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A role for mrgA, a DPS family protein, in the internal transport of Fe in the cyanobacterium *Synechocystis* sp. PCC6803

Sigal Shcolnick [a], Yeala Shaked [b], Nir Keren [a,*]

[a] *Department of Plant and Environmental Sciences, The Alexander Silberman Institute of Life Sciences, Hebrew University, Givat Ram, Jerusalem 91904, Israel*
[b] *Inter University Institute for Marine Sciences and Earth Sciences Institute, Hebrew University, Jerusalem 91904, Israel*

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Abstract

The mrgA protein of the cyanobacterium *Synechocystis* sp. PCC6803 is a member of the DPS Fe storage protein family. The physiological role of this protein was studied using a disruption mutant in the *mrgA* gene (*slr1894*) and by measuring intracellular Fe quotas, 77K chlorophyll fluorescence and growth rates. It was found that the deletion of the *mrgA* gene did not impair the Fe storage capacity, as the intracellular Fe quotas of the Δ*mrgA* cells were comparable to those of the wild type. Furthermore, the cellular response to decreasing external Fe concentrations, as detected by the emergence of the CP43′ 77K fluorescence band, was similar in wild type and mutant cultures. On the other hand, a considerable slow down in the growth rate of Δ*mrgA* cultures was observed upon transfer from Fe replete to Fe depleted medium, indicating impeded utilization of the plentiful intracellular Fe. Based on these results, we suggest that *mrg*A plays an important role in the transport of intracellular Fe from storage (within bacterioferritins) to biosynthesis of metal cofactors throughout the cell's growth.

Keywords: Cyanobacteria; DPS family protein; Fe; *Synechocystis* sp.

1. Introduction

The emergence of oxygenic photosynthesis in early cyanobacterial cells approximately 2.8 billion years ago represents a turning point in the natural history of the Earth [1]. Prior to the evolution of oxygenic photosynthesis, life on earth depended mainly on reduced sulfur compounds. Oxygenic photosynthesis, on the other hand, utilized water as an abundant source of reducing power [2]. As a result, di-oxygen levels started rising and the reductive atmosphere of Earth became oxidative [1]. Following this change Fe(II) which was present at large quantities in the ancient oceans was eventually oxidized to Fe(III). While Fe(II) is soluble and easy to transport and utilize, Fe(III) is insoluble in aqueous solution at neutral pH values. Thus, the cyanobacterial induced oxidation of the planet turned biologically essential Fe from an abundant resource to a limiting factor [3]. In addition, organisms were exposed for the first time to reactive oxygen species due to the interaction of oxygen with various compounds, including Fe [4].

In the new oxidative environment of Earth, organisms had to contend with the problem of transporting, storing and assembling Fe into active cofactors and, at the same time, protect against oxidative damage generated by the interaction of Fe and oxygen. The problem of balancing Fe homeostasis and oxidative stress is most acute in the organisms which are at the source of the problem, the cyanobacteria. Their photosynthetic electron transfer chain utilizes radicals and reduced metal species as part of its normal catalysis. Moreover, their photosynthetic apparatus imposes Fe requirements that far exceed that of non-photosynthetic bacteria. The Fe quota of the cyanobacterium *Synechocystis* sp. PCC 6803 is in the range of 10^6 atoms/cell [5], one order of magnitude higher than that of the similarly sized non-photosynthetic *Escherichia coli* [6].

Metal transport pathways are composed of membrane transporters, chaperones and storage proteins [7]. In the case of the cyanobacterium *Synechocystis* 6803 a number of these components were identified and studied. The major Fe(II)

* Corresponding author. Tel.: +972 2 658 5233; fax: +972 2 658 4425.
E-mail address: nirkeren@vms.huji.ac.il (N. Keren).

