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Iron-uptake in the Euryarchaeon *Halobacterium salinarum*

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Abstract Iron-uptake is well studied in a plethora of pro- and eukaryotic organisms with the exception of Archaea, which thrive mainly in extreme environments. In this study, the mechanism of iron transport in the extremely halophilic Euryarchaeon *Halobacterium salinarum* strain JW 5 was analyzed. Under low-iron growth conditions no siderophores were detectable in culture supernatants. However, various xenosiderophores support growth of *H. salinarum*. In [^{55}Fe]–[^{14}C] double-label experiments, *H. salinarum* displays uptake of iron but not of the chelator citrate. Uptake of iron was inhibited by cyanide and at higher concentrations by Ga. Furthermore, a K_M for iron uptake in cells of 2.36 μM and a V_{max} of approximately 67 pmol Fe/min/mg protein was determined. [^{55}Fe]-uptake kinetics were measured in the absence and presence of Ga. Uptake of iron was inhibited merely at very high Ga concentrations. The results indicate an energy dependent iron uptake process in *H. salinarum* and suggest reduction of the metal at the membrane level.

Keywords Archaea · *Halobacterium salinarum* · Iron uptake · Xenosiderophore

Introduction

A variety of iron uptake processes in pro- and eukaryotes have been identified. A major iron transport mechanism employs siderophores, high-affinity ferric iron chelators produced by bacteria, fungi, and monocotyledoneous plants (Andrews et al. 2003; Howard 1999; Ratledge and Dover 2000; Matzanke 2005). Ferric siderophore complexes are bound by receptor proteins located in the membrane and exhibiting high selectivity for specific siderophores. The uptake itself is mediated by a periplasmic iron binding protein and the subsequent transport across the cytoplasmic membrane requires an ABC-transport system (Winkelmann 2001). Iron is released at the cell surface or intracellularily either by reduction (Fe^{2+} is only weakly bound to siderophores) (Matzanke et al. 2004; Matzanke 2005) or by enzymatic degradation of the siderophore backbone as shown for example for ferric enterobactin, one of the siderophores produced by *Escherichia*

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coli (Raymond et al. 2003). Some siderophores are not excreted but remain membrane- or cell-surface bound. They are part of an uptake mechanism called ''ligand exchange'' where the membrane bound siderophore takes over ferric iron delivered by a second soluble type of siderophore. Such a process was shown for *Aeromonas* sp., *Pseudomonas* or *Mycobacterium* sp. (de Voss et al. 1999; Matzanke et al. 1997; Stintzi et al. 2000). Membrane association of siderophores prevents their dilution thus providing various advantages as e.g. discussed for marine microorganisms (Butler 1998; Martinez et al. 2003; Morel and Price 2003; Tortell et al. 1999). Some organisms carry on ferric siderophore piracy as well. *E. coli* can take up iron from at least eight different iron–chelate complexes: four endogenously produced siderophores and four siderophores produced exogenously by other organisms (xenosiderophores). Biosynthesis of siderophores is missing in the yeast *Saccharomyces cereviseae*, but the expression of at least four different siderophore receptor proteins and the uptake of the corresponding siderophores was described (Winkelmann 2001; Kosman 2003; Moore et al. 2003). In certain fungi, siderophores function—in addition to their role as iron carrier—as iron storage compounds (Matzanke et al. 1987).

The reductive uptake system is best described for *S. cereviseae* where ferric iron is reduced to the ferrous form by one of the Fre-proteins and subsequently either taken up as the ferrous species by a low affinity transport system (Fet4p) or as the ferric form by a high affinity system composed of a multicopper ferroxidase (Fet3p) which reoxidises the ferrous iron and the respective transport protein, the ferric iron permease (Ftr1p) (Kosman 2003; Van Ho et al. 2002). Ferric reductase systems have also been described for bacteria and dicotyledoneous plants.

