Identification of a Novel Regulator for the Escherichia coli fit Iron Transport System

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Abstract: The Escherichia coli fit iron transport system consists of 6 genes, fitA, B, C, D, E and fitR. Based on in silico analysis, FitA-E composes a typical bacterial iron transporter, while FitR was deduced to be a regulator. In this paper the regulation of fit expression by FitR was studied using a quantitative RT-PCR technique and a lacZ reporter assay. It was found that fit expression was repressed when FitR was over-expressed and de-repressed when fitR was knocked out by mutation. When the mutation in fitR was complemented in trans- with the wild type fitR gene, repression of fit expression by FitR was restored. Finally, recombinant FitR was found to bind to the fit promoter DNA when employed in an electrophoretic mobility-shift assay. These results demonstrated that fitR encodes an auto-repressor for the E. coli fit system.

INTRODUCTION

Iron is an essential element for bacterial growth and pathogenesis. Although there is sufficient iron in the environment, iron is not readily available to bacteria in animal or human hosts [1]. To support growth, bacteria have evolved to possess multiple iron transport systems to acquire iron from the environment or hosts. In a previous study, a novel putative ABC iron transport system, fit, was identified in an E. coli strain causing human septicemia [2]. The E. coli fit system consists of 6 genes designated fitA, -B, -C, -D, -E and fitR, encoding an outer-membrane receptor protein (FitA), a periplasmic binding protein (FitE), two permease proteins (FitC and -D), an ATPase (FitB), and a hypothetical protein (FitR) [2]. Gene fitA,B,C,D,E encode a typical bacterial iron transporter. Although there is no match for the predicted FitR protein through BLAST analysis, sequence analysis found that an N-terminal helix-turn-helix DNA binding motif and a C-terminal sugar isomerase domain were present in the predicted FitR. This structure is conserved in many bacterial transcriptional regulators, such as E. coli RpiR, LpcA, GutQ, and Glms [3, 4]. In this paper, quantitative RT-PCR and a fit-lacZ reporter analysis were employed to study the expression of fit system under conditions of fitR over-expression, and mutation and complementation of fitR. It was found that fitR repressed fit expression.

METHODS

Bacterial Strains and Media

The bacterial strains and plasmids used in this study are listed in Table 1. All strains were grown in Luria-Bertani (LB) media. All reagents and media were made with deionized water after passage through a Millipore Cartridge system (Millipore, USA). All glassware was treated with 8M HCl and then rinsed 3 times with double distilled H2O. When appropriate, supplements were added to media at following concentrations: ampicillin, 100 μg/ml; tetracycline, 15 μg/ml; kanamycin, 50 μg/ml; trimethoprim, 100 μg/ml.

Recombinant DNA Techniques

General genetic techniques including PCR, genomic or plasmid DNA purification, ligation and transformation were performed as described previously [2]. Restriction endonuclease and DNA-modifying enzymes were used according to the manufacturer’s recommendations. DNA fragments were purified from agarose gels using Qiaquick gel extraction kit (Qiagen, USA). DNA cloning and manipulation were conducted in E. coli DH5α cells. All oligonucleotide primers were commercially synthesized by IDT (Integrated DNA Technologies, USA) and listed in Table 2. DNA sequencing work was done at the Advanced Genetic Analysis Center (AGAC) at the University of Minnesota.

Construction of Transposon-Insertional Mutants

The EZ::TN transposons (Epigen, Madison, Wisconsin, USA) were used to create gene disruption mutants in E. coli i484 through in vitro transposon insertional mutagenesis according to the protocols provided by the manufacturer [5, 6]. Briefly, the particular gene to be mutated was amplified by PCR. After purification, an EZ::TN transposon was inserted into the DNA fragment through in vitro transposition using the EZ::TN transposase. The disrupted DNA was then electroporated as linear DNA into a recipient strain containing the plasmid pKD46 [5]. Mutants were screened for antibiotic resistance encoded on the inserted EZ::TN transposon and the presence of the transposon in the mutated gene was confirmed by PCR amplification and DNA sequence analysis. Plasmid pKD46 was then eliminated from these cells by growth at 42°C.
RNA Extraction and Real-Time RT-PCR Analysis

Bacteria were grown in LB with appropriate supplements at 37°C with shaking. After 3 hours, cells were harvested, lysed, and RNA was isolated using the Qiagen RNeasy Mini Kit (Qiagen, USA). Reverse transcription was performed using the SuperScript™ III Reverse Transcriptase (Invitrogen, USA). Real-time PCR (qPCR) using Platinum® SYBR® Green qPCR SuperMix-UDG (Invitrogen, USA) was then performed to measure the level of gene expression. The genes $gst$ and $polA$ were used as endogenous controls.

