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ORIGINAL ARTICLE

New insights into iron acquisition by cyanobacteria: an essential role for ExbB-ExbD complex in inorganic iron uptake

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Cyanobacteria are globally important primary producers that have an exceptionally large iron requirement for photosynthesis. In many aquatic ecosystems, the levels of dissolved iron are so low and some of the chemical species so unreactive that growth of cyanobacteria is impaired. Pathways of iron uptake through cyanobacterial membranes are now being elucidated, but the molecular details are still largely unknown. Here we report that the non-siderophore-producing cyanobacterium *Synechocystis* sp. PCC 6803 contains three *exbB-exbD* gene clusters that are obligatorily required for growth and are involved in iron acquisition. The three *exbB-exbD*s are redundant, but single and double mutants have reduced rates of iron uptake compared with wild-type cells, and the triple mutant appeared to be lethal. Short-term measurements in chemically well-defined medium show that iron uptake by *Synechocystis* depends on inorganic iron (Fe′) concentration and ExbB-ExbD complexes are essentially required for the Fe′ transport process. Although transport of iron bound to a model siderophore, ferrioxamine B, is also reduced in the *exbB-exbD* mutants, the rate of uptake at similar total [Fe] is about 800-fold slower than Fe′, suggesting that hydroxamate siderophore iron uptake may be less ecologically relevant than free iron. These results provide the first evidence that ExbB-ExbD is involved in inorganic iron uptake and is an essential part of the iron acquisition pathway in cyanobacteria. The involvement of an ExbB-ExbD system for inorganic iron uptake may allow cyanobacteria to more tightly maintain iron homeostasis, particularly in variable environments where iron concentrations range from limiting to sufficient.
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Introduction

Low concentrations of iron in surface waters of the open sea are maintained by efficient uptake systems of planktonic biota and limited solubility of the inorganic forms (Morel and Price, 2003). Complexation by siderophores and other uncharacterized organic ligands increases the solubility of the dissolved iron (Mawji *et al.*, 2008) but reduces its availability to many taxa. At the extreme, such reduced iron bioavailability can impair phytoplankton growth (Boyd *et al.*, 2007; Falkowski and Raven, 2007; Chappell *et al.*, 2012; Wilhelm *et al.*, 2013) and is thought to limit the primary productivity in as much as 40% of global ocean (Martin *et al.*, 1994; Falkowski *et al.*, 1998; Achilles *et al.*, 2003) and some freshwater habitats (North *et al.*, 2007; Havens *et al.*, 2012).

Siderophores are strong iron chelators, secreted by many organisms, including bacteria, fungi, yeast and monocotyledonous plants to solubilize, bind and make available iron in the environment. Generally, organisms synthesize and secrete these low molecular weight chelators to bind Fe(III) and then transport the ferri-siderophore complex through the cell membrane. Unlike other organisms, Gram-negative bacteria possess an outer membrane (OM) as well as a cytoplasmic membrane (CM), which presents an additional barrier to the exchange of solutes. As ferri-siderophores are too large to passively diffuse through the OM porins, they must be actively transported across the membrane by specific receptor proteins (Miethke and Marahiel, 2007; Noinaj *et al.*, 2010). The OM receptors/transporters bind the ferri-siderophore complexes and directly interact with the energizing TonB-ExbB-ExbD complex in the inner membrane to allow the iron complex to be transported into the periplasmic space. This transport process involves

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three components: (i) OM localized transporters; (ii) a CM-localized TonB-ExbB-ExbD complex, and (iii) ion electrochemical potential (Noinaj *et al.*, 2010). Over the past three decades, many aspects of this TonB-ExbB-ExbD-dependent transport system have been revealed. The crystal structures of several OM transporters and their complexes with TonB are now known (Ferguson *et al.*, 1998, 2002; Buchanan *et al.*, 1999; Ferguson and Deisenhofer, 2004; Pawelek *et al.*, 2006; Shultis *et al.*, 2006; Krieg *et al.*, 2009), the signal transduction of OM transporters by interaction with TonB has been elucidated (Ferguson *et al.*, 2007; Kim *et al.*, 2007) and the rotational mechanism of TonB motion has been reported (Jordan *et al.*, 2013). However, with regard to the substrates of the transport system, we are probably only seeing the 'tip of the iceberg' (Schauer *et al.*, 2008). Originally, iron complexes and vitamin B_{12} were thought to be the main substrates of the TonB-ExbB-ExbD system, but more and more new substrates have been found to be transported, including citrate, transferrin, hemoproteins, heme, phages, colicins, maltodextrins, nickel chelators and sucrose (Schauer *et al.*, 2008; Noinaj *et al.*, 2010).

Cyanobacteria are globally important primary producers and dominate some iron-limited marine environments, such as the equatorial Pacific Ocean (Kolber *et al.*, 1994). However, the siderophore-mediated iron uptake pathway described in non-photosynthetic bacteria has not been fully confirmed in cyanobacterial species (Stevanovic *et al.*, 2012). The following observations are pertinent: (i) although cyanobacteria possess an OM and are commonly considered as Gram-negative bacteria, their cell envelopes are partly characteristic of Gram-positive bacteria, which do not possess the TonB-ExbB-ExbD system (Hoiczyk and Hansel, 2000); (ii) while some species produce strong siderophores, most cyanobacteria do not (Ito and Butler, 2005; Hopkinson and Morel, 2009; Mirus *et al.*, 2009); and (iii) some cyanobacteria can use the iron bound to siderophores of other organisms (Kranzler *et al.*, 2011). Hopkinson and Morel (2009) suggested that the role of siderophores in marine environments is probably overestimated but could reach no definitive conclusion, because the iron acquisition mechanisms of cyanobacteria are poorly understood. Kranzler *et al.* (2011, 2014) proposed an alternative reduction-based uptake strategy by which cyanobacteria can sequester iron from multiple complexes in dilute aquatic environments.

Overall, the iron uptake mechanism of cyanobacteria remains largely unclear, especially how iron crosses the cyanobacterial OM as well as the role of TonB-ExbB-ExbD system in cyanobacterial OM transport. In a siderophore-producing strain of *Anabaena* sp. PCC 7120, putative *tonB*, *exbB* and *exbD* genes have been identified, and their inactivation induces an iron starvation phenotype (Stevanovic *et al.*, 2012), but direct evidence of their participation in iron transport is lacking. As most cyanobacteria do not produce siderophores, generalizing the results from *Anabaena* to other species may be inappropriate. Here we identify and characterize the ExbB-ExbD complexes in a non-siderophore-producing cyanobacterium, *Synechocystis* sp. PCC 6803, and find that they are required for inorganic iron (Fe′) uptake. Although substrates for the TonB-ExbB-ExbD-mediated transport pathway in non-photosynthetic bacteria are exclusively organic, our results suggest that cyanobacteria use the ExbB-ExbD complexes to activate a different class of OM transporter involved in inorganic iron uptake. These finding may be helpful in understanding how cyanobacteria acquire iron in nature and survive in iron-limited environments.

