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Functional Interaction of Region 4 of the Extracytoplasmic Function Sigma Factor FecI with the Cytoplasmic Portion of the FecR Transmembrane Protein of the *Escherichia coli* Ferric Citrate Transport System†

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Transcriptional regulation of the ferric citrate transport genes of *Escherichia coli* is initiated by the binding of ferric citrate to the outer membrane protein FecA. This binding elicits a signal that is transmitted by FecR across the cytoplasmic membrane into the cytoplasm, where the sigma factor FecI directs the RNA polymerase to the promoter upstream of the *fecABCDE* genes. An in vivo deletion analysis using a bacterial two-hybrid system assigned the interaction of the FecR and FecI proteins to the cytoplasmic portion of the FecR transmembrane protein and region 4 of FecI. Missense mutations randomly generated by PCR were localized to region 4 of FecI, and the mutants were impaired with regard to the interaction of FecR with FecI and *fecB-lacZ* transcription. The cloned region 4 of FecI interfered with *fecB-lacZ* transcription. Interaction of N-proximal regions of predicted FecR homologs with region 4 of predicted FecI homologs of *Pseudomonas aeruginosa* was demonstrated. The interaction was specific in that only cognate protein pairs interacted with each other; no interactions occurred between heterologous combinations of the *P. aeruginosa* proteins and between a *P. aeruginosa* FecI homolog and *E. coli* FecR. The results demonstrate that region 4 of FecI specifically binds FecR and that this binding is necessary for FecI to function as a sigma factor.

The ferric citrate transport system transports iron into the cytoplasm of *Escherichia coli*. Fe^{3+} is bound to citrate in the culture medium and probably forms an Fe^{3+}-dicitrate complex (30, 37). Fe^{3+}-dicitrate is bound to the outer membrane protein FecA, which plays a dual role (3, 12, 16). It transports Fe^{3+}-dicitrate across the outer membrane and, independently of transport, induces transcription of the *fecABCDE* transport genes in the presence of Fe^{3+}-dicitrate. Transport-negative *fecA* mutants fully retain the inducing activity. The signal transmitted by FecA loaded with Fe^{3+}-dicitrate across the outer membrane is then transmitted across the cytoplasmic membrane by FecR, which is oriented such that residues 1 to 85 are in the cytoplasm, residues 86 to 100 form a transmembrane segment, and residues 101 to 317 are in the periplasm (44). The signal activates the sigma factor FecI, which directs the RNA polymerase to the promoter upstream of the *fecA* gene (1, 7). The *fecI* and *fecR* genes are located further upstream of *fecA* and are regulated by the intracellular iron concentration through the Fur protein, which when loaded with Fe^{2+} represses *fecIR* transcription (2). Fe^{2+}-Fur also directly represses *fec* transport gene transcription by binding to the promoter upstream of *fecA* (2). Lack of intracellular iron results in the synthesis of FecIR; in the presence of extracellular Fe^{3+}-dicitrate, this results in the synthesis of the FecABCDE transport proteins, which in turn bring the required iron into the cells.

The model of a signaling cascade from the cell surface into the cytoplasm is supported by the finding that the N-proximal end of FecA interacts with the periplasmic portion of FecR and that the cytoplasmic portion of FecR interacts with FecI (8, 28). A FecA derivative lacking the N-terminal portion no longer has the inducing activity but fully retains the transport activity (16). $FecR_{1–85}$ (which consists of the 85 N-proximal residues of FecR) causes a constitutive transcription of the *fec* transport genes (27, 44), and single amino acid replacements in $FecR_{1–85}$ abolish induction and interaction with FecI (38).

FecI belongs to the extracytoplasmic function (ECF) sigma factors (1, 7), which respond to extracellular signals (21). The Fec system is the only system in which the signal and the signaling cascade have been completely determined (3). Regulatory systems homologous to FecIR have been identified in *Shigella flexneri* (FecIR, encoded on a novel pathogenicity island) (22), *Pseudomonas putida* (PupIR) (18), *Bordetella avium* (RhuIR) (17), *Bordetella pertussis* (HurIR) (42), and *Bordetella bronchiseptica* (BupIR) (31) and additional homologs have been deduced from the genome sequences of these organisms and from those of *Pseudomonas aeruginosa* and *Caulobacter crescentus* (27, 39).

The regulatory proteins of the FecIR type form a subgroup among the ECF sigma factors. ECF sigma factors are usually regulated by anti-sigma factors (10, 13, 14, 26, 45). This is apparently not the case for FecIR, since FecR or $FecR_{1–85}$ has to be present in vivo for transcription of the *fec* transport genes to occur. At present, it is not clear whether the inducing signal changes the conformation of FecR and whether the altered FecR activates FecI by altering the FecI conformation similar to the way allosteric enzymes are regulated. Alternatively, and closer to the anti-sigma factor concept, FecR could bind FecI

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and prevent FecI from being inactivated by precipitation or proteolytic degradation. When the inducing signal arrives at FecR, FecI might dissociate from FecR and immediately interact with the RNA polymerase. Biochemical studies to decide between these alternatives are hampered by the presence of very low amounts of FecR and FecI [below 1% of σ^{70} (23)], the insolubility of overexpressed FecI, and degradation of FecR (1, 45, 46).

In this report, we further studied the interaction between FecR and FecI and identified the region of FecI that binds to FecR. As found for the soluble anti-sigma factors Rsd and AsiA that bind to σ^{70} (6, 15, 41), the transmembrane FecR interacted with region 4 of FecI.

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 previously described by Miller (24). Antibiotics were added to the media to the following concentrations: ampicillin, 50 μg/ml; chloramphenicol, 40 μg/ml; tetracycline, 12 μg/ml.

