Bioinformatic prediction and experimental verification of Fur-regulated genes in the extreme acidophile *Acidithiobacillus ferrooxidans*

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ABSTRACT

The γ-proteobacterium *Acidithiobacillus ferrooxidans* lives in extremely acidic conditions (pH 2) and, unlike most organisms, is confronted with an abundant supply of soluble iron. It is also unusual in that it oxidizes iron as an energy source. Consequently, it faces the challenging dual problems of (i) maintaining intracellular iron homeostasis when confronted with extremely high environmental loads of iron and (ii) of regulating the use of iron both as an energy source and as a metabolic micronutrient. A combined bioinformatic and experimental approach was undertaken to identify Fur regulatory sites in the genome of *A. ferrooxidans* and to gain insight into the constitution of its Fur regulon. Fur regulatory targets associated with a variety of cellular functions including metal trafficking (e.g. *feoPABC*, *tdr*, *tonBexbBD*, *copB*, *cdf*), utilization (e.g. *fdx*, *nif*), transcriptional regulation (e.g. *phoB*, *irr*, *iscR*) and redox balance (*grx*, *trx*, *gst*) were identified. Selected predicted Fur regulatory sites were confirmed by FURTA, EMSA and *in vitro* transcription analyses. This study provides the first model for a Fur-binding site consensus sequence in an acidophilic iron-oxidizing microorganism and lays the foundation for future studies aimed at deepening our understanding of the regulatory networks that control iron uptake, homeostasis and oxidation in extreme acidophiles.

INTRODUCTION

Iron availability is an environmental stimulus to which organisms respond by regulating the expression of many genes, termed the iron stimulon (1). The iron stimulon includes clusters of genes under the control of a number of different transcriptional regulators of which Fur is the best characterized (2). Fur recognizes and binds specifically to a DNA sequence, known as the Fur box, that is typically located in proximity to the −10 and/or −35 σ_{70} promoter elements of target genes (3). Genes, whose expression is under the control of Fur, constitute the Fur regulon. To date, more than 90 genes in *Escherichia coli* (2,4), 87 in *Pseudomonas aeruginosa* (5) and 46 in *Bacillus subtilis* (1) are known to be regulated by Fur.

Although less data exists for other microorganisms, Fur typically binds target DNA that has recognizable sequence similarity to the *E. coli* Fur box consensus. For example, canonical Fur boxes have been described in *Legionella pneumoniae* (6), *Campylobacter jejuni* (7), *P. aeruginosa* (8), *Neisseria gonorrhoeae* (9), *Vibrio anguillarum* (10), *V. cholerae* (11) and *Yersinia pestis* (12) among others. There are a few exceptions where Fur-binding sites have been reported to exhibit an incomplete target DNA consensus (13,14).

Since Fur is a global regulator controlling a relatively common set of genes in different microorganisms, both the search for conserved target genes and for conserved Fur-binding sites have proved useful for the identification of genes belonging to the Fur regulon in a wide variety of bacteria. Several experimental strategies have been employed to find Fur regulated loci: *in vivo* FURTA assays in *E. coli*, *Salmonella typhimurium* and *Helicobacter pylori* genomes (15–18), *in vitro* cycle-selection procedures in *P. aeruginosa* (8) and more recently microarray transcriptional profiling in *Shewanella oneidensis*, *P. aeruginosa*, *B. subtilis*, *E. coli*, *S. enterica*, *N. meningitidis* and *H. pylori* (1,4,5,19–22). Once Fur-binding sites have been recognized for common shared genes in genomes, the information can then be used to mount searches for Fur-binding sites associated with species-specific targets.

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As the complete genome sequences of more microorganisms become available, the prediction of transcriptional regulatory DNA sites by computational analysis of sequence data, including phylogenetic footprinting, is quickly becoming an indispensable tool in biological research. Quantitative models based on information theory have been constructed for predicting the DNA recognition sequence for the OxyR, PurR, Lpr, Fis transcription factors, among others (23–26) and used to search for additional or new regulator target sequences. Consensus-building algorithms have also been employed to conduct comparative genomic analyses of the Lex regulon (27) and the Fur regulons of *E. coli*, *S. typhi*, *Y. pestis* and *V. cholerae* (28) and *B. subtilis* (29).

We are interested in understanding how the bacterium *Acidithiobacillus ferrooxidans* copes with iron uptake and homeostasis considering that it grows at acidic pHs in environmental situations where soluble iron is abundant ($[FeIII]_{pH2} = 0.1\,M$) compared with its bioavailability in circum-neutrophilic aerobic conditions ($[FeIII]_{pH7} = 10^{-18}\,M$). Another special feature of *A. ferrooxidans* is its use of iron as an energy source that may make its requirement for iron particularly demanding given its abundant repertoire of iron-dependent respiratory enzymes. It also suggests that *A. ferrooxidans* must have developed mechanisms to coordinately regulate iron homeostasis and iron oxidation.

In this article, an *in silico* approach, fed with experimentally confirmed Fur boxes, has been used to identify candidate Fur-binding sites in the *A. ferrooxidans* genome. The identification of Fur boxes associated with several transporters, iron-containing proteins and miscellaneous functions, in addition to typical Fur targets that are involved in iron assimilation in other bacteria, provides the first insight into the nature of the Fur regulon in an acidophilic, iron oxidizing microorganism. A role for Fur in coordinating metal homeostasis responses, beyond iron uptake can be suggested. A first glimpse into how iron homeostasis and iron oxidation could be coordinated through the Fur-dependent repression of Fe(II)/Fe(III) uptake systems and de-repression/activation of Fe(II) oxidizing and Fe(III) uptake systems in response to iron availability is presented and a framework is suggested for future investigations into the biology of less well-studied Fur targets that could form part of the iron stimulon in *A. ferrooxidans*.

MATERIALS AND METHODS

Weight-matrix-based Fur-binding site prediction

A set of 66 experimentally confirmed Fur boxes from *E. coli*, *S. typhimurium*, *P. aeruginosa* and *Staphylococcus aureus* was used to generate an alignment matrix and a weight matrix by the information content method of Schneider (30) (Supplementary Data S1). The weight matrix was initially tested against known Fur-box-containing DNA segments from several microorganisms. The method consistently predicted the DNAse I protected and/or gel-shifted sites by Fur binding in each tested example (data not shown). The matrix was used to search the genome of *A. ferrooxidans* using a 19-bp sliding window. Each window was scored for its degree of matching the Fur weight matrix and only those with scores higher than the lowest accepted *E. coli* Fur box were retained in the analysis. To further reduce the number of false positives, this initial pool of candidate Fur boxes was culled by including only those that: (i) were located <600 nt from the proposed initiation of translation of the potential target gene and (ii) exhibited conservation of key nucleotides known to be protected by Fur binding in *E. coli* (31,32). Near-symmetric sites with high scores in both the forward and reverse direction were counted only once, and the higher of the two scores was retained.

Hidden Markov Model-based Fur binding site prediction

A group of experimentally validated Fur-binding sites from *A. ferrooxidans* derived from electrophoretic mobility shift assays (EMSA) was used to build HMM profiles and to search for additional sites in the genome of *A. ferrooxidans*. For this purpose, the Fur-binding promoters were aligned with ClustalW (www.ebi.ac.uk/ClustalW) and an HMM was built from this alignment (Supplementary Data S1) using *hmmb* in the HMMER package 1.8.4 programs (33). Subsequently, *hmmls* was used for finding multiple non-overlapping matching hits in an *A. ferrooxidans* intergenic sequence database constructed from the annotated genome sequence (www.tigr.org). HMMER bit scores assigned to each candidate Fur box reflect whether the sequence is a better match to the profile model (positive score) or to the null model (negative score), the magnitude of which shows how well the sequence matches the HMM. Scores above log2 of the number of sequences in the target database are likely to be true functional Fur-binding sites. Our cutoff value was set on the basis of the worst scored training sequence at 9.00 bits. This strong search criteria is bound to overlook intrinsically weak binding sites (or sites significantly divergent to the ones tested experimentally herein which may not accurately reflect the statistical distribution of all true targets), but was chosen to reduce biases introduced by false positives.

