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1 The interplay between iron limitation, light, and
2 carbon in the proteorhodopsin containing
3 *Photobacterium angustum* S14

4
5 Coco Koedooder^{1,2*}, Rémy Van Geersdaële¹, Audrey Guéneuguès¹, François-Yves Bouget¹,
6 Ingrid Obernosterer¹, Stéphane Blain¹

7
8 ¹*Sorbonne Université, UPMC Univ Paris 06, CNRS, Laboratoire d'Océanographie*
9 *Microbienne (LOMIC), Observatoire Océanologique, Banyuls/mer, France*

10 ²*The Fredy and Nadine Herrmann Institute of Earth Sciences, Hebrew University of*
11 *Jerusalem, Jerusalem, Israel*

12
13 **Keywords:** proteorhodopsin, Fe-limitation, *Photobacterium angustum* S14,
14 photoheterotrophy

15
16 **Abstract**

17 *Fe-limitation is known to affect heterotrophic bacteria within the respiratory electron*
18 *transport chain, therefore strongly impacting the overall intracellular energy production. We*
19 *investigated whether the gene expression pattern of the light-sensitive proton pump,*
20 *proteorhodopsin (PR), is influenced by varying light, carbon and Fe-concentrations in the*
21 *marine bacterium Photobacterium angustum S14 and whether PR can alleviate the*
22 *physiological processes associated with Fe-starvation. Our results show that the gene*
23 *expression of PR increases as cells enter the stationary phase, irrespective of Fe-replete or Fe-*
24 *limiting conditions. This upregulation is coupled to a reduction in cell size, indicating that PR*
25 *gene regulation is associated with a specific starvation-stress response. We provide*
26 *experimental evidence that PR gene expression does not result in an increased growth rate,*
27 *cell abundance, enhanced survival or ATP concentration within the cell in either Fe-replete or*
28 *Fe-limiting conditions. However, independent of PR gene expression, the presence of light did*
29 *influence bacterial growth rates and maximum cell abundances under varying Fe-regimes.*
30 *Our observations support previous results indicating that PR phototrophy seems to play an*
31 *important role within the stationary phase for several members of the Vibrionaceae family, but*
32 *that the exact role of PR in Fe-limitation, remains to be further explored.*
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* **corresponding author details:**

Coco Koedooder

cocokoedooder@gmail.com

The Fredy and Nadine Herrmann Institute of Earth Sciences, Hebrew University of Jerusalem, Jerusalem,
Israel

36 **1. Introduction**

37 Iron (Fe) is an essential trace element in organisms due to its ability to harbour two major
38 oxidation states, and the various enzymes that require Fe for their functioning are responsible
39 for major cellular processes including photosynthesis, respiration, DNA synthesis and nitrogen
40 fixation (Merchant and Helmann 2012; Ilbert and Bonnefoy 2013). Fe in the ocean is present
41 in various chemical forms and redox states, and experiences a multitude of transformations
42 mediated by chemical, physical and biological processes that will ultimately determine both its
43 solubility and bioavailability towards the microorganisms present within the water column (Lis
44 *et al.* 2015; Blain and Tagliabue 2016; Tagliabue *et al.* 2017). Heterotrophic bacteria, like all
45 other organisms, are affected by Fe-limitation which will subsequently impact the
46 biogeochemical cycling of carbon through their role as principal remineralizers of dissolved
47 organic carbon (Azam, F. *et al.* 1983). Within heterotrophic bacteria, the majority of Fe-
48 containing enzymes (~90%) are located within the complexes of the respiratory electron
49 transport chain in the form of various cytochromes and Fe-sulfur clusters (Holms 1996; Tortell
50 *et al.* 1999; Alberts B, Johnson A, Lewis J 2002; Andrews, Robinson and Rodríguez-Quñones
51 2003; Almaas, Oltvai and Barabási 2005). Consequently, Fe-limitation, has been shown to
52 particularly affect heterotrophic bacteria in their ability to produce ATP (Tortell, Maldonado
53 and Price 1996).

54

55 Proteorhodopsin (PR), a light-driven transmembrane proton pump, was discovered through the
56 genomic analysis of an uncultivated gammaproteobacterial strain of the ubiquitous SAR86
57 clade (Béjà *et al.*, 2000; 2001). PR requires the chromophore retinal which, in the presence of
58 light, enables it to undergo a conformational change that allows for the absorption and release
59 of a proton across the cellular membrane. The formation of a proton gradient can be harboured
60 for several different physiological functions (Fuhrman, Schwalbach and Stingl 2008), and

61 when coupled to ATP synthase, PR activity can result in the transformation of light energy to
62 ATP. The low energetic production costs of PR in comparison to other light harvesting
63 complexes (Raven 2009; Kirchman and Hanson 2013), together with the widespread
64 prevalence of PR in the surface waters of the ocean (Gómez-Consarnau *et al.* 2019), hereby
65 suggested the presence of an important form of photoheterotrophy (Béjà *et al.* 2000, 2001;
66 Pinhassi *et al.* 2016; Song *et al.* 2020).

67

68 While the biological function of PR as a light-driven proton pump has been well characterized
69 (Martinez *et al.* 2007; Song *et al.* 2020), the exact physiological function and role of PR has
70 only been tested in a handful of cultivatable strains (Pinhassi *et al.*, 2016). Thus far,
71 experiments conducted on culturable PR-containing bacteria (Supplementary Table 1) have
72 shown that the gene expression pattern of PR can differ between strains, and that PR activity
73 can be attributed to a wide range of physiological functions, including the promotion of growth
74 and its use as a survival mechanism under stressful conditions such as periods of carbon
75 starvation and other forms of nutrient limitation (Fuhrman, Schwalbach and Stingl 2008;
76 Pinhassi *et al.* 2016; Song *et al.* 2020).

77

78 Considering that Fe-limitation typically results in a decline in energy levels within the cell
79 (Tortell *et al.* 1999), a possible link may exist between PR-based phototrophy and the ability
80 to enhance energy production within Fe-limited cells (Raven 2009; Marchetti *et al.* 2012, 2015;
81 Sun *et al.* 2017; Cohen *et al.* 2018). The formation of a proton gradient by the PR photosystem
82 does not require Fe, while phototrophic systems containing Type I and Type II reaction centers,
83 such as those found in oxygenic phototrophs, require Fe-containing cytochromes in order to
84 sustain electron flow (Blankenship 2010). The comparison of three closely related *Roseobacter*
85 strains, indicated that the two strains isolated from Fe-replete regions contained a Type II

86 reaction centre (bacteriochlorophyll complex), while the strain isolated from an environment
87 characterised by Fe-limitation (Antarctic waters) contained a PR gene (Sun *et al.* 2017). It was
88 hereby suggested that the presence of a PR gene was a potential adaptation for a Roseobacter
89 strain subjected to low Fe-concentrations. PR genes have also been identified in several marine
90 eukaryotes (Marchetti *et al.* 2012) and were shown to be upregulated under Fe-limitation
91 compared to Fe-replete conditions within both the oceanic diatom *Pseudo-nitzschia granii*
92 and the polar sea-ice diatom *Fragilariopsis cylindrus* (Marchetti *et al.* 2015; Cohen *et al.*
93 2018). Interestingly, this upregulation in both light and dark conditions, did not result in
94 significant differences in the growth rate of *P. granii*. PR was hereby predicted to enable
95 the continued survival of *P. granii* until Fe becomes available again to allow cellular growth
96 (Marchetti *et al.* 2015). Whether a similar prediction can be made for PR-containing bacteria
97 subjected to Fe-limitation has, to our knowledge, not been tested. In this study, we aimed to
98 elucidate the potential role of PR in the presence of Fe-limitation using the model marine
99 bacterium *Photobacterium angustum* S14.

100

101 **2. Material and Methods**

102 **2.1. Strains and Maintenance**

103 A culture of *P. angustum* strain S14 was provided by Prof. S. Kjelleberg (UNSW, Sydney,
104 NSW, Australia) and stored under cryopreservation in the Microbial Observatory of the
105 Laboratoire Arago (MOLA) culture collection (BBCC7451). For the bacterial transformation
106 of *P. angustum* S14, cultures were grown and maintained in ZoBell and LB medium or agar as
107 described previously (Bertani 1951; Oppenheimer H. C. and ZoBell 1952; Koedooder *et al.*
108 2018). *Escherichia coli* strains Π 3813 and β 3914 were used as plasmid hosts for amplification
109 and transformation by bacterial conjugation respectively (Le Roux *et al.* 2007). Both *E. coli*
110 strains were grown in LB medium and LB agar at 37°C. When necessary 5 μ g/mL of

111 chloramphenicol (Cm) for *P. angustum* S14 and 25 µg/mL for *E. coli* strains, thymidine (0.3
112 mM) and diaminopimelate (0.3 mM) were added as supplements to the medium. Induction of
113 the P_{BAD} promoter was achieved through the addition of 0.2% L-arabinose to the growth
114 medium. Conversely, its repression was obtained by the addition of 1% D-glucose.

115

116 For the experimental setup, all strains and genetic constructs were grown in 30 mL Thermo
117 Scientific™ Nalgene™ polycarbonate bottles containing 12 mL Aquil medium supplemented
118 with 6 mM[C] glucose and 5.4µM Fe:EDTA (1:1 ratio, Price *et al.* 1989) and placed in the
119 dark at 20°C, agitated with a stirring rate of 110 rpm. Batches were refreshed at least 2 times
120 prior to the start of the experiment (25 µL of a culture was transferred to a fresh 12 mL batch
121 of Aquil medium every 2 days) in order to remove any traces of Fe and carbon that could be
122 carried over from growth on Zobell or LB medium.

123

124 **2.2. Genetic Constructs**

125 Construction of both a PR gene bioreporter (ptr-luc) and a PR gene knockout (Δ pr) was
126 performed in *P. angustum* S14 by allelic exchange using the R6K based pSW7848T suicide
127 vector, as described previously (Le Roux *et al.* 2007; Val *et al.* 2012; Lemire *et al.* 2015;
128 Koedooder *et al.* 2018). Briefly, the pSW7848T plasmid contains the gene insert for allelic
129 exchange which, for the construction of the gene knockout, consists of the upstream and
130 downstream region (500 bp) that encompasses the PR gene of *P. angustum* S14 (EAS63552.1)
131 and, for the construction of the bioreporter, consists of the PR gene fused together with a
132 luciferase gene. The pSW7848T plasmid further contains the RP4 replication origin oriT, the
133 pir-dependent R6K replication origin oriR6K and the resistance gene for the antibiotic Cm, as
134 a selection marker. For the construction of Δ pr, the pSW7848T plasmid additionally contains

135 the inducible CcdB toxin which is under control of the P_{BAD} promoter and induced in the
136 presence of arabinose. The assembly of the plasmid construct was performed according to the
137 Gibson Assembly protocol (New England Biolabs, NEB) according to the manufacturer's
138 instructions and inserted into the *E. coli* strain Π3813 for amplification by electroporation.
139 After amplification, the plasmid was extracted and purified prior to its insertion into *E. coli*
140 strain β3914 (Le Roux *et al.* 2007; Lemire *et al.* 2015). The plasmid was subsequently
141 transferred by conjugation from *E. coli* β3914 to the recipient *P. angustum* S14 by a filter
142 mating procedure as described previously (Le Roux, Davis and Waldor 2011) with a
143 donor/recipient ratio of 1:10 ml. The plasmid is integrated into *P. angustum* S14 via
144 homologous recombination and selected for in media containing Cm. The gene knockout Δpr,
145 which contains the P_{BAD} promoter, undergoes a second homologous recombination enabling
146 the removal of the plasmid backbone which is selected for in media containing arabinose. The
147 formation of Δpr and ptr-luc was verified via PCR for the presence or absence of the gene
148 fragment and by sequencing the gene products with the wildtype serving as positive or negative
149 control (Supplementary Fig 1).