Construction of plasmids. Plasmids carrying *fecI* with various deletions were constructed by insertion of a *Sac*I restriction site and by outward PCR amplification. Plasmid pMMO203 was used as the DNA template for plasmids pSM39, pSM40, pSM41, pSM42, pSM43, and pSM44. For construction of plasmids pSM20, pSM21, pSM22, pSM23, pSM24, and pSM25, plasmid pSM173 was used as the DNA template. Plasmids pSM39 and pSM20 were obtained with primers FecI2.1SACRP (5′-CTCGAACGTTAAGAGCTCCGCGGTAGTGGC-3′) and FecI2.1PSAC (5′-GCTGGCTGACGGCGGAGCTCCAGTCTGC-3′), respectively. Plasmids pSM40 and pSM21 were constructed using primers FecI2.2NRPSAC (5′-GCATCAAAAGCAGACTGGAGCTCGCGCGTCAG-3′) and FecI2.2NPSAC (5′-GCGGGTAATGGTCAGCGAAGAGCTCTCGAC GATCC-3′), respectively. Plasmids pSM41 and pSM22 were created with primers FecI2.3RPSAC (5′-CGAGAGCGTGAGCTCCGCCATTACCCG-3′) and FecI2.3PSAC (5′-CGCTCCTTCCTCGCGGAGCTCGCCAAACGC-3′), respectively. Plasmids pSM42 and pSM23 were constructed using primers FecI2.4RPSAC (5′-GGCGATAGTGCAGAGCTCCGCGCGAGGATCGCG-3′) and FecIP2.4SAC (5′-CGCCGAAACGCGGAGCTCAAAGCGTATCTGG −3′), respectively. Plasmids pSM43 and pSM24 were created with primers FecI3RPSAC (5′-CCAGATACGCTTTGAGCTCCGCGTTTCGGCG-3′) and FecI3PSAC (5′-CGAGACCCTAGCGGAGCTCGACAGCATGCTGGACGG-3′), respectively. Plasmid pSM44 was obtained using primers FecI4.2PPSAC (5′-CAGACCATCGAGCTCCGCAAGCAGAAACGC-3′) and FecI4.2PSAC (5′-CGTCTGGAGTATGCGGAGCTCATCCTTTGTTAACCG-3′). Plasmid pSM25 was created using primers FecI4.1NRPSAC (5′-CCGTCCAGCATGCT GTCGAGGAGCTCTAGGGTCTC-3′) and FecI4.1NPSAC (5′-GCGTTTCTG CTTTCGGAGCTCGATGGTCTG-3′). Plasmid pSM26 was constructed using primers Fec*Ibst*EII (5′-GATGCAGGTGACCATGTCTGACCGCGCC-3′) and Fec*IPst*I (5′-GGTTAACACTGCAGCGAAAGCAGAAACGC-3′). The resulting *fecI* fragment was digested with *Bst*EII/*Pst*I and cloned into *Bst*EII/*Pst*I-restricted pSM604.

fecI was randomly mutagenized by PCR using primers 2.4BstEIIPr (5′-CCG CGATCCGGTCACCTTCCTCTGCACTATCGCC-3′) and Fec*IPst*I (5′-GGTT AACACTGCAGCGAAAGCAGAAACGC-3′), and plasmid pSV66 was used as the DNA template. Each of the mutated *fecI* fragments was cloned into pMS604 restricted with *Bst*EII and *Pst*I, resulting in plasmids pSM45, pSM46, pSM47, pSM49, pSM51, and pSM52.

The *fecI* mutations described above were introduced by site-directed mutagenesis into plasmid pMMO203. The mutated *fecI* fragments were amplified by PCR with primers FecIL114P (5′-GCCAACTCGAGACCCTACAACCCCTCGACAGC-3′), L137P_rev (5′-CTCGCTGTATGTCGGACCATCCAGTTGCG-3′), L137R_rev (5′-CTCGCTGTATGTGCGACCATCCAGTTGCG-3′), TAQL146P (5′-GCGCA CAAACCGGGTGTTTCCATCA-3′), TAQL146R (5′-GCGCACAAACGGGGT GTTTCCATCA-3′), FecI_L166P_rev (5′-GGATTCATATGCCATACTCCAGA CGGAACGGCAGGC-5′), FecI_L166R_rev (5′-GGATTCATATGCCATACTC CAGACGGAACCTCAGGC-3′), Fec*IXho*I_for (5′-GCCAACTCGAGACCCTA CAACTCC-3′) and Fec*INde*I_rev (5′-GGATTCATATGCCATACTCCAGACG G-3′), and plasmid pSV66 was used as the DNA template. The PCR fragments were digested with *Xho*I and *Nde*I and cloned into plasmid pMMO203 cleaved with XhoI/*Nde*I, resulting in plasmids pSM53, pSM54, pSM55, pSM56, pSM57, pSM59, and pSM60.

Truncated fragments of *fecI* were synthesized by PCR. For construction of plasmid pSM61, the *fecI* fragment was amplified by PCR with primers FecIReg4BstEII (5′-GCCAACTGGTGACCCTACAACTCCTCG-3′) and Fec*IPst*I (5′-GGTTAACACTGCAGCGAAAGCAGAAACGC-3′), and plasmid pSV66 was used as the DNA template. The amplified fragment was cleaved with *Bst*EII and *Pst*I and ligated into *Bst*EII/*Pst*I-restricted pMS604. Plasmid pSM74 was constructed using primers FecI4.2RPSACI (5′-CAGACCATCGAGCTCC GCAAGCAGAAACGC-3′) and FecI_1.Helix (5′-GCGCAAACTCGAGCTCT CCATCAGCTCCG-3′), and plasmid pSM75 was created with FecI4.2PPSAC (5′-CAGACCATCGAGCTCCGCAAGCAGAAACGC-3′) and FecI4.2PSAC (5′-CGTCTGGAGTATGCGGAGCTCATCCTTTGTTAACCG-3′); in both cases, plasmid pSM173 was used as the DNA template.