A somehow special uptake-system was described for the marine algae *Dunaliella salina* employing a membrane bound transferrin-homologue for iron uptake (Fisher et al. 1997; 1998). This protein was detected during a screening procedure of proteins induced by elevated salt concentrations in the medium. In a second report on salt-shock induced genes in *Bacillus subtilis*, it was shown that elevated salt concentration induced genes of the iron uptake pathway (Hoffmann et al. 2002) the authors concluded that a high-salt environment correlates with iron limitation. The *D. salina* mechanism is interesting because *Halobacterium salinarum* succeeds growth of *D. salina*, when the salt concentration of the environment raises to a value higher than 2.5 molar. The first ''halophilic'' siderophore produced by *Halomonas campisalis* (Aiken et al. Biometals 2004) was described very recently. Very little is known about iron uptake mechanisms in the third domain of life, the Archaea. Only a ferric reductase of the archaeon *Archaeoglobus fulgidus* was identified and its structure solved. Unfortunatly it is still unclear, if the enzyme fulfills its function in the assimilation of iron or in the dissimilatory reduction (Chiu et al. 2001; Vadas et al. 1999).

Within a major project aimed on the analysis of iron metabolism in Archaea, we decided to study iron transport in the extremely halophilic Euryarchaeon *Halobacterium salinarum*. There are few reports dealing with the effects of iron on growth of *Halobacteria* (Dundas et al. 1963; Sehgal and Gibbons 1960). Recently we published preliminary data on iron metabolism (Hubmacher et al. 2002), providing evidence for significant changes in the membrane protein pattern due to iron limitation. DpsA, the first archaeal ferritin like iron storage protein was isolated from the cytosol of *H. salinarum* as well (Reindel et al. 2002) and its regulation was analyzed (Reindel et al. 2005). Previously published results indicated that *H. salinarum* can thrive even in environments with an iron concentration below 0.5 μM by altering the respiratory chain (Hubmacher et al. 2003). Here, we report experiments analyzing iron transport mechanisms of *Halobacterium salinarum*.

Material and methods

Cell growth and growth promotion experiments with xenosiderophores

Halobacterium salinarum (DSM 3754) strain JW5 was a kind gift from Dr. Peter Palm, Max Planck Institut für Biochemie, Martinsried and was

grown as described previously (Hubmacher et al. 2003). In brief, low-iron growth medium was prepared by treatment with the metal chelator Chelex-100. The residual amount of iron was determind colorimetrically as described (Makino et al. 1988). Iron concentration in the media was below 0.1 μM. Determination of iron by atomic absorbance spectroscopy was not possible due to the high salt concentration in the growth medium and buffers (>4 M), as well as in membrane and cytosolic fractions. All glassware for the experiments with iron-limited cultures was subsequently soaked in Methanol/KOH, HCl and EDTA for at least 8 h.

Xenosiderophore promoted growth was tested in iron-deficient media by the addition of 10 μM of iron complexed by an excess of the corresponding desferri-siderophore. Subsequently, the final optical density of the culture was determined. Viable counts as a measure for the cell number could not be applied to *H. salinarum*, because salt precipitates very soon after incubation of solid media on plates at 37°C. In order to eliminate possible iron complexing effect of citrate, normally used in *H. salinarum* medium, low-iron media for siderophore uptake experiments were prepared with 1.8 g glucose per litre instead of citrate. Iron siderophore complexes were prepared as a stock solution of 10 mM Fe and 20 mM desferri-siderophore in the case of a 1:1 complex, 25 mM desferri-siderophore in the case of the 2:3 complex of rhodotorulate and 50 mM of the desferri-siderophore in the case of the 1:3 complex of dihydroxy benzoic acid.

Siderophore enrichment and detection

The supernatant (1 l) of *H. salinarum* cells grown under iron limitation was passed over a XAD-2 (BioRad) column to enrich hydrophobic organic macromolecules, to which most siderophores belong. The organic molecules were eluted with methanol, after the removal of the solvent by roti evaporation dissolved in water and probed for the presence of siderophores. In a second experiment, the culture supernatant was dialyzed against water, using a cellulose ester membrane with a molecular weight cut off of 100 Da (Spectrapor). After reducing the volume, the residual retendate was tested for the presence of siderophores.

Presence of siderophores in solution was tested with Chromeazurol-S as described (Schwyn and Neilands 1987). In addition culture supernatants were analyzed spectrophotometrically after the addition of ferric chloride, because ferric siderophores display typical absorption patterns between 400–600 nm.

