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Regulation of iron-siderophore uptake in a diazotrophic cyanobacterium *Anabaena anabaena 7120*

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ABSTRACT

The regulation of ^{55}Fe-siderophore uptake of was studied in a diazotrophic cyanobacterium *Anabaena 7120*. The uptake up to 20 min was found to be biphasic, a rapid first and steady second phase. A single kinetic system mediated the uptake of iron-siderophore with an apparent K_m of 200 µM and a V_{max} of 20 nmol iron taken up mg^{-1} protein min^{-1}. 3-(3,4-dichlorophenyl)-1,1-dimethyl urea (DCMU), 2-n-heptyl-4-hydroxy quinoline (HOQNO), dicyclohexyl carbodiimide (DCCD), carbonyl cyanide p-fluoromethoxy phenyl hydrazone (FCCP) and carbonyl cyanide-m-chlorophenyl hydrazone (CCCP) inhibited the iron-siderophore uptake significantly, whereas KCN inhibited marginally. No iron-siderophore uptake was observed under dark-aerobic and dark-anaerobic conditions. The siderophore schizokinen supported the maximum iron-siderophore uptake. However, the siderophore aerobactin and desferal could support only 53 and 3.4% iron-siderophore uptake, respectively as compared to its schizokinen counterpart.

Key words : *Anabaena 7120,* Cyanobacterium, , Iron- siderophore uptake

Iron acts as a cofactor for many enzymes and is often crucial for the catabolic activity. Iron deficiency in cyanobacteria impairs several biochemical pathways (Saxena *et al.* 2006, Raghuvanshi *et al.* 2007a, Xing *et al.* 2007). The most notable is the effect of iron deficiency on expression of Fur (Katoh *et al.* 2001, Martin-Luna *et al.* 2006), IsiAB and IdiA proteins which take part in rendering protection to PS II and PS I from oxidative damage (Michel and Pistorius 2004). The uptake of iron has long been recognized as a central problem in microbial nutrition. Under laboratory conditions, iron availability is ensured by synthetic chelators such as EDTA. Many microorganisms produce high affinity ferric-ion transport compounds collectively known as siderophores (Crumbliss 1991). A number of cyanobacteria also release extracellular ferric chelating agents (siderophores) during conditions of low iron availability (Wilhelm *et al.* 1996, Raghuvanshi *et al.* 2007b, Gress *et al.* 2004).

Cyanobacterial siderophores are thought to function as a part of high-affinity iron acquisition systems (Kerry *et al.* 1988). Although many cyanobacteria are capable of producing siderophores, some strains do not produce siderophores and therefore depend on siderophores produced by bacteria or other cyanobacteria to continue growth in iron deficient environments. Since, nitrogenase, the key enzyme responsible for N_2-fixation in diazotrophic cyanobacteria contains up to 36 iron atoms per enzyme complex (Averil and Orme-Johnson 1978), a better understanding of iron uptake and its metabolism attains importance in enhancing their contribution to soil fertility.

Whereas, a considerable amount of research work has been done on iron acquisition and its uptake in bacteria and other microorganisms (Bailey and Taub 1980, Braun *et al.* 1998, Ratledge and Dover 2000, Stinzi *et al.* 2000, Budzikiewicz 2004), little attention has, however, been paid on the mechanisms of iron uptake in cyanobacteria (Boyer *et al.* 1987, Katoh *et al.* 2001). Particularly the regulation of iron uptake in cyanobacteria has not yet been fully understood. Using ^{55}Fe-siderophore, we have deciphered the iron-siderophore uptake and its regulation in a diazotrophic cyanobacterium *Anabaena 7120.*

MATERIALS AND METHODS

Experiment were conducted in triplicate and analysis of varience was performed

Axenic culture of *Anabaena 7120*, obtained from the National Center for Conservation and Utilization of Blue-Green Algae, Indian Agricultural Research Institute, New Delhi, was grown in BG-11_0 medium (Rippka *et al.* 1979)

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without citrate, EDTA and iron. Iron contamination was further minimized by passing the medium through a Chelex 100 column (Biorad) and filtered through an acid-washed polycorbonate filter (nucleopore) with pore size of 0.4 mm. The medium was buffered to *p*H 7.5 with 10 mM 4-(2-hydroxyethyl)-1-peperazine ethane sulphonic acid (HEPES)/ NaOH. The cultures were routinely grown in a culture room at 25°C and illuminated with day-light fluorescent tubes having the photon flounce rate of 50 µmol/m^2/s on the surface of the vessels with a 16:10 light-dark rhythm.

