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Sites of Interaction between the FecA and FecR Signal Transduction Proteins of Ferric Citrate Transport in *Escherichia coli* K-12

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Transcription of the *fecABCDE* ferric citrate transport genes of *Escherichia coli* K-12 is initiated by a signaling cascade from the cell surface into the cytoplasm. FecR receives the signal in the periplasm from the outer membrane protein FecA loaded with ferric citrate, transmits the signal across the cytoplasmic membrane, and converts FecI in the cytoplasm to an active sigma factor. In this study, it was shown through the use of a bacterial two-hybrid system that, in the periplasm, the C-terminal $FecR_{237-317}$ fragment interacts with the N-terminal $FecA_{1-79}$ fragment. In the same C-terminal region, amino acid residues important for the interaction of FecR with FecA were identified by random and site-directed mutagenesis. They were preferentially located in and around a leucine motif (residues 247 to 268) which was found to be highly conserved in FecR-like proteins. The degree of residual binding of FecR mutant proteins to FecA was correlated with the degree of transcription initiation in response to ferric citrate in the culture medium. Three randomly generated inactive FecR mutants, FecR(L254E), FecR(L269G), and FecR(F284L), were suppressed to different degrees by the mutants FecA(G39R) and FecR(D43E). One FecR mutant, FecR (D138E, V197A), induced *fecA* promoter-directed transcription constitutively in the absence of ferric citrate and bound more strongly than wild-type FecR to FecA. The data showed that FecR interacts in the periplasm with FecA to confer ferric citrate-induced transcription of the *fec* transport genes and identified sites in FecR and FecA that are important for signal transduction.

In *Escherichia coli* K-12, transcription of the ferric citrate transport genes *fecABCDE* is controlled by a signal transduction mechanism that starts from the cell surface (3, 4, 12). Binding of ferric citrate to the outer membrane FecA protein initiates a signal that is transmitted across the cytoplasmic membrane by FecR (15), resulting in an active FecI sigma factor that directs the RNA polymerase core enzyme to the promoter of the *fecABCDE* transport genes (1, 6, 22, 23). The C-terminal domain of FecR (Fig. 1) is located in the periplasm (32), interacts with FecA (7, 15), and receives the signal from ferric citrate-loaded FecA. The N-terminal region of FecR is located in the cytoplasm and interacts with FecI (7, 17, 27). FecR contains a stretch of hydrophobic amino acids between residues 85 and 100 that spans the cytoplasmic membrane (32).

FecI belongs to the class of sigma factors that respond to extracytoplasmic stimuli (ECF) (10, 13, 16, 20, 33). ECF sigma factors are usually controlled by anti-sigma factors. No role as an anti-sigma factor has been uncovered for FecR. Instead, FecR is necessary for FecI to function as a sigma factor. To support this finding further, we generated point mutations in *fecR* by random and site-directed mutagenesis; the mutants obtained showed reduced or no transcription of the *fecABCDE* operon and were affected in binding of FecR to FecA. The mutations also revealed sites of interaction between FecR and FecA. One FecR mutant displayed a constitutive phenotype and bound more strongly than wild-type FecR to FecA. Many of the mutations were located within and close to a region that is conserved in FecR-like proteins. The motif is composed of repeating heptapeptides flanked by three leucine residues and one valine residue (Fig. 1). It resembles leucine zipper motifs contained in certain prokaryotic and eukaryotic gene-regulatory proteins (2, 14, 35), and they are also highly conserved in FecR-like proteins (Fig. 1). However, since the leucine zipper is not perfectly conserved (valine replaces leucine at one site, and proline is contained in several repeats) and the motif is located in the periplasm and does not bind to DNA, we use the term leucine motif. The data further support the involvement of FecR in signal transduction in a way that cannot be reconciled with a simple anti-sigma factor activity.

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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 medium (TY) or nutrient broth medium (NB) as described previously (1). Antibiotics were used at the following concentrations: ampicillin, 50 μg/ml; chloramphenicol, 40 μg/ml; and tetracycline, 12 μg/ml.

Construction of plasmids. The truncated $fecA_{1-79}$ fragment was synthesized by PCR. Plasmid plexFecA was obtained with primers A1XhoI (5′-CCACGGTAG ATCTTTATTCTTTTGGTGCG-3′) and A79BglII (5′-CCGCTTTTGCTCTCG AGGTTAATATCGCAC-3′). The resulting *fecA* fragment was digested with *Xho*I and *Bgl*II and cloned into *Xho*I- and *Bgl*II-restricted pDP804.

Plasmids pLZ1A and pLZ1 were constructed by site-directed mutagenesis of plasmid pAA70 with primers 2490A (5′-GAAGGATATCGCGACGTTCAGC G-3′) and 2490 (5′-GAAGGATATCCCGAGCTTCAGCG-3′), respectively, and the reverse primer 2490REV (5′-TCAGGATATCCTTCGTCCAGCTTG-3′) for introduction of the leucine substitutions at amino acid 247 and an *Eco*RV cleavage site at position 2490. The resulting PCR fragments were cleaved with *Eco*RV and religated. For construction of plasmid pLZ2 by site-directed mutagenesis, primers 2511 (5′-ACCGCCCGGGGAGGTGATAGCCACGCTAA-3′) and 2511REV (5′-CCTCCCCGGGCGGTTTATCGCTCAA-3′) were used to introduce the leucine-to-proline substitution at residue 254 and a *Sma*I cleavage site at position 2511.

