have a similar packing arrangement with respect to each other in their membranes.

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Ferric Enterobactin Transport System in *Escherichia coli* K-12. Extraction, Assay, and Specificity of the Outer Membrane Receptor†

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ABSTRACT: An outer membrane preparation from cells of *Escherichia coli* K-12 grown in low iron medium was found to retain ferric enterobactin binding activity following solubilization in a Tris-HCl, Na_2EDTA buffer containing Triton X-100. Activity was measured by means of a DEAE-cellulose column which separated free and receptor bound ferric enterobactin. The binding activity was greatly reduced in preparations obtained from cells grown in iron rich media or from cells of a colicin B resistant mutant grown in either high or low iron media. Sodium dodecyl sulfate–polyacrylamide gel electrophoresis analysis enabled correlation of this lack of activity to a single band missing in the outer membrane profile of the colicin B mutant. Evidence was obtained for in vitro competition between ferric enterobactin and colicin B for the extracted receptor. The binding specificity of the extracted receptor was examined by competition between ferric enterobactin and several iron chelates including a carbocyclic analogue of enterobactin, *cis*-1,5,9-tris(2,3-dihydroxybenzamido)cyclododecane. The ferric form of the latter compound supported growth of siderophore auxotrophs, apparently without hydrolysis to dihydroxybenzoic acid and resynthesis into enterobactin. These data may require revision of the accepted mechanism of enterobactin mediated iron utilization.

Several active transport systems have been identified in *Escherichia coli* which require an outer membrane component to bind the specific substrate, generally a hydrophilic molecule of molecular weight exceeding 600. Each such binding protein thus far identified also acts as a receptor site for bacteriophage and/or colicins. Those systems known are for vitamin B-12 (Bradbeer et al., 1976), maltose (Hazelbauer, 1975), ferrichrome (Wayne & Neilands, 1975), nucleosides (Hantke, 1976), and ferric enterobactin (Wayne & Neilands, 1976). Studies of these receptors relate to mechanisms of phage in-

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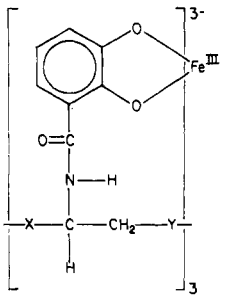

FIGURE 1: Structure of ferric enterobactin (X = C=O; Y = O) and its ferric carbocyclic analogue (X = Y = CH_2).

fection and colicin action, as well as to basic transport phenomena and outer membrane structure.

One system under study in this laboratory functions in the uptake of ferric enterobactin (enterochelin). Enterobactin is a siderophore (microbial high affinity iron carrier) which is overproduced by *E. coli* and related enteric bacteria under low iron stress. Its large formation constant for iron(III), equal to 10^{52} (McCardle, J. V., Avdeef, A., Cooper, H. S., & Raymond, K. N., in preparation),[1] enables it to compete effectively at physiological pH for ferric oxyhydroxide polymers (Langmuir & Whittemore, 1971). Enterobactin contains an inner ring consisting of three residues of L-serine condensed as a macrocyclic lactone. Each α-amino group of the series is acylated by a residue of 2,3-dihydroxybenzoic acid (Pollack & Neilands, 1970; O'Brien & Gibson, 1970). Enterobactin binds iron(III) in an octahedral structure containing all six catechol oxygens; the resulting complex has a net charge of minus three (Figure 1). The carbocyclic analogue[2] is believed to bind iron(III) in an analogous manner (Corey & Hurt, 1977).

The mechanism of transport of ferric enterobactin is not known in detail, but several genetic loci are required for the operation of the system. Transport begins with binding of the siderophore to an outer membrane protein. The *cbr* (Pugsley & Reeves, 1976) and *feuB* (Hantke & Braun, 1975) genes are believed to affect this step. The model currently does not require a periplasmic component. Active transport of the siderophore across the cytoplasmic membrane is required, and *fep* (Cox et al., 1970) and *tonB* (Konisky et al., 1976; Braun et al., 1976; Pugsley & Reeves, 1976) have been shown to affect ferric enterobactin transport, possibly at this step. Cox et al. (1970) also reported the requirement of the *fes* gene for utilization of ferric enterobactin as an iron source. Mutations in this gene have been associated with the loss of an enterobactin esterase activity (Langman et al., 1972). Cleavage of the ester bonds of the ligand by this enzyme is thought to weaken the ligand-metal binding and to facilitate removal of the iron.

