

Gene regulation by transmembrane signaling

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Abstract

Studies of the ferric citrate transport genes in *Escherichia coli* K-12 have revealed a novel type of transcriptional regulation. The inducer, ferric citrate, binds to an outer membrane protein and must not be transported into the cells to initiate transcription of the ferric citrate transport genes. Rather, a signaling cascade from the cell surface across the outer membrane, the periplasm, and the cytoplasmic membrane into the cytoplasm transmits information on the presence of the inducer in the culture medium into the cytoplasm, where gene transcription occurs. The outer membrane protein FecA serves as a signal receiver and as a signal transmitter across the outer membrane. The FecR protein serves as a signal receiver in the periplasm and as a signal transmitter across the cytoplasmic membrane into the cytoplasm, where the FecI sigma factor is activated to bind RNA polymerase and specifically initiate transcription of the *fecABCDE* transport genes by binding to the promoter upstream of the *fecA* gene. Transcription of the *fecI fecR* regulatory genes is repressed by Fe^{2+} bound to the Fur repressor protein. Under iron-limiting conditions, Fur is not loaded with Fe^{2+} , the *fecI* and *fecR* genes are transcribed, and the FecI and FecR proteins are synthesized and respond to the presence of ferric citrate in the medium when ferric citrate binds to the FecA protein. Regulation of the *fec* genes represents the paradigm of a growing number of gene regulation systems involving transmembrane signaling across three cellular compartments.

FecA is a transport protein

Citrate-mediated transport of iron into *E. coli* involves four cellular compartments: the outer membrane, the periplasm, the cytoplasmic membrane, and the cytoplasm (Figure 1). Diferric dicitrate (Figure 2), hitherto designated ferric citrate, binds to the FecA protein at a site well above the cell surface (Figure 3).

FecA belongs to a group of outer membrane proteins that transport rare substrates across the outer membrane by an energy-coupled mechanism. The crystal structures of six of these transporters reveal the same basic design. Twenty-two anti-parallel β -strands form a β -barrel. The pore within the β -barrel is closed by a globular domain, designated as the cork, plug, or hatch. Transport

of substrates requires movement of the cork so that a pore is opened in the β -barrel. In addition, the substrates must be released from their binding sites, at which they strongly bind with a K_D in the nanomolar range. Movement of the cork and movement of amino acid side chains at the substrate binding site to reduce substrate affinity require energy, which is provided by the proton motive force (pmf) of the cytoplasmic membrane. The energy stored as the pmf is transferred into the outer membrane by a protein complex consisting of the TonB, ExbB, and ExbD proteins, which are anchored in the cytoplasmic membrane and extend into the periplasm. The protein complex somehow measures the pmf and reacts by assuming an energized conformation (Braun 1997; Braun *et al.* 2002; Postle & Kadner 2003). TonB directly

