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Analysis of the Ferric Citrate Transport Gene Promoter of *Escherichia coli*

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FecI, an extracytoplasmic-function σ factor, is required for initiation of transcription of the ferric citrate transport genes. A mutational analysis of the *fecA* promoter revealed that the nonconserved −10 region and a downstream regulatory element are important for *fecA* promoter activity. However, nucleotide substitutions in the well-conserved −35 region also have an effect on the *fecA* promoter activity. Titration of FecI suggests that the FecI-RNA polymerase holoenzyme does not bind strongly to the downstream regulatory element, which is therefore probably involved in a subsequent step of transcription initiation.

In *Escherichia coli*, transcription of the ferric citrate transport genes *fecABCDE* is controlled by a signal transduction mechanism that starts at the cell surface. $(Fe^{3+}$ citrate$)_2$ binds to the outer membrane protein FecA and without further transport into the cell induces transcription of the *fec* transport genes (9, 14). The signal transmitted by FecA loaded with $(Fe^{3+}$ citrate$)_2$ across the outer membrane is transmitted across the cytoplasmic membrane by FecR, a transmembrane protein (21, 28) that interacts with FecA in the periplasm and with the FecI extracytoplasmic-function (ECF) σ factor in the cytoplasm (7, 8, 18, 24). FecR enables FecI to bind to the RNA polymerase core enzyme; this complex then binds to the *fecA* promoter to initiate transcription of the *fec* transport genes (1, 2). The only promoter known to be recognized by FecI is that of the *fecABCDE* operon; no other σ factor of *E. coli* is endowed with such a narrow promoter specificity (17).

ECF σ factors belong to a subfamily of the σ^{70} class, based on their sequence conservation and function across bacterial species (3, 10, 16, 20). Comparisons of sequences indicate that the genomes of *Caulobacter crescentus*, *Pseudomonas aeruginosa*, *Nitrosomonas europaea*, and *Streptomyces coelicolor* are particularly rich in ECF σ factors and contain 13, 19, 22, and 50 predicted ECF σ factors, respectively. σ factors share four conserved regions which can be further subdivided. Region 4.2 recognizes the −35 element, and region 2.4 recognizes the −10 element of promoter DNA. A bacterial promoter consists of at least about 60 bp spanning the positions −40 to +20 in relation to the +1 start site of transcription (12). A comparison of ECF σ factors reveals that the −35 sequence and the spacing but not the sequence between the −35 and −10 regions are well conserved (Table 1) (4, 5, 10, 11, 19, 20, 23, 29). The −10 sequences show less homology (Table 1), which is reflected by the low homology of regions 2.4 in the ECF σ factors (16). The characteristic feature of region 2.4 of σ^{70}, a set of hydrophobic residues that form an amphipatic α-helix, is not present in the corresponding region of ECF σ factors (16). The diversity of the −10 sequences and region 2.4 probably accounts for the coexistence of multiple members of the ECF σ-factor subfamily in the same species.

Previously, it was shown that transcription of the *fec* transport genes starts at nucleotide 2741 (7) of the *fec* sequence (22), resulting in a polycistronic mRNA (7). Binding of the FecI RNA polymerase holoenzyme to a 75-bp DNA fragment (from positions −61 to +13) was demonstrated by DNA band shift experiments (2). The smallest plasmid-encoded DNA fragment studied that inhibited chromosomal *fec* transport gene expression by binding the FecI RNA polymerase extended from positions −82 to +62. In vitro transcription by FecI RNA polymerase was shown with a 650-bp promoter DNA fragment (2). Footprinting scans revealed that the Fur repressor protein loaded with Mn^{2+} (less prone to oxidation than Fe^{2+}) covered 38 nucleotides (from positions −38 to −1) of the coding strand of the *fecA* promoter in which the Fur consensus sequence was localized (positions −36 to −17) (7). In addition, preliminary evidence was obtained for the involvement of the promoter downstream region for *fecA* transcription (2).

