A Two-component Signal Transduction Pathway Regulates Manganese Homeostasis in *Synechocystis* 6803, a Photosynthetic Organism*

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Elemental manganese is essential for the production of molecular oxygen by cyanobacteria, plants, and algae. In the cyanobacterium *Synechocystis* sp. PCC 6803, transcription of the *mntCAB* operon, encoding a high affinity Mn transporter, occurs under Mn starvation (nM Mn) conditions but not in Mn-sufficient (μM Mn) growth medium. Using a strain in which the promoter of this operon directs the transcription of the *luxAB* reporter genes, we determined that inactivation of the *slr0640* gene, which encodes a histidine kinase sensor protein component of a two-component signal transduction system, resulted in constitutive high levels of lux luminescence. Systematic targeted inactivation mutagenesis also identified *slr1837* as the gene encoding the corresponding response regulator protein. We have named these two genes *manS* (manganese-sensor) and *manR* (manganese-regulator), respectively. A polyhistidine-tagged form of the ManS protein was localized in the *Synechocystis* 6803 cell membrane. Directed replacement of the conserved catalytic His-205 residue of this protein by Leu abolished its activity, although the mutated protein was present in cyanobacterial membrane. This mutant also showed suboptimal rates of Mn uptake under either Mn-starved or Mn-sufficient growth condition. These data suggest that the ManS/ManR two-component system plays a central role in the homeostasis of manganese in *Synechocystis* 6803 cells.

Manganese is an essential transition metal in almost all organisms. It plays a critical role for the phototrophic life style of cyanobacteria, algae, and plants. During oxygenic photosynthesis, a cluster of four Mn atoms in the photosystem II complex in thylakoid membranes catalyzes photolysis of water to produce molecular oxygen (1). Despite this importance of Mn in the biosphere, the regulatory details of cellular Mn homeostasis remain poorly understood (2, 3).

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We have identified previously MntABC, an ABC-type permease that mediates high affinity Mn transport in the cyanobacterium *Synechocystis* sp. PCC 6803 (4, 5). The protein components of this transporter are encoded by three neighboring genes, *mntA*, *mntB*, and *mntC*, organized in an operon *mntCAB*. This transporter functions when *Synechocystis* 6803 cells are grown under Mn starvation conditions. A second high affinity Mn transporter functions in *Synechocystis* 6803 cells grown under Mn-sufficient conditions (5). Furthermore, a low affinity Mn transporter also operates in these cells. It is evident that Mn uptake in these cyanobacterial cells is controlled via a carefully modulated regulatory process.

The maintenance of homeostasis in a cell often requires complex sets of components that regulate the balance of metabolism. Such a regulatory cascade of events is initiated with the perception of the status of the environment with regard to specific metabolites or nutrients. Almost all cells utilize signaling cascades to respond to both positive and negative environmental stimuli. During recent years, two-component signal transduction has been recognized as a widely used strategy by which cells adapt and respond to their environment (6–10). This means of sensing the environment is utilized by bacteria, as well as plants, and involves at least two separate protein components. At the beginning of the signal transduction chain is a protein containing a sensor domain that is typically a histidine kinase with a His residue that is essential to a phosphorylation cascade. The second component is the response regulator that contains a receiver regulatory domain with a critical aspartic acid residue, an acceptor of the phosphate group from the His group in the histidine kinase. Often, a second domain of the response regulator protein binds directly to DNA and interacts with the transcription machinery to regulate the expression of a set of genes (7–9). Recent analysis has shown that among the various bacterial species with completely sequenced genomes, cyanobacteria have the largest numbers of two-component sensor regulator pairs (10, 11). In *Synechocystis* 6803 there are 43 proteins containing the canonical histidine kinase sensor domains and 40 proteins containing the response regulator signature (11). In the recently sequenced genome of the filamentous N2-fixing cyanobacterium *Anabaena* sp. PCC 7120, 195 genes encode components of such two-component signal transduction systems (12). To date, functional roles have been determined for only a limited number of such proteins in cyanobacteria. These include two-component systems for responses to extreme environmental conditions such as general nutrient limitation and high light stress (13), phosphate limitation (14), and cold stress (15, 16). One of the first two-component sensor-regulator pairs to be identified in *Synechocystis* 6803 was the Cph1/Rcp1 proteins that are
Sensing of Manganese by a Cyanobacterial Two-component System