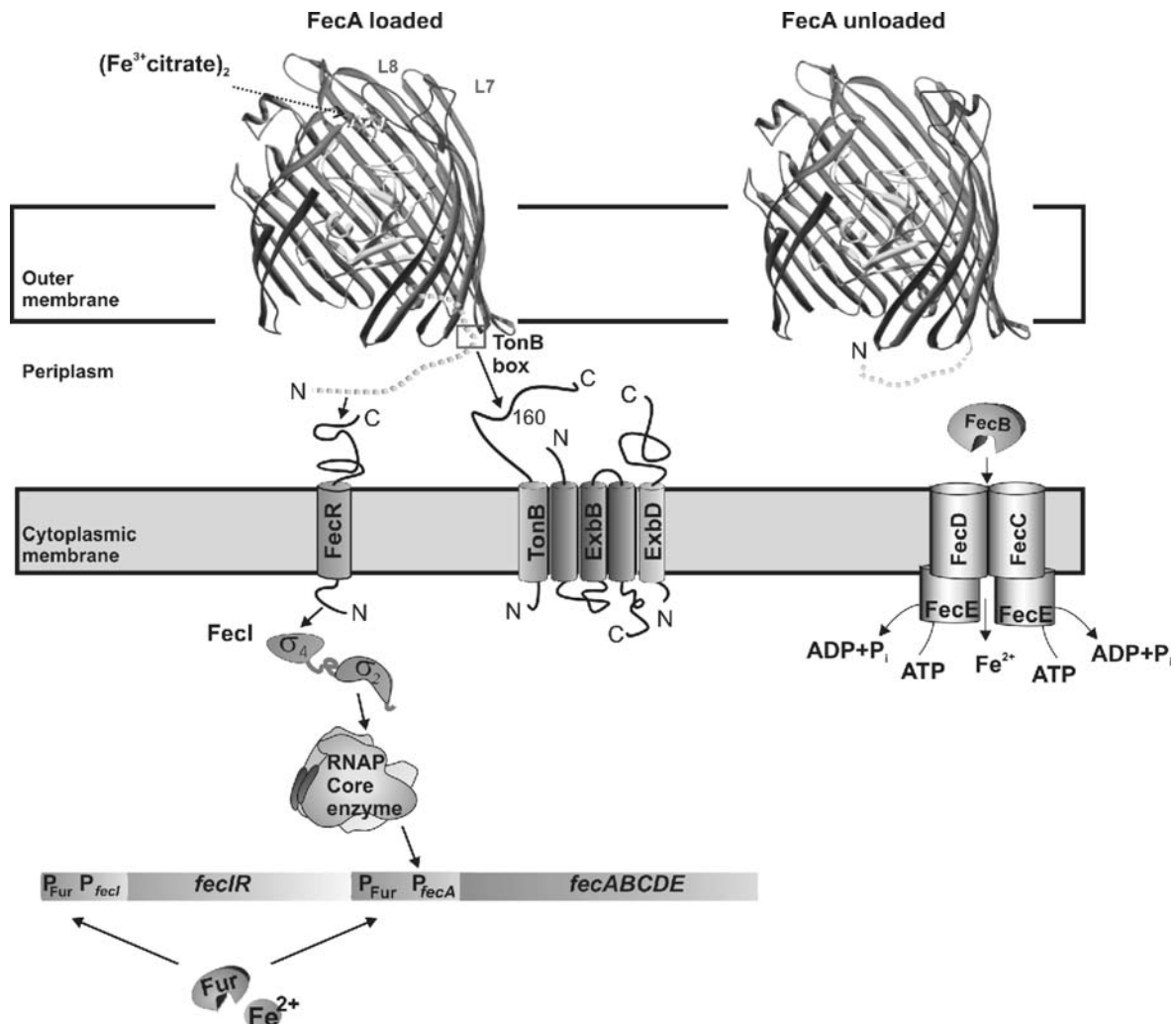


Figure 1. The ferric citrate transport and regulatory system. The signaling pathway from FecA to FecI; the involvement of TonB, ExbB, and ExbD in signaling and transport; and transport of iron through the periplasmic FecB protein and the ABC transporter FecCDE proteins are shown. Fe^{2+} -loaded Fur repressor binds to the promoter upstream of *fecI* and *fecA* and dissociates from the promoter under low iron conditions. Interactions between the FecA TonB box and TonB and between the FecA signaling domain and FecR are indicated. N indicates the N-terminal end, C the C-terminal end of the proteins. σ_2 and σ_4 indicate FecI domains involved in binding to FecR and DNA, respectively. (See text for details)

interacts with the outer membrane transporters. TonB in the energized conformation changes the conformation of the transporters such that the substrates are released from their binding sites and move through the open channel into the periplasm. This latter concept must still be experimentally proved.

Interaction of TonB with FecA was shown by cross-linking through cysteine disulfide bonds (Figure 4). The cysteine residues were introduced into the TonB box of FecA and region 160 of TonB. These regions were selected because earlier

genetic studies suggested that both are important for interactions between TonB and transporters: mutations in the TonB box of various other outer membrane transporters are suppressed by mutations in region 160 of TonB. For example, the TonB box mutation I9P (isoleucine at position 9 replaced by proline) and mutation V11D inactivate FhuA in all TonB-dependent activities, such as transport of ferrichrome and functioning as a receptor for phages T1, $\Phi 80$, and colicin M, but not for the TonB-independent phage T5, and activity is partially restored by mutations Q160L, Q160K,

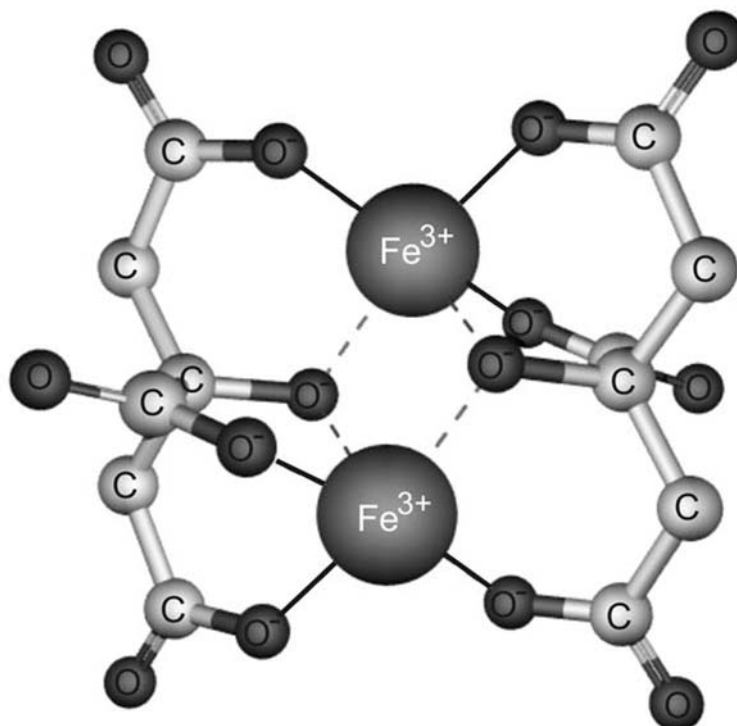


Figure 2. Structure of diferric dicitrate, the biologically active form of ferric citrate as bound to FecA

