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1 **Characterization of an unconventional rhodopsin from the freshwater Actinobacterium**

2 ***Rhodoluna lacicola***

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12 **Abstract**

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

25

26 **Importance**

27 Up to 70% of microbial genomes in some environments are predicted to encode
28 rhodopsins. Because most microbial rhodopsins are light-activated proton pumps, the prevalence
29 of this gene suggests that in some environments, most microorganisms respond to or utilize light
30 energy. Actinorhodopsins were discovered in an analysis of freshwater metagenomic data and
31 subsequently identified in freshwater Actinobacterial cultures. We hypothesized that
32 actinorhodopsin from the freshwater actinobacterium *Rhodoluna lacicola* was a light-activated
33 proton pump, and confirmed this by expressing actinorhodopsin in retinal-producing *Escherichia*
34 *coli*. Proton pumping in *R. lacicola* was only induced after providing both light and retinal,

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35 suggesting that the cells lacked the retinal cofactor. These results indicate that photoheterotrophy
36 in this organism may require cofactors obtained from the environment.

37 **Introduction**

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

51 Microbial rhodopsins consist of seven transmembrane alpha-helices with a photosensitive
52 chromophore, retinal, linked to a lysine residue of the protein via a retinylidene Schiff base (11).
53 Upon absorption of a photon, the retinal isomerizes and induces a conformational change in the
54 protein, which in turn initiates the ion transport or signal transduction activity of the rhodopsin
55 (21). The first eubacterial rhodopsin identified was the proton-pumping proteorhodopsin (PR)
56 found in a marine metagenomic survey (5). Homologs of PR and other microbial rhodopsins,
57 such as bacteriorhodopsin (22), halorhodopsin (23), sensory rhodopsin (24), and

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58 xanthorhodopsin (XR; (18)) have been identified in many lineages of archaea, bacteria,
59 dinoflagellates, algae and viruses (25). Some estimates of microbial rhodopsin abundance, based
60 on metagenomic sequence analysis, suggest that from 48% to 70% of cells in some marine
61 environments (4, 26) and up to 60% of microbes from estuarine and freshwater habitats may host
62 rhodopsins (7, 27).

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

79 The freshwater actinobacterium, *Rhodoluna lacicola*, has an actinorhodopsin gene, but
80 lacks efficient pathways for CO_2 fixation, and thus relies on organic carbon (29). To investigate

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81 if, under conditions of suitable illumination, the actinorhodopsin could contribute to
82 photoheterotrophy in this organism, we recently cloned actinorhodopsin (*actR*) from *R. lacicola*
83 and overexpressed it in retinal-expressing *Escherichia coli* (30). An amino acid alignment of *R.*
84 *lacicola* ActR with other characterized rhodopsins suggests ActR is a proton-pumping rhodopsin,
85 since it contains the conserved acidic residues required for proton transport and the Schiff base
86 linkage Lys-231 (2). While analysis of homology can suggest rhodopsin protein function, it
87 cannot predict under what conditions the rhodopsin will be expressed nor whether the rhodopsin
88 will be active and functional. To date, no study has characterized the function of the
89 actinorhodopsins, either through heterologous expression or the use of cultivated isolates. It is
90 important to understand the physiological function of diverse rhodopsins in order to correctly
91 determine the contribution(s) of these proteins to metabolic processes within the host organism
92 and to estimate the extent of solar energy utilization in the environment. In this study, we
93 investigate ActR from *R. lacicola*. Functional studies with heterologously expressed *R. lacicola*
94 ActR demonstrate that it is indeed capable of light-activated proton transport. Surprisingly,
95 experiments in *R. lacicola* show that actinorhodopsin is constitutively expressed, but does not
96 pump protons in response to light stimulation until provided with exogenous retinal.
97
98 Methods
99 *Strains and growth conditions.* The red-pigmented actinobacterium *R. lacicola* strain MWH-
100 Ta8^T (1, 2, 29) was grown in 3 g L^{-1} NSY (nutrient broth/soytone/yeast extract; (31)) medium at
101 room temperature with gentle shaking and 8-12 hr natural sunlight. The gene *actR*, encoding
102 actinorhodopsin, was amplified from *R. lacicola* genomic DNA, cloned into plasmid pMCL200
103 as described previously, and sequenced (30). The actinorhodopsin-encoding plasmid (pTAR)