MATERIALS AND METHODS

Bacterial Growth Conditions—Wild-type and mutant cells of *Synechocystis* 6803 were grown at 30 °C in BG11 medium (21), buffered at pH 8.0, and bubbled with air. Solid medium for cyanobacterial growth was BG11, supplemented with 1.5% agar and 15 mM sodium thiosulfate. Continuous illumination was provided by fluorescent lamps at 50 μE·m⁻²·s⁻¹. For Mn-uptake assays, *Synechocystis* 6803 cells were conditioned in Mn-free liquid BG11 medium, in which ferric ammonium citrate was replaced by ferric nitrate, and Mn was omitted from the trace element components. To starve *Synechocystis* 6803 membranes for Mn, colonies was assayed using a VIM camera system (model C-1400-47; Hamamatsu Photonics Co., Hamamatsu, Japan) and processed on an LS 5000 TD scintillation counter (Beckman Instruments).

RT-PCR Analysis of Expression of Metal Transporter Genes—The relative amounts of transcripts from various genes were evaluated by the RT-PCR method (29). Total RNA from *Synechocystis* 6803 cells cultured in normal or Mn-free BG11 medium was extracted according to Ref. 30. cDNA was prepared with the cDNA Synase kit (Roche Molecular Biochemicals), and then phenyl-chloroform extraction and ethanol precipitation. A reverse transcription reaction was performed using Superscript II enzyme (Invitrogen). The products were amplified by PCR and then analyzed by electrophoresis on 0.8% agarose gels. Primers were designed so that the amplified products would be internal to the coding region of each gene. All of the forward primers were designed for sequences 130 nucleotides downstream of the translation initiation codon, and the reverse primers were designed to obtain 350- to 450-bp-long PCR products from each gene. The RNaseP gene was used as a control template with constitutive expression levels (31).

RESULTS

Isolation of Mutants with Unregulated Expression of the pmnt::luxAB Reporter Gene—The pmnt::luxAB operon encodes a high affinity ABC transporter protein complex found in *Synechocystis* 6803 cells grown under Mn starvation conditions (4, 5). Expression of this transporter is controlled by the concentration of available Mn. RT-PCR experiments have shown that when grown in the BG11 medium (containing 9 μM Mn), *Synechocystis* 6803 cells do not have any detectable level of the *mntCAB* transcript (data not shown). The presence of this transcript was, however, detected within 15 min of incubation of the same cells in Mn-deficient BG11 medium. The expression of these genes is evidently under tight transcriptional control.

To identify factors that mediate such Mn-mediated regulation, a pmnt::luxAB reporter strain was constructed (Fig. 1A; also see “Materials and Methods”). In this strain, the promoter of the *mntCAB* operon directs the transcription of the *luxAB* reporter genes. It is noteworthy that in this strain the endogenous *mntCAB* operon was not transcribed. In these cells, the expression of the reporter gene and that of the *mntCAB* operon were similarly regulated by Mn (data not shown). This reporter strain was mutagenized randomly by transformation with a transposon inactivation library (see “Materials and Methods”), and the resultant CmR colonies were screened for mutants that exhibited expression of the reporter gene under Mn-sufficient conditions.
conditions (Fig. 1, B and C). Of nearly 20,000 CmR colonies, we identified two such colonies. They were called manS-1 and manS-2, respectively.

Identification of a Genetic Locus That Regulates Expression of the pmnt Promoter—Analysis of the manS-1 and manS-2 strains showed that in both mutants, the Cm R gene was inserted in the same open reading frame, slr0640 (also termed hik27) (Cyanobase; www.kazusa.or.jp/cyano/cyano.html), although at two different positions (Fig. 2A). This gene encodes a histidine kinase sensor protein that belongs to a two-component signal transduction system in Synechocystis 6803 cells (11). Because this protein is involved in manganese sensing (see below), we have named it ManS, and we have named the corresponding gene manS. The ManS protein has 441 residues with a predicted molecular mass of 49.2 kDa. COG (www.ncbi.nlm.nih.gov/COG/) and SMART (smart.embl-heidelberg.de/) analysis indicated that ManS is a histidine kinase sensor protein (10), with two transmembrane domains, a HisKA domain that includes the conserved and catalytically important His-205 residue, a HAMP dimerization domain, and a HAT-Pase domain (Fig. 2B).