and R158L in TonB (Schöffler & Braun, 1989; Günter & Braun, 1990). However, FecA TonB box mutations are not suppressed by TonB mutations, but the TonB-box double mutant DTLTR (wild-type: DALTV) combined with TonB(R158L) is not active, whereas these mutations combined with wild-type TonB, TonB(Q160L), or TonB(Q160K) result in fully active derivatives. This result suggests an allele-specific interaction of FecA(DTLTR) with TonB(R158L). Mutants with the TonB-box mutations DAPTV, DALTG, DALTR, DANTV, or GTNTV do not grow with low concentrations of ferric citrate as the sole iron source, and *fec* gene transcription is not inducible by ferric citrate (Habeck 1998). However, DALTV of FecA replaced by the TonB box of FhuA (DTITV) or FepA (DTIVV) results in fully inducible and transport-active FecA derivatives (Ogierman & Braun 2003). The valine residue and a hydrophobic leucine or isoleucine seem to be essential for a functional TonB box. Replacement of aspartate by cysteine greatly diminishes the transport and induction activity of FecA, whereas replacement of the other TonB box residues by cysteine results in fully active FecA derivatives.

Spontaneous *in vivo* cross-linking between all cysteine residues in the FecA TonB box and TonB(Q160C), TonB(Q162C), or TonB(Q163C) has been observed. Therefore, the FecA TonB box is in close physical contact with region 160 of TonB. Since the yields of all cross-linked proteins were similar, the two interacting regions must be flexible, which results in various amino acid contacts. Cross-linking is independent of energization, as revealed by the inactive ExbD(D25N) mutant, whose outer membrane transport and receptor activities cannot be energized (Braun *et al.* 1996). Disulfide bond formation is also independent of the presence of ferric citrate, which upon binding to FecA apparently does not cause binding of FecA to TonB. This does not exclude the possibility that binding is enhanced by ferric citrate, which was not disclosed by the assay used.

FecA is a signaling protein

Independent of its transport activity, FecA also functions as a signaling protein. Results obtained

