Iron uptake in a cyanobacterium: Role of Siderophore and other modifying factors.

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ABSTRACT

The type of siderophore and its role in iron uptake in the diazotrophic cyanobacterium *Nostoc calcicola* Breb has been investigated. A typical dihydroxamate type of siderophore was found be present in the cyanobacterium. 200µM Fe^{3+} was found to be optimal iron concentration for the formation of siderophore while 150µM Fe^{3+} used for routine culturing of *N. calcicola* exhibited lower siderophore production. However, higher Fe^{3+}concentration inhibited siderophore production. Iron uptake was negligible in absence of siderophore. Iron uptake was found to be typically biphasic (a) rapid binding upto Ist 20 min followed by (b) slower metabolism-dependent uptake upto 50 min. The reduced Fe^{3+} uptake in dark reflected the role of ATP in iron transport and DCMU (5µM) reflected the vital role of ATP to support this process. Fe^{3+} uptake was lowered by p- chloromericuic benzoate (*p*CMB), N,N´-dicyclohexycariimide (DCCD), and sodium Azide (NaN_3) thus suggesting the role of membrane potential –SH groups and ATP hydrolysis in regulating Fe^{3+} transport. Fe shizoknien (Dihydroxamate) supported maximum Fe^{3+} uptake while aerobactin showed insignificant change in Fe^{3+} uptake.

Keywords: Iron, cyanobacteria and Siderophore

INTRODUCTION:

Iron is fourth abundant metal on the earth crust. It occurs in two ionic forms namely ferrous (Fe^{2+}) and ferric (Fe^{3+}). Fe^{3+} form of iron is the main form of Fe^{3+} utilized by microbes including cyanobacteria [15,25]. Iron is essential micronutrient and it occurs in many enzymes, acts as cytochorome, and is involved in biochemical reactions [3,425]]. At neutral pH it precipitates and remains not available to cyanobacteria. In this situation microbes produced siderophores to take

up iron from surroundings medium [24]. The role of iron in animal cells very much explored and its role in microbes including cyanobacteria is well documented [9, 11,25,27,34,36]. The presence of siderophore in cyanobacteria was dealt before [6,23,31,32] , but no body has attempted so far to investigate Fe^{3+} uptake and its regulation in cyanobacteria. The present work is an attempt to investigate the role of siderophore in iron uptake process and its regulation by light, dark and various metabolic inhibitors in the cyanobacterium *Nostoc calcicola* Bréb and evidence has been provided that hydroxamate type of siderophore is strictly required for Fe^{3+} uptake in *Nostoc calcicola* cells.

MATERIALS AND METHODS:

Experimental organism and growth condition: *Nostoc calcicola* Bréb, a local isolate was grown axenically in[1] medium devoid of any combined nitrogen source with A6 trace elements devoid of Fe^{3+} (EDTA is used in place of Fe EDTA). The culture medium was passed through chelax 100 column (Biorad, USA) and filtered through 0.4µM size pore size acid washed polycarbonate filter to remove traces of iron. Different iron concentration (0-500 µM as $FeCl_3$) was added directly to filtered basal medium. Cultures were routinely maintained at 25 $\pm$ 1^{o}C and 50µe m^{-2} s^{-1} light intensity on the surface of culture vessels with and 18 /6 light and dark cycle. Protein content was determined by the method of Lowry *et al* (1951) as modified by Herbart *et al*., (1971) using lysozyme (Sigma, St Louis Missouri, USA) as the standard.

Detection of Siderophore: Fe^{3+} (0-500µM) incubated cyanobacterial cells were harvested at 2nd, 5th and 10th day .such cells were centrifuged (5000 rpm,Remi, India) and cell free supernatant was used for the assay of the nature of siderophore by following the method of [18].

Detection of the type of Siderophores;

(i) **Hydroxamate type**: 1ml of culture supernatant 4ml of freshly prepared 2% of aqueous $FeCl_3$ solution was added and the absorbance between 200-600 nm was recorded. A peak at 430 nm indicates the presence of hydroxamate type of siderophore (Neiland, 1981). .

