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Transcriptional Regulation from the Cell Surface: Conformational Changes in the Transmembrane Protein FecR Lead to Altered Transcription of the Ferric Citrate Transport Genes in *Escherichia coli*

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Ferric citrate induces the ferric citrate transport system in *Escherichia coli* without being taken up into cells. The cytoplasmic transmembrane protein FecR, required for the response to ferric citrate, was found to be cleaved by a cellular protease. FecR protein produced by *fecR* mutants impaired or constitutive in *fecA* transcription was protease resistant, indicating that conformational changes affect proper functioning of FecR.

Transcription induction of the ferric citrate transport genes *fecABCDE* occurs by a novel mechanism, in that the inducer, ferric citrate, does not have to enter the cell but acts from the cell surface via the outer membrane receptor FecA and the energy transduction system composed of TonB, ExbB, and ExbD. *fecA* missense mutants which (i) induced *fecA* transcription in the absence of ferric citrate, (ii) exhibited *fecA* transcription which was induced by ferric citrate but did not transport ferric citrate, and (iii) transported ferric citrate but displayed no induction have been isolated (3). All *Escherichia coli* cells with mutations in *fecBCDE*, genes encoding binding-protein-dependent transport across the cytoplasmic membrane, were fully inducible (14).

Two regulatory genes, *fecI* and *fecR*, are located upstream of the *fec* transport genes and are controlled by their own promoter. The transport genes *fecABCDE* are transcribed from a promoter upstream of *fecA* (13). Mutants with Mud1 (Ap *lac*) insertions in *fecI* do not transcribe *fecA* and *fecB* and are no longer inducible (13), while mutants with deletions in *fecR* constitutively express *fecA* as long as FecR contains the 59 N-terminal amino acids (total, 317 residues) (9). Further truncation of FecR abolishes *fecA* transcription, ruling out the possibility that FecR is a repressor (9). FecR is found in the cytoplasmic membrane fraction and probably spans the membrane by a single stretch of hydrophobic amino acids extending from residues 85 to 100 (9). Response to ferric citrate requires the entire FecR, which presumably activates FecI. FecI displays homology to a recently recognized subgroup of σ^{70}-factors (5) and specifically stimulates binding of the RNA polymerase core enzyme to the *fecA* promoter (1).

In this study, we isolated missense mutations in *fecR* to investigate its role in transcription induction further. We obtained mutants showing no response to ferric citrate and others in which *fecA* transcription was partially constitutive. Cellular proteolysis of wild-type FecR provided a convenient tool to investigate the effect of conformational changes on the activity of FecR from *fecR* mutants.

Mutants with altered *fec* transport gene regulation were isolated in *E. coli* AA93 Δ*lacU169* Δ*fec aroB*, in which the entire *fec* locus was deleted (9) and which would not produce a siderophore because of a mutation in *aroB*, which is required for enterobactin synthesis. *E. coli* AA93 was transformed with plasmid pIS1034 carrying a *fecA-lacZ* gene fusion and wild-type *fecA* (9). Wild-type *fecA* was provided for induction; *tonB*, *exbB*, and *exbD* were on the chromosome. *E. coli* AA93(pIS1034) transformed with the low-copy-number plasmid pIS135 carrying the wild-type *fecI* and *fecR* genes (9) forms white colonies on MacConkey agar and red colonies on MacConkey agar supplemented with 1 mM citrate (9). The *fecA-lacZ* fusion was used to isolate red colonies on MacConkey plates in the absence of ferric citrate and white or pink colonies in the presence of ferric citrate. The *fecR* gene was randomly mutagenized by PCR with *Taq* polymerase at suboptimal concentrations of dATP (4). To replace wild-type *fecR* by mutated *fecR*, an *Nde*I restriction site was introduced by PCR (10) into the 5′-terminal start codon of *fecR* on pIS135.

