JOURNAL OF BACTERIOLOGY, Jan. 1981, p. 156-163
0021-9193/81/010156-08$02.00/0

Vol. 145, No. 1

Ferric Citrate Transport in *Escherichia coli* Requires Outer Membrane Receptor Protein FecA

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Mutants of *Escherichia coli* K-12 AB2847 and of *E. coli* K-12 AN92 were isolated which were unable to grow on ferric citrate as the sole iron source. Of 22 mutants, 6 lacked an outer membrane protein, designated FecA protein, which was expressed by growing cells in the presence of 1 mM citrate. Outer membranes showed an enhanced binding of radioactive iron, supplied as a citrate complex, depending on the amount of FecA protein. The FecA protein was the most resistant of the proteins involved in ferric irion iron translocation across the outer membrane (FhuA = TonA, FepA, Cir, or 83k proteins) to the action of pronase P. It is also shown that previously isolated *fec* mutants (G. C. Woodrow et al., J. Bacteriol. **133**:1524-1526, 1978) which are cotransducible with *argF* all lack the FecA protein. They were termed *fecA* to distinguish them from the other ferric citrate transport mutants, now designated *fecB*, which mapped in the same gene region at 7 min but were not cotransducible with *argF*. *E. coli* W83-24 and *Salmonella typhimurium*, which are devoid of a citrate-dependent iron transport system, lacked the FecA protein. It is proposed that the FecA protein participates in the transport of ferric citrate.

Three high-affinity iron transport systems have been characterized in *Escherichia coli* K-12. The substrates of these systems include the ferric iron complexes of enterochelin, ferrichrome, and citrate (see references 2, 4, 12, 15, 17, and 22 for reviews). For the uptake of ferric enterochelin and ferrichrome, receptor proteins in the outer membrane are required. A citrate-dependent iron transport system was induced when cells were grown in media containing 1 mM citrate (7). It was also shown that under these conditions an additional protein appeared in the outer membrane (9). It seems obvious to assume that this protein, formerly designated Cit and in this paper called FecA protein, serves as receptor for citrate-dependent iron transport as has been demonstrated for the FepA and FhuA (formerly *tonA* [13]) proteins in the transport of ferric enterochelin and ferrichrome, respectively. For this reason, we isolated mutants which were unable to grow on ferric citrate as the sole iron source. Such mutants were mapped earlier close to 7 min on the linkage map of *E. coli* (25). Previously, these mutants were not characterized further. We show here that 25% of the mutants lacked the FecA protein and that binding of ferric citrate to outer membranes was enhanced by the FecA protein.

MATERIALS AND METHODS

Bacterial strains and media. The *E. coli* K-12 strains used are listed in Table 1. Mutants of strain AB2847, of its *fepA* derivative BR10, and of strain AN92 (7) were isolated. The method used for the isolation of mutants followed basically a previously described procedure (25) but was substantially improved in the following way. After mutagenesis with *N*-methyl-*N'*-nitro-*N*-nitrosoguanidine, 10^3 cells were spread on agar plates which contained M9 medium (3), 1 mM citrate, 0.1 mM bipyridyl, 0.4% glucose, the required amino acids (20 mg/liter), and vitamins (4 μM). The colonies which had grown after 4 days of incubation at 37°C were marked, and then 0.2 ml of a 2 mM solution of 2,3-dihydroxybenzoate (DHB) was distributed between the colonies. After another 4 days of incubation, the additional colonies were picked and tested on agar plates with M9 medium containing 100 μM bipyridyl and a supplement of either 1 mM citrate or 20 μM DHB. Cells which grew on DHB but not on citrate plates were selected and studied further. The use of bipyridyl restricted the iron supply to the iron chelators citrate or DHB. DHB supports growth under iron-limiting conditions because it is the required precursor for enterochelin synthesis in the *aroB* strains used. DHB donates iron also to *fepA* strains which are unable to take up ferric enterochelin across the outer membrane (8, 10). For the isolation of citrate-dependent iron-uptake mutants of *fepA* strains, the concentration of bipyridyl was reduced to 50 μM, and the plates were inspected after 2 days of growth.

