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ORIGINAL PAPER

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FieF (YiiP) from *Escherichia coli* mediates decreased cellular accumulation of iron and relieves iron stress

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Abstract The *Escherichia coli yiiP* gene encodes an iron transporter, ferrous iron efflux (FieF), which belongs to the cation diffusion facilitator family (CDF). Transcription of *fieF* correlated with iron concentration; however, expression appeared to be independent of the ferrous iron uptake regulator Fur. Absence of FieF led to decreased growth of *E. coli* cells in complex growth medium but only if *fur* was additionally deleted. The presence of EDTA was partially able to relieve this growth deficiency. Expression of *fieF in trans* rendered the double deletion strain more tolerant to iron. Furthermore, *E. coli* cells exhibited reduced accumulation of ^{55}Fe when FieF was expressed *in trans*. FieF catalyzed active efflux of Zn(II) in antiport with protons energized by NADH via the transmembrane pH gradient in everted membrane vesicles. Using the iron-sensitive fluorescent indicator PhenGreen-SK encapsulated in proteoliposomes, transmembrane fluxes of iron cations were measured with purified and reconstituted FieF by fluorescence quenching. This suggests that FieF is an iron and zinc efflux system, which would be the first example of iron detoxification by efflux in any organism.

Keywords Iron · Zinc · Efflux · Cation diffusion facilitators · *E. coli* · Fluorescence quenching

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Abbreviations CDF: Cations diffusion facilitator · FieF: Ferrous ion efflux

Introduction

Iron is the most abundant transition metal in nearly all organisms, followed by zinc and other metals of the first elemental transition period (Nies 2004). These heavy metals are essential at low concentrations, but toxic at higher concentrations, forcing living cells to carefully maintain intracellular homeostasis of these trace elements. In most cases, heavy-metal homeostasis involves efflux pumps that export excess metal cations. Efflux pumps detoxify the cations of cobalt, nickel, copper, zinc, silver, cadmium and lead (Nies 2003), but none are yet known to export iron.

Nevertheless, high intracellular iron concentrations pose a severe threat to living cells. Iron can mediate cellular damage by catalyzing the Haber-Weiss reaction, which generates hydroxyl radicals from superoxide and hydrogen peroxide. Moreover, oxidation of ferrous iron by hydrogen peroxide (the Fenton reaction) also results in the production of hydroxyl radicals (reviewed in: Halliwell and Gutteridge 1992). Both processes are the consequence of the redox potential of the Fe(III)/Fe(II) pair [$E_0' = +356$ mV (Weast 1984)], which allows redox reactions under biochemical conditions and is also the reason for the importance of iron as a bio-element. Copper is very similar to iron in its ability to perform one-electron transfer reactions under biochemical conditions [Cu(II)/Cu(I), $E_0' = -262$ mV (Weast 1984)] and is therefore of biological importance as a bio-element as well as a toxic heavy metal. However, cellular homeostasis of copper is tightly controlled and employs metal chaperones (O'Halloran and Culotta 2000) and metal efflux systems. In *Escherichia coli*, copper efflux is accomplished by two independent determinants, *cusCFBA* and *cueO*/*copA*, as reviewed recently (Grass and Rensing 2001; Outten et al. 2001; Rensing and Grass

2003). In contrast, no efflux systems are known that export excess iron, neither in prokaryotes nor in eukaryotes. Thus, it was the accepted view that cells answer iron overload and iron-dependent oxidative stress by down-regulating iron uptake and depositing surplus iron in ferritin as well as by their general response against reactive oxygen species (Zheng et al. 1999).

One of the protein families that might encompass iron-exporting members (Nies 2003) is the cation diffusion facilitator (CDF) family of membrane-bound transporters (Paulsen and Saier 1997). CDF proteins, found in all three domains of life (Nies 2003), are exclusively metal-specific pumps. Frequent substrates are zinc, cobalt, cadmium, and nickel (Anton et al. 1999; van der Zaal et al. 1999; Persans et al. 2001; Bloss et al. 2002). Eukaryotic members, such as ZnT6, function in transporting cytoplasmic zinc into the Golgi apparatus and into the vesicular compartment; they are also important components of zinc homeostasis in mammals (Huang et al. 2002). Recently, the ShMTP1 protein from the manganese-tolerant plant *Stylosanthes hamata* was shown to confer Mn^{2+} tolerance in yeast (Delhaize et al. 2003), expanding the substrate spectrum of CDF proteins to manganese. In yeast mitochondria, two CDF proteins, MFT1 and MFT2 (MMTp1, 2), might have a redundant function affecting cytosolic iron levels. When cells overexpressed these transporters in iron-rich medium, the mitochondrial iron content was increased, suggesting that the proteins are involved in the redistribution of iron from intracellular compartments (Li and Kaplan 1997; Lange et al. 1999).

A specific subset of frequently occurring CDF proteins are of unknown physiological function (Nies 2003). One of these proteins is encoded by *yiiP* (accession no. NP_418350) in *E. coli*, now named *fieF*, for ferrous iron efflux. In a previous study, transcription of this gene was induced by zinc and, to a far lesser extent, by cadmium but not by nickel, cobalt, or copper (Grass et al. 2001a). However, in contrast to the second CDF protein from *E. coli*, ZitB, no involvement of FieF in zinc homeostasis could be detected (Grass et al. 2001a). In this study, we demonstrate that FieF is a novel component in iron homeostasis in *E. coli* and may be the first example of an iron-exporting efflux pump.

Materials and methods

Bacterial strains, growth media

Strains used in this work are listed in Table 1. *E. coli* was grown in Luria-Bertani (LB) medium or in Tris-buffered mineral salts medium (Mergeay et al. 1985) containing 2 ml glycerol and 3 g casamino acids per liter. Metal salts, EDTA or antibiotics [chloramphenicol (15–20 μg/ml), kanamycin (25 μg/ml), ampicillin (100 μg/ml)] were added where appropriate.

