The Fur regulon in anaerobically grown *Salmonella enterica* sv. Typhimurium: identification of new Fur targets

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**Abstract**

**Background:** The Ferric uptake regulator (Fur) is a transcriptional regulator that controls iron homeostasis in bacteria. Although the regulatory role of Fur in *Escherichia coli* is well characterized, most of the studies were conducted under routine culture conditions, i.e., in ambient oxygen concentration. To reveal potentially novel aspects of the Fur regulon in *Salmonella enterica* serovar Typhimurium under oxygen conditions similar to that encountered in the host, we compared the transcriptional profiles of the virulent wild-type strain (ATCC 14028s) and its isogenic Δfur strain under anaerobic conditions.

**Results:** Microarray analysis of anaerobically grown Δfur *S*. Typhimurium identified 298 differentially expressed genes. Expression of several genes controlled by Fnr and NsrR appeared to be also dependent on Fur. Furthermore, Fur was required for the activity of the cytoplasmic superoxide dismutases (MnSOD and FeSOD). The regulation of FeSOD gene, sodB, occurred via small RNAs (i.e., the *ryhB* homologs, *rfrA* and *rfrB*) with the aid of the RNA chaperone Hfq. The transcription of sodA was increased in Δfur; however, the enzyme was inactive due to the incorporation of iron instead of manganese in SodA. Additionally, in Δfur, the expression of the gene coding for the ferritin-like protein (*ftnB*) was down-regulated, while the transcription of the gene coding for the nitric oxide (NO) detoxifying flavohemoglobin (*hmpA*) was up-regulated. The promoters of *ftnB* and *hmpA* do not contain recognized Fur binding motifs, which indicated their probable indirect regulation by Fur. However, Fur activation of *ftsB* was independent of Fnr. In addition, the expression of the gene coding for the histone-like protein, H-NS (*hns*), was increased in Δfur. This may explain the observed down-regulation of the *tdc* operon, responsible for the anaerobic degradation of threonine, and *ftnB* in Δfur.

**Conclusions:** This study determined that Fur is a positive factor in *ftnB* regulation, while serving to repress the expression of *hmpA*. Furthermore, Fur is required for the proper expression and activation of the antioxidant enzymes, FeSOD and MnSOD. Finally, this work identified twenty-six new targets of Fur regulation, and demonstrates that H-NS repressed genes are down-regulated in Δfur.

**Background**

The Ferric uptake regulator (Fur) is a metal-dependent regulator of transcription and post-transcription in bacteria, which senses metal concentration and/or the redox state of the cells (reviewed in [1]). The classical model of the regulatory role of Fur depicts transcriptional repression through ferrous iron that results in Fur-Fe^{2+} binding to the operator site of a target gene [2,3]. Fur-Fe^{2+} binding to DNA are presumed to be homodimeric; however, multimeric complexes have been reported [4,5]. In addition, the metal cofactor present in vivo is controversial, due to the ability of the Fur protein to bind different divalent cations, in vitro [6]. For example, Fur represses aerobactin biosynthesis using ferrous iron, cobalt, or manganese [2]. Moreover, most researchers studying Fur binding to promoter sequences, in vitro, employ manganese instead of ferrous iron due to the reactivity of ferrous iron with oxygen. However, evidence exists that Fur regulates specific genes differently in the presence of ferrous iron or manganese [7].
Fur also contains zinc for protein stability [8,9]. This indicates that the availability of the metal cofactor to pathogens residing in the host dictates the activity of Fur.

The regulatory role of Fur has been demonstrated in numerous pathogenic and non-pathogenic organisms growing in the presence of ambient oxygen [10–19]. However, research has shown that the oxygen concentration in the host is low. For example, the oxygen sensitive [20], Fnr (Fumarate nitrate reduction) was shown to be essential for virulence in Salmonella enterica serovar Typhimurium (S. Typhimurium) [21], Shigella flexnari [22], Neisseria meningitidis [23], and Pseudomonas aeruginosa [24]. In addition, the expression of the dimeric Cu-Zn superoxide dismutase (SodCl), one of the virulence determinants in S. Typhimurium, within the J774.1 cell line was shown to be Fnr-dependent [25]. Fnr is a transcriptional regulator that is active as a homodimer and contains an oxygen labile iron sulfur cluster (4Fe-4S) [26]. Fnr can serve either as an activator or as a repressor of transcription, depending on the target gene. For instance, under anaerobic conditions, Fnr represses the cytochrome c oxidase (cyoABCDE) and the cytochrome bd complex (cydAB), while activating genes important for utilizing alternative electron acceptors such as fumarate [21]. Therefore, it is reasonable to conclude that the O₂ concentration within the host is low enough to activate Fnr in S. Typhimurium residing within cells of the innate immune system. This in vivo low oxygen concentration appears to be sufficient to cause a shift in the redox state of iron from ferric to ferrous. Indeed, when S. Typhimurium is within macrophages, repression of the Fur regulated iroABCDE promoter occurs regardless of the presence of the host metal transporter Nram1 [27,28]. This demonstrates that during intracellular growth of S. Typhimurium, the state of oxygen tension and iron valence are adequate for the activation of both Fnr and Fnr, respectively. Recently, we demonstrated the role of Fur in HilA expression and virulence in S. Typhimurium, which is mediated by the negative regulation of H-NS by Fur under anaerobic conditions [29].

H-NS is a DNA binding protein that is associated with the nucleoid of Gram-negative enteric bacteria (reviewed in [30]). Deletion of hns is considered lethal unless an additional mutation occurs in either the alternative sigma factor, rpoS, or the transcription factor, phoP [31]. H-NS binding can alter the topology of DNA and influence gene regulation [32]. Typically, H-NS exhibits a repressive role in gene regulation, especially of genetic loci associated with virulence [31,33-35]. H-NS preferentially binds to AT rich segments of DNA, which are characteristic of horizontally acquired Salmonella pathogenicity islands (SPIs) [36]. Interestingly, H-NS also represses genes associated with anaerobic metabolism including those responsible for the degradation of L-threonine, encoded by the tdc operon, and are induced under anaerobic conditions [37]. H-NS binds the tdc locus and represses its transcription [31], thereby linking amino acid catabolism with H-NS regulation. In addition, Fur is known to activate SPI1 via the activation of the positive regulators of SPI1 (i.e., HilA and HilD) [38,39]. This activation is, in part, indirect where Fur represses the expression of hns, which represses the expression of hilA and hilD [29]. Thus, Fur indirectly activates SPI1 via its repression of hns, demonstrating that iron metabolism can influence genes regulated by H-NS.

Our goal here was to compare the transcriptome of wild-type (WT) S. Typhimurium to an isogenic strain lacking the fur gene (Δfur) in cells growing under anaerobic conditions (i.e., conditions resembling that encountered by the pathogen during infection [40]). To accomplish that goal, we used DNA microarray analysis and operon reporter fusions. We found that Fur directly or indirectly regulates 298 genes (~6.5% of the genome) of these, 49 contained a putative Fur binding site. Interestingly, Fnr controls 15 of these 49 genes [21] and 12 of the 15 genes contain putative binding sites for both Fur and Fnr. This suggests a regulatory link between oxygen and iron availability through the action of these two global regulators, Fur and Fnr. Furthermore, Fur was required for the activity of both cytoplasmic superoxide dismutases (MnSOD and FeSOD). We also found that the anaerobic expression of fitnB (encoding a ferritin-like protein) and hmpA (encoding the NO- detoxifying flavohemoglobin) was dependent on both Fur and Fnr. However, the promoters of fitnB and hmpA do not contain recognizable Fur binding motifs indicating their indirect regulation by Fur. Increased expression of H-NS, a known repressor of fitnB, tdc operon, and other genes, in Δfur may account for their activation by Fur. Finally, we have also identified twenty-six genes as new targets of Fur regulation in S. Typhimurium.

