JOURNAL OF BACTERIOLOGY, Jan. 2001, p. 162–170 Vol. 183, No. 1
0021-9193/01/$04.00+0 DOI: 10.1128/JB.183.1.162–170.2001

Control of the Ferric Citrate Transport System of *Escherichia coli*: Mutations in Region 2.1 of the FecI Extracytoplasmic-Function Sigma Factor Suppress Mutations in the FecR Transmembrane Regulatory Protein

ALFRED STIEFEL, SUSANNE MAHREN, MARTINA OCHS, PETRA T. SCHINDLER, SABINE ENZ, AND VOLKMAR BRAUN*

Mikrobiologie/Membranphysiologie, Universität Tübingen, 72076 Tübingen, Germany

Received 26 July 2000/Accepted 13 October 2000

Transcription of the ferric citrate transport genes is initiated by binding of ferric citrate to the FecA protein in the outer membrane of *Escherichia coli* K-12. Bound ferric citrate does not have to be transported but initiates a signal that is transmitted by FecA across the outer membrane and by FecR across the cytoplasmic membrane into the cytoplasm, where the FecI extracytoplasmic-function (ECF) sigma factor becomes active. In this study, we isolated transcription initiation-negative missense mutants in the cytoplasmic region of FecR that were located at four sites, L13Q, W19R, W39R, and W50R, which are highly conserved in FecR-like open reading frames of the *Pseudomonas aeruginosa*, *Pseudomonas putida*, *Bordetella pertussis*, *Bordetella bronchiseptica*, and *Caulobacter crescentus* genomes. The cytoplasmic portion of the FecR mutant proteins, $FecR_{1–85}$, did not interact with wild-type FecI, in contrast to wild-type $FecR_{1–85}$, which induced FecI-mediated *fecB* transport gene transcription. Two missense mutations in region 2.1 of FecI, S15A and H20E, partially restored induction of ferric citrate transport gene induction of the *fecR* mutants by ferric citrate. Region 2.1 of σ^{70} is thought to bind RNA polymerase core enzyme; the residual activity of mutated FecI in the absence of FecR, however, was not higher than that of wild-type FecI. In addition, missense mutations in the *fecI* promoter region resulted in a twofold increased transcription in *fecR* wild-type cells and a partial restoration of *fec* transport gene transcription in the *fecR* mutants. The mutations reduced binding of the Fe^{2+} Fur repressor and as a consequence enhanced *fecI* transcription. The data reveal properties of the FecI ECF factor distinct from those of σ^{70} and further support the novel transcription initiation model in which the cytoplasmic portion of FecR is important for FecI activity.

Citrate does not serve as a carbon source for *Escherichia coli* K-12 since it is not taken up by the cells; however, Fe^{3+} delivered as a citrate complex is actively transported by the FecA protein across the outer membrane (46) and by the FecBCDE proteins (ATP binding cassette transporter) across the cytoplasmic membrane (33, 40). Transport studies using radiolabeled [$^{55}Fe^{3+}$][^{14}C]citrate revealed uptake of iron and only minimal amounts of citrate, indicating that only iron and not the iron complex enters the cytoplasm (21). Yet ferric citrate induces transcription of ferric siderophore transport genes and is the only ferric siderophore of *E. coli* K-12 known to do so (46). Intracellular ferric citrate does not serve as an inducer, and *fecBCDE* mutants impaired in the transport of iron across the cytoplasmic membrane are fully inducible (51).

The question arose as to how the inducer initiates transcription of transport genes in the cytoplasm when only iron and not citrate enters the cytoplasm. Mutants in the *fecA*, *tonB*, *exbB*, or *exbD* gene (the latter two in combination with *tolQ* or *tolR* mutations) are devoid of ferric citrate transport across the outer membrane and are not inducible (51). The obvious conclusion that entry of ferric citrate into the periplasm is required for induction has been ruled out by supplying to a *fecA* null mutant growth-promoting concentrations of ferric dicitrate (molecular mass, 434 Da) that enter the periplasm by diffusion through the porins. No induction of *fec* transport genes is observed (19), and the transport genes encoding cytoplasmic membrane transport activities have to be constitutively overexpressed from a multicopy plasmid to provide sufficient amounts of transport proteins.

A direct involvement of FecA in induction has been shown with *fecA* missense mutants that induce *fec* transcription constitutively in the absence of ferric citrate (19) but do not transport ferric citrate. In contrast to other *E. coli* ferric siderophore receptors, the mature FecA protein contains an N-terminal extension. When a portion of the extra peptide (residues 14 to 68) is removed by genetic means, induction is abolished, but FecA fully retains transport activity. An overexpressed N-terminal $FecA_{1–67}$ fragment inhibits induction but not transport (22). This proves that the N terminus of mature FecA specifically participates in signal transduction but is dispensable for transport.

The N-proximal portion of mature FecA is located in the periplasm (22) and most likely interacts with the FecR regulatory protein; residues 101 to 317 of FecR were localized to the periplasm, residues 86 to 100 were localized to the cytoplasmic membrane, and residues 1 to 85 were localized to the cytoplasm (31, 47). The transmembrane topology of FecR suggests that it transmits the signal, elicited by binding of ferric citrate to FecA, across the cytoplasmic membrane into the

* Corresponding author. Mailing address: Mikrobiologie/Membranphysiologie, Universität Tübingen, Auf der Morgenstelle 28, D-72074 Tübingen, Germany. Phone: 49-7071-2972096. Fax: 49-7071-295843. E-mail: volkmar.braun@mikrobio.uni-tuebingen.de.

cytoplasm. FecR does not directly act on the promoter upstream of the *fecA* gene that regulates transcription of *fecA* and of the *fecBCDE* genes downstream of *fecA* (2). Rather, FecR is required for the activity of the FecI sigma factor, which belongs to extracytoplasmic-function (ECF) sigma factors (28). No other ECF regulatory system has been studied with respect to the entire sequence of events, chemical entity of the signal, signal recognition, signal transmission, and signal response, to the same extent as the ferric citrate regulation system. Purified FecI mediates specific binding of the RNA polymerase core enzyme to the promoter region upstream of *fecA*, as revealed by DNA mobility band shift experiments, and promotes *fecA* transcription in vitro (2). *fecA* and *fecBCDE* mRNA formation is dependent on FecI under iron-limiting conditions in the presence of ferric citrate, as shown by Northern hybridization studies (12).

Interaction between the FecA, FecR, and FecI regulatory proteins has been demonstrated using two methods. In in vitro binding assays, FecA retained by FecR His tagged at the N terminus (His_{10}-FecR) and bound to a Ni-nitrilotriacetic acid agarose column is coeluted with His_{10}-FecR; FecI retained by FecR His tagged at the C terminus (FecR-His_6) bound to the column and is coeluted with FecR-His_6 from the column. An N-terminally truncated, induction-negative but transport-active FecA protein does not bind to His_{10}-FecR. In an in vivo assay, the FecA-, FecR-, and FecI-interacting regions have been determined using the bacterial two-hybrid Lex-based system. $FecA_{1–79}$ interacts with $FecR_{101–317}$, and $FecR_{1–85}$ interacts with $FecI_{1–173}$ (13).

The regulatory *fecIR* genes and the *fecABCDE* transport genes form separate transcripts (12). Transcription of *fecIR* is repressed by iron and the Fur protein but is unaffected by ferric citrate, while *fecABCDE* transcription is regulated by iron and Fur and by ferric citrate via FecI and FecR (3, 12). FecI and FecR regulate *fec* transport genes transcription, but they display no autoregulation (32). The iron transport genes are regulated by the internal iron concentration and by external ferric citrate. This is a very economical way of using ferric citrate as an iron source. When iron is not needed or when ferric citrate is not present, the transport system is almost totally shut off by cytoplasmic iron. When the iron concentration in the cytoplasm falls below a certain limit, the *fecIR* genes are transcribed. To turn on the ferric citrate transport system, the carrier has to be in the culture medium. In addition to regulatory functions, the FecA, FecR, and FecI proteins also have vectorial activities in that they transmit information through three cell compartments. Binding of ferric citrate to FecA induces a signal that is transferred from the cell surface into the periplasm and across the cytoplasmic membrane into the cytoplasm. It is thought that the information flux involves coupled conformational changes of FecA and FecR.