Wild-type *fecR* was amplified by PCR with primers Fec*RXho*I (5′-GGAGTA CTCGAGATGAATCCTTTGTTAA-3′) and LexFecR2 (5′-GGAAGATCTTC CACCTAGTTTACAGTGGTGAAATGTT-3′), with plasmid pSV66 as the DNA template. The resulting *fecR* fragment was cloned into *Xho*I/*Bgl*II-digested pDP804.

The wild-type *pa2468* and *pa3899* genes were amplified by PCR with primers Con48Fec*IBst*EII_for (5′-GTGTCGCCCCGAGGTGACCATGTCCGCCCCGAT CC-3′), and Con48Fec*IXho*I_rev (5′-CGTCCACGGCTCGAGTCATTCGCCGT AGCG-3′) and with Con52Fec*IBst*EII_for (5′-CGTTAATCCTTGGTGACCGGG AATGTCCAGGTG-3′) and Con52Fec*IBst*EII4_for (5′-GCGAAGAGACGGTG ACCATCGTCCTGGAGACC-3′), respectively. The truncated *pa2468* and *pa3899* genes were amplified by PCR with primers Con48FecI4BstEII_for (5′-CCGAGGC GCGGGAACTGGTGACCGAACTGCTG-3′) and Con48Fec*IXho*I_rev (5′-CGT CCACGGCTCGAGTCATTCGCCGTAGCG-3′) and with Con52Fec*IBst*EII4_for (5′-GCGAAGAGACGGTGACCATCGTCCTGGAGACC-3′) and Con52FecI-*Xho*I_rev (5′-CTTGGGTGCTCGAGGCAGGCTCATGGCAGCTCGG-5′), respectively. The PCR fragments were digested with *Bst*EII and *Xho*I and ligated into *Bst*EII/*Xho*I-restricted pMS604, resulting in plasmids pSM65, pSM66, pSM68, and pSM69.

To obtain plasmids pSM67 and pSM70, the truncated *pa2467* and *pa3900* gene fragments were amplified by PCR using primers Con48Fec*RXho*I (5′-CCTGCTG CGCTCGAGCGAATGAGCGGAGCCGTG-3′) and Con48Fec*RBgl*II (5′-CAGC AGCGAAGACTCTTACAGCGCCTGGCGCCGCTG-3′) and using Con52FecR-*Xho*I (5′-CTTCGCCGACTCGAGATGAGCCTGCCCGCCGCACC-3′) and Con52Fec*RBgl*II (5′-GGACCAGCAGAGATCTCTACAGCGCGCGCCGGC G-3′), respectively. The *Xho*I/*Bgl*II-restricted PCR fragments were cloned into *Xho*I/*Bgl*II-cleaved pDP804. For construction of plasmids pSM65, pSM66, pSM67, pSM68, pSM69, and pSM70, the chromosomal DNA of strain PAO1 was used as the DNA template.

All plasmids constructs were confirmed by DNA sequencing.

Recombinant DNA techniques. Standard techniques (33) or the protocols of the suppliers were used for isolation of plasmid DNA, digestion with restriction endonucleases, ligation, transformation, and agarose gel electrophoresis. DNA was sequenced by the dideoxy chain-termination method (34) using the Auto-Read sequencing kit (Pharmacia Biotech, Freiburg, Germany). The reaction products were sequenced on an A.L.F. DNA sequencer (Pharmacia Biotech). 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 followed the method previously described (20). Site-directed mutagenesis was carried out according to the methods of Landt et al. (19).

Western blotting. To estimate the amounts of wild-type FecI and mutated FecI obtained, a Western blot analysis was employed using anti-LexA antibodies (Invitrogen, Karlsruhe, Germany). In brief, transformants of *E. coli* SU202 carrying the *fecI* genes were grown in TY medium supplemented with 1 mM IPTG (isopropyl-β-D-thiogalactopyranoside) to a density of 8×10^8 cells per ml. Cell lysates were treated with sample buffer (50 mM Tris-HCl [pH 6.8], 2% sodium dodecyl sulfate, 10% glycerol) and separated on 15% polyacrylamide gels. Proteins were electroblotted onto nitrocellulose membranes (Schleicher and Schuell, Dassel, Germany). Blots were blocked overnight in 3% bovine serum albumin in TNT buffer (20 mM Tris-HCl, 500 mM NaCl, 0.05% Tween 20), probed with the anti-LexA antibodies, washed with TNT buffer, and incubated with anti-rabbit immunoglobulin G conjugated with alkaline phosphatase (Sigma). The blots were developed with nitroblue tetrazolium–5-bromo-4-chloro-3-indolylphosphate (NBT-BCIP; Serva, Heidelberg, Germany).

Determination of β-galactosidase activity. β-galactosidase activity was determined according to the methods of Miller (24) and Giacomini et al. (9). To determine the induction level, cells were grown in NB medium with no additions