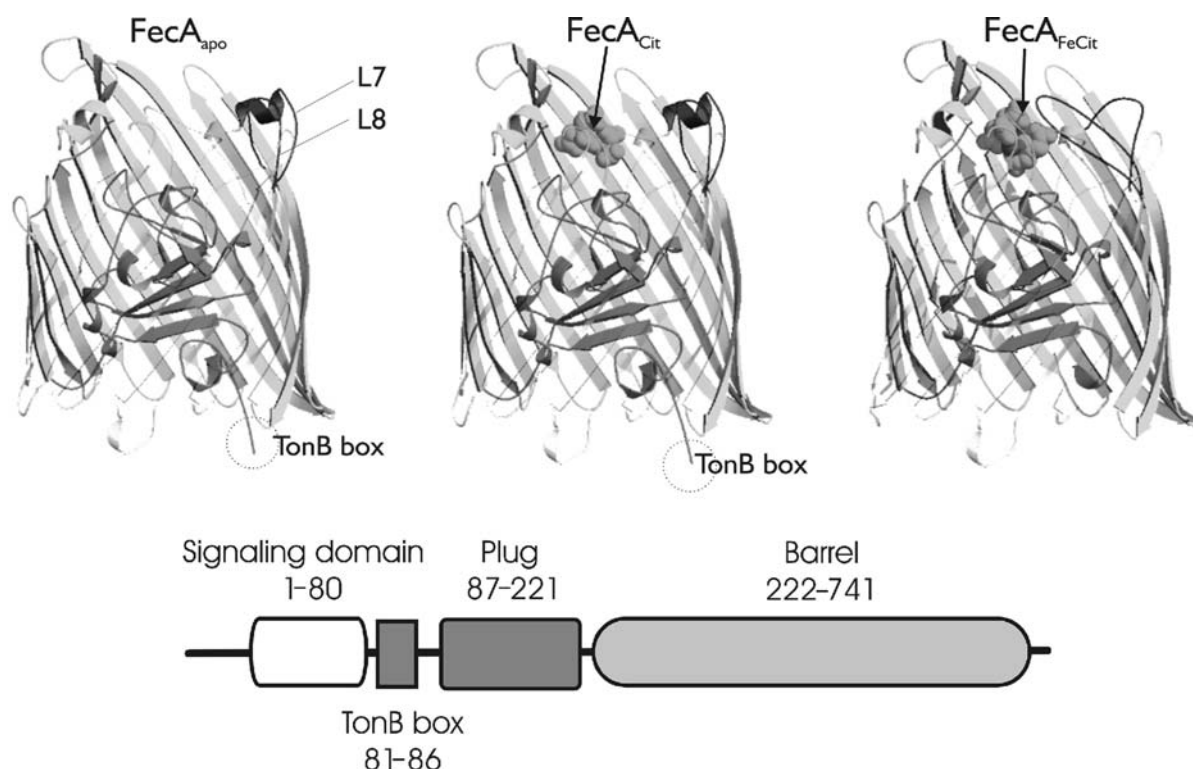


Figure 3. Crystal structures of unloaded FecA (FecA_{apo}), FecA loaded with dicitrate (FecA_{Cit}), and FecA loaded with diferric dicitrate (FecA_{FeCit}). Note the movement of loops 7 and 8 upon binding of diferric dicitrate. The domain structure of FecA, composed of the signaling domain, the TonB box, the plug (cork), and the β -barrel are shown (modification of figure from Yue *et al.* 2003)

clearly demonstrate direct involvement of FecA in induction. *fecA* deletion mutants are not inducible, even when ferric citrate concentrations high enough for ferric citrate to diffuse through the porins into the periplasm are used and proteins of the plasmid-encoded *fecBCDE* genes catalyze iron transport across the cytoplasmic membrane (Härle *et al.* 1995). Two FecA mutants with single mutations, I593F and W122C, constitutively transcribe *fec* transport genes (Härle *et al.* 1995; U. Stroehrer, personal communication). The *fecA4* mutant induces *tonB*-independent *fec* transport gene transcription, but does not transport ferric citrate.

Mature FecA contains a 79-residue peptide region N-terminal to the TonB box. This region is designated the signaling domain since its deletion abolishes induction of *fec* transport gene transcription but retains ferric citrate transport (Kim *et al.* 1997). The signaling domain is not seen in the crystal structures of FecA with bound ferric citrate or citrate, or without ligand (Figure 3). This absence in the structure could be caused by the flexi-

bility of the domain relative to the rest of the molecule or to the lack of a defined fold. The issue was resolved by NMR analysis of the structure in solution, which showed a defined unique fold (A. Ferguson and H. Vogel, personal communication; see also the contribution of H. Vogel in this volume).

Ferric citrate but not citrate serves as transcription inducer

Ferric citrate induces transcription of the *fec-ABCDE* transport genes. Removal of iron by deferric-ferrichrome, which is a much stronger chelator than citrate, abolishes induction (Hussein *et al.* 1981; V. Braun and C. Herrmann, unpublished results). Binding of ferric citrate (Ferguson *et al.* 2002) but not of citrate (Yue *et al.* 2003) causes strong movements of loops 7 and 8 by 11 and 15 Å, respectively. This movement closes the entrance of FecA to the diferric dicitrate and dicitrate binding sites. The two

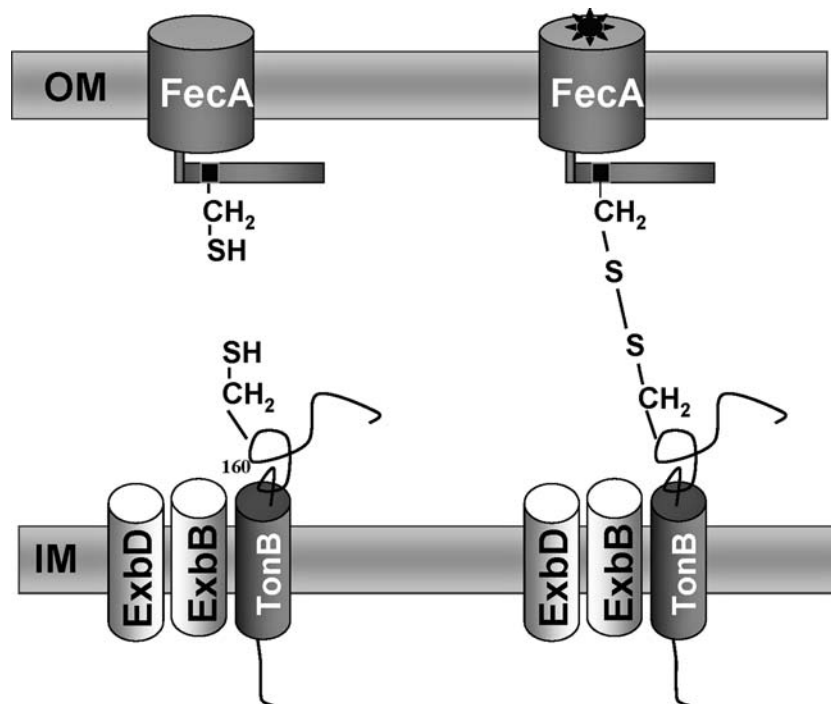


Figure 4. The interaction of the FecA TonB box with region 160 of TonB by spontaneous *in vivo* cross-linking of cysteine residues introduced into both regions (Ogierman & Braun 2003)