Materials and methods

Strains, culture conditions and general methods

A glucose-tolerant, axenic strain of *Synechocystis* sp. (PCC 6803) was cultured in BG11 medium at 30 °C under continuous illumination (40 μmol photons $m^{-2} s^{-1}$). All growth media, buffers and solutions used during the experiments were autoclave sterilized. To analyze growth in iron-starved medium, ammonium ferric citrate was omitted from the BG11 medium and replaced by the same concentration of ammonium citrate. Glassware used for iron-starved conditions was soaked in 6 M HCl for about 12 h and rinsed seven times with Milli-Q water to remove residual iron. Before the experiment, exponential cells were harvested by centrifugation at 6000 *g* and 30 °C for 5 min and washed three times with BG11–Fe medium to remove extracellular iron from the cell surfaces. The specific growth rate and chlorophyll *a* content were assayed as described in previous studies (Jiang *et al.*, 2010, 2012).

Construction of mutants, complementation and overexpression strains of Synechocystis 6803

The single mutants of the three *exbB-exbD* gene clusters were generated by introducing a single C.K2 (Km^r), C.CE2 (Em^r) or Omega (Sp^r) resistance cassette into the open-reading frame of *sll1404-sll1405*, *slr0677-slr0678* or *sll0477-sll0478-sll0479*, respectively, by the homologous recombination method (Jiang *et al.*, 2010, 2012). The double mutants were generated by transforming two of the resistance cassettes into the genome. The triple mutant was generated by sequentially transforming all three cassettes into wild-type cells in different order. In the complementation and overexpression strains, the genes or gene clusters were expressed by a P*psbAII* expression vector (Jiang *et al.*, 2012). The resulting plasmids were used to transform the gene fragments into *Synechocystis* 6803 wild-type or mutant strains. The plasmid construction and primers used are listed in Supplementary Table S1.

Extraction of RNA and reverse transcriptase–PCR
The cells of *Synechocystis* strains grown in standard BG11 medium or 24 h after transfer into iron-deficient BG11 medium were collected and quickly frozen in liquid nitrogen. The RNA extraction and reverse transcriptase–PCR methods are described previously (Jiang *et al.*, 2012).

Determination of cellular iron contents and measurement of iron uptake rates
Cells were grown from OD_{730} (optical density at 730 nm) 0.2 to 2.0 in standard BG11 medium. Iron contents were measured according to Nicolaisen *et al.* (2008). Briefly, the cells were collected and washed three times in 20 mM EDTA, then dried and digested for element determination by an atomic absorption spectrometer (AA240FS, Varian, Palo Alto, CA, USA). The $^{55}Fe^{3+}$ uptake rates shown in Figure 1f were measured as described previously (Jiang *et al.*, 2012) in the presence of 1 mM Ferrozine. To determine the inorganic iron or desferrioxamine (DFB)-Fe uptake rates shown in Figures 4a and b, cells were resuspended in iron-free BG11 medium lacking citric acid and ferric ammonium citrate (the medium contained 2.68 μM EDTA). For measuring the inorganic iron uptake rate, 100 nM (3.7 kBq ml^{-1}) $^{55}FeCl_3$ was added to the cell solutions. For measuring the Fe-siderophores uptake rate, 100 nM $^{55}FeCl_3$ complexed with 133 nM DFB was added to the cell solutions. To clarify the role of ExbB-ExbD in Fe′ uptake, more rigorous experiments were carried out using trace metal clean techniques and precomplexed FeEDTA solution, as described by Kranzler *et al.* (2011) with minor modification. Cells were grown to logarithmic phase in BG11 medium, harvested and washed three times in a modified BG11 medium lacking EDTA, citric acid, ferric ammonium citrate and trace metals (this medium contained no organic ligands). The samples were then resuspended in different concentrations of precomplexed EDTA-Fe buffered with the modified BG11 medium. Samples (0.2 ml) were collected at the indicated times, filtered on 0.22-μm pore size polycarbonate filters (Poretics, Livermore, CA, USA) and washed with 3 ml oxalate–EDTA washing solution (Tang and Morel, 2006). Fe′ concentrations were calculated using the Mineql speciation software (Westall *et al.*, 1976; http://www.mineql.com/). The uptake experiment of Figure 4e was prepared by the same methods as Figures 4c and d. To obtain two higher Fe′ concentrations, 1 μM $FeCl_3$ was buffered with insufficient 1.6 μM EDTA (yielding 12.8 nM Fe′), and 0.1 μM $FeCl_3$ was added to the modified BG11 medium lacking organic ligands (yielding 99.9 nM Fe′) before measurement.

Yeast two-hybrid assays
In the yeast two-hybrid assays, the protein–protein interaction of Sll1404 and Sll1405 was detected by a Matchmaker GAL4 Two-Hybrid System 3 (Clontech, Palo Alto, CA, USA). The *sll1404* and *sll1405* gene fragments were cloned into pGBKT7 and pGADT7, respectively. The resulting plasmids were co-transformed into *Saccharomyces cerevisiae* AH109 and selected on SD/-Trp-Leu-His agar plates. The transformants were then grown on SD/-Trp-Leu-His-Ade plates for 3 days.

Localization of ExbB and ExbD proteins in Synechocystis 6803
Total protein was extracted from *Synechocystis* 6803 cells and centrifuged at 50 000 *g* for 1 h to isolate soluble and membrane proteins. Cytoplasmic and thylakoid membranes were isolated by the 2D-separation method combined with sucrose density centrifugation (Norling *et al.*, 1998; Huang *et al.*, 2006). Samples were resolved on 12% sodium dodecyl sulfate polyacrylamide gel and immunoblotted with corresponding antibodies. The marker proteins of cytoplasmic and thylakoid membranes were NrtA and CP47, respectively.

Results

Three exbB-exbD gene clusters in the Synechocystis 6803 genome were induced by iron starvation, and their inactivation stimulated known iron uptake genes
Previously, we identified a *Synechocystis* 6803 strain with a mutant putative *exbB-exbD* gene cluster (*Δsll1404-sll1405*) that grew more slowly than the wild type under iron-deficient conditions (Jiang *et al.*, 2012). At toxic iron concentrations (1 mM), the same mutant grew more rapidly than the wild type (Supplementary Figure S1A), indicating a positive role of the gene cluster in iron homeostasis. Bioinformatic analysis revealed a further two putative *exbB-exbD* gene clusters (*slr0677-slr0678* and *sll0477-sll0478-sll0479*) in the genome of *Synechocystis* 6803 (Supplementary Figure S2A). The three clusters, respectively, encoded putative ExbB proteins Sll1404, Slr0677 and Sll0477, with 57–61% similarity in their amino-acid sequences, and putative ExbD proteins Sll1405, Slr0678, Sll0478 and Sll0479, with 46–58% amino-acid similarity (Supplementary Figure S2B). Genes encoding TonB-resembling proteins, *slr1484*, and a putative OM transporter, *sll1406*, were located close to the *sll1404-sll1405* gene cluster (Supplementary Figure S2A). It should be noted that, in *Synechocystis* 6803, the three clusters that encode putative ExbB-ExbD also show similarity with *Escherichia coli* TolQ-TolR (data not shown). Although the TolA-TolQ-TolR and TonB-ExbB-ExbD energy-coupled import systems show strong sequence homology and mutual functional substitution, no uptake functions for TolA-TolQ-TolR have been described other than for certain bacteriophages and colicins (Braun and Herrmann, 1993).