(ii) **Catechol type**: 1ml cell free supernatant was mixed with 2% $FeCl_3$. The formation of wine colour indicates the presence of catechol type of siderophore as done earlier (Arnow, 1937).

(iii) Detection of mono, di, and trihydroxamate nature of siderophore: pH dependent absorption maxima of ferrate siderophores the mono, di, and trihydroxamate type of siderophore as described by earlier [24]. As it showed wide shift at pH 4-5 so the presence of dihydroxamate type of siderophore in *N. calcicola* was confirmed.

Fe^{3+} uptake Experiment: *N.calcicola* cells were Fe^{3+} starved for 7 days prior to Fe^{3+} uptake experiment and uptake was monitored as described by Scott and Nicholas (1980). Such mid-log phage grown cells were centrifuged (5000 rpm, 5 min.), washed with sterile distilled water and suspended in phosphate buffer (0.1 M pH 8.0). Fe^{3+} uptake was monitored at increasing Fe^{3+} concentrations (0-2.5mM as $^{55}FeCl_3$ specific activity 0.1mci, BARC, India) to account for saturating iron concentrations. Samples 400µl were removed at different time in travels during 1hr and separated from buffer by micro centrifugation through silicon oil/ dioxylthelate 945:55 vol/vol) perchloric acid: water (15:85 vol/ vol).samples of perchloric acid fractions were withdrawn and the Fe^{3+} uptake was determined with a liquid scintillation counter (Wallac 1409, Finland) and expressed as µmol Fe^{3+} mg^{-1} protein. Slope of linear portion of the curve was used to calculate the Fe^{3+} uptake rate. The kinetic constants (K_m and V_{max}) were calculated from line-weaver-Burk Plots.

Role of light and exogenous ATP: Mid log phase growing cyanobacterial cells were dark incubated (24 ± 1^oC) for 24 h to render them in ATP and during Fe^{3+} uptake experiments. Non growth inhibitory concentration of ATP (10µM ; Sodium salt sigma, St Lousis, Missouri, USA) was applied to dark incubated cells as well as photoautrophically grown cells exposed to Fe^{3+} (2.0 mM: saturating concentration for uptake).

Inhibitors/ uncouplers: DCMU [3,(3,4-dichlorophenyl)-1.1-dimethyl urea], DCCD (N, N´-dicyclohexylcarbodimide) and FCCP(carboxyl cyanide p- nitrofluromethoxylphenyl hydrazone) were purched from Sigma Chemical Company; USA), PCMB (p-chloromercuric benzoate) from Koch light Lab (U.K), NaN_3 from BDH (Delhi, India). DCCD, DCMU and FCCP were solubilized in ethanol and PCMB in medium in such a way that the final concentration of each could not exceed 0.1 %.

Experiment with different siderophores: Hydroxamate , Catechol were purchased from Sigma Chemical Co., and added separately in Fe^{3+} uptake medium (20µM each; non growth inhibitory concentration) and Fe^{3+} uptake was monitored as described above.

Statistical analysis: All uptake experiments were carried at in triplicate and mean value were found to be < 5%.

The data of Fe^{3+} uptake under different treatment and the incubation period were verified by two factor analysis of variance (ANOVA) and variance ratio (F) was calculated by the following equation;

$$F = \frac{\text{Treatment mean square}}{\text{Residual mean square}}$$

RESULS AND DISCUSSION

Table 1 shows the presence and nature of siderophore present in test organism *Nostoc calcicola.* The positive response of culture supernatant at 430 nm suggested the presence of siderophore. Also the culture supernatants of *N. calcicola* cells showed positive signal against Neilands test [18], a test for hydroxamate type of siderophores and thus, provides an evidence for the production of hydroxamate type of siderophores by *N. calcicola* (table 1). The pattern of ^{55} Fe uptake in *N. calcicola* cells reflected that it follows (a) rapid binding of metal cations for Ist 20 min followed by slower phase at least upto 40 min (Fig 1a). Iron uptake is dependent as metal concentration (0.5-2.5mM) as well as the time (0-60 min). These findings are similar with those of reported with other metals in cyanobacteria [19.21]. The doubling in $^{55}Fe^{3+}$ concentration at lower doses (0.5 mM) increased the metal uptake in corresponding manner in contrast to higher doses (1.5-2.5 mM). Almost the similar ^{55}Fe uptake in 2.0 mM and 2.5 mM iron incubated cells (205.16 n mol $^{55}Fe^{3+}$ mg^{-1} protein) suggested the metal saturation at 2.0 mM. Similar concentration-dependent metal uptake were also present in cyanobacteria (21,19,28, Sheta 30, 33].

