O₂ availability impacts iron homeostasis in Escherichia coli

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The ferric-uptake regulator (Fur) is an Fe²⁺-responsive transcription factor that coordinates iron homeostasis in many bacteria. Recently, we reported that expression of the Escherichia coli Fur regulon is also impacted by O₂ tension. Here, we show that for most of the Fur regulon, Fur binding and transcriptional repression increase under anaerobic conditions, suggesting that Fur is controlled by O₂ availability. We found that the intracellular, labile Fe²⁺ pool was higher under anaerobic conditions compared with aerobic conditions, suggesting that higher Fe²⁺ availability drove the formation of more Fe³⁺–Fur and, accordingly, more DNA binding. O₂ regulation of Fur activity required the anaerobically induced FeoABC Fe³⁺ uptake system, linking DNAase Fur activity to ferrous import under iron-sufficient conditions. The increased activity of Fur under anaerobic conditions led to a decrease in expression of ferric import systems. However, the combined positive regulation of the feoABC operon by ArcA and FNR partially antagonized Fur-mediated repression of feoABC under anaerobic conditions, allowing ferrous transport to increase even though Fur is more active. This design feature promotes a switch from ferric import to the more physiological relevant ferrous iron under anaerobic conditions. Taken together, we propose that the influence of O₂ availability on the levels of active Fur adds a previously undescribed layer of regulation in maintaining cellular iron homeostasis.

Significance

Our understanding of how cells regulate intracellular iron pools has been largely shaped by studying cells grown under aerobic conditions, in which the barrier to iron acquisition is dominated by O₂-dependent insolvency. However, less is known about how bacteria meet their iron demands in the O₂-limiting or anaerobic environments that are common to many ecosystems and reflective of the ancient atmosphere of early Earth. Because the transcription factor ferric-uptake regulator (Fur) plays a central role in controlling iron homeostasis in many bacterial species, we use Fur activity as a surrogate for understanding how anaerobiosis alters iron homeostasis. Our finding that levels of active Fur are increased during anaerobiosis emphasizes fundamental differences in bacterial iron requirements between aerobic and anaerobic growth conditions.


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occurs, which involves the up-regulation of Fe$^{3+}$ uptake under anaerobic conditions.

**Results**

**Fur DNA Occupancy Increases During Anaerobiosis.** A previous genome-wide study of Fur-DNA interactions using ChIP-seq technology (ChIP-seq) revealed that under anaerobic conditions, Fur bound more regions in the *E. coli* K12 genome than under aerobic growth conditions (6). This finding led us to hypothesize that during anaerobiosis, an increase in the amount of active Fe$^{3+}$-Fur could explain Fur binding to DNA sites that are more varied from the consensus Fur binding motif (6) than those occupied by Fur in both the presence and absence of O$_2$. Here, we build upon these results to address whether the O$_2$-dependent changes in expression observed for the Fur regulon (6) can be attributed to changes in Fur DNA occupancy genome-wide.

By quantifying the ratio of Fur enrichment for 74 of the highest intensity iron-dependent ChIP-seq peaks (6) using the statistics-based differential binding algorithm DBChIP (18), we found that, overall, the ratio of anaerobic to aerobic Fur ChIP-seq signal increased, ranging from ~1.5- to 10-fold, with a median of threefold (Tables S1 and S2). The increase in the ratio of anaerobic to aerobic Fur ChIP-seq signal at each site suggested an increase in Fe$^{3+}$-Fur binding at these sites.

