Regulation of Manganese Uptake in *Synechocystis* 6803 by RfrA, a Member of a Novel Family of Proteins Containing a Repeated Five-Residues Domain†

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ABSTRACT: The *rfrA* gene was identified in a suppressor screen of a *Synechocystis* sp. PCC 6803 strain deficient in both *mntC*, encoding a component of an ABC transport system for manganese, and *psbO*, encoding the extrinsic manganese stabilizing protein of photosystem II (PSII). A spontaneous suppressor mutant (Δ*C*Δ*O rfrA-Sup*) has a point mutation in *rfrA*, which restores photosynthetic activity to the Δ*mntC*Δ*psbO* double mutant. Manganese transport and photosynthesis are related in that manganese is essential to the function of PSII, and the state of cellular manganese availability influences the rate of oxygen evolution mediated by PSII. Oxygen evolution experiments with the Δ*C*Δ*O rfrA-Sup* mutant revealed that the mechanism of suppression is not through a direct modification of PSII. Instead, radioactive manganese uptake experiments indicated that RfrA is a regulator of a high affinity manganese transport system different from the more thoroughly characterized manganese ABC transport system in *Synechocystis* 6803. RfrA was named for the repeated five-residues domain in the amino terminus of the protein. The RFR domain defines a 16-member family in *Synechocystis* 6803. Predicted proteins with RFR domains have also been identified in other organisms, but RfrA is the first member of this family to be linked to a physiological process.

Manganese is an essential trace element in all organisms. In prokaryotes and eukaryotes manganese is a cofactor in the active site of many enzymes, such as manganese-superoxide dismutase, manganese-catalase, and ribonucleotide reductase (*1*). Even though manganese is essential to most organisms, it can be toxic at high levels (*2*−*4*). Therefore, homeostasis of this metal ion is important for survival. Prokaryotic organisms lack internal compartments, so homeostasis is maintained through tight regulation of metal ion flux across the cytoplasmic membrane.

In plants and cyanobacteria, manganese is required for the light-dependent oxidation of water into molecular oxygen by photosystem II (PSII).[1] Therefore, manganese plays a critical role in these photosynthetic organisms. We have recently reported a light-dependent mechanism for the accumulation of large amounts of manganese in the cyanobacterium, *Synechocystis* sp. PCC 6803 (*5*). This pool of manganese does not appear to be present in other types of bacteria, suggesting a complexity in manganese regulation unique to the demands for this ion during photosynthesis.

One of the first manganese transport systems to be genetically characterized is encoded by the *mntCAB* operon in the cyanobacterium *Synechocystis* 6803 (*6*), and many other manganese transport systems have since been described (*7*). Most manganese uptake systems fall into one of two groups: the ATP-binding cassette (ABC) transporters and the natural resistance-associated macrophage proteins (Nramp) (*7*). In addition, a transporter in *Lactobacillus plantarum* is the only identified P-type ATPase with high specificity for manganese (*8*).

Since manganese is essential for the survival of a cyanobacterium such as *Synechocystis* 6803, there is an intricate genetic network for controlling manganese homeostasis in this organism. The ABC transporter system (MntABC) encoded by the *mntCAB* operon is the only genetically identified manganese uptake system. Recently, we reported that the transcription of the *mntCAB* operon is tightly regulated by a two-component sensor/regulator system encoded by the *manS* and *manR* genes (*9*). It was also shown that the ManR protein binds to the *mntCAB* operon (*10*) to support the suggestion that MntABC is directly regulated by the two-component system. The *mntCAB* operon is expressed in cells grown in manganese-deficient (submicromolar levels of Mn^{2+}) medium and functions in high-affinity manganese uptake (*6*). In contrast, cells grown under micromolar concentrations of Mn^{2+} transport this metal by a second high affinity system (*11*). However, the gene(s) encoding this system are yet to be identified.

A unique need for manganese in cyanobacteria and plants is in their photosynthetic apparatus. There are four manganese ions necessary for each active PSII complex, and it has been observed that deficiencies in manganese uptake lead to decreased photosynthetic oxygen evolution in *Synechocystis* 6803 (*6*). Therefore, changes in photosynthetic activity can reflect changes in the ability of the cells to take up manganese. In this study, a deletion mutation at the *mntC* locus was combined with an interruption mutation in *psbO*

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[1] Abbreviations: PSII, photosystem II; MntABC, ATP binding cassette manganese transport system; RFR domain, repeated five-residues domain.

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Table 1: *Synechocystis* 6803 Strains Used in This Study

strain	characteristic	source
WT	wild type, glucose tolerant	*14*
Δ*mntC*	Δ*mntC*::Km^r	*11*
Δ*psbO*	Δ*psbO*::Sp^r	this paper
Δ*mntC*Δ*psbO*	Δ*mntC*::Km^r; Δ*psbO*::Sp^r	this paper
Δ*C*Δ*O rfrA-Sup*	Δ*mntC*::Km^r; Δ*psbO*::Sp^r; *rfrA* suppressor	this paper
Δ*rfrA*	Δ*rfrA*::Gm^r	this paper

that encodes the manganese stabilizing protein of PSII. When grown in manganese-deficient medium, this double mutant has no active PSII complex. A suppressor mutation in *rfrA* restores PSII activity in the Δ*mntC*Δ*psbO* strain. In this paper, we have characterized the functional role of RfrA through analysis of the spontaneous point mutation allele that suppresses the Δ*mntC*Δ*psbO* phenotype, as well as examining a deletion at the *rfrA* locus. Our data indicate that RfrA is not a manganese transporter. Instead, RfrA regulates the manganese transport system that functions in *Synechocystis* 6803 cells grown in high concentrations of external manganese.