**Methods**

**Bacterial strains, plasmids, growth conditions, and reagents**

S. Typhimurium (ATCC 14028s) was used throughout this study, and for the constructing gene knockouts. Bacterial strains and plasmids used are listed in Table 1. Primers used were purchased from Integrated DNA Technologies (Coralville, IA) and are listed (Additional file 1: Table S1).

All knockouts were constructed using λ. Red mediated methodologies in the host strain carrying pKD46. The cells were grown in Luria-Bertani (LB) medium to an optical density (OD₆₀₀) of 0.3 at which point 50 mM
arabinose was added for 90 min [41]. The culture was centrifuged, electroporated with 1 μg PCR product of the gene of interest, recovered in SOC media (20 g tryptone, 5 g yeast extract, 0.5 g NaCl, per liter plus 20 mM glucose) for 3 h, plated on LB agar with the appropriate antibiotic, and incubated at 37°C. Transformants were verified by PCR followed by DNA sequencing. P22 phage transduction was used to move the mutations into the specified genetic backgrounds of S. Typhimurium 14028s. Colony PCR was used to confirm the genotype(s). Transductants were purified on Evans-Blue-Uranine (EBU) agar plates.

The medium used throughout this study was a buffered (pH = 7.4) LB containing 100 mM MOPS and 20 mM xylose (LB-MOPS-X) [21,29,42,43]; where indicated, kanamycin and ampicillin were used at 55 μg ml⁻¹ and 100 μg ml⁻¹, respectively. Anaerobic conditions were maintained in a Coy anaerobic chamber (Coy Laboratory Products, Grass Lake, MI) filled with anaerobic gas mixture (10% H₂, 5% CO₂, and 85% N₂). Media were equilibrated in the anaerobic chamber for at least 48 h prior to use. Anaerobic conditions were maintained by shaking at 200 RPM at 37°C in a New Brunswick gyratory water bath. Growth was determined by measuring changes in OD₆₀₀ over time. The ferrous iron chelator, 2, 2’ dipyridyl (dip), was purchased from Sigma-Aldrich (St. Louis, MO) and used at 200 μM. PCR reagents were from Promega (Madison, WI).

RNA isolation
For the microarray experiments, independent anaerobic cultures of 14028s and Δfur (KLM001) were used to inoculate three independent flasks (150 ml of anoxic LB-MOPS-X) for each strain. The three independent cultures of 14028s and Δfur were grown to an OD₆₀₀ of 0.30 to 0.35 (~ four generations) and treated with RNA-later (Qiagen) to fix the cells and preserve the quality of the RNA as described previously [21,43]. Total RNA was extracted and its quality was assured before aliquots of the RNA samples were stored at -80°C for use in the microarray as previously described [21,43].

Microarray studies
Serovar Typhimurium microarray slides were prepared and used as previously described [21,43,44]. The SuperScript Indirect cDNA labeling system (Invitrogen, Carlsbad, CA) was used to synthesize the cDNA for the hybridizations. Each experiment consisted of two hybridizations, on two slides carried-out at 42°C overnight. Dye swapping was performed to avoid dye-associated effects on cDNA synthesis. The slides were washed at increasing stringencies and the microarrays were scanned for the Cy3 and Cy5 fluorescent signals with a ScanArray 4000 microarray scanner from GSI Lumonics (Watertown, MA). The intensity of each spot was expressed as the sum of the intensities of the pixels included in a circle positioned over the spot. The

| Strains | Genotype | Reference/Source |
|---------|----------|------------------|
| Salmonella enterica Typhimurium 14028s | 'wild-type' | American Type Culture Collection |
| KLM001 | Δfur::bla | [79] |
| NC 997 | Δfnr::cat | This work |
| NC 1006 | Δfur::bla Δfnr::cat | This work |
| NC1016 | Δhfq::FRT | [29] |
| NC 1067 | fnb::lacZY | This work |
| AV0305 | hmpA::lacZY | [125] |
| NC 1065 | Δfur::bla fnb::lacZY | This work |
| NC 1066 | Δfur::bla hmpA::lacZY | This work |
| NC 1068 | Δfnr::cat hmpA::lacZY | This work |
| NC 1069 | Δfur::bla Δfnr::cat hmpA::lacZY | This work |
| NC 1077 | Δfnr::cat fnb::lacZY | This work |
| NC1078 | Δfur::bla Δfnr::cat fnb::lacZY | This work |
| NC1020 | Δfur::bla Δhfq::FRT | This work |

### Plasmids
- pKD46: Phage λ gam-bet-exo under P_{cat} [41]
- pCP20: bla cat cI857 λPR flp pSC101 oriTS [46]
- pCE36: ahp FRT lacZY+ oriR6K [41]
- pKD3: bla FRT ahp FRT P51 PS2 oriR6K [41]
- pKD4: bla FRT cat FRT P51 PS2 oriR6K [41]
- pKD13: bla FRT ahp FRT P51 PS4 oriR6K [41]
background was the sum of the intensities of an identical number of pixels surrounding the circled spot.

Data analysis
Values of Cy3 and Cy5 for each spot were normalized over the total intensity for each dye to account for differences in total intensity between the scanned images. The data from the microarray analysis were evaluated by two methods as previously described [21,43]. Briefly, the data were evaluated by a pair-wise comparison, calculated with a two-tailed Student’s t test and analyzed by the MEAN and TTEST procedures of SAS-STAT statistical software (SAS Institute, Cary, NC) the degrees of freedom for the t test were calculated as described previously [21,43]. The t statistic was performed using the two-tailed, heteroscedastic TTEST function of Excel software (Microsoft Corporation, Redmond, WA). The signal intensity at each spot from Δfur and the WT was analyzed and used to calculate median expression ratios and standard deviations for ORFs showing at least 2.5-fold change and p < 0.05 [21,43].

Microarray data
The microarray data are accessible via GEO accession number GSE18441 at http://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE18441.

Logo graph and promoter analysis
The information matrix for the generation of the Fur logo was produced using the alignment of the Escherichia coli Fur binding sequences, available at http://arep.med.harvard.edu/ecoli_matrices/. To account for slight variation in nucleotide usage between E. coli and Salmonella, a second alignment for S. Typhimurium was built using the 5’ regions of the homologous genes used to build the E. coli information matrix. The new alignment was used to generate an information matrix specific for S. Typhimurium. A graphical representation of the matrix through a logo graph was obtained with Weblogo software (version 2.8.1, 18 October 2004), available at http://weblogo.berkeley.edu. The information matrix was used to scan the 5’ region (from the position -400 to +50) of the genes with significant variations of transcripts using the Patser software (version 3d), available at http://rsat.ulb.ac.be/rsat/.

If a sequence corresponding to a Fur binding motif was identified, then this sequence was given a weighted score [45].

Construction of transcriptional lacZ fusions
Single-copy genomic transcriptional lacZ fusions were constructed as described previously [46]. Briefly, 300 ng of pCP20 was transformed into mutant strains; cultures were transferred twice at 30°C, and checked for loss of the antibiotic marker. Plasmids with a single FRT site upstream of promoterless lacZY were transformed into mutant strains carrying pCP20 and incubated at 37°C on an LB-agar plate with kanamycin. Transformants were transferred three times at 40°C, verified by PCR, and transduced into appropriate background(s).

