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Iron regulates transcription of the *Escherichia coli* ferric citrate transport genes directly and through the transcription initiation proteins

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Abstract Ferric citrate induces transcription of the ferric citrate transport genes *fecABCDE* in *Escherichia coli* by binding to the outer-membrane receptor protein FecA without entering the cell. Replete iron concentrations inhibit transcription of the *fec* transport system via the iron-loaded Fur repressor. Here we show that the Fur repressor activated by Mn^{2+} (used instead of Fe^{2+}) binds to the promoter of the regulatory genes *fecIR* and to the promoter of *fecABCDE*. DNase I footprint analysis revealed that Mn^{2+}–Fur (50 nM) protected 30 nucleotides of the coding strand and 24 nucleotides of the noncoding strand of the *fecIR* promoter. Higher amounts of Mn^{2+}–Fur (100 nM) covered 41 nucleotides of the coding strand of the *fecIR* promoter and 38 nucleotides of the coding strand of the *fecA* promoter. The corresponding region of the noncoding strand of the *fecA* promoter was hypersensitive to DNase I. The results of a deletion analysis of the *fecA* promoter supported the previously assigned –35 and –10 regions and nucleotide position +11 for FecI–RNA polymerase interaction. Induction of *fecIR* transcription by iron limitation increased *fecB-lacZ* transcription 3.5-fold, whereas under constitutive *fecIR* transcription, iron limitation increased *fecB-lacZ* transcription twofold. The two iron-regulated sites of *fec* transport gene transcription suggest a fast response to sufficient intracellular iron concentrations by repression of *fecABCDE* transcription and a slower adaptation as the result of *fecIR* transcription inhibition.

Key words *Escherichia coli* · Ferric citrate transport · Transcription regulation · Surface signaling

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Introduction

Escherichia coli K-12 expresses six transport systems for Fe^{3+}, which due to its insolubility is not taken up as a metal ion but is taken up in the form of ferric siderophore complexes. One of the siderophores, ferric citrate, is transported into cells by the ferric citrate transport system. The five transport genes *fecABCDE* (Pressler et al. 1988; Staudenmaier et al. 1989) are transcribed from a promoter upstream of *fecA* (Angerer et al. 1995). The *fecA* gene product catalyzes transport across the outer membrane, and the *fecBCDE* gene products catalyze binding-protein-dependent transport across the cytoplasmic membrane.

The ferric citrate transport system is the only ferric siderophore transport system in *E. coli* K-12 that is induced by the substrate. Most interestingly, ferric citrate does not have to be taken up into the cell in order to induce transcription of the *fec* transport genes. Binding to FecA is sufficient for induction to occur (Härle et al. 1995; Braun 1997). In addition to FecA, the Ton system (consisting of the TonB, ExbB, and ExbD proteins) and the electrochemical potential of the cytoplasmic membrane are required for transfer of the transcription initiation signal across the outer membrane (Kim et al. 1997).

The regulatory genes *fecIR* (Van hove et al. 1990) are encoded upstream of *fecABCDE* and form a transcription unit independent of *fecABCDE*. The C-terminus of FecR is located in the periplasm (Ochs et al. 1995) and is thought to interact with the N-terminus of FecA, which is essential for signal transfer but dispensable for transport (Kim et al. 1997). The N-terminus of FecR is located in the cytoplasm and serves to transmit the signal of the presence of ferric citrate bound to FecA to the FecI σ-factor (Lonetto et al. 1994; Angerer et al. 1995; Enz et al. 1995; Ochs et al. 1996), which in turn mediates transcription of the *fec* transport genes by the RNA polymerase.

Primer-extension experiments determined nucleotide 2738 (Angerer et al. 1995) or 2741 (Enz et al. 1995) as the transcription initiation site of *fecABCDE*. [These and the following nucleotide numbers refer to the published nucleotide sequence of *fecI* and *fecR* and the region up-

stream of *fecIR* (Van hove et al. 1990).] A 75-bp DNA fragment containing the *fecA* promoter has been shown to bind the FecI-RNA polymerase (Angerer et al. 1995). Isolated *fecA* promoter mutants with altered FecI–RNA polymerase binding have mutations clustered around position +11 (relative to nucleotide 2741) of the *fecA* transcription start site (Angerer et al. 1995; Enz et al. 1995), which indicates that FecI interacts most strongly with the +11 region. Since this is an unusual binding site for a σ^{70}-factor, we analyzed transcription of *fecA-lacZ* under the control of various DNA fragments of the region upstream of *fecA*.

In addition to induction by ferric citrate, the *fec* transport genes are repressed by the iron-loaded Fur protein if sufficient amounts of iron are present in the cell (Van hove et al. 1990; Enz et al. 1995; Ochs et al. 1996). Transcription of the *fecIR* genes is derepressed by iron limitation, and the increased amounts of FecIR accelerate ferric-citrate-mediated *fecABCDE* transcription. Fur is a 16.8-kDa protein rich in histidine (Schäffer et al. 1985) that binds 1 mol of Fe^{2+} or Mn^{2+} per mol of protein (De Lorenzo et al. 1987, 1988; Escolar et al. 1997). Comparison of the sequences of many iron-regulated promoters identified a similar nucleotide sequence (Fur box) to which Fe^{2+}–Fur binds (De Lorenzo et al. 1987, 1988). A Fur box is present in the promoter of *fecIR* and in the promoter of the *fecABCDE* transport genes (Van hove et al. 1990), which match 15 and 11 out of 19 residues of the Fur consensus sequence GATAATGATAATCATTATC, respectively. Iron regulation of *fecIR* transcription and, consequently, of *fecABCDE* transcription was demonstrated, but whether the *fecABCDE* promoter responds to Fe^{2+}–Fur has not been studied. Since iron regulation of *fecIR* transcription interferes with the examination of the direct (non-FecIR-mediated) iron regulation at the *fecABCDE* promoter, iron regulation at the *fecABCDE* promoter was examined in this study in an *E. coli* mutant that executes Fur-independent *fecIR* transcription. DNA footprinting with purified Fur protein on the *fecIR* and *fecABCDE* promoters was performed to determine whether both promoters bind metal-activated Fur repressor and to identify the DNA sequences that interact with Fur.