Growth of the mutants was tested on M9 medium-bipyridyl agar plates with filter paper disks impregnated with 10 μl of 100 mM citrate, 2 mM DHB, or 1 mM ferrichrome. The diameter of the growth zone around the disks (minus 6 mm of the disk diameter) was measured. Growth in liquid culture was determined in M9 medium from which iron had been ex-

TABLE 1. *Strains used*

Strain	Genotype[a]	Source
E. coli K-12		
AB2847	F⁻ *aroB tsx malT thi*	9
WA176	As AB2847, but *fecA11*	This study
WA380	As AB2847, but *fecA12*	This study
WA2	As AB2847, but *fecB30*	This study
WA14	As AB2847, but *fecB31*	This study
WA20	As AB2847, but *fecB32*	This study
WA26	As AB2847, but *fecB33*	This study
WA27	As AB2847, but *fecB34*	This study
WA28	As AB2847, but *fecB35*	This study
WA37	As AB2847, but *fecA13*	This study
WA38	As AB2847, but *fecB36*	This study
WA63	As AB2847, but *fecB37*	This study
WA79	As AB2847, but *fecB38*	This study
WA83	As AB2847, but *fecB39*	This study
WA107	As AB2847, but *fecB40*	This study
WA140	As AB2847, but *fecB41*	This study
WA760	As AB2847, but *fecB42*	This study
WA810	As AB2847, but *fecB43*	This study
BR10	As AB2847, but *fepA*	9
WA1024	As BR10, but *fecA14*	This study
WA1031	As BR10, but *fecA15*	This study
WA1069	As BR10, but *fecB45*	This study
AN92	F⁻ *aroB proA argE pheA tyrA try rpsL*	I. G. Young
AN298	As AN92, but *fec401*	I. G. Young
AN299	As AN92, but *fec402*	I. G. Young
AN300	As AN92, but *fec403*	I. G. Young
AN302	As AN92, but *fec405*	I. G. Young
AN303	As AN92, but *fec406*	I. G. Young
AN306	As AN92, but *fec409*	I. G. Young
AN299-21	As AN299, but *fepA tonA*	I. G. Young
E. coli W		
WA83-24	*aroB*, derivative of *E. coli* W (ATCC 9637)	11
W83-24/971	As 83-24, but *fhuA fepA*	This study
S. typhimurium		
SL1027	*try met str*	B. A. D. Stocker
LT2	*aroB* derivative	This laboratory

[a] The gene symbols follow those of Bachmann and Low (1).

tracted by passing the salt solution through a column of Chelex-100 (20) and which was supplemented with bipyridyl to 100 μM and citrate to 1 mM or with DHB to 20 μM.

FepA mutants were isolated with colicin B; *fhuA* (*tonA*) mutants were isolated with phage T5.