The outer membrane receptor for ferric enterobactin is also reported to be the binding site for colicins B and D (Wayne & Neilands, 1976), which have been shown to compete with the siderophore for the receptor site. Mutants resistant to colicins B and D do not bind the colicins (Davies & Reeves, 1975) nor do they transport ferric enterobactin efficiently (Pugsley & Reeves, 1977). Relative to iron rich cultures, *E. coli* K-12 strains grown in low iron media show an increased amount of two or three bands, depending on the strain, in the 80K molecular weight region on $NaDodSO_4$-polyacrylamide electrophoresis gels of the outer membrane (McIntosh & Earhart, 1976; Uemura & Mizushima, 1975). Despite some variation in size assignments between laboratories, probably due to differences in strains or techniques, the upper band in strains with two bands is believed to be an outer membrane component of the enterobactin transport system. Colicin B resistant mutants lack this band. Some previous work on this protein band included Triton X-100–EDTA preparations of the membrane (Braun et al., 1976), but none of these preparations has been shown to be active in binding enterobactin.

We report here the extraction, assay, and some properties of the ferric enterobactin binding protein. Our data suggest that the extracted receptor resembles its in vivo counterpart and hence may be studied in regard to its structure and biological mechanism of action.

TABLE 1: Summary of Bacterial Strains.[a]

Strain	Properties	Source
E. coli K-12		
RW193	F^-, thi^-, $proC^-$, leu^-, trp^-, *entA*	R. Wayne
RWB18	*Colicin B resistant*, from RW193	R. Wayne
AN92	proA2, argE3, pheA1, tyrA4, trp 401, *aroB 351*	I. G. Young
AN270	*fep*$^-$, from AN92	I. G. Young
AN272	*fes*$^-$, from AN92	I. G. Young
R2.1/Vλ	str, met^-, λ^s, lysogenic for $\phi 80/\lambda$ hybrid, *colicinogenic for B*	S. Guterman
S. typhimurium LT-2		
enb-7	DHBA dependent enterobactin synthesis	B. Ames
enb-1	DHBA independent block in enterobactin synthesis	B. Ames

[a] Italicized properties are those of significance in this report. Unless an appropriate precursor is provided, such as DHBA, strains with either $entA^-$ or $aroB^-$ markers are unable to synthesize enterobactin.

Experimental Section

Materials. [^{55}Fe]$FeCl_3$, specific activity 33.5 Ci/g, in 0.5 M HCl was obtained from ICN Corp. Enterobactin was isolated by published methods (Wayne & Neilands, 1975). The carbocyclic analogue was obtained from E. J. Cory and the DHBA methylamide from S. Salama. The scintillation fluid was comprised of 2.14 L of toluene (sulfur free), 1.25 L of Triton X-100, and 9.1 g of Omnifluor, the latter obtained from New England Nuclear. DEAE-cellulose was the Whatman product DE-22. The 2% TTE buffer (pH 8.0) contained 2% Triton X-100, 0.1 M Tris-HCl, and 0.005 M Na_2EDTA, while 2% TTES buffer contained in addition to these ingredients, 0.1 M NaCl. Tryptone broth included 1.3% Tryptone (Difco) and 0.7% NaCl. Tryptone agar contained in addition to these 1.3% agar.

The bacterial strains used are listed in Table I.

Experimental Methods. Radioactive counting was performed on a Searle Delta 300 counter with window settings of 0.16–1.3; the efficiency was 27%.

Cultures were grown in Tris medium (Simon & Tessman, 1963) supplemented with 60 mg/L of L-proline, L-tryptophan,

[1] Fe(III) + enterobactin^{6-} $\rightleftarrows$ ferric enterobactin^{3-}; $K = 10^{52}$.

[2] Abbreviations used: carbocyclic analogue, *cis*-1,5,9-tris(2,3-dihydroxybenzamido)cyclododecane; DHBA, 2,3-dihydroxybenzoic acid; $NaDodSO_4$, sodium dodecyl sulfate; +Fe and −Fe after strain names mean high or low iron cultures, respectively, e.g., RW193 + Fe; Hepes, *N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid; TTES, Triton X-100–trihydroxymethylaminomethane–ethylenediaminetetraacetate–sodium chloride; TTE, Triton X-100–trihydroxymethylaminomethane–ethylenediaminetetraacetate.

L-leucine, 2 mg of thiamin hydrochloride, and 0.5% glucose. High iron cultures contained 5 μM $FeSO_4$, added after autoclaving. The inoculum was a fully grown nutrient broth culture added at 2% by volume.

Protein was determined by a modification (Wang & Smith, 1975) of the Lowry technique (Lowry et al., 1951).

Unless otherwise noted, all experiments with the extracted receptor were performed at room temperature.

Membrane Preparation. Cells were grown at 37 °C, collected in late log phase and sonicated in 1.0 M Tris-HCl, pH 8.0. Membranes were prepared by the Schnaitman procedure (Schnaitman, 1971). The term "outer membrane" is used here to refer to the pellet recovered after treatment of whole membranes with 2% Triton X-100, 10 mM $MgCl_2$, 0.1 M Tris-HCl; pH 8.0. The supernatant from treatment of the outer membranes with 2% TTE is referred to as extracted outer membrane or extracted receptor.

