3-2-2009

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Role of \textit{mrgA} in peroxide and light stress in the cyanobacterium \textit{Synechocystis} sp. PCC 6803

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Running Title: Oxidative stress and \textit{mrgA} in \textit{Synechocystis} sp.

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Abstract

In the unicellular cyanobacterium *Synechocystis* sp. PCC 6803, the *mrgA* gene is part of the PerR regulon that is upregulated during peroxide stress. We determined that an Δ*mrgA* mutant was highly sensitive to low peroxide levels and that the mutant upregulated a gene cluster (*sll1722-24*) that encoded enzymes involved with exopolysaccharide (EPS) production. We made mutants in this EPS cluster in both a wild type and Δ*mrgA* background and studied the responses to oxidative stress by measuring cell damage with LIVE/DEAD stain. We show that *Synechocystis* sp. PCC 6803 becomes highly sensitive to oxidative stress when either *mrgA* or the *sll1722-24* EPS components are deleted. The results suggest that the deletion of the EPS cluster makes a cell highly susceptible to cell damage, under moderate oxidative stress conditions. Mutations in either *mrgA* or the EPS cluster result in cells that are larger and more light- and peroxide-sensitive than normal and the EPS cluster may help determine cell size. MrgA is now known to be involved in the storage or mobilization and the cells can be damaged by iron-induced oxidative stress when MrgA is missing. We suggest that the wild type cells attempt to produce more EPS to prevent such damage.
Introduction

Iron is a critical nutrient for the growth of all life, but it is particularly important for photosynthetic organisms. Iron can have two oxidation states (II and III) and it is a key component in those reactions involved with redox cycling, such as respiration and photosynthesis. The more soluble Fe(II) reacts with $O_2$ to create reactive oxygen species that can damage DNA and proteins, thus inhibiting many physiological processes. This inhibition is particularly critical in photosynthetic organisms such as cyanobacteria where iron is required in photosystem I (PSI) which consumes up to 25% of the cellular iron (Keren et al., 2004). Thus, it is of great importance for all organisms to inhibit the deleterious side effects of Fe (II) and to store the excess Fe (II) in a bioavailable form. It is for this reason that most organisms produce ferritin or ferritin-like proteins as Fe storage and detoxifying units.

*Synechocystis* sp. PCC 6803 (*Synechocystis* 6803) contains two types of ferritin storage complexes: two proteins that resemble bacterioferritin (Bfr) and a protein termed MrgA. The importance of the Bfr proteins was shown by inactivation mutants in either of the two genes which resulted in the loss of about 50% of the total cellular iron, as well as a reduction in the PSI content and induction of the Fe stress pathway; even under Fe sufficient conditions (Keren et al., 2004). In *Synechocystis* 6803, there is another ferritin-like protein encoded by the gene *slr1894* (*mrgA*, metal regulated gene). MrgA is related to the Dps proteins that are members of the ferritin family, but which lack the fifth C-terminal helix that are present in other ferritins. We have adopted the MrgA designation because the gene was first identified as a peroxide-responsive gene in our study of peroxide sensitivity in *Synechocystis* 6803 (Li et al., 2004).
In this present study, we are interested in understanding the role of MrgA in peroxide resistance in *Synechocystis* 6803. Previously, it has been shown that *mrgA* was induced by peroxide in differential transcription studies using a full genome microarray (Li *et al*., 2004). In the presence of 1.5mM hydrogen peroxide (H$_2$O$_2$), the *mrgA* gene was induced ~2-fold, and although it had a putative *perR* box, it was still up-regulated 2-fold in a $\Delta$*perR* mutant (Li *et al*., 2004). However, a knockout mutant in *mrgA* was highly sensitive to peroxide and currently represents the most H$_2$O$_2$-sensitive strain of *Synechocystis* yet identified (Li *et al*., 2004). Previous studies have shown that in comparisons of $\Delta$mrgA and wild type in the presence and absence of 75µM H$_2$O$_2$, there are a number of significant transcriptional changes in the mutant (Singh *et al*., 2007). These differentially regulated genes included one major cluster (sll1722-24) that appeared to be involved with the biosynthesis of exopolysaccharides and several other proteins destined for the periplasm or the cell wall (Singh *et al*., 2007). Here, we examine the physiological alterations of mutants defective in $\Delta$mrgA and cluster sll1722-24 during oxidative and light stress, so that we can determine some of the characteristics that lead to peroxide sensitivity in this strain. Based on previous DNA microarray studies (Singh *et al*., 2007), we propose that the production of exopolysaccharides in the *Synechocystis* 6803 acts as a protective cellular response to potential stress damage by agents such as H$_2$O$_2$ and light. Furthermore, we postulate that $\Delta$mrgA cells are less sensitive to H$_2$O$_2$ damage as the culture approaches stationary phase than while it is growing exponentially. In this study, we test these predictions and discuss the potential cellular strategies for protection against oxidative stress.
Materials and methods

