Identification and Unusual Properties of the Master Regulator FNR in the Extreme Acidophile Acidithiobacillus ferrooxidans

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The ability to conserve energy in the presence or absence of oxygen provides a metabolic versatility that confers an advantage in natural ecosystems. The switch between alternative electron transport systems is controlled by the fumarate nitrate reduction transcription factor (FNR) that senses oxygen via an oxygen-sensitive [4Fe-4S]\textsuperscript{2+} iron-sulfur cluster. Under O\textsubscript{2} limiting conditions, FNR plays a key role in allowing bacteria to transition from aerobic to anaerobic lifestyles. This is thought to occur via transcriptional activation of genes involved in anaerobic respiratory pathways and by repression of genes involved in aerobic energy production. The Proteobacterium Acidithiobacillus ferrooxidans is a model species for extremely acidophilic microorganisms that are capable of aerobic and anaerobic growth on elemental sulfur coupled to oxygen and ferric iron reduction, respectively. In this study, an FNR-like protein (FNR\textsubscript{AF}) was discovered in At. ferrooxidans that exhibits a primary amino acid sequence and major motifs and domains characteristic of the FNR family of proteins, including an effector binding domain with at least three of the four cysteines known to coordinate an [4Fe-4S]\textsuperscript{2+} center, a dimerization domain, and a DNA binding domain. Western blotting with antibodies against Escherichia coli FNR (FNR\textsubscript{EC}) recognized FNR\textsubscript{AF}. FNR\textsubscript{AF} was able to drive expression from the FNR-responsive \textit{E. coli} promoter P\textsubscript{narG}, suggesting that it is functionally active as an FNR-like protein. Upon air exposure, FNR\textsubscript{AF} demonstrated an unusual lack of sensitivity to oxygen compared to the archetypal FNR\textsubscript{EC}. Comparison of the primary amino acid sequence of FNR\textsubscript{AF} with that of other natural and mutated FNRs, including FNR\textsubscript{EC}, coupled with an analysis of the predicted tertiary structure of FNR\textsubscript{AF} using the crystal structure of the related FNR from Aliivibrio fischeri as a template revealed a number of amino acid changes that could potentially stabilize FNR\textsubscript{AF} in the presence of oxygen. These include a truncated N terminus and amino acid changes both around the putative Fe-S cluster.
coordinating cysteines and also in the dimer interface. Increased O\textsubscript{2} stability could allow \textit{At. ferrooxidans} to survive in environments with fluctuating O\textsubscript{2} concentrations, providing an evolutionary advantage in natural, and engineered environments where oxygen gradients shape the bacterial community.

Keywords: fumarate nitrate reductase, anaerobic regulation, transcriptional regulation, DNA binding, iron-sulfur cluster, biomining, microbial ecology

INTRODUCTION

A central challenge in microbial ecology is to understand how microorganisms interact in complex communities, including how they respond to dynamically changing environments. Answers to this challenge are important for addressing issues such as the role of biogeochemical reactions in nutrient and energy cycling and in understanding ecosystem functioning in earth, ocean, and atmospheric environments (Widder et al., 2016). However, it is difficult to model such systems because of their complexity as well as that their experimental investigation in the field may require long time scales, measured in years to centuries, e.g., ecological succession.

Hyperacidic environments (pH < 3) provide an advantage over neutral milieu for addressing these issues as they generally exhibit low microbial diversity (Mendez-Garcia et al., 2015; Teng et al., 2017). This facilitates data collection, observation, and experimental exploration of ecological models over periods measured in weeks or months and simplifies model building of microbial interactions. One such environment is bioleaching heaps (termed “bioheaps”) that exploit acidophilic microorganisms’ metabolism (Bonnefoy and Holmes, 2012; Dopson and Johnson, 2012) to catalyze commercial metal recovery from sulfide minerals in many parts of the world (Brierley and Brierley, 2013; Vera et al., 2013). Bioheaps offer additional advantages for studying microbial community function and dynamics as they are subjected to dynamically changing conditions including levels of heavy metals, acidity, CO\textsubscript{2}, temperature, nutrients, and available redox couples for growth (Dopson et al., 2009; Valdés et al., 2010; Riekkola-Vanhainen, 2013; Tupikina et al., 2013; Dopson and Holmes, 2014). In addition, bioheap microbes are challenged by a gradient of O\textsubscript{2} availability when thick biofilms are formed (Baker-Austin et al., 2010), due to limitations of O\textsubscript{2} gas–liquid mass transfer that are exacerbated at higher temperatures (Petersen, 2010), and decreasing O\textsubscript{2} concentrations in the center of the bioheap (Yin et al., 2011).

\textit{Acidithiobacillus ferrooxidans} is a keystone bioheap species that is especially prevalent during early stage bioleaching (Demergasso et al., 2005; Remonsellez et al., 2009; Halinen et al., 2012). This is likely as it is able to grow at higher pH values than e.g., \textit{Leptospirillum ferrophilum} (Dopson, 2016) and as it fixes carbon that aids in the subsequent growth of heterotrophic acidophilic species, as has been demonstrated during co-culture of autotrophic, and heterotrophic biomining species (Nancucheo and Johnson, 2010). \textit{At. ferrooxidans} is an acidophilic, obligatory chemolithoautotrophic mesophile that gains its energy from the oxidation of ferrous iron, elemental sulfur, inorganic sulfur compounds, and hydrogen (Bonnefoy and Holmes, 2012; Dopson and Johnson, 2012; Hedrich and Johnson, 2013). \textit{At. ferrooxidans} is a facultative anaerobe that grows under aerobic and anaerobic conditions and the main electron transport components couple the aerobic oxidation of iron and sulfur to the reduction of O\textsubscript{2} (Quatrini et al., 2009) and the anaerobic oxidation of elemental sulfur to reduction of ferric iron (Pronk et al., 1991; Ohmura et al., 2002; Osorio et al., 2013). This switch from aerobic to anaerobic growth is expected to require a regulator of gene expression, which has not been studied in detail in \textit{At. ferrooxidans}.