Table 1 Bacterial strains and plasmids used in this study

Strain or plasmid	Genotype	Source of reference
Strains *(Escherichia coli)*		
AB2847	*aroB malT tsx thi*	This institute
ZI418	*fecB*::Mud1 (Ap, *lac*) *araD139 ΔlacU169 rpsL150 relA1 flbB5301 deoC1 ptsF25 rbsR thi aroB*	Van hove et al. (1990)
MO704	ZI418 *fecI*::kan^R ′*fecR*	Ochs et al. (1996)
Plasmids		
pMLB1034	pBR322 derivative for cloning of *lacZ* gene fusion, Ap^R	Silhavy et al. (1984)
pMMO1034	pMLB1034 *fecA-lacZ*	Ochs et al. (1995)
pMAA11	pMLB1034 *fecA-lacZ*	This study
pMAA4	pMLB1034 *fecA-lacZ*	This study
pMAA19	pMLB1034 *fecA-lacZ*	This study
pMAA20	pMLB1034 *fecA-lacZ*	This study
pMAA22	pMLB1034 *fecA-lacZ*	This study
pMAA23	pMLB1034 *fecA-lacZ*	This study
pMAA1024	pMLB1034 *fecA24-lacZ*	This study
pMAA1083	pMLB1034 *fecA83-lacZ*	This study
pFPS24	pBCSK, *fecA24*	This study
pFP83	pBCSK, *fecA83*	This study
pSU18	*ori* p15A, Cm^R	Martinez et al. (1988)
pNR1	pSU18 *fecI*′	This study
pBCSK+	pColE1-derived cloning vector, Cm^R	Stratagene
pMMO10	pBCSK *fecA*′	Ochs et al. (1995)
pHSG576	pSC101 derivative, Cm^R	Takeshita et al. (1987)
pIS135	pHSG576 *fecIR*	Ochs et al. (1995)
pSUSK1	p15A origin, Cm^R	Kim et al. (1997)
pCM135	pSUSK1 ′*fecIR*	This study
pMMO1035	pMLB1034 *fecI-lacZ*	Ochs et al. (1996)
pSV66	pHSG576 *fecIRA*	Härle et al. (1995)

Materials and methods

Bacterial strains, growth conditions, and plasmids

The *E. coli* strains and plasmids used are listed in Table 1. *E. coli* cells were grown in nutrient broth (NB), tryptone-yeast extract medium (TY), or M9 minimal medium (Miller 1972). For growth of *aroB* mutants, M9 medium was supplemented with 2 mM 4-hydroxybenzoate, 2 mM 4-aminobenzoate, and 0.1 mg each per ml of tryptophan, phenylalanine, and tyrosine. Where indicated, citrate (1 mM), dipyridyl (100 or 200 μM), Fe_2SO_4 (0.1 mM), ampicillin (50 μg ml^{-1}), or chloramphenicol (40 μg ml^{-1}) were added to the media.

To construct the *fecA-lacZ* plasmids pMAA11, pMAA4, pMAA19, pMAA20, pMAA22, and pMAA23, *fecA* promoter fragments of different sizes were cloned into the *Eco*RI-*Bam*H1 site of the *lacZ* fusion vector pMLB1034. The *fecA* promoter fragments were synthesized by PCR. Primer AA21 (5′-CGGGATCC-CGAATGGTGTTAACCAAAGG-3′) annealed downstream of the *fecA* promoter at nucleotides 2843–2823 and contained a *Bam*H1 restriction site. Primers AA11 and AA4 (Angerer et al. 1995), AA19 (5′-CCGGAATTCTGGATAAACATTTCACCACTGT-3′, nucleotides 2681–2702), AA20 (5′-GGAATTCTTATTTCGAT-TGTCC-3′, nucleotides 2712–2731), AA22 (5′-GGAATTCGAT-TGTCCTTTTTACCC-3′, nucleotides 2721–2740), and AA23 (5′-GGAATTCTCGTTCGACTCATAGC-3′, nucleotides 2741–2759) were complementary to the *fecA* promoter and introduced an *Eco*R1 restriction site.

Plasmids pFP83 and pFPS24 contain mutated *fecA* promoter fragments. PCR-mutagenized *fecA* promoter fragments (Angerer et al. 1995) were cloned into the *Eco*RI-*Sal*I sites of vector pBCSK+ (Stratagene). The *fecA* promoter fragment in pFPS24 displays the same FecI-RNA polymerase binding as the wild-type *fecA* promoter, whereas the fragment in pFP83 shows no FecI–RNA polymerase binding (Angerer et al. 1995). Plasmids pMAA1083 and pMAA1024 were constructed by insertion of the *Sma*I–*Hpa*I fragment of pFP83 and pFPS24 into vector pMLB1034. The orientation and correctness of the cloned fragments were examined by DNA sequencing.