Table 1 *Escherichia coli* strains

Strain	Genotype	Source/reference
W3110	Wild-type	Bachmann (1972)
GG193	Δ*lacZYA*::*kanφ*(*fieF-lacZ*)	Grass et al. (2001a)
GG203	Δ*fur*::*cat* Δ*lacZYA*::*kanφ* (*fieF-lacZ*)	This study
GG196	Δ*fieF*::*cat*	This study
GG199	Δ*fur*::*cat*	This study
GG200	Δ*fieF*::*cat* Δ*fur*	This study
GR362	Δ*zntA*::*kan* Δ*zntB*::*cat* Δ*zitB* Δ*zupT* Δ*znuABC*	Grass et al. (2002)

Gene disruptions, deletions and plasmid construction

Genes were disrupted by the insertion of Cam^R cassettes employing a protocol developed in the laboratory of B. Wanner that is based on the λ Red-recombinase system as described previously (Datsenko and Wanner 2000). The primer pairs 5′-CCGTTATACTAGCGTCAGTTGATAGCGGGAGTATTT*ATG*GCGATTGTGTAGGCTGGAGCT-3′, 5′-TACACAAAATGCGGGTCTGGCTCTCTTTTATACTGA*TTA*CCATGGTCCATATGAATATCCTCC-3′ for *fieF* and 5′-CGC *ATG*ACTGATAACAATACCGCCCTAAAGAAAGCTGGCGCGATTGTGTAGGCTGGAGCT-3′, 5′-CGCAGGTTGGCTTTTCTCGTTCAGGCTGGC*TTA*TTTGCCCCATGGTCCATATGAATATCCTCC-3′ for *fur* were employed. For each primer, start or stop (inverse) codons of *fieF* or *fur*, respectively, are indicated in italic and underlined sequences are homologous to a *cat* resistance cassette. Initial deletions comprising a chloramphenicol resistance cassette (*cat*) were transduced by general transduction with phage P1 into *E. coli* strain W3110. Multiple deletions were constructed by elimination of the respective resistance cassette (Datsenko and Wanner 2000) and subsequent general phage-P1 transduction.

Plasmid pFIEF was constructed as follows. *fieF* was amplified by PCR from chromosomal DNA of *E. coli* wild-type strain W3110 using forward primer 5′-GTA GAATTC AAT CAA TCT TAT GGA CGG CTG GTC-3′ (*Eco*RI site underlined) and reverse primer 5′-TGA CTGCAG AAG CAT TGT CCG TTT ACC CTC CCT-3′ (*Pst*I site underlined) and cloned into the vector plasmid pASK-IBA3 (IBA, Göttingen, Germany) providing the ATG start codon, a Strep-TagII to the amino-terminus and the *tetAp* promoter. The resulting pFieF plasmid was checked by restriction and DNA sequence analysis.

^{55}Fe uptake

Uptake experiments were done by filtration. Cells were grown overnight in LB medium, diluted 400-fold into minimal medium, grown overnight and again diluted to 30 Klett units in fresh prewarmed minimal medium. The cells were grown to an optical density of 60 Klett units and *fieF* was induced with 200 μg anhydrotetracycline

(AHT) per liter. After growth for 30 min, ascorbate (1 mM final concentration) and $FeSO_4$, labeled with $^{55}FeCl_3$ (5 μM final concentration), were added. Cells were incubated at 37°C with shaking and 0.5-ml aliquots were filtered through nitrocellulose membranes (0.45 μm) at various times and immediately washed with 5 ml of 0.1 mM LiCl. The membranes were dried, and radioactivity was measured using a liquid scintillation counter (LS6500, Beckman, Munich, Germany). The dry weight was determined from the optical density with a calibration curve. $^{55}FeCl_3$ was from Perkin-Elmer.

Transport assays

Everted membrane vesicles were prepared essentially as previously described (Ambudkar et al. 1984) using a buffer consisting of 25 mM Tris–HCl, pH 7.5, with 200 mM KCl, and 250 mM sucrose. Use of strain GR362(Δ*zntA*::*kan* Δ*zntB*::*cat* Δ*zitB* Δ*zupT* Δ*znuABC*) is advantageous for transport assays because the multiple deletions of zinc transport systems reduce interference by reuptake systems during transport assays. Vesicles were stored at −80°C after flash freezing in liquid nitrogen. Transport assays were done at room temperature in a reaction buffer consisting of 75 mM NaHEPES pH 7.5, with 50 mM KCl, 250 mM sucrose, with added 2 mM $MgCl_2$ and 2 μM of a $^{65}ZnCl_2$–$ZnCl_2$ mix. In 1-ml reaction mixes, 0.2 mg of membrane protein were preincubated for 5 min, and the reactions were initiated with the addition of 5 mM NADH. A proton gradient was built up via the electron transport chain using NADH, thereby energizing the vesicles. At timed intervals, 0.1-ml samples were withdrawn and filtered through 0.2-μm-pore-size cellulose nitrate filters (Whatman, Maidstone, England). Filters were immediately washed with 5 ml of reaction buffer to which 10 mM $ZnCl_2$ and 10 mM $MgCl_2$ were added (final pH 6.3). The radioactivity of the filters was quantified using a Tri-carb 2100TR liquid scintillation counter (Packard, Meriden, Conn., USA). A background value was determined by performing the assays without NADH. This value was subtracted from the values for the corresponding time points of the NADH-energized assays.

FieF purification

FieF was purified using Strep-TagII technology (IBA , Göttingen, Germany). *fieF* was expressed from plasmid pASK-IBA3 in *E. coli* strain BL21 cells (Stratagene Europe, Amsterdam, The Netherlands). Cells were cultivated overnight at 30°C in LB-medium, diluted 1:100 into 1 l of fresh TB-medium and cultivated with shaking at 30°C up to an optical density at 600 nm of 1.5. Expression of *fieF* was induced with 200 μg AHT/l , and incubation continued for 3 h. Cells were harvested by centrifugation (7,650×*g*, 4°C, 15 min). suspended in 30 ml buffer W (100 mM Tris–HCl, pH 8.0) and broken twice via French press (SLM Aminco, SOPRA, Germany, 138 kPa) in the presence of protease inhibitor cocktail (Sigma-Aldrich, Deisenhofen, Germany) and DNaseI (10 g/l). Debris was removed by centrifugation (23,400×*g*, 15 min, 4°C) and the membrane fraction was isolated by ultracentrifugation (100,000×*g*, 2 h, 4°C). The membrane pellet was suspended in buffer W to a final protein concentration of 5 g/l. FieF was solubilized with 1 mg *n*-dodecyl maltoside per 1 mg membrane protein and residual membrane fragments were removed by ultracentrifugation (100,000×*g*, 30 min, 4°C). The resulting solubilized protein fraction (100 mg total protein) was applied to a Strep-Tactin-sepharose affinity chromatography column (bed volume 3 ml) that was washed with 30 ml of buffer W containing 0.05 g/l *n*-dodecyl maltoside. Finally, FieF was eluted with 100 mM Tris–HCl buffer (pH 8.0) containing 2.5 mM desthiobiotin and 0.05 g *n*-dodecyl maltoside/l.