Preparation of [^{55}Fe]Ferric Enterobactin. Exactly 400 μL (1.09 μmol) of [^{55}Fe]ferric chloride solution was added to 5 mL of ethanol and the volume reduced on a rotary evaporator to about 0.2 mL. Evaporation to dryness was repeated from ethanol and methanol. The residue was dissolved in 5 mL of methanol, 1.09 μmol of enterobactin in 450 μL of methanol was added, and the mixture taken to dryness. After addition of 0.5 mL of water and 0.5 mL of 0.76 M Tris-HCl, pH 7.4, a wine-colored solution resulted. The solution was removed and the remaining residue treated as in the previous step. The combined extractions were placed on a 1.5 × 8 cm Bio-Rex 70 (Na^+) column and eluted with water. The colored zone was placed on a 1.5 × 6 cm alumina column and eluted with 20% methanol–water (McCardle et al., in preparation). The colored peak was evaporated and dissolved in 1.0 mL of water. The solution was stored at −15 °C to protect against hydrolysis and oxidation of the ligand. Purity was confirmed by paper electrophoresis at pH 6.5. The yield was 63.2% of label with no significant change in specific activity.

Preparation of Ferric Catecholates. The ferric complexes of the catechol compounds studied in this paper were prepared as follows. Exactly 0.10 mL of ethanol, 0.08 mL of a neutral solution of 5 mM ferric triacethydroxamate, 0.08 mL of a solution of the catechol compound 15 mM in catechol sites, and 0.10 mL of 10 mM Hepes-Na^+ buffer (pH 7.2) were mixed and allowed to stand at room temperature for 30 min. The entire 0.36 mL of solution was then applied to a sheet of filter paper impregnated with 0.1 ionic strength phosphate buffer, pH 6.5. Separation was performed by electrophoresis at 15 mA and 1 kV for about 1 h. Ferric enterobactin moved with a mobility ca. 1.2 times that of ferrichrome A. The wine-colored zones were excised and a stock solution prepared by eluting the ferric catecholate into a small volume of water. The concentration of ferric catecholate was determined by use of the extinction coefficients at the absorption maxima in the visible: ferric enterobactin, 495 nm, a_{mM} = 5.6 (Anderson et al., 1976); ferric carbocyclic analogue of enterobactin, 495 nm, a_{mM} = 5.5 (Corey & Hurt, 1977). Unlike ferric enterobactin, solutions of the ferric carbocyclic analogue were decolorized upon freezing and thawing.

Colicin B Preparation. R2.1/Vλ was grown at 37 °C in a 10-L aerated tryptone broth culture until late log, at which time 2 mg of mitomycin C was added. After a further 8 h the cells were collected and washed with 0.05 M potassium phosphate, pH 7.0. The washed cells were suspended in four times their wet weight of phosphate buffer, and sonicated at 0 °C until a 90% decrease in the OD_{650} was obtained. The sonicate was centrifuged 10 min at 40 000g, and the supernatant was then spun 1 h at 250 000g. Ammonium sulfate was added to the supernatant to 20% saturation and the precipitate discarded. The supernatant was brought to 50% saturation in $(NH_4)_2SO_4$ and the precipitate redissolved in a minimum amount of potassium phosphate buffer and dialyzed in the cold overnight against the same buffer. The colicin B was stored in small samples at −20 °C. After thawing, the colicin B activity remained constant for several weeks when stored at 4 °C. The preparations used in this report had an activity of 1024 units/mL. Activity was assayed by applying 2-μL samples of a series of 1:2 dilutions of the colicin in phosphate buffer, to a 1.3% Tryptone agar plate. After the samples had soaked in, 50 μL of RW193 culture (OD_{650} = 0.6, in nutrient broth) in 2.5 mL of plain top agar was poured onto the plate. Plates were scored after incubation overnight at 37 °C. Units/mL = 2^N with N = highest number of dilutions giving clear killing (Guterman, 1971).

Preparation of DEAE Microassay Column. A 12 cm × 4 mm o.d. piece of standard wall glass tubing, scored at the midpoint, was inserted tightly in a 23G 1-in. Yale disposable hypodermic needle. A small volumetric measure fashioned from a short piece of the same tubing was used to induce ~30 mg of sand. The base of the column was sealed with melted parafin. From a well-stirred suspension of DEAE-cellulose, equilibrated with 2% TTES buffer and originating from a 1:4 ratio of packed to supernatant volume, 0.4 mL was added to the column by means of a syringe with a 18G needle. The column was washed with 0.4 mL of 2% TTES buffer. Using melted paraffin, the column was sealed at the top leaving a small volume of buffer above the bed.