150

151 **2.3. Trace metal-controlled conditions**

152 All experiments were conducted under trace metal clean conditions in a clean room (class
153 10000) and under a laminar flow hood (ADS class 100; Morel *et al.* 2008). Briefly, all plastic
154 materials were made trace metal free by soaking them for 24 h in 10% HCl. This was followed
155 by rinsing and soaking the materials for another 24 h in an ultra-pure water (UPW) bath that
156 was produced with an 18.2 MΩ cm, Elga system equipped with a 0.2 μm final filter (Thermo
157 Fisher Scientific). Nalgene™ polycarbonate flasks dedicated to the culture experiments were
158 additionally microwaved 3 times at 750 W until boiling with UPW. All plastic materials were
159 placed under the laminar flow hood to dry and sterilized by UV light (30 min) before use. Aquil

160 medium was prepared according to Price et al. (1989) where Synthetic Ocean Water (SOW) is
161 passed through a column containing a Chelex 100 resin (Bio-Rad) in order to remove all trace
162 metals including Fe (Davey *et al.* 1970). Carbon sources were chelexed separately to form
163 stock solutions of 3 M[C] and were added to the medium as single carbon sources (6 mM[C]
164 final concentration). SOW was filtered (0.2 μm) and further sterilized by microwaving it 3
165 times at 750 W until boiling before the addition of nutrients, vitamins and metals all filtered
166 through a 0.2 μm syringe Acrodisc filter (Pall Corporation). Fe-concentrations in the medium
167 were derived from a stock solution of FeCl_3 complexed with EDTA at a 1:1 ratio for at least
168 24 h before being subsequently filtered (0.2 μm) and added to the Aquil medium (5.4 μM final
169 concentration).

170

171 Bacteria were grown in 30 mL Thermo Scientific™ Nalgene™ polycarbonate bottles with 12
172 mL of Aquil medium containing 6 mM[C] glucose and transferred into fresh growth medium
173 every 2 days with a starting dilution of approximately 1.8×10^4 cells mL^{-1} . When Fe-
174 contamination could not be accounted for, which occurred when using medium containing Cm
175 or luciferin, the addition of 50 μM of the strong Fe-chelator Desferrioxamine-B (DFOB) was
176 used to obtain Fe-deplete conditions in *P. angustum* S14. An overview of all the conducted
177 experiments and their aim can be seen in Table 1.

178 **2.4. Ptr-luc PR gene expression experiments**

179 The bioreporter ptr-luc was used to measure the gene expression of PR over time via
180 luminescence (arbitrary units, a.u.) under various different conditions. Ptr-luc was grown under
181 different wavelengths of red (570-638 nm), green (485-570 nm) and blue light (434-502 nm),
182 and light intensities (0, 15, 30, 60 $\mu\text{mol quanta m}^{-2} \text{s}^{-1}$) using light-emitting diodes (LEDs)
183 coupled to a ventilation system to regulate temperature, and grown under different single

184 carbon sources (acetate, glucose, serine) in varying concentrations (60, 600, 6000 $\mu\text{M}[\text{C}]$). Fe-
185 deplete conditions were obtained through the addition of 50 μM DFOB.

186

187 Luminescence was measured every hour (5 s per well) using an automated microplate
188 luminometer (Berthold LB Centro LB; Thommen *et al.* 2015). The obtained luminescence
189 measurements were corrected for by subtracting the observed background noise derived from
190 the blanks (Supplementary Fig 2) from the sample measurements. In order to obtain a
191 luminescence signal with a high enough resolution for the observation of possible changes
192 during the start of the experiment, ptr-luc was diluted in Aquil media to a starting concentration
193 of $\sim 1.0 \times 10^6$ cells mL^{-1} . 200 μL of the culture was pipetted into each well of a clear-bottom
194 black 96-well microplate (Greiner Bio-One) containing luciferin (final concentration 250 μM)
195 and covered with a clear diamond film seal (Thermo Fisher ScientificTM). Bacterial cell counts
196 were performed on samples fixed with formaldehyde (0.2% final concentrations) and stained
197 with SYBR Green I (15 min) using a Becton Dickinson Accuri C6 flow cytometer (Marie *et*
198 *al.* 1997).

199

200 **2.5. Wt and Δpr experiments**

201 In order to address the question of how PR gene expression can affect growth under different
202 light and Fe regimes, comparative experiments were conducted between the *P. angustum* S14
203 wildtype (wt) and Δpr . The strains were grown at a starting abundance of $\sim 1.8 \times 10^5$ cells
204 mL^{-1} in a range of blue light (LEDs; 434-502 nm range) intensities (0, 15, 30 and 60 μmol
205 $\text{quanta m}^{-2} \text{s}^{-1}$) and Fe concentrations (5.4 μM , 0.54 μM , 54 nM, 5.4 nM).

206

207 For the construction of bacterial growth curves, cell counts were performed as described above.

208 In parallel, the mean cell volume (μm^3) was determined for several time points using a coulter

209 counter (CASY Cell Counter, Roche) with a 45 μm capillary and a cellular size range between
210 0.7 and 30 μm and analyzed according to the manufacturer's instructions. The mean volume
211 was calculated from the measured particles within each sample ($>0.7 \mu\text{m}$).

212

213 Respiration rates and growth rates of wt and Δpr were obtained from O_2 measurements
214 conducted using a SensorDish reader (Presens) equipped with 24 glass vials of 4.8 mL
215 containing non-invasive O_2 sensors (OxoDish) and placed in an incubator at a constant
216 temperature of 20°C for a period of 24 hours as described previously (Koedooder et al., 2018;
217 Supplementary Text).

218

219 ATP measurements (fmol per 1000 cells) were taken from wt subjected to Fe-replete and Fe-
220 deplete (50 μM DFOB) conditions using the ATP Biomass Kit HS (BioThema) by a
221 luminometer and following the manufacturer's instructions (Lundin, 2000).

222

223 The presence of reactive oxygen species (ROS) from ptr-luc was measured using the
224 CellROX[®] Deep Red reagent according to the manufacturer's instructions (Lifetechnologies,
225 Switzerland) in bacterial cells (Manoil and Bouillaguet 2018). Briefly, CellROX[®] Deep Red
226 is a cell permeable dye that detects oxidative stress in cells by reacting to ROS in the cells and
227 becoming brightly fluorescent upon oxidation (excitation/emission of 640/665 nm). Ptr-luc was
228 grown in their respective media and pipetted into 200 μL wells of a clear-bottom 96-well
229 microplate (Greiner Bio-One) and covered with a clear diamond film seal (Thermo Fisher
230 Scientific[™]). Cells were incubated for 30 minutes with 5 μM CellROX[®] Deep Red and then
231 consequently fixed with formaldehyde (0.2% final concentration) and stained with SYBR
232 Green I (15 min). Fluorescence from CellROX[®] Deep Red was measured from the

233 allophycocyanin (APC)-H channel using a BD FACS-Canto II. The geometric mean was taken
234 and expressed in relative fluorescence units (rfu).

235

236 **2.6. Statistical Analysis**

237 All statistical analyses were conducted in R software (Version 3.5.1) and consisted of a one-
238 way or two-way Analysis of Variance (ANOVA) once the assumptions of normality (Shapiro-
239 Wilk) and homogeneity of variance (Levene Test) were met. A Tukey HSD test was conducted
240 to further elucidate significant interactive results amongst the different conditions*.

241

242 **3. Results**

243 **3.1. PR gene expression patterns in *P. angustum* S14**

244 The PR gene expression pattern in *P. angustum* S14 was determined under different growth
245 conditions using the luminescent bioreporter ptr-luc (Table 1). In order to determine whether
246 PR gene expression was stimulated by a certain wavelength of light, ptr-luc was subjected to
247 continuous dark, red, green and blue light conditions ($30 \mu\text{mol quanta m}^{-2} \text{s}^{-1}$) and the total
248 luminescence was observed over time (Figure 1). A sudden increase in luminescence was
249 observed in both the dark and all light conditions after 8h of incubation. The luminescence
250 continues to increase thereafter in blue light but not in the other light conditions, which
251 indicates that *P. angustum* S14 specifically induces its PR gene under blue light but not under
252 red or green light or in the dark.

253

254 Ptr-luc was further subjected to 12-hour light:12-hour dark or 12-hour dark:12-hour light cycles
255 in order to test how sensitive PR gene expression was to the presence or absence of blue light

* The outcome of all statistical analyses has been deposited under the following github link:
https://github.com/cocokoedooder/STATS_PR.P.angS14

256 (Supplementary Fig 3). As shown previously, an increase in luminescence was observed in all
257 conditions after 8-hours, followed by a further increase of luminescence over time in the
258 presence of blue light that was absent under dark conditions. Importantly, after ~24 hours, PR
259 gene expression was stimulated by the presence of blue light and turning off or turning on the
260 light respectively resulted in a decrease or increase of the luminescent signal.

261

262 When normalizing the total luminescence by cell counts (Figure 2), ptr-luc grown under Fe-
263 replete conditions and continuous blue light, was shown to express a low luminescent signal
264 within the exponential growth phase. Upon approaching the stationary phase at around ~30
265 hours, the luminescence signal increased per cell in both light and dark conditions. The
266 increased luminescent signal became significantly higher in the presence of blue light
267 compared to the dark at ~48 hours ($p=0.0006$) and ~57 hours ($p<0.0001$). Luminescence
268 peaked around ~60 hours and then gradually declined over time in both light and dark
269 conditions. When comparing the growth rates of ptr-luc grown in Fe-replete conditions in the
270 presence ($7.4 \pm 0.1 \text{ day}^{-1}$) or absence ($7.1 \pm 0.4 \text{ day}^{-1}$) of blue light (Table 2), no significant
271 differences were observed ($p=0.9081$). The growth rates further coincided with a maximum
272 cell abundance of $1.31 \pm 0.29 \times 10^8$ cells per mL in the presence and $1.26 \pm 0.09 \times 10^8$ cells
273 per mL in the absence of blue light and was not found to be statistically different ($p=0.2345$).