The transition from oxic, to hypoxic (low concentrations of O\textsubscript{2}), and finally anoxic environments may require gene regulation systems to respond to the varying O\textsubscript{2} concentrations (Osorio et al., 2009). In addition, \textit{At. ferrooxidans} requires iron homeostasis systems (such as for the ferric iron utilized as electron acceptor) as its concentration can reach $10^{18}$-fold higher than in pH neutral environments (Osorio et al., 2008a,b). Bacterial O\textsubscript{2} sensing systems include the direct interaction of O\textsubscript{2} with membrane sensors such as FixL and the cytoplasmic transcription factor FNR (Fumarate and Nitrate Reduction), along with redox responsive regulatory systems that include, but are not limited to, ArcBA and Rex (reviewed in (Green et al., 2009; Bueno et al., 2012; Mettert and Kiley, 2018). The FNR transcription factor is a member of the cyclic AMP (cAMP) receptor protein (Crp) superfamily and plays a major role in altering gene expression between oxic and anoxic conditions (Constantinidou et al., 2006). The \textit{Escherichia coli} FNR protein (termed FNR\textsubscript{Esch}) senses O\textsubscript{2} via four cysteine residues that ligate an O\textsubscript{2}-sensitive [4Fe-4S]\textsuperscript{2+} iron-sulfur cluster in the N-terminal region and affects its regulatory function via the C-terminal helix-turn-helix (HTH) DNA binding domain (Fleischhacker and Kiley, 2011; Mettert and Kiley, 2018). In anoxic conditions, FNR is activated by Isc protein-dependent acquisition of the [4Fe-4S]\textsuperscript{2+} cluster that promotes dimerization. The dimer binds to target DNA sequences, and induces or represses transcription. As O\textsubscript{2} levels increase, the FNR [4Fe-4S]\textsuperscript{2+} cluster is degraded and the protein is converted to a monomeric form, which is no longer active in gene regulation (Crack and Le Brun, 2018; Mettert and Kiley, 2018). \textit{At. ferrooxidans} gene clusters predicted to be under the control of FNR are suggested to be involved in carbon and energy metabolism along with nitrogen fixation (Osorio et al., 2009). However, the predicted role of FNR and the mechanisms of adaption to changing O\textsubscript{2} concentrations in \textit{At. ferrooxidans} have not been experimentally tested.

Due to the importance of \textit{At. ferrooxidans} as a model organism in natural and man-made acidic environments, it is important to understand the regulation of growth, adaptation,
and extracellular electron transfer under anoxic and acidic conditions. Here, we characterized the *At. ferrooxidans* FNR master gene regulator and examined the effect of changing O$_2$ concentrations on its Fe-S cluster ligand.

## MATERIALS AND METHODS

### Bacterial Strains and Growth Conditions

*Acidithiobacillus ferrooxidans* ATCC 23270 was obtained from the American Type Culture Collection (Table 1). The strain was maintained in sterile 9K basal salts medium (sterilized by Tyndallization) adjusted to pH 3.5 with H$_2$SO$_4$ (Silverman and Lundgren, 1959) containing 0.5% (wt/vol) S$^0$ and incubated under aerobic conditions at 30°C with shaking. Anaerobic (S$^0$/Fe$^{3+}$) cultures of *At. ferrooxidans* were grown in identical medium with the exception of the addition of 25 mM ferric iron (sterile filtered through a 0.22 μm filter) as electron acceptor and the pH was adjusted to 1.8. *At. ferrooxidans* was pre-grown in aerobic conditions before transferring the cells to an anaerobic jar using the Anaeroclus A system (Merck).

*Escherichia coli* strains (Table 1) were grown on a rotary shaker in sterile LB medium at 37°C. The following antibiotics were added as required: spectinomycin (Sp; 25 μg/mL), streptomycin (Sm; 25 μg/mL), ampicillin (Ap; 50 μg/mL), and tetracycline (Tc; 10 μg/mL). For β-galactosidase assays, *E. coli* strains were grown under aerobic or anaerobic conditions at 37°C in minimal medium M9 containing 0.1% (wt/vol) glucose with the respective antibiotics. Anaerobic cultures were carried out in anaerobic jars (as described for *At. ferrooxidans*) on M9 minimal medium containing 0.1% (wt/vol) glucose.

### Ferrous Iron Production During Anaerobic Cultures

The formation of ferrous iron in anaerobic cultures was determined by titration with 2,2'-dipyridyl. Samples (1 mL) of culture medium were passed through a 0.2 μm membrane filter and 160 μL aliquots of the filtrate were added to 40 μL of 5 mM 2,2'-dipyridyl. The ferrous iron concentration was determined via a calibration curve of FeSO$_4$ × 7H$_2$O at an absorbance of 510 nm in a spectrophotometer. *At. ferrooxidans* growth was quantified by counting in a Petroff-Hausser chamber. The cells in aerobic and anaerobic cultures were quantified in triplicate cultures to construct the respective growth curves [data presented are means (n = 3) ± standard deviations].

### Bioinformatics

The amino acid sequence of FNR from *E. coli* K12 (FNR$_{EC}$; accession number, WP_000916335) was used in a BlastP search against the genome of *At. ferrooxidans* ATCC 23270. A potential FNR candidate (FNR$_{AF}$; locus tag, AFE_0270) was identified with 28% identity. Using FNR$_{AF}$ in a reciprocal best Blast hit against the NCBI nr database recovered hits against the Crp-Fnr family (domain architecture ID 11429533) from multiple organisms. A conserved domain analysis of FNR$_{AF}$ was carried out (Marchler-Bauer et al., 2013). Multiple sequence alignments of FNR$_{AF}$, FNR$_{EC}$ and FNR from *Alivibrio fisheri* (FNR$_{AFI}$, QSE593) were carried out using Clustal Omega (Sievers and Higgins, 2018) and Swiss-Model (Waterhouse et al., 2018). Where there was a difference between the two alignment methods, the alignment by Swiss-Model was chosen.