EXPERIMENTAL PROCEDURES

Bacterial Strains and Culture Conditions. Bacterial strains used in this study are described in Table 1. *Synechocystis* 6803 strains were grown at 30 °C under 20 μmol m^{-2} s^{-1} white fluorescent light in BG11 medium (*12*). For growth on solid medium, BG11 was supplemented with 1.5% (w/v) agar. BG11 without added manganese (BG11-Mn) was prepared as described in ref *11*, and sterilized $MnCl_2$ was added to a final concentration of 5 μM, unless otherwise stated. DNA disruptions used to engineer the mutants used in this study are shown in Figure 1. Growth medium for Δ*mntC*, Δ*psbO*, and Δ*rfrA* was supplemented with 10 μg/mL kanamycin, 10 μg/mL spectinomycin, and 2 μg/mL gentamycin, respectively. Cyanobacterial cell growth was monitored by measuring light scattering at 730 nm on a DW2000 spectrophotometer (SLM-Aminco, Urbana, IL).

For generating growth curve data, the strains were grown as 3 mL cultures in 12-well microtiter plates in either BG11-Mn or BG11 with 5 μM $MnCl_2$ added. At each time point, 150 μL was removed from each replicate for absorbance determination in a μQuant plate reader (Bio-Tek Instruments, Inc., Winooski, VT). Measurements were typically taken once every 20−24 h for 120 h, and the growth rate was determined between 46 and 72 h.

Transformation and DNA Manipulations. Routine DNA manipulations were performed essentially as described in ref *13*. Enzymes for recombinant DNA techniques were from New England Biolabs (Beverly, MA). DNA sequencing was performed with dideoxynucleotide chain termination using custom-made oligonucleotide primers ordered from Integrated DNA Technologies (Coralville, IA). Synechocystis 6803 cells were transformed as previously described (*14*). For the complementation analysis, linear fragments of chromosomal DNA from Δ*mntC*Δ*psbO* suppressor mutant were spotted onto a lawn of Δ*mntC*Δ*psbO* mutant cells to determine which fragment restored photoautotrophy under low Mn^{2+} concentrations (*6*, *15*).

Measurement of Oxygen Evolution. As previously described, a Clark-type electrode was used to determine the rate of photosynthetic oxygen evolution (*16*). Cells were harvested after 6 days of growth in BG11 with 5 μM Mn^{2+} and resuspended in fresh BG11 to a final chlorophyll concentration of 5 μg/mL. PSII-mediated O_2 evolution was measured in the presence of 0.5 mM 2,6-dichloro-*p*-benzoquinone (Eastman-Kodak, Rochester, NY) and 1 mM K_3FeCN_6 (Sigma, St. Louis).

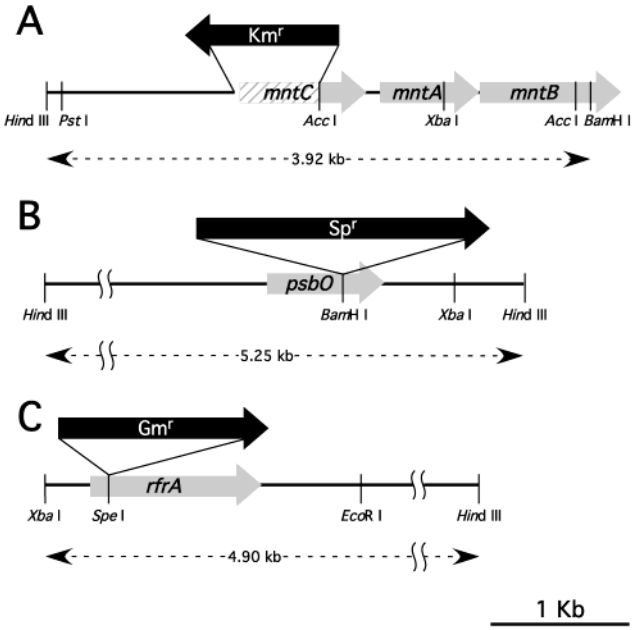


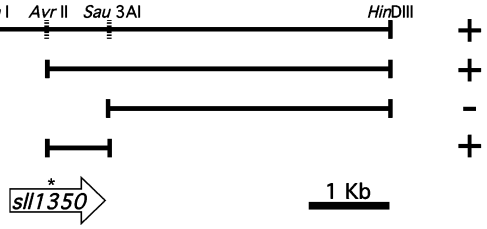
FIGURE 1: Constructs used for the generation of *Synechocystis* 6803 mutants examined in this study. (A) Restriction enzyme map of the portion of the *mntCAB* operon used to generate a partial deletion of the *mntC* gene. (B) Restriction map of the construct used for an insertional mutation of *psbO*. *Synechocystis* 6803 Δ*mntC* cells were transformed with the Δ*psbO* construct to make the Δ*mntC*Δ*psbO* double mutant. (C) Restriction map of the construct used to generate an insertional mutation at the *rfrA* locus. Block arrows, direction of transcription; Km^r, kanamycin resistance cassette; Sp^r, spectinomycin/streptomycin resistance cassette; and Gm^r, gentamycin resistance cassette.