The anaerobic increase in genome-scale Fur DNA binding appears to be transcriptionally relevant since, for most of the Fur regulon, the ratio of anaerobic to aerobic ChIP-seq signal correlated positively with the fold increase in Fur-mediated repression calculated from anaerobic and aerobic transcriptomic data, respectively (Table S1). For example, Fur binding to the promoter regions of *exbB* and *tonB* (both involved in Fe$^{3+}$-siderophore transport) and *bflD* (involved in iron storage/release from bacterioferritin) increased 6.2-, 5.2-, and 3.9-fold under anaerobic conditions, respectively (Fig. 1 and Table S1). This corresponded to a 6.0-, 3.3-, and 7.0-fold respective increase in Fur-mediated repression of these genes under anaerobic conditions compared with aerobic conditions (Table S1). In instances where the ChIP-seq ratio measured for Fur occupancy in anaerobic and aerobic cells was closer to 1, smaller O$_2$-dependent changes in gene expression were detected (e.g., *entC* and *fluE*, involved in Fe$^{3+}$-siderophore transport; *rhyB*, encodes a regulatory small RNA) (Table S1). This latter group of promoters represents some of the more strongly repressed Fur genes (6, 19), and the lower ratio likely stems from nearly comparable Fur binding under both aerobic and anaerobic conditions. In summary, these data indicate that under anaerobic conditions, Fur binding is increased at many promoters, which can lead to comparable changes in Fur-mediated gene repression.

A Fur Binding Site Is Sufficient to Confer Regulation by O$_2$. To test if the presence of a Fur DNA binding site is sufficient to confer O$_2$-dependent regulation as suggested by our genome-wide analysis, we created a synthetic Fur-dependent promoter (P$_{fepBS}$), in which the tac promoter (P$_{tac}$) contains the Fur binding site from P$_{fepB}$ (which drives expression of FepB, an enterobactin binding protein). This binding site is predicted to bind two Fur dimers (20, 21) and was positioned in P$_{tac}$ to maintain the same spacing (~19 to +21 bp) with respect to the promoter ~35 hexamer from P$_{fepB}$. In a strain containing P$_{fepBS}$ driving expression of a chromosomal lacZ reporter gene, β-galactosidase activity was significantly decreased when Fur was present (Fig. S1), indicating that Fur mediates repression of P$_{fepBS}$ in vivo. Furthermore, Fur-dependent repression was increased threefold under anaerobic growth conditions compared with aerobic growth conditions (Fig. 2), demonstrating a direct effect of O$_2$ availability on Fur-dependent regulation of P$_{fepBS}$. Taken together, the enhancement in Fur occupancy at genomic sites, along with the increase in Fur repression under anaerobic conditions, provides evidence for a direct role of Fur in the global response to anaerobiosis.

**Additional Transcription Factors Can Tailor O$_2$-Mediated Expression of Some Members of the Fur Regulon.** For a few members of the Fur regulon, we hypothesized from previous results that the impact of O$_2$ on their transcription might also require the O$_2$-regulated transcription factors FNR and ArcA (22, 23). Therefore, we measured expression of lacZ fusions to P$_{exbB}$, P$_{fepB}$, P$_{cirA}$, or P$_{fepA}$ in strains lacking one or more of these transcription factors. In the absence of any mutations, expression of P$_{exbB}$, P$_{fepA}$, and P$_{cirA}$ decreased under anaerobic conditions (Fig. 3 A–C), in agreement with transcriptomic data (6). For P$_{exbB}$, we found that deletion of fur increased expression to comparable levels under both aerobic and anaerobic conditions, indicating Fur-mediated repression was sufficient to explain its decreased expression under anaerobic growth conditions (Fig. 3A). Deletion of fur had no effect on P$_{exbB}$ expression, despite a reported FNR binding site (22). In contrast, for P$_{fepA}$ and P$_{cirA}$, we found that decreased expression of these promoters under anaerobic conditions was only partially dependent on Fur (Fig. 3 B and C). For both promoters, we found that ArcA also contributes to anaerobic repression (Fig. 3 B and C), in agreement with ArcA binding these promoter regions in vivo (23). This additional role of ArcA likely explains why the increase in Fur binding to P$_{cirA}$ and P$_{fepA}$ anaerobically did not correlate well with the fold increase in Fur-mediated repression (Table S1).