Ferric iron reduction assays

Ferrireductase experiments with whole cells were performed with bathophenantroline or ferrozine as cheletors in a high salt buffer (4.28 mM NaCl, 0.027 mM KCl, 0.081 mM $MgSO_4$, pH 7.4) at 37°C as described (Timmerman and Woods 1999; Worst et al. 1998). As assay for the presence of multicopper oxidases, the colorimetric detection of the reaction with *p*-phenylenediamin was used (Grass and Rensing 2001). Purified membranes were resuspended in 3 M KCl, 10 mM MOPS, 250 μM $CuSO_4$, pH 7.2 and the increase of the absorption at 570 nm at 37°C was monitored.

Uptake-assay for [^{55}Fe]/[^{14}C]-citrate

Cells from 100 ml culture were harvested (15 min, 9,500 g, 4°C) and resuspended in 51.5 ml of low-iron medium. The culture was then preincubated in a gyratory shaker at 37°C for 1 h at 140 rpm. 2×1 ml suspensions were collected for protein determination. 0.5 ml of a Fe-citrate stock solution (84 kBq [^{55}Fe], 42 kBq[^{14}C]-citrate, 1 mM Fe: 20 mM citrate) was added to start the uptake assay. The first sample ($t = 0$) was taken directly after the addition of the radioactivity (time required: approximately 15 s) and used to quantify unspecific absorption which was then subtracted from all the other sample values. 1 ml samples were sucked through a nitro-cellulose filter (0.45 μm pore size, Sartorius, Göttingen), washed with 10 ml of basal salt solution (250 g NaCl, 3 g Na_3citrate, 2 g KCl, 20 g $MgSO_4$ per litre, pH 7.4), placed on the bottom of a scintillation vial, incubated over night with 8 ml of scintillation liquid (OptiPhase ''HiSafe'' 3, Perkin-Elmer) and measured in a scintillation counter (Wallac Winspectral α/β).

Concentration dependence of iron uptake was determined in a glass test tube by adding 0.5 ml of ''double concentrated'' cells (200 ml batch was harvested and resuspended in 52 ml of low iron medium, 2 × 1 ml were removed for protein determination) to 0.5 ml of low iron medium containing [^{55}Fe]-citrate ranging from 0 μM to 100 μM. The test tubes were incubated at 37°C in a gyratory shaker for exactly 30 min at 160 rpm. The uptake process was stopped by adding 7 ml of ice cold basal salt solution followed by the immediate transfer on ice until the samples were further sucked through a nitro-cellulose filter and processed as described above.

For protein determination the above 2 × 1 ml samples were harvested (13,000 rpm, RT, table desk centrifuge), washed with basal salt solution (250 g NaCl, 20 g $MgSO_4 \times 7\ H_2O$, 3 g Na_3Citrate, 2 g KCl), centrifuged again, resuspended in 500 μl of Tris-maleate-buffer (3.5 M NaCl, 50 mM maleate, pH 7.2) and stored at –20°C over night. After thawing, cells were disrupted on ice with a Branson sonifier, equipped with a microtip for 5 × 30 s with 30 s break and the total amount of protein was determined with an Lowry based protein assay (DC Protein Assay, BioRad).

Estimate of iron accumulation in *H. salinarum*

Protein content per 10^9 cells is 200 μg, protein content per cell 2×10^{-13} g. Assuming a cylindric shape with 6×10^{-6} m length and 1.8×10^{-6} m in diameter, the cytoplasmic volume is 15.27×10^{-12} [cm^3]. This corresponds to 13.1 mg protein per [cm^3]. Assuming an iron accumulation 3 μMol within 2 h (Fig. 3), the cytoplasmic concentration of Fe will be 49 mM i.e., an over 1000-fold increase compared to the concentration in the medium.

Chemicals

[^{55}Fe], [1,5-^{14}C]-citric acid and the scintillation liquid was purchased from PerkinElmer (Rodgau-Jügesheim, Germany), HBED was supplied by Dojindo (Gaithersburg, USA). All other chemicals were purchased from Sigma or Merck.