DNA sequence Logos

Logos were generated using the web-based application available at http://weblogo.berkeley.edu/logo.cgi. The height of each letter corresponds to its relative abundance at each position in bits.

Bacterial strains and growth conditions

Bacterial strains and plasmids are described in Table 1. *Acidithiobacillus ferrooxidans* ATCC23270 was grown in modified 9K basal salt media (0.1 g of $(NH_4)_2SO_4$, 0.04 g of K_2HPO_4, 0.4 g of $MgSO_4$-$7H_2O$ per liter) containing 200 mM $FeSO_4$; adjusted to pH 1.6 with H_2SO_4) at 30°C under aerobic conditions on a rotary shaker at 150 rpm. *E. coli* was grown at 37°C in LB broth with the appropriate antibiotics: ampicillin (Amp: 100 µg/ml), streptomycin (Sm: 100 µg/ml) and/or kanamycin (Km: 30 µg/ml) as indicated in Table 1.

Table 1. Bacterial strains and plasmids used in this study

Strains	Relevant genotype	Reference or source
A. ferrooxidans		
ATCC23270	Type strain, genome sequenced by TIGR and Integrated Genomics	ATCC
Escherichia coli		
H1717	*fhuF::lacZ*, Sm^r	(34)
QC-1732	GC4468, F- $\triangle$(*lacZYA-argF*)U169 *rpsL* $\triangle$*fur*, Sm^r, Km^r	(35)
Plasmids		
pUC-18	plasmid vector, Amp^r	Gibco, BRL
pGEM-T	plasmid vector, Amp^r	Promega
pAFH	*fur* from *A. ferrooxidans* ATCC23270 expressed from pGEMT-Easy *lacZ* promoter, Amp^r	(36)

General DNA techniques and cloning procedures

Acidithiobacillus ferrooxidans cultures, to be used for nucleic acid or protein purification, were centrifuged at 600 g to remove solid sulfur or iron precipitates prior to cell harvest. The cell pellet was resuspended in 9 K salt solution for further washing. Washed cells were collected by centrifugation at 10,000 g for 10 min.

DNA isolation and routine manipulations were carried out following standard protocols as described (37) 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). Polymerase chain reaction (PCR) products 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 of template DNA, 0.5 μM of required primers and 0.2 mM of each deoxyribonucleotides in a volume of 25 μl of 1 × PCR buffer containing 1.5 mM MgCl$_2$ (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. DNA sequencing was carried out by Agencourt, Inc. (Beverley, MA, USA)

Fur titration assay (FURTA)

Fur titration assays was performed according to Stojiljkovic *et al.* (15). A pUC-18-based randomly cloned genomic library from *A. ferrooxidans* (pGTF) containing *Sau*3AI DNA fragments (average weight of 0.5 kb) was electroporated into *E. coli* H1717 (*fur$^+$*, *fhuF::lacZ*, Table 1). A total of 3.1×10^5 Amp^r clones were recovered in selective MacConkey indicator plates containing 40 μM FeSO$_4$. The cloning vector pGEMT-Easy and the *E. coli* Fur box upstream of the *fhuF* gene cloned in the same vector (FB-FhuF1 + 2, Table 2) were employed as negative and positive controls, respectively. Clones exhibiting red color in FeSO$_4$-MacConkey plates after 12–24 h of incubation at 37°C were isolated and further re-streaked. DNA inserts of candidate clones were subsequently subjected to DNA sequencing. The recovered sequences were assessed for the presence of putative promoter elements and Fur box-like

Table 2. Oligonucleotide primers used in this study

Primers	Sequence (5′–3′)
Vector	
pUC-1(2006971)*	AACAGCTATGACCATC
pUC-2(2006972)*	GTTTTCCCAGTCACGAC
A. ferrooxidans	
FeoP-1*	GAATTCCGTCATTACCGTCATGGCAAC
FeoP-2*	TGGATCCCCAGCGCAGATGATGAGCAC
GloA-1*	ACGCAAGCGGTAACAAAGC
GloA-2*	ACGGCAACAACGACACATC
Fdx1-1*	TTATTGGGCAGACTGTTGCC
Fdx1-2*	TTGATCACTGAGAGGCAGGAC
HppH-1*	TCAAGGCGACCTGGTATTCC
HppH-2*	TCTTTCCTTTCTGTGGATGCC
IscR-1*	CGTCAGTTTCATATCTGGTGC
IscR-2*	TCGTCAATAAGGGTGAGCATAC
PhoB-1*	CACTTCAGTAATTCACTGATC
PhoB-2*	TGCTGCCTTGCGGTATGG
CopB-1*	CAGGCTACCCAATGGGTAG
CopB-2*	CCAAGGCAGCAGTAGAAGC
AbcS4-1*	CGCAGTTTCAAAAAGCCTCTC
AbcS4-2*	GTGTTTCACGGGCTCTACTCC
FeoP-3♦	GACCCCATCGCTAGTCATG
FeoP-4♦	TAGCTCACTGTCGGCATG
GloA-3♦	ACGCAAGCGGTAACAAAGC
GloA-4♦	TCGGGAAACCGCATATCC
CopB-3♦	CCAAGGCAGCAGTAGAAGC
CopB-4♦	GGCGGCATGGTTGTGACC
PhoB-3♦	CACTTCAGTAATTCACTGATC
PhoB-4♦	AAATCGGGCAGACCAGTCC

*Oligonucleotides used for amplification of EMSA probes; ♦Oligonucleotides used for amplification of IVTA probes.

sequences using bioinformatics approaches described above. FURTA-positive clones were ranked for further analysis based on the following criteria: (i) presence of a predicted Fur-binding site in the insert; (ii) that the target gene is Fur-regulated in other bacteria and/or (iii) that the target gene is related to known genes involved in iron metabolism in other organisms.

Overexpression and purification of *A. ferrooxidans* Fur

The *E. coli* strain QC1732/pAFH carrying the recombinant plasmid pAFH expresses the *fur* regulator from *A. ferrooxidans* 23270 (36). The 500 ml cultures of this strain were grown in Luria-Bertani broth containing 100 μg/μl of ampicillin and 100 μg/μl of streptomycin to an optical density at 590 nm of 0.8–1.0. The cells were isolated by centrifugation and were resuspended in 50 ml of buffer Tris-HCl 50 mM pH 8.0 containing 5% glycerol,

1 mM phenylmethylsulfonyl fluoride and 100 µg/ml of lysozyme. The cells were incubated to 4°C for one hour and sonicated, and the mixture was centrifuged at 25 000 g for 15 min. The soluble extract was applied to a DEAE-Sephacel (Pharmacia Biotech, Uppsala, Sweden) column with a 50-ml bed volume. The bound proteins were eluted with a 50–500 mM NaCl linear gradient, and 2-ml fractions were collected. The majority of Fur eluted between 150 and 200 mM NaCl as determined by western blot analysis of fractions with anti-Fur antibody. Elution fractions containing Fur were pooled and applied to a chelating Sepharose (Pharmacia Biotech) column (5-ml bed volume) charged with $ZnSO_4$ 0.2 M as recommended by the manufacturer. The bound proteins were eluted with 1M imidazole in 50 mM Tris-HCl (pH 7.4), 150 mM NaCl and 0.5-ml fractions were collected. Fractions containing Fur were pooled and dialyzed at 4°C for 1 h in 50 mM Tris-HCl pH 7.4, 1 mM DTT, 25 mM EDTA, 1 h in 50 mM Tris-HCl pH 7.4, 1 mM DTT and finally overnight in 50 mM Tris-HCl pH 7.4, 1 mM DTT, 30% glycerol, 0.1 mM $MnSO_4$. The protein fractions were analyzed by SDS-PAGE (15% gel) and either silver stained or western blotted. Polyclonal antibodies against *A. ferrooxidans* Fur were obtained by immunizing white rabbits with 200 µg of a Fur protein preparation following standard procedures (37).