Secondary structure analysis of FNR$_{AF}$ was carried out using homology modeling by comparing the predicted protein with the FNR crystal structure from *A. fisheri* (PDB 5e44) with a sequence identity of 27.35% and full coverage of the complete protein (Volbeda et al., 2015). The homology model was constructed using Modeler V.9 (Eswar et al., 2008) and validated using the ADIT! Validation Server from PDB (Richarson et al., 2013).

### RNA Extraction and Real-Time PCR

Cells were harvested from *At. ferrooxidans* cultures (maximum 1 × 10$^9$ total cells) by centrifugation at 12000 × g for 10 min at 4°C. The pellet was washed with 10 mM H$_2$SO$_4$ and then with TE buffer pH 8.0 and finally resuspended in 100 μL of TE pH 8.0. To this mixture, 10 μL RNAse-free lysis buffer (0.5 M TrisHCl, 20 mM EDTA, 10% SDS, pH 6.8) was added and mixed gently. The tubes were incubated at 100°C for 3 min and allowed to cool to room temperature. The previous steps were sufficient to guarantee the rupture of the cells without damaging

### Table 1

| Strain or plasmid | Genotype | References or sources |
|-------------------|-----------|-----------------------|
| *At. ferrooxidans* | ATCC 23270 | Wild type |
| *Escherichia coli* | JM109 | endA1, recA1, gyrA96, thi, hsdR17 (k-, mK+), relA1, supE44, Δlac-proAB, [F’ trdA36, proAB, lacIqZAM15] |
| | BL21 | F-, ompT, hsdSB (F- mB-), gal, dcm, m13E1, (DE3) |
| | PK22 | hsdS, gal, ΔDE53, Δcrp-bs990, rpsL, Δfnr, zcu-3061::Tn10 |
| | RZ7350 | lacZ Δ145, narG234::MudI1734 |
| | RZ8480 | Δfnr, lacZ Δ145, narG234::MudI1734 |
| Plasmids | pET100/D-TOPO | Expression vector |
| | pET100/D-TOPO FNR$_{AF}$ | Expression vector, FNR$_{AF}$ protein |
| | pKK223-3 | Plasmid vector, Amp' |
| | pKK223-3 fla$_{AF}$ | fla$_{AF}$ coding region cloned into pKK223-3 vector with a tac promoter |
| | pET11A | Expression vector, Amp' with a T7 promoter |
| | pET11A fla$_{AF}$ | fla$_{AF}$ coding region cloned into pET11A vector with a T7 promoter |
the RNA. RNA was isolated using RNasy Mini Kit (Qiagen®) and contaminant DNA removed using RNase free DNase I (Fermentas) according to the manufacturer’s recommendations. The RNA was resuspended in five volumes of RNA later solution (Qiagen) and subsequently frozen at −80°C until use. RNA samples were reverse-transcribed using Revertaid M-MuLV (Fermentas) and specific oligonucleotides (Table 2) according to the manufacturer’s recommendations and 0.5 μg of total RNA for each reaction. The real-time PCR reactions were performed using an iCycler thermal cycler (Bio-Rad) and the KAPA SYBR FAST qPCR kit (KAPABIOSYSTEMS). The 20 μL PCR reactions contained 2 μL of a 1:100 diluted cDNA sample, 200 nM of each primer (Table 2), and 1 × KAPA SYBR FAST qPCR Master Mix. The reference dye ROX was included at a final concentration of 5 nM. The cycling protocol was as follows: initial denaturation for 10 min at 95°C followed by 40 cycles of 30 s each at 95°C, 56°C, and 72°C. Fluorescence was measured after the extension phase at 72°C and specific amplification was confirmed by a single peak in the melting curve. For each experimental condition, total RNA was extracted from replicate *At. ferrooxidans* cultures and the real-time PCR reactions were performed in triplicate and thus, the data sets consist of six values per gene. Relative expression levels of *At. ferrooxidans fnr* (amplified with qPCR fnr FF and qPCR fnr REV primers; Table 2) were normalized with the expression of the stable reference gene *rpoC* (amplified with qPCR rpoC FF and qPCR rpoC REV primers; Table 2). The rpoC gene has previously been demonstrated to be expressed at a constant level and is a valid choice as a reference (Nieto et al., 2009). Stationary phase genomic DNA (10-fold dilutions ranging from 10 ng to 1 pg) was used to generate a five-point standard curve for each gene by using the Cycle Threshold (Ct) value vs. the logarithm of each dilution factor. Reaction efficiency (E = [10−(−1/slope)]−1) for every gene was derived from the slope of the corresponding standard curves. A one-way ANOVA (multiple comparison analysis) or a two-way ANOVA was carried out under anaerobic conditions in a Coy anaerobic jar until the cell density reached an optical density of 400 μM for 1 h to induce FNR biosynthesis. After induction, cells were sparged overnight at 4°C with argon to remove the presence of O₂. All subsequent steps in FNR purification were carried out under anaerobic conditions in a Coy anaerobic chamber with an atmosphere of 90% N₂ and 10% H₂ or in sealed tubes. The cells were harvested by centrifugation at 7,900 g for 15 min at 4°C and concentrated 200-fold in buffer A [50 mM potassium phosphate (pH 6.8), 0.1 mM

### Cloning Procedures

*Acidithiobacillus ferrooxidans* genomic DNA was prepared using the Wizard®-Genomic DNA Purification Kit (Promega Corp.). Plasmid DNA was prepared from *E. coli* JM109 cultures with the QIAprep®Spin MiniPrep Kit (Qiagen). PCR products for cloning were amplified using oligonucleotides in Table 2 and purified from agarose gels with the SpinPrepTM Gel DNA Kit (Novagen).

