Evidence That a Respiratory Shield in *Escherichia coli* Protects a Low-Molecular-Mass Fe-\(\text{II}\) Pool from \(\text{O}_2\)-Dependent Oxidation

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Evidence that a respiratory shield in *Escherichia coli* protects a low-molecular-mass Fe\textsuperscript{II} pool from O\textsubscript{2}-dependent oxidation

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Iron is critical for virtually all organisms, yet major questions remain regarding the systems-level understanding of iron in whole cells. Here, we obtained Mössbauer and EPR spectra of *Escherichia coli* cells prepared under different nutrient iron concentrations, carbon sources, growth phases, and O\textsubscript{2} concentrations to better understand their global iron content. We investigated WT cells and those lacking Fur, FtnA, Bfr, and Dps proteins. The coarse-grain iron content of exponentially growing cells consisted of iron–sulfur clusters, variable amounts of nonheme high-spin Fe\textsuperscript{III} species, and an unassigned residual quadrupole doublet. The iron in stationary-phase cells was dominated by magnetically ordered Fe\textsuperscript{III} ions due to oxyhydroxide nanoparticles. Analysis of cytosolic extracts by size-exclusion chromatography detected by an online inductively coupled plasma mass spectrometer revealed a low-molecular-mass (LMM) Fe\textsuperscript{II} pool consisting of two iron complexes with masses of \(\sim 500\) (major) and \(\sim 1300\) (minor) Da. They appeared to be high-spin Fe\textsuperscript{II} species with mostly oxygen donor ligands, perhaps a few nitrogen donors, and probably no sulfur donors. Surprisingly, the iron content of *E. coli* and its reactivity with O\textsubscript{2} were remarkably similar to those of mitochondria. In both cases, a “respiratory shield” composed of membrane-bound iron-rich respiratory complexes may protect the LMM Fe\textsuperscript{II} pool from reacting with O\textsubscript{2}. When exponentially growing cells transition to stationary phase, the shield deactivates as metabolic activity declines. Given the universality of oxidative phosphorylation in aerobic biology, the iron content and respiratory shield in other aerobic prokaryotes might be similar to those of *E. coli* and mitochondria.

Iron is critical for virtually all living systems, including *Escherichia coli*, the most extensively studied single-celled organism on the planet (1). This redox-active transition metal is at the active site of numerous enzymes, including respiratory complexes that contain iron–sulfur clusters (ISCs)\textsuperscript{2} and heme centers (2, 3). Mononuclear Fe\textsuperscript{II} complexes are particularly dangerous to cells because they react with O\textsubscript{2} or O\textsubscript{2}-derived species (e.g., H\textsubscript{2}O\textsubscript{2}) to generate reactive oxygen species (ROS) such as hydroxyl radicals. Regulating iron traffic is critical to minimizing the dangers of this metal in aerobic organisms (4).

The iron content of whole *E. coli* cells has been studied sporadically over the past 4 decades by Mössbauer (MB) spectroscopy, in which transitions from the \(I = \frac{1}{2}\) ground state of an \(^{57}\)Fe nucleus are induced to the \(I = \frac{3}{2}\) excited state using \(\gamma\) radiation (5, 6). The energies of these transitions are sensitive to the type of iron center (ISCs, hemes, nonhemes, etc.) as well as to oxidation state, spin state, and ligand environment. The technique can decompose the major iron-containing species in whole cells and in organelles such as mitochondria into groups of iron centers. This partial or coarse-grain resolution is better than can be achieved by any other iron-sensitive spectroscopy; however, MB spectroscopy cannot resolve individual iron species. The inherent spectral intensity of each iron in a sample is approximately the same, such that spectral percentages of a center can be converted into absolute iron concentration if the overall iron concentration of the sample is known (e.g. as determined by ICP-MS). The most typical spectral feature, called a quadrupole doublet, consists of two equal-intensity lines extending down from the baseline. In a magnetic field, a six-line sextet is generally observed. In cases where unpaired electrons generate an internal magnetic field, the sextet “collapses” into a doublet at sufficiently high temperatures.

For magnetically ordered materials, such as the Fe\textsuperscript{III} cores of ferritins, the critical temperature for a related phenomenon is called the blocking temperature \(T_B\) (7). At temperatures below \(T_B\), magnetically ordered Fe\textsuperscript{III} exhibits a magnetic spectrum, whereas above \(T_B\), this form of iron exhibits a broad quadrupole doublet. *E. coli* cells contain three types of ferritins that store iron under iron-replete conditions. These include ferritin (FtnA), bacterioferritin (Bfr), and miniferritin (Dps) (8). The main iron storage protein is FtnA, a 24-subunit multimer that can bind thousands of iron ions as inert ferric aggregates. Expression of FtnA and Bfr increases dramatically as cells transition from exponential growth to stationary phase (9).

\textsuperscript{2}The abbreviations used are: ISC, iron–sulfur cluster; FTS, flow-through solution; LC-ICP-MS, liquid chromatography with on-line detection by an inductively coupled plasma mass spectrometer; LIP, labile iron pool; LMM, low-molecular-mass; MB, Mössbauer; NHHS, nonheme high-spin; OXPHOS, oxidative phosphorylation; ROS, reactive oxygen species; \(T_B\), blocking temperature; T, tesla.

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In 1980, Bauminger et al. (10) reported that MB spectra of E. coli are dominated by a broad quadrupole doublet characteristic of magnetically ordered FeIII ions. Matzanke et al. (11) identified a second doublet, representing up to half of the spectral intensity, with parameters typical of nonheme high-spin (NHHS) FeII complexes coordinated by oxygen and nitrogen ligands. Hudson et al. (12) recognized that the FeII doublet was composed of two subcomponents.

FtnA has a $T_H$ of $\sim 20$ K, which is substantially higher than that of the magnetically ordered FeIII species in E. coli cells ($<3.5$ K). Because of this, Bauminger et al. (10) suggested that ferritin was not the source of the observed magnetically ordered iron. In contrast, Abdul-Tehrani et al. (13) concluded that the magnetically ordered iron arose from FtnA.

Iron regulation and metallation of metalloproteins in E. coli involve a poorly characterized labile iron pool (LIP) (14–16). The LIP is presumably composed of one or more low-molecular-mass (LMM) nonproteinaceous FeII complexes in the cytosol. Estimates of the LIP concentration in aerobic bacteria are variable; 1 $\mu$m (17), 10 $\mu$m (18, 19), 26 $\mu$m (20), 15–30 $\mu$m (21), and 140 $\mu$m (22) have been reported. The size of the LIP increases (to $\sim 177$ $\mu$m) under anaerobic conditions (20) and reportedly with increasing iron levels in the growth medium (23).

The Fur (ferric uptake regulator) system regulates cellular iron by binding FeII ions from the LIP and regulating transcription of more than 100 genes involved in iron import, trafficking, and storage as well as in iron-dependent enzyme catalysis and cellular metabolism (24). Fur also controls expression of proteins involved in the TCA cycle and respiration via a small RNA RyB (25). Fur expression is induced during oxidative stress, which represses iron uptake and limits Fenton chemistry. $\Delta$fur cells reportedly contain less iron than WT cells and a higher LIP (13, 18, 21). $\Delta$fur cells cannot respire effectively because they are deficient in many iron-containing proteins (25, 27).