TABLE 1. Bacterial strains and plasmids

Strain or plasmid	Genotype	Reference or source
Strains		
E. coli		
DH5α	*endA1 hsdR17* (r_K^- m_K^+) *supE44 thi1 recA1 gyrA relA1* Δ(*lacZYA-argF*)*U169* ϕ80Δ*lacZ*M15	11
ZI418	*araD139* Δ*lacU169 rpsL 150 relA1 flbbB5301 deoC1 ptsF25 rbsR thi aroB fecB*::Mud1 (Ap, *lac*)	43
MO704	*fecB*::Mud1 (Ap, *lac*) *fecI*::Kan^r *aroB araD139* Δ*lacU169 rpsL 150 relA1 flbB5301 deoC1 ptsF25 rbsR thi*	43
SU202	*lexA71*::Tn*5 sulA211 sulA*(op408/op+)::*lacZ* Δ(*lacIPOZYA*) *169*/F′ *lacI*^{q} *lacZ*ΔM15::Tn*9*	5
P. aeruginosa		
PAO1	Wild type	Lab stock
Plasmids		
pHSG576	pSC101 derivative, Cm^r	40
pSV66	pHSG576 *fecI fecR fecA*	12
pMMO202	pHSG576 *Nde*I/*Bgl*I (cleavage sites removed)	38
pMMO203	pMMO202 *fecI fecR*	38
pSM39	pMMO202 *fecI*Δ9–32 *fecR*	This study
pSM40	pMMO202 *fecI*Δ32–55 *fecR*	This study
pSM41	pMMO202 *fecI*Δ52–67 *fecR*	This study
pSM42	pMMO202 *fecI*Δ65–83 *fecR*	This study
pSM43	pMMO202 *fecI*Δ79–114 *fecR*	This study
pSM44	pMMO202 *fecI*Δ114–134 *fecR*	This study
pSM53	pMMO202 *fecI*(T1570C)(L113P) *fecR*	This study
pSM54	pMMO202 *fecI*(T1642C)(L137P) *fecR*	This study
pSM55	pMMO202 *fecI*(T1642G)(L137R) *fecR*	This study
pSM56	pMMO202 *fecI*(T1669C/C1670G)(L146P) *fecR*	This study
pSM57	pMMO202 *fecI*(T1669G/C1670G)(L146R) *fecR*	This study
pSM59	pMMO202 *fecI*(T1729C)(L166P) *fecR*	This study
pSM60	pMMO202 *fecI*(C1728A/T1729G)(L166R) *fecR*	This study
pMS604	*ori*ColE1 Tet^r $lexA_{1–87}$-*fos* zipper	5
pSM173	pMS604 $lexA_{1–87}$-*fecI*	8
pSM20	pMS604 $lexA_{1–87}$-*fecI* Δ9–32	This study
pSM21	pMS604 $lexA_{1–87}$-*fecI* Δ32–55	This study
pSM22	pMS604 $lexA_{1–87}$-*fecI* Δ52–67	This study
pSM23	pMS604 $lexA_{1–87}$-*fecI* Δ65–83	This study
pSM24	pMS604 $lexA_{1–87}$-*fecI* Δ79–114	This study
pSM25	pMS604 $lexA_{1–87}$-*fecI* Δ114–134	This study
pSM26	pMS604 $lexA_{1–87}$-*fecI* Δ133–173	This study
pSM76	pMS604 $lexA_{1–87}$-$fecI_{64–173}$	This study
pSM45	pMS604 $lexA_{1–87}$-$fecI_{64–173}$(T1570C)(L113P)	This study
pSM49	pMS604 $lexA_{1–87}$-$fecI_{64–173}$(T1642C)(L137P)	This study
pSM46	pMS604 $lexA_{1–87}$-$fecI_{64–173}$ (T1669C)(L146P)	This study
pSM52	pMS604 $lexA_{1–87}$-$fecI_{64–173}$ (A1613G/T1669G/T1702C)(E127E/L146R/V161A)	This study
pSM47	pMS604 $lexA_{1–87}$-$fecI_{64–173}$ (T1729C)(L166P)	This study
pSM51	pMS604 $lexA_{1–87}$-$fecI_{64–173}$ (T1729C/T1749G)(L166P/L173V)	This study
pSM61	pMS604 $lexA_{1–87}$-$fecI_{109–173}$	This study
pSM74	pSM604 $lexA_{1–87}$-*fecI* Δ132–148	This study
pSM75	pSM604 $lexA_{1–87}$-$fecI_{1–153}$	This study
pDP804	*ori* p15A Amp^r $lexA_{1–87}$408-*jun* zipper	5
pSM85	pDP804 $lexA_{1–87}$408-$fecR_{1–85}$	8
pSM84	pDP804 $lexA_{1–87}$408-*fecR*	This study
pSM65	pMS604 $lexA_{1–87}$-$pa2468_{1–172}$	This study
pSM66	pMS604 $lexA_{1–87}$-$pa2468_{110–172}$	This study
pSM68	pMS604 $lexA_{1–87}$-$pa3899_{1–170}$	This study
pSM69	pMS604 $lexA_{1–87}$-$pa3899_{105–170}$	This study
pSM67	pDP804 $lexA_{1–87}$408-$pa2467_{1–90}$	This study
pSM70	pDP804 $lexA_{1–87}$408-$pa3900_{1–85}$	This study

or supplemented with 50 μM 2,2′-dipyridyl or 1 mM citrate. For the LexA-based repression system, cells were grown in TY medium supplemented with 1 mM IPTG.

Sequence alignments and prediction of secondary structure. Sequences of *Pseudomonas aeruginosa* were obtained from the *Pseudomonas* Genome Project (http://www.pseudomonas.com/current_annotation.asp). The sequence of *E. coli* FecI was translated from the GenBank entries given in parentheses. Amino acid sequences were aligned using ClustalW multiple sequence alignment. The helix-turn-helix motif of region 4 of *E. coli* FecI was predicted using the PredictProtein program (http://www.embl-heidelberg.de/predictprotein).

RESULTS

Binding sites of FecR on FecI. The bacterial two-hybrid LexA system was used to identify sites of interaction of FecR

TABLE 2. Interaction of FecI deletion derivatives with complete FecR and $FecR_{1–85}$

Plasmid	Protein	Region deleted in FecI	β-Galactosidase activity (Miller units)[a]	
			$FecR_{1–85}$	Wild-type FecR
None	No FecI		241	216
pSM173	Wild-type FecI		25	36
pSM20	FecI Δ9–32	2.1	29	27
pSM21	FecI Δ32–55	2.2	28	29
pSM22	FecI Δ52–67	2.3	23	28
pSM23	FecI Δ65–83	2.4	25	32
pSM24	FecI Δ79–114	3	29	71
pSM25	FecI Δ114–134	4.1	225	209
pSM26	FecI Δ133–173	4.2	213	216
pSM61	$FecI_{109–173}$	1–3	32	31