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104 was transformed into *E. coli* epi300 (Epicentre Biotechnology, catalog number EC300105)
105 containing a plasmid for retinal biosynthesis (pRET04; (30)) to create a strain co-expressing
106 ActR and its cofactor, retinal (*E. coli*/pRET04/pTAR). An empty-vector control strain was
107 produced by transforming pMCL200 into *E. coli* epi300 with pRET04.
108
109 *Proton pumping experiments with* E. coli *or* R. lacicola. *E. coli* epi300/pRET04/pMLC200
110 (retinal-expressing cells) and *E. coli* epi300/pRET04/pTAR (retinal- and ActR-expressing cells)
111 were grown in LB with 50 µg mL^{-1} ampicillin, 34 µg mL^{-1} chloramphenicol and 0.2% L-
112 arabinose overnight at 37 ^{o}C with shaking. *R. lacicola* was grown in 3 g L^{-1} NSY at room
113 temperature with 8-12 hrs natural sunlight for 10 days. Cells were harvested by centrifugation
114 and washed once with 10 mM NaCl/10 mM $MgCl_2$/100 µM $CaCl_2$. Cells were concentrated 20-
115 fold (*E. coli*) or 100-fold (*R. lacicola*) in 1 mL of 10 mM NaCl/10 mM $MgCl_2$/100 µM $CaCl_2$
116 and immediately assessed for light-induced proton pumping activity. Initial pH ranged from 5.8
117 to 6.5. An aliquot of concentrated *R. lacicola* was vortexed with 10 µg mL^{-1} all-*trans* retinal
118 (Sigma-Aldrich) and incubated for 2 hrs under illuminated conditions. Following the retinal
119 treatment, cells were harvested by centrifugation, resuspended in 1 mL 10 mM NaCl/10 mM
120 $MgCl_2$/100 µM $CaCl_2$ and immediately assessed for proton pumping activity.
121 The pH was measured using a Sper Scientific pH SD card Datalogger equipped with a
122 Mettler Toledo InLab Micro electrode. Light was provided by a 250-watt halogen lamp placed
123 10 cm from the sample, and the two-min ON/OFF intervals were controlled with a digital timer
124 (Leviton LT112). Irradiance at the sample was ~550 µmol photons m^{-2} sec^{-1}. The pH meter and
125 electrode were protected from direct illumination with a foil shield, and the sample was

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126 incubated in a water bath to prevent heating. Sample was constantly stirred with a small

127 magnetic stirrer.

128 For inhibition experiments, concentrated cell solutions of *E. coli*, *R. lacicola*, or *R.*

129 *lacicola* with retinal were incubated with 100 μM carbonylcyanide *m*-chlorophenylhydrazone

130 (CCCP; Sigma-Aldrich) for 1.5 hrs in the dark, then proton pumping was measured as described

131 above.

132

133 *RNA isolation and reverse transcriptase-polymerase chain reaction (RT-PCR). R. lacicola* was

134 grown in 600 mL cultures of 3 g L^{-1} NSY in constant light (30-420 μmol photons m^{-2} sec^{-1}) or

135 dark. At day 3, 5, 7, and 10 after inoculation, ten milliliters were removed from each flask,

136 centrifuged at 5000 rpm for 45 min at 4 ^{o}C, and stored in RNAlater (Ambion) at -20 ^{o}C until use.

137 For RNA isolation, the RNAlater was discarded and the pellet was washed with 1X PBS. Cells

138 were resuspended in 30 mM Tris/1 mM EDTA pH 8.0/15 mg mL^{-1} lysozyme with 10 μL

139 Proteinase K (Thermo Scientific), vortexed for 10 sec, and incubated at room temperature for 10

140 min with shaking. The remaining RNA isolation procedure was followed per the Qiagen RNEasy

141 Miniprep protocol with beta-mercaptoethanol added to the RLT buffer. Genomic DNA

142 contamination was removed with both an on-column DNAse digestion (Qiagen) and a Turbo

143 DNAse treatment (Ambion). Total RNA was quantified using a Nanodrop spectrophotometer.

144 A primer set was designed to amplify a 330-bp fragment of the *R. lacicola*

145 actinorhodopsin mRNA. The sequence of the forward (sense) primer was 5′ – GGA TAC CGC

146 TAC GTT GAC TGG – 3′ and the sequence of the reverse (antisense) primer was 5′ – GGT

147 AAA CGC CCC AGG TTG – 3′. Total RNA was used to reverse-transcribe actinorhodopsin

148 mRNA into cDNA using the antisense primer and the two-step protocol of the RETROscript Kit

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149 (Ambion). To denature any secondary structure, the RNA extracts and the antisense primer were
150 incubated at 75 ^{o}C for 3 min, then immediately transferred to ice. The final reaction solutions of
151 the reverse transcriptase (RT) Buffer, dNTP mix, RNase Inhibitor, and with (RT +) or without
152 (RT -) MMLV-RT enzyme were added to the denatured template and primer mixture. The
153 reverse transcription steps were 44 ^{o}C for 1 hr, followed by 92 ^{o}C for 10 min. An aliquot of the
154 RT reaction product (2.5 μL) was used as a template for the subsequent PCR. The PCR
155 amplification conditions were: 94 ^{o}C for 1 min, then 26 cycles of 94 ^{o}C for 20 sec, 52 ^{o}C for 30
156 sec, 72 ^{o}C for 30sec, and a final step of 72 ^{o}C for 5 min with Taq polymerase (Sigma-Aldrich).
157 All RT negative reactions showed no bands after the PCR step, indicating no genomic DNA
158 contamination in the samples. As positive RT-PCR controls, the mouse RNA and primers
159 provided with the kit were used. PCR products were electrophoresed on a 2% agarose-TBE gel
160 run at 90 V for 1.5 hrs with a 100-bp standard (Life Technologies SM0241).

