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Purification of outer membrane iron transport receptors from *Escherichia coli* by fast protein liquid chromatography: FepA and FecA

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Fast protein liquid chromatography (FPLC) with DEAE–Sepharose Fast Flow, PBE-94 and Q–Sepharose Fast Flow columns are applied to the purification of the ferric enterobactin protein receptor (FepA). The apparent single band of FepA on SDS–PAGE is isolated and purified into two proteins with very similar molecular weights. The two proteins are identified to be FepA and ferric citrate protein receptor (FecA) by N-terminus amino acid determination and a computer search with the Gene Bank file. The assay of binding activities of these proteins shows that both FepA and FecA bind ferric enterobactin, with the former having about double the activity of the latter. Competition studies shows that Fe–MECAM is competitively bound to both proteins and that ferric parabactin only slightly competes with [^{55}Fe]ferric enterobactin. It is found that ferrichrome A has no effect on the binding of the receptor proteins with ferric enterobactin.

Keywords: binding competition, ferric enterobactin receptor protein (FepA), ferric citrate receptor protein (FecA), iron transport receptors, purification of membrane proteins, specificity of binding

Introduction

It is known that iron transport in microorganisms is mediated by low molecular weight iron chelating compounds called siderophores. In *Escherichia coli* iron uptake is mediated by enterobactin which is produced by the organism. The ferric complex of enterobactin has a stability constant of 10^{52} (Harris *et al.* 1981). Outer membrane proteins have been found which are involved in uptake of ferric enterobactin and its synthetic structural analogs in *in vivo* studies (Neilands *et al.* 1980, Ecker *et al.* 1986). Studies have shown that ferric enterobactin is specifically taken up by an 81 kDa outer membrane protein receptor, FepA (McIntosh *et al.* 1979, Ozenberger *et al.* 1987). The siderophore is subsequently transported by a periplasmic protein, FepB. Transport of iron from the periplasm into the cytosol involves a cytoplasmic membrane ferric enterobactin permease FepC (Ozenberger *et al.* 1987) and two other proteins in the cytoplasmic membrane, which are identified as FepD and FepG (Chenault & Earhart 1991). The FepA protein is thus the first step in the transport of ferric enterobactin and it is important to study the properties of the isolated protein. The protein was first isolated in 1978 (Hollifield & Neilands 1978, Fiss *et al.* 1982, Jalal & van der Helm 1989). At that time the N-terminus of FepA was determined to be phenylalanine (F) (Fiss *et al.* 1982). This is in contrast with the results on the DNA sequence determination of the *E. coli fepA* gene carried out by Lundrigan & Kadner (1986). This study shows that the polypeptide has a molecular weight of 79 908 and consists of 723 amino acids with glutamine as the N-terminus. A purification procedure of FepA from UT5600/pBB2 was set up in this laboratory in 1989 (Jalal & van der Helm 1989). Purified FepA receptor, possibly with a small amount of impurity, was obtained and crystallized. However, the N-terminus and binding activity of the receptor were not determined at that time and the protein was not further characterized.

Unlike the purification of soluble proteins (Zhou *et al.* 1989), the purification of membrane proteins is more difficult since detergents have to be used for their solubilization. The purification procedure, therefore, involves a larger number of columns and longer periods of time. For high level purification

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and analysis of these proteins, fast protein liquid chromatography (FPLC) is an attractive method, because it provides easy operation, sensitive detection, high resolution and reproducibility.

The identity of any protein can always be established from its N-terminal amino acid sequence once the full sequence is known. The binding activity may or may not be lost during purification and in the latter case can also be used to identify the protein. Both characterizations are described. The present study shows the separation and characterization of two receptor proteins, FepA and FecA, produced by strain UT5600/pBB2, with closely similar molecular weights and p*I* points.

Materials and methods

Materials

DEAE–Sepharose Fast Flow, PBE-94, Q–Sepharose Fast Flow Sephadex G-10 and Sephadex G-25 were obtained from Pharmacia LKB. β-D-Octylglucoside, pyroglutamate aminopeptidase and Triton X-100 were purchased from Mannheim Boehringer GmbH. Benzamidine–HCl was obtained from Fluka Chemika, MECAM was provided by Dr K. N. Raymond, University of California at Berkeley and L-parabactin was provided by Dr R. J. Bergeron, University of Florida at Gainesville. [^{55}Fe]ferric chloride (39 m Ci/mg, in 0.5 M hydrochloric acid) was purchased from DuPont (NEN). PVDF membranes (Immobilon transfer), 0.45 mm pore size, were obtained from Millipore. All other chemicals used in this study were analytical reagent grade.

Microorganisms and culture conditions

Bacteria (*Escherichia coli* UT5600/pBB2: *leu*$^-$, *proC* , *trpE*$^-$, *rpsL*$^-$, Δ(*ompT-fepA*)$^-$/*Ampr, fepA*) was provided by Dr M. D. Lundrigan, University of Mississippi Medical Center, Jackson, MS. *E. coli* strains AN102 and AN311 were provided by Dr J. B. Neilands, University of California at Berkeley and Dr Winkelmann, University of Tübingen, Germany, respectively.

E. coli strain (UT5600/pBB2) was grown in a trypton/yeast extract (TY) medium (Hoffman *et al.* 1986), containing ampicillin (4 mg ml^{-1}), to an optical density (OD) at 660 nm of 0.300 at 37 °C. The cell suspension in TY medium was stored frozen at −70 °C in 1 ml quantities as a 30% solution in glycerol until ready to use. For FepA production, the cells were thawed at room temperature and grown in 50 ml volume of TY medium with antibiotic to an OD at 660 nm of 0.150. Portions of the cell suspension (5 ml) were transferred to 500 ml of minimal growth medium in 2-l volume acid washed Fernbach flasks. Minimal culture medium contained (per liter of distilled water): 10.5 g K_2HPO_4; 4.5 g KH_2PO_4; 1.0 g $(NH_4)_2SO_4$; 0.5 g sodium citrate (dehydrate); 0.25 g $MgSO_4 \cdot 7H_2O$; 0.01% L-proline; 0.01% L-leucine; 0.01% L-tryptophan; 0.4 mg ampicillin; 0.2% glucose; and 25 μg thiamine–HCl (Vitamin B_1). Ampicillin was sterilized by filtering through a 0.2 mm disposable filter assembly. Other media components were prepared separately and sterilized by autoclaving. Cells were grown in the minimal medium at 37 °C and shaken at 200 r.p.m. for 16–18 h after which the cells were chilled to 4 °C with ice and harvested by centrifugation. The cells were washed once with buffer A (50 mM Tris–HCl buffer containing 10 mM benzamidine with a pH of 7.5).