β-Galactosidase Assays

Bacteria carrying lacZ reporter vectors grown in appropriate media were harvested when bacterial growth reached exponential growth phase. Ortho-nitrophenyl-β-D-galactopyranoside (ONPG, Sigma, USA) was used as the substrate to measure β-Galactosidase activities and the Miller Units was calculated as described [7]. Each experiment was repeated 3 times, and the results were statistically analyzed using the student’s $t$ test.

### Table 1. Bacterial Strains and Plasmids Used in this Study

| Strain and Plasmid | Description | Reference |
|--------------------|-------------|-----------|
| **E. coli strains** |             |           |
| i484               | O25:H autoagglutinating, human isolate | 17        |
| oy077              | i484, fitR :: EZ-TN5 < Kan-2 > | This study |
| oy016              | i484, lacZ :: EZ-TN5 < DHFR-1 > | This study |
| oy021              | oy016 carrying pMP-fitB | This study |
| oy022              | oy016 carrying pMP-fitA | This study |
| oy026              | oy016, fitR :: EZ-TN5 < Kan-2> | This study |
| oy028              | oy026 carrying pMP-fitB | This study |
| oy029              | oy026 carrying pMP-fitA | This study |
| oy071              | i484, fur :: EZ-TN5 <DHFR> | This study |
| oy078              | oy077, fur :: EZ-TN5 <DHFR> | This study |
| DH5α               | F-, φ80d lacZAM15 Δ(lacZYA-argF) U169, endA1, recA1, hsdS17 (rK–mK+), deoR, thi-1, supE44, λ-, gyrA96, relA1 | Invitrogen |
| **Plasmids**       |             |           |
| pMP220             | Contains a promoterless lacZ gene, Tet$^R$ | 19        |
| pMP-fitA           | fit promoter was cloned into pMP220 with a direction of fitA transcription | 2         |
| pMP-fitB           | fit promoter was cloned into pMP220 with a direction of fitB transcription | 2         |
| pBAD22             | overexpression vector, Amp$^R$ | 8         |
| pBAD-fitR1         | DNA fitR1 cloned into pBAD22 at EcoR I and Hind III sites | This study |
| pBAD-fitR2         | DNA fitR2 cloned into pBAD22 at EcoR I and Hind III sites | This study |
| pCR                | Cloning vector pCR-XL-TOPO | Invitrogen |
| pCR64              | DNA fitR1 cloned into pCR-XL-TOPO | This study |

Construction of Tightly-Controlled FitR Expression Plasmid

To control expression of fitR, a fitR expression plasmid, pBAD-fitR1, was constructed by cloning the fitR gene into the expression vector pBAD22 [8]. Primers (Table 2) were used to PCR amplify fitR using high-fidelity DNA pfu polymerase (Stratagene, USA). The forward primer contained an EcoR I site, and the reverse primer contained a Hind III site. The fragment was cloned into pBAD22 cleaved with EcoR I and Hind III. Protein expression from pBAD-fitR1 was tightly controlled by the arabinose pBAD promoter and was induced by addition of arabinose to the growth medium.

Cloning and Purification of FitR

A his-tagged fitR expression plasmid was constructed by cloning fitR into the expression vector pBAD22. A 6 × His tag sequence was included in the reverse primer, which produced a C-terminal 6 X His tag for protein purification (listed in supplemental materials Table 2). Then the amplified fragment was digested with EcoR I and Hind III, and
ligated into plasmid pBAD22. Positive clones were identified through PCR and sequence analysis. In order to express the recombinant Fur protein, DH5α cells carrying the positive fitR expression plasmid pBAD-fitR2 were grown in LB media. When bacterial growth reached an A_600 of 0.6, arabinose was added to the culture to a final concentration of 10mM to induce protein expression. After 5 hours of induction, cells were harvested by centrifugation (4000g for 20min at 4°C). Protein was purified by Ni-nitrilotriacetic acid (NTA) affinity chromatography, using Ni-NTA spin columns (Qiagen, USA), under denaturing conditions according to the manufacturer's instructions. Then the purified FitR was refolded by dialysis against 10mM Tris (pH 7.0). After the pellet was removed by centrifugation at 10,000 rpm for 10min, supernatant was collected, analyzed by SDS-polyacrylamide gel electrophoresis, quantified using the Bradford assay kit (Bio-Rad, USA) and used as active FitR in the following assays.

