Sll1263, a Unique Cation Diffusion Facilitator Protein that Promotes Iron Uptake in the Cyanobacterium Synechocystis sp. Strain PCC 6803

Hai-Bo Jiang1, Wen-Jing Lou1, Han-Ying Du1, Neil M. Price2,* and Bao-Sheng Qiu1,*

1College of Life Sciences, and Hubei Key Laboratory of Genetic Regulation and Integrative Biology, Central China Normal University, Wuhan 430079 Hubei, PR China
2Department of Biology, McGill University, 1205 Docteur Penfield, Montreal, Québec H3A 1B1, Canada
*Corresponding authors: Neil M. Price: E-mail, neil.price@mcgill.ca; Bao-Sheng Qiu: E-mail, bsqiu@mail.ccnu.edu.cn
(Received August 11, 2011; Accepted May 24, 2012)

Cyanobacteria are known to survive in iron-deficient environments, but the ways in which they acquire Fe and acclimate are not completely understood. Here we report a novel gene sll1263 that is required for Synechocystis sp. strain PCC 6803 to grow under iron-deficient conditions. sll1263 encodes a putative cation diffusion facilitator protein (CDF) that shows 50% amino acid similarity with ferrous iron efflux protein (FieF) of heterotrophic bacteria. In bacteria, the gene product is involved in metal export from the cell, but in Synechocystis sp. strain PCC 6803 plays a role in iron uptake. The results show that expression of sll1263 was induced by iron-deficient conditions and its inactivation significantly decreased the growth rate of an sll1263– mutant. Other genes known to be required for Fe acquisition were also strongly up-regulated in the mutant even in the presence of high Fe. Overexpression of sll1263 increased growth under iron deficiency but reduced growth under high-iron stress, suggesting that the gene product was involved in iron uptake rather than detoxification. Expression of FieF in the sll1263– mutant was unable to rescue the Fe-deficient phenotype, but Sll1263 completely restored it. Measurements of cellular iron content and the iron uptake rate showed that they were significantly less in the sll1263– mutant than in the wild type, consistent with a role for sll1263 in iron uptake. We hypothesize that the low-iron habitats and high-iron requirements of cyanobacteria may be the reason why cyanobacterial CDF protein functions in Fe uptake and not efflux as in non-photosynthetic bacteria.

Keywords: CDF protein • Cyanobacteria • Iron deficiency • Iron uptake.

Abbreviations: ABC, transporter; ATP, -binding cassette transporter; CDF, cation diffusion facilitator; FieF, ferrous iron efflux protein; OD, optical density; RT–PCR, reverse transcription–PCR.

Introduction

Iron is an essential element required for the growth and development of all organisms that acts as a catalytic cofactor in multiple metabolic pathways (Hantke 2001, Walker and Connolly 2008). In photosynthetic organisms, such as higher plants and algae, it plays crucial roles in electron transport in photosynthetic and respiratory chains (Jeong and Guerinot 2009). Although iron is abundant in the earth’s crust, limited solubility of Fe(III) in aerobic aquatic environments keeps the concentrations of inorganic species at extremely low levels estimated to be 10−9–10−18 M (Palyada et al. 2004, Schalk et al. 2004, Miethke and Marahiel 2007). These levels are far below those required for growth of most photosynthetic organisms, such as cyanobacteria (Shcolnick et al. 2009, Kranzler et al. 2011).

Because of low environmental concentrations, many organisms have evolved complex systems to capture iron efficiently. Plants acclimate to iron-deficient conditions in two ways. Non-graminaceous plants use the strategy I response, in which a H+-ATPase acidifies the rhizosphere, making Fe(III) more soluble (Robinson et al. 1999). Grasses use an alternative mechanism, the strategy II response, and release Fe-binding ligands into the soil that are subsequently internalized in the iron-bound state via specific transporters (Romheld and Marschner 1986, Curie et al. 2001). Many bacteria also use a similar strategy to acquire iron by synthesizing and exporting Fe(III) chelators called siderophores (Miethke and Marahiel 2007). Some species reduce ferric iron to ferrous iron via a surface reductase and subsequently transport it across the plasma membrane (Schroder et al. 2003). Cyanobacteria are prominent members of the marine biosphere that account for a significant percentage of global primary productivity. Despite the obvious environmental importance of cyanobacteria and the observation that iron limits their growth in nature...
(Falkowski et al. 1998, Morel and Price 2003, Chappell et al. 2012), the iron uptake system of cyanobacteria is still not completely understood.