Electrophoretic mobility shift assays (EMSAs)

Probes (representing selected candidate Fur boxes) were obtained by PCR amplification using the pairs of oligonucleotides shown in Table 2 and were end labeled with [γ^{32}P]-ATP using T4 polynucleotide kinase (Invitrogen). Unincorporated nucleotides were removed with Bio-Gel P10 Micro Bio-spin chromatography columns (BioRad). EMSA assays were performed as described by de Lorenzo et al. (31) with the following modifications. A 300 nM (or otherwise stated concentrations) of purified Fur protein form *A. ferrooxidans* obtained as described above was equilibrated in 20 µl final volume of gel mobility shift buffer (20 mM Tris-HCl pH 8.0, 40 mM KCl, 1 mM $MgCl_2$, 0.1 mM $MnSO_4$, 1mM DTT, 0.1 mg/ml bovine serum albumen, 5% (v/v) glycerol). The labeled probes (50–75 pM; 10,000 c.p.m.) and non-specific competitor salmon sperm DNA (50 µg/µl) were added, and the reactions incubated for 10 min at 30°C. In supershift experiments, a 1:500 dilution of Fur-specific antiserum was added to the reaction and incubated for an additional 5 min. A 50-fold excess of cold probe was used to challenge each of the labeled probes. Bound and unbound probes were separated by non-denaturing polyacrylamide (4% w/v) gel electrophoresis at 100 V for 1 h in Tris acetate buffer at 4°C. Retardation was assessed after exposure of the dried gel to Imaging Screen-K (Kodak) by scanning on a PhosphorImager (Molecular Imagen FX Pro Plus, BioRad). The image was captured and analyzed with Quantity One version 4.1.1 (BioRad). Purified Fur protein concentration to be used in EMSA assays (300 nM) was determined after titrating the amount of protein that produced a shift of a non-specific probe corresponding to

a low G + C PCR fragment spanning the multicloning region of pUC18 (amplified with primers shown in Table 2), which is 500 nM (Figure 3F—lane 6).

In vitro transcription (IVTA)

In vitro transcription assays were performed according to Friedman and O'Brian (14) with minor modifications. Promoter-containing template sequences (average size = 400 bp) were PCR-amplified using the primers specified in Table 2. Templates (5–7 nM) were incubated for 10 min at 37°C with 300 nM Fur protein from *A. ferrooxidans* (Fur$_{AF}$) in IVTA buffer (20 mM Tris-HCl pH 8.0, 40 mM KCl, 10 mM $MgCl_2$, 0.1 mg/ml bovine serum albumin, 0.1 mM $MnSO_4$, 5 mM dithiothreitol) in a final volume of 10 µl. One unit of *E. coli* RNA polymerase (Amersham) was added to the reaction in the presence of 40 U of RNAse inhibitor (Fermentas) and incubated at 37°C for 5 min, before adding 10 µl of a preheated NTP mix (250 µM ATP, CTP and GTP, 30 µM UTP and 12.5 µM of UTP-αP^{32}) in IVTA buffer. Incubation at 37°C was carried out for 10 min. Transcripts were resolved by polyacrylamide gel electrophoresis (10%) under denaturing conditions (7 M urea) in Tris borate EDTA electrophoresis buffer. Transcript sizes were determined using an RNA ladder (Invitrogen) labeled with (γ^{32}-P)-ATP using T4 polynucleotide kinase (Fermentas).

RESULTS AND DISCUSSION

Strategy for the identification of candidate Fur-binding sites in *A. ferrooxidans*

In order to identify *A. ferrooxidans* genes directly targeted by the Fur regulator, a genome-wide search for conserved Fur boxes was carried out using the computational and experimental approach outlined in Figure 1.

The strategy involves the following steps:

(i) A Fur recognition weight matrix was derived from a pool of recognized Fur-binding sites of several bacteria as described in Methods (Supplementary Data S1A). This matrix was used to locate potential Fur-binding sites by computing the information content of each 19 base pair sequence of a sliding window passed over the complete genome of *A. ferrooxidans*. After reducing potential false positives, a set of 90 candidate sites (Supplementary Data S1B) was selected for further analysis.

(ii) A gene library of *A. ferrooxidans* was transformed into the *E. coli* FURTA strain H1717 carrying a Fur regulated *lacZ* fusion as a reporter gene (*fhuF::lacZ*) in order to identify *A. ferrooxidans* sequences capable of sequestering *E. coli* Fur. After re-streaking positive candidates three times to fresh media, clones with reproducible LacZ+ phenotypes were sequenced and potential Fur-binding sites were identified using information theory (Table 3).

(iii) A set of candidate Fur-binding sequences derived from bioinformatic predictions (Information Theory) and *in vivo* isolated candidate Fur-regulated
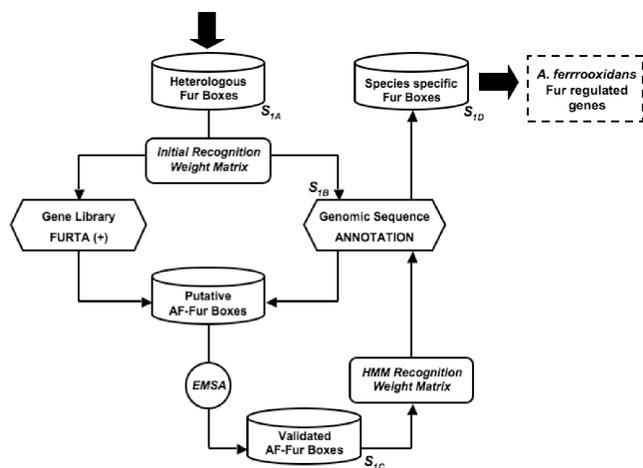
Figure 1. Pipeline for the computational and experimental steps used to identify candidate Fur-binding sites in *A. ferrooxidans*. Additional information and results can be found in Supplementary Data S1.

promoters (FURTA) were evaluated for *in vitro* binding of Fur from *A. ferrooxidans* (Fur$_{AF}$) using the EMSA (Supplementary Data S1C).

(iv) The EMSA-validated sequences were used to construct a HMM profile for further screening of the *A. ferrooxidans* genome. This strategy aimed to set a more stringent search-criteria in order to locate potential 'species specific' Fur-binding sites and to reduce the rate of false positives. The DNA sequences of binding sites with scores higher than the weakest experimentally confirmed Fur box are provided in Supplementary Data S1D.

First, Fur boxes were identified upstream of conserved *A. ferrooxidans* genes whose orthologs encode Fur-regulated functions in other microorganisms. This set of genes not only provides interesting insights into the iron uptake strategies of *A. ferrooxidans* but also strengthens the validity of the prediction pipeline. Second, through this genome-wide search, Fur regulatory targets were predicted that are associated with a variety of other cellular functions (metal uptake, utilization and efflux; phosphate utilization; transcriptional regulators and redox balance) providing the first model of the Fur-dependent iron regulon in *A. ferrooxidans*. Selected predicted targets were functionally evaluated for Fur binding *in vitro* through (i) gel shift assays (EMSA) and (ii) Fur-mediated metal-dependent control determined by *in vitro* transcription analysis in the presence of purified Fur from *A. ferrooxidans* and the metal co-repressor.

Identification of *A. ferrooxidans* candidate Fur-binding sites and associated genes

Genes associated with predicted Fur-binding sites were classified into five functional categories (Table 3). The first two categories include several genes orthologous to well characterized iron and/or Fur-regulated genes from other bacteria, encoding iron acquisition and iron utilization functions, respectively. Most Fur regulons described to

date overlap at the level of iron uptake and iron utilizing protein functions. The fact that these two gene categories are also well represented in the *A. ferrooxidans* candidate target gene list, attests to the efficacy of the prediction pipeline outlined above.

The following predicted gene function categories correspond to those listed in Table 3.

Iron acquisition includes a predicted GTP-driven FeoB ferrous iron uptake transporter, one ortholog of the NRAMP family of proton-coupled FeII/MnII transporters known in bacteria as MntH and a number of genes predicted to be involved in the uptake of ferric iron by means of TonB dependent outer membrane ferri-siderophores receptors (Tdr) (Figure 2). Among the latter Fur regulatory targets are two adjacent genes encoding TonB-dependent OMRs with probable affinity for catechol siderophores (*tdr* of the *cirA*-type), a 13 gene cluster encoding for all necessary siderophore mediated ferric iron uptake functions and four loci encoding TonB/ExbBD biopolymer transport proteins.

