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Iron-limited growth and kinetics of iron uptake in *Magnetospirillum gryphiswaldense*

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Abstract Growth and magnetite formation in *Magnetospirillum gryphiswaldense* MSR-1 were found close to the maximum at an extracellular iron concentration of 15–20 μM. Ferrous iron was incorporated by a slow, diffusion-like process. Several iron chelators including various microbial siderophores were unable to promote transport of iron into the cells. In contrast, spent culture fluids stimulated the uptake of ferric iron in iron-depleted cells at a high rate, whereas fresh medium and transport buffer were unable to promote iron uptake. However, no siderophore-like compound could be detected in spent culture fluids by the Chrome Azurol S assay. Ferric iron uptake followed Michaelis-Menten kinetics with a K_m of 3 μM and a V_{max} of 0.86 nmol min^{-1} (mg dry weight)$^{-1}$, suggesting a comparatively low-affinity, but high-velocity transport system. Iron incorporation was sensitive to 2,4-dinitrophenol and carbonylcyanide-*m*-chlorophenylhydrazone, indicating an energy-dependent transport process.

Key words Magnetic bacteria · *Magnetospirillum gryphiswaldense* · Magnetite biomineralization · Iron uptake

Abbrevations *CCCP* Carbonylcyanide-*m*-chlorophenylhydrazone · *CAS* Chrome Azurol S · *DNP* 2,4-Dinitrophenol · *NTA* Sodium nitrilotriacetate

Introduction

Iron is an essential element for bacterial growth because numerous proteins including cytochromes, catalases, peroxidases, superoxide dismutases, ribonucleotide reductases, and nitrogenases contain iron (Guerinot 1994). Because of its insolubility in oxic environments at neutral pH, iron is not easily available. Aerobic bacteria have developed various high-affinity iron-uptake pathways that function under iron-deficient conditions. Many bacteria produce iron chelators, termed "siderophores". In gram-negative cells, these are incorporated by means of specific receptor proteins located in the outer membrane (Neilands 1995). Assimilation of iron is strictly controlled because of the potential harmful effects of excess intracellular iron (Touati et al. 1995).

In magnetic bacteria, iron as a nutrient is peculiar not only because it is required as "biochemical" iron bound to proteins, but also because large amounts of the element are compartmentalized as "inorganic" iron within the magnetosomes, which are intracellular enveloped crystals of a magnetic iron mineral (Balkwill et al. 1980; Bazylinski 1995). While, for example, the total iron content in *Escherichia coli* is documented as being 0.005–0.022% of the dry weight (Hartmann and Braun 1981; Matzanke et al. 1989; Hudson et al. 1993), the iron content in magnetic bacteria may exceed 2% of the dry weight (Blakemore et al. 1979). Because of the extreme amount of iron that has to be transported, magnetic bacteria can be expected to use very efficient uptake systems that might be different from known transport mechanisms in other bacteria. To understand the biomineralization of magnetic particles, a detailed knowledge of iron uptake in magnetic bacteria is necessary. The small number of studies of iron uptake in magnetic bacteria does not correspond to its importance.

Paoletti and Blakemore (1986) initially reported the production of a hydroxamate-type siderophore under iron-rich conditions in *Magnetospirillum* (formerly *"Aquaspirillum"*) *magnetotacticum*. In contrast, *Magnetospirillum* sp. AMB-1 (Nakamura et al. 1993) did not produce siderophores under either iron-deficient or iron-rich conditions. Most recently, an iron-regulated gene that might be involved in iron transport in *Magnetospirillum* sp. AMB-1 has been described (Nakamura et al. 1995). However, the metabolism of iron in magnetic bacteria is still poorly understood, and the mechanisms of iron uptake remain to be elucidated.

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We are studying the biomineralization of magnetite (Fe_3O_4) in the magnetic bacterium *Magnetospirillum gryphiswaldense* MSR-1, which has been isolated from a freshwater sediment (Schüler and Köhler 1992). *M. gryphiswaldense* is a member of the α-subdivision of gram-negative eubacteria and has been found to contain intracellularly up to 60 cubo-octahedral magnetite crystals that are 45 nm in diameter and enveloped by a membrane (Schleifer et al. 1991). Magnetite biomineralization in *M. gryphiswaldense* involves several steps. To accumulate iron inside the magnetosome vesicles, the iron must be transported across the outer, cytoplasmic, and magnetosome membranes. This study was initiated to investigate the iron supply in *M. gryphiswaldense* and to understand the initial steps involved in the uptake and subsequent precipitation of this element.