In this paper, we report further investigation of the unusual mechanism of transcription initiation mediated by FecA, FecI, and FecR. Induction-inactive missense mutants in the cytoplasmic portion of FecR and missense mutants in FecI which restore FecR-dependent FecI transcription initiation were isolated. The suppressing mutations obtained in FecI were located close to each other in region 2.1 of FecI. In addition, we isolated mutations in the *fecI* promoter which upregulated *fecI* transcription because they displayed a reduced binding of the Fe^{2+} Fur repressor.

MATERIALS AND METHODS

Bacterial strains, plasmids, and media. The *E. coli* strains and plasmids used in this study are listed in Table 1. Cells were grown in tryptone-yeast extract (TY) or nutrient broth (NB) as described previously (2). The antibiotics ampicillin (50 μg/ml), chloramphenicol (40 μg/ml), and tetracycline (12 μg/ml) were added to media as required.

Construction of plasmids. Plasmid pMMO202 was obtained by removing the cleavage sites for *Nde*I and *Bgl*I in pHSG576. To construct plasmid pMMO203, the *Bam*HI-*Hin*dIII *fecIR* fragment of pKW135 (containing an *Nde*I restriction site at the start codon of *fecR*) was cloned into *Bam*HI/*Hin*dIII-digested pMMO202.

fecR was randomly mutagenized by PCR using primers FecR1 (5′-CTGGAG TATGGCATATGAATC-3′) and FecIR3. The mutated *fecR* fragments were cloned into pMMO203, restricted with *Nde*I and *Bgl*I to replace the N-terminal 69 amino acids of wild-type FecR, resulting in plasmids pMMO222, pMMO247, pMMO252, and pMMO277. Site-directed mutagenesis of these plasmids by PCR was performed to introduce double nucleotide replacements using primers PTS222B (5′-CGCTGGCAACAGAAGTATGAACAGGATCAGG-3′), PTS47A (5′-AGCTGAACGTTGCGCTCGACGGCGGG-3′), PTS52A (5′-CACGGCATA TCTGTGGGAAGCTG-3′), and PTS277B (5′-GATAACCAGTGGGCCCGCCA GCAGGTTGAAAACC-3′), yielding plasmids pPTS222, pPTS247, pPTS252, and pPTS277 respectively.

fecI was randomly mutagenized by PCR using primers SVP1 (5′-CCGACAC ATGCCAGAAGCAGAGGATCCATCCC-3′) and FecIR3. The resulting *fecIR* fragments were cleaved with *Bam*HI/*Nde*I and cloned into the *Bam*HI/*Nde*I-restricted mutant *fecR* plasmids pPTS222, pPTS247, pPTS252, and pPTS277. To obtain plasmids pASc1, pASc2, and pASc3, the mutated *fecI* fragments were cloned into *Bam*HI/*Nde*I-cleaved pPTS252, pPTS222, and pPTS247, respectively. Plasmids pASc11, pASc12, and pASc13 were constructed by replacing the mutated *fecR* fragments in pASc1, pASc2, and pASc3 with wild-type *fecR*.

Wild-type and mutated *fecI* promoter regions were amplified by PCR using primers PromIEco (5′-GCGAATTCCCATCCCATTTTATACCTACC-3′) and PromIBam (5′-CGGGATCCGGAGTGCATCAAAAGTTAATTATC-3′). To construct plasmids pGFPI, pGFPI1, pGFPI2, and pGFPI3, the resulting PCR fragments were digested with *Eco*RI/*Bam*HI and cloned into *Eco*RI/*Bam*HI-restricted pFPV25.

To avoid mutations in the *fecI* promoter region, the mutated *fecIR* fragments were restricted with *Ase*I and *Nde*I and ligated into the *Ase*I/*Nde*I-cleaved mutant *fecR* plasmids pAS222, pAS247, pAS252, and pAS277, resulting in plasmids pAS321, pAS312, pAS323, and pAS344, respectively. Plasmid pAS202 was obtained by removing the cleavage site for *Ase*I in pMMO202. To construct plasmid pAS203, the *Bam*HI-*Hin*dIII *fecIR* fragment of pKW135 was cloned into *Bam*HI/*Hin*dIII-digested pAS202. For construction of plasmids pAS222, pAS247, pAS252, and pAS277, the *Nde*I-*Hin*dIII *fecR* fragments of pPTS222, pPTS247, pPTS252, and pPTS277 were ligated into *Nde*I/*Hin*dIII-cleaved pAS203.

To replace the *fos* zipper motif of plasmid pMS604, plasmids pAS203, pAS312, pAS323, and pAS344 were amplified by PCR with primers FecIBstEII (5′-GA TCGAGGTGACCATGTCTGACCGCGCC-3′) and FecIPst (5′-GGTTAACA CTGCAGTCATAACCCATACTC-3′). The resulting fragments were cloned into *Bst*EII/*Pst*I- or *Bst*EII/*Xho*I-cleaved pMS604, resulting in plasmids pSM173, pSM1731, and pSM1732 or plasmids pSM852, pSM853, and pSM854.

Plasmids pSM85, pSM8539, and pSM8550 were constructed by PCR amplification using primers FecRXhoI (5′-GGAAGTCTCGAGATGAATCCTTTGT TAACC-3′) and FecRBglII (5′-CAACAGAATCTTCATTTCATCACACGTG ACG-3′) and plasmids pAS203, pAS222, and pAS277 as DNA templates. To replace the *jun* zipper motif of plasmid pDP804, the resulting *Xho*I/*Bgl*II $fecR_{1–58}$ and mutated $fecR_{1–58}$ fragments were ligated into *Xho*I/*Bgl*II-digested pDP804.

For construction of plasmid pLCIRA, the *Eco*RI-*Bam*HI *fecA* fragment of pIS711 was ligated into *Eco*RI/*Bam*HI-cleaved pAS203. Plasmids pSM10, pSM11, and pSM12 were constructed by PCR using primers FecR1 and FecR85HindIII (5′-GAGCAAAAGCTTTAATCATTTCATCACGTGACG-3′) with the DNA templates pAS203, pAS222, and pAS277, respectively. The resulting *Nde*I-*Hin*dIII $fecR_{1–58}$ fragments were ligated into *Nde*I/*Hin*dIII-cleaved pLCIRA. To obtain plasmids pSM13, pSM14, and pSM15, the mutated *Bam*HI/*Nde*I *fecI* fragment of plasmid pAS323 was cloned into *Bam*HI/*Hin*dIII-digested pSM10, pSM11, and pSM12, respectively.

Recombinant DNA techniques. Standard techniques (34) or the protocols of the suppliers were used for the isolation of plasmid DNA, PCR, digestion with restriction endonucleases, ligation, transformation, and agarose gel electro-