Growth conditions

*Synechocystis* sp. PCC 6803 (hereafter *Synechocystis* 6803) wild-type (WT) and ΔmrgA were grown at 30°C in the chemically defined media BG-11 (Rippka et al., 1981). Cultures were inoculated with 1 x 10^9 cells per ml of media and shaken at 125 rpm under three light regimes: low (30 µmol photons m^−2 s^−1), medium (50 µmol photons m^−2 s^−1), and high (100 µmol photons m^−2 s^−1). All light treatments utilized using cool white fluorescent lights (Phillips 30W). Cell growth was monitored using a spectrophotometer at A_{750} and direct cell counting using a Petroff-Hausser cell counter. Where appropriate, cells were also cultivated in BG-11 media with variable levels of iron. Cells were grown in either 1X (20 µM Fe), 0.1X (2 µM Fe), 0.01X (0.2 µM Fe), or 0.001X (20 nM Fe) BG-11 media. All chemicals for reagents supplied by Sigma-Aldrich (St. Louis, MO) unless otherwise notified.

Construction of *Synechocystis* 6803 mutants

The cluster *sll1722-24* was PCR amplified using Taq Polymerase (Takara Bio, Shiga, Japan) using following primers (Forward Primers: ATGCACTCCATTCAATCACC and reverse primer: CTAATTTGCTTCCATCCCAAG). The amplified product was cloned in pGEM-T vector (Promega, Madison, WI). The *sll1722-24* genes were replaced by Sp′ cassette following digestion with AflII and blunting of the cohesive ends with T4 DNA polymerase (WT-A and ΔmrgA-A construct; Fig. 1)
**Bacterial viability staining**

To assess the effects of hydrogen peroxide ($H_2O_2$) and light stress on membrane permeability, *Synechocystis* 6803 WT, WT-A, $\Delta mrgA$ and $\Delta mrgA$-A were stained with the LIVE/DEAD Bacterial Viability kit (Molecular Probes, Invitrogen, Carlsbad, CA) as previously described (Foster *et al.*, 2004). Briefly, the viability kit visualizes the integrity of the plasma membrane and consists of two fluorescence markers: 1) SYTO 9, a green stain that is indicative of intact membranes; and 2) propidium iodide (PI), a red stain that permeates cells that have undergone cell death or membrane damage. Cells undergoing peroxide treatment were cultivated under medium light levels (50 $\mu$mol photons m$^{-2}$s$^{-1}$) exposed to 2 mM $H_2O_2$ for 30 min and then stained for 5 min with a LIVE/DEAD solution containing 1.67 $\mu$M of SYTO 9 and 10 $\mu$M of PI. In those cells exposed to light stress, cells cultivated at medium light were incubated for 30 min at low (30 $\mu$mol photons m$^{-2}$s$^{-1}$) or high (100 $\mu$mol photons m$^{-2}$s$^{-1}$) light levels and stained for 5 min in the LIVE/DEAD solution. In both treatments the stained cells were examined with an epifluorescence Zeiss Axioscope microscope (Carl Zeiss, Jena, Germany). Since cyanobacterial autofluoresce (650 nm), overlaps with the emission spectrum of PI, a 515 nm long pass filter (Zeiss) was used to simultaneously visualize the emission spectra of SYTO 9 and PI, thereby removing autofluorescence.