DEAE Column Microassay. [^{55}Fe]Ferric enterobactin was mixed with 5× concentrated 2% TTE buffer and water to form a solution of 20 μM [^{55}Fe]ferric enterobactin in 2% TTE. The solution was used immediately or frozen soon after preparation. Exactly 10 μL of the solution was mixed with 90 μL of soluble receptor. The seal on an assay column was broken at the scored mark in order to allow buffer to reach the bed about 5 min after mixing [^{55}Fe]ferric enterobactin with receptor. A microsyringe was used to place 20 μL of the reaction mix on the bed surface. After the sample entered the bed, the column was washed with 0.2 mL of 2% TTES. The eluate was collected in a vial containing 300 μL of water and counted after addition of 5 mL of scintillation fluid. Two or three columns were used per sample. Blanks were run with 2% TTE in place of a receptor preparation.

Colicin B–Receptor Binding Assay. A qualitative assay for colicin B binding to extracted receptor was employed. Fresh tryptone plates were used. Plain top agar (2.5 mL) containing 10 μL of a 1:10 dilution of standard colicin B solution, the minimum amount to kill all cells, was poured onto the plate. Samples 2–5 μL in volume were placed on top of the colicin B layer and allowed to seep into the agar. Indicator cells were introduced via a second 2.5 mL of top agar and the plates were incubated overnight. A sample was scored positive if growth above background was observed where it had been applied. Serial dilution of the receptor with 2% TTE or use of plates with varied amounts of colicin B provided a semiquantitative measure of receptor concentration.

Colicin B–Ferric Enterobactin Receptor Competition Assay. Plates containing colicin B were prepared as above using 5 μL of standard colicin B solution. Receptor concentration was adjusted with 2% TTE so that a further 1:4 dilution showed little or no protection. Receptor solution was mixed 1:1 with various ferric enterobactin solutions. Samples of the mixtures 2 μL in volume were placed on different plates. Controls (see Results) for each mixture were run on the corresponding plate. After the samples had soaked in, plain top

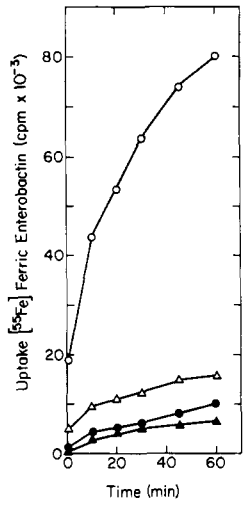

FIGURE 2: Uptake of [^{55}Fe]ferric enterobactin by cells of RW193 ± Fe and RWB18 ± Fe. Cells were grown in the appropriate Tris media until late log, collected, washed with and resuspended in low iron Tris media at the original culture volume. Cultures were shaken at 37 °C. [^{55}Fe]ferric enterobactin was added at time zero to a final concentration of 1 μM. Volumes equal to 2.5×10^9 cells at $t = 0$ were collected on Millipore filters previously soaked in 1 μM unlabeled ferric enterobactin. The filters were washed twice with 5 mL of Tris medium, dried, and counted in 5 mL of scintillation fluid. Symbols: RW193 + Fe (●—●); RW193 − Fe (○—○); RWB18 + Fe (▲—▲); RWB18 − Fe (△—△).

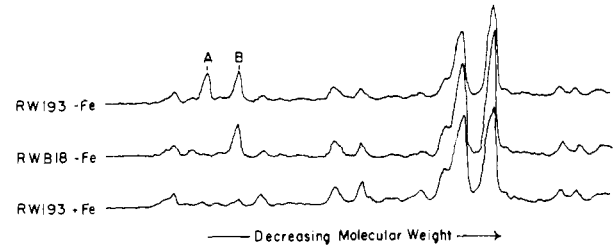

FIGURE 3: NaDodSO$_4$-polyacrylamide gel electrophoresis densitometer profiles of whole membranes. Sixty micrograms of protein samples was run on 11% acrylamide Lugtenberg gel system (Lugtenberg et al., 1976), stained with Coomassie Brilliant Blue R. Gels were read on a Transdidyne General RFT scanning densitometer at 600 nm, on the logarithmic scale.

agar (2.5 mL) was poured on each plate. After 30 min at room temperature, the plate was poured with indicator cells in the usual way and incubated overnight at 37 °C.

Results

E. coli RW193 was chosen as the source from which to extract the ferric enterobactin receptor in view of the existence of mutant RWB18, which had been shown not to bind colicin B. Lack of colicin binding had been attributed to absence of an outer membrane receptor, which had also been implicated in ferric enterobactin transport (Wayne & Neilands, 1975; Wayne et al., 1976). If RWB18 indeed lacked the outer membrane receptor, then it would serve as the ideal control for experiments on RW193. Further experiments to confirm this assumption were performed.

Growth and Transport. It was shown that ferric enterobactin supported the growth of RW193 on minimal plates but not RWB18, while ferrichrome stimulated both strains (Wayne & Neilands, 1976). RW193 and RWB18 when grown in Tris media for 18 h gave an $OD_{650} = 0.4$–0.6 in low iron cultures and an $OD_{650} = 1.3$ in high iron cultures. No differences in growth curves between these strains were observed under these conditions. If 20 mg/L DHBA was added to the high iron cultures, the OD_{650} was 0.5 for RWB18 and 1.3 for RW193. In the presence of DHBA both strains synthesized enterobactin which would chelate all of the iron in the culture. The lack of growth response of RWB18 implied that it was unable to utilize this ferric enterobactin. That this failure was related to the inability of RWB18 to efficiently transport ferric enterobactin was confirmed by studies on the uptake of [^{55}Fe]ferric enterobactin (Figure 2).