Manganese Uptake Assays. Synechocystis 6803 cells were maintained on solid BG11 plates from which a loop full of cells was used to inoculate 50 mL of liquid BG11-Mn cultures. After 3−4 days of growth, the cells were harvested by centrifugation and resuspended in fresh BG11-Mn with the addition of 5 μM $MnCl_2$ where indicated. As described previously (*11*), cells were harvested and washed after 20−24 h of growth and resuspended in fresh BG11-Mn to a concentration of 4×10^8 cells/mL. The cells were preincubated at 28 °C and under 30 μmol m^{-2} s^{-1} white light for at least 20 min before the experiment. $^{54}Mn^{2+}$ (as $MnCl_2$) was added at ∼0.4 μCi/mL (∼0.4 pmol of Mn^{2+}). In some experiments, divalent cadmium (as $CdCl_2$ to 10 μM) was added just prior to $^{54}Mn^{2+}$. After the indicated time intervals, 50 μL samples were removed and diluted 100-fold in a 10 mM $MnCl_2$ solution and immediately filtered through nitrocellulose membrane filters. The filters were suspended in scintillation mixture (EcoScint) and counted on an LS 5000 TD scintillation counter (Beckman Instruments).

RESULTS

Isolation of the ΔCΔO rfrA-Sup Mutant. A suppressor screen was designed to identify genetic loci, separate from

A



B

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MPNELSNLPP VLGSEETIAP RRSLPQWVFQ ESVEPAMPWL  40
LVVTVVGIGL GLIFQSSWLG FTSAVLALMF SLQVILPTIK  80
SWVQHYLTVQ ERRSLLGGLG FVLAIGALAH YLGVYSSLKY 120
WLNQFKYDEF GSWAEWVGAL GQIMIAVLAV YVAWQQYVIS 160
KDLTIQQNRI TQQQTVDAYF QGISDLALSD EGMLEDWPQE 200
RAFAEGRTAA ILASVDSGGK AKILRFLSQS RLLTPLKRDY 240
YLGRPIFDGM GGYQEDRIHG LRVINLGVML VAADLVGQDL 280
RWVDLSEIYL IRANLSQADL VKANLSRTVL YEANLDGADL 320
KGTRLFYGTW DQASPRSRNH IPNYETGEYT GAVVENANFS 360
KTKNMSEEQR CYCCAWGGTL TRKTIPGGCE GIENRLDR*  398
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FIGURE 2: Cloning by complementation and predicted amino acid sequence of *Synechocystis* 6803 RfrA. (A) Restriction enzyme fragments used to complement the Δ*mntC*Δ*psbO* mutant. +, complementation; −, no complementation; *, mutation in ORF *sll1350* (represented by the arrow) in the Δ*C*Δ*O rfrA-Sup* strain. (B) Predicted amino acid sequence of RfrA with four membrane-spanning domains (underlined). The 12 pentapeptide repeats in the RFR domain are shown in bold with alternate repeats italicized. The mutation in Δ*C*Δ*O rfrA-Sup* resulted in a T171A change (boxed).

mntCAB, which influence manganese transport in *Synechocystis* 6803. The genes of interest could be transporters or regulators of transport activity. The strain used for the suppressor screen contained mutations at the *mntC* and *psbO* loci. The Δ*mntC* strain was described previously (*11*), and deficiencies at the *psbO* locus have been described in several studies (*17*−*19*). The Δ*mntC*Δ*psbO* double deletion mutant lacks the high-affinity manganese transporter whose expression is induced when the Mn^{2+} concentration in the medium falls below 5 μM, conditions that decrease the photosynthetic activity of PSII by 85% (*20*). The double mutant also lacks the manganese stabilizing protein (PsbO), one of the extrinsic subunits of PSII, whose absence decreases the activity of PSII by 60% (*18*). The presence of both mutations was necessary because neither the Δ*mntC* nor the Δ*psbO* mutation completely inhibits PSII activity. In contrast, the Δ*mntC*Δ*psbO* double mutant is not photoautotrophic when grown in submicromolar concentrations of Mn^{2+} because there is insufficient manganese to support PSII activity. Δ*mntC*Δ*psbO* cells were maintained on plates containing 5 mM glucose and were plated onto BG11-Mn without glucose to screen for spontaneous mutants allowing photoautotrophic growth. One suppressor mutant strain was chosen for further analysis.