TABLE 1. Bacterial strains and plasmids used

Strain or plasmid	Genotype	Reference or source
E. coli strains		
H1717	*fhuF*::λp*lac*Mu *aroB araD139* Δ*lacU169 rpsL150 relA1 flbB5301 deoC1 ptsF25 rbsR*	41
DH5α	*endA1 hsdR17*(r_K^- m_K^+) *supE44 thi1 recA1 gyrA relA1* Δ(*lacZYA-argF*)*U169* ϕ80 Δ*lacZ*M15	18
ZI418	*fecB*::Mud1 (Ap *lac*) *aroB araD139* Δ*lacU169 rpsL150 relA1 flbB5301 deoC1 ptsF25 rbsR thi*	45
MO704	*fecB*::Mud1 (Ap *lac*)*fecI*::Kan^r *'fecR aroB araD139* Δ*lacU169 rpsL150 relA1 flbB5301 deoC1 ptsF25 rbsR thi*	45
AA93	Δ*fec aroB araD139* Δ*lacU169 rpsL150 relA1 flbB5301 deoC1 ptsF25 rbsR thi*	31
SU202	*lexA71*::Tn*5 sulA211 sulA::lacZ* Δ(*lacIPOZYA*)*169*/F′*lacI*^{q} *lacZ*ΔM15::Tn*9*	9
Plasmids		
pMLB1034	*ori* ColE1 Amp^r contains promoterless *lacZ*	39
pMMO1034	pMLB1034 *fecA-lacZ* fusion	31
pHSG576	pSC101 derivative Cm^r	43
pMMO202	pHSG576 *Nde*I/*Bgl*I cleavage sites removed	This study
pKW135	pHSG576 *fecI fecR*	50
pMMO203	pMMO202 *fecI fecR*	This study
pMMO222	pMMO202 *fecI fecR*(T1865A)(W39R)	This study
pMMO247	pMMO202 *fecI fecR*(T1788A)(L13Q)	This study
pMMO252	pMMO202 *fecI fecR*(T1805A)(W19R)	This study
pMMO277	pMMO202 *fecI fecR*(T1898C)(W50R)	This study
pPTS222	pMMO202 *fecI fecR*(T1865A/G1866A)(W39R)	This study
pPTS247	pMMO202 *fecI fecR*(T1788A/G1789A)(L13Q)	This study
pPTS252	pMMO202 *fecI fecR*(T1805A/G1807A)(W19R)	This study
pPTS277	pMMO202 *fecI fecR*(T1898C/G1900C)(W50R)	This study
pASc1	pMMO202 *fecI*(A1120G *fecR*T1805A/G1807A)(W19R)	This study
pASc2	pMMO202 *fecI*(A1174T/A1211G *fecR*T1865A/G1866A)(W39R)	This study
pASc3	pMMO202 *fecI*(A1180G *fecR*T1788A/G1789A)(L13Q)	This study
pASc11	pMMO203 *fecI*(A1120G) *fecR*	This study
pASc12	pMMO203 *fecI*(A1174T/A1211G) *fecR*	This study
pASc13	pMMO203 *fecI*(A1180G) *fecR*	This study
pFPV25	*ori* ColE1 Amp^r contains promoterless *gfp*mut3	44
pGFPI	pFPV P_{fecI}-*gfp* fusion	This study
pGFP1	pFPV $P_{fecI(A1120G)}$-*gfp* fusion	This study
pGFP2	pFPV $P_{fecI(A1174T/A1211G)}$-*gfp* fusion	This study
pGFP3	pFPV $P_{fecI(A1180G)}$-*gfp* fusion	This study
pAS202	pMMO202 *Ase*I cleavage site removed	This study
pAS203	pAS202 *fecI fecR*	This study
pAS222	pAS202 *fecI fecR*(T1865A/G1866A)(W39R)	This study
pAS247	pAS202 *fecI fecR*(T1788A/G1789A)(L13Q)	This study
pAS252	pAS202 *fecI fecR*(T1805A/G1807A)(W19R)	This study
pAS277	pAS202 *fecI fecR*(T1898C/G1900C)(W50R)	This study
pAS321	pAS202 *fecI*(A1266T/T1275G)(S15A/T12S) *fecR*(T1788A/G1789A)(L13Q)	This study
pAS312	pAS202 *fecI*(C1246G/T1275G)(S15A/A5G) *fecR*(T1865A/G1866A)(W39R)	This study
pAS323	pAS202 *fecI*(T1275G)(S15A) *fecR*(T1788A/G1789A)(L13Q)	This study
pAS344	pAS202 *fecI*(T1274G/C1295A)(H20E) *fecR*(T1898C/G1900C)(W50R)	This study
pMS604	*ori* ColE1 Tet^r $lexA_{1–87}$-*fos* zipper	9
pSM173	pMS604 $lexA_{1–87}$-$fecI_{1–173}$	13
pSM1732	pMS604 $lexA_{1–87}$-$fecI_{1–173}$(T1274G/C1295A)(H20E)	This study
pSM1731	pMS604 $lexA_{1–87}$-$fecI_{1–173}$(T1275G)(S15A)	This study
pDP804	*ori* p15A Amp^r $lexA_{1–87}$408-*jun* zipper	9
pSM85	pDP804 $lexA_{1–87}$408-$fecR_{1–85}$WT	13
pSM8539	pDP804 $lexA_{1–87}$408-$fecR_{1–85}$(T1865A/G1866A)(W39R)	This study
pSM8550	pDP804 $lexA_{1–87}$408-$fecR_{1–85}$(T1898C/G1900C)(W50R)	This study
pT7-7	*ori* ColE1 phage T7 gene promoter	42
pIS711	pT7-7 *fecA*	22
pLCIRA	pAS202 *fecI fecR fecA*	This study
pSM10	pAS202 *fecI* $fecR_{1–85}$ *fecA*	This study
pSM11	pAS202 *fecI* $fecR_{1–85}$(T1865A/G1866A)(W39R) *fecA*	This study
pSM12	pAS202 *fecI* $fecR_{1–85}$(T1898C/G1900C)(W50R) *fecA*	This study
pSM13	pAS202 *fecI*(T1275G)(S15A)$fecR_{1–85}$ *fecA*	This study
pSM14	pAS202 *fecI*(T1275G)(S15A) $fecR_{1–85}$(T1865A/G1866A)(W39R) *fecA*	This study
pSM15	pAS202 *fecI*(T1275G)(S15A) $fecR_{1–85}$(T1898C/G1900C)(W50R) *fecA*	This study

phoresis. DNA was sequenced by the dideoxy chain termination method (35) using an AutoRead Sequencing kit (Pharmacia Biotech, Freiburg, Germany). The reaction products were sequenced on an A.L.F. DNA sequencer (Pharmacia Biotech).

PCR techniques. PCR amplification was carried out using *Taq* polymerase (Qiagen, Hilden, Germany) and standard conditions. DNA was initially denatured by heating to 94°C for 3 min. This was followed by 30 cycles consisting of denaturing at 94°C for 1 min, annealing at 54°C for 2 min, and extension at 72°C for 3 min. Random mutagenesis by PCR was carried out as previously described (26). Site-directed mutagenesis was performed according to Feinberg et al. (14).

Determination of β-galactosidase activity. β-Galactosidase activity was determined according to Miller (30) and Giacomini et al. (16). For measurement of induction activity, the cells were grown in NB medium without additions or supplemented as indicated with 0.2 mM 2,2′-dipyridyl or 1 mM citrate. For the LexA-based repression system, the cells were grown in TY medium supplemented with 1 mM IPTG (isopropyl-β-D-thiogalactopyranoside).

GFP measurements. Cells were grown in TY or NB medium as indicated. Green fluorescent protein (GFP) was quantified by fluorometry in an Bio-Tek FL500 microplate fluorescence reader (Bio-Tek Instruments Inc., Winooski, Vt.). Specific activity of GFP in bacterial cultures was expressed as relative fluorescence intensity at 530 nm of cells adjusted to an optical density at 578 nm of 0.5 in phosphate-buffered saline (44).

Similarity searching and sequence alignments. A global similarity search of the current National Center for Biotechnology Information nucleic acid databases with the advanced BLAST search and the specialized BLAST search of finished and unfinished microbial genomes was used to look for amino acid sequences homologous to the FecR sequence. Preliminary sequence data for *Bordetella pertussis* and *Bordetella bronchiseptica* were obtained from The Institute for Genomic Research website at http://www.tigr.org. Sequence data for *Pseudomonas putida* and *Caulobacter crescentus* were from the Sanger Center and can be obtained from ftp://ftp.sanger.ac.uk/pub/yyy. Sequences of *Pseudomonas aeruginosa* were obtained from the Pseudomonas Genome Project at http://www.pseudomonas.com/data.html. The sequences of *E. coli* FecR (M63115), *P. aeruginosa* HasR (AF127223) and PigE (AF060193), and *P. putida* PupR (X77918) were translated from the GenBank entries given in parentheses. Protein sequences were aligned by using CLUSTAL W.

RESULTS

Isolation of induction-negative *fecR* mutations. To identify functionally important residues of FecR and to determine regions of interaction between FecR and FecI, we isolated inactive *fecR* point mutants and examined restoration of transcription initiation of the *fec* transport genes by *fecI* point mutants. *fecR* was mutagenized by PCR, and the fragments encoding residues 1 to 69 of FecR were cloned into the low-copy-number plasmid pMMO203 *fecIR* to replace residues 1 to 69 of wild-type FecR. *E. coli* MO704 *fecI*::Kan *′fecR fecB-lacZ* was used to select *fecR* mutants. Insertion of the kanamycin resistance box resulted in the deletion of the 3′ end of *fecI* and the 5′ end of *fecR* (32). Transformants with the mutagenized pMMO203 plasmids were screened on MacConkey agar plates containing 1 mM citrate, which forms ferric citrate with iron contained in the nutrient agar. Under these conditions, *fecB-lacZ* transcription is induced by wild-type *fecIR*, and red colonies are formed; mutated *fecIR* does not induce *fecB-lacZ* transcription, and white and pink colonies are formed. The *fecR* genes of plasmids from white and pink colonies were sequenced; four missense mutations with a leucine-to-glutamine change at position 13 and three different tryptophan-to-arginine changes at residues 19, 39, and 50 were identified (Table 2).

