothesized that actinorhodopsin from the freshwater actinobacterium *Rhodoluna lacicola* was a light-activated proton pump, and confirmed this by expressing actinorhodopsin in retinal-producing *Escherichia coli*. Proton pumping in *R. lacicola* was only induced after providing both light and retinal,
suggesting that the cells lacked the retinal cofactor. These results indicate that photoheterotrophy in this organism may require cofactors obtained from the environment.

Introduction

Rhodopsin-containing photoheterotrophic microbes are common inhabitants of marine, terrestrial, and freshwater environments, where they have been identified by cultivation (1-3), metagenomic sequencing (4-6), targeted amplicon sequencing (7-9), and quantitative PCR (9, 10). The cosmopolitan distribution of rhodopsin-containing microbes in diverse habitats is reflected in the variety of effects the rhodopsins have on their hosts. For instance, certain rhodopsins transport protons or other ions, while others affect gene expression through signaling networks (11). Within a single organism, multiple rhodopsins can be present and perform different roles (12, 13). Closely related rhodopsin-containing organisms have been shown to react to light differently: in *Dokdonia* spp., light exposure has been shown to provide a growth advantage for one species, while offering no measurable benefit to another (14-16). In addition, rhodopsins may differ in their maximum absorption peak (480-560 nm; (17)), or their ability to bind additional carotenoids (18, 19), and thus affect light intensity or wavelength preference of the host microbe (20).

Microbial rhodopsins consist of seven transmembrane alpha-helices with a photosensitive chromophore, retinal, linked to a lysine residue of the protein via a retinylidene Schiff base (11). Upon absorption of a photon, the retinal isomerizes and induces a conformational change in the protein, which in turn initiates the ion transport or signal transduction activity of the rhodopsin (21). The first eubacterial rhodopsin identified was the proton-pumping proteorhodopsin (PR) found in a marine metagenomic survey (5). Homologs of PR and other microbial rhodopsins, such as bacteriorhodopsin (22), halorhodopsin (23), sensory rhodopsin (24), and
xanthorhodopsin (XR; (18)) have been identified in many lineages of archaea, bacteria, dinoflagellates, algae and viruses (25). Some estimates of microbial rhodopsin abundance, based on metagenomic sequence analysis, suggest that from 48% to 70% of cells in some marine environments (4, 26) and up to 60% of microbes from estuarine and freshwater habitats may host rhodopsins (7, 27).

The first rhodopsins identified from a freshwater environment were found exclusively associated with Actinobacteria and were named actinorhodopsins (ActR; (2, 7)). The revelation that some of the most abundant members of the freshwater bacterioplankton could potentially utilize sunlight was intriguing. Recent work has extended the hosts of actinorhodopsin-like sequences to include organisms from the freshwater Verrucomicrobia, Proteobacteria (Alpha-, Beta-, Gamma-, and Delta-), and Sphingobacteria (27). Actinorhodopsins belong to the xanthorhodopsin (XR)-like family of rhodopsins (7). Some characterized members of this family are capable of light-activated proton pumping, and utilize both a retinal chromophore and an accessory antenna carotenoid, such as echinone or salinixanthin (18, 20, 28). In a recent study, the XR-like family was subdivided into two groups, and the actinorhodopsins were assigned to Subgroup I of the XR family. Characteristics of Subgroup I members include meso- and thermophilic non-marine habitats, highly divergent gene clusters, and a hypothesized binding pocket to accommodate antenna carotenoids (20). However, unlike the well-studied Subgroup I xanthorhodopsins from *Salinibacter ruber* and *Gloeobacter violaceus*, actinorhodopsins are found predominantly in freshwater environments, and their biochemical function is uncharacterized.

The freshwater actinobacterium, *Rhodoluna lacicola*, has an actinorhodopsin gene, but lacks efficient pathways for CO₂ fixation, and thus relies on organic carbon (29). To investigate
if, under conditions of suitable illumination, the actinorhodopsin could contribute to
photoheterotrophy in this organism, we recently cloned actinorhodopsin (actR) from *R. lacicola*
and overexpressed it in retinal-expressing *Escherichia coli* (30). An amino acid alignment of *R.
lacicola* ActR with other characterized rhodopsins suggests ActR is a proton-pumping rhodopsin,
since it contains the conserved acidic residues required for proton transport and the Schiff base
linkage Lys-231 (2). While analysis of homology can suggest rhodopsin protein function, it
cannot predict under what conditions the rhodopsin will be expressed nor whether the rhodopsin
will be active and functional. To date, no study has characterized the function of the
actinorhodopsins, either through heterologous expression or the use of cultivated isolates. It is
important to understand the physiological function of diverse rhodopsins in order to correctly
determine the contribution(s) of these proteins to metabolic processes within the host organism
and to estimate the extent of solar energy utilization in the environment. In this study, we
investigate ActR from *R. lacicola*. Functional studies with heterologously expressed *R. lacicola*
ActR demonstrate that it is indeed capable of light-activated proton transport. Surprisingly,
experiments in *R. lacicola* show that actinorhodopsin is constitutively expressed, but does not
pump protons in response to light stimulation until provided with exogenous retinal.

