Reactive oxygen species are involved in the morphology-determining mechanism of *Fremyella diplosiphon* cells during complementary chromatic adaptation

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*Fremyella diplosiphon* modifies the pigment composition of its major light-harvesting complexes, i.e. phycobilisomes, and cell and filament morphology according to ambient light quality in a process termed complementary chromatic adaptation (CCA). The cells are red in colour and rectangular shaped, and filaments are longer under green light (GL), in contrast with blue-green, spherical cells and shorter filaments under red light (RL). In this study, we report that wild-type (WT) UTEX 481 and WT-pigmented, shortened filament strain SF33 of *F. diplosiphon* accumulate reactive oxygen species (ROS) under both GL and RL, with the level of oxidative stress being higher under RL as compared with GL. During CCA, higher levels of ROS under RL are correlated with the RL-specific spherical cell shape and filament fragmentation – cells exhibiting elevated levels of ROS under RL have reduced cell length, yet the width of cells is not affected. Addition of ascorbic acid to RL-grown cultures resulted in lower ROS levels and a concomitant shift to GL-associated cellular morphology, i.e. an increased cell length. This observation identifies an RL-dependent oxidative-stress-mediated regulation of morphogenesis in a bacterial system. Spherical cell morphology may result from ROS-dependent changes in the cell membrane integrity or cell wall loosening and associated cell expansion.

INTRODUCTION

Cyanobacteria, the first oxygen-evolving photosynthetic bacteria, are a phylogenetically primitive group of Gram-negative prokaryotes that have a diverse distribution occupying most of the niches in both terrestrial and aquatic ecosystems (Fischer, 2008; Stanier & Cohen-Bazire, 1977). Cyanobacteria are major primary producers and support the food web in both aquatic and terrestrial ecosystems. In addition to biomass production, they can also fix atmospheric nitrogen into accessible forms for themselves, as well as for plants and animals, to produce biomass (Häder et al., 2011; Zehr, 2011). Both photosynthesis and nitrogen fixation are energy-dependent processes driven by solar energy between light wavelengths of 400 and 700 nm.

However, in aquatic ecosystems both light quality and quantity change significantly at different depth levels to affect both of the aforementioned ecologically important processes (Postius et al., 2001). Thus, light is a limiting factor for benthic cyanobacteria, as light absorbed by chlorophyll molecules is reduced significantly at different depth levels. However, these organisms have evolved a major light-harvesting complex called the phycobilisome (PBS) to support photosynthesis in blue- or green-light-enriched benthic environments (Gutu & Kehoe, 2012). Many cyanobacteria have the ability to change the pigment or protein composition of their PBSs in response to available wavelengths of light. This ecologically important phenomenon is known as complementary chromatic adaptation (CCA; Gutu & Kehoe, 2012; Kehoe, 2010).

The PBS, which transfers absorbed energy to the photosynthetic reaction centres within the thylakoid membrane to fuel photosynthesis, consists of pigmented phycobiliproteins (PBPs) and largely non-pigmented linker proteins (Bogorad, 1975). There are three major classes of PBPs: allophycocyanin (AP; $\lambda_{\text{max}}=650$ nm), phycocyanin (PC; $\lambda_{\text{max}}=620$ nm) and phycoerythrin (PE; $\lambda_{\text{max}}=565$ nm). AP

Abbreviations: AP, allophycocyanin; CCA, complementary chromatic adaptation; DCFH-DA, 2′,7′-dichlorodihydrofluorescein diacetate; DIC, differential interference contrast; GL, green light; PBP, phycobiliprotein; PBS, phycobilisome; PC, phycocyanin; PE, phycoerythrin; RL, red light; ROS, reactive oxygen species; RR, response regulator; WL, white light; WT, wild-type.

Supplementary methods and two supplementary figures are available with the online version of this paper.
is contained in the core of the PBS, whereas PC and PE make up the rod component of the PBS that radiates outwards from the core (Kehoe & Gutu, 2006). AP and PC are blue in colour and absorb red light (RL) efficiently, whereas PE is red in colour and efficiently absorbs green light (GL). The ratio of PE to PC in PBSs depends on the ambient light quality, i.e. GL promotes the biosynthesis of PE, whereas RL promotes the biosynthesis of PC (Gutu & Kehoe, 2012).

