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SHORT COMMUNICATION

In situ light responses of the proteorhodopsin-bearing Antarctic sea-ice bacterium, *Psychroflexus torques*

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Proteorhodopsin (PR) is a wide-spread protein found in many marine prokaryotes. PR allows for the potential conversion of solar energy to ATP, possibly assisting in cellular growth and survival during periods of high environmental stress. PR utilises either blue or green light through a single amino acid substitution. We incubated the PR-bearing bacterium *Psychroflexus torquis* 50 cm deep within Antarctic sea ice for 13 days, exposing cultures to diurnal fluctuations in light and temperature. Enhanced growth occurred most prominently in cultures incubated under irradiance levels of ~50 µmol photons $m^{-2} s^{-1}$, suggesting PR provides a strong selective advantage. In addition, cultures grown under blue light yielded over 5.5 times more live cells per photon compared to green-light incubations. Because *P. torquis* expresses an apparently 'green-shifted' PR gene variant, this finding infers that the spectral tuning of PR is more complex than previously thought. This study supports the theory that PR provides additional energy to bacteria under sub-optimal conditions, and raises several points of interest to be addressed by future research.
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Introduction

The protein proteorhodopsin (PR) utilises solar irradiance to translocate protons across cell membranes of many prokaryotes (Béjà *et al.*, 2000, 2001; Finkel *et al.*, 2013), which allows for ATP synthesis. Consequently, the growth-rate under visible light increases for some PR-containing bacteria (Gómez-Consarnau *et al.*, 2007; Palovaara *et al.*, 2014; Gomez-Consarnau *et al.*, 2016), but, surprisingly, not for others (Giovannoni *et al.*, 2005; Lami *et al.*, 2009; Steindler *et al.*, 2011). This variation in response led Fuhrman *et al.* (2008) to suggest that PR may be utilised to buffer environmental stress. Antarctic sea ice harbours wide gradients of temperature (Petrich and Eicken, 2009), salinity (Kottmeier and Sullivan, 1988) and (predominantly blue) light (Petrich and Eicken, 2009; Ryan *et al.*, 2009). When cultured under salinity stresses similar to those in sea-ice brine channels (Arrigo *et al.*, 1997; Petrich and Eicken, 2009), the growth of the Antarctic sea-ice PR-bacterium *Psychroflexus torquis* (Bowman *et al.*, 1998) was stimulated by light (Feng *et al.*, 2013).

PR can be spectrally tuned through the substitution of a single amino acid at PR-position 105 (Béjà *et al.*, 2001; Man *et al.*, 2003). A glutamine ensures maximum absorption at 490 nm, whereas other amino acids shift it to 530 nm (Béjà *et al.*, 2001; Man *et al.*, 2003; Riedel *et al.*, 2010). In sea ice, blue light in upper sections typically favour blue-shifted PR-bacteria (Koh *et al.*, 2010), while green-shifted species populate lower sections, where high chlorophyll concentrations ensure the only available light is green (Koh *et al.*, 2010). To assess how *in situ* conditions affect the PR-response of *P. torquis,* we incubated monocultures within Antarctic sea ice, exposing them to various wavebands and intensities of visible light under natural diurnal variations in temperature and solar irradiance. We expected to observe an increase in growth under illumination, and as *P. torquis* possesses a methionine at position 105 (Bowman *et al.*, 1998), we expected the highest light-induced growth under green light.

Materials and methods

P. torquis ATCC 700755^T was cultured within annual sea ice at Cape Evans, Antarctica (77°38′S, 166°24′E). A 100 µl aliquot of stock culture was inoculated into

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10 ml of sterile Marine Broth 2216 (Becton Dickinson, Auckland, New Zealand) in 15 ml tubes wrapped with polycarbonate filters (Wellington Photographic Supplies, Wellington, New Zealand). Treatments were: ambient photosynthetically active radiation (PAR; no filter), reduced PAR (50% Neutral Density), blue (141-Bright Blue), green (122-Fern Green), red (021-Gold Amber) or darkness (aluminium foil). A series of 5 cm-diameter holes were drilled in 1.7 m-thick annual ice (Kovacs, Roseburg, OR, USA). Each sample was randomly allocated to a single hole and incubated at 50 cm depth within the sea ice for up to 13 days ($N = 5$ per treatment). A one-way analysis of variance (ANOVA; $\alpha = 0.05$) was used to compare each treatment and Tukey_b *post hoc* tests differentiated subgroups within the population. Subgroups were compared again using a secondary series of one-way analysis of variances, thus avoiding the use of harmonic mean group sizes (Smith, 1971).