Determination of ferric citrate binding to membranes. A membrane preparation enriched in the outer membrane was obtained as follows. Cells were grown in 400 ml of iron-extracted M9 minimal medium to 5×10^8 cells per ml. They were centrifuged and washed once with 100 ml of 10 mM HEPES (*N*-2-hydroxyethylpiperazone-*N'*-2-ethanesulfonic acid) buffer at pH 7.4. The pellet was suspended in 18 ml of HEPES buffer containing 0.75 M sucrose, 0.75 mM EDTA, and 10 μg of lysozyme per ml. The cells were passed once through a French pressure cell (Aminco; Travenol Laboratories) at 1,260 lb/in² pressure, and the homogenate was centrifuged at 5,000 × *g* for 15 min at 4°C. The supernatant was centrifuged for 90 min at 170,000 × *g* at 4°C, and the membrane pellet was suspended in 30 ml of doubly distilled water. The suspension was centrifuged again, and the membranes were taken up in 200 μl of water (5 to 10 mg of protein/ml). The membrane suspension was diluted to a protein concentration of 2 mg/ml either with 20 mM Na-K (3:1)-phosphate buffer at pH 6.9, 20 mM Tris-hydrochloride, pH 6.9, or 20 mM HEPES, pH 6.9. The 3:1 ratio of sodium to potassium ions seems to be important for preventing precipitation of ferric iron phosphate complexes (H. Rosenberg, Australian National University, Canberra, personal communication). Since the phosphate buffer gave the lowest unspecific binding of ferric citrate to membranes without the FecA protein, it was used for all of the data presented in this paper. A solution containing $^{55}FeCl_3$ (2 μM), citrate (2 mM), or nitrilotriacetic acid (0.8 mM, controls without citrate) was mixed and incubated for 30 min before being mixed with an equal volume (0.25 ml) of the membrane suspension (or as a control with the phosphate buffer alone). After 15 min of incubation at 0°C, 200-μl samples were filtered through cellulose nitrate membrane filters (BA83, pore size, 0.2 μm; Schleicher & Schüll) which had been pretreated with a solution of 40 μM NH_4^+-Fe^{3+}-EDTA in 0.9% NaCl and then washed three times with doubly distilled water. An iron stock solution was prepared by mixing 200 mM (final concentration) $FeNH_4SO_4$ with 200 mM (final concentration) EDTA. The pH was adjusted to 7 by adding 1 N NaOH. The solution was filtered before use (P. Wookey, personal communication). The filters were washed once with 1 ml of the phosphate buffer and then dried at 60°C, and then radioactivity was measured by using 5 ml of Rotiszint 11 (C. Roth, Karlsruhe) and a liquid scintillation counter. The measured values of the binding of the ^{55}Fe-citrate complex to membranes of citrate-induced cells (for example, 3,935 cpm) and uninduced cells (2,215 cpm) were corrected for the binding of the ^{55}Fe-nitrilotriacetate and ^{55}Fe-citrate complexes (200 to 300 cpm) to the filters.

Miscellaneous methods. The protein concentration was determined with Fluram (Hoffmann-La Roche A.G.) by using serum albumin as the standard.

For the isolation of purified outer and cytoplasmic membranes, the method of Osborn et al. (19) was employed. The protein composition of membranes was analyzed by sodium dodecyl sulfate-gel electrophoresis basically as described by Lugtenberg et al. (16). The following alterations were introduced to improve the resolution of the proteins in the molecular-weight range of 80,000 daltons; the concentration of bisacrylamide in the stock solution for the running gel was lowered from 0.8 to 0.3%, and the concentration of acrylamide in the stock solution for the stacking gel was raised from 30 to 50%. Straight protein bands were obtained when the initial current was adjusted to 12 mA until the tracking dye had entered the running gel. Then the current was increased to 15 mA.

For the proteolytic degradation of outer membrane proteins, 10^{11} cells, freshly grown in 200 ml of M9 minimal medium, were suspended in 5 ml 10 mM HEPES buffer at pH 7.4 and treated with 25 mg of pronase P (Serva, Heidelberg) for 30 min at 37°C on a gyratory water bath shaker at 400 rpm. The cell suspension was then diluted 10 times with 0.9% NaCl

and centrifuged at 5,000 × *g* for 10 min. The sedimented cells were washed twice with 50 ml of 0.9% NaCl and then suspended in 9 ml of 10 mM HEPES buffer, pH 7.4, containing 0.75 M sucrose. Membranes enriched in the outer membrane were prepared as described above and then analyzed by gel electrophoresis and the ferric citrate binding assay.

RESULTS

Isolation of mutants. Mutants which were unable to grow with ferric citrate as the sole iron source were isolated from *E. coli* strains AB2847, BR10 (*fepA*), and AN92, all of which were *aroB* mutants that could not synthesize enterochelin unless they were provided with DHB. The selection plates contained 50 or 100 μM 2,3-bipyridyl, which completely suppressed the low-affinity iron uptake, so that iron uptake occurred only via the citrate-dependent transport system.