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transporter across the plasma membrane is the FutABC–protein complex. Inactivation mutants in the different genes encoding for components of this transporter can only survive on high Fe concentrations [8]. After transport of Fe(II) through the plasma membrane, it is stored as Fe(III) oxides in the center of ferritin complexes [9]. *Synechocystis* 6803 contains two ferritin type storage complexes, bacterioferritin and the DPS family protein MrgA. *Synechocystis* 6803 bacterioferritins, like other ferritin family proteins, store Fe in a cavity at the center of their 24-mer ultrastructure. *Synechocystis* 6803 *bfr* genes belong to a subfamily of bacterioferritin genes in which one gene codes for a protein with conserved heme ligands and the other codes for a protein with conserved di-iron center ligands [5].

DPS proteins are a subgroup of the ferritin family which lack the fifth helix found in other ferritins [9]. Evolutionarily, DPS proteins represent a more diverse group than the other ferritin families with members functioning as Fe storage proteins, DNA binding proteins protecting against oxidative stress, cold shock proteins, neutrophile activators and pili components [9]. Unlike other ferritins, DPS proteins utilize hydrogen peroxide to oxidize Fe(II) [10]. In the cyanobacterium *Synechococcus sp*. PCC7942 a heme binding DpsA protein was identified [11]. Inactivation of this protein resulted in slow growth rates on Fe depleted media [12]. Interestingly, the *Synechococcus* 7942 DpsA protein was found to be associated with thylakoid membranes [13].

In *Synechocystis* 6803 the transcription of the *mrgA* gene was found to be under the control of the PerR regulator and therefore induced under oxidative stress [14]. In this work we demonstrate the importance of MrgA proteins for Fe homeostasis in *Synechocystis* 6803 cells suggesting a specific role for these proteins in intracellular Fe trafficking, rather than in Fe storage.

2. Materials and methods

2.1. Bioinformatics analysis

A BLASTP search was used to collect DPS proteins from the cyanobacterial genome database (http://www.kazusa.or.jp/cyanobase/). Sequences were aligned using the ClustalW method [15] and edited by hand to remove the highly divergent flanking sequences. Tree-puzzle 5.2 program (http://www.tree-puzzle.de/) was used to construct and bootstrap the neighbor-joining phylogenetic tree.

2.2. Medium and growth conditions

Cultures of wild type and Δ*mrgA* cells [14], were grown in standard BG11 medium [16] or in the modified metal buffered YBG11 medium. Detailed description of the composition of YBG11 can be found in the supplemental data file. Briefly, iron precipitation in YBG11 is prevented by lowering the concentration of iron, adding excess EDTA as compared to iron and other trace metals, and pre-complexing the iron stocks with EDTA. While BG11 contains only 2.8 μM EDTA, which is barely sufficient to complex trace metals other than iron, the EDTA concentration in YBG11 was increased to 16 μM. Standard YBG11 media contained 6 μM Fe. Cells were then spun down and re-suspended in YBG11 containing no added iron (YBG11-Fe) twice to eliminate carryover of Fe before transfer into different medium Fe concentrations. The Δ*mrgA* strain was grown in the presence of 10 μg/ml of Kanamycin. Cultures were grown in 250 ml Erlenmeyer flasks at 30 °C with constant shaking. Light intensity was set at 50 μmol photons m^{-2} s^{-1}.

2.3. Sample preparation and analytical techniques

Samples for cellular Fe quotas were processed in a trace metal clean-room as described in Keren et. al [5]. Briefly, extracellular Fe was removed by two subsequent 15-min washes (in 20 mM MES, 10 mM EDTA, pH 5). The cells were digested at 100 °C with distilled HNO_3, evaporated to dryness, and reconstituted in double distilled water. Subsamples were reduced in 0.1 M ascorbic acid over night and the total Fe(II) was determined spectrophotometrically with Ferrozine (at 562 nm, with pH 7 ammonium acetate buffer, [17]). For verification, Fe quotas were also determined using an Inductively Coupled Plasma Mass Spectrometer (ICP-MS; Perkin Elmer SCIEX, CDR II). The ICP-MS results agreed well with those if the colorometric method and were within the standard deviation of the duplicate samples.