Methods

*Strains and growth conditions.* The red-pigmented actinobacterium *R. lacicola* strain MWH-
Ta8^T* (1, 2, 29) was grown in 3 g L^-1 NSY (nutrient broth/soytone/yeast extract; (31)) medium at
room temperature with gentle shaking and 8-12 hr natural sunlight. The gene *actR*, encoding
actinorhodopsin, was amplified from *R. lacicola* genomic DNA, cloned into plasmid pMCL200
as described previously, and sequenced (30). The actinorhodopsin-encoding plasmid (pTAR)
was transformed into *E. coli* epi300 (Epicentre Biotechnology, catalog number EC300105) containing a plasmid for retinal biosynthesis (pRET04; (30)) to create a strain co-expressing ActR and its cofactor, retinal (*E. coli*/pRET04/pTAR). An empty-vector control strain was produced by transforming pMCL200 into *E. coli* epi300 with pRET04.

Proton pumping experiments with *E. coli* or *R. lacicola*. *E. coli* epi300/pRET04/pMLC200 (retinal-expressing cells) and *E. coli* epi300/pRET04/pTAR (retinal- and ActR-expressing cells) were grown in LB with 50 µg mL⁻¹ ampicillin, 34 µg mL⁻¹ chloramphenicol and 0.2% L-arabinose overnight at 37 °C with shaking. *R. lacicola* was grown in 3 g L⁻¹ NSY at room temperature with 8-12 hrs natural sunlight for 10 days. Cells were harvested by centrifugation and washed once with 10 mM NaCl/10 mM MgCl₂/100 µM CaCl₂. Cells were concentrated 20-fold (*E. coli*) or 100-fold (*R. lacicola*) in 1 mL of 10 mM NaCl/10 mM MgCl₂/100 µM CaCl₂ and immediately assessed for light-induced proton pumping activity. Initial pH ranged from 5.8 to 6.5. An aliquot of concentrated *R. lacicola* was vortexed with 10 µg mL⁻¹ all-trans retinal (Sigma-Aldrich) and incubated for 2 hrs under illuminated conditions. Following the retinal treatment, cells were harvested by centrifugation, resuspended in 1 mL 10 mM NaCl/10 mM MgCl₂/100 µM CaCl₂ and immediately assessed for proton pumping activity.

The pH was measured using a Sper Scientific pH SD card Datalogger equipped with a Mettler Toledo InLab Micro electrode. Light was provided by a 250-watt halogen lamp placed 10 cm from the sample, and the two-min ON/OFF intervals were controlled with a digital timer (Leviton LT112). Irradiance at the sample was ~550 µmol photons m⁻² sec⁻¹. The pH meter and electrode were protected from direct illumination with a foil shield, and the sample was
incubated in a water bath to prevent heating. Sample was constantly stirred with a small magnetic stirrer.

For inhibition experiments, concentrated cell solutions of E. coli, R. lacicola, or R. lacicola with retinal were incubated with 100 µM carbonylcyanide m-chlorophenylhydrazone (CCCP; Sigma-Aldrich) for 1.5 hrs in the dark, then proton pumping was measured as described above.

RNA isolation and reverse transcriptase-polymerase chain reaction (RT-PCR). R. lacicola was grown in 600 mL cultures of 3 g L⁻¹ NSY in constant light (30-420 µmol photons m⁻² sec⁻¹) or dark. At day 3, 5, 7, and 10 after inoculation, ten milliliters were removed from each flask, centrifuged at 5000 rpm for 45 min at 4 °C, and stored in RINalater (Ambion) at -20 °C until use. For RNA isolation, the RINalater was discarded and the pellet was washed with 1X PBS. Cells were resuspended in 30 mM Tris/1 mM EDTA pH 8.0/15 mg mL⁻¹ lysozyme with 10 µL Proteinase K (Thermo Scientific), vortexed for 10 sec, and incubated at room temperature for 10 min with shaking. The remaining RNA isolation procedure was followed per the Qiagen RNEasy Miniprep protocol with beta-mercaptoethanol added to the RLT buffer. Genomic DNA contamination was removed with both an on-column DNAse digestion (Qiagen) and a Turbo DNAse treatment (Ambion). Total RNA was quantified using a Nanodrop spectrophotometer.

