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Signal transfer through three compartments: transcription initiation of the *Escherichia coli* ferric citrate transport system from the cell surface

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Transport of ferric citrate into cells of *Escherichia coli* K-12 involves two energy-coupled transport systems, one across the outer membrane and one across the cytoplasmic membrane. Previously, we have shown that ferric citrate does not have to enter the cytoplasm of *E.coli* K-12 to induce transcription of the *fec* ferric citrate transport genes. Here we demonstrate that ferric citrate uptake into the periplasmic space between the outer and the cytoplasmic membranes is not required for *fec* gene induction. Rather, FecA and the TonB, ExbB and ExbD proteins are involved in induction of the *fec* transport genes independent of their role in ferric citrate transport across the outer membrane. The uptake of ferric citrate into the periplasmic space of *fecA* and *tonB* mutants via diffusion through the porin channels did not induce transcription of *fec* transport genes. Point mutants in FecA displayed the constitutive expression of *fec* transport genes in the absence of ferric citrate but still required TonB, with the exception of one FecA mutant which showed a TonB-independent induction. The phenotype of the FecA mutants suggests a signal transduction mechanism across three compartments: the outer membrane, the periplasmic space and the cytoplasmic membrane. The signal is triggered upon the interaction of ferric citrate with FecA protein. It is postulated that FecA, TonB, ExbB and ExbD transfer the signal across the outer membrane, while the regulatory protein FecR transmits the signal across the cytoplasmic membrane to FecI in the cytoplasm. FecI serves as a sigma factor which facilitates binding of the RNA polymerase to the *fec* transport gene promoter upstream of *fecA*. Signal transfer presumably involves a sequence of conformational changes in FecA, FecR and FecI.

Key words: *E.coli*/ferric citrate/transcription initiation/transmembrane

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Introduction

Ferric citrate is one of the ferric siderophores that serve as iron sources for *Escherichia coli* K-12 (Frost and Rosenberg, 1973). The ferric citrate transport system (Fec system) is determined by seven Fec-specific genes (Figure 1), of which the *fecA* gene product catalyses transport across the outer membrane and the *fecBCDE* gene products catalyse binding protein-dependent transport across the cytoplasmic membrane (Pressler *et al.*, 1988; Staudenmaier *et al.*, 1989; Van hove *et al.*, 1990). Binding of ferric citrate to FecA was inferred from binding to outer membranes related to the degree of the FecA concentration (Wagegg and Braun, 1981). Two regulatory proteins, FecR anchored in the cytoplasmic membrane and exposed to both the cytoplasm and the periplasm, and FecI located in the cytoplasm, regulate transcription of the *fec* transport genes (Van hove *et al.*, 1990; Ochs *et al.*, 1995). FecI displays sequence similarities to a subgroup of σ^{70} factors and binds to the *fec* transport gene promoter upstream of *fecA* (Ochs *et al.*, 1995; S.Enz, V.Braun and J.Crosa, unpublished data). The Fec transport system, like all seven ferric siderophore transport systems of *E.coli* K-12, is repressed by the Fur protein when it is loaded with iron (Fe^{2+} Fur). Unique among the ferric siderophore transport systems of *E.coli* is the induction of the Fec system by ferric citrate (Hussein *et al.*, 1981; Zimmermann *et al.*, 1984), as the other ferric siderophores do not influence transcription of the related transport systems. However, under aerobic conditions exogenous citrate does not serve as a carbon source due to the lack of citrate uptake. When supplied as a ferric complex, at least 10 times more iron than citrate enters *E.coli*, which led to the hypothesis that ferric citrate does not have to reach the cytoplasm to induce transcription of the *fec* transport genes. This theory was proved with mutants in *fecB* which were inducible. In contrast, mutants in the genes *fecA*, *tonB*, *exbB* and *exbD*, which encode proteins involved in outer membrane transport, were no longer inducible (Zimmermann *et al.*, 1984). It was concluded that ferric citrate has to enter the periplasmic space, from where it induces transcription of the *fec* transport genes.

Active transport across the outer membrane is unusual because no energy source is known to exist in this compartment or in the adjacent periplasmic space. An idea about the transport mechanism was obtained by isolating deletion derivatives of two outer membrane proteins which may act similarly to FecA. FhuA, responsible for the transport of ferrichrome, and FepA, responsible for the transport of ferric enterobactin, were converted into open channels by the removal of cell surface loops comprising segments of 34 and 135 amino acid residues, respectively (Rutz *et al.*, 1992; Killmann *et al.*, 1993). Cells containing the deletion derivatives took up ferrichrome and ferric enterobactin independently of the TonB, ExbB and ExbD proteins (Ton complex), and were sensitive to certain antibiotics which are excluded from entering wild-type cells by the permeability barrier of the outer membrane. These data led to the proposal that the outer membrane proteins form closed channels which are opened through interaction with the TonB−ExbB−ExbD complex. The energy required is provided by the electrochemical potential of the cytoplasmic membrane