Materials and methods

Organism and growth conditions

Magnetospirillum gryphiswaldense strain MSR-1 (DSM 6361), which has been isolated previously from a freshwater sediment (Schüler and Köhler 1992), was used throughout all experiments. Cells were grown at 30° C in the following medium containing (per liter) 0.5 g KH_2PO_4, 1.0 g sodium acetate, 1.0 g soy bean peptone (Merck), 0.1 g NH_4Cl, 0.1 g $MgSO_4 \times 7\ H_2O$, and 0.1 g yeast extract. The concentration of trace iron in the growth medium was below 1 μM. This was confirmed spectrophotometrically by α,α′-dipyridyl (Spectroquant Iron-Test, Merck) and by atomic absorption spectroscopy. When necessary, extra iron was added as $FeSO_4$ in various amounts.

For iron-uptake experiments, cells were grown with moderate iron depletion, i.e., the additional iron source was omitted from the growth medium without added chelating compound. Under these conditions, lower growth rates and final cell yield were achieved, but initial uptake rates were found to be two to three times higher than those of iron-sufficient cells grown in the presence of an added iron source.

For iron-limited growth studies, α,α′-dipyridyl was added to the medium in various amounts to enhance the effect of iron deprivation (Chart et al. 1986). Cells were moderately aerated by shaking during exposure to air.

Iron transport assay

Cells of a 24-h culture (mid-exponential growth phase) were harvested, washed, and diluted in transport buffer [5 mM Mops, 6 mM Na-acetate (pH 7.0)] to an OD_{400nm} = 0.5. Mops was used because of its weak complexing properties with iron. Alternatively, in some experiments transport buffer was replaced by either spent or fresh growth medium. Spent growth medium was obtained from a stationary culture by removing the cells by centrifugation and adjusting to pH 7.0. With the transport buffer and media described above, cells retained their viability, while other buffer compounds tested were found to inhibit growth.

After 5 min preincubation of the cells at 30° C, the transport was started by adding the radioactive iron to a final concentration between 0.5 and 100 μM. ^{55}Fe was applied as $FeCl_3$ or as a chelate. Citrate, 2,3-dihydroxybenzoate, pyrophosphate, 1,3,4,5-tetrahydroxycyclohexane carboxylate (quinate), and EDTA were used as chelating compounds in 100- to 500-fold excess to ^{55}Fe. In addition, various ferric siderophores including coprogen, ferrioxamine B, ferricrocin, ferrichrome, and rhodotorulic acid were used in the uptake experiments. Siderophores were kindly provided by K. Hantke and B. Matzanke (University of Tübingen, Germany). The ^{55}Fe-labeled iron chelates were prepared as previously described (Müller et al. 1984). To study the uptake of ferrous iron, 0.1 M ascorbic acid was added to the transport assay and to the iron-stock solution as a reductant.

At time intervals, samples of 0.2 ml were withdrawn, added to 5 ml of 0.1 M LiCl, 5 mM EDTA, filtered on 0.45-μl-pore-size filters (Sartorius), and washed once with 5 ml of 0.1 M LiCl, 5 mM EDTA. The filters were dried at 70° C, and the radioactivity was determined in a liquid scintillation counter.

Inhibition studies were performed in the same way with increasing concentrations of inhibitor. Carbonylcyanide-*m*-chlorophenylhydrazone (CCCP) and 2,4-dinitrophenol (DNP) were incubated with the cells for 60 min prior to addition of ^{55}Fe. In some experiments, 0.1 M sodium nitrilotriacetate (NTA) was used to suppress low-affinity uptake as is done routinely in iron-transport assays (Frost and Rosenberg 1973).

Detection of siderophores

Spent culture fluids were checked for the occurrence of siderophores by the Chrome Azurol S (CAS) assay of Schwyn and Neilands (1987). In this test, a decrease in the absorbance at 630 nm indicates a positive reaction.

Determination of growth and magnetite formation

Growth of cells was determined by measurement of the optical density at 400 nm. The average magnetic orientation of cell suspensions was assayed by an optical method as described previously (Schüler et al. 1995). According to this method, cells are aligned at different angles relative to the light beam by means of an external magnetic field. The ratio of the resulting maximum and minimum scattering intensities (C_{mag}) was previously demonstrated to be well correlated with the average number of magnetic particles and can be used for the semi-quantitative evaluation of magnetite formation (C_{mag} = 1 corresponds approximately to 10 particles per cell).