Table 1 Mean midday irradiance (± SEM) of each treatment at 50 cm depth in Antarctic sea ice (n = 13)

Light treatment (μmol photons m^{-2} s^{-1})					
Ambient	*Reduced*	*Blue*	*Green*	*Red*	*Dark*
103.5 (±1.88)	48.6 (±0.88)	45.6 (±0.83)	54.4 (±0.99)	9.5 (±0.17)	0 (±0)

Measurements were made with a 4-channel broadband sensor—PAR (400–700 nm), blue (430–470 nm), green (495–540 nm) and red (640–680 nm; Skye Instruments, UK). The total light transmittance and percentage transmission of each waveband at 50 cm deep was estimated in similar sea ice at 77°0′S, 162° 54′E in 2009, by lowering the metre angled at 90°, into a hole in the ice, measuring through-ice light scattering. The percentage transmission of each waveband at 50 cm was estimated. The PAR transmitted by each polycarbonate filter was also estimated by comparing ambient PAR measurements to PAR measurements made with each filter directly over the light sensor. The midday light exposure of each treatment at 50 cm depth in the ice was calculated as $E \times d \times f$ for each day, where E = the midday surface PAR during the experiment, d = the proportion of each wavelength that penetrates to 50 cm depth in the sea ice, f = the proportion of PAR intensity transmitted by each filter.

Results and discussion

The mean surface PAR at midday was ~1150 μmol photons m^{-2} s^{-1}, although irradiances fell below this value over daily cycles. The estimated mean midday irradiance (μmol photons m^{2-} s^{-1}) at 50 cm was 103.5 (ambient), 48.6 (reduced), 45.6 (blue), 54.4 (green), 9.5 (red) and 0 (dark), taking account of the differential attenuation of these wavelengths in ice, and the filter transmission properties (Table 1). Thus, reduced PAR, blue and green treatments provided approximately equal photon fluxes at 50 cm depth (analysis of variance $F_{2,39} = 1.55$, $P = 0.22$), at about half that of the ambient treatment ($F_{1,54} = 96.58$, $P < 0.001$), and over five times greater than the red treatment ($F_{1,54} = 124.32$, $P < 0.001$).

The number of live cells in cultures exposed to reduced PAR increased linearly throughout the incubation (Figure 1a), whereas those under ambient light had no additional growth after 10 days ($F_{1,9} = 2.198$, $P = 0.177$), thus yielding ~35% less live cells than reduced PAR treatments at day 13 ($F_{1,8} = 11.6257$, $P = 0.011$). As these treatments had equal abundances at day 10, their nutrient consumption and rates of gas exchange would be similar. Therefore, the increase in light exposure must account for the lower abundance in ambient PAR treatments indicating a photo-inhibitory response somewhere above 48.6 μmol photons m^{-2} s^{-1}. Similarly, Feng *et al.* (2013) observed photo-oxidative stress on *P. torquis* at 27.7 μmol photons m^{-2} s^{-1}. At day 13, reduced PAR treatments contained over 40% more live cells ($F_{1,12} = 11.224$, $P = 0.006$) than in ambient or blue treatments ($F_{1,8} = 0.794$, $P = 0.399$). Green treatments differed from ambient and blue ($F_{1,13} = 29.289$, $P < 0.001$), with only one third of the live cells in reduced PAR samples. Red exposed cultures were similar to dark treatments ($F_{1,8} = 2.730$, $P = 0.137$), both of which yielded even fewer live cells than in green treatments ($F_{1,13} = 35.758$, $P < 0.001$).

The efficiency of each wavelength in promoting cellular growth was visualised by estimating the number of light-generated live cells per μmol photon (Figure 1b). Reduced PAR and blue light were the most efficient treatments ($F_{1,7} = 3.827$, $P = 0.091$) with over three times more light-generated live cells than samples exposed to ambient PAR ($F_{1,12} = 24.476$, $P < 0.001$). Green light however, was much less efficient, with 5.5 × fewer cells per photon than reduced PAR and blue-light samples and ~45% fewer than ambient PAR treatments ($F_{1,8} = 7.856$, $P = 0.023$). This indicates that cellular growth was not directly correlated with spectral intensity.

P. torquis PR contains methionine in position 105 as in other *Bacteroidetes* (Bowman *et al.*, 1998; Koh *et al.*, 2010), suggesting it should preferentially absorb green light (Riedel *et al.*, 2010). Our results are therefore surprising. Blue and green treatments were exposed to a similar number of photons, removing differential photon exposure as a potential contributing factor. Furthermore, reduced PAR incubations were exposed to the same level of irradiation, and yielded a similar number of light-generated live cells as blue-light treatments. Because *P. torquis* is present throughout the entire ice column (Bowman *et al.*, 1998) the utilisation of blue light rather than green would be more beneficial. The preference of this bacterium for blue light suggests that the capacity for PR to absorb a particular wavelength of light (Béjà *et al.*, 2001; Man *et al.*, 2003; Riedel *et al.*, 2010; Mao *et al.*, 2014), is controlled by more factors than this single amino acid shift. One such factor may be the abundance of carotenoids (such as β-carotene and zeaxanthin) within *P. torquis* (Bowman *et al.*, 1998). These pigments have a major spectral absorbance between 425 and 525 nm (Feng *et al.*, 2013). *P. torquis* carotenoids increase under

