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Transport and utilization of ferrioxamine-E-bound iron in *Erwinia herbicola* (*Pantoea agglomerans*)

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Summary. We have analyzed ferrioxamine-E-mediated iron uptake and metabolization in *Erwinia herbicola* K4 (*Pantoea agglomerans*) by means of in vivo Mössbauer spectroscopy and radioactive labeling techniques. A comparison of cell spectra with the spectrum of ferrioxamine clearly demonstrates that ferrioxamine E is not accumulated in the cell, indicating a fast metal transfer. Only two major components of iron metabolism can be detected, a ferric and a ferrous species. At 30 min after uptake, 86% of the internalized metal corresponded to a ferrous ion compound and 14% to a ferric iron species. Metal transfer apparently involves a reductive process. With progressing growth, the oxidized species of the two major proteins becomes dominant. The two iron metabolites closely resemble species previously isolated from *Escherichia coli*. These components of iron metabolism differ from bacterio-ferritin, cytochromes and most iron-sulfur proteins. All other iron-containing cellular components are at least one order of magnitude lower in concentration. We suggest that the ferrous and ferric iron species correspond to two different oxidation states of a low-molecular mass protein.

Key words: *Erwinia herbicola* (*Pantoea agglomerans*) – Ferrioxamine E – In vivo Mössbauer spectroscopy – Iron metabolism – Iron transport – Siderophores

Introduction

The extreme insolubility of ferric ion at neutral pH ($K_{SP} = 10^{-38.7}$ M) severely restricts the bioavailability of iron (Raymond et al. 1984). Thus microbes have evolved iron-complexing agents, termed siderophores, which are synthesized under conditions of iron deficiency (reviewed e.g. by Raymond et al. 1984; Matzanke et al. 1989a). A concomitant response to low-iron stress is the synthesis of several outer-membrane proteins which are essential for specific tansport of a variety of siderophores. In the case of *Escherichia coli*, these include receptors for enterobactin (FepA) (Lundrigan and Kadner 1986), ferrichrome (FhuA) (Fecker and Braun 1983), aerobactin (Iut) (Krone et al. 1985), coprogen and ferric rhodotorulate (FhuE) (Sauer et al. 1990), citrate (FecA) (Wagegg and Braun 1981; Staudenmaier et al. 1989) and of two unknown substrates (Fiu and Cir) (Hantke 1990). This multitude of siderophore transport systems reveals that an enormous effort is devoted by microbial systems to providing sufficient iron for growth. Within enterobacteria the typical siderophore synthesized under conditions of iron deficiency is enterobactin. However, several strains of the enterobacterial group *Erwinia herbicola*, now named *Pantoea agglomerans* (Gavini et al. 1989), produce the cyclic trihydroxamate siderophore ferrioxamine E (Fig. 1) (Berner et al. 1988). In a recent study it has been shown that ferrioxamine-E-mediated iron transport requires a 76-kDa outer-membrane protein (FoxA) (Berner and Winkelmann 1990). In previous papers we have demonstrated that in vivo Mössbauer spectroscopy is a powerful tool to analyze siderophore-mediated ^{57}Fe uptake and metabolization in procaryotic (Matzanke et al. 1986; Matzanke 1987; Matzanke et al. 1989a, 1989b) and eucaryotic systems (Matzanke 1987; Matzanke et al. 1987a, 1987b, 1988 and 1990). Thus, we have employed in vivo Mössbauer spectroscopy and

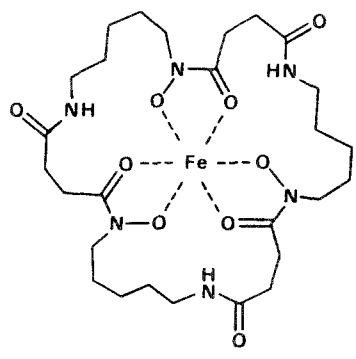

Fig. 1. Structure of ferrioxamine E

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^{55}Fe-labeling techniques to disclose the mechanism of ferrioxamine-E-mediated iron uptake and to characterize the main iron metabolites of *E. herbicola*.