β-Galactosidase activity of *E. coli* MO704 cells transformed with the pMMO203 *fecR* mutant derivatives and grown in NB medium with and without citrate supplementation was determined to verify the results obtained on the MacConkey plates. All four mutants with point mutations in *fecR* displayed very low ferric citrate-dependent *fecB-lacZ* transcription compared to the 35-fold increase of the wild-type *fecR*$^+$ strain in the presence of citrate (Table 2). The threefold increased activity of the *fecR* mutants in the presence of citrate may result from iron starvation, which derepresses *fecIR* and *fecABCDE* transcription, caused by the lack of citrate-mediated iron uptake of the *fecB-lacZ* transport mutant. The increased levels of mutant FecR and wild-type FecI could enhance the residual functional interaction between the proteins. Synthesis of the mutant FecR proteins was confirmed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis after overexpression in an IPTG-inducible T7 promoter system (data not shown). Since the *fecR* point mutants reverted to wild type in the subsequent suppression analysis with *fecI* mutants, a second nucleotide replacement was introduced into the mutated *fecR* codons (Table 1).

TABLE 2. *fecB-lacZ* transcription in *fecR* mutants

Plasmid	Mutation	Amino acid substitution	β-Galactosidase activity (Miller units)[a]	
			NB medium	NB medium + citrate
pMMO203	None	None	27	943
pMMO247	T1788A	L13Q	19	59
pMMO252	T1805A	W19R	15	54
pMMO222	T1865A	W39R	20	64
pMMO277	T1898A	W50R	18	59

[a] Determined in *E. coli* MO704 *fecI*::Kan *fecB-lacZ* transformed with plasmids carrying wild-type *fecI* and mutated *fecR*.

Mutations in the *fecI* coding region that restore transcription initiation by the *fecR* mutants. To determine sites of FecI that might interact with FecR, *fecI* mutations that suppress the *fecR* missense point mutations were isolated. To prevent mutations in the *fecI* promoter region, *fecI* lacking the promoter region was randomly mutagenized by PCR, and the DNA fragments were inserted into the pMMO203 *fecIR* derivatives encoding the *fecR* mutants with two mutations in one codon. Red colonies of *E. coli* MO704 transformants were screened on MacConkey plates, plasmids were isolated, and the *fecI* regions were sequenced. Four mutants were identified (Fig. 1B). Three of the four mutants showed a serine-to-alanine change at position 15 (S15A); one was a single mutation, one contained an additional alanine-to-glycine change (A5G), and one contained an additional threonine-to-serine change (T12S) (Table 3). These differences showed that all three S15A mutations resulted from independent events. All three mutants had similar citrate-dependent β-galactosidase activities, 21 to 28% of wild-type activity (Table 3), which suggests that the S15A mutation causes the partial restoration of transcription initiation (regarding A5G, see Discussion). In comparison to the β-galactosidase values of cells with wild-type *fecI* (Table 2), the *fecI* mutants displayed a three- to fivefold-higher citrate-dependent activity. The fourth mutant, with a single histidine-to-glutamic acid change at position 20, had β-galactosidase activity twofold higher than that of FecR(W50R) combined with wild-type FecI.

Since the *fecI* suppressor mutations were observed in combination with distinct *fecR* point mutations, we tested allele specificity. Each of the four *fecI* suppressor mutations was combined with each of the four *fecR* mutant genes in *E. coli* MO704. The β-galactosidase activities of the FecI derivatives with each of the FecR mutants were similar, 244 ± 10 U for

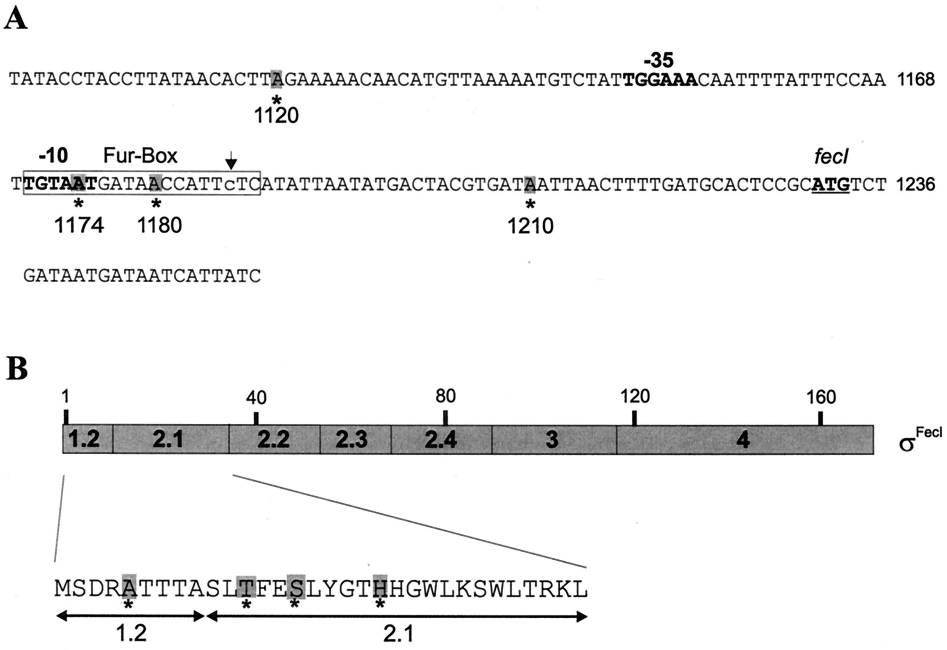

FIG. 1. (A) Promoter sequence upstream of the *fecI fecR* operon. Positions of mutations are indicated by asterisks, positions of −35 and −10 promoter regions are illustrated in bold letters, and the transcription start point is indicated by an arrow. The consensus sequence of the Fur box is illustrated below. (B) Schematic map of σ^{FecI} illustrating regions homologous to σ^{70}. The sites of amino acid substitutions are indicated below σ^{FecI}.

FecI(S15A, T12S), 198 ± 6 U for FecI(S15A, A5G), 267 ± 7 U for FecI(S15A), and 159 ± 6 U for FecI(H20E), which indicated no allele specificity.

FecI(S15A), FecI(S15A, W12S), and FecI(H20E) are located close together in region 2.1 of the sigma factor; this region in σ^{70} is implicated in RNA polymerase core binding (25, 38). Therefore, the missense mutations in *fecI* could have increased the affinity of FecI to the RNA polymerase core enzyme, resulting in a higher level of *fec* transport gene transcription. To examine this possibility, the *fecI* suppressor mutations were combined with the wild-type *fecR* gene. The β-galactosidase activities measured were similar to those of wild-type *fecIR*, on average 18 U in NB medium and 797 U in NB medium supplemented with citrate. In addition, the β-galactosidase activities of the FecI mutants in the absence of FecR were measured; the activities were lower than those of wild-type FecI (8 to 10 U and 20 U, respectively). These results provide no evidence that the S15A and H20E mutations alter the interaction of FecI with the RNA polymerase core enzyme or increase the amount of active FecI.

TABLE 3. *fecB-lacZ* transcription of *fecI fecR* mutants

Plasmid	*fecI* mutation	Amino acid substitution in:		β-Galactosidase activity (Miller units)[a]	
		FecI	FecR	NB medium	NB medium + citrate
pMMO203	None	None	None	27	943
pAS301	A1266T T1275G	S15A T12S	L13Q	8	254
pAS302	C1246G T1275G	S15A A5G	W39R	11	201
pAS303	T1275G	S15A	L13Q	15	265
pAS304	C1295A	H20E	W50R	40	157

[a] Determined in *E. coli* MO704 *fecI*::Kan *fecB-lacZ* transformed with plasmids carrying mutated *fecI* and *fecR*.