Plasmid pNR1 was constructed by cloning the 393-bp *fecI* promoter fragment (nucleotides 997–1389) of plasmid pMMO1035 into the *Eco*RI site of vector pSU18. To construct plasmid pCM135 (bp 1204–2705 of the *fec* sequence; Vanhove et al. 1990), the *fecIR* genes of pSV66, synthesized by PCR without the corresponding promoter and Fur binding site, were inserted into the *Bam*H1-*Hin*dIII cloning sites of vector pSUKS1. Primer AA80 (5′-CGGGATCCGTGATAATTAACTTTTGATGC-3′) annealed upstream of the *fecIR* genes (nucleotides 1204–1225) and contained a *Bam*H1 restriction site; primer IR3 (Ochs et al. 1995) annealed downstream and contained a *Hin*dIII restriction site. To confirm the correctness of the inserted fragment, the *Sph*I-*Hin*dIII fragment was replaced by the wild-type plasmid-encoded fragment, whereas the *Bam*H1-*Sph*I fragment was sequenced.

Recombinant DNA techniques

DNA isolation from bacteria, recovery of DNA fragments from agarose gels, cloning of restriction fragments, and transformation were performed according to standard protocols. All of the PCR-amplified DNA fragments were sequenced by the enzymatic dideoxy chain-termination method (Sanger et al. 1977) using fluorescein-15-dATP for labeling. The reactions were analyzed with an A. L. F. DNA Sequencer (Pharmacia Biotech, Freiburg, Germany).

DNase footprint analysis

DNase I footprinting was essentially carried out as described previously (De Lorenzo et al. 1987; Desai et al. 1996) with minor modifications and using the DNase I footprint analysis system of BRL (Grand Island, NY, USA). *fecA* promoter fragments encompassing the putative Fur boxes were amplified by PCR from plasmid pSV66 *fecIRA*. The + and – strands were labeled with different fluorescein-labeled primers. To label the + strand, the 377-nucleotide *fecA* promoter fragment was synthesized with primer Aflu1 (5′-fluorescein-d[GGCTCAGGCGAATGGTGTTAACC]), which annealed downstream of the *fecA* promoter at nucleotides 2851–2829, and with primer AA11, which annealed upstream at nucleotides 2475–2494. To label the – strand, the 456-nucleotide *fecA* promoter fragment was synthesized with primer Aflu2(5′-fluorescein-d[GCCACGCTAACCCGTTACCGC]), which is complementary to the *fecA* promoter at nucleotides 2525–2545, and with primer SVP2 (Ochs et al. 1995), which annealed at nucleotides 2980–2951.

fecI promoter fragments encompassing the putative Fur boxes were amplified by PCR from plasmid pNR1 (pSU18 *fecI′*). To label the + strand, the 393-nucleotide *fecI* promoter fragment was synthesized with fluorescein-labeled M13 reverse primer and unlabeled M13 universal primer. To label the – strand, the 393-nucleotide *fecI* promoter fragment was synthesized with unlabeled M13 reverse primer and fluorescein-labeled M13 universal primer. The M13 primers (Pharmacia Biotech) annealed at the multiple cloning site of vector pSU18, upstream or downstream of the cloned *fecI* promoter. The resulting 5′-labeled fragments were purified by using a PCR purification kit (Quiagen, Hilden, Germany) and were diluted to a concentration of 2 ng/µl in sterile, distilled H_2O. Purified *E. coli* Fur protein (kindly provided by M. Coy, University of California at Berkeley, USA through P. J. Desai) was reconstituted in a buffer containing 1 mM dithiothreitol, 20 mM Mops [3-(*N*-morpholino)-propanesulfonic acid] (pH 7.0), 0.1 M sodium acetate, 0.01 M EDTA, and 200 µg bovine serum albumin per ml to retain Fur in an active form. Increasing concentrations of the Fur protein (50–450 nM) in the presence and absence of 0.15 mM $MnCl_2$ were incubated in a solution containing 10 mM Tris-HCl (pH 8.0), 0.1 mM EDTA, 5 mM dithiothreitol, and 10% glycerol for 20 min at room temperature. Fluorescein-labeled DNA (6 ng *fecI* promoter DNA or 15 ng *fecA* promoter DNA) was then added to each of the reaction tubes, and the mixture was further incubated for 10 min. Following the addition of DNase I (0.05–0.2 ng), digestion was carried out for 2 min at 37 °C, followed by phenol extraction and ethanol precipitation. The resulting DNA pellets were dissolved in 100% deionized formamide containing Dextran Blue 2000 (5 mg/ml), denatured, and electrophoresed on an A. L. F. Automatic DNA Sequencer (Pharmacia Biotech). DNA sequencing using the Fragment Manager version 1.1 (Pharmacia Biotech) provided the external standard for the assignment of the DNase-I-resistant region protected by Mn^{2+}–Fur. DNA sequencing was carried out with plasmid pNR1 (*fecI* promoter) and the fluorescein-labeled universal or reverse primer, or with plasmid pMMO10 *fecA′* and the Aflu1 or Aflu2 primer. The dideoxy chain-termination method (Auto Read Sequencing Kit; Pharmacia Biotech) was used.

Enzyme assay

β-Galactosidase was determined according to Giacomini et al. (1992) and Miller (1972). Cells were cultured in NB or M9 medium, and the media were supplemented with 1 mM citrate and antibiotics as indicated.