Plasmids pLZ3 and pLZ4 were obtained by PCR with primers 2532 (5′-ACC CGGTACCGCAACGGCGTCGT-3′) and 2552 (5′-ACCCGGTACCGCAACG GCCCGCTGCGCT-3′), respectively, and reverse primers 2532REV (5′-TTGC GGTACCGGGTTGGCGTGGCTAT-3′) and 2552REV (5′-TTGCGGTAC

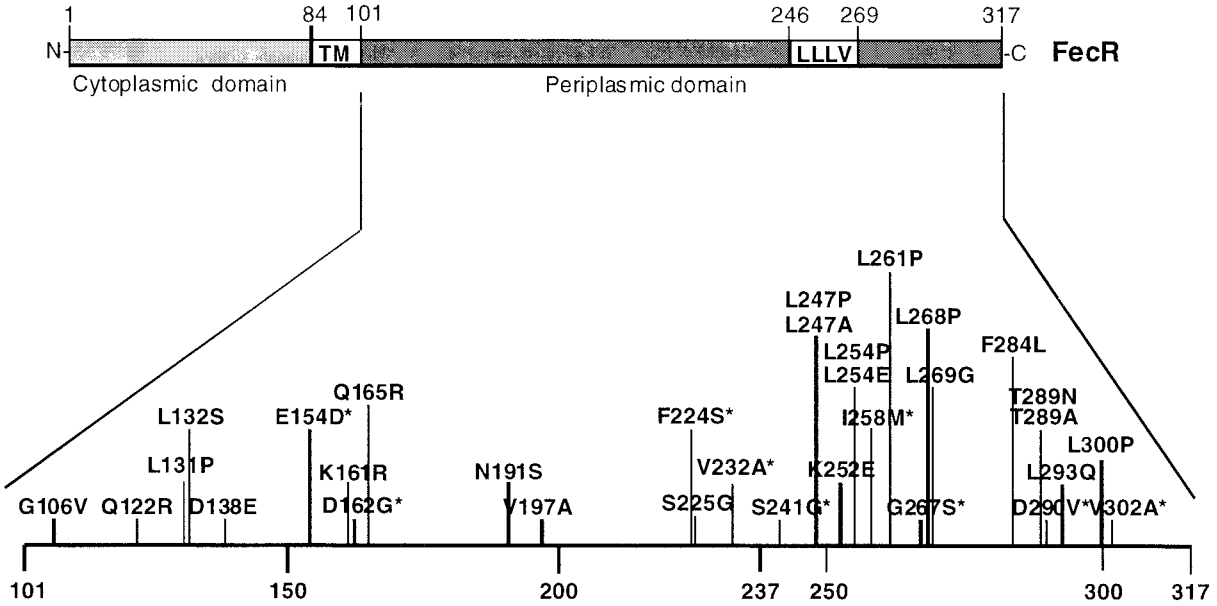

FIG. 1. Model of functional domains of FecR. TM, transmembrane-spanning segment between residues 85 and 100.

CGGGTTAGCGTGGCTAT-3′), respectively, replacing residues 261 and 268, respectively, with proline residues and introducing a *Kpn*I cleavage site.

Plasmid plexAN was constructed by *Bst*EII and *Xho*I restriction of plasmid pMS604 (5) and religation of the plasmid treated with Klenow polymerase. For the LexA repressor fusion proteins, the *fos* zipper motif of plasmid pMS604 was replaced with the wild-type or a mutated $fecR_{101\text{-}317}$ fragment. $fecR_{101\text{-}317}$ from plasmids pAA70, pLZ1A, pLZ1, pLZ2, pLZ3, and pLZ4 was amplified by PCR with primers lexfecR8 and lexfecR9 (7), cleaved with *Bst*EII and *Xho*I, and cloned into *Bst*EII- and *Xho*I-restricted pMS604, yielding plasmids plexRC, plexLZ1A, plexLZ1, plexLZ2, plexLZ3, and plexLZ4, respectively.

For construction of plasmid pGFPA′, the *fecA* promoter region was amplified by PCR with primers AA11 (1) and PA2769 (5′-GCCCTAGGTTGTGTTCAG CTATG-3′). The resulting PCR fragment was cleaved with *Eco*RI and *Bam*HI and cloned into *Eco*RI- and *Bam*HI-digested pFPV25 (30). The complete *fecA*-containing fragment was obtained by PCR with primers AA11 and FA5160 (5′-CGGAATTCTAATCACATTCCAGC-3′), restricted with *Eco*RI, and ligated into *Eco*RI-cleaved pGFPA′ in the opposite orientation to P_{fecA}::*gfp*, resulting in plasmid pGFPAA′. To replace wild-type *fecR* with the leucine zipper mutants, the *Nde*I-*Hin*dIII fragment of plasmids pLZ1A, pLZ1, pLZ2, pLZ3, and pLZ4 was cloned into *Nde*I- and *Hin*dIII-restricted pMMO203 (27), yielding plasmids pLCLZ1A, pLCLZ1, pLCLZ2, pLCLZ3, and pLCLZ4, respectively.

The region of *fecR* encoding $fecR_{237\text{-}317}$ was obtained by PCR amplification with primers RBstE237 (5′-GCTGGTGACCGAGAGTACAAGCTGGACGA A-3′) and lexfecR9 (5′-CCCCTCGAGTTACAGTGGTGAAATGTTTAT-3′) and plasmid pAA70 as the template. The resulting PCR fragment was digested with *Bst*EII and *Xho*I and ligated into *Bst*EII- and *Xho*I-restricted pMS604, yielding plasmid plex237. Plasmid plex288 was obtained by PCR amplification of $fecR_{101\text{-}288}$ with primers lexfecR8 (5′-GAACCGGTGACCTCGGAAACCGGC GAAGGT-3′) and RXhoI288 (5′-GGATCTCGAGTCAATTTTTCAGCGGG AACGTCC-3′) and plasmid pAA70 as the template. Plasmid plex278 encoding $fecR_{101\text{-}278}$ was obtained by PCR amplification with primers lexfecR8 and RXhoI278 (5′-CCCCTCGAGTCACAGCCCGGCAACGGCGGGATC-3′) and plasmid pAA70 as the template. Plasmid plexLZG encoding $fecR_{227\text{-}288}$ was obtained by PCR amplification with primers RBstEII227 (5′-GTTGGTGACCT CTGAGTTTGGCGCAGTG-3′) and RXhoI288 and plasmid pAA70 as the template. The resulting PCR fragments were digested with *Bst*EII and *Xho*I and cloned into *Bst*EII- and *Xho*I-restricted pMS604.

$fecR_{224\text{-}317}$ was randomly mutagenized by PCR with primers FecR1 (5′-CTG GAGTATGGCATATGAATC-3′) and FecIR3 (5′-GGGAATTATTAAGCTT ACAGTGG-3′) and plasmid pSV66 as the template. The resulting *fecR* fragments were cleaved with *Pst*I and *Hin*dIII and cloned into the *Pst*I- and *Hin*dIII-restricted plasmid pIS712 *fecIRA*, yielding plasmids pOR603, pOR601, and pOR600.