Based on the characterization of CCA mutants and complementation experiments, a plant phytochrome-related photoreceptor RcaE (regulator of chromatic adaptation E) was identified in the model cyanobacterium *Fremyella diplosiphon* and was shown to regulate CCA under RL and GL (Kehoe & Grossman, 1996; Terauchi et al., 2004). Based on genetic studies, it has been proposed that RcaE controls CCA by acting as a kinase under RL and a phosphatase under GL (Kehoe & Grossman, 1997). In RL-grown cultures, RcaE has been proposed to phosphorylate the cognate response regulator (RR) RcaF, which further activates the RR RcaC by the transfer of a phosphate group. Activated RcaC then acts as an activator for the transcription of genes involved in PC biosynthesis, while acting as a repressor for the transcription of PE genes (Li et al., 2008).

In addition to pigment changes, the morphology of the cells and filaments of *F. diplosiphon* is also altered during CCA. Cells are brick shaped or rectangular and red in colour under green wavelengths of light, while they are spherical and bluish-green under red wavelengths (Bennett & Bogorad, 1973). Furthermore, the length of the filaments is also reduced under RL in comparison with longer filaments under GL (Bennett & Bogorad, 1973; Bordowitz & Montgomery, 2008). So far, studies of the molecular mechanisms employed for regulating CCA have largely focused on changes in pigment content in response to RL or GL. However, insight into the biochemistry and molecular mechanism(s) of the regulation of cell shape and filament length alteration during CCA is emerging (Bordowitz & Montgomery, 2008; Bordowitz et al., 2010; Pattanaik & Montgomery, 2010; Pattanaik et al., 2011b). In this study, we investigated the association of reactive oxygen species (ROS) levels with the regulation of cellular morphology in *F. diplosiphon* during CCA. Here we report that higher levels of ROS under RL are associated with the observed spherical cellular morphology and shorter filaments in *F. diplosiphon* during CCA.

**METHODS**

**Strains and culture conditions.** The wild-type (WT) *F. diplosiphon* UTEX 481, WT-pigmented, shortened-filament mutant strain SF33 (Cobley et al., 1993) and a *rcaE* mutant (Kehoe & Grossman, 1996) were used in this study. Strains were grown under axenic conditions in autoclaved BG-11 medium (Fluka) containing 20 mM HEPES at pH 8.0 under continuous white fluorescent light at an initial intensity of ~15 μmol m⁻² s⁻¹. The experimentally growing cultures, which were diluted to an initial optical density of 0.2 at 750 nm, were transferred to either monochromatic GL or monochromatic RL growth chambers at an intensity of ~10–12 μmol m⁻² s⁻¹ with continuous shaking at 175 r.p.m. at 28 °C. GL and RL sources were those previously reported (Bordowitz & Montgomery, 2008). For filament length measurements, the cultures of UTEX 481 were exposed to 35 μmol m⁻² s⁻¹ GL or RL provided by green LED light bars (λmax at 530 nm; Geneva Scientific LLC) or red LEDs (λmax at 660 nm; LED Wholesalers), respectively. The intensity of the light used in this study was measured with a LI-250 light meter (LI-COR) equipped with a quantum sensor (model LI-190SA).

**ROS detection and antioxidant treatment.** The detection of ROS was performed by using the fluorescent dye 2’,7’-dichlorodihydrofluorescein diacetate (DCFH-DA; EMD chemicals) as described by He & Häder (2002a). Cells were incubated with 10 μM DCFH-DA (final concentration) for 1 h at room temperature in the dark and fluorescence was excited at 520 nm with excitation at 485 nm by using a SpectraMax M2 microplate reader (Molecular Devices). The antioxidant ascorbic acid (Sigma-Aldrich) was added to the cultures at a concentration of 0.5, 1 or 2 mM and cultures were grown as described above to determine the effect of the antioxidant on oxidative stress and subsequent cellular morphology. To see the effect of removing ascorbic acid from the growth medium on cellular morphology under RL, the experiment was performed by diluting cells that had been grown in the absence (control) or presence (treated) of 2 mM ascorbic acid for 4 days, then resuspending the treated cells in BG-11 medium without ascorbic acid and allowing further growth for 4 days, as shown in Fig. 3.