Table 1. Types of siderophore produced by *N.calcicola* Breb

Type of siderophore	Neilands	Arnow	Shift in λ_{max}(*p*H 4-7)
Hydroxamate			
(i) Dihydroxamate	+	-	+
(ii) Trihydroxamate	+	-	-
Catecholate	-	-	-
+ = Positive test.			
- = Negative test.			

The iron uptake kinetics was found to be hyperbolic with a k_m of 1.33 and V_{max} of 66.66 nmol $^{55}Fe^{3+}$ mg^{-1} protein min^{-1} and followed the Michaels-Menton kinetics (fig.1b). In similar experiments with Hg^{2+} [21]reported a K_m of 1.0 µm and V_{max} of 0.21 n mol mg^{-1} protein min^{-1}.Such a variance reflects the more requirement of Fe^{3+} as it is essential nutrient where as former is the non essential one. The higher V_{max} in present case compared to Ca^{2+} and Cu^{2+} [19,33] suggested that Fe^{3+} was taken in more rapidly than those metals. It may be due to accumulation of cations through carriers lacking any specificity in contrast to specific low uptake under conditions of low cations concentration [16].

The $^{55}Fe^{3+}$ uptake was mainly dependent on light generated energy is deplicated in Fig 2, as the dark incubated cells during metal uptake represented more than 6 fold decline in Fe^{3+} accumulation. It is also recorded that same cells under light incubation showed increasing trend of metal accumulation with increased Fe^{3+} uptake rate accompanying exposure time. Dark incubation on the other hand, reflected only a slight initial rise within 10 min. The photosynthetically generated energy-dependent metal uptake in present case are in agreement with those reported for Cu^{2+} in *N. calcicola* [33] Hg^{2+} in a cyanobacterium [21], Ni in *Anabaena Cylindrica* [7], Ca^{2+} in *Nostoc* Mac[19] and in contrast to Al uptake in *A .cylindrica* (Petersson et al., 1986) where light has no role in regulating metal uptake. The addition of DCMU (5.0µM) to light grown cells resulted in more than 3 folds decline in Fe^{3+} uptake rate as well as total metal uptake thus suggesting the initial role of energy generated via PS-II to support the metal ion influx. Addition of ATP (10µM) to dark grown cells showed almost 4 folds enhancement in Fe^{3+} uptake over dark-grown cells devoid of ATP, thus reflecting the energy –deficient status of such

cells a very light energy is left to drive the metal uptake initially as heavy metals also caused the depletion of ATP [20,29]. However, the same ATP concentration (10 µM) when added to light grown cells failed to bring at any significant increase over. Light grown cells (control cells) thus indicating that cation uptake cannot be enhanced beyond a certain limit even by additional supply of ATP.