Since the ECF promoters deviate in several respects from the σ^{70} promoters, we examined the contribution of the −35, −10, and the downstream region for transcription of the *fecA* transport gene. Using site-directed mutagenesis, we constructed single-nucleotide changes in the *fecA* promoter (Fig. 1). To analyze the effects of the nucleotide substitutions, the mutated and the wild-type *fecA* promoters were fused to the *gfp* reporter gene. Plasmid pGFPA′ is a derivative of pFPV25 that contains a promoterless *gfp*mut3 gene (26) fused to the *fecA* promoter. Each construct was transferred into the *fec* deletion mutant *E. coli* AA93 transformed with plasmid pSV66 *fecIRA*, and the relative fluorescence was assayed in nutrient broth (NB) medium in the presence and absence of ferric citrate. The fluorescence was measured with the FL600 fluorescence reader (Biotek, Bad Friedrichshall, Germany) at an excitation wavelength of 485 nm and an emission wavelength of 530 nm.

Point mutations in the −35 region affect *fecA* promoter activity. All mutated *fecA* promoters with nucleotide substitutions in the −35 element (−35 to −30) were less active than the *fecA* wild-type promoter, but none decreased *fecA* promoter activity more than threefold (Table 2). Mutations of the

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TABLE 1. Promoter sequences recognized by ECF σ factors

ECF σ factor	Nucleotide sequence		Reference
	−35 element	−10 element	
FecI	GAAAAT	TGTCCT	
PA2468	GAAAAT[a]	TGTCGG[a]	25
PA3899	GATATT[a]	TTTTTC[a]	25
SigR	GGGAAT	GTT	10
SigB	GGgaac[b]	cGTTa[b]	10
AlgU/RpoE	GAAC	TCT	29
CarQ	GAAAC	CGT	29
SigW	TGAAAC	CGTA	29
SigX	TGTAACT	CGAC	29
PvdS	TAAAT	CGT	29

[a] Alignment of sequences of the putative *pa2466* and *pa3901* promoters with that of the *fecA* promoter. The amino acid sequences of the PA2468 and PA3899 σ factors in the region 4.2 are very similar to that of FecI.
[b] Lowercase letters indicate sites of lower homology.

most conserved nucleotides A_{-34} and A_{-33} reduced the relative fluorescence no more than to 81 and 69%, respectively, of wild-type activity. Nucleotide substitutions next to the −35 element from positions −40 to −36 had a small effect on the activity of the *fecA* promoter, which shows that the putative −35 element with the conserved sequence GAAAAT begins at position G_{-35}. Since none of the single mutations abolished *fecA* promoter activity, double mutations were introduced at a few sites. Combination of the single mutation $G_{-35}T$ (57% activity) with $T_{-30}G$ (41%) or $A_{-34}C$ (81%) reduced the activity to 32 and 29%, respectively. The double mutant C_{-32} G_{-30} was also less active (21%) than the C_{-32} (43%) and G_{-30} (41%) single mutants (Table 2). It appears that nucleotide substitutions in the −35 region are tolerated to some extent, which is not unexpected since different ECF σ factors bind to similar −35 regions. A comparable result was obtained with the *sigX* promoter of the SigX σ factor (13). However, the finding that the *crtL* promoter of *Myxococcus xanthus* to which the CarQ ECF σfactor binds is critically dependent on a pentanucleotide sequence centered at the −31 position (19) indicates differences in the structural requirements of ECF σ factor promoters. Upstream deletions in the *fecA* promoter extending to nucleotides −28 and −19 reduced β-galactosidase activity of a plasmid-encoded *fecA-lacZ* operon fusion to 23 and 25% of wild-type activity, respectively, whereas a deletion covering the entire promoter region to +1 completely abolished *fecA-lacZ* transcription (1). These data demonstrate that the −35 region can be partially replaced by other nucleotide sequences. This finding is consistent with the lack of complete promoter inactivation by the point mutations.

Point mutations in the −10 region strongly reduce *fecA* promoter activity. To determine the −10 element and its boundaries, single-nucleotide substitutions were introduced from −18 to −5. The mutated promoters were analyzed as described above for the −35 region. Mutations between positions −14 and −9 greatly reduced the activity of the *fecA* promoter (Table 2). Mutant *fecA* promoters with the nucleotide substitutions $G_{-13}T$, $C_{-10}A$, and $T_{-9}G$ almost completely abolished the activity of the *fecA* promoter, whereas mutations at C_{-12} strongly reduced *fecA* promoter activity. The $G_{-13}T$ substitution was replaced by the less extreme C and A substitutions. These mutants displayed a higher *fecA* promoter activity than did T_{-13}. The mutations that completely inactivated the *fecA* promoter activity all had extreme nucleotide changes. The high *fecA-gfp* transcription rates of the −17 to −15 mutants define well the 5′ boundary of the −10 element, in contrast to the lower transcription of the −6 to −4 mutants (Table 2), which define less clearly the 3′ boundary. The data strongly indicate that the −10 region is essential for the activity of the *fecA* promoter and agree with data for SigX promoters and the *crt* promoter indicating that single-nucleotide replacements also abolished promoter activity.