**Cellular morphology analysis.** The cellular morphology of *F. diplosiphon* strains grown under GL or RL was analysed by an inverted Axiovert 200 Zeiss LSM 510 Meta confocal laser scanning microscope (Carl Zeiss MicroImaging) using differential interference contrast (DIC) optics and fluorescence excitation and emission filters as described previously (Bordowitz & Montgomery, 2008, 2010). Slides of live, immobilized *F. diplosiphon* cells were prepared according to the method described by Bordowitz & Montgomery (2010). The length and width of the cells were measured by using LSM FCS Zeiss 510 Meta AIM imaging software following instructions provided by the manufacturer.

**Statistical analysis.** All experiments were performed with at least three biological replicates. The length and width measurements of cells were conducted on 50–100 cells, whereas filament length data are presented as a mean value of 50 measured filaments. All data were analysed by one- or two-way analysis of variance using OpenStat statistical software [version 10.01.08; W. G. Miller (http://www.statprograms4U.com)]. Once a significant difference was detected, post hoc multiple comparisons were made by using the Tukey test. The level of significance was set at 0.05 for all tests.

**RESULTS**

**RL induces high levels of ROS in *F. diplosiphon***

Oxidative stress measurements indicated that ROS levels were significantly higher after 24 h of exposure to GL or RL than for cells grown under white light (WL) prior to light transition. Furthermore, the ROS level was significantly...
higher in RL-exposed SF33 samples than in GL-exposed samples (Fig. 1a). The ROS level increased transiently in RL-exposed samples after 48 and 72 h of exposure. The ROS level was significantly decreased after 96 h of exposure under RL; however, in GL-exposed samples, no significant increase in the ROS level was noticed throughout the study after 24 h of exposure (Fig. 1a). Notably, ROS levels were always significantly higher in RL-exposed samples than in GL-exposed samples at each time interval. We also tested the cellular component fluorescence at 520 nm to reject the possibility that values we obtained for the ROS measurements were caused by the fluorescence of cellular components after excitation at 485 nm (Fig. 1b). There was no significant increase in the cellular component fluorescence at 520 nm in GL-exposed samples after 24, 48 and 72 h, despite the fact that cell density was increased during this time period as measured by the scattering of light at 750 nm (Fig. S1, available with the online version of this paper). The cellular component fluorescence was significantly higher after 24 h of exposure under RL; however, no further significant increase in fluorescence was seen throughout the experiment. Thus, it is clear that fluorescence values in Fig. 1(a) reflect the light-dependent generation of ROS under GL or RL exposure at different time intervals. Furthermore, RL exposure results in significantly higher ROS generation than GL.

**RL-dependent ROS elevation is associated with spherical cell morphology in *F. diplosiphon***

Once it was clear that both GL and RL exposure resulted in the accumulation of ROS in *F. diplosiphon* SF33 and that RL caused higher ROS levels than GL, we developed a hypothesis that spherical cellular morphology under RL...
during CCA could be associated with the higher level of ROS. Cells that had been grown under WL prior to transition to GL or RL were largely rectangular (data not shown). To test the hypothesis that RL-elevated ROS levels are correlated with spherical cell shape, different concentrations of ascorbic acid, which has been shown to function as an ROS scavenger (He & Häder, 2002b), were added to the SF33 cultures at a final concentration of 0.5, 1 or 2 mM. ROS levels and cellular morphology were then analysed after 72 h of exposure to GL or RL, as ROS levels were induced maximally after 72 h of exposure (Fig. 1a).

Although absolute values of ROS-dependent DCF fluorescence in Fig. 1(c) are slightly higher due to a slight increase in the intensity of the growth light relative to the values in Fig. 1(a), all concentrations of ascorbic acid used in this study significantly reduced the ROS level under both GL and RL. However, the 2 mM concentration was found to maximally reduce ROS levels in SF33 as well as in the parental strain of SF33, i.e. UTEX 481 (Fig. 1c, e, results not shown for 0.5 and 1 mM ascorbic acid). Thus, further studies were carried out with 2 mM ascorbic acid. Addition of ascorbic acid had no effect on the cellular component