Results

Dependence of growth and magnetite formation on extracellular iron concentration

In order to establish iron-deficient and iron-limited growth conditions, cells were grown in the presence of various iron concentrations. Added $FeSO_4$ became rapidly oxidized but remained soluble in the growth medium throughout the incubation time up to a concentration of 100 μM. The rapid decrease of Fe(II) concentrations was followed by the Ferrozine test (Stookey 1970), a spectrophotometric method for determining ferrous iron.

When added iron was omitted, the growth medium was found to contain this element only in trace amounts less than 1 μM, a concentration generally considered to be sufficiently low for an iron-limited growth of bacteria (Neilands 1995). To enhance the effect of iron starvation, variable amounts of α,α′-dipyridyl, a chelator commonly used to restrict the physiological availability of iron (Chart et al. 1986), were added.

Growth and magnetism were found to be limited by the extracellular iron concentration (Fig. 1). In the presence of more than 40 μM α,α′-dipyridyl, growth was totally inhibited while chelator concentrations of between 1 and 10

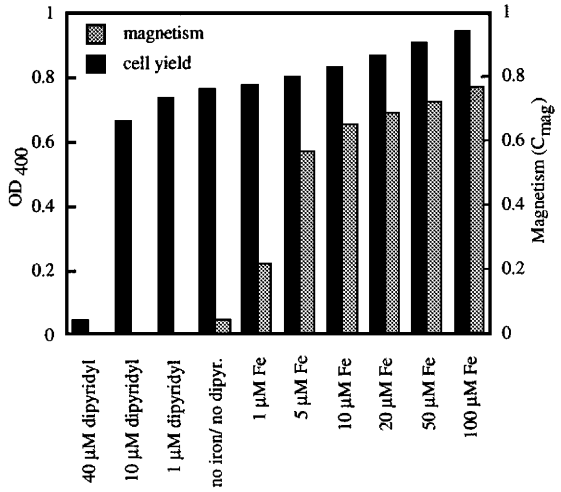


Fig. 1 Final cell yield and magnetism in cells of *Magnetospirillum gryphiswaldense* grown at various extracellular concentrations of α,α′-dipyridyl and iron. After 36 h incubation, growth and magnetism were determined by measurement of optical density (OD_{400}) and differential light scattering (C_{mag}), respectively

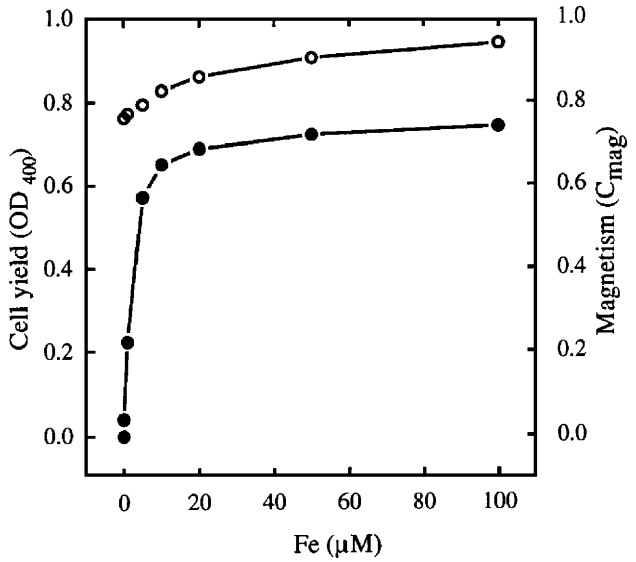


Fig. 2 Magnetism (●) and cell yield (○) in *M. gryphiswaldense* shown as functions of the extracellular iron concentration. Cells were grown for 36 h in the presence of various amounts of added iron

µM resulted in poor growth with no detectable magnetism. Growth in the presence of α,α′-dipyridyl could be restored by addition of excess iron. Hence, it can be excluded that growth inhibition was caused by toxic effects of the chelator and scarcity of nutrients other than iron. When α,α′-dipyridyl was omitted, increasing the iron concentration up to 100 µM resulted in faster growth and higher magnetite content, which could be monitored by differential light-scattering measurements. However, extracellular iron concentrations higher than 200 µM also prevented cells from growing.