Restriction fragments from the Δ*mntC*Δ*psbO* suppressor mutant genome were used in a complementation study with the Δ*mntC*Δ*psbO* strain to determine the location of the mutation (Figure 2A). This is possible because *in situ* dot transformations can be used to screen pools of linear or plasmid DNA (*15*). Sequencing of a 0.5 Kb complementing fragment revealed a point mutation in the *sll1350* (Cyanobase notation; www.kazusa.or.jp/cyano/cyano.html) open reading frame (ORF). This point mutation results in a Thr 171 to Ala amino acid change in the predicted protein sequence. Further *in silico* analysis of the Sll1350 sequence predicted the presence of four membrane-spanning regions within the N-terminal half (Top Pred2, ref *21* and a repeated five-residues (RFR) domain within the soluble C-terminal half of this protein. Thus, the gene was named *rfrA.* As shown in Figure 2B, the RFR domain has 12 repeats of AXLXX (X being any amino acid), with strong conservation at the third position and usually a small hydrophobic residue in the first position. A deletion mutation was generated at the *rfrA* locus (Figure 1) to compare the loss of function allele to the suppressor allele.

Photoautotrophic Growth of the rfrA Mutants. Even thought the suppressor mutant exhibited photoautotrophic growth on solid medium, growth in liquid medium was examined to determine relative growth rates. Table 2 summarizes the results of the growth data. Growth rates for all of the strains were decreased under manganese limiting conditions, and as expected, the Δ*psbO* and Δ*mntC* mutants were more severely affected than the wild-type cells. Under manganese limiting conditions, there was a slight increase in growth rate in the suppressor mutant as compared to the Δ*mntC*Δ*psbO* double mutant. Interestingly, there was a decrease in the photoautotrophic growth rate of the Δ*rfrA* mutant under manganese limiting conditions but an increase relative to wild type under sufficient manganese conditions. There are many factors that contribute to photoautotrophic growth rate, so additional experiments were designed to test the mechanism of suppression more directly.

Photosynthetic Activity of the rfrA Mutants. Next, we investigated whether the Δ*C*Δ*O rfrA-Sup* strain acquired photoautotrophic growth by overcoming the Δ*mntC* phenotype or the Δ*psbO* phenotype. Three hypotheses could explain the mechanism of suppression in the Δ*C*Δ*O rfrA-Sup* strain. Suppression could be (1) caused by a modification of PSII, (2) the result of a mutation in a manganese transport protein, or (3) caused by the aberrant regulation of a manganese transport system that allows sufficient manganese uptake to restore photoautotrophic growth.

First, we examined the activity of PSII in the Δ*C*Δ*O rfrA-Sup* mutant relative to control strains by measuring the rate of photosynthetic oxygen evolution. Δ*C*Δ*O rfrA-Sup* consistently had the same rate of oxygen evolution as the Δ*psbO* strain, about 50% of wild type (Table 3). The cells were grown in BG11 containing 5 μM Mn^{2+} so that the suppressor mutant could be compared to Δ*mntC*, Δ*psbO*, as well as the Δ*mntC*Δ*psbO* double mutant, which does not grow well enough to test under manganese-limiting conditions. Under manganese deficient conditions, all of the other strains had less PSII activity. However, it is important to note that compared to the wild-type strain, the relative PSII activity in either the Δ*C*Δ*O rfrA-Sup* or the Δ*rfrA* strain was similar under both Mn-deficient (data not shown) and Mn-sufficient conditions. The Δ*rfrA* mutant was also tested to determine if the RfrA directly influences PSII activity. Δ*rfrA* consistently evolved the same amount of oxygen as the wild-type cells (Table 3), suggesting that the absence of RfrA had no direct effect on PSII activity. If RfrA were influencing PSII, then the suppressor mutant would have been expected to have greater activity than the Δ*psbO* mutant, or the Δ*rfrA* mutant should have had less PSII activity than wild type. Because the suppressor mutant had a similar rate of oxygen evolution as Δ*psbO*, we concluded that the mechanism of suppression is most likely not due to a modification in PSII.

Table 2: Growth Data for *Synechocystis* 6803 Cells

growth conditions[a]	−Mn		+Mn	
	rate (%)[b]	final A_{730}[c]	rate (%)	final A_{730}
strain				
WT	100 ± 8.9[d]	0.641 ± 0.037	100 ± 19.3[e]	1.164 ± 0.168
Δ*mntC*	10.8 ± 3.0	0.152 ± 0.008	105 ± 10.2	1.302 ± 0.129
Δ*psbO*	75.3 ± 1.8	0.395 ± 0.032	94.0 ± 7.3	0.741 ± 0.063
Δ*mntC*Δ*psbO*	0	0.090 ± 0.017	118 ± 34.0	1.182 ± 0.132
Δ*C*Δ*O rfrA-Sup*	10.1 ± 0.6	0.079 ± 0.002	72.6 ± 17.1	0.918 ± 0.090
Δ*rfrA*	30.0 ± 10.5	0.344 ± 0.010	162 ± 24.8	1.596 ± 0.246

[a] Cells were grown with (+Mn) or without (−Mn) added 5 μM $MnCl_2$ for 120 h under 20 μmol photons/m^2 sec at 30 °C on a platform shaker. [b] Growth rate was determined during log phase. [c] The maximum culture density in the 120-h experiment. [d] Values are given as the percentage of the wild-type growth rate (100% = 0.00368 ΔA_{730}/h) with standard deviation between three replicates. [e] 100% = 0.00874 ΔA_{730}/h. [f] These data are representative of several experiments.