Reconstitution of FieF in proteoliposomes

FieF was reconstituted according to the procedure published recently (Chao and Fu 2004a) for ZitB. Fifty mg chloroformic phospholipid extract from *E. coli* (Avanti Polar Lipids, Alabaster, Ala., USA) was dried and rehydrated in 1 ml bisTris buffer (20 mM, pH 6.8, buffer A) under vortexing for 1 min and subsequent ultrasonic treatment at 40°C for 5 min. An aliquot of the phospholipid suspension (corresponding to12.5 mg phospholipid) was dissolved in buffer A with octylglycoside (1% w/v final detergent concentration), mixed with a dilution of the purified FieF transporter (0.25 mg/ml), dissolved in the same buffer with 1% (w/v) octylglycoside and incubated for 20 min at room temperature. The detergent excess of the mixture was adsorbed on an Amberlite XAD-16 column ($d = 2$ cm, $h = 7$ cm, bed volume 6 ml, equilibrated with buffer A and collected in fractions of 2 ml (HighLoad System, Amersham Biosciences, Freiburg, Germany). The proteoliposomes formed were eluted in the void volume. Turbid fractions were combined, diluted to 20 ml with buffer A and sedimented by centrifugation for 90 min at 125,000×*g* (Ti 60, L8-70M Ultracentrifuge, Beckman). The sediment was resuspended in buffer A (400 μl) and 40 μl of a 2 mM PhenGreen-SK solution (Molecular Probes, Ore., USA) were added. The mixture was sonicated at 40°C for 10 s and subjected to one freeze–thaw cycle in liquid nitrogen. The free dye was separated from the liposome-encapsulated dye by passage over a desalting column (PD-10, Amersham Sciences, Freiburg, Germany), preequilibrated with Tris/HCl buffer (20 mM, pH 7.8, buffer B, measurement buffer). The turbid and stained fractions were combined, and centrifuged for 90 min at 125,000×g (Ti 70, L8-70M Ultracentrifuge, Beckman Coulter, Krefeld, Germany).

Fluorescence measurement of iron transport

PhenGreen-SK fluorescence was measured with excitation at 500 nm and emission at 530 nm on a F-2000 Fluorescence Spectrometer (Hitachi, Tokyo, Japan). PhenGreen-SK is membrane-impermeable and is quenched by addition of Fe^{2+} ions (Esposito et al. 2002; Shingles et al. 2002). An iron solution (ammonium iron-II-sulfate, Alfa Aesar, Karlsruhe, Germany) at a concentration of 20 mM was prepared in buffer B and stabilized by ascorbic acid (1% w/v) to prevent transition to the ferric oxidation state. Fe^{2+}-concentrations (final concentrations) in the range of 0.1–10 μM were made from this solution in buffer B. Fifty μl proteoliposomes with encapsulated fluorescence dye was diluted in buffer B (2 ml final volume) and preequlibrated under stirring until the baseline remained stable. The reaction was started by addition of Fe^{2+} and fluorescence measured for 10 min.

Immunoblotting

Protein samples were separated on SDS-polyacrylamide gels, blotted (SemiDry-Blot; Biometra, Göttingen, Germany) onto a polyvinylidene difluoride membrane and developed using a chromogenic substrate as described previously (Lee et al. 2002).

Miscellaneous

Standard molecular genetic techniques were used (Sambrook 1989). PCR was carried out in the presence of *Pwo* or *Taq* DNA polymerase (Roche, Mannheim, Fermentas, St. Leon-Rot, Germany). Plasmid pASK-IBA3, Strep-Tactin-sepharose and AHT were from IBA (IBA GmbH, Göttingen, Germany). *β*-Galactosidase activity in permeabilized cells was determined as published previously (Miller 1992; Grass et al. 2001b).

Results

Gene expression of *fieF* is iron dependent but Fur is probably not involved

If FieF is involved in iron homoeostasis in *E. coli*, it should be preferentially expressed at high iron concentrations. Transcription of the Φ(*fieF-lacZ*) operon fusion on the chromosome of *E. coli* strain GG193 (Grass et al. 2001a) was indeed induced by iron in a concentration-dependent manner (Fig. 1). Up to a concentration of 10 μM iron in the medium, the Φ(*fieF-lacZ*) operon fusion showed a steep increase in ß-galactosidase activity, but a further increase in iron concentration did not lead to a significant increase in *fieF* expression. Mn(II) did not induce transcription above basal level nor did hydrogen peroxide or paraquat (data not shown).

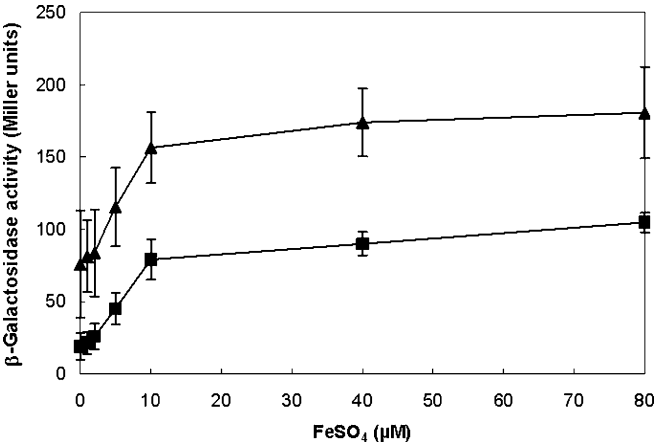

Fig. 1 Iron-dependent induction of *fieF* in *Escherichia coli*. Cells of *E. coli* strains containing a Φ(*fieF-lacZ*) operon fusion on the bacterial chromosome were grown overnight in LB broth, diluted 1:400 into minimal medium, grown overnight and diluted 50-fold into fresh minimal medium. $FeSO_4$ and ascorbate (1 mM final concentration) were added after 2 h of growth and incubation was continued for 2 h with shaking at 37°C. The *β*-galactosidase activities of strains GG193(Δ*lacZYA*::*kan* Φ(*fieF-lacZ*)) (*squares*) and GG203(Δ*fur*::*cat* Δ*lacZYA*::*kan* Φ(*fieF-lacZ*)) (*triangles*) were determined as Miller units. Experiments were carried out in triplicate and the average and standard deviation calculated