FepA purification

A typical purification scheme of FepA from *E. coli* is described below. Dialysis and redissolution were carried out at 4 °C.

Preparation of crude extract. Cells harvested from 6 l of culture were suspended in 200 ml of buffer A and disrupted (in 10 ml batches) by sonication (3 min, in 30 s bursts) in an ice bath. The homogenate was fractionated and extracted by a Triton X-100 EDTA/octylglucoside solubilization procedure previously reported. (Jalal & van der Helm 1989). Fractions rich in FepA determined by SDS–PAGE were pooled and dialyzed over 24 h with two changes of 1 l of buffer B (25 mM Tris–HCl, 10 mM benzamidine, 2% Triton X-100, pH 7.5).

Ammonium sulfate fractionation. The clarified aqueous extract (65 ml) was brought to 4.8% of ammonium sulfate and the pH of solution was adjusted with NH_4OH to 7.5. The precipitate was collected and washed, with buffer B (without Triton X-100), twice by centrifugation (12000 × *g*, 15 min at 4 °C), and then redissolved in 200 ml of buffer B.

DEAE–Sepharose Fast Flow (FPLC) column chromatography. FepA solution (200 ml) was applied to a FPLC DEAE–Sepharose Fast Flow column (1.6 × 1.6 cm) which was previously equilibrated with buffer C (25 mM Tris–HCl, 10 mM benzamindine, 0.1% Triton X-100, pH 7.5). After the column was washed with 400 ml of buffer C and a base line was reached, proteins were eluted at a flow rate of 4 ml min^{-1} with a 150 min FPLC gradient from 0 to 10, 12.5 and then 15% of 1 M NaCl in buffer C. The remainder of the heterogeneous protein was eluted with a final wash using 1 M NaCl in buffer C (Figure 1). The proteins were detected at 280 nM. Every second fraction was examined by SDS–PAGE to identify the bottom band ($FepA_B$) or top band ($FepA_T$) according to the known mobility of FepA.

PBE-94 anion exchange FPLC chromatography. After the pooled $FepA_B$ solution was dialyzed against buffer C for 1 h, it was loaded onto a PBE-94 column (1.6 × 16 cm) anion exchanger, which was pre-equilibrated with the same buffer. $FepA_B$ was eluted with a linear FPLC gradient of 0–0.5 M NaCl in buffer C at a flow rate of 2 ml min^{-1} and monitored at 280 nm.

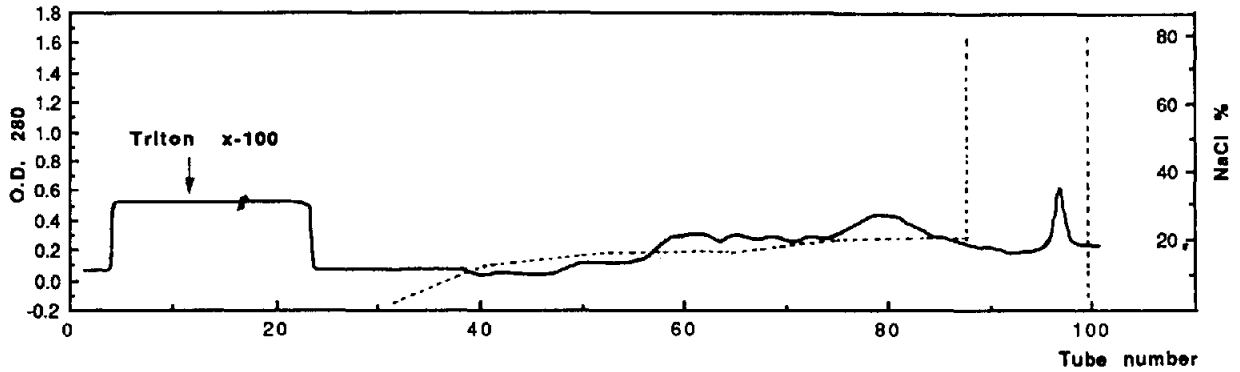


Figure 1. Separation of FepA from outer membrane extraction of *E. coli* UT 5600/pBB2 on FPLC DEAE–Sepharose Fast Flow. A whole batch of 200 ml of outer membrane extraction (250–270 mg) was applied to a FPLC DEAE–Sepharose Fast Flow column (1.6 × 16 cm). After sample loading, the column was washed with mobile phase A until the absorbance at 280 nm (—) had returned to baseline and then proteins were eluted by a gradient of NaCl in mobile phase A (- - - - -). Full details are given in Materials and methods.

Q-Sepharose Fast Flow FPLC column chromatography. After DEAE–Sepharose column chromatography, the fractions containing $FepA_T$ were pooled and dialyzed against 2 l of buffer C and then applied onto a Sepharose Fast Flow FPLC column (1.6 × 10 cm) equilibrated with buffer C. $FepA_T$ was eluted with a linear FPLC gradient of 0–0.5 M NaCl in buffer C at a flow rate of 2 ml min^{-1}.

PBE-94 anion exchange rechromatography. Partially purified $FepA_B$ and $FepA_T$ were further purified by passing each through a PBE-94 anion exchange FPLC column (1 × 16 cm) which was pre-equilibrated with buffer C (pH 7.5). After washing with 50 ml of the same buffer, the column was eluted with a linear gradient of 0–0.5 M NaCl in buffer C at a flow rate of 2 ml min^{-1}. The fractions with $FepA_B$ or $FepA_T$ were pooled and stored at −20 °C.