Iron utilization functions contain several genes predicted to encode redox proteins and/or Fe–S binding proteins, including electron transporting ferredoxins, oxido/reductases (NuoI, NoxA), heme biosynthesis coproporphyrinogen III dehydrogenase (HemN), a high potential iron protein (HppH) and a subunit of the Hup reductase involved in hydrogenase maturation (DrsK-like), and several genes implicated in Fe–S cluster assembly such as a cysteine desulfurase NifS, accessory protein NifV and an Fe–Mo nitrogen fixation protein (NifX) (Figure 2).

Transporters were identified belonging to diverse functional families (Figure 2) including two distinct efflux permeases of the ATP-dependent family of transmembrane efflux pumps (secretion ABC). One of these systems consists of adjacent genes encoding, respectively, a protein with a permease motif characteristic of the FatC Fe(III) -anguibactin efflux permease of *Bartonella quintana* (CAF26500) and an uncharacterized permease similar to YvsF (CAB69806) from *Bacillus cereus*. The other permease transporter is similar to CvaB of *E. coli* (P22520), and forms part of a predicted operon encoding a microcin exporter system that has been shown to be iron regulated via a well conserved Fur box in *E. coli* (38). Shared organization and associated Fur box suggest a similar regulatory behavior in *A. ferrooxidans*. Two gene clusters encoding resistance-nodulation-cell division (RND) family efflux pumps and one cluster encoding for a translocase of the major facilitator superfamily (MSF) are preceded by Fur boxes in *A. ferrooxidans*. RND complexes typically transport cations (39) or a chemically diverse group of organic substances (40) from the cytoplasm across the periplasmic space to the outside of the cell, driven by proton motive force. Additionally, trans-envelope efflux pumps of the RND have been proposed to be involved in the secretion of iron siderophores under conditions of iron starvation in *P. aeruginosa* (41,42) and *Acinetobacter baumannii* (43). Recently, several MSFs sharing the signature COG0477 of the EmrB/QacA subfamily of MFS have been implicated in the proton motive force-driven and iron-regulated

Table 3. Genes associated with HMM- or IT-predicted Fur boxes

Prediction METHOD	Validation FURTA	EMSA	ITVA	Fur box	Score (bits)	Distance to ATG (bp)	Gene or Gene cluster	Annotated function (TIGR)	TIGR#
Iron acquisition									
IT	T	S	R	TGTAATAAGACTCATTCGT	11.7	21	*feoP/feoABC*	OprB family porin -Feo FeII uptake transporter	AFE0580
HMM				GTAATAAGACTCATTCGT	10.56	32			
HMM				CTAATATTAGTCATTAGT	9.69	238			
HMM	T	S(36)		TAAACGGGAATCATTCTC	10.73	28	*mntH*	Metal ion transporter, NRAMP family	AFE2920
IT				ATAAACGGGAATCATTCTC	11.3	46			
IT	T			CAATATCGTTATCATCCTT	6.4	35	*tdrA*	TonB-dependent receptor	AFE0174
IT				TACTATCAGATTTATAATT	6.2	521	*tdrB*	TonB-dependent receptor	AFE0173
HMM	T	S	R	GATATTATAGTTATTCTT	11.33	161	*gloA*	Conserved globin domain protein	AFE1602
IT				TGATATTATAGTTATTCTT	9.7	161			
IT	T			GATATCCAGAATTTTTATC	8.7	55	*abcP1*	ABC iron(III) dicitrate transport system permease, FecCD family	AFE1594
HMM				AAAATGACAATTTTATGA	9.38	109	*exbBDtonB2*	TonB Biopolymer transport system	AFE0827
IT	T			TAAATTCATTCCCATTTTC	13.0	1	*tonB4*	TonB family protein	AFE2270
IT	T			GAAAATAAAACTGTGTTC	5.6	113	*tonB4*	TonB family protein	AFE1114
IT				TGAAATGAGAATCAAATCT	9.7	20	*exbD4*	Bipolymer transport ExbD protein	AFE0106
Iron utilization									
HMM	T	S		AGAATTGTTAATTTGTCT	11.95	255	*hyp/fdx1*	Ferredoxin (4Fe-4S)	AFE1138
IT				CACAAAGAGAATCAATATC	10.5	304	*fdx2*	Ferredoxin (Fe-S)	AFE2230
IT				GAGAATCATATGCAATTTC	13.0	−10	*nuoI*	NADH-quinone oxidoreductase, I subunit-2(4Fe-4S)	AFE0482
IT				AAAAACAATAATATATATT	7.4	273	*noxA*	Pyridine nucleotide-disulfide oxidoreductase (Fe-S)	AFE1282
IT	T			CGAAACCAGATCCATTTCC	5.1	583	*hemN*	O2-indep coproporphyrinogen III DH (4Fe-4S)	AFE2891
HMM	T	S		ATATATGTAATTATTCTT	12.5	74	*hppH*	MBH NiFe hydrogenase high potential iron-sulfur protein (4Fe-4S)	AFE2333
	T						*dsrK*	Hup reductase (Fe-S)	AFE3048
IT	T			GATAACGCGTTTCGTCTTT	5.3	434	*hdrC*	Heterosulfide reductase subunit (4Fe-4S)	AFE0551
IT				ATCATTGGATAACAATTATC	10.1	26	*nifV*	Fe-S cluster assembly accessory protein (Fe-S)	AFE1553
IT				TGGAAGGATTGGCATATTC	5.1	−20	*nifS*	Nitrogenase cofactor synthesis protein	AFE1547
IT				GGAAATCGCATTTATCATC	5.6	95	*fdx4*	Ferredoxin (2Fe-2S)	AFE1543
IT				GAAAATGGTATTCTTCTGC	7.5	−87	*nifX*	Nitrogenase cofactor processing protein (Fe-Mo)	AFE1569
IT				GAAAAAGCGTTTAATTTTT	6.7	21	*hyp/hyp/fdx7*	Hypothetical in nitrogenase gene cluster	AFE1570
Predicted transporters									
ABC transporters									
HMM				ATAATAATAGCTAGTAAT	9.53	−19	*fatC*	ABC transporter, permease protein, putative	AFE0990
HMM				ATAATAATAGCTAGTAAT	9.53	−19	*ywsF*	ABC transporter, permease protein	AFE0989
IT				TATAATAAGTGTCATTATT	14.0	301	*hyp/cvaA*	Hypothetical protein	AFE0932
HMM				ATAATAAGTGTCATTATT	9.98	302			AFE0932
IT				TAAAATGACAATCGATACT	10.6	410	*cvaA*	CvaA colicin V exporter MFP family	AFE0931
									AFE0931
IT	T			TGTTTGTATACTTATTATC	5.3	573	*abcA*	ABC transport protein, ATP-binding subunit	AFE0803.1
IT				AGTAATGGTAACTGTTCTG	9.3	266	*nikB*	Di/oligopeptide/nickel permease	AFE0119
HMM	T	S		TACTGGTAACTATTTTTG	10.59	40	*abcS4*	ABC transporter, periplasmic SO4/MoFeIII-binding protein	AFE1586