![Fig. 2. Confocal laser scanning microscopy analyses of the cellular morphology of F. diplosiphon SF33 and UTEX 481 strains grown with or without ascorbic acid under GL or RL. (a, c) Representative optical slices from a Z-series of DIC images and corresponding maximum intensity projection PBP autofluorescence (Auto) images indicating the effect of ascorbic acid (0 or 2 mM final concentration) on cellular morphogenesis in F. diplosiphon SF33 (a) or UTEX 481 (c) grown under GL or RL for 72 h. Images were acquired using a x40 oil immersion objective with x2 zoom setting. AA, ascorbic acid; bars, 5 μm. (b, d) Cell length and width measurements of F. diplosiphon SF33 (b) or UTEX 481 (d) cells grown under GL or RL with or without 2 mM ascorbic acid. Identical letters over bars represent a homogeneous mean group (P>0.05), whereas a line over bars indicates no significant difference between the length and width of cells (P>0.05). No symbol over the bar indicates a significant difference (P<0.05) from the others. Black bars, length; grey bars, width. Error bars indicate SD. (e) DIC images of terminal cells of F. diplosiphon SF33 filaments grown under GL or RL with or without 2 mM ascorbic acid.](image-url)
fluorescence at 520 nm, similar to what we observed in its absence (Fig. 1d, f).

The results from morphological analyses confirmed the rectangular cell shape for *F. diplosiphon* under GL, in contrast with the spherical shape under RL (Fig. 2a, c; Bennett & Bogorad, 1973; Bordowitz & Montgomery, 2008). However, when 2 mM ascorbic acid was added to the growth medium, the cell shape under RL was altered from spherical to rectangular, resulting in a phenotype similar to GL-grown cells in the absence of exogenous ascorbic acid (Fig. 2a, c). In contrast, no effect on cellular morphology was observed with the addition of the ascorbic acid under GL (Fig. 2a, c). Thus, the present study clearly shows that spherical morphology observed under RL during CCA is associated with a higher level of ROS in comparison with GL.

The length of *F. diplosiphon* cells was significantly reduced in RL-grown cultures in comparison with GL-grown cultures; however, the width of the cells was not affected under either light condition (Fig. 2b, d). There was no significant difference between the length and width of cells under RL, resulting in a spherical morphology and indicating that RL-dependent reduction in the length of cells results in a spherical morphology during CCA. The addition of 2 mM ascorbic acid significantly increased the length of cells under RL, which resulted in there being no significant difference between GL-grown and RL-grown cells in the presence of ascorbic acid (Fig. 2b, d). Addition of ascorbic acid had no effect on the length or width of the cells under the GL growth condition (Fig. 2b, d). Thus, the higher level of ROS under RL may act as a signal to reduce the cell length, which consequently results in spherical cell morphology.

The terminal cells of filaments were also different under GL and RL growth conditions. Terminal cells were longer under the GL growth condition, while shorter and more rounded terminal cells were characteristic of the RL growth condition (Fig. 2e). Similar to other cells, the morphology of the terminal cells under RL was also altered to that of the GL-associated growth condition on addition of ascorbic acid, while there was no major effect of ascorbic acid on terminal cellular morphology under the GL growth condition (Fig. 2e).

![Flow chart showing the steps involved in the reversal experiment of removing ascorbic acid from the growth medium and its subsequent effect on cellular morphology in *F. diplosiphon* SF33 under RL. *F. diplosiphon* cells were grown in RL without (control) or with (treated) 2 mM ascorbic acid for 4 days. After this initial growth period, cells were washed with sterile water (ddH₂O) three times and once with BG-11 medium before resuspending them in BG-11 for control cells, or in BG-11 either with 2 mM ascorbic acid or without for treated cells. Following 4 days of additional growth, cells were analysed by confocal microscopy.](http://mic.sgmjournals.org/2018/11/SF33/flow-chart-reversal-experiment-ascorbic-acid.jpg)

**Fig. 3.** Flow chart showing the steps involved in the reversal experiment of removing ascorbic acid from the growth medium and its subsequent effect on cellular morphology in *F. diplosiphon* SF33 under RL. *F. diplosiphon* cells were grown in RL without (control) or with (treated) 2 mM ascorbic acid for 4 days. After this initial growth period, cells were washed with sterile water (ddH₂O) three times and once with BG-11 medium before resuspending them in BG-11 for control cells, or in BG-11 either with 2 mM ascorbic acid or without for treated cells. Following 4 days of additional growth, cells were analysed by confocal microscopy.