Materials and methods

Chemicals. Ethylenediaminetetraacetic acid, Na_4 salt (EDTA) and XAD-2 were purchased from Serva (Heidelberg, FRG), Chelex 100 from Bio-Rad (Munich, FRG), and ^{57}Fe (95% isotopically pure) was from Rohstoffeinfuhr (Düsseldorf, FRG). Bovine serum albumin and ethylenediamine-di(*o*-hydroxyphenylacetic acid) (EDDHA) were from Sigma Chemical Co. (St. Louis, MO, USA). Ferrioxamine E was isolated from *Streptomyces pilosus* (Meiwes 1989). Desferrioxamine B was provided by Ciba-Geigy (Basel, Switzerland). All other reagents were of analytical grade and purchased from Merck (Darmstadt, FRG). Cellulose nitrate filters (0.45 µm) were from Sartorius (Göttingen, FRG) and Delrin rods from E.I. du Pont de Nemours & Co., Inc. (Wilmington, DE, USA). Delrin Mössbauer sample holders were manufactured in the machine shop of the Institute für Biologie II (Tübingen). Doubly distilled water was used throughout. All glassware used during the experiments was washed with KOH, HCl, EDTA and then triply rinsed with water to eliminate adventiously bound iron.

Desferri-ferrioxamine E was prepared by the 8-hydroxyquinoline method (Wong et al. 1983). ^{55}Fe-labeled ferrioxamine E was prepared by adding a solution of desferri-ferrioxamine E (10 µl, 1 nmol) and $^{55}FeCl_3$ (10 µl, carrier-free, in 0.1 M HCl, 22.2 MBq/ml, Amersham, UK) to a solution of unlabeled ferrioxamine E (1 ml, 0.1 µmol/ml). A ^{57}Fe(III) stock solution was obtained by dissolving ^{57}Fe in a small volume of HNO_3/HCl (1:2, by vol.). The pH of the solution was adjusted to 1.0 with KOH and the Fe(III) concentration determined spectrophotometrically with desferrioxamine B at 428 nm. Synthesis of [^{57}Fe]ferrioxamine E was achieved by mixing equimolar solutions of ^{57}Fe(III) and aqueous desferri-ferrioxamine E. Due to poor solubility of the desferri-compound, the solution has to be kept warm (45° C) during this procedure (Keller-Schierlein and Prelog 1961). The red-brown reaction product was passed through an XAD-2 column and purity was checked by thin-layer chromatography on silica gel (chloroform/methanol/water, 70:24:4; $R_f = 0.45$) (Berner et al. 1988).

Bacterial strains and growth conditions. The strain K4 of *Erwinia herbicola*, isolated from an infected wound, was kindly provided by U. Ullmann (Department of Medical Microbiology, University of Kiel, FRG). The strain was maintained on agar slants containing 0.4% yeast extract, 1% malt extract, and 0.4% glucose. Rich medium contained 0.8% nutrient broth (Difco, Detroit, MI, USA). For uptake studies, cells were grown in M9 minimal salts medium (Miller 1972). The iron concentration of the medium and of the glucose stock solution was kept $\leq 10^{-6}$ M by passing the solutions separately through a Chelex 100 column to remove Fe^{3+}. An alternative route to make the M9 medium iron-deficient was to add EDDHA, a strong iron chelator which cannot be utilized by *Erwinia* (Berner and Winkelmann 1990). Trace metals (1 mM $MgSO_4$, 0.1 mM $CaCl_2$) were prepared separately.

Transport experiments. Cells were grown in a M9 medium (100 ml) with and without 50 µM EDDHA at 27° C to an $A_{578} = 0.5$, collected by centrifugation and washed twice with M9. The cells were finally suspended in M9 medium to a density of $A_{578} = 0.5$. Aliquots of this suspension were taken for the transport assay and preincubated for 10 min at 27° C and 120 rpm. Transport was started by the addition of 0.05, 0.1, 0.3, 0.5, 0.7, 0.9, 2, 5, and 7 µM [^{55}Fe]ferrioxamine E (specific activity 2.2 kBq/nmol). Samples (1 ml, corresponding to 0.2 mg dry mass) were taken at 1-min intervals over a time range of 10 min, filtered on cellulose nitrate membrane filters (0.45 µm) and washed twice with ice-cold 0.9% NaCl solution. The radioactivity on the filters was measured in a liquid scintillation counter.