To carry out β-galactosidase assays, the coding region of the *At. ferrooxidans fnr* gene (termed *fnrAF*) was amplified with primers containing embedded EcoRI restriction sites: pKK FF *fnr*-EcoRI and pKK REV *fnr*-EcoRI (Table 2). Cloning and transformation was carried out using standard techniques as described by Miller (1972). The amplification product was cloned into the multiple cloning site of pKK223-3 carrying the P*lac* promoter (Pharmacia Biotech), generating pKK223-3 *fnrAF*.

pKK223-3 *fnrAF* was transformed into *E. coli* strain RZ8480

| Name | Sequence (5’−3’) | Function |
|------|-----------------|----------|
| pKK FF *fnr*-EcoRI | ATCGATGATTCTACTGGACGACGGCGACC | Cloning |
| pKK REV *fnr*-EcoRI | ATCGATGTTCTAGGCGGGGTCG | Cloning |
| *fnr* FF-Ndel | CATATTGACGCGGAGACC | Cloning |
| *fnr* REV-Ndel | CATATTGTCATTGACATATCCAA | Cloning |
| qPCR *fnr* FF | AGCTGTCGAAGTCTGTCGCCAAC | RT-qPCR |
| qPCR *fnr* REV | TGCGGTCAAGTAATGCACTAT | RT-qPCR |
| qPCR *rpoC* FF | ATTGGCTGGTGGGATGGAAAC | RT-qPCR |
| qPCR *rpoC* REV | AGGTACGTGTCCTGCTTAAG | RT-qPCR |

which is Δfnr and contains lacZ under control of the PnarG promoter (Lazazzera et al., 1993). Finally, the *fnrAF* coding region was cloned from PKK223-3 FNRAF into the pET100/D-TOPO expression vector to generate pET100/D-TOPO FNRAF that was subsequently used as a control in the Western blot analysis.

For the construction of the plasmid pET11A *fnrAF* the *At. ferrooxidans fnr* gene coding region was amplified with primers containing embedded Ndel restriction sites *fnr* FF-Ndel and *fnr* REV-Ndel (Table 2). The product was cloned into pET11A (Novagen), generating plasmid pET11A *fnrAF* (Table 2). The construct was then transformed into *E. coli* strain PK22 (Lazazzera et al., 1993) for FNRAF purification experiments. *E. coli* strain PK22 was used because it lacks both FNRREC and the structurally related CRP (cAMP-activated global transcriptional regulator) that could potentially contaminate the preparation of FNRAF (Lazazzera et al., 1993).

### β-Galactosidase Assays

*Escherichia coli* was pre-grown aerobically overnight in M9-glucose medium (Sambrook et al., 1989). The medium (10 mL) was inoculated with 1% (vol/vol) seed culture and incubated in anaerobic jars until the cell density reached an optical density at 600 nm (OD600) of 0.8. The cultures had not yet achieved stationary phase since the cell mass increased at least twofold with further incubation. β-galactosidase assays were performed as described by (Miller, 1972) using chloroform and 0.1% sodium dodecyl sulfate to permeabilize the cells. All β-galactosidase assay results are the average of triplicate samples for each strain ± standard deviations.

### Purification of FNRAF

FNR-like protein was purified from strain PK22 carrying pET11A-*fnrAF* using the anaerobic protocol developed for FNRREC. The cells were grown aerobically in 4 L of M9 minimal medium plus 0.2% (wt/vol) glucose and ampicillin at 37°C to an OD600~0.3, and IPTG was added to a final concentration of 400 μM for 1 h to induce FNR biosynthesis. After induction, cells were sparged overnight at 4°C with argon to remove the presence of O₂. All subsequent steps in FNR purification were carried out under anaerobic conditions in a Coy anaerobic chamber with an atmosphere of 90% N₂ and 10% H₂ or in sealed tubes. The cells were harvested by centrifugation at 7,900 g for 15 min at 4°C and concentrated 200-fold in buffer A [50 mM potassium phosphate (pH 6.8), 0.1 mM

http://www.graphpad.com/scientific-software/prism/
EDTA, 0.1 M KCl, 10% glycerol, 1 mM dithiothreitol (DTT), and 0.1 mM phenylmethanesulfonylfloride (PMSF), and passed once through a French press at 20,000 psi. The extracts were centrifuged at 139,000 g for 1 h to remove the membrane fraction. Cell extracts were passed over a 5 mL Bio-Rex 70 cation-exchange column (BioRad Laboratories) at a flow rate of 0.17 mL/min and eluted with a 70 mL linear gradient of 0.1 to 1 M KCl in buffer A. Fractions containing a green color were pooled and diluted 1:4 with buffer C [50 mM phosphate (pH 6.8) plus 10% (vol/vol) glycerol] and loaded onto a 1 mL BioRex-70 gravity column, washed with 2 column volumes of buffer A, and eluted with 1 column volume of buffer B [50 mM phosphate (pH 6.8), 10% (vol/vol) glycerol, and 1 M KCl]. The purity of the FNR protein preparations was estimated from Coomassie-stained SDS-polyacrylamide gels. The protein concentration was estimated by a Bradford assay using the Coomassie Blue protein assay [Bio-Rad Laboratories] using BSA as standard.

Iron determinations in FNRAF were performed by the TPTZ method which forms a deep blue-purple color with ferrous iron that is spectrophotometrically measured at 562 nm as previously described (Yan and Kiley, 2009).