274

275 In order to test whether the gene expression pattern of PR could be affected by the carbon
276 concentration in the medium, ptr-luc was grown in a range of glucose concentrations (60, 600
277 6000 $\mu\text{M}[\text{C}]$) in the presence and absence of light (Supplementary Fig 4). The gene expression
278 pattern that was observed previously under Fe-replete conditions became less distinct.
279 Specifically, the clear distinction in PR gene expression between light and dark conditions

280 became less pronounced in lower carbon concentrations (600 $\mu\text{M}[\text{C}]$) and was absent in the
281 lowest carbon concentration (60 $\mu\text{M}[\text{C}]$).

282

283 *Ptr-luc* was further grown under Fe-limiting conditions (50 μM DFOB) in order to see if the
284 gene expression pattern of PR would change compared to Fe-replete conditions. Growth rates
285 respectively declined to 4.0 ± 0.1 and 5.1 ± 1.0 (day^{-1}) in the presence or absence of light (Table
286 2), but again were not found to be significantly different ($p=0.1451$). The decline in growth
287 rate respectively coincided with a decline in maximum cell abundance to 0.11 ± 0.08 and 0.11
288 $\pm 0.13 \times 10^8$ cells per mL in dark or blue light conditions which was not significantly different
289 ($p=0.9948$). When looking at the total luminescence normalized by cell counts, the gene
290 expression experiments again revealed that as *ptr-luc* approached the stationary phase at ~ 20
291 hours, an increase in luminescence was observed in both light and dark conditions (Figure 3).
292 Unlike what was observed for Fe-replete conditions, under Fe-limitation, no significant
293 differences in the luminescence per cell was found between light and dark conditions
294 ($p=0.8866$).

295

296 Lastly, in order to determine whether single-carbon sources other than glucose could change
297 the PR gene expression pattern in *P. angustum* S14, *ptr-luc* was grown in either 6mM[C]
298 acetate or 6mM[C] serine in the presence of blue light (Supplementary Fig 5). Compared to
299 glucose, a lower luminescence signal was observed in serine while the signal completely
300 flatlined in the presence of acetate (Supplementary Fig 5). For cells grown in acetate, the low
301 luminescent signal in the presence of light, was due to the absence of cell growth. Under further
302 investigation, the presence of light for cells grown in acetate was coupled to a significantly
303 higher presence of reactive oxygen species (ROS) which doubled in the presence of blue light
304 in comparison to cells grown in the dark (Supplementary Table 2).

305

306 **3.2. Comparison between the wt and Δ pr**

307 Growth experiments of wt and Δ pr in a range of Fe and light concentrations allowed us to
308 establish the effect of a well-defined Fe-concentration gradient regarding growth rates and total
309 cell abundance (Supplementary Fig 6, Table 1). Overall, decreasing Fe-concentrations resulted
310 in a significant and gradual decline in both the growth rate ($p < 0.0001$) and maximum cell
311 abundance ($p < 0.0001$) of both wt (Supplementary Table 3) and Δ pr (Supplementary Table 4).

312

313 In Fe-replete conditions ($5.4 \mu\text{M}$), the growth rates and maximum cell abundance of wt were
314 similar to what was obtained for ptr-luc, reaching values between 7.1 ± 0.1 and 7.4 ± 0.1 (day^{-1})
315 and 1.4 ± 0.1 and $1.1 \pm 0.1 \times 10^8$ cells per mL respectively. These values decreased with
316 lower Fe-concentrations to growth rates between 3.8 ± 0.1 and 4.3 ± 0.2 (day^{-1}) and maximum
317 cell abundances between 0.2 ± 0.1 and $0.3 \pm 0.1 \times 10^8$ cells per mL) under 5.4 nM Fe,
318 respectively. Furthermore, the maximum cell abundance obtained from wt grown under 5.4
319 μM and $0.54 \mu\text{M}$ of Fe was similar to each other, indicating that, at these concentrations, Fe is
320 not considered to be a limiting factor within the medium and that at $5.4 \mu\text{M}$ Fe is in excess. Fe-
321 limitation caused a similar decline in both the growth rate ($p < 0.0001$) and maximum cell
322 abundance ($p < 0.0001$) in Δ pr (Supplementary Figure 6; Supplementary Table 4).

323

324 The presence of light significantly affected both the growth rate ($p = 0.0004$) and maximum cell
325 abundance ($p = 0.0007$) of wt (Figure 4, Supplementary Table 3). Under 5.4 nM of Fe, a small
326 but significantly lower growth rate was observed in the dark compared to 25, 50 and 100%
327 blue light. The opposite response was observed in Fe-replete conditions ($5.4 \mu\text{M}$) where a small
328 but higher growth rate was observed in the dark compared to the light treatments. Under high

329 light intensities, significantly lower maximum cell abundances were observed for wt grown in
330 5.4 μM of Fe ($p=0.0013$). Similar results were obtained from growth experiments conducted
331 on Δpr . A significant interactive effect between light and Fe-concentrations occurred on both
332 the growth rate ($p=0.0001$) and maximum cell abundance ($p=0.0029$) (Supplementary Table
333 4). A Tukey Test revealed that under Fe-replete conditions, no negative effect of light was
334 observed on the growth of Δpr under high light intensities (100% blue light) as was previously
335 shown for wt. Similar to wt, under Fe-limiting conditions an increase in the maximum cell
336 abundance was observed under high light intensities and under 54 nM a significant increase in
337 the growth rate was observed ($p=0.0022$).

338

339 Growth and respiration rates derived from O_2 measurements were taken for both the wt and
340 Δpr grown under Fe-limiting conditions (100 μM DFOB) in the presence and absence of light
341 (Table 3). In the presence of light, no significant differences were observed in the respiration
342 rate ($p=0.999$). Significant differences in the growth rate between the wt and Δpr ($p=0.045$)
343 were observed but concerned a respectively small difference of 0.2 (2.6 ± 0.1 and 2.8 ± 0.05
344 day^{-1}).

345

346 Changes in cell volume were measured for wt grown in both Fe-replete (5.4 μM) and Fe-
347 limiting (50 μM DFOB) conditions in the presence or absence of light over time (Figure 5,
348 Supplementary Table 5). Cell volumes were always slightly lower in constant blue light
349 compared to dark-conditions. The mean cell volume declined over time from $1.44 \pm 0.01 \mu\text{m}^3$
350 and $1.35 \pm 0.01 \mu\text{m}^3$ in Fe-limiting and Fe-replete conditions to $0.76 \pm 0.01 \mu\text{m}^3$ and $0.69 \pm$
351 $0.01 \mu\text{m}^3$ respectively.

352

353 Lastly, ATP measurements were taken for wt grown in Fe-replete (5.4 μ M) and Fe-limiting
354 (50 μ M DFOB) conditions that coincided with cells entering the stationary phase (Table 4).
355 ATP measurements were significantly lower in Fe-limiting conditions compared to Fe-replete
356 conditions, but no significant differences were observed in the presence or absence of light
357 under both Fe-conditions.

358

359 4. Discussion

360 4.1. PR gene expression pattern of *P. angustum* S14 is linked to a starvation state

361 The gene expression pattern of PR is known to differ amongst various bacterial strains as
362 several studies have been able to demonstrate that the PR gene can be either facultatively or
363 continuously expressed within the cell (Supplementary Table 1). Our results established that
364 *P. angustum* S14 facultatively expresses its PR gene as cells approach the stationary phase and
365 that this occurs in both Fe-replete and Fe-limiting conditions. The gene expression pattern of
366 PR in *P. angustum* S14 was similar to other members of the Vibrionaceae family including
367 *Vibrio sp.* AND4 and *Vibrio campbelli*, both of which contain green-tuned PR molecules
368 (Gómez-Consarnau *et al.* 2010; Wang *et al.* 2012; Akram *et al.* 2013). Due to the presence of
369 glutamine in position 105 of its amino acid sequence, the PR present in *P. angustum* S14 is
370 expected to be tuned towards blue light which coincides with our results. Thus far, *P. angustum*
371 S14 is the only known bacterial strain in culture containing a blue-tuned PR molecule of which
372 the PR gene expression pattern has been elucidated.

373

374 In the stationary phase, significant differences in the PR gene expression of *P. angustum* S14
375 were observed in the presence and absence of light and these differences only became apparent
376 under high glucose concentrations of 600 μ M[C] and 6mM[C] but not 60 μ M[C]. For other

377 members of the Vibrionaceae family, significant differences in the PR gene expression in the
378 presence or absence of light have been observed in *V. campbelli* (132 $\mu\text{M}[\text{C}]$; Wang et al.,
379 2012), but not for *Vibrio* sp. AND4 (2 $\text{mM}[\text{C}]$; Akram et al., 2013). While it is important to
380 note that the two experiments are not strictly comparable due to differences in their culture
381 media and carbon source, the carbon concentration present within the medium can be relevant
382 in these expression analyses. In *Dokdonia* sp. MED 134 (Gómez-Consarnau et al. 2007), for
383 example, significant differences in the gene expression of PR in the presence or absence of
384 light only appeared under DOC concentrations ranging between 140 $\mu\text{M}[\text{C}]$ and 1.1 $\text{mM}[\text{C}]$
385 but not in lower (60 $\mu\text{M}[\text{C}]$) or higher (242 $\text{mM}[\text{C}]$) carbon concentrations. Unlike members
386 of the Vibrionaceae family, however, *Dokdonia* sp. MED 134 has been shown to constitutively
387 express PR in the presence of light (Palovaara et al. 2014; Gómez-Consarnau et al. 2016).

388

389 As cells approached the stationary phase, the increased gene expression of PR was coupled
390 with a strong decrease in cellular biovolume over time under both Fe-replete and Fe-limiting
391 conditions. The ability to starkly reduce cellular biovolume is a characteristic trait that occurs
392 when cells are capable of inducing a stringently controlled starvation state upon energy and
393 carbon deprivation (Kjelleberg, Humphrey and Marshall 1982; Nyström and Kjelleberg 1989;
394 Nyström, Olsson and Kjelleberg 1992). The induction of a starvation state is a phenomenon
395 commonly observed in several members of the Vibrionaceae family including *P. angustum*
396 S14 (Hild et al. 2000; Lauro et al. 2009; Lever et al. 2015). The gene expression pattern of PR
397 in both *V. campbelli* and *Vibrio* sp. AND4 has previously been linked to the alternative sigma
398 factor RpoS1 which instigates the induction of a starvation state (Wang et al. 2012; Akram et
399 al. 2013). It is therefore likely that a similar form of regulation may be occurring in *P. angustum*
400 S14 under both carbon-limitation and Fe-limitation.