Mutated *fecA* genes were amplified by PCR with primers AA4 (5′-CCGTTA GAATTCAGTCTATTACGC-3′) and AA13 (5′-GGCGTGGCGGATCCCCA GCAGCAGGCC-3′) and pIS712 as the template. The *fecA* fragments were digested with *Eco*RI and *Dsa*I and ligated into *Eco*RI- and *Dsa*I-cleaved plasmid pOR600, yielding plasmids pOR600D1 and pOR600C3. The mutated *fecA* fragments of plasmids pOR600D1 and pOR600C3 were cleaved with *Eco*RI and *Dsa*I and cloned into *Eco*RI- and *Dsa*I-restricted plasmids pIS712, pOR603, and pOR601, yielding plasmids pORD1, pORC3, pOR603D1, pOR601D1, pOR603C3, and pOR601C3, respectively.

The periplasmic domain of FecR, representing the region from amino acids 101 to 317, was randomly mutagenized by PCR with primers LexFecR1 (5′-CGCCTCGAGGGATCTAGATCGGAAACCGGCGAAGGT-3′) and LexFecR2 (5′-GGAAGATCTTCCACCTAGTTTACAGTGGTGAAATGTT-3′) and plasmid pSV66 as the template. The mutated *fecR* fragments were cloned into pDP804 by replacing the *Xho*I-BglII fragment containing the Jun zipper motif, resulting in plasmids pHBlexR2, pHBlexR7, pHBlexR12, pHBlexR13, pHBlexR15, pHBlexR16, pHBlexR21, and pHBlexR22. The *fecR* point mutations were identified by DNA sequencing.

Plasmid $pMS604fecA_{1\text{-}79}$ encodes the N-terminal region of FecA from amino acids 1 to 79. This plasmid was obtained by replacing the *Bst*EII-*Xho*I fragment containing the Fos zipper on plasmid pMS604 with sequence encoding the N-terminal region of the mature FecA protein. The gene was amplified from plasmid pSV66 with oligonucleotides LexFecA3 (5′-GAACCGGTGACCGGA TCTAGAGCACAGGTTAATATCGGA-3′) and LexFecA2 (5′-TTCCCCCT CGAGTCCACTAGTTTCTTTTGGTGCGGGCGC-3′). For construction of plasmids pHBlcR2, pHBlcR7, pHBlcR12, pHBlcR13, pHBlcR15, pHBlcR16, pHBlcR21, and pHBlcR22, the DNA encoding the N-terminal region of the mature FecR protein was amplified with primers fecR1 (5′-CTGGAGTATGG CATATGAATC-3′) and AgeIWTfecR (5′-ATCCCTACCGGTTTCCCGCTGC CAGAGCTGCCA-3′) with plasmid pSV66 as the template. The mutated periplasmic part of FecR was amplified by PCR with oligonucleotides AgemutFecR (5′-ATCCCTACCGGTGAAGGTCTGCGGGCAGATTAC-3′) and the reverse primer pDP804HindIII (5′-CGTTGCCAAGCTTCTTTTACCCCTGCATCTT TG-3′) with plasmid pHBlexR2, pHBlexR7, pHBlexR12, pHBlexR13, pHBlexR15, pHBlexR16, pHBlexR21, or pHBlexR22 as the template. The two *Age*I-digested PCR fragments were ligated and cloned into plasmid pLCIRA, replacing the *Nde*I-*Hin*dIII region encoding wild-type FecR.

To obtain plasmids pHBhcR2, pHBhcR7, pHBhcR12, pHBhcR13, pHBhcR15, pHBhcR16, pHBhcR21, and pHBhcR22, wild-type *fecR* was cut out of plasmid pHCIR with *Nde*I and *Hin*dIII and replaced with the *Nde*I- and *Hin*dIII-digested mutated *fecR* of plasmids pHBlcR2, pHBlcR7, pHBlcR12, pHBlcR13, pHBlcR15, pHBlcR16, pHBlcR21, and pHBlcR22, respectively.

Recombinant DNA techniques. Standard techniques (25) 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. *E. coli* strains and plasmids used in this study