Point mutations in the region downstream of the *fecA* transcription start site strongly reduce transcription. The *fecA* downstream promoter element plays a critical role, as mutations $C_{+9}T$ and $A_{+11}C$ strongly reduced competition of a *fecA* promoter DNA fragment with the corresponding wild-type *fecA* promoter fragment for FecI-induced *fecA-lacZ* transcription. In addition, the $T_{+7}G$ mutation strongly reduced FecI-mediated binding of RNA polymerase to a 95-bp *fecA* DNA promoter fragment (2). In this study the level of *fecA* transcription was not determined. To further support this finding, we constructed by PCR mutagenesis single-nucleotide substitutions between positions +1 and +20. The promoter activity of the mutated downstream fragments was monitored by measuring *gfp* expression as indicated above. Mutants with the nucleotide substitutions $C_{+9}T$, $G_{+10}T$, and $A_{+11}C$ strongly reduced the relative fluorescence, resulting in 5, 1, and 7%, respectively, of wild-type promoter activity (Table 2). The mutation $G_{+10}A$ abolished *fecA-gfp* transcription as strongly as did $G_{+10}T$ (1% activity), whereas transcription by the $G_{+10}C$ promoter was 54% of wild-type activity (Table 2). Mutations at positions T_{+8}, T_{+16}, and T_{+20} affected *fecA* promoter activity much less, ranging between 45 and 66%.

DNA downstream of the transcription start site was shown to be important for other ECF σ factor-controlled promoters. DNA from nucleotides +50 to +120 of the *crtL* promoter is required for promoter activity (19). The DNA downstream of *crtL* contains an enhancer-like element that remains active when displaced to a site upstream of the promoter. The ECF σ factor PvdS is required for transcription of the pyoverdin synthesis genes of *P. aeruginosa*. For *pvdF* transcription the smallest fragment retaining promoter activity extended from nucleotides −91 to +34, indicating that a sequence element in the downstream region is required for maximal expression from this promoter (27). However, DNA downstream from the *pvdE* +1 site to nucleotide +195 did not increase the PvdS-dependent *pvdE* promoter activity (29). A study of the PvdS-dependent *pvdA* promoter finds that DNA downstream of the transcription start site is required for promoter activity in *P. aeruginosa* (15).

In order to relate promoter activity to promoter binding of

FIG. 1. Nucleotide sequence of the *fecA* promoter analyzed. The −35 and −10 elements are underlined, and the sites of nucleotide substitutions are in bold letters.

TABLE 2. Site-directed mutations introduced at the −35 element (−40 to −25), the −10 element (−18 to −5), and the downstream regulatory element[a]

