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Identification and Characterization of a Novel ABC Iron Transport System, *fit*, in *Escherichia coli*[▽]†

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A putative ABC transporter, *fit*, with significant homology to several bacterial iron transporters was identified in *Escherichia coli*. The *E. coli fit* system consists of six genes designated *fitA*, *-B*, *-C*, *-D*, *-E*, and *-R*. Based on DNA sequence analysis, *fit* encodes an outer membrane protein (FitA), a periplasmic binding protein (FitE), two permease proteins (FitC and -D), an ATPase (FitB), and a hypothetical protein (FitR). Introduction of the *E. coli fit* system into *E. coli* strain K-12 increased intracellular iron content and transformed bacteria were more sensitive to streptonigrin, which suggested that *fit* transports iron in *E. coli*. Expression of *fit* was studied using a *lacZ* reporter assay. A functional, bidirectional promoter was identified in the intergenic region between genes *fitA* and *fitB*. The expression of the *E. coli fit* system was found to be induced by iron limitation and repressed when Fe^{2+} was added to minimal medium. Several *fit* mutants were created in *E. coli* using an in vitro transposon mutagenesis strategy. Mutations in *fit* did not affect bacterial growth in iron-restricted media. Using a growth promotion test, it was found that *fit* was not able to transport enterobactin, ferrichrome, transferrin, and lactoferrin in *E. coli*.

Iron is an essential microelement for bacteria (12). It is required as a cofactor for a lot of important enzymes which are involved in many fundamental cellular processes, including electron transfer, cell respiration, and superoxide metabolism. Iron is also an important factor for bacterial pathogenesis (5, 24, 36). Although the concentration of iron in the environment is sufficient to sustain the viability of microbes, most iron is present as ferric hydroxide, which is insoluble and biologically inaccessible for bacteria. In the host, iron is bound by high-affinity iron binding proteins, such as transferrin and lactoferrin (12). In human and animal body fluids, the concentration of free iron at neutral pH is estimated to be about 10^{-18} M, which is far too low to support bacterial growth (10, 12). However, under iron starvation, bacteria produce high-affinity iron binding molecules, such as siderophores, to scavenge iron from the environment. The iron-siderophore complexes are transported into bacteria by specific iron transport systems. Most iron transport systems consist of an outer membrane receptor, a periplasmic binding protein, an ABC transporter formed by permease, and ATPase proteins (7, 10). In *Escherichia coli*, there are at least nine known iron transport systems, including ferrichrome (*fhu*), enterobactin (*fep*), ferric citrate (*fec*), aerobactin (*iut*), heme (*chu*), rhodotorulic acid and coprogen (*fhuE*), salmochelin (*iro*), yersiniabactin, and ferrous iron (*feo*) transport systems (6, 7, 10, 17, 24, 25, 27, 32, 37). Besides these iron systems, other putative iron transport systems have been identified by sequence analysis of the *E. coli* genome (23, 35, 39).

In a previous study that employed in vivo gene expression technology, a novel gene, ivi932, was identified in a clinical *E. coli* isolate (18). Gene ivi932 was shown to be expressed in vivo in a mouse septicemia infection model. Furthermore, when a mutation was introduced into ivi932, the strain was attenuated about fourfold in the infection model (M. A. Khan and R. E. Isaacson, unpublished data). DNA sequence analysis suggested that ivi932 is probably related to iron transport. We report here that the open reading frames flanking ivi932 appear to encode a novel ABC iron transport system, which was termed *fit* (ferric or ferrous iron transport). In this report, we studied the effect of mutations in the *E. coli fit* system on bacterial response to iron limitation. In addition, we studied *fit* expression under various conditions using *lacZ* reporter assays. The data in this report support our hypothesis that *fit* encodes a novel iron transport system.

MATERIALS AND METHODS

Bacterial strains and media. The bacterial strains and plasmids used in this study are listed in Table 1. *E. coli* was grown in Difco LB broth (Becton Dickson and Company, Maryland) medium, a defined α-MEM with 10 μM $FeCl_3$ (Invitrogen), or MM9 minimal medium (0.03% KH_2PO_4, 0.1% NH_4Cl, 0.05% NaCl, 1 mM $MgSO_4$, 0.3% Casamino Acids, 0.2% thiamine-HCl). Glucose was added to MM9 medium (0.2%) as a carbon source. For iron-rich medium, a solution of freshly prepared $FeSO_4$ was added to LB medium at a final concentration of 20 μM. For iron-restricted medium, the iron chelator 2,2′-dipyridyl (DIP; Sigma) was added into LB medium at a final concentration of 200 μM.

All reagents and media were made with deionized water after passage through a Millipore reverse osmosis system (Millipore). All glassware was treated with 8 M HCl and then rinsed three times with water. When appropriate, supplements were added to media at the following concentrations: ampicillin, 100 μg/ml; tetracycline, 15 μg/ml; kanamycin, 50 μg/ml; trimethoprim, 100 μg/ml.