β-galactosidase assay and “Differential Plot” presentation of the data
The β-galactosidase assay was used to assess expression of transcriptional fusions in cultures growing at steady state. This was accomplished by 50-fold dilution of anaerobically grown overnight (~17 hr) cultures into fresh medium and once a steady state of growth was established, the cells were re-inoculated into fresh LB-MOPS-X medium to an OD600 ~0.02. β-galactosidase assays were conducted during growth and the activity (U/ml) [47] was plotted against changes in OD600 in the form of a differential plot [48,49]; which are usually recommended for determining the rate of synthesis of an mRNA or a protein relative to the total rate of synthesis in the cell. The slope of the linear regression of this type of plot represents the differential rate of synthesis (i.e., Specific Activity, Units/OD600) during the steady state of growth. The intrinsic advantages of using this method (i.e., differential rate) over the commonly used method (i.e., one-time point assays) are well documented [50-53]. Data shown were from three independent cultures with standard deviation.

Preparation of cell-free extracts and SOD activity gels
Cultures were grown anaerobically overnight, diluted to ~0.02 OD600 in LB-MOPS-X, and cells were harvested at OD600 ~0.25. Further cell growth and de novo protein synthesis were minimized by adding chloramphenicol (50 μg ml⁻¹) and ice to the cultures. In addition, 50 μg ml⁻¹ chloramphenicol was included at each step of sample preparation and handling. The cultures were sealed anaerobically and the cells collected by centrifugation at 5,000 × g at 4°C. Cells were washed with phosphate buffer (pH 7.8, 50 mM potassium phosphate containing 0.1 mM EDTA, KPi), centrifuged again, and resuspended in the same buffer. Cells were sonicated on ice for 15 sec on and 30 sec off for 15 min of total sonication time. Cell debris was cleared by centrifugation at 19,000 × g for 30 min at 4°C, and the supernatant was dialyzed against KPi in dialysis membranes with an 8,000 molecular weight cut-off. Dialyzed cell-free extracts were centrifuged at 20,000 × g for 30 min at 4°C, and the supernatant was stored at -80°C until use. Protein concentration was determined by the Lowry method [54]. Superoxide dismutase activity gels were performed using native 10% acrylamide gels as described previously [55].
Fumarate reductase activity
Fumarate reductase activity (FRD) was assayed from cell-free extracts as described previously [56]. Briefly, cells were grown, cell-free extracts were prepared as described above, and the fumarate dependent oxidation of reduced benzyl viologen was determined. Specific activity of FRD is expressed as μmole of reduced benzyl viologen oxidized per minute per milligram of total protein.

Measurements of total [Mn]
Independent anaerobic cultures were diluted to OD₆₀₀ ~0.02 and grown until OD₆₀₀ 0.35 in a Coy anaerobic chamber. Chloramphenicol was added at 50 μg ml⁻¹, samples were sealed anaerobically, and centrifuged at 12,000 × g for 20 min at 4°C. Samples were washed with KPi as above, centrifuged, and resuspended in 2 ml of buffer. Samples were dried and treated with 3 M nitric acid overnight at room temperature then quickly boiled. Total manganese content was determined by Inductively Coupled Plasma Optical Emission Spectroscopy (ICP-OES) at North Carolina State University Analytical Service Laboratory. Total manganese and iron was measured in LB medium as above using a 5X concentration of medium.

Results
Growth of Δfur under anaerobic and aerobic conditions
Iron is an essential element for redox reactions in biology. However, it is an important factor in oxygen toxicity due to its involvement in hydroxyl radicals (HO⁻) formation via Fenton chemistry [57]. Therefore, we compared the effects of a deletion of fur on growth kinetics under both anaerobic and aerobic conditions. Data in Figure 1 demonstrate that Δfur was not compromised in its growth kinetics under either anaerobic or aerobic conditions.

Effect of Fur on the anaerobic transcriptome of S. Typhimurium
Under anaerobic conditions, the absence of fur resulted in the differential expression of 298 genes (Additional File 2: Table S2). These genes were organized by Cluster of Orthologous Groups (COGs) and the numbers of genes within each COG are shown in Table 2. The absence of fur resulted in increased expression (i.e., Fur acted as a repressor) of 226 genes. However, the absence of Fur resulted in decreased expression (i.e., Fur acted as an activator) of 72 genes, most likely via an indirect mechanism.

A Fur information matrix, specific for S. Typhimurium, was generated (Figure 2), and used to scan the upstream regions of the 298 genes identified as differentially expressed in response to deletion of fur. We identified 49 genes that contain a putative Fur binding site (Table 3 - columns 1 & 2 and Additional file 2: Table S2).

a. Fur as a repressor
Genes associated with metal homeostasis were up-regulated in Δfur. These included the well characterized genes/operons involved in iron homeostasis (i.e., entA-BEC, iroBCDE, iroN, fis, tonB, fepA, bf, hfd), Mn²⁺ transport genes (i.e., sitABC), and copper resistance (i.e., cutC) [58-65] (Additional file 2: Table S2).

Expressions of genes involved in xylose metabolism (xylBR) were increased 3.7 and 2.9-fold, respectively, in Δfur relative to the WT (Additional file 2: Table S2). In addition, the glycolytic genes pfkA and gpmA were 3.3- and 5.6-fold higher in Δfur, respectively (Additional file 2: Table S2). Two genes, STM1586 (coding for a periplasmic protein) and sitA were up-regulated 76.1 and 53.8-fold, respectively, in Δfur (Additional file 2: Table S2). These two genes exhibited the highest differential expression in Δfur. Intriguingly, the microarray data showed that the gene for adenlosuccinate synthetase (purA), which is required for adenosine 5’ monophosphate synthesis, was up-regulated 3.5-fold in Δfur. Incidentally, purA mutants are known to be highly attenuated and have been used in developing in vivo expression technology (IVET) to detect promoters activated during S. Typhimurium infection [66,67].

Transcription of the cytochrome-o ubiquinol oxidase operon (cyaABCDE) and the high affinity cytochrome-d terminal oxidase genes (cydAB) was repressed by Fur (Additional file 2: Table S2). Interestingly, aerobic expression of cydAB is repressed by H-NS, which is relieved by the response regulator ArcA [68]. In addition, we detected increased expression of hns in Δfur (Additional file 2: Table S2), and earlier work detected in vivo binding of Fur to the upstream region of hns [29]; this strongly indicates that Fur directly represses hns under anaerobic conditions. How or if H-NS may interact in the anaerobic regulation of cydAB under our conditions is not clear at the present time.
conditions is unknown, since the repression of cydAB by H-NS does not appear to occur under anaerobic conditions [68].

Genes associated with DNA repair and purine metabolism (nrdAB, nth, recA, and nei) were repressed by Fur under anaerobic conditions (Additional file 2: Table S2), thus implicating Fur as a regulator of DNA repair and de novo synthesis. Fur was found to repress ydiE (STM1346) and a putative Fur binding site was found upstream of the start codon, where the expression of the gene was 7.4-fold higher in the mutant than in the wild-type (Additional file 2: Table S2). In S. Typhimurium, YdiE has a conserved HemP (COG4256) domain, and is encoded within the hemin uptake operon [69]. Although S. Typhimurium is not known to utilize host’s heme, previous work has established a Fur binding site upstream of ydiE and hemP in S. Typhimurium and Y. enterocolitica, respectively [16,69]. This indicates that our bioinformatic analyses indeed agree with experimentally identified Fur binding sites.