Aqueous FeII ions bind Fur with a $K_D$ of $\sim 1$ $\mu$m (17, 23, 28) or $\sim 10$ $\mu$m (19) for one-iron binding and $K_{D1} = 30$ $\mu$m and $K_{D2} = 280$ $\mu$m for two-iron binding (22). Weakly coordinated ligands in the LIP undergo fast ligand exchange, making such complexes difficult to isolate and study. Böhnhke and Matzanke (15) isolated and characterized a soluble nonproteinaceous negatively charged iron species from E. coli extracts that accounted for 40% of the cellular LIP; they reported a mass of $\sim 2.2$ kDa.

Using MB spectroscopy, Abdul-Tehrani et al. (13) estimated the concentration of the FeII species in the cell at $\sim 200$ $\mu$m, higher than most other estimates and higher than implied by the FeII-Fur dissociation constant. To explain this, they proposed that the FeII species observed by MB represented a different pool than is used to bind Fur.

Under aerobic conditions, the LIP is thought to enter cells via any of a number of ferric or ferrous iron transport systems that are largely regulated by Fur. Ferric ions, brought into the cell by siderophore transport systems, are likely reduced to FeII prior to entry into the cytosolic LIP. Under anaerobic conditions, the feo ferrous iron transport system dominates iron import, leading to an increased LIP (20, 29). FNR is an $O_2$-sensing transcription factor that regulates the shift between aerobic and anaerobic metabolism by regulating $\geq 300$ genes (30). In the absence of $O_2$, FNR increases expression of the feo iron uptake operon to increase cellular FeII (20).

Using MB spectroscopy, Hristova et al. (31) reported a fourth quadrupole doublet in whole E. coli cells, representing $\sim 60\%$ of spectral intensity in their samples. This doublet was attributed to [Fe$_3$S$_4$]$^{2+}$ and [Fe$_5$S$_5$]$^{2+}$ clusters, low-spin FeII hemes, and possibly fast-relaxing high-spin FeIII species. Beilschmidt et al. (32) assigned the same doublet exclusively to ISCs.

In summary, previous MB studies have decomposed the iron content of E. coli into four major groups. These include magnetically ordered FeIII (which may represent the iron core of the ferritin FtnA), two nonheme high-spin FeII species (some of which may represent the LIP), and a group of overlapping iron centers that include [Fe$_3$S$_4$]$^{2+}$ and [Fe$_5$S$_5$]$^{2+}$ clusters, low-spin FeII hemes, and possibly fast-relaxing high-spin FeIII species.

In this study, we used MB spectroscopy (and EPR) to investigate the iron content of E. coli. We show that the dominant magnetically ordered FeII doublet does not arise from ferritins. We use an anaerobic LC-ISC-MS system to detect and partially characterize the LMM FeII species that may constitute the LIP in these cells. We report a dramatic change in the size of the LMM FeII pool due to oxygenation state of the growth medium. Our results suggest a major reinterpretation of iron homeostasis in this organism, and they reveal an unexpected and intriguing connection to the iron content of mitochondria and other prokaryotes.

**Results**

**Initial objective: To better understand the iron content of WT E. coli**

Although the iron content of E. coli has been investigated sporadically using MB spectroscopy over the past 4 decades, many fundamental issues remain unresolved. Most previous studies were focused on the MB of E. coli strains in which an iron-containing protein of interest was overexpressed; WT cells served only as the controls. Here, we focused on WT E. coli, with mutant strains assisting in that investigation. The word “content” not only refers to the concentration of iron in such cells but to a semiquantitative description of the major iron species contained therein. $^{57}$Fe-enriched cells were grown on minimal medium using two different carbon sources, three different nutrient iron concentrations, and variable levels of $O_2$ exposure. Some batches were harvested during exponential growth, whereas others were harvested in stationary phase. Our motivation was to gain a foundational understanding of iron trafficking and regulation in WT E. coli, as our previous MB investigations of whole cells have focused on eukaryotic systems. These studies were only performed using minimal medium, and we are uncertain whether results would differ using other growth media.

**Exponentially growing E. coli cells contain 400–1600 $\mu$m iron, depending on conditions**

The iron concentration of E. coli cells grown on glucose minimal medium and harvested under exponential growth conditions increased with increasing iron concentration in the growth medium. Cells grown in media supplemented with 1, 10, and 100 $\mu$m $^{57}$Fe citrate contained 350 ± 120 ($n = 4$),
A respiratory shield to protect low-mass Fe\textsuperscript{II} from oxidation

**Figure 1.** Low-temperature low-field (5 K, 0.05 T) Mössbauer spectra of WT \textit{E. coli} harvested during exponential growth. A, glucose medium supplemented with 100 \(\mu\text{M} \text{Fe}^\text{II}\) citrate; B, acetate medium supplemented with 1 (top) and 100 \(\mu\text{M} \text{Fe}^\text{II}\) citrate (bottom). Gold, blue, green, and pink lines simulate Fe\textsuperscript{II}LMM, ISC, Fe\textsuperscript{II}LMM, and residual doublets, respectively. The red lines in this and other figures represent composite simulations assuming components, parameters, and percentages given in Table 1. The presence of the Fe\textsuperscript{II}LMM doublet in the spectrum from acetate-grown cells with 1 \(\mu\text{M} \text{Fe}^\text{II}\) added is evident from the shift in the high-energy line of the NHHS Fe\textsuperscript{II} doublet. The spectrum in C is the sum of all six spectra in Fig. 1 and Fig. S1, after removing contributions from the four major doublets. The red line in C is a simulation assuming parameters typical of S = 1/2 \text{Fe}^8\text{II} or ferritins (the two cannot be distinguished) with 5% of overall spectral intensity. Unless specified otherwise, the magnetic field was applied parallel to the \(\gamma\) radiation.

540 \(\pm\) 70 \((n = 2)\), and 990 \(\pm\) 20 \(\mu\text{M}\) iron \((n = 2)\), respectively. WT \textit{E. coli} cells harvested during exponential growth using acetate as the carbon source and in media supplemented with 1, 10, and 100 \(\mu\text{M} \text{Fe}^\text{II}\) citrate contained 600 \(\pm\) 200 \((n = 2)\), 730 \(\pm\) 490 \((n = 3)\), and 1600 \(\pm\) 400 \(\mu\text{M}\) iron \((n = 2)\), respectively. These concentrations are \(\sim\)1.6 times those in glucose-grown cells. See Table S1 for a compilation of other element concentrations. The trends observed were similar to those reported previously (33).

**Mössbauer spectra of exponentially growing cells exhibit the ISC and NHHS Fe\textsuperscript{II} doublets, as observed previously, as well as a previously undetected (residual) quadrupole doublet**

Low-temperature low-field MB spectra of glucose-grown and exponentially harvested cells were similar regardless of nutrient iron concentration (Fig. 1A and Fig. S1, A and B). The doublet simulated by the solid \textcolor{blue}{blue} line in Fig. 1 and referred to as the ISC doublet had parameters \((\delta = 0.45 \text{ mm/s and } \Delta E_Q = 1.15 \text{ mm/s; Table 1})\) typical of \(S = 0\) [Fe\textsuperscript{III}S\textsuperscript{2+}] clusters and low-spin Fe\textsuperscript{III} hemes (the two cannot be resolved by MB). The ISC doublet represented as much as 30% of the overall spectral intensity. The species simulated by the pink line in Fig. 1B will be referred to as the residual doublet. In some spectra, this doublet represented as much as 40% of the overall intensity \((\delta = 0.47 \text{ mm/s and } \Delta E_Q = 0.72 \text{ mm/s; Table 1})\). Its isomer shift \(\delta\) was near to that of \(S = 0\) [Fe\textsuperscript{III}S\textsuperscript{2+}] clusters, but the quadrupole splitting \(\Delta E_Q\) was smaller than normal; thus, we leave this species unassigned. The combined simulation of the ISC and residual doublets, as they would appear at 6 \(\text{T}\), was overlaid on the experimental 6 \(\text{T}\) spectrum in Fig. S2A. Although the spectrum was noisy, the fit provides evidence that both ISC and residual doublets arise from diamagnetic \(S = 0\) centers, as would be the case for [Fe\textsuperscript{III}S\textsuperscript{2+}] clusters and low-spin Fe\textsuperscript{III} hemes.