DNA manipulation techniques. General genetic techniques were performed as described previously, including genomic or plasmid DNA purification, ligation, and transformation (29). PCR amplification using *Taq* polymerase (Promega) or high-fidelity *Pfu* DNA polymerase (Stratagene) was performed according to the manufacturers' instructions. Restriction endonuclease and DNA-modifying enzymes were used according to manufacturers' protocols. *E. coli* DH5α cells were used as standard competent cells for DNA cloning. The oligonucleotide primers used in this study for PCR, cloning, and sequencing were commercially synthesized by Integrated DNA Technologies and are listed in Table S1 of the supple-

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TABLE 1. Bacterial strains and plasmids used in this study

Strain or plasmid	Description	Source or reference
E. coli strains		
i484	O25:H autoagluttinating; human isolate	18
xy006	i484, *fitA*::EZ-TN5 <Kan-2>	This study
xy005	i484, *fitB*::EZ-TN5 <Kan-2>	This study
xy009	i484, *fepA*::EZ-TN5 <DHFR>	This study
xy008	i484, *fepC*::EZ-TN5 <DHFR>	This study
xy007	i484, *fhuA*::EZ-TN5 <DHFR>	This study
oy016	i484, *lacZ*::EZ-TN5 <DHFR>	This study
AA93	F^- *araD139* Δ*lacU169 rpsL150 relA1 deoC1 flbB5301 ptsF25 rbsR aroB fecB*::Mud1 (Ap *lac*)	26
oy097	AA93; *feoB*::EZ-TN5 <DHFR>	This study
AB1515.199	*purE42 proC14 leu-6 trpE38 thi-1 fhuA23 lacY1* (*fepC*::Tn*5*)	6
123	Field isolate	Pig
124	Field isolate	Pig
252	Field isolate	Pig
EcoR33	Field isolate	Pig
263	Field isolate	Pig
431	Field isolate	Pig
987	Field isolate	Pig
1413	Field isolate	Pig
16	Field isolate	Human
17	Field isolate	Human
18	Field isolate	Human
EcoR40	Field isolate	Human
EcoR60	Field isolate	Human
EcoR62	Field isolate	Human
EcoR8	Field isolate	Human
EcoR26	Field isolate	Human
EcoR42	Field isolate	Human
EcoR45	Field isolate	Human
DH5α	*F*$^-$ ϕ*80*d*lacZ*Δ*M15* Δ(*lacZYA-argF*)*U169 endA1 recA1 hsdS17* (r_K^- m_K^+) *deoR thi-1 supE44* λ$^-$ *gyrA96 relA1*	Invitrogen
Plasmids		
pMP220	Contains a promoterless *lacZ* gene, Tet^r IncP	33
pMP-*fitA*	*fit* promoter cloned into pMP220 with direction of *fitA* transcription	This study
pMP-*fitB*	*fit* promoter cloned into pMP220 with direction of *fitB* transcription	This study
pCR	Cloning plasmid pCR-XL-TOPO	Invitrogen
pCR63	*lacZ* cloned into pCR-XL-TOPO	This study
pCR64	*fitR* cloned into pCR-XL-TOPO	This study
pfit1	Whole *fit* region cloned into pCR-XL-TOPO	This study

mental material. Primers used to amplify and sequence each of the *fit* genes were designed based on the sequence of *E. coli* CFT073 (39). DNA sequencing was performed by the Advanced Genetic Analysis Center at the University of Minnesota.

Cloning of the *E. coli fit* system. The whole *fit* system was PCR amplified using the Elongase amplification system (Invitrogen). After DNA was purified using a QiaQuick gel extraction kit (QIAGEN), it was cloned into plasmid pCR-XL-TOPO (Invitrogen). Positive clones were identified by colony PCR, restriction endonuclease digestion, and sequence analysis.

Functional annotation of the *E. coli fit* system. Gene prediction and annotation were performed by Open Reading Frame Finder analysis and BLAST sequence similarity searching against the GenBank database (National Center for Biotechnology Information) (40) and confirmed by analyzing with the GenQuiz server (1) and the fold recognition server GenThreader (16). Protein domains were assigned by searching against the Conserved Domain Database (CDD) (40), clusters of orthologous groups (34), and the PFAM (2) and SMART (30) databases and by sequence motif analysis (E-motif) (15). A SOSUI analysis (14) was carried out to identify transmembrane segments.

Construction of transposon-insertional mutants. The EZ::TN transposons (Epicenter, Madison, Wis.) were used to create gene disruption mutants in *E. coli* i484 according to the protocols provided by the manufacturer (8, 9, 11). Briefly, the particular gene to be mutated was amplified by PCR using high-fidelity *Pfu* DNA polymerase. After purification of the amplified DNA, an EZ::TN transposon was inserted into the DNA fragment through in vitro transposition that employs addition of exogenous transposase. The DNA was then transformed as linear DNA by electroporation into *E. coli* strains containing the plasmid pKD46 (8). The lambda recombinase system carried on pKD46 promotes the homologous recombination of the linear, mutated DNA into the chromosome. Mutants were screened for antibiotic resistance carried on the inserted EZ::TN transposon and confirmed by PCR amplification and sequence analysis. Plasmid pKD46 was then eliminated from these cells by growth at 42°C.

Growth promotion test. A plate bioassay (37) was performed to determine the substrate of the *E. coli fit* system. Briefly, LB agar plates were depleted of iron by adding 400 μM and 250 μM 2,2′-dipyridyl for *E. coli* i484 and AA93, respectively. Approximately 10^5 (for *E. coli* i484) or 10^7 (for *E. coli* AA93) CFU/ml of cells in log phase were seeded onto the plate. Discs containing 10 μl of various iron-binding or iron-containing compounds were placed on the plates. The concentrations of the compounds were as follows: 50 μM 2,3-dihydroxybenzoic acid, 10 μM ferrichrome, 10 μM heme, 10 μM heme-bovine serum albumin (BSA) complex, 10 μM lactoferrin, 25 μM transferrin, 50 μM rhodotorulic acid (RA), 50 μM desferal, iron citrate, 10 mM $FeSO_4$, and 10 mM $FeCl_3$. Culture supernatant from *E. coli* AB1515.199 grown in LB with 100 μM DIP was used as a source of enterobactin (6). Bacterial growth was monitored after 24 h of incubation at 37°C. If there was bacterial growth around the disc, it demonstrated that the bacteria could utilize the compound on the disc as an iron source.