401

402 Previous experiments have shown that the reduction in cellular biovolume observed in *P.*
403 *angustum* S14 is absent under other forms of limitation such as low phosphorus and nitrogen
404 concentrations (Holmquist and Kjelleberg 1993). Our results, hereby, indicate that Fe-
405 limitation, like carbon limitation, induces a starvation-like state in *P. angustum* S14. The lower
406 biovolume observed in the presence of Fe-limitation was in contrast to what was shown for *E.*
407 *coli* (Hubbard *et al.* 1986) and a small oceanic *A. macleodii* strain (Fourquez *et al.* 2014), the
408 latter of which resulted in a 2-fold increase in its biovolume under Fe-limiting conditions.
409 While carbon-limitation will directly affect energy production within the cell and is known
410 from previous studies to induce a starvation state in *P. angustum* S14 (Holmquist and
411 Kjelleberg 1993), the effect of Fe-limitation on the energy status of the cell is inherently more
412 complex. Fe-limitation is known to indirectly affect energy production within the cell by
413 affecting the synthesis of Fe-containing enzymes. These include enzymes located within the
414 respiratory electron transport chain, and enzymes involved in the TCA cycle and DNA
415 synthesis (Tortell, Maldonado and Price 1996).

416

417 A large fraction of the heterotrophic bacteria are considered to be present in a starvation-like
418 state until specific nutrients become available for them to grow (Burgess 1997; Gray *et al.*
419 2019) and, for members of the Vibrionaceae family, PR is currently considered to be an
420 important mechanism to maintain sufficient energy-levels to keep basal metabolic functioning
421 within the cell under unfavourable and starved conditions (Gómez-Consarnau *et al.* 2010;
422 Akram *et al.* 2013). Extended growth experiments conducted for both wt and Δpr in the
423 presence or absence of light also did not result in any further significant differences which is
424 in contrast to what was observed in *Vibrio sp.* AND4 where an enhanced survival upon
425 starvation conditions occurred by means of a series of dilution experiments (Gómez-Consarnau
426 *et al.* 2010; Akram *et al.* 2013). The effect of light on *P. angustum* S14 in the context of

427 maintenance energy and carbon yield had previously been studied using continuous culture
428 experiments (Courties *et al.* 2015). When correcting for variations in the dilution rates, Courties
429 *et al.* (2015) reported an increase in the carbon yield for cells subjected to light and dark cycles
430 compared to dark controls. It was predicted that higher carbon yields resulted from a lower
431 maintenance coefficient that could be attributed to PR activity although PR gene expression
432 had not been measured in these experiments. As continuous culture experiments aim to target
433 the exponential growth phase, these predictions were in contrast to our finding that PR activity
434 in *P. angustum* S14 specifically occurs as cells enter the stationary phase and appears to be
435 linked to a starvation-like state.

436

437 **4.2. PR gene expression pattern of *P. angustum* S14 grown in alanine and serine**

438 Experiments conducted by Palovaara *et al.* (2014) for *Dokdonia* sp. MED 134 compared the
439 effect of PR under different single carbon sources. Growth of *Dokdonia* sp. MED 134 under a
440 “poor” carbon source such as alanine resulted in a stronger difference in the enhanced bacterial
441 growth- and uptake rates in the presence of light when compared to glucose. In previous
442 experiments, *P. angustum* S14 has been shown to grow on “poor” carbon sources such as serine
443 and acetate (Koedooder *et al.* 2018). When *P. angustum* S14 was grown under carbon sources
444 other than glucose, such as serine and acetate, we hereby predicted that any affect from PR
445 activity in the presence of light may be enhanced. Instead, growth under acetate or serine was
446 hindered in the presence of light and appeared to heighten the susceptibility of *P. angustum*
447 S14 towards photo-oxidative stress as a significant build-up of ROS was observed within the
448 cell that increased in the presence of light. It is well established that the various methods in
449 which a bacterial strain can combat (photo)oxidative stress are strain specific (Cabiscol,
450 Tamarit and Ros 2000). *P. angustum* S14 is particularly known to contain a wide diversity of
451 pathways to counter oxidative stress (Matallana-Surget *et al.* 2012, 2013). Our results

452 emphasize that the sensitivity of ptr-luc grown in acetate and serine towards continuous blue
453 light ultimately outweighed any potential benefit for the cells in terms of PR activity.

454

455 **4.3. PR gene expression pattern of *P. angustum* S14 in Fe-limitation**

456 In *P. angustum* S14, the PR gene expression was shown to increase over time in the presence
457 of both light and dark conditions under Fe-limitation. This is in line with experiments
458 conducted by Marchetti *et al* (2015) which showed that the presence of Fe-limitation increased
459 the gene expression of PR in the oceanic diatom *P. granii* compared to Fe-replete conditions.
460 Importantly, Marchetti *et al* (2015) did not link these expression experiments to a particular
461 growth phase and whether the observed increase in PR gene expression in *P. granii* specifically
462 occurs as cells were entering the stationary phase could not be determined from these
463 experiments.

464

465 Our results highlight that care must be taken when attributing changes in growth rate and cell
466 yield to the presence of PR within the cell. Under Fe-limitation, a small but significant
467 enhanced growth rate and maximum cell abundance was observed in the presence of light
468 compared to the dark. Based on our expression experiments and on further investigation using
469 Δpr , the enhanced growth rate and maximum cell abundance was not due to the presence of PR
470 in *P. angustum* S14. Instead, it is likely due to an enhanced bioavailability of Fe through the
471 photo-reduction of the Fe:EDTA complex within the medium (Sunda and Huntsman 2003;
472 Barbeau 2006). In contrast, under Fe-replete conditions, the presence of light resulted in a
473 slightly lower growth rate and maximum cell abundance which may be attributed to an
474 enhancement of photo-oxidative stress by means of the Fenton reaction (Touati 2000; Dixon
475 and Stockwell 2014; Mullarky and Cantley 2015). Similar growth rates and maximum cell

476 abundances were, for example, obtained for *P. angustum* S14 grown in the presence of both
477 5.4 μM and 0.54 μM of Fe and is an indication that *P. angustum* S14 is grown under Fe-excess.
478 Furthermore, while Fe-limitation clearly reduced ATP concentrations and respiration rates
479 within the cell, no significant differences were observed in *P. angustum* S14 in the presence or
480 absence of light. Similarly, the experiments conducted with *P. granii* did not result in additional
481 differences in the growth rate or maximum cell abundances (Marchetti *et al.* 2015).

482

483 In conclusion, our results contribute to the growing body of PR gene expression studies that
484 have been conducted over the past decade. While our observations strengthen the finding that
485 PR seems to play an important role within the stationary phase for several members of the
486 Vibrionaceae family, the exact role of PR, and in particular under Fe-limitation, still remains
487 unclear. Another potential mechanism by which PR can aid a cell within the stationary phase
488 is in the search and uptake of available nutrients. Copiotrophs are often characterized by a wide
489 diversity in transporters that enables the uptake of various nutrients (Ho, Lonardo and Bodelier
490 2017). Under carbon starvation, *P. angustum* S14 has the ability, for example, to induce a high-
491 affinity uptake system for glucose (Lauro *et al.* 2009; Lever *et al.* 2015) and has also been
492 shown to evoke a chemotaxis response towards several carbon sources (Malmcrona-Friberg,
493 Goodman and Kjelleberg 1990). Both the observed chemotaxis response towards glucose in
494 the presence of carbon-starvation and the ability of *P. angustum* S14 to upregulate high-uptake
495 mechanisms for glucose are all potential physiological functions that could ultimately require
496 the use of PR activity and remains to be further elucidated (Fuhrman, Schwalbach and Stingl
497 2008; Gómez-Consarnau *et al.* 2016).

498

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503

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511

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702

703 7. Table Legends

704 **Table 1.** Overview and aim of the different experiments performed with the *P. angustum* S14
705 wildtype (wt), the PR luminescent bioreporter (*ptr-luc*) and PR gene knockout (Δ pr).
706

707 **Table 2.** Growth rates (μ) and maximum cell abundance of *ptr-luc* grown in Fe-replete and
708 Fe-limiting conditions, in the presence or absence of light. (**n=3**)
709

710 **Table 3.** Growth rates (μ) and cellular respiration rates derived from O_2 measurements of *P.*
711 *angustum* S14 (wt) and Δpr grown in Fe-limiting conditions (100 μM DFOB) and the
712 presence of constant light or dark conditions. Statistically significant values are marked in
713 bold. (n=3)

714

715 **Table 4.** ATP measurements taken at 20 h (coinciding with the exponential phase) for *P.*
716 *angustum* S14 (wt) grown in Fe-replete and Fe-limiting conditions and p-values of a 1-way
717 ANOVA comparing differences between light and dark conditions after a ln transformation in
718 order to meet the assumptions of homogeneity of variance. (n=4)

719

720 8. Figure Legends

721 **Figure 1.** *Ptr-luc* grown in Aquil medium and 6mM[C] glucose subjected to either (a) no
722 light, (b) red-light, (c) green-light and (d) blue-light. Light intensity for all wavelengths was
723 set at 30 μmol quanta $m^{-2} s^{-1}$. An increase in luminescence occurs at ~8 hours and continues
724 to increase in blue light but not in the other light conditions. (n=5)

725

726 **Figure 2.** *Ptr-luc* grown in Fe-replete conditions in Aquil medium and 6mM[C] glucose
727 subjected to either dark (dark red circles) or constant blue light (light red circles; 30 μmol
728 quanta $m^{-2} s^{-1}$). (a) Total luminescence where vertical dashed lines represent the time points
729 when cell counts were taken, (b) cell counts and (c) luminescence per cell. Total
730 luminescence increases after ~25 hours in the presence of both light and dark, which
731 coincides with the time cells are approaching the stationary phase. In the presence of light,
732 luminescence continues to increase and peaks at ~60 hours which coincides with slightly
733 elevated cell counts in the presence of light compared to dark conditions, albeit this is not
734 significantly different. (n=3)

735

736 **Figure 3.** *Ptr-luc* grown in Fe-limiting conditions (50 μM DFOB) in Aquil medium and
737 6mM[C] glucose subjected to either dark conditions (dark blue circles) or constant blue light
738 (light blue circles; 30 μmol quanta $m^{-2} s^{-1}$). (a) total luminescence where vertical dashed
739 lines represent the time points when cell counts were taken, (b) cell counts and (c)
740 luminescence per cell. Total luminescence shows an increase in expression after ~20 hours in
741 the presence of both light and dark, which coincides with the time cells are approaching the
742 stationary phase. No clear difference between light and dark conditions were observed. (n=3)

743

744 **Figure 4.** The relationship between the growth rate (day^{-1}) of *P. angustum* S14 (wt) for a
745 range of Fe-concentrations grown in either 0, 25, 50, 100% blue light (dark grey, grey, light
746 grey and white circles) where 100% light was equivalent to 60 μmol quanta $m^{-2} s^{-1}$. Under
747 low Fe-concentrations (5.4 nM), the presence of light positively influences the growth rate
748 while under high Fe-concentrations (5.4 μM), the presence of light negatively influences the
749 growth rate. (n=3)

750

751 **Figure 5.** Mean cell volume (μm^3) of *P. angustum* S14 (wt) at several time points grown in
752 either 6mM[C] of glucose in Fe-limiting (blue) or Fe-replete (red) conditions and subjected
753 to constant darkness (dark blue or red) or blue light (light blue or light red; 30 μmol quanta
754 $m^{-2} s^{-1}$). At 1 hour, cell size was measured from acclimated cells grown overnight in glucose
755 and Fe-replete conditions. 24 hours coincides with the end of the exponential growth phase

756 and start of the exponential phase. 50 hours coincides with peak proteorhodopsin expression
 757 and 75 hours when the expression of proteorhodopsin is in decline. (n=3)
 758

759 **9. Tables**

Table 1. Overview and aim of the different experiments performed with the *P. angustum* S14 wildtype (wt), the PR luminescent bioreporter (*ptr-luc*) and PR gene knockout (Δpr).