Growth of the mutants was tested on plates and in liquid culture with iron-extracted minimal medium. Only 50 μM bipyridyl was added to the *fepA* strain BR10 and its derivatives and to AN299-21 (Table 1) since they grew very poorly on DHB due to the lack of the outer membrane receptor protein for the uptake of ferric enterochelin. The growth zones around filter paper disks with citrate had diameters of 15 to 20 mm on plates seeded with the parent strains and 0 mm on plates seeded with the mutants, except for strain WA1031 (6 mm). Growth in liquid culture with ferric citrate reached an optical density of 2.5 after 24 h for the parent strains and 0.15 to 0.34 for the mutants. For comparison, growth with DHB and ferrichrome as iron sources was determined. The mutants showed essentially the same growth zones as the parent strains. The isolated mutants and their genealogies are listed in Table 1.

Changes in membrane proteins of ferric citrate transport mutants. Of the 22 transport mutants, 6 lacked the citrate-inducible outer membrane protein. In Fig. 1 and 2, representative examples of outer membrane proteins after electrophoretic separation are shown. The section which contains the proteins required for the uptake of ferric iron complexes is shown in the figures. The FepA protein is involved in ferric enterochelin uptake. The 83,000 apparent-molecular-weight (83k) and Cir proteins (colicin I receptor) appear under iron-limited growth conditions (3). This is demonstrated in Fig. 1, A to D, and in Fig. 2, A and B, where cells have been grown in iron-extracted minimal medium. These proteins were only barely observable when cells had been grow in nutrient broth (Fig. 1, E to K; Fig. 2, C and D). In both media, an additional protein, designated here as FecA (formerly Cit [9]) was expressed in response to the presence of 1 mM citrate during growth (Fig. 1, Ab, Bb, Cb, Ea, Ha, and Ia; Fig. 2, Aa and Da). This protein was absent when citrate was omitted. Some of the mutants, designated *fecA*, did not

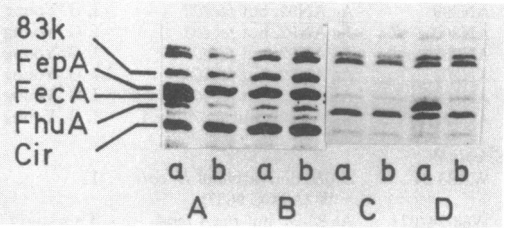

FIG. 2. *Sections of two electropherograms as in Fig. 1. The four probes to the left originated from cells grown in M9 minimal medium containing 0.1 μM $FeCl_3$. The four probes to the right were derived from cells grown in nutrient broth. (a) Cells grown with 1 mM citrate and 20 μM DHB. (b) Cells grown with 20 μM DHB. The mutants were derivatives of strain AB2847. (A) Strain WA79 fecB; (B) strain WA380 fecA; (C) strain WA37 fecA; (D) strain WA83 fecB.*

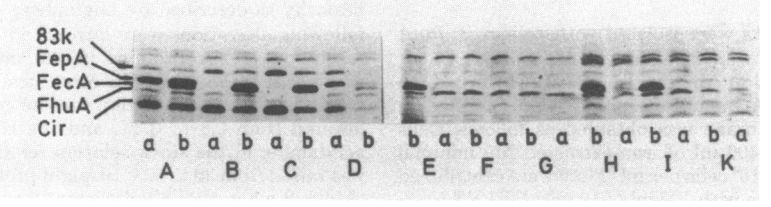

FIG. 1. *Sections of two electropherograms showing outer membrane proteins in the molecular-weight range of 80,000 obtained after polyacrylamide gel electrophoresis in the presence of 0.1% sodium dodecyl sulfate. The eight probes to the left originated from cells grown in M9 minimal medium containing 1 μM $FeCl_3$. The 12 probes to the right originated from cells grown in nutrient broth. (a) Cells grown in the presence of 20 μM DHB. (b) Cells grown with 1 mM citrate and 20 μM DHB. (A) Strain AN92, wild type; (B) strain AN299-23 fecB fepA; (c) strain AN299-21 fecB fepA fhuA; (D) strain WA9219, which in the presence of citrate did not form any of the iron transport-related proteins; (E) strain AN298 fecB; (F) strain AN300 fecA; (G) strain AN302 fecA; (H) strain AN299 fecB; (I) strain AN303 fecB; (K) strain AN306 fecA.*

contain the protein under any condition (Fig. 1, D, F, G, and K; Fig. 2, B and C). Other ferric citrate transport mutants contained the FecA protein in the outer membrane (Fig. 1, B, C, E, H, and I; Fig, 2, A and D). It is assumed that they were not altered in the FecA protein (see below), and, therefore, they were called *fecB* mutants.

