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Specificity and Mechanism of Ferrioxamine-Mediated Iron Transport in *Streptomyces pilosus*†

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Although the ferrioxamines are an important and well-characterized class of siderophores produced by several species of *Nocardia*, *Streptomyces*, *Micromonospora*, *Arthrobacter*, *Chromobacterium*, and *Pseudomonas*, no studies of the mechanism of ferrioxamine-mediated iron uptake have been performed for an organism which produces the siderophore. This is the first report of metal transport in *Streptomyces pilosus* mediated by the native ferrioxamines B, D_1, D_2, and E. ^{55}Fe accumulation in these ferrioxamines was dependent on metabolic energy and was a saturable process with increasing complex concentration. The apparent K_m for [^{55}Fe]ferrioxamine B uptake was ~0.2 μM. Both chromic desferriferrioxamine B and [^{67}Ga]desferriFerrioxamine B were transported at rates similar to those of the ^{55}Fe complexes: this implies that no decomplexation or reduction of the metal ion is required for transport, since the chromic complexes are kinetically inert and the gallium complexes have no stable divalent state as a possible reduction product. In addition, isomers of inert chromic desferriferrioxamine B complexes were used to probe the stereospecificity of the ferrioxamine uptake system. The chromic complexes were separated into three fractions by cationic exchange chromatography and assigned as two *cis* and a (mixture of) *trans* geometrical isomer(s) by their visible spectra. [^{55}Fe]ferrioxamine B uptake was equally inhibited by each isomer, suggesting that no differentiation between *cis* and *trans* geometrical isomers occurs. In the presence of chromic desferriferrioxamine B isomers, the uptake rates for ^{55}Fe-labeled ferrioxamines E, D_1, and D_2 were even more strongly reduced than was that for [^{55}Fe]ferrioxamine B itself. From these results we conclude that all the ferrioxamines tested are transported into the cells by the same uptake system.

The ferrioxamines are an important class of iron transport agents that are produced by several species of *Nocardia* (37, 42), *Streptomyces* (4, 41, 42), *Micromonospora* (9), *Arthrobacter* (25), *Chromobacterium* (25), and *Pseudomonas* (24). Although ferrioxamines have never been isolated from fungi, in growth promotion tests they can act as fungal growth factors (3, 31). The ferrioxamines are also antagonists to the structurally related ferrimycins, which are potent antibiotics (4, 5, 30, 43). For example, ferrioxamine B is capable of reversing ferrimycin A antibiotic activity against organisms such as *Bacillus subtilis* (5, 43). A characteristic structural feature of the ferrioxamines (Fig. 1) is repeating units of α-amino-ω-hydroxyaminoalkane and succinic or acetic acid, so that a thermodynamically stable octahedral ferric complex may be formed with three hydroxamate groups.

Streptomyces and *Nocardia* species are gram-positive soil bacteria which are famous for contributing the majority of the structurally characterized antibiotics (35). These bacteria are evolutionarily the closest to the fungi. Two main classes of siderophores having α-amino-ω-hydroxyaminoalkane in common as one building block have been isolated from these groups: the lipophilic and water-insoluble mycobactins and the water-soluble ferrioxamines (29). Although mycobactins and ferrioxamines never have been isolated from the same organism, the question arises whether there is any mechanism of uptake in which ferrioxamine acts as an extracellular solubilizing agent and transfers iron to a membrane-bound mycobactin.

Uptake of labeled ferrioxamine B has been studied in strains of *Salmonella* (19), *Bacillus* (13), and *Neurospora* (40), none of which excrete desferriferrioxamines. In each case no evidence was found for uptake of the intact metal siderophore complex. However, transport behavior has not previously been tested in a producing strain. In this study, the mechanism and stereospecificity of ferrioxamine uptake was investigated in *Streptomyces pilosus*. This strain was chosen because of the great variety of desferriferrioxamines excreted: desferrioxamines A_1, A_2, B, C, D_1, D_2, E, F, G, and H (1, 3, 5, 15–17). Of this natural group of structurally similar siderophores, two linear and two cyclic ones were tested for their uptake characteristics, providing a probe of the specificity of the uptake system(s). Ferrioxamines B and D_1 differ only in one side chain: the positively charged amino group of ferrioxamine B is acetylated in ferrioxamine D_1. The two cyclic ferrioxamines E and D_2 differ in the 1-amino-5-hydroxyaminopentane unit, which is replaced in ferrioxamine D_2 by 1-amino-4-hydroxyaminobutane (Fig. 1).

In principle, siderophore recognition and uptake rate could be sensitive to the geometry of the metal coordination sites as well as to the ligand structure. Studies of synthetic mirror images of ferrichrome (39), enterobactin (28), and rhodotorulic acid (B. F. Matzanke, G. I. Müller, and K. N. Raymond, submitted for publication) have shown that there is discrimination between the optically active siderophore complex and its synthetic enantiomer. However, the question of whether siderophore uptake systems are capable of distinguishing different stereochemistry at the metal center rather than that of the combined metal ligand has not been resolved (19, 20, 34). The high-spin d^5 ferric ions in siderophore complexes are kinetically too labile in aqueous solution to allow separation of geometrical isomers. Replacing the ferric ion with the kinetically inert chromic ion (which has a large ligand field stabilization energy due to its d^3 electronic configuration) leads to slow ligand substitution

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and isomerization reactions. This allows the separation of coordination isomers which can be used as probes for the stereospecificity of uptake and determination of whether ligand exchange during uptake is a rate-limiting step (32, 33). For this purpose, chromic desferriferrioxamine B complexes were synthesized and the isomers were separated as described previously (22) (Fig. 2).

Although reduction is a known mechanism for removing iron from ferric siderophores within the microbial cell, there are no examples of reductases which are responsible for the rate-limiting step of transport. To obtain evidence for a reductive or a nonreductive uptake mechanism, we prepared Ga(III) desferriferrioxamine B complexes, which are kinetically labile and have the same charge and ionic radius ratio as Fe(III) but cannot be reduced under physiological conditions (9).