Strain or plasmid	Genotype[a]	Source or reference
E. coli		
DH5α	Δ(*argF lac*) *U196 endA1 recA1 hsdR17*(r_K^- m_K^+) *supE44 thi-1 gyrA1 relA1* (F′ ϕ80 Δ*lacZ*ΔM15)	11
AA93	Δ*fec aroB* Δ(*argF lac*)*U169 araD139 rspL150 relA1 deoC1 flbB5301 ptsF25 rbsR*	22
SU202	*lexA71*::Tn*5 sulA211 sulA*(op408/op+)::*lacZ* Δ(*lacIPOZYA*)*169*/F′ *lacI*^{q} *lacZ*ΔM15::Tn*9*	5
Plasmids		
pSV66	pHSG576 *fecI fecR fecA*	12
pMS604	*ori* ColE1 Tet^r *lexA*$_{1–87}$-*fos* zipper	5
pDP804	*ori* p15A Amp^r *lexA*$_{1–87}$408-*jun* zipper	5
plexFecA	pDP804 *lexA*$_{1–87}$408-*fecA*$_{1–79}$	This study
plexAN	pMS604 *lexA*$_{1–87}$	This study
plexRC	pMS604 *lexA*$_{1–87}$-*fecR*$_{101–317}$	This study
plex237	pMS604 *lexA*$_{1–87}$-*fecR*$_{237–317}$	This study
plex288	pMS604 *lexA*$_{1–87}$-*fecR*$_{101–288}$	This study
plex278	pMS604 *lexA*$_{1–87}$-*fecR*$_{101–278}$	This study
plexLZG	pMS604 *lexA*$_{1–87}$-*fecR*$_{227–288}$	This study
pT7-7	*ori* ColE1 phage T7 gene promoter	28
pAA70	pT7-7 *fecR*	34
pLZ1A	pT7-7 *fecR*(L247A)	This study
pLZ1C	pT7-7 *fecR*(L247C)	This study
pLZ1	pT7-7 *fecR*(L247P)	This study
pLZ2	pT7-7 *fecR*(L254P)	This study
pLZ3	pT7-7 *fecR*(L261P)	This study
pLZ4	pT7-7 *fecR*(V268P)	This study
plexLZ1A	pMS604 *lexA*$_{1–87}$-*fecR*$_{101–317}$(L247A)	This study
plexLZ1C	pMS604 *lexA*$_{1–87}$-*fecR*$_{101–317}$(L247C)	This study
plexLZ1	pMS604 *lexA*$_{1–87}$-*fecR*$_{101–317}$(L247P)	This study
plexLZ2	pMS604 *lexA*$_{1–87}$-*fecR*$_{101–317}$(L254P)	This study
plexLZ3	pMS604 *lexA*$_{1–87}$-*fecR*$_{101–317}$(L261P)	This study
plexLZ4	pMS604 *lexA*$_{1–87}$-*fecR*$_{101–317}$(V268P)	This study
pFPV25	*ori* ColE1 Amp^r contains promoterless *gfp*mut3	30
pGFPA′	pFPV25 P$_{fecA}$-*gfp*	This study
pGFPAA′	pFPV25 *fecA* P$_{fecA}$-*gfp*	This study
pHSG576	pSC101 derivative, Cm^r	29
pMMO203	pHSG576 *fecI fecR*	27
pLCLZ1A	pHSG576 *fecI fecR*(L247A)	This study
pLCLZ1C	pHSG576 *fecI fecR*(L247C)	This study
pLCLZ1	pHSG576 *fecI fecR*(L247P)	This study
pLCLZ2	pHSG576 *fecI fecR*(L254P)	This study
pLCLZ3	pHSG576 *fecI fecR*(L261P)	This study
pLCLZ4	pHSG576 *fecI fecR*(V268P)	This study
pIS712	pHSG576 *fecI fecR fecA*	15
pOR603	pHSG576 *fecI fecR*(T2511C)(L254E) *fecA*	This study
pOR601	pHSG576 *fecI fecR*(C2255G/T2256C)(L269G) *fecA*	This study
pOR600	pHSG576 *fecI fecR*(T2600C/C2602G)(F284L) *fecA*	This study
pOR603D1	pHSG576 *fecI fecR*(T2511C)(L254E) *fecA*(C3054A)(G39R)	This study
pOR601D1	pHSG576 *fecI fecR*(C2255G/T2256C) (L269G) *fecA*(C3054A) (G39R)	This study
pOR600D1	pHSG576 *fecI fecR*(T2600C/C2602G) (F284L) *fecA*(C3054A) (G39R)	This study
pOR603C3	pHSG576 *fecI fecR*(T2511C)(L254E) *fecA*(G3004C)(D43E)	This study
pOR601C3	pHSG576 *fecI fecR*(C2255G/T2256C) (L269G) *fecA*(G3004C) (D43E)	This study
pOR600C3	pHSG576 *fecI fecR*(T2600C/C2602G) (F284L) *fecA*(G3004C) (D43E)	This study
pORD1	pHSG576 *fecI fecR fecA*(C3054A) (G39R)	This study
pORC3	pHSG576 *fecI fecR fecA*(G3004C) (D43E)	This study
pLCIRA	pHSG576 *fecI fecR fecA*	27
pIS135	pHSG576 *fecI fecR*	22
pHCIR	pBCKS$^+$ *fecI fecR*$_{1–110}$	This study
pAS103	pBCKS$^+$ *fecI fecR*	27
pMS604*fecA*$_{1–79}$	pMS604 *lexA*$_{1–87}$-*fecA*$_{1–79}$	This study
pUS11	pDP804 *lexA*$_{1–87}$408-*fecR*$_{101–317}$	7
pHBlexR2	pDP804 *lexA*$_{1–87}$408-*fecR*$_{101–317}$ (T2164A/T2340C) (D138E/V197A)	This study
pHBlexR7	pDP804 *lexA*$_{1–87}$408-*fecR*$_{101–317}$ (A2235G/A2471G/A2619T) (D162G/S241G/D290V)	This study
pHBlexR12	pDP804 *lexA*$_{1–87}$408-*fecR*$_{101–317}$ (A2115G/T2142C/A2145G/A2232G/A2322G/A2504G/T2628A) (Q122R/L131P/N132S/K161R/N191S/K252E/L293Q)	This study
pHBlexR13	pDP804 *lexA*$_{1–87}$408-*fecR*$_{101–317}$ (T2445C/G2548A) (V232A/G267S)	This study
pHBlexR15	pDP804 *lexA*$_{1–87}$408-*fecR*$_{101–317}$ (A2423G/A2615G) (S225G/T289A)	This study
pHBlexR16	pDP804 *lexA*$_{1–87}$408-*fecR*$_{101–317}$ (C2616A/T2649C) (T289N/L300P)	This study
pHBlexR21	pDP804 *lexA*$_{1–87}$408-*fecR*$_{101–317}$ (A2212T/T2421C/A2524G/T2655C) (E154D/F224S/I258M/V302A)	This study
pHBlexR22	pDP804 *lexA*$_{1–87}$408-*fecR*$_{101–317}$ (G2067T/A2244G) (G106V/Q165R)	This study
pHBlcR2	pHSG576 *fecI fecR* (T2164A/T2340C) (D138E/V197A) *fecA*	This study
pHBlcR7	pHSG576 *fecI fecR* (A2235G/A2471G/A2619T) (D162G/S241G/D290V) *fecA*	This study

Continued on following page

TABLE 1—*Continued*

Strain or plasmid	Genotype[a]	Source or reference
pHBlcR12	pHSG576 *fecI fecR* (A2115G/T2142C/A2145G/A2232G/A2322G/A2504G/T2628A) (Q122R/L131P/N132S/K161R/N191S/K252E/L293Q) *fecA*	This study
pHBlcR13	pHSG576 *fecI fecR* (T2445C/G2548A) (V232A/G267S) *fecA*	This study
pHBlcR15	pHSG576 *fecI fecR* (A2423G/A2615G) (S225G/T289A) *fecA*	This study
pHBlcR16	pHSG576 *fecI fecR* (C2616A/T2649C) (T289N/L300P) *fecA*	This study
pHBlcR21	pHSG576 *fecI fecR* (A2212T/T2421C/A2524G/T2655C) (E154D/F224S/I258M/V302A) *fecA*	This study
pHBlcR22	pHSG576 *fecI fecR* (G2067T/A2244G) (G106V/Q165R) *fecA*	This study
pHBhcR2	pBCKS$^+$ *fecI fecR* (T2164A/T2340C) (D138E/V197A)	This study
pHBhcR7	pBCKS$^+$ *fecI fecR* (A2235G/A2471G/A2619T) (D162G/S241G/D290V)	This study
pHBhcR12	pBCKS$^+$ *fecI fecR* (A2115G/T2142C/A2145G/A2232G/A2322G/A2504G/T2628A) (Q122R/L131P/N132S/K161R/N191S/K252E/L293Q)	This study
pHBhcR13	pBCKS$^+$ *fecI fecR* (T2445C/G2548A) (V232A/G267S)	This study
pHBhcR15	pBCKS$^+$ *fecI fecR* (A2423G/A2615G) (S225G/T289A)	This study
pHBhcR16	pBCKS$^+$ *fecI fecR* (C2616A/T2649C) (T289N/L300P)	This study
pHBhcR21	pBCKS$^+$ *fecI fecR* (A2212T/T2421C/A2524G/T2655C) (E154D/F224S/I258M/V302A)	This study
pHBhcR22	pBCKS$^+$ *fecI fecR* (G2067T/A2244G) (G106V/Q165R)	This study

