

The ferric iron uptake regulator (Fur) from the extreme acidophile *Acidithiobacillus ferrooxidans*

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Acidithiobacillus ferrooxidans is a Gram-negative bacterium that lives at pH 2 in high concentrations of soluble ferrous and ferric iron, making it an interesting model for understanding the biological mechanisms of bacterial iron uptake and homeostasis in extremely acid conditions. A candidate *fur*_{AF} (Ferric Uptake Regulator) gene was identified in the *A. ferrooxidans* ATCC 23270 genome. *Fur*_{AF} has significant sequence similarity, including conservation of functional motifs, to known Fur orthologues and exhibits cross-reactivity to *Escherichia coli* Fur antiserum. The *fur*_{AF} gene is able to complement *fur* deficiency in *E. coli* in an iron-responsive manner. *Fur*_{AF} is also able to bind specifically to *E. coli* Fur regulatory regions (Fur boxes) and to a candidate Fur box from *A. ferrooxidans*, as judged by electrophoretic mobility shift assays. *Fur*_{AF} represses gene expression from *E. coli* Fur-responsive promoters *fiu* and *fhuF* when expressed at high protein levels. However, it increases gene expression from these promoters at low concentrations and possibly from other Fur-regulated promoters involved in iron-responsive oxidative stress responses.

INTRODUCTION

Acidithiobacillus ferrooxidans is an important component in the consortia of micro-organisms used in biomining operations for metal recovery (Rawlings, 2002). It is an acidophilic, chemolithoautotrophic, γ -proteobacterium capable of aerobic growth via the oxidation of Fe(II) to Fe(III) or reduced inorganic sulfur to sulfuric acid. Direct consequences of this metabolism are the generation of a substantial extracellular proton concentration (pH 1–3) and the release of high concentrations of soluble metals, including iron; the latter can reach values as high as 6 g l⁻¹. Such values are in sharp contrast with the scarcity of soluble iron in near-neutral environments [10⁻¹⁷ M solubility limit for Fe(III) at pH 7].

Because of its redox properties, iron is an essential micronutrient for almost all organisms. However, given its limited bioavailability, most micro-organisms have

developed sophisticated mechanisms to scavenge this metal from their environment (Braun & Killmann, 1999). On the other hand, the redox properties of iron also turn it into a biological hazard when acquired in excess, and bacteria have developed tight intracellular homeostatic controls to balance iron uptake and storage against possible oxidative stress (Hantke & Braun, 2000; Touati, 2000).

We are interested in understanding the mechanisms by which *A. ferrooxidans* copes with iron uptake and homeostasis given the unusually abundant supply of soluble iron in its acid environment. Also, since it uses iron as an energy source, it has presumably developed novel regulatory mechanisms to balance iron requirements for assimilation versus those needed for energy production. Very little is known about these postulated regulatory mechanisms, although evidence is beginning to emerge that implicates the use of alternate electron transport pathways depending on the energy source being used (iron or sulfur) (Brasseur *et al.*, 2004) and a role for Fe(II) in the regulation of the rusticyanin operon, the expression of which is required for the oxidation of iron (Yarzabal *et al.*, 2004).

In most bacteria, the iron-responsive transcriptional regulator Fur is responsible for coordinating the expression of iron uptake and storage functions (Andrews *et al.*, 2003; Hantke, 2001). Although originally thought to be a typical apo-repressor of gene expression, activated in the presence of Fe(II) and binding to a conserved 19 bp sequence element

Abbreviations: EMSA, electrophoretic mobility shift assay; Q-PCR, quantitative PCR.

The GenBank/EMBL/DDBJ accession number for the sequence reported in this paper is AY465905.

A comparison of the predicted amino acid sequence of *A. ferrooxidans* *Fur*_{AF} with that of other micro-organisms, revealing the conservation of several motifs and structural features typical of the Fur family, is available with Supplementary Fig. S1 with the online version of this paper at <http://mic.sgmjournals.org>.

in the promoter region of its target genes (Escobar *et al.*, 1999), it is currently recognized to act as a global control switch adjusting substantial parts of bacterial metabolism in response to iron availability (Baichoo *et al.*, 2002; McHugh *et al.*, 2003; Ochsner *et al.*, 2002).

The function of Fur has been investigated in many micro-organisms, including the dissimilatory ferric iron-reducing bacterium *Shewanella oneidensis* strain M-1 (Thompson *et al.*, 2002). However, there are no studies regarding its role in *A. ferrooxidans* or in any other acidophilic, iron-oxidizing micro-organism. This deficiency motivated the investigations reported herein.

METHODS

Bioinformatic analysis. A candidate *fur* gene was identified by BLASTP and TBLASTX searches in the partial genome sequence of *A. ferrooxidans* ATCC 23270 deposited in the TIGR (www.tigr.org) and Integrated Genomics (www.integratedgenomics.com) databases. The predicted amino acid sequence of the candidate *fur* gene was then used to formulate a BLASTP (<http://www.ncbi.nlm.nih.gov/BLAST/>) search of the non-redundant database at NCBI and bidirectional best hits were accepted as evidence for putative homologues. The candidate gene and the translated protein were further characterized employing the following bioinformatic tools available online: primary structure similarities (www.ebi.ac.uk/ClustalW), secondary structure predictions (http://npsa-pbil.ibcp.fr/cgi-bin/npsa_automat.pl?page=npsa_multalin.html) and domain/motif recognition (www.ebi.ac.uk/InterProScan). The GenBank accession number of Fur_{AF} is AY481558.

Bacterial strains and growth conditions. Bacterial strains and plasmids are described in Table 1. *A. ferrooxidans* was grown in modified 9K basal salt media [per litre: 0.4 g (NH₄)₂SO₄, 0.1 g

K₂HPO₄, 0.4 g MgSO₄·7H₂O] containing iron (9K+Fe: 100–400 mM FeSO₄; adjusted to pH 1.6 with H₂SO₄) or sulfur (9K+S: 0.5% ethanol-sterilized powdered sulfur, adjusted to pH 3.5 with H₂SO₄) at 30 °C under aerobic conditions on a rotary shaker at 150 r.p.m. *E. coli* was grown at 37 °C in LB broth with the pertinent antibiotics: ampicillin (Amp, 100 µg ml⁻¹), streptomycin (Sm, 100 µg ml⁻¹) and/or kanamycin (Km, 30 µg ml⁻¹), as indicated in Table 1. LB medium was supplemented with 40 µM FeSO₄ to generate iron-plentiful growth conditions or with the iron chelator *o*-phenanthroline (200 µM) to achieve iron deprivation.

Growth curves. 9K+Fe or 9K+S media were inoculated with fresh mid-exponential-phase cells of *A. ferrooxidans* ATCC 23270 pre-grown in 9K+Fe or 9K+S, respectively, and incubated at 30 °C. Samples were removed daily and counted with a Petroff–Hausser counting chamber under a phase-contrast microscope at 400 × magnification. The number of cells per millilitre was plotted against the incubation time. All experiments were performed at least twice with two to three replicates per treatment.