Table 3: Rates of Oxygen Evolution under 5 μM $MnCl_2$

strain	oxygen evolution (μmol O_2 mg chlorophyll^{-1} h^{-1})	% wild type
WT	305 ± 71[a]	100
Δ*mntC*	313 ± 93	102 ± 19
Δ*psbO*	161 ± 43	54 ± 13
Δ*mntC*Δ*psbO*	184 ± 34	62 ± 15
Δ*C*Δ*O rfrA-Sup*	143 ± 42	49 ± 18
Δ*rfrA*	323 ± 88	106 ± 10

[a] Each value is mean ± standard deviation of five measurements.

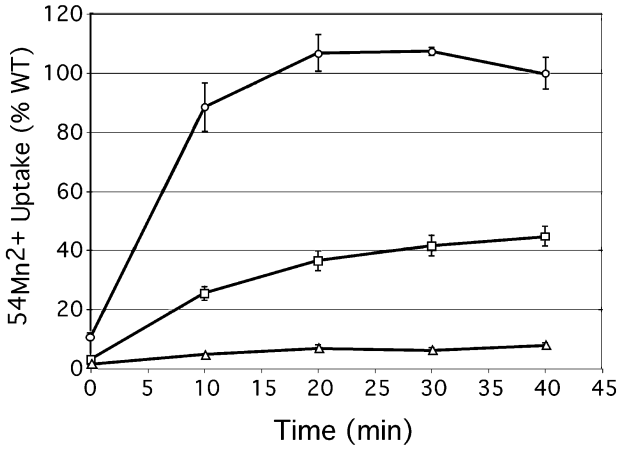

FIGURE 3: $^{54}Mn^{2+}$ uptake in cells grown in Mn-deficient medium. Circles, wild type; squares, Δ*C*Δ*O rfrA-Sup*; triangles, Δ*mntC*. 100% was taken as the amount of $^{54}Mn^{2+}$ taken up by the wild-type cells after 40 min incubation (corresponding to 0.013 pmol). The data are from one representative experiment with three replicates of each time point.

Examining the Rate of Manganese Uptake. Since the first hypothesis for a mechanism of suppression was ruled out, we then examined if a change in manganese uptake was causing suppression. Quantitative measurements of manganese uptake were made using $^{54}Mn^{2+}$ uptake assays. As described in ref *11*, the MntABC transporter is induced under submicromolar concentrations of Mn^{2+} and is repressed under higher concentrations of this metal. This study demonstrated that a second manganese transporter is active during growth in high manganese-containing medium (*11*). Figure 3 shows that Δ*C*Δ*O rfrA-Sup* cells take up more manganese than the Δ*mntC* strain but have only about 45% of the activity as compared to the wild-type cells. The suppressor mutant could not be compared to the Δ*mntC*Δ*psbO* double deletion mutant because that strain does not grow under the experimental conditions. This result indicates that the mechanism of suppression is due to an alteration in manganese uptake activity.

Table 4: Radioactive Manganese Uptake by *Synechocystis* 6803 Cells

growth conditions[a]	−Mn		+Mn	
	−Cd	+Cd[b]	−Cd	+Cd[b]
strain				
WT	100	11 ± 1[c]	48 ± 7	43 ± 4
Δ*mntC*	8 ± 1	NT[d]	38 ± 5	50 ± 3
Δ*C*Δ*O rfrA-Sup*	45 ± 3	46 ± 7	52 ± 5	63 ± 2
Δ*rfrA*	100 ± 7	48 ± 6	64 ± 6	62 ± 17

[a] Cells were grown with (+Mn) or without (−Mn) added 5 μM $MnCl_2$ for 24 h prior to the assays. [b] 10 μM $CdCl_2$ added just prior to initiation of uptake assay. [c] Values are given as the percentage $^{54}Mn^{2+}$ taken up by the wild-type control ± standard deviation of the mean of three samples (100% = 0.013 pmol in 40 min). [d] Cd^{2+} inhibition was not tested because there was essentially no uptake in the Δ*mntC* strain in −Mn growth medium.

Two possibilities for altered manganese entry into the cells are that (1) a transport protein is aberrantly facilitating manganese uptake or (2) the activity of a regulator of manganese transport has been altered. The rate of manganese uptake in the Δ*C*Δ*O rfrA-Sup* strain (Figure 3) suggests a different transporter than the MntABC system, which takes up manganese much more efficiently under these conditions. Manganese uptake in the Δ*rfrA* strain still takes place at a rate similar to the Δ*C*Δ*O rfrA-Sup* mutant (Table 4), which indicates that RfrA is not a transport protein. The following experiment was designed to distinguish which of the two manganese transport systems in *Synechocystis* 6803 RfrA regulates.