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

166

167 *Membrane fraction preparation*. Membranes from either one-liter cultures of *R. lacicola* grown
168 for ten days at room temperature with 8-12 hrs natural sunlight, concentrated *R. lacicola*
169 provided with exogenous retinal as described above, or *E. coli*/pRET04/pTAR were partially
170 purified as described (30). Briefly, the cells were lysed with an osmotic lysis buffer containing
171 lysozyme (0.075 M Tris pH 8.0, 2.0 mM $MgSO_4$, 0.4 M sucrose, 10 mg mL^{-1} lysozyme),

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172 followed by incubation in a high salt buffer (50 mM Tris pH 7.6, 10 mM $MgSO_4$, 0.8 M NaCl)
173 and sonication. After broken cells were centrifuged at 25000 × *g* for 30 min at 4 ^{o}C, the brightly-
174 colored membrane film was removed and resuspended in 3% beta-octylglucopyranoside (β-OG;
175 Amresco) in 10 mM HEPES, pH 7.1 by vortexing overnight at 4 ^{o}C in the dark. The detergent-
176 solubilized membrane was centrifuged at 11000 × *g* for 10 min at 4 ^{o}C to remove insoluble
177 material. Absorption spectra from 250 – 900 nm were recorded using a Thermo Scientific
178 BioMate 3S UV-Visible Spectrophotometer. Membranes prepared in this manner were used for
179 SDS-PAGE, extraction of carotenoids for analysis by HPLC, and oxidation with ammonium
180 persulfate (see below).
181
182 *Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE).* Membrane
183 preparations to be analyzed by SDS-PAGE were incubated 1:1 in 2X loading buffer (250 mM
184 Tris, 2% SDS, 30% glycerol, 10% 2-mercaptoethanol, 0.002% bromophenol blue) for 1 hr at
185 room temperature. Samples were loaded on a 10% Tris-buffered polyacrylamide resolving gel,
186 topped with a 5% polyacrylamide stacking gel and electrophoresed according to the method of
187 Laemmli (32). The gel was washed with DI water, fixed with 10:25:65 glacial acetic
188 acid:methanol:water for 15 min, and stained with LabSafe GEL Blue (G-Biosciences). The
189 molecular weight standard was PageRuler Prestained Protein Ladder 10-170 kDa (Thermo
190 Scientific).
191
192 *Mass spectrometry (MS) identification of actinorhodopsin.* The expected actinorhodopsin band
193 was excised for analysis. The enzymatic digestion procedure was performed with trypsin
194 (Promega) at 37 ^{o}C as previously described (33) and included reduction/alkylation with

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195 dithiothreitol (BioRad) and iodoacetamide (Sigma), respectively. Subsequently, the sample was
196 desalted and concentrated using Ziptips (Millipore) and applied to a target plate with α-cyano-4-
197 hydroxycinnamic acid matrix (Sigma). Data were collected on a 4800 MALDI TOFTOF
198 Analyzer (ABSciex) in positive ion, reflector mode over a mass range of 900-4000 m/z, with
199 internal calibration. Select peaks were further analyzed by MSMS at 1kV with default
200 calibration. The combined MS and MSMS data were submitted to Mascot v2.2 (Matrix Science)
201 and searched against NCBI. The protein identification was made based on a database match of
202 95% confidence or greater, including four MSMS matches with >99% confidence.
203
204 *Pigment analysis by high-performance liquid chromatography (HPLC).* Previous work suggested
205 disruption of the C=N double bond was necessary for retinal release from rhodopsin proteins
206 (34). Membranes, prepared as described above, in 10 mM HEPES pH 7.1 and 3% β-OG, were
207 treated with 100 mM hydroxylamine (Alfa Aesar) on ice for two hrs, then extracted with 7:2
208 acetone:methanol (v/v). Following centrifugation for 2 min at 10000 rpm, the supernatant was
209 removed and dried. Dried pigments were resuspended in methanol and filtered through 0.2 μm
210 polytetrafluoroethylene syringe filters (Thermo Scientific) prior to injection into the HPLC. The
211 HPLC system was a Shimadzu Prominence system with solvent degasser (DGU-20A5),
212 quaternary pump (LC-20AT), and 996-element diode array detector (SPD-M20A) fitted with a
213 Supelco Ascentis reverse-phase C18 column (100 × 3 mm, 3 μm beads; Sigma-Aldrich catalog
214 number 581308-U). Solvent A was methanol:water (3:1 v/v) and solvent B was
215 methanol:dichloromethane (4:1 v/v; (35)). The gradient was as follows (min, %B): (0, 0), (20,
216 100), (40,100) with a flow rate of 0.5 mL min^{-1}. The column was kept at a constant temperature
217 of 30 °C.

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219 *Oxidation of pigments with ammonium persulfate*. Ammonium persulfate has been shown to
220 selectively oxidize carotenoid pigments while leaving rhodopsin-bound retinal intact (36).
221 Membranes prepared from *R. lacicola* in 10 mM HEPES pH 7.1 and 3% β-OG were diluted with
222 the same buffer until the absorbance peaks of the carotenoids were well-resolved. The
223 absorbance spectra were recorded, then 5 mM ammonium persulfate (Amresco) was added. After
224 addition of ammonium persulfate, the pH decreased slightly (on average from 7.4 to 6.8).
225 Following addition of ammonium persulfate, the membranes were incubated in the dark and
226 absorbance spectra were recorded at regular intervals.

227

228 Results

229 In previous work, the gene encoding actinorhodopsin was cloned from *R. lacicola* MWH-
230 Ta8^T and expressed in *E. coli* that co-expressed retinal biosynthesis genes (30). Based on the
231 conserved lysine at position 234, which functions as the Schiff base linkage, we predicted that
232 ActR was capable of binding retinal. The leucine at position 100 was characteristic of green-light
233 absorbing rhodopsins (37), suggesting that ActR would have an absorption maximum in the 490-
234 560 nm range. In addition, actinorhodopsin possesses the conserved residues His-62 and Asp-92,
235 likely as a hydrogen bonded pair, and Glu-103, which are necessary for proton transport through
236 the protein channel in response to light-induced conformational changes (38, 39). Thus, we
237 hypothesized that ActR was a green-light-absorbing, proton pumping rhodopsin with a retinal
238 cofactor. Our previous results showed that when expressed in *E. coli*, actinorhodopsin bound
239 retinal, localized to the membrane, and had a maximum absorbance peak at 528 nm (30).