Similar analysis of the promoter driving transcription of *feoABC*, encoding a ferrous uptake system, revealed several distinctive features (Fig. 3D). First, in contrast to the ferric uptake systems, P$_{feoA}$ is poorly expressed aerobically even in the absence of Fur. Second, unlike the ferric uptake systems (e.g., *cirA, exbB, fhu*), expression of P$_{feoA}$ increased a small amount under anaerobic conditions compared with aerobic conditions, despite increased anaerobic Fe$^{3+}$-Fur levels. Third, we found that this increased anaerobic expression of P$_{feoA}$ required both of the anaerobic transcription factors FNR and ArcA, as suggested by genome-wide and other data (22-24). This positive effect of both ArcA and FNR appears to limit the negative effect of Fur under anaerobic conditions. Thus, FNR and ArcA produce an expression pattern for P$_{feoA}$ under anaerobic conditions opposite to the expression pattern of the ferric transport systems. Taken together, these data support the notion that Fur plays a central role in reprogramming gene expression profiles in response to O$_2$ because of changes in levels of Fe$^{3+}$-Fur available for DNA binding and by Fur acting in concert with other transcription factors.

**The Labile Fe$^{3+}$ Pool Increases During Anaerobiosis.** To determine the basis for increased levels of Fe$^{3+}$-Fur under anaerobic conditions, we measured the levels of both Fur protein and iron. Western blot analysis revealed that Fur protein levels are comparable under aerobic (22 μM) and anaerobic (17 μM) growth conditions (Fig. S2). To test if increased intracellular Fe$^{3+}$.
availability could explain the increase in active Fe$^{2+}$-Fur protein levels under anaerobic conditions, we used chelator-assisted electron paramagnetic resonance (EPR) spectroscopy (25) to measure the intracellular chelatable Fe$^{2+}$ pool. This labile pool is estimated to be 1.0% of cellular iron (14) and is defined as iron not tightly bound to cellular proteins and possibly associated with metabolites, such as glutathione, citrate, or phosphorylated sugar compounds (25–27). We found that the amount of chelatable Fe$^{2+}$ was approximately sevenfold higher in anaerobic cells compared with aerobic cells (Fig. 4A and B). When total cellular iron levels (which encompass both Fe$^{2+}$ and Fe$^{3+}$ in proteins and the labile iron pool) were measured by inductively coupled plasma mass spectrometry, there was only a slight increase in the total iron content of anaerobically grown cells (Fig. 4C). These results indicate that while total cellular iron in anaerobically and aerobically grown cells is comparable, the labile iron pool (that which is accessible for Fur binding) is higher in the absence of O$_2$.

To provide further support for an elevated labile Fe$^{2+}$ pool in anaerobic cells, we assayed the sensitivity of cells to hydrogen peroxide (H$_2$O$_2$). H$_2$O$_2$ is reduced by Fe$^{2+}$, resulting in the formation of hydroxyl radicals via the Fenton reaction (28). Hydroxyl radicals are highly detrimental to the integrity of cellular components, such as DNA (29, 30), and can lead to cell death. We determined the sensitivity of aerobic and anaerobic cells to H$_2$O$_2$ using a strain lacking RecA, which is defective in DNA repair and thus more sensitive to H$_2$O$_2$ compared with a wild-type strain (31). Anaerobically grown cells showed 10-fold more sensitivity to killing by H$_2$O$_2$ than aerobically grown cells (Fig. 4D), suggesting that Fe$^{2+}$ is more readily available in anaerobic cells to promote hydroxyl radicals and DNA damage. These results agree with previous findings that anaerobic cells are highly sensitive to H$_2$O$_2$ (31, 32).