Results

Mapping of the *fecA* promoter

The regulatory activity of the DNA region upstream of *fecA* was analyzed by determination of the expression of *fecA-lacZ* gene fusions linked to various DNA fragments of the *fecA* promoter (Fig. 1). *E. coli* AB2847 was transformed with the plasmids, and β-galactosidase activities were determined in M9 medium supplemented with 1 mM citrate, which complexes iron in the medium (Fig. 1). In *E. coli* AB2847 *fec*$^+$, the transcription initiation signal elicited by ferric citrate is mediated by FecA, TonB, ExbB, ExbD, FecR, and FecI. Plasmid pMMO1034 which encodes the entire *fecA* promoter, the intergenic region between *fecR* and *fecA*, and a 3′-fragment of *fecR* (nucleotides 2414–2832) upstream of *fecA-lacZ* conferred high β-galactosidase activities in the presence of citrate (Fig. 1), whereas transformants carrying the vector expressed only five units of β-galactosidase (data not shown). Removal of 73 nucleotides from –326 to –253 relative to the transcriptional start site (pMAA11; Fig. 1) reduced β-galactosidase activity by 23% (Fig. 1). Computer analysis of the sequence of the removed region did not reveal obvious binding sites for a transcriptional activator. Further deletions (Fig. 1; pMAA4 and pMAA19) did not further reduce β-galactosidase activity (Fig. 1). Deletion of the –35 region (pMAA20) and of the main part of the Fur consensus sequence (pMAA22) led to a 60% reduction of β-galactosidase activity. Further removal of the –10 region (pMAA23) diminished *fecA-lacZ* transcription activity to background level.

E. coli AB2847 transformed with plasmid pMAA1083, which contained a single-base-pair substitution (A2751C, +11) in the otherwise complete *fecA* promoter (as in pMAA4), showed only background-level β-galactosidase activity (Fig. 1). *E. coli* AB2847 transformed with plasmid pMAA1024, which comprised the complete *fecA* promoter and two base pair substitutions (T2700G, –40, and A2771G, +31), showed the same wild-type activity as *E.*

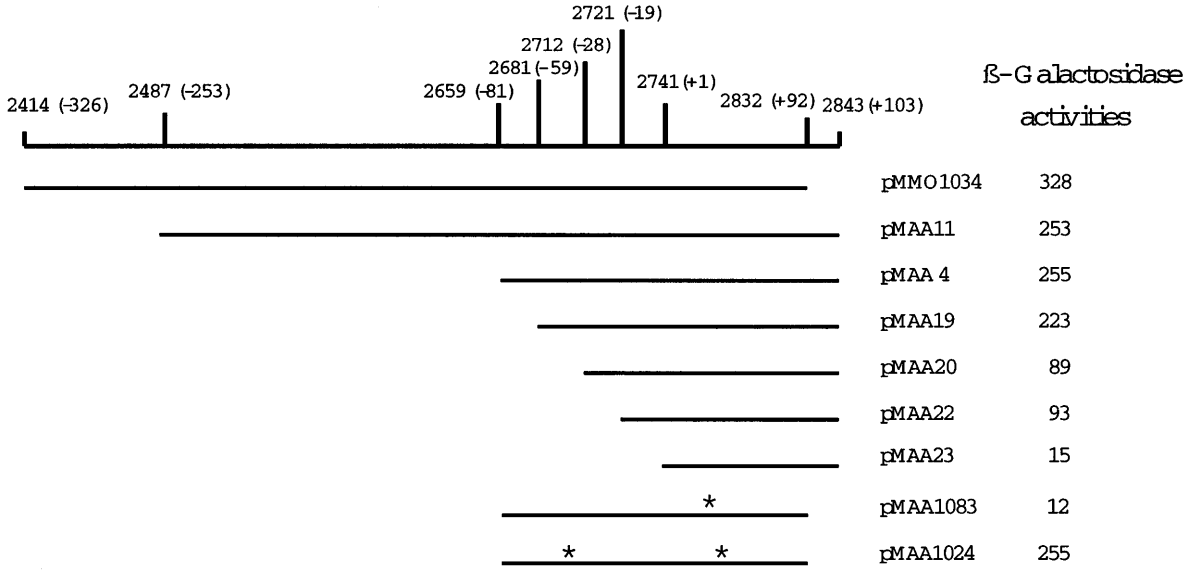


Fig. 1 Upstream and downstream regions of the *fecA* promoter encompassing nucleotides from –326 to +103 relative to the transcription start point (*in parentheses*; Enz et al. 1995) that correspond to nucleotides 2414–2843 of the published sequence (Van hove et al. 1990). The β-galactosidase activities of the plasmid-encoded *fecA-lacZ* gene fusions are listed. Cells were grown in M9 medium supplemented with 40 μg ampicillin ml^{-1} and 1 mM Na_3-citrate. The β-galactosidase activities given are the average of five determinations in duplicate (standard deviation, 5%). Single base pair substitutions are indicated by *

coli AB2847 (pMAA4) (Fig. 1). These results support previous findings in which the *fecA* promoter with the A2751C substitution did not compete with the wild-type *fecA* promoter for FecI-mediated RNA polymerase binding (Angerer et al. 1995), whereas the *fecA* promoter with the double mutation competed with the wild-type *fecA* promoter (A. Angerer, unpublished work).