Cell density was measured as previously described [18], using a Uvikon 860 spectrophotometer (Uvikon corporation, Switzerland). The 77K chlorophyll fluorescence spectra were measured using a Fluoromax-3 spectrofluorometer (Jobin Ivon, Longjumaeu, France).

3. Results

3.1. Phylogenetic analysis of the DPS protein family in cyanobacteria

Proteins of the DPS family can be found in many cyanobacterial species for which a complete genome sequence is available. Phylogenetic analysis of the sequenced cyanobaterial DPS proteins (Fig. 1) identified three subgroups within the family. A number of cyanobacterial species, including *Synechococcus elongatus* PCC7942, *Gloeobacter violaceus*, *Anabaena* sp. PCC7120 and *Nostoc punctiforme* PCC73012 contain genes encoding for DPS proteins from two or three groups (Fig. 1). The *Synechococcus* 7942 which was studied by Pena and coworkers [11] as well as the *Trichodesmium* IMS101 protein studied by Castruita and coworkers [19] cluster in a different group than the *Synechocystis* 6803 protein studied here (Fig. 1). Since DPS family proteins are known to carry out many different functions in the cellular metabolism [9], it is possible that each distinct phylogenetic group clusters DPS proteins with specific physiological role. This hypothesis requires further study, but should be taken into account when comparing data obtained from different cyanobacterial species.

3.2. Chemically defined growth medium YBG11

One of the roles of DPS proteins is in Fe homeostasis. In order to elucidate the role of mrgA in *Synechocystis* 6803, tight control of intracellular and external Fe concentrations are required. This was accomplished by modifying the standard BG11 medium. BG11 medium contains approximately 15 μM of Fe(III) supplied as ferric-ammonium-citrate. Since the solubility of Fe(III) in oxygenated aqueous solutions is in the sub-nanomolar range [3], most of this Fe eventually precipitates from the medium, making control of Fe levels a difficult task. In order to overcome this limitation we have used a trace metal buffered medium along the lines proposed by Sunda and coworkers for marine photosynthetic organisms [20]. The major difference between the modified YBG11 used here and the standard BG11 is the introduction of excess EDTA to the medium so that all of the iron (and the other trace metals) is