The other major quadrupole doublet of Fig. 1A had parameters of NHHS Fe\textsuperscript{II} species coordinated by 4 – 6 oxygen, 0 – 2 nitrogen, and few if any sulfur donor ligands. The doublet was broader than expected for one species, so two subcomponents (Fe\textsuperscript{II}_{\text{RET}} and Fe\textsuperscript{II}_{\text{LMM}}) were assumed \((\delta = 1.17 \text{ mm/s and } \Delta E_Q = 2.99 \text{ mm/s (Fe\textsuperscript{II}_{\text{RET}})}\ and \(\delta = 1.33 \text{ mm/s and } \Delta E_Q = 3.47 \text{ mm/s (Fe\textsuperscript{II}_{\text{LMM}}))}\). The **green** and **gold lines** in Fig. 1 (A and B) simulate the two subcomponents (Table 1). The subscripts RET and LMM are explained below.

Acetate-grown and exponentially harvested cells exhibited the MB spectra shown in Fig. 1B and Fig. S1 (C and D). Cells grew less than half as fast on acetate as they did on glucose; the average exponential growth rate \(\alpha\) was 0.40 \(\pm\) 0.04 \(\text{h}^{-\text{1}}\) for glucose-grown cells and 0.17 \(\pm\) 0.03 \(\text{h}^{-\text{1}}\) for acetate-grown cells \((n = 3\) for each). \textit{E. coli} converts acetate directly to acetyl-CoA, which is then sent to the TCA cycle without involving glycolysis (34). In contrast, cells first convert glucose to acetyl-CoA via glycolysis (and pyruvate dehydrogenase). Thus, acetate-grown cells respire exclusively, whereas glucose-grown cells both respire and ferment, depending on local oxygen and glucose concentrations.

### Table 1 Components assumed in simulating Mössbauer spectra of \textit{E. coli} and the percentages of each component included in simulations

In addition to the parameters listed for the sextet species, the following parameters were used in simulations: \(D = 0.5 \text{ cm}^{-1}, E/D = 0.33, \eta = 2, A_{\text{iso/g}}/g_B = -228 \text{ K}\).

<table>
<thead>
<tr>
<th>Mössbauer Component</th>
<th>ISC</th>
<th>Residual</th>
<th>Fe\textsuperscript{I\textsubscript{LMM}}</th>
<th>Fe\textsuperscript{II\textsubscript{RET}}</th>
<th>Fe\textsuperscript{II\textsubscript{LMM}}</th>
<th>S = 5/2 Fe\textsuperscript{II} sextet</th>
</tr>
</thead>
<tbody>
<tr>
<td>(S) (mm/s)</td>
<td>0.45 (\pm) 0.03</td>
<td>0.47 (\pm) 0.03</td>
<td>1.33 (\pm) 0.01</td>
<td>1.17 (\pm) 0.05</td>
<td>0.55 (\pm) 0.01</td>
<td>0.54 (\pm) 0.01</td>
</tr>
<tr>
<td>(\Delta E_Q) (mm/s)</td>
<td>0.72 (\pm) 0.13</td>
<td>3.47 (\pm) 0.01</td>
<td>2.99 (\pm) 0.05</td>
<td>0.55 (\pm) 0.01</td>
<td>0.39 (\pm) 0.01</td>
<td></td>
</tr>
<tr>
<td>(\Gamma) (mm/s)</td>
<td>0.6 (\pm) 0.1</td>
<td>0.6 (\pm) 0.1</td>
<td>0.6 (\pm) 0.3</td>
<td>0.7 (\pm) 0.1</td>
<td>0.5 (\pm) 0.1</td>
<td>0.4 (\pm) 0.1</td>
</tr>
</tbody>
</table>

**Spectrum** | % | % | % | % | % | % |
---|---|---|---|---|---|---|
Fig. 1A | 22 | 36 | 07 | 29 | 0 | 05 |
Fig. 1B (top) | 28 | 38 | 11 | 18 | 0 | 05 |
Fig. 1B (bottom) | 35 | 42 | 00 | 34 | 0 | 05 |
Fig. 1C | 0 | 00 | 0 | 0 | 0 | 05 |
Fig. 1A | 17 | 00 | 40 | 45 | 00 | 00 |
Fig. 1B | 11 | 00 | 30 | 54 | 00 | 00 |
Fig. 1C | 19 | 18 | 37 | 27 | 00 | 00 |
Fig. 1D | 21 | 7 | 24 | 55 | 00 | 00 |
Fig. 1E | 18 | 4 | 31 | 51 | 00 | 00 |
Fig. 1F | 12 | 7 | 31 | 43 | 00 | 00 |
Fig. 1G | 10 | 29 | 15 | 44 | 00 | 00 |
Fig. 1H | 15 | 00 | 00 | 10 | 61 | 00 |
Fig. 1I | 30 | 32 | 09 | 15 | 00 | 10 |
Fig. 1J | 26 | 39 | 06 | 15 | 00 | 06 |
Fig. 1K | 17 | 00 | 00 | 10 | 76 | 00 |
Fig. 1L | 23 | 12 | 35 | 18 | 12 | 00 |
Fig. 1M | 31 | 15 | 13 | 37 | 00 | 00 |
Fig. 1N | 17 | 20 | 29 | 21 | 16 | 00 |
Fig. 1O | 17 | 20 | 29 | 21 | 16 | 00 |
Fig. 1P | 0 | 00 | 13 | 00 | 13 | 00 |
Fig. 1Q | 11 | 31 | 10 | 48 | 00 | 00 |
Fig. 1R | 0 | 00 | 100 | 00 | 00 | 00 |
Fig. 1S | 13 | 29 | 32 | 35 | 00 | 00 |
Fig. 1T | 13 | 29 | 00 | 35 | 00 | 00 |
MB spectra of acetate-grown cells were similar to those grown in glucose, except that the ISC and residual doublets were slightly more intense, consistent with a higher iron concentration. Acetate-grown cells probably contain a higher concentration of iron-rich respiration-related proteins. We also observed a shift in the position of the Fe²⁺ doublet, depending on the iron concentration in the growth medium. For the sample grown with 1 μM ⁵⁷Fe³⁺ citrate, 11% of spectral intensity was Fe²⁺ LMM (Fig. 1B, top spectrum). For samples grown with 10 or 100 μM ⁵⁷Fe³⁺ citrate (Fig. 1B, bottom spectrum), no Fe²⁺ LMM was evident.