**Changes in cell diameter during growth cycle**

Cultures of *Synechocystis* 6803 WT, WT-A and $\Delta mrgA$, and $\Delta mrgA$-A were monitored for changes in cell diameter during growth at the exponential and stationary phase growth (see Foster *et al.*, 2007). Cells diameters were measured and compared at
exponential growth phase (72 h) and in stationary growth phase (144 h). The cell
diameters (µm²) were measured using the Zeiss MicroImaging software package (Carl
Zeiss, Germany). For each strain, the diameters of ten cells from three replicate cultures
were measured and statistically compared (MiniTab, State College, PA).

**EPS extraction and quantification**

To examine the effect of light stress on exopolymeric substance (EPS)
production, the EPS material was isolated and quantified from WT, WT-A, ΔmrgA and
ΔmrgA-A mutants. The EPS extraction was performed in triplicate using a modified
procedure of Kawaguchi and Decho (2000). Cultures were grown to stationary phase
for 144 h under either low (30 µmol photons m⁻²s⁻¹) or high (100 µmol photons m⁻²s⁻¹)
light and centrifuged to pellet the cells. Pellets were then vortexed for 30 min in a 1.5%
NaCl solution then re-centrifuged. Subsequently, the supernatant was removed and
heated at 80°C for 15 min, and then briefly centrifuged to remove any remaining cells.
To precipitate the EPS, 4 volumes of 95% ethanol was added then placed at -80°C for 1
h. The EPS material was collected by centrifuging, washed once with 95% ethanol and
then allowed to air dry. The dry pellet was then resuspended in sterile water and the
carbohydrate content evaluated by the anthrone quantification method (Laurentin and
Edwards, 2003). EPS material from each extraction (40 µl) was added to an untreated
96-well microtiter plate and chilled for 10 min. A solution (100 µl) of 10.3 mM (2mg/ml)
of anthrone solublized in sulfuric acid was added to each well. The plate was then
sealed and briefly vortexed, then heated for 3 min at 90°C. After heating, the plate was
cooled for 5 min at room temperature then incubated at 45°C for 15 min. The
absorbance of each well was measured using a Synergy HT microtiter plate reader (BioTek, Winooski, WT) at 630 nm. The absorption of the EPS material was compared to a glucose standard curve. The glucose standard curve ranged from 0 - 400 mg/ml at 50 mg/ml increments.

Results and Discussion

Changes in membrane sensitivity to peroxide stress at different growth phases

The growth of the four strains (WT, WT-A, $\Delta mrgA$ and $\Delta mrgA-A$) was examined under a variety of different conditions, including different light intensities and in media with 0.1, 0.01 and 0.001 the normal levels of iron. The strains displayed comparable growth rates in regular BG-11 at low light intensities, but the WT-A mutant had slower growth rates when the light intensity was increased to 100 $\mu$mol photons m$^{-2}$s$^{-1}$. In addition, under Fe-deficient conditions, WT-A grew at only 30 - 40% the WT level, even at 0.1X Fe. Both $\Delta mrgA$ (and $\Delta mrgA-A$) responded as previously described (Shcolnick et al., 2007) and grew poorly under iron deficiency, also growing to about 30% of the level seen in normal BG-11 medium (data not shown).

To examine the sensitivity of WT and $\Delta mrgA$ to oxidative stress throughout the growth cycle, *Synechocystis* 6803 cells were grown at 50 $\mu$mol photons m$^{-2}$s$^{-1}$ and exposed to hydrogen peroxide (H$_2$O$_2$) during the logarithmic (3 d) and stationary (6 d) phases. To visualize the effects of H$_2$O$_2$ on *Synechocystis* 6803, cells were stained with the membrane permeability stain LIVE/DEAD Viability kit (Fig. 2). In untreated cells, both the parent and the mutant exhibited a 2-fold higher background level of cell damage in logarithmic growth than in stationary phase growth. The mean percentage of
damaged cells per 1000 \( \mu \text{m}^2 \) was 27\% (± 13.6) in WT and 36\% (± 9.5) in \( \Delta \text{mrgA} \) cultures (Fig. 2a, b, i), whereas in stationary phase the mean percentage of dying or damaged cells was 11\% (± 3.2) in WT and 8.7\% (± 1.4) in \( \Delta \text{mrgA} \) (Fig. 2e, f, i). Although differences were detected in the two growth phases in untreated cells, there was no statistical difference in the number of dead or damaged cells between the parent WT and mutant \( \Delta \text{mrgA} \) strains during logarithmic (P < 0.25) or stationary (P < 0.15) phase growth (Fig. 2).

