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# Characterization of ferrioxamine E as the principal siderophore of *Erwinia herbicola (Enterobacter agglomerans)*

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**Summary.** Several strains of the enterobacterial group *Erwinia herbicola (Enterobacter agglomerans)* were screened for siderophore production. After 3 days of growth in a low-iron medium, all strains studied produced hydroxamate siderophores. The retention values of the main siderophore during thin-layer chromatography on silica gel plates and on HPLC reversed-phase columns were identical with those of an authentic sample of ferrioxamine E (norcardamine). Gas-chromatographic analysis of the HI hydrolyzate yielded succinic acid and 1,5-diaminopentane in equimolar amounts; fast-atom-bombardment (FAB) mass spectroscopy showed a molecular mass of 653 Da. Iron from $^{55}$Fe-labelled ferrioxamine E was well taken up by iron-starved cells of *E. herbicola* ($K_m = 0.1$ μM, $V_{max} = 8$ pmol mg$^{-1}$ min$^{-1}$). However, besides ferrioxamine E (100%), several exogenous siderophores such as enterobactin (94.5%), ferric citrate (78.5%), coprogen (63.5%) and ferrichrome (17.5%) served as siderophores, suggesting the presence of multiple siderophore receptors in the outer membrane of *E. herbicola*.

**Key words:** *Erwinia herbicola* — Siderophore transport — Ferrioxamine E

## Introduction

The typical enterobacterial siderophore is the cyclic trimer of 2,3-dihydroxy-*N*-benzoyl-L-serine, called enterobactin or enterochelin. Enterobactin has been detected in a variety of enterobacterial species, such as *Escherichia coli* (O'Brien and Gibson 1970). *Salmonella* (Pollack and Neilands 1970) *Shigella* (Payne 1980). Members of the family Enterobacteriaceae are also able to synthesize hydroxamate siderophores. Thus, the dihydroxamate aerobactin was isolated from *Aerobacter aerogenes* 62-I (Gibson and Magrath 1969), now designated as *Enterobacter aerogenes*. Plasmid and chromosomally encoded aerobactin synthesis has subsequently been shown to occur in strains of *E. coli* (Warner et al. 1981), *Salmonella* (Colonna et al. 1985) and *Shigella* (Lawlor and Payne 1984), indicating that the ability to synthesize hydroxamate siderophores is common among the family of Enterobacteriaceae.

Ferrioxamines have so far only been detected in the genus *Streptomyces* (Bickel et al. 1960, Yang and Leong 1982), *Arthrobacter, Chromobacterium* (Müller and Zähner 1968) and *Pseudomonas* (Meyer and Abdallah 1980). The present report on the occurrence of ferrioxamines in the familiy of Enterobacteriaceae is surprising and may be an important trait for the distinction of the *E. herbicola* group from other *Erwinia* groups. The biosynthetic capacity for ferrioxamine production among the *Erwinia* group may also help to elucidate their biogenetic relationship to other bacterial genera.

## Materials and methods

*Bacterial strains and growth condition.* Clinical isolates of *E. herbicola (Enterobacter agglomerans)* from wound infections and from tracheal exudates were kindly provided by U. Ullmann (Department of Medical Microbiology, University of Kiel, FRG). These strains were biochemically identical with strains which we have isolated from lake water, such as *E. herbicola* A 111 and B 111 (Table 1). Strain A 111 has been shown to produce an antifungal and antimycoplasmatic glycolipodepsinonapeptide, named herbicolin (Aydin et al. 1985). Strain B 111 produces an antibacterial agent which is presently under investigation in our laboratory. All strains were maintained on

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agar slants containing 0.4% yeast extract, 1% malt extract and 0.4% glucose (YMG). Production of siderophores was carried out in liquid cultures containing M9 salts and glucose without further iron removal. Preparative-scale production of siderophores was carried out in 5-l conical flasks containing 2.5 l medium, inoculated with a 100-ml preculture and incubated for 3 days at 27° C.