MATERIALS AND METHODS

Materials. Reagent-grade chemicals were used throughout. Desferrioxamine B (Desferal) was obtained from Ciba-Geigy Corp. Summit, N.J. Samples of the ferrioxamines D_1, D_2, and E were generous gifts of W. Keller-Schierlein. Their isolation and characterization have been described previously (3, 6, 15, 16). Ion exchange chromatography was performed on Chelex-100 and AG 50W-X5 resins, minus 400 mesh (Bio-Rad Laboratories); XAD-2 (200 to 250 mesh, Serva) was used for adsorption chromatography. The metal complexes were chromatographed on aluminum-backed cellulose plates and with C_{18} reverse-phase thin-layer chromatography plates (Merck & Co.). The $CrCl_3 \cdot 3$ tetrahydrofuran (THF) was prepared by the method of Collman and Kittlerman (7). $^{55}FeCl_3$ and $^{67}GaCl_3$ were obtained from New England Nuclear Corp.

Organism and growth conditions. *S. pilosus* ATCC 19797 stock cultures were kept on agar slants containing 2.1% YM broth (Difco Laboratories) with 2% agar and stored at 4°C.

Preparation and harvesting of spores. Growing *S. pilosus* in a continuous culture was unsuitable for uptake experiments; the colonies formed large clumps and could not be pipetted in equal portions. However, by starting each liquid culture with a large number of spores, a method was developed which resulted in very fine homogeneous suspensions of pseudomycelia. A high yield of spores was obtained as follows: 500-ml Erlenmeyer flasks with 100 ml of YM broth (2.1%) and agar (2%) were inoculated with 1 ml of a spore suspension (10^3 to 10^4 spores per ml) or with a thick cell suspension. Spores or cells were evenly spread on the surface with a sterile bent glass rod. After incubation for 8 days at 27°C, a white layer of spores developed confluently on the surface of the agar. The spores were harvested as follows: 50 ml of medium or buffer (0.05 M Tris-hydrochloride [pH 7.5], 0.05% Tween 80) was added to the flasks. For fast and quantitative removal and separation of the spores, which are very lipophilic and grow as long chains, the flasks were placed into an ultrasonic water bath cleaner. The water level of the bath was adjusted to the same level as the spore surface. Within 5 to 10 s, all the spores moved into the liquid layer. Ultrasonic treatment also resulted in removal of some colonies from the agar surface. They were separated from the spores by pouring the suspension into a graduated cylinder. The colonies sedimented within a few minutes, whereas the spores remained floating in the supernatant, which was cautiously decanted and centrifuged at 8,000 rpm for 10 min. The pellet was then washed twice with buffer or medium. Since storage at −20°C resulted in loss of viability of the spores and storage at 4°C in buffer resulted in germination, the spores were freshly harvested for each experiment.

Quantification of spores. The spore concentration was determined by measuring the optical density of the spore suspensions at 578 nm. The calibration curve was obtained by plating and counting the number of colonies after 3 days of incubation at 27°C.

Cultivation of cells. For most experiments, cells were grown in a defined iron-deficient medium. The water used was deionized twice and then glass distilled. The medium contained (per liter): 2 g of K_2SO_4, 3 g of K_2HPO_4, 1 g of NaCl, and 5 g of NH_4Cl. These salts were made iron deficient by passage through a column of Chelex-100 (Na^+ form). Thiamine (2 mg) and trace elements were added: 0.005 mg of $CuSO_4$, 0.035 mg of $MnSO_4 \cdot H_2O$, 2 mg of $ZnSO_4 \cdot 7H_2O$, 80 mg of $MgSO_4 \cdot 7H_2O$, and 100 mg of $CaCl_2 \cdot 2H_2O$. Spectroscopic-grade glycerol, 2.5% (autoclaved separately as a 50% stock solution), served as an energy source. All glassware was washed with nitric acid. For uptake experiments, 200 ml of iron-deficient salt medium was inoculated with 5×10^9 to 10^{10} spores and incubated for 6 to 8 h at 27°C in a water bath shaker (140 rpm). During this period the spores started to germinate, and the colonies grew as a homogeneous suspension ideal for pipetting aliquots. The cells were then centrifuged at 8,000 rpm for 10 min, washed twice, and resuspended in iron-deficient medium. After incubation for an additional hour, the cells were

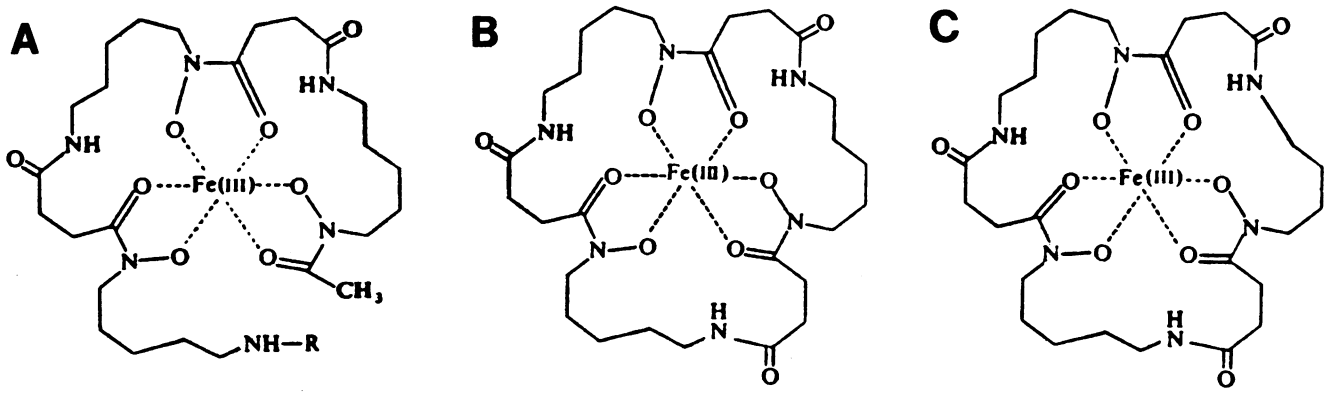

FIG. 1. Structure of ferrioxamines B and D_1 (A) (for B, R = H; for D_1, R = $COCH_3$), E (B), and D_2 (C).