In cultures treated with 2 mM \( \text{H}_2\text{O}_2 \), there were significant differences between the two strains in the number of propidium iodide stained cells during logarithmic phase growth. In peroxide-treated WT cultures, an average of 45\% of the cells exhibited signs of cell membrane damage or death, whereas 77\% of the \( \Delta \text{mrgA} \) treated exhibited signs of cell death or damage (Fig. 2c,d, i). Although significant differences between the parent and mutant strain were detected at logarithmic growth (P < 0.01), these differences did not persist into stationary phase growth. The average number of peroxide sensitive cells significantly decreased during stationary phase in both the WT (26\%) and \( \Delta \text{mrgA} \) (27\%) cells and were not significantly different from each other (P < 0.74; Fig. 2g, h, i). The staining results indicated that, during exponential growth, mutants defective in \textit{mrgA} were more susceptible to peroxide stress than in stationary phase growth. These results are consistent with the one hypothesis that \( \Delta \text{mrgA} \) is more easily damaged than the WT in the exponential growth phase and that this stress is relieved as the culture proceeds toward stationary phase growth.

These results strongly correlate to our previous study in which there was an upregulation of the PerR regulon, including \textit{mrgA}, under peroxide stress (Li et al.,
PerR and MrgA resembled proteins induced by the peroxide stress response in *Bacillus subtilis* where *mrgA* protected against oxidative killing (Chen and Helmann 1995; Chen *et al*., 1995; Helmann *et al*., 2003). The *mrgA* gene also has a strong relationship to *dpsA* in *Synechococcus* sp. PCC 7942 and a strain lacking *dpsA* was more sensitive to peroxide (Dwivedi *et al*., 1997; Sen *et al*., 2000).

**Loss of *mrgA* and *sll1722-24* causes increased sensitivity to oxidative and light stress**

In our previous DNA microarray analyses of *Synechocystis* 6803 cells after exposure to low levels of peroxide throughout the diel cycle, one of the major, up-regulated gene clusters was *sll1722-24* (Singh *et al*., 2007). Genes within this cluster exhibited homology to genes associated with the biosynthesis of exopolysaccharides and proteins designated for the periplasm or cell wall. Mutants defective in these genes (WT-A and Δ*mrgA*-A) were generated and tested under different oxidative stress conditions. The results in Fig. 3a demonstrated that both Δ*mrgA* and Δ*mrgA*-A were highly sensitive to 2 mM H$_2$O$_2$ and that ~60% of cells were damaged. The two strains showed similar sensitivity to this stress. On the other hand, the control WT cells had very few damaged cells, whereas WT-A showed a significant increase, up to 25% damaged cells. The WT-A strain manifested greater variability under all stress conditions, suggesting that they are more easily damaged even under modest stresses.

Based on these results, cells were grown to stationary phase (6 d) and then exposed to low- (30 μmol photons m$^{-2}$s$^{-1}$) and high-light (100 μmol photons m$^{-2}$s$^{-1}$) conditions. LIVE/DEAD fluorescent staining indicated that, under low light conditions, all
tested strains had low background levels of damaged or dying cells (Fig. 3b). Of the four tested strains, only WT-A had a significantly higher number of damaged or dying cells compared to its parent strain WT (P < 0.0001) in low light. Strains $\Delta mrgA$ and $\Delta mrgA$-A showed no difference in the numbers of dead or damaged cells (P < 0.18). However, under high light, all three of the mutant strains had significantly more damaged cells than the WT parent strain. In WT cultures, only 8% of the cells exhibited signs of cell damage. This was significantly lower than 29% of the WT-A (P < 0.0001), 24% of the $\Delta mrgA$ (P < 0.0001), and 27% of the $\Delta mrgA$-A cells (P < 0.0001). These results clearly indicate that mutants defective in $mrgA$ and $sll1722$-24 have a decreased tolerance to high-light stress.