For Mössbauer measurements cells of an M9 preculture were suspended in 2 l medium in a baffle flask ($A_{578} = 0.1$), grown in M9 medium at 27° C to an $A_{578} = 0.7$ and supplemented with 2 µM [^{57}Fe]ferrioxamine E. After 30, 60 and 90 min of incubation, cells were cooled to 4° C within 2 min, washed, and transferred to Delrin Mössbauer sample holders. All sample volumes were about 0.8 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 performed. In a second set of Mössbauer experiments the inoculum was supplied with [^{57}Fe]ferrioxamine E and the cultures were grown aerobically to an A_{578} of 0.6, 1.0 and 1.3. Mössbauer samples were prepared as described above.

Mössbauer measurements. The Mössbauer samples were either frozen cells or frozen aqueous solutions. The Mössbauer spectra were recorded in the horizontal transmission geometry using a constant-acceleration spectrometer operated in conjunction with a 512-channel analyzer in the time-scale mode. The source was at room temperature and consisted of 1.85 GBq ^{57}Co diffused in Rh foil (Amersham Buchler). The spectrometer was calibrated against a metallic α-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 γ-beam was applied to the tail of the bath cryostat using a permanent magnet. Isomer shift δ, quadrupole splitting ΔE_Q, and percentage of the total absorption area were obtained by least-squares fits of Lorentzian lines to the experimental spectra.

Results and Discussion

In a first set of experiments, the uptake kinetics of [^{55}Fe]ferrioxamine E in *E. herbicola* (strain K4) was characterized. *E. herbicola* was grown either in M9 minimal salts medium or in M9 medium supplemented with EDDHA. EDDHA was added as an iron scavenger, enabling low-iron growth conditions because Fe-EDDHA is not transported in *Erwinia*. Prior to the experiments, cells were washed and resuspended in fresh M9 medium without EDDHA. Transport studies of [^{55}Fe]ferrioxamine E in *E. herbicola* showed rapid accu-

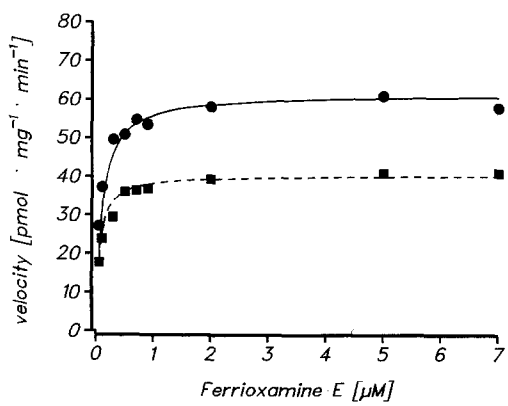

Fig. 2. Transport kinetics of ferrioxamine-E-mediated ^{55}Fe accumulation in *E. herbicola*. (■) Growth in M9 minimal salts medium; (●) growth in M9 medium supplemented with 50 µM EDDHA. Time-dependent uptake studies were performed in the concentration range 0.05–7 µM [^{55}Fe]ferrioxamine E. Between 2–6 min of siderophore uptake transport rates were constant. From these data the transport kinetics were derived

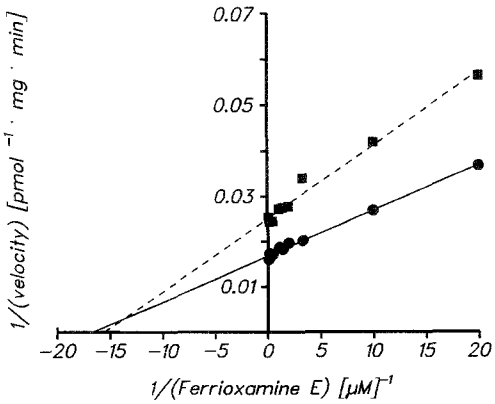

Fig. 3. Lineweaver-Burk plots of ^{55}Fe uptake kinetics in M9-grown (■) ($V_{max}=39.5$ pmol·mg^{-1}·min^{-1}, $K_m=0.063$ μM, $r=0.9910$) and in M9/EDDHA-grown (●) ($V_{max}=59.3$ pmol·mg^{-1}·min^{-1}, $K_m=0.059$ μM, $r=0.9964$) *E. herbicola* cells