Synechocystis grown in iron-replete (21 μM Fe) BG11 medium (standard conditions) showed low expression of *sll1404*, *sll0477* and *slr0677*, but the

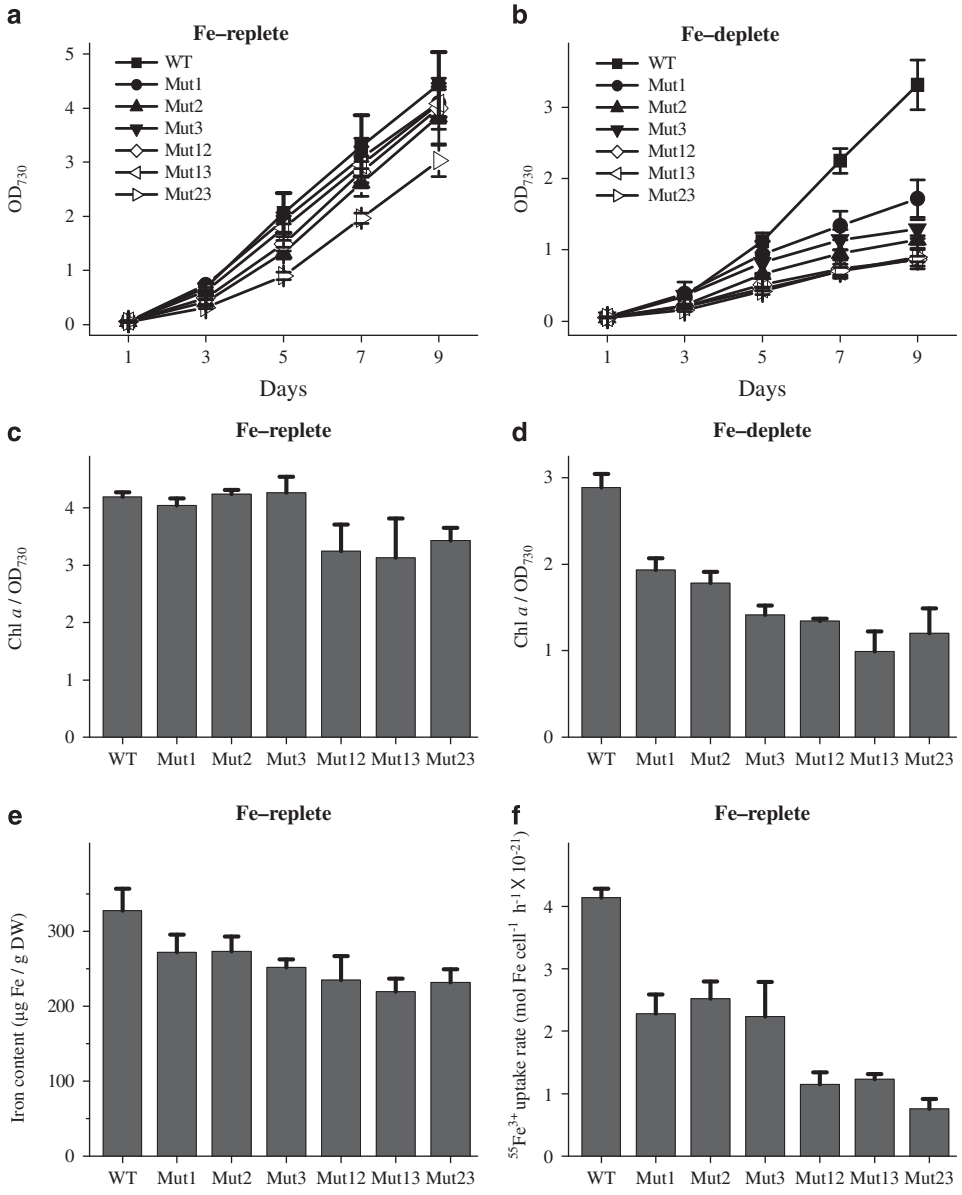

Figure 1 Physiological phenotypes of *Synechocystis exbBD* mutants. Cells were grown in BG11 medium under standard iron concentrations (Fe-replete) (**a**, **c**, **e** and **f**) or iron-deficient conditions, in which ammonium ferric citrate was replaced with ammonium citrate (Fe-deplete) (**b** and **d**). The growth characteristics (**a** and **b**), pigment content (**c** and **d**), cellular iron content (**e**) and short-term $^{55}Fe^{3+}$ uptake rate (**f**) were determined as previously described (Jiang *et al.*, 2012).

transcript abundance of all genes dramatically increased following 24 h iron starvation (Supplementary Figure S2C). The three putative *exbB* genes were highly expressed in a cation diffusion facilitator iron transport mutant (*sll1263*::C.K2) (Jiang *et al.*, 2012) in the same iron-enriched medium (21 μM Fe) and were further induced after 24 h iron starvation (Supplementary Figure S2C). As genes in the putative *exbB-exbD* clusters were highly expressed in iron-starved cells, their products are likely involved in iron uptake. Indeed, the expression of several known iron uptake genes (*fut* and *feo* genes) (Katoh *et al.*, 2001) is elevated in *exbB-exbD* mutants (see Supplementary Figure S3 for mutant constructions), even during growth in standard iron-replete conditions (Supplementary Figure S2D). These genes are further expressed in iron-deficient medium (Supplementary Figure S2D), and each *exbB* gene is enhanced when other homologs are knocked out (Supplementary Figure S2E). This result suggests that the three *exbB-exbD* gene clusters are redundant and perform a similar function.

Mutation of any one of the exbB-exbD gene clusters resulted in a low-iron-sensitive phenotype, which was reinforced in double mutants

Three single mutants and three double mutants of the putative *exbB-exbD* gene clusters were constructed

(Supplementary Figure S3). For convenience, we refer to gene clusters *sll1404-sll1405*, *slr0677-slr0678* and *sll0477-sll0478-sll0479* as *exbBD1*, *exbBD2* and *exbBD3*, respectively, and their corresponding single mutants are denoted Mut1, Mut2 and Mut3, respectively. Double mutants were deficient in *exbBD1* and *exbBD2* (Mut12), *exbBD1* and *exbBD3* (Mut13) and *exbBD2* and *exbBD3* (Mut23). As shown in Figures 1a and c, the growth and chlorophyll *a* concentration of all three single mutants grown in iron-replete BG11 medium was similar to that of wild-type cells, while that of the double mutants was slightly decreased. In iron-deficient medium, however, growth and chlorophyll *a* concentration of the mutants was significantly lower than wild-type levels (Figures 1b and d). The iron-deficient phenotype was more severe in double mutants than in single mutants (Figures 1b and d).

Although their growth characteristics were indistinguishable in iron-replete medium (see Figure 1a), the mutants contained about 17–33% less cellular iron than the wild type (Figure 1e), with double mutants containing the lesser iron quotas (Figure 1e). These reduced cellular iron levels were consistent with the upregulation of iron uptake-related genes in the mutants growing in standard BG11 medium (see Supplementary Figure S2D). To evaluate whether the reduced iron content resulted from slower rates of iron transport and/or reduced iron storage capacity, the short term $^{55}Fe^{3+}$ uptake rates were measured in mutant and wild-type strains. Cells were grown in standard BG11 medium (21 μM iron) and the Fe^{2+} produced by photochemical or cellular reduction was trapped by the iron reagent ferrozine. Fe^{3+} uptake rates were found to be significantly slower in the single and double mutants than in wild-type cells (39–46% and 70–82% reduction, respectively; see Figure 1f).