Only ~5% of MB intensity could be due to ferritins in exponentially grown cells

We searched for low-intensity features emanating from the baselines associated with the spectra of Fig. 1 and Fig. S1. Such features, if present, might arise from paramagnetic centers, such as S = ½ Fe³⁺ hemes, or from superparamagnetic centers, such as ferritins (at temperatures below their Tₚ). To probe this, we summed the six spectra and removed the major spectral features described above. The resulting difference spectrum (Fig. 1C) exhibited weak spectral absorption, which could be simulated using parameters of either S = ½ Fe³⁺ hemes and nonheme species or of ferritins below their Tₚ. Magnetic features with S = ½ may have also been present but obscured by features of the central spectral region. Collectively, these magnetic baseline features constituted ~5% of overall spectral intensity. We include this estimate in the composite simulations of Fig. 1, A and B (Table 1).

EPR spectra confirm Mössbauer analysis; exponentially grown cells contain minor levels of paramagnetic ISCs and heme centers

Glucose- and acetate-grown whole cells exhibited EPR signals in the low-field (g = 4–6) and high-field (g ~2) regions (Fig. 2). Spectral decomposition identified three low- and three high-field signals (Fig. S3). Low-field signals were typical of S = ½ hemes and nonheme Fe³⁺ species. High-field signals included two gave = 1.94 type signals arising from reduced S = ½ [Fe₃S₄]¹⁻ and/or [Fe₂S₂]¹⁻ clusters, as well as an unassigned isotropic g = 2.00 signal. Similar spectra of whole E. coli cells have been reported (25) except that signals from [Fe₃S₄]¹⁻ centers were not observed in our spectra, and the low-field signals in our reductant-free spectra were more typical of signals of previous dithionite-reduced cells. All current signals except the radical were 2–10 times more intense in acetate-grown cells than in glucose-grown cells (the intensity of the radical was similar in both spectra). The overall spin concentration in the g = 2 region was ~60 μM for the acetate-grown sample and 20 μM for the glucose-grown sample. We did not quantify the signals in the low-field region, but concentrations are likely of the same magnitude or less. We assumed that these paramagnetic centers contributed <5% to the central regions of the MB spectra and a similar percentage in the “wings.” This makes the EPR signals consistent with the minor intensity associated with paramagnetic centers in the MB of Fig. 1C, and it suggests that most ISCs in WT E. coli cells are diamagnetic and oxidized (i.e. [Fe₃S₄]²⁺ and [Fe₂S₂]²⁺ clusters).

The intensity of the NHHS Fe²⁺ doublets was correlated to O₂ levels during growth

In some spectra, the NHHS Fe²⁺ doublets were significantly more intense than in Fig. 1, reaching as high as 85% of overall intensity for the MB spectra (Fig. 3). This variation was initially puzzling because batches were grown on the same medium, using the same concentration of nutrient ⁵⁷Fe³⁺ citrate, and harvested at about the same Aₕₒₒ. Like Beauchene et al. (20), we discovered that the concentration of NHHS Fe²⁺ in cells was affected by the O₂ concentration in the culture. Subtle changes in aerobicity (e.g. caused by differences in rotation rates of the shaker or in volume ratios of liquid culture to flask capacity) affected the intensity of the Fe²⁺ spectral features. Higher concentrations of NHHS Fe²⁺ species correlate with lower O₂ concentrations.

Δfur cells have dysregulated Fe²⁺ LMM

We were surprised that modest changes in O₂ had a significant effect on the concentration of NHHS Fe²⁺ in WT cells; we had expected that Fe²⁺ concentrations would be tightly regulated by Fur. To investigate further, Δfur cells were grown on glucose media supplemented with 1, 10, and 100 μM ⁵⁷Fe³⁺ citrate. Like WT cells, Δfur cells exhibited MB spectra dominated by NHHS Fe²⁺ and ISC doublets (Fig. 4). Iron concentrations of a different set of Δfur cells but grown in the same way were 420 ± 10, 1460 ± 240, and 4300 ± 70 μM, respectively (n = 2 for each). The most significant spectroscopic difference relative to WT was the increased intensity of the Fe²⁺ LMM doublet relative to other features as the concentration of nutrient iron increased. This effect was not observed in WT cells; it

![Figure 2. Low-temperature X-band EPR spectra of whole packed E. coli cells grown on glucose (G100E) or acetate (A100E) and harvested under exponential growth conditions. Samples were grown in media supplemented with 100 μM Fe³⁺ citrate. Composite simulations are the red lines overlaying the data (black lines). Individual simulations are shown in Fig. S3. Temperature was 10 K, microwave frequency was 9.38 GHz, microwave power was 0.2 milliwatt, time constant was 0.293 s, and modulation amplitude was 10 G.](http://www.jbc.org/content/294/1/50.full)
implies that the species responsible for the Fe$^{II}_{\text{LMM}}$ doublet is not well regulated in /H9004$^{fur}$ cells (13, 18). The presence of higher concentrations of iron in /H9004$^{fur}$ versus WT cells also implies poorer regulation in /H9004$^{fur}$ cells. These results differ from a previous report (13) in which the iron concentration in /H9004$^{fur}$ cells was less than that of WT cells. Perhaps the difference arises because we harvested these cells during exponential phase, whereas Abdul-Tehrani et al. (13) harvested them during stationary phase. The mechanism of Fur regulation implies that the concentration of iron in /H9004$^{fur}$ cells should be higher than in WT cells, as we observed.

\textbf{Mössbauer spectra of cells harvested at stationary phase were dominated by magnetically ordered Fe$^{III}$}  

WT cells harvested in stationary phase exhibited a broad quadrupole doublet with parameters typical of magnetically ordered Fe$^{III}$ (Fig. 5B). This doublet was largely absent in spectra of cells harvested during exponential growth (Fig. 5A). The Fe$^{III}$ doublets were stronger in spectra from exponentially growing cells, whereas the ISC and residual doublet intensities were almost unchanged regardless of growth phase. This suggests that the species in the cell that gives rise to these latter doublets are less sensitive to the metabolic changes associated with the exponential → stationary shift than are the cell’s Fe$^{II}$ species. The overall percentage effect nearly doubled in the MB spectrum of stationary-phase cells relative to that of exponentially growing cells. This implies that stationary-phase cells import iron even when the cells are not growing. We observed a similar phenomenon in yeast (35).
Magnetically ordered Fe\textsuperscript{III} arises from oxyhydroxide nanoparticles rather than ferritins

The magnetically ordered \(\text{Fe}^{\text{III}}\) doublet was similar to that arising from \(\text{Fe}^{\text{III}}\) oxyhydroxide nanoparticles in mitochondria for which ISC assembly is impaired \((36)\) (see Fig. 5A). Also, the high-field spectrum of the magnetically-ordered material in \textit{E. coli} (Fig. 5 of Popescu et al. 37) was remarkably similar to that of mitochondrial nanoparticles \((38, 39)\). Thus, we assign this spectral feature to \(\text{Fe}^{\text{III}}\) oxyhydroxide nanoparticles.

Some past investigators assigned this feature to iron bound in ferritin cores \((8, 15, 36)\), whereas others doubted such an assignment \((10, 11, 32, 39)\). To settle the issue, we repeated the experiment using three genetic strains of \textit{E. coli} (\textit{Δ}ftnA, \textit{Δ}bfr, and \textit{Δ}bfr\textit{Δ}dps) in which ferritin-related genes were deleted. As above, a portion of a growing culture was harvested during exponential phase, and the remainder was harvested later in stationary phase. In the experiments involving \textit{Δ}ftnA and \textit{Δ}bfr, samples harvested during exponential growth exhibited little if any intensity from the magnetically ordered doublet (see Fig. 5 (C and D) for \textit{Δ}ftnA and Fig. S5A for \textit{Δ}bfr), whereas the corresponding samples harvested during stationary phase were dominated by this material (Fig. 5E for \textit{Δ}ftnA and Fig. S5B for \textit{Δ}bfr). The iron concentration of \textit{Δ}ftnA cells increased from 1.2 to 2.0 mM as cells transitioned from exponential to stationary phase. The corresponding iron concentration shifts for \textit{Δ}bfr and WT cells were from 1.6 to 1.8 mM and from 1.1 to 1.3 mM, respectively.