In vivo interaction of the FecI suppressor mutants with mutated FecR. We have recently shown that FecI interacts with the cytoplasmic N terminus of FecR ($FecR_{1–85}$) by using a bacterial two-hybrid system (13). To determine the physical interactions between the mutants of FecI and FecR, translational fusions were constructed between $LexA_{1–87}$408, which binds to one site of the *sulA* promoter (9), and the wild-type $FecR_{1–85}$ ($FecR_{1–85}$WT) derivatives, and between $LexA_{1–87}$, which binds to another site of the *sulA* promoter, and the FecI derivatives (Table 4). $FecR_{1–85}$ had to be used because it represents the cytoplasmic portion of FecR. Mutated $FecR_{1–85}$ combined with mutated FecI or wild-type FecI did not repress *sulA-lacZ* transcription, indicating that the mutated $FecR_{1–85}$ fragments did not interact with FecI. This could explain inactivity of the mutated FecR proteins. In contrast, $FecR_{1–85}$WT combined with mutated or wild-type FecI repressed *sulA-lacZ* transcription (Table 4).

Since the complete FecR and FecR mutant proteins combined with the FccJ mutant proteins induced transcription of *fecB-lacZ*, the induction of transcription of the truncated $FecR_{1–85}$ and mutant $FecR_{1–85}$ proteins was studied. β-Galactosidase activities were determined in *E. coli* AA93 Δ*fec*/pMMO1034 *fecA-lacZ* transformed with low-copy-number plasmids encoding *fecA* and the $fecR_{1–85}$ and *fecI* derivatives (Table 5). No transcription induction was recorded with the $FecR_{1–85}$ mutant proteins. These results agree with the lack of interaction of the truncated FecR mutant proteins in the bac-

TABLE 4. Binding of the cytoplasmic region of wild-type FecR and mutated FecR to wild-type FecI and mutated FecI

Protein combination	β-Galactosidase activity (Miller units)[a]	Protein interaction
$LexA_{1–87}$WT-Fos zipper and $LexA_{1–87}$408-Jun zipper	16	+
$LexA_{1–87}$WT-Fos zipper and $LexA_{1–87}$408-$FecR_{1–85}$WT	246	–
$LexA_{1–87}$408-Jun zipper and $LexA_{1–87}$WT-FecI	253	–
$LexA_{1–87}$408-$FecR_{1–85}$WT and $LexA_{1–87}$WT-FecI	42	+
$LexA_{1–87}$408-$FecR(W39R)_{1–85}$ and $LexA_{1–87}$WT-FecI	241	–
$LexA_{1–87}$408-$FecR(W50R)_{1–85}$ and $LexA_{1–87}$WT-FecI	221	–
$LexA_{1–87}$408-$FecR_{1–85}$WT and $LexA_{1–87}$WT-FecI(S15A)	44	+
$LexA_{1–87}$408-$FecR_{1–85}$WT and $LexA_{1–87}$WT-FecI(H20E)	32	+
$LexA_{1–87}$408-$FecR(W39R)_{1–85}$ and $LexA_{1–87}$WT-FecI(S15A)	251	–
$LexA_{1–87}$408-$FecR(W39R)_{1–85}$ and $LexA_{1–87}$WT-FecI(H20E)	231	–
$LexA_{1–87}$408-$FecR(W50R)_{1–85}$ and $LexA_{1–87}$WT-FecI(S15A)	236	–
$LexA_{1–87}$408-$FecR(W50R)_{1–85}$ and $LexA_{1–87}$WT-FecI(H20E)	247	–

[a] Determined by the bacterial two-hybrid LexA-based system in *E. coli* SU202 *sulA-lacZ*.

terial two-hybrid system. In the control, $FecR_{1–85}$WT combined with wild-type or mutated FecI displayed constitutive *fecA-lacZ* transport gene transcription, as found previously for the wild-type combination (31).

***fecI* promoter mutants that restore induction of the *fecR* mutants.** Originally we randomly mutagenized complete *fecI* by PCR. *E. coli* MO704 carrying one of the *fecR* mutant genes (Table 2) was transformed with the pooled *fecI* plasmids, and red colonies were isolated on MacConkey agar plates supplemented with 1 mM citrate. They displayed β-galactosidase activities two- to threefold higher (Table 6) than the values obtained with wild-type FecI (Table 2), which indicated a citrate-independent transcription initiation by the mutated *fecI* region.

TABLE 5. Induction of *fecA-lacZ* transcription by cytoplasmic FecR derivatives combined with wild-type and mutant FecI

Plasmid	Encoded Fec protein(s)[a]	β-Galactosidase activity (Miller units)[b]	
		NB medium + 50 μM dipyridyl	NB medium + 1 mM citrate
pHSG576	None	8	6
pLCIRA	IRA	54	219
pSM10	$IR_{1–85}$A	312	264
pSM11	$IR_{1–85}$(W39R)A	16	14
pSM12	$IR_{1–85}$(W50R)A	13	12
pSM13	I(S15A)$R_{1–85}$A	435	469
pSM14	I(S15A)$R_{1–85}$(W39R)A	9	9
pSM15	I(S15A)$R_{1–85}$(W50R)A	7	7

[a] I, R, and A denote the FecI, FecR, and FecA proteins.
[b] Determined in *E. coli* AA93 Δ*fec* carrying pMMO1034 *fecA-lacZ* and the listed plasmids.

TABLE 6. *fecB-lacZ* transcription in *fecI* promoter mutants combined with *fecR* mutations or wild-type *fecR*

Plasmid	*fecI* promoter mutation	Amino acid substitution in FecR	β-Galactosidase activity (Miller units)[a]	
			NB medium	NB medium + citrate
pMMO203	None	None	27	943
pASc1	A1120G	W19R	42	112
pASc2	A1174T A1210G	W39R	68	135
pASc3	A1180G	L13Q	105	150
pASc11	A1120G	None	330	1471
pASc12	A1174T A1210G	None	782	2,291
pASc13	A1180G	None	1,141	2,286

[a] Determined in *E. coli* MO704 *fecI*::Kan *fecB-lacZ* transformed with plasmids carrying mutated promoter *fecI* and mutated or wild-type *fecR*.

An additional two- to threefold increase was recorded in the presence of 1 mM citrate in the growth medium; this increase could be caused by iron limitation and ferric citrate induction.

Sequencing of the mutated *fecI* regions revealed nucleotide replacements upstream of the *fecI* open reading frame (Table 6). The A1120G replacement is located 28 bp upstream of the postulated −35 region; A1174T is in the −10 region and together with A1180G rests in the predicted Fur box to which the Fe^{2+} Fur repressor binds under iron-replete conditions (Fig. 1A). A1210G is located 24 bp downstream of the transcription start site. The *fecI* promoter mutants combined with wild-type *fecR* displayed in the absence of ferric citrate 12-, 29-, and 42-fold, and in the presence of ferric citrate 1.6-, 2.4-, and 2.4-fold, increases in *fecB-lacZ* transcription (Table 6).

Constitutive transcription by the *fecI* promoter mutants was also determined in *E. coli* AA93 Δ*fec* grown in NB medium with and without addition of 1 mM citrate. Since the Δ*fec* strain cannot take up ferric citrate, addition of citrate results in trapping of iron, making it unavailable to cells. Ferric citrate cannot act as an inducer since the cells lack FecA and FecR. Transcription initiation controlled by the mutated *fecI* promoter regions was examined by inserting the promoter regions upstream of a promoterless *gfp* gene. Two of the promoter mutants, A1174T, A1210G, and A1180G, displayed a two- to threefold increase in fluorescence relative to that of the strain carrying nonmutated *fecI*, whereas the A1120G mutation increased transcription only slightly (Table 7). In the latter mutant, iron starvation by addition of citrate increased transcription 2.4-fold, whereas the already strongly induced mutants showed only 1.2- and 1.6-fold enhancements of transcription.

TABLE 7. *fecI-gfp* transcription of *fecI* promoter and *fecR* mutants

Plasmid	*fecI* promoter mutation	Relative fluorescence[a]	
		NB medium	NB medium + citrate
pGFPI	None	416	946
pGFPI1	A1120G	461	1,098
pGFPI2	A1174T A1210G	773	1,220
pGFPI3	A1180G	1,163	1,408

[a] Determined in *E. coli* AA93 Δ*fec* transformed with plasmids carrying mutated *fecI* promoter and promoterless *gfp*.