SDS–PAGE

The process was carried out on 10 and 7% polyacrylamide slab gels, respectively, using modified Laemmli buffer system (Laemmli 1970). Coomassie brilliant blue R-250 was used for staining.

Protein determination

Protein was determined by a modification of the Lowry procedure (Lowry *et al.* 1951). In order to minimize the interference of Triton X-100, deoxychlorate was used during the measurement of protein (Dudley & Grieve 1975). Bovine serum albumin was used as the standard.

Preparation and purification of enterobactin

E. coli AN311 strain was grown at 37 °C for 16 h in a supplemented minimal medium. The sterilized minimal medium (5.8 g NaCl, 3.7 g KCl, 1.1 g NH_4Cl, 0.15 g $CaCl_2 \cdot 2H_2O$, 0.1 g $MgCl_2 \cdot 6H_2O$, 0.14 g Na_2SO_4, 0.27 g KH_2SO_4, 12.1 g Trisma base in 1 l of distilled water, pH 7.4) was supplemented with 4 g glucose, 25 mg thiamine–HCl, 0.1 g casamino acids, and 0.4 g each of L-proline, L-leucine and L-tryptophan. The supplements were sterilized separately before being added to the mineral salt medium. Cells were harvested by centrifugation at 7000 r.p.m. and the supernatant was extracted three times with one-third volume ethylacetate. The ethylacetate extract was washed with water (pH 4) and dried with $MgSO_4$ (Pecoraro *et al.* 1983). The extract was concentrated to approximately 5 ml volume, and enterobactin was precipitated by slow addition of hexane and dried under vacuum.

The crude enterobactin sample was partially purified with Sephadex LH-20 column (1.6 × 0.50 cm) and further purified with a FPLC reverse-phase column (ODS C-18). The purity of enterobactin was examined by silica gel thin layer chromatography using a chloroform:methanol:water (35:12:1 by volume) solvent system. Spots were enhanced by a spray of $FeCl_3$ in pure ethanol.

Preparation of ferric–ligand complexes

Ferric complexes of enterobactin, MECAM and parabactin ligands were dissolved in pure methanol and then mixed with distilled water to 40% methanol. The concentration of ligands was determined spectrophotometrically prior to use: enterobactin ($\varepsilon_{319} = 11\,200$), MECAM ($\varepsilon_{321} = 10\,000$), parabactin ($\varepsilon_{309} = 10\,300$ or $\varepsilon_{250} = 27\,000$) (Peterson & Neilands 1979, Raymond *et al.* 1984). Equimolar amounts of ferric chloride or a slight excess were added and the solution pH carefully adjusted to 8 with concentrated aqueous KOH to minimize solution volume. The ferric complexes was fractionated by loading the mixtures on a Sephadex G-10 column (0.9 × 4.5 cm). The complexes were eluted with 0.1 M Tris–HCl buffer, pH 7.4. Labeled complexes were prepared similarly by adding equimolar amounts of [^{55}Fe]ferric chloride (39 m Ci/mg in 0.5 M HCl). Ferrichrome and ferrichrome A were isolated from *Ustilago sphaerogena* and purified as described by Emery (1971). The purity of the ferric

complexes was checked by silica gel thin layer chromatography using chloroform:methanol (6:5 by volume). The concentration of the ferric complexes including ferrichrome and ferrichrome A were determined again using previously determined extinction coefficients ferric enterobactin (ε_{495} = 5600), ferric MECAM (ε_{516} = 3900), ferric parabactin (ε_{516} = 3100), ferrichrome (ε_{425} = 2895) and ferrichrome A (ε_{440} = 3360) (Peterson & Neilands 1979, Raymond *et al.* 1984). The purified complexes were stored at −20 °C.

Assay of FepA binding activity

For the comparison of binding activities of $FepA_B$ and $FepA_T$, 100 μg (6 μM) of protein and 3 μM of [^{55}Fe]ferric enterobactin were introduced to a reaction buffer (0.1 M Tris–HCl buffer with a pH of 7.4 containing 0.1% Triton X-100) in a total volume of 200 μl. This was incubated at room temperature for 30 min. A Sephadex G-25 (fine) column in a 1 ml hypodermic syringe was used to isolate the receptor–ligand complex from the free ligand. Eight approximately 140 μl fractions were collected following application of the reaction mixture to the column and stepwise elution with 200 μl portions of reaction buffer. Control experiments were carried out by determining the elution profile of [^{55}Fe]ferric enterobactin in a reaction mixture without protein. Radioactivity was measured by counting 50 μl aliquots of eluent from each fraction in 4 ml of Eculome in a Beckmann liquid scintillation counter (Model LS 5801).

Competition studies

These were performed similarly with the same Sephadex G-25 column. First, 10 μg (0.6 μM) of protein with a fixed concentration of labeled ferric enterobactin (0.3 μM) and 60-fold excess of the unlabeled ferric complexes in a 200 μl reaction buffer were used. Twelve approximately 120 μl fractions were collected and 50 μl aliquots of eluent from each fraction was applied to the liquid scintillation counter for radioactivity determination.

N-terminal sequence determination of receptors

This was carried out by the method of Matsudaira (1987). After SDS slab-gel electrophoresis of 30 μg of purified protein, the gel was soaked in transfer buffer (25 mM Tris, 192 mM glycine) with 0.1% SDS for 15 min. During this time a PVDF membrane was rinsed with 100% methanol for 5 s and stored in transfer buffer. The gel, sandwiched between a sheet of PVDF membrane and several sheets of blotting paper, was assembled into a blotting apparatus (Bio-Rad) and electrotransferred overnight at 80 V (0.3 A). The PVDF membrane was washed in deionized water for 5 min, stained with 0.1% Coomassie Blue R-250 in 50% methanol for 5 min and then destained in 50% methanol, 10% acetic acid for 10 min at room temperature. The membrane was finally rinsed in distilled water for 10 min to remove glycine and Tris, and air dried. The protein bands were cut out with a clean razor and either loaded onto the sequenator or stored at −20 °C.