The three exbB-exbDs gene clusters were functionally redundant, and the triple-mutant appeared to be lethal

To elucidate whether the iron-deficient phenotypes resulted from inactivation of the *exbB-exbD* gene clusters, we constructed several complementation and overexpression strains. Growth assays of the strains in iron-deficient medium (Table 1) showed that the iron-deficient phenotype of each mutant was complemented by the gene cluster itself, ruling out possible second point mutations and polar effects. Moreover, each gene cluster corrected the low-iron-sensitive phenotypes of mutants lacking both alternative gene clusters (Table 1). This result showed that the three gene clusters are functionally interchangeable and probably undertake a similar function. The mutant lacking all three *exbB-exbD* gene clusters was never completely segregated, even after 6 months culturing of the transformants in BG11 medium supplemented with the corresponding antibiotics (in this test, cells were alternately streaked on BG11 solid medium and cultured in liquid BG11 medium). As shown in Figure 2a, PCR amplification of the triple mutant genome yielded wild-type gene copies of *exbB-exbD.* Nonetheless, this triple mutant knockdown line scarcely survived in standard BG11 medium (21 μM iron) (Figure 2b). A triple mutant was alternatively acquired by supplementing the medium with 5 mM glucose to allow mixotrophy, thereby reducing the growth requirement of cellular iron. Although levels of wild-type copies were lower in the mixotrophic than in photoautotrophic cells, the fragments remained incompletely segregated on the gel (Figure 2c). This 'approximate' triple-mutant scarcely survived under photoautotrophic conditions but performed moderately well in glucose-supplemented medium (Figure 2d), presumably because of the reduced iron growth costs in this medium. To exclude possible polar effects while obtaining a triple mutant from Mut13, we attempted to knockout *exbBD1* in Mut23 and *exbBD3* in Mut12 but to no avail (Figure 2e). Collectively, the results confirmed that the three *exbB-exbD* gene clusters have similar functions and are essential to the survival of *Synechocystis* 6803.

E. coli homologs can complement the phenotype of cyanobacterial ExbB-ExbD mutant

Although the encoding products of the *exbB-exbD* gene clusters possess similar amino-acid sequences to known ExbB and ExbD protein homologs, whether they are functional homologs requires further genetic or biochemical evidence. Thus, *Synechocystis* 6803 Mut1 and Mut2 were transformed with the expression plasmid containing *exbB-exbD* from *E. coli* K-12. Growth assays confirmed that *E. coli* ExbB-ExbD restored the iron-deficient phenotypes of *Synechocystis* 6803 (Figure 3c), providing direct genetic evidence that the putative gene clusters in *Synechocystis* 6803 are indeed ExbB-ExbD-encoding genes. To further elucidate the role of these ExbB-ExbD proteins in *Synechocystis* 6803, we investigated their subcellular location and interactions. Analysis of soluble and membrane protein fractions revealed that both ExbB (Sll0477) and ExbD (Slr0678) were localized at the CM (Figure 3a). Yeast two-hybrid assay showed that the yeast *Saccharomyces* AH109 transformed with plasmids pAD-Sll1405 and pBD-Sll1404 could grow on synthetic defined (SD) plates lacking tryptophan, leucine, histidine and adenine (SD/-Trp-Leu-His-Ade). This suggests a protein–protein interaction between Sll1404 (ExbB) and Sll1405 (ExbD) (Figure 3b). Given the high degree of similarity among the ExbB-ExbD homologs (Supplementary Figure S2B), we propose that the three *exbB-exbD* gene clusters encode three CM-located ExbB-ExbD complexes with overlapping functions in *Synechocystis* 6803.

ExbB-ExbD complexes are required for Fe′ uptake in Synechocystis 6803

Hopkinson and Morel (2009) have reported that *Synechocystis* 6803 possesses neither siderophore

biosynthesis nor siderophore transport genes within its genome. Kranzler *et al.* (2011) have proved direct evidence that *Synechocystis* 6803 does not produce siderophore during iron starvation. Interestingly, the species grows well in minimal medium without organic substrates (Supplementary Figure S1B). Like other photosynthetic microbes, *Synechocystis* 6803 is unable to transport Fe-EDTA complexes directly (Kranzler *et al.*, 2011). Short-term measurements revealed that iron uptake rates were strongly affected by complexation. For example, at the same total iron concentration (100 nM), iron uptake was roughly 800-fold faster when added as an FeEDTA than when added as a DFB complex (Figures 4a and b). Unexpectedly, iron uptake rates under both conditions were two to three times slower in the mutant than in the wild-type cells. As FeEDTA is unavailable for transport, the substrates sequestered by the cells are probably the inorganic complexes and free Fe^{3+} (collectively Fe′) in equilibrium with FeEDTA or produced by cellular reduction of FeEDTA species. Thus the ExbBs and ExbDs of *Synechocystis* are required for iron uptake from both inorganic and organic (ferrioxamine) complexes. These results also indicated that Fe′ is a more available iron resource for *Synechocystis* 6803 than Fe-DFB.

TonB-ExbB-ExbD-dependent OM transporters are thought to be involved in uptake of organically chelated iron, such as siderophore-iron (Noinaj *et al.*, 2010). To our knowledge, the involvement of this transport system in Fe′ uptake has not been experimentally verified. Therefore we evaluated the relationship between iron uptake and [Fe′] in *exbB-exbD* mutant and wild-type cells, using an EDTA-buffered system (Maldonado and Price, 1999). Cultures were grown to exponential phase in standard BG11 medium, and ^{55}Fe was added (as $FeCl_3$) at a total concentration of 0.993 μM preequilibrated with EDTA to achieve [Fe′] of 0.049, 0.130 and 0.496 nM (Figure 4c). Under these conditions, we calculated that >99% of the added iron was bound to EDTA. As shown in Figure 4c, the iron uptake rate increased

Table 1 Specific growth rates of the wild-type, mutants, overexpression and complementation strains of *Synechocystis* 6803 cultured in iron-deficient medium

Strains (Synechocystis PCC 6803)	*Specific growth rates*[a]
WT	0.748 ± 0.025
Mut1	**0.605 ± 0.024**
exbBD1-OE	0.688 ± 0.018
Mut1 complemented by *exbBD1*	0.667 ± 0.026
Mut1 complemented by *exbBD2*	0.688 ± 0.015
Mut1 complemented by *exbBD3*	0.761 ± 0.005
Mut2	**0.556 ± 0.010**
exbBD2-OE	0.674 ± 0.012
Mut2 complemented by *exbBD1*	0.725 ± 0.002
Mut2 complemented by *exbBD2*	0.692 ± 0.013
Mut2 complemented by *exbBD3*	0.749 ± 0.020

Abbreviation: WT, wild type. The values in bold show the significantly reduced growth rates of the mutants.
[a]The values are the means ± s.ds. of triplicate cultures.