Strain	Fe-regime	Carbon	Light regime	Aim
PR gene expression Pattern				
<i>ptr-luc</i>	+Fe	glucose	dark, red, green, blue	PR gene expression in dark, red, green, or blue light
	+Fe	glucose	dark, 12:12DL, 12:12LD, blue	PR gene expression and sensitivity towards switching-on or switching-off the light
	+Fe, -Fe	glucose	dark, blue	PR gene expression under Fe-replete and Fe-limited conditions
	+Fe	glucose (gradient)	dark, blue	PR gene expression under different glucose concentrations
	+Fe	acetate, serine	dark, blue	PR gene expression under different carbon sources
Comparison between wt and Δpr				
wt, Δpr	Fe-gradient	glucose	blue (gradient)	Effect of light and Fe on growth rates and cell yield
wt, Δpr	-Fe	glucose	dark, blue	Effect of light on growth rates and respiration rates using O ₂ measurements
wt	+Fe, -Fe	glucose	dark, blue	Effect of light on cell volume
wt	+Fe, -Fe	glucose	dark, blue	Effect of light on ATP concentrations

760

Table 2. Growth rates (μ) and maximum cell abundance of *ptr-luc* grown in Fe-replete and Fe-limiting conditions, in the presence or absence of light. (n=3)

Conditions	μ (day⁻¹)	Bacteria (10⁸ cells mL⁻¹)
Fe-rep + dark	7.4 ± 0.1	1.26 ± 0.09
Fe-rep + light	7.1 ± 0.4	1.31 ± 0.29
p-value	p=0.9081	p=0.2345
Fe-lim + dark	4.0 ± 0.1	0.11 ± 0.08
Fe-lim + light	5.1 ± 1.0	0.11 ± 0.13
p-value	p=0.1451	p=0.9948

761

Table 3. Growth rates (μ) and cellular respiration rates derived from O_2 measurements of *P. angustum* S14 (wt) and Δpr grown in Fe-limiting conditions (100 μM DFOB) and the presence of constant light or dark conditions. Statistically significant values are marked in bold. (n=3)

μ (day ⁻¹)	wt	Δpr	p-value
light	2.6 \pm 0.1	2.8 \pm 0.1	p=0.045
dark	2.6 \pm 0.1	3.0 \pm 0.2	p=0.108

Respiration rate
(fmol cell⁻¹ h⁻¹)

light	0.08 \pm 0.02	0.13 \pm 0.02	p=0.999
dark	0.11 \pm 0.02	0.12 \pm 0.03	p=0.993

762

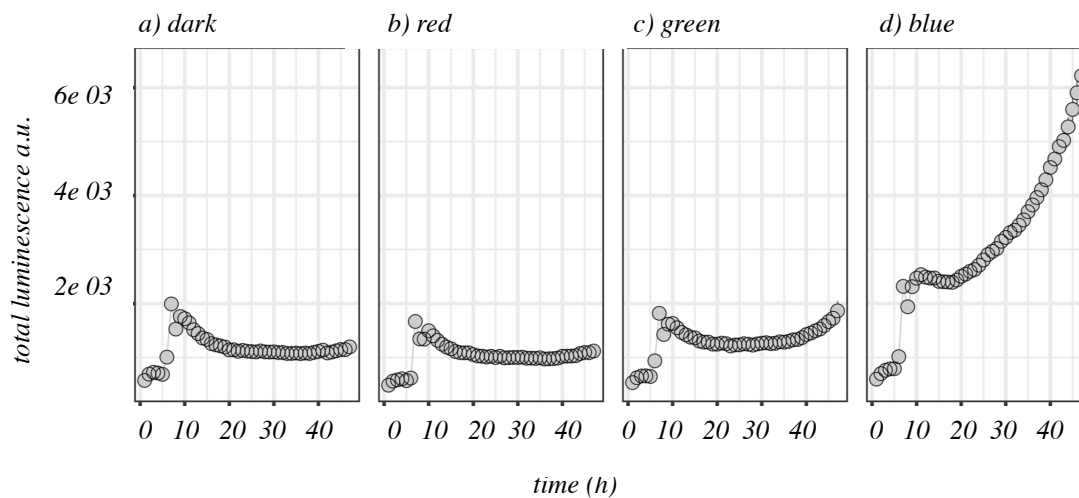
Table 4. ATP measurements taken at 20 h (coinciding with the exponential phase) for *P. angustum* S14 (wt) grown in Fe-replete and Fe-limiting conditions and p-values of a 1-way ANOVA comparing differences between light and dark conditions after a \ln transformation in order to meet the assumptions of homogeneity of variance. (n=4)

ATP (fmol per 1000 cells)	Dark	Light	p-value
Fe-replete	11.8 \pm 1.47	8.2 \pm 2.99	p = 0.1357
Fe-limiting	0.9 \pm 0.04	0.9 \pm 0.05	p = 1.0000

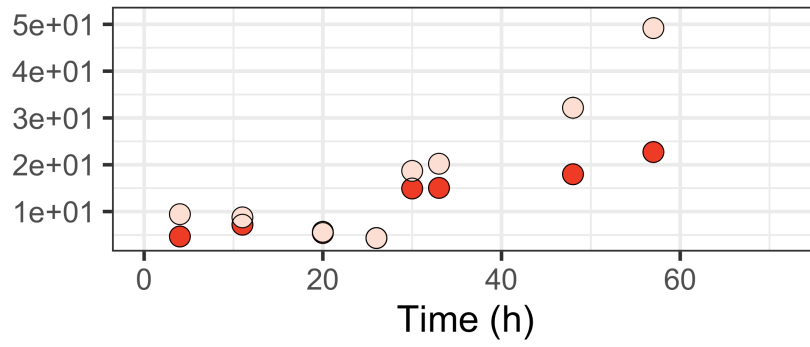
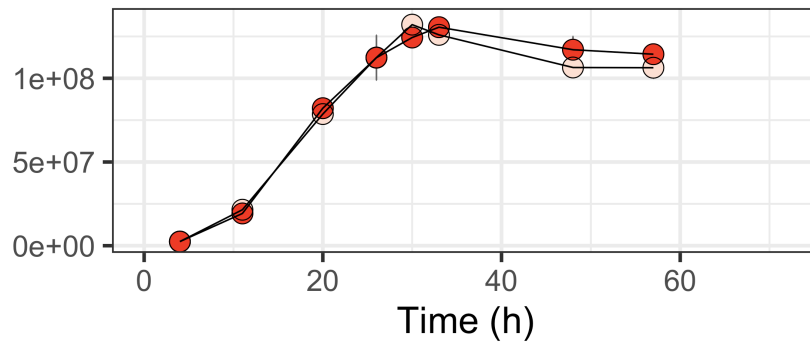
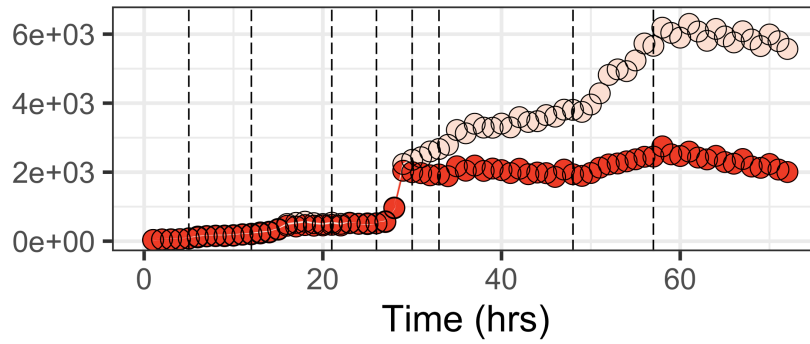
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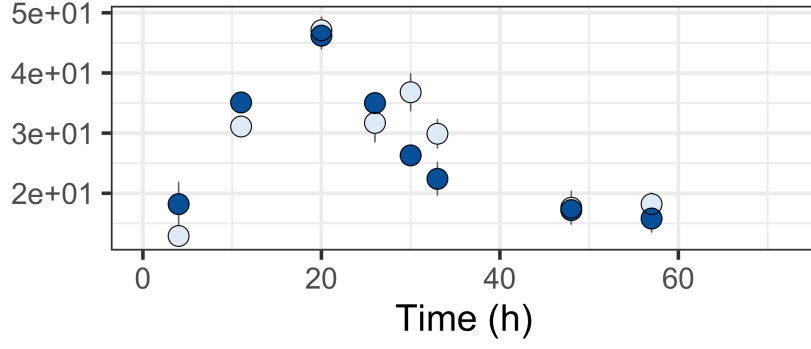
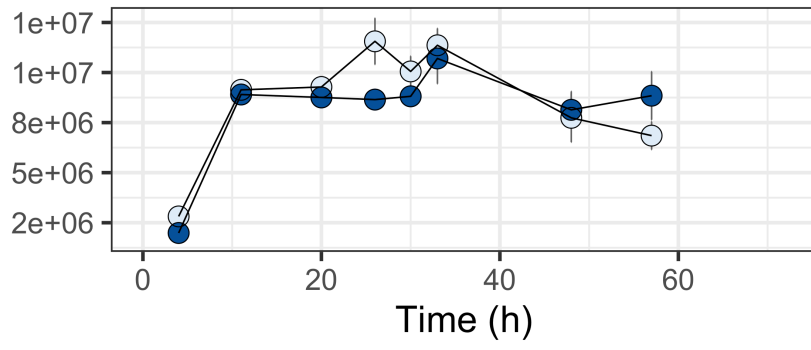
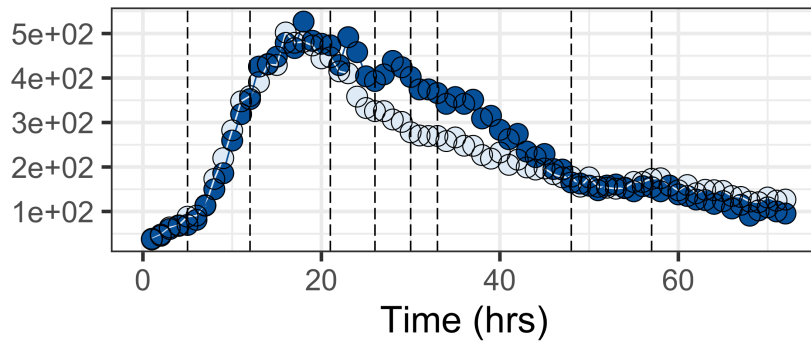
765 **10. Figures**