### b. Fur as an activator

Anaerobic transcription of the fumarate reductase (frdABD) operon and the aspartase gene (aspA) was significantly lower in Δfur (i.e., Fur is serving as an activator); however, the genes coding for the alpha and beta subunits of succinyl-CoA synthetase (sucCD) were upregulated 4.1 and 2.7-fold, respectively (Additional file 2: Table S2). These genes (i.e., frdABD, aspA, sucCD) and fumAB (fumarate hydratase) are members of the

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**Table 2 Number of Differentially Expressed Genes in Δfur**

| Cluster of Orthologous Groups | Number of Genes **Fur Repressed**<sup>a</sup> | Number of Genes **Fur Activated**<sup>b</sup> | Total |
|--------------------------------|-----------------------------------------------|-----------------------------------------------|-------|
| No COG                        | 30                                            | 9                                             | 39    |
| Energy Production and Conversion | 16                                            | 18                                            | 34    |
| Cell Cycle Control            | 3                                             | 0                                             | 3     |
| Amino Acid Metabolism and Transport | 7                                             | 16                                            | 23    |
| Nucleotide Metabolism and Transport | 7                                             | 4                                             | 11    |
| Carbohydrate Metabolism and Transport | 9                                             | 4                                             | 13    |
| Coenzyme Metabolism and Transport | 6                                             | 0                                             | 6     |
| Lipid Metabolism and Transport | 5                                             | 0                                             | 5     |
| Translation                   | 46                                            | 0                                             | 46    |
| Replication, Recombination, and Repair | 9                                             | 2                                             | 11    |
| Cell Wall/Membrane/Envelope Biogenesis | 14                                            | 3                                             | 17    |
| Cell Motility                 | 1                                             | 0                                             | 1     |
| Post-Translational Modification, Protein Turnover, Chaperone Functions | 10                                            | 1                                             | 11    |
| Inorganic Ion Transport and Metabolism | 20                                            | 2                                             | 22    |
| Secondary Metabolite Biosynthesis, Transport, and Catabolism | 5                                             | 4                                             | 9     |
| General Functional Prediction Only | 15                                            | 4                                             | 19    |
| Function Unknown              | 9                                             | 2                                             | 11    |
| Signal Transduction Mechanisms | 5                                             | 2                                             | 7     |
| Intracellular Trafficking and Secretion | 3                                             | 0                                             | 3     |
| Defense Mechanisms            | 1                                             | 0                                             | 1     |
| **Total**                     | **226**                                       | **72**                                        | **298** |

Categorized According to Cluster of Orthologous Groups (COGs)

<sup>a</sup> Genes with increased expression in the absence of fur

<sup>b</sup> Genes with decreased expression in the absence of fur

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![Fur Logo for S. Typhimurium](image.png)

**Figure 2** Logo graph of the information matrix from the alignment of Fur-regulated genes in S. Typhimurium. The height of each column of characters represents information, measured in bits, for that specific position and the height of each individual character represents the frequency of each nucleotide.
reductive branch of the TCA cycle. We assayed for fumarate reductase (FRD) in cell-free extracts from anaerobic cultures and found that Fur is required for the anaerobic transcription and activity of FRD in *S. Typhimurium* (Additional file 3: Table S3). In *E. coli*, the transport of C4-dicarboxylates occurs via two seemingly redundant genes encoded by *dcuA* and *dcuB* [70]. In the present study, the *dcuB-fumB* operon was unaffected by Fur, while the *aspA-dcuA* operon was significantly down regulated in *Δfur* and both genes contained a putative Fur box 5’ of the start codon (Additional file 2: Table S2).

Genes involved in anaerobic respiration (*dmsABC*) and ethanolamine utilization (*eutSPQTDMEJGHABCLK*) were activated by Fur (Additional file 2: Table S2). The mechanism for reduced expression of *dmsABC* is unclear. Ethanolamine is a significant source of carbon and nitrogen during *Salmonella* infection [71].

One metabolic pathway that appears impacted by Fur is that required for glycerol metabolism. The genes for glycerol metabolism are located throughout the genome. For instance, *glpQT* and *glpABC* are divergently transcribed in two predicted operons. All of these genes were significantly down regulated in *Δfur* (Additional file 2: Table S2). Furthermore, *glpD*, and *glpKF* were all down regulated in *Δfur* (Additional file 2: Table S2). The down-regulation of these genes suggests that the *Δfur* strain may be unable to utilize glycerol or transport glycerol-3 phosphate. The mechanism of this regulation is unclear, but the absence of Fur binding sites in the promoters of any of these genes suggests an indirect mode of regulation. The contribution of glycerol metabolism to infection is unknown.

Another metabolic pathway, the *tdc* operon (required for the anaerobic transport and metabolism of L-threonine and L-serine [72,73]) was activated by Fur. The genes in this operon (*tdcBCDEG*) are activated by *tdcA* [74]. *TdcA* is a member of the LysR family of transcriptional activators [75]. Our data showed that the expression of all genes in this operon, *tdcA*B*C*D*E*G*, was significantly down-regulated in *Δfur* (Additional file 2: Table S2). However, a Fur binding site was not

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**Table 3 Newly Identified Genes Regulated by Fur That Contain a Predicted Fur Binding Site**

| Gene   | Function                                      | Fold Changea | Predicted Fur Binding Sequenceb |
|--------|-----------------------------------------------|--------------|---------------------------------|
| rlgA   | Putative resolvase                            | 2.8          | AAAATTAAATCGTTGGC               |
| map    | Methionine aminopeptidase                      | 2.6          | AAATGGAATCATCTTT                |
| rpsB   | 30S ribosomal subunit protein S2              | 4.0          | AAATGGAATCATCTTT                |
| yajC   | Tranlocase protein, IISF family               | 3.2          | GTATGCAAAGCATAAAA               |
| mntR   | Putative transcriptional regulator            | 2.5          | GAAACGGTAAAAATTACC              |
| sucC   | Succinyl-CoA synthetase, beta subunit          | 4.1          | CTAAGATAAACGATTAC               |
| cmk    | Cytidine monophosphate kinase                 | 2.7          | AAAAGTAAATCATGTGC               |
| STM1013| Gifsy-2 prophage, regulatory protein           | 2.8          | AAAATCAAATCATGAAC               |
| STM113S| Putative dehydrogenase                        | -4.2         | ATATGAGTGAATTTTG                |
| nth    | Endonuclease III                              | 2.9          | GAAAGCCTACCATCCTC              |
| ldha   | Fermentative D-lactate dehydrogenase          | -4.0         | AATATGTTTAAATTTAC              |
| ynaF   | Putative universal stress protein             | -37.3        | GAAATAATTAAATTATTT              |
| hns    | Histone like protein                          | 5.8          | AAAAAATGAATAAATAC               |
| STM179S| Homolog of glutamic dehydrogenase             | -8.8         | AAATGGAAGATTATTT                |
| STM2186| Putative glutamate synthase                   | -4.1         | AATATGCCCCATGTTC                |
| eutC   | Ethanolamine ammonia lyase                    | -3.2         | AATATGGAATTAATAC               |
| eutB   | Ethanolamine ammonia lyase                    | 2.6          | GAAATTCGAATAAAAT                |
| yrdF   | Putative glutaredoxin                         | 9.1          | CTAATGATAATATATC                |
| ygaF   | Cytoplasmic protein                           | 3.5          | AATATGTTTAAATTTAT              |
| STM3600F| Putative sugar kinase                         | -6.8         | CTATGGCTCATCATTT                |
| STM3690| Putative lipoprotein                          | -4.2         | AAATGATAATATATCT                |
| rpoZ   | RNA polymerase, omega subunit                 | 3.9          | AATATGATAATATCT                 |
| ucp   | Uridine phosphorylase                         | -5.4         | CAATATGATAATATCT               |
| yjcD   | Putative xanthine/uracil permease             | 2.8          | AAAAAAGCAAACGATTAC              |
| dcaA   | Anaerobic dicarboxylate transport protein     | -5.8         | CAATATGATAATATCT               |

a Ratio of mRNA, *Δfur*/*14028s*  
b Predicted Fur binding site located within -400 to +50 bp relative to ATG  
c Indicates the predicted Fur binding site is located on the reverse strand
identified in the promoters of any of the genes in the *tdc operon*, suggesting its indirect regulation by Fur. Importantly, H-NS is known to directly bind and repress this operon [31,76]. Therefore, the increased expression of *hns* in Δ*fur* (Additional file 2: Table S2), may account for the observed effect of Fur on the *tdc* operon. Mutations in the *tdc* operon have been shown to reduce invasion and virulence in *S. Typhimurium* [77,78]. In addition to the reduced expression of the *eut* operon, the reduced expression of the *tdc* operon and *hilA* may contribute to the observed attenuation of the Δ*fur* strain of *S. Typhimurium* [29,79].