Cell collection. *A. ferrooxidans* cultures, to be used for nucleic acid or protein purification, were centrifuged at 6000 g to remove solid sulfur or iron precipitates prior to cell harvest. The cell pellet was resuspended in 9K salt solution (ice cold solution in the case of RNA purification) for further washing. Washed cells were collected by centrifugation at 10 000 g for 10 min.

General DNA techniques and cloning procedures. DNA isolation and routine manipulations were carried out following standard protocols as described by Sambrook *et al.* (1989) or by the manufacturers of the reagents. Plasmid DNA was prepared with the Wizard Plasmid Miniprep Kit (Promega) or the QIAprep Spin Mini-kit (Qiagen). PCR products for cloning were amplified with proofreading DNA polymerase Elongase (Invitrogen) and were purified from agarose gels with the QiaEx DNA purification kit (Qiagen). Oligonucleotide primers used in this study are listed in Table 2. Each PCR reaction contained 10 ng template DNA, 0.5 µM required primers and 0.2 mM each deoxyribonucleotide in a volume of 25 µl of

Table 1. Bacterial strains and plasmids used in this study

Strain or plasmid	Relevant genotype/phenotype	Reference or source
<i>A. ferrooxidans</i>		
ATCC 23270	Type strain, genome sequenced by TIGR and Integrated Genomics	ATCC
<i>E. coli</i>		
MG1655	Type strain K12, F ⁻ <i>λ</i> ilvG <i>rfb50 rph1</i>	ATCC
H1780	<i>fur</i> , <i>fiu</i> :: <i>lacZ</i> , Sm ^r , Km ^r	Hantke (1987)
H1681	<i>fur</i> , <i>fhuF</i> :: <i>lacZ</i> , Sm ^r , Km ^r	Hantke (1987)
QC-1732	GC4468, F ⁻ Δ (<i>lacZYA-argF</i>)U169 <i>rpsL</i> Δ <i>fur</i>	Touati <i>et al.</i> (1995)
Plasmids		
pUC-18	Plasmid vector, Amp ^r	Gibco, BRL
pGEMT-Easy	Plasmid vector, Amp ^r	Promega
pMH15	<i>fur</i> from <i>E. coli</i> K-12	Hantke (1987)
pECH	<i>fur</i> from <i>E. coli</i> K-12 expressed from pGEMT-Easy <i>lacZ</i> promoter, Amp ^r natural RBS-ATG spacer of 10 nt (amplified with primer pair Fur5 and Fur6)	This study
pECL	<i>fur</i> from <i>E. coli</i> K-12 expressed from pGEMT-Easy <i>lacZ</i> promoter, Amp ^r RBS-ATG spacer of 11 nt (amplified with primer pair Fur7 and Fur6)	This study
pAFH	<i>fur</i> from <i>A. ferrooxidans</i> ATCC 23270 expressed from pGEMT-Easy <i>lacZ</i> promoter, Amp ^r RBS-ATG spacer of 10 nt (amplified with primer pair Fur3 and Fur2)	This study
pAFL	<i>fur</i> from <i>A. ferrooxidans</i> ATCC 23270 expressed from pGEMT-Easy <i>lacZ</i> promoter, Amp ^r RBS-ATG spacer of 11 nt (amplified with primer pair Fur1 and Fur2)	This study

1 × PCR buffer containing 1.5 mM MgCl₂ (Invitrogen). PCR conditions were as follows: initial denaturing step at 95 °C for 5 min followed by 28–30 amplification cycles (denaturation at 95 °C for 30 s, annealing at the appropriate temperature depending on the specific primer pairs for 30 s and elongation at 72 °C) and a final elongation step at 72 °C for 10 min.

The *A. ferrooxidans* ATCC 23270 529 bp *fur* coding region was amplified from chromosomal DNA preparations with primers Fur1 and Fur2 (Table 2). The primer oligonucleotide pair Fur5 and Fur6 (Table 2) was used to amplify the *E. coli* K-12 *fur* gene from plasmid pMH15. Forward primers Fur1, Fur3, Fur5 and Fur7 included a consensus Shine–Dalgarno sequence placed at 11 or 10 bp from the start codon in varying sequence contexts (Table 2). PCR products were purified by gel electrophoresis and cloned into the pGEMT-Easy plasmid at the *EcoRV* site generating plasmids pAFH, pAFL, pECH and pECL, respectively (Table 1). Ligation mixtures were transformed into *E. coli* DH5 α strain by electroporation and transformants were selected on LB plates containing the appropriate antibiotic. Clones selected for further analysis were validated by PCR analysis followed by DNA sequencing.

RNA preparation, RT-PCR and quantitative PCR. RNA was isolated from *E. coli* grown to an OD₆₀₀ of 0.5, as previously described by Aiba *et al.* (1981), and from 0.5 l *A. ferrooxidans* grown to mid-exponential phase in 9K basal salts medium in the presence of iron (100 and 200 mM) or sulfur (0.5%), as described previously by Guacucano *et al.* (2000). Contaminating DNA was removed using a High Pure RNA Isolation Kit (Roche). Reverse transcription and coupled PCR amplification (RT-PCR) using the appropriate primers (Table 2) and including controls (Guacucano *et al.*, 2000) were performed, respectively, with Superscript II reverse transcriptase (Gibco-BRL) and *Taq* Polymerase (Invitrogen), according to the manufacturer's recommendations. One microgram of total cellular RNA was used for each reaction.

Transcriptional expression levels of *fur* and control genes were estimated through real-time quantitative PCR (Q-PCR) on reversed transcribed cDNA. cDNA was prepared from 2–5 μ g total RNA utilizing random hexamers and Superscript II reverse transcriptase. Specific primers (Table 2) amplifying mean products of 300 bp were used to quantify *fur* and pertinent control gene transcripts. The Fast Start DNA Master SYBR Green I PCR kit (Roche Applied Biosystems) and the LightCycler System (Roche Applied Biosystems) were utilized according to the manufacturer's protocol. Quantifications were performed twice, with both independent total RNA and cDNA preparations, by the comparative threshold cycle method. The calculated threshold cycle (Ct) for each gene was normalized to Ct of the 16S rRNA gene (which has invariant expression under different growth conditions; Yarzabal *et al.*, 2004) amplified from the corresponding sample before calculating the fold change between growth conditions (S, Fe₁₀₀, Fe₂₀₀).

Complementation of *fur* mutations. Complementation was performed by transformation of *fur* mutant strains H1780 or H1681 (Hantke, 1987) with the constructs presented in Table 1 and was assessed by three methods: phenotypic analysis on MacConkey-lactose agar plates (Hantke, 1987), β -galactosidase activity determination (Miller, 1972) or survival to H₂O₂ challenge (Touati *et al.*, 1995).

