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Iron Supply to *Escherichia coli* by Synthetic Analogs of Enterochelin

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Synthetic analogs of enterochelin (enterobactin) were tested for their ability to support the growth of *Escherichia coli* K-12 under iron-limiting conditions. The cyclic compound MECAM [1,3,5-*N*,*N*′,*N*″-tris-(2,3-dihydroxybenzoyl)-triaminomethylbenzene] and its *N*-methyl derivative Me_3MECAM promoted growth, whereas the 2,3-dihydroxy-5-sulfonyl derivatives MECAMS and Me_3MECAMS were inactive. The same results were obtained with TRIMCAM [1,3,5-tris(2,3-dihydroxybenzoylcarbamido)-benzene] and TRIMCAMS (the 2,3-dihydroxy-5-sulfonyl derivative of TRIMCAM). However, the sulfonic acid-containing linear compound LICAMS [1,5,10-*N*,*N*′,*N*″-tris(5-sulfo-2,3-dihydroxybenzoyl)-triazadecane] supported growth. In contrast, LIMCAMC, in which the sulfonyl groups at the five position of LICAMS are replaced by carboxyl groups at the four position, was inactive. The uptake of the active analogs required the functions specified by the *fepB*, *fesB*, and *tonB* genes. Surprisingly, growth promotion of mutants lacking the enterochelin receptor protein in the outer membrane was observed. Only MECAM protected cells against colicin B (which kills cells after entering at the enterochelin uptake sites) and transported Fe^{3+} at about half the enterochelin rate.

The concentration of iron(III) required for microbial growth is in the range of 0.05 to 1 μM. The extremely low concentration of ferric ions in equilibrium with ferric hydroxide (10^{-12} μM), transferrin (10^{-18} μM), and lactoferrin ($<10^{-18}$ μM) (1) demands the production of strong extracellular iron-chelating compounds by microorganisms to satisfy their iron requirements. Enterochelin (20), also designated enterobactin (22), is the strongest ferric ion-chelating agent known. At pH 7.4, the concentration of unchelated hexaaquoiron(III) in a solution which is 10 μM ligand–1 μM metal has been calculated to be 10^{-30} μM (8), i.e., 1 iron ion in 10^{12} liters.

Enterochelin has been found in all enterobacteria studied. The biochemistry and genetics of enterochelin synthesis and of ferric enterochelin transport have been determined in great detail with *Escherichia coli* (2, 18, 25). Four genes have been identified for ferric enterochelin transport: *fepA*, *fepB*, *fes*, and *tonB* (2, 18, 25). *fepA* specifies a protein in the outer membrane which also serves as a receptor for colicin B (5–7, 11, 15, 16, 23). *fepB* probably controls a function required to translocate the iron complex across the cytoplasmic membrane. The product of *fes* serves for the esterolytic degradation of enterochelin after delivery of iron(III) into the cell (13). Although the mode of action of the *tonB* gene product is unknown, its function appears to be required in all high-affinity iron transport systems (2, 18, 25).

The structure of enterochelin is shown in Fig. 1. The molecule contains three dihydroxybenzoyl groups covalently attached via amide linkages to a central triester ring (composed of three L-serine moieties). Upon formation of the ferric complex, at least two potential protein recognition sites are generated. First, the catecholate coordination about the metal ion yields a distinct site on the molecule in terms of shape and size. Moreover, since isomers of absolute configuration are possible, a further degree of specificity may be operative. Indeed, there is some evidence for just such specificity with hydroxamate siderophores (33) and enterochelin (19). Second, the change in conformation of the central ring may be of critical importance for ferric enterochelin recognition.

One can synthesize analogs of enterochelin which selectively modify certain sites of similar molecules. In this way, those features which are critical to the iron acquisition process of the microbe may be explored. The synthetic enterochelin analogs used in this study are shown in Fig. 2. The rationale for the selection of these

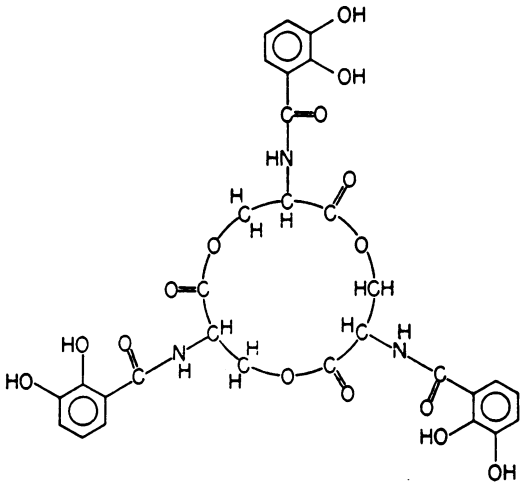

FIG. 1. Structure of enterochelin.

compounds was as follows. MECAM (The full chemical name of each compound may be found in the legend to Fig. 2.) (9, 27, 30), the analog closest to enterochelin, retains the ferric ion coordination geometry and modifies the central ring structure. Me_3MECAM (21) probes the importance of the amide that is bound, by alkylation of the amide nitrogen. TRIMCAM (8), a structural isomer of MECAM in which the methylene and carbonyl functions have been interchanged, probes the effect of the change in the electronic structure of the catechol ring and the importance of hydrogen bonding with the protein via the carbonyl moiety. In the sulfonated derivatives (MECAMS, Me_3MECAMS, and TRIMCAMS), steric constraints to the transport protein binding sites and the alteration of the electronic character of the catecholate ring are tested. With the linear compounds LICAMS (8, 30), LICAMC (32), and Diisp-3,4-LICAM (31), the importance of the ring structure and steric hindrance are probed. Finally, LICAMC modifies LICAMS by shifting the site of ring substitution from the five position to the four position and interchanges a carboxylate for a sulfonate group.

We used *E. coli* to study the activity of these structural analogs of enterochelin, since many mutants are available for all known high-affinity iron uptake systems. Our aim was to obtain further insights into the mechanism of the supply of iron(III) via enterochelin and to determine whether the synthetic enterochelin analogs are suitable for the treatment of iron overload diseases or as potential antimicrobial agents.

MATERIALS AND METHODS

Chemicals. The synthesis and iron-binding properties of the enterochelin analogs have been described

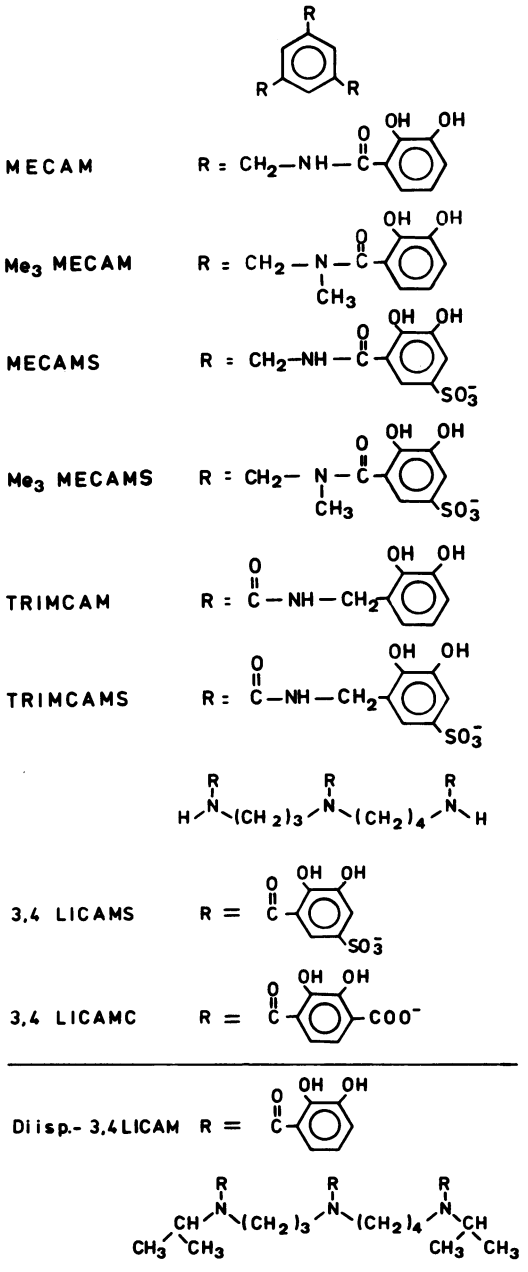

FIG. 2. Synthetic enterochelin analogs used in this study: MECAM [1,3,5-*N*,*N*′,*N*″-tris-(2,3-dihydroxybenzoyl)-triaminomethylbenzene], Me_3MECAM (*N*-methyl derivative of MECAM), MECAMS and Me_3MECAMS (2,3-dihydroxy-5-sulfonyl derivatives of MECAM and Me_3MECAM), TRIMCAM [1,3,5-tris(2,3 - dihydroxybenzoylcarbamido)-benzene], TRICAMS (2,3-dihydroxy-5-sulfonyl derivative of TRIMCAM), LICAMS [1,5,10-*N*,*N*′,*N*″-tris(5-sulfo-2,3-dihydroxybenzoyl)-triazadecane], LICAMC (5-sulfo group of LICAMS replaced by a 4-carboxy group), and Diisp-3,4-LICAM [1,10-*N*,*N*′-di(isopropyl)-*N*,*N*′,*N*″-tris(2,3-dihydroxylbenzoyl)-1,5,10-triazadecane].

previously (4, 8, 9, 21, 27, 29, 30). All ferric complexes were prepared as follows. The unchelated ligand was dissolved in 5 ml of degassed water (pH 10) under inert atmosphere (nitrogen). Iron was added from a standardized ferric nitrate solution (pH 1.1), and the pH was raised until the characteristic wine-red color of a ferric triscatecholate was observed. This solution was then taken to dryness under vacuum at room temperature. The solid was redissolved just before the assay.

Bacterial strains. Table 1 lists the bacterial strains used. The strains used for the first time in this study were selected as resistant to phage T5 (*tonA*), colicin Ib (*cir*), or colicin B (*fepA*) (5).

Growth studies. Growth promotion by enterochelin analogs was studied on 10-ml plates consisting of 1.5% agar and the following medium (per liter): 30 mM sodium succinate, 1.36 g of KH_2PO_4, 0.2 g of $(NH_4)_2SO_4$ (adjusted with KOH to pH 7), 1 mM $MgSO_4$, 100 mg of tryptophan, 100 mg of phenylalanine, 100 mg of tyrosine, 0.2 mM *p*-aminobenzoic acid, 0.2 mM *p*-hydroxybenzoic acid, 1 mg of thiamine, 10 μM sodium citrate, and, if required, 50 mg of proline and 50 mg of arginine.

Cells (10^8) in 2.5 ml of 0.6% agar in water (42°C) were spread over the plates. Growth around filter paper disks, each of which contained 200 nmol of an enterochelin analog, was scored after 1 or 2 days of incubation at 37°C.

Inhibition of colicin B action. Plates with 10 ml of 1.5% agar–0.8% tryptone–0.5% yeast extract–0.5% NaCl were overlaid with 2×10^8 cells of strain AB2847 in 2.5 ml of the above tryptone-yeast extract-NaCl medium with 0.6% agar at 42°C. Isolated colicin B (5 μl of a 1:10-diluted colicin B solution [titer, 10^4]) was spotted onto the plates, which were then incubated for 26 h at 4°C to allow diffusion of the colicin B without growth of the cells. This procedure resulted in a larger diffusion zone and hence in a less steep concentration gradient, so that inhibition of colicin B by the enterochelin analogs could be observed more clearly. Three filter paper disks (6-mm diameter) with either 20 or 200 nmol of a ferric enterochelin analog were then placed at a distance of 1.3 cm around the colicin B application spot. After 1 h of incubation at 20°C, the plates were kept at 37°C and inspected after 20 h.

For growth in liquid cultures, 2.5×10^8 cells of strain AB2847 were incubated at 37°C in 5 ml of tryptone-yeast extract-NaCl medium to which 25 μl of an aqueous solution of 1 mM ferric MECAM and 100 μl of colicin B solution had been added. Growth was followed by measuring the absorbance at 578 nm. Colicin B was partially purified by chromatography of a cell homogenate supernatant on a carboxymethyl cellulose column, as described previously (26).

Transport assays. Cells were grown and transport was measured in the following medium: 37 mM Na_2HPO_4, 19 mM KH_2PO_4, 7 mM NaCl, 16 mM NH_4Cl, 0.87 mM $MgSO_4$, 87 μM $CaCl_2$, 0.17 mM *p*-aminobenzoic acid, 0.17 mM *p*-hydroxybenzoic acid, 3.5 g of glucose per liter, 87 mg of tryptophan per liter, 87 mg of phenylalanine per liter, 87 mg of tyrosine per liter, and 8.7 mg of thiamine per liter.

The radioactive ferric complexes were prepared before the transport assays were started by mixing a 1.2 mM solution of MECAM or TRIMCAM in methanol, or of Me_3MECAM, LICAMS, or Diisp-3,4-LICAM in water, with 1 mM $^{55}FeCl_3$ (0.25 nCi/ml) in water. To 5 ml of the above transport medium 10 μl of the ferric chelator solution and 0.1 mg of nitrilotriacetate were added. Nitrilotriacetate was supplied to suppress iron uptake via the poorly understood, so-called low-affinity uptake system (25). The solution was shaken for 10 min at 37°C before 5 ml of cells harvested from a logarithmically growing culture were added (final concentration, 5×10^8 cells per ml). Samples (0.5 ml) were taken, filtered through Schleicher & Schuell BA 85 filters, washed twice, each time with 4 ml of 0.1 M LiCl, and dried, and the radioactivity was counted in a liquid scintillation counter. Control values of iron absorbed to the filters without cells were determined and subtracted from the values obtained with cells.

TABLE 1. *E. coli* K-12 strains used

Strain	Genotype	Reference or source
AB2847	*aroB thi tsx malT*	6
IR20	As AB2847, but *fepA*	7
IR20A	As IR20, but *tonA*	This study
IR20/111	As IR20A, but *cir*	This study
VR42/B9	As AB2847, but *fepA cir*	6
BR158	As AB2847, but *tonB*	7
WA1024	As AB2847, but *fecA14 fepA*	28
VR42	As AB2847, but *cir*	7
AN92	*aroB proA argE pheA tyrA try rpsL thi*	34
HE92/B3	As AN92, but *fepA*	6
AN260	As An92, but *fepB*	13
AN272	As AN92, but *fesB*	13
AN299	*fecB*	34
HE299/23	As AN299, but *fecB fepA*	This study

RESULTS

Growth promotion. To test the iron supply activities of the enterochelin analogs (Fig. 2), cells were grown in a minimal medium with succinate as the carbon source. In this medium, cell metabolism depends on respiration, which requires the iron-containing cytochromes and nonheme iron proteins. All of the strains used were unable to synthesize their own enterochelin owing to the mutation in the *aroB* gene.

The results were straightforward with strains AB2847 and AN92 (Table 2), which had functional ferric enterochelin transport systems and which were not deficient in any other uptake system. The iron complexes of MECAM, Me_3MECAM, TRIMCAM, and LICAMS supported growth, whereas the other compounds were inactive (Table 2). It is noteworthy that introduction of a sulfonyl group rendered the first three compounds inactive but that the linear LICAMS promoted growth. However, LICAMC was inactive.

To test whether the ferric compounds were taken up by the ferric enterochelin transport system, mutants of the ferric enterochelin transport system were included in the assay. The

TABLE 2. Growth promotion of *E. coli* strains by enterochelin analogs[a]

Strain	Genotype	Growth promotion by the following:[a]								
		MECAM	Me_3MECAMS	MECAMS	Me_3MECAMS	TRIMCAM	TRIMCAMS	LICAMS	LICAMC	Diisp-3,4-LICAM	DHB[b]
AB2847	*aroB*	+	+	−	−	+	−	+	−	+	+
AN92	*aroB*	+	+	−	−	+	−	+	−	−	+
AN260	*fepB*	−	−	−	−	−	−	−	−	−	−
AN272	*fesB*	−	−	−	−	−	−	−	−	−	−
BR138	*tonB*	−	−	−	−	−	−	−	−	−	+
IR20	*fepA*	+	+	−	−	+	−	+	−	−	+
HE92/B3	*fepA*	+	(+)	−	−	+	−	+	−	−	+
IR20A	*fepA tonA*	+	+	−	−	+	−	+	−	−	+
WA1024	*fepA fecA*	(+)	(+)	−	−	(+)	−	(+)	−	−	(+)
IR20/111	*fepA tonA cir*	+	(+)	−	−	+	−	−	−	−	+
VR42	*cir*	+	+	−	−	+	−	+	−	+	+
VR42/B9	*fepA cir*	+	+	−	−	+	−	−	−	−	+
HE299/23	*fepA fecB*	+	+	+	−	+	−	+	−	+	+

[a] Each compound was tested twice. +, Growth promotion; −, no growth promotion; (+), weak growth.
[b] DHB, 2,3-Dihydroxybenzoate.

fepB, *fesB*, and *tonB* mutants did not grow with the ferric enterochelin analogs as the sole iron source. This result demonstrated that uptake across the cytoplasmic membrane (*fepB* function), release of the ferric ions from the complexes (*fesB* function), and the use of the *tonB* function were identical with ferric enterochelin transport. Unexpectedly, growth of the *fepA* mutants was promoted, showing that the receptor protein in the outer membrane could be bypassed by the ferric enterochelin analogs.

We then tested whether receptors of other iron transport systems (2, 18, 25) were used instead of the fepA protein by the ferric enterochelin analogs. The *fepA fecA* double mutant (*fecA*, ferric citrate transport) responded weakly to the otherwise active compounds. The *fepA* strain IR20A, which was, in addition, mutated in the *tonA* gene (ferrichrome transport), responded to the active compounds. However, when a third mutation, *cir*, was introduced (strain IR20/111), Me_3MECAM supported growth only weakly, and LICAMS was inactive. The outer membrane protein specified by the *cir* gene is synthesized at iron-limiting growth conditions, but no iron transport system has been identified which uses the cir protein (12). The *cir* mutation alone had no effect (strain VR42). The double mutant *cir fepA* was only unresponsive to LICAMS. These results indicate that the accumulation of mutations in the outer membrane proteins related to the iron supply reduced the efficiency of the enterochelin analogs, especially those which are poor iron donors.

Some inconsistent results were found. Although LICAMC did not support the growth of most strains, we sometimes obtained growth of mutants HE299/23 and VR42 (listed in Table 2 as negative). It is interesting that the growth of these strains was also supported by Diisp-3,4-LICAM. The latter compound was also active with strain AB2847 but not with strain AN92. We included in the growth assays 2,3-dihydroxybenzoate, from which all of the *aroB* strains could synthesize enterochelin (25). As shown previously (6), the *fepA* and the *tonB* functions were not required for growth promotion, but the *fepB* and *fesB* functions have to be active.

Transport assay. To compare the various compounds in a more quantitative way, we measured transport of radioactive $^{55}Fe^{3+}$ supplied to the assay mixtures as a complex with the enterochelin analogs. Only MECAM transported iron in amounts comparable with those found with enterochelin (Fig. 3; see also reference 25). Although TRIMCAM supported growth on iron-poor minimal plates, it transported iron very poorly (Fig. 3). Even less uptake of iron was obtained with Me_3MECAM, LICAMS, and Diisp-3,4-LICAM (Fig. 4; note the different

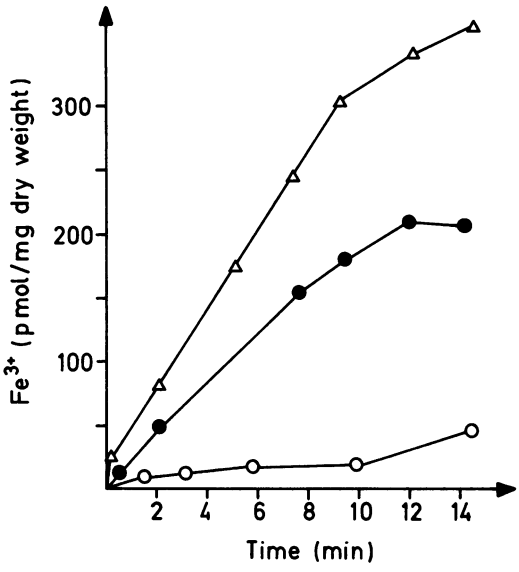

FIG. 3. Uptake of iron(III) into *E. coli* AB2847 as enterochelin (△), MECAM (●), and TRIMCAM (○) complexes.

scale in Fig. 4 compared with that in Fig. 3). Even this amount of iron delivery was apparently sufficient to support growth.

Inhibition of colicin B action by ferric enterochelin analogs. Since enterochelin and colicin B use the same receptor protein (FepA) for initial binding to cells, the ferric complexes of the synthetic analogs were tested to determine whether they inhibited binding of colicin B. Ferric MECAM (20 nM) counteracted the action of colicin B, a counteraction which was observed as a reduction of the colicin B inhibition zone at the ferric MECAM diffusion zone. The other analogs did not inhibit colicin B at 200 nM. Ferric Me_3MECAMS, Me_3MECAM, and TRIMCAMS (200 nM) inhibited growth around the filter paper disk and added to the growth inhibition of colicin B. In liquid culture, 5 μM ferric MECAM partially prevented growth inhibition by colicin B (Fig. 5). The tests on plates and in liquid cultures demonstrated that ferric MECAM, the analog closest to enterochelin, interfered with the killing action of colicin B.

DISCUSSION

The active enterochelin analogs required the same gene products as enterochelin to supply cells of *E. coli* with iron. This could be shown with mutations in the *fepB*, *fesB*, and *tonB* genes. The first two genes must be active for ferric ion transport via 2,3-dihydroxybenzoate (6), which is the precursor in enterochelin synthesis (25). The requirement for the *fepB* and

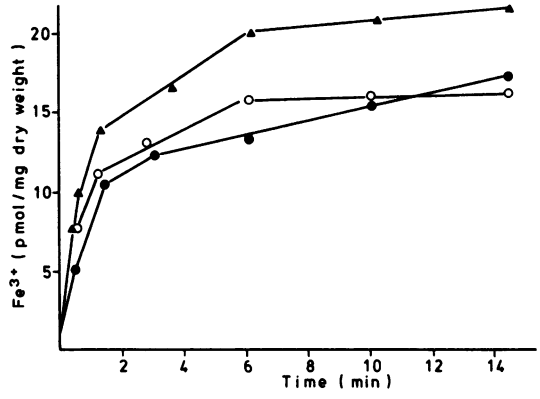

FIG. 4. Uptake of iron(III) into *E. coli* AB2847 as Diisp-3,4-LICAM (▲), 3,4-LICAMS (○), and Me_3MECAM (●) complexes.

tonB gene functions is understandable if both are involved in transport across the cytoplasmic membrane (35). These functions demonstrate that the iron is not released from the ligands at the cell surface. Rather, uptake into the cells is required for all of the complexes that promote growth, even for those with a very low uptake rate (Fig. 4). The inability of *fesB* mutants to grow in the presence of the ferric enterochelin analogs was unexpected. These mutants lack the function of the smaller subunit of enterochelin esterase (13). Either the esterase also hydrolyzes amide bonds, or the smaller subunit has another enzymatic activity which releases iron from the complexes. It has been shown that ferric enterochelin is the superior substrate over enterochelin for the esterase (13), which could mean that reduction from Fe^{III} to Fe^{II} and hydrolysis are performed by one enzyme consisting of two different polypeptides. Another

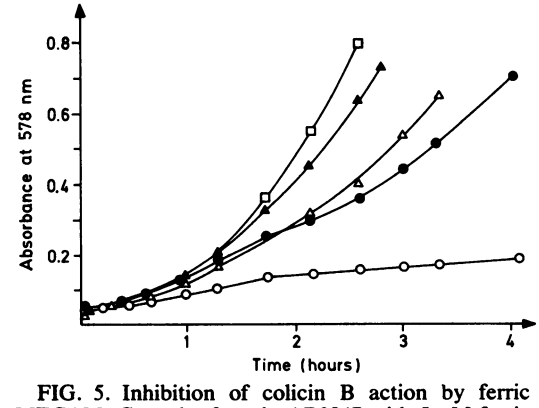

FIG. 5. Inhibition of colicin B action by ferric MECAM. Growth of strain AB2847 with 5 μM ferric MECAM in the medium (□); with colicin B (diluted $1:10^5$) without (○) and with (●) 5 μM ferric MECAM; and with colicin B (diluted $1:5 \times 10^5$) without (△) and with (▲) 5 μM ferric MECAM (▲).

enterochelin analog, *cis*-1,5,9-tris(2,3-dihydroxybenzamido)cyclododecane, also failed to support growth of an *fes* mutant (10). Furthermore, a ferrisiderophore reductase system that released iron from ferric enterochelin, MECAM, and Me_3MECAMS was demonstrated in a cell extract of *Bacillus subtilis* WB2802 (14). Despite the low redox potential (−750 mV at pH 7) of ferric enterochelin (20), enzymatic reduction of Fe^{III} to Fe^{II} is apparently possible without hydrolysis of the chelator. The dependence of the iron supply by the enterochelin analogs on the *fesB* gene product indicates that the iron complexes enter the cytoplasm or reach the cytoplasmic side of the cytoplasmic membrane. The esterase is a soluble enzyme (13) and cannot be released by osmotic shock treatment (W. Wagegg and V. Braun, unpublished data).

Growth in the presence of the ferric enterochelin analogs was also observed with *fepA* mutants lacking the receptor protein in the outer membrane. This finding agrees with the result in reference 27, in which growth promotion with ferric MECAM was observed with an enterochelin-synthesizing strain lacking receptor activity. In the *fepA* strains used in our study, no FepA protein could be observed after polyacrylamide gel electrophoresis (data not shown; see also reference 5). It required the accumulation of several mutations in outer membrane proteins related to iron transport to render cells unresponsive to the active ferric enterochelin analogs. However, one has to take into account that the growth assay is extremely sensitive. Growth promotion was still observed when very low transport activity was measured (compare MECAM with TRIMCAM in Fig. 3, and with Me_3MECAM, LICAMS, and Diisp-3,4-LICAM in Fig. 4). In *fepA* mutants, amounts of the ferric analogs apparently sufficient to support growth pass through the outer membrane. The subsequent active transport across or into the cytoplasmic membrane is, however, a necessary step, since *fepB* mutants showed no growth (see also reference 10).

Another significant observation from these studies is the importance of the general enterochelin structure for recognition of the ferric chelate by the FepA protein. Only ferric MECAM is sufficiently similar to ferric enterochelin to bind to the FepA protein, a result that is shown by the competition of ferric MECAM with colicin B, which also adsorbs to the FepA protein. All of the active compounds, including MECAM, pass through the outer membrane without the receptor protein specified by the *fepA* gene. Nonspecific diffusion across the outer membrane sufficient to support growth is questionable since the *fepA fecA* double mutants lacking two of the receptor proteins for iron chelates responded only weakly to the iron complexes of the enterochelin analogs. However, all compounds required the *fepB* gene product, which indicates that the cytoplasmic membrane forms a more restrictive permeability barrier than does the outer membrane. Since the growth assay is very sensitive, it can be concluded that cytoplasmic membranes lacking the *fepB* gene product are impermeable to the active compounds.

The transport activity of enterochelin and of the cyclic and linear enterochelin analogs via the same uptake system may arise from the common geometry around the iron(III) ion which may be recognized by the proteins specified by the *fep* and *fes* genes. The observation that ferric MECAM but not the derivative ferric MECAMS, sulfonated at the 5-position of the benzene ring, protected cells against colicin B shows for the first time that the tricatechol iron portion of the molecule is specifically recognized by the receptor site of the protein. The activity pattern of the compounds tested is very interesting with regard to their possible practical application. All should be much more resistant to hydrolysis than is enterochelin. The inactive sulfonated cyclic compounds have additional favorable properties in that they are more soluble and more oxygen resistant than the unsubstituted ligands (8, 21). The compounds are strong iron chelators and thermodynamically capable of removing iron from transferrin. In the case of MECAMS, the competition equilibrium with transferrin favors the catecholate ligand by a factor of about 10^6 (8). The compounds are also kinetically capable of withdrawing iron from transferrin. At a ligand/transferrin ratio of 40:1, LICAMS complexed 50% of the transferrin-bound iron in 30 min (4). The sulfonated compounds, which do not support growth of *E. coli* cells, are therefore worthy candidates for the treatment of iron overload syndromes (24). They should also be tried as supplements to dialysis fluids to remove iron released by hemolysis.

The stability of these compounds renders them suitable agents with which to raise enterochelin-specific antibodies. These antibodies would trap the released enterochelin in the growth environment and could bind to the microbial cell surface receptor loaded with ferric enterochelin, thus blocking iron uptake (3). Naturally occurring enterochelin-specific immunoglobulins against enterochelin have been identified in normal human serum (17).

ACKNOWLEDGMENTS

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