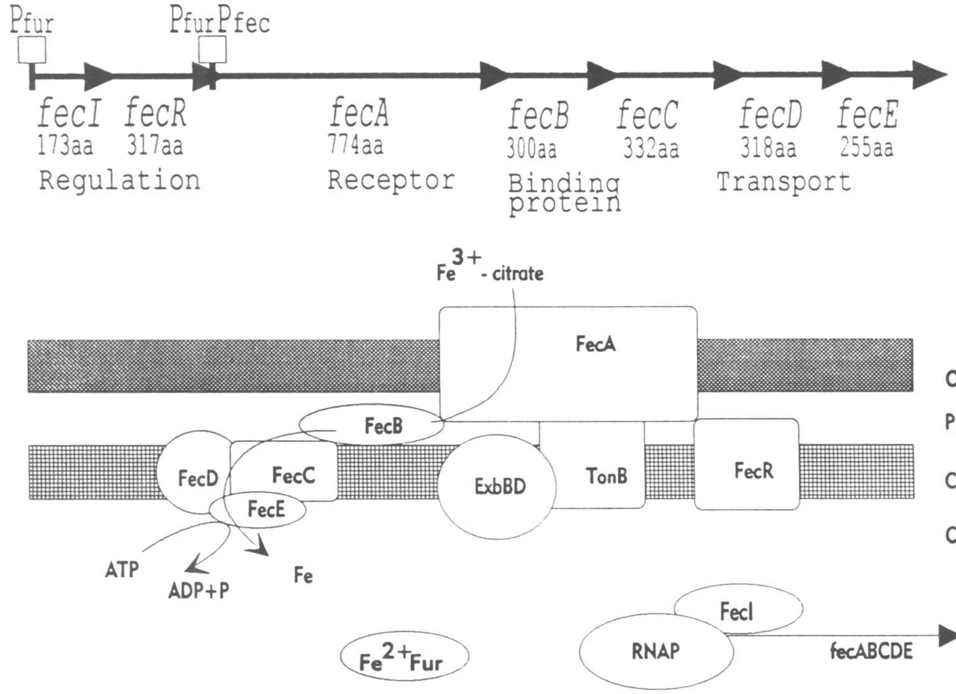

Fig. 1. Scheme of the ferric citrate regulatory and transport system. P_{fur} and P_{fec} denote operator regions which are controlled by the Fe^{2+} Fur repressor and the FecI FecR regulatory proteins in response to ferric citrate, respectively. OM, outer membrane; PP, periplasmic space; CM, cytoplasmic membrane; CP, cytoplasm; RNAP, RNA polymerase.

(Bradbeer, 1993). It is thought that energy flows from the cytoplasmic into the outer membrane via the Ton complex. TonB (Postle and Skare, 1988; Hannavy *et al.*, 1990) and ExbD (Kampfenkel and Braun, 1992) are anchored to the cytoplasmic membrane by their N-terminal ends, while the larger portions are located in the periplasmic space. In contrast, ExbB spans the cytoplasmic membrane three times and large loops are exposed to the cytoplasm (Kampfenkel and Braun, 1993). Genetic (Heller *et al.*, 1988; Schöffler and Braun, 1989) and biochemical (Günter and Braun, 1990; Skare *et al.*, 1993) evidence suggests that TonB interacts with the outer membrane proteins involved in the translocation of ferric siderophores.

In the presence of a 20-fold surplus of citrate over Fe^{3+}, the precipitation of Fe^{3+} as polymeric ferric hydroxide is prevented and a soluble complex is formed (Spiro *et al.*, 1967), which consists predominantly of ferric dicitrate (Hussein *et al.*, 1981). Ferric dicitrate, with a molar mass of 434, is small enough to diffuse through the channels formed by the porins, which exclude hydrophilic compounds >600 Da. If ferric dicitrate diffuses through the porin channels, induction of the Fec transport system should only depend on FecA and the Ton complex at low ferric citrate concentrations where the transport of ferric citrate is faster than the diffusion through the porin channels. At higher ferric citrate concentrations the diffusion rate should be sufficient to provide enough ferric dicitrate in the periplasm for induction.

In this study we examined the concentration dependence of *fec* induction in mutants of outer membrane transport. We also constructed *fecA* point mutants with the aim of uncoupling induction and transport. The results obtained demonstrate that FecA and the Ton complex display dual activity: they are required for induction of the *fec* transport genes and for transport of ferric citrate into the periplasmic space. Transport of ferric citrate into the periplasmic space is not required for induction. These results suggest a signal transduction pathway from the cell surface into the cytoplasm.

Results

Bypass of the outer membrane transport system consisting of FecA and the TonB, ExbB and ExbD proteins

We examined the involvement of FecA in the induction of *fec* transport gene transcription, independent of FecA and Ton-coupled ferric citrate transport into the periplasmic space. All experiments were performed with *aroB* mutants which did not form enterobactin, the only siderophore synthesized by *E.coli* K-12. Therefore, the iron supply depended on added citrate which forms a ferric dicitrate complex when the citrate concentration in the culture medium exceeds the iron concentration by at least 20-fold. Citrate concentrations that are sufficiently high to support growth should diffuse through the porin channels into the periplasmic space and then be transported across the cytoplasmic membrane through the activities of the *fecBCDE* genes. A *fecA* mutant that displayed no substantial reduction in the expression of the *fecBCDE* genes located downstream of *fecA* was required to determine *fecBCDE* transcription rates and to measure the uptake of iron independent of FecA. *E.coli* WA1031 was unable to grow on Fec plates, which consisted of nutrient agar in which 0.2 mM 2,2′-dipyridyl reduced the available iron so that added 1 mM citrate served as the sole iron source (Wagegg and Braun, 1981). Transformation of WA1031 with plasmid pSV6 *fecA* restored growth on Fec plates, showing that WA1031 was mutated in *fecA* with no apparent polar effect on *fecBCDE*. Sequencing of *fecA*

Table I. FecA and porin-mediated growth stimulation by ferric citrate as the sole iron source

Strain	Growth zone in mm				
	1 mM[a]	10 mM[a]	50 mM[a]	100 mM[a]	1 M[a]
AB2847	21	25	ND	40	48
WA1031	0	0	((11))	((15))	(25)
WA1031 pSV6 *fecA*	15	24	30	36	48
WA1031 pUP40 *fecBCDE*	0	0	(10)	(15)	(25)
WA1031 pIS125 *fecIR*	0	0	(10)	(15)	28
WA1031 pMMO126 *fecI*	0	0	(11)	(15)	26
AA93	0	0	0	0	0
AA93 pSV662 *fecIRABCDE*	10	15	30	40	50
AA93 pIS200 *fecIRBCDE*	0	0	(11)	(15)	25
AA93 pUP40 *fecBCDE*	0	0	(10)	(15)	26

Growth promotion was tested on NBD agar plates around filter paper disks which were loaded with 20 µl of citrate solution as indicated. Numbers in parentheses indicate weak and in double parentheses very weak growth promotion. ND = not determined.
[a]Citrate concentration on paper disks.