A primer set was designed to amplify a 330-bp fragment of the R. lacicola actinorhodopsin mRNA. The sequence of the forward (sense) primer was 5′ – GGA TAC CGC TAC GTT GAC TGG – 3′ and the sequence of the reverse (antisense) primer was 5′ – GGT AAA CGC CCC AGG TTG – 3′. Total RNA was used to reverse-transcribe actinorhodopsin mRNA into cDNA using the antisense primer and the two-step protocol of the RETROscript Kit.
To denature any secondary structure, the RNA extracts and the antisense primer were incubated at 75 °C for 3 min, then immediately transferred to ice. The final reaction solutions of the reverse transcriptase (RT) Buffer, dNTP mix, RNase Inhibitor, and with (RT +) or without (RT -) MMLV-RT enzyme were added to the denatured template and primer mixture. The reverse transcription steps were 44 °C for 1 hr, followed by 92 °C for 10 min. An aliquot of the RT reaction product (2.5 µL) was used as a template for the subsequent PCR. The PCR amplification conditions were: 94 °C for 1 min, then 26 cycles of 94 °C for 20 sec, 52 °C for 30 sec, 72 °C for 30 sec, and a final step of 72 °C for 5 min with Taq polymerase (Sigma-Aldrich). All RT negative reactions showed no bands after the PCR step, indicating no genomic DNA contamination in the samples. As positive RT-PCR controls, the mouse RNA and primers provided with the kit were used. PCR products were electrophoresed on a 2% agarose-TBE gel run at 90 V for 1.5 hrs with a 100-bp standard (Life Technologies SM0241).

For RT-PCR with rpoB, primers were designed to amplify a 936 bp region. The forward primer was 5′ - ACA ACT TCG AGG ACG CGA TC - 3′, and the reverse primer was 5′ - GCG TGG ATC TTG TCG TC - 3′, and the PCR conditions were changed to 94 °C for 1 min, then 26 cycles of 94 °C for 20 sec, 49 °C for 30 sec, 72 °C for 1 min, and a final step of 72 °C for 5 min with Taq polymerase (Sigma-Aldrich).

Membrane fraction preparation. Membranes from either one-liter cultures of R. lacicola grown for ten days at room temperature with 8-12 hrs natural sunlight, concentrated R. lacicola provided with exogenous retinal as described above, or E. coli/pRET04/pTAR were partially purified as described (30). Briefly, the cells were lysed with an osmotic lysis buffer containing lysozyme (0.075 M Tris pH 8.0, 2.0 mM MgSO₄, 0.4 M sucrose, 10 mg mL⁻¹ lysozyme),
followed by incubation in a high salt buffer (50 mM Tris pH 7.6, 10 mM MgSO$_4$, 0.8 M NaCl) and sonication. After broken cells were centrifuged at 25000 × g for 30 min at 4 °C, the brightly-colored membrane film was removed and resuspended in 3% beta-octylglucopyranoside (β-OG; Amresco) in 10 mM HEPES, pH 7.1 by vortexing overnight at 4 °C in the dark. The detergent-solubilized membrane was centrifuged at 11000 × g for 10 min at 4 °C to remove insoluble material. Absorption spectra from 250 – 900 nm were recorded using a Thermo Scientific BioMate 3S UV-Visible Spectrophotometer. Membranes prepared in this manner were used for SDS-PAGE, extraction of carotenoids for analysis by HPLC, and oxidation with ammonium persulfate (see below).

**Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE).** Membrane preparations to be analyzed by SDS-PAGE were incubated 1:1 in 2X loading buffer (250 mM Tris, 2% SDS, 30% glycerol, 10% 2-mercaptoethanol, 0.002% bromophenol blue) for 1 hr at room temperature. Samples were loaded on a 10% Tris-buffered polyacrylamide resolving gel, topped with a 5% polyacrylamide stacking gel and electrophoresed according to the method of Laemmli (32). The gel was washed with DI water, fixed with 10:25:65 glacial acetic acid:methanol:water for 15 min, and stained with LabSafe GEL Blue (G-Biosciences). The molecular weight standard was PageRuler Prestained Protein Ladder 10-170 kDa (Thermo Scientific).