mulation of ^{55}Fe. Time-dependent transport was determined at siderophore concentrations ranging over 0.05–7 μM. Within this concentration range, the ferrioxamine-E-mediated ^{55}Fe transport process was linear between 2–6 min. The transport rate, obtained from the slope of this interval, was used to calculate the transport kinetics. [^{55}Fe]Ferrioxamine E is taken up via a saturable process, indicating that the transport is not diffusion-controlled (Fig. 2). The kinetics can be described formally according to Michaelis and Menten. Linear regression analysis of Lineweaver-Burk plots (Fig. 3) yielded the following values: $K_m=0.059$ μM, $V_{max}=59.3$ pmol·mg^{-1}·min^{-1}, $r=0.9964$ for cells grown with EDDHA supplement and $K_m=0.063$ μM, $V_{max}=39.5$ pmol·mg^{-1}·min^{-1}, $r=0.9910$ for cells grown without EDDHA supplement. The low K_m values indicate a strong affinity of ferrioxamine E for its transport system. The maximum transport rate of cells grown with EDDHA is approximately 50% higher than for cells grown without EDDHA. Since the K_m values differ by only 7%, we conclude that the same transport system is employed. The difference in V_{max} could be explained by changes in the uptake system involved causing different uptake rates or by a different number of uptake systems per cell. In fact, iron deficiency is expected to be more pronounced in cultures grown with EDDHA and, hence, more transport systems could be expressed under these conditions.

In additional sets of experiments we followed the time dependence of ferrioxamine-E-mediated ^{57}Fe transport and metabolization by Mössbauer spectroscopy. Fig. 4A shows a Mössbauer spectrum of a frozen aqueous solution of [^{57}Fe]ferrioxamine E recorded at 4.2 K. This spectrum exhibits a complex hyperfine pattern which is typical of paramagnetic $^6S_{5/2}$ ferric iron in the slow relaxation limit. In Fig. 4B–D spectra are depicted of *E. herbicola* cells, grown in a low-iron M9 medium, supplemented with 3 μM [^{57}Fe]ferrioxamine E at $A_{578}=0.7$ and harvested after 30 min (Fig. 4B), 60 min (Fig. 4C) and 90 min (Fig. 4D) of additional growth. Cell spectra and the siderophore spectrum display strikingly different features. Only two quadrupole