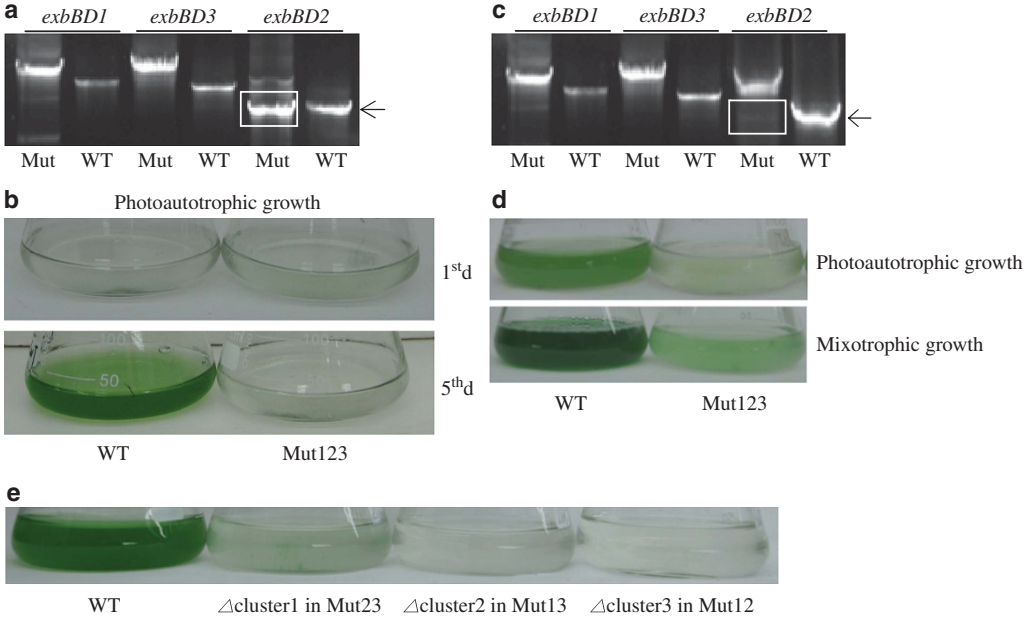

Figure 2 *Synechocystis* 6803 triple mutant lacking all three functional *exbBD* gene clusters (Mut123) cannot be fully segregated. (**a**) The knockdown line of Mut123 is not completely segregated on the PCR gel after several months' incubations and transfers in standard BG11 medium supplemented with antibiotics. The arrow indicates the presence of the wild-type *exbBD* gene cluster. (**b**) Photograph of the knockdown line Mut123 strain grown in standard BG11 medium. Labels 1st d and 5th d represent the first and fifth days of growth, respectively. (**c**) PCR result of a putative triple mutant Mut123 obtained from mixotrophic growth conditions. (**d**) Photographs of the wild-type and the putative triple mutant on the fifth day of growth in photoautotrophic and mixotrophic growth conditions. (**e**) Photographs of the transformants of triple mutants grown in standard BG11 medium for 5 days. The triple mutants (left to right) were obtained by knocking out *exbBD1* in Mut23, *exbBD2* in Mut13 or *exbBD3* in Mut12, respectively.

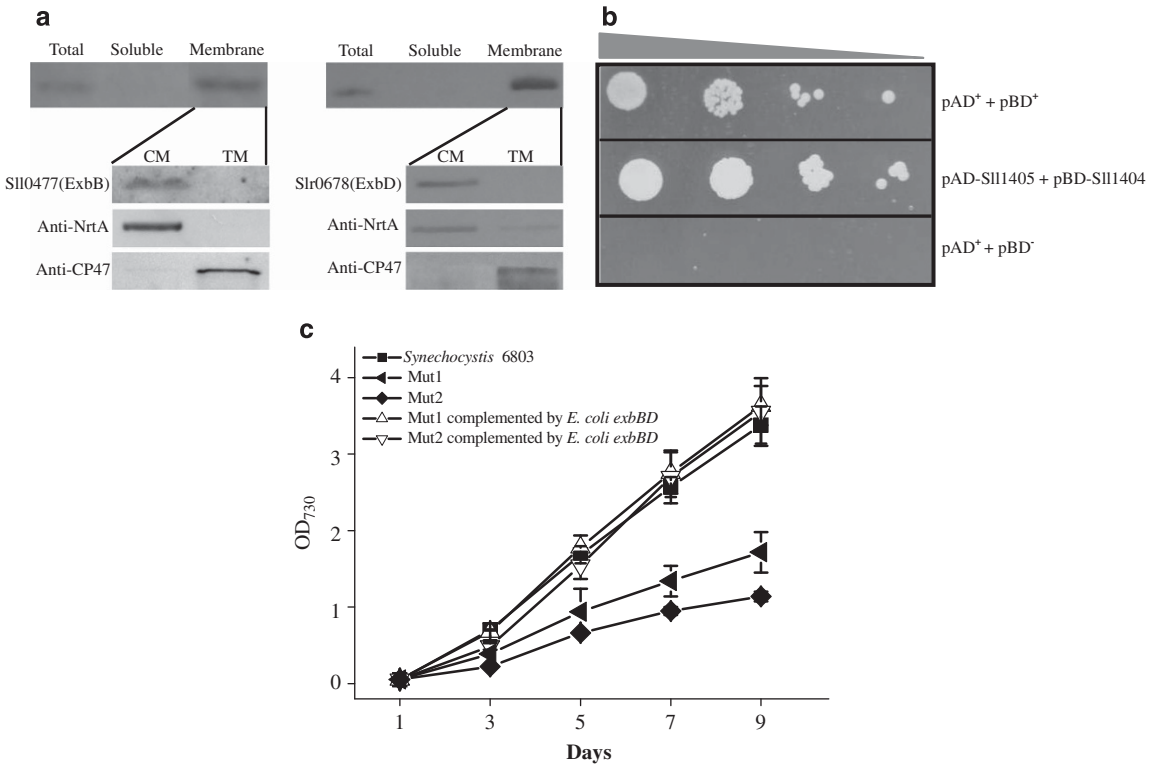

Figure 3 Localization and protein–protein interactions of ExbB and ExbD in *Synechocystis* 6803 and their functional comparison with *Escherichia coli* homologs. (**a**) Localization of ExbB and ExbD in *Synechocystis* 6803. NrtA and CP47 were selected as cytoplasmic membrane (CM) and thylakoid membrane (TM) marker proteins, respectively. (**b**) Protein–protein interaction analysis of ExbB and ExbD in *Synechocystis* 6803. The yeast transformants (20 μl each of cell suspensions diluted from optical density at 600 nm (OD_{600}) 0.1 to 0.0001) expressing positive control plasmids ($pAD^+ + pBD^+$), negative control plasmids ($pAD^+ + pBD^-$) and detected plasmids (pAD-Sll1405 + pBD-Sll1404) were grown on SD/-Trp-Leu-His-Ade agar plate for 3 days. (**c**) The growth curves of *Synechocystis* wild-type, Mut1, Mut2, Mut1 complemented by *E. coli exbBD* and Mut2 complemented by *E. coli exbBD* cultured under iron-deficient conditions. A full color version of this figure is available at *The ISME Journal* online.

with increasing [Fe′] in both the wild type and Mut13 but was suppressed in the mutants, being 67–75% that of the wild type under all conditions. Iron uptake rate was independent of FeEDTA concentration, confirming that this substrate is not transported (Figure 4d). These results strongly suggest that iron uptake by *Synechocystis* 6803 depends on [Fe′] rather than on total iron concentration and that ExbB-ExbD complexes are involved in Fe′ transport.