[a] Subscripts denote amino acid residues of the encoded proteins. Numbers in parentheses indicate amino acids replaced.

phoresis. DNA was sequenced by the dideoxy chain termination method (26) with the AutoRead sequencing kit (Pharmacia Biotech, Freiburg, Germany). The reaction products were sequenced on an A.L.F. DNA sequencer (Pharmacia Biotech).

Determination of β-galactosidase activity. β-Galactosidase activities were determined according to Miller (19) and Giacomini et al. (9). To determine the induction level, cells were grown in NB medium with no additions 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 isopropylthiogalactopyranoside (IPTG).

GFP measurements. Cells were grown in NB medium containing supplementations as indicated. Green fluorescent protein (GFP) was quantified by fluorometry in a 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 of 0.5 at 578 nm in phosphate-buffered saline (30).

Similarity search 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 *Pseudomonas syringae* were obtained from the Institute for Genomic Research website at http://www.tigr.org. The sequence data for *Pseudomonas* 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. Protein sequences were aligned with ClustalW.

RESULTS

Binding sites of FecR on FecA. The N-terminal domain of FecA (residues 1 to 79) interacts in vivo and in vitro with the C-terminal domain of FecR (residues 101 to 317) (7). To localize the region of FecR that interacts with FecA specifically, a bacterial LexA-based two-hybrid system was used. LexA is a transcriptional repressor that binds as a homodimer to the *sulA* promoter. It consists of an N-terminal DNA-binding domain and a C-terminal dimerization domain. To determine heterodimerization, the C-terminal domain can be replaced with the dimerization domains of other proteins. To prevent homodimerization of LexA hybrid proteins, the promoter of *sulA* is mutated so that wild-type LexA binds to one site and mutated LexA408 binds to the other site. Dimerization of the hybrid proteins was assessed by repression of chromosomal P_{sulA}::*lacZ* transcription of the reporter strain *E. coli* SU202. The $LexA_{1\text{-}87}$408 DNA-binding domain was fused to the N terminus of $FecA_{1\text{-}79}$ and the $LexA_{1\text{-}87}$ DNA-binding domain was fused to the N terminus of $FecR_{101\text{-}317}$ and to N- and C-terminally truncated derivatives of $FecR_{101\text{-}317}$. Control measurements involved LexA-Fos combined with LexA-$FecA_{1\text{-}79}$ as a negative control and LexA-$FecR_{101\text{-}317}$ combined with LexA-$FecA_{1\text{-}79}$ as a positive control (Table 2).

The truncated $FecR_{237\text{-}317}$ derivative combined with $FecA_{1\text{-}79}$ repressed P_{sulA}::*lacZ* transcription (Table 2). A more central fragment, $FecR_{101\text{-}288}$, and $FecA_{1\text{-}79}$ showed less repression (4.9-fold higher β-galactosidase activity than fully repressed cells). Deletion of a further 10 residues at the C terminus, resulting in $FecR_{101\text{-}278}$, and also $FecR_{227\text{-}288}$ led to no repression. The data indicate that a region encompassing residues 237 to 317 of FecR is required for the interaction with $FecA_{1\text{-}79}$.

Point mutations in FecR leucine motif reduce binding to FecA. $FecR_{237\text{-}317}$, which was sufficient for binding to $FecA_{1\text{-}79}$, contains a conserved leucine motif within residues 247 to 268 (Fig. 1 and 2). To determine whether the leucine motif is important for binding of FecR to $FecA_{1\text{-}79}$, the leucine and valine residues were replaced with proline residues. In addition, the first leucine residue was replaced with alanine and cysteine residues.

E. coli SU202 was transformed with plasmids carrying *lexA-fecA*$_{1\text{-}79}$ and the mutated *lexA-fecR*$_{101\text{-}317}$ fusion genes. All leucine-to-proline mutations resulted in higher levels of

TABLE 2. Interaction of truncated $FecR_{101–317}$ derivatives with $FecA_{1–79}$

Plasmid	Proteins	β-Galactosidase activity[a] (Miller units)
pMS604	$LexA_{1–87}$WT-Fos	478
plexRC	$LexA_{1–87}$WT-$FecR_{101–317}$	45
plex237	$LexA_{1–87}$WT-$FecR_{237–317}$	65
plex288	$LexA_{1–87}$WT-$FecR_{101–288}$	221
plex278	$LexA_{1–87}$WT-$FecR_{101–278}$	453
plexLZG	$LexA_{1–87}$WT-$FecR_{227–288}$	453

[a] Determined by using the bacterial two-hybrid LexA-based system in *E. coli* SU202 *sulA-lacZ*, which, in addition to FecR derivatives, synthesized $LexA_{1–87}$ 408-$FecA_{1–79}$ encoded by plexFecA. WT, wild type.

FIG. 2. Alignment of leucine motifs of FecR homologues. Similarity search and sequence alignment were done as described in Materials and Methods. Note the highly conserved leucine residues of the leucine motif corresponding to positions 247, 254, 261, and 268 of *E. coli* FecR. See additional information in Martinez-Bueno et al. (18), Nelson et al. (21), and Visca et al. (31).

P_{sulA}::*lacZ* transcription than wild-type $FecR_{101-317}$ (Table 3). Proline residues introduced at positions 247 and 268 reduced binding to $FecA_{1-79}$ less than proline substitutions at positions 254 and 261 in the middle of the leucine zipper-like motif (Table 3). Proline distorts an α-helix, particularly when it is located in the middle of an α-helix. Alanine at position 247 slightly reduced binding to $FecA_{1-79}$, and cysteine exerted a somewhat stronger effect. To eliminate the possibility that differences in activity were caused by different amounts of protein, Western analysis was performed with an anti-LexA antibody. The expression level of the hybrid proteins was low, but similar amounts of the proteins were observed (data not shown). The amounts of the plasmid-encoded FecR derivatives were higher than the amounts of chromosomally encoded wild-type FecR. These results demonstrate the important role played by the conserved leucine residues in binding of FecR to FecA.