Iron supply was found close to saturation between 10 and 20 µM iron. Iron concentrations above 20 µM iron only slightly increased cell yield and magnetism (Fig. 2). In the absence of both a chelator and an added iron source ("moderate iron depletion"), growth was found to be clearly limited by the trace amounts of residual iron. Nevertheless, cells still exhibited a weak magnetic orientation, as could be detected by differential light scattering. In electron micrographs, cells under these conditions were found to contain low numbers of small crystalline particles of an imperfect shape and prevalently in the superparamagnetic size range (not shown).

When cells were harvested by centrifugation, the pellet color differed, depending on the concentration of iron used for growth. Cells grown in the presence of α,α′-dipyridyl were pale white to pinkish, apparently due to a lack of magnetosomes and a reduced cytochrome content. Cells grown at 1 µM were colored orange to rusty red, while cells with a high magnetite content were dark brown to blackish.

Table 1 Initial rates of iron uptake in *Magnetospirillum gryphiswaldense* at different extracellular concentrations of $^{55}Fe(II)$

Fe(II) concentration (µM)	Initial uptake rate [nmol min^{-1} (mg dry weight)$^{-1}$]
5	0.06
10	0.12
20	0.16
50	0.2

$^{55}Fe(II)$ uptake

Uptake of ferrous iron was detected only at a very low rate in the presence of 0.1 M ascorbate (Table 1). Metabolic inhibitors had little or no effect, but uptake was completely inhibited by 100 µM sodium nitrilotriacetate (NTA), an iron chelator commonly used to abolish low-affinity uptake (Frost and Rosenberg 1973). A saturation of initial uptake rates at higher iron concentrations was not observed.

Uptake in the presence of chelators and spent culture fluids

Citrate, dihydroxybenzoate, EDTA, pyrophosphate, quinate, and the microbial siderophores ferioxamine B, ferricrocin, ferrichrome, rhodotorulic acid, and coprogen were tested for the stimulation of ferric iron uptake. Under experimental conditions described in this paper, iron uptake in the presence of any of the complexing compounds could not be detected at a significant rate in either transport buffer or fresh medium.

In contrast, spent culture fluids promoted an efficient uptake of ferric iron (Fig. 3). Addition of 100 µM NTA resulted in a significant reduction of the uptake rate but did not completely inhibit the incorporation of iron.

Effects of metabolic inhibitors

To determine if the uptake of ferric iron in the presence of spent culture fluid involves energy coupling, cells were

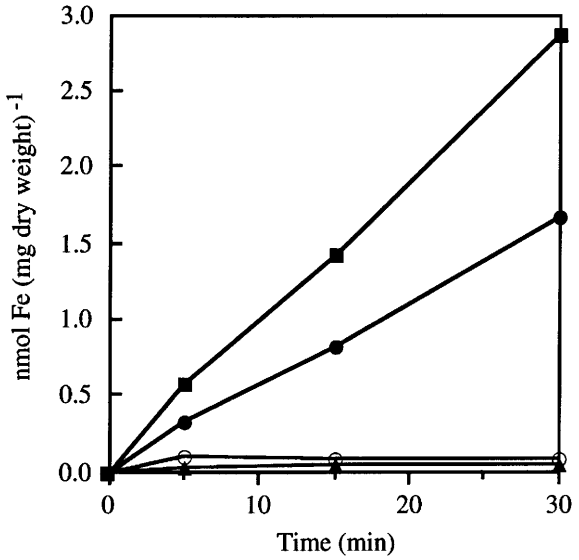


Fig. 3 Uptake of ^{55}Fe(III) by cells of *M. gryphiswaldense* from transport buffer (▲), fresh growth medium (○), spent culture fluids (■), and spent culture fluids plus 0.1 mM sodium nitrilotriacetate NTA (●). The extracellular concentration of iron used in the experiment was 2.5 μM

Table 2 Influence of metabolic inhibitors on initial rates of ^{55}Fe(III) uptake in *M. gryphiswaldense* from spent culture fluids (*CCCP* carbonylcyanide-*m*-chlorophenylhydrazone, *DNP*, 2,4-dinitrophenol)

Inhibitor	Concentration (mM)	Inhibition (%)
DNP	0.25	85–90
	1.0	92
	5.0	96
CCCP	0.025	55–60
	0.1	80–85
	0.5	94

incubated with various metabolic inhibitors prior to and during the transport experiments. Inhibition rates are shown in Table 2. Uptake was almost totally inhibited by 500 μM CCCP and 5 mM DNP.