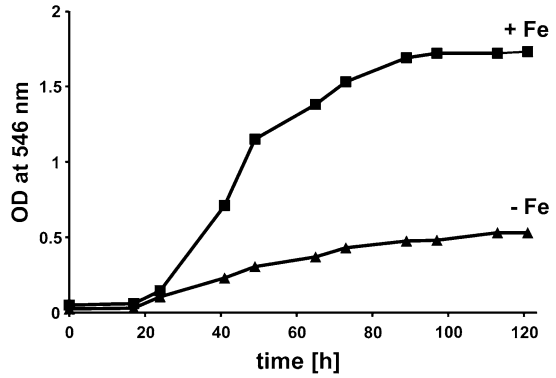

Fig. 1 Growth under iron-limiting and iron-sufficient growth conditions. *H. salinarum* was grown in the presence of 180 μM of iron (+Fe) and under iron-limiting conditions (< 0.5 μM as determined by colorimetric assay) (-Fe). The optical density of the cell culture was monitored at 546 nm as a measure of growth

Results

Role of iron concentration for growth of *H. salinarum*

Growth of *H. salinarum* was limited due to the lack of iron, but in contrast to *E. coli*, which survives at maximum four to five subsequent passages in low-iron medium (Hubbard et al. 1986), *H. salinarum* throve under low iron conditions for months (cells transferred to new batch culturers after 4 days) (Fig. 1).

A selection of iron-II and iron-III complexes was tested for the ability to promote or inhibit growth of *H. salinarum*. We added 10 μM biphenyl, bathophenantrolin, ferrozin, ferrioxamine E and HBED (*N*,*N*′-Bis(2-hydroxybenzyl) ethylendiamine-*N*,*N*′-diacetic acid) to iron-free medium and monitored the optical density over 136 h. HBED, like the other chelators was added in a roughly 20-fold excess over iron. The ferric complex of ferrioxamine E was used because it does not support growth of *H. salinarum*. The siderophore binds iron with high affinity leaving no adventitious iron in the medium. Because growth (measured as optical density) was not further lowered than in an iron-limited medium we could exclude the presence of significant amounts of trace iron.

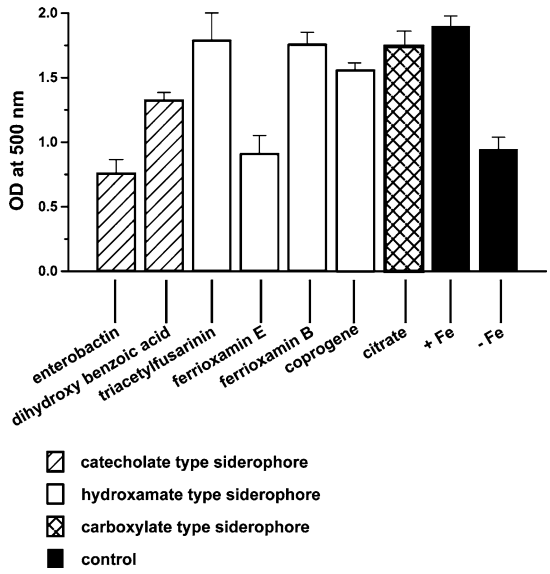

Fig. 2 Growth promotion in the presence of xenosiderophores. Low-iron cultures were supplied with xenosiderophores ($[Fe^{3+}]$ = 10 μM, excess of ligand) Siderophores were picked exhibiting different chelating groups and complex formation constants. In the control batch, iron was added as $FeCl_3$

Biosynthesis of siderophores

Experiments to detect siderophores in culture-supernatants from *H. salinarum* failed. Analyses of supernantants, of XAD-2 chromatographed fractions of the supernantant and of supernatant concentrates did not show CAS-reactivity.

Iron transport mediated by xenosiderophores

Growth promotion of *H. salinarum* was achieved with xenosiderophores (Fig. 2). We tested iron chelates belonging to three major classes of siderophores: catecholate siderophores like enterobactin, hydroxamates like ferrioxamine B, coprogen or triacetylfusarinin and carboxylate-type siderophores like rhizoferrin and citrate. Enterobactin and ferrioxamin E did not promote growth in comparison to growth under conditions of iron deficiency. The best growth promotion was achieved employing triacetylfusarinine, a hydroxamate based siderophore, produced by fungi of the Fusarium group. Class-specific siderophore-mediated iron transport was not observed. An uptake mechanism involving a receptor for a special siderophore class can be, therefore, ruled out. The stability constants of the ferric siderophore complexes employed in this study range from 10^{52} for enterobactin to 10^{12} for citrate (depending on the metal/ligand ratio). It is important to note, that ferrioxamine B supports growth of *H. salinarum* whereas the cyclic form, ferrioxamine E, did not. Ferrioxamine B is a linear hydroxamate trimer with a terminal amino group which is likely charged at an pH to be expected at the cell membrane. In contrast, ferrioxamine E is a cyclic trimer where the metal is less exposed to the exterior and an exchange kinetics is expected to slower than for the linear counterpart. The compound is not charged at acidic pH values between 4 and 7.