β -Galactosidase activity was assayed by the ONPG assay as described by Miller (1972) using SDS and chloroform-permeabilized cells. β -Galactosidase activity was calculated in Miller units. For this purpose, freshly inoculated cultures were grown to OD₆₀₀ 0.4–0.5 in LB plus antibiotics and divided into two 15 ml replicate aliquots to be either supplemented with 40 μ M FeSO₄ or depleted of iron by addition of 200 μ M of the iron chelator *o*-phenanthroline. Treatments were for 3 h at 37 °C with agitation (200 r.p.m.) and were terminated by chilling on ice. Duplicate samples of each culture were assayed in at least three repetitions of each experiment.

Table 2. Primers used in this study

Primers	Sequence (5'–3')
<i>A. ferrooxidans</i>	
Fur1*†‡	TTAAGGAGACTCGAGCGAATGATCGACGAACG
Fur2*	ACCGTGACAACCTGGTGC
Fur3*‡	ACAGGACCTCGAGCGCATGATCGACGAACG
Fur4†	CACCATATGATCGTGGTGG
Igr1*§	GTCAAAAAGCACCAGAACC
Igr2*§	TCAAAAATGCCGAGTATCT
FB-MntH1§	CTCTAGAATAAACGGGAATCATTCTCTCTAGAG
FB-MntH2§	CTCTAGAGAGAATGATTCCCGTTTATTCTAGAG
<i>E. coli</i>	
Fur5*‡	ACAGGACCTCGAGCGCATGACTGATAAC
Fur6*	TATTTGCCTTTCGTGCGC
Fur7*‡	TTAAGGAGACTCGAGCGAATGACTGATAAC
FB-FhuF1§	CTCTAGAGATAATGATAACCAATATCTCTAGAG
FB-FhuF2§	CTCTAGAGATATTGGTTATCATTATCTCTAGAG
Vector	
pUC-2(2006972)	GTTTTCCCAGTCACGAC

*Oligonucleotides used for PCR amplification of inserts.

†Oligonucleotides used for Q-PCR.

‡Underlined features indicate RBS and start codon.

§Oligonucleotides used as probe in EMSA after annealing.

H₂O₂ challenge was performed as described by Touati *et al.* (1995), with minor modifications. LB-grown cells (OD₆₀₀ 0.4–0.5) were distributed into 5 ml aliquots, and treated with 0, 1, 5, 15, 30 mM H₂O₂. After 20 min incubation with shaking (200 r.p.m.) at 37 °C, treatment was stopped by chilling on ice and dilution into fresh LB. Dilutions of each treatment were immediately plated on LB agar and colonies were counted after overnight incubation at 37 °C. Killing by H₂O₂ was assessed as the percentage of surviving colonies compared with unchallenged cells. Experiments were performed twice with duplicate samples.

Construction of vectors expressing low concentrations of Fur. The *fur*_{AF} gene was cloned into pGEMT-Easy such that its transcription was controlled by the vector promoter but its translational control was exerted by a synthetic ribosome-binding sequence (RBS) introduced upstream of the ATG start site of *fur*_{AF} (Table 2). The new construct was termed pAFL (L, low expression). This sequence contains a translation initiation region with a predicted secondary structure twice as stable as that in the equivalent structure of pAFH (H, high expression) and this modification significantly reduces the efficiency of translation (de Smith & van Duin, 1990; Gross *et al.*, 1990; Hartz *et al.*, 1991). A similar construct containing Fur_{EC} under the control of the sequence that reduces translational efficiency was prepared and termed pECL.

Western blotting. Samples of *E. coli* cultures (1.5 ml) grown to OD₆₀₀ 0.5 in LB broth were centrifuged, suspended in sample loading buffer (100 µl) and heat denatured (10 min) in the presence of reducing agents (Sambrook *et al.*, 1989). Aliquots of 4–10 µl of the total protein extract were separated by SDS-PAGE in 15 or 18% acrylamide and transferred onto nitrocellulose filters by standard methods with a Bio-Rad blotting apparatus. The blotted proteins were subsequently screened using a polyclonal rabbit anti-Fur serum generated against Fur from *E. coli* (obtained from M. L. Vasil, University of Colorado Health Sciences Center). When necessary, antiserum was pre-adsorbed against *E. coli* H1780 crude extracts as described by Sambrook *et al.* (1989). Filters were blocked overnight at 4 °C with agitation in blocking solution [5% skimmed milk, 0.05% Triton X-100, Tris-buffered saline (TBS)], incubated 1 h with a 1:500 dilution of the primary antibody in TBS/Tween 20 (0.05%) and further incubated in a 1:15000 dilution of peroxidase-conjugated anti-rabbit immunoglobulin in TBS/Tween 20 (0.05%) for another hour. Immunoreactive proteins were detected using the Supersignal West Pico chemiluminescent substrate (Pierce). Pre-stained broad-range molecular mass protein standards from Bio-Rad were used. Protein concentrations were determined with Bio-Rad Protein Assay, using BSA as standard.

Immunoprecipitation. *E. coli* was grown to OD₆₀₀ 0.8 in LB broth and cells were collected by centrifugation at 12000 g. Pellets were washed with 25 mM Tris/HCl, pH 8.0, and suspended in 10 vols lysis buffer (50 mM glucose, 10 mM EDTA, 25 mM Tris/HCl, pH 8.0, 1 mM PMSF) containing 4 mg lysozyme ml⁻¹. After 30 min incubation on ice, cells were sonicated for 10 s per pulse (5 pulses alternating with 1 min incubation in ice). Following centrifugation for 10 min at 12000 g, the supernatant was diluted in 2 × radioimmunoprecipitation assay (RIPA) buffer [100 mM Tris/HCl, pH 8.0, 2 mg SDS ml⁻¹, 10 mg deoxycholate ml⁻¹, 300 mM NaCl, 2% (v/v) Nonidet P40, 2 mM PMSF] and incubated 5 min on ice by the method of Qi *et al.* (1999). The samples were treated with polyclonal rabbit anti-Fur_{EC} antibodies (dilution 1:500) for 2 h at 4 °C and immediately incubated with stirring for 1 h at 4 °C with 300 µl protein A-agarose (Invitrogen) pre-equilibrated in 1 × RIPA buffer. Immunoprecipitated proteins were released from the collected beads in 8 M urea and resuspended in gel loading buffer for subsequent electrophoresis in 15% polyacrylamide gels and Western blotting (20 µl per well).