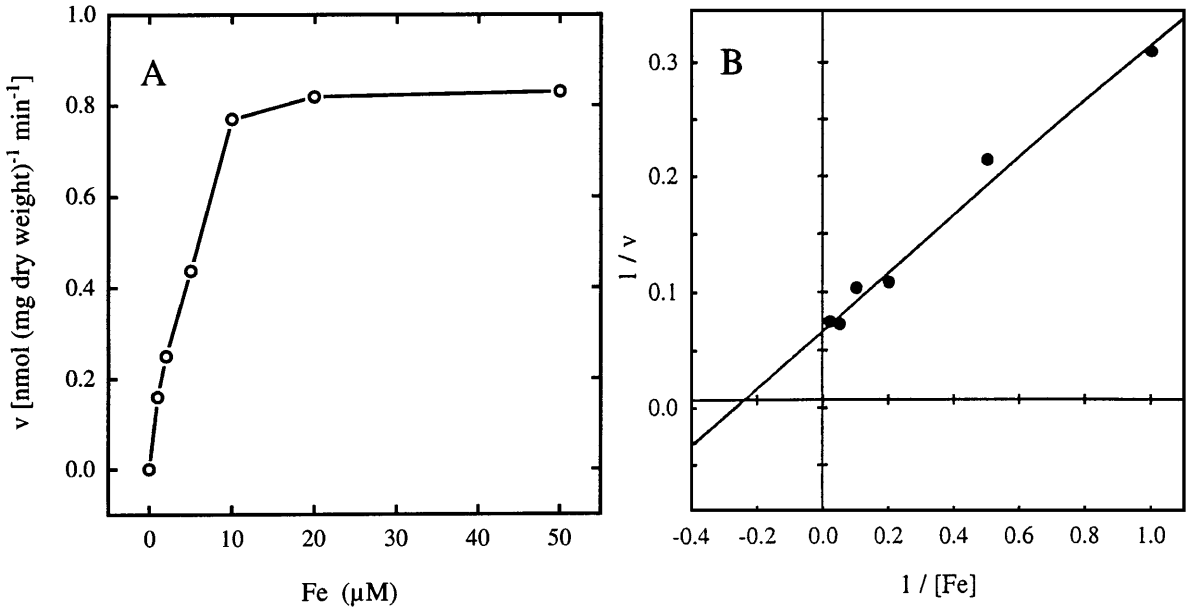
Kinetics of ferric iron uptake

Uptake experiments were performed at various ^{55}Fe(III) concentrations to determine saturation kinetics. A saturation in uptake velocity was observed at an extracellular iron concentration of 15–20 μM (Fig. 4A). Uptake of ferric iron in the presence of spent culture fluids was found to obey simple Michaelis-Menten saturation kinetics (Fig. 4B). The following apparent kinetic constants were calculated from the Lineweaver-Burk plot: K_m = 3 μM Fe (III), V_{max} = 0.86 nmol min^{-1} (mg dry weight)$^{-1}$.

Tests for siderophores

Spent culture fluid efficiently stimulated the uptake of iron and magnetic formation in the presence of 10–20 μM iron whereas fresh growth medium was unable to promote iron incorporation. We assumed that such a high transport rate for iron might not be correlated to a tight-binding iron siderophore. Nevertheless, we used three different test systems to exclude these siderophores: (1) the Chrome Azurol S (CAS) assay (Schwyn and Neilands 1987), (2) the iron(III) perchlorate assay for hydroxamate sideropho-

Fig. 4 **A** Concentration-dependent kinetics of ^{55}Fe(III) uptake into *M. gryphiswaldense* from spent culture fluids. **B** Lineweaver-Burk plot of kinetic data of ^{55}Fe(III) uptake. Initial iron-uptake rates were measured at various concentrations in the presence of spent culture fluids. The following apparent kinetic constants were calculated from the Lineweaver-Burk plot: K_m = 3 μM Fe(III), V_{max} = 0.86 nmol min^{-1} (mg dry weight)$^{-1}$



