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# Transport and utilization of rhizoferrin bound iron in *Mycobacterium smegmatis*

Berthold F. Matzanke[1], Rudolf Böhnke[2], Ute Möllmann[3,*], Volker Schünemann[4], Gisbert Schumann[3], Alfred X. Trautwein[4] & G. Winkelmann[2]

[1]*Isotopenlabor, Technisch-Naturwissenschaftliche Fakultät, Medizinische Universität, D-23538 Lübeck, Germany*
[2]*Mikrobiologie/Biotechnologie, Eberhard-Karls-Universität, D-72076 Tübingen, Germany*
[3]*Hans-Knöll-Institut für Naturstoff-Forschung e.V. (HKI), D-07708 Jena, Germany*
[4]*Institut für Physik, Medizinische Universität, D-23538 Lübeck, Germany*
[*]*Address for correspondence (Tel.: (+49)3641-656656; Fax: (+49)3641-656652; E-mail: moellman@pmail.hki-jena.de)*

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**Abstract**

Transport and metabolization of iron bound to the fungal siderophore rhizoferrin was analyzed by transport kinetics, Mössbauer and EPR spectroscopy. Saturation kinetics ($v_{max} = 24.4$ pmol/(mg min), $K_m = 64.4\mu$M) and energy dependence excluded diffusion and provided evidence for a rhizoferrin transport system in *M. smegmatis*. Based on the spectroscopic techniques indications for intracellular presence of the ferric rhizoferrin complex were found. This feature could be of practical importance in the search of novel drugs for the treatment of mycobacterial infections. EPR and Mössbauer spectroscopy revealed different ferritin mineral cores depending on the siderophore iron source. This finding was interpreted in terms of different protein shells, i.e. two types of ferritins.

## Introduction

Iron is an indispensible cofactor for a wide range of enzymatic reactions in almost any organism. Under aerobic conditions the bioavailability of this metal is, however, extremely restricted due to the low solubility of iron minerals (Matzanke 1991; Neilands 1982; Raymond *et al.* 1984). Thus, microorganisms have evolved siderophores, low-molecular-mass complexing agents exhibiting extraordinary high complex formation constants for ferric iron (Matzanke *et al.* 1989; Matzanke 1994; Neilands 1982; Winkelmann 1991). The major role of siderophores is extracellular solubilization from minerals or organic substrates and transport of the metal into microbial cells. Siderophore mediated iron uptake is both a receptor- and energy-dependent process which is strictly regulated (Braun & Hantke 1991; Bagg & Neilands 1987; Ecker *et al.* 1986; Hall *et al.* 1987).

In mycobacteria membrane-bound siderophores (mycobactins) and extracellular siderophores (exochelin MS and MN) are present (Barclay & Ratledge 1983; Macham *et al.* 1975; Ratledge 1982; Snow 1970; Sharman *et al.* 1995a,b). Unfortunately, the siderophore iron-transport mechanisms in these medically important Gram positive bacteria are not well understood (Hall & Ratledge 1987; Wheeler & Ratledge 1994). In addition, the intracellular iron-transfer pathways remain to be uncovered.

Not only those siderophores that are produced by the organism itself can be utilized but also siderophores that are synthesized by other microorganisms (xenosiderophores). In the search of novel antibiotics for mycobacterial infections we have previously analyzed and could identify a variety of iron uptake and intracellular iron transfer mechanisms of xenosiderophores in *Mycobacterium smegmatis* (Matzanke *et al.* 1998). In the present study

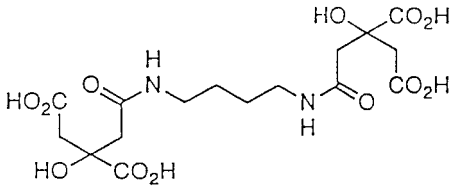

*Figure 1.* Structural formula of rhizoferrin.

we have focussed our interest on rhizoferrin, which has been isolated recently from low-iron culture filtrates of *Rhizopus* strains and other members of the zygomycetes (Figure 1) [Drechsel *et al.* 1992; Thieken & Winkelmann 1992). In this compound two citric acid residues are linked to putrescin. Rhizoferrin is very interesting because a relatively facile synthesis of antibiotic aducts based on this compound seems to be feasible.The results presented in this paper indicate that ferric rhizoferrin is transported as a whole into *M. smegmatis.*