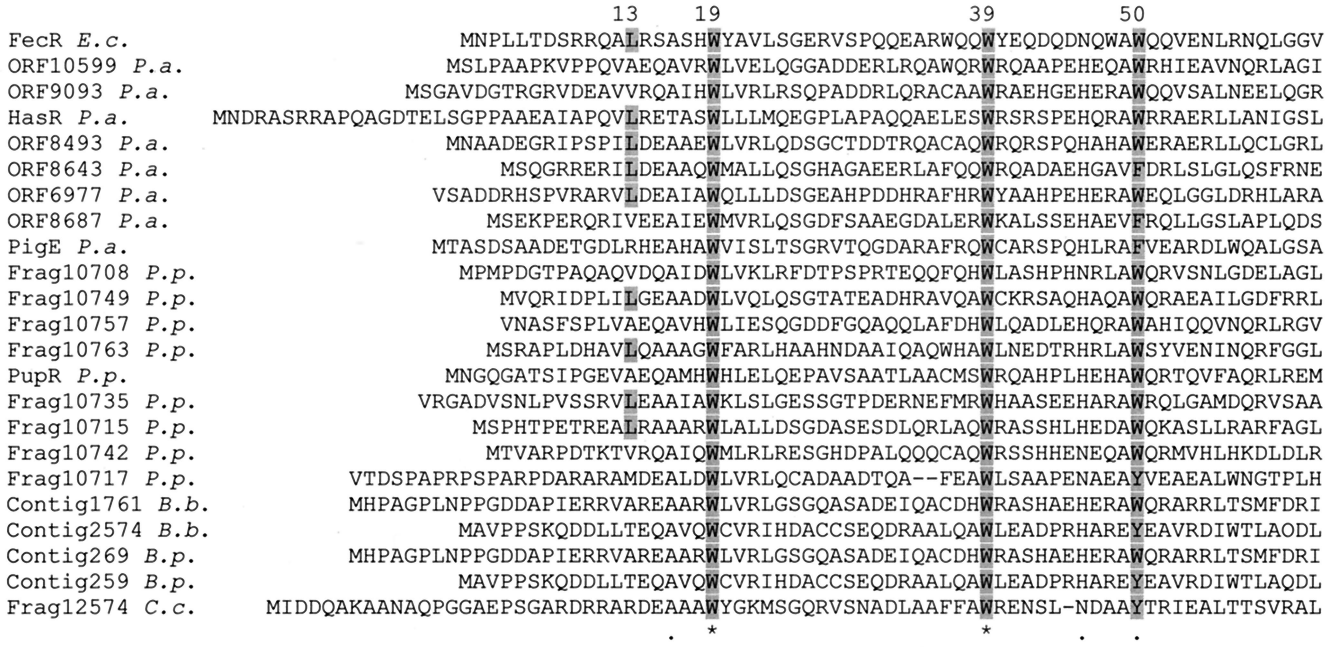

FIG. 2. Alignment of the N termini of *E. coli* FecR homologs. Similarity search and sequence alignment were done as described in Materials and Methods. *E. c.*, *E. coli*; *P. a.*, *P. aeruginosa*; *P. p.*, *P. putida*; *B. b.*, *B. bronchiseptica*; *B. p.*, *B. pertussis*; *C. c.*, *C. crescentus*. Note the highly conserved tryptophan (W) residues corresponding to positions 19, 39, and 50 of *E. coli* FecR.

To demonstrate differences in Fe^{2+} Fur repressor binding to the mutants as the cause of their distinct induction levels, Fe^{2+} Fur repressor binding to *fecI* wild-type and *fecI* mutant promoters was examined in a Fur titration assay using *E. coli* H1717 *fhuF-lacZ*; transcription of *fhuF* is particularly sensitive to the level of the iron supply (41). TY medium, which provides sufficient iron to cells, was used to eliminate experimentally imposed iron limitation. As shown in Table 8, the promoters of *fecI*(A1174T, A1210G) and *fecI*(A1180G) bound less Fe^{2+} Fur than the *fecI*(A1120G) and wild-type *fecI* promoters, resulting in an increased concentration of free Fe^{2+} Fur, which could then bind to the *fhuF* promoter and repress *fhuF-lacZ* transcription. Since also *fecR* transcription is controlled by the promoter upstream of *fecI*, an increase of one or both proteins resulted in the suppression phenotype. It is conceivable that residual activities of the mutated FecR proteins together with an increased level of FecI suffice to initiate transcription of the *fec* transport genes. The titration results do not explain the slight increase of *fecI* transcription by the A1120G mutation.

TABLE 8. Fe^{2+} Fur titration with *fecI* promoter mutants

Plasmid	*fecI* promoter mutation	β-Galactosidase activity (Miller units)[a]	Fe^{2+} Fur binding
pFPV25	Vector	78	−
pGFPI	None	253	+
pGFPI1	A1120G	258	+
pGFPI2	A1174T A1210G	104	−
pGFPI3	A1180G	96	−

[a] Determined in *E. coli* H1717 *fhuF-lacZ* transformed with plasmids carrying mutated promoter *fecI* and promoterless *gfp*.

DISCUSSION

Since no transcription initiation mechanism that starts at the cell surface and requires a cytoplasmic membrane protein for induction has been characterized to date, we further investigated our proposed model by examination of FecR missense mutants. Point mutants with amino acid replacements in the cytoplasmic portion of FecR were isolated and failed to induce transcription of *fecB-lacZ*. The mutations L13Q, W19R, W39R, and W50R are located in the region from residues 9 to 49, which was previously shown to interact with FecI (13). Database searches of complete and incomplete sequenced bacterial genomes revealed genes homologous to the *fec* regulatory genes *fecI* and *fecR* in *P. aeruginosa*, *P. putida*, *B. bronchiseptica*, *B. pertussis*, and *C. crescentus* (Fig. 2). Of 23 complete and partial FecR homologous sequences available, all contained W19, W39, and W50 with the exception of three F and four Y replacement in W50. Since the replacements conserved the aromatic nature of the amino acids, it is conceivable that aromatic amino acids are essential at these sites. Random mutagenesis used in this study clearly identified these highly conserved amino acid residues as important for FecR activity. L13 is less conserved and is replaced mostly by apolar amino acids but in two sequences also by charged arginine. The sequences align perfectly with FecR with two exceptions where sequence gaps of only one and two amino acids, respectively, had to be introduced. The identity between *E. coli* K-12 FecR and the putative FecR sequences range from 24 to 37%, with an average identity of 30%. In the various genomes, *fecI* and *fecR* are arranged as in *E. coli* and are presumably transcriptionally coupled. Furthermore, downstream of the regulatory genes are sequences that show similarity to ECF sigma factor-

dependent promoters. Only the PupI-PupR system of *P. putida* has been studied to the extent that the outer membrane transporter, PupI, and PupR were shown to be required for induction (23). In the absence of inducer, PupR seems to repress synthesis of the outer membrane transporter, as one would expect from an anti-sigma factor and in agreement with other ECF sigma factor systems in which the regulator functions negatively as an anti-sigma factor. No evidence exists for a negative FecR function in the *fec* system. For the homologous genes other than *pup*, it is not known whether sequence similarity reflects similar functions and whether the mechanism of *fec* regulation represents the paradigm of other gene transcription regulatory devices.

Restoration of transcription induction of the FecR mutants depended on the FecI mutants and ferric citrate in the medium. Wild-type FecI was inactive. We assume that the FecI mutant proteins can be converted to an active sigma factor more easily than wild-type FecI, so that the presumed residual activities of the mutated FecR derivatives are sufficient for FecI activation. The mutant $FecR_{1–85}$ derivatives were not active, in contrast to $FecR_{1–85}$WT, which together with FecI initiated *fec* transport gene transcription in the absence of ferric citrate. This result suggests that $FecR_{1–85}$ does not entirely reflect the conformation of the cytoplasmic portion of the complete FecR once it has received the signal from FecA occupied by ferric citrate. This interpretation is supported by the lack of interaction of $FecR_{1–85}$(W39R) and $FecR_{1–85}$(W50R) with either wild-type FecI, FecI(S15A), or FecI(H20E), as revealed by the two-hybrid system.

The *fecI* mutations were confined to a narrow region at the FecI N terminus, even though the entire *fecI* gene was mutagenized by PCR. The S15A mutation was obtained three times and represents a conservative alteration from a polar to an apolar amino acid, both of which require a similar amount of space. The average β-galactosidase activity induced by the FecI S15A mutants amounted to 25% of the activity displayed by wild-type FecI; the mutations increased the *fecB-lacZ* β-galactosidase activity of the FecR missense mutants three- to fivefold. The FecI H20E mutant was less active; it had 16% of the β-galactosidase activity of the wild-type and a twofold increase in *fecB-lacZ* β-galactosidase activity of the FecR mutant cells.