from WA1031, after amplification by PCR, revealed in three independently isolated PCR products a G/T substitution resulting in His which replaced wild-type Arg at position 413 (Arg413His) of the published FecA sequence of *E.coli* K-12 (Van hove *et al.*, 1990). One PCR product contained additional Val262Ala and Phe742Leu substitutions, and another contained a Tyr521His substitution. The mutations found only in one PCR product were probably introduced by PCR, which is also supported by the finding that the Arg413His substitution determines the phenotype (see below). All three PCR products contained the following nucleotide replacements: A at position 4227 replaced by G (A4227G), G4485T and T4917C. The same nucleotide substitutions were found when additional mutated *fecA* genes were sequenced (described later in Figure 4). These nucleotide substitutions do not change the FecA amino acid sequence, although the published nucleotide sequence has to be corrected. Val262, Arg413, Tyr521 and Phe742 were contained in the mutated FecA proteins (Figure 4), supporting their presence in wild-type FecA.

Growth stimulation of WA1031 was tested on citrate-containing filter paper disks on nutrient broth agar supplemented with 0.2 mM dipyridyl (NBD plates). Citrate diffused from the filter paper, dissolved iron contained in the nutrient broth and competed for iron bound to dipyridyl. Only very weak growth zones appeared when the filter paper disks (6 mm diameter) contained 20 µl of 50 mM, 100 mM and 1 M citrate (Table I). If ferric citrate in the periplasm did not induce transcription of the *fecBCDE* transport genes, poor growth could be caused by a low transport rate through the cytoplasmic membrane. Indeed, growth was improved by transforming WA1031 with the plasmid pUP40 *fecBCDE*. A similar result was obtained by transforming WA1031 with the multi-copy plasmids pIS125 *fecI fecR* and pMMO126 *fecI* which overexpressed FecI and increased transcription of the chromosomal *fecBCDE* genes. Apparently, enough ferric citrate entered the periplasmic space to support growth, provided that transport through the cytoplasmic membrane was sufficiently active.

Additional experiments were performed with strain AA93 Δ*fec* in which the entire *fec* region was deleted. AA93 and WA1031 are derived from distinct parents so that any strain specificity influencing the results could be ruled out. AA93 did not grow on Fec plates and formed no growth zones around filter paper disks soaked with citrate solutions up to 1 M, showing that the response to citrate absolutely required the *fec* transport genes. Transformants carrying the entire *fec* operon *fecIRABCDE* on pSV662 grew on Fec plates and formed dense growth zones on NBD plates around filter paper disks soaked with 1 mM citrate (Table I). Transformants expressing *fecIRBCDE* on pIS200 and *fecBCDE* on pUP40 developed small colonies on Fec plates and displayed weak growth zones only at citrate concentrations of 50 and 100 mM; they showed a dense growth zone at 1 M citrate. The growth zone of AA93 pIS200 *fecIRBCDE* was similar to the growth zone of WA1031 transformed with pUP40 *fecBCDE*, showing that in both strains growth was promoted in the absence of *fecA* but required overexpressed *fecBCDE*.

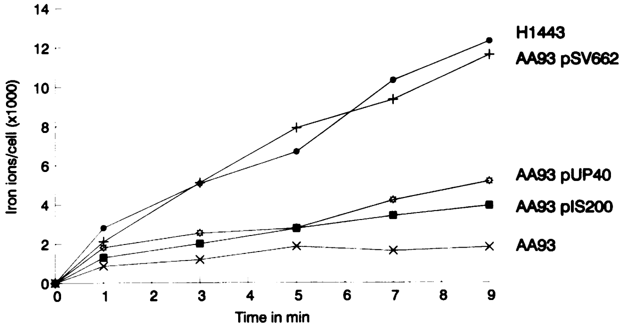

Fig. 2. Transport of $^{55}Fe^{3+}$ (1 µM) in the presence of 10 mM citrate into cells of *E.coli* H1443 *fec*$^+$ (●), AA93 Δ*fec* (×), AA93 Δ*fec* transformed with pSV662 *fecIRABCDE* (+), pUP40 *fecBCDE* (○) and pIS200 *fecIRBCDE* (■).

The growth promotion data were corroborated by transport determinations of $^{55}Fe^{3+}$ in the presence of a surplus of citrate (Figure 2). AA93 pIS200 and AA93 pUP40, both lacking *fecA*, transported iron at rates which were sufficient to support growth (Hussein *et al.*, 1981). AA93 pSV662 *fecIRABCDE* transported iron at a rate identical to H1443 *fec* wild-type (Figure 2).

Induction of fec transport gene transcription in fecA mutants

The above experiments demonstrated the entry of ferric citrate, if supplied at high concentrations, into the peri-

Table II. Uncoupling of ferric citrate induction and transport in *fecA* point mutants

Strain	Induction		Growth[a]	
	−Cit	+Cit	1 mM	10 mM
pSV66 *fecA*$^+$	11	1900	17	23
pIS135 Δ*fecA*	7	10	0	0
pCO3 *fecA3*	9	13	0	12
pCO38 *fecA38*	44	70	0	0
pCO4 *fecA4*	135	269	8	15
pCO4 *fecA4*[b]	189	214	0	0
pCO30 *fecA30*	208	1076	9	20
pCO27 *fecA27*	1009	2110	18	26

Induction was studied in strain AA93 Δ*fec* pMMO1034 *fecA*−*lacZ*. The values given reflect the β-galactosidase activities expressed in Miller units. Growth was determined in strain WA1031 *fecA*$^-$ (*fecB*$^+$*C*$^+$*D*$^+$*E*$^+$).

[a] Growth zone in mm around filter paper disks to which 20 μl of a 1 and 10 mM sodium citrate solution were added.