Fur titration assay (FURTA). The Fur titration assay was performed according to Stojiljkovic *et al.* (1994). A pUC18-based randomly cloned genomic library from *A. ferrooxidans* ATCC 19859 (pGTF) containing *Sau*3A DNA fragments (averaging 1 kb in size) was electroporated into the *E. coli* strain H1717 (*fur*⁺, *fhuF*::*lacZ*, Table 1). A total of 3.1 × 10⁵ Amp^R clones were recovered on selective MacConkey indicator plates containing 40 µM FeSO₄. The cloning vector pGEMT-Easy and the *E. coli* Fur box upstream of the *fhuF* gene (FB-FhuF1+2, Table 2) were employed as negative and positive controls, respectively. Clones exhibiting red colour in MacConkey-Fe plates after 24 h incubation at 37 °C were isolated and further restreaked. Inserts were subsequently subjected to DNA sequencing.

Electrophoretic mobility shift assays (EMSA). Double-stranded DNA probes (33 bp), containing the well-characterized Fur box of the *fhuF* gene from *E. coli* and the putative Fur box of the *mntH* gene from *A. ferrooxidans*, were generated by annealing two perfectly complementary oligonucleotides designed after de Lorenzo *et al.* (1988) (Table 2). Equal parts of both oligonucleotides (50 pmol) were heated to 65 °C for 10 min and allowed to cool slowly to room temperature. The probe was end labelled with [³²P]ATP using T4 polynucleotide kinase (Invitrogen). Unincorporated nucleotides were removed through a Bio-Gel P10 Micro Bio-spin chromatography column (Bio-Rad). *E. coli* QC-1732 carrying pAFH and pECL was grown to OD₆₀₀ 0.8, harvested at 12000 g and suspended at 1/10 volume in gel mobility buffer [20 mM Tris/HCl, pH 8.0, 0.5% (v/v) glycerol, 1 mM PMSF, 2 µg aprotinin ml⁻¹] containing 100 µg lysozyme ml⁻¹. After 40 min in ice, the cell suspension was sonicated for 10 s per pulse (5 pulses alternating with 1 min incubations in ice). Following centrifugation for 10 min at 12000 g, the supernatant was stored at -20 °C until use.

EMSA assays were performed as described by de Lorenzo *et al.* (1988), with the following modifications: 80–100 µg total protein was equilibrated in 20 µl final volume of gel mobility shift buffer [20 mM Tris/HCl, pH 8.0, 40 mM KCl, 1 mM MgCl₂, 0.1 mM MnSO₄, 0.1 mg BSA ml⁻¹, 5% (v/v) glycerol]. The labelled probe (50–75 pM; 10000 c.p.m.) and non-specific competitor salmon sperm DNA (50 µg µl⁻¹) were added, and the reactions incubated for 10 min at 30 °C. A 50-fold excess of cold annealed oligonucleotide was used to challenge the labelled probe. In supershift experiments, the target DNA and a 1:500 dilution of Fur-specific antiserum were added to the reaction and incubated for an additional 5 min. Mixtures were resolved by non-denaturing polyacrylamide (4%, w/v) gel electrophoresis at 200 V for 1 h in Tris/acetate buffer at 4 °C. Retardation was examined by exposing the gel after drying to Kodak X-OMAT film (Sigma) or analysed with a PhosphorImager (Molecular Imagen FX Pro Plus, Bio-Rad).

RESULTS

Identification of the *fur* homologue in *A. ferrooxidans*

Examination of the partial genomic sequence of *A. ferrooxidans* ATCC 23270 revealed the presence of a candidate gene encoding a potential Fur protein with 60% amino acid sequence identity to the predicted Fur of *Alteromonas* sp. O-7 (gi: BAB13366) and 51% identity to the experimentally verified Fur of *E. coli* (gi: P06975). The nucleotide sequence of the candidate *fur*_{AF} and the amino acid sequence of its hypothetical protein product were deposited in GenBank (accession no. AY465905). The candidate Fur_{AF}

has a predicted length of 158 amino acids (molecular mass 17.9 kDa) and an isoelectric point of 5.0.

Comparison of the predicted amino acid sequence of Fur_{AF} with that of other micro-organisms revealed the conservation of several motifs and structural features typical of the Fur family (see Supplementary Fig. S1, available with the online version of this paper at <http://mic.sgmjournals.org>). Fur_{AF} exhibits a conserved helix–turn–helix motif near the N-terminus known to be involved in DNA binding in well-documented Fur proteins (Gonzalez de Perédo *et al.*, 2001; Holm *et al.*, 1994). It also presents conservation of all residues demonstrated or suggested to be involved in iron binding and structural zinc coordination (Adrait *et al.*, 1999; Bsai & Helmann, 1999; Coy *et al.*, 1994; Jacquamet *et al.*, 1998; Pohl *et al.*, 2003). On the basis of these similarities, it is proposed that Fur_{AF} is a member of the Fe-responsive subfamily of Fur regulators.

Inspection of the genome sequence of *A. ferrooxidans* around the candidate *fur*_{AF} gene revealed an ORF potentially encoding the putative outer-membrane protein OmlA, 66 bp upstream from the suggested start site of Fur_{AF}, but oriented in the opposite direction. The juxtaposition of this gene pair is conserved in several α - and β -Proteobacteria, including *Burkholderia*, *Ralstonia*, *Neisseria*, *Bordetella*, *Pseudomonas*, *Xylella* and *Xanthomonas* (Lowe *et al.*, 2001; Ochsner *et al.*, 1999). Although divergent genes frequently share common regulatory mechanisms (Escobar *et al.*, 1998), experimental evidence suggests that transcription of *Pseudomonas aeruginosa omlA–fur* is not co-regulated (Ochsner *et al.*, 1999). In addition, none of the bacteria where the *omlA–fur* context is conserved are capable of the otherwise frequently observed autoregulation of Fur (Loprasert *et al.*, 1999; Barton *et al.*, 1996; Thomas & Sparling, 1994).

Complementation of Fur deficiency in *E. coli* by Fur_{AF}

To determine if Fur_{AF} is a functional Fur homologue, complementation of *fur* deficiency in the *E. coli* reporter

strains H1780 and H1681 was evaluated as described by Hantke (1987). H1780 and H1681 (Table 1) each carry a chromosomally embedded *lacZ* gene fused to a Fur-dependent promoter such that, in the presence of a functional Fur and the co-repressor Fe(II), β -galactosidase synthesis is significantly inhibited. The candidate *fur*_{AF} from *A. ferrooxidans* was cloned into the expression vector pGEMT-Easy and the resulting plasmid was termed pAFH (Table 1). Similarly, the experimentally validated *E. coli fur* gene, *fur*_{EC}, was cloned into pGEMT-Easy and the resulting plasmid was termed pECH (Table 1). pAFH and pECH were each transformed into the *E. coli* reporter strains H1780 and H1681 and β -galactosidase activity determined in the presence or absence of iron (Fig. 1).

