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Iron transport systems in the Cyanobacterium *Synechocystis sp*. strain PCC 6803

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Introduction

Iron serves as an essential component of heme and iron sulfur centers integrated into a variety of proteins that function in basic physiological processes such as photosynthesis, respiration, and nitrogen metabolism (Straus, 1994). Molecular analysis of iron transport systems has been carried out mostly on nonphotosynthetic bacteria. However, little is known about the molecular mechanism of iron transport in photoautotrophic organisms. We have recently identified *fut* genes (*futA1*, *futA2*, *futB*, *futC*) and *feoB* gene essential to ferric and ferrous iron transport, respectively, in the cyanobacterium *Synechocystis* sp. strain PCC 6803 (Katoh, et al., 2000, Katoh, et al., 2001a). In this report we show the properties of Fut and Feo systems in iron transport and ferric iron-binding activity of recombinant FutA1 protein.

Materials and Methods

Cells of wild-type (WT) and mutants (Δ*futA1*/Δ*futA2*, Δ*futB*, Δ*futC*, Δ*futB*/Δ*futC*, Δ*futA1*, Δ*futA2*, and Δ*feoB*) of *Synechocystis* PCC 6803 were grown at 30 ˚C in BG-11 medium (Stanier, et al., 1971) or iron-free BG-11 medium (Katoh, et al., 2000) buffered at pH 8.0 during aeration with 3 % CO_2 (vol/vol) in air. Continuous illumination was provided at 60 μmol photosynthetically active radiation/m^2s (400-700 nm).

Growth characteristics of wild-type and mutants cells on iron-free BG-11 medium (pH 8.0) were determined (Katoh, et al., 2001a). Iron-deprived wild-type and mutants cells were collected by centrifugation and resuspended in fresh iron-free BG-11 medium to optical densities at 730 nm (OD_{730}) of 0.1, 0.01, and 0.001. The OD_{730} of the cell culture was determined using a recording spectrophotometer (model UV2200; Shimadzu Co., Kyoto, Japan). Two μl each of the cell suspensions was spotted onto iron-free BG-11 agar-plates. The plates were incubated in 3 % (vol/vol) CO_2 in air for 7 days with continuous illumination by fluorescent lamps providing photosynthetically active radiation at 60 μmol of photons/m^2s.

Uptake of iron by wild-type and mutants cells was measured by using the radioactive tracer $^{59}FeCl_3$ (Amersham Pharmacia Biotech) as described previously (Katoh, et al., 2000, Katoh, et al., 2001a). The uncoupler,

carbonylcyanide-p-trifluoro-methoxyphenilhydrazone (FCCP), the ATP synthase inhibitor, dicyclohexyl-carbodiimide (DCCD) or the respiration inhibitor, KCN, were added 30 min before the labelled substrate to final concentrations of 10 μM, 100 μM and 40 mM, respectively. For metal competition studies, stock solution of Zn^{2+}, Mn^{2+}, Cu^{2+}, Co^{2+}, Ni^{2+}, Cd^{2+}, and Cr^{3+} chloride salts were diluted with the assay medium to give a final concentration of 1 μM, 10 μM, and 100 μM, respectively. The metal salts were added immediately before the addition of ^{59}Fe to the incubation medium.

The recombinant FutA1 (rFutA1) protein was expressed in *Escherichia coli* as a GST-tagged protein that lacks predicted signal sequence region of FutA1. rFutA1 was isolated from the cells using GST purification Kit (Amersham Pharmacia Biotech). Solution containing purified rFutA1 was acidified by adding 0.1 volume of 0.1 % acetic acid, and iron was chelated by adding 2,000-fold molar excess of sodium citrate (pH 8.0) over rFutA1. Excess citrate and iron-citrate were removed by using an Econo-10DG microbiospin column (Bio-Rad) and fractions containing deferrated rFutA1 were collected in acid-washed tubes. Ferric chloride was added stepwise to solutions containing 4.2 mg/ml (70 μM) of deferrated rFutA1, and the mixture was incubated at room temperature for 15 min. The amount of Fe^{3+}-rFutA1 in the solution was estimated from the absorbance at 453 nm. A453 nm was measured by using a UV/VIS spectrophotometer V-550 (Jasco, Tokyo, Japan).