**Role of Fur in regulation of antioxidant genes**

Reactive oxygen and nitrogen species (ROS and RNS, respectively) are important host defense responses during bacterial infection. Our array data (Additional file 2: Table S2) revealed differential regulation of some important antioxidant genes whose products are essential for protecting the cells against ROS and RNS (i.e., superoxide dismutases, ferritin-like protein, and flavohemoglobin). Therefore, we decided to study the expression of these genes in greater detail.

*a. Regulation of sodA and sodB*

There is plethora of information about the regulation of *sodA* and *sodB* in *E. coli* [80-85], but there is little knowledge about the regulation of these genes in *S. Typhimurium* [86]. In the present study, the microarray data showed that the anaerobic expression of *sodA* and *sodB* in Δ*fur* was > 9-fold higher and > 3-fold lower, respectively, than in the parent WT strain (Additional file 2: Table S2). SodA (MnSOD) and SodB (FeSOD) are the cytosolic superoxide dismutases of *S. Typhimurium* and they require the cofactors manganese and iron, respectively. These SODs are homodimers, and are fully functional when metalated with the appropriate metals (i.e., manganese for SodA and iron for SodB). However, a heterodimer consisting of SodA(Mn)/SodB(Fe) can still exhibit SOD activity, albeit at a reduced level compared to the homodimer [87]. Thus, in order to see an active hybrid SOD, both SodA and SodB must be expressed. Data in Figure 3A demonstrated that, as in anaerobic *E. coli*, the WT strain (Lane 1) lacked the activity of both Mn- and Hybrid-SODs, but possessed an active FeSOD. However, Δ*fur* (Figure 3A - Lane 2) was devoid of all three SOD-isozymes. The lack of FeSOD in Δ*fur* was of no surprise, as previous studies in *E. coli* [83,84] have established that Fur is indirectly required for the translation of sodB via its repression of the small RNA, *ryhB*, which works in conjunction with the RNA chaperon protein, Hfq [88,89]. Indeed, a strain harboring deletions in both Fur and Hfq (Δ*furΔhfq*) resulted in restoration of SodB activity (Figure 3A - Lane 4). Furthermore, the high degree of sequence identity in the promoter and the gene sequence of *ryhB* of *E. coli* with the two *ryhB*-like small RNAs, *rfrA* and *rfr* of *S. Typhimurium* [39], suggested that the regulation of *sodB* in *S. Typhimurium* is similar to that reported in *E. coli* [88,89]. Interestingly, expression of the hybrid SOD appears up-regulated in Δ*hfq* and Δ*furΔhfq* (Figure 3A - Lane 3 and 4). The reason for this is unclear, but may be due to the activation of the Hfq-binding small RNA (*fnrS*) by Fnr, which subsequently represses the expression of *sodA* [90,91].

The WT strain of *S. Typhimurium* possessed neither an active SodA (MnSOD) nor the hybrid enzyme (SodA/SodB), which is not surprising since this is normally the case in WT *E. coli* [92]. What was surprising is the lack of MnSOD activity in the anaerobic cell-free extracts from Δ*fur* (Figure 3A - Lane 2) in spite of the > 9-fold increase in the transcription of *sodA* (Additional file 2: Table S2). Therefore, we reasoned that the...
increased intracellular concentration of free iron in Δfur [93] could result in competition of iron with manganese for the active site of SodA. This would lead to the formation of a non-active form of the enzyme, i.e., SodA-Fe instead of the active SodA-Mn (MnSOD). Analysis of total iron and manganese concentrations in our media showed that it contained ~40-fold more iron than manganese (i.e., ~7.5 μM iron vs. ~0.2 μM manganese). Additionally, the manganese content of anaerobic cultures of the parent strain and of the Δfur strain were low, 0.09 ± 0.01 and 0.08 ± 0.04 μmoles manganese per gram of dry weight, respectively. Therefore, we supplemented the growth media with 1 mM MnCl₂ and determined the SOD activities (Figure 3B). If our reasoning was correct, we expected that excess Mn²⁺ added to the growth media would reveal increased MnSOD activity in Δfur. Indeed, this was the case, as a dramatic increase in MnSOD was observed in Δfur, but not in the parent strain (Figure 3B - lanes 1 vs. 4). Also, cultures grown in presence of 1 mM MnCl₂ contained 47.2 ± 2.7 and 48.8 ± 2.0 μmoles of manganese per gram of dry weight for the parent strain and for Δfur, respectively. Altered MnSOD activity in Δfur was due entirely to the lack of a functional fur gene since the introduction of a plasmid carrying the fur gene (i.e., pfur-ha) diminished MnSOD activity to that of the parent strain (Figure 3B - lane 5). In addition, the plasmid pfur-ha restored FeSOD activity (Figure 3A - lane 5) as well as the phenotypic appearance of the WT strain observed on a Tris buffered chrome azurol agar plates (CAS plates) [94] containing 0.3% xylose [29]. These results indicated that increased transcription of sodA in Δfur did not result in a corresponding increased MnSOD activity due to the excess intracellular free iron and that the addition of Mn²⁺ negated this effect. On the other hand, the inclusion of excess Mn²⁺ in the growth medium of the parent strain did not increase MnSOD activity, which indicated that Mn²⁺ was not a signal for sodA induction. Furthermore, these findings demonstrated an important aspect of metalloenzyme regulation, i.e., the availability of the correct cofactor has a profound impact on enzyme activity.

b. Regulation of ftnB

Microarray data (Additional file 2: Table S2) revealed a 7-fold reduction in the expression of ftnB in Δfur as compared to the parent strain. The expression of ftnB was shown to be activated by Fnr [21]. Therefore, we used a chromosomal ftnB-lacZ transcriptional fusion in Δfur and in Δfnr genetic backgrounds to determine the contribution of each regulator in the expression of ftnB. The deletion of fur reduced the aerobic rate of synthesis of the reporter gene by > 2-fold compared to the parent strain (Figure 4A). 2',2'-dipyridyl (dip) reduced the rate of synthesis of the reporter gene in aerobic conditions (Figure 4A). Although induction of the reporter fusion occurred earlier in the growth phase with dip treated cultures, the rate of synthesis was reduced compared to untreated parent strain. This indicates inhibition by dip
As expected, the oxygen sensitive regulator Fnr did not impact regulation of ftnB in aerobic conditions (Figure 4A). This indicated that Fur is required for ftnB expression, independent of Fnr. Data in Figure 4B show that the absence of fur resulted in a 2-fold reduction in the rate of synthesis (U/OD600) of ftnB-lacZ under anaerobic conditions. Furthermore, the ferrous iron chelator, dip, reduced the rate of anaerobic synthesis of ftnB-lacZ in the WT strain by > 2-fold (Figure 4B). In Δfur, the rate of synthesis was further reduced (> 10-fold) when compared to the WT parent strain treated with dip (Figure 4B). In addition, the rate of synthesis in the parent strain was greatest under anaerobic conditions due to the active roles of both Fnr and Fur (Figure 4). Collectively, full expression of ftnB is dependent on Fur in aerobic and anaerobic conditions, whereas Fnr is a strong activator in the absence of O2.