*Isolation and purification of siderophores.* The cells were removed from the culture filtrate by means of a Millipore tangential filtration implement (Pellicon). After adding ferrous sulfate and aeration, to allow complex formation, the brown culture filtrate was then passed through a Servachrome XAD-2 column. After washing with two volumes of water, the siderophore iron complex was desorbed by one volume of methanol and purified by gel filtration on Sephadex LH20 in methanol. On silica gel columns using chloroform/methanol/water (50:50:4), three bands were observed from which the main band was further purified by gel filtration on Sephadex LH20 using methanol as a solvent. The purity was checked by thin-layer chromatographs (TLC) using chloroform/methanol/water (70:24:4) and by HPLC on an RP-18 column using an acetonitrile-water (10 mM phosphate, pH 3) gradient as a solvent system (Konetschny-Rapp et al. 1988). Desferri-siderophores were prepared by the 8-hydroxyquinoline method (Wiebe and Winkelmann 1975). As ferrioxamine E and its desferri form both possess poor solubility in water and cold methanol (Keller-Schierlein and Prelog 1961), the solutions have to be kept warm during this procedure.

*Gas chromatography.* The isolated desferri-siderophore (1 mg) was reductively hydrolysed with 57% HI (1 ml) at 110° C for 12 h. After removal of the HI, the hydrolysate was dissolved in water and extracted twice with chloroform, firstly after acidification with HCl and then after adding NaOH. The extracts were dried by evaporation, dissolved in dry pyridine (0.1 ml) and mixed with an equal volume of *N,O*-bis(trimethylsilyl)trifluoracetamide (Serva). After a 1-h reaction time at room temperature, the mixture was analyzed by gas chromatography on an SE 30 capillary column (10 m) using a temperature program 80–150° C, 4° C/min and with trimethylsilyl derivatives of succinic acid and diaminopentane as a reference.

*FAB mass spectrometry.* Fast-atom-bombardment (FAB) spectra were recorded with a Varian MAT 711 A instrument combined with an SS 200 data system with glycerol as a solvent and a xenon ionizing beam produced with a saddlefield primary atom gun (Ion Tech, England) at an ion source temperature of 25° C.

*$^{55}$Fe-labelled siderophores.* Labelled siderophores were prepared by adding solutions of desferri-siderophores (10 µl, 1 nmol) and $^{55}FeCl_3$ (10 µl, carrier-free, in 0.1 M HCl, 0.6 mCi/ml, Amersham, UK) to a solution of unlabelled siderophores (1 ml, 0.1 µmol/ml).

*Transport assay.* Transport of $^{55}$Fe-labelled siderophores was carried out according to our earlier protocol described for *E. coli* (Braun and Winkelmann 1981). Iron-deficient cells of *E. herbicola* (A 111) were grown in M9 medium without iron salts to a density of $A = 1.2$, centrifuged at 6000 *g* and washed twice with M9 medium (+0.4% glucose and 100 µM nitrilotriacetate). The cells were finally suspended in M9 medium (18 ml), preincubated for 10 min at 27° C and then incubated with 10 nmol (0.5 µM final concentration) of the $^{55}$Fe-labelled siderophore (specific activity 130 000 dpm/nmol) under gentle rotation. Samples of 1 ml (2.2 mg dry mass) were taken at intervals and filtered through cellulose acetate membrane filters (0.45 µm). The radioactivity of the filters was measured in a liquid scintillation counter. Corrections were made for adsorption of radioactivity on the membrane filters. Concentration-dependent transport of [$^{55}$Fe]ferrioxamine E was carried out as described for the transport of [$^{55}$Fe]ferrichrome in *E. coli* (Braun and Winkelmann 1981).