**RESULTS AND DISCUSSION**

**Anaerobic Growth of *At. ferrooxidans* ATCC 23270**

A comparison of *At. ferrooxidans* growth in oxic and anoxic conditions was performed in which elemental sulfur oxidation was coupled, respectively to reduction of O2 or Fe3+ as final electron acceptors. Despite the fact that both cultures reached similar levels of cell density, a reduced growth rate was observed when *At. ferrooxidans* used Fe3+ anaerobically instead of O2 as terminal electron acceptor (Figure 1). During anaerobic reduction of Fe3+, the amount of Fe2+ rose to a maximum of 228 ± 4 µM at stationary phase in the presence of *At. ferrooxidans* compared to 4 ± 4 µM Fe2+ in the un-inoculated control. These growth curves confirm and extend earlier observations (Osorio et al., 2013).

**Western Blotting**

Aliquots of total protein extract and purified FNR protein (approximately 10 µM of protein) were separated by SDS-PAGE with either 15% or 18% acrylamide (total acrylamide/bisacrylamide) 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-FNR serum generated against FNRSC. Filters were blocked overnight in blocking solution [5% skimmed milk, 0.05% Triton X-100, and Tris-buffered saline (TBS)] at 4°C with agitation, incubated for 1 h with a 1:500 dilution of the primary antibody in TBS/Tween 20 (0.05%) and further incubated in a 1:15,000 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.

**UV-Visible Spectroscopy**

To measure the absorbance of the FNR, 1 mL of the protein stored under anaerobic conditions was used at a concentration of 10 µM in 50 mM phosphate buffer pH 6.8 containing 0.4 M KCl and its absorbance was recorded between 200 to 700 nm in a Lambda 25 UV/Vis spectrophotometer (PerkinElmer). The impact of O2 on the spectral characteristics of FnraF was evaluated by exposure of the sample to air for 0, 30, 90, 120, 150, 180, and 210 min and the absorbance spectra between 200 and 700 nm was recorded. As a control, the absorbance of the protein under anaerobic conditions was measured over the same time period in order to rule out other environmental factors causing a change in the protein’s spectral properties.

Iron determinations in FNRAF were performed by the TPTZ method which forms a deep blue-purple color with ferrous iron that is spectrophotometrically measured at 562 nm as previously described (Yan and Kiley, 2009).
Primary and Secondary Structure Analysis of FNR\textsubscript{AF}

The amino acid sequence of FNR\textsubscript{EC} from \textit{E. coli} K12 (accession number: WP_000916335) was used in a BlastP search against the genome of \textit{At. ferrooxidans} ATCC 23270. A potential FNR\textsubscript{AF} candidate (A0E_0270) was identified with 28% identity. Using FNR\textsubscript{AF} in a reciprocal best Blast hit against the NCBI nr database recovered hits against the Crp-Fnr family of transcriptional regulators (domain architecture ID 11429533) from multiple organisms.

A conserved domain analysis of FNR\textsubscript{AF} (Marchler-Bauer et al., 2013) and a comparison of its primary amino acid sequence with FNR\textsubscript{EC} [as reviewed in (Crack and Le Brun, 2018; Mettert and Kiley, 2018)] showed that it contained the following motifs characteristic of an FNR-like protein:\textsuperscript{1} (i) three of the four cysteines (positions 20, 23, and 122, using a numbering system based on the FNR\textsubscript{EC} sequence) potentially forming part of an Fe-S cluster binding domain involved in coordinating an [4Fe-4S]\textsuperscript{2+} center; (ii) a dimerization helix; and (iii) a DNA binding domain (Figure 2).

FNR\textsubscript{AF} Can Drive Expression From the FNR-Responsive \textit{E. coli} Promoter P\textsubscript{narG}

Although bioinformatic analyses strongly support the contention that FNR\textsubscript{AF} is an FNR-like protein, we investigated whether FNR\textsubscript{AF} could complement a mutant strain that lacked \textit{fnr}, providing evidence for its function. It is difficult to generate a \textit{Δfnr} mutant of \textit{At. ferrooxidans} as the organism is challenging to manipulate genetically (Inaba et al., 2018) as reviewed in (Gumulya et al., 2018). Therefore, we chose to complement an \textit{E. coli} strain RZ8480 lacking \textit{fnr} (\textit{Δfnr}). To accomplish this, a plasmid pKK223-3 \textit{fnrAF} was constructed containing the predicted \textit{fnrAF} coding sequence fused to the IPTG inducible promoter Ptac, and was transformed into RZ8480 (\textit{Δfnr}, P\textit{narG}-lacZ). This strain contains lacZ, under the control of the FNR inducible \textit{PnarG} promoter (Figure 3A). Therefore, when a functional FNR is cloned and expressed in \textit{E. coli} strain RZ8480, it can induce the expression of lacZ, giving rise to a measurable β-galactosidase activity.

\textit{Escherichia coli} strain RZ8480 transformed with pKK223-3 \textit{fnrAF} was grown anaerobically until mid-log and β-galactosidase activity was measured after induction with 0.5 mM IPTG for 1 hr (Figure 3B). This activity was compared to that of \textit{E. coli} strain RZ7350 that contains a native \textit{fnr} and the \textit{PnarG}-lacZ allele. We observed that β-galactosidase was expressed in the recombinant strain harboring FNR\textsubscript{AF}, indicating FNR\textsubscript{AF} is able to drive expression from the \textit{PnarG} promoter. However, expression from \textit{PnarG} is about 70% of that produced by FNR\textsubscript{EC}. One-way ANOVA (multiple comparisons analysis) was used to test the statistical significance yielding \(p < 0.05\). Possible explanations for the observed decrease in expression are that there are important amino acid and/or structural differences between the respective FNRs or the architecture of the respective FNR binding sites are different. Alternatively, since \textit{Ptac} is a strong promoter, a certain amount of expressed FNR\textsubscript{AF} could be present in the cell in a non-soluble form potentially accounting, at least in part, for the lower activity.

As expected, little β-galactosidase activity was detected in the \textit{E. coli} strain RZ8480 transformed with the vector only control (pKK223-3 lacking \textit{fnrAF}). Thus, \textit{fnrAF} is driving the expression of β-galactosidase and hence is capable of complementing \textit{Δfnr}\textsubscript{EC}.