**Electrophoretic Mobility-Shift Assay**

The electrophoretic mobility-shift assay was performed as follows. The 347bp intergenic region between fitA and fitB, including the fit promoter, was PCR amplified using pfu polymerase (Stratagene, USA) with synthesized primers (listed in Table 2). The purified DNA was end-labeled with digoxigenin using recombinant terminal transferase (Roche Applied Science, USA). Three nanograms of labeled DNA and various amounts of purified FitR protein were mixed and incubated at 37°C for 20min. The standard binding buffer was: 20mM Bis-Tris (pH 7.0), 2.5µg/ml sonicated salmon

### Table 2. Oligonucleotide Primers Used in this Study

| Gene | Primer, 5'-3' |
|------|--------------|
| **PCR and cloning** | |
| fitR1 | forward | CGGAATTCGGGAGTCAGTTAACGCCT |
| | reverse | TATAAGCTTGGCCATTTGCTTTCCTCCG |
| fitR2 | forward | CGGAATTCATGATCCAGAAAGATAAAGTGCG |
| | reverse | TATAAGCTTGGATGATGGTGATGGTGATGCCATTTTCCTCC |
| fit promoter | forward | GTGCCAGGTTAGGCAAGGACCTTAAT |
| | reverse | TCGCGGATTGTCGAGTGAAG |
| **qPCR** | |
| fitA | forward | ACCATGCTCTATCCCATC |
| | reverse | GGCAGTTTCGCTGTAAGGTC |
| fitB | forward | GCACTTGCACGGTATCATTC |
| | reverse | ATACGGCTCTGTATGGTGTC |
| fitC | forward | CAAGCAGGTTAAATGTCG |
| | reverse | TCTGCTGTCCTACTCATGTTG |
| fitD | forward | AACAACGGCCAGCAGAATC |
| | reverse | TATCGGATTTACGCAAAACAG |
| fitE | forward | GTAATACCGTCCCAGGAATGAT |
| | reverse | ATAGCCCCTGATACTGTC |
| fitR | forward | ATAGCCGATTTAC |
| | reverse | AATCCTTCCTGAATC |
| fepA | forward | GCGGATTGATTCCTTC |
| | reverse | ATCGTGTGTGATTAACACT |
| gst | forward | GAAGCTGCAAATGTGAGG |
| | reverse | AGCCGAGAACCCTAAACAGATA |
| polA | forward | TGGATCAAACGCTGAGT |
| | reverse | TCTGCCAACTCTGTTTCC |

Restriction enzyme sites were depicted as underlined. In the reverse primer for fitR2, the 6×his tag sequence was indicated in bold.
sperm DNA, 50μg/ml poly[d(I-C)], 5% (w/v) glycerol, 100μg/ml BSA, 1mM MgCl₂, 40mM KCl and 100μM Mn²⁺. Protein-DNA complexes were separated from unbound DNA on 5% nondenaturing polyacrylamide gels run at 50V for 2 to 3h. The DNA was transferred onto a positively charged Nylon membrane (Roche Applied Science, USA) by electrophoretic transfer. The DNA was then analyzed by Southern blotting. The digoxigenin-labeled probes were subsequently hybridized to the membranes, and the chemiluminescent substrate disodium 3-(4-methoxy)methoxy-1,2-dioxetane-3,2’-(5’-chloro)tricyclo[3.3.1.1³,⁷]decan-4-yl) luminous substrate disodium 3-(4-methoxyspiro{1,2-quinuclidine}3,3.1.1³,⁷]decan-4-yl) phenyl phosphate (CSPD) (Roche Applied Science, USA).