It is known that *E. coli* W contains no ferric citrate transport system (22). We isolated *aroB fhuA fepA* mutants from *E. coli* W83-24 to eliminate interference with the ferric enterochelin transport system and to remove the FepA and FhuA proteins close to the electrophoretic position of the FecA protein. The cells grew well in iron-extracted minimal medium in the presence of 20 μM DHB and 1 mM citrate. Citrate alone inhibited growth. No FecA protein could be detected.

When *E. coli* BR10 was grown anaerobically in M9 medium with 1% KNO_3 and 1 mM citrate, no citrate-dependent iron transport ($^{55}Fe^{3+}$) could be measured aerobically and none, or only a barely detectable amount, of the FecA protein was observed on polyacrylamide gels (data not shown).

Salmonella typhimurium is devoid of a ferric citrate transport system but grows on citrate as sole carbon source (14). Whether this organism was grown in 0.4% glucose with 1 mM citrate or on 20 mM citrate alone with 0.1 μM or 1 μM $FeCl_3$, no additional protein was detected in the outer membrane in response to citrate. The three proteins OM1 to OM3 (6) and the Sid protein (equivalent to TonA and FhuA) described before (5) were present (data not shown).

The protein composition of the cytoplasmic membrane was analyzed to identify citrate-inducible proteins and to characterize the *fecB* mutants. Half of the mutants lacked a protein with an apparent molecular weight of 50,000 (Table 2). However, the presence of this protein was independent of the addition of citrate to the growth medium.

Binding of ferric citrate to membranes. To test the function of the FecA protein in ferric

TABLE 2. *Properties of E. coli mutants unable to grow with ferric citrate*

Strain	Presence of outer membrane FecA protein	Binding of ferric citrate (pmol/mg of protein)	Presence of cytoplasmic membrane 50K protein	Additional protein alterations
AB2847 (wild type)	+	28, 35, 35, 38, 39, 54	+	
WA37	−	−8, −15	−	
WA176	−	−15	−	
WA1024	−	−5	−	OmpF reduced
WA380	−	−11	+	
WA1031	−	4	+	
WA14	+	19	−	
WA20	+	ND	−	
WA38	+	61	−	
WA79	+	46	−	
WA107	+	ND	−	
WA760	+	38	−	
WA2	(+)	ND	−	OmpF reduced
WA140	(+)	ND	−	
WA26	+	16	+	OmpF reduced
WA27	+	ND	+	
WA28	+	39	+	
WA63	+	ND	+	
WA83	+	ND	+	
WA1069	+	ND	+	
WA810	(+)	ND	+	

[a] +, Protein present; (+), weaker presence than in the parent strain; −, undetectable; ND, not determined. The measured counts per minute of $^{55}Fe(III)$ bound to membranes in the presence of 1 mM citrate and 0.4 mM nitrilotriacetate were corrected for the binding of $^{55}Fe(III)$ to filters under the same conditions but without membranes. The same procedure was used to measure iron binding to membranes and filters in the absence of citrate in the assay mixture. The value obtained was subtracted from the corrected value above. The data listed represent the difference between binding to outer membranes of citrate-induced and uninduced cells. Therefore, each value given is the result of eight determinations. The range of values obtained for citrate-induced parent cells was 40 to 89 pmol per mg of protein; that of uninduced cells was 0.8 to 26 pmol per mg of protein.