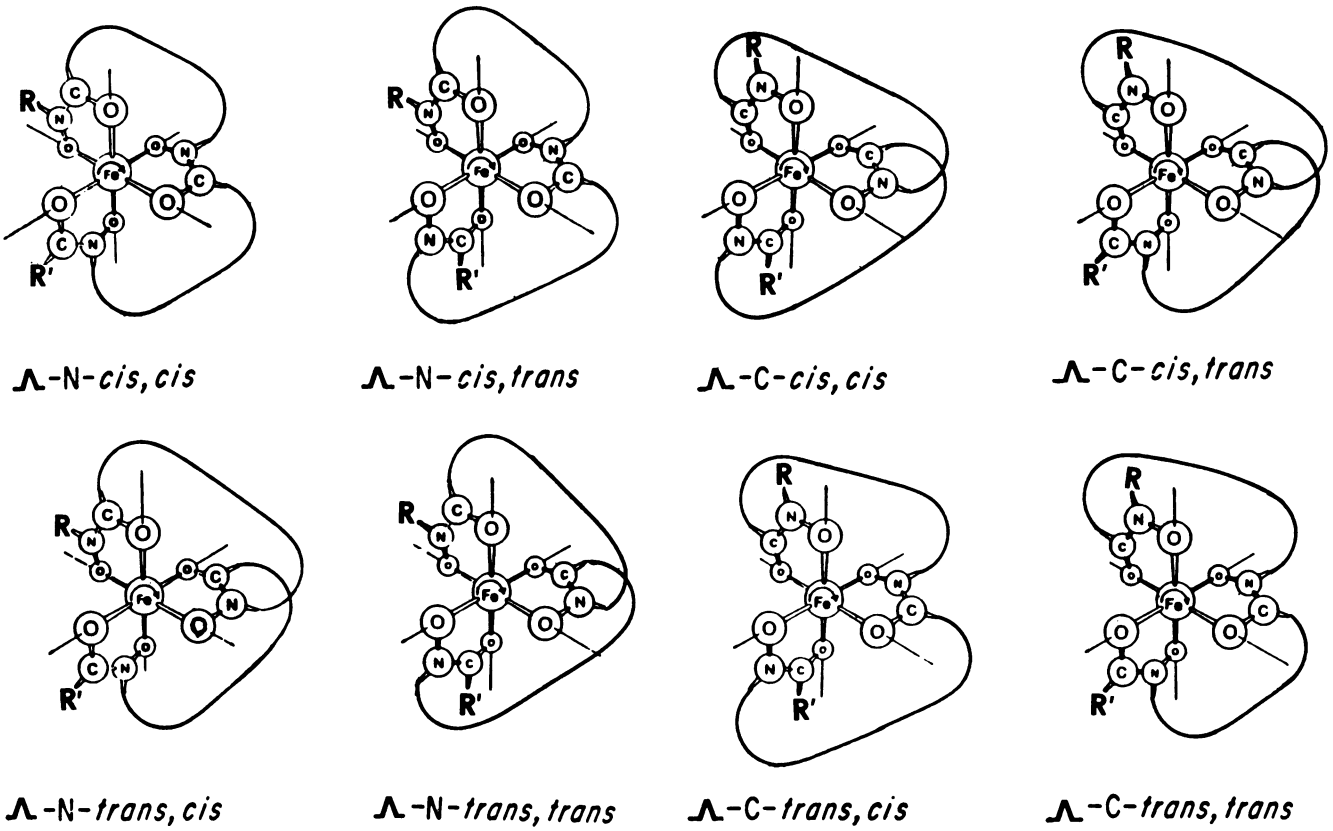

FIG. 2. Eight distinct diastereomeric geometrical isomers of ferrioxamine B. Only the Δ optical isomers are shown in each case (see reference 20 for the nomenclature of these isomers); visible and UV spectra are sensitive only to the immediate environment at the metal site, either *cis* (C-*cis*,*cis* or N-*cis*-*cis*) or *trans* (all of the others). From molecular models it appears that all of the structures shown are possible.

aliquoted in several flasks; these were put on ice and reincubated at 27°C before each experiment.

Transport assays. ^{55}Fe transport was measured by incubating cell suspensions with [^{55}Fe]ferrioxamine in a water bath shaker at 27°C and 140 rpm. Time course measurements were made with 20 ml of a cell culture in 125-ml flasks. At time zero, [^{55}Fe]ferrioxamine was added to the culture, and 1-ml aliquots of cell suspension were removed at regular intervals (usually 2 min), diluted with 5 ml of ice-cold saline (0.9%), filtered through nitrocellulose membrane filters (3-μm pore size; Schleicher & Schuell, Inc.), and washed twice with 5 ml of cold saline. The filters with cells were counted in Aquasol 2 scintillation fluid with a Searle Mark III liquid scintillation counter after homogenization of the sample in the fluid, which contains detergent.

For concentration-dependent kinetic measurements at time zero, 0.8-ml cell suspensions were added to test tubes containing various concentrations of [^{55}Fe]ferrioxamine in a volume of 0.2 ml; 2 and 8 min after initiating uptake, 5 ml of ice-cold saline was added to each test tube. The cells were then filtered, washed, and counted as described above.

Inhibition studies were performed in the same way, but with the addition of chromic desferriferrioxamine B. Although the isomers were stable over the duration of the uptake experiments, they were always kept in liquid nitrogen and added to the assay shortly before the cells were added to prevent isomerization of the chromic complexes during long storage. The cell dry weight was 0.1 to 0.3 mg/ml. All transport studies were repeated two to four times.

The uptake of chromic desferriferrioxamine B was measured by atomic absorption: 10 μM chromic complex was added to a suspension of cells (200 ml), and every 20 min, 30 ml of the cell suspension was removed, filtered through a membrane filter (3-μm pore size), and washed twice with saline (20 ml). The cells with the filter were dried and then dissolved in 1 ml of nitric acid (78%). In a parallel assay, the uptake of [^{55}Fe]ferrioxamine B was determined for the same time intervals as a control.