Exponentially growing (7 days old) cultures of *A. 7120* were harvested by centrifugation, washed and resuspended in 10 mM HEPES/NaOH buffer (*p*H 7.0) to a final cell density of 400 µg protein/ml. The cells were then equilibrated for 30 min at 25°C and at 50 µmol/m^2/s photon fluence rate prior to the addition of [^{55}Fe] ferric siderophore. Solutions of [^{55}Fe] ferric siderophore were prepared by mixing $^{55}FeCl_3$ in 0.1 N HCl with two fold molar excess of appropriate siderophore dissolved in uptake buffer. The *p*H was adjusted to 7.0. The two fold molar excess of siderophore to iron was utilized to ensure that all the added iron was coordinated.

Assays were initiated by the addition of [^{55}Fe] ferric schizokinen (50, 100, 150, 200, 250 and 300 µM) and the cells were incubated at 25°C and 50 µmol/m^2/s photon fluence rate unless otherwise stated. Aliquots of the samples (400 µl) were withdrawn at regular time intervals ranging from 10-60 min. Cells were separated by microcentrifugation through silicon oil/dinonylphthalate (45:55, v/v) into perchloric acid/ water (15:85, v/v) as described by Scott and Nicholls (1980). Samples of the perchloric acid fractions were pooled and the incorporation of iron was determined using a Liquid Scintillation Counter (Wallac 1409, Finland). The linear portions of the curves were used to calculate the rate of iron-siderophore uptake.

The nonspecific adsorption of iron was measured by following [^{55}Fe] ferric siderophore incorporation in toluene-treated cells. The cells were harvested, resuspended in 1% (v/v) toluene in 10 mM HEPES/NaOH buffer (*p*H 7.0) and the suspension was vortexed to avoid the formation of separate toluene layer. After 15 min, the cells were sedimented by centrifugation, resuspended in the fresh buffer, and ^{55}Fe incorporation was determined as stated above. These values were substracted from all other samples before plotting the data. The values of the kinetic constants (K_m and V_{max}) were calculated using the double reciprocal plots.

100x stock solutions of metabolic inhibitors DCMU, HOQNO, DCCD, FCCP, CCCP and KCN were prepared in 95% ethanol and aliquots were added to uptake assay buffer. Assay buffer devoid of metabolic inhibitors but with equivalent ethanol served as controls. Assays were initiated after 1 h pre-incubation. Anaerobic condition was achieved by replacing air of the vessels with N_2. For dark incubation, the flasks containing cultures were wrapped in black papers lined with aluminium foil during the period of experimentation and assays were carried out in complete darkness.

Cellular protein was estimated following the method of Lowry *et al.* (1951).

Radiolabelled $^{55}FeCl_3$ was obtained from BARC (Mumbai, India). HEPES, DCMU, HOQNO, DCCD, CCCP, FCCP and KCN were purchased from Sigma Chemical Co. (St. Louis, Missouri, USA). Silicon DC 550 and dinonyl-phthalate were purchased from Fluka AG (Buchs, Switzerland). The siderophores, schizokinen, aerobactin and desferal were obtained from Ciba-Geigy Chemical Co. (USA). All other chemicals were of analytical grade available from BDH (Poole).

RESULTS AND DISCUSSION

Fig 1 shows the time dependent iron-siderophore uptake in *A. anabaena 7120*. The uptake appeared to be biphasic - a rapid first phase and a slower second phase until uptake was stopped after an interval of 20- 30 min.

In order to rule out a non-specific mode of iron-siderophore uptake or adsorption, the assay was carried out in toluenized cells (Fig 1). The toluene-treated cells showed a small amount of iron –incorporation.