RESULTS AND DISCUSSION

FitR Represses fit Expression

A lacZ reporter assay was employed to determine if FitR regulated fit expression. The intergenic DNA containing the bidirectional fit promoter, located between fitA and fitB, was cloned in two directions into the lacZ reporter vector, pMP220. The two fit-lacZ reporter plasmids were named pMP-fitA and pMP-fitB, and were used to measure the expression of fitA and fitB, respectively [2]. β-galactosidase activities were measured from exponential growth phase cells as described [7]. As shown in Fig. (1A), the expression of fitB (Fig. 1A) in strains containing pMP-fitB was very similar to the strain carrying pMP-fitB and the cloning vector pBAD22. The wild type strain (oy021) containing the plasmid pMP-fitB expressed 139 units of β-galactosidase (Fig. 1A). When FitR was expressed from pBAD-fitR1 in wild type strains, this strain expressed 43 units of β-galactosidase, which showed that expression of fitB was repressed 3.2 fold. Further, cells expressed 211 units of β-galactosidase in the fitR knockout mutant, indicating that fitB was de-repressed 4.9 fold in the fitR mutant (oy028). Finally, when the fitR mutation was complemented in trans by pBAD-fitR1, cells expressed 55 units of β-galactosidase, which confirmed the observation that fit was repressed by FitR. A similar regulation pattern was obtained for strains containing pMP-fitA (Fig. 1B). These data demonstrated that FitR does repress fit expression.

The repression of fit by FitR was also found even when FitR expression was not induced (0mM arabinose, Fig. 1), which might be the result of leaky expression of fitR from pBAD-fitR1. To test this, we cloned fitR into the plasmid pCR-XL-TOPO, a cloning vector without a ribosome binding site (RBS). The new plasmid was designated pCR64. When this plasmid was introduced into wild type strains oy021 or fitR mutant oy028, strains oy021/pCR-XL-TOPO, oy021/pCR64, oy028/pCR-XL-TOPO and oy028/pCR64 expressed 129, 120, 228 and 217 units of β-galactosidase, respectively, which demonstrated that fit expression was not repressed without fitR expression (Fig. 1C). Further, we examined fitR transcription using RT-PCR. As shown in Fig. (1D), the fitR transcript was detected in cells carrying pBAD-fitR1 grown under non-induced condition (lane 2). Thus we concluded that the repression of fit by pBAD-fitR1 when cells were grown in LB containing 0mM arabinose resulted from the leaky expression of FitR from pBAD promoter. The fact that pBAD22 is quite leaky has been found in other studies [8, 9]. The regulation of the fit system by FitR was also examined using a quantitative RT-PCR analysis. Compared to gene expression in wild type strain E. coli i484, fitB was repressed 1.8 fold when FitR was expressed from pBAD-fitR1 and de-repressed 3.2 fold when fitR was mutated. In addition, when the fitR mutation was comple-
Iron is not required for repression of fit expression by Fur. E. coli strains carrying pMP-fitB (A) or pMP-fitA (B) were grown in LB (open bar) or LB with 100μM 2, 2’-dipyridyl (filled bar). Star (*) represents statistical significant (p < 0.05). C), Gene expression levels measured using quantitative RT-PCR analysis. RNA was isolated from bacteria grown in LB for 3 hours. Strains used were: E. coli i484; oy071 (i484, fur::DHFR-1), oy077 (fitR::kan-2), and oy078 (fur and fitR double mutant). Results were shown as fold of change: ratio of gene expression levels under other conditions to the expression level in E. coli i484 (open bar). Values are the means from three independent experiments.

In E. coli, for those well-studied iron transporters, such as f ep, fhu, fec, feo, they are all regulated by the global regulator, Fur (ferric uptake regulator), through binding of Fur-Fe³⁺ complex to the Fur box in the gene promoters [10, 11, 12]. And except for the fec system, most E. coli iron transporters do not have their own regulators [13, 14, 15, 16]. Interestingly, the E. coli fit system is regulated by two independent repressors, an auto-regulator FitR and a global regulator Fur. This elegant regulation may indicate the important role of fit in E. coli metabolism. So far we don’t know if FitR is a global regulator or if any other factor is involved in the regulation of the fit system. Further studies, for example, identification of the exact binding site of FitR or Fur on fit
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promoter, whether there is a synergy in binding by addition of Fur and FitR, and whether other proteins might be involved in the expression of fit, etc, may elucidate the detailed regulation mechanism of this novel iron transporter.

CONCLUSION

In conclusion, an auto-repressor, FitR, was identified for the E. coli fit iron transport system in this report.

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