![Cell morphological analyses showing the effect of the removal of ascorbic acid on cellular morphology of *F. diplosiphon* SF33 grown under RL.](http://mic.sgmjournals.org/2018/11/SF33/ascorbic-acid-cellular-morphology.jpg)

**Fig. 4.** Cell morphological analyses showing the effect of the removal of ascorbic acid on cellular morphology of *F. diplosiphon* SF33 grown under RL. (a) Representative confocal optical slices from a Z-series of DIC images and corresponding maximum intensity projection PBP autofluorescence (Auto) images indicating the effect of no ascorbic acid (control), 2 mM ascorbic acid (final concentration) or reversal of growth in ascorbic acid (reversed) on cellular morphogenesis in *F. diplosiphon* SF33 grown under RL for 96 h. Images were acquired using a ×40 oil immersion objective with ×2 zoom setting. Bars, 5 μm. (b) Cell length and width measurements of *F. diplosiphon* SF33 cells grown under RL under the conditions described in (a). Identical letters over bars represent a homogeneous mean group (*P* > 0.05), while lines over bars indicate no significant difference between the length and width of cells (*P* > 0.05). No symbol over the bar indicates a significant difference (*P* < 0.05) from the others. Black bars, length; grey bars, width. Error bars indicate SD.
We tested the reversibility of the cellular morphology of cells previously grown in the presence of ascorbic acid under RL and then shifted to growth medium without ascorbic acid after washing, as shown in Fig. 3. The images obtained from confocal microscopy showed that the presence of ascorbic acid in the growth medium prevented spherical morphology in the SF33 strain under RL (Fig. 4). However, cells that were previously grown in the presence of ascorbic acid and then depleted for ascorbic acid by its removal from the growth medium exhibited RL-characteristic spherical morphology, similar to the control samples that were always grown without ascorbic acid under RL (Fig. 4a). The length and width of the cells were not significantly different for individual samples or when comparing the control to the reversed sample that was previously grown in the presence of ascorbic acid and then depleted for it (Fig. 4b). Contrary to this, the cell length was significantly higher than the width in samples grown in the presence of ascorbic acid under RL and the cell length was also significantly higher than those for the control and

Fig. 5. Confocal laser scanning microscopy analyses of filament morphology of F. diplosiphon UTEX 481 grown with or without ascorbic acid under GL or RL. (a) Representative optical slices from a Z-series of DIC images indicating the effect of ascorbic acid (0 or 2 mM final concentration) on filament length in F. diplosiphon UTEX 481 grown under GL or RL for 72 h. Images were acquired using a ×2.5 objective with ×2 zoom setting. Bars, 100 µm. (b) Filament length measurements of F. diplosiphon UTEX 481 cells grown under GL or RL with or without 2 mM ascorbic acid. Identical letters over bars represent a homogenous mean group (P>0.05), whereas no symbol over the bar indicates a significant difference (P<0.05) from the others. Error bars represent SD. AA, ascorbic acid.

Fig. 6. Measurement of ROS accumulation in the RcaE-deficient mutant (ΔrcaE) strain of F. diplosiphon grown in the presence or absence of ascorbic acid under GL and RL. (a) ROS-dependent DCF fluorescence and (b) cell component fluorescence at 520 nm in the ΔrcaE strain after different time intervals of growth under GL or RL. (c) ROS-dependent fluorescence in the ΔrcaE strain after 72 h of growth under GL or RL with different concentrations of ascorbic acid added to the growth medium. The negative control represents BG-11 medium + DCFH-DA fluorescence without cells added. Asterisks indicate a significant difference (P<0.05) from 0 h and identical letters over bars represent homogeneous mean groups (P>0.05), while no symbol over a bar means a significant (P<0.05) difference from other treatments. The line over bars indicates no significant (P>0.05) difference between GL- and RL-grown samples treated with the same concentration of ascorbic acid. AU, arbitrary units. Light grey bars, GL; dark grey bars, RL. Error bars represent SD.
reversed samples. The width of the cells was unchanged among all treatments (Fig. 4b).

**RL-dependent higher ROS levels impact on filament morphology in *F. diplosiphon* UTEX 481**

The length of filaments was also reported to change during CCA in *F. diplosiphon* (Bennett & Bogorad, 1973; Bordowitz & Montgomery, 2008), so we were interested to know whether fragmentation of UTEX 481 filaments under RL is also associated with the RL-dependent higher levels of ROS. To test this, *F. diplosiphon* UTEX 481 was grown for 3 days in the presence or absence of the antioxidant under GL and RL growth conditions, and thereafter, filament length in UTEX 481 populations was measured (Fig. 5). We used 35 μmol m⁻² s⁻¹ intensity of GL and RL to study the filament fragmentation instead of lower light intensities because lower RL intensity was found to be unable to induce fragmentation in contrast with its effect on cellular morphology in UTEX 481 (data not shown). We observed filament fragmentation, i.e. shorter filaments, under RL, whereas under GL longer filaments were observed, similar to earlier reports (Fig. 5a; Bennett & Bogorad, 1973; Bordowitz & Montgomery, 2008). However, the presence of ascorbic