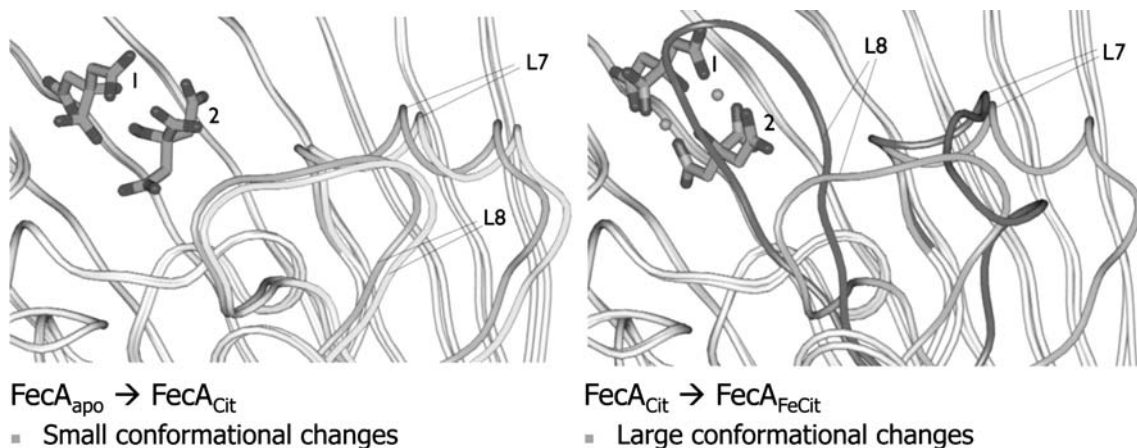


Figure 5. The similar but not identical binding of citrate and ferric citrate to FecA (Yue *et al.* 2003) (see also Figure 3). FecA_{Cit} → FecA_{FeCit} indicates that a large surplus of citrate over iron is used to obtain a defined diferric dicitrate solution. Under these conditions, dicitrate binds to FecA and is replaced by diferric dicitrate. Whether this has a physiological meaning is unknown

binding sites overlap, but they are not identical (Figure 5). Both loops are essential for transport of and induction by ferric citrate (Sauter & Braun 2004). In contrast, deletion of loops 7 and 8 in FhuA reduces but does not abolish FhuA activities (Endriß & Braun 2004). The FhuA crystal

structures do not reveal strong movements of loops 7 and 8 upon binding of ferrichrome (Ferguson *et al.* 1998; Locher *et al.* 1998). Despite the great similarity in the structures of this class of outer membrane transporters, they do not function identically.

In signal transduction, FecA interacts with FecR

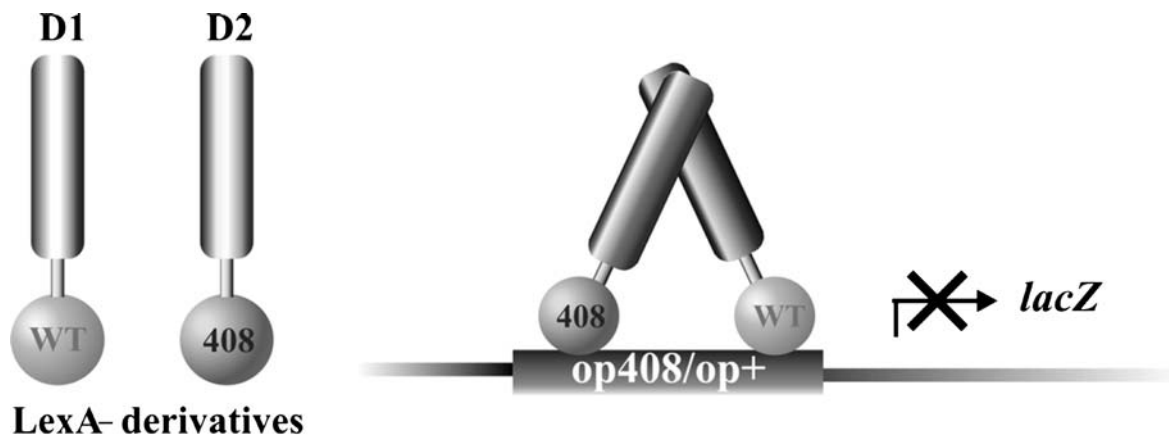
FecR is a transmembrane protein in the cytoplasmic membrane with the N-proximal end in the cytoplasm and the C-proximal end in the periplasm (Welz & Braun 1998). The signaling domain of FecA interacts with the C-proximal region of FecR in the periplasm, as shown *in vitro* by binding of FecA to FecR adsorbed to a Ni-agarose column by an N-terminal His₁₀ tag (Enz *et al.* 2000). Removal of the signaling domain abolishes binding of the FecA deletion derivative to FecR(His)₁₀. FecR(His)₆ does not retain FecA on the column, presumably because the C-terminal (His)₆ tag interferes with FecR binding.