Results and Discussion

Growth characteristics of wild-type and mutants cells.

Figure 1 shows the growth characteristics of wild-type, *fut*, and *feo* mutants

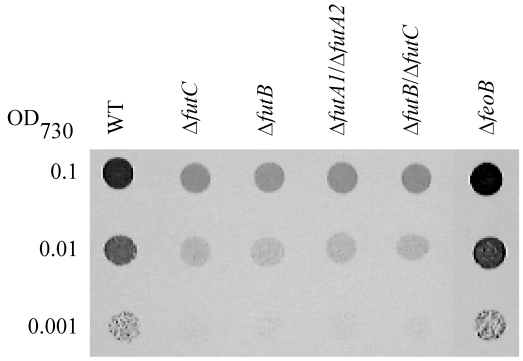


Figure 1. Growth of the wild-type and mutants on solid iron-free BG-11 medium. Cell suspensions of wild-type and mutants cells were spotted on agar plates containing iron-free BG-11 buffered at pH 8.0, and the plates were incubated under 3 % (vol/vol) CO_2 in air for 7 days.

cells on iron-free BG-11 agar-plate. WT and Δ*feoB* cells actively grew because these two strains could utilize very low level of iron in the plate. However, Δ*futC* and Δ*futB* cells grow very slowly on iron-free BG-11 plate. Δ*futA1*/Δ*futA2* double mutant showed

similar growth characteristics as that of Δ*futC* and Δ*futB*, although Δ*futA1* and Δ*futA2* single mutants cells actively grew like the wild-type (data not shown). Additional inactivation of *futB* gene in the Δ*futC* mutants did not affect the growth characteristics at all. These results suggest that four *fut* genes are involved in iron transport of *Synechocystis*. Furthermore, *futB* and *futC* genes are essential and *futA1* and *futA2* genes play a redundant role for iron utilization.

Ferric and ferrous iron uptake by the WT, fut and feo mutants.

Figure 2A shows uptake of Fe^{3+} by wild-type (circles) and *ΔfutC* (triangles) mutant cells grown in nutrient-replete medium (open symbols) or in iron-free medium (solid symbols). Fe^{3+} uptake by the *ΔfutC* strain was very low in both types of cells. Other *fut* mutants (Δ*futA1*/Δ*futA2*, Δ*futB*, Δ*futC*, Δ*futB*/Δ*futC*) showed the same phenotype as *ΔfutC* (data not shown). Δ*feoB* cells took up ferric iron as much as wild type. The activity of Fe^{3+} uptake by wild-type cells grown in nutrient-replete medium was about five times that in the *ΔfutC* mutant. Fe^{3+} transport activity increased more than threefold in wild-type cells after iron deprivation. These results suggest that four *fut* genes encode subunits of a single ferric iron transporter and that the Fut transport system are expressed both in nutrient-replete and iron-deficient conditions.

Figure 2B shows uptake of Fe^{2+} by wild-type (circles) and Δ*feoB* (triangles) mutant cells grown in nutrient-replete (open symbols) or iron-free (solid symbols) medium. The activity of Fe^{2+} uptake was very low in the *ΔfeoB* cells grown in both types of medium and in the wild-type cells grown in nutrient-replete medium. The