The membrane with the protein band was centered on the Teflon seal and placed in the cartridge of the sequenator. Proteins were sequenced on an Applied Biosystems Sequenator (Model 470) equipped with online phenylthiohydantoin (PTH) analysis using the regular program O3RPTH. The PTH–amino acid produced at each cycle was converted automatically and identified in a model 120-A amino acid PTH analyzer. Each HPLC chromatogram of amino acid PTH output of the instrument was analyzed according to the retention times of standard PTH–amino acid derivatives.

Removal of N-terminal pyroglutamate from FepA

This was performed by a modification of the method of Podell & Abraham (1978). First, 25 μg of pyroglutamate aminopeptidase (sequencing grade) was dissolved in 50 μl of milliQ water and then mixed with 50 μl of 50 mM Tris–HCl buffer, pH 7.5, with 0.1% Triton, which containing 40 μg of purified $FepA_T$. The reaction solution was incubated for 18 h at 4 °C followed by 4 h at 25 °C. After the enzymatic reaction, the reaction solution was checked on SDS–PAGE. For N-terminal determination of $FepA_T$, the protein in the gel was electrotransferred to a PVDF membrane by the method described above and the membrane with $FepA_T$ protein was loaded into the Sequenator for analysis.

Computer search

In order to identify the purified proteins, the determined partial N-terminal sequences of the proteins were loaded into a VAX terminal computer to search for identical proteins from the Gene Bank by using the Netblast program (Altschul & Lipman 1990).

Results

Purification of FepA and FecA

The manner in which detergents are used in the purification of membrane proteins plays an important role. The detergents are needed to increase the solubility of membrane protein. However, when a high concentration of detergent is used, the resolution is reduced because different types of proteins carried in the same detergent micelle prevent separation. If, however, a low concentration of detergent is used, the solubility of protein is decreased and may precipitate on the column. When the organism is grown on a citrate medium as described in Methods, simple ion exchange chromatography will yield an apparent pure protein. However, when the protein is examined in different concentrations by SDS–PAGE, a double band is observed which indicates that the protein is not homogenous

(Figure 2). Initially it was assumed that these proteins were FepA and a degradation product of FepA ($FepA_T$ and $FepA_B$, respectively). In this study, a concentration of 0.1% Triton X-100 was used during the whole purification procedure with FPLC. The double band can be separated with the use of a DEAE–Sepharose Fast Flow FPLC column. The results of SDS–PAGE show that the bottom band ($FepA_B$) starts in fraction 48 and stops in fraction 64. The top band ($FepA_T$) appears in fraction 53 and continues until fraction 91 (Figure 3). The double band mixture accounts for 70% of $FepA_B$ and $FepA_T$ (see the brackets in the column of 'Total protein' in Table 1). After further fractionation with PBE-94 and Q–Sepharose FPLC columns, $FepA_B$ and $FepA_T$ can be completely purified into single bands (Figure 4). In this procedure for purification of the outer membrane proteins, salting out is used as the first step for the separation from other outer membrane proteins (lane A and lane B in Figure 4). The purification of $FepA_B$ and $FepA_T$ is summarized in Table 1, and protein recovery is typically 2% for $FepA_B$ and 1% for $FepA_T$.

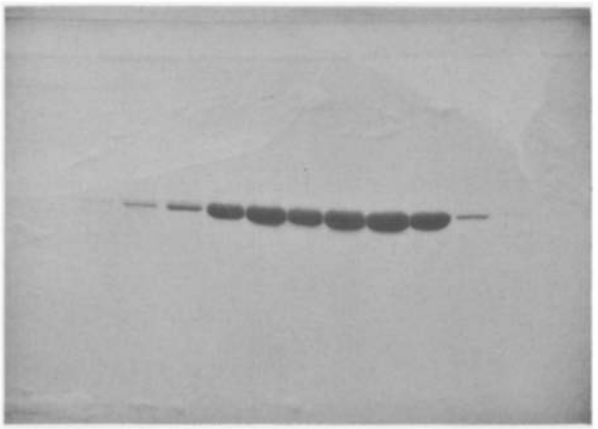


Figure 2. SDS–PAGE analysis of previously purified FepA. Full details are given in Materials and methods.

Purity and N-terminal sequences of FepA and FecA

The purities of the two proteins were judged on 7 and 10% SDS–PAGE using various concentration of the proteins. The purified proteins were electrotransferred onto a PVDF membrane for N-terminal determination. In this way, Triton X-100 used during purification can be readily removed from the proteins in order to avoid any interference of the detergent in the determination of the N-terminal sequence. $FepA_B$ (30 μg) produces a strong single sequence which indicates the purity of the protein. This protein has a N-terminal 19 amino acid sequence: AQVNIAPGSLDKALNQYAA–. This exactly matches with the N-terminus of mature FecA protein (a iron dicitrate transport protein) using a computer search of the Gene Bank with the method of Altschul & Lipman (1990). However, $FepA_T$ only shows very weak peaks for proline in position 3 and

