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Iron binding to *Azotobacter salinestris* melanin, iron mobilization and uptake mediated by siderophores

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Iron-sufficient *Azotobacter salinestris* cells bound large amounts of ^{55}Fe to cell-associated catechol melanin in an energy-independent manner. Iron was mobilized from the cell surface by citric acid and transported into the cell in a process that was inhibited by azide, carbonyl cyanide *m*-chlorophenyl-hydrazone (CCCP), KCl or RbCl, the latter two known to inhibit Na^+-dependent activities in *A. salinestris*. Iron-limited cells produced a hydroxamate compound (HDX) which promoted ^{55}Fe-uptake into iron-limited cells in a two step process. Initial uptake was inhibited by azide or CCCP, but not by KCl, while subsequent uptake was blocked by all inhibitors. Citric acid also mediated energy-dependent ^{55}Fe-uptake in iron-limited cells, but initial iron-uptake was less sensitive to CCCP than HDX-mediated iron-uptake. The results show that melanin serves as an iron trap, probably to protect the cells from oxidative damage mediated by H_2O_2 and the Fenton reaction. A model for HDX siderophore-mediated iron-uptake is proposed which requires energy to concentrate iron in the periplasm and H^+/Na^+-dependent events to bring iron into the cell.

Keywords: catechol melanin, hydroxamate uptake, iron binding, microaerophile, oxidative damage, siderophore

Introduction

Azotobacter salinestris is a micoraerophilic nitrogen-fixing bacterium (Page & Shivprasad 1991). This bacterium resembles *Azotobacter chroococcum*, one of the most common of the obligate aerobic soil azotobacters, but differs in that it has very low catalase activity and extreme sensitivity to H_2O_2 as found in other microaerophiles (Page *et al.* 1988, Krieg & Hoffman 1986). However, *A. salinestris* can adapt to grow and fix nitrogen under aerobic conditions despite its apparent sensitivity to toxic oxygen products. This aeroadaptation is mediated by a switch in C-source preference, to support respiratory protection of the oxygen-labile nitrogenase (Shivprasad & Page, unpublished data), and by melanization of the cells (Shivprasad & Page 1989). In the latter case, the catechol-melanin is thought to act as a trap for free radicals formed during aerobic growth (Shivprasad & Page 1989).

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A. salinestris requires at least 1 mM Na^+ for optimal growth (Page 1986). This requirement is quite like that of marine bacteria in that Li^+ or Mg^{2+} can substitute for some of the required Na^+, but K^+ or Rb^+ are growth inhibitory (Page 1986, 1991). *A. salinestris* also requires at least 0.25 μM Fe for growth to occur since iron is required for the activity of enzymes that protect the cells from toxic oxygen products (Page 1987; Page *et al.* 1988). Under low iron conditions (0.25–1.0 μM Fe), the cells produce a hydroxamate compound (HDX) that promotes *A. salinestris* growth in media containing insoluble iron minerals. Citric and succinic acids also are excreted by the cells constitutively and may serve to solubilize the iron sources and act as siderophores.

The *A. salinestris* HDX differs from the hydroxamate siderophores of *A. chroococcum* (Fekete *et al.* 1989) because the former does not form a red Fe^{3+} complex, it cannot be extracted into benzyl alcohol and its ability to reverse the inhibition of growth caused by ethylene-diamine-di(*o*-hydroxyphenylacetic acid) (EDDHA) is weak (Page 1987). Although HDX production is repressed by more than 5 μM Fe, there is no direct proof of its function as a siderophore. In fact HDX production corresponds more directly to O_2 stress (Shivprasad & Page 1989) than iron limitation (Page 1987), so it is possible that

it acts as an iron chelator to prevent an iron-catalyzed Fenton reaction (Gutteridge 1987, Halliwell & Gutteridge 1988, Shivprasad & Page 1989), rather than acting as a siderophore *per se*. Alternatively, citric acid could be the true siderophore of *A. salinestris*. Therefore, this study examines iron-sufficient and iron-limited *A. salinestris* to determine how iron is taken up by these cells.