The observation that \textit{fnrAF} can regulate expression from the \textit{E. coli} \textit{PnarG} promoter provides evidence that it could potentially be involved in the regulation of anaerobic metabolism in \textit{At. ferrooxidans} as has been observed in a number of organisms. However, as yet, there is no experimental evidence to test this hypothesis because of the difficulties involved in genetic manipulation of this organism.

Transcription Levels of \textit{At. ferrooxidans fnr} in Different Growth Conditions

Having demonstrated that FNR\textsubscript{AF} is functional in a surrogate host, we wished to evaluate whether it was expressed in cell cultures of \textit{At. ferrooxidans} and if so, under what conditions. Whole cell RNA was prepared from cells in four culture conditions: (i) anaerobic exponential growth; (ii) aerobic exponential growth; (iii) anaerobic stationary phase; and (iv) aerobic stationary phase and was quantified by RT-qPCR using the housekeeping gene \textit{rpoC} mRNA as an internal standard.

\textsuperscript{1}www.uniprot.org/uniprot/P0A9E5
the two FNRs have similar structural regions that the antibody into E. coli FNR EC Antibodies prepared against FNR Purification and Biochemical is not auto-regulated. Frontiers in Microbiology | www.frontiersin.org 7 July 2019 | Volume 10 | Article 1642

RNA transcripts of FNR AF (Osorio et al. FNR in Exponential), in the stationary phase in both anaerobic and aerobic conditions fnr for regulating the level of fnr the growth phase and the presence or absence of O₂ fnr known transcription factor binding sites of Figure 4). In anaerobic stationary phase, the number of RNA transcripts depending on fnr AF transcripts in aerobic and anaerobic conditions. The ability to sense and adapt to changes in O₂ concentration is critical for the regulatory function of FNR proteins. The ability of FNR to function as a transcription factor depends on the integrity of the [4Fe-4S]²⁺ cluster, which promotes a conformation amenable for dimerization, site-specific DNA binding, and transcriptional regulation [reviewed in (Crack and Le Brun, 2018; Mettert and Kiley, 2018)]. The O₂ sensitivity of FNR is mediated by the [4Fe-4S]²⁺ cluster whereby in the presence of O₂, the [4Fe-4S]²⁺ cluster is converted to [2Fe-2S]²⁺ both in vitro and in vivo. The [2Fe-2S]²⁺ form of FNR is monomeric in solution and is inactive for DNA binding and transcriptional regulation (Jordan et al., 1997; Khoroshilova et al., 1997; Popescu et al., 1998). To test whether FNR AF is O₂ sensitive, the UV-visible spectrum of anaerobically purified FNR AF was recorded after it was exposed to air. A progressive decrease in absorbance was observed consistent with the degradation of the [4Fe-4S]²⁺ by O₂ and with complete degradation occurring by 210 min (Figure 6). The cluster decay was much slower than that observed for wild type FNR EC (Crack et al., 2014) and other naturally O₂-stable FNRs from Neisseria meningitidis (Edwards et al., 2010), Pseudomonas putida [FNR PP_3233;
FIGURE 5 | Biochemical validation and properties of FNR<sub>AF</sub>. (A) Western blot, antibodies prepared against FNR<sub>EC</sub> were used to detect FNR<sub>AF</sub>, which was overproduced from plasmid pET100/D-TOPO fnr<sub>AF</sub> cloned into E. coli strain BL21. The gel shows the molecular mass marker (MM), crude cell free extract (approximately 10 µg of total protein) prepared prior to induction of fnr<sub>AF</sub> expression with IPTG (lane 1), 10 µg crude cell free extract 1 h after induction of fnr<sub>AF</sub> expression with IPTG (lane 2), and elution's 1 to 4 of 10 µg purified FNR<sub>AF</sub> protein after elution from the nickel column (lanes 3–6). (B) Overproduction and purification of FNR<sub>AF</sub> from the pET11A fnr<sub>AF</sub> plasmid as shown by SDS-PAGE analysis. Gel lane 1 shows crude cell free extract (approximately 10 µg of total protein) prepared prior to induction of fnr<sub>AF</sub> expression with IPTG; lane 2, 10 µg of crude cell free extract 1 h after induction of fnr<sub>AF</sub> expression with IPTG; and lane 3, 10 µg FNR<sub>AF</sub> after the second cationic interchange chromatographic column. M, molecular mass marker (Mr are indicated). (C) Ultraviolet/visible absorption spectrum of a representative result of biological duplicates for purified FNR<sub>AF</sub> from anaerobically grown At. ferrooxidans with an inset of the ultraviolet/visible spectrum of anaerobically purified FNR<sub>EC</sub> taken from Lazazzera et al. (1993). (D) Calculation of the FNR<sub>AF</sub> iron content by spectrophotometric assay.

The primary amino acid sequences were then compared to three dimensional models of FNR<sub>AF</sub> and FNR<sub>EC</sub> built using the crystal structure of FNR from A. fisheri (FNR<sub>AFi</sub>) as a...
template [PDB 5CVR (Volbeda et al., 2015)]. In agreement with the amino acid sequence evidence, the model shows that FNR$_{AF}$ shares similar global protein structure with important functional domains of FNR$_{EC}$, displaying a similar spatial distribution with an acceptable QMEAN score of −1.71 (Figure 8). These domains include the sensor domain that comprises a series of structural $\beta$-sheets with a [4Fe-4S]$^{2+}$ coordination site, an $\alpha$-helix promoting protein dimerization, and a DNA-binding domain composed of an HTH motif that allows recognition and binding to transcription factor binding sites (Myers et al., 2013).

