Defined Inactive FecA Derivatives Mutated in Functional Domains of the Outer Membrane Transport and Signaling Protein of *Escherichia coli* K-12

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The FecA outer membrane protein of *Escherichia coli* functions as a transporter of ferric citrate and as a signal receiver and signal transmitter for transcription initiation of the *fec* transport genes. Three FecA regions for which functional roles have been predicted from the crystal structures were mutagenized: (i) loops 7 and 8, which move upon binding of ferric citrate and close the entrance to the ferric citrate binding site; (ii) the dinuclear ferric citrate binding site; and (iii) the interface between the globular domain and the β-barrel. Deletion of loops 7 and 8 abolished FecA transport and induction activities. Deletion of loops 3 and 11 also inactivated FecA, whereas deletion of loops 9 and 10 largely retained FecA activities. The replacement of arginine residue R365 or R380 and glutamine Q570, which are predicted to serve as binding sites for the negatively charged dinuclear ferric citrate, with alanine resulted in inactive FecA, whereas the binding site mutant R438A retained approximately 50% of the FecA induction and transport activities. Residues R150, E541, and E587, conserved among energy-coupled outer membrane transporters, are predicted to form salt bridges between the globular domain and the β-barrel and to contribute to the fixation of the globular domain inside the β-barrel. Mutations E541A and E541R affected FecA induction and transport activity slightly, whereas mutations E587A and E587R more strongly reduced FecA activity. The double mutations R150A E541R and R150A E587R nearly abolished FecA activity. Apparently, the salt bridges are less important than the individual functions these residues seem to have for FecA activity. Comparison of the properties of the FecA, FhuA, FepA, and BtuB transporters indicates that although they have very similar crystal structures, the details of their functional mechanisms differ.

FecA serves as a transport protein for ferric citrate across the outer membrane of *Escherichia coli* and as a signal receiving and signal transduction protein (4, 24, 28). Binding of ferric citrate to FecA induces transcription of the ferric citrate transport genes *fecABCDE* (4, 5, 7). Transport of ferric citrate is not required for induction. The N terminus of FecA is located in the periplasm and interacts with the FecR signal-transducing protein across the cytoplasmic membrane (17). Deletion of the N terminus abolishes induction, but transport is fully retained (28). FecR interacts with the FecI sigma factor in the cytoplasm and controls transcription initiation of the *fec* transport genes (18, 43). Comparisons with genome sequences of other bacteria reveal homologs of FecIRA whose encoding genes are arranged in the same order as *fecIRA* of *E. coli* (7). Particularly abundant are *fecIRA* homologs in *Pseudomonas aeruginosa* (46), *Pseudomonas putida* (34), *Nitrosomonas europaea*, and *Bacteroides thetaiotaomicron*, for which 10, 11, 15, and 23 *fecIRA* gene clusters, respectively, are predicted (V. Braun and S. Mahren, unpublished results). The *E. coli fecIRA* type of transmembrane transcriptional control thus seems to represent a paradigm of a large number of similar transcriptional control devices in many gram-negative bacteria.

The crystal structure of FecA is similar to the crystal structures of FhuA (20, 33), FepA (8), and BtuB (12, 13). They all consist of a β-barrel composed of 22 antiparallel β-strands that form a channel closed by the N-terminal globular domain. Only FecA contains, in addition to the globular domain located inside the β-barrel, a periplasmic 79-residue N-terminal extension through which it interacts with FecR for *fec* transport gene transcription initiation (Fig. 1). Moreover, it is the only transporter for which the crystal structure revealed a strong movement of surface loops 7 and 8 upon binding of the substrate, dinuclear ferric citrate. It is thought that this movement closes the entrance to the ferric citrate binding site and thus prevents escape of ferric citrate into the medium when it is released from the binding site during transport through the β-barrel into the periplasm. Interestingly, when FecA is incubated with iron-free citrate, two citrate molecules bind to the binding site of dinuclear ferric citrate but do not induce movement of loops 7 and 8 (48). Not all citrate binding sites are identical to the ferric citrate binding site, and the configuration of the two citrate molecules differs from that of ferric citrate. Citrate alone does not induce synthesis of the ferric citrate transport system (25), as confirmed by measuring transcription of a *fecA-lacZ* reporter gene (V. Braun and C. Herrmann, unpublished results).

Since closure of the entry cavity upon binding of the substrate is seen only in the crystal of FecA (21) but not in the crystals of FhuA (20, 33) and BtuB (12, 13), whether loops 7 and 8 are essential for FecA activity was studied. It was conceivable that deletion of these loops would reduce, but not abolish, transport. Furthermore, the signaling activity of FecA requires TonB, ExbB, and ExbD, as does the transport activity.

* Corresponding author. Mailing address: Mikrobiologie/Membranphysiologie, Auf der Morgenstelle 28, D-72076 Tübingen, Germany. Phone: 49 7071 2972096. Fax: 49 7071 5843. E-mail: volkmar.braun @mikrobio.uni-tuebingen.de.