The ferric iron uptake regulator, Fur, is an important component of iron homeostasis in *E. coli* and other bacteria (Andrews et al. 2003; Pojl et al. 2003). Deletion of *fur* did not alter the expression profile of Φ(*fieF-lacZ*) per se (Fig. 1), i.e. the slope of induction remained very similar. Thus, Fur is neither an activator nor a repressor of iron-dependent transcription of *fieF*. Moreover, no putative Fur binding site could be found upstream of *fieF* making it unlikely that Fur is directly involved in *fieF*-regulation (data not shown). However, the basic expression of *fieF* without additional iron in the medium was elevated in the absence of Fur, and so was *fieF* expression at any given iron concentration (Fig. 1). Since Fur mostly represses iron uptake genes (Andrews et al. 2003), the absence of Fur should lead to increased iron uptake resulting in elevated cellular free iron concentration which, as a consequence, could create the need for expression of iron detoxification systems.

A double deletion of *fieF* and *fur* results in decreased iron tolerance

If FieF is involved in iron detoxification, its deletion should lead to decreased iron tolerance. However, the growth yield of a Δ*fieF* deletion strain in time-course growth experiments was similar to that of the wild-type (Fig. 2a, b), even in the presence of iron. A single deletion of *fur* led to a decrease in growth yield regardless whether the cells were grown in the presence of iron, EDTA or without additive (Fig. 2c). However, addition of iron resulted in a shorter lag phase and a slightly increased final growth yield. The Δ*fieF* Δ*fur* double

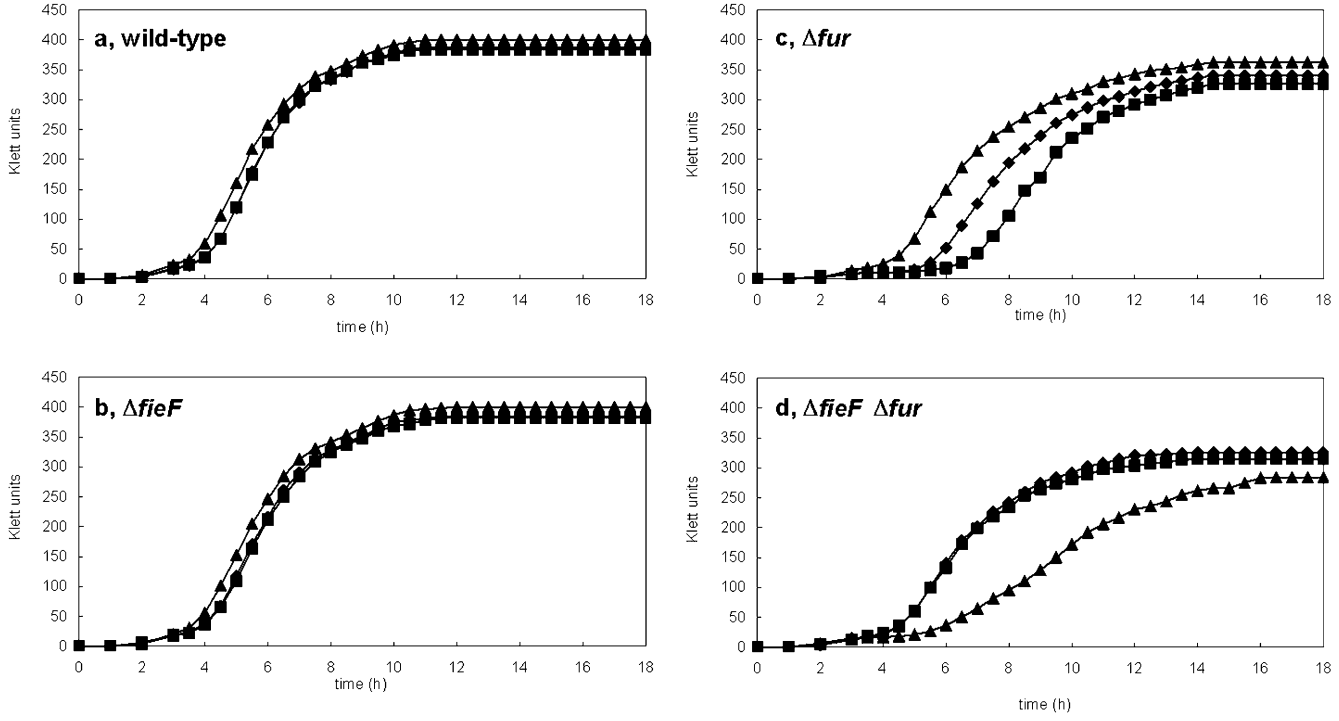

Fig. 2 Growth of *E. coli* strains in the presence of iron or EDTA. Overnight cultures grown in LB medium were diluted 1:400 into fresh LB medium and grown for 2 h to early exponential phase. Cells were diluted 1:400 into fresh LB medium containing 1 mM ascorbate with 25 μM EDTA (*filled diamonds*), 50 μM $FeSO_4$ (*filled triangles*) or no additives (*filled squares*) and growth was monitored as turbidity in Klett units from *E. coli* strains W3110 (wild-type, **a**), GG196(Δ*fieF*::*cat*) (**b**), GG199(Δ*fur*::*cat*) (**c**) and GG200(Δ*fieF*::*cat* Δ*fur*) (**d**). Experiments were done in triplicate and the average is shown

deletion strain also exhibited a decrease in final growth yield (Fig. 2d). Yet, the lowest growth yield was observed in the presence of iron. In the presence of iron, the lag-phase was prolonged and stationary phase was reached 4 h later than without added iron. Thus, FieF was important for the growth of *E. coli* cells when the Fur regulator was absent and iron was added.