citrate uptake, binding of $^{55}Fe(III)$ as a citrate complex to the outer membrane dependent on the presence of the FecA protein was determined. A rather high level of nonspecific binding in the absence of the FecA protein was observed. Several buffers (HEPES, Tris-hydrochloride, and phosphate) and various procedures of membrane preparations (opening of the cells with the French press or by treatment with ultrasound and separation of membranes by differential or isopycnic sucrose density-gradient centrifugation) were tried to find the optimal conditions for specific binding. Only weak, if any, binding to the cytoplasmic membrane was observed. Contaminating cytoplasmic membrane could therefore be neglected in the binding assays to the outer membrane. Binding of ferric citrate to the filter could largely be avoided by pretreating the filters with 40 μM NH^{4+}-Fe^{3+}-EDTA in a 0.9% NaCl solution and subsequently washing them with 20 mM Na-K (3:1)-phosphate buffer, pH 6.9. The ferric iron solutions were prepared immediately before the binding experiments to prevent formation of insoluble iron aggregates. For the same reason, the radioactive ferric chloride solution was first dissolved in 0.8 mM nitrilotriacetate and citrate before buffer was added. The highest specific binding was observed when cells were first converted to spheroplasts and then opened mechanically. Under these conditions, specific binding to isolated outer membranes prepared as described by Osborn et al. (19) and to membranes which were enriched in the outer membrane by differential centrifugation (see above) was obtained.

Six independent determinations were performed for the wild-type strain AB2847 to show the variations of the values measured (Table 2). The *fecA* mutants lacked specific binding. The negative values arose from stronger binding to cells which had not been grown in the presence of citrate compared with the citrate-grown cells. All of the *fecB* mutants tested showed a binding activity comparable to that of the parent strain. This observation justifies the differentiation of the *fecB* mutants from the *fecA* mutants because none of the latter contained a citrate-inducible but inactive protein. The absence of the 50k protein (Table 2) had no effect on the binding of $^{55}Fe(III)$-citrate to whole cell envelopes (data not shown).

For comparison, we measured binding of ferric enterochelin to outer membranes. We obtained values of 137 and 170 pmol of $^{55}Fe(III)$ per mg of protein and no binding to mutants which lacked the FepA receptor protein. There was no unspecific binding of iron when it was supplied as an enterochelin complex.

To strengthen further the correlation between the presence of the FecA protein and ferric citrate binding, the time course of the appearance of both functions was measured. In outer membranes of cells grown in the presence of 1 mM citrate, the FecA protein was first visible after 20 min (Fig. 3), which is also the time it takes to induce the ferric citrate transport system (7; our own experiments). The amount of FecA protein increased in parallel with the increase of ferric citrate transport. Binding of ferric citrate could clearly be demonstrated only after 60 min due to the unspecific binding discussed above and then increased further (Fig. 3).

The location of the FecA protein in the outer membrane was also tested by treating whole cells with pronase P. The cells were then repeatedly washed to remove the protease before the outer membrane was prepared and analyzed by gel electrophoresis and the ferric citrate binding assay. As shown in Fig. 4, h, h', and h'', (citrate-induced) and Fig. 4, i (uninduced) the amount of FecA protein was only slightly reduced, whereas other proteins related to iron transport had virtually disappeared. The FecA protein apparently was the most resistant protein to pronase treatment among the iron receptors. Additional protein bands with low molecular weight showed up which resulted from the partial degradation of the proteins. The specific binding of ferric citrate was retained with values of about 34 pmol of Fe(III) per mg of protein after exposure to pronase. For comparison, binding of ferric enterochelin to the FepA protein went below 10% of the value of membranes not treated with pronase.