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240 *E. coli* co-expressing retinal and actinorhodopsin was assayed for light-activated proton
241 transport. Light-dependent decreases in pH were observed in *E. coli* cells expressing ActR, but
242 not in those with an empty vector control plasmid (Fig. 1). This result demonstrated that the
243 retinal-bound ActR translocated protons out of the cell in response to light. In addition, we tested
244 the effects of carbonylcyanide *m*-chlorophenylhydrazone (CCCP), a proton ionophore, on proton
245 pumping in *E. coli*/pRET04/pTAR. In the presence of CCCP, the light-activated decrease in pH
246 was completely abolished (Fig. 1). This result confirmed that protons were the ions pumped out
247 of the cell in response to light.

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

260 Since the gene was constitutively expressed, *R. lacicola* cultures in both mid-exponential
261 and stationary phase were assayed for light-activated proton transport. However, no light-
262 induced pH change was observed in either culture (Fig. 3A). To confirm the presence of the

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263 protein in the cultures that were utilized for proton pumping experiments, membranes were
264 prepared from 10-day cultures by osmotic lysis followed by extraction with β-
265 octylglucopyranoside. Samples were analyzed by SDS-PAGE (Fig. 3B). A band corresponding
266 to the putative actinorhodopsin was extracted from the gel and analyzed by mass spectrometry.
267 The masses of peptides obtained from fragmentation were matches to the *R. lacicola*
268 actinorhodopsin protein sequence, confirming that the actinorhodopsin apoprotein was produced
269 by the cells (Table S1).

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

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286 absorption spectra to retinal oxime (Fig. 3C, bottom trace). Membranes extracted from *R.*
287 *lacicola* grown under a variety of other conditions, including dilutions of full-strength media,
288 constant dark, anoxia, and into very late stationary phase also lacked the retinal pigment (data
289 not shown).

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

300 These results demonstrate that there was no retinal or retinal-like product in the whole
301 cells or membrane preparations of *R. lacicola*, even though the actinorhodopsin protein was
302 present. Since the protein was present but appeared to lack any cofactor, all-*trans* retinal was
303 provided exogenously to concentrated *R. lacicola* cells. Following the retinal treatment, cells
304 were washed several times to remove unbound retinal, then membrane fractions were prepared.
305 The membranes were treated with hydroxylamine, the pigments were extracted with acetone and
306 methanol, then analyzed by HPLC. A peak corresponding to retinal oxime was detected in these
307 preparations, indicating that the exogenously added retinal was incorporated into cellular
308 membranes (Fig. 4A). To confirm that the retinal was bound to the actinorhodopsin apoprotein,

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309 membrane fractions were prepared from cells incubated with all-*trans* retinal, and carotenoid
310 pigments were oxidized using ammonium persulfate (Fig. 4B). In the presence of exogenous
311 retinal, a peak at ~530 nm was revealed after oxidation of the other pigments (Fig. 4C). This
312 peak is characteristic of retinal-bound actinorhodopsin (30).
313 Cultures of *R. lacicola* that were provided with retinal were tested for light-activated
314 proton pumping. When the cells were provided with exogenous retinal, proton translocation was
315 detected. Under illuminated conditions, the pH of the cell solution decreased, while in dark
316 conditions, the pH of the solution increased (Fig. 4D). As in *E. coli*, treatment of retinal-fed *R.*
317 *lacicola* with CCCP abolished light-induced proton transport (Fig. 4D, dashed line).
318
319 Discussion
320 *Actinorhodopsin is a green-light absorbing, proton pumping rhodopsin.* The amino acid
321 sequence of actinorhodopsin from *R. lacicola* suggests it should have an absorbance maximum in
322 the green light range (490-560 nm) due to the leucine at position 100. Additionally, the acidic
323 residues at positions 92 and 103 suggest that it should be capable of proton translocation. As
324 predicted, ActR heterologously expressed in retinal-expressing *E. coli* has a maximum
325 absorbance at 528 nm (30) and pumps protons in response to light (Fig. 1). Similarly, several
326 other characterized XR-family members are capable of proton pumping in response to light (18,
327 20, 28), and all known eubacterial xanthorhodopsin-like rhodopsins encode a leucine at the
328 equivalent position. However, the absorbance maximum of *R. lacicola* ActR is slightly blue-
329 shifted relative to the characterized examples from two *Octadecabacter* species (533±1 nm and
330 535±1 nm; (20)), *Salinibacter ruber* (560 nm; (18)), and *Gloeobacter violaceus* (540 nm;, (19)).
331 While the leucine residue is primarily responsible for spectral tuning to green light, the

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332 additional variability in observed absorbance maxima of different XR-family proteins may be
333 attributed to differences in solution pH (17), which can affect the protonation state of the
334 rhodopsin, and the retinal-binding pocket protein microenvironment (44).