E. coli fur-host strains H1780 and H1681 express *fiu/fhuF–lacZ* constitutively in a manner unaffected by iron (200 Miller units). In both hosts, transformation with pAFH resulted in decreased β -galactosidase activity in the presence of iron. Addition of the iron chelator *o*-phenanthroline diminished this reduction. Similar results were obtained when the positive control pECH was introduced into H1780 and H1681. Under the conditions of the experiment, the activity of Fur_{AF} in *E. coli* is similar to that of Fur_{EC}, strongly suggesting that Fur_{AF} is a functional Fur homologue capable of iron-dependent repression.

Fur_{AF} binds to the *E. coli fhuF* Fur box

The ability of *A. ferrooxidans* Fur to bind the *E. coli fhuF* Fur box *in vitro* was investigated using EMSA (Fig. 2a). As expected, a whole-cell protein extract from the *E. coli* host strain QC-1732 (Δfur , Table 2) was unable to retard the radioactively labelled *E. coli fhuF* Fur box in an EMSA (Fig. 2a, lane 2). In contrast, proteins derived from the same host harbouring pAFH were able to shift the labelled *E. coli fhuF* Fur box (Fig. 2a, lane 3). Furthermore, the addition of anti-Fur antibodies to the reaction produced a supershift in migration, indicating that the shift reaction was caused specifically by the binding of Fur_{AF} to the *E. coli fhuF* Fur box (Fig. 2a, lane 4). Consistent with this interpretation, a complete loss of the shift was observed when excess

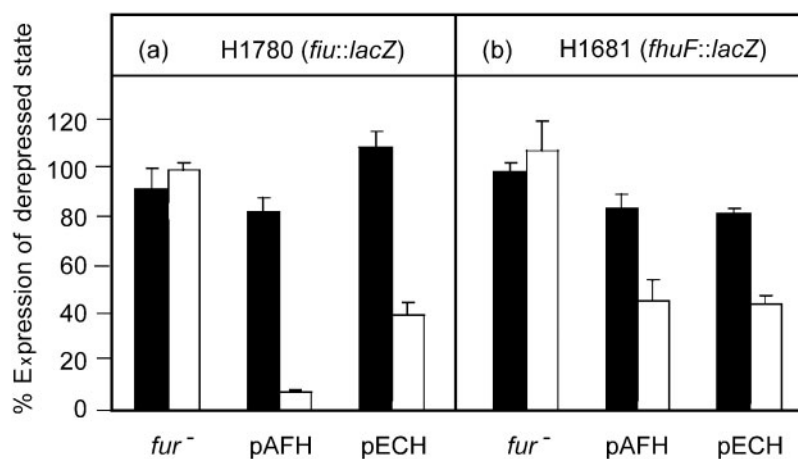


Fig. 1. Complementation of Fur repression activity by Fur derived from *A. ferrooxidans* in (a) *E. coli* H1780 *fur*⁻ and (b) *E. coli* H1681 *fur*⁻. Fur activity (mean of three experiments) was determined by β -galactosidase activity in Miller units (Miller, 1972) for cells grown in either the presence of 40 mM FeSO₄ (white columns) or 200 μM *o*-phenanthroline (black columns) and is reported as the percentage activity of *E. coli fur*⁻. pAFH, construct expressing *A. ferrooxidans* Fur; pECH, construct expressing *E. coli* Fur.

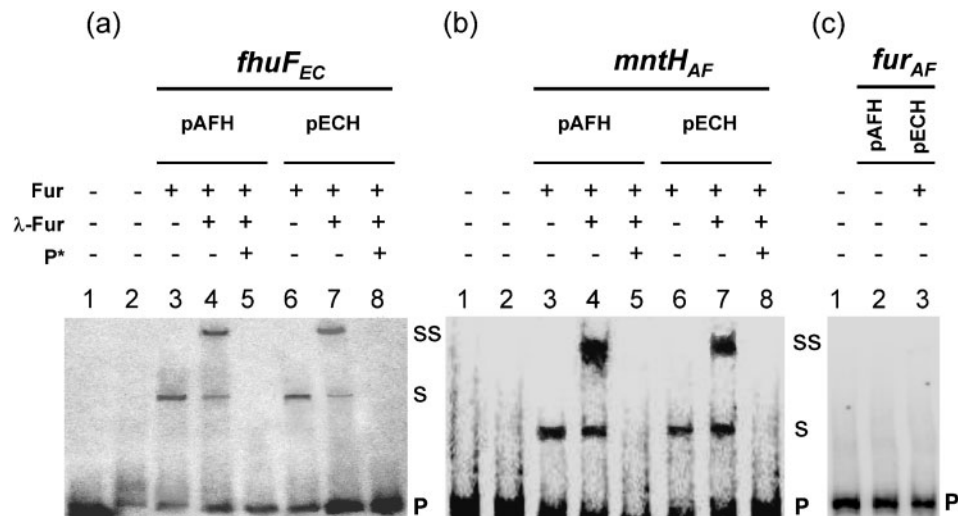


Fig. 2. Determination of the ability of Fur_{AF} to bind Fur boxes using the EMSA and supershift assay. The probe DNA Fur boxes were: (a) 33 bp oligonucleotide containing the *fhuF_{EC}* Fur box, (b) 33 bp oligonucleotide containing the *mntH_{AF}* Fur box and (c) 428 bp PCR product containing the *fur_{AF}* promoter region. Probe DNA was incubated with extracts from *E. coli* QC-1732 (Δfur) carrying construct pECH or construct pAFH. Abbreviations: S, shift; SS, supershift; P, probe DNA; P*, competing excess unlabelled probe DNA; λ -Fur, anti-Fur antibody; Fur, Fur protein in cell extract; +, addition; -, no addition.

unlabelled DNA probe was used to outcompete the labelled probe (Fig. 2a, lane 5). As a positive control, these reactions were repeated using proteins derived from *E. coli* QC-1732 transformed with pECH (Fig. 2a, lanes 6, 7 and 8), confirming that the homologous Fur_{EC} as well as the heterologous Fur_{AF} was capable of binding to the labelled *fhuF E. coli* Fur box.

Fur_{AF} binds to the *A. ferrooxidans mntH* Fur box

Having demonstrated that Fur_{AF} is able to recognize and bind to the heterologous *E. coli fhuF* Fur box *in vitro*, we investigated whether it could also recognize and bind a Fur box from *A. ferrooxidans* (Fig. 2b). A search for functional Fur box sequences in *A. ferrooxidans* was carried out by subjecting a randomly cloned genomic library of *A. ferrooxidans* to the Fur titration assay (Stojiljkovic *et al.*, 1994). A candidate target DNA fragment carrying the region upstream of the putative Mn(II)/Fe(II) transporter gene *mntH* (Makui *et al.*, 2000) was identified and selected for further study based on two lines of evidence: 1) homologues of this gene, including one in *E. coli*, have been shown to be regulated by upstream Fur boxes (Kehres *et al.*, 2000, 2002) and 2) alignment of the upstream regions of the respective *mntH* genes of *E. coli* and *A. ferrooxidans* reveals a 12 bp region of 100% identity (AATCATCTCG) that partially corresponds to the known Fur box of the *E. coli mntH* gene.