The mechanisms of iron uptake in marine and freshwater cyanobacteria have been studied in several *Synechococcus* spp., *Synechocystis* sp. strain PCC 6803 (hereafter *Synechocystis* 6803) and *Anabaena* sp. strain PCC 7120 (also named *Nostoc* sp. strain PCC 7120). Collectively, the results show that when iron is limiting to growth, cyanobacteria induce expression of the iron stress-induced operon isiAB. The products of the isiAB include a chlorophyll antenna protein CP43\(^+\) (IsiA), which protects cyanobacteria from photo-oxidative stress, and flavodoxin (IsiB), which functionally replaces ferredoxin under conditions of iron stress (Burnap et al. 1993, Kutzki et al. 1998, Park et al. 1999, Bibby et al. 2001, Havaux et al. 2005, Wilson et al. 2007). *Anabaena* sp. strain PCC 7120 is reported to release siderophores in response to low environmental iron concentrations, which chelate Fe(III) and are transported into the periplasmic space by a TonB-dependent outer membrane transporter (Nicolaissen et al. 2008, Nicolaissen et al. 2010). However, not all cyanobacteria species possess genes for the biosynthesis of siderophores (Muris et al. 2009). Furthermore, the identities of the outer membrane receptor proteins that interact with TonB are still unknown in many cyanobacteria. In *Synechocystis* 6803, inactivation of four putative outer membrane receptor proteins has no effect on the iron uptake capability of the cells, suggesting that other transporters in the outer membrane remain to be discovered. Once Fe\(^{3+}\) is transported into the periplasmic space, it is recognized and transported into the cytoplasm by an ATP-binding cassette (ABC) transporter system in the plasma membrane. The major ABC-type ferric iron transporter of *Synechocystis* 6803 is encoded by four ftr genes, futA1 (slr1295), futA2 (slr0513), futB (slr0327) and futC (slr1878) (Katoh et al. 2001, Koropatkin et al. 2007, Waldron et al. 2007, Badarau et al. 2008, Brandt et al. 2009), but mutants lacking the transporter still take up Fe\(^{3+}\), indicating the presence of other Fe\(^{3+}\) transporters. Iron reduction has been reported to be an important process preceding uptake by *Synechocystis* 6803 (Kranzler et al. 2011), although the mechanism of ferrous iron uptake remains unclear. A mutant of the putative ferrous iron transporter gene (fetO, slr1392) of *Synechocystis* 6803 grows as well as the wild type under iron deficiency conditions, indicating the presence of other Fe\(^{2+}\) transporters, or that transport of Fe\(^{2+}\) is not essential for iron acquisition in this species as the authors suggested (Katoh et al. 2001).

To investigate the pathways of iron uptake and assimilation in cyanobacteria, microarrays were carried out by Singh et al. (2003) and Nodop et al. (2008) in *Synechocystis* 6803 and *Synechococcus elongatus* PCC 7942, respectively. Singh et al. (2003) identified dozens of genes with unknown functions that were strongly up-regulated following Fe resupply to iron-starved cultures. Using genetic transformation, we knocked out 24 of these genes in *Synechocystis* 6803, to identify those with essential functions in iron uptake or assimilation. A putative cation diffusion facilitator (CDF) was identified in *Synechocystis* 6803 that was required for growth under low Fe conditions. In bacteria and plants, CDF proteins such as ferrous iron efflux protein (FieF) are typically involved in metal detoxification and reduce metal toxicity by transporting Zn\(^{2+}\) or Fe\(^{2+}\) out of the cell or into the vacuole (Nies 2003, Grass et al. 2005), but the functions of cyanobacterial CDF protein are not yet known. Here we report, for the first time, that the CDF protein in *Synechocystis* is involved in iron uptake and required for acclimation to Fe-limiting conditions.

### Results

#### Six novel genes and one gene cluster were identified to be required by *Synechocystis* 6803 to adapt to iron starvation

To identify genes required for acclimation to low-iron conditions in cyanobacteria, 24 genes or gene clusters from *Synechocystis* 6803 were knocked out (Supplementary Table S1). The genes were chosen based on the results of microarray experiments performed by Singh et al. (2003) and Nodop et al. (2008). We selected genes that were up-regulated by changes in iron availability or had a high similarity to genes known to be involved in iron uptake systems of other bacteria. All the genes and gene clusters were inserted or replaced with a kanamycin-resistant cassette fragment C.K2, as described previously (Jiang et al. 2010). Fig. 1A illustrates an example of such gene knockout, taking *slr1263* as an example.

Using genetic transformation and mutant phenotype analyses, six genes (*slr1263, slr1484, slr1036, slr0376, slr0772 and slr0964*) and a gene cluster (*slr1404–slr1405*) were found to be crucial for *Synechocystis* 6803 survival under iron starvation. The PCR results shown in Fig. 1B confirmed that all the mutants were completely segregated. Growth of the mutants in BG-11 medium containing the normal iron concentration (21 \(\mu M\)) was identical to that of the wild-type control, a kanamycin-resistant *Synechocystis* strain WT1188 (Williams 1988) (Table 1). However, when the mutants were cultured under iron starvation conditions, their growth was significantly reduced compared with the wild type (Table 1, Fig. 1C).

The putative functions of the inactivated genes were identified from a BLAST procedure using the NCBI database (http://blast.ncbi.nlm.nih.gov) (Table 1). Analysis showed that two of the genes encode a TonB-like protein (Slr1484) and ExbB–ExbD dimer-like protein (Slr1404–Slr1405), suggesting a possible role for a TonB-dependent Fe\(^{3+}\) transport in *Synechocystis*. The exact roles of these proteins are currently being studied in our lab. Inactivation of a putative porin (Slr0772) and a putative high-affinity iron permease FTR1 (Slr0964) also reduced growth under iron starvation. Two other proteins, Slr0376 and Slr1036, known to be highly expressed under a variety of stress conditions, including iron starvation (Singh and Sherman 2002, Singh et al. 2003), were also identified.
To investigate the function of the putative CDF protein in *Synechocystis*, we monitored expression of *sll1263* under varying iron concentrations. Cells cultured at the standard BG-11 iron concentration (21 μM) expressed the *sll1263* gene at a very low level, but after 24 h of iron starvation the gene was strongly up-regulated (Fig. 3A). The increased expression in iron-starved cells suggested that the product of the *sll1263* gene could play a positive role in iron uptake. Indeed, expression of several other important iron uptake genes was greatly increased in the mutant compared with the wild type under low and high Fe conditions (Fig. 3B).

**The sll1263 gene of Synechocystis 6803 is induced by iron starvation, and inactivation of sll1263 stimulates known iron uptake genes**

Of particular interest was the observation that Sll1263 was required to adapt to iron-limiting conditions. This gene was identified as a putative CDF, a member of a class of membrane proteins known to play a role in metal detoxification (Montanini et al. 2007). Members of the CDF family are ubiquitous in bacteria and eukaryotes, but until now no functional role for CDF protein has been identified in cyanobacteria species. According to the *Synechocystis* 6803 database (NCBI or Cyanobase, http://genome.kazusa.or.jp/cyanobase), *Sll1263* is the only CDF protein in this species and is predicted to encode a protein with six transmembrane domains, typical of proteins in this family (Fig. 2).