The magnetically ordered iron in these spectra could not have originated from the major ferritin or bacterioferritin in \textit{E. coli} because the corresponding genes had been deleted. The \textit{Δ}ftnA 5 K spectral baseline in Fig. 5C suggested a hint of magnetic material (\(~5\%\) of total intensity) in the wings. We collected a spectrum at 100 K and found that about half of the intensity remained, suggesting that no more than \(~3\%) of cellular iron might be due to ferritins. We cannot identify the ferritin-like species that could be involved, but it cannot be FtnA. The EPR spectrum suggests that most of the magnetic material emanating from the baseline arose from high-spin \(S = \frac{5}{2}\) \text{Fe}^{\text{III}}\) hemes and nonheme Fe\textsuperscript{III} (see above). In summary, our results indicate that no more than \(~3\%) of cellular iron (\(~50\ \mu\text{M}\)) could be due to ferritins in any experiment performed in this entire study.

The results of two experiments with the \textit{Δ}bfr\textit{Δ}dps strain differed from those of WT, \textit{Δ}ftnA, or \textit{Δ}bfr cells. In duplicate experiments, the spectra of exponential and stationary phase were similar (Fig. 5 (\textit{F} and \textit{G}) and Fig. S5 (\textit{C} and \textit{D})); nanoparticles did not form under what was ostensibly stationary phase. However, these cells grew slowly and may not have reached true stationary phase when they were harvested. Also, the iron concentration of \textit{Δ}bfr\textit{Δ}dps cells declined slightly (from 980 to 880 \(\mu\text{M}\)) during the exponential \(\rightarrow\) stationary transition, which differed from the other ferritin mutants.

The iron content of \textit{E. coli} is similar to that of mitochondria

We have studied mitochondria from yeast and human cells extensively using MB and EPR spectroscopies (\textit{e.g.} see Refs. 36, 38, and 40) and were surprised by the remarkable similarity to
that the cytosol of exponentially growing *E. coli* cells is sufficiently reducing to maintain the Fe^{II} state of a LMM Fe^{III} complex called Fe_{550} (see below) and that this space is sufficiently oxidizing in stationary-phase cells to support O_{2}-dependent oxidation of Fe^{III} \rightarrow Fe^{II} oxyhydroxide nanoparticles.

For *E. coli* transitioning into stationary phase, the low activity of respiratory complexes is caused, not by insufficient ISC or heme centers, but by low metabolic levels. The flow of electrons through respiratory centers can as readily be restricted by insufficient reducing equivalents as it can by insufficient iron centers. We suggest that the respiratory complexes on the periplasmic membrane of *E. coli* constitute a “shield” that partially blocks O_{2} from penetrating the cytosol. This shield, when operational, maintains the cytosol in a microaerobic (although not fully anaerobic) state. When exponentially growing cells transition to stationary phase, metabolic activity declines and the shield deactivates such that the cytosol becomes more oxygenated.

**Inhibiting respiratory complex IV with cyanide generates nanoparticles from NHHS Fe^{II} in *E. coli*, suggesting a protective “respiratory shield” against O_{2} or O_{2}-derived species**

To test this hypothesis, we inhibited cytochrome *c* oxidase activity in exponentially growing cells by treating cells with sodium cyanide, a well-known inhibitor of this respiratory complex. Prior to treatment, cells grew rapidly (with a specific growth rate of 0.26 h^{-1}), and after treatment, they quickly stopped growing (with a specific growth rate of 0 h^{-1}). MB samples of each state were prepared. After 90-min incubation, 13% of cellular iron in the form of NHHS Fe^{II} had converted to nanoparticles, as observed in the difference MB spectrum (Fig. 7C). The parameters needed to simulate the observed NHHS Fe^{III} doublet were those of Fe^{II}_{LMM}. The other doublet was simulated using parameters typical of nanoparticles (Table 1). That only the Fe^{II}_{LMM} doublet was affected suggests that the iron species giving rise to this doublet is particularly susceptible to oxidation by O_{2} or its ROS derivatives. We cannot exclude the possibility that the nanoparticle doublet might include other forms of Fe^{III} that have similar isomer shift and quadrupole splitting parameters; nor can we exclude the possibility that some effects of cyanide treatment may have been due to the binding of cyanide to cellular species other than cytochrome *c* oxidase.

**Mössbauer spectra of LMM flow-through solutions and retentate of *E. coli* soluble extracts distinguish Fe^{II}_{RET} from Fe^{II}_{LMM}**

We wondered whether the NHHS Fe^{III} species in *E. coli* that give rise to the Fe^{II}_{RET} and/or Fe^{II}_{LMM} doublets were bound to Fe^{III}-containing proteins or whether they were nonproteinaceous LMM Fe^{III} complexes. Such structural differences would suggest different physiological roles. Small Fe^{III} complexes might be associated with the LIP and function in iron trafficking and regulation, in Fenton chemistry, and as substrates for ISC/heme assembly (13, 18, 20, 21, 27, 43). In contrast, Fe^{III}-containing proteins often serve catalytic roles.

To distinguish the species giving rise to these two Fe^{II} doublets, three MB samples were brought into an anaerobic glove box after their spectra had been collected. The averaged spectrum exhibited a strong NHHS Fe^{III} doublet (Fig. 8A), indicating a high concentration of Fe^{III} species. Samples were thawed, diluted with buffer, and lysed. The supernatant was then passed through a 10-kDa cutoff membrane. The retentate and flow-through solutions (FTSs) were collected and transferred to MB cups. The retentate should have contained soluble iron-bound proteins, whereas the FTS should have been largely protein-free. The MB spectrum of the FTS (Fig. 8B) exhibited a sharp NHHS Fe^{III} doublet, which was simulated using parameters of the Fe^{II}_{LMM} doublet associated with whole-cell spectra (Table 1). This suggests that Fe^{III}_{LMM} is a LMM Fe^{II} complex that passes through the 10-kDa cutoff membrane (which explains our nomenclature). A MB spectrum of the FTS was also collected at 6 T and 4.2 K (Fig. S2B). Although noisy, it could be simulated using a high-spin Fe^{II} Hamiltonian.

Figure 7. Mössbauer spectra (5 K, 0.05 T) of cyanide-treated *E. coli* cells. A, before treatment; B, after treatment. C, a difference spectrum of B − A. The solid red line in C is a simulation in which the Fe^{II}_{LMM} doublet in A is replaced by the nanoparticle doublet in B (13% of spectral intensity for each).