Detection of ManS and Analysis of Expression of the pmnt::luxAB Reporter Gene—To detect the ManS protein, we added both a polyhistidine tag and a c-Myc epitope tag at the C-terminal end of this protein (Fig. 3A). This gene encodes a histidine kinase sensor protein that belongs to a two-component signal transduction system in Synechocystis 6803 cells (11). Because this protein is involved in manganese sensing (see below), we have named it ManS, and we have named the corresponding gene manS. The ManS protein has 441 residues with a predicted molecular mass of 49.2 kDa. COG (www.ncbi.nlm.nih.gov/COG/) and SMART (smart.embl-heidelberg.de/) analysis indicated that ManS is a histidine kinase sensor protein (10), with two transmembrane domains, a HisKA domain that includes the conserved and catalytically important His-205 residue, a HAMP dimerization domain, and a HAT-Pase domain (Fig. 2B).

1 The abbreviation used is: WT, wild-type.
idue was changed to Leu. Such a modification still allowed the accumulation of the ManS protein in cell membrane, although at a reduced level (Fig. 3B). Finally, to evaluate the potential role of the periplasmic domain of this protein in binding Mn, a conserved loop (residues 60 to 132) was deleted from the protein. However, such a mutation resulted in the absence of the conserved loop (residues 60 to 132) was deleted from the protein.

Fig. 3C shows the time-dependent changes in the lux luminescence intensity from the pmntlux reporter strain transformed with wild-type (lane 1), WT/His/Myc (lane 2), H205L (lane 3), and Δ60–132 (lane 4) constructs, using anti c-Myc antisera. 20 μg of protein-containing sample was loaded in each lane. C, time of course of luminescence from the pmntC::luxAB reporter gene in Synechocystis 6803 cells. ○, wild-type; □, WT/His/Myc; △, Δ60–132; and ▲, H205L.

data demonstrated that the ManS protein is a strong determinant in Mn-mediated regulation of transcription of the pmnt promoter, and the His-205 residue plays an important role in this process.

Identification of the Cognate Response Regulator ManR—As described above, extensive mutagenesis of the pmntlux reporter strain using the cosmid inactivation library identified the ManS sensor but not the corresponding response regulator protein. The known response regulator genes in Synechocystis 6803 (11) that are not represented in this cosmid library were inactivated systematically in the reporter strain. Among such inactivation mutants, only the slr1837 (Fig. 2C) mutant cells exhibited high levels of lux luminescence, grown under either Mn-sufficient or Mn-depleted conditions (data not shown), similar to the observations with the mntS mutant cells described above. We concluded that the slr1837 gene encodes the response regulator that interacts with ManS and named it ManR. The ManR protein is 234 residues long with a predicted molecular mass of 25.6 kDa. COG and SMART analysis (see above) indicated that ManR is a member of the OmpR subfamily of response regulators and has an N-terminal CheY-like receiver domain (Fig. 2D) that includes the conserved phosphate acceptor Asp-52 residue (10).

Manganese Uptake Activities in the manS Mutant Cells—Synechocystis 6803 cells have at least two high affinity Mn-uptake systems (3, 5). Among them, the MntABC transporter is present and active when cells are grown under Mn starvation conditions. As a consequence, wild-type cells had high Mn-uptake activities when grown under both Mn-sufficient and Mn-deficient conditions (Fig. 4). As reported previously (5), the ΔmntC mutant lacking any functional MntABC transporter showed poor uptake activity under Mn-deprived conditions. However, it had normal Mn-uptake activity when grown in Mn-sufficient condition. In contrast, the Mn-uptake activity of the H205L mutant, as well as in the original manS-1 mutant (data not shown), was unaffected by the presence or absence of
domain of ManS is involved in the physical interaction with the Mn$^{2+}$ cation.