**Mass spectrometry (MS) identification of actinorhodopsin.** The expected actinorhodopsin band was excised for analysis. The enzymatic digestion procedure was performed with trypsin (Promega) at 37 °C as previously described (33) and included reduction/alkylation with
Dithiothreitol (BioRad) and iodoacetamide (Sigma), respectively. Subsequently, the sample was desalted and concentrated using Ziptips (Millipore) and applied to a target plate with α-cyano-4-hydroxycinnamic acid matrix (Sigma). Data were collected on a 4800 MALDI TOF/TOF Analyzer (ABSciex) in positive ion, reflector mode over a mass range of 900-4000 m/z, with internal calibration. Select peaks were further analyzed by MSMS at 1kV with default calibration. The combined MS and MSMS data were submitted to Mascot v2.2 (Matrix Science) and searched against NCBI. The protein identification was made based on a database match of 95% confidence or greater, including four MSMS matches with >99% confidence.

Pigment analysis by high-performance liquid chromatography (HPLC). Previous work suggested disruption of the C=N double bond was necessary for retinal release from rhodopsin proteins (34). Membranes, prepared as described above, in 10 mM HEPES pH 7.1 and 3% β-OG, were treated with 100 mM hydroxylamine (Alfa Aesar) on ice for two hrs, then extracted with 7:2 acetone:methanol (v/v). Following centrifugation for 2 min at 10000 rpm, the supernatant was removed and dried. Dried pigments were resuspended in methanol and filtered through 0.2 µm polytetrafluoroethylene syringe filters (Thermo Scientific) prior to injection into the HPLC. The HPLC system was a Shimadzu Prominence system with solvent degasser (DGU-20A5), quaternary pump (LC-20AT), and 996-element diode array detector (SPD-M20A) fitted with a Supelco Ascentis reverse-phase C18 column (100 × 3 mm, 3 µm beads; Sigma-Aldrich catalog number 581308-U). Solvent A was methanol:water (3:1 v/v) and solvent B was methanol:dichloromethane (4:1 v/v; (35)). The gradient was as follows (min, %B): (0, 0), (20, 100), (40,100) with a flow rate of 0.5 mL min⁻¹. The column was kept at a constant temperature of 30 °C.
Oxidation of pigments with ammonium persulfate. Ammonium persulfate has been shown to selectively oxidize carotenoid pigments while leaving rhodopsin-bound retinal intact (36). Membranes prepared from R. lacicola in 10 mM HEPES pH 7.1 and 3% β-OG were diluted with the same buffer until the absorbance peaks of the carotenoids were well-resolved. The absorbance spectra were recorded, then 5 mM ammonium persulfate (Amresco) was added. After addition of ammonium persulfate, the pH decreased slightly (on average from 7.4 to 6.8). Following addition of ammonium persulfate, the membranes were incubated in the dark and absorbance spectra were recorded at regular intervals.

Results

In previous work, the gene encoding actinorhodopsin was cloned from R. lacicola MWH-Ta8T and expressed in E. coli that co-expressed retinal biosynthesis genes (30). Based on the conserved lysine at position 234, which functions as the Schiff base linkage, we predicted that ActR was capable of binding retinal. The leucine at position 100 was characteristic of green-light absorbing rhodopsins (37), suggesting that ActR would have an absorption maximum in the 490-560 nm range. In addition, actinorhodopsin possesses the conserved residues His-62 and Asp-92, likely as a hydrogen bonded pair, and Glu-103, which are necessary for proton transport through the protein channel in response to light-induced conformational changes (38, 39). Thus, we hypothesized that ActR was a green-light-absorbing, proton pumping rhodopsin with a retinal cofactor. Our previous results showed that when expressed in E. coli, actinorhodopsin bound retinal, localized to the membrane, and had a maximum absorbance peak at 528 nm (30).
**E. coli** co-expressing retinal and actinorhodopsin was assayed for light-activated proton transport. Light-dependent decreases in pH were observed in *E. coli* cells expressing ActR, but not in those with an empty vector control plasmid (Fig. 1). This result demonstrated that the retinal-bound ActR translocated protons out of the cell in response to light. In addition, we tested the effects of carbonylcyanide *m*-chlorophenylhydrazone (CCCP), a proton ionophore, on proton pumping in *E. coli/pRET04/pTAR*. In the presence of CCCP, the light-activated decrease in pH was completely abolished (Fig. 1). This result confirmed that protons were the ions pumped out of the cell in response to light.