High affinity manganese transport is inhibited by cadmium in *Bacillus subtilis* (*22*), *Lactobacillus plantarum* (*23*), *Staphylococcus aureus* (*24*), as well as *Synechocystis* 6803 (*11*). When grown at submicromolar levels of Mn^{2+}, Cd^{2+} severely inhibits manganese uptake in the *Synechocystis* 6803 cells, but uptake in the wild type, as well as the Δ*mntC*, strain is insensitive to Cd^{2+} when grown in medium containing 5 μM Mn^{2+} (*11*). This means that the MntABC transporter is sensitive to cadmium and the second transporter is not.

As shown in Table 4, the suppressor mutant is insensitive to Cd^{2+} inhibition when grown in the absence of manganese, demonstrating that the second transporter is aberrantly functional in this strain. Moreover, in the Δ*rfrA* strain 48% of the activity was insensitive to Cd^{2+} implying that both transport systems were functioning under these conditions.

Table 5: *rfr* Genes in *Synechocystis* 6803

gene	AAs	predicted location	name (similarity; organism)
sll1350	398	membrane	*rfrA*
sll0183	259	membrane	*rfrB*
sll0274	196	membrane	*rfrC*
slr0516	166	membrane	*rfrD*
slr0719	388	membrane	*rfrE*
slr1519	245	putative membrane	*rfrF* (*hglK*; *Anabaena* 7120)
slr1697	574	membrane	*rfrG*/*spkB*
slr1851	162	membrane	*rfrH*
sll0414	286	cytoplasmic	*rfrI*
sll1011	270	cytoplasmic	*rfrJ*
slr1152	331	cytoplasmic	*rfrK*
sll1446	320	cytoplasmic	*rfrL*
slr1819	331	cytoplasmic	*rfrM*
sll0301	169	lumenal/periplasmic	*rfrN*
sll0577	169	lumenal/periplasmic	*rfrO*
slr0967	150	lumenal/periplasmic	*rfrP*

It is noteworthy that the uptake activity in the suppressor strain was restored to only 45% when the cells were grown without manganese, which is the same level of activity of the wild-type cells grown in the presence of manganese. It is well-established that when the external metal concentration is high, the transporters active under such a condition take up the metal at a much lower rate than transporters needing to scavenge metals from low external availability (*25*). The rate of uptake by the second transporter in the wild-type strain is the same as the aberrant activity in the suppressor mutant, which supports the notion that regulation of the second transporter is altered in the Δ*C*Δ*O rfrA-Sup* mutant. Table 4 also shows that under low manganese growth conditions, the cadmium-insensitive activity in the wild type cells was only 11% of the normal activity, as opposed to 45% in the mutant. This result suggests that the second transporter is normally repressed under low manganese growth conditions.

For the cells grown in the presence of manganese, both Δ*C*Δ*O rfrA-Sup* and Δ*rfrA* take up more manganese than the wild type or Δ*mntC* strains. This result indicates that even though the second transporter is normally induced under high manganese growth conditions, its activity is still regulated to prevent too much manganese uptake. In contrast, the second, Cd^{2+}-insensitive, manganese transporter in both the Δ*C*Δ*O rfrA-Sup* and the Δ*rfrA* strains is deregulated under high manganese growth conditions but subject to further control under low manganese conditions.

Analysis of the RFR Domain. Searching Cyanobase (www.kazusa.or.jp/cyano/cyano.html) with the RfrA amino acid sequence uncovered a family of genes with 16 members in *Synechocystis* 6803; 15 of these are not homologous to any gene with a predicted function (Table 5). One *Synechocystis* 6803 RFR protein is similar to the *Anabaena* sp. PCC 7120 *hglK* gene. On the basis of the predicted amino acid sequence of the RFR-containing ORFs, the genes were divided into three major groups: putative membrane spanning, cytoplasmic, and thylakoid lumen or periplasm targeted (*26*). After *rfrA*, the genes were arranged by group and named *rfrB*-*rfrN*. The gene *rfrG* has also been called *spkB* because of its kinase activity exhibited after overexpression in *Escherichia coli* (*27*). RfrG is the only *Synechocystis* 6803 protein with a kinase domain, so its kinase activity does not explain the role of other members in the family.

Further analysis using BLAST to search all Genbank sequences revealed that RFR domains are abundant in photosynthetic organisms (Table 6). The RFR domain was called a pentapeptide repeat when it was first recognized in the *hglK* gene in *Anabaena* 7120, which is involved in glycolipid localization during heterocyst formation (*28*). However, *Synechocystis* 6803 does not form heterocysts; hence, the function of the corresponding RFR protein in *Synechocystis* 6803 remains elusive. We are not using the pentapeptide repeat name for nomenclature because PPR is already used to define the large family of proteins with over 200 members in *Arabidopsis thaliana* containing a pentatricopeptide repeat motif (*29*).