Proteins derived from *E. coli* QC-1732, transformed with either pAFH (Fig. 2b, lane 3) or pECH (Fig. 2b, lane 6), were able to shift the DNA probe containing the postulated *A. ferrooxidans mntH* Fur box. These reactions were

demonstrated to be Fur specific by supershifting in the presence of anti-Fur antibody (Fig. 2b, lanes 4 and 7) and by competition with cold probe DNA (Fig. 2b, lanes 5 and 8). No effect on the migration of the *mntH_{AF}* probe could be detected when utilizing the host strain QC-1732 protein extracts (Fig. 2b, lane 2) that do not contain Fur.

Given the frequently observed capacity of Fur to affect its own expression, we also evaluated the migration shift of a probe carrying the *olmA-fur_{AF}* intergenic region, containing the divergent promoters for both genes. No retardation could be detected using either the pAFH or the pECH protein extracts (Fig. 2c, lanes 2 and 3). The inability of Fur_{AF} to shift its own promoter region *in vitro* is in agreement with previously reported evidence indicating that none of the bacteria sharing the conserved gene context *olmA-fur* are capable of autoregulation (Barton *et al.*, 1996; Loprasert *et al.*, 1999; Thomas & Sparling, 1994).

Expression of Fur_{AF} in *A. ferrooxidans* in different growth conditions

Fur_{AF} was detected by Western blotting in whole-cell extracts of *A. ferrooxidans* grown on either S⁰ or on standard 9K+Fe media [100 mM Fe(II); Fig. 3b, lanes 2 and 3]. However, increasing the Fe(II) concentration to 200 mM significantly decreased Fur_{AF} (Fig. 3b, lane 4) and growth in 400 mM Fe(II) almost abolished it (Fig. 3b, lane 5). This decrease was not paralleled by a general reduction in total cell protein yield or by an observable change in the distribution of molecular masses of the proteins as determined by SDS-PAGE (data not shown).

Quantification of Fe(II) by the *o*-phenanthroline method in

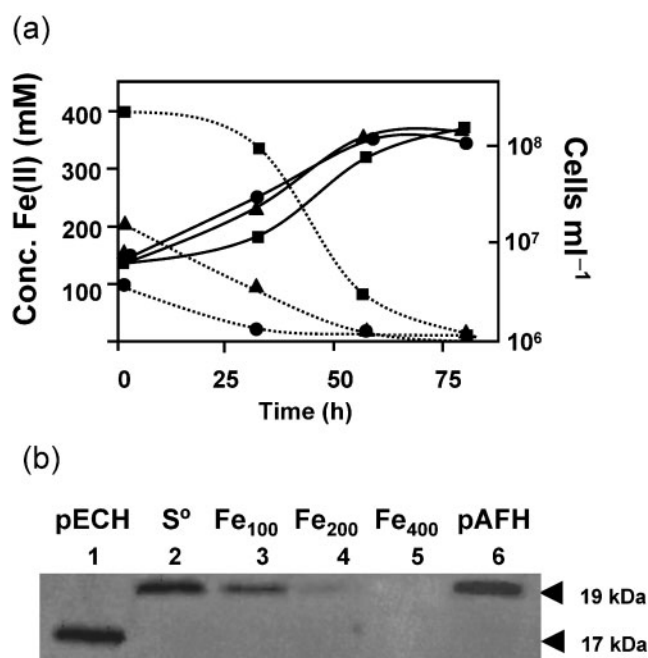


Fig. 3. (a) Fe(II) concentration remaining (dotted lines) in the media at each time point plotted against the growth (solid lines) of *A. ferrooxidans* (cells ml⁻¹). A mid-exponential-phase starting culture grown at 100 mM FeSO₄ with 9K salts was inoculated into fresh 9K basal media containing increasing concentrations of FeSO₄ (●, 100 mM; ▲, 200 mM; ■, 400 mM) and incubated at 30 °C with agitation (150 r.p.m.). (b) Western blot (SDS-PAGE, 18%) with antiserum raised against *E. coli* Fur of *A. ferrooxidans* total proteins from cells cultured either in sulfur-containing medium or in medium containing different Fe(II) concentrations. Arrows mark the migration of proteins with molecular masses of approximately 17 kDa and 19 kDa. Lanes: 1, Fur_{EC} expressed from pECH construct as positive control; 2, *A. ferrooxidans* total protein extracts from 9K+S grown cells; 3, *A. ferrooxidans* total protein extracts from 100 mM Fe(II)-grown cells; 4, *A. ferrooxidans* total protein extracts from 200 mM Fe(II)-grown cells; 5, *A. ferrooxidans* total protein extracts from 400 mM Fe(II)-grown cells; 6, Fur_{AF} expressed from pAFH construct as a cross-reactivity control.

the culture medium at the time of cell harvest (Fig. 3a) revealed that cultures initiated with 100 mM Fe(II) lacked detectable amounts of Fe(II) after 50 h growth, indicating that all the available Fe(II) had been oxidized to Fe(III). In contrast, samples expressing diminished amounts of Fur_{AF} protein obtained from cultures grown at either 200 or 400 mM Fe(II) had oxidized only part of the initial Fe(II) content of the medium (5 and 20 %, respectively). The results are thus consistent with a pattern in which Fur levels diminish in cells exposed to increasingly higher concentrations of Fe(II) and this is consistent with the observed presence of Fur in S⁰-grown cells.

Having demonstrated that Fur concentrations diminished with increasing Fe(II) concentration, we wanted to evaluate

Table 3. Fold change in gene expression levels determined by Q-PCR

Values shown are mean ± SD.

Gene	S ⁰ /S ⁰	Fe ₁₀₀ /S ⁰	Fe ₂₀₀ /Fe ₁₀₀
<i>pgm</i>	1	1.42 ± 0.54	1.11 ± 0.23
<i>sdrAI</i>	1	58.66 ± 17.99	0.87 ± 0.29
<i>fur</i>	1	2.26 ± 0.75	1.00 ± 0.34

whether the decrease resulted from a reduction of transcription of *fur*_{AF}. Quantitative RT-PCR experiments were designed to measure *fur* mRNA levels. Table 3 shows the mean fold difference in *fur* mRNA levels between iron and sulfur (Fe₁₀₀/S⁰) and between the two iron concentrations employed (Fe₂₀₀/Fe₁₀₀). No significant decrease in *fur* mRNA was detected with increasing Fe(II) concentration and levels of *fur* mRNA were similar (± twofold) in both Fe(II)- and S⁰-grown cells, indicating that the observed variation in Fur concentrations does not result from substantial differences in transcriptional activity. Control genes *pgm* (no variation) and *sdrAI* (iron induced) were included for comparative purposes.