TABLE 3. Binding of mutated $FecR_{101–317}$ to $FecA_{1–79}$

Plasmid	FecR proteins	β-Galactosidase activity[a] (Miller units)
pSM604	$LexA_{1–87}$WT-Fos	446
plexRC	$LexA_{1–87}$WT-$FecR_{101–317}$	36
plexLZ1A	$LexA_{1–87}$WT-FecR (L247A)	54
plexLZ1C	$LexA_{1–87}$WT-FecR (L247C)	94
plexLZ1	$LexA_{1–87}$WT-FecR (L247P)	170
plexLZ2	$LexA_{1–87}$WT-FecR (L254P)	367
plexLZ3	$LexA_{1–87}$WT-FecR (L261P)	376
plexLZ4	$LexA_{1–87}$WT-FecR (L268P)	112

[a] Determined by using the bacterial two-hybrid LexA-based system in *E. coli* SU202 *sulA-lacZ*, which, in addition to FecR derivatives, synthesized $LexA_{1–87}$ 408-$FecA_{1–79}$ encoded by plexFecA. WT, wild type.

TABLE 4. Binding of mutated $FecR_{101–317}$ to $FecA_{1–79}$

Plasmid	FecR protein	β-Galactosidase activity[a] (Miller units)
pDP804	$LexA_{1–87}$408-Jun	636
pUS11	$LexA_{1–87}$408-$FecR_{101–317}$	134
pHBLexR2	$LexA_{1–87}$408-$FecR_{101–317}$ (D138E, V197A)	93
pHBLexR7	$LexA_{1–87}$408-$FecR_{101–317}$ (D162G, S241G, D290V)	542
pHBLexR12	$LexA_{1–87}$408-$FecR_{101–317}$ (Q122R, L131P, N132S, K161R, N191S, K252E, L293Q)	607
pHBLexR13	$LexA_{1–87}$408-$FecR_{101–317}$ (V232A, G267S)	516
pHBLexR15	$LexA_{1–87}$408-$FecR_{101–317}$ (S225G, T289A)	537
pHBLexR16	$LexA_{1–87}$408-$FecR_{101–317}$ (T289N, L300P)	688
pHBLexR21	$LexA_{1–87}$408-$FecR_{101–317}$ (E154D, F224S, I258M, V302A)	754
pHBLexR22	$LexA_{1–87}$408-$FecR_{101–317}$ (G106V, Q165R)	731

[a] Determined by using the bacterial two-hybrid LexA-based system in *E. coli* SU202 *sulA-lacZ*, which, in addition to FecR derivatives, synthesized $LexA_{1–87}$-$FecR_{1–79}$ encoded by pMS604fec$A_{1–79}$.

Randomly generated point mutations in FecR affect binding to FecA. To determine additional FecR residues that are important for the interaction with $FecA_{1-79}$, binding of PCR-mutated $FecR_{101-317}$ to FecA was studied in the LexA-based two-hybrid system. The $LexA_{1-87}$ DNA-binding domain was fused to the N terminus of $FecA_{1-79}$, and the $LexA_{1-87}$ 408 DNA-binding domain was fused to the N terminus of $FecR_{101-317}$. Blue colonies on TY agar plates supplemented with IPTG and 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside (X-Gal) were selected from *E. coli* SU202 transformed with mutagenized *fecR*$_{101-317}$ and wild-type *fecA*$_{1-79}$ because they indicated lack of repression of P_{sulA}::*lacZ* and consequently lack of heterodimerization. All FecR mutants except FecR(D138E, V197A) displayed high transcription levels (Table 4). The mutations were clustered in the regions from residues 131 to 165 and from 224 to 302 (Fig. 1).

FecR point mutants display reduced activation of FecI. The site-directed and randomly generated FecR point mutants were tested to determine whether reduction of binding to FecA affected transcription initiation of the *fec* transport genes. The DNA fragment encoding the wild-type leucine zipper-like motif of FecR was replaced with the mutated fragment, and the resulting *fecR* mutant genes were cloned on the low-copy-number plasmid pHSG576 together with wild-type *fecI*. After confirming that the FecR mutant proteins were contained in the membrane fraction in amounts similar to that of wild-type FecR (data not shown), transcription of the *fec* transport genes was determined in *E. coli* AA93 lacking the *fec* genes but carrying the reporter plasmid pGFPAA′, which encodes complete *fecA* and P_{fecA}::*gfp*, the green fluorescence protein gene under the control of the *fecA* promoter. The promoter upstream of *fecA* controls transcription of the *fecABCDE* transport genes (6). *E. coli* AA93/pGFPAA′ was transformed with plasmids carrying the *fecR* mutant genes and the *fecI* wild-type gene.

The FecR mutant proteins in the leucine motif induced ferric citrate-dependent *fecA* transcription less than wild-type FecR (low fluorescence activity; Table 5). The mutations that most strongly reduced P_{fecA}::*gfp* transcription also most strongly reduced dimerization (high β-galactosidase activity; Table

TABLE 5. Induction activity of FecR leucine motif mutants[a]

Plasmid	FecR protein	Relative fluorescence	
		NB medium	NB medium + citrate
pHSG576	None	3	4
pMMO203	FecR	22	356
pLCLZ1A	FecR(L247A)	21	285
pLCLZ1C	FecR(L247C)	21	235
pLCLZ1	FecR(L247P)	18	107
pLCLZ2	FecR(L254P)	25	28
pLCLZ3	FecR(L261P)	10	36
pLCLZ4	FecR(V268P)	11	174

[a] Induction was determined in *E. coli* AA93 Δ*fec*(pGFPAA′ *fecA*, P_{fecA}::*gfp*). The plasmids listed encoded FecI in addition to the listed FecR derivatives.

3). Induction depended on the inducer, ferric citrate (Table 5). Only background fluorescence was detected in *E. coli* AA93 (pGFPAA′) transformed with the vector plasmid pHSG576. This result supports the involvement of the leucine motif in *fecA* promoter activity mediated by FecI.