[b] Determinations in the *tonB* derivatives CO93 (induction) and CO1031 (growth). The plasmids listed contained in addition the *fecI fecR* regulatory genes.

plasmic space through the porin channels in growth-promoting quantities. Therefore, the induction of *fec* transcription was examined under growth-promoting conditions using a *fecA*−*lacZ* gene fusion on plasmid pMMO1034 so that the *fecA* transcription rate determined the level of *lacZ* expression. WA1031 was made *lac*$^-$ by inserting the transposon Tn*10* into the *lac* operon. The resulting strain IS1031 carrying pMMO1034 *fecA*−*lacZ* formed white colonies on MacConkey plates around a filter paper strip soaked with a 1 M citrate solution. Transformation of IS1031 pIS1034 carrying, in addition to *fecA*−*lacZ*, wild-type *fecA* resulted in red colonies at 1 mM citrate. The quantitative determination of β-galactosidase activity gave 4 U (background level, expressed in Miller units) in strain IS1031 pMMO1034 and 117 U in strain IS1031 pIS1034 at 1 mM citrate concentration, which increased to 210 U at 50 mM citrate while the 4 U in IS1031 pMMO1034 remained unchanged. Strain AA93 pIS200 *fecIRBCDE* pMMO1034 *fecA*−*lacZ* showed no *lacZ* expression (6 U), even at high citrate concentrations. AA93 *fecIRABCDE* pMMO1034 *fecA*−*lacZ* expressed 70 U of LacZ at 1 mM citrate and 104 U at 100 mM citrate. These experiments demonstrated that FecA was required for *fecA* induction, and that ferric citrate in the periplasm did not induce *fecA* transcription.

To prove that the lack of transport and induction of WA1031 were confined to the *fecA* Arg413His mutation, *fecA1031* containing only the Arg413His substitution was cloned on plasmid pIS332 downstream of *fecIR* to achieve sufficient *fecA1031* expression. AA93 pMMO1034 *fecA*−*lacZ fecA1031* formed white colonies on MacConkey−citrate plates, while transformants carrying *fecA* wild-type were red.

FecA mutants displaying constitutive fecA expression

The above experiments demonstrate that ferric citrate in the periplasm does not induce *fecA* transcription. FecA is the only known candidate in the outer membrane displaying ferric citrate binding specificity. The *fecA* gene was randomly mutagenized by PCR with the aim of isolating FecA derivatives with altered induction properties. Mutated *fecA* genes were cloned in pIS135 downstream of the two regulatory genes *fecIR* to study induction by ferric citrate. AA93 pMMO1034 *fecA*−*lacZ* was transformed with the mutated pIS135 derivatives and *lacZ* expression was screened on MacConkey−citrate agar and MacConkey containing 0.1 mM dipyridyl to reduce the available iron. Colonies which were pink and red on MacConkey−dipyridyl indicated *fecA* transcription in the absence of ferric citrate. Colonies which were white and pink on MacConkey−citrate indicated impaired induction. Representative examples of each type, which were obtained in different PCR mutagenesis experiments, are listed in Table II.

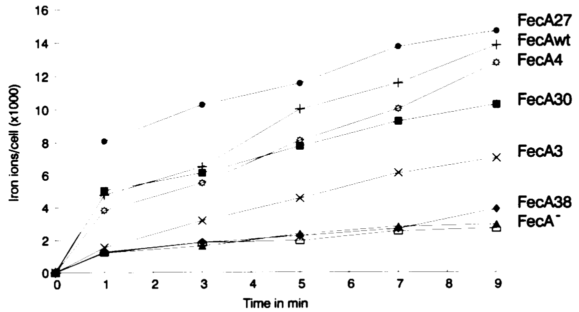

Fig. 3. Transport of $^{55}Fe^{3+}$ (1 μM) in the presence of 10 mM citrate into *E.coli* WA1031 *fecA* (□), transformed with pSV66 *fecIRA* (+), pCO27 *fecIRA27* (●), pCO4 *fecIRA4* (○), pCO30 *fecIRA30* (■), pCO3 *fecIRA3* (×), pCO38 *fecIRA38* (◆) and pIS135 *fecIR* (▲).

AA93 pMMO1034 carrying *fecA3* on pCO3 formed white colonies on MacConkey−citrate plates and showed only background expression of *fecA*−*lacZ* (Table II). However, growth of WA1031 transformed with pCO3 *fecA3* was stimulated by 10 mM ferric citrate (Table II). WA1031 pCO3 *fecA3* also displayed substantial transport activity (Figure 3). Citrate-mediated iron transport of WA1031 pCO3 *fecA3* was not reduced to an extent where induction could not occur. The mutation of *fecA3* affected induction much more strongly than transport, suggesting that induction did not necessarily follow transport. $^{55}Fe^{3+}$ transport was determined in a salt solution containing 0.1% glucose as an energy source. Prior to the transport assay, cells were grown for 2 h in nutrient broth supplemented with 1 mM ferric citrate to induce the transport system.

Additional mutants in *fecA* were obtained which demonstrated the uncoupling of induction from transport. *fecA38* conferred a 5-fold higher induction than *fecA3* in the presence and absence of ferric citrate, but it did not stimulate growth (Table II) and showed no transport (Figure 3), suggesting a direct function of FecA in induction independent of its transport activity. The strongest induction in the absence of ferric citrate was observed in *fecA27* transformants (Table II). *fecA27* conferred in the absence of ferric citrate a *fecA*−*lacZ* expression level which was half the value of the full induction seen with wild-type *fecA* in the presence of ferric citrate. WA1031 pCO27 also showed a high transport rate which was superior to the transport of WA1031 pSV66 expressing wild-type *fecA* (Figure 3). WA1031 pCO4 and WA1031 pCO30 showed intermediary induction in the absence of