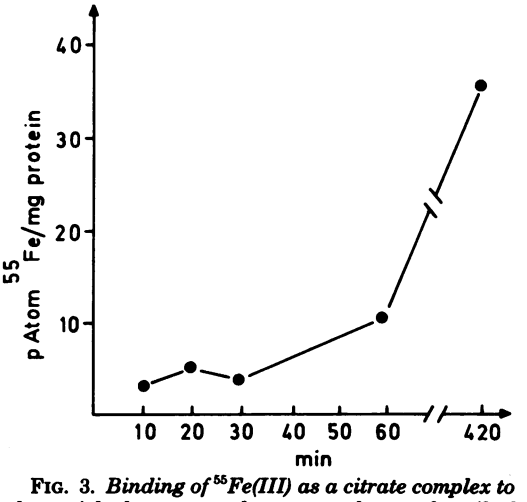

FIG. 3. *Binding of $^{55}Fe(III)$ as a citrate complex to the enriched outer membrane samples, as described in Fig. 4, during the course of the induction of the FecA protein. The difference between binding to membranes of citrate-induced and uninduced cells is shown.*

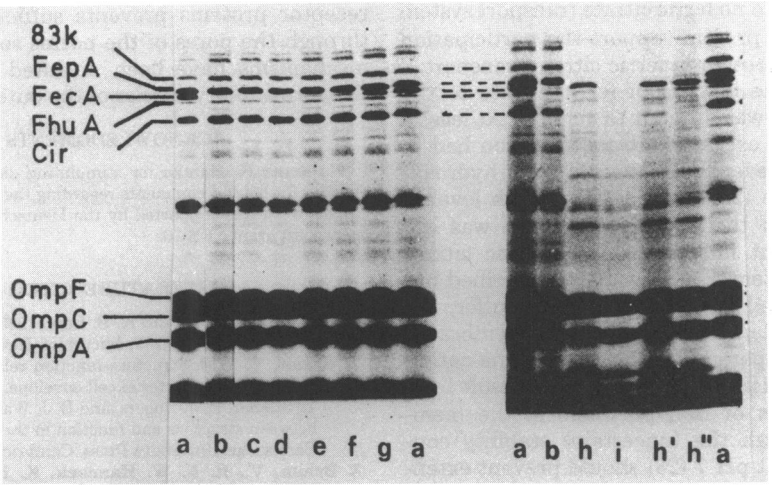

FIG. 4. *Polyacrylamide gel electrophoresis of outer membranes of cells of E. coli K-12 AB2847 during the expression of the FecA protein in the presence of 1 mM citrate in the growth medium. Parallel cultures of 200 ml of M9 minimal medium containing 0.1 μM $FeCl_3$ and 20 μM DHB were grown from an absorbance (at 578 nm) of 0.02 to 0.4. Then citrate was added to 1 mM, and growth was stopped after 0, 10, 20, 30, and 60 min by pouring the cultures on crushed ice. Membranes enriched in the outer membrane were prepared by differential centrifugation as described in the text. (a) Membranes of cells grown for 420 min with citrate and DHB and (b) with DHB alone from an absorbance of 0.02 to 0.5. Cells grown with citrate and DHB for (c) 0, (d) 10, (e) 20, (f) 30, and (g) 60 min. In addition, the protein patterns of the outer membranes of cells are shown which had been treated with pronase P (h, h', H"; i). (h) Citrate-induced cells; (i) uninduced cells.*

DISCUSSION

Five outer membrane proteins of *E. coli* K-12 have been related to the transport of iron. The FepA protein serves as a receptor for ferric enterochelin, and the FhuA protein serves as a receptor for ferrichrome (2–6, 11–13, 15, 17). Two other proteins, the Cir protein (colicin I receptor protein) and the 83k protein, are expressed under iron-limiting growth conditions but no iron complexes are yet known which use these proteins as receptors (3, 15). The fifth protein was induced (9), as was the ferric citrate transport system (7), by growing cells in the presence of 1 mM citrate. In this paper, it was shown that one-fourth of the mutants unable to grow with ferric iron in the presence of a large excess of citrate were lacking this outer membrane protein. The presence of this protein strongly enhanced binding of ferric citrate to outer membranes.