Each of the single *fecI* mutations is located in region 2.1 of FecI. Deletion of subregion 2.1 of σ^{70} reduces σ^{70} binding to the RNA polymerase core enzyme (25, 38). The recently determined structure of a large part of σ^{70} revealed that regions 2.1 and 2.2 form two α-helices at the surface of σ^{70} that are linked by a loop and interact primarily through hydrophobic contacts (29). We found no evidence for an improved interaction between mutated FecI with RNA polymerase core subunits, which might have explained restoration of induction in the FecR mutants. The FecI mutants did not show higher activity with wild-type FecR and did not display higher residual activity in the absence of FecR.

Since the FecI S15A and H20E mutations are located close to each other and the FecR mutations L13Q, W10R, W39R, and W50R are confined to a short region that may fold such that the side chains are positioned close to each other, it is tempting to assume that these two sites disclose regions of interaction between the two proteins. The lack of allele specificity does not rule out this interpretation since, for example, the sites of interaction between the TonB protein (around residue 160) and the so-called TonB box of the BtuB (17) and FhuA proteins (36), as identified by suppressor analyses, also lack allele specificity, yet recent cysteine cross-linking studies established beyond doubt interaction of the TonB box of BtuB with region 160 of TonB (8). Since no specific side chain recognition was observed, it has been concluded that the mutations distort the secondary structure of the interacting regions and thus impair functional interaction (17). The same conclusion could apply to the FecI-FecR interaction.

The *fecI* promoter mutations that partially restored induction of *fecB* gene transcription of the inactive *fecR* mutants bound less Fe^{2+} Fur and consequently led to a higher *fecI* and *fecR* transcription than in the wild-type *fecIR* strain under the same conditions. Derepression could still be enhanced by withdrawing iron by addition of citrate or dipyridyl to the medium. The same explanation as given above may account for this finding. Increase of FecI and mutant FecR proteins increases the probability of a functional interaction that leads to induction-active FecI. A comparable situation exists in the case of σ^E which is one of the stress response σ factors of *E. coli* that belongs to the ECF family and is negatively regulated by the transmembrane anti-sigma factor RseA. The activity of σ^E is determined by the relative levels of σ^E and RseA, and RseA is regulated by controlled proteolysis (1). FecI is positively regulated by FecR, and the amount of active FecI certainly depends on the amounts of active FecR. Activation of FecI does not necessarily imply an alteration in the conformation of FecI or a chemical modification of FecI. Rather, if FecI is synthesized in an active form but rapidly loses activity, stabilization of the active FecI form by active FecR would also account for the data hitherto collected. FecI while bound to FecR would be inactive. When FecR receives the transcription initiation signal from ferric citrate-loaded FecA, it changes the conformation in the cytoplasmic portion, and FecI dissociates from FecR and immediately binds to the RNA polymerase.

The ECF σ factors lack large portions of the conserved region 1 (28), which in σ^{70} appears to primarily affect DNA binding by region 4. This led to the proposal that binding of the σ factors to the core subunits induces a conformational change that exposes the DNA binding region to allow promoter recognition by the holoenzyme (11). FecI completely lacks region 1.1 and has only nine amino acids in region 1.2. Deletion of residues 2 to 8 did not affect FecI activity (data not shown), and therefore region 1 is not essential for ferric citrate-dependent *fec* transport gene transcription. This result makes it unlikely that the A5G mutation of pAS302 transformants contributed to the phenotype. This conclusion is supported by our data on SigX of *Bacillus subtilis*, which completely lacks regions 1.1 and 1.2 and can replace FecI in that it initiates transcription of the *fec* transport genes in *E. coli* in the absence of ferric citrate and FecR (6).

ACKNOWLEDGMENTS

We thank A. Angerer, U. H. Stroeher, and K. Hantke for helpful discussions and K. A. Brune for critical reading of the manuscript.

This work was supported by the Deutsche Forschungsgemeinschaft (SFB 323, project B1, Graduiertenkolleg Mikrobiologie) and the Fonds der Chemischen Industrie.