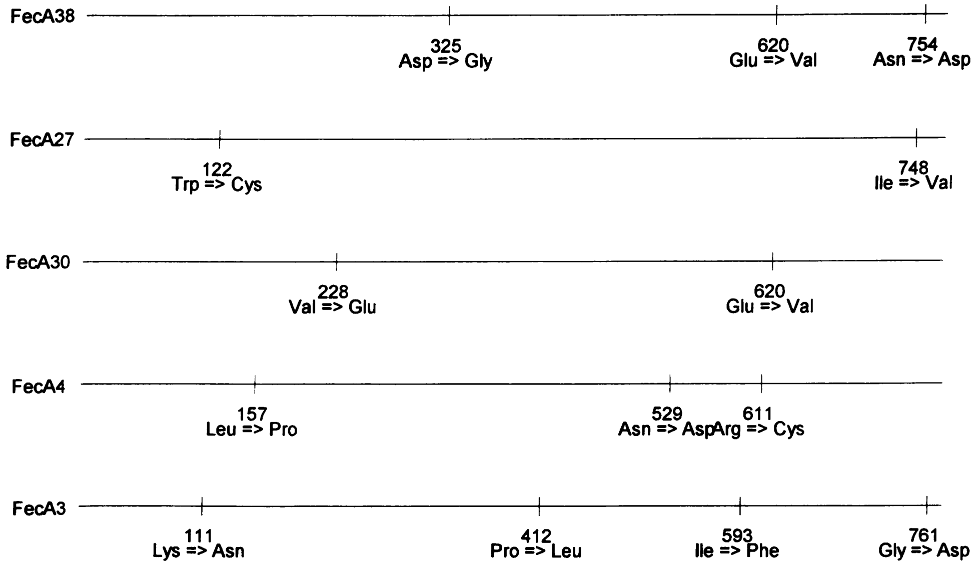

Fig. 4. Amino acid replacements at the positions indicated in the mutated *fecA* derivatives.

citrate, growth promotion by citrate and iron transport activities.

To exclude that the constitutive phenotype arose from citrate excreted by the mutants, citrate was determined in M9 culture medium. The values obtained were the same for wild-type and mutant strains and did not exceed 15 μM.

The rather high initial values after 1 min transport of the most active transformants probably arose from the strong transcription of the *fec* transport genes, so that binding to overexpressed FecA was increased. It appears that overexpression of the *fecBCDE* genes was less important, reflecting the observation made in the Fhu system that transport through the outer membrane was rate-limiting (Killmann *et al.*, 1993). Untransformed WA1031 and WA1031 pIS135 carrying only *fecI fecR* and no *fecA* exhibited no transport activities.

As induction was uncoupled from transport in the medium lacking ferric citrate, it was of interest to discover whether TonB was still required for induction. *tonB* mutants of AA93 and WA1031 were isolated and transformed with wild-type *fecA* and mutated *fecA* genes. The *tonB* transformants carrying wild-type and mutated *fecA* showed no induction, growth stimulation or transport (data not shown). The exception was *fecA4* (see footnote [b] in Table II) which conferred on the *tonB*$^-$ strain the same degree of *fecA*–*lacZ* transcription as on the *tonB*$^+$ strain. Ferric citrate only slightly increased transcription and supported growth only at high citrate concentrations (weak growth promotion at 50 mM, dense growth zone at 100 mM and 1 M citrate). No transport was observed which is typical for *tonB* mutants. Isolation of *tonB* mutants using highly active samples of colicin B and albomycin results in *tonB* knockout mutants, as demonstrated by the resistance of the *tonB* mutants obtained to phage ϕ80. Induction of *fecA* transcription in the absence of ferric citrate transport in the *tonB* mutant expressing *fecA4* further supports the direct participation of FecA in induction.

The constitutive fecA mutants carry missense mutations

The mutated *fecA* genes, conferring partially constitutive *fecA*–*lacZ* transcription, were sequenced to determine the kinds of mutation. All the mutants in the *fecA* gene carried only missense point mutations (Figure 4) which did not give rise to termination codons so that full-size FecA proteins and normal amounts could be expected. Only one of the mutated *fecA* genes, *fecA30*, carried an additional mutation (T2711C) in the promoter region of *fecA* proposed to be the binding site of the Fe^{2+} Fur repressor. No clustering of the mutations was observed, although an accumulation in the C-terminal region around residue 600 was apparent. FecA38 and FecA30 contained the same Glu to Val replacement but differed in additional mutations. No mutated *fecA* gene contained only a single mutation, so that the FecA phenotype could not be ascribed to an amino acid substitution although the strongest induction exerted by FecA27 was probably due to the replacement of Trp by Cys and not caused by the substitution of Val for Ile.

Synthesis of the mutated FecA proteins

In addition to growth promotion and *fecA*–*lacZ* transcription reflecting the degree of *fecABCDE* induction, the amount of FecA protein in the outer membrane of the FecA derivatives was determined. Transformants of *E.coli* AA93 carrying the wild-type *fecA* plasmid pSV66 and the mutant plasmids pCO30 *fecA30*, pCO4 *fecA4*, pCO27 *fecA27*, pCO38 *fecA38* and pCO3 *fecA3* were grown in M9 minimal medium in the presence and absence of 1 mM citrate. Because the plasmids also carried the *fecI fecR* genes, the expression was citrate-regulated. In each of the mutants a FecA protein of the size of wild-type FecA was expressed (Figure 5). FecA27 moved a little more slowly than the other FecA proteins, probably reflecting a conformational change caused by the mutation(s). FecA production was induced by ferric citrate; the relative amounts produced by the strains, as well as the increase obtained by citrate, agreed with the various transcription rates listed in Table II. FecA30 (Figure 5, lanes 1 and 2) and FecA27 (lanes 5 and 6) were the strongest bands in induced and uninduced cells; they produced more FecA than AA93 pSV66 *fecA* wild-type (lanes 9 and 10). The moderate expression of FecA4 and FecA38, induced and