Streptonigrin sensitivity test. Streptonigrin sensitivity was monitored for cells grown in LB, LB supplemented with 200 μM 2,2′-dipyridyl, or α-MEM. Overnight cultures were harvested by centrifugation, washed three times, resuspended in LB or MEM, and adjusted to an A_{600} of 1.0. The washed cells were then inoculated into fresh medium (1:100). Cell growth was monitored by measure-

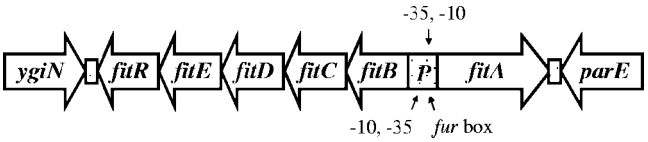

FIG. 1. Organization and orientation of the *E. coli fit* system. The block arrow indicates the gene transcription direction. Intergenic regions are shown as boxes. P, promoter. Arrows indicate the locations of a putative *fur* box and the −10 and −35 boxes of *fitA* and *fitB*, respectively.

ment of the A_{600} at intervals, and growth curves were drawn. To measure sensitivity to streptonigrin, three experimental groups were designed. One was supplemented with 0.4% (vol/vol) dimethyl formamide (control), and the other two groups were supplemented with 1 or 5 μg/ml streptonigrin (dissolved in dimethyl formamide).

Iron content measurement. Bacteria were grown in α-MEM supplemented with 10 μM $FeCl_3$. When bacterial growth reached an A_{600} of 0.4 to 0.6, 20 ml of the culture was harvested by centrifugation at 5,000 rpm at 4°C for 15 min. The pellet was washed twice with 20 ml of ice-cold 0.1 M Tris buffer and once with metal-free double-distilled water. The pellet was resuspended in 1 ml of 37% HCl (TraceMetal grade; Fisher Scientific), lightly vortexed, and heated at 78°C for 5 min until cells were completely lysed. Nine milliliters of metal-free double-distilled water was added to the lysed cells and mixed by vortex. Samples were centrifuged at 13,000 rpm for 10 min, and the supernatant was analyzed for iron content by inductively coupled plasma atomic emission spectrometry at the Research Analytical Laboratory, University of Minnesota. One ppm yttrium was used as an internal standard. The data were normalized by bacterial dry weights, which were determined after drying cell pellets at 87°C for 18 h.

Construction of *fit-lacZ* transcriptional fusions. The plasmid pMP220 (33), which contains a promoterless *lacZ* gene, was used to create *fit-lacZ* transcriptional fusion vectors. The putative *fit* promoter region, presumed to be located between *fitA* and *fitB*, was amplified by PCR with primers listed in Table S1 in the supplemental material. These primers contain an EcoRI site. After digestion of the PCR product with EcoRI, the fragment was cloned into plasmid pMP220. Blue colonies on LB plates containing tetracycline and 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside were picked. Positive clones were identified using PCR and confirmed by DNA sequence analysis.

Assay of β-galactosidase. Bacteria were grown at 37°C in LB broth or MM9 minimal medium. β-Galactosidase activities were measured using ortho-nitrophenyl-β-D-galactopyranoside (Sigma) as the substrate, and Miller units were calculated as described elsewhere (21). Each experiment was repeated in triplicate, and the results were analyzed using Student's *t* test.

Nucleotide sequence accession numbers. GenBank accession numbers are DQ885342 to DQ885347 for *fitA*, *fitB*, *fitC*, *fitD*, *fitE*, and *fitR*, respectively.

RESULTS

Sequencing and annotation of the *E. coli fit* system. In a previous study, a putative iron transport gene, ivi932, was identified in *E. coli* i484 by using an in vivo expression technology protocol (18). Preliminary sequence analysis showed that ivi932 was present in *E. coli* O157:H7 strain EDL933 and in the uropathogenic strain CFT073, but not in K-12 strain MG1655 (3, 13, 28, 39). In addition to ivi932, five additional genes are located in the vicinity of ivi932 in the genomes of *E. coli* EDL933 and CFT073. Based on PCR amplification, these six genes were found to be present as a gene cluster in the *E. coli* i484 genome. The entire gene cluster in strain i484 was amplified and sequenced. Sequence comparison demonstrated that the gene cluster in *E. coli* i484 was almost identical (96%) to those of the other two *E. coli* strains. These six genes, designated *fitA*, *-B*, *-C*, *-D*, *-E*, and *-R*, form a novel putative iron transport system which was named *fit* (ferric or ferrous iron transport) (Fig. 1).