![Diagram of sll1263](image)

**Fig. 1.** Mutants of *Synechocystis* 6803 sensitive to iron starvation conditions identified in this study. (A) Sketch map of knocked-out genes depicted with the example of *sll1263*. (B) PCR results confirmed that all the mutants were completely segregated. PCR was carried out using the same primers of related genes with the genomic DNA of the mutants (left band) and wild type (right band) as templates. (C) Photographs of the mutants and wild type (WT1188) cultured for 12 d in iron-starved BG-11 medium, except for the first strain from the left, which is WT1188 grown in normal BG-11 medium.

**Table 1.** Mutants of *Synechocystis* 6803 found to be sensitive to iron starvation.

<table>
<thead>
<tr>
<th>Strains</th>
<th>Growth (OD730 value) of the strains cultured in BG-11 with or without Fe for 8 d</th>
<th>Putative encoded products of the inactivated genes</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>+Fe</td>
<td>-Fe</td>
</tr>
<tr>
<td>WT1188</td>
<td>1.563 ± 0.143*</td>
<td>0.738 ± 0.077*</td>
</tr>
<tr>
<td>sll1263::C.K2</td>
<td>1.613 ± 0.146*</td>
<td>0.386 ± 0.058*</td>
</tr>
<tr>
<td>slr0376::C.K2</td>
<td>1.536 ± 0.125*</td>
<td>0.427 ± 0.095*</td>
</tr>
<tr>
<td>sll1036::C.K2</td>
<td>1.501 ± 0.106*</td>
<td>0.372 ± 0.083*</td>
</tr>
<tr>
<td>sll1404-1405::C.K2</td>
<td>1.591 ± 0.110*</td>
<td>0.512 ± 0.064*</td>
</tr>
<tr>
<td>slr0964::C.K2</td>
<td>1.639 ± 0.121*</td>
<td>0.517 ± 0.082*</td>
</tr>
<tr>
<td>sll0772::C.K2</td>
<td>1.636 ± 0.145*</td>
<td>0.524 ± 0.069*</td>
</tr>
</tbody>
</table>

The growth of the cells is presented by the changes of optical density at 730 nm from the similar initial values at about 0.05. The possible function of the knocked-out gene of *sll1263*, we monitored expression of the putative encoded products of related genes with the genomic DNA of the mutants (left band) and wild type (right band) as templates. The primers are listed in Table 3. (C) Photographs of the mutants and wild type (WT1188) cultured for 12 d in iron-starved BG-11 medium, except for the first strain from the left, which is WT1188 grown in normal BG-11 medium.

![Diagram of primers](image)
medium, suggesting that it could be a member of the iron uptake gene family.

The sll1263::C.K2 mutant of Synechocystis 6803 is abnormally sensitive to iron starvation, but its growth rate can be restored by adding iron.

Detailed physiological experiments were performed to characterize the iron-starved phenotype of the sll1263::C.K2 mutant. As shown in Fig. 4A (left), B and E, the sll1263::C.K2 mutant showed a similar growth and Chl a concentration in Fe-replete BG-11 medium to the wild-type Synechocystis 6803.

When the cells were cultured in iron-starved medium, growth of the mutant was slightly decreased after 6 d compared with the wild type (Fig. 4A, right). The differences between the two cultures became more pronounced following the second transfer of the strains into iron-starved medium (Fig. 4A, C), probably because of depletion of intracellular iron due to growth. On the 12th day of iron starvation, the optical density of the sll1263::C.K2 mutant culture at 730 nm (OD 730) was about 0.4, less than half the density of the wild type. The concentration of Chl a in the sll1263::C.K2 mutant was also reduced relative to the wild type (Fig. 4E), although when normalized to...
the OD levels they were similar. Other stresses such as low temperature and light had no significant effect on the growth of the sl1263::C.K2 mutant compared with the wild type (Supplementary Fig. S1), suggesting that the mutant phenotype was only expressed under Fe-deficient conditions.

To confirm that iron deficiency was the direct reason for the growth reduction, 21 μM ammonium ferric citrate (the normal iron concentration in BG-11 medium) was added to an iron-starved culture of the sl1263::C.K2 mutant. As shown in Fig. 4D and E, the growth and Chl concentration of the mutant

Fig. 4 Growth characteristics and pigment contents of Synechocystis 6803 wild-type and sl1263::C.K2 mutant cultures grown under different iron concentrations. (A) Photographs of the wild type and sl1263::C.K2 mutant grown in BG-11 medium with normal iron concentrations (+Fe, left) or iron starvation conditions created by replacing ammonium ferric citrate by ammonium citrate (−Fe, right). Three photographs are shown for each strain: G1-1st d represents the first day of growth of the first transfer; G1-6th d represents the sixth day of growth of the first transfer; G2-12th d represents the 12th day of growth of the second transfer. Growth curves (determined by the OD730) of the wild type and the sl1263::C.K2 mutant of Synechocystis 6803 cultured under normal BG-11 (B) and iron starvation conditions (C). (D) Growth curves of the iron-starved cells after the resupply of 21 μM ammonium ferric citrate to the iron-starved medium. (E) Chl a concentration of the wild type and sl1263::C.K2 mutant of Synechocystis 6803 cultured under normal iron concentrations, iron starvation and iron resupply (Restore) conditions.
culture were restored to the wild-type level following Fe resupply. Thus, we conclude that the phenotypic differences between the sll1263::C.K2 mutant and the wild type were due to the Fe concentration in the culture medium.