Materials and methods

Strains and growth conditions

This study used the type strain of *A. salinestris* sp. nov., strain 184 (ATCC 49674; Page & Shivprasad 1991). Strains with either no melanization (white) or heavy melanization (black) were picked as spontaneous variants from the 184 culture. These variants bred true for the course of the experiments described, but were unstable variants that appear to result from a switching event that also regulates melanin synthesis in *A. salinestris* (Shivprasad 1991). The strains were grown in Burk's medium under the conditions described previously (Page 1991), except that the aeration rate was decreased (shaking rate 176 r.p.m.). The iron content of the medium was adjusted using ferric citrate. Burk's buffer is the 5 mM phosphate buffer, pH 7.2, and mineral salts component of this medium. Inhibitors used were the energy poisons 5 mM sodium azide and 10 μM carbonyl cyanide *m*-chlorophenylhydrazone (CCCP) or the Na^+ antagonists 3 mM KCl and 3 mM RbCl (Page 1986, 1991).

Hydroxamate compounds used in feeding experiments were Desferal® obtained from Ciba-Geigy Canada (Mississauga, Ontario, Canada), ferrichrome prepared from *Ustilago sphaerogena* (Neilands 1952) and schizokinin prepared from *Bacillus megaterium* ATCC19213 (Byers *et al.* 1967). Ferrichrome was deferrated as described by Emery & Neilands (1960).

Conditions for the assay of ^{55}Fe uptake into A. salinestris

In our previous experience with aerobic nitrogen-fixing bacteria there is considerable non-specific binding of iron to the cells (Page & Huyer 1984, Collinson & Page 1989) so ^{55}Fe uptake assays have contained 10 mM sodium citrate, pH 7.0, to reduce this non-specific binding (Knosp *et al.* 1984). However, in the present study such a large amount of sodium citrate would preclude any determination of the role of Na^+ or the role of citrate in ^{55}Fe uptake into *A. salinestris*.

Therefore, the cells were grown in iron-sufficient (10 mM ferric citrate) or iron-limited (0.35 mM ferric citrate) medium under the conditions described by Knosp *et al.* (1984). The cells were harvested by centrifugation and suspended in 0Fe (no added ferric citrate) Burk's buffer containing 3 mM potassium citrate. These cells were resedimented and washed in 1 mM potassium citrate before resuspension in 0Fe Burk's buffer containing 0.5% glucose to an OD_{620} of about 9. When the cells in this suspension were diluted into the final assay solution (0Fe Burk's buffer containing 0.5% glucose and appropriate additions) the potassium citrate concentration was decreased to 0.16 mM. The $^{55}FeCl_3$ used in the assay was prepared as perviously described (Knosp *et al.* 1984) using 10 mM potassium citrate rather than sodium citrate. The Na^+ in the final assay solution was growth limiting at below 0.05 mM, the K^+concentration was insufficient to inhibit growth and sufficient citrate remained to keep the 1 μM $^{55}FeCl_3$ from precipitating during the assay.

Analysis of cells and culture fluids

Culture growth was estimated by viable counts for ^{55}Fe uptake assays or by protein assays (Page 1986, Page *et al.* 1988), as noted in the Results. HDX production was estimated by the Csaky (1948) assay. The iron content of cells was estimated by an ascorbate-bipyridyl assay after digestion of the cells in perchloric acid (Page 1993).

Results and discussion

^{55}Fe binding and uptake in iron-sufficient A. salinestris

^{55}Fe uptake into iron-sufficient (10 μM Fe) cells followed a two-step process: an initial uptake of iron that occurred in the first 15 min, followed by continued uptake that was only observed in the cultures containing NaCl (Figure 1). Na^+-dependent iron uptake occurred at a linear rate of 0.11 ng Fe min^{-1} per 10^8 cells. The addition of excess (3 mM) sodium citrate to the *A. salinestris* assay eliminated the initial binding of ^{55}Fe to the cells and decreased the Fe uptake rate to 0.02 ng Fe min^{-1} per 10^8 cells (Figure 1). This uptake was linear and Na^- dependent, because 3 mM potassium citrate failed to promote significant iron uptake.