Proton pumping experiments with whole cell cultures of *E. coli* heterologously expressing rhodopsins has been widely reported (5, 13, 35, 40), but less success has been obtained with whole cell cultures of native producers (3, 41, 42). This disparity can be attributed to the limited number of rhodopsin producers in culture, their inability to grow to high enough density for pumping experiments, and unknown requirements for rhodopsin expression (43). Before testing whole cells of *R. lacicola* for proton pumping activity, it was necessary to determine whether *R. lacicola* was synthesizing actinorhodopsin under the culture conditions utilized. Gene expression was analyzed in light- and dark-grown cultures during mid- and late-exponential growth phase and in stationary phase by reverse-transcriptase-mediated polymerase chain reaction (RT-PCR) directed at *actR* (Fig. 2A), with *rpoB* as a positive control (Fig. 2B). The mRNA of *actR* was detected in all samples collected over a ten-day period, in cells grown in either constant light or constant dark conditions (Fig. 2A).

Since the gene was constitutively expressed, *R. lacicola* cultures in both mid-exponential and stationary phase were assayed for light-activated proton transport. However, no light-induced pH change was observed in either culture (Fig. 3A). To confirm the presence of the
protein in the cultures that were utilized for proton pumping experiments, membranes were prepared from 10-day cultures by osmotic lysis followed by extraction with β-octylglucopyranoside. Samples were analyzed by SDS-PAGE (Fig. 3B). A band corresponding to the putative actinorhodopsin was extracted from the gel and analyzed by mass spectrometry. The masses of peptides obtained from fragmentation were matches to the *R. lacicola* actinorhodopsin protein sequence, confirming that the actinorhodopsin apoprotein was produced by the cells (Table S1).

These results demonstrated that actinorhodopsin was both actively transcribed and translated in *R. lacicola*, and accumulated in membrane fractions. However, these same cells, even when concentrated to high density, do not pump protons. Therefore, we hypothesized that the protein lacked the light-responsive retinal cofactor. Two distinct carotenoid biosynthesis gene clusters are found in the genome of *R. lacicola* (NCBI accession number NZ_CP007490.1; Table S2), but these genes were not located in a cluster near actinorhodopsin, nor did the genome encode any genes with homology to known carotenoid cleavage oxygenase genes. The pigments produced by *R. lacicola* were analyzed by HPLC to determine whether retinal might be present but produced by a previously unknown pathway. Retinal is only released from rhodopsin when hydroxylamine or other reagents disrupt the C=N double bond between retinal and lysine (34). Membranes were prepared from *E. coli* expressing retinal and actinorhodopsin (*E. coli/pRET04/pTAR*) as a positive control, treated with hydroxylamine and extracted with acetone:methanol (7:2 v/v), then analyzed by HPLC (Fig. 3C, top trace). In *E. coli/pRET04/pTAR* membranes, retinal was detected as retinal oxime. Membranes from *R. lacicola* grown in standard NSY media were prepared in the same manner as the *E. coli* membranes. *R. lacicola* synthesizes several pigments, but none with similar retention times and
absorption spectra to retinal oxime (Fig. 3C, bottom trace). Membranes extracted from *R. lacicola* grown under a variety of other conditions, including dilutions of full-strength media, constant dark, anoxia, and into very late stationary phase also lacked the retinal pigment (data not shown).

As seen in the HPLC chromatogram, several pigments co-purified with membrane fractions containing actinorhodopsin. Ammonium persulfate has been used to selectively oxidize carotenoid pigments both in the presence and absence of another rhodopsin, xanthorhodopsin (XR). When XR is present, the rhodopsin-bound retinal is not affected by ammonium persulfate (36). Ammonium persulfate treatment of membrane fractions prepared from *E. coli* expressing actinorhodopsin and retinal verified that the oxidation does not affect actinorhodopsin-bound retinal (data not shown). Ammonium persulfate was used to oxidize the co-purifying pigments in membrane fractions from *R. lacicola*. After approximately one hour of incubation, all the carotenoids in *R. lacicola* membrane fractions were completely oxidized; however, no peak corresponding to retinal-bound actinorhodopsin was revealed (Fig. 3D).