Four pink colonies from MacConkey-citrate plates (designated FecR2, -3, -5, and -7) and two red colonies from MacConkey plates (no citrate) (FecR6 and -8) obtained from different PCR mutagenesis experiments were studied further. A third type of regulatory mutant was pink on MacConkey plates and red on MacConkey-citrate plates (FecR1 and -4). Addition of $FeCl_3$ (40 μM) suppressed *fecA* transcription in all mutants (white colonies). Expression of *fecA-lacZ* was determined quantitatively by measuring β-galactosidase activity. *E. coli* AA93(pIS1034) lacks the *fec* genes required for transport of ferric citrate across the cytoplasmic membrane; therefore, added citrate enhances iron starvation. For this reason, 50 μM dipyridyl was added to nutrient broth to reduce the available iron to an extent similar to that found in citrate-supplemented nutrient broth. The enzyme activities obtained agreed with the phenotype observed on the MacConkey plates (Table 1). FecR2, -3, -5, and -7 showed low β-galactosidase activities and were not inducible by ferric citrate. FecR6 and FecR8 displayed enhanced *fecA* transcription in the absence of citrate, at an even higher level than after ferric citrate induction; FecR1 and FecR4 were still partially inducible.

Since truncated FecR derivatives render cells constitutive for *fecA-lacZ* expression (9), the sizes of the mutated FecR derivatives were determined. FecR was barely detectable by autoradiography even when its gene was cloned downstream of the phage T7 gene 10 promoter and transcribed by the T7 RNA polymerase (13). To improve the *fecR* ribosome binding site, wild-type *fecR* and the mutated *fecR* genes were each

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TABLE 1. Expression of *fecA-lacZ* in *E. coli* AA93 Δ*fec aroB* pIS1034 *fecA fecA-lacZ* transformed with plasmids carrying mutated *fecR* genes[a]

FecR protein	β-Galactosidase activity (Miller units)	
	Dipyridyl	Citrate
FecR wild-type	8	559
FecR1	20	451
FecR2	14	11
FecR3	7	3
FecR4	19	284
FecR5	135	37
FecR6	136	96
FecR7	41	13
FecR8	426	201

[a] Cells were grown in 8 g of nutrient broth–5 g of NaCl per liter, pH 7, supplemented with 0.05 mM dipyridyl or 1 mM citrate. Cells were harvested at a density of about 5×10^8 cells per ml and β-galactosidase activity was determined as previously described (2, 8).

cloned behind the ideal Shine-Dalgarno sequence of pAA70 (9). The resulting plasmids pKW11 to pKW18 carrying *fecR1* to *fecR8*, respectively, were expressed in *E. coli* BL21 DE3 (11) by the T7 RNA polymerase. Under these conditions, FecR was the most prominent protein of *E. coli* and was identified by staining with Serva blue (Fig. 1). Wild-type FecR was cleaved into two fragments (Fig. 1, lane 2), even though unfractionated whole cells were suspended in the sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) sample buffer (6) and were dissolved by boiling for 5 min. Recovery of the fragments from the gel and determination of the amino acid sequence by automatic Edman degradation in a gas phase sequencer (7) revealed a specific cleavage after residue 181 (glycine-threonine bond) by an unknown cellular protease. In

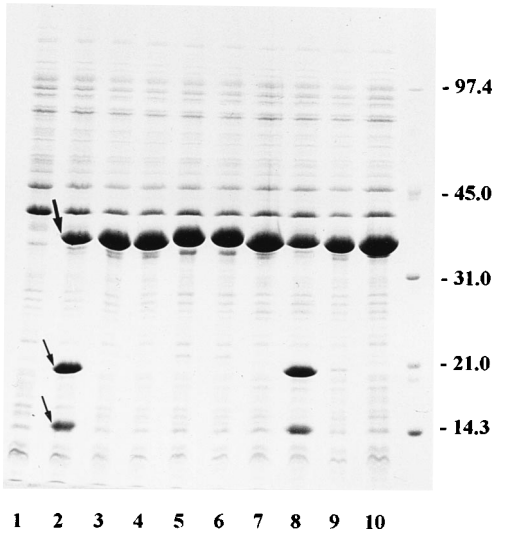

FIG. 1. Identification of wild-type FecR (lane 2) and mutated FecR1 (lane 3), FecR2 (lane 4), FecR3 (lane 5), FecR4 (lane 6), FecR5 (lane 7), FecR6 (lane 8), FecR7 (lane 9), and FecR8 (lane 10) by staining with Serva blue after SDS-PAGE of whole cells. Transformants of *E. coli* BL21 DE3 carrying the *fecR* genes were grown in tryptone-yeast extract medium to a density of 5×10^8 cells per ml. Transcription of *fecR* was initiated by induction of T7 RNA polymerase during 1.5 h of growth in the presence of 1 mM isopropyl-β-D-galactopyranoside. Cells applied to lane 1 had been transformed with the vector pT7-7 (12) used to construct pAA70. The molecular weights (in thousands) of standard proteins are indicated on the right.