Iron uptake rates were also measured at much higher Fe′ concentrations: 1 μM $FeCl_3$ buffered with 1.6 μM EDTA (12.8 nM [Fe′]); and 0.1 μM $FeCl_3$ without EDTA (99.9 nM [Fe′]). Under these conditions, iron speciation was less well controlled than at high EDTA levels, but the transport rates showed the same pattern of dependence on ExbB-ExbD (Figure 4e). At the highest [Fe′] tested, the uptake rate by the mutant was reduced to 11% that of the wild type (see the points marked with an asterisk '*' in Figure 4e).

The distribution of ExbB-ExbD complexes shows their functional universality in diverse cyanobacterial species

Putative homologs of ExbB-ExbD proteins are found in almost all the cyanobacterial species whose genomes have been fully sequenced. The exceptions are some *Prochlorococcus marinus* and marine *Synechococcus* strains (Cyanobase, http://genome.kazusa.or.jp/cyanobase) (Table 2). The ExbB proteins show high amino-acid sequence similarity with the known ExbB protein Slr1404 (E-value $< 2 \times e^{-8}$). The genes encoding ExbB and ExbD are closely located in the genomes of diverse cyanobacteria and range in copy number from 1 to 5 (Table 2). To determine whether cyanobacterial *exbB-exbD* genes perform the same function as in *Synechocystis* 6803, we complemented the mutant strains with homologs from a filamentous, multi-cellular, heterocyst-forming strain *Anabaena* sp. PCC 7120 and a unicellular marine strain *Synechococcus* sp. PCC 7002. Growth bioassays show that both of the ExbB-ExbD homologs rescued the iron-deficient phenotype of *Synechocystis* 6803 ExbB-ExbD mutant (Mut1) (Supplementary Figure S1C). Thus ExbB-ExbD protein complexes likely perform a similar function in Fe′ uptake of most cyanobacteria.

Discussion

The widespread distribution of ExbB-ExbD proteins among cyanobacteria and their functional

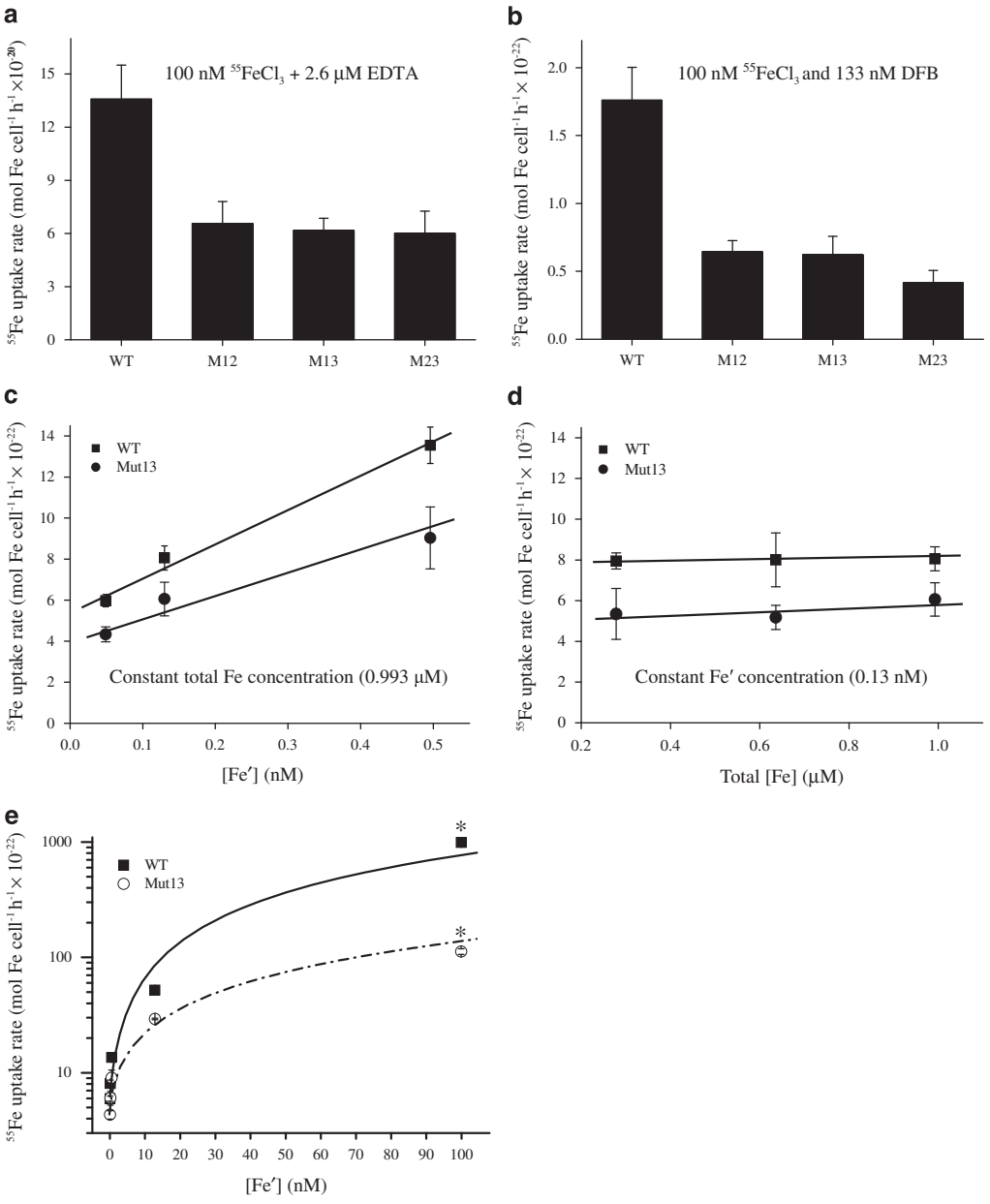

Figure 4 ^{55}Iron uptake rate of the *Synechocystis* 6803 *exbBD* double-mutant strains cultured in standard BG11 medium. (**a**, **b**) Cells were grown in BG11 medium to logarithmic phase, harvested, washed and re-suspended in iron-free BG11 medium lacking citric acid and ferric ammonium citrate. For free iron uptake rate measurement, $^{55}FeCl_3$ was added to the cell solutions at 100 nM (3.7 kBq ml^{-1}) complexed with 2.6 μM EDTA (**a**). For measurement of iron-siderophores uptake rate, 100 nM $^{55}FeCl_3$ and 133 nM DFB were added (**b**). (**c**, **d**) Cells were grown in BG11 medium to logarithmic phase, and iron uptake experiments were conducted using the trace metal clean technique. The ^{55}iron was precomplexed with EDTA over 24 h, as described in Kranzler *et al.* (2011). (**c**) Uptake rates of *Synechocystis* wild-type and Mut13 supplied with the same total iron concentration (0.993 μM) but varying free Fe′ concentrations. (**d**) Uptake rates of cells supplied with the same free Fe′ concentrations (0.13 nM) but different total iron concentration. (**e**) Uptake rates of *Synechocystis* wild-type and Mut13 supplied with different iron concentrations. Note the two additional measurements. The data marked with asterisk '*' were obtained using the same experimental procedure as the data of panels (**c**) and (**d**), but the medium lacked EDTA and all organic compounds.