335 The rhodopsins from *S. ruber* and *G. violaceus* have binding pockets on the outside of the
336 protein for the ketocarotenoids salinixanthin and echinenone, respectively (19, 39). Recent work
337 on the *Octadecabacter* strains suggested that even though these rhodopsins did not bind keto-
338 carotenoids, though the amino acids necessary for ketocarotenoid binding are conserved in these
339 proteins (20). Similarly, while the ActR of *R. lacicola* also possesses the glycine residue and a
340 majority of the other amino acids assigned to the keto-carotenoid binding pocket (19, 20), it is
341 unlikely that ActR binds these pigments, since *R. lacicola* lacks both the *crtW* and *crtO*
342 carotenoid ketolases and is thus unlikely to synthesize carotenoids similar to echinenone or
343 salinixanthin (Table S2; (45)).
344
345 R. lacicola *constitutively transcribes actinorhodopsin and accumulates ActR in membrane*
346 *fractions*. Because the conditions required for expression of rhodopsins are often unknown,
347 characterization of their function(s) *in vivo* has been difficult. No research has reported the
348 expression pattern of a XR-like rhodopsin in the producing microbe. A few studies have
349 examined the expression of proteorhodopsin (PR) in cultivated marine bacteria, and have found
350 varying results. In RT-PCR and transcriptomic analysis of *Dokdonia* sp. MED134, PR
351 expression was found to occur preferentially in media with extremely low carbon content and
352 under illuminated conditions (14, 15). However, in a study on the expression of PR in
353 *Candidatus* Pelagibacter ubique HTCC1062, PR expression was constant during logarithmic
354 growth in both light and dark cultures (46). Here, we show that *actR* is constitutively expressed

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355 in *R. lacicola* in our standard laboratory growth media, in cells grown under both light and dark
356 conditions (Fig. 2A). In addition, we detect the ActR protein in membrane fractions prepared
357 from *R. lacicola*, where it appears to be the most abundant protein in the solubilized membranes
358 (Fig. 3B).
359
360 R. lacicola *does not produce the retinal cofactor*. Despite the constitutive expression of
361 actinorhodopsin in *R. lacicola* and production of abundant carotenoids (Fig. 3D) that could serve
362 as retinal precursors, this organism does not appear to synthesize retinal. In whole cells of *R.*
363 *lacicola*, ActR does not function as a light-activated proton pump until provided with exogenous
364 retinal (Fig. 3A and Fig. 4D). This is the first report in a producing organism of a microbial
365 rhodopsin expressed without its retinal cofactor. However, we cannot exclude the possibility that
366 *R. lacicola* produces retinal via an unknown biosynthetic pathway and with unknown growth
367 requirements that do not coincide with conditions found for actinorhodopsin production in
368 culture.
369 There is genomic evidence that other microorganisms may similarly encode a rhodopsin
370 but not retinal biosynthesis genes. Bioinformatic analysis of two nearly-complete genomes
371 assembled from metagenomes and two partial genomes from single-cell sequencing efforts of the
372 uncultivated, abundant marine bacterial clade, SAR86, found all four assemblies lack a pathway
373 for retinal biosynthesis despite containing at least one proteorhodopsin gene (47). Likewise, a
374 draft genome (~97% complete) from a single cell of the acI lineage of Actinobacteria was
375 recently published and found to contain a rhodopsin homolog, but no identified carotenoid
376 cleavage oxygenases (48). The genomes of *Roseiflexus* sp. RS-1 (NCBI accession NC_009523.1)
377 and *Thermus* sp. CCB_US3_UF1 (49) both encode xanthorhodopsin homologs but no predicted

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378 carotenoid cleavage enzymes. On the other hand, the draft genome of another actinobacterium
379 with an actinorhodopsin, Candidatus *Aquiluna* sp. (50) encodes the carotenoid cleavage enzyme,
380 *blh,* necessary for the final step of retinal biosynthesis.
381
382 *Potential physiological role(s) of actinorhodopsin in* R. lacicola. A light-responsive rhodopsin
383 requires the presence of both apoprotein and cofactor. Because *R. lacicola* was isolated from
384 Lake Taihu, China (1), a hyper-eutrophic lake with high bacterial and phytoplankton density
385 (between 10^6 and 10^8 cells ml^{-1};(51)), a variety of cofactors are likely to be available as cells die
386 and lyse. Many organisms, especially those with streamlined genomes, rely on additional
387 molecules produced by community members, emphasizing the importance of community
388 structure in microbial ecology and even in cellular physiology and function. For example, recent
389 research has demonstrated the inability of *Candidatus* Pelagibacter ubique to synthesize certain
390 B vitamins and reduced sulfur compounds that are both required for its growth (52, 53), and
391 other work has found sediment-associated candidate phyla with metabolic dependence on other
392 organisms for essential amino acids, nucleotides, and lipids (54). Since *R. lacicola* shares with
393 *Cand.* Pelagibacter strains a small genome size (1.4 Mb), it is not surprising that the strain also
394 relies on the uptake of organic substances. If members of the microbial community where *R.*
395 *lacicola* lives release retinal, *R. lacicola* could incorporate retinal into actinorhodopsin and pump
396 protons in response to light. This activity would contribute directly to the proton motive force
397 and provide the cells with additional ATP for biomass production or for driving energy-
398 consuming uptake processes. A similar mechanism of scavenging retinal from the environment
399 has been proposed for the proteorhodopsin-containing members of the marine clade SAR86 that
400 lack biosynthetic pathways for retinal (47).

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401 *R. lacicola* cells synthesize so much of the actinorhodopsin apoprotein it appears to be
402 the most abundant protein in membrane preparations. However, because the cells do not
403 synthesize retinal, an alternative role for the actinorhodopsin apoprotein cannot be ruled out.
404 Many microbial rhodopsins oligomerize in the membrane and form large aggregates (55-60).
405 Without a cofactor, actinorhodopsin might still aggregate in the membrane, providing structural
406 stability against membrane stresses.