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

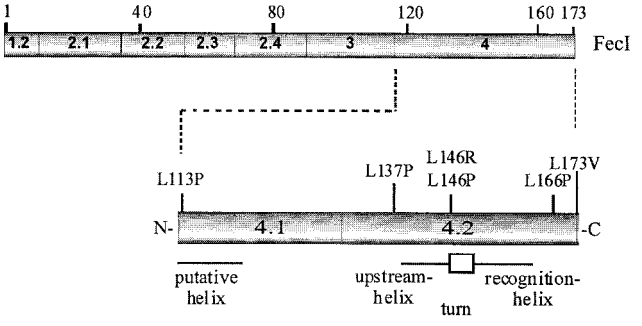

FIG. 1. Domain structure of FecI and location of the amino acid substitutions within the putative helix-turn-helix motif of FecI.

with FecI in vivo. The dimer of LexA represses *sulA* transcription. The dimerizing C-proximal end can be replaced by heterologous protein fragments that are thought to dimerize. The promoter of *sulA* has been mutagenized such that one half binds the wild-type N-proximal end of LexA ($LexA_{1–87}$) and the other half binds a mutated N-proximal end of LexA ($LexA_{1–87}$408) (5). Complete FecR and $FecR_{1–85}$ (residues 1 to 85 of FecR) were fused to $LexA_{1–87}$408, and FecI and deletion derivatives of FecI were fused to $LexA_{1–87}$. When $FecR_{1–85}$ interacts with FecI, the *fec* transport genes are constitutively transcribed, whereas when complete FecR interacts with FecI, transcription initiation of the *fec* transport gene requires ferric citrate and FecA (8, 27, 38, 44).

E. coli SU202, which contains *lacZ* under the control of the mutated *sulA* promoter, was transformed with plasmids carrying the truncated *lexA-fecR* and *lexA-fecI* fusion genes. $LexA_{1–87}$-$FecI_{109–173}$, which contained only region 4 of FecI combined with FecR and $FecR_{1–85}$, repressed *lacZ* transcription (Table 2). Deletions were introduced into regions 2, 3, and 4 of FecI but not into region 1, since region 1.1 of FecI is lacking and deletion of region 1.2 does not affect FecI activity (38). FecI with deletions covering regions 2 and 3 formed dimers with FecR and $FecR_{1–85}$, as evidenced by the background activity of β-galactosidase, in contrast to FecI with deletions in region 4, which showed high β-galactosidase activity (Table 2). Cells expressing FecIΔ79-114 and wild-type FecR had a 2.5-fold-higher β-galactosidase activity than fully repressed cells. These results are consistent with the proposal that region 4 of FecI binds FecR and that the cytoplasmic N-terminal end of FecR is sufficient for binding. Regions 2 and 3 of FecI did not contribute directly to binding or influence binding of region 4 to FecR.

Transcription activity of the FecI deletion derivatives. To determine transcription levels of the *fec* transport genes in the *fecI* deletion mutants, wild-type *fecI* on plasmid pMMO203 *fecI fecR* was replaced by mutated *fecI*. Transcription levels were measured in *E. coli* MO704, which carried on the chromosome a kanamycin resistance gene in *fecI* and a *fecB-lacZ* fusion. *E. coli* MO704(pMMO203), grown in NB supplemented with 50 μM dipyridyl to limit the available iron, displayed approximately 8% (8 to 12 Miller units) of the β-galactosidase activity of cells grown in the presence of 1 mM citrate (291 Miller units). *E. coli* MO704 transformed with the vector pHSG576 displayed only 1.5% of the β-galactosidase activity of cells grown in the presence of 1 mM citrate; this value was also determined for cells that synthesized the FecI deletion derivatives listed in Table 2. All regions in which the deletions were found—2.1, 2.2, 2.3, 2.4, 3, and 4.2 (4.1 was not determined)—were important for sigma factor activity.

If FecR interacts with region 4 of FecI, a FecI fragment comprising region 4 should inhibit interaction of FecR with wild-type FecI and hence transcription of *fecB-lacZ. E. coli* ZI418, which carries on the chromosome a complete *fec* transport operon with the exception of *fecB*, which is fused to *lacZ,* was transformed with plasmid pSM61, which encodes $FecI_{109–173}$. Transcription of the *fecI* fragment gene was induced by adding IPTG. The β-galactosidase activity of the citrate-induced culture decreased from 177 units without IPTG induction to 51 units after induction of $FecI_{109–173}$ synthesis. This result further supports the involvement of region 4 in the FecR-mediated FecI activity.

Point mutations in FecI that affect binding to FecR. To further localize FecI regions that interact with FecR, point mutants were introduced into FecI. Inactive mutant proteins could then be used to correlate impaired interaction with FecR with the ability to induce transcription of *fec* genes. A *fecI* fragment comprising regions 2.4 to 4 was mutagenized via PCR to isolate FecI mutants that no longer interacted with $FecR_{1–85}$. The formation of red colonies on MacConkey agar plates of *E. coli* SU202 transformed with the cloned mutagenized *fecI* fragments indicated impaired repression of *sulA-lacZ*. Of 3,000 colonies tested, only 6 were red. All mutations were located in region 4 (Fig. 1). Those FecI mutants with the highest *lacZ* transcription levels (Table 3) had a replacement of leucine by proline (L146P and L166P). The mutant with the L113P replacement, which is close to the border of region 3, displayed the least increase in β-galactosidase activity. The mutations are located in the predicted helix-turn-helix motif, and the L→P substitutions most likely affect the secondary structure.