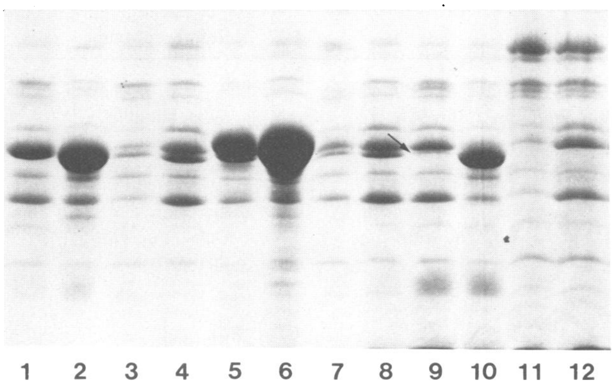

Fig. 5. Section of a polyacrylamide gel after separation of the outer membrane proteins of *E.coli* AA93 transformed with plasmids pCO30 (lanes 1 and 2), pCO4 (lanes 3 and 4), pCO27 (lanes 5 and 6), pCO38 (lanes 7 and 8), pSV66 (lanes 9 and 10) and pCO3 (lanes 11 and 12). Cells were grown in M9 medium unsupplemented (odd numbers) and supplemented with 1 mM citrate (even numbers). The proteins were stained with Serva blue.

uninduced (lanes 3 and 4, and 7 and 8 respectively), and the low expression of FecA3 (lanes 11 and 12), also corresponded with the transcription rate of the *fecA*−*lacZ* fusions. In AA93 pSV66 *fecA* a very weak FecA band was observed when cells were grown in the absence of citrate (lane 9, the arrow indicates uninduced FecA).

Discussion

Citrate and iron were required for the induction of the citrate-dependent Fe^{3+} transport system (Fec system) of *E.coli*. The trapping of iron in a siderophore which could not be taken up abolished citrate induction (Hussein *et al.*, 1981). Too much iron repressed the Fec system via the Fur protein loaded with Fe^{2+}. The main, or only, promoter of the *fec* transport genes is located upstream of *fecA*, which responds to ferric citrate induction and Fur Fe^{2+} repression. The *fec* transport genes were almost fully inducible by 0.1 mM citrate. The same concentration was required to keep the system induced, which argued against uptake of ferric citrate for induction because the inducing ferric citrate concentration would become lower due to improved transport by the higher amounts of the transport proteins. *In vitro* studies on *fec* mRNA synthesis revealed that transcription of the *fec* regulatory genes *fecI* and *fecR* was regulated by Fur Fe^{2+} but did not respond to ferric citrate (S.Enz, V.Braun and J.Crosa, unpublished results).

Transcriptional control by an inducer which does not enter the cytoplasm suggested a regulatory mechanism via the two-component system in which the cytoplasmic membrane-bound sensor protein phosphorylates the receiver protein in the cytoplasm, which in turn induces transcription (Stock *et al.*, 1989). As FecI and FecR contain no amino acid sequences which are found around the conserved phosphorylated histidine and aspartate residues of sensors and receivers, a two-component mechanism for *fec* regulation was unlikely (Van hove *et al.*, 1990).

In this paper we show that *fec* transport gene regulation is different from the two-component regulatory system. The bypass of outer membrane transport by diffusion through porins resulted in no induction. Growth-promoting concentrations of ferric citrate in the periplasm did not induce *fecA* transcription. The possibility that the ferric citrate concentration required for induction is higher than the concentration necessary for growth does not make sense because it would mean that the transport system is already active before its induction. The need to overexpress *fecBCDE* for stimulating growth in a *fecA* mutant came from the lack of induction by ferric citrate entering the periplasmic space through the porins.

The direct involvement of FecA in induction, independent of its transport activity, was demonstrated by *fecA* point mutants which conferred induction of *fecA*−*lacZ* transcription in the absence of ferric citrate. In addition, transformants carrying *fecA3* transported iron via citrate but were not induced by ferric citrate; transformants carrying *fecA38* were inducible by ferric citrate but did not transport iron. The various *fecA* mutants displayed different degrees of *fecA* transcription. Mutant *fecA27* showed a very high constitutive *fecA* expression which in the absence of ferric citrate was almost as high as a fully-induced wild-type *fec* strain. In this strain and in the other constitutive *fecA* strains the expression of *fecA* could be increased only ~2-fold by ferric citrate. The exception was *fecA30* which exhibited a 5-fold induction. However, *fecA30* contains, in addition to the point mutations in *fecA*, a point mutation in the *fecA* promoter region which may cause the stronger inducibility. We hypothesized that the altered FecA proteins assume conformations which are close to the inducing conformation of wild-type FecA. In this model, a low response of the mutated FecA derivatives to citrate would be expected, as has been observed. The system works autocatalytically, in that induction increases the level of FecA, and FecA increases induction. However, the transport system is adapted to the iron supply since FecA also determines the iron transport rate, so that increasing concentrations of intracellular iron shut off *fecIRABCDE* transcription via the Fur repressor.

Induction of the *fec* transport genes was dependent on the TonB and ExbB proteins because mutants in *tonB* and *exbB* were no longer inducible (Zimmermann *et al.*, 1984). In the latter case, the *exbB* gene used carried a Tn*10* insertion that displayed a strong polar effect on the expression of *exbD*, which is located downstream of *exbB* (Braun and Herrmann, 1993); thus *exbB* and *exbD* may be required for *fec* transport gene induction. The constitutive *fecA* mutants still required *tonB* (*exbB* and *exbD* were not determined), except for mutant *fecA4* which induced *fecA* transcription independently of *tonB*. The latter mutant also showed a similar *fecA* transcription level regardless of the presence of ferric citrate. Apparently, FecA4 partially assumes a conformation which wild-type FecA adopts by interaction with TonB. Since *tonB* mutants do not transport ferric citrate, mutant *fecA4* supports the conclusion that induction does not require transport of ferric citrate across the outer membrane.