## Materials and methods

### *Chemicals*

$^{57}$Fe (95% isotopically pure) was from Wissenschaftliche Elektronik GmbH (Starnberg, Germany) and [$^{55}$Fe]$Cl_3$ (carrier-free, in 0.1 M HCl, 0.6 mCi/ml) from Amersham Buchler (Braunschweig, Germany). Ethylenediaminetetraacetic acid $Na_4$-salt (EDTA) was purchased from SERVA (Heidelberg, Germany) and CHELEX 100 from Biorad (Munich, Germany). All other reagents were of analytical grade and purchased from Merck (Darmstadt, Germany) if not stated otherwise. Cellulose nitrate filters (0.45 $\mu$m) were from Sartorius (Göttingen, Germany). Delrin (E.I. du Pont de Nemours & Co., Inc. Wilmington, Del., USA) Mössbauer sample holders were manufactured in the machine shop of the local department. Doubly distilled water was used throughout. All glassware used during the experiments was washed with KOH, HCl, EDTA and triply rinsed with water to eliminate adventitiously bound iron. Rhizoferrin was purified as described (Drechsel *et al.* 1992; Thieken & Winkelmann 1992). [$^{55}$Fe]-labeled rhizoferrin was prepared by adding a solution of desferri-rhizoferrin (60 $\mu$l, 2 mM) and [$^{55}$Fe]$Cl_3$ (60 $\mu$l, carrier-free, in 0.1 M HCl, 22.2 MBq/ml, Amersham, UK) to a solution of unlabelled rhizoferrin (500 $\mu$l, 2 $\mu$mol/ml). After mixing the pH of the solution was adjusted with MOPS (final concentration 1 mM) to 7. A [$^{57}$Fe](III) stock solution was obtained by dissolving metallic [$^{57}$Fe] in a small volume of $HNO_3$/HCl (1:2[vol/vol]). The pH of the solution was adjusted to 1.0 with KOH and the Fe(III)-concentration determined spectrophotometrically with desferrioxamine B in 1 mM MOPS buffer (pH 7) at $A_{428}$. Synthesis of [$^{57}$Fe]rhizoferrin was achieved by mixing of [$^{57}$Fe](III) with an fivefold molar excess of aqueous desferri-rhizoferrin. The pH was adjusted to 7 and the solution was sterile filtered.

### *Strain and growth conditions*

Wild type strain *M. smegmatis* HKI 0056 taken from the institute's culture collection was maintained on nutrient broth (NB) agar slants (DIFCO, Detroit, USA). Precultures were grown in baffled Erlenmeyer flasks with gentle shaking at 37 °C in NB medium (per liter: NB, 8 g; NaCl, 5 g). The same conditions were employed for growth in mineral salts medium (MM) which was prepared according to Hall and Ratledge (1982) supplemented (per L) with $ZnSO_4 \cdot 7H_2O$, 2.02 mg; $MnSO_4 \cdot 4H_2O$, 0.4 mg; $MgSO_4 \cdot 7H_2O$, 0.4 g. For uptake studies cells were grown in low-iron mineral salts medium. Glass beads were added in order to minimize the formation of aggregates. The iron concentration of the medium and of the glycerol stock solution was kept at $10^{-6}$ M by passing the solutions separately through a CHELEX 100 column to remove $Fe^{3+}$.

### *Transport experiments and spectroscopic measurements*

Cells were taken from agar slants and grown in NB medium for 5 days. After transfer to MM (2% inoculum) cells were grown for additional 5 days. Taking a 5% inoculum of this culture *M. smegmatis* was cultivated for 6 days in iron-deficient MM, washed by centrifugation, resuspended in fresh medium and grown for additional 4 h. Aliquots of this suspension were taken for the transport assay. Transport was started by the addition of various quantities of [$^{55}$Fe]rhizoferrin (specific activity 1.33 kBq/nmol). Samples (1 ml, corresponding to approx. 1.2 mg dry mass) were taken 30 s after addition of radiolabel and then at 30 min intervals over a time range of 3 h. The cell suspension was filtered on cellulose nitrate membrane filters (0.45 $\mu$m) and washed twice with ice-cold 0.9% LiCl solution. The radioactivity on the filters was measured in a $\beta$-counter. Cell bound activity after 30 s was subtracted from the other values in order to eliminate