## Results

All strains of *Erwinia herbicola (Enterobacter agglomerans),* studied in the present investigation, either isolated from infected wounds (K2–K7, AC, BC) or from lake water (A 111, B 111), were homogenous with respect to the characteristic biochemical reactions shown in Table 1, but differed in various other metabolic activities. All strains were found to produce hydroxamate siderophores during growth under iron limitation. When the crude siderophores were separated on silica gel columns with chloroform/methanol/water (50:50:4) as an eluting solvent, generally one major and two minor bands were observed. As strain K4 produced the highest amount of siderophores, the main siderophore component of this strain was further purified and characterized in the present investigation.

Thin-layer chromatography on silica gel 60, with chloroform/methanol/water (70:24:4) as

**Table 1.** Biochemical reactions common to all investigated strains of *E. herbicola* (A 111, B 111, K2, K3, K4, K5, K6, K7, AC, BC)

| Test | Reaction |
|---|---|
| Gram reaction | — |
| OF test (oxidative) | + |
| OF test (fermentative) | + |
| Galactosidase | + |
| Arginine dihydrolase | — |
| Lysine decarboxylase | — |
| Ornithine decarboxylase | — |
| Indole production | — |
| Acetoin production | + |
| Acid from glucose (without gas) | + |
| Acid from mannose | + |
| Acid from inositol | + |
| Acid from galactitol | — |
| Acid from sorbitol | — |
| Acid from L-rhamnose | + |
| Acid from L-arabinose | + |
| Resistance to ampicillin | + |
| Resistance to Cefalotin | + |
| Ferrioxamine E production | + |

API 20E System (Bio Merieux, Nürtingen, FRG) was used and incubated for 24 h at 27° C. All strains produce a water-insoluble yellow pigment. Strain A 111 produces additionally the glycolipodepsinonapeptide 'herbicolin' (Aydin et al. 1985). Strain K4 was selected for a preparative-scale isolation and characterization of ferrioxamine E and strain A 111 was used for the transport measurements. OF = oxidation or fermentation degradation of glucose

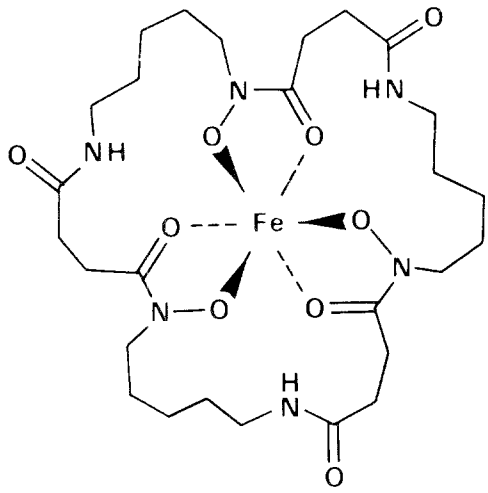


**Fig. 1.** Structural formula of ferrioxamine E

solvent system, revealed $R_f = 0.46$ for the major component, which corresponded with an authentic sample of ferrioxamine E (Fig. 1). HPLC analysis of the purified siderophore on a $C_{18}$ reversed-phase column (Nucleosil $C_{18}$, 5 μm), using a 10 mM phosphate buffer(pH 3)/acetonitrile gradient, gave a retention time of the principle peak of 18.5 min. The identity of this component with an authentic sample of ferrioxamine E (Fig. 2b) was confirmed by coinjection of the reference compound together with the isolated siderophore mixture (Fig. 2a). There are several other minor peaks to be seen in the isolated siderophore mixture (Fig. 2a) which have not been analysed so far but which seem to represent further members of the ferrioxamine family. No amino acids could be detected in an amino analyzer after HCl or HI hydrolysis. Gas chromatography of the silylated HI hydrolysis products on an SE 30 glass capillary column (10 m) using a temperature program 80–150° C, 4 °C/min, revealed two peaks possessing retention times identical with succinic acid (1.96 min) and diaminopentane (12.93 min, major silyl derivative, 3.54 min and 15.0 min, minor silyl derivatives) confirming the presence of ferrioxamines. A final proof for the identity of the isolated siderophore with ferrioxamine E was obtained by FAB mass spectroscopy (Fig. 3). The molecular mass peak was found at $m/z$ 654 $[M+H]^+$, corresponding to a molecular mass of 653 Da for ferrioxamine E (nocardamine). An additional peak at 692 Da was assigned to $[M+K]^+$. The peak at $m/z$ 601 was assigned to $[M+H]^+ - 53$, which corresponded to the iron-free ligand. Thus the molecular ion region of the positive FAB spectra of the isolated ferrioxamine E corresponded to