Uptake of [^{55}Fe]- and [^{14}C]- double labelled ferric citrate by iron deficient *H. salinarum*

First of all our data show that iron is transported in the cell but citrate, which represents the siderophore is not (Fig. 3). Citrate mediated iron uptake results in an intracellular iron concentration of

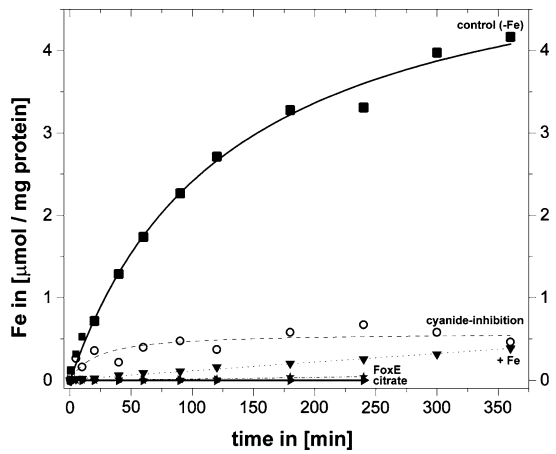

Fig. 3 Uptake of double-labelled [^{55}Fe]–[^{14}C]-citrate and inhibition with cyanide. [^{55}Fe] was added in a final concentration of 10 μM and two samples were withdrawn at the indicated timepoints. Here typical sample curves were shown. Fe-citrate: ^{55}Fe complexed with an excess of citrate, filled squares: 55Fe-uptake in an iron-deficient culture(-Fe), filled stars 14C-citrate uptake; CN: [^{55}Fe]-citrate + 2 mM KCN, open circles; Fe: iron uptake with cells grown under iron sufficient conditions, filled triangles (tip down); FoxE: [^{55}Fe] complexed with an excess of ferrioxamine E, filled triangles (tip up)

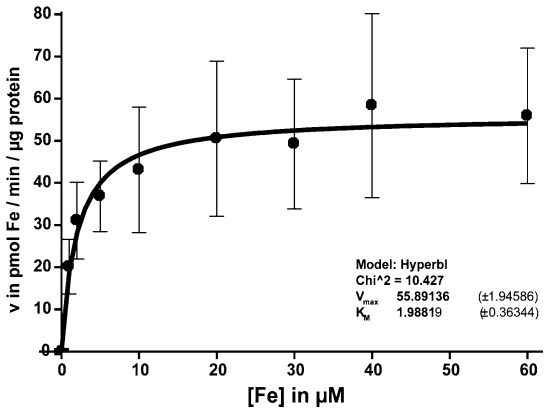

Fig. 4 Concentration dependent uptake of iron into halobacterial cells. Iron-uptake shows saturable kinetics: Determination of K_M and V_{max} (numerical values: see Table 1) was based on a hyperbolic least squares fit (χ^2 = 10.22). The mean of n = 3 experiments with double determinations is shown including standard deviations

approximately 40 mM (see Materials and methods). When iron is supplied as complex with ferrioxamine E no internalisation of iron into the cells could be measured. This observation was in line with the results of our growth experiments, where ferrioxamine E did not promote growth of *H. salinarum*. Moreover the uptake of iron into halobacterial cells, which had been grown in the presence of iron showed merely a slight linear increase which indicates that unspecific diffusion or binding to the cell wall accounts only for a very small part of the overall radioactive iron on or in the cell. The addition of 2 mM of cyanide blocks respiration of *H. salinarum* to 88.7% (Hubmacher et al. 2003) as determined by the reduced oxygen consumption and inhibits iron uptake at around 90%. This showed that iron uptake is an energy-dependent process either dependent on the respiratory chain or on the existence of the membrane potential.