To study the kinetic behaviour of iron-siderophore uptake system in *A. 7120*, the half saturation value (K_m) and maximum rate (V_{max}) of iron-siderophore uptake were calculated by using the double reciprocal plot (Fig 2). It is evident that a single kinetic system mediated the iron-siderophore uptake. The estimated K_m was 200 µM and V_{max} 20 nmol iron taken up/mg protein/min.

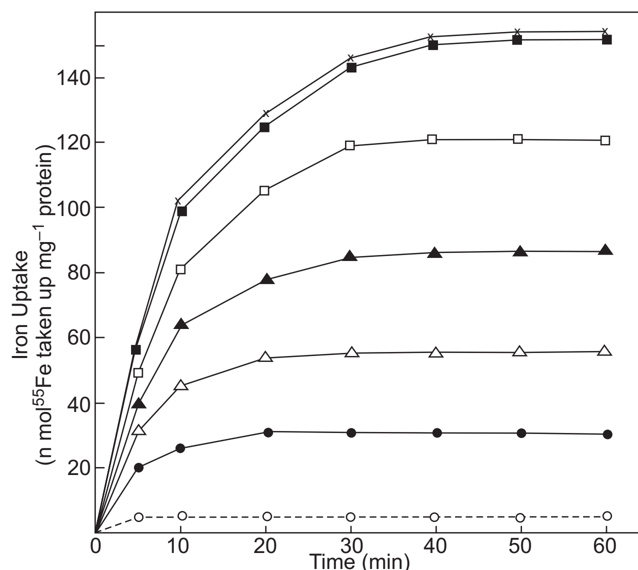

Fig. 1 Effect of iron concentration on iron-schizokinen uptake by *Anabaena 7120.* Iron (in µ*M*) : (○),50; (●),100; (△), 150; (▲), 200; (□), 250; (■), 300; (x), 350; cells pretreated with 1% toluene for 15 min. Data are means of two independent experiments with four replicates The maximum variation from the mean value was less than 5%

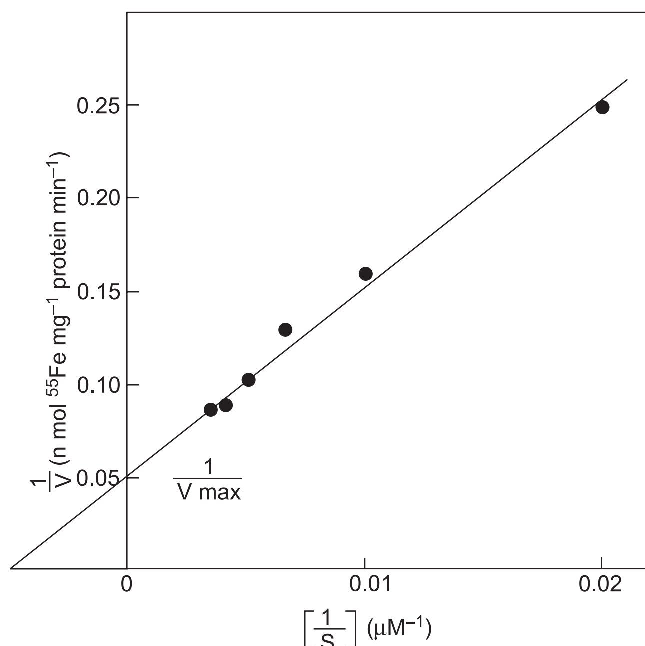

Fig 2 Lineweaver – Burk plot of iron- schizokinen uptake in *Anabaena 7120*. (Data are derived from Fig 1)

Table 1 Effect of different metabolic situations and inhibitors on iron- schizokinen uptake in *Anabaena 7120*

Addition/Incubation conditions	Metabolic inhibitors	Iron-schizokinen uptake (nmol/mg protein/min) (μM)
Control (Light aerobic)	0.0	9.76
Dark aerobic	0.0	0.91
Dark anaerobic	0.0	0.59
DCMU	10.0	0.0
HOQNO	10.0	0.0
DCCD	100.0	0.0
KCN	100.0	8.78
CCCP	25.0	0.0
FCCP	10.0	0.0
CD	0.654	
SE(d)	0.309	
SE(m)	0.218	
CV	16.985	

Iron-schizokinen uptake was assayed with 200 μ*M* iron in N_2-grown cultures. *A. 7120* cells were incubated for 1 h with the inhibitors prior to the uptake experiments. From the uptake data, toluene data have been substracted to correct for the non-specific binding. Data in each column are means of two independent experiments with four replicates each. The maximum variation from the mean value was less than 5%.