TABLE II: Properties of the Receptor Preparations from RW193 and RWB18.

Source	[^{55}Fe]Ferric enterobactin binding[a]	
	Whole membrane[b]	Extracted membrane
RW193 + Fe	26%	0–8%
RW193 − Fe	100%	100%
RWB18 + Fe	35%	0–15%
RWB18 − Fe	26%	0%

[a] Given in terms of the activity observed for RW193 − Fe. Ranges given include the maximum values observed in any experiment. Results less than blanks were scored as 0%. [b] Whole membrane binding assayed using 0.45-μm Millipore filters 25HA, 47-mm diameter. Membranes were incubated 5 min at 37 °C in 0.1 M Tris-HCl, pH 8, with 5×10^{-6} M [^{55}Fe]ferric enterobactin. Samples collected on filters soaked with 10^{-6} M ferric enterobactin (unlabeled). The filters were washed with 2 mL of Tris-HCl, dried, and counted in 5 mL of scintillation fluid.

NaDodSO$_4$-Polyacrylamide Gel Electrophoresis Analysis. When membranes of both strains were examined on NaDodSO$_4$-polyacrylamide gel electrophoresis in this laboratory and that of Charles Earhart (personal communication), two bands were found to increase in RW193-Fe, but only one (B in Figure 3) increased in RWB18-Fe relative to the respective high iron cultures. Figure 3 shows whole membrane profiles; high iron profiles are equivalent for both strains. The missing band in RWB18-Fe had been assigned to the ferric enterobactin-colicin B binding protein. The apparent molecular weight was determined as 90 000 (P. Klebba, personal communication). Outer membranes and outer membrane extracts retained this single difference in appearance between strains on NaDodSO$_4$-polyacrylamide gel electrophoresis. The other iron repressed band (B) is believed to correspond to the colicin Ia receptor (Konisky et al., 1976).

Receptor-Colicin B Binding. In vitro binding of the extracted receptor to colicin B was demonstrated by the plate assay. The indicator cells only grew above samples in which receptor molecules bound the colicin and prevented it from killing the cells. Only extracted outer membrane from RW193-Fe provided this protection; RW193 + Fe and RWB18 ± Fe were negative as was 2% TTE.

Assay for Ferric Enterobactin Binding by Extracted Receptor. Since the 2% TTE extraction of RW193 outer membrane showed differential binding of colicin B relative to RWB18, parallel results with ferric enterobactin binding were anticipated. Membrane bound receptor had been studied by retaining it on filters, while unbound substrate was washed away (Pugsley & Reeves, 1976). Preliminary experiments showed no binding activity in extracted preparations using this approach. Since ferric enterobactin has a three minus charge, it would be tightly bound by DEAE-cellulose, while the receptor would not be so retained in 2% TTES (Braun et al., 1976). Ferric enterobactin associated with receptor was not expected to bind to DEAE-cellulose. This was shown to be the case and receptor-dependent release of [^{55}Fe]ferric enterobactin 12 times blank values was demonstrated. An activity profile of the columns (not shown) indicated the entire peak