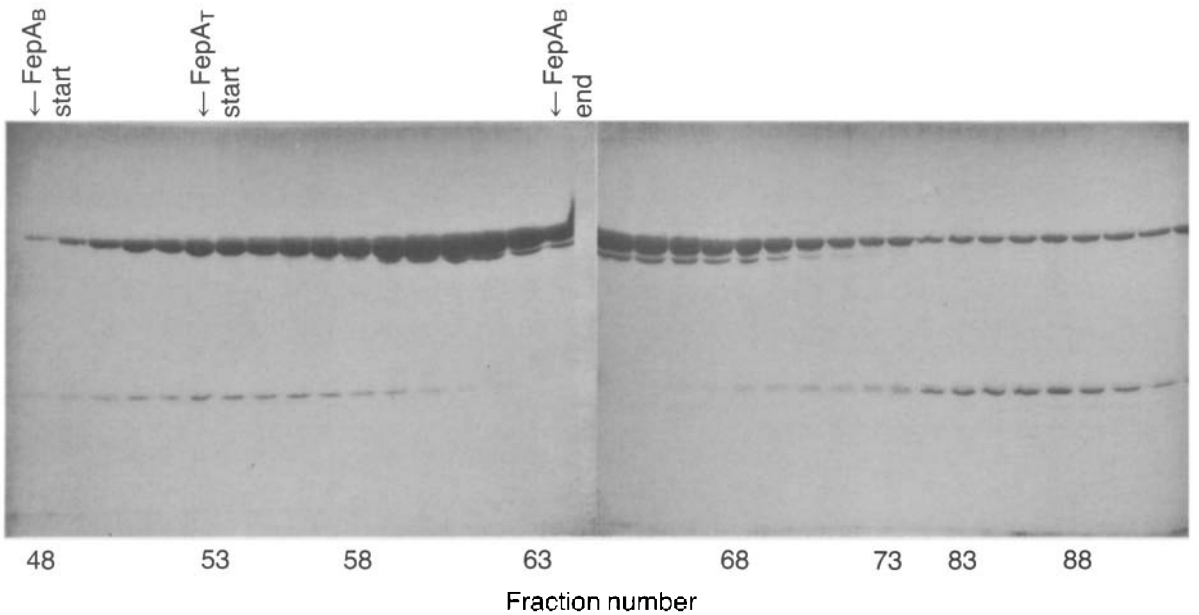


Figure 3. SDS–PAGE analysis of partially purified FepA after DEAE–Sepharose Fast Flow separation. The partially purified samples of FepA by DEAE–Sepharose Fast Flow were analyzed by SDS–PAGE. Lane numbers correspond to the fractions (4 ml each) eluted from the column.

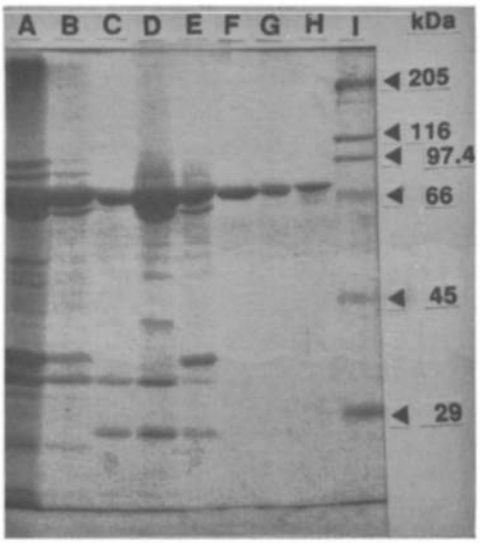


Figure 4. SDS–PAGE of purified FepA and FecA. Samples from each step of the purification as described in Results were analyzed by SDS–PAGE as described in Materials and methods. Lane A, crude FepA material after extraction; lane B, crude FepA after salting out. Lanes C, D and E represent partially purified FecA, a mixture of FepA and FecA, and partially purified FepA, respectively, after DEAE–Sepharose Fast Flow column. Lane F, purified FecA after chromatography on PBE-94 as anion exchange column; Lane H, purified FepA after Q–Sepharose Fast Flow column. Lane G represents the double bands when purified FecA was mixed with the same amount of FepA. Lane I represents molecular mass markers.

7, suggesting that the protein is blocked at the N-terminus. After removal of pyroglutamate from the N-terminus by pyroglutamate amino peptidase, a weak sequence is found: (Q)XPTDNPPS–. This closely matches the N-terminal sequence: QEPTDTPVS– for the FepA receptor predicted from the analysis of the *fepA* gene in *E. coli* (Lundrigan and Kadner 1986). It indicates that $FepA_T$ is indeed the FepA receptor protein even though the residues in position 6 and 8 are not identical with the ones in the FepA N-terminus predicted by Lundrigan & Kadner (1986). The reason for the incomplete match is most likely that Triton X-100 decreases the activity of pyroglumate aminopeptidase, thus yielding only a weak sequence.

Receptor binding activity

It was found that both FepA and FecA bind ferric enterobactin. In a typical binding experiment using 3 μM of [^{55}Fe]ferric enterobactin, approximately 50% of the total radioactivity is bound to FepA while only 32% of the total radioactivity is found to bind to FecA. It indicates that FepA has a 1.5-fold higher binding activity for ferric enterobactin than FecA (Figure 5). Ferric enterobactin binding to FepA and FecA reaches equilibrium at room temperature within 15 min of incubation (including time required for filtration of the assay mixture). The binding assay is reproducible and, for a given

Table 1. Purification of FepA and FacA receptors from *E. coli* UT5600/pBB2

Step	Volume (ml)	Protein content (mg ml^{-1})	Total protein (mg)	Recovery (%)
Extraction	75	3400	255	100
Salting out	200	720	144	56
DEAE–Sepharose Fast Flow				
FecA	56	240	13 (16)	5
mixture	64	950	61 (71)	24
FepA	36	319	12 (13)	4
PBE-94 for FecA	28	242	7	3
Q–sepharose Fast Flow for FepA	20	190	4	2
PBE-94				
FecA	20	250	5	2
FepA	20	150	3	1