Citrate-mediated $^{55}Fe^{3+}$ transport rates into WA1031 expressing the various *fecA* mutant genes were similar, but started at different levels and correlated with the degree of *fecA*−*lacZ* transcription and the amounts of FecA protein in the outer membrane. Apparently,

Table III. Strains of *E.coli* K-12 and plasmids used

	Genotype	Reference/source
Strains		
H1443	*araD*139 Δ(*argF-lac*)U169 flb5301 *deo*C1 *pts*F25 *rbsR aroB rpsL150 relA1*	this institute
AB2847	*aroB malT tsx thi*	this institute
WA1031	AB2847 *fepA fecA*	Wagegg and Braun (1981)
IS1031	WA1031 *lac*::Tn*10*	this study
CO1031	WA1031 *tonB*	this study
AA93	F^{-}*araD139 ΔlacU169 rpsL150 relA1 flbB5301 deoC1 ptsF25 rbsR thi aroB Δfec*	Ochs *et al.* (1995)
CO93	AA93 *tonB*	this study
Plasmids		
pSU18	*ori* p15A Cm^{R}	Martinez *et al.* (1988)
pMc2-54	*ori* ColE1 Cm^{R}	H.-J.Fritz
pHSG576	pSC101 derivative Cm^{R}	Takeshita *et al.* (1987)
pBR328	*ori* ColE1 Ap^{R}, Cm^{R}, Tet^{R}	Soberon *et al.* (1980)
pACYC184	p15A, Cm^{R}	Chang *et al.* (1978)
pIS135	pHSG576 *fecIR*	Ochs *et al.* (1995)
pMMO126	pSU18 *fecI*	Ochs *et al.* (1995)
pIS125	pSU18 *fecIR*	Ochs *et al.* (1995)
pIS200	pHSG576 *fecIRBCDE*	this study
pMMO1034	pMLB1034 *fecA–lacZ* fusion	Ochs *et al.* (1995)
pIS1034	pMMO1034 *fecA*	Ochs *et al.* (1995)
pSV662	pHSG576 *fecIRABCDE*	S.Veitinger
pSV66	pHSG576 *fecIRA*	S.Veitinger
pSV7	pBR328 *fecA*	S.Veitinger
pUP40	pACYC184 *fecBCDE*	Pressler *et al.* (1988)
pIM92	pHSG576 *tonB*	I.Traub
pIS332	pHSG576 *fecIRA1031*	this study
pCO38	pHSG576 *fecIRA38*	this study
pCO27	pHSG576 *fecIRA27*	this study
pCO30	pHSG576 *fecIRA30*	this study
pCO4	pHSG576 *fecIRA4*	this study
pCO3	pHSG576 *fecIRA3*	this study

radioactive ferric citrate bound to the cells according to the amount of FecA protein in the outer membrane.

We propose a model in which transcriptional regulation of the *fec* transport genes is initiated by a conformational change in FecA triggered by the binding of ferric citrate. Transmission of the signal across the outer membrane involves the Ton system which presumably opens a channel in FecA. FecR either binds to or dissociates from FecA in the open conformation. This induces a conformational change in FecR which is transmitted across the cytoplasmic membrane to FecI, which in turn facilitates binding of the RNA polymerase to the *fecA* promoter. According to this model the transcriptional signal is transmitted from the cell surface across the outer membrane, periplasmic space and cytoplasmic membrane to the cytoplasm.

A regulatory device similar to that of the ferric citrate transport system was proposed recently for control of the expression of the outer membrane protein PupB which facilitates transport of pseudobactin BN7 and BN8 into *Pseudomonas putida* WCS358 (Koster *et al.*, 1994). Ferric pseudobactin induced synthesis of PupB. Two genes (*pupI* and *pupR*), located upstream of the *pupB* gene, were sequenced, and displayed high sequence homology to FecI and FecR. Mutants in *pupI* were no longer inducible, and *pupI* carried on a plasmid-induced *pupB* expression irrespective of the presence of pseudobactin. Mutants in *pupR* synthesized PupB independent of pseudobactin. Transformants of the *pupR* mutant carrying plasmid-encoded *pupI* showed increased *pupB* expression, whereas in *pupI pupR* transformants regulation by ferric pseudobactin was restored. These data supported the induction of *pupB* expression by PupI and repression by PupR, as proposed for the activities of FecI and FecR (Van hove *et al.*, 1990). In the Fec system FecR does not serve as a repressor but takes part in transcription activation in response to ferric citrate (Ochs *et al.*, 1995). A direct role for PupB in regulation independent of its transport activity was derived from experiments in which the N-terminal 86 residues of PupB were replaced with the corresponding region of PupA. The PupAB hybrid protein showed pseudobactin BN8 transport activity but conferred no *pupB* induction. Conversely, a PupBA hybrid displayed pseudobactin 358 transport activity and conferred *pupB* induction in response to pseudobactin 358. The N-terminal end of PupB conferred the induction specificity with regard to *pupB* but in response to pseudobactin 358 which is recognized by the PupBA hybrid. These experiments showed signal transduction from PupB upon transport of the ferric siderophore, and assigned the region important for induction to the N-terminal end of PupB which was predicted to be located in the periplasmic space.

It can be expected that the Fec and Pup regulatory systems will not remain the only examples where an external transcriptional signal crosses three compartments of a Gram-negative bacterial cell.

Materials and methods

Bacterial strains

The *E.coli* K-12 strains and plasmids used are listed in Table III. *E.coli* CO93 *tonB* and *E.coli* CO1031 *tonB* were obtained by isolating mutants of strain AA93 Δ*fec* and WA1031 *fepA* which were simultaneously resistant to colicin B and albomycin. The *tonB* mutants obtained were also resistant to phage ϕ80, showing an absence of any residual TonB activity. CO93 and CO1031 could be complemented to $tonB^+$ by transformation with plasmid pIM92 *tonB*. Strain IS1031 was constructed by P1 transduction of *lac*:Tn*10* into *E.coli* WA1031.

Media

Tryptone–yeast extract (TY) medium, nutrient broth (NB) medium and M9 minimal medium were used as described by Miller (1972). For the growth of $aroB^-$ mutants, M9 medium was supplemented with 2 mM 4-hydroxybenzoic acid, 2 mM 4-aminobenzoic acid and 0.1 mg/ml tryptophan, phenylalanine and tyrosine. Growth on ferric dicitrate was tested on Fec agar plates containing nutrient agar, 0.2 mM 2,2′-dipyridyl and 1 mM citrate.