The six open reading frames were annotated using various software packages described in Materials and Methods. *fitA* was deduced to encode a 78-kDa outer membrane receptor protein with homology (44%) to the outer membrane receptor of the *Vibrio cholerae* ferrichrome transporter FhuA. FitA has a TonB-dependent ligand-gated channel domain, which is found in the outer membrane receptors of many iron transport systems, such as FepA, FecA, FhuE, CirA, and Fiu (4, 22). *fitB* was deduced to encode a 30-kDa ATPase protein with homology (63%) to the ATPase of the *E. coli* enterobactin transporter FepC. The characteristic ATP binding domain (Walker A and B motifs) (38) was predicted in the protein. *fitC* and *fitD* were predicted to encode two permease proteins of 34 and 37 kDa, respectively. FitC had homology (55%) to *Escherichia coli* FepD, and FitD had homology (59%) to *Yersinia enterocolitica* HemU. Based on SOSUI analysis, both FitC and FitD were predicted to be strongly hydrophobic, and each protein contains nine transmembrane motifs. *fitE* encodes a 35-kDa periplasmic binding protein with homology (46%) to the *Bacillus subtilis* FhuD protein. These five components, FitA, -B, -C, -D, and -E, form a typical bacterial iron transport system. Besides these five genes, there is an extra open reading frame in the *E. coli fit* system, *fitR*, which encodes a 32-kDa hypothetical protein. The predicted FitR protein contains an RpiR domain that has been associated with other regulators and thus might be a regulatory protein (31). Between the genes *fitA* and *fitB* is a 373-bp A/T-rich (70%) region. Based on DNA sequence analysis, it is predicted to be the promoter of the *fit* system, containing two −35 and −10 signal sequences (Fig. 1). The −10 and −35 sequences of *fitA* overlap the −35 and −10 sequences of *fitB*, respectively, but they are oriented in opposite directions. In addition, a putative *fur* box has been predicted in this region.

Distribution of the *fit* locus in *E. coli* isolates. PCR amplification was performed to detect the presence of each *fit* gene among 18 *E. coli* isolates. As shown in Table 2, when any of the *fit* genes was found, all the other *fit* genes were also detected. Of the strains investigated, *fit* was found only in *E. coli* isolates of human origin and not in pig isolates (including fecal commensal strains and enterotoxigenic strains).

Intracellular iron content measurement. To determine if the *E. coli fit* system is involved in iron transport, the *fit* system was introduced into *E. coli* K-12 strain AA93 and we then asked if *fit* contributed to intracellular iron accumulation in this strain. The intracellular iron content was measured indirectly using streptonigrin as a probe. Streptonigrin interacts with intracellular iron to form reactive oxygen species that lead to DNA damage and eventual cell death (41). Thus, sensitivity to streptonigrin can be used as a measure of the intracellular iron content in strains AA93/pCR and AA93/pfit1. It was expected that if *fit* mediated iron accumulation, AA93/pfit1 would be more sensitive to streptonigrin than AA93/pCR. As shown in Fig. 2A, there was no difference between these two strains when cells were grown in LB containing no streptonigrin. However, in LB supplemented with 1 μg/ml streptonigrin (dissolved in dimethyl formamide), AA93/pCR grew very well while the growth of AA93/pfit1 was inhibited, which indicated that AA93/pfit1 was more sensitive to streptonigrin. Similar phenomena were observed when both strains were grown in α-MEM (Fig. 2B) or in iron-restricted medium (Fig. 2C). The *E. coli feo* system is a major ferrous iron transporter (17). To

TABLE 2. Distribution of the *E. coli fit* system among *E. coli* clinical isolates

E. coli strain source and type	*fit* system
Pig commensal strains	
123	−
124	−
252	−
EcoR33	−
Pig enterotoxigenic strains	
263	−
431	−
987	−
1413	−
Human extraintestinal pathogenic strains	
16	+
17	−
18	+
EcoR40	−
EcoR60	+
EcoR62	+
Human commensal strains	
EcoR8	−
EcoR26	−
EcoR42	+
EcoR45	−

decrease background iron uptake in AA93, we created a *feoB* knockout (oy097) and then introduced the *fit* system into this strain. Strain oy097/pCR grew well in LB medium with 5 μg/ml streptonigrin, while the growth of oy097/pfit1 was completely inhibited (Fig. 2D). These results showed that oy097/pfit1 was more sensitive to streptonigrin than oy097/pCR.

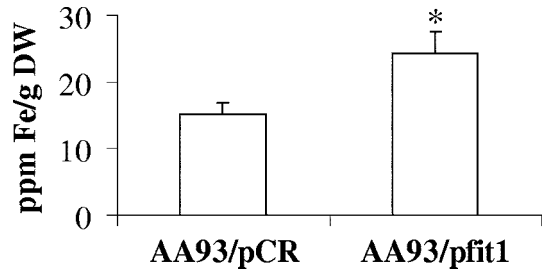

FIG. 3. Measurement of the iron content of the *E. coli* AA93 derivatives by inductively coupled plasma atomic emission spectrometry. Cells were grown in α-MEM supplemented with 10 μM $FeCl_3$. The data were calculated from three independent replicates. Error bars indicate standard deviations. The asterisk indicates statistical significance using Student's *t* test ($P < 0.05$).

To confirm the above results, the intracellular iron content was measured directly from lysed cells using inductively coupled plasma atomic emission spectrometry. As shown in Fig. 3, there is a significantly higher iron content in *E. coli* AA93/pfit1 (25 ppm iron/g [dry weight]) than in AA93/pCR (15 ppm iron/g [dry weight]).