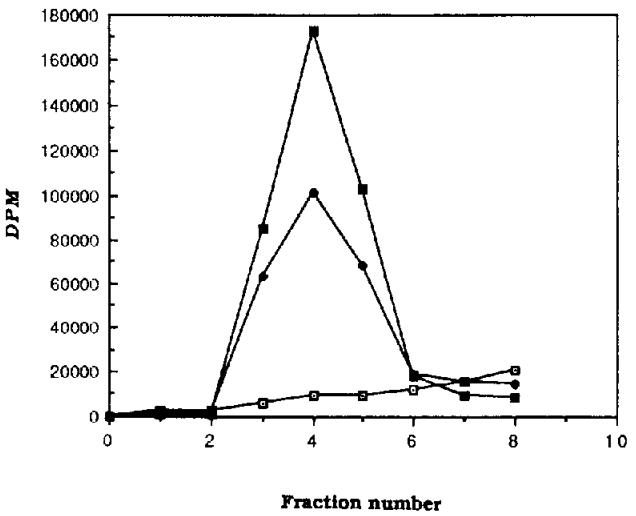


Figure 5. Determination of binding activity of FepA and FecA using [^{55}Fe]ferric enterobactin. Binding tests were performed using 1 ml of a Sephadex G-25 column at room temperature with a fraction size of 115 μl; 20 μl of which was counted in a Beckmann liquid scintillation counter. □, [^{55}Fe]ferric enterobactin alone; ■, $FepA_T$ plus [^{55}Fe]ferric enterobactin; ●, $FepA_B$ plus [^{55}Fe]ferric enterobactin.

preparation of protein and ligand, the variation is less than 5% in assays performed in triplicate. The Triton X-100 solubilized proteins are quite stable in storage at 4 °C, losing about 10% of their activity after 30 days of storage.

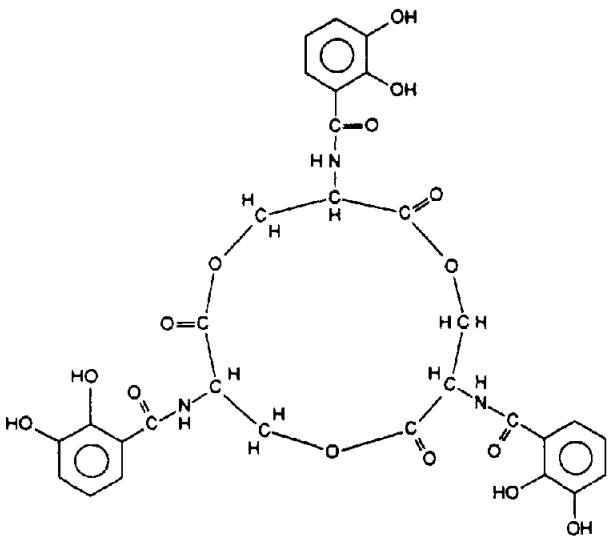
Specificity of ligand binding

All binding studies were carried out with 0.3 μM solution of [^{55}Fe]ferric enterobactin (Figures 6 & 7). Panel A shows the elution profile from the Sephadex G25 column for [^{55}Fe]ferric enterobactin itself while panel B shows the profile when 0.6 μM protein, respectively FepA and FecA, are added. The results show that both proteins bind ferric enterobactin.

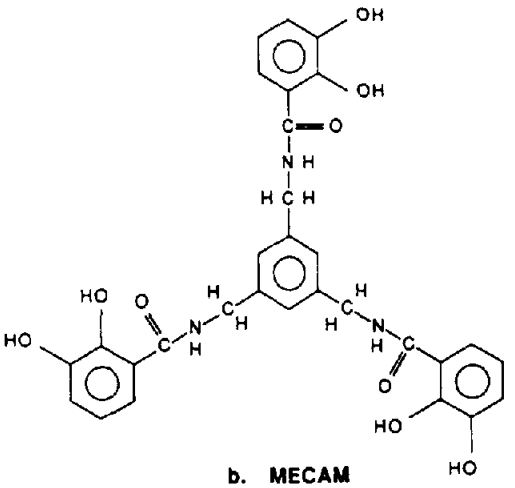
In order to show the specificity of binding, inhibition studies were carried out. In these studies the solutions were 0.3 μM in [^{55}Fe]ferric enterobactin and 0.6 μM in protein (FepA or FecA) while the inhibitor was 20 μM. Panel C shows the effect when 20 μM unlabeled ferric enterobactin is added. The conclusion is that ferric enterobactin inhibits itself and that the binding of both FepA and FecA with the siderophore is specific. In order to delineate this specificity further, inhibition studies were carried out with siderophores which have some similarity to ferric enterobactin. MECAM is a synthetic analog of enterobactin in which the triester ring is replaced by a benzene ring (Figure 6) (Harris *et al.* 1979). Ferric MECAM delivers iron to the cell in *in vivo* studies (Ecker *et al.* 1986). The present *in vitro* studies (panel D) show that ferric MECAM inhibits ferric enterobactin binding to both proteins but not to the extent of ferric enterobactin itself. The stereochemistry for the iron surrounding in ferric enterobactin is Δ (Harris *et al.* 1979) while in ferric MECAM it is a Λ/Δ (50/50) mixture. It is, therefore, possible that only the Δ form of ferric MECAM competes with ferric enterobactin. This possibility is reinforced by the results on the competition studies with ferric parabactin (Figure 6), another catecholate siderophore but with Λ stereochemistry (Harris *et al.* 1979). The results show only a small amount of inhibition of ferric enterobactin binding caused by ferric parabactin. Another property of ferric enterobactin is its triply negative charge and it is, therefore, of interest to study the competition of binding with another triply negatively charged siderophore, ferrichrome A (Figure 6). Ferrichrome A (Panel F) shows no inhibition on the ferric enterobactin binding. Ferrichrome is a siderophore which is not produced by *E. coli* but still taken up by this organism (Hoffman *et al.* 1986). It uses a different uptake system (FhuA) (Hoffman *et al.* 1986) from ferric enterobactin. The result with ferrichrome (Panel G) is not clear and may indicate a small amount of exchange of label between the two siderophores.