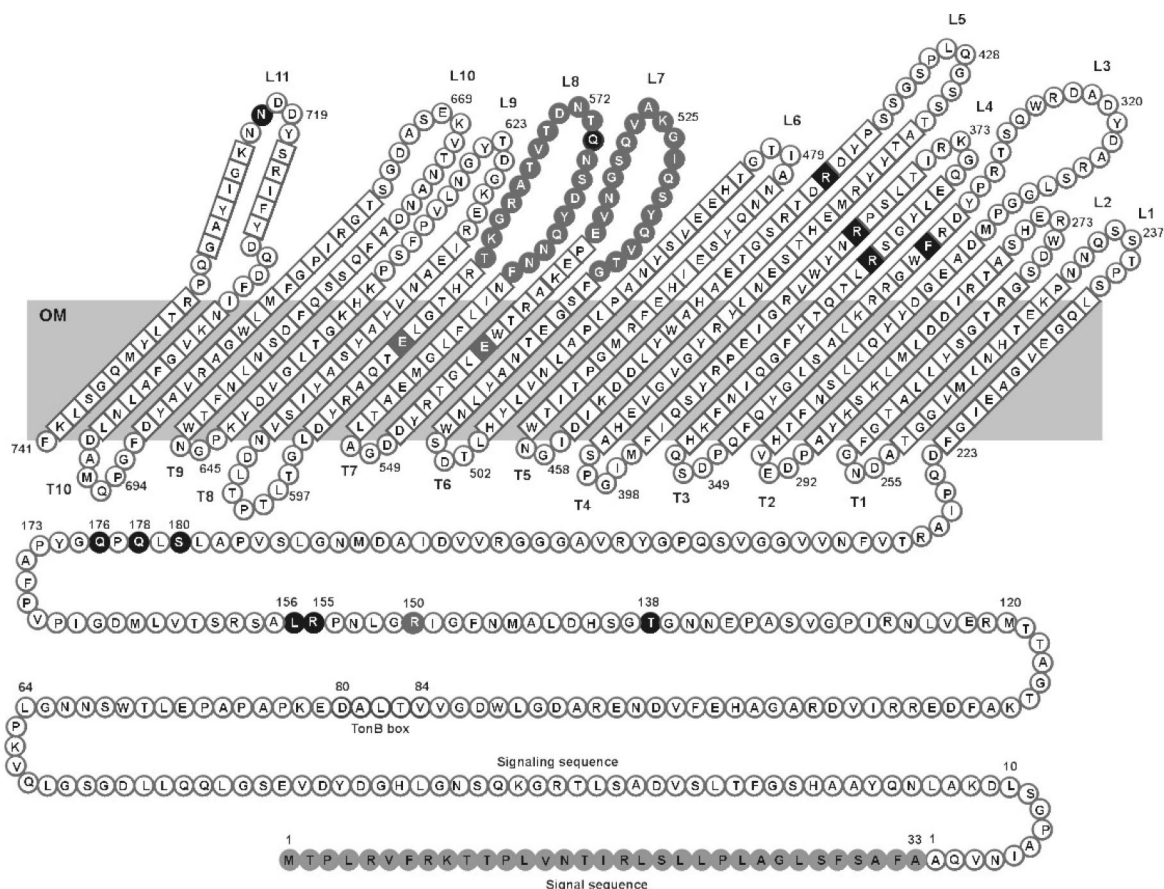FIG. 1. Topology of the FecA polypeptide chain across the outer membrane of *E. coli*. The outer membrane (OM) transmembrane antiparallel β-strands of the unfolded β-barrel are connected at the cell surface by loops 1 to 11 (L1 to L11). The residues of the globular domain, the β-barrel, and the loops that are predicted to bind dinuclear ferric citrate (21) are shaded black. Residues R150, E541, and E581 at the interface between the globular domain and the β-barrel are shaded grey. The signal sequence released upon secretion of FecA across the cytoplasmic membrane and the TonB box are indicated. The globular cork domain (residues 80 to 221) that is inserted in the β-barrel is shown as linear sequence.

Whether signaling has the same FecA structural requirements as transport, in particular with regard to loops 7 and 8, was addressed. Loops 7 and 8 were deleted to determine whether they are essential for ferric citrate induction and transport. For comparison, other loops were deleted, and the activities of the FecA deletion derivatives were tested. Moreover, amino acids in the predicted ferric citrate binding site, composed of residues from the globular domain and the β-barrel, were replaced by alanine. The basic amino acids lysine (K) in loop 7 and arginine (R) in loop 8 were replaced by alanine to determine whether they form primary ferric citrate binding sites that facilitate entry of ferric citrate into the tight binding site inside FecA.

MATERIALS AND METHODS

Bacterial strains, plasmids, and growth conditions. The strains and plasmids used are listed in Table 1. Cells were grown at 37°C in tryptone-yeast extract and nutrient broth (NB) (Difco Laboratories) and supplemented, as indicated, with 1 mM citrate and where required with ampicillin (40 μg/ml) or chloramphenicol (20 μg/ml).

Construction of plasmids. Plasmids were constructed by PCR amplification of DNA fragments with appropriate primer pairs, the sequences of which are available upon request. *E. coli* K-12 strain AB2847 was used as a PCR template, unless indicated otherwise.

To construct plasmids pASA30 to pASA37, two *fecA* fragments lacking the codons for the amino acids to be deleted were amplified by PCR. Introduced restriction sites were used to ligate the fragments to form mutant *fecA* genes, which were then PCR amplified. The PCR products were digested with EcoRI and BamHI, which flank *fecA*, and cloned into vector pT7-7 cleaved with EcoRI and BamHI.