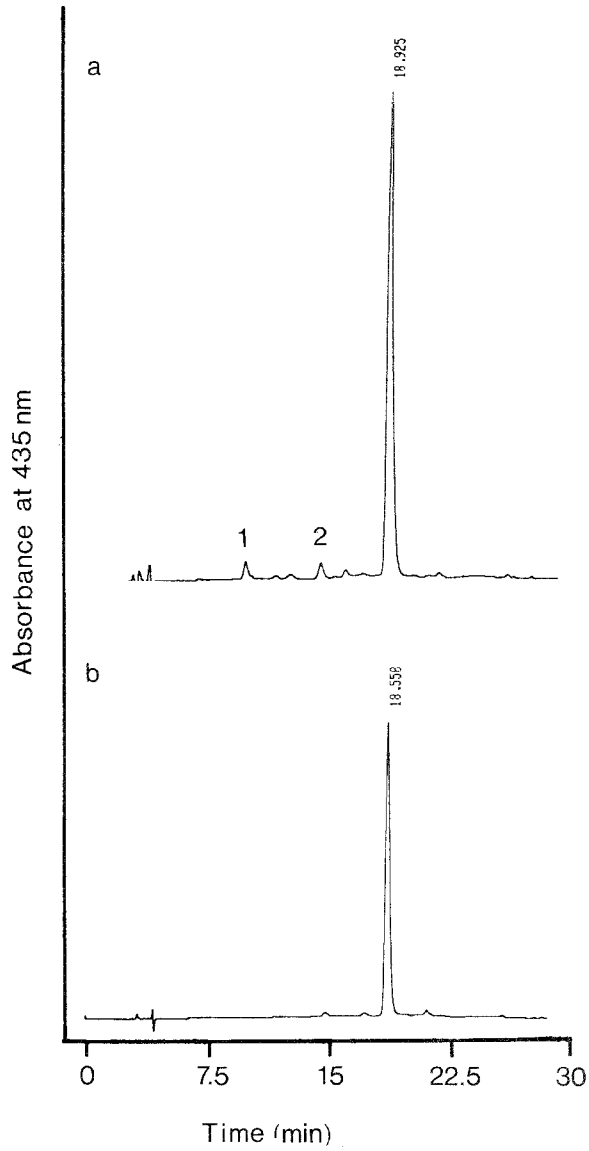


**Fig. 2a, b.** HPLC separation **(a)** of the isolated siderophore mixture and ferrioxamine E as a reference (coinjection) and **(b)** of the reference (ferrioxamine E) using a $C_{18}$-reversed phase column (Nucleosil $C_{18}$ 5 μm) and a 10 mM phosphate buffer (pH 3)/ acetonitrile gradient as described in Konetschny-Rapp et al. 1988). Peaks 1 and 2 represent unidentified siderophores of *E. herbicola*

the general signal pattern reported for ferrioxamine B (Dell et al. 1982).