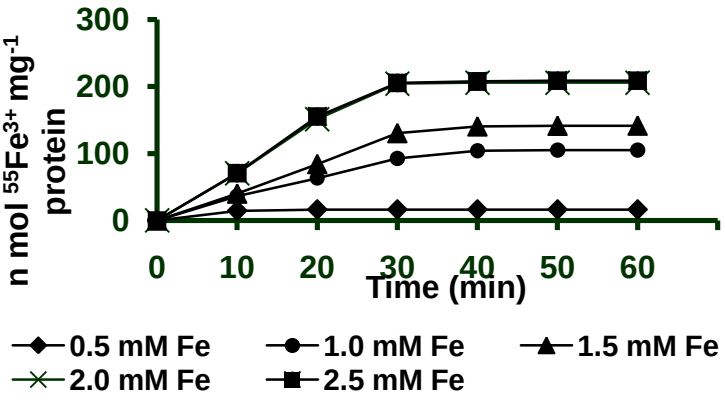

Figure: 1a. Iron uptake in *N. calcicola* cells in relation to varying Fe concentration. $Fe^{3+}_{conc.}$ $_{4,24}$ = 48.36; $F_{min\ 6,24}$ = 37.29; p < 0.005). Data are mean of three independent experiments with four replicates each. (Also for Fig. 2 & 3).

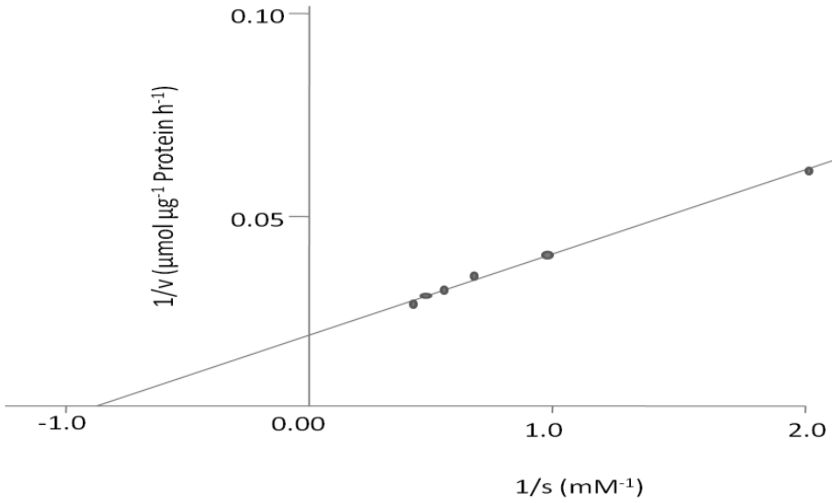

Fig 1 b: Concentration Dependence of Iron Uptake In *Nostoc calciocola* cells during 60 min(Based on Fig 1 a)

Figure 3 showed the relative contribution of different inhibitors / uncoupler as Fe^{3+} in *N.calcicola* cells is metabolism dependent. NaN_3 (Sodium azide), a well known inhibitor of respiratory electron transport and uncoupler of oxidative phosphorylation (Hewitt and

Nicholas,1963) reduced Fe^{3+} uptake by 50 %. The least inhibition in Fe^{3+} uptake might be owing to that azide was also used as substrate for nitrogenase. The inhibition (75%) by DCCD (10µM) signifies the role of membrane potential in regulating Fe^{3+} uptake. The strong inhibition (86%) by FCCP, a strong proton phase uncoupler and electron chain blocker suggested the vital role of membrane potential, ATP hydrolysis and proton gradient in driving Fe^{3+} uptake.

Complete inhibition (100%) in *p*CMB added in terms of Fe^{3+} uptake in cyanobacterial cells can be explained as the interaction of *p*CMB with –SH groups present in cell membrane [5] in a way signifying the intergrity of membrane, as well as the –SH groups containing enzyme sectors might be affected as *p*CMB enters through a damaged cell membrane resulting the observed inhibition (100%) in iron uptake.