Overexpression of sll1263 in Synechocystis 6803 enhances the ability to adapt to iron deficiency

The sll1263 gene was overexpressed in Synechocystis 6803, using a stable overexpression platform ‘PsbaAll expression vector’ illustrated in Supplementary Fig. S2. In this vector, a psbaAll promoter lacking the high-light-responsive region (Agrawal et al. 2001) was cloned downstream of a spectinomycin-resistant cassette, Omega. The PsbaAll expression vector has proved to be a stable heterologous gene overexpression platform in Synechocystis 6803 (for more details, see the Materials and Methods and the Supplementary data).

The growth and pigment composition of the wild type, sll1263::C.K2 mutant and sll1263 overexpression (sll1263-OE) strains were measured in Fe starvation medium (Table 2, Fig. 5). Under iron-limiting conditions, the sll1263-OE strain grew significantly faster than the wild-type Synechocystis 6803 and mutant strains, with a specific growth rate of 0.413 ± 0.008 and 0.312 ± 0.012 d⁻¹, respectively (P < 0.05, Tukey multiple comparison). The sll1263-OE culture contained a higher pigment concentration under iron-starved conditions than the wild type and sll1263::C.K2 mutant (Fig. 5A, B). Based on the analysis of gene mutation and overexpression, the sll1263 gene appears to be necessary for low-iron acclimation in Synechocystis.

Sll1263, the only CDF protein in Synechocystis 6803, is not a ferrous iron efflux protein, and plays roles other than heavy metal detoxification

Sll1263 is predicted to have six transmembrane-spanning domains like other CDF family proteins and is highly similar to the CDF protein in Escherichia coli (Fig. 2), previously identified as an FieF (Grass et al. 2005). Comparison of the amino acid sequences of these two proteins showed a 50% similarity and 29% identity, with an E-value of 3 x e⁻³¹. To test if Sll1263 was involved in metal detoxification, sll1263-OE, sll1263::C.K2 mutant and wild-type Synechocystis cells were grown in BG-11 medium containing elevated iron (1 mM) or zinc (1 mM) concentrations. Growth of the wild-type strain was greatly reduced under these conditions compared with normal BG-11 medium (Fig. 4), confirming that the Fe concentration used in these experiments was indeed inhibitory (Fig. 6A).

The results showed that sll1263-OE actually grew more slowly (0.404 ± 0.02 d⁻¹) than both the wild type (0.454 ± 0.02 d⁻¹) and the sll1263::C.K2 mutant (0.496 ± 0.02 d⁻¹), suggesting that overexpression of Sll1263 aggravated high-iron stress. Enhanced intracellular iron accumulation in the sll1263-OE strain probably increased iron toxicity. No significant differences in growth were observed among the three types of cells cultured in high-Zn²⁺-enriched medium (data not shown). The results imply that Sll1263, the only CDF family protein in Synechocystis 6803, promotes uptake of iron and not efflux of potentially toxic metals.

We explored the functional differences between Sll1263 and the FieF in E. coli, by overexpressing the E. coli fief gene in the Synechocystis sll1263::C.K2 mutant using the PsbaAll expression vector described above. At the same time, the sll1263 gene (as a control) was also expressed by the same vector in the sll1263::C.K2 mutant, to test if it could rescue the mutant phenotype. As shown in Fig. 6B, the sll1263 gene expressed under the psbaAll promoter completely complemented the iron-deficient phenotype of the sll1263::C.K2 mutant. Thus, the iron-deficient phenotype was indeed caused by the inactivation of the sll1263 gene and not by a second point mutation. The fief gene from E. coli was unable to complement the sll1263::C.K2 mutant, as expected from its known role in metal efflux. Collectively, our results (Fig. 6A, B) suggest that the sll1263 gene in Synechocystis 6803 does not regulate ferrous iron efflux, but is needed for cells to grow in iron-deficient medium.

The sll1263::C.K2 mutant shows a reduced cellular iron content and decreased iron uptake rate compared with wild-type cells

If sll1263 is involved in the Fe uptake system, the cellular Fe concentration should be influenced by the inactivation of this gene. To test this hypothesis, we measured the Fe content of wild-type and mutant strains grown in the normal BG-11 iron concentration. Growth of both strains was identical under these conditions (Fig. 4), but the sll1263::C.K2 mutant contained roughly 40% less cellular iron (Fig. 7A). The cellular iron concentration of the sll1263::C.K2 mutant was only 168.9 ± 9.7 µg g⁻¹ DW of cells, compared with 281.9 ± 7.9 µg g⁻¹ DW of cells in the wild type (Fig. 7A). We note that the reduced cellular iron content of the mutant is consistent with up-regulation of other related iron uptake genes in normal BG-11 medium (Fig. 3B).

The reduced iron content of the mutant strain could be due to slower rates of iron transport and/or a reduced ability for iron storage. We thus determined iron uptake rates of the strains directly using 1 µM ⁵⁵FeCl₃ according to Katoh et al. (2001). The short-term ⁵⁵Fe uptake rate was significantly

Table 2 The growth characteristics of the wild type, sll1263-OE and the sll1263::C.K2 mutant of Synechocystis 6803 in iron-starved BG-11 medium for a period of 12 d

<table>
<thead>
<tr>
<th>Strains</th>
<th>OD at 730 nm</th>
<th>Specific growth rates (d⁻¹)</th>
</tr>
</thead>
<tbody>
<tr>
<td>WT6803</td>
<td>From 0.05 to 1.272 ± 0.088</td>
<td>0.389 ± 0.008³</td>
</tr>
<tr>
<td>sll1263-OE</td>
<td>From 0.05 to 1.555 ± 0.197</td>
<td>0.413 ± 0.015³</td>
</tr>
<tr>
<td>sll1263::C.K2</td>
<td>From 0.05 to 0.672 ± 0.063</td>
<td>0.312 ± 0.012³</td>
</tr>
</tbody>
</table>