Transport of [$^{55}$Fe]ferrioxamine E in iron-deficient cells of *E. herbicola* (strain A 111) revealed rapid accumulation of $^{55}$Fe during a 30-min incubation (Fig. 4). Approximately 185 pmol/mg dry mass was taken up after 30-min incubation at 27° C. Time-dependent uptake of $^{55}$Fe from labelled ferrioxamine E revealed three different phases: an initial non-linear rapid-uptake phase

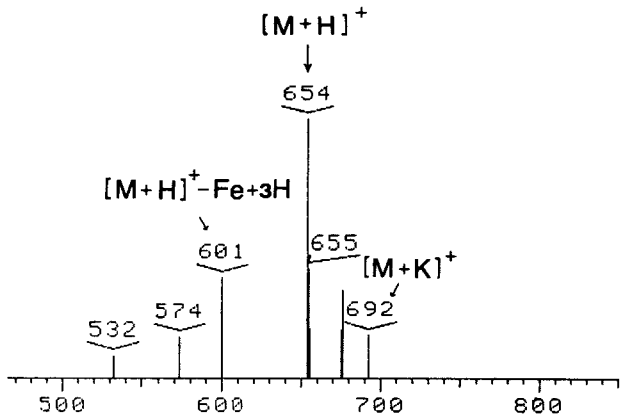


**Fig. 3.** Fast-atom-bombardment spectrum of the isolated siderophore (ferrioxamine E, iron complex) showing the molecular peak area. For assignment see text

**Table 2.** Iron uptake from $^{55}$Fe-labelled siderophores by iron-starved cells of *E. herbicola* A 111

| Siderophore | Uptake after 10-min incubation (pmol mg$^{-1}$) | Comparative uptake (%) |
|---|---|---|
| Ferrioxamine E | 120.5 | 100 |
| Enterobactin | 113.8 | 94.5 |
| Ferric citrate | 94.6 | 78.5 |
| Coprogen | 63.5 | 63.5 |
| Ferrichrome | 21.0 | 17.5 |

Cells were grown in M9 medium at 27° C without additional iron and incubated with $^{55}$Fe-labelled siderophores as described in Materials and methods. Samples of the cell suspension were filtered, washed and radioactivity measured in a liquid scintillation counter. Results are compared to those for Ferrioxamine E taken as 100%

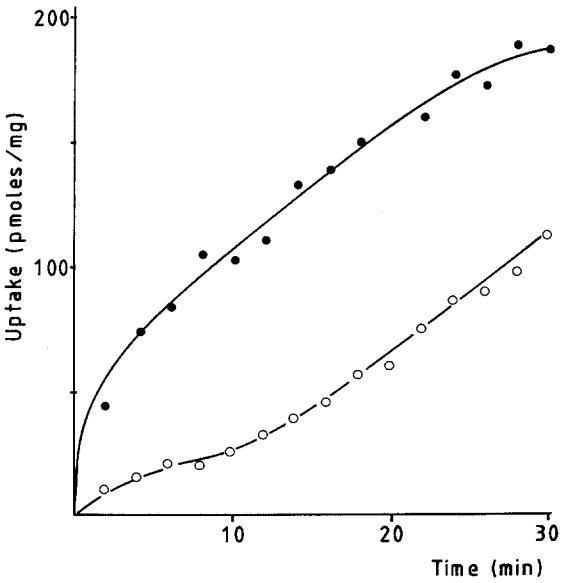


**Fig. 4.** Time-dependent uptake of iron from [$^{55}$Fe]ferrioxamine E in iron-starved cells of *Erwinia herbicola* A 111. Cells were collected by centrifugation from a low-iron culture grown overnight in M9 medium. The cells were suspended in fresh medium and incubated with [$^{55}$Fe]ferrioxamine E (10 nmol, specific activity 130000 dpm/nmol). Samples were taken at intervals, filtered, washed and radioactivity measured as described in Materials and methods