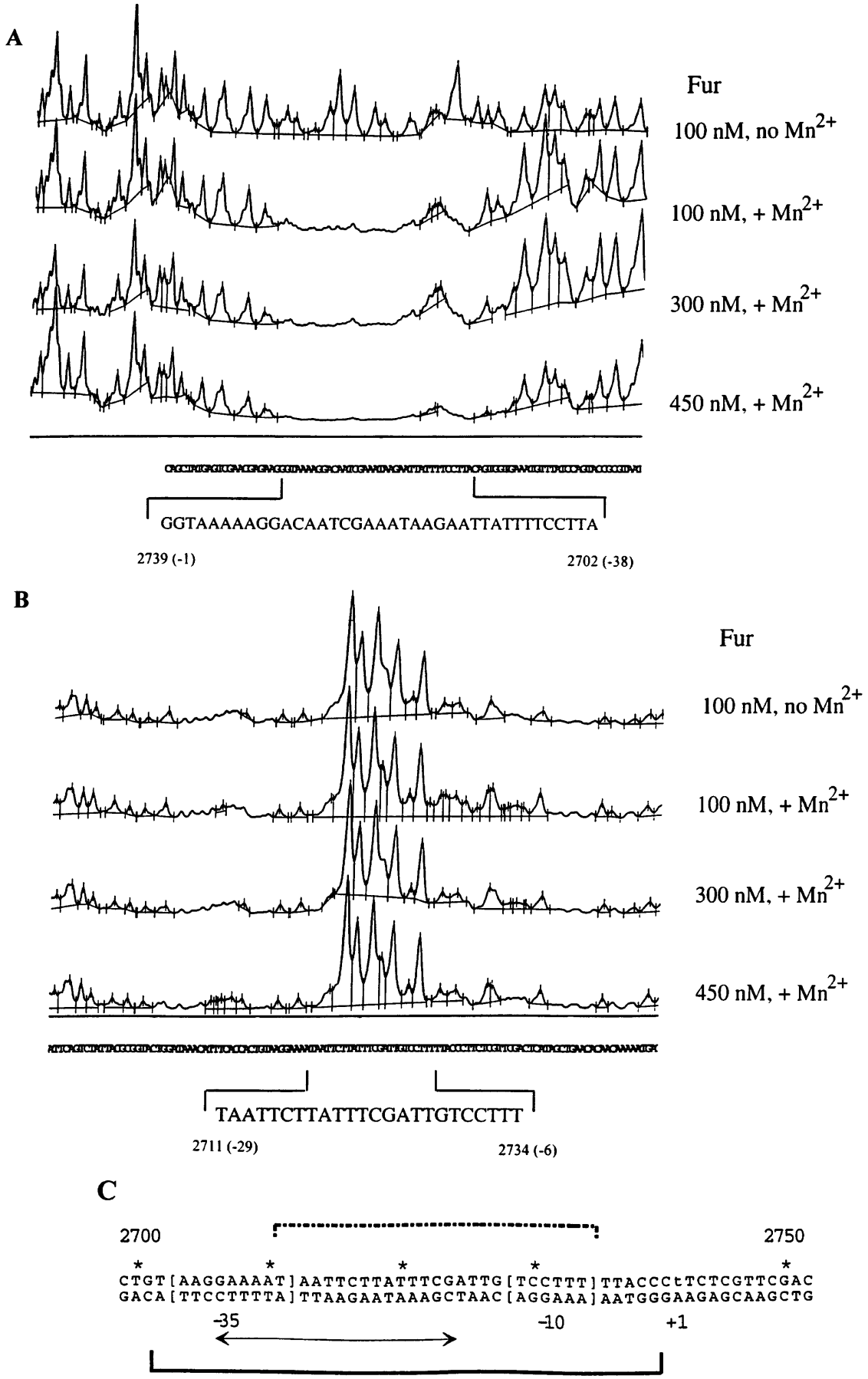
Fur regulation at only the *fecA* promoter

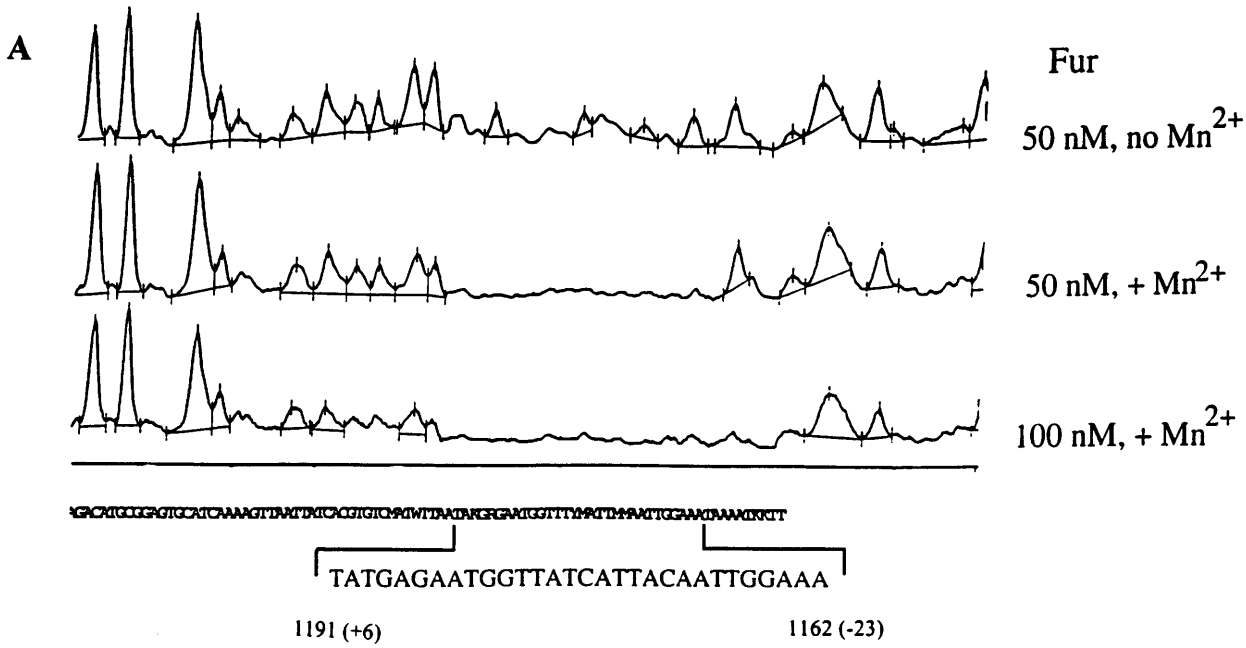
The promoter of *fecIR* with the –10 and –35 regions and the Fur box was deleted from pSV66, resulting in plasmid pCM135, in which *fecIR* was transcribed from the *lac* promoter of the vector pSUSK1. Strain MO704 *fecIR fecB*::Mud1 (Ap, *lac*) devoid of the *lac* repressor was transformed with plasmid pCM135 and acted as a control with plasmid pIS135, which carried wild-type *fecIR*. Cells were grown in NB medium under iron-replete conditions (1 mM citrate and 0.1 mM Fe_2SO_4) or under iron-deplete conditions (1 mM citrate and 100 or 200 μM dipyridyl). Under iron limitation, pCM135 reproducibly conferred a twofold increase of *fecB-lacZ* transcription with respect to that of iron-replete conditions (Table 2), which indicated Fur regulation at only the *fecA* promoter. Plasmid pIS135 conferred a 3.5-fold increase of *fecB-lacZ* transcription, which was caused by derepression of *fecIR* and *fecB* transcription.