The results show that there was significant non-specific

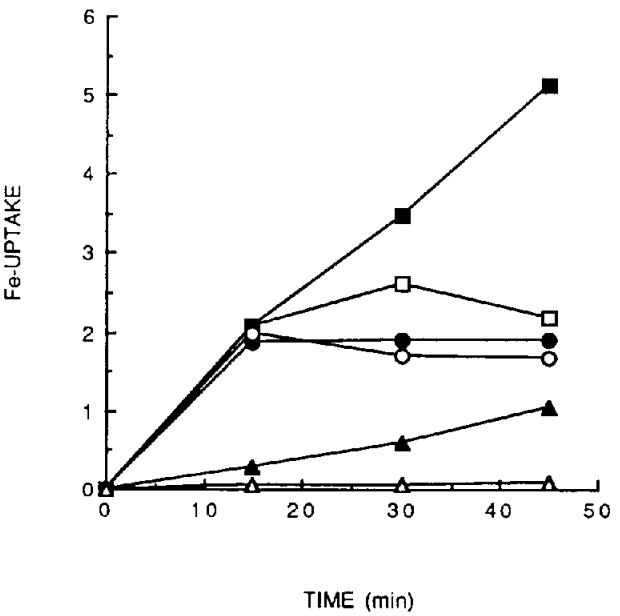

Figure 1. Iron uptake by iron-sufficient *A. salinestris*. The uptake of ^{55}Fe (ng min^{-1} per 10^8 cells) was followed in iron-limited uptake buffer (□) containing 3 mM NaCl (■), KCl (●) or RbCl (○), sodium citrate (▲) or potassium citrate (△).

iron binding to *A. salinestris* cells. This binding of iron was not very strong. ^{55}Fe that would have been bound to the cell was chelated by 3 mM sodium citrate and the majority of this was not transported into the cell. Initial binding of ^{55}Fe to the cells during the first 15 min was not prevented by 5 mM sodium azide or 10 μM CCCP, but subsequent uptake was decreased 34 and 72% respectively (data not shown).

One of the characteristics of *A. salinestris* grown aerobically is the production of cell-bound catechol-melanin (Shivprasad & Page 1989). Melanin is found in the cell membrane and the outer membrane (unpublished data) and can be expressed differentially in variants of *A. salinestris* strain 184 (Shivprasad 1991). Iron uptake was assayed in iron-sufficient strain 184, which is normally tan colored, and in a white variant and a black variant of strain 184. In 15 min, 10^8 cells of strain 184 bound about 2 ng ^{55}Fe (as in Figure 1), while cells of the white and black variants bound about 1.5 ng and 38 ng ^{55}Fe, respectively. ^{55}Fe binding to 10^8 black variant cells continued in the presence of KCl and reached 44 ng ^{55}Fe by 45 min, while ^{55}Fe bound to the other cell types increased to only 1.7 ng ^{55}Fe (white) and 2.3 ng ^{55}Fe (strain 184). During 15 min incubation on ice, 10^8 black variant cells bound 14 ng ^{55}Fe, while the white variant and strain 184 bound only 1.6 ng and 2.3 ng ^{55}Fe, respectively. ^{55}Fe binding to the black variant increased 61% during a further 30 min incubation on ice.

These results show that there were two stages of iron uptake into iron-sufficient *A. salinestris*: (i) and energy-independent stage, followed by (ii) an energy-dependent stage. The energy-independent binding of iron to *A. salinestris* cells is to the polyphenolic melanin found in the cell envelope and heavily melanized cells bind much greater amounts of iron. Excess citrate (3 mM) was sufficient to prevent the energy-independent binding of iron to the cell and promoted iron uptake into the cell in an Na^+-dependent manner.

