JOURNAL OF BACTERIOLOGY, July 1992, p. 4783–4789
0021-9193/92/144783-07$02.00/0

Vol. 174, No. 14

Ferric Iron Uptake in *Erwinia chrysanthemi* Mediated by Chrysobactin and Related Catechol-Type Compounds

MAGNUS PERSMARK,[1]† DOMINIQUE EXPERT,[2] AND J. B. NEILANDS[1]*

Department of Molecular and Cell Biology, 401 Barker Hall, University of California, Berkeley, California 94720,[1] and Laboratoire de Pathologie Végétale, Institut National de la Recherche Agronomique, 75231 Paris Cedex 05, France[2]

Received 21 January 1992/Accepted 28 April 1992

***Erwinia chrysanthemi* 3937 possesses a saturable, high-affinity transport system for the ferric complex of its native siderophore chrysobactin, [*N*-α-(2,3-dihydroxybenzoyl)-D-lysyl-L-serine]. Uptake of ^{55}Fe-labeled chrysobactin was completely inhibited by respiratory poison or low temperature and was significantly reduced in rich medium. The kinetics of chrysobactin-mediated iron transport were determined to have apparent K_m and V_{max} values of about 30 nM and of 90 pmol/mg · min, respectively. Isomers of chrysobactin and analogs with progressively shorter side chains mediated ferric iron transport as efficiently as the native siderophore, which indicates that the chrysobactin receptor primarily recognizes the catechol-iron center. Free ligand in excess only moderately reduced the accumulation of ^{55}Fe. Chrysobactin may therefore be regarded as a true siderophore for *E. chrysanthemi*.**

The enterobacterial phytopathogenic bacterium *Erwinia chrysanthemi* 3937 was recently shown to produce in vitro a low-molecular-weight ferric ion chelator, a siderophore, which was excreted in response to low-iron stress. The siderophore, termed chrysobactin (Cb) (Fig. 1), was characterized as *N*-α-(2,3-dihydroxybenzoyl)-D-lysyl-L-serine (38). As in other siderophore systems (4), Cb synthesis was accompanied by membrane protein synthesis. Three low-iron-induced proteins were detected in the outer membrane of *E. chrysanthemi* 3937, and one of these, an 80-kDa polypeptide, has been identified as the ferric Cb receptor (Fct) (17, 18, 22). The integrity of this iron transport system was essential for virulence of *E. chrysanthemi* 3937 on saintpaulia plants (17, 19), a phenomenon well established in animals but not previously observed in plants (32, 37). The gene encoding Fct, as well as the *cbsABCE* genes involved in the four primary stages of Cb biosynthesis, are integral parts of an operon (21, 22).

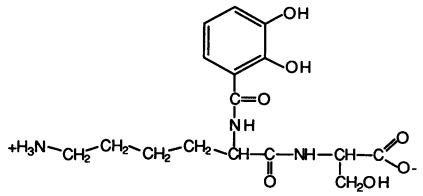
Many siderophores possess six ligands, allowing full octahedral coordination of hexacoordinate Fe(III) (25, 33). However, siderophores with fewer ligands have been described. The iron-dicitrate transport system in *Escherichia coli* is the perhaps most thoroughly studied (46). Other examples are the hydroxamates rhodotorulic acid (2), dimerum acid (14), and fusarinine (14) and the thiazoline-containing ferrithiocin (23) and pyochelin (13). A relatively large number of compounds, including Cb, represent yet another class of nonhexadentate ligands. This class contains amino acids or peptides, mono- or disubstituted with dihydroxybenzoic acid (DHBA) (5, 10, 28, 29, 36, 38 [and references therein], 44).

Although several reports on monocatecholates have associated them with iron transport, some factors have served to call into question their function as true siderophores. (i) Many bacteria, e.g., *E. coli* and *Salmonella typhimurium*, produce hexadentate siderophores, such as aerobactin and enterobactin, which are extremely efficient ferric iron chelators. Enterobactin, which may be the most common enterobacterial siderophore, is a hydrolytically labile cyclic triester of dihydroxybenzoyl serine. Furthermore, receptors for exogenous hexadentate ferric siderophores are widespread in bacterial genera. (ii) Bidentate ligands are far less efficient chelators than their hexadentate counterparts, especially at low concentrations. Monocatecholates found in culture media have thus tended to be regarded as degradation products. The same reasoning has been applied to some monohydroxamates, such as the fusarinines (26). Other studies have pointed towards a true role for the monocatecholates as siderophores. *E. coli* was postulated to contain a transport system for complexes containing two molecules of DHBA (42), and biological activity has been associated with several of the monocatecholates mentioned above. The low-iron-inducible outer membrane proteins Cir and Fiu of *E. coli* were recently shown to function as cell surface receptors for monocatecholates (24, 34).

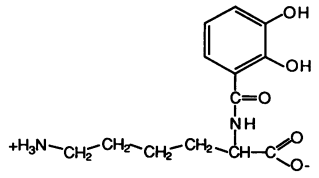
E. chrysanthemi 3937 was found to utilize the exogenous hexadentate siderophores ferrichrome and enterobactin, and two low-iron-inducible outer membrane proteins were identified as their receptors. However, *E. chrysanthemi* appeared to synthesize only Cb in vitro (18, 38). Furthermore, in contrast to *Erwinia amylovora* (8), *E. chrysanthemi* is unable to utilize ferric citrate, the main iron carrier in higher plants, and thus would have to rely on an endogenous siderophore(s) for iron acquisition in planta. The intercellular fluid appeared sufficiently low in iron that Cb synthesis could be induced in planta (18).

Recently, the genes involved in ferric iron transport in *E. chrysanthemi* have been studied in depth (21, 22). To further understand the physiology of this system, we investigated the transport of iron in wild-type cells, as well as in two mutants, with isomers of Cb and catecholate derivatives (Fig. 1). It was found that *E. chrysanthemi* 3937 possesses a high-affinity Cb-mediated transport system, by which Fe(III) is accumulated by a saturable, energy-requiring process. Only the catecholate moieties appear to be important in receptor recognition, since enantio-Cb, DHBA-lysine, *N*-

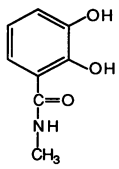
* Corresponding author.
† Present address: Department of Biochemistry, Center in Molecular Toxicology, Vanderbilt University, Nashville, TN 37232.



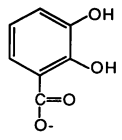
Chrysobactin



N-α-(2,3-dihydroxybenzoyl)-lysine



N-(methyl)-2,3-dihydroxybenzamide



2,3-dihydroxybenzoic acid

FIG. 1. Structure of the catecholate derivatives used in this study. Cb (*N*-α-(2,3-dihydroxybenzoyl)-D-lysyl-L-serine) is an endogenous siderophore of *E. chrysanthemi* 3937.

methyl-2,3-dihydroxybenzamide (MDHB), and DHBA were taken up as efficiently as the native siderophore.

MATERIALS AND METHODS

Materials. When applicable, glassware was washed in 6 N HCl and rinsed excessively with double-distilled water prior to use. D-Serine methyl ester hydrochloride was from Chemical Dynamics Corp. CytoScint was from ICN Biomedicals Inc. $Ga_2(SO_4)_3$ was obtained from Fluka AG, and $^{55}FeSO_4$ (specific activity, 19.63 and 24.22 Ci/g) in 0.1 N H_2SO_4 was from Du Pont, NEN Research Products.

Spectroscopy. Bausch and Lomb Spectronic 2000 and Shimadzu UV160 spectrophotometers were used for UV and visible spectrophotometry. Nuclear magnetic resonance (NMR) spectra were obtained on a Bruker AM spectrometer, operating at a 400.13-MHz proton frequency, at the University of California, Berkeley, NMR facility. Mass spectra (MS) were obtained at the Mass Spectrometry Facility, University of California, Berkeley.

Chemical synthesis. Cb [*N*-α-(2,3-dihydroxybenzoyl)-D-lysyl-L-serine] (D-L-Cb), its L-lysine-D-serine (L-D-Cb) and L-lysine-L-serine (L-L-Cb) isomers, and the two isomers of *N*-α-(2,3-dihydroxybenzoyl)-lysine (DHBA-D-Lys and DHBA-L-Lys) were synthesized as described previously (38, 39).

The analog MDHB was prepared by condensation of methylamine with DHBA. Three millimoles of DHBA was dissolved in a small volume of tetrahydrofuran, and 1,3-dicyclohexylcarbodiimide in 10% molar excess was added. Methylamine (40% in H_2O) was added in excess, and the coupling was allowed to proceed at room temperature overnight. Acetic acid (300 μl) was added, and the dicyclohexylurea was removed by filtering. Purification was followed by thin-layer chromatography ($CHCl_3$-methanol, 4:1), in which MDHB moved faster than DHBA and was identified as a blue fluorescent spot which turned red after being sprayed with $FeCl_3$ in 10 mM HCl. After extraction with 10% (wt/vol) citrate, pH 5.5, the organic phase was evaporated to dryness. The residue was dissolved in ethyl acetate and chromatographed on a silica column in this solvent. Material adhering to the column was eluted with ethyl acetate-ethanol (9:1). Fractions containing MDHB were again taken to dryness, dissolved in $CHCl_3$-methanol (9:1), and chromatographed on a silica column. After removal of organics by rotary evaporation, the residue was taken up in acetone and impurities were precipitated with water. MDHB was obtained as a whitish powder after filtration and evaporation of the acetone. Analyses were as follows. MS (high-resolution EI) *m/z* (deviation (Δ, mmu), relative intensity): $[M]^+$ 167.0586 (Δ 0.4, 50); $[M - NH_2CH_3]^+$ 136.0181 (Δ 2.0, 100); $[M - C(=O)NH_2CH_3]^+$ 108.0228 (Δ 1.6, 23). ^1H NMR (CD_3OD): δ = 2.90 (s, 3 H), 6.70 (t, ^3J = 8.0 Hz, 1 H), 6.91 (dd, ^3J = 7.9 Hz, ^4J = 1.5 Hz, 1 H); 7.16 (dd, ^3J = 8.1 Hz, ^4J = 1.4 Hz, 1 H). A minor impurity was observed at 3.48 ppm.

Bacterial strains. The wild-type strain *E. chrysanthemi* 3937, the mutant *E. chrysanthemi* PMV 4089 (Cbs^-), blocked in the synthesis of Cb between DHBA and the final product, and the biosynthesis and transport mutant *E. chrysanthemi* PMV 4082 (Cbs^- Fct^-) have been described previously (17). The bacteria were kept at room temperature on Luria-Bertani (LB) plates with biweekly restreakings.

Preparation of radiolabeled complexes. ^{55}Fe complexes were prepared by adding $^{55}FeSO_4$ to each ligand in H_2O at a metal-to-ligand (M/L) ratio of 1:2.5, 1:3, or 1:4. The solutions were neutralized with NaOH and the pH was adjusted to 6.5 (or 7.4) by the addition of 1 M MOPS (morpholinepropanesulfonic acid) (or 1 M Tris) to give stock solutions ranging from 100 to 600 μM ^{55}Fe complex (0.11 to 0.80 mCi/ml; 1 Ci = 37 GBq). Degassed and nitrogen-purged solvents were used throughout.

Uptake experiments. All incubations were done at 30°C on a rotary shaker set at 300 rpm. Single colonies from LB plates were used to inoculate 10 ml of LB medium, which was shaken overnight. A 2% inoculum of this culture was made into MM9 medium (43) with 2.5 μM added $FeCl_3$ and buffered with either 0.1 M MOPS (pH 6.5) or 0.1 M Tris (pH 7.4). After one transfer in MM9 (at least two for kinetic experiments), 2.5 ml of the culture was used to inoculate 22.5 ml of MM9 in a 250-ml Erlenmeyer flask. This final culture medium contained 2.5 μM $FeCl_3$ for *E. chrysanthemi* PMV 4082 and PMV 4089 and 0.5 μM $FeCl_3$ for *E. chrysanthemi* 3937.

After 4 to 6 h ($A_{600} \approx 1.3$) cells were harvested by centrifugation at 7,000 × *g* for 10 min. With *E. chrysanthemi* PMV 3937 the supernatant was positive for catechols with the Arnow molybdate test (1) (A_{515} = 0.07 to 0.1), and the culture thus was iron starved. For kinetic and time-dependent-uptake studies, the cells were centrifuged at 4°C and washed with cold transport medium (modified MM9 without Fe) in the absence of glucose. Washed cells were suspended in transport medium to an A_{600} of 0.35 or 0.50 (kinetic measurements) or an A_{600} of 0.80 (time-dependent-uptake studies) and kept on ice until use. In all cases, cells were used in less than 2 h.

For the time-dependent-uptake experiments, 2.8 ml of a cell suspension with an A_{600} of 0.80 was placed in a 50-ml conical polypropylene tube and preincubated for 30 min in

the rotary shaker. Radiolabeled chelate, as a stock solution diluted to 200 μl in sterile transport medium, was then added to give a final concentration of 2 μM ^{55}Fe-ligand. At intervals from 2.5 to 30 min, 300-μl portions were withdrawn and immediately filtered through 0.45-μm-pore-size Millipore HA filters which had been presoaked for at least 24 h in 50 μM unlabeled ferric complex (50 μM Fe, 250 μM ligand) and washed before use. Filter-bound cells were immediately washed twice with 5 ml of ice-cold transport buffer without glucose. The filters were placed in empty scintillation vials and air dried overnight. Ten milliliters of CytoScint ES was added, and after equilibration for 24 h, cell-associated radioactivity was measured by liquid scintillation counting. Four 100-μl portions of each sample were also counted to verify the total amount of radioactivity. Energy-dependent metabolism was inhibited by adding sodium azide to 20 mM at the onset of the preincubation or by keeping the cell suspension at 0°C during both the preincubation and the uptake experiment. In experiments performed at pH 6.5 the M/L ratio was 1:2.5, whereas the ratio was 1:4 for experiments carried out at pH 7.4. The latter pH was used in a comparison of D-L-Cb with MDHB and DHBA, the higher pH enhancing the solubility of the catecholates lacking amino functions.

In a study of transport as a function of growth medium, the following media were used: MM9 plus 2.5 μM Fe (0.5 μM for the final culture), MM9 plus 25 μM $FeCl_3$, MM9 plus 25 μM $FeCl_3$ plus 0.3% Casamino Acids, LB, and LB plus 0.2% glucose. Cells were harvested at room temperature, washed twice in transport buffer without glucose, and suspended to an A_{600} of 0.85. The cells were assayed for transport competence at pH 6.5 as described for the time-dependent study above, except that the radiolabeled chelate (2.0 μM final concentration) was added after only a 5-min preincubation on the rotary shaker.

For kinetic measurements 2.8 ml of cell suspension was preincubated for 30 min before the addition of 200 μl of radiolabeled Cb to final concentrations ranging from 10 to 100 nM. Samples (400 μl) were withdrawn every 20 s for 2 min, filtered, and treated as described above. The kinetic experiment was carried out with two M/L ratios, 1:2.5 and 1:3, and *E. chrysanthemi* PMV 4089 was used throughout.

To measure the effect of free ligand concentration on uptake, initial rates were measured with 100 nM $^{55}Fe^{3+}$ and Cb concentrations from 200 nM to 5 μM. Experiments were performed as described for kinetic measurements.

RESULTS

Uptake of ferric Cb. Iron supplied to *E. chrysanthemi* in the form of ferric Cb was very efficiently taken up by the cells. An energy dependence for the uptake process was shown by an almost complete loss of transport competence by cells pretreated with the respiratory poison sodium azide or by cells which were kept on ice during the experiment (data not shown).

The most rapid uptake of ferric Cb was observed with cells grown in the modified low-iron MM9 medium, whereas in complex LB medium the uptake rates were greatly reduced (Fig. 2). This difference may be ascribed to the repression of synthesis of low-iron-induced ferric Cb transport functions in rich medium. However, in MM9 with 25 μM added iron, significant uptake was observed. The supplementation of LB and MM9 with glucose and Casamino Acids, respectively, did not significantly affect uptake (Fig. 2).

Specificity of iron transport. Cb and its analogs are derivatives of a chiral molecule, D- or L-lysine. These chelators

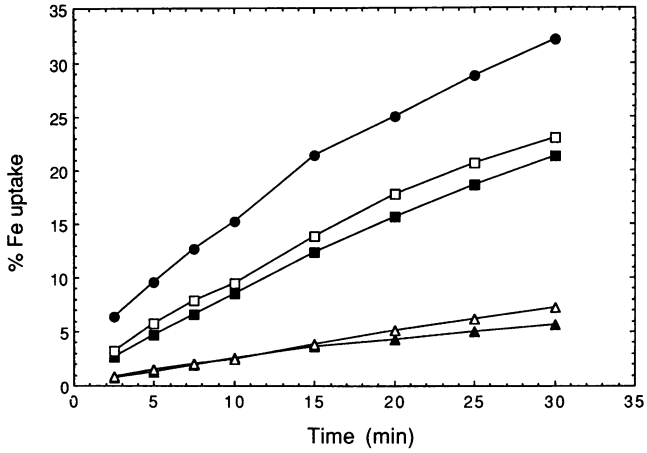


FIG. 2. Variation of iron uptake by *E. chrysanthemi* 3937 grown in different media: MM9 plus 2.5 μM Fe (●), MM9 plus 0.3% Casamino Acids plus 25 μM Fe (□), MM9 plus 25 μM Fe (■), LB plus 0.2% glucose (△); and LB (▲). Cells were washed twice with transport buffer at room temperature, suspended in transport medium, and assayed for uptake of $^{55}Fe(Cb)_{2.5}$ at 2.0 μM after a 5-min preincubation. Data shown are average values from two experiments.

could therefore potentially form optically active metal complexes. Time-dependent-uptake experiments at pH 6.5 were performed with D-L-Cb and the L-D-Cb and L-L-Cb isomers and with DHBA-D-Lys and DHBA-L-Lys. As shown in Fig. 3, no difference in the uptake rates between the native D-L-Cb and any of the DHBA-lysine derivatives was observed. The chirality at the two α carbons of the peptide backbone was apparently not recognized by the receptor. On the basis of the results with DHBA-lysine, we furthermore conclude that the serine moiety was not important in receptor recognition. The same results, within experimental error, were obtained with the Cbs$^-$ PMV 4089 and, surprisingly, with the Cbs$^-$ Fct$^-$ PMV 4082 (data not shown). As both mutants are Cbs$^-$, the possibility of ligand exchange during the preincubation could be excluded.

The importance of net charge and the positively charged lysine side chain for receptor recognition of ferric catecholates was probed with MDHB and DHBA. The ferric complex of MDHB has the same net charge as ferric Cb, −1 for the dimer and −3 for the trimer, whereas ferric DHBA has one more negative charge per catecholate moiety. MDHB lacks both the negative and positive charges of Cb but contains an amide bond, which for an analog of enterobactin was found to be crucial for receptor recognition (16). Both MDHB and DHBA mediated Fe(III) transport as efficiently as did Cb (Fig. 3); therefore, it may be concluded that the catecholate moieties and/or the metal center are the most important structural features of ferric Cb in receptor recognition.

Kinetics of ferric Cb uptake. To further investigate the presence of a specific carrier-mediated uptake process for ferric Cb, its kinetics of assimilation were studied. The transport of ^{55}Fe-Cb was characterized by a rapid phase, lasting less than 30 s, followed by a slower linear phase. The former may represent association of labeled ferric siderophores and cells, whereas the latter phase presumably represents true transport. Results in Fig. 4 show that at ferric Cb concentrations between 10 and 100 nM, transport occurs via a saturable process. Initial uptake rate values were

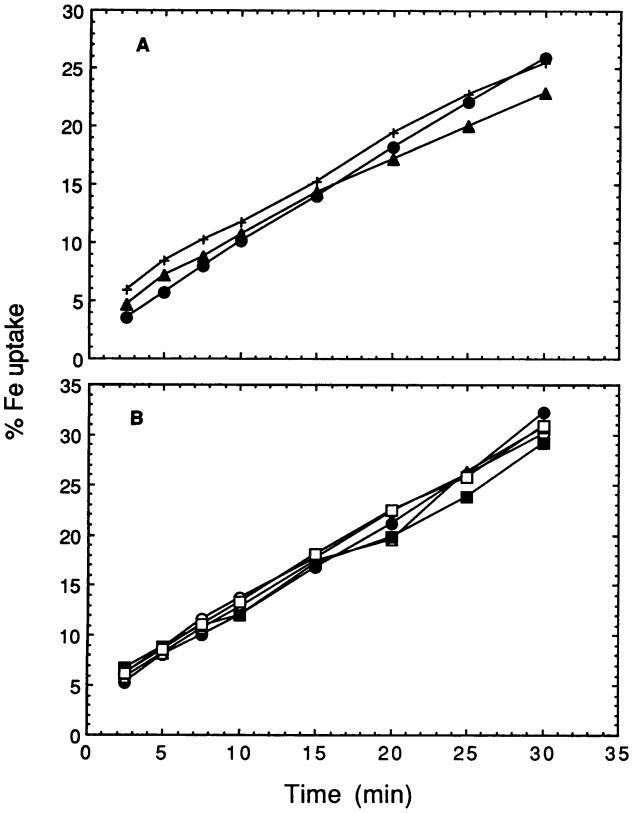


FIG. 3. Time-dependent uptake of ferric catecholates by *E. chrysanthemi* PMV 4089 (M/L = 1:4, pH 7.4) (A) and *E. chrysanthemi* 3937 (M/L = 1:2.5, pH 6.5) (B). Uptake was measured at 2.0 μM ^{55}Fe(chelate) with the following DHBA derivatives: D-L-Cb (●), L-D-Cb (○), L-L-Cb (▲), DHBA-D-Lys (■), DHBA-L-Lys (□), MDHB (△), and DHBA (+). Data shown are average values from at least two experiments.

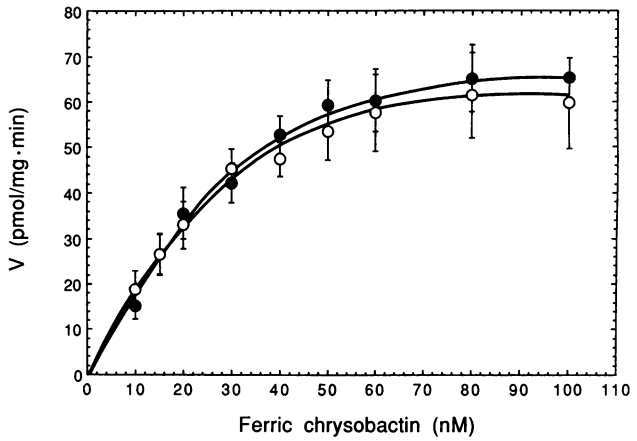


FIG. 4. Concentration-dependent kinetics of ^{55}Fe(Cb) uptake by iron-starved cells of *E. chrysanthemi* PMV 4089 (Cbs$^-$) at M/L ratios of 1:2.5 (○) and 1:3.0 (●). Data are presented as average values with standard deviation bars for five and six experiments, respectively.

essentially constant over a concentration range of ferric Cb from 50 nM to 100 μM and linear, with A_{600} values from 0.35 to 0.80, over a period of up to 8 min for the highest concentrations (data not shown). Furthermore, no difference was observed between the two ferric Cb complexes in which the M/L ratios were 1:2.5 and 1:3. In the former it is theoretically not possible to quantitatively form the Tris complex. Cb-mediated ^{55}Fe uptake obeyed simple Michaelis-Menten kinetics, as indicated by a straight line in the Hanes-Woolf plot (not shown). The initial uptake rate values, V_0, were generated from the first 2 min of uptake. Data points were fitted by linear regression to a line whose slope was equal to the initial uptake rate. As the initial phase of very rapid cell association may not represent true transport, the first datum point for each experiment was omitted. Furthermore, as the chelate pool became depleted within the time course of the experiment for the four lowest concentrations, initial uptake rates were generated from three and four datum points for 10 to 20 nM and 30 nM, respectively. The apparent K_m and V_{max} values were generated with the Fortran program of Cleland (11). For the ferric Cb complexes with 1:2.5 and 1:3 M/L ratios, K_m values (nanomolar) were found to be 29.9 ± 3.6 and 34.6 ± 4.2, respectively. Values for V_{max} (picomoles per milligram of dry weight per minute) were found to be 86.7 ± 4.0 and 93.3 ± 4.7, respectively. All data were corrected for growth during the assay period. The value for V_{max} is comparable to those for other bacterial ferric siderophore transport systems (6, 7, 9, 33). Remarkably, the K_m value of about 30 nM represents one of the lowest for a ferric siderophore-receptor system, although similar values have been reported (9, 12, 30). Notable is that, of these, pyochelin is also a nonhexadentate siderophore, and the K_m value for the weak chelator citrate was in the same study measured to be 100 nM (12). The only reported kinetic data for a monocatecholate siderophore gave a K_m value of 0.23 μM (3). For fungi that frequently produce large amounts of siderophores, transport systems with considerably lower affinities have been described (33, 49).

The influence of free Cb on the uptake of the ferric complex was probed by measuring initial uptake rates at 100 nM Fe and Cb concentrations from 200 nM to 5 μM. The initial uptake rates were slightly reduced by increasing the ligand concentration (Fig. 5). However, at an M/L ratio of 1:50 the uptake rate was still approximately 70% of the rate at an M/L ratio of 1:2.

DISCUSSION

Siderophore-mediated iron acquisition has for a long time been recognized as an important factor for the virulence of bacterial pathogens to higher organisms (37, 48). For phytopathogenic species this correlation is thus far restricted to the enterobacterium *E. chrysanthemi* 3937. This strain requires a functional iron assimilation system for systemic virulence on saintpaulia plants (17). The monocatecholate derivative Cb was identified as a native siderophorelike compound of *E. chrysanthemi* 3937 (38). Recently the Cb system has been extensively studied genetically. Four genes (*cbsABCE*) are involved in the biosynthesis of Cb, and a fifth gene (*fct*) encodes the ferric Cb receptor. These genes are clustered in the *E. chrysanthemi* 3937 genome, forming an operon spanning about 8 kb (21, 22). The function of each *cbs* gene product appears to be completely interchangeable with a counterpart from the *E. coli* enterobactin *ent* system (15, 22). Furthermore, *E. coli* EntB$^-$ cells transformed with a plasmid carrying the *fct* gene not only expressed high amounts of Fct in the outer membrane but were also able to

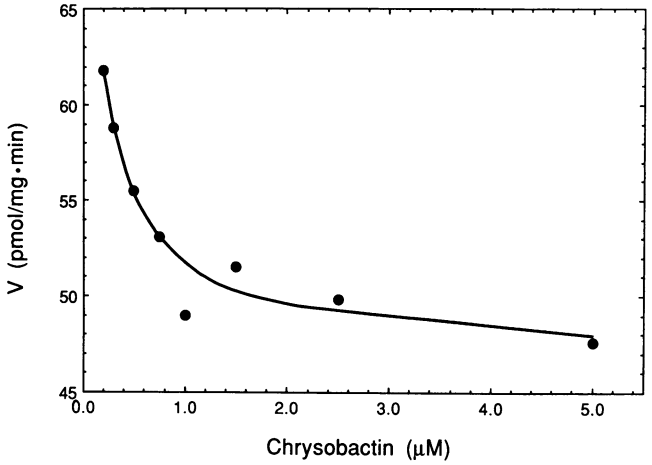


FIG. 5. Inhibition by free ligand of uptake of ^{55}Fe(Cb) by iron-starved *E. chrysanthemi* PMV 4089 cells. Initial uptake rates were determined from the first 2 min of uptake at 0.1 μM ^{55}Fe and 0.2 to 5.0 μM chrysobactin. Data shown are average values from three experiments.

utilize ferric Cb as an iron source (22). In the light of these results, we investigated the biological properties of ferric Cb.

We have in this study shown that Cb very efficiently mediates iron transport in *E. chrysanthemi* 3937. The uptake process was a saturable energy-requiring process, completely inhibited by the respiratory poison sodium azide or 0°C temperatures. Uptake was also significantly reduced when cells were grown in rich medium containing approximately 10 μM iron. However, cells grown in minimal medium with 25 μM added iron were, surprisingly, found to transport ferric Cb at a considerable rate. Previous results have indicated that the Cb operon is turned off under such conditions of iron repletion (22). The Fct protein is not detectable in the outer membrane (17), and no catecholates are produced according to results of the Arnow test (detection limit, approximately 10 nM) (38). Although there is no clear explanation for these findings, they may be related to the unexpected behavior of the Fct$^-$ mutant, which retained the ability to take up the ferric Cb complex. Similar observations were also made in bioassays, in which growth of iron-deprived Fct$^-$ PMV 4082 cells could be stimulated by supernatants from low-iron cultures of *E. chrysanthemi* 3937 (38) as well as by high concentrations of Arnow test-positive intercellular fluid from saintpaulia plants infected by *E. chrysanthemi* 3937 (18). Growth could not, however, be stimulated by the purified free ligand at equal concentrations (38), although it is conceivable that this observation may be due to an inability of Cb to successfully compete for Fe(III) with EDDA present in the agar at 100 μg/ml. Of relevance for these results may be the presence of a receptor for ferric enterobactin in *E. chrysanthemi* 3937; it was recently determined that *E. coli* could transport both dihydroxybenzoyl serine and DHBA by three receptors, albeit with varying efficiency (24). Furthermore, other reports have described siderophore transport systems which consisted of more than one receptor, or mechanism, with different affinities for one ferric siderophore species (6, 31, 41). It appears possible that our results may likewise reflect the presence of a second, perhaps less stringently regulated, ferric catecholate transport system, and the question will be addressed experimentally.

Peripheral groups have been shown to affect transport for several siderophores (33, 45, 49). The sensitivity of the *E. chrysanthemi* ferric catecholate transport system to structural variations of the Cb backbone was therefore probed by a successive shortening of the molecule, as well as by using isomers of Cb and DHBA-lysine. The ferric complexes of Cb, DHBA-lysine, and MDHB have identical iron-binding ligands and net charge; any uptake rate changes should thus be due to reduced receptor affinity and not to nonspecific electrostatic interactions. However, all the DHBA derivatives tried were taken up as efficiently as Cb. This included ferric DHBA, which in addition to having one more negative charge per catechol moiety, lacks an amide bond. Furthermore, results obtained with the isomers of Cb and DHBA-lysine show that the two chiral centers of Cb are not important for receptor recognition. Our data thus indicate that the specificity of the Cb-mediated ferric uptake system of *E. chrysanthemi* is restricted to the iron-catecholate center. The reason for the presence of the D-lysine-L-serine backbone, if it is not required for receptor recognition, is thus an open question. It seems possible that resistance to extracellular degradation, increased solubility, and decreased uptake by other microorganisms could all be valid explanations.

The ferric catecholate transport system in *E. chrysanthemi* appears to be functionally related to other systems. The genetic similarity to *ent* was mentioned above, and there are also biological precedents. From a study utilizing inert Rh complexes it was concluded that only the carbonyl group of the DHBA moiety and the Tris-catechol portion of ferric enterobactin were essential for receptor recognition (16). In relation to monocatecholate-mediated iron transport, we may again refer to the study with receptor mutants of *E. coli* (24), as well as to an early study in which DHBA-D-serine was found to be equally effective as a growth promoter as the natural L isomer (35). In *B. subtilis*, which produces the monocatecholate siderophore itoic acid (DHBA-glycine), both amino acid-substituted DHBA compounds and DHBA were determined to transport Fe(III) (40). Furthermore, DHBA-substituted β-lactam antibiotics were taken up via the *cir* and *fiu* gene products in *E. coli* (34). It would be interesting to determine if this broad specificity is of general occurrence in microbes which produce monocatecholate siderophores.

At the concentrations, M/L ratios, and pH used here, ferric bis-Cb would be the almost exclusively present species (39). The high efficiency of Cb in mediating Fe(III) transport under these conditions indicates that Fe(III) $(Cb)_2$ is a biologically active species. This idea was supported by the lack of difference in the kinetic studies with M/L ratios of 1:2.5 and 1:3, as almost all label was taken up during the course of the experiments and the 1:2.5 mix cannot quantitatively form a Tris complex. Stereospecific recognition of the metal center has been regarded as of major importance for several ferric siderophore receptors (6, 33, 45, 49). Although the Cb analogs used in this study formed optically active ferric Tris complexes, whose conformation around the iron center depended on the chirality of the amino acid to which DHBA was attached, the bis complexes were optically inactive (39). No conclusion can therefore be drawn regarding the sensitivity of the ferric Cb receptor to the handedness of the metal center propeller of an Fe(III) $(catecholate)_3$ complex. To determine this and to unequivocally prove if both ferric bis- and Tris-Cb are biologically active, complexes of a kinetically inert metal ion could be used. Another unanswered question is the precise mecha-

nism, of several proposed models (33), by which Cb mediates Fe(III) transport.

In a report discussing the roles of the *cir* and *fiu* gene products (34), it was determined that the free ligand was taken up at a rate exceeding even that of the ferric complex, and a "salvage" role for the putative monocatecholate receptors was proposed. Although *E. chrysanthemi* does not appear to produce hexadentate siderophores, the inhibition of Cb-mediated Fe(III) transport by the free ligand was investigated. Initial uptake rates were only moderately reduced, even at a 50-fold excess of ligand over iron. This result thus showed that free Cb is not recognized by Fct. There are at least three possible explanations for the observed reduction. First, the receptor may have some, albeit low, affinity for the free ligand. Second, contaminating unlabeled Fe(III) in the medium may be chelated, thus competing with the isotopically labelled ligand. Finally, the possibility exists that ferric bis-Cb is the only biologically active species or that the receptor has significantly lower affinity for ferric Tris-Cb and that the excess of free ligand shifted the equilibrium towards the Tris complex. Nevertheless, we conclude that the presence of the monocatecholate Cb in the culture medium of iron-starved *E. chrysanthemi* 3937 cells reflects a siderophore function of this compound. Other nonhexadentate catecholates have been found to possess biological activity in both gram-positive and gram-negative bacteria (3, 24, 27, 40, 47). Dihydroxybenzoyl serine was also shown to efficiently remove iron from human transferrin (20). Furthermore, the fungus *Gliocladium virens* produces several different mono-, di-, and trihydroxamates, which all showed biological activity (26). Interestingly, the mono- and dihydroxamate ferric complexes (*cis*- and *trans*-fusarinine, rhodotorulic acid, and dimerum acid) were taken up at higher rates than the trihydroxamates. It thus appears possible that some microorganisms have applied a strategy of using less-efficient siderophores, which may be biologically active either by being produced in very large amounts (hydroxamates) or by rapid removal from the $Fe(OH)_3$-ferric siderophore equilibrium by efficient receptors (catecholates). Bidentate siderophores may also be less metabolically expensive than hexadentate siderophores with regard to both synthesis and removal of iron from the ferric complex.

In conclusion, our results indicate that Cb may be regarded as a true siderophore for *E. chrysanthemi*. Recently, intercellular fluid from *E. chrysanthemi*-infected saintpaulia plants was found to contain Arnow test-positive material, which also was active in a bioassay with iron-starved Cbs^- cells (18). Although direct evidence for the identity of the Arnow test-positive material and Cb is lacking, it is tempting to speculate on a biological role for Cb in iron acquisition in planta.

ACKNOWLEDGMENTS

We gratefully acknowledge the staff at the University of California, Berkeley, Mass Spectrometry Facility for technical assistance, the use of computer facilities in the laboratory of J. F. Kirsch, and C. Doyle for critically reading the manuscript.

This work was supported in part by grants AI04156 and CRCR-1-1633 from the NIH and USDA, respectively.

REFERENCES

1. **Arnow, L. E.** 1937. Colorimetric determination of the components of 3,4-dihydroxyphenylalanine-tyrosine mixtures. J. Biol. Chem. **118**:531–537.
2. **Atkin, C. L., and J. B. Neilands.** 1968. Rhodotorulic acid, a diketopiperazine dihydroxamic acid with growth-factor activity. I. Isolation and characterization. Biochemistry **7**:3734–3739.
3. **Bachhawat, A. K., and S. Ghosh.** 1987. Iron transport in *Azospirillum brasilense*: role of the siderophore spirilobactin. J. Gen. Microbiol. **133**:1759–1765.
4. **Bagg, A., and J. B. Neilands.** 1987. Molecular mechanism of regulation of siderophore-mediated iron assimilation. Microbiol. Rev. **51**:509–518.
5. **Barghouthi, S., R. Young, M. O. J. Olson, J. É L. Arceneaux, L. W. Clem, and B. R. Byers.** 1989. Amonabactin, a novel tryptophan- or phenylalanine-containing phenolate siderophore in *Aeromonas hydrophila*. J. Bacteriol. **171**:1811–1816.
6. **Bergeron, R. J., and W. J. Weimar.** 1990. Kinetics of iron acquisition from ferric siderophores by *Paracoccus denitrificans*. J. Bacteriol. **172**:2650–2657.
7. **Berner, I., S. Konetschny-Rapp, G. Jung, and G. Winkelmann.** 1988. Characterization of ferrioxamine E as the principal siderophore of *Erwinia herbicola* (*Enterobacter agglomerans*). Biol. Metals **1**:51–56.
8. **Berner, I., and G. Winkelmann.** 1990. Ferrioxamine transport mutants and the identification of the ferrioxamine receptor protein (FoxA) in *Erwinia herbicola* (*Enterobacter agglomerans*). Biol. Metals **2**:197–202.
9. **Braun, V., and G. Winkelmann.** 1988. Microbial iron transport. Structure and function of siderophores. Prog. Clin. Biochem. Med. **5**:67–99.
10. **Chakraborty, R. N., H. N. Patel, and S. B. Desai.** 1990. Isolation and partial characterization of catechol-type siderophore from Pseudomonas stutzeri RC 7. Curr. Microbiol. **20**:283–286.
11. **Cleland, W. W.** 1967. The statistical analysis of enzyme kinetic data. Adv. Enzymol. **29**:1–32.
12. **Cox, C. D.** 1980. Iron uptake with ferripyochelin and ferric citrate by *Pseudomonas aeruginosa*. J. Bacteriol. **142**:581–587.
13. **Cox, C. D., K. L. Rinehart, Jr., M. L. Moore, and J. C. Cook, Jr.** 1981. Pyochelin: novel structure of an iron-chelating growth promoter for *Pseudomonas aeruginosa*. Proc. Natl. Acad. Sci. USA **78**:4256–4260.
14. **Diekmann, H.** 1970. Stoffwechselprodukte von Mikroorganismen. 81. Vorkommen und Strukturen von Coprogen B und Dimerumsäure. Arch. Mikrobiol. **73**:65–76.
15. **Earhart, C. F.** 1987. Ferrienterobactin transport in *Escherichia coli*, p. 67–84. *In* G. Winkelmann, D. van der Helm, and J. B. Neilands (ed.), Iron transport in microbes, plants and animals. VCH Verlagsgesellschaft, Weinheim, Germany.
16. **Ecker, D. J., L. D. Loomis, M. E. Cass, and K. N. Raymond.** 1988. Substituted complexes of enterobactin and synthetic analogues as probes of the ferric-enterobactin receptor in *Escherichia coli*. J. Am. Chem. Soc. **110**:2457–2464.
17. **Enard, C., A. Diolez, and D. Expert.** 1988. Systemic virulence of *Erwinia chrysanthemi* 3937 requires a functional iron assimilation system. J. Bacteriol. **170**:2419–2426.
18. **Enard, C., T. Franza, C. Neema, P. R. Gill, M. Persmark, J. B. Neilands, and D. Expert.** 1991. The requirement of chrysobactin dependent iron transport for virulence incited by *Erwinia chrysanthemi* on *Saintpaulia ionantha*. Plant Soil **130**:263–271.
19. **Expert, D., and A. Toussaint.** 1985. Bacteriocin-resistant mutants of *Erwinia chrysanthemi*: possible involvement of iron acquisition in phytopathogenicity. J. Bacteriol. **163**:221–227.
20. **Ford, S., R. A. Cooper, R. W. Evans, R. C. Hider, and P. H. Williams.** 1988. Domain preference in iron removal from human transferrin by the bacterial siderophores aerobactin and enterochelin. Eur. J. Biochem. **178**:477–481.
21. **Franza, T., C. Enard, F. van Gijsegem, and D. Expert.** 1991. Genetic analysis of the *Erwinia chrysanthemi* 3937 chrysobactin iron-transport system: characterization of a gene cluster involved in uptake and biosynthetic pathway. Mol. Microbiol. **5**:1319–1329.
22. **Franza, T., and D. Expert.** 1991. The virulence-associated chrysobactin iron uptake system of *Erwinia chrysanthemi* 3937 involves an operon encoding transport and biosynthetic functions. J. Bacteriol. **173**:6874–6881.
23. **Hahn, F. E., T. J. McMurry, A. Hugi, and K. N. Raymond.** 1990. Coordination chemistry of microbial iron transport. 42. Structural and spectroscopic characterization of diasteromeric

Cr(III) and Co(III) complexes of desferrithiocin. J. Am. Chem. Soc. **112**:1854–1860.
24. **Hantke, K.** 1990. Dihydroxybenzoylserine—a siderophore for *E. coli*. FEMS Microbiol. Lett. **67**:5–8.
25. **Hider, R. C.** 1984. Siderophore mediated absorption of iron. Struct. Bonding **58**:25–87.
26. **Jalal, M. A. F., S. K. Love, and D. van der Helm.** 1987. Siderophore mediated iron(III) uptake in *Gliocladium virens*. 2. Role of ferric mono- and dihydroxamates as iron transport agents. J. Inorg. Biochem. **29**:259–267.
27. **Knosp, O., M. von Tigerstrom, and W. J. Page.** 1984. Siderophore-mediated uptake of iron in *Azotobacter vinelandii*. J. Bacteriol. **159**:341–347.
28. **Kobaru, S., M. Tsunakawa, M. Hanada, M. Konishi, K. Tomita, and H. Kawaguchi.** 1983. Bu-2743E, a leucine aminopeptidase inhibitor, produced by *Bacillus circulans*. J. Antibiot. **35**:1396–1398.
29. **Kunze, B., N. Bedorf, W. Kohl, G. Höfle, and H. Reichenbach.** 1989. Myxochelin A, a new iron chelating compound from *Angiococcus disciformis* (Myxobacteriales). J. Antibiot. **42**:14–17.
30. **Lammers, P. J., and J. Sanders-Loehr.** 1982. Active transport of ferric schizokinen in *Anabaena* sp. J. Bacteriol. **151**:288–294.
31. **Leong, J., W. Bitter, M. Koster, V. Venturi, and P. J. Weisbeek.** 1991. Molecular analysis of iron transport in plant growth-promoting *Pseudomonas putida* WCS358. Biol. Metals **4**:36–40.
32. **Leong, S. A., and D. Expert.** 1989. Siderophores in plant-pathogen interactions, p. 62–83. *In* E. Kosuge and E. Nester (ed.), Plant-microbe interactions—a molecular genetic perspective. McGraw-Hill, New York.
33. **Matzanke, B. F., G. Müller-Matzanke, and K. N. Raymond.** 1989. Siderophore-mediated iron transport, p. 3–121. *In* T. M. Loehr (ed.), Iron carriers and iron proteins. VCH Verlagsgesellschaft, Weinheim, Germany.
34. **Nikaido, H., and E. Y. Rosenberg.** 1990. Cir and Fiu proteins in the outer membrane of *Escherichia coli* catalyze transport of monomeric catechols: study with β-lactam antibiotics containing catechol and analogous groups. J. Bacteriol. **172**:1361–1367.
35. **O'Brien, I. G., G. B. Cox, and F. Gibson.** 1969. 2,3-Dihydroxybenzoylserine: chemical synthesis and comparison with the natural product. Biochim. Biophys. Acta **177**:321–328.
36. **Patel, H. N., R. N. Chakraborty, and S. B. Desai.** 1988. Isolation and partial characterization of phenolate siderophore from *Rhizobium leguminosarum* IARI 102. FEMS Microbiol. Lett. **56**:131–134.
37. **Payne, S. M.** 1988. Iron and virulence in the family Enterobacteriaceae. Crit. Rev. Microbiol. **16**:81–111.
38. **Persmark, M., D. Expert, and J. B. Neilands.** 1989. Isolation, characterization, and synthesis of chrysobactin, a compound with siderophore activity from *Erwinia chrysanthemi*. J. Biol. Chem. **264**:3187–3193.
39. **Persmark, M., and J. B. Neilands.** 1992. Iron(III) complexes of chrysobactin, a siderophore of *Erwinia chrysanthemi*. BioMetals **5**:29–36.
40. **Peters, W. J., and R. A. J. Warren.** 1970. The mechanism of iron uptake in *Bacillus subtilis*. Can. J. Microbiol. **16**:1285–1291.
41. **Poole, K., L. Young, and S. Neshat.** 1990. Enterobactin-mediated iron transport in *Pseudomonas aeruginosa*. J. Bacteriol. **172**:6991–6996.
42. **Rogers, H. J.** 1987. Bacterial iron transport as a target for antibacterial agents, p. 223–233. *In* G. Winkelmann, D. van der Helm, and J. B. Neilands (ed.), Iron transport in microbes, plants and animals. VCH Verlagsgesellschaft, Weinheim, Germany.
43. **Schwyn, B., and J. B. Neilands.** 1987. Universal chemical assay for the detection and determination of siderophores. Anal. Biochem. **160**:47–56.
44. **Skorupska, A., A. Choma, M. Derylo, and Z. Lorkiewicz.** 1988. Siderophore containing 2,3-dihydroxybenzoic acid and threonine formed by *Rhizobium trifolii*. Acta Biochim. Pol. **35**:119–130.
45. **van der Helm, D., M. A. F. Jalal, and M. B. Hossain.** 1987. The crystal structures, conformations, and configurations of siderophores, p. 135–165. *In* G. Winkelmann, D. van der Helm, and J. B. Neilands (ed.), Iron transport in microbes, plants and animals. VCH Verlagsgesellschaft, Weinheim, Germany.
46. **van Hove, B., H. Staudenmaier, and V. Braun.** 1990. Novel two-component transmembrane transcription control: regulation of iron dicitrate transport in *Escherichia coli* K-12. J. Bacteriol. **172**:6749–6758.
47. **Wang, C. C., and A. Newton.** 1969. Iron transport in *Escherichia coli*: roles of energy-dependent uptake and 2,3-dihydroxybenzoylserine. J. Bacteriol. **98**:1142–1150.
48. **Weinberg, E. D.** 1984. Iron withholding: a defense against infection and neoplasia. Physiol. Rev. **64**:65–101.
49. **Winkelmann, G., and H. Huschka.** 1987. Molecular recognition and transport of siderophores in fungi, p. 317–336. *In* G. Winkelmann, D. van der Helm, and J. B. Neilands (ed.), Iron transport in microbes, plants and animals. VCH Verlagsgesellschaft, Weinheim, Germany.