The same procedure was used to construct pASA39, with pASA34 as a template. Plasmid pASA29 was constructed by introducing an SphI restriction site and a BsiWI restriction site before and after the deleted amino acids of loop 7, respectively, in plasmid pASA32. pASA38 was constructed by replacing the EcoRI/BamHI fragment of vector pT7-7 with the EcoRI/BamHI *fecA* PCR fragment encoding a FecA protein with amino acids of loop 11 deleted.

To construct plasmids pASA40 to pASA53, the QuikChange site-directed mutagenesis kit (Stratagene Europe, Amsterdam, The Netherlands) was used to introduce point mutations. pASA49 and pASA51 were used for construction of pASA52 and pASA53 by PCR.

To obtain plasmids pASA54 to pASA57, SphI/BsiWI *fhuA* fragments were amplified from plasmid pHK763 and cloned into pASA29 cleaved with SphI and BsiWI.

Plasmids pASA58 to pASA85 were constructed by cloning *fecA* excised with EcoRI and BamHI from plasmids pASA30 to pASA57 into plasmid pMMO203 cleaved with EcoRI and BamHI.

The sequences of all constructed *fecA* derivatives were confirmed by nucleotide sequencing.

Recombinant DNA techniques. Standard techniques (41) or the protocols of suppliers were used for the isolation of plasmid DNA, PCR, digestion with restriction endonucleases, ligation, transformation, and agarose gel electro-

TABLE 1. *E. coli* strains and plasmids used in this study

Strain or plasmid	Genotype	Reference or source
Strains		
AB2847	*aroB malT tsx thi*	6
IS1031	*fecA aroB thi malT tsx fepA lac*::Tn*10*	24
AA93	F⁻ Δ*fec araD139* Δ*lacU169 rpsL150 relA1 flbB5301 deoCI ptsF25 rbsR thi aroB*	38
Plasmids		
pT7-7	*ori* ColE1 phage T7 gene promoter Amp^r	44
pMON37	*fecBCDE* on pT7-7	39
pASA29	pASA32 with SphI and BsiWI restriction sites	This study
pASA30	pT7-7 *fecA* Δ313–328	This study
pASA31	pT7-7 *fecA* Δ423–433	This study
pASA32	pT7-7 *fecA* Δ517–533	This study
pASA33	pT7-7 *fecA* Δ519–533 V518G	This study
pASA34	pT7-7 *fecA* Δ563–577 F562P	This study
pASA35	pT7-7 *fecA* Δ568–577 D567P	This study
pASA36	pT7-7 *fecA* Δ616–630	This study
pASA37	pT7-7 *fecA* Δ662–673	This study
pASA38	pT7-7 *fecA* Δ713–727	This study
pASA39	pT7-7 *fecA* Δ517–533 Δ563–577 F562P	This study
pASA40	pT7-7 *fecA* T138A	This study
pASA41	pT7-7 *fecA* R365A	This study
pASA42	pT7-7 *fecA* R380A	This study
pASA43	pT7-7 *fecA* R438A	This study
pASA44	pT7-7 *fecA* Q570A	This study
pASA45	pT7-7 *fecA* D573A	This study
pASA46	pT7-7 *fecA* K525A	This study
pASA47	pT7-7 *fecA* R578A	This study
pASA48	pT7-7 *fecA* E541A	This study
pASA49	pT7-7 *fecA* E541R	This study
pASA50	pT7-7 *fecA* E587A	This study
pASA51	pT7-7 *fecA* E587R	This study
pASA52	pT7-7 *fecA* R150A E541R	This study
pASA53	pT7-7 *fecA* R150A E587R	This study
pT7-6	*ori* ColE1 phage T7 gene promoter, Amp^r	44
pHK763	pT7-6 *fhuA*	27
pASA54	pT7-7 *fecA* Δ516–535 replaced by *fhuA* 243–273	This study
pASA55	pT7-7 *fecA* Δ516–535 replaced by *fhuA* 318–339	This study
pASA56	pT7-7 *fecA* Δ516–535 replaced by *fhuA* 394–419	This study
pASA57	pT7-7 *fecA* Δ516–535 replaced by *fhuA* 502–515	This study
pHSG576	pSC101 derivative, Cm^r	45
pMMO203	pHSG576 *fecIR*	38
pMMO1034	*ori* Col E1 Amp^r *fecA-lacZ*	38
pASA58	pMMO203 *fecA* Δ313–328	This study
pASA59	pMMO203 *fecA* Δ423–433	This study
pASA60	pMMO203 *fecA* Δ517–533	This study
pASA61	pMMO203 *fecA* Δ519–533 V518G	This study
pASA62	pMMO203 *fecA* Δ563–577 F562P	This study
pASA63	pMMO203 *fecA* Δ568–577 D567P	This study
pASA64	pMMO203 *fecA* Δ616–630	This study
pASA65	pMMO203 *fecA* Δ662–673	This study
pASA66	pMMO203 *fecA* Δ713–727	This study
pASA67	pMMO203 *fecA* Δ517–533 Δ563–577 F562P	This study
pASA68	pMMO203 *fecA* T138A	This study
pASA69	pMMO203 *fecA* R365A	This study
pASA70	pMMO203 *fecA* R380A	This study
pASA71	pMMO203 *fecA* R438A	This study
pASA72	pMMO203 *fecA* Q570A	This study
pASA73	pMMO203 *fecA* D573A	This study
pASA74	pMMO203 *fecA* K525A	This study
pASA75	pMMO203 *fecA* R578A	This study
pASA76	pMMO203 *fecA* E541A	This study
pASA77	pMMO203 *fecA* E541R	This study
pASA78	pMMO203 *fecA* E587A	This study
pASA79	pMMO203 *fecA* E587R	This study
pASA80	pMMO203 *fecA* R150A E541R	This study
pASA81	pMMO203 *fecA* R150A E587R	This study
pASA82	pMMO203 *fecA* Δ516–535 replaced by *fhuA* 243–273	This study
pASA83	pMMO203 *fecA* Δ516–535 replaced by *fhuA* 318–339	This study
pASA84	pMMO203 *fecA* Δ516–535 replaced by *fhuA* 394–419	This study
pASA85	pMMO203 *fecA* Δ516–535 replaced by *fhuA* 502–515	This study

phoresis. DNA was sequenced commercially using the dideoxy chain-termination method.