c. Regulation of hmpA

The gene coding for the flavohemoglobin (hmpA), a NO· detoxifying protein [95-98], was differentially expressed in Δfur (Additional file 2: Table S2). Expression of hmpA is repressed by Fnr and another DNA binding protein that contains an iron sulfur cluster, NsrR [21,95-97,99]. Repression of hmpA by two regulators that are sensitive to RNS allows derepression of this gene under conditions of increased RNS. Indeed, regulation of hmpA-lacZ was induced ~80-fold by the nitro-sating agent sodium nitroprusside in aerobic conditions (B. Troxell and H.M. Hassan, unpublished data). Under anaerobic conditions, hmpA was up-regulated 4-fold in Δfur. Thus, we examined its anaerobic regulation with a chromosomal hmpA-lacZ transcriptional fusion. Figure 5 shows that the WT exhibited very little expression of hmpA-lacZ under anaerobic conditions (Figure 5A); suggesting regulation may be oxygen dependent. Indeed, expression was ~14-fold higher under aerobic conditions than anaerobic conditions (B. Troxell and H.M. Hassan, unpublished data). However, the addition of the iron chelator, dip, resulted in an increased rate of synthesis ~81-fold (Figure 5A). The increased expression of hmpA-lacZ by the addition of dip could have been due to inactivation of Fnr, Fur, and/or NsrR. We narrowed our focus to the roles of Fur and Fnr in regulation of this gene. In Δfur, the reporter activity was up-regulated > 9-fold (Figure 5A), which confirmed the microarray data. The addition of dip increased the rate of synthesis by 25-fold in Δfur. One known repressor of hmpA is Fnr [21,95-97]. Therefore, we combined the fur and the fnr deletions (ΔfurΔfnr) in the hmpA-lacZ background to determine the role of Fur and Fnr in the regulation of hmpA. Deletion of fnr increased the rate of hmpA-lacZ synthesis by 216-fold as compared to the parent strain (Figure 5B). The synthesis of hmpA-lacZ in the Δfur mutant background was similar to that seen in the Δfur treated with dip (i.e., 1253 ± 107 and 1403 ± 280 - U/OD600). The lack of an obvious Fur binding motif upstream of hmpA indicates that reporter activity seen in Δfur was likely indirect. The combined deletion of fur and fnr in the hmpA-lacZ strain increased the rate of synthesis 746-fold as compared to the WT strain (i.e., 4328 ± 90 vs. 5.8 ± 2.4 - U/OD600) (Figure 5). Thus, the rate of synthesis of hmpA-lacZ in ΔfurΔfnr was ~3.5-fold higher than the rate of synthesis in Δfnr (i.e., 4328 ± 90 vs. 1253 ± 107 - U/OD600). Since we did not...
identify a discernable Fur binding site in hmpA, the fact that there is no published report showing Fur binding to the regulatory region of hmpA, and that the expression of hmpA-lacZ in ΔfurΔfur was ~3.5-fold higher than in Δfur demonstrates that under anaerobic conditions, Fur is indirectly regulating hmpA-lacZ independent of Fnr.

Identification of new Fur targets
Table 3 shows genes differentially regulated in Δfur that contain a putative Fur binding site located within -400 to +50 nucleotides relative to the translational start site. The putative translocase subunit, yajC, was up-regulated 3.2-fold in Δfur. This gene is predicted to be in the Sec-dependent pathway of protein export. At least one other gene of the Sec-dependent pathway of protein export was up-regulated in Δfur, secY. This gene, secY, is a direct target of Fur regulation in Neisseria meningitides [100,101]. Indeed, we detected a putative Fur binding site upstream of secY (Additional file 2: Table S2). The role of yajC during infection is unknown, but our results suggest Fur controls Sec-dependent protein secretion.

NrdR is a global transcriptional regulator that controls expression of oxygen-dependent and independent ribonucleotide reductases [102-104]. Expression of nrdR was up-regulated in Δfur and a putative Fur binding site was identified. Although, deletion of fur results in up-regulation of nrdHIIEF [105], a class Ib ribonucleotide reductase, we did not detect increased expression of this operon in our conditions. However, we did detect up-regulation of the class Ia ribonucleotide reductase, nrdAB, in Δfur (Additional file 2: Table S2). The class III oxygen sensitive ribonucleotide reductase, encoded by nrdDG, is encoded in an operon. Expression of nrdD, the first gene of this operon, was down-regulated in Δfur 2.5-fold. (Additional file 2: Table S2). Our data indicate that Fur controls the class Ib and III ribonucleotide reductases, either directly or indirectly, under anaerobic conditions.

A putative dehydrogenase (STM1133) was down-regulated 4.2-fold in the Δfur (Table 3). This gene contains a putative Fur binding site on the reverse DNA strand. STM1133 is the final gene in an apparent four gene operon of unknown function (STM1130-1133). The first gene of this operon, STM1130, was also down-regulated 7.9-fold in Δfur (Additional file 2: Table S2); however, a Fur binding site was not identified upstream of STM1130. Interestingly, this operon is composed of the putative N-acetylneuraminic acid mutarotase (STM1130), a putative outer membrane protein (STM1131), a putative sialic acid transporter (STM1132), and a putative NAD (P) binding dehydrogenase (STM1133). Thus, our results suggest Fur controls at least a portion of this operon that may be localized to the bacterial membrane. The importance of these genes during infection is unknown.

Several putative genes appear to be under direct control of Fur. Genes that exhibited reduced expression in Δfur were the putative universal stress protein encoded by ynaF, the putative glutamate synthase (STM2186), the putative sugar kinase (STM3600), and the putative lipoprotein (STM3690). The putative Fur binding site for ynaF and STM3600 is located on the reverse strand for these genes. The mechanism of Fur activation of these putative genes is unknown. In addition, several putative genes exhibited up-regulation in Δfur. A putative glutamic dehydrogenase (STM1795), a putative glutaredoxin (yffB), and a putative protein (yggL), were all up-regulated in Δfur. Interestingly, yffB is predicted to be a glutathione-dependent thiol reductase. The contribution of these genes to infection is unknown.

The TonB siderophore receptor gene, iroN, was up-regulated 9.1-fold in Δfur. Despite the widespread study of siderophores (salmonochelins) in Salmonella virulence, we were unable to find any published report that Fur represses iroN. Although Fur repression of the iroBCDE loci is known [59], iroN is encoded downstream of this operon and is transcribed in the opposite orientation. Our results confirm the prediction by Baumler et al that iroN is regulated by Fur [58].

Discussion
Iron is essential in most pathogenic bacteria, which compete rigorously with the host for this element. S. Typhimurium is no exception. The 17-kDa transcriptional regulator, Fur, plays an important role in bacterial iron homeostasis. Although publications of Fur regulation in E. coli and other bacteria are numerous, this is the first report on the global role of Fur in anaerobically grown S. Typhimurium. Indeed, anaerobic metabolism has been shown to be important for pathogens and pathogenesis [21-24,29].

In this study, we found that, under anaerobic conditions, Fur directly or indirectly affected the expression of 298 genes (Additional file 2: Table S2). A putative Fur binding motif was identified in 49 genes (Table 4. column #1). Also, Table 4 shows evidence of published data demonstrating the role of Fur in their regulation (column #3) and published experimental evidence for Fur binding to the regulatory region of these genes (column #4). The role of other co-regulators is also shown (Table 4, column #5). Interestingly, twelve of the 49 genes contained the binding motifs for both Fnr and Fur (Additional file 4: Table S4).