These results demonstrate that there was no retinal or retinal-like product in the whole cells or membrane preparations of *R. lacicola*, even though the actinorhodopsin protein was present. Since the protein was present but appeared to lack any cofactor, all-trans retinal was provided exogenously to concentrated *R. lacicola* cells. Following the retinal treatment, cells were washed several times to remove unbound retinal, then membrane fractions were prepared. The membranes were treated with hydroxylamine, the pigments were extracted with acetone and methanol, then analyzed by HPLC. A peak corresponding to retinal oxime was detected in these preparations, indicating that the exogenously added retinal was incorporated into cellular membranes (Fig. 4A). To confirm that the retinal was bound to the actinorhodopsin apoprotein,
membrane fractions were prepared from cells incubated with all-trans retinal, and carotenoid pigments were oxidized using ammonium persulfate (Fig. 4B). In the presence of exogenous retinal, a peak at ~530 nm was revealed after oxidation of the other pigments (Fig. 4C). This peak is characteristic of retinal-bound actinorhodopsin (30).

Cultures of R. lacicola that were provided with retinal were tested for light-activated proton pumping. When the cells were provided with exogenous retinal, proton translocation was detected. Under illuminated conditions, the pH of the cell solution decreased, while in dark conditions, the pH of the solution increased (Fig. 4D). As in E. coli, treatment of retinal-fed R. lacicola with CCCP abolished light-induced proton transport (Fig. 4D, dashed line).

Discussion

Actinorhodopsin is a green-light absorbing, proton pumping rhodopsin. The amino acid sequence of actinorhodopsin from R. lacicola suggests it should have an absorbance maximum in the green light range (490-560 nm) due to the leucine at position 100. Additionally, the acidic residues at positions 92 and 103 suggest that it should be capable of proton translocation. As predicted, ActR heterologously expressed in retinal-expressing E. coli has a maximum absorbance at 528 nm (30) and pumps protons in response to light (Fig. 1). Similarly, several other characterized XR-family members are capable of proton pumping in response to light (18, 20, 28), and all known eubacterial xanthorhodopsin-like rhodopsins encode a leucine at the equivalent position. However, the absorbance maximum of R. lacicola ActR is slightly blue-shifted relative to the characterized examples from two Octadecabacter species (533±1 nm and 535±1 nm; (20)), Salinibacter ruber (560 nm; (18)), and Gloeobacter violaceus (540 nm.; (19)).

While the leucine residue is primarily responsible for spectral tuning to green light, the
additional variability in observed absorbance maxima of different XR-family proteins may be attributed to differences in solution pH (17), which can affect the protonation state of the rhodopsin, and the retinal-binding pocket protein microenvironment (44).

The rhodopsins from *S. ruber* and *G. violaceus* have binding pockets on the outside of the protein for the ketocarotenoids salinixanthin and echinenone, respectively (19, 39). Recent work on the *Octadecabacter* strains suggested that even though these rhodopsins did not bind ketocarotenoids, though the amino acids necessary for ketocarotenoid binding are conserved in these proteins (20). Similarly, while the ActR of *R. lacicola* also possesses the glycine residue and a majority of the other amino acids assigned to the keto-carotenoid binding pocket (19, 20), it is unlikely that ActR binds these pigments, since *R. lacicola* lacks both the *crtW* and *crtO* carotenoid ketolases and is thus unlikely to synthesize carotenoids similar to echinenone or salinixanthin (Table S2; (45)).

*R. lacicola* constitutively transcribes actinorhodopsin and accumulates ActR in membrane fractions. Because the conditions required for expression of rhodopsins are often unknown, characterization of their function(s) in vivo has been difficult. No research has reported the expression pattern of a XR-like rhodopsin in the producing microbe. A few studies have examined the expression of proteorhodopsin (PR) in cultivated marine bacteria, and have found varying results. In RT-PCR and transcriptomic analysis of *Dokdonia* sp. MED134, PR expression was found to occur preferentially in media with extremely low carbon content and under illuminated conditions (14, 15). However, in a study on the expression of PR in *Candidatus Pelagibacter* ubique HTCC1062, PR expression was constant during logarithmic growth in both light and dark cultures (46). Here, we show that *actR* is constitutively expressed
in *R. lacicola* in our standard laboratory growth media, in cells grown under both light and dark conditions (Fig. 2A). In addition, we detect the ActR protein in membrane fractions prepared from *R. lacicola*, where it appears to be the most abundant protein in the solubilized membranes (Fig. 3B).