Expression of *fieF in trans* restores iron tolerance of a Δ*fieF* Δ*fur* double deletion strain

If FieF detoxifies iron by efflux, its expression in *E. coli* should lead to decreased iron accumulation within the cells. In order to characterize FieF-mediated metal transport, *fieF* was cloned into the inducible expression vector pASK-IBA3, creating the plasmid pFieF. Expression of pFieF in *E. coli* wild-type strain W3110 or in either of the single gene deletion strains (Δ*fieF* or Δ*fur*) did not result in increased metal tolerance (data not shown). However, *in trans* expression of *fieF* in the Δ*fieF* Δ*fur* double mutant strain resulted in enhanced growth in complex medium (Fig. 3) compared to the control strain that only harbored the vector plasmid. In this control strain, addition of 100 μM EDTA increased the growth yield, while addition of 100 μM Fe(II) decreased it (Fig. 3), similar to the results obtained with the plasmid-free double mutant strain (Fig. 2). However, expression of FieF *in trans* in the double mutant strain led to cells that were no longer stimulated by the addition of EDTA, and no longer inhibited by the addition of Fe(II). Thus, *in trans* expression of FieF counteracted the toxic effect of iron on cells of the Δ*fieF* Δ*fur* double deletion strain.

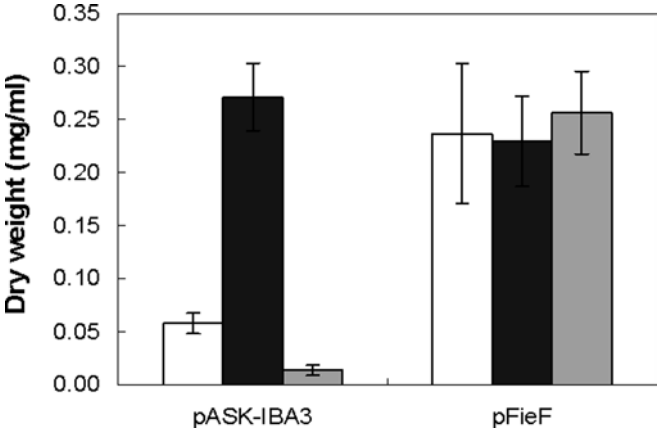

Fig. 3 Complementation of *fieF in trans*. *E. coli* strain GG200(Δ*fieF*::*cat* Δ*fur*) with *fieF* on plasmid pASK-IBA3 was grown for 6 h as described in Fig. 2. Anhydrotetracycline (200 μg/l) was added to induce *fieF* from a *tetAp* with no additives (*white bars*), 100 μM EDTA (*black bars*) or 100 μM $FeSO_4$ (*gray bars*). Experiments were done in quadruple and the average and standard deviation calculated

Expression of *fieF in trans* leads to decreased accumulation of ^{55}Fe in an *E. coli* Δ*fieF* deletion strain

Iron uptake experiments were done by the filtration assay, using intact cells of the Δ*fieF* single mutant strain complemented *in trans* with FieF. The Δ*fieF* Δ*fur* double deletion strain was not used for this purpose in order to avoid possible interference with uptake systems. These systems are under negative control of Fur and are turned on constitutively when Fur is absent (Andrews et al. 2003) while cellular iron-storage proteins are additionally negatively regulated in the absence of Fur by the small regulatory mRNA *ryhB* (Masse and Gottesman 2002).

When levels of cell-associated iron in *E. coli* strain GG196(Δ*fieF*::*cat*), with or without expressed *fieF*, were compared, cells harboring plasmid pFieF accumulated about half as much iron as their respective control cells (Fig. 4). Therefore, FieF is part of the cellular defense against excess iron since expression of FieF led to decreased accumulation of iron by *E. coli* cells.

Increased iron uptake does not result in elevated iron concentration in *E. coli* when *fieF* is expressed

Like iron, gallium forms trivalent cations. However, Ga(III) is soluble and leads to increased iron uptake into *E. coli* cells (Hubbard et al. 1986). If FieF functions as an iron-efflux transporter, addition of gallium would increase the amount of iron accumulated only in strains not expressing *fieF*.

Comparison of cell-associated iron levels in *E. coli* strain GG196(Δ*fieF*::*cat*), with or without expressed *fieF in trans*, showed that the total accumulation of iron into cells harboring plasmid pFieF was significantly lower, about 50% that of the control, and that the total accumulation of iron was nearly unaffected by various gallium concentrations (Table 2). In contrast, the total iron concentration increased with increasing Ga(III) concentrations, reaching a maximum at 25 μM, in the control strain lacking FieF. At this Ga(III) concentration, the concentration of iron taken up was more than fourfold higher than that of the *fieF* expressing strain (Table 2). This suggests that FieF might counteract increased cellular iron concentrations probably by efflux of iron from the cytoplasm of *E. coli*.

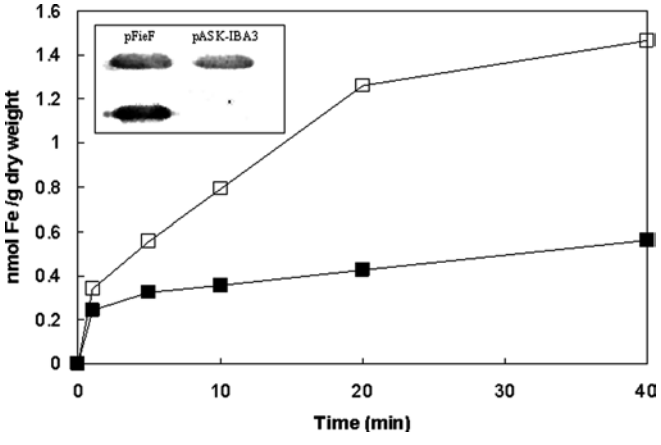

Fig. 4 ^{55}Fe uptake by cells of *E. coli* GG196 (Δ*fieF*::*cat*) pFieF expressing *fieF*. Cells of *E. coli* strains GG196(Δ*fieF*::*cat*) pFieF (*closed squares*) and GG196(Δ*fieF*::*cat*) pASK-IBA3 (*open squares*) were grown in Tris-buffered minimal medium to an turbidity of 60 Klett and *fieF* expression was induced with 200 μg AHT per liter. After growth for 30 min, $FeSO_4$ labeled with $^{55}FeCl_3$ was added to a final concentration of 5 μM. Ascorbate (1 mM final concentration) was added in order to reduce the iron. The cells were incubated at 37°C with shaking and 0.5-ml aliquots were filtered through nitrocellulose membranes (0.45 μm) at various times and immediately washed with 5 ml of 0.1 mM LiCl. Radioactivity was measured using a liquid scintillation counter and the dry weight was determined from the optical density with a calibration curve. Shown is the average of four independent experiments. *Insert* Western blot of StreptagII-labeled FieF proteins expressed from plasmid pFieF in *E. coli* (10 μg total protein each):*right* vector control, *left* FieF (*bottom*). An unspecific signal appearing in both lanes was used as loading control (*top*)