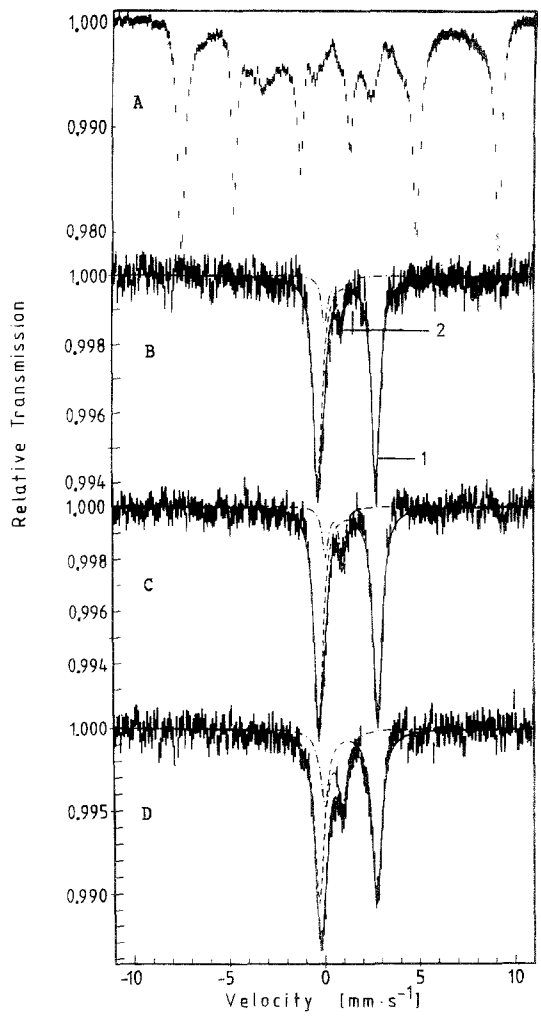

Fig. 4. Mössbauer spectra of frozen aqueous [^{57}Fe]ferrioxamine E (2 mM) (A) and of frozen *E. herbicola* cells (B–D), measured at 4.2 K, in a perpendicular field of 20 mT. Cells were grown in low-iron M9 minimal salts medium to an $A_{578}=0.7$. Then [^{57}Fe]ferrioxamine E was added to the suspension (3 μM final concentration). Cells were harvested and washed after 30 min (B), 60 min (C), and 90 min of additional growth. Solid and broken lines are obtained by least-squares fits of Lorentzian lines to the experimental spectra yielding the Mössbauer parameters listed in Table 1

doublets are visible in the cell spectra. Other iron metabolites must have an intracellular abundance which is at least one order of magnitude lower. The Mössbauer parameters are summarized in Table 1. The Mössbauer parameters of component 1 do not fit those found for cytochromes nor those typical for iron-sulfur proteins. This species corresponds to a hexacoordinated Fe^{2+} high-spin complex. Component 2 fits high-spin Fe^{3+}, but is not consistent with cytochromes and iron-sulfur proteins containing ferric high-spin iron. Since iron may participate in the oxidative damage of cells by activating oxygen, it must be bound sufficiently inertly by low- or high-molecular-mass enzymes, carriers and storage compounds. Accordingly Fe^{2+} must be bound specifically by a low- or high-molecular-mass chelator. In a previous study of *E. coli* we have detected Mössbauer species very similar to those presented here. In *E.*

Table 1. Mössbauer parameters of the iron species in cell spectra of *E. herbicola*

Component	Time (min)	A_{578}	δ (mm/s)	ΔE_Q (mm/s)	Γ (mm/s)	Absorption area (% total)
1. (Fe^{2+})	30		1.28 (2)	3.10 (2)	0.55 (2)	86.4 (14)
	60		1.24 (2)	3.06 (2)	0.53 (2)	86.7 (18)
	90		1.25 (2)	3.04 (2)	0.60 (2)	70.4 (12)
		0.6	1.26 (2)	2.95 (2)	0.62 (3)	57.8 (13)
		1.0	1.23 (2)	2.86 (3)	0.67 (3)	49.9 (9)
		1.3	1.26 (4)	2.88 (5)	0.78 (4)	47.2 (10)
2. (Fe^{3+})	30		0.47 (4)	0.85 (5)	0.38 (6)	13.6 (33)
	60		0.53 (4)	0.88 (4)	0.37 (6)	13.3 (25)
	90		0.49 (4)	0.91 (4)	0.60 (6)	29.6 (16)
		0.6	0.46 (2)	0.96 (2)	0.49 (3)	42.2 (11)
		1.0	0.43 (2)	0.97 (2)	0.62 (2)	50.1 (7)
		1.3	0.44 (3)	1.02 (3)	0.63 (2)	52.8 (7)

Data were obtained by least-squares fits of Lorentzian lines to the experimental spectra in Figs. 4 and 5. [^{57}Fe]Ferrioxamine E was added either with the inoculum for monitoring growth-phase-dependent changes of the cellular iron pool or at log growth to follow the [^{57}Fe]ferrioxamine E uptake process and the initial intracellular reaction on a short time scale

coli we could demonstrate that the two Mössbauer species arise from 17-kDa and 15-kDa proteins binding approximately 10 metal ions/molecule. An analysis of cell fractions of *Erwinia* will be reported elsewhere. However, we would like to mention that fluorograms of a ^{55}Fe-labeled cytoplasmic fraction (not shown here) exhibit one single band at 6 kDa comprising 70% of the applied radioactivity.