Method	Assay	Sequence			Gene	Protein	AFE
Cation diffusion facilitator transporters							
HMM	T	TACTGGTAACTATTTTTG	10.59	40	cdf1	CDF family protein*	AFE1588
RND efflux transporter							
HMM		TACTGGTAACTATTTTTG	10.59	40	rnd1	Heavy metal RND efflux outer membrane protein	AFE1587
HMM	T	GAATTGTTAATTTGTCTA	11.95	445	hyp/omf/mfp/rnd2	Conserved hypothetical protein TIGR00481	AFE1140
IT		TATAGCGGTATTGCACTTG	6.5	247	omf/mfp/rnd3	Heavy metal RND efflux outer membrane protein	AFE1057
MFS Transporters							
HMM		GAATGAATAAGTTTTATT	9.05	276	msfB	Drug resistance transporter, EmrB-QacA family	AFE2984
P-type ATPase							
IT		GCAAATGATAAGCATCCTT	13.1	144	copA	Copper-translocating P-type ATPase	AFE1073
		CAAATGATAAGCATCCTT	9.75	143			AFE0329
HMM	S R	GAAACGATAATTTTATTG	9.89	244	copB	Copper-translocating P-type ATPase	AFE2222
HMM		ATGATGGTAAATATTCAG	9.54	391	hat1	Plasma-membrane proton-efflux P-type ATPase	
Transcriptional regulators							
HMM	T	ATAAGGGTGAGCATACGG	10.64	272	iscR	Fe-S cluster assembly	AFE2368
HMM	T	ATAACTATAACTAGTTTA	9.5	50	irr	Transcriptional regulator Fur family	AFE1137
HMM	S R	AAAATGACAATTTTATGA	9.38	109	phoB	Transcriptional regulator PhoB family	AFE0827
HMM		TTTTTGGTAATTATAAGC	9.04	31	arsR	Transcriptional regulator ArsR family	AFE0734
HMM		AAAATAGTAAGTATATTT	10.93	56	marR	Transcriptional regulator MarR family	AFE0507
HMM		TCAAAAGTAATTATTTTAG	9.68	148	ntrC	Sigma-54 dependent transcriptional regulator NtrC family	AFE0505

FURTA, Fur titration assay (T, positive titration); EMSA, Electrophoretic mobility shift assay (S, positive shift); IVTA, *in vitro* transcription assay (R, transcriptional repression); IT, Information Theory; HMM, Hidden Markov Models.

transport of siderophores in a number of bacteria (43–48). The gene context of RND-1 and the presence of Fur boxes in the promoters of both the RND-1 and the MSF gene clusters suggest that their expression is regulated by iron and by Fur in *A. ferrooxidans* and suggests a role for these proteins in the secretion of xenosiderophores. In addition, a hybrid operon (Figure 2) consisting of an unusual combination of transport components including an ABC solute-binding protein (AbcS4), an RND-type outer membrane factor (Rnd1) similar to OprD and a cation diffusion facilitator (Cdf1), was found in *A. ferrooxidans*. The solute-binding protein shares 70% similarity to the sulfate/molybdate-binding protein ModA (COG0725) and weak similarity (<30%) to the ABC-type Fe(III)-binding protein AfuA (COG1840), while the CDF (pfam01545) carries a C-terminal signature (MTH1175) found in several uncharacterized proteins belonging to the Fe–Mo cluster binding proteins. Since members of the CDF family are metal-specific pumps that serve as secondary filters for various divalent cations (48–50), a role for Mo or Fe efflux facilitation can be envisioned. Finally, three predicted ATP dependent translocases of charged ions of the P-type ATPases superfamily were found to have upstream Fur boxes. These membrane pathways can either actively take up or extrude inorganic monovalent cations such as $Cu(I)/Ag(I)$, divalent $Zn(II)/Cd(II)/Pb(II)$ or $H(I)/Na(I)/K(I)$ (48). One of the *A. ferrooxidans* candidate Fur-regulated P-type ATPases potentially encodes a plasma-membrane proton-efflux transporter [EC 3.6.3.6], and the other two are predicted to be copper P-type ATPases (CPx-type; EC 3.6.1.3) with similarity, respectively, to the well-studied CopA (61% S to P32113) and CopB (68% S to P05425) copper homeostasis proteins in *Enterococcus hirae*.
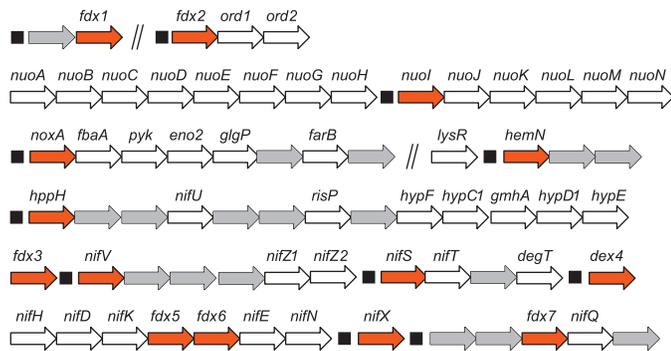
Transcriptional regulators were identified belonging to six different protein families: IscR, Irr, PhoB, NtrC, MarR and ArsR (Figure 2). Some of these regulators exhibit iron and/or Fur-dependent expression in other bacteria and therefore are reasonable targets for Fur control in *A. ferrooxidans*. For example, the IscR regulator of *E. coli* controls the negative feedback expression of a housekeeping Fe–S assembly gene-cluster (51) and the Irr regulator of α-proteobacteria is known to control heme biosynthesis (52). The PhoB-like transcriptional regulator in *A. ferrooxidans* is the first gene of a predicted operon containing the three components of the TonB iron acquisition system known to be a Fur target in other organisms (53) and an acid phosphatase similar to AcpA of *Burkholderia sp.* The other three transcriptional regulators are located upstream (ArsR) or divergent to (NtrC and MarR) gene clusters encoding proteins that have predicted roles in redox balance maintenance (e.g. rhodanases, glutaredoxins, thiorredoxins, GST). The presence of these Fur boxes suggests the necessity to control intracellular iron-mediated effects on oxidative stress.

In addition, several genes that encode a variety of functions such as ribosomal proteins, transposase etc., or genes with unknown functions were identified with predicted Fur boxes. Their possible role in Fur-regulated activities remains to be addressed.
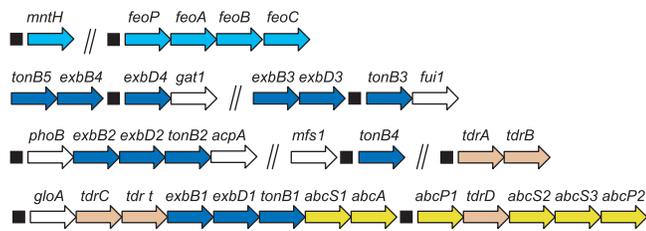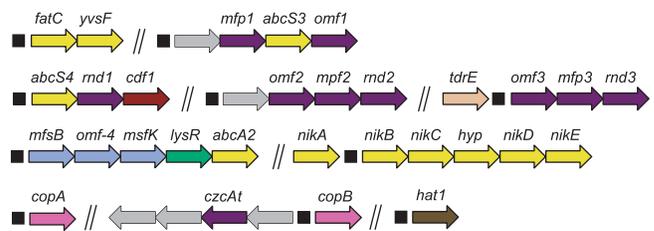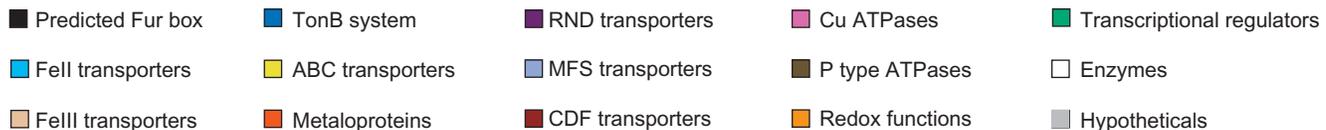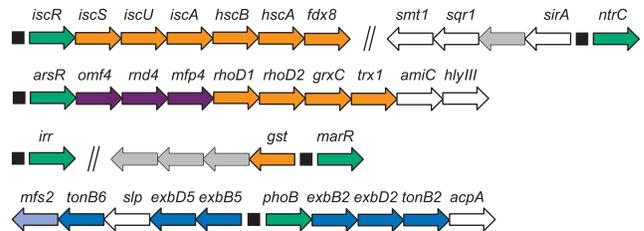
Figure 2. Predicted Fur-regulated gene clusters and associated predicted Fur boxes grouped into four main functional categories: iron acquisition, iron utilization, transporters and transcriptional regulators. Arrows representing each gene indicate direction of transcription and are not drawn to scale. The double-hashed line separating independent gene clusters indicates that they are not contiguous in the genome.