FieF enhances ^{65}Zn(II) accumulation in everted membrane vesicles of *E. coli* strain GR362 that are energized by NADH

Attempts to measure direct transport of ^{55}Fe in everted (inside-out) membrane vesicles derived from *E. coli* strain GG196(Δ*fieF*::*cat*) expressing *fieF* from a plasmid were not successful. Therefore, everted membrane vesicles were prepared from the Zn(II)-sensitive *E. coli* strain GR362(Δ*zntA*::*kan* Δ*zntB*::*cat* Δ*zitB* Δ*zupT* Δ*znuABC*), transformed with pFieF or the control plasmid. FieF-containing vesicles exhibited increased Zn(II) uptake in response to NADH addition, while vesicles from cells carrying the control plasmid did not (Fig. 5). No active transport of ^{65}Zn(II) was detected in vesicles without added NADH, regardless of the presence of FieF (data not shown), indicating that a proton gradient is necessary for FieF activity. The concentration of intravesicular Zn(II) achieved by NADH- and FieF-dependent uptake was estimated to be 1 nmol mg^{-1} membrane protein.

Table 2 Iron accumulation in *E. coli* in the presence of $GaCl_3$. Cultures were grown and iron content measured as described in Fig. 4 except that $GaCl_3$ was added at $t=0$. Total iron accumulation was measured after 1 h by the filtration method. Experiments were performed in triplicate and the average with the standard deviation is shown

$GaCl_3$ (μM)	Iron accumulated (μmol Fe/g dry weight)	
	GG196(Δ*fieF*::*cat*) pASK-IBA3	GG196(Δ*fieF*::*cat*) pFieF
0	1.206 ± 0.17	0.570 ± 0.03
5	1.518 ± 0.2	0.630 ± 0.14
25	2.508 ± 0.49	0.582 ± 0.08
100	1.902 ± 0.53	0.828 ± 0.35
1000	1.716 ± 0.3	0.600 ± 0.06

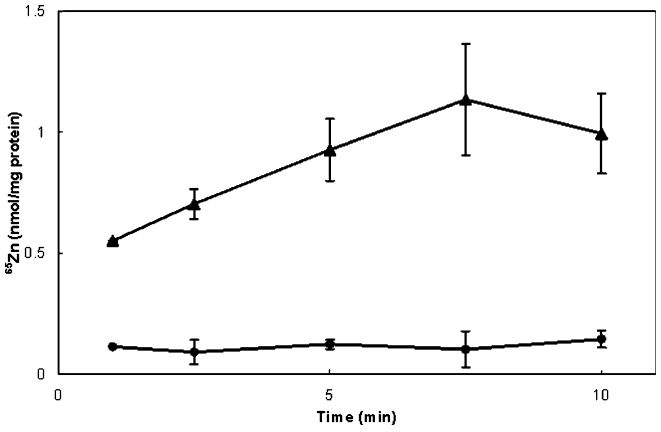

Fig. 5 NADH-driven Zn(II) accumulation in everted membrane vesicles. ^{65}Zn(II) accumulation into everted membrane vesicles prepared from strain GR362(Δ*zntA*::*kan* Δ*zntB*::*cat* Δ*zitB* Δ*zupT* Δ*znuABC*) carrying the plasmids pFieF (*triangles*) or pASK-IBA3 (*circles*) was measured using a liquid scintillation counter. Membranes (0.2 mg protein ml^{-1}) were energized with 5 mM NADH at time point 0. Background values from identical, but non-energized, assays have been subtracted from the plotted values. Assays were performed in triplicate, with the standard deviation represented by the *error bars*

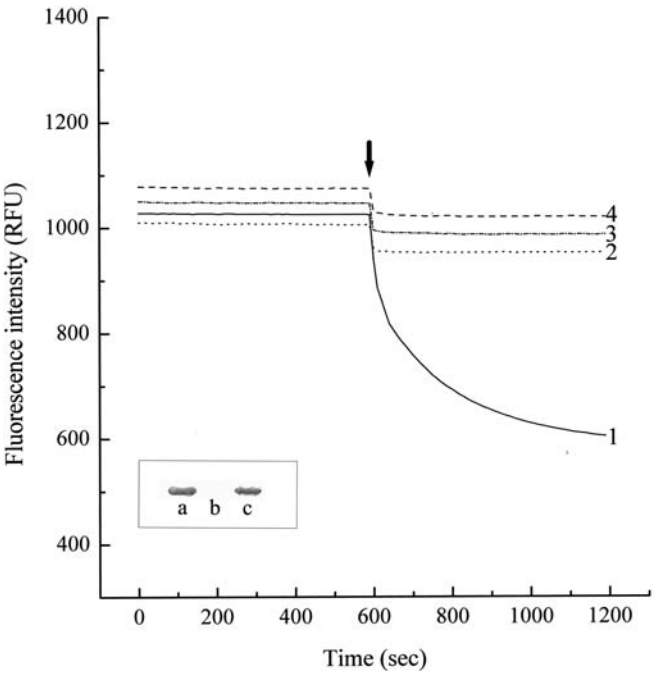

Fig. 6 Fluorescence quenching of PhenGreen SK by FieF-mediated iron transport in reconstituted in proteoliposomes. Transport of ferrous iron was measured using a fluorescence spectrophotometer (λ_{Ex} 500 nm, λ_{Em} 530 nm) under stirring for 10 min (final volume 2 ml, buffer B). Fifty microliters of proteoliposomes (corresponding to 28 μg reconstituted FieF) with the encapsulated dye PhenGreen-SK were preequilibrated (*dotted line*) for 10 min. The transport reaction was started by the addition of 1 μM Fe(II) (line 1, *arrow*) or not (line 2) and quenching of fluorescence dye by iron was continuously measured over 10 min. Additionally, liposomes without FieF were treated accordingly (lines 3, 4). *Insert* Western-blot of FieF(*a*) purified from *E. coli*, (*b*) in liposomes prior to FieF-reconstitution and (*c*) proteoliposomes after FieF-reconstitution