Discussion

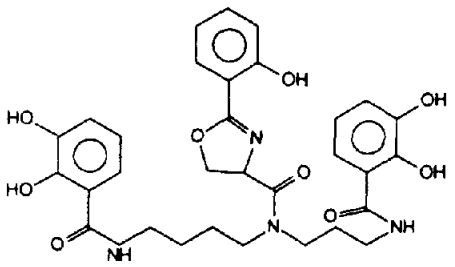
The purity of purified iron-related receptors from *E. coli* membrane is significant because the envelope of *E. coli* contains at least six outer membrane receptors related to the transport of iron. They include FepA, FecA, FhuA, FhuE, Cir protein and the 83 K protein (Pressler *et al.* 1986). All of the proteins are in the 80 kDa range of molecular weight with similar isoelectric points. It is extremely difficult to identify the specific receptor mobilities on SDS–PAGE. The measurement of the partial N-terminal sequence of the receptor, therefore, is the best assay method for the purity of the protein because the DNA sequences of the receptors are available in the literature. By purification with FPLC described in this study, FepA and FecA can be separated with a high purity and examined by N-terminal sequence determination. The purity of FecA is particularly good. The 19 amino acid sequence in the N-terminus is clearly observed and possible contamination of FepA is excluded because no proline peak is observed at position 3. This is clear evidence that there is no FepA contamination in purified FecA. For the FepA receptor, the N-terminus amino acid is determined to be glutamine, which supports the



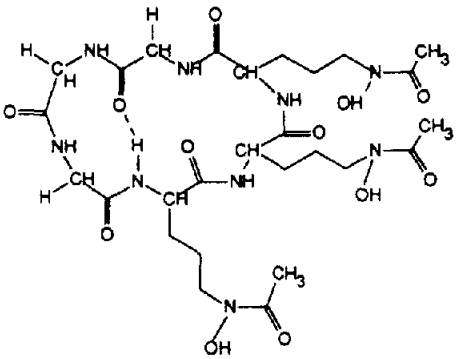
a. Enterobactin



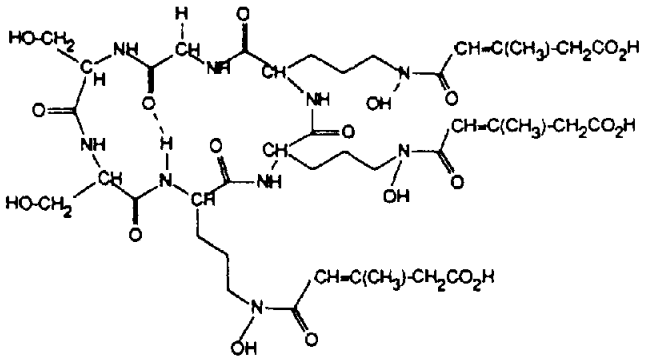
b. MECAM



c. L-parabactin



d. Ferrichrome



e. Ferrichrome A

Figure 6. Structures of catecholate ligands used in this study.