[4Fe-4S]$^{2+}$ Center Coordination in FNR$_{AF}$

A notable difference in amino acid sequence between FNR$_{AF}$ and FNR$_{EC}$ is in the coordinating ligands of the [4Fe-4S]$^{2+}$ center. In FNR$_{EC}$, coordination is carried out by four cysteines located at positions 20, 23, 29, and 122 (Figure 7). In FNR$_{AF}$, cysteines are conserved at positions 20, 23, and 122 and, based on the 3D model, these are predicted to be located in positions that could potentially allow them to participate in coordinating the [4Fe-4S]$^{2+}$ center (Figure 8). There is no corresponding cysteine at position 29 in FNR$_{AF}$; there is, however, a Ser at this position that could potentially serve as a cluster ligand (Fujinaga et al., 1993; Vassiliev et al., 1995; Bentrop et al., 1996; Mansy et al., 2002) naturally occurring serine ligands are rare. Nevertheless, it is worth noting that the LipA enzyme contains an auxiliary [4Fe-4S] cluster that contains a 3Cys/Ser cluster ligation (Harmer et al., 2014).

An alternative hypothesis is that a Cys in FNR$_{AF}$ at position 40 could assume the function of the missing Cys$_{29}$. Although this hypothesis cannot be rejected, we do not favor it. Inspection of the three-dimensional model of FNR$_{AF}$ suggests that the protein is unlikely to be able to fold to bring Cys$_{40}$ into sufficient proximity to the [4Fe-4S]$^{2+}$ center to facilitate the required coordination.

Amino Acid/Structural Changes That Could Help Explain the Observed Stability of FNR$_{AF}$ in Air

One of the major differences of FNR$_{AF}$ compared to FNR$_{EC}$ is the increased stability of the Fe-S cluster in air. Here, we inspect the primary amino acid sequence and postulated 3D structure of FNR$_{AF}$ in order to propose hypotheses for explaining this unusual property.

Amino acid changes around the cluster ligand Cys$_{23}$ have been shown to alter the $O_2$ response of FNR in a number of organisms. In FNR$_{EC}$, replacement of Ser$_{24}$, located immediately adjacent
to the cluster ligand Cys322, by Pro results in increased aerobic FNR activity (Jervis et al., 2009). A natural variant of FNR from *P. denitrificans* has Pro in the position equivalent to Ser24 in FNR_{EC} and is at least six times less sensitive to O_{2} than FNR_{EC} (Crack et al., 2016). In *P. putida* a natural variant of FNR has an Arg in position 24 and is more stable to O_{2} than FNR_{EC} (Ibrahim et al., 2015). In *At. ferrooxidans* there is Leu in position 24 (Figure 7) and by analogy, this amino acid substitution could at least partially account for the lower O_{2} reactivity of FNR_{AF}.

Amino acid changes at other positions next to the cluster-coordinating Cys residues are also known to influence the aerobic reactivity of FNR_{EC}. For example, substitution of Asp22 by Ala (Ibrahim et al., 2015) or by Gly (Kiley and Reznikoff, 1991) increased O_{2} activity of FNR. FNR_{AF} has an Asp to His substitution at position 22. Interestingly, it has been found that juxtaposition of His to the cysteine-coordinated [4Fe-3S]^{2+} center of a subgroup of Ni hydrogenases provides stability in the presence of O_{2} (Frielingsdorf et al., 2014; Flanagan et al., 2018). Also, substitution of Leu28 by the positively charged His has been shown to stabilize the [4Fe-4S]^{2+} center in FNR_{EC} in the presence of O_{2} (Bates et al., 2000) perhaps by hindering conformational flexibility of the region (Volbeda et al., 2015). FNR_{AF} has the bulky, polar, neutral amino acid Gln in this position and perhaps, like the L_{22}F variant of FNR_{EC} (Jervis et al., 2009), this could hinder conformational flexibility by steric hindrance that results in greater activity in O_{2}.

Changes in the dimerization helix may also alter the stability of the FNR dimer in O_{2}. Two charged residues Arg140 and Asp130 have been reported to play a key role in the monomer-dimer equilibrium in FNR_{EC} (Moore and Kiley, 2001). An examination of the crystal structure of FNR_{AF} indicates that these residues could form a salt bridge between the a-C helix (Arg140) of one monomer with the opposite a-B helix (Asp130) of the other monomer, perhaps modulating monomer-dimer equilibrium in FNR (Volbeda et al., 2015). Both Arg140 and Asp130 are conserved in FNR_{AF} implying conservation of the salt bridge and its role in monomer-dimer equilibrium in changing O_{2} environments (Figures 7, 8).

Of particular importance is the observation that when Asp154 in the dimer interface of FNR_{EC} is replaced with Ala_{154}, FNR_{EC} exhibits increased activity under aerobic conditions (Moore et al., 2006). In FNR_{AF} position 154 is occupied naturally by Ala, strongly suggesting that this change could, at least partially, explain its increased activity in O_{2} (Figures 7, 8).

Hydrophobic interactions have also been shown to be involved in dimer interaction and stabilization. These include Met_{144}, Met_{147}, Ile_{151}, and Ile_{154} that lie on the dimer interface of FNR_{EC} as shown in the three-dimensional model (Figure 8; Moore and Kiley, 2001; Volbeda et al., 2015). Ile_{158} is conserved in FNR_{AF}, but the other equivalently positioned residues in FNR_{AF} are Phe_{144}, Trp_{147}, and Leu_{151}, respectively. All are hydrophobic and potentially play a role in dimer stabilization. Of interest is the possibility that in FNR_{AF} Trp_{147} of one monomer helix and Phe_{144} of the complementary monomer helix could interact through stacking of their respective aromatic rings, potentially providing additional stability to the interacting helices as has been observed in other proteins (McGaughey et al., 1998; Budyak et al., 2013; Madhusudan Makkwana and Mahalakshmi, 2015).