Taken together, these data show that increased levels of Fe$^{2+}$-Fur under anaerobic conditions are not due to elevated Fur protein levels, but rather to an increase in the labile Fe$^{2+}$ pool. Consequently, these higher Fe$^{2+}$ levels would allow for increased formation of Fe$^{2+}$-Fur, and thus explain increased Fur DNA binding and transcriptional repression during anaerobiosis.

**Regulation of the Labile Iron Pool by O$_2$.** To determine how the labile iron pool is increased under anaerobic conditions, we asked whether the source of iron (Fe$^{2+}$ or Fe$^{3+}$) or media composition (rich versus minimal) influences Fur-mediated repression under aerobic or anaerobic conditions. Fur-mediated repression was assayed using the synthetic $P_{feoB}$-lacZ fusion. We did not find any changes in O$_2$-dependent regulation of Fur activity when the media were varied from our standard 3-(N-morpholino)propanesulfonic acid (MOPS) minimal media (Fig. S3). We also considered the possibility that decreased repression by Fur under aerobic conditions could be explained if iron uptake was less efficient due to the decreased solubility of iron in the presence of O$_2$. However, increasing external iron levels 10-fold resulted in no change in the levels of Fur-mediated repression under aerobic conditions, suggesting that iron availability was not limiting (Fig. S3). In contrast, under anaerobic conditions, repression by Fur was increased even further, suggesting that iron transport could be contributing to Fur regulation under anaerobic conditions.

Since the FeoABC system has a major role in anaerobic Fe$^{2+}$ uptake (17, 33, 34), we investigated whether anaerobic regulation of Fur was disrupted in a strain lacking feoB. Indeed, we found that elimination of the FeoB iron transporter caused a threefold defect in $P_{fepB}$ repression by Fur under anaerobic conditions (Fig. 2). Furthermore, O$_2$-dependent regulation of Fur required FeoB since Fur repressed $P_{feoB}$ to a similar extent under aerobic and anaerobic conditions in the ΔfeoB mutant (Fig. 2). The effect of feoB on Fur activity did not appear to reflect a general defect in iron-containing transcription factors since we did not find any change in activity of two Fe-S cluster-containing transcription factors (FNR and IscR) when similarly assayed under anaerobic conditions (Fig. S4). These data suggest that FeoABC contributes to the O$_2$-dependent regulation of Fur by increasing the anaerobic labile iron pool available for Fur binding.
uptake was disrupted

Intracellular labile Fe\textsuperscript{2+} levels increase during anaerobiosis. EPR spectra showing the signal at g = 4.3, normalized for OD\textsubscript{600} from aerobic or anaerobic cells grown in MOPS minimal glucose media containing 1.0 mM FeSO\textsubscript{4}. Aerobic (red) or anaerobic (blue) cells were treated with deferoxamine mesylate (DFO) or were left untreated (black). (A) Four to five replicates of each experiment are shown. (B) Chelatable Fe levels in aerobic (gray) or anaerobic cells (white) calculated from EPR spectra. (C) Inductively coupled plasma mass spectrometry was used to measure whole-cell Fe levels in three biological replicates of aerobic (gray) or anaerobic cells (white), which were grown in MOPS minimal glucose media containing 1.0 mM FeSO\textsubscript{4} and normalized for cell pellet (milligrams). (D) Viable cell counts (assayed in triplicate) for aerobic (gray) or anaerobic (white) cells grown in MOPS minimal glucose media containing 1.0 mM FeSO\textsubscript{4}, with and without treatment of 2.5 mM H\textsubscript{2}O\textsubscript{2} for 15 min. For \(B, C,\) and \(D\), error bars represent the SE.

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We also tested whether other iron transport systems affected Fur activity (3). Enteroactin-mediated Fe\textsuperscript{2+} uptake was disrupted by deletion of \(entA\). The inability to synthesize the enterobactin siderophore caused a twofold loss in the ability of Fur to repress \(P\text{\textsubscript{fepBS}}\) under both aerobic and anaerobic conditions (Fig. 2). However, the \(EntA\) mutant retained O\textsubscript{2}-dependent regulation of Fur, presumably because of the continued function and regulated expression of FecoABC (Fig. 2). As a control, we showed that Fur-mediated repression was not affected in a strain lacking \(fecC\), involved in transport of ferric citrate, which was not added to our media (Fig. 2).