The low-field 5 K MB spectrum of the retentate (Fig. 8C) also exhibited the Fe^{II}_{LMM} doublet along with other features. The presence of the Fe^{II}_{LMM} doublet in the retentate was expected because we did not wash this solution (e.g. by adding buffer and reconcentrating) to remove all traces of LMM Fe^{II}_{LMM}. To highlight the other features in the retentate spectrum, we subtracted the Fe^{II}_{LMM} doublet. The Fe^{II}_{RET} doublet was present in the resulting spectrum (Fig. 8D), as was another broad doublet near the central region. This latter doublet was composed of the ISC and residual doublets. The species giving rise to these doublets should be high-molecular-mass and probably proteinaceous, in that protein-bound iron cofactors should be retained by the 10-kDa cutoff membrane.
LC-ICP-MS chromatograms of flow-through solutions reveal that the LIP consists of two iron complexes

In some samples, a portion of the FTS was analyzed for metal concentrations. For one sample, the concentration of LMM iron within the cell was back-calculated from the iron concentration in the FTS. After multiplying by all dilution factors involved in preparing the sample from whole cells, the concentration of LMM iron species within whole cells was calculated to be \( \frac{1}{200} \) M.

We then used LC in conjunction with an online ICP-MS to detect LMM iron species in soluble E. coli extracts. FTSs from such extracts were separated on a size-exclusion column designed to resolve LMM peptides, and the eluent fractions flowed into an online ICP-MS. Iron-detected chromatograms are shown in Fig. 9; those of the other elements are presented in Fig. S6.

One major LMM iron species was detected in all chromatographs, with a mass of \( \sim 500 \) Da (to be referred to as \( \text{Fe}_{500} \)). A minor LMM iron species at \( \sim 1300 \) Da was evident in all chromatograms but was more intense in FTSs from acetate-grown cells. The intensities of \( \text{Fe}_{500} \) peaks did not vary systematically with the concentration of nutrient iron in cells grown on glucose, but in acetate-grown cells, \( \text{Fe}_{1300} \) increased with increasing nutrient iron levels. In one experiment, the cells contained 370 \( \mu \)M iron (at a collective volume of 1.42 ml), whereas the 600 \( \mu \)l of FTS contained 230 \( \mu \)M iron. This suggests that \( \sim 26\% \) of total cellular iron was LMM; this compares nicely to the MB percentage of \( \text{Fe}^{\text{II}}_{\text{LMM}} \) for this sample, namely 20%. We conclude that \( \text{Fe}_{500} \) and \( \text{Fe}_{1300} \) collectively give rise to the \( \text{Fe}^{\text{II}}_{\text{LMM}} \) doublet in the MB spectra of E. coli cells. We hypothesize that these two LMM species constitute the LIP.

**Discussion**

**Composition and properties of the LMM Fe\(^{\text{II}}\) pool in E. coli**

In this study, we have isolated two LMM Fe\(^{\text{II}}\) complexes from E. coli, which we call \( \text{Fe}_{500} \) and \( \text{Fe}_{1300} \) (subscripts indicate approximate masses in Da). \( \text{Fe}_{500} \) was the major component in all samples investigated. The concentration of \( \text{Fe}_{1300} \) was variable, with higher levels in samples grown on acetate and under iron-replete conditions. The concentration of this pool depended on the level of \( \text{O}_2 \) exposure during cell growth. Under more aerobic conditions, the LMM Fe\(^{\text{II}}\) concentration in WT E. coli cells was only \( \sim 50 \) \( \mu \)M, whereas under less aerobic conditions, it exceeded 500 \( \mu \)M in some samples.

Our results suggest that these LMM Fe\(^{\text{II}}\) complexes are not artifacts of isolation. The \( \delta \) and \( \Delta E_Q \) parameters for the Fe\(^{\text{II}}_{\text{LMM}} \) doublet exhibited by isolated FTS were the same as those for MB spectra of intact E. coli cells. We generated the FTS from whole cells rapidly in a refrigerated anaerobic glove box to avoid oxidation to the Fe\(^{\text{III}}\) state and to slow potential ligand-exchange reactions. The intensity of the Fe\(^{\text{II}}_{\text{LMM}} \) doublet in whole cells varied with \( \text{O}_2 \) levels similar to the effect observed by Kiley and co-workers (20). Also, the FTS Fe\(^{\text{II}}\) quadrupole doublet was sharper than would be expected for adventitious Fe\(^{\text{II}}\), which would generally be broadened due to heterogeneity. Also, the
simplicity of the LMM chromatograms, which reproducibly exhibited only peaks from Fe500 and Fe1300 is inconsistent with our expectation that adventitious iron would show batch-to-batch variation with multiple irreproducible peaks. Finally, aqueous FeII/FerII solutions, which would likely be a component of adventitious iron, adsorb strongly on our columns (44), whereas the LMM iron complexes observed here do not. We hypothesize that the physiological labile iron pool in E. coli consists mainly of one NHHS FeII complex with an approximate mass of 500 Da. A second minor species with a mass of ~1300 Da is also present. The low mass of these complexes suggests that they are not proteins. We do not know the functions of Fe500 or Fe1300 but speculate that they are involved in iron trafficking, sensing, and/or regulation. They might serve as feedstock for building ISCs and/or hemes for metallating various apoenzymes. Further studies are required to evaluate these possibilities.

Comparing δ and ΔEQ parameters for the FeII_LMM doublet with those from chemically defined high-spin FeII complexes (Table 2) provides some insight into the coordination environment of Fe500. An FeII ion coordinated to six oxygen donors affords similar parameters. The parameters for FeII_RET are closer to those of FeII complexes with a mixture of nitrogen/oxygen donor ligands.

### A new perspective for iron regulation in E. coli

A popular notion is that only a small portion of cellular iron is present as labile FeII, only enough for regulatory sensing and trafficking so as to minimize Fenton chemistry (14, 23). Consequently, the LIP is thought to be buffered in the low micromolar range (35, 43, 45). This view is supported by our results for cells grown under aerobic conditions; here, the concentration of the LMM FeII pool was 50 μM or less. However, the situation is different under microaerobic conditions in which the LMM FeII concentration can exceed 500 μM. If KD for binding of FeII to Fur equals ~1 μM (17, 23, 28), virtually all Fur in these cells should be bound with FeII under most nutrient iron conditions. How might Fur serve as a regulator under these conditions? One possibility is that the KD is actually weaker than 1 μM; higher values have been reported (19, 22), including one as high as KD = 280 μM. There is more than one binding site on Fur; perhaps sites that bind FeII weakly may be physiologically relevant. Another possibility is that only certain FeII complexes within the LIP bind Fur, and yet another possibility is that LIP levels in anaerobic/microaerobic cells are not as tightly regulated as is generally assumed.

Beauchene et al. (20) suggested that expression of the feo genes that control iron import under anaerobic conditions might be controlled more by FNR than by Fur. However, our results suggest that the concentration of FeII_LMM is Fur-dependent. Its concentration in Δfur cells was significantly affected by the concentration of iron in the medium, whereas its concentration in WT cells was not. Without Fur, FeII_LMM does not seem to be well regulated.

### A modified role of ferritins in E. coli

Abdul-Tehrani et al. (13) concluded from their MB study of iron-replete WT E. coli that half of the iron in cells harvested in stationary phase was bound to FtnA ferritin. They assumed that the magnetically ordered FeIII material observed in MB spectra of such cells arose from ferritin iron cores. During exponential growth, the concentration of iron in the cell was about half of what it was at stationary state, due, they suggested, to the absence of the magnetically ordered FeIII (i.e. FtnA-bound iron). This conclusion was reasonable because expression of FtnA increases 10-fold as exponentially growing cells transition to stationary phase (13).