Plasmid	Sequences of the *fecA* promoter and the mutant *fecA* promoters	Relative fluorescence units (% of wild-type)	
		NB + 50 μM dipyridyl	NB + 1 mM citrate
	−35 element		
pGFPA′	GTAAGGAAAATAATTC	403 (2)	17,170 (100)
pGFP40	**T**TAAGGAAAATAATTC	363 (2)	13,471 (78)
pGFP39	G**G**AAGGAAAATAATTC	247 (1)	10,816 (61)
pGFP03	GT**C**AGGAAAATAATTC	669 (4)	7,555 (44)
pGFP37	GTA**C**GGAAAATAATTC	391 (2)	16,101 (93)
pGFP36	GTAA**T**GAAAATAATTC	958 (6)	13,661 (79)
pGFP06	GTAAG**T**AAAATAATTC	704 (4)	9,787 (57)
pGFP07	TTAAGG**C**AAATAATTC	1,287 (8)	13,907 (81)
pGFP08	TTAAGGA**C**AATAATTC	1,013 (6)	11,847 (69)
pGFP09	GTAAGGAA**C**ATAATTC	635 (4)	7,383 (43)
pGFP10	GTAAGGAAA**C**TAATTC	446 (3)	6,353 (37)
pGFP11	GTAAGGAAAA**G**AATTC	481 (3)	7,039 (41)
pGFP29C	GTAAGGAAAAT**C**ATTC	369 (2)	6,122 (35)
pGFP388	GTAAGGAAAATA**G**TTC	351 (2)	6,705 (39)
pGFP14	GTAAGGAAAATAA**G**TC	601 (4)	9,272 (54)
pGFP26	GTAAGGAAAATAAT**G**C	293 (2)	6,745 (39)
pGFP25	GTAAGGAAAATAATT**A**	1,071 (6)	8,035 (46)
pGFP3530	GTAAG**T**AAAA**G**AATTC	482 (3)	5,736 (32)
pGFP3534	GTAAG**TC**AAATAATTC	287 (2)	5,199 (29)
pGFP3230	GTAAGGAA**C**A**G**AATTC	369 (2)	3,764 (21)
pGFP3231	GTAAGGAA**CC**TAATTC	262 (2)	6,633 (37)
	−10 element		
pGFPA′	CGATTGTCCTTTTT	400 (2)	17,926 (100)
pGFP19	**A**GATTGTCCTTTTT	244 (1)	16,935 (94)
pGFP18	C**T**ATTGTCCTTTTT	211 (1)	12,934 (72)
pGFP17	CG**C**TTGTCCTTTTT	244 (1)	13,639 (76)
pGFP16	CGA**G**TGTCCTTTTT	348 (2)	7,147 (39)
pGFP27	CGAT**G**GTCCTTTTT	430 (2)	3,764 (21)
pGFP28	CGATT**T**TCCTTTTT	36 (0)	179 (1)
pGFP28C	CGATT**C**TCCTTTTT	235 (1)	8,097 (45)
pGFP28A	CGATT**A**TCCTTTTT	525 (3)	8,502 (47)
pGFP29	CGATTG**G**CCTTTTT	322 (2)	2,151 (12)
pGFP30	CGATTGT**A**CTTTTT	502 (3)	4,660 (26)
pGFP31	CGATTGTC**A**TTTTT	54 (0)	717 (4)
pGFP32	CGATTGTCC**G**TTTT	72 (0)	1,255 (7)
pGFP8G	CGATTGTCCTT**G**TT	186 (1)	11,228 (62)
pGFP7G	CGATTGTCCTTT**G**T	208 (1)	5,563 (31)
pGFP6G	CGATTGTCCTTT**G**	470 (3)	8,869 (49)
	Downstream regulatory element		
pGFP41	**C**TCTCGTTCGACTCATAGCT	1,470 (8)	15,954 (89)
pGFP44	TTC**C**CGTTCGACTCATAGCT	1,308 (7)	14,162 (79)
pGFP48	TTCTCGT**C**CGACTCATAGCT	968 (5)	11,831 (66)
pGFP49	TTCTCGTTT**T**GACTCATAGCT	89 (0)	896 (5)
pGFP50	TTCTCGTTC**T**ACTCATAGCT	36 (0)	179 (1)
pGFP50C	TTCTCGTTC**C**ACTCATAGCT	153 (1)	9,793 (54)
pGFP50A	TTCTCGTTC**A**ACTCATAGCT	211 (1)	220 (1)
pGFP51	TTCTCGTTCG**C**CTCATAGCT	107 (0)	1,254 (7)
pGFP57	TTCTCGTTCGACTCAT**C**GCT	609 (3)	8,066 (45)
pGFP60	TTCTCGTTCGACTCATAGC**C**	753 (4)	10,397 (58)

[a] The plasmids carry *gfp* fused to either the wild-type or mutated *fecA* promoter. Values (in Miller units) were determined for *E. coli* AA93 (Δ*fec*) pSV66 (*fecIRA*) transformed with plasmids carrying the wild-type or mutant promoter fused to *gfp*. The bold letters indicate the changed nucleotides. The first base of each sequence given for the downstream regulatory element is at position +1.