Finally, we tested whether iron storage proteins affected the amount of iron available for Fur binding by measuring Fur-mediated repression in iron storage-deficient strains. The only known mechanisms of iron storage in \textit{E. coli} require O\textsubscript{2} or H\textsubscript{2}O\textsubscript{2} (35), suggesting that less iron might be stored anaerobically, and potentially contributing to the increase in the labile iron pool. However, elimination of iron storage proteins by deletion of \(fna\), \(bfr\), and \(dps\) did not alter the level of Fur-mediated repression under either aerobic or anaerobic conditions (Fig. 2).

**Fur Activity Responds to a Shift in O\textsubscript{2} Availability.** Adapting to changes in \(O_2\) tension is key for most organisms. Given the significant differences in the bioavailability of iron in the presence and absence of \(O_2\), we wanted to test if the sudden introduction of \(O_2\) to anaerobic cells altered Fur activity. To do this, we assayed \(\beta\)-galactosidase from \(lacZ\) fusions to either our synthetic promoter (\(P\text{\textsubscript{fepBS}}\)) or the \(fepA\) promoter (\(P\text{\textsubscript{fepA}}\)), which drives expression of the FepA Fe\textsuperscript{2+}-enterobactin transporter. Upon exposure to either \(O_2\) or \(\alpha,\alpha′\)-dipyridyl, a known cell-permeable iron chelator, we observed an increase in \(P\text{\textsubscript{fepBS}}\) and \(P\text{\textsubscript{fepA}}\) expression (Fig. 5 and Fig. S5). Although the rate of increase in expression of these promoters after exposure to \(O_2\) was slower than with \(\alpha,\alpha′\)-dipyridyl, de-repression of these promoters occurred on a similar time scale.

To determine how other genes that are directly repressed by Fur respond to \(O_2\) exposure, we analyzed time series transcriptomic data from RNA isolated from \textit{E. coli} at 0.5, 1, 2, 5, and 10 min after a shift from anaerobic to aerobic conditions [first reported by von Wulffen et al. (36)]. Similar to what we observed for \(P\text{\textsubscript{fepBS}}\) and \(P\text{\textsubscript{fepA}}\), expression of these genes showed an immediate response to the addition of \(O_2\), with almost all RNA levels increasing by 2 min (Table S3). Thus, these data indicate that expression of the Fur regulon responds to sudden changes in environmental \(O_2\) conditions in which \textit{E. coli} would experience in its natural habitat.

**Discussion**

Our data provide insight into how \(O_2\) availability alters iron homeostasis in the facultative bacterium \textit{E. coli}. Overall, we propose that an increase in the intracellular labile Fe\textsuperscript{2+} pool, mediated by the FecoABC ferrous transport system, leads to an increase in Fur-mediated repression under anaerobic conditions. The rise in anaerobic Fe\textsuperscript{2+} availability increases the population of Fur protein that is bound to Fe\textsuperscript{2+} and, accordingly, drives Fur DNA binding and transcriptional repression. The global change in expression of the Fur regulon under iron-sufficient anaerobic conditions promotes a shift in expression from Fe\textsuperscript{3+} to Fe\textsuperscript{2+} transport systems, allowing \textit{E. coli} to use the most physiologically relevant form of iron.