Transport assays. Quantitative transport of $^{55}Fe^{3+}$ citrate by plasmid-encoded FecA was determined in freshly transformed *E. coli* K-12 strain IS1031 *fecA* and with *E. coli* AA93 Δ*fec*, which in addition to the various *fecA* genes was transformed with pMON37 *fecBCDE*. Cells were grown overnight at 37°C in NB medium and used to inoculate 7-ml NB medium supplemented with 0.4% glucose and 1 mM sodium citrate. The culture was incubated for 3 h to an optical density at 578 nm (OD_{578}) of 0.5 to 1 to fully induce the transport system. After 1.5 h, 50 μM dipyridyl was added to complex free iron(II) ions in the medium and to induce the *fec* transport genes. Cells were suspended to an OD_{578} of 0.5 in 5 ml of transport medium (2.07 g $NaH_2PO_4 \cdot H_2O$, 0.68 g of KH_2PO_4, 0.66 g of $(NH_4)_2SO_4$, 47 mg of $MgCl_2$, 22 mg of $CaCl_2$, 1 g of glucose per liter, adjusted to pH 6.9). Sodium nitrilotriacetate (50 μl, 10 mM) was added, and the mixture was incubated for 5 min at 37°C to remove free iron. After the addition of 50 μl of radioactive ferric citrate (10 μM $^{55}Fe^{3+}$, 1 M sodium citrate [pH 6.8]), 0.7-ml samples were taken after 1, 6, 11, 16, 21, and 26 min of incubation at 37°C and filtered through cellulose nitrate filters (pore size, 0.45 μm; Sartorius AG, Göttingen, Germany); the filters were then washed twice with 5 ml of 0.1 M LiCl. The filters were dried, and the radioactivity was determined by liquid scintillation counting.

Iron citrate binding assays. Cells of Δ*fec E. coli* AA93 were freshly transformed with the plasmids to be tested and grown overnight at 37°C in NB medium. *fecA* transcription was induced by growing cells first in 7 ml of NB medium supplemented with 0.4% glucose and 1 mM sodium citrate. After 1.5 h, 50 μM dipyridyl was added to complex free iron(II) ions in the medium. Cells were suspended to an OD_{578} of 0.5 in 5 ml of transport medium as described in the transport assays. After addition of 50 μl of the $^{55}Fe^{3+}$ citrate mix (10 μM $^{55}Fe^{3+}$, 1 M sodium citrate [pH 6.8]), 0.7-ml samples were taken after 1, 6, 11, 16, and 21 min of incubation at 37°C. After 21 min, a 100-fold surplus of nonradioactive ferric citrate (1 mM) was added to chase radiolabeled iron that has not entered the cytoplasm. Two chase samples were measured. Probes were filtered through cellulose nitrate filters (pore size, 0.45 μm; Sartorius AG) and washed twice with 5 ml of 0.1 M LiCl, the filters were dried, and the radioactivity was determined by liquid scintillation counting.

Protein analytical methods and Western blotting. *E. coli* AA93 transformed with one of the various plasmids was grown in NB medium with or without 1 mM sodium citrate at 37°C. Cells were harvested by centrifugation at an OD_{578} of 1.

Outer membranes were prepared by lysing cells with lysozyme-EDTA, followed by solubilization of the cytoplasmic membrane with 0.2% Triton X-100 and differential centrifugation (23). The proteins were separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) (11% polyacrylamide) (32) and then transferred to nitrocellulose membranes (Schleicher and Schuell, Dassel, Germany) for Western blot analysis to estimate the amount of wild-type FecA and mutant FecA. Blots were blocked in 5% bovine serum albumin in TNT buffer (20 mM Tris-HCl, 500 mM NaCl, 0.05% Tween 20 [pH 7.5]), probed with polyclonal anti-FecA antibodies (diluted 1:20,000) overnight, and then washed with TNT buffer and incubated with anti-rabbit immunoglobulin G conjugated with alkaline phosphatase (Sigma, München, Germany). The blots were developed with nitroblue tetrazolium-5-bromo-4-chloro-3-indolylphosphate (Serva, Heidelberg, Germany).

Determination of β-galactosidase activity. β-Galactosidase activity was measured according to published methods (22, 35). To determine the induction level, cells were grown in NB medium with or without 1 mM sodium citrate.