complementarity suggests that inorganic iron maybe an important iron source for growth of many species. All the freshwater cyanobacterial strains whose genomes have been fully sequenced and many ecologically relevant marine cyanobacteria have this active transport system. For example, the coastal strain *Synechococcus* sp. PCC 7002 has two putative *exbB-exbD* gene clusters as does *Trichodesmium erythraeum* IMS101, an important nitrogen fixer. *Prochlorococcus marinus* MIT9202, isolated from low-iron waters near the equatorial Pacific Ocean, also has an ExbB-ExbD active transport system possibly reflecting the importance of inorganic iron in this habitat. Interestingly, most open ocean *Synechococcus* and *Prochlorococcus* strains do not have ExbB-ExbD homologs and so may rely on different mechanisms for inorganic iron

Table 2 Putative *exbB* and *exbD* genes in selected cyanobacterial species/strains

Species/strains	*Number of genes*		*Accession number*		*E values*	
	exbB	*exbD*	*exbB*	*exbD*	*exbB*	*exbD*
Freshwater cyanobacteria						
Anabaena sp. PCC 7120	3	2	all5047	all5046	4e–65	4e–27
			alr0643	alr0644	6e–33	6e–13
			alr4587	—	1e–60	—
Anabaena variabilis ATCC 29413	3	2	Ava_2306	Ava_2305	3e–65	5e–27
			Ava_4574	Ava_4575	3e–33	7e–13
			Ava_2465	—	1e–61	—
Arthrospira platensis NIES-39	1	1	NIES39_E01480	NIES39_E01470	2e–34	2e–17
Chlorobium tepidum TLS	2	2	CT0633	CT0634	1e–09	2e–05
			CT1586	CT1584	8e–14	7e–04
Cyanothece sp. PCC 8801	2	2	PCC8801_3017	PCC8801_3016	7e–77	3e–33
			PCC8801_3262	PCC8801_3261	7e–36	8e–16
Cyanothece sp. PCC 7424	2	2	PCC7424_2391	PCC7424_2392	3e–78	9e–35
			PCC7424_2011	PCC7424_2012	9e–34	6e–16
Cyanothece sp. PCC 7425	2	2	Cyan7425_2418	Cyan7425_2417	2e–67	1e–33
			Cyan7425_0843	Cyan7425_0844	4e–35	1e–16
Gloeobacter violaceus PCC 7421	3	3	glr2402	glr2403	1e–41	8e–18
			glr1387	glr1388	1e–41	1e–21
			gll1141	gll1140	4e–13	6e–11
Microcystis aeruginosa NIES-843	2	1	MAE08540	MAE08550	8e–36	5e–16
			MAE43740	—	7e–16	—
Nostoc punctiforme ATCC 29133	3	2	Npun_R0782	Npun_R0781	4e–65	5e–28
			Npun_R5174	Npun_R5173	2e–31	1e–13
			Npun_R4966	—	1e–63	—
Synechococcus elongates PCC 6301	1	1	syc_1677_d	syc_1678_d	1e–33	9e–37
Synechococcus elongates PCC 7942	1	1	Synpcc7942_2429	Synpcc7942_2428	1e–33	1e–16
Synechococcus sp. JA-3-3Ab	1	1	CYA_2815	CYA_2816	6e–54	2e–26
Synechococcus sp. JA -2-3B′a (2-13)	1	1	CYB_0819	CYB_0820	3e–53	2e–25
Thermosynechococcus elongatus BP-1	1	1	tll0316	tlr0055	5e–32	2e–19
Marine cyanobacteria						
Acaryochloris marina MBIC11017	5	6	AM1_B0122	AM1_B0121	5e–60	1e–29
			AM1_3411	AM1_3410	9e–60	3e–31
			AM1_0171	AM1_1070	9e–33	7e–10
			AM1_4903	AM1_4902	2e–29	8e–15
			AM1_A0165	AM1_A0164	1e–59	2e–28
			—	AM1_0169	—	3e–09
Cyanothece sp. ATCC 51142	3	3	cce_3054	cce_3053	5e–79	2e–34
			cce_1116	cce_1115	6e–36	4e–15
			cce_1159	cce_1160	7e–32	4e–11
Prochlorococcus marinus MIT9202	1	1	P9202_71 (gb: EEE39300.1)	Unnamed (gb: EEE41044.1)	3e-27	1e-15
Synechococcus sp. PCC 7002	2	2	SYNPCC7002_G0137	SYNPCC7002_G0136	6e–70	2e–32
			SYNPCC7002_A1318	SYNPCC7002_A1317	4e–29	2e–14
Synechococcus sp. RCC307	1	—	SynRCC307_1279	—	2e–14	—
Synechococcus sp. WH7803	1	—	SynWH7803_0707	—	2e–09	—
Trichodesmium erythraeum IMS101	1	1	Tery_4448	Tery_4449	6e–35	1e–17

uptake or on different chemical species of iron for nutrition. We hypothesize that the use of an ExbB-ExbD system for inorganic iron uptake may allow cyanobacteria to more tightly maintain iron homeostasis, particularly in variable environments where iron concentrations may range from limiting to sufficient. A regulated OM transporter may allow cyanobacteria to buffer fluctuating levels of iron in their environment and to more efficiently capture low levels of iron when it is limiting. Areas of the ocean experiencing upwelling, dust input and freshwater runoff as well as the epilimnia of lakes exposed to iron-rich hypolimnetic waters may be habitats where inorganic iron is variable and where such an inorganic iron transport system may be adaptive.

In this study, we experimentally identified CM-localized ExbB-ExbD complexes in the non-siderophore-producing cyanobacterium, *Synechocystis* 6803, and provided direct evidence that cyanobacteria sequester iron through the OM via an ExbB-ExbD-dependent transport system. In bacteria Ton transport systems, the ExbB-ExbD membrane protein is not a transporter, but a molecular complex that harvests energy from the ion electrochemical potential generated across the CM and transmits it to the TonB subunit to open a channel in the OM transporter. Our results suggest that ExbB-ExbD complexes from *Synechocystis* 6803 operate like those in bacteria, because *E. coli* homologs restore the *Synechocystis* mutants. Low sequence similarity

between putative TonBs and OM transporters of *Synechocystis* and well-characterized models in Gram-negative bacteria have hampered our attempts to describe all the interacting protein partners of ExbB-ExbD complexes in the cyanobacteria. A mutant of the putative TonB protein, Slr1484, only showed moderate decline in iron uptake rate compared with the wild type (data not shown), suggesting that other unknown TonB proteins may exist in *Synechocystis* 6803. Identifying the OM transporters may be equally elusive, judging from the work of Katoh *et al.* (2001) who knocked out four putative OM candidates in the same cyanobacterial species but did not find an obvious phenotype compared with the wild type. Regardless of the complexity of TonB proteins and OM transporters, the interesting finding in the present study is that ExbB-ExbD complexes are essentially required by *Synechocystis* 6803 for Fe′ uptake. Our results also show that this ExbB-ExbD-mediated iron uptake strategy is probably prevalent among most cyanobacteria species. The Ton transport system has been investigated for over 30 years in non-photosynthetic bacteria, and it is generally thought to be involved in transport of organic compounds such as Fe chelators, as the compounds are too large to pass through porins (Noinaj *et al.*, 2010). For the first time, we have shown that the ExbB-ExbD-dependent transport system has an essential role in inorganic iron uptake in cyanobacteria.