contributions caused by unspecific binding. For Mössbauer measurements more medium (2 l in 5 l baffled Erlenmeyer flasks) was required in order to harvest sufficient cell material (approximately 1 $cm^3$of packed cells). A short-time experiment (6 days of growth in iron deficient MM, 2 h of incubation with 20 $\mu$M [$^{57}Fe^{3+}$]rhizoferrin) as well as a long-time experiment has been performed. In the latter case the culture was grown 6 days in iron deficient MM, however, the inoculum was supplied with [$^{57}Fe^{3+}$]-rhizoferrin. After incubation, cells were cooled down to 4 °C within 2 min, washed, and transferred to Delrin Mössbauer sample holders. All sample volumes were about 1 ml. Sample thickness did not exceed 9 mm. The containers were quickly frozen in liquid nitrogen and kept in a liquid nitrogen storage vessel until measurement was done. The Mössbauer samples were either frozen cells or frozen solutions. The Mössbauer spectra were recorded in transmission geometry using a constant acceleration spectrometer and a 512-channel analyzer in the time-scale mode. The source (1.45 GBq [$^{57}$Co] diffused in Rh foil (Amersham Buchler)) was kept at room temperature. The spectrometer was calibrated against an $\alpha$-iron foil at room temperature yielding a standard line width of 0.24 mm/s. The Mössbauer cryostat was a helium bath cryostat (MD306, Oxford Instruments). A small field of 20 mT perpendicular to the $\gamma$-beam was applied to the tail of the bath cryostat using a permanent magnet. Isomer shift, quadrupole splitting $\Delta E_Q$, and percentage of the total absorption area were obtained by least-squares fits of Lorentzian lines to the experimental spectra. EPR-measurements were performed at X-band (9.4 GHz) using a cw-EPR spectrometer (Bruker ER200D SRC) equipped with a helium-flow cryostat (Oxford Instruments ESR9110). The data acquisition system was a local development based on a personal computer.

## Results

In a first set of experiments rhizoferrin uptake in *M. smegmatis* was analyzed by time and concentration dependent [$^{55}$Fe] transport assays. The transport was effectively inhibited by the respiratory poison azide (100 $\mu$M) (data not shown). The time window (45 min) for the transport assay differs from other bacterial and fungal siderophore transport systems (4 to 15 min) indicating lower uptake rates. The concentration dependent uptake rates of [$^{55}$Fe]-rhizoferrin in *M. smegmatis* are shown in Figure 2. They display

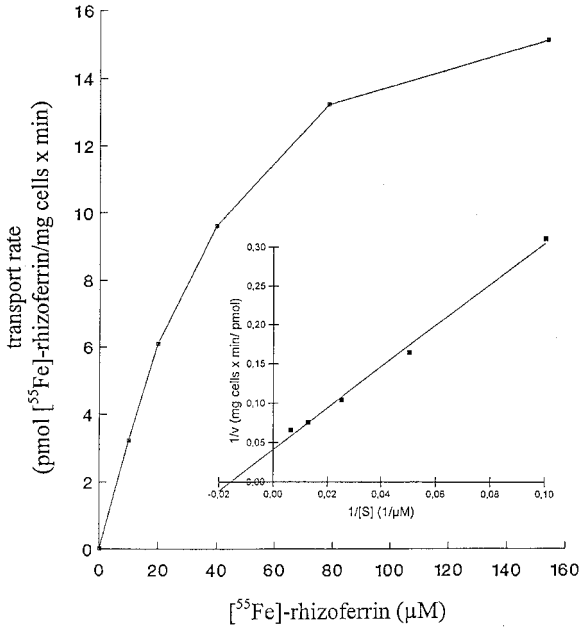


*Figure 2.* Concentration dependent uptake rates (30 min-value) of [$^{55}$Fe]-rhizoferrin in *M.smegmatis*. The insert shows a Lineweaver-Burk plot ($r = 0.987$) yielding $v_{max} = 24.39$ [pMol $^{55}$Fe/mg dry weight × min) and $K_m = 64.4$ $\mu$M.

saturation kinetics and the transport, therefore, can be formally described according to Michaelis–Menten. The insert represents a double reciprocal Lineweaver-Burk plot of the concentration dependent uptake rates enabling linear regression ($r = 0.987$). Michaelis-Menten constants. are: $v_{max} = 24.39$ pmol/(mg min) (maximum velocity) and apparent $K_m = 64.39$ $\mu$M. Saturation kinetics and energy dependence of rhizoferrin transport exclude diffusion and provide evidence for a transport system. The $v_{max}$ value is well within the range observed for bacterial systems (5 to 300 pmol/mg min) (Winkelmann 1986; Matzanke *et al.* 1989). However, the $K_m$-value is one order in magnitude higher than for the endogenous siderophore exochelin of *M. smegmatis*. From this the conclusion can be drawn that [$^{55}$Fe]-rhizoferrin exhibits a considerably lower affinity to its transport system than the endogenous exochelin. It is not clear on this level whether the two siderophores share the same transport system or whether a specific rhizoferrin receptor-transport system exists.