Effect of Fur titre variation on (heterologous) gene expression

In contrast to what has been described in other systems where Fur levels are known to be regulated but do not drop below a threshold (Delany *et al.*, 2002, 2003), Fur_{AF} levels varied dramatically in *A. ferrooxidans*. This prompted the question whether varying Fur_{AF} concentration would differentially affect gene expression in *E. coli*. To address this issue, expression of *lacZ* from the reporter strain H1780 was monitored in *E. coli* to provide insight into the effect of Fur_{AF} on an established promoter *fhuF*. In addition, *E. coli* cell survival was measured as a function of increasing concentrations of H₂O₂. This latter assay has been used as evidence that *E. coli* Fur provides protection against iron-induced oxidative damage both by binding iron in a direct manner and also by repressing the transcription of genes involved in iron uptake and homeostasis (Touati *et al.*, 1995).

For this purpose, a construct was designed expressing low levels of Fur_{AF} (pAFL) or Fur_{EC} (pECL), to be compared with the previously described high-expression counterparts (pAFH and pECH). The four constructs were transformed into the *E. coli fur*⁻ strain H1780, and Fur expression at both protein and mRNA levels was monitored by Western blotting and RT-PCR, respectively (Fig. 4a, b). A comparison of protein and mRNA expression demonstrated that the absence of detectable Fur protein, in cells containing the low-expression constructs pAFL or pECL, results from reduced translational (Fig. 4a, lanes 4 and 7) and not transcriptional efficiency (Fig. 4b, lanes 4 and 7). Complete absence of Fur_{AF} in H1780 containing pAFL was discounted

because Fur could be detected in the extracts after immunoprecipitation (Fig. 4a, lane 6).

β -Galactosidase activity assays showed that the high-expression constructs pAFH and pECH (Fig. 4c) reduced expression of the reporter by 60 and 90 %, respectively (with respect to basal expression of the host strain H1780 in the absence of Fe), supporting the observations of Fig. 1. Strikingly, while the construct expressing low levels of Fur from *E. coli* pECL reduced reporter gene expression by nearly 90 % in the presence of iron, pAFL increased expression of the *lacZ* reporter gene by more than 70 %. Similar results were obtained with the host H1681 containing the *fhuF-lacZ* fusion (data not shown). The positive effect on the expression of *lacZ* from these *E. coli* promoters does not appear to be significantly influenced by the presence or absence of iron. One possible explanation for this observation is that at such low Fur concentrations, all the Fur regulator is in an iron-complexed state.

Results consistent with this interpretation were obtained in

the H₂O₂ survival tests. Whereas nearly 90 % of *E. coli* K-12 cells containing wild-type *fur* survived exposure to H₂O₂ up to 10 mM, growth of *E. coli* H1780 lacking *fur* was severely impaired (less than 20 % survival) (Fig. 4d). Partial restoration of survival was achieved by complementation of *fur* deficiency with either Fur_{EC} (pECH) or Fur_{AF} (pAFH). However, the incorporation of pAFL into *E. coli* H1780 rendered it appreciably more sensitive to oxidative stress by H₂O₂, with no cells surviving exposure above 10 mM. In contrast, low-expression levels of Fur_{EC} (pECL) restored survival to the same levels as the high-expression construct pECH.

Taken together, these results raise the possibility that Fur_{AF} is able to act as a positive gene regulator of Fur targets in *E. coli*, inducing expression from *E. coli* *fur* (H1780) and *fhuF* (H1681) promoters and probably that of other iron-uptake genes, thereby disturbing iron homeostasis and rendering the organism more sensitive to H₂O₂. The mechanism by which these effects are exerted will only be clarified by the development of appropriate genetic tools for *A. ferrooxidans*.

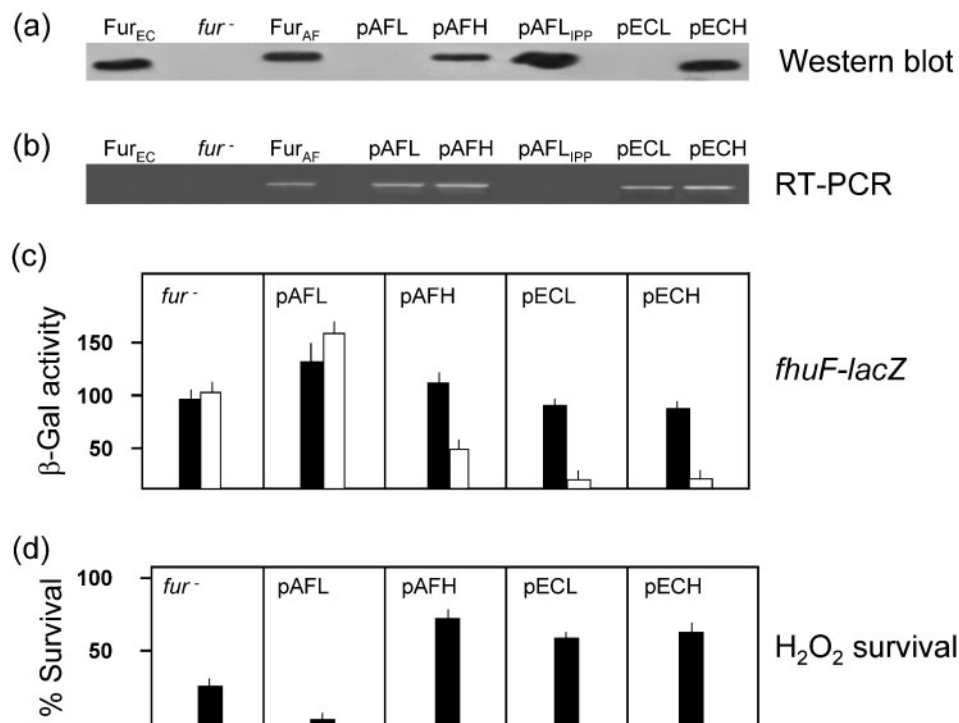


Fig. 4. Effect of Fur_{AF} protein expression levels in *E. coli* H1780 (*fur*⁻), as indicated by (a) Western blot detection of Fur in *E. coli* crude extracts, (b) RT-PCR detection of *fur* mRNAs in *E. coli* total RNA preparations, (c) the expression of *lacZ* in the *E. coli* reporter strain H1780 and (d) the survival of *E. coli* cells as a function of increasing concentrations of H₂O₂. In (c), the quantitative determination of β -galactosidase (β -Gal) activity for clones grown in either the presence (white columns) or absence (black columns) of Fe(II) is shown. The mean (\pm SD) β -galactosidase activity (OD₄₂₀/OD₆₀₀ ml min) from at least three independent experiments is expressed as a percentage of the derepressed condition for strain H1780. In (d), aerobically grown cultures were challenged for 20 min with 10 mM H₂O₂. The mean (\pm SD) c.f.u. ml⁻¹ after overnight incubation from at least three independent experiments is expressed as a percentage of the counts for wild-type *E. coli* K-12. Abbreviations: Fur_{EC}, Fur from *E. coli* K-12 (*fur*⁺); *fur*⁻, *E. coli* H1780; Fur_{AF}, Fur from *A. ferrooxidans* grown in S⁰. pAFL, pAFH, pAFL_{IPP}, pECL and pECH refer to *E. coli* H1780 carrying plasmids of the same names (described in text).