Regulation of expression of the *E. coli fit* system. To study the regulation of *fit* expression, a *lacZ* reporter assay was developed by insertion of the predicted *fit* promoter into the *lacZ* reporter plasmid pMP220 in two directions. Transcriptional fusion plasmids pMP-fitA and pMP-fitB were used to measure the expression of *fitA* and *fitB*, respectively. We first asked

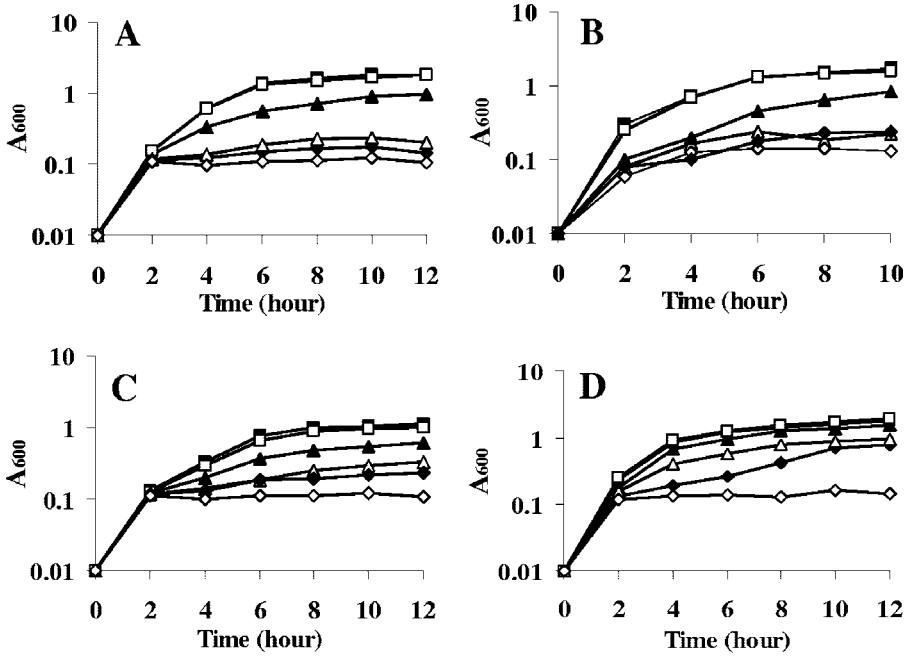

FIG. 2. Effect of streptonigrin on growth of *E. coli* AA93 derivatives. Growth of strain AA93 with plasmids in LB (A), α-MEM (B), or LB with 200 μM dipyridyl (C) and growth of strain AA93/*feoB* with plasmids in LB (D) were measured. Open symbols, strains carrying pfit1; filled symbols, strains carrying pCR; squares, growth in medium without streptonigrin; triangles, growth in medium with 1 μg/ml streptonigrin; diamonds, growth in medium with 5 μg/ml. All experiments were performed at least three times to assess reproducibility. The figure presents the results of one typical experiment.

TABLE 3. Expression of β-galactosidase from *E. coli* oy016 derivatives grown in LB or MM9 medium

E. coli derivative	β-Galactosidase activity (Miller units, mean ± SD)	
	LB	MM9-glucose
oy016	0.0	0.0 ± 0.3
pMP220	2.4 ± 0.3	0.75 ± 0.1
pMP-*fitA*	4.2 ± 0.3	11.7 ± 1.2
pMP-*fitB*	153 ± 12	759 ± 82

whether β-galactosidase was produced from strains containing either of the fusion plasmids. As shown in Table 3, β-galactosidase activity was not detected in cells containing pMP220 while pMP-fitA and pMP-fitB expressed β-galactosidase, although *fitB* was expressed at a much higher level (about 36-fold) than *fitA*. When cells were grown in MM9 minimal medium, expression of *fitA* and *fitB* was induced 2.8- and 4.9-fold, respectively, compared with gene expression levels in cells grown in LB medium. These results indicated that there is a bidirectional promoter in the intergenic region between *fitA* and *fitB*.

To determine if *fit* expression was affected by bacterial growth phase or iron depletion, β-galactosidase activity was measured for *E. coli* oy016 carrying pMP-fitA or pMP-fitB. Cells were first grown in LB for 3 h. The culture was split in two groups. DIP (200 μM) was added to one group. The cells were further incubated, and samples were collected at intervals. As shown in Fig. 4, there was no significant difference among the β-galactosidase levels at different cell growth phases. When iron was depleted from the medium by addition of DIP, expression of *fitA* and *fitB* was increased about four- and fivefold, respectively. This increase was observed at all time points after the first 3-hour period. To determine if metal ions other than iron affected expression of *fit*, β-galactosidase activities were measured from bacteria grown in MM9-glucose medium supplemented with different metal ions. Our results showed that pMP-fitB expressed 531 units of β-galactosidase when cells were grown in this minimal medium without adding any metal ion (Fig. 5A). Addition of Fe^{2+}, Co^{2+}, or Cd^{2+} resulted in significant reduction of *fitB* expression. Similar trends were observed for pMP-fitA (Fig. 5B). These results demonstrated that both *fitA* and *fitB* are significantly repressed by Fe^{2+}, Co^{2+}, and Cd^{2+}.

Previous studies reported that some *E. coli* iron transporters are regulated by oxidative stress and pH (19, 42). To measure the pH effect on *fit* expression, LB medium was buffered by the addition of 100 mM homopiperazine-*N*,*N*′-bis(2-ethanesulfonic acid), and the pH was adjusted to 5.0, 6.0, 7.0, or 8.0. When cells were grown in LB of pH 5.0, 6.0, 7.0, or 8.0, expression levels of *fitB* and *fitA* were increased with increasing pH (Fig. 6A and B). To measure the effect of H_2O_2 on *fit* expression, mid-log-phase cells grown in LB medium were split into four groups. One group was used as the control group. The other three groups were exposed to 0.1, 0.5, or 1.0 μM H_2O_2 for 10 min. Our results showed that expression of *fitB* was increased about 2.0-, 2.8-, and 2.2-fold when cells were exposed to 0.1, 0.5, or 1.0 μM H_2O_2, respectively (Fig. 6C). This result indicated that *fitB* was induced significantly by H_2O_2. Similar results were obtained for pMP-fitA (Fig. 6D).