Construction of plasmids

Plasmid pSV662 was obtained by cloning the 10.5 kb *Hin*dIII fragment of the Kohara λ phage 7G7 (Kohara *et al.*, 1987), which encompasses the entire *fec* region, into low-copy vector pHSG576. The same fragment cloned in the opposite direction was contained in pSV266. Plasmid pSV66 *fecIRA* contained the 10.4 kb *Sal*I fragment of plasmid pSV266. Plasmid pSV6 *fecA* contained the 7.9 kb *Xmn*I fragment of pSV66. To construct plasmid pSV7 *fecA*, the 4.2 kb *Hin*dIII–*Bam*HI fragment of plasmid pSV6 was cloned into vector pBR328. To construct plasmid pIS100 *fecIRBCDE*, a 4.4 *Kpn*I–*Bam*HI fragment, comprising the *fecBCDE* genes, was cloned into the *Kpn*I–*Bam*HI site of plasmid pIS125 *fecIR*. Plasmid pIS200 encompasses the same *fec* genes (4.6 kb *Pvu*II fragment) but in the low-copy vector pHSG576.

Recombinant DNA techniques

The isolation of plasmid DNA, use of restriction enzymes, ligation and agarose gel electrophoresis were performed by standard techniques.

Random mutagenesis of the fecA gene by PCR

The *Eco*RI site upstream of the *fecA* gene and the *Bam*HI site adjacent to the 3′ end of *fecA* were created by PCR using primers AA4 (5′-CCGTTAGAATTCAGTCTATTACGC-3′) and AA13 (5′-GGCCTGCTGCTGGGGATCCGCCACGCC-3′), which annealed upstream of *fec*A at 2652–2675 bp and downstream of *fecA* at 5216–5190 bp, respectively. The reaction mixture for mutagenesis was composed of 0.1 μg DNA of plasmid pSV7 containing the *fecA* sequence (Van hove *et al.*, 1990), 2 nmol of each primer, 0.25 mM dGTP, dTTP and dCTP, 0.05 mM dATP, 2.5 mM $MgCl_2$ and 1 U *Taq* polymerase in reaction buffer (Promega). For amplification of the DNA, the mixture was heated for 1 min at 94°C, 2 min at 58°C and 3 min to 72°C in a DNA Thermal Cycler TC1 (Perkin-Elmer Cetus, Norwalk, CT). After 30 cycles the mixture was heated for 10 min at 72°C. The resulting PCR product was cleaved with *Eco*RI–*Bam*HI and cloned into the *Eco*RI–*Bam*HI sites of pIS135 carrying the *fecIR* wild-type genes.

Screening for fecA mutants

The screening for *fecA* mutants was carried out in strain AA93 pMMO1034 on lactose MacConkey plates supplemented with 0.1 mM dipyridyl or 1 mM citrate. Red colonies on lactose MacConkey plates with dipyridyl indicated an enhanced *fecA–lacZ* expression, in contrast to cells expressing wild-type *fecA*. Pink colonies on lactose MacConkey plates with citrate indicated a lower induction than wild-type *fecA*.

Cloning of the fecA gene of strain WA1031

The chromosomal *fecA* gene WA1031 was amplified by PCR using primers AA4 and AA13 (see above; Güssow and Clackson, 1989; Ochs *et al.*, 1995). Three PCR experiments were carried out. The 2.53 kb products were examined by electrophoresis and the products recovered from the agarose gel and cloned into the *Eco*RI–*Bam*HI site of vector pMc2-54. After sequencing, the *fecA* genes were cloned into plasmid pIS135 *fecIR* for determination of the phenotypes.

DNA sequencing

DNA was sequenced according to the dideoxy-chain termination method (Sanger *et al.*, 1977) by using the Auto Read Sequencing kit (Pharmacia Biotech, Uppsala, Sweden). Fluorescein-15-dATP was used for labelling. Oligonucleotides complementary to certain sites of the *fecA* gene were used for sequencing. The reactions were analysed with an A.L.F. DNA sequencer (Pharmacia Biotech).

Determination of β-galactosidase activity

β-Galactosidase assays were performed according to Miller (1972) and Giacomini *et al.* (1992). Cells were cultured in M9 medium, or M9 medium supplemented with 1 mM citrate.

SDS–PAGE

Proteins of outer membranes were separated by electrophoresis on 8% polyacrylamide gels (PAGE) in the presence of SDS (Lugtenberg *et al.*, 1975). Outer membranes were prepared from cells grown to an OD_{578} of 0.4 in M9 medium supplemented with 5 μM $(NH_4)_2Fe(SO_4)_2$ or with 5 μM $(NH_4)_2Fe(SO_4)_2$ and 1 mM citrate, as described previously (Hantke, 1981).

Growth promotion assay

Growth promotion by ferric citrate as the sole iron source and the intensity of the growth zone were determined on NBD plates (NB containing 0.2 mM dipyridyl) seeded with 0.2 ml of an overnight culture of the strain to be tested in 3 ml overlay NBD agar supplemented with the appropriate antibiotics. Filter paper disks (6 mm diameter) were placed onto the NBD agar which contained 20 μl of sodium citrate solutions (1, 10, 50, 100 mM and 1 M).

Transport assays

Cells were grown in NB medium with 0.4% glucose and suspended in minimal medium supplemented with 0.1 mM nitrilotriacetate to an OD of 0.5 (5×10^8 cells/ml; Hussein *et al.*, 1981). The radioactive $^{55}Fe^{3+}$ solution consisted of 0.1 μM $^{55}Fe^{3+}$, 1 μM $FeCl_3$ in 0.02 N HCl, 10 mM sodium citrate, pH 6.7. Samples (0.45 ml) were withdrawn after 1, 3, 5, 7 and 9 min, filtered, washed twice with 2 ml of 0.1 M LiCl, dried and counted in a liquid scintillation counter.

Citrate determination

Citrate was determined in the M9 culture medium after 3 h growth to an OD of 0.5 at 578 nm using the citrate UV test of Boehringer (Mannheim, Germany).

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