Another difference is the presence of a truncated and divergent N terminal region of FNR_{AF} compared to FNR_{EC}, in which
FNR$_{AF}$ has only 7 amino acids just prior to the first Cys involved in $[4\text{Fe}-4\text{S}]^{2+}$ center coordination instead of the 19 observed in FNR$_{EC}$ (Figure 7). These amino acids form part of a flexible region with no predicted secondary structure. The truncation in FNR$_{AF}$ does not appear to be a result of sequence mis-annotation e.g., incorrect translation start site. Interestingly, deletion of N-terminal amino acid residues 2 to 16 and 2 to 17 FNR$_{EC}$, increased FNR activity under aerobic conditions (Yan and Kiley, 2008). These results suggest that the N-terminal region also contributes to the lability of the $[4\text{Fe}-4\text{S}]$ cluster of FNR to O$_2$ and that the removal of amino acids in this region may act to increase the stability of the cluster to O$_2$. How these changes operate is not known.

**ADDITIONAL DISCUSSION**

Anaerobic culturing of *At. ferrooxidans* confirmed previous reports that it is capable of growth using Fe$^{3+}$ as the final electron acceptor (Ohmura et al., 2002; Osorio et al., 2013) and that anaerobic growth is slower than with O$_2$ as the electron acceptor (Osorio et al., 2013; Figure 1). The slower rate of growth for anaerobic cultures may be due to the greater amount of energy available from sulfur oxidation using O$_2$ as terminal electron acceptor (−124 kcal/atom S$^0$) compared with Fe$^{3+}$ reduction (−75 Kcal/atom S$^0$).

The ability to transition from aerobic reduction of O$_2$ to utilizing ferric iron as a terminal electron acceptor suggests that *At. ferrooxidans* must regulate the expression of alternative electron transfer chains used in energy conservation. In this study, we provide evidence that *At. ferrooxidans*, a model organism for studying life at extremely low pH, contains a FNR-like protein (FNR$_{AF}$) that is a member of the CRP FNR superfamily of regulators. FNR$_{AF}$ exhibits sequence (Figure 2) and structural similarity (Figure 8) with the archetypal FNR from *E. coli* (FNR$_{EC}$) that was recently deduced from the crystal structure of FNR from *Al. fisheri* (Mettert and Kiley, 2018). FNR$_{AF}$ reacts with antibodies prepared against FNR$_{EC}$ (Figure 5A) and is able to drive expression from the FNR-responsive *E. coli* *PnarG* promoter, suggesting that it is functionally active as an FNR-like protein at least in the surrogate host *E. coli*.

Despite high levels of structural and protein sequence similarity, FNR$_{AF}$ exhibits several properties that differ from FNR$_{EC}$. First, RNA encoded by *fnr$_{AF}$*, although detected in aerobic cultures, increases in amount in the stationary phase of anaerobically grown cultures (Figure 4), suggesting that depletion of O$_2$, and/or culture age upregulate *fnr* expression or modifies post-transcriptional processing of *fnr* RNA. Upregulation of *fnr* in anaerobic conditions has also been observed in *B. subtilis* (Cruz Ramos et al., 1995). In contrast, although there is negative auto-regulation of FNR$_{EC}$ expression (Mettert and Kiley, 2007), in *E. coli* it has been demonstrated that FNR activity is predominantly regulated at the protein level where FNR appears to cycle between active $[4\text{Fe}-4\text{S}]^{2+}$, inactive $[2\text{Fe}-2\text{S}]$, and apomorphic, with the level of O$_2$ determining which form predominates and therefore, the extent to which FNR is transcriptionally active. Such a mechanism requires that the levels of FNR in the cell are tightly controlled (Spiro and Guest, 1987; Sutton et al., 2004; Mettert and Kiley, 2007; Jervis et al., 2009). The suggestion that expression of *fnr$_{AF}$* is regulated opens up opportunities to investigate the underlying mechanism(s) involved.

Another important consideration is the significantly increased stability of FNR$_{AF}$ compared to FNR$_{EC}$. What could be the evolutionary advantage of this strategy? We hypothesize that it allows the control of genes in its network over a wide range of O$_2$ concentrations without the need to resort to recycling between active and inactive forms of FNR, as in *E. coli*, or to differential transcriptional regulation of FNR as exhibited by *B. subtilis, P. putida*, *H. seropedicae*, *B. cenocepacia*, and *R.eutropha*. These mechanisms are energetically costly and time consuming. Speed of response to increased environmental concentrations of O$_2$ might be particularly critical for *At. ferrooxidans* as it needs to transition rapidly from anaerobic to highly oxidizing Fe-rich environments at very low pH such as found in bioheaps for industrial copper recovery (Jerez, 2008) and in biofilms in naturally occurring acidic environments (Wilmes et al., 2009; Liljeqvist et al., 2015) and these responses need to be made in an organism with a relatively slow growth rate and whose energy budget allocation is restricted by living at the thermodynamic edge of life.

**DATA AVAILABILITY**

Publicly available datasets were analyzed in this study. This data can be found here: https://www.ncbi.nlm.nih.gov/genome/1014?genome_assembly_id=300479.

**AUTHOR CONTRIBUTIONS**

DH, EJ, and PK designed the study. HO and EM carried out the experiments. All authors analyzed the data. DH and MD drafted the manuscript and all authors agreed on the final version.

**FUNDING**

This work was supported by the Programa de Apoyo a Centros con Financiamiento Basal AFB 17004 to Fundación Ciencia & Vida and Fondecyt 1181717 to DH. The work in the Kiley lab was supported by the NIH grants R01-GM115894 and R01-GM045844 to PK.

**ACKNOWLEDGMENTS**

HO thanks the doctoral program Mecesup UAB0802 for supporting a stay in the laboratory of PK. The authors wish to thank a reviewer for drawing our attention to additional literature on the replacement of Cys by Ser as one of the potential coordinating ligands of the Fe-S cluster.
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**Conflict of Interest Statement:** The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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