REFERENCES

1. **Ades, S. E., L. E. Connolly, B. A. Alba, and C. A. Gross.** 1999. The *Escherichia coli* σ^E-dependent extracytoplasmic stress response is controlled by the regulated proteolysis of an anti-σ factor. Genes Dev. **13:**2449–2461.
2. **Angerer, A., S. Enz, M. Ochs, and V. Braun.** 1995. Transcriptional regulation of ferric citrate transport in *Escherichia coli* K-12. FecI belongs to a new subfamily of σ^{70}-type factors that respond to extracytoplasmic stimuli. Mol. Microbiol. **18:**163–174.
3. **Angerer, A., and V. Braun.** 1998. Iron regulates transcription of the *Escherichia coli* ferric citrate transport genes directly and through the transcription initiation proteins. Arch. Microbiol. **169:**483–490.
4. **Braun, V.** 1997. Surface signaling: novel transcription initiation mechanism starting from the cell surface. Arch. Microbiol. **167:**325–331.
5. **Braun, V., K. Hantke, and W. Köster.** 1998. Bacterial iron transport: mechanisms, genetics, and regulation, p. 67–145. *In* A. Sigel and H. Sigel (ed.), Metal ions in biological systems, vol. 35. Marcel Dekker, New York, N.Y.
6. **Brutsche, S., and V. Braun.** 1997. SigX of *Bacillus subtilis* replaces the ECF sigma factor FecI of *Escherichia coli* and is inhibited by RsiX. Mol. Gen. Genet. **256:**416–425.
7. **Buchanan, S. K., B. S. Smith, L. Venkatramani, D. Xia, L. Esser, M. Palnitkar, R. Chakraborty, D. van der Helm, and J. Deisenhofer.** 1998. Crystal structure of the outer membrane active transporter FepA from *Escherichia coli*. Nat. Struct. Biol. **6:**56–63.
8. **Cadieux, N., and R. J. Kadner.** 1999. Site-directed disulfide bonding reveals interaction site between energy-coupling protein TonB and BtuB, the outer membrane cobalamin transporter. Proc. Natl. Acad. Sci. USA **96:**10673–10678.
9. **Dmitrova, M., G. Younes-Cauet, P. Oertel-Buchheit, D. Porte, M. Schnarr, and M. Granger-Schnarr.** 1998. A new LexA-based genetic system for monitoring and analyzing protein heterodimerization in *Escherichia coli*. Mol. Gen. Genet. **257:**205–212.
10. **Dombroski, A. J., W. A. Walter, M. T. Record, D. A. Siegele, and C. A. Gross.** 1992. Polypeptides containing highly conserved regions of the transcription initiation factor σ^{70} exhibit specificity of binding to promoter DNA. Cell **70:**501–512.
11. **Dombroski, A. J., W. A. Walter, and C. A. Gross.** 1993. Amino-terminal amino acids modulate σ-factor DNA-binding activity. Genes Dev. **7:**2446–2455.
12. **Enz, S., V. Braun, and J. Crosa.** 1995. Transcription of the region encoding the ferric dicitrate transport system in *Escherichia coli*: similarity between promoters for *fecA* and for extracytoplasmic function sigma factors. Gene **163:**13–18.
13. **Enz, S., S. Mahren, U. H. Stroeher, and V. Braun.** 2000. Surface signaling in ferric citrate transport gene induction: interaction of the FecA, FecR, and FecI regulatory proteins. J. Bacteriol. **182:**637–646.
14. **Feinberg, B. B., S. Berman, and J. Politch.** 1995. PCR-Ligation-PCR mutagenesis: a protocol for creating gene fusions and mutations. BioTechniques **18:**746–750.
15. **Ferguson, A. D., E. Hofmann, J. W. Coulton, K. Diederichs, and W. Welte.** 1998. Siderophore-mediated iron transport: crystal structure of FhuA with bound lipopolysaccharide. Science **282:**2215–2220.
16. **Giacomini, A., B. Corich, F. J. Ollero, A. Squartini, and M. P. Nuti.** 1992. Experimental conditions may affect reproducibility of the β-galactosidase assay. FEMS Microbiol. Lett. **100:**87–90.
17. **Gudmundsdottir, A., P. E. Bell, M. D. Lundrigan, C. Bradbeer, and R. J. Kadner.** 1989. Point mutations in a conserved region (TonB box) of *Escherichia coli* outer membrane BtuB affect vitamin B_{12} transport. J. Bacteriol. **171:**6526–6533.
18. **Hanahan, D.** 1983. Studies in transformation of *Escherichia coli* with plasmids. J. Mol. Biol. **166:**557.
19. **Härle, C., K. Insook, A. Angerer, and V. Braun.** 1995. Signal transfer through three compartments: transcription initiation of the *Escherichia coli* ferric citrate transport system from the cell surface. EMBO J. **14:**1430–1438.
20. **Heller, K. J., R. J. Kadner, and K. Günther.** 1988. Suppression of the *btuB451* mutations by mutations in the *tonB* gene suggests a direct interaction between TonB and TonB-dependent receptor proteins in the outer membrane of *Escherichia coli*. Gene **64:**147–153.
21. **Hussein, S., K. Hantke, and V. Braun.** 1981. Citrate dependent iron transport system in *Escherichia coli* K-12. Eur. J. Biochem. **117:**431–4337.
22. **Kim, I., A. Stiefel, S. Plantör, A. Angerer, and V. Braun.** 1997. Transcription induction of the ferric citrate transport genes via the N-terminus of the FecA outer membrane protein, the Ton system and the electrochemical potential of the cytoplasmic membrane. Mol. Microbiol. **23:**333–344.
23. **Koster, M., W. van Klompenburg, W. Bitter, J. Leong, and P. Weisbeek.** 1994. Role of the outer membrane ferric siderophore receptor PupB in signal transduction across the bacterial cell envelope. EMBO J. **13:**2805–2813.
24. **Laemmli, U. K.** 1970. Cleavage of structural proteins during the assembly of the head of bacteriophage T4. Nature **227:**680–685.
25. **Lesley, S. A., and R. R. Burgess.** 1989. Characterization of the *Escherichia coli* transcription factor sigma 70: localization of a region involved in the interaction with core RNA polymerase. Biochemistry **28:**7728–7734.
26. **Leung, D. W., E. Chen, and D. V. Goeddel.** 1989. A method for random mutagenesis of a defined DNA segment using a modified polymerase chain reaction. Technique **1:**11–15.
27. **Locher, K. P., B. Rees, R. Koebnik, A. Mitschler, L. Moulinier, J. P. Rosenbusch, and D. Moras.** 1998. Transmembrane signaling across the ligand-gated FhuA receptor: crystal structures of free and ferrichrome-bound states reveal allosteric changes. Cell **95:**771–778.
28. **Lonetto, M. A., K. L. Brown, K. E. Rudd, and M. J. Buttner.** 1994. Analysis of the *Streptomyces coelicolor sigE* gene reveals the existence of a subfamily of eubacterial RNA polymerase σ factors involved in the regulation of extracytoplasmic functions. Proc. Natl. Acad. Sci. USA **91:**7573–7577.
29. **Malhotra, A., E. Severinova, and S. Darst.** 1996. Crystal structure of a σ^{70} subunit fragment from *E. coli* RNA polymerase. Cell **87:**127–136.
30. **Miller, J. H.** 1972. Experiments in molecular genetics. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, N.Y.
31. **Ochs, M., S. Veitinger, I. Kim, D. Welz, A. Angerer, and V. Braun.** 1995. Regulation of citrate-dependent iron transport of *Escherichia coli*: FecR is required for transcription activation by FecI. Mol. Microbiol. **15:**119–132.
32. **Ochs, M., A. Angerer, S. Enz, and V. Braun.** 1996. Surface signaling in transcriptional regulation of the ferric citrate transport system of *Escherichia coli*: mutational analysis of the alternative sigma factor FecI supports its essential role in *fec* transport gene transcription. Mol. Gen. Genet. **250:**455–465.
33. **Pressler, U., H. Staudenmaier, L. Zimmermann, and V. Braun.** 1988. Genetics of the iron dicitrate transport system of *Escherichia coli*. J. Bacteriol. **170:**2716–2724.
34. **Sambrook, J., E. F. Fritsch, and T. Maniatis.** 1989. Molecular cloning, 2nd ed. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, N.Y.
35. **Sanger, F., S. Nicklen, and A. R. Coulson.** 1977. DNA sequencing with chain-terminating inhibitors. Proc. Natl. Acad. Sci. USA **74:**5463–5467.
36. **Schöffler, H., and V. Braun.** 1989. Transport across the outer membrane of *Escherichia coli* via the FhuA receptor is regulated by the TonB protein of the cytoplasmic membrane. Mol. Gen. Genet. **217:**378–383.
37. **Sharp, M. M., C. L. Chan, C. Z. Lu, M. T. Marr, S. Nechaev, E. W. Merrit, K. Severinov, J. W. Roberts, and C. A. Gross.** 1999. The interface of a σ with core RNA polymerase is extensive, conserved, and functionally specialized. Genes Dev. **13:**3015–3026.
38. **Shuler, M. F., K. M. Tatti, K. H. Wade, and C. P. Moran.** 1995. A single amino acid substitution in sigma E effects its ability to bind core RNA polymerase. J. Bacteriol. **153:**597–603.
39. **Silhavy, T. S., M. L. Bermann, and L. W. Enquist.** 1994. Experiments with gene fusions. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, N.Y.
40. **Staudenmaier, H., B. Van Hove, Z. Yaraghi, and V. Braun.** 1989. Nucleotide sequences of the *fecBCDE* genes and locations of the proteins suggest a periplasmic-binding-protein-dependent transport mechanism for iron(III) dicitrate in *Escherichia coli*. J. Bacteriol. **171:**2626–2633.
41. **Stojiljkovic, I., A. J. Bäumler, and K. Hantke.** 1994. Fur regulon in Gram-negative bacteria: identification and characterization of new iron-regulated *Escherichia coli* genes by a Fur titration assay. J. Mol. Biol. **1236:**531–546.
42. **Tabor, S., and C. C. Richardson.** 1985. A bacteriophage T7 RNA polymerase/promoter system for controlled exclusive expression of specific genes. Proc. Natl. Acad. Sci. USA **82:**1074–1078.
43. **Takeshita, S., M. Sato, M. Toba, W. Masahashi, and T. Hashimoto-Gotoh.** 1987. High-copy-number and low-copy-number plasmid vectors for *lacZ* α-complementation and chloramphenicol- or kanamycin-resistance selection. Gene **61:**63–74.
44. **Valdivia, R. H., and S. Falkow.** 1996. Bacterial genetics by flow cytometry: rapid isolation of *Salmonella typhimurium* acid-inducible promoters by differential fluorescence induction. Mol. Microbiol. **22:**367–378.
45. **Van Hove, B., H. Staudenmaier, and V. Braun.** 1990. Novel two-component transmembrane transcription control: regulation of iron dicitrate transport in *Escherichia coli* K-12. J. Bacteriol. **172:**6749–6758.
46. **Wagegg, W., and V. Braun.** 1981. Ferric citrate transport in *Escherichia coli* requires outer membrane receptor protein FecA. J. Bacteriol. **145:**156–163.
47. **Welz, D., and V. Braun.** 1998. Ferric citrate transport of *Escherichia coli*: functional regions of the FecR transmembrane regulatory protein. J. Bacteriol. **180:**2387–2394.
48. **Wilson, C., and A. J. Dombroski.** 1997. Region 1 of sigma 70 is required for efficient isomerization and initiation of transcription by *Escherichia coli* RNA polymerase. J. Mol. Biol. **267:**60–74.
49. **Wilson Bowers, C., and A. J. Dombroski.** 1999. A mutation in region 1.1 of σ^{70} affects promoter DNA binding by *Escherichia coli* RNA polymerase holoenzyme. EMBO J. **18:**709–716.
50. **Wriedt, K., A. Angerer, and V. Braun.** 1995. Transcriptional regulation from the cell surface: conformational change in the transmembrane protein FecR lead to altered transcription of the ferric citrate transport genes in *Escherichia coli*. J. Bacteriol. **177:**3320–3322.
51. **Zimmermann, L., K. Hantke, and V. Braun.** 1984. Exogenous induction of the iron dicitrate transport system of *Escherichia coli*. J. Bacteriol. **159:**271–277.