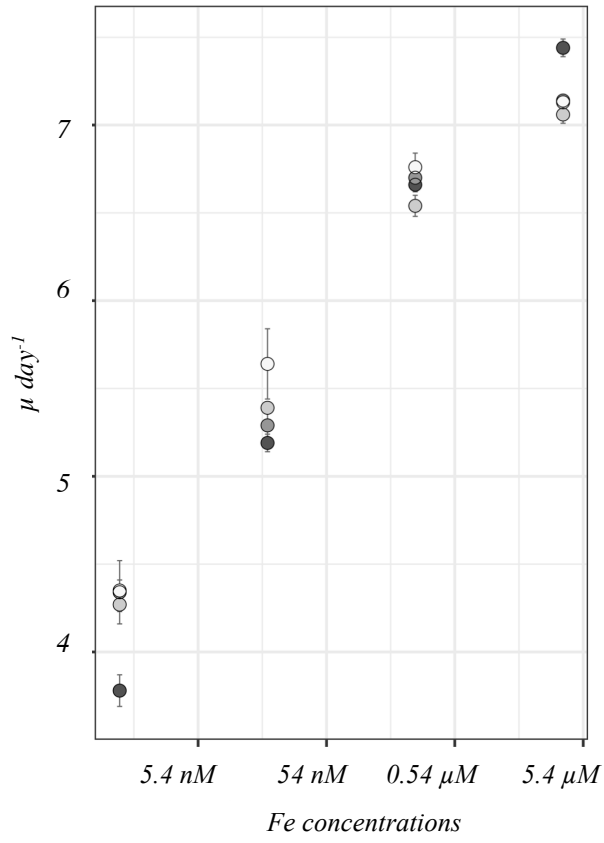
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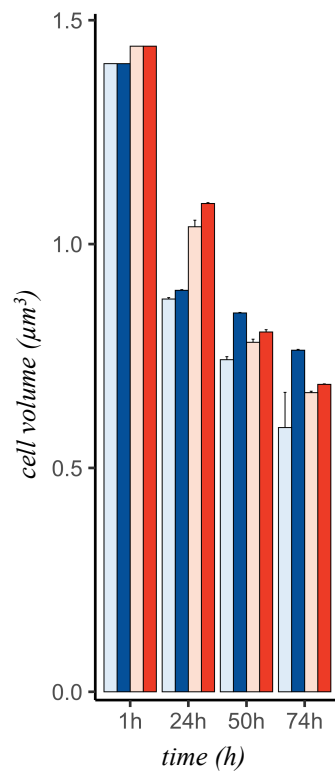
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771 11. Supplementary Information

772 11.1. Supplementary Text

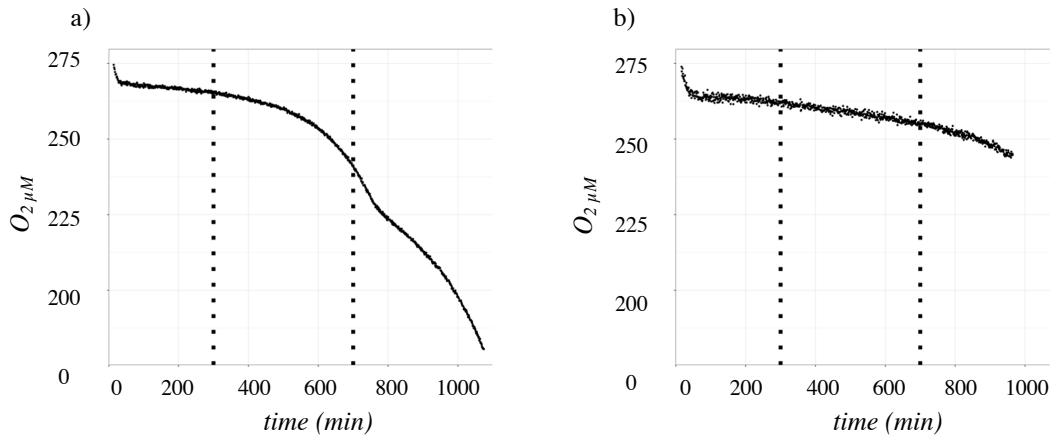
773 *Deriving growth rates and respiration rates from O₂ measurements*

774 When bacterial cells are in exponential growth, both the bacterial respiration rate and growth
775 rate are assumed to be constants. Therefore, if the initial bacterial cell abundance (N_0) is known,
776 both the growth (μ) and respiration rates per cell (R_{cell}) can be derived from continuous
777 dissolved oxygen concentration (O_2) measurements (Figure 15). While the relevance in the
778 growth rate has been explained in the previous paragraphs, the respiration rate is an additional
779 important parameter which provides an indication of the effect of Fe-limitation as 99% of Fe
780 is expected to reside within the electron transport chain of heterotrophic bacteria and during
781 respiration, a large portion of O_2 is consumed through the electron transport chain* where O_2
782 acts as the final electron acceptor.

783

784 O_2 measurements were taken from batch cultures using a SensorDish reader (Presens) equipped
785 with 24 glass vials of 4.8 mL containing non-invasive O_2 sensors (OxoDish). All growth and
786 respiration experiments were conducted in triplicate (where one replicate represents one O_2
787 sensor vial). The entire setup was placed in an incubator at a constant temperature of 20°C. O_2
788 concentrations were derived from 10-min averages of quenched fluorescence measurements
789 taken every 30 s over a period of 24 h. Each vial was calibrated using a two-point protocol. A
790 0 μM O_2 solution was prepared with a 1% Na_2SO_3 in Milli-Q water. A 100% oxygenated
791 solution was prepared by bubbling air at a constant temperature for 2 h in Milli-Q water and
792 left overnight with the lid open to allow exchange with air. O_2 concentrations in Aquil medium
793 were further corrected for by taking into account the salinity of the medium ($S = 35$) using the
794 solubility equation of Garcia and Gordon (1992). Blanks consisting of filtered Aquil medium
795 (0.2 μm) were used to monitor for any significant drift from the O_2 sensors for the entire
796 duration of the experiments (± 24 h). A time window was carefully chosen in order to capture
797 the exponential phase. In order to meet the assumption of a constant growth and respiration
798 rate, care was taken to avoid the inclusion of any metabolic switches (Wolfe, 2005) that would
799 result in a change in the oxygen consumption rate.

* other oxygen consuming entities are also known to occur such as NADH-oxidases but can be considered to be almost negligible in comparison to the electron transport chain.



Supplementary Text Figure i. O_2 consumption curves for *P. angustum* S14 grown in either Fe-replete (a) and Fe-limiting (b) conditions. Notice that two respiration curves can be observed for *P. angustum* S14 grown in Fe-replete conditions. Horizontal lines depict the window that was chosen to calculate the growth and respiration rate.

800 An average of every 10 min was taken to derive the growth and respiration rates from the
 801 selected O_2 measurements. Changes in ΔO_2 concentrations over time were derived from the
 802 integral of the rate of change in O_2 over time using the following equation below:

803
$$\frac{dO_2}{dt} = -N_0 R_{cell} \exp(\mu t)$$

804 The integration of the above equation gives:

805
$$\Delta O_2 = [O_2]_{ti} - [O_2]_{tf} = A (1 - \exp(\mu t))$$

806 with

807
$$A = \frac{-N_0 R_{cell}}{\mu}$$

808 where $[O_2]_{ti}$ and $[O_2]_{tf}$ are the respective O_2 concentrations at the starting time point (ti) and
 809 at the final time point (tf). $\Delta O_2 = f(t)$ was fitted to the equation using sigmaplot software to
 810 derive μ and $A = \frac{-N_0 R_{cell}}{\mu}$. A was then used to calculate R_{cell} .

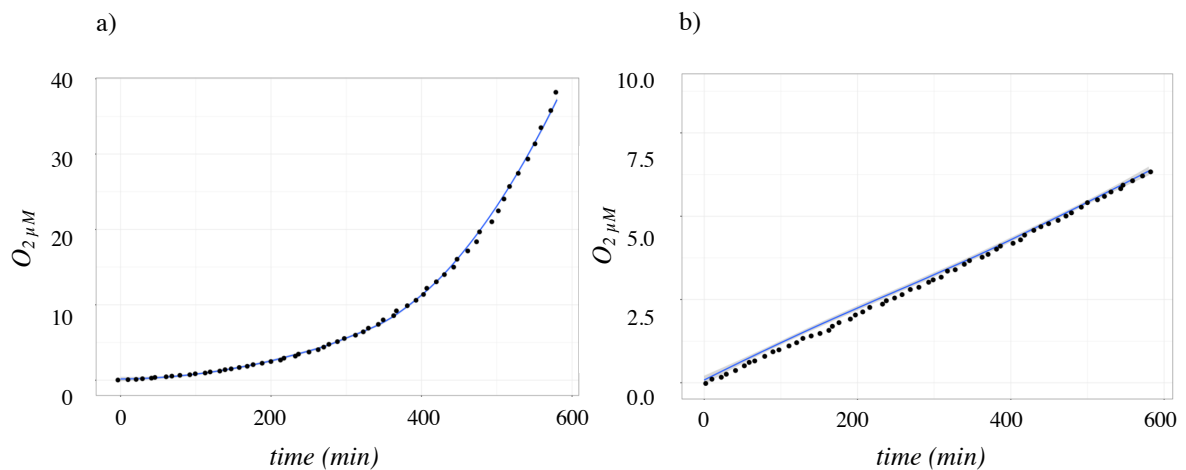
811 Under Fe-limiting conditions, ΔO_2 was small and therefore the equation leads to an
 812 approximately linear relationship:

813
$$\Delta O_2 = N_0 R_{cell} t$$

814 where R_{cell} is derived from the slope of $\Delta O_2 = f(t)$ measurements which in this case can be
 815 defined from the following equation:

816
$$\mu = \frac{\ln(N_f - N_0)}{(N_0 t_f)}$$

817 Where N_f is the final cell abundance at t_f derived from flow cytometry measurements. The
 818 agreement between experimental data and model fitting was then plotted in order to verify that
 819 the main assumptions of the model concerning a constant growth and respiration rate were met
 820 (Figure 16).



Supplementary Text Figure ii. ΔO_2 consumption curves derived from the selected time window for *P. angustum* S14 grown in Fe-replete (left) and Fe-limiting (right) conditions. Fitting of the model (blue) with the experimental values shows the model's prediction for both the growth and respiration rates. The curves meet the assumption of a constant growth and respiration rate.

821

822 **11.2. Supplementary Tables**

Supplementary Table 1. A non-exhaustive list of culture-based studies testing the function of proteorhodopsin in heterotrophic bacteria and a brief summary of their findings. (G-green tuned, B-blue tuned, N-no, Y-Yes, n/a-not available).