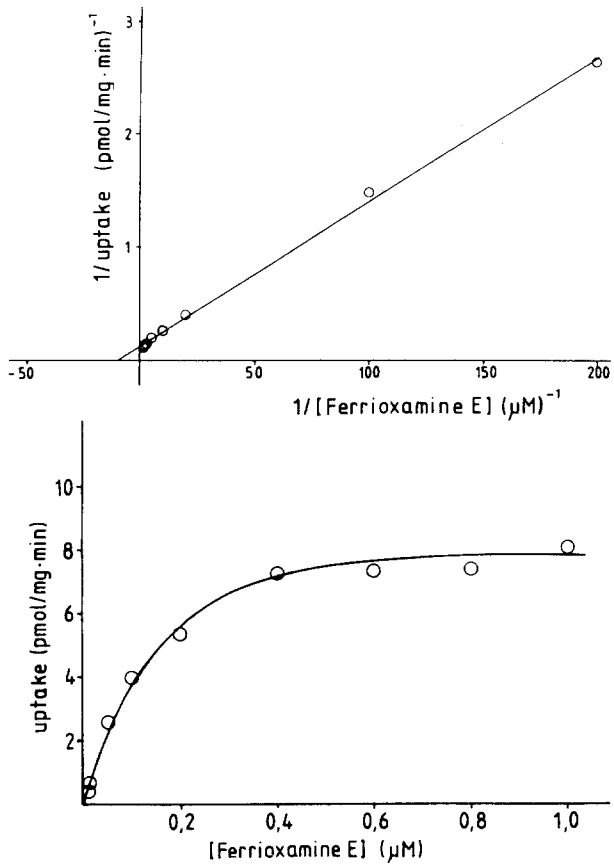


**Fig. 5.** Concentration-dependent uptake of iron from [$^{55}$Fe]ferrioxamine E in iron-starved cells of *Erwinia herbicola* A 111 (saturation kinetics) and the corresponding Lineweaver-Burk diagram. Initial uptake values of labelled ferrioxamine E were determined by incubating cells for 5 min with increasing concentrations of labelled ferrioxamine E and by calculating the amount (pmol) taken up per dry mass (mg) and time (min)

during the first 2 min followed by a linear-uptake phase and a second non-linear phase at the end of the uptake experiment. From the total 10 nmol ferrioxamine E supplied to the cell suspension (18 ml), nearly 5.94 nmol (60%) were taken up after 30 min.

Transport of $^{55}$Fe-labelled enterobactin, ferric citrate, coprogen and ferrichrome was also observed (Table 2). As can be inferred from these results, ferrioxamine E and enterobactin were the most effective siderophores followed by ferric citrate and coprogen showing medium transport rates and ferrichrome with only 17% efficiency

compared to ferrioxamine E (100%). Since the cells continue to produce desferrioxamine E during the transport assay, iron-exchange events between added siderophores and excreted desferrioxamine E cannot be excluded. Thus, a comparison of transport of different siderophores in a siderophore-producing organism is not unequivocal.

Concentration-dependent uptake of [$^{55}$Fe]ferrioxamine E resulted in hyperbolic uptake curves (Fig. 5). Transformation of the saturation kinetics to a Lineweaver-Burk diagram showed a half-maximal transport rate at $K_m = 0.1$ μM and a maximal initial velocity of $V_{max} = 8$ pmol mg$^{-1}$ min$^{-1}$.