RESULTS

Activity of loop deletion mutants. Loops 3, 5, 7, 8, 9, 10, and 11 were each deleted such that at least three amino acid residues remained to connect the β-strands with a turn in the periplasm. In loop 7 and 8 two different deletion mutants were constructed. Two fragments in loop 7 were excised that differed by two residues in length, and two fragments in loop 8 were excised that differed by five residues in length, to examine whether the size of the turns remaining affected integration into the outer membrane and FecA activity. The small loops L1, L2, L4, and L6 were not shortened. The *fecA* wild-type and mutant genes were each cloned on the low-copy-number plasmid pHSG576 and introduced into *E. coli* AA93 Δ*fecIRABCDE* by transformation. To measure *fecA* transcription, strain AA93

TABLE 2. Properties of FecA mutants

Mutation	β-Galactosidase activity [Miller units (%)][a]	$^{55}Fe^{3+}$ transport rate (%)[b]	
		Medium copy	Low copy
None (vector)	12 (0.7)	11	10
None (wild type)	1,898 (100)	100	100
ΔL3 (Δ313–328)	15 (0.8)	10	ND
ΔL5 (Δ423–433)	136 (7)	23	16
ΔL7₁ (Δ517–533)	16 (0.9)	7	ND
ΔL7₂ (Δ519–533)	15 (0.8)	6	ND
ΔL8₁ (Δ563–577)	15 (0.8)	7	ND
ΔL8₂ (Δ568–577)	15 (0.8)	8	ND
ΔL9 (Δ616–630)	695 (37)	74	76
ΔL10 (Δ662–673)	952 (50)	48	33
ΔL11 (Δ713–727)	23 (1.2)	16	8
ΔL7ΔL8 (Δ517–533 Δ563–577)	8 (0.4)	9	ND
T138A	1,198 (63)	46	58
R365A	28 (1.5)	8	ND
R380A	15 (0.8)	8	ND
R438A	797 (42)	53	73
Q570A	13 (0.7)	11	ND
D573A	1,177 (62)	97	40
K525A	1,895 (100)	94	126
R578A	1,556 (82)	53	82
E541A	1,708 (90)	112	81
E541R	1,594 (84)	91	72
E587A	721 (38)	85	87
E587R	626 (33)	50	47
R150A E541R	285 (15)	27	42
R150A E587R	76 (4)	25	29

[a] Transcription induction of *fecA* was measured in *E. coli* AA93 (Δ*fec* pMMO1034 *fecA-lacZ*) grown in NB containing 1 mM citrate. The values are the averages of three independent experiments. The uninduced values (cells grown in NB lacking citrate) were between 8 (0.4%) and 18 (1%) Miller units.

[b] Transport was determined in *E. coli* IS1031 (*fecA*) and in *E. coli* AA93. The transport rate was calculated by subtracting the values after a 1-min incubation from the values after a 16-min incubation; the values given are relative to the wild-type rate (100%). The values are the averages of two independent experiments.

contained a plasmid-encoded *fecA-lacZ* reporter gene. Ferric citrate did not induce *fecA* transcription of the loop 7 and loop 8 deletion mutants and the loop 7/8 double mutant (Table 2). The loop 3 and loop 11 deletion mutants were also inactive. The loop 5 mutant showed low activity, and the loop 9 and 10 mutants displayed an intermediate level of activity (Table 2).

Citrate-mediated $^{55}Fe^{3+}$ transport was determined for *E. coli* IS1031, which carries a chromosomally encoded FecA R413H mutation that renders cells inactive for citrate-mediated iron transport and exhibits no polar effect on the downstream *fecBCDE* genes (24, 47). IS1031 transformants carried the *fecA* genes on the medium-copy-number plasmid pT7-7. IS1031 transformed with the wild-type *fecA* gene transported iron (Table 2). IS1031 synthesizing the loop 7 or loop 8 FecA deletion protein did not transport iron (Table 2). The loop 3 and loop 11 mutants were also inactive in citrate-mediated iron transport. The loop 5, 9, and 10 mutants displayed 23, 74, and 48% of the *fecA* wild-type transport rate, respectively.

In addition, $^{55}Fe^{3+}$ transport into *E. coli* AA93, which was the strain used for the induction experiments, was determined. AA93 was transformed with the *fecA* wild-type and mutant genes on the low-copy-number plasmid pMMO203 which were used for the induction experiments. The level of transport could thus be directly compared with the level of induction. For transport across the cytoplasmic membrane, AA93 was transformed with plasmid pMON37 carrying the *fecBCDE*
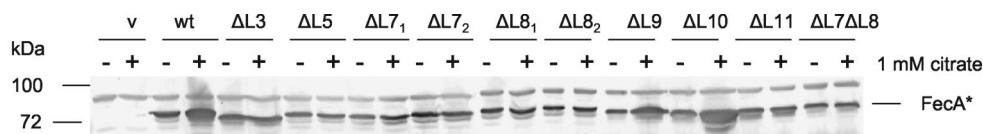
FIG. 2. Anti-FecA Western blot of outer membranes of *E. coli* IS1031 (*fecA*) transformed with pT7-7 carrying wild-type *fecA* or one of a number of mutant *fecA* genes encoding FecA loop deletion proteins. +, induction with 1 mM citrate; −, uninduced; v, vector; wt, wild-type FecA. The sizes of the marker proteins are indicated on the left in kilodaltons.

wild-type genes. Transport of $^{55}Fe^{3+}$ into AA93 was determined with those *fecA* mutant genes, which displayed intermediary transport rates in IS1031. Related to the transport rate of the *fecA* wild-type transformant, the *fecA* mutant transformants showed rates comparable with those obtained with the IS1031 transformants (Table 2). The plasmid-encoded *fecABCDE* transport genes in AA93 conferred transport rates which were comparable with those of the chromosomally encoded *fecBCDE* transport genes in IS1031 complemented by the *fecA* mutant genes. The similar results obtained with AA93 and IS1031 indicated that gene copy numbers did not strongly influence the mutant values related to wild-type FecA and that recombination between chromosomal and plasmid-encoded *fecA* in IS1031 played no role. Since the studied mutations were in the β-barrel domain and IS1031 is also mutated in the β-barrel domain, no reconstitution of active FecA could occur of the kind observed with FhuA, where wild-type cork of one FhuA molecule could complement the missing cork of a cosynthesized FhuA β-barrel (3). In fact, such a reconstitution does not occur with FecA (V. Braun, unpublished results).