Experimental validation of selected candidate Fur boxes

Fur titration assays (FURTA). Evidence for the capacity of Fur from *E. coli* to recognize and bind target DNA sequences in our model system was obtained by transforming a gene library of *A. ferrooxidans* into the *E. coli* FURTA reporter strain H1717 (15). Clones with LacZ-positive phenotype were recovered and the plasmids purified for DNA sequencing. Isolated genome fragments were inspected bioinformatically for the presence of ORFs, promoter regions and Fur-binding sites. FURTA positive clones retained after filtering as likely Fur targets in *A. ferrooxidans* are shown in Table 3.

Sequence analysis of FURTA-positive clones revealed sequences with genes and/or promoters known to be regulated by Fur in other organisms such as the ferrous iron transporters *feoPABC* and *mntH*, several ferri-siderophore transport components (*tdr*, *tonB*, *abc*) and a variety of proteins that use iron as a cofactor in the form of heme groups or Fe–S clusters (*fdx1*, *hemN*, *hppH*, *dsrK*, *hdrC*, *iscR*). In addition, the majority of these sequences contained predictions for Fur boxes according to information theory screens, based on a heterologous Fur box weight matrix, and/or by HMM profile searches

based on EMSA-validated *A. ferrooxidans* Fur boxes. These experimental results provide evidence that (i) there are several genomic sequences in *A. ferrooxidans* carrying Fur-binding site motifs, (ii) that at least a part of the population of Fur-binding sites present in *A. ferrooxidans* are recognizable by the Fur protein of *E. coli in vivo* and (iii) that several of the Fur-binding sites found within these genomic fragments coincide with the bioinformatically predicted Fur boxes presented above (Table 3).

Fur_{AF} binding to candidate Fur boxes (EMSA). Gel shift assays with purified regulatory proteins is a well recognized method to reveal putative DNA-binding sites in a DNA probe that represent direct targets for the concerned regulator. To evaluate the functionality of the predicted Fur boxes, we assayed the capacity of *A. ferrooxidans* purified Fur protein to form Fur_{AF}–DNA complexes *in vitro* using the EMSA.

Fur_{AF} was able to shift the promoter regions of the iron acquisition genes *gloA* and *feoB* (Figure 3A). Probes encompassing the Fur box predicted upstream of the transporter genes *copB* and *abcS4* (Figure 3B), the transcriptional regulators *iscR* and *phoB* (Figure 3C) and the iron-containing protein encoding genes *hppH*
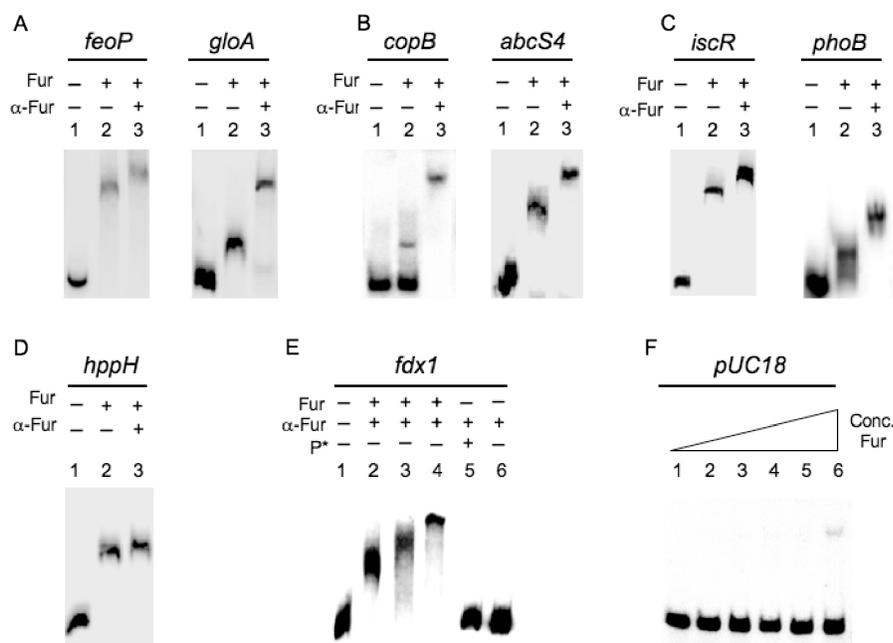
Figure 3. EMSAs of ^{32}P-labeled DNA fragments (probes) containing predicted Fur boxes and promoters of (**A**) *gloA* and *feoP*, (**B**) *copB* and *abcS4*, (**C**) *iscR* and *phoB*, (**D**) *hppH* and (**E**) *fdx1* in the presence of Fur$_{AF}$ (Fur), Fur$_{AF}$ plus anti-Fur serum (α-Fur) or cold probe (P*) as indicated. (**F**) EMSA of a ^{32}P-labeled probe containing no predicted Fur box and corresponding to a low G + C DNA fragment of pUC18 vector. 1 = probe DNA, (A–D) 2 = shift with 300 nM Fur$_{AF}$, 3 = supershift with anti-Fur antibody; (E) 2 = shift with 300 nM Fur$_{AF}$, 3 = shift with 400 nM Fur$_{AF}$, 4 = supershift, 5 = cold probe competition, 6 = absence of shift in the presence of the anti-Fur antibody alone. (F) absence of shift in the presence of: 2 = 100 nM Fur$_{AF}$, 3 = 200 nM Fur$_{AF}$, 4 = 300 nM Fur$_{AF}$, 5 = 400 nM Fur$_{AF}$ and occurrence of shift in the presence of 6 = 500 nM Fur$_{AF}$.

(Figure 3D) and *fdx1* (Figure 3E) were bound and shifted *in vitro* by 300 nM purified Fur$_{AF}$. No Fur$_{AF}$-dependent shift was achieved with the probe for the predicted *copA* Fur box, indicating that Fur$_{AF}$ is unable to bind the region under the conditions used or else that this Fur box prediction is a false positive.

These reactions were demonstrated to be Fur specific by supershifting in the presence of anti-Fur antiserum (Figure 3, A–D lane 3; E lane 4). A complete loss of the shift was also observed when excess unlabeled DNA probe was used to compete out the labeled probe (Figure 3, E lane 5). No effect on the migration of the labeled probes could be detected in the absence of purified Fur$_{AF}$ (Figure 3, A–E lane 1) or in the presence of the antiserum alone (Figure 3, E lane 6). Purified Fur$_{AF}$ up to 400 nM was unable to shift a non-specific probe (no Fur box prediction) (Figure 3, F lanes 2–5). At 500 nM Fur$_{AF}$ non-specific binding was observed to occur (Figure 3, F lane 6). Therefore, an upper limit of 400 nM Fur$_{AF}$ was set for specific binding assays.

EMSA results confirm that DNA fragments containing the predicted Fur boxes for *gloA*, *feoB*, *copB*, *abcS4*, *iscR*, *phoB*, *hppH* and *fdx1* could be recognized by purified Fur$_{AF}$ *in vitro* and can thus be considered as *bona fide* sites. An additional predicted Fur box associated with *mntH* has been previously validated by EMSA (36).

Transcriptional repression of Fur-dependent promoters by Fur$_{AF}$ using in vitro transcription assays (IVTA). To evaluate the effect of Fur$_{AF}$ binding on transcription expression, predicted EMSA Fur targets were analyzed

by *in vitro* transcription in the presence of the metal co-repressor Mn(II) (Figure 4).

All Fur boxes evaluated overlap with predicted -10 or -35 elements of the corresponding promoters (Figure 4A). In the presence of 300 nM purified Fur$_{AF}$ protein, 100 µM Mn(II) and *E. coli's* RNA polymerase, disappearance of the expected transcripts for the iron transporter genes *feoP* and *gloA*, the copper transporter *copB* and the transcriptional regulator *phoB* is observed (Figure 4B). These results indicate that *A. ferrooxidans* regulator is involved in a classical divalent metal dependent Fur-mediated repression of transcription of the genes directly linked to the tested promoters and, by implication, also of the genes clustered with them in predicted operons.