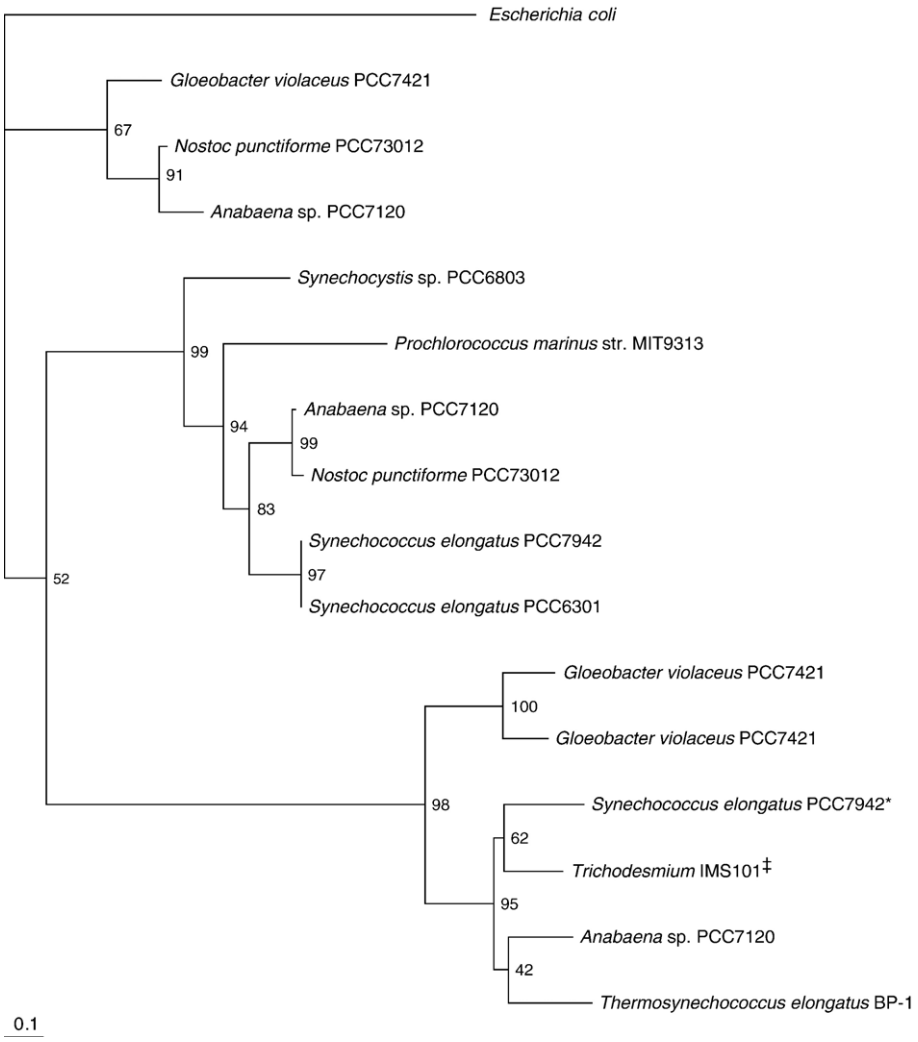

Fig. 1. Phylogenetic analysis of the DPS family proteins. Cyanobacterial DPS protein family members were analyzed using the neighbor-joining method. The numbers at the branch points represent bootstrap values. The tree is rooted with *Escherichia coli* DpsA sequence. The *Synechococcus* protein studied by Pena and coworkers [11] is marked by an asterisk and the *Trichodesmium* sequence studied by Castruita and coworkers [19] is marked by a double cross. The complete list of annotations in the order of appearance in the figure (top to bottom) is: gi|16128780, gll2819, gi|2312454, all0458, slr1894, PMT2218, all4145, gi|23129807, gi|53763190, gi|56686363, glr2566, gll0337, gi|862383, *Trichodesmium* sequence from Castruita et al. [19], alr3808, tll0614. The scale bar on the bottom represents 0.1 mutations/site.

complexed by EDTA and remain in solution. This chemically defined medium allowed for a better control over soluble Fe concentrations and diminished the troublesome precipitation of Fe on the cell surface [21].

Cyanobacterial cultures grew as well in YBG11 medium, containing 6 μM of Fe, as in BG11 medium containing more than double total Fe (Fig. 2). EDTA itself showed no adverse effects on growth at all concentrations tested up to 40 μM EDTA (data not shown). Furthermore, transfer of cells from medium containing 6 μM Fe to medium with no added Fe resulted in an immediate decrease in growth rate (Fig. 2). Elaborate washing procedures or rounds of sub-culturing were not required.

3.3. Cellular response to different medium Fe concentrations

Using this chemically defined medium we were able to test the effect of the medium Fe concentrations on the growth rates

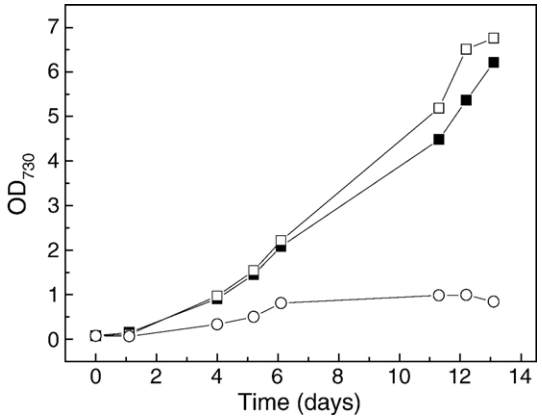

Fig. 2. Growth of Synechocystis cultures in BG11 and in modified YBG11 media. Wild type *Synechocystis* cultures were inoculated into YBG11 containing 6 μM Fe and 16 μM EDTA (□); 0 μM Fe and 16 μM EDTA (○); and BG11 containing 15 μM Fe (■). The growth of the cultures was measured as OD_{730}.