Values with different superscript letters are significantly different (P < 0.05, Tukey multiple comparison). The values are means ± SDs and were calculated using the results of at least three parallel cultures.
slower by 30% in the sll1263::C.K2 mutant compared with the wild-type cells, confirming the role of the gene sll1263 in Fe transport (Fig. 7B, C). The iron uptake rates were 27.2 ± 0.9 and 19.6 ± 1.7 pmol Fe 10⁻⁸ cells 5 min⁻¹ for the wild type and sll1263::C.K2 mutant, respectively (Fig. 7C). Additional Fe uptake experiments were conducted with ferrozine (Fe²⁺ transport inhibitor) and ascorbate (Fe³⁺ reducing agent) to differentiate between Fe³⁺ and Fe²⁺ transport. Using these reagents, we found that the Fe³⁺ uptake rate of the mutant (1.9 ± 0.1 pmol Fe 10⁻⁸ cells 5 min⁻¹) was reduced by 38% compared with the wild type (3.1 ± 0.4 pmol Fe 10⁻⁸ cells 5 min⁻¹), whereas Fe²⁺ uptake rates of the strains were not significantly different (Fig. 7C).

**Discussion**

Iron is known to catalyze a wide range of biochemical reactions essential for most organisms. However, its redox properties also lead to oxidative interactions, resulting in the formation of highly reactive hydroxyl radicals (OH⁻). When intracellular iron exceeds certain limits, these toxic radicals are formed through the Fenton reaction in the presence of oxygen and destroy DNA, RNA, proteins and membranes (Masse et al. 2007). Thus, organisms must overcome the problem of transporting and storing iron in active cofactors and, at the same time, protect themselves against oxidative damage. Hence, even though iron is required for respiration, photosynthesis and nitrogen assimilation complexes in cyanobacteria, their intracellular level and iron uptake have to be tightly controlled (Shcolnick and Keren 2006).

CDF proteins are members of a protein superfamily of metal transporters found in bacteria, plants and animals. Their primary substrates are Zn, but some are also able to transport cations such as Co, Ni, Cd, Mn and Fe (Nies 2003). CDF proteins have been widely reported to transport metal from the cytoplasm into various intracellular eukaryotic organelles such as the plant vacuole, or to catalyze efflux from the cytoplasm into the extracellular medium as in *E. coli* (Delhaize et al. 2003, Montanini et al. 2007, Gustin et al. 2011). However, in at least one instance, a CDF protein was shown to be involved in Zn uptake rather than Zn efflux across the plasma membrane in human intestinal cells, indicating diverse roles for this class of proteins (Cragg et al. 2002).

Although they probably play essential roles in cyanobacterial cellular cation homeostasis, cyanobacterial CDF proteins have not been identified yet. The results of this study show clearly that Sll1263, the unique CDF in *Synechocystis* 6803, plays an essential role in iron uptake, and not in iron efflux as expected. According to bioinformatic analysis, all cyanobacteria species that have been sequenced contain only one single CDF protein homolog with high similarity to Sll1263. Thus other cyanobacterial CDF proteins may play similar roles to Sll1263 in promoting iron uptake. *Escherichia coli* possesses in addition to a FieF (also named YiiP), another CDF protein, ZitB, that exports zinc outside the bacterial cells (Grass et al. 2001). According to our results, the FieF from *E. coli* performs functions different from those of the cyanobacterial CDF protein Sll1263.

Differences in CDF function in photosynthetic cyanobacteria and heterotrophic bacteria may be related to the iron requirements of the organisms and the availability of iron in
Cyanobacteria are thus faced with a challenge of fulfilling their large iron requirement in an environment where iron exists in extremely low concentration. Indeed, the multiple pathways of Fe uptake in these organisms suggest that Fe limitation has been a major selective force during their evolution. As shown here, CDF protein provides yet another way by which cyanobacteria have adapted to acquire Fe for growth. In iron-replete conditions such as BG11 medium, sll1263 displays very low basic expression. Only when iron concentrations reach starvation levels is it sharply induced (Fig. 3). Katoh et al. (2001) predicted the presence of other Fe\(^{3+}\) transporters in *Synechocystis* since mutants lacking all ABC-type transporters continue to show Fe\(^{3+}\) uptake activity. Thus, Sll1263 probably complements the role of the ABC transporter system in facilitating Fe uptake under conditions of reduced Fe bioavailability.

The iron uptake results reported here provide preliminary evidence that Sll1263 is an Fe(III) transporter, although we note that methodological issues preclude a definitive conclusion. Currently, it is still unknown whether or not Fe\(^{3+}\) transport is also affected by the CDF homolog in *E. coli*, since only Fe\(^{2+}\) transport was analyzed in the study of Grass et al. (2005).

Although ferrozine is commonly used to differentiate Fe(III) from Fe(II) uptake by complexing any free Fe(II) in solution, it may incompletely block Fe(II) transport and could facilitate abiotic photoreduction of Fe(III), complicating a simple interpretation of the Fe(III) results. We note that the sum of the ferric (+ ferrozine) and ferrous (+ ascorbate) uptake rates we measured were not equal to the total Fe uptake rate (Fig. 7C). This discrepancy may arise because of differences in the total inorganic iron concentration (Fe\(^{2+}\)) in the experimental treatments even though the total Fe added in each case was the same (Kranzler et al. 2011). The iron uptake rate in *Synechocystis* is a hyperbolic function of Fe\(^{2+}\) which will be strongly affected by the high concentrations of ferrozine and ascorbate that were added in our Fe uptake experiments. Regardless of the confounding issues of Fe chemistry, our results show clearly that Fe uptake by the sll1263 mutant is significantly reduced compared with the wild type and support the conclusion that the gene product is involved in Fe uptake.