Table 6: RFR Proteins Found in Different Organisms

organism	Rfr homologues[a]
Photosynthetic Organisms	
cyanobacteria	
Synechocystis sp. PCC 6803	16
Anabaena sp. PCC 7120	31
Synechococcus WH 8102 (marine)	4
Thermosynechococcus elongatus BP1	15
Nostoc punctiforme ATCC 29133	≥32
Gloeobacter violaceaous PCC 7421	≥18
Prochlorococcus sp. MIT9313	5
Prochlorococcus sp. MED4	1
Arabidopsis thaliana	4
Oryza sativa	1
Nonphotosynthetic Organisms	
Escherichia coli	2
Bacillus subtilis	2
Caulobacter crescentus	6
Mycobacterium tuberculosis	1
Clostridium acetobutylicum	4
Pseudomonas areuginosa	4
Streptomyces coelicolor	2
Drosophila melanogaster	2
Homo sapiens	1
Saccharomyces cerevisiae	0
Caenorhabditis elegans	0

[a] Criteria for homology was eight consecutive repeats per RFR domain.

The RFR family was hypothesized to be involved in bacterial metabolism. Representatives from numerous bacterial genomes have been aligned and compared (*30*). A total of 352 individual repeats were aligned to identify a five-residue consensus (ANLSG) and to predict that the repeated elements form a right-handed parallel β-helix with the leucine side chains forming aliphatic stacks in the interior of the helix (*30*). Information on this family of proteins can be found in the Pfam database (http://www.sanger.ac.uk/cgi-bin/Pfam/getacc?PF00805). As in *Synechocystis* 6803, the RFR genes found in other organisms have no defined function.

DISCUSSION

We have presented evidence that RfrA is a novel regulator of manganese uptake in *Synechocystis* 6803. We recently reported that the MntABC transporter in *Synechocystis* 6803 is regulated by a two-component system that regulates the transcription of the *mntCAB* operon (*9*). This raises questions as to how RfrA regulates the second manganese transporter and why *Synechocystis* 6803 contains such an intricate network of control over manganese uptake. Two hypotheses for the mode of RfrA regulation of manganese uptake are (1) transcriptional by altering the expression of the second

transporter and (2) posttranslational through reversible protein modification. It is necessary to consider both what is known about protein regulation in bacteria as well as unique factors in *Synechocystis* 6803 that would demand multiple manganese transport systems under complex regulation.

Two well-known metalloregulatory protein families are represented by the *E. coli* Fur (ferric uptake repressor) and *Corynebacterium diptheriae* DtxR (diptheria toxin repressor) proteins. Regulators in the Fur family control iron uptake, Zn^{2+} transport, and peroxide stress response (*31*−*33*). Fur-mediated regulation takes place through transcriptional repression when the regulator, in the Fur-Fe^{2+} state, binds iron-regulated promoters (*32*). DtxR family regulators control iron uptake and manganese transport. An example of a DtxR regulator is the *B. subtilis* bifunctional manganese regulator, MntR, which transcriptionally represses an Nramp transporter when external manganese concentrations are high and activates a manganese ABC transporter under low external manganese concentrations (*34*). Recently, it was shown in *Salmonella enterica* serovar Typhimurium that both MntR and Fur mediate manganese repression at the *mntH* promoter (*35*). RfrA has no sequence or structural similarities to these manganese-regulated transcription factors, and it does not have any known DNA-binding domain. Hence, it is more plausible that RfrA regulates the second transporter through a mechanism other than transcriptional control.

Reversible modification of proteins is a well-established mode of regulation. An example of this type of regulation is through phosphorylation and dephosphorylation to activate or inactivate a protein. Interestingly, the Δ*C*Δ*O rfrA-Sup* mutant T171A modification changes a potential phosphorylation site (Thr) to a residue that cannot be phosphorylated (Ala). A reversible modification of this residue could be required for RfrA-mediated regulation of the second manganese transporter in *Synechocystis* 6803, especially under low external manganese concentrations. Further experiments are required to determine the exact mode of such regulation.

We have observed the deregulation of the second high affinity manganese uptake system, but this makes up only one part of the complex network of manganese uptake regulation in *Synechocystis* 6803. The 45% cadmium-insensitive activity in the suppressor mutant and the Δ*rfrA* mutant grown under low manganese conditions indicates constitutive activity of the MntABC-independent transporter, which normally functions only under high manganese growth conditions (Table 4). The MntABC transport system, which is expressed under submicromolar concentrations of external Mn^{2+}, is controlled by a two-component manganese sensor/regulator system, ManS/ManR (*9*). This regulatory system is the first two-component regulatory system identified for manganese. Interestingly, when a mutant form of the ManS protein is unable to down-regulate MntABC activity, there is still only ∼60% of the normal manganese uptake activity (*9*). These data support our hypothesis that manganese uptake by both the MntABC system and the second transporter is subject to regulation, and there may be cross talk between the regulatory pathways.

Synechocystis 6803 has two exquisitely regulated manganese transport systems. The activity of the two systems was established in ref *11*. MntABC transport is controlled by a two-component sensor/regulator system (*9*), and we have shown that RfrA regulates the second transport system. Cultured *Synechocystis* 6803 can tolerate an enormous range of external manganese concentrations (0.1 μM to 1 mM; unpublished observation). An intricate network of regulation would provide adequate, but not toxic, manganese uptake regardless of the external concentration. The pool of manganese needed to maintain active PSII provides a demand for manganese unique to these photosynthetic organisms. Such a demand could be a sufficient cause for the network of manganese uptake regulation. Nevertheless, further investigation is needed to determine how cyanobacteria regulate their functions over such a broad range of external manganese conditions.