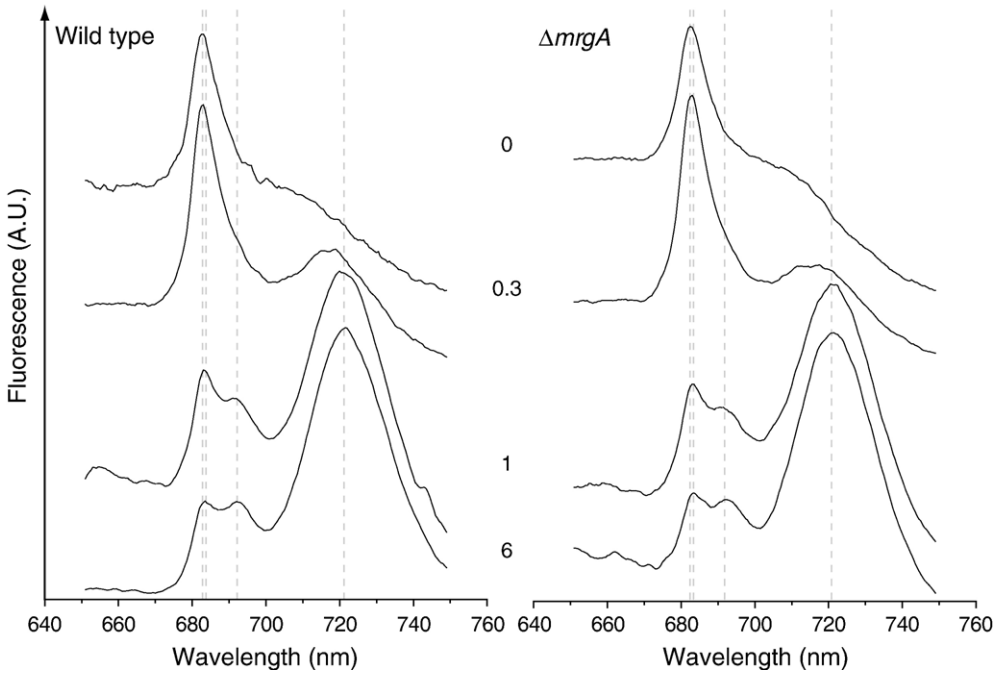

Fig. 3. 77 K chlorophyll fluorescence emission spectra of wild type and Δ*mrgA* cells. Chlorophyll fluorescence spectra at 77 K from wild type cells (left panel) and Δ*mrgA* cells (right panel). Cultures were grown in YBG11 media containing 0–6 μM Fe as indicated between the panels. The excitation wavelength was set at 420 nm ± 5 nm. The fluorescence bands at 682, 684, 692, and 720 nm, result from the emission of the CP43′ antenna complex, the PSII proximal antenna, the PSII reaction center core and the PSI reaction center core, respectively. The positions of these fluorescence bands are marked with dashed gray lines. Curves are baseline shifted for clarity.

of wild type and mutant cultures in which the *mrgA* gene was disrupted by an antibiotic resistance cassette [14]. Medium Fe concentrations ranging from 0.3 to 6 μM had little effect on either wild type or Δ*mrgA* cultures growth rates (data not shown). Nevertheless, while growth rates were barely affected, a major change in the cellular response to Fe availability was observed in the 77K fluorescence emission spectra (Fig. 3). Under Fe replete conditions (6 μM Fe) fluorescence bands rising from PSII and PSI appear at 684, 692 and 720 nm (Fig. 3). With decreasing medium Fe concentrations the CP43′ fluorescence band at 682 nm increased, indicating Fe limitation [22]. In medium containing 0.3 μM Fe or less this band is the most prominent in the spectrum. Nonetheless, the response of wild type and mutant cultures to the different external Fe concentrations is very similar.