Fur-binding site consensus sequence and organization in *A. ferrooxidans*

Candidate *A. ferrooxidans* Fur-binding sites derived from both bioinformatic prediction routines (Figure 5C and D) and from experimental validations (Figure 5E) were aligned and used to derive the corresponding logos. *Acidithiobacillus ferrooxidans* HMM-predicted species-specific Fur boxes, and the nine targets bound *in vitro* by Fur$_{AF}$ (including previously validated *mntH*), are conserved with respect to the *E. coli* Fur box consensus (Figure 5A) in 10–12 out of 19 positions including those deemed critical for Fur binding (31,32,54).

Each of the candidate *A. ferrooxidans* Fur box sequences contains a conserved core region encompassing
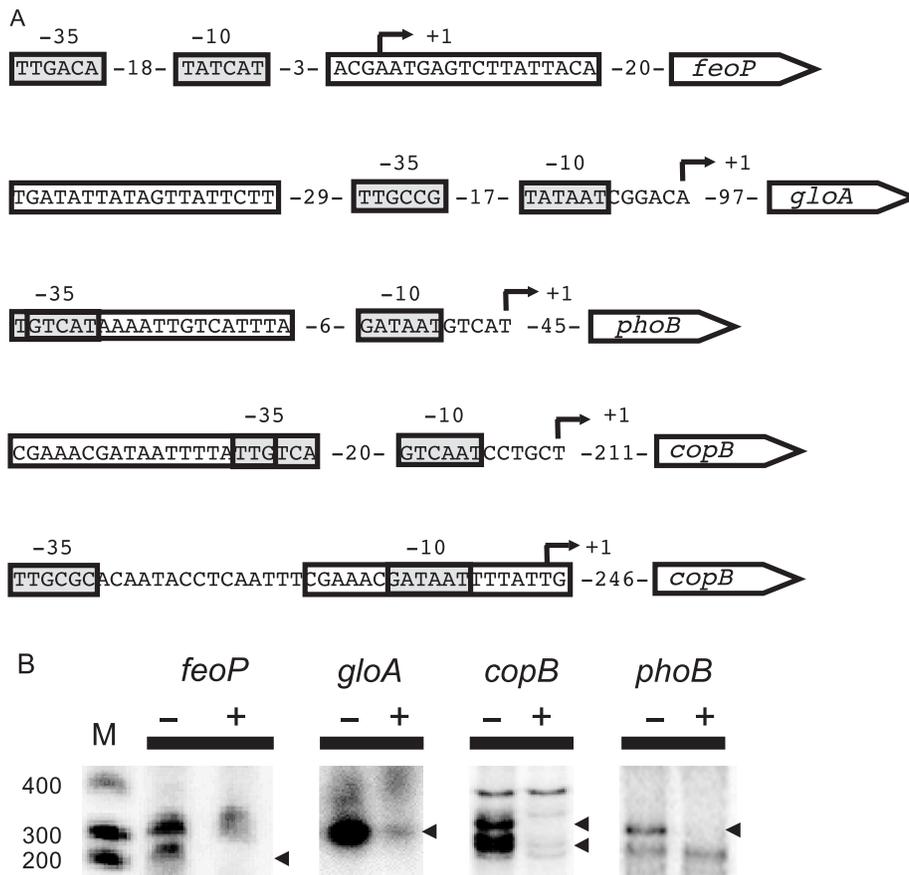
Figure 4. *In vitro* transcription assays (IVTAS) of genes with predicted Fur boxes. (**A**) Scheme of each gene showing the sequence and relative position of the predicted Fur box with respect to cognate promoters. (**B**) IVTAs in the presence (+) or absence (−) of 300 nM Fur_{AF} and 100 μM Mn(II). Primers used for IVTAs are listed in Table 2. M = molecular standards in bps. Arrow heads indicate where a transcript is absent after the addition of Fur_{AF} and Mn(II).

two contiguous imperfect repeats with additional bases present in a poorly conserved third repeat offset either to the right or left of the central hexamer (Figure 5F). Although the key residues known to interact with Fur are conserved between *E. coli* and *A. ferrooxidans*, the latter Fur box consensus exhibits a less conserved third flanking hexamer suggesting either that a single dimer of Fur binds to the box or else that the architecture of the box in *A. ferrooxidans* might alter the capacity of Fur to polymerize along the site.

ADDITIONAL DISCUSSION

As part of an effort to understand iron management in the acidophilic iron-oxidizing bacterium *A. ferrooxidans*, a combined computational search and experimental analysis to identify Fur-regulated genes was undertaken (Figure 1). This approach is particularly important given the lack of amenable genetic systems to manipulate *A. ferrooxidans* that impair classical genetic approaches to study Fur regulation. However, experimental validation of candidate Fur boxes is also essential to overcome the limitations of bioinformatic approaches imposed by the reduced information content and considerable sequence variability of Fur boxes. However, once a predicted site has been demonstrated to be functional, then that site can be employed to generate a revised binding site weight

matrix to be used in a new global search for the identification of stronger regulatory signatures. Such a pipeline has been successfully introduced in the work described herein.

Fur-control is a common feature of ferrous iron and ferri-siderophore transporting genes in other bacteria (55–58). Thus, the occurrence of a functional Fur regulator (36) and of several predicted specialized high-affinity systems for the acquisition of iron (59) provided initial evidence that a Fur-dependent regulatory network was present in *A. ferrooxidans*. The strategy devised herein enabled the recognition of cognate Fur-binding sites upstream of these previously identified iron acquisition genes and also uncovered several novel Fur targets specific for *A. ferrooxidans*. This new evidence not only provides a better understanding of the Fur regulon and the iron homeostasis control mechanisms in *A. ferrooxidans*, but also provides the first consensus Fur box in acidophilic iron-oxidizers.

Among the high affinity iron acquisition systems that constitute part of the *A. ferrooxidans* Fur regulon, the following were identified herein: a FeoB Fe(II) trans-porter, a *gloA*-linked TonB-dependent Fe(III)-siderophore transporter and several additional Fe(III)-siderophore transport components. A well conserved Fur box was recognized upstream of a predicted *feoPABC* gene cluster (Table 3, Figure 2) and was shown to bind Fur (Figure 3)
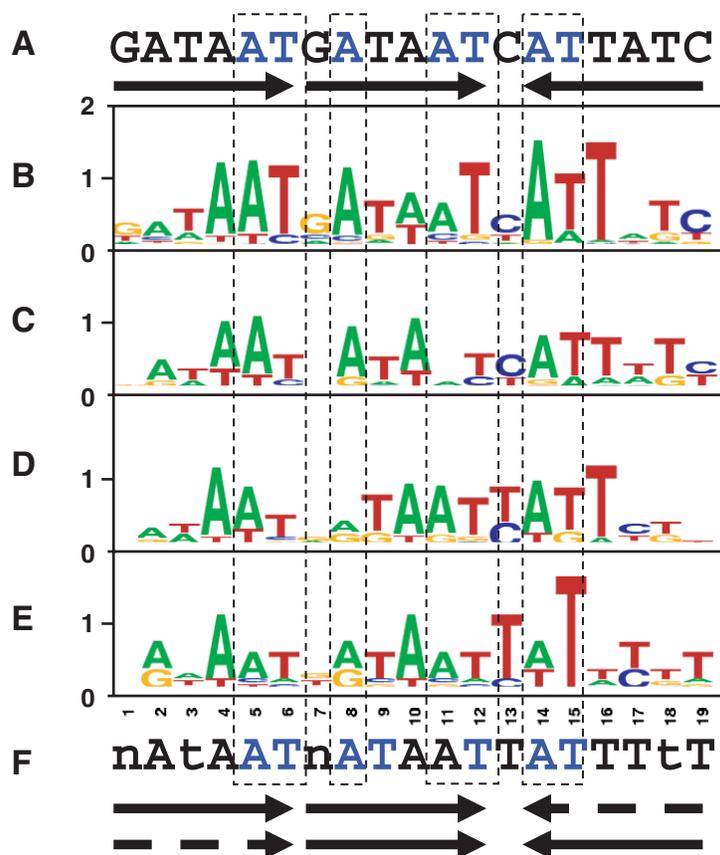
Figure 5. DNA sequence logos of *A. ferrooxidans* Fur-binding sites derived from several bioinformatic and experimental prediction strategies. (**A**) *E. coli* Fur box consensus sequence (3), (**B**) Heterologous training set logo (66 Fur boxes, Supplementary Data S1A), (**C**) *A. ferrooxidans* information-theory-based logo (90 Fur boxes, Supplementary Data S1B), (**D**) *A. ferrooxidans* HMM-based logo (79 Fur boxes, Supplementary Data S1D), (**E**) *A. ferrooxidans* Fur box logo derived for EMSA-validated genes (9 Fur boxes), (**F**) *A. ferrooxidans* consensus Fur-binding sequence. Boxed and blue letters represent bases that are protected by Fur DNA binding in *E. coli* (31,32,54).