Of great interest is how the sll1263 gene product identified here evolved to function in Fe uptake when most other known members of the CDF family are metal exporters. Analysis of CDF structure and function in other organisms should allow us to unravel the phylogenetic relationship among these important transport proteins. For example, cyanobacteria are thought to be the ancestor of chloroplasts of higher plants, so we might expect CDF proteins of chloroplasts to play a role in iron uptake. Indeed, some CDF-like proteins in Arabidopsis are reported to contain a transit peptide for the chloroplast, suggesting that these kinds of proteins may operate in the transport of metals into the organelle (Grotz et al. 1998).

In conclusion, for the first time, we have identified a unique role for cyanobacterial CDF protein in iron homeostasis. The protein promotes iron uptake and is of critical importance for cellular acclimation to low-iron concentrations. Since the
mutant and overexpression strains have identical growth phenotypes to the wild-type cells under Zn toxicity, the role of CDF protein in the homeostasis of Zn or other cations such as Co, Ni, Cd in cyanobacteria needs to be further investigated.

Materials and Methods
Cyanobacterial strains, culture conditions and general methods
A glucose-tolerant *Synechocystis* 6803 was cultured in BG-11 medium without shaking, at 30°C under continuous illumination of 30 μmol photons m⁻² s⁻¹. BG-11 plates were prepared by adding 1.4% agar, 8 mM TES (pH 8.2) and 0.3% Na₂S₂O₃. In addition to *Synechocystis* 6803, a kanamycin-resistant derivative strain showing no phenotypic change under different conditions as described in previous reports (Williams, 1988, Kunert et al. 2000) was used as wild-type control with an antibiotic resistance marker. According to the culture requirements of the mutants or complemented strains, kanamycin (25 mg l⁻¹) or spectinomycin (20 mg l⁻¹), or both were added into BG-11 medium.

For the analysis of growth in iron-starved media, ammonium ferric citrate was omitted from the BG-11 medium and replaced by the same concentration of ammonium citrate. Glassware used for iron-starved experiments was soaked with 6 M HCl.
and 1 mM EDTA to remove residual iron and then rinsed with Milli-Q water. To starve *Synechocystis* cells for iron, wild-type and mutant strains were grown in iron-starved BG-11 medium for 6–8 d. Before cultivating in fresh iron-starved BG-11 medium, the cells were collected by centrifugation and washed twice with iron-starved medium.

The growth of cyanobacterial strains was detected by their turbidity (OD<sub>730</sub>) calculated from the results for 3–4 parallel cultures. The specific growth rates (d<sup>−1</sup>) were calculated as follows: \((\log \text{OD}_{t_2} - \log \text{OD}_{t_1})/(t_2 - t_1)\), where OD<sub>t1</sub> signifies the OD<sub>730</sub> on day \(t_1\) and OD<sub>t2</sub> is the OD<sub>730</sub> on day \(t_2\). Chl \(a\) content was determined spectrophotometrically at OD<sub>664</sub> in 90% methanol extracts and calculated with the formula: Chl \(a\) (mg l<sup>−1</sup>) = 13.34 \(\times\) \(A_{664}\). The whole-cell absorption spectrum from 400 to 800 nm was determined by using a Varian Cary 300 Bio UV-Visible spectrophotometer.

**Construction of plasmids**

Molecular manipulations were performed by standard protocols. Enzymes were used according to the instructions provided by the manufacturers. Sticky ends of DNA fragments were blunted using T4 DNA polymerase (Promega). Restriction enzymes and T4 DNA ligase were purchased from TAKARA. Plasmids used or constructed and primers used in PCRs are listed in **Table 3**.

Construction of the plasmids for targeted gene inactivation. Taking the *sll1263::C.K2* mutant as an example: a 2,093 bp DNA fragment containing the *sll1263* gene was generated by PCR using primers *sll1263*−1 and *sll1263*−2 with *Synechocystis* 6803 chromosomal DNA as template, cloned into pMD18-T, and confirmed by sequencing. The C.K2 fragment excised from pRL446 (NCBI GenBank accession No. Eu346690) (Elhai and Wolk 1988) by *Pvu*ll was inserted into the *Ball* site of that plasmid, resulting in pHS091 for the inactivation of *sll1263* in *Synechocystis* 6803.

Construction of a stable overexpression vector in *Synechocystis* 6803 under the *psbAll* promoter (PsbsAll expression vector). The *psbAll* promoter without the high-light-responsive region was generated by PCR using primers PpsbAlloe−1 and PpsbAlloe−2, with *Synechocystis* 6803 chromosomal DNA as template cloned into pBluescript II SK(−) and confirmed by sequencing, resulting in pHS211. A spectinomycin resistance cassette ‘Omega’ excised by *Dra*I from pRL57 was inserted in the *CloI* site of *pHS211*, resulting in pHS215. The two transcription terminators at the two ends of Omega ensure that the *psbAll* promoter is not influenced by upstream sequences. The *omega-PpsbAll* fragment was excised by *XhoI*/*SpeI* from pHS215, and cloned into the *EcoRI* sites of Platform0168, a similar vector to pKW1188 (Williams 1988) but without *Smal* and *Ndel* sites. Platform0168 contained an *sll0168* gene, which was regarded as a platform for inserting the exogenous gene by homologous recombination. The resulting PsbsAll expression vector (pHS298) has only one *Ndel* site at the end of the *psbAll* promoter. Heterologous genes can be easily cloned at the *Ndel* site and can be expressed under the control of *psbAll* promoter.