Further evidence for the interaction of the periplasmic portion of FecR with the N-terminal signaling domain of FecA is derived from *in vivo* binding studies using a bacterial two-hybrid system (Figure 6). FecA₁₋₇₉ binds to FecR₁₀₁₋₃₁₇, and interaction is abolished by amino acid replacements in the periplasmic domain of FecR (Enz *et al.* 2003). The C-proximal FecR region between residues 237 and 317 is sufficient for binding to FecA₁₋₇₉. FecR(D138E, V197A) induces *fecA* transcription in the absence of ferric citrate and binds more strongly to FecA. In addition, L259G and F284L mutations in FecR are suppressed by G39R and D43D mutations in FecA (Enz *et al.* 2003). The FecA mutations are located on one side of the recently determined NMR solution struc-

ture of the FecA signaling domain (A. Ferguson and H. Vogel, personal communication). Other mutations impair the interaction between FecR and the FecA signaling domain. They are all located in the region of the suppressor mutations (E. Breidenstein, S. Mahren, and V. Braun, unpublished results), which provides strong evidence for a specific contact surface in FecA₁₋₇₉ that binds FecR.

FecR interacts with FecI for signal transfer to FecI

FecR is required for ferric-citrate-induced *fec* transport gene transcription. Synthesis of FecI in the absence of FecR results in a *fecA* transcription level only a few percent of that of the wild-type. FecI belongs to the extracytoplasmic sigma factors (ECF) or type 4 sigma factors of the large σ^{70} family (Gruber & Gross 2003; Paget & Helman 2003). σ^{70} factors bind to the -10 and -35 promoter regions and form multiple contacts to RNA polymerase. The structure of these sigma factors can be subdivided into various domains (Figure 2). Domain 2 is important for binding to the -10 promoter region and the coiled coil of the β' -subunit of RNA polymerase. Domain 4 contains a helix-turn-helix motif and a major RNA polymerase binding determinant, recognizes the -35 promoter region, and is involved in binding of anti-sigma factors. Crystal structures support the



LexA- derivatives

Figure 6. The bacterial two-hybrid system used for studying *in vivo* interaction between FecA (D2) and FecR (D1), and FecR (D2) with FecI (D1). The system is designed such that wild-type LexA repressor and a derivative of LexA bind to different regions of the *sulA* promoter. LexA dimerization is required for repressor activity, which is tested with suspected dimerizing proteins genetically linked to the DNA binding regions of LexA wild-type (WT) and mutant LexA (408). Only heterodimers and no homodimers can bind to the *sulA* promoter (Dmitrova *et al.* 1998)

modular design and show that the domains are connected by flexible linkers (Murakami *et al.* 2002; Vassilyev *et al.* 2002). Upon DNA binding of sigma factors with RNA polymerase, conformational changes occur in domains 2 and 4 and further structural transitions occur during transcription initiation that alter the contact sites to the RNA polymerase and to DNA.

DNA mobility band-shift experiments have demonstrated binding of a FecI-RNA polymerase complex to the promoter of *fecA*, and competition experiments between wild-type and mutant promoter regions have identified the region to which the complex binds (Angerer *et al.* 1995; Enz *et al.* 1995). Mutational studies of FecI support the importance of domains 2 and 4 in FecI activity and demonstrate that FecI belongs to the group 4 sigma factors (Ochs *et al.* 1996; Enz *et al.* 2000; Mahren *et al.* 2002, and unpublished results). Mutants in domains 2.2 and 4 exhibit a strongly reduced activity (Figure 7). Use of a bacterial two-hybrid system (Figure 6) has demonstrated that complete FecI₁₋₁₇₃ binds to the cytoplasmic domain of FecR, FecR₁₋₈₅, and that mutants in domain 4 of FecI and mutants in FecR₁₋₈₅ are impaired in FecI-FecR interaction. FecI binds to the β' subunit of RNA polymerase, and mutants in the FecI domain 2.2 are impaired in binding to the β' subunit (Mahren & Braun 2003). His-tagged FecI loaded on a Ni- agarose column binds FecR.

Mutations in *fecR* randomly generated by PCR abolish activation of *fec* transport gene transcription. The mutations generated are located at positions 19, 39, and 50 and all are tryptophan-to-arginine replacements. These tryptophan residues are highly conserved in homologs of FecR (Stiefel *et al.* 2001) and are located in the

cytoplasmic portion of FecR that interacts with FecI. Expression of FecR₁₋₈₅ induces constitutive transcription of the *fec* transport genes. Transcription initiation does not require ferric citrate, FecA, and TonB. The authors concluded that binding of FecR₁₋₈₅ to FecI either induces the active conformation of FecI or prevents degradation of FecI. FecR₁₋₈₅ mimics the active state of complete FecR after FecR has received the signal from FecA loaded with ferric citrate. Another FecR mutation, S127F, is located in the periplasmic portion and displays a partial constitutive phenotype. Ferric citrate induces *fec* transcription threefold above the constitutive level, which reaches only 64% of the wild-type level. This phenotype suggests that FecR(S127F) assumes a conformation similar to the ferric-citrate-induced FecR wild-type conformation (Ochs *et al.* 1995).