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**Fig. 7.** Confocal laser scanning microscopy analyses of cellular morphology of *F. diplosiphon* ΔrcAE strain grown with or without ascorbic acid under GL or RL. (a) Representative optical slices from a Z-series of DIC images and the corresponding maximum intensity projection PBP autofluorescence (Auto) images indicating the effect of ascorbic acid (AA) at different final concentrations (0, 2, 4 or 6 mM) on cellular morphogenesis in *F. diplosiphon* ΔrcAE strain grown under GL or RL for 72 h. Images were acquired using a ×40 oil immersion objective with ×2 zoom setting. AA, ascorbic acid; bars, 5 μm. (b) Cell length and width measurements of *F. diplosiphon* ΔrcAE cells grown identically to those in (a). Identical letters over bars represent a homogeneous mean group (P>0.05). No symbol over the bar indicates a significant difference (P<0.05) from the others. Black bars, length; grey bars, width. Error bars represent SD.
acid in the growth medium prevented the fragmentation or shortening of filament length under RL, and under these conditions filament length was similar to the UTEX 481 population grown under GL (Fig. 5b). There was no significant effect of adding ascorbic acid on the filament length under GL. Thus, our present study indicates that the filament length changes associated with RL in F. diplosiphon during CCA may be signalled by higher levels of ROS generated by the cyanobacterium under RL.

**RcaE function is required for light-dependent differential in ROS levels in F. diplosiphon**

RcaE has been shown previously to have a photoregulatory role in controlling cellular morphology and filament length in *F. diplosiphon* (Bordowitz & Montgomery, 2008). To determine whether the impact of light on ROS levels and the correlation with increased ROS levels under RL depends on RcaE function, we assessed ROS levels and the impact of ascorbic acid treatment on ΔrcaE null mutant cells. We observed overall higher levels of ROS in ΔrcaE cells, with relatively equal ROS levels under RL relative to GL, with the exception of the 48 h time point, when ROS levels were higher under RL than GL (Fig. 6). Thus, ΔrcaE cells grown in WL and then switched to either GL or RL exhibited a similar transient increase in ROS levels, which peaked at 72 h after transfer (Fig. 6a). This response differed from cells of the parental SF33 strain, which exhibited differential responses for GL versus RL exposure (Fig. 1a). The light-induced increase in ROS in ΔrcaE cells could be decreased by adding increasing concentrations of ascorbic acid (Fig. 6c); however, no ascorbic-acid-induced impact on cellular morphology was observed (Fig. 7a). The lengths or widths of ΔrcaE cells treated with ascorbic acid did not differ significantly from cells lacking ascorbic acid (Fig. 7b).

**DISCUSSION**

Cyanobacteria have been subjected to a myriad of environmental factors or stressors since their appearance on the Earth and they have developed several adaptive mechanisms to support their survival under the pressure of natural selection. Their genome has evolved in such a way that they can sense and respond to different environmental stimuli (Los *et al.*, 2010). Due to their photosynthetic nature, they have also developed an ability to fine-tune their photosynthetic machinery in response to ambient light quality. CCA is a well-known, ecologically important example of the adjustment of the cyanobacterial photosynthetic machinery to ambient light quality (Bogorad, 1975), which maximizes their photosynthetic efficiency (Campbell, 1996). In addition to pigment changes, the morphology of the cells and filaments of *F. diplosiphon* were also found to change during CCA (Bennett & Bogorad, 1973). Many advances have been made in our understanding of the biochemistry and molecular mechanisms regulating the pigment changes of CCA (Gutu & Kehoe, 2012; Kehoe, 2010); however, information about the causes of morphological changes during CCA is limited (Bordowitz & Montgomery, 2008; Bordowitz *et al.*, 2010; Pattanaik & Montgomery, 2010; Pattanaik *et al.*, 2011a, 2012).

In this study, we report that morphological changes occurring in *F. diplosiphon* during CCA are correlated with higher levels of ROS experienced by the organism under RL. This study shows that *F. diplosiphon* accumulates ROS under both GL and RL during CCA. However, ROS accumulates to a greater extent under RL in comparison with GL. This higher level of ROS under RL may act as a signal for inducing a spherical morphology of the cells as the reduction of ROS levels by the addition of an antioxidant results in an alteration of shape under RL to the GL-associated rectangular cellular morphology (Fig. 2a, c).