**Construction of the plasmids for overexpressing sll1263, fieF and egfp by the PpsbAll expression vector.** The *sll1263* gene from the *Synechocystis* 6803 genome, the *fieF* gene from *E. coli* DH5α and the *egfp* gene from plasmid pEGFP-1 were generated by PCR using their corresponding primers, *sll1263*−1/*sll1263*−2, *FieF−1/FieF−2* and *egfp−5/egfp−3*, respectively. The PCR products were inserted into the *Ndel* site (blunt end) of pHS298, making the genes in the same orientation as the *psbAll* promoter. The resulting pHS248, pHS303 and pHS304 were used to transform *Synechocystis* 6803, in which the three genes were expressed under the control of the *psbAll* promoter.

**Construction of the mutant, overexpression and complementation strains of *Synechocystis***

To construct the *Synechocystis* 6803 *sll1263::C.K2* mutant, pHS091 was introduced into wild-type *Synechocystis* 6803 by natural transformation according to Williams (1988). Homologous double-crossover recombinants were generated between the plasmid and genomic DNA under the positive selection of kanamycin. Complete segregation of the mutant was confirmed by PCR using the primer pair *sll1263*−1/*sll1263*−2, with PCR of DNA from wild-type *Synechocystis* 6803 as a positive control. To complement the *sll1263::C.K2* mutant, pHS303 and pHS304 were introduced into the *Synechocystis* 6803 *sll1263::C.K2* mutant.

**Extraction of RNA and RT–PCR**

About 200 ml of *Synechocystis* 6803 strains grown in BG-11, or 24 h after transfer into Fe-deficient BG-11, was collected by centrifugation, and quickly frozen in liquid nitrogen. Total RNA was extracted using a TRIzol Reagent Kit (Invitrogen), according to the manufacturer’s instructions, and treated with RNase-free DNase I (TAKARA) three times until no PCR product could be detected with the primers for reverse transcription–PCR (RT–PCR).

For RT–PCR, first-strand cDNA was synthesized in a 25 μl reaction mixture containing 50 mM Tris–HCl (pH 8.3), 75 mM KCl, 3 mM MgCl₂, 10 mM diithiothreitol (DTT), 50 mM dNTPs, 25 U of RNasin, 200 U of M-MLV reverse transcriptase (Promega), 2 μg of total RNA and 0.5 μg of random primers at 37°C for 60 min. The relative concentration of cDNA was evaluated after serial dilutions by PCR using primers *rnpB*−1 and *rnpB*−2 and adjusted to the same level according to the brightness of PCR bands. Primers of *sll1263* or the related iron uptake genes used in RT–PCR are listed in **Table 3**. Two independent experiments were performed, which showed consistent results.

**Determination of cellular iron contents**

Iron contents were measured as described in Keren et al. (2004) and Nicolaelsen et al. (2008). The *Synechocystis* 6803 cells were treated with EDTA wash solution to remove the iron attached.
Table 3 Cyanobacterial strains, plasmids and primers used in this study