**The Labile Iron Pool Increases Under Anaerobic Conditions.** It is generally considered that the affinity of a metal sensor for its metal, and the subsequent homeostatic control the regulator exerts on its regulon, defines intracellular free metal concentrations (37–39). Thus, before this work, one would expect a priori that the intracellular free (labile) Fe\textsuperscript{2+} pool would be comparable in aerobically and anaerobically grown cells. Despite this expectation, we found that intracellular chelatable iron levels increased nearly sevenfold (∼26 μM in aerobic cells and ∼177 μM in anaerobic cells) under anaerobic conditions. Previous studies have shown that the FecoABC transporter, which is widely distributed in bacteria (17), contributes to anaerobic iron uptake (33, 34). Therefore, our finding that FecoABC was required for increased Fur activity under anaerobic conditions provided a link between increased iron import under anaerobic conditions and the anaerobic labile Fe\textsuperscript{2+} pool. The ability of FecoABC to mediate an increase in the iron pool under anaerobic conditions appears to be due, in part, to its unique mechanism of transcriptional regulation (discussed below).

**Fur Fe\textsuperscript{2+} Occupancy Varies with \(O_2\) Tension.** Since the affinity of Fur for Fe\textsuperscript{2+} is estimated at 1.2–55 μM (11–13), and the cellular Fur

![Fig. 4](image-url). Intracellular labile Fe\textsuperscript{2+} levels increase during anaerobiosis. EPR spectra showing the signal at g = 4.3, normalized for OD\textsubscript{600} from aerobic or anaerobic cells grown in MOPS minimal glucose media containing 1.0 mM FeSO\textsubscript{4}. Aerobic (red) or anaerobic (blue) cells were treated with deferoxamine mesylate (DFO) or were left untreated (black). (A) Four to five replicates of each experiment are shown. (B) Chelatable Fe levels in aerobic (gray) or anaerobic cells (white) calculated from EPR spectra. (C) Inductively coupled plasma mass spectrometry was used to measure whole-cell Fe levels in three biological replicates of aerobic (gray) or anaerobic cells (white), which were grown in MOPS minimal glucose media containing 1.0 mM FeSO\textsubscript{4} and normalized for cell pellet (milligrams). (D) Viable cell counts (assayed in triplicate) for aerobic (gray) or anaerobic (white) cells grown in MOPS minimal glucose media containing 1.0 mM FeSO\textsubscript{4}, with and without treatment of 2.5 mM H\textsubscript{2}O\textsubscript{2} for 15 min. For \(B, C,\) and \(D\), error bars represent the SE.

![Fig. 5](image-url). Anaerobic Fur activity decreases upon exposure to \(O_2\). A strain containing \(P\text{\textsubscript{fepBS}}\)-lacZ was grown anaerobically and was then either shifted to aerobic growth conditions (●) or treated with an iron chelator (200 μM \(\alpha,\alpha′\)-dipyridyl, ▲) at time 0. Samples were removed and assayed for \(\beta\)-galactosidase at various time points. Untreated cells (●) are indicated. Cultures were grown in MOPS minimal glucose media containing 1.0 mM FeSO\textsubscript{4}. Error bars represent the SE from three experiments. (Inset) Expanded view of \(O_2\)- and \(\alpha,\alpha′\)-dipyridyl-treated cells from time 0–20 min.
concentration is comparable between aerobic (~22 μM) and anaerobic (~17 μM) cells, our data suggest that the fraction of Fur bound by Fe⁴⁺ is impacted by O₂-dependent changes in Fe²⁺ levels. We propose that under aerobic conditions even when iron levels are sufficient, Fur is not completely occupied with Fe²⁺, whereas under anaerobic conditions, Fur-Fe²⁺ occupancy increases due to the increase in the labile iron pool (Fig. 6). It was also surprising that the decrease in iron available for Fur binding observed with the ΔfeoB mutant, did not impact gene regulation mediated by the transcription factors FNR and IscR that ligate Fe-S clusters. This result could imply that the affinity for iron for Fe-S cluster biogenesis machinery is much stronger than the binding affinity to Fur, creating a hierarchy of iron usage in the cell.