The appropriate metal cofactor was shown to be essential for detection of MnSOD activity, in spite of the 9-fold increase in sodA transcript for Δfur. Therefore, genetic backgrounds that alter the steady-state [Mn^2+]
Table 4 Comparison of Differentially Expressed Genes in Δfur That Contain a Putative Fur Binding Site with Confirmed Data of Fur Regulation from other Studies and the Possible Involvement of other Transcription Regulators

| Genes Regulated by Fur and containing a putative Fur motifb | Fold Changeb | Published Evidence of Fur Regulation [Ref.] | Published Evidence of Fur Binding [Ref.1] | Published Evidence of Control By Other Regulators [Ref]d |
|-----------------------------------------------------------|--------------|---------------------------------------------|------------------------------------------|-------------------------------------------------------|
| rlgA                                                      | 2.8          | No                                          | No                                       |                                                       |
| map                                                       | 2.6          | No                                          | No                                       |                                                       |
| rpsB                                                     | 4.0          | No                                          | No                                       |                                                       |
| yajC                                                      | 3.2          | No                                          | No                                       |                                                       |
| rnrR                                                     | 2.5          | No                                          | No                                       |                                                       |
| cyoE                                                      | 3.1          | Yes [12]                                    | No                                       |                                                       |
| cyoD                                                      | 7.1          | Yes [12]                                    | No                                       |                                                       |
| cyoB                                                      | 8.2          | Yes [12]                                    | No                                       |                                                       |
| cyoA                                                      | 3.2          | Yes [12]                                    | No                                       |                                                       |
| fepA                                                      | 10.7         | Yes [12,15,16,126-129]                      | Yes [128,129]                            |                                                       |
| fes                                                       | 39.8         | Yes [12,16,127-129]                         | Yes [128,129]                            |                                                       |
| entC                                                      | 6.8          | Yes [12,15,130]                             | No                                       |                                                       |
| succC                                                     | 4.1          | No                                          | No                                       | Fnr [21]                                              |
| gpmA                                                      | 5.6          | Yes [12]                                    | No                                       |                                                       |
| cmk                                                       | 2.7          | No                                          | No                                       |                                                       |
| STM1013                                                   | 2.8          | No                                          | No                                       |                                                       |
| STM1133                                                   | -4.2         | No                                          | No                                       | Fnr [21]                                              |
| ydeE                                                      | 7.4          | Yes [12,15]                                 | No                                       | Rcs [131]                                             |
| nth                                                       | 2.9          | No                                          | No                                       |                                                       |
| STM1586                                                   | 76.1         | Yes [15]                                    | No                                       |                                                       |
| ldhA                                                      | -4.0         | No                                          | No                                       | Fnr [21]                                              |
| ynaF                                                      | -37.3        | No                                          | No                                       | Fnr [21]                                              |
| tonB                                                      | 11.4         | Yes [12,15]                                 | Yes [132]                                |                                                       |
| hns                                                       | 3.1          | Yes [29]                                    | Yes [29]                                 |                                                       |
| STM1795                                                   | 5.8          | No                                          | No                                       | Fnr [21]                                              |
| STM2186                                                   | -8.8         | No                                          | No                                       | Fnr [21]                                              |
| cirA                                                      | 4.0          | Yes [12,15]                                 | Yes [133]                                |                                                       |
| eutC                                                      | -4.1         | No                                          | No                                       | Fnr [21]                                              |
| eutB                                                      | -3.2         | No                                          | No                                       | Fnr [21]                                              |
| yfB                                                       | 2.6          | No                                          | No                                       |                                                       |
| iroB                                                      | 4.6          | Yes [15,59]                                 | No                                       |                                                       |
| iroN                                                      | 9.1          | No                                          | No                                       |                                                       |
| sitA                                                      | 53.8         | Yes [15,46,61,134-138]                      | No                                       | MntR [61]                                             |
| yggU                                                      | 3.5          | No                                          | No                                       |                                                       |
| yqhH                                                      | 3.8          | Yes [12]                                    | No                                       |                                                       |
| secY                                                      | 4.0          | Yes [101]                                   | Yes [100]                                |                                                       |
| bfr                                                       | 3.2          | Yes [14,79,88]                              | No                                       |                                                       |
| bflD                                                      | 5.9          | Yes [12,14,15]                              | No                                       |                                                       |
| fecB                                                      | 11.8         | Yes [12,14,63,134,139,140]                  | No                                       | ArcA and Fnr [141]                                     |
| STM3600                                                   | -6.8         | No                                          | No                                       | Fnr [21]                                              |
| STM3690                                                   | -4.2         | No                                          | No                                       | Fnr [21]                                              |
| rpoZ                                                      | 3.9          | No                                          | No                                       |                                                       |
| udq                                                       | -5.4         | No                                          | No                                       | IscS [142]                                             |
| sodA                                                      | 9.1          | Yes [14,55,82,88,143-148]                   | Yes [85,146,148]                         | Fnr, ArcA, IHF, SoxRS [53,81]                         |
| yjcD                                                      | 2.8          | No                                          | No                                       |                                                       |
or its competitor [Fe^{2+}] may have dramatic effects on MnSOD activity. Indeed, we were only able to discern the role of Fur in \textit{sodA} and MnSOD expression with the addition of excess MnCl$_2$ to the growth media. These data are summarized in Figure 6, which depicts the transcriptional, translational, and post-translational role of Fur in \textit{sodA} and \textit{sodB}. This implies that disruption of iron homeostasis is likely to have a two-pronged effect, increase in Fenton chemistry and a decrease in MnSOD activity due to iron overload. It appears that the inhibition of MnSOD by iron is evolutionarily conserved. Thus, the mitochondrial Mn$^{2+}$-cofactored SOD2 has been shown to be inactivated in a similar manner when iron homeostasis was disrupted in yeast [106]. In addition, supplementation of the medium with Mn$^{2+}$ reduced oxidative stress in a murine model of hemochromatosis [107]. It is unknown if this is due to enhanced MnSOD or if Mn$^{2+}$ supplementation reduces oxidative stress in other pathological states of altered iron homeostasis.

Our decision to further study \textit{ftnB} and \textit{hmpA} was due to our previous findings, where we found that \textit{ftnB} and \textit{hmpA} were activated and repressed by Fnr, respectively [21]. The Fnr-dependent expression of \textit{ftnB} was apparent from the reduced activity in \textit{Δfnr} under anaerobic conditions, and the reduced activity in the WT strain in presence of oxygen. In addition, iron chelation and the deletion of \textit{fur} results in increased transcription of \textit{sodA}, but incorporation of Fe$^{2+}$ into the active site of SodA resulting in SodA-Fe and an inactive enzyme. Addition of excess Mn$^{2+}$ to the culture media can out compete Fe$^{2+}$ for the active site of SodA resulting in SodA-Mn and an active enzyme.