To date, the ManS/ManR pair constitutes the only known two-component signal transduction system for manganese. Among various transition metals, such two-component systems have been identified for copper and silver. Two different copper-responsive two-component systems are present in *E. coli*, namely, CusS/CusR (34), and PcoS/PcoR (35). In *Pseudomonas syringae*, a plant pathogen, the CopS/CopR two-component system provides copper resistance (36), whereas in *Salmonella*, the SiiS/SiiR system provides resistance to silver ions (37). During the preparation of this manuscript, Reyes and co-workers (38) reported that in *Synechocystis* 6803 cells, the RpaA/RppB two-component system identified originally by Li and Sherman (20) as a redox regulation system also has a role in nickel sensing. In all of these examples, the genes encoding the sensor His kinase and the response regulator are organized in operons. In contrast, the manS gene is located far away from the *manR* gene in the chromosome of *Synechocystis* 6803 (www.kazusa.or.jp/cyano/cyano.html).

During recent years, homologs of the MntABC transporter have been implicated to have significant roles in various bacterial infectious processes (39, 40). A number of regulator proteins for bacterial Mn homeostasis have also been identified. Notably, Que and Helmann (41) have studied MntR, a member of the DtxR diphtheria toxin repressor protein family, in *Bacillus subtilis* cells (41). *B. subtilis* cells have two distinctly different Mn transporter systems. Among them, MntABCD is an ABC-type transporter, similar to the MntABC transporter in *Synechocystis* 6803. A second Mn transporter is MntH, a member of the Nramp family of transporters (42). MntR is a bifunctional protein. In Mn-starved conditions, it activates transcription of the *mntABCD* operon, whereas in Mn-sufficient conditions, MntR represses expression of the *mntH* gene. In the presence of manganese, ScaR, a homolog of MntR in *Streptococcus gordonii*, acts as a repressor for the *sca* operon that encodes a Mn permease similar to the MntABC transporter (43). Interestingly, in *E. coli* cells, both Fur, an iron-dependent regulator, and MntR, a manganese-dependent regulator, control the expression of the *mntH* gene (44). It is noteworthy that none of these organisms has any known two-component signal transduction system for Mn.

As shown in Fig. 5, the ManS His kinase sensor protein appears to have some regulatory effect on the expression of the *feoB* and *znuA* genes. In other bacterial systems, expression of the iron transporter FeoB is known to be regulated by the well known regulator Fur. In *Synechocystis* 6803 cells, expression of the *znuA* gene is transcriptionally regulated by the Zur repressor protein, encoded by the *sll1938* gene. It is known that the Mn$^{2+}$ cation can bind to Fur (and presumably Zur) (45). It is possible that in the absence of ManS activity, an unregulated supply of Mn inside the cyanobacterial cells may lead to binding of this metal to Fur and Zur, with consequent transcriptional repression of the *feoB* and *znuA* genes. However, the dominant effect of ManS is on the *pmnt* promoter (Fig. 3C), indicating that the primary function of this protein is in sensing Mn.

Because of their oxygenic photosynthetic lifestyle, cyanobacterial cells must monitor carefully the available levels of Mn. The data presented in this manuscript demonstrate that the ManS/ManR two-component system in *Synechocystis* 6803 cells is an important determinant in the sensing of external Mn concentration. An additional interesting finding during this

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study is that in the H205L mutant strain, Mn-uptake activity is suboptimal under both Mn-sufficient and Mn-deficient conditions (Fig. 4), raising the question whether pmnt is the only promoter in Synechocystis 6803 cells that is regulated by ManS. It is possible that the Mn-responsive signal transduction pathway initiating with the ManS His kinase has more than one cognate response regulator, one of which (ManR) acts on pmnt, whereas the other(s) may control the expression of the second high affinity Mn transporter, as well as that of the Mn-efflux system(s) in these cyanobacterial cells. Which other promoters are regulated by the ManS sensor, as well as how and where Mn binds to this protein, are being investigated currently.

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