The randomly generated *fecR* mutant genes were cloned into the low-copy-number plasmid pLCIRA *fecIRA* to replace the sequence of wild-type *fecR* encoding residues 101 to 317. The transcription levels were measured in *E. coli* AA93 carrying the reporter plasmid pGFPA′ P_{fecA}::*gfp*. All FecR mutants conferred lower transcription levels than wild-type FecR, which depended on ferric citrate (Table 6). Only FecR(D138E, V197A) displayed constitutive P_{fecA}::*gfp* transcription that was only slightly increased by ferric citrate. This FecR mutant bound to FecA even more strongly than wild-type FecR (Table 4). The level of P_{fecA}::*gfp* transcription was correlated with binding of FecR to FecA with the exception of FecR(S225G, T289A), which showed strongly reduced binding to $FecA_{1-79}$ but a rather high transcription level (58%) in the presence of ferric citrate. Inactivity of the mutated FecR could be caused by instability of the point mutants. Therefore, their amounts were estimated in uninduced and induced cells by Western blot analysis with anti-FecR antibodies. FecR(D162G, S241G, D290V), FecR(V232A, G267S), and FecR(E154D, F224S, I258 M, V302A) were not detectable in the blot (data not shown), which implied that their low binding to FecA and low induction of P_{fecA}::*gfp* were caused by small amounts of protein.

Mutations in *fecA* that restore transcription initiation of *fecR* mutants. Another approach to identify interacting regions of FecA and FecR used genetic suppression analysis. *fecR* point mutants were isolated and analyzed for restoration of transcription initiation of the *fec* transport genes by *fecA* point mutants. A *fecR* fragment encoding $FecR_{224-317}$ was mutagenized by PCR, and the *fecR* genes were cloned into the low-copy-number plasmid pIS712 *fecIRA*. *E. coli* AA93 Δ*fec* (pGFPA′ P_{fecA}::*lacZ*) was transformed with the mutagenized plasmids, and white and pink transformant colonies were picked on MacConkey agar plates containing 1 mM citrate. Citrate and iron in the nutrient agar form ferric citrate. Transformants containing the wild-type plasmid pIS712 formed red colonies. The mutated *fecR* genes of three white colonies were sequenced and shown to contain a leucine-to-glutamine change at position 254 (L254E), 269 (L269G), or 284 (F284L) (Table 7). To confirm the results obtained on the MacConkey agar plates, the inducing activity of the mutated *fecR* genes was determined in *E. coli* AA93 Δ*fec*(pGFPA′ P_{fecA}::*gfp*) transformed with the *fecR* mutant plasmids. Cells were grown in NB medium with and without added citrate, and their relative fluorescence was determined. All three mutants displayed a fluorescence of 7 to 9% of that of wild-type *fecR* in the presence of citrate (Table 7).

To identify residues in the FecA N terminus that bind FecR, mutants with mutations in *fecA* that suppressed the *fecR* missense mutations were isolated. *fecA* was mutagenized by PCR, and the fragments comprising residues 1 to 156 were inserted into plasmid pIS712 *fecIRA* encoding each of the *fecR* mutations. *E. coli* AA93 Δ*fec* harboring the reporter plasmid pMMO1034 P_{fecA}::*lacZ* was transformed with each of the *fecIRA* derivatives. Transformants that formed red colonies on MacConkey agar plates were picked, plasmids were isolated from them, and the isolated *fecA* fragments were sequenced. Two mutants were isolated; one contained a G39R replacement, and the other contained a D43E replacement. The relative fluorescence of *E. coli* AA93(pGFPA′ P_{fecA}::*gfp*) and of the same strain transformed with each of the *fecIRA* mutant derivatives was measured to verify the results obtained on the MacConkey agar plates and to obtain quantitative data. The mutated FecA proteins increased P_{fecA}::*gfp* transcription 1.6- to 4.8-fold (Table 7). Some allele specificity was observed; for example, FecR(L269G) combined with FecA(G39R) yielded a relative fluorescence of 318, whereas FecR(L269G) combined with FecA(D43E) yielded a relative fluorescence of 124. Both FecA derivatives were highly active in combination with wild-type FecA (Table 7).

DISCUSSION

FecR consists of three domains: the periplasmic signal sensor, the transmembrane signal transmitter, and the cytoplasmic signal receiver that conveys the signal to the FecI sigma factor. Previously, we have reported that the C terminus of $FecR_{101-317}$ binds to the N terminus of $FecA_{1-79}$ (7). In this

TABLE 6. Induction activity of FecR mutants[a]

Plasmid	Protein	Relative fluorescence (% of wild-type)		Stability
		NB medium	NB medium + citrate	
pIS135	FecR	7	8	Wild type
pLCIRA	FecR	17	100	Wild type
pHBlcR2	FecR(D138E, V197A)	43	74	As wild type
pHBlcR7	FecR(D162G, S241G, D290V)	7	8	Unstable
pHBlcR12	FecR(Q122R, L131P, N132S, K161R, N191S, K252E, L293Q)	7	10	As wild type
pHBlcR13	FecR(V232A, G267S)	22	18	Unstable
pHBlcR15	FecR(S225G, T289A)	15	58	As wild type
pHBlcR16	FecR(T289N, L300P)	16	14	As wild type
pHBlcR21	FecR(E154D, F224S, I258M, V302A)	10	12	Unstable
pHBlcR22	FecR(G106V, Q165R)	7	10	As wild type

[a] Induction was determined in *E. coli* AA93 Δ*fec*(pGFPA′ *fecA*, P_{fecA}::*gfp*). The plasmids listed encoded FecA and FecI in addition to the listed FecR derivatives except pIS135, which encoded FecI but no FecA.