res (Atkin et al. 1970), and (3) the Arnow assay for catecholate siderophore (Payne 1994). In the CAS assay, the presence of high-affinity chelating compounds in the test solution is indicated by a decrease in the absorbance at 630 nm. Siderophores at different concentrations were used for positive controls. The assay revealed that complexing properties of the spent culture fluids were only slightly altered as compared to the fresh growth medium, i.e., no siderophores could be detected in significant amounts. Likewise, no siderophores (including no α-Keto acids; see Drechsel et al. 1993) were detected using the latter two assays (M. Gassmann, unpublished results). In addition, it was impossible to establish an agar plate assay with *Magnetospirillum gryphiswaldense* which generally cannot be grown on the surface of solid media. It is very difficult to find and enrich an unknown probable metabolic product of a complex medium. We, therefore, are trying to establish growth and magnetite formation of *M. gryphiswaldense* in minimal media and hope to identify the compound involved in iron transport.

Discussion

Growth and magnetite formation of *Magnetospirillum gryphiswaldense* depended on the extracellular iron concentration over a wide range. The maximum in growth and magnetite formation occurred at 100 μM Fe, but both were found close to saturation at 15–20 μM. Raising the iron concentration beyond this value resulted only in a minor increase in final cell yield and magnetosome content, while iron concentrations higher than approximately 200 μM were growth-inhibiting. We cannot exclude the possibility that life-threatening radicals were generated by oxidation in the presence of high concentrations of Fe(II); our aim was to find the high concentration limits of iron with respect to cell growth and magnetism and not to elucidate the mechanism of cell toxicity. The iron concentration sufficient for unlimited growth was approximately tenfold higher than, for example, that of *Escherichia coli*, where concentrations of 0.36–2 μM have been reported to compensate for growth limitation by iron (Lankford 1973; Hartmann and Braun 1981). The higher iron demand appears to be a consequence of the considerable amounts of iron required in magnetite biomineralization.

Most remarkably, imperfect magnetite particles were also formed inside the cells under conditions of moderate iron starvation (< 1 μM Fe), indicating that cells are able to accumulate iron from a very poor environment. At the same time, the formation of inorganic iron oxide seemed to compete with the metabolic iron requirement at the expense of reduced growth. Once iron has entered the cell, there is apparently no strict priority for its „biochemical" use, which might reflect a lack of intracellular control. The ecological significance of imperfect magnetite crystals that are not functional as navigational devices is not obvious under conditions of low iron stress.

These results demonstrate that iron supply in *M. gryphiswaldense* is crucial for growth and magnetite synthesis, both of which were optimal only in a narrow range of extracellular iron concentrations.

Magnetic bacteria are often found in sediments close to the oxic-anoxic transition zone, which is characterized by areas of low redox potential (Frankel and Bazylinski 1994). In such an environment, sufficient iron may exist in the more soluble ferrous form, which makes it easily available for microbial acquisition. We found that ferrous iron was incorporated by *M. gryphiswaldense* at low rates in the presence of 0.1 M ascorbate. At an extracellular concentration of 5 μM, the initial uptake rate for ferrous iron was 0.06 nmol min^{-1} (mg dry weight)$^{-1}$, whereas at the same extracellular concentration, ferric iron was incorporated at 0.43 nmol min^{-1} (mg dry weight)$^{-1}$. Uptake of Fe(II) was unaffected by metabolic uncouplers, implying a diffusion-like process. This process cannot explain the vast accumulation of the metal in fast-growing cells. However, it cannot be excluded that under conditions occurring in nature, where cells multiply at very low rates, slow diffusion and subsequent magnetite formation might contribute to iron accumulation over a prolonged time period. In addition, such an unregulated influx of iron by diffusion, which might lead to harmful effects, may explain the sensitivity of cells to higher extracellular concentrations of iron.