Functional reconstitution of FieF

FieF was reconstituted in proteoliposomes for in vitro measurement of FieF-mediated iron transport. Since our studies on Zn(II) transport by FieF in everted membrane vesicles suggested that the proton gradient energizes Zn(II)/proton antiport, the interior of the liposomes was set at pH 6.8 and the exterior at 7.8. Thus, proton antiport would transport Fe(II) into the proteoliposomes and quench PhenGreen-SK fluorescence. The proteoliposomes were stable since the fluorescence did not change for at least 10 min under our experimental conditions without added iron (Fig. 6). The presence of FieF in proteoliposomes was confirmed by Western blotting (Fig. 6, insert). Addition of ferrous iron quenched PhenGreen-SK fluorescence in a time-dependent manner indicative of proton-dependent transport of ferrous iron by FieF (Fig. 6). Vesicles without FieF did not change fluorescence upon addition of iron (data not shown). Thus, FieF is probably a cytoplasmic membrane iron-efflux transporter utilizing the proton gradient generated by the respiratory chain.

Discussion

The *fieF* gene from *E. coli* encodes a protein that belongs to the CDF family, which comprises membrane-bound divalent heavy-metal cation transporters from all three domains of life (Nies 2003). Direct transport dependency on the proton- or potassium-gradient was recently demonstrated for the zinc-efflux CDF protein CzcD from *Bacillus subtilis* (Guffanti et al. 2002). Data from that study indicated that the double Zn(II) efflux mutant *E. coli* GR362 possesses a Zn(II)/proton antiporter as part of its remaining Zn(II) efflux capacity and that this antiporter uses K^+ poorly, relative to protons, under the conditions tested (Guffanti et al. 2002).

Previously FieF was identified as a part of the zinc regulon in *E. coli*, but even though *fieF* expression was induced by zinc up to a similar level observed for iron in this study, its role remained obscure (Grass et al. 2001a). In the present study, it was demonstrated that *fieF* expression alleviated iron toxicity in a Δ*fur* strain. Furthermore, transport assays clearly showed that FieF is able to transport Zn(II) in a proton-dependent manner, suggesting that FieF is probably the second Zn(II)/proton antiporter postulated by Guffanti et al. (2002).

In the light of the observed FieF-dependent phenotype, the expression profile of *fieF* appears odd: both zinc and iron induce *fieF* (*yiiP*), while other metal cations do not (Grass et al. 2001a). Conversely, expression of *zitB* but not *fieF* (*yiiP*) in zinc-sensitive *E. coli* strain GG48 restored zinc resistance. Thus, FieF is probably not involved in zinc homeostasis in *E. coli*, in contrast to ZitB, the other CDF protein from *E. coli* (Grass et al. 2001a) which probably is a zinc-specific transporter

(Chao and Fu 2004a). By contrast, FieF (YiiP) was shown to bind Zn(II), Cd(II) or Hg(II) in vitro to a set of non-competitive binding sites within the polypeptide (Chao and Fu 2004b). It may be that a cytoplasmic metal chaperone delivers the metal cations to the respective CDF transporter in vivo and thereby determines their substrate specificity. However, as long as the mechanisms of *fieF* and *zitB* regulation are unknown, including the respective regulators of gene expression, the contradictory data on FieF cannot be resolved.

Using FieF reconstituted in proteoliposomes, we could demonstrate that FieF mediated iron transport in antiport with protons. This is only the second report that a CDF protein was reconstituted in proteoliposomes and proton-dependent transport demonstrated with both ZitB and FieF coming from *E. coli* (Chao and Fu 2004a). The reconstitution results support our notion that FieF is indeed an iron-efflux transporter.

Moreover, additional but indirect results support the idea that FieF is an iron-efflux pump: (1) expression of FieF led to decreased accumulation of iron in *E. coli* cells, (2) FieF was needed for iron detoxification in Δ*fur* cells that were forced to accumulate too much iron and, (3) transcription of *fieF* was induced by increasing iron concentrations. Furthermore, the proton-dependency of FieF-mediated transport is supported by FieF being able to mediate increased Zn(II) uptake into everted membrane vesicles in the presence of a NADH-energized proton gradient over the cytoplasmic membrane.

With Fe(II) and Mn(II) now included (Delhaize et al. 2003), the substrate spectrum of CDF proteins comprises these two cations in addition to Co(II), Ni(II), Zn(II) and Cd(II). The ionic radius of Co(II) (72 pm) is the smallest of the latter four while Cd(II) (97 pm) is the largest (Weast 1984). Iron and manganese cations, with ionic radii of 76 and 80 pm respectively, fit well into the range of the size of the four previously known CDF substrates, but two divalent cations, Mg(II) (65 pm) and Ca(II) (99 pm), that are major bio-elements do not (Weast 1984). This may point to an effective strategy for how cells deal with divalent cations: quickly and without too much discrimination accumulate them all, sort out the possibly dangerous transition metals having a size of between 72 and 97 pm radius with a filter that refuses magnesium and calcium, and then export those transition metals if their amount is in surplus.