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

413

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421

422 **Figure Captions**

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423 Figure 1. Proton translocation in response to light by *E. coli* expressing actinorhodopsin. *E. coli*
424 co-expressing retinal and actinorhodopsin (*E. coli*/pRET04/pTAR; solid black line) were
425 resuspended in an unbuffered salt solution (10 mM NaCl/10 mM $MgCl_2$/100 µM $CaCl_2$) and
426 exposed to light. When the light was ON (white bars), the pH of the solution decreased. When
427 light was OFF (gray bars), the pH of the solution increased. *E. coli* with the retinal biosynthesis
428 plasmid and the empty vector pMCL200 (*E. coli*/pRET04/pMCL200; gray line) were monitored
429 for proton pumping under the same conditions, and no proton translocation was observed. *E.*
430 *coli*/pRET04/pTAR were resuspended in 10 mM NaCl/10 mM $MgCl_2$/100 µM $CaCl_2$, treated
431 with 100 µM CCCP, and exposed to light/dark cycles. Treatment with CCCP abolished the light
432 induced proton transport (dashed line).
433
434 Figure 2. Presence of actinorhodopsin mRNA in *R. lacicola*. (**A**) Expression of *actR* was
435 analyzed by RT-PCR. Cell cultures were grown under either 24-hr light or 24-hr dark conditions
436 for ten days. Aliquots of cells were removed on days 3, 5, 7, and 10 and stored at -20 °C in
437 RNAlater until analysis. The partial *actR* transcript was present in both the light and dark
438 cultures at all time points. No genomic DNA contamination was detected in any sample, and a
439 representative example of the RT negative control is presented in the lane marked "RT-". A 100-
440 bp ladder was used for size analysis and is visible in lanes marked L. (**B**) Expression of *rpoB* was
441 monitored by RT-PCR as a loading control. See Figure S1 for images of full-sized gels.
442
443 Figure 3. Actinorhodopsin in *R. lacicola.* (**A**). Proton pumping assay. A stationary phase culture
444 of *R. lacicola* was concentrated and resuspended in an unbuffered salt solution (10 mM NaCl/10
445 mM $MgCl_2$/100 µM $CaCl_2$) and exposed to two-minute cycles of light (white bars) and dark

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446 (gray bars). No proton translocation was observed. No proton pumping was observed for a mid-
447 exponential phase culture either (data not shown). (**B**) ActR protein expression. Cell cultures
448 were grown for ten days. Membrane fractions were purified from the harvested cells and
449 analyzed by SDS-PAGE. Lane 1: protein present in *R. lacicola* membrane fractions. Lane 2:
450 positive control of protein from *E. coli*/pRET04/pTAR membrane preparations. The band
451 corresponding to ActR at ~22 kDa is labeled. See Figure S1 for image of intact gel. (**C**). HPLC
452 chromatograms of pigments extracted from *E. coli*/pRET04/pTAR (top panel) and *R. lacicola*
453 (bottom panel), monitored at 360 nm. Membrane fractions were treated with 100 mM
454 hydroxylamine to hydrolyze the Schiff base linkage, then extracted in acetone:methanol (7:2
455 v/v). Retinal, detected as retinal oxime here, is only present in the *E. coli* culture (top panel). (**D**).
456 Oxidation of pigments in suspensions of *R. lacicola* membranes. Absorption spectra of
457 suspensions measured before (1) and after (2-9) addition of 5 mM ammonium persulfate and
458 incubation for 0, 7, 13, 21, 28, 38, 57, and 160 min, respectively.
459
460 Figure 4. Pigment analysis and proton pumping of *R. lacicola* cultures with exogenous retinal.
461 (**A**). HPLC chromatogram of pigments extracted from membranes prepared from *R. lacicola* fed
462 with retinal and monitored at 360 nm. Membrane fractions were treated with 100 mM
463 hydroxylamine, then extracted in 7:2 acetone:methanol. Retinal was detected as retinal oxime,
464 and the absorbance spectrum is shown in the inset. (**B**). Oxidation of pigments in suspensions of
465 *R. lacicola* membranes containing actinorhodopsin and exogenous retinal. Absorption spectra of
466 suspensions were recorded before (1) and after (2-9) addition of 5 mM ammonium persulfate and
467 incubation for 0, 5, 10, 15, 20, 30, 45, and 126 min, respectively. (**C**). The last three time-points
468 from the pigment oxidation of *R. lacicola* cultures with exogenous retinal (curves 7-9, Fig. 4B)