[$^{55}$Fe] transport measurements provide merely limited information about the mechanisms of transport. No information about intracellular iron utilization can be derived therefrom. However, EPR and in particular Mössbauer spectroscopy allow, in principle, nonde-

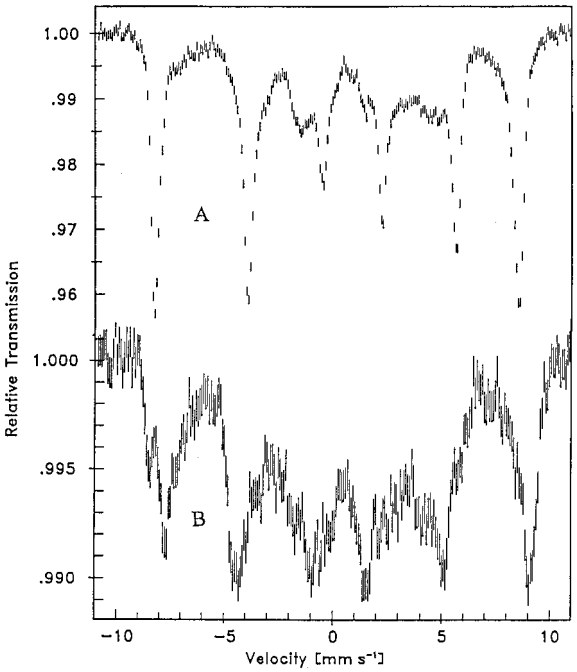


*Figure 3.* Mössbauer spectra of frozen solutions of the siderophores [$^{57}$Fe]-rhizoferrin (A) and of [$^{57}$Fe]-mycobactin J (B). The spectra were measured at 4.2 K in a field of 20 mT perpendicular to the $\gamma$-beam. The spectral patterns of the two siderophores differs enabling their discrimination in cell spectra.

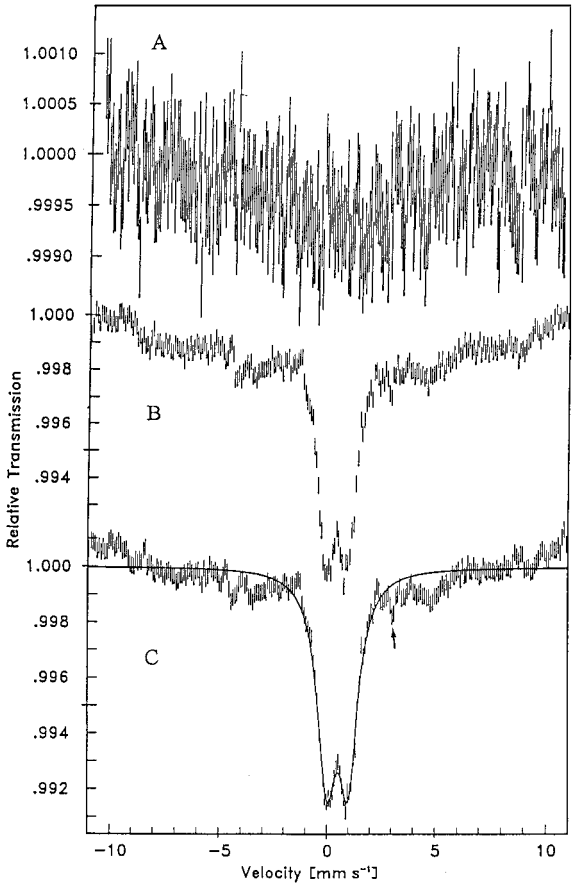


*Figure 4.* Mössbauer spectra of *M. smegmatis* grown for 6 days in iron deficient mm and supplemented with 20 $\mu$M [$^{57}$Fe]-rhizoferrin either for two hours prior to harvest (A) or with the inoculum (B). (C) represents (B) after subtraction of 7.5% rhizoferrin.

structive investigations of iron metabolism in situ, if the spectra are not too complex, i.e., if only few main metabolites are visible and discernible in the spectra. In fact, a wealth of information about the redox states of the metal centers, their spin configuration, symmetries of the ligand fields and types if ligands can be derived in general from Mössbauer spectra. In various studies we have demonstrated the potential of in situ Mössbauer spectroscopy (Matzanke 1994; Matzanke *et al.* 1991b, 1989b). In this work [$^{57}$Fe]-Mössbauer and EPR spectroscopy was employed in order to obtain more detailed information about the mechanisms of iron tranport and metabolization in *M. smegmatis*.