In order to study the limitations imposed by the intracellular Fe availability, we have conducted transient growth experiments in which cultures grown with different Fe concentrations were transferred to no added Fe medium (YBG11-Fe; Fig. 4). Δ*mrgA* cells, transferred from 10 μM Fe medium, grew at a much slower rate than wild type cells (Fig. 4). Wild type and Δ*mrgA* cultures, grown in medium containing only 0.3 μM, exhibited comparably slow growth rates upon transfer to YBG11-Fe (Fig. 4).

We have also measured the internal Fe quota of the cells prior to transfer YBG11-Fe so that the growth response can be better interpreted (Fig. 5). Both wild type and Δ*mrgA* cells grown in the presence of 0.3 μM Fe had low and statistically indistinguishable intracellular Fe quotas (Fig. 5). The intracellular Fe quota of wild type cells grown in the presence of 10 μM Fe (Fig. 5) is about 4 times higher than that of cells grown in 0.3 μM Fe which fits well with slower growth rates observed upon transfer of these cells to YBG11-Fe. Interestingly, the internal Fe quota of Δ*mrgA* cells, in the presence of 10 μM Fe, is slightly higher than that of wild type cells (Fig. 5), the difference

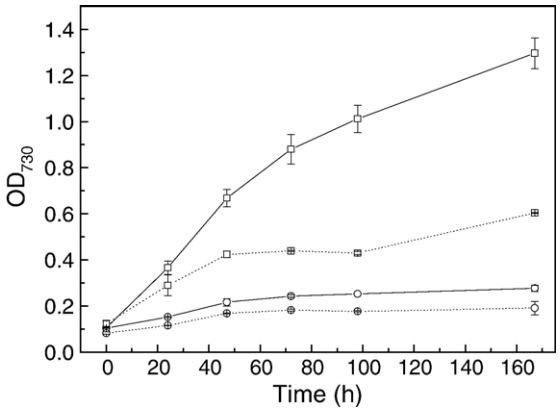

Fig. 4. Transient growth of wild type and Δ*mrgA* cultures in YBG11-Fe. Cultures grown in YBG11 containing 0.3 or 10 μM Fe, were harvested at late logarithmic phase at concentrations of $3–6\times10^8$ cells/mL. Cells were washed twice with YBG11-Fe to eliminate carryover of external Fe, adjusted to an equal concentration and transferred to YBG11-Fe (time zero). Growth was monitored as OD_{730}. Iron concentrations in the growth medium prior to the transfer are marked by open symbols: 0.3 μM (○) and 10 μM (□). Wild type cultures are marked with solid lines and Δ*mrgA* by dashed lines. Bars represent standard deviation based on two replicate experiments.

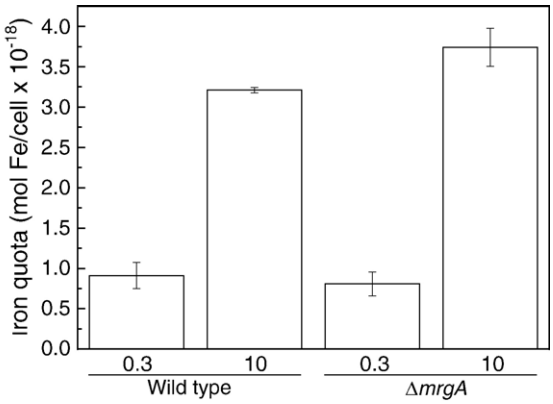

Fig. 5. Iron quotas of wild-type and mutant Δ*mrgA* cells. Late logarithmic phase cells grown in the presence of 0.3 or 10 μM Fe were harvested at $3–6\times10^8$ cells/mL and treated with EDTA to remove external Fe. Bars represent standard deviation, based on two different cultures, each measured in two replicates. The Fe quota of Δ*mrgA* cells grown with 10 μM Fe was significantly higher than that of wild type cells grown under the same conditions at $p<0.05$ based on a two sample *t*-test. No significant difference between cultures grown at 0.3 μM Fe was found.