Fe-uptake kinetics and Ga-inhibition

A Michalis-Menten type of concentration dependence was found for iron accumulation in *H. salinarum* (Fig. 4). Obviously, the uptake of iron shows saturation in the concentration range from 0 to 60 micromolar iron. Our results are in the range of other data originating for example from *E. coli* (Table 1). On the other hand extreme K_M values are reported for *N. crassa* or *M. smegmatis*. *P. denitrificans* is interesting, because it showed two different uptake systems for the same siderophore, a high-affinity system, up to 10 µM iron and a low-affinity system as well.

In order to obtain evidence for ferric ion reduction at the cell surface, uptake kinetics were performed in the presence of increasing amounts of gallium (Fig. 5). Gallium-III forms structurally very similar complexes with siderophores as ferric iron. However, the reduction potential of Ga^{3+} is outside the range of biological reductants. Therefore, Ga–citrate should inhibit uptake of iron if there is a reduction step at the cell-surface or the cytoplasmic membrane involved. In addition, the metal exchange kinetics of Ga complexes is much slower than of the ferric ion. If metal removal from the complex would be the velocity determining step of the uptake process one would expect inhibition at high Ga–citrate concentrations only. If, however, the reduction would be velocity determining, a strong inhibition effect of Ga should be expected. Figure 4 shows that no

Table 1 Kinetic parameters of siderophore transport in various systems

Organism	K_M [µM]	Vmax [pmol/(min × mg)]	Siderophore	References
H. salinarum	1.74 ± 0.74	53.5 ± 15.6	Citrate (xenosiderophore)	This work
E. coli	0.23	73	Enterobactin	(Ecker et al. 1986)
	1.4	22.9	Rhodotorulate	(Matzanke et al. 1984)
M. smegmatis	24.8	3.6	Ferricrocin	(Matzanke et al. 1999)
	6	–	Exochelin	(Stephenson and Ratledge 1979)
P. denitirifcans	0.24	108 (high affinity)	Parabactin	(Bergeron and Weimar 1990)
	3.9	494 (low affinity)	Parabactin	
N. crassa	5	200	Coprogen	(Huschka et al. 1985)
S. cerevisiae	0.5	2 (per OD at 600 nm)	Ferrioxamine B	(Lesuisse et al. 1998)
	0.2	9 (per OD at 600 nm)	Ferricrocin	

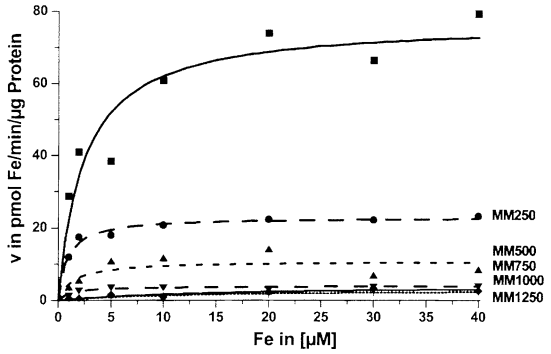

Fig. 5 Inhibition of iron-uptake with Ga–citrate To compete for a potential reductive iron uptake system, Ga–citrate (excess of ligand) was added in increasing amounts up to 1250 µM and the uptake kinetics of ^{55}Fe (10 µM with an excess of citrate as ligand) was determined

inhibition of iron-uptake with Ga can be achieved up to 125 µM. However, at concentrations of 259 µM and higher an inhibitory effect was observed (Fig. 5).