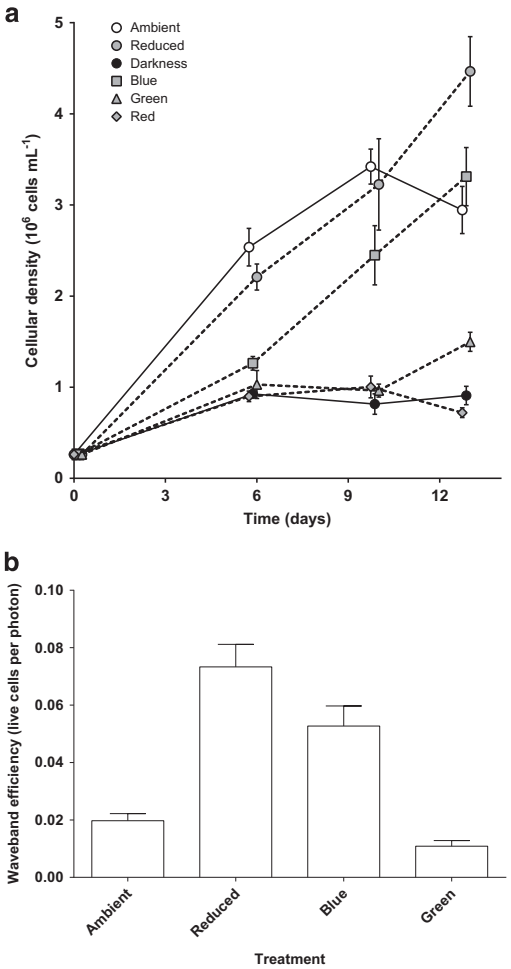

Figure 1 (**a**) Mean number (± s.e.m.) of live *in situ* incubated *P. torquis* cells. Treatments include exposure to ambient photosynthetically active radiation (○), complete darkness (●), reduced photosynthetically active radiation (◐), blue (□), green (△) or red light (◇). Data points are offset (±3 h) for ease of viewing. Following incubation for 6, 10 or 13 days, 2 ml samples were fixed in 1% formaldehyde (Sigma-Aldrich, Auckland, New Zealand) and stored at − 20 °C. After thawing, 1 ml of each was centrifuged (5000 *g*, 12 min), and the pellets air dried for ~10 min. Cells were re-suspended in 350 µl of 0.22 µm filtered phosphate buffered saline (Life Technologies, Auckland, New Zealand) diluted 10 × and 350 µl was transferred to a 5 ml tube containing 35 µl of TruCount bead solution (Becton Dickinson). Each sample was incubated at room temperature for 15 min in 3 µM propidium iodide (Life Technologies). Prepared samples were analysed using a FACSCanto II flow cytometer (Becton Dickinson), acquiring data until 1000 TruCount bead events were recorded. Total cell counts were obtained through particle sizing, and intact and membrane-permeablised cells (live and dead at the time of fixation) were differentiated via the uptake of propidium iodide. (**b**) Mean number (± s.e.m.) of light-generated live cells per µmol photon present in each waveband treatment on day 13. The number of light-generated live cells of each sample was calculated by subtracting the number of live cells present in each light-exposed sample from the mean number of live cells present in dark incubations. The number of live cells under red light was similar to that in darkness, and so the red-light treatment was not included in this analysis. This value was then divided by the irradiance of their corresponding treatment to provide an estimate of the light-generated live cells per µmol photon for each light treatment.

light exposure (Feng *et al*., 2013), and PR function is dependent on the carotenoid derived chromophore, retinal (Béjà *et al*., 2001). *P. torquis* possesses both an unidentified carotenoid and retinal (Feng *et al*., 2013), similar to the retinal protein-carotenoid complex, xanthorhodopsin (Balashov *et al*., 2005). However, xanthorhodopsin has not been reported in *P. torquis*. Therefore, the carotenoids present in *P. torquis* may have a functional effect on the wavelength tuning of PR and merit further investigation.

The vast distribution of PR-bacteria indicates that PR may confer a selective advantage. However, their varied light responses suggest that this advantage is not as simple as increased growth under higher irradiances. The rate of cellular growth and the light–dark differences observed are similar to the experimental work performed by Feng *et al.* (2013). However, the growth differences in light and dark treatments were more extreme than expected, which indicates that environmental stressors such as temperature, may be having a larger influence on PR than initially thought. This work supplements the growing volume of evidence that PR provides additional energy to bacteria growing under sub-optimal conditions (Fuhrman *et al*., 2008; Feng *et al*., 2013). To our knowledge, this is the first study to demonstrate light-enhanced growth of a PR-bacterium using a realistic natural habitat. This insight into spectral tuning provides an exciting area for future research, potentially shifting the current understanding of the ecological significance of PR-bacteria.

Conflict of Interest

The authors declare no conflict of interest.

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