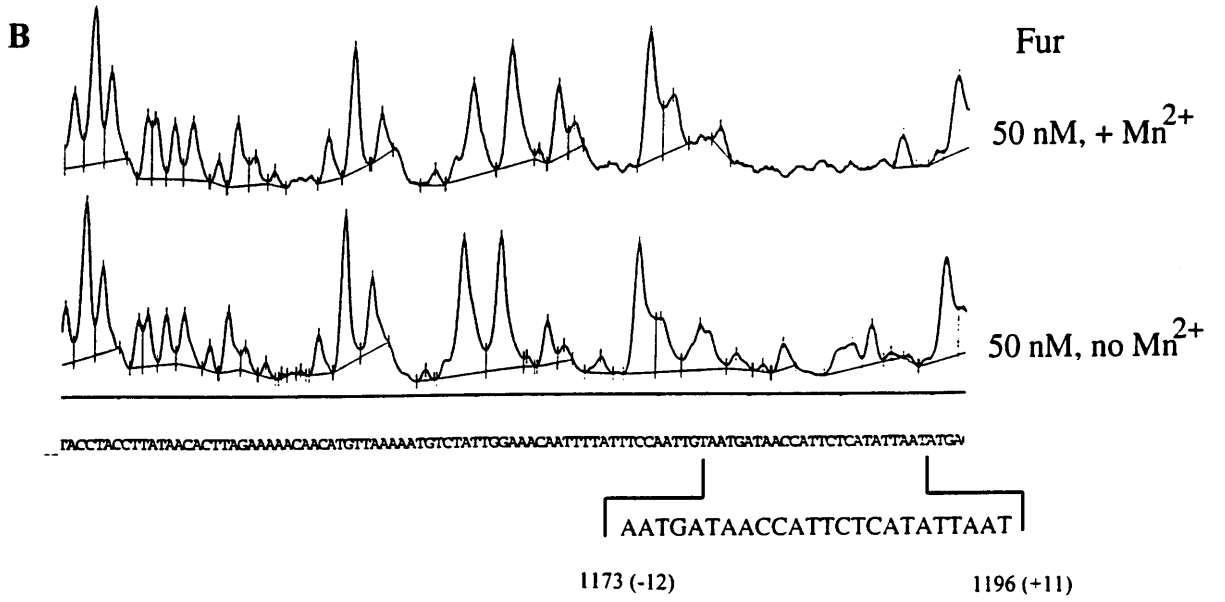
Table 2 Regulation of *fecB-lacZ* expression in *Escherichia coli* strain MO704 *fecI fecR*. Cells were grown in nutrient broth medium with chloramphenicol (40 μg/ml), 1 mM citrate, and increasing amounts of dipyridyl (0, 100, and 200 mM) or Fe_2SO_4 (100 μM). β-Galactosidase activities are expressed in Miller units and are an average of three measurements (standard deviation, 8%)

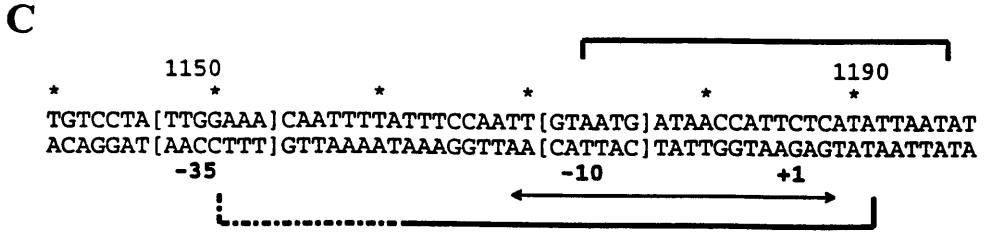
	100 μm Fe_2SO_4	100 μm Dipyridyl	200 μm Dipyridyl
MO704 (pIS135 *fecIR*)	74	238	264
MO704 (pCM135 *′fecIR*)	106	162	212