Bacterial growth in iron-restricted medium. To determine if *fit* is involved in iron transport in *E. coli*, the growth of *fit* mutants in LB containing 200 μM DIP was compared with that of the parental strain, *E. coli* i484. As shown in Fig. 7, mutations in *fitA* or *fitB* did not affect bacterial growth. In this report, the growth of a *fepC* (enterobactin transport) mutant and *fhuA* (ferrichrome transport) mutant in the iron-restricted medium also was studied. Results showed that when *fepC* was mutated, bacterial growth in this medium was significantly in-

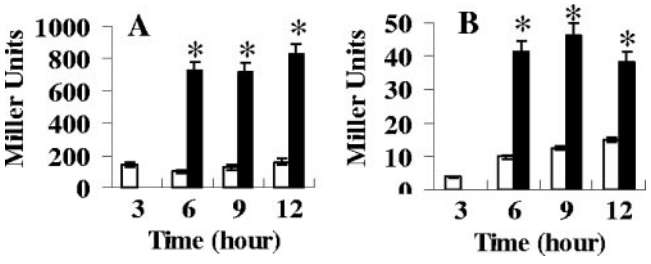

FIG. 4. Expression of *fit* was induced by iron depletion. *E. coli* oy016 cells carrying pMP-fitB (A) or pMP-fitA (B) were first grown in LB medium for 3 h. Then the culture was split into two groups. Into one group, DIP was added at a final concentration of 200 μM, and into the other group no DIP was added. Cells were then allowed to grow, samples were taken at various time points, and β-galactosidase activities were measured as described elsewhere (21). Open bars, cells grown in LB; filled bars, cells grown in LB with DIP. Values are the means from three independent experiments. Error bars indicate standard deviations. Asterisks indicate statistical significance using Student's *t* test ($P < 0.05$).

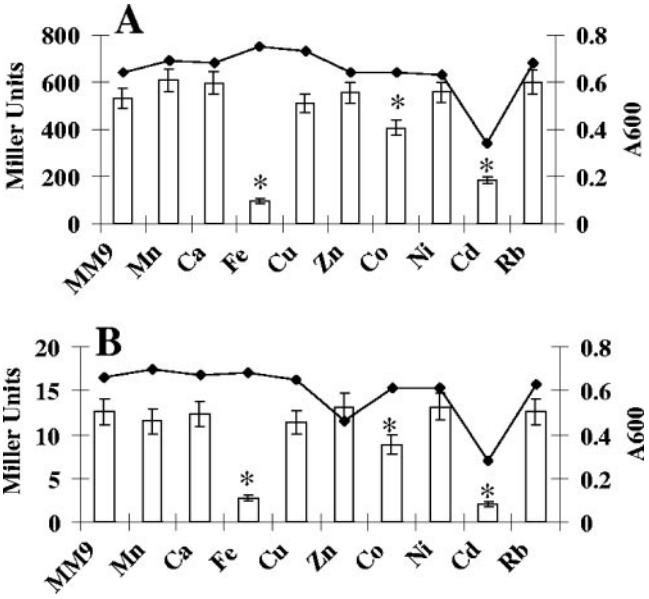

FIG. 5. Expression of *fit* was repressed by iron. *E. coli* oy016 cells carrying pMP-fitB (A) and pMP-fitA (B) were grown in MM9 medium with various metal ions. A 100 μM concentration each of Mn^{2+}, Ca^{2+}, Fe^{2+}, Cu^{2+}, Ni^{2+}, and Rb^{+}, 50 μM each of Zn^{2+} and Co^{2+}, and 10 μM Cd^{2+} were used in the experiment. Cells were harvested at mid-log phase, and β-galactosidase activities were measured as described previously (21). Error bars indicate standard deviations ($n = 3$). Asterisks indicate statistical significance using Student's *t* test ($P < 0.05$).

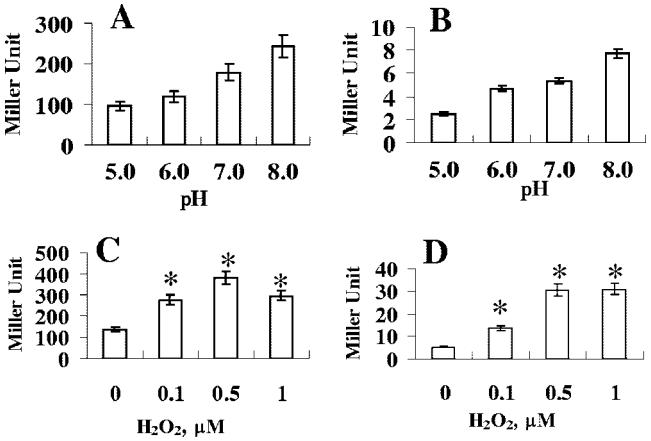

FIG. 6. Effects of pH and H_2O_2 on *fit* expression, based on β-galactosidase activities of oy016 cells carrying pMP-fitB (A and C) and pMP-fitA (B and D). (A and B) Cells were grown in LB at various pHs. (C and D) Cells were grown in LB, and when bacterial growth reached an A_{600} of 0.6, various amounts of H_2O_2 were added to the medium. After 10 min, cells were collected and β-galactosidase activities were measured as described elsewhere (21). Values are the means from three independent experiments. Error bars indicate standard deviations ($n = 3$). Asterisks indicate statistical significance using Student's *t* test ($P < 0.05$).

hibited compared to the parental strain, while a mutation in *fhuA* did not affect bacterial growth.