Inactivity of the mutated FecI derivatives linked to LexA could arise from instability of the hybrid proteins. Therefore, their amounts after IPTG induction were estimated by Western blot analysis using anti-LexA antibodies. All samples contained proteins which showed electrophoretic mobilities corresponding to expectations formed on the basis of their sizes (Fig. 2). The amounts of mutated FecI carrying point muta-

TABLE 3. Binding of mutated $FecI_{64–173}$ to wild-type $FecR_{1–85}$

Plasmid	*fecI* mutation(s)	Amino acid substitution(s) in FecI	β-Galactosidase activity (Miller units)[a]
pSM76	None	None	25
pSM45	T1570C	L113P	58
pSM49	T1642C	L137P	72
pSM46	T1669C	L146P	180
pSM52	A1613G, T1669G, T1702C	E127E, L146R, V161A	71
pSM47	T1729C	L166P	178
pSM51	T1729C, T1749G	L166P, L173V	205

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

tions and deletions were somewhat smaller than the amount of wild-type FecI, and the L→P mutants revealed a defined degradation product whose amount, however, was much less than those of the original products. The amounts of the mutated LexA-FecI hybrid proteins were still large and certainly sufficient to repress transcription of the chromosomal *sulA-lacZ* reporter gene.

Sigma factor activity of FecI point mutants. To determine whether the L→P substitutions that impaired binding to $FecR_{1–85}$ affected FecI sigma factor activity, *fecB-lacZ* transcription levels were determined in *E. coli* MO704 carrying the *fecI* mutant genes. In addition, these mutated leucine residues—L137, L146, and L166—were replaced by arginine via site-directed mutagenesis. All L→P mutants showed very low levels of *fecB-lacZ* transcription, including L113P and L137P (Table 4), which displayed residual interaction with $FecR_{1–85}$ (Table 3). After induction with ferric citrate, the L→R mutants L137R and L166R showed levels of activity equal to that of wild-type FecI and L146R showed 22% of the wild-type FecI activity. These results demonstrate a correlation between FecR binding to FecI and the level of transcription and suggest that at least in the case of FecI(L137P) and FecI(L166P), secondary structure alterations disrupt interaction of FecI with FecR and hence sigma factor activity.

Interactions of proteins of *P. aeruginosa* homologous to FecI and FecR. The genome of *P. aeruginosa* (39) encodes a number of proteins homologous to FecI and FecR of *E. coli* (38), among them PA2468 (172 residues) and PA3899 (170 residues), which are 46 and 47% identical to FecI (173 residues), respectively, and PA2467 (332 residues) and PA3900 (317 residues), which are 17 and 35% identical to FecR (317 residues), respectively. Genes *pa2468* and *pa2467* are seemingly controlled by the same promoter, as are genes *pa3899* and *pa3900*. The region upstream of *pa2467* contains a putative *fur* box, which points to iron-regulated transcription. The sequence similarities suggest that each of these sets of genes encodes an ECF sigma factor of the FecI type and a regulatory protein of the FecR type. To support this assumption and to obtain further data on the specificity of the interaction between FecI-type sigma factors and their regulatory proteins, the genes were fused to $LexA_{1–87}$ and $LexA_{1–87}$408 in various combinations and β-galactosidase activity resulting from *sulA-lacZ* transcription was determined. The fragments of the *P. aeruginosa* proteins used were homologous either to region 4 of FecI or to the cytoplasmic segment of FecR. The *E. coli* SU202 transformant that synthesized PA2468 and $PA2467_{1–90}$ and the transformant that synthesized PA3899 and $PA3900_{1–85}$ exhibited low β-galactosidase activity (Table 5), which indicated repression of *sulA-lacZ* transcription as a result of protein dimerization. When PA2468 was replaced by $PA2468_{110–172}$ and PA3899 by $PA3899_{105–170}$, both of which represent region 4 of the proteins, repression was even stronger (Table 5). However, the heterologous combinations, PA2468 with $PA3900_{1–85}$ and PA3899 with $PA2467_{1–90}$, did not repress *sulA-lacZ* transcription. The different amino acid sequences prevented interaction of the proteins (PA2468 is 48% identical to PA3899 and PA2467 is 42% identical to PA3900; see also Fig. 3). Furthermore, $FecR_{1–85}$ combined with PA2468 did not repress *sulA-lacZ* transcription; this result in addition demonstrated that the identical turns (GVS) between the two helices in region 4 of PA2468 and of FecI do not determine the specificity of interaction between the sigma factors and the cognate regulatory proteins.

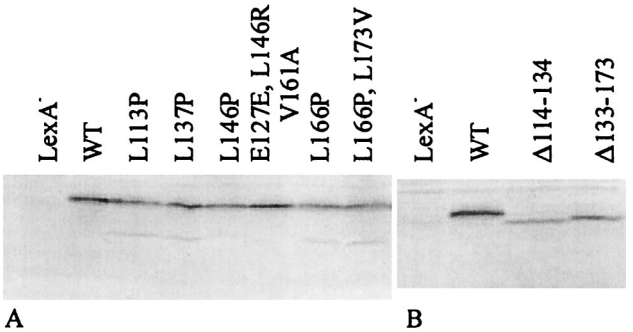

FIG. 2. Western blot analysis of *E. coli* SU202 cell extracts. LexA hybrid proteins of wild-type FecI, mutated FecI (A), and truncated FecI (B) were detected with anti-LexA antibodies.

DISCUSSION

The activity of those ECF sigma factors studied in some detail has been shown to be controlled by anti-sigma factors (13, 14). Exogenous signals are thought to activate the inactive sigma factors by causing dissociation of the anti-sigma factors from the sigma factors or by causing conformational changes in the sigma factors. In the absence of the anti-sigma factors, the ECF sigma factors initiate transcription without extracytoplasmic signals. This holds true for the PupIR regulatory system of *P. putida*, which shows sequence similarity to FecIR. *lacZ* fused to the promoter of the *pupB* gene, which encodes an outer membrane protein for the uptake of pseudobactin BN8, is strongly transcribed in $pupI^+$ $pupR^-$ cells (18). In addition, the

TABLE 4. *fecB-lacZ* transcription by mutant FecI

Plasmid	*fecI* mutation(s)	Amino acid substitution in FecI	β-Galactosidase activity (Miller units)[a]	
			NB medium + 50 μM dipyridyl	NB medium + 1 mM citrate
pHSG576	Vector	Vector	5	6
pMMO203	Wild type	None	23	376
pSM53	T1570C	L113P	6	6
pSM54	T1642C	L137P	12	13
pSM55	T1642G	L137R	26	333
pSM56	T1669C, C1670G	L146P	9	7
pSM57	T1669G, C1670G	L146R	13	82
pSM59	T1729C	L166P	5	6
pSM60	C1728A, T1729G	L166R	41	393

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

B. avium BhuR outer membrane protein, which is required for heme uptake, is synthesized in high amounts when *rhuI*, a *fecI* homolog, is overexpressed in the absence of heme as inducer (17).