*R. lacicola* does not produce the retinal cofactor. Despite the constitutive expression of actinorhodopsin in *R. lacicola* and production of abundant carotenoids (Fig. 3D) that could serve as retinal precursors, this organism does not appear to synthesize retinal. In whole cells of *R. lacicola*, ActR does not function as a light-activated proton pump until provided with exogenous retinal (Fig. 3A and Fig. 4D). This is the first report in a producing organism of a microbial rhodopsin expressed without its retinal cofactor. However, we cannot exclude the possibility that *R. lacicola* produces retinal via an unknown biosynthetic pathway and with unknown growth requirements that do not coincide with conditions found for actinorhodopsin production in culture.

There is genomic evidence that other microorganisms may similarly encode a rhodopsin but not retinal biosynthesis genes. Bioinformatic analysis of two nearly-complete genomes assembled from metagenomes and two partial genomes from single-cell sequencing efforts of the uncultivated, abundant marine bacterial clade, SAR86, found all four assemblies lack a pathway for retinal biosynthesis despite containing at least one proteorhodopsin gene (47). Likewise, a draft genome (~97% complete) from a single cell of the acl lineage of Actinobacteria was recently published and found to contain a rhodopsin homolog, but no identified carotenoid cleavage oxygenases (48). The genomes of *Roseiflexus* sp. RS-1 (NCBI accession NC_009523.1) and *Thermus* sp. CCB_US3_UF1 (49) both encode xanthorhodopsin homologs but no predicted
carotenoid cleavage enzymes. On the other hand, the draft genome of another actinobacterium with an actinorhodopsin, Candidatus *Aquiluna* sp. (50) encodes the carotenoid cleavage enzyme, *blh*, necessary for the final step of retinal biosynthesis.

*Potential physiological role(s) of actinorhodopsin in R. lacicola.* A light-responsive rhodopsin requires the presence of both apoprotein and cofactor. Because *R. lacicola* was isolated from Lake Taihu, China (1), a hyper-eutrophic lake with high bacterial and phytoplankton density (between $10^6$ and $10^8$ cells ml$^{-1}$ (51)), a variety of cofactors are likely to be available as cells die and lyse. Many organisms, especially those with streamlined genomes, rely on additional molecules produced by community members, emphasizing the importance of community structure in microbial ecology and even in cellular physiology and function. For example, recent research has demonstrated the inability of *Candidatus* Pelagibacter ubique to synthesize certain B vitamins and reduced sulfur compounds that are both required for its growth (52, 53), and other work has found sediment-associated candidate phyla with metabolic dependence on other organisms for essential amino acids, nucleotides, and lipids (54). Since *R. lacicola* shares with *Cand.* Pelagibacter strains a small genome size (1.4 Mb), it is not surprising that the strain also relies on the uptake of organic substances. If members of the microbial community where *R. lacicola* lives release retinal, *R. lacicola* could incorporate retinal into actinorhodopsin and pump protons in response to light. This activity would contribute directly to the proton motive force and provide the cells with additional ATP for biomass production or for driving energy-consuming uptake processes. A similar mechanism of scavenging retinal from the environment has been proposed for the proteorhodopsin-containing members of the marine clade SAR86 that lack biosynthetic pathways for retinal (47).
R. lacicola cells synthesize so much of the actinorhodopsin apoprotein it appears to be the most abundant protein in membrane preparations. However, because the cells do not synthesize retinal, an alternative role for the actinorhodopsin apoprotein cannot be ruled out. Many microbial rhodopsins oligomerize in the membrane and form large aggregates (55-60). Without a cofactor, actinorhodopsin might still aggregate in the membrane, providing structural stability against membrane stresses.

The experiments presented here demonstrate that biochemically, actinorhodopsin is a proton pump; however, its physiological role is still unknown. Despite being unable to synthesize its own cofactor de novo, R. lacicola is primed for any physiological benefit from actinorhodopsin by incorporating scavenged retinal into the apoprotein present in the membranes. We propose that R. lacicola may be a photoheterotroph if it is able to acquire retinal from environmental sources and if enough light is available.