In contrast, we found virtually no evidence for iron bound to ferritin in any E. coli sample studied, including iron-replete WT samples harvested at stationary state. We observed an intense magnetically ordered FeIII doublet in 5 K MB spectra of stationary-phase cells, and indeed this material represented over half of the iron in those samples. However, this material does not arise from FtnA-bound iron, as evidenced by the following results. First, the same doublet was observed in stationary-state cells lacking FtnA and Bfr. The doublet had δ and ΔEq typical of FeIII oxihydroxide nanoparticles in mitochondria. The Tg of the doublet, like that of mitochondrial nanoparticles, is <5 K. This explains why we observed (in E. coli spectra) a broad doublet at 5 K, whereas others observed magnetic interactions for the same material at 1.7 K (13).

Although the baselines of our 5 K MB spectra were largely devoid of any features, some spectra exhibited very low-intensity features that could be due to ferritins. The intensity of these features represented ~5% or less of total cellular iron. Such features had characteristics (at 5 K and 0.05 T) of either ferritin-associated iron or high-spin FeIII heme or nonheme centers; these possibilities could not be distinguished. However, similar features were evident in spectra of samples in which FtnA had been deleted, in which case the features could not have originated from that ferritin. Also, EPR spectra of packed E. coli cells exhibited three low-field signals arising from mononuclear S = ½ FeIII hemes and nonheme species. Such spin systems could have given rise to the MB baseline features. The 100 K high-temperature MB spectra of ΔftnA cells exhibited similar magnetic features as at 5 K (but with reduced intensity). Collectively, our results demonstrate that no more than half of these minor MB features, barely distinguishable from baseline, could have arisen from FtnA-bound iron.
Evolution of respiratory shields as a strategy for dealing with $O_2$ and iron

Chemiosmotic coupling is as ancient a process as transcription and translation, and it is universally employed to generate energy in living systems (46). We propose that this process has important consequences for iron metabolism. Prior to the dramatic increase in atmospheric $O_2$ due to the evolution of photosynthetic organisms, early prokaryotes used molecules other than $O_2$ as terminal electron acceptors in chemiosmotic coupling. During that era, aqueous Fe$^{II}$ dominated the environment, so these ancestral cells imported Fe$^{II}$ and trafficked these ions through the cytosol for use in ISC assembly. This was followed by installation into various proteins, including nonoxy- genic respiratory complexes located on the periplasmic membrane. Once $O_2$ appeared in the environment, it replaced these other molecules as the terminal electron acceptor in chemiosmotic energy coupling. This boosted the thermodynamics of the process such that more ATP could be generated per electron transferred. However, it also had negative consequences for iron trafficking and ROS damage.

We hypothesize that the newly evolved OXPHOS process provided a “respiratory shield” for these early prokaryotes. Such a shield is not a new concept; previous studies have provided evidence for it in aerobic bacteria. Azotobacter vinelandii uses a respiratory shield to protect nitrogenase, an extremely $O_2$-sensitive enzyme (47). In fact, the nitrogenase iron protein remains active when expressed in the mitochondrial matrix of yeast (48), which should be encapsulated by a respiratory shield on the inner membrane.

We hypothesize that during early stages of evolution, the shield maintained the cytosol at low $O_2$ concentrations as the pressure in the atmosphere increased. This allowed cytosolic Fe$^{II}$ ions to continue to be used in trafficking because such ions were shielded from oxidation to the poorly soluble Fe$^{III}$ state. For this reason, hexaqua Fe$^{III}$ complexes could not be used in iron trafficking. Maintaining the respiratory shield simply required that the organisms metabolize nutrients fast enough to generate a rapid flow of electrons into the respiratory complexes, which in turn reduced $O_2$ fast enough to limit its diffusion into the cytosol.

Respiratory shields provide “safe spaces” for $O_2$-sensitive biochemistry in aerobic prokaryotes and mitochondria

At a later stage in evolution, an ancestor of $\alpha$-proteobacteria was engulfed by a proto-eukaryotic host, culminating in a symbiotic relationship in which mitochondria provided chemiosmotic energy to the host via OXPHOS (49). Mitochondria and their bacterial ancestors share similar iron-rich respiration-related proteins as well as a suite of proteins involved in ISC biosynthesis (50, 51).

We report here that the iron content of $E$. coli and mitochondria are remarkably similar, at least at the coarse-grain level accessible by our methods. We recently proposed that the mitochondrial matrix in healthy eukaryotic cells is microaerobic and that this provides a safe space for $O_2$-sensitive enzymes (41, 42). This is especially important for ISC biosynthesis, an $O_2$-sensitive process that occurs in the matrix. The matrix contains a pool of labile LMM NHFS Fe$^{II}$ (called Fe$^{580}$) that gives rise to a quadrupole doublet in MB spectra (Fig. S4, blue line) (52). Fe$^{580}$ in mitochondria and Fe$^{590}$ in $E$. coli may both serve as feedstock for ISC biosynthesis and possibly for the iron insertion step of heme biosynthesis. Although the two LMM complexes have different names, we cannot exclude the possibility that they are the same complex. Isolated mitochondria also exhibit an ISC doublet (Fig. S4, green line) with parameters that are nearly identical to those of the ISC doublet in $E$. coli spectra.

Previous results from the Lindahl laboratory (42, 52) suggest that nanoparticles form in mitochondria when $O_2$ penetrates the matrix and reacts with Fe$^{580}$ as illustrated in Fig. 6 (top). $O_2$ penetrates ISC-deficient mitochondria because the respiratory shield is disabled. Under these conditions, the respiratory complexes are probably not fully loaded with ISCs and heme centers, and are thus unable to transfer sufficient electrons from the tricarboxylic acid cycle to cytochrome $c$ oxidase and then to $O_2$. In healthy mitochondria, more of the $O_2$ that diffuses to the IM is reduced to $H_2$O so that this diatomic molecule cannot overwhelm the matrix. We now propose an equivalent mechanism in $E$. coli and other aerobic bacteria (Fig. 6, bottom). Electrons generated by the metabolic activity of the cell are delivered to the inner membrane complexes and eventually to cytochrome $c$ oxidase. This rapidly reduces some of the $O_2$ that would otherwise diffuse into the cytosol. When the metabolic activity of the organism declines, as when cells transition to stationary phase, more $O_2$ penetrates the cytosol, where it can react with Fe$^{590}$ to form nanoparticles. The content and $O_2$-associated reactivity of iron in mitochondria and bacteria are similar largely because both have respiratory shields.

Examining the iron content of all bacteria is not possible, but published MB spectra of various bacteria support a common iron content, at least at the coarse-grain level of our analysis. The MB spectra of Pseudomonas aeruginosa whole cells (harvested after 44 h of growth) exhibit the same features as we observed for $E$. coli and mitochondria, namely a magnetically ordered Fe$^{III}$ doublet, an ISC doublet, and a NHHS Fe$^{II}$ doublet (53). The 82 K MB spectrum of Proteus mirabilis consists of a broad magnetically ordered Fe$^{III}$ doublet as well as a NHHS Fe$^{II}$ doublet (54). MB spectra of Erwinia chrysanthemi (grown on glucose, harvested in stationary state) exhibit a magnetically ordered Fe$^{III}$ doublet and a NHHS Fe$^{II}$ doublet (55).