Since the loop 3, 7, 8, and 11 mutants did not induce *fecA-lacZ* transcription, transport inactivity could be caused by the lack of transport proteins. Induction was determined with the mutant genes cloned on the low-copy-number plasmid pHSG576, whereas transport was determined with the mutant genes cloned on the medium-copy-number plasmid pT7-7. The presence of the mutant FecA proteins in *E. coli* IS1031 carrying the pT7-7 derivatives, used for the transport assays, was determined by SDS-PAGE. Western blotting with anti-FecA antiserum revealed that all the mutants synthesized the FecA protein in amounts sufficient to mediate transport (Fig. 2). Transformants that synthesized wild-type FecA or mutant FecA with a deletion in loop 9 or 10 produced more FecA protein when grown in ferric-citrate-containing medium than in citrate-free medium (Fig. 2). No ferric-citrate-dependent increase of FecA was observed in the loop 3, 5, 7, 8, and 11 mutants and the loop 7/8 double mutant (Fig. 2). *E. coli* IS1031 carrying the pT7-7 vector synthesized chromosomally encoded mutant FecA; the amount of FecA did not increase when ferric citrate was present in the medium, as was found previously (47). The results indicated that the lack of citrate-mediated iron transport was not caused by the lack of FecA protein.

The FecA loop 7 cannot be replaced by FhuA loops. To examine whether loop 7 must assume a certain structure to fulfill its function, residues 516 to 535 of FecA were replaced by residues 502 to 515 (loop 7), residues 243 to 273 (loop 3), residues 318 to 339 (loop 4), or residues 394 to 419 (loop 5) of FhuA. None of the mutants showed induction and transport activity. SDS-PAGE revealed wild-type amounts of uninduced FecA mutant proteins (data not shown). Apparently, movement of loop 7 upon binding of ferric citrate and/or closure of the entry to the binding site depends on a certain loop structure.

Activity of ferric-citrate-binding-site mutants. In FecA, residues T138, R365, R380, R438, and Q570 are in closest proximity, 3.0, 3.0, 2.6, 3.3, and 2.5 Å, respectively, to bound dinuclear ferric citrate (21, 48; A. D. Ferguson, personal communication). These residues were individually replaced by alanine, and ferric-citrate-mediated induction of *fecA-lacZ* transcription and citrate-mediated iron transport were determined. FecA(R365A), FecA(R380A), and FecA(Q570A) expressed in *E. coli* IS1031 were induction and transport inactive, whereas FecA(T138A) and FecA(R438A) displayed approximately half-maximal induction and transport activities (Table 2). Of particular importance are the positively charged R residues for binding of the negatively charged dinuclear ferric citrate. In addition, residue K525 of loop 7 or residue D573 or R578 of loop 8 was replaced by alanine to determine whether these residues play a role in initial adsorption of ferric citrate to FecA, from which ferric citrate could enter the tight binding site within FecA. We postulated that access to the final binding site might be facilitated when ferric citrate first binds to the loops and then is transferred to the final binding site by the movement of loops 7 and 8. FecA(K525A) and FecA(R578A) showed high induction and transport rates, whereas FecA(D573A) displayed a reduced induction but a high transport rate (Table 2; Fig. 3). Binding of ferric citrate to FecA(D573A) was decreased, as revealed by the low 1-min value (Fig. 3), which reflects binding and initial transport. Since the distance of D573 to dinuclear ferric citrate is 3.5 Å, D573 might contribute to the binding of dinuclear ferric citrate; the mutation D573A might reduce binding and therefore induction.

Binding of radiolabeled $^{55}Fe^{3+}$ citrate could not reliably be determined. Initial binding (1-min value) to Δ*fec* *E. coli* AA93 transformed with wild-type *fecA* was low at 600 ions per cell, which increased to 1,000 ions after 20 min. Chase with a surplus of unlabeled ferric citrate reduced binding to only 600 ions per cell. Mutant K525A gave wild-type values, mutants T138A and D573A gave 400 ions per cell, and the other mutants gave below 200 ions per cell, which was also obtained with AA93 transformed with the vector. It is possible that binding of ferric citrate is not strong enough to withstand washing of the filters with 0.1 M LiCl. In addition, the molecular form of ferric citrate in the assay is not clearly defined, since it polymerizes into numerous molecular forms (40). The results give no indication that closing of loops 7 and 8 prevent release of ferric citrate from the FecA binding site. Under similar conditions, 20,000 iron ions supplied as ferrichrome complex are bound to FhuA, and after chase 2,000 ions remain bound to the cells.
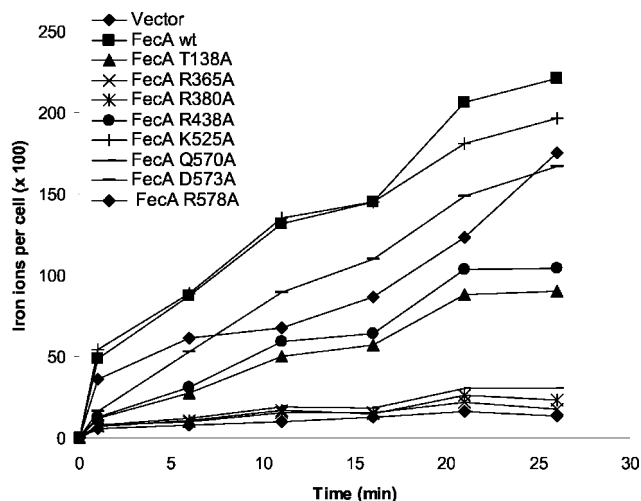
FIG. 3. Transport of $^{55}Fe^{3+}$ citrate into *E. coli* IS1031 (*fecA*) transformed with pT7-7 carrying wild-type *fecA* or one of a number of mutant *fecA* genes. The mutant *fecA* genes encode FecA derivatives mutated in the ferric citrate binding site.