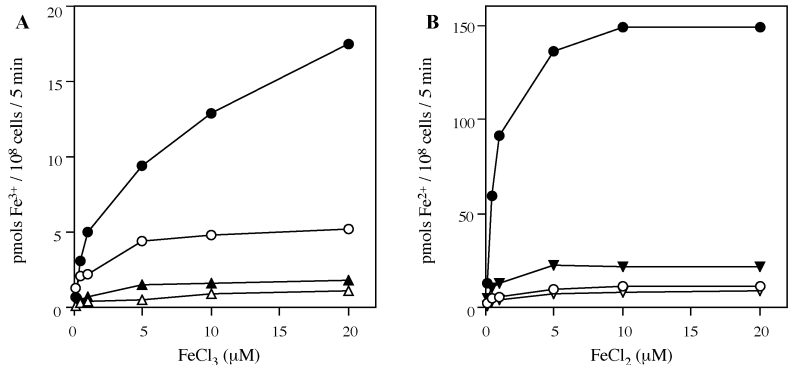


Figure 2. Uptake of $^{59}Fe^{3+}$ or $^{59}Fe^{2+}$ by wild-type and *ΔfutC* (A) or wild-type and *ΔfeoB* (B) mutant cells grown in complete medium or in iron-free medium. Cells were incubated for 5 min in the light with various concentrations of $FeCl_3$ in the presence of 1 mM ferrozine (A) or 5 mM ascorbate (B).

activity increased more than tenfold in the wild-type cells after iron deprivation. These results demonstrate that *feoB* gene encode a ferrous iron transporter and is induced under iron deprived conditions. The *fut* mutants cells showed similar characteristics for

ferrous iron uptake as wild-type cells.

Energization and substrate specificity.

In the wild-type cells uptake of Fe^{3+} and Fe^{2+} was inhibited by FCCP, a protonophore, and by DCCD, an inhibitor of ATP synthesis (Table 1). These results are consistent with ATP hydrolysis being the driving force for ferric and ferrous iron transport. The activities of Fe^{3+} and Fe^{2+} uptake in the dark were about 90% of those in the light and KCN strongly inhibited the uptake (Table 1). Thus, respiration and other dark metabolic reactions supply ATP sufficient to energize the iron transport.

Table 1. Effects of metabolic inhibitors on ferric and ferrous iron uptake.

Addition	Fe^{3+} uptake (%)	Fe^{2+} uptake (%)
None (light)	100	100
None (dark)	92.6	87.5
FCCP (10 μM)	61.6	41.1
DCCD (100 μM)	56.2	35.5
KCN (40 mM)	7.5	0.4

. The specificity of the Fut and Feo transporters to their substrates was investigated by examining the ability of cations, such as Zn^{2+}, Mn^{2+}, Cu^{2+}, Co^{2+}, Ni^{2+}, Cd^{2+} and Cr^{3+}, to inhibit ^{59}Fe accumulation in the wild-type. The metal salts were added at a concentration of 100 μM, and the concentration of $^{59}FeCl_3$ was 1 μM (Figure 3. A, B). Mn^{2+}, Ni^{2+}, and Cr^{3+} had no significant effect on the uptake of Fe^{3+} and Fe^{2+}. Zn^{2+} inhibited Fe^{3+} uptake by 77 % and Cu^{2+} inhibited Fe^{2+} uptake by 96 %. Co^{2+} and Cd^{2+} inhibited both transport systems to a similar degree, which appeared to be toxicity effect. No significant inhibition was observed by all metal ions tested at the

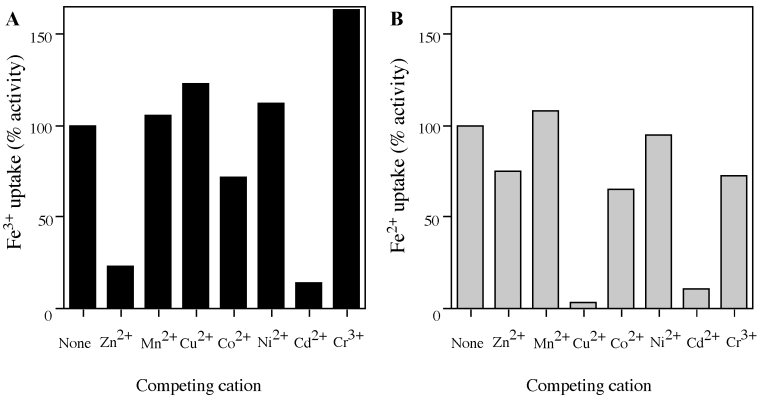


Figure 3. Effect of transition metals on Fe^{3+} (A) or Fe^{2+} (B) uptake. The activities are shown in percentages of the control.

concentrations of 1 μM and 10 μM (data not shown). Thus, the specificity of the Fut and Feo systems for their substrates appears to be very high.