Since no sextet component is visible in the cell spectra, we conclude that ferrioxamine E is apparently not accumulated inside the cells. This indicates a rapid metal transfer inside the cells. Moreover, 86% of the internalized metal corresponds to ferrous iron and 14% to ferric iron species after 30 min of incubation. This finding suggests that metal transfer involves a reductive process. After 90 min of incubation with ferrioxamine E, the contribution of ferric iron increases to 30% of the total absorption area and, correspondingly, ferrous high-spin decreases to 70%. This indicates an interconversion from ferrous to ferric iron. Similar observations have been made in *E. coli* under conditions of glucose deficiency and prolonged growth. Therefore, in a second set of Mössbauer experiments growth-phase-dependent changes of the main iron metabolites were analyzed.

Fig. 5 (A, B and C) shows cell spectra of *E. herbicola* at $A_{578} = 0.6$, 1.0 and 1.3, respectively. The corresponding Mössbauer parameters are listed in Table 1. The cell suspensions were supplemented with [^{57}Fe]ferrioxamine E at $A_{578} = 0.01$. Within the growth range observed, only two major components of iron metabolism can be detected. Again, all other iron-requiring functionalities of *Erwinia* must be much less abundant. Compared to short-time uptake studies (Fig. 4), the contribution of ferric iron is considerably higher (42% of total absorption area in Fig. 5A) and increases with prolonged growth (53% at $A_{578} = 1.3$). Correspondingly the ferrous iron contribution decreases. Thus, as cell

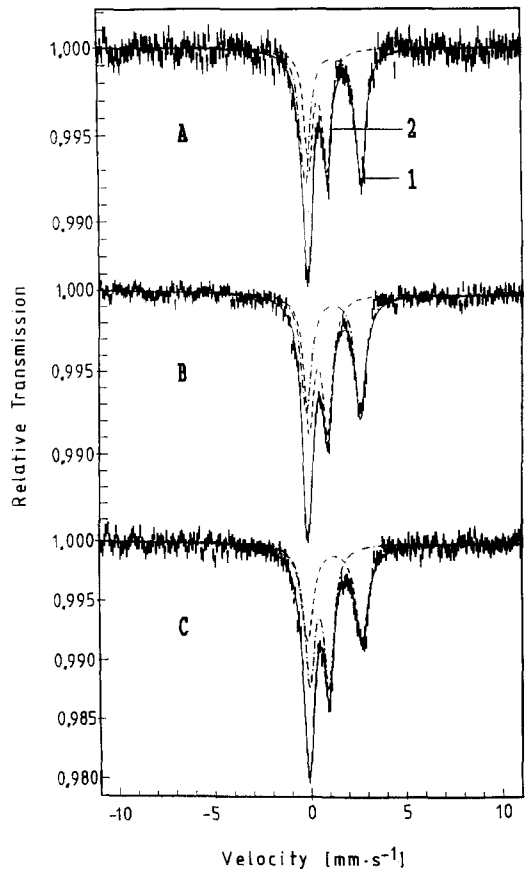

Fig. 5. Mössbauer spectra of *E. herbicola* at different growth states. Measurements were performed at 4.2 K in a perpendicular field of 20 mT. Cells were grown in a M9 minimal salts medium, supplemented with 3 μM [^{57}Fe]ferrioxamine E. Cells were harvested at $A_{578} = 0.6$ (A), 1.0 (B), and 1.3 (C). With the inoculum, the medium was supplemented with [^{57}Fe]ferrioxamine E. The iron metabolite spectra were fitted with two quadrupole doublets of Lorentzian line shape. The Mössbauer parameters and the corresponding percentage of the absorption areas are listed in Table 1

growth proceeds, increasing amounts of the ferrous species are converted into the ferric component which therefore seems to represent the acceptor for processed ferrous iron or, directly, the oxidized form of ferrous iron (see Table 1).

While the isomer shifts for both the ferric and the ferrous iron species exhibit only minor changes under the various conditions employed, a shift of the quadrupole splittings is observed. The quadrupole splitting of the ferric iron species varies from 0.85 mm/s to 1.02 mm/s and that of the ferrous species from 3.10 mm/s to 2.88 mm/s, concomittant to the increased ferric/ferrous ratio. These changes, however, are only marginal due to the relatively large linewidths obtained. This finding, and the observation of a single band at 6 kDa in fluorograms, suggest that ferrous and ferric iron represent interconvertible forms of the same low-molecular-mass protein.

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