Preparation of labeled complexes. The radioactive ferric complexes of desferriferrioxamines B, D_1, and E were prepared by adding a $^{55}FeCl_3$ solution in 0.1 N HCl to 0.12 mM ligand solution, which was then diluted and the pH adjusted to pH 7 to give a stock solution of 0.01 mM [^{55}Fe]ferrioxamine B complex (5 to 10 μCi/ml) with 20% excess ligand. Desferrioxamine B was dissolved in water, desferrioxamine E was dissolved in 50% methanol (MeOH), and desferrioxamine D_1 was dissolved in MeOH. The MeOH was evaporated after complexation of the iron. Ferrioxamine D_2 was labeled by adding $^{55}FeCl_3$ in 0.1 N HCl to a 0.1 mM solution of the ferric complex at pH 3.5. The mixture was stirred for 5 h, the pH was adjusted to 7, and excess iron was removed by passing the solution over a column of Chelex-100. The [^{67}Ga]desferrioxamine B was prepared in the same way as described for the ^{55}Fe complexes. Radiopurity was tested with aluminum-backed cellulose thin-layer chromatography plates, which were cut into squares after development and counted in the liquid scintillation counter.

Ferrioxamines D_1 and D_2 were quantitated by determining

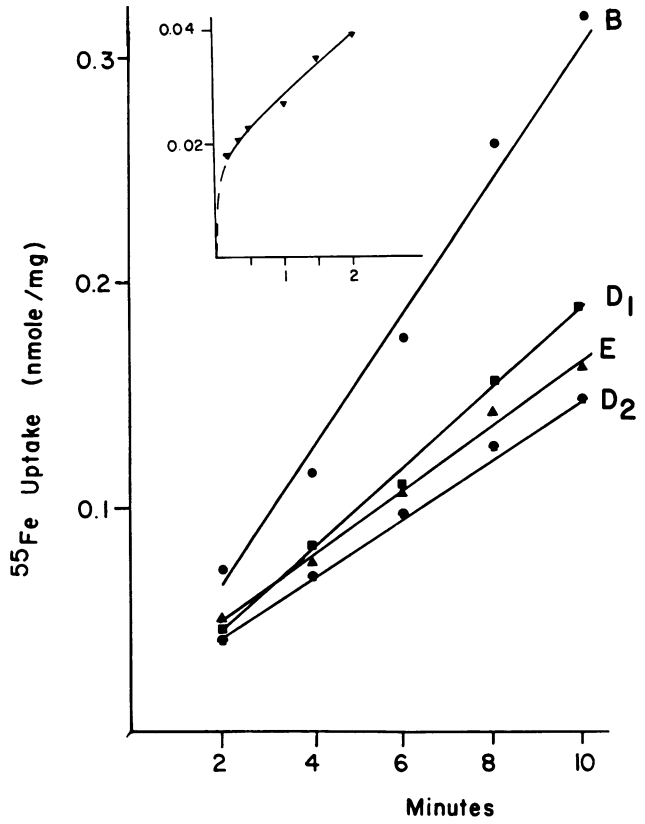

FIG. 3. Time-dependent uptake of ^{55}Fe-labeled ferrioxamines B, D_1, D_2, and E, added at 2 μM. (Inset) Uptake of [^{55}Fe]ferrioxamine E during the first 2 min.

the iron content of nonlabeled solutions by atomic absorption and measuring the visible spectrum with maxima at 434 nm (ε = 2,840 M^{-1} cm^{-1}) and 436 nm (ε = 2,830 M^{-1} cm^{-1}), respectively.

Chromic desferriferrioxamine B. Chromic desferriferrioxamine B was prepared by the method of Leong and Raymond (22). Methanol was distilled from magnesium methoxide, and all chemicals were dried over P_2O_5. To a solution of 300 mg of desferrioxamine B (0.457 mmol) and 449.7 mg (5.48 mmol) of anhydrous sodium acetate in 90 ml of MeOH was added 222 mg of $CrCl_3 \cdot 3THF$ (0.59 mmol) dissolved in MeOH, heated to reflux under dry air, and concentrated to dryness in vacuo. The solid was then suspended in a small volume of glass-distilled water, applied to an XAD-2 resin (200 to 250 mesh), and washed several times with water. The adsorbed chromic complex was eluted with 50% MeOH-H_2O. The MeOH was evaporated, and the aqueous solution of the chromic desferriferrioxamine B was freeze-dried in the dark. Both the chromic complex and ferrioxamine B had identical R_f values on reverse-phase thin-layer chromatography (C_{18}) of 0.84 with 70% MeOH–30% 0.035 M formic acid as the solvent.

Separation of geometrical isomers. Isomers were isolated by a previously described method of sodium cation exchange column chromatography (22). A 100-mg sample of chromic desferriferrioxamine B in 2 ml of water was applied to a column of AG 50W X-r (0.7 mm inner diameter; bed volume, 12 ml) which was equilibrated with 0.3 N NaCl. By collecting 1-ml fractions in a 4°C cold room under N_2 pressure, three major blue-green bands were eluted. The sodium was removed by adsorption chromatography over XAD-2 (200 to 250 mesh) in the dark. The chromic complexes were eluted with 50% MeOH–H_2O, the MeOH was evaporated, and the aqueous solutions were kept in liquid nitrogen for physical measurements and uptake experiments to prevent isomerization.

Physical measurements. The visible spectra of the chromic complexes were determined in aqueous solution at room temperature with a Hewlett Packard 8450A UV-VIS spectrophotometer. The solution concentration of chromium(III) was determined spectrophotometrically as CrO_4^{2-} (ε_{372}max = 4,815 liters mol^{-1} cm^{-1}) as previously described (12). The atomic absorption studies were performed by the Microanalytical Laboratory, Department of Chemistry, University of California, Berkeley.