being statistically significant. Therefore, iron storage by itself cannot explain the slower growth rate observed upon transfer of iron replete Δ*mrgA* cells to YBG11-Fe.

4. Discussion

The results presented in this paper demonstrate that mrgA proteins play an important role in Fe homeostasis in *Synechocystis* 6803 cells. The Δ*mrgA* mutant growth rate is slowed down upon transfer from Fe replete medium to Fe depleted medium, clearly indicating that the cells experience iron limitation. On the other hand, the internal Fe quota of mutant cells and the development of the CP43′ fluorescence band, as a function of decreasing medium Fe concentrations, are not different from those observed in wild type cells. Based on these observations we suggest that mrgA has no role in Fe storage *per se*. Nonetheless, it is required for the mobilization of the stored iron within the cell.

Combining the data presented here with the results obtained for bacterioferritin mutants in the same organism [5], we can further support this idea and draw a flow scheme for Fe homeostasis in *Synechocystis*. In their study, Keren and co-workers [5] report a significant slow down in growth rates of bacterioferritin mutants upon transfer from Fe replete to Fe depleted conditions, similar to that observed for Δ*mrgA* cultures here. However, unlike Δ*mrgA* cells, the bacterioferritin mutants accumulate only 50% of the wild type Fe quota and develop the CP43′ fluorescence even under Fe replete conditions [5]. Therefore, we can hypothesize that in *Synechocystis* 6803 the storage of Fe takes place in bacterioferritin complexes, while its utilization requires mrgA complexes. It is important to note that the slow down of growth in the transient experiments, which was found for all mutants, represents a different underlying physiological mechanism: impeded Fe mobilization in the Δ*mrgA* cells versus defected Fe storage capacity for the bacterioferritin mutants.

In addition to the Fe homeostasis deficiency reported here, Δ*mrgA* cells were found to be extremely sensitive to hydrogen peroxide [14], a phenotype that was not observed in bacterioferritin mutants [5]. The interaction of free Fe and hydrogen peroxide can lead to detrimental results through Fenton reactions [4]. The importance of mrgA complexes to oxidative stress response can be a direct result of their peroxidase activity. It can, in addition, be related to the role of mrgA complexes in Fe relocation inside the cells. The risk of Fenton reactions is higher in Δ*mrgA* cells than in wild type or in the bacterioferritin mutant cells, in which cellular iron quotas are lower.

Evidence from microarray analysis indicates that the translation of the *mrgA* gene is controlled by Fe availability [23]. Similar results were reported in a number of bacterial species where DPS transcripts were found to be regulated by Fe [12,24]. These results correspond well with the role proposed for mrgA complexes here, as intermediary Fe shuttles downstream of the bacterioferritin storage complexes and upstream of the cofactor assembly processes. The division of the DPS sequences into phylogenetic subgroups raises the possibility that each subgroup perform a different function. However, further research will be required in order to determine the expression pattern of DPS genes and the function of DPS proteins across a wide range of cyanobacterial species.

Mobilization of Fe from cellular storage proteins will be required mostly during transition from high to low Fe concentrations, conditions which severely inhibit the growth of Δ*mrgA* cells. It is important to note that in aqueous environments bioavailable Fe is supplied during transient events of dust deposition or upwelling [25]. It is under such conditions that the function of mrgA in particular and DPS family proteins in general will be crucial.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.bbabio.2006.11.015.

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