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469 and *R. lacicola* cultures without exogenous retinal (curves 7-9, Fig. 3D) were averaged, then the
470 spectrum from the unsupplemented cultures was subtracted from that of the retinal-fed cultures.
471 The difference spectrum obtained shows a peak at ~530 nm, corresponding to retinal-containing
472 actinorhodopsin. (**D**). *R. lacicola* expressing actinorhodopsin and provided with exogenous
473 retinal were resuspended in an unbuffered salt solution (10 mM NaCl/10 mM $MgCl_2$/100 µM
474 $CaCl_2$) and exposed to light/dark cycles (solid lines). When the light was ON (white bars), the
475 pH of the solution decreased. When light was OFF (gray bars), the pH of the solution increased.
476 *R. lacicola* provided with retinal was prepared in the same way, and incubated with 100 µm
477 CCCP for 1.5 hours in the dark (dashed lines). Treatment with CCCP abolished the light induced
478 proton transport.
479
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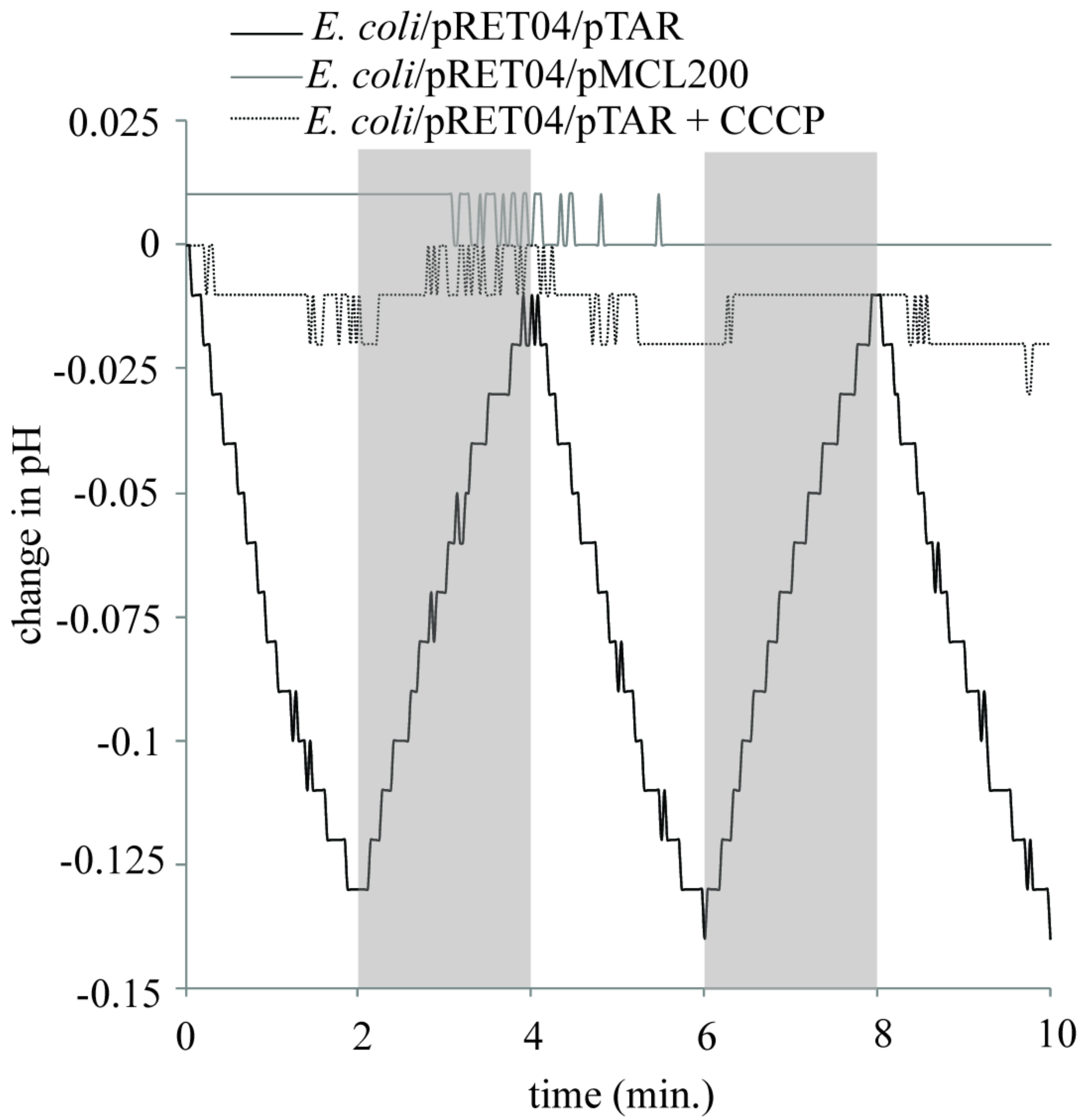

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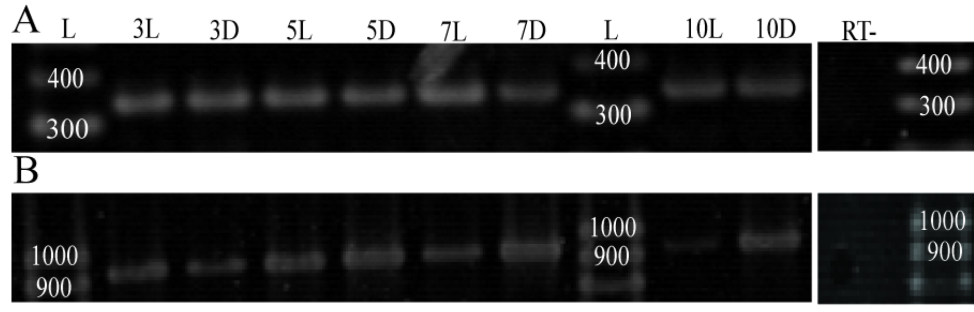