The phytochrome-like RcaE photoreceptor impacts on cellular morphology, as previously reported (Fig. 7; Bordowitz & Montgomery, 2008). However, morphological changes in *F. diplosiphon* are also associated with a RL-dependent higher level of ROS (Figs 2 and 4). In addition to morphological changes, *F. diplosiphon* has been shown to differentiate hormogonia at an early stage of CCA when shifted from GL to RL. Additionally, instead of the RcaE photosensor, redox potential imbalance in the photosynthetic electron transport system was found to signal this biological process (Campbell *et al.*, 1993). The filaments of *F. diplosiphon* are also fragmented under RL and cellular lysis at the site of necridia or separation discs was proposed for the fragmentation (Bennett & Bogorad, 1973). In this study, we report that fragmentation of the filament under RL is also induced by the RL-dependent higher levels of ROS. UV-radiation-dependent oxidative stress has also been shown to induce the fragmentation of filaments in *Anabaena variabilis* PCC 7937 and *Arthrospira platensis* and this oxidative-stress-induced

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**Fig. 8.** Model indicating the role of RcaE and RL or GL on the regulation of ROS levels and the corresponding cellular morphology in *F. diplosiphon*. Under GL, an RcaE-dependent decrease in ROS levels is associated with an elongated, rod-shaped cell and RcaE-independent effects on cellular morphology are also supported. Under RL, independent of the presence of RcaE, ROS levels are significantly higher and are correlated with a spherical cell shape in *F. diplosiphon*. 

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morphological change was found to improve photosynthetic efficiency in *Arthrospira platensis* (Ma & Gao, 2009; Rastogi *et al.*, 2010). Thus, our results are in accordance with Bennett & Bogorad (1973) and here we propose that selective lysis of cells by oxidative stress results in fragmentation of the filaments under RL.

Bacterial systems are known to change their cellular morphology in response to different environmental stressors and the rod-to-spherical cellular morphological change, which reduces the exposed surface area under different stress factors, including oxidative stress, is a common mitigation strategy adopted by these organisms to protect themselves (Singh & Montgomery, 2011; Young, 2006). Similarly, reduced filament length also results in a smaller exposed area and therefore, spherical morphology and shorter filaments in *F. diplosiphon* under RL may protect the organism from excessive oxidative stress by reducing the exposed surface area. This may be critical in natural environments where red-enriched light is generally associated with higher light intensity near the surface of the water, rather than deeper in the water column, where green-enriched light is lower in overall intensity (Postius *et al.*, 2001). Indeed, we have recently demonstrated that increased light intensity can lead to rod-to-spherical shape changes in *F. diplosiphon* UTEX 481 (Pattanaik *et al.*, 2012). In addition, oxidative-stress-induced shorter filaments under RL may also help in diluting out the GL-absorbing PE pigment after shifting cultures from GL to RL (Bennett & Bogorad, 1973). The level of ROS was found to increase until 72 h of exposure under RL and thereafter it started decreasing (Fig. 1a). This may be explained by the fact that cyanobacteria possess several enzymic and non-enzymic ROS-scavenging mechanisms to reduce oxidative stress and these mechanisms could be responsible for the observed lower oxidative stress after 72 h of exposure to RL (Singh *et al.*, 2010).

The results from our study show that the length of the cell is reduced under RL, giving spherical morphology; however, the width of cells was unaffected (Fig. 2b, d). In bacterial systems, the length of the cell is regulated by the prokaryotic cytoskeleton (Young, 2010). The present study demonstrates that ROS levels are correlated with morphological changes during CCA in *F. diplosiphon*; however, how this signal is translated into morphological changes by regulating cytoskeletal proteins or components of the cytoskeleton to give final morphological changes is still unknown. Notably, cyanobacterial genomes possess several sensor kinases to sense oxidative stress (Los *et al.*, 2010) and it will be interesting to determine whether morphological changes in *F. diplosiphon* during CCA involve a sensor kinase of oxidative stress that directly affects the integrity of the cytoskeletal proteins to give the spherical morphology under RL.