Bacterial Isolate	Phylum	PR type	Enhanced Growth?	Additional Findings	References
<i>HTCC2207 (SAR92)</i>	γ -proteobacteria	G	N	- No differences in growth or cell yield	Stingl et al., 2007
<i>Vibrio campbellii AND4</i>	γ -proteobacteria	G	Y	- Longterm survival in sterile seawater - PR expression is determined by nutrient limitation (dilutions) - Sigma factor RpoS plays key regulatory role	Gómez-Consarnau et al., 2010 Akram et al., 2013
<i>Vibrio campbellii BAA-1116</i>	γ -proteobacteria	G	Y	- Longterm survival under respiratory stress (sodium azide) - Enhanced ATP production - Sigma factor RpoS1 plays key regulatory role	Wang et al., 2012
<i>Pelagibacter ubique HTCC1062 (SAR11)</i>	α -proteobacteria	G	N	- Constitutively expressed - No differences in growth or cell yield	Giovannoni et al., 2005 Steindler, 2005
<i>Dokdonia sp. MED134</i>	Bacteroidetes	G	Y	- Upregulated in light - Enhanced growth rate and cell yield under low (0.6 mM[C]) and intermediate (1.1 mM[C]) carbon concentrations - Enhanced Vitamin B1 acquisition - Upregulation of the glyoxylate shunt in light - Upregulation in transporters (alanine and phosphate) - Upregulation of Complex I of the electron transport chain	Gómez-Consarnau et al., 2007 Gómez-Consarnau et al., 2016 Palovaara et al., 2014 Kimura et al., 2011
<i>Nontabens marinus S1-08</i>	Bacteroidetes	n/a	Y	- Enhanced growth rate and cell yield under 0.14 mM[C] - 3 rhodopsin types (H ⁺ , Na ⁺ and Cl ⁻ pumps)	Yoshisawa et al., 2014
<i>Polaribacter sp. MED152</i>	Bacteroidetes	n/a	N	- Enhanced bicarbonate fixation and uptake	González et al., 2012
<i>Psychroflexus torquis</i>	Bacteroidetes	G	Y	- Enhanced growth and cell yield under hyposalinity stress	
<i>ATCC 700755</i>				- Constant expression post transcriptional upregulation by light and salinity - Increased yield in natural Antarctic sea ice in the presence of blue vs green light.	Feng et al., 2013 Feng et al., 2015 Burr et al., 2017
<i>Hymenobacter nivis P3T</i>	Bacteroidetes	n/a	Y	- Enhanced growth and cell yield	Terashima et al., 2018
<i>P. angustum S14</i>	γ -proteobacteria	B	N	- No difference in growth and cell yield - Decrease in Pirt's maintenance coefficient in light under high carbon concentrations - Increase in carbon yield	Courties et al., 2016

823

Supplementary Table 2. Cell counts and ROS measurements taken at ± 96 hours from *ptr-luc* (2n) grown in Fe-replete conditions in Aquil medium and acetate (6 mM[C]) and subjected to either dark conditions or constant blue light (n=2).

Conditions	Bacteria (cells mL ⁻¹)	ROS (r. f. u)	ROS 1000 cells ⁻¹
Acetate dark	8.29 x10 ⁶	7716	0.93
	7.94 x10 ⁶	8276	1.04
Acetate light	3.13 x10 ⁶	15388	4.92
	3.53 x10 ⁶	13919	3.94

824

Supplementary Table 3. Growth rate (day^{-1}) and maximum cell abundance (abund.) of *P. angustum* S14 grown in a range of Fe-concentrations and subjected to either constant darkness (0%-blue) or 25, 50, 100% blue light in a white 96-well plate. ($n=3$).

Growth rate (day^{-1})	0% Blue	25% Blue	50% Blue	100% Blue
5.4 μM	7.4 \pm 0.1	7.1 \pm 0.1	7.1 \pm 0.1	7.1 \pm 0.1
0.54 μM	6.7 \pm 0.1	6.7 \pm 0.1	6.5 \pm 0.1	6.8 \pm 0.1
54 nM	5.2 \pm 0.1	5.3 \pm 0.1	5.4 \pm 0.1	5.6 \pm 0.2
5.4 nM	3.8 \pm 0.1	4.3 \pm 0.2	4.3 \pm 0.1	4.3 \pm 0.1
Abund. (10^8 cells mL^{-1})	0% Blue	25% Blue	50% Blue	100% Blue
5.4 μM	1.4 \pm 0.1	1.2 \pm 0.1	1.3 \pm 0.1	1.1 \pm 0.2
0.54 μM	1.3 \pm 0.1	1.2 \pm 0.1	1.2 \pm 0.1	1.2 \pm 0.1
54 nM	0.7 \pm 0.1	0.7 \pm 0.1	0.8 \pm 0.1	0.6 \pm 0.1
5.4 nM	0.2 \pm 0.1	0.3 \pm 0.1	0.2 \pm 0.1	0.2 \pm 0.1

825

Supplementary Table 4. Growth rate (day^{-1}) and maximum cell abundance (abund.) of Δpr grown in a range of Fe-concentrations and subjected to either constant darkness (0%-blue) or 25, 50, 100% blue light in a black 96-well plate. ($n=3$).

Growth rate (day^{-1})	0% Blue	25% Blue	50% Blue	100% Blue
5.4 μM	6.8 \pm 0.2	7.0 \pm 0.2	6.9 \pm 0.1	7.0 \pm 0.2
0.54 μM	5.8 \pm 0.1	6.2 \pm 0.2	5.7 \pm 0.3	5.4 \pm 0.4
54 nM	4.6 \pm 0.1	4.6 \pm 0.2	4.9 \pm 0.2	5.5 \pm 0.1
5.4 nM	3.3 \pm 0.1	3.3 \pm 0.1	3.3 \pm 0.1	3.2 \pm 0.1
abund. (10^8 cells mL^{-1})	0% Blue	25% Blue	50% Blue	100% Blue
5.4 μM	1.2 \pm 0.1	1.3 \pm 0.1	1.1 \pm 0.1	1.5 \pm 0.2
0.54 μM	1.2 \pm 0.1	1.2 \pm 0.1	1.1 \pm 0.1	1.3 \pm 0.1
54 nM	0.3 \pm 0.1	0.4 \pm 0.1	0.3 \pm 0.1	0.5 \pm 0.1
5.4 nM	0.1 \pm 0.1	0.2 \pm 0.1	0.2 \pm 0.1	0.3 \pm 0.1

826

Supplementary Table 5. Mean cell volume (μm^3) of *P. angustum* S14 at several time points grown in 6mM[C] of glucose in either Fe-replete or Fe-limiting conditions and subjected to constant darkness or blue light. At 1 hour, cell size was measured from acclimated cells grown overnight in glucose and Fe-replete conditions. 24 hours coincides with the end of the exponential growth phase and start of the exponential phase. 50 hours coincides with peak proteorhodopsin expression and 75 hours when the expression of proteorhodopsin is in decline. Significant differences between light and dark conditions for each time point are shown in bold. (n=3).

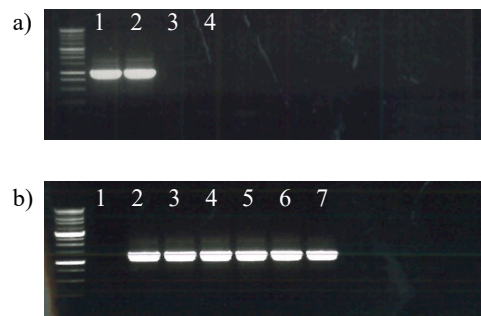
Conditions	1h	24h	50h	74h
Fe-rep + dark	1.44	1.09 ± 0.00	0.80 ± 0.01	0.69 ± 0.00
Fe-rep + light	1.44	1.04 ± 0.02	0.78 ± 0.01	0.67 ± 0.00
		p= 0.0056	p= 0.0707	p= 0.9860
Fe-lim + dark	1.35	0.90 ± 0.00	0.85 ± 0.00	0.76 ± 0.00
Fe-lim + light	1.35	0.88 ± 0.00	0.74 ± 0.01	0.59 ± 0.08
		p= 0.3343	p<0.0001	p= 0.0573

827

828

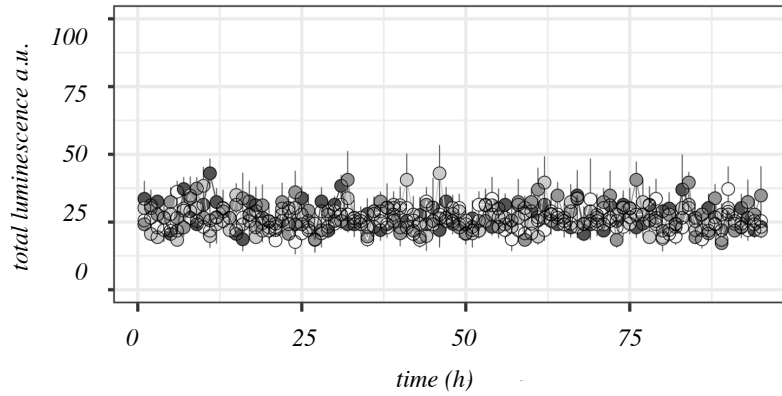
11.3. Supplementary Figures

829



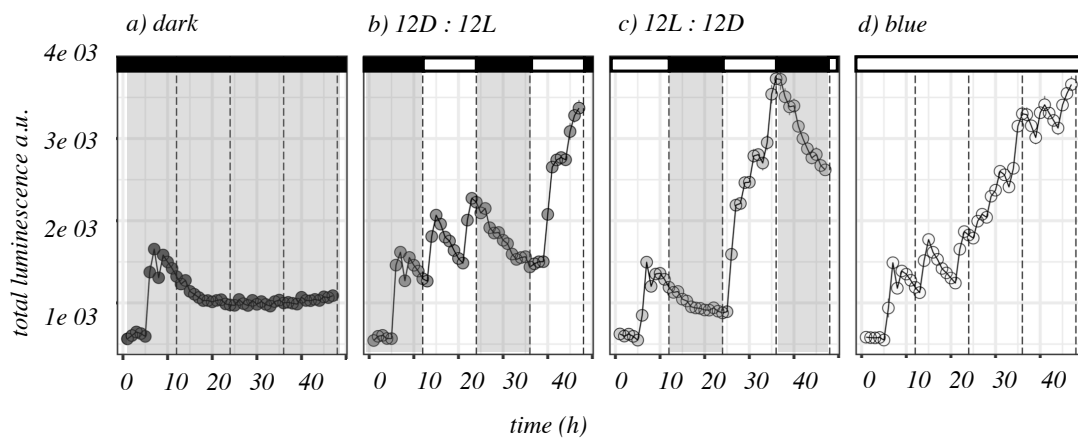
Supplementary Figure 1. PCR results confirming the formation of the mutants Δpr (a) and ptrluc (b). **a)** PCR products of the presence or absence of the proteorhodopsin gene (800bp) where columns 1-2 represents the wt with the presence of the proteorhodopsin gene and columns 3-4 represents the Δpr with the absence of that gene. **b)** PCR products of the proteorhodopsin-luciferin construct ptrluc (1169 bp) where the first column represents the wt (with the absence of luciferin) and columns 2-7 represents ptrluc containing the construct.