In contrast to the yeast mitochondrial CDF proteins that are implicated in iron uptake (Li and Kaplan 1997; Lange et al. 1999), FieF does not possess histidine-rich motifs with a potential function in metal binding. Histidine-rich metal-binding motifs can often be identified in zinc transporters (Paulsen and Saier 1997; Eng et al. 1998). However, while these motifs were important for function in the Cd(II)-, Zn(II)-, Co(II)-translocating CDF transporters from *Ralstonia metallidurans* CH34 (Anton et al. 2004), they were not necessary for function in zinc-translocating ZitB (Lee et al. 2002). Specific iron-binding sites in transporters remain elusive at this time. In the yeast ferric-iron-uptake transporter FTR1, one of its RExxE-motifs was found to be necessary for iron transport (Stearman et al. 1996), and in the sensor kinase PmrB from *Salmonella*, a similar motif might be necessary for iron sensing (Wosten et al. 2000). However, these ExxE-type motifs are probably responsible for ferric rather than for ferrous iron binding. The single ExxE motif found in FieF is $EE_{214}RQE$ and is located in the large cytoplasmic C-terminus. Since a similar motif ($NE_{211}LLE$) is present in the zinc transporter ZitB from *E. coli* (Grass et al. 2001a; Lee et al. 2002) but not in MamB, a putative iron-translocating CDF protein required for magnetosome synthesis in *Magnetospirillum gryphiswaldense* (Grunberg et al. 2001), this motif might not constitute an iron-specific binding site in CDF proteins. Moreover, all CDF proteins characterized so far are Me(II) transporters; therefore, the substrate of FieF probably is ferrous rather than ferric iron.

Under physiological conditions, ferrous iron is easily oxidized to ferric iron. However, in the cytoplasm cells can counteract oxidation by providing reducing compounds, such as cysteine, glutathione, or ascorbate. It has been demonstrated that thiols and NAD(P)H are capable of transferring electrons to free iron in vitro (Rowley and Halliwell 1982a, b), leading to a mixed free Fe(II)/(III) pool within the cell. Within *E. coli*, the concentration of free, not protein- or heme-integrated, iron was determined by EPR analysis to be equivalent to 10–20 μM (Touati et al. 1995; Keyer and Imlay 1996). Efflux of ferrous iron, mediated by the integral membrane protein FieF, may alter the steady state of free iron and thus diminish cellular damage caused by metal-catalyzed generation of reactive oxygen species.

Deletion of the gene for the global iron homeostasis regulator Fur did not change the expression profile of *fieF*. No Fur-binding sites, comprising the three repeats of the hexameric motif GATAAT (Escolar et al. 1998), could be found upstream of *fieF*. Therefore it seems unlikely that Fur is directly involved in *fieF* expression. In *E. coli*, deletion of *fur* leads to derepression of iron-uptake systems (Hantke 1984). Counter-intuitively, this does not result in elevated, but reduced, total iron concentrations within the cytoplasm. Since genes for iron storage proteins and some iron-containing enzymes are also down-regulated, the total iron content is decreased (Abdul-Tehrani et al. 1999; McHugh et al. 2003). In light of the elevated basal expression of *fieF* in a Δ*fur* background, this model can be refined. With the iron-uptake systems all turned "on", a low capacity of iron storage in the cytoplasm would raise the "free" iron concentration (McHugh et al. 2003), resulting in *fieF* expression, and thus iron efflux, keeping a low steady-state iron concentration in the cell. Previously it could be demonstrated that a deletion of *fur* produced a 2.5-fold iron overload in *E. coli* and subsequently increased generation of hydroxyl radicals and DNA lesions (Nunoshiba et al. 1999). As a result, with *fur* deleted, lower concentrations of iron are needed to induce *fieF* than in the wild-type strain. This indicated that the influence of Fur on *fieF* expression is indirect by raising the intracellular free iron concentration. Since

the presence of cellular iron is connected to oxidative stress, the inducing signal for *fieF* expression could be reactive oxygen species or their effects on cellular components. However, hydrogen peroxide or paraquat did not induce *fieF* (data not shown). Moreover, zinc is also an inducer of *fieF*. Zinc is not redox active and thus does not directly promote oxidative stress. Copper, by contrast, is also able to cause metal-catalyzed generation of hydroxyl radicals but copper was not an inducer of *fieF* (*yiiP*) (Grass et al. 2001a). With Fur excluded as a direct regulator for *fieF* expression, other regulators of *fieF* must be responsible.

A paradigm of iron-research is that most microorganisms suffer iron deficiency, especially when they interact with higher eukaryotes. Mammals produce iron-binding proteins, such as lactoferrin or transferrin, in order to further reduce the free iron available for microorganisms (Bullen et al. 1978). However, in its natural habitat, the gut, *E. coli* might be predominantly exposed to iron in the ferrous form stabilized by anoxic conditions. Determination of available iron in the duodenal lumen of mice fed a standard diet yielded a concentration of up to 60 μM (Simpson and Peters 1990). Furthermore, *E. coli* might encounter high iron concentrations in soil after leaving the gut and this could easily induce *fieF* expression (Ratering and Schnell 2001). Thus, while iron is scarce most of the time, it might nevertheless be beneficial for *E. coli* to possess an iron-efflux system when iron is abundant.

In contrast to other transition metals, such as zinc or copper, there is a very high cellular demand for iron. Therefore, storage proteins in *E. coli* exist only for iron (ferritin, bacterioferritin and Dps) but not for copper or zinc. Iron storage proteins are down-regulated (through *ryhB*) only when *fur* is deleted, and the free iron concentration subsequently rises, causing oxidative stress (Masse and Gottesman 2002). While FieF is probably also active in a *fur* wild-type background, a FieF-dependent phenotype could only be observed when *fur* was additionally deleted. This is reminiscent of a recent functional analysis of ZitB, the second CDF protein from *E. coli*: a ZitB-dependent phenotype is only observed when the Zn(II) efflux ATPase ZntA is not present (Grass et al. 2001a).

FieF is the first characterized transporter responsible for iron detoxification by efflux. A quasi-efflux-transporter family is known in mammals, initially described by Abboud and Haile (2000). These ferroportin1 (also known as MTP1 or Ireg1) proteins transport iron from the epithelial cell layer of the duodenum, via the basolateral membrane, into the plasma, eventually reaching all of the body (Roy and Enns 2000), or mediate delivery from a mother to the embryo (Donovan et al. 2000). Yet the functional transport by ferroportin1 results in uptake of iron into the body rather than real efflux.

In *E. coli*, the level of iron uptake was reduced when FieF was present, and expression of *fieF in trans* rendered the Δ*fieF* Δ*fur* double mutant tolerant to elevated iron concentrations in the growth medium. This makes FieF an ideal candidate for studying iron homeostasis mediated by CDF proteins.

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