Both observations suggest that the citrate-inducible protein is involved in ferric citrate transport. Woodrow et al. (25) have isolated six mutants deficient in ferric citrate transport and termed them *fec*. Examination of their mutants with regard to the expression of the outer membrane protein showed that all three mutants which were cotransducible by phage P1 with the *argF* gene were devoid of the outer membrane protein. In accordance with the nomenclature of the other ferric complex receptor proteins mentioned above, we designated the ferric citrate receptor protein FecA and the mutation *fecA*. The three other ferric citrate transport mutants they studied were mapped by interrupted mating experiments in the same gene region at 7 min of the linkage map of *E. coli* K-12, but transduction experiments failed to show cotransduction between the *proA* and the *fec* genes (25). Our data demonstrate that they comprise a second class of ferric citrate transport mutants, and we term them *fecB*. Furthermore, half of the *fecA* mutants and half of the *fecB* mutants lacked a protein with an apparent molecular weight of 50k in the cytoplasmic membrane. Although we found no evidence that this protein was formed in response to citrate in the growth medium, the high frequencies these mutants attained in independent selections suggest a role of the 50k protein in ferric citrate transport across the cytoplasmic membrane. This interpretation implies that half of the *fecA* mutants should be regulatory mutants or that polarity effects cause the concomitant loss of the FecA and 50k proteins. Among the *fecA* and the *fecB* mutants there may be mutations affecting structural and regulatory genes.

The fact that the *E. coli* W83-24 and *S. typhimurium*, which have no ferric citrate transport system, lack the FecA protein, and the finding that under anaerobic growth conditions *E. coli*

K-12 expressed no ferric citrate transport system and no FecA protein support the participation of the FecA protein in ferric citrate transport.

Proof of the binding of ferric citrate to the FecA protein was difficult to achieve. At least a 20-fold molar excess of citrate over iron had to be used to prevent formation of large hydroxy-iron polymers (24). Still, a rather high level of adsorption to the outer membranes was obtained without induction of the ferric citrate transport system. The procedure described has been optimized with regard to the buffer, the membrane filters used, and the way membranes have been prepared. We do not know the nature of the "unspecific binding." It could result from the deposition of iron polymers in the membrane, although the (uncertain) stability constant of 10^{25} at pH 7 (23) should prevent extensive dissociation of the ferric citrate complex. We cannot exclude additional binding sites for ferric citrate, which, however, would not be citrate-inducible. The other proteins related to iron transport, especially the Cir and the 83k proteins, could serve as receptors. In fact, binding of ferric citrate to membranes of uninduced cells was decreased after treatment with pronase, which degraded these proteins. No problems were encountered in binding studies with ferric enterochelin, where binding was high in *fepA*$^+$ strains and zero in *fep* strains. Binding of ferric citrate to the cytoplasmic membrane, whether citrate-induced or uninduced, was only in the range of 10 to 15% of the binding to outer membranes. The low binding prevented study of the function of the 50k protein in ferric citrate transport.

Under the conditions of the iron supply of cells via citrate, the predominant form of the complex is probably the ferric dicitrate (S. Hussein, unpublished data). The size of this complex (molecular weight, 443) is below the exclusion limit of the pores formed by the porins (18). The specific receptor protein may therefore not be required for permeating the outer membrane if only the size of the solute is the limiting factor. The evolution of the receptor may therefore have been caused by the low availability of ferric iron due to its extreme insolubility (21). The receptor may thus extract the soluble portion of the iron from the medium and concentrate it at the cell surface. This argument may also apply to ferric enterochelin and ferrichrome, whose sizes (722 and 740, respectively) are not so much above the exclusion limit that they could not diffuse with a sufficiently fast rate through the porins if one takes into account the fact that the requirement for iron is at least a factor of 1,000 below the amount needed for a carbon source. However, binding of the ferric complexes to their receptor proteins prevents sufficient diffusion through the pores of the porins so that specific mechanisms have been invented to overcome the permeability barrier of the outer membrane.

ACKNOWLEDGMENTS

We thank K. Hantke for stimulating discussions and P. Wookey for helpful comments regarding the manuscript.

This work was supported by the Deutsche Forschungsgemeinschaft grant SFB 76.

LITERATURE CITED

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