To provide further evidence for a common (coarse-grain) iron content in prokaryotes, we examined the MB spectrum of Gram-positive Bacillus subtilis harvested under exponential growth conditions. As predicted, the spectrum (Fig. S7) was similar to those of $E$. coli and mitochondria. The MB spectrum of B. subtilis exhibited more magnetic iron than in $E$. coli or mitochondria, but the difference is only one of degree. Viewed collectively, these results provide strong support for a common (coarse-grain) iron content in aerobic prokaryotes and mitochondria.

The respiratory shield, NHHS Fe$^{II}$ LMM, and nanoparticles might collectively serve to regulate and store iron in prokaryotes

Eukaryotes protect against Fenton chemistry by minimizing the concentration of Fe$^{II}$ in the cytosol and by storing excess iron in ferritin (or in vacuoles, for fungi and plants). It was
A respiratory shield to protect low-mass FeII from oxidation

reasonable to assume that E. coli and other prokaryotes use the same strategies. However, this does not appear to be the case. As strange as it sounds, metabolically active aerobic E. coli simply do not store much iron, certainly not in ferritin cores. Under lower O₂ conditions, E. coli does not minimize the concentration of labile FeIII species in the cytosol; in fact, they might store iron as labile FeII. Metabolically active E. coli avoid Fenton chemistry by reducing the amount of O₂ that diffuses into the cytosol via the respiratory shield. Metabolically dormant E. coli store iron mainly as FeIV oxyhydroxide nanoparticles. Although we have not determined whether E. coli can utilize such iron when they reactivate metabolically, yeasts are able to do this (56). The implication is that ferritins play more specialized and limited roles in storing iron in E. coli cells than has been considered previously.

The love-hate relationship of cells with O₂ involves respiratory shields and antioxidant swords

The concentration of O₂ in the mitochondrial matrix, E. coli, and other aerobic prokaryotes has not been measured directly, so estimates vary. Unden and co-workers (57) measured O₂ consumption rates of bacterial cultures and calculated O₂ diffusion rates. They concluded that the O₂ concentration in the cytosol of bacteria is essentially the same as in external aerobic environments. This conclusion implies that the respiratory shield is weak and ineffective, and it supports the view that a multilayered antioxidant system (involving SOD, catalases, peroxidases, etc.) is continuously and vigorously fighting against ROS damage in healthy metabolically active cells and rapidly repairing any damage that occurs. We stress that the respiratory shield is not completely effective in preventing O₂ from entering the cytosol or matrix, and we recognize the critical importance of the antioxidant system. These protected spaces certainly contain some O₂ and O₂-derived ROS, as evidenced by enzymes (e.g. dioxygenases, superoxide dismutases, and FNR) that are in these spaces and use O₂ as substrates. However, we suggest that cells use both a respiratory “shield” and an antioxidant “sword” in their battle with O₂. By locating the respiratory shield near their peripheries, cells reap the energetic benefits of using O₂ in OXPHOS while at the same time minimizing exposure to O₂-sensitive cytosolic species. We look forward to probing further this intriguing love-hate relationship between aerobic cells and the molecule that they so desperately need but also the one that poses an existential threat to their very survival.

Experimental procedures

Construction of cell strains

The Δfur::kanR, ΔtnaA::kanR, and Δbfr::kanRΔdps::cmR mutations were constructed by replacing the indicated open reading frames in strain DY330 with the kanamycin resistance cassette (kanR) from pKD4 or the chloramphenicol resistance cassette (cmR) from pKD3 using the A RED system as described (58). Mutations were moved by P1 transduction into the MG1655 or other strain backgrounds. MG1655 Δbfr was generated by removal of the kanR cassette from the MG1655 Δbfr::kanR strain using the pCP20 plasmid as described (58).

Cells and growth

A single colony of each strain was inoculated into 50 ml of M9 minimal medium containing 0.2% (w/v) glucose. Cultures were incubated overnight at 37 °C and ∼200 rpm. Grown cultures were used to inoculate 1–2 liters of the same medium but supplemented with 1, 10, or 100 μM FeIII citrate. For exponential growths, cells were harvested at A600 = 0.5–0.8. Stationary-phase samples were harvested at A600 ~1.2.

The same procedure was used for cells grown on acetate except that the medium contained 0.4% (w/v) sodium acetate rather than glucose. For MB or EPR samples, harvested cells were centrifuged and washed with 50 ml of 50 mM EDTA, 100 mM sodium oxalate, 100 mM NaCl, and 10 mM KCl. Washed cells were packed into either MB cups or EPR tubes and then frozen in liquid N₂. WT B. subtilis cells (a generous gift of Jennifer K Herman, Texas A&M University (TAMU)) were grown in glucose-containing M9 medium under aerobic conditions and supplemented with 100 μM Fe citrate. Cells were harvested during exponential phase (A600 = 0.6) and a MB sample was prepared as above.

For experiments involving cyanide, WT cells were inoculated into 2 liters of minimal medium containing glucose and 100 μM iron citrate in a 2.8-liter baffled flask and incubated as above. Half of the cells were harvested during exponential phase (A600 ~0.65) and used to prepare a MB sample. The other half was incubated with 3.0 mM KCN (final concentration) and then used to prepare an MB sample after 1.5 h of incubation. Metal analysis and LC-ICP-MS experiments were performed essentially as described (42, 44) using MB samples after spectra had been collected. For metal analyses, a packing efficiency of 0.7 volume of cells/volume of packed wet cells was assumed, as was a density of 1.1 g/ml.

For LC-ICP-MS studies, samples (~800 μl) were thawed in the glove box and suspended in a 15-ml Falcon tube containing 5.0 ml of 20 mM ammonium bicarbonate, pH 8.5, ~100 mg of lysozyme (Sigma-Aldrich), and 2.0 ml of 0.1-mm diameter acid-washed glass beads (Sigma-Aldrich). The suspension was vortexed for 5 min and then placed in an ice bath for 5 min. This process was repeated twice. The lysate was then centrifuged for 15 min at 12,000 × g (Sorval Evolution RC centrifuge, GSA rotor). One ml of supernatant was mixed with 1.0 ml of 2% (v/v) Triton X-100 (Sigma-Aldrich), affording a 1% final concentration of Triton. The solution was vortexed for 30 min. (The remaining supernatant was frozen at −80°C.) The Triton-containing lysate was spun at 12,000 × g for 15 min. The resulting supernatant (~2 ml) was passed through an Ultracel 10-kDa ultrafiltration membrane using a stirred cell concentrator (Amicon, Millipore). Three hundred microliters of each FTS was injected onto two Superdex Peptide 10/300 GL (GE Healthcare) columns connected in series, equilibrated in 20 mM ammonium bicarbonate, pH 8.5.

Mössbauer spectra were collected on an MS4 WRC or LHe6T spectrometer (SEE Co., Edina, MN). Spectra were simulated with WMOSS software, and calibrated at room temperature with a-iron foil. EPR spectra were recorded using an X-band Elexys E500 spectrometer (Bruker Biospin Corp., Billerica, MA). EPR spectra were simulated using SpinCount.
A respiratory shield to protect low-mass FeII from oxidation


References
A respiratory shield to protect low-mass Fe\textsuperscript{II} from oxidation


60. Reiff, W. M., Frankel, R. B., and Abeledo, C. R. (1973) Orbital ground state inversion in [Fe(H\textsubscript{2}O)\textsubscript{6}](ClO\textsubscript{4})\textsubscript{2}. Chem. Phys. Lett. 22, 124–126 CrossRef


Evidence that a respiratory shield in *Escherichia coli* protects a low-molecular-mass Fe II pool from O2-dependent oxidation

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