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REFERENCES

1. Dismukes, G. C. (1996) *Chem. Rev. 96*, 2909−2926.
2. Gerber, G. B., Leonard, A., and Hantson, P. (2002) *Crit. Rev. Oncol. Hematol. 42*, 24−34.
3. Mergler, D. (1999) *Environ. Res. 80*, 99−102.
4. Reaney, S. H., Kwik-Uribe, C. L., and Smith, D. R. (2002) *Chem. Res. Toxicol. 15*, 1119−1126.
5. Keren, N., Kidd, M. J., Penner-Hahn, J. E., and Pakrasi, H. B. (2002) *Biochemistry 41*, 15085−15092.
6. Bartsevich, V. V., and Pakrasi, H. B. (1995) *EMBO J. 14*, 1845−1853.
7. Jakubovics, N. S., and Jenkinson, H. F. (2001) *Microbiology 147*, 1709−1718.
8. Hao, Z., Chen, S., and Wilson, D. B. (1999) *Appl. Environ. Microbiol. 65*, 4746−4752.
9. Ogawa, T., Bao, D. H., Katoh, H., Shibata, M., Pakrasi, H. B., and Bhattacharyya-Pakrasi, M. (2002) *J. Biol. Chem. 277*, 28981−28986.
10. Yamaguchi, K., Suzuki, I., Yamamoto, H., Lyukevich, A., Bodrova, I., Los, D. A., Piven, I., Zinchenko, V., Kanehisa, M., and Murata, N. (2002) *Plant Cell 14*, 2901−2913.
11. Bartsevich, V. V., and Pakrasi, H. B. (1996) *J. Biol. Chem. 271*, 26057−26061.
12. Allen, M. (1968) *J. Phycol. 4*, 1−4.
13. Sambrook, J., Fritsch, E. F., and Maniatis, T. (1989) *Molecular Cloning: A laboratory manual*, 2nd ed., Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY.
14. Williams, J. G. K. (1988) *Methods Enzymol. 167*, 766−778.
15. Dzelzkalns, V. A., and Bogorad, L. (1988) *EMBO J. 7*, 333−338.
16. Mannan, R. M., and Pakrasi, H. B. (1993) *Plant Physiol. 103*, 971−977.
17. Chu, H. A., Nguyen, A. P., and Debus, R. J. (1994) *Biochemistry 33*, 6150−6157.
18. Burnap, R. L., Shen, J. R., Jursinic, P. A., Inoue, Y., and Sherman, L. A. (1992) *Biochemistry 31*, 7404−7410.
19. Philbrick, J. B., Diner, B. A., and Zilinskas, B. A. (1991) *J. Biol. Chem. 266*, 13370−13376.
20. Meetam, M., Keren, N., Ohad, I., and Pakrasi, H. B. (1999) *Plant Physiol. 121*, 1267−1272.
21. von Heijne, G. (1992) *J. Mol. Biol. 225*, 487−494.
22. Laddaga, R. A., Bessen, R., and Silver, S. (1985) *J. Bacteriol. 162*, 1106−1110.
23. Archibald, F. S., and Duong, M. N. (1984) *J. Bacteriol. 158*, 1−8.
24. Weiss, A. A., Silver, S., and Kinscherf, T. G. (1978) *Antimicrob. Agents Chemother. 14*, 856−865.
25. Van Ho, A., Ward, D. M., and Kaplan, J. (2002) *Annu. Rev. Microbiol. 56*, 237−261.
26. Kieselbach, T., Mant, A., Robinson, C., and Schroder, W. P. (1998) *FEBS Lett. 428*, 241−244.
27. Kamei, A., Yuasa, T., Geng, X., and Ikeuchi, M. (2002) *DNA Res. 9*, 71−78.
28. Black, K., Buikema, W. J., and Haselkorn, R. (1995) *J. Bacteriol. 177*, 6440−6448.

29. Small, I. D., and Peteers, N. (2000) *Trends Biochem. Sci. 25*, 46−47.
30. Bateman, A., Murzin, A. G., and Teichmann, S. A. (1998) *Protein Sci. 7*, 1477−1480.
31. Chen, L., Keramati, L., and Helmann, J. D. (1995) *Proc. Natl. Acad. Sci. U.S.A. 92*, 8190−8194.
32. Escolar, L., Perez-Martin, J., and de Lorenzo, V. (1999) *J. Bacteriol. 181*, 6223−6229.
33. Gaballa, A., and Helmann, J. D. (1998) *J. Bacteriol. 180*, 5815−5821.
34. Que, Q., and Helmann, J. D. (2000) *Mol. Microbiol. 35*, 1454−1468.
35. Kehres, D. G., Janakiraman, A., Slauch, J. M., and Maguire, M. E. (2002) *J. Bacteriol. 184*, 3151−3158.

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