Fig. 2 **A** Footprinting scans of the 377-bp *fecA* promoter (coding strand). Sequencing of the protected DNA region following incubation with Fur and Mn^{2+}–Fur was carried out as described in the text. Automated evaluation was carried out with the A. L. F. DNA Fragment Manager Program. The concentration of added Fur is listed *to the right* of each scan. The nucleotide sequence is given *at the bottom*; the protected region is shown *expanded*, and the *numbers* correspond to the published sequence (Van hove et al. 1990); the *numbers in brackets* are relative to the transcription start point (Enz et al. 1995). **B** Footprinting scans of the 456-bp *fecA* promoter (noncoding strand). Sequencing of the protected DNA region following incubation with Fur and Mn^{2+}–Fur was carried out as described in the text. Automated evaluation was carried out with the A. L. F. DNA Fragment Manager Program. The concentration of added Fur is listed *to the right* of each scan. The nucleotide sequence is given *at the bottom*; the protected region is shown *expanded*, and the *numbering* corresponds to the published sequence; the *numbering in brackets* is relative to the transcription start point. **C** Nucleotide sequence of the *fecA* promoter. *Brackets* indicate the –10 and –35 regions; *+1* indicates the transcriptional start site. The Fur binding region as deduced from the DNase protection experiments is indicated by a *solid line* for the coding (*bottom*) strand. The protected Fur box is consistent with the Fur box predicted by the MATSCAN program (PC/GENE Release 6.7 Intelli Genetics Software Package). On the noncoding (*top*) strand, the region of DNase hypersensitivity is indicated (*dashed line*). The Fur box within the *fecA* promoter deduced with the MATSCAN program is represented by a *thin arrow*


A
Fur
100 nM, no Mn2+
100 nM, + Mn2+
300 nM, + Mn2+
450 nM, + Mn2+
GGTAAAAAGGACAATCGAAATAAGAATTATTTTCCTTA
2739 (-1)
2702 (-38)
B
Fur
100 nM, no Mn2+
100 nM, + Mn2+
300 nM, + Mn2+
450 nM, + Mn2+
TAATTCTTATTTCGATTGTCCTTT
2711 (-29)
2734 (-6)
C
2700
2750
CTGT[AAGGAAAAT]AATTCTTATTTCGATTG[TCCTTT]TTACCCtTCTCGTTCGAC
GACA[TTCCTTTTA]TTAAGAATAAAGCTAAC[AGGAAA]AATGGGAAGAGCAAGCTG
-35
-10
+1


A
Fur
50 nM, no Mn2+
50 nM, + Mn2+
100 nM, + Mn2+
TATGAGAATGGTTATCATTACAATTGGAAA
1191 (+6)
1162 (-23)


B
Fur
50 nM, + Mn2+
50 nM, no Mn2+
AATGATAACCATTCTCATATTAAT
1173 (-12)
1196 (+11)


C
1150
1190
TGTCCTA[TTGGAAA]CAATTTTATTTCCAATT[GTAATG]ATAACCATTCTCATATTAATAT
ACAGGAT[AACCTTT]GTTAAAATAAAGGTTAA[CATTAC]TATTGGTAAGAGTATAATTATA
-35
-10
+1

◀ **Fig. 3** **A** Footprinting scans of the 393-bp *fecI* promoter (coding strand). Sequencing of the protected DNA region following incubation with Fur and Mn^{2+}–Fur was carried out as described in the text. Automated evaluation was carried out with the A. L. F. DNA Fragment Manager Program. The concentration of added Fur is listed *to the right* of each scan. The nucleotide sequence is given *at the bottom*; the protected region is shown *expanded*, and the *numbering* corresponds to the published sequence (*numbering in brackets* is relative to the transcription start point). **B** Footprinting scans of the 393-bp *fecI* promoter (noncoding strand). Sequencing of the DNase hypersensitive region following incubation with Fur and Mn^{2+}–Fur was carried out as described in the text. Automated evaluation was carried out with the A. L. F. DNA Fragment Manager Program, which also introduced the numbering below the scans (which does not correspond to the published sequence). The concentration of added Fur is listed *to the right* of each scan. The nucleotide sequence is given *at the bottom*; the protected region is shown *expanded*, and the *numbering* corresponds to the published sequence (*numbers in brackets* are relative to the transcription start point). **C** Nucleotide sequence of the *fecI* promoter. *Brackets* indicate the –10 and –35 regions; *+1* indicates the transcriptional start site. The region on the coding (+) and on the noncoding (–) strand protected by Fur (50 nM) are indicated by a *solid line*. The region additionally protected by 100 nM Fur on the + strand is indicated by a *broken line*. The Fur box within the *fecA* promoter deduced with the MATSCAN program is represented by a *thin arrow*

Identification of the Mn^{2+}–Fur binding site of the *fecA* promoter

To determine the binding site of the iron repressor, Fe^{2+} was replaced by Mn^{2+}, which acts like Fe^{2+} but is less prone to oxidation (De Lorenzo et al. 1987). The Mn^{2+}–Fur binding site of the *fecA* promoter was determined by DNase I footprint analysis using a protein-to-DNA ratio of 200:1. In the absence of added Fur protein, the + strand showed a relatively uniform distribution of DNA fragments (data not shown) similar to the pattern observed with Fur in the absence of Mn^{2+} (Fig. 2). A clear zone of protection appeared with 100 nM Fur (Fig. 2A); the protected zone comprised 38 nucleotides in a region upstream of the +1 transcription initiation site, covering the –10 and –35 sequences of the *fecA* promoter. At 450 nM Fur, the protected region was somewhat larger at the 3′-end (Fig. 2A). On the – strand, no protection zone could be identified at all Fur and Mn^{2+} concentrations tested (Fig. 2B). In contrast, at the potential Mn^{2+}–Fur binding site, more and higher amounts of DNA fragments were obtained; this result indicates a region of DNase I hypersensitivity. Hypersensitivity to DNase I was independent of the addition of Fur. The results of the protection experiments are summarized in Fig. 2C.