**The Strength of Fur DNA Recognition Sites May also Contribute to O₂ Regulation of the Fur Regulon.** The effect of O₂ on Fur-mediated repression varied for individual members of the Fur regulon. Some genes exhibited only small differences in Fur-mediated regulation between aerobic and anaerobic conditions, whereas others showed a much stronger dependence on anaerobic conditions to observe regulation by Fur. To explain these findings, we propose that the increase in Fe⁺⁺-Fur levels under anaerobic conditions allows binding to Fur sites of a wider range of affinities. In contrast, under aerobic conditions, the reduced levels of Fe⁺⁺-Fur binding to stronger affinity Fur sites. In support of this model, bioinformatic analysis of sites bound by Fur only under anaerobic conditions was much further from consensus than that of sites bound by Fur both in the presence and absence of O₂ (6). Although, there is no resource to compare Fur affinities obtained under similar solution conditions for individual sites across the regulon, information theory predictions of Fur binding site strength suggest that many of the DNA sites, which bound Fur well both in the presence and absence of O₂ (e.g., rhbB, entC, cirC, yijZ, fepA), should be high-affinity sites (21). Thus, the strength of the Fur DNA binding site likely contributes to how much of an effect O₂ has on an individual regulon member. For the Fur homolog Zur, the affinity of the transcription factor for its DNA sites also correlates to the fold repression (40). Further, it has been shown that stepwise metallation of the Zur Zn binding sites fine-tunes the degree of Zur repression of its target promoters (41, 42).

**Physiological Significance of O₂ Regulation of Fur.** Our data reveal that the O₂-dependent changes in levels of active Fur play a role in tailoring expression of iron uptake systems to the growth conditions in which their substrate (Fe²⁺ or Fe³⁺) is most likely to be found. In most cases, Fur-mediated repression was sufficient to explain decreased expression of Fe²⁺ uptake systems under anaerobic conditions. However, for a few Fe²⁺ uptake systems (e.g., fur, cirC), decreased expression in under anaerobic conditions required repression by both ArcA and Fur. In contrast, anaerobic expression of the FeoABC Fe²⁺ uptake system is under positive control of ArcA and FNR. This regulatory mechanism prevents additional Fur-mediated repression of PFeo (unlike that of the ferric uptake systems) and explains how expression increases under anaerobic conditions even when Fur is more active. By wiring *feoABC* expression in this manner, Fe⁺⁺ can enter cells through a transport system whose expression is not strictly controlled by Fur under anaerobic growth conditions. In turn, anaerobic up-regulation of *feoABC* facilitates the increase in the iron pool and recalibrates iron homeostasis.

It might be counterintuitive that even small changes in Fur-mediated repression between aerobic and anaerobic conditions could be physiologically significant. However, it is important to note that under iron-sufficient conditions, the low expression levels of Fur-repressed iron uptake systems are sufficient to drive iron uptake. Iron uptake systems only become further induced when external iron becomes scarce. Thus, it is logical to assume that even small changes in levels of these transport systems will affect uptake rates.

In summary, our data show that O₂ has a direct effect on levels of active Fur in vivo and that several factors contribute to the expression profile of the Fur regulon in response to O₂. The expression output of each gene is a summation of Fur activity, which depends on the extent of Fur Fe⁺⁺ occupancy, the Fur DNA binding sequence, and coordinated regulation by other transcription factors. Our data highlight the complexity of coordinating a central cellular activity like iron homeostasis to multiple environmental cues. This fine-tuning of transcriptional regulation ensures that the necessary gene products are available for cellular function. Finally, we predict that the differential effect of O₂ on iron homeostasis observed in this study is likely to be conserved in other Fur-containing facultative bacteria since it reflects an adaptation to the intrinsic sensitivity of Fe⁺⁺ to oxidation in aerobic environments.

**Methods**

Detailed materials and methods can be found in SI Methods.