RESULTS

Iron uptake via ferrioxamines. It has previously been found that several different siderophores produced by an organism can each act as an iron transport agent for the producing organism (8, 38). Iron supplied as ferrioxamine B, D_1, D_2, or E (2 μM) was taken up by *S. pilosus* (Fig. 3). ^{55}Fe from the cyclic ferrioxamines E and D_2 was taken up at the same rate as from the linear ferrioxamine D_1. Ferrioxamine B, which unlike the others has a positive charge at physio-

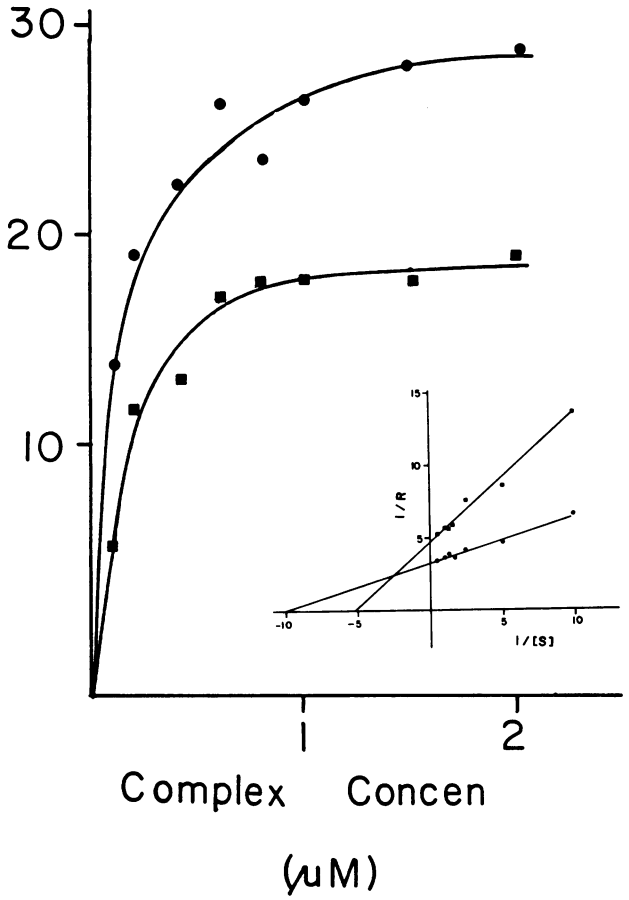

FIG. 4. Concentration-dependent kinetics of ^{55}Fe-labeled ferrioxamine B (●) and E (■) uptake; shown in picomoles per milligram per minute. (Inset) Lineweaver-Burke plot of 1/R (in units of 10^4 milligram-minutes per mole) versus s^{-1}, the inverse complex concentration (in units of 10^6 liters per mole).

TABLE 1. Temperature dependence of [^{55}Fe]ferrioxamine B uptake

Incubation temp (°C)	^{55}Fe uptake[a] (nmol/mg)
0	0.0015
15	0.034
25	0.086
35	0.116
40	0.132
45	0.094

[a] Uptake was measured after 10 min.

logical pH, supplied iron at a 50% higher rate than the other ferrioxamines tested. In all four cases, the accumulation of iron was biphasic. During the first 10 to 20 s, there was a rapid initial rate of ^{55}Fe accumulation which was considered to be specific and nonspecific adsorption to the cell surface (adsorption to filters was subtracted). This rapid phase was followed by a slower rate of transport which was linear at least between 2 and 10 min (Fig. 3).

Evidence for the involvement of specific carriers in the transport of ferrioxamines was obtained by studying the kinetics of iron uptake as a function of the concentration. These were interpreted with a model formally derived from Michaelis-Menten kinetics (2, 24, 38). The ^{55}Fe uptake kinetics from ferrioxamines B and E were measured to assure that uptake was linear between the 2- and 8-min points. This was also done for the lowest concentration applied, which could be achieved by using only a small amount of cells per assay (0.1 mg [dry weight] of cells per ml). The uptake rate was calculated from this linear accumulation phase. The results show that uptake occurred via a saturable system (Fig. 4). Double-reciprocal plots (Fig. 4, inset) yielded an apparent K_m of ~0.1 μM for ferrioxamine B and ~0.2 μM for E, D_1, and D_2 (data for D_1 and D_2 not shown). The low K_m values give an indication of the high affinity of the transport system for its substrate.

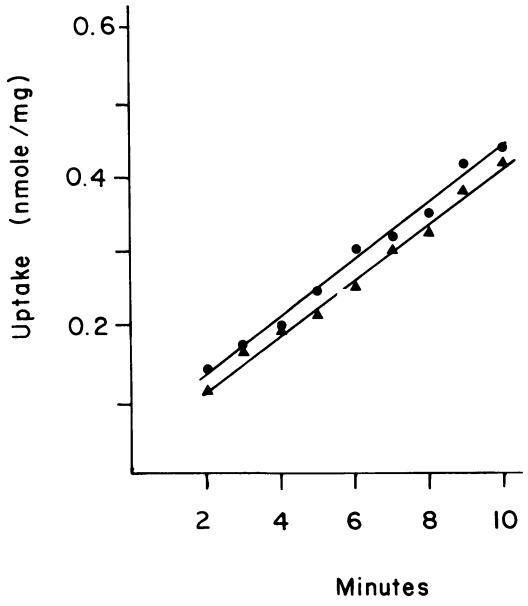

FIG. 5. Uptake of [^{67}Ga]ferrioxamine B (●) and [^{55}Fe]ferrioxamine B (▲). The metal complexes were added at 5 μM concentration.

TABLE 2. Uptake of [^{55}Fe]ferrioxamine B and chromic desferriferrioxamine B as determined by atomic absorption in 1 μM solutions

Time (min)	Uptake (nmol/mg [dry wt] of cells) of:	
	Cr(III)	^{55}Fe
30	2.0	1.73
60	3.1	2.65
90	3.9	3.40

Effect of pH, temperature, and iron concentration in the medium. During growth under iron-deficient conditions, the pH of the culture dropped to 5.8 after 24 h of incubation. To test whether this was of any importance for the uptake rate of ferrioxamine B, the effect of the growth medium pH on iron uptake was measured. For this purpose, 10-ml aliquots of cells were incubated in medium supplemented with 50 mM Tris–morpholineethanesulfonate buffer at different pH at 27°C for 20 min (pH range from 5.5 to 7.5), and then [^{55}Fe]ferrioxamine B uptake was measured for 10 min. Within this range, pH had no effect on the uptake rate (data not shown).