Identification of the Mn^{2+}–Fur binding site of the *fecIR* promoter

To precisely define the Fur binding site of the *fecIR* promoter, a 393-bp end-labeled fragment that contained the promoter region was analyzed by DNase I protection experiments using purified *E. coli* Fur protein in the absence and presence of an excess of Mn^{2+}. Fur binding to the *fecIR* promoter was studied at a protein-to-DNA ratio of 250:1. In the absence of added Fur protein, DNase I digestion of the fluorescein-labeled fragment produced a uniform distribution of DNA fragments of the + strand (data not shown), as was observed with 50 nM Fur in the absence of Mn^{2+} (Fig. 3A). On the + strand, a clear zone of protection appeared with 50 nM Fur in the presence of Mn^{2+} (Fig. 3A, nucleotides 1191–1162). The protected zone comprised 30 nucleotides in a region upstream and downstream of the +1 region of the *fecIR* promoter. At a Fur concentration of 100 nM, 11 additional nucleotides were protected (Fig. 3A). The increase in the protected region may be caused by the polymerization of the Fur protein. At 250 nM Fur, an even larger DNA region was protected (data not shown). On the – strand, protection by Fur was also Mn^{2+}-dependent (Fig. 3B; compare the scan in the presence of Mn^{2+} with the scan in the absence of Mn^{2+}). At 50 nM Fur, the protection zone comprised 24 nucleotides (1196–1173). The results of the protection experiments are summarized in Fig. 3C.

Discussion

This communication shows with two methods that control of *fec* transport gene transcription by iron occurs not only at the *fecIR* promoter, but also at the *fecABCDE* promoter. DNase I footprint analysis revealed binding of Fur to the *fecABCDE* and *fecIR* promoters that depended on the presence of the co-repressor Mn^{2+}, which had been used instead of Fe^{2+}. Mn^{2+}–Fur protected 38 nucleotides on the coding strand of the *fecABCDE* promoter, 30 nucleotides on the coding strand of the *fecIR* promoter, and 24 nucleotides on the noncoding strand. Since prokaryotic repressor dimers typically occupy approximately 20 base pairs of two consecutive DNA turns, probably one repressor dimer binds to the *fecIR* promoter and two repressor dimers bind to the *fecABCDE* promoter. At higher concentrations, Fur binding upstream of the predicted Fur box of *fecI* was observed; this binding may be related to polymerization of the protein, as has been observed previously by high-resolution electron and atomic force microscopy at the promoter of the aerobactin synthesis genes (Le Cam et al. 1994). The Fur box of the noncoding strand of the *fecABCDE* promoter was hypersensitive to DNase I cleavage, which presumably prevented the detection of DNA zones protected by Mn^{2+}–Fur. The results of the DNase I footprint analysis (Figs. 2C and 3C) agree with the Fur boxes, as was predicted by the MATSCAN program.

A twofold increase of *fecB-lacZ* transcription was observed under iron-limiting conditions in strain MO704 *fecIR*, which transcribed plasmid-encoded *fecIR* independent of iron. Since *fecB-lacZ* transcription was controlled by the promoter of the *fecABCDE* transport genes, it is concluded that transcription of the *fec* transport genes is inhibited by Fe^{2+}–Fur binding to the promoter upstream of *fecA*, which agrees with the DNase I footprint analysis. In the presence of the *fecIR* promoter, derepression of *fecB-*

lacZ transcription was 3.5-fold. If there is enough iron in the cells, Fe^{2+}–Fur shuts off transcription of the *fec* transport genes at the promoters of *fecIR* and *fecABCDE*. Iron limitation sets the Fur box free in both promoters, the FecIR proteins are synthesized, and FecI directs the RNA polymerase to the *fecABCDE* promoter in the presence of ferric citrate. If sufficient amounts of iron have been transported into the cells, Fe^{2+}–Fur presumably interferes with the FecI-mediated binding of the RNA polymerase to the *fecABCDE* promoter and immediately inhibits *fec* transport gene transcription. It takes longer for the inhibition of FecIR transcription to affect *fec* transport gene transcription because FecI and FecR have to be diluted out by growth of the cells. It is not known whether proteolysis assists in the removal of FecI and FecR. Transcription of *fecIR* is not autoregulated (Ochs et al. 1996).

The unusual involvement of the +11 region of the *fecABCDE* promoter in FecI-mediated binding of the RNA polymerase, which had been previously deduced from competition experiments between wild-type and mutated *fecABCDE* promoters (Angerer et al. 1995), was confirmed by the lack of *fecA-lacZ* transcription of the mutant plasmid that carried the A2751C replacement at position +11. As has been demonstrated for other types of σ^{70}-RNA polymerase complexes (De Haseth and Helman 1995; Eick et al. 1995), this site is probably part of the approximately 60 base pairs, that are covered by the FecI-RNA polymerase. Progressive removal of the –35 and –10 regions resulted in a strong decrease in transcription from the *fecA* promoter until only background levels of the reporter β-galactosidase were obtained.

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