<table>
<thead>
<tr>
<th>Strain, plasmid or primer</th>
<th>Derivation and/or relevant characteristics</th>
<th>Reference(s) or source</th>
</tr>
</thead>
<tbody>
<tr>
<td>PCC 6803</td>
<td>Wild type</td>
<td>This study</td>
</tr>
<tr>
<td>WT1188</td>
<td>Km(^r), Synechocystis sll0168::C.K2; result of transformation with pKW1188</td>
<td>Williams (1988)</td>
</tr>
<tr>
<td>sll263::C.K2</td>
<td>Km(^r), Synechocystis 6803 mutant</td>
<td>This study</td>
</tr>
<tr>
<td>sll1484::C.K2</td>
<td>Km(^r), Synechocystis 6803 mutant</td>
<td>This study</td>
</tr>
<tr>
<td>slr1036::C.K2</td>
<td>Km(^r), Synechocystis 6803 mutant</td>
<td>This study</td>
</tr>
<tr>
<td>slr1404–1405::C.K2</td>
<td>Km(^r), Synechocystis 6803 mutant</td>
<td>This study</td>
</tr>
<tr>
<td>slr0376::C.K2</td>
<td>Km(^r), Synechocystis 6803 mutant</td>
<td>This study</td>
</tr>
<tr>
<td>slr0964::C.K2</td>
<td>Km(^r), Synechocystis 6803 mutant</td>
<td>This study</td>
</tr>
<tr>
<td>sll1263::C.K2</td>
<td>Km(^r), Synechocystis 6803 mutant</td>
<td>This study</td>
</tr>
<tr>
<td>/C10-PsbAII-eGFP</td>
<td>Sp(^r), Omega-P(_{\text{psbAII}})-egfp integrated into sll0168 in the genome of Synechocystis wild type</td>
<td>This study</td>
</tr>
<tr>
<td>/C10-PsbAII-Sll1263 (sll1263-OE)</td>
<td>Sp(^r), Omega-P(_{\text{psbAII}})-sll1263 integrated into sll0168 in the genome of Synechocystis wild type</td>
<td>This study</td>
</tr>
<tr>
<td>sll1263::C.K2 (Ω-PsbAll-FieF)</td>
<td>Km(^r)Sp(^r), Omega-P(_{\text{psbAII}})-fieF integrated into sll0168 in the genome of mutant Synechocystis sll1263::C.K2</td>
<td>This study</td>
</tr>
<tr>
<td>sll1263::C.K2 (Ω-PsbAll-Sll1263)</td>
<td>Km(^r)Sp(^r), Omega-P(_{\text{psbAII}})-sll1263 integrated into sll0168 in the genome of mutant Synechocystis sll1263::C.K2</td>
<td>This study</td>
</tr>
<tr>
<td>Plasmids</td>
<td></td>
<td></td>
</tr>
<tr>
<td>pH5091</td>
<td>Ap(^r)Km(^r), PCR fragment containing the sll1263 gene cloned into pMD18-T, and C.K2 inserted in its Boll site</td>
<td>This study</td>
</tr>
<tr>
<td>pH5092</td>
<td>Ap(^r)Km(^r), PCR fragment containing the slr1484 gene cloned into pMD18-T, and C.K2 inserted in its Apal site</td>
<td>This study</td>
</tr>
<tr>
<td>pH5093</td>
<td>Ap(^r)Km(^r), PCR fragment containing the slr1036 gene cloned into pMD18-T, and C.K2 inserted in its Ncol site</td>
<td>This study</td>
</tr>
<tr>
<td>pH5094</td>
<td>Ap(^r)Km(^r), PCR fragment containing the slr1404-sll1405 gene cloned into pMD18-T, which was excised by Ncol and then inserted by C.K2</td>
<td>This study</td>
</tr>
<tr>
<td>pH5095</td>
<td>Ap(^r)Km(^r), PCR fragment containing the slr0964 gene cloned into pMD18-T, and C.K2 inserted in its Hpal site</td>
<td>This study</td>
</tr>
<tr>
<td>pH5096</td>
<td>Ap(^r)Km(^r), PCR fragment containing the slr0964 gene cloned into pMD18-T, which was excised by Boll and then inserted by C.K2</td>
<td>This study</td>
</tr>
<tr>
<td>pH5211</td>
<td>Ap(^r), PCR fragment containing the psbAll promoter region was cloned into pBluescript II SK(−)</td>
<td>This study</td>
</tr>
<tr>
<td>pH5215</td>
<td>Ap(^r)Sp(^r), omega cassette from pRL57 inserted into pH5211 at the Clal site</td>
<td>This study</td>
</tr>
<tr>
<td>pH5248</td>
<td>Ap(^r)Sp(^r), PCR fragment containing the eGFP fragment from pEGFP-1 cloned in the PsbAll expression vector, pH5298</td>
<td>This study</td>
</tr>
<tr>
<td>pH5298</td>
<td>Ap(^r)Sp(^r), PsbAll expression vector, Omega-P(_{\text{psbAII}})-fragment from pH5215 inserted into Platform0168 at the EcoRI site</td>
<td>This study</td>
</tr>
<tr>
<td>pH5303</td>
<td>Ap(^r)Sp(^r), PCR fragment containing the sll1263 gene cloned in the PsbAll expression vector, pH5298</td>
<td>This study</td>
</tr>
<tr>
<td>pH5304</td>
<td>Ap(^r)Sp(^r), PCR fragment containing the fieF gene from E. coli DH5(^a) cloned in the PsbAll expression vector, pH5298</td>
<td>This study</td>
</tr>
<tr>
<td>pKW1188</td>
<td>Ap(^r)Km(^r), plasmid bearing an integrative platform for Synechocystis 6803</td>
<td>Williams (1988)</td>
</tr>
<tr>
<td>Platform0168</td>
<td>Ap(^r)Km(^r), pKW1188 derivation without Smal and Ndel sites</td>
<td>This study</td>
</tr>
<tr>
<td>pRL466</td>
<td>Km(^r), a cloning vector with a kanamycin resistance marker (C.K2)</td>
<td>Elhai and Wolk (1988)</td>
</tr>
<tr>
<td>pRL57</td>
<td>Sp, cloning vector with the spectomycin resistance cassette omega</td>
<td>Black et al. (1993)</td>
</tr>
</tbody>
</table>

Primers used in this study

<table>
<thead>
<tr>
<th>Primer</th>
<th>Sequence</th>
</tr>
</thead>
<tbody>
<tr>
<td>sll1263-1</td>
<td>5'-CCAAGTTGCCTCGTTATTCCGC-3'</td>
</tr>
<tr>
<td>sll1263-2</td>
<td>5'-ACCACAGGGCCACGCAATCAGC-3'</td>
</tr>
</tbody>
</table>

(continued)
to the cell surface. The samples were then digested in 80% HNO₃ in a microwave oven (Xintuo XTIII). After digestion, the samples were reconstituted in fresh 5% HNO₃ solution. Iron element contents were determined with an atomic absorption spectrometer (AA240SS, Varian).

**Measurement of iron uptake rates**
The amounts of iron taken up by wild-type and mutant cells were measured using the radioactive tracer ⁵⁵FeCl₃ (PerkinElmer Inc.) according to Katoh et al. (2001) and Nicolaisen et al. (2008) with minor modification. Cells were grown in 50 ml of BG-11 medium to the logarithmic stage of growth, harvested, washed and resuspended with 2 ml of iron-free BG-11 medium (cell densities about 8 × 10⁸ cells ml⁻¹). For total iron uptake rate measurement, ⁵⁵FeCl₃ was added to the cell solutions at a concentration of 1 mM (37 kBq ml⁻¹). The ferric iron uptake rate was measured in the presence of 1 mM ferrozine, and ferrous iron uptake was measured in the presence of 1 mM ascorbate. The uptake reaction was performed under 30 μmol photons m⁻² s⁻¹ continuous light illumination. Samples of 0.2 ml were taken at the indicated times, filtered on 0.2 μm pore size polycarbonate filters (Poretics) and washed with 2 ml of 20 mM TES-KOH (pH 8.0) containing 10 mM EDTA. Then, the samples were placed in 5 ml of scintillation fluor (Ultima Gold, Beckman) before counting on a Packard Tri-Carb scintillation counter.

**Supplementary data**
Supplementary data are available at PCP online.

**Funding**
This study was supported by the National Natural Science Foundation of China [No. 31100184]; the National Basic
Research Program [973 Program, No. 2008CB418004]; Natural Sciences and Engineering Research Council of Canada.

Acknowledgments

We are grateful to Professor C. Peter Wolk (Michigan State University) and Professor Xudong Xu (Institute of Hydrobiology, Chinese Academy of Sciences) for kindly providing the plasmids for the use of cyanobacterial gene knockout. We also thank Natasha Dudek (McGill University) for her kind help in language improvement of this manuscript.

References