Iron-binding activities of rFutA1

The absorption spectrum of the solution containing purified rFutA1 and ferric chloride shows the broad band with a peak at 453 nm (data not shown, Katoh, et al., 2001b). The absorbance at this wavelength rose linearly as the amount of iron bound to rFutA1 increased to reach a plateau when the molar ratio of iron to rFutA1 became unity (Figure 4). This indicates that absorbance at 453 nm is an accurate measure of the ferration state of this protein and that 1 molecule of rFutA1 is capable of incorporating 1 molecule of ferric ion. A similar absorption band has been observed with the ferric iron-binding protein HitA of *Haemophilus influenzae* and attribute to the absorption by the coordinated Fe^{3+} atom (Adhikari, et al., 1995).

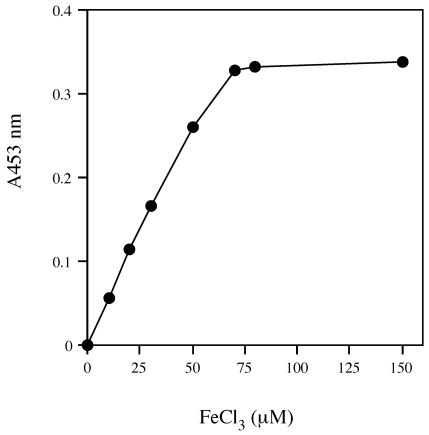
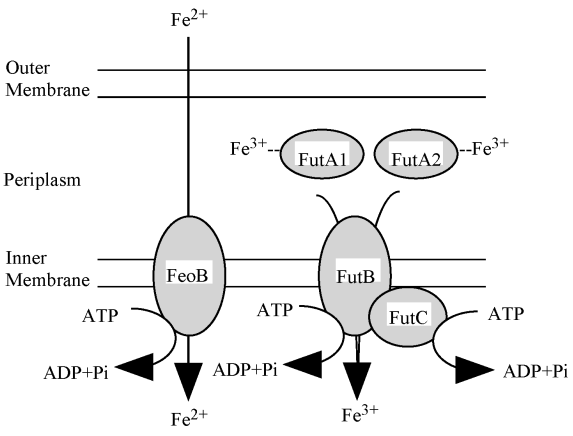


Figure 4. Absorbance at 453 nm as a function of concentration of $FeCl_3$ added to the solution containing rFutA1 (70 μM).

A model for iron uptake in Synechocystis.

FutA1 and FutA2 have a redundant role for ferric iron transport and are homologous to the periplasmic iron-binding protein HitA. Purified rFutA1 expressed in *E. coli* binds ferric ion without any iron-chelator. These observations support the view that *futA1* and *futA2* encode periplasmic iron binding proteins of the Fut transporter (Figure 5). Deduced amino-acid sequences of *futB* and *futC* implie that these genes encode membrane-bound or membrane-associated ATP-binding subunits of the Fut transporter (Figure 5). All the *fut* mutants exhibited the same characteristics of ferric iron uptake and all the *fut* genes showed the same expression properties (data not shown). In *Anabaena* PCC7120, an N_2-fixing cyanobacterial strain, the homologues for *fut* genes constitute operon structure, although *fut* genes are located away from one another in the genome of *Synechocystis* 6803. These results support the view that four *fut* genes encode subunits of an ABC-type single ferric iron transporter. This is the first finding of the presence of high affinity Hit-type iron acquisition system in photosynthetic bacteria.

Figure 5. A model for iron uptake in *Synechocystis*.



Deduced amino-acid sequence of *feoB* contains ATP-binding motif and *feoB* in *Synechocystis* 6803 is homologous to *feoB* in *E. coli*, which encodes an

ATP-binding subunit of the ferrous iron transporter (Kammler, et al., 1993). Thus, *feoB* in *Synechocystis* 6803 also encode a subunit of ferrous iron transporter.

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