Several pigment mutants have been isolated in *F. diplosiphon* and their morphological analysis led to the proposal that several molecular entities are involved in the process of morphology regulation. Based on morphological analysis of an RcaE-deficient mutant, it was proposed that RcaE regulates cell shape during CCA, in addition to pigment changes (Bordowitz & Montgomery, 2008). However, cell shape regulation by RcaE was found to be different from pigment regulation as the cognate RRs for light-dependent pigment changes, i.e. RcaF and RcaC, were found to be required only for RL-specific spherical morphology (Bordowitz & Montgomery, 2008; Bordowitz *et al.*, 2010). Here we see that RcaE-deficient cells differ most from WT cells in the higher levels of ROS detected under GL in *ArcaE* cells (compare Figs 1a and 6a). Notably, the mitigation of ROS levels by ascorbic acid treatment had no effect on the morphology of RcaE-deficient cells (Fig. 7). Taken together, these results suggest that RcaE is essential for repressing ROS levels under GL, which may be linked to the cells' ability to elongate under these conditions. The impact of RcaE on the regulation of cell shape, however, diverged to a great degree from its impact on ROS levels, as ascorbic-acid-induced mitigation of ROS levels did not result in a significant change in morphology in *ArcaE* cells (Fig. 7). This latter result indicates that functional RcaE is required for light-dependent changes in cell shape, independent of ascorbic acid treatment. Collectively, these results lead to a model, under which RcaE mediates a GL-dependent mechanism for lowering ROS levels or inhibiting an increase in ROS levels, that is associated in part with a molecular response of cellular elongation (Fig. 8). Yet under GL, RcaE also appears to be critical for photoregulation of morphology, as distinct from its impact on ROS levels (Fig. 8).

The impact of ROS on cellular morphology is probably occurring through its effect on the cell wall structure as spherical cells, i.e. UTEX 481, SF33 cells under RL or ArcaE cells independent of light condition, are more susceptible to lysis by lysozyme treatment than rod-shaped cells, i.e. UTEX 481 or SF33 cells under GL (Fig. S2). Increased lysozyme susceptibility of bacterial cells indicates a disruption of the lipopolysaccharide layer (Johnson & Larson, 2005), which is associated with cell shape changes.

Recently, CpeR, which is a regulator of PE biosynthetic genes, was found to impact on morphology, as a ΔcpeR null mutant was found to have altered cellular morphology under both GL and RL (Pattanaik *et al.*, 2011b). By contrast, mutants lacking PE subunit-encoding genes, i.e. cpeB and cpeA, and PE linker gene cpeD, were found to be defective only in PE accumulation and their cell shape was not much affected (Pattanaik *et al.*, 2011b). These findings support the results of the present study that signal transduction for changes in pigmentation and cellular morphology during CCA diverge, as here we see impacts of ROS on cellular morphology, but not on pigment levels (data not shown).

In other biological systems, including a number of photosynthetic organisms, ROS accumulation has been associated with the regulation of cellular morphology. For example, ROS accumulation is associated with the regulation of hyphal morphology in *Aspergillus nidulans* (Semighini & Harris, 2008). ROS accumulation has also been associated
with pollen germination and tube emergence/elongation in plants (Potocky et al., 2007; Speranza et al., 2012). ROS production in roots has been associated with root elongation (Foreman et al., 2003) and in the root hair tip growth mechanism (Carol & Dolan, 2006). Mechanically, the functions of ROS in cell wall loosening during germination and radicle elongation have been reported (Muller et al., 2009). ROS-mediated cell expansion has also been implicated in cotton fibre cell elongation (Li et al., 2007). Thus, ROS production in response to RL in F. diplosiphon may be associated with cell wall loosening that, in combination with other factors such as osmotic pressures, may result in a shift towards spherical morphology. In this regard, the impact of ascorbic acid, which has been shown to protect against lipid peroxidation in other cyanobacterial systems (He & Hader, 2002b), on cellular morphology in F. diplosiphon supports a mechanism for the association of the light-dependent regulation of ROS levels with an ROS-dependent regulation of morphology.

ACKNOWLEDGEMENTS

This research was supported by the US Department of Energy (Chemical Sciences, Geosciences and Biosciences Division, Office of Basic Energy Sciences, Office of Science, grant no. DE-FG02-91ER20021 to B.L.M.). Support for S.P.S. is from a CAREER award from the National Science Foundation (grant no. MCB–0643516 to B.L.M.). We thank Dr Juliana Bordowitz, University of California, San Diego, for conducting lysozyme sensitivity assays.

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Edited by: C.-C. Zhang