Discussion

This is the first study on the iron uptake system of the extremely halophilic archaeon *H. Salinarum.* Siderophores could not be detected in culture supernatants. However, *H. salinarum* is able to utilize a variety of xenosiderophores from different siderophore-classes and with different stability constants. A similar phenomenon was described for the marine bacterium *Aeromonas* (Stintzi et al. 2000). In that study 20 different heterologous siderophores were reported to show transport using a single uptake system. The data presented in this investigation indicate, that *H. salinarum* exhibits no preference for a special class of siderophores. In addition, siderophore mediated growth promotion in *H. salinarum* seems not to be dependent on the stability constant of the various ferric siderophore complexes. An uptake via ligand exchange is, therefore, unlikely. Moreover, it was shown by [^{55}Fe]/[^{14}C] double-label experiments, that the chelator citrate remains outside the cell and only the metal is taken up. This result implies that iron-III is released from its chelator at the membrane level. Taking into account that growth promotion was observed by adding various structurally very different siderophores exhibiting a wide range of complex formation constants, it is tempting to suggest one common iron transport mechanism across the cell membrane of *H. salinarum.* This process could be achieved by two different strategies: either iron transport across the cell membrane includes a reductive step which is very likely located at the cell surface or there exists a ligand exchange mechanism at the cell wall or at the membrane.

The question then arises, what are the components of this hypothetical redox-system and how are the different types of siderophores recognized? It is interesting to note here that in the genome of Halobacterium NRC 1 a gene (Pan1) exists, which is homologous to the Fet3p from *Saccharomyces cerevisiae* and other multi copper oxidases. Unfortunately, experiments to detect a ferric reductase activity failed. In general, there are big problems to apply standard enzymatic assays in a high-salt environment, which is normally essential to maintain optimal enzymatic activity of *H. salinarum.* A piece of information was provided by inhibition experiments with Ga. A 25-fold excess over iron was necessary to inhibit uptake of labelled iron significantly. The most likely explanation for this surprising finding is the reduced kinetic stability of the Fe–citrate complex compared to gallic citrate. In Fe^{3+}-high-spin complexes, the metal exhibits a d5 electronic configuration. Therefore, ferric ion is transferred much faster to the active site of a potential reductase than Ga. A second hint came from a comparison of iron uptake in *H. salinarum* mediated by the siderophores ferrioxamine B and ferrioxamine E. The latter one did not promote growth and did not contribute to iron accumulation. Ferrioxamine E has a cyclic structure with the iron atom buried inside, whereas in the linear structure of ferrioxamine B the metal is much more exposed to the medium. Therefore, an explanation for the described preference could be that the cyclic form of ferrioxamine E provides unfavourable access for a potential reductase. Similarly, in the more stable Ga–citrate, the metal-coordination site is much less accessable to attack by external agents than in the kinetically considerably more labile ferric–citrate complex.

The membrane protein patterns of membranes prepared from cells grown in the presence of the different types of ferric siderophores displayed no differences (gels not shown here). Moreover, membrane proteins of the low-iron control showed the same pattern as those prepared from cells grown with different siderophores. This indicates that no siderophore specific protein was expressed on the membrane level under low-iron growth conditions. Therefore, a non-specific membrane-level reductase might represent the general siderophore-iron acceptor. An additional indication for iron uptake by reduction is that growth of *H. salinarum* was strongly inhibited by tetrathiomolybdate, an inhibitor for multi copper oxidases like ceruloplasmin (Chidambaram et al. 1984).

If there would exist a ligand exchange mechanism at the cell wall or membrane site and if a protein would be involved in this process, the situation would be comparable to the reduction scenario. However, if iron would be transferred from a soluble to a membrane bound siderophore the process would be dependent on the differences in the complex formation constants as well as on the kinetics of the exchanging ligands (Stintzi et al. 2000). A membrane based ligand should posses a very high binding constant for iron-III. Initial experiments to isolate a ligand by extraction from halobacterial membranes failed.

A survey of the *Halobacterium* NRC1 (Ng et al. 2000) genome sequence uncovered various genes showing homology to iron uptake systems e.g. to iron uptake permeases and to ferrichrome and ferric enterobactin transport proteins. However, none of them was characterized on a functional level. Although there exists a homologue of a ferric enterobactin transport protein, we showed, for example, that enterobactin does not support growth of *H. salinarum* and accordingly it was not a source of iron. The same was shown for ferrichrome. Most of the annotated genes are homologous to ABC-transporters located in the inner, the cytoplasmic membrane, lacking the high substrate specificity of siderophore receptors located in the outer membrane of Gram negative bacteria. In a very recent report on a proteomic approach to elucidate expressed proteins in *H. salinarum* under iron-sufficient growth conditions, only one ''iron-binding'' protein with two predicted transmembrane helices was found (Goo et al. 2003).

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