In contrast, overexpression of FecI in the absence of FecR results in a very low level of transcription of chromosomal *fecB-lacZ* (29). Transcription of the *fecABCDE* transport genes requires FecR and ferric citrate for FecI to function as a sigma factor. Overexpression of plasmid-borne *fecI* in a chromosomal $fecI^+$ $fecR^+$ strain leads to high levels of *fecB-lacZ* transcription in the absence of ferric citrate; for example, in uninduced and in ferric-citrate-induced $FecI^+$ $FecR^+$ cells, the β-galactosidase activity is 185 and 190 units, respectively. Overexpression of plasmid-encoded *fecR* does not reduce uninduced or ferric-citrate-induced *fecB-lacZ* transcription in chromosomal $fecI^+$ $fecR^+$ cells (52 versus 51 units and 192 versus 199 units, respectively). C-terminally truncated FecR derivatives confer constitutive *fecA-lacZ* transcription in $fecI^+$ $fecR^-$ cells (27, 38, 44). The electrophoretic mobility of a *fecA* promoter DNA fragment on a polyacrylamide gel is retarded when purified FecI and isolated RNA polymerase core enzyme are added together. When a crude cell extract of a FecIRA-producing cell is used, band shifting occurs at much lower levels of FecI than of purified FecI (1). However, since a detergent had to be used to solubilize FecI, it remains unclear whether a substantial fraction of FecI was denatured. The crude cell extract caused band shifting only when the cells expressed the *fecIRA* genes and were grown in the presence of ferric citrate. When FecR was omitted, no band shifting was observed (1). These data indicate the requirement of the presence of FecR for FecI activity.

TABLE 5. Interaction of the complete and truncated FecI homologs PA2468 and PA3988 of *P. aeruginosa* with the truncated FecR homologs PA2467 and PA3900 of *P. aeruginosa* and $FecR_{1–85}$ of *Escherichia coli*

Plasmids	Protein combination	β-galactosidase activity (Miller units)[a]
pMS604, pDP804	Fos and Jun[b]	25
pSM65, pSM67	PA2468 and $PA2467_{1–90}$	28
pSM66, pSM67	$PA2468_{110–172}$ and $PA2467_{1–90}$	14
pSM68, pSM70	PA3899 and $PA3900_{1–85}$	39
pSM69, pSM70	$PA3899_{105–170}$ and $PA3900_{1–85}$	23
pSM65, pSM70	PA2468 and $PA3900_{1–85}$	397
pSM68, pSM67	PA3899 and $PA2467_{1–90}$	269
pSM65, pSM85	PA2468 and $FecR_{1–85}$	181

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

[b] Dimerization of Jun and Fos was used to develop the LexA-based dimerization assay (33).

The deviation of FecIR-mediated regulation from regulation by anti-sigma control of ECF sigma factors prompted this study on the sites of interaction between FecR and FecI. FecR and $FecR_{1–85}$ interacted with complete FecI, as evidenced by repression of *sulA-lacZ* transcription through the FecI and FecR or $FecR_{1–85}$ LexA fusions. Repression was retained when fragments of FecI were sequentially excised from the N terminus to the C terminus up to region 4.1. Deletion of residues 114 to 134 (region 4.1) and 133 to 173 (region 4.2) resulted in C-terminally truncated FecI derivatives that no longer combined with FecR or $FecR_{1–85}$ to repress *sulA-lacZ* transcription. The finding that region 4 interacted with FecR was supported by results obtained with randomly generated *fecI* mutants, which in combination with $FecR_{1–85}$ displayed lower repression of *sulA-lacZ* transcription and were exclusively mutated in region 4.1 or 4.2. Impaired dimerization correlated with lack of induction. None of the four FecI L→P mutants initiated transcription of *fecB-lacZ*. The finding that fragments covering regions 1.2 to 3 could be excised from FecI without affecting binding of region 4 to FecR supports the exclusive involvement of region 4 in FecR binding and indicates the structural and functional independence of region 4 with regard to FecR binding.

The FecI protein displays a helix-turn-helix motif from residues 139 to 158 (Fig. 1). Secondary structure analysis predicts an amphipathic helix for the first portion of the motif, of which Tyr139, Ile142, Ala143, and Leu146 form the hydrophobic side and Ser140, Glu141, His144, and Lys145 form the hydrophilic side (27, 43). The L→P mutations in region 4 probably changed the conformation of FecI, since leucine was replaced by proline in all of the mutations (proline inserted in an α-helix disrupts the secondary structure). This hypothesis was supported by the results obtained with mutants in which the leucine residues were replaced by arginine instead of proline. Despite the strong alteration in charge and steric requirements, two of the three examined mutants exhibited activities close to that of the wild type and only one, L146R, showed a lower activity (22% of wild-type FecI activity). These results

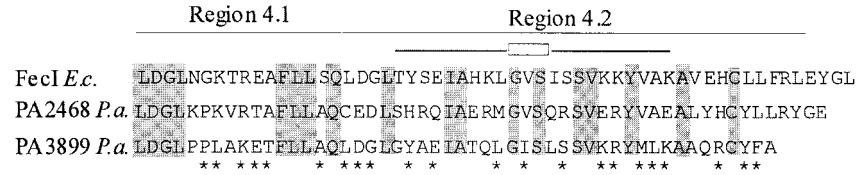

FIG. 3. Alignment of the conserved region 4 of *E. coli* (*E.c.*) FecI with *P. aeruginosa* (*P.a.*) FecI homologs. Shaded positions indicate three identical residues, and asterisks indicate two identical residues. The helix-turn-helix motif in Region 4.2 is indicated above the *E. coli* sequence.

clearly associate functional interactions of FecR with region 4 of FecI.