The results suggest that the deletion of the $sll1722$-24 cluster makes the cells highly sensitive to cell damage, even under moderate oxidative stress conditions, such as 100 $\mu$mol photons m$^{-2}$s$^{-1}$ of light. This cluster is one of the important up-regulated gene clusters in the $\Delta mrgA$ strain and may act, in conjunction with other gene products, to protect the cell. The WT-A strain did not have the benefit of additional differential gene transcription that occurs after the deletion of $\Delta mrgA$ (Singh and Sherman, 2007) and became hypersensitive to both peroxide and the higher light used for growth. We conclude that $sll1722$-24 is an important gene cluster for maintaining cellular integrity. The increased sensitivity to even moderate-light stress of $\Delta mrgA$ and $\Delta mrgA$-A may be a direct result of the inability to mobilize or utilize iron. Previous studies have shown that MrgA is required to access and mobilize the stored Fe within the cells (Shcolnick et al., 2007). High light may trigger an increase in the Fenton reactions in cells that lack $mrgA$,
thus increasing the extent of oxidative damage in the cell membranes as detected by
the LIVE/DEAD staining.

**Increase in cell diameter in mutants defective in mrgA and sll1722-24**

Mutants defective in *mrgA* and *sll1722-24* were compared to WT to determine if there
were any growth or morphological phenotypes. Growth curves for WT and all mutant
strains (WT-A, Δ*mrgA*, Δ*mrgA*-A) showed no statistically significant differences in
growth rate (data not shown). However, the size properties of the WT and the mutants
differed in three important ways. First, the cell diameter of the WT (Fig. 4a), Δ*mrgA*
(Fig. 4b) and *sll1722-24* mutants were quite different from each other. When comparing
strains in the logarithmic growth phase, the cell diameter of WT was smaller than all of
the other tested strains (Fig. 4c, P < 0.001). Second, there was a significant increase in
the cell diameter of each *Synechocystis* 6803 strain between logarithmic (3-d) and
stationary phase growth (6-d; P < 0.002) in all tested strains. Moreover, Δ*mrgA* was the
strain that became the largest after 6d growth (although it was also the most variable).
Third, there were no statistical differences between the WT and WT-A strains in the
stationary phase and WT cells were the smallest after 6d growth (P < 0.002). These
results suggest that mutants defective in the EPS cluster increase in cell size during
logarithmic growth when compared to the normal wild type cells and that cells that are
also defective in Δ*mrgA* become even larger, especially after long-term growth.
Production of exopolymeric substances under low and high light

The levels of extracellular exopolymeric substances (EPS) were compared in the WT, WT-A, and \textit{mrgA} mutant strains cultivated under low and high light levels. Anthrone colorimetric staining revealed that in WT cells there was a two-fold higher level of extracellular EPS compared to mutants WT-A, \textit{\textDelta mrgA}, and \textit{\textDelta mrgA-A} at low light levels (P < 0.0001). At high light levels, the EPS production was less in WT cells; however, it still was significantly higher than in the other tested mutants (P < 0.0001). When WT-A was cultivated under high light there was less extracellular EPS material than in the WT-A cultures grown under low light (P < 0.01). In all the \textit{mrgA} mutants, there was no statistical difference between the strains when grown at low or high light levels (P < 0.30). These results demonstrated the importance of these EPS components for the WT strain and the drop in EPS concentration was consistent with the sensitivity of the WT-A strain to oxidative stresses. The levels of EPS in WT versus the other three strains was also consistent with the results in Fig. 3 and Fig. 4 and indicate the importance of this EPS for maintaining cell size and resistance to oxidative stress. It is evident that the enzymes encoded by the \textit{sll1722-24} cluster are only responsible for less than half of the extracellular EPS (Fig. 5) and that the \textit{\textDelta mrgA} is already deficient in this other component.