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FigureCaptions
Figure 1. Proton translocation in response to light by *E. coli* expressing actinorhodopsin. *E. coli* co-expressing retinal and actinorhodopsin (*E. coli*/pRET04/pTAR; solid black line) were resuspended in an unbuffered salt solution (10 mM NaCl/10 mM MgCl₂/100 µM CaCl₂) and exposed to light. When the light was ON (white bars), the pH of the solution decreased. When light was OFF (gray bars), the pH of the solution increased. *E. coli* with the retinal biosynthesis plasmid and the empty vector pMCL200 (*E. coli*/pRET04/pMCL200; gray line) were monitored for proton pumping under the same conditions, and no proton translocation was observed. *E. coli*/pRET04/pTAR were resuspended in 10 mM NaCl/10 mM MgCl₂/100 µM CaCl₂, treated with 100 µM CCCP, and exposed to light/dark cycles. Treatment with CCCP abolished the light induced proton transport (dashed line).

Figure 2. Presence of actinorhodopsin mRNA in *R. lacicola*. (A) Expression of *actR* was analyzed by RT-PCR. Cell cultures were grown under either 24-hr light or 24-hr dark conditions for ten days. Aliquots of cells were removed on days 3, 5, 7, and 10 and stored at -20 °C in RNAlater until analysis. The partial *actR* transcript was present in both the light and dark cultures at all time points. No genomic DNA contamination was detected in any sample, and a representative example of the RT negative control is presented in the lane marked “RT-”. A 100-bp ladder was used for size analysis and is visible in lanes marked L. (B) Expression of *rpoB* was monitored by RT-PCR as a loading control. See Figure S1 for images of full-sized gels.

Figure 3. Actinorhodopsin in *R. lacicola*. (A). Proton pumping assay. A stationary phase culture of *R. lacicola* was concentrated and resuspended in an unbuffered salt solution (10 mM NaCl/10 mM MgCl₂/100 µM CaCl₂) and exposed to two-minute cycles of light (white bars) and dark cycles.
(gray bars). No proton translocation was observed. No proton pumping was observed for a mid-exponential phase culture either (data not shown). (B) ActR protein expression. Cell cultures were grown for ten days. Membrane fractions were purified from the harvested cells and analyzed by SDS-PAGE. Lane 1: protein present in *R. lacicola* membrane fractions. Lane 2: positive control of protein from *E. coli/pRET04/pTAR* membrane preparations. The band corresponding to ActR at ~22 kDa is labeled. See Figure S1 for image of intact gel. (C). HPLC chromatograms of pigments extracted from *E. coli/pRET04/pTAR* (top panel) and *R. lacicola* (bottom panel), monitored at 360 nm. Membrane fractions were treated with 100 mM hydroxylamine to hydrolyze the Schiff base linkage, then extracted in acetone:methanol (7:2 v/v). Retinal, detected as retinal oxime here, is only present in the *E. coli* culture (top panel). (D).

Oxidation of pigments in suspensions of *R. lacicola* membranes. Absorption spectra of suspensions measured before (1) and after (2-9) addition of 5 mM ammonium persulfate and incubation for 0, 7, 13, 21, 28, 38, 57, and 160 min, respectively.

Figure 4. Pigment analysis and proton pumping of *R. lacicola* cultures with exogenous retinal. (A). HPLC chromatogram of pigments extracted from membranes prepared from *R. lacicola* fed with retinal and monitored at 360 nm. Membrane fractions were treated with 100 mM hydroxylamine, then extracted in 7:2 acetone:methanol. Retinal was detected as retinal oxime, and the absorbance spectrum is shown in the inset. (B). Oxidation of pigments in suspensions of *R. lacicola* membranes containing actinorhodopsin and exogenous retinal. Absorption spectra of suspensions were recorded before (1) and after (2-9) addition of 5 mM ammonium persulfate and incubation for 0, 5, 10, 15, 20, 30, 45, and 126 min, respectively. (C). The last three time-points from the pigment oxidation of *R. lacicola* cultures with exogenous retinal (curves 7-9, Fig. 4B)
and *R. lacicola* cultures without exogenous retinal (curves 7-9, Fig. 3D) were averaged, then the spectrum from the unsupplemented cultures was subtracted from that of the retinal-fed cultures. The difference spectrum obtained shows a peak at ~530 nm, corresponding to retinal-containing actinorhodopsin. (D). *R. lacicola* expressing actinorhodopsin and provided with exogenous retinal were resuspended in an unbuffered salt solution (10 mM NaCl/10 mM MgCl₂/100 µM CaCl₂) and exposed to light/dark cycles (solid lines). When the light was ON (white bars), the pH of the solution decreased. When light was OFF (gray bars), the pH of the solution increased. *R. lacicola* provided with retinal was prepared in the same way, and incubated with 100 µm CCCP for 1.5 hours in the dark (dashed lines). Treatment with CCCP abolished the light induced proton transport.

References