Growth promotion test. It was hypothesized that if the *E. coli* *fit* system were responsible for transporting iron compounds, the ability of bacteria to transport this substrate would be attenuated or lost by creating mutations in *fit*. To address this question, a growth promotion test was performed. As shown in Table 4, the parental strain *E. coli* i484 was able to utilize all the compounds, with the exception of transferrin and lactoferrin, as external iron sources. When the *fep* system was mutated (strains xy008 and xy009), the mutants lost their abilities to utilize enterobactin (the growth supernatant of *E. coli* strain AB1515.199 under iron limitation). It also was found that the

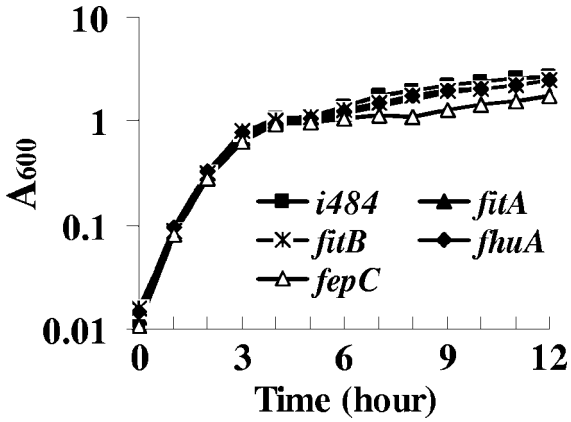

FIG. 7. Bacterial growth in iron-restricted medium. LB medium was made iron depleted by adding DIP to a final concentration of 200 μM. Bacteria were grown at 37°C with shaking, and the A_{600} was measured during growth. All tests were repeated three times, and the results of one typical experiment are presented here.

TABLE 4. Growth promotion test of *E. coli* strains[a]

Substrate	Growth of strain							
	i484	fitA- (xy006)	fitB- (xy005)	fhuA- (xy007)	fepC- (xy008)	fepA- (xy009)	AA93/ pfit1	AA93/ pCR
S_{i484}	+	+	+	+	+	+	+	+
S_{AB1515}	+	+	+	+	−	−	+	+
Heme	+	+	+	ND	ND	ND	−	−
Heme-BSA	+	+	+	ND	ND	ND	−	−
DHBA	+	+	+	ND	ND	ND	−	−
Lactoferrin	−	−	−	ND	ND	ND	−	−
Transferrin	−	−	−	ND	ND	ND	−	−
Desferal	+	+	+	ND	ND	ND	−	−
RA	+	+	+	ND	ND	ND	+	+
Ferrichrome	+	+	+	−	ND	ND	+	+
Iron citrate	+	+	+	ND	ND	ND	−	−
$FeSO_4$	+	+	+	+	+	+	+	+
$FeCl_3$	+	+	+	+	+	+	+	+
dH_2O	−	−	−	−	−	−	−	−

[a] Concentrations of iron compounds used are reported in Materials and Methods. S_{i484} and S_{AB1515} represent supernatants of *E. coli* i484 and AB1515.199 cells, respectively, grown in iron-restricted media. Heme-BSA was made by mixing 100 μM heme and 100 μM BSA at a 1:1 molar ratio. Iron citrate was made by mixing fresh $FeSO_4$ with sodium citrate at a molar ratio of 1:1,000. +, positive; −, negative; ND, not determined; DHBA, dihydroxybenzoic acid; dH_2O, distilled water.

fhuA mutant (xy007) lost its ability to utilize ferrichrome. When *fitA* or *fitB* was mutated, bacteria were still able to utilize enterobactin, ferrichrome, heme, heme-BSA, RA, desferal, $FeSO_4$, or $FeCl_3$ as the only iron source to support their growth. This test was also performed in *E. coli* AA93, and similar results were found (Table 4). It was found that AA93/pfit1 did not utilize heme, heme-BSA, transferrin, lactoferrin, desferal, or iron citrate. These data suggested that the *E. coli* *fit* system does not transport enterobactin, ferrichrome, transferrin, lactoferrin, heme, or iron citrate.

DISCUSSION

Iron is an essential element for almost all bacteria. In the host, iron is nearly unavailable for bacteria due to the binding of transferrin and lactoferrin, etc. To support their growth, pathogenic bacteria have developed elaborate mechanisms to overcome this iron deficiency. One of these mechanisms involves the production of siderophores, which are low-molecular-weight chelators that have high affinities for iron (10, 22). Once siderophores bind iron, the siderophore-iron complexes are transported into cells by specific transport systems.

In this paper, we described the discovery of a new *E. coli* iron transport system. The *fit* operon was discovered by using an in vivo expression technique coupled with directed DNA sequencing in an *E. coli* strain that causes human septicemia. Based on DNA sequence analysis, the likely functions of the genes in this cluster include a TonB-dependent outer membrane receptor, a periplasmic binding protein, two permease proteins, and an ATPase. These components form a typical iron transport system in gram-negative bacteria (7). However, unlike most iron transport systems, the *fit* operon contains a sixth gene that encodes a hypothetical protein. Based on CDD analysis, the predicted protein FitR has an RpiR domain, which suggested that it might be a regulator.

To determine if mutations in the *fit* operon affected bacterial

growth under iron limitation, a collection of *fit* mutants were created. Under in vitro iron-limiting conditions, no significant differences in growth characteristics between the parental strain and the *fit* mutants were found. It is likely that this is because *E. coli* strain i484 encodes other iron transport systems, including *fep*, *fhu*, *fec*, *iro*, *fhuE*, *feo*, heme, aerobactin, and yersiniabactin transport systems (their presence in *E. coli* i484 was confirmed by PCR amplification [data not shown]). These iron transporters probably abrogate the effects of *fit* mutation on iron uptake.