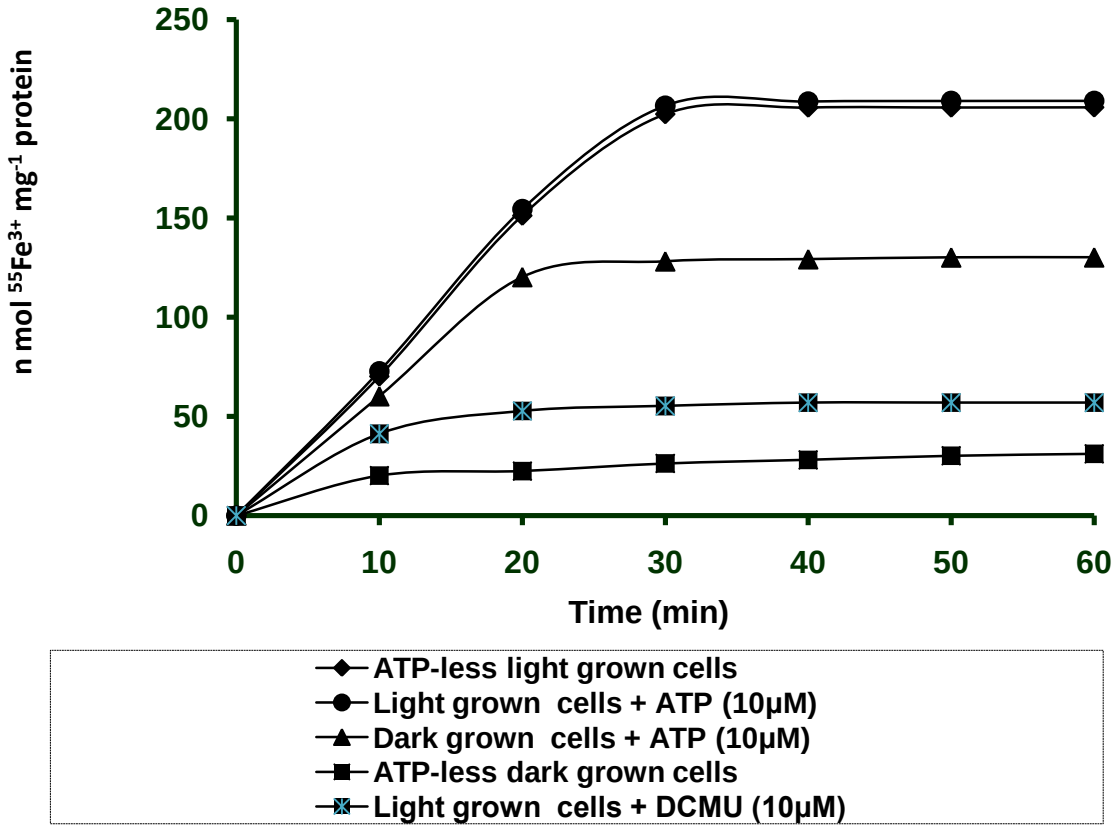

Figure: 2. Fe uptake in *N. calcicola* cells as a function of light, DCMU and ATP. F parameters $_{4,24}$ = 28.03; $F_{min\ 4,24}$ = 24.24; p < 0.005).