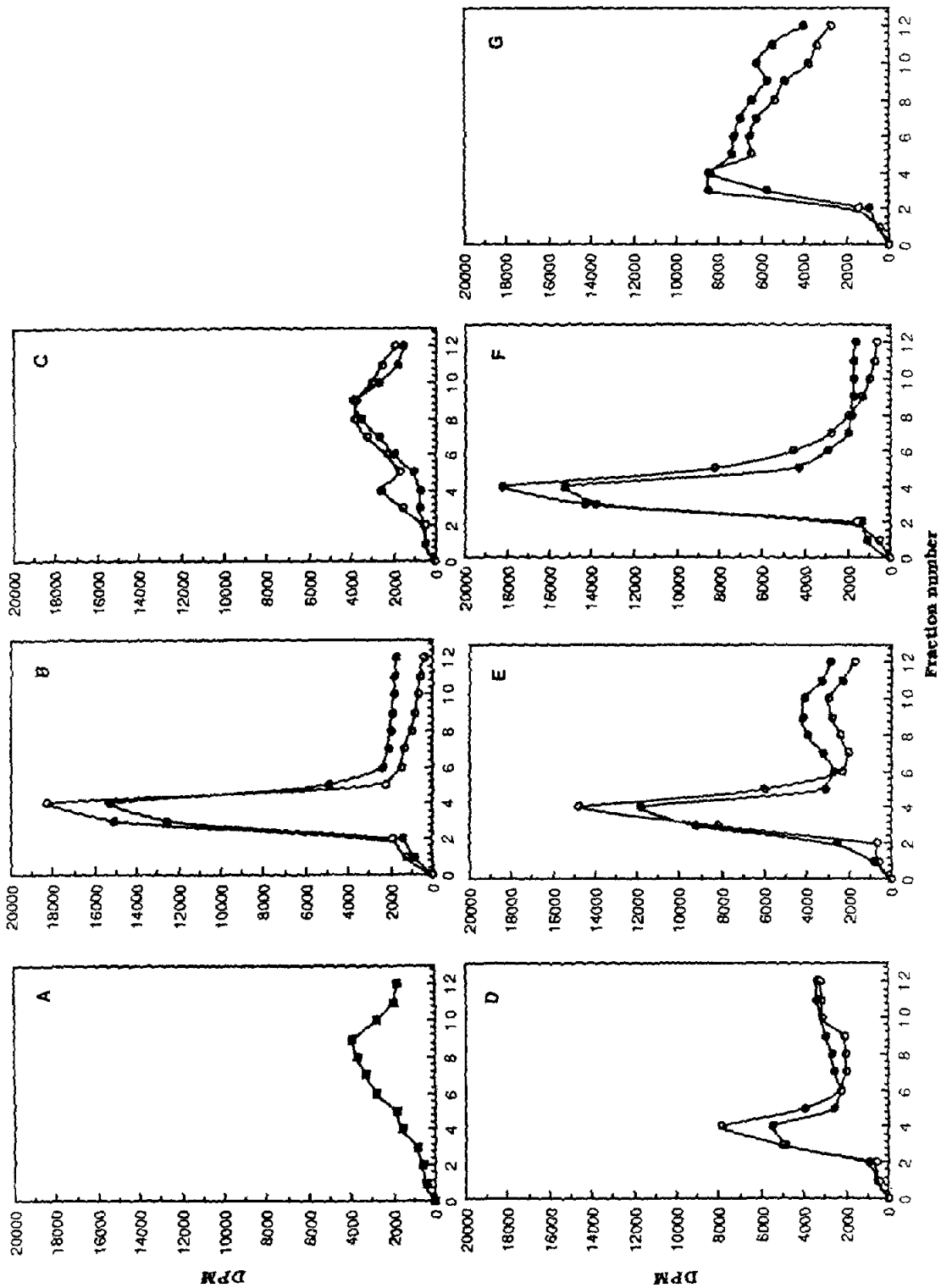


Figure 7. Binding of [^{55}Fe]ferric enterobactin to FepA and FecA in the presence of excess ferric–ligand complexes. Elution profile of [^{55}Fe]ferric enterobactin (0.3 μM) in a 0.1 M Tris–HCl reaction buffer containing 0.1% Triton X-100 (panel A); 10 μg of FepA (●) or FecA (●) was added to each of the other reaction mixtures. Except in panel (B), excess of the following non-labelled complexes was included in the reaction mixture: panel (C), ferric enterobactin; panel (D), ferric MECAM; panel (E), ferric parabactin; panel (F), ferrichrome A and panel (G), ferrichrome.

results of Lundrigan & Kadner (1986), rather than phenylalanine as determined by Fiss *et al.* (1982).

In our earlier report (Jalal & van der Helm 1989) FepA receptor was purified according to the mobility of SDS–PAGE. The protein obtained at that time most likely was FepA because citrate was not used in growing up this plasmid strain and the FecA protein was not apparent on SDS–PAGE. Different concentrations of the purified FepA were examined by SDS–PAGE, each time only showing a single band. However, difficulties were encountered in growing the organism in this fashion and a decision was made to switch from a TY medium to a minimal citrate medium which consistently yielded good production of FepA. Simultaneously, however, FecA was produced as well, as is shown in Figure 2.

The present study shows that UT5600/pBB2 produces a large amount of plasmid encoded FepA; however, in addition, an approximately equal amount of FecA is produced. Presumably this latter protein is produced by the chromosome and induced by citrate (Hancock *et al.* 1976, Pressler *et al.* 1986) added to the medium. The similarity in the physical characteristics of FepA and FecA complicates their purification and reduces the final yields of purified proteins. FepA can be produced, with only small amounts of FecA, using UT5600/pBB2 by eliminating citrate from the medium (results not shown).

In this study, $FepA_T$ is identified as the real FepA receptor protein, not only from the results of the N-terminal sequence determination, but also from the binding experiments of this protein with ferric enterobactin. When [^{55}Fe]ferric enterobactin and FepA are incubated with 67-fold of non-labeled ferric enterobactin, the radioactive profile is found to be the same as the one of [^{55}Fe]ferric enterobactin alone. This shows that the binding of ferric enterobactin with the FepA receptor is specific and saturable. Ferric MECAM competes with this binding and, to a smaller extent, ferric parabactin, while ferrichrome A does not compete. The *in vitro* binding of FepA with ferric enterobactin is quite specific. It also clearly indicates that the structural integrity of the protein is maintained after purification and that, therefore, a structural study of the isolated protein is meaningful. Our *in vitro* binding results are in agreement with several earlier reports (Hancock *et al.* 1976; Pugsley & Reeves 1977, Hollifield & Neilands 1978, Fiss *et al.* 1982) indicating that ferric enterobactin uptake is mediated by the FepA receptor protein.

FecA has been demonstrated to be the iron citrate receptor protein in the outer membrane of *E. coli* (Hancock *et al.* 1976, Pugsley & Reeves 1977, Pressler *et al.* 1986). The surprising result we report here is that FecA shows similar binding behavior for ferric enterobactin as FepA does. Previous results reveal a possible linkage between FepA and FecA. Raymond's group (Ecker *et al.* 1986) found that when RW193, an enterobactin synthesis deficient mutant (*pro*$^-$, *leu*$^-$, *trp*$^-$, *thi*$^-$, *purE*$^-$, *ent*A), was grown in an iron-deficient medium supplemented with 5 mM ferric enterobactin, no growth was observed when citrate was left out of the medium. This observation implies that the outer membrane receptor, FecA, which is induced by citrate, assists in the uptake of ferric enterobactin by FepA using some unknown mechanism. Nothing is known about the location of FepA and FecA on the outer membrane surface *E. coli*. A coordinated mechanism is possible when the two receptors are histologically located close to each other. Earhart's group (McIntosh *et al.* 1978) found that iron uptake in RW193 and UT2300, a spontaneous *Cbr*$^-$ (*fepA*$^-$) derivative of RW193, was completely dependent on added ferric enterobactin while uptake occurs in UT2300, but was reduced compared with RW193. This indicates that another receptor exists in *E. coli* for uptake of ferric enterobactin, albeit weaker than FepA. In 1979, the same group found another *fepA*$^-$ mutant, UT500, derived from RW193 (McIntosh *et al.* 1979). The protein SDS–PAGE pattern showed that this mutant did not have any 81 kDa protein, but instead a protein band with a mobility the same as FecA was observed. Experiments demonstrated that this mutant, UT500, was still capable of transporting ferric enterobactin but the activity of uptake was reduced compared to RW193. They concluded, therefore, that this strain possessed an outer membrane protein which was defective in colicin B absorption but still functioned in ferric enterobactin transport.

Our *in vitro* results and the results from Raymond's group, however, are not in agreement with the report by the Neilands group (Hollifield & Neilands 1978). They found that, without citrate, RW193 was readily grown up in Tris medium containing different concentrations of ferric enterobactin. No growth was found when the mutant strain AN270 (*fepA*$^-$) was incubated under the same conditions, but experiments using addition of citrate to induce FecA were not carried out. It is clear that the binding and uptake of ferric enterobactin by FecA requires further investigation and characterization. Our *in vitro* binding test may differ from the *in vivo* transport, because in an *in vitro* study, the membrane dynamics and protein–lipid interactions are completely destroyed due to the purification procedure. It is significant that the binding in the

present *in vitro* studies is still specific. However, *in vivo* experiments study transport rather than binding and it is, therefore, important to combine *in vitro* studies with *in vivo* transport experiments or experiments with reconstituted membranes.

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