TABLE 2. Mutations in *fecR* generated by PCR

Mutated *fecR* gene	Nucleotide exchange	Amino acid replacement
fecR1[a]	A2492G	Ser248Gly
fecR2	T1917C	Leu56Pro
	T1972C	Asp74[b]
	T2118C	Leu123Pro
	T2655C	Val302Ala
fecR5	A2112G	Gln121Arg
	A2181G	His144Arg
	A2389G	Lys213
	T2415C	Leu222Pro
fecR6	T2081C	Tyr111His
	A2087G	Thr113Ala
	T2259C	Val170Ala
	T2300C	Phe184Leu
	A2630T	Asn294Tyr
fecR8	G1790A	Arg14His
	T2026C	Ala92
	A2211G	Glu154Gly
	T2214C	Ile155Thr
	C2266T	Thr172
	A2337G	Asp196Gly
	T2340C	Val197Ala
	T2367C	Leu206Pro
	A2486G	Ile246Pro
	T2515C	Gly255

[a] Adenine replaced by guanine at position 2492 of the published *fecR* sequence (13) results in replacement of serine by glycine at position 248.
[b] Nucleotide exchange does not result in an amino acid replacement.

contrast to wild-type FecR, altered FecR derivatives were not cleaved proteolytically (Fig. 1, lanes 3 through 7, 9, and 10). Only FecR6 was cleaved into the same fragments as wild-type FecR (Fig. 1, lane 8). It is unlikely that the lack of proteolysis per se caused the constitutive phenotype, since genetically constructed N-terminal fragments of wild-type FecR of the size obtained by cellular proteolysis of FecR lead to constitutive expression of *fecA-lacZ* (9). Although half of overexpressed wild-type FecR is cleaved, the cells show no constitutive expression of the *fec* transport genes but induce *fec* expression in response to ferric citrate. Presumably, wild-type FecR is dominant over the truncated FecR derivatives with regard to interaction with FecI.

Five mutated *fecR* genes were sequenced to examine whether lack of proteolysis indicates a conformational change rather than a mutation at the cleavage site. None of the uncleaved FecR derivatives contained an amino acid replacement at, or close to, residue 181 (Table 2). With the exception of FecR1, which contained a single mutation, all FecR derivatives contained several mutations that were scattered over the polypeptide and were located in the periplasmic C-terminal portion and in the cytoplasmic N-terminal portion of the molecule. No amino acid replacement was located in the transmembrane region, which would be expected if this segment plays an important role in transduction of the signal from the periplasm into the cytoplasm. However, the number of mutants tested was too low to exclude a functional role of the transmembrane region. In three of the mutants, a leucine was replaced by proline, which very likely changes the conformation of the protein and renders FecR protease resistant. FecR6, which was protease sensitive, contained no leucine-to-proline substitution. On the other hand, FecR1 with the single substitution of glycine for serine at position 248 (Ser248Gly) was protease resistant, showing that any of the amino acid replacements could have caused proteolytic resistance. The high frequency of protease resistance among the mutants sug-

gests that extended conformational changes of FecR rather than localized changes in amino acid side chains caused the altered FecR regulatory activity.

This paper describes for the first time *fecR* point mutations with an impaired *fecA* transcription induction. The constitutive mutants indicate that mutations in FecR affect the response to ferric citrate as inducer. The data support the essential role displayed by FecR in initiating transcription of the *fec* transport genes and in transduction of the signal from the cell surface into the cytoplasm. Previously, we described a chromosomally encoded FecR derivative (HG504) in which serine at position 127 was replaced by phenylalanine, causing a constitutive expression of a *fecB-lacZ* operon fusion (9). Cells expressing a chromosomally encoded (RE2847) or one of several plasmid-encoded C-terminally truncated FecR derivatives all show high levels of transcription of *fecA* that are little affected by ferric citrate (9). Apparently, the cytoplasmic N-terminal segment of FecR is able to activate FecI without ferric citrate, which in turn directs RNA polymerase to the *fec* transport gene promoter upstream of *fecA*.

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