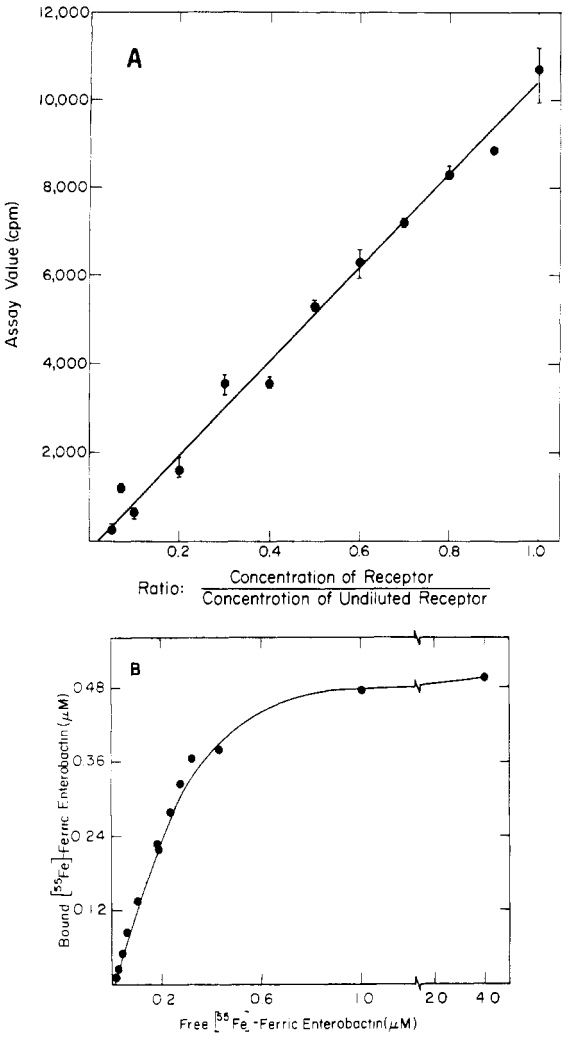

FIGURE 4: (A) Dependency of DEAE-cellulose column assay on receptor concentration. Standard receptor solution diluted with 2% TTE. Three columns run per concentration (0.9 dilution = single column); range of values indicated by error bars. (B) Saturation of receptor dependent elution of [^{55}Fe]ferric enterobactin with increasing concentration of [^{55}Fe]ferric enterobactin. Blanks were run for each different concentration. Free [^{55}Fe]ferric enterobactin concentration was calculated from the original concentration minus the concentration of receptor bound [^{55}Fe]ferric enterobactin measured in the assay.

was eluted by the wash and $NaDodSO_4$ gel electrophoresis analyses of the activity peak showed no unexpected differences between RW193 and RWB18. Using the micro column assay (Experimental Section), extracted outer membranes from both strains were assayed for binding activity. These results, along with those from an experiment on [^{55}Fe]ferric enterobactin binding to whole membranes for comparison, are shown in Table II. The activity shown for iron rich cultures may be the result of some form of contamination. Preparations from high iron cultures, involving extra washing or extraction steps prior to treatment with 2% TTE, often showed no activity above blank values. The results found with RW193 and RWB18 preparations from low iron cultures were conclusive. No activity was found in the strain apparently lacking the receptor. This difference paralleled and was as definitive as those found in the previous procedures.

Column Assay Characteristics. A study of the dependency of the assay on receptor concentration is shown in Figure 4A, which also provides an example of the variance observed between columns for identical samples. The lower practical limit of the assay, 30% above the blank, was equivalent to 0.3 pmol

TABLE III: Ferric Enterobactin–Colicin B Competition for Extracted Receptor.

Fe-enterobactin concn (M)	Receptor + H_2O	Receptor + Fe-enterobactin	2% TTE + Fe-enterobactin	2% TTE + H_2O
$>10^{-4}$	+	+++	+++	−
10^{-4} to 10^{-5}	+	−	−	−
$<10^{-5}$	+	+	−	−

[a] Each range of concentrations represents a summary of several experiments. In any one experiment competition occurred at a particular concentration between 10^{-4} and 10^{-5} M, while other concentrations in this range may have showed no competition. Symbols: +++ = 14–23-mm diameter region of diffuse growth; + = 7-mm diameter region of heavy growth; − = no growth (see Experimental Section).

of bound [^{55}Fe]ferric enterobactin, or a 15 nM concentration of binding sites in the sample. The dependency of binding on substrate concentration was investigated. Apparent breakdown of the assay at ferric enterobactin concentrations $<10^{-7}$ or $>5 \times 10^{-6}$ M, combined with technical difficulties in interpretation, made difficult the analysis of these data. Using both saturation (Figure 4B) and Scatchard plots of the data, saturation was found at high substrate concentration, and a $K_{dissociation}$ in the range 0.2–0.5 μM was indicated. Figure 4B is an example of the results from one such experiment.

Agarose Gel Chromatography. On A-0.5m and A-5m agarose gel molecular sieving columns, run with 2% TTE, the peak of ferric enterobactin binding activity eluted slightly behind the ferritin standard, indicating a molecular weight of 300 000–400 000 (Ellen Fiss, personal communication). This magnitude and range of molecular weight in the presence of Triton X-100 argues against the binding activity being in the form of membrane particles such as those described by Ichihara & Mizushima (1977).

Ferric Enterobactin–Colicin B Competition. An attempt to reproduce the competition between ferric enterobactin and colicin B for receptor in vivo was made using extracted receptor in the competition assay described in the Experimental Section (Table III). With ferric enterobactin concentration greater than 0.1 mM, similar results were obtained in the presence and absence of receptor. This protection was provided by the competition between ferric enterobactin and colicin B for the receptors of the indicator cells. At less than 10 μM ferric enterobactin the response of the experimental sample equaled that of the receptor control. At concentrations between these a third response was observed. The receptor–ferric enterobactin sample offered little or no protection for the indicator cells. This can be attributed to ferric enterobactin binding the extracted receptor and thus preventing the binding of colicin B, which diffuses through the receptor layer to kill the indicator cells. The ferric enterobactin concentration was low enough that diffusion lowered it below the level necessary to protect the cells during incubation. Ferric chloride in similar concentrations had no effect.