E. coli i484 produces four kinds of siderophores (enterobactin, aerobactin, salmochelin, and yersiniabactin) under iron starvation (data not shown), and it possesses multiple iron transport systems. An *E. coli* K-12 strain, AA93, does not produce enterobactin and salmochelin due to an *aroB* mutation (26). Genes for synthesis of aerobactin and yersiniabactin were not detected in this strain (data not shown). AA93 also is a *fec* deletion mutant, and the *fit* system is not present in its genome. Thus, *fit*-mediated iron uptake in *E. coli* i484 is likely to be masked due to the redundancy of iron transport systems. Therefore, to study the contribution of the *fit* system to iron uptake, the whole *fit* system was cloned and introduced into *E. coli* AA93. The relative intracellular iron content was determined indirectly by measuring sensitivity to streptonigrin. Streptonigrin is an antibiotic whose toxicity is dependent on intracellular iron. Increased sensitivity to this drug is a sign of increased availability of intracellular free iron. Our results showed that introduction of the *E. coli fit* system into strain AA93 resulted in the strain being much more sensitive to streptonigrin, which suggested that there is more intracellular iron in AA93/pfit1 than in AA93/pCR. The plasmid pCR is a high-copy-number cloning vector. Thus, we assume that pfit1 was present in high copy numbers in *E. coli* AA93. The presence of a high-copy-number plasmid in bacteria may cause metabolic burden effects, which induce a significant shift in the normal metabolism and a reduced bacterial growth. Thus, the accumulation of iron observed in *E. coli* AA93/pfit1 possibly is an indirect effect which is mediated through the metabolic burden effects due to the presence of the high-copy-number plasmid. To address this question, a *fitR* clone, pCR64, and a *lacZ* clone, pCR63, were used as controls in this test. It was found that *E. coli* AA93/pCR64 and AA93/pCR63 had growth curves similar to that of AA93/pfit1, while AA93/pfit1 was more sensitive to streptonigrin than AA93/pCR64 and AA93/pCR63 (data not shown). This suggested that the iron accumulation effect in AA93/pfit1 was not due to an increased burden from the presence of a high-copy-number plasmid. To confirm the results from the streptonigrin sensitivity test, inductively coupled plasma atomic emission spectrometry was performed to directly measure the iron content within the cells. Our data showed that *E. coli* AA93/pfit1 had significantly higher iron content than AA93/pCR. These results suggested that the *E. coli fit* system transports iron when it is expressed from the plasmid pfit1.

To identify the substrate of the *E. coli fit* system, a growth promotion test was performed using various iron compounds. BLAST and CDD analyses suggested that *fit* was most closely homologous to ferrichrome (*fhu*) or enterobactin (*fep*) iron transport systems. Our results showed that when *fepC* (encoding the ATPase of the *fep* system), or *fhuA* (encoding the receptor of the *fhu* system) was mutated, these mutants lost their abilities to utilize enterobactin or ferrichrome, respectively, as iron sources. These results demonstrated that, contrary to our expectation, enterobactin and ferrichrome are not the substrates of the *E. coli fit* system. We also studied if *fit* transported ferric citrate, since FitC and FitD had FecCD domains, which are conserved in the permease proteins of the ferric citrate transport (*fec*) system (26). We found that strain AA93/pfit1 did not use ferric citrate as the only iron source, which suggested that *fit* did not transport ferric citrate.

Gene regulation information can provide clues to the function of the *fit* system. In this study, a *lacZ* reporter assay was employed to study the expression of *fit*. Sequence data suggested that the intergenic region between *fitA* and *fitB* contained a putative bidirectional promoter for the *E. coli fit* system. The experimental results indicated that this was true, since both pMP-fitA and pMP-fitB expressed β-galactosidase. Our data showed that the β-galactosidase activity from the *fitA* promoter was much lower than that of the *fitB* promoter, which suggested that *fitB* was expressed at a higher level than *fitA*. However, the responses of *fitA* to iron depletion, pH, or H_2O_2 were of a magnitude similar to those of *fitB*. Both genes were regulated at similar levels under these conditions.

Of those well-characterized *E. coli* iron transporters mentioned above, all are regulated by iron (4, 20). Since *fit* encodes a putative iron transport system, we hypothesized that *fit* was regulated by iron. Our data demonstrated that the expression of *fit* was induced by depletion of iron and repressed by addition of exogenous iron. This result serves as strong evidence that *fit* is involved in *E. coli* iron metabolism.

We further examined if other environmental condition regulators were involved in the regulation of *fit* expression. For example, *E. coli* responds to pH changes encountered in the digestive tract by regulating different sets of genes. At low pH, for example, acid consumption, proton export, and oxidative stress responses are induced. At high pH, proton import is induced while the energy-expensive systems of flagellar biosynthesis and chemotaxis are repressed (19). Some iron transporters are found to be regulated by pH. For example, *fecAB* and *fhuD* are induced at high pH (19). Similarly, we found that the *E. coli fit* system also was induced at high pH (8.0). In this study, we also found that *fit* was induced by hydrogen peroxide. Since sequence analysis indicated that the *fit* promoter region lacks an OxyR binding site, currently the mechanism of this induction is unknown.

We have described here a novel *E. coli* iron transport system, *fit*, which has homology to many bacterial iron transporters. Our data showed that the *fit* system contributes to iron accumulation in *E. coli* AA93. We also found that the expression of the *E. coli fit* system was repressed by addition of exogenous iron and derepressed by depletion of iron. Based on these experimental data, combined with the sequence information, we believe that the *E. coli fit* system is involved in iron acquisition.

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