Of those FecA mutants that transported iron in strain IS1031, transport was also determined in strain AA93 to test correlation of transport with induction in the same strain. Qualitatively the results obtained were similar to the results obtained with strain IS1031, but quantitative differences were noted (Table 2). With half of the mutants the level of induction correlated somewhat better for AA93 than for IS1031.

Activity of globular domain and β-barrel contact-site mutants. The four crystal structures of TonB-dependent outer membrane transporters indicate strictly conserved residues at the interface between the globular domain and the β-barrel. In FecA, these residues are R150 and R196 in the globular domain and E541 and E587 in the β-barrel, which are close enough and oriented such that they form salt bridges (R150 to E541 and E587 and R196 to E587). R150, E541, and E587 were each mutated, and induction and transport activities of the mutant proteins were determined. Induction and transport by FecA(E541A) and FecA(E541R) were close to that of wild-type FecA (Table 2), which indicated that the salt bridge to R150 was dispensable and that even repulsion of R150 by R541 did not impair FecA structure and function. In contrast, induction by FecA(E587A) and FecA(E587R) was reduced to one-third of that of wild-type FecA. Transport was also reduced with FecA(E587A) and even more strongly reduced with FecA(E587R) (Table 2). Induction by the double mutants

FecA(R150A E541R) and FecA(R150A E587R) was abolished or nearly abolished, and transport was strongly reduced (Table 2). Outer membrane fractions of cells expressing FecA(E541A) or FecA(E541R) contained reduced amounts of ferric-citrate-induced FecA, and the double mutants contained only uninduced amounts (Fig. 4). The data suggested that the conserved interface residues play a role in FecA structure and function and not just for fixation of the globular domain inside the β-barrel.

DISCUSSION

Crystal structures provide a precise but static view of a protein in the form in which it crystallizes. Postulations derived from the crystal structure as to how a protein might function have to be tested by studying appropriate mutants and by using biophysical methods that monitor the dynamics of a protein. In this study, three FecA regions with predictable functional roles were selected for further study: (i) loops 7 and 8, (ii) the dinuclear ferric citrate binding site, and (iii) the interface between the globular domain and the β-barrel.

Deletion of loops 7 and 8 abolished induction and transport, which showed that both loops are essential for both FecA activities and suggested that movement of the two loops, as observed in the crystal structures, is important. The difference in size of loop 7 (FecA 18, FhuA 14, and FepA 38 residues) and loop 8 (FecA 20, FhuA 7, and FepA 13 residues) suggest that loops 7 and 8 do not function the same way in all transporters. Loop 7 and 8 deletion mutants of FepA display no ferric enterobactin binding and transport (37). FecA and FepA are similar in that deletion of loop 7 or 8 abolishes all activities; in contrast, FhuA loop 7 or 8 deletion mutants retain all activities (15). FecA is similar to FhuA in that deletion of loop 3 or 11 abolishes all activities; in contrast, a loop 3 deletion mutant of FepA retains fully ferric enterobactin transport and sensitivity to colicin B (37). Comparison of the mutant phenotypes clearly indicates that each loop has different roles in the three transporters.

Surface loops in BtuB have still other properties and functions. In the BtuB crystal, the extracellular loops 2, 3, and 4 are disordered. Upon binding of two calcium ions, loop 2 becomes ordered and loop 3 becomes partially ordered. Upon additional binding of cyanocobalamin, loops 3 and 4 become fully ordered (12, 13). BtuB tolerates duplication of large parts of the sequence to a surprising degree, e.g., duplications extending from β-strand 1 into loop 2, loop 3 to β-strand 6, or β-strand 10 to β-strand 12. Such duplication mutants display 64 to 100% of the wild-type level of vitamin B_{12} transport activity
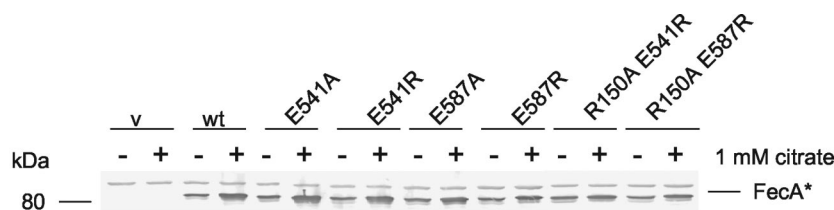

FIG. 4. Anti-FecA Western blot of outer membranes of *E. coli* IS1031 (*fecA*) transformed with pT7-7 carrying wild-type *fecA* or one of a number of mutant *fecA* genes encoding FecA derivatives mutated in the interface between the globular domain and the β-barrel. +, induction with 1 mM citrate; −, uninduced; v, vector; wt, wild-type FecA. The sizes of the marker proteins are indicated on the left in kilodaltons.