and to overlap with its promoter (Figure 4). Divalent metal-Fur-dependent repression of *feoP* was similar to that observed in other bacteria where expression of the *feo* gene cluster has been shown to vary in accordance with ferrous iron bioavailability in a Fur-dependent manner (60–63) under anaerobic (64) or acidic-microaerobic growth conditions (65). In addition, a high scoring Fur box associated with a *gloA*-linked ferri-siderophore transporter (Table 3, Figure 2) was bound by Fur *in vitro* (Figure 3) and Fur binding to this site down regulated transcription of the transporter (Figure 4). This evidence shows that the *A. ferrooxidans* TonB-dependent iron uptake system is probably regulated in a classical iron and Fur dependent way and suggests that *A. ferrooxidans* is able to scavenge iron from the environment if necessary as has been described for well-studied neutrophilic bacteria (66). This novel finding raises the question as to what these iron-scarce environmental conditions might be, given that it is generally considered that *A. ferrooxidans* inhabits principally iron-rich acidic environments. It might be that *A. ferrooxidans* needs to compete occasionally for Fe(III) with other microorganisms in iron-poor environments while using sulfur as its principal energy source or else that these TonB-regulated systems confer *A. ferrooxidans* with a special capacity

to acquire Fe(III) when Fe(II) concentrations begin to drop due to its oxidation. This property, might in turn, render *A. ferrooxidans* more sensitive to the high Fe(III) concentrations that are generated after prolonged bio-oxidation of minerals in tank reactors (67). Both possibilities require exploration.

The occurrence of predicted Fur boxes upstream of a number of *nif* and *hup* genes (Table 3) suggests that Fur acts specifically in *A. ferrooxidans* to modulate the use of iron as a cofactor by the FeMo nitrogenase and the NiFe hydrogenase. These results are in agreement with the evidence presented for *Shewanella oneidensis* (19) and *E. coli* (4) showing (positive) Fur regulation of *nifS* and the hydrogenase-2 operon *yybOA-G*, respectively. Other Fur target genes, coding for redox active proteins that carry iron as Fe–S clusters or heme groups (e.g. certain ferredoxins), provide evidence that Fur-dependent control of the expression of iron-containing proteins also constitutes part of the global iron homeostatic mechanism in *A. ferrooxidans* as has been found in other microorganisms (4). Interestingly, the transcriptional regulators Irr and IscR, that control heme biosynthesis and Fe–S cluster assembly and/or repair respectively, also constitute part of the *A. ferrooxidans* Fur regulon. This finding suggests that Fur$_{AF}$, in addition to controlling metaloprotein

expression, could be indirectly adjusting cofactor production in response to changes in iron availability through coordinated control of specific secondary regulators.

In addition, the results presented herein are in agreement with reports in other microorganisms showing that Fur controls the expression of genes belonging to several functional classes besides iron transport and utilization (2). Our global search of Fur boxes in the genome of *A. ferrooxidans* yielded a substantial number of putative boxes that were associated with functions that are not *a priori* related to iron metabolism but which can be clearly related to metal homeostasis. Several of the Fur regulatory targets identified in *A. ferrooxidans* are efflux transporters driven either by ATP or by protons, with specificities other than iron. As a strict acidophile *A. ferrooxidans* has to face the high concentrations of iron required for its energetic metabolism, but also it has to deal with the high concentrations of most other metals found in its environment, notably copper.

The most important metal recovered in industrial bioleaching operations is copper (68). Copper is bioleached as soluble copper sulfate from insoluble copper sulfides by acid and ferric iron. *A. ferrooxidans*, and other acidophilic iron oxidizers, contribute to the solubilization of copper by generating ferric iron and sulfuric acid as by-products of metabolism and in a typical bioleaching operation are confronted with soluble copper concentrations that may exceed 600 mM (69). Copper-adapted cell shows decreased Cu(II) accumulation (70), suggesting that the Cu(II) is excluded from the cell possibly via an inducible efflux system. The Cu efflux P-type ATPase CopB and the Cus-like RND system identified herein could contribute to this task.

Physiologically, copper is an essential metal ion required as a cofactor for enzymes such as the copper-heme family of cytochrome c oxidases (that also carry Fe–S clusters) and electron transporting blue copper proteins like rusticyanin (5% of the total soluble protein in *A. ferrooxidans*). However, as with other redox active metals, use of copper in aerobes is inevitably accompanied by oxidative stress in the absence of tightly regulated homeostatic mechanisms (71,72). In this context, a coordinated homeostatic response to both iron and copper mediated by Fur would be beneficial both for energetic metabolism and for cell survival. Alternatively, Fur could be regulating the expression of the copper trafficking genes via metal-independent mechanisms, such as in response to acid shock (73,74). Two independent observations support this view: (i) studies in the acid tolerant *Lactobacillus bulgaricus* (75) and *H. pylori* (76) have shown that acidification of the growth medium significantly induced the expression of *cop* orthologs by an unknown mechanism and (ii) a Fur box has been described for *copA* in *H. pylori* (77).

Several of the Fur target genes identified in *A. ferrooxidans*, are transcriptional regulators. Thus, many genes are probably controlled indirectly by Fur. This finding implies that Fur acts as a master regulator of iron-dependent gene expression in *A. ferrooxidans*. An iron-responsive global regulator, like Fur, could aid in the control of metal transport functions that in general

entail a risk for *A. ferrooxidans* by coordinating the expression of a variety of metal uptake and efflux systems when iron, and probably other metals are in excess, either directly or through a set of secondary regulators. Consistent with this suggestion is the evidence indicating that metal toxicity for *A. ferrooxidans* is dependent on the growth substrate; for example, cultures growing on Fe(II) rather than on thiosulfate are more metal resistant (78,79).

During chemolithoautotrophic growth, *A. ferrooxidans* oxidizes ferrous iron to ferric iron as an energy source. Therefore, iron uptake systems involved in the assimilation of iron for biosynthesis must be fine-tuned with iron utilizing proteins implicated in ferrous iron oxidation. In the initial stages of growth at pH 2, when ferrous iron is highly abundant, sensitive control of the expression levels of these systems by Fur is expected to allow a sufficient provision of iron for biosynthesis yet avoiding intracellular overloads that would cause severe oxidative stress (80). Intracellular copper levels might be fine-tuned with intracellular iron level, through Fur-dependent balancing of the uptake and efflux of this metal. Such a coordinated response might ensure that both metals, needed as cofactors for many energy-metabolism proteins, are simultaneously available in appropriate concentrations. However, as iron levels increase above a certain 'repression-threshold,' Fur might turn off high affinity Fe(II) uptake systems and copper transporters to escape oxidative threats. In addition, as growth proceeds and ferrous iron is oxidized to Fe(III), *A. ferrooxidans* might need to switch to the uptake of ferric iron. Alternatively, during growth of *A. ferrooxidans* via the oxidation of sulfur at higher pHs (3.5–5.5), where iron availability might become limiting both as an energy source and as a nutrient, it is hypothesized that the ferrous iron oxidizing machinery will need to be turned off while ferric iron acquisition systems might be activated to scavenge for iron traces and efficiently compete with other microorganisms present in its niche. Under these conditions efficient intracellular removal of xenosiderophores might become necessary, either to efficiently recycle these high affinity chelators or for cell detoxification. Fur-dependent regulation, coupling the expression levels of all these systems driven by iron oxidation during *A. ferrooxidans* growth, is likely to be a key aspect for iron homeostasis and survival in this bacterium.

SUPPLEMENTARY DATA

Supplementary Data are available at NAR online.

ACKNOWLEDGEMENTS

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