Figure 3 displays the Mössbauer spectra of frozen aqueous solutions of $^{57}$Fe-rhizoferrin and for comparison methanolic $^{57}$Fe-mycobactin J measured at 4.2 K. The spectra are typical for Fe(III) S=5/2 systems in the slow relaxation limit. The overall magnetic splittings of the ferric rhizoferrin complex is slightly larger (51.6 T) than that of mycobactin (50.6 T) enabling their discrimination in cell spectra. Figure 4a shows a Mössbauer spectrum of *M. smegmatis* grown for 6 days in iron-deficient medium and incubated for two hours with $^{57}$Fe-rhizoferrin. Merely a very broad and featureless resonance absorption is found indicating that little iron is accumulated in the cells. This parallels the results of $^{55}$Fe labeled transport experiments. Figure 4b displays the spectrum of cells grown for 6 days with $^{57}Fe^{3+}$-rhizoferrin. At a first glance one or two broad sextet ferric iron species can be observed, a quadrupole doublet in the center of the cell spectrum and a trace of high-spin ferrous iron (arrow). The quadrupole doublet in the center of the spectrum ($\delta = 0.51$ mm/s, $\Delta E_Q = 0.98$ mm/s) exhibits Mössbauer parameters typical of high-spin ferric iron. Employing a strip program we analyzed the magnetic subspectra for the presence of mycobactin and rhizoferrin. Attempts to subtract a mycobactin contribution from the cell spectrum was not successful. In contrast, subtraction of a rhizoferrin contribution from the cell spectra was possible (contribution 7.5±2% of total absorption area). However, the rhizoferrin contribution

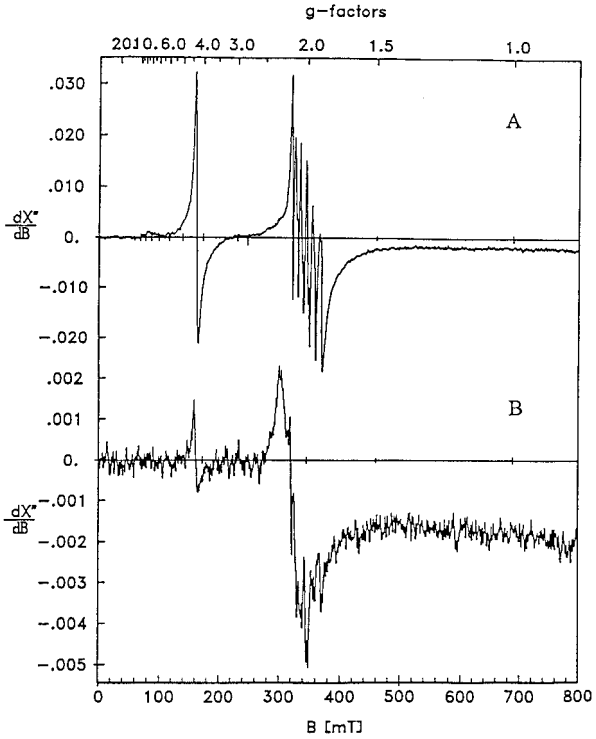


*Figure 5.* EPR spectrum of the sample shown in Figure 4B at 10 K (A) and 150 K (B). See text for detailed description.

does not affect very much the shape of the cell spectrum. Therefore additional evidence for the presence of a siderophore is required in order to validate this assignment (vide infra). The residual spectrum, shown in Figure 4C, is dominated by a broad sack. We attribute this feature, together with the ferric doublet, to a superparamagnetic transition, i.e., to a ferritin-type structure.