**Differential Fur DNA Binding Analyses.** Differential binding of Fur between aerobic and anaerobic conditions was assessed using the ChIP-seq approach (16). This analysis was performed on 74 previously identified iron-dependent Fur ChIP-seq peaks from anaerobically grown *E. coli* with read counts >4,000 at the peak summit (6). Read counts of each dataset were normalized to 20 million reads. The fold change between aerobic and anaerobic Fur DNA occupancy, reported here as the ChIP ratio, was calculated by dividing the average ChIP signal from three anaerobic replicates by that of three aerobic replicates. Fur was annotated as differentially bound if the adjusted P value of the fold change was <0.05.

**Growth Conditions.** For the β-galactosidase assays (Figs. 2, 3, and 5 and Figs. 51 and 53–55) and H₂O₂ sensitivity assays (Fig. 4D), strains were grown at 37 °C in MOPS minimal 0.2% glucose media (43) containing either 1.0 μM or 10 μM FeSO₄ under aerobic conditions (by shaking) or anaerobic conditions (using filled screw-capped tubes) as described previously (44). In Fig. 53, different media and iron sources are indicated in the figure legend. β-galactosidase assays were performed as described (6).

For the in vivo O₂ shift experiments (Fig. 5 and Fig. 55), strains bearing *pO₂-lacZ* or *pO₂-lacZ* were grown at 37 °C in MOPS minimal 0.2% glucose media containing 1.0 μM FeSO₄ by sparging with an anaerobic gas mix of 95% N₂ and 5% CO₂. At time 0 min, which corresponded to an OD₆₀₀ of 0.1, cultures were either shifted to aerobic conditions by switching the gas mix to 70% N₂, 5% CO₂, and 25% O₂, or were treated with o-dipyridyl (final concentration of 200 μM). Samples were removed at various time points for measurement of OD₆₀₀ and β-galactosidase activity as described by Beauchene et al. (6).

For EPR spectroscopy and Western blot analysis, cultures were grown at 37 °C in MOPS minimal glucose media containing 1.0 μM FeSO₄ to an OD₆₀₀
of −0.4 (Lambda 25 UV/Vis Spectrophotometer; PerkinElmer) by sparging with either an aerobic (70% N2, 5% CO2, 25% O2) or anaerobic (95% N2, 5% CO2) gas mix (44).

**Chelator-Assisted EPR Spectroscopy.** A MG1655 culture volume of 450 mL (OD600 of −0.4) was pelleted, resuspended in 10 mL of fresh media containing either 20 mM membrane-permeable iron chelator desferoxamine mesylate (DFO; Sigma Aldrich) and 10 mM membrane-impermeable iron chelator diethylthlenetriaminepentaacetic acid (DTPA; Sigma Aldrich) or just 10 mM DTPA (to remove contaminating iron) as described previously (25). After a 15-min incubation at 37 °C, cells were washed twice with 5 mL of 20 mM Tris HCl (pH 7.4) and resuspended in 350 μL of 20 mM Tris HCl (pH 7.4) and 30% glycerol. A portion of these cells (200 μL) was transferred to EPR tubes (4-mm Thin Wall Quartz EPR sample tube with a length of 250 mm; Wilma-Lab Glass) and frozen at −80 °C. The remaining cells were diluted to measure the final OD600 (Lambda 25 UV/Vis Spectrophotometer; PerkinElmer). All manipulations were completed in a Coy anaerobic chamber. Four to five replicates of each sample were analyzed.

**H2O2 Sensitivity Assays.** Cells were treated with 2.5 mM H2O2 (equilibrated in an anaerobic chamber for anaerobic cultures) for 15 min at 37 °C (31) and then immediately diluted 1,000-fold into fresh MOPS glucose minimal media containing 1.0 μM FeSO4. Treated and nontreated cultures were plated on TYE agar plates to determine the number of viable cells. Anaerobic conditions were maintained by use of a Coy anaerobic chamber and GasPak anaerobic jar.

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