Temperature usually has a dramatic effect on the uptake of siderophores because it strongly influences the fluidity of membranes (23). [^{55}Fe]ferrioxamine B uptake increased between 0 and 40°C but dropped at higher temperatures (Table 1). This is consistent with results for other siderophore uptake systems (16). Although iron transport still increased above 30°C, the uptake measurements were made at 27°C; this is considered the optimal temperature, since incubation at temperatures above 30°C inhibited sporulation on agar medium.

Energy dependence. Incubating the cell suspensions with 10 mM KCN or 10 mM NaN_3 for 5 min before the initiation of uptake resulted in complete inhibition of ^{55}Fe uptake from all ferrioxamines tested (data not shown). This is further evidence that an active transport system is involved in the iron uptake process.

Mechanism of uptake. Since Ga(III) cannot be reduced by reductases and Cr(III) also cannot be reduced and is kinetically inert to ligand exchange as well, substitution of iron by these metal ions is an excellent probe for investigation of the uptake mechanism (34). Both ^{67}Ga and ^{55}Fe were taken up at similar rates when added to the culture as desferrioxamine B complexes (5 μM) (Fig. 5). The transport of chromium(III) as the desferrioxamine B complex was similar to the uptake of ^{55}Fe (Table 2). These results suggest that the metal ferrioxamine complex is taken up as an intact complex, but that neither ligand exchange nor reduction is a rate-determining process.

It has been shown in many microorganisms that siderophore uptake decreases with increased iron concentration in the growth medium (18). This kind of regulation was found for ^{55}Fe uptake from ferrioxamine B in *S. pilosus* (Fig. 6). The 20-ml portions of iron-deficient cell suspensions were incubated for 3 h with various concentrations of iron citrate (0 to 10 μM) at 27°C. After the cells were washed and incubated for 10 min, 5 μM [^{55}Fe]ferrioxamine B was added and uptake was measured at 2 and 8 min to eliminate adsorption. The cell dry weight was determined for each flask. The maximal uptake rate was reached when no iron was added to the medium (Fig. 6).

It can be assumed that when spores are harvested from

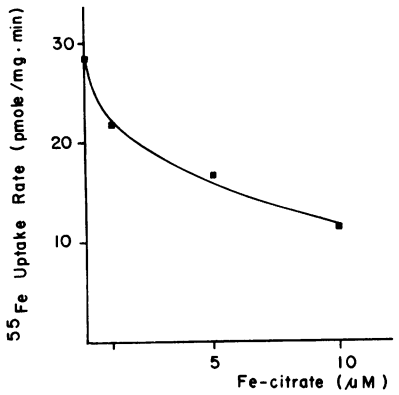

FIG. 6. Dependence of [^{55}Fe]ferrioxamine B uptake rates on the iron concentration in the medium.

iron-sufficient medium, as was done in our experiments, they have stored a considerable amount of iron. Nevertheless, the siderophore transport system is already induced at this very early stage of growth. Attempts to manipulate the iron content of spores by growing and harvesting them in iron-deficient salt medium with iron-deficient agar (Difco) failed. Spore production in this medium was poor (0.01%) compared with that in the complex medium; not enough inoculum could be obtained for an uptake assay.

Separation of geometrical isomers of chromic desferriferrioxamine B. Cation exchange chromatography of the chromic desferriferrioxamine B resulted in three blue-green bands (Table 3 and Fig. 7). The first and the third fractions eluted had extinction coefficient maxima very similar to those of the previously assigned *cis* isomer and *trans* (or mixture of *trans*) isomer(s) (22). In addition, the λ_{max} values of the presently resolved *cis* isomer were bracketed by those of the *trans* isomer. The ratio of the absorption maxima was 0.96 for the *cis* isomer and 0.70 for the *trans* isomers. However, another band, which eluted as the second main fraction, was collected which had different characteristics. The absorption peak maxima at 420 nm (ε = 74.7) and 582 nm (ε = 71.1) had a ratio of 1.05; the high-energy peak showed higher extinction than the low-energy peak compared with these peaks in the *cis* isomer. Since fractions 2 and 3 eluted very near each other, their difference in polarity has to be small compared with that of the *trans* isomer, which eluted much faster. The features of the visible spectra were typical for a *cis* isomer. Previously, only one *cis* isomer and four *trans* isomers have been proposed as sterically allowed structures (22). However, by an examination of molecular models, we found that not only an N-*cis,cis* isomer is possible, but also a C-*cis,cis* isomer and two more *trans* isomers: that is, all of the isomers shown in Fig. 2 are possible. Since fraction 2 did inhibit [^{55}Fe]ferrioxamine B uptake as did the other isomers isolated (Fig. 8), it can be assumed that this fraction was not an artifact. Ferrichrome and ferrichrysin only form N-*cis,cis* isomers due to the rigidity of the molecule.

TABLE 3. Characterization of chromic desferriferrioxamine B isomers

Assigned chromic desferriferrioxamine B isomer[a]	Absorption maxima (ϵ)[b] at indicated wavelength
N-*cis,cis*	67.6 (420 nm), 70.2 (586 nm)
C-*cis,cis*	74.7 (420 nm), 71.1 (582 nm)
trans	50.4 (412 nm), 71.8 (592 nm)

[a] See the text and Fig. 2 for descriptions and a discussion of these assignments.
[b] Units are liters mol^{-1} cm^{-1}.

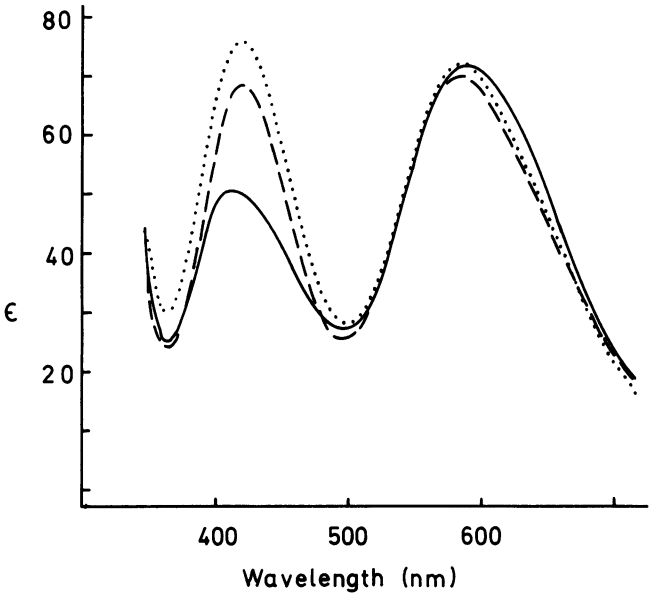

FIG. 7. Visible absorption spectra of the separated geometrical isomers of chromic desferriferrioxamine B. The spectra were recorded as aqueous solutions at pH 7.0. Spectra are shown for the C-*cis,cis* (· · ·), N-*cis,cis* (– – –), and *trans* (or a mixture of *trans*) (———) isomers.

The visible spectra for the chromic desferriferrioxamine and desferrichrysin complexes are the same as those for the third fraction of chromic desferriferrioxamine B in this study (21). Therefore, it was assigned as the N-*cis,cis* isomer. However, the second band eluted also had features of the visible spectra which were more similar to a *cis* isomer. (For a discussion of the assignments of the visible spectra of geometrical isomers of chromic siderophore complexes, see reference 22.) Although only a crystal structure determination could give absolute certainty about the coordination geometry, the second fraction was tentatively assigned as a C-*cis,cis* isomer (Table 3).

Inhibition of iron uptake by geometrical isomers of chromic desferriferrioxamine B. An interesting question which has not yet been resolved (34) is whether an uptake system is so highly specific that it can distinguish between isomers of siderophores which differ only in the stereochemistry at the metal ion. Various concentrations of the three geometrical isomers or mixtures of isomers of chromic desferriferrioxamine B complexes inhibited uptake of ^{55}Fe-labeled ferrioxamines B and E (at 1 μM) (Fig. 8). The three isomers showed the same degree of inhibition of ^{55}Fe uptake at all concentrations tested. At an inhibitor concentration as low as 10 μM, uptake of [^{55}Fe]ferrioxamine B was already reduced to 60 to 70% of the uptake without inhibitor. A remarkable difference was observed in comparing the inhibition of ferrioxamines B and E. At an inhibitor concentration of 10 μM, the rate of ferrioxamine E iron transport decreased to 20% for the *trans* isomer, in contrast to 60% for ferrioxamine B. The same strong decrease in uptake occurred for ferrioxamines D_1 and D_2. When 10 μM *trans* chromic desferriferrioxamine B was

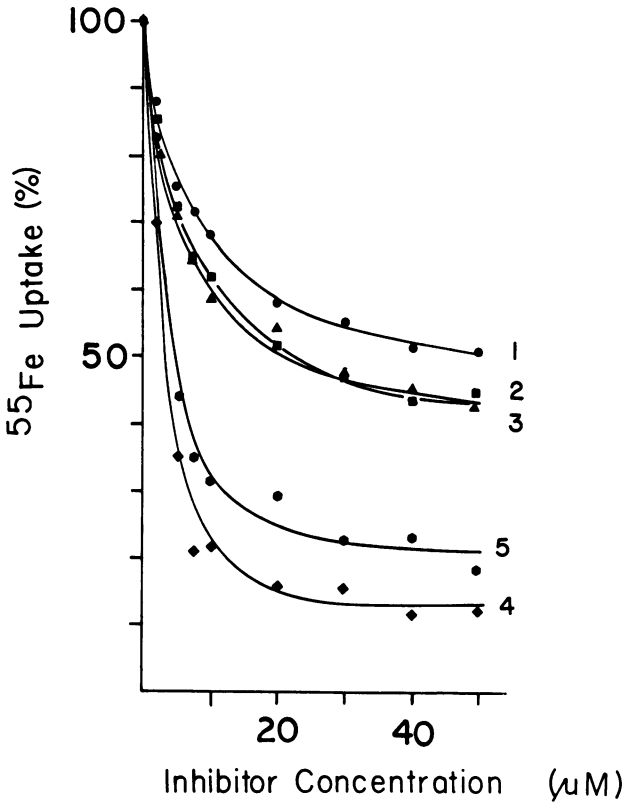

FIG. 8. Inhibition of uptake of [^{55}Fe]ferrioxamines B and E by coordination isomers of chromic desferriferrioxamine B. Inhibition of [^{55}Fe]ferrioxamine B uptake by the N-*cis*,*cis* isomer (line 1), C-*cis*,*cis* isomer (line 2 [■]), and *trans* (or mixture of *trans*) isomers (line 3 [▲]) and inhibition of [^{55}Fe]ferrioxamine E uptake by the *trans* (or mixture of *trans*) isomers (line 4) and N-*cis*,*cis* isomer (line 5). The ^{55}Fe-iron complexes were added at 1 μM concentrations. The 100% values were determined from the uptake rates of [^{55}Fe] ferrioxamines B and E when no chromic complexes were added.

added, ^{55}Fe accumulation was only 25% for ferrioxamine D_2 and 32% for ferrioxamine D_1 (data not shown).

DISCUSSION

Freshly germinated spores of *S. pilosus* can accumulate iron from ferrioxamines B, E, D_1, and D_2 when cultivated under iron-deficient conditions. Iron supplied by these four chelating agents is taken up by an energy-dependent process which displays saturation kinetics. The maximal uptake rate was reached at ~1 μM substrate concentration; the K_m values were low (~0.1 μM for ferrioxamine B and ~0.2 μM for E, D_1, and D_2), indicating that a high-affinity transport system is involved, with no diffusion into the membrane (Fig. 4). The kinetic parameters and uptake rates are within the range reported for other bacterial siderophore transport systems (10, 11).

The exact sequence of events in hydroxamate iron assimilation is still obscure for most microorganisms. However, it is clear that it is a complex process, involving many steps, including recognition and binding, translocation, and decomplexation. Desferrioxamine complexes of ^{67}Ga(III) and Cr(III) are excellent probes of the transport mechanism: ^{67}Ga(III) cannot be reduced, and Cr(III) is inert to ligand exchange. In *S. pilosus*, ^{67}Ga(III), Cr(III), and ^{55}Fe(III) as desferrioxamine complexes were taken up at comparable rates. This result demonstrates that neither reduction nor decomplexation is the rate-limiting step during the transport process (Fig. 5 and Table 2). The intact complexes are accumulated by the cells.

Three separated, kinetically inert chromium complexes of desferriferrioxamine B were assigned as the N-*cis*,*cis*, C-*cis*,*cis*, and a *trans* (or mixture of *trans*) isomers, by the UV-visible spectra. These all showed substantially the same inhibition of [^{55}Fe]ferrioxamine B uptake. From this result it can be concluded that all three isomer fractions interact with the ferrioxamine B transport system. This in turn suggests that the uptake mechanism does not discriminate between geometrical isomers. The same result was obtained in an experiment with *Rhodotorula pilimanae*, in which inhibition of ^{55}Fe-uptake as mediated by rhodotorulic acid was impeded equally by a *cis* and a *trans* isomer and by a mixture of *trans* isomers (G. Müller, Y. Isowa, and K. N. Raymond, submitted for publication). It must be pointed out, however, that no differentiation between uptake (which includes recognition, binding, and translocation) and binding alone (without subsequent transport) can be made by inhibition experiments.

Since no siderophore receptor mutants of *S. pilosus* are available, the chromic isomers of desferriferrioxamine B were also used to gain information about the mutual interactions of ferrioxamines at one or more receptor sites. If ferrioxamines E, D_1, and D_2 are accumulated in the cells by a different system(s) than ferrioxamine B, no inhibition of ^{55}Fe uptake from ferrioxamines E, D_1, and D_2 should occur. Surprisingly, chromic desferriferrioxamine B isomers inhibited iron uptake from ferrioxamines E, D_1, and D_2 more dramatically than from ferrioxamine B itself. This is evidence that all the ferrioxamines tested provide iron to the cells by the same receptor and transport system. The higher K_m values of ferrioxamine E, D_1, and D_2 uptake (which means lower affinity to the transport system) are consistent with the resulting stronger inhibition. However, these experiments do not exclude the possibility that there exists one or more uptake systems for ferrioxamines E, D_1, and D_2 in which ferrioxamine B is nevertheless transported at a good rate or only adsorbed and thus acts as inhibiting agent. This latter possibility is not considered likely from the viewpoint of efficient supply of iron to the cell.

In nature it is not unique that several structurally related siderophores of one family deliver iron by the same transport system, as demonstrated by *Escherichia coli* with its ferrichrome receptor (31). Conclusive proof for the number of uptake systems can only be obtained by characterizing siderophore receptor-deficient mutants of *S. pilosus*.

Assuming that iron from all four siderophores tested in *S. pilosus* is accumulated by the same carrier system, it appears that no discrimination is made between linear (D_1) and cyclic (E, D_2) ferrioxamines. In addition, minor differences in the chain length (D_2 had one methylene group less than E) of the ligand do not affect uptake. However, the positive charge of ferrioxamine B seems to play an important role and results in higher uptake rates than those for its derivative D_1, which is acetylated at the amino group (Fig. 3).

The geometrical isomers of ferrioxamine B display changes of three features. There were differences in (i) the charge distribution at the ferric Tris hydroxamate side, (ii) the relative position of R and R', and (iii) the hydroxamate linking chains. By examining the models of ferrioxamine B isomers, it can be seen that the relative position of the NH_3^+-$(CH_2)_5$- side arm changes in each isomer (Fig. 2).

However, these differences do not affect uptake or inhibition of the chromium complexes. Since the long side arm of the $-NH_3^+$ group is free to rotate and assume almost any geometry with regard to the complex, this may explain the lack of specificity. Thus, it is not clear how recognition and transport take place, whether the positive $-NH_3^+$ group has a corresponding binding site at the receptor system or simply brings ferrioxamine B into the proper position of recognition or is a factor for increasing the translocation rate. At least for the latter two possibilities, any charge distribution effect at the metal octahedral coordination site might have the same role as the positive charge of the amino group in ferrioxamine B. Therefore, isomers of the uncharged ferrioxamine D_1 would be a more accurate probe for use in future investigations.

Since the neutral complexes are taken up at significant rates, the $-NH_3^+$ group certainly is only one important part for recognition. In ferrioxamine B the groups bound to the hydroxamate C and N atoms are methylenes [which are part of the flexible hydroxamate-linking $-(CH_2)_5-CONH-(CH_2)_2$ chains] or the N-terminal aminopentane. The C-terminal residue is a methyl group. The cyclic ferrioxamines E and D_2 have only CH_2 groups adjacent to the hydroxamate C and N atoms. When the different geometrical isomers at the outer surface of the complex are compared, the rim of CH_2-/CH_3-groups is seen to remain the same. This could explain the lack of distinction between the *cis* and *trans* isomers, as well as the considerable uptake of the uncharged ferrioxamine B homologs.

In another investigation (26), it was shown that even ferrichrome is taken up by *S. pilosus*. Since the inhibition of ferrichrome uptake by chromic desferriferrioxamine B is similar to the inhibition of ferrioxamine E uptake, it is concluded that ferrichrome is accumulated by the same uptake system as the ferrioxamines. If we exclude the possibility of a multifunctional receptor (which has adsorption sites specialized for each type of siderophore), then it must be concluded that the common features of recognition are the iron center, its adjacent hydrophobic CH_2 and CH_3 units, and the positive charge.

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