## Discussion

Ferrioxamines have so far not been reported to occur in the family of Enterobacteriaceae. According to *Bergey's Manual of Systematic Bacteriology* (1984), bacteria of the genus *Erwinia* include gram-negative, facultative anaerobic rods, which are generally motile by peritrichous flagella. They are associated predominantly with plants as pathogens, saprophytes, or as constituents of the epiphytic flora. The plant diseases of *Erwinia* species include blights, cankers, die back, leaf spots, wilts, discoloration of plant tissue and soft rots. The genetics of *Erwinia* has been reviewed comprehensively (Chatterjee and Starr 1980; Kotoujansky 1987). The taxonomy of the genus *Erwinia* is in a preliminary stage and includes three groups: (i) the highly plant-pathogenic species *E. amylovora, E. salicis, E. tracheiphilia* that cause necrotic or wilt diseases; (ii) the soft-rotting pathogens *E. carotovora* and *E. chrysanthemi,* which are also collectively termed *Pectobacteria;* and (iii) the *E. herbicola* group which includes yellow and non-pigmented strains originating from plant lesions, surfaces, soil and water. According to Ewing and Fife (1972), strains isolated from clinical sources have been designated *Enterobacter agglomerans*, because of their conformity with the genus *Enterobacter.* The *Erwinia herbicola* group may be separated from other Enterobacteriaceae by the absence of the arginine dihydrolase, ornithine and lysine decarboxylases and several other biochemical reactions (Table 1). Our strains also revealed a positive inositol (bromthymol blue) and a negative galactitol (dulcitol) reaction, although these reactions are reversed in Table 5.27. of *Bergey's Manual.* A significant resistance towards ampicillin, cefalotin, cefalozin and low concentrations of carbenicillin (32 μg/ml) was also observed in the present investigation which corresponds to the description of Enterobacter agglomerans in *Bergey's Manual.* However, the biochemical identification of *Erwinia* isolates is still a tedious venture (Slade and Tiffin 1984; Mergaert et al. 1984). Therefore, the production of ferrioxamine E may be used as an additional trait in the identification of *E. herbicola* strains.

Only a few papers have so far dealt with the siderophores of plant-pathogenic *Erwinia* strains (Expert and Toussant 1985; Leong and Neilands 1982) and only in a few cases have siderophores of the catechole type been detected. As shown in the present investigation, ferrioxamine E (norcardamine) is the principle siderophore of the *E. herbicola* group and iron from ferrioxamine E is rapidly taken up by iron-deficient cells ($K_m = 0.1$ μM, $V_{max} = 8$ pmol mg$^{-1}$ min$^{-1}$).

The apparent $K_m$ value corresponded roughly to that measured in *E. coli* for the uptake of ferrichrome ($K_m = 0.06$ μM, $V_{max} = 50$ pmol mg$^{-1}$ min$^{-1}$; Braun and Winkelmann 1987), suggesting that the ferrioxamine E transport system in *E. herbicola* is as effective as the ferrichrome transport system in *E. coli.* However, the $V_{max}$ values of ferrioxamine E uptake are significantly lower than those measured for ferrichrome uptake in *E. coli* wich may due to a different degree of iron deficiency. As has been shown earlier (Matzanke et al. 1984), ferrioxamine E is not taken up by *E. coli* (RW 193, ATCC no. 33475; genotype: *pro leu trp entA purE*), a strain unable to synthesize enterobactin, while ferrioxamine B was taken up to a certain extent. The present investigation has shown that the transport of iron from exogenous siderophores such as ferrichrome, coprogen, enterobactin, ferric dicitrate is also observed in *E. herbicola* strains, indicating that several non-produced siderophores can also be used for iron acquisition. The use of exogenous siderophores has also been reported for *E. coli* (Braun et al. 1987). It is, however, uncertain whether in *E. herbicola* wild-type cells all tested exogenous siderophores are taken up by specific routes and/or receptors because iron-exchange events render a clear interpretation of uptake difficult. Exogenous siderophores seem not to be required by wild-type strains of *E. herbicola* since desferrioxamine E is rapidly biosynthesized and excreted in high amounts during iron limitation. However, the utilization of exogenous siderophores may be beneficial when new ecosystems have to be colonized and competition from different microorganisms is to be expected.

Crystal data have shown that ferrioxamine E is a relatively flat molecule with a total thicknes

of 0.36 nm in which the iron is coordinated in a *cis*-configuration (racemic mixture of optical isomers *Λ-cis* and *Δ-cis*) enabling a molecular distinction of both sides (van der Helm and Poling 1976). Further work is in progress to identify the iron-regulated outer-membrane receptor for ferrioxamine E and to investigate its recognition capability.

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