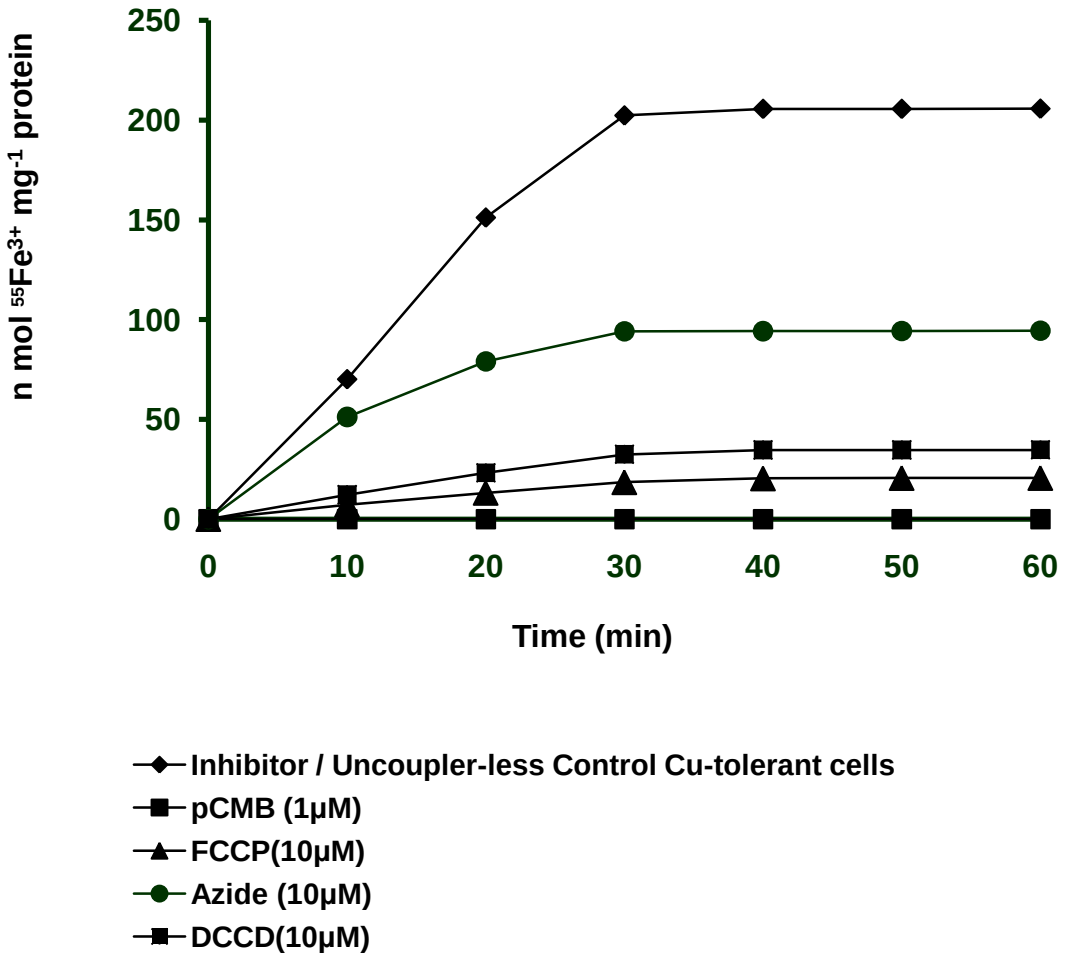

Figure: 3. Regulation of Fe uptake in *N. calcicola* cells by different metabolic inhibitors/ uncouplers. $F_{parameters3,18} = 34.32$ $p < 0.005$; $F_{min\ 6,21} = 4.5$ $p < .025$.

As it was confirmed that a hydroxamate type of siderophore is present in cyanobacteria as also reported [24], so the role of hydroxamate and catechol type siderophore on Fe^{3+} uptake were observed and result was deplicated in table 2. The control cells (devoid of any externally added of siderophore) reflected a Fe^{3+} uptake rate 8.0 nmol $^{55}fe^{3+}$ protein min^{-1} (calculated at 10min). Addition of hydroxamate type (20 µM) resulted in 30 % enhancement in Fe^{3+} uptake rate (10.8 n mol $^{55}Fe^{3+}$ mg^{-1} protein min^{-1}) suggesting that internally

Table 2. Effect of externally added siderophore in iron uptake in *Nostoc calcicola* cells.

Siderophore	Iron uptake rate (at 10 min). (n mol. $^{55}Fe^{3+}$ mg^{-1} protein min^{-1})
Control – (external siderophore)	8.0
Dihydroxamate (20µm)	10.8
Catechol (20 µm)	8.1

The data are means of two independent experiments with four replicates each (also from table 1).the maximum variation from mean value was less than 5 %.

present siderophore is not able to drive Fe^{3+} uptake maximally as also reported in other cyanobacteria and bacteria [9,10,14]. Catechol type siderophore (20 µM) on the other hand was not able to enhance Fe^{3+} uptake significantly thus indicating no definite role of such type of siderophore in Fe^{3+} uptake process in present organism. .Similar catechol independent Fe^{3+} uptake is also reported in the cyanobacterium *Anabaena flos-aquae* [8,35] in contrast to presence of catechol type of siderophore and its role in iron uptake in *Synechococcous* [34].

ACKNOWLEDGEMENTS

We are grateful to Hon'ble Vice-chancellor for providing lab infrastructure. We are also thankful to DBT, Govt. of India for financial assistance for instruments used in present study.

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