### Table 4 Comparison of Differentially Expressed Genes in ΔΔ?fur That Contain a Putative Fur Binding Site with Confirmed Data of Fur Regulation from other Studies and the Possible Involvement of other Transcription Regulators (Continued)

| Gene | Fold Change | Direct Fur Binding | Other Transcription Factors |
|------|-------------|---------------------|----------------------------|
| \textit{dcuA} | -5.8 | No | No |
| \textit{aspA} | -3.6 | Yes [13,15] | No |
| \textit{ytfE} | 10.0 | Yes [13] | No |
| \textit{ftuF} | 8.5 | Yes [12,13,15] | Yes [11,152,153] |

$^a$ Genes from the present study that are regulated by Fur and possess a putative Fur-binding motif

$^b$ Fold change of expression in Δfur relative to the wt 14028s

$^c$ Evidence of direct Fur binding the regulatory region of the gene

$^d$ Regulation by other transcription factors besides Fur

![Figure 6 Role of Fur in the transcriptional, translational and post-translational regulation of \textit{sodA} and \textit{sodB}](image-url)
oxygen tension. These results indicated that Fur controlled regulation of ftNB is independent of Fnr. Our results are in agreement with earlier work that demonstrated dependence of ftNB expression on Fur [15]. However, they are contrary to a previous report, which determined that Fur exhibited a repressive role on ftNB expression [79]. The reason for this discrepancy is unclear. It is evident from work reported herein and in a previous study in E. coli that ftNB exhibits a strong dependence on low O2 conditions [108]. Furthermore, the earlier study [108] determined that Fnr bound the promoter of ftNB in E. coli and that the Fnr binding site was further upstream than in known Fnr regulated genes. The same investigators [108], postulated that Fnr was unable to induce ftNB and that other regulators were required. However, we have determined that Fnr alone contributes to the activation of ftNB and that Fur is required for full induction of the gene, with Fnr exhibiting a more pronounced role. The lack of a predicted Fur binding site in ftNB indicated that Fur regulation was indirect. The following scenario is proposed to explain these findings and to suggest that the observed regulation of ftNB by Fur is mediated by the histone-like protein H-NS. First, the microarray data showed that Fur negatively regulates the expression of hns and has a predicted Fur binding site (Table 3). Second, we recently demonstrated that Fur binds upstream of hns in a metal dependent fashion [29]. Third, whole genome ChiP analysis demonstrated that H-NS binds to ftNB and the expression of ftNB is up-regulated in the absence of hns [31]. Fourth, the tdc operon is a known target for H-NS repression [31,76] and was significantly reduced in the absence of fur. Therefore, we propose that the positive regulation ftNB by Fur is mediated by the negative regulation of hns by Fur. Thus removal of Fur (i.e., as in Δfur) results in repression of ftNB by H-NS (see Figure 7). A second possibility is reduced Fnr function (or an additional activator) in Δfur since several Fnr regulated genes were differentially expressed in Δfur. However, our data rule-out this possibility in ftNB regulation by showing the involvement of Fur in the regulation of ftNB under aerobic conditions, where Fnr is inactive.

H-NS controls diverse functions within the cell and forms complex structures when binding DNA that indicates a central role in DNA topology [109-113]. Similar to Fur, H-NS is a repressor of transcription [31,34,35,114]. This implies that genes controlled by H-NS are regulated by iron through Fur. This interaction also demonstrates interaction between two regulators (Fur and H-NS) functioning in highly conserved physiological events, regulating a potentially toxic, but needed metal and regulating foreign DNA in a concerted manner. Thus, our results provided additional insight into iron-dependent regulation of H-NS.

Another gene regulated by Fnr or Fur was the NO detoxifying flavohemoglobin protein encoded by the hmpA. This gene (hmpA) is repressed by Fnr and contained a putative Fnr binding site, but did not contain a predicted Fur binding site [21,95,96]. Previous work determined that Fur was a repressor of hmpA [115]. However, it was later revealed that the reporter fusion was to the Fur repressed hmpA and not to the hmpA [116]. Additionally, a previous report did not reveal a role for Fur in regulation of hmpA [97], while two other studies found a modest effect of Fur on hmpA expression [98,117]. NsrR is another repressor of hmpA [97]. Thus, hmpA is repressed by two regulators that contain an iron-sulfur cluster. Despite contradictory reports, increased hmpA expression was detected in Δfur. Our initial hypothesis was that this was due to reduced Fnr function in Δfur. To support this hypothesis, we expected reporter activity to be similar in Δfur and

![Figure 7](http://www.biomedcentral.com/1471-2180/11/236)
\(\Delta fur\) backgrounds. However, our results did not support this initial hypothesis since \(\Delta fur\) exhibited ~3.5-fold increased expression compared to \(\Delta fur\); indicating that Fur regulation was FnR-independent.

A striking finding was the shared regulation of several genes by Fur and FnR. Microarray and bioinformatic studies indicated that 12 of the 298 Fur-dependent genes contained a predicted binding site for both Fur and FnR. Thus, these two global regulators may be directly involved in regulation of these 12 genes (Additional file 4: Table S4). The expression data indicated that Fur and FnR cooperate in the regulation of these 12 genes. For instance, each gene was regulated in the same manner in \(\Delta fur\) or \(\Delta fnr\); a gene activated by Fur was also activated by FnR. Lastly, our investigations indicate that Fur indirectly regulates genes that are under control of FnR or additional regulators with an iron sulfur cluster (i.e., \(ftnB\) and \(hmpA\)). Furthermore, the observed reduced expression of the ethanolamine operon, \(frdABD\), and \(dmsABC\) in \(\Delta fur\), suggest altered regulation of operons induced under anaerobiosis (Additional file 2: Table S2). Thus, Fur is an activator of genes that are typically induced under anaerobic conditions. Ethanolamine utilization within the host is important for \(S.\) Typhimurium and the Gram-positive pathogen \(Listeria\ monocyctogenes\) [118,119]. In addition, FnR is an activator of the \(frd\) and \(dms\) operons, which are responsible for anaerobic utilization of fumarate and dimethyl sulﬁde as alternative electron acceptors, respectively [120–123]. Our study of the anaerobic expression of \(hmpA\) suggests that it is regulated by Fur, independent of FnR. Clearly, these results suggest FnR is functional in \(\Delta fur\) and that Fur is regulating genes of anaerobic metabolism (\(eut, frd\), and \(dms\) operons) through an unknown mechanism.

**Conclusions**

We demonstrated that Fur is an activator of \(ftnB\) in \(S.\) Typhimurium, which is likely due to the de-repression of \(hns\) in \(\Delta fur\). The strong dependence of \(ftnB\) expression on \(O_2\) indicates that FnR is crucial in its regulation. Additionally, we presented evidence that Fur indirectly controls \(hmpA\), independent of FnR. We determined that Fur represses \(sodA\) transcription, but is required for the maturation of SoxA into an active enzyme, MnSOD. Finally, we identified new target genes regulated by Fur in \(S.\) Typhimurium, and our data support the increasing evidence of enhanced H-NS expression in \(\Delta fur\).

**Additional material**

| Additional file 1: Table S1. Primer table | This file contains the sequence of primers used in this study. |
| Additional file 2: Table S2. Fur Regulated Genes | This file contains the genes that were differentially expressed between 14028s and \(\Delta fur\) under anaerobic conditions. |
| Additional file 3: Table S3. Fumarate reductase activity under anaerobic conditions | This file contains the specific activity of fumarate reductase in cell-free extracts isolated from 14028s and \(\Delta fur\) under anaerobic conditions. |
| Additional file 4: Table S4. Genes regulated by Fur and FnR under anaerobiosis and contain putative binding sites for both regulators | This file contains genes that were differentially expressed in 14028s, \(\Delta fur\), and the fnr, which contain a putative binding site for Fur and for FnR. |

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**Authors’ contributions**

All authors have read and approved this work. BT, RCF, HMH designed and conducted the experiments and contributed to the writing and editing of the manuscript. RCF conducted the microarrays, constructed the Fur Logo, and contributed to the editing of the manuscript. MM and SP constructed and provided the microarray slides and reviewed the manuscript. BT and HMH conceived the research idea, directed the research, and contributed to the writing and editing of the manuscript.

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