Stability of the Extracted Receptor. Receptor stored at −196 °C was stable indefinitely. Storage at −15 °C resulted in no loss of activity over one month. Repeated freezing and thawing was not possible without loss of activity. At 4 °C after 1 week the receptor was still at least 95% active, though stability varied with different preparations at this and higher temperatures. Some preparations showed significant decay in activity after 2 days at room temperature. At 37 °C, 87% or more of the ferric enterobactin binding activity was lost after

TABLE IV: Competition between Ferric Chelates and [^{55}Fe]Ferric Enterobactin for Receptor Binding.

Competitor	cpm	% of standard	K_{comp}/K_{ent}[a]	Colicin B protection
None	10 550	100	*b*	*b*
Ferric enterobactin (unlabeled)	5 690	54	1.2	+
Ferric carbocyclic analogue	7 336	70	2.5 (1.24)[c]	+
Ferric tris(DHBA)	10 198	97	35	*b*
Ferric tris(DHBA methylamide)	8 970	85	6.7	−
Ferrichrome	10 056	95	25	−

[a] K_{comp}/K_{ent} = [rec-ent][comp]/[rec-comp][ent]; [rec-ent] refers to receptor bound [^{55}Fe]ferric enterobactin. This was determined by the column assay; [ent] = 2 μM − [rec-ent], refers to unbound [^{55}Fe]ferric enterobactin; [rec-comp] = $[\text{rec-ent}]_{standard} - [\text{rec-ent}]_{+competitor}$, refers to receptor bound competitor; [comp] = 2 μM − [rec-comp], refers to unbound competitor. [b] Not applicable. [c] See discussion on specificity of receptor binding.

24 h. However, no loss in colicin B binding activity was seen. The significance of this result will be the subject of further research.

Specificity of Substrate Binding. The specificity of the receptor was studied by competition between various iron chelates and [^{55}Fe]ferric enterobactin. Receptor competitively binding ferric chelates would elute less labeled substrate as measured with the DEAE-cellulose column assay. The assay mixture was made up in the usual way except the [^{55}Fe]ferric enterobactin stock solution also contained the iron chelate so that the final concentrations were 20 μM for both compounds in the stock solution. Blanks were run on each ferric chelate assay mixture. The receptor solution used was approximately 0.4 μM in binding sites. Based on the assumption of a nearly saturated receptor, a ratio of the apparent dissociation constant (K_{comp}) of the competitor–receptor complex to the dissociation constant (K_{ent}) of the [^{55}Fe]ferric enterobactin–receptor complex was calculated. This ratio should be 1.0 for equivalent molecules and should be greater than 1.0 for less tightly bound compounds.

Table IV presents the results of these experiments along with a study of the ability of these compounds, at a concentration of 0.4 mM, to protect against colicin B. The K_{comp}/K_{ent} ratio for ferric enterobactin was greater than the ideal value of 1.0. This was probably the result of a combination of small variations in the assay along with some deviation from the assumed saturation of the receptor.

This result is a measure of the limitations of this approach. The ratio shown by the ferric carbocyclic analogue means that it is strongly bound by the receptor but is distinguishable from ferric enterobactin. Ferric tris(DHBA-methylamide), a less ideal analogue of enterobactin, shows correspondingly less affinity for the receptor. DHBA has minimal binding, in agreement with the proposed mechanism of DHBA mediated iron uptake (Hancock et al., 1977). Ferrichrome also gave little inhibition of ferric enterobactin binding, which would be expected since the ferrichrome uptake system requires a different outer membrane receptor (Luckey et al., 1975). Experiments run with the complexes exposed to the labeled substrate for longer periods of time showed no differences in results, and along with preliminary experiments on ligand exchange kinetics, ruled out the possibility of label exchange.

Growth Studies with the Carbocyclic Analogue. Since esterase cleavage of the lactone ring in enterobactin was believed to be required for release of the iron, and since relative to ferrichrome the carbocyclic analogue appeared to bind iron as strongly as enterobactin (data not shown), it was not expected that the ferric carbocyclic analogue would support growth of cells blocked in enterobactin synthesis. As Table V indicates, however, it supported growth to about the same extent as ferric enterobactin. RWB18 (lacking the receptor), AN270 (fep$^-$), and AN272 (fes$^-$) all showed no growth on either catechol-based siderophore, confirming the common mechanism of uptake for the compounds. The tests with the *S. typhimurium* LT-2 strains enb-1 and enb-7 were necessary to eliminate the possibility of either DHBA contamination or ligand breakdown acting as a source of DHBA which could be used to synthesize enterobactin. The ability of the ferric carbocyclic analogue to support growth of both strains obviated illicit enterobactin synthesis as the mechanism of action. Ferric tris(DHBA-methylamide) did not support growth of any strain.