HDX-mediated ^{55}Fe uptake into iron-limited cells

A. salinestris formed a HDX compound optimally in medium containing 0.25–1 μM Fe (Figure 2). HDX production was repressed at higher iron concentrations, more in response to growth than to the iron content of the cells (Figure 2). The amount of HDX in the iron-limited culture supernatant fluids used in the ^{55}Fe uptake assays was about 2 μg hydroxamate-N ml min^{-1}. Therefore, if the HDX compound was a trihydroxamate there would be more than sufficient (48 μM) HDX available for chelation of the 1 μM ^{55}Fe in the assay.

The rate of iron uptake promoted by HDX into iron-limited cells was 0.64 ng ^{55}Fe min^{-1} per 10^8 cells (Figure 3). Iron uptake was inhibited by KCl after 15 min (Figure 3), as shown for iron-sufficient cells (Figure 1). Although iron-limited strain 184 cells were not more melanized than cells grown in iron-sufficient medium, they appeared to bind about five-times more ^{55}Fe initially (compare Figures 1 and 3). The uptake of ^{55}Fe promoted by HDX was

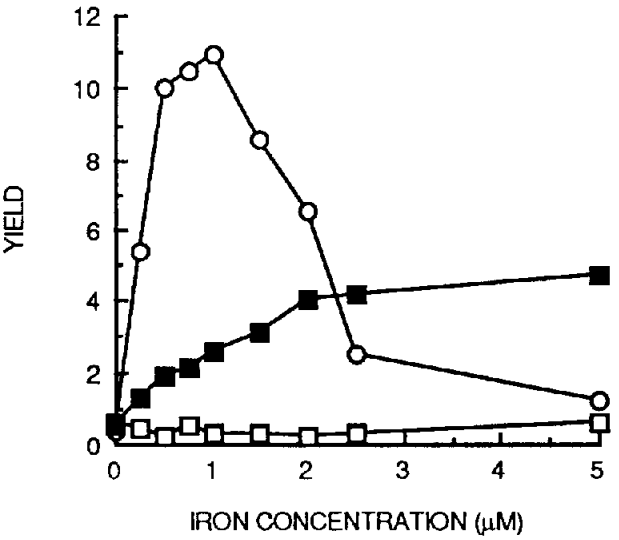

Figure 2. Growth and hydroxamate production by *A. salinestris*. The cell protein (mg/10 ml, ■), μg Fe per mg cell protein (□) and the production of hydroxamate (μg HDX-N per mg cell protein, ○) was measured after 24 h incubation in medium containing varied iron concentrations.

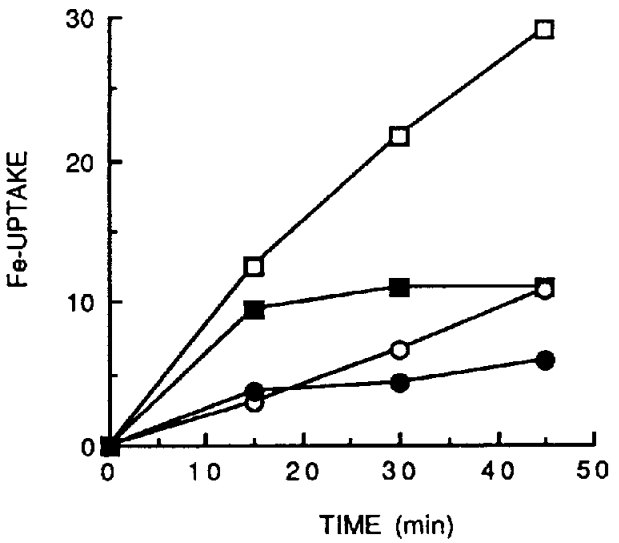

Figure 3. Iron-uptake into iron-limited or iron-sufficient *A. salinestris* promoted by HDX. The uptake of ^{55}Fe (ng min^{-1} per 10^8 cells) was followed using iron-limited cells in iron-limited culture fluid containing 3 mM NaCl (□) or containing 3 mM KCl (■), or into iron-sufficient cells in iron-limited culture fluid containing 3 mM NaCl (○) or containing 3 mM KCl (●).

energy dependent (Figure 4). However, the initial binding of ^{55}Fe was prevented by the addition of azide or CCCP (Figure 4), which was not the case in iron-sufficient cells. In fact, both iron-sufficient and iron-limited cells bound about 2 ng ^{55}Fe per 10^8 cells after 15 min in the presence of CCCP or the amount attributed to energy-independent binding to melanin.