Control of the *fecI fecR* regulatory genes and the *fecABCDE* transport genes by the Fe^{2+} Fur repressor

Transcription of the *fecIR* genes is not induced by ferric citrate, and there is no autoregulation. Transcription is repressed by binding of the Fur protein loaded with Fe^{2+} to the *fecI* promoter. Under iron-limiting growth conditions, Fur is unloaded and becomes inactive, and the *fecIR* genes are transcribed. However, the FecIR proteins remain inactive until the signal elicited from FecA loaded with ferric citrate reaches the cytoplasm. FecI then becomes active, recruits the RNA polymerase, and directs it to the promoter upstream of *fecA*. *fecI* promoter mutants show an

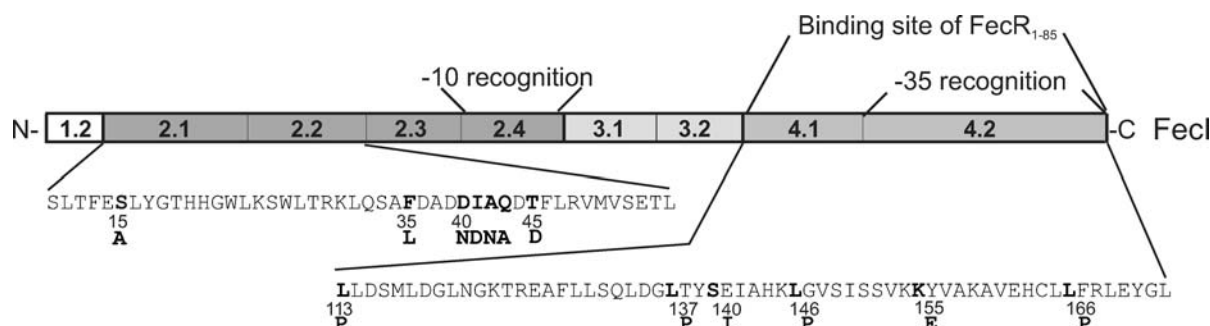


Figure 7. Domain structure of FecI with single amino acid replacements that strongly reduce the activity of FecI. For example serine (S) at position 15 is replaced with alanine (A)

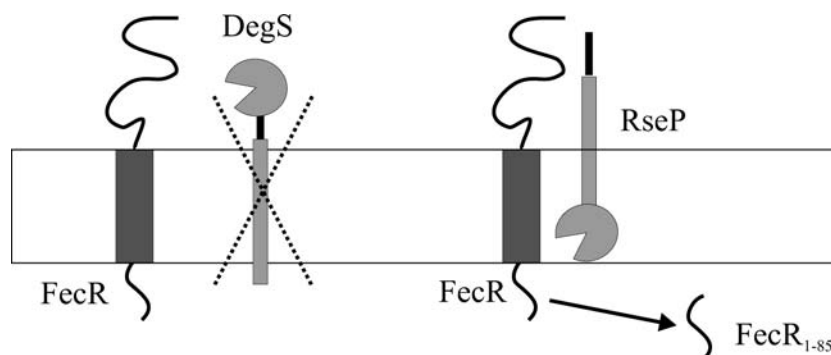


Figure 8. The predicted cleavage of FecR by the RseP protein in the cytoplasmic membrane of *E. coli*. Upon receiving the signal from FecA loaded with diferric dicitrate, FecR₁₋₈₅ or a somewhat larger FecR fragment is released from FecR by RseP. FecR₁₋₈₅ activates FecI, which recruits RNA polymerase and directs it to the *fecA* promoter, resulting in the transcription of the *fecABCDE* genes. DegS does not play a role in *fec* transcription initiation as it usually does for RseP activated systems

increased *fecI* transcription, which is caused by a reduced binding of Fe²⁺-Fur (Stiefel *et al.* 2001).

Fe²⁺-Fur controls *fecABCDE* transcription not only via *fecIR* repression but also by binding to the *fecA* promoter, thereby repressing the *fecABCDE* operon (Angerer & Braun 1998). Double control of *fecABCDE* transcription guarantees a rapid response to the iron status of the cells. With sufficient iron in the cells, Fe²⁺-Fur immediately represses *fecABCDE* transcription. If only *fecIR* repression would occur, the existing FecIR proteins would have to be diluted out by cellular growth until transcription ceases. The system is economically designed. Cells first recognize iron starvation, which results in synthesis of the FecIR regulatory proteins. However, the ferric citrate transport system is only synthesized when the cognate ferric-citrate-iron complex is present in the medium.

Is FecR activated by proteolytic cleavage through the RseP protease in the cytoplasmic membrane?