As shown in Table 1 the iron-siderophore uptake system is energy-dependent, as the process was inhibited to the photosynthesis inhibitors, DCMU and HOQNO. The experiments were also conducted under dark anaerobic condition where the endogenous energy was presumably low. No significant iron-siderophore uptake was observed under dark anaerobic condition. The iron-siderophore uptake was also significantly reduced in the cells kept under dark-aerobic condition. This indicated the involvement of ATP derived through photophosphorylation.

Furthermore, the inhibition of iron-siderophore uptake by DCCD, an inhibitor of bacterial ATPase (Kozlov and Skulachev 1977), H^+ adenosine triphosphate and membrane, also suggested the involvement of ATP in iron-siderophore uptake. Cyanide is a well known inhibitor of electron transport in bacterial mitochondria due to strong binding of cyanide to the oxygen-binding site in the terminal cytochrome oxidase of the electron transport chain. The persistence of significant level of iron-siderophore uptake (90%) even in the presence of KCN in the light indicates that *A. 7120* depends on photophosphorylation for light uptake of iron-siderophore. Similar KCN caused inhibition was also reported in cyanobacteria (Lammers and Sandera-Loehr 1982). CCCP, an uncoupler of cyclic electron flow (Heytler and Prichard 1962) significantly inhibited the iron-siderophore uptake, suggesting the vital role of cyclic photophosphorylation in iron-siderophore uptake. FCCP, an uncoupler of photosynthetic and oxidative phosphorylation almost abolished the iron-siderophore uptake. Since, FCCP, also collapses the proton gradient (Grevillegd 1969), it is proposed that iron-siderophore uptake in *A. 7120* may be linked to proton gradient as well.

To ascertain and also to find our more conclusive evidence regarding the relative contribution of different siderophores on iron-siderophore uptake, the iron-siderophore uptake was measured in the presence of siderophores schizokinen, aerobactin and desferal (Table 2). Siderophore schizochinen supported the maximum iron-siderophore uptake in *A. 7120,* suggesting that *A. 7120* cells exhibited a high affinity for ferric schizokinen. Aerobactin, which acts as an alternate iron chelator in the absence of schizokinen, supported only 53% iron-siderophore uptake. In contrast, the siderophore desferal, however, supported only 3.4% iron-siderophore uptake as compared to its schizokinen counterpart. These results suggest that *A. 7120* cells apart from using schizokinen

Table 2 Relative contribution of different siderophores on iron-siderophore uptake in *Anabaena 7120*

Siderophores	Iron-siderophore uptake (nmol/mg protein/min)
Schizokinen	9.76 (100)
Aerobactin	5.17 (53)
Desferal	0.34 (3.4)

Data in each column are means of two independent experiments with four replicates each. The maximum variation from the mean value was less than 5%. The values in parentheses indicate the percent iron-siderophore uptake

can also utilize aerobactin as an alternate siderophore for uptake of iron. However, the excess negative charge on aerobactin as compared to schizokinen may be one of the reasons for the reduced aerobactin-mediated iron-siderophore uptake in *A. 7120*. The replacement of one of the two acetylhydroxamate group by a bulkier substituent in desferal (Goldman *et al*. 1983) may be responsible for its ineffectiveness in iron-siderophore uptake in *A. 7120*.

Thus, overall in can be concluded that (i) iron-siderophore uptake system in *A. 7120* is an energy-dependent process and the energy required to support the iron-siderophore uptake is derived via photophosphorylation (ii) *A. 7120* cells apart from having high affinity for ferric schizokinen can also utilize aerobactin as an alternate siderophore for the uptake of iron in the absence of schizokinen.

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