(31). In FhuA, insertions of 4 to 16 heterologous residues in loops 4, 5, 7, or 10 result in derivatives that support growth on ferrichrome as the sole iron source and sensitivity to the FhuA ligands albomycin, colicin M, microcin J25, and the phages T1, T5, ϕ80, and UC1 (30, 36). The loops, therefore, display a complex behavior. On one hand, they are highly flexible and show large changes in position upon binding of substrate and in response to TonB and energy input (26); on the other hand, they assume rather rigid structures, e.g., loops 3, 5, and 11 in FhuA form a sturdy protuberance extending approximately 35 Å above the membrane surface (33). Multiple interactions between loops occur, and these interactions might change during transport.

Only the crystal structures of FecA reveal closure of loops 7 and 8 upon binding of dinuclear ferric citrate (21). No such movement is seen in the crystal structures of FhuA (20, 33). However, ferrichrome-induced fluorescence quenching of fluorescein-labeled FhuA at residue D336C indicates movement of loop 4 or movement of neighboring loops such that the environment of the label changes (2). The crystal structure of FepA does not reveal the entire loops 4, 5, and 8 and bound ferric enterobactin (8). However, spectroscopic and cross-linking studies have disclosed movement of loops during ferric enterobactin transport (10, 26, 29, 42).

Biphasic binding kinetics and mutant analyses point to there being two ferric enterobactin binding sites on FepA (9, 10). It is conceivable that ferric enterobactin first binds reversibly to peripheral residues, followed by firm binding inside FepA. With such a model in mind, potential initial binding sites in loops 7 and 8 of FecA were replaced. These are the only positively charged residues—K525 in loop 7 and R578 in loop 8—that could initially bind the negatively charged dinuclear ferric citrate. The induction and transport activities of FecA(K525A) were similar to or even higher than those of wild-type FecA; the activities of FecA(R578A) were diminished. The level of transport reduction suggests that R578 serves as an initial ferric citrate binding site or that the replaced A alters the structure of loop 8 such that it affects movement of loop 8.

Of the binding site mutants studied, R365A, R380A, and Q570A most strongly reduced citrate-mediated iron transport. These residues are the residues closest to dinuclear ferric citrate. The negatively charged dinuclear ferric citrate is probably most strongly bound by the positively charged R365 and R380 residues, suggesting decreased binding of ferric citrate to the R365A and R380A mutants. However, weak binding does not necessarily lead to slow transport, as examples of FhuA (15) and FepA (37) demonstrate. Replacement of R81, which is highly conserved in FhuA homologues, leads to a very strong reduction in binding but fully retained transport (14).

The FecA crystal structure predicts salt bridges of R150 with E541 and E587 and of R196 with E587. Disruption of the salt bridges in the E541A mutant and even repulsion in the E541R mutant had no strong effects on FecA-mediated induction and ferric citrate transport. Apparently, the salt bridge between R150 and E541 is not essential for FecA structure and function. The E587A and E587R mutants displayed less induction, and transport was more strongly reduced in the E587R mutant than in the E587A mutant. Strong reduction of induction and transport was observed in the R150A E541R and R150A

E587R double mutants. These results partially differ from those obtained with FhuA (16)—disruption of the salt bridges of R93 to E522 and E571 and of R133 to E571 and repulsion between these residues have either no effects or only small effects on FhuA transport and receptor activities. However, in contrast to FecA, the FhuA(E522R) mutant is inactive and the FhuA(E571R) mutant is nearly inactive, even in the double mutant FhuA(R93E E522R), in which a salt bridge could be formed. R is not tolerated at the E sites, but E is not required, since E-to-A replacements yield FhuA wild-type activities. In FhuA, 60 hydrogen bonds and 7 salt bridges (33) either directly or through intermediate water molecules (19) are predicted to contribute to the fixation of the globular domain to the β-barrel; and the situation is similar for FecA. Disruption of one or two such bridges might not be sufficient to affect the structures to a degree that functions are seriously reduced. There must be other reasons in addition to fixation of the globular domain in the β-barrel to explain why these residues are highly conserved in TonB-dependent transporters and why E587 is important in FecA and E522 and E571 cannot be replaced by R without loss of FhuA activity. A similar conclusion can be drawn from results obtained with FepA in which R75, R126, E511, and E567 constitute the polar interaction sites between the globular domain and the β-barrel. The R75L, R75P, and R126H derivatives resulting from random mutation are strongly reduced in ferric enterobactin transport (1), the site-directed mutant R75Q displays a 10-fold increased K_m and a threefold lower V_{max} than wild-type FepA, the E511Q mutant has wild-type activities, and the E567A mutant has a V_{max} of 17% of that of wild-type FepA, but the E567A E511Q double mutant has 32% of the wild-type level of FepA transport activity (11). The distinct phenotypes of the mutants suggest that they affect the functions of the transporters, such as binding of the substrate, release of the substrate, opening of the β-barrel channel, and restoration of the closed state, in a complex manner.

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