FecI RNA polymerase, in vivo titration experiments were carried out. An excess of plasmid-encoded wild-type *fecA* promoter DNA binds the FecI-RNA polymerase complex, resulting in lesser availability of the holoenzyme for transcription of the chromosomal *fecB-lacZ* operon fusion in *E. coli* ZI418 (1). Mutations in the −10, −35, and downstream regions were examined. The wild-type *fecA* promoter and mutant *fecA* promoters fused to *gfp* were cloned into the high-copy-number vector pBCSK$^+$. The β-galactosidase activity of *E. coli* ZI418 transformed with pBCSK$^+$ and grown in the presence of the inducer $(Fe^{3+}\ citrate)_2$ amounted to 325 Miller units (Table 3). β-Galactosidase activity was reduced to 192 Miller units in cells with plasmid-encoded wild-type *fecA* promoter. Even though the plasmid-encoded *fecA* promoter did not completely reduce expression of chromosomal *fecB-lacZ*, this reduction was taken as 100%, in relation to which the reduction caused by the

TABLE 3. FecI-binding and induction activity of the wild-type and the mutated *fecA* promoter regions[a]

Plasmid	Base changes	β-Galactosidase activity (percent FecI-binding)		Relative fluorescence (percent induction)	
		NB medium	NB medium + 1 mM citrate	NB medium	NB medium + 1 mM citrate
pBCSK+		108	325 (0)	181	224
pHCA	Wild type	94	192 (100)	258	2,636 (100)
pHC10	$A_{-31}C$	105	246 (60)	255	1324 (50)
pHC29	$T_{-12}G$	138	307 (14)	178	909 (34)
pHC50	G_{+10} T	125	241 (63)	160	234 (9)

[a] Values (in Miller units) were determined for *E. coli* ZI418 *fecB-lacZ* transformed with high-copy-number plasmids carrying the *fecA* wild-type promoter or *fecA* mutant promoter fused to *gfp*.

mutated *fecA* promoters was determined. The $G_{+10}T$ mutation reduced β-galactosidase activity only by 63% (Table 3). The $T_{-12}G$ mutation reduced β-galactosidase activity by 14%, while the $A_{-31}C$ mutation reduced β-galactosidase activity by 60%. The mutation in the −10 region most strongly impaired binding of the FecI RNA polymerase complex to the plasmid-encoded *fecA* promoter.

Activity of the wild-type and the mutated *fecA* promoter fragments on the high-copy-number plasmids in ZI418 was determined by *gfp* expression (Table 3). Despite the relatively high FecI RNA polymerase binding ability of the $G_{+10}T$ promoter mutant (63%), the induction activity was low (9%) (Table 3). The relative activities of the promoter mutants on the high-copy-number plasmids were higher (Table 3) than on the low-copy-number plasmids (Table 2). However, the absolute fluorescence values listed in Table 3 are lower than those listed in Table 2, which may be caused by the reduced availability of FecI RNA polymerase due to its binding to the chromosomal *fecA* promoter of ZI418, which is not present in the AA93 *fec* deletion mutant used for the data presented in Table 2. Induction levels by $(Fe^{3+}$ citrate$)_2$ via the wild-type promoter were comparable (10-fold) in the two *E. coli* strains.

The results described here confirm and extend previous findings on the importance of the downstream region of the *fecA* promoter for *fec* transport gene transcription (2). Since binding of the FecI-RNA polymerase complex to the downstream region did not play a major role, the downstream region may be involved in a subsequent step of transcription initiation. For the initiation process in *E. coli* the closed RNA polymerase holoenzyme (RPc) turns into an open complex conformation. DNase I footprint assays showed that several *E. coli* promoters exist in two forms; one form displays protection of the promoter from −55 to −5 (RPc_1) against DNase I, and the other displays it from −55 to + 20 (RPc_2) (6). Although it is not clear how the conformational changes of RPc_1 to RPc_2 occur, it seems that the downstream DNA is placed into its binding site (12).

Mutations in the *fec*-specific −10 region strongly reduce FecI polymerase binding and *fecA* transcription. FecI RNA polymerase specifically binds to the −10 *fecA* promoter region, and for this reason mutations in the −10 region reduce promoter activity. This may apply to other ECF σ factors for which different −10 regions exist. In contrast, the *fecA* −35 region tolerates nucleotide replacements.

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