Cells grown at moderate iron depletion took up ferric iron very efficiently in the presence of spent culture fluids. The apparent V_{max} was 0.86 nmol Fe min^{-1} (mg dry weight)$^{-1}$. This is relatively as high compared to that of *E. coli* and other gram-negative bacteria, where V_{max} for high-affinity iron transport has generally been reported to be between 0.05 and 0.09 nmol Fe min^{-1} (mg dry weight)$^{-1}$ (Frost and Rosenberg 1973; Hancock et al. 1977; Winkelmann 1991). In contrast, V_{max} for a low-affinity iron-uptake system in *Paracoccus denitrificans* was determined with 0.494 nmol min^{-1} (mg dry weight)$^{-1}$ (Bergeron and Weimar 1990). The apparent K_m = 3 μM Fe falls within the range of values reported for siderophore-mediated iron transport. These extend from the value for the extremely high affinity system for ferric schizokinen (K_m = 0.04 μM) in the cyanobacterium *Anabaena* sp. (Lammers and Sanders-Loehr 1982) to those for comparatively low affinity systems, such as that for ferric-coprogen transport in the fungus *Neurospora crassa* (K_m = 5 μM; Huschka et al. 1985). As is characteristic for a low-affinity system, iron uptake was reduced in the presence of the chelator NTA, and saturation could be achieved at comparatively high iron concentrations of 10–20 μM. This finding is consistent with the maximum growth rate observed at similar concentrations. Strikingly, these results match the approximate abundance of free iron in the natural habitat of magnetic bacteria, a concentration which has been reported for freshwater sediments with 20 μM (Blakemore et al. 1979; Schüler 1994). This might reflect adaptation to the relevant abundance of iron under conditions occurring in nature. The uptake of ferric iron in *M. gryphiswaldense* was an active process. The strong inhibition by CCCP and 2,4-DNP suggests that energy coupling was involved.

None of the tested chelators was able to stimulate the incorporation of iron. To some extent, this was an unexpected result because ferric citrate, ferric quinate, and other iron complexes are reported to serve as the sole iron source for cultivation of *Magnetospirillum ("Aquaspirillum") magnetotacticum* MS-1 (Blakemore et al. 1979) and *Magnetospirillum* sp. AMB-1 (Nakamura et al. 1993). When added to the growth medium of *M. gryphiswaldense*, both ferric citrate and ferric quinate provided growth and moderate magnetism. However, final magnetosome content was found to be higher when the complexing compounds were omitted and iron was added to the medium as free salt (not shown here). Our data suggest that iron cannot be taken up as complexes of citrate or quinate. It is, therefore, possible that iron is released from the complex or that the chelator is degraded during growth of the cells. A possible explanation might be that, although the chelator keeps the iron soluble, its relatively low affinity for iron produces a transient pool of free Fe(III). This free iron might be transported by a system that does not recognize the ferric complex itself, as is reported for ferric citrate utilization in *Rhodobacter sphaeroides* (Moody and Dailey 1984).

The competence of the growth medium in supporting the uptake of iron was found to be altered by the metabolic activity of *M. gryphiswaldense*. Spent culture medium from cells grown at moderate iron depletion promoted uptake of ferric iron, while fresh growth medium did not. Initially, it was assumed that this growth-dependent modification of the medium was caused by the excretion of siderophore-like compounds, as similarly reported for hydroxamate production under iron-rich conditions in *Magnetospirillum ("Aquaspirillum") magnetotacticum* (Paoletti and Blakemore 1986). However, so far we have been unable to demonstrate that siderophores are formed by cells of *M. gryphiswaldense*. None of the siderophores tested in the transport assay was found to be efficient in the promotion of iron uptake. The CAS assay for siderophores revealed that the complexing properties of the medium were not significantly modified, whereas the addition of siderophores to the medium yielded a strong positive reaction. This is consistent with the finding of Nakamura et al. (1993), who could not detect any siderophores in spent culture medium of the related *Magnetospirillum* sp. AMB-1. The possibility remains that *M. gryphiswaldense* may produce a siderophore under some undetermined growth conditions. However, it seems unlikely that the incorporation of high amounts of iron such as that required for magnetite synthesis is mediated by siderophore-dependent systems, which usually are involved in high-affinity but low-capacity transport (Winkelmann 1991). Therefore, it can be speculated that the iron uptake relevant to magnetite formation is of a different type, i.e., without the involvement of siderophore production known from other bacteria (Wee et al. 1988; Guerinot 1994).

Since iron was found to exist in the ferric form throughout the growth period of bacteria, as analyzed by the Ferrozine method (data not shown), effects of the redox state of iron can be excluded. Aside from siderophore-mediated iron transport, some naturally occurring organic acids such as pyruvate and malate or α-ketoacids are also able to bind external iron and facilitate its assimilation by bacteria (Briat 1992; Drechsel et al. 1993). Thus, modification of the medium might be a nonspecific effect caused by cellular metabolism. During growth, acetate and amino acids are consumed from the complex medium and replaced by excreted organic compounds with weak complexing activity that keep the iron easily available or that might serve as carriers for cellular uptake systems.

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