In order to confirm the presence of rhizoferrin and of a ferritin-type structure we have applied in situ EPR spectroscopy of the sample shown in Figure 4b. Ferritins cannot be detected in EPR at low temperatures because their EPR signals will broaden and decrease in intensity below the Nee′l (ordering) temperature and eventually will disappear (Matzanke 1994; Aasa & Vänngard 1975; BoasTroup 1971). Above the ordering temperature, however, EPR spectra of ferritins/bacterioferritins display a broad resonance near $g = 2.00$. Therefore, in situ EPR can be employed to identify ferritin/bacterioferritin. Figure 5A shows an EPR spectrum of the cell spectrum (Figure 4b) at 10 K. Two components are visible. The signal at $g = 4.3$ is typical of rhombic iron as found in siderophores and many other ferric iron compounds. In conjunction with the Mössbauer data it is plausible to assign this signal to rhizoferrin. Secondly, there is a signal around $g = 2.00$ with hyperfine splittings typical of $Mn^{2+}$. Figure 5B displays the EPR spectrum of the sample at 150 K. The same components as at 10 K are observed, although with a considerably lower signal intensity. In addition the signal at $g = 2.00$ is superimposed by a broad resonance which was not visible at 10 K. From this we conclude that a ferritin-type compound is present in *M. smegmatis* grown with rhizoferrin as sole iron source.

## Discussion

In a previous paper we have demonstrated that siderophores in Mycobacteria are taken up by diverse mechanisms which include ligand exchange (citrate/mycobactin) or rapid reductive removal (ferricrocin) (Matzanke *et al.* 1998). The results presented in this paper imply an uptake mechanism of rhizoferrin which excludes mycobactin as a temporary iron carrier. Moreover, the intracellular presence of small amounts of ferric rhizoferrin strongly suggests that the complex is transported as a whole to the mycobacterial cell interior. This adds a third siderophore transport mechanism in *Mycobacterium*. Even more important is the possible application which might arise from this observation. Worldwide an increase of mycobacterial infections is observed as well as a growing resistance against the few available antimycobacterial drugs (Bloom 1992; Kaufmann & Van Embden 1993; WHO 1993; Zhang *et al.* 1992). The observed permeation of the cell envelope of *M. smegmatis* by ferric rhizobactin might be utilized as a possible platform for novel antibiotics. Via synthetic or semisynthetic rhizoferrin-antibiotic-conjugates the problem of the relatively impermeable, hydrophobic cell envelope of mycobacteria might be circumvented.

The major component of the cell spectra corresponds to a ferritin-type compound. In fact, heme-containing ferritins (HCF) have been detected recently as major membrane proteins in a variety of mycobacteria including *M. paratuberculosis*, *M. leprae* and *M. avium* (Inglis *et al.* 1994, Pessolini *et al.* 1994). Moreover, bacterioferritin was identified by in situ Mössbauer spectroscopy as the major cellular iron pool of *M. smegmatis* after long-term growth with ferric citrate as sole iron source (Matzanke *et al.* 1998). However, the Mössbauer parameters of the bacterioferritin after growth with ferric citrate ($\delta = 0.53$ mm/s,

$\Delta E_Q$ = 0.71 mm/s) and after growth with rhizoferrin ($\delta$ = 0.51 mm/s, $\Delta E_Q$ = 0.98 mm/s) are distinct. In addition, the ferritin-type compound of the current study exhibits a magnetic contribution already at 4.2 K, whereas in the previous investigation superparamagnetic transition could be observed only below 4.2 K which is typical of *E. coli* type HCF (Bauminger *et al.* 1980). From this finding the conclusion can be drawn that the formed iron mineral core is different depending on the iron source in the medium. The difference can be caused either by different iron nucleation processes within the same protein shell or by nucleation in different proteins. Indeed, variation of the phopshate concentration was demonstrated to affect the mineral core structure *in vitro* (St. Pierre et all 1986; Mann *et al.* 1987; Rohrer *et al.* 1990). We exclude such a possibility in our systems, because the nutrient media employed were the same in all experiments. On the other hand, there is evidence for the coexistence of different classes of the ferritin superfamily within one organism. In *E. coli*, for example, a second type of ferritin is found which does not cross-react with, and exhibits only 14% identity to HCF (Izuhara *et al.* 1991; Hudson *et al.* 1993). Similar ferritins were detected in a variety of bacteria (Doig *et al.* 1993; Evans *et al.* 1995; Wai *et al.* 1995). We interpret, therefore, the different ferritin mineral core in cells grown with rhizoferrin compared with those grown with ferric citrate in terms of different protein shells. This in turn implies (i) siderophore dependent iron metabolization pathways and (ii) two types of ferritins in *M. smegmatis*.

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