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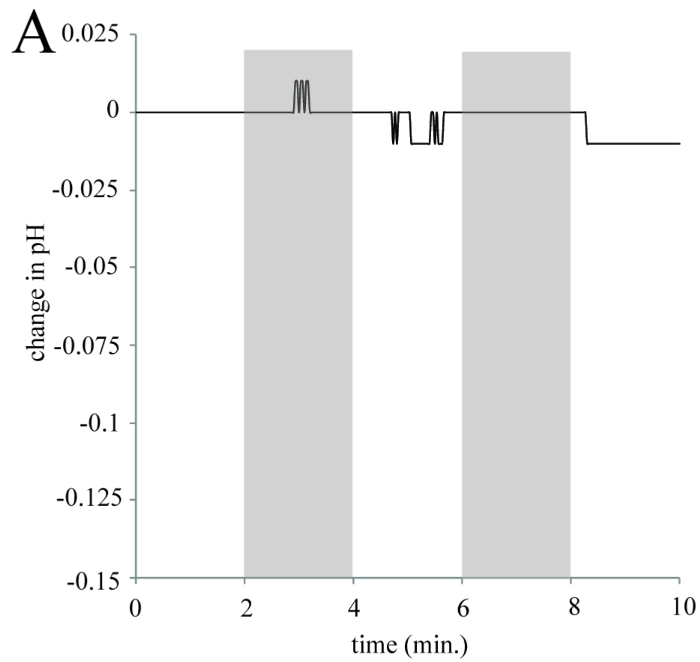

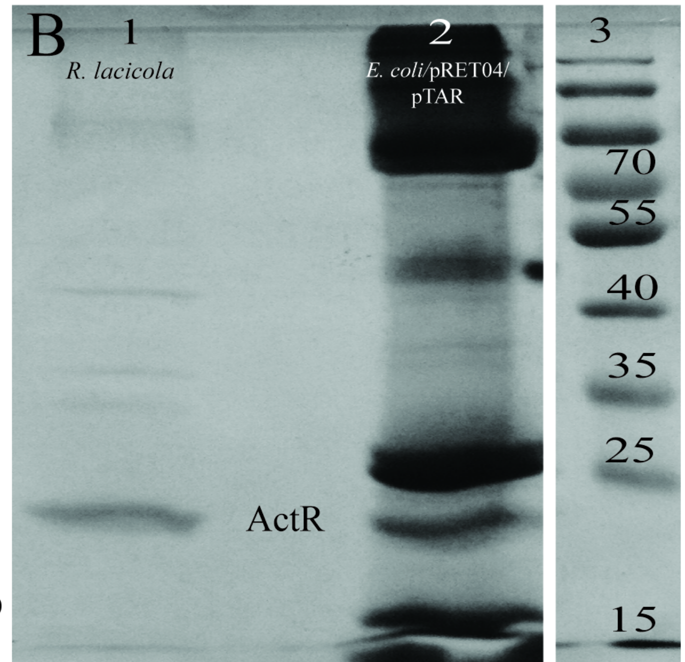

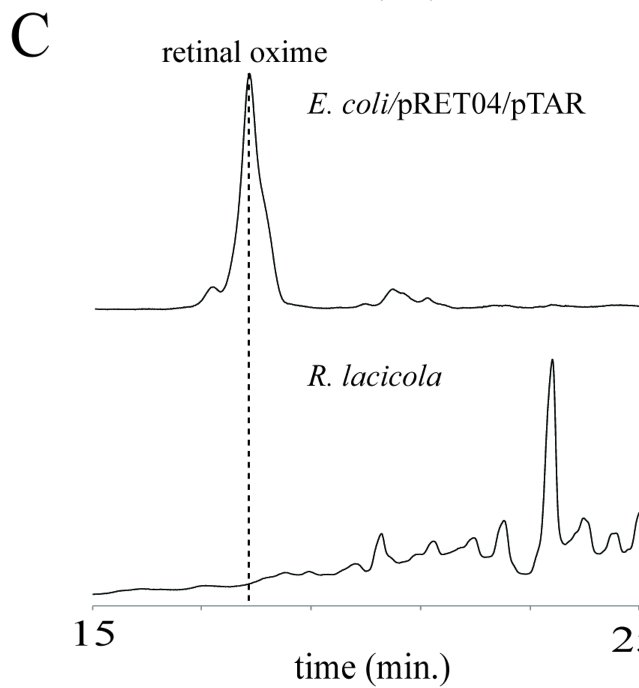

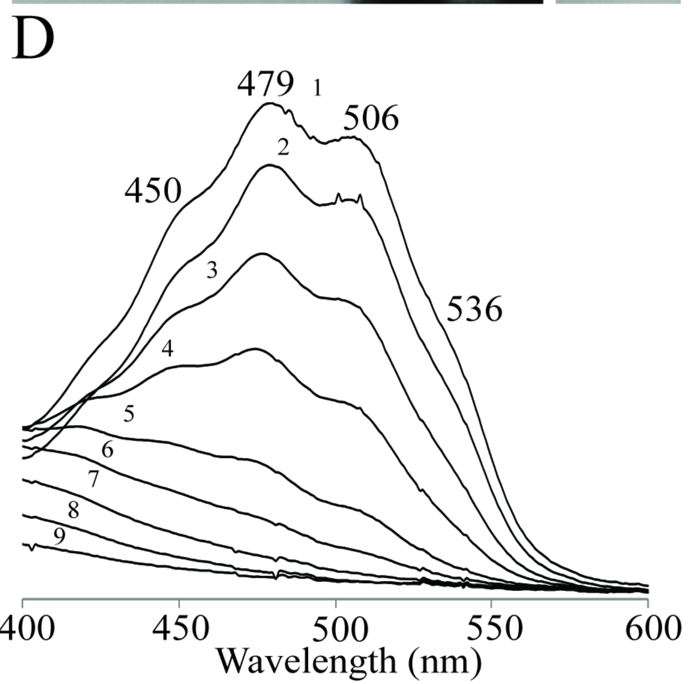

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