ExbB-ExbD complexes may be more essential for iron uptake in the photosynthetic cyanobacterium *Synechocystis* 6803 than in non-photosynthetic Gram-negative bacteria. In *E. coli*, for example, the mutant RA1051 (*ΔexbBD::kan ΔtolQR*), lacks the single ExbB-ExbD complex as well as another active transport system, TolQ-TolR, but nonetheless grows well in Luria–Bertani broth medium and only shows growth inhibition under low iron bioavailability (Brinkman and Larsen, 2008). *Synechocystis* 6803, on the other hand, has three essential *exbB-exbD* gene clusters that are functionally redundant and are regulated in a compensatory manne when one of the gene clusters is inactivated (Supplementary Figure S2E). Two factors may explain why ExbB-ExbD is essential in cyanobacteria but not in other Gram-negative bacteria. First, iron demand is much higher in photosynthetic cyanobacteria than in non-photosynthetic bacteria because of its use in the photosynthetic electron transport chain. Indeed, estimates are that the cyanobacteria require 10-fold more iron than bacteria of similar cell size to maintain photosynthetic activity (Raven *et al.*, 1999; Shcolnick *et al.*, 2009). Without TonB-ExbB-ExbD-dependent active uptake pathway, cyanobacterial cells might rely on passive transport of iron through porins, which may provide insufficient iron for photosynthesis. Mutant cells survived in glucose, albeit with reduced growth, indicating a relatively lower iron requirement under mixotrophic culture conditions (Figure 2d). Second, the substrate preference of the TonB-ExbB-ExbD system differs between cyanobacteria and non-photosynthetic bacteria. In *E. coli*, vitamin B_{12}, nickel chelates and carbohydrates are also taken up by this active transport system as well as iron, and colicins and phages exploit the same transport system to gain entry to the cells (Miethke and Marahiel, 2007; Schauer *et al.*, 2008). As autotrophic organisms, cyanobacteria require much more iron but few organic compounds for growth. The recipe of standard cyanobacterial BG11 medium usually excludes or contains very low concentration of cobalt, nickel and carbohydrates (Allen and Stanier, 1968; Stanier *et al.*, 1971). In summary, cyanobacteria require ExbB-ExbD complexes for Fe′ transport for three reasons: they have a high iron demand for photosynthesis, they inhabit low-iron environments, and they have high substrate specificity for iron.

Several reports have suggested that cyanobacteria can extract free iron with high efficiency if free iron is available (Fujii *et al.*, 2010, 2011; Kranzler *et al.*, 2011), but until now, direct evidence that Fe′ uptake by cyanobacteria requires TonB-ExbB-ExbD-dependent transport system has been lacking. Although Fe-siderophores can be slowly sequestered by *Synechocystis* 6803 (Figure 4b), the role of siderophores in iron acquisition by cyanobacteria has probably been overrated in natural water systems (Hopkinson and Morel, 2009; Wirtz *et al.*, 2010). As many cyanobacterial species other than *Synechocystis* 6803 do not excrete siderophores, the importance of siderophore-mediated iron uptake in cyanobacteria has been questioned. Hopkinson and Morel (2009) and Kranzler *et al.* (2011, 2014) suggested that cyanobacteria probably use uptake pathways involving a reduction step, rather than direct internalization of siderophores and other iron chelates. We hypothesize that organic chelators such as siderophores may provide iron pools, which maintain iron solubility and recruit iron in dilute water environments, while cyanobacteria prefer free iron and efficiently sequester it with high rate via TonB-ExbB-ExbD-dependent active transport system (see a hypothesized model shown in Figure 5). Thermal dissolution and photochemical reduction of siderophore-bound iron may provide a source of free Fe′ for uptake. Although both siderophore-mediated and reductive iron uptake pathway have been reported in cyanobacteria (Kranzler *et al.*, 2012; Stevanovic *et al.*, 2012), the latter seems to be the dominant pathway. OM transport of both siderophore-Fe (despite its low rate) and Fe′ are under the control of the TonB-ExbB-ExbD-dependent transport system (Figures 4a and b). Fe′ may be able to enter the cells by passive transport via OM porins at a very low rate, but the majority would be absorbed by energy-coupled active transport. As it crosses the OM, Fe′ maybe reduced in the periplasmic space before transport across the cyanobacterial plasma membrane (Kranzler *et al.*, 2011, 2014). The findings in the present study provide new

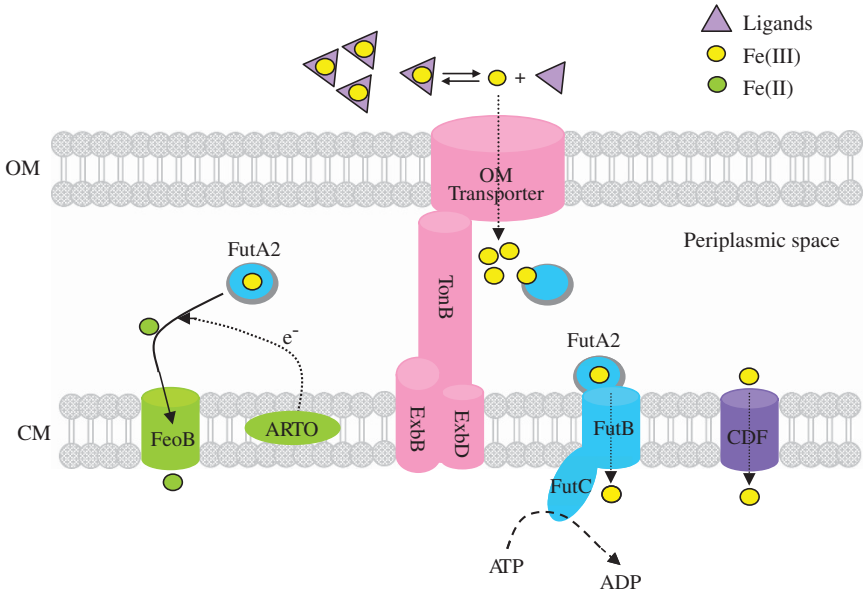

Figure 5 Iron uptake pathway model for *Synechocystis* sp. PCC 6803. In oxygenic aquatic environments, iron exists primarily in organic complexes that increase the solubility of dissolved iron and buffer an extraordinarily low concentration of Fe′. *Synechocystis* cells efficiently sequester Fe(III) that in equilibrium with the organically complexed Fe pool through active transport via TonB-ExbB-ExbD-dependent transport system. Outer membrane (OM) transport of siderophore-Fe is also under control with the TonB-ExbB-ExbD-dependent transport system despite its low rate. Once Fe(III) crosses the OM, it is probably bound to periplasmic protein FutA2 and then reduced to Fe(II) by a reductive iron uptake pathway (Kranzler *et al.*, 2014). Immediately, Fe(II) could be transported into CM by CM-located FeoB transporter. As the half-life of Fe(II) is very short, Fe(III) will be transported into CM by an ATP-binding cassette transporter FutB and FutC. The cation diffusion facilitator protein (CDF) will improve the Fe(III) transport across CM when cells were under iron deficiency (Jiang *et al.*, 2012).

insights into the iron acquisition pathway of photosynthetic cyanobacteria in aqueous environments. The presence of unique OM receptors, TonBs and the mechanism of the TonB-ExbB-ExbD dependent Fe′ uptake system in cyanobacteria are currently under investigation in our lab.

Conflict of Interest

The authors declare no conflict of interest.

Acknowledgements

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