TABLE 7. Induction activity of FecR and FecA mutants[a]

Plasmid	Protein	Relative fluorescence	
		NB medium	NB medium + citrate
pHSG576	None	40	45
pIS712	FecR FecA	65	856
pOR603	FecR(L254E) FecA	67	70
pOR601	FecR(L269G) FecA	59	66
pOR600	FecR(F284L) FecA	58	63
pOR603D1	FecR(L254E) FecA(G39R)	65	78
pOR601D1	FecR(L269G) FecA(G39R)	63	318
pOR600D1	FecR(F284L) FecA(G39R)	48	108
pOR603C3	FecR(L254E) FecA(D43E)	65	139
pOR601C3	FecR(L269G) FecA(D43E)	65	124
pOR600C3	FecR(F284L) FecA(D43E)	47	289
pORD1	FecR FecA(G39R)	45	801
pORC3	FecR FecA(D43E)	43	764

[a] Induction was determined in *E. coli* AA93 Δ*fec*(pGFPA′ *fecA*, P_{fecA}::*gfp*). The plasmids listed encoded FecI in addition to the listed FecR and FecA derivatives.

study, the site of FecR to which FecA binds was more accurately delineated. The region comprising residues 237 to 317 of FecR was found to be sufficient for binding to $FecA_{1-79}$. Further-truncated FecR C termini displayed low or no interaction with $FecA_{1-79}$. The binding site of FecR contains the leucine motif, whose role was assessed by replacing the leucine residues with alanine or proline residues. Interaction, as evidenced by repression by the mutated $LexA_{1-87}$-$FecR_{101-317}$ proteins, was 1.5- to 10-fold lower than repression by wild-type $LexA_{1-87}$-$FecR_{101-317}$, depending on the nature of the introduced amino acid and the position within the motif. The strongest effects were observed with proline substitutions in the center of the motif, as found previously for prokaryotic leucine zipper motifs (2, 14, 35). The transcription-inducing activity of the FecR mutant proteins was correlated with the degree of binding of mutated $FecR_{101-317}$ to $FecA_{1-79}$. The FecR leucine zipper mutant proteins that showed the lowest degree of dimerization showed the lowest level of P_{fecA}::*gfp* transcription.

Suppression of *fecR* mutations by *fecA* mutations supported the functionally relevant binding of the periplasmic regions of FecA and FecR. The independently isolated inactive FecR mutations L254E, L269G, and F284L in FecA wild-type cells were located within or near the leucine motif (residues 247 to 268), even though the entire periplasmic fragment, $FecR_{101-317}$, was randomly mutagenized. Restoration of transcription induction of the FecR mutants depended on the FecA mutants and the presence of ferric citrate. The FecA mutations were located close to each other at the beginning of the N terminus of FecA. FecA(G39R), with the mutation of a small neutral residue to a large basic residue, combined with FecR(L254E) and FecR(L269G) increased the transcription activity at least to 1.5-fold compared to that of wild-type FecA. The FecA (D43E) mutant is particularly noteworthy because the difference between wild-type and mutant FecA is just a CH_2 group. Despite this small change, FecA(D43E) restored the transcription activity of the FecR point mutations, especially of FecR (F284L). The complementing mutants did not display strict allele specificity, which leads us to conclude that the mutations mainly affect the conformations of the interacting regions and do not reveal interacting amino acid side chains.

The two-hybrid system offered the possibility to screen for FecR mutants that no longer interact with FecA. Such mutants should not repress P_{sulA}::*lacZ* transcription. Of the eight FecR mutants isolated, three were not identifiable on Western blots and were therefore unstable. One mutant, FecR(D138E, V197A), bound to FecA better than wild-type FecR; it repressed P_{sulA}::*lacZ* transcription more strongly than wild-type FecR. This mutant transcribed P_{fecA}::*gfp* constitutively, which implied that it did not require ferric citrate for induction. FecR(D138E, V197A) in the absence of ferric citrate already assumes a conformation that activates FecI. FecR(D138E, V197A) might reflect the conformation of FecR activated by FecA in the presence of ferric citrate. This could mean that in the ferric citrate-dependent wild-type FecR, ferric citrate induces binding of FecR to FecA.

The data presented in this paper further support the concept that FecR does not act as a simple anti-sigma factor. FecR is involved in signal transduction from the cell surface and is required for FecI activity. A model consistent with the data proposes interaction of FecR with FecA, as has been demonstrated previously with a bacterial two-hybrid system (7). Upon binding of ferric citrate, FecA undergoes major and minor conformational changes that involve mainly extracellular loops of the β-barrel and the central plug domain, as revealed by the FecA crystal structure (8). Connected to the plug domain is the TonB box, which is involved in the interaction with TonB (24), and the N-terminal extension, which interacts with FecR (7, 15). Unfortunately, the crystal structure does not disclose the conformation of the N-terminal extension located in the periplasm; the extension is flexible in the ferric citrate-loaded and unloaded structure. It does, however, show that upon binding of ferric citrate, the TonB box region becomes disordered. The structural change in FecA is conveyed to FecR, which presumably reacts with a structural change. The structural change in FecR is communicated into the cytoplasm, where interaction with FecI produces active FecI, which subsequently acts as a sigma factor. FecI is released from FecR and binds to the *fec* promoter. The dependence of FecI activity on FecR could be caused by instability of FecI in the absence of FecR or by FecI's assuming a different, active conformation when it dissociates from FecR. The high constitutive inducing activity of $FecR_{1-85}$, which binds to FecI (27), might be caused by the stabilization of FecI and spontaneous dissociation of FecI from FecR in the absence of the signal.

Analysis of microbial genome sequences reveals at least 52 *fecI* and *fecR* homologs in more than 20 different genera with high abundance in certain bacteria, such as *Pseudomonas*, *Caulobacter*, and *Nitrosomonas*. FecIR of *E. coli* might become the paradigm for these transcription regulatory systems. As in *E. coli*, the genes homologous to *fecR* are preceded by genes homologous to *fecI* and are followed by *fecA* homologs. The promoters of the *fecA* homologs resemble extracytoplasmic sigma factor (ECF)-dependent promoters, and the encoded proteins contain an N-terminal extension that is not present in other TonB-dependent outer membrane transport proteins that have no role in transcription initiation. The pairwise identity between *E. coli* FecR and the FecR homologues ranges from 24 to 37%. They exhibit the highest

sequence identity in the C-terminal region: residues 242 to 317 of FecR, in the leucine motif, with hydrophobic residues at all four conserved positions (Fig. 2). Motif search programs do not reveal the FecR leucine-like zipper motif, probably because of the α-helix-breaking proline at position 253 of FecR. As this study shows, the leucine motif is part of the FecR binding site for binding to FecA. Other FecR homologs that contain proline or arginine instead of proline might bind to FecA through the leucine motif. Another signature of FecR proteins is the tryptophan residues in the N-proximal end, which in *E. coli* are essential for FecR activity. These tryptophan residues are highly conserved among FecR-like proteins from a variety of gram-negative bacteria and are only occasionally replaced with other aromatic amino acids (27).

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