Regions 4.1 and 4.2 of FecI proved to be important for FecR binding, as shown by the abolishment of FecR binding caused by individual deletions in either region and the impairment of transcription of *fecB-lacZ* caused by missense mutations in either region. The involvement of regions 4.1 and 4.2 of FecI is similar to the situation with the AsiA anti-sigma factor of phage T4 and σ^{70}; binding of AsiA to σ^{70} not only involves region 4.2 of σ^{70} (4, 25, 35, 36) but also region 4.1 (41). AsiA inhibits transcription of *E. coli* genes and early T4 genes and enhances transcription of middle T4 genes. In contrast to single alanine mutations in synthetic peptides of σ^{70}, which alone do not strongly affect binding to AsiA (25), the FecI L→P substitutions reduced binding of $FecR_{1-85}$ and abolished FecI activity. As with $FecR_{1-85}$, an N-terminal fragment of AsiA of 20 residues was necessary and sufficient for binding of AsiA to σ^{70}. In both FecR and AsiA, the sigma factor binding domain is located in the first quarter of the polypeptides [FecR contains 317 residues, and 68 residues are sufficient (8, 27); AsiA consists of 90 residues].

Region 4 of FecI participates in recognition of the −35 promoter sequence, which is rather well conserved in ECF-regulated promoters (21). The sequence of the −35 region of the promoter upstream of the *fecABCDE* transport genes is homologous to that of the ECF-regulated promoters, whereas the −10 region shows poor sequence similarity (7, 32). FecI mediates binding of the *E. coli* RNA polymerase core enzyme to a 75-bp fragment that encompasses the −10 and −35 *fecA* promoter regions (1). It does not seem that FecR binding to region 4 of FecI interferes with binding of FecI to the −35 region, as has been suggested for AsiA (4, 35), since overexpressed FecR does not affect FecI-mediated *fecB-lacZ* transcription (43). An additional binding site for the FecI-RNA polymerase apoenzyme has been revealed by randomly generated mutations in a *fecA* promoter DNA fragment. The nucleotide replacements that reduced binding are clustered around position +13 (1) relative to the *fecA* transcription initiation site (7). This unusual binding site is another indication that *fec* transcription regulation has certain properties distinct from those of other sigma regulatory mechanisms.

Binding of FecR to region 4 of FecI concurs with the binding of anti-sigma factors to binding sites on sigma factors. Anti-sigma-factor activity of FecR cannot be evaluated because FecI shows only very low levels of activity in the absence of FecR, and therefore FecI inhibition by FecR cannot be determined. If nascent FecI is rapidly inactivated by proteolysis or aggregation, binding to FecR could keep it in an active conformation. The signal exerted by ferric citrate bound to FecA could dissociate FecI from FecR, followed by immediate binding of FecI to RNA polymerase core enzyme and transcription initiation of the *fecABCDE* genes. In this model, FecR would act as a membrane-bound chaperone. This model is more appealing than a model that proposes binding of the FecI-RNA polymerase via FecR to the cytoplasmic membrane, because in the latter case a portion of the RNA polymerase would have to be sequestered in an inactive form as long as no induction by ferric citrate occurs—only when induction occurs would the FecI-RNA polymerase dissociate from the membrane and initiate transcription. In a third model, the signal would not dissociate FecR and FecI but rather would bind FecIR to the RNA polymerase core enzyme. Since FecR is inserted into the cytoplasmic membrane, this model would imply that transcription of the *fec* transport genes occurs while the RNA polymerase is bound to the membrane.

We have previously identified tryptophan residues 19, 39, and 50 of FecR as being important for interaction with FecI. In randomly generated mutants containing arginine in place of the tryptophan residues, binding of mutated $FecR_{1-85}$ to FecI and *fecB-lacZ* transcription mediated by mutated FecR were abolished. Two mutations in FecI (S15A and H20E) that partially suppressed the FecR W→R mutations are clustered in region 2.1 (38). Since no allele specificity is observed and the mutated FecI suppressor proteins do not restore constitutive *fecB-lacZ* transcription of $FecR_{1-85}$ W→R mutants, a direct interaction between FecR and region 2.1 of FecI is unlikely. Rather, the FecI suppressor mutants increase FecI activity, presumably by improving interaction with the RNA polymerase core enzyme.

Tryptophan residues 19 and 39 are strictly conserved in 22 FecR homologs found in GenBank, whereas tryptophan residue 50 is replaced by phenylalanine or tyrosine in a few proteins. Among these proteins are FecI and FecR homolog pair PA2468 and PA2467 and homolog pair PA3899 and PA3900 of *P. aeruginosa*. In this report, we showed that these pairs interact with each other but that heterologous pair PA2468 and PA3900 and heterologous pair PA3899 and PA2467 do not. Similar to the truncated FecI and FecR derivatives, the N-terminally truncated $PA2468_{110-172}$ and the C-terminally truncated $PA2467_{1-90}$ interacted with each other. Since $PA2468_{110-172}$ consists of predicted region 4 and binds even more strongly to $PA2467_{1-90}$ than complete PA2468, region 4 represents the binding site of the FecR homolog. These data strongly support the prediction that these proteins belong to the FecIR subgroup of ECF σ^{70} factors, and they underline the importance of region 4 for the interaction of the two regulatory proteins.

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