DISCUSSION

In this paper, we report the identification and preliminary functional characterization of the first Fur orthologue from the Fe(II)-oxidizing acidophilic bacterium *Acidithiobacillus ferrooxidans*. Despite considerable effort, *A. ferrooxidans* is recalcitrant to standard genetic manipulation techniques, although a promising report for establishing a conjugation system for genetic exchange with *E. coli* has been published (Liu *et al.*, 2000). This experimental deficiency seriously limits the exploration of the functional role of the postulated Fur by mutation analysis in *A. ferrooxidans*.

To address this limitation, we analysed a potential *fur* gene and a *fur*-responding Fur box in the available *A. ferrooxidans* genome and experimentally tested predictions of the bioinformatic analysis, both *in vitro* (EMSA experiments) and in heterologous *E. coli* mutant strains (Fig. 1).

Genome analysis and experimental evidence support the tentative conclusion that the *fur* gene of *A. ferrooxidans* encodes a functional Fur orthologue Fur_{AF}. Fur_{AF} exhibits significant sequence similarity to well-characterized Fur proteins, including conserved motifs involved in DNA recognition and binding. As demonstrated for other Fur orthologues sharing more than 50% sequence identity with Fur from *E. coli*, Fur_{AF} displays cross-reactivity with *E. coli* Fur antiserum, which allowed informative supershift EMSA (Fig. 2) and Western blot experiments (Fig. 4) to be carried out. Fur_{AF} overcomes *fur* deficiency in *E. coli* mutant strains by downregulating the expression of *fiu* and *fluF* genes in an iron-dependent manner, as has been described for well-characterized Fur orthologues (Fig. 1). This suggests that Fur_{AF} is capable of recognizing and binding *in vivo* to the *fiu* and *fluF* Fur boxes. Both Fur_{AF} from *A. ferrooxidans* and Fur_{EC} from *E. coli* are capable of binding *in vitro* to a proposed *A. ferrooxidans* Fur box postulated to reside upstream of the putative gene *mntH* that potentially encodes a Mn(II)/Fe(II) transporter (Fig. 2). This supports the conclusion from sequence analysis that the DNA binding helices and DNA sequence-recognition motifs of Fur_{AF} and Fur_{EC} are similar. Such similarity has been documented for conserved Fur proteins from many other bacteria that generally also share well-conserved Fur regulons.

Despite their similarities, Fur_{AF} and Fur_{EC} exhibit an unexpected and important difference in their ability to control transcription in *E. coli*. Low cellular concentrations of Fur_{AF} stimulated transcription not only from reporter genes regulated by two well-established *E. coli* Fur boxes, *fiu* and *fluF*, but also promoted H₂O₂-related cell death (Fig. 4d), an observation that is consistent with the idea that Fur_{AF} can upregulate Fur target genes that contribute to increased intracellular oxidative stress.

Several reports have identified conditions in which Fur promotes gene expression rather than represses it. These results have been largely attributed to indirect mechanisms

involving RNA antisense regulation (Massé & Gottesman, 2002) and regulatory cascades that do not implicate the involvement of Fur boxes (Hall & Foster, 1996; Touati, 2000; Vasil & Ochsner, 1999). In *Helicobacter pylori*, Fur regulates Fe-activated promoters by a mechanism of derepression (Fe-dependent derepression) (Delany *et al.*, 2001). Only recently was evidence found for the direct participation of Fur in iron-responsive transcriptional activation in *Neisseria meningitidis* (Delany *et al.*, 2004). In this organism, Fur-activated genes have been shown to have canonical Fur operators centred upstream of the -35 promoter signature. This mechanism is likely to be shared by other Fur orthologues, and among them possibly Fur_{AF}.

The fact that Fur_{EC} at equivalently low intracellular titres remained a repressor of target genes raises an intriguing question: is this property of dual regulatory activity inherent to *A. ferrooxidans* Fur_{AF} or is it a result of heterologous mixing of Fur_{AF} with *E. coli* Fur-dependent promoters? The answer to this question awaits the development of genetic tools for *A. ferrooxidans*.

A dual role for Fur_{AF} is appealing in light of the measurements showing that Fur levels vary in *A. ferrooxidans* depending on the concentration of Fe(II) (Fig. 3). Typically, Fur is expressed in other bacteria at constitutively high levels, reaching protein titres that can amount to as many as 10 000 molecules per cell (Barton *et al.*, 1996; Watnick *et al.*, 1997; Zheng *et al.*, 2001). Such abundance, presumably beyond the need for stoichiometric binding to Fur-box promoters, constitutes a distinguishing feature of the regulatory strategy of Fur that may be explained by the need to control the large number of genes of the Fur regulon and by its polymerization-dependent mechanism of repression that requires the cooperative binding of several Fur molecules per regulatory site (Escolar *et al.*, 2000). Small variations in the level of Fur protein that respond to variations in iron availability have previously been documented in other bacteria (Hernández *et al.*, 2002; de Lorenzo *et al.*, 1988; Delany *et al.*, 2002) but in no case do these drop below protein detection limits as occurred in *A. ferrooxidans*.

The possibility that Fur exhibits a dual capacity as a gene activator or repressor in *E. coli*, depending on its intracellular concentration, suggests a mechanism by which *A. ferrooxidans* can simultaneously control the expression of genes involved in iron uptake, which need to be down-regulated in the presence of unusually high iron loads to diminish oxidative stress, and genes involved in the energy-producing oxidation of iron, which need to be upregulated in these conditions, such as the rusticyanin operon (Yarzabal *et al.*, 2004) and the alternate cytochrome *bc*₁ complex thought to be involved in reverse electron flow (Brasseur *et al.*, 2004). These suggestions are currently under experimental investigation.

Although our results are based on *in vitro* assays (Fig. 2) and measurements of the activity of Fur_{AF} in a heterologous system (Figs 2 and 4), the evidence obtained suggests

that in addition to classical Fe-dependent repression, Fe-sensitive derepression, as reported by Delany *et al.* (2001), and Fe-dependent indirect activation exerted by Fur in other systems (Hall & Foster, 1996; Massé & Gottesman, 2002), variations in the levels of Fur might add to the complexity of regulatory strategies of the ferric uptake regulator in *A. ferrooxidans*.

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