Constitutive activation of FecI by the genetically constructed cytoplasmic fragment of FecR suggests that proteolytic release of this fragment might be the natural mechanism by which the signal generated by conformational changes in FecA and FecR finally activates FecI. Intramembrane proteolysis is a widespread mechanism in prokaryotes and eukaryotes by which cells react to regulatory signals. *E. coli* responds to periplasmic stress elicited by misfolded outer membrane proteins by activating the DegS protease, which cleaves in the periplasmic portion of the RseA

regulatory protein anchored to the cytoplasmic membrane (Figure 8). This in turn leads to further RseA proteolysis within or at the inner side of the cytoplasmic membrane by the RseP protease. RseA is thus inactivated and no longer prevents σ^E from activating stress-dependent promoters (Alba *et al.* 2002; Young & Hartl 2003; Akiyama *et al.* 2004); To examine whether this proteolytic cascade also applies to activation of FecI, mutants in the proteases were used to determine synthesis of FecA by immunoblotting. Mutants in DegS and RseA did not alter the amounts of FecA, but mutants in RseP strongly reduced the amounts of FecA. Complementation of the *rseP* mutants by plasmid-encoded wild-type *rseP* restored FecA expression. RseP did not influence constitutive synthesis of FecA elicited by FecR₁₋₈₅. These results suggest that FecR is cleaved within or at the cytoplasmic membrane by RseP and that the cleavage product released into the cytoplasm activates FecI. The size of FecR₁₋₈₅ may closely resemble the natural RseP-generated FecR activation fragment. It is proposed that ferric citrate induction changes the conformation of FecR such that it becomes sensitive to specific RseP cleavage.

Occurrence of the *fecIR* regulatory mechanism

Table 1 lists the ferric citrate regulatory systems in *E. coli* and closely related bacteria. *Shigella flexneri* 2a YSH6000 carries the *fec* system on a plasmid pathogenicity island. The large virulence plasmid pLVPK of *K. pneumoniae* encodes a complete FecI, a C-terminally truncated inactive

Table 1. Similarity of the Fec proteins in Enterobacteria

Strain	FecI	FecR	FecA
<i>Escherichia coli</i> B	0	0	2
<i>Klebsiella pneumoniae</i>	0	0	0
Plasmid pLVPK	20	(31)	139
<i>Shigella flexneri</i>	0	0	0
<i>Enterobacter aerogenes</i>	0	0	^a
<i>Photobacterium luminescens</i>	42	18	97

Number of amino acid replacements related to those of the *E. coli* K-12 Fec proteins.

^ano FecA

Table 2. Frequent occurrence of the FecIRA type regulatory device

Strain	FecI	FecIR	FecIRA
<i>Bacteroides thetaiotaomicron</i>	24	4	23
<i>Nitrosomonas europaea</i>	22	20	15
<i>Pseudomonas aeruginosa</i>	2	4	8
<i>Pseudomonas putida</i>	11	1	11

FecR, and a FecA protein with an unusually long signal sequence of 71 residues. The overlong signal sequence is released during FecA secretion, and mature, transport-active FecA is incorporated into the outer membrane. However, FecA does not induce *fec* transport gene transcription since it lacks the signaling domain (Mahren *et al.* 2005). The genomes of *E. coli* O157:H7, *E. coli* CFT073, and *Salmonella enterica* do not encode *fec* genes. Those strains that carry *fec* genes seem to have acquired them by horizontal gene transfer. The *fec* genes are frequently encoded on mobile genetic elements. In *E. coli* K-12, the *fec* genes are flanked upstream by an IS1 element and downstream by an IS911 element that is disrupted by an IS10 element and a truncated IS2 insertion, and in *fec*⁺ strains, the degree of *fec* gene identity is higher than that of most genes.

Evidence for gene regulation by transmembrane signaling of the *fec* type is derived from sequenced annotated genomes of gram-negative bacteria (Wandersman & Stojiljkovic 2000; Visca *et al.* 2002; Braun *et al.* 2003; Schalk *et al.* 2004; Braun & Mahren 2005). A few systems have been studied experimentally. Two groups have been revealed. In one group, activity of the FecI homolog requires the FecR homolog, as in the *fec* system. In the other group, the FecI homolog is active in the absence of the FecR homolog, and the FecR

homolog functions as an anti-sigma factor. FecR could be an anti-sigma factor provided it binds FecI and prevents it from inactivation by proteolytic degradation or aggregation and precipitation. When FecR receives the signal, it dissociates from FecI, which immediately binds to RNA polymerase prior to its degradation. The constitutive activity of FecR₁₋₈₅ is difficult to reconcile with such a mechanism and favors more an activation of FecI by FecR.

Regulation by ECF sigma factors occur frequently in gram-negative bacteria. Those with strong homologies to FecI are listed in Table 2. For example, in the gut bacterium *Bacteroides thetaiotaomicron* there are 24 *fecI* homologs, 4 additional *fecIR* homologs where both genes are adjacent and 23 *fecIRA* homologs where all three genes are arranged as in *E. coli* K-12. It is predicted that the *fecIR* and *fecIRA* homolog function similar to the *fecIR* and *fecIRA* genes of *E. coli* K-12 which represent the paradigm for this type of gene regulation.

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