In conclusion, our results indicate that \textit{Synechocystis} sp. PCC 6803 becomes highly sensitive to oxidative stress when either \textit{mrgA} or the EPS components produced by \textit{sll1722-24} are deleted. The lack of \textit{mrgA} results in the production of more EPS via the \textit{sll1722-24} genes to try to prevent Fe-induced cell damage. This material is also
critical for the WT cells in maintaining cell size, membrane integrity, and for preventing cell damage under stressful conditions.

Acknowledgements
The authors would like to thank Kevin Hetherington and Matoya Robinson for their technical assistance. This work was supported by a Florida Space Research and Education grant awarded to JSF by the Florida Space Grant Consortium (UCF Project #UCF-0000127787) and by grant DE-FG02-99ER20342 from the Dept. of Energy (LAS).

References


**Figure legends**

**Figure 1.** Gene map and construction of WT and \( \Delta \text{mrgA} \) mutants defective in genes sll1722-26. In mutants denoted with the letter A, the sll1722 – 24 genes were replaced with a spectinomycin cassette (Sp\(^r\)) using the restriction enzyme AflII. Mutants with the entire gene segment of sll1722 – 26 removed using the restriction enzyme BstEII are denoted with the letter B, whereas mutants labeled S have only had genes sll1722 and sll1723 removed using the restriction enzyme SmaI. In all cases the deleted genes were replaced with a spectinomycin cassette (Sp\(^r\)). Since the results from all three mutant strains were comparable, we only reported on the mutant designated A.

**Figure 2.** *Synechocystis* 6803 cells exposed to peroxide stress throughout the growth cycle. (a – b) Epifluorescent micrograph of untreated wild type (WT) and \( \Delta \text{mrgA} \) cells. (c) WT cells exposed to 2 mM H\(_2\)O\(_2\). (d) Mutants defective in mrgA exposed to 2 mM H\(_2\)O\(_2\) in logarithmic growth. (e – f) Micrographs depicting WT and \( \Delta \text{mrgA} \) cells in
stationary phase (6 d). (g – h) WT and \(\Delta mrgA\) cells treated with 2 mM \(H_2O_2\). (i) Graphical representation of epifluorescent micrographs depicting the increased levels of membrane damage and cell death during logarithmic (log) and stationary (stat) growth in wild type (WT) \(\Delta mrgA\) mutants under peroxide stress. Bars represent the standard deviation within the ten replicate cultures examined for each strain.

**Figure 3.** Effects of peroxide and high-light stress on *Synechocystis* 6803 WT and \(\Delta mrgA\) mutants defective in sll1722–24 (\(\Delta WT-A\) and \(\Delta mrgA-A\)) using the LIVE/DEAD fluorescent staining kit. (a) Cells exposed to 2 mM \(H_2O_2\) at 50 \(\mu\)mol photons m\(^{-2}\)s\(^{-1}\). (b) Cells exposed to low (30 \(\mu\)mol photons m\(^{-2}\)s\(^{-1}\)) and high (100 \(\mu\)mol photons m\(^{-2}\)s\(^{-1}\)) light for 30 min. Bars represent the standard deviation of the ten representative cultures examined.

**Figure 4.** Changes in cell size of *Synechocystis* 6803 wild type (WT) and \(\Delta mrgA\) mutants throughout the growth cycle. Light micrographs of (a) WT and (b) \(\Delta mrgA\) cells in logarithmic growth. (c) Graphical representation of the differences in cell diameter in WT, \(\Delta mrgA\), and mutants defective in the genes sll1722-24 (\(\Delta WT-A\) and \(\Delta mrgA-A\)) in logarithmic and stationary phase growth. Error bars reflect the standard deviation within the ten replicate samples.

**Figure 5.** Levels of extracellular exopolymeric substances (EPS) detected in cultures of *Synechocystis* 6803 wild type (WT), \(\Delta mrgA\), and mutants defective in the sll1722-1724 (\(\Delta WT-A\), and \(\Delta mrgA-A\)) grown for 1 week under low and high light growth conditions.
The EPS was extracted from each culture as described in the Materials and Methods and then compared to a glucose standard curve. Bars represent the standard deviation in the ten replicate extractions of EPS material from each treatment.