830



Supplementary Figure 2. Blanks containing Aquil medium, luciferin (μM) and a range of light concentrations (0, 10, 50, 100 %) placed in a black 96-well plate for a period of 96-hours. The use of a black plate removed the differences found in the presence or absence of light when using a white plate.

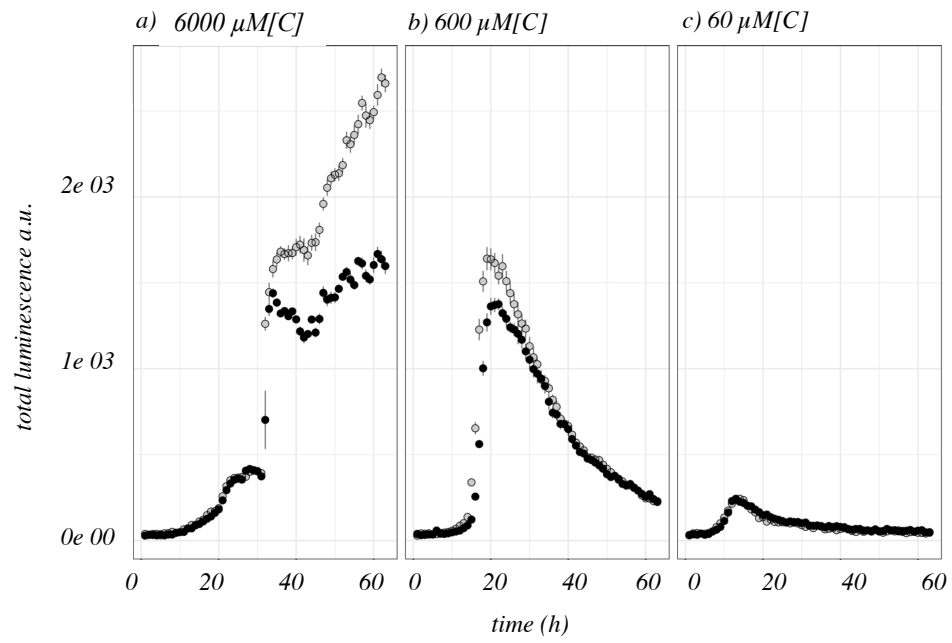
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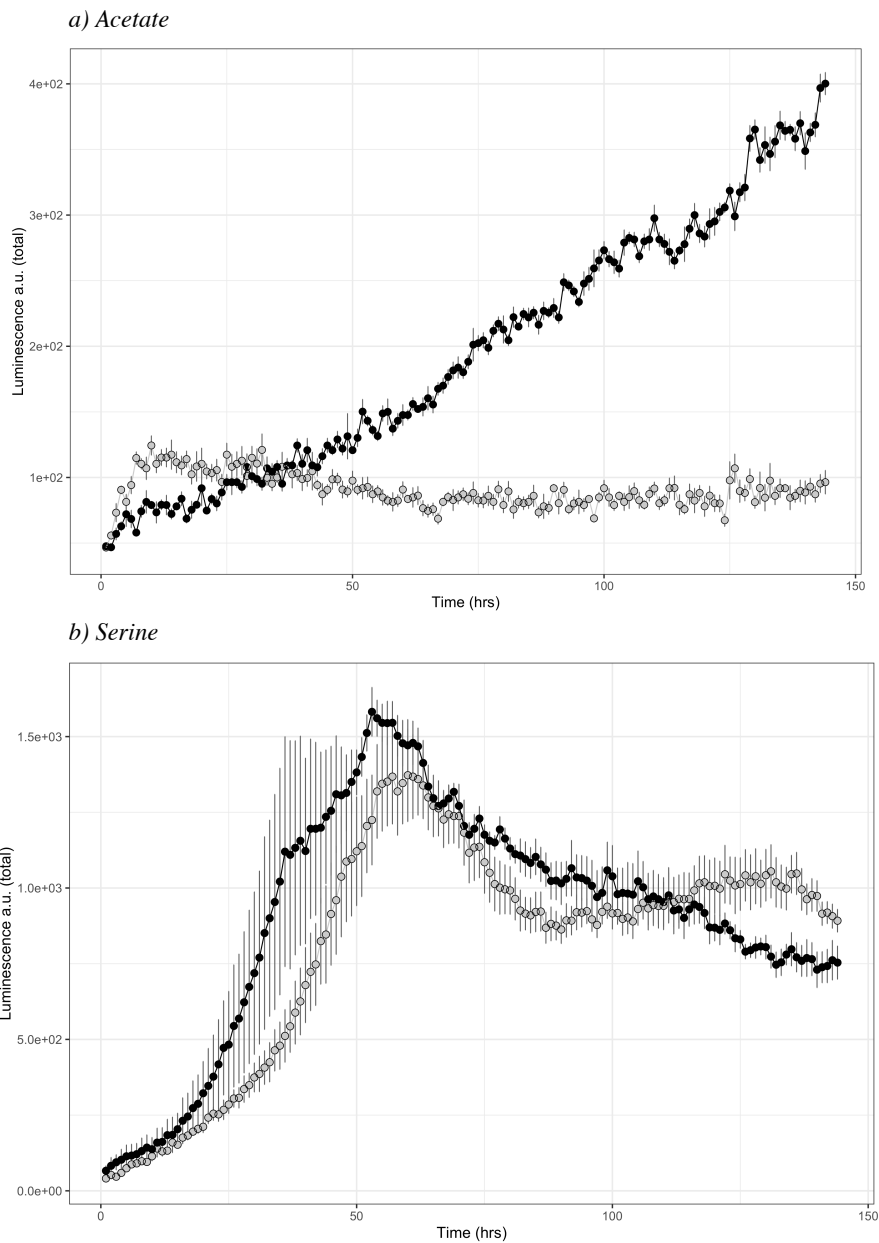
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Supplementary Figure 3. PTR-luc in Aquil medium and 6mM[C] glucose, subjected to either (a) no light, (b) 12 h dark – 12 h light cycles, (c) 12 h light – 12 h dark cycles (12L:12D) and (d) constant blue light (50%). After 8 hours, an increase in luminescence is observed regardless of the presence or absence of light. After 24 hours, respective increase or decrease in luminescence is observed when light is either present or absent. ($n=5$)

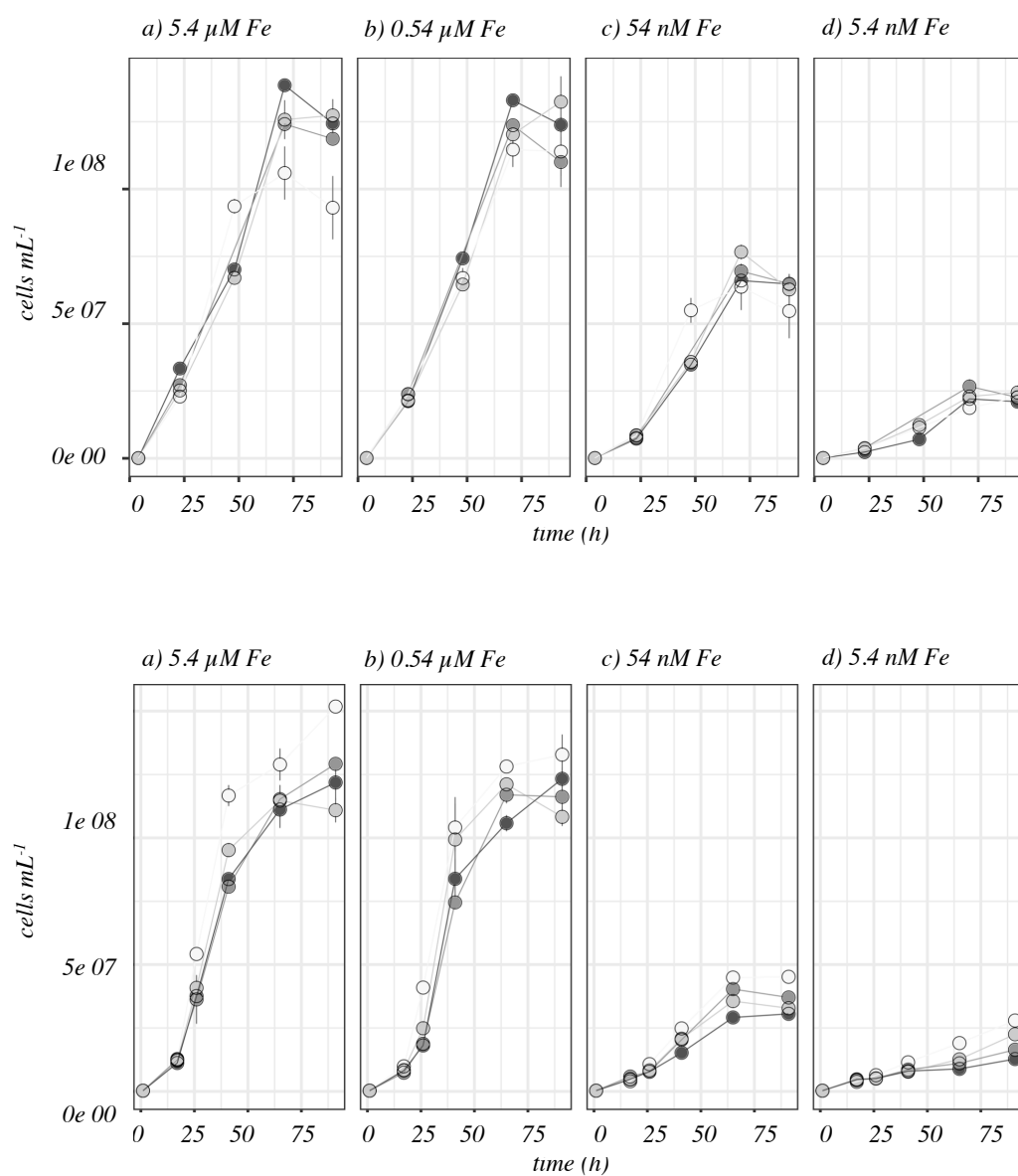
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Supplementary Figure 4. PTR-luc grown in Fe-replete conditions in Aquil medium and (a) (6000 μM[C]) (b) 600 μM[C], and (c) 60 μM[C] glucose and subjected to either dark conditions (grey circles) or constant blue light (white circles). ($n=5$)



Supplementary Figure 5. PTR-luc grown in Fe-replete conditions in Aquil medium containing (a) 6mM[C] acetate (b) 6mM[C] serine and subjected to either dark (black) or constant blue light (grey). The high standard deviation present in serine is likely a result of a high production of EPS which formed particles that interfered with the luminescence measurements.



Supplementary Figure 6. Growth curves of *P. angustum* S14 wt (top) and Δpr (bottom) grown in Aquil with 6mM[C] glucose, subjected to (a) 5.4 μM , (b) 0.54 μM , (c) 54 nM, (d) 5.4 nM of Fe and 0, 25, 50, 100% blue light (dark grey, grey, light grey and white circles). ($n=3$)