Therefore, ^{55}Fe uptake into iron-limited cells appeared to be a two step process. During the first 15 min, ^{55}Fe or

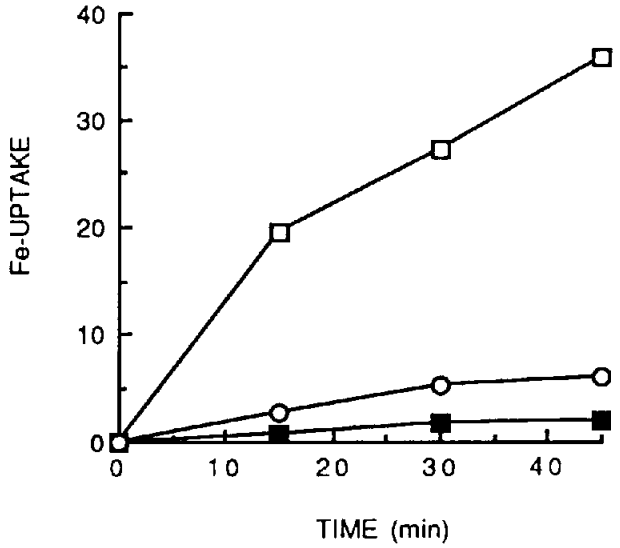

Figure 4. Inhibition of iron uptake by iron-limited *A. salinestris*. The uptake of ^{55}Fe (ng min^{-1} per 10^8 cells) was followed using iron-limited cells in iron-limited culture fluid containing 3 mM NaCl (□) alone or with 5 mM sodium azide (■) or with 10 μM CCCP (○).

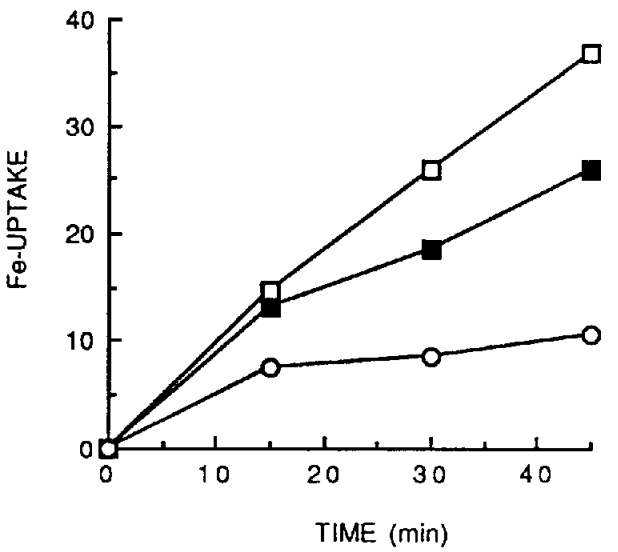

Figure 5. Iron uptake into iron-limited *A. salinestris* promoted by citrate. The uptake of ^{55}Fe (ng min^{-1} per 10^8 cells) was followed using iron-limited cells suspended in iron-limited culture fluid containing 3 mM NaCl (□), or suspended in iron-limited Burk's buffer containing 3 mM NaCl and 0.16 mM sodium citrate (■) alone or with 10 μM CCCP (○).

^{55}Fe-HDX was concentrated in the cells (about 8 ng Fe per 10^8 cells). This concentration step was not possible in the presence of CCCP of azide, but was not prevented by KCl (compare Figures 3 and 4). Further uptake of ^{55}Fe into the cell was blocked by KCl (Figure 3) or CCCP (data not shown) and the HDX in the assay was not able to remove the cell bound ^{55}Fe.

^{55}Fe uptake into iron-sufficient cells mediated by HDX

Iron-limited culture fluid containing HDX and 3 mM NaCl (plus 0.16 mM citrate from the assay buffer) promoted the uptake of 0.24 ng $^{55}Fe\,min^{-1}$ per 10^8 cells into iron-sufficient cells (Figure 3). This rate was twice as fast as that obtained in Figure 1 with 3 mM NaCl (plus 0.16 mM citrate from the assay buffer), which suggests that the HDX siderophore receptors were not fully repressed by 10 mM Fe. Similar results have been obtained with *A. macrocytogenes*, which also fails to fully repress siderophore receptors at 95 μM Fe (Collinson & Page 1989). This appears to be due to the rapid oxidation of iron under these aerobic growth conditions, so that the added iron is not readily available (Page 1993).

Thus the higher rate of ^{55}Fe uptake seen in iron-sufficient cells with NaCl (Figure 1) may be the result of endogenous production of undetectable and limiting amounts of HDX during the assay period. The slower rate of ^{55}Fe uptake obtained with excess sodium citrate may be more representative of low affinity (or citrate-promoted) iron-uptake in iron-sufficient cells (Figure 1).

Citrate-mediated ^{55}Fe uptake into iron-limited cells

Citrate also mediated ^{55}Fe uptake into iron-limited *A. salinestris* in an energy dependent manner (Figure 5). In this experiment, the rate of HDX-promoted uptake was 0.81 ng $^{55}Fe\,min^{-1}$ per 10^8 cells, while the rate with citrate was 50% of that. The initial binding of ^{55}Fe-citrate to iron-limited cells was relatively insensitive to CCCP (Figure 5) compared to the binding of ^{55}Fe-HDX (Figure 4), while subsequent uptake was inhibited by CCCP (Figure 5) or KCl (data not shown).

Use of heterologous ligands to promote ^{55}Fe uptake

Ferrichrome, Desferal, or shizokinin (all at 10 μM) did not promote ^{55}Fe uptake into iron-limited *A. salinestris* during a 45 min assay, even when suspended in iron-limited culture fluid containing HDX and up to 10 mM sodium citrate (data not shown). These heterologous hydroxamates inhibited the growth of iron-limited *A. salinestris* when the iron content of the medium was low. As iron became more available, it appeared that the HDX of *A. salinestris* could compete for iron (Figure 6).

Iron and A. salinestris *growth*

Since melanization correlates with oxygen-stress (Shivprasad & Page 1989), it appears perfectly logical for *A. salinestris* to use melanin as an iron trap. *A. salinestris* has superoxide dismutase activity, but very little catalase activity (Page *et al.* 1988). This means that the Fenton reaction (2) will be a very real threat to these bacteria during aeroadaptation:

$$Fe^{3+} + {}^{\cdot}O_2^- \rightarrow Fe^{2+} + O_2 \qquad (1)$$

$$Fe^{2+} + H_2O_2 \rightarrow Fe^{3+} + OH^- + {}^{\cdot}OH \qquad (2)$$

Once $^{\cdot}OH$ is formed it will react immediately with neighboring biomolecules, so the binding of iron to melanin distributed through the cell envelope would

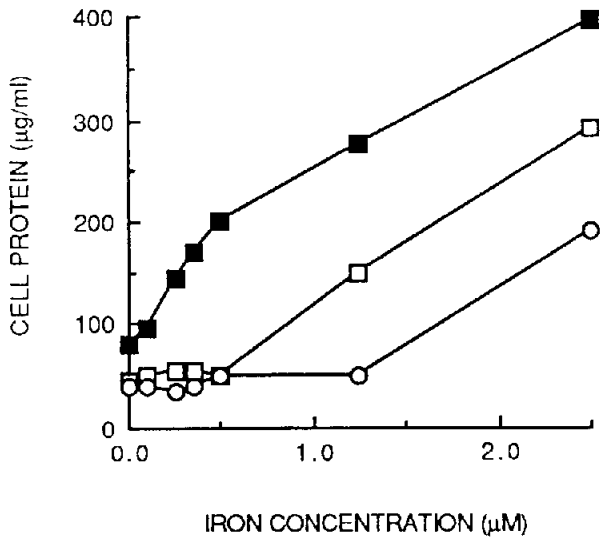

Figure 6. Inhibition of *A. salinestris* growth by Desferal. Culture growth (μg cell protein ml^{-1}) was measured after 24 h incubation in medium containing Desferal added at 0 μM (■), 10 μM (□) or 50 μM (○) and varied iron concentrations.

ensure the localization of the Fenton reaction within this polymer, a useful free radical trap (Mason *et al.* 1960). Furthermore, the reduction of Fe^{3+} in reaction (1) and the subsequent reaction (2) may be diminished by the pseudo-superoxide dismutase and catalase activities detected in melanins (Sichel *et al.* 1987, Geremia *et al.* 1989). The catechol groups in melanin could reduce Fe^{3+} and promote the Fenton reaction, while subsequent binding of Fe^{2+} to melanin may ultimately inhibit the reaction (Pilas *et al.* 1988). These radical trapping and protective roles also have been proposed for melanins in animal tissues (Scalia *et al.* 1990, Porebska-Bundy *et al.* 1992).

Mobilization of iron from the iron-sufficient cell surface is most likely by the 20 μM citric acid and 6 mM succinic acid that is constitutively produced by *A. salinestris* (Page 1987) and by small amounts of the HDX siderophore that does not appear to be fully repressed. Transport of iron into the cells is dependent on energy and Na^+. K^+ is a competitive inhibitor of an essential Na^+ site required for cell viability and growth and Na^+-limited cells lose viability quickly (Page 1986, 1991). These data suggest that this essential site could be one involved in iron uptake.

Both HDX and citrate appear to promote iron uptake into iron-limited cells. Since citrate production is not iron repressible and appears to promote iron uptake into iron-sufficient cells, it could be argued that it is not a true siderophore (Neilands 1981). Iron uptake proceeds in a two-step energy-dependent manner. Initial uptake is inhibited by CCCP, but not by KCl, while subsequent uptake is inhibited by CCCP or KCl. Since HDX does not solubilize the iron that is cell bound in the first step, it is likely that the iron has been internalized. A model to explain these results is that initial uptake across the outer membrane is energized by proton motive force. This could be analagous to the uptake of ferrichrome into *Escherichia coli*, through the *FhuA* receptor which is energized by proton motive force via the *TonB* complex of the cytoplasmic membrane (Postle 1990, Nikaido 1993). A periplasmic binding protein could aid in iron concentration. Subsequent uptake of periplasmic iron or Fe-HDX would be mediated by a Na^+- or Na^+/H^+-dependent site on the cytoplasmic membrane. The uptake of ferric citrate appears to differ from this scheme in that initial uptake is not as susceptible to CCCP. This may indicate permeation of ferric citrate through the outer membrane is not energized and only subsequent uptake is dependent on a Na^+- or Na^+/H^+-dependent site on the cytoplasmic membrane.

The results of this study are consistent with previous speculations (Page 1987) that *A. salinestris* has citrate- and HDX siderophore-mediated iron uptake systems. Both of these systems appear to have a low affinity for iron as they are easily inhibited by EDDHA and other hydroxamate siderophores, especially when the iron content of the medium is low. Mutants defective in siderophore production or iron uptake would be useful for the analysis of iron uptake, but this species has proven to be very recalcitrant to genetic exchange and transposon-mediated mutagenesis. However, *A. salinestris* genes can be expressed in *E. coli* (Shivprasad 1991), so complementation of *E. coli* mutants by cosmids containing *A. salinestris* DNA may lead to the identification of genes involved in iron uptake. A scheme for the purification of the HDX siderophore is also being developed (S. Shivprasad, unpublished data) for future studies of HDX-mediated iron uptake and for further examination of the role of HDX in protecting cells from the Fenton reaction. The nature of the Na^+-dependent site required for iron uptake is also being investigated.

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