TABLE V: Growth Response Displayed by Ferric Catecholates in Enterobactin Requiring and Ferric Enterobactin Utilization Deficient Strains of Enteric Bacteria.[a]

	Diameter of exhibition of growth (mm)			
	E. coli			
Concn(μM)	RWB18 RW193	AN270 AN272	*S. typhimurium* enb-1	*S. typhimurium* enb-7
Ferric enterobactin				
25.0	21.5	Nil	18.0	16.0
2.5	15.0	Nil	12.0	10.5
0.25	9.0	Nil	Nil	Nil
Ferric carbocyclic analogue				
25.0	16.5	Nil	16.0	14.0
2.5	11.0	Nil	8.0	8.0
0.25	7.0	Nil	Nil	Nil
Ferrichrome				
50.0	ca. 20	20	20	20
DHBA				
100	ND[b]	ND	Nil	16.0

[a] In each case a 10-μL volume was pipetted onto a 6-mm filter disk. [b] ND, not done.

Discussion

The results reported herein support the conclusion that the activity measured by the DEAE-cellulose column assay was the binding of [^{55}Fe]ferric enterobactin to an extracted protein of the *E. coli* outer membrane and that this binding was representative of the physiological function of the protein. The data from the temperature inactivation experiments emphasize the importance of following substrate binding activity during studies of Triton extractions of the binding protein. They demonstrate that colicin B binding activity is insufficient for defining the intact receptor system. An apparent parallel loss of substrate binding without loss of phage binding was reported in the ferrichrome-T5-receptor system (Luckey et al., 1975).

The competition experiments indicate that while a ring structure may be an important requirement for tight binding, the affinity of the ferric carbocyclic analogue for the receptor argues strongly against the importance of the macro cyclic lactone moiety in determining receptor specificity. The importance of the lactone ring for receptor binding to the substrate may be related to the constraints it places on the structure of catechols around the iron. Ferric tris(DHBA) and ferric

tris(DHBA-methylamide), unlike ring-mounted catecholates, may exist in cis and trans forms. This, plus the increased charge of ferric DHBA, may account for the decreased affinity these compounds have for the receptor. Unlike ferric enterobactin, which exists only as the Δ-cis isomer (Isied et al., 1976), the ferric carbocyclic analogue must be present as a racemic mixture. The assumption of a stereospecific binding site on the receptor yields a K_{comp}/K_{ent} ratio of the correct stereoisomer very close to that calculated for ferric enterobactin (see Table IV, c). This calculation is only speculative and the final resolution of receptor specificity awaits the availability of more analogues, particularly a pair of diastereomers.

The results in Table V cast doubt on the accepted mechanism for utilization of the iron atom of ferric enterobactin. The iron release mechanism for ferric enterobactin is believed to require the participation of an esterase to hydrolyze the ester bonds of the ligand which, in turn, labilizes the iron, possibly through raising its oxidation-reduction potential. Strains which are *fes*$^-$ lack the esterase and are unable to obtain iron from ferric enterobactin. Although the iron-salvage mechanism would seem to require that the esterase hydrolyze the complex and not the ligand, controversy has surrounded its substrate specificity. One group claims it prefers the complex (Langman et al., 1972) while other workers found that it only hydrolyzes the free ligand (Bryce & Brot, 1972). While it is not surprising—in view of their many common properties—that the ferric carbocyclic analogue of enterobactin can interact with the ferric enterobactin receptor, the fact that the substance is practically as potent as the native siderophore in supplying iron to RW193 questions the role of an esterase in the metabolic event(s) involved in the "out-transfer" of the coordinated metal ion.[3]

A possibility that springs to mind is that hydrolysis of the ester bonds is only coincidentally related to the iron delivery step. In this regard, the experiments of Emery with ester bond containing hydroxamate siderophores from fungi are of interest (Emery, 1976). He found hydrolysis of the ester bond to be dependent on the type of metal ion in the complex and proposed that its reduction might precede hydrolysis.

The carbocyclic analogue may be a valuable tool in the future for studying the ferric enterobactin transport system. Its synthetic origins should permit the creation of useful derivatives which might be difficult to reproduce with enterobactin due to its lability under many reaction conditions. These derivatives could be useful in many ways including affinity labeling or detail mapping of the protein–ligand binding domains.

The molecular mechanical details of the ferric enterobactin transport system in *E. coli* are still largely unknown. As this paper has shown, some of the fundamental aspects of the current model need to be reexamined. However, the stability of the extracted receptor and the large quantities available from derepressed cells render this an ideal system for the study of outer membrane dependent transport in bacteria. The ability to quantitatively assay for ferric enterobactin binding and the use of derivatives such as the carbocyclic analogue should facilitate isolation of the active outer membrane receptor, the determination of its structure and the mode of ferric enterobactin transport.

[3] Recent work in this laboratory has demonstrated in vitro reduction of the iron in ferric enterobactin by thiol compounds under special conditions. This reduction did not require prior hydrolysis of the esters in the ligand. Alternatively, the increased hydrophobicity of the carbocyclic analogue could indirectly impart to it a higher oxidation reduction potential by virtue of its greater